

Randomized Multicenter Trial of the Effects of Melanoma-Associated Helper Peptides and Cyclophosphamide on the Immunogenicity of a Multi-peptide Melanoma Vaccine

Craig L. Slingluff Jr, Gina R. Petroni, Kimberly A. Chianese-Bullock, Mark E. Smolkin, Merrick I. Ross, Naomi B. Haas, Margaret von Mehren, and William W. Grosh

A B S T R A C T

Purpose

This multicenter randomized trial was designed to test whether melanoma-associated helper peptides augment CD8⁺ T-cell responses to a melanoma vaccine and whether cyclophosphamide (CY) pretreatment augments CD4⁺ or CD8⁺ T-cell responses to that vaccine.

Patients and Methods

In all, 167 eligible patients with resected stage IIB to IV melanoma were randomly assigned to four vaccination study arms. Patients were vaccinated with 12 class I major histocompatibility complex–restricted melanoma peptides (12MP) to stimulate CD8⁺ T cells and were randomly assigned to receive a tetanus helper peptide or a mixture of six melanoma-associated helper peptides (6MHP) to stimulate CD4⁺ T cells. Before vaccination, patients were also randomly assigned to receive CY pretreatment or not. T-cell responses were assessed by an ex vivo interferon gamma ELISpot assay. Clinical outcomes and toxicities were recorded.

Results

Vaccination with 12MP plus tetanus induced CD8⁺ T-cell responses in 78% of patients and CD4⁺ T-cell responses to tetanus peptide in 93% of patients. Vaccination with 12MP plus 6MHP induced CD8⁺ responses in 19% of patients and CD4⁺ responses to 6MHP in 48% of patients. CY had no significant effect on T-cell responses. Overall 3-year survival was 79% (95% CI, 71% to 86%), with no significant differences (at this point) by study arm.

Conclusion

Melanoma-associated helper peptides paradoxically decreased CD8⁺ T-cell responses to a melanoma vaccine ($P < .001$), and CY pretreatment had no immunologic or clinical effect. Prior work showed immunologic and clinical activity of 6MHP alone. Possible explanations for negative effects on CD8 responses include modulation of homing receptor expression or induction of antigen-specific regulatory T cells.

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INTRODUCTION

Most peptide-based cancer vaccines have incorporated class I major histocompatibility complex (MHC)–restricted peptides to activate CD8⁺ cytotoxic T lymphocytes. However, activation of tumor-specific CD4⁺ helper T cells may also be critical for elimination of tumor.¹⁻³ Adoptive therapy with CD4⁺ T cells can induce tumor protection or regression,^{1,2} and depletion of CD4⁺ T cells inhibits vaccine-induced protective immunity.⁴ Immune responses to pathogens integrate cytotoxic T lymphocyte responses to epitopes presented by class I MHC molecules and CD4⁺ T-cell responses to epitopes presented by class II MHC molecules.⁵ CD4⁺ cells can activate dendritic cells for heightened antigen

presentation. Th1 CD4⁺ cells produce a cytokine milieu critical for induction of immune-mediated tumor destruction.^{6,7} CD4⁺ T-cell responses are also implicated in establishing immunologic memory.⁸ In patients with HIV, induction of CD4⁺ responses to HIV antigens augments HIV-specific CD8⁺ T-cell reactivity.⁹ Despite the acknowledged importance of CD4⁺ helper T cells in active immunotherapy, there has been a paucity of experience, in patients who have cancer, with tumor-associated peptides that stimulate CD4⁺ T-cell (helper peptides), the investigation of which has been limited to pilot or nonrandomized studies with only a few peptides.¹⁰⁻¹²

In prior studies,¹³⁻¹⁷ we included an HLA-DR–restricted helper peptide from tetanus toxoid in melanoma vaccines, which activated CD4⁺ T cells

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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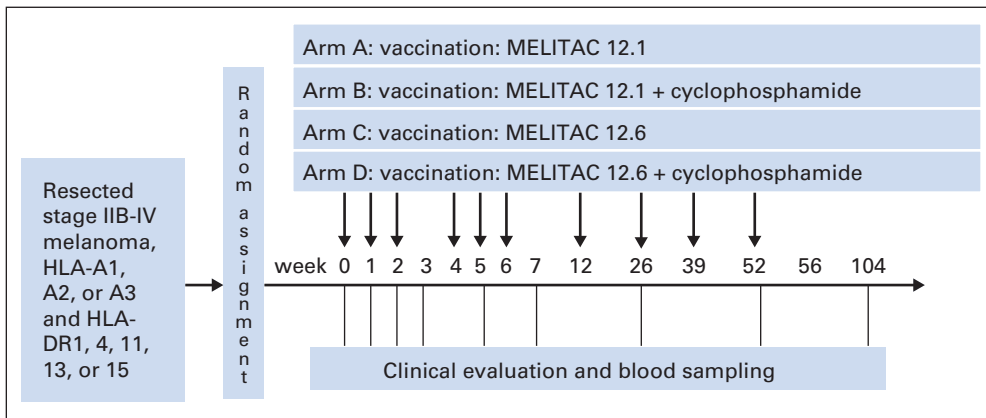


Fig 1. Mel44 protocol schema. MELITAC 12.1, vaccines with 12 class I major histocompatibility complex–restricted melanoma peptides (12MP) plus the tetanus peptide AQYIKANSKFIGITEL; MELITAC 12.6, vaccines incorporating 12MP plus a mixture of six melanoma-associated helper peptides (6MHP).

nonspecifically. Class II MHC-restricted helper peptides from melanoma-associated proteins now promise to activate CD4⁺ T cells in a melanoma-specific manner.¹⁸ In a phase I/II clinical trial, we found that a mixture of six melanoma helper peptides (6MHP) derived from *cancer-testis antigens* (CTAs) and *melanocytic differentiation proteins* (MDPs) was immunogenic in more than 80% of patients and was associated with durable clinical responses (12%) and durable stable disease (12%).¹⁹ This study was designed to address whether melanoma-associated helper peptides augment induction and persistence of CD8⁺ T-cell responses to a multi-peptide melanoma vaccine administered in the adjuvant setting to melanoma patients with a predicted high risk for recurrence.

A second question addressed in this study was whether pretreatment with cyclophosphamide (CY) enhanced CD4⁺ or CD8⁺ T-cell responses to a multi-peptide vaccine. CY doses lower than those used for tumor lysis have been reported to augment immune responses in mice and humans²⁰⁻²⁴ through several potential mechanisms.^{23,25-31} Prior studies^{20,24,29,30,32,33} have tested immunomodulatory properties of 75 to 1,000 mg/m² CY with varied results. For patients with melanoma, CY pretreatment was associated with augmented delayed-type hypersensitivity responses to an autologous melanoma cell vaccine in nonrandomized studies.^{20,21} However, studies of the effects of CY on immune responses to vaccines have been limited by small sample size, nonrandomized designs, and/or lack of defined antigens. The largest study is with 300 mg/m² dosing, which we evaluated.

In this article, we report findings from a multicenter randomized trial designed to test whether melanoma-associated helper peptides augment CD8⁺ T-cell responses to a melanoma vaccine and whether CY pretreatment augments CD4⁺ or CD8⁺ T-cell responses.

PATIENTS AND METHODS

Patients

Eligible patients had American Joint Committee on Cancer (AJCC; 6th edition)⁽³⁴⁾ stage IIB to IV melanoma from cutaneous, mucosal, or unknown primary sites, rendered clinically free of disease by surgery or stereotactic radiosurgery. Inclusion criteria included expression of HLA-A1, -A2, or -A3; expression of HLA-DR1, -DR4, -DR11, -DR13, or -DR15; age 18 years and older; Eastern Cooperative Oncology Group (ECOG) performance status 0 to 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; multiple brain metastases; use of steroids; class III to IV heart disease; or severe autoimmune disease. Patients provided informed consent, and the study was approved by the institutional review board and the US Food and Drug Administration.

Vaccine Composition

Patients received a vaccine comprising 12 melanoma peptides restricted by HLA-A1, -A2, or -A3 (12MP), as described,¹⁶ plus peptides to stimulate helper T cells. Vaccines with 12MP plus the tetanus peptide AQYIKANSKFIGITEL¹³ are called MELITAC 12.1; vaccines incorporating 12MP plus a mixture of 6MHP¹⁹ are called MELITAC 12.6. Peptide sequences are provided in the Data Supplement.

Each vaccine was administered as 2 mL of stable water-in-oil emulsion prepared by the two-syringe method and consisting of 100 µg of each of the 12 melanoma peptides, 190 µg of each helper peptide, and 1 mL Montanide ISA-51 VG adjuvant (Seppic, Paris, France/Fairfield, NJ).³⁵ A drop of each emulsion was tested for stability in water before injection. Vaccines were administered, half subcutaneously and half intradermally, on days 1, 8, 15, 29, 36, and 43 and then at 3, 6, 9, and 12 months (Fig 1).

Clinical Trial Design

This was an open-label, multicenter phase I/II study with random assignment to MELITAC 12.1 or MELITAC 12.6, with or without CY pretreatment (Fig 1). Primary goals were to test safety and immunogenicity. The study was designed to assess a partial ordering of immune response magnitudes among the four study arms, specifically arm D (12.6 + CY) greater than B (12.1 + CY) or C (12.6), and B or C greater than A (12.1). The study included interim safety assessments. Sample size determination was based on differences in cumulative immune response measured in the peripheral blood mononuclear cells (PBMCs) over six vaccines compared with baseline. We were interested in detecting at least a 30% increase in the four main comparisons of interest. The study was designed to test these comparisons at the two-sided 2.5% significance level (10% overall) with 90% power at the alternative, requiring 40 participants per arm (total, 160 eligible). Maximum accrual was adjusted up to 173 participants to allow for 5% ineligibility and 3% overenrollment. Patients were accrued at three participating institutions, were stratified by HLA type and institution (Table 1), and were randomly assigned within strata with various block sizes. Randomization lists were generated by the study statisticians and stored in the database, with arm assignment being released to the study coordinator only at the time of registration. The study design is presented schematically in Figure 1.

Toxicity Assessment and Stopping Rules

The trial was monitored continuously for treatment-related adverse events by using National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Toxicities were recorded by patients in a daily toxicity diary and reviewed weekly by interview with a study clinician. Protocol treatment was to be discontinued for all grade 3, ocular grade 1,

Table 1. Demographics and Clinical Characteristics of Eligible Patients

Characteristic	Study Arm									
	A (n = 41)		B (n = 41)		C (n = 42)		D (n = 43)		All (N = 167)	
	No.	%	No.	%	No.	%	No.	%	No.	%
Sex										
Male	28	68	25	61	29	69	30	70	112	67
Race										
Black/African American	0		0		0		0		0	
Hispanic	1		0		1		0		2	1.2
Hospital										
Fox Chase Cancer Center	9		6		7		6		28	
MD Anderson Cancer Center	12		13		13		14		52	
University of Virginia	20		22		22		23		87	
Disease status										
Initial diagnosis	17		20		24		24		85	
Recurrence	24		21		18		19		82	
Stage at enrollment										
IIB	2		2		5		4		13	
IIC	2		3		3		2		10	
IIIA	4		8		5		4		21	
IIIB	11		10		11		18		50	
IIIC	10		10		9		8		37	
IV	12		8		9		7		36	
ECOG PS										
0	34		39		40		38		151	
1	7		2		2		5		16	
Median age, years	59		60		55		58		58	
HLA expression										
HLA-A1	12		13		13		14		52	
HLA-A2	23		21		22		19		85	
HLA-A3	15		17		20		20		72	
HLA-A31	4		1		0		1		6	

NOTE. Total enrollment for study arms A through D was 41, 43, 42, and 44, respectively.
Abbreviations: ECOG, Eastern Cooperative Oncology Group; PS, performance status.

allergic grade 2, or higher, unexpected treatment-related adverse events. Patients with disease progression requiring other therapy were removed from treatment.

End Points

The primary end point was the maximum cumulative circulating CD8⁺ T cell response to 12MP measured by ELISpot assay over the first six vaccines (to day 50). This is reported both as a binary measure of immune response (yes/no) and a continuous measure based on fold-increase over background corrected for any prevaccine reactivity. Secondary end points included CD8⁺ T-cell responses to 12MP during and after booster vaccines (> day 50 to 2 years), T-cell response to helper peptides, disease-free survival, and overall survival.

Collection of PBMCs

At times shown in Figure 1, peripheral blood (120 mL week 0, then 75 mL) was drawn into heparinized tubes, and 20 mL was drawn for serum studies. Blood drawn at other participating institutions was shipped in insulated containers at near room temperature for overnight delivery. PBMCs were isolated by using Ficoll gradient centrifugation and were cryopreserved in 10% dimethylsulfoxide/90% serum by the Biorepository and Tissue Research Facility at the University of Virginia.

ELISpot Assays

ELISpot assays (for interferon gamma) were performed on PBMCs collected through day 50 (weeks 0, 1, 2, 3, 5, and 7). Assays were performed directly ex vivo after cryopreservation (direct ELISpot), as reported^{17,36} (de-

tails in Data Supplement). Patients were designated immunologic responders if vaccine antigen-specific responses represented increases of at least 0.02% of CD4⁺ or CD8⁺ T cells over negative control and were at least two-fold above the negative control and above any prevaccine response, and if standard deviations of the antigen-reactive responses and negative controls did not overlap. These criteria match those we used previously¹⁷ and are in the range of other published criteria (Data Supplement). Interassay coefficients of variation (CVs) were calculated for normal donor PBMC responses to the pool of 32 peptides from cytomegalovirus, Epstein-Barr virus, and influenza proteins,³⁷ testing high and low responders in each assay. For two high-responder donors (mean spots per 100,000 cells: 233, 485), CVs were 15% and 7%, respectively (weighted mean, 10%). For two low-responder donors (mean spots per 100,000 cells: 29, 96), CVs were 47% and 28%, respectively (weighted mean, 34%).

Primary analyses were performed on the basis of eligible patients. For hypothesis testing, patients who discontinued protocol therapy early were considered to have immune response failures if no response was observed in evaluable samples. Immune response rates were calculated on the basis of the proportion of patients whose data met the stated criteria. Point estimates and 95% CIs were calculated for summary parameters. Differences between study arms by binary measures of immune response and measures of cumulative response were assessed by χ^2 tests and Kruskal-Wallis tests, respectively. Evaluation of immune response persistence to year 2 is not yet complete and will be reported in the future. The product-limit method was used to estimate overall survival and disease-free survival.

RESULTS

Eligibility Review

In all, 170 patients were enrolled beginning in March 2005 until meeting the accrual goal in January 2008. Three (1.8%) were ineligible on post review (one thrombocytopenia, one tumor progression before first treatment, and one insufficient time after radiation therapy). These patients had been randomly assigned to arms B (two) and D (one). Immune responses were evaluable for 161 (96%) of the 167 eligible patients. Patient demographics and clinical presentations were similar across study arms (Table 1).

CD8 T-Cell Responses to Class I MHC-Restricted Peptides: Direct ELISpot Assay

Immune responses were induced by 11 of the 12 peptides by day 50. Patients in arm A or B (MELITAC 12.1 with or without CY) had the highest immune response rates for nine of the peptides (Table 2). Rates of immune response to at least one peptide restricted by each HLA allele were also highest in arms A and B: 69%, 83%, and 68%, for HLA-A1, -A2, and -A3, respectively (Table 2). CD8⁺ T-cell response rates to the 12MP mixture were 78% and 71% for arms A and B, respectively (*P* = .45, not significant), and 19% and 28% for arms C and D, respectively (*P* = .34, not significant; Fig 2 and Table 2). For this end point, the predicted ordering of D more than (C or B) more than A was the opposite of what was observed. For most peptides tested, the observed ordering was effectively A = B more than C = D,

with marked decreases in reactivity observed in arms C and D compared with arms A and B (*P* < .001 for both).

Magnitudes of CD8⁺ T-cell responses to 12MP are shown in Figure 3, both by fold-increase (Fig 3A) and by percent of CD8⁺ cells (Fig 3B). For arms A and B, these values were equivalent at approximately 5× and 0.2% of CD8⁺ cells, but for arms C and D, median values were only 1×, and 0% of CD8⁺ cells. Differences across all four arms were significant (*P* < .001) for both analyses. For arms A and B, 75th percentile values were approximately 0.5% to 0.7%, and the highest responses exceeded 1% (Fig 3A). Summaries of raw ELISpot data are provided in the Data Supplement.

CD4⁺ T-Cell Responses to Tetanus Helper Peptide and 6MHP: Direct ELISpot Assay

Th1 CD4⁺ T-cell responses were measured by direct interferon gamma ELISpot assay. Immune responses to tetanus peptide were detected in 91% of patients vaccinated with tetanus peptide (average across arms A and B) and in only 18% of patients vaccinated with 6MHP (arms C and D; Fig 2 and Table 2). Responses to 6MHP were detected in only 1% of patients vaccinated with tetanus peptide (arms A and B) but in 52% of patients vaccinated with 6MHP (arms C and D; Fig 2 and Table 2). CD4⁺ T-cell response magnitudes were greater to tetanus peptide (medians, approximately 0.25% for arms A and B; Fig 3C) than to 6MHP (medians < 0.1% for arms C and D; Fig 3D). Differences across all four arms were significant (*P* < .001). However,

Table 2. Percent of Patients (167 evaluable patients) With Immune Responses to Each Peptide or to Peptide Mixtures (direct ELISpot data through day 50)

Individual Class I MHC Peptides*	Source Protein	Study Arm				Pairs of Study Arms	
		A (12.1) (n = 41)	B (12.1 + Cy) (n = 41)	C (12.6) (n = 42)	D (12.6 + Cy) (n = 43)	A+B (12.1) (n = 82)	C+D (12.6) (n = 85)
HLA-A1							
DAEKSDICTDEY	Tyrosinase	42	54	23	14	48	19
EADPTGHSY	MAGE-A1	8	8	8	0	8	4
EVDPIGHLY	MAGE-A3	25	15	8	0	20	4
SSDVIPIGTY	Tyrosinase	8	0	0	7	4	4
HLA-A2							
GLYDGM EHL	MAGE-A10	39	48	9	11	43	10
IMDQVPFSV	gp100	78	57	18	21	68	20
YLEPGPVTA	gp100	9	0	5	0	5	2
YMDGTMSQV	Tyrosinase	4	0	5	5	2	5
HLA-A3							
ALLAVGATK	gp100	42	47	5	0	44	2
ASGPGGGAPR	NY-ESO-1	16	6	0	0	11	0
LIYRRRLMK	gp100	0	0	0	0	0	0
SLFRAVITK	MAGE-A1	53	59	5	14	53	10
Mixtures of class I MHC peptides							
Maximum of HLA-A1 peptides		42	69	23	21	56	22
Maximum of HLA-A2 peptides		83	57	23	26	70	24
Maximum of HLA-A3 peptides		68	59	5	14	64	10
Maximum of all 12 peptides		73	68	17	26	71	21
12MP mixture		78	71	19	28	74	24
Helper peptides							
6MHP mixture		2	0	48	56	1	52
Tetanus peptide		93	90	12	23	91	18

NOTE. Bold values represent the highest immune response rates per study arm (or pair of study arms).

Abbreviations: Cy, cyclophosphamide; MAGE, melanoma-associated gene; MHC, major histocompatibility complex; 6MHP, mixture of six melanoma-associated helper peptides; 12MP, 12 class I MHC-restricted melanoma peptides.

*Immune response rates for individual peptides or for groups of peptides restricted by an HLA allele are based on the subset of patients expressing that HLA allele.

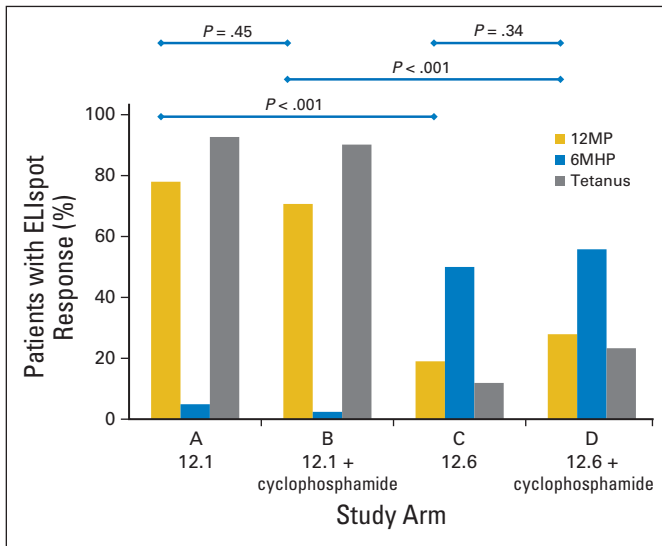


Fig 2. Immune response rates for 12 class I major histocompatibility complex-restricted melanoma peptides (12MP), a mixture of six melanoma-associated helper peptides (6MHP), and tetanus peptide by ELISpot assay are shown for each study arm. These are based on evaluation of peripheral blood mononuclear cells at weeks 1, 2, 3, 5, and 7 (through day 50). *P* values for the response to 12MP are noted for four pairs of study arms. 12.1 [MELITAC 12.1], vaccines with 12MP plus the tetanus peptide AQYIKANSKFIGITEL; 12.6 [MELITAC 12.6], vaccines incorporating 12MP plus 6MHP.

there is no evidence of association between immune responses and relapse-free or overall survival.

Summary of Clinical Toxicities

Treatment-related adverse events are detailed in the Data Supplement for 170 treated patients and were similar to those from our prior peptide-based trials.^{16,17,19} There was one treatment-related grade 4 toxicity (hypoglycemia; < 1%), but no treatment-related deaths or deaths on study. Fourteen patients (8%) had unexpected treatment-related grade 3 adverse events that required reporting to the institutional review board: four (arm A), seven (arm B), two (arm C), and one (arm D). They included injection site ulceration (*n* = 10); injection site reaction/induration (*n* = 7); and auditory toxicity, lymphopenia, vomiting, hypoglycemia, muscle pain, pain (not otherwise specified), dyspnea, pneumonitis, and cytokine release syndrome (one each). Treatment-related autoimmune toxicities were reported in 10 patients (6%; details and study completion rates in Data Supplement).

Clinical Outcome

For the entire patient cohort, estimates were approximately 88% (95% CI, 81% to 92%) for 2-year overall survival and 56% (95% CI, 48% to 63%) for 2-year disease-free survival and were 79% (95% CI, 71% to 86%) for 3-year overall survival and 52% (95% CI, 44% to 60%) for 3-year disease-free survival. There were no significant differences in outcome by study arm at this early follow-up. In a global test across the four study arms, there was no difference in overall survival (*P* = .95) or disease-free survival (*P* = .26). Kaplan-Meier curves are shown in Figure 4.

DISCUSSION

Cancer immunotherapy is coming of age, with a cancer vaccine approved for prostate cancer,³⁸ improved survival with CTLA4 (CD152)

blockade for melanoma,³⁹ clinical benefit of adding a peptide vaccine to high-dose interleukin-2 (IL-2),⁴⁰ and durable responses to adoptive T-cell therapy for melanoma and other cancers.⁴¹ These treatments increase T-cell activation, and most target defined T-cell antigens. In addition, most effective immune therapies can also induce severe toxicity. Cancer vaccines offer the potential to activate T cells with specificity and low morbidity to bring benefits of immune therapy to patients treated in the adjuvant setting.

We previously found that vaccination with either 12MP or 6MHP induced antitumor CD8⁺^{16,17} and CD4⁺ T cells,¹⁹ respectively. In this study, we tested the hypothesis that adding 6MHP to 12MP would increase the magnitude and persistence of CD8⁺ T-cell responses to 12MP, but it paradoxically decreased CD8⁺ T-cell responses compared with MELITAC 12.1 vaccination. Adding 12MP did not appear to inhibit helper T-cell responses: CD4⁺ T-cell response rates to tetanus peptide exceeded 90%, and response rates to 6MHP were approximately 50%. The latter result is comparable with the 57% immune response rate in PBMCs when vaccinating with 6MHP alone.¹⁹ However, responses to 6MHP were lower than responses to tetanus peptide. One might conclude that the negative effect of 6MHP simply reflects a weak helper T-cell response. However, in a separate study, we found that vaccination with 12MP + 6MHP led to lower CD8⁺ T-cell responses than vaccination with 12MP alone.⁴²

Possible explanations for the observed negative effect as measured in PBMCs may include induction of antigen-specific regulatory T cells, Th2 cytokine induction by 6MHP, increased homing of T cells to tumor deposits, or sequestration of T cells at the vaccine site.⁴³ Prior vaccination with 6MHP has induced Th1-dominant responses and was not associated with increases in total regulatory T cells.¹⁹ If clinical outcome were improved with addition of 6MHP, it might suggest that melanoma-specific helper T-cell responses support better homing to tumor; however, these patients had no clinically evident disease at study entry, and at current early follow-up, a survival effect has not been observed. There is a weak trend to shorter disease-free survival for patients on arm A (MELITAC 12.1 alone), leaving open some possibility that there may be a clinical impact with longer follow-up. Future studies to define the mechanism of this observation will include characterizing cytokines and chemokines produced in the vaccine site microenvironment and draining nodes, changes in antigen-specific regulatory T cells, and differences in homing receptors expressed on T cells induced by each vaccination strategy.

In addition to testing the effect of melanoma-associated helper peptides, this study also evaluated the effect of CY pretreatment. CY provided no detectable improvement in CD4⁺ or CD8⁺ T-cell responses (Figs 2 and 3) or in clinical outcome (Fig 4). In preclinical studies, immunopotentiality has been reported with CY administered 1 to 7 days before vaccination.⁴⁴⁻⁴⁷ Prior experience in humans suggested that CY increased immunogenicity when administered 3 days before a cell-based vaccine, but those studies were nonrandomized and were limited by semiquantitative immunologic end points.^{20,21,24} Other human experience failed to identify changes in regulatory T cells with CY treatment,⁴⁸ and recent data identified negative effects of CY (200 mg/m² or greater) pretreatment on cellular immune responses to a breast cancer cell vaccine.³³ This study was unique in evaluating effects of CY on immune responses to defined antigens as well as effects on both CD4⁺ and CD8⁺ responses. Other doses or timing of CY pretreatment may have effects different from those observed in this study; however, the dose and timing here is in

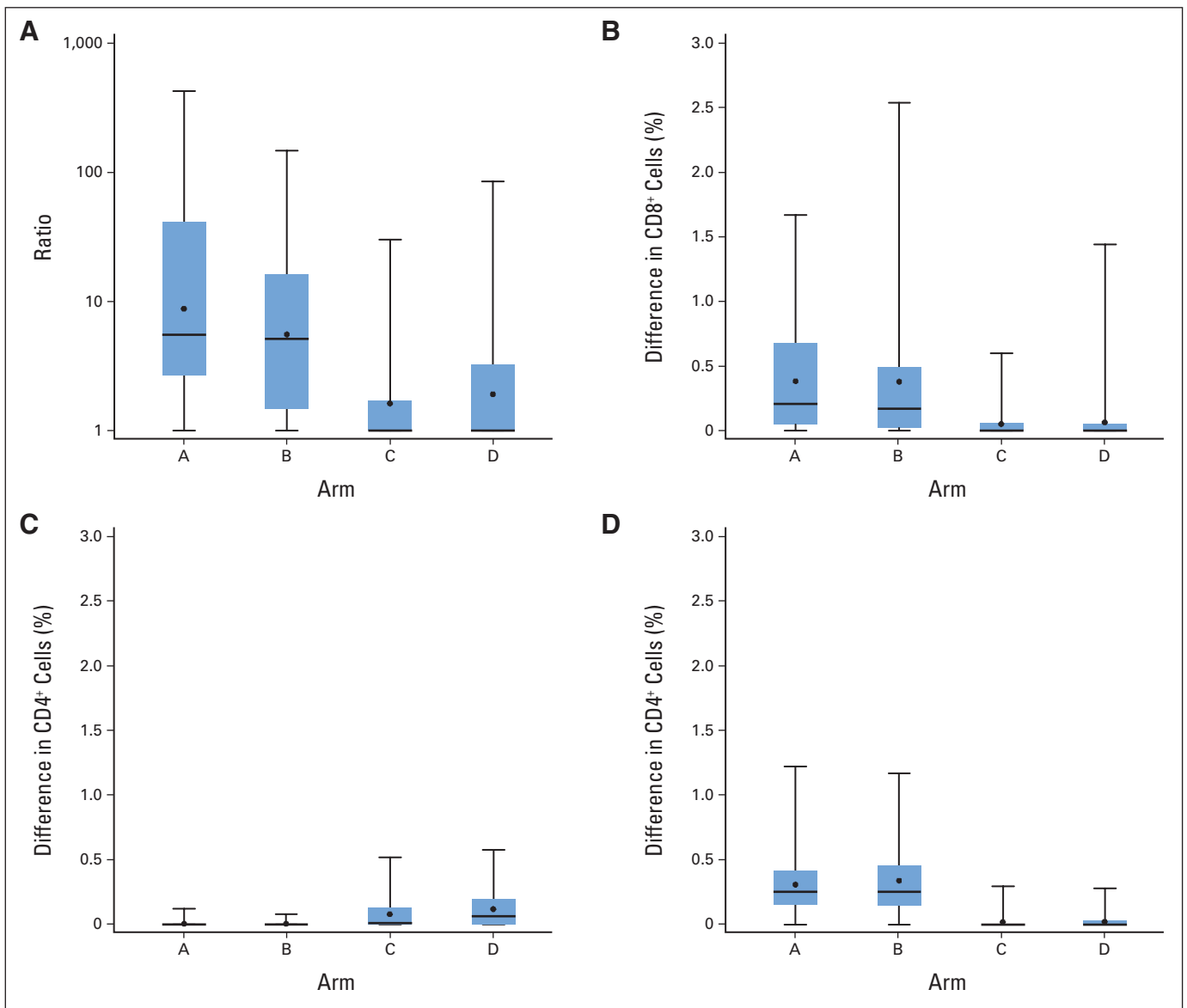


Fig 3. The magnitudes of immune responses to 12 class I major histocompatibility complex–restricted melanoma peptides (12MP) by patient, grouped by study arm. Box plots represent the magnitude of ELISpot reactivity to the 12MP mixture, mixture of six melanoma-associated helper peptides (6MHP), or tetanus peptide through day 50. Boxes represent 25th through 75th percentile values, with mean (black circle) and median (horizontal line) marked, and with maximum and minimum values at the ends of the stems. Maximum responses to the 12MP pool per patient are plotted as fold-increase over baseline (A) and as increase in percent of CD8⁺ cells (B). Maximum responses to the 6MHP pool (C) and tetanus peptide (D) are plotted as increase in percent of CD4⁺ cells responding to each peptide in interferon gamma ELISpot assays. The mean background reactivity across all assays was 19.3 spots (95% CI, 15.9 to 22.7 spots). CD8⁺ cells and CD4⁺ cells on average represented 20% (95% CI, 19% to 21%) and 36.3% (95% CI, 34.5% to 38.2%) of peripheral blood mononuclear cells, respectively. Thus, on average, 100 spots per well above negative controls represent approximately 0.25% of CD8⁺ cells.

the range of what has been previously tested. Thus, this study and others challenge the value of CY as a vaccine adjuvant for induction of CD8⁺ or Th1 CD4⁺ responses.

In summary, this study supports the immunogenicity of 12MP for CD8⁺ T cells and both tetanus peptide and 6MHP for CD4⁺ T cells. However, the combination of 6MHP with 12MP paradoxically reduced the circulating CD8⁺ T-cell response, and CY (300 mg/m²) pretreatment had no measurable effect on CD8⁺ or CD4⁺ responses. Clinical outcome was not increased by adding melanoma-associated helper peptides or by adding CY, although there is a trend to shorter disease-free survival in patients who received neither (arm A). Nonetheless, the immunogenicity of 6MHP in this study, and its clinical

activity in prior work,¹⁹ support further investigation of vaccines by using melanoma-associated helper peptides, and the interaction of CD8⁺ and CD4⁺ T-cell responses needs to be understood. We plan to pursue whether the combination vaccination induces antigen-specific regulatory T cells, regulatory cytokines, or homing receptors that may alter homing to tumor, vaccine sites, or other peripheral tissues.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject

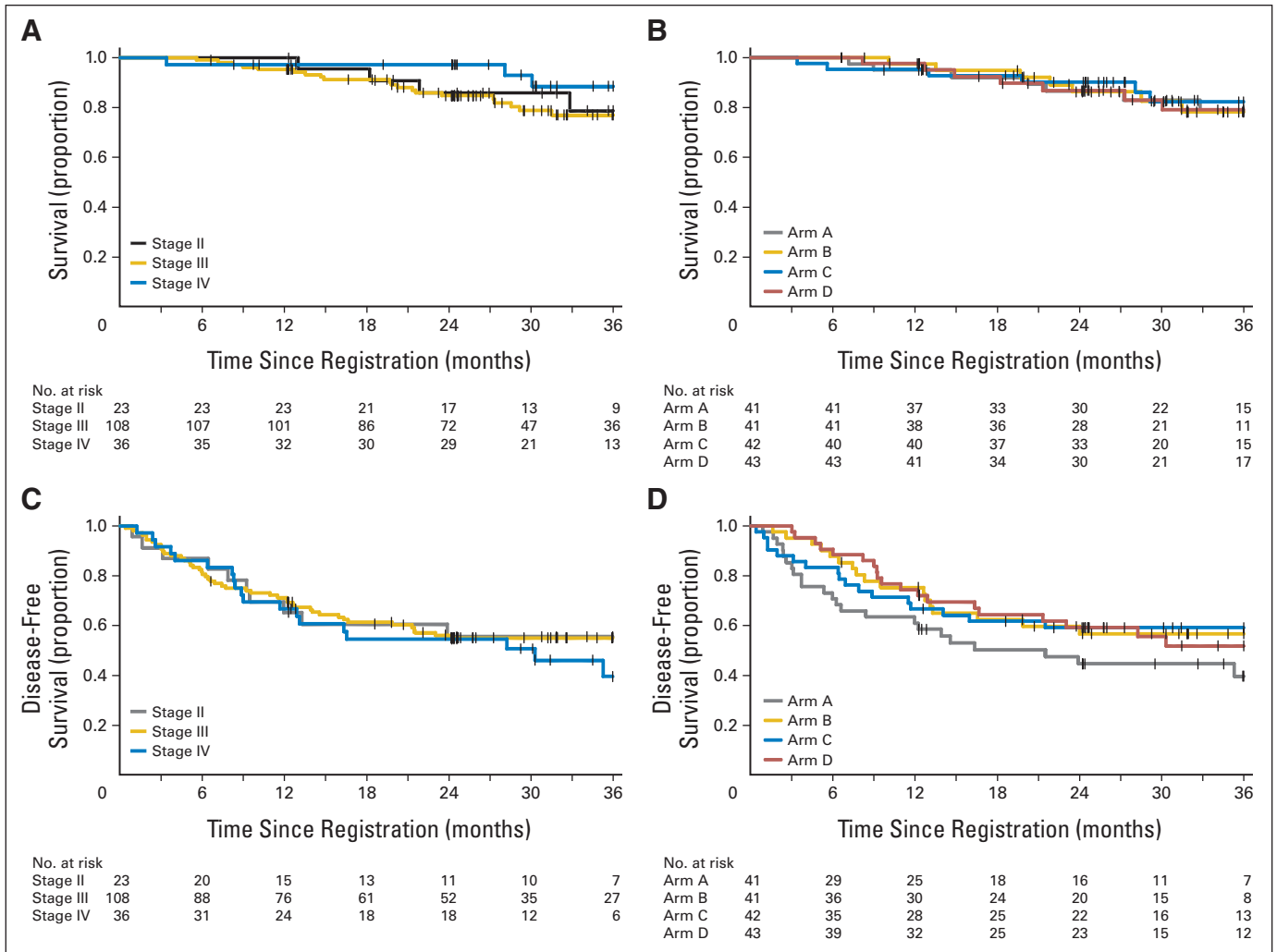


Fig 4. Clinical outcome. Overall survival by stage (A) and study arm (B) and disease-free survival by stage (C) and study arm (D) are shown as Kaplan-Meier curves from the time of study enrollment.

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Glossary Terms

Cancer-testis antigen (CTA): Proteins expressed on the surface of cancer and testicular cells capable of eliciting an immune response outside of the immunologically shielded testis.

CD4: The surface antigen that characterizes CD4⁺ T lymphocytes, CD4 is associated with the T-cell receptor and major histocompatibility complex, necessary for antigen recognition.

CTLA4 (CD152): Receptor on activated T cells that binds B7 molecules with a higher affinity than CD28, downregulating T-cell responses by inhibiting CD28 signaling.

ELISpot: Enzyme-linked immunospot that is exquisitely sensitive to assay minute amounts of mediators that are produced by cells. Typically, cells are deposited on a membrane coated with an antibody specific for a given protein. The protein of interest is captured directly around the secreting cell and is detected with an antibody specific for a different epitope. Coupled with colorimetry, the cells are visualized by specialized plate readers. Thus, the molecule is assayed before it is diluted in the supernatant, captured by receptors of adjacent cells, or degraded.

Helper peptide: A peptide that binds to class II MHC molecules and thereby creates an epitope recognized by CD4⁺ helper T cells.

Melanocytic differentiation protein (MDP): Protein expressed by melanocytes and by melanoma cells, typically functioning as part of the melanin synthetic pathway. Examples include tyrosinase, gp100, and MART-1/MelanA.

Th1: A categorization of helper (CD4⁺) T-cell responses, manifested typically by production of cytokines, including interferon gamma, interleukin-2, and tumor necrosis factor α , and with functional importance in supporting generation of cytotoxic T-cell responses.

Th2: A categorization of helper (CD4⁺) T-cell responses, manifested typically by production of cytokines, including interleukin-4, interleukin-5, and interleukin-10, and with functional importance in supporting generation of B-cell and antibody responses.