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IMMUNOGENICITY FOR CD8+ AND CD4+ T CELLS OF TWO FORMULATIONS OF AN INCOMPLETE FREUND'S ADJUVANT FOR MULTYPEPTIDE MELANOMA VACCINES

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Abstract

An incomplete Freund's adjuvant (IFA) commonly used in experimental cancer vaccines has recently been reformulated. Oleic acid used in the surfactant was purified from a vegetable source (olives, IFA-VG) rather than an animal source (beef tallow, IFA-AN). To provide insight into adjuvant properties of the new formulation, we reviewed T cell responses, by ELISPOT assay, to multipeptide vaccines in two sequential clinical trials that spanned this transition of adjuvants. Analyses included 19 patients who received either IFA-AN or IFA-VG for all vaccines, and a subset of 93 patients best matched by study arm for vaccine antigens (12 melanoma peptides restricted by MHC Class I, 12MP; plus a tetanus helper peptide, tet) administered with IFA but without GM-CSF. Inflammation was observed at vaccine sites clinically for almost all patients, even including ulceration in a subset with each IFA formulation. CD8⁺ T cell response rates to the 12 melanoma peptides were 53% (95% CI (44, 61%)) for IFA-AN and 46% (95% CI (32, 59%)) for IFA-VG. In the 93 patient subset, those rates were 73% (95% CI (61, 83%)) and 70% (95% CI (47, 87%)), respectively. CD4⁺ T cell responses to tetanus helper peptide were identified in 94%

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CLS is listed as an inventor for several peptides used in the melanoma vaccines used in the clinical trials reported in this manuscript, but those patents were filed by the University of Virginia Patent Foundation and have been licensed to Glaxo Smith Kline. All other authors have declared there are no financial conflicts of interest in regards to this work.

(95% CI (86, 98%)) and 96% (95% CI (78, 100%)), respectively. Responses to individual HLA-A1, A2, and DR associated peptides were largely preserved, but reactivity trended lower for some HLA-A3 associated peptides. Despite the necessarily retrospective nature of the analysis and limitations of multiple comparisons, our summary data support use of IFA-VG as an adjuvant with multi-peptide vaccines in melanoma patients.

Keywords

cancer vaccines; T cells; adjuvants; CD4⁺ T cells; CD8⁺ T cells; peptides

Introduction

Numerous vaccines use an incomplete Freund's adjuvant, and many cancer vaccines use the incomplete Freund's adjuvant Montanide ISA-51 (Seppic, Inc., Paris, France). This is a clear liquid consisting of mineral oil Drakeol 6 VR plus an emulsifying agent mannide monooleate.¹ Mannide monooleate is a surfactant glycolipid comprised of oleic acid and a sugar. In formulations provided prior to 2006, the oleic acid was derived from beef tallow, but due to concerns internationally with risk of mad cow disease from bovine tissue, the source of oleic acid was changed. In the newer formulation, oleic acid was made from a vegetable source (olives). This replaced the animal form in 2006 for clinical trials and clinical use worldwide. The original adjuvant using oleic acid from beef tallow was simply referred to as Montanide ISA-51 and will be identified in this manuscript as Montanide ISA-51 AN (IFA-AN). The newer vegetable source Montanide ISA-51 is referred to as Montanide ISA-51 VG¹ (IFA-VG).

We have had substantial experience with Montanide ISA-51 in both of its formulations, in a series of clinical trials of melanoma peptide vaccines²⁻¹⁰ (and manuscripts in preparation). In the more recent trials, we have used a mixture of 12 Class I MHC restricted peptides (12MP), and are thus able to provide data about their immunogenicity both in IFA-AN and in IFA-VG. The 12MP include peptides of melanocyte differentiation proteins gp100 and tyrosinase and of cancer testis antigens MAGE-A1, -A3, -A10 and NY-ESO-1.⁸ The present report summarizes data on immune responses to 12MP for 194 patients enrolled in either of two clinical trials, in whom the assay methods were consistent. These vaccines also included a tetanus toxoid peptide designed to stimulate CD4⁺ helper T cells,² and data on its immunogenicity, with each of the two adjuvant formulations, are also provided.

Materials and Methods

Patients were enrolled in two clinical trials of multi-peptide melanoma vaccines, Mel43 and Mel44. Details about the Mel43 trial have been reported.¹¹ Many of the approaches in the Mel44 trial were similar, and have been reported in abstract form¹² and will be published in full separately.

Patients

For both trials, patients with resected AJCC stage IIB-IV (6th edition) melanoma arising from cutaneous, mucosal or unknown primary sites were eligible. Inclusion criteria included expression of HLA-A1, A2, or A3 (~80% of patients screened, data not shown), ECOG performance status 0-1, adequate liver and renal function, and ability to give informed consent. Exclusion criteria included: ocular melanoma; pregnancy; cytotoxic chemotherapy, interferon, or radiation within the preceding 4 weeks; known or suspected allergies to vaccine components; multiple brain metastases; use of steroids; Class III-IV heart disease; or significant autoimmune disease. Patients were studied following informed consent, and

with Institutional Review Board (HIC#10524 and 11491) and FDA approval (BB-IND #9847 and 12191) for Mel43 and Mel44, respectively. The studies differed in their lower age limit: age 12 for Mel43 and age 18 for Mel44. They also differed in that HLA-DR typing was required only for Mel44, and patients on that trial had to express HLA-DR1, 4, 11, 13, or 15 (~80% of melanoma patients screened). Toxicities were recorded in accordance with NIH common toxicity criteria v.3.

Peptides used in vaccines

All patients in both trials received a vaccine comprising 12 melanoma peptides restricted by HLA-A1, -A2, or -A3, as described⁸: A1 peptides: DAEKSDICTDEY (Tyrosinase_{240–251}, which has a substitution of S for C at residue 244), SSDVIPIGTY (Tyrosinase_{146–156}), EADPTGHSY (MAGE-A1_{161–169}), EVDPIGHLY (MAGE-A3_{168–176}); A2 peptides: YMDGTMSQV (Tyrosinase_{369–377D}), IMDQVPFSV (gp100_{209–217}, 209-2M), YLEPGPVTA (gp100_{280–288}), GLYDGMEHL (MAGE-A10_{254–262}); and A3 peptides: ALLAVGATK (gp100_{17–25}), LIYRRRLMK (gp100_{614–622}), SLFRVITK (MAGE-A1_{96–104}), and ASGPGGGAPR (NY-ESO-1_{53–62}). The NY-ESO-1 peptide ASGPGGGAPR, originally reported to be immunogenic in association with HLA-A31, is naturally processed and presented also by HLA-A*0301 (A3) on human cells.¹³ For all patients on Mel43 and for those on arms A and B of Mel44, a tetanus helper peptide AQYIKANSKFIGITEL² was used. For arms C and D on Mel44, the vaccines included a mixture of six melanoma-associated helper peptides (6MHP).⁵

Peptide synthesis and GMP vialing of peptides for vaccines

Peptides for vaccines were synthesized and purified (> 95%) under GMP conditions (Multiple Peptide Systems, now NeoMPS, San Diego, CA). After solubilization, each peptide was sterile-filtered, mixed, vialled and lyophilized under GMP conditions by Merck Biosciences AG Clinalfa (L'ouvelfingen, Switzerland) in single-use vials. The 12 melanoma peptides 12MP were combined and vialled together. The tetanus peptide was vialled as one preparation. The 6 melanoma helper peptides 6MHP were combined and vialled together. Each preparation was stored as a lyophilized preparation in sterile single-use vials. DMSO was not used in the preparations. Vials were submitted to quality-assurance studies including sterility, identity, purity, potency, general safety, pyrogenicity, and stability in accordance with CFR guidelines and BB-IND #9847 and 12191.

Vaccine emulsion preparation and administration

Each vaccine was 2 ml of a stable water-in-oil emulsion consisting of 100 mcg of each of the 12 Class I MHC restricted peptides, 190 mcg of the tetanus helper peptide (or 6MHP), and 1 ml Montanide ISA-51 adjuvant (Seppic, Inc., Paris, France/ Fairfield, NJ). For patients in arms B and D in Mel43, the emulsion also contained 110 mcg GM-CSF (Leukine/ Sargramostim; Berlex, Seattle, WA; now Genzyme). For Mel43 arms A and B, the full emulsion was administered to one extremity skin location, whereas for Mel43 arms C and D and for Mel44, the emulsion was divided in half, with half delivered to each of two extremities. The emulsions were made by use of the two-syringe method. A drop of each emulsion was tested for stability in water. The vaccines were administered on days 1, 8, 15, 29, 36, and 43 (weeks 0, 1, 2, 4, 5, and 6), then at 3, 6, 9, and 12 months. At each injection site, half of the dose was administered subcutaneously (s.c.), and half intradermally (i.d.).

Clinical trial design

The Mel43 study was designed to estimate: (1) whether GM-CSF administered locally changes the immunogenicity of vaccination with multiple synthetic melanoma peptides in an emulsion with incomplete Freund's adjuvant, and (2) whether vaccination at two extremity

sites induces different immunogenicity than vaccination at a single site. The Mel44 study was designed to test (1) whether melanoma-associated class II MHC-restricted helper epitopes included in the vaccine regimen augment the magnitude of CD8⁺ T cell responses and contribute to the development of persistent cytotoxic T cell responses to the class I MHC-restricted peptides in the vaccine, and (2) whether pre-treatment with a single dose of cyclophosphamide prior to vaccination enhances the immunogenicity of a peptide-based vaccine.

Patients on both studies were randomized to one of four vaccine regimens. Patients were stratified on stage of disease (IIB/C vs. III vs. IV), and randomization was based on a random assignment within strata with varying block sizes. For Mel44, patients were stratified by HLA type and participating institution.

Collection of peripheral blood mononuclear cells (PBMC)

Peripheral blood (60 ml) was drawn into heparinized tubes for isolation of PBMC, at multiple time points during the vaccine regimen. Lymphocytes were isolated using Ficoll gradient centrifugation, and cryopreserved in 10% DMSO/90% serum by the Tissue Procurement Facility at the University of Virginia. Blood drawn at other participating institutions was shipped in insulated containers near room temperature for overnight delivery and was processed as above.

ELISpot assays

ELISpot assays were performed directly *ex vivo*, after cryopreservation (direct ELISpot). Methods for these direct ELISpot assay have been reported.¹¹ Briefly, 200,000 PBMC were plated per well, and pulsed with synthetic peptide (10 mcg/ml), in quadruplicate. The peptides used in the assays corresponded to those used for vaccination. Two peptides in the vaccines (DAEKSDICTDEY and IMDQVPFSV) are modified from their original sequences. The natural sequence DAEKCDICTDEY is problematic to use in assays because the two cysteine residues can cause cyclization by internal disulfide bonds of the free peptide,¹⁴ thus, the assays were performed against the modified peptide which is cross-reactive with the native sequence.¹⁴ The IMDQVPFSV peptide is modified at the 2nd position from ITDQVPFSV.¹⁵ The ELISpot assays were done against the modified peptide, but tetramer assays have also been performed and reported, for which the natural sequence peptide ITDQVPFSV was used.¹¹ Controls included irrelevant peptides, a mixture of viral peptides (CEF peptide pool), PMA-ionomycin and PHA. Assessment of immunologic response was based upon a fold-increase over the maximum of two negative controls. Evaluation of T-cell responses was based on the following definitions:

N_{vax} = number T-cells responding to vaccine peptide; N_{neg} = number T-cells responding to maximum negative control; $R_{\text{vax}} = N_{\text{vax}}/N_{\text{neg}}$.

For evaluations of PBMC, a patient was considered to have a T-cell response to vaccination (binary yes/no), by direct ELISpot assay only if all of the following criteria were met: (1) N_{vax} exceeded N_{neg} by at least 20 cells / 100,000 CD4⁺ or CD8⁺ cells (0.02%), where CD8 and CD4 counts were based on flow cytometric evaluations of the PBMC samples. (2) $R_{\text{vax}} \geq 2$, (3) $(N_{\text{vax}} - 1 \text{ SD}) \geq (N_{\text{neg}} + 1 \text{ SD})$, and (4) R_{vax} after vaccination $\geq 2 \times R_{\text{vax}}$ pre-vaccine, as described. The same criteria applied for stimulated ELISpot assays except that the threshold for criterion (1) was higher: such that N_{vax} had to exceed N_{neg} by at least 100 cells / 100,000 CD4⁺ or CD8⁺ cells (0.1%). Fold-increases less than one (e.g., control counts exceed number of responding T-cells, or fold response compared to baseline is less than one) were set equal to one to indicate no response and to prevent overinflating adjusted fold-increases due to pre-vaccine ratios less than one, or division by zero, while not affecting the determination of response. These methods are consistent with our prior analyses.⁷

Continuous measures of immune response denoted as fold-increase must satisfy conditions (1)–(3), and were defined as the amount of R_{vax} . Cumulative response over all HLA-appropriate peptides, $\text{Cum}R_{\text{time}}$, was defined, at each time point, as 1 + the sum of fold-increase exceeding 1 over all patient-specific peptides (eg. at week 3, $\text{Cum}R_3 = 1 + (\text{sum over each } (R_{\text{vax}} - 1) \text{ for each peptide for which a response was detected})$). When making comparisons across HLA types, this cumulative response is also calculated for the four peptides restricted by each HLA-Class I allele. When making comparisons across patients overall, this is calculated for all HLA-appropriate peptides in the 12MP, which may be 4 or 8 peptides, depending on HLA type.

Interassay CVs were calculated for normal donors in their response to the CEF peptide pool. A high responder and a low responder were tested in each assay. For Mel43, the weighted mean CVs were 17% for high responders and 34% for low responders; for Mel44, the respective values were 10% for high responders and 34% for low responders.

Maximal response was based upon responses in the blood to the first 6 vaccinations (through day 50). Patients who discontinued protocol therapy prior to collection of all blood samples for allergic reactions or adverse events, disease progression, or noncompliance were considered immune response failures if no response was observed in evaluable samples. Immune response was a binary indicator of whether or not the criteria listed above were met, and immune response rates were calculated as the proportion of participants with an immune response.

Statistical analyses

The data from these studies have been reviewed by two statisticians (GP and MS) but are presented without p-values because these analyses were not preplanned or powered for evaluation of differences. Non-significant statistical differences could occur because the sample sizes were small and not necessarily because there is no difference in immune response. Rates of immune response, 95% exact binomial confidence intervals and confidence intervals of differences in rates of immune response were calculated using SAS 9.1.

Results

Eligibility review and patient subsets

Final enrollment was 121 eligible patients on Mel43 and 167 eligible patients on Mel44 (total 288). Information on the Montanide ISA-51 formulation used for each vaccine was available for most patients. For 197 patients, one formulation was used for all vaccines, with the remainder receiving both formulations. These 197 are the basis of the current report, of which 194 were evaluable for CD8+ T cell responses to 12MP and one additional patient was evaluable for CD4+ T cell response to tetanus peptide only. All patients on the Mel43 trial received IFA-AN formulation. Among 76 patients on the Mel44 trial where only one formulation was used, 19 received IFA-AN, and 57 received the newer IFA-VG. (Table 1) Patients on both trials were randomized to 4 study arms, and numbers on each are shown in Table 2. On Mel43, patients vaccinated with peptides in Montanide ISA-51 + GMCSF (arms B and D) had significantly lower immune response rates than those on Mel43 vaccinated without GM-CSF.¹¹ Mel43 also tested whether vaccination in one skin site (A, B) or two (C, D) affected immune responses, but this did not have an impact.¹¹ Patients on Mel44 were all vaccinated with Montanide ISA-51 without GM-CSF, but were evaluated for the effect of pretreatment with one dose of 300mg/m² cyclophosphamide (Cytosan), which had no significant effect on immune responses (manuscript in preparation).¹² Mel44 patients were also evaluated for the immunologic effect of helper peptides derived from tetanus

toxoid² or from melanoma proteins (6MHP).⁵ Overall, immunogenicity of the 12MP was significantly greater for the patients vaccinated with tetanus helper peptide than with the 6 melanoma helper peptides (manuscript in preparation).¹²

A subset of patients on two arms of Mel43 (A and C) and on two arms of Mel44 (A and B) received the same vaccines (12MP + tetanus peptide, without GM-CSF), and differed only in whether they were vaccinated in one or two vaccine sites, and whether or not they received cyclophosphamide, neither of which significantly impacted immune responses to 12MP. Thus, a separate analysis has been performed for this subset of patients who had these comparable immunizations. This group comprises 60 Mel43 patients and 33 Mel44 patients (total 93, Tables 1 and 2) evaluable for response to 12MP, plus one Mel43 patient evaluable only for response to tetanus peptide.

Injection site reactions

Clinicians involved in administering vaccines on both trials have had the impression of similar injection site reactions with the transition from IFA-AN to IFA-VG. We have commonly observed ulceration at the vaccine sites in some of our patients. For the 19 patients on Mel44 with IFA-AN, injection site reactions were reported in 100%, with ulceration in 21% (4/19). For the 57 patients on Mel44 with IFA-VG, injection site reactions were reported in 99%, with ulceration in 14% (8/57).

CD8 T cell response to 12MP

The overall rate of immune responses to the pool of 12 melanoma peptides (12MP) restricted by Class I MHC molecules was 50.5%. Similar immune response rates were observed for the two Montanide formulations on the Mel44 trial and overall (Figure 1A). Patient treatments were particularly well-matched for the subset of patients on Mel43 arms A+C and Mel44 arms A+B. For this patient subset, the immune response rates for the two adjuvant formulations were 70.0% (95% CI (35, 93%)) for IFA-AN and 69.6% (47, 87%) for IFA-VG for patients on Mel44, and 72.9% (61, 83%) vs. 69.6% (47, 87%) overall (Figure 1B).

CD8 T cell response by peptide and by HLA allele

The CD8⁺ T cell response rate to each of the 12 peptides restricted by HLA-A1, A2, or A3 is shown in Table 3 for animal or vegetable source Montanide ISA-51. Overall, the ratio of the CD8⁺ T cell response rates (IFA-VG/IFA-AN) was greater than 0.8 for 7 of the 12 peptides, including all 4 HLA-A1 peptides and 3 of the 4 HLA-A2 peptides (Table 3). The HLA-A3 peptides were all less immunogenic (ratio < 0.4) in the vegetable formulation, but this could be explained partly by an imbalance in the number of HLA-A3 patients randomized to groups C and D of Mel44 compared to groups A and B (17 vs. 11, respectively).

In the same assessment for the subset of patients vaccinated with 12MP + tetanus peptide, without GM-CSF, the ratio of the CD8⁺ T cell response rates (IFA-VG / IFA-AN) was greater than 1.0 for 5 of the 12 peptides and at least 0.6 for 7 of the 12 peptides, including 3 of the HLA-A1 peptides and 3 of the HLA-A2 peptides (Table 3). The HLA-A3 peptides were all less immunogenic in the vegetable formulation, but with higher ratios than for the complete dataset (ratios 0.35 to 0.64), not considering LIY.

The CD8⁺ T cell response rates were evaluated by HLA allele, for the whole data set broken down by clinical trial and type of adjuvant formulation (Figure 2A), and for the subset of patients vaccinated with 12MP + tetanus peptide, without GM-CSF, divided into two groups by formulation of Montanide ISA-51 (Figure 2B). Here, as above, the immunogenicity of

HLA-A1 peptides and A2 peptides are quite similar, with a suggestion of lower immunogenicity in IFA-VG only for the A3 peptide responses.

CD4 T cell response to the tetanus helper peptide

The CD4⁺ T cell response rate to the tetanus helper peptide, restricted by HLA-DR is shown in Table 3 and Figure 2C for animal or vegetable source Montanide ISA-51, specifically for the subset of patients vaccinated with 12MP + tetanus peptide, without GM-CSF. Immune response rates were comparable (RR 1.01).

Actual peptide-specific T cell percentages

The range of the CD8⁺ or CD4⁺ T cell responses to each of the 12 class I restricted peptides and to tetanus peptide varied across the study population, as a percentage of total CD8⁺ or CD4⁺ T cells, but these magnitudes were similar for the IFA-AN and IFA-VG types of adjuvant, for the HLA-A1 and HLA-A2-restricted peptides and tetanus peptide, but trended lower for HLA-A3-restricted peptides (Figure 3).

Discussion

Defined antigen vaccines used clinically to induce protective antiviral immunity routinely contain adjuvants; however, optimal adjuvants for cancer vaccines are not yet known. Traditional adjuvants such as alum and incomplete Freund's adjuvants are effective for induction of protective antiviral immunity, which can be mediated by induction of neutralizing antibodies. A newer challenge is to induce therapeutic immunity against cancer antigens recognized by T cells, especially CD8⁺ T cells. Not only is this complicated by tumor-induced tolerance, but also, it is made more difficult by inadequate understanding of the critical functions of adjuvants. Though other promising adjuvants exist, they remain to be optimized. Thus, numerous vaccine trials for cancer use adjuvants with which there has been decades of experience. One of the most commonly used such adjuvants is the incomplete Freund's adjuvant Montanide ISA-51 (Seppic), which is not FDA-approved as an adjuvant but is available now in a GMP formulation for experimental clinical studies and is used globally in veterinary vaccines. It is particularly relevant that addition of a peptide vaccine to high-dose IL-2 therapy in melanoma patients increased progression-free survival (the primary endpoint of the study, $p = 0.01$) and clinical response rate, and that this vaccine used Montanide ISA-51 as its adjuvant.¹⁶ This is the first successful randomized phase III trial of a peptide vaccine in any cancer and the first successful randomized phase III trial of any vaccine in melanoma. Thus, recent questions about the impact of changes in the formulation of this adjuvant are important to progress in the field.

The older formulation of the adjuvant (Montanide ISA-51 AN), which included oleic acid from an animal source, was replaced with the current formulation (Montanide ISA-51 VG) using oleic acid from a vegetable source. There was no formal process for comparing the adjuvants side-by-side in a randomized manner prior to the transition. Instead, in the present report we have evaluated immune responses to multi-peptide vaccines in two sequential trials that represented our transition between these two adjuvant formulations. We have observed similar injection site reactions clinically, with similar rates of ulceration between the Mel43 and Mel44 trials, though a trend to slightly lower severity of injection site reactions is suggested and cannot be ruled out. In analysis of the ELISpot data on immune responses, findings are numerical increases in immune response rates to some peptides and decreases in response rates to others (Table 3), but the summary CD8⁺ T cell response rates to the 12-peptide mixture were very similar for those vaccinated with IFA-AN and for those vaccinated with IFA-VG. The estimated difference overall in immune response rates to 12MP (VEG-ANIMAL) was -7% (95% CI (-22, 8%)) for IFA-VG (Figure 1A). When

excluding study groups that were vaccinated with less immunogenic strategies, and evaluating the subset of patients in groups that received the same immunogens (12MP + tetanus peptide) without the negative effects of GM-CSF, the immune response rates to 12MP were very similar (Figure 1B), with an estimated difference for VG-AN of -3% (95% CI (-25, 18%)). In addition to the effect on CD8⁺ T cell responses, rates of helper T cell responses were >90% and were almost identical for the two formulations across more than 90 patients (Table 3, Figure 2C).

A peptide of particular interest that was included in this vaccine preparation is the one used by Schwartzenuber et al in their successful vaccine trial.¹⁶ This peptide from gp100 (residues 209–217, with substitution of methionine for threonine at position 2) has the sequence IMDQVPFSV, and its native sequence counterpart is ITDQVPFSV. We did not detect any decrement in the immunogenicity of that peptide in our trials, with a ratio of immunogenicity between the two adjuvant formulations of 1.00 (Table 3). A weakness of the analysis for that peptide is that the ELISpot assays were done with the modified peptide IMDQVPFSV as a target, rather than the native sequence. Nonetheless, the data do not suggest a change in immunogenicity of this peptide with the new adjuvant formulation. All but two of the peptides in the 12MP mixture are natural sequences; only the gp100 209-2M peptide and the Tyrosinase 240–251 peptide restricted by HLA-A1 have been modified from the natural sequence to improve immunogenicity. Regardless, the lack of detectable change in reactivity to the gp100–209-2M peptide, and the immunogenicity of numerous other peptides, both for CD4⁺ and for CD8⁺ responses, suggests that this newer adjuvant formulation Montanide ISA-51 VG is useful for multi-peptide vaccines, at least as we have administered it. We acknowledge also that there are differences in the way the various clinical trials are designed in their preparation and administration of melanoma vaccines. Features of our approach that have a potential to affect immune response to vaccines containing IFA are listed in Table 4, along with some potential implications.^{17–20}

In summary, the present manuscript provides data that suggests that the new formulation of Montanide ISA-51 incomplete Freund's adjuvant is associated with decreased immunogenicity only of a few HLA-A3 associated peptides among a panel of 12 melanoma peptides, and with preservation or increased immunogenicity of others, including some peptides commonly used in melanoma vaccines restricted by HLA-A1 or HLA-A2. Overall, immunogenicity of the 12MP mixture is quite stable with the new VG formulation compared to the older formulation, and this consistency is evident also for the CD4⁺ T cell response to a tetanus helper peptide. Obviously these clinical trials were not originally designed to test for a difference in formulation, and with only 57 receiving the VG formulation, care should be taken in interpreting the results. P values for differences between groups are not provided because of their inadequacy in ruling out type 2 errors for small differences, and the challenge of defining a significant cutoff for a p value in the setting of multiple comparisons and in a non-randomized comparison that was not pre-designed. However, upon review, the aggregate data do not seem to support that immune responses decrease drastically with the newer vegetable formulation. It is important to understand more about the effects of adjuvants that are critical to induction of anti-tumor T cell responses to a vaccine, and also to use this information to develop a set of adjuvants that are optimized for varied types of vaccines. However, for the present, the current GMP-grade formulation of Montanide ISA-51 VG does appear to have adjuvant properties for peptide vaccines in melanoma patients.

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Figure 1. CD8 T cell responses to 12MP

Immune response rates, by study, to one or more of 12 MHC Class I restricted melanoma peptides were assayed by Direct ELIspot assay and are shown for patients vaccinated with peptides in Montanide ISA-51 AN (white bars) and in Montanide ISA-51 VG (black bars). The data in (A) represent 137 patients receiving Montanide ISA-51 AN (118 on Mel43, and 19 on Mel44) and 57 patients receiving Montanide ISA-51-VG on the Mel44 trial. The data in (B) represent the subset of 93 patients receiving 12MP + tetanus peptide, in Montanide ISA-51, without GM-CSF (Mel43 arms A+C and Mel44 arms A+B).

A **B** **C**

Figure 2. CD8 and CD4 T cell responses by HLA allele

The percentage of patients with a T cell response to any of the peptides restricted by HLA-A1, A2, or A3 are shown in panels A and B, where the data in (A) represent Mel43 patients receiving Montanide ISA-51 AN (vertical hatched bars), Mel44 trial receiving Montanide ISA-51 AN (white bars), and Mel44 patients receiving Montanide ISA-51 VG (black bars). The data in (B) represent the subset of 93 patients vaccinated with 12MP + tetanus peptide, without GM-CSF (Mel43 arms A+C and Mel44 arms A+B), for patients receiving Montanide ISA-51 AN (white bars) and Montanide ISA-51 VG (black bars). Note that the for the IFA-VG group, the HLA-A1 data represent just 6 patients. In (C), the percentage of patients with a CD4+ T cell response to the tetanus peptide, also detected by direct IFN-

gamma ELISpot assay, is shown for the subset of patients vaccinated with 12MP + tetanus peptide, without GM-CSF (Mel43 arms A+C and Mel44 arms A+B), for patients receiving Montanide ISA-51 AN (white bars) and Montanide ISA-51 VG (black bars).

Figure 3. The absolute magnitude of T cell responses

The percentage of CD4⁺ or CD8⁺ T cells producing IFN-gamma in response to each of the 12 peptides in 12MP (abbreviated with 3–4 letter abbreviations), or to the tetanus helper peptide (TET), are shown. For each peptide, the maximum value across multiple time points was used for each patient. These are shown for the subset of patients vaccinated with the 12MP + tetanus peptides, without GM-CSF, with IFA-AN (**A**) or IFA-VG (**B**). In this box plot format, the box represents the 25th to 75th percentile values, the horizontal line inside the box is the median, the symbol in the box is the mean, and the tails of the stems represent the minimum and maximum values.

Table 1

Distribution of patients by IFA formulation

Clinical trial	Type of Montanide ISA-51	No. of patients Mel43 + Mel44*				No. of patients with 12MP + tetanus peptide, no GMCSE			
		Total	HLA-A1+	HLA-A2+	HLA-A3+	Total	HLA-A1+	HLA-A2+	HLA-A3+
Mel43	IFA-AN	118	46	59	38	60	24	30	22
Mel44	IFA-AN	19	9	10	8	10	5	7	3
	IFA-VG	57	15	31	31	23	6	13	13
Total	IFA-AN	137	55	69	46	70	29	37	25
	IFA-VG	57	15	31	31	23	6	13	13
Total	Total	194	70	100	77	93	35	50	38

* Some patients express two of the HLA alleles A1, A2, or A3; so the total number of patients is less than the sum of the number expressing each of those alleles.

Table 2

Distribution of patients by study arm evaluable based on type of IFA used

Clinical Trial	Study arm	Peptide antigens	Local adjuvant	Systemic adjuvant	IFA-AN	IFA-VG	Mixed	Unkn	Total
Mel43	A	12MP + tet	IFA	--	<u>30</u>	0	0	0	30
	B	12MP + tet	IFA + GMCSF	--	30	0	0	0	30
	C	12MP + tet	IFA	--	<u>30</u>	0	0	0	30
	D	12MP + tet	IFA + GMCSF	--	28	0	0	0	28
Mel44	A	12MP + tet	IFA	--	<u>6</u>	<u>11</u>	3	21	41
	B	12MP + tet	IFA	Cy*	<u>4</u>	<u>12</u>	5	20	41
	C	12MP + 6MHP	IFA	--	3	19	0	20	42
	D	12MP + 6MHP	IFA	Cy	6	15	2	20	43
Total	All	All	IFA +/- GMCSF		137	57	10	81	285
Mel43	A+C	12MP + tet	IFA	--	<u>60</u>	0	0	0	60
	B+D	12MP + tet	IFA + GMCSF	--	58	0	0	0	58
Mel44	A+B	12MP + tet	IFA	--/Cy	<u>10</u>	<u>23</u>	8	41	82
	C+D	12MP + 6MHP	IFA	--/Cy	9	34	2	40	85
Subset*	--	12MP + tet	IFA	--/Cy	70	23	8	41	142

Note: In Mel44, skew toward more patients in less immunogenic arms in the IFA-VG group

* Cy = cyclophosphamide

** Subset = patients on Mel43 Arms A and C plus Mel44 arms A and B, who all received 12MP + tetanus peptide, in IFA, without GM-CSF (underlined).

Table 3
Immune response to each of 12 peptides for Mel43 and Mel44 studies

Rates of CD8 T cell responses to each of the 12 peptides in 12MP and of CD4 T cell responses to the tetanus peptide are shown for patients receiving Montanide ISA-51 consisting of oleic acid from animal or vegetable sources. The relative risk of immune response for vegetable over animal is als listed.

HLA	Peptide sequence	Source protein	All patients Mel43 and Mel44			Subset: Mel43 A+C and Mel44 A+B		
			AN % pos	VG % pos	Ratio VG/AN	AN % pos	VG % pos	Ratio VG/AN
A1	DAEKSDICTDEY	Tyrosinase	40 %	33 %	0.83	55 %	33 %	0.60
	EADPTGHSY	MAGE-A1	5 %	7 %	1.22	3 %	17 %	4.91
	EVDPIGHLY	MAGE-A3	13 %	13 %	1.05	17 %	33 %	1.94
	SSDYVIPIGTY	Tyrosinase	7 %	7 %	0.92	7 %	0 %	0.00
A2	GLYDGMHL	MAGE-A10	26 %	26 %	0.99	35 %	46 %	1.32
	IMDQVPFSV	Gp100 _{209-2M}	42 %	42 %	1.00	60 %	69 %	1.16
	YLEPGPVT A	Gp100 ₂₈₀	7 %	6 %	0.89	5 %	8 %	1.43
	YMDGTMSQV	Tyrosinase	12 %	6 %	0.56	16 %	8 %	0.48
A3	ALLAVGATK	Gp100	24 %	6 %	0.27	44 %	15 %	0.35
	ASGPGGAPR	NY-ESO-1	9 %	3 %	0.37	12 %	8 %	0.64
	LIYRRRLMK	Gp100	7 %	0 %	0.00	8 %	0 %	0.00
	SLFRAVITK	MAGE-A1	48 %	16 %	0.34	72 %	31 %	0.43
DR	AQYIKANSKFIGITEL	Tetanus				94 %	96 %	1.01

Table 4

Details of vaccine preparation and administration

Feature	Detail	Implications
Vaccine preparation	Multiple peptides administered together	A non-randomized study suggested that addition a tyrosinase peptide decreased immunogenicity of the gp100 209-2M peptide. ¹⁷ However, concerns about competitive inhibition of immunogenicity were not supported in a randomized trial. ⁸
Peptide solubilization	Peptides were solubilized in an aqueous solution, without DMSO	DMSO has not been evaluated for its effect on emulsions or on immunogenicity, but has been avoided for concerns about possible impacts on the emulsions.
Peptide synthesis and vialing	GMP-grade peptides were solubilized, sterile-filtered, mixed, vialled, and lyophilized under GMP conditions, and quality assurance studies confirm identity, stability, purity and stability over this interval. ¹⁸	The gp100 209-2M peptide contains a methionine residue at position 2, which is susceptible to oxidation. This occurs to a very limited extent in peptide preparations used in this study based on mass-spectrometry confirmation of molecular identity of the peptides. ¹⁸
Storage of peptide vials	Lyophilized, -80oC.	These conditions maintain stability and identity of the peptides for years, in our hands. ¹⁸
Creation of vaccine emulsions	The two-syringe method was used, with confirmation of emulsion stability by the water drop test	In our hands, the vortex method does not produce stable emulsions (unpublished observations).
Peptide dose	100 mcg per class I MHC restricted peptide, 190 mcg per helper peptide	This is at the lower range of doses used in other vaccine trials.
Vaccination frequency	Weekly for most of the vaccines	Weekly vaccines in the same site may induce cumulative changes in the vaccine site microenvironment that differ from vaccines rotated from one site to another with each vaccine (manuscript submitted). ¹⁹ Also, in that study, the vaccine site microenvironments of the subcutaneous location and the intradermal location appear to differ. ¹⁹ One or more vaccines with IFA may create a depot effect that acts as a sink for antigen-reactive T cells. ²⁰
Vaccine location	Each vaccine is given into the same skin site.	
Depth of vaccine injection	Half intradermal and half subcutaneous.	
Use of helper peptides	All vaccines in this report included one or more peptides to stimulate helper T cell responses	It is conceivable that responding CD4+ T cells may change the nature of the CD8 T cell response and/or alter the microenvironment induced by the adjuvant.