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ROLE OF MACROPHAGES IN ADAPTIVE RESISTANCE TO ANTI-VEGF THERAPY

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ROLE OF MACROPHAGES IN ADAPTIVE RESISTANCE TO ANTI-VEGF THERAPY

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ROLE OF MACROPHAGES IN ADAPTIVE RESISTANCE TO ANTI-VEGF THERAPY

A

THESIS

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The University of Texas
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Graduate School of Biomedical Sciences
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for the Degree of

MASTER OF SCIENCE

by

Heather J. Dalton, M.D.
Houston, Texas

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Acknowledgements

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ROLE OF MACROPHAGES IN ADAPTIVE RESISTANCE TO ANTI-VEGF THERAPY

Heather J. Dalton, M.D.

Advisory Professor: Anil K. Sood, M.D.

**Background:** The clinical implementation of therapies targeting the VEGF pathway in cancer has been limited by acquired resistance; yet, the mechanisms by which this occurs is unclear. We investigated the role of macrophages in the development of acquired resistance to anti-VEGF antibody (AVA) therapy.

**Materials and Methods:** We first established a murine ovarian cancer model of resistance to anti-VEGF therapy. Using this model we investigated changes in macrophage infiltration during AVA sensitive and resistant phases. We also investigated the *in vivo* effects of macrophage depletion at the emergence of anti-VEGF resistance and in upfront combination with AVA therapy. *In vitro*, we assessed differences in viability and invasion/migration in AVA sensitive and resistant macrophages. We also investigated macrophage VEGF receptor expression in response to AVA therapy. Finally, we performed high throughput analyses to determine pathways important in modulating macrophage response to AVA.

**Results:** We show that macrophages are actively recruited to the tumor microenvironment, where their accumulation correlates with the emergence of anti-VEGF resistance. Importantly, depletion of macrophages at the emergence of anti-VEGF resistance halts tumor growth and significantly prolongs survival in murine models. Additionally, the upfront combination of anti-VEGF therapy with macrophage depletion is synergistic, decreasing tumor growth *in vivo*. We found
downregulation of macrophage VEGFR-1 expression in conjunction with upregulation of alternative angiogenic and anti-apoptotic pathways at the emergence of resistance, possibly facilitating escape from VEGF-directed therapies.

**Conclusions:** After establishing murine ovarian cancer models of anti-VEGF resistance, we demonstrate a previously unrecognized role for macrophages in adaptive resistance to anti-VEGF therapy. Depletion of macrophages restores sensitivity to AVA therapy and reduces tumor growth in combination with VEGF blockade. Collectively, this study highlights macrophages as catalysts in the development of anti-VEGF resistance and offers strategies to modulate the influence of macrophages, thus improving the effectiveness of anti-VEGF therapy.
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Background and Introduction

Epithelial ovarian cancer

In 2014, an estimated 14,270 new cases of epithelial ovarian cancer will be diagnosed, while approximately 21,980 women will die of this disease, making it the fifth leading cause of cancer death among women and the most lethal gynecological malignancy (1). Improvements in surgical approaches and the utilization of platinum and taxane-based cytotoxic agents has resulted in a 1.6 year gain in life expectancy over the last thirty years (2, 3). Despite these improvements and an initial response to chemotherapy in up to 80% of patients with advanced disease, the majority of these women will relapse after first-line treatment and eventually succumb to their disease (4, 5).

Treatment options following recurrence often depend on the elapsed interval from when the patient last received platinum-based therapy. In patients with platinum-resistant disease (i.e., cancer recurrence or progression within 6 months after receiving platinum therapy), single-agent chemotherapy is most commonly recommended. Such agents include docetaxel, topotecan, pegylated liposomal doxorubicin, gemcitabine, or weekly paclitaxel (6). Response to these second-line treatments has yielded disappointing results of approximately 20% (7-10).

These findings highlight the need for more efficacious chemotherapy regimens in both the up-front and recurrent settings. Increasingly, attention has been focused on targeting the biological pathways that fuel ovarian cancer growth. One attractive strategy is directed at targeting tumor blood vessel growth, a process known as anti-angiogenesis therapy (6).
Angiogenesis and tumor growth

Angiogenesis is a central hallmark of cancer and is essential for tumor growth and metastasis (11). While the mechanisms regulating blood vessel formation in cancer are complex, the vascular endothelial growth factor (VEGF) family is known to be a predominate pathway (12). VEGF (VEGF-A) interacts with the tyrosine receptor kinase, VEGF Receptor-2 (VEGFR-2), and is the central promoter of tumor angiogenesis (13, 14). VEGF also has known angiocrine and intracrine functions, where it has been shown to modulate cancer cell survival (15, 16). Cofactors Neuropilin 1 and 2 (NRP1, NRP2), potentiate the activity of VEGFR-2 and can also signal independently. VEGF can exist as both soluble and matrix-bound isoforms, with the former controlling vessel enlargement and the later regulating branching morphogenesis (17).

VEGF-C is another member of the VEGF family capable of interaction with both VEGFR-2 and VEGFR-3. VEGF-C regulates the activation of blood vessel tip cells (18). VEGFR-3 is essential for blood vessel formation during embryogenesis and later plays important roles in the regulation of lymphangiogenesis (19). Importantly, VEGFR-3 can augment VEGF-induced angiogenesis and sustains blood vessel growth, even in the presence of VEGFR-2 inhibitors (19).

VEGF Receptor-1 (VEGFR-1) interacts with VEGF-B and also binds to VEGF with high affinity. VEGFR-1 may serve as an alternative receptor for VEGF, thereby regulating the amount of VEGF available to activate VEGFR-2. In support of this concept, loss of VEGFR-1 results in vessel overgrowth (20). VEGFR-1 also plays roles in the pathologic angiogenesis seen in tumor growth. Tumor cells are known
to express VEGFR-1, which may facilitate VEGF interaction through autocrine mechanisms (21). Additionally, VEGFR-1 is capable of inducing the growth of VEGFR-1-expressing tumor cells (22).

Placental growth factor (PlGF) is another member of the VEGF family, acting as a cytokine to stimulate angiogenesis. PlGF activates bone-marrow derived myeloid cells and endothelial progenitors, in addition to directly activating tumor cells (20). PlGF can also directly bind to VEGFR-1 (17).

In addition to the VEGF family, other factors make substantial contributions to aberrant angiogenesis, including the notch-deltalike ligand 4 (Dll4) pathway. Vascular endothelial cells express the notch 1 and 4 receptors, as well as the ligands jagged 1, Dll1, and Dll4. This pathway has also been implicated in the establishment of a perivascular niche for colon cancer stem cells by endothelial cells (23). Notch-Dll4 signaling is critical for embryonic angiogenesis, as its haploinsufficiency in knock-out experiments is lethal (24). Dll4 is upregulated in tumor vasculature, in part by VEGF, which may allow it to serve as a negative feedback mechanism for sustained angiogenesis (25).

The angiopoietin-Tie pathway also plays roles in tumor angiogenesis. Tie-1 and Tie-2 are tyrosine kinases predominantly found on vascular endothelium and serve as receptors for the ligands, ANG-1, ANG-2, and ANG-4. ANG-1 works through Tie-2 to regulate endothelial cell quiescence and vessel tightness (26). In response to angiogenic stimuli, sprouting endothelial cells release ANG-2, which antagonizes the activity of ANG-1 and Tie-2 and increases vascular permeability and vessel sprouting (27). In cooperation with VEGF, ANG-2 works to stabilize and
mature new capillaries (25). ANG-2 is also released by tumor cells, resulting in the recruitment of pro-angiogenic Tie-2 expressing monocytes and macrophages (28).

Therapies targeting angiogenic pathways in cancer

Given the importance of angiogenesis in tumor growth, therapies targeting VEGF and other pro-angiogenic pathways have been the subject of intense investigation. The first targeted anti-angiogenic agent was approved by the Food and Drug Administration (FDA) in 2004. Bevacizumab, a humanized monoclonal VEGF antibody, demonstrated activity in metastatic colorectal cancer in combination with standard chemotherapy, resulting in a survival benefit in a randomized phase 3 trial (29). Bevacizumab was subsequently approved by the FDA for the treatment of advanced non-small cell lung cancer, renal cell carcinoma, and recurrent glioblastoma (30). This agent was also initially approved for the treatment of metastatic breast cancer; however, this approval was withdrawn in 2011 after failing to improve overall survival. Bevacizumab has also been use in both the up-front and recurrent ovarian cancer settings, with improvement in progression-free survival, which may be reasonable endpoint given the prolonged survival of these patients following progression (31). In the setting of recurrent disease, historical response rates to bevacizumab as monotherapy range from 16% to 21%. Combination with other chemotherapeutic agents increases response rates to 24-31% (32).

Several small-molecule receptor-tyrosine-kinase inhibitors (RKTIs) have been approved as targeted anti-angiogenic agents. Sunitinib targets multiple tyrosine kinases, including VEGFR-1, VEGFR-2, Platelet-derived growth factor
receptors (PDGFRs), and macrophage colony stimulating factor receptor (CSF1R), with demonstrated activity in pancreatic neuroendocrine tumors, advanced renal cell carcinoma, and gastrointestinal stromal tumors (25, 33). Sorafenib is FDA-approved for renal cell carcinoma, hepatocellular carcinoma, and differentiated thyroid cancer and inhibits VEGFR-1, VEGFR-2, and VEGFR-3, as well as PDGFR-β (34). Alternative methods to target VEGF include aflibercept, a fusion protein composed of the VEGFR-1 and VEGFR-2 extracellular domains with high affinity for VEGF-A, VEGF-B, and PIGF(30). Compounds directed at the ANG-TIE pathway are also in development.
These graphs show a 30 year period of ovarian cancer incidence and mortality. On left you can see a slight decrease in the overall incidence of ovarian cancer. Improvements in surgical approaches and the utilization of platinum and taxane-based cytotoxic agents has resulted in a 1.6 year gain in life expectancy over the last thirty years. Despite these improvements and an initial response to chemotherapy in up to 80% of patients with advanced disease, the majority of these women will relapse after first-line treatment and eventually succumb to their disease. Thus there is a continued impetus for new treatment options, including the development of targeted agents.

Angiogenesis is a central hallmark of cancer and is essential for tumor growth and metastasis. While the mechanisms regulating blood vessel formation in cancer are complex, the vascular endothelial growth factor (VEGF) family has been found to be a predominate pathway. This family of glycoproteins and type III tyrosine kinases and associated molecules are shown on the figure.

**VEGF (VEGF-A)** binds to VEGFR-1 and VEGFR2
- Endothelial cell proliferation
- Migration, invasion, and survival

**VEGF-B** binds to VEGFR1
- Blood vessel survival factor

**VEGF-C** binds to VEGFR2 and VEGFR3
- Angiogenesis, lymphangiogenesis and vascular permeability

**VEGF-D** binds to VEGFR2 and VEGFR3
- Angiogenesis, lymphangiogenesis and vascular permeability

Bevacizumab, a humanized monoclonal VEGF antibody, demonstrated activity in metastatic colorectal cancer in combination with standard chemotherapy, resulting in a survival benefit in a randomized phase 3 trial. Bevacizumab was subsequently approved by the FDA for the treatment of advanced non-small cell lung cancer, renal cell carcinoma, and recurrent glioblastoma. This agent was also initially approved for the treatment of metastatic breast cancer; however, this approval was withdrawn in 2011 after failing to improve overall survival. Bevacizumab has also been used in both the up-front and recurrent ovarian cancer settings, with improvement in progression-free survival.

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As angiogenesis is a critical component for tumor growth and VEGF is constitutively overexpressed in numerous cancers, therapies targeting this pathway were eagerly anticipated additions to standard treatment.
In ovarian cancer, numerous agents targeting angiogenesis are being investigated. AMG 386 is an inhibitor of ANG-1 and ANG-2 with activity in recurrent ovarian cancer currently in clinical trials. Others include the multi-kinase inhibitor, cediranib, which targets VEGFR-1, -2, and -3, PDGFR-α/β, FGFR-1, and c-kit; and nintedanib, directed against VEGFR-1, -2, and -3, PDGFR-α/β, FGFR-1, -2, and -3, as well as the sarcoma viral oncogene homolog (Src) family. Pazopanib, targeting VEGFR-1, -2, and -3, PDGFR-α/β, FGFR-1 and -3, and c-kit; and sorafenib are also being evaluated (5).

**Adaptive resistance to anti-angiogenic therapies**

As angiogenesis is a critical component for tumor growth and VEGF is constitutively overexpressed in numerous cancers, therapies targeting this pathway were eagerly anticipated additions to standard chemotherapy (35, 36). Yet, clinical survival benefits have been modest, usually measured in months (25). Resistance to angiogenic blockade often develops (17). Rebound tumor growth, along with rapid revascularization following termination of anti-angiogenic therapies, has been demonstrated (37, 38). Intriguingly, collapse of survival curves and clinical benefit is observed following cessation of agents such as bevacizumab (39-41). Several mechanisms for this phenomenon have been proposed. VEGF blockade produces hypoxia at the tumor level, which can increase the production of alternative pro-angiogenic factors. Tumors may also utilize other methods of vascularization, such as vasculogenic mimicry or vessel co-option. Further, stromal components of the tumor microenvironment, including macrophages, may offer alternative angiogenic avenues in the setting of VEGF blockade (17). T-helper type 17 cells, with their
major cytokine IL-17, have been implicated in such mechanisms of resistance to anti-VEGF therapy through induction of G-CSF and recruitment of immature myeloid cells, including macrophages (42).

**Characterization of Macrophages**

Macrophages arise from differentiated monocytes, which enter the circulation after development from a myeloid progenitor in the bone-marrow. The transcriptome of macrophages is complex, allowing them to serve diverse and often tissue-specific functions (43). Historically, macrophages have been described as classically activated M1 or alternatively activated M2 phenotypes. These phenotypes are now thought to represent extremes in a continuum of macrophage activation, and have been reversed, highlighting their plasticity in response to environmental signals (44-46).

Pro-inflammatory macrophages, or the classical M1 phenotype, react to STAT1 signaling pathways through response to INF-γ and activation of Toll-like receptors (TLRs) (47). TNFα, IL-1β, IL-6, IL-12, type 1 INF, as well as reactive oxygen species contribute to the pro-inflammatory properties of this phenotype (Table 1) (48). Arginine metabolism leads to inducible nitric oxide production. Major histocompatibility complex II (MHC II) is elevated, allowing interactions with Th1 cells (49). STAT3 and STAT6 are activated by IL-4 and IL-13, with subsequent transcription of characteristic genes of pro-angiogenic macrophages, (50). The arginase pathway results in the production of ornithine and polyamines (51). Characteristic features are seen in Table 1.
**Table 1.** Associated chemokines and cytokines of macrophage phenotypes.

<table>
<thead>
<tr>
<th></th>
<th><strong>Pro-inflammatory Macrophages</strong></th>
<th><strong>Pro-angiogenic Macrophages</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines and Chemokines</strong></td>
<td>CCL3, CCL4, CCL5, CCL8, CXCL9, CXCL10, CXCL11</td>
<td>CCL2, CCL16, CCL17, CCL18, CCL22, CCL24, CXCL1, CXCL2</td>
</tr>
<tr>
<td><strong>Other factors</strong></td>
<td>TNF-α, IL-1β, IL-6, IL-12, INF, Reactive O₂ species</td>
<td>MMPs, IL-8, VEGF-A, VEGF-C, VEGF-D, FGF-2, ARG-1, Wnt5a, Wnt7b</td>
</tr>
<tr>
<td><strong>Surface Receptors</strong></td>
<td>CCR7, CD14, CD16, CD32, CD64, CD84, CD86, MHCII, TRL2/TRL4, CSF1-R</td>
<td>CD14, CD23, CD163, CXCR1, CXCR2, CXCR4, CCR2, IL-1Ra, VEGFR-1, VEGFR-2, VEGFR-3, Tie-2, mannose receptor</td>
</tr>
</tbody>
</table>

**Macrophages in tumor angiogenesis**

Tumor associated macrophages (TAMs) are abundant in established tumors, where they were initially thought to be tumoricidal. Contemporary evidence, however, demonstrates a much more sinister purpose. Macrophage infiltration is associated with increased tumor invasion, migration and poor clinical prognosis in 80% of solid tumors (46, 52). Tumor and stromal cells produce numerous factors that actively recruit macrophages to the microenvironment, including VEGF (47, 53). Tumors are also rich in hypoxia, a known macrophage chemoattractant. While most macrophages influxing into the cancerous microenvironment are believed to originate from the circulating monocyte population and are, therefore, bone-marrow derived, recent studies demonstrate the ability of resident tissue macrophages to proliferate. Proliferation of resident tissue macrophages has been described as the dominant mechanism for the establishment of peritoneal macrophages in the postnatal period. Specifically, in the context of inflammation, both resident peritoneal macrophages and recruited bone-marrow derived macrophages have
been demonstrated to proliferate in the microenvironment, adding new complexity to our previous understandings of macrophage differentiation (54). This proliferative ability has recently been linked to the transcription factor Gata6 (55).

Once in the tumor microenvironment, macrophages secrete numerous pro-angiogenic factors including VEGF-A, VEGF-C, VEGF-D, IL-8, and FGF-2; which help flip the angiogenic switch regulating the transformation to malignancy (21, 46, 56). Increased capillary density is correlated with TAM infiltration (21). Macrophages also express VEGFR-1, VEGFR-2, and VEGFR-3, permitting interaction with ligands of the VEGF family (56, 57). TAMs are transcriptionally similar to the pro-angiogenic macrophage phenotype, with high levels of IL-8, TGF-β, ARG-1 and the mannose receptor in conjunction with low levels of pro-inflammatory cytokines (53, 58). In response to hypoxia, macrophages upregulate HIF-1α and HIF-2α, which facilitate the transcription of genes involved in angiogenesis (53). Tie2 is also upregulated, enhancing pro-angiogenic polarization.

Response to anti-cancer therapies can be altered by TAMs. Radiotherapy induces CSF1, leading to recruitment of CSF1R-expressing macrophages and enhanced tumor regrowth via their associated pro-angiogenic properties, while CSF1R inhibitors have resulted in improved response to radiation (59). Tumor regrowth following radiotherapy is linked to macrophage recruitment by CXL12 in response to hypoxia. Platinum therapy increases pro-angiogenic macrophages in tumor samples (60). Further, macrophages are associated with the development of resistance to anti-tumor therapies, including platinum-based chemotherapy and radiotherapy (59-61). The development of resistance to anti-VEGF therapy has
been linked to macrophages, secondary to their ability to activate pro-angiogenic pathways (62). Macrophages are also directly tied to vascular regrowth following therapy-induced vascular injury (61).

**Mechanisms to target macrophages**

Numerous methods of targeting macrophage-driven angiogenesis are currently being investigated. Bisphosphonates, used clinically for the treatment of osteoporosis and bony metastases, are one potential approach. Multiple large-scale studies have demonstrated the reduced risk of breast and colon cancers in patients receiving bisphosphonates (46, 63). Bisphosphonates reduce bone metastasis in breast cancer patients, and in those with prostate or renal cell carcinoma and pre-existing bone metastasis, result in a trend towards increased survival (64). Bisphosphonates work by inhibiting of osteoclast activity, which contributes to the growth of solid tumors by liberating bone marrow-derived growth factors such as TGF-β and IGF. Importantly, they also have been found to directly induce apoptosis in TAMS with a resultant decrease in tumor infiltration and associated pro-angiogenic factors that aid tumor growth and spread. This apoptotic effect is mediated through the inhibition of farnesyl diphosphate synthase, which prevents prenylation of small GTPase signaling proteins required for normal cellular function (65). Bisphosphonate treatment has also significantly reduced angiogenesis in several murine cancer models (66).

The DNA binding agent, trabectedin, has activity against macrophages. Treatment with trabectedin reduced TAM infiltration, resulting in significantly reduced tumor growth and metastasis, as well as decreased angiogenesis (67).
These effects are mediated through induction of the extrinsic apoptosis pathway, with specificity for cells of monocyctic lineage. The expression of functional TRAIL receptors seen on monocytes and TAMs is responsible for this specificity, as neutrophils and lymphocytes have decoy TRAIL receptors, which impart protection from the effects of trabectedin (67).

Inhibition of the CSF1 receptor (CSF1R) is also being investigated, as its ligand, CSF1, is a potent recruiter of macrophages and monocytes and is unregulated in the tumor microenvironment. In prostate cancer models, the CSF1R inhibitor, GW2580, reduced tumor regrowth following irradiation and reduced infiltration of TAMs (68). This selective small kinase inhibitor competitively binds to CSF1R, thereby preventing CSF-1-dependent macrophage growth (69). Reduced TAM recruitment and decreased vascular density was seen in breast cancer models, along with reduced tumor growth in prostate cancer models following treatment with the small molecule CSF1R kinase inhibitor, PLX3397. Additionally, treatment with PLX3397 enhanced CD8\(^+\) T cell response, resulting in improved chemosensitivity (70). AC708 is another high-affinity CSF1R inhibitor with demonstrated activity in breast cancer models currently in clinical development (work yet unpublished, poster presented at AACR 2013).

PF-04136309, a CCR2 inhibitor, depletes TAMs, reduces metastasis, and enhances chemosensitivity in pancreatic cancer models (71). Anti-STAT3 agents offer additional possibilities for macrophage modulation (71). Anti-Ang2 antibodies produced regression of tumor vasculature and decreased tumor progression in murine models of pancreatic and breast cancer (62).
Macrophage reprogramming is being explored through the PD-1 (programmed death-1) pathway. This inhibitory factor is secreted by macrophages in the tumor microenvironment, with a subsequent reduction in CD8\(^+\) cytotoxic T cell activity and induced immune tolerance. In an ovarian cancer model, PD-L1 blockade, in combination with whole tumor antigen vaccination, increased immune activity and facilitated tumor rejection through stimulation of CD8\(^+\) T cells (46, 72). This tumoricidal macrophage phenotype is achieved through administration of an agonistic CD40 antibody for “priming,” followed by a “triggering” signal mediated through toll-like receptors (TLRs). This method of macrophage reprogramming has produced tumor regression in vivo (73).

Tumor cells express “don’t eat me” signals through expression of CD47. This surface receptor interacts with signal regulatory protein \(\alpha\) (SIRP\(\alpha\)) on the surface of TAMs, inducing a powerful anti-phagocytosis signal. CD47 inhibition increases the phagocytic capability of macrophages with resultant tumoricidal response. These anti-CD47 antibody therapies are currently in clinical development (73).

Macrophage-derived exosomes are also being utilized to achieve targeted drug delivery to the tumor microenvironment. These endogenous nanovesicles are capable of transferring biological information between cells. Recently, macrophages have been broken down into nanovesicles mimicking exosomes, with retention of plasma membrane proteins, thus maintaining the inherent targeting ability of the original macrophage. These macrophage-derived nanovesicles have been loaded with various chemotherapeutic agents and demonstrated to track to the tumor
microenvironment *in vivo*, resulting in decreased tumor growth without the adverse effects associated with administration of free drug (74).

The ability of macrophages to directly affect angiogenesis prompted us to specifically consider the role of macrophages in adaptive resistance to VEGF blockade. Given the clinical significance of adaptive resistance to anti-VEGF therapy, the biological roles and underlying mechanisms by which macrophages contribute to adaptive resistance to anti-VEGF therapy are the focus of the present study.
Hypotheses and Specific Aims

The overall hypotheses of this project are:

1) Depletion of macrophages in the tumor microenvironment will alter adaptive tumor responses and improve the efficacy of anti-VEGF therapy.

2) Prolonged exposure to anti-VEGF therapy results in down-regulation of VEGF receptors on macrophages, allowing them to persist in the microenvironment and aid tumor growth through the release of pro-angiogenic factors.

These hypotheses can be pictorially unified into a central hypothesis, seen in Figure 1. Tumors are initially responsive to anti-VEGF therapy, where treatment is associated with a reduction both macrophage infiltration and tumor growth. With prolonged treatment and the development of resistance, macrophage infiltration increases, with a reduction in VEGFR expression. During this phase, tumor growth rapidly increases through the liberation of other macrophage-derived pro-angiogenic factors. The following specific aims will test this hypothesis:

**Specific Aim 1:** To determine the biological role of macrophages in adaptive resistance to anti-VEGF therapy

**Specific Aim 2:** To identify the mechanisms by which macrophages contribute to adaptive resistance to anti-VEGF therapy.
Figure 1. Central Hypothesis. VEGFR-expressing (green) and VEGFR-negative (red) macrophages are found in the tumor microenvironment. The initiation of anti-VEGF therapy reduces both tumor size and macrophage infiltration. In this phase, macrophages expressing VEGFR predominate. With continued anti-VEGF therapy, resistance emerges and VEGFR-negative macrophages are found in abundance in the microenvironment.
Methods

Cell lines and tissue culture

IG10 cells were maintained in DMEM-F12 supplemented with 5% fetal bovine serum, 1x insulin-transferrin-sodium selenite supplement (Roche Diagnostics, Indianapolis, IN), and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). OVCAR5 was maintained in DMEM with 10% fetal bovine serum, and 0.1% gentamicin sulfate. SKOV3ip1 was maintained in RPMI 1640 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate. All cell lines were routinely screened for mycoplasma and experiments were performed at 60-80% cell confluence.

Immortomouse macrophages

Immortomouse macrophages, a kind gift from Dr. Robert Langley, were maintained in DMEM with 10% fetal bovine serum, and 0.1% gentamicin sulfate. These conditionally immortalized cells are derived from the immortomouse (Jackson Laboratory, Bar Harbor, ME) and bear a transgene which allows interferon-inducible expression of a thermolabile large tumor antigen (TAg) (and the small tumor antigen) from the SV40 thermosensitive A58 (tsA58) strain directed to widespread tissues by the interferon-inducible Class I antigen promoter from the mouse H-2Kb locus. The tsA58 TAg gene product is functional at the 33°C, but is rapidly degraded at 39.5°C (75). In this way, immortomouse macrophages could be cultured at 33°C, where they proliferate as an immortalized cell line, but fail to proliferate after incubation at 39.5°C.

Animal studies
All animal work was done in accordance with protocols approved by the MD Anderson Institutional Animal Care and Use Committee. Female athymic nude mice and immune competent (C57BL/6) mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). All animals were cared for in accordance to the guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the US Public Health Service policy on Human Care and Use. All animals used were 8-12 weeks old at the time of injection.

**In vivo model of ovarian cancer and tissue processing**

For all animal experiments, cells were harvested using trypsin-EDTA, neutralized with FBS-containing media, washed, and re-suspended to the appropriate cell number in HBSS prior to injection. IG10 (1x10^6) cells were transduced with lentivirus-encoding luciferase and injected into C57BL/6 mice. Mice were imaged once weekly for luminescent signals using a Xenogen IVIS system. For syngeneic experiments, B20 mAb, a murine monoclonal VEGF-A and VEGFR-2 antibody (Genentech Inc, San Francisco, CA) was administered intraperitoneally at 5mg/kg, twice weekly. For nude models, bevacizumab was given intraperitoneally at 6.25 mg/kg, twice per week. Zoledronic acid was given intraperitoneally at 1 mg/kg, once weekly. At the time of necropsy, the weight, number, and distribution of tumors were recorded. Individuals who performed necropsies were blinded to the treatment group assignments. Tissue specimens were fixed with either formalin or optimal cutting temperature compound (OCT) (Miles, Elkhart, IN), or snap frozen in liquid nitrogen.
Quantitative real-time PCR

The total RNA from either cell lines or tumor tissue was extracted using a Qiagen RNeasy Kit (Qiagen, Valencia, CA). Using 1 µg of RNA, cDNA was synthesized using a Verso cDNA kit (Thermo Scientific, Houston, TX) per the manufacturer’s instructions. cDNA was then subjected to amplification by real-time PCR using specific primer sequences (100 ng/µL) as specified in Table 2. For real-time RT-PCR, we obtained quantitative values (each sample was normalized on the basis of its 18S content) as previously described (76).

Table 2. PCR primer sequences.

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<th>Reverse Sequence</th>
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<td>5’-CTTCACGCACACGCTCTAGA-3’</td>
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<td>VEGFR3</td>
<td>5’-CCCCCGTGTCATACATATA-3’</td>
<td>5’-CTCTGCCTCGGACTCCTC-3’</td>
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Methylation-specific PCR

MethPrimer software (http://www.urogene.org/methprimer/) was used for the prediction of the CpG islands of the murine VEGFR1 promoter regions and for design of methylation-specific primers. CpG islands of the promoter are seen in Figure 2.

Figure 2. VEGFR-1 promoter CpG islands. VEGFR-1 promoter CpG islands are
shown above, as predicted using MethPrimer software. Actual CpG sequences are shown along the bottom as red dashes.

Using this software, appropriate primers were designed (VEGFR1 methylated sense: 5’- GGAGTTTGTAAGGATTGAGC-3’, VEGFR1 methylated antisense: 5’- CGACACCTCCTTCTAATAACGTC-3’, VEGFR1 un-methylated sense: 5’- GGAGTTGTAGGATTTTTTGAGTG-3’, VEGFR1 un-methylated antisense: 5’- CCAACACCTCCTTCTTAATAACATC-3’). Total DNA was isolated from control, AVA sensitive, and AVA resistant immortomouse macrophages cells using Phenol:Chloroform extraction, followed by treatment with bisulphite using a methylation kit (EZ DNA Methylation-Gold; Zymo Research, Orange, CA). Using real-time PCR, as described above, quantification of methylation in AVA resistant samples was compared to AVA sensitive samples.

**Immunoblotting**

For immunoblotting, lysates from cultured cells were prepared using modified RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton, 0.5% deoxycholate) plus 25 µg/mL leupeptin, 10 µg/mL aprotinin, 2 mM EDTA, and 1 mM sodium orthovanadate. Protein concentrations were determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL). Lysates were loaded and separated on 8% sodium dodecyl sulfate—polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane by semidry electrophoresis (Bio-Rad Laboratories, Hercules, CA) overnight, blocked with 5% milk for 1 hour and then incubated at 4°C with primary antibody overnight. After washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)—conjugated horse
anti-mouse IgG (1:2000, GE Healthcare, UK) for 2 hours. HRP was visualized by use of an enhanced chemiluminescence detection kit (Pierce). To confirm equal sample loading, the blots were probed with an antibody specific for beta-Actin (0.1 µg/mL; Sigma). Densitometric analysis was performed using ImageJ.

**Gene Expression Microarray**

Immortalized murine macrophages were treated with AVA for 2 weeks (anti-VEGF sensitive) and 6 weeks (anti-VEGF resistant) then RNA was extracted using mirVana RNA isolation labeling kit (Ambion, Grand Island, NY). Five hundred nanograms of total RNA were used for labeling and hybridization on a MurineWg-6 v2 Beadchip (Illumina, San Diego, CA) according to the manufacturer’s protocol. After the bead chips were scanned with an Illumina BeadArray Reader (Illumina), the microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data (LIMMA) package in the R language environment. The expression level of each gene was transformed into a log2 base before further analysis.

**Reverse Phase Protein Arrays (RPPA)**

Immortalized murine macrophages were treated with AVA for 2 weeks (sensitive) and 6 weeks (resistant). Cells were harvested at 80% confluence and lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholate, 25 µg/mL leupeptin, 10 µg/mL aprotinin, 2 mmol/L EDTA, and 1 mmol/L sodium orthovanadate). RPPA analysis was performed at the University of Texas, M.D. Anderson Cancer Center RPPA Core Facility using the methods described at the following web address:
http://www.mdanderson.org/education-and-research/resources-for-professionals/scientific-resources/core-facilities-and-services/functional-proteomics-rppa-core/index.html. Samples were probed with 161 antibodies by CSA amplification approach and visualized by DAB colorimetric reaction. Slides were scanned on a flatbed scanner to produce a 16-bit TIFF image. Spots from TIFF images were identified and the density was quantified by MicroVigene. Relative protein levels for each sample were determined by interpolation of each dilution curves from the "standard curve" (supercurve) of the slide (antibody). All data presented is in fold-change compared to the baseline (control treatment). Positive fold-change was calculated by dividing each linear value (>1.0) with average control linear value for each antibody tested, while negative fold-change (for linear values <1.0) was also calculated (using the following formula: [-1/linear fold-change]) as in log 2.0 value.

**Cytokine Assay**

Supernatant from cultured control, AVA sensitive and AVA resistant murine macrophages were stored at −20°C for batch analyses to measure cytokines. Supernatants were evaluated for cytokines/chemokines using the Milliplex MAP murine cytokine/chemokine panel (Millipore, MA). Cytokine levels were measured in 50 µL of supernatant by Multiplex cytometric bead array (Multiplex) assay on a Luminex 100 Analyzer (Luminex Corp., Austin, Texas). The inter-variability for all inflammatory cytokines tested was less than 10%, indicating the highly reliability of the Multiplex-Luminex method of cytokine assay.

**Migration and invasion assays**
Modified Boyden chambers (Coster, Boston, MA) were coated with 0.1% gelatin (migration) or extracellular matrix components (invasion). Untreated, AVA sensitive and AVA resistant immortomouse macrophages cells were suspended in 100 µL of serum-free media following one hour of exposure to AVA and added into the upper chamber. Complete media for cells containing 10% FBS (500 µL) was added to the bottom chamber as a chemo-attractant. The chambers were incubated at 37°C in 5% CO2 for 6 hours (migration) or overnight (invasion). After incubation, cells were fixed, stained, and counted in 5 random fields using light microscopy at 200x.

Cell Viability Assay

Immortalized murine macrophages sensitive and resistant to AVA therapy (1 x 10⁴ in 100 µL) were plated in a 96-well plate. After 24 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT, was added to each well. The plate was incubated at 37°C for 20 minutes and then absorbances were read at 570 nm (Ceres UV 900C; Bio-Tek Instrument Inc, Winooski, VT).

Immunostaining

All staining was performed in formalin-fixed, paraffin-embedded 8-µm thick tumor sections or OCT-embedded frozen tissue sections. Following deparaffinization, rehydration, and antigen retrieval or fixation, 3% H2O2 was used to block endogenous peroxidase activity for 10 minutes. Protein blocking of non-specific epitopes was done using either 5% normal horse serum, 1% normal goat serum, or 4% fish gelatin in either PBS or TBS-T for 20 minutes. Slides were incubated with primary antibody for CD68 (Santa Cruz, 1:400), VEGFR1 (AbCam,
1:500), CD-31 (Pharmingen, 1:800 for mouse tissue), overnight at 4 °C. For
immunohistochemistry, after primary antibody was washed with PBS, the
appropriate amount of horseradish peroxidase-conjugated secondary antibody was
added and visualized with 3,3'-diaminobenzidine chromogen and counterstained
with Gill's hematoxylin #3. For immunofluorescence, secondary antibody staining
was performed with either Alexa 594 or 488 (Molecular Probes). Nuclear staining
was performed with Hoechst 33342 (1:10,000; Molecular Probe H3570). Light field
images were obtained using a Nikon Microphot FXA microscope and Leica DFC320
digital camera, and immunofluorescent images were obtained using a Zeiss
Axioplan 2 microscope and Hamamatsu ORCA-ER digital camera. To quantify
microvessel density, we examined 5-10 random fields at 100x magnification for
each tumor (5 tumors per group) and counted the microvessels within those fields
as previously described (77). A vessel was defined as an open lumen with at least
one adjacent CD31-positive cell. Multiple positive cells beside a single lumen were
counted as one vessel. Quantification was performed by two investigators in a
blinded fashion. For immunofluorescent quantification, VEGFR1 expression was
determined using Photoshop by calculating the mean pixel density for each
representative image.

**Statistical analysis**

Differences in continuous variables such as tumor weight were analyzed
using the Mann-Whitney rank sum test. Two-tailed \( P \) values of no more than 0.05
were deemed statistically significant. Normally distributed continuous variables were
compared using the student \( t \)-test. Differences in variables that were not normally
distributed were compared using a nonparametric test (Mann-Whitney $U$ test). Only two-tailed values are reported in this study. We considered $P$ values less than 0.05 to be significant. Statistical analysis of the clinical data was performed using 2-sample t-test and Fisher’s exact test. Survival analysis was performed using Kaplan-Meier analysis.
Results

Macrophage numbers increase with development of anti-VEGF resistance

To evaluate the role of immune cells in the development of resistance to VEGF blockade, we first established a syngeneic mouse model of anti-VEGF resistance. After intraperitoneal injection of luciferase-labeled IG10 ovarian cancer cells and following confirmation of tumor establishment with bioluminescence imaging, immune competent C57BL/6 mice were randomized to two treatment groups: 1) control and 2) Anti-VEGF antibody (AVA). Treatment mice received AVA twice weekly and both groups underwent weekly bioluminescence imaging to monitor tumor growth. Mice receiving AVA were subsequently divided into AVA-sensitive or AVA resistant groups based on imaging. AVA resistant mice were defined as those with increased tumor growth, by increased bioluminescence intensity in previously stable tumor burden (Figure 3). This point marked clinical resistance to anti-VEGF therapy. All mice were subsequently sacrificed and tumors collected for immune cell profiling.
Figure 3. Establishment of a model of AVA resistance. Following injection of luciferase-labeled IG10 cells, AVA treatment was initiated and continued until increased tumor burden was demonstrated by bioluminescence imaging.

Immune cells were isolated from the collected tumors of control, AVA-sensitive, and AVA-resistant mice then subjected to FACS profiling. Compared to control samples, tumors from AVA-sensitive mice showed decreased macrophage infiltration. In contrast, macrophages were increased in the tumors of AVA-resistant mice, while other immune cell populations remained unchanged (p<0.0001, Figure 4).
Figure 4. **FACS myeloid cell profiling of tumor samples.** (A) Myeloid cells were isolated from tumor samples of control, AVA sensitive, and AVA resistant groups and sorted by flow-assisted cytometry according to CD11b+F4/80+ expression. (B) The proportion of macrophage is expressed as a percentage of the total CD45+ cells. **** indicates p<0.0001.

Additionally, tumors from AVA-resistant mice showed increased vessel density compared to either control or AVA-sensitive tumors, as measured by CD31 staining (p<0.001, Figure 5a, b). The marked increase in macrophages seen in coordination with increased blood vessel density lead us to consider macrophages as potential catalysts in resistance to anti-VEGF therapy.
Depletion of macrophages at the time of anti-VEGF resistance restores sensitivity

As anti-VEGF resistance was associated with a significant increase in tumor macrophages in our model, we next investigated effects of their depletion using bisphosphonates at the emergence of resistance. Bisphosphonates, such as zoledronic acid and clodronate, are clinically approved for the treatment of osteoporosis and bony metastases, but also induce macrophage depletion (78-80). (Figure 6)
Figure 6. CD68 staining of tumor sections after treatment with zoledronic acid. Representative SKOV3ip1 tumor sections of control and zoledronic acid-treated tumors are shown following CD68+ immunohistochemical staining. Magnification is at 400x. *** indicates p<0.001.

C57Bl/6 mice were again injected with luciferase-labeled IG10 ovarian cancer cells. Following bioluminescence imaging to confirm establishment of tumor, mice were randomized to: 1) control; 2) AVA only; or 3) AVA plus zoledronic acid. Controls received placebo until becoming moribund and were then sacrificed. In the AVA only group, treatment continued twice weekly until resistance developed, with sacrifice as mice became moribund. In the AVA or plus zoledronic acid group,
mice received anti-VEGF therapy alone until resistance was documented, as measured by an increase in previously stable disease burden by bioluminescence imaging. At the emergence of resistance, weekly zoledronic acid was added to anti-VEGF treatment (Figure 7).

**Figure 7. Treatment schema of resistance model with zoledronic acid.** Mice received intraperitoneal injection of luciferase-labeled IG10 cells. Twenty-one days later, mice were assigned to either AVA alone or AVA with the addition of zoledronic acid at the emergence of resistance, as demonstrated by bioluminescence imaging.

The combined treatment was then continued until mice became moribund. The addition of zoledronic acid at the emergence of resistance halted tumor growth and significantly prolonged survival, as compared to either control or anti-VEGF therapy only (p<0.001, Figure 8).
Macrophage depletion increases the effectiveness of anti-VEGF therapy

In light of our data implicating macrophages in the development of resistance to VEGF blockade, we investigated the upfront combination of macrophage depletion with anti-VEGF therapy. Nude mice were injected with either SKOV3ip1 or OVCAR5 and then assigned to receive: 1) no treatment; 2) AVA only; 3) zoledronic acid only; or 4) AVA plus zoledronic acid. Mice were sacrificed when any group became moribund and tumors were harvested. The combination of AVA plus zoledronic acid dramatically reduced tumor weight and nodules in both the
SKOV3ip1 (p<0.0001 and p<0.05, Figure 9a) and OVCAR5 models (p<0.0001 and p<0.05, Figure 9b).

**Figure 9. Zoledronic acid increases the effectiveness of AVA therapy.** Total tumor weight and number of nodules are shown in SKOV3ip1 and OVCAR5 ovarian cancer models following treatment with AVA, zoledronic acid, or the combination. **** indicates p<0.0001, * indicates p<0.05.
To ensure that our results were not specific to zoledronic acid, we repeated the experiment using an additional bisphosphonate, clodronate. Nude mice were injected with SKOV3ip1 and then randomly assigned to: 1) no treatment; 2) AVA only; 3) clodronate only; or 4) AVA plus clodronate. Again, the combination of AVA plus clodronate significantly reduced tumor growth (weight p<0.0001, nodules p<0.01, Figure 10).

![Figure 10. Clodronate increases the effectiveness of AVA therapy.](image)

**Figure 10. Clodronate increases the effectiveness of AVA therapy.** Total tumor weight and nodules are shown following treatment with clodronate, AVA, or the combination. **** indicates p<0.0001, *** indicates p<0.01.

Additionally, the combination groups demonstrated significantly reduced macrophage numbers as compared to the other groups (zoledronic acid p<0.0001, clodronate p<0.001, Figure 11a-d). This decrease in macrophage infiltration mirrors the results seen in anti-VEGF sensitive tumors in our initial immune profiling.
Figure 11. **Immunohistochemical staining of macrophages.** Macrophages from SKOV3ip1 tumor samples treated with either zoledronic acid (A), or clodronate (B), were stained for CD68+ and numbers compared between respective groups (C, D). Graphs represent the mean number of macrophages per 5 randomly selected 400x high power fields (HPF) ± SEM (A) or per 5 randomly selected 200x high power fields (HPF) ± SEM (C). Representative photomicrographs of macrophage density are shown following treatment with zoledronic acid (A) or clodronate (B). **** indicates p<0.0001, *** indicates p<0.001.
Anti-VEGF resistant macrophages display increased viability

Given the marked differences in macrophage infiltration in the AVA sensitive and resistant settings, we wondered whether these observations may be reflective of two distinct populations of macrophages. To investigate phenotypic differences between AVA sensitive and resistant macrophages that might contribute to resistance, we compared cell viability using an MTT assay. Following exposure to either two weeks (anti-VEGF sensitive) or six weeks (anti-VEGF resistant) of AVA treatment, macrophages were exposed to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cell viability was assessed. As compared to anti-VEGF sensitive macrophages, resistant macrophages demonstrated a 57% increase in cell viability (p<0.001, Figure 12).
**Figure 12. Viability is increased in AVA resistant macrophages.** MTT assays were performed on untreated, AVA sensitive, and AVA resistant macrophages. *** indicated p<0.001.

Invasion/migration of anti-VEGF resistant macrophages not affected by anti-VEGF treatment

On the basis of the increased macrophages seen at the emergence of resistance, we wondered if AVA resistant macrophages were better adapted to invade and migrate despite AVA therapy. To address this question, we investigated differences in the ability of anti-VEGF sensitive and resistant macrophages to invade and migrate following exposure to an anti-VEGF agent. Both groups were exposed to AVA for one hour before being plated into modified Boyden chambers. Migration and invasion were subsequently assessed at 6 hours and 24 hours, respectively. As predicted, AVA-sensitive macrophages displayed significantly inhibited migration following exposure to AVA (121 vs 91.4 cells per HPF, p<0.001). In contrast, the ability of AVA-resistant macrophages to migrate was increased by 57% after exposure to AVA. (p<0.001, Figure 13a). While there was a trend towards decreased invasion of AVA-sensitive macrophages following exposure to AVA, this difference was not significant. Invasion of AVA-resistant macrophages was not affected by AVA exposure (Figure 13b).
Figure 13. Invasion and Migration Assays. Control, AVA-sensitive, AVA-resistant macrophages were exposed to AVA treatment for one hour and then plated into modified Boyden chambers to assess (A) migration (6 hours) and (B) invasion (24 hours). Cell numbers per high power field (HPF, 200x) were then counted. *** indicates p<0.001.

Anti-VEGF resistant macrophages secrete alternative pro-angiogenic cytokines

To assess phenotypic differences between anti-VEGF sensitive and resistant macrophages, we performed a cytokine array. Supernatant was collected from murine macrophages exposed to either two weeks (AVA sensitive) or six weeks
(AVA resistant) of AVA treatment and evaluated for cytokines/chemokines using the Milliplex MAP murine cytokine/chemokine panel (Millipore, MA). Compared to anti-VEGF sensitive macrophages, those resistant to VEGF blockade secreted significantly less VEGF and instead show a trend toward increased G-CSF and dramatically increased levels of the pro-angiogenic platelet-derived growth factor-aa (Figure 14).

![Cytokine Array](image)

**Figure 14. Cytokine array of AVA sensitive and resistant macrophages.**

Cytokines altered are shown above. Significant differences are indicated. * indicates p<0.05, *** indicates p<0.001.

High throughput analyses reveal changes in VEGFR expression

Given the significant differences in macrophage numbers in the anti-VEGF sensitive and resistant settings, we sought to investigate the genotypic differences
in these populations. Macrophages were cultured in vitro and treated with AVA twice weekly. Based on our previous in vivo studies, macrophages were collected at two weeks and six weeks to reflect anti-VEGF sensitive and resistant conditions, respectively, and gene expression profiling was performed on isolated RNA. Pathway enrichment analysis revealed upregulation of pro-angiogenic pathways, including the molecules shown below (p<0.00007, Figure 15).

![Pro-angiogenic genes upregulated in AVA resistant macrophages.](image)

**Figure 15. Pro-angiogenic genes upregulated in AVA resistant macrophages.** Netwalker© software was used to analyze pathways up- and downregulated in AVA treated macrophages. Pro-angiogenic pathways were found to be significantly upregulated.

Concurrently, we also analyzed differences between macrophage populations in the anti-VEGF sensitive and resistant conditions using reverse phase
protein array (RPPA). Macrophages were harvested and protein extracted following 2 weeks (anti-VEGF sensitive) and 6 weeks (anti-VEGF resistant) of treatment with AVA in vitro. Analysis revealed upregulation of anti-apoptotic pathways including proteins such as CAV1, CTNNB1, ESR1, AKT1, MCL1, BCL2, MDM2, MAPK8, BAX, GSK3B, BCL2L1, IGF1R, and CDKN1A (Tables 3,4; Figure 16).

Table 3. Associated functions of proteins significantly altered in RPPA

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<th>Functional Annotation</th>
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<th>p-value</th>
<th>Genes</th>
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Table 4. Expanded table of RPPA network functions

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<p>| negative regulation of programmed cell death | 13 | 4.06E-14 | CAV1, CTNNB1, ESR1, AKT1, MCL1, BCL2, MDM2, MAPK8, BAX, GSK3B, BCL2L1, IGF1R, CDKN1A |</p>
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Figure 16. Proteins significantly altered in AVA resistant macrophages. Proteins either significantly up- or downregulated are shown. Netwalker© software was used to create networks of related molecules.

Decreased macrophage VEGFR-1 expression with resistance to anti-VEGF therapy

In the context of therapy directed at the VEGF pathway, we investigated whether VEGFR expression changes could be modulating the differences in behavior observed between AVA sensitive and resistant macrophages. We assessed the effects of AVA therapy on macrophage VEGF receptor expression in *vitro*. Cultured macrophages were treated with AVA twice weekly. Subsequently,
VEGFR expression was assessed at baseline, two weeks (AVA sensitive), and six weeks (AVA resistant). We found that, following an initial period of upregulation during the AVA sensitive phase, VEGFR-1 expression was significantly downregulated in the setting of AVA resistance (p<0.001, Figure 17). Because VEGFR-1 expression was the most significantly altered in AVA resistant macrophages, we chose to focus on this receptor.

![Graph showing VEGFR expression levels](image)

**Figure 17. Macrophage VEGFR expression.** Macrophage VEGFR-1, VEGFR-2, and VEGFR-3 expression were assessed in untreated, AVA sensitive and AVA resistant macrophages. VEGFR-1 expression was significantly altered in AVA resistant macrophages compared to those that were AVA sensitive. *** indicates p<0.001.

VEGFR-1 protein levels from untreated, AVA sensitive, and AVA resistant macrophages were compared using western blot. AVA resistant macrophages
demonstrated significantly less protein expression of VEGFR-1, as seen in Figure 18. These results were confirmed by immunofluorescent staining (Figure 19).

**Figure 18. VEGFR-1 expression by Western Blot.** VEGFR-1 expression was compared in untreated, AVA sensitive, and AVA resistant macrophages using Western Blot. β-actin served as a loading control.
Figure 19. Immunofluorescent staining of macrophage VEGFR-1 expression.

Following treatment with AVA, expression of VEGFR-1 was quantitated in sensitive and resistant macrophages. Representative micrographs are shown at 400x magnification.

In parallel, we assessed co-localization of CD68 and VEGFR-1 in AVA sensitive and AVA resistant tumor samples. Again, we noted decreases in macrophage expression of VEGFR-1 (Figure 20) in AVA resistant tumors compared to AVA sensitive tumors.
Figure 21. Co-localization of macrophages and VEGFR-1 in tumor samples.
Macrophage expression of VEGFR-1 was assessed in tumor samples (IG10) using CD68+ and VEGFR-1 antibodies in AVA sensitive (top panel) and AVA resistant (bottom panel) tumors. Representative photomicrographs are shown at 400x magnification.

Downregulation of VEGFR-1 promoter with AVA resistance

We sought to uncover the mechanism by which macrophage VEGFR-1 downregulation occurs. Since VEGFR-1 methylation has been linked to the expression of VEGF and as methylation is known to play a role in drug resistance and can be induced by hypoxia, we assessed methylation of the promoter region of VEGFR-1 following AVA treatment, a known generator of hypoxia (81-83). DNA samples from AVA sensitive and resistant macrophages were treated with bisulphite...
and methylation-specific polymerase chain reaction (MSP) analysis was performed, revealing a significant increase in methylation at the VEGFR-1 promoter region (p<0.05, Figure 21).

Figure 21. Relative VEGFR-1 CPG methylation. Following treatment with bisulphite, VEGFR-1 methylation was assessed by methylation-specific polymerase chain reaction (MSP). * indicates p<0.05.
Summary

This work makes several contributions to the current understanding of resistance to AVA therapy, with important clinical implications.

1) Increases in tumoral macrophages are associated with the emergence of resistance to AVA therapy. The depletion of macrophages at this transition point restores sensitivity to AVA therapy and prolongs survival in murine models. The upfront combination of bisphosphonates with AVA therapy is synergistic in reducing tumor growth.

2) Prolonged AVA therapy induces adaptive changes in macrophages, with upregulation of angiogenic and anti-apoptotic pathways, which may facilitate tumor growth in the AVA resistant microenvironment.

3) AVA therapy results in downregulation of macrophage VEGFR-1. This decreased expression of macrophage VEGFR-1 is secondary to increased VEGFR-1 promoter methylation.
Discussion

Biological significance of macrophages in anti-VEGF resistance

While macrophages have been previously implicated in the development resistance to chemotherapy, their role in resistance to VEGF blockade is not well studied (59, 84). Previously, targeting of the Ang/Tie2 pathway has impaired the angiogenic activity of Tie2 expressing macrophages and diminished the emergence of resistance in vivo (62). In our detailed investigation into the contribution of macrophages to anti-VEGF resistance, we first show decreased TAM infiltration in tumors sensitive to AVA therapy. This mirrors previously published data showing that AVA therapy can decrease tumoral macrophages (85). Next, we demonstrate, for the first time, chronological increases in tumoral macrophages with the emergence of resistance to anti-VEGF therapy.

This influx of macrophages has important biological significance, as macrophages are capable of secreting numerous angiogenic factors, including VEGF, PI GF, and others seen in Table 1(86). Indeed, in coordination with elevated macrophage counts, we show dramatically increased blood vessel density in AVA resistant tumors. The significance of macrophages in AVA resistance is most dramatically illustrated by the prolonged survival of mice receiving zoledronic acid following the emergence of resistance. As compared to mice receiving only AVA therapy, who all developed resistance and quickly became moribund, those mice receiving zoledronic acid had stable or reduced disease burden and lived for up to six months following tumor cell injection. We also show the potential additive combination of bisphosphonate with AVA therapy in the upfront setting. While mice
in all groups were sacrificed when the control groups became moribund, those receiving AVA therapy with bisphosphonates demonstrated no evidence resistance and had very little disease burden.

Several other experiments need to be performed to definitely prove the catalytic role of macrophages in AVA resistance. First, we are presently creating a murine Csf1 knockout model. Homozygous mice lack Csf1, a critical growth factor for development of the monocyte and macrophage lineage, resulting in a dramatic reduction of systemic macrophage counts (87). Csf1\textsuperscript{op}/Csf1\textsuperscript{op} mice will be injected intraperitoneally with IG10 cells and assigned to 1) no treatment, or 2) AVA alone. Matched groups of wild-type C57Bl/6 will also be injected with IG10 cells to serve as additional controls. As compared to wild-type mice, we expect Csf1\textsuperscript{op}/Csf1\textsuperscript{op} mice receiving no treatment to have a prolonged disease course, while those receiving AVA will fail to develop resistance to VEGF blockade.

We will also investigate whether re-introduction of macrophages into our Csf1 knockout model will restore the wild-type pattern of AVA resistance. Csf1\textsuperscript{op}/Csf1\textsuperscript{op} knockout mice will be injected with IG10 cells and assigned to 1) AVA therapy alone, or 2) AVA therapy plus macrophage transfusion. Matched groups of wild-type C57Bl/6 will be injected with IG10 cells to serve as controls. We predict the survival of Csf1\textsuperscript{op}/Csf1\textsuperscript{op} receiving macrophage infusion therapy with AVA therapy to recapitulate that of untreated wild-type mice. Untreated Csf1\textsuperscript{op}/Csf1\textsuperscript{op} mice should fail to develop resistance to AVA therapy.

In light of recent data suggesting that both resident and bone marrow-derived macrophages can proliferate, the origin of influxing macrophages must be
delineated (54). We are currently investigating whether the increase in macrophages seen at the emergence of resistance is secondary to resident macrophage proliferation or increased recruitment from bone marrow populations. In this experiment, bone marrow was isolated from GFP-labeled FVB.Cg-Tg(CAG-EGFP)B5Nagy/J mice. These mice express a GFP label in all tissues, including the bone marrow and its derived cells. Following cell sorting to confirm GFP labeling, harvested bone marrow cells were injected into irradiated wild-type C57Bl/6 mice (n=15). Successful bone marrow transplant will be confirmed by hematologic profiling 4 weeks post-transplant, including verification of GFP expression in bone marrow-derived cells. Recipient mice will then be injected with luciferase-labeled IG10 cells and tumor establishment verified 21 days after injection. Mice will receive AVA therapy twice weekly in conjunction with weekly bioluminescent imaging. Mice will be while sensitive and resistant to VEGF blockade, as demonstrated by imaging. Macrophages of bone marrow origin will be GFP-labeled, while resident tissue macrophages will not be labeled, allowing them to be distinguished. Injection with BRDU prior to sacrifice will allow us to determine if the increase in macrophages with AVA resistance is secondary to proliferation.

Mechanisms

In this study, we prove that AVA sensitive and resistant macrophages are phenotypically distinct. AVA resistant macrophages display increased cell viability and increased migratory ability, as compared to their AVA sensitive counterparts. Additionally, AVA resistant macrophages have altered cytokine secretion, with significantly increased production of PDGF-AA and decreased VEGF. PDGF-AA is
implicated in both the autocrine and paracrine angiogenic switch in solid tumors (88). To our knowledge, this is the first time a distinctly different macrophage population has been shown to emerge in response to AVA therapy.

Additionally, we use high-throughput studies to show that pro-angiogenic and anti-apoptotic pathways are upregulated in AVA resistant macrophages. While many studies have focused on tumor cell adaptation to AVA therapy, this is the first to specifically show macrophage adaptation to AVA therapy. Collectively, we believe these changes allow macrophages to continue to aid tumor growth in the face of AVA therapy, where they might otherwise be depleted.

We demonstrate modulation of macrophage VEGFR-1 in response to AVA therapy. The reduction in expression of macrophage VEGFR-1 is secondary to increased VEGFR-1 promoter methylation. Promoter methylation is a known mechanism for drug resistance (89, 90). We are currently exposing AVA resistant macrophages to the de-methylating agent, azacitidine. Restoration of AVA sensitivity would support VEGFR-1 methylation as a mechanism of drug resistance in macrophages.

Macrophage VEGFR-1 expression has also been linked to phenotypic behavior, as antibody blockade of VEGFR-1 reduces monocyte and macrophage VEGF-induced migration (91, 92). It is possible that macrophage VEGFR-1 is downregulated in response to an AVA therapy-induced reduction of VEGF levels in the microenvironment.

Other potential explanations for decreased macrophage VEGFR-1 exist. Our observed effect could be in response to hypoxia, which is known to be induced by
AVA therapy (93). Previous data from our laboratory shows that AVA therapy leads to hypoxia and increased EZH2 expression (94). EZH2 decreases vasohibin 1 (VASH1), leading to increased angiogenesis. Data not published in our original study also demonstrates a reduction in VEGFR-1 in response to increased EZH2 levels. We currently have several experiments underway to determine if hypoxia alone can downregulate macrophage VEGFR-1. Together these experiments will elucidate the complete mechanisms responsible for the VEGFR-1 reduction described in this work.

Clinical implications

Our study has important clinical implications. We show depletion of macrophages at the emergence of anti-VEGF resistance using bisphosphonates can halt tumor growth and prolong survival in murine models. Additionally, the combination of bisphosphonates plus anti-VEGF therapy can prevent the development of resistance and improve the effectiveness of anti-angiogenic therapy.

These findings offer direct support to previous clinical observations regarding the tumor-modifying ability of bisphosphonates, which have been shown to reduce bone metastasis in breast cancer patients, and in those with prostate or renal cell carcinoma and pre-existing bone metastasis, result in a trend towards increased survival (64, 66). As further evidence, we investigated the effects of bisphosphonate use on overall cancer mortality using the Federal Drug Administration (FDA) Adverse Event Reporting System. Of approximately 17,000 patients with a cancer diagnosis co-medicated with a bisphosphonate, overall
reported death rate was 36% lower (17.6% vs 27.7%, \( p < 0.0001 \)) than those not receiving bisphosphonates, seen below in Figure 23. Additionally, we are in the process of obtaining IRB approval to investigate outcomes of cancer patients receiving bisphosphonates at M.D. Anderson Cancer Center.

Figure 22. Overall mortality in cancer patients co-medicated with bisphosphonates. Using the FDA Adverse Event Reporting System, 191,387 cancer patients who did not receive bisphosphonates and 16,952 patients co-medicated with a bisphosphonate were identified. Overall mortality is shown above as a percentage as a percentage of each group. *** indicates \( p < 0.0001 \).

Given the role of macrophages in resistance to VEGF blockade, strategies to modify macrophage response should be investigated in combination with anti-VEGF therapy in patients. Possible approaches include bisphosphonates, as described here, CSF-1 inhibitors, CCR2 inhibitors, and trabectedin (67, 70, 71). Consideration
should be given to the upfront combination of these therapies with VEGF blockade, thereby diminishing the opportunity of macrophages to contribute to anti-VEGF resistance. Our group is in the preliminary stages of trial design to investigate the use of bisphosphonates or a CSF-1 inhibitor, AC708 (Ambit Biosciences; San Diego, CA), in patients initially responsive to bevacizumab. A detailed treatment schema is seen below in Figure 24.

**Figure 23. Proposed trial design to investigate macrophage depletion in combination with bevacizumab.** Patients initially responsive to bevacizumab will be randomized to receive either a bisphosphonate or a CSF-1 inhibitor, AC708.

Additionally, we are investigating macrophage VEGFR-1 expression as a potential as a predictor of response to AVA therapy. We have obtain human
ovarian cancer samples from patients treated with bevacizumab and are currently performing immunohistochemical staining to assess VEGFR-1 expression in macrophages. We will then retrospectively correlate this with patients’ response to bevacizumab treatment. We predict that patients with low macrophage VEGFR-1 expression will have diminished bevacizumab efficacy compared to those with high macrophage VEGFR-1 expression.

**Limitations and Conclusions**

In the current study, we demonstrate the previously unrecognized role of macrophages in resistance to VEGF blockade. We show that macrophage accumulation in the tumor microenvironment correlates with the emergence of anti-VEGF resistance. The downregulation of VEGFR-1 is seen in conjunction with upregulation of alternative angiogenic and anti-apoptotic pathways, facilitating escape from VEGF-directed therapies.

While the evidence presented in this study offers new insights into AVA resistance, more work remains to be done to clearly elucidate the mechanisms behind our observations. Further, bisphosphonates are known to have effects not specific to macrophages, including decreasing endothelial cell migration and cytokine secretion (article in press; Reusser N, et al, Clodronate Inhibits Tumor Angiogenesis in Mouse Models of Ovarian Cancer, Cancer Biology & Therapy). As such, the addition of a more specific method of macrophage depletion, such as a CSF-1 inhibitor, would strengthen the evidence presented here. The use of only a bisphosphonate at the emergence of AVA resistance, instead of AVA plus a
bisphosphonate, should serve as an additional control and is being incorporated into our future experiments.

In summary, we describe the previously unrecognized role of macrophages in resistance to anti-VEGF therapy. We offer strategies to modulate the influence of macrophages, thus improving the effectiveness of VEGF blockage. These readily translatable findings warrant further clinical investigation.
Bibliography


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Vita

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