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ROLE OF THE ANG-TIE2 PATHWAY IN THE INVASIVE RECURRENCE OF GBM FOLLOWING ANTI-VEGF THERAPY

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DEDICATION

To my mother, Ivette Santiago and my father, Jose R. Cortés.

Without you, none of this would've happened. You have made all my dreams come true.

This is for you. I love you.

And to my grandmother, Blanca Cuevas. Your warm food, faith and prayers kept me going. I love you with all my heart.

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Role of the Ang-Tie2 pathway in the invasive recurrence of GBM following anti-VEGF therapy

by

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Strong pre-clinical and clinical data supporting the effectiveness of bevacizumab, a humanized monoclonal anti-VEGF antibody, for the treatment of gliomas led to its accelerated approval for the treatment of patients with recurrent glioma. However, despite strong anti-tumor effects, upon treatment with bevacizumab, patients will invariably recur with a tumor characterized by enhanced invasiveness and resistance to therapy. This study aims to elucidate the mechanisms leading to this enhanced malignancy with the hope of uncovering new potential therapeutic targets for combined treatment. Using tissue sections from U87-derived glioma bearing mice treated with or without aflibercept (another anti-VEGF antibody) we have gathered evidence of a significant increase in the number of Tie2 expressing monocytes (TEMs) within the tumor after treatment, particularly around areas of invasion. TEMs are a pro-angiogenic subset of circulating monocytes that express the Tie2 receptor. Our data demonstrates that TEMs have higher expression and activity of pro-invasive molecules, such as MMP2 and MMP9, than their Tie2 negative counterparts and are able to increase glioma cell invasion *in vitro,* suggesting an active role of these cells in the invasive process observed after treatment. Furthermore, we have shown that expression of angiopoietin 2 (Ang2), a Tie2 ligand, is dramatically increased in the periphery of the tumor after aflibercept

treatment. Interestingly, our data provides evidence that Ang2 is chemoattractant to TEMs, both *in vitro* and *in vivo* suggesting a potential mechanism of recruitment of these cells to the tumor. These data suggests an active role of the Ang-Tie2 pathway, and in particular Ang2, in the invasive recurrence of GBM following anti-VEGF therapy and that targeting it in combination with anti-VEGF therapy could lead to improved outcomes. Supporting this idea, we were able to observe a dramatic decrease in the invasive phenotype after anti-VEGF treatment when we inhibited the Ang-Tie2 pathway in combination with an anti-VEGFR antibody. These results have important implications for patients with GBM since a decrease in the invasive pattern upon recurrence after anti-angiogenesis therapy could potentially render the tumors amenable to surgical excision upon recurrence. This in turn can lead to improved patient survival.

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I. Glioblastoma

Glioblastoma (GB) is the most common and aggressive (grade IV) primary brain malignancy in adults. Despite therapeutic advances within the last ten years, patients diagnosed with GB have a grim prognosis with median survival ranging between15-18 months and a 5 year survival rate of less than 5%.[1-2] Even with aggressive therapy with the standard of care, which consists of maximal surgical resection coupled with radiotherapy and chemotherapy with temozolomide, GB patients inevitably present with recurrent disease.[3]

Pathologically, it is characterized by extensive necrosis and microvascular proliferation, which makes them highly vascular tumors.[2] One of the most important mechanisms through which these tumors acquire such prominent vascularity is through activation of hypoxia inducible factor (HIF) which drives the expression of vascular endothelial growth factor (VEGF).[4] Along this line, bevacizumab, an anti-VEGF antibody, was recently granted accelerated approval by the FDA for the treatment of recurrent GB due to unprecedented results in clinical trials.[5-6]

II. Angiogenesis

The generation of stable, functional blood vessels and vascular networks is an essential biological process, without which complex organisms such as ourselves could not exist. To date, two main processes through which this can occur have been described: vasculogenesis, which is the process of *de novo* vessel formation from

endothelial precursors, and angiogenesis, which is the formation of new blood vessels from pre-existing ones.[7] Vasculogenesis mainly occurs during embryogenesis, in which a primitive vascular network is established from endothelial precursor cells named angioblasts.[7] Following this initial step, angiogenesis takes over and allows for the ramification of this primitive vascular network and establishment of complex vascular networks that deliver nutrients and oxygen throughout the human body.[7] In healthy adults, most vasculature is quiescent, and angiogenic processes are circumscribed to organ systems in which vascular remodeling is essential for its function (i.e. female reproductive tract) or for the purposes of wound healing.[8] However, pathological angiogenesis has been described for several disease processes (i.e. inflammatory disease states, cancer) and the role of active angiogenesis in tumor development has been extensively studied (see below).[8]

The VEGF-VEGFR pathway in angiogenesis

The establishment of new functional blood vessels is a complex multistep process. Physiological stimuli, such as hypoxia, initiate this process mainly through upregulation of vascular endothelial growth factor (VEGF). VEGF, was initially described and purified as a tumor secreted factor capable of inducing vascular permeability and was therefore named vascular permeability factor (VPF).[9] Several years after, Leung *et al.* independently reported the discovery of vascular endothelial growth factor, a potent endothelial cell mitogen capable of inducing angiogenesis.[10] VPF and VEGF were described to be the same molecule by Keck *et al*. shortly after the discovery of VEGF.[11]

The VEGF family is composed of five ligands, namely VEGF-A (from now on referred to as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor. VEGF-A mRNA can be alternatively spliced to produce four main different isoforms: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$.[12-13] Three tyrosine kinase receptors have been described so far, namely VEGFR1, VEGFR2 and VEGFR3 with other co-receptors described such as neuropilins. Evidence gathered so far demonstrate that VEGFR2 is the most important mediator of endothelial cell responses to VEGF and therefore therapeutic approaches aimed at targeting the receptor are directed against VEGFR2.[14-15]

As described above, VEGF is a potent mitogen and pro-survival molecule for endothelial cells. Its expression is upregulated by environmental stimuli such as hypoxia and upon binding to VEGFR leads to endothelial cell proliferation and migration, two key events without which angiogenesis cannot occur.[8] Hypoxia also leads to increased matrix metalloproteinase (MMP) expression, such as MMP9, which degrade the extracellular matrix (ECM) around the cell. This has a dual effect: releasing VEGF bound to ECM making it available for interaction with its receptor and degrading the ECM providing a path for endothelial cell migration.[8] In parallel to VEGF upregulation, loosening of pericyte/endothelial cell interactions occurs mainly through the activity of angiopoietin 2 (Ang2), a Tie2 ligand (see below). This "uncovers" endothelial cells causing them to become more sensitive to VEGF. In the presence of high VEGF levels, endothelial cells begin to proliferate and migrate forming a cell column that eventually fuses forming a lumen through which blood can flow. Elongation of this tubular structure occurs through endothelial cell proliferation and migration in response to a VEGF gradient and continues to occur as long as the pro-angiogenic stimuli are present.

Eventually, pericyte cells are recruited, and pericyte/endothelial cell interactions are established leading to the formation of a functional new vessel.[16] The Ang-Tie2 pathway plays an essential role in this process (see below).

The Ang-Tie2 pathway

The Ang-Tie2 pathway is extremely important for normal blood vessel physiology. It is composed of two type I tyrosine kinase receptors, namely Tie1 and Tie2. Tie2 was initially described by *Dumont et al.* as an endothelial cell specific orphan receptor.[17] This changed in the following years, with the discovery of its currently known ligands, the angiopoietins (Ang).[18-20] The importance of the Tie2 receptor in the vascular system was highlighted by loss of function studies that showed that lack of Tie2 led to embryonic lethality at E10.5 to E12.5. These mice were unable to form a vascular network beyond the primary capillary plexus. These early vessels failed to mature and showed fewer endothelial cells and diminished branching, as well as decreased pericyte and smooth muscle cell coverage.[21-22] Consistently, gain of function studies in which Ang1, the main Tie2 agonist (see below), led to opposite changes. Overexpression of Ang1 in skin led to numerous blood vessels that were highly branched, with decreased intrinsic, or induced, leakiness due to enhanced supporting cell coverage (pericytes and smooth muscle cells).[23-24]

The ligands of the Tie2 receptor are referred to as angiopoietins. There are three human angiopoietins (Ang), namely Ang1, Ang2 and Ang4 (Ang3 is an Ang4 mouse ortholog).[18-20] Ang1 and Ang2 are the most widely studied human Tie2 ligands. Ang1 is considered to be the main Tie2 agonist and as such, loss of function studies led to similar

outcomes as knockdown of Tie2.[25] Upon binding to Tie2, it leads to dimerization or multimerization, and auto-phosphorylation of tyrosine residues located in the intracellular domain of the receptor. This leads to binding and activation of downstream targets ultimately leading to endothelial cell survival and vessel stability.[26] Tie2 and Ang1 are widely expressed in the adult vasculature and are required for the maintenance of a functional quiescent vascular network.[8]

Ang2 is considered to be a natural Ang1 antagonist. This notion came from gain of function studies in which mice engineered to overexpress Ang2 had similar vascular defects as mice deficient for Ang1.[19] Further evidence of its antagonistic functions came later when mice with conditional overexpression of Ang2 were shown to be unable to phosphorylate Tie2, the idea being that when Ang2 is present it binds to the Tie2 receptor effectively preventing binding of Ang1.[27] This in turn leads to loosening of pericyte coverage and vessel destabilization. The end result of Ang2 activity on vascular structures will largely depend on the presence or absence of VEGF. Loosening of pericyte coverage sensitizes endothelial cells to the effects of VEGF, and therefore, in the context of high VEGF expression, Ang2 potentiates its effects and functions as a pro-angiogenic molecule leading to enhanced endothelial cell migration and sprouting. On the other hand, in the absence of the pro-survival and mitogenic stimuli conferred by VEGF, lack of pericyte coverage causes endothelial cells to undergo apoptotic cell death. In this context, high Ang2 expression leads to vessel regression.[28] Interestingly, the role of Ang2 in the Ang-Tie2 pathway has proven to be complex and in the absence of Ang1, Ang2 has been shown to partially activate the Tie2 receptor.[29] Furthermore, Ang1 has

been shown to reverse the effects of Ang2 inhibition in colon, prostate and epidermoid carcinomas, suggesting Ang2 can function as a Tie2 agonist under certain conditions.[30]

There are other roles of the Ang-Tie2 pathway outside blood vessel physiology and pathology. For example, loss-of-function studies have shed light into other physiological roles for Ang2. Although normal embryonic development is not hampered by lack of Ang2, mice with knocked down Ang2 expression develop chylous ascites that is lethal in the 129/J genetic background.[31] This result suggests an important agonistic role of Ang2 in the development of lymphatic vasculature. Furthermore, mice deficient for Ang2 are incapable of mounting a rapid inflammatory reaction in response to TNF-α, suggesting a key role for this cytokine in inflammation. In depth studies on how Ang2 is capable of regulating the inflammatory response have shown that late stages of leukocyte recruitment, which involve firm adhesion and transmigration are affected by the absence of Ang2, whereas initial stages, namely chemoattraction and selectin-mediated rolling and tethering, are not affected.[32] The Ang-Tie2 system has also been shown to be crucial for the maintenance of the hematopoeitc stem cell (HSC) niche. HSC expressing the Tie2 receptor were found to be a side population within the bone marrow with a quiescent phenotype and anti-apoptotic features. Osteoblasts and stromal cells within the bone marrow produce Ang1 that binds to and activates Tie2 within HSC in a paracrine manner. This constitutive activation of the Ang-Tie2 pathway has been shown to be essential for maintenance and repopulation of the stem cell niche within the bone marrow.[33] Finally, Ang1 has neuro-protective effects through inhibition of apoptotic cell death in neurons. Whether the latter is mediated by Tie2 still needs to be clarified.[34]

Angiogenesis in cancer

Angiogenesis is a highly complex process requiring a tightly regulated interplay of many molecular pathways, both pro- and anti-angiogenic. An imbalance towards either one of the extremes of the spectra can lead to dysfunctional vasculogenesis and angiogenesis. For example, genetic knockdown of even one VEGF allele leads to early embryonic death due to abnormal vasculature formation.[35] On the other hand, a pro-angiogenic environment lacking appropriate regulation can lead to disease, cancer being an example of the latter.[8] The effective generation of a vascular network is considered to be a rate-limiting step in tumor progression. The growth of any solid mass beyond a few millimeters requires the concomitant formation of blood vessels that allow oxygen and nutrient delivery to tumor cells, as well as waste removal from the tumor bed.[36]

Tumor progression has historically been described in two major phases, namely avascular and vascular. The avascular phase of tumor growth is characterized by the acquisition and accumulation of genetic alterations that confer a given cell a proliferative and survival advantage. This leads to uncontrolled cell proliferation and the resulting formation of a small lesion. Eventually, the lack of blood supply to central areas of the tumor hampers expansion and the mass reaches a point where apoptosis equals cell proliferation and no further growth occurs.[37-38] In the absence of angiogenesis, these lesions can remain dormant for years and cause no disease. Underlining the importance of vascular formation to generate a clinically significant tumoral lesion, there have been reports of accidental findings of small, occult lesions in autopsies from patients that died from a different cause.[39] This supports the idea that only a subset of tumors eventually enters the second, vascular phase in which exponential tumor growth occurs. Although

extensive research on the subject has shed some light into the mechanisms underlying this "angiogenic switch", the subject remains obscure.

Angiogenesis is the main mechanism through which tumors acquire a vascular network. However, some solid tumors have been shown to induce *de novo* vessel formation through the recruitment of endothelial precursor cells from the bone marrow, a process called vasculogenesis.[40] Adding to the complexity of tumor vascularity, cancer cells have been shown to integrate in the vessel wall.[41] Similar to physiologic angiogenesis, initiation of tumor angiogenesis can be triggered by the sole overexpression of VEGF. However, the imbalance of pro- and anti-angiogenic stimuli within the tumor microenvironment leads to the formation of chaotic vascular networks, with deregulated maturation and diminished or absent pericyte coverage.[42] This leads to vessels containing features of capillaries, venules and arterioles altogether. Lack of pericyte coverage leads to enhanced sensitivity to VEGF stimulation, which generates a vicious cycle of ongoing angiogenesis and the production of dilated leaky vessels that differ in great extent to their normal counterparts.

III. Anti-angiogenesis therapy: development and challenges

The idea that targeting angiogenesis for cancer treatment could be useful was first proposed by Judah Folkman in 1971.[43] The discovery of VEGF and the understanding of its essential role in angiogenesis provided the basis for the development of several types of inhibitors of this pathway to target angiogenesis. Antibodies against VEGF and its receptor (VEGFR), tyrosine kinase inhibitors with selectivity towards VEGFR and soluble VEGFR formulations have been developed, and in some cases FDA approved, for

the treatment of disease, including cancer. The idea behind these efforts being that without an active VEGF pathway, angiogenesis cannot occur and tumors would "starve" to death.

Bevacizumab, one of the currently FDA approved anti-angiogenesis agents, is a humanized monoclonal antibody that binds vascular endothelial growth factor and traps it in the circulation preventing it from interacting with the VEGF receptor (VEGFR). Its precursor, a murine monoclonal antibody (muMAb 4.6.1) was developed by *Kim et al.* in an attempt to better understand the physiological roles of VEGF shortly after its discovery.[44] It was shown to bind and effectively inhibit VEGF-mediated endothelial cell proliferation and vascular permeability. A year later, the same group published a study demonstrating that VEGF induced angiogenesis *in vivo* and that targeting VEGF *in vivo* with mAB 4.6.1 led to decreased vessel density and inhibition of tumor growth in three tumor models: leiomyosarcoma, GBM and rhabdomyosarcoma.[45] This was the first study to demonstrate that targeting VEGF *in vivo* led to decreased angiogenesis and tumor growth inhibition, establishing a precedent for further drug development. Even though the biggest attention was given to the potential of targeting VEGF in cancer, this strategy was also investigated in other models of disease. For example, diabetic retinopathy is characterized by hypervascularization of the retina, and studies were able to show that targeting VEGF in this context led to diminished vessel density in models of this disease.[46] Furthermore, rheumatoid arthritis also has a prominent vascular component and some studies suggested that inhibiting VEGF in this context could be useful.[47]

In 1997, five years after its development, *Presta et al.* published the humanization of mAb 4.6.1, now named bevacizumab.[48] Bevacizumab had similar binding affinities to

VEGF, as well as tumor growth inhibitory properties as those observed for mAb 4.6.1. With this, a tool for testing the effectiveness of targeting VEGF in human disease was established. The first placebo controlled trial was published by *Yang et al.*[49] Patients with metastatic renal carcinoma were given placebo, low-dose bevacizumab or high dose bevacizumab. Primary end points were time to progression of disease and response rate, with overall survival being a secondary endpoint. Results demonstrated a significant increase in progression free survival, particularly in the high-dose bevacizumab group when compared with placebo. No change in overall survival was observed. Since then, many clinical trials have been conducted in several types of solid tumors leading to the approval of bevacizumab for the treatment of non-small cell lung carcinoma, metastatic colorectal and renal cell carcinoma and, of particular interest to us, second line treatment for GBM.

The first report on the use of bevacizumab in GBM was done by Stark-Vance and was presented as an abstract at the 2005 European Association of Neuro-Oncology.[50] She treated 11 patients who had recurrent GBM with 5mg/kg of bevacizumab combined with 125mg/m^2 irinotecan (CPT-11) and was able to show anti-tumor activity. This opened the door for clinical trials with bevacizumab for the treatment of recurrent GBM. Historically, patients treated with conventional therapy had radiographic responses of 9% and 6-month progression free survival ranging between 9-15%. Clinical trials performed with bevacizumab demonstrated an improvement of both these measures, producing radiographic responses ranging between 24-57% and 6-month PFS of 25-65%. Furthermore, its vascular normalization effects led to decreased cerebral edema and intracranial pressure, which resulted in a decrease in corticosteroid use in these patients

and a consequent improvement in quality of life.[51] The FDA granted accelerated approval of bevacizumab in 2009 for the treatment of recurrent GBM due to the outcomes reported on clinical trials[5-6] and it is currently standard of care for this patient population. Data on overall survival (OS), the most important parameter used to evaluate therapeutic efficacy, is not available for recurrent GBM. However, two recent phase III clinical trials in which the efficacy of bevacizumab as a first line agent for GBM was evaluated concluded that bevacizumab did not lead to increased OS when compared to conventional therapy.[52-53] Whether this translates to the setting of recurrent GBM is undetermined since no large-scale clinical trial with OS as a primary endpoint has been done for bevacizumab in this context. Importantly, progression free survival was improved in both studies, although the pre-specified improvement target was not achieved in one of them.[53]

Even though bevacizumab led to an improvement in quality of life, progression free survival and radiographic responses in GBM, the idea of it being a miracle drug is far from reality. Despite its benefits, patients treated with bevacizumab invariably present with recurrent disease and upon recurrence, the tumors acquire a highly invasive phenotype that is resistant to all currently available therapies. At this point, prognosis is measured in short weeks. The notion that bevacizumab treatment leads to enhanced invasion in gliomas dates to the year 2000, way before it was tested in the clinic and many years before its approval for the treatment of recurrent GBM. *Rubenstein et al.* reported the use of bevacizumab for the treatment of intracranial glioma for the first time in an animal model.[54] They demonstrated the effectiveness of bevacizumab at increasing survival, decreasing vessel density and increasing apoptosis of intracranial glioma, and discuss the infiltrative behavior of the tumor upon recurrence as an adaptation to anti-

angiogenic therapy. Since then, concerns regarding this highly aggressive and infiltrative behavior after anti-VEGF therapy has been raised in several studies, both in animal models as well as in patients.[55-56] Evidence gathered demonstrated that targeting either VEGF or VEGFR led to similar outcomes, suggesting that this phenomena was a result of targeting the pathway, and did not necessarily stem from the approach taken to do so (targeting VEGF or VEGFR). In 2009, *Paez-Ribes et al.* established the unequivocal link between inhibiting VEGF and highly aggressive and infiltrative behavior.[55] In their study, they demonstrated that both, pharmacological inhibition of the VEGF pathway as well as genetic knockdown of VEGF, led to tumor progression with increased vessel cooption and enhanced invasion in a GBM model and pancreatic neuroendocrine carcinoma model, the latter having higher rates of distant metastasis in the context of VEGF inhibition. Understanding how the tumors acquire this invasive and resistant phenotype following anti-VEGF therapy will be essential for improving GBM treatment and outcomes.

IV. The tumor microenvironment

Historically, research on cancer has focused on understanding the events occurring within tumor cells for tumorigenesis to take place. Intensive efforts led to the identification of mutational events required for an epithelial cell to be transformed and become tumorigenic. A prime example of such efforts is the characterization of the genetic alterations that occur in the progression from benign epithelial hyperplasia to invasive carcinoma in colon cancer. However, tumors are not composed of an isolated mass of transformed epithelial cells, but of a composite of extracellular matrix, blood

vessels and immune cells, among other stromal components. These components outside of the epithelial compartment are known as the tumor microenvironment.[57] It is now widely accepted that communication and interactions between transformed epithelial cells and its microenvironment are essential for development, progression and therapeutic response of any neoplastic growth, and that components within this microenvironment can be exploited therapeutically.[58] Immune cells are one of the most widely represented cells in the tumor microenvironment, and within this group, cells of the myeloid lineage, particularly monocyte/macrophages (tumor associated macrophages – TAMs), are of the most abundant.

Monocytes and macrophages

Macrophages are characterized by their remarkable plasticity. Depending on the micro-environmental needs, their functions can range from immune activation and pathogen recognition and clearance to scavenging, immune suppression and wound healing. This wide variety of, and sometimes opposing, functions underline the ability of macrophages to sense cues from the microenvironment and adapt to these so that appropriate immunological responses can be mounted. In this way, macrophages function as switches of the immune system and can develop into different subsets to tailor and regulate immune responses.[59]

The most widely accepted model of macrophage differentiation is the M1-M2 polarization spectrum. In one end, M1 macrophages are considered to be pro-inflammatory monocytes that activate the immune system via inflammatory cytokine production and antigen presentation. This subset of macrophages is activated by

microbial products such as lipopolysaccharide (LPS) and pro-inflammatory molecules such as IFNγ and they aid in the process of pathogen recognition and clearance.[59] On the other end, M2 macrophages are activated by IL4 and IL13 and they function to keep the immune system in check by producing large amounts of anti-inflammatory molecules that regulate and terminate inflammatory reactions initiated by M1 polarized macrophages. Furthermore, M2 polarized macrophages have scavenging and pro-angiogenic properties that are important in the normal process of wound healing.[60-61] At any given moment, macrophages exist somewhere in the middle of this spectrum with environmental signals tilting the pro- and anti-inflammatory balance to one side or the other.

Although macrophages can potentially have anti-tumor effects, the bulk of the evidence available so far suggests that tumor associated macrophages (TAMs) enhance tumor survival and hamper response to therapy in a wide variety of solid tumors, including breast carcinoma, prostate carcinoma, bladder carcinoma and gliomas.[62-67] In gliomas, monocyte/macrophages are one of the most widely represented cells within the tumor microenvironment.[68] Studies aimed at understanding the characteristics of these cells within gliomas have shown that the bulk of these tumor-associated macrophages are M2-polarized, a profile consistent with a pro-tumorigenic phenotype.[69] The mechanisms through which these cells can promote tumor progression are varied and include diminished anti-tumor activity, immune suppression and the production of cytokines and chemokines that induce glioma cell proliferation, angiogenesis and invasion. Furthermore, glioma cells are capabable of producing soluble mediators that perpetuate

this pro-tumorigenic phenotype in macrophages establishing this way a vicious cycle leading to enhanced malignancy.[70]

Tie2 expressing monocytes

As the name implies, Tie2 expressing monocytes (TEMs) are a subpopulation of circulating monocytes expressing the Tie2 receptor. They were discovered in 2005 by *De Palma et. al*. as an effort to characterize the Tie2 expressing cell population within tumors.[71] Through the use of a transgenic mouse model in which GFP expression was driven by transcriptional regulatory elements present within the Tie2 gene [TgN(Tie2-GFP)], they identified a population of Tie2 expressing cells within the stromal compartment of N202 mammary tumors that co-express the monocytic marker CD11b, i.e Tie2 expressing monocytes. Co-injection experiments in which nude mice were implanted with N202 mammary carcinoma cells in conjunction with TEMs isolated from TgN(Tie2-GFP) mice, demonstrated that TEMs have remarkable pro-angiogenic properties, with tumors co-injected with TEMs having a 4-fold higher average vascular area than controls. As a continuation of this work, in 2007 *Venneri et al.* described the presence of TEMs in peripheral blood of healthy donors, as well as in human tumor samples, establishing a link between the findings in the mouse mammary tumor model and human disease.[72] Tie2 expressing monocytes have also been studied in the context of hepatocellular carcinoma. *Matsubara et al.* showed that TEMs increase in patients suffering from HCC and that their numbers correlate with response to therapy, i.e. surgery, as well as tumor recurrence.[73] Given these data, the authors highlight the

potential of using TEMs as biomarker for HCC. Finally, their selective recruitment to tumors has been exploited as a tool for drug delivery.[74]

Due to their relatively recent discovery, reports on TEMs and their biological properties are still limited. The bulk of the research performed so far has been directed to further elucidate their role in tumor angiogenesis and progression. In 2009, *Pucci et al.* analyzed the gene signature of TEMs in comparison to TAMs, endothelial cells and other myeloid derived cells, such as neutrophils.[75] Their work further confirmed that TEMs are part of the myeloid lineage, since they posses higher expression of myeloid genes such as *csf1, Fc* receptors and *cd14*, while having negligible amounts of endothelial derived genes. Their expression profile is also consistent with their pro-angiogenic role, having higher expression of mRNAs classically associated with angiogenesis, such as vegfa, vegfb, and pdgf. Furthermore, expression of M2 polarization markers such as arginase and mannose receptor placed TEMs in this end of the monocyte/macrophage polarization spectra, further supporting their pro-tumorigenic properties.[69, 75] The role of the Tie2 receptor in this monocyte subpopulation is still unclear. However, the Tie2 ligand Ang2, has been shown to promote their pro-angiogenic and pro-tumorigenic properties. In their work published in 2009, *Coffelt et al.* provided evidence that Ang2 can recruit TEMs to tumors and enhance their pro-angiogenic properties.[76] In their follow up work, they extended the role of TEMs to that of an immunomodulatory cell capable of inducing an immune suppressed microenvironment through the expression of IL10. Interestingly, Ang2 was capable of further enhancing their expression of this cytokine, which then led to suppression of T cell proliferation, increase in the CD4+/CD8+ T cell ratio and expansion of Tregs.[77] Therefore, although literature on TEMs is still limited, the bulk of

the evidence heavily supports the notion that this cell population support tumor progression and their presence could be associated with worse patient prognosis

Glioblastoma multiforme is one of the deadliest malignancies known to man. Extensive research efforts have only led to modest advances in therapy and patient outcomes. The purpose of this investigation is to better understand the events leading to recurrence following anti-VEGF therapy (i.e. bevacizumab) in GBM with the ultimate goal of identifying new therapeutic targets for combined treatment. Based on the available evidence, we hypothesize that the Ang-Tie2 pathway plays a key role in the recurrence of GBM after anti-angiogenesis treatment. A corollary of this hypothesis being that targeting this pathway in combination with anti-VEGF therapy could lead to improved outcomes in GBM.

Chapter II: Materials and Methods

Cell Culture. U87 malignant glioma and THP-1 monocytic leukemia cell lines were obtained from the American Type Culture Collection. GSC20 glioma stem cells were established from a human glioblastoma surgical specimen at MD Anderson Cancer Center (Houston, TX). GL261 mouse glioma cells were obtained through the NCI-Frederick Cancer Research Tumor Repository (Frederick, MD). GL261.GFP and GL261.sTie2 established cell lines were a kind gift from Luc Vallières (Quèbec, CA), Ph.D. U87 MG cells were cultured in Minimum Essential Medium Eagle (MEM) with 10% FBS and 1% essential amino acids under the same conditions. THP-1 monocytic leukemia cell line was cultured in RPMI1640 media supplemented with 2mM L-glutamine and 10% FBS. GSC20 glioma stem cells were cultured using Dulbecco's Modified Eagle Medium/F12 supplemented with 1X B27 and 20ng/mL bFGF and 20ng/ml EGF. GL261, GL261.GFP and GL261.sTie2 cell lines were cultured in Dulbecco's Modified Eagle Medium/F12 supplemented with 10% FBS. All media was supplemented with 100μg/ml penicillin and 100μg/ml streptomycin.

Immunohistochemistry. Five μm sections were deparaffinized in three washes of xylene for 5 minutes each, followed by rehydration in serial dilutions of ethanol as follows: 100% ethanol twice for 5 minutes, 95% ethanol twice for 5 minutes and 70% ethanol once for 5 minutes. Following a brief wash in PBS, endogenous peroxidase activity was quenched using 3% H_2O_2 in 100% methanol for 10 minutes at room temperature. Heat-induced antigen retrieval was performed in a pre-warmed solution of 10mM citrate

buffer, pH6 for 10 minutes in a pressure cooker. Slides were allowed to cool down at room temperature for 30 minutes before washing and blocking using a 5% BSA in PBS solution. Primary antibodies were diluted using IHC Tek diluent pH 7.4 (IHC World, Cat. # IW1000) and placed at 4°C overnight. Biotin-conjugated secondary antibodies (Vector Labs) were diluted 1:400 in IHC Tek diluent pH 7.4 and applied to tissue sections for 1 hour at room temperature. This was followed by incubation with ABC system (Vector labs, Cat. # PK4000) for one hour at room temperature. 3, 3' diaminobenzidine (Sigma, Cat. # D4418) was used for signal detection. Nuclei counterstain was done using hematoxilin, after which slides were dehydrated in 100% ethanol. Following a brief wash in xylene slides were mounted using CytoSeal (Thermo Scientific, Cat. # 23-244257). Images were taken using a brightfield, Zeiss Axioskop 40 microscope at the designated magnification.

Immunofluorescence. Five μm sections were deparaffinized in three washes of xylene for 5 minutes each, followed by rehydration in serial dilutions of ethanol as follows: 100% ethanol twice for 5 minutes, 95% ethanol twice for 5 minutes and 70% ethanol once for 5 minutes. Blocking was done with a 5% BSA in PBS solution followed by primary antibody diluted in IHC Tek diluent at 4°C overnight. Biotin-conjugated secondary antibodies were diluted 1:500 in IHC Tek diluent pH7.4 and placed on tissue sections for one hour at room temperature. Dylight-conjugated streptavidin was used for signal detection. When required, signal was amplified by an additional step of biotin-conjugated anti-streptavidin antibody (Vector Labs, Cat. # BA0500, 1:1000) for 30 minutes at room temperature followed by an additional step of Dylight-conjugated

streptavidin (Vector Labs, Cat. # SA-5488-1 and SA-5549-1, 1:1000) for 30 minutes at room temperature. DAPI was applied for 15 minutes at room temperature for nuclear counterstaining. Slides were then mounted using Dako Fluorescence Mounting Medium (DAKO, Cat. # S3023). Images were taken in a fluorescent Zeiss Axiovert 200M microscope at 20x magnification. For quantification purposes, at least eight pictures of random fields were taken and manually quantified per slide.

Primary antibodies used for immunohistochemistry and immunofluorescence are Ang2 (IHC World, ready-to-use), Ang1 (R&D, 1:100), VEGF (Santa Cruz, 1:500), Iba1 (Millipore, 1:1000), CD31 (R&D, 1:100), SMA (Dako, 1:100), hNuMA (abcam, 1:500),. Secondary antibodies used are biotinylated anti-rabbit (Vector Labs), biotinylated anti-mouse (Vector Labs), biotinylated anti-goat (Vector Labs). Dylight-594 and Dylight-488 conjugated streptavidin (Vector Labs) was used for immunofluorescence detection.

M2 polarization and induction of Tie2 expression in THP-1 monocytic leukemia cell line. THP-1 cells were placed in serum free media in the presence of 20ng/ml IL4 (R&D, Cat. #204-IL-010) and 20ng/ml IL13 (R&D, Cat. #213-ILB-005) and placed in hypoxic conditions (1% O_2, 5% CO_2 and 94% N_2) for 48-72 hours. Tie2 expression was measured via flow cytometric analysis following polarizing conditions.

Flow cytometric analysis. Cells of interest were harvested and washed three times with a solution of PBS containing 0.5% BSA. Incubation with primary antibodies was performed at 4°C for 30 minutes in the dark, after which cells were washed twice with PBS. Cells were resuspended in 500ul-2mL of PBS depending on total number of cells.

Flow cytometry was done using a FacsCalibur flow cytometer from Beckton Dickinson machine and expression was analyzed using CellQuest software. In experiments requiring sorting, expression was analyzed and sorting was performed using a FACSAriaII cell sorter from Beckton Dickinson.

Primary antibodies used were phycoerythrin-conjugated mouse anti-human Tie2 (1:50, R&D, Cat. #FAB3131P), fluorescein-isothyocyanate conjugated mouse anti-human CD11b (1:50, Abcam, Cat. # ab25827-100), allophycocyanin conjugated mouse anti-human CD14 (1:50, R&D, Cat. #FAB3832A).

Isolation of monocytes from peripheral blood of healthy donors. Peripheral blood mononuclear cells (PBMCs) were prepared from healthy donor blood (Gulf Coast Regional Blood Center, Houston, TX) by centrifugation on a Ficoll-Paque Plus (GE Healthcare, Cat. # 17-1440-02) density gradient at 950g for 20 minutes, with deceleration set at zero. PBMCs were incubated in the presence of 20µl per $1x10^7$ cells CD14 MicroBeads (Miltenyi Biotech, Cat. # 120-000-305) for 20 minutes at 4°C after which cells were run through a magnetic column to isolate CD14 positive monocytes. Monocyte-enriched population was then double stained with anti-human CD14-APC (R&D Systems, Cat. # FAB3832A) and anti-human Tie2-PE (R&D Systems, Cat. # FAB3131P). Stained cell suspension was then subjected to flow cytometric analysis and cell sorting (FACSAria, Becton Dickinson) to obtain Tie2-negative ($CD14^+/Tie2^-$) and Tie2-expressing monocytes ($CD14^+/Tie2^+$).

Migration assays. For migration assays, THP-1 cells were placed in either normoxia or hypoxia conditions in the presence or absence of 20ng/ml IL-4 and 20ng/ml IL-13 for 48 hours. In some experiments, cells were sorted according to Tie2 expression prior to migration assay. For this purpose, cells were stained using phycoerythrin-conjugated anti-Tie2 antibody (R&D, Cat. #FAB3131P) and subjected to flow cytometric analysis and cell sorting using Becton Dickinson FACS Aria II.

24-well plates were used for migration assay with a transwell containing a 5μm pore polycarbonate membrane (Corning, Cat. # 3422). Depending on the experiment, $1x10^5$ treated or sorted THP-1 cells, were placed in a transwell, which was then placed over a well containing 500μl of either 400ng/ml Ang2 (R&D, Cat. #623-AN-025) in serum-free media or the equivalent amount of BSA. Cells were allowed to migrate for 24 hours, after which total number of migrated cells in the lower chamber was counted with a hemocytometer using Trypan Blue. Results shown are the mean from three separate migrations.

Invasion assays. For invasion assays, THP-1 cells were placed in normoxia or hypoxia conditions in the presence or absence of 20ng/ml IL-4 and 20ng/ml IL-13 for 48 hours. In some experiments, cells were stained using phycoerythrin conjugated anti-Tie2 antibody (R&D, 1:50) and subjected to flowcytometric analysis and cell sorting using Becton Dickinson FACS Aria II. Following sorting or polarizing conditions, cells were cultured in RPMI media containing 1% fetal bovine serum for 24 hours. Conditioned media from Tie2 negative and Tie2 positive cells were then collected and used for invasion assay.

24-well plates were used for invasion assay with a transwell containing an 8μm pore polycarbonate membrane (Corning, Cat. #3422). Membrane was coated with 0.3mg/ml LDEV free, growth factor reduced matrigel matrix (BD Biosciences, Cat #356230). Matrigel was allowed to solidify at room temperature for 2 hours prior to the experiment. Depending on the experiment, $1x10^5$ U87 glioma cells or GSC20 glioma stem cells were seeded on the transwell, which was then placed over a well containing 500μl of conditioned media from either Tie2-negative or Tie2-positive monocytes. Cells were allowed to invade for 24 hours, after which cells in the transwell were fixed and stained using a solution of 0.1% crystal violet in 20% methanol at room temperature for 20 minutes. Non-invading cells within the upper portion of the transwell were removed using a cotton swab and invading cells attached to the bottom side of the transwell were quantified.

Gelatinase activity assay. Tie2 negative and Tie2 positive monocytes were obtained from donor blood as described above. After sorting, cells were cultured in serum free media for 24 hours after which conditioned media was used to detect gelatinase activity using EnzCheck® Gelatinase/Collagenase Assay Kit (Life Technologies, Cat. #E12055) as per manufacturer's instructions. Briefly, conditioned media was incubated in the presence of fluorescein-conjugated DQ-gelatin from pig skin at room temperature overnight in the dark. Fluorescence was measured at 495nm.

Enzyme-linked immunosorbent assay. Tie2-positive and Tie2-negative monocytes were isolated from peripheral blood as described above. After sorting, cells were cultured in

serum free media for 24 hours after which conditioned media was collected. MMP2 and MMP9 were detected using Quantikine ELISA Kit (R&D, Cat#MMP200 and DMP900) as per manufacturer's instructions. Briefly, conditioned media was diluted with Assay Diluent provided and added to each well. After a 2 hour incubation at room temperature, wells were washed and conjugated antibody against the protein of interest was added to each well. After a 2 hour incubation period, wells were washed and a mixture of Color Reagent A and Color Reagent B was added to the wells for at least 30 minutes. The reaction was stopped using a sulfuric acid based solution and absorbance was read at 450nm.

BrdU incorportation assay. Cells were plated in a 96-well plate and allowed to attach overnight. BrdU incporation assay was performed using BrdU Cell Proliferation Assay Kit (Cell Signaling, Cat #6813) as per manufacturer's instructions. Briefly, cells were incubated at 37°C overnight in the presence of labeling agent. Media was aspirated and cells were exposed to Fixing/Denaturing solution followed by incubation in detection antibody solution at room temperature. Cells were then incubated in HRP-conjugated secondary antibody followed by TMB substrate. Reaction was stopped after 30 minutes using the provided Stop solution and absorbance was measured at 450nm.

In vivo experiments. All animal studies were performed in the Department of Veterinary Medicine at The University of Texas MD Anderson Cancer Center (Houston, TX) in accordance with institutional and ethical guidelines for experimental animal care and approved by the MD Anderson IACUC.

U87MG cells ($5x10^5$) were implanted intra-cranially in the caudate nucleus of athymic mice as described before. Briefly, a 2.6-mm guide screw with a 0.5-mm diameter central hole is implanted into a drill hole made 2.5 mm lateral and 1 mm anterior to the bregma. It is capped with a stylet between treatments. Tumor cells were injected using a 26-gauge needle of a Hamilton syringe with a cuff to determine the depth of injection. VEGF Trap was administered subcutaneously at 25mg/kg, twice a week starting 10 days after implantation and continued for either 3 weeks or 6 weeks. Bevacizumab treatment was administered intraperitoneally at 10mg/kg starting on day 5 post-implantation and continued for 6 weeks. Temozolomide treatment was administered intraperitoneally at 7.5 mg/kg/day on days 4-8 and 11-22 post-implantation. Mice were sacrificed when signs of high tumor burden were apparent. Brains were extracted, formalin fixed and paraffin embedded. Five µm sections were cut and mounted on slides for histological and protein expression analysis.

GL261, GL261.Ctrl or GL261.sTie2 mouse glioma cells ($2x10^5$) were implanted intracranially into the caudate nucleus of C57/BL6 mice as described above. DC101 treatment was administered intraperitoneally at 800µg/dose three times a week for a total of 4 weeks. Mice were sacrificed when signs of high tumor burden were apparent. Brains were extracted, formalin fixed and paraffin embedded. Five µm sections were cut and mounted on slides for histological and protein expression analysis.

Statistical Analysis. The two-tailed Student *t*-test was used to determine the statistical significance in *in vitro* experiments. Log-rank test was used to determine statistical

significance of *in vivo* survival. Only p values less than 0.05 were accepted as statistically significant.

Chapter III: Tie2 expressing monocytes are recruited to the tumor following anti-VEGF therapy

Rationale and expectations

In the process of angiogenesis, there is a tight interplay between the VEGF pathway and the Ang-Tie2 pathway. This led us to hypothesize that in the context of anti-angiogenesis therapy, the Ang-Tie2 pathway could be upregulated as a means to escape VEGF blockade. To test this, we initially evaluated the presence of Tie2 within tumors following anti-VEGF therapy. We hypothesized that there would be an increase in Tie2 following anti-angiogenesis treatment.

Results

Tie2-expressing cells accumulate in the periphery of the tumor after anti-VEGF therapy. Tumor sections from glioma-bearing mice treated with either control hFc or aflibercept were obtained and immunostaining for Tie2 was performed (Figure 1). We tested two different VEGF-Trap/aflibercept schedules: a short 3-week schedule that did not lead to an enhanced invasive pattern and a prolonged 6-week schedule that led to the appearance of invasive nodules. As can be observed, there is a marked increase in the number of Tie2-expressing cells in tumors treated with anti-VEGF therapy, particularly in the periphery of the tumor and surrounding areas of invasion. Quantification of positive cells shows this increase in Tie2-positive cells to be significant and circumscribed to the periphery of the tumor. Interestingly, this significant increase in Tie2 positive cells is only observed after prolonged (6-week) anti-VEGF therapy, whereas 3-week treatment does not lead to an increase in these cells.

The morphological features of the Tie2 positive cells suggested they were not U87 glioma cells. They were characterized by small, rounded and normal appearing nuclei with scant cytoplasm, which suggests they could be circulating immune cells infiltrating the tumor or resident cells, such as microglia, proliferating from within the tumor.

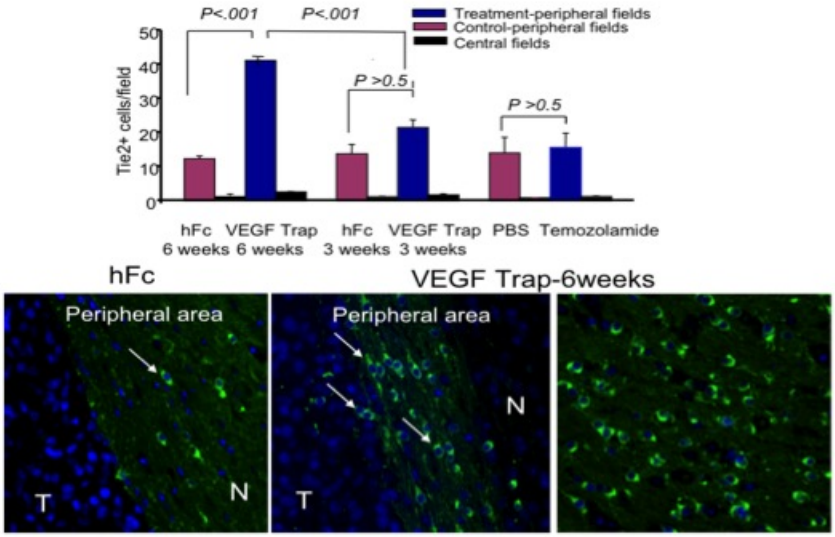

Figure 1. Tie2 expressing cell population increases significantly within the periphery of the tumor following anti-VEGF therapy. Nude mice were implanted with U87 glioma cells and treated with hFc or VEGF-Trap/aflibercept for 3 or 6 weeks and PBS or temozolomide as described in materials and methods. Immunostaining for Tie2 was performed. Upper panel: representative image of Tie2 staining as labeled. Lower panel: quantification of Tie2 positive cells within tumor sections and fields as labeled.

Tie2 expressing monocytes are recruited to the tumor following anti-VEGF therapy. Monocytes/macrophages are one of the most abundant cells within the tumor microenvironment in gliomas.[68] Given the morphological features, we hypothesized that these Tie2-expressing cells were in fact Tie2 expressing monocytes that are recruited to the tumor following anti-VEGF therapy. To test our hypothesis, we obtained tissue sections from glioma-bearing mice treated with VEGF-Trap/aflibercept for 3 or 6 weeks, bevacizumab or temozolomide. Double staining for Iba1, a monocyte/microglia marker, and Tie2 was performed and double positive cells were quantified. Our results confirmed our hypothesis showing a marked and significant increase in the number of double positive cells following anti-VEGF therapy with VEGF-Trap/aflibercept or bevacizumab. TEMs were mainly localized around the tumor periphery and areas of invasion. Again, this increase TEMs was only observed after a prolonged schedule of VEGF-Trap/aflibercept when invasion occurs. Furthermore, treatment with temozolomide did not lead to a similar outcome, providing evidence that TEMs are being selectively recruited as a response to anti-angiogenic treatment.

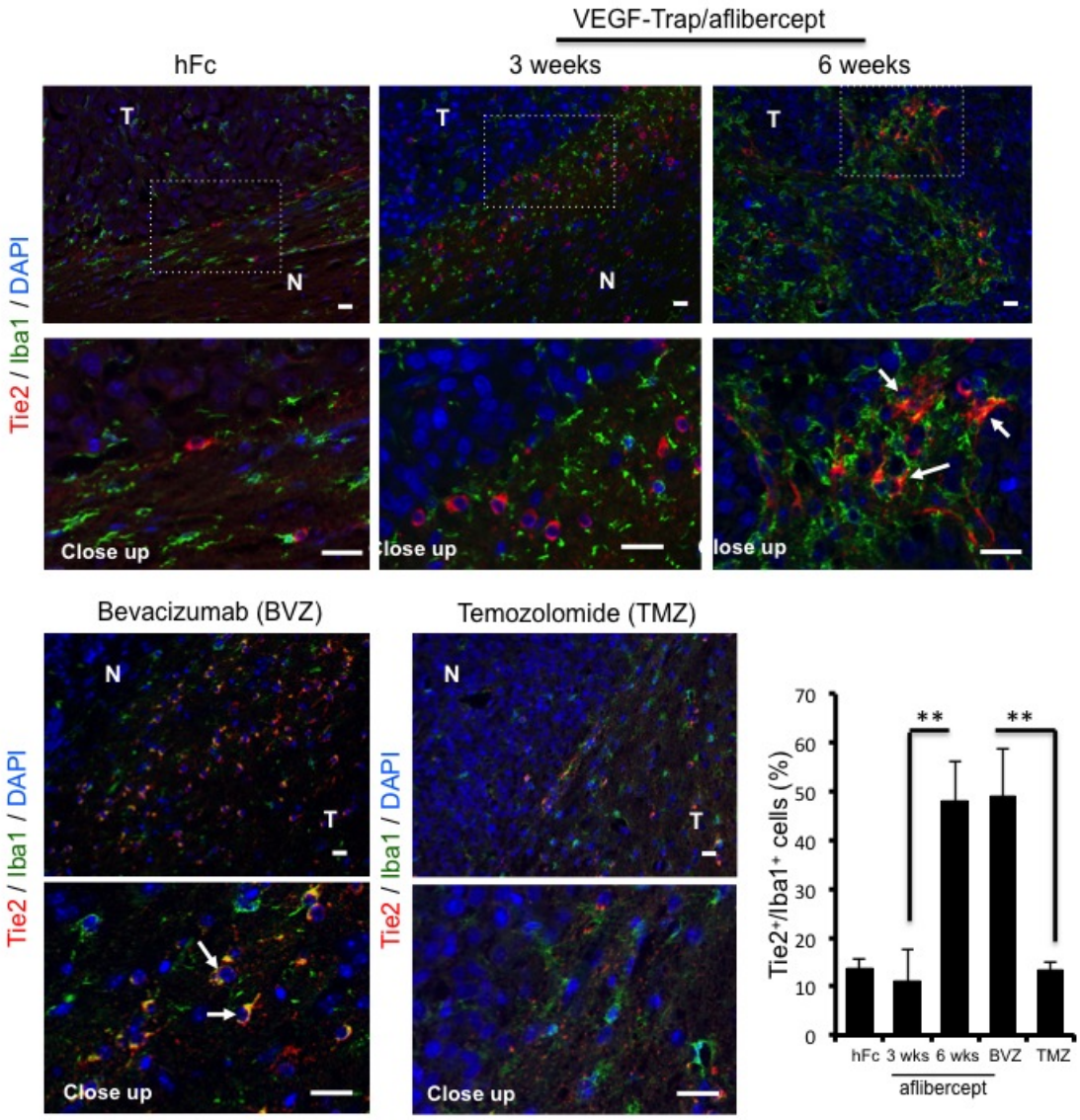

Figure 2. Tie2-expressing monocytes are recruited to the tumor following anti-VEGF therapy. Nude mice were implanted with U87 glioma cells and treated with hFc, VEGF-Trap/aflibercept for 3 or 6 weeks, bevacizumab or temozolomide as described in materials and methods. Immunostaining for Tie2 and Iba1 was performed. Upper panel: representative image of Tie2/Iba1 staining as labeled. Lower panel: Representative images of Tie2/Iba1 staining as labeled. To the right is the quantification of double positive cells presented as a percentage of the total monocyte (Iba1+) population. p-values were calculated using Student t-test. ** $p<0.01$

Conclusions

Tie2-expressing cells, and Tie2-expressing monocytes in particular, are selectively recruited to the tumor following prolonged anti-VEGF therapy. This recruitment occurs only after a prolonged schedule of anti-VEGF therapy, when invasion appears. Furthermore, they accumulate around the peripheral areas of the tumor, and particularly around invasive nodules. This result suggests a role for this cell population in the invasive phenotype observed after anti-angiogenic therapy.

Chapter IV: Increased Ang2 expression is associated with the development of invasion following anti-VEGF therapy

Rationale and expectations

We have shown an accumulation of Tie2-expressing cells, and Tie2-expressing monocytes in particular, around the periphery of the tumor following anti-VEGF therapy. We wanted to further elucidate the status of the Ang-Tie2 pathway following anti-VEGF therapy, hypothesizing that there was an upregulation of angiopoietin expression leading to recruitment of Tie2-expressing monocytes to the tumor and potentially modulating their behavior to aid in the invasive process. At the moment of this study, there were various reports correlating Ang2 expression with overexpression of MMP2 and invasion in glioma.[78-79] Furthermore, Ang2 has been shown to be expressed in human GBM surgical samples to a greater extent than in lower grade astrocytomas, particularly in peripheral areas nad invasive cells.[78] Therefore, we expected to find an upregulation of Ang2 following anti-VEGF therapy and that this had an important role in TEM recruitment as well as the invasive recurrence of gliomas after treatment.

Results

Ang2 expression is upregulated after prolonged treatment with VEGF-Trap/aflibercept. Using tissue sections from glioma-bearing mice treated with either control hFc or VEGF-Trap/aflibercept for 3 or 6 weeks, we performed immunostaining for Ang1 and Ang2 (Figure 3). Ang1 expression, as assessed by immunohistochemical staining, is below detection levels regardless of treatment status. On the other hand, Ang2 expression dramatically increases following anti-VEGF therapy. Interestingly, the increased expression is mainly circumscribed to the periphery of the tumor and invasive nodules, following the same pattern observed for TEMs. We proceeded to perform immunofluorescence analysis for quantification purposes (Figure 4). Results obtained show a significant increase in Ang2 positive cells following anti-VEGF therapy when compared to control treated tumors or tumors treated for 3 weeks, when invasion does not occur.

Since VEGF has been associated with invasion in gliomas, we were interested in evaluating the effect of inhibiting VEGF in the expression of this ligand within the tumor (Figure 3). Interestingly, we saw an upregulation of VEGF after VEGF/Trap aflibercept. However, we begin to see this upregulation after only 3 weeks of treatment when invasion does not occur. This result suggests that increased VEGF expression is not sufficient to cause the invasive phenotype we observe after prolonged treatment.

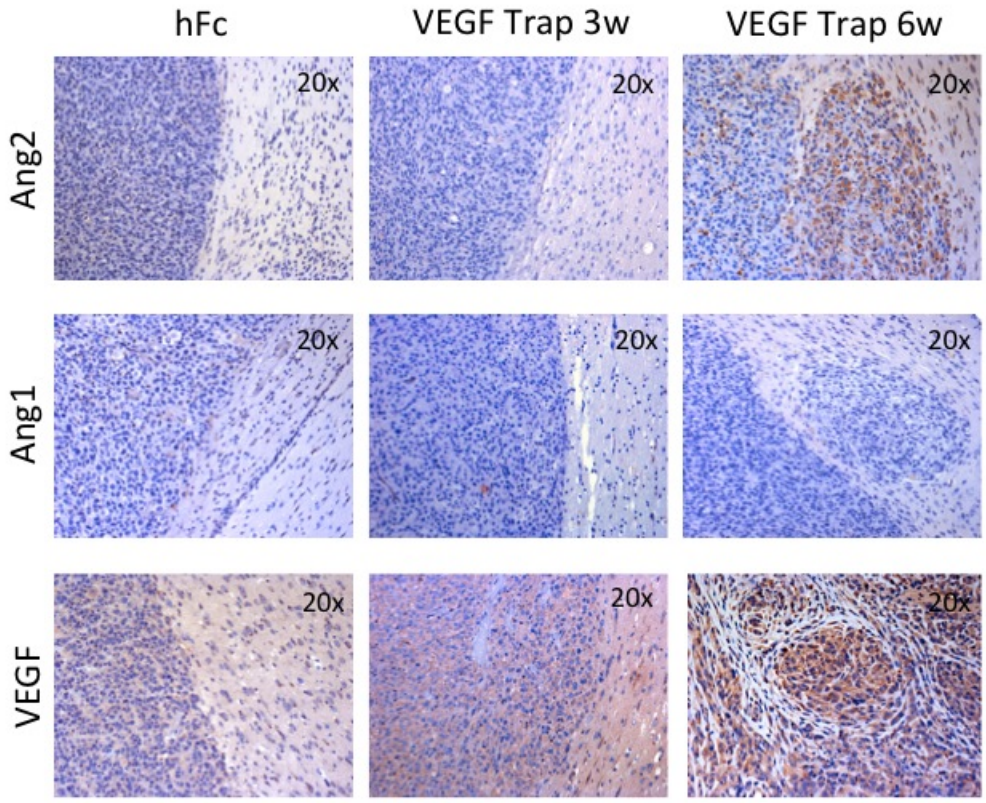

Figure 3. Expression of Ang2, a Tie2 ligand, increases in the periphery of the tumor after anti-VEGF therapy, particularly around areas of invasion. Nude mice were implanted with U87 glioma cells and treated with hFc or VEGF-Trap/aflibercept for 3 or 6 weeks and sacrificed when signs of high tumor burden were apparent. Immunohistochemical analysis was performed to detect changes in the expression of molecules associated with the Tie2 and VEGF pathway. Shown are representative images.

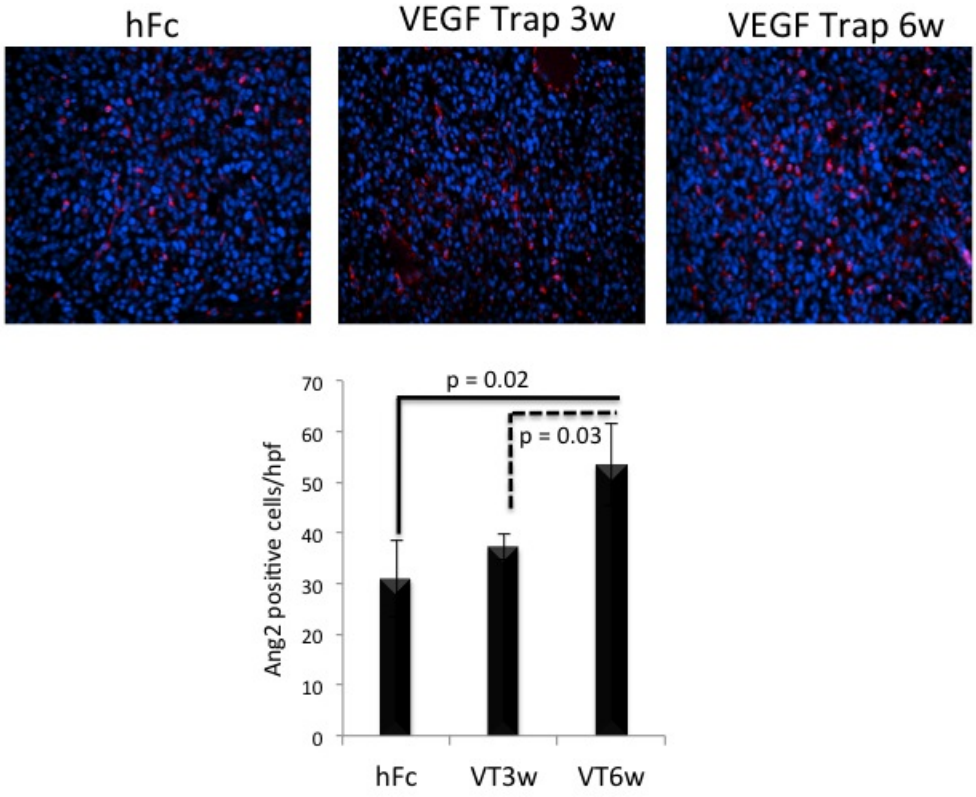

Figure 4. Increased in Ang2 expression following anti-VEGF therapy is statistically significant as assessed by immunofluorescence analysis. Nude mice were implanted with U87 glioma cells and treated with hFc or VEGF-Trap/aflibercept for 3 or 6 weeks and sacrificed when signs of high tumor burden were apparent. Immunoflurescence staing for Ang2 was performed. Upper panel: representative images of immunofluorescence staining of Ang2 (red) expression after hFc control treatment, or a short (3 week) or prolonged (6 week) regimen of anti-VEGF therapy. Lower panel: quantification of Ang2 expression is graphed as average number of positive cells per high power field.

Ang2 upregulation is a common phenomena to all VEGF targeted approaches. VEGF-Trap/aflibercept is a chimeric antibody composed of the VEGF binding domain of the VEGFR1 and VEGFR2 attached to the constant portion (Fc) of an IgG for solubilization.[80] Therefore, it works a soluble ligand trap. It is currently in clinical trials for the treatment of recurrent GBM at MD Anderson Cancer Center. Bevacizumab, on the other hand, is an anti-VEGF antibody that is currently FDA approved for the treatment of recurrent GBM. To determine if bevacizumab treatment led to similar increases in Ang2 as VEGF-Trap/aflibercept, we performed immunostaining for Ang2 in tissue sections from glioma-bearing mice treated with bevacizumab or control hFc (Figure 5). Our results show a dramatic increase in Ang2 expression in bevacizumab treated tumors in a similar pattern as that observed for aflibercept. These data support that upregulation of Ang2 is a global phenomenon for VEGF targeted therapeutic approaches regardless of the means used to achieve inhibition.

We then obtained fresh tissue protein lysate from tumors treated with either control treatment or bevacizumab treatment and performed ELISA to determine Ang2 concentration within the tumor and normal brain from the contralateral hemisphere (Figure 6). Our data show an increase in Ang2 expression in tumors treated with bevacizumab when compared to control treated tumors. This change was not found to be statistically significant, possibly due to the inherent variability of *in vivo* models. Interestingly, Ang2 was barely detectable in normal brain tissue regardless of treatment.

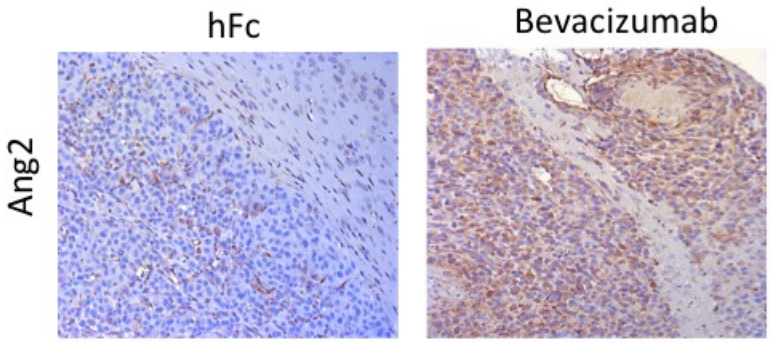

Figure 5. Enhanced Ang2 expression is common to all anti-VEGF therapeutic approaches. Tissue sections from glioma bearing mice treated with either hFc or bevacizumab for 6 weeks were obtained and staining for Ang2 was performed. Shown are representative images of the staining.

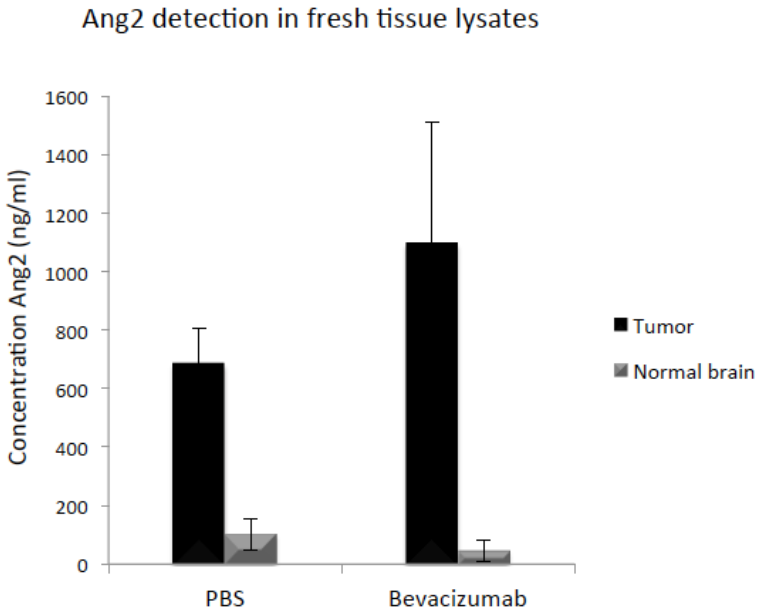

Figure 6. Ang2 concentration increases within tumor tissue following anti-VEGF therapy with bevacizumab. Nude mice were implanted with U87 glioma cells and treated with PBS or bevacizumab 6 weeks and sacrificed when signs of high tumor burden were apparent. Fresh tissue protein lysates was obtained from tumor and normal brain from contralateral hemisphere. ELISA was performed to assess Ang2 concentration. Shown is concentration of Ang2 within tissues as labeled.

Temozolomide treatment does not lead to increased Ang2 expression. Temozolomide (TMZ) is the currently approved chemotherapeutic agent for patients with newly diagnosed glioblastoma. We wanted to see if upregulation of Ang2 expression was specific for anti-VEGF approaches or if other therapeutic regimes could also lead to similar effects. To evaluate this, we performed immunohistochemical staining for Ang2 in tissue sections from glioma-bearing mice treated with either control PBS or TMZ (Figure 7). Our results show that following TMZ treatment, Ang2 remains localized mainly in vascular structures and it is not increased as observed for anti-VEGF therapy.

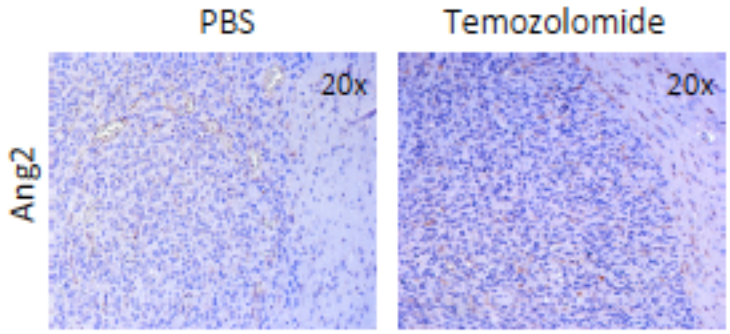

Figure 7. Temozolomide treatment does not lead to increased Ang2 expression. Nude mice were implanted with U87 glioma cells and treated with PBS or temozolomide as described in material and methods. They were sacrificed when signs of high tumor burden were apparent. Immunohistochemical staining for Ang2 was performed. Shown are representative images.

Characterization of Ang2-expressing cells following anti-VEGF therapy. Given the varied morphological features of the cells expressing Ang2 after anti-VEGF therapy, we proceeded to characterize the major contributing cell to the Ang2 pool. For this purpose, we performed double immunofluorescence for Ang2 in combination with α-SMA as a marker for pericytes, CD31 as a marker of endothelial cells, Iba1 as a marker of monocytes or NuMA as a marker of cells from human origin to distinguish glioma cells. As expected, our results show that pericytes are not a significant source of Ang2, regardless of treatment. As can be seen in the inset the two dyes do not colocalize (Figure 8). On the other hand, the vast majority of endothelial cells within the tumor bed express Ang2 as can be shown by the colocalization of the two dyes in the representative picture (Figure 9). However, the relative contribution of endothelial cells to Ang2 does not change significantly with treatment as appreciated in the quantification (lower panel, Figure 9). Interestingly, monocytes are a significant source of Ang2 (Figure 10), but upon quantification, their relative contribution decreases with treatment. Given the morphological features of the Ang2 expressing cells, we hypothesized that glioma cells upregulate their expression of Ang2 becoming an important source following anti-angiogenic therapy. Since it is very difficult to obtain a cell marker that homogeneously stains all glioma cells, we took advantage of the fact that we are using a human glioma cell line on a mouse model. We obtained an antibody that detects only the human form of the nuclear mitotic apparatus protein (NuMA) and used it to differentiate glioma cells from all other cells in the tumor micorenvironment. Using this antibody, we performed double immunofluorescence staining with Ang2 to determine the contribution of glioma

cells to the Ang2 pool (Figure 11). Taken together, our results show that only within the glioma compartment is there a significant increase in the relative contribution of Ang2.

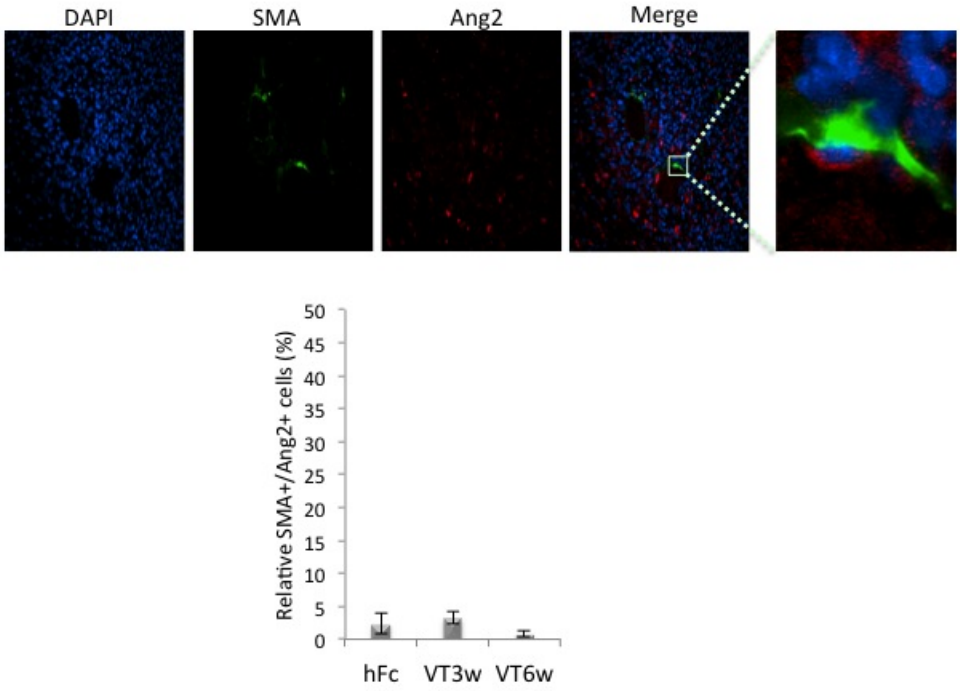

Figure 8. Pericytes are not a significant source of Ang2 following anti-VEGF therapy. Nude mice were implanted with U87 glioma cells and treated with hFc or VEGF-Trap/aflibercept for 3 or 6 weeks. They were sacrificed when signs of high tumor burden were apparent. Upper panel: representatve image of immunofluorescence analysis for SMA (green) and Ang2 (red) of tissue sections from glioma bearing mice treated with anti-VEGF therapy for 6 weeks. Nuclei counterstaining was done with DAPI (blue). Lower panel: graph depicting percentage of Ang2 coming from pericytes (i.e. SMA + cells) following control hFc treatment or a short (3 week) or prolonged (6 week) schedule of anti-VEGF therapy.

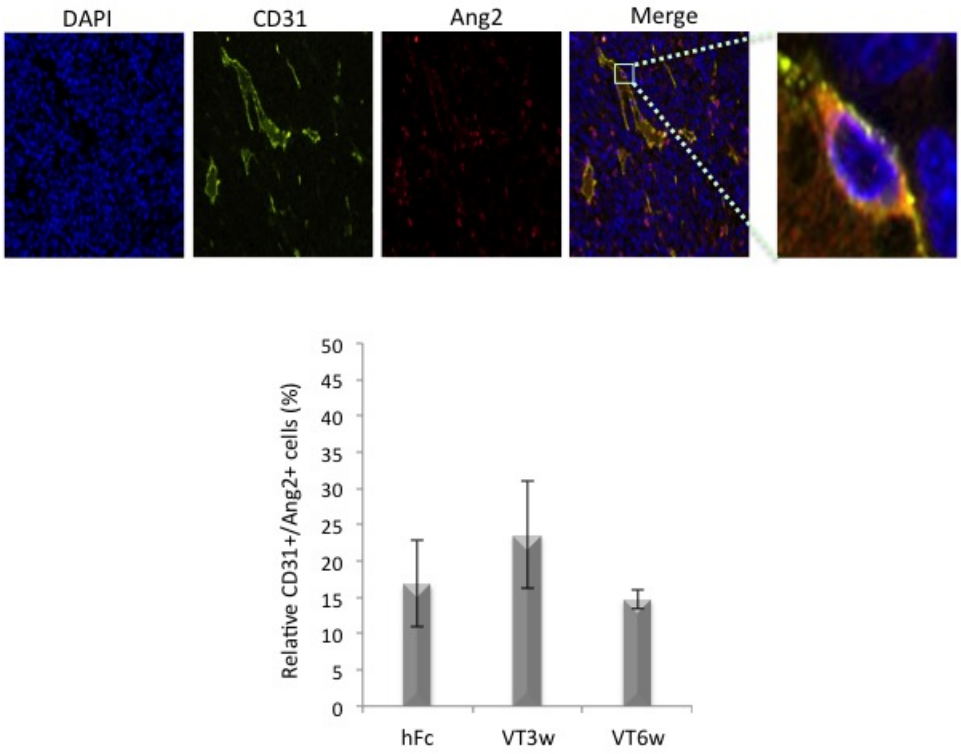

Figure 9. Endothelial cells are an important source of Ang2 following anti-VEGF therapy but their relative contribution remains unchanged regardless of treatment. Nude mice were implanted with U87 glioma cells and treated with hFc or VEGF-Trap/aflibercept for 3 or 6 weeks. They were sacrificed when signs of high tumor burden were apparent. Upper panel: representatve image of immunofluorescence analysis for CD31 (green) and Ang2 (red) of tissue sections from glioma bearing mice treated with anti-VEGF therapy for 6 weeks. Nuclei counterstaining was done with DAPI (blue). Lower panel: graph depicting percentage of Ang2 coming from endothelial cells (i.e. CD31 + cells) following control hFc treatment or a short (3 week) or prolonged (6 week) schedule of anti-VEGF therapy.

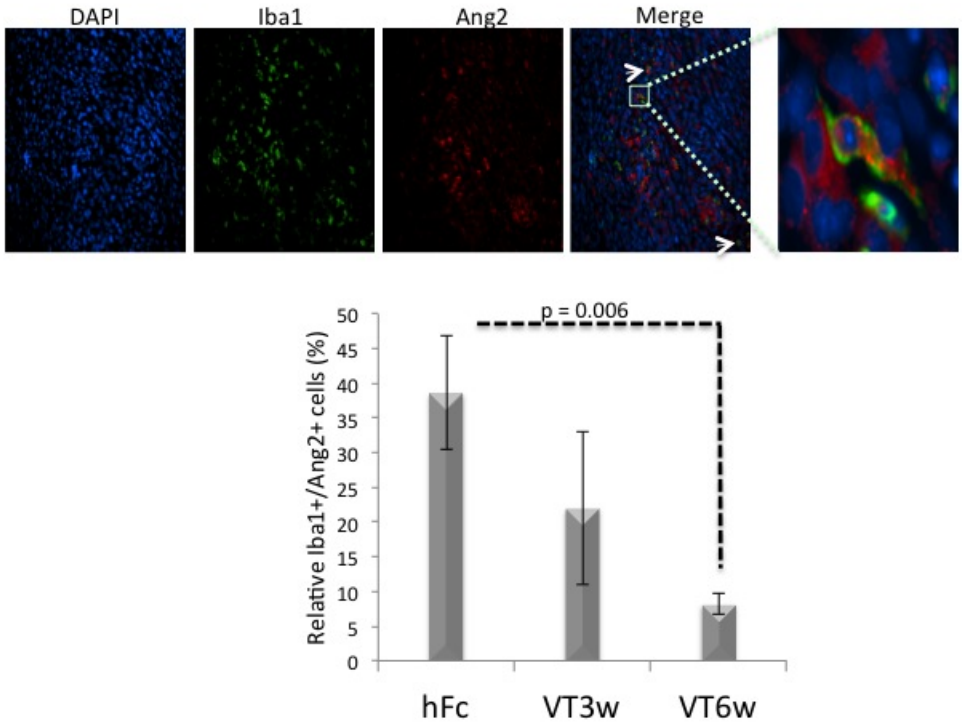

Figure 10. Monocytes are a significant source of Ang2 following anti-VEGF therapy, but their relative contribution decreases with treatment. Nude mice were implanted with U87 glioma cells and treated with hFc or VEGF-Trap/aflibercept for 3 or 6 weeks. They were sacrificed when signs of high tumor burden were apparent. Upper panel: representatve image of immunofluorescence analysis for Iba1 (green) and Ang2 (red) of tissue sections from glioma bearing mice treated with anti-VEGF therapy for 6 weeks. Nuclei counterstaining was done with DAPI (blue). Lower panel: graph depicting percentage of Ang2 coming from monocytes (i.e. Iba1 + cells) following control hFc treatment or a short (3 week) or prolonged (6 week) schedule of anti-VEGF therapy.

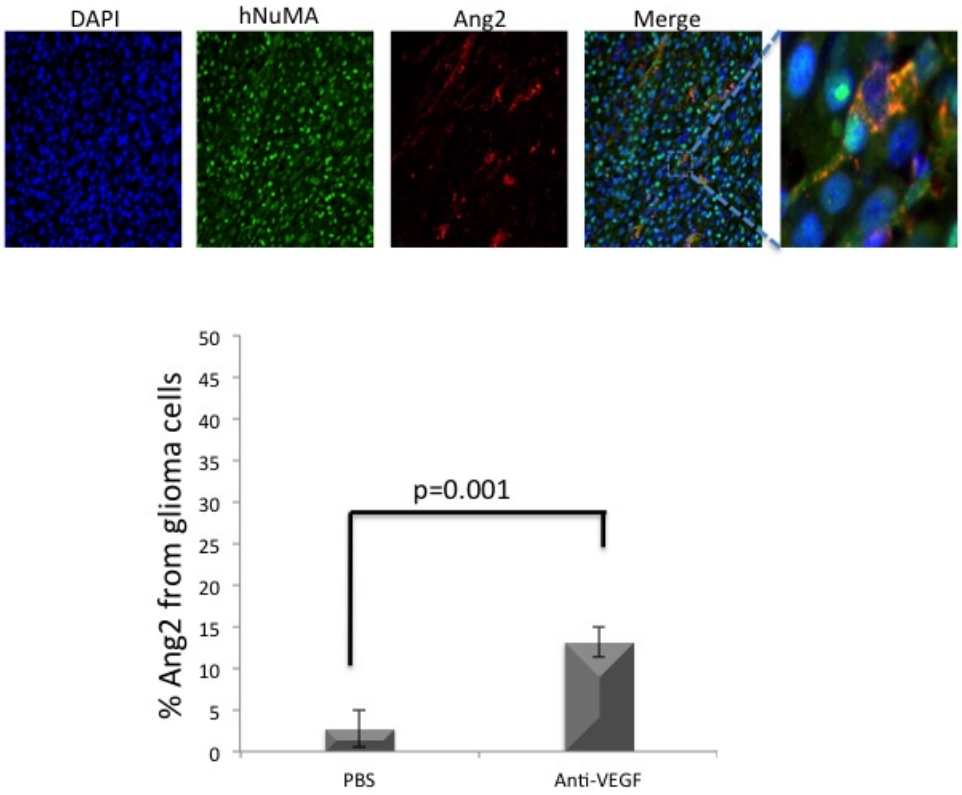

Figure 11. Glioma cells significantly increase their production of Ang2 following anti-VEGF therapy. Nude mice were implanted with U87 glioma cells and treated with hFc or VEGF-Trap/aflibercept for 3 or 6 weeks. They were sacrificed when signs of high tumor burden were apparent. Upper panel: representatve image of immunofluorescence analysis for NuMA (green) and Ang2 (red) of tissue sections from glioma bearing mice treated with anti-VEGF therapy for 6 weeks. Nuclei counterstaining was done with DAPI (blue). Lower panel: graph depicting percentage of Ang2 coming from human cells (i.e. NuMA + cells, glioma cells) following control hFc treatment or a short (3 week) or prolonged (6 week) schedule of anti-VEGF therapy.

Conclusions

Ang2, a Tie2 ligand, is upregulated following anti-VEGF therapy following the same spatial pattern as that observed for TEMs. This increase in Ang2 is specific for anti-VEGF targeted approaches, since treatment with temozolomide did not lead to similar changes. Furthermore, characterization of the cells expressing Ang2 showed that the glioma compartment significantly upregulates expression in response to anti-VEGF therapy. These data suggest that Ang2 is an important mediator of glioma-acquired resistance to anti-VEGF therapy and the invasive phenotype observed upon recurrence.

Chapter V: Ang2 leads to TEM recruitment after anti-VEGF therapy

Rationale and expectations

Ang2 is one of the most studied ligands of Tie2 and it is known to cooperate with VEGF in the process of angiogenesis. Given our data on TEM recruitment and Ang2 overexpression after anti-VEGF therapy, we hypothesized that Ang2 is responsible for the recruitment of TEMs to the tumor following anti-VEGF therapy. We expect Tie2 expressing monocytes to have an enhanced migratory response in the presence of Ang2 than their Tie2 negative counterparts *in vitro* and *in vivo.*

Results

Ang2 induces migration in polarized THP-1 cells, and in particular Tie2 expressing THP-1 cells. Studying cells of monocytic origin can be very difficult due to their short duration in culture once isolated from buffy coats. Additionally, TEMs represent 10% or less of the monocytic population of a normal patient, adding to the difficulty of studying them. Given these problems, we designed a method to generate TEMs using the THP-1 monocytic leukemia cell line. As described in the materials and methods section, THP-1 cells were M2 polarized and induced to express Tie2 by exposure to hypoxia for 48 hours in the presence of M2 polarizing cytokines, IL4 and IL13. As controls, cells exposed to normoxia, normoxia with IL4 and IL13 and hypoxia alone were also evaluated for the expression of Tie2 (Figure 12). Following 48-72 hours in these conditions, cells were either used directly and compared to cells placed in normoxia, or sorted for Tie2 expression before migration experiments were done.

After verifying the increase in Tie2 expression following polarizing conditions (Figure 12), migration towards Ang2 of cells exposed to hypoxia with interleukins was tested and compared to cells placed in normoxia (Figure 13). Our results show a significant increase in the number of migrating cells in the cells exposed to hypoxia with IL4 and IL13 compared to cells placed in normoxia. To determine whether this increased migratory response is due to the presence of Tie2 expressing monocytes, we proceeded to sort THP-1 cells after Tie2 induction and polarizing conditions. Migration of Tie2 negative and Tie2 positive cells towards Ang2 was then evaluated (Figure 14). Our

results show a significantly higher number of migrating cells in the Tie2 positive THP-1 group than their Tie2 negative counterparts.

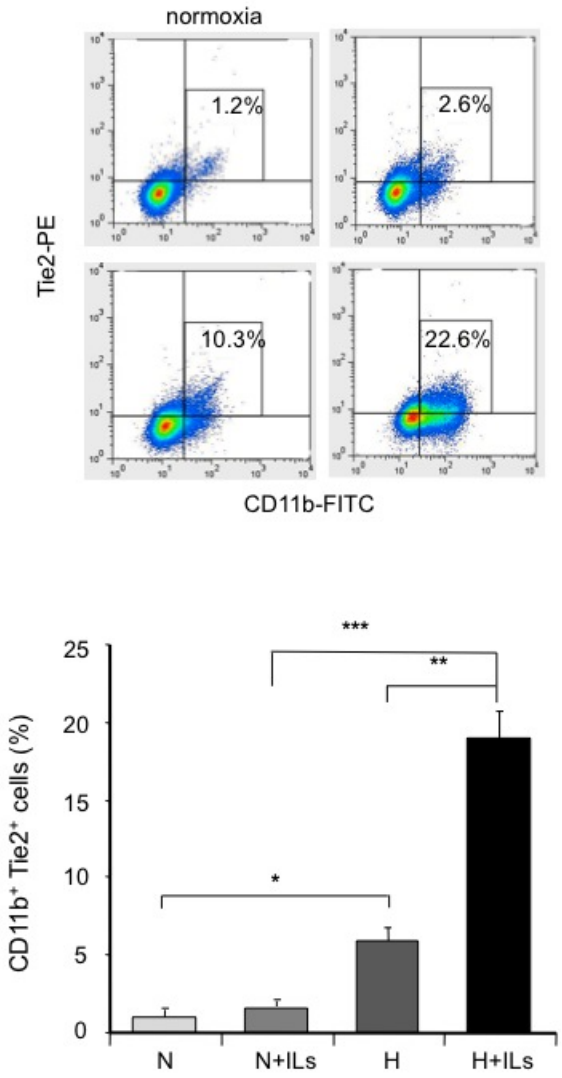

Figure 12. Tie2 expression increases in THP-1 cells following hypoxia with IL4 and IL13 treatment. THP-1 cells were polarized and induced to express Tie2 using hypoxia with IL4 and IL10 treatment for 48 hours. Upper panel: increased Tie2 expression in the presence of hypoxia with IL4 and IL10 compared to all other conditions. CD11b was used as a monocytic marker. Lower panel: the average expression of Tie2 after exposure of THP-1 cells to each condition was graphed. p-values were calculated using Student t-test.

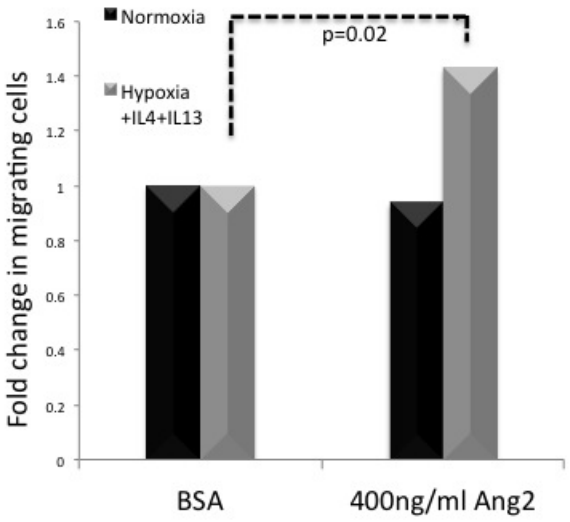

Figure 13. Ang2 is chemoattractant to polarized THP-1 cells. THP-1 cells were polarized using hypoxia with IL4 and IL10 treatment and their migration through a transwell towards Ang2 was quantified. Graph shows fold change in migration when compared to migration in the presence of vehicle alone.

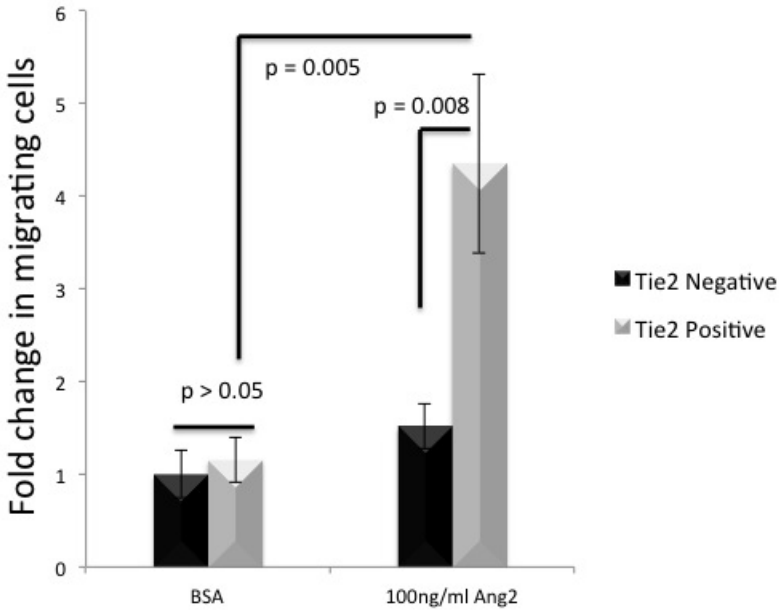

Figure 14. Ang2 is chemoattractant to Tie2 expressing THP-1 cells. THP-1 cells were sorted according to Tie2 expression following polarizing conditions. Cells that migrated through a transwell in the presence of an Ang2 gradient were quantified. Graph shows fold change in migration when compared to migration by Tie2 negative THP-1 cells in the presence of vehicle alone. p-values were calculated using Student t-test statistical analysis.

Ang2 leads to TEM recruitment *in vivo*. To evaluate whether our *in vitro* findings had any relevance in the *in vivo* setting, we proceeded to determine if high Ang2 expression within the tumor led to increased number of TEMs. For this purpose, tissue sections from glioma-bearing mice infected with an adenovirus overexpressing Ang2 (AdAng2) were obtained. To detect and quantify TEMs, double immunofluorescence for Tie2 and Iba1 was performed (Figure 15). Our results show a dramatic and highly significant increased number of TEMs in tumors treated with AdAng2 when compared to tumors treated with AdCMV (empty adenovirus). This confirms that Ang2 is chemoattractant to TEMs both *in vitro* and *in vivo*.

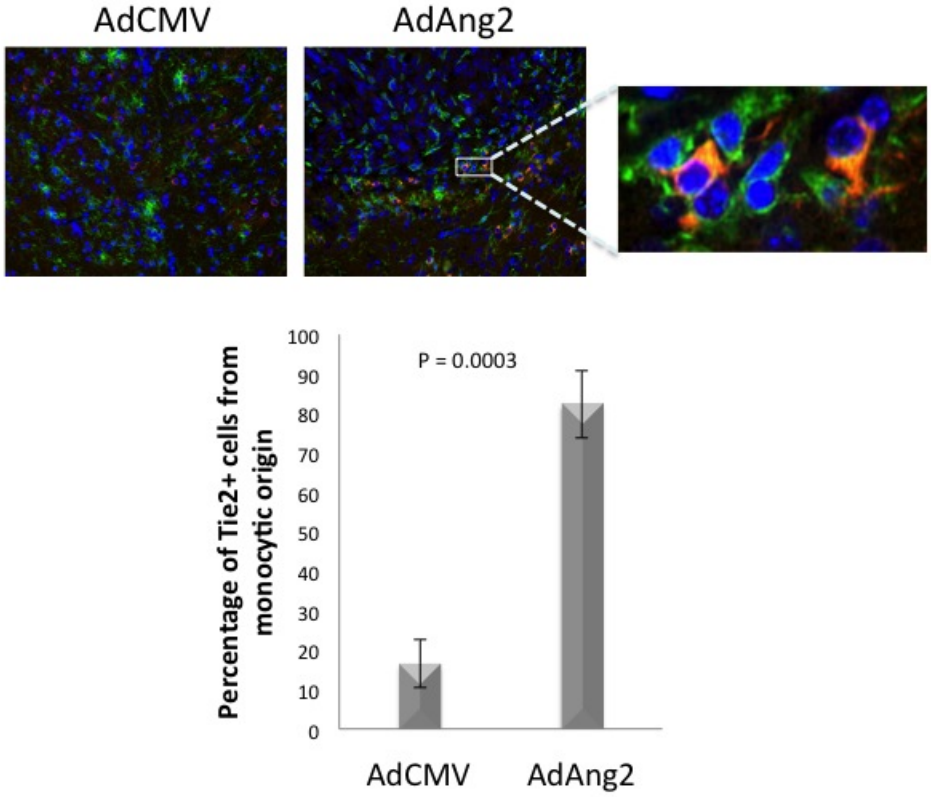

Figure 15. Ang2 is chemoattractant to Tie2 expressing monocytes in vivo. U87-glioma bearing mice were treated with an adenovirus expressing high levels of Ang2. Double immunofluorescence for Tie2 and Iba1 was performed to detect Tie2 expressing monocytes. Upper panel: representative image of double immunofluorescence staining. Lower panel: quantification of the number of double positive cells (i.e. TEMs) expressed as a percentage of monocyte population. p-values were calculated using Student t-test analysis.

Conclusions

Ang2 stimulates migration of Tie2 expressing monocytes, both *in vitro* and *in vivo.* These data provide evidence that Ang2 is capable of recruiting Tie2 expressing monocytes following anti-VEGF therapy. It is worth noting that since our experiments were performed, another group published similar results in an *in vitro* setting, which further validates our data.[72]

Chapter VI: Tie2 expressing monocytes promote an environment conducive for invasion

Rationale and expectations

Tie2 expressing monocytes are a recently discovered subset of circulating monocytes that express the Tie2 receptor.[71] They have been shown to be highly pro-angiogenic and Ang2 has been shown to further enhance their pro-angiogenic properties.[72] There is a tight interplay between the process of angiogenesis and the process of invasion, with extensive overlapping in terms of molecules required and associated with each. For example, MMPs are required to partially degrade the ECM to allow for endothelial migration during the process of angiogenesis. But, they are also known to promote tumor invasion and metastasis. Based on the distribution and temporal appearance of TEMs, we hypothesized they play an important role in the invasive phenotype after anti-VEGF therapy. We hypothesized a higher production of pro-invasive molecules, such as MMPs, in Tie2 positive monocytes when compared with their Tie2 negative counterparts. Furthermore, we expect Ang2 to further enhance the secretion of said molecules.

Results

TEMs secrete higher amounts of pro-invasive molecules than their Tie2 negative counterparts. So far we have shown that anti-VEGF therapy leads to an increase in Ang2 expression which then leads to recruitment of TEMs. We hypothesized that this increased representation of TEMs is generating an environment conducive for invasion through the secretion of pro-invasive molecules that facilitate the process. To test this hypothesis, we obtained TEMs from peripheral blood of healthy donors as described in the material and methods section. Figure 16 shows a representative sorting experiment. Once Tie2 positive and Tie2 negative monocytes were obtained, we cultured them in serum free media for 24 hours, time after which media was collected and the secretion of MMP2 and MMP9 was assessed via ELISA (Figure 17). Our results show a highly significant increased level of expression of both of these molecules by TEMs, when compared to their Tie2 negative counterparts, supporting the idea that the presence of TEMs is at least partially responsible for the pattern of recurrence following anti-VEGF therapy.

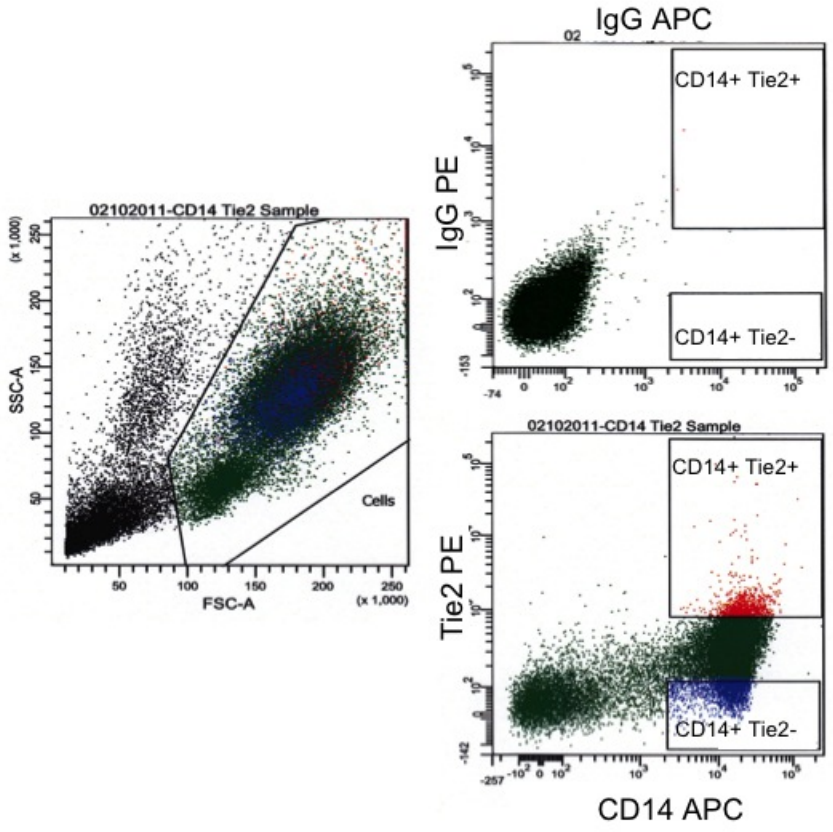

Figure 16. Flowcytometric analysis and cell sorting of TEMs and Tie2 negative monocytes obtained from buffy coats from healthy donor. Representative image of a sorting experiment in which Tie2 positive and Tie2 negative monocytes were isolated from peripheral blood of healthy donors. CD14 was used as a monocytic marker.

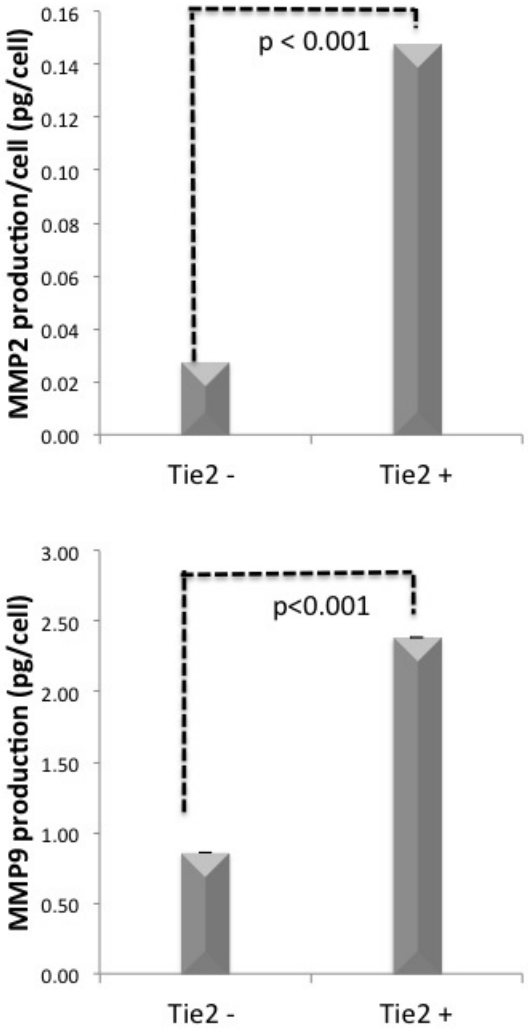

Figure 17. TEMs secrete higher levels of pro-invasive molecules. TEMs and Tie2 negative monocytes were isolated from peripheral blood of healthy donors. They were cultured for 24 hours in serum free media after which media was collected and MMP2 and MMP9 was detected via ELISA. Graphs depicts production of MMP2 (upper panel) and MMP9 (lower panel) per cell. p-values were calculated using Student t-test.

Tie2 expressing cells, potentially TEMs, express MMP9 *in vivo*. Given the selective recruitment of TEMs to the periphery of the tumor following anti-VEGF therapy, and their high expression of MMP9 we wanted to determine whether MMP9 expression was upregulated within the tumor following anti-VEGF therapy. To evaluate this we performed MMP9 immunohistochemistry in tissue sections from glioma-bearing mice treated with control hFc or VEGF-Trap/aflibercept for 6 weeks. Our results show a significant increase in the expression of MMP9 following anti-VEGF treatment (Figure 18).

To determine if Tie2 expressing monocytes could be a source of MMP9 *in vivo* we performed double immunostaining to detect Tie2 and MMP9 in tissue sections from glioma-bearing mice treated with bevacizumab (Figure 19). We evaluated dye colocalization and were able to detect double positive cells around the periphery of tumors treated with bevacizumab. Analysis through confocal microscopy shows colocalization of the two colors within one cell (lower panel, Figure 19). Morphological features of these cells support the idea of them being Tie2-expressing monocytes. However, triple staining adding Iba1 as a monocyte/microglia marker would be helpful to determine the exact contribution from this cell population to the MMP9 pool.

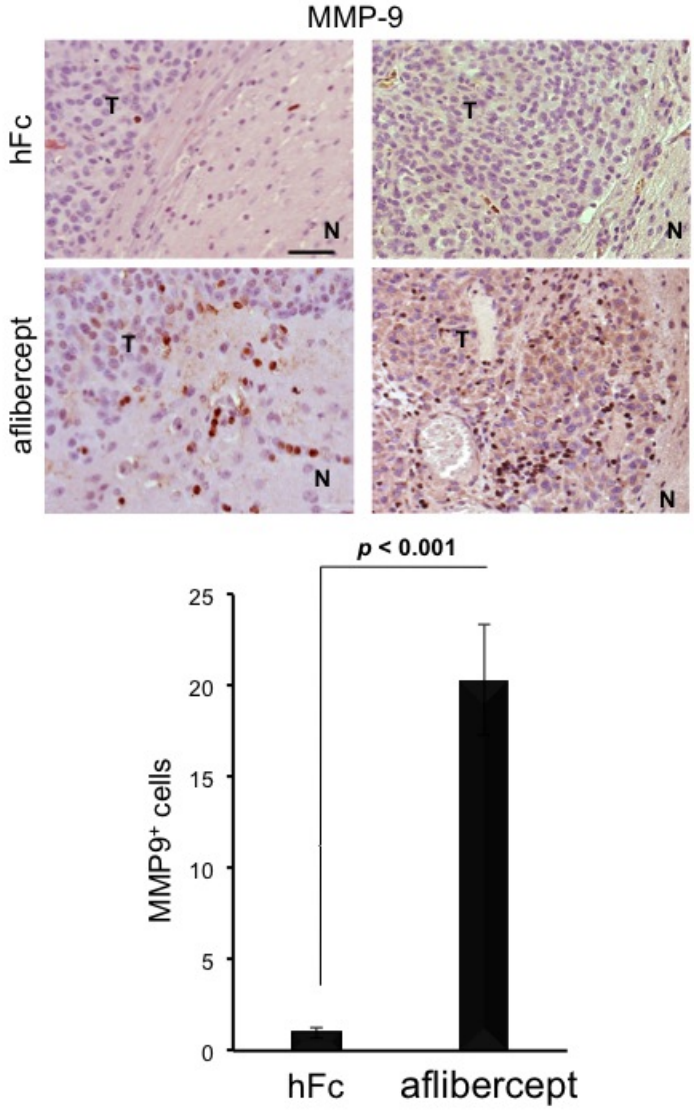

Figure 18. MMP9 expression increases significantly following anti-VEGF therapy with VEGF-Trap/ aflibercept. Tissue sections from glioma bearing mice treated with either hFc control treatment of 6 weeks of VEGF-Trap/aflibercept and MMP9 immunohistochemistry was performed. Upper panel: representative image of immunohistochemical staining. Lower panel: quantification of MMP9 positive cells. Statistical significance was calculated using Student t-test.

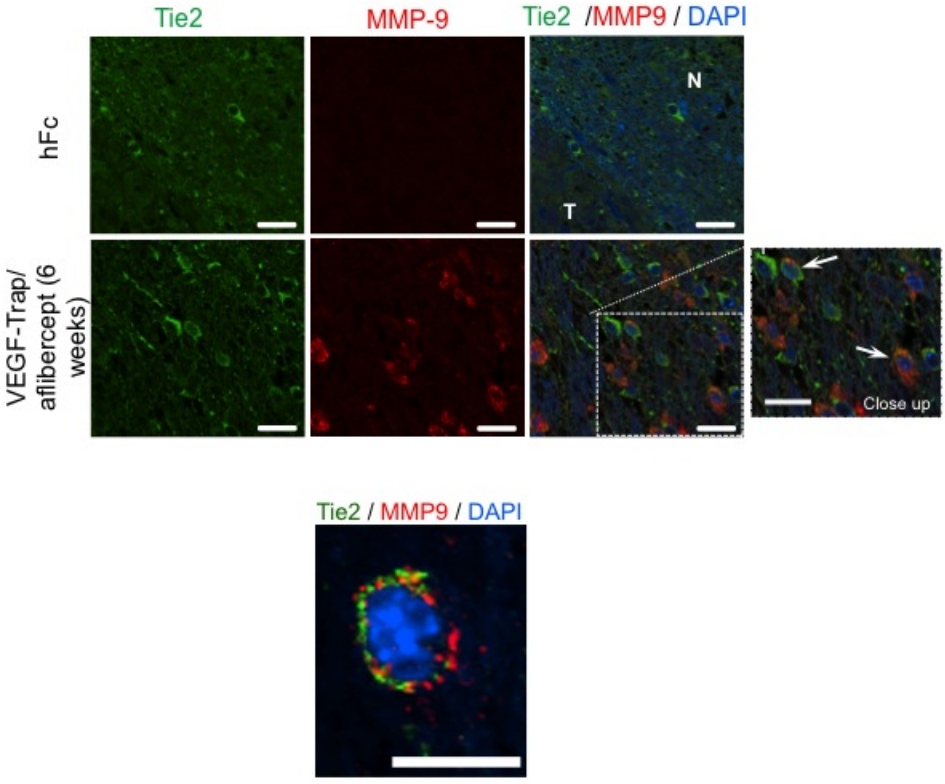

Figure 19. Tie2 expressing cells, potentially TEMs, produce MMP9 in vivo. Nude mice were implanted with U87 glioma cells and treated with hFc or VEGF-Trap/aflibercept for 6 weeks. They were sacrificed when signs of high tumor burden were apparent. Double immunofluorescence staining for Tie2 and MMP9 was performed to determine if Tie2 positive cells were a source of MMP9 *in vivo*. Upper panel: colocalization of the two dyes, i.e. MMP9 producing Tie2 positive cells, can be appreciated following anti-VEGF treatment with VEGF-Trap/aflbercept. Lower panel: confocal microscopy shows colocalization of the two dyes within a single cell.

Conditioned media from TEMs induces glioma cell invasion *in vitro*. Given the significantly higher expression of MMPs observed for TEMs, we hypothesized that glioma cells would exhibit higher invasion in the presence of conditioned media from these cells. To test this possibility we initially polarized THP-1 cells in hypoxia with IL4 and IL13 treatment for 48 hours. After 48 hours, Tie2 expression was confirmed through flow cytometric analysis (Figure 12) and conditioned media was harvested and used for invasion assays with U87 glioma cells (Figure 20). We observed a significantly higher number of invading U87 glioma cells when conditioned media from THP-1 cells grown under hypoxia conditions was used. This increased invasion was further enhanced when conditioned media from cells also exposed to IL4 and IL13 M2 polarizing cytokines was used. This further enhancement was also shown to be significant.

To determine if this increased invasion was due to the presence of TEMs, we exposed THP-1 cells to hypoxia with IL4 and IL13 for 48 hours after which cells were sorted according to Tie2 expression. After sorting, cells were cultured in serum free media for 24 hours after which conditioned media was harvested and used for invasion assays (Figure 21). Our results show a significant increase in the number of invading U87 glioma cells and invading GSC20 glioma stem cells in the presence of conditioned media from TEMs when compared to those in the presence of conditioned media from Tie2 negative monocytes.

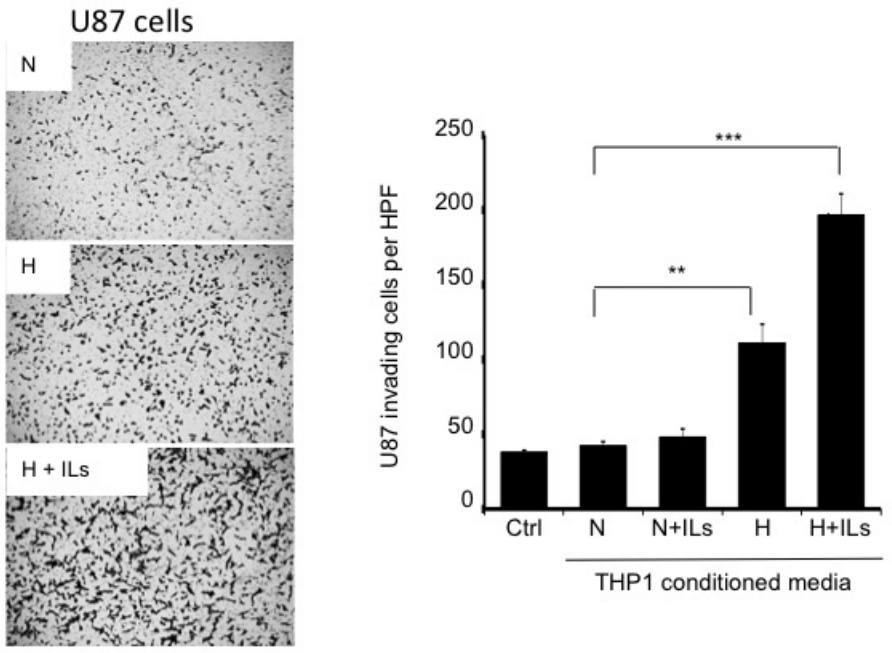

Figure 20. Conditioned media from polarized THP-1 cells induces glioma cell invasion in vitro. THP-1 cells were polarized using hypoxia with IL4 and IL10 treatment. Following polarizing conditions cells were cultured in SFM for 24 hours after which conditioned media was collected and used for invasion assay. U87 cells were placed in a transwell containing a membrane with 8um pores coated with 0.3mg/ml of growth factor reduced matrigel. Transwell was placed over a well containing conditioned media from THP-1 cells exposed to different conditions as labeled. Left panel: representative pictures of invading U87 cells attached to bottom of transwell membrane in the presence of conditioned media from monocytes exposed to labeled conditions. Right panel: quantification of invading U87 cells is graphed. p-values were calculated using Student t-test.

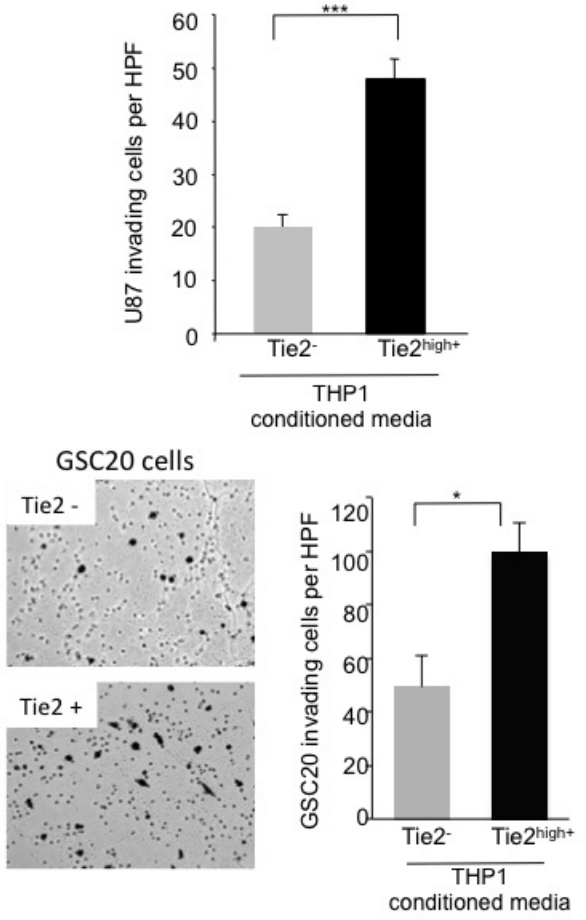

Figure 21. Conditioned media from sorted Tie2-expressing THP-1 cells induces glioma cell invasion in vitro. THP-1 cells were polarized using hypoxia with IL4 and IL10 treatment, and then sorted according to Tie2 expression. After sorting, cells were cultured in SFM for 24 hours after which conditioned media was collected and used for invasion assay. U87 glioma cells or GSC20 glioma stem cells were placed in a transwell containing a membrane with 8um pores coated with 0.3 mg/ml of growth factor reduced matrigel. Transwell was placed over a well containing conditioned media from Tie2 negative or Tie2 positive THP-1 cells. Upper panel: quantification of invading U87 cells is graphed as average number of cells per high power field. Lower left panel: representative pictures of invading GSC20 glioma stem cells attached to bottom of transwell membrane in the presence of conditioned media from Tie2 – or Tie2+ THP-1 cells. Lower right panel: quantification of invading GSC20 stem cells was graphed as average number of cells per high power field. p-values were calculated using Student t-test.

Ang2 further enhances the secretion of pro-invasive molecules by TEMs. We have already shown that Ang2 is at least partially responsible for the increased representation of TEMs within the tumor after anti-VEGF therapy. We hypothesized that Ang2 is capable of further enhancing their pro-invasive capabilities. To test this, we obtained Tie2 positive and Tie2 negative monocytes from peripheral blood of healthy donors as described in the material and methods section. Once cells were obtained, both Tie2+ and Tie2- monocytes were cultured in serum free media in the presence or absence of Ang2 for 24 hours. At 24 hours, media was collected and gelatinase activity was measured using the EnzChek Gelatinase/Collagenase Assay (Figure 22). This kit uses a fluorescein conjugated DQ-gelatin that is so highly labeled that fluorescence is quenched. Gelatinase activity digests the substrate and produces highly fluorescent fragments. Fluorescence levels correlate with gelatinase activity. Results obtained show that media obtained from TEMs has higher gelatinase activity as shown by the significantly higher fluorescence in the absence of Ang2 when compared to their Tie2 negative counterparts. Furthermore, our data confirms our hypothesis that Ang2 further enhances this secretion as the gelatinase activity significantly increased in the presence of Ang2. Interestingly, Ang2 only enhanced the pro-invasive activity in the Tie2 positive population which suggests its effect is mediated by the Tie2 receptor.

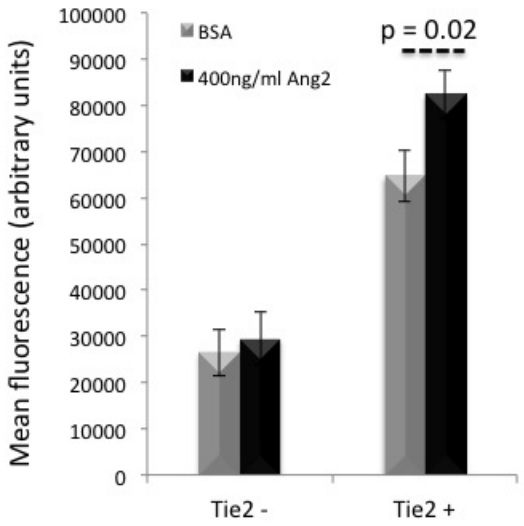

Figure 22. Ang2 further enhances the secretion of pro-invasive molecules by TEMs. Tie2 positive and Tie2 negative monocytes were obtained from peripheral blood of healthy donors. They were cultured for 24 hours in the presence or absence of Ang2 and collagenase activity was measure using EnzChek Gelatinase/Collagenase Assay. Fluorescence produced by degradation of gelatin was measured and graphed. p-values were calculated using Student t-test.

Conclusions

Through the expression of pro-invasive molecules, such as MMP2 and MMP9, TEMs are capable of modifying the tumor microenvironment making it one conducive for invasion. There is an increase in the expression of MMP9 following anti-VEGF therapy and our data suggests that TEMs are at least partially responsible for this phenomena. Highlighting the significance of these findings, we were able to show that glioma cells display enhanced invasive capabilities in the presence of conditioned media from TEMs. These data demonstrates the importance of TEMs and the Ang-Tie2 pathway in the invasive recurrence of GBM after anti-VEGF therapy.

Chapter VII: Targeting the Ang-Tie2 pathway in combination with anti-VEGF therapy leads to improved outcomes in syngeneic mouse glioma model.

Rationale and expectations

GBM is a highly aggressive cancer characterized by infiltration into the normal brain. Recently, bevacizumab was added to the battery of treatments offered to patients with recurrent GBM and although it has important anti-tumor effects, patients treated with bevacizumab invariably present with recurrent disease.[56] Upon recurrence, these tumors acquire a highly malignant behavior characterized by enhanced invasion into adjacent normal brain which renders surgical excision ineffective. Throughout this work we have provided evidence for the involvement of Ang-Tie2 pathway in the pattern of recurrence of gliomas following anti-VEGF therapy. We hypothesized that by targeting the Ang-Tie2 pathway in combination with anti-VEGF therapy we will observe improved outcomes in GBM. We expect to obtain improved survival outcomes as well as a decrease in the invasive phenotype seen with anti-VEGF monotherapy.

Results

Targeting VEGFR also leads to an invasive phenotype in a mouse glioma model. To target the Ang-Tie2 pathway we obtained a GL261 mouse glioma cell line overexpressing a soluble form of the Tie2 receptor. Upon release of sTie2, it will bind all the angiopoietins in the media preventing their binding to, and activation of the cell-bound Tie2 receptor. However, given that this is a mouse glioma cell line and bevacizumab targets only human VEGF, we needed to find a compound that effectively targets the mouse VEGF pathway. We obtained an antibody named DC101 that targets mouse VEGFR2 and performed a pilot study to determine if DC101 led to similar changes as those observed for VEGF-Trap/aflibercept.

We implanted GL261 mouse glioma cells intracranially in C57/BL6 mice and treated them with either control or DC101 three times a week. Mice were sacrificed when signs of high tumor burden became apparent and brain was extracted, formalin fixed and paraffin embedded for morphological studies and protein expression analysis. Upon histological assessment, GL261 tumors were characterized by a well circumscribed tumor, with dilated blood vessels and tendency to hemorrhage. No overt necrosis could be appreciated in either group. Tumors in the control group had a sharp tumor-normal brain interface and no signs of invasive nodules. On the other hand, tumors treated with DC101 showed disruption of the interface between tumor and normal brain with finger-like projections into the normal brain and some areas showing initial detachment from the main tumor mass, suggesting early stages of invasion (Figure 23).

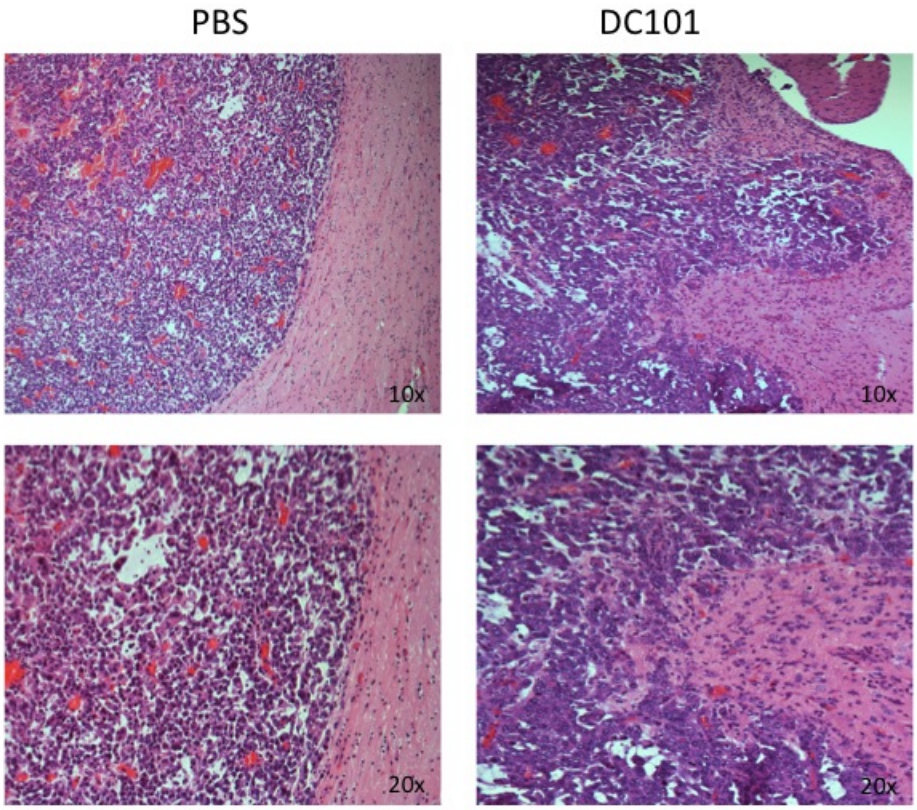

Figure 23. Anti-VEGF therapy with DC101 (anti-mouse VEGFR antibody) causes disruption of the tumor/normal brain interface. C57/BL6 mice were implanted with GL261 mouse glioma cells and treated with 800ug/dose of DC101 or control treatment three times a week for 4 weeks. Mice were sacrificed when signs of high tumor burden and histology was evaluated. Representative images of H&E stain are shown.

DC101 treatment leads to decreased microvascular density. Since targeting the VEGF-VEGFR pathway disrupts the process of angiogenesis, a way to measure its effectiveness is quantifying microvascular proliferation within the tumor. For this purpose, we performed CD31 staining to label endothelial cells and proceeded to quantify vascular structures, defined as a cluster of 3 or more endothelial cells having a lumen (Figure 24). To be considered a discrete vascular structure, the lumen cannot connect with any other vascular structure within the field. Our results show a decrease in MVD of more than 50% in tumors treated with DC101 versus control treated tumors. This provides evidence that DC101 is effectively inhibiting the process of new vessel formation within the tumor.

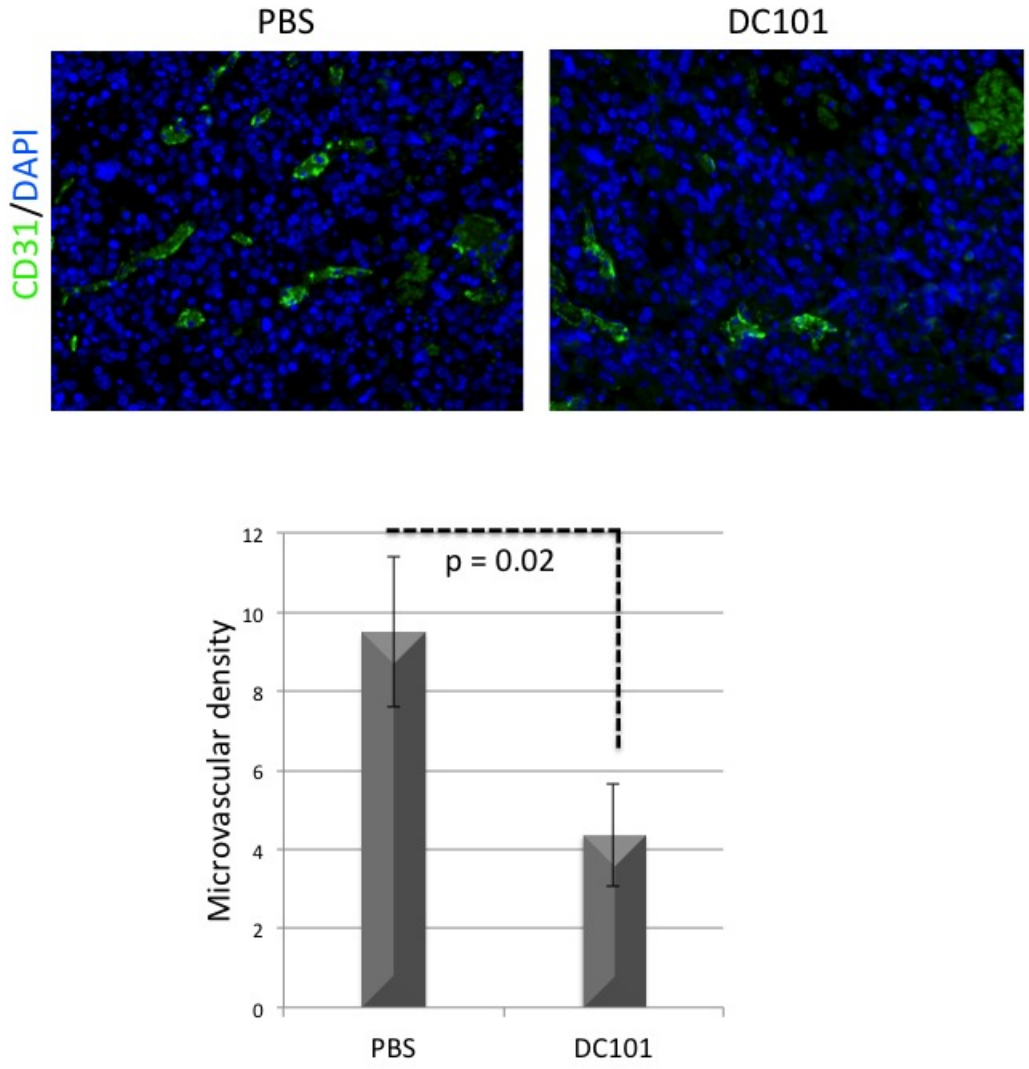

Figure 24. Anti-VEGF therapy with DC101 causes a reduction in microvascular density within the tumor. C57/BL6 mice were implanted with GL261 mouse glioma cells and treated with 800ug/dose of DC101 or control treatment three times per week for 4 weeks. Mice were sacrificied when signs of high tumor burden were apparent. Single stain with CD31 was performed to detect and quantify vascular structures. Upper panel: representative images of CD31 stain. Lower panel: quantification of vascular units shown as average number per high power field. p-values were calculated using Student t-test.

DC101 treatment leads to recruitment of macrophages, Tie2 expressing cells and TEMs to the periphery of the tumor. We next determined if treatment with DC101 led to macrophage recruitment, as does bevacizumab and aflibercept. To evaluate this, we performed Iba1 staining in tissue sections from mice treated with either control treatment or DC101 (Figure 25). Quantification of Iba1 positive cells revealed a highly significant increase in macrophage recruitment to the periphery of the tumor. As can be observed in the image, these cells tend to accumulate right at the interface between tumor and normal brain.

Treatment with DC101 leads to Tie2 expressing cell accumulation around the periphery of the tumor and TEM recruitment. We have already shown that anti-VEGF therapy with bevacizumab and aflibercept leads to Tie2 expressing cell accumulation around the periphery of the tumor, and in particular TEM recruitment around areas of invasion. To determine if DC101 had a similar effect we performed double immunofluorescence staining for Iba1 and Tie2 (Figure 26). Quantification of double positive cells shows a significant increase in the fraction of macrophages expressing the Tie2 receptor following treatment with DC101. Taken together, these data confirms that DC101 leads to similar changes in the tumor microenvironment as other human anti-VEGF therapeutic approaches and therefore is a sensible model to study the effect of macrophage depletion on the responsiveness of tumors to anti-VEGF therapy.

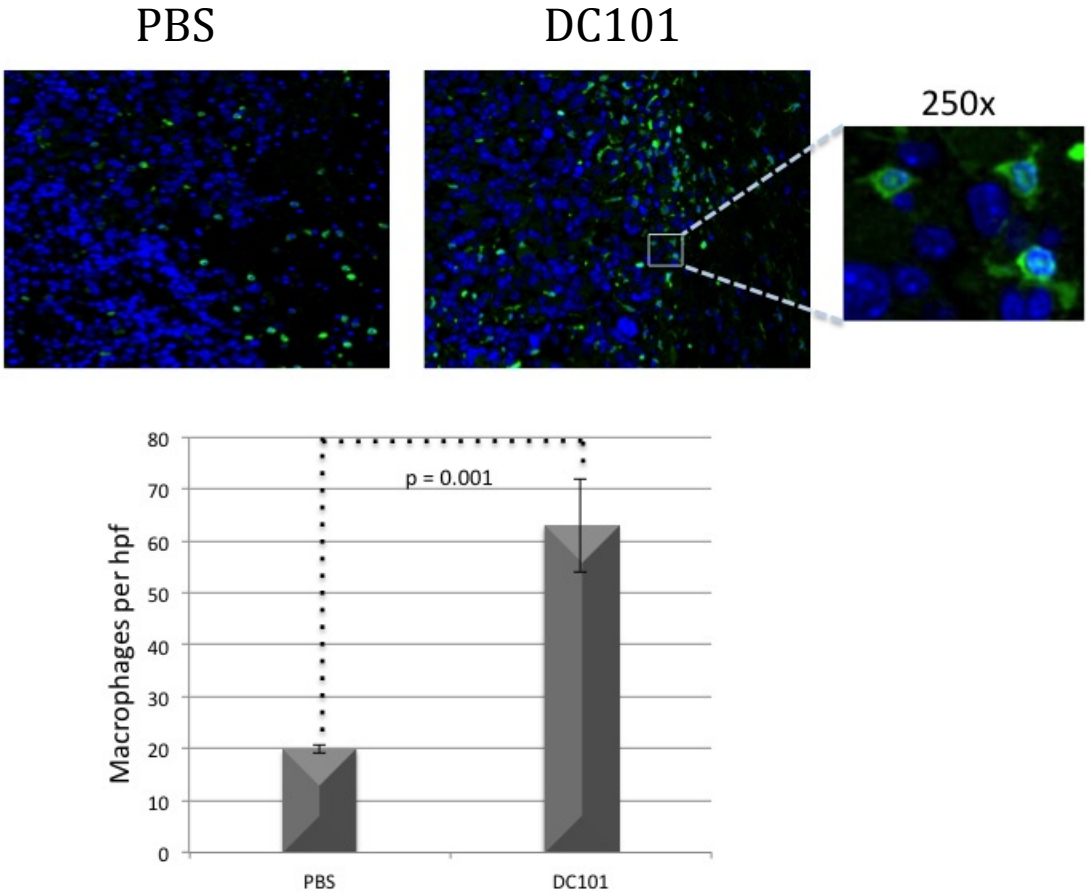

Figure 25. Anti-VEGF therapy with DC101 leads to macrophage recruitment along the tumor/normal brain interface. C57/BL6 mice were implanted with GL261 mouse glioma cells and treated with 800ug/ dose of DC101 or control treatment three times per week for 4 weeks. Mice were sacrificied when signs of high tumor burden were apparent. Iba1 stain was performed to quantify macrophages. Upper panel: representative images of GFP stain. Lower panel: quantification of GFP positive cells shown as average number of cells per high power field. p-values were calculated using Student t-test.

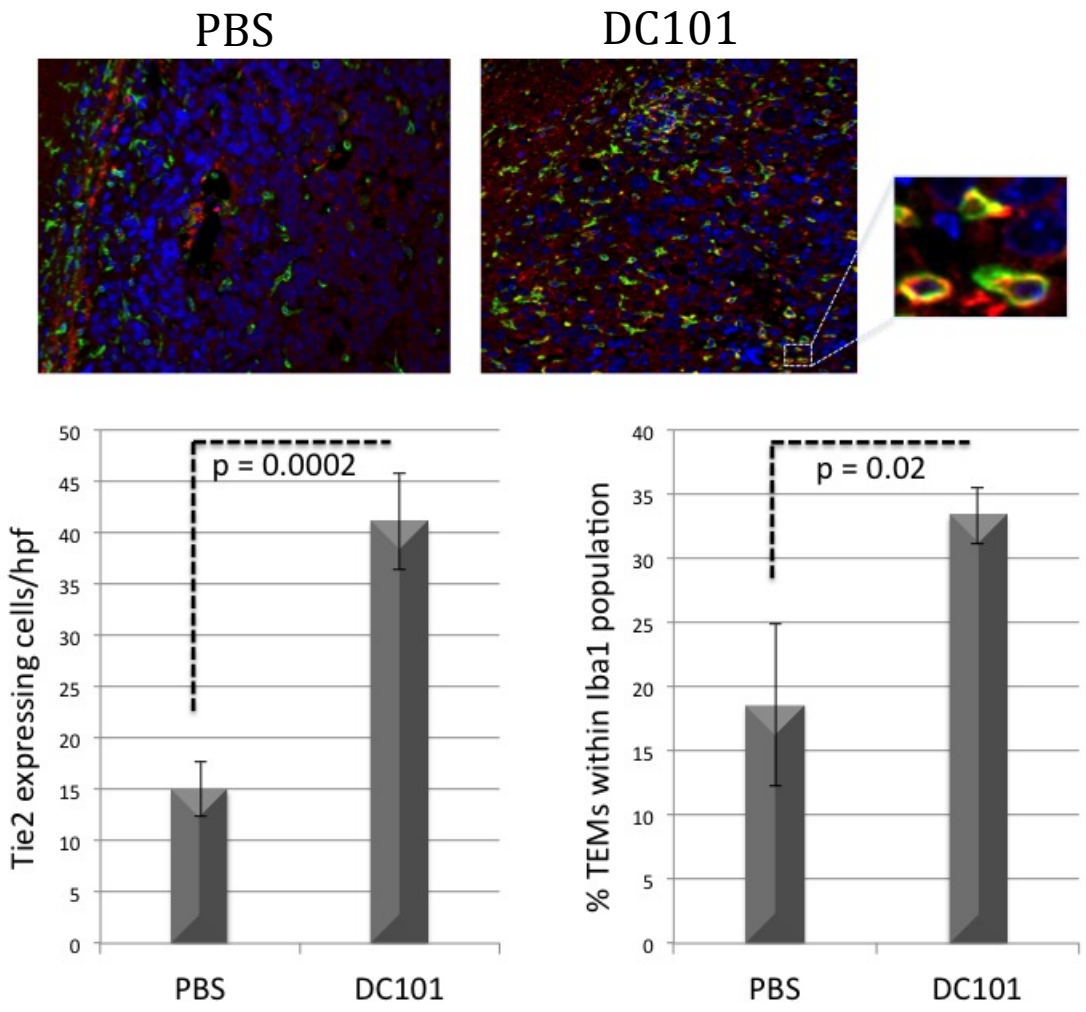

Figure 26. Anti-VEGF therapy with DC101, anti-mouse VEGFR antibody, leads to TEM recruitment. C57/BL6 mice were implanted with GL261 mouse glioma cells and treated with 800ug/dose of DC101 or control treatment three times per week for 4 weeks. Mice were sacrificied when signs of high tumor burden were apparent. Double stain with Iba1 and Tie2 was performed to detect and quantify Tie2 expressing monocytes. Upper panel: representative images of the stain. Lower panel: quantification of Tie2 positive cells (left) shown as average number per high power field and Iba1/Tie2 double positive cells (right) expressed as percentage of monocyte population.

GL261sTie2 cell line has similar growth kinetics as the control GL261.GFP cell line. Before proceeding to evaluate whether in the presence of sTie2 gliomas responded better to anti-VEGF therapy, we wanted to determine if sTie2 expression led to differences in tumor growth and survival. For this purpose we performed a proliferation assay *in vitro* and studied the tumor growth *in vivo* in C57/BL6 mice (Figure 27). Cells showed no difference in BrdU incorporation *in vitro*, demonstrating that there is no significant difference in their rate of proliferation. Upon implantation, no differences in tumor size or overall survival were observed with the expression of sTie2. Upon histological examination, both GL261.GFP and GL261.sTie2 displayed circumscribed borders with no invasion towards adjacent normal brain. However, GL261.sTie2 had more extensive necrotic foci highlighting the importance of an active Ang-Tie2 pathway for the formation of vascular structures and tissue oxygenation.

Targeting the Ang-Tie2 pathway does not lead to improved survival in a GL261 syngeneic glioma model. One of the most important outcome measures in cancer treatment is overall survival. Therefore, we were very interested in evaluating whether targeting the Ang-Tie2 pathway in combination with anti-VEGF therapy led to improved survival in mice. Mice were implanted with GL261.GFP or GL261.sTie2 and treated with either control treatment or DC101 three times a week for 4 weeks. Mice were sacrificed when signs of high tumor burden and overall survival was evaluated for each group (Figure 28). DC101 treatment alone led to a significant increase in survival when compared to control therapy. Unfortunately, adding sTie2 to the treatment regimen did not further increase the overall survival in mice. There could be several reasons for this.

There is a possibility that targeting the Ang-Tie2 pathway does not lead to improved survival. However, because this cell line overexpresses sTie2 from the moment of implantation and prior to DC101 treatment administration, it is possible that the cells were able to adapt more quickly to DC101 treatment.

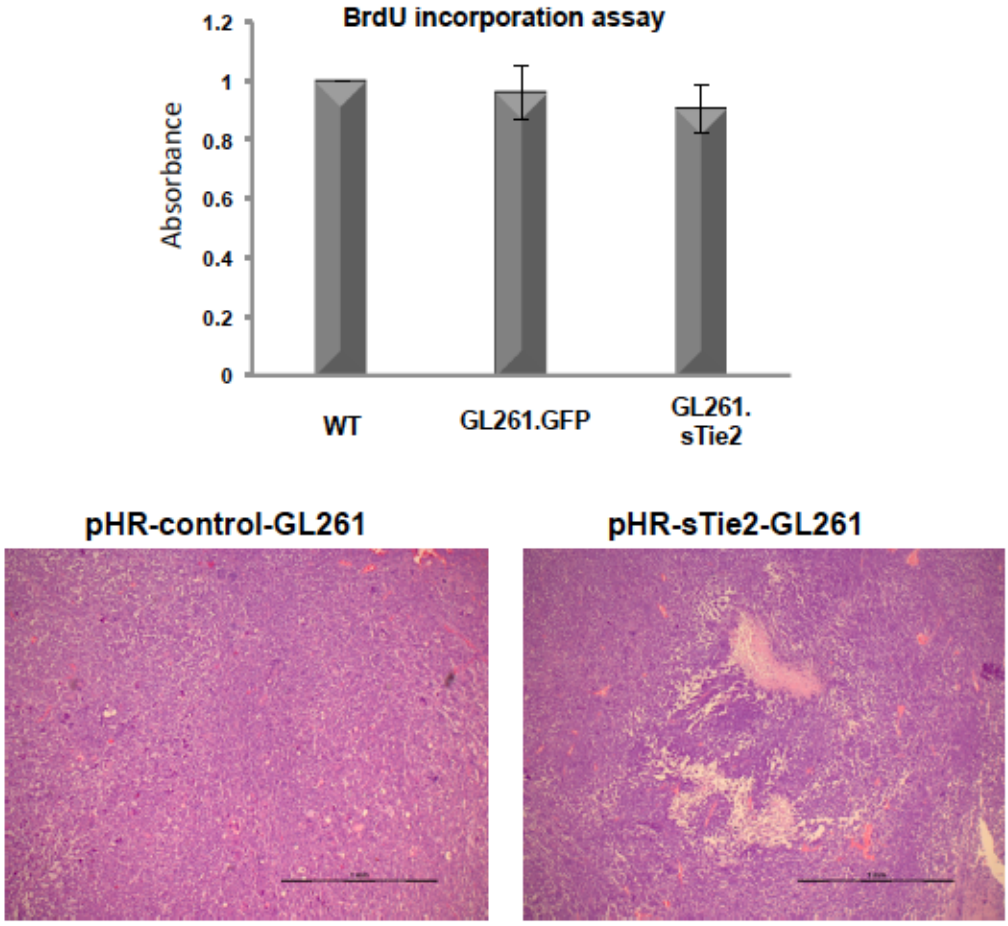

Figure 27. Determining effect of sTie2 overexpression in the proliferative rate and in vivo growth of GL261 mouse glioma cell line. Upper panel: BrdU incorporation assay for wild type (WT), GFP or sTie2 mouse glioma cell lines. Lower panel: C57/BL6 mice were implanted with GL261.GFP or GL261.sTie2 mouse glioma cell line and sacrificed when signs of high tumor burden were apparent. Representative images of H&E staining are shown.

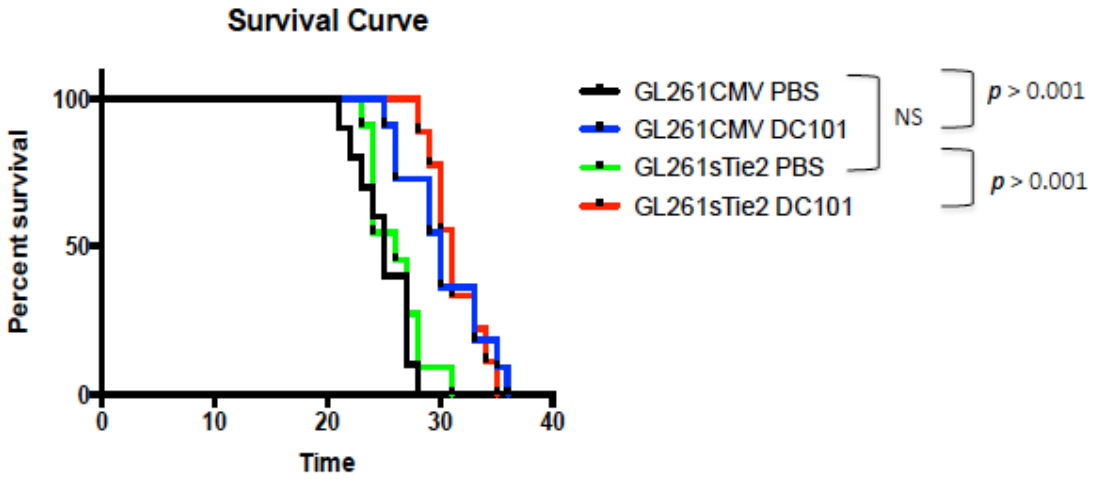

Figure 28. Targeting the Ang-Tie2 pathway in combination with anti-VEGF therapy does not lead to improved survival. C57/BL6 mice were implanted with GL261.GFP or GL261.sTie2 mouse glioma cell line and treated with either PBS as control or 800ug/dose of DC101 three times a week for 4 weeks. Mice were sacrificed when signs of high tumor burden were apparent. Kaplan Meier survival curve is shown.

Targeting the Ang-Tie2 pathwat leads to a significant decrease in the invasive phenotype after anti-VEGF therapy. One of the main problems arising from anti-VEGF therapeutic approaches is the enhancement of invasion observed after therapy. This renders surgical excision ineffective, which coupled with the highly resistant nature of GBM leaves patients with little to no therapeutic alternatives. At this point survival is measured in weeks. Therefore, another important outcome measure in this setting is preventing the development of invasive feature after anti-angiogenic treatment so that surgical excision becomes an option in this patient population. We were interested in evaluating whether targeting the Ang-Tie2 pathway in combination with anti-VEGF therapy was effective at achieving this goal. Mice were implanted with GL261.GFP or GL261.sTie2 and treated with either control treatment or DC101 three times a week for 4 weeks. Mice were sacrificed when signs of high tumor burden and brains were extracted, formalin fixed and paraffin embedded for histological and protein expression analysis. Histological analysis of tumor sections revealed a striking increase in the number of invasive nodules when DC101 was administered in GL261.GFP derived tumors. In areas where no overt invasive nodules could be observed, the tumor-normal brain interface was disrupted and finger-like projections into the normal brain became to appear. There was a striking and statistically significant decrease in the number of invasive nodules in GL261.sTie2 derived tumors treated with DC101 compared to GL261.GFP derived treated tumors. There were virtually no invasive nodules present and no disruption of the tumor-normal brain interface (Figure 29).

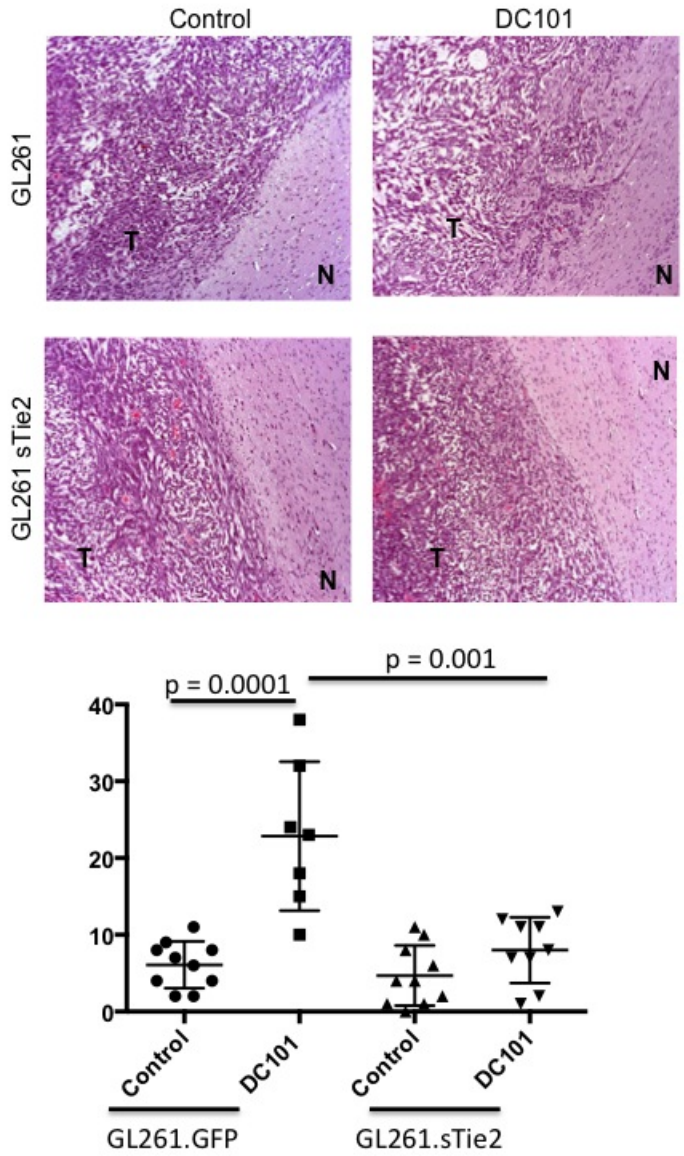

Figure 29. Targeting the Ang-Tie2 pathway in combination with anti-VEGF therapy leads to a significant decrease in invasion. C57/BL6 mice were implanted with GL261.GFP or GL261.sTie2 mouse glioma cell line and treated with either PBS as control or 800ug/dose of DC101 three times a week for 4 weeks. Mice were sacrificed when signs of high tumor burden were apparent. Histological analysis was performed. Upper panel: representative image of H&E stain of tumor sections from each group. Lower panel: invasive nodules were quantified and graphed in a box plot. Student t-test was used for p-value calculations.

Targeting the Ang-Tie2 pathway prevents recruitment of TEMs to the tumor following anti-VEGF therapy. Our data supports an active role for Ang2 in the recruitment and modulation of TEMs which then promote an invasive microenvironment conducive for glioma cell invasion into the normal brain. We wanted to evaluate whether targeting the angiopoietins effectively prevented the recruitment of TEMs. For this purpose we obtained tissue sections from GL261.GFP or GL261.sTie2 glioma-bearing mice treated with either control or DC101 treatment. Double staining for Iba1, a monocyte/microglia marker, and Tie2 was performed and double positive cells were quantified (Figure 30). Our results show a significant decrease in the number of double positive cells, i.e. TEMs, in GL261.sTie2 derived tumors treated with DC101 when compared to treated GL261.GFP derived tumors. This result highlights the importance of available Ang2 for the recruitment of these cells and further supports their role in the development of invasion following anti-VEGF therapy

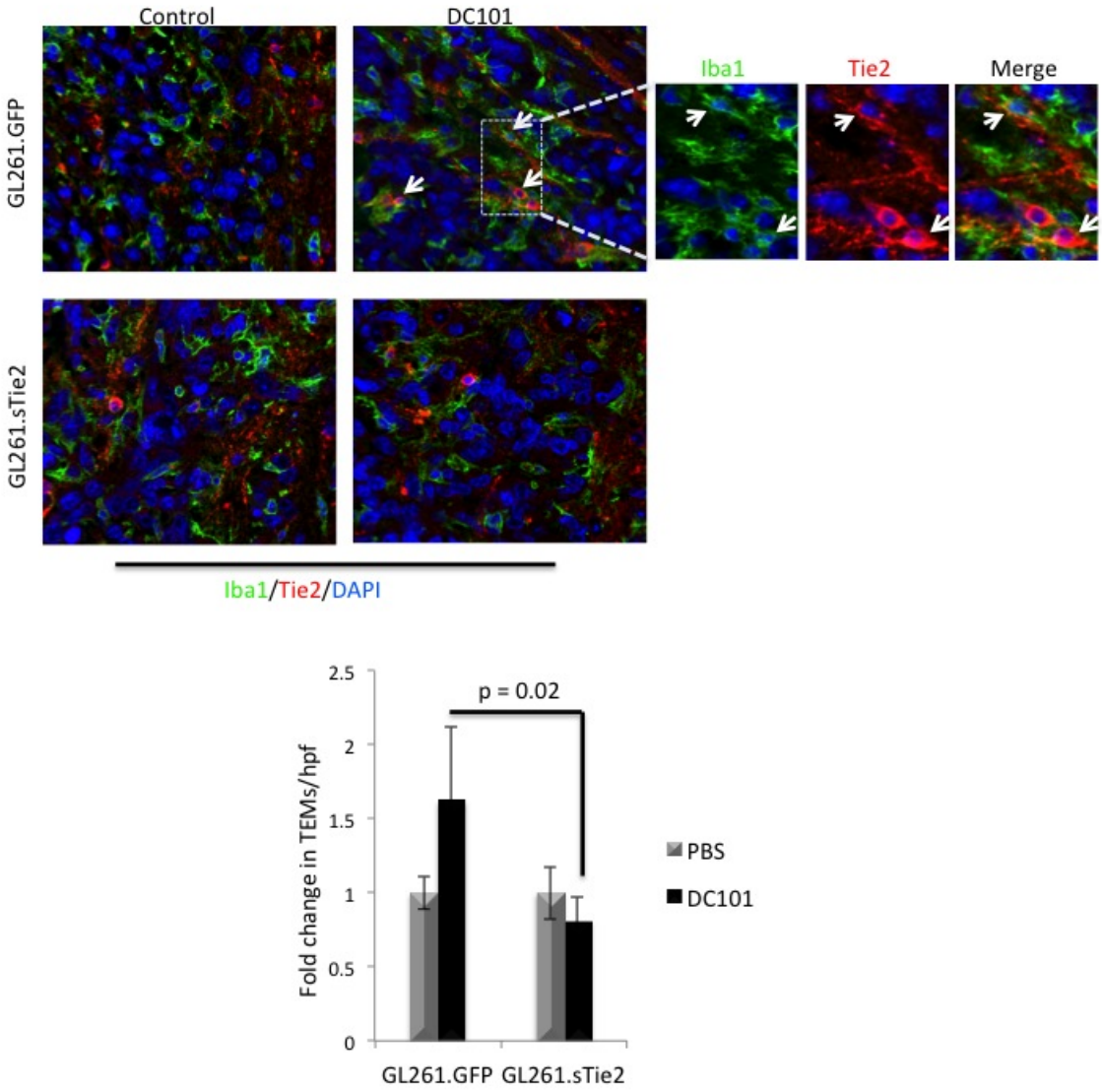

Figure 30. Targeting the Ang-Tie2 pathway in combination with anti-VEGF therapy leads to a significant reduction in TEM recruitment. C57/BL6 mice were implanted with GL261.GFP or GL261.sTie2 mouse glioma cell line and treated with either PBS as control or 800ug/dose of DC101 three times a week for 4 weeks. Mice were sacrificed when signs of high tumor burden were apparent. Double immunofluorescence staining against Iba1 and Tie2 to detect TEMs. Upper panel: representative image of double staining for each group. To the right is an amplification of designated area. Lower panel: quantification of double positive cells, i.e. TEMs, is graphed as fold change from control (GL261.GFP treated with PBS). p-values calculated using Student t-test.

Conclusions

Targeting the Ang-Tie2 pathway in combination with anti-VEGF therapy leads to an impairment in TEM recruitment and a consequent significant decrease in the invasive phenotype observed after anti-VEGF therapy. This has important implications for patients for GBM. By preventing this enhanced invasive phenotype, we can potentially render the tumor amenable to surgical excision and this way improve patient survival. Infiltration of glioma cells into the normal brain is characteristic of GBM and it is important to understand that curative therapy will require the effective targeting of this cell population. However, by removing the bulk of the tumor after anti-VEGF therapy we can significantly improve patient survival, one of the main goals in GBM.

Chapter VIII: Discussion

GB continues to be a devastating disease, with a median survival of only 15 months despite aggressive chemotherapy.[1-2] One of the main features of GBM contributing to its highly malignant behavior is its infiltrative nature, which makes complete surgical resection virtually impossible. This, coupled with its highly resistant nature, makes GB an incredibly difficult cancer to treat.

In 2009, bevacizumab, an anti-VEGF antibody, was FDA approved for the treatment of recurrent GBM. Treatment with bevacizumab consistently leads to dramatic radiographic responses and improved progression free survival.[47] However, despite its anti-tumor effects, patients treated with bevacizumab invariably present with recurrent disease, and upon recurrence the tumor acquires a highly aggressive phenotype characterized by enhanced invasion and resistance to therapy.[52-53,56,79,80] Once patients present with this stage of disease, there is little to no effective therapy that can be offered.[80] Furthermore, surgical removal is ineffective due to further enhancement of its already infiltrative behavior. Therefore, understanding the mechanisms behind the development of such a malignant disease state will lead to the development of improved combined therapeutic approaches that can result in better patient outcomes.

The results obtained throughout this project implicate the Ang-Tie2 pathway as a key player in the recurrence of GBM following anti-VEGF therapy and in particular, the acquisition of its highly invasive behavior. An accumulation of Tie2 expressing cells accompanies the development of invasive nodules. Further characterization of these cells demonstrated them to be TEMs. Shortly after their discovery, TEMs were shown to be detectable in the blood of healthy donors, comprising less than 10% of circulating

mononuclear cells in these cases. Furthermore, they comprise the main monocytic population, other than tumor-associated macrophages, within certain types of solid tumors. Interestingly, their presence is undetectable in non-neoplastic healthy tissue.[73] In hepatocellular carcinoma (HCC), there is one report correlating the occurrence of TEMs in the blood of patients with the presence and recurrence of HCC.[74] Taken together, these data suggests that TEMs play a role in cancer development and progression, and supports the notion that their recruitment following anti-VEGF therapy is an important event during recurrence. There have been reports where accumulation of tumor associated macrophages has been linked to anti-angiogenic treatment and correlated with worse patient survival.[82-83] However, the authors did not further characterize this myeloid population and did not follow up on the association.

MMP9 is shown to be upregulated following anti-VEGF therapy and our data shows that TEMs have higher secretion of MMP2 and MMP9 than their Tie2 negative counterparts, suggesting that TEMs are at least partially responsible for the upregulation of MMP9 following anti-VEGF therapy. Interestingly, the Tie2 receptor has been shown to be necessary for the secretion of MMP9 by TEMs *in vitro*[81], and we gathered data showing that Ang2 is capable of further enhancing the secretion of gelatinases by TEMs, but had no effect in Tie2 negative monocytes. Our data strongly supports that TEMs have an important role in the invasive phenotype observed after anti-VEGF therapy and that targeting this population can prove to be beneficial for patients with recurring GBM. Furthermore, our data highlights the potential benefit of inhibiting the Ang-Tie2 pathway in the context of anti-VEGF treatment by two distinct mechanisms: 1) TEMs cannot be recruited to the tumor due to lack of functionally available Ang2 and, 2) Ang2 is not

available to modulate TEMs that might already be present within the tumor and enhance their pro-invasive features.

Ang2 has been shown to promote invasion of cancer cells independent of the tumor microenvironment. Ang2 expression has been correlated with invasion in GBM and breast cancer surgical samples, and with distant metastasis in the latter.[84-85] In glioma, Ang2 was shown to induce MMP2 expression and increase invasion *in vitro.* Furthermore, implantation of U87 glioma cells over-expressing Ang2 led to the production of invasive tumors in nude mice, a feature largely absent in U87-derived tumors.[86] In this study, authors report on the increased expression of MMP2 *in vitro* and *in vivo* and its contribution to glioma invasion *in vitro*, but do not characterize the nature of the infiltrating myeloid cells so their contribution to the invasive features observed under these conditions remains unexplored. The results obtained in this work provide evidence that Ang2-mediated TEM recruitment is at least partially responsible for the invasive features observed after anti-VEGF therapy, and potentially of MMP2 upregulation. However, several mechanisms are likely to be in place and it is possible that inhibition of the effect of angiopoietins, and in particular Ang2, can also have effects within the tumor compartment (i.e. cancer cells). It is worth noting that some of the effects of Ang2 have been shown to be mediated by integrins, particularly $\alpha_5\beta_1$, suggesting that targeting the ligand would be a better approach than targeting the receptor.[86-87]

An important limitation to our approach is that by using sTie2 as a means to inhibit the Ang-Tie2 pathway, all angiopoietins present within the environment will be inhibited, which does not allow us to differentiate between the effect of inhibiting each

one individually. Glioma cells have been shown to express Ang1 which in turn leads to chemoresistance through upregulation of ABC transporters.[88] ABC transporters are not likely to play a role in resistance to bevacizumab since its mechanism of action does not require entry into tumor cells. However, it cannot be ruled out that some of the effects observed in the combination treatment group can be caused by inhibition of Ang1 within the tumor and tumor vasculature. We were unable to detect Ang1 in glioma tissue sections, independent of treatment status. Furthermore, research on TEMs has not supported a role for Ang1 in their recruitment or modulation, supporting the notion that it has little to no role in the invasive phenotype observed after anti-VEGF therapy.[77] However, this cannot be completely ruled out since we have observed activation of Tie2 with experimentally undetectable levels of Ang1. Furthermore, the Ang1-Tie2 axis has been shown to enhance adhesion of glioma cells to endothelial cells and induce glioma cell invasion *in vitro*.[89]

Understanding the mechanisms of resistance to anti-VEGF therapy has been the focus of extensive research since its development. The results depicted throughout this project suggest a role for the Ang-Tie2 pathway in the modulation of the tumor microenvironment for the development of invasion as a means to escape VEGF blockade in gliomas. Several groups, however, have reported on the role of different signaling pathways in the development of resistance to anti-VEGF therapy in gliomas. For example, Carbonell *et al*. report on the upregulation of integrin β_1 following anti-VEGF therapy in gliomas and provide evidence that targeting integrin β_1 potentiates the effects of bevacizumab in a glioma xenograft and inhibit the development of invasion after therapy.[92] Interestingly, Ang2 has been shown to act through integrins, in particular

integrin $\alpha_5\beta_1$, and in gliomas it has been shown to enhance MMP2 expression and invasion through interactions with this integrin.[86-87] This suggests a potential interplay between these two pathways in the recurrent phenotype observed in gliomas. A potential mechanism of sTie2 at inhibiting invasion after anti-VEGF therapy could be through inhibition of Ang2-mediated activation of $\alpha_5\beta_1$ integrin.

Another interesting study found an inverse relationship between activation of the VEGF/VEGFR pathway and c-met phosphorylation and activation in gliomas.[93] They provide evidence that VEGF leads to an inhibition of c-met phosphorylation and activation and that in the absence of VEGF there is enhanced phosphorylation of this receptor within glioma cells. Furthermore, this increased phosphorylation led to enhanced migration of glioma cells *in vitro* and enhanced vessel cooption *in vivo*. Consequently, they demonstrate enhanced phosphorylation of c-met accompanying invasion following VEGF blockade, and successfully inhibit the invasive phenotype observed with anti-VEGF monotherapy with the concurrent inhibition of c-met. Interestingly, this approach has been shown to be successful at inhibiting invasion and metastasis in pre-clinical models of other types of solid tumors.[94] These studies, along with others associating a multitude of pathways to anti-VEGF therapy resistance, highlight the multiplicity of mechanisms used by cancer cells to acquire resistance, and potentially argue against the use of highly selective therapy (i.e. targeted therapy). More generalized approaches might be more successful than attempting to target one or several pathways within the transformed epithelial compartment. Targeting the tumor microenvironment, for example through immune modulation, can be such an approach.

Currently, there are several inhibitors of the Ang-Tie2 pathway either under development or under pre-clinical or clinical evaluation. Selectively targeting Ang2 as monotherapy has been shown to be effective at inhibiting tumor growth in several solid malignancies, including colon carcinoma, renal cell carcinoma, ovarian carcinoma, pancreatic carcinoma and breast carcinoma.[30,90] Added to this, several studies have explored the value of targeting Ang2 in combination with anti-VEGF therapy and have shown that the combination therapy has greater anti-tumor effects than either approach alone.[30,91] Currently, there is no preclinical or clinical data assessing the efficacy of targeting the Ang-Tie2 pathway alone or in combination with bevacizumab in glioma. We show here that by targeting both, the Ang-Tie2 pathway and the VEGF pathway, we are able to circumvent the development of invasion in the GL261 syngeneic glioma model. This has very important implications for GBM patients. As stated above, the enhanced invasive features observed in recurring tumors after anti-VEGF therapy render surgical excision ineffective. By effectively preventing the development of enhanced invasion, we could potentially render the tumors amenable to surgical removal upon recurrence. It is important to note that GBM is naturally a highly invasive tumor and complete removal of all tumor cells is virtually impossible at any stage of disease. Curative therapy will require effectively targeting remaining tumor cells after surgical removal of the bulk of the tumor. Having said this, being able to improve survival in patients with GBM is one of the main therapeutic goals and the approach suggested here has the potential of doing so.

Chapter IX: Future Directions

Besides upregulation of Ang2, we were also able to see an increase in VEGF expression after anti-VEGF therapy. Interestingly, we detected a dramatic increase in binding of VEGF to its receptor within the glioma compartment following bevacizumab treatment (Figure 31). The hypoxic environment that ensues after anti-VEGF therapy due to decreased microvascular density is a possible reason for VEGF upregulation in the context of anti-VEGF therapy.[8] Added to this, certain VEGF isoforms bind the extracellular matrix until protease activity releases them making them available to bind cell bound receptors.[95] The enhanced expression of MMPs observed after anti-VEGF therapy can lead to release of ECM-bound VEGF upon ECM degradation, which in turn can explain the enhanced binding to its receptor within the tumor compartment. Interestingly, VEGF expression has been associated with higher tumor grade in gliomas.[96] Furthermore, it has been associated with increased invasion and metastasis in several solid malignancies.[97-98]

The observation of enhanced VEGF binding to its receptor within glioma cells might seem counterintuitive in the context of bevacizumab treatment, given the fact that we are administering an anti-VEGF antibody that prevents this same interaction. However, antibodies (i.e. bevacizumab) generally do not cross an intact blood brain barrier[99] and although the BBB within gliomas is compromised, vessel abnormalities are thought to impede diffusion of chemotherapeutic drugs to the tumor. Factors such as high interstitial pressure and chaotic blood flow lead to uneven drug distribution and lack of drug delivery to some areas of the tumor.[100-101] In the presence of bevacizumab, some of these abnormalities regress, a process called vessel normalization. Although this

process has been shown to improve delivery of chemotherapeutic drugs, in the context of GBM vessel normalization is accompanied by restoration of the low permeability BBB.[102] Our observation suggests that regardless of the status of the vascular network within GBM, bevacizumab is generally not able to reach cancer cells in the brain in sufficient amounts to cause inhibition outside of the vascular compartment. Given the tight interplay of the VEGF and the Ang-Tie2 pathway in vessel physiology and pathology, it would be interesting to explore whether Ang2 can potentiate the effects of VEGF-VEGFR activation in gliomas or viceversa. Furthermore, in a recent publication by *Niewoehner et al.,* they report on the development of a modified antibody against β-amyloid that has facilitated transport through the BBB via transferrin receptor mediated transcytocis.[103] It would be interesting to apply this technology to anti-VEGF targeted approaches in GB to improve delivery to the tumor bed.

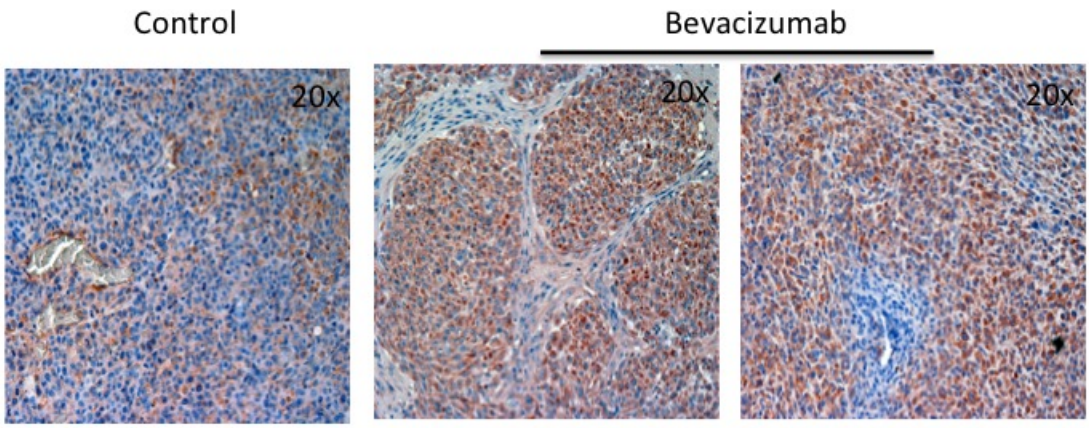

Figure 31. Anti-VEGF therapy leads to enhanced binding of VEGF to VEGFR within the tumoral compartment. Nude mice were implanted with U87 glioma cells and treated with PBS or bevacizumab for 6 weeks. They were sacrificed when signs of high tumor burden were apparent. Immunohistochemical staining for the VEGF/VEGFR complex was performed. Shown are representative images of immunohistochemical staining of VEGF bound to VEGFR following control or anti-VEGF therapy with bevacizumab.

Finally, we were interested in understanding the mechanisms through which Ang2 is upregulated following anti-VEGF therapy. Uncovering the molecular players leading to this phenomenon can lead to the identification of other therapeutic targets to be combined with anti-VEGF therapy. The Ets family transcription factors Ets-1 and Elf-1 have been reported to act as strong enhancers of endothelial cell Ang-2–promoter activity.[104] In particular, Ets-1 has been shown to strongly induce Ang2 promoter activity in cervical cancer cells.[105] Furthermore, Ets-1 has been associated with malignancy in gliomas and has been shown to promote invasion and migration in glioma cells.[106-107] Therefore, we hypothesized that activation of Ets-1 was responsible for inducing expression of Ang2 and enhancing the invasive phenotype of gliomas after anti-angiogenic treatment. Ras-mediated phosphorylation of Ets1 has been shown to contribute to its transactivation.[108] We obtained an antibody that recognizes the phosphorylated form of Ets-1 to test our hypothesis. We analyzed tumor cell lysates from glioma-bearing mice treated with control PBS or bevacizumab for 6 weeks (Figure 32). We observed a significant increase in phospho-Ets1 levels, suggesting an increase in its transactivation activity as a response to bevacizumab treatment. Future studies on further understanding the role of Ets-1 in the regulation of Ang2 within glioma cells, as well as its role in the invasive recurrence of gliomas after anti-VEGF therapy can shed light on to the molecular events leading to the highly malignant behavior of gliomas following anti-VEGF therapy.

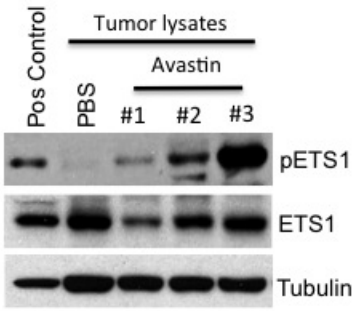

Figure 32. Anti-VEGF therapy leads increased phosphorylation of Ets-1 transcription factor. Nude mice were implanted with U87 glioma cells and treated with PBS or bevacizumab for 6 weeks. They were sacrificed when signs of high tumor burden were apparent. Fresh tumor tissue was extracted and lysed for protein extraction. Western blot analysis of tumor lysates for p-Ets-1 (Thr38) was performed.

Chapter X: List of Conclusions

1) Tie2 expressing cells are recruited to the tumor following anti-VEGF therapy in gliomas.
2) Temozolomide treatment does not lead to recruitment of Tie2 expressing cells.
3) TEMs are recruited to the tumor following anti-VEGF therapy in gliomas.
4) TEMs accumulate in the periphery of the tumor and around areas of invasion following anti-VEGF therapy.
5) Temozolomide does not lead to recruitment of TEMs.
6) Ang2 is upregulated within the tumor after anti-VEGF therapy in gliomas.
7) Ang2 upregulation after anti-VEGF therapy occurs mainly in the periphery of the tumor and around areas of invasion.
8) Glioma cells significantly increase their expression of Ang2 after anti-VEGF therapy.
9) Temozolomide treatment does not lead to Ang2 upregulation.
10) THP-1 cells significantly increase their expression of Tie2 when exposed to hypoxia in the presence of IL4 and IL13 M2-polarizing cytokines.
11) Ang2 is chemoattractant to TEMs *in vitro* and *in vivo.*
12) TEMs secrete higher amounts of MMP2 and MMP9 than their Tie2 negative counterparts.
13) MMP9 is upregulated in the tumor following anti-VEGF therapy in gliomas.
14) TEMs are a source of MMP9 within the tumor following anti-VEGF therapy.
15) Conditioned media of polarized THP-1 cells induces invasion of glioma cells *in vitro.*

16) Conditioned media from Tie2-expressing THP-1 cells induces invasion of glioma cells and glioma stem cells *in vitro.*
17) Targeting the VEGF receptor leads to a similar invasive pattern as that observed for VEGF-Trap/aflibercept and bevacizumab.
18) Targeting the VEGF receptor leads to a reduction in microvascular density.
19) Targeting the VEGF receptor leads to macrophage recruitment.
20) Accumulation of macrophages following anti-VEGFR treatment occurs mainly in the peripheral areas.
21) Targeting the VEGF receptor leads to TEM recruitment.
22) Accumulation of TEMs after anti-VEGFR treatment occurs mainly around the peripheral areas of the tumor.
23) GL261 mouse glioma cells overexpressing soluble Tie2 produce tumors *in vivo.*
24) GL261.sTie2 and GL261.GFP control cells produce non-invasive tumors.
25) GL261.sTie2 produces more necrotic tumors than GL261.GFP control cells.
26) Targeting the Ang-Tie2 pathway using a sTie2 in combination with anti-VEGF therapy does not lead to improved survival.
27) Targeting the Ang-Tie2 pathway using sTie2 in combination with anti-VEGF therapy leads to decreased invasion.
28) Targeting the Ang-Tie2 pathway using sTie2 in combination with anti-VEGF therapy prevents recruitment of TEMs to the tumor.

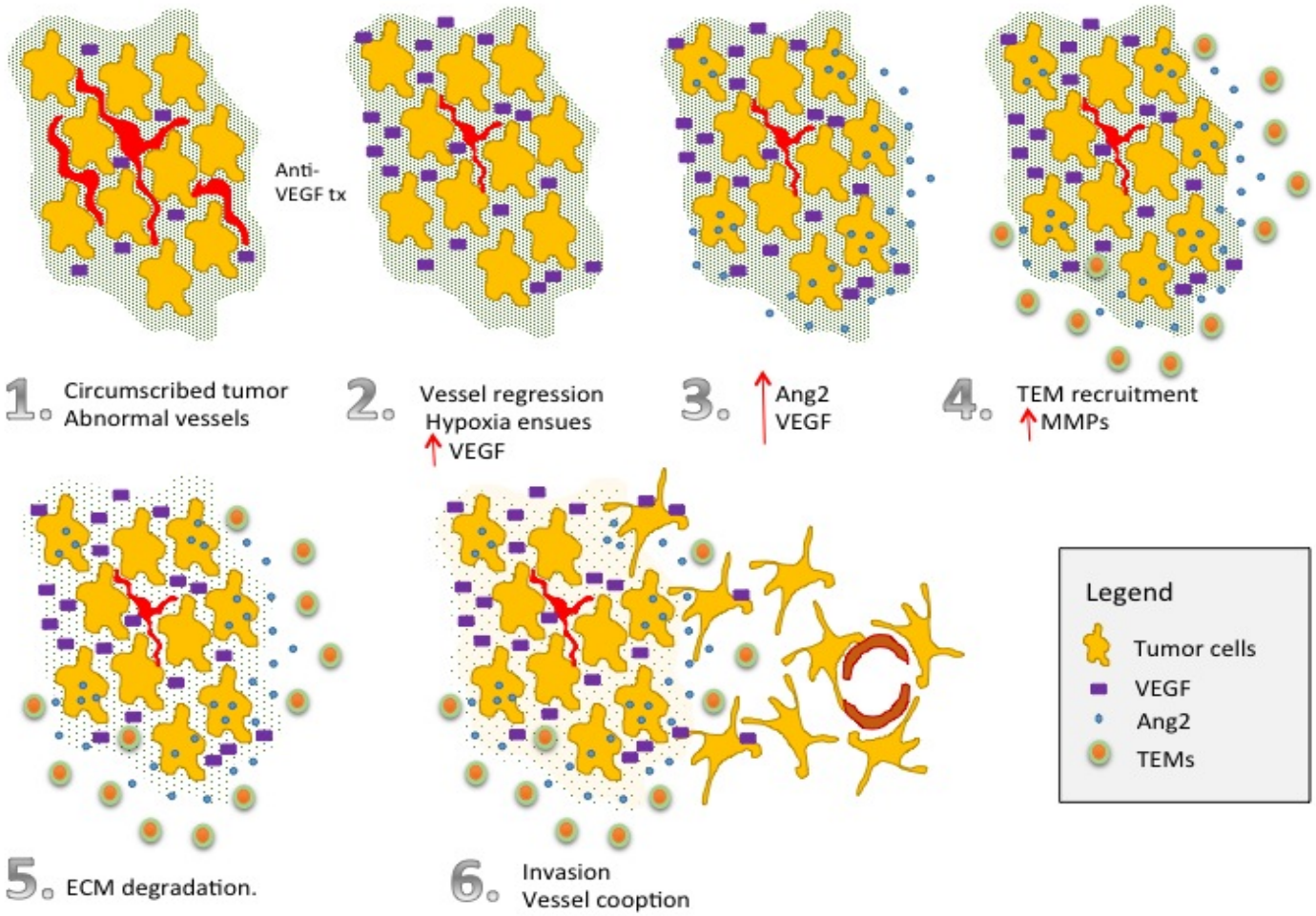

Figure 33. **Role of the Ang-Tie2 pathway in the invasive recurrence of glioblastoma following anti-VEGF therapy.** An inititally circumscribed tumor is exposed to an agent targeting the VEGF pathway. This leads to vessel regression and hypoxia, causing an increase in the expression of VEGF. Through mechanisms not yet identified, Ang2 is upregulated within glioma cells. Ang2 upregulation leads to recruitment of Tie2-expressing cells, and TEMs in particular. TEMs secrete high amounts of pro-invasive molecules such as MMPs, and this activity is further enhanced by Ang2. This leads to degradation of the extracellular matrix providing a pathway for tumor cell migration and invasion into adjacent structures. The driving forces behind glioma cell migration and invasion remain obscure.

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Vita

Nahir Cortés Santiago was born in Ponce, Puerto Rico on October 3, 1982, and raised by her parents Ivette Santiago Cuevas and Jose Rafael Cortés Soto. Her passion for the biological world began as a child and after graduating from Academia Santo Tomás de Aquino, Nahir attended The University of Puerto Rico, Mayagüez Campus in Mayagüez, Puerto Rico. She graduated with a Bachelor in Biology and entered the University of Puerto Rico Medical Sciences Campus in Río Piedras, Puerto Rico where she completed her degree of Doctor in Medicine. During the third year of medical school, Nahir pondered the possibility of continuing her studies and pursuing a degree of Doctor in Philosophy. During her fourth year of medical school, she spent four months doing research at the University of Texas – Graduate School of Biomedical Sciences studying the role of the Src family of tyrosine kinases in the resistance of prostate cancer to taxane therapy under the tutelage of Dr. Gary Gallick. During these four months, Nahir confirmed her interest in research and applied for admission to The University of Texas – Graduate School of Biomedical Sciences where she was admitted in May, 2009. She joined the laboratory of Dr. Gomez-Manzano to investigate the mechanisms of resistance of gliomas to anti-VEGF therapy in January, 2010. Nahir has been awarded with the Rosalie B. Hite Fellowship, which has paid her stipend for three consecutive years. She was also awarded the Minority Scholar in Cancer Research Award by the American Association for Cancer Research, which allowed her to assist and participate in the AACR Special Conference in Tumor Invasion and Metastasis held in San Diego, California in January, 2013. Starting July 1st, 2014, Nahir will begin a residency in Anatomic and Clinical Pathology at Baylor College of Medicine in Houston, Texas.