Immune Correlates of GM-CSF and Melanoma Peptide Vaccination in a Randomized Trial for the Adjuvant Therapy of Resected High-Risk Melanoma (E4697)

Lisa H. Butterfield, University of Pittsburgh
Fengmin Zhao, Dana Farber Cancer Institute
Sandra Lee, Dana Farber Cancer Institute
Ahmad A. Tarhini, University of Pittsburgh
Kim A. Margolin, Stanford University
Richard L. White, Levine Cancer Institute
Michael B. Atkins, Georgetown Lombardi Comprehensive Cancer Center
Gary I. Cohen, Greater Baltimore Medical Center
Theresa L. Whiteside, University of Pittsburgh
John M. Kirkwood, University of Pittsburgh

Only first 10 authors above; see publication for full author list.

Journal Title: Clinical Cancer Research
Volume: Volume 23, Number 17
Publisher: American Association for Cancer Research | 2017-09-01, Pages 5034-5043
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1158/1078-0432.CCR-16-3016
Permanent URL: https://pid.emory.edu/ark:/25593/tdb9x

Final published version: http://dx.doi.org/10.1158/1078-0432.CCR-16-3016

Copyright information:
© 2017 American Association for Cancer Research.
Accessed January 30, 2020 11:16 PM EST
Immune correlates of GM-CSF and melanoma peptide vaccination in a randomized trial for the adjuvant therapy of resected high-risk melanoma (E4697)

Lisa H. Butterfield¹, Fengmin Zhao², Sandra Lee², Ahmad A. Tarhini¹, Kim A. Margolin³, Richard L. White⁴, Michael B. Atkins⁵, Gary I. Cohen⁶, Theresa L. Whiteside¹, John M. Kirkwood¹, and David H. Lawson⁷

¹University of Pittsburgh, Pittsburgh, Pennsylvania ²Dana Farber Cancer Institute – ECOG-ACRIN Biostatistics Center, Boston, Massachusetts ³Stanford University, Stanford, California (current location), Seattle Cancer Care Alliance, Seattle, Washington (former location) ⁴Levine Cancer Institute, Carolinas Healthcare System, Charlotte, North Carolina ⁵Georgetown Lombardi Comprehensive Cancer Center Washington, DC (current location) Beth Israel Deaconess Medical Center, Boston, Massachusetts (former location) ⁶Greater Baltimore Medical Center, Baltimore, Maryland ⁷Winship Cancer institute of Emory University, Atlanta, Georgia

Abstract

Purpose—E4697 was a multi-center intergroup randomized placebo-controlled Phase III trial of adjuvant GM-CSF and/or a multi-epitope melanoma peptide vaccine for patients with completely resected, high-risk stage III/IV melanoma.

Experimental Design—815 patients were enrolled from 12/99 to 10/06 into this 6-arm study. GM-CSF was chosen to promote the numbers and functions of dendritic cells (DC). The melanoma antigen peptide vaccine (Tyrosinase368-376 (370D), gp100209-217 (210M), MART-127-35) in Montanide was designed to promote melanoma specific CD8⁺ T cell responses.

Results—Although the overall RFS and OS were not significantly improved with the vaccine or GM-CSF when compared with placebo, immunomodulatory effects were observed in peripheral blood and served as important correlates to this therapeutic study. Peripheral blood was examined to evaluate the impact of GM-CSF and/or the peptide vaccine on peripheral blood immunity and to investigate potential predictive or prognostic biomarkers. 11.3% of unvaccinated patients and 27.1% of vaccinated patients developed peptide-specific CD8⁺ T cell responses. HLA-A2⁺ patients who had any peptide-specific CD8⁺ T cell response at day +43 tended to have poorer OS in univariate analysis. Patients receiving GM-CSF had significant reduction in percentages of circulating myeloid dendritic cells (mDC) and plasmacytoid DC (pDC) at day +43. In a subset of patients who received GM-CSF, circulating myeloid-derived suppressor cells (MDSC), and anti-
GM-CSF neutralizing antibodies (Nabs) were also modulated. The majority of patients developed anti-GM-CSF Nabs, which correlated with improved RFS and OS.

Conclusions—The assessment of cellular and humoral responses identified counter-intuitive immune system changes correlating with clinical outcome.

Keywords
melanoma; vaccine; GM-CSF; T cells; MDSC

Introduction
Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) is a hematopoietic cell growth factor established in practice for its utility in promoting development of neutrophils and macrophages. It plays a key role in dendritic cell (DC) development and maturation (1). Substantial preclinical studies have shown that tumor cell transfection with a GM-CSF gene could render tumors more immunogenic in part by mobilizing mononuclear cells into the site of tumor (2). GM-CSF is also a critical growth factor for dendritic cell homeostasis (3) and can promote increases in circulating DC frequencies and maturation (4). Administration of melanoma vaccines has improved immune responses and promoted clinically significant anti-tumor responses in some studies (5-7). As recently reviewed (8), results of the clinical trials testing different doses, routes and combinations of GM-CSF in melanoma have been mixed. A previous single arm study in 48 stage III/IV melanoma patients indicated that median survival was significantly longer in those receiving adjuvant GM-CSF compared to historical controls, supporting further clinical testing (9). Another previous cooperative group randomized phase II study (E1696) evaluated the effect of systemically administered GM-CSF or IFNα2b on immune responses to a melanoma antigen multi-epitope peptide vaccine comprised of the same peptides, in patients with inoperable metastatic melanoma. The ability to mount an immune response to at least one vaccinating peptide, as determined by IFNγ ELISPOT, was associated with improved survival (10). There were positive but non-significant trends toward enhancement of the immune response with both GM-CSF and IFNα2b. Of the 115 analyzable patients in E1696, there were 2 complete and 4 partial responses, and there was no significant difference in terms of best overall response amongst the 4 treatment arms.

E4697 was the first randomized trial of adjuvant GM-CSF in patients with resected stage III and IV melanoma. The median overall survival (OS) was 10.3 months longer (69.6 vs. 59.3 months, hazard ratio 0.94, not significant), and the median relapse-free survival (RFS) for GM-CSF was 11.4 vs. 8.8 months (for placebo, hazard ratio of 0.88 (not significant). These results are regardless of HLA status and whether the patients received peptides or peptide placebo. There are several immune measures, however, that may help to elucidate the function of GM-CSF in antitumor immunity and the types of immune modulation that might be beneficial.

Analysis of results by pre-specified clinical stratification factors suggested differential effects of both GM-CSF and peptide vaccine according to the sites of metastases (visceral vs. non-visceral). These results are hypothesis-generating and, if real, they could possibly be

Clin Cancer Res. Author manuscript; available in PMC 2018 September 01.
explained by differences in the immune status of patients. Visceral metastases that persist past resection, the effect of resection itself and possible differential effects of immunomodulatory agents on visceral vs. non-visceral tumor microenvironments could impact immunity and trial outcomes.

In order to examine the immunologic impact of GM-CSF and the peptide vaccine, peripheral blood was drawn to test for cellular and humoral responses over time, as well as circulating GM-CSF levels. Based on the earlier E1696 study, we hypothesized that evidence of developing peptide specific, CD8+IFNγ+ T cell responses by ELISPOT and increased DC activity could be biomarkers of improved outcome, and that GM-CSF-mediated enhancement of circulating myeloid-derived suppressor cells (MDSC) or inhibition of GM-CSF activity by development of neutralizing anti-GM-CSF antibodies might be associated with worse outcome.

Materials and Methods

The clinical trial details, eligibility, toxicities and clinical outcomes have been published (11). A descriptive diagram is shown in Supplementary Figure 1. GM-CSF (or placebo) dosing schedule was: 250 μg/d, s.c., days 1-14 of each 28 day cycle. Peptide (or placebo) dosing schedule was: emulsified peptides were mixed and administered s.c., into 3 different sites on days 1 and 15 of cycle one, then on day 1 of subsequent cycles. Enrollment occurred between Dec. 29, 1999, and Oct. 31, 2006.

Peripheral blood (in heparin anticoagulant for peripheral blood mononuclear cells (PBMC) and without anticoagulant for serum) from patients was sent by overnight courier to the ECOG-ACRIN Immunology Reference Laboratory at the University of Pittsburgh Cancer Institute. The laboratory is CAP and CLIA certified, which requires personnel testing, reagent qualification and other laboratory function regulation adherence. PBMC were isolated by a Ficoll gradient centrifugation and cryopreserved after removing an aliquot for any fresh flow cytometric testing performed (below). After completion of the treatment regimen, samples from baseline and the day 43 and 85 time points were evaluated simultaneously for each patient.

For flow cytometry, 218 PBMC samples were available for analysis of at least some phenotypic markers which were pre-specified in the study protocol (Table 1, Supplementary Figure 2). Fresh whole blood (from between 128-218 patients, depending on antibody panel and timepoint, tested between Oct. 3, 2002, and May 7, 2008) was stained for CD3, CD11c, CD19, CD56, CD86, CD123 and HLA-DR. A total of 10 measures of immune cells were assessed by relative percentage, including total dendritic cells (large, granular, lineage-negative, HLA-DR+ cells), myeloid DC (mDC, lin/HLA-DR+/CD11c+), plasmacytoid DC (pDC, lin/HLA-DR+/CD123+), T cells (CD3+), NK cells (CD56+) and B cells (CD19+). In addition, using quantitation beads, the absolute counts of total lymphocytes, T, NK and B cells were calculated from whole blood as cells/mL. Cell levels were measured at baseline, day 43 and day 85. Banked PBMC were tested after trial closure for suppressor cell frequencies in a pilot study (tested in 2012). MDSC (48 patients) were identified as either
HLA-DR\textsuperscript{low}/CD14\textsuperscript{+} or lineage\textsuperscript{-}/HLA-DR\textsuperscript{+}/CD11b\textsuperscript{+}/CD33\textsuperscript{+}. Regulatory T cells (Treg) (48 patients) were identified by staining for CD3\textsuperscript{+}/CD4\textsuperscript{+}/CD25\textsuperscript{hi}/FOXP3\textsuperscript{+}/CD39\textsuperscript{+}.

For direct IFN\(\gamma\) ELISPOT analysis of CD8\textsuperscript{+} T cell responses to peptides (pre-specified in the study protocol), 277 patients had at least some data available. Assays were performed as described for E1696 (10) beginning Aug. 6, 2003, and completing Feb. 25, 2009. No \textit{in vitro} sensitization of lymphocytes was performed. The IFN\(\gamma\) ELISPOT was performed by trained technologists using standard operating procedures and validated reagents. Performance of the assay by different technologists was cross-compared. All plates were read by automated ELISPOT plate readers. Each assay contained wells in which normal healthy donor cells (made in large, multi-use batches) were tested for spontaneous and phorbol 12-myristate 13-acetate/ionomycin-stimulated cytokine release, serving as an assay control. Medium-only wells served as controls for nonspecific spots from assay reagents. A doubling of the net baseline spot count (with 10 or more spots per well counted) was considered a positive response.

Banked serum samples were tested for circulating GM-CSF by ELISA among 47 patients (23 patients with long term survival [\(\geq 60\) months] and 24 patients with poor survival) in a pilot case-control study designed after the completion of the trial (tested in 2012). Circulating anti-GM-CSF neutralizing antibodies were detected with a TF-1 erythroleukemia cytokine-dependent cell line (ATCC CRL-2003) proliferation inhibition assay. The TF-1 cell proliferation assay was developed and standardized, in which the growth factor-dependent cell line was cultured in the presence of GM-CSF with increasing amounts of patient serum to test for antibodies that would block the function of GM-CSF and reduce TF-1 cell proliferation (12, 13).

**Statistical Analysis**

For the secondary objectives of laboratory correlative studies (analysis of T cell reactivity and circulating DC and immune response), the sample size calculation was based on projected number of cases who will provide lab samples. For the two pre-specified correlative studies (i.e., examining circulating lymphocyte and myeloid cell frequencies and CD8\textsuperscript{+} T cell IFN\(\gamma\) response to peptides), all patients enrolled to the main study were eligible to participate (Supplementary Table 1). For the pilot study designed after completion of the trial to examine circulating MDSC, Treg, GM-CSF, and anti-GM-CSF neutralizing antibody levels, 48 patients (24 patients with good survival [\(\geq 63\) months] and 24 patients with poor survival [\(\leq 60\) mo.]) were included, all of whom received GM-CSF (+/- peptides).

CD8\textsuperscript{+} immune response was defined as: doubling of numbers of spots over at least 10 per 5 \times 10^4 cells at day 43 or day 85 from the baseline measurement. If the baseline spot was zero, an increase by at least 10 per 5 \times 10^4 cells was considered as the immune response. This is consistent with the definition used in E1696. Fisher exact test was used to compare CD8\textsuperscript{+} immune response between patients with and without peptide vaccines. Log rank test used a landmark method (14) in which the time of 1.45 months (i.e., 43 days) was used to evaluate the association between CD8\textsuperscript{+} immune response at Day +43 and clinical outcomes. Levels of circulating lymphocytic and myeloid cells were compared between groups receiving or not receiving GM-CSF using Wilcoxon rank sum tests. For evaluating the prognostic value
of these measures, the log rank test was used to examine the association between baseline level and change in cell levels between baseline and Day +43 (all dichotomized at the median, with the same landmark method used for the evaluation of cell level changes) and clinical outcomes.

For the pilot study, the prognostic value of baseline circulating MDSC and Treg levels (dichotomized at the median) and change between baseline and Day +43 (defined as follow up level- baseline level, and dichotomized as increase vs. no change/ decrease, via landmark method) were explored in relation to RFS and OS. For anti-GM-CSF neutralizing antibody analysis, the presence of antibody at baseline (TF-1 proliferation below expected level with GM-CSF cytokine added) and development of anti-GM-CSF neutralizing antibodies during treatment (defined as serum-mediated inhibition of TF-1 proliferation at any time point) was examined. The association between presence of neutralizing GM-CSF antibody and clinical outcomes was explored using the log rank test. The association between detectable circulating GM-CSF levels during the trial (defined as rhGM-CSF greater than 14 at any time point after pre) and clinical outcomes were also explored using log rank test.

STATA 13.1 was used for all analyses, and all tests were two-sided with significance level of 0.05. No adjustment was made for multiple comparisons due to the exploratory nature of the analysis.

Results

To elucidate the immunomodulatory effects of the GM-CSF and peptide vaccine and to potentially identify peripheral blood biomarkers of clinical benefit, serial samples of peripheral blood were tested. One objective of this trial was to assess the activity of the melanoma lineage antigen peptide vaccine in HLA-A2–positive patients (Arms A, B, C and D). In this cohort, the median RFS was 1.7 months longer in patients receiving peptide vaccine vs. peptide vaccine placebo (11.5 vs. 9.8 months), which was not statistically significant. Among these HLA-A2–positive patients, median OS and RFS were longer in patients receiving both GM-CSF and peptide vaccine, compared to patients on the other three arms, but not significantly so, which is consistent with the findings from trial E1696 conducted in patients with active disease (10).

IFNγ ELISPOT analysis of responses to peptides

To determine whether immunization with these HLA-A2-restricted, well characterized melanoma lineage antigen peptides, with or without GM-CSF, elicited a measurable T cell response, CD8+ response was assessed at baseline, Day 43 (post vaccination), Day 85 and end of 1 year or relapse (if available). As with the E1696 study, a standardized direct IFNγ ELISPOT assay was performed. A total of 277 patients had data about their immune response to peptides gp100209-217, MART-127-35 and Tyrosinase368-376 at baseline, Day 43 and/or Day 85. The overall CD8+ T cell response rate was 19.5% (95% CI: 15.0, 24.7), and as expected, it was higher in patients who received peptide vaccination (27.1%, 95% CI: 20.0, 35.1) than in patients without peptide vaccination (11.3%, 95% CI: 6.4, 17.9). The difference was statistically significant (p = 0.001, Fisher exact test), indicating that the peptide vaccination was moderately immunogenic across patients. Gp100209-217 was the
most immunogenic at days 43 and 85, followed by MART-1\textsubscript{27-35} and Tyrosinase\textsubscript{368-376} was least commonly responded to. The data also indicate that 11% of patients had a spontaneous immune response to these commonly expressed melanoma lineage antigens. At day +43, 11.3% of patients in Arm A (peptides + GM-CSF) had an immune response, while in Arm B (peptides + GM-CSF placebo) this was significantly higher at 25.0% (p = 0.004). The data suggest that GM-CSF did not improve patients' responses to the peptide vaccine and may have inhibited responses.

**Association between vaccine CD8\(^+\) T cell response and clinical outcome**

Using a landmark method with a landmark time of 1.45 months (i.e., 43 days), the median OS for patients with any vaccine peptide-specific CD8\(^+\) T cell response at Day +43 was 39.8 months (95% CI: 22.6,-), and it was 89.6 months (95% CI: 60.4,-) for patients without a CD8\(^+\) response. The 5-year OS rate was 46.4% (95% CI: 32.5, 59.2) for patients with CD8\(^+\) response and 57.2% (95% CI: 50.3, 63.5) for patients without CD8\(^+\) response. Figure 1 shows the Kaplan-Meier estimates of OS by CD8\(^+\) response at Day +43. While the difference was not statistically significant (log-rank test p = 0.200 in all patients and p = 0.077 in patients receiving both peptide vaccine and GM-CSF), it was in disagreement with previous results from E1696 in which patients all had measurable active disease. Figure 1B shows the analysis for RFS, which was not significantly different (log-rank test p = 0.672 in all patients, and p = 0.332 for arms A and B). The responses to each of the three vaccine peptides are shown in Supplementary Table 2, showing that the gp100\textsubscript{2092M} peptide was the most immunogenic.

**Interaction of stage and CD8\(^+\) response**

CD8\(^+\) response and staging data were available for 267 patients, of whom 64 had resected M1b/M1c (visceral metastases) disease. Overall CD8\(^+\) response was 28.1% in M1b/M1c patients vs. 17.1% in patients with other stages (p=0.054). At day 43, the CD8\(^+\) response was 23.2% in patients with resected visceral metastases vs. 10.0% in patients who had non-visceral disease (p=0.011). A CD8\(^+\) response was associated with worse outcome even after adjusting for stage, indicating no imbalance in disease stage in the analysis population.

**Effect of GM-CSF on circulating lymphocyte and myeloid cells, absolute counts and percentages**

Another correlative objective of the study was to determine the effect of GM-CSF on circulating myeloid (especially DC) and lymphocyte numbers in fresh peripheral blood. A total of 581 patients had flow cytometric data about their levels of the different cell subsets at one or more time points, and 97 patients had data for all measures of cells at all three time points (Table 1, Supplementary Table 1, Supplementary Figure 2, all arms). Because whole blood was tested, we collected data on absolute lymphocyte counts as well as percentages.

Overall, total DC, mDC, CD19\(^+\) B and CD123\(^+\)(pDC) cell percentages significantly decreased with GM-CSF treatment, and mean changes in levels of cells were negative in both Day 43 and Day 85 assessments for patients with GM-CSF, while they were positive for patients without GM-CSF (p < 0.05 for comparing change in cell levels during Day 43 and Day 0 between GM-CSF groups for all of them) (example in Supplementary Figure 3).
T cell percentages, total absolute lymphocyte, absolute T cell and NK cell counts significantly increased with GM-CSF treatment, and the mean changes in cell levels were positive in both Day 43 and Day 85 assessments for patients with GM-CSF (p < 0.05 for comparing change in cell levels during Day 43 and Day 0 between GM-CSF groups for all of them) (Supplementary Figure 3). Percent NK cells and absolute B cell counts were relatively stable over time and there was no significant difference between GM-CSF groups (Table 1, Supplementary Figure 2).

Correlation between circulating cell changes and clinical outcome

Baseline cell levels and change in cell levels between baseline and day +43 were dichotomized at the median for each measure. For the mDC and pDC, lower baseline levels of the CD11c+ mDC and of CD123+ pDC were associated with better RFS (p = 0.048 and p = 0.041, respectively) and a decrease in pDC at day +43 showed a trend towards improved OS (p = 0.059) (Fig 2A). Other DC measures were not significantly associated with either RFS or OS.

Of the measures of lymphocytes, a decrease in B cells at day +43 was significantly associated with improved OS (p = 0.006) (Fig 2B) and also showed a trend towards improved RFS (p = 0.053). As stated above, T cell absolute counts and percentages increased with GM-CSF, and a lack of decrease in CD3+ T cells was significantly associated with improved OS (Fig 2C). No other measures correlated with RFS or OS.

Examination of circulating MDSC and Treg

GM-CSF may directly impact MDSC. There are several populations of these suppressive myeloid cells, and we examined two subsets (15-17) that we have examined previously in other melanoma clinical trials. At the same time, we also tested for the frequency of circulating regulatory T cells (Treg), which could also negatively impact antitumor immunity (in patients receiving GM-CSF, Amrs A, C, E).

Forty-five of the 47 patients had Treg and/or MDSC data for at least one time point and included in the analysis (21 patients were in the poor survival group, and 24 patients in the good survival group) (Supplementary Table 3). The results showed that at baseline, no measures of Treg and only one subset of immature myeloid cells (lin-/HLA-DR-/CD33+ cells, (whether larger in size (p = 0.042) or smaller in size (p = 0.065)) correlated with or showed a trend towards improved outcome (Fig 3A, 3B). Examining changes over time, increases in smaller size lin-/HLA-DR-/CD33+/CD11b+ (trend at p = 0.059) and in immature lin-/HLA-DR+ myeloid gate cells (p = 0.045) correlated with poorer outcome (Fig 3C, 3D). Interestingly, an increase in lin-/HLA-DR+ monocytes was correlated with improved OS (p = 0.045). Changes in Treg (both FoxP3+ and CD39+ Treg) did not correlate with either RFS or OS.

Interaction of stage with circulating cell subsets

Baseline circulating lymphocytes and myeloid cells (dichotomized at the median) were not associated with stage. M1b/M1c disease was, however, associated with less of a decrease in CD123+ (pDC) cells than seen in other stages (p=0.042). There was no significant
association between baseline MDSC or Treg and stage or with changes in these cells over time. There was a trend towards less increase in lin-/HLA-DR- monocytes in patients with stage M1b/M1c disease compared to other stages (14.3 vs 41.7%, p=0.055).

**Examination of anti-GM-CSF neutralizing antibody levels**

In the pilot study, we hypothesized that the effects of the administered GM-CSF could be reduced by the biologic activity of anti-GM-CSF neutralizing antibodies (Nabs). Overall, 47 patients were included in the analysis, and 8/47 (17%) tested patients had detectable Nabs at baseline and 37/47 (78%) of these showed detectable circulating Nabs on treatment. 7/8 (87%) patients who were baseline-positive remained positive on treatment; 30/39 (77%) who were baseline-negative became antibody-positive on treatment, suggesting that development of Nabs was a common phenomenon in this study. The Kaplan-Meier plots (Figure 4) show that presence of Nabs at either point in time on treatment correlates with superior OS (p = 0.045) and that presence of Nabs on treatment correlates with superior RFS (p = 0.048). Patients who had Nabs at baseline tended to have lower levels of circulating GM-CSF, but the sample size was small (p = 0.085, not significant).

It was possible that those patients with higher titers of anti-GM-CSF antibodies had fewer MDSC. Therefore, we correlated titers of anti-GM-CSF antibodies with the frequency of MDSC subsets, but none of these results were statistically significant. There was no association between stage and development of neutralizing antibodies.

**Serum GM-CSF levels**

The levels of circulating GM-CSF at baseline, day +43 and day +85 were also tested in the above 47 patients included in the pilot study. Overall, 13 of the 47 patients tested had detectable circulating GM-CSF cytokine (> = 14 pg/mL) at baseline, and 21/47 showed detectable circulating GM-CSF on treatment; however, neither was related to clinical outcome (OS or DFS). To examine a correlation between development of Nabs and reduction in serum GM-CSF, results showed that 21/47 had both detectable GM-CSF and Nabs above the LLD. Of those, 17/21 showed a pattern of circulating GM-CSF reduction after Nab development (not significant). Circulating GM-CSF could also potentially support an increase in circulating monocyte subsets (and as noted above, increases in lin-/HLA-DR+ monocytes was associated with improved OS), however, there are no data suggesting correlation with circulating MDSC levels.

**Discussion**

After two decades of careful exploration the role of GM-CSF as an immunotherapy for patients with cancer and a modulator of the immunotherapeutic effects of vaccines and immune checkpoint blockade, the contribution of GM-CSF remains uncertain. Studies have demonstrated modest effects in both directions with respect to enhancement or reduction of immune antitumor responses (9, 11, 18). The present study presents the immunological corollary analysis of the largest prospective trial that has evaluated the role of GM-CSF as adjuvant therapy for patients with resected high-risk melanoma.
The vaccine peptide-specific CD8+ T cell ELISPOT immune response results in this study were not consistent with those obtained earlier in E1696, in which the T cell response correlated with improved outcome (10). This is despite the assays testing responses to the same peptides being performed in the same core laboratory at the same time points, by the same SOPs with similar assay control results. A possible reason for this difference relates to the adjuvant setting evaluated in this trial, in comparison to the metastatic setting of the previous study (E1696). Another is that the shared, non-mutated antigen targets may only be effective when they can promote tumor lysis and determinant spreading to additional, potentially mutated antigens, which may not occur to the same extent in the adjuvant setting as in advanced disease (19). Possible reasons for this lack of a significant impact measured across the entire patient population includes baseline immune status of the patients (inability to respond due to immune tolerance, limited repertoire or peripheral suppression) or limitations of the vaccine and immune adjuvant platform for effective activation of T cell responses. Patients with resected visceral disease were significantly more likely to develop a CD8+ response than those with non-visceral disease. The impact of CD8+ response persisted even within the M1b/M1c patients. In addition, a larger number of different institutions participated in E4697 than E1696, which could contribute to variability at several points.

To address the issue of peripheral tolerance, we examined the circulating immunosuppressive cell types, Treg and MDSC. Treg are known to inhibit the proliferation and function of antigen-specific T cells that could negatively impact the effectiveness of the peptide vaccine (20, 21). We also tested whether the GM-CSF dosages used in this study (slightly higher than in some others (8)) might have increased MDSC. In addition, MDSC levels have also been shown to correlate with clinical outcome (17) in patients with renal cell cancer and melanoma treated with CTLA-4 blocking antibodies (22-25) and the combination of PD-1 and CTLA-4 blocking antibodies (26). We did not see significant changes in MDSC frequency with GM-CSF as administered in this study that would support this hypothesis, although an increased circulating frequency of some subsets of immature myeloid cells and MDSC was negatively correlated with OS. In the pilot study, among patients receiving GM-CSF, a minority showed an increase in circulating MDSC.

The decrease in circulating mDC observed in this study was unexpected. A previous study in resected stage III B/C and stage IV melanoma patients (n = 39) receiving a slightly lower dosage (125 μg/m^2/d) of GM-CSF found a transient increase in both frequency and maturation status of DC subsets (27). In addition, the time point at which the significant increase in DC frequency and maturation was detected was week 2 post GM-CSF. Our first measure was taken at d+43 (1 day after the end of the second 14-day course of GM-CSF); hence, a transient increase may have been missed. GM-CSF was previously shown to inhibit pDC development in mice (28).

The presence of anti-GM-CSF neutralizing antibodies at baseline is rare and has been associated with autoimmunity (12); however, this study did not track any clinical measures of autoimmunity. The development of an anti-GM-CSF antibody response after cytokine administration may be a biomarker of successful treatment with GM-CSF, or may simply represent immune competence that in itself correlates with improved endpoints like OS and RFS. A previous study testing adjuvant therapy with GM-CSF in melanoma patients (29)
and utilizing 125 μg/m²/d GM-CSF found that 0/43 patients had nAb at baseline, 42% developed nAb during GM-CSF treatment, and development of Nab correlated with reduced effects of GM-CSF, including WBC counts.

GM-CSF levels, in combination with the levels of other cytokines, have been identified as correlates of outcome in patients with CLL or colorectal cancer (30, 31). While GM-CSF may have influenced the numbers of neutrophils, the numbers and functions of these cells were not tested experimentally. We did observe that most patients who had detectable GM-CSF in their serum who subsequently developed Nabs had a subsequent reduction in circulating growth factor.

The adjuvant setting of this trial means that there is no tumor and tumor microenvironment to examine. While measures of biomarkers in blood can correlate with tumor response, there are limitations to assaying this compartment. In addition, cytokines, by their nature, act in close proximity as cell-cell communication, and their half lives are limited. The setting of this large, multi-institution trial allowed for limited blood sampling, and the kinetics of cytokine impact are not yet known.

The biomarker studies performed in this study were designed for consistency with the goal of validating a candidate biomarker of clinical response from E1696 in E4697. Given the results, future analysis of trials with peptide vaccines and with GM-CSF may consider more detailed phenotyping of mDC and pDC subsets, including activation, maturation, trafficking and functional markers. Likewise, MDSC and Treg phenotyping has evolved to include markers of different subsets, and functional testing is now more accessible. The single parameter IFNγ ELISPOT for CD8+ T cell response may be insufficient to shed light on the functional state of the tumor-specific effector cell response, and testing of costimulatory and checkpoint molecules may provide important information.

GM-CSF may find its greatest role in the therapy of melanoma when administered in combination with other agents or as a component of autologous or allogeneic cancer cell vaccine (32). In addition, the GM-CSF encoding vaccinia T-VEC has also shown clinical efficacy (33). Recent data from ECOG-ACRIN suggests that adding GM-CSF at the dose and schedule used in the present study to ipilimumab (10 mg/kg) may improve both efficacy and safety of this regimen in patients with metastatic disease (18). These data have led to an ongoing trial (EA6141) that will further test this hypothesis with the recently approved combination immunotherapy (ipilimumab + nivolumab +/- GM-CSF) for advanced melanoma (18). If GM-CSF improves outcomes and mitigates toxicity from this combination, the role of GM-CSF may then warrant testing with these agents in the adjuvant setting.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

This study utilized the University of Pittsburgh Cancer Institute’s Immunologic Monitoring Laboratory shared facility, supported in part by award P30 CA047904. We want to thank all the coordinators who ensured proper handling of specimens and data, as well as the patients and their families.

Financial Support: This study was coordinated by the ECOG-ACRIN Cancer Research Group (Robert L. Comis, MD and Mitchell D. Schnall, MD, PhD, Group Co-Chairs) and supported in part by Public Health Service Grants CA180802, CA180794, CA180844, CA180888, CA180816, CA189858, CA180821, CA180802, CA180864, and from the National Cancer Institute, National Institutes of Health and the Department of Health and Human Services. Its content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute.

References


Statement of Translational Relevance

This multicenter, randomized Phase III trial tested a shared melanoma antigen peptide vaccine and/or GM-CSF for high-risk operable melanoma in the adjuvant setting. This study investigated a range of immunotherapy biomarkers in the blood of patients, including cellular and humoral responses. The clinical impact of GM-CSF and antigen-specific CD8\(^+\) T cell responses was counter to what had been hypothesized based on a previous phase II study in the metastatic setting, with significant decreases in circulating dendritic cells with GM-CSF and poorer outcomes for those with peptide-specific CD8\(^+\) T cell responses. The optimal setting for peptide vaccines and GM-CSF remains to be identified.
Figure 1.
Overall survival (OS) by CD8+ response in HLA-A2+ patients. OS by CD8+ response in all patients with data (1A) or RFS (1B) is plotted in the Kaplan Meier graphs.
Figure 2.
Circulating DC and lymphocyte subsets. A: Kaplan Meier curve for OS by pDC change between Day 43 and Day 0 (dichotomized at median)—landmark method. B: Kaplan Meier curve for OS by B cell change between Day 43 and Day 0 (dichotomized at median)—landmark analysis. C: KM curve for OS by CD3 change between Day 43 and Day 0 (dichotomized at median)—landmark analysis.
Figure 3.
Circulating Monocyte and MDSC subsets. A: OS by small (lymphocyte gate) Lin1+/HLA-DR+/CD33+ at baseline. B: OS by Lin1+/HLA-DR+/CD33+ at baseline. C: OS by change (increased vs. no change/reduced) in small Lin1+/HLA-DR+/CD33+/CD11b+. D: OS by change (increased vs. no change/reduced) in larger Lin1+/HLA-DR+. 
Figure 4.
<table>
<thead>
<tr>
<th>Change in cell levels</th>
<th>No GM-CSF</th>
<th>GM-CSF</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># of patients</td>
<td>Mean change</td>
<td>SD</td>
</tr>
<tr>
<td>Total dendritic cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>103</td>
<td>0.007</td>
<td>0.261</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>95</td>
<td>0.007</td>
<td>0.274</td>
</tr>
<tr>
<td>mDC (CD11c+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>98</td>
<td>0.120</td>
<td>6.148</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>81</td>
<td>-0.642</td>
<td>6.060</td>
</tr>
<tr>
<td>CD11c+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>103</td>
<td>0.020</td>
<td>0.284</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>95</td>
<td>0.035</td>
<td>0.284</td>
</tr>
<tr>
<td>pDC (CD123+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>98</td>
<td>-0.624</td>
<td>4.268</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>81</td>
<td>1.442</td>
<td>21.090</td>
</tr>
<tr>
<td>CD123+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>101</td>
<td>0.013</td>
<td>0.357</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>94</td>
<td>0.050</td>
<td>0.307</td>
</tr>
<tr>
<td>B cells (CD19+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>171</td>
<td>0.544</td>
<td>4.118</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>158</td>
<td>0.430</td>
<td>3.500</td>
</tr>
<tr>
<td>Percent T cells (CD3+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>167</td>
<td>-11.323</td>
<td>304.937</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>155</td>
<td>-49.916</td>
<td>268.096</td>
</tr>
<tr>
<td>Absolute T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>171</td>
<td>-0.667</td>
<td>5.380</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>158</td>
<td>-1.006</td>
<td>4.802</td>
</tr>
<tr>
<td>Total absolute lymphocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>167</td>
<td>0.970</td>
<td>382.695</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>155</td>
<td>-43.445</td>
<td>349.389</td>
</tr>
<tr>
<td>Change in cell levels</td>
<td>No GM-CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td># of patients</td>
<td>Mean change</td>
<td>SD</td>
</tr>
<tr>
<td>Absolute NK cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>167</td>
<td>4.210</td>
<td>80.765</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>155</td>
<td>7.084</td>
<td>70.634</td>
</tr>
<tr>
<td>Percent NK cells (CD56+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>171</td>
<td>0.222</td>
<td>4.915</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>158</td>
<td>0.734</td>
<td>3.860</td>
</tr>
<tr>
<td>Absolute B cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change between Day 43 and Day 0</td>
<td>167</td>
<td>11.287</td>
<td>76.757</td>
</tr>
<tr>
<td>Change between Day 85 and Day 0</td>
<td>155</td>
<td>2.381</td>
<td>67.698</td>
</tr>
</tbody>
</table>

Note: mean change>0 indicated an increase in cell levels between time points, and mean change<0 indicated a decrease in cell levels between time points.