Phenotypic and Functional Analysis of Dendritic Cells and Clinical Outcome in Patients With High-Risk Melanoma Treated With Adjuvant Granulocyte Macrophage Colony-Stimulating Factor


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Authors’ disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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ABSTRACT

Purpose

Granulocyte macrophage colony-stimulating factor (GM-CSF) can induce differentiation of dendritic cells (DCs) in preclinical models. We hypothesized that GM-CSF–stimulated DC differentiation may result in clinical benefit in patients with high-risk melanoma.

Patients and Methods

We conducted a prospective trial in patients with high-risk (stage III B/C, IV), resected melanoma, with GM-CSF 125 μg/m²/d administered for 14 days every 28 days. Patients underwent clinical restaging every four cycles, with DC analysis performed at baseline and at 2, 4, 8, and 12 weeks.

Results

Of 42 patients enrolled, 39 were assessable for clinical outcome and DC analysis. Median overall survival was 65 months (95% CI, 43 to 67 months) and recurrence-free survival was 5.6 months (95% CI, 3 to 11 months). GM-CSF treatment caused an increase in mature DCs, first identified after 2 weeks of treatment, normalizing by 4 weeks. Patients with decreased DCs at baseline had significant increases in DC number and function compared with those with “normal” parameters at baseline. No change was observed in the number of myeloid-derived suppressor cells (MDSCs). Early recurrence (< 90 days) correlated with a decreased effect of GM-CSF on host DCs, compared with late or no (evidence of) recurrence.

Conclusion

GM-CSF treatment was associated with a transient increase in mature DCs, but not MDSCs. Greater increase of DCs was associated with remission or delayed recurrence. The prolonged overall survival observed warrants further exploration.

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INTRODUCTION

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a polypeptide that can stimulate differentiation of hematopoietic progenitor cells. In patients with high-risk, resected melanoma, GM-CSF appears to improve overall survival by almost three-fold compared with matched historical controls. On the basis of these results, the Eastern Cooperative Oncology Group is currently conducting a randomized trial of GM-CSF in patients with high-risk melanoma.

One of the major immunostimulatory mechanisms of GM-CSF is through the stimulation of the professional antigen-presenting cells, such as dendritic cells (DCs). GM-CSF increases the mobilization, differentiation, and function of DCs. However, the effect of GM-CSF on DC function in cancer patients has not been well studied. Both quantitative and functional defects in DCs have been described in patients with cancer; these defects may contribute to tumor “escape” from immune surveillance. In this study, we examined the effects of GM-CSF on DC phenotype and function and its association with clinical outcome in patients with resected, high-risk melanoma.

PATIENTS AND METHODS

Patient Selection and Treatment

Patients had documented stage IIIB/C or IV melanoma. All disease was grossly resected before enrollment. No prior treatment with GM-CSF was allowed. Patients may have had any prior interferon or chemotherapy provided treatment was completed and WBC and platelet
counts had recovered. A maximum of 60 days from the last surgery that rendered them disease-free was allowed. All patients provided written informed consent, and the institutional review board at the H. Lee Moffitt Cancer Center (Tampa, FL) approved this protocol.

GM-CSF was administered at 125 μg/m² subcutaneously daily for 14 consecutive days followed by 14 days of no treatment for a total of 13 cycles unless there was unacceptable toxicity or recurrence. A physical exam was performed every 4 weeks, and restaging was performed after every 4 cycles.

Evaluation of Cell Phenotype

Peripheral blood was collected before starting and after 2, 4, 8, and 12 weeks of GM-CSF treatment. Peripheral-blood mononuclear cells (PBMCs) were isolated and cryopreserved in freezing media containing 50% RPMI-1640-1640, 40% FBS, and 10% DMSO and stored in liquid nitrogen. All samples from each patient were analyzed simultaneously. Briefly, PBMCs were thawed, cultured overnight in complete culture medium (RPMI-1640-1640/10% FCS) and labeled with the appropriate antibodies for 40 minutes on ice. The cocktail of lineage-specific antibodies included phycoerythrin-conjugated antibodies against CD3, CD19, CD56, and CD14 (all from BD Pharmingen, San Jose, CA). In addition, we used peridinin chlorophyll protein–conjugated anti–human leukocyte antigen (HLA)-DR antibody, allopheocyanin-conjugated anti-CD3 and CD11c antibodies, fluorescein isothiocyanate–conjugated anti-CD86, CD83, and CD40 antibodies (all from BD Pharmingen). Fluorescein isothiocyanate–conjugated antibodies against CCR7 and CD123 were obtained from R&D Systems (Minneapolis, MN) and Miltenyi (Auburn, CA), respectively. The phenotype of the cells was evaluated by multicolor flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). At least 25,000 cells were examined from each parameter, with subsequent analysis of the samples described previously. An example of the analysis is shown in Appendix Figure A1 (online only).

**Evaluation of DC function**

Allogeneic mixed leukocyte reaction (MLR) was used to evaluate DC function. Mononuclear cells from healthy donors were seeded in U-bottom 96-well plates (10⁵ cells per well). Mononuclear cells from patients were isolated as described earlier in this article, irradiated at 20 Gy, washed, and then mixed in triplicates at different ratios (from 1:1 to 1:8) with mononuclear cells isolated from healthy donors. Cell proliferation was assessed after 5-day culture by uptake of [³H]-thymidine. One micromurie of [³H]-thymidine was added to each well 18 hours before cell collection and thymidine uptake was measured in liquid scintillation counter (Beckman Coulter, Fullerton, CA) and expressed as counts per minute (cpm). Each patient’s sample was tested against two different donors, and average for each cell ratio was calculated. All samples from each patient were analyzed simultaneously.

**Statistical Analysis**

The distribution of the DC parameters at each time point was explored with the Wilcoxon signed rank test. The changes from the baseline were further analyzed using the same method by the following stratified subgroups: disease status, tumor stage (stage III v stage IV), baseline scores (low v high divided by median value), and time to recurrence (early recurrence [< 90 days] v late recurrence [≥90 days]). The correlation among all immunologic markers was evaluated using Spearman correlation coefficients. We analyzed the association of clinical response to the levels of immunologic markers at each time point utilizing Fisher’s exact test. Kaplan–Meier curves were used to plot survival data, and the log-rank test was used to compare survival between subgroups. All statistical tests were two sided, with statistical significance

**Table 1. Baseline Patient Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study Patients (N = 39)</th>
<th>Spalter et al² (%)</th>
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<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIB</td>
<td>7</td>
<td>18</td>
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<tr>
<td>IIIC</td>
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<td>28</td>
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<tr>
<td>IVA</td>
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<td>IVB</td>
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<tr>
<td>IVC</td>
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<tr>
<td>Age, years</td>
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<tr>
<td>&gt; 65</td>
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<td>46</td>
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<tr>
<td>Median</td>
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<tr>
<td>Range</td>
<td>36-80</td>
<td>†</td>
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<tr>
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<tr>
<td>Male</td>
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<td>72</td>
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<tr>
<td>Female</td>
<td>11</td>
<td>28</td>
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<tr>
<td>Location of primary</td>
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<tr>
<td>Skin</td>
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<td>Unknown</td>
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<td>18</td>
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<tr>
<td>Breslow thickness, mm</td>
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<td></td>
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<tr>
<td>&lt; 1</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>1.01-2.0</td>
<td>7</td>
<td>18</td>
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<tr>
<td>2.01-4.0</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>&gt; 4</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>‡55 years.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>†Range, 23-81 years.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>§1.5-4.0 mm.</td>
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</table>

*55 years.
†Range, 23-81 years.
§1.5 mm.

**Table 2. Toxicities by Grade**

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<tr>
<th>Adverse Event</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Any Grade</th>
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<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
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<tr>
<td>Anemia</td>
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<td>4.9</td>
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<td>Lymphopenia</td>
<td>4</td>
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<tr>
<td>Pain</td>
<td>20</td>
<td>48.8</td>
<td>4</td>
<td>9.8</td>
<td>1</td>
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<tr>
<td>Fever</td>
<td>7</td>
<td>17.1</td>
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<td>0</td>
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<td>Flu-like symptoms</td>
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<td>36.6</td>
<td>1</td>
<td>2.4</td>
<td>0</td>
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<td>Arthralgia/myalgia</td>
<td>19</td>
<td>46.3</td>
<td>4</td>
<td>9.8</td>
<td>2</td>
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<tr>
<td>Injection-site reaction</td>
<td>22</td>
<td>53.7</td>
<td>14</td>
<td>34.2</td>
<td>0</td>
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<tr>
<td>Fatigue</td>
<td>24</td>
<td>58.5</td>
<td>1</td>
<td>2.4</td>
<td>0</td>
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<tr>
<td>Abdominal cramps</td>
<td>7</td>
<td>17.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>GERD/indigestion</td>
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<td>14.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Insomnia</td>
<td>9</td>
<td>22</td>
<td>1</td>
<td>2.4</td>
<td>1</td>
</tr>
<tr>
<td>Cough</td>
<td>4</td>
<td>9.8</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Sweating</td>
<td>6</td>
<td>14.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
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Abbreviation: GERD, gastroesophageal reflux disease.
accepted at the \( P < .05 \) level. Because of the exploratory nature of this study, multiple comparisons were not adjusted for in the analyses.

**RESULTS**

Of 42 patients enrolled, 39 patients were assessable for disease status and for at least one DC analysis. Staging and demographic characteristics are described in Table 1. Comparable data from the Spitzer et al trial\(^2\) is shown in Table 1. GM-CSF treatment was well tolerated in this trial, as described in (Table 2). The most common adverse events reported were injection-site reactions (88%). Despite the high-risk characteristics of the patient population under study, the median overall survival was 65 months (95% CI, 43 to 67 months; Fig 1A). This figure is significantly higher than that reported for essentially the same population by Spitzer et al\(^2\) or by the American Joint Committee on Cancer staging committee. Median recurrence-free survival was a more modest 5.6 months (95% CI, 3.7 to 11 months; Fig 1B). Patients with stage III B or IIIC disease had a clinical outcome that was similar to those with stage IV disease (median survival, 65 months for stage III, 56.8 months for stage IV; \( P = .7455 \)), as shown in Figure 2. Patients with recurrent disease had significantly shorter survival compared with those who remained in remission (Appendix Fig A2, online only). Control values for the analysis of different DC subsets were established previously using a small cohort of eight healthy volunteers.\(^6\) In the current study, analysis of different subsets of DCs was performed before and during treatment with GM-CSF (a typical DC phenotype analysis is shown in Fig A1). Patients enrolled on this study did not show significant differences in DC parameters compared with healthy volunteers (data not shown). The time course of DC changes is shown in Figure 3. Two weeks of GM-CSF treatment resulted in a significant increase in the proportion of the total population of mature DCs, as defined by the expression of CD86, CD83, CCR7, and CD40 molecules (Fig 3, and Appendix Table A2, online only). GM-CSF did not affect the proportion of immunosuppressive, myeloid-derived suppressor cells (MDSCs) at the early or late time points (Fig 3). Two weeks after GM-CSF was stopped, the proportions of DCs, as well most of their subsets, returned to a pretreatment level. Subsequent cycles of GM-CSF treatment (weeks 8 and 12) did not change the DC presence in peripheral blood (Fig 3). It is important to point out that the number of patients evaluated before the start of treatment and after 2 and 4 weeks of GM-CSF treatment was the same (\( n = 39 \)), whereas analysis could be performed only in 20 patients 8 weeks after start of treatment and in only 16 patients 12 weeks after start of treatment. The total number of DCs was evaluated before and 4 weeks after start of the treatment. No differences from pretreatment levels were found (data not shown).

The allogeneic MLR, a hallmark of DC activity, was used to evaluate the function of these cells. Patients enrolled on this study did not show significant differences in DC function compared with healthy volunteers (data not shown). GM-CSF did not cause significant changes in DC function in total cohort of patients (Fig 3).

Although the mean values of DC parameters in patients before starting treatment were not different from healthy controls, there was a substantial patient-to-patient variability. Therefore asked whether the effect of GM-CSF on DCs depended on the pretreatment levels of these DC phenotype markers and functional activity. To address this question, patients were split into two groups. Group 1 included half of the patients with pretreatment levels of DC populations above median values established for the control group. Thus,
these patients could be considered as having control level of DC parameters. Group 2 included half of the patients with pretreatment levels of DC subsets below median values established for control group. GM-CSF treatment did not affect any of the tested cell populations in patients with normal pretreatment levels of DC (Fig 4). In contrast, the proportions of total DCs; CCR7⁺, CD40⁺, and CD83⁺ mature DCs; and allogeneic MLR were significantly increased in patients with low pretreatment levels of these parameters (Fig 4). GM-CSF did not affect the presence of MDSCs regardless of pretreatment levels of these cells (Fig 4). Thus, it appears that GM-CSF treatment increased proportion and function of DCs only in patients who had a low level of these cells before start of the treatment.

Next, we asked whether the effect of GM-CSF on DCs could be associated with the clinical outcome of melanoma patients. The first clinical evaluation after 4 weeks of treatment showed no evidence of recurrence for all patients. However, six of 38 patients experienced recurrence within 60 days after the start of GM-CSF therapy, with 11 patients experiencing recurrence within 90 days after the start of the treatment. These were considered early recurrences. A total of 27 patients had no evidence of recurrence during the first 3 months of GM-CSF therapy. These were considered late recurrences. By the time of this analysis, 16 patients eventually experienced recurrence, and 11 patients still have no evidence of recurrence. We evaluated the effect of GM-CSF on DC after 2 weeks of treatment in patients with early and late recurrence. No difference was found in the level of DC subsets between the groups before starting GM-CSF therapy. There was a trend to higher level of DC function in patients with late recurrence (58,847 cpm) compared with patients with early recurrence (49,409 cpm). However, those differences did not reach statistical significance (P = .20). Two weeks of GM-CSF treatment resulted in a significant increase in the proportion of DCs and DC:MDSC ratio in patients with late recurrence, but not in those with early recurrence (Fig 5).

To investigate the association between DC response to GM-CSF treatment and clinical outcome, we further analyzed patients after 2 weeks of GM-CSF treatment. All patients were split into groups on the basis of the presence of different DC subsets; patients with control levels of DC subsets (greater than median values) and patients with low levels of DC subsets (less than median values). The frequency of patients with favorable course of the disease (late recurrence) was calculated in each group and compared. Patients with control levels of total population of DCs and CD40⁺ mature DCs had significantly higher frequency of late recurrence than patients with low levels of these parameters (P = .05 and .016, respectively).

It is well established that different populations of DCs are biologically connected with each other. We evaluated this connection using a correlation matrix of 159 pairs of parameters analyzed in this study (parameters are listed in Appendix Table A2). Before starting treatment, we identified statistically significant correlations in 43 pairs of immunologic parameters in patients who had no evidence of recurrence for more than 90 days after starting treatment. The number of pairs with significant correlation remained stable during the first 4 weeks of the GM-CSF treatment.

**Fig 3.** Effect of granulocyte macrophage colony-stimulating factor treatment on (A) dendritic cells (DCs; Lin-HLA-DR cells), (B) myeloid-derived suppressor cells (MDSCs; Lin-HLA-DR-CD33), (C) CD86⁺ cells among DCs, (D) CD40⁺ cells among DCs, (E) CD83⁺ cells among DCs, (F) CCR7⁺ cells among DCs, and (G) allogeneic mixed leukocyte reaction (MLR) in patients with high-risk melanoma. *1P values of differences from pretreatment level are shown only if they were less than .05. Lin, lineage; HLA, human leukocyte antigen; cpm, counts per minute.
In preclinical studies, GM-CSF has demonstrated a positive effect on DC differentiation. There are, however, limited and conflicting data on GM-CSF effects on DC in patients with cancer. Therefore, detailed pharmacodynamic analysis of the effect of GM-CSF treatment on DC subsets in cancer patients is needed to help clarify its potential clinical utility. Several groups have examined the role of GM-CSF therapy in high-risk melanoma patients. Notably, detailed pharmacodynamic analysis of the effect of GM-CSF treatment on DC subsets in cancer patients is needed to help clarify its potential clinical utility. Several groups have examined the role of GM-CSF therapy in high-risk melanoma patients.

Our results showed a 65-month median overall survival (95% CI, 43 to 67 months) for patients with high-risk characteristics similar to those treated by Spitler et al (Table 1 and Appendix Table A1, online only). Compared with other melanoma data sets, in this high-risk population, our data show a markedly prolonged survival. Although stage migration, patient selection, lead-time bias, and better medical care may potentially account for such differences, our data would argue for a potential positive effect of GM-CSF in melanoma patients.

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Our results showed a 65-month median overall survival (95% CI, 43 to 67 months) for patients with high-risk characteristics similar to those treated by Spitler et al (Table 1 and Appendix Table A1, online only). Compared with other melanoma data sets, in this high-risk population, our data show a markedly prolonged survival. Although stage migration, patient selection, lead-time bias, and better medical care may potentially account for such differences, our data would argue for a potential positive effect of GM-CSF in melanoma patients.

In contrast, patients with "early" recurrence had only 19 pairs with significant correlation before start of GM-CSF treatment. This was significantly lower compared with patients with more favorable disease outcomes (P = .001). After 4 weeks of treatment, significant correlations were detected in only eight of 159 pairs, significantly lower than the number before treatment (P = .043) and in those patients with no evidence of recurrence for more than 90 days (P < .001).

DISCUSSION

(P = .14). In contrast, patients with “early” recurrence had only 19 pairs with significant correlation before start of GM-CSF treatment. This was significantly lower compared with patients with more favorable disease outcomes (P = .001). After 4 weeks of treatment, significant correlations were detected in only eight of 159 pairs, significantly lower than the number before treatment (P = .043) and in those patients with no evidence of recurrence for more than 90 days (P < .001).
Because no patient demonstrated clinical evidence of recurrence at 4 weeks after the start of GM-CSF treatment, those differences most likely cannot be directly attributed to the disease progression at the time of peripheral-blood collection, suggesting that increased proportions of DCs may have positive impact on disease progression. Although this difference cannot be considered prognostic on the basis of this small data set, changes in DC populations should be further analyzed in large randomized trials. How a transient increase in DCs in peripheral blood could be associated with a positive clinical outcome is not entirely clear. It is possible that DCs migrate from peripheral blood and accumulate in tissues where they can pick up tumor antigen with subsequent stimulation of T cells. In this case a transient increase of DCs in peripheral blood may cause a long-lasting effect. More detailed studies, including biopsies of tissues in addition to circulating DCs are needed to directly test this possibility.

It appears that the effect of GM-CSF depends on basal levels of DC subsets. GM-CSF increased the proportion of DC only in patients who initially had a low level of these cells, having no effect for those patients with a normal level of DGs. These results suggest that GM-CSF treatment may be beneficial only to those patients with reduced DC numbers at baseline. The total tumor burden seems to directly affect the proportion of DCs in cancer patients. A recent study evaluated the effect of GM-CSF alone or in combination with autologous tumor-derived heat shock protein peptide vaccine in 16 patients with metastatic melanoma. The authors observed an accumulation of immunosuppressive CD14+ HLA-DR−negative/low cells. This accumulation was not observed with vaccine alone. We did not find such an effect of GM-CSF on MDSCs in our study. There are two important differences between this study and our trial, namely that our patients were tumor free at the start of GM-CSF treatment and that the dose and schedule of GM-CSF used in our trial was different.

Although in this study we have independently replicated the clinical results observed by Spitler et al, ultimately, the Eastern Cooperative Oncology Group E4697 trial, which recently randomly assigned more than 800 patients with resected, high-risk melanoma in a 2 × 2 design to adjuvant GM-CSF versus placebo and (for HLA-A2–positive patients only) to a peptide vaccine versus placebo, will establish whether or not adjuvant GM-CSF therapy influences the natural history of these patients. Given both our findings and those of Spitler et al, it will be of particular interest to see whether the overall survival impact of GM-CSF therapy may be greater than the recurrence-free survival benefit—a situation at odds with the findings in adjuvant interferon trials. Our observations regarding the effect of this agent on DC phenotype and function could provide potential surrogate markers for future trials and offer avenues to further extend this benefit. Our results also suggest that perhaps only a subset of patients treated with GM-CSF obtain a benefit.

Because of the relatively short-lived effect of GM-CSF on DCs, it may have a direct impact on clinical outcome of melanoma patients. We failed to show a response to GM-CSF in patients who had early recurrence of the disease (<90 days), whereas patients with late or no recurrence had significant increases in several DC markers. These data were obtained after 2 weeks of treatment.
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Stock Ownership: None
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Research Funding: Adil I. Daud, Berlex Oncology
Expert Testimony: None
Other Remuneration: None

AUTHOR CONTRIBUTIONS

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Final approval of manuscript: Adil Daud, Stephanie Andrews, Vernon K. Sondak, Adam I. Riker, Dmitry Gabrilovich

Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).