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Immunization of Patients with Metastatic Melanoma Using Both Class I- and Class II-Restricted Peptides from Melanoma-Associated Antigens

Giao Q. Phan, Christopher E. Touloukian, James C. Yang, Nicholas P. Restifo, Richard M. Sherry, Patrick Hwu, Suzanne L. Topalian, Douglas J. Schwartzentruber, Claudia A. Seipp, Linda J. Freezer, Kathleen E. Morton, Sharon A. Mavroukakis, Donald E. White, and Steven A. Rosenberg

Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD

Summary:

Cancer vaccines targeting CD8⁺ T cells have been successful in eliciting immunologic responses but disappointing in inducing clinical responses. Strong evidence supports the importance of CD4⁺ T cells in “helping” cytotoxic CD8⁺ cells in antitumor immunity. We report here on two consecutive clinical trials evaluating the impact of immunization with both human leukocyte antigen class I- and class II-restricted peptides from the gp100 melanoma antigen. In Protocol 1, 22 patients with metastatic melanoma were immunized with two modified class I A * 0201-restricted peptides, gp100:209-217(210M) and MART-1:26-35(27L). In Protocol 2, 19 patients received the same class I-restricted peptides in combination with a class II DRB1 * 0401-restricted peptide, gp100:44-59. As assessed by in vitro sensitization assays using peripheral blood mononuclear cells (PBMC) against the native gp100:209-217 peptide, 95% of patients in Protocol 1 were successfully immunized after two vaccinations in contrast to 50% of patients in Protocol 2 ($P_2 < 0.005$). Furthermore, the degree of sensitization was significantly lower in patients in Protocol 2 ($P = 0.01$). Clinically, one patient in Protocol 2 had an objective response, and none did in Protocol 1. Thus, the addition of the class II-restricted peptide gp100:44-59 did not improve clinical response but might have diminished the immunologic response of circulating PBMC to the class I-restricted peptide gp100:209-217. The reasons for this decreased immune reactivity are unclear but may involve increased CD4⁺CD25⁺ regulatory T-cell activity, increased apoptosis of activated CD8⁺ T cells, or the trafficking of sensitized CD8⁺ reactive cells out of the peripheral blood. Moreover, the sequential, nonrandomized nature of patient enrollment for the two trials may account for the differences in immunologic response.

Keywords

gp100; MART-1; CD4⁺; T cell; CD8⁺ T cell; CD4⁺CD25⁺ T cell

The 5-year survival of patients with metastatic melanoma is less than 2% in most published series.¹ Treatment options for these patients are limited. Chemotherapy can induce transient responses but is rarely, if ever, curative. Immunotherapy with high-dose intravenous interleukin-2 (IV IL-2) has been associated with durable, and probably curative, responses,²⁻⁴ but the overall response rate is only 16%.⁵ The identification of tumor-associated antigens

and the ability to manufacture pharmaceutical-grade peptides have led to vaccination trials evaluating the extent to which immunotherapy can be used for the treatment of patients with metastatic melanoma. Many of these vaccines have employed human leukocyte antigen (HLA) class I-restricted peptides, and although they can generate high levels of CD8⁺ T cells capable of recognizing melanoma cells, clinical responses have been unpredictable and remain an elusive goal.⁶

In a pilot study⁶ in which patients with metastatic melanoma were vaccinated with either the native class I-restricted gp100:209-217 peptide or a peptide with a methionine substitution for threonine at the second position, gp100:209-217(210M) (IMDQVPFSV), the modified peptide was superior to the native peptide in eliciting immune reactivity (91% versus 25% of patients, $P = 0.006$). A modified epitope for the MART-1 peptide in which lysine substitutes for the native alanine, MART-1:26-35(27L) (ELAGIGILTV), has also been noted in preliminary in vitro studies⁷ to be more immunologic than the native MART-1:27-35 peptide.

A potential explanation for the paucity of in vivo clinical responses to these class I-restricted cancer peptide vaccinations is lack of appropriate “help” from CD4⁺ T lymphocytes. CD4⁺ T cells can regulate the growth, differentiation, and function of immunologic effector cells and thus may play a significant role in antitumor immunity^{8,9} by elaborating cytokines and by inducing expression of adhesion and costimulatory molecules on the surface membrane of antigen-presenting cells.¹⁰⁻¹³ Recognition of the importance of CD4 “help” has stimulated attempts to immunize cancer patients with class II-restricted epitopes derived from tumor antigens.

The class II allele HLA-DRB1 * 0401 is present in approximately 15% of patients with metastatic melanoma.¹⁴ We have identified a DRB1 * 0401-restricted human gp100 epitope, gp100:44-59 (WNRQLYPEW-TEAQRDL), using mice transgenic for chimeric humanmurine DR4-IE.¹⁵ CD4⁺ T cells from these transgenic mice vaccinated with gp100:44-59 could recognize human tumors expressing gp100 and DRB1 * 0401 but not tumors lacking expression of either molecule. Human CD4⁺ T cells sensitized with gp100:44-59 in vitro specifically recognized DRB1 * 0401-expressing Epstein-Barr virus-transformed B cells pulsed with this peptide.

We thus hypothesized that simultaneous immunization of melanoma patients with both class I- and class II-restricted melanoma peptides would generate both CD8⁺ and CD4⁺ T-cell activation and thus lead to improved clinical responses. In this publication, we report on the clinical and immunologic responses of two consecutive phase 2 trials: In the first protocol (Protocol 1), 22 patients with metastatic melanoma were vaccinated with two modified class I-restricted melanoma peptides, gp100:209-217(210M) and MART-1:26-35(27L). In the subsequent protocol (Protocol 2), 19 patients with metastatic melanoma were immunized with the same modified class I-restricted peptides, gp100:209-217(210M) and MART-1:26-35(27L), in conjunction with a class II-restricted peptide, gp100:44-59. Unexpectedly, as evaluated in peripheral blood lymphocytes, the addition of the class II peptide appeared to decrease immunity against the class I peptides.

PATIENTS AND METHODS

Patients

For both protocols, HLA-A * 0201⁺ patients with evaluable metastatic melanoma, no previous exogenous exposure to gp100 or MART-1, and an expected survival of greater than 3 months were eligible. All patients in Protocol 2 were also HLA-DRB1 * 0401⁺. Exclusion criteria were serum creatinine greater than 2.0 mg/dL, total bilirubin greater than 2.0 mg/dL, alanine aminotransferase and aspartate aminotransferase greater than three times the upper limit of

normal, white blood cell count less than 3000/mm³, platelet count less than 90,000/mm³, current pregnant or lactating status, Eastern Cooperative Oncology Group performance status greater than 2, the presence of any active systemic infection, symptomatic cardiac disease, autoimmune or immunodeficiency disease, and positivity for hepatitis BsAg or human immunodeficiency virus antibody. At least 3 weeks were required between enrollment into this protocol and any previous systemic therapy.

All patients were enrolled in these sequential trials between April 1999 and August 2000, and all signed an informed consent prior to protocol enrollment. Both protocols were approved by the Institutional Review Board of the National Cancer Institute. All patients were treated in the Surgery Branch at the Warren G. Magnuson Clinical Center of the National Institutes of Health in Bethesda, Maryland.

Vaccine Therapy

In Protocol 1, patients received 1 mg of gp100:209-217(210M) peptide emulsified in incomplete Freund adjuvant (IFA) injected subcutaneously in one extremity and 1 mg of MART-1:26-35(27L) peptide emulsified in IFA in another extremity every 3 weeks.

In Protocol 2, patients received the same peptides in IFA that were administered in the same fashion every 3 weeks as in Protocol 1, but each emulsion also contained 5 mg of the class II-restricted peptide gp100:44-59. All peptides were prepared under Good Manufacturing Practice by Multiple Peptide Systems (San Diego, CA).

All patients underwent apheresis before treatment and 3 weeks after every two vaccinations to obtain peripheral blood lymphocytes for in vitro immunologic monitoring. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (ICN, Aurora, OH) separation and were cryopreserved at 10⁸ cells/vial in heat-inactivated human AB serum with 10% dimethyl sulfoxide and stored at -180°C until further use.

Each course of treatment consisted of four cycles. Patients who completed one course of treatment and experienced either a minor, mixed, partial or complete response (as defined below) or stable disease received a second course. Patients who had progressive disease after receiving vaccines alone had the option of receiving IL-2 therapy.

IL-2 Therapy

Patients who opted for IL-2 therapy after disease progression with vaccines alone, and who had no contraindications to IL-2 administration, were given high-dose IV IL-2. For each cycle of treatment, IL-2 was started the day after vaccination and was administered as described in previous publications.^{5,16} Briefly, 720,000 IU/kg of recombinant IL-2 (provided by Cetus Oncology Division, Chiron Corp., Emeryville, CA) was reconstituted from lyophilized powder in 5% human serum albumin and given as a 15-minute IV infusion for each dose. IL-2 was administered every 8 hours as tolerated for up to a maximum of 12 doses or until the development of a grade III or IV toxicity not easily reversed by supportive therapy, any evidence of neurologic toxicity, or patient refusal.

Clinical Response Evaluation

All patients underwent magnetic resonance imaging of the brain and computed axial tomography of the chest, abdomen, and pelvis within 4 weeks before starting treatment and subsequently after every two cycles of therapy. Radionuclide bone scans were also used for pretreatment staging: If they were positive for bony metastases, they were repeated at each evaluation point; if they were negative, they were not repeated again unless suggested by

clinical symptoms. Photographs, plain radiographs, or other radiologic modalities were also used as needed to evaluate disease sites.

For every patient, the product of the maximum perpendicular diameters of all tumors before and after treatment was calculated. A partial response was defined as the reduction of $\geq 50\%$ (but $< 100\%$) in the sum of the products of the maximum perpendicular diameters of all evaluable metastases lasting at least 1 month with no new or enlarging tumors; a minor response was the reduction of $\geq 25\%$ but $< 50\%$; a complete response was the disappearance of all evaluable tumor sites for at least 1 month. A mixed response was defined as regression of some tumors but growth in others. Patients not achieving these criteria were deemed as having progressive disease. For final analysis, patients not having either a partial or complete response were deemed nonresponders.

In Vitro Sensitization Assay

In vitro sensitization (IVS) assay was used to assess immunologic reactivity as previously described.⁶ Cryopreserved PBMC were thawed into complete media (CM: Iscove's Modified Dulbecco Media with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 50 $\mu\text{g/mL}$ gentamicin), plated at 1.5×10^6 cells/mL with 1 $\mu\text{mol/L}$ of native gp100:209-217 or MART-1:27-35 peptide and incubated overnight at 37°C and 5% CO₂. IL-2 was added on the following day for a final concentration of 300 IU/mL. Cells were split (and fresh media with 300 IU/mL IL-2 added) as needed and were harvested 11 to 13 days after initiation of the culture. Then 10^5 of these cells were co-incubated with 10^5 peptide-pulsed T2 cells in 200 μL CM per well in 96-well plates. The T2 cells were pulsed by incubating T2 cells at 2×10^6 cells/mL with 1 $\mu\text{mol/L}$ peptide for 2 to 3 hours at 37°C and 5% CO₂. Alternatively, 10^5 melanoma cells were also used as stimulators. After 18 to 24 hours of co-incubation, interferon- γ (IFN- γ) release in the supernatant was measured using standard enzyme-linked immunosorbent assays (ELISA) (Pierce-Endogen, Rockford, IL).

The stimulation index (SI) was calculated using the ratio of IFN- γ released by PBMC due to co-incubation with T2 cells pulsed with the stimulating peptide compared with an irrelevant control gp100:280-288(288V) peptide. IVS assays were performed on PBMC pretreatment, post-2-vaccinations and post-4-vaccinations; each assay was repeated at least once. A positive assay is defined as IFN- $\gamma \geq 100$ pg/mL, ≥ 2 times greater when compared with a control peptide (ie, when $SI \geq 2$) and ≥ 2 times greater than preimmunization samples.

Enzyme-Linked Immunosorbent Spot Assay

To quantify the frequency of cells reactive to class I-restricted peptides, cryopreserved PBMC were thawed in an enzyme-linked immunosorbent spot (ELISPOT) assay media (EAM: RPMI 1640 with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 25 mmol/L HEPES, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin) at 2×10^6 cells/mL and rested overnight at 37°C and 5% CO₂. MultiScreen-HA plates (Millipore, Bedford, MA) were also incubated overnight at room temperature with 100 μL of anti-human IFN- γ antibody (BioSource, Camarillo, CA) at 10 $\mu\text{g/mL}$. The plates were washed and blocked with EAM the following day for 1 hour to prevent nonspecific binding. Irradiated C1R-A2 cells (at 10^6 cells/mL) were pulsed with either 5 $\mu\text{mol/L}$ influenza peptide:58-66 or 1 $\mu\text{mol/L}$ melanoma peptide for 2 to 3 hours at 37°C and 5% CO₂; native gp100:209-217 or MART-1:27-35 peptide was used to assess for immunization status while irrelevant gp100:280-288(288V) was used as control. Peptide-pulsed C1R-A2 cells (10^5) were subsequently co-incubated with 10^5 PBMC in 200 μL EAM in each preblocked IFN- γ antibody-coated well. After 24 hours of co-incubation at 37°C and 5% CO₂, the cells were lysed by washing the wells with phosphate buffered saline (PBS) with 0.05% Tween-20. Biotinylated anti-IFN- γ antibody, 100 μL (BD Pharmingen, San

Diego, CA), diluted to 2 µg/mL in PBS with 1% bovine serum albumin and 0.05% Tween-20, was added to each well and incubated at 4°C overnight. The plates were washed and developed the following day with avidinalkaline phosphatase (Invitrogen, Carlsbad, CA) and then stained with BCIP/NBT Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Dots per well were scanned and computed in a “blinded” manner using an ImmunoSpot reader (Cellular Technology Ltd, Cleveland, OH).

The number of ELISPOTs per experiment were averaged over quadruplicate wells and were corrected by subtracting the background ELISPOTs due to PBMC coincubation with unpulsed C1R-A2 cells. The corrected number of ELISPOTs (N: the number of peptide-reactive CD8⁺ T cells) was then used to calculate the frequency of T-cell precursors by dividing into 10⁵ (PBMC used per well): one precursor per (10⁵/N).

To measure class II-reactive T-cell precursors, the ELISPOT assay was performed as above except that 50 µmol/L gp100:44-59 peptide was used directly to stimulate 10⁵ PBMC in 200 µL EAM per well. A T-cell clone with known reactivity against the gp100:44-59 vaccine was used as a positive control.

RESULTS

Patients and Clinical Response

Patient characteristics are shown in Table 1 and were similar in both protocols, although more patients with a prior history of chemotherapy were enrolled in Protocol 2. No patient in Protocol 1 had an objective response to vaccine therapy alone (Table 2). One patient in Protocol 2 with a 6-year history of multiple resections of metastases to the brain, lymph nodes, and subcutaneous tissue entered the protocol with a 1-cm biopsy-proven inguinal node metastasis (as his only measurable disease) that disappeared gradually over two courses of vaccination, associated with the development of vitiligo. He remained without disease for a total of 18 months until he developed an adrenal mass and recurrence of the inguinal node metastasis.

Eleven nonresponding patients in Protocol 1 subsequently received IL-2 therapy, and two had objective clinical responses (18%), comparable with our previous experience with IL-2 alone. Of the 19 nonresponders to vaccines alone in Protocol 2, 11 were subsequently treated with IL-2, and none responded.

Immunologic Response

Of 19 patients in Protocol 1 with PBMC available for testing using the IVS assay, 18 patients (95%) developed new anti-gp100:209-217 reactivity (Fig. 1) after two vaccinations while one patient already had pretreatment reactivity. Eight (44%) of 18 patients had pretreatment reactivity against the MART-1:27-35 peptide, and little change was seen after two or four vaccinations (Fig. 2).

Of 16 patients in Protocol 2 with PBMC available for IVS assays, eight patients (50%) developed new anti-gp100:209-217 reactivity (data not shown). In 10 patients who received four vaccinations with both class I- and II-restricted peptides, seven patients (70%) were reactive against gp100:209-217. As in Protocol 1, little new anti-MART-1:27-35 reactivity was generated as a consequence of vaccination in Protocol 2.

The decreased immunization rate against gp100:209-217 found in patients receiving both class I- and II-restricted peptides in Protocol 2 was surprising. To examine this phenomenon further, we directly compared the 16 patients in Protocol 2 with the 19 patients on Protocol 1 with PBMC available. To ensure that any differences in the IVS assay results were not due to reagent differences, the assays on patients in both protocols were repeated and performed at the same

time using cryopreserved samples. As seen in Figure 3, the SI of patients receiving both class I- and class II-restricted peptides was significantly decreased compared with the SI of those receiving only class I-restricted peptides ($P = 0.01$ by Mann-Whitney U test).

The level of immunization in both protocols was at the limit of detection of the ELISPOT assay, and the majority of patients generated fewer than 10 ELISPOTs per 10^5 cells. As seen in Table 3, using PBMC obtained after four vaccinations, a slight trend toward decreased frequency of gp100:209-210-reactive CD8⁺ T-cell precursors was noted in the group receiving both class I- and II-restricted peptides ($P = 0.16$ by Mann-Whitney U test); however, the number of ELISPOTs per 10^5 PBMC was too small to draw reliable conclusions. Results from the ELISPOT assay evaluating reactivity against the MART-1:27-35 peptide were also below the limit of detection (data not shown).

To assess whether the decreased reactivity toward gp100:209-217 seen in patients receiving both the class I and II-restricted peptides was due to physical factors from having the two peptides mixed prior to vaccination, “mixing studies” were performed as follows: In one experiment (Table 4), a T-cell clone with known anti-gp100:209-217(210M) reactivity was incubated overnight with serial dilutions of either 1) 1 $\mu\text{mol/L}$ gp100:209-217(210M), 2) 1 $\mu\text{mol/L}$ gp100:209-217(210M) mixed with 5 $\mu\text{mol/L}$ gp100:44-59 or 3) 5 $\mu\text{mol/L}$ gp100:44-59. IFN- γ release measured using ELISA found no evidence of inhibition of gp100:209-217(210M)-reactive CD8⁺ T cells by in vitro coinubation with the mixture of gp100:44-59 and gp100:209-217(210M). In another “mixing” experiment, the IVS assay was performed on reactive PBMC (of several patients from Protocol 1) that were stimulated with either 1 $\mu\text{mol/L}$ gp100:209-217(210M) alone or 1 $\mu\text{mol/L}$ gp100:209-217(210M) mixed with 5 $\mu\text{mol/L}$ gp100:44-59. No differences were seen between PBMC stimulated with only gp100:209-217(210M) or with gp100:209-217(210M) mixed with gp100:44-59 (data not shown).

Both IVS and ELISPOT assays failed to demonstrate any reactivity against the gp100:44-59 peptide in either pretreatment or post-4-vaccinations PBMC (data not shown).

DISCUSSION

Immunization with HLA class I-restricted tumor peptides in cancer patients can result in the generation of circulating CD8⁺ cytotoxic T lymphocytes (CTL) with antitumor reactivity, although tumor regression in these patients is uncommon. CD8⁺ cells, although crucial, are only part of the complex ensemble of participants involved in mediating tumor regression. CD4⁺ T cells have been shown to be essential in providing “help” to CD8⁺ cells.⁸⁻¹³ Activation of CD4⁺ cells by recognition of the appropriate epitopes presented by the HLA class II molecules on antigen presenting cells (APC) causes the expression of CD40L, which engages CD40 on APC, in turn “conditioning” these APC to express costimulatory molecules (such as B7-1 and B7-2). These activated APC subsequently can further activate CD8⁺ T cells not only through the HLA class I-epitope-T-cell receptor pathway, but also through B7-CD28 costimulation. Furthermore, activated CD4⁺ helper cells also release cytokines (such as IL-2) important in CTL proliferation and differentiation and in recruitment of other effector cells, such as eosinophils and macrophages, which may be important in mediating antitumor reactivity.^{8,9}

In an effort to evaluate “help” from CD4⁺ cells, we vaccinated melanoma patients using either class I-restricted epitopes alone or both class I- and class II-restricted epitopes from melanoma-associated antigens. Analysis of PBMC from patients in the two sequential protocols presented here suggested that the successful immunization against a class I-restricted gp100 melanoma peptide, as assessed in circulating lymphocytes, was unexpectedly decreased by simultaneous immunization with a class II-restricted gp100 peptide. Only 50% of patients (8 of 16) receiving both class I- and II-restricted peptides showed evidence of new immunization against

gp100:209-217 after two vaccinations, in contrast to 95% of patients (18 of 19) receiving only class I-restricted peptides ($P_2 < 0.005$; Fisher's exact test). A pilot trial evaluating the same gp100:209-217(210M) peptide had shown previously that 91% of patients (10 of 11) receiving only this peptide were successfully immunized after two vaccinations.⁶ Although there were two outliers in the group immunized with both class I- and II-restricted epitopes who had high SI values (241 and 2112; Fig. 3) against gp100:209-217, these patients were among the clinical nonresponders. With the ELISPOT data, there was a hint that patients vaccinated with both class I- and II-restricted peptides had fewer class I-reactive CD8⁺ T-cell precursors, but overall values were too close to the limits of detection by this assay for accurate assessment. With the MART-1:26-35(27L) peptide, we were not successful in vaccinating patients on either protocol.

What are possible explanations for the seemingly subdued clinical and immunologic responses in patients immunized with both class I- and II-restricted epitopes? The sequential, nonrandomized conduct of the two trials could have resulted in an undetected referral and selection bias in the enrollment of specific patients to the protocols. In particular, since the patients in Protocol 2 were required to be HLA-DRB1 * 0401⁺, the decreased anti-gp100:209-217 reactivity seen in these patients might be due to intrinsically decreased responsiveness of people with this genotype. Perhaps the prior history of chemotherapy (which was seen in more patients on Protocol 2) negatively affected vaccine responsiveness, although all patients were required to be at least 3 weeks from any prior therapy and had normal cell counts and differential prior to protocol enrollment. The decrease in class I-restricted reactivity when the gp100:44-59 class II-restricted peptide was added does not appear to be due to direct physical interaction between the peptides (Table 4). Increasing evidence has shown the importance of CD4⁺CD25⁺ T cells with regulatory capabilities¹⁷⁻²¹ that are naturally suppressive and antigen-nonspecific in their effector functions.²² Recent studies have also shown that engagement of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on these CD4⁺CD25⁺ T cells by APC activates these cells,²³ leading to increased suppressive activities. Woo et al.²⁴ have shown that a high percentage of tumor-infiltrating lymphocytes from lung cancer specimens were CD4⁺CD25⁺ T cells; moreover, 80% of these CD4⁺CD25⁺ T cells expressed CTLA-4 and suppressed proliferation of autologous PBMC. It is possible that the unintended immunization and activation of these CD4⁺CD25⁺ regulatory T cells may have inhibited the development of class I-restricted reactivity. In pilot studies, no differences were seen in the levels of CD4⁺CD25⁺ T cells between pre- and posttreatment cryopreserved PBMC samples in our patients on Protocol 2 (data not shown); however, it is possible that the majority of these regulatory T cells are located at sites of inflammation, such as tumor sites, and not in the peripheral blood, thus limiting our ability to identify and enumerate them. It is also possible that immunization with the gp100:44-59 peptide affected CD8⁺ cell survival by triggering activation-induced cell death, mediated possibly through cytokines or Fas/FasL. On the other hand, we were not able to demonstrate, using IVS and ELISPOT assays, that vaccination with the class II-restricted gp100:44-59 elicited a CD4⁺ response against the gp100:44-59 peptide. Furthermore, the assessment of immune reactivity in patients receiving peptide immunization reported here was conducted using peripheral circulating lymphocytes, and thus these results may not reflect immune reactions at the tumor site. Further studies of lymphocytes infiltrating into tumors following immunization are required.

This study has not detected a beneficial impact of adding immunization with a class II-restricted peptide to simultaneous immunization with a class I-restricted peptide. Indeed, a negative impact may exist with the addition of the class II-restricted peptide. Randomized trials will be required to answer this question.

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FIGURE 1. Reactivity against gp100:209-217 using PBMC of patients receiving only class I-restricted peptides using IVS assays. IFN- γ release was measured after PBMC co-incubation with T2 pulsed with 1 μ mol/L of either gp100:209-217 or irrelevant gp100:280-288(288V). The horizontal line shows the median value.

FIGURE 2.

Reactivity against MART-1:27-35 using PBMC of patients receiving only class I-restricted peptides using IVS assays. IFN- γ release was measured after PBMC co-incubation with T2 pulsed with 1 μ mol/L of either MART-1:27-35 or irrelevant gp100:280-288(288V). The horizontal line shows the median value.

FIGURE 3.

Comparison of the reactivity against gp100:209-217 between patients receiving only class I-restricted peptides and those receiving both class I- and II-restricted peptides. IFN- γ release was measured after PBMC co-incubation with T2 pulsed with 1 $\mu\text{mol/L}$ of either gp100:209-217 or irrelevant gp100:280-288(288V). The horizontal line shows the median value; $P = 0.01$ by Mann-Whitney U test.

TABLE 1

Patient demographics

Patient trait	Protocol	
	1	2
Vaccine received	Class I only	Class I + II
Total no. of patients (%)	22 (100%)	19 (100%)
Gender		
Male	14 (64%)	15 (79%)
Female	8 (36%)	4 (21%)
Age (y)		
Average	52.1	50.6
Range	31-77	28-79
ECOG score		
0	21 (95%)	18 (95%)
1	1 (5%)	1 (5%)
prior therapy		
Surgery	22 (100%)	19 (100%)
Chemotherapy	5 (23%)	11 (58%)
Immunotherapy	15 (68%)	11 (58%)
Radiotherapy	4 (18%)	6 (32%)
Hormonal	2 (9%)	0 (0%)
Any 2 or more	17 (77%)	15 (79%)
Any 3 or more	8 (36%)	10 (53%)

Class I = gp100:209-217(210M) and MART = MART-1:26-35(27L); Class II = gp100:44-59; ECOG, Eastern Cooperative Oncology Group.

TABLE 2

Clinical response

Protocol	Total no. of patients	NR	PR	CR	PR + CR
1: Class I only	22	22	0	0	0
Moved to IL-2	11	9	2	0	2 (18%)
2: Class I + II	19	18	0	1	1 (5%)
Moved to IL-2	11	11	0	0	0

Class I = gp100:209-217(210M) and MART-1:26-35(27L); Class II = gp100:44-59; NR, no response; PR, partial response; CR, complete response.

TABLE 3CD8⁺ T cell precursors reactive against gp100:209-217

Patients	N = corrected ELISPOTs per 10 ⁵ PBMC	1 Precursor per (10 ⁵ /N)
Class I only	Median = 4.6 [*]	
1	4.8	20,833
2	4.1	24,390
3	0.3	333,333
4	0	—
5	5.1	19,608
6	4.4	22,727
7	2.9	34,483
8	12.9	7,752
9	8.3	12,048
10	4.3	23,256
Class I + II	Median = 1.5 [*]	
1	0	—
2	1.2	83,333
3	2.8	35,714
4	0.4	250,000
5	1.8	55,556
6	178.3	561
7	8.2	12,195
8	0.1	1,000,000
9	0.5	200,000
10	2.3	43,478

* $P = 0.16$ by Mann-Whitney U test. Class I = gp100:209-217(210M) and MART-1:26-35(27L); Class II = gp100:44-59; corrected = the background number of ELISPOTs due to PBMC co-incubation with unpulsed C1R-A2 cells were subtracted from the number of ELISPOTs due to co-incubation with gp100:209-217-pulsed C1R-A2.

TABLE 4
IFN- γ (pg/mL) released by anti-gp100:209-217(210M) T-cell clone with serial dilutions of gp100:209-217(210M) $\pm$ gp100:44-59

Dilution of peptides	gp100:209-217(210M) alone		gp100:209-217(210M) + gp100:44-59		gp100:44-59 alone	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
1	2289	2428	2624	2466	1	0
1:100	1532	2022	2182	1851	1	0
1:500	913	1123	1205	1132	5	1
1:2500	512	670	702	600	0	0
1:12,500	45	83	255	96	5	0
1:62,500	0	2	61	4	0	2
1:31,250	0	0	8	0	0	2

Dilution of “1” = 1 μ mol/L gp100:209-217(210M); 1 μ mol/L gp100:209-217(210M) mixed with 5 μ mol/L gp100:44-59; or 5 μ mol/L gp100:44-59. Rep, replicate.