Granulocyte Macrophage Colony-stimulating Factor and Interleukin 4 Enhance the Number and Antigen-presenting Activity of Circulating CD14+ and CD83+ Cells in Cancer Patients

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ABSTRACT
Antigen-presenting cells (APCs) are essential for stimulating antigen-specific immunity, including immunity against tumor cells. We hypothesized that systemic administration of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, which promote monocytes to differentiate into dendritic cells in vivo, might enhance the number and antigen-presenting activity of CD14+ cells in vivo. Patients with metastatic solid malignancies were treated with daily s.c. injections of either GM-CSF alone (2.5 μg/kg/day) or GM-CSF in combination with IL-4 (0.5–6.0 μg/kg/day) in a multicohort study. When given alone, GM-CSF increased the number of CD14+ cells but did not enhance the cells’ expression of APC markers or antigen-presenting activity. In contrast, combination therapy with GM-CSF and IL-4 stimulated CD14+ cells to acquire several APC characteristics including increased expression of HLA-DR and CD11c, decreased CD14, increased endocytotic activity, and the ability to stimulate T cells in a mixed leukocyte reaction. Combination therapy also induced a dose-dependent increase in the number of CD14+/CD83+ cells with APC activity. Clinically significant and sustained tumor regression was observed in one patient. Systemic therapy with GM-CSF and IL-4 may provide a mechanism for increasing the number and function of APCs in patients with cancer.

INTRODUCTION
Professional APCs, such as DCs, play a central role in stimulating antitumor immune responses (1–3). Using chimeric mice, Huang et al. (2) observed that immunogenic tumors expressing the B7-1 transgene were incapable of stimulating an immune response in the absence of MHC-matched, bone marrow-derived APCs. In a more direct approach, murine bone marrow-derived DCs have been loaded with tumor antigen peptides (3, 4), antigenic proteins (5), tumor lysates (6), or tumor antigen genes (7) and have been shown in each case to stimulate antitumor activity when used to vaccinate naive mice. APCs appear to play a similar role in the response against human cancer. Histopathology studies have long reported a correlation between the number of tumor-associated APCs and patient survival (8–11). As in the murine models, in vitro-generated DCs have also been loaded with tumor antigens and used to vaccinate patients with a variety of cancers including B-cell lymphoma (12), melanoma (13), or prostate cancer (14, 15). Antitumor immunity and objective clinical responses were demonstrated in all three studies. This success with DC therapy prompted us to examine other approaches for increasing the number and/or function of APCs in cancer patients.

Whereas little is known about the in vivo origins and trafficking of human DCs, in vitro studies have identified two cell populations that can act as DC precursors: (a) CD34+ stem cells originating from bone marrow (16, 17); and (b) circulating CD14+ monocytes (18–20). CD34+ stem cells mature into DCs in response to GM-CSF and tumor necrosis factor α, an effect that can be enhanced by a variety of other factors including IL-4, flt3 ligand, and CD40 ligand (21, 22). CD14+ monocytes mature into DCs primarily in response to the combination of GM-CSF and IL-4 (19, 23), although similar effects have been reported when GM-CSF is combined with IFN-α, CD40 ligand or IL-13 (24–26). Based on evidence that viable DC progenitors exist in cancer patients (27) and can respond to cytokine stimulation, we set out to determine whether systemic therapy with the combination of GM-CSF and IL-4 would work in vivo as it does in vitro to increase the number and/or function of circulating APCs. A total of 21 patients with metastatic solid tumors were enrolled in a Phase I, dose-escalating, multicohort study to evaluate the effects of daily therapy with GM-CSF alone versus the combination of GM-CSF and IL-4 on the number, phenotype, and function of circulating APCs.

MATERIALS AND METHODS

Subject Selection. Patients with metastatic solid tumors who had failed or refused standard treatment were enrolled. No other cancer therapy was allowed for 4 weeks before or during this study. Primary exclusion criteria included current central nervous system metastases, an Eastern Cooperative Oncology Group performance status greater than 1, cardiac insufficiency/infarction within the last 12 months, acute or chronic infection, bleeding peptic ulcer disease within the last 6 months, dependence on corticosteroids, organ transplantation, or laboratory evidence of organ insufficiency. Written informed consent was obtained in accordance with the University of California at Los Angeles Institutional Review Board.

Clinical Protocol. Patients were recruited into a total of six successive treatment cohorts (cohorts AI, AII, B, C, D, and E) in a serial manner. Patients were treated with daily s.c. injections of sterile human recombinant GM-CSF (specific activity = 1.125 × 107 units/mg) and IL-4 (specific activity = 2.414 × 107 units/mg) provided by Schering-Plough Research Institute (Kenilworth, NJ). For cohorts AI–D, the 28-day protocol included 14 continuous days of therapy followed by 14 days of observation. For cohort E, the 28-day protocol alternated 7-day periods of cytokine therapy with 7-day periods of observation. Injections were self-administered s.c. to the skin of the thigh and/or abdomen as a home-based therapy. Patients in cohort AI received a fixed dose of GM-CSF (2.5 μg/kg/day), whereas patients in subsequent cohorts received the same dose of GM-CSF in combination with IL-4 at 0.5 (cohort AI), 1.0 (cohort B), 2.0 (cohort C), 4.0 (cohort D), or 6.0 (cohort E) μg/kg/day. All patients successfully completing the AI cohort were enrolled as subjects in the AII cohort, but new subjects were enrolled into all subsequent groups (cohorts B–E). Patients achieving objective clinical responses or stable disease were eligible to continue therapy without further determination of biological activity. Patients were monitored twice weekly for evidence of...
toxicity according to the National Cancer Institute Common Toxicity Criteria. Blood was drawn on days 0, 7, 14, and 21 for in vitro testing to assess the biological effects of therapy. To assess antitumor efficacy, radiological imaging of measurable and evaluable disease and tumor marker serologies (when applicable) were obtained serially for as long as patients remained on therapy. Standard Southwest Oncology Group response criteria were used to determine objective responses.

Laboratory Reagents and Antibodies. CM was composed of RPMI 1640 supplemented with glutamine (Irvine Scientific, Irvine, CA), 0.01 M HEPES buffer, antibiotic-antimycotic mixture (Life Technologies, Inc., Grand Island, NY), and 10% heat-inactivated human AB serum (Gemini Bioproducts, Inc., Calabasas, CA). Dulbecco’s PBS (Life Technologies, Inc.) was supplemented with 2% human AB serum. M450 magnetic beads coated with goat antimouse IgG were obtained from Dynal Inc. (Lake Success, NY). Fluorescein-labeled dextran (M, 40,000) was purchased from Molecular Probes (Eugene, OR). Fluorochrome-conjugated mAbs used for FACS analysis included anti-CD3, anti-CD13, anti-CD14, anti-CD15, anti-CD20, anti-CD40, and anti-CD87 from Caltag Laboratories (South San Francisco, CA); anti-HLA-DR and anti-CD80 (B7-1) from Becton Dickinson (San Jose, CA); anti-CD11c and anti-CD86 (B7-2) from PharMingen (San Diego, CA); anti-CD1a from Serotec (Raleigh, NC); and anti-CD83 (clone HB15a) from Beckman Coulter (Fullerton, CA).

FACS Analysis. Peripheral blood samples from study days 0, 7, 14, and 21 were collected in heparinized tubes, and leukocytes were isolated by differential centrifugation over Ficoll-Paque gradients (Pharmacia Biotech, Inc., Alameda, CA). Leukocytes were analyzed for their expression of cell surface markers by three-color FACS analysis using a single FACScan II flow cytometer that was calibrated daily with CaliBRITE beads and AutoCOMP software (all from Becton Dickinson). Between 5,000 and 300,000 events (depending on the cell population and marker) were acquired for each sample using CellQuest software (Becton Dickinson) that simultaneously acquired data for forward scatter, side scatter, FL1 (FITC label), FL2 (PE label), and FL3 (either tricolor or PerCP label). The settings for all of these parameters were optimized at the initiation of the study and maintained constant during all subsequent analyses to allow direct comparison between samples analyzed on different days. For cohort E, a new lot of anti-CD14 mAb resulted in lower expression as compared to prior cohorts. The relative expression of a given marker was expressed by the LFI. The number of circulating CD14⁺ or CD83⁺ cells per milliliter of blood was determined by multiplying the percentage of leukocytes staining with a given marker (as determined by FACS analysis) by the number of leukocytes recovered per milliliter of blood.

Endocytosis Assay. The temperature-dependent uptake of FITC-labeled dextran was used to measure endocytotic function according to a modification of the procedure described by Sallusto et al. (28). Fresh leukocytes from study patients in the D cohort were resuspended in 0.5 ml of CM (containing 25 mM HEPES) and cultured with FITC-labeled dextran (1 mg/ml) for 60 min at either 0°C or 37°C. Cells expressing CD14 were stained by adding anti-CD14-PE during the last 5 min of the assay, and the reaction was terminated by adding 3 ml of ice-cold PBS containing 0.1% azide. Cell pellets were washed four times, and the CD14⁺ population was analyzed immediately for the intracellular accumulation of the FITC label by FACS analysis. The degree of endocytosis was determined by comparing the intracellular uptake at 37°C with the nonspecific binding that occurred at 0°C.

Allogeneic MLR. APCs collected on different days of therapy were evaluated for their ability to stimulate the proliferation of allogeneic T cells using a one-way MLR. This approach, as compared to a soluble antigen presentation assay, was specifically chosen to prevent any treatment- or cancer-related effects on the patient’s own T cells from interfering with the measurement of APC function (29, 30). APCs were prepared either by depleting contaminating neutrophils from PBMCs with endotoxin-free anti-CD24 mAb (29) or by preparing purified CD14⁺ or CD14⁻/CD83⁺ cells by fluorescence-activated cell sorting analysis using a FACStarPlus flow cytometer and Lysys II software (Becton Dickinson) as described previously (19). APCs were irradiated with 30 Gy (cesium source) and cocultured with 1 × 10³ responder T cells for 6 days at APC:T cell ratios of 1:5 to 1:100. Wells were pulsed with 1 µCi of [³H]thymidine (DuPont New England Nuclear, Boston, MA) and harvested 18 h later using an automated cell harvester (PhD cell harvester; Cambridge Technology, Cambridge, MA). cpm were determined by liquid scintillation counting, and each data point represented the average ± SD of three to six wells.

Data Analysis. Data from replicate measurements of a single assay are expressed as the mean value ± 1 SD, and data representing entire cohorts are represented as the mean value for all of the subjects in a cohort ± 1 SE. The hypothesis that treatment either increased or decreased an outcome variable within a given cohort or between different cohorts was evaluated using either a paired Student’s t test or an unpaired Student’s t test, respectively. A two-way ANOVA with correction for multiple comparisons was used to compare MLR results for the same subject when measured on different days of the treatment. A P of ≤0.05 was considered significant.

RESULTS

Cohort Characteristics. Twenty-one patients with primary tumors including cancers of the lung, kidney, colon, and prostate were enrolled into the six cohorts (Table 1). Reversible, cytokine-induced leukocytosis occurred in all patients with maximal day 14 peripheral blood counts averaging 22,300 ± 10,070 in cohort A and 30,300 ± 7,238 in cohort D (no significant differences). Maximal day 7 counts in the E cohort averaged 15,100. Side effects consisted primarily of grade 1 and 2 constitutional symptoms: fever; bone pain; local injection site erythema; nausea and vomiting; thrombocytopenia; or transaminitis. Grade 3 hepatic toxicity (elevated alkaline phosphatase) occurred in one subject each in cohorts C and D, and grade 3 headache occurred in one subject in cohort E, all of which resolved spontaneously without interruption in therapy. No dose-limiting toxicity was observed, but one patient in cohort AI receiving GM-CSF alone voluntarily withdrew from the study after developing a rash at the injection site.

Effects of Systemic GM-CSF and IL-4 on the Number, Pheno-
type, and Endocytotic Function of Circulating CD14⁺ Cells. GM-
CSF increased the number of circulating CD14⁺ cells, an effect that peaked on day 7 (average, 6.29 × 10⁶ cells/ml) and returned to baseline levels by day 21 (average, 3.5 × 10⁶ cells/ml). A similar result occurred when low doses of IL-4 (0.5–2.0 µg/kg/day) were administered in combination with GM-CSF. However, when patients were treated with ≥4.0 µg/kg/day IL-4, there was a further increase in the number of circulating CD14⁺ cells beyond that observed with GM-CSF alone (Fig. 1). On day 7, peripheral blood CD14⁺ counts averaged 5-fold higher in cohort D (P < 0.05) and 2.2-fold higher in cohort E as compared with the increase that resulted from GM-CSF alone. The number of CD14⁺ cells returned to baseline within 7 days of completing cytokine therapy in all cohorts.

As observed when GM-CSF and IL-4 were used to generate DCs in vitro, the administration of these cytokines in vivo to patients resulted in an IL-4-dependent decrease in the intensity of CD14 staining.

### Table 1 Cohort characteristics

<table>
<thead>
<tr>
<th>Cohort</th>
<th>GM-CSF (µg/kg/day)</th>
<th>IL-4 (µg/kg/day)</th>
<th>Age (yr ± SE)</th>
<th>Primary tumor (no. per type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.5</td>
<td>0</td>
<td>56 ± 4</td>
<td>Lung: 1</td>
</tr>
<tr>
<td>AII</td>
<td>2.5</td>
<td>0.5</td>
<td>52 ± 3</td>
<td>Kidney: 3</td>
</tr>
<tr>
<td>B</td>
<td>2.5</td>
<td>1</td>
<td>65 ± 5</td>
<td>Colon: 1</td>
</tr>
<tr>
<td>C</td>
<td>2.5</td>
<td>2</td>
<td>60 ± 9</td>
<td>Kidney: 3</td>
</tr>
<tr>
<td>D</td>
<td>2.5</td>
<td>4</td>
<td>69 ± 3</td>
<td>Lung: 1</td>
</tr>
<tr>
<td>E</td>
<td>2.5</td>
<td>6</td>
<td>59 ± 6.3</td>
<td>Prostate: 1</td>
</tr>
</tbody>
</table>

* Patients received daily s.c. injections of GM-CSF and IL-4 at the indicated doses for either 14 consecutive days (cohorts A–D) or for two 7-day periods interrupted by 7 days of observation (cohort E).
on days 0, 7, 14, and 21 for their capacity to take-up FITC-labeled dextran. Cells collected from days 7 and 14 of therapy showed a 5–6-fold greater uptake of FITC-labeled dextran as compared with CD14+ cells collected from either day 0 or day 21, suggesting functional activation of receptor-mediated endocytosis (Fig. 4).

Effects of Systemic GM-CSF and IL-4 on the Number of Circulating CD14+/CD83+ Cells. In addition to its effect on CD14+ cells, the combination of GM-CSF and IL-4 may play a role in the differentiation of DCs from stem cells (16, 21). This pathway of DC maturation is associated with the early expression of several markers, including CD83 and CD1a, which are only expressed on monocyte-derived DCs at terminal stages in their development (17, 31). This pathway of DC maturation was monitored by staining peripheral blood samples with fluorescence-labeled anti-CD83 and anti-HLA-DR mAb and enumerating the percentage and number of CD83+/HLA-DR+ cells by FACS analysis (Figs. 1B and 5). The administration of GM-CSF, alone or in combination with 0.5 or 1.0 μg/kg/day IL-4, did not significantly increase the percentage of CD83+/HLA-DR+ cells. However, there was a dose-dependent increase in the percentage of CD83+/HLA-DR+ cells at higher IL-4 doses, with the maximal effect observed in the D cohort (2.35 ± 1.8% of PBMCs in the D cohort versus 0.09 ± 0.09% of PBMCs in the A1 cohort, P < 0.05). In terms of the total number of circulating CD83+/HLA-DR+ cells recovered per milliliter of blood, there was an average 130-fold increase by day 7 in patients treated with 4 μg/kg/day IL-4 (range, 26–399-fold increase; Fig. 1B). This CD83+/HLA-DR+ population was comprised of two distinct subsets, one containing smaller cells with minimal autofluorescence, and the other containing larger cells with a relatively high autofluorescence (Fig. 6). Both subsets expressed HLA-DR and CD1a in the absence of CD14, a pattern consistent with DCs of stem cell origin.

Effects of Systemic GM-CSF and IL-4 on in Vitro Measures of Antigen-presenting Activity and in Vivo Measures of Antitumor Activity. PBMCs collected from patients on days 0, 7, 14, and 21 of therapy were evaluated for their ability to stimulate T-cell proliferation in an allogeneic one-way MLR using T cells from a single normal donor as responders. No consistent treatment effect was observed in patients treated with GM-CSF alone or with GM-CSF in combination with low doses of IL-4 (cohorts AII or B). However, PBMCs collected from one patient in cohort C demonstrated increased MLR activity on treatment days (days 7 and 14) as compared with pre- and posttreatment days (Fig. 7). In cohorts D and E, the CD14+ populations were purified by cell sorting and compared for their allostimulatory activity. A treatment-related increase in activity was observed in two of the four patients in cohort D and in four of the four patients in cohort E (Fig. 7). In one patient (patient D4), CD83+ cells were also prepared by cell sorting and found to stimulate greater T-cell proliferation than either PBMCs or sorted CD14+ cells collected from day 0.

All patients were assessed for therapy-associated changes in tumor burden. Two of three patients (one in cohort D and one in cohort E)

![Fig. 1. Circulating CD14+ cells and CD83+/HLA-DR+ cells are increased in patients receiving GM-CSF and IL-4.](image)

Fig. 1. Circulating CD14+ cells and CD83+/HLA-DR+ cells are increased in patients receiving GM-CSF and IL-4. Patients were treated for 14 days with either GM-CSF alone at 2.5 μg/kg/day (cohort A) or GM-CSF in combination with IL-4 at 0.5, 1.0, 2.0, or 4.0 μg/kg/day (cohorts AII–D, respectively). Peripheral blood leukocytes collected on days 0, 7, 14, and 21 were stained with fluorescence-labeled anti-CD14 or a combination of anti-CD83 and anti-HLA-DR, and the percentage of cells expressing these markers was determined by FACS analysis. This value was multiplied by the number of cells isolated per milliliter of blood to calculate the number of CD14+ (A) or CD83+/HLA-DR+ (B) cells/ml. Values represent the mean ± SE for each cohort. * P < 0.05 compared to the same cohort on day 0, paired t test. † P < 0.05 compared to cohort AII or AIII on the same day, unpaired t test.

(Table 2). This effect was detectable on day 7 in the C cohort and was statistically significant at both days 7 and 14 in the D cohort. Similar effects were observed by day 7 in the E cohort, where CD14 expression was decreased by 65% compared to the day 0 value. Concurrent with the decrease in CD14, there was an IL-4-dependent increase in the expression of HLA-DR in the same cells (Fig. 2). By day 7 of treatment, HLA-DR expression increased to 229 ± 87% of day 0 levels in the D cohort (P < 0.05) and 308 ± 83% of day 0 levels in the E cohort (P < 0.05). Significant increases in the expression of HLA-DR were not observed in patients receiving GM-CSF alone. A similar trend was observed for the up-regulation of CD11c (Fig. 2).

However, in contrast to DCs generated from monocytes in vitro (19), expression of CD80 was only modestly increased (Fig. 3), and expression of CD86 was not increased at all on circulating CD14+ cells from treated patients. To evaluate this further, peripheral blood leukocytes were isolated from cohort E on day 7 of in vivo therapy and cultured for 24 h in vitro in the absence of any exogenous cytokines. This short-term culture resulted in a dramatic up-regulation of CD80, CD86, CD1c, and HLA-DR on the CD14dim+ cells, producing a pattern of expression similar to that observed when monocytes are cultured in vitro with GM-CSF and IL-4 for a full 7 days (19). Overnight culture also resulted in expression of CD83, a marker usually expressed only on functionally mature DCs.

The maturation of monocytes into DCs is associated with an increase in their ability to take-up and process antigens. As a measure of this function, CD14+ cells from cohort D were serially evaluated in an in vitro test.

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### Table 2  CD14 expression is down-regulated by GM-CSF in combination with increasing doses of IL-4

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI</td>
<td>1193 ± 312</td>
<td>911 ± 35</td>
<td>1025 ± 231</td>
<td>1017 ± 221</td>
</tr>
<tr>
<td>AII</td>
<td>1181 ± 336</td>
<td>1503 ± 203</td>
<td>1489 ± 181</td>
<td>1302 ± 200</td>
</tr>
<tr>
<td>B</td>
<td>1770 ± 454</td>
<td>1284 ± 203</td>
<td>1300 ± 301</td>
<td>1299 ± 114</td>
</tr>
<tr>
<td>C</td>
<td>1908 ± 194</td>
<td>982 ± 123</td>
<td>1299 ± 114</td>
<td>1536 ± 143</td>
</tr>
<tr>
<td>D</td>
<td>1782 ± 96</td>
<td>629 ± 50</td>
<td>717 ± 53</td>
<td>1695 ± 320</td>
</tr>
</tbody>
</table>

|       | Average ± SE for each cohort, n = 3 for cohort AI, and n = 4 for cohorts AII, B, C, and D. |
|       | * P ≤ 0.10 compared to day 0 for the same cohort, paired t test. |
|       | † P ≤ 0.05 compared to day 0 for the same cohort, paired t test. |
with prostate cancer demonstrated objective responses. Patient D1, who had hormone-refractory prostate cancer metastatic to two mediastinal lymph nodes and multiple bones (confirmed by computed tomography scans, positron emission tomography scans, and bone scans), experienced a partial response with a marked improvement in bone pain and a lasting decrease in serum PSA from 49 (pretreatment) to 0.8 ng/ml after four cycles of therapy and to 2.3 ng/ml after 1 year. After four cycles of therapy, he also experienced a 50% reduction in the size of involved mediastinal lymph nodes (which continued to decrease over 1 year of observation) and a persistent decrease in the size and intensity of all bone lesions as determined by bone scan.

Patient E4, who had a rising postoperative PSA, responded with a decrease in PSA from 13 to 5.2 ng/ml after the first cycle of therapy. After three cycles of therapy, PSA remained at 5.0 ng/ml but rose to

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**Fig. 2.** In vivo exposure to GM-CSF and IL-4 down-regulates the expression of CD14 and up-regulates the expression of HLA-DR and CD11c in a dose-responsive manner. Peripheral blood was collected from each subject on days 0 (white histograms) and 7 (black histograms), and the CD14+ population was evaluated for expression of CD14, HLA-DR, and CD11c by FACS analysis. Numbers indicate the average marker expression (mean ± SE for the entire cohort) on day 7 as a percentage of each individual’s marker expression on day 0. Representative histograms for a single individual from each cohort are shown. *, $P < 0.05$ comparing day 7 to day 0, paired t test. †, $P < 0.10$ comparing day 7 to day 0, paired t test.

**Fig. 3.** Circulating CD14+ cells from patients treated with GM-CSF and IL-4 rapidly mature into DCs in culture. Circulating CD14+ cells collected from cohort E on day 7 expressed less CD14 but increased levels of HLA-DR, CD80, and CD11c compared to CD14+ cells collected from the same subjects on day 0. When cultured in vitro in CM (no exogenous cytokines) for 24 h, day 7 cells rapidly up-regulated expression of HLA-DR, CD80, CD86, CD11c, and CD83 in a pattern consistent with mature DCs. A representative experiment is shown (subject E4).
Fig. 4. In vivo exposure to GM-CSF and IL-4 increases receptor-mediated endocytosis. Peripheral blood leukocytes from cohort D were collected on days 0, 7, 14, and 21 and cultured for 60 min at either 0°C (white histograms) or 37°C (black histograms) in the presence of FITC-labeled dextran (1 mg/ml). Cells were counterstained with anti-CD14-PE, and the intracellular accumulation of FITC-labeled dextran on the CD14+ population was determined by FACS analysis. Numbers indicate LFI for the FITC label at 37°C minus that at 0°C. Representative results are shown (subject D2).

19.9 within 2 weeks of stopping treatment, and the patient was switched to hormonal therapy. He continues with stable disease after 10 months of follow-up. No other patients demonstrated objective tumor responses.

**DISCUSSION**

DCs are bone marrow-derived APCs that express high levels of MHC, adhesion molecules, and other important costimulatory molecules required for antigen presentation (32, 33). Their ability to take-up antigens and induce antigen-specific immunity has stimulated considerable interest in using them to treat cancer. However, naturally occurring DCs are exceptionally rare, comprising only 0.01–0.5% of circulating and tumor-infiltrating mononuclear leukocytes (19, 34). Even when present, DCs harvested from cancer patients often fail to express normal levels of antigen-presenting molecules and lack the ability to stimulate effective immune responses (27, 34, 35). Enk et al. (35) purified CD83+ DCs from the tumors of patients with both regressing and progressing melanoma metastases. Whereas the DCs from regressing metastases expressed CD86 and functioned as APCs, the DCs recovered from progressing metastases expressed little CD86 and induced T-cell anergy instead of stimulation. Similarly, Gabrilovich et al. (27) found that DCs isolated from the circulation of breast cancer patients were defective and that DC function inversely correlated with tumor stage. A variety of tumor-derived factors, including vascular endothelial growth factor, macrophage colony-stimulating factor, IL-6, and IL-10, are believed to alter the maturation and function of DCs (36–38). Despite the poor function of mature DCs in cancer patients, DC precursors appear to be relatively unaffected. By isolating PBMCs from cancer patients and culturing them with cytokines in vitro, Gabrilovich et al. (27) and many others (13–14, 39) have been able to generate large numbers of functionally normal DCs. Using this strategy, DCs expanded from precursors in vitro have been loaded with antigenic tumor peptides and used to vaccinate patients. This approach has produced approximately 30% response rates in initial trials for metastatic melanoma (13) and prostate cancer (14, 15) but has produced no responses in a trial for colon cancer (40).

Whereas the ex vivo generation of cytokine-derived DCs offers an important new approach to cancer therapy, it requires considerable resources and expertise. A leukopheresis is required to obtain precursors, recovered cells need to be purified and differentiated in vitro under sterile conditions, and mature DCs need to be loaded with antigen and returned to patients. As an alternative to ex vivo therapy, we hypothesized that the combination of GM-CSF and IL-4 might be administered directly to cancer patients to generate cytokine-derived APCs in situ. This approach has several potential advantages in terms of the large number of CD14+ and CD34+ precursors that exist in vivo; the capacity to activate APCs directly within lymphoid organs, tissues, and tumor sites; and the potential for a simplified home-based therapy. Patients in this study quickly learned how to administer their own injections at home. The expansion of APCs in this manner could also be combined with standard vaccine approaches to target responses against specific antigens. However, even in the absence of antigen targeting, the placement of cytokine-derived APCs directly within murine tumors has been shown to induce tumor regression (41). Similarly, the in vivo expansion of APCs with systemic FLT3 ligand induces antitumor immunity in a variety of tumor models (42–44). These animal models, in combination with human studies that find a correlation between the number of tumor-associated APCs and clinical outcome (8–11), support the hypothesis that increasing the number of APCs in vivo might activate antitumor immunity.

The feasibility of combining GM-CSF and IL-4 as an in vivo therapy was based on past experience with these cytokines. As others have reported (45, 46), we found that a 2-week course of GM-CSF was well tolerated and induced a 5–20-fold increase in the number of circulating CD14+ cells. Although there is less experience with IL-4, a prior study observed 5–6 μg/kg/day to be the maximum tolerable dose when given alone as a daily injection for 2 weeks (47). Pharmacokinetic analysis documented blood levels in the range of 1–12

**Fig. 5.** In vivo exposure to GM-CSF and IL-4 promoted a dose-responsive increase in the percentage of PBMCs expressing CD83. Peripheral blood leukocytes collected from cohorts AI–D on day 7 were stained with anti-CD83 and anti-HLA-DR to determine the percentage of cells expressing the CD83+ /HLA-DR+ phenotype. Representative two-dimensional dot-plots (CD83-stained cells versus unstained cells) for each cohort are shown, and the average percentage (± SE) of PBMCs expressing the CD83+ /HLA-DR+ phenotype for that cohort is displayed numerically. *P < 0.05 compared to cohort AI, unpaired t test.
ng/ml after a 4 μg/kg/day injection of IL-4 and suggested that peak levels might be sustained for up to 8 h (48). Preliminary in vitro studies (data not shown) confirmed that as little as 1.25 ng/ml IL-4 was sufficient, when combined with GM-CSF, to promote human monocytes to mature into APCs, with higher levels producing more rapid and complete DC maturation. The fact that neither GM-CSF nor IL-4 has produced marked antitumor responses in the past when used as single agents is not surprising (45, 49, 50). The capacity for GM-CSF and IL-4 to differentiate precursors into DCs is highly synergistic, requiring both cytokines to achieve an effect (20). This type of synergism was readily apparent in our study when the combination of GM-CSF and IL-4, but not GM-CSF alone, dramatically increased the number of CD14^+ and CD83^+ cells with APC characteristics. In terms of tolerability, combined treatment with GM-CSF and IL-4 at doses of ≤4 μg/kg was similar to GM-CSF alone and was not associated with any dose-limiting toxicity. At 6 μg/ml, patients experienced a higher frequency and severity of constitutional symptoms, which prompted us to shorten the treatment interval to 7 days.

Using the characteristics of in vitro monocyte-derived DCs as a guide, we carefully evaluated circulating CD14^+ cells from patients for evidence of maturation along a DC pathway. Down-regulation of CD14 expression was used as a hallmark to identify monocyte-derived APCs and allowed us to identify the concurrent up-regulation of HLA-DR and CD11c on these cells. In contrast to the phenotypic changes that occurred under optimal in vitro conditions, the cells from treated patients retained modest CD14 expression and did not express high levels of either CD80 or CD86. One conclusion would be that in vivo cytokine therapy resulted in an intermediate stage of DC differentiation. This would not be surprising because cytokine-induced DC maturation occurs in a continuum that is both dose- and time-dependent. Higher dosing with IL-4 or secondary stimulation by CD40 ligand or other activating factors might be required to produce more mature DCs. Alternatively, more mature DCs that developed in response to therapy might have exited the circulation before our sampling. We obtained blood only once a week and, in each case, 24 h after the last cytokine dose. Rapid margination and trafficking of cytokine-activated cells to peripheral sites is a common phenomenon after cytokine therapy, including therapy with GM-CSF (45, 46). Unfortunately, biopsies from tumor sites and/or lymph nodes were not obtained in this study and could not be evaluated for DC infiltration. Finally, the failure to see full expression of CD80 and CD86 on circulating APCs could have resulted from tumor-derived immunosuppressive factors interfering with DC maturation.
To address these issues, we recovered circulating cells from treated patients and placed them into short-term culture in the absence of any exogenous cytokines. Within 24 h, we observed a dramatic up-regulation of HLA-DR, CD80, CD86, CD11c, and CD83 on the CD14dim population, a phenotype almost identical to that generated by culturing monocytes for 7–8 days in high concentrations of GM-CSF and IL-4. This rapid in vitro maturation is similar to that reported when naturally occurring DCs are isolated from the peripheral blood of control subjects (51, 52). In addition to acquiring the phenotypic characteristics of APCs, CD14+ cells from patients treated with higher doses of IL-4 also increased their capacity to take-up FITC-labeled dextran, a well-characterized marker of functionally active DCs (28), as well as their capacity to stimulate T cells in a MLR. Collectively, these findings suggest that combined therapy with GM-CSF and higher doses of IL-4 induced myeloid precursors to differentiate into functional APCs consistent with immature DCs. Whether these circulating cells migrate into tissue and lymphoid organs and terminally differentiate, as they did when cultured for 24 h in vitro, remains to be determined.

In addition to their effects on peripheral blood monocytes, GM-CSF and IL-4 are involved in the expansion of DCs directly from bone marrow precursors. DCs generated in this manner mature either through a CD14+ intermediate stage or directly into DCs as identified by the earlier acquisition of CD83 and CD1a in the absence of CD14 (16, 17). As with CD14+ cells, we observed that GM-CSF in combination with higher doses of IL-4 produced a significant increase in the number of circulating CD83+ cells. These cells lacked CD14, expressed CD1a and high levels of HLADR, and were active APCs in preliminary sorting experiments. Two distinct CD83+ populations emerged at higher IL-4 doses, one composed of smaller cells, and one composed of larger cells. A preliminary sorting experiment suggests that these CD83+ cells are able to stimulate T-cell proliferation, but further work is required to confirm their origins and functional role. Although increased up to 300-fold in response to GM-CSF and IL-4, the CD83+ population was still outnumbered 10-fold by CD14dim APCs.

From a clinical perspective, it appears likely that the administration of GM-CSF and IL-4 can be used to generate large numbers of APCs in vivo. Although this Phase I study enrolled only four patients in each course of GM-CSF alone (Leukine; 250 μg/m2/day). Ten of 22 patients treated with this regimen developed intermitternt decreases in serum PSA, but their values uniformly rose to levels higher than the pretreatment levels after each treatment cycle. High levels of GM-CSF were found to directly down-regulate PSA expression when added to the LNCaP cell line in culture, offering one potential mechanism for this phenomenon. In contrast to that report with GM-CSF alone, our prostate cancer patients treated with GM-CSF and IL-4 exhibited a progressive decline in PSA and tumor burden over time. Other studies have either found minimal antitumor effects of GM-CSF therapy alone (45, 50) or documented significantly fewer patient responses when GM-CSF was administered concurrently as an adjuvant with peptide vaccines or peptide-pulsed DCs (54, 55).

In conclusion, this Phase I study suggests that the combined administration of GM-CSF and IL-4 to patients with advanced cancer produces an IL-4-dependent increase in the number of circulating CD14+ and CD83+ APCs with many of the phenotypic and functional characteristics associated with DCs. Systemic cytokine administration aimed at generating APCs in situ, either alone or in combination with the administration of targeting antigens, may provide an additional approach to cancer immunotherapy.

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Granulocyte Macrophage Colony-stimulating Factor and Interleukin 4 Enhance the Number and Antigen-presenting Activity of Circulating CD14$^+$ and CD83$^+$ Cells in Cancer Patients

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