

Safety and immunogenicity of a human and mouse gp100 DNA vaccine in a phase I trial of patients with melanoma

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A differentiation antigen commonly expressed on melanoma cells, gp100 is the target of infiltrating T cells. We conducted a phase I randomized cross-over trial of melanoma patients with either xenogeneic (mouse) or human gp100 plasmid DNA injected intramuscularly at three dosages (100, 500 or 1,500 µg) every three weeks for three doses. After the first three injections, patients were then immunized three times with gp100 from the other species. Peripheral blood samples were analyzed at various time points following 10-day culture with gp100 peptides using multi-parametric flow cytometry. A total of 19 patients were enrolled, with 18 assessable for immune function and survival. 14 (74%) were male, with a median age of 56 years (range, 20-82). All patients had no evidence of disease; 10 (53%) had stage III disease, 3 each (16%) had stage IIB and IV disease, 2 (11%) had choroidal and 1 (5%) had anal mucosal involvement. With a median follow-up of 30 months, median progression-free survival (PFS) is 44 months. Median survival is not reached. There was no grade 3/4 toxicity; the most common grade 1/2 toxicity was an injection site reaction in 12 patients (63%, all grade 1). Five patients developed CD8+ cells binding gp100₂₈₀₋₂₈₈ HLA-A2-restricted tetramer. One patient had an increase in CD8+ IFN-γ+ cells. This xenogeneic immunization strategy was safe and associated with minimal toxicity. There was also evidence of immune response.

Keywords: phase I clinical trial, melanoma, gp100, DNA vaccine, cellular immunity

Introduction

Melanoma is one of several cancers whose incidence has increased in recent years (1). Unfortunately, therapy in the metastatic and adjuvant setting remains toxic and sub-optimal. Because of the disappointing nature of current therapy, interest has focused on cancer vaccines as potential therapy in melanoma. One reason that immunotherapy is attractive in treating melanoma is the fact that melanoma cells selectively express a number of differentiation antigens that can serve as targets for a vaccine strategy (2, 3).

One such differentiation antigen is gp100, a glycoprotein that is a structural component of the melanosome and which is expressed only by melanoma cells and melanocytes (4). Some investigators have also detected gp100 mRNA in glioma cells, an observation that is plausible given that both tissues share a common embryonic origin from the neural crest (5).

In patients with metastatic melanoma, CD8+ cells that are specific for gp100 have been detected (6, 7). Our group has also previously demonstrated that immunization of stage III/IV melanoma patients with the relatively immunogenic gp100₂₀₉₋₂₁₇ peptide and various adjuvants led to an increased number of CD8+ T cells that recognized the peptide (8).

A challenge of immunization with a self-antigen is its lack of immunogenicity due to immune tolerance or ignorance. A potential strategy to overcome this lack of immunogenicity is to immunize with a xenogeneic form of the antigen that shares significant homology but is also sufficiently different so as to generate a cross-response to the self-antigen. Our group previously demonstrated in mouse models that immunization with a xenogeneic form of gp100 results in protection from a lethal syngeneic melanoma challenge and causes autoimmune depigmentation (9-11). Mouse gp100 is 76% identical to human gp100 at the amino acid level (12).

While there are a number of distinct vaccine strategies, immunization with a plasmid encoding DNA for a tumor antigen is a relatively new strategy to induce humoral and cellular immune responses. Such an approach has several advantages. The presence of the full-length DNA presents multiple epitopes and avoids the need for major histocompatibility complex restriction. In addition, bacterial plasmid DNA contains unmethylated CpG motifs that stimulate the innate immune response via toll-like receptor-9 and may serve as a potent adjuvant for the innate immune system (13, 14).

In this paper, we report the results of a phase I trial of a human and mouse gp100 DNA vaccine. Such vaccination was associated with minimal toxicity and produced an increase in gp100-specific tetramer-positive cells and cytokine responses.

Results

Patient demographics

Nineteen patients were enrolled, with 18 receiving all six injections and evaluable for survival and immune function. One patient only received three injections before discontinuing therapy due to progressive disease. All patients were evaluable for toxicity. Patient characteristics are summarized in Table 1.

Fourteen patients (74%) were male, with a median age of 56 years (range 20-82). All patients had no evidence of disease and had excellent performance statuses. The majority – 10 of 19 or 53% – had stage III disease, while 3 patients (16%) had completely resected stage IV disease. Two patients (11%) had a primary choroidal melanoma while one patient (5%) had a primary anal mucosal melanoma. In addition to surgical resection, prior therapy included chemotherapy with temozolomide (5 patients or 26%), while 2 patients each had also received adjuvant immunotherapy with high-dose IFN- α .

Table 1
Patient demographics.

Characteristic	Patients (n = 19)	
	No.	%
Age (yr)		
Median	56	
Range	20-82	
Sex		
Male	14	74
Female	5	26
Karnofsky performance status		
90	9	47
100	10	53
Stage		
IIB	3	16
III	10	53
IV (without disease)	3	16
Choroidal	2	11
Anal mucosa	1	5
LDH within normal limits	19	100
Prior therapy		
Chemotherapy (temozolomide)	5	26
Interferon- α therapy	2	11
Radiotherapy	2	11

Toxicity and survival

There was no dose-limiting toxicity (DLT). The most common grade 1/2 toxicity was a local injection site reaction in 12 patients (63%, all grade 1). The only other grade 1/2 toxicities that were attributable to the vaccinations included fatigue and myalgias in 2 patients each (11%) and fever, chills and pruritis in 1 patient each (5%). With a median follow-up of 30 months, median PFS is 44 months. Median survival is not reached.

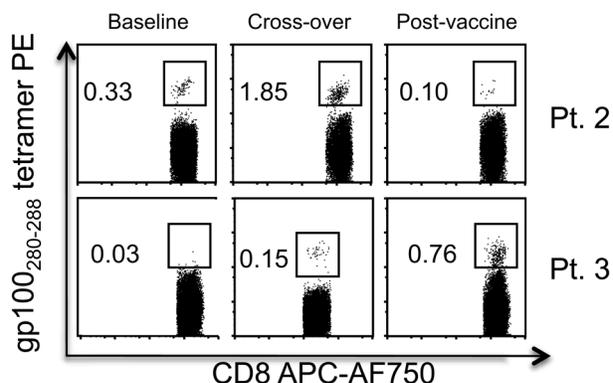
Increases in gp100-specific tetramer-reactive CD8+ cells after immunization

We performed multi-parametric flow cytometry on patient PBMC samples at baseline, at the time of cross-over to the other species vaccine at dose four and after completion of all six doses using tetramers for the HLA*A201 restricted peptides gp100₂₀₉₋₂₁₇ and gp100₂₈₀₋₂₈₈.

We noted that five of the 18 evaluable patients (28%; IDs 2, 3, 6, 8 and 12) developed an increase in the frequency of gp100₂₈₀₋₂₈₈ tetramer-reactive CD8+ cells either at the time of cross-over and/or upon completion of all six vaccinations. Representative dot plots are shown in Figure 1, while Figure 2 depicts the

change in frequency of tetramer-reactive CD8+ cells for each patient.

Figure 1



Change in gp100₂₈₀₋₂₈₈ HLA*A201-restricted tetramer CD8+ cells following mouse and human gp100 DNA vaccination. Multi-parameter flow cytometry was performed at baseline, at the time of cross-over to the other species DNA vaccine (after three doses) and after receiving all six vaccinations. Representative dot plots reveal an increase in the frequency of tetramer-reactive CD8+ cells in two patients, defined as an increase 3 or more standard deviations greater than the mean value at baseline and an absolute value >0.1%.

Chemokine receptor 7 (CCR7), CD45RA, CD27 and CD28 subtypes in the tetramer-reactive CD8+ cells reflect an effector cell population

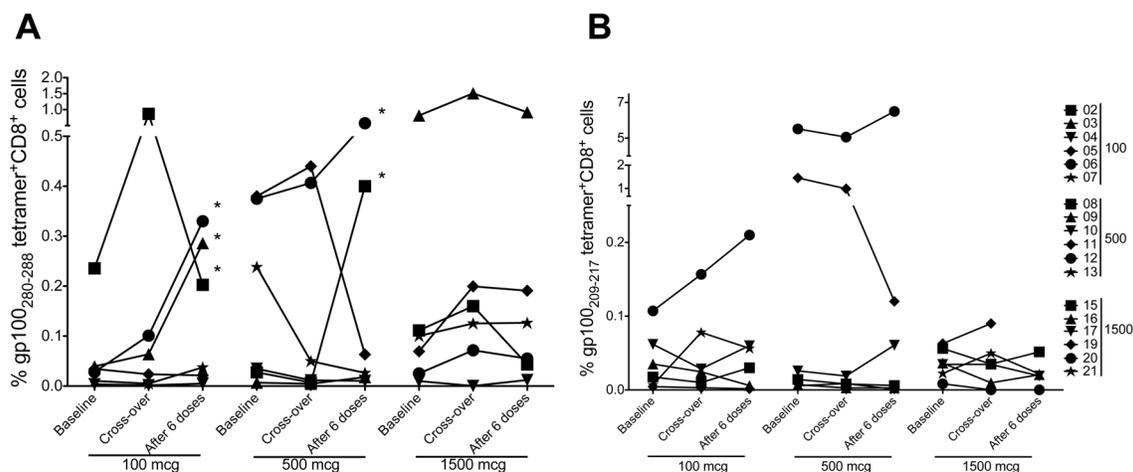
We then proceeded to analyze the gp100-specific tetramer-reactive CD8+ cells by characterizing the CCR7 and CD45RA expression of these cells. These two markers have been proposed (15) to classify CD8+ cells into four subtypes: naïve cells (CCR7+ CD45RA+), central memory cells (CCR7+ CD45RA-), effector memory cells (CCR7- CD45RA-) and effector cells (CCR7- CD45RA+). The tetramer-reactive CD8+ cells of all five patients with an increase in the frequency of tetramer-reactive CD8+ cells were also CCR7^{lo} CD45RA^{lo}, consistent with an effector memory phenotype.

In addition, it has recently been proposed (16) that CD27 and CD28 are markers that further characterize the CCR7- CD45RA- population into four subpopulations with different effector/cytolytic function: EM1 (CD27+ CD28+), EM2 (CD27+ CD28-), EM3 (CD27- CD28-) and EM4 (CD27- CD28+). The EM1 subtype has a phenotype very similar to the CCR7+ CD45RA- central memory cells, while the EM2 and EM3 subtypes express mediators associated with effector cells. Of the five patients who were CCR7^{lo} CD45RA^{lo}, two patients were CD27^{hi} CD28^{lo} (EM2 subtype; IDs 2 and 3), again consistent with cells having strong effector functions. The other patients (IDs 6, 8 and 12) were CD27^{hi} CD28^{int}. Representative dot plots are shown in Figure 3.

Increases in CD8+ IFN- γ + cells after immunization and evidence of polyfunctional cytokine responses

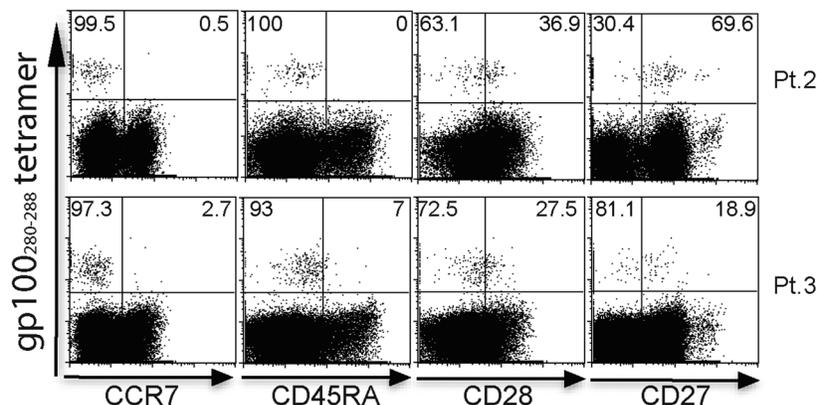
We also performed intracellular cytokine staining (ICS) to examine the intracellular cytokine profile of CD8+ cells at the time points previously described. One patient (ID 21) was found to have an increase in the frequency of CD8+ IFN- γ + cells after immunization with the gp100 DNA vaccine. Representative dot plots are shown in Figure 4.

Figure 2



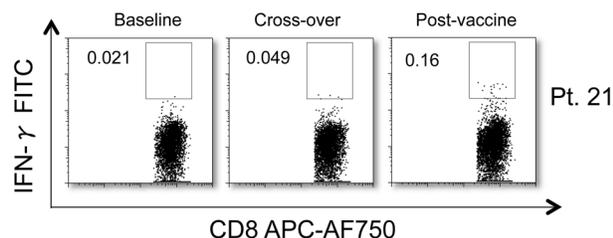
Changes in (A) gp100₂₈₀₋₂₈₈ and (B) gp100₂₀₉₋₂₁₇ HLA*A201-restricted tetramer CD8+ cells following mouse and human gp100 DNA vaccination. Each point refers to the mean of triplicate values. Most patients also underwent two peripheral blood draws at baseline one week apart prior to receiving vaccination. The values at baseline represent the mean of both of these time-points. The asterisks (*) refer to patients with an increase in the number of tetramer-reactive CD8+ cells.

Figure 3



CCR7, CD45RA, CD27 and CD28 subpopulations in gp100₂₈₀₋₂₈₈ HLA*A201-restricted tetramer CD8+ cells following mouse and human gp100 DNA vaccination. Representative dot plots of two patients (IDs 2 and 3) following gp100 DNA vaccination. Both patients had an increase in the frequency of tetramer-reactive CD8+ cells. Further characterization revealed that these cells were CCR7- CD45RA- CD27^{hi} CD28^{lo}, consistent with an effector phenotype.

Figure 4



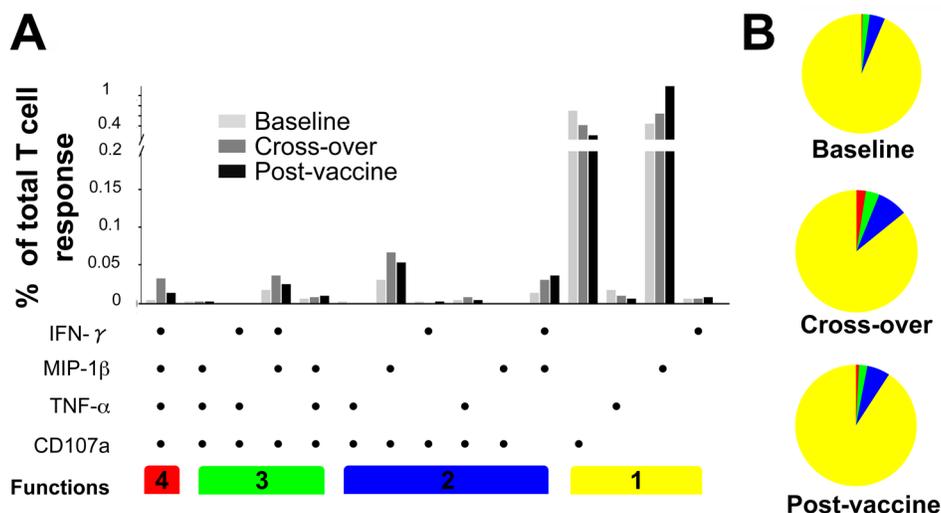
Intracellular cytokine staining (ICS) of patient PBMCs following gp100 DNA vaccination. ICS was performed by multi-parameter flow cytometry. These are representative dot plots of the patient (ID 21) who had an increase in CD8+ IFN-γ+ cells following vaccination.

In addition, there were two other patients (IDs 2 and 3) who had an increase in CD8+ IFN-γ+ cells that was <3 standard deviations higher than the baseline value. However, we did note that these patients had an increase in polyfunctional cytokine/chemokine responses – consisting of combinations of IFN-γ, macrophage inflammatory protein-1β, tumor necrosis factor-α and surface CD107a – following vaccination. These data are shown in Figure 5.

Lack of correlation between immune responses and dose levels or survival

Overall, we did not note any significant difference in the development of gp100-specific tetramer positivity or an increase in intracellular IFN-γ production between the different gp100 DNA dose levels or based on the sequence in which patients received the human or mouse DNA vaccines. There did appear to be a trend towards increased tetramer positivity in patients

Figure 5



Polyfunctional cytokine responses following gp100 DNA vaccination. Two patients (IDs 2 and 3) had an increase in CD8+ IFN- γ + that was <3 standard deviations higher than the mean of the baseline value. However, these patients had evidence of an increase in polyfunctional cytokine responses. The following results represent the average of values for both patients at baseline, the time of cross-over from one species vaccine to the other and post-vaccination. In (A), all possible combinations of four selected functional responses are shown on the x-axis. Responses are grouped and color-coded. In (B), each slice of the pie charts represents the fraction of the total response that is positive for a given number of functions.

Table 2
Changes in gp100-specific tetramer-reactive CD8+ cells and CD8+ IFN- γ + cells at baseline and following immunization with gp100 DNA vaccine.

Dose Level (μ g)	Patient No.	Increase in gp100 Tetramer-reactive Cells	Increase in CD8+ IFN- γ + Cells	Clinical Status ¹
100	2	gp100 ₂₈₀₋₂₈₈ +		Died
	3	gp100 ₂₈₀₋₂₈₈ +		Developed stage IV NSCLC
	4	-		NED
	5	-		NED
	6	gp100 ₂₈₀₋₂₈₈ +		NED
	7	-		PD
500	8	gp100 ₂₈₀₋₂₈₈ +		PD
	9	-		PD
	10	-		PD
	11	-		NED
	12	gp100 ₂₈₀₋₂₈₈ +		PD
	13	-		Died of NSCLC
1500	15	-		NED
	16	-		NED
	17	-		PD
	19	-		NED
	20	-		NED
	21	-		NED

¹ Abbreviations: NED, no evidence of disease; NSCLC, non-small cell lung cancer; PD, progressive disease.

who received the two lower vaccine doses (100 and 500 μ g; 5 of 12 patients) versus those who received the highest vaccine dose (1,500 μ g; 0 of 6 patients) but this did not reach statistical significance ($P = 0.11$).

Similarly, we did not observe a difference in PFS or overall survival based on the development of an immune response by either tetramer positivity or increased cytokine expression. These patient data are summarized in Table 2.

Discussion

The strategy presented in this paper to overcome the low immunogenicity of the self-antigens found in many human cancers is to immunize patients with the xenogeneic counterpart of that antigen. Proof-of-concept for such an approach stems from a prior parallel effort by our group. In conjunction with the Animal Medical Center in New York, NY, we previously demonstrated that immunizing dogs with melanoma with a human tyrosinase DNA vaccine produced clinically significant and durable responses (17, 18).

Based on these novel findings, a human tyrosinase DNA vaccine has been issued a conditional license by the United States Department of Agriculture – the body that regulates vaccines in animals – for the treatment of canine melanoma. This licensure represents the first approval of a vaccine strategy for the treatment of cancer in any species in the U.S. In this phase I study, we demonstrated that vaccination with a human and mouse gp100 DNA vaccine is safe and associated with minimal toxicity. Importantly, no autoimmune manifestations were noted.

In addition, we also noted evidence of an immune response. Firstly, we observed an increase in the frequency of gp100-specific tetramer-reactive CD8+ cells in 5 of 18 patients following immunization. Further characterization of these tetramer-reactive CD8+ cells by the phenotype markers CCR7, CD45RA, CD27 and CD28 suggest that these cells have significant effector functions. Secondly, we also noted that one patient developed an increase in the frequency of CD8+ IFN- γ + cells following immunization.

There are several findings in our study that were somewhat surprising. The five patients who developed tetramer positivity did so only for the HLA*A201 restricted gp100₂₈₀₋₂₈₈ peptide but not the gp100₂₀₉₋₂₁₇ peptide, even though both peptides are thought to have comparable immunogenicity and intermediate binding affinity for the HLA*A201 molecule (19, 20). The small patient numbers preclude any definitive conclusions but it may be that the mouse gp100 DNA vaccine is more effective in generating tetramer-specific responses against the gp100₂₈₀₋₂₈₈ peptide than the gp100₂₀₉₋₂₁₇ peptide. A similar observation was made in a report where dendritic cells were infected with a recombinant vaccinia viral construct expressing gp100 protein (21). Although gp100 protein was expressed, it was found that the ability to present the gp100₂₀₉₋₂₁₇ peptide was poor. Together, these data would suggest that the processing of a whole antigen to produce specific immunogenic peptides of interest that are then presented in the appropriate context to elicit an immune response is a variable phenomenon that remains incompletely understood.

In addition, only one patient developed an increase in CD8+ IFN- γ + T cells recognizing the gp100 peptide following vaccination. These results are in contrast to our prior report of a phase I evaluation of a tyrosinase DNA vaccine (22). In that trial, 5 of 18 patients developed an increase in CD8+ IFN- γ + T cells recognizing the tyrosinase₃₆₉₋₃₇₇ peptide (YMDGTSQV), while 3 patients developed tyrosinase tetramer-specific responses. One must make comparisons across different phase I trials with extreme caution but these divergent findings may offer insight into the relative immunogenicity and immune effects of similar DNA vaccines directed against different melanoma differentiation antigens.

We did note that two patients who had an increase in CD8+ IFN- γ + cells following vaccination that was <3 standard deviations higher than the baseline value did have an increase in polyfunctional cytokine responses. While a single parameter – most commonly IFN- γ – has previously been used to assess the effector function of T cells, there are increasing data that polyfunctional responses are in fact associated with improved viral control in pre-clinical models of infectious diseases, in patients infected with human immunodeficiency virus (23) or immunized using vaccinia constructs (24). Whether polyfunctional versus single effector function assessments are preferable for monitoring in cancer vaccine development is very much an unanswered question.

Finally, we also noted a non-statistically significant trend towards increased tetramer positivity in patients who received the two lower doses of the gp100 DNA vaccine versus those who received the highest vaccine dose. While there is no ready explanation for this unexpected observation, one intriguing possibility is that the higher dose of the gp100 DNA vaccine may have generated a stronger regulatory T cell (Treg) response that dampened any measurable immune effects. Such a possibility would have significant implications for the development of whole DNA or protein vaccines and should be addressed in future immune monitoring efforts that evaluate Treg populations to determine if they are augmented following DNA vaccination.

While we did find evidence of an immune response to the xenogeneic vaccination strategy, we did not note a difference in PFS between patients with and without an immune response. Clearly, this was a phase I study that was not designed to evaluate survival, much less differences in survival between either patients at different dose levels or between immune responders and non-responders. At the same time, it is plausible that the immune responses we assessed are necessary but not sufficient for an anti-tumor effect in the metastatic setting or tumor protection in the adjuvant setting. For example, it may be that a more robust and durable immune response that can be detected weeks or months following vaccination in a majority of patients is required.

In order to enhance the immune responses produced by vaccination with this mouse gp100 construct, we are currently comparing a more prolonged administration of the DNA vaccine by intramuscular injection or via particle-mediated delivery with gold particles into the dermis.

Other potential evaluations include the co-administration of adjuvants to potentially boost the immune response. One particularly attractive strategy is the administration of the vaccine with simultaneous antibody blockade of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which serves as a negative checkpoint to regulate immune responses and prevent autoimmunity (25, 26). Antibodies against CTLA-4 have been shown to produce durable clinical responses in patients with advanced melanoma in a number of phase II trials and are currently undergoing phase III evaluation (27, 28). In addition, our group has recently shown that anti-CTLA4 therapy in patients with metastatic melanoma can generate or enhance antigen-specific CD8+ and polyfunctional cytokine responses, providing an immunological basis to combine it with a vaccine strategy (29).

In conclusion, this phase I study established the safety of a human and mouse gp100 DNA vaccine, with no DLT noted at the pre-planned highest dose of 1,500 μ g administered every three weeks for six doses. We also saw evidence of an immune response – in terms of an increase in the frequency of gp100-specific tetramer-reactive and IFN- γ -expressing CD8+ cells – in 6 of 18 evaluable patients. Further evaluation of this mouse gp100 DNA vaccine is ongoing.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008; **58**: 71-96. (PMID: 18287387)
2. Houghton AN, Eisinger M, Albino AP, Cairncross JG, Old LJ. Surface antigens of melanocytes and melanomas. Markers of melanocyte differentiation and melanoma subsets. *J Exp Med* 1982; **156**: 1755-1766. (PMID: 7175440)
3. Houghton AN, Taormina MC, Ikeda H, Watanabe T, Oettgen HF, Old LJ. Serological survey of normal humans for natural antibody to cell surface antigens of melanoma. *Proc Natl Acad Sci U S A* 1980; **77**: 4260-4264. (PMID: 6933476)
4. Thomson TM, Real FX, Murakami S, Cordon-Cardo C, Old LJ, Houghton AN. Differentiation antigens of melanocytes and melanoma: analysis of melanosome and cell surface markers of human pigmented cells with monoclonal antibodies. *J Invest Dermatol* 1988; **90**: 459-466. (PMID: 3280698)
5. Chi DD, Merchant RE, Rand R, Conrad AJ, Garrison D, Turner R, Morton DL, Hoon DS. Molecular detection of tumor-associated antigens shared by human cutaneous melanomas and gliomas. *Am J Pathol* 1997; **150**: 2143-2152. (PMID: 9176405)
6. Kammula US, Lee KH, Riker AI, Wang E, Ohnmacht GA, Rosenberg SA, Marincola FM. Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J Immunol* 1999; **163**: 6867-6875. (PMID: 10586088)
7. Riker A, Cormier J, Panelli M, Kammula U, Wang E, Abati A, Fetsch P, Lee KH, Steinberg S, Rosenberg S, Marincola F. Immune selection after antigen-specific immunotherapy of melanoma. *Surgery* 1999; **126**: 112-120. (PMID: 10455872)
8. Schaed SG, Klimek VM, Panageas KS, Musselli CM, Butterworth L, Hwu WJ, Livingston PO, Williams L, Lewis JJ, Houghton AN, Chapman PB. T-cell responses against tyrosinase 368-376(370D) peptide in HLA*A0201+ melanoma patients: randomized trial comparing incomplete Freund's adjuvant, granulocyte macrophage colony-stimulating factor, and QS-21 as immunological adjuvants. *Clin Cancer Res* 2002; **8**: 967-972. (PMID: 12006508)
9. Bowne WB, Srinivasan R, Wolchok JD, Hawkins WG, Blachere NE, Dyall R, Lewis JJ, Houghton AN. Coupling and uncoupling of tumor immunity and autoimmunity. *J Exp Med* 1999; **190**: 1717-1722. (PMID: 10587362)
10. Hawkins WG, Gold JS, Dyall R, Wolchok JD, Hoos A, Bowne WB, Srinivasan R, Houghton AN, Lewis JJ. Immunization with DNA coding for gp100 results in CD4 T-cell independent antitumor immunity. *Surgery* 2000; **128**: 273-280. (PMID: 10923004)
11. Weber LW, Bowne WB, Wolchok JD, Srinivasan R, Qin J, Moroi Y, Clynes R, Song P, Lewis JJ, Houghton AN. Tumor immunity and autoimmunity induced by immunization with homologous DNA. *J Clin Invest* 1998; **102**: 1258-1264. (PMID: 9739060)
12. Zhai Y, Yang JC, Spiess P, Nishimura MI, Overwijk WW, Roberts B, Restifo NP, Rosenberg SA. Cloning and characterization of the genes encoding the murine homologues of the human melanoma antigens MART1 and gp100. *J Immunother* 1997; **20**: 15-25. (PMID: 9101410)
13. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci U S A* 1996; **93**: 2879-2883. (PMID: 8610135)
14. Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, Silverman GJ, Lotz M, Carson DA, Raz E. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 1996; **273**: 352-354. (PMID: 8662521)
15. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004; **22**: 745-763. (PMID: 15032595)
16. Romero P, Zippelius A, Kurth I, Pittet MJ, Touvrey C, Iancu EM, Corthesy P, Devedre E, Speiser DE, Rufer N. Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. *J Immunol* 2007; **178**: 4112-4119. (PMID: 17371966)
17. Bergman PJ, Camps-Palau MA, McKnight JA, Leibman NF, Craft DM, Leung C, Liao J, Riviere I, Sadelain M, Hohenhaus AE, Gregor P, Houghton AN, Perales MA, Wolchok JD. Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. *Vaccine* 2006; **24**: 4582-4585. (PMID: 16188351)
18. Liao JC, Gregor P, Wolchok JD, Orlandi F, Craft D, Leung C, Houghton AN, Bergman PJ. Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. *Cancer Immunol* 2006; **6**: 8. URL: <http://www.cancerimmunity.org/v6p8/060308.htm>
19. Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, Robbins PF, Sette A, Appella E, Rosenberg SA. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol* 1995; **154**: 3961-3968. (PMID: 7706734)
20. Salgaller ML, Marincola FM, Cormier JN, Rosenberg SA. Immunization against epitopes in the human melanoma antigen gp100 following patient immunization with synthetic peptides. *Cancer Res* 1996; **56**: 4749-4757. (PMID: 8840994)
21. Nagorsen D, Panelli M, Dudley ME, Finkelstein SE, Rosenberg SA, Marincola FM. Biased epitope selection by recombinant vaccinia-virus (rVV)-infected mature or immature dendritic cells. *Gene Ther* 2003; **10**: 1754-1765. (PMID: 12939642)
22. Wolchok JD, Yuan J, Houghton AN, Gallardo HF, Rasalan TS, Wang J, Zhang Y, Ranganathan R, Chapman PB, Krown SE, Livingston PO, Heywood M, Riviere I, Panageas KS, Terzulli SL, Perales MA. Safety and immunogenicity of tyrosinase DNA vaccines in patients with melanoma. *Mol Ther* 2007; **15**: 2044-2050. (PMID: 17726460)
23. Duvall MG, Precopio ML, Ambrozak DA, Jaye A, McMichael AJ, Whittle HC, Roederer M, Rowland-Jones SL, Koup RA. Polyfunc-

- tional T cell responses are a hallmark of HIV-2 infection. *Eur J Immunol* 2008; **38**: 350-363. (PMID: 18200635)
24. Precopio ML, Betts MR, Parrino J, Price DA, Gostick E, Ambrozak DR, Asher TE, Douek DC, Harari A, Pantaleo G, Bailer R, Graham BS, Roederer M, Koup RA. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 2007; **204**: 1405-1416. (PMID: 17535971)
 25. Brunner MC, Chambers CA, Chan FK, Hanke J, Winoto A, Allison JP. CTLA-4-Mediated inhibition of early events of T cell proliferation. *J Immunol* 1999; **162**: 5813-5820. (PMID: 10229815)
 26. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995; **182**: 459-465. (PMID: 7543139)
 27. Ribas A. Overcoming immunologic tolerance to melanoma: targeting CTLA-4 with tremelimumab (CP-675,206). *Oncologist* 2008; **13** Suppl 4: 10-15. (PMID: 19001146)
 28. Wolchok JD, Saenger Y. The mechanism of anti-CTLA-4 activity and the negative regulation of T-cell activation. *Oncologist* 2008; **13** Suppl 4: 2-9. (PMID: 19001145)
 29. Yuan J, Gnjatic S, Li H, Powel S, Gallardo HF, Ritter E, Ku GY, Jungbluth AA, Segal NH, Rasalan TS, Manukian G, Xu Y, Roman RA, Terzulli SL, Heywood M, Pogoriler E, Ritter G, Old LJ, Allison JP, Wolchok JD. CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit. *Proc Natl Acad Sci U S A* 2008; **105**: 20410-20415. (PMID: 19074257)
 30. Goldberg SM, Bartido SM, Gardner JP, Guevara-Patiño JA, Montgomery SC, Perales MA, Maughan MF, Dempsey J, Donovan GP, Olson WC, Houghton AN, Wolchok JD. Comparison of two cancer vaccines targeting tyrosinase: plasmid DNA and recombinant alphavirus replicon particles. *Clin Cancer Res* 2005; **11**: 8114-8121. (PMID: 16299244)
 31. Perales MA, Yuan J, Powel S, Gallardo HF, Rasalan TS, Gonzalez C, Manukian G, Wang J, Zhang Y, Chapman PB, Krown SE, Livingston PO, Ejadi S, Panageas KS, Engelhorn ME, Terzulli SL, Houghton AN, Wolchok JD. Phase I/II study of GM-CSF DNA as an adjuvant for a multi-peptide cancer vaccine in patients with advanced melanoma. *Mol Ther* 2008; **16**: 2022-2029. (PMID: 18797450)
 32. Yuan J, Gallardo HF, Rasalan T, Ranganathan R, Wang J, Zhang Y, Panageas K, Stan R, Young JW, Houghton AN, Wolchok JD. In vitro expansion of Ag-specific T cells by HLA-A*0201-transfected K562 cells for immune monitoring. *Cytotherapy* 2006; **8**: 498-508. (PMID: 17050255)

Materials and methods

Eligibility criteria

Eligibility criteria included American Joint Committee on Cancer stage IIB, IIC, III and IV melanoma. Patients with stage IIIC/IV disease were eligible if they were free of disease after surgical resection while those with choroidal melanoma were eligible if they met one of the following criteria: basal diameter of 16 mm or more, height of 8 mm or more, or involvement of the ciliary body. All pathology was confirmed at the Memorial

Sloan-Kettering Cancer Center (MSKCC). Other eligibility criteria included a Karnofsky performance status of 80% or more, normal organ and bone marrow function and HLA A2 positivity. Exclusion criteria included brain metastases, prior chemotherapy or radiotherapy within four weeks of study entry, prior gp100 vaccine therapy or history of autoimmune disease. The study was approved by the Institutional Research Board and all patients gave informed consent.

Study design and treatment plan

This was a phase I study, in which cohorts of six patients were randomized (three per each arm) to receive a DNA vaccine first with either human or mouse gp100 via intramuscular injection every three weeks for three doses. After the first three injections, patients then received another three doses with the DNA vaccine from the other species, e.g. patients who received the mouse DNA vaccine first then received the human DNA vaccine. Intramuscular injection was administered via the Biojector2000 jet delivery device (Bioject, Tualatin, OR). The injection site was rotated for each immunization and no injection was given at a site where draining lymph nodes had been removed.

The vaccine was administered at one of three dose levels – 100, 500 or 1,500 µg. Dose escalation occurred only after the last patient in each preceding cohort completed all six injections without dose-limiting toxicity (DLT). DLT was defined as any grade 3/4 toxicity or allergic/immunologic toxicity of grade 2 or more, according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 2.0.

DNA vaccine construct

Mouse and human gp100 complementary DNA were previously sequenced by our group and introduced into the pING vector (30), a standard eukaryotic expression vector used extensively in pre-clinical evaluations and in clinical trials by our group (17, 22). The vector conforms to criteria specified in the Food and Drug Administration points to consider for DNA vaccines. Clinical-grade material was manufactured by Althea Technologies (San Diego, CA).

Evaluations at baseline and during therapy

At baseline, patients underwent a complete history and physical examination, as well as ophthalmologic evaluation to rule out uveitis or an ineligible primary choroidal melanoma. They also underwent routine blood work and a chest X-ray or computed tomography scan.

In order to perform immune function monitoring, blood samples were also collected peripherally at baseline, prior to cross-over to the other series of vaccinations at the fourth dose and at the completion of all six doses. Whenever possible, patients underwent leukapheresis at baseline and after completion of the six doses to ensure that sufficient peripheral blood mononuclear cells (PBMCs) were collected. After completion of therapy, patients underwent repeat radiographic imaging if warranted. Further evaluation was then performed as clinically indicated to monitor disease course/recurrence.

Immune function monitoring

Tetramer and intracellular cytokine staining (ICS) assays were performed using multi-parameter flow cytometry, as previously described (22, 31, 32). The assays were performed on frozen PBMC specimens collected at baseline, prior to the fourth dose and after receiving all six doses.

The following tetramers and fluorochrome-labeled antibodies were used: HLA-A*0201-PE-labeled tetramers loaded with gp100₂₀₉₋₂₁₇ (ITDQVPFSV) and gp100₂₈₀₋₂₈₈ (YLEPGPVTA) (Tetramer Core, Lausanne Branch, Ludwig Institute of Cancer Research, Lausanne, Switzerland), PE-Cy7-CD3, APC-CD27, PerCPCy5.5-CD28, APC-interleukin (IL)-2, PE-macrophage inflammatory protein (MIP)-1 β , and FITC-interferon (IFN)- γ (BD Pharmingen, San Jose, CA), Pacific blue-CD3, APC-AF750-CD8, PE-Cy7-tumor necrosis factor (TNF)- α (eBioscience, San Diego, CA), ECD-CD4, ECD-CD45RA (Beckman Coulter Inc., Fullerton, CA) and FITC-CCR7 (R&D Systems, Minneapolis, MN).

Briefly, thawed PBMCs were incubated at a 1:1 ratio with irradiated autologous PBMCs pulsed with the following peptide pool at 10 μ g/ml each: HLA A2-restricted gp100₂₀₉₋₂₁₇ (ITDQVPFSV) and gp100₂₈₀₋₂₈₈ (YLEPGPVTA), HLA DQ6-restricted gp100₁₇₅₋₁₈₉ (GRAMLGHTHTMEVTVY), HLA DR4-restricted gp100₄₄₋₅₉ (WNRQLYPEWTEAQRDL), HLA DR7-restricted gp100₄₂₀₋₄₃₇ (TTEWVETTARELPIPEPE) and gp100₁₇₄₋₁₉₀ (TGRAMLGHTHTMEVTVYH), and HLA DR53-restricted gp100₁₇₅₋₁₈₉ (GRAMLGHTHTMEVTVY) (JPT Peptide Technologies, Berlin, Germany). The PBMCs were incubated with IL-2 (Chiron, Emeryville, CA) at 10 IU/ml and IL-15 (R&D Systems, Minneapolis, MN) at 10 ng/ml.

The cells were harvested on day 10. They were analyzed immediately by tetramer staining. For ICS, they were additionally incubated for 20 minutes with PE-Cy5-CD107a (5 μ l/ml; BD Pharmingen) prior to re-stimulation with the preceding gp100 peptide pool for 2 hours. 5 μ g/ml each of Brefeldin A and monensin (BD Biosciences, San Jose, CA) were then added for another 4 hours. Cells were analyzed using a CYAN flow cytometer with Summit software (Dako Cytomation California Inc., Carpinteria, CA). Analysis was performed using FlowJo software (version 8.8; TreeStar, Inc., Ashland, OR).

Statistical analysis

A T cell response at any post-vaccination time-point was considered positive if it was 3 or more standard deviations greater than the mean value at baseline and had an absolute value >0.1%. Differences between groups were analyzed using Fisher's exact test. Progression-free survival (PFS) and overall survival were estimated using the Kaplan-Meier method.

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