

# Macrophage Effector Mechanisms in Melanoma in an Experimental Study

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**Background:** The tumor-bearing state is known to induce immune dysfunction that contributes to increased infectious complications and tumor progression. However, the mechanisms underlying this immunosuppression remain unclear.

**Hypothesis:** Macrophage (MO) dysfunction may play a role in tumor-induced immunosuppression.

**Design and Main Outcome Measures:** Using a murine model, this study investigated the effects of melanoma growth on peritoneal macrophage effector molecule and prostaglandin production, MO-mediated cytotoxicity, and candidacidal mechanisms. Female C57BL/6 mice were inoculated with 106 B16 melanoma cells or a salt solution subcutaneously. Mice were euthanized 3 weeks later and peritoneal MOs were harvested and assayed for nitric oxide, superoxide anion, tumor necrosis factor  $\alpha$ , and prostaglandin E<sub>2</sub> production. Macrophage-mediated cytotoxicity against B16 melanoma

targets and MO candidacidal mechanisms were also measured.

**Results:** Macrophage production of nitric oxide, superoxide anion, and tumor necrosis factor  $\alpha$  were significantly decreased, while prostaglandin E<sub>2</sub> production was increased in MOs from melanoma-bearing mice. Concomitantly, MO-mediated cytotoxicity and candidacidal mechanisms were significantly impaired.

**Conclusions:** Melanoma growth leads to decreased MO effector molecule production, increased prostaglandin E<sub>2</sub> production, and impaired MO cytotoxic and candidacidal mechanisms. These results may help explain the observed increased infectious complications in the tumor-bearing host. Strategies aimed at restoring MO function may have therapeutic potential.

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THE INCIDENCE of melanoma has increased dramatically over the last few decades among white populations.<sup>1-3</sup> Novel therapeutic approaches are being tested based on better understanding of the immune response to melanoma and the isolation of melanoma-specific antigens.<sup>4,5</sup> However, clinical responses have been less than encouraging. Further refinement in our understanding of melanoma immunology is needed,<sup>6,7</sup> specifically of the mechanisms involved in immunosuppression induced by melanoma and other tumors. This immunosuppression may account for the host's inability to respond to immunotherapeutic protocols and increased incidence of infectious complications in addition to continued tumor growth.<sup>8,9</sup> The macrophage (MO) plays an important role in the immune response to pathogens and tumors, and is one of the first cell populations to appear with neutrophils in the acute phase of the inflammatory response. Macrophage-mediated tumor killing and killing of intracellular

organisms has been shown to be dependent on the elaboration of reactive intermediates such as nitric oxide (NO), superoxide (SO) anion, and cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ).<sup>10-13</sup> Conversely, the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) has been shown to be associated with immunosuppressive states and decreased MO effector functions.<sup>14</sup> The purpose of this study was to examine the effect of B16 melanoma growth on peritoneal MO (PMO) function in melanoma-bearing mice.

## RESULTS

### TUMOR MODEL CHARACTERISTICS

The inoculation of B16 subcutaneously into C57BL/6 mice resulted in progressive tumor growth. No differences in food intake between the groups were noted during the study period. Mean food intake during the study period averaged  $2.9 \pm 0.3$  g/d per mouse for controls compared with  $2.8 \pm 0.3$  g/d per mouse for B16 melanoma-bearing

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## MATERIALS AND METHODS

### TUMOR

The B16 melanoma tumor line was derived from a malignant melanoma that arose spontaneously in C57BL/6 mice at the Jackson Laboratories (Bar Harbor, Me). This tumor was obtained from the Tumor Depository, Division of Cancer Treatment, National Cancer Institute (Frederick, Md) and is characterized as being nonimmunogenic. The B16 cell lines were free of mycoplasma (tested by ATCC, Rockville, Md) and free of the following viruses and microorganisms: pneumonia virus of mice, Sendai, mouse hepatitis virus, Theiler, minute virus of mice, parvovirus, lymphocytic choriomeningitis, epizootic diarrhea of infant mice, lactate dehydrogenase-elevating virus, reo type 3, polyoma, ectromelia, K virus, mouse adenovirus, mouse cytomegalovirus, *Mycoplasma pulmonis*, and cilium-associated respiratory bacillus (tested by Microbiological Associates, Rockville). The cells were grown in vitro in Dulbecco Modified Eagle Medium (high glucose) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah), 2mM of glutamine, 100 U/mL of penicillin G sodium, 100 µg/mL of streptomycin sulfate, 0.25 µg/mL of amphotericin B, and 50µM 2-mercaptoethanol in 6% carbon dioxide to 95% air at 37°C. Cells were passaged biweekly by removing the old medium, rinsing the cells with Hank balanced salt solution (HBSS) (calcium- and magnesium-free), and briefly exposing them to 0.05% trypsin in 0.53mM edetic acid solution at 37°C for 2 to 3 minutes. The flask was tapped sharply against a solid surface to dislodge the cells, Dulbecco Modified Eagle Medium was added, and the cell suspension was repeatedly pipetted to produce a single cell suspension.

### ANIMALS

Virus-free female C57BL/6 mice aged 8 to 12 weeks (average weights, 20-22 g) were purchased from Charles Rivers Laboratories (Wilmington, Mass). Mice were given food and water ad libitum and given a week to acclimate before being used in experiments. For in vivo tumor studies, mice (n=20) were randomized to receive melanoma cells ( $5 \times 10^5$  B16 cells) subcutaneously into the interscapular region (n=10) or to receive HBSS injection only (control mice) (n=10). Body weight and food intake were measured on alternate days. Measurements of tumor size (longest and shortest diameters) were done biweekly from the time tumors became palpable using electronic digital calipers (Fowler Ultra-cal II; Sylvac, Switzerland). Tumor volume was calculated by the formula for a prolate spheroid according to the equation  $(L [\text{longest diameter}] \times S^2 [\text{shortest diameter}] \times 0.52) = \text{tumor volume (cm}^3\text{)}$ . This

technique has proved to be a reliable measure of tumor volume in many previous studies and to correlate closely with tumor weight.<sup>15</sup> Mice were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care (Weill Medical College, Cornell University, New York, NY), and all animal protocols were approved by the Institutional Animal Care and Use Committee at Weill Medical College of Cornell University.

### HARVESTING OF PERITONEAL EXUDATE CELLS

Elicited PMOs were obtained by injecting mice with 2 mL of 4% thioglycollate solution intraperitoneally 4 days prior to euthanization. Mice were killed by carbon dioxide (CO<sub>2</sub>) asphyxiation and the peritoneum was exposed. Ten milliliters of cold sterile HBSS (calcium- and magnesium-free) was injected intraperitoneally. The solution was agitated in the peritoneal cavity for 1 minute and aspirated. The aspirate was centrifuged at 200g for 10 minutes at 4°C. The supernatant was decanted, the pellet was resuspended in HBSS, and the cells were counted. Viability (trypan blue exclusion) was usually 95% to 100%. The cells were adjusted to a final concentration of 106 cells/mL. Peritoneal exudate cells were plated, depending on the experiment, in 24- or 96-well plates and incubated for 2 hours in 6% CO<sub>2</sub>/95% room air at 37°C. Non-adherent cells were aspirated and adherent cells were washed twice with HBSS. This protocol yielded a greater than 90% pure PMO population as documented by morphological and fluorescent-activated cell sorter analysis using MO-specific F4/80 monoclonal antibody (Harlan, Indianapolis, Ind). All MO studies were performed in Dulbecco Modified Eagle Medium (high glucose) supplemented with 3% fetal calf serum (Hyclone), 2 mM of glutamine, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 0.25 µg/mL of amphotericin B, and 50µM of 2-ME in 6% CO<sub>2</sub>/95% room air at 37°C. This complete medium was tested for endotoxins using the chromogenic Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, Md) and was found to contain an endotoxin concentration of less than 0.2 E U/mL.

### STIMULATION FOR NO PRODUCTION AND NITRITE MEASUREMENT

Adherent PMOs ( $2 \times 10^5$  per well) were stimulated with lipopolysaccharide (LPS) (1 µg/mL) and interferon gamma (100 U/mL). Preliminary studies demonstrated that these concentrations stimulate maximal NO production under the experimental conditions used. Seventy-two hours later supernatants were removed and assayed for NO production

Continued on next page

mice. Mice were euthanized 3 weeks after tumor inoculation and the time points were chosen to ensure that all animals in the study had palpable tumors. At the time of euthanization the mean tumor volume was  $3.2 \pm 0.8 \text{ cm}^3$ .

### MELANOMA GROWTH INHIBITS PMO NO PRODUCTION

To determine the effects of progressive melanoma growth on PMO NO production, PMOs from control and B16 melanoma-bearing mice were stimulated with

LPS (1 µg/mL) and interferon gamma (100 U/mL) for 72 hours and NO production was measured. **Figure 1** shows PMOs from B16 melanoma-bearing mice with significantly less mean NO production compared with controls ( $4.4 \pm 1.4$  vs  $24.6 \pm 0.7 \text{ nM}/2 \times 10^5 \text{ MO}$ ;  $P < .001$ ). The decreased ability of PMOs from melanoma-bearing mice to produce NO was not owing to decreased viability, since PMOs from control and melanoma-bearing mice had similar viability on the results of trypan blue dye-exclusion tests and the methylthiotetrazole viability assay (not shown).

by measuring the accumulation of nitrite in supernates using the Griess reaction as described previously.<sup>16</sup> Briefly, in a 96-well plate, 100  $\mu$ L of medium was mixed with 100  $\mu$ g of an equal mixture of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid and incubated at room temperature for 10 minutes. Absorbance at 550 nm was measured using a UVmax microplate reader (Molecular Devices, Menlo Park, Calif) and data were calculated by comparison to standard curves generated using sodium nitrite standards.

#### SO ANION MEASUREMENTS

Superoxide anion was assayed according to the method of Pick and Mizel as the superoxide dismutase-inhibitable reduction of cytochrome c method.<sup>17</sup> Adherent PMOs ( $2 \times 10^5$  per well) in 96-well flat-bottom plates were incubated with a 160  $\mu$ M solution of ferricytochrome c in phenol red-free HBSS alone, with superoxide dismutase (300 U/mL), or with a stimulus (1-mg/mL phorbol myristate acetate [PMA]). Plates were incubated at 37°C for 90 minutes. Absorbance at 550 nm was measured using a microplate reader. Values (mean of triplicate wells) were converted to nanomoles of SO anion per  $2 \times 10^5$  cells per 90 minutes based on the extinction coefficient of (reduced minus oxidized) cytochrome c.

#### TNF- $\alpha$ ENZYME-LINKED IMMUNOSORBENT ASSAY

Adherent PMOs ( $2 \times 10^5$  per well) were stimulated with 1  $\mu$ g/mL of LPS and supernates were harvested 24 hours later. Tumor necrosis factor  $\alpha$  was measured by enzyme-linked immunosorbent assay using specific antibody pairs according to the manufacturer's recommendation (Pharmingen, San Diego, Calif). Plates were read in a microplate reader, unknown values were calculated by extrapolation from standard curves generated using recombinant TNF- $\alpha$ , and analysis was performed using Delta Soft (Biometallics Inc, Princeton, NJ) analysis software on a Macintosh (Apple Inc, Cupertino, Calif) computer.

#### PGE<sub>2</sub> ASSAYS

Adherent PMOs ( $2 \times 10^5$  per well) were stimulated with 1  $\mu$ g/mL of LPS and supernates were harvested 24 hours later. Prostaglandin E<sub>2</sub> levels were assayed using an enzyme immunoassay kit according to the manufacturer's protocol (Cayman, Ann Arbor, Mich).

#### MO CANDIDACIDAL ACTIVITY

Macrophage-mediated candidacidal assay was performed according to the method of Cenci et al.<sup>18</sup> Adherent PMOs

( $2 \times 10^5$  per well) were incubated with *Candida albicans* in 96-well flat-bottom plates at an MO to *Candida* ratio of 10:1 at 37°C. After 4 hours of incubation, Triton X-100 (Sigma, St Louis, Mo) was added to give a final concentration of 0.1% and the contents of the wells were removed by vigorous pipetting and washing with sterile deionized H<sub>2</sub>O. Complete removal of MOs and *Candida* was confirmed using an inverted microscope. Serial dilutions from each well were made in sterile deionized H<sub>2</sub>O and cultured on Sabouraud dextrose agar plates at 37°C. The number of colony-forming units (cfu) was determined after 12 to 18 hours of incubation at 37°C. Macrophage candidacidal activity was calculated as:

$$\frac{[\text{cfu control well} - \text{cfu experimental well}]}{\text{cfu control well}} \times 100$$

#### CYTOTOXICITY ASSAY

Adherent PMOs ( $2 \times 10^5$  per well) in 96-well flat-bottom plates were stimulated with interferon gamma (100 U/mL) and LPS (1  $\mu$ g/mL) for 16 hours and the activation medium was replaced with fresh medium. B16 melanoma cells were added to the wells at an MO to melanoma ratio of 20:1. Cells were then incubated at 37°C and supernatants were harvested 48 hours later. Macrophage-mediated cytotoxicity was then assayed by measuring the amount of lactate dehydrogenase released from lysed target cells according to the method of Decker and Lohmann-Mattes.<sup>19</sup> Specific lysis was calculated using the formula:

$$\% \text{ Specific Lysis} = \frac{A [\text{Experimental}] - A [\text{Spontaneous}]}{A [\text{Maximal}]} \times 100,$$

where A is the absorbance at 490 nm subtracted from background absorbance (medium alone); A (experimental) is the net absorbance of supernatant from wells containing activated macrophages and melanoma cells; A (spontaneous) represents the net absorbance by wells containing melanoma cells alone; and A (maximal) represents the net absorbance of melanoma cells that have been freeze-thawed to lyse all the cells, representing the total amount of lactate dehydrogenase that can be released from melanoma targets.

#### STATISTICAL ANALYSES

The data are presented as the mean  $\pm$  SE for control (n = 10) and melanoma-bearing mice (n = 10). Experiments were repeated 2 to 3 times. When comparing 2 groups, the student t test was used for parametric data and the Wilcoxon rank sum test was used for nonparametric data analysis. Calculations were performed using a statistical package (InStat for MacIntosh; GraphPad Software, San Diego, Calif).

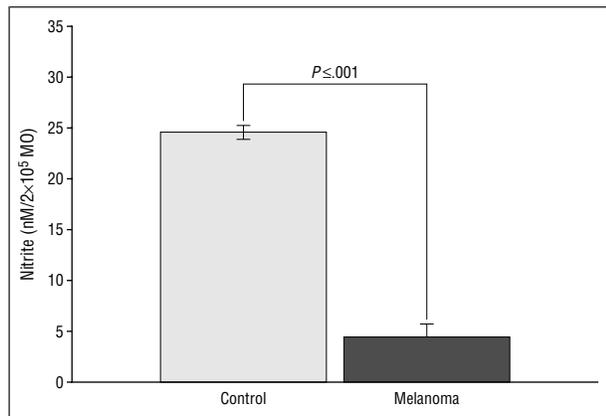
### MELANOMA GROWTH INHIBITS PMO SO ANION PRODUCTION

To determine the effects of melanoma growth on PMO SO anion production, PMOs from control and B16 melanoma-bearing mice were stimulated with PMA (1 mg/mL) for 90 minutes and SO anion production was measured. **Figure 2** shows that PMOs from B16 melanoma-bearing mice had significantly less mean SO anion production compared with controls ( $0.11 \pm 0.08$  vs

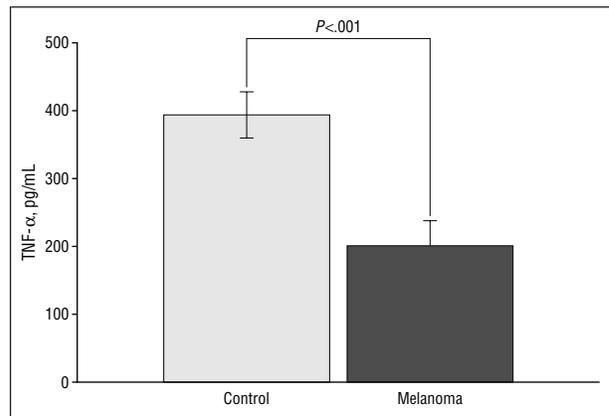
$0.31 \pm 0.05$  nM/ $2 \times 10^5$  MO;  $P < .01$ ). These data indicate that PMOs from melanoma-bearing mice had a significant decrease in SO anion production.

### MELANOMA GROWTH INHIBITS PMO TNF- $\alpha$ PRODUCTION

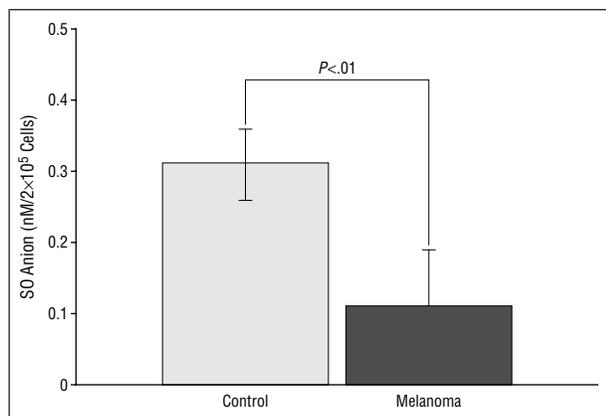
To determine the effects of melanoma growth on PMO TNF- $\alpha$  production, PMOs from control and B16 melanoma-bearing mice were stimulated with LPS (1  $\mu$ g/



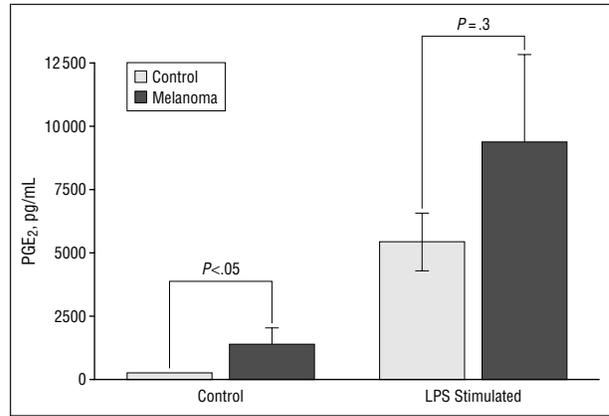
**Figure 1.** Production of nitric oxide by peritoneal macrophages (PMOs) from control and B16 melanoma-bearing mice. Peritoneal MOs were harvested from control and B16 melanoma-bearing mice 3 weeks after tumor injection and stimulated in vitro with interferon gamma and lipopolysaccharides. Supernatants were collected 72 hours later and assayed for nitrite production as detailed in the “Materials and Methods” section.



**Figure 3.** Production of tumor necrosis factor α (TNF-α) by peritoneal macrophages from control and B16 melanoma-bearing mice. Peritoneal macrophages were harvested from control and B16 melanoma-bearing mice 3 weeks after tumor injection and stimulated in vitro with lipopolysaccharides for 24 hours.



**Figure 2.** Production of superoxide (SO) anions by peritoneal macrophages from control and B16 melanoma-bearing mice. Peritoneal MOs were harvested from control and B16 melanoma-bearing mice 3 weeks after tumor injection and stimulated in vitro with phorbol myristate acetate. Superoxide anion production was measured as detailed in the “Materials and Methods” section.



**Figure 4.** Production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) by peritoneal macrophages from control and B16 melanoma-bearing mice. Peritoneal macrophages were harvested from control and B16 melanoma-bearing mice 3 weeks after tumor injection and stimulated in vitro with lipopolysaccharides for 24 hours.

mL) for 24 hours and TNF-α production was measured by enzyme-linked immunosorbent assay. **Figure 3** shows that PMOs from B16 melanoma-bearing mice had significantly less mean TNF-α production compared with controls (200±38 vs 394±34 pg/mL;  $P < .001$ ).

#### MELANOMA GROWTH INCREASES PMO PGE<sub>2</sub> PRODUCTION

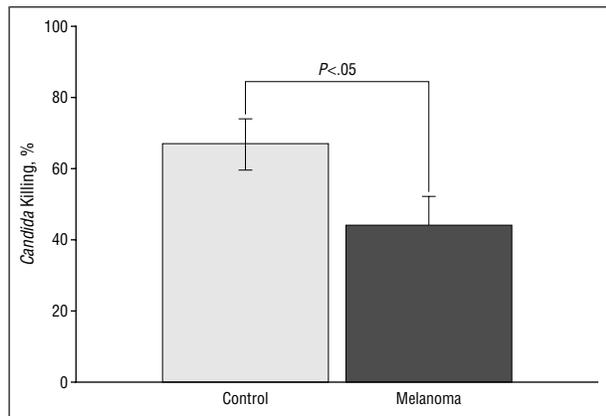
To determine the effects of melanoma growth on PMO PGE<sub>2</sub> production, PMOs from control and B16 melanoma-bearing mice were stimulated with LPS (1 μg/mL) for 24 hours and PGE<sub>2</sub> production was measured by immunoassay. **Figure 4** shows that PMOs from B16 melanoma-bearing mice had significantly more basal PGE<sub>2</sub> production compared with controls (249±17 vs 1368±676 pg/mL;  $P < .003$ ). Similarly, stimulated PGE<sub>2</sub> production was also increased in MOs from melanoma bearing mice compared with controls, but this did not reach statistical significance (5430±1149 vs 9337±3481 pg/mL;  $P = .3$ ).

#### MELANOMA GROWTH DECREASES PMO CANDIDACIDAL ACTIVITY

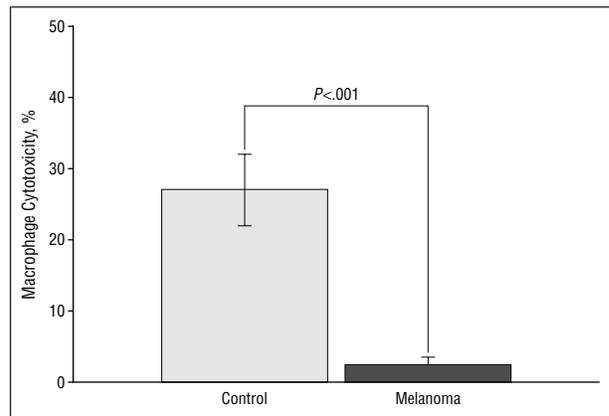
Since the mediators tested above are critical MO effector mechanisms against microbial infections, we investigated the functional candidacidal capacity of PMOs from melanoma-bearing mice. Peritoneal macrophages from control and B16 melanoma-bearing mice were coincubated with *C albicans* as described and PMO killing of *Candida* was measured. **Figure 5** shows that PMOs from B16 melanoma-bearing mice were significantly less able to kill *Candida* species in vitro compared with controls (44%±8.3% vs 67%±7.2%;  $P < .05$ ).

#### MELANOMA GROWTH DECREASES PMO CYTOTOXICITY

To determine the effects of melanoma growth on PMO-mediated killing of B16 melanoma, PMOs from control and B16 melanoma-bearing mice were activated with LPS (1 μg/mL) and interferon gamma (100 U/mL) and



**Figure 5.** Macrophage (MO) candidacidal capacity in control and B16 melanoma-bearing mice. Peritoneal MOs were harvested from control and B16 melanoma-bearing mice 3 weeks after tumor injection and assayed for candidacidal capacity as described.



**Figure 6.** Macrophage-mediated cytotoxicity against B16 melanoma targets. Peritoneal macrophages were harvested from control and B16 melanoma-bearing mice 3 weeks after tumor injection and stimulated in vitro with interferon gamma and lipopolysaccharides for 16 hours. B16 melanoma targets were then added and allowed to incubate for 48 hours and the supernates harvested and assayed for lactate dehydrogenase release as described.

PMO cytotoxicity was measured. **Figure 6** shows that PMOs from melanoma-bearing mice had significantly decreased cytotoxicity against syngenic B16 melanoma targets compared with controls ( $2.3\% \pm 1.2\%$  vs  $27\% \pm 5\%$ ;  $P < .001$ ).

#### COMMENT

The incidence of melanoma among white populations has increased dramatically over the last few decades.<sup>2,3</sup> While many aspects of the immunobiology of melanoma are known, the basis for immunosuppression induced by melanoma and other tumors remains unclear. Tumor-induced immunosuppression results in defective cellular immune responses and probably contributes to an increased incidence of perioperative and infectious complications, poor responses to immunotherapy, and ultimately, accelerated tumor growth.<sup>9</sup> The MO plays an important role in immune regulation, tumor cytotoxicity, and microbicidal immune response.<sup>20,21</sup> The antimicrobial capability of the MO is well documented, especially against facultative and obligate intracellular bacteria, protozoa, and fungi.<sup>22</sup> Several effector molecules are known to be important in MO-mediated cytotoxicity and microbicidal activity. These include SO anion, NO, proteases, acid hydrolases, cationic proteins, and lysozymes. In addition, the MO secretes several cytokines, bioactive lipid products, and complement components that act in an autocrine and paracrine manner to modulate the immune response to a pathogen.<sup>10</sup>

We have characterized in this murine melanoma model significant suppression in MO production of effector molecules and the impaired MO-mediated killing of melanoma targets and candidacidal mechanisms that result. Our study demonstrates that MOs from melanoma-bearing mice have significantly reduced production of major effector molecules, including NO, SO anion, and TNF- $\alpha$  production. Both basal and stimulated PGE<sub>2</sub> production were increased in MOs from melanoma-bearing mice. The functional consequences of these alterations were significant decreases in MO-mediated killing of melanoma targets in vitro and significant im-

pairment of candidacidal mechanisms by MOs from melanoma-bearing mice. This study provides a comprehensive examination of effector molecules (NO, SO anion, and TNF- $\alpha$ ), suppressive molecule (PGE<sub>2</sub>), and functional capacity (cytotoxicity and microbicidal mechanisms) in PMOs from melanoma-bearing mice. Other investigators, focusing on PMO functions, have demonstrated increased PGE<sub>2</sub> production and decreased cytotoxicity in MOs from tumor-bearing hosts.<sup>23-27</sup> It is possible that different results might be seen with other fixed MO populations. Human circulating monocytes and macrophages may exhibit different patterns than seen in this study. Further studies are required to elucidate these differences.

Our results may provide an explanation for the increased susceptibility to infections (especially fungal) in tumor-bearing hosts. Similarly, the defective MO-mediated cytotoxicity against tumor targets may provide an explanation for ineffective responses to immunotherapy. Strategies aimed at reversing MO immunosuppression may prove useful adjuvants to immune therapy.

While not directly tested in this study, the inhibition of PGE<sub>2</sub> production may prove beneficial in reversing MO dysfunction, especially with the availability of selective cyclooxygenase inhibitors.<sup>28</sup> Current studies examine the mechanisms and cellular pathways involved in mediating the observed defects. In in vitro studies we have been able to reproduce some of the defects in MO function by coinoculating MOs with a melanoma-conditioned medium. Preliminary characterization studies point to a secreted melanoma-derived protein that may mediate the observed suppression in vivo.

In summary, MOs from melanoma-bearing mice demonstrate significant defects in NO, SO anion, and TNF- $\alpha$  production. This was associated with increased PGE<sub>2</sub> production. Concomitantly, MO-mediated cytotoxicity and killing of *Candida* species were significantly decreased. Augmenting MO function or inhibit-

ing PGE<sub>2</sub> production in the tumor-bearing host may restore MO immune competence and host immunity.

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## Correction

**Reference Error.** In the first reference of the article titled "Colorectal Hepatic Metastases: Resection, Local Ablation, and Hepatic Artery Infusion Pump Are Associated With Prolonged Survival" published in the March issue of the ARCHIVES (2001;136:318-323), the first author, Fong Y, was omitted from the list.