Selective in Vivo Mobilization with Granulocyte Macrophage Colony-stimulating Factor (GM-CSF)/Granulocyte-CSF as compared to G-CSF Alone of Dendritic Cell Progenitors from Peripheral Blood Progenitor Cells in Patients with Advanced Breast Cancer Undergoing Autologous Transplantation

David Avigan, Zekui Wu, Jianlin Gong, Robin Joyce, James Levine, Anthony Elias, Paul Richardson, Joya Milano, Leslie Kennedy, Kenneth Anderson, and Donald Kufe
Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215 [D. A., R. J., J. L., J. M., L. K.], and Cancer Pharmacology, Dana-Farber Cancer Institute, Boston, Massachusetts 02215 [Z. W., J. G., A. E., P. R., K. A., D. K.]

ABSTRACT
Dendritic cells (DCs) are potent antigen-presenting cells that are essential for the initiation of T cell-mediated immunity. DCs develop from myeloid progenitor populations under the influence of granulocyte macrophage colony-stimulating factor (GM-CSF) and pass through an intermediate stage of maturation that is characterized by CD14 expression. Interest has focused on generating human-derived DCs for antigen-specific tumor vaccines to be used as adjuvant immunotherapy in minimal disease settings, such as after autologous transplantation. In the present study, mobilized peripheral blood progenitor cells (PBPCs) were obtained from 18 patients with locally advanced or metastatic breast cancer preparing to undergo autologous stem cell transplantation. PBPCs mobilized in 10 patients with GM-CSF for 1 week, followed by the combination of GM-CSF and G-CSF, were compared with those obtained from patients receiving G-CSF alone with respect to the presence of DC progenitors and the capacity to generate functionally active mature DCs. PBPCs mobilized with GM-CSF/G-CSF were markedly enriched for CD14+ DC progenitor cells as compared with those mobilized with G-CSF alone. Consistent with an immature progenitor population, the CD14+ cells express Ki-67 antigen but not nonspecific esterase. CD14+ cells purified by fluorescence-activated cell sorting from PBPCs mobilized with either regimen and cultured for 1 week in GM-CSF and interleukin-4 generated nearly pure populations of cells with characteristic DC phenotype and function. The addition of GM-CSF to the mobilization regimen resulted in greater yields of functionally active DCs for potential use in posttransplant immunotherapy.

INTRODUCTION
DCs are bone marrow-derived leukocytes that excel in antigen presentation and the initiation of T cell-mediated immunity (1). DCs express costimulatory molecules [such as CD80 (B7-1), CD86 (B7-2), ICAM-1, and LFA-3] that are essential for activation of primary cellular immune responses (2, 3). Tumor cells that present antigens in the absence of costimulation induce loss of immune recognition (4–6). By contrast, anergy is reversed through immunization with DCs manipulated to express tumor-specific antigens. Recent studies in animal models have shown that DCs pulsed with tumor peptides or fused with malignant cells are effective in protecting animals from tumor challenge and mediate tumor regression in cancer-bearing hosts (7–9). Moreover, a clinical study using idiotypype-pulsed DCs for the treatment of B-cell lymphoma has demonstrated efficacy without significant morbidity (10). The development of DC vaccines for immunization in minimal disease settings, such as after autologous transplantation, represents another focus for investigation.

DCs develop from myeloid progenitor populations under the influence of cytokines, most notably GM-CSF (11–17). Previous studies have shown that DC precursors isolated from murine peripheral blood and bone marrow produce DC progeny when exposed to GM-CSF (18). Human CD34+ cells derived from bone marrow, cord blood, or PBPCs generate significant numbers of DCs when cultured in the presence of GM-CSF, TNFα, and/or IL-4 (19–21). Mature DCs can also be generated from partially differentiated progenitor populations. In this context, significant yields of peripheral blood-derived DCs have been obtained by culturing peripheral blood precursor populations in GM-CSF and IL-4 (22). As DCs mature from early...
precursor populations they pass through intermediate stages of development defined by distinctive functional and phenotypic characteristics. Immature cells exemplified by LCs of the skin are capable of antigen uptake, but demonstrate poor stimulatory capacity of T cells (23). LCs characteristically express CD1a, E-cadherin, and Lag antigen and contain cytoplasmic inclusion bodies known as Birbeck granules (15). On culture with GM-CSF, these cells develop a mature phenotype associated with diminished ability to process exogenous antigen. In addition, the up-regulated expression of costimulatory and adhesion molecules on mature DCs is associated with increased activity in allogeneic MLRs. DCs may also be derived from precursor cells related to the monocytic lineage. This category of intermediately differentiated DCs express the CD14 antigen (19, 24). These cells are distinguished from mature monocytes by faint expression of nonspecific esterase and by a proliferative capacity, as documented by expression of Ki-67 antigen.

A potential clinical source of DC precursors is PBPCs mobilized with cytokines from patients before undergoing high-dose chemotherapy with stem cell rescue. Treatment with myeloid CSFs results in a 20- to 40-fold increase in circulating progenitors produced a nearly pure population of mature DCs with a characteristic phenotype. These results demonstrate that DC progenitors can be selectively mobilized with GM-CSF/G-CSF in vivo.

PATIENTS AND METHODS

Patient Population. Samples were obtained from 18 women with locally advanced or metastatic breast cancer. Patients were treated with induction chemotherapy consisting of paclitaxel, doxorubicin, or cyclophosphamide and then enrolled in an Institutional Review Board approved protocol for high-dose chemotherapy with stem cell rescue. Treatment with myeloid CSFs results in a 20- to 40-fold increase in circulating colony-forming unit-GM per kilogram (25). In the present study, we show that PBPCs mobilized in vivo with GM-CSF/G-CSF are enriched for CD14+ DC progenitors as compared with those mobilized with G-CSF alone. When cultured in vitro with GM-CSF and IL-4, CD14+ progenitors produced a nearly pure population of mature DCs with a characteristic phenotype. The sorted populations were then cultured in GM-CSF and IL-4 for 1 week.

Preparation of Peripheral Blood Monocytes and T Cells. Peripheral blood mononuclear cells were derived from leukopaks obtained from healthy donors that were subjected to Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation (d = 1.077, 400 × g). Cells were suspended in RPMI 1640 containing 10% heat-inactivated human AB serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin for 1 h. Nonadherent cells were harvested, and T cells from that fraction were purified using a T-cell column or nylon wool (R&D Systems, Minneapolis, MN). The adherent population was incubated for 12–16 h, and the loosely adherent cells were removed by gentle washing of the culture flask. The strongly adherent cells represented a mature monocyte population and were maintained in media with human sera in the absence of cytokines.

Monoclonal Antibodies and FACS Analysis. Phenotypic characterization of the cell populations before, as well as after, in vitro culture was performed using monoclonal antibodies specifically reactive with the cell surface antigens CD14, CD80, CD86, CD83, CD54, and HLA-DR (Coulter). Cells were incubated with primary murine antihuman antibody for 30 min on ice. After two washes, the cells were then incubated with a secondary goat antimurine antibody conjugated with FITC or PE (Sigma Chemical Co.) for indirect fluorescence labeling. The cells were subjected to FACS sorting and segregated into CD14-positive and -negative populations. The sorted populations were then cultured in GM-CSF and IL-4 for 1 week.

Immunocytochemistry Staining

Analysis of DR Expression. Cells were spun onto glass slides using a cytocentrifuge. The cell preparations were fixed in acetone for 5 min and treated with 100 μg/ml avidin/1% normal horse serum in PBS (pH 7.2) for 30 min at 4°C to block endogenous avidin-binding activity. The cells were then incubated for 1 h at room temperature with 10 μg/ml murine antihuman DR antigen (Coulter). Hydrogen peroxide solution (0.3%) containing 20 μg/ml biotin was used to quench endogenous peroxidase activity for 30 min at 4°C. The sample was incubated with 1:100 biotinylated F(ab)’2 fragment of horse antimouse IgG (Vector Laboratories, Burlingame, CA) for 45 min at room temperature, washed with PBS, and incubated for 30 min at room temperature with ABC reagent solution (Vector Laboratories). Specific binding was detected with 3-amino-9-
Table 1  Impact of mobilization regimen on DC progenitor yields

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mobilization</th>
<th>Stage/site of disease</th>
<th>CD34+ cells × 10⁶/kg</th>
<th>Nonadherent CD14+*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G-CSF³</td>
<td>Stage IV/Inflammatory, pleura</td>
<td>2.0</td>
<td>1%</td>
</tr>
<tr>
<td>2</td>
<td>G-CSF³</td>
<td>Stage II/+ axial lymph nodes</td>
<td>2.4</td>
<td>5%</td>
</tr>
<tr>
<td>3</td>
<td>G-CSF³</td>
<td>Stage IV/lung</td>
<td>2.3</td>
<td>3%</td>
</tr>
<tr>
<td>4</td>
<td>G-CSF³</td>
<td>Stage IV/+ axial lymph nodes</td>
<td>7.34</td>
<td>10%</td>
</tr>
<tr>
<td>5</td>
<td>G-CSF³</td>
<td>Stage IV/pleura</td>
<td>1.8</td>
<td>3%</td>
</tr>
<tr>
<td>6</td>
<td>G-CSF³</td>
<td>Stage IV/bone</td>
<td>0.5</td>
<td>14%</td>
</tr>
<tr>
<td>7</td>
<td>G-CSF³</td>
<td>Stage IV/lung</td>
<td>1.6</td>
<td>9%</td>
</tr>
<tr>
<td>8</td>
<td>G-CSF³</td>
<td>Stage III/locally advanced</td>
<td>1.1</td>
<td>20%</td>
</tr>
<tr>
<td>9</td>
<td>GM-CSF/G-CSF³</td>
<td>Stage III/inflammatory</td>
<td>2.6</td>
<td>74%</td>
</tr>
<tr>
<td>10</td>
<td>GM-CSF/G-CSF³</td>
<td>Stage III/inflammatory</td>
<td>4.0</td>
<td>67%</td>
</tr>
<tr>
<td>11</td>
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<td>Stage III/locally advanced</td>
<td>12.3</td>
<td>66%</td>
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<tr>
<td>12</td>
<td>GM-CSF/G-CSF³</td>
<td>Stage IV/bone, bone marrow</td>
<td>1.9</td>
<td>61%</td>
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<tr>
<td>13</td>
<td>GM-CSF/G-CSF³</td>
<td>Stage IV/bone, liver</td>
<td>1.0</td>
<td>33%</td>
</tr>
<tr>
<td>14</td>
<td>GM-CSF/G-CSF³</td>
<td>Stage IV/lungs, pleura, liver</td>
<td>3.6</td>
<td>51%</td>
</tr>
<tr>
<td>15</td>
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<td>Stage IV/skin, locally recurrent</td>
<td>2.9</td>
<td>55%</td>
</tr>
<tr>
<td>16</td>
<td>GM-CSF/G-CSF³</td>
<td>Stage IV/skin, locally recurrent</td>
<td>3.4</td>
<td>35%</td>
</tr>
<tr>
<td>17</td>
<td>GM-CSF/G-CSF³</td>
<td>Stage IV/bone</td>
<td>3.5</td>
<td>17%</td>
</tr>
<tr>
<td>18</td>
<td>GM-CSF/G-CSF³</td>
<td>Stage III inflammatory</td>
<td>4.3</td>
<td>53%</td>
</tr>
</tbody>
</table>

* Mean CD14+ cells: G-CSF, 8.1%; GM-CSF/G-CSF, 51.2%; P < 0.0001.
³ Cyclophosphamide.
³ Taxane.
³ None.

RESULTS

Characterization of PBPCs Mobilized in Vivo with GM-CSF and G-CSF as Compared to G-CSF Alone. PBPCs obtained from 10 patients with advanced breast cancer undergoing stem cell mobilization with GM-CSF and G-CSF were compared with those from 8 patients whose stem cells were mobilized with G-CSF alone. Loosely adherent cells were separated from nonadherent B cells, T cells, and granulocytes and the firmly adherent mature monocytes, and were then subjected to FACS analysis. The mean total CD34+ cells/kg was not statistically different in the PBPC collections mobilized with GM-CSF/G-CSF as compared with those obtained with G-CSF (4.0 versus 2.3, P = 0.22; Table 1). By contrast, the population of CD14+ cells was significantly increased in PBPCs mobilized with GM-CSF/G-CSF (range, 17–74%; mean, 51%) as compared with those mobilized with G-CSF alone (range, 1–20%; mean, 8%; P < 0.0001). DR, CD80, CD54, and CD86 expression was also more pronounced in PBPCs mobilized with GM-CSF/G-CSF, whereas CD83 was absent in both populations (Fig. 1). CD14+ cells obtained after either mobilization failed to show evidence of nonspecific esterase activity. Immunocytochemical staining of the CD14+ cells for Ki-67 was uniformly strongly positive, whereas adherent monocytes showed no evidence of Ki-67 expression (Fig. 2).

Phenotypic Characterization of Cells Generated from CD14+, CD14−, and Unselected PBPCs. Loosely adherent PBPCs were segregated into CD14+ and CD14− fractions by FACS. Unselected and sorted PBPCs were cultured in GM-CSF and IL-4 for 1 week. CD14+ PBPCs obtained from either GM-CSF/G-CSF- or G-CSF-mobilized patients yielded a nearly uniform population of cells that displayed a mature DC phenotype, grew as loosely adherent aggregates, and exhibited a veiled morphology characteristic of DCs. The cells prominently expressed DR, CD80, and CD86, whereas CD14 was predominately absent. CD83, a marker characteristic of mature DCs, was expressed by >90% of cells (Fig. 3). There was no expansion of the CD14+ PBPCs after 1 week of culture. The yield of DCs as a percentage of the initial PBPC harvest was significantly higher in patients mobilized with GM-CSF/G-CSF, as compared with G-CSF alone (Table 2).

Compared with PBPCs obtained with GM-CSF/G-CSF, those mobilized with G-CSF alone contained a significantly higher percentage of loosely adherent cells that did not express CD14. CD14− PBPCs mobilized with both regimens and cultured for 1 week in GM-CSF and IL-4 produced a population in which CD83 expression was absent and CD86 was found in only a minority of cells. Unselected PBPCs mobilized with GM-CSF/G-CSF or G-CSF alone generated a mixed population in which CD83 and CD86 expression was found in a moderate...
proportion of the cells, but significantly less than that seen with the progeny of CD14\(^+\) selected PBPCs. Immunocytochemistry analysis confirmed the presence of a heterogeneous population of cells, including those with a characteristic DC morphology that prominently stained for class II molecules (Fig. 4). After two washes, the cells were incubated with goat antimouse immunoglobulin conjugated to FITC and then fixed in paraformaldehyde. Histograms were generated by cytometric analysis.

**Fig. 1**  Phenotypic profile of PBPCs generated from patients mobilized with GM-CSF and G-CSF as compared with G-CSF alone. PBPCs were mobilized in vivo with chemotherapy, followed by G-CSF alone versus GM-CSF for 1 week, followed by the combination of GM-CSF and G-CSF. After collection, PBPCs were plated for 1 h and the nonadherent fraction was removed. The adherent population was incubated overnight in human sera. Loosely adherent cells were subsequently harvested, and an aliquot was taken for FACS analysis. The cells were then incubated with murine antibodies against the indicated antigens. After two washes, the cells were incubated with goat antimouse immunoglobulin conjugated to FITC and then fixed in paraformaldehyde. Histograms were generated by cytometric analysis.

**Fig. 2**  Phenotypic characterization of CD14\(^+\) sorted PBPCs. A, nonspecific esterase stain. CD14\(^+\) cells were separated from nonadherent PBPCs using FACS and spun onto glass slides. Cytospins were fixed in citrate-acetone, air dried, incubated with ethylene glycol-naphthyl acetate solution, and counterstained in Mayer’s hematoxylin. B, positive control. Peripheral blood containing monocytes and mature T cells were subjected to nonspecific esterase staining as a positive control. C, Ki-67 antigen. CD14\(^+\) PBPCs were spun onto glass slides, treated with heated citrate buffer, and incubated with murine antihuman Ki-67 antibody. Purified monocytes used as a negative control failed to demonstrate reactivity with anti-Ki-67.

**Functional Analysis of CD14\(^+\), CD14\(^-\) and Unselected PBPCs Cultured in Vitro with GM-CSF and IL-4.** Before in vitro culture, PBPCs mobilized with GM-CSF/G-CSF or G-CSF alone did not show significant activity in allogeneic MLRs as compared with monocyte controls (data not shown). After 1 week of culture in GM-CSF and IL-4, CD14\(^+\) PBPCs mobilized with either regimen generated a nearly uniform population of DCs that were potent stimulators of allogeneic T-cell proliferation (Fig. 5). By contrast, CD14\(^-\) PBPCs cultured for 1 week with cytokines exhibited significantly less allostimulatory activ-
ity. These findings suggest that loosely adherent CD14+ cells are preferentially mobilized by GM-CSF and that this population represents primary DC precursors in the PBPC product.

**DISCUSSION**

Immunization with DCs that present tumor antigens has resulted in disease regression in cancer-bearing animals (7–9). The clinical use of DCs to stimulate antitumor immune responses has necessitated defining the most effective source of functionally active DCs. DCs mature from myeloid progenitor populations under the influence of GM-CSF. GM-CSF promotes DC survival and supports the differentiation of immature precursors into cells with a mature phenotype (11–17). Approaches used to generate DCs in sufficient quantity for clinical use include isolation of CD34+ precursors from bone marrow, cord blood, or PBPC and subsequent culture with appropriate cytokines, particularly GM-CSF (19–21). As DCs mature, they progress through an intermediate stage of development that is often associated with CD14 expression. CD34+ cells derived from cord blood have been shown to mature into CD1a+ LCs or CD14- cells of monocytic lineage before terminally differentiating into mature DCs (19). Other studies have shown that CD14+ intermediates generated from bone marrow-derived CD34+ cells differentiate into either mature monocytes or DCs depending on the cytokine exposure (24). Once terminally differentiated, the cell phenotype is no longer reversible by altering...
cytokine exposure. In contrast, another study has shown that terminally differentiated CD14+ monocyte populations can be induced to adopt a DC phenotype by culturing for 1 week in GM-CSF, IL-4, and TNFα (26).

Cytokine-mobilized PBPCs are rich in myeloid progenitor populations (25). Several studies have demonstrated that CD34+ cells derived from G-CSF mobilized PBPCs and cultured with GM-CSF and TNFα generate mixed populations of cells that include large numbers of functionally active LCs and mature DCs (21). G-CSF-mobilized PBPCs depleted of CD34+ cells also produce significant yields of DCs when cultured in GM-CSF and IL-4 (27). However, little is known about whether PBPCs mobilized with GM-CSF are enriched for the presence of partially differentiated DC precursors. The present study demonstrates that the addition of GM-CSF to the mobilization regimen markedly increases the number of nonadherent CD14+ DC progenitors in the stem cell product, as compared with that obtained with G-CSF alone. Unlike mature monocytes, these cells do not express nonspecific esterase. Moreover, nuclear staining for Ki-67 demonstrates that the CD14+ cells represent a precursor population with proliferative capacity. These cells exhibit moderate class II expression, whereas costimulatory molecules CD80 and CD86 were largely absent and CD83 was undetectable.

When cultured for 1 week in vitro with GM-CSF and IL-4, CD14+ PBPCs generated a nearly pure population of cells with a characteristic DC phenotype, as demonstrated by FACS analysis. These cells expressed the relatively specific DC marker CD83 and potentially stimulated allogeneic T-cell proliferation. CD14+ PBPCs represented a greater percentage of cells mobilized with G-CSF alone. CD14+ PBPCs cultured for 1 week in GM-CSF and IL-4 generated cells that did not express CD83 and were less active in MLR studies. The final yield of DCs as a percentage of the starting PBPC population was significantly greater for patients mobilized with GM-CSF/G-CSF. Therefore, although CD14+ DC precursors were present in PBPCs mobilized with either GM-CSF/G-CSF or G-CSF alone, the use of GM-CSF was associated with significantly higher yields of these DC progenitors and, as such, resulted in an increase in mature DCs generated in vitro.

Previous studies have demonstrated that DCs produced from CD34+ PBPCs from cancer patients are potent stimulators of allogeneic MLR (28). In another study, DCs derived from PBPCs mobilized from patients with multiple myeloma were shown to be functionally active (27). In the present study, DCs generated from heavily pretreated patients with advanced breast cancer undergoing stem cell mobilization were also found to be functionally active. Thus, the postautologous transplant period may be an ideal setting for the effective use of antitumor DC-based vaccines. Patients treated with high-dose chemotherapy are often able to achieve a complete remission, but sub-
quenty relapse. These findings have suggested that patients achieving a minimal disease state retain tumor clones that are chemotherapeutic resistant. These residual tumor cells may, however, be susceptible to active specific immunotherapy with DC-based vaccines. Our study is the first to demonstrate that chemotheraphy followed by the combination of GM-CSF and G-CSF may selectively mobilize DC progenitors in vivo in the stem cell product and that these cells can generate relatively pure populations of functionally active mature DCs. This approach to stem cell mobilization, therefore, results in an excellent source of DC progenitors that could be used for immunotherapeutic strategies in the posttransplant period.

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