**Abstract**

**Purpose:** Prior studies show that i.m. injection of xenogeneic orthologues of melanosomal antigens (tyrosinase, gp100) induces CD8+ T-cell responses to the syngeneic protein. To further define the optimal vaccination strategy, we conducted a pilot clinical trial comparing i.m. injection with particle-mediated epidermal delivery (PMED).

**Experimental Design:** Human leukocyte antigen (HLA)-A*0201+ disease–free melanoma patients were randomized to the PMED or i.m. arm, receiving eight vaccinations over 4 months. Patients received 4 μg or 2,000 μg per injection, respectively, of mouse gp100 DNA. Peripheral blood mononuclear cells were collected, cultured with gp100 peptides, and analyzed by tetramer and intracellular cytokine staining for responses to HLA-A*0201–restricted gp100 epitopes [gp100209-217 (ITDQVPFSV) and gp100280-288 (YLEPGPVTA)].

**Results:** Twenty-seven patients with stage IIB-IV melanoma were analyzable for immune response. The only common toxicity was grade 1 injection site reaction in nine patients with no intergroup difference, and one dose-limiting toxicity of acute hypersensitivity occurred in a PMED patient with undiagnosed gold allergy. Four of 27 patients produced gp100 tetramer+CD8+ T cells, all carrying the CCR7loCD45RAlo effector-memory phenotype. Five of 27 patients generated IFN-γ+CD8+ T cells, one who was also tetramer-positive. Overall, vaccination induced a response in 30% of patients, which was not significantly associated with study arm or clinical outcome. However, the PMED group showed a trend toward increased IFN-γ+CD8+ T-cell generation ($P = 0.07$).

**Conclusion:** A comparable efficacy and safety profile was shown between the i.m. and PMED arms, despite a significantly decreased dose of DNA used for PMED injection. Clin Cancer Res; 16(15); 4057–65. ©2010 AACR.

Currently, the only Food and Drug Administration–approved adjuvant therapy for melanoma is high-dose IFN-α, which does not produce a significant prolongation in overall survival but does produce relapse-free survival (1). Therefore, there is a substantial interest in alternative adjuvant therapy including tumor vaccines such as DNA vaccines. Evidence for immunotherapy efficacy continues to mount with immunomodulatory antibodies, including anti–CTLA-4 antibodies (2–5). Melanoma provides an ideal setting for evaluating tumor antigen-specific immune responses, given selective expression of differentiation antigens.

**Gp100**, a melanocytic differentiation protein present mainly in melanocytes and melanoma, provides a specific immunization target. In fact, preexisting gp100 antigen-specific T cells have been shown in patients with melanomas. This supports the goal of expanding functional tumor antigen-specific T-cell responses with antitumor immunity. Our group and others have compared gp100 vaccination using the human leukocyte antigen (HLA)-A*0201 gp100209-217 (ITDQVPFSV) or gp100 cDNA, comparing different adjuvants and i.m. gp100 DNA, with the demonstration of enhanced antigen-specific T-cell responses (6, 7). Prior work has defined adjuvant criteria for peptide immunization. In addition, xenogeneic DNA vaccination has provided sufficient antigenic disparity to overcome immune tolerance or ignorance (8). However, much of the preclinical DNA vaccination development in mouse models was done using particle-mediated epidermal delivery (PMED). It has been shown that genetic immunization through PMED could generate potent immune responses (9). We have previously shown that DNA vaccination by PMED could result in frequent tumor-specific T-cell responses in mice (10). However, clinical
Translational Relevance

This article, “Immunologic Response to Xenogeneic gp100 DNA in Melanoma Patients: Comparison of Particle-Mediated Epidermal Delivery with Intramuscular Injection”, presents the results of a pilot clinical trial directly comparing two vaccination methods in terms of safety and immunogenicity. From a laboratory and scientific perspective, it provides insight into the human immune response to xenogeneic plasmid DNA, and through comprehensive monitoring of antigen-specific CD8+ T-cell response, provides a better understanding of the mechanism of action. Designed as a randomized clinical trial in patients with resected American Joint Committee on Cancer stage IIB-IV melanoma, it has clear translational applications with respect to the safety and efficacy of using DNA vaccination as a means of clinical management of metastatic melanoma. Perhaps most importantly, this is the first cancer vaccine clinical trial to directly compare the standard technique of intramuscular injection with another such as particle-mediated epidermal delivery, providing a foundation for the optimization of vaccination strategies.

Materials and Methods

Eligibility criteria
To be eligible to participate in this study, patients must have already undergone initial standard therapy (surgery), and all patients free of disease after surgical resection were only eligible if they had refused IFN-α therapy or had a recurrence while on it. Patients with choroidal melanoma required a basal diameter of ≥16 mm, height ≥8 mm, or involvement of the ciliary body. Additional eligibility criteria included a Karnofsky performance status of ≥80%, HLA-A*0201 positivity, the absence of detectable brain metastases, a negative anti–double-stranded DNA serum antibody screen, and adequate organ and marrow function as defined by prior standards. Exclusion criteria included prior chemotherapy, immunotherapy, or radiotherapy within 4 weeks of participation in the study, previous immunization with a gp100-containing vaccine, preexisting choroidal eye disease, pregnancy or nursing, allergy to gold, and any comorbidity or medication (e.g., corticosteroids) that could interfere with the treatment course. The Institutional Review Board approved this study and consent was received by all patients (NCT00398073).

Study design and treatment plan
In this study, patients were randomized to receive mouse gp100 DNA vaccine by means of either i.m. injection or PMED. Patients receiving i.m. delivery were given 1,000 μg of plasmid DNA per injection using the Biojector 2000 jet delivery device (Bioject), whereas those in the PMED subgroup were vaccinated with 2 μg of plasmid DNA coated on 1,000 μg of gold per actuation via the ND10 delivery system (PowderMed/Pfizer). Regardless of delivery method, all patients were treated with two injections per day every 2 weeks for 4 months, with rotation of injection sites and avoidance of location with prior draining regional lymph node removal. Treatment was discontinued in the presence of a dose-limiting toxicity or with the development of progressive or recurrent disease requiring systemic treatment or radiation therapy. Dose-limiting toxicity was defined as any grade 3/4 toxicity or grade ≥2 allergic/immunologic toxicity, as per the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

DNA vaccine construct
Mouse gp100 plasmid DNA had been previously sequenced and introduced into the pING vector by our group (13), which has extensively used this conventional eukaryotic expression vector in both preclinical studies and clinical trials (14, 15). This vector contains a cytomegalovirus promoter and a kanamycin resistance selection marker (KanR). KanR is the antibody selection gene, in accordance with the Food and Drug Administration’s Points to Consider for DNA vaccination. The mouse gp100 plasmid DNA was subsequently tested for endotoxin, sterility, and animal safety. Production of clinical grade material was accomplished by Althea Technologies.
Evaluations at baseline and during therapy

Prior to initializing therapy, a complete history and physical examination was performed for all patients, along with a baseline ophthalmologic examination to evaluate for preexisting retinal or choroidal eye disease. Routine blood work, chest imaging (X-ray or computed tomography), and a brain magnetic resonance imaging was also completed, in addition to appropriate radiographic imaging of lesions in patients with measurable disease.

For immune function monitoring, blood samples were drawn at 1 week before and immediately prior to the first vaccination and at weeks 18 and 30, associated with 3 and 15 weeks postvaccination completion, respectively. To ensure the acquisition of a sufficient quantity of peripheral blood mononuclear cells (PBMC), leukapheresis was performed at baseline and at week 18, if possible. In addition, patients underwent clinical and radiologic monitoring as indicated to assess for disease recurrence and adverse events.

Immune function monitoring

Assessment of T-cell response was made using tetramer and intracellular cytokine staining (ICS) assays coupled with multiparameter flow cytometry. Frozen PBMC samples were used for such assays, originally obtained at baseline and weeks 18 and 30, as described above. In short, thawed PBMCs were incubated at a 1:30 ratio with HLA-A*0201–transfected K562 cells pulsed with either of the following peptides at 10 μg/mL each: HLA A2-restricted gp100209-217 (ITDQVPFSV) and gp100280-288 (YLEPGPVTA; JPT Peptide Technologies). Every 2 days, the cells were re-fed with complete medium (10% pooled human serum and RPMI), 10 units/mL of interleukin 2 (IL-2) and 10 units/mL of interleukin 1 (IL-1), 10 units/mL of interleukin 2 (IL-2) and 10 units/mL of interleukin 1 (IL-1), and 10 units/mL of interleukin 2 (IL-2) and 10 units/mL of interleukin 1 (IL-1).

Immune function monitoring continued

the acquisition of sufficient quantity of peripheral blood mononuclear cells (PBMC), leukapheresis was performed at baseline and at week 18, if possible. In addition, patients underwent clinical and radiologic monitoring as indicated to assess for disease recurrence and adverse events.

Statistical methods

To determine positive T-cell responses, we calculated the SD of the prevaccination replicate values taken at two time points (1 week prior to vaccination and the value immediately before vaccination). A T-cell response at any postvaccination time point was considered positive if it had a value ≥3 SD greater than the mean value at baseline and having an absolute value of >0.1%. Differences between groups were analyzed using Fisher’s exact test. Progression-free survival and overall survival were estimated using the Kaplan-Meier method.

Results

Patient demographics

Thirty-four patients were enrolled with equal numbers in both the i.m. and PMED arms, all evaluable for survival. Twenty-nine patients received all eight vaccinations, 27 of whom were assessable for immune function (15 i.m. and 12 PMED). Reasons for termination of vaccination or lack of immune assessment included progression of disease during vaccination course (1 i.m. and 4 PMED), development of multiple myeloma (1 i.m.), and an acute hypersensitivity reaction following the initial actuation (1 PMED). Postvaccine PBMC samples were not collected on these seven patients. Patient demographics are listed in Table 1.

The median age of the patients was 55 (37-79) in the i.m. arm and 47 (26-78) in the PMED arm, with an overall male predominance of 74%. All patients had a Karnofsky performance status of 90% or greater at the time of initial

### Table 1. Patient demographics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Overall (n, %)</th>
<th>I.m. (n, %)</th>
<th>PMED (n, %)</th>
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<tbody>
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<td>17 (50)</td>
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</tr>
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<tr>
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<td>37-79</td>
<td>26-78</td>
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<td>47</td>
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<td>III</td>
<td>25 (74)</td>
<td>10 (59)</td>
<td>15 (88)</td>
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<tr>
<td>IV</td>
<td>5 (15)</td>
<td>4 (24)</td>
<td>1 (6)</td>
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<td>Karnofsky performance status</td>
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<td>90%</td>
<td>14 (41)</td>
<td>7 (41)</td>
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<td>100%</td>
<td>20 (59)</td>
<td>10 (59)</td>
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<td>None</td>
<td>22 (65)</td>
<td>8 (47)</td>
<td>14 (82)</td>
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<tr>
<td>Temozolomide</td>
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<td>2 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>3 (9)</td>
<td>2 (12)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>IFN alone</td>
<td>4 (12)</td>
<td>3 (18)</td>
<td>1 (6)</td>
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<tr>
<td>Multiple therapies</td>
<td>3 (9)</td>
<td>2 (12)</td>
<td>1 (6)</td>
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<td>Radiation + IFN-α</td>
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<td>1 (6)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>IFN-α + interleukin 2</td>
<td>1 (3)</td>
<td>1 (6)</td>
<td>0 (0)</td>
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</table>
vaccination. Eighty-eight percent (30 of 34) of the patients were stage III or IV, with all patients having no evidence of disease. Twelve patients had received prior therapy, including temozolomide (2), radiation alone (3), IFN-α alone (4), or combination therapy of radiation plus IFN-α (2), or IFN-α plus interleukin-2 (1).

Toxicity and survival
Toxicity was assessed in all patients receiving at least one vaccination. Therapy was generally well-tolerated with 10 patients (59%) in the i.m. arm versus 6 patients (35%) in the PMED arm having no adverse effects. There was one incident of dose-limiting toxicity, with one patient in the PMED arm developing an acute hypersensitivity reaction after the first injection. This was evaluated by a dermatologist who felt this was most consistent with gold sensitivity. The most common toxicity otherwise was grade 1 local injection site reactions in four patients in the i.m. arm and five patients in the PMED arm. Other potentially vaccine-associated toxicities included fatigue, watery eyes, and abdominal pain in two patients each (6%), and nausea, arthralgia, myalgia, pruritus, and rash in one patient each (3%). In addition, one patient with a prior history of gout had an exacerbation after the third injection, and another previously irradiated patient experienced radiation recall after the first injection. The median follow-up was 24 months with a median progression-free survival of 17 months. Median survival was not yet reached.

Postimmunization increase in gp100-specific tetramer-reactive CD8+ cells
Following a 10-day culture, patient PBMC samples were stained with HLA*A0201-restricted gp100209-217 and gp100280-288 peptides at two baseline measurements and at weeks 18 and 30, again associated with 3 and 15 weeks after the final vaccination. We have found this 10-day culture necessary to detect low frequency self-antigen-specific T cells, and it is also known that this relatively short in vitro culture does not cause in vitro priming (16). Multiparameter flow cytometry was then performed to analyze these samples.

Overall, 4 of the 27 assessed patients experienced an increase in gp100 tetramer-reactive CD8+ cells postvaccination when compared with baseline, corresponding to 1 and 3 patients in the i.m. and PMED groups, respectively. Three patients were positive for gp100209-217 alone, and one patient was positive for both gp100209-217 and gp100280-288. Figure 1 shows dot plots for a representative positive patient, and Fig. 2 shows all patients’ changes in frequency of tetramer-reactive CD8+ cells from baseline to both postvaccination time points.

Phenotypic analysis of the tetramer-positive CD8+ cells: indicative of an effector cell population
Chemokine receptor 7 (CCR7) and CD45RA are often used to subtype CD8+ T-cell populations, providing the following phenotypes: naïve cells (CCR7-CD45RA+), central memory cells (CCR7+CD45RA+), effector memory cells (CCR7+CD45RA-), and effector cells (CCR7-CD45RA-). We therefore further characterized the gp100 tetramer-positive CD8+ cells based on analyzing the expression of the above markers. Every tetramer-positive sample carried the phenotype of an effector memory cell, exhibiting the above-described CCR7lo CD45RAlo expression. Specifically, the tetramer+ T cells were, on average, 80.2% CCR7lo (range, 60.2-96.1) and 76.3% CD45RAlo (range, 67.7-94.9). See Fig. 3 for representative dot plots.

Postimmunization increases in CD8+ IFN-γ+ cells and polyfunctionality
ICS was also performed on all samples and time points to assess the effect of vaccination on the intracellular cytokine constitution (Fig. 4). Five of the 27 patients analyzed showed an increase in CD8+ IFN-γ+ cells postvaccination when compared with baseline, with one and four patients in the i.m. and PMED groups, respectively. All five of these patient samples were restimulated with the gp100280-288 peptide, whereas none of the samples restimulated with
the gp100209-217 peptide showed such a response. These five patients were further analyzed for the intracellular presence of macrophage-inhibitory protein-1β, TNF-α, and CD107a. These are common cytokines used in vaccine response assessment and formed the basis of polyfunctionality analyses in our prior study (17). An increase in polyfunctionality (two or more intracellular cytokines) was observed in four of the five patients, with positive values corresponding to the time points of IFN-γ positivity. Figure 5 shows representative dot plots. Figure 6 shows the trends in each intracellular cytokine of the five patients who generated CD8+IFN-γ+ cells.

Correlation between immune responses, vaccination method, and clinical outcome

All demographic characteristics as well as treatment arm were analyzed for association with immune response and clinical outcome. There were no associations between any demographics and tetramer response or clinical outcome. However, two borderline associations became evident when assessing the ICS data which failed to reach statistical significance. First, all of the patients producing IFN-γ+CD8+ cells had no previous treatment (P = 0.06). In addition, this response may have been associated with the treatment arm, as 80% of these responders were in the PMED group (P = 0.07). Nevertheless, neither demographic information nor study arm was associated with overall immune response or clinical outcome, nor was the presence of an immune response associated with clinical outcome. An overall summary of specific immune response and clinical outcomes by study arm can be seen in Table 2.

Discussion

This randomized clinical trial was designed to validate and expand on the understanding of methods for DNA vaccination, both in comparing i.m. injection to PMED and in assessing the safety and feasibility of using PMED as an administration technique. With these end points in mind, we showed the ability of PMED to provide an equal or even enhanced tumor antigen–specific immune response at a significantly lower DNA dose. Furthermore, we showed that using PMED is a safe tool for DNA vaccination, providing minimal toxicity, aside from one patient with a presumed unknown gold allergy, and no autoimmune manifestations.

Previous research from our laboratory and from others has explored the use of xenogeneic vaccination to overcome poor immunogenicity of self-antigens. Preclinical models have shown the effectiveness of inducing tumor immunity using homologous DNA from other species (18), which has been further shown with a number of different antigens (18–21). This hypothesis has been validated by the U.S. Department of Agriculture licensure of the gp100 DNA Vaccine for Melanoma Patients

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first approved therapeutic cancer vaccine in the United States, a human tyrosinase DNA vaccine for the treatment of melanoma-afflicted dogs (14, 22). Currently, there are also ongoing DNA vaccination trials in multiple human malignancies including prostate, breast, lymphoma, and melanoma.

This is the first clinical comparison of PMED as a vaccination method compared with i.m. injection as far as we are aware. At first, it was shown that genetic vaccination using PMED generated immune responses (9). We then observed that DNA vaccination using PMED induced tumor-specific T-cell responses (10). There are a number of theories as to why PMED could have a superior potency to i.m. injection. First, it allows for direct transfection of resident APCs in the epidermis, including dendritic and Langerhans cells, by the gold particles. Furthermore, the particulate nature of this type of vaccine generates a stronger inflammatory response compared with that which would be produced from liquid i.m. injection. This increased inflammation might induce dendritic cell migration to local draining lymph nodes and enhance cytokine and chemokine production (23). Finally, by vaccinating directly into the epidermis and dermis, a rich supply of resident APCs are available, optimizing the opportunity for direct APC transfection. Preclinical models have explored this comparison, demonstrating an increased transgene expression with PMED, producing protective immunity in a variety of animal models of infection and cancer (9, 24).

Consistent with our previous gp100 DNA vaccination trial, there was an increased immune response compared with baseline. Four of 27 patients (15%) generated a gp100-specific tetramer-reactive CD8+ T-cell response. This was comparable with results in our tyrosinase367-377 peptide vaccine trial, in which 3 of 18 patients (17%) produced a positive CD8+tetramer+ response (15). Further analysis in this study revealed that these tetramer-positive CD8+ T cells carried an effector memory phenotype. In addition, 5 of 27 patients showed an increased postvaccination CD8∗IFN-γ+ cell production, 4 of which were not tetramer positive. Therefore, 30% (8 of 27) of our patients produced at least one marker of gp100 immune response after gp100 DNA vaccination.

One patient generated gp100209-217 tetramer-reactive cells, compared with none previously. This remained fewer than the four patients in the prior study who generated gp100280-288 tetramer-reactive cells, and interestingly, one of which was positive for both gp100 peptides. This supports the theory that whole antigen vaccination might
produce a tiered peptide-specific response with the dominance of a specific epitope which, however, does not prevent the codevelopment of alternate epitopes such as gp100209-217. Also remarkable was that one patient in the i.m. injection arm produced a CD8+IFN-γ response, similar to that of the previous trial. However, four patients in the PMED arm produced such a response, potentially indicative of a higher level of immunogenicity related to the mechanism of delivery. Within these groups, however, there did not seem to be a significant difference in inducing a polyfunctional cytokine response. In addition, the polyfunctionality of the cytokine response was not as robust as that which was seen in our prior DNA vaccine trials, including gp100 and granulocyte macrophage colony-stimulating factor DNA vaccines (6, 25).

There was also no progression-free survival advantage when comparing both arms of the study, nor when comparing who had a detectable immune response to treatment. It should be noted, however, that this is a pilot trial and was not designed to analyze survival. Nevertheless, it could be presumed that an immunologic response to vaccination is required for an effect on clinical outcome, but it is not enough.

Although the immune response following DNA vaccination might not be sufficient for clinical response, it provides a backbone for enhancement with immunomodulatory antibodies. In fact, much research has been in development, showing the detrimental outcomes of multiple vaccinations as a result of immunosuppression. A recent study using a mouse model showed that this immune suppression is often mediated by regulatory T-cells, which must be eliminated in order for vaccines to retain their efficacy (26). Potential adjuvants which may accomplish this task include the anti-OX40, anti-4-1BB, anti-CD25, anti-PD-1, and anti-CTLA-4 antibodies (27, 28).

Perhaps the most studied of these therapeutics is the antibody against CTLA-4 (ipilimumab/tremelimumab), a cell surface molecule which normally serves to control immune responses and avoid autoimmunity (29, 30). As monotherapy, it has been shown to be an effective

<table>
<thead>
<tr>
<th>Arm</th>
<th>Patient no.</th>
<th>Increase in gp100 tetramer–reactive cells</th>
<th>Increase in CD8+IFN-γ+ cells</th>
<th>Clinical status</th>
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<tr>
<td>i.m.</td>
<td>17</td>
<td>gp100280-288+</td>
<td>+</td>
<td>POD</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td></td>
<td></td>
<td>POD</td>
</tr>
<tr>
<td>PMED</td>
<td>4</td>
<td>gp100280-288+</td>
<td>+</td>
<td>POD</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>gp100280-288+</td>
<td>+</td>
<td>NED</td>
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<td></td>
<td>9</td>
<td>gp100209-217+; gp100280-288+</td>
<td>+</td>
<td>POD</td>
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<td></td>
<td>27</td>
<td>gp100280-288+</td>
<td>+</td>
<td>NED</td>
</tr>
</tbody>
</table>

Abbreviations: NED, no evidence of disease; POD, progression of disease.
treatment in patients with advanced melanoma, producing beneficial and lasting clinical responses in phase II (31, 32). Anti–CTLA-4 therapy could also produce or augment antigen-specific CD8+ responses both quantitatively and qualitatively with respect to polyfunctional cytokine production (17). Using immunomodulatory antibody therapy as an adjuvant in conjunction with DNA vaccination may augment immunologic potency.

Moreover, the above vaccine strategies have to be compared at different dosing levels. For instance, this trial only used i.m. injection at a dose of 2,000 μg, whereas our prior trial comparing different doses of gp100 DNA vaccine administered i.m. found that the lowest dose, 100 μg, was potentially more immunogenic than the highest dose, 1,500 μg/injection, although no definitive dose-response was found. A consensus in the field is that one reason for the drop-off in immunity to DNA immunization seen going from mouse studies to human studies could be a dose-scaling effect. Therefore, we used the highest practical dose for both i.m. and PMED administration. For i.m., this was based on volume and number of injections, and for PMED, this was based on the manufacturing methods developed by PowderMed. Determination of an optimal vaccination strategy may prove critical in effective antitumor immunization and treatment.

In summary, this pilot trial compared two methods of delivery for our gp100 DNA vaccine, demonstrating comparable efficacy in both study arms in terms of immune response and clinical outcome. Both mechanisms shared a good safety profile, aside from a hypersensitivity reaction following PMED injection in a patient with previously undiagnosed gold allergy. Both techniques induced a measurable immune response, either via HLA-A*0201–restricted gp100 tetramer+ CD8+ T cells carrying an effector memory phenotype or by producing IFN-γ+CD8+ T cells, with variable effects on polyfunctionality within the intracellular cytokine profile. Therefore, with the PMED delivery method’s ability to generate immune response (with a possible trend towards enhanced immunogenicity compared with i.m. injection) combined with the small quantity of DNA required to generate such a response, this study supports further exploration in the use of this approach in additional human studies, including in combination with other adjuvants.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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