Defective Antitumor Function of Monocyte-Derived Macrophages from Epithelial Ovarian Cancer Patients

Ilyssa O. Gordon¹,² and Ralph S. Freedman²

Abstract

Purpose: Monocytes/macrophages (MO/MA) are an important but heterogeneous population of immune inflammatory cells that have diverse effector functions. We examined and compared these differences in peripheral blood and ascites of epithelial ovarian cancer patients with peripheral blood of normal donors.

Experimental Design: Comparisons were made of cell surface subsets, cytokine production, and FcR-dependent cytotoxicity of CD14⁺ MO/MA and the CD14brightCD16⁺ HLA-DR⁺ MO/MA subset in normal donor peripheral blood and peripheral blood and ascites from epithelial ovarian cancer patients. Studies were done on monocyte-derived macrophages cultured with macrophage colony-stimulating factor and activated with lipopolysaccharide or a combination of lipopolysaccharide plus recombinant IFN-γ.

Results: We determined that MO/MA or its subset from epithelial ovarian cancer patients had altered morphology and significantly less antibody-dependent cell-mediated cytotoxicity and phagocytic activity than did MO/MA from normal donors. Our findings also showed that monocyte-derived macrophages from both epithelial ovarian cancer patients and normal donors produce macrophage colony-stimulating factor – stimulated cytokines, including interleukin-8, tumor necrosis factor-α, and interleukin-6.

Conclusions: These findings highlight for the first time the defective antibody-dependent cell-mediated cytotoxicity and phagocyte functions of epithelial ovarian cancer – associated MO/MA, which could have implications for immunobiotherapeutic strategies.

Over the past decade, clinical trials have tested immunologic agents for the treatment of epithelial ovarian cancer. Although some clinical trials of these agents, either alone or in combination with chemotherapy, have yielded encouraging results (1), barriers remain to the development of such treatments for epithelial ovarian cancer. A more complete understanding of the interactions between the tumor microenvironment and effector cells of the immune system therefore could pave the way for improvements in immunotherapeutic regimens for this cancer.

Activated monocytes and macrophages (MO/MA), mononuclear leukocytes important to the innate immune system, can be directly cytotoxic to tumor cells, either through antibody-mediated mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis (2) or through direct cellular mechanisms. In contrast, a subset of MO/MA in the ascites of epithelial ovarian cancer patients has been shown to suppress T-cell proliferation through the production of interleukin (IL)-10 and transforming growth factor-β (3), suggesting that this subset might contribute to regulatory activities. MO/MA that seem to be immobilized in hypoxic areas at the tumor site (4) may also enhance tumor progression by facilitating neoangiogenesis and invasion (5, 6). In epithelial ovarian cancer, tumor necrosis factor-α (TNF-α) can also be produced by MO/MA (7) and may contribute to the proliferation of malignant ovarian epithelial cells (8).

The effector function of MO/MA in epithelial ovarian cancer remains poorly understood. Because MO/MA are a heterogeneous population, it seems useful to examine their functional properties at both the whole population and subset levels.

Here, we report that MO/MA isolated from epithelial ovarian cancer patient peripheral blood and ascites have different subsets from normal donors and that MO/MA differentiated in vitro in macrophage colony-stimulating factor (M-CSF) are deficient in certain antitumor activities. In culture, these MO/MA have an altered morphology compared with the same population obtained from normal donors. In addition, compared with similar cells from normal donors, Fcγ receptor–mediated cytotoxicity and phagocytosis are deficient in activated monocyte-derived macrophages (MDM) derived from both the total CD14⁺ population and an isolated subpopulation of CD14brightCD16⁻ HLA-DR⁺ MO/MA from...
epithelial ovarian cancer patients. In contrast, either activated whole MDM or the CD14^{bright}CD16^{low} HLA-DR^{+} subset produced elevated levels of IL-8, IL-6, and TNF-α, similar to those seen in normal donors.

**Patients and Methods**

**Monocyte and macrophage isolation and differentiation into MDM in culture.** Buffy coats from anonymous normal donors were obtained from the Gulf Coast Regional Blood Center in Houston, Texas. Peripheral blood (30–60 mL) and heparinized ascites were obtained from epithelial ovarian cancer patients under a protocol approved by the Institutional Review Board at The University of Texas M.D. Anderson Cancer Center. Mononuclear leukocytes were isolated by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich Co., St. Louis, MO) and RBC were lysed with 10 times volume 1× ammonium chloride for 5 to 7 minutes at room temperature. Platelets were removed by centrifugation at 540 rpm for 10 minutes. MO/MA from normal donor peripheral blood were further purified by centrifugation over a 47% RediGrad gradient (Amersham Biosciences AB, Uppsala, Sweden) according to the method of Gmelig-Meyling and Waldmann (9). Tumor cells were removed if needed from ascites using Epithelial Enrich Ber-EP4–coated microsize beads (Dynal Biotech, Oslo, Norway) according to the instructions of the manufacturer. Cell number and viability were determined by trypan blue exclusion. Gradient-purified mononuclear leukocytes or MO/MA were labeled with anti-CD14 phycoerythrin (BD Biosciences, San Jose, CA) or anti-CD16 phycoerythrin, anti-CD16 FITC (BD Biosciences, and anti-HLA-DR, phycoerythrin-Cy5 (BD Cy-Chrome, BD PharMingen, San Diego, CA) at 10× 10^6 cells per 200 μL fluorescence-activated cell sorting (FACS) buffer [1× PBS, 2% heat inactivated fetal bovine serum (FBS), 0.02% sodium azide] for 30 to 60 minutes on ice. FACS was done on a Becton Dickinson Vantage Turbo SE Flow Cytometer (BD Immunocytometry Systems, San Jose, CA). In some cases, gradient-purified mononuclear leukocytes or MO/MA were frozen for future use at 20× 10^6 cells/mL in 95% heat-inactivated FBS with 5% DMSO. Cryopreserved cells were rapidly thawed in a 37°C water bath, immediately placed on ice, washed once in cold 2% heat-inactivated FBS in 1× PBS, twice in cold 1× FACS, and counted, and then sorted by FACS.

MDM were induced by culturing-sorted MO/MA at 1× 10^5 cells per well (except where noted) for 7 days in serum-free medium [RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with well (except where noted) for 7 days in serum-free medium [RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% heat-inactivated FBS in 1× ammonium chloride for 5 minutes at room temperature. RBCs were lysed with 1× ammonium chloride for 5 minutes at room temperature. Labeled cells were washed with FACS buffer, fixed with 2% paraformaldehyde, and analyzed using a FACSscan flow cytometer (BD Biosciences). Events totaling 100,000 were collected in list mode and analyzed with CellQuest software (BD Biosciences). MO/MA were identified by size and granularity characteristics and by positivity for both CD14 and CD45 surface antigens. Gated MO/MA subpopulations were further identified by the intensity of CD14 expression as well as the presence of CD16 and HLA-DR. Proportions of MO/MA subpopulations were determined from total CD14^+ MO/MA (i.e., CD14^{bright} plus CD14^{dim}).

**Cytokine analysis.** Sorted MO/MA were cultured at 2× 10^5 cells per well in 96-well flat-bottomed plates for 7 days in M-CSF as described above. Supernatants were collected 0 to 72 hours after addition of activating agent. Cytokine analysis for IL-10, IL-12p70, IL-6, IL-8, and TNF-α in 50 μL culture supernatant was determined by the Luminex 100 Analyzer (Luminex Corp., Austin, TX). The sensitivity of the assay is in the range of 10 to 15 pg/mL per cytokine.

**ADCC.** ADCC was determined in a thymidine release assay modified from the method of Munn and Armstrong (10). Target cells were the allogeneic ovarian tumor cell line SKOV3 (American Type Culture Collection, Manassas, VA), which overexpresses Her2/neu. The humanized immunoglobulin (Ig) G1 monoclonal antibody trastuzumab (Herceptin, Genentech, Inc., South San Francisco, CA), which recognizes Her2/neu (11), was the mediating antibody. One million target cells were labeled with 50 μg/mL [methyl-3H]thymidine in RPMI plus 10% heat-inactivated FBS in a 37°C shaking water bath for 18 to 22 hours. Half the cells were then labeled with 5 μg/mL trastuzumab for 30 minutes in a 37°C shaking water bath. Cells were washed, counted on a hemocytometer, and added in duplicate to wells containing activated MDM in U-bottomed plates at a previously determined optimum effector-to-target cell ratio of 20:1. This test was previously standardized in our laboratory and provides results comparable with the 51Cr release assay (12). Plates were centrifuged at 980 rpm for 1 minute and then placed in 5% CO2 at 37°C for 72 hours. Plates were then centrifuged at 1,000 rpm for 1 minute, and 100 μL of supernatant was transferred from each well into scintillation vials. Scintillation fluid (Safety Solve, Research Products International Mount Prospect, IL) was added to each vial and β-emission was analyzed using a WinSpectral β-emission counter and software (Wallac Oy, Turku, Finland). Maximum release was determined by lysis of target cells alone using 0.5% SDS. Spontaneous release, determined in wells with labeled target cells alone, was always <5% (mean ± SD, 13 ± 6%). Percentage specific lysis was determined from the following equation: (experimental release – spontaneous release) / (maximum release – spontaneous release) × 100%.

**Opsonized phagocytosis.** For phagocytosis measurements, 0.5 × 10^6 sheep RBCs were labeled in 0.5 mL of 1× PBS with 92 μg/mL rabbit anti-sheep IgG (Nordic Immunology Laboratories, Tilburg, the
Netherlands) for 1 hour at 37°C. Sheep RBCs were then washed and added to wells of activated MDM in flat-bottomed plates at an effector-to-target cell ratio of 1:100 for 30 to 40 minutes at 37°C. Uningested sheep RBCs were lysed with 200 µL 1 × ammonium chloride per well for 3 minutes at room temperature. Cells were washed gently in distilled H2O and fixed in 0.3% glutaraldehyde. In some experiments, cells were stained with Wright’s stain before being counted. Phagocytosis was determined by counting 200 MDM with two or more ingested sheep RBCs, expressed as a percentage.

Cytotoxicity studies. Sorted MO/MA were cultured and activated in 96-well flat-bottomed RepCell plates (1 × 10^5 cells per well; CellSeed, Inc., Tokyo, Japan) as described above. Plates were then cooled to 4°C for 15 minutes to allow cell detachment. MDM from like conditions were pooled, and cytocentrifuge preparations with 0.8 × 10^5 to 1.0 × 10^5 cells per slide were made. Slides were fixed in acetone for 5 minutes at room temperature and stored at −20°C. In some experiments, sorted MO/MA were allowed to adhere to RepCell plate wells for 1 hour at 37°C before cell detachment and cytocentrifuge preparation.

Slides were thawed at room temperature for 30 to 45 minutes and blocked with 10% normal goat serum for 30 minutes. The cells were labeled for 1 hour with one of the following primary antibodies under humidified culture conditions: anti-CD16, anti-CD64, anti-CD32, IgG1 isotype (all 5 µg/mL; BD Biosciences), or anti-CD32B (2B6, 1.5 µg/mL; MacroGenics Inc., Rockville, MD). Slides were washed thrice in 1 × PBS followed by the addition for 30 minutes of peroxidase-conjugated secondary antibody diluted 1:250 in normal goat serum. Slides were washed and 3-amino-9-ethylcarbazole substrate (Vector Laboratories, Burlingame, CA) was added according to the instructions of the manufacturer. The reaction was allowed to proceed for 15 to 30 minutes to enable the full color to develop. The reaction was stopped by exposure of the slides to 1× PBS for 5 minutes, followed by a wash in distilled H2O. Slides were stained with hematoxylin for 4 to 5 minutes, rinsed in tap water, and dipped 10 times in 2% glacial acetic acid. Then, the slides were dipped 10 times in tap water, submerged in bluing solution (1.5% NH4OH in 75% ethanol) for 1 minute, and dipped 10 more times in fresh tap water. Slides were air-dried for 5 minutes, covered with three drops of crystal mount (Biomeda Corp., Foster City, CA), and dried overnight.

Macrophage-mediated cytotoxicity. A thymidine release assay modified from the method of Melichar et al. (13) was done using the allogeneic ovarian tumor cell line 2774. One million target cells were labeled with 50 µCi thymidine in RPMI plus 5% heat-inactivated FBS in a 37°C shaking water bath for 18 to 22 hours, washed, counted on a hemocytometer, and added in duplicate to wells of activated MDM in U-bottomed plates at an effector-to-target cell ratio of 20:1. Plates were centrifuged at 980 rpm for 1 minute and placed at 37°C in 5% CO2 for 48 hours. Plates were then centrifuged at 1,000 rpm for 1 minute, and 100 µL of supernatant were transferred from each well into scintillation vials. β-Emission was analyzed using a WinSpectral β-emission counter and software. Maximum release was determined by lysis of target cells alone using 0.5% SDS. Spontaneous release was determined in wells with labeled target cells alone (mean ± SD, 26 ± 6%). Percentage specific lysis was calculated as described for ADCC.

Cytostasis. Because peripheral MO/MA do not undergo sizable increases in MO/MA numbers following exposure to growth factors, such as M-CSF (14), thymidine uptake closely parallels the proliferation response of the tumor cells. The ability of activated MDM to inhibit the growth of tumor cells (cytostasis) was measured in a thymidine incorporation assay modified from the method of Melichar et al. (13). Sorted MO/MA were cultured and activated at 1 × 10^5 cells per well in 96-well U-bottomed plates as described above. Target cells (ovarian tumor cell line 2774) were added in duplicate to activated MDM at an effector-to-target cell ratio of 20:1. Control wells containing either target cells alone or activated MDM alone were also prepared in duplicate. After 48 hours of coculture, 10 µCi/mL [methyl-3H]thymidine was added to each well for an additional 24 hours. Cocultures were washed with warm 10% FBS in 1× PBS, and the cells were lysed by exposure for 15 minutes at room temperature to 0.5% SDS. Supernatants were transferred to scintillation vials, scintillation fluid was added, and radioisotope incorporation was analyzed by a WinSpectral β-emission counter. Percentage cytostasis was determined by the following equation: (2774 alone − [(2774 + MDM) − MDM alone]) / 2774 alone × 100%

**Results**

**Demographics of patients with epithelial ovarian cancer.** Thirty-four peripheral blood and 19 ascites samples from epithelial ovarian cancer patients were collected and used. Of the peripheral blood donors, 29 were Caucasian, 3 were Hispanic, and 2 did not identify ethnicity. Of the ascites donors, 15 were Caucasian, 2 were Hispanic, 1 was Asian, and 1 did not identify ethnicity. Fifteen peripheral blood donors (44%) had received chemotherapy at least 3 months (median, 13 months; range, 3-96 months) before participating in this study, two for unrelated cancers. Twenty-one peripheral blood donors (62%) were newly diagnosed with epithelial ovarian cancer, and 5 (15%) provided ascites and peripheral blood. All ascites donors had newly diagnosed epithelial ovarian cancer or peritoneal cancer and were chemotherapy naive. Peripheral blood donors included the following histopathologies: papillary-serous (n = 20), mixed (n = 7), clear cell (n = 1), peritoneal (n = 3), and other (n = 3). Histopathology of ascites donors included papillary-serous (n = 11), mixed (n = 2), endometrioid (n = 1), clear cell (n = 2), peritoneal (n = 2), and other (n = 1).

**Proportions of monocyte and macrophage subpopulations are different in epithelial ovarian cancer patients and healthy subjects.** MO/MA subpopulation proportions were compared in whole peripheral blood of normal donor and epithelial ovarian cancer patients, and in epithelial ovarian cancer ascites and tumor by three-color flow cytometry (Table 1). Gated MO/MA subpopulations were identified by the intensity of expression of CD14 (the LPS receptor), CD16 (FcγRIII, the low-affinity receptor for IgG), and HLA-DR (the MHC class II receptor). Proportions of CD14brightCD16−HLA-DR+ MO/MA in epithelial ovarian cancer peripheral blood (57%), epithelial ovarian cancer ascites (33%), and epithelial ovarian cancer tumor-associated macrophages (62%) were less than in normal donor peripheral blood (88%; P < 0.01 for each). In contrast, proportions of CD14brightCD16+HLA-DR− MO/MA in epithelial ovarian cancer peripheral blood (36%) and epithelial ovarian cancer ascites (31%) were higher than in normal donor peripheral blood (3%; P < 0.01 for each) and epithelial ovarian cancer tumor-associated macrophages (6%; P = 0.02 and P = 0.03, respectively). Proportions of CD14brightCD16+HLA-DR+ were similar in epithelial ovarian cancer peripheral blood, ascites, and tumor-associated macrophages and proportions of CD14brightCD16−HLA-DR− MO/MA were similar in epithelial ovarian cancer peripheral blood and ascites. However, proportions of CD14brightCD16+HLA-DR− MO/MA were higher in epithelial ovarian cancer ascites (19%) than epithelial ovarian cancer peripheral blood (2%; P = 0.04) and normal
donor peripheral blood (4%; \( P = 0.02 \)), and were also higher in epithelial ovarian cancer tumor-associated macrophages (18%) than in epithelial ovarian cancer peripheral blood (\( P = 0.02 \)) and normal donor peripheral blood (\( P < 0.01 \)). In addition, proportions of MO/MA expressing CD14\textsuperscript{bright}CD16\textsuperscript{HLA-DR+} were higher in epithelial ovarian cancer ascites (11%) than epithelial ovarian cancer peripheral blood or normal donor peripheral blood (<1% and \( P < 0.01 \) for each). These findings show an increased frequency of MO/MA in epithelial ovarian cancer peripheral blood, which have the appearance of a less-differentiated effector phenotype (i.e., expressing neither CD16 nor HLA-DR) compared with normal donors, whereas MO/MA from the tumor environment (ascitic macrophages and tumor-associated macrophages) show a more differentiated effector phenotype (expressing both CD16 and HLA-DR surface antigens at high frequency) compared with cells from the systemic circulation of epithelial ovarian cancer patients and normal donors. When the seven epithelial ovarian cancer peripheral blood samples were separated into a group that had previous chemotherapy (\( n = 3 \)) and one that had not (\( n = 4 \)), the proportions of MO/MA subpopulations were not found to be statistically different.

Morphology of MDM from epithelial ovarian cancer patients is variable. MO/MA were isolated from the peripheral blood of normal donors and epithelial ovarian cancer patients as well as from the ascites of epithelial ovarian cancer patients and their morphology compared by light microscopy both before and after in vitro culture and activation. MO/MA, which were freshly isolated by immunofluorescence sorting of CD14\textsuperscript{+} cells, were morphologically similar and consisted of small, round, nongranular adherent cells. MO/MA from normal donors that were cultured for 7 days in M-CSF then stimulated with LPS or a combination of LPS plus IFN-\( \gamma \), consisted of larger, oval or spindle-shaped, adherent granular cells. However, MO/MA from epithelial ovarian cancer peripheral blood that were cultured and stimulated under identical conditions predominately remained small and round, with or without granules, with only a few large cells developing. MDM retained their adherence properties throughout the culture period. The frequency of large, oval, or spindle cells varied from essentially none to the frequency seen in normal donors. MO/MA from epithelial ovarian cancer ascites that were cultured and stimulated under identical conditions often resembled those of normal donor peripheral blood, but, like those from the epithelial ovarian cancer peripheral blood, there was a high frequency of small, round cells, with or without granules, depending on the patient (Fig. 1). The viability of fresh and cultured cells was >98% as determined by trypan blue exclusion.

ADCC is deficient in MDM from epithelial ovarian cancer patients. ADCC capacity of activated MDM derived from CD14\textsuperscript{bright}CD16\textsuperscript{HLA-DR+} MO/MA was comparable with that of the heterogeneous group of CD14\textsuperscript{+} cells for both epithelial ovarian cancer patients and normal donors (Fig. 2). Overall, however, the percentage of tumor cells lysed by MDM from epithelial ovarian cancer patients compared with normal donors was lower. The mean \( \pm \) average deviation percentage of tumor cells lysed by LPS-activated MDM derived from CD14\textsuperscript{+} MO/MA from normal donor peripheral blood was 53 \( \pm \) 19% (\( n = 4 \)), compared with 16 \( \pm \) 4% from epithelial ovarian cancer peripheral blood and 10 \( \pm \) 12% from epithelial ovarian cancer ascites (\( P = 0.0045, n = 3 \) and \( P < 0.0001, n = 5 \), respectively; Fig. 2B). Also, the percentage of tumor cells lysed by LPS-activated

<table>
<thead>
<tr>
<th>MO/MA subpopulation</th>
<th>Percentage of CD14\textsuperscript{+} MO/MA ( * )</th>
<th>( P ) value</th>
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<tbody>
<tr>
<td></td>
<td>ND PB</td>
<td>EOC PB</td>
</tr>
<tr>
<td>CD14\textsuperscript{bright}CD16\textsuperscript{HLA-DR+}</td>
<td>88%</td>
<td>57%</td>
</tr>
<tr>
<td>CD14\textsuperscript{bright}CD16\textsuperscript{HLA-DR-}</td>
<td>3%</td>
<td>36%</td>
</tr>
<tr>
<td>CD14\textsuperscript{bright}CD16\textsuperscript{HLA-DR+}</td>
<td>4%</td>
<td>2%</td>
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<tr>
<td>CD14\textsuperscript{bright}CD16\textsuperscript{HLA-DR+}</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
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Abbreviations: ND, normal donor; PB, peripheral blood; EOC, epithelial ovarian cancer patient; asc, ascites; TAM, tumor-associated macrophage; NS, not significant.

* Percentages represent proportions of the total number of CD14\textsuperscript{+} MO/MA.

**Fig. 1.** Morphology of epithelial ovarian cancer ascites MO/MA. Before culture, CD14\textsuperscript{+} (A) and CD14\textsuperscript{bright}CD16\textsuperscript{HLA-DR+} (B) ascitic MO/MA are morphologically similar and consisted of small, round, nongranular adherent cells. Following culture for 8 days in M-CSF, CD14\textsuperscript{+} (C), and CD14\textsuperscript{bright}CD16\textsuperscript{HLA-DR+} (D), ascitic MO/MA varied from oval, spindle-shaped cells (arrowheads) to small, round, nongranular cells (arrow).
MDM derived from CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{+} MO/MA from normal donor peripheral blood was 41 ± 6% (n = 3), compared with 15 ± 8% from epithelial ovarian cancer peripheral blood and 5 ± 6% from epithelial ovarian cancer ascites (P = 0.039, n = 3 and P < 0.0001, n = 4, respectively; Fig. 2B). The percentage of tumor cells lysed by LPS-activated MDM from epithelial ovarian cancer ascites and epithelial ovarian cancer peripheral blood were similarly low and were not significantly different (Fig. 2B). The percentage lysis by MDM from epithelial ovarian cancer peripheral blood activated with M-CSF alone (Fig. 2A) or with LPS plus IFN-γ (Fig. 2C) was also significantly lower than for similar populations of MDM from normal donor peripheral blood and was similar to values from epithelial ovarian cancer ascites MDM activated under the same conditions.

**MDM from epithelial ovarian cancer patients are deficient in phagocytosis.** The ability of activated MDM to function as phagocytes was determined using IgG-opsonized sheep RBCs. As seen in Table 2, MDM derived from CD14\textsuperscript{+} and CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{+} cells from epithelial ovarian cancer peripheral blood or ascites showed a significantly lower percentage (P ≤ 0.0015) of phagocytosis than did MDM from normal donor peripheral blood, regardless of the activation condition or cell type (n = 4 or 5 for each mean value). The percentage of epithelial ovarian cancer ascites MDM showing phagocytosis, which was always <30%, was not significantly different from that for epithelial ovarian cancer peripheral blood MDM (n = 4 or 5 for each mean value; Table 2).

**Expression of Fcγ receptors is altered in MDM from epithelial ovarian cancer patients.** Because Fcγ receptors mediate both ADCC and phagocytosis, the presence of Fcγ receptors was determined on cultured MO/MA by immunocytochemistry (Table 3). The low-affinity FcγRIII (CD16) was rarely present on cultured CD14\textsuperscript{+} MDM from normal donor peripheral blood activated with LPS and was undetectable on the CD14\textsuperscript{+} MDM from epithelial ovarian cancer peripheral blood and ascites. CD16 was also absent on LPS-activated MDM derived from CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{+} MO/MA from normal donor peripheral blood, epithelial ovarian cancer peripheral blood, and epithelial ovarian cancer ascites.

CD14\textsuperscript{+} and CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{+} adherent MO/MA from normal donor peripheral blood and epithelial ovarian cancer ascites were rarely positive for CD16, in contrast to CD14\textsuperscript{+} and CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{+} adherent MO/MA from epithelial ovarian cancer peripheral blood that were weakly positive in 50% and 100%, respectively. These results suggest that MO/MA from epithelial ovarian cancer peripheral blood, including those initially isolated as CD16\textsuperscript{−}, developed expression of CD16 after 1 hour of adherence, but lost expression of CD16 by the end of the 8-day culture period.

The high-affinity FcγRI (CD64) was diffusely positive on all 1-hour adherent MO/MA, as well as on CD14\textsuperscript{+} and CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{+} LPS-activated MDM derived from normal donor peripheral blood, but not in either subpopulation of LPS-activated MDM from epithelial ovarian cancer peripheral blood (Table 3). Epithelial ovarian cancer ascitic CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{−} MDM also lacked expression of CD64; however, 50% of epithelial ovarian cancer ascitic CD14\textsuperscript{−} MDM did express CD64. Thus, CD64 expression in epithelial ovarian cancer MDM declines after 8 days in culture.

**Fig. 2.** ADCC of activated MDM. ADCC is deficient in epithelial ovarian cancer peripheral blood (EOC PB) and ascites MDM compared with normal donor peripheral blood (ND PB) MDM derived from CD14\textsuperscript{+} (black columns) and CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{+} (white columns) MO/MA. Similar results were obtained when cells were activated with M-CSF alone (A), LPS (B), or LPS + IFN-γ (C). Columns, mean of three to five different MDM samples per group; bars, average deviation. *, P ≤ 0.05.

In normal donor peripheral blood, total CD32 and CD32B were diffusely positive on LPS-activated MDM derived from CD14\textsuperscript{+} or CD14\textsuperscript{bright}CD16\textsuperscript{−} HLA-DR\textsuperscript{+} MO/MA (Table 3). The same cell populations derived from epithelial ovarian cancer peripheral blood and epithelial ovarian cancer ascites had a low frequency of CD32 positivity. 2B6 was positive in 10% to 50% of epithelial ovarian cancer ascitic MDM and in <10% of epithelial ovarian cancer peripheral blood MDM. One hour adherent MO/MA from normal donor peripheral blood were diffusely positive for CD32 and only rarely positive for 2B6, whereas both CD32 and 2B6 were diffusely positive on adherent MO/MA from epithelial ovarian cancer peripheral blood and ascites (Table 3). These results again suggest differences in FcγR expression on both adherent MO/MA and cultured MDM.
Macrophage-mediated cytotoxicity is low in MDM from epithelial ovarian cancer patients. Both CD14+ and CD14brightCD16−HLA-DR− derived MDM cultured and activated with M-CSF alone had higher mean percentages of macrophage-mediated cytotoxicity than those activated with LPS or LPS plus IFN-γ for normal donor peripheral blood, epithelial ovarian cancer peripheral blood, and epithelial ovarian cancer ascites (Fig. 3A). Interestingly, CD14+-derived MDM from normal donor peripheral blood had significantly greater percentage specific lysis regardless of activation condition (P = 0.015). This was the only functional experiment in which the heterogeneous group of MDM behaved differently from the isolated subpopulation of CD14brightCD16−HLA-DR−-derived cells.

MDM from epithelial ovarian cancer patients can inhibit tumor cell growth. The ability of activated MDM to inhibit the growth of tumor cells was measured in a thymidine incorporation assay (Fig. 3B). Mean percentage cytostasis by activated CD14+-derived MDM was 38%; this was 39% for MDM derived from epithelial ovarian cancer peripheral blood and 31% for MDM derived from epithelial ovarian cancer ascites. Of the activated CD14brightCD16−HLA-DR−-derived MDM, mean percentage cytostasis was 36%; this was 22% for MDM derived from epithelial ovarian cancer peripheral blood and 29% for MDM derived from epithelial ovarian cancer ascites. Statistical analysis showed no significant differences in cytostatic activity among cell sources, subpopulations, or activation conditions.

MDM from epithelial ovarian cancer patients produce IL-10, IL-8, IL-6, and TNF-α. Median concentrations of certain cytokines was determined by Luminex Multiplex Bead Immunoassay in supernatants of normal donor peripheral blood (n = 4), epithelial ovarian cancer peripheral blood (n = 3), and epithelial ovarian cancer ascites (n = 4). IL-12 levels were near the lower limit of detection for all MDM tested (Fig. 4). IL-10 levels were increased up to 5.2-fold in normal donor peripheral blood 24 hours after activation, particularly when activated with LPS or LPS plus IFN-γ (Fig. 4A). IL-10 levels remained near the lower limit of detection after the addition of M-CSF, LPS, or LPS plus IFN-γ in CD14+ MDM derived from epithelial ovarian cancer ascites, but were increased up to 2.0-fold in CD14brightCD16−HLA-DR− MDM 24 hours after activation with LPS (data not shown). Epithelial ovarian cancer peripheral blood MDM derived from CD14+ and CD14brightCD16−HLA-DR− MO/MA had up to 2.3-fold higher concentrations of IL-10 24 hours after activation with LPS or LPS plus IFN-γ than with M-CSF alone (Fig. 4B).

Median concentrations of IL-8 were elevated in MDM supernatants from normal donor peripheral blood, epithelial ovarian cancer peripheral blood, and epithelial ovarian cancer ascites with medians (ranges) of 7,559 pg/mL (3,007-270,105

<table>
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<th>Subpopulation</th>
<th>Activation condition</th>
<th>ND PB (%)</th>
<th>EOC PB (%)</th>
<th>EOC ascites (%)</th>
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<td>CD14+</td>
<td>M-CSF alone</td>
<td>75.8 (58.0-85.0)</td>
<td>13.7 (0-57.0)</td>
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<td>3.2 (0-15.9)</td>
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<td>CD14+</td>
<td>LPS + IFN-γ</td>
<td>68.8 (63.5-73.5)</td>
<td>13.6 (0-41.5)</td>
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Table 2. Mean and range of phagocytosis in MDM

Table 3. Expression of Fcγ receptors on MO/MA and LPS-activated MDM

<table>
<thead>
<tr>
<th>Fcγ receptor</th>
<th>Culture period</th>
<th>ND PB</th>
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NOTE: −, no positive cells; +, <10% positive; ++, 10% to 50% positive; ++++, 50% to 75% positive; ++++, >75% positive.
IFN-γ increases, respectively, 24 hours after activation with LPS plus M-CSF alone (Fig. 4B), produced TNF-α with M-CSF alone (20.4-fold and 14-fold, respectively, compared with M-CSF alone).

Ascending ovarian cancer peripheral blood MDM 24 hours after activation was similar to that by epithelial ovarian cancer ascites MDM (Fig. 4B), with increased levels of TNF-α after activation with LPS and LPS plus IFN-γ produced by epithelial ovarian cancer peripheral blood MDM derived from CD14+ MO/MA (23.9-fold and 12.1-fold, respectively, compared with M-CSF alone) and from CD14brightCD16+ HLA-DR+ MO/MA (20.4-fold and 14-fold, respectively, compared with M-CSF alone).

**Conclusion**

This study shows certain deficiencies in epithelial ovarian cancer patient MO/MA cultured in M-CSF. M-CSF is produced by malignant ovarian cells (15) and can be detected in the blood (16) and ascites (17) of ovarian cancer patients and is a chemoattractant for MO/MA (18). Studies in rats have shown that previous exposure to cytokines in vivo may determine the course of in vitro differentiation and that cells may not respond to further in vitro cytokine exposure (19). Epithelial ovarian cancer MO/MA, which have previous exposure to M-CSF (16), and other cytokines (15) in vivo, may therefore not have the capacity to respond to further in vitro exposure. This was suggested by data from our in vitro experiments, which showed that although normal donor peripheral blood MO/MA cultured with M-CSF display a similar profile of cytokine production to that of epithelial ovarian cancer patients, they retain antitumor and other activities, which is not the case for M-CSF cultured MO/MA from epithelial ovarian cancer patients.

We also showed that epithelial ovarian cancer patients have different proportions of MO/MA found in their peripheral blood and ascites, and in their tumor stroma, than normal donors. These differences may be related to factors produced by the tumor environment but have not been explained. Ascitic macrophages are the most abundant mononuclear population in the peritoneal cavity, where they are in contact with invading tumor cells. Because ascitic macrophages can be reproducibly isolated, they were used for further functional experiments. CD14brightCD16+ HLA-DR+ MO/MA were the most abundant subpopulation in epithelial ovarian cancer patients; they were, therefore, selected for further in vitro study of their morphology and function. Previous publications also indicated that MO/MA from normal donor peripheral blood express CD16 under adherent culture conditions and function as effective antitumor agents for IL-8 or IL-6.

TNF-α production by normal donor peripheral blood MDM (Fig. 4A) was near the lower limit of detection at all time points for cells activated with M-CSF alone and for cells just before activation with any agent (i.e., 0 hour time point). Normal donor peripheral blood MDM derived from CD14+ and CD14brightCD16+ HLA-DR+ MO/MA had 97.7-fold and 11.8-fold increases, respectively, in TNF-α production 24 hours after activation with LPS and 6.4-fold and 249.0-fold increases, respectively, 24 hours after activation with LPS plus IFN-γ. Production of TNF-α by epithelial ovarian cancer ascites MDM had a similar pattern as normal donor peripheral blood MDM. Epithelial ovarian cancer ascites MDM activated with M-CSF alone (Fig. 4B) produced TNF-α near the lower limit of detection, as did the same MDM just before activation with any agent (data not shown). Activation with LPS or LPS plus IFN-γ caused increased production of TNF-α at 24 hours by epithelial ovarian cancer ascites MDM derived from CD14+ MO/MA (118.0-fold and 30.3-fold, respectively) and from CD14brightCD16+ HLA-DR+ MO/MA (57.1-fold and 24.9-fold, respectively). TNF-α production by epithelial ovarian cancer peripheral blood MDM 24 hours after activation was similar to that by epithelial ovarian cancer ascites MDM (Fig. 4B), with increased levels of TNF-α after activation with LPS and LPS plus IFN-γ produced by epithelial ovarian cancer peripheral blood MDM derived from CD14+ MO/MA (23.9-fold and 12.1-fold, respectively, compared with M-CSF alone) and from CD14brightCD16+ HLA-DR+ MO/MA (20.4-fold and 14-fold, respectively, compared with M-CSF alone).

**Fig. 3.** Non-Fcγ receptor–mediated functions of MDM. Macrophage-mediated cytotoxicity (A) and cytostatic capacity (B) of MDM derived from CD14+ (black columns) and CD14brightCD16+ HLA-DR+ (white columns) MO/MA. Normal donor peripheral blood MDM derived from CD14+ MO/MA have significantly greater macrophage-mediated cytotoxicity than epithelial ovarian cancer peripheral blood MDM derived from CD14+ MO/MA. Epithelial ovarian cancer peripheral blood and ascites MDM and normal donor peripheral blood MDM could equivalently inhibit tumor cell growth. Columns, mean of two to five different MDM samples per group; bars, average deviation. *, P = 0.015.

ppg/mL, 6,502 pg/mL (3,562-70,770 pg/mL), and 11,062 pg/mL (2,911-23,714 pg/mL), respectively. Concentrations of IL-6 were also elevated in MDM supernatants from normal donor peripheral blood, epithelial ovarian cancer peripheral blood, and epithelial ovarian cancer patients cultured in M-CSF. These differences may be related to factors produced by the tumor environment but have not been explained. Ascitic macrophages are the most abundant mononuclear population in the peritoneal cavity, where they are in contact with invading tumor cells. Because ascitic macrophages can be reproducibly isolated, they were used for further functional experiments. CD14brightCD16+ HLA-DR+ MO/MA were the most abundant subpopulation in epithelial ovarian cancer patients; they were, therefore, selected for further in vitro study of their morphology and function. Previous publications also indicated that MO/MA from normal donor peripheral blood express CD16 under adherent culture conditions and function as effective antitumor agents for IL-8 or IL-6.

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The morphology of MO/MA from epithelial ovarian cancer peripheral blood and ascites differs from normal donor peripheral blood after culture in M-CSF. The MO/MA of normal donor peripheral blood exhibit the large, spindle morphology as previously described (26, 27). In contrast, the morphology of MDM of epithelial ovarian cancer patients showed some variability between patient specimens. MDM from epithelial ovarian cancer peripheral blood were frequently smaller and rounded, with relatively fewer spindle cells, whereas MDM from epithelial ovarian cancer ascites included smaller, rounded cells, and larger, spindle cells. The smaller, rounded morphology of MDM from epithelial ovarian cancer patients is consistent with that seen in M-CSF-starved cells (26, 27). This may be explained by the down-regulation of the M-CSF receptor, which has previously been described in MO/MA upon exposure to continuous levels of M-CSF in vivo (28–30). Decreased responsiveness to M-CSF might also explain the reduced FcγR-mediated cytotoxicity of MDM from epithelial ovarian cancer peripheral blood and ascites. Although the ADCC activity of M-CSF-derived MDM from normal donor peripheral blood is consistent with previous reports (21, 31–33), MDM from epithelial ovarian cancer ascites and peripheral blood produced under the same M-CSF conditions resulted in a reduced ADCC effect. In addition, we found for the first time that the phagocytic activity of M-CSF-derived epithelial ovarian cancer MDM was also decreased. Although the mechanism of ADCC seems to depend on culture conditions (34), MDM matured by M-CSF may accomplish ADCC through a phagocytosis-related mechanism (2, 35). This may explain our findings that show a decrease in both phagocytosis and ADCC in epithelial ovarian cancer MDM, whereas both activities were observed in normal donor MDM. The impaired phagocytosis might also be explained, at least in part, by the altered morphology of MO/MA. The smaller, rounded MDM from epithelial ovarian cancer peripheral blood cultures could be less efficient at performing phagocytic ingestion because of their physical characteristics. This may not be the entire explanation, however, because, in certain ascites samples, ingested sheep RBCs were detected in both small, round cells and in large, spindle-shaped cells.

Both the high- and low-affinity Fcγ receptors can mediate ADCC (35–37) and phagocytosis (37–39). The absence of CD16 and the markedly reduced expression of CD64 in epithelial ovarian cancer peripheral blood and ascites MDM leave only CD32, the FcγRII, as a mediator of FcγR functions. However, in contrast to normal donor MDM, CD32 is only weakly positive in MDM from epithelial ovarian cancer peripheral blood and ascites, whereas the inhibitory CD32B isotype is also expressed to a similar degree on the cultured cells. This FcγR surface marker profile suggests that epithelial ovarian cancer patient MDM are unable to mediate FcγR-mediated functions effectively. In contrast, normal donor peripheral blood MDM strongly express both CD64 and CD32 and may therefore mediate FcγR functions through either of these receptors. This indicates that the FcγRIIB receptor may have a negative regulatory role in FcγR-mediated events in epithelial ovarian cancer patients. The reason for altered FcγR expression in MDM of epithelial ovarian cancer patients is unknown; however, there is evidence that two cytokines circulating in ovarian cancer patients, transforming growth factor-β and TNF-α, can cause down-regulation of FcγR (40–42). The altered expression of FcγR on MDM of epithelial ovarian cancer patients and the associated impairment of ADCC and phagocytosis might be important factors in the poor responsiveness of patients with ovarian cancer seen in clinical studies of monoclonal antibodies, such as trastuzumab (43). Trastuzumab mediates ADCC activity in addition to down-regulating tumor cell growth (11).

Interestingly, MDM of epithelial ovarian cancer patients were shown to effectively inhibit the growth of an ovarian cancer cell line. The specific mechanism for human MO/MA cytostatic activity has not been defined, although a murine study has suggested that certain cytokines, such as TNF-α, may be
involved (44). Because cytostasis is not a FcR-mediated activity of MO/MA, it could involve different pathways from ADCC.

The cytokine profile of the MDM of epithelial ovarian cancer patients suggests that these MDM might also support tumor growth. Indeed, cultures of MO/MA from normal donor and epithelial ovarian cancer patients in M-CSF induce the production of TNF-α, IL-6, and IL-8. TNF-α and IL-6 can stimulate the proliferation of malignant epithelial cells in culture (8). TNF-α also has a blocking effect on the chemotactic function of M-CSF (45). In addition, IL-8 promotes tumor angiogenesis (6) and its expression is inversely correlated with survival in murine models of human epithelial ovarian cancer (46). Further, in a recent microarray study of the peritoneum in the context of epithelial ovarian cancer, we found that IL-8 occupies a central stage among the gene profile alterations detected in these tissues and was associated with a profile of MO/MA activation and differentiation (47).

It is interesting that the cytokine profile exhibited by the MDM of epithelial ovarian cancer patient MDM as a result of continued previous in vivo exposure, these cells might be capable of binding to M-CSF in amounts sufficient to induce cytokine production but not antitumor activity. Our results would seem to suggest this.

In contrast, normal donor peripheral blood showed ADCC and phagocytosis and expressed cytokines in response to M-CSF. Normal donor peripheral blood MDM would not normally be exposed to high levels of M-CSF in vivo, and therefore M-CSF receptor expression would not be downregulated. In addition, M-CSF-induced IL-6 production in vitro may also result in increased functional M-CSF receptor expression and ultimately increased M-CSF uptake (48), enabling these cells to perform the M-CSF functions associated with higher M-CSF exposure. This may not happen in epithelial ovarian cancer patient MDM because previous cytokine exposure in vivo might impair their response to autocrine IL-6 (19), or perhaps as speculated for the M-CSF receptor, overexposure to IL-6 in vivo may have led to down-regulation of the IL-6 receptor, thereby impairing responses to IL-6.

We conclude that MO/MA from epithelial ovarian cancer patients have impaired antitumor activity but retain other activity that might be important in tumor formation. These findings provide additional support for a pervasive immune suppressive environment in epithelial ovarian cancer patients in vivo.

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Defective Antitumor Function of Monocyte-Derived Macrophages from Epithelial Ovarian Cancer Patients

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