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# Phase I/II study of GM-CSF DNA as an adjuvant for a multipeptide cancer vaccine in patients with advanced melanoma

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## Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances immune responses by inducing dendritic cell proliferation, maturation, and migration and B and T lymphocyte expansion and differentiation. The potency of DNA vaccines can be enhanced by the addition of DNA encoding cytokines, acting as molecular adjuvants. We conducted a phase I/II trial of human GM-CSF DNA in conjunction with a multipeptide vaccine (gp100 and tyrosinase) in stage III/IV melanoma patients. Nineteen human leukocyte antigen (HLA)-A*0201(+) patients were treated. Three dose levels were studied: 100, 400, and 800 mcg DNA/injection, administered subcutaneously (SQ) every month with 500 mcg of each peptide. In the dose-ranging study, 3 patients were treated at each dose level. The remaining patients were then treated at the highest dose. Most toxicities were grade 1 injection site reactions. Eight patients (42%) developed CD8+ T-cell responses, defined by a ≥3 SD increase in baseline reactivity to tyrosinase or gp100 peptide in tetramer or intracellular cytokine staining assays. There was no relationship between dose and T-cell response. Responding T cells had an effector memory cell phenotype. Polyfunctional T cells were also demonstrated. At a median of 31 months follow-up, median survival has not been reached. Human GM-CSF DNA was found to be a safe adjuvant.

## Introduction

Granulocyte-macrophage colony stimulating factor (GM-CSF) has become an attractive vaccine adjuvant because of its immune modulation effects and low toxicity profiles.[1] There is significant pre-clinical and clinical data demonstrating the adjuvant effects of GM-CSF in a variety of cancer vaccine approaches, including cellular vaccines, viral vaccines, peptide and proteins vaccines, and DNA vaccines.[2-11]



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These studies have been based on pre-clinical mouse studies that showed that vaccination with syngeneic mouse melanoma cells secreting GM-CSF stimulated more potent and long-lasting anti-tumor immunity than vaccines that produced other cytokines.[2] Similar results were observed in other tumor models, including lung, colon, renal cell, prostate, lymphoma and leukemia.[3-7] The ability of GM-CSF to act as a growth factor to stimulate and recruit dendritic cells (DC), thus augmenting the survival and density of antigen presenting cells (APC), may explain its adjuvant role.[12,13] After being stimulated by exogenous antigens, DCs migrate to regional lymph nodes, where they present antigen to T cells.

We are currently investigating the efficacy of DNA vaccines in patients with solid tumor and hematologic malignancies.[14,15] One of the advantages of DNA vaccines is the ability to combine them with cytokine genes as molecular adjuvants. The use of molecular adjuvants allows more efficient co-administration with DNA vaccines, the inclusion of cytokines for which there is no clinical grade recombinant protein, and gene modifications to increase potency such as fusion to immunoglobulin Fc domains.[16]

We have shown that administration of mouse GM-CSF DNA by particle bombardment into skin induces an inflammatory response that leads to a significant increase in DCs at the inoculation site and in draining lymph nodes.[8,17] GM-CSF DNA expression increases T-cell responses following peptide immunization and antibody responses following xenogeneic DNA immunization.[8] It also provides increased tumor protection in mice immunized with human tyrosinase-related protein-1 (TRP-1/gp75/TYRP1) or tyrosinase-related protein-2 (a DOPAchrome tautomerase, DCT, also known as TRP-2).[8,9] We also showed in studies in companion animals that human GM-CSF DNA enhanced the effects of a DNA vaccine targeting tyrosinase.[10] Based on these pre-clinical studies, we performed a phase I/II study of human GM-CSF DNA combined with a multiepitope peptide vaccine (tyrosinase and gp100) in patients with advanced melanoma.

## Results

### Clinical Trial Design

The endpoints of this study were safety and immunogenicity of human GM-CSF DNA given in combination with tyrosinase and gp100 peptides (500 mcg each). In the dose ranging part of the study, patients were treated in three different cohorts with escalating doses of 100, 400 and 800 mcg of GM-CSF DNA. Further dose escalation was not planned, since the goal of the study was not to define a maximum tolerated dose of GM-CSF DNA, but to identify a biologically active dose. DNA was administered subcutaneously with the Biojector2000® needle-free delivery system. Three patients were treated at each dose level. An additional patient was treated at the highest dose as two patients had consented simultaneously. In the second part of the trial, 10 additional patients were treated at the highest dose of GM-CSF DNA. One patient was removed from the study and replaced due to progression of disease prior to administration of the second vaccine. This patient was evaluable for toxicity. A total of 19 patients completed the study.

## Patient Demographics

The trial enrolled mainly stage III melanoma patients who were free of disease after surgery but at high risk for recurrence (Table 1). All patients with stage III disease either progressed on high-dose IFN, had a medical contraindication to high-dose IFN or refused IFN therapy after a complete discussion of the data from the relevant Eastern Cooperative Oncology Group (ECOG) trials with a physician investigator. One patient with stage III discontinued adjuvant interferon after 3 months due to poor tolerance. One patient had a large mucosal melanoma of the nasal cavity. Three patients had stage IV disease. One patient was classified as stage IV with no evidence of disease (NED), after receiving neo-adjuvant chemotherapy prior to resection of a metastatic jejunal lesion. Two additional patients with stage IV disease had low volume disease. One had a left upper lobe pulmonary nodule present at the time of enrollment that was subsequently found to be metastatic disease when it increased in size 7 months after study completion and was resected. A second patient had a similar evolution of a liver lesion. Patients were enrolled within 6 months of definitive surgery for resection of primary tumor, and/or regional lymph nodes or metastatic disease. There were 11 male and 8 female patients. The age range was 12-77 (median 60). All patients had Karnofsky performance status ≥ 80 with minimal or no laboratory abnormalities at study entry.

## Toxicity

No patient enrolled in this study developed a dose-limiting toxicity, defined as any event in the NCI Common Toxicity Criteria (CTC v2) of grade 3 or 2 allergic/immunologic toxicity. Side effects consisted primarily of grade I injection site reactions. Given that patients were injected with double-stranded DNA, we measured anti-nuclear anti-DNA antibodies (Hep-2 indirect fluorescence assay) during and after treatment. Similar to prior DNA vaccination trials,[14,18] we did not detect any persistent elevation of anti-DNA antibodies. In accordance with FDA guidelines for gene transfer studies, all patients are being followed for 15 years as surveillance for second malignancies, neurologic or autoimmune disease.

## Pharmacokinetics

After injection of GM-CSF DNA, we measured GM-CSF levels in the serum. Unlike our studies in mice,[17] in which we detected transient low levels of GM-CSF in the serum after injection of plasmid, no GM-CSF was detected in patients on the clinical trial.

## Evaluation of $CD8^+$ T-Cell Responses

Peripheral blood mononuclear cells (PBMCs) were collected and stored at -120° C at two time points before the first immunization (A, B), at 7 weeks (C), 11 weeks (D) and 17 weeks (E) following the first immunization. Since we did not observe any responses on freshly thawed PBMCs without prior in vitro stimulation in our study of tyrosinase DNA,[14] we used a more sensitive assay, in which PBMCs are incubated with peptide-pulsed K562 cells expressing HLA-A* 0201 for 10 days to expand previously activated CD8+ T cells,[19] prior to tetramer and intracellular cytokine staining (ICS) analysis. We defined a positive response as one in which: (1) the population of responding cells was >0.1% of total CD3+CD8+ cells, and (2) the post-vaccination specimen was ≥ 3 SDs above the pre-vaccination specimens.

Eight of 19 patients demonstrated a positive response to tyrosinase$_{369\text{-}377}$ or gp100$_{209\text{-}217}$ at one or more post-vaccination time points by either assay (Fig 1, Table 2). In the tetramer assay, the peak response ranged 1.7-90.2-fold greater than the respective pretreatment values, while the range in the ICS assay was a 1.3-7.1 fold increase. Positive responses were seen as early as week 7, two weeks prior to the last immunization and up to week 17, two months after the final immunization. Positive responses were divided among all three dose levels. We have previously validated the reproducibility of the assays,[19] and confirmed it in two of the patients in the present study.

## Phenotype of responding CD8+ T cells

We assessed the phenotype of responding CD8+ T cells (Figs 2 and 3, Table 2). The majority of specimens positive by tetramer assay were CD45R0$^{high}$ and CD62L$^{low}$ or CD62L$^{int}$, indicating an effector memory phenotype.[20] Expression of CCR7 was also low, consistent with the phenotype. One patient had a positive response by tetramer staining with a predominantly CD62L$^{high}$ population and co-expression of CCR7, consistent with a central memory phenotype. Two of four responses scored positive by ICS comprised T cells that were CD45R0$^{high}$ CD62L$^{low}$, indicative of an effector memory population. In two of these responders, we also detected expression of the effector molecule granzyme B in responding T cells (Fig 3). Surface expression of CD107a in these specimens, which indicates degranulation and lytic function, was however low in three of four responders.[21] CD107a expression was only detected in the patient analyzed in the polyfunctional assay (see below, Fig 4), in which both Brefeldin A and Monensin were used to detect intracellular cytokines. We have recently shown that combining the two agents increases detection of CD107a compared to using Brefeldin A alone (J. Yuan, unpublished observation). Expression of CD127 (CD127Rα) was generally low on tetramer positive or IFNγ-secreting cells, consistent with an effector phenotype.[22-24]

## Polyfunctional responses are observed in some patients

We were able to analyze polyfunctional responses[25,26] in patient 12, who had a response to gp100 in the ICS assay (Fig 4). We examined CD8+ T cell secretion or expression of IFNγ, MIP1β, TNF, CD107a and IL-2 (Fig 4A). Prior to immunization, the majority of CD8+ T cells only secreted a single cytokine (Fig 4B, time points A+B). In contrast, 7 weeks after the 1$^{st}$ immunization (time point C), a significant percent of CD8+ T cells were polyfunctional, expressing up to 3 or 4 markers, including CD107a. This time point also corresponded to the peak response to the gp100 vaccine. At the following time point, the percentage of polyfunctional CD8+ T cells decreased (time point D) and at the last time point (time point E), a majority of T cells had no detectable function, although we cannot exclude other cytokines being produced, since we only studied 5 markers.

## Clinical Observations

Although this study was not designed to evaluate clinical efficacy, we have continued to follow these patients for a median of 31 months since enrollment. Fourteen of 19 patients remain alive and we have not reached a median overall survival as of yet.

## Discussion

We have previously shown that GM-CSF DNA acts as a potent adjuvant for melanoma DNA vaccines in pre-clinical mouse models as well as in companion animal studies.[8-10] In a recent study in companion animals, we treated groups of dogs with melanoma on three different arms, mouse tyrosinase DNA alone, human GM-CSF DNA alone, or the combination of the two constructs. Dogs were treated on the same dose escalation of GM-CSF DNA used in the present study. Although the animals were treated sequentially rather than in a randomized fashion due to the later availability of the GM-CSF DNA construct, study entry criteria were similar in the three groups. A significantly improved overall survival was observed in the combination arm compared to the other two arms.

In the present study, we treated 19 patients with human GM-CSF DNA in combination with a multiepitope peptide vaccine (tyrosinase and gp100) and observed CD8+ T cell responses in eight patients either by tetramer or ICS assay. The majority of the responding CD8+ T cells had an effector memory phenotype, similar to observations in our recent study of tyrosinase DNA.[14] Furthermore, in one of the patients that responded in the ICS assay, we were able to study polyfunctional responses using polychromatic flow.[25,26] Polyfunctional T cells have been demonstrated in a number of infectious diseases pre-clinical models as well as in patients infected with HIV-1 or immunized using vaccinia constructs.[26-28] These studies indicate that polyfunctional T cell responses correlate with improved control of viral replication, suggesting that an effective vaccine should attempt to elicit these types of responses.[29] A recent study in mice showed polyfunctional responses following a DNA prime adenoviral vector boost immunization with a bicistronic vector expressing GM-CSF and OVA.[30] In the patient we studied, we demonstrated polyfunctional CD8+ T cells to the gp100 peptide, with almost 75% of the T cells demonstrating 3 or more cytokines or markers at time point C, which was the peak response determined by IFNγ secretion. Interestingly, only time point C would be considered a positive response to the vaccine when using 3 SD above the mean as a definition of a positive response. However, clear differences in the type of polyfunctional response were also noted at time point D compared to pre-vaccine time points, indicating that this assay may be biologically more informative than studying IFNγ alone as is routinely done in most vaccine studies. To our knowledge, this is the first demonstration of a polyfunctional CD8+ T cell response to a cancer vaccine in humans. Future studies in which this approach is used systematically will help determine whether it provides a better assessment of immune responses to cancer vaccines.

One of the primary aims of this study was to investigate the safety and feasibility of injection of GM-CSF DNA as a vaccine adjuvant. We found that SQ jet injection of all three doses of DNA was well tolerated, with transient grade 1 injection site reactions the only reproducibly reported side effect. No significant induction or exacerbation of anti-DNA antibodies was noted. These results are consistent with those of other reports of early clinical trials of GM-CSF DNA.[31-33] Timmerman et al.[31] performed a Phase I/II clinical trial of DNA vaccines encoding autologous idiotype linked to mouse immunoglobulin (MsIg) heavy- and light-chain constant regions chains in patients with follicular B-cell lymphoma. Patients received 3 series of vaccines, including DNA given intramuscularly, then intramuscularly and intradermally, and finally in combination with 500 mcg of GM-CSF

DNA. Addition of GM-CSF DNA appeared to boost the B and T cell responses to the mouse immunoglobulin in some of the patients. In another study, patients with melanoma were immunized with gp100 DNA alone or with GM-CSF DNA.[33] Although increased dendritic cell infiltration was seen at the injection site of patients who received GM-CSF DNA, no conclusions could be drawn on the effect of GM-CSF DNA on T cell responses, as they were only studied in a small subset of patients. Finally, in a study of a DNA vaccine against Plasmodium falciparum, addition of GM-CSF DNA (20, 100 or 500 mcg) had a negative effect on class I-restricted but not class II restricted responses.[32] Although injection of peptides alone without any adjuvant or carrier should not elicit a response, we cannot exclude that we would be able to detect some responses with the sensitive T cell assays we used in the present study. Nevertheless, we think the superior survival we observed in companion animals treated with GM-CSF DNA as an adjuvant and the detection of vaccine-specific T cell responses in the present study indicate that GM-CSF DNA provided an adjuvant effect.

The results of this study and those of others demonstrate that GM-CSF DNA is a safe and potentially effective adjuvant. In future studies we plan to combine human GM-CSF DNA (800 mcg/dose) with DNA vaccines targeting cancer antigens in patients with melanoma or other malignancies. We will use separate plasmid vectors for the GM-CSF DNA and the antigen, as this makes practical sense allowing the use of the molecular adjuvant with a number of different vaccines. Furthermore, recent studies have demonstrated that the use of fusion gene constructs where GM-CSF is linked to an antigen can lead to anti-GM-CSF auto-antibodies and decreased immune responses.[30,34]

## Materials and Methods

### Patients

Patients with AJCC stage IIb-IV melanoma, HLA-A*0201$^{+}$, and confirmed pathologic diagnosis were eligible to participate. Patients must have had any potentially curative surgery before being allowed entry. Patients with stages IIb-III disease must have either progressed on, been ineligible for or refused high dose interferon-alfa after a complete discussion of the results of the relevant ECOG trials.[35-37] Patients with stage IV disease had at most five anatomic sites of metastasis and no evidence of brain metastases. All patients signed an informed consent approved by the Memorial Sloan-Kettering Cancer Center (MSKCC) IRB. The protocol was approved by the NIH Recombinant DNA Advisory Committee and the Food and Drug Administration.

### Plasmid design

Human GM-CSF cDNA was cloned at MSKCC and inserted into the pING vector,[38] a standard eukaryotic expression plasmid used extensively in preclinical models and in clinical trials by our group,[10,14,39] which conforms to criteria specified in the FDA points to consider for DNA vaccines. Clinical grade material was manufactured by Althea Technologies, Inc (San Diego, CA). The potency of the plasmid was confirmed by detection of human GM-CSF protein by ELISA in the skin of mice injected with human GM-CSF DNA using the gene gun (data not shown).

### Peptide

The vaccine consisted of tyrosinase 368–376(370D) peptide (YMDGTMSQV) and gp100 209–217(210M) peptide (IMDQVPFSV), which were prepared and administered separately. Both peptides are HLA-A2.1 restricted. The gp100 209–217(210 M) peptide was modified at the second position to increase the affinity of binding to HLA-A2.1 and to enhance induction of T cells against the native peptide expressed by melanoma.[40] Both peptides were provided by Cancer Treatment Evaluation Program/NCI under an Investigational New Drug application held by the NCI.

### Immunization

Patients were injected with GM-CSF DNA subcutaneously monthly for a total of three immunizations using the Biojector2000 needle-free delivery system with the #3 syringe (Tualatin, OR). On day 5 or 6 following GM-CSF DNA administration, 500 mcg each of the tyrosinase and gp100 peptides were injected at the same site using the same method of injection. Injection sites were rotated for each immunization and no injection was given to a site where draining lymph nodes had been removed.

### Pharmacokinetics

Following GM-CSF DNA administration, serum was collected at specific time points (6 hours, 24 hours, 48 hours and 120 hours). GM-CSF concentrations in serum were determined using a commercial ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

### T-cell stimulation in vitro

Thawed PBMCs were stimulated in triplicate with K562-A*0201 cells pulsed with peptides (tyrosinase$_{369\text{-}377}$ YMDGTMSQV, gp100$_{209\text{-}217}$ ITDQVPFSV, EBV-BMLF1$_{280–288}$ GLCTLVAML)[40-42] at 10 μg/ml as previously described.[19]

### Immune Response Assays

The tetramer and ICS assays were performed as previously described with some modifications.[14,19] The following tetramers and fluorochrome-labeled antibodies were used: HLA-A*0201-PE labeled tetramers loaded with EBV$_{369\text{-}377}$, gp100$_{209\text{-}217}$, and tyrosinase$_{369\text{-}377}$ peptides (Tetramer Core, Lausanne Branch, Ludwig Institute of Cancer Research), CD45RO, CD3 and anti-IFNγ (BD Biosciences, San Jose, CA), CD127, CD45RO, MIP-1β, CD107a, TNFα, CD28, CD27, CD8 and Granzyme B (BD, Pharmingen, San Jose, CA), CD3 (Caltag Laboratories, Burlingame, CA), CD122 (Sanquin, NL), CD127, CD4, CD45RA and CD8 (Beckman Coulter Inc., Fullerton, CA.), CD62L (eBioscience San Diego, CA) CCR7 (R&D Systems, Minneapolis, MN). Brefeldin A (1 mcg/ml, BD Biosciences) was added two hours after peptide stimulation in the ICS assay. Cells were analyzed by flow cytometry using a CYAN flow cytometer with Summit software (DakoCytomation California Inc., Carpinteria, CA). The percentage of positive cells was determined by gating on the population of cells that were viable (forward scatter$^{low}$ and side scatter$^{low}$), CD3$^{high}$ and CD8$^{high}$. 4',6-diamidino-2- phenylindole (DAPI, Invitrogen, Carlsbad, CA) was used to gate out dead cells for tetramer staining.

For the polyfunctional assay, thawed PBMCs were stimulated at a 1:1 ratio with irradiated autologous PBMCs pulsed with the tyrosinase and gp100 peptides (10 mcg/ml). Cells were harvested at day 10 and analyzed by polyfunctional intracellular cytokine staining.[25,26] Brefeldin A (1 mcg/ml) and Monensin (5 mcg/ml, Sigma, St. Louis, MO) were added after peptide stimulation. Unstimulated samples were used as negative controls to set up single function gates. The data analysis program Simplified Presentation of Incredibly Complex Evaluations (version 4.1.6; kindly provided by M.E. Roederer, NIH, Bethesda, MD) was used to analyze and generate graphical representations of T cell responses detected by polychromatic flow cytometry. All values used for analyzing proportionate representation of responses were used after the background was subtracted.

### Assay validation

For the tetramer and ICS assays, standard operating procedures were established in the Ludwig Center for Cancer Immunotherapy Immunologic Monitoring Facility for use in clinical trial monitoring. Freshly-thawed HLA-A*0201 tyrosinase$_{369\text{-}377}$, gp100$_{209\text{-}217}$ and EBV-BMLF1$_{280–288}$ tetramer positive T-cell lines were used for each tetramer staining as a positive control. We have subjected these methods to validation criteria outlined by the FDA for cell based assays (http://www.fda.gov/CBER/summaries/120600bio10.htm).

### Statistical methods

In order to determine positive T-cell responses, we calculated the SD of the pre-vaccination replicate values. Patients were considered to have positive T-cell responses if at any post-vaccination time point there was an increase of ≥ 3 SDs from the baseline mean and the response was at least 0.1%. Overall survival was estimated using the Kaplan-Meier method.

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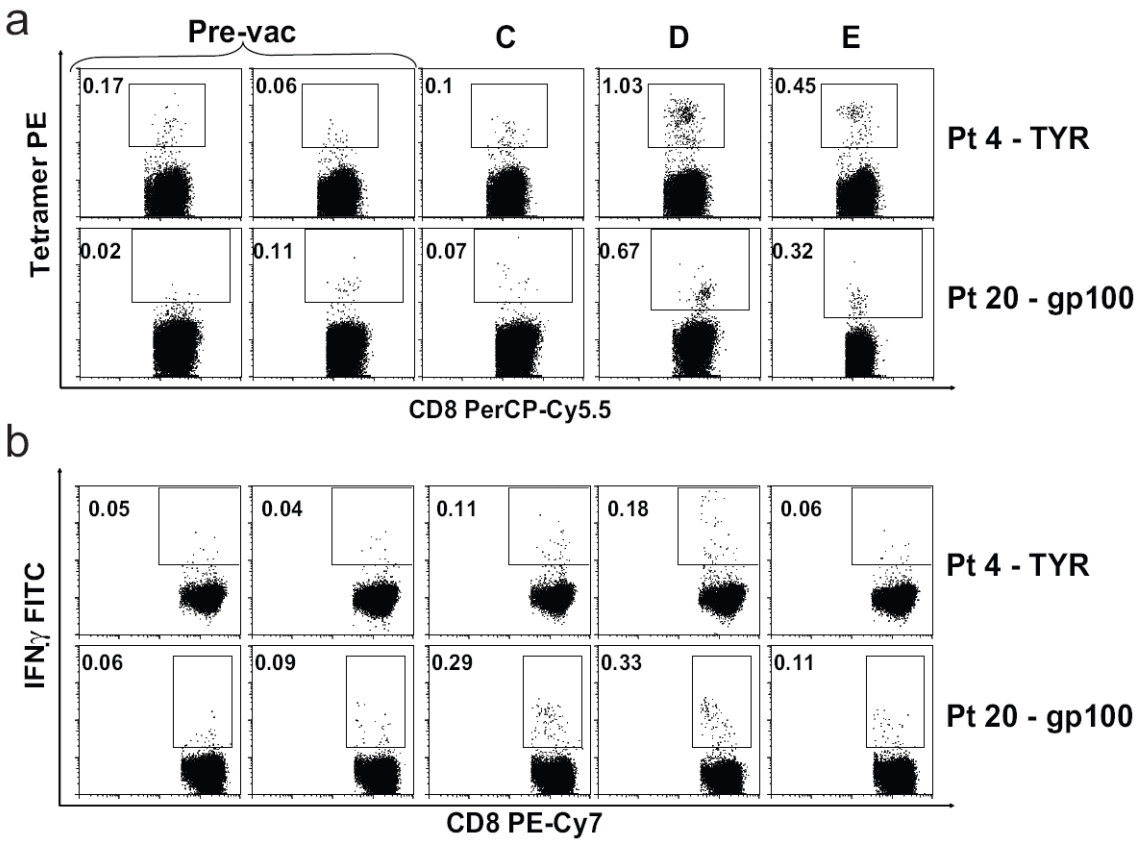


**Figure 1. Immunization with GM-CSF DNA followed by tyrosinase and gp100 peptides induced peptide-specific CD8+ T cells assessed by tetramer binding and IFNγ production**

PBMCs were collected pre-vaccination, and at several time points during or after vaccination (C = week 7, D = week 11, and E = week 17) and analyzed by tetramer and ICS IFNγ assays. (**a**) Two patients with positive tetramer assays – patient # 4, Tyrosinase time points D and E; patient # 20 gp100 time point D, and (**b**) the corresponding ICS IFNγ assays are shown. Only patient # 20 – time points C and D – are positive. Pre-vac, pre-vaccination.

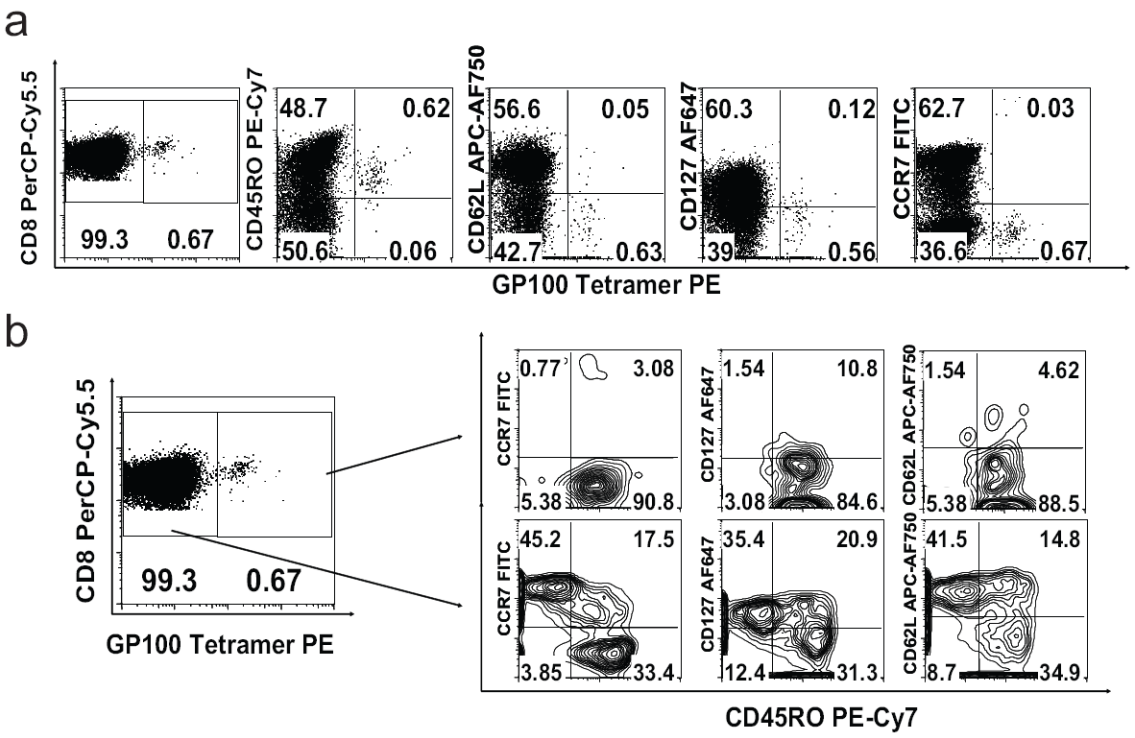


**Figure 2. GP100 tetramer-reactive CD8+ cells in the responder population have an effector memory phenotype**

PBMCs were analyzed by tetramer assay after in vitro culture using $gp100_{209\text{-}217}$ ITDQVPFSV peptide. (**a**) Dot plots from patient # 20 at time point D are shown. (**b**) Contour plots from patient # 20 at time point D show CD3+CD8+ T cells analyzed for tetramer reactivity. Upper plots gated CD3+CD8+tetramer+ T cells; lower row plots gated on CD3+CD8+tetramer-T cells.

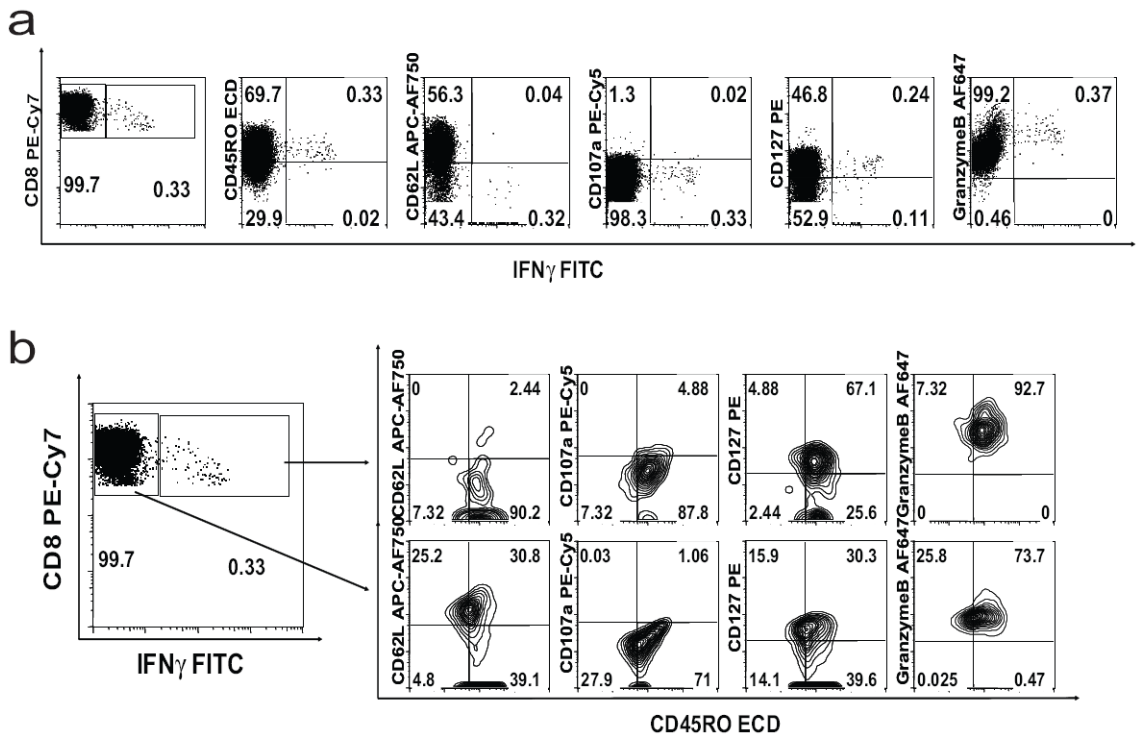


**Figure 3. Phenotypic characterization of cells secreting IFNγ in ICS assays**

ICS assays were performed with CD45RO, CD62L, CD127, CD107a, and granzyme B. (**a**) Representative dot plots from patient # 20 at time point D are shown. (**b**) Contour plots from patient # 20 at time point D show the gated CD3+CD8+IFN-γ+ T cells. Upper plots gated on CD3+CD8+IFNγ+ T cells; lower row plots gated on CD3+CD8+IFNγ- T cells.

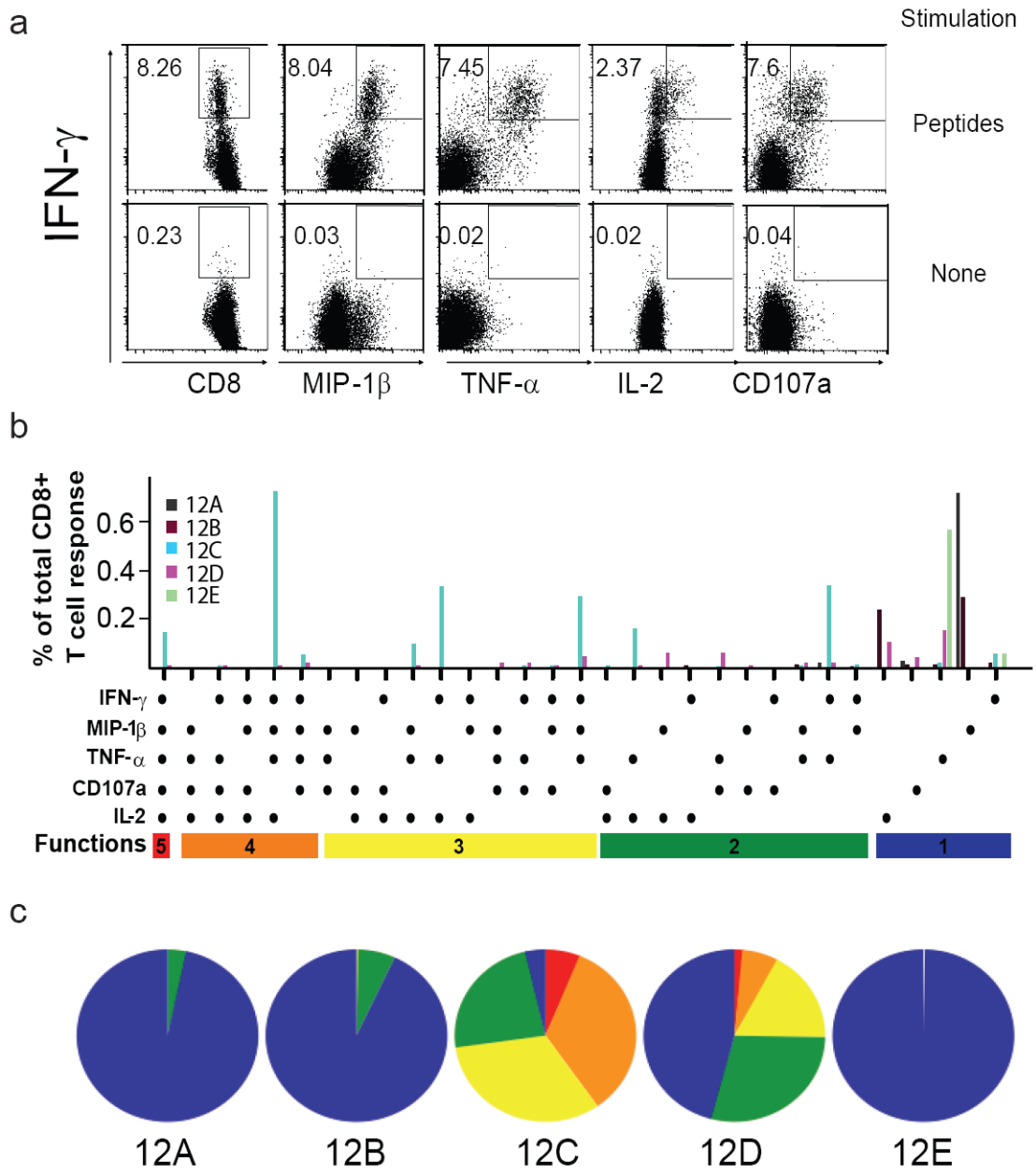


**Figure 4. Polyfunctional antigen specific CD8+ T cells are induced following immunization**

(**a**) Representative dot-plots from sample patient #12 at time point C. Single function gates were set based on negative control (unstimulated sample, bottom row) and were placed consistently across samples. (**b**) Responses in patient #12 are shown at all 5 time points. Every possible combination of responses is shown on the x axis. Responses are grouped and color coded according to the number of functions. Bars indicate the percentage of the total response contributed by CD8+ T cells with a given functional response. (**c**) Each pie represents a time point in patient #12 and each slice of the pie represents the fraction of the total response that consists of CD8+ T cells positive for a given number functions.

**Table 1**

Patient Demographics and T-cell Responses

| Patient | Stage | Prior Therapy | Dose level (mcg) | T-cell Response | PFS (months) | OS (months) |
|---|---|---|---|---|---|---|
| GM 01 | III | None | 100 | Yes | >49 | >49 |
| GM 02 | IV | None | 100 | No | 9 | >46 |
| GM 03 | III | None | 100 | Yes | >44 | >44 |
| GM 04 | III | None | 400 | Yes | 39 | >41 |
| GM 05 | III | None | 400 | No | >40 | >40 |
| GM 06 | III | None | 400 | No | 14 | 29 |
| GM 08 | IV | None | 800 | Yes | 3 | 11 |
| GM 09 | Mucosal melanoma 2.6 cm | None | 800 | No | >35 | >35 |
| GM 10 | III | IFN | 800 | No | 9 | 17 |
| GM 11 | IIb | None | 800 | No | >32 | >32 |
| GM 12 | III | IFN | 800 | Yes | >28 | >28 |
| GM 13 | III | None | 800 | No | 3 | 9 |
| GM 14 | III | None | 800 | Yes | 3 | 8 |
| GM 15 | III | None | 800 | No | 4 | >30 |
| GM 16 | III | None | 800 | N/A | 1 | 8 |
| GM 17 | IIT4 vs. III in transit | None | 800 | No | 4 | >28 |
| GM 18 | III | None | 800 | No | >28 | >28 |
| GM 19 | III | None | 800 | No | 1 | >29 |
| GM 20 | III | None | 800 | Yes | >28 | >28 |
| GM 21 | IV | Neo-adjuvant CVT | 800 | Yes | >23 | >23 |

CVT: Cisplatin, vinblastine and temozolomide

## Table 2

Immune Responses[a]

| Patient No. | Positive Assay | Time | Phenotype | Clinical Status |
|---|---|---|---|---|
| GM 01 | Gp100 Tetramer | W7, W17 | CD45RO$^{high}$; CD62L$^{low}$; CCR7$^{low}$; CD127$^{low}$ | NED |
| GM 03 | gp100 Tetramer | W7 | ND | NED |
| | gp100 ICS | W7 | CD45RO$^{low}$; CD62L$^{low}$; CD107a$^{low}$; Gran$^{high}$ | |
| GM 04 | gp100 Tetramer | W7 | CD45RO$^{high}$; CD62L$^{int}$; CCR7$^{low}$; CD127$^{low}$ | NED |
| | Tyr Tetramer | W11, W17 | CD45RO$^{high}$; CD62L$^{int}$; CCR7$^{low}$; CD127$^{low}$ | |
| GM 08 | gp100 Tetramer | W11 | CD45RO$^{high}$; CD62L$^{int}$; CCR7$^{low}$; CD127$^{low}$ | DOD |
| | gp100 ICS | W11 | CD45RO$^{high}$; CD62L$^{low}$; CD107a$^{low}$; Gran$^{high}$; CD127$^{low}$ | |
| GM 12 | gp100 Tetramer | W7, W11, W17 | CD45RO$^{high}$; CD62L$^{low}$; CCR7$^{low}$; CD127$^{low}$ | NED |
| | Tyr Tetramer | W11 | CD45RO$^{high}$; CD62L$^{int}$; CCR7$^{low}$; CD127$^{low}$ | |
| | gp100 ICS | W7 | ND | |
| GM 14 | gp100 Tetramer | W11 | ND | DOD |
| | Tyr Tetramer | W7 | CD45RO$^{high}$; CD62L$^{high}$; CCR7$^{int}$; CD127$^{low}$ | |
| GM 20 | gp100 Tetramer | W11 | CD45RO$^{high}$; CD62L$^{low}$; CCR7$^{low}$; CD127$^{low}$ | NED |
| | gp100 ICS | W7, W11 | CD45RO$^{high}$; CD62L$^{low}$; CD107a$^{low}$; CD127$^{high}$ | |
| GM 21 | Tyr Tetramer | W7b | CD45RO$^{int}$; CD62L$^{low}$; CCR7$^{int}$; CD127 $^{low}$ | NED |

Abbreviations: DOD, died of disease; ICS, intracellular cytokine staining; ND, not determined; NED, no evidence of disease; W, week after initiation of study.

[a] Markers not indicated were not examined. Low = 0–30%, intermediate = 30–60%, and high = 60–100% of cells that are positive for above-mentioned markers.

[b] Patient was unavailable at time of scheduled W11 and W17 visits.