

Effect of Granulocyte/Macrophage Colony-Stimulating Factor on Circulating CD8⁺ and CD4⁺ T-Cell Responses to a Multi-peptide Melanoma Vaccine: Outcome of a Multicenter Randomized Trial

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Abstract Purpose: Granulocyte/macrophage colony-stimulating factor (GM-CSF) administered locally together with vaccines can augment T-cell responses in animal models. Human experience has been limited to small and uncontrolled trials. Thus, a multicenter randomized phase II trial was done to determine whether local administration of GM-CSF augments immunogenicity of a multi-peptide vaccine. It also assessed immunogenicity of administration in one versus two vaccine sites.

Experimental Design: One hundred twenty-one eligible patients with resected stage IIB to IV melanoma were vaccinated with 12 MHC class I-restricted melanoma peptides to stimulate CD8⁺ T cells plus a HLA-DR-restricted tetanus helper peptide to stimulate CD4⁺ T cells, emulsified in incomplete Freund's adjuvant, with or without 110 µg GM-CSF. Among 119 evaluable patients, T-cell responses were assessed by IFN-γ ELISpot assay and tetramer analysis. Clinical outcomes were recorded.

Results: CD8⁺ T-cell response rates to the 12 MHC class I-restricted melanoma peptides (by day 50) with or without GM-CSF were 34% and 73%, respectively ($P < 0.001$), by direct ELISpot assay. Tetramer analyses corroborated the functional data. CD4⁺ T-cell responses to tetanus helper peptide were higher without GM-CSF (95% versus 77%; $P = 0.005$). There was no significant difference by number of vaccine sites. Three-year overall and disease-free survival estimates (95% confidence interval) were 76% (67-83%) and 52% (43-61%), respectively, with too few events to assess differences by study group.

Conclusions: High immune response rates for this multi-peptide vaccine were achieved, but CD8⁺ and CD4⁺ T-cell responses were lower when administered with GM-CSF. These data challenge the value of local GM-CSF as a vaccine adjuvant in humans. (Clin Cancer Res 2009;15(22):7036-44)

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Sharp, and the Patients and Friends Research Fund of the University of Virginia Cancer Center. GM-CSF (made by Berlex/now Bayer) and Montanide ISA-51 (made by Seppic) were used in the vaccines in this trial, but these were paid for by the University of Virginia. No corporate funding support was provided for this study. Berlex has provided support (in the form of GM-CSF at no charge) for a different clinical trial for which C.L. Slingluff, Jr., is the study chair, so he has a relationship with Berlex related to that separate support.

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Translational Relevance

Cancer vaccines hold promise for the treatment of melanoma and other cancers; however, their promise is not yet fully realized. Cytokines may modulate the effectiveness of cancer vaccines. This article reports outcomes of a multicenter randomized prospective clinical trial of a melanoma vaccine with or without the cytokine granulocyte/macrophage colony-stimulating factor and with administration of vaccines in either one or two extremity sites. The findings are based on human clinical experience and include laboratory studies of the immune response to the vaccines. The findings have direct clinical translational relevance.

Vaccines of all kinds use adjuvants to increase immune responses; however, the molecular and cellular mechanisms of adjuvants are poorly understood. Mechanisms believed to be important include dendritic cell activation and maturation and activation of innate immunity through Toll-like receptor activation. The cytokine granulocyte/macrophage colony-stimulating factor (GM-CSF) has been reported to have potent adjuvant effects, presumed to be mediated through effects on dendritic cells and other antigen-presenting cells. In murine studies, GM-CSF has been an effective adjuvant for cancer and viral vaccines. A syngeneic whole-cell vaccine for melanoma was most effective at inducing protective immunity when B16 melanoma cells were transduced with a gene encoding GM-CSF (1). Similarly, immunogenicity of a peptide vaccine for HIV was augmented by inclusion of GM-CSF in the adjuvant emulsion in which the peptides were administered (2).

In humans, we found that vaccination with peptides in an emulsion of GM-CSF plus incomplete Freund's adjuvant was more immunogenic than vaccination with peptides pulsed on dendritic cells (3). Other peptide- and cell-based vaccines have been administered with local GM-CSF, with encouraging immunologic and clinical outcomes (4–8). On the other hand, a study comparing several adjuvants in sequentially treated cohorts challenged the adjuvant effect of GM-CSF (9). In another study, adding GM-CSF to an adjuvant appeared to increase CXCR3 expression by vaccine-induced T cells (10). The present study was designed to test formally whether immunogenicity of a multi-peptide vaccine emulsion was increased by including GM-CSF in the vaccine emulsion. The vaccine includes 12 MHC class I-restricted melanoma peptides (12MP) plus a tetanus helper peptide restricted by HLA-DR molecules. Thus, this study was designed to test effects of GM-CSF on both CD8⁺ and CD4⁺ T-cell responses to a synthetic vaccine. The study also was designed to address whether vaccination at one versus two extremity sites affects vaccine immunogenicity.

Materials and Methods

Patients. Patients with resected American Joint Committee on Cancer stage IIB to IV melanoma arising from cutaneous, mucosal, or unknown primary sites were eligible. Inclusion criteria included expression of HLA-A1, HLA-A2, or HLA-A3 (~80% of patients screened; data not shown), ages ≥12 years, Eastern Cooperative Oncology Group performance status 0 to 1, adequate liver and renal function, and ability

to give informed consent. Exclusion criteria included ocular melanoma; pregnancy; cytotoxic chemotherapy, IFN, or radiation within the preceding 4 weeks; known or suspected allergies to vaccine components; multiple brain metastases; use of steroids; class III to IV heart disease; or systemic autoimmune disease with visceral involvement. Patients were studied following informed consent and with institutional review board (HIC#10524) and Food and Drug Administration (BB-IND#9847) approval.

Vaccine composition. All patients received a vaccine comprising 12 melanoma peptides restricted by HLA-A1, HLA-A2, or HLA-A3 as described (11): A1 peptides: DAEKSDICTDEY (Tyrosinase₂₄₀₋₂₅₁, which has a substitution of S for C at residue 244), SSDVIPIGTY (Tyrosinase₁₄₆₋₁₅₆), EADPTGHSY (MAGE-A1₁₆₁₋₁₆₉), and EVDPIGHLY (MAGE-A3₁₆₈₋₁₇₆); A2 peptides: YMDGTMSQV (Tyrosinase_{369-377D}), IMDQVPFSV (gp100₂₀₉₋₂₁₇, 209-2M), YLEPGPVTA (gp100₂₈₀₋₂₈₈), and GLYDGMHL (MAGE-A10₂₅₄₋₂₆₂); and A3 peptides: ALLAVGATK (gp100₁₇₋₂₅), LIYRRRLMK (gp100₆₁₄₋₆₂₂), SLFRAVITK (MAGE-A1₉₆₋₁₀₄), and ASGPGGGAPR (NY-ESO-1₅₃₋₆₂). 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 (12). The tetanus helper peptide used was AQYIKANSKFIGITEL (13). This combination of 12 MHC class I-restricted peptides plus 1 class II-restricted peptide is called MELITAC 12.1.

Peptides for vaccines were synthesized and purified (>95%) under GMP conditions (Multiple Peptide Systems, now NeoMPS). After solubilization, each peptide was sterile-filtered, mixed, vialled, and lyophilized under GMP conditions by Merck Biosciences AG Clinalfa in single-use vials. 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.

Each vaccine was 2 mL of a stable water-in-oil emulsion consisting of 100 µg of each of the 12 MHC class I-restricted peptides, 190 µg of the tetanus helper peptide, and 1 mL Montanide ISA-51 adjuvant (Seppic). For patients in arms B and D, the emulsion also contained 110 µg GM-CSF (Leukine/Sargramostim; Berlex, now Bayer). For arms A and B, the full emulsion was administered to one extremity skin location, whereas, for arms C and D, 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) and then at 3, 6, 9, and 12 months. At each injection site, half of the dose was administered subcutaneously and half intradermally.

Clinical trial design. Primary objectives of this study were to estimate (a) whether GM-CSF administered locally changes the immunogenicity of vaccination with multiple synthetic melanoma peptides in an emulsion with incomplete Freund's adjuvant and (b) whether vaccination at two extremity sites induces different immunogenicity than vaccination at a single site. Secondary goals were to obtain preliminary data on (c) whether booster vaccination every 3 months maintains immune responses to a peptide vaccine and (d) whether cellular immune responses to a 12-peptide vaccine correlate with clinical outcome. This article addresses the first two objectives.

This was a phase II study with randomization to one of four vaccine regimens. A 2 × 2 factorial design was used to obtain preliminary estimates of possible difference in measures of immune response by factor (a) inclusion/exclusion of GM-CSF and factor (b) number of vaccination sites (1 or 2). It was assumed that there were no clinical or statistical interactions between the two factors. Patients were stratified on stage of disease (IIB/C versus III versus IV), and randomization was based on a random assignment within strata with varying block sizes. This is presented schematically in Fig. 1.

For this study, we were interested in detecting at least a 2-fold difference in maximum immune response within a patient between the two primary study groups (GM-CSF versus not; one versus two vaccine sites). Sample size determination was based on maximum immune

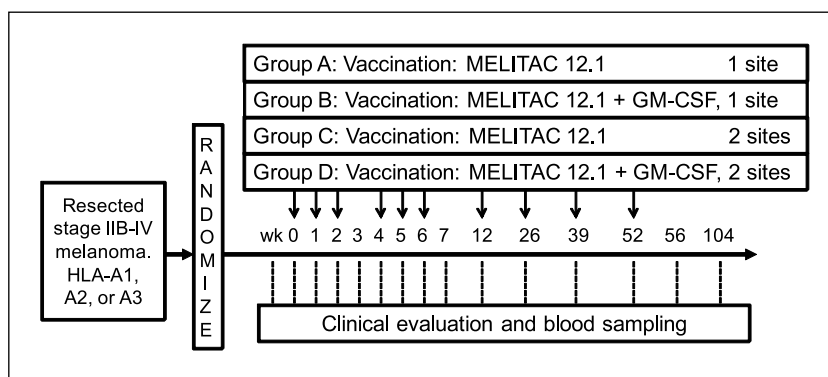


Fig. 1. Mel43 protocol schema.

response within a patient. For design and analysis purposes, the natural logarithmic transformation is used to stabilize the variance. Assuming equal variances, normality, and equal SDs of $\ln(\text{fold change})$ of 1.29, ~29 eligible patients per arm (116 total) were required to detect at least a 2-fold change in maximum immune response using a t test at the two-sided 5% level of significance with ~80% power. Adjustment of the sample size for a 7.5% ineligibility rate resulted in a target accrual goal of 125 patients. Patients were accrued at the University of Virginia and at four other participating institutions.

Toxicity assessment and stopping rules. The trial was monitored continuously for treatment-related adverse events using National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. Dose modification of GM-CSF was indicated if grade 3 adverse events occurred other than grade 3 local vaccine site reactions. Protocol treatment was to be discontinued for any grade 4 toxicity or for grade 3 toxicities that failed to respond to dose reductions of GM-CSF. Toxicities were recorded by patients using a daily toxicity diary reviewed by interview with a study clinician weekly. Patients with disease progression requiring other therapy were removed from treatment.

Endpoints for the study. The primary endpoint was the CD8⁺ T-cell response to the 12 melanoma peptides in the vaccine over the first six vaccines (to day 50). T-cell response was assessed by using both a binary indicator of immune response (yes/no) and a continuous measure based on amount of increase over background (fold increase) relative to any prevaccine reactivity. Secondary endpoints included CD8⁺ T-cell responses to the 12 melanoma peptides during and after the booster vaccines (>day 50 to 2 years), T-cell response to the tetanus helper peptide, disease-free survival, and survival.

Collection of peripheral blood mononuclear cells. Peripheral blood (60 mL) was drawn into heparinized tubes for isolation of peripheral blood mononuclear cells (PBMC), and 10 mL were drawn for serum studies at the time points shown in Fig. 1. 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 done directly *ex vivo*, after cryopreservation (direct ELISpot), or after one *in vitro* sensitization (stimulated ELISpot). Methods for the stimulated ELISpot assay have been reported (11). For direct ELISpot assays, 200,000 PBMCs were plated per well, and pulsed with synthetic peptide (10 $\mu\text{g}/\text{mL}$), in quadruplicate. Controls included irrelevant peptides, a mixture of viral peptides (CEF peptide pool), phorbol 12-myristate 13-acetate/ionomycin, and phytohemagglutinin. Assessment of immunologic response was based on a fold increase over the maximum of two negative controls. Evaluation of T-cell responses was based on the following definitions: N_{vax} = number of T cells responding to vaccine peptide; N_{neg} = number of T cells responding to maximum negative control; $R_{\text{vax}} = N_{\text{vax}} / N_{\text{neg}}$. For evaluations of PBMCs, a patient was considered to have a T-cell re-

sponse to vaccination (binary yes/no) by direct ELISpot assay only if all of the following criteria were met: (a) 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; (b) $R_{\text{vax}} \geq 2$; (c) $(N_{\text{vax}} - 1 \text{ SD}) \geq (N_{\text{neg}} + 1 \text{ SD})$; and (d) R_{vax} after vaccination $\geq 2 \times R_{\text{vax}}$ prevaccine 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 <1 (e.g., control counts exceed number of responding T cells or fold response compared with baseline is <1) were set equal to 1 to indicate no response and to prevent overinflating adjusted fold increases due to prevaccine ratios <1, or division by zero, while not affecting the determination of response. These methods are consistent with our prior analyses (14). Continuous measures of immune response denoted as fold increase must satisfy conditions 1 to 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 $1 + [\text{sum over each } (R_{\text{vax}} - 1) \text{ for each peptide for which a response was detected}]$. 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 coefficients of variation were calculated for each of 6 normal donors in their response to the CEF peptide pool. A high responder and a low responder were tested in each assay. For three high responder normal donors (mean number of spots per 100,000 cells plated ranged from 123 to 484), coefficients of variation were 8%, 24%, and 31%. For three low normal donors (mean 54-128), coefficients of variation were 28%, 30%, and 61%. The weighted mean coefficients of variation were 17% for high responders and 34% for low responders.

Primary analyses were based on eligible patients. Maximal response was based on responses in the blood to the first 6 vaccinations (through day 50). For hypothesis testing, patients who discontinued protocol therapy before 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 the criteria listed above were met, and immune response rates were calculated as the proportion of participants with an immune response. Point estimates and 95% confidence intervals were calculated for all summary parameters. Differences between groups by binary measures of immune response and cumulative response measures were assessed by χ^2 tests and t tests, respectively.

Analysis for antigen-specific T cells using MHC-peptide tetramers. Enumeration of antigen-specific T-cell responses was done using tetrameric HLA class I/peptide reagents (tetramers; Beckman Coulter). HLA-A2 tetramers used were MAGE-A10₂₅₄₋₂₆₂ (GLYDGM EHL),

gp100₂₀₉₋₂₁₇ (IMDQVPFSV), gp100₂₈₀₋₂₈₈ (YLEPGPVTA), and Tyrosinase_{369-377D} (YMDGTMSQV); A3 tetramers used were gp100₁₇₋₂₅ (AL-LAVGATK) and gp100₆₁₄₋₆₂₂ (LIYRRRLMK). Tetramers for the other six peptides were not studied: two do not recognize functional antigen-reactive T cells (DAEKSDICTDEY and SLFRAVITK), one could not be synthesized (ASGPGGGAPR), two others were not used because of the low rates of T-cell response observed in ELISpot assay (EADPTGHSY and SSDVIPIGTY), and one worked but was not evaluated (EVDPIGHLY). Samples from weeks 0 and 6 were selected; if week 6 samples were not available, samples from week 5 or 7 were used. After thawing, PBMCs were enriched for CD8⁺ cells (Miltenyi Biotec), washed twice, suspended in PBS/2% FCS (Sigma-Aldrich), and incubated with tetramer for 15 min at room temperature before adding a mixture of CD8 FITC (clone: SFC121Thy2D3), CD4 PE-Cy5, CD13 PE-Cy5, and CD19 PE-Cy5 (Beckman Coulter). Samples were transferred to 4°C, incubated 30 min, and washed. Cells were incubated in 7-amino-actinomycin D (Calbiochem; ref. 15) for 20 min, washed, fixed in 0.5% paraformaldehyde (Sigma-Aldrich) containing actinomycin D (Calbiochem; ref. 16), and acquired on a CyAn ADP LX 9 color flow cytometer (DAKOCytometry/Coulter) maintained by the Flow Cytometry Core Facility at the University of Virginia. Gates were set on singlet viable CD8⁺ T cells. Data were analyzed with FlowJo (Treestar) software. Proportion tetramer⁺ cells were calculated as (number tetramer⁺CD8⁺ cells) / (total number CD8⁺ T cells), although it was the square root transformation of this quantity that was used for statistical analysis. Changes were summarized as pre-to-post changes for each peptide and negative control and cumulatively by summation of changes for each peptide tetramer across each HLA-A allele. *t* tests were done to determine whether changes in tetramer proportions differed by groups.

Clinical outcome. The product-limit method was used to estimate 2- and 3-year survival and disease-free survival rates.

Results

Eligibility review. One hundred twenty-five patients were enrolled. Four (3.2%) were found ineligible on postreview (low platelet counts ($n = 2$), palpable disease developed before first treatment ($n = 1$), and insufficient time between radiation therapy and registration ($n = 1$). Analyses of immune response and clinical outcome are limited to 121 eligible patients, which exceeds the target of 116 eligible patients. Patient demographics and clinical presentations were similar across study arms (Supplementary Table S1). Median age was 55 years, 69% were male and 0.8% African/American; stage at enrollment was IIB to IIC (14%), IIIA (16%), IIIB/C (55%), or IV (16%). Eastern Cooperative Oncology Group performance status was 0 in 88% and 1 in 12%.

Summary of clinical toxicities. Treatment-related adverse events are detailed in Supplementary Table S2 for 124 treated patients and were similar to those from our prior experience (11). There were no treatment-related grade 4 toxicities or treatment-related deaths. There were no deaths on study, but one patient died ~30 days after coming off study because of tumor progression. Nine (7%) patients had unexpected grade 3 treatment-related adverse events requiring institutional review board reporting: arm A, fever (1); arm B, syncope (1); arm C, rash (2), headache/myalgias/arthritis (1), injection site reaction with pain (1), and fever/cellulitis (1); and arm D, fatigue (1) and diarrhea (1). Grade 3 injections site reactions occurred in 6, 9, 13, and 6 patients, respectively, for arms A to D, representing 24% and 31% of those with one and two vaccine sites, respectively, and 31% and 24% of those without and with GM-CSF, respectively. Arm C patients had the highest estimated rate of treatment-related grade 3 adverse events, but there were no

statistical differences in these grade 3 toxicities when arms were evaluated in pairs by the use of GM-CSF or number of vaccine sites.

Seventy-seven (64%) patients completed all protocol treatment. Forty-four came off study before completion of all 10 vaccines (within 1 year of study entry): 38 for disease progression (31%), 4 for adverse events (3%), 1 for refusing further therapy (1%), and 1 for noncompliance (1%). There were no significant differences in rates of completion of treatment or in interrupting treatment for disease progression between groups (GM-CSF or not; one versus two vaccine sites). All four patients who stopped treatment early for adverse events were in arm C (no GM-CSF, two vaccine sites).

Autoimmune toxicities. Autoimmune toxicities were observed in 27 (24%) patients, including increased ANA serum levels (8 patients, 7%), increased RF serum levels (7 patients, 6%), vitiligo (12 patients, 10%), and other autoimmune reactions (15 patients, 14%). These were similar for all study groups: 22% and 26% for those without and with GM-CSF, respectively, and 21% and 27% for one or two vaccine sites, respectively.

CD8 T-cell responses to MHC class I-restricted peptides: direct ELISpot assay. Of 121 eligible patients, 119 (98%) were evaluable for immune responses in PBMCs. Immune responses were evaluated by ELISpot assay for production of IFN- γ in response to each of the 12 peptides restricted by MHC class I alleles expressed by the patient. ELISpot data are summarized in Supplementary Table S3. These are analyzed based on the fold increase over background, corrected for any prevaccine response. Considering the complete patient population, each of the 12 peptides was immunogenic, with immune response rates ranging from 7% to 50% for each peptide, measured by direct ELISpot assay (Supplementary Table S3). Among the 12 peptides, 8 were immunogenic in >10% of patients, and those 8 were more immunogenic for the patients in arms A and C (no GM-CSF) than those in arms B and D (GM-CSF; Fig. 2A).

Immune response rates for subsets of four peptides relevant for each HLA-A allele (A1, A2, and A3) also were higher for vaccines without GM-CSF as was the response to the mixture of 12MP *in vitro* assayed in a subset of 66 patients (Fig. 2A). For patients vaccinated without GM-CSF, immune response rates for the 12 peptides ranged from 4% to 73% (Fig. 2A; Supplementary Table S3), whereas they ranged from 0 to 27% for those vaccinated with GM-CSF (Fig. 2A). Immune reactivity was greatest to the HLA-A3-restricted peptides, with 82% in arms A and C having reactivity to one or more of the A3 peptides.

Overall, 54% of patients responded to at least one of the 12 melanoma peptides, and the response rate was significantly higher for vaccines without GM-CSF than for those with GM-CSF (73% and 34%, respectively, $P < 0.001$, χ^2 ; Table 1). There was a trend to a better response rate with vaccines at two sites rather than one ($P = 0.093$, χ^2).

CD8⁺ T-cell responses to MHC class I-restricted peptides: stimulated ELISpot assay. Stimulated ELISpot assays are expected to be more sensitive than direct ELISpot assays and may be compared with our prior experience (3, 11, 17). They were done on a subset of 45 patients on this study. Example data for one patient show transient responses by direct ELISpot for some peptides but a durable response to another peptide (Fig. 3A), whereas more of those responses were durable in stimulated ELISpot assays (Fig. 3B). Raw data and controls for this patient

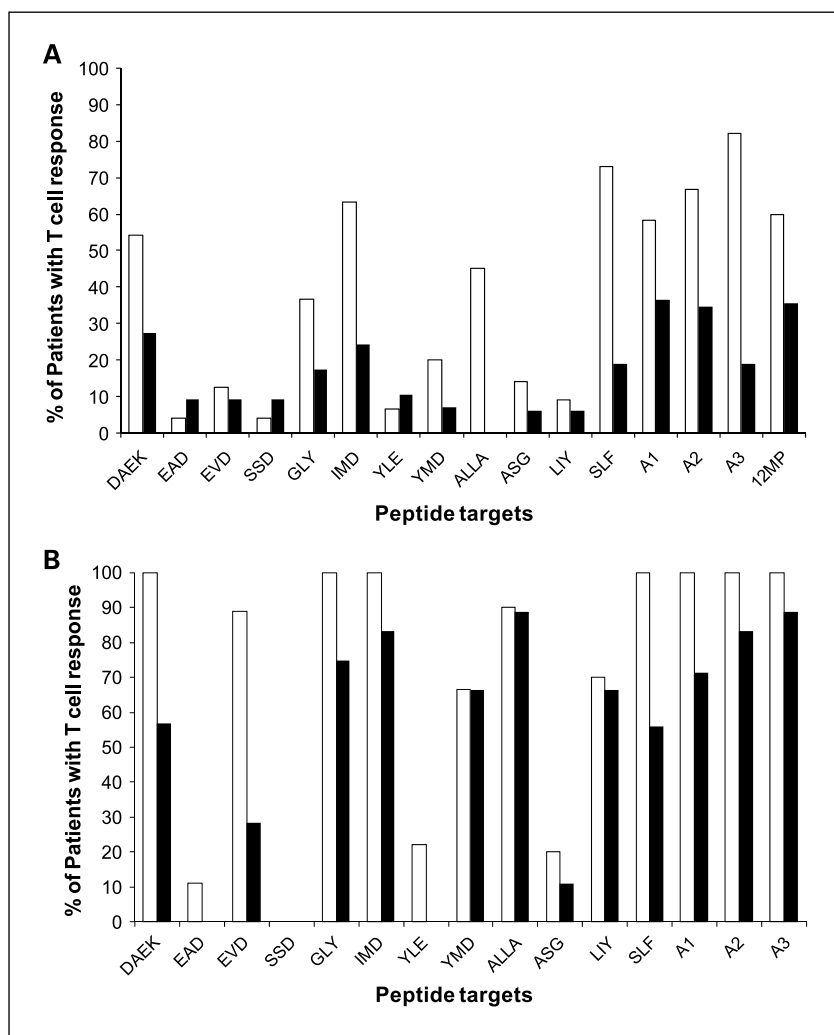


Fig. 2. Immune response rates by peptide (3-4 letter abbreviation), by HLA allele (A1, A2, or A3), and overall (12MP) for patients evaluated by direct ELISpot assay (A) or by stimulated ELISpot assay (B). The proportion of patients meeting criteria for an ELISpot response is plotted for those in groups A and C (no GM-CSF; white columns) and those in groups B and D (with GM-CSF; black columns). Response rates to each of the 12 HLA class I peptides are shown along with data for response rates to one or more peptides per HLA class I allele (A1, A2, or A3) or, for the direct ELISpot, to one or more of the 12MP.

are provided in Supplementary Fig. S1. Results of the stimulated assays also reveal numerically higher immune response rates in the arms without GM-CSF than in those with GM-CSF (Fig. 2B; Supplementary Table S3), with immunogenicity rates of 90% to 100% for 5 of the 12 peptides: Tyrosinase_{240-251S} (DAEKSDICTDEY), MAGE-A10₂₅₄₋₂₆₂ (GLYDGMEHL), gp100_{209-2M} (IMDQVPFSV), gp100₁₇₋₂₅ (ALLAVGATK), and MAGE-A1₉₆₋₁₀₄ (SLFRAVITK). Immune response rates across each HLA-A allele were 100% for patients vaccinated without

GM-CSF but were only 71%, 83%, and 89%, respectively, for those with GM-CSF (Fig. 2B).

CD8⁺ T-cell responses to HLA-A-restricted peptides: tetramer analyses. For 6 of the 12 MHC class I-restricted peptides, responses were also assessed by quantification of CD8⁺ T-cell staining with MHC class I tetramers. In some patients, tetramer⁺ T cells approached and exceeded 1% of circulating CD8⁺ T cells (examples; Fig. 3C). Summary data are presented in Fig. 4 for each of the four study arms. Higher tetramer-detected

Table 1. Measures of immune response to one or more of the 12 melanoma peptides (Sum 12MP) and to the tetanus helper peptide (119 evaluable patients): direct ELISpot data through day 50

Antigen	Group	n (n pos)	Rate (%)	Median cumulative fold increase (median of positives)	P (χ ² for rates)
12MP	No GM	60 (44)	73	5.8 (11.6)	<0.001
12MP	GM-CSF	58 (20)	34	1.0 (7.9)	
12MP	1 site	60 (28)	47	1.7 (10.9)	0.093
12MP	2 sites	58 (36)	62	3.4 (7.9)	
Tetanus	No GM	61 (58)	95	35.4 (38.6)	0.005
Tetanus	GM-CSF	57 (44)	77	10.6 (20.2)	
Tetanus	1 site	59 (53)	90	21.3 (23.5)	0.28
Tetanus	2 sites	59 (49)	83	14.3 (24.8)	

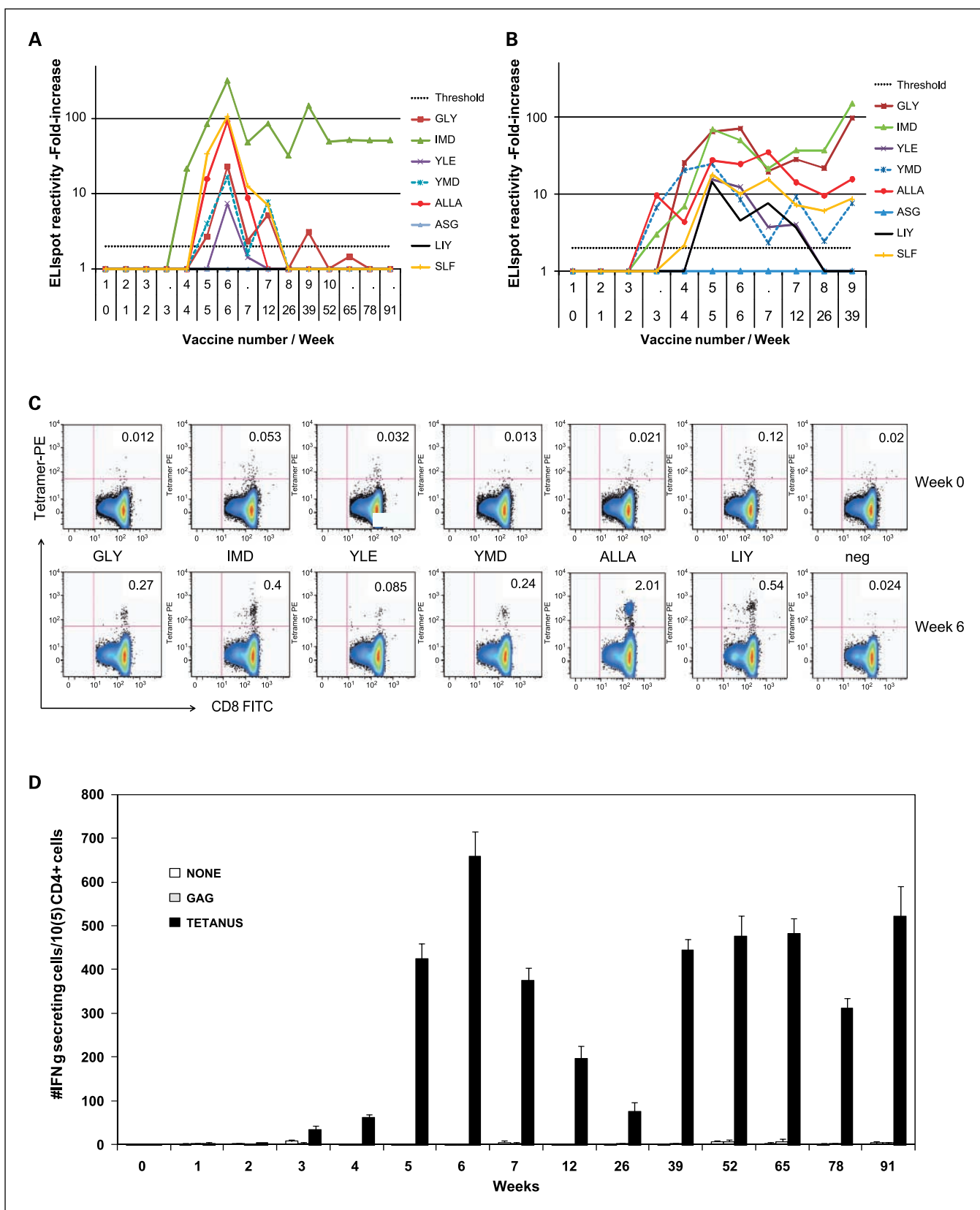


Fig. 3. Immunologic data for an immune responder VMM526. Summary data for the CD8⁺ T-cell reactivity to eight peptides evaluated for this patient are shown for the calculated fold increase over time for direct ELISpot data (A) and stimulated ELISpot data (B). The horizontal dotted line represents the 2-fold increase threshold for a positive response. Flow cytometry data on weeks 0 and 6 for tetramer⁺ cells are shown in C for the six evaluable tetramers plus negative control tetramer. Numbers in the top right quadrant of each plot represent the percent of CD8⁺ cells staining with the tetramer. CD4⁺ T-cell responses to the tetanus helper peptide are shown in D as measured by direct ELISpot assay.

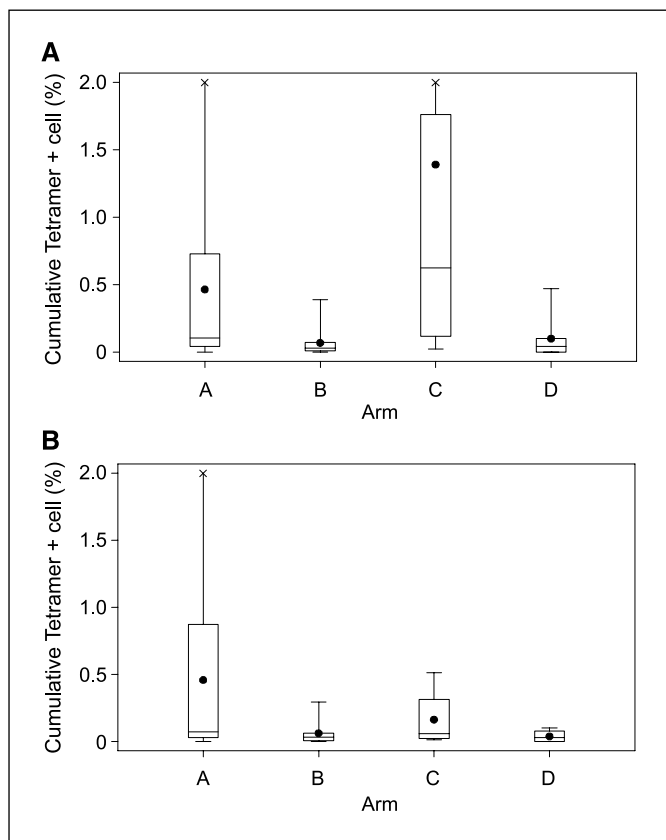


Fig. 4. Tetramer data Mel43 through day 50, sum of responses to evaluated MHC class I-restricted peptides. PBMC staining for MHC-peptide tetramers reactive to T-cell receptors for (A) four HLA-A2-associated peptides ($n = 48-53$ each) or for (B) two HLA-A3-associated peptide ($n = 36$). Box plots represent the cumulative percent of tetramer⁺ T cells, among CD8⁺ cells, for the four HLA-A2 and two HLA-A3 peptide tetramers by study arm, respectively. Values represent the difference between postvaccine and prevaccine values for each peptide (corrected to 0 if negative), summed among peptides subsetted by HLA restriction. Negative controls were consistently low with median $\sim 0.02\%$. The boxes represent the 25th to 75th percentile values, with the mean (symbol) and median (horizontal line) marked and with the maximum and minimum values at the end of the stems at either end of the boxes. X marks represent high maximal values clipped for (A) 2.55% for arm A and 7.02% for arm C and for (B) 2.41% for arm A.

responses were in patients in arms A and C (without GM-CSF), for four of the six tetramers studied, for HLA-A2 peptides overall ($P < 0.001$) and with a strong trend for HLA-A3 peptides overall ($P = 0.053$; Table 2).

CD4⁺ T-cell responses to HLA-DR-restricted tetanus helper peptide: direct ELISpot assay. The tetanus helper peptide (AQYI-KANSKFIGITEL) was used in all patients, and Th1 CD4⁺ T-cell responses were measured by direct IFN- γ ELISpot assay. Responses were detected in 86% of patients, with higher response rates in patients vaccinated without GM-CSF than with GM-CSF (95% versus 77%; $P = 0.005$; Table 1). There were no significant differences in CD4⁺ immune response rates in patients with vaccination based on the number of vaccine sites ($P = 0.28$; Table 1).

Clinical outcome. For the entire study population, 2-year overall and disease-free survival estimates (95% confidence interval) were $\sim 84\%$ (76-89%) and 56% (47-64%; data not shown). Three-year overall survival and disease free survival estimates were 76% (67-83%) and 52% (43-61%), respectively. There have been too few events to assess differences in clinical outcome by study group at this time.

Discussion

In prior work, we found that melanoma vaccines using mixtures of synthetic MHC-associated peptides are immunogenic and that 10 of the 12 melanoma peptides in MELITAC 12.1 were individually immunogenic (11). The present study enrolled 119 eligible patients who were evaluable for immune responses, and each of the 12 peptides was immunogenic, when assayed directly, without *ex vivo* stimulation. However, some were weakly immunogenic, and those patterns match our prior studies. Overall, 54% of patients developed immune responses detectable by direct ELISpot assay, which compares favorably to a 37% immune response rate measured in a multicenter clinical trial using three HLA-A2 peptides using a very similar assay system (18). For the 61 patients vaccinated with peptides in Montanide ISA-51 without GM-CSF, immune responses to the 12 melanoma peptides were detected in 73% of patients (Table 1), which exceeds response rates in prior studies using direct ELISpot.

Also, in prior work, we used an ELISpot assay on lymphocytes stimulated *ex vivo*, and immune responses detected using this assay did correlate with disease-free survival (11). A direct ELISpot assay is widely considered a more biologically relevant assay; however, that remains to be determined. A stimulated ELISpot assay may be relevant in measuring, in part, proliferative capacity of T cells, which may have some connection with memory responses. In a subset of patients on this trial ($n = 45$), we performed stimulated ELISpot assays also, which were associated with immune response rates on the order of 90% for the study overall and 100% for patients vaccinated without GM-CSF (Table 2). In those assays, even for individual peptides, immune response rates were 100% for four peptides and close to 90% for two others (Fig. 2B; Supplementary Table S3). These findings highlight the immunogenicity of peptide vaccines administered in an incomplete Freund's adjuvant.

Table 2. Measures of immune response to six melanoma peptides by median change in percentage of tetramer⁺ cells from weeks 0 to 6

Antigen	No GM-CSF		With GM-CSF		P for differences by group	
	n	Median	n	Median	GM-CSF	No. sites
GLY	25	0.020	26	0.002	0.011	0.53
IMD	26	0.081	26	0.011	0.004	0.91
YLE	24	0.002	24	0	0.14	0.036
YMD	26	0.024	26	0.004	0.005	0.18
ALLA	21	0.046	15	0.016	0.014	0.47
LIY	21	0.025	15	0	0.10	0.31
Neg	46	0	45	0	0.76	0.22
A2	26	0.23	26	0.04	<0.001	0.49
A3	21	0.07	15	0.03	0.053	0.64

NOTE: Differences between postvaccine and prevaccine tetramer⁺ cells are presented as a percentage of CD8⁺ cells and are >0 if there was an increase after vaccination. P values for the differences between groups are shown when considering the role of GM-CSF or of the number of vaccine sites. Allowing a limited correction for multiple comparisons, those with P values ≤ 0.025 are boldfaced.

One question addressed by the present study is whether vaccination in two extremity sites leads to better immune responses than vaccination in just one site. There was a trend to greater CD8⁺ T-cell response with two vaccine sites and a weak trend to a greater CD4⁺ T-cell response with one vaccine site, neither of which was statistically significant (Table 1). Thus, we conclude that there is insufficient evidence in the present data to claim that response rates are increased by vaccinating at two sites. However, other data strongly suggest that trafficking of T cells to tumor may depend on where they are primed by a vaccine (19); thus, there is still more work to do to answer the question of where to vaccinate depending on the location of melanoma metastases.

The main question that was addressed in the present study was whether GM-CSF administered locally in the vaccine emulsion would increase immunogenicity. Prior murine studies found that this strategy increased immune responses to a peptide vaccine (2) and supported the value of GM-CSF delivered in whole-cell vaccines (1). However, recent studies are conflicting on the role of GM-CSF as an immune potentiator or therapeutic agent (20). GM-CSF administered with a heat shock protein vaccine has been implicated in the induction of myeloid-derived suppressor cells in melanoma patients (21), and GM-CSF at high dose, in murine models, may increase myeloid-derived suppressor cells (22). However, daily subcutaneous administration of GM-CSF (125 µg/m²/d) increased circulating mature dendritic cells but did not increase myeloid-derived suppressor cells in melanoma patients (23).

Prior studies of GM-CSF as a vaccine adjuvant were primarily small nonrandomized studies or did not include defined antigens. We designed the present study to address the role of GM-CSF administered locally as a vaccine adjuvant, in a randomized multicenter design, with adequate power to detect a significant immunologic effect. The resulting data show that the circulating T-cell response to the multi-peptide vaccine is significantly lower in patients whose vaccines included GM-CSF. This was true both for CD8⁺ T-cell responses to 12 melanoma peptides and for CD4⁺ T-cell responses to a tetanus helper peptide. Reasons for this remain to be defined, but the findings cast doubt on the benefit of GM-CSF protein as local adjuvant. This finding is consistent with a prior small study with local administration of GM-CSF using a different delivery strategy (9). It has been suggested that negative immunologic effects of GM-CSF may be associated primarily with high doses of GM-CSF in the range of 100 to 500 µg/d (20). The dose used in this trial was 110 µg administered only once a week. Because it was given in a slow-release emulsion, the effective daily dose is arguably <20 µg/d. Thus, even low doses of GM-CSF administered with a multi-peptide vaccine may have negative immunologic effects. A possible mechanism by which GM-CSF may interfere with immune function is through milk fat globule EGF-8 (MFG-E8; refs. 24, 25). GM-CSF induces expression of MFG-E8 by macrophages and dendritic cells; MFG-E8 can maintain regulatory T cells

and can downregulate Th1 responses (24, 25). This mechanism deserves further investigation as a possible explanation for some negative effects of GM-CSF as an adjuvant. Additional studies are also needed to determine if GM-CSF alters the quality of function of vaccine-induced T cells and whether inclusion of GM-CSF with the vaccine may have an effect on clinical outcome.

In addition to questions about whether GM-CSF may have value as an immune adjuvant, it has been suggested that GM-CSF may have therapeutic value when administered subcutaneously as a single-agent therapy for melanoma (26), and that finding is being tested in a randomized phase III trial of systemic subcutaneous GM-CSF for the adjuvant therapy of high-risk melanoma alone or combined with a multi-peptide vaccine (Eastern Cooperative Oncology Group trial E4697). That study has been closed to accrual, and final analysis is pending. In a recent nonrandomized study, clinical outcome of 39 patients with stage III to IV melanoma treated with subcutaneous GM-CSF has been presented as additional support for the therapeutic potential of single-agent GM-CSF (23). However, the results of nonrandomized clinical studies of small size has been notoriously subject to selection bias, and the clinical outcome in the present study with and without GM-CSF compares favorably with what was reported in that study (23). With longer follow-up, we will be able to address whether clinical outcome mirrors immunologic outcomes.

In summary, we have found that immunization with the MELTAC 12.1 multi-peptide melanoma vaccine is immunogenic in a majority of patients with high-risk melanoma, that vaccination in two extremities does not increase T-cell response, and that addition of GM-CSF to the vaccine decreases circulating CD4⁺ and CD8⁺ T-cell responses to the vaccine. Caution should be taken in future use of GM-CSF as a local vaccine adjuvant.

Disclosure of Potential Conflicts of Interest

C.L. Slingsluff, Jr., commercial research grant and other commercial research support, Berlex; consultant, Immatix; patents on peptides, inventor, and royalties received from licensing to Ludwig Institute and from contract with National Cancer Institute; part of royalties from a licensing agreement from Ludwig Institute to Glaxo-Smith Kline. The other authors disclosed no potential conflicts of interest.

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