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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

Kang-Hun Lee,* Ena Wang,† Mai-Britt Nielsen,† John Wunderlich,† Steven Migueles,§ Mark Connors,§ Seth M. Steinberg,‡ Steven A. Rosenberg,† and Francesco M. Marincola[1]*†

Although in vitro sensitization assays have shown increased melanoma Ag (MA)-specific CTL reactivity after vaccination with MA peptides, clinical responses have been uncommon. This paradox questions whether data obtained from the in vitro stimulation and expansion of T cells lead to an overestimation of the immune response to vaccines. Using HLA/peptide tetramer (tHLA), we enumerated MA-specific T cell precursor frequency (TCPF) directly in PBMC from 23 melanoma patients vaccinated with gp100:209–217(210M) (g209–2M) peptide. Vaccine-specific TCPF was higher in postvaccination PBMC from seven of seven patients treated with peptide alone and four of five patients treated with peptide plus IL-12 (range of postvaccination TCPF, 0.2–2.4% and 0.2–2.5%, respectively). The increased TCPF correlated with enhanced susceptibility to in vitro stimulation with the relevant epitope. Paradoxically, no increase in postvaccination TCPF was observed in most patients who had been concomitantly treated with IL-2 (1 of 11 patients; range of postvaccination TCPF, 0.02–1.0%), a combination associated with enhanced rates of tumor regression. The lack of increase in TCPF seen in these patients corresponded to inability to elicit expansion of vaccine-specific T cells in culture. This study shows that a peptide-based vaccine can effectively generate a quantifiable T cell-specific immune response in the PBMC of cancer patients, though such a response does not associate with a clinically evident regression of metastatic melanoma. *The Journal of Immunology,* **1999, 163: 6292–6300.**

The identification of melanoma Ags (MA)[2] recognized by T cells and their respective HLA class I-restricted epitopes stimulated peptide-based vaccination efforts as a new approach to tumor therapy (1, 2). Although comparative ex vivo sensitization of pre- and postvaccination PBMC has identified reproducible, vaccine-specific systemic T cell responses to immunization, in the majority of cases no regression of tumor is seen (2–4). We have previously published the results of a vaccination protocol in which patients with metastatic melanoma were sensitized against the gp100 MA by administration of the modified gp100 epitope gp100:209–217(210M) (g209–2M) that is presented in association with the HLA-A*0201 allele. The modification increases ligand affinity to HLA-A*0201/β_2-microglobulin complexes and is associated with higher immunogenicity in vitro (5) and in vivo (4) compared with the native epitope. Based on in vitro sensitization assays, 91% of patients that had been injected s.c. with the g209–2M peptide emulsified in IFA demonstrated successful immunization. However, no clinical responses were observed while 13 of 31 patients (42%) treated with the g209–2M peptide plus the systemic administration of high-dose IL-2 demonstrated objective cancer responses (2). Interestingly, only 16% of patients in this second cohort developed immune reactivity. It was suggested that the decreased precursor cell frequency observed when IL-2 was administered could have been due to activation of the vaccine-specific T cells and trafficking to the tumor site. Alternatively, differential susceptibility of vaccine-specific T cells to in vitro sensitization could have been responsible for the discrepant results in the two cohorts of patients rather than different frequency of vaccine-specific T cells in PBMC.

Therefore, the paradoxical behavior of vaccine-elicited immune responses questions, among other variables, the validity of data obtained by ex vivo stimulation and expansion of T cells. These assays require repeated stimulation with arbitrarily chosen concentrations of exogenous epitopes and cytokines, which could alter the functional and phenotypic characteristics of the T cells. Therefore, other assays directly measuring T cell reactivity in PBMC have been fashioned. Among them, the enzyme-linked immunospot (ELISPOT) assay can detect MA-specific CTL directly in PBMC (6) but may overlook inactive epitope-specific T cells that are unable to respond to epitope-specific stimulation. Tetrameric HLA/peptide complexes (tHLA) allow for direct measurement of epitope-specific T cell precursor frequency (TCPF) without in vitro manipulation (7). This method has been successfully used to measure T cell responses to viral infections (8, 9) and has been shown to estimate higher TCPF in PBMC than other assays (10). Furthermore, as this method does not rely on functional T cell responses, supplementary information to functional assays can be obtained.

*Department of Transfusion Medicine, Clinical Center, †Surgery Branch, National Cancer Institute, ‡Biostatistics and Data Management Section, National Cancer Institute, and §Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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[1] Address correspondence and reprint requests to Dr. F. M. Marincola at the Surgery Branch, National Cancer Institute, Building 10, Room 2B42, 10 Center Drive MSC 1502, Bethesda, MD 20892-1502. E-mail address: marincola@nih.gov

[2] Abbreviations used in this paper: MA, melanoma Ag; tHLA, tetramer HLA; g209, gp100:209–217; g209–2M, gp100:209–217(210M); ELISPOT, enzyme-linked immunospot; MART-1, MART-1:26–35 (27L); Flu, FluM1:58–66; TCPF, precursor frequency; TIL, tumor infiltrating lymphocyte; f_c, calculated frequency; KW, Kruskal-Wallis; P, peptide.

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We analyzed g209- and g209–2M-specific TCPF in pre- and posttreatment PBMC from 23 melanoma patients vaccinated with g209–2M peptide emulsified in IFA. We also evaluated whether enhancement in TCPF secondary to vaccination corresponded to increased susceptibility to in vitro stimulation with the relevant epitope. Furthermore, as the frequency of MA-specific T cells at tumor site might be of greater relevance than in PBMC, we compared, when available, g209-specific TCPF in PBMC and tumor infiltrating lymphocyte (TIL) pairs simultaneously obtained after vaccination.

Materials and Methods

Patients' selection

HLA-A*0201 patients received the g209–2M peptide in IFA. Representative PBMC were obtained from patients treated with peptide in IFA (n = 7, P1–P7), peptide with IL-12 s.c. (IL-12, n = 5, P8–P12), or with high dose (720,000 IU/kg every 8 h) IL-2 i.v. (IL-2, n = 11, P13-P23). These PBMC were selected according to previous in vitro sensitization, suggesting different vaccination outcomes in relation to concomitant cytokine treatment (2). Postvaccination PBMC from the patients treated with peptide alone or peptide with IL-12 had demonstrated tumor specificity after in vitro expansion, whereas PBMC from patients who had been treated with peptide and IL-2 did not. Vaccinations were administered at 3-wk intervals, and blood samples were obtained three weeks after vaccination unless otherwise specified. The HLA class I phenotype of patients was determined on PBMC using sequence specific primer-PCR (11). PCR was also used for molecular subtyping of HLA-A2 (12).

Cells and cultures

Samples were obtained from blood draws and leukapheresis of melanoma patients before and after vaccination with g209–2M peptide. PBMC were isolated by Ficoll gradient separation and frozen until analysis. For analysis of TCPF in TIL, excised tumor samples were enzymatically digested and frozen without separation of mononuclear cells from tumor cells as previously described (13). 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. This procedure allowed depletion of adherent monocytes. PBMC were also analyzed after 10 days of in vitro culture following stimulation with exogenous peptide. This was achieved by the administration of 1 μM peptide in complete medium to the PBMC at the time of thaw and the addition of IL-2 (300 IU/ml) the following day and every third day thereafter.

Epitope-specific T cell staining using HLA-A2 tetramer

Tetrameric peptide-HLA-A*0201 complexes were produced as described previously (7). Recombinant HLA-A*0201 heavy chain containing a biotinylation site and recombinant β_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: gp100:209–217 (ITDQVTCPFSV, g209); gp100:209–217 (210M) (IMDQVTCPFSV, g209–2M); and FluM1:58–66 (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 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 μg/ml for g209 and g209–2M tHLA, and to 1 μg/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 1×10^6 cells/50 μl ice-cold FACS buffer (phosphate buffer plus 5% inactivated FCS; Biofluids) and cells from day 10 CTL cultures were washed and resuspended at 2×10^5 cells/50 μl cold FACS buffer. Cells were incubated on ice with 1 μg tHLA for 15 min, and then continued 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). Fifty thousand events were acquired for CTL cultures and 200,000 events for PBL samples. Staining of tumor preparations was performed similarly to the preparation of PBMC with overnight resting in complete medium and direct staining of nonadherent cells.

FACS analysis for intracellular expression of IFN-γ

Nonadherent PBMC (1×10^6 cells) were stimulated for 6 h with peptide (1 μg/ml) pulsed T2 cells (1×10^6 cell/ml). After 2 h, Brefeldin A (10 μg/ml) (Sigma, Deisenhofen, Germany) was added. After four additional hours, the cells were treated with 4500 U DNase I (Calbiochem, La Jolla, 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. Cells were blocked overnight with PBS/5% milk on ice in cold room. Permeabilization of cells was performed with PBS. After staining with mAb for 30 min on ice, cells were washed in PBS. Staining with tetramers was performed before fixation of cells. The rest of the procedure was conducted according to the same protocol as all other stains. All samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer using the CellQuest software. Live-gating on lymphocytes, $CD3^+$, and $CD8^+$ was performed during acquisition. The following mAbs were used: allophyocyanin-conjugated mouse anti-human CD3 (IgG1), peridinin-chlorophyll protein-conjugated mouse anti-human CD8 (IgG1), and fluorescein (FITC)-conjugated mouse anti-human CD45RA were purchased from Becton Dickinson (Heidelberg, Germany), and fluorescein (FITC)-conjugated mouse anti-human IFN-γ (IgG1) and fluorescein (FITC)-conjugated mouse anti-human CD45RO (PharMingen, San Diego, CA).

Statistical analysis

In the light scatter, the lymphocyte population was gated in for evaluation. The frequency (f) of peptide-specific T cells per 10^6 $CD8^+$ cells was calculated using the following formula: f = URQ/(URQ + LRQ) $\times$ 10^6 $CD8^+$ cells, with URQ containing the $tHLA^+$, $CD8^+$ cells and LRQ containing all other $CD8^+$ cells. From these frequencies, the background with $CD8^+$ staining only was subtracted for each sample to obtain the corrected frequency (f_c). The f_c is presented as the number of peptide-specific T cells per 1×10^6 $CD8^+$ T cells.

For statistical comparison, the basic unit of analysis was the $\log_{10}$ of change between posttreatment vs pretreatment f_c for each day, staining, and

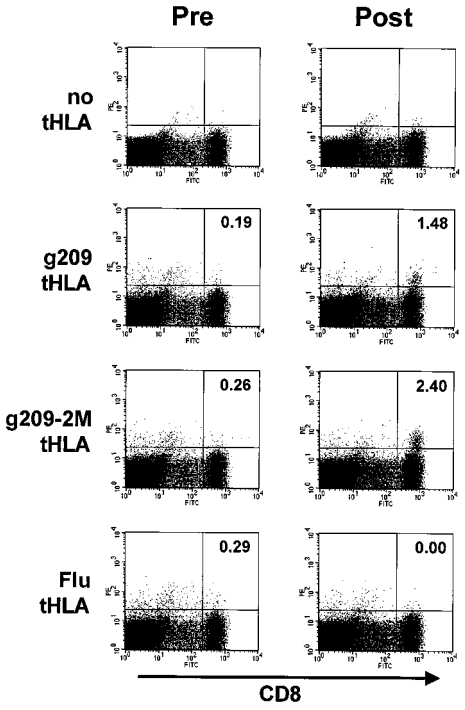

FIGURE 1. Detection of vaccine-specific T cells in PBMC. A patient (P4) who had received two vaccinations with g209–2M peptide demonstrated marked in vivo expansion of g209/g209–2M-specific $CD8^+$ cells in the postvaccination PBMC. Numbers in the URQ indicate the percentage of tHLA staining $CD8^+$ T cells calculated according to the formula: URQ/(URQ + LRQ) $\times$ 100.

stimulation condition. The $\log_{10}$ was chosen because of the wide range of f_c values observed in different patients. Because of varying intervals between pre- and posttreatment samples, Spearman correlation analyses and scatter plots were constructed to determine whether there was a relationship between interval (days) from pretreatment to posttreatment and $\log_{10}$ change in f_c for each experimental condition.

For each condition, the statistical significance of the $\log_{10}$ change from pre- to posttreatment f_c was determined by the Wilcoxon signed rank test, separately for each treatment group. In addition, specific analyses comparing changes under one experimental condition to changes with another condition were also done by the Wilcoxon signed rank test after subtracting one change [$\log_{10}$ (postpre)] from the other.

Finally, the Kruskal-Wallis (KW) test was used to determine the significance of the difference among the three treatment groups with respect to any of the changes or comparisons of the changes from pre- to posttreatment within a particular condition. These *p* values are indicated as KW in the figures. All *p* values are two sided and have not been explicitly adjusted for multiple comparisons because all analyses are being done on small groups of patients and with exploratory intent; thus, the results should be interpreted as hypothesis generating until confirmed by other studies.

Results

Frequency of vaccine-specific T cells in pre- and postvaccination PBMC

The TCPF of g209- and g209–2M-specific $CD8^+$ cells were determined in PBMC by g209 and g209–2M tHLA staining as shown in the patient's sample illustrated in Fig. 1. The precursor frequencies calculated (f_c) were then calculated as shown in Table I. Detection of Flu-specific $CD8^+$ cells was included as a control. Because of varying intervals, ranging from 40 to 283 days, between pre- and posttreatment PBMC samples, the effect of the time was assessed by Spearman correlation analysis. The results of the correlation analysis suggested that slight variations in f_c could be considered a negligible factor in the interpretation of the results

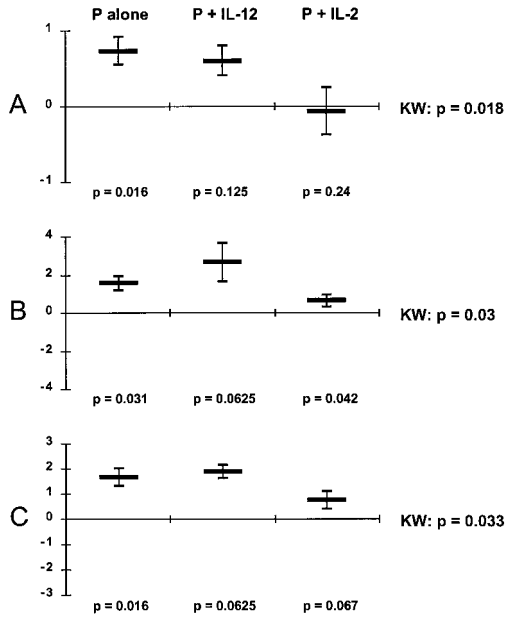

FIGURE 2. Frequency differences of g209 $tHLA^+$ $CD8^+$ cells in different treatment groups. The $\log_{10}$ differences of frequency were calculated for each pre- and postvaccination sample pair and analyzed with the Wilcoxon signed rank test. The means of the $\log_{10}$ difference are depicted as thick bars; positive means indicate higher post- than prevaccination frequencies. The SEs of the mean (thin bars) are displayed symmetrically. As there was insufficient evidence for normal distribution in some sample groups, this figure is an approximation. The treatment groups were P alone ($n = 7$), P + IL-12 ($n = 5$), and P + IL-2 ($n = 11$). *A*, Analysis of PBMC on day 1. *B* and *C*, T cell cultures 10 days after in vitro stimulation with g209 or g209–2M peptide, respectively. The KW values indicate the significance of the difference among the three treatment groups.

Table I. *Calculated precursor frequencies[a] of peptide-specific $CD8^+$ cells in PBMC on day 1 (peptide-specific/10e6 $CD8^+$ cells)*

		g209[b]		g209-2M		Flu		
		Pre	Post	Pre	Post	Pre	Post	Days[c]
Peptide alone[d]	P1	356	2,109	638	9,664	357	2,254	41
	P2	667	2,863	2,218	4,916	2,177	3,064	41
	P3	2,083	3,451	1,558	4,301	1,115	1,274	223
	P4	1,854	14,806	2,587	23,952	2,870	1[e]	43
	P5	104	4,402	1,416	3,440	4,732	5,703	46
	P6	4,779	9,137	5,473	12,723	6,820	7,901	94
	P7	454	1,988	408	1,564	415	2,389	41
Peptide + IL-12	P8	508	4,177	500	5,609	2,002	1,237	42
	P9	497	2,199	401	1,714	480	667	90
	P10	282	3,135	243	3,488	836	1,386	40
	P11	8,566	6,834	12,490	13,618	2,054	4,164	42
	P12	4,770	14,549	2,274	24,576	21,583	16,650	42
Peptide + IL-2	P13	2,141	121	1,735	853	1,992	1	283
	P14	1,895	1,714	1,607	2,335	663	1,026	199
	P15	1,842	9,524	1,791	10,043	1,519	4,941	89
	P16	7,225	4,887	4,620	2,771	2,228	1,473	184
	P17	1	433	300	533	14,496	6,309	45
	P18	2,345	971	1,755	272	421	1	145
	P19	616	271	1,051	251	1,690	1,656	54
	P20	2,558	950	769	979	908	2,687	50
	P21	1,870	239	895	306	838	260	94
	P22	3,134	1,813	4,507	1,338	1,838	1,341	41
	P23	2,427	1,057	2,426	869	1,374	465	85

[a] Frequency calculation: $[URQ/(LRQ + URQ) \times 10e6]_{epitope\text{-}specific} - [URQ/(LRQ + URQ) \times 10e6]_{background}$.
[b] Staining with g209-, g209-2M-, or Flu-tHLA.
[c] Time interval between the pre- and postvaccination sample.
[d] Treatment group.
[e] Calculated frequencies below or equal to zero were arbitrarily set to 1 to permit statistical analysis involving $\log_{10}$.

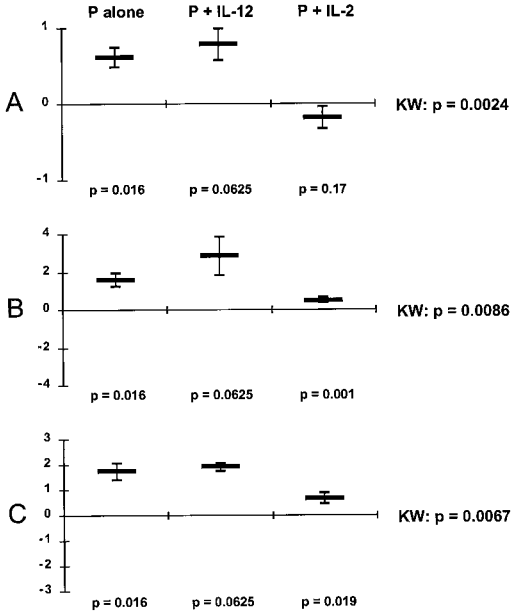

FIGURE 3. Frequency differences of g209–2M tHLA$^+$ CD8$^+$ cells in different treatment groups. The $\log_{10}$ differences of frequency were calculated for each pre- and postvaccination sample pair and analyzed with the Wilcoxon signed rank test. The means of the $\log_{10}$ difference are depicted as thick bars; positive means indicate higher post- than prevaccination frequencies. The SEs of the mean (thin bars) are displayed symmetrically. As there was insufficient evidence for normal distribution in some sample groups, this figure is an approximation. The treatment groups were P alone ($n = 7$), P + IL-12 ($n = 5$), and P + IL-2 ($n = 11$). *A*, Analysis of PBMC on day 1. *B* and *C*, T cell cultures 10 days after in vitro stimulation with g209 or g209–2M peptide respectively. The KW values indicate the significance of the difference among the three treatment groups.

(data not shown). The range of f_c noted was from undetectable above background, particularly in the peptide + IL-2 group to 14,806/10^6 CD8$^+$ cells for g209 (P4, postvaccination), 24,576/10^6 CD8$^+$ cells for g209–2M (P12, postvaccination), and 21,583/10^6 CD8$^+$ cells for Flu (P12, prevaccination). The variability of the results and the details for patients in the various cohorts are shown in Table I. Statistical comparison of the pre- and postvaccination f_c for each patient in the peptide alone (P alone) treatment group showed a significant increase of g209- and g209–2M-specific CD8$^+$ cells (g209: mean $\log_{10}$ difference (Δ) = 0.73, $p = 0.016$; g209–2M: $\Delta = 0.61$, $p = 0.016$) (Figs. 2*A* and 3*A*). A similar change was observed in the peptide + IL-12 (P + IL-12) treatment group (g209: $\Delta = 0.60$, $p = 0.125$; g209–2M: $\Delta = 0.78$, $p = 0.0625$). The peptide + IL-2 group (P + IL-2) did not show any obvious trends (g209: $\Delta = -0.07$, $p = 0.240$; g209–2M: $\Delta = -0.19$, $p = 0.17$). The differences of trends among various groups were statistically significant (g209: KW = 0.018; g209–2M: KW = 0.0024). Flu f_c values were not significantly different between pre- and postvaccination PBMC samples in all treatment groups (P alone: $\Delta = -0.22$, $p = 0.30$; P + IL-12: $\Delta = 0.07$, $p = 0.44$; P + IL-2: $\Delta = -0.58$, $p = 0.28$) (Fig. 4*A*). Thus, vaccination-dependent enhancement of TCPF could be detected in peptide treated patients who had not received i.v. IL-2. Sample collection in this study was dictated by the clinical protocol structure, which provided for patient return to our institution for re-evaluation and vaccine administration at 3-wk intervals. However, to investigate whether the relatively low TCPF observed were due to the distance between the last vaccination and sample collection, we tested patients' PBMC at shorter intervals after vaccine administration.

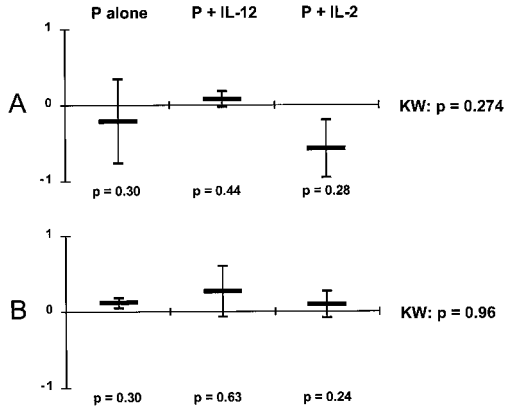

FIGURE 4. Frequency differences of Flu tHLA$^+$ CD8$^+$ cells in different treatment groups. The $\log_{10}$ differences of frequency were calculated for each pre- and postvaccination sample pair and analyzed with the Wilcoxon signed rank test. The means of the $\log_{10}$ difference are depicted as thick bars; positive means indicate higher post- than prevaccination frequencies. The SEs of the mean (thin bars) are displayed symmetrically. As there was insufficient evidence for normal distribution in some sample groups, this figure is an approximation. The treatment groups were P alone ($n = 7$), P + IL-12 ($n = 5$), and P + IL-2 ($n = 11$). *A*, Analysis of PBMC on day 1. *B*, T cell cultures 10 days after in vitro stimulation with Flu peptide. The KW values indicate the significance of the difference among the three treatment groups.

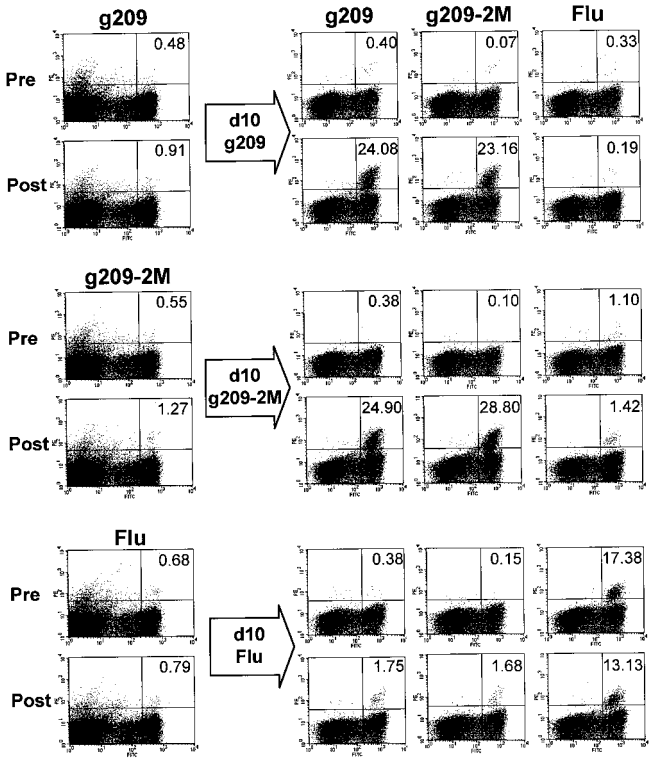

FIGURE 5. Comparison of TCPF determined on PBMC and after ex vivo stimulation. TCPF of pre- and postvaccination samples from P6 were determined in PBMC and compared with TCPF of 10-day cultures stimulated with g209, g209–2M, and Flu as specified by the open arrows. Titles of columns refer to the tHLA used for staining. PBMC were stained with g209 tHLA (*first column*, top two dot plots), g209–2M (*first column*, middle two dot plots), and Flu (*first column*, bottom two dot plots). T cell cultures stimulated with peptide were stained with g209 (*second column*), g209–2M (*third column*), and Flu (*fourth column*) tHLA. Numbers in the URQ indicate the percentage of tHLA staining CD8$^+$ T cells calculated according to the formula: URQ/(URQ + LRQ) × 100.

Table II. *Calculated precursor frequencies[a] of g209-specific CD8$^+$ cells on day 10 (peptide-specific/10e6 CD8$^+$ cells)*

		d1		g209 Stimulation[b]		g209-2M Stimulation	
		Pre	Post	Pre	Post	Pre	Post
Peptide alone[c]	P1	356	2,109	538	18,291	429	20,655
	P2	667	2,863	2,895	2,323	1,211	2,595
	P3	2,083	3,451	460	133,698	585	98,962
	P4	1,854	14,806	189	196,025	379	197,705
	P5	104	4,402	540	12,206	1,569	16,637
	P6	4,779	9,137	4,036	240,755	741	231,598
	P7	454	1,988	175	971	370	3,779
Peptide + IL-12	P8	508	4,177	1[d]	107,871	613	123,606
	P9	497	2,199	659	4,018	1,015	42,937
	P10	282	3,135	268	1,078	586	6,242
	P11	8,566	6,834	1,321	47,490	279	73,518
	P12	4,770	14,549	1	184,158	1,397	146,721
Peptide + IL-2	P13	2,141	121	16,140	25,141	16,304	13,802
	P14	1,895	1,714	3,519	2,587	4,370	3,538
	P15	1,842	9,524	1,073	4,203	233	9,625
	P16	7,225	4,887	2,283	3,417	1,654	1,282
	P17	1	433	628	987	596	1,123
	P18	2,345	971	16,380	19,702	6,425	11,965
	P19	616	271	1	2,949	129	725
	P20	2,558	950	3,663	2,039	1,388	725
	P21	1,870	239	564	7,454	182	14,645
	P22	3,134	1,813	1,142	1,327	172	508
	P23	2,427	1,057	124	2,129	1	3,023

[a] Frequency calculation: $[URQ/(LRQ + URQ) \times 10e6]_{epitope\text{-}specific} - [URQ/(LRQ + URQ) \times 10e6]_{background}$.
[b] Ten-day in vitro cultures stimulated with g209 or g209-2M.
[c] Treatment group.
[d] Calculated frequencies below or equal to zero were arbitrarily set to 1 to permit statistical analysis involving $\log_{10}$. Control cultures stimulated with Flu or without any peptide did not demonstrate an increase in g209/g209-2M-specific TCPF (data not shown).

Samples were collected before and 3, 7, 10, and 14 days after vaccination. At no time point were TCPF fluctuations noted (data not shown). Thus, it is unlikely that significantly higher frequencies were missed in the cohort of patients studied because of the delay in which the blood samples had been collected.

Differential susceptibility to ex vivo stimulation corresponds to differences in vaccine-specific pre- and postvaccination TCPF

Because determination of vaccine-specific TCPF does not yield information about their ability to respond to epitope-specific stimulation, we performed an in vitro expansion of the same PBMC in all 23 patients as exemplified for patient 6 (Fig. 5). The selection of PBMC as APC emphasizes a bias toward expansion of memory T cells elicited by the vaccination. Furthermore, this method was selected because it is the one previously described for the assessment of response to vaccination. Thus, analysis of CTL expansion after in vitro stimulation using tHLA can be directly compared with results previously reported by our group obtained by evaluating cytokine release and cytotoxicity of vaccine-induced in vitro-expanded T cells (2–4). As shown in this patient, an enrichment of vaccine-specific T cells could be easily demonstrated after in vitro stimulation of post- but not prevaccination PBMC. Concomitant stimulation of T cells specific for an epitope irrelevant to the one used for in vitro stimulation was occasionally noted. In patient 6, we noted persistence and/or minor expansion of Flu-specific CTL in cultures stimulated with g209–2M. Conversely, g209 and g209–2M-specific T cells could be identified in postvaccination cultures stimulated with Flu. In no case could the concomitant T cell expansion be noted in unstimulated control cultures (data not shown).

TCPF determined after 10 days of in vitro culture and after one stimulation with g209, g209–2M, or Flu peptides are shown in Tables II, III, and IV**,** respectively. After in vitro stimulation with g209 or g209–2M, all treatment groups demonstrated a peptide-specific expansion in the postvaccination samples. The TCPF increased dramatically in most patients treated with P alone or P + IL-12, and reached up to 29% of CD8$^+$ cells (P12 in Table III). The increase was less pronounced in the P + IL-2 group. In fact, most patients in the P + IL-2 group did not show any increase. The amplified f_c differences between pre- and postvaccination cultures underlined the trends observed in PBMC within each treatment group. However, f_c differences in pre- and postvaccination PBMC of single patients were not reflected quantitatively by f_c differences in the corresponding in vitro cultures.

Statistically significant differences in specific T cell expansion between pre- and postvaccination samples could be detected in the patient group receiving P alone (Fig. 2, *B* and *C*; Fig. 3, *B* and *C*, and Fig. 4*B*). The P + IL-12 group demonstrated the same trend but did not reach statistical significance. Measurement of vaccine-specific T cell expansion in patients receiving peptide plus IL-2 also revealed a modest but significant increase in post- compared with prevaccination samples. Thus, it appears that the inability to detect an enhancement of vaccine-specific T cell in these patients was in part due to a limitation in the sensitivity of tHLA staining rather than an absolute lack of effect of the vaccine. This analysis, therefore, demonstrates that the enhancement of TCPF detected in

Table III. *Calculated precursor frequencies[a] of g209-2M-specific CD8$^+$ cells on day 10 (peptide-specific/10e6 CD8$^+$ cells)*

		d1		g209 Stimulation[b]		g209-2M Stimulation	
		Pre	Post	Pre	Post	Pre	Post
Peptide alone[c]	P1	638	9,664	427	7,203	986	11,271
	P2	2,218	4,916	749	798	1,582	4,576
	P3	1,558	4,301	674	129,367	854	113,703
	P4	2,587	23,952	283	249,081	280	296,596
	P5	1,416	3,440	537	15,161	2,780	60,936
	P6	5,473	12,723	3,842	248,973	1,000	287,991
	P7	408	1,564	289	3,140	318	13,412
Peptide + IL-12	P8	500	5,609	1[d]	137,740	1,202	226,460
	P9	401	1,714	422	4,935	1,085	43,216
	P10	243	3,488	540	3,291	710	19,966
	P11	12,490	13,618	1,318	58,345	696	72,815
	P12	2,274	24,576	1	287,954	2,058	291,347
Peptide + IL-2	P13	1,735	853	22,517	25,930	26,037	19,794
	P14	1,607	2,335	3,849	4,860	7,221	2,924
	P15	1,791	10,043	873	5,313	563	11,763
	P16	4,620	2,771	872	2,877	1,863	3,066
	P17	300	533	365	1,227	270	1,502
	P18	1,755	272	18,272	23,677	7,404	15,465
	P19	1,051	251	753	3,466	355	3,379
	P20	769	979	2,855	3,466	1,385	3,379
	P21	895	306	556	16,699	686	41,414
	P22	4,507	1,338	754	935	349	715
	P23	2,426	869	542	3,738	260	10,406

[a] Frequency calculation: $[URQ/(LRQ + URQ) \times 10e6]_{epitope\text{-}specific} - [URQ/(LRQ + URQ) \times 10e6]_{background}$.
[b] Ten-day in vitro cultures stimulated with g209 or g209-2M.
[c] Treatment group.
[d] Calculated frequencies below or equal to zero were arbitrarily set to 1 to permit statistical analysis involving $\log_{10}$. Control cultures stimulated with Flu or without any peptide did not demonstrate an increase in g209/g209-2M-specific TCPF (data not shown).

PBMC in response to vaccination is associated with an enhanced susceptibility to in vitro epitope-specific stimulation.

Enhancement of vaccine-specific T cell frequency is paralleled by enhanced T cell reactivity toward the vaccine and susceptibility to ex vivo expansion but not to clinical regression of tumor

In one patient (P4), TCPF was monitored throughout treatment with multiple vaccinations (before and after 1, 2, 4, 6, 8, and 10 vaccinations) (Fig. 6). One vaccination was not sufficient to elicit a detectable increase in TCPC. However, a strong enhancement was noted 3 wk after the second vaccination (Fig. 1) at which time point there was no evidence of tumor regression. Starting from the third vaccination, IL-2 was added. Surprisingly, the TCPF decreased and tumors began to shrink. Thus, the detection of vaccine-specific T cells in PBMC after two vaccinations did not correlate with clinical outcome. Rapid expansion in response to vaccine-specific stimulation in vitro makes it unlikely that functional unresponsiveness was responsible for tumor progression. To more directly assess the functional state of g209/g209–2M-specific T cell, pre- and postvaccination PBMC were compared for intracellular IFN-γ expression in response to stimulation with T2 cells exogenously pulsed with g209 or g209–2M (Fig. 7). This analysis demonstrated a specific enhancement of g209/g209–2M reactive T cells only in postvaccination PBMC, which correlated with specific down-regulation of TCR as judged by tetramer staining. Interestingly the number of IFN-γ-expressing T cells in the postvaccination PBMC sample was noted to be roughly half the number of tetramer positive T cells. It is possible that tetramer staining of postvaccination PBMC picks up a fraction of vaccine-induced T cells that do not produce IFN-γ in response to Ag stimulation in the in vitro conditions exercised used for this study.

After eight vaccinations, the patient developed a new s.c. metastasis. Analysis of T cells obtained from a fine needle aspirate of that lesion failed to demonstrate evidence of localization of vaccine-specific CD8$^+$ T cells at the tumor site. Immunocytochemical analysis of the tumor at this time point revealed loss of gp100, which might explain the clinical outcome and, at the same time, the failure to identify vaccine-specific T cells.

Comparison of PBMC/tumor pairs

The low TCPF observed in the P + IL-2 group, which has been associated with clinical responses, may be explained by a migration of tumor-specific T cells from the systemic circulation to the tumor site. To address whether this phenomenon really occurs, we analyzed PBMC and tumor preparations, which had been obtained at the same time point after vaccination. Seven such pairs were available for analysis (Table V). In two of seven cases (L.R. and D.W.), a suggestive evidence of localization was seen. Interestingly, these lesions demonstrated high expression of the target Ag gp100 by immunocytochemistry. It should be emphasized, however, that this analysis could be strongly biased by the selection of lesions that had persisted after immunization.

Discussion

Systemic and local T cell-based immune responses against melanoma have been repeatedly identified in humans (1, 13–17). Rarely, however, are detectable immune responses sufficient to arrest tumor growth as apparent by the grim prognosis of advanced melanoma and the frequent identification of immune reactivity in

Table IV. *Calculated precursor frequencies[a] of Flu-specific CD8$^+$ cells on day 10 (peptide-specific/10e6 CD8$^+$ cells)*

		d1		Flu Stimulation[b]	
		Pre	Post	Pre	Post
Peptide alone[c]	P1	357	2,254	7,280	6,538
	P2	2,177	3,064	19,347	33,450
	P3	1,115	1,274	497	1,334
	P4	2,870	1[d]	43,229	72,034
	P5	4,732	5,703	17,348	18,676
	P6	6,820	7,901	173,757	131,309
	P7	415	2,389	30,492	32,211
Peptide + IL-12	P8	2,002	1,237	46,275	27,266
	P9	480	667	12,150	12,462
	P10	836	1,386	436	13,785
	P11	2,054	4,164	15,343	30,713
	P12	21,583	16,650	228,989	121,649
Peptide + IL-2	P13	1,992	1	8,189	8,288
	P14	663	1,026	63,546	21,965
	P15	1,519	4,941	43,607	78,601
	P16	2,228	1,473	34,709	2,864
	P17	14,496	6,309	75,455	99,607
	P18	421	1	1,612	2,285
	P19	1,690	1,656	38,732	53,972
	P20	908	2,687	20,011	53,972
	P21	838	260	65,978	115,133
	P22	1,838	1,341	7,071	7,373
	P23	1,374	465	261	4,267

[a] Frequency calculation: $[URQ/(LRQ + URQ) \times 10e6]_{epitope\text{-}specific} - [URQ/(LRQ + URQ) \times 10e6]_{background}$.
[b] Ten-day in vitro cultures stimulated with Flu.
[c] Treatment group.
[d] Calculated frequencies below or equal to zero were arbitrarily set to 1 to permit statistical analysis involving $\log_{10}$. No increase in g209/g209-2M-specific TCPF (data not shown).

patients whose disease is rapidly leading to their demise. Yet the ease with which MA-specific TIL can be generated from tumors (13, 14) illustrates the awareness of cancer cells by the immune system. Thus, a paradoxical coexistence of immune competent T cells and their respective targets appears to occur in vivo as judged from reagents characterized ex vivo.

The identification of MA epitopes recognized by T cells has led to the utilization of minimal peptide sequences for the in vivo induction or amplification of systemic, tumor-specific T cell responses (1, 2). Results from pilot studies testify for the high spec-

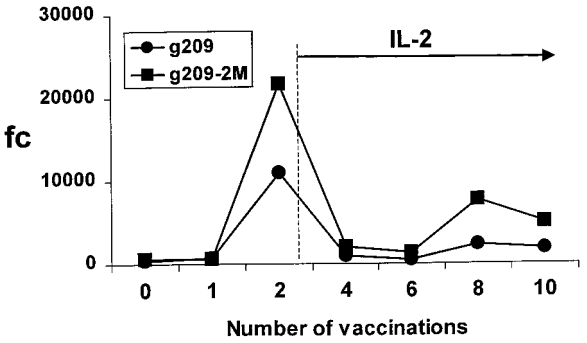

FIGURE 6. Evaluation of vaccine-specific T cell f_c in a patient throughout an entire vaccination treatment. The frequency of vaccine-specific T cell is shown at different time points. Number of vaccination refers to the vaccine that had been administered before the analysis of a given specimen. The arrow shows the period in which IL-2 was added to the patient's treatment. The PBMC samples were stained with g209–2M and g209 tHLA as well as Flu tHLA. Flu-specific TCPF did not change significantly throughout the vaccination treatment (data not shown).

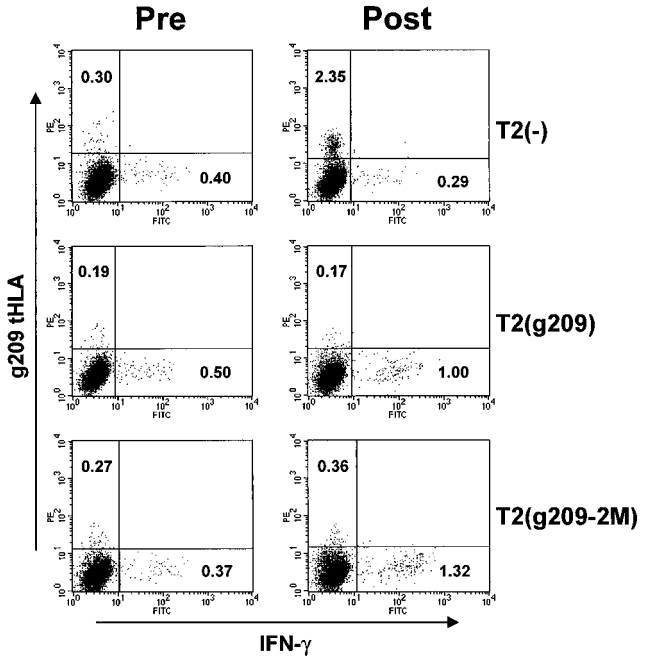

FIGURE 7. FACS analysis for intracellular IFN-γ expression in pre- and postvaccination PBMC from patient 4. The PBMC were obtained after two vaccinations before that patient had received concomitant systemic IL-2 therapy and were stimulated with unpulsed T2 cells (T2(−)), or pulsed with g209 (T2(g209)) or g2092M (T2(g209–2M)) peptides (1 μM). CD3$^+$/CD8$^+$ cells were gated for analysis and numbers indicate the percentage of cells in the quadrants over the total gated cells. g209 tHLA staining decreased upon stimulation due to down-regulation of TCR, as previously noted analyzing epitope-specific clonal populations (data not shown).

Table V. *Calculated precursor frequencies[a] of g209-specific CD8$^+$ cells in PBMC/tumor pairs (g209-specific/10e6 CD8$^+$ cells)*

Patient	gp100	HLA-A2	PBMC	Tumor
D.G.	NA[b]	NA	279	63
F.K.	Negative	Positive	2283	3492
L.R.	>75%, 2+	Positive	528	3134
J.R.	NA	NA	249	0[c]
D.W.	>75%, 3+	Positive	596	2448
A.C.	>75%, 2+	Positive	1545	659
S.B.	<25%, 2+	Positive	563	0

[a] Frequency calculation: $[URQ/(LRQ + URQ) \times 10e6]_{epitope\text{-}specific} - [URQ/(LRQ + URQ) \times 10e6]_{background}$.
[b] NA, not available.
[c] Calculated frequencies below or equal to zero were arbitrarily set to 0.

ificity of the induced T cell responses against HLA-matched tumor cells (2–4). These studies gave the impression that vaccines induce powerful immunizations comparable to those demonstrable against common pathogens such as the influenza virus to which individuals are repeatedly exposed throughout their lifetime. In most cases, this vaccine-induced T cell reactivity still does not lead to tumor regression.

Most studies thus far have measured T cell response to epitope-specific vaccination by comparative assessment of pre- and post-vaccination PBMC for tumor-specific T cell expansion in vitro in response to stimulation with relevant and irrelevant epitopes, as originally described by Vitiello et al. (18) in a viral system. These assays are excellent for qualitative assessment of T cell responses at two time points in an individual's life or for nonparametric comparison of treatment outcomes in different patient groups. However, they cannot provide quantitative insight about the strength of the observed response because of the arbitrary nature of the stimulus applied ex vivo and the addition of secondary proliferative stimuli, which most commonly consists of IL-2. Thus, as for TIL, it is likely that the immune responses judged after ex vivo expansion of postvaccination PBMC overestimate quantitatively the strength of the immune reaction within the organism.

Therefore, other methods of analysis have been fashioned to evaluate T cell responses directly without the need for ex vivo amplification. Among these, the ELISPOT assay has enjoyed notable popularity because of its simplicity, relative accuracy and sensitivity (19). Analysis of postvaccination PBMC from patients treated with g209–2M peptide in IFA could identify T cells specific for the altered epitope in four of six patients. In the four patients, the estimated frequencies ranged between 1/1000 and 1/2000 epitope-specific T cells (6). In no patient was it possible to identify T cells recognizing the natural epitope (g209) by the ELISPOT assay, whereas limiting dilution assays estimated precursor frequencies for g209-reactive T cells to range between 1/3000 and 1/6000 (2). Because of the dependency upon cytokine secretion/proliferation, these assays may underestimate the actual frequency of CTL precursors by not identifying T cells with a threshold for cytokine expression/proliferation above the stimulus applied in the assays (10). Moreover, naive T cells, less responsive to epitope-specific stimulation, might be missed by these functional assays (20). Recently, the use of HLA/epitope tetramers (7) has offered a tool to directly measure the frequency of CTL precursors presumably independently of their functional state. Measurements by this assay demonstrated CTL precursor frequencies considerably higher than those suggested by ELISPOT or limiting dilution assays (10).

T cell responses to epitope-specific vaccination have not been measured with this direct assay, yet. Several studies have measured the response of T cells to acute or chronic viral infections or during ongoing autoimmune episodes (8–10, 21–23). So far studies on tumor reactivity have been presented by few groups of investigators and have been primarily limited to the analysis of MART-1 and tyrosinase. In melanoma patients with *vitiligo*, MART-1-specific CTL have been identified at a frequency up to 0.67% of CD8$^+$ T cells (24). Furthermore, MART-1-specific T cells could be identified in melanoma infiltrated lymph nodes with a frequency ranging from 0.22 to 1.8% of CD8$^+$ T cells and correlated with MA expression (25). Characterization of circulating T cells demonstrated identifiable MA-specific T cells in approximately half of patients affected with metastatic melanoma (26). Functional characterization of MA-specific T cells from one patient lead to the generalization that some of the T cells identified with tHLA are unresponsive to Ag stimulation and, thus, unable to control tumor growth. This study is the first quantitative evaluation of the response to an HLA class I-restricted epitope vaccination. Epitope-specific vaccination yielded significant differences between the pre- and posttreatment CTL precursor frequencies. Vaccine-specific T cell frequency increased up to 2.5% of CD8$^+$ cells after vaccination, and the frequency of T cell recognizing the natural gp100 epitope (g209) was enhanced up to 1.4%. In a significant proportion of patients, frequency of g209 recognizing T cell after vaccination ranged between 0.2 to 0.9%. Thus, a significant conceptual finding of this study is the limited extent of vaccine-specific response. We were surprised at the relatively low numbers of CTL precursors after vaccination even in patients' samples that boasted an exceptional epitope-specific expansion in vitro. An inverse correlation has been reported between HIV-specific CTL frequency and viral RNA load in HIV infected individuals (8). Furthermore, TCPF as high as 2% of CD8$^+$ T cells have been reported in HIV-infected patients, who remained asymptomatic (22). 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.

Clonal deletion, exhaustion, or senescence (27–34) have been implicated in the induction of systemic, epitope-specific immune tolerance. However, because g209-specific T cell could be identified after vaccination in this study, deletion of tumor-reactive T cells may not be as significant in humans as suggested by preclinical models (27, 30, 33–35). Inadequate immune responses in patients with cancer and other chronic illnesses have been attributed to decreased TCR signaling capacity (36, 37) or circulating immune-suppressive cytokines (37). Finally, analysis of MA-specific T cells from one patient has lead to the generalization that tumor-specific T cells may be anergic in vivo (26). However, in this study, T cells elicited by the vaccine could be readily stimulated with the cognate epitope to rapidly proliferate in culture. Furthermore, analysis of postvaccination PBMC identified a significant percentage of vaccine-specific T cells capable to secrete IFN-γ in response to vaccine-specific stimulation. Taken together, these data suggest that the extent rather than the quality of the response might be the more significant limitation of the vaccination protocol analyzed in this study.

Although differences in TCPF were detectable between pre- and postvaccination PBMC selected from patients that had been treated with peptide alone, no significant differences could be detected in patients who had received peptide plus systemic IL-2 therapy and had shown no enhanced reactivity by in vitro sensitization assays (2). The association of undetectable vaccine-specific T cells with the enhanced frequency of clinical responses after systemic administration of IL-2 remains mysterious. One possibility is that the responsiveness of T cell to in vitro restimulation might be reduced in patients who received IL-2. This study demonstrates instead that

the number rather than susceptibility to in vitro expansion is decreased in these patients. It has been suggested that tumors will induce tolerance by presenting epitope-specific stimulation (signal one) without costimulation (signal two) to wandering MA-recognizing T cells (38). By increasing vascular permeability, IL-2 might facilitate encounters between T cells and cancer cells that lead to reduced tumor burden and T cell number at the same time. Study of seven simultaneously obtained PBMC and tumor samples demonstrated a slightly increased frequency of vaccine-induced T cells at tumor site in only two of the pairs studied in correlation with expression of gp100. It is possible that insufficient localization and/or poor survival of vaccine-induced T cells at tumor site may be the reason why the lesions analyzed in this study did not regress in response to therapy.

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