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Los Angeles

Colony-Stimulating Factor-1 Receptor Inhibition Reverses the Vascular Leakage that Causes  
Malignant Ascites in Late-Stage Epithelial Ovarian Cancer

A dissertation submitted in partial satisfaction  
of the requirements for the degree Doctor of Philosophy in  
Molecular & Medical Pharmacology

by

Diana Louise Moughon

2015

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## ABSTRACT OF THE DISSERTATION

# Colony-Stimulating Factor-1 Receptor Inhibition Reverses the Vascular Leakage that Causes Malignant Ascites in Late-Stage Epithelial Ovarian Cancer

by

Diana Louise Moughon

Doctor of Philosophy in Molecular & Medical Pharmacology

University of California, Los Angeles, 2015

Professor Lily Wu, Chair

Malignant ascites is a common complication in the late stages of epithelial ovarian cancer (EOC), which greatly diminishes the quality of life of patients. Although malignant ascites is known to be the result of vascular dysfunction, current approved treatments are not effective in stopping its accumulation. This study took on an alternative strategy of targeting the macrophage functions to reverse the vascular pathology of malignant ascites. We analyzed the content of immune cells and macrophages in ascites fluid of human patients and in a murine immunocompetent model (ID8) of EOC. This model developed progressive vascular disorganization and leakiness that culminated in massive ascites, mirroring the human disease. The macrophage content in ascites fluid from human patients and ID8 model directly correlates with vascular permeability. To further substantiate macrophages' role in the pathogenesis of malignant ascites, a colony-stimulating

factor 1 receptor (CSF1R) kinase inhibitor (GW2580) was used to block the functions of macrophages in the ID8 model. GW2580, administered in the late stages, was able to reduce the number of protumorigenic (M2) macrophages infiltrating the ascites and lower ascites volume dramatically. Disorganized peritoneal vasculature became normalized and vascular permeability assays showed that GW2580-treated ascites sera protected against endothelium permeability. Because these results were seen with just ascites sera, the conclusion was that soluble factors in the ascites are responsible, at least in part, for the regulation of the peritoneal vasculature in late-stage EOC. Preliminary mass spectrometry analysis revealed some interesting candidates including apolipoproteins, CD5 antigen-like, glutathione peroxidase, and inter-alpha-trypsin inhibitor heavy chains. Future directions focus on identifying these factors. This macrophage-targeted blocking treatment may be a promising strategy towards a safe and effective means to control malignant ascites of EOC.

The dissertation of Diana Louise Moughon is approved.

Luisa Iruela-Arispe

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2015

## **Dedication**

This work is dedicated to my grandmother, Marie Ann Redmond. She was beautiful inside and out, and to know her was to love her. She could bring out the good in every person and situation with her kind, gentle, generous and loving nature. In 1998 she was diagnosed with stage IV cancer and, after battling it for over four years, she slipped away from us. It is in her memory that I seek ways to lessen the pain and suffering of other cancer patients and all those who love them.



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Pricema SJ, Sung JL, Shaposhnik Z, Burton JB, Torres-Collado AX, **Moughon DL**, Johnson M, Lusis AJ, Cohen DA, Iruela-Arispe ML, Wu L. Targeting Distinct Tumor-Infiltrating Myeloid Cells by Inhibiting CSF-1 Receptor: Combating Tumor Evasion of Antiangiogenic Therapy. *Blood*, 2010 Feb 18;115:1461-1471.

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#### Awards and Grants

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## I. Introduction

Ovarian cancer is the ninth most common cancer in the United States, but it is the fifth leading cause of cancer death among women in the United States (1). With a 5-year survival rate of 45%, ovarian cancer is the most deadly of the female reproductive cancers. Each year, approximately 22,500 women will be diagnosed with ovarian cancer in the United States, and approximately 14,000 will die of the disease (2).

There are three main subtypes of ovarian cancer: germ cell, sex cord stromal, and epithelial (3). Germ cell tumors make up about 3% of cases and are very curable with 5-year survival rates of 96% in early stages and 75% in late stages of the disease (4,5). Sex cord stromal tumors make up a little over 1% of cases and are usually (approximately 90%) diagnosed early on in the disease when the survival rate is 90-100% (4,6,7). Epithelial ovarian cancer (EOC) makes up about 95% of all diagnosed cases of ovarian cancer, and is by far the most deadly of the subtypes (4). If diagnosed early, the 5-year survival rate is actually quite good at ~90% and ~70% for stages I and II, respectively (8). However, only 30% of patients are diagnosed at these stages. 70% of patients are diagnosed at stages III and IV, where the survival rates are 34% and 18%, respectively (8–10) (Figure 1.1).

Ascites likely	Stage of EOC	5-year survival rate	30% of patients diagnosed here
	I	90%	
	II	70%	70% of patients diagnosed here
	III	34%	
	IV	18%	

**Figure 1.1:** General statistics and information about EOC

There are many reasons why EOC is rarely detected in the early, more treatable stages. The position of the ovaries is quite proximal to the other organs in the abdomen. The initial route of metastasis is by dissemination around the abdominal cavity to these other organs (11). This method of metastasis is easy for the cancer to accomplish, and results in the progression of the disease to stage III (12). The symptoms of EOC, such as bloating, back pain, digestion problems, and abdominal cramps, are nonspecific and do not inspire worry in the average busy woman (13–15). Women who do go to the doctor tend not to go to an obstetrician-gynecologist because they are not aware that they have a gynecological issue. Thus, they may receive a variety of diagnoses (and treatments for the incorrect diagnoses) such as irritable bowel syndrome, depression, and constipation (16). A major symptom that is more specific to ovarian cancer is ascites. Unfortunately, ascites accumulation starts in stages III and IV, and thus the prognosis for these women is poor (17) (Figure 1.1).

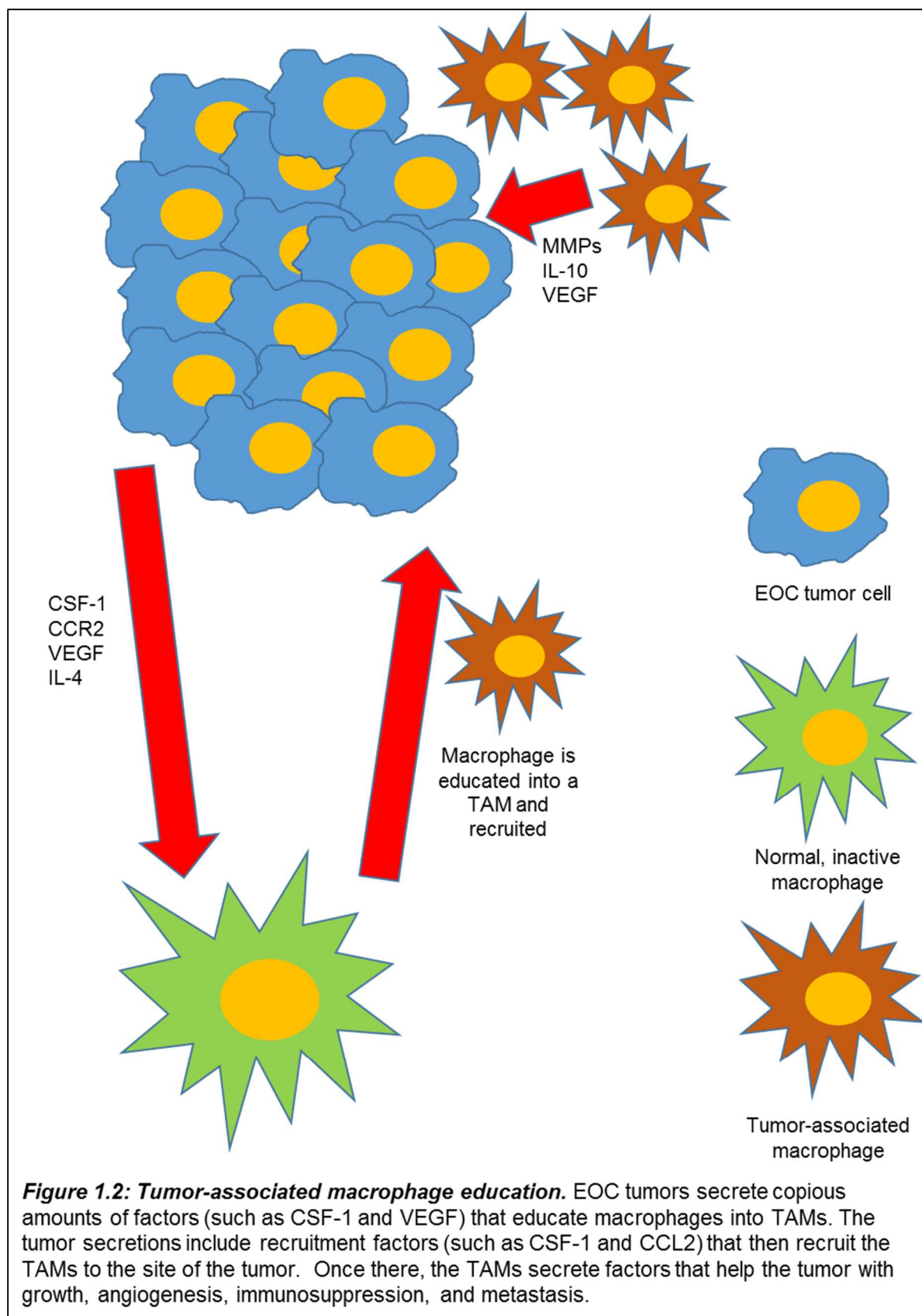
The first line of treatment for EOC is surgical debulking (2). The surgeon will try to remove all of the tumor mass, though depending on how extensively the tumor cells have disseminated and onto which organs, it may be impossible to collect all of it (2,18). After the surgery, a combination chemotherapy regimen will be prescribed, usually paclitaxel and a platinum drug (19,20). Unfortunately, recurrence of the cancer is common, and the new tumors are much harder to treat (21). The patient may undergo another surgery and/or be prescribed some different chemotherapies or antiangiogenic drugs (17). The patient may also have the option of joining clinical trials (21). However, the majority of EOC mortality is due to resistance to therapies, especially resistance to platinum drugs (22).

Throughout this battle against EOC, women are often fighting another battle against ascites. About 50% of women present with ascites, and almost all women will have ascites at the time of recurrence (11,23). Ascites is thought to form because of leaky peritoneal vasculature, blocked lymph drainage from the peritoneum, or a combination of both (24,25). Current treatments for

ascites include paracentesis, diuretics, and peritoneovenous shunts (26–28). None of these treatments cure the root of the problem causing ascites however, so recurrence and continual drainage is a reality that the patients must contend with (24,26,27). The actual cause of ascites is currently unknown, and research focusing on finding the cause continues. Vascular endothelial growth factor (VEGF) was originally described in ascites and found to have great vascular permeability and angiogenesis-stimulating properties (29–31). However, therapies developed to target VEGF or its receptor have effectively eliminated ascites in only a small fraction of patients. The vast majority of patients either still have recurring ascites or suffer a host dangerous side effects, including death by bowel perforation in about 10% of patients (32–35). The unacceptable outcome of worsening the quality of patients' lives by a therapy supposedly designed to give patients better quality of life through ascites reduction means that the search continues for a correct target.

EOC shows highly immunosuppressive microenvironments both in the tumors and in the peritoneal cavity, i.e. the ascites (36–38). This immunosuppression is largely due to the infiltration of macrophages into these areas (37–39). Although one might think that the presence of macrophages in the tumors and ascites might mean that the body is mounting an assault against the cancer (as classically-activated “M1” macrophages would (40,41)), the opposite is actually true (41,42). Once in contact with the tumor and secretions from the tumor, these macrophages are educated into tumor-associated macrophages (TAMs, a spinoff of wound-healing alternatively activated “M2” macrophages (40,41)) and begin to assist the tumor (41,42) (Figure 1.2). They secrete immunosuppressive, pro-angiogenic, tissue remodeling, and growth factors, thus explaining the worse prognosis seen in the clinic when more macrophages are present in the tumors (43–45) (Figure 1.2).

The macrophage-associated cytokine seen elevated specifically in ovarian cancer patients is colony-stimulating factor 1 (CSF-1) (46,47). Copious amounts of this ligand is secreted by EOC



tumors, thus highly elevating the CSF-1 levels both in circulation and in ascites and indicating a poor prognosis (48,49). Using CSF-1 as a biomarker along with CA-125 improves the sensitivity of initial EOC diagnosis over CA-125 detection alone, implicating CSF-1 in the oncogenesis of EOC (50,51). CSF1R, the receptor for CSF-1, is expressed by macrophages, and this axis is responsible for the survival, growth, proliferation, differentiation, and motility of monocytes and macrophages (52,53). It is used to recruit macrophages to tissues and can affect the differentiation, immune function, and cytokine production of the macrophages (52,54). In fact, it is known that activation of this axis results in a macrophage with an M2 profile (55,56) (Figure 1.2). It is no surprise that tumors hijack this essential pathway and use it to recruit macrophages to its site and educate them into TAMs.

In Chapter II of this thesis, we explore how specifically inhibiting this axis in a mouse model of late-stage EOC specifically blocks the M2 macrophages from the ascites, resulting in vascular normalization and a reduction of ascites without invasive paracentesis. We then discovered that ascites serum alone could make an endothelial layer permeable (control mouse serum) or highly resistant to permeability (M2-macrophage depleted ascites serum). This finding gives rise to Chapter III of this thesis, where we attempt to find the soluble factor(s) in the ascites that so greatly affect late-stage EOC malignant ascites. Multiple studies have already led us to the conclusion that VEGF levels are unchanged after M2 macrophage depletion, thus we begin to use unbiased discovery-based proteomics techniques in Chapter III of this thesis to discover if there is indeed a better target.



## **II. NON-INVASIVE THERAPEUTIC INTERVENTION FOR MALIGNANT ASCITES**

### **Colony-Stimulating Factor-1 Receptor Inhibition Reverses the Vascular Leakage That Causes Malignant Ascites in Late-Stage Epithelial Ovarian Cancer**

#### **Abstract**

Malignant ascites is a common complication in the late stages of epithelial ovarian cancer (EOC), which greatly diminishes the quality of life of patients. Although malignant ascites is known to be the result of vascular dysfunction, current approved treatments are not effective in stopping its accumulation. This study took on an alternative strategy of targeting the macrophage functions to reverse the vascular pathology of malignant ascites. We analyzed the content of immune cells and macrophages in ascites fluid of human patients and in a murine immunocompetent model (ID8) of EOC. This model developed progressive vascular disorganization and leakiness that culminated in massive ascites, mirroring the human disease. The macrophage content in ascites fluid from human patients and ID8 model directly correlates with vascular permeability. To further substantiate macrophages' role in the pathogenesis of malignant ascites, a colony-stimulating factor 1 receptor (CSF1R) kinase inhibitor (GW2580) was used to block the functions of macrophages in the ID8 model. GW2580, administered in the late stages, was able to reduce the number of protumorigenic (M2) macrophages infiltrating the ascites and lower ascites volume dramatically. Disorganized peritoneal vasculature became normalized and vascular permeability assays showed that GW2580-treated ascites sera protected against endothelium permeability. The macrophage-targeted blocking treatment may be a promising strategy towards a safe and effective means to control malignant ascites of EOC.

## INTRODUCTION

Malignant ascites is a common side effect of epithelial ovarian cancer (EOC), characterized by the accumulation of fluid in the abdomen (26). It has been estimated that approximately 70% of EOC patients will develop ascites, particularly in the disseminated or recurrence stage of the disease. Although it is debated whether malignant ascites contributes to a poor prognosis or is merely indicative of the advanced stage of progression for EOC patients, this complication clearly compromises their quality of life (57). Current treatment methods, such as paracentesis and peritoneous shunts, physically drain the accumulated ascites fluid but do not address the root cause of this complication. Hence, the ascites fluid reaccumulates after the procedure. Furthermore, a significant risk of side effects due to infection or fluid and electrolyte imbalance are associated with physical drainage of malignant ascites (26,57).

In the pursuit of new, effective pharmaceutical remedies to manage ascites of EOC, vascular endothelial growth factor (VEGF) emerged as an excellent target for several reasons (24,32). VEGF is a factor originally isolated from ascites fluid that was recognized for its ability to induce vascular permeability (29). Subsequent clinical validation showed that VEGF is markedly elevated in the ascites fluid of ovarian cancer patients (58,59). Furthermore, increased VEGF secretion in ovarian cancer has been shown to be a prognostic parameter for ascites, advanced disseminated disease, and decreased survival (60–62). Numerous therapeutic studies in xenograft mouse EOC models have demonstrated the ability of anti-VEGF treatments to effectively suppress tumor growth and reduce ascites formation (63,64). Corroborating these preclinical findings are two recent reports of phase II clinical trials showing that treatment with VEGF trap Aflibercept significantly reduces ascites buildup in patients with advanced ovarian cancer (34);(35). However, the enthusiasm for this VEGF blockade treatment is dampened by a host of grade 3 and 4 treatment-related adverse vascular events, such as hypertension, venous thrombosis and

congestive heart failure. The most concerning of the adverse events is fatal intestinal perforation, which affected 10% of Aflibercept-treated patients in the randomized, controlled study (35). Therapies with anti-VEGF antibody, bevacizumab, also have similar severe side effects (33). These life-threatening side effects of VEGF-targeted therapies raise significant concerns of their use to merely gain symptomatic relief of EOC without clear long term survival benefits. The search for safe and effective treatments to manage malignant ascites of EOC continues.

Another tumor microenvironment component that has received great attention in recent years is the infiltrating myeloid cells, such as macrophages (43). A large volume of evidence supports that once recruited to and “educated” by the tumor, these macrophages promote cancer progression (65) by various mechanisms such as heightening the immunosuppressive conditions, angiogenesis and tissue remodeling, which in turn leads to enhanced tumor growth and metastasis (43,65). The tumor-promoting tumor-associated macrophages (TAMs) are commonly designated as “M2” in contrast to the classical-activated inflammatory “M1” macrophages (43,65). In EOC a large infiltrating population of macrophages has been observed within tumor nodules as well as in the ascites fluid (38,66). However, their phenotypes and functions have not been well studied. A distinctive feature of many human EOC tumors is that they secrete copious amounts of colony-stimulating factor 1 (CSF-1). CSF-1, also known as M-CSF, is a critical cytokine that regulates the differentiation, growth and function of macrophages by binding to and activating its cognate receptor CSF1R (*c-fms*) present on monocytes and macrophages (67). CSF-1 is also known to play a role in educating macrophages into M2 macrophages (55,56). Not only is CSF-1 known to be elevated in patient ascites, but an elevated level of this cytokine is associated with poor prognosis (68,69). These findings suggest that the CSF-1/CSF1R axis might promote oncogenic effects on tumor cells directly or modulate tumorigenesis through the recruitment and function of TAMs found in EOC tumors, or both.

In this study, we characterized the progression of the murine ID8 EOC model with special attention paid to the evolution of TAMs in this context. Mirroring the characteristics of human EOC, the ID8 tumor-bearing mice developed massive malignant ascites in the late stages. We observed a great expansion in macrophages within the ascites that correlated with vascular dysregulation. To demonstrate a causative role of TAMs in the vascular pathology of malignant ascites, we employed a selective CSF1R kinase inhibitor, GW2580, to block macrophage function. GW2580 lowered “M2” TAMs and also dramatically reduced ascites fluid accumulation. Findings from this study support the notion that TAMs are key players in causing or perpetuating the vascular leakiness of EOC ascites.

## **MATERIALS AND METHODS**

### **Cell Culture, lentiviral cell marking and RT-PCR**

All cell lines were cultured at 37°C with 5% CO<sub>2</sub> in DMEM with 100 U/mL penicillin and specific media components for each cell line as follows. Murine ID8 epithelial ovarian cancer cells (kind gift from Dr. Oliver Dorigo, Stanford University) require 4% FBS and 1% Insulin-Transferrin-Selenium (ITS, Gibco 100X solution). Liver sinusoidal endothelial cells from Immortomice® (IMECs) required 10% FBS and 20U/mL interferon gamma, while OVCAR3 cells required 20% FBS and SVEC4-10 cells required 10% FBS. HUVECs required MCDB-131 medium (VEC Technologies) with 10% FBS. Cell lines were PCR-tested for the absence of mycoplasma contamination.

Renilla luciferase lentiviral vector was propagated as previously described (70). ID8 cells were transduced at MOI 2, which resulted in 97% GFP+ transduction.

### ***In vivo* tumor models and bioluminescent imaging**

All animal experiments were approved by the UCLA IACUC and conformed to national animal care guidelines, ethics, and regulations. For the intraperitoneal (IP) model: Renilla luciferase-marked ID8 cells ( $10 \times 10^6$ ) in 500ul PBS were injected intraperitoneally into C57BL/6 female mice (Jackson Laboratory (Bar Harbor)). GW2580 (LC Labs) treatment (160mg/kg) or control diluent (0.1% hydroxypropyl methylcellulose, Sigma-Aldrich; 0.1% Tween20 in distilled H<sub>2</sub>O) was given daily starting 10-12 weeks post-injection.

Briefly, the method to establish the intraovarian, orthotopic model is as the following: A small incision was made through the skin and peritoneal wall on the lower-middle back of C57BL/6

female mice. The left ovary was exposed under a dissecting microscope and a Hamilton syringe was used to place  $1 \times 10^6$  ID8 cells under the ovarian bursa.

For the OVCAR3 model, cells were initially grown subcutaneously in female nude mice (Jackson Laboratories, Ben Harbor) to obtain enough tumor cells for the IP model. Tumors were harvested and cells disassociated, and  $2.5 \times 10^6$  cells were injected IP into female nude mice. Animals develop signs of late-stage EOC after 3 months, and GW2580 treatment or diluent was given as described above.

Renilla-marked ID8 tumor-bearing mice were imaged using the IVIS Lumina II as previously described (70), and bioluminescent signals were analyzed using Living Image 4.0 software.

### **Perfusion Assay and Whole Mount Immunohistochemistry**

Mice were injected IV with 60ug biotinylated lectin (Vector) and 60ug streptavidin Cy3 (Invitrogen). After 5 minutes, mice were anesthetized and perfused with 20ml of PBS and 20ml of 3% paraformaldehyde injected into the left ventricle.

Tissues were fixed with 2% PFA for 5-6 hours, then washed with PBS overnight at 4°C. Mesentery was fragmented into 0.2-0.5 cm and blocked in PBST (0.05% tween20 in PBS) with 3% donkey serum for 30 min. Samples were then incubated with primary antibodies for 1 hour and washed with PBST, followed by secondary antibodies (1:200) for 1 hour. Antibodies used are anti-mouse CD31 (1:50, BD Biosciences), anti-mouse isolectin (1:75, Invitrogen), anti-mouse CD11b (1:50, BD Biosciences), and anti-mouse CD206 (1:50, BD Biosciences).

### **Miles Assay**

1mg of Evans Blue in 100ul sterile PBS was IV injected into mice. After 30 minutes, mice were sacrificed and mesentery tissue was removed. Tissue was weighed and placed in 500ul formamide for 48 hours at 55°C. The OD of the extracted Evans Blue was read at 620nm (BioTek Synergy H1 plate reader) and converted to ng/mg tissue.

### **Flow cytometry**

Harvested tumors were minced into fragments and digested with 80 U/mL collagenase (Invitrogen) in PBS containing 2% FBS for 1 hour at 37°C. Minced tumors were then ground through a 70um cell strainer (BD). Spleens and lymph nodes were gently dissociated between the rough surfaces of 2 glass slides for single-cell isolation. Peripheral blood was isolated by retro orbital bleed. Ascites was completely drained from the peritoneum with a syringe. After red blood cell (RBC) lysis (Sigma-Aldrich), single-cell suspensions were filtered and incubated for 30 minutes on ice with the following: APC-CD45, e450-CD11b, PerCP-Cy5.5-Gr-1, PE-Cy7-F4/80, a700-MHCII, PE-CD4, e450-CD8, FITC-CCR2 (eBioscience , 1:500). Intracellular staining was performed for PE-Cy7-IFN $\gamma$  and PE-IL-12. Samples were run on the BD LSR-II flow cytometer (BD). Data was analyzed with FlowJo software (TreeStar).

### **Processing of patient samples**

Patient materials were collected under a UCLA Gyn-Onc Tissue Bank protocol approved by the institutional review board (IRB). All patients' identification was blinded in this study. 50ml of freshly harvested ascites fluid were spun down at 1500rpm and sera were frozen immediately at -80 degrees C. Ascites cells were subjected to red blood cell lysis, filtered, and incubated for 30 minutes in ice with the following flow cytometry antibodies: PE-Cy7-CD33, PE-CD68, APC-HLA-DR, FITC-CD4, APC-e780-CD8a (eBioscience, 1:500), PerCP e710-CD206, Alexa Fluor 488-

Muc-1 (eBioscience 1:200). Cells were fixed for 15 minutes in 3%PFA at RT and run on the BD LSR-II flow cytometer (BD). Data was analyzed with FlowJo software (TreeStar).

### **In Vitro Permeability assays**

Liver sinusoidal endothelial cells from Immortomice® (IMECs) and human umbilical vein endothelial cells (HUVECs) were plated on stabilized 8W10E+ PET Electric Cell-substrate Impedance Sensing (ECIS) Cultureware™ Disposable Electrode Arrays (Applied Biophysics). 300ul of murine serum was added to each well containing confluent IMECs and 300ul patient serum was added to each well containing confluent HUVECs. Arrays were connected to and read by an ECIS 1600R instrument (Applied Biophysics) for four hours. Values recorded are in terms of resistance to permeability. Data was analyzed using ECIS software (Applied Biophysics) and GraphPad Prism (GraphPad Software).

SVEC4-10 murine lymphatic endothelial cells were incubated in normal media or media with 5% ascites sera for 24 hours. Cells were then collected, fixed in 3%PFA for 15 minutes, permeabilized with 90% methanol for 30 minutes, and stained according to our flow cytometry protocol with Alexa Fluor488 anti-VE-Cadherin (1:100, eBioscience).

### **Statistical analysis**

Data are presented as mean plus or minus SEM. Statistical comparisons between groups were performed using the Student *t* test.



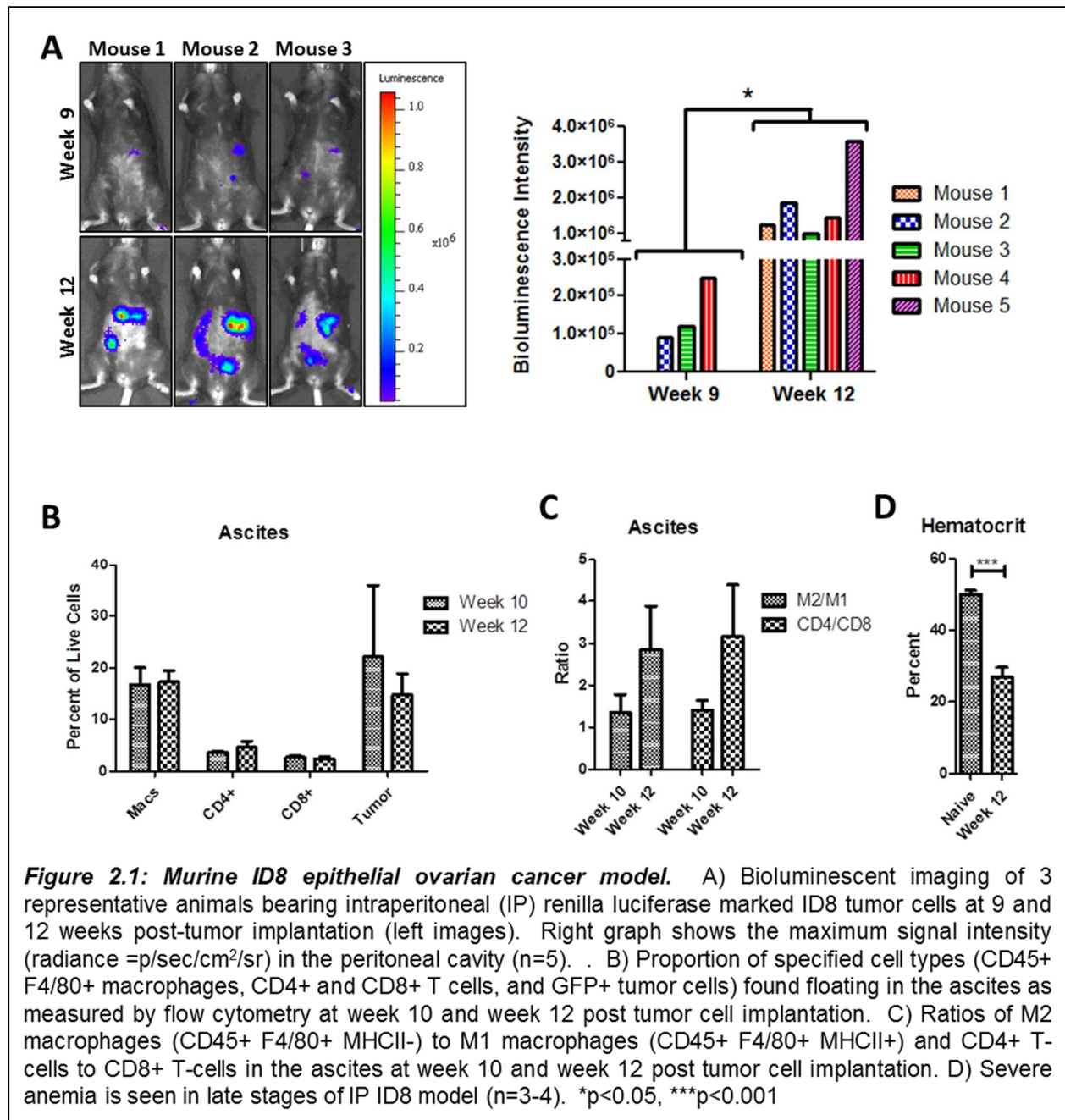
## RESULTS

### **The ID8 Murine EOC Model Mirrors Late-Stages of Human Disease with Malignant Ascites**

The ID8 is a well-studied murine serous EOC model (71–73). The full complement of immune system of this model is particularly favorable to investigate the innate immune response of the myeloid cells. The ID8 cells were marked with renilla luciferase, enabling longitudinal monitoring of tumor growth and dissemination by non-invasive bioluminescent imaging. For the intraperitoneal model, bioluminescent signals from the tumor cells first became detectable at around week 9 post-injection, which also corresponded to the time that ascites began to accumulate (Figure 2.1A), and the tumors grew rapidly from this point onward. By week 12, the tumors had grown throughout the peritoneal cavity (Figure 2.1A) and ascites greatly distended the abdomen. The substantial ascites found in each mouse was hemorrhagic and tumor nodules had spread to the mesenteries, peritoneal wall, liver, and fat pads (Figure 2.2A). The same pattern of peritoneal dissemination and ascites formation were also observed in the orthotopic ID8 model, implanted into the ovarian bursa (Figure 2.2B). Taken together, both the intraperitoneal and intrabursal ID8 model recapitulate important characteristics of human EOC with slow initial growth that progressed to dispersed peritoneal metastasis, and massive ascites.

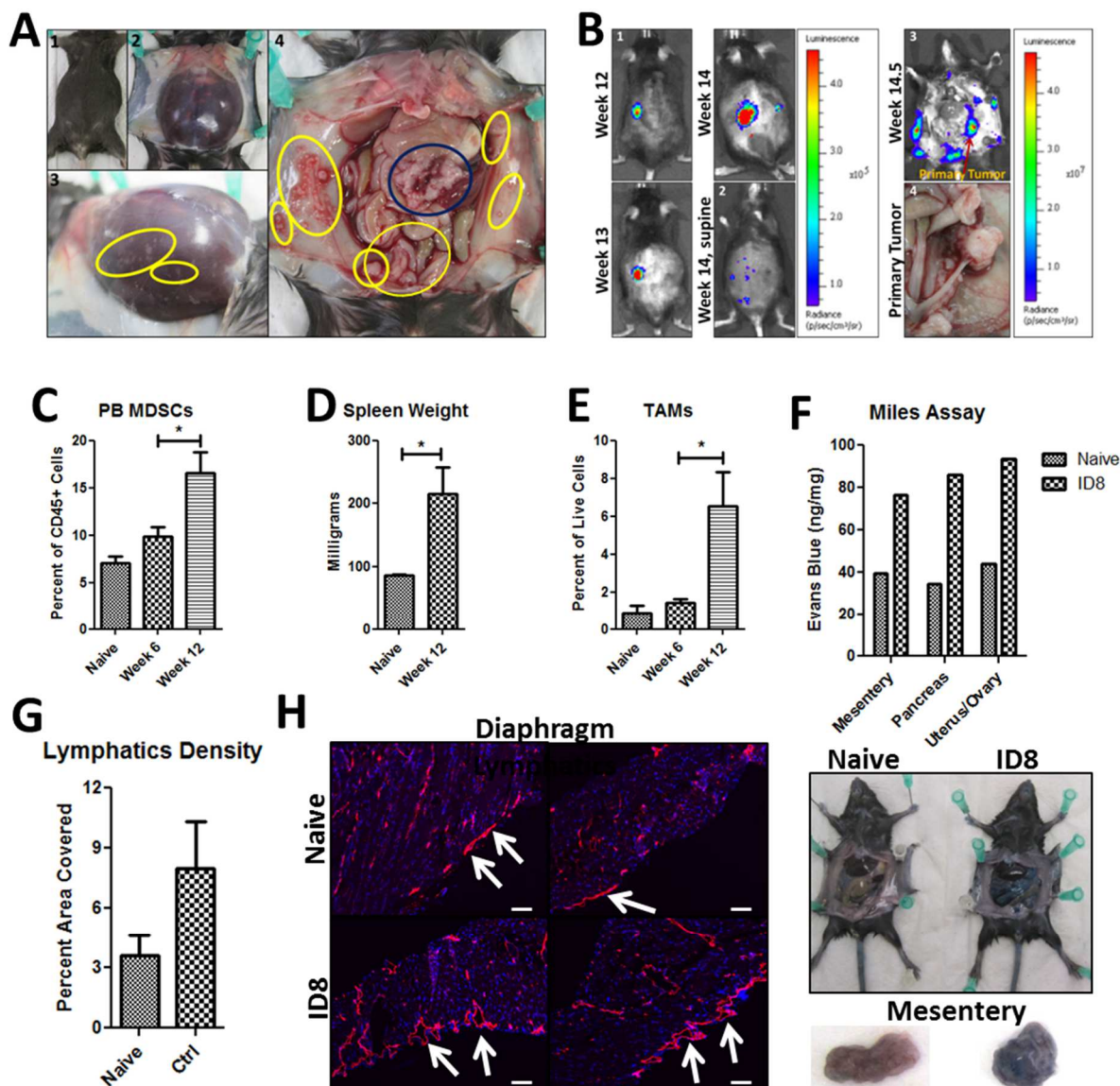
### **Immune and Vascular Dysregulation Worsen As EOC Progresses**

Immune dysregulation in cancer patients often results in the systemic expansion of myeloid cell populations that can be observed in the peripheral blood, lymphoid organs and at the tumor (74). This expansion of circulating and infiltrating myeloid cells is also associated with worse prognosis (43). Consistent with this finding, we observed the progressive increase in the immature myeloid cells also known as myeloid-derived suppressor cells (MDSCs) in the peripheral blood in the ID8 model from weeks 6 to 12 post tumor injection (Figure 2.2C). Increases of MDSCs and macrophages in the spleen and lymph nodes (data not shown) and splenomegaly (Figure 2.2D)



were also observed consistently. The systemic expansion of the myeloid population was also manifested in the tumor, resulting in a significant increase in TAM content over time (Figure 2.2E).

Next we analyzed the immune cell content in the ascites fluid and found that macrophages and floating tumor cells were the majority of viable cells in the ascites fluid at both week 10 (the

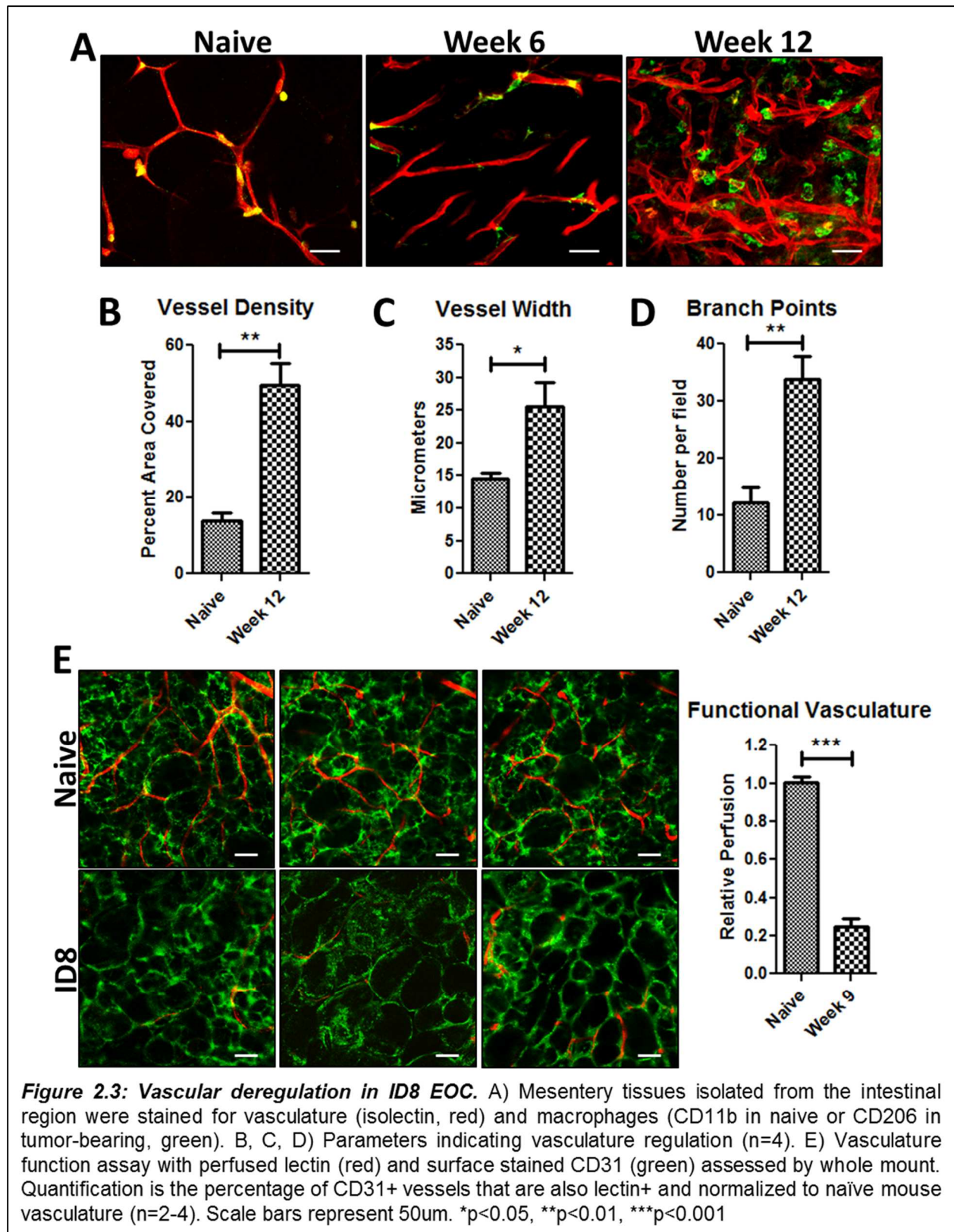


**Figure 2.2: Murine ID8 EOC dissemination and systemic effects.** A) Gross observations of the IP model at end stage. Picture 1.) Ascites bloats the peritoneal cavity. Picture 2.) The ascites is hemorrhagic. Picture 3.) Tumor nodules can be seen attached to the peritoneal wall (yellow circles). Picture 4.) Nodules, circled in yellow, are spread throughout peritoneal wall and cavity. B) Gross observations of the orthotopic model. Picture 1.) Bioluminescent signal becomes visible around week 12 post-implantation. Picture 2.) By week 14, metastases throughout the abdomen were seen, which is confirmed by *in situ* bioluminescent imaging (Picture 3). Picture 4 is the gross image of orthotopic primary tumor *in situ*. C) An accumulation of MDSCs (CD45+ CD11b+ Gr-1+) is found in the peripheral blood (n=3-4). D) Splenomegaly is consistently seen in the late stages of ID8 cancer. E) During the progression of IP ID8 cancer, flow cytometry shows an accumulation of macrophages (CD45+ F4/80+) in the area that becomes the "primary" tumor (n=3-4). F) Results of Miles assay on naïve and tumor-bearing mice. Pictures below show the visible increase of accumulated blue dye in tissues in the peritoneum of the tumor-bearing mouse *in situ* and *ex vivo* mesentery, in comparison to the naïve animal. G) Density of mesentery lymphatic vessels. H) Diaphragm immunofluorescence stain showing lymphatic vessel (LYVE1, red) and DAPI stains. Arrows point out representative vessels. Scale bars represent 100um. \*p<0.05

eventual start time of treatments) and week 12 (Figure 2.1B). The immune cells in ID8 ascites consist of a high proportion of immunosuppressive and protumorigenic subtype, as the MHCII<sup>+</sup>M2 to MHCII<sup>+</sup>M1 macrophage and CD4 to CD8 T cell ratios were both around 3 to1 at week 12, an increase from the ratios at week 10 (Figure 2.1C). Large numbers of macrophages and a high ratio of CD4 to CD8 T cell infiltration have previously been associated with poor prognosis in breast cancer (75). We modified this immune cell signature by including the cell ratio of M2 to M1 macrophages in the ascites fluid. High ratios of M2/M1 and CD4/CD8 are likely to be indicative of cancer-supporting immune dysregulation in the host with EOC.

Dysfunction of vasculature is one of the known etiologies of malignant ascites (57). The hemorrhagic nature of the ascites in the ID8 model indicates that vascular leakage and extravasation of red blood cells is occurring. Hence, the ID8 tumor-bearing mice developed severe anemia in the late stages of the disease (Figure 2.1D). Close examination of mesentery blood vasculature revealed that by week 12 the vasculature was highly disorganized (Figure 2.3A), with greatly increased vessel density (Figure 2.3B), vessel width (Figure 2.3C), and number of branch points (Figure 2.3D). The *in vivo* vascular function in the animals were further examined with the Miles assay, which assess vascular leakage by the extravasation of Evans Blue dye from circulation into tissues, and a lectin perfusion assay. ID8 tumor-bearing mice displayed clear vascular leakage compared to naïve animals (Figure 2.2F). In contrast to the robust lectin perfusion observed in the mesentery capillaries of naïve animals, the vessel perfusion function in the mesentery capillaries of tumor-bearing mice was significantly decreased, to about 25% of normal (Figure 2.3E). No notable difference in perfusion in the larger mesentery arterioles was observed between tumor-bearing and naïve animals (data not shown), indicating that the leakage of blood and ascites fluid is occurring at the capillary level. Parallel the dysregulated blood vasculature, tumor-bearing mice displayed increased lymphatic density in the mesentery (Figure



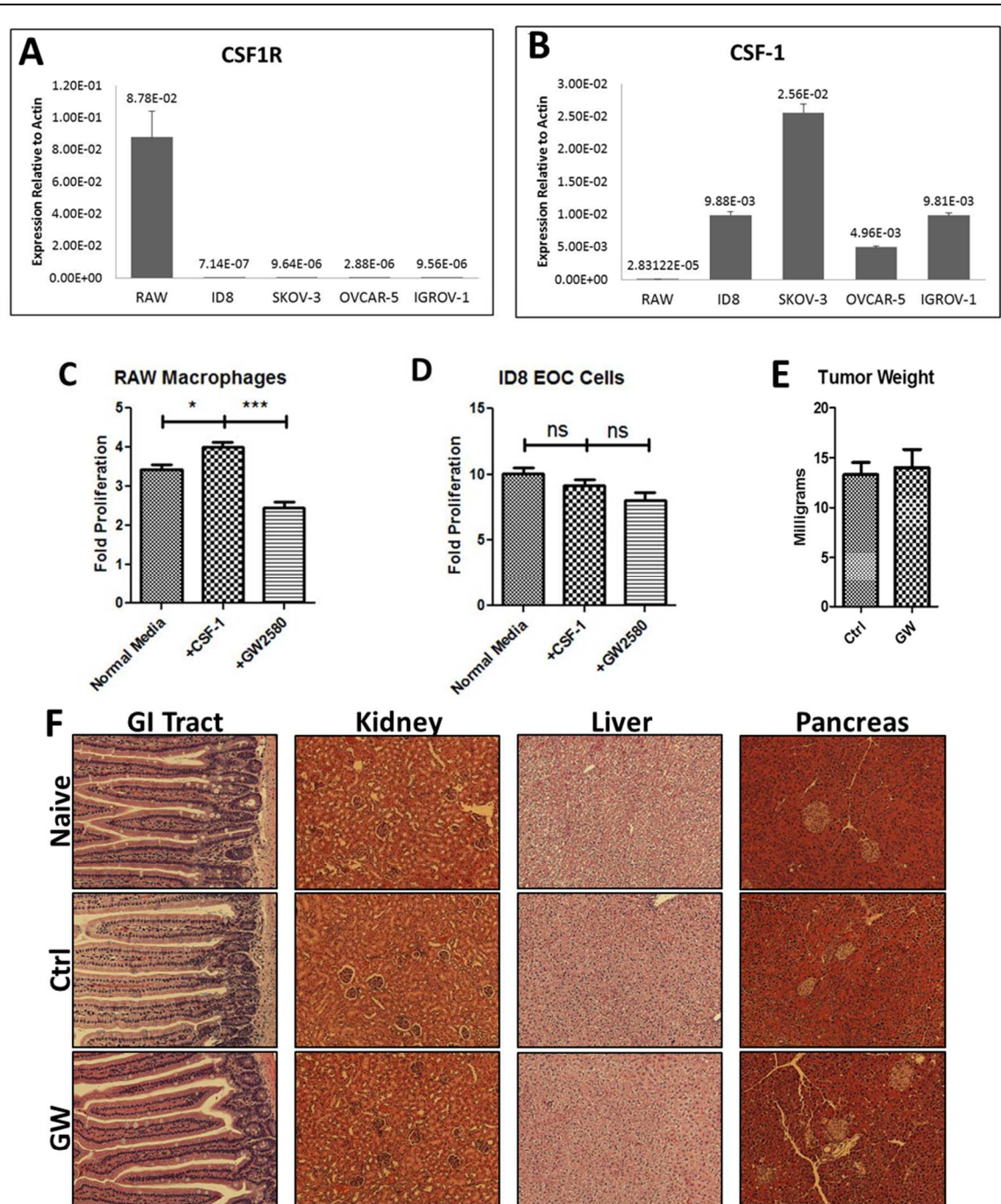


2.2G), and tortuous lymphatics with enlarged lumen (Figure 2.2H). Although these findings are

consistent with known lymphatic vascular and lymph drainage dysfunction in EOC malignant ascites (76,77), they require further functional verification. Suffice to say, the preclinical data presented so far support that macrophages are playing a pivotal role in the pathogenesis of EOC malignant ascites. To further verify this assertion, we pursued a therapeutic approach to block macrophage function in the ID8 EOC model.

### **Suppressing Macrophage Function with CSF1R Blockade Ameliorated the Vascular Dysfunction of Malignant Ascites of EOC**

Since the CSF-1/CSF1R axis is known to be a critical pathway in the development and function of myeloid cells and macrophages, we used a highly selective CSF1R inhibitor, GW2580 (78) to treat mice during the late stages of ID8 EOC. We and others have shown that GW2580 is able to selectively and effectively inhibit the protumorigenic functions of TAMs in several tumor models, including prostate, breast, and lung cancer (79–81). A confounding issue in EOC is that CSF-1 and CSF1R were found to be expressed in human ovarian cancer and this signaling pathway has been implicated to have a tumor-intrinsic role in promoting EOC oncogenesis (68). Although ID8 tumor cells express a moderate level of CSF-1 comparing to several other human EOC lines (Figure 2.4A), this model expresses negligible level of CSF1R that is more than 5 orders of magnitude below that expressed in a macrophage cell line and bone marrow derived macrophages (data not shown), and 5 to 10 fold lower than 3 other human ovarian cancer lines (Figure 2.4B). Furthermore, unlike macrophages, ID8 cells were not responsive to CSF1 induction or CSF1R blockade *in vitro* (79) (Figure 2.4C, D) and subcutaneous ID8 tumor growth was not affected by GW250 treatment (Figure 2.4E). There were also no off-target effects or organ toxicity with the treatment (Figure 2.4F) (78,79). Thus, we conclude that the therapeutic action of CSF1R inhibition is directed at macrophages and not at the ID8 tumor cells.



**Figure 2.4: CSF1R and CSF-1 in ovarian cancer cells and off-target effects.** A) RT-PCR showing negligible CSF1R expression in ID8 cells and three human EOC cell lines as compared to positive control RAW macrophages. B) RT-PCR showing all EOC cell lines express CSF-1. C) Proliferation assay showing that having functional CSF1R makes RAW macrophages sensitive to both CSF-1 and GW2580 (n=4). D) GW2580 does not significantly alter CSF1R negative ID8 cell proliferation. E) Subcutaneous ID8 tumor weight after two weeks of diluent or GW2580 treatment (n=3). F) Histology of gastrointestinal tract, kidney, liver, and pancreas of naive, control, and GW2580-treated mice with IP tumors. \*p<0.05, \*\*\*p<0.001

Female mice bearing intraperitoneal implanted ID8 tumors were allowed to progress to late stage,  
20

when ascites developed, and then treated with diluent or GW2580 for two more weeks (Figure 2.5A). Control diluent-treated mice continued to accumulate ascites while GW2580 treatment resulted in a significant reduction of ascites, down from an average volume of 6.2 ml/control animal to 1.9 ml/ treated animal (Figure 2.5B), and prevented the development of severe anemia (Figure 2.5C). Notably, GW2580 treatment significantly altered the content of macrophages in the ascites. Over the two weeks of treatment course, the percentage of floating ascites macrophages in the control cohort increased significantly while those in the GW2580-treated ascites reduced significantly (Figure 2.5D). For instance, in 1 experiment, the absolute number of M2 macrophages decreased from  $41.9 \pm 15.9$  million to  $2.9 \pm 1.7$  million ( $p < 0.05$ ), the absolute number of M1 macrophages was not significantly affected by the GW2580 treatment, and CD8 T cell increased from  $0.68 \pm 0.16$  million to  $2.2 \pm 1.4$  million in ascites by GW2580 treatment (Figure 2.5E, Figure 2.6A). Notably, the high 3:1 ratio of M2:M1 macrophage and CD4:CD8 T cell were reduced to approximately 1:1 by GW2580 treatment (Figure 2.5F). Significantly more of the GW-treated ascites macrophages expressed CCR2 (Figure 2.5G), and many more of those macrophages expressed interferon gamma and IL-12 compared to control ascites macrophages (Figure 2.6B, C). These results indicate that inhibiting macrophage function with CSF1R blockade was able to reverse the protumorigenic, immunosuppressive phenotypes of the ascites immune cells.

Given the significant reduction of ascites fluid volume, the critical issue to unveil is the impact of CSF1R blockade on the peritoneal vasculature. Upon staining of the mesentery blood vasculature, a clear normalization was seen in GW2580-treated mice (Figure 2.7A). Along with the reduction in vessel density (Figure 2.7B), there is a concomitant decrease in vessel width (Figure 2.7C), tortuosity and branch points (Figure 2.7D). We further explored the blood vascular dysregulation in a second EOC model, namely the human OVCAR3 xenograft model. As seen in



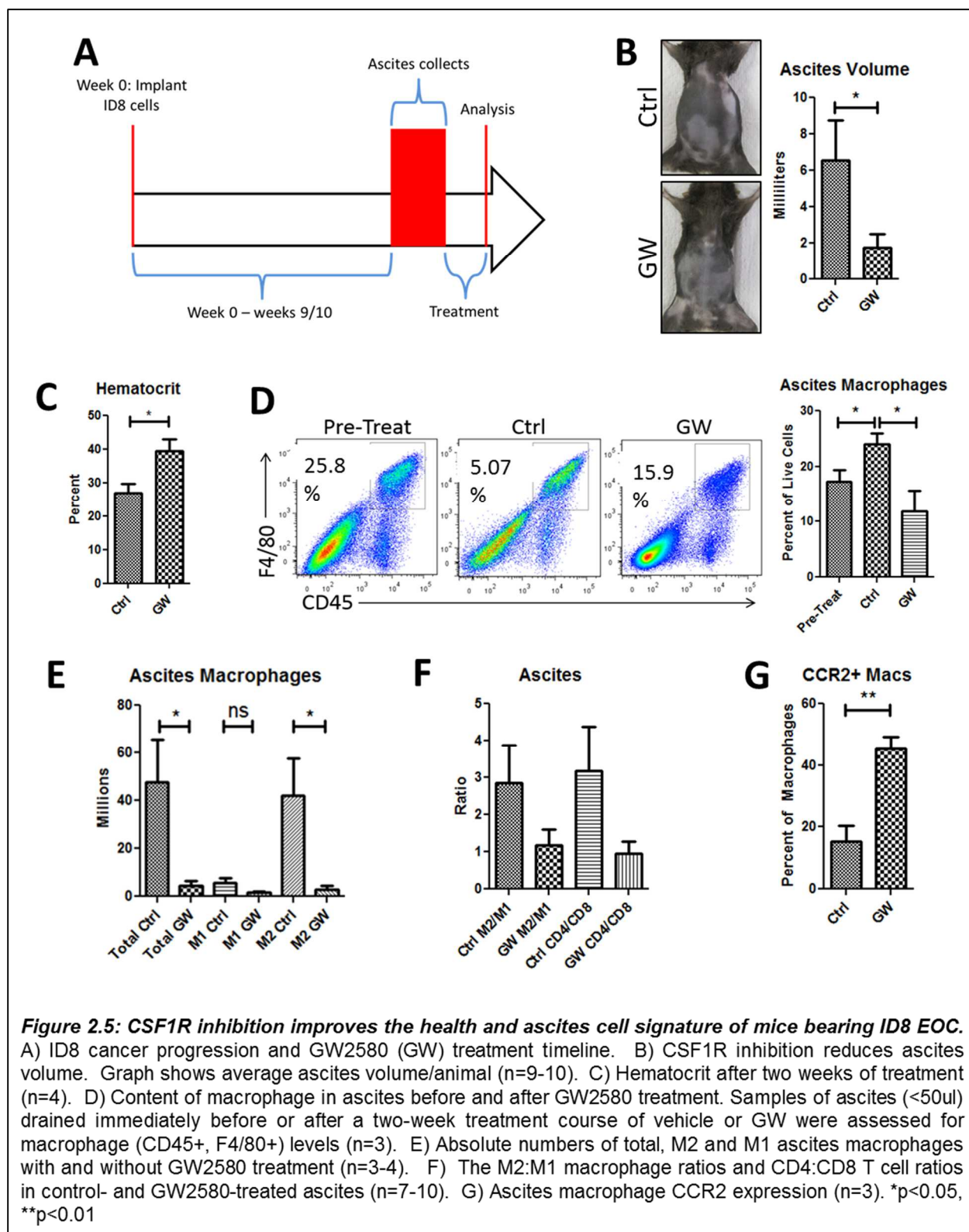
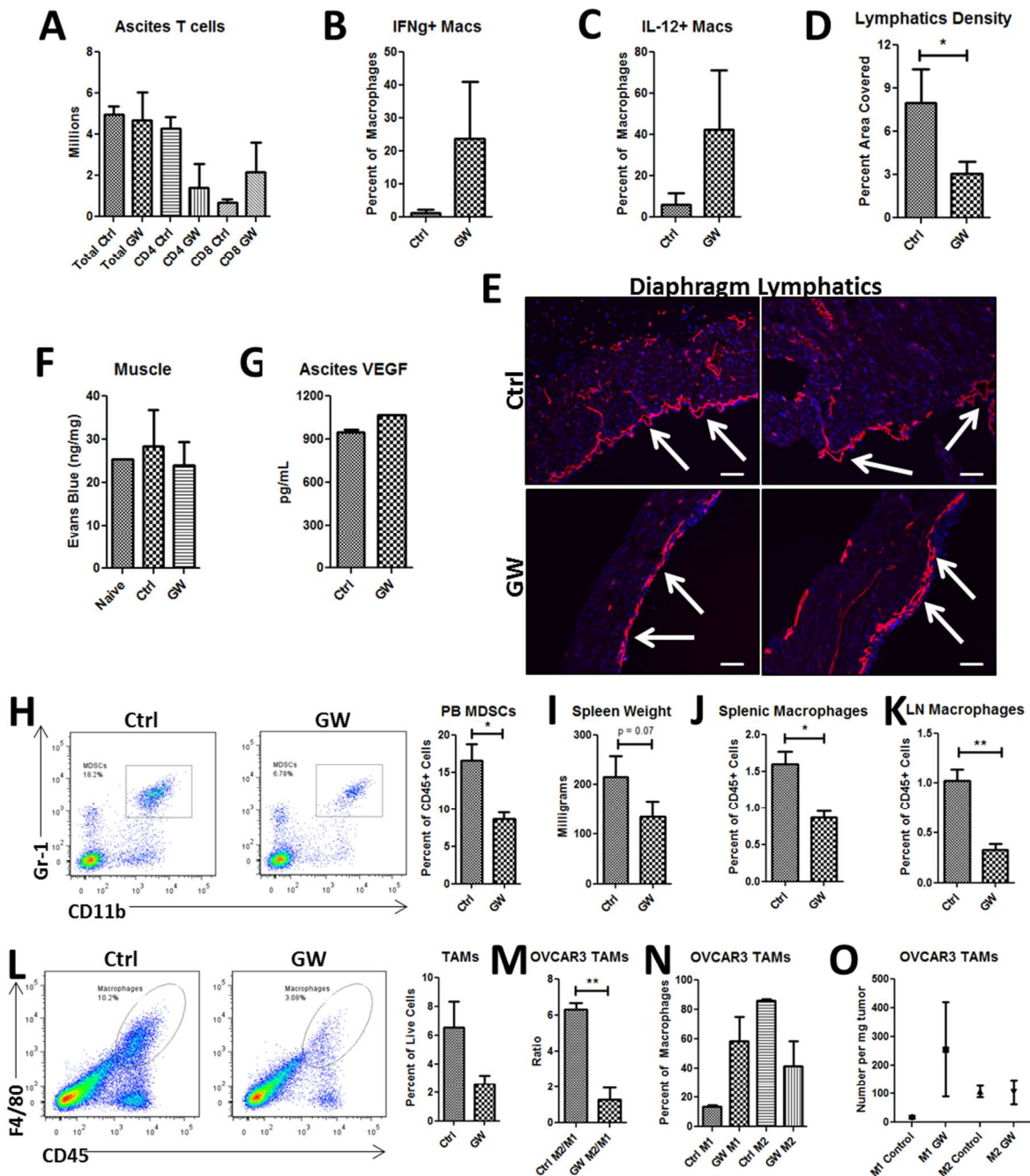


Figure 2.7E, GW2580 treatment again normalized the dysregulated mesentery blood vasculature

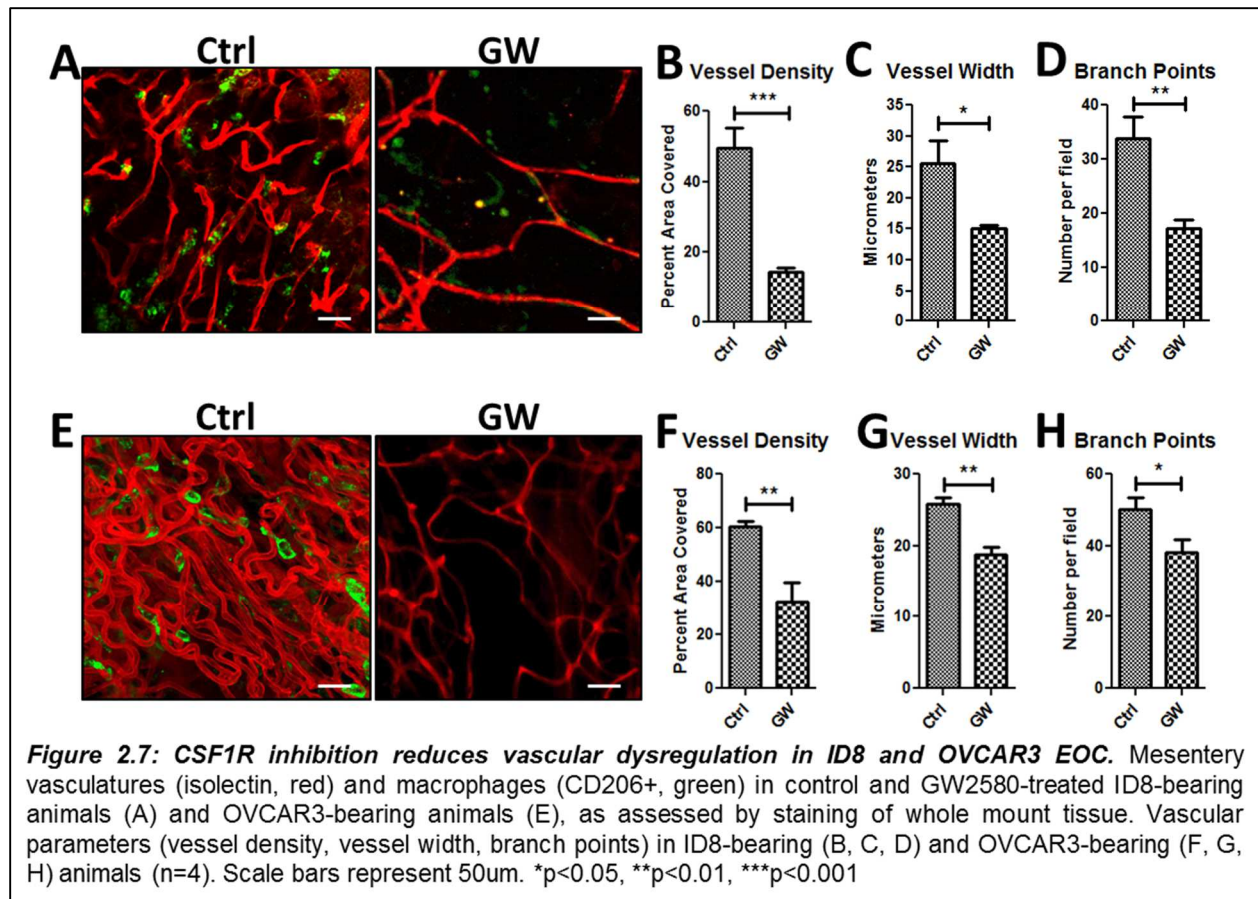


**Figure 2.6: Systemic effects of GW2580 treatment.** A) With GW2580 treatment, the absolute numbers of total ascites T cells do not change, while the absolute number of CD4+ T cells decreases and the absolute number of CD8+ T cells increases (n=2-3). B) Interferon gamma expression in ascites macrophages (n=3). C) IL-12 expression in ascites macrophages (n=3). D) Density of mesentery lymphatic vessels changed with GW2580 treatment. E) Immunofluorescence stain of diaphragm showing lymphatic vessel (LYVE1, red) and nuclei (DAPI). Arrows point out representative vessels.

**Figure 2.6 continued.** F) No change seen in muscle compartment with Evans Blue extravasation. G) VEGF ELISA performed on ascites H) Circulating MDSC levels are high during late-stage ID8 ovarian cancer, but they are reduced by CSF1R inhibition (n=3- 4). I) CSF1R inhibition lessens splenomegaly. J and K) Macrophages (CD45+ F4/80+) are elevated in the lymph nodes and spleen in late-stage ID8 cancer (data not shown), but are significantly reduced with CSF1R inhibition (n=4). L) CSF1R blockade reduced macrophage infiltration in tumors of GW2580-treated animals when compared to vehicle-treated animals. (n=4). M and N) The polarization of OVCAR3 TAMs as assessed by CD45+, F4/80+, and MHCII+ (M1) and CD45+, F4/80+, MHCII- (M2). O) Number of OVCAR3 TAMs per mg tumor, with polarization assessed in L and M. Scale bars represent 100um. \*p<0.05, \*\*p<0.01

as indicated by a significant reduction of vessel density, width, and branch points (Figures 2.7F, 2.7G, 2.7H). The lymphatic vasculature density as well as the tortuosity and patent lumen of the lymphatics are all decreased with GW2580 treatment (Figure 2.6D,E).

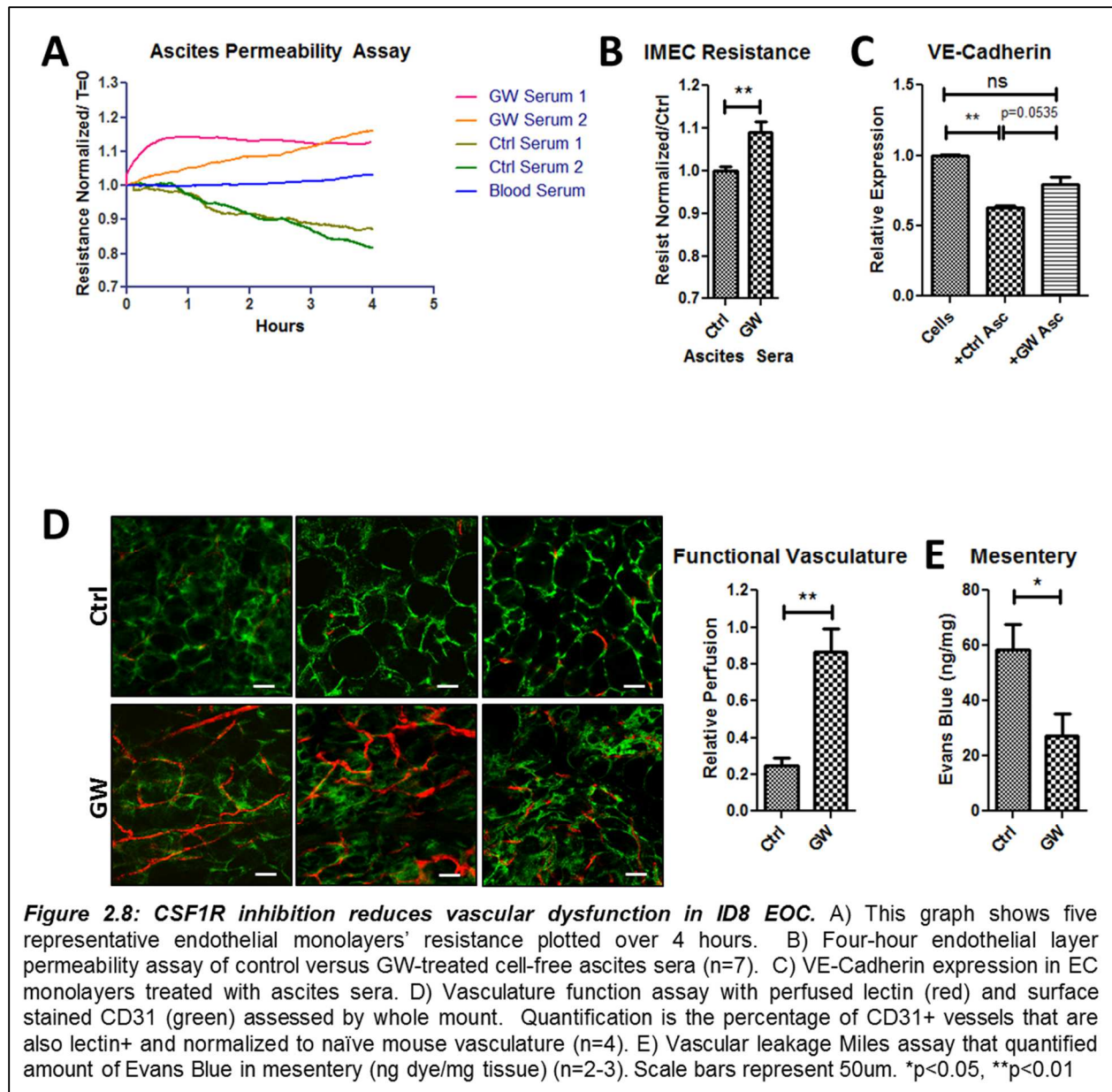
To further assess the vascular leakage-causing potential of malignant ascites, we performed additional EC permeability assays with control or GW2580-treated mouse ascites sera. As shown in Figure 2.8A, unlike normal murine blood serum that did not alter the (ECIS) EC permeability over 4 hours, the ascites serum from control (untreated) ID8 tumor bearing animals induced a reduction in EC resistance, reaching a level that is about 15-20% below normal blood serum. In contrast, the addition of ascites sera from GW2580-treated mice resulted in an immediate increase in EC resistance reaching a level that is 15% higher than control mouse sera. Although the magnitude of change in EC resistance was not large, the direction of change was very consistent. Analyses on ascites sera from seven control and seven GW-treated mice across 3 different studies showed a significant increase in EC resistance in the GW-treated over control mice (\*\*p<0.01) (Figure 2.8B). Endothelial permeability is regulated by cell-cell adherens junctions, which are largely composed of vascular endothelial cadherin (82). The downregulation of this endothelium-specific cadherin from the plasma membrane of ECs leads to increased vascular permeability (83). The ability of ascites sera to induce endothelial permeability was further assessed by their impact on the surface VE-cadherin expression on the ECs. Incubating ECs with control (untreated) ascites serum led to a significant downregulation of VE-cadherin



expression (relative to normal media), while GW-treated ascites did not significantly reduced VE-cadherin expression (Figure 2.8C).

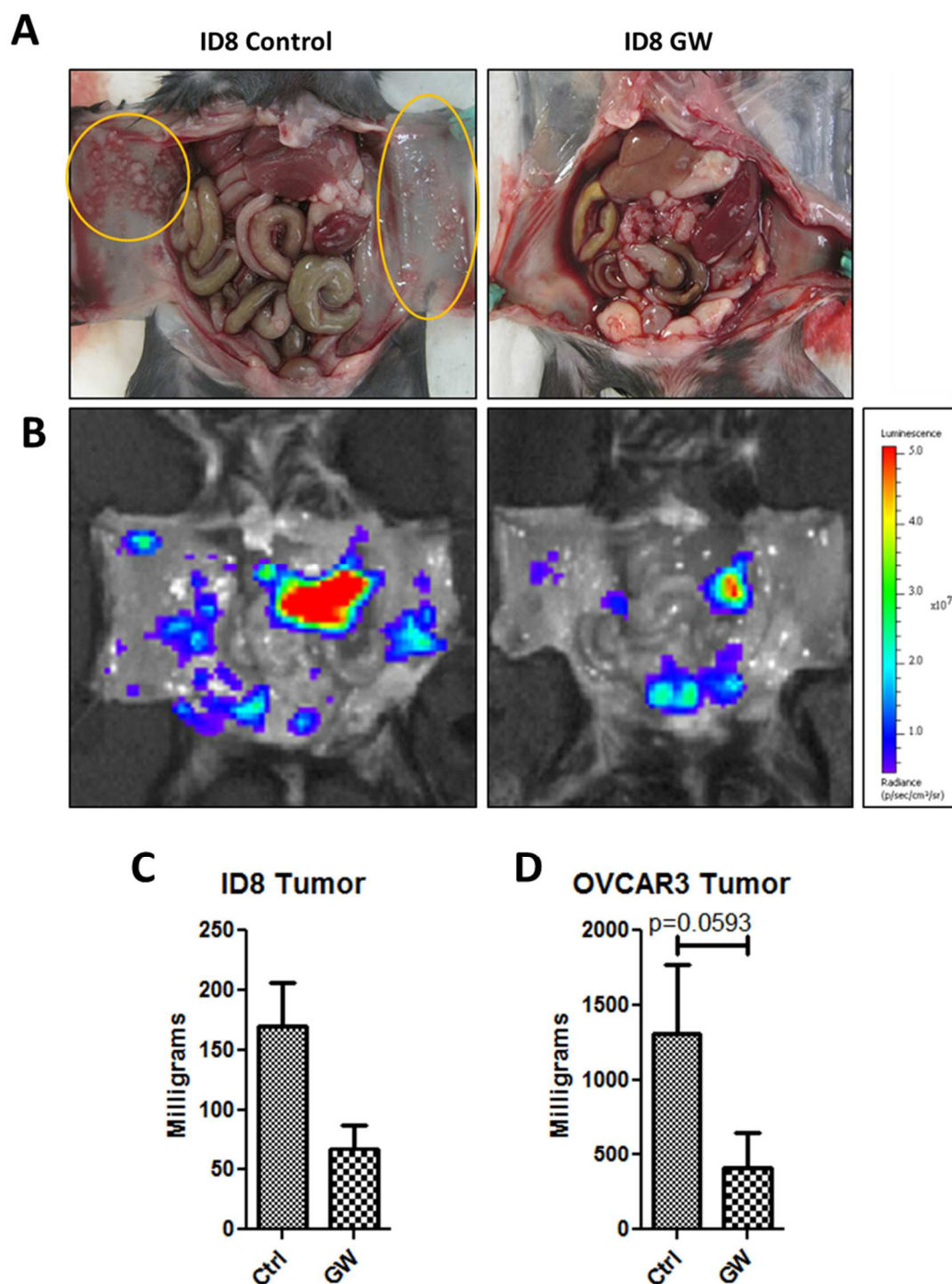
The improvement of *in vivo* vascular function upon CSF1R blockade was further verified by the significant increase in the number of perfused, blood-carrying capillaries (Figure 2.8D) and the reduced vascular leakage assessed by the Miles assay (Figure 2.8E) in the mesentery of GW2580-treated mice compared to that from the untreated control. Notably, the increased vascular leakage was largely limited to the tumor-bearing peritoneal compartment as no significant difference was observed in the muscles of naïve, control- or GW-treated animals (Figure 2.6F). Although VEGF is well-known to contribute to both blood and lymphatic vascular dysregulation in cancer, the vascular normalization observed with GW2580 treatment happened





without a change in the very high VEGF levels observed in the ascites serum of ID8 EOC model (Figure 2.6G). This result suggests that other factor(s) may be counteracting the impact of VEGF as a result of the CSF1R blocking treatment.

Although the main focus here is to decipher the influences of myeloid cells/macrophages on malignant ascites, it is clear that the CSF1R blockade treatment has a major systemic impact. As



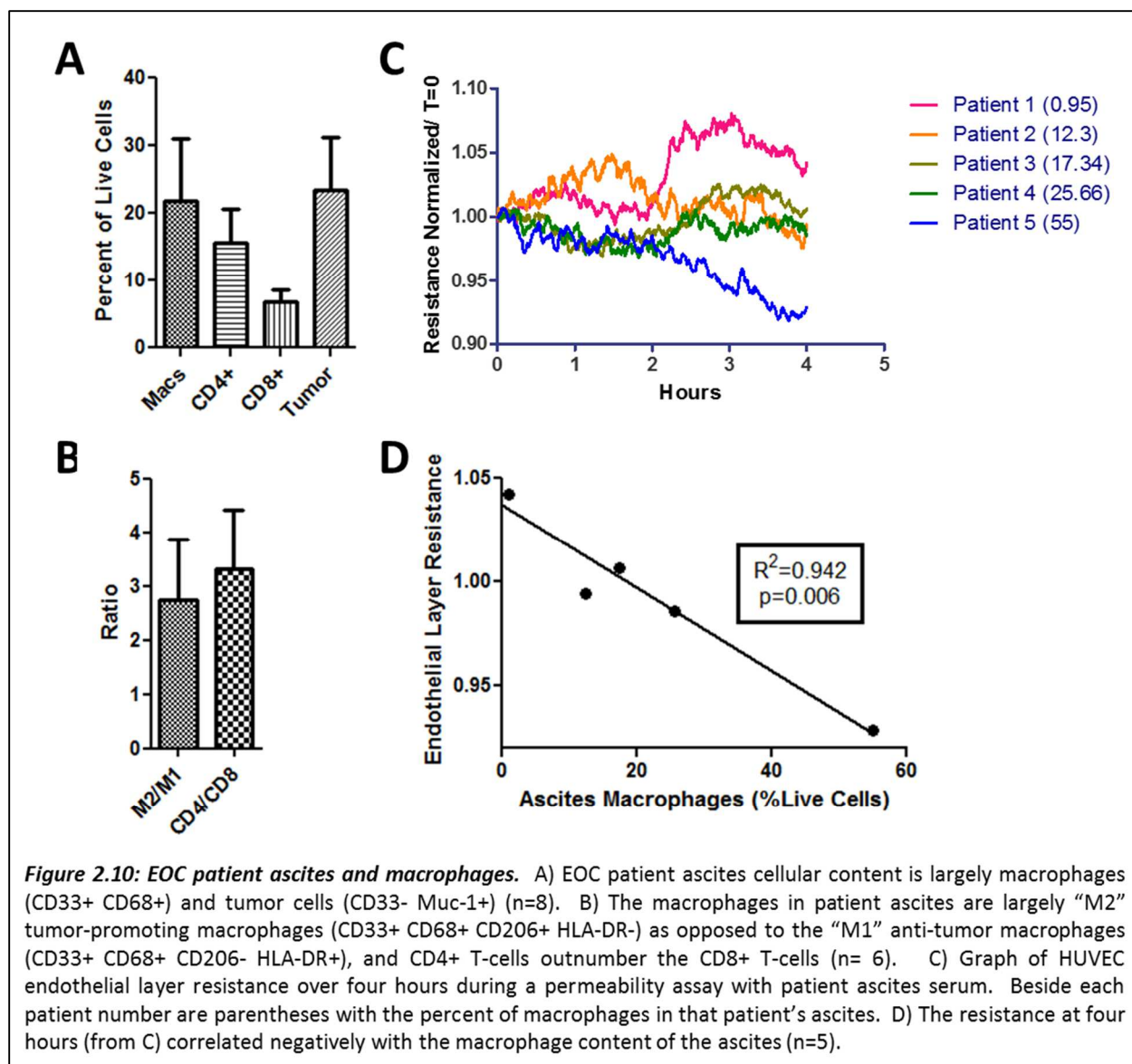
**Figure 2.9: Tumor growth and spread is reduced with CSF1R inhibition.** A) Control ID8-bearing mice have widespread disseminated metastasis, while GW-treated mice have fewer visible nodules. B) This lessened tumor burden in the ID8 model is confirmed by *in situ* bioluminescent imaging. C) This trend of decreasing tumor burden in the ID8 model is quantified to the right from one study (n=4). D) Decreased tumor burden is also observed in the OVCAR3 model (n=2-4).

shown in Figure 2.6, GW2580 treatment significantly reduced peripheral blood MDSCs (Figure 2.6H), spleen weight (Figure 2.6I) and splenic myeloid cells (Figure 2.6J), as well as macrophages in lymph nodes (Figure 2.6K). CSF1R blockade also significantly reduced TAMs in the tumor (Figure 2.6L). Similar to what was seen in the ID8 model, the TAMs in the OVCAR3 model showed significant polarization toward a less pro-tumorigenic phenotype with GW2580 treatment (Figure 2.6M and 2.6N). This appeared to be due to an influx of the M1 macrophages upon treatment (Figure 2.6O). Of interest, the short 2-week GW2580 treatment appeared to reduce the overall intraperitoneal tumor burden in both the ID8 model (Figure 2.9A, B, C) and the OVCAR3 model (Figure 2.9D). In light of the same treatment having no effect on subcutaneous tumor burden (Figure 2.4E) we conclude that the ascites microenvironment becoming unfavorable for tumor growth could be the cause.

### **Increased Macrophage Presence in Patient Ascites Predicts Endothelial Cell Permeability**

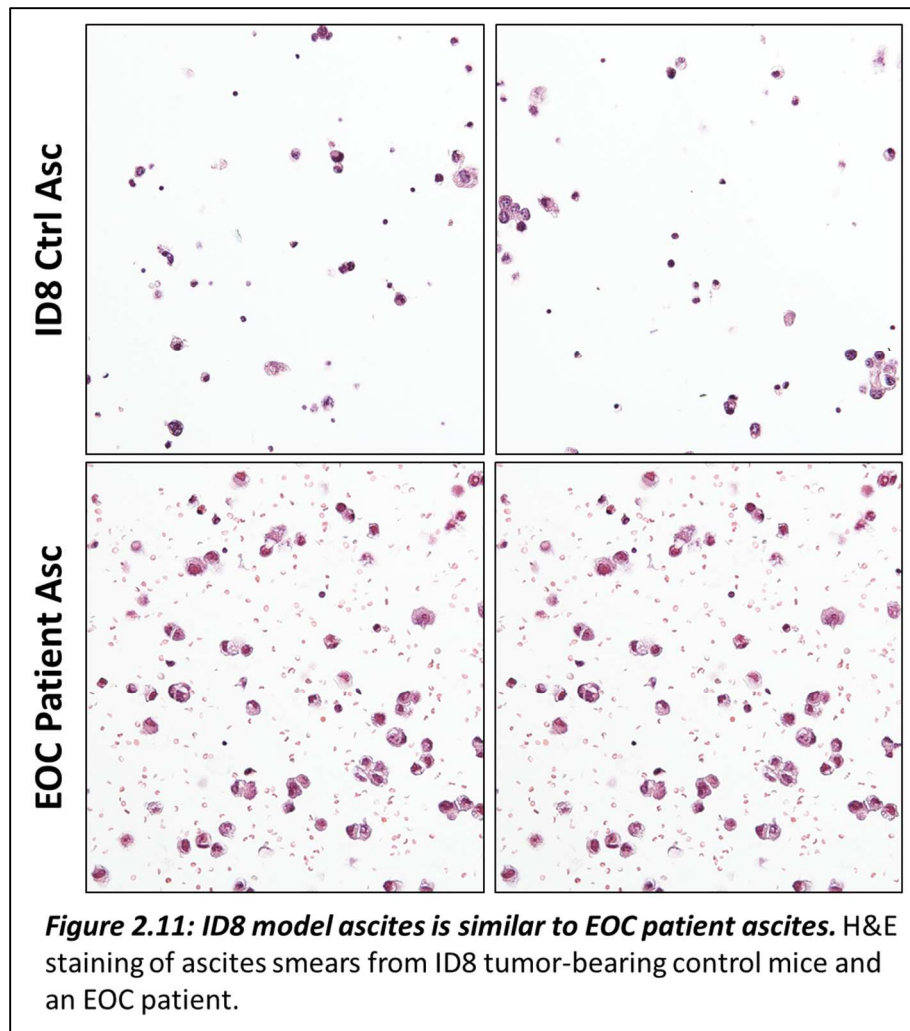
Next, we explored whether the macrophage findings in the murine models corroborate those in EOC patients. In freshly isolated samples of ascites fluid from patients, macrophages and floating tumor cells again constituted the majority of viable cells in the ascites fluid (Figure 2.10A). The M2 macrophages (CD33+ CD68+ MHCII- CD206+) outnumber the M1 macrophages (CD33+ CD68+ MHCII+ CD206-) by almost 3:1 and CD4 T cells outnumber CD8 T cells by more than 3:1 (Figure 2.10B). Interestingly, the proportion and phenotype of the patients' ascites immune cells is almost identical to that of the ID8 model (Figure 2.1B and 2.1C, Figure 2.11).

The ability of patients' ascites serum to induce vascular permeability of HUVECs was measured by the ECIS assay. Analyses on five patient ascites sera showed that the two-hour mark represent the inflection point of this assay, where the endothelial cell (EC) resistance began to change (Figure 2.10C). Interestingly, the macrophage content in the ascites (as % of live cells)



significantly correlated with the inverse of EC resistance (Figure 2.10D). In another words, the higher the number of macrophages in a patient's ascites, the higher the vascular permeability (i.e. loss of EC resistance) when the ascites serum is placed on ECs. These results from clinical specimens are suggestive of a pathological contribution of macrophages to vascular dysfunction, reminiscent of our findings in the murine models.





Collectively, the results from this therapeutic study support that protumorigenic macrophages are playing an instrumental role in the vascular dysfunction causing EOC malignant ascites. Inhibiting the macrophages' function with selective CSF1R blockade not only dramatically reversed the vascular pathology but also improved systematic environment that might be more favorable to reject the tumor. Thus, inhibiting the protumorigenic influences of myeloid cells/macrophages could be a part of a comprehensive treatment plan, to improve the outcome of EOC.

## DISCUSSION

Malignant ascites is a devastating complication of EOC that greatly lowers the quality of life of patients at late stages of the disease (57). Current treatment options for malignant ascites are largely ineffective and have high rate of complications (57). Recent research and results from clinical trials showed that VEGF is a promising therapeutic target, especially for malignant ascites (34,35,63,64). However, the notable risk of severe and even deadly side effects coupled with the lack of long term survival benefits of VEGF-targeted therapies raised significant concern on their use. In this study, we postulate that broadening the therapeutic target to a particular immune cell population, namely macrophages, could be advantageous over VEGF-specific approaches. The protumorigenic TAMs are known to promote angiogenesis through VEGF-dependent and independent means as well as heightening the immunosuppressive state of tumor microenvironment (43,65). Thus, simultaneously blocking multiple prongs of TAMs' influences might be more effective than block the single VEGF axis. Furthermore, therapeutic resistance to overcome the blockade of a single pathway is much more straight-forward than against a multi-prong-targeted therapy.

This study provides supportive evidence that protumorigenic M2 macrophages promote the vascular leakage causing EOC malignant ascites. We observed that higher macrophage content in human ascites sera was also associated with increased vascular permeability. Detailed characterization of the murine ID8 EOC model also showed that M2 macrophages expanded and infiltrated the peritoneal vasculature over time and were linked to progressive vascular dysregulation, leaky vessels and ascites formation. Using a selective CSF1R inhibitor, GW2580, to block macrophage function in late stages of EOC reversed the vascular dysfunction and greatly reduced ascites accumulation. The vascular normalization is likely secondary to the lowered macrophage/myeloid cell contributions in tumor bearing sites throughout the peritoneum as well

as systematically, after GW2580 treatment. We deduced that one component of the macrophage-mediated vascular dysfunction is dictated through soluble factors as cell-free ascites sera from untreated animals induced higher EC permeability. In this exploratory study we did not address the specific soluble factor(s) induced by macrophages that might be promoting or protecting vascular permeability in malignant ascites of EOC. However, we believe VEGF is not the culprit. VEGF protein levels in the ID8 ascites is very high, several orders of magnitude higher than in peripheral blood. In GW2580-treated ascites sera, VEGF levels remained very high, often exceeding that of untreated sera. It is interesting to note that ascites serum from GW2580-treated mice consistently induced a higher EC resistance when compared to normal blood serum. This result could indicate the presence of a protective factor(s) against vascular leak in the GW-treated sera, especially to counteract the permeability effects of VEGF.

Another layer of complexity in this macrophage-induced vascular dysfunction is the close cell-cell contact and cross-talk between macrophages and EC cells that have been reported in developmental and pathological settings. Our recent study showed that ECs provide a specific niche for the proliferation and differentiation of macrophages (84) and macrophages are often recruited to sites of vascular remodeling during embryonic development (85). Kubota et al (86) showed that macrophages play a key role in pathological neovascularization of ischemic retinopathy. Reduction of the macrophages' number and function either by genetic deletion of CSF-1 (M-CSF) or by a CSF1R (c-fms) kinase inhibitor, Ki20227, was able to correct the vascular pathology. In oncology, physical interactions between macrophages and endothelial cells has been described in the context of breast cancer metastasis facilitation (87,88). Clearly, the functional crosstalk between macrophages and ECs is an important topic in cancer and vascular biology.

Although the precise molecular mechanism of the macrophage-driven vascular dysfunction is yet to be determined, our results support that therapies that inhibit macrophage functions will be a rational strategy to manage ascites. Besides the CSF1/CSF1R pathway, several other cytokine/chemokine axes have been reported to regulate myeloid cell recruitment and function. For instance, inhibiting G-CSF, CCL2 and CXCR4 have been shown to be effective in abrogating the functions of tumor-infiltrating myeloid cells (43,89,90). Of note, we and others consistently observed the downregulation of CCL2 after inhibition of CSF1R signaling in different cancer cell lines and tumor models (80,91). We further demonstrated that myeloid cells within the tumor of GW2580-treated mice showed reduced CCL2 expression, suggesting a direct link between CSF1 and CCL2 (92). These data suggest that CSF1/CSF1R could be a dominant driver pathway in the function of TAMs. From the drug development perspective, the CSF1/CSF1R axis is a promising target due to the wealth of pharmacological agents available. For instance, selective monoclonal antibodies that target either the ligand or the receptor, and numerous small molecule CSF1R tyrosine kinase inhibitors, exhibiting variable target selectivity, have been developed in the last 10 years (78,93,94). Many of these agents are in early phase clinical investigation for inflammatory diseases and cancer. The CSF1R inhibitor PLX3397 is the furthest along in clinical testing, especially in oncology application. Paralleling our finding that daily oral GW2580 regimen (160 mg/kg) given to mice over 1 month resulted in no discernable toxicity, early clinical trial findings showed that PLX3397 appeared to be well-tolerated in patients with advanced cancer (94).

Recent therapeutic developments for EOC favored intraperitoneal-directed cisplatin delivery as this route was shown to prolong the survival of patients over systemic delivery (95). We believe that it might not be beneficial to administer the TAMs blockade therapy described here by intraperitoneal route even though it is directed mainly to relieve a peritoneal-based complication. Recent reports published by our group and others demonstrated that the benefit of inhibiting

TAMs infiltration and function is systemic and not tumor or tissue-confined as this treatment can greatly improve the outcomes of conventional cancer therapies, such as anti-angiogenesis therapy, radiotherapy, chemotherapy and vaccine therapy in prostate cancers (79,80), lung cancers and breast cancers (75). Here, we showed that CSF1R blockade resulted in systemic reduction of immunosuppressive MDSCs and thus lowering the tumor supportive environment, which in turn could contribute to the lowered tumor burden observed. Given these promising findings, the combination of TAMs blockade with conventional chemotherapy treatment might be particularly fruitful for EOC.

Given the very heterogeneous nature of human EOC and the known plasticity of myeloid cell and macrophage subtypes in cancer, a critical issue for clinical translation of TAM blockade strategy is to determine which patients might be responsive to CSF1/CSF1R- or other TAM-targeted therapy. Clearly, the promising prospects of this therapeutic strategy warrant more study and attention pay to myeloid cells' contribution to the aggressive and resistant nature of EOC. This knowledge could lead to more rationale therapeutic strategies to improve the current poor outcome for EOC.

### **III. SOLUBLE TARGETS IN ASCITES THAT AFFECT VASCULATURE FUNCTION**

#### **Isolating Soluble Factors in Malignant Ascites that Regulate Vascular Permeability in Late-Stage Epithelial Ovarian Cancer**

##### **Abstract**

Malignant ascites, a buildup of fluid in the abdominal cavity caused by dysfunctional vasculature, is a common complication of stage III and IV epithelial ovarian cancer. Previous studies have found that the largest proportion of cells in the ascites is macrophages, and that when protumorigenic M2 macrophages are depleted from the ascites with a CSF1R inhibitor (GW2580), the vasculature normalizes and ascites reduces. *In vitro* studies revealed that cell-free ascites serum, when placed on a monolayer of endothelial cells, will cause the layer to become permeable. Ascites serum from GW2580-treated mice allows the monolayer to increase its resistance. The conclusion is that a soluble factor is, at least in part, affecting the permeability of the endothelium. In this study, we used fractionation by ion-exchange chromatography and mass spectrometry to isolate which factor(s) may be responsible for the effects on the vasculature.

## INTRODUCTION

Malignant ascites is a common and devastating symptom of epithelial ovarian cancer (EOC) that greatly reduces quality of life. About 50% of women diagnosed with EOC present with ascites (11). After the first line of treatment, most of these patients' EOC will recur and almost 100% of the recurrent disease is accompanied by ascites (11). The most common treatment for ascites is paracentesis – puncturing the abdomen and manually draining the fluid (24,26–28,57). However, this does not solve the cause of the ascites and only provides temporary relief. The fluid often builds up again and the drainage must be continually repeated (28,96).

Currently, the best way to treat the cause of ascites is to treat the underlying EOC (24). This may work fairly well while the cancer is responding to treatments, but the largest cause of mortality associated with EOC is resistance to therapies (24). At this stage, most patients will have ascites and the underlying EOC does not respond to treatments (24). Non-invasive therapies that directly target ascites are needed to improve these patients' quality of life. In order to develop these, the molecular and mechanistic cause of ascites needs to be teased out.

Ascites is known to be result of leaky peritoneal blood vessels and sluggish or block lymphatic drainage from the peritoneum (24,25). In Chapter II of this thesis, we detailed a study that showed the increasing deregulation and dysfunction of peritoneal vasculature during the progression of ID8 EOC, a syngeneic murine model of EOC that mirrors human disease. When we depleted specifically M2 macrophages with a highly selective colony-stimulating factor 1 receptor (CSF1R) inhibitor (GW2580), we saw normalization of the vasculature and return of vasculature functionality to almost naïve levels even though peritoneal EOC tumors remained.

When we analyzed the ascites from control and GW2580-treated mice closer, we found that specifically the serum from control mice had endothelium permeability-inducing properties and the serum from treated mice had endothelium permeability-reducing properties in two different *in*

*vitro* assays. This implicated a soluble factor(s) as at least partially responsible for the regulation of the vasculature in late-stage EOC. The most popular soluble ascites factor that is currently studied in relation to ascites-reducing therapies is VEGF. It is true that VEGF does have angiogenesis and vasculature permeability-inducing properties, it is highly elevated in ascites, and that blocking it does reduce ascites in a percentage of patients (32,34,35). However, most patients on anti-VEGF therapies have severe side effects that are even fatal 10% of the time (33–35). Interestingly though, when we looked at VEGF levels in control versus treated mouse ascites sera, there was no difference. We conclude that there is another factor(s) that is acting upon the vasculature apart from VEGF.

In order to find this factor in an unbiased manner, we turn to proteomics. We fractionated the ascites sera by ion exchange chromatography and studied the resulting fractions with gel electrophoresis. We analyzed the bands of interest with mass spectrometry and found several interesting potential candidates. We are currently confirming these and other candidates through a more rigorous mass spectrometry analysis and *in vitro* permeability assays.



## **MATERIALS AND METHODS**

### **Cell culture and permeability assay**

Murine endothelial cells (SVEC4-10 cells), a kind gift from Dr. Hui Sun, were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO<sub>2</sub>.

For the permeability assay, SVEC4-10 cells were allowed to form a monolayer. Each well received DMEM with 5% of a different ascites serum and 1% penicillin/streptomycin for 24 hours. Cells were then collected, fixed in 3%PFA for 15 minutes, permeabilized with 90% methanol for 30 minutes, and stained according to our flow cytometry protocol with Alexa Fluor488 anti-VE-Cadherin (1:100, eBioscience).

### **Animal model and ascites collection**

All animal experiments were approved by the UCLA IACUC and conformed to national animal care guidelines, ethics, and regulations. Renilla luciferase-marked ID8 cells ( $10 \times 10^6$ ) in 500ul PBS were injected intraperitoneally into C57BL/6 female mice (Jackson Laboratory (Bar Harbor)). GW2580 (LC Labs) treatment (160mg/kg) or control diluent (0.1% hydroxypropyl methylcellulose, Sigma-Aldrich; 0.1% Tween20 in distilled H<sub>2</sub>O) was given daily starting 10-12 weeks post-injection, once ascites became visible. After two weeks of treatment, animals were euthanized and total ascites was collected. Ascites cells were pelleted and the serum collected into aliquots that were stored at -80°C.

### **Ion exchange chromatography**

To prepare the columns, Q-Sepharose beads (GE Healthcare) were added to a poly-prep chromatography column (Bio-Rad) so that the beads were ~1mL thick when packed. Columns were washed with 2mL 1M sodium acetate pH 5.1 and then equilibrated with 20mL PBS.

Albumen and IgG were depleted from 100ul of each ascites serum sample with the Qproteome Murine Albumin Depletion Kit (Qiagen). All subsequent steps allowed for flow-through by gravity.

The resultant albumin-depleted samples were added to prepared columns and flow-through were collected. 5mL Phosphate-buffered saline was added to the columns and the flow-throughs combined with the previous flow-throughs (Fraction 1). 2mL of 100mM NaCl were added to each column and allowed to flow through (Fraction 2). 200mM, 300mM, 400mM, 500mM, and 2M NaCl were added sequentially to each column in the same way, and became Fractions 3-7, respectively.

### **Fraction Concentration**

Depending on the volume of each fraction, a 15mL or 4mL Amicon Ultra 3,000 MWCO centrifugal filters (Millipore) was used to condense the fractions into ~500ul. Then 500ul Amicon Ultra 3,000 MWCO centrifugal filters were used to desalt the fractions and concentrate them to a final volume of ~100ul in PBS.

### **Gel electrophoresis and band isolation**

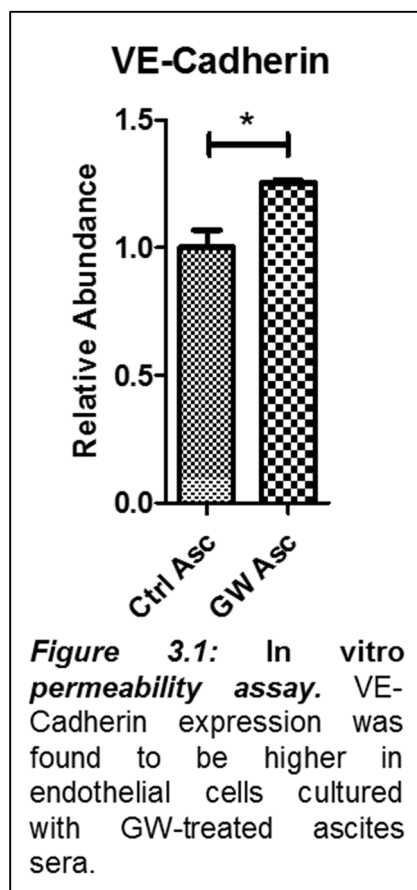
Equal amounts of protein from each fraction of two control and two GW2580-treated animals were denatured with SDS and boiling, and loaded onto a 4%-12% gradient gel for SDS-PAGE. The gel was then washed for 2x30 minutes in 50% methanol and 10% acetic acid. Gels were then placed overnight on a shaker with Sypro Ruby (Life Technologies) and washed 2x30 minutes in 10% methanol and 7% acetic acid. The band patterns in the gel were then imaged with UV light and excised with a scalpel.

### **Mass spectrometry**

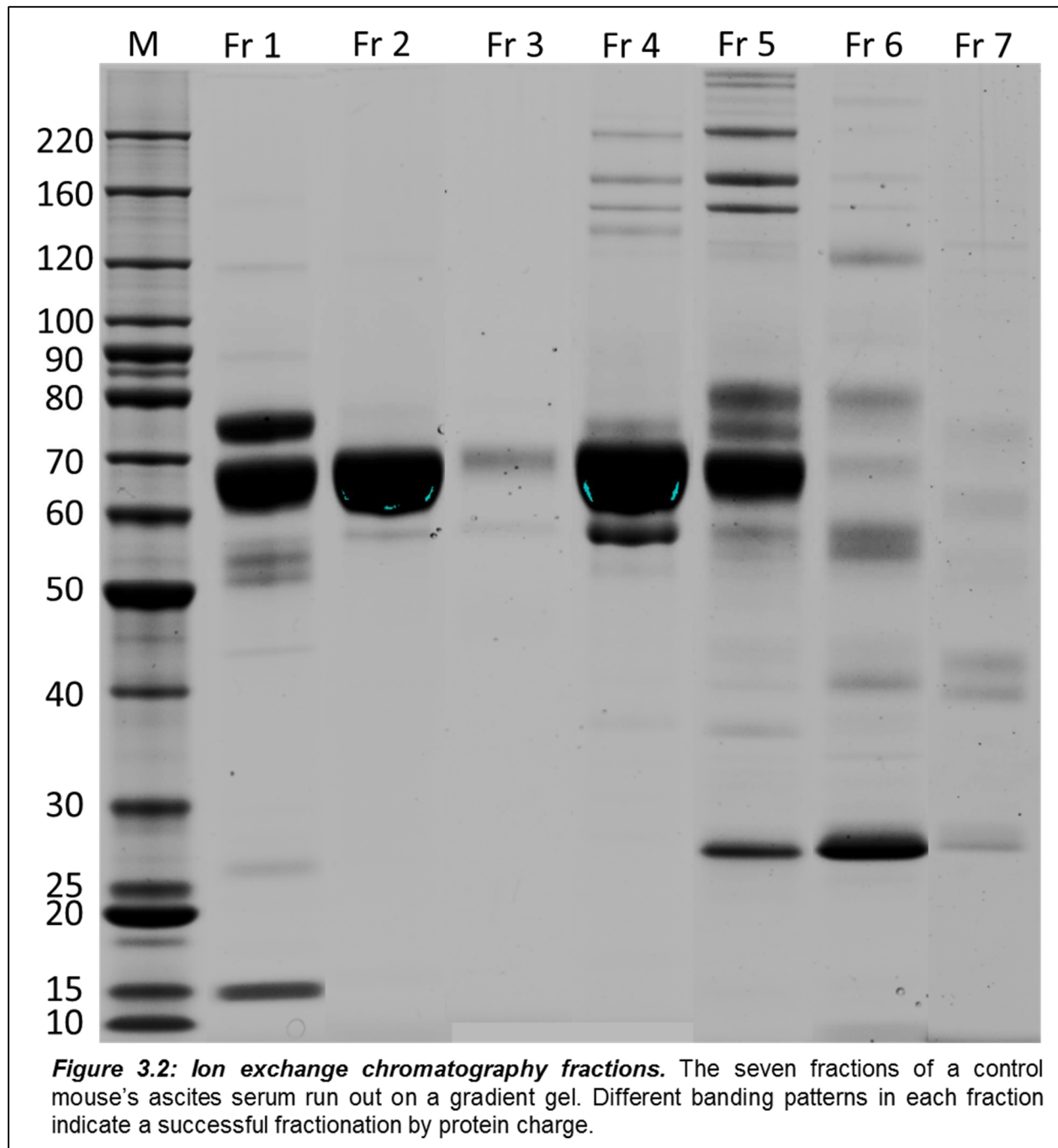
All in-gel digestion and mass spectrometry procedures were done according to standard protocols by the UCLA Pasarow Mass Spectrometry Laboratory.

## RESULTS/DISCUSSION

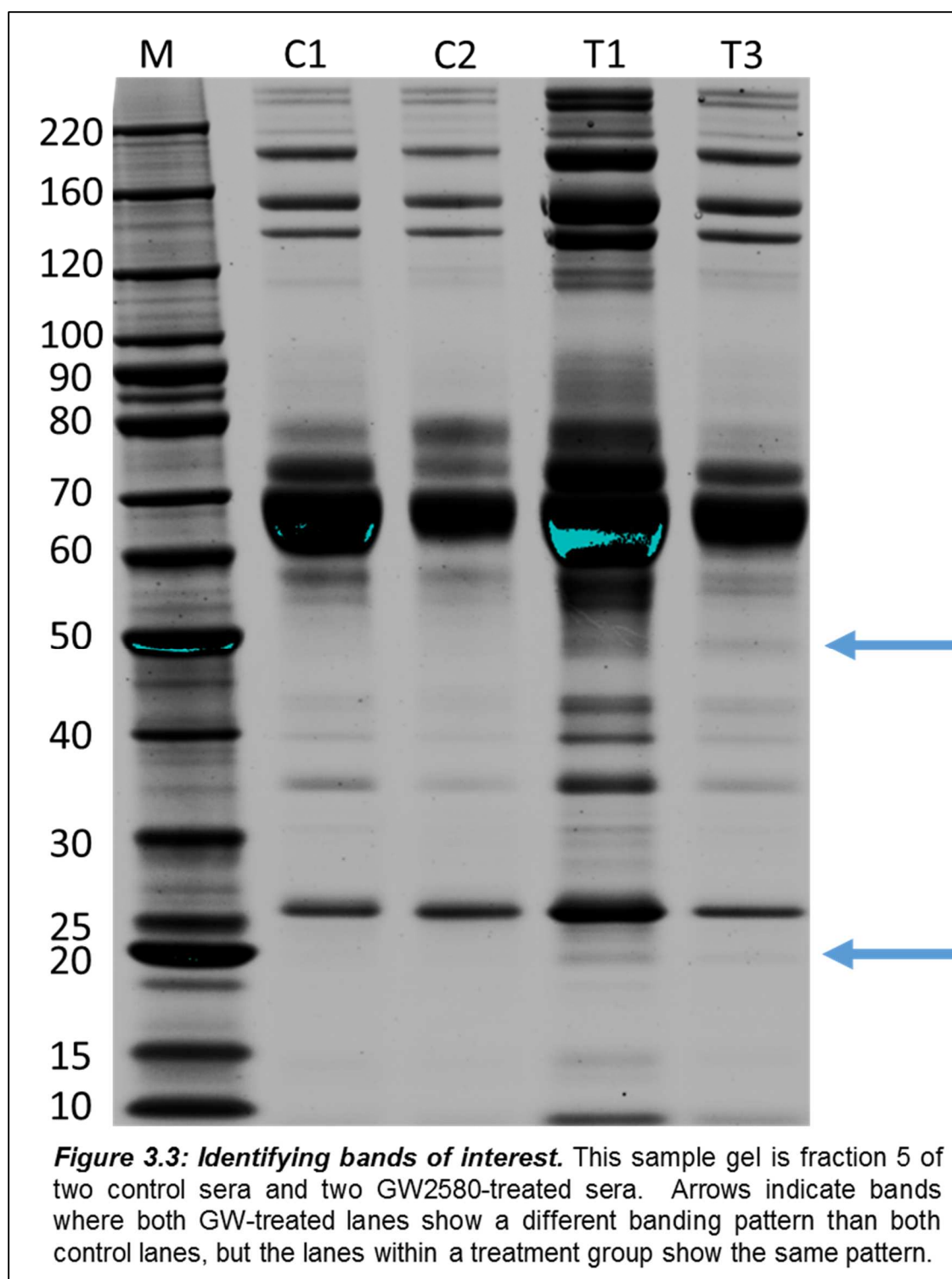
In order to study soluble factors in ascites serum without bias, we needed a large quantity of serum. Female C57BL/6 mice were intraperitoneally implanted with the previously described ID8 syngeneic murine EOC cells. Once the disease reached late stages and the mice had undergone two weeks of treatment, total ascites was collected and the sera were isolated. These ascites sera were used in a VE-Cadherin assay to determine if we did indeed still see less endothelium permeability with GW-treated ascites sera. Endothelial cells did show significantly more VE-Cadherin expression when cultured with the GW-treated sera than with the control sera (Figure 3.1).



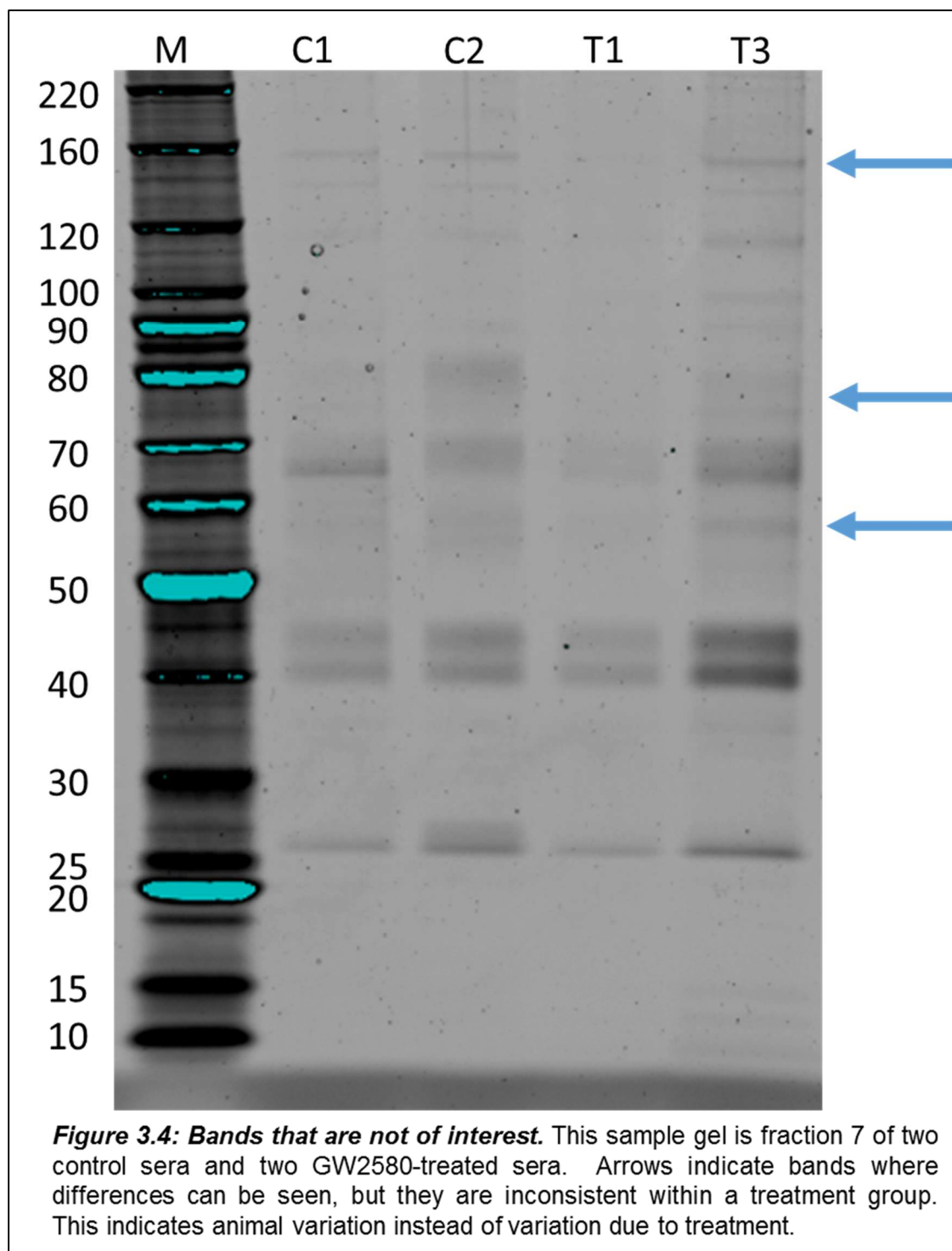
Next, it was necessary to make the complex ascites sera simpler by fractionation so that individual proteins in the mixture could be studied more easily. Albumen and IgG were depleted from the



sera so as not to contaminate the fractionation process and the view of other more important but less abundant proteins. The sera were fractionated by ion exchange chromatography into seven fractions, the protein concentrations of each fraction were determined, and equal amounts of protein per fraction were loaded onto gels for SDS-PAGE.



The resulting gels showed distinct banding patterns in each fraction, indicating successful fractionation (Figure 3.2). Careful scrutiny of the gels reveals several bands among the



fractions that showed different intensity in both treated animals versus both control animals

(Figure 3.3). These were the bands of interest. Many bands showed different intensities even among the same treatment group, but these are likely due to animal variations rather than the result of treatment (Figure 3.4).

The bands of interest from Figure 3.3 were excised from the gel, underwent in-gel digestion, and then were run through a mass spectrometer to discover what proteins were present in the bands. Several potential candidates were identified.

Many apolipoproteins were increased in abundance with the GW2580 treatment. Interestingly, some apolipoproteins have already been associated with EOC, and some (Apo A-I and Apo A-IV) have even been noted as inhibitory of the EOC tumors or downregulated in EOC patients (97–99). If that is the case, then the increase of apolipoproteins with GW2580 treatment, including Apo A-I, Apo A-II, Apo C-I, Apo C-III, ApoE, and Apo M, may be indicative of the better prognosis we see with the treatment. It will be interesting to tease out whether these proteins are also playing a role in the reversal of vascular dysfunction, as nothing has been found yet regarding this possibility.

Another set of proteins that increase with GW2580 treatment are the inter-alpha-trypsin inhibitor heavy chains and the related alpha-1-microglobulin/bikunin precursor (AMBIP). These proteins have been associated with inhibition of tumor progression, inhibition of metastasis, and are downregulated in many solid tumors, including ovarian tumors (100–102). As with the apolipoproteins, seeing an increase of these factors with GW2580 treatment is indicative of the better prognosis, but they have never been studied with regard to vascular function in cancers. It is not fully known how these proteins are inhibiting cancer progression and metastasis. It will be interesting to see if it is because they affect vasculature, as this could indicate a potential role in reducing ascites in the ID8 model.

A few proteins known to be involved in immunomodulation were upregulated with GW2580 treatment. CD5 antigen-like is a secreted glycoprotein that, when acting on macrophages, inhibits TNF-alpha secretion and increases IL-10 secretion (103). Interestingly, we do see that the macrophages in ascites after GW2580 treatment are producing more IL-10 (data not shown) even though they are more M1-like than the macrophages from control mice. TNF-alpha is an inflammatory protein that causes permeable vasculature (104), and the decrease of this protein, potentially through CD5 antigen-like, is an interesting hypothesis to pursue. There was also an increase of the endogenous antioxidant, glutathione peroxidase, which can not only lower reactive oxygen species, but can inhibit activation of NF kappa B in macrophages, block macrophage NO generation, and lessen the production of iNOS by macrophages (105,106). This broad immunomodulation may involve changes in macrophage secretions/phenotype that helps normalize vasculature. Similar hypotheses can be made for the upregulation of serum paraoxinase seen with GW2580 treatment. This protein has anti-inflammatory properties in macrophages that may modulate proteins responsible for vascular permeability (107,108).

In conclusion, there are many avenues that the mass spectrometry analysis has opened up for exploration. More quantitative mass spectrometry analysis of the samples is currently underway and will help determine which future directions may be the most impactful to identifying the factor(s) that regulate vascular dysfunction and normalization in late-stage EOC.



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