Macrophages Mediate Inflammation-Enhanced Metastasis of Ovarian Tumors in Mice

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Abstract

The tumor microenvironment is known to have a profound effect on tumor progression in a highly context-specific manner. We have investigated whether peritoneal inflammation plays a causative role in ovarian tumor metastasis, a poorly understood process. Implantation of human ovarian tumor cells into the ovaries of severe combined immunodeficient mice resulted in peritoneal inflammation that corresponds temporally with tumor cell dissemination from the ovaries. Enhancement of the inflammatory response with thioglycollate accelerated the development of ascites and metastases. Suppression of inflammation with acetyl salicylic acid delayed ascites development and reduced tumor implant formation. A similar prometastatic effect for inflammation was observed when tumor cells were injected directly into the peritoneum of severe combined immunodeficient mice, and in a syngeneic immunocompetent mouse model. Inflammation-modulating treatments did not affect primary tumor development or in vitro tumor cell growth. Depletion of peritoneal macrophages, but not neutrophils or natural killer cells, reduced tumor progression, as assessed by ascites formation and peritoneal metastasis. We conclude that inflammation facilitates ovarian tumor metastasis by a mechanism largely mediated by macrophages, and which may involve stromal vascular endothelial growth factor production. The confirmation of these findings in immunocompetent mice suggests relevance to human disease. Identifying the mechanisms by which macrophages contribute to tumor metastasis may facilitate the development of new therapies specifically targeting immune cell products in the tumor microenvironment.

Introduction

Ovarian cancer represents ~3% of newly diagnosed cancers in women, but accounts for almost 6% of female cancer deaths. More than 75% of patients have late-stage disease at the time of diagnosis, and the 5-year survival rate for these patients is <25% due to the rapid emergence of drug-resistant disease. As a result, much of our knowledge of the disease is derived from end-stage patient tissue, and less is known of the early disease processes that lead to tumor progression.

The host immune response comprises a multitude of highly developed interconnected biological processes involving both cellular and humoral responses that coordinate to eliminate foreign bodies and repair the site of injury. The innate arm of the immune response provides rapid responses prior to the development of highly specific adaptive responses. In the context of a malignant tumor, many of the degradative and stimulatory properties of innate immunity may influence tumor progression in both positive and negative ways (reviewed in ref. 1). Immune cells have long been known for their roles primarily in immune surveillance and tumor cell elimination, and many tumor cell types secrete immunosuppressive cytokines such as transforming growth factor-β, interleukin (IL)-10 and IL-13, and chemokines that recruit cells that negatively regulate immunity such as T-regulatory cells, myeloid suppressor cells, natural killer (NK) cells, and macrophage subsets. A mutant strain of mice possessing enhanced innate immune responses is almost completely resistant to tumor development (2), indicating the protective capacity of innate immunity against tumors.

Despite the clear evidence for an antitumor function of the immune system, it is increasingly recognized that the role of immune cells in tumor initiation and progression is more complicated. A widely accepted model of tumor and immune cell interaction, termed immunoediting, describes an initial restriction of tumor cell growth, but maintains that the immune system ultimately selects for tumor cells with reduced immunogenicity that subsequently prevail over the host immune system (3). Therefore, whereas the immune system may initially be protective against tumor development, its efficacy may diminish over time and it may ultimately facilitate tumor progression. In addition, chronic inflammation is associated with initiation and/or progression of the most common cancer types including lung, gastric, pancreatic, prostate, and colorectal cancers. Inflammatory cells provide proteases that facilitate invasion and matrix remodeling, along with growth factors, chemokines, angiogenic and lymphangiogenic factors (4), and can be directly tumoricidal (5). The paradoxical roles of immune cells in tumor initiation and progression underscore the need for more detailed study in particular tumor contexts.

Inflammation has been suggestively linked with the development of ovarian cancer by several epidemiologic studies that showed a higher incidence in women with prior pelvic inflammatory disease, exposure to inflammatory irritants, and endometriosis (6). In addition, self-reported use of nonsteroidal anti-inflammatory drugs has been shown to have a small negative effect on the incidence of ovarian tumor development (7, 8), although the effect was not consistently seen in all studies (9). Even though the presence of T lymphocytes in ovarian tumors correlates with improved prognosis, T cells in malignant ascites are less likely to be activated...
than in peripheral blood, suggestive of a local immunosuppressed state (10). Innate immune cells, including macrophages, neutrophils, and NK cells are frequently present in both primary ovarian tumors and malignant ascites (11) and may be important in driving tumor progression. Macrophages can contribute to tumor dissemination by increasing tumor cell adhesion molecules on the peritoneal mesothelium and by releasing growth factors and invasive proteases (12, 13). Macrophages are frequently detected in the tumors of patients with ovarian cancer, and their recent identification in metaplastic inclusion cysts, a possible precursor lesion to ovarian tumors, is an interesting finding (14). However, no difference in macrophage density has been found between benign and malignant patient ovarian tumor samples (15), leaving their role in tumor initiation and progression unclear. The proximity of inflammatory cells to both the primary tumor and to malignant ascites, along with the epidemiologic evidence, illustrates the need for a precise understanding of the particular roles of specific inflammatory cell types in ovarian tumor progression or inhibition. Ovarian tumor cell expression of tumor necrosis factor α may be a key factor in amplifying the inflammatory response and promoting tumor progression (16). Knowledge of how inflammation, in the context of the fluid, growth factor–rich peritoneal microenvironment, affects tumor metastasis may provide promising targets for clinical intervention.

Materials and Methods

Cell culture. The ES-2 ovarian clear cell carcinoma and NIH-OVCAR3 ovarian serous papillary cell lines were purchased from American Type Culture Collection and maintained in DME with 10% and 20% FCS, respectively, and without antibiotics in an atmosphere of 5% CO2. Routine testing indicated that the cells were free of Mycoplasma. Red fluorescent protein (RFP; DsRed Express, Clontech) was stably transfected into ES-2 cells with Escort V Transfection Reagent (Sigma) followed by selection with Geneticin (Invitrogen). RFP-expressing ES-2 cells (ES-2–RFP) resembled the parental cell line both morphologically and in frequency of passage. I8 cells were provided by Dr. H. Naora, M.D. Anderson Cancer Center, and Dr. K. Roby, University of Kansas Medical Center (17), and were maintained in DME with 10% FCS.

Mice. CB-17 severe combined immunodeficient (SCID) mice and C57Bl6 mice (Charles River Laboratories) and Beige mice on C57Bl6 (The Jackson Laboratory) and SCID backgrounds (Charles River) were housed under specific pathogen–free conditions. To prevent opportunistic infections, antibiotics were routinely administered in the drinking water of all mice. Amoxicillin (2.7 mmol/L; Sigma) and neomycin sulfate (1.1 mmol/L; Neo-Sections) were given every second week and enrofloxacin (0.28 mmol/L; Fluka) every other week. No effect on tumor progression was seen in our model when compared with mice not given antibiotics (data not shown).

Generation of in vivo tumors. In the spontaneous metastasis model, ES-2–RFP cells were suspended in Matrigel (mouse basement membrane proteins; BD Biosciences) for implantation in female SCID mice. A transverse lateral incision was made in the abdominal wall of anesthetized mice and ~5 μL of tumor cells (2 × 106 cells) were injected into the parenchyma of the left ovary. The experimental metastasis model involved re suspending 1 × 106 cells in PBS/Matrigel (1:1) in a total volume of 0.5 mL, which was then injected into the peritoneum of female mice. Ip. lavage and blood collection were done weekly in SCID mice and every 2 weeks in C57Bl6 mice.

Modulation of inflammation. Mice were treated with ASA (100 mg/kg, pH 5) by daily s.c. injection, or thiglucose (0.5 mL of a 3% solution) by ip. injection twice per week. Control mice were administered PBS with matched pH at both dosage routes and frequencies, and a separate group was maintained without a weekly peritoneal lavage procedure or blood collection. No differences in tumor progression variables were observed among any of these control groups (data not shown), so data were merged in the analyses. Blood smears were prepared from anti-coagulated blood, and peritoneal lavage cells were cytospun onto slides and stained by Kwik-Diff (Thermo Electron). Differential counts were done by a hematologist (C.A. Mercer). Total cell counts were done by hemocytometer after BBC lysis (PharM Lyse, BD Biosciences). Peripheral blood plasma and peritoneal lavage fluid were stored at ~20°C until assay. Mouse IL-6 and serum amyloid A ELISAs were done on undiluted samples according to the instructions of the manufacturer (E Bioscience and Invitrogen).

Assessment of tumor progression. Mice were monitored daily for signs of tumor progression, including abdominal distension, wasting, or other morbidity, and were sacrificed when any of these signs became apparent. The volume of ascites fluid was determined by aspirating with a needle and syringe. RFP-expressing tumors were imaged with a panoramic macrofluorescence imaging system (Lightools Research). Images were analyzed with ImagePro Express software (Image Processing Solutions) to assess fluorescent tumor coverage of the diaphragm as a percentage of total diaphragm area. Tumor diaphragm coverage was assessed in non-RFP-expressing tumors from a photomicrograph taken at the time of necropsy. Data were analyzed for significance by Mann-Whitney U test.

Tumor burden was evaluated by several methods. First, unstained cytospin preparations were evaluated by fluorescence microscopy, and Kwik-Diff–stained slides were evaluated based on morphology. In addition, flow cytometry (FacsAria, BD Biosciences) was carried out to determine the proportion of total RFP-expressing cells as a percentage of total lavage cells. Finally, we used real-time reverse transcription-PCR analysis (Eppendorf Mastercycler Realplex 4S) to determine the proportions of RNA for human and mouse housekeeping genes (L32), as compared with standard curves of differing proportions of human and mouse cDNA, as previously described (including primer sequences; ref. 18). At least two methods were used for each sample.

Immunodepletion studies. To determine their contribution to the progression of ovarian tumors, innate immune cells were depleted with the following treatments (further details are provided in the Supplementary Methods). Neutrophils were depleted with anti-GR1 (RB6-8C5) antibody, peritoneal macrophages were depleted by injection of clodronate-containing liposomes, and NK cells were depleted with polyclonal anti-asialo-GM1 antibody (Cedarlane Laboratories; Supplementary Fig. S1). SCID-Beige mice, which have a profound deficiency in NK cell activity (in addition to the SCID mutation), and C57Bl6-Beige mice were also used. Metastatic scores were estimated, through blinded observations, based on the number of organs with metastases and the extent of tumor coverage of organs.

Histology, zymography, and ELISA. After antigen retrieval and blocking, sections were incubated with polyclonal rabbit anti-mouse MMP-9 (Chemicon), rabbit anti-human vascular endothelial growth factor (VEGF; Biomeda), or rat anti-mouse F4/80 antigen (eBioscience). Secondary antibodies included biotinylated goat anti-rabbit IgG and biotinylated rabbit anti-rat IgG (Vector). Detection was carried out with ABC reagent, rabbit anti–rat IgG (Vector), or biotin-labeled goat anti-rabbit IgG (Vector). In the analyses. Blood smears were prepared from anti-coagulated blood, and peritoneal lavage cells were cytospun onto slides and stained by Kwik-Diff (Thermo Electron). Differential counts were done by a hematologist (C.A. Mercer). Total cell counts were done by hemocytometer after BBC lysis (PharM Lyse, BD Biosciences). Peripheral blood plasma and peritoneal lavage fluid were stored at ~20°C until assay. Mouse IL-6 and serum amyloid A ELISAs were done on undiluted samples according to the instructions of the manufacturer (E Bioscience and Invitrogen).
were done at 95°C for 10 min, then 50 cycles of 95°C for 15 s and 60°C for 1 min. Melt-curve analysis was done after each run to confirm the production of a single product of the correct size.

Results

Increased local and systemic inflammation accompanies tumor progression. As we have previously shown, our orthotopic mouse model of ovarian cancer generates a pattern of tumor progression that resembles human ovarian cancer in many respects (18). Here, we used an aggressive RFP-expressing human ES-2 ovarian clear cell carcinoma cell line implanted directly into the ovaries of SCID mice. Mice developed large ovarian tumors, malignant ascites, and numerous metastases to the greater omentum, diaphragm, peritoneal wall, and mesentery within 22 to 26 days (Fig. 1A). Systemic and local inflammatory responses were elevated within 2 weeks after tumor implantation as assessed by total and differential cell counts of peripheral blood and peritoneal lavage fluid (Fig. 1B and C; Supplementary Fig. S2A). IL-6 and serum amyloid A production (Fig. 1D and Supplementary Fig. S2B). This increase in inflammatory cells coincided with the appearance of tumor cells in the peritoneum as determined by cytologic analysis (see below). These data indicate that robust local and systemic host inflammatory responses accompanied ovarian tumor progression, similar to those observed in patients with ovarian cancer (11, 19).

The extent of inflammation corresponds with disease progression. We did a series of experiments using our orthotopic mouse model of ovarian cancer to determine whether inflammatory responses affected the development or progression of ovarian tumors. Initially, we showed that suppression of inflammation with ASA (100 mg/kg) reduced systemic and local inflammation, and that thioglycolate enhanced inflammation (data not shown; Fig. 1E). Once we had confirmed our ability to modulate inflammation, we next sought to quantify the effects of modulating inflammation on tumor progression after orthotopic administration of tumor cells. The progression-free interval was defined as the experimental period between tumor administration and development of symptoms such as ascites or other tumor-related morbidity. This interval was shortest in thioglycolate-treated mice (median, day 22; range, days 21–23), intermediate in PBS-treated mice (median, day 24; range, days 23–27), and longest in ASA-treated mice (median, day 27; range, days 27–31; Fig. 2A). In a parallel experiment, mice were treated as before but the experiment was terminated at day 23 to assess the effects of inflammation on the extent of tumor spread. At this time point, all of the thioglycolate-treated mice had developed ascites (11 of 11; 100%) compared with 9 of 11 (82%) PBS-treated mice, and 3 of 12 (25%) ASA-treated mice. Of the mice that had developed ascites, the thioglycolate-treated mice had the greatest volume of ascites fluid (Fig. 2B). In addition, a greater extent of tumor spread was evident within the peritoneal cavity of thioglycolate-treated mice, including more frequent tumor implants and more metastatic foci, including greater coverage of the diaphragm (Fig. 2C; Supplementary Fig. S3A–C). Despite dramatic differences in the extent of tumor spread, the size of the primary ovarian tumor did not differ among the groups (PBS, 0.31 ± 0.07 g; ASA, 0.27 ± 0.01 g;
thioglycolate, 0.28 ± 0.01 g). Consistent with other studies (20), clinically relevant doses of ASA and thioglycolate had no direct effect on tumor cells, as assessed in vitro by MTT assay (data not shown). Additionally, tumor-suppressive effects due to cyclooxygenase-2 (COX-2) inhibition are unlikely because a majority of studies have shown that ovarian tumor cells, including the ES-2 cell line, lack the receptor for COX-2 (21, 22). These data indicate that any potential effects of ASA and thioglycolate in vivo are more likely to arise through their known effects on inflammatory cell recruitment and activation, rather than through direct action on tumor cells.

As a measure of tumor cell dissemination, we determined the proportion of tumor cells among the cells in the peritoneal cavity lavage fluid. We used real-time reverse transcription-PCR analyses to determine the amount of human (tumor cell) RNA and mouse RNA, and compared the results with a standard curve of known proportions of human and mouse RNA, as previously described (18). These results were confirmed by flow cytometric analyses of RFP-expressing cells in lavage fluid and cytologic examination of the infiltrates. Human tumor cells were detected in lavage fluid in all groups at 2 weeks, and their percentage increased at 3 weeks (Fig. 2D). Absolute numbers of tumor cells increased after 2 weeks and were greatest in the thioglycolate-treated mice (thioglycolate, 98.9 ± 15.0 cells x 10^5/mL; PBS, 3.1 ± 2.1 cells x 10^5/mL; ASA, 2.0 ± 2.0 cells x 10^5/mL). Further increases were evident at 3 weeks (Fig. 2D). These data, when compared with the results in Fig. 1, indicate that inflammatory cell infiltration in the tumor microenvironment was proportional to the number of disseminating tumor cells.

Two further experiments were done to further test whether modulating inflammation predominantly affected the dissemination of tumor cells, rather than primary tumor development. In the first experiment, we delayed the administration of ASA and thioglycolate until 1 week after tumor implantation, the time corresponding with the earliest stages of tumor spread from the ovaries. Delayed immunomodulating treatment did not change the time course of ascites development and tumor dissemination, as compared with mice treated immediately after tumor implantation (data not shown). Thus, the level of inflammation prior to tumor cell dissemination affected tumor cell dissemination. Figure 2. The rate and extent of disease progression corresponds with the magnitude of the inflammatory response. A, the progression-free interval after ovarian tumor implantation was assessed in PBS- (n = 7), ASA- (n = 6), and thioglycolate-treated (n = 8) mice by monitoring the development of ascites or other tumor-related symptoms. In a separate experiment in which all mice were sacrificed at day 23, the volume of ascites (B) and the percentage of the diaphragm that was covered with tumor (C) were measured in PBS-, ASA-, and thioglycolate-treated mice (n = 4 in each group; * P < 0.05; ** P < 0.001). D, the proportion of human tumor cells in the peritoneal fluid was assessed by weekly lavage and real-time PCR analysis of the proportion of mouse and human RNA, as compared with a standard curve of known proportions of each (*, P < 0.05). E, mice administered i.p. tumor cells were treated with PBS, ASA, or thioglycolate (n = 4 in each group), and the progression-free interval was measured.
cell dissemination did not affect ascites formation. In the second experiment, we injected tumor cells directly into the peritoneal cavity. In this model of experimental metastasis, growth and exfoliation of the primary tumor was not required for the processes of tumor dissemination. In addition, a more rapid course of disease progression occurs, as compared with ovarian implantation, due to a greater number of tumor cells implanted. Consistent with our findings using orthotopic tumor grafts (Fig. 2A and C), we observed enhanced tumor progression in thioglycolate-treated mice and delayed tumor development in ASA-treated mice (Fig. 2E; Supplementary Fig. S3D). To confirm our findings in an ovarian serous papillary cell line, mice were injected with NIH:OVCAR3 cells and treated with ASA. After 10 weeks, PBS-treated mice (n = 3) had all developed ascites and had significant peritoneal metastases. ASA-treated mice had not developed ascites and had fewer peritoneal implants, indicating a protective effect for ASA in this less aggressive model. Together, these experiments show an association between increased inflammation and enhanced tumor progression, and indicate that treatment with inflammation-modulating agents can alter the progression of ovarian tumors in these models. In addition, the tumor-promoting effects of inflammation in the peritoneal cavity seem to have a greater effect on the dissemination of tumor cells than on primary tumor development.

**Modulation of inflammation alters tumor progression in the presence of adaptive immunity.** Because SCID mice have only innate immunity, we next sought to examine the effects of modulating inflammation in mice with both innate and adaptive immune responses. Using a previously described tumor cell line derived from spontaneously transformed C57Bl6 mouse ovarian surface epithelial cells (1D8; ref. 17), we injected cells into the peritoneal cavity of syngeneic female C57Bl6 mice, as previously described (17). Cells from lavage fluid once again indicated that thioglycolate enhanced inflammation and ASA reduced inflammation in a tumor setting (Fig. 3A). At 8 to 9 weeks after tumor initiation, ascites was present in thioglycolate-treated but not ASA-treated mice. At 9 weeks, thioglycolate-treated mice had a greater volume of ascites and an increased area of diaphragm covered by tumor. The difference between PBS- and ASA-treated mice was not significant, most likely because tumor spread was at an early stage in both groups. To better define any potential difference between these two groups, a similar experiment was carried out in which mice were not killed until 12 weeks after tumor initiation. Significantly greater volumes of ascites were recovered from PBS-treated mice, and the percentage of the diaphragm covered with tumor was also greater than in ASA-treated mice. These results indicate that modulating the activity of innate inflammatory cells can alter tumor progression irrespective of the presence of adaptive immunity.

**Depletion of innate cell populations.** To define the cell types responsible for the tumor-promoting effect of an enhanced inflammatory response, we depleted specific innate immune cell populations in the peritoneal cavity. Because this effect was related to innate immunity, we used the SCID mouse orthotopic xenograft model in these experiments. Specific depletion of neutrophils, NK cells, or macrophages was carried out using standard protocols (as described in Supplemental Methods), and the efficacy of depletion was confirmed by cytologic analysis and flow cytometry of peritoneal lavage fluid (Supplementary Fig. S1). Beige/SCID mice, which have a profound deficit in NK cell activity, were also used in the analysis.

No effect on tumor progression was observed in neutrophil-depleted mice as compared with mice treated with control antibody (rat IgG) or PBS (Table 1). We found increased tumor coverage of the diaphragm in NK cell–depleted mice, but these
values did not reach significance. Macrophage depletion, however, had a profound effect on both primary tumor development and on tumor progression, including diaphragm coverage (Table 1). Only one of these mice developed ascites (0.5 mL) during the experimental period, and all mice in this group had dramatically fewer tumor implants in the peritoneum. Although antibody-mediated depletions could not be carried out in the syngeneic C57Bl6 immunocompetent model due to the extended time frame, clodronate liposomes were administered to these mice to achieve macrophage depletion. Ascites formation was not significantly different between PBS and clodronate-treated mice, but tumor progression was again significantly retarded in macrophage-depleted mice (Table 1). These experiments indicate that macrophages have a profound effect on tumor dissemination in a manner similar to ASA treatment, but in contrast, there is an additional effect on primary tumor development.

**Macrophage depletion leads to decreased VEGF expression.** Immunohistochemical detection of macrophage F4/80 antigen indicated almost complete depletion of macrophages in the ovaries of clodronate-treated mice, and abundant macrophages in the peritoneal cavity of PBS-treated mice (Fig. 4A and B). Previous studies have indicated deficits in vascular density, reticular fiber formation, and MMP-9 production as a result of macrophage depletion in tumors (4, 23). In addition, MMP-9 activity releases VEGF, hence, triggering the angiogenic switch in a tumor (24). In contrast to these studies, we found abundant smooth muscle actin–positive (Fig. 4C and D, insets) and von Willebrand factor–positive (data not shown) vessels in tumors of macrophage-depleted mice. Enumeration of vessels indicated a significant increase in vessel counts in macrophage-depleted mice (38.0 ± 7.0 vessels per section) compared with control mice (10.9 ± 3.2 vessels). No differences in mature collagen fibers, lymphatic vessels, or veins were detected (Masson trichrome–stained sections), or in reticular fiber development (silver-stained sections; data not shown). Slight increases in MMP-9 expression were detected in both the tumors and the lavage fluid of macrophage-depleted mice, as shown by immunohistochemistry (Fig. 4A and B, insets) and zymography (Supplementary Fig. S3E), respectively. Further immunohistochemical analyses were done on cytospin preparations of lavage fluid to identify the cell types expressing MMP-9. Both neutrophils and mesothelial cells in tumor-bearing peritonea were positive for MMP-9 production. As expected, because ovarian tumors secrete VEGF, immunostaining for human VEGF was abundant in tumors. Analysis of human VEGF expression in lavage fluid similarly indicated that VEGF levels reflected the amount of human tumor present, with the highest levels in thioglycolate-treated mice (data not shown). Interestingly, despite abundant MMP-9 expression in the lavage fluid of macrophage-depleted mice, species-specific ELISA indicated decreased levels of mouse VEGF protein compared with control mice (Fig. 4E). ASA-treated mice also had reduced mouse VEGF protein levels, and thioglycolate-treated mice had the highest levels. Therefore, although we were unable to find any stromal deficit to account for decreased primary tumor growth, peritoneal macrophage depletion did result in reduced stromal production of VEGF in the ascites fluid, and this may be associated with decreased tumor spread.

**Discussion**

Ovarian tumors may spread by direct extension to adjacent organs, by exfoliation and implantation on peritoneal surfaces, or by lymphatic dissemination. Many of the mechanisms that are important in the metastases of other tumor types may or may not be relevant to the fluid environment of ovarian and peritoneal cancers. Many recent studies have elucidated the importance of the tumor microenvironment in influencing the malignancy of ovarian tumors (19, 25). Ascites provides for an efficient exchange of soluble factors from inflammatory cells and mesothelial cells to assist in tumor cell growth and invasion (13). The current study was designed to determine whether inflammatory responses that accompany the dissemination of ovarian tumors play a role in facilitating tumor progression. Our *in vivo* approach allowed us to determine the effect of modulating inflammation in a controlled and physiologically relevant environment. We found that innate immunity plays an important role in facilitating human ovarian tumor dissemination in the mouse peritoneum and that macrophages largely accounted for this prometastatic effect.

Many studies show a role for inflammation in tumor initiation and promotion, although effects on metastasis are less clear. Here, we sought to specifically determine whether inflammation affects the dissemination, rather than the incidence, of established tumors in the ovary. ASA reduces inflammation by inhibition of COX-1 and COX-2, muted tumor necrosis factor α production, prevention of

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**Table 1. Tumor progression in mice with depletion of innate immune cell populations**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment/mutation</th>
<th>Ascites volume (mL)</th>
<th>Ovary weight (g)</th>
<th>Tumor metastasis</th>
<th>Diaphragmatic coverage (%)</th>
<th>n</th>
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<tr>
<td>SCID*</td>
<td>PBS</td>
<td>1.43 ± 0.27</td>
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<td>+++</td>
<td>33.13 ± 6.9</td>
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<td></td>
<td>Control antibody</td>
<td>1.65 ± 0.32</td>
<td>0.42 ± 0.04</td>
<td>+++</td>
<td>35.43 ± 9.7</td>
<td>11</td>
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<td></td>
<td>PBS liposomes</td>
<td>1.20 ± 0.75</td>
<td>0.20 ± 0.05</td>
<td>+++</td>
<td>32.40 ± 8.4</td>
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<td>Neutrophil depletion</td>
<td>1.50 ± 0.35</td>
<td>0.34 ± 0.02</td>
<td>+++</td>
<td>41.33 ± 16.9</td>
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<td></td>
<td>NK cell depletion</td>
<td>0.91 ± 0.28</td>
<td>0.31 ± 0.03</td>
<td>+++</td>
<td>51.66 ± 12.5</td>
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</tr>
<tr>
<td></td>
<td>PBS (beige mice)</td>
<td>1.03 ± 0.27</td>
<td>0.42 ± 0.04</td>
<td>+++</td>
<td>42.17 ± 8.7</td>
<td>8</td>
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<td>Macrophage depletion</td>
<td>0.50 ± 0.18</td>
<td>0.13 ± 0.03</td>
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<tr>
<td>C57Bl6†</td>
<td>PBS</td>
<td>0.90 ± 0.32</td>
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<td>++</td>
<td>27.00 ± 13.1</td>
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<td>N/A</td>
<td>+</td>
<td>3.51 ± 1.06†</td>
<td>4</td>
</tr>
</tbody>
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*SCID mice were orthotopically implanted with human ES-2 cells and sacrificed after 21 d.

†P < 0.05.

†C57Bl6 mice were implanted with syngeneic ID8 cells and sacrificed after 9 wks.
inflammatory cell activation, and increased suppressor of cytokine signaling-2 expression (26). Suppression of inflammation with ASA delayed all aspects of tumor progression, including the development of ascites and the formation of peritoneal implants and metastases. In vitro studies showed that ASA did not cause apoptosis of ovarian tumor cells unless supraphysiologic doses were used, as reported by others (20, 27). Such doses are unattainable clinically and induce apoptosis via unknown COX-2–independent mechanisms (27). Additionally, the absence of COX-2 receptors on most human ovarian patient tumors and cell lines, including ES-2, makes direct tumor cell–suppressive effects of ASA unlikely even at physiologic doses (21, 22, 28). Instead, ASA seems to be altering tumor progression through its known suppression of innate inflammatory cell activity. Similarly, thioglycolate dramatically altered inflammation and tumor outcome in our model without altering tumor cell growth in vitro. The inflammatory cell–enriched environment in thioglycolate-treated mice enhanced the dissemination of ovarian tumors. Previous studies have shown the increased growth of s.c. tumors and lung metastasis in mice pretreated with thioglycolate, and the effect was attributed to inflammatory cells (29). Interestingly, we did not find that modulating inflammation affected primary tumor growth, despite the systemic effects of inflammation modulation. Nor did treatment in the early phase of disease progression alter subsequent tumor dissemination, indicating that the protumor effects of thioglycolate treatment occurred when tumor cells were in contact with the peritoneal inflammatory cells.

The tumor-promoting effects of inflammation were seen in C57BL6 mice given syngeneic tumors despite the presence of adaptive immunity. This may indicate local suppression of lymphocytes by tumor cells via secreted factors, such as IL-10, IL-23, and HSP10 (30, 31), and the recruitment of immunosuppressive cells (32, 33). Considering the lymphocyte suppression shown by others, and our results indicating that tumor outcomes are unrelated to the presence of adaptive immunity, innate immune cells may play a greater role in early ovarian cancer progression than lymphocytes. Of the innate immune populations studied, neither neutrophil nor NK cell depletion significantly affected either ovarian tumor growth or peritoneal metastasis.

Macrophage depletion had a profound suppressive effect on both primary tumor development and tumor progression in our model. ASA treatment, however, did not affect primary tumor

Figure 4. Macrophage depletion leads to decreased stromal VEGF expression. A and B, immunohistochemistry for macrophages (anti-F4/80; Nova Red substrate) in ovarian tumors of PBS-treated (A) and clodronate-treated (macrophage-depleted) mice (B). Insets, similar MMP-9 immunostaining (brown, 3,3′-diaminobenzidine) in both groups of mice despite the difference in macrophage numbers (A and B). Immunohistochemistry for human VEGF (Nova Red) indicated high levels in both PBS-treated (C) and macrophage-depleted (D) mice. Insets, slightly elevated levels of smooth muscle actin–positive vessels (pink, Fast Red) in macrophage-depleted mice (C and D). E, mouse VEGF expression is decreased in ASA-treated and macrophage-depleted SCID mice after 2 and 3 wks, as assessed by species-specific ELISA. Inset, mouse VEGF levels in C57Bl6 mice 9 wks after i.p. implantation of ID8 tumor cells (E).
development, and this difference may reflect that ASA has broader effects than macrophage depletion, and that it less effectively ablates macrophage activity. The effect on primary tumor development after peritoneal macrophage depletion was unexpected. However, external penetrance of the ovary by clodronate-containing liposomes has previously been shown by others (34), and likely accounts for macrophage depletion in ovarian tumors in our study and subsequent impaired tumor growth. Nonetheless, reduced tumor dissemination in macrophage-depleted mice could not have been solely due to decreased primary tumor growth because the effect was also seen in the experimental metastasis model.

Despite well-established tumoricidal activities, good correlations exist between macrophage density and poor prognosis in many tumor types, including breast, cervix, and bladder cancer. There are many mechanisms by which macrophages may contribute to tumor progression. Ovarian tumor macrophage products include growth, angiogenic, and survival factors such as IL-6 and IL-8, and VEGF-C, which promotes lymphatic metastasis (19, 35). Differences in macrophage behavior may result from differing activation stimuli. Classic activation by microbial products or IFN-γ generates M1-type macrophages with activities such as cell lysis and antigen presentation. Alternate activation by IL-4, IL-10, and IL-13 induces M2 type macrophages, the predominant type found in neoplasias, with enhanced tissue remodeling and debris scavenging activity (36). In vitro coculture of macrophages with ovarian tumor cells results in the induction of a characteristic M2 phenotype, including enhanced production of cytokines and expression of the scavenger receptor (37). At least five distinct subpopulations of macrophages have been identified in malignant ascites, and both protumor and antitumor properties have been identified (5, 11). Our studies showed an overall protumor effect for macrophages in tumor cell dissemination.

The effect of macrophage deficiency on tumor development has previously been investigated in mice lacking colony-stimulating factor 1 (CSF-1). CSF-1 is a major regulator of macrophage activity, and most macrophage populations are absent in lesions of CSF-1 null mice. These mice show delayed s.c. tumor growth and impaired PyMT-induced breast cancer metastasis (23, 38). Reduced vascularity and collagenous fibers in the tumors of CSF-1–null mice indicated inadequate tumor stroma formation, and resulted in increased necrosis and a lower mitotic index (23). We were unable to detect any stromal deficit in tumors of macrophage-depleted mice, including mature or immature collagen fibers, vascular development, or necrosis. Microvascular density was actually increased in the tumors of these mice, a finding consistent with many patient studies that revealed negative correlations between macrophage density and vascularity in ovarian tumors, and positive correlations between microvascular density and increased progression-free survival (15, 39–41). A dissociation between angiogenesis and tumor growth has been shown in other contexts (42), and indicates the complexity of the relationship between the tumor stroma and angiogenesis. Despite the increased vascularity of tumors, other macrophage properties, such as destruction of extracellular matrix or release of tumor stimulatory factors, are evidently required for tumor progression. Specifically, Hagemann et al. have shown that coculture of ovarian tumor cells with macrophages increased tumor cell nuclear factor κB and c-Jun NH2-kinase signaling (43). Our study provides in vivo validation of this protumor effect for macrophages in ovarian cancer progression, and the magnitude of the effect that we observed suggests that the role of macrophages in tumor cell signaling could be important.

The macrophage products MMP-9 and VEGF have previously been implicated in ovarian tumor progression (4, 44). However, we did not observe differences between macrophage-depleted and control mice in MMP-9 production or activity due to neutrophil and mesothelial cell production. Tumor cell VEGF production has previously been shown to be augmented by macrophage products (45) and may account for reduced human VEGF in macrophage-depleted mice. Tumor production of (human) VEGF in the ascites fluid reflected tumor burden and may be an indirect effect of macrophages enhancing the number of peritoneal tumor cells or up-regulation of their VEGF production. Solid tumors of both groups of mice contained abundant VEGF. Peritoneal macrophages are apparently major contributors to VEGF production in our model, and reduced levels of host (mouse) VEGF in the ascites of macrophage-depleted mice may suggest a mechanism for the reduced tumor dissemination. VEGF production has been shown to induce ascites production, tumor angiogenesis, and metastasis (46–50), and it is reasonable to assume that reduced VEGF levels would at least in part contribute to reduced tumor dissemination in macrophage-depleted mice.

These studies provide the first in vivo evidence of a role for inflammation in human ovarian tumor dissemination using a mouse model of spontaneous metastasis. The results suggest that macrophages and their products are logical targets for new antitumor therapies. Further investigations to identify appropriate targets and effective strategies are currently under way. Such interventions could complement traditional chemotherapy by focusing on agents that target the stromal microenvironment in addition to those that directly target tumor cells.

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