FUNCTIONAL SIGNIFICANCE AND THERAPEUTIC TARGETING OF CHEMOKINES AND CHEMOKINE RECEPTORS IN GLIOBLASTOMA

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2011

To my wife and everyone I love

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Jeffrey K. Harrison, for educating and training me regarding research, culture, and life. I appreciate Dr. Brent A. Reynolds, Dr. Brian K. Law, and Dr. Wolfgang J. Streit for serving on my committee and for the advice they have provided. I also wish to thank my colleagues Defang Luo, Kathy Laughlin, Kien Pham, and Cyrus Bhadha for everything they did along these years. I especially thank Neal Benson and Amy Poirier for their technical help on my flow cytometry experiments. I am also thankful to everyone who has helped me in every respect.

Most important, I would like to thank my wife Wei, and my parents. Without you I couldn't go this far. Thank you for your support, patience, and unconditional love.

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of the University of Florida in Partial Fulfillment of the
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May 2011

Chair: Jeffrey K. Harrison
Major: Medical Sciences – Physiology and Pharmacology

Human glioblastoma multiforme (GBM) is the most common primary brain tumor in adults. The goal of this study is to investigate the functions of several chemokine systems in glioblasotma biology. First, the role of the chemokine CX3CL1 and its receptor CX3CR1 in the GL261 murine model of malignant glioma was investigated. In situ hybridization analysis identified CX3CL1 and CX3CR1 expression in GL261 tumors. With CX3CR1 gene-disrupted C57BL/6 mice, a slight increase in the tumor growth rate in $CX3CR1^{-/-}$ mice was evident with similar numbers of microglia and $CD4^{+}$, $CD8^{+}$, $Foxp3^{+}$, or $Ly49G2^{+}$ lymphocytes within tumors established in $CX3CR1^{+/-}$ and $^{-/-}$ mice. These data indicate that CX3CR1 has little or no effect on either gliomagenesis or the migration of microglia and lymphocytes into GL261 tumors.

Next, we examined the role of CXCR3 in glioma progression. Intracranial GL261 tumors express CXCL9 and CXCL10 *in vivo*. Glioma-bearing CXCR3-deficient mice had significantly shorter median survival time and reduced numbers of tumor-infiltrated natural killer and natural killer T cells as compared with control. In contrast, antagonism of CXCR3 with NBI-74330 prolonged median survival times of both tumor-bearing WT and CXCR3-deficient mice when compared with vehicle-treated groups. NBI-74330

treatment did not impact tumor infiltration of lymphocytes and microglia. A small percentage of GL261 cells were identified as $CXCR3^{+}$, which was similar to the expression of CXCR3 in several grade IV human glioma cell lines. When cultured as gliomaspheres (GS), the human and murine lines increased CXCR3 expression; CXCR3 expression was also found in a primary human GBM-derived GS. Additionally, CXCR3 isoform A was expressed by all lines, whereas CXCR3-B was detected in T98G-, U118- and U138-GS cells. CXCL9 or CXCL10 induced *in vitro* glioma cell growth in GL261- and U87-GS as well as inhibited cell loss in U138-GS cells and this effect was antagonized by NBI-74330. The results suggest that CXCR3 antagonism exerts a direct anti-glioma effect and this receptor may be a potential therapeutic target for treating human GBM.

In the last part of the project, we demonstrated that CCR3, CXCR3, CXCR4 and CXCR7 are expressed in primary human GBM cell lines. GBM cell lines can be grouped as $CXCR4^{high}$-$CXCR7^{low}$ (GBM L0, L2, L3) and $CXCR4^{low}$-$CXCR7^{high}$ (GBM L1, S3, S7). In addition, CCL5 and CXCL11 are also expressed by GBM L0 and L1. When GBM cell subtypes were isolated, all of them were capable of restoring their parental phenotypes regarding cell surface expression of CXCR4 and CXCR7. Moreover, CXCL12 induced cell migration while CXCL11 and CXCL12 promoted cell growth in GBM L0 and L1 cells.

CHAPTER 1
INTRODUCTION

Glioblastoma Multiforme (GBM)

Glioma is tumor that arises from the glial tissue of the brain. One type of glioma, which arises from astrocytes is called astrocytoma. Astrocytomas are graded using a scale of I to IV according to their degree of malignancy. On this scale, grade IV astrocytoma, also known as glioblastoma multiforme (GBM) or glioblastoma, are the most aggressive tumor that contains areas of necrosis. GBM is the most common primary glioma in adults and about 50 % of these gliomas are GBM. In addition, 10 % of pediatric gliomas are also GBM. The integrity of the brain is influenced by the tumor growth and the accompanied increase in intracranial pressure, which leads to several symptoms that include headaches, seizures, memory loss, and changes in behavior.

Treatment

Surgery is the first procedure in the treatment of GBM in order to remove as much tumor as possible and to provide tissues for confirming the diagnosis. Due to the highly infiltrative character of GBM, it is nearly impossible to surgically remove the tumor completely. To overcome this situation, radiation and chemotherapy are then utilized to target the remaining tumor cells. Despite the substantial investigation to search for potential novel chemotherapeutic agents, only a few drugs have made it into clinical practice. An oral methylating cytotoxic drug, temozolomide, has shown a certain level of effectiveness and is now a standard chemotherapeutic agent to treat newly diagnosed GBM[1]. Bevacizumab, a monoclonal antibody against the vascular endothelial growth factor A (VEGF-A), is another drug that has been approved by the FDA[2]. Immunotherapy with immune response modulating reagents, like vaccines, antibodies,

cytokines, and drugs, has also been well studied. The mutated and aberrantly expressed proteins by tumor cells are potential targets that could be identified by the immune system. The marked presence of glioma-infiltrating microglia and lymphocytes supports the concept of targeting the immune system to treat GBM. Interferons and TGFβ are examples of cytokines studied in tumor immunotherapy[3]. Cancer vaccines[4] accompanied with dendritic cell (DC) based immunotherapy[5,6] and adoptive transfer of T cells[7,8] are other potential immune-based therapies under investigation.

Despite the continuous efforts from researchers to look for different approaches for treating GBM, the outcomes of treatment have not significantly improved. The median survival of GBM patients with multi-faceted therapies is less than 16 months[9,10]. The 2 year-survival of patients treated with temozolomide is only 25% and recurrent brain tumors tend to be more aggressive than their parental tumors before treatment. The effect of bevacizumab does not last very long as the median duration of effect ranges from 3 to 6 months[2,11]. In addition, the bevacizumab based treatment for recurrent GBM has not shown any improvement on patient survival[12]. Although immunotherapies have achieved some successes in treating certain types of cancers, like melanoma, renal cell cancer, and hematologic malignancies[13], the blood-brain barrier (BBB) of the brain prevents immunotherapy-based reagents from penetrating brain tissue, which results in the ineffectiveness of immunotherapy reagents. Most important, successful immunotherapy of GBM will need to overcome the highly immunosuppressive environment created by the tumor, that includes immunosuppressive cytokines such as TGFβ and interleukin 10[14] and immune cells such as the regulatory T cell[15]. Thus, more

thorough investigations to understand GBM biology are necessary to 1) decipher the mechanisms contributing to therapeutic resistance of GBM and 2) discover novel therapeutic targets to develop better drugs and treatments.

Therapeutic Resistance and Heterogeneity of GBM

One of the main reasons that GBM is resistant to multiple therapies is the high level of heterogeneity within GBM cell populations. The realization that GBMs with similar histopathologic features can have different subtypes, and the heterogeneity of the molecular profile in GBM has lead to variable responses to traditional brain tumor therapies. For instance, the heterogeneity of GBM contributes to temozolomide resistance. The MGMT gene, which encodes a DNA alkylating-repair enzyme, has been found to be methylated in 45% of GBM tissues examined[16]. The methylation state of the MGMT promoter determines whether tumor cells are responsive to temozolomide as tumor cells without MGMT promoter methylation are insensitive to temozolomide treatment. In the combination of temozolomide and radiation therapy, patients with MGMT-promoter methylation showed an improved 2 year-survival rate when compared with patients without MGMT promoter methylation (46 % v.s. 14 %)[16]. Therefore, MGMT methylation status may be one predictive marker for GBM and confirms that GBM with different molecular profiles may have varying levels of therapy resistance. Epidermal growth factor receptor (EGFR) is another example. EGFR has been examined as a therapeutic target since EGFR amplification has been reported in GBM[17]. However, only 41 % of GBM samples showed EGFR gene amplification[17], and mutated EGFR variants are commonly found in GBM with EGFR overexpression[18]. Therefore, EGFR heterogeneity could result in varied responses of GBM patients to EGFR-based treatments. The heterogeneity of GBM provides a challenge for targeting a single

molecule as a therapeutic approach. To date continuous efforts are being made to understand the molecular profile of GBM. Several micro-array analyses comparing gene expression patterns of GBM from patients have established that GBMs can be divided into 3 distinct molecular groups based on their gene expression profile: proneural, proliferative, and mesenchymal[19,20]. It was shown that patients with proliferative or mesenchymal glioblastomas have shorter life spans than those characterized by the proneural type, and glioblastomas tend to shift toward a mesenchymal subtype at recurrence. A more recent publication reported a higher diversity of molecular profiles of GBMs[21]. According to Verhaak et al., GBMs can be classified into four subtypes, namely classical, mesenchymal, proneural, and neural[21]. Interestingly, they reported that recurrent secondary tumors do not change their subtype class, which differs from what was suggested previously[19]. In addition, each subtype shows similar gene sets to distinct neural cell types. The proneural class is highly similar to an oligodenrocytic gene signature while the classical subtype is associated with an astrocytic signature. The mesenchymal group is associated with cultured astroglial signature, whereas the neural class shows the association with oligodendrocytic, astrocytic, and neuronal gene expression patterns. These differences in genetic profiles within GBMs indicate the necessity of developing a personalized and molecular subtype-specific therapeutic approach to treat GBM patients.

Cancer Stem-Like Cells

Another finding that provides understanding of GBM heterogeneity is the discovery of cancer stem-like cells (CSCs). Cancer stem-like cells are a small subset of cancer cells that share several similar properties with normal stem cells. It is believed that CSCs are also responsible for heterogeneity and therapeutic resistance of cancers.

CSCs were first found in leukemia and multiple myeloma[22-24]. It was hypothesized that only a specific small population of cancer cells have tumor initiating capacity[24] and are able to differentiate, which could increase the diversity of cancer cell populations. Growing evidence suggests that CSCs exist among different types of cancer, such as malignant melanoma[25], colorectal cancer[26], and brain tumors[27]. CD133, a neural and hematopoietic stem cell marker, is found on the cancer stem-like cells and is widely used to identify CSCs among different cancers. Other markers that are present in normal stem cells such as CD44, CD24, CD15, nestin, and SOX2 are also utilized in the identification and characterization of CSCs. Previous efforts to characterize $CD133^+$ CSCs have realized that $CD133^+$ levels correlated to the malignancy of human glioma and are associated with poor prognosis, with higher grade (III and IV) gliomas having enhanced CD133 expression[28]. Moreover, $CD133^+$ CSCs isolated from human GBM were capable of tumor initiation while the $CD133^-$ GBM cells from the same patients were incapable of forming solid tumors[27,29-31]. These findings lead to the hypothesis that CSCs are the cells responsible for tumor initiation *in vivo*. However, recent studies from colorectal cancer[32] and glioma[33-35] recognized that $CD133^-$ CSCs, with their lower proliferation index and different molecular profiles, also had tumor initiatimg capacity. These studies raised controversy of CD133 as a CSC marker and revealed higher heterogeneity of CSCs than previously thought, suggesting the existence of different CSC populations. Studies to identify the CSC populaiton with other stem cell markers once again confirmed the heterogeneous characteristics of CSCs. Challenges to the CSC hypothesis have also been proposed. Quintana et al reported that when human melanomas were tranplanted into non-obese diabetic/severe combined

immunodeficiency (NOD/SCID) interleukin-2 receptor gamma chain deficient (*Il2rg*$^{-/-}$), aks NSG mice, which are more highly immune-compromised than regular NOD/SCID mice, the detectable frequency of tumorigenic cells in melanoma increased by an average of 27 %[36]. This study suggests that the tumor initiating cells are actually more common in the cancer cell population. More important, this study showed that the assessment techniques and assays could dramatically affect results of CSC studies. In addition, the same group showed in a recent publication that none of the 22 stem cell markers tested, including CD271 (neural crest nerve growth factor receptor), are capable of enriching tumor initiating cells[37]. Interestingly, Boiko et al. reported that in a different animal model (T-, B- and natural-killer-deficient *Rag2*$^{-/-}\gamma c^{-/-}$ mice), CD271^{+} cells, isolated from 90 % of melanomas tested, successfully formed tumors, but CD271^{-} cells failed to form solid melanomas *in vivo*[38]. These data once again fueled the debate about the accuracy of assays used to characterize CSCs and whether tumor initiation is an exclusive property of these cells.

Targeting Cancer Stem-Like Cells

While controversy surrounds the concept of tumor initiating cells (CSCs), CSCs are still worthy targets in treating GBM. Firstly, GBM stem-like cells showed greater tumor initiating efficiency than non-stem cells. In the mouse model of GL261 glioma, as few as 100 CD133^{+} cells were capable of initiating gliomas while it required 10,000 CD133^{-} cells to form a solid tumor[33]. The impact of different immune-compromised animals on the frequency of tumor initiating cells also indicated that CSCs are more efficient to form tumors in the less immune-compromised environment[36-39]. Several studies have reported that CSCs exhibit properties of enhanced resistance to standard

therapies in GBMs. After radiation, $CD133^+$ cells were enriched *in vitro* and *in vivo*. In addition, $CD133^+$ cells preferentially activate enhanced DNA damage checkpoint mechanisms after radiation[9]. Moreover, CSCs tend to express higher levels of genes associated with resistance to chemotherapy, such as *BCRP1* and *MGMT*[16] as well as the ATP binding cassette drug transporter[40]. Together, these findings suggest that GBM stem-like cells contribute to therapeutic resistance of GBM.

Several signaling pathways involved in stem cell maintenance have been reported and their therapeutic importance has been investigated. Inhibition of the Notch signaling pathway depleted $CD133^+$ cells and reduced gliomasphere formation of GBM stem-like cells[41]. EGF mediated growth signaling is another critical pathway in GBM. EGFR kinase inhibitors lowered GBM stem-like cell proliferation and gliomasphere formation *in vitro*[42,43]. However, EGFR antagonism-based clinical trials have not provided promising results given the heterogeneity of EGFR in GBM. Inhibitors of STAT3, BMP, TGFβ, and Wnt signaling pathways are either in clinical trials or under current consideration. The forced differentiation of GBM stem-like cells has also been investigated[44] as differentiated cells usually lose their long-term repopulation capacity and are unable to initiate tumors *in vivo*. Thus, promoting cell differentiation could lead to successful development of GBM therapies. A previous study has shown that BMP4 treatment led to GBM differentiation and reduced tumor growth and invasion[45]. Other studies suggested that PTEN, a well-known tumor suppressor, is involved in GBM cell differentiation with inactivation of PTEN promoting an undifferentiated state of GBM[46,47]. Currently the mechanisms that underlie maintenance of GBM stem-like cells are unclear which prompts further study. Since heterogeneity is a typical feature in GBM, future treatment

might require combination of therapies targeting the various signaling pathways and proteins mentioned above.

Chemokines and Chemokine Receptors

Chemokines (chemotactic cytokines) are small proteins that were initially discovered in association with inflammatory responses and are now known to comprise a large family consisting of more than 40 proteins. Chemokines can be classified by the position of their first two cysteine residues (CC, CXC, C and CX3C) in their sequences. Chemokines are attractive molecules to mediate the migration of responsive cells, such as immune cells, by inducing chemotaxis through G-protein coupled receptors expressed on the cell surface. Therefore, chemokines and their receptors control the homing of immune cells and draw great attention in the context of immune therapy to many diseases, including cancer. Both the CC and CXC families have many members, while the C family has only two chemokines (XCL1, 2) and one receptor (XCR1), and the CX3C family has one chemokine (CX3CR1) and one receptor (CX3CL1). Members of CXC chemokine family can be further classified according to the presence or absence of a Glu-Leu-Arg (ELR) motif[48]. This ELR motif is located at the N-terminus adjacent to the first cysteine amino acid residue. ELR^+ CXC chemokines have opposite functions to ELR^- CXC chemokines regarding angiogenesis[49]. The ELR^+ chemokines promote angiogenesis by regulating neutrophil migration[49-51]. On the other hand, ELR^- chemokines are angiostatic peptides[49,52,53]. Most chemokines exert their function as secreted proteins with the exception of CX3CL1 and CXCL16[54]. Chemokines show redundancy in terms of their binding specificity, and activation of chemokine receptors results in activation of many downstream signaling pathways, such as ERK, PI3/AKT, p38, and JNK. The complexity of chemokine systems and the evidence that chemokines

and their receptors are widely expressed by different types of cancers raise the possibility of targeting them in tumor treatments.

Chemokines, Chemokine Receptors, and Cancer

In cancers, chemokines have effects on tumor growth, angiogenesis, metastasis, and immune cell trafficking. Most efforts of researchers have been focused on the functions of chemokines and their receptors in tumor metastasis, immune cell trafficking, and angiogenesis. However, more studies suggest that tumor cells utilize chemokines and receptors to provide growth signals or modulate cell status in an autocrine or paracrine manner. Further understanding of the functions of chemokine systems in all aspects of tumor progression could provide future therapeutic possibilities.

CX3CR1-CX3CL1

The chemokine receptor system CX3CR1 and its ligand CX3CL1 are known to be involved in immune responses that underlie various human diseases and their corresponding animal models. For instance, CX3CR1 is responsible for recruiting dendritic cells and a subset of monocytes in models of atherosclerosis[55,56]. CX3CR1 deficiency results in impaired microglia migration in a mouse model of age-related macular degeneration[57]. Enhanced neuronal cell loss is also evident in CX3CR1 deficient mice after systemic lipopolysaccharide injection, in toxin-induced Parkinsonism, and the SOD1-G93A transgenic mouse model of motor neuron disease[58]. A role for CX3CL1/CX3CR1 system in tumorigenesis has also been established. CX3CL1 has been shown to mediate both natural killer cell-dependent and T cell-dependent antitumor activity[59-61]. CX3CL1 also has angiogenic activity. Several groups have suggested that CX3CL1 increases angiogenesis through endothelial cell activation in the pathogenesis of rheumatoid arthritis[62-64]. Together, these data suggest that this

chemokine system may be similarly involved in tumor angiogenesis. Thus CX3CL1/CX3CR1 might be a suitable target in the development of novel therapies to treat cancer.

CXCR3 and CXCL9, 10, 11

CXCR3 belongs to the CXC chemokine receptor sub-family and has three endogenous ligands, CXCL9 (MIG), CXCL10 (IP10) and CXCL11 (ITAC). This chemokine system has been reported to be involved in tumor growth, metastasis, angiogenesis, and immune cell infiltration into tumors. CXCR3 has been shown to be expressed by tumor cells such as melanoma[65], ovarian[66] and renal carcinoma[67], breast cancer cells[68-70], B-cell leukemia[71], prostate[72], colorectal[73], and brain tumor cell lines[74]. In addition, the level of CXCR3 expression has been reported to correlate with poor prognosis of breast cancer patients[75] and with tumor thickness in melanoma[76]. CXCR3 activation enhances tumor cell proliferation of myeloma[77] and osteosarcoma[78], and CXCR3 inhibition induces caspase-independent cell death[78]. Collectively, the data suggest that CXCR3 is involved in tumor growth in a variety of cancers.

With respect to immune cell recruitment, CXCR3 is expressed by activated T cells, natural killer (NK), NKT cells and, within the central nervous system, microglia[79-81]. $CXCR3^+$ lymphocyte recruitment, directed by CXCL10, can promote spontaneous regression of melanoma[82], while CXCL11 increases tumor-infiltrating lymphocytes and inhibits tumor growth in both breast cancer and T cell lymphoma[83,84]. Therefore, CXCR3-mediated homing of immune cells represents a potential target for tumor therapy investigation. In addition to immune cell trafficking, CXCR3 also mediates cancer cell metastasis by stimulating matrix metalloproteinase (MMP) production[78]. It

has been known to regulate metastatic activity of melanoma[75], breast cancer[68], osteosarcoma[78] and colorectal carcinoma[85].

As a receptor for ELR$^-$ chemokine family members, CXCR3 has been demonstrated to block angiogenesis. For instance, CXCL10 is capable of attenuating CXCL8 and FGF-2 induced angiogenesis[52]. *In vivo* delivery of CXCL9 or CXCL10 inhibited angiogenesis within the tumor[86,87]. In humans, different isoforms of CXCR3, including CXCR3A, CXCR3B, CXCR3-alt, have been reported. Endothelial cells were found to express CXCR3B, which mediates the angiostatic response caused by CXCL9-11[88]. CXCR3B activation has been reported to induce apoptosis, which might explain the angiostatic effect of CXCR3 activation in endothelial cells.

The recent demonstration that CXCL10 is expressed by murine[89] and human glioma[74] cell lines suggests that this chemokine could play important roles in brain tumor biology. CXCL10 is up-regulated in grade III and grade IV human glioma cells as compared to normal astrocytes[74]. Additionally, CXCR3 is also elevated in both grade III and grade IV human glioma cells and its activation can increase DNA synthesis of these cells *in vitro*[74]. The DNA synthesis enhancing effect of CXCL10 on glioma cells is abolished by CXCL10 neutralizing antibody[74]. While these *in vitro* results support a role for CXCR3 in malignant glioma, investigations of this receptor in glioma progression *in vivo* are absent and further study is necessary.

CXCR4-CXCR7

CXCR4 and its ligand CXCL12 is one of the common chemokine receptor/chemokine pairs studied in tumor growth and metastasis of many tumors. CXCR4 and/or CXCL12 have been shown to be up-regulated in pancreatic cancer[90], colon cancer[91], ovarian cancer[92], lymphoma[93], medulloblastoma and glioma[94-97].

CXCL12 is also constitutively expressed in tissues such as liver, lung, lymph nodes, adrenal glands and bone marrow, which may explain the important role of CXCL12/CXCR4 in metastasis[98]. Inhibition of CXCR4/CXCL12 decreases the metastasis of osteosarcoma and melanoma[99], as well as the growth of medulloblastoma and glioma[96]. In the context of glioma, CXCR4 is elevated in GBM and grade III glioma compared with grade II glioma[100]. Inhibition of CXCR4-mediated pathway can inhibit human glioma growth, invasion, and pro-MMP2 activation[101,102]. Although CXCL12 belongs to the ELR^- family, several studies have shown that CXCL12 induces the migration, proliferation, capillary tube formation as well as VEGF production in endothelial cells[103-105]. Furthermore, inhibition of CXCL12 and CXCR4 reduces tumor growth by blocking angiogenesis[106].

Recently, CXCL12 has been reported to be a ligand of another receptor, which is termed CXCR7[107]. CXCR7 also binds CXCL11[107], which complicates deciphering the role of CXCR7 in tumor progression. CXCR7 is expressed by a variety of cancers, including breast cancer[108,109], lung cancer[109], and glioma[110,111]. Breast cancer lines stably overexpressing CXCR7 form larger tumors while other lines with CXCR7 silencing show decreased tumor volumes[109]. In lung cancer, CXCR7 not only promotes tumor growth but also enhances tumor metastasis[109]. Several publications suggest that CXCR7 contributes to tumor progression indirectly via regulation of CXCR4-dependent activities. It has been demonstrated that CXCR7 regulates acute CXCR4 activation by depleting extracellular CXCL12 via CXCR7 internalization[108,112]. On the other hand, it has been shown that CXCR7 is a functional receptor and induces cell adhesion of malignant hematopoietic cells through ERK 1/2 and AKT pathway activation[113]. Another

study of CXCR7 in glioma also suggests that CXCR7 is functional and exhibits anti-apoptotic activity and thus promotes glioma tumor growth[111]. Therefore, the CXCL12-CXCR4-CXCR7 axis in cancers could be more complicated and the direct and indirect activities of CXCR7 may play critical roles in tumor progression.

Other Chemokines

Other chemokines, such as CXCL1 and CXCL8, are increased in a variety of cancers[114,115], and have been suggested to promote tumor growth by stimulating angiogenesis[114,116]. CXCL8 is one of the most thoroughly studied chemokines in the field of angiogenesis[117,118]. CXCL8 can induce proliferation and chemotaxis of human umbilical vein endothelial cells (HUVEC)[117]. In addition, CXCL8 can directly promote survival, proliferation, and capillary tube formation, and these effects can be inhibited by an anti-CXCL8 monoclonal antibody[119,120]. In addition, in ovarian cancer, levels of CXCL8 are correlated with blood vessel formation and poor survival[121]. Serum levels of CXCL8 are increased in prostate and breast cancer patients, which suggests an important role of CXCL8 in these diseases[122,123].

CCR7/CCL21 are critical for a variety of tumors in terms of metastasis into lymph nodes, including breast cancer[124], melanoma[125], colorectal cancer[126], gastric carcinoma[127], and esophageal cell carcinoma[128]. CCL21 is abundant in the lymph nodes and results in the trafficking of CCR7-expressing tumor cells toward the lymph nodes.

Chemokines and receptors also play important roles in tumor-associated immunosuppression through the recruitment of immunosuppressive cells. In gastric cancer, the levels of chemokines CCL17 and CCL22 are correlated with the frequency of $Foxp3^+$ Treg cells, a type of immunosuppressive cells, in the tumor[129]. CCL17 and CCL22 are able to induce the migration of $Foxp3^+$ Treg cells in a concentration-

dependent manner *in vitro*[129]. CCL22 and CCL2 are also expressed by human glioma cell lines and Treg cells from GBM patients have a more elevated level of CCR4 (CCL2 receptor) than Treg cells from control tissues[130]. Others have shown that both human and mouse pancreatic cancers express higher levels of CCR5 ligand CCL5, and Treg cells in the tumor microenvironment show CCR5 expression[131]. Disruption of the CCL5-CCR5 axis by systemic administration of a CCR5 antagonist slows tumor growth via a Treg cell-mediated mechanism[131].

Gliomasphere Model

In the field of cancer research, *in vitro* culture of tumor cell lines is an essential and important tool to investigate mechanisms related to tumorigenesis and progression. For decades, researchers have been using tumor cell lines cultured as a monolayer and supplemented with bovine serum. However, recent studies have raised doubts about cells cultured under these traditional conditions and indicate that they are not the best model system to understand mechanisms of tumor formation *in vivo*. Howard Fine and colleagues have proposed that GBM stem cells from the gliomasphere culture model have a considerable advantage over traditional serum-cultured models[132]. They compared primary human GBM cells cultured in stem cell-enriched gliomasphere conditions (serum free medium supplemented with B27, human recombinant EGF, and bEGF) with GBM cells grown in the presence of DMEM supplemented with 10% fetal bovine serum. In their study they reported that gliomasphere culture conditions successfully maintained the tumorigenic potential of GBM cells while serum supplemented GBM cells lost this potential[132]. Moreover, primary GBM gliomaspheres kept their parental phenotypes and genotypes when compared with serum-cultured GBM cells and glioma cell lines[132]. This concept was further investigated in a more

recent study that examined the effect of long-term gliomasphere culture of 7 GBM cell lines from biopsies of glioblastoma patients[133]. It was found that long-term (10 passages) gliomasphere culture had only a modest affect on overall expression patterns of these cells, and each cell line maintained the individual characteristics of their parental biopsies[133]. Therefore, each cell line could serve as a personalized model of the glioblastoma it was derived from, even after multiple passages. These findings indicate that the gliomasphere model has a greater genetic stability than the serum supplemented culture model. Thus, the gliomasphere model is useful in studying the biology of the primary GBMs and may lead to more clinically relevant results than serum supplemented cell culture models. However, it has been reported that relapsed glioblastomas have different genetic signatures from the paired primary tumors[19,134]. Thus, even the gliomasphere culture of primary GBM may not reflect the genetic alteration(s) in the paired recurrent GBM because of the genetic stability of gliomasphere *in vitro* model. Therefore, establishing relapsed GBM-derived gliomaspheres and comparing them with the paired primary GBM-derived gliomaspheres may help elucidate the mechanisms that underlie GBM recurrence.

Murine Glioma 261 (GL261) Model

The GL261 mouse model of malignant glioma is one of the most frequently used brain tumor animal models. GL261 tumors were orginally generated from an intracranial injection of 3-methylcholantrene into C57BL/6 mice[135]. The GL261 model shows similar features to human GBM. They are hypervascular and highly proliferative and there is necrosis accompanied with VEGF and hypoxia-inducible factor 1 (HIF1) induction. $CD133^+$ cancer stem cells have also been found in the GL261 cell line[33], similar to human GBM stem-like cells. In addition, the GL261 model shows a substantial presence

of immune cells, including microglia and lymphocyte subsets, that is similar to human GBM[89]. Although several studies have used the GL261 model for studying gliomagenesis and identifying potential therapeutic targets, there are few published studies reporting the roles of chemokine and chemokine receptors in the GL261 mouse model of malignant glioma. For this reason, we have established this animal model to investigate the role of chemokines and receptors in glioblastoma tumorigenesis and progression.

Significance and Specific Aims

Chemokines and chemokine receptors play important roles in almost all step of tumorigenesis and tumor progression. As reviewed, chemokines can enhance tumor cell proliferation, prevent apoptotic activity, promote metastasis, and induce angiogenesis. In addition, the recruitment of immunosuppressive cells into the tumor is also mediated by chemokines. In contrast, chemokines also mediate physiological activities that block tumor growth, such as the infiltration of inflammatory and antitumor immune cells into the tumor as well as exert angiostatic effects. Thus, investigating the functions of chemokines and receptors in cancer is beneficial for developing novel therapeutic methods for cancer treatment. Although chemokines have been widely studied in metastatic cancers like breast cancer and colorectal cancer, their importance in GBM biology is still unclear. The fact that a single chemokine receptor can pair up with multiple ligands and one chemokine can bind to more than one receptor increases the complexity of chemokine system in GBM and prompts further investigations. The focus of this dissertation is to provide a more thorough understanding of the functions of chemokines and chemokine receptors in GBM biology and reveal potential therapeutic targets for future treatments of GBM patients.

The specific aims in this study included:

1. **Determine the expression of chemokines and chemokine receptors in murine and human GBM cells *in vitro* and *in vivo*.**

   The first question we addressed related to identifying chemokine and chemokine receptor expression in GBM in order to look for potential drugs. In this study, we utilized the murine GL261 glioma cell line and measured its expression pattern of chemokines and receptors. GL261 implantation and in situ hybridization allowed us to determine the expression profile *in vivo*. To translate the results from GL261 cells into human, the data were compared with several well-established human GBM cell lines as well as GBM patient-derived primary cell lines. In addition, *in vitro* gliomasphere and serum-cultured models were compared side by side regarding the expression of chemokine and chemokine receptors.

2. **Evaluate the effect of host chemokine receptor deficiency on glioma-bearing animal survival.**

   One phenotype influenced by tumor growth is the host survival rate. Through GL261 cell implantation, we analyzed the impact of host chemokine receptor disruption on animals with intracranial tumors. Kaplan-Meyer survival analysis reflected tumor growth in wild type and chemokine receptor gene deficient mice while H&E staining of tumor sections provided information on tumor sizes.

3. **Address the influence of chemokine receptor dysfunction on GBM-infiltrated microglia and lymphocytes.**

Certain chemokine receptors are expressed and mediate the migration of microglia and lymphocytes into tumors. The chemokine expression profile determined in aim 1 provided us candidate chemokine receptors to study the recruitment of tumor-infiltrating immune cells. Using multiple chemokine receptor deficient mice, we examined the number of microglia and lymphocytes within GBM tumors from wild type and chemokine receptor-disrupted animals.

4. **Examine the growth effect of chemokine stimulation on murine and human GBM cells.**

   Chemokines are known to evoke proliferative and survival signaling pathways via activation of phospho-ERK and phospho-AKT pathway in a variety of cancers. In this study we determined the *in vitro* cell growth rates of murine and human GBM cells using the gliomasphere model with and without chemokine stimulation. Total cell numbers were counted to quantify cell growth.

5. **Study the effect of chemokine receptor antagonism on tumor growth *in vitro* and *in vivo*.**

   Our final goal evaluated inhibiting chemokine receptors pharmacologically in order to test the possibility of using chemokine receptor antagonists as chemotherapy drugs. Murine and human GBM cells were co-incubated with chemokines and paired receptor inhibitors to determine the tumor cell growth rates *in vitro*. In addition, glioma-bearing animals were treated with a specific chemokine receptor antagonist and Kaplan-Meyer survival analysis was performed to address this aim.

CHAPTER 2
MATERIALS AND METHODS

Animals

Wild type (WT) C57BL/6 mice were obtained from either Charles River Laboratories or Jackson Laboratories. CX3CR1-deficient (−/−) mice, backcrossed to the C57BL/6 background for greater than 10 generations, were obtained from JAX Laboratories. The generation of these mice has been previously described[136]. The protein coding sequence of the CX3CR1 gene was exchanged with GFP in heterozygous (one allele replaced) and homozygous (both alleles replaced) mice. In these mice, all cells normally expressing CX3CR1 express GFP. Colonies of $CX3CR1^{-/-}$ and $^{+/-}$ mice were maintained at the University of Florida. All mice used in studies presented herein were derived from breeding $CX3CR1^{+/-}$ and $^{-/-}$ mice; hence all comparisons were made between littermates. $CXCR3^{-/-}$ mice, backcrossed 16 generations to the C57BL/6 back- ground were generated as described previously[137]. All procedures involving mice were carried out in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee (IACUC).

Cell Culture

The GL261 glioma cell line was maintained in RPMI-1640 medium supplemented with 10 % heat- inactivated FBS, 1 % penicillin–streptomycin, 4 mM L-glutamine. The A172 and U118 glioma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % penicillin–streptomycin, 2 mM L-glutamine. The T98G, U118 and U138 glioma cell lines were maintained in Eagle's minimum essential medium supplemented with 10 % heat-inactivated FBS, 1 % penicillin– streptomycin, 1 % sodium pyruvate and 2 mM L-

glutamine. All gliomaspheres (GS) were cultured in DMEM/F12 medium supplemented with 2 % B27, 20 ng/ml of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), 5 µg/ml of heparin and 1 % penicillin–streptomycin. All the cells were grown in a humidified incubator at 37 °C with 5 % CO2. DMEM, Eagle's minimum essential medium, RPMI-1640, DMEM/F12 medium, B27, EGF, bFGF, L-glutamine and antibiotics were obtained from Gibco-BRL (Invitrogen). Sodium pyruvate and heparin were purchased from Sigma–Aldrich. FBS was from HyClone (Thermo Scientific).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from glioma cells with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA contamination was removed by RQ1 RNase-free DNase treatment (Promega). Total RNA was then quantified and stored at -80°C. RNA (1 µg) was retrotranscribed with iScript complementary DNA (cDNA) synthesis kit (Bio- Rad). Synthesized cDNA was subjected to polymerase chain reaction analysis. Polymerase chain reaction (PCR) was performed by heating for 96 °C for 2 min, followed by amplification for 35 cycles: 96 °C for 30s, 56 °C for 1 min and 72 °C for 1 min. Touchdown PCR was utilized in some cases. For touchdown PCR, the annealing temperature started at 65 °C and was decreased by 1 °C every cycle for 15 cycles and reached 50 °C. 50 °C was then used for the remaining number of cycles. The parameters of denaturing and elongating temperatures were the same as regular PCR protocol. The following primers were used: murine CXCL9: 5'-CTCGGATCCGCCATGAAGTCCGCTGTTCTTTTC-3'(forward), and 5'-TATGAATTCAAATTAACACTTTATGTTTTGTAG-3'(reverse); murine CXCL10: 5'-CCGGAATTCTCCCCATCAGCACCATGAACCC-3'(forward), and 5'-CTGCTCGAGGAGTAGCAGCTGATGTGACC-3'(reverse); murine CXCL11: 5'-

GCAGAATTCTGCAGCGGCTGCTGAGATGAACAG-3'(forward), and 5'-GGACCTTCTAGAAAGTTCTGCAGC-3'(reverse); murine CXCR3: 5'-GAGGTTAGTGAACGTCAAGTG-3'(forward), and 5'-GGGGTCCCTGCGGTAGATCTG-3'(reverse); murine GAPDH: 5'-AAATGGTGAAGGTCGGTGTG-3'(forward) and 5'-TCTCCATGGTGGTGAAGACA-3'(reverse); human CXCL9: 5'-TGCTGGTTCTGATTGGAGTG-3'(forward) and 5'-CTGTTGTGAGTGGGATGTGG-3'(reverse); human CXCL10: 5'-AACCTCCAGTCTCAGCACCA-3'(forward), and 5'-TTTGAAGCAGGGTCAGAACA-3'(reverse); human CXCL11: 5'-CCTGGGGTAAAAGCAGTGAA-3'(forward), and 5'-TGGGGAAAGAAGTGTGTATTTG-3'(reverse); human CXCR3-A and -B: 5'-ACCCAGCAGCCAGAGCAC-3'(forward), and 5'-GTTCAGGTAGCGGTCAAAGC-3'(reverse); human GAPDH: 5'-CGAGATCCCTCCAAAATCAA-3'(forward), and 5'-TGCTGTAGCCAAATTCGTTG-3'(reverse).Predicted PCR product sizes are listed in Table 2-1.

Intracranial Injection of GL261 Glioma Cells

GL261 glioma cells (2×10^5 cells in CX3CR1$^{+/-}$ and $^{-/-}$ mice; 1.6×10^5 cells in wild type and CXCR3$^{-/-}$ mice) in a total volume not exceeding 3 µL were injected 3 mm deep into the right cerebral hemisphere (1 mm posterior and 2 mm lateral from Bregma) of wild type C57/B6, CX3CR1$^{+/-}$ and $^{-/-}$, and CXCR3$^{-/-}$ mice. To determine tumor growth, glioma-bearing mice (3 weeks after GL261 cell injection) were euthanized using sodium pentobarbital (32 mg/kg) and subsequently perfused with 0.9 % saline followed by buffered 4 % paraformaldehyde (PFA). Brains were surgically removed and post-fixed with 4 % PFA. After fixation, tissues were incubated in 30 % sucrose solution at 4 °C overnight followed by liquid nitrogen freezing. Frozen brains were then sectioned, thaw

mounted on Superfrost/Plus slides (Fischer Scientific), and subjected to either hematoxilin and eosin (H&E) staining, in situ hybridization, or immunohistochemistry.

NBI-74330 Treatment

NBI-74330 was synthesized according to Medina et al. (Patent WO02083143, USA, 24 October 2002) and the dosing protocol was performed as described previously[138]. Briefly, animals received 100 mg/kg/day of NBI-74330 in 1 % sodium docusate in 0.5 % 400Cp methylcellulose, injected subcutaneously, beginning from day 3 after surgery, for 12 days. A control group of mice were treated with vehicle only.

Kaplan–Meier Survival Analysis

For Kaplan–Meier survival analysis, percentages of surviving mice in each group of animals were recorded daily after GL261 glioma implantation. The endpoint was defined by a lack of physical activity and a body weight reduction of greater than 15 %. The data were subjected to Log-rank analysis in order to determine if significant differences existed in survival between the experimental groups.

In Situ Hybridization (ISH)

In situ hybridization probes were generated by PCR using cDNA synthesized from total RNA extracted from GL261 glioma cells. DNA fragments were cloned into pGEM-7 (Promega). To generate the antisense and sense (c)RNA hybridization probes, plasmids were linearized and then subjected to *in vitro* transcription using either T7 or SP6 RNA polymerase in the presence of [33P]UTP. Brain sections were hybridized separately with antisense and sense probes. In all cases, no signals were detected in sections probed with “sense” riboprobes. After ISH, sections were apposed to film and subsequently dipped in LM-1 emulsion and stored at 4 °C. Slides were developed (after exposure for 1–4 weeks), fixed, and counterstained with hematoxylin and eosin.

Immunohistochemistry

For immunohistochemistry, brain sections were permeabilized with 0.5 % of Triton X-100 in phosphate-buffered saline (PBS) for 15 min at room temperature followed by blocking with 10 % goat serum in PBS for 30 min. In some cases (anti-Foxp3 immunohistochemistry), sections underwent an antigen retrieval treatment. In brief, slides were first permeabilized with 0.5 % Triton X-100 followed by heating slides (immersed in a boiling water bath for 25 min) in a buffer containing 10 mM Sodium Citrate, 0.05 % Tween 20, pH 6.0. Slides were then cooled to room temperature for 20 min, washed with PBS three times, and finally subjected to standard immunohistochemistry procedures. The sections were incubated in primary antibodies at 4 °C overnight. The following antibodies were used: rat anti-CD4 (dilution 1:50, BD Pharmingen), rat anti-CD8 (dilution 1:50, Serotec), rat anti-Foxp3 (dilution 1:50, eBioscience), and rat anti-Ly49G2 (dilution 1:50, BD Pharmingen). The following day, sections were washed three times with PBS and incubated subsequently in goat anti-rat Alexa 594 (dilution 1:1000, Invitrogen) The sections were then washed three times with PBS and finally counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich). For quantification of $CD4^+$, $CD8^+$, $CD11b^+$, $Foxp3^+$ and $Ly49G2^+$ cells, the number of cells per high-powered field in several sections from multiple animals were determined and the mean and standard error of the means calculated. The data were subjected to statistical analysis.

CXCL10 Enzyme-Linked Immunosorbent Assay

To quantitate CXCL10 protein secreted by GL261 cells, 10^5 cells were plated in a 12-well plate and grown for 24 h. Cells were then washed with PBS twice and incubated with 500 µL serum-free medium. Conditioned medium was collected at 24 and 48 h and

the CXCL10 concentration was measured by Mouse CXCL10/IP-10/CRG-2 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's protocol. CXCL10 concentration was normalized to the number of cells in the well and expressed as nanograms per milliliter per 10^6 cells.

FACS Analysis

Adherent (AD) glioma cells and GS were harvested with 0.01 % ethylenediaminetetraacetic acid (EDTA) in PBS, pH 7.4, washed with ice cold 0.5 % to 1 % bovine serum albumin (BSA) in PBS and subsequently blocked with 5 µg/mL of mouse and rat IgG mixture for 15 min at room temperature. Cells were then incubated with specific antibody for 30 min on ice. Mouse anti-human CXCR3-PE (dilution 1:12, BD Biosciences), rat anti-mouse CXCR3-APC (dilution 1:12, R&D Systems), mouse anti-human Nestin-APC (dilution 1:40, R&D Systems), mouse anti-Nestin-Alexa 647 (dilution 1:12, BD Biosciences), mouse anti-human/mouse SOX2 (dilution 1:12, R&D Systems) were used. Samples were then washed and analyzed with BD LSR II system (BD Biosciences). Dead cells were excluded by 7-AAD (eBioscience) or DAPI staining. All data were analyzed by FlowJo software version 7.6 (Tree Star).

In Vitro Growth Analysis

GS cells were plated in 12-well plates at different density and treated with either CXCL9 (1 nM or 10 nM), CXCL10 (1 nM or 10 nM) or the combination of EGF and bFGF (each at 20 ng/mL). Cells cultured in medium without growth factor supplements served as the control. Cell numbers were determined at days 3, 6 and 9. To investigate the effect of CXCR3 inhibition, cells cultured as described above with 1 µM of NBI-74330 were compared with samples without NBI-74330. Cell numbers were determined on day 6 or 9. All experiments were performed in triplicate and are representative of

three independent experiments. Recombinant mouse and human CXCL9 and CXCL10 were purchased from R&D Systems.

For the *in vitro* growth of CXCL11 and CXCL12, primary human GBM cells were plated in 96-well plates at 2000 cells/wee and treated with either CXCL11 (10 nM), CXCL10 (10 nM) or without any chemokines as the control. All conditions contained 2 ng/ml EGF. Cell numbers were determined at day 10. Recombinant mouse and human CXCL11 and CXCL12 were purchased from R&D Systems.

Migration Assay

Human primary GBM cell were trypsinized, counted, and 2000 cells were transferred to uncoated 8-μm cell culture inserts (BD Bioscience) in medium containing 2ng/ml of EGF and the assembly placed into 24-well plates containing 2ng/ml of EGF and 10nM of CXCL11 or CXCL12. After 48 h, non-migrating cells were removed from the top of the filter with a cotton swab, and migrating cells on the bottom of the filter were fixed with 4 % paraformaldehyde, stained with DAPI, and counted.

Primary Sphere Formation Assay

Primary sphere formation assays were performed to quantify stem-like cell frequency within primary GBM cells. Cells were plated in 96-well plates at a density of 2000 cells per well per 200 μl medium containing either CXCL11 (10 nM) or CXCL10 (10 nM). Cells cultured in medium without chemokines served as the control. All conditions were supplemented with 2ng/ml EGF. Number of spheres formed at day 10 were counted.

Statistical Analysis

All statistical analyses were calculated using either Microsoft Excel or GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). All data are presented as mean ±

standard error of the mean. P-values were calculated using Student's t-test with two-tailed distribution. Survival data were subjected to log-rank test to determine statistically significant differences between groups. A P value <0.05 was considered significant and is indicated with asterisks in figures.

Table 2-1. Predicted PCR product sizes (bp)

	CXCL9	CXCL10	CXCL11	CXCR3-A	CXCR3-B	GAPDH
Murine	526	467	441	479	N/A	314
Human	651	603	768	484	728	727

N/A: not applicable.

CHAPTER 3
ROLE OF CX3CR1 AND CX3CL1 IN GBM

The chemokine receptor system CX3CR1 and its ligand CX3CL1 are known to be involved in immune responses that underlie various human diseases and their corresponding animal models. For instance, CX3CR1 mediates recruitment of dendritic cells and a subset of monocytes in atherosclerosis[55,56]. CX3CR1 deficiency results in impaired microglia migration in age-related macular degeneration[57]. The function of CX3CL1/CX3CR1 system in tumorigenesis has also been examined. CX3CL1 has been shown to regulate natural killer cell-dependent and T cell-dependent antitumor activity[59-61]. Several groups have suggested that CX3CL1 increased angiogenesis through endothelial cell activation in the pathogenesis of rheumatoid arthritis[62-64], which suggests the potential of this chemokine system to be similarly involved in tumor angiogenesis. Thus CX3CL1/CX3CR1 might be a suitable target in the development of novel therapies to treat cancer.

The specific functions of CX3CL1/CX3CR1 in gliomagenesis have not been established. In this study, we sought to determine the role of CX3CR1 in glioma formation and the associated recruitment of microglia and lymphocytes, using the GL261 murine model of glioma[135,139]. CX3CL1 and CX3CR1 expression were determined in GL261 tumors established in its syngeneic host, the C57BL/6 mouse. The role of this chemokine system was then characterized in CX3CR1 deficient C57BL/6 mice. The results indicate that CX3CR1 has little to no effect on glioma growth. Moreover the migration of microglia and $CD4^+$, $CD8^+$, $Foxp3^+$, and $Ly49G2^+$ lymphocytes into the tumor tissue was not impacted by the lack of CX3CR1.

Results

CX3CL1 and CX3CR1 Expression in GL261 Tumors

CX3CR1 and CX3CL1 expression in GL261 glioma *in vivo* was established using the technique of in situ hybridization analysis. The results indicated that both CX3CR1 and CX3CL1 were expressed in GL261 gliomas analyzed from wild type C57BL/6 mice. Strong hybridization signals for CX3CR1 were evident throughout the tumor mass (Figure 3-1, panels A–C) and indicated that levels of CX3CR1 mRNA are elevated within the tumor as compared to the surrounding normal brain tissue. Higher resolution analysis showed that these tumor infiltrated CX3CR1-expressing cells were relatively abundant (Figure 3-1, panel E) and similar in number to $CD11b^{+}$ cells (Figure 3-1, panel G). These correlative results suggested that tumor infiltrating microglia were the primary source of CX3CR1. In contrast, CX3CL1 hybridization signals were less prevalent. When present, CX3CL1-expressing cells were found near the perimeter of the tumor mass (Figure 3-2). These CX3CL1-expressing cells were therefore hypothesized to be important for directing CX3CR1-expressing microglia into the tumor from the brain parenchyma.

Tumor Growth and Animal Survival Were Not Affected by CX3CR1 Deficiency

The effects of CX3CR1-deficiency on GL261 glioma formation *in vivo* was then determined by characterizing tumor growth and animal survival in CX3CR1 deficient C57BL/6 mice. Tumor sections from GL261 bearing $CX3CR1^{+/-}$ and $^{-/-}$ mice, obtained 3 weeks after GL261 cell implantation, indicated that tumor size was slightly larger in homozygous (−/−) animals as compared to the heterozygous mice (Figure 3-3A). This result suggested that GL261 tumor growth rate was slightly faster in $CX3CR1^{-/-}$ mice. Consistent with the histological examination, Kaplan–Meier analysis of tumor bearing

mice indicated a slightly shorter life span of glioma-bearing CX3CR1$^{-/-}$ mice than the life span of CX3CR1$^{+/-}$ mice (Figure 3-3B). The median survival time of CX3CR1$^{-/-}$ mice after GL261 cell implantation was 19 days, while that of CX3CR1$^{+/-}$ mice was 20 days ($p = 0.0332$). These survival times are also similar to what is observed in glioma-bearing wild type C57BL/6 mice (data not shown).

CX3CR1 Did Not Mediate Microglia Migration into Glioma Tissue

Microglia are the major CX3CR1-expressing cells in the brain[140,141]. To track the tumor infiltrating CX3CR1-expressing microglia we visualized GFP-expressing cells using fluorescence microscopy. Figure 3-4 shows abundant CX3CR1-expressing cells were found inside the tumors from both CX3CR1$^{+/-}$ and $^{-/-}$ mice. The CX3CR1$^{-/-}$ mice showed similar numbers of microglia within the tumors as compared to tumors from heterozygous (+/−) mice. Moreover, the microglia in the normal brain parenchyma from both $^{+/-}$ and $^{-/-}$ mice exhibited a comparable ramified morphology, while microglia inside the tumors from both groups of mice displayed similar morphological characteristics consistent with an activated phenotype (insets to Figure 3-4, panels A–D). In both CX3CR1$^{+/-}$ and $^{-/-}$ mice, most GFP-expressing cells also expressed CD11b (Figure 3-4, panels E and F); no obvious differences in the CD11b expression pattern were observed between the two groups of mice. Quantitative analysis of both CD11b^{+} and GFP^{+} cells in the two animal groups indicated that numbers of these cells did not significantly differ between CX3CR1$^{+/-}$ and $^{-/-}$ mice (Table 3-1). These collective observations suggested that CX3CR1 deficiency had no substantial effects on the recruitment, morphology, and level of expression of CD11b by CX3CR1-expressing microglia.

CX3CR1 Was Not Necessary for Lymphocyte Infiltration into GL261 Gliomas

To address the lymphocyte response to the glioma in CX3CR1 deficient mice, immunohistochemical analysis using several T lymphocyte markers was performed and the numbers of these cells were quantified in CX3CR1$^{+/-}$ and $^{-/-}$ mice. Figure 3-5 depicts a series of representative sections subjected to immunohistochemistry from the two groups of animals, while Table 3-2 summarizes the quantitative analysis of several sections from multiple animals. CD4^{+}, CD8^{+}, Foxp3^{+}, and Ly49G2^{+} cells were all present within GL261 tumors in both types of mice (Figure 3-5). CD4^{+}, CD8^{+}, and Ly49G2^{+} cells were all GFP negative and indicated that these tumor infiltrating lymphocyte populations do not express CX3CR1. The regulatory T cell (Treg) subpopulation of CD4^{+} T cells, identified by staining sections with the anti-Foxp3 antibody, comprised about half of the numbers of CD4^{+} cells. These data are consistent with two previous reports on the presence of the Treg population in the GL261 model[142,143]. While there were no significant differences in the numbers of the specific tumor infiltrated CD4^{+}, CD8^{+}, Foxp3^{+}, and Ly49G2^{+} cells between CX3CR1$^{+/-}$ and $^{-/-}$ glioma-bearing mice (Table 3-2), quantitative analysis of each of these cells indicated that tumors from CX3CR1$^{-/-}$ mice exhibited a tendency toward fewer CD4^{+}, CD8^{+}, Foxp3^{+}, and Ly49G2^{+} cells inside the tumors compared with numbers found in tumors from $^{+/-}$ mice. Nonetheless, CX3CR1 does not appear to be necessary for the recruitment of these lymphocyte subsets into GL261 gliomas.

Discussion

In this study we have shown that CX3CR1 deficiency resulted in a slightly shorter life span of tumor bearing mice with no significant differences in numbers of tumor infiltrated microglia and lymphocytes. These results favor a lack of effects of CX3CR1

signaling on antiglioma activity as well as in the intratumoral recruitment of microglia and lymphocytes. Previous studies done in CX3CR1 deficient mice have pointed out that CX3CL1 and CX3CR1 are important for migration of macrophages, microglia and lymphocytes *in vivo*. For example, CX3CR1-deficient mice show an aberrant accumulation of microglia at subretinal areas that might contribute to age-related macular degeneration[57,144]. In addition, a reduction in the lesion size and accumulation of immune cells during atherosclerosis were found in CX3CR1$^{-/-}$ mice. Moreover, CX3CR1 deficient animals showed impaired recruitment of NK cells in both EAE and in tumorigenesis[60,145]. Thus, it was somewhat surprising to find that CX3CR1 deficiency did not impact glioma infiltration of CX3CR1-expressing microglia *in vivo*. Equally unanticipated was the lack of morphological alterations in tumor-associated microglia in CX3CR1$^{-/-}$ mice given the results of Cardona et al. (2006)[58] in which microglia from CX3CR1 deficient animals display a greater extent of activation in various models of neurotoxicity.

While Log-rank statistical analysis indicated that the two survival curves were significantly different, suggesting that CX3CR1 deficiency may actually favor glioma growth, the difference in median survival time of 1 day indicates that CX3CR1 plays a small role in GL261 tumorigenesis. The four types of lymphocytes we examined here, though not significantly different between the two groups of mice, all showed slightly fewer numbers in $^{-/-}$ mice than in $^{+/-}$ mice. This raises the possibility that CX3CR1 deficiency might have a global impact on the host's immune system and its ability to suppress glioma growth.

One reasonable explanation for our results is that the function of CX3CR1 is masked by a highly immunosuppressive environment created by the GL261 glioma. TGFβ is one of the major immunosuppressive cytokines produced by tumors and contributes to immune tolerance of malignant cells[146]. Inhibition of TGFβ prevented the growth of EL-4 thymoma, B16.F10 melanoma[147], and SMA-560 glioma[147] *in vivo*. TGFβ is known to be expressed in human and rodent gliomas[148-150] and we have determined that TGFβ is present in GL261 gliomas *in vivo* (data not shown). Previous published results from our lab have shown that TGFβ up-regulates CX3CR1 expression in rat microglia although the signaling efficiency of this receptor was markedly inhibited after CX3CL1 stimulation[151]. Therefore, CX3CR1 signaling in tumor infiltrated microglia from wild type or $CX3CR1^{+/-}$ mice might be blocked by the high levels of TGFβ present within the GL261 tumors. If CX3CR1 function is inhibited under these conditions, a lack of a microglial cell phenotype in CX3CR1 deficient GL261 tumor bearing mice might be expected. Studies have shown that microglia under an immunosuppressive environment can still mediate phagocytosis and non-MHC restricted cytotoxicity but lack the ability to secrete IL-1β, IL-6, and TNF-α[152,153]. Given that CX3CR1 can regulate pro-inflammatory cytokine secretion as indicated by previous studies[58, 154], the relationship between CX3CR1 signaling blockade and cytokine secretion by glioma infiltrating microglia should be further studied. It is possible that microglia from CX3CR1 deficient animals secrete higher levels of factors that might facilitate glioma growth and invasiveness more directly, e.g. tumor growth factors and extracellular proteases.

Table 3-1. Numbers of tumor infiltrated GFP^+ and $CD11b^+$ cells

	GFP+	CD11b+
$CX3CR1^{+/-}$	544 ± 79 (6)	384 ± 28 (6)
$CX3CR1^{-/-}$	561± 54 (6)	396 ± 62 (6)
p value	0.42	0.5

Note: Shown are mean (± S.E.M.) numbers of tumor-infiltrated cells expressing either GFP or CD11b. Animal numbers for each group are indicated in the brackets.

Table 3-2. Numbers of tumor-infiltrated lymphocytes

	CD4	CD8	Foxp3	Ly49G2
$CX3CR1^{+/-}$	112 ± 13 (13)	72.2 ± 11.6 (11)	58.4 ± 5.5 (12)	26.5 ± 4.6 (10)
$CX3CR1^{-/-}$	102 ± 14 (11)	53.9 ± 8.8 (11)	48.3 ± 6.5 (12)	23.8 ± 4.9 (12)
p value	0.618	0.214	0.319	0.692

Note: Shown are mean (± S.E.M.) numbers of tumor-infiltrated lymphocytes. Animal numbers for each group are indicated in the brackets.

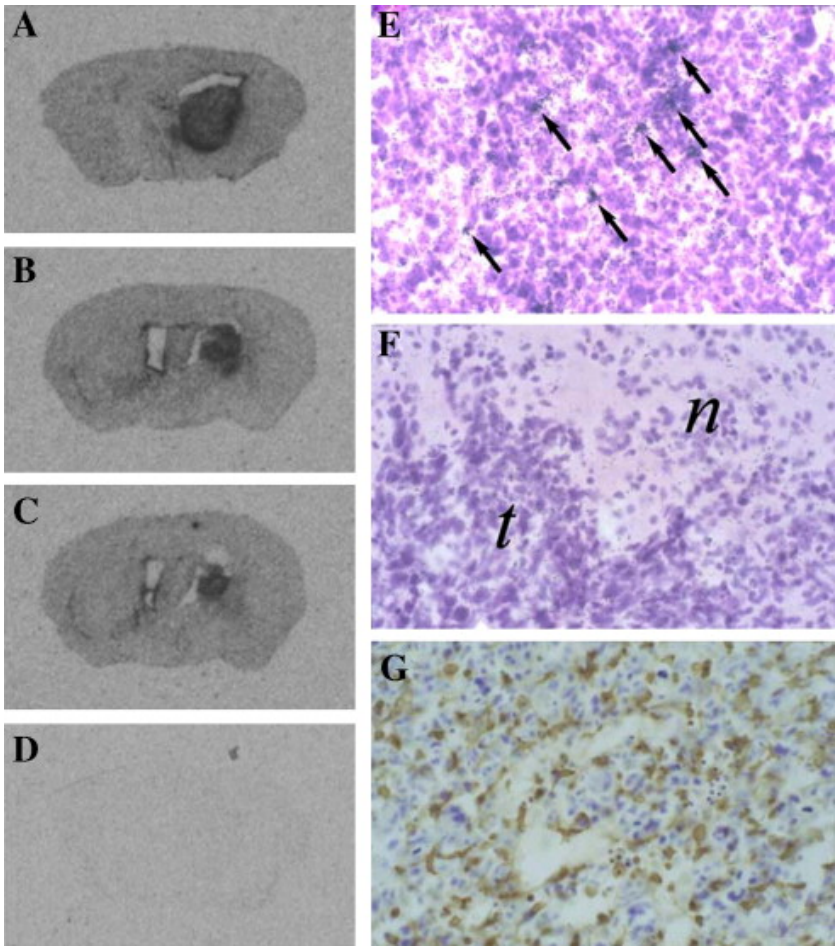

Figure 3-1. CX3CR1 was expressed in GL261 tumors. A–F) In situ hybridization (ISH) analysis of GL261 tumors using anti-sense (A–C, E) and sense (D, F) CX3CR1 riboprobes. Panels A–C are representative autoradiographs from three different tumor bearing mice, implanted with either 200,000 (A) or 100,000 (B,C) GL261 cells. Panels E and F depict representative fields from developed emulsion dipped slides. Arrows in panel E identify some of the specific hybridization signals. Normal (*n*) and tumor (*t*) tissues are indicated in the sense riboprobed section (panel F). G) A representative tumor section stained with anti-CD11b.

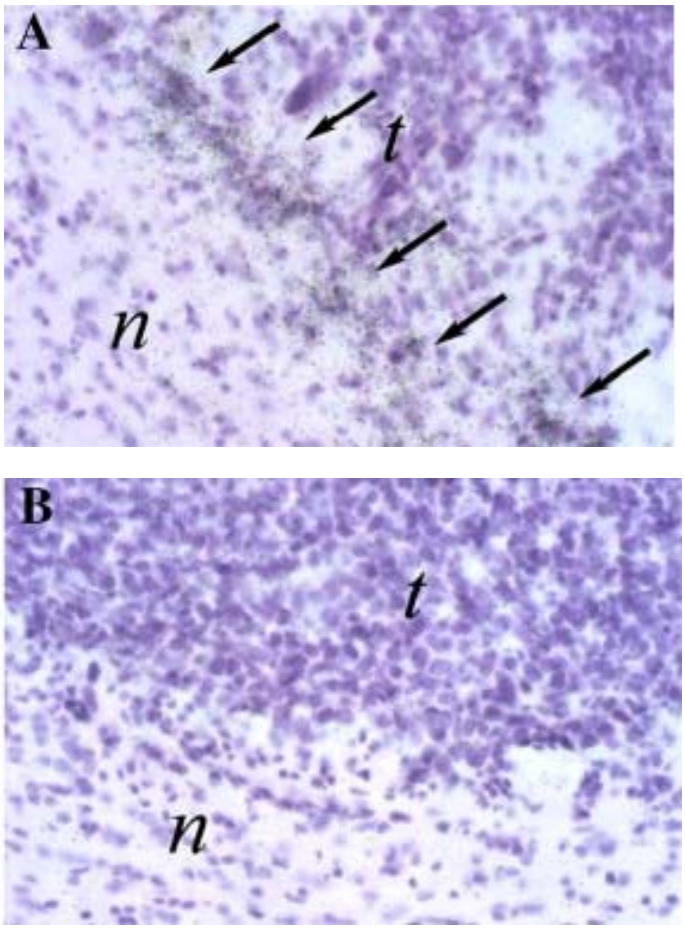

Figure 3-2. CX3CL1 was expressed in GL261 tumors. ISH analysis of GL261 tumors using anti-sense (A) and sense (B) CX3CL1/FKN riboprobes. Arrows show hybridization signals. Normal (*n*) and tumor (*t*) tissues are depicted in each figure panel.

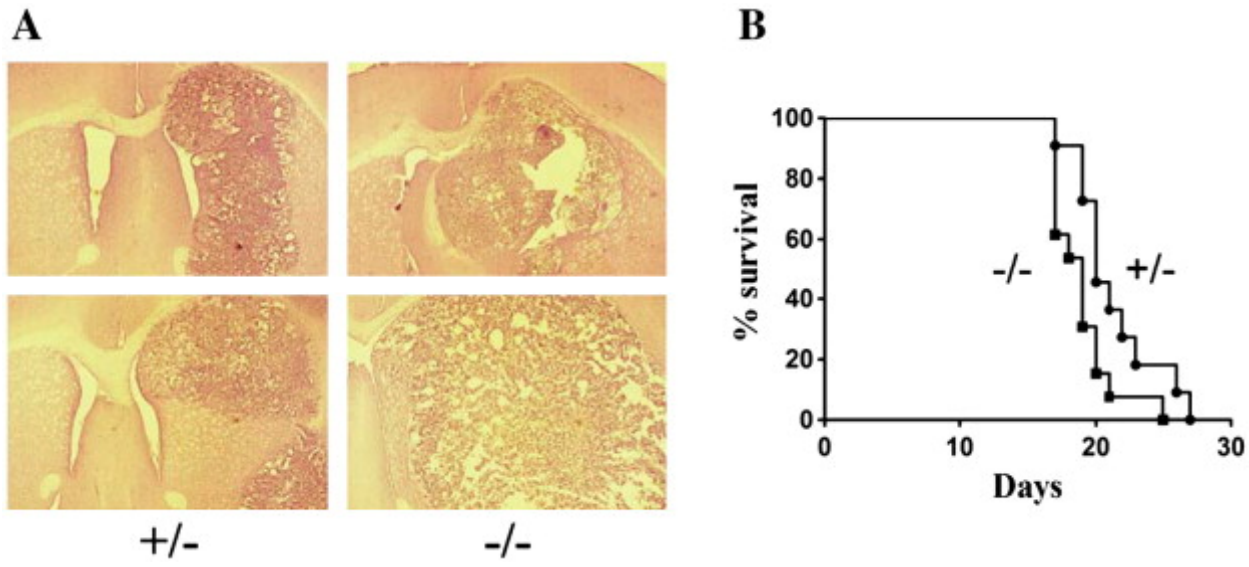

Figure 3-3. Tumorigenesis in CX3CR1$^{+/-}$ and $^{-/-}$ mice. A) Representative H&E stained sections from GL261 tumor bearing CX3CR1$^{+/-}$ and $^{-/-}$ animals. Two representative sections from at least eight different animals in each group are shown. B) Kaplan–Meier survival analysis of GL261 tumor bearing CX3CR1$^{+/-}$ and $^{-/-}$ animals. The median survival of the tumor bearing $^{+/-}$ (N = 11) and $^{-/-}$ (N = 13) mice were 20 and 19 days, respectively. Log-rank analysis determined that the two curves were different (p = 0.0332).

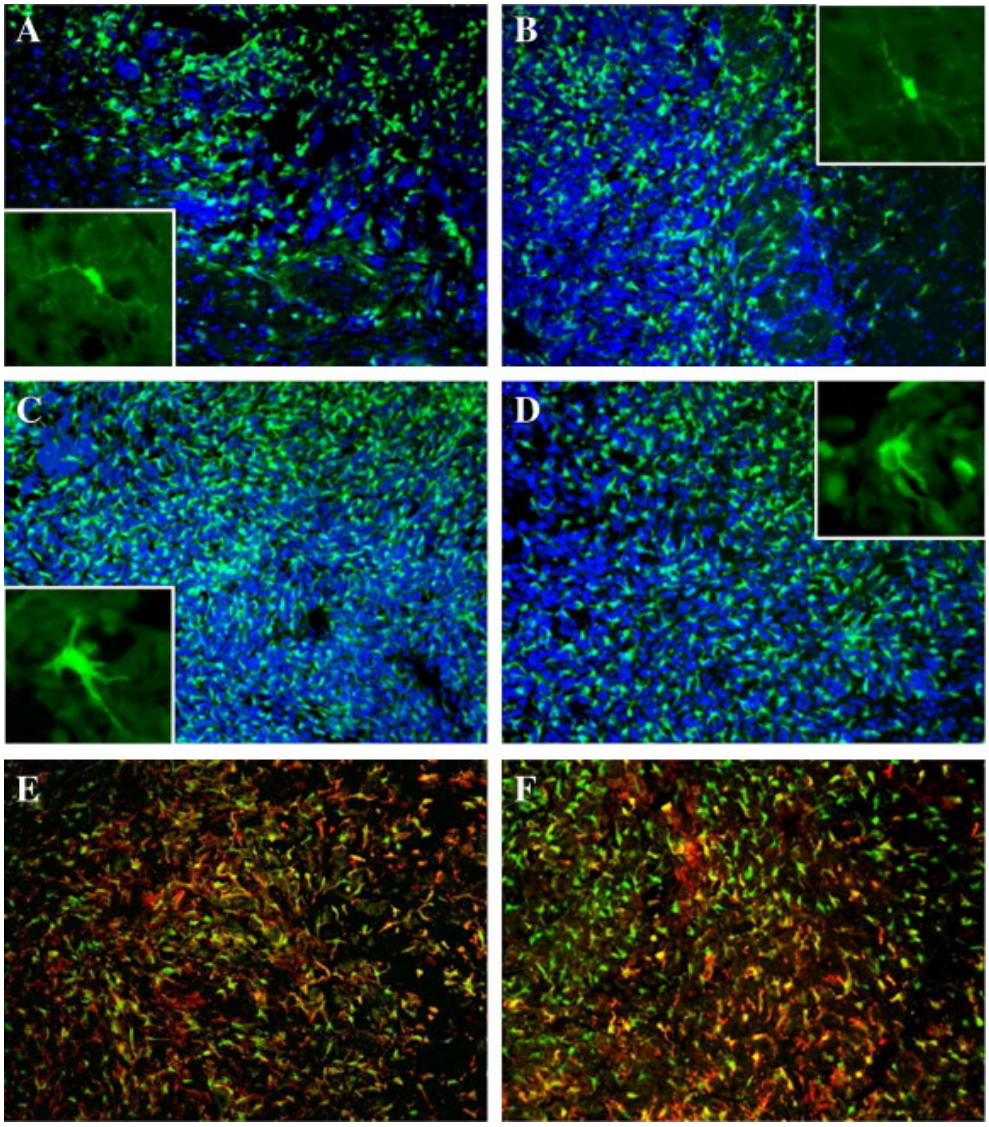

Figure 3-4. Infiltration of CX3CR1^{+}/Cd11b^{+} cells into GL261 gliomas. GFP-expressing cells at the perimeter of the tumor in $^{+/-}$ (A) and $^{-/-}$ (B) mice. Panels C and D show GFP-expressing cells inside the tumor in $^{+/-}$ and $^{-/-}$ mice, respectively. Insets of panels A–D show higher magnifications of GFP-expressing microglia in normal brain parenchyma adjacent to the tumor (panels A and B) and GFP-expressing cells within the tumors (panels C and D). Sections depicted in panels A–D were counterstained with DAPI and the final pictures are a result of the merged images. Panels E and F depict CD11b expression by intratumoral GFP-expressing cells in $^{+/-}$ (E) and $^{-/-}$ (F) mice.

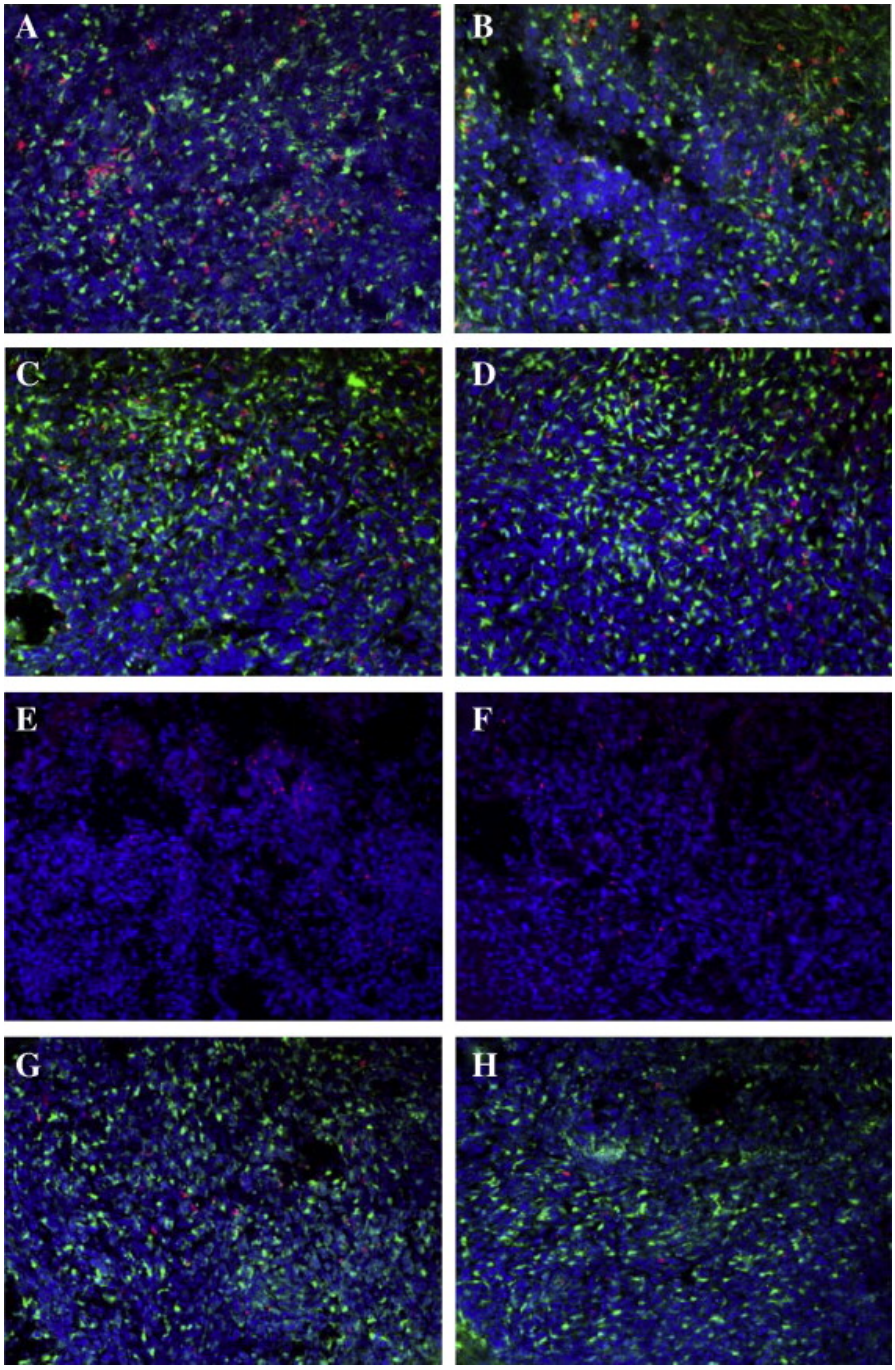

Figure 3-5. $CD4^+$, $CD8^+$, $Foxp3^+$, and $Ly49G2^+$ cells were present in GL261 tumors from $CX3CR1^{+/-}$ and −/− mice. Representative fluorescence micrographs depicting tumor infiltration of lymphocytes from +/− (A, C, E, G) and −/− (B,D, F, H) mice. A, B) $CD4^+$ cells; C, D) $CD8^+$ cells; E, F) $Foxp3^+$ cells; G, H) $Ly49G2^+$ cells. Lymphocyte markers are defined by red fluorescence. Sections were counterstained with DAPI.

CHAPTER 4
ROLE OF CXCR3 AND ITS LIGANDS IN GBM

Recently, CXCR3 and CXCL10 have been reported to be up-regulated in grade III and grade IV human glioma cells as compared to normal astrocytes. The activation of CXCR3 can increase DNA synthesis of these cells *in vitro*. While these *in vitro* results support a role for CXCR3 in malignant glioma, investigations of this receptor in GBM progression *in vivo* are absent and further study is necessary.

In this study, we investigated the role of CXCR3 in glioma progression using the GL261 murine model of malignant glioma[135,139,155]. CXCL9 and CXCL10 expression were determined in GL261 cells and GL261 tumors established in syngeneic C57BL/6 mice. CXCR3-deficient mice and a CXCR3 antagonist, NBI-74330, were utilized to address the role of this receptor in glioma progression. NBI-74330 is a small molecule selective CXCR3 antagonist[156,157] and has been shown to attenuate atherosclerotic plaque formation by blocking the migration of $CD4^+$ T cells and macrophages, as well as enhancing the immune suppression controlled by $Foxp3^+$ Treg cells[138]. We found that CXCR3 deficiency in the host and CXCR3 antagonism with NBI-74330 had different effects on GL261 glioma progression. However, NBI-74330 exerted an anti-tumor progression effect not dependent on host expression of CXCR3, supporting a role for this receptor directly on glioma cells. The glioma expression of CXCR3 was confirmed thru *in vitro* studies of the murine GL261 cells and then extended by characterization of several human glioma cells. Functional characterization of tumor-expressed revealed a role for CXCR3 in promoting glioma proliferation. Taken together, our results indicate that CXCR3 is involved in glioma progression and is a potential therapeutic target for glioma.

Results

Murine Glioma GL261 Cells Expressed CXCL10 *In Vitro* and GL261 Tumors Expressed CXCL9 and CXCL10 *In Vivo*

Murine GL261 glioma cells are known to express CXCL10 as previously established using microarray analysis[74] Thus, we utilized the GL261 murine model of malignant glioma to address the role of CXCR3 in glioma progression *in vitro* and *in vivo*. By using RT-PCR, we determined that CXCL10 mRNA was expressed by cultured GL261 cells (Figure 4-1A); CXCL9 mRNA was undetectable. Since the *in vivo* expression of CXCR3 system has not been reported, we implanted GL261 cells into wild type C57BL/6 mice, and the expression of CXCL9 and CXCL10 mRNAs were detected by in situ hybridization analysis (Figure 4-1C). These data indicate that CXCL9 and 10 are expressed in GL261 glioma and might be involved in GL261 glioma progression.

Reduced Numbers of Tumor-Infiltrated Ly49G2$^+$ NK and NKT Cells in GL261 Gliomas from CXCR3-Deficient Mice Was Associated with Decreased Animal Survival

To address the functional significance of host-expressed CXCR3 in glioma progression, we evaluated the effect of CXCR3 deficiency on GL261 tumor growth and tumor-bearing animal survival using CXCR3-deficient mice. Kaplan-Meier survival analysis was carried out by comparing animal survival rates of wild type and CXCR3-deficient mice implanted with GL261 cells. The results demonstrated that tumor-bearing CXCR3 deficient mice succumbed to tumor growth more rapidly than wild type mice (Figure 2A, $p<0.0001$). The median survival time of tumor-bearing CXCR3 deficient mice was significantly decreased to 18 days as compared with wild type mice (23 days).

To access the possibility that the augmented GL261 tumor growth in the CXCR3-deficient mice resulted from malfunction of CXCR3 mediated homing of lymphocytes and/or microglia into the gliomas, we investigated numbers of tumor-infiltrated lymphocytes ($CD4^+$, $CD8^+$, $Ly49G2^+$, $Foxp3^+$ cells) as well as tumor-infiltrated $CD11b^+$ microglia. The numbers of $CD4^+$, $CD8^+$ cells and microglia inside the tumor were not significantly different between wild type and CXCR3-deficient groups (Figure 4-2B). However, the number of $Ly49G2^+$ cells inside the tumors from CXCR3-deficient mice was significantly reduced when compared to cell numbers in tumor sections from the wild type group (KO: 7.7 cells ± 1.3 cells per high powered field, n=9; WT: 28.9 cells ± 6.9 cells per high powered field, n=7, p=0.0041)(Figure 4-2B). While there was a tendency for decreased numbers of $Foxp3^+$ cells in tumors from CXCR3-deficient mice, the difference did not reach statistical significance (Figure 4-2B). In situ hybridization analysis determined that CXCL9 and CXCL10 mRNAs were detected in tumors from CXCR3-deficient mice, indicating that CXCR3 deficiency did not affect CXCL9 and CXCL10 expression (Figure 4-2C).

CXCR3 Antagonism Suppressed GL261 Tumor Growth and Increased Animal Survival Independent of Host CXCR3 Expression

To determine if pharmacological antagonism of CXCR3 mimicked CXCR3 deficiency in promoting GL261 glioma growth, we evaluated the effect of a CXCR3 antagonist, NBI-74330, on GL261 glioma progression. Wild type and CXCR3-deficient mice implanted with GL261 cells were treated with either NBI-74330 or vehicle as a control. Kaplan-Meier survival analysis showed that this CXCR3 antagonist prolonged survival of glioma-bearing WT animals (Figure 4-3A, p=0.0212) and increased median survival days from 20 days (vehicle) to 24 days (NBI-74330). Moreover, in CXCR3-

deficient animals, CXCR3 antagonism overcame the effect of CXCR3-deficiency and increased the rate of animal survival (Figure 4-3B, p=0.0028). The median survival time for CXCR3 antagonist treated group was increased to 23 days from a median survival time for vehicle treated group of 17 days. Tumor-infiltrated cells in tumors from vehicle- and NBI-74330-treated wild type mice were also evaluated, and no significant differences in the numbers of tumor-infiltrated $CD4^+$, $CD8^+$, $Foxp3^+$, $Ly49G2^+$ cells or $CD11b^+$ microglia were found (Figure 4-4A). In addition, expression of CXCL9 and CXCL10 was unaltered by NBI-74330 treatment (Figure 4-4B).

CXCR3 and Its Ligands Were Expressed by Murine and Human Glioma Cells

The effect of CXCR3 antagonism on tumor-bearing animal survival, which was independent of host CXCR3 expression, lead to the hypothesis that NBI-74330 inhibits glioma growth by exerting its effect on GL261 cells. Thus, we elucidated the expression of CXCR3 in GL261 cells by FACS analysis. A small fraction (8.4 % ± 0.5 %) of GL261 cells were determined to express CXCR3 (Figure 4-5B). To extend the results in the murine model to human GBM, we examined the expression of CXCR3, and its ligands, in five commonly studied grade IV human glioma cell lines, namely the A172, T98G, U87, U118, and U138 cell lines. With RT-PCR, we determined that two human glioma cell lines, T98G and U87, expressed CXCL10 mRNA (Figure 4-5A). T98G showed the highest level of CXCL10 mRNA expression and U87 had a lower level of CXCL10. CXCL10 was undetectable in the other three human cell lines and all lines lacked both CXCL9 and CXCL11 mRNAs. In addition, CXCR3 was expressed on all of the human glioma cells, as assessed by FACS analysis (Figure 4-5B). The percentage of $CXCR3^+$ cells in the various lines ranged from approximately 3 % to 8 %.

It has been reported that human glioma cell lines, cultured as adherent cells in the presence of fetal calf serum, are phenotypically different from their matched primary human tumor-derived tumor stem cells[158]. In contrast, gliomaspheres (GS), derived from culturing cells under more defined conditions that include bFGF and EGF, have higher resemblance to primary human GBM[132] and exhibit a stem cell phenotype characterized by nestin and SOX2 expression (Figure 4-6). Thus, we evaluated the expression of CXCR3, its ligands (CXCL9/10/11) in a patient GBM tissue-derived gliomasphere, GBM L0, and compared it with GL261 and other human glioma cell lines cultured as gliomaspheres. CXCL10 was expressed by GL261-GS and three of the human gliomasphere lines, including T98G-, U87-, U118-GS (Figure 4-7A). In addition, CXCL11, while undetectable in cells grown in media containing serum, was expressed by GL261-, T98G-, U87-, and U118-GS (Figure 4-7A). CXCR3 expression in the various gliomaspheres was determined by FACS analysis. All gliomasphere cell lines as well as the GBM L0 expressed CXCR3, albeit at various levels (Figure 4-8). When compared with their matched serum-supplemented cell lines, the percentage of CXCR3-expressing cells significantly increased in GL261, A172, T98G, U87, U118, and U138 gliomaspheres (Table 4-1). Two CXCR3 isoforms (CXCR3-A and -B) have been identified in human[88,159] while only one form of CXCR3, most similar to the human A isoform, exists in the mouse genome. The presence of CXCR3 isoforms in human gliomaspheres was characterized by RT-PCR. CXCR3-A was expressed by all cell lines examined with A172-, T98G-, U87-GS expressing the highest levels of CXCR3-A (Figure 4-7B). CXCR3-B was detected in T98G-, U118-, and U138-GS cells (Figure 4-7B).

CXCL9 and CXCL10 Stimulated Growth of Murine and Human Gliomaspheres.

Because expression of CXCR3 was enhanced in gliomaspheres as compared to serum-supplemented cell lines and CXCR3 isoforms were found in some of these cells, we compared the effects of CXCR3 activation on the cell growth of GL261-, U87-, U118-, U138-GS, and GBM L0 cells. Both CXCL9 and CXCL10 stimulated growth of GL261- and U87-GS by day 6 of incubation as compared to control (Figure 4-9); an impact on proliferation was not evident at the earliest time point measured (3 days). The growth effect of these chemokines was sustained thru day 9 (Figure 4-9). Cell numbers of U118- and U138-GS continuously decreased thru day 9, and both CXCL9 and CXCL10 attenuated the cell loss of U138-GS by day 9 while no significant effect was observed in U118-GS cells (Figure 4-9). In GBM L0, CXCL9-treated group had significant higher cell numbers by day 9 when compared with control group (Figure 4-9). CXCL10-treated group, showed similar tendency as the CXCL9-treated group, did not reach statistic significance ($p=0.11$). Adherent T98G cells were able to form spheres when initially seeded into the serum free, defined growth factor conditions but did not survive subsequently in the presence of CXCL9, CXCL10 or EGF/bFGF. A172-GS cells also formed spheres but did not proliferate in the presence of the CXCR3 ligands or EGF/bFGF. Co-incubation of CXCL9 or CXCL10 with NBI-74330 blocked the response to either CXCL9 or CXCL10 stimulation in GL261- and U87-GS cells by day 6 and U138-GS cells by day 9 (Figure 4-10). A trend for NBI-74330 to attenuate the CXCL9 or CXCL10 responses in GBM L0 cells was observed (CXCL9: $p=0.14$; CXCL10: $P=0.16$). NBI-74330 did not affect cell growth in either the control or growth factor(s)-supplemented groups, suggesting that NBI-74330 selectively inhibited CXCR3 activation.

Discussion

The role of CXCR3 in a variety of cancers has gained considerable attention. However, the importance of CXCR3 and its ligands in tumorigenesis of human GBM is still unclear. Previous studies have shown that CXCR3 and CXCL10 are expressed by several human glioma cells lines[74], while CXCL10 mRNA is also detected in the murine GL261 glioma cell line[89]. In human glioma cells, CXCL10 stimulates DNA synthesis and cell proliferation *in vitro*[74]. These data suggest an involvement of CXCR3 in glioma formation and progression, although an *in vivo* relationship of this chemokine system and glioma progression has not yet been established. The results reported here indicate that human and murine glioma cell lines and tumors express components of the CXCR3 chemokine system. More important, the increased presence of $CXCR3^+$ cells in cultures enriched in glioma-initiating cells and suppression of the *in vitro* and *in vivo* growth of glioma by pharmacological antagonism of CXCR3 supports future consideration of this receptor as a target for GBM therapy.

To investigate the *in vivo* function of CXCR3 in glioma progression, we initially established that GL261 cells expressed CXCL10 *in vitro* and CXCL9 and CXCL10 in vivo. The intra-glioma expression of these chemokines prompted us to study the role of CXCR3 in glioma progression using two approaches, specifically CXCR3-deficeint mice and pharmacological antagonism. Glioma-bearing CXCR3-deficient mice had lower survival rate and significantly fewer numbers of NK and NKT cells inside the tumor when compared to WT mice. The absence of tumor-infiltrating NK and NKT cells is the likely reason for the enhanced tumor growth and shorter median survival time in the tumor-bearing CXCR3-deficient animals. The reduction of NK and NKT cells in CXCR3-deficient mice has also been documented in ocular herpes simplex virus type 1

infection[160] as well as pulmonary fibrosis[161]. However, it has been reported that CXCR3-deficiency results in an impaired homeostasis of NK and NKT cells, with unchallenged CXCR3-deficient mice having significantly reduced numbers of NK and NKT cells in lung, liver, and peripheral blood[161]. Thus, the reduction of NK and NKT cells in GL261 gliomas we observed is likely a result from a defect in NK and NKT cell homeostasis, and not from a specific alteration of CXCR3 mediated cell migration into the tumor. Altered migration of $Foxp3^+$ T regulatory (Treg) cells in CXCR3-deficient mice has also been reported. In an experimental autoimmune encephalomyelitis model of multiple sclerosis, Treg cells are decreased in number and dispersed in lesions from CXCR3-deficient mice[162]. These data, coupled with results showing an involvement of $CD4^+CD25^+$ regulatory T cells in suppression of anti-glioma immune responses in the GL261 model[143,162], suggested a Treg phenotype in the glioma bearing CXCR3-deficient animals might have been observed. While we found a tendency of $Foxp3^+$ Treg cell reduction in tumors from CXCR3-deficient mice, the difference was not statistically significant.

To circumvent the NK/NKT defect in the CXCR3-deficient mice, a pharmacological approach, using NBI-74330, was undertaken. The selectivity of NBI-74330 for CXCR3 has been determined in previous studies. NBI-74330 inhibits CXCR3-agonist binding and CXCR3-mediated PLC activation but has little or no effect on other chemokine and histamine receptors[163]. Our results are consistent with the pharmacological properties of this antagonist since NBI-74330 attenuated the responses of the gliomashepre cells to both CXCL9 and CXCL10 but had no effect on either control or EGF/bFGF supplemented groups. In contrast to the outcomes from CXCR3-deficient mice, NBI-

74330 enhanced the survival rate of tumor bearing wild type mice with no impact on microglia and lymphocyte(s) infiltration. Tumor bearing CXCR3-deficient mice also displayed prolonged survival with NBI-74330 treatment, a result that suggested a CXCR3 inhibitory effect directly on the tumor cells. Indeed, we found that GL261 cells express CXCR3 as do several human glioma cells. The lack of effect of CXCR3 antagonism on the numbers of tumor-infiltrating microglia and lymphocytes, cells known to express CXCR3, suggests that this receptor system is not the primary means by which these immune cells traffic into glioma. Given that multiple chemokine systems have been shown to mediate microglia and lymphocytes infiltration into the brain[164-167], the influx of these cells into the glioma is likely mediated by other pro-migratory systems.

The applicability of the murine GL261 model to human GBM was further validated with analysis of 5 different human GBM cells lines, A172, T98G, U87, U118, and U138. All of the human lines express CXCR3 protein but showed varying levels of expression of CXCL10. CXCL10 was only detectable in T98G and U87. The variation of CXCL10 expression in human glioma lines had been documented that the lack of constitutive NF-κB activity in A172 results in undetectable CXCL10 expression, even with IFNγ stimulation[168]. On the other hand, T98G has been shown to have constitutive NF-κB activity and IFNγ treatment enhances CXCL10 expression in these cells. In addition, we analyzed the expression patterns of CXCR3 and its ligands in GL261 and human GBM lines cultured in a serum free, stem cell-enriched condition. It has been suggested that phenotypes of these cells better recapitulate the cells in the glioma environment than cell lines cultured in the presence of serum[132]. Interestingly, we found that GL261 gliomaspheres and all of the human gliomaspheres had greater $CXCR3^+$ population

when compared to their matched serum-supplemented cultures; a patient GBM tissue-derived primary gliomasphere, GBM L0, also contained a CXCR3 population. The positive correlation of CXCR3 expression and glioma malignancy has been suggested in human gliomas[74], and GL261 gliomaspheres were confirmed to be more malignant than their matched adherent (AD) cell lines with serum supplement[89]. Moreover, the expression of CXCR3 ligands was also enhanced in some of the gliomasphere cell lines. For example, CXCL10 expression was detected in U118-GS but not in U118-AD cells. Furthermore, CXCL11 expression was detected in GL261, T98G, U87, and U118 gliomaspheres as well as the patient-derived GBM L0 cells, while this chemokine was undetectable in all of the adherent cells. Thus, the CXCR3 system may contribute to glioma malignancy.

Insights into the role of CXCR3 in glioma progression came from *in vitro* studies where we determined that both CXCL9 and CXCL10 were able to enhance GL261 and U87 gliomasphere cell growth. This result is consistent with the DNA synthesis promoting effect found in other adherent human glioma cell lines[74]. In addition, CXCL9 and CXCL10 decelerated the lost of cells in U138 gliomaspheres by day 9. The growth and/or survival effects of CXCR3 activation on human gliomaspheres are correlated with their expression pattern of CXCR3 mRNA isoforms. Two human CXCR3 isoforms, CXCR3-A and -B, have been identified[159,169]. Stimulation of CXCR3-A activates ERK and AKT pathway, enhancing cell proliferation[159], while CXCR3-B activation exerts proliferation inhibitory and angiostatic effects through p38[88]. In human myeloma cells, CXCL10 stimulation results in an anti-apoptotic effect on cell lines that have high CXCR3-A expression but not on cell lines with a predominance of CXCR3-B[77]. Our data,

consistent with previous studies, indicates that CXCR3 enhances cell growth in cell lines that express only CXCR3-A (U87-GS). In lines expressing both isoforms, the functional responses to CXCR3 agonists are more complex. The ratio of CXCR3-A and –B isoforms has been postulated to determine the outcome. For example, CXCR3 activation reduces the lost of cells in U138-GS cells (expresses higher level of CXCR3-A than CXCR3-B) but has little or no effect on U118-GS cells (only shows slight difference of expression between CXCR3-A and -B). . CXCL9 and CXCL10 stimulation also show tendency of increasing numbers of GBM L0 cells although it is not statistically significant. While GBM L0 also expresses CXCR3-A but not CXCR3-B, its mRNA level of CXCR3-A is relatively lower than other human gliomaspheres, which could potentially result in the difficulty of observing the effect of CXCL9 and CXCL10 on GBM L0 gliomaspheres.

In summary, components of the CXCR3 system are expressed by glioma cells *in vitro* and *in vivo*. The increased expression of CXCR3 in the more malignant population of gliomasphere cells, and the CXCR3 antagonist sensitive effects on the *in vitro* and in vivo growth of glioma, suggest that this chemokine system could be a unique target for human GBM therapy.

Table 4-1. % $CXCR3^+$ population in adherent cells and gliomaspheres

	GL261	A172	T98G	U87	U118	U138	GBM L0
AD	8.4±0.5	4.7±1.2	3.3±0.7	3.8±1.0	4.0±0.5	5.1±0.8	N/A
GS	14.6±2.7	12.4±1.4	11.5±2.7	7.8±1.4	8.7±1.2	10.0±1.7	5.6±0.6
p value	0.07	0.0013	0.02	0.03	0.01	0.04	N/A

Note: Results are shown in mean ± S.E.M. from at least three independent experiments. Adherent cells (AD); Gliomaspheres (GS)

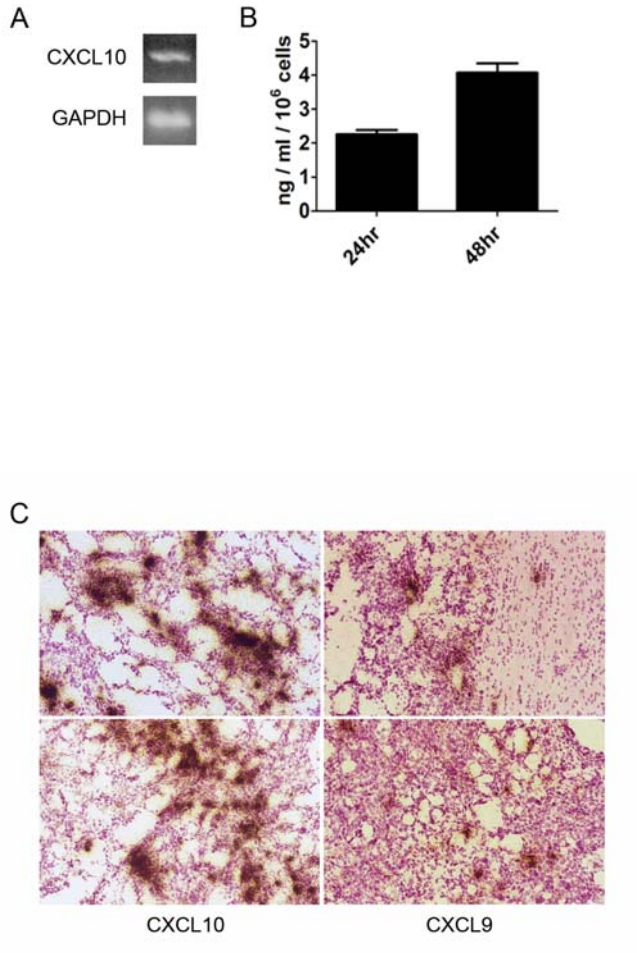

Figure 4-1. CXCL9 and CXCL10 were expressed in GL261 glioma cells and/or tumors. (A) RT–PCR identified CXCL10 mRNA in GL261 cells *in vitro*. GAPDH was used as a control. (B) CXCL10 ELISA showing CXCL10 protein secretion by GL261 cells *in vitro* at 24 and 48 h (C) CXCL9 and CXCL10 were expressed in intracranial GL261 tumors *in vivo* as determined by in situ hybridization analysis. Two representative sections, depicting expression of each chemokine, are shown.

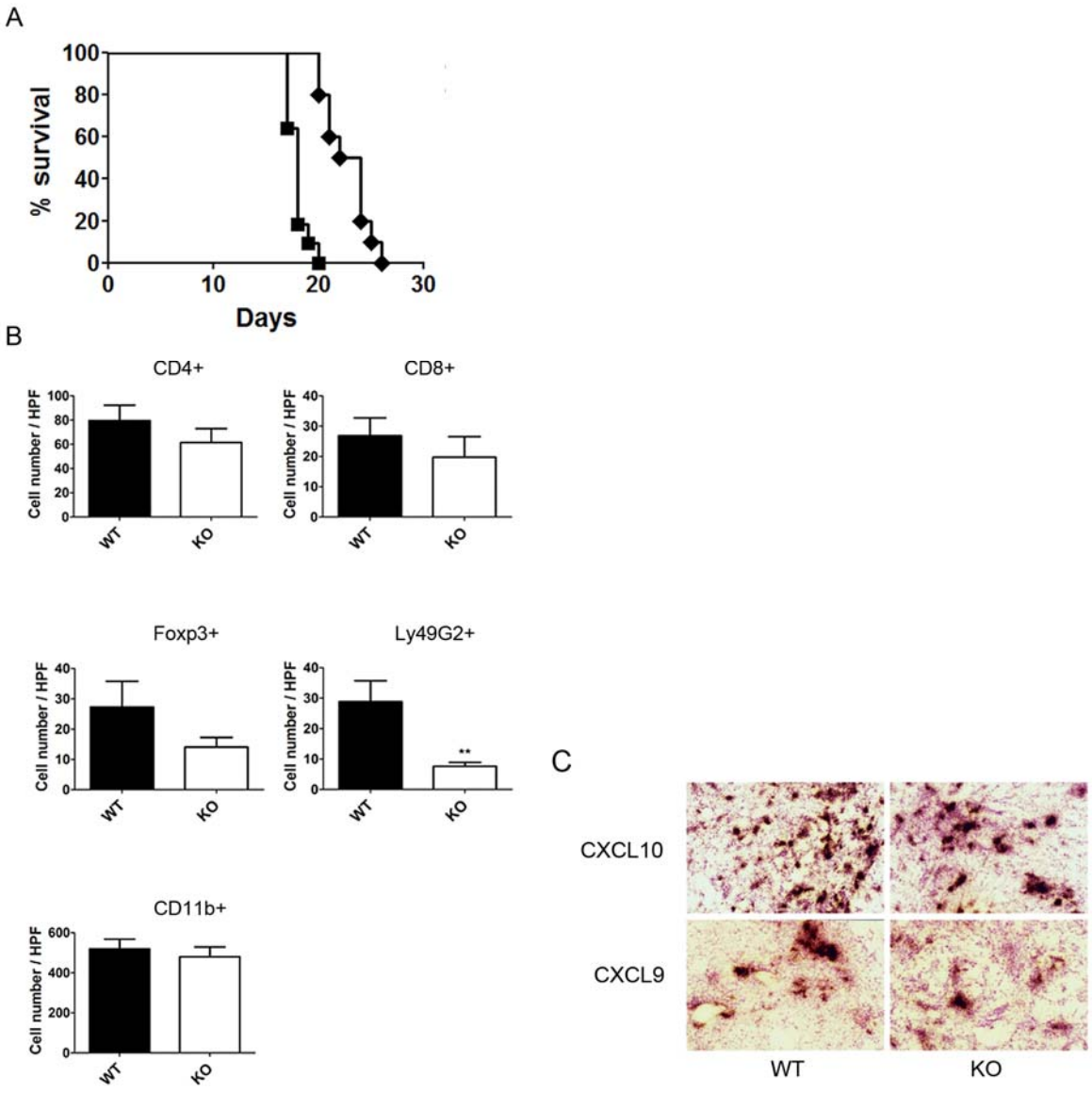

Figure 4-2. GL261 tumor-bearing CXCR3-deficient mice had decreased survival rates and tumor-infiltrated NK and NKT cells. (A) Kaplan–Meier survival analysis indicated that CXCR3-deficient mice (n = 10) have shorter life span than WT mice (n = 10, $P < 0.0001$). Filled squares: CXCR3-deficient mice; filled diamonds: WT mice. (B) Numbers of tumor-infiltrated $CD4^+$, $CD8^+$, $Foxp3^+$, $Ly49G2^+$ and $CD11b^+$ cells were evaluated by immunohistochemistry. Gliomas from CXCR3-deficient mice had a significant reduction of $Ly49G2^+$ (NK and NKT) cells in the tumor as compared with WT mice (**$P < 0.01$). WT: wild-type; KO: CXCR3-deficient mice. (C) Intratumoral expression of CXCL9 and CXCL10 mRNA was not altered by host CXCR3 deficiency. Shown are representative sections from WT and CXCR3-deficient glioma-bearing mice subjected to in situ hybridization analysis.

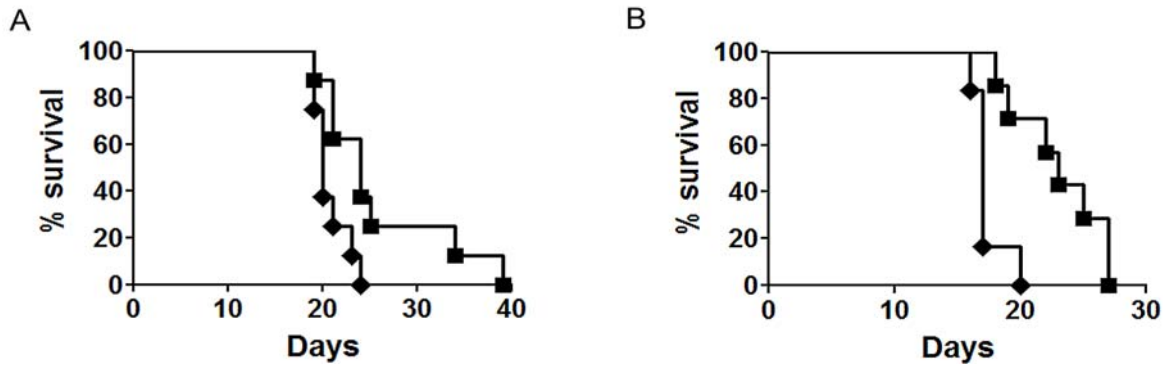

Figure 4-3. NBI-74330 suppressed tumor growth in both WT and CXCR3-deficient mice. (A) Kaplan–Meier survival analysis of glioma-bearing WT mice shows that NBI-74330 prolonged animal survival (n = 8), as compared with vehicle-treated mice (n = 8) (P = 0.0212). Filled squares: NBI-74330 treated; filled diamonds: vehicle treated. (B) Kaplan–Meier survival analysis shows that glioma-bearing CXCR3-deficient mice treated with NBI-74330 (n = 7) had a higher survival rate than vehicle-treated glioma-bearing CXCR3-deficient mice (n = 6, P = 0.0028). Filled squares: NBI-74330 treated; filled diamonds: vehicle treated.

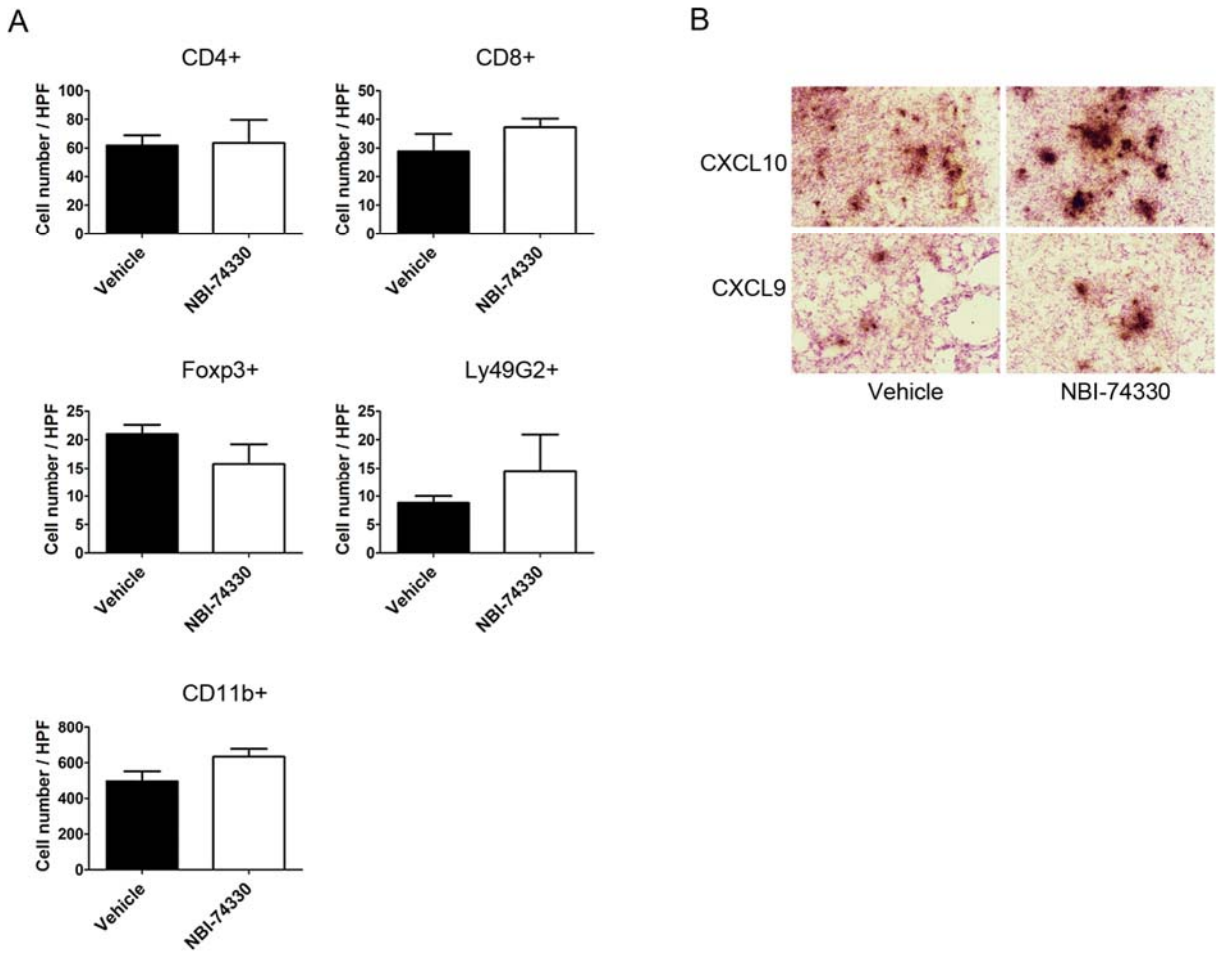

Figure 4-4. NBI-74330 did not alter numbers of tumor-infiltrated cells nor CXCL9 and CXCL10 expression. (A) Similar numbers of tumor-infiltrated lymphocytes and microglia in GL261 gliomas from NBI-74330- and vehicle-treated WT mice. Numbers of tumor-infiltrated $CD4^+$, $CD8^+$, $Foxp3^+$, $Ly49G2^+$ and $CD11b^+$ cells were not affected by NBI-74330 when compared with vehicle treatment. (B) *In vivo* expression of CXCL9 and CXCL10 was not altered by NBI-74330 treatment. Shown are representative sections from vehicle- and NBI-74330-treated glioma-bearing mice subjected to in situ hybridization analysis.

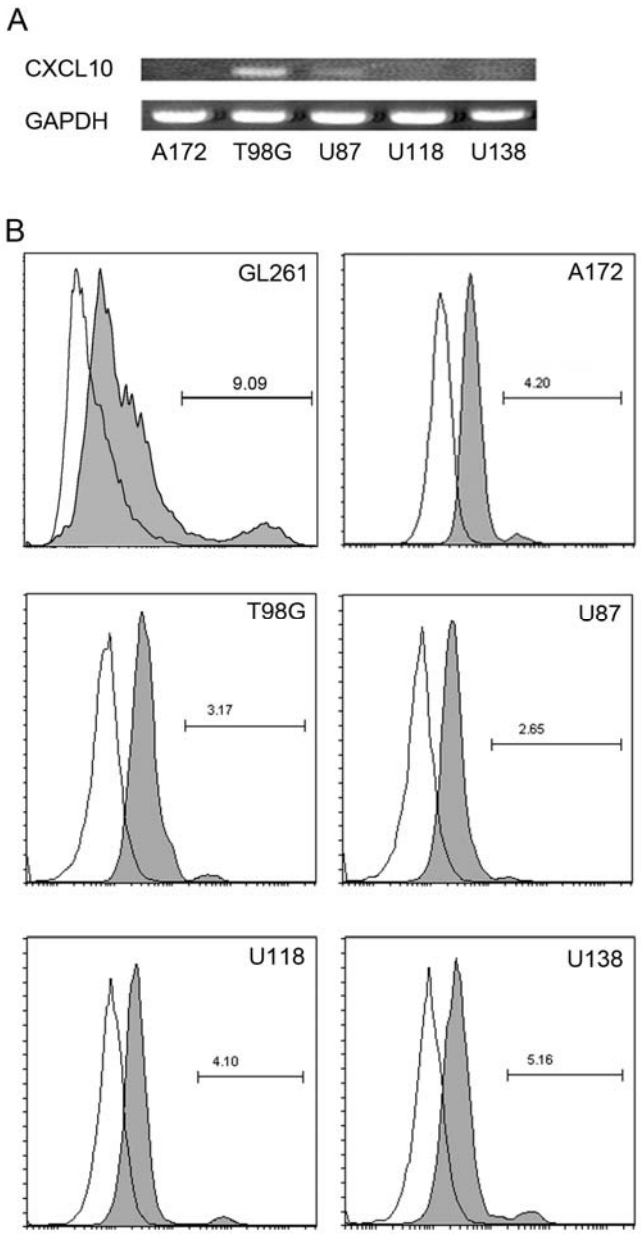

Figure 4-5. CXCR3 and CXCL10 expression in murine and human glioma cell lines cultured in serum-containing media. (A) RT–PCR identified CXCL10 mRNA in T98G and U87 cells *in vitro*. GAPDH was used as a control. (B) Representative histograms from fluorescence-activated cell sorting analysis showing CXCR3 expression by GL261, A172, T98G, U87, U118 and U138 cells *in vitro*. Gray filled area, anti-CXCR3-APC (mouse) or anti-CXCR3-PE (human) staining; blank area: isotype controls. GL261 had the highest CXCR3 expression level among all the glioma cell lines.

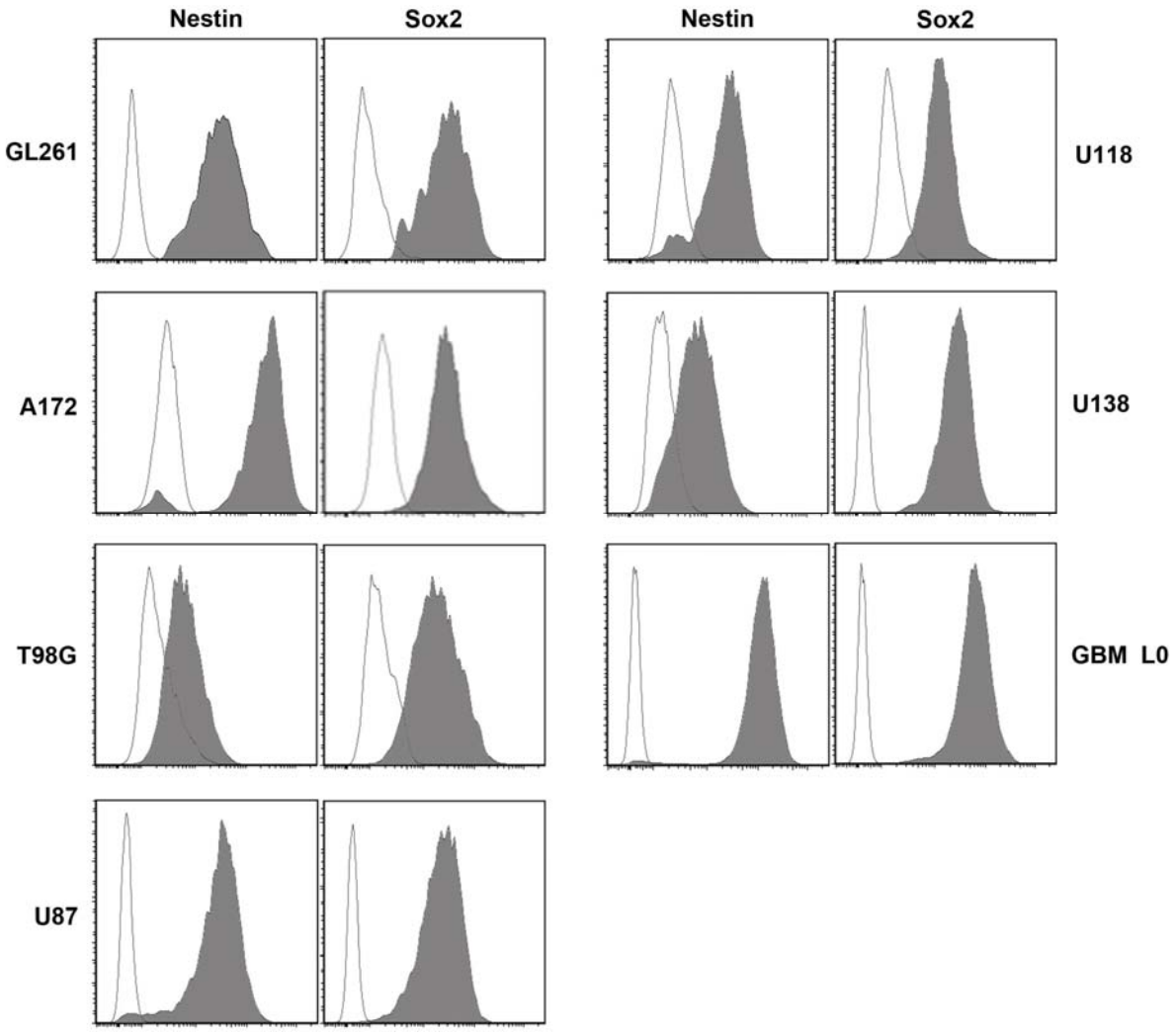

Figure 4-6. Gliomaspheres derived from culturing cells under more defined conditions that include bFGF and EGF, had higher resemblance to primary human GBM and exhibited a stem cell phenotype characterized by nestin and SOX2 expression.

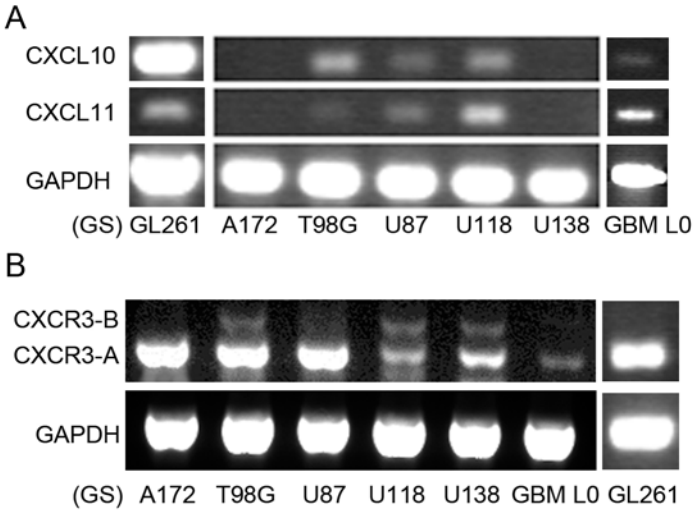

Figure 4-7. CXCL10, CXCL11 and CXCR3 expression in GS cells. (A) RT–PCR identified *in vitro* expression of CXCL10 mRNA in GL261-, T98G-, U87- and U118-GS cells and a patient GBM tissue-derived primary GS (GBM L0). In addition, CXCL11 is expressed by GL261-, T98G-, U87-, U118-GS and GBM L0 cells. GAPDH was used as a control. (B) RT–PCR analysis identified *in vitro* expression of CXCR3 mRNA isoforms in murine and human GS cells. CXCR3-A was detected in all cells with A172-, T98G- and U87-GS cells expressing the highest levels. CXCR3-B was detected in T98G-, U118- and U138-GS cells. One form of CXCR3 mRNA was detected in GL261-GS cells; only the equivalent of CXCR3-A exists in mouse.

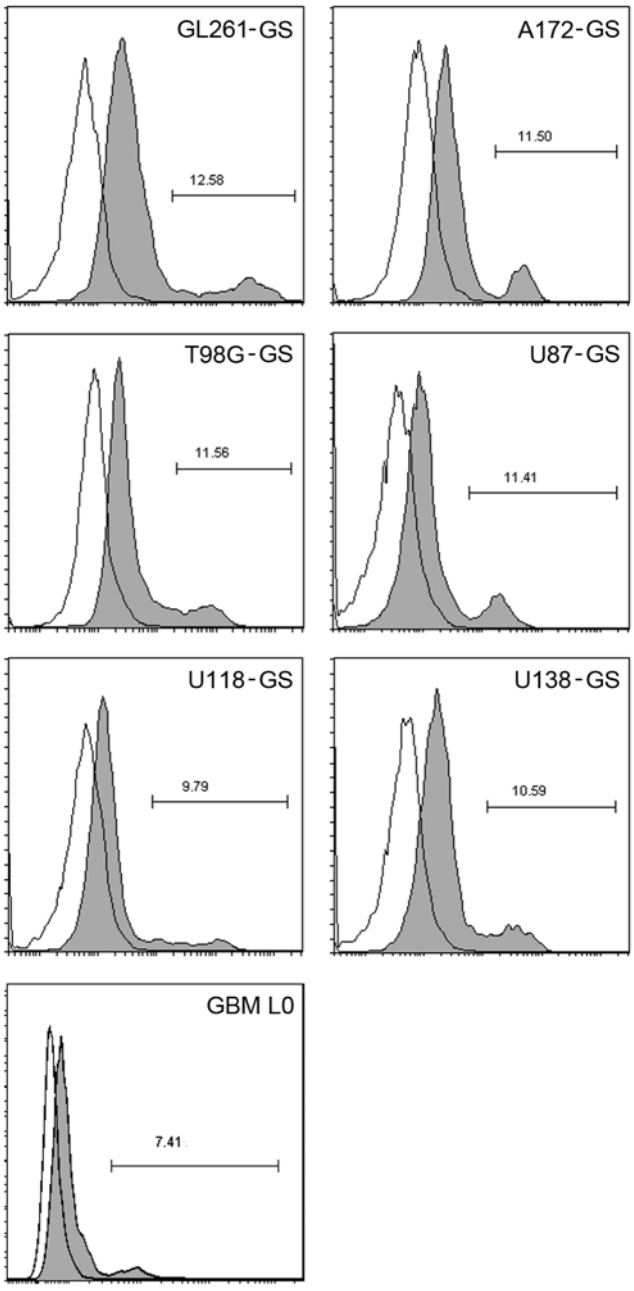

Figure 4-8. Representative histograms from fluorescence-activated cell sorting analysis showing CXCR3 expression by GL261-, A172-, T98G-, U87-, U118-, U138-GS and GBM L0 cells. Gray filled area: anti-CXCR3-APC (mouse) or anti-CXCR3-PE (human) staining. Blank area: isotype controls.

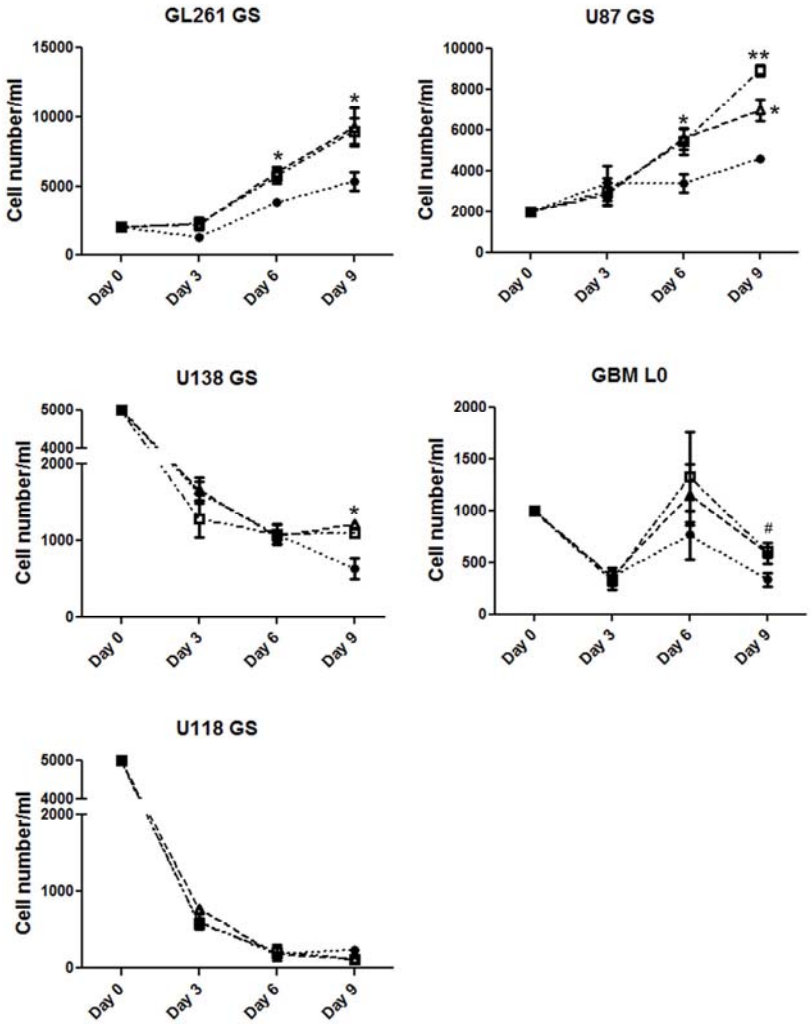

Figure 4-9. GL261- and U87-GS cells (2000 cells/ml) were incubated with 1 nM CXCL9 (open squares) or 1 nM CXCL10 (open triangles); U118-, U138-GS cells (5000 cells/ml) and GBM L0 (1000 cells/ml) were incubated with 10 nM CXCL9 (open squares) or 10 nM CXCL10 (open triangles). The control group was cultured in medium without chemokines or growth factors (filled circles). All conditions contained 0.1% dimethyl sulfoxide (NBI-74330 vehicle). Representative results of three individual experiments performed in triplicate are shown. CXCL9 and CXCL10 significantly enhanced GL261- and U87-GS growth at day 6 and 9 (*P< 0.05, **P < 0.01) and prevented U138-GS cell loss at day 9. CXCL9 stimulation significantly increased cell numbers of GBM L0 at day 9 (#P < 0.05); CXCL10-stimulated group was not statistically significant as compared with control.

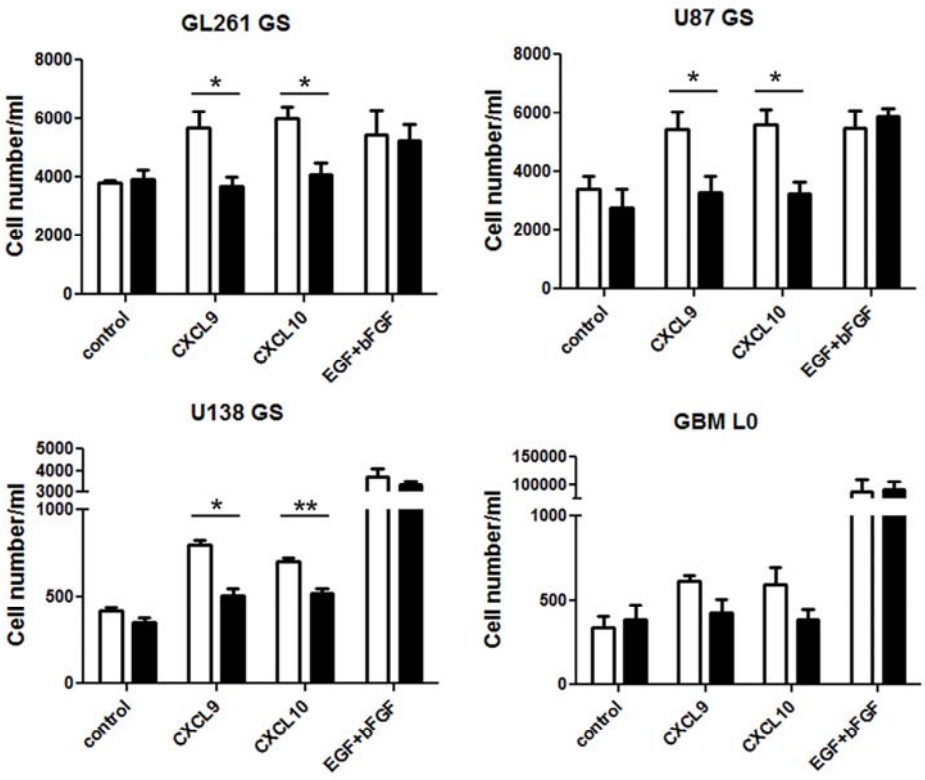

Figure 4-10. Effect of 1 μM NBI-74330 (black filled bars) and 0.1% dimethyl sulfoxide (open bars) on chemokine- and growth factor-stimulated GS growth. Representative results of three individual experiments performed in triplicate are shown. NBI-74330 attenuated response of GL261-, U87- and U138-GS to CXCL9 and CXCL10 but did not affect either control or growth factor-supplemented groups.

CHAPTER 5
CXCR4 AND CXCR7 IN GBM

As reported in chapter 4, we determined that several gliomasphere lines, including the primary GBM line 0 (GBM L0), expressed CXCL11 (Figure 4-7). This prompted our interest to further evaluate the function of CXCL11 in glioblastoma since it has been shown to be a versatile chemokine. As mentioned previously, CXCL11 is well known to be one of the ligands of CXCR3. In addition, CXCL11 has been demonstrated to interact with CCR3[170], CCR5[171] and CXCR7[107]. CCR3, CCR5, and CCR1 are three chemokine receptors that also share the same ligand, namely CCL5. Previous studies have shown that CXCL11 acted as an antagonist of CCR3[170] and CCR5[171], and inhibited CCL5-mediated cell migration[171]. CXCR7 binds CXCL11 and CXCL12 while CXCL12 also binds CXCR4. The activation of CXCR7 by CXCL11 exerts an inhibitory effect on CXCL12-CXCR4 mediated tumor cell transendothelial migration[172]. Thus, CXCL11 may have multiple functions through different chemokine receptor systems it interacts with.

It has been reported that CXCR7 was expressed by “differentiated” GBM cells and exhibited anti-apoptotic activity while CXCR4 was expressed in GBM stem-like cells[111]. However, with substantial evidence of interactions between CXCR4 and CXCR7 through CXCL11 and CXCL12, we hypothesized that the CXCR4-CXCR7 axis has multiple roles in GBM biology. In this study, we sought to determine the roles of CXCR4 and CXCR7 in GBM by using primary patient-derived GBM cells. The expression of CXCR4 and CXCR7 as well as CCR1, 3, 5 was determined in 6 primary GBM lines (GBM L0, L1, L2, L3, S3, S7). Heterogeneous expression of CXCR4 and CXCR7 was documented in GBM cells while all lines tested showed a small fraction of CCR3- and

CXCR3-expressing populations. GBM L0 and L1 were then chosen as two model lines, and we found substantial levels of CXCL11, CXCR4, and CXCR7 mRNA as well as intracellular CXCR4 and CXCR7 proteins in GBM L0 and L1 cells. When cells were isolated according to their CXCR4 and CXCR7 cell surface expression patterns, all subpopulation were able to form spheres. In addition, these subpopulations recapitulated the parental heterogeneous expression pattern of CXCR4 and CXCR7. Results from migration assay indicated that CXCL12 induced the migration of GBM L0 and L1 cells. Moreover, CXCL11 and CXCL12 enhanced GBM L0 and L1 cell growth but did not promote primary sphere formation. Taken together, the data indicate that CXCL11 and CXCL12 promote GBM progression. The results suggest that these chemokine systems should be considered as therapeutic targets for future drug development in GBM therapy.

Results

Primary GBM Cell Lines Had a Small Fraction of CCR3- and CXCR3-Expressing Cells

To investigate the functional significance of several chemokine receptors in GBM, we studied 6 GBM patient-derived primary cell lines, namely GBM L0, L1, L2, L3, S3, S7. By FACS analysis, we determined that all lines contained a small percentage of $CCR3^+$ cells (Figure 5-1). In addition, there were also a small fraction of $CXCR3^+$ cells in all of the GBM lines (Figure 5-1). Co-staining with CCR3 and CXCR3 specific antibodies revealed that amongst CCR3- and CXCR3-expressing cells, around 50% of them were $CCR3^+CXCR3^+$ (Figure 5-1), i.e. double positive for both receptors. Table 5-1 summarizes the percentage of CCR3- and CXCR3-expressing cell populations in GBM

cell lines. CCR1 and CCR5 were undetectable in all of the GBM cell lines (data not shown).

Primary GBM Cell Lines Showed Differential Expression of CXCR4 and CXCR7 on the Cell Surface

To determine the expression of CXCR4 and CXCR7 in the GBM cell lines, we utilized FACS analysis with CXCR4- and CXCR7-specific antibodies and found that all GBM cell lines expressed CXCR4 and CXCR7 on their cell surface (Figure 5-2). Interestingly, GBM cells showed different expression patterns of relative levels of CXCR4 and CXCR7 within each individual line. GBM L0, L2, and L3 had a relatively high level of CXCR4 and a low level of CXCR7 while GBM L1, S3, and S7 exhibited low levels of CXCR4 and high levels of CXCR7 (Figure 5-2; Table 5-1). Each cell line consisted of four subpopulations: $CXCR4^{-}CXCR7^{-}$; $CXCR4^{+}CXCR7^{-}$; $CXCR4^{+}CXCR7^{+}$; $CXCR4^{-}CXCR7^{+}$ (Figure 5-2). Therefore, the expression patterns of CXCR4 and CXCR7 are heterogeneous within and amongst several human GBMs. For the following experiments, we used GBM L0 cells as a representative model GBM line for $CXCR4^{high}$-$CXCR7^{low}$ and the GBM L1 line as a model for $CXCR4^{low}$-$CXCR7^{high}$ GBMs.

CXCL11, CXCR4, CXCR7, but Not CXCL12 Were Expressed by GBM L0 and L1 Cells Independent of Their Surface CXCR4-CXCR7 Heterogeneity

To determine the expression of chemokines CCL5, CXCL11, and CXCL12 in GBM L0 and L1, RT-PCR was performed and demonstrated that CCL5 and CXCL11 were abundantly expressed by both cell lines while CXCL12 was undetectable (Figure 5-3A). RT-PCR analysis showed that CXCR4 and CXCR7 mRNAs were present in the GBM L0 and L1 lines (Figure 5-3A), and the amount of mRNAs for these receptors did not correlate with the surface receptor levels of CXCR4 and CXCR7. To further address this phenomenon, we examined the intracellular levels of CXCR4 and CXCR7 proteins in

GBM L0 and L1 cells. FACS analysis of permeabilized cells determined that CXCR4 and CXCR7 were substantially expressed in the cytoplasm of most L0 and L1 cells (Figure 5-3B).

GBM L0 and L1 Subpopulations Formed Spheres and Retained the Parental Surface CXCR4-CXCR7 Heterogeneity

To test the hypotheses that 1) different $CXCR4^+$ and/or $CXCR7^+$ subpopulations within L0 and L1 are distinct from each other or 2) the differential expression of surface CXCR4-CXCR7 are homeostatic and reversible, we isolated $CXCR4^+CXCR7^-$ and $CXCR4^-CXCR7^-$ populations from L0 cells as well as $CXCR4^-CXCR7^+$ and $CXCR4^-CXCR7^-$ populations from L1 cells (Figure 5-4A) and compared them with unsorted cells after further culture for 7 days. All the subpopulations isolated from L0 and L1 were able to form spheres (Figure 5-4C) and repopulate the four different subtypes of surface CXCR4-CXCR7 after 7 days (Figure 5-4B). In addition, cells from GBM L0 retained the $CXCR4^{high}$-$CXCR7^{low}$ receptor profile while cells from GBM L1 restored the $CXCR4^{low}$-$CXCR7^{high}$ expression pattern (Figure 5-4B). The progeny of $CXCR4^+CXCR7^-$ GBM L0 cells had more $CXCR4^+$ cells than cells from $CXCR4^-CXCR7^-$ and unsorted populations. There were also a higher percentage of $CXCR7^+$ cells in cells from the L1 $CXCR4^-CXCR7^+$ cells when compared with double negative and unsorted populations.

CXCL12 Induced GBM L0 and L1 Cell Migration

CXCR4 and CXCR7 have been reported to regulate cell migration in a variety of cell types[112,172,173]. To investigate the effects of CXCR4 and CXCR7 on GBM L0 and L1 cell migration, we measured the number of GBM cells that migrated through the membrane of a standard Boyden chamber assay under the stimulation of either 10 nM CXCL11 or CXCL12. CXCL12 (10 nM) significantly induced cell migration of both GBM

L0 and L1 cells when compared to the non-treated controls (Figure 5-5). In contrast, CXCL11 treated groups in L0 and L1 showed a slight increase in the numbers of migrated cells but it was not statistically significant (Figure 5-5).

CXCL11 and CXCL12 Promoted GBM Cell Growth but Had No Effect on Primary Sphere Formation *In Vitro*

Next, we evaluated the effects of CXCL11 and CXCL12 on GBM cell growth and sphere forming capacity. GBM L0 or L1 cells (2000) were seeded in 96 well plates and incubated with either 10 nM CXCL11 or CXCL12. Cells without either of the two chemokines served as the control. The numbers of primary spheres were counted after 10 days and neither CXCL11 nor CXCL12 impacted primary sphere formation of GBM L0 and L1 cells (Figure 5-6A). In contrast, CXCL11 and CXCL12 significantly enhanced cell growth of GBM L0 and L1 cells (Figure 5-6B). In GBM L0, the response to CXCL11 was greater than the response to CXCL12 in terms of cell growth while the effect of CXCL11 and CXCL12 were comparable in GBM L1 cells (Figure 5-6B).

Discussion

As the role of CXCR4 in a variety of cancers has been well studied, the recently reported receptor of CXCL12, namely CXCR7, has started to gain considerable attention. However, the importance of CXCR4-CXCR7 axis in tumorigenesis of human GBM is still unclear. A previous study has shown that CXCR4 is upregulated in stem-like GBM cells while CXCR7 is elevated in differentiated GBM cells[111]. The proposed function of CXCR7 is to prevent GBM cells from apoptosis, which may contribute to the resistance to therapy. However, it has also been suggested that CXCR7 could impact CXCR4-dependent by depleting extracellular CXCL12[108,112]. In addition, CXCR7 dimerizes with CXCR4 and impairs CXCR4-regulated $G_{\alpha i}$ activation[174]. Therefore, the

CXCR4-CXCR7 axis could be involved in multiple phenotypes of GBM biology. The results reported here indicate that human primary GBM lines express CXCR4 and CXCR7 in a heterogeneous manner on the cell surface while keeping intracellular levels of CXCR4 and CXCR7 constant. In addition, the cell surface expression of CXCR4 and CXCR7 are homeostatic. More important, CXCL12 induces cell migration of GBM L0 and L1 as CXCL11 and CXCL12 enhances L0 and L1 cell growth. Taken together, these data support further investigation of CXCR4-CXCR7 axis as a target for GBM therapy.

In the primary GBM lines we examined, all have small fractions of CCR3- and/or CXCR3-expressing cells. Moreover, RT-PCR showed that CCL5, the ligand of CCR3, and CXCL11, which binds both CCR3 and CXCR3, were expressed by GBM L0 and L1 cells. Since CXCL11 has been shown to be an antagonist of CCR3[170], our data suggest that CCR3-CXCR3 axis might play important role in GBM biology.

Next, when analyzed with extracellular and intracellular FACS, the differential expression of CXCR4 and CXCR7 on the cell surface of L0 and L1 was documented and an abundant level of intracellular CXCR4 and CXCR7 proteins were observed. The expression of CXCL11 and CXCR7 by GBM cells indicates that CXCL11 exerts its effects on tumor cells in an autocrine manner. Although CXCL12 was undetectable in GBM cells by RT-PCR, it is known that CXCL12 is expressed by vascular endothelial cells in human GBM tissues[100] Therefore, CXCL12 secreted by non-tumor cells is the likely source of this chemokine.

When GBM cells were isolated according to the cell surface heterogeneity of CXCR4 and CXCR7, all sub-populations of cells were able to restore the original

CXCR4-CXCR7 cell surface expression pattern. These results suggest that CXCR4 and CXCR7 on the cell membrane are not markers of distinct cell subpopulations. Instead, the differential CXCR4-CXCR7 expression on the cell membrane might be an indicator of cell status when cells respond to environmental stimuli. Indeed, the reversible state of tumor cells has been documented. In human melanoma, cell subtypes defined by 22 different markers are all capable of recapitulating the tumor heterogeneity[37]. Other studies suggest that several genes in melanoma are reversibly turned on and off, and this phenomenon is related to cell function[175,176]. Environmental changes, such as hypoxia and differentiation status, could alter the level of CXCR4 and CXCR7 in different models[111,177-179], which also suggests the transitional expression of these two chemokine receptors in a manner related to cell function. Interestingly, the primary human GBM cell lines we studied can be classified by their differential CXCR4-CXCR7 level on the cell surface, namely $CXCR4^{high}$-$CXCR7^{low}$ (GBM L0, L2, L3) and $CXCR4^{low}$-$CXCR7^{high}$ (GBM L1, S3, S7). An important question to understand is does the differential expression of CXCR4-CXCR7 reflect corresponding responses and functional significance of CXCL11 and CXCL12 in GBM? Further elucidation of CXCR4 and CXCR7 in GBM will help us in future development of novel therapies.

To investigate the effects of CXCL11 and CXCL12 on GBM cells, GBM L0 and L1 cells were incubated with either CXCL11 or CXCL12 (each at 10 nM), and their effects on cell migration and growth were investigated. Here we determined that regardless of the surface CXCR4-CXCR7 heterogeneity between GBM L0 and L1 lines, only CXCL12 significantly induced tumor cell migration. On the other hand, CXCL11 treated groups exhibited slight, but insignificant migratory responses. Our results are consistent with

other previously published studies[180,181]. The migration effect is more likely being mediated by CXCR4 instead of CXCR7 since CXCL11 is ineffective in cell migration as we and other groups have shown[113]. However, recent studies revealed that CXCR7 is as important as CXCR4 in regulating cell migration. One hypothesis is that CXCR7 removes CXCL12 from the surrounding cellular environment to create the concentration gradient so that cells can migrate via CXCR4[112]. Other studies indicate that CXCR7 controls the CXCR4-mediated migration by sustaining CXCR4 protein level[173], dimerizing with CXCR4 on the cell surface to alter the intracellular G-protein coupling[174], and potentially interacting with CXCR4 by β-arrestin2 recruitment[172]. Since we showed here that CXCL12 could induce the migration of GBM cells, it is critical to investigate the mechanism and functional outcomes that underlie the interaction of CXCR4 and CXCR7 in GBM.

With the exception of cell migration, our data suggests that both CXCL11 and CXCL12 promote cell growth of GBM L0 and L1 cells. In gliomas, CXCR4 has been well studied with respect of its cell growth enhancement capacity[181,182]. However, these studies did not examine the expression of CXCR7 in gliomas, which may have lead to an oversimplified conclusion. Indeed, our results indicate that not only CXCL12, but also CXCL11 is able to increase GBM cell growth *in vitro*, which suggests a possible involvement of CXCR7 in cell growth. Although CXCL11 can bind to CXCR3, the growth effect of CXCL11 is more likely to be mediated by CXCR7 since we have shown that neither CXCL9 nor CXCL10 promote GBM L0 growth (Figure 4-9). To further address this question, we will use the CXCR4 specific antagonist (AMD3100) and CXCR7

specific inhibitor (CCX733) to establish the functional significance of either of these receptors.

A previous publication suggests that CXCR4 is highly associated with the glioma stem-like cells while CXCR7 is found on the more abundant population of GBM cells that display a differentiated phenotype[111]. The proposed functions of CXCR7 involve protection of the predominant population of glioma cells from apoptosis. However, since GBM L1 cells show a relatively high level of CXCR7, when cultured under conditions favoring stem cells, this model seems oversimplified. The functions of CXCR4 and CXCR7 in GBM stem-like cells are still unclear. CXCR4 is known to regulate stem cell mobilization[183,184] as well as maintain the stem cell pool through cell cycle regulation[185]. However, the importance of CXCR7 alone and with CXCR4 in cancer stem-like cells is under studied. Given the accumulated evidence of CXCR7 involvement in CXCR4-mediated activities, it would be important to examine the effect of CXCR7 on cancer stem-like cell biology in concert with CXCR4. Here we found that CXCL11 and CXCL12 did not increase primary sphere formation in GBM L0 and L1 cells. In this experiment cells were divided into the various conditions and, as such, would be expected to have the same frequency of stem-like cells since they were derived from one culture flask. Since CXCL11 and CXCL12 promoted GBM cell growth, which may have included symmetric and asymmetric cell division of stem-like cells, alterations in the stem-like cell frequencies, by CXCL11 and CXCL12 stimulation, might not be reflected in the primary sphere formation assay. Therefore, the secondary sphere formation assay, which measures the stem cell frequency after chemokine treatments, will more likely inform us if either CXCL11 or CXCL12 promotes GBM stem-like cell renewal.

Table 5-1. % $CCR3^+$, $CXCR3^+$, $CXCR4^+$, and $CXCR7^+$ populations in primary GBM cell lines

	GBM L0	GBM L2	GBM L3	GBM L1	GBM S3	GBM S7
$CCR3^+$	7.4±2.8	5.6±1.5	3.2±0.6	13±6.6	9.1±3.1	7.7±1.8
$CXCR3^+$	3.9±1.4	2±0.3	5±0.6	8.9±3.3	9.4±3.3	8±1.6
$CXCR4^+$	43.9±9.2	42.2±3.7	37.8±11	7±1.5	8.4±2.3	12.2±5.3
$CXCR7^+$	15±2.3	14.2±5.9	6.1±3.2	45.8±7.7	18±2.5	23.2±7.3

Note: Results are shown in mean ± S.E.M. from at least three independent experiments.

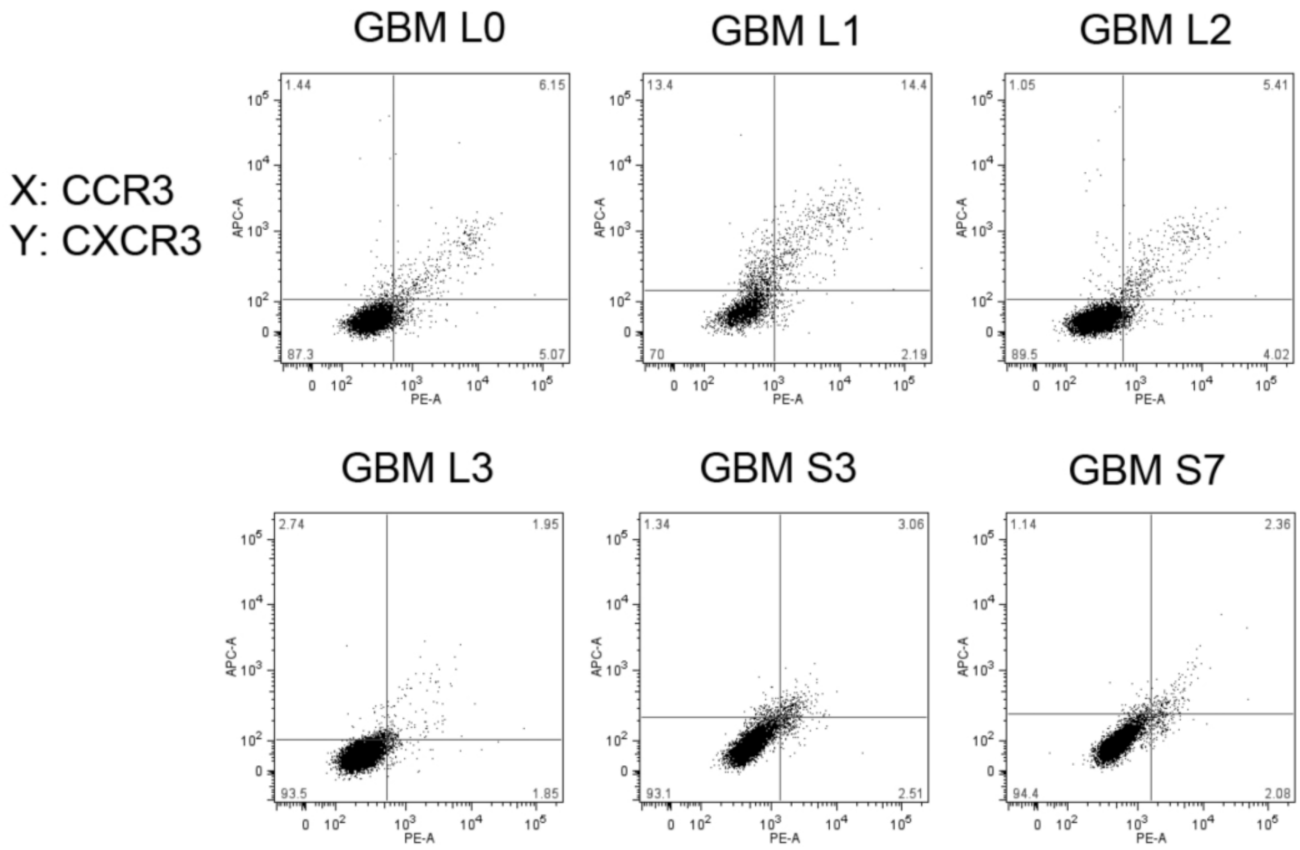

Figure 5-1. CCR3 and CXCR3 expression in primary human GBM cell lines. Representative histograms from fluorescence-activated cell sorting analysis showing the expression of CCR3 and CXCR3 by all GBM cell lines. A fraction of $CCR3^{+}CXCR3^{+}$ cells were present in all GBM cell lines.

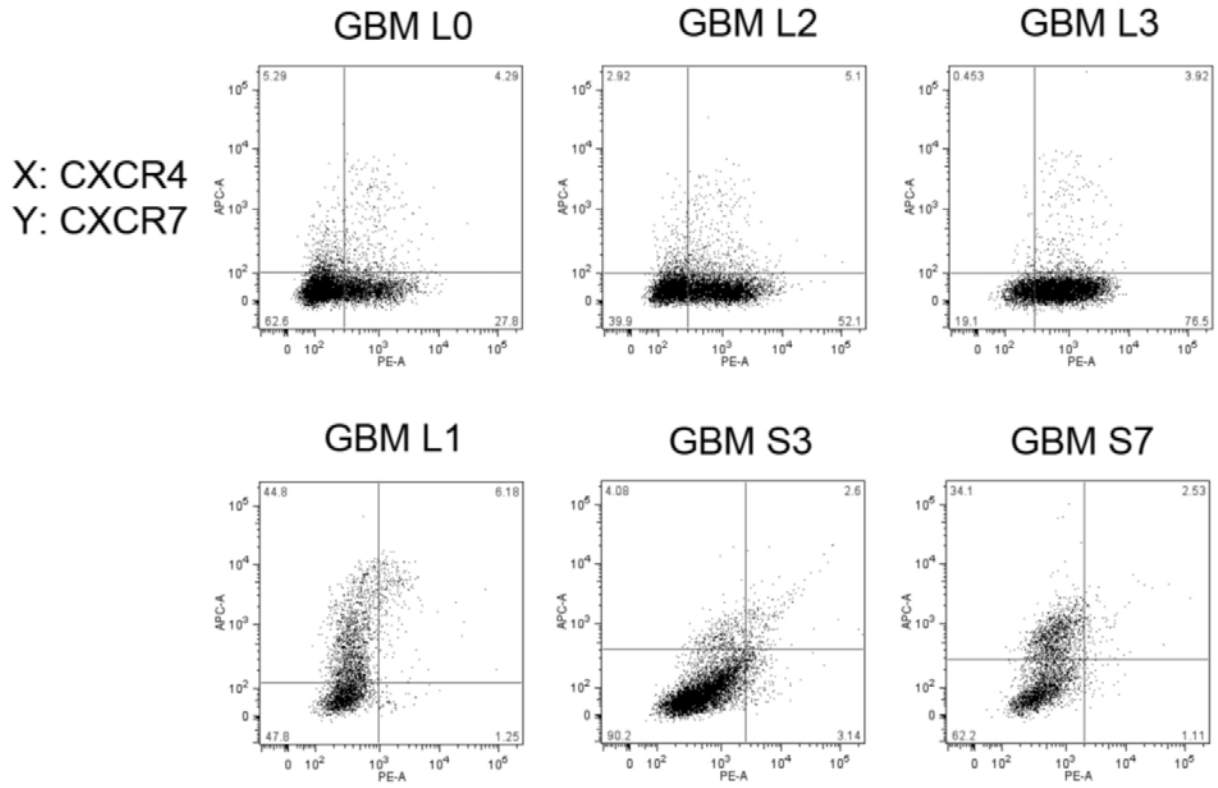

Figure 5-2. CXCR4 and CXCR7 expression in primary human GBM cell lines. Representative histograms from fluorescence-activated cell sorting analysis showing the expression of CXCR4 and CXCR7 in all GBM cell lines. Heterogeneous expression of CXCR4 and CXCR7 were evident in GBM cells *in vitro*. GBM L0, L2 and L3 are $CXCR4^{high}$-$CXCR7^{low}$ while GBM L1, S3, and S7 are $CXCR4^{low}$-$CXCR7^{high}$.

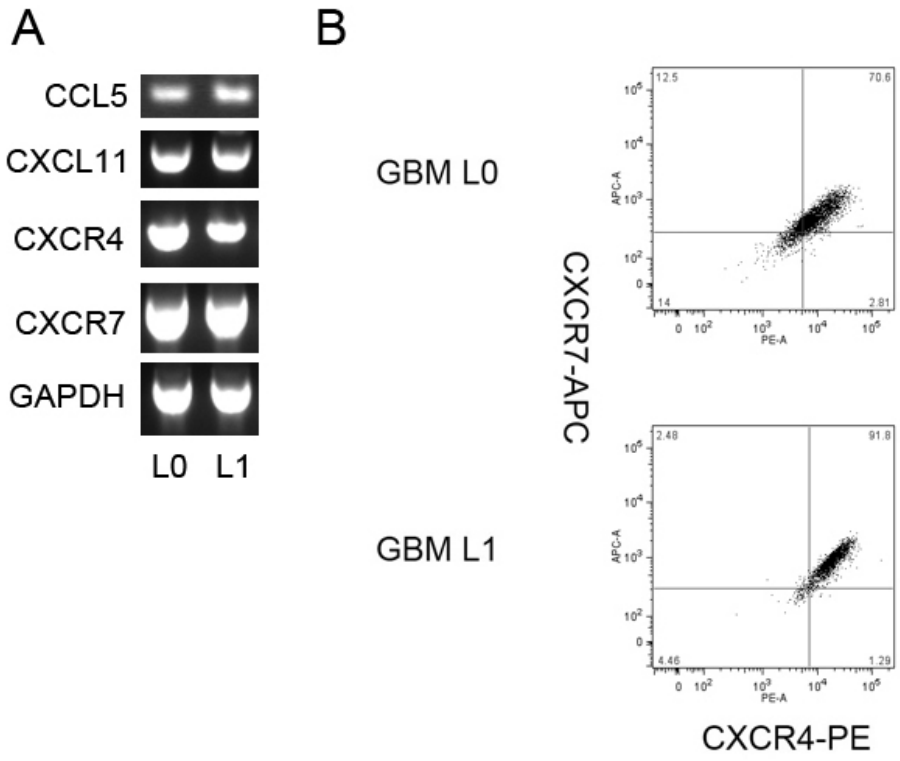

Figure 5-3. CXCL11, CXCR4, and CXCR7 mRNA and intracellular CXCR4 and CXCR7 protein expression in primary human GBM cell lines. (A) RT–PCR analysis identified CCL5, CXCL11, CXCR4, CXCR7 mRNAs in GBML0 and L1 cells *in vitro*. GAPDH was used as a control. (B) Representative histograms from fluorescence-activated cell sorting analysis showing intracellular CXCR4 and CXCR7 expression by GBM L0 and L1 cells *in vitro*. Despite the differential expression of surface CXCR4 and CXCR7 on GBM L0 and L1, both cell lines showed substantial coexistence of intracellular CXCR4 and CXCR7 in the majority of the cells.

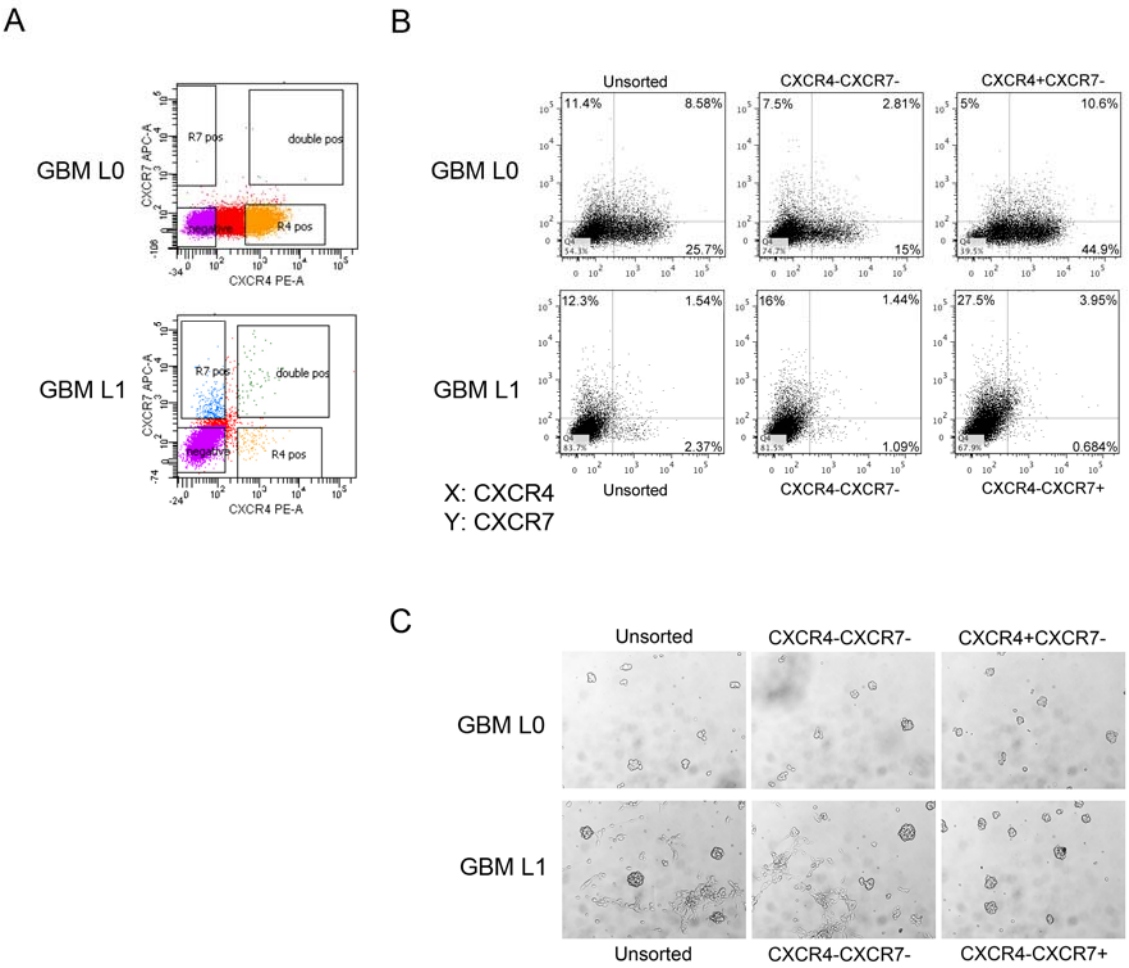

Figure 5-4. GBM cell subtypes recapitulated the original heterogeneous expression of CXCR4 and CXCR7 on cell membrane. (A) Cell subpopulations were isolated from GBM L0 and L1 based on their surface CXCR4-CXCR7 expression (B) Representative histograms from fluorescence-activated cell sorting analysis showed that after 7 days, each isolated subpopulation was capable of restore their parental nature regarding cell surface CXCR4-CXCR7 expression. (C) Representative images showing that isolated subtypes of GBM L0 and L1 cells were able to form spheres *in vitro*.

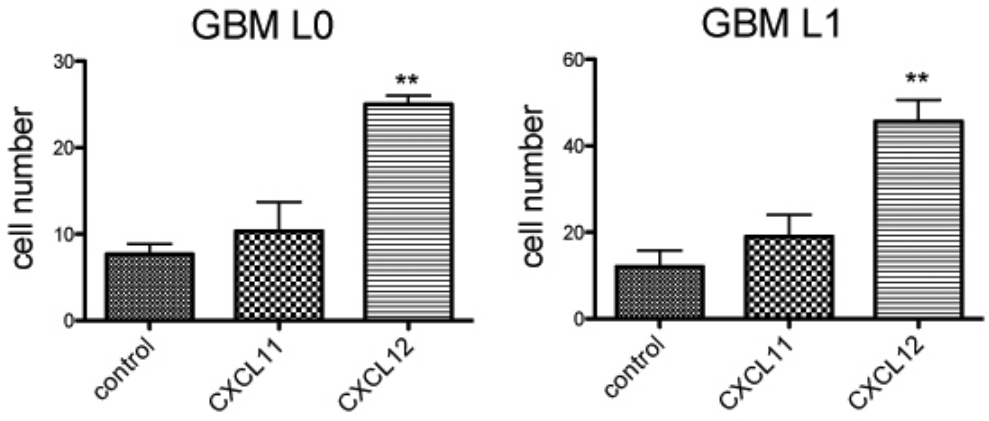

Figure 5-5. Effect of 10 nM CXCL11 or CXCL12 on GBM L0 and L1 cell migration. CXCL12 enhanced cell migration in GBM L0 and L1 cells while CXCL11 did not significantly promote cell migration of GBM cells.

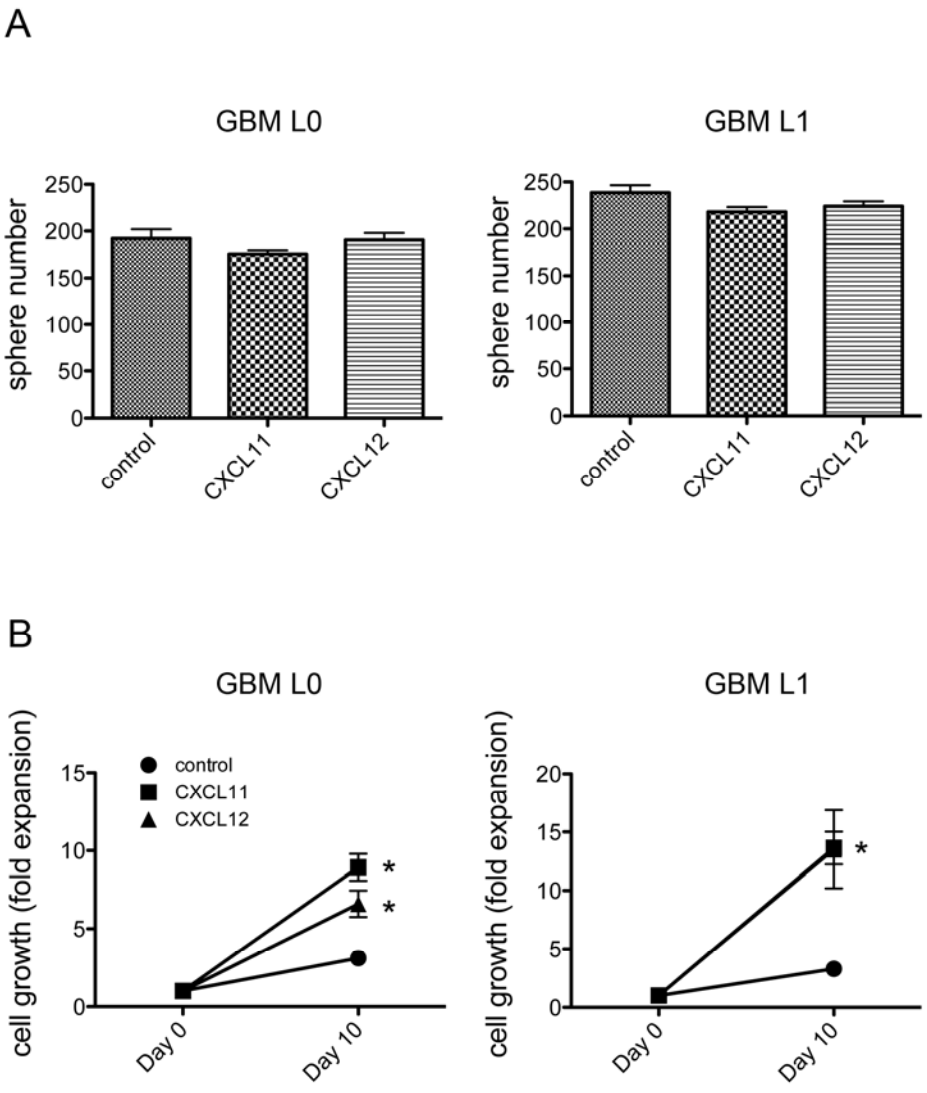

Figure 5-6. Effect of 10 nM CXCL11 or CXCL12 on GBM L0 and L1 primary sphere formation and cell growth. (A) CXCL11 and CXCL12 had no effect on primary sphere formation of GBM L0 and L1 cells. (B) CXCL11 and CXCL12 promoted cell growth of GBM L0 and L1 cells.

CHAPTER 6
GENERAL DISCUSSION

Glioblastoma multiforme, a WHO grade IV glioma, is characterized by behavioral aggressiveness, sites of necrosis within the tumor, and a poor prognosis despite multimodality therapies. Factors such as intratumoral heterogeneity, mutational evolution, and a highly immunosuppressive microenvironment are involved in the resistance to therapy of GBM. The main goals of researchers who are looking for novel therapeutic targets are identifying genotypic and phenotypic markers and factors that create heterogeneity and therapeutic resistance.

Chemotactic cytokines (chemokines) mediate a variety of functional activities in cancers, such as tumor growth, angiogenesis, metastasis, and recruitment of immune and effector cells. In this study, we addressed the functions and therapeutic potentials of several chemokine systems and revealed that GBM cells show differential expression of chemokines and chemokine receptors in the manner of protein isoforms and cell surface receptor levels. The effects of chemokines on immune cell trafficking, tumor cell growth, and tumor cell migration of GBM were evaluated.

Summary of Findings

In this study, we found that CX3CR1 and CXCR3 deficiency/blockade does not affect trafficking of tumor-infiltrating immune cells and microglia. Functional characterization revealed that CXCR3 exerts a growth-promoting effect directly on GBM cells, and a CXCR3 inhibitor successfully attenuated tumor growth *in vitro* and in *vivo*. Thus, CXCR3 is a potential therapeutic target to treat human GBM. In addition to CXCR3, the chemokine receptor CCR3, CXCR4, and CXCR7 were detected in primary human GBM cells. The GBM cells possess a differential CXCR4 and CXCR7

expression profile on the cell surface. Our results indicate that CXCL12 induced GBM cell migration while CXCL11 and CXCL12 promoted GBM cell growth. Therefore, the therapeutic targeting of CXCR4 and CXCR7 in GBM requires further study.

Chemokines and Tumor-Infiltrated Immune Cells in GBM

In this study, we utilized CX3CR1-deficient and CXCR3 deficient mice as well as a CXCR3 specific antagonist with the GL261 mouse model of glioma to investigate the functions of CX3CR1 and CXCR3 in intratumoral immune cell recruitment. Our data suggest that dysfunction of single chemokine receptor has little to no effect on immune cell migration into gliomas. Although CXCR3-deficient mice had reduced numbers of intratumoral natural killer (NK) and natural killer T (NKT) cells, this decrease is likely due to the impaired homeostasis of NK and NKT cell levels in the CXCR3-deficient mice[161]. The lack of an immune cell phenotype from chemokine receptor-deficient mice and antagonist treated mice may be explained by the redundancy of multiple chemokine receptors that coexist in immune cells and the variety of chemokines produced by tissues[186]. For example, lymphocytes can express multiple chemokine receptors. Th1 cells express CXCR3 and CCR5 when Th2 cells have CCR3, CCR4, and CCR8[187], and they respond and migrate toward the relevant chemokines[188,189]. Microglia have also been reported to express more than one chemokine receptor, such as CXCR1, CXCR3, CCR3, and CX3CR1[190,191]. Therefore, blocking only one specific chemokine receptor may not lead to a beneficial effect. Indeed, the combination of different chemokine receptor inhibitors has shown a greater impact on lymphocyte migration and immune responses than using a single chemokine receptor antagonist treatment[192].

Heterogeneity and Redundancy of Chemokine Receptors in GBM

In this study, we demonstrated that mouse and human GBM cells exhibit differential expression of chemokine receptors. $CXCR3^+$ and $CXCR3^-$ cells are observed in mouse GL261 cells and human A172, T98G, U87, U118, U138, and GBM L0 cells. In primary GBM cell lines GBM L0, L1, L2, L3, S3, S7, heterogeneous levels of cell surface CXCR4 and CXCR7 are also documented. The cell surface levels of chemokine receptors show a flexibility that is influenced by environmental stimuli. For example, our study indicates that serum supplemented culture medium will decrease CXCR3 expression in GBM cells. Serum supplemented changes in CXCR4 and CXCR7 levels in GBM have also been documented[111]. By intracellular FACS, we found a significant intracellular presence of CXCR4 and CXCR7 that was independent from their surface expression pattern. Similar substantial intracellular expression of CXCR3 has also been reported[193,194]. Taken together, GBM cells may be able to form reversible functional subtypes of cells by chemokine receptor transportation and internalization mechanisms. Interestingly, when we isolated $CXCR4^-CXCR7^-$ population from GBM L0 and L1 cell lines, both populations looked identical in their CXCR4-CXCR7 surface level and, when cultured under identical conditions, recapitulated their distinct parental differential expression of CXCR4 and CXCR7 on the cell surface. In addition, we found that other subpopulations restored the unique CXCR4-CXCR7 surface profile of the lines they were derived from. These results indicate a more complicated mechanism in regulating cell surface chemokine receptor levels, which shows a “memory-like” characteristic.

Another chemokine receptor heterogeneity we identified in several GBM cells included different variants of CXCR3, namely CXCR3A and CXCR3B. The expression of CXCR3 isoforms in GBM may increase the difficulty to target CXCR3 as a standard

therapeutic approach since CXCR3A enhances cell growth while CXCR3B induces apoptosis. Therefore, we should be careful when using CXCR3 antagonists to treat GBM patients and the screening of CXCR3 isoforms in GBM patients may be necessary to determine if an antagonist will have the desirable beneficial effect.

The existence of chemokine system redundancy raises difficulties for researchers to understand the function of chemokine systems and makes targeting chemokine systems as a therapeutic approach more complicated. For example, CXCR3 ligands have been considered as potential anti-tumor drugs due to their angiostatic activity through CXCR3B[88]. However, CXCR3 ligands also inhibited CCR5-mediated monocyte migration[171] and hence may interfere with the host immune response against cancer. Therefore, it is critical to consider the redundancy and interaction between chemokine systems when studying the functional significance of chemokine receptors. For instance, activation of CXCR7 alone does not induce cell migration[107], but CXCR7 is involved in cell migration by interacting with CXCR4[173]. Thus, investigation of multiple chemokine systems simultaneously may lead to more reliable understanding of their roles in human GBM.

Future Directions

The heterogeneity of CXCR4 and CXCR7 on the cell surface of GBM cells indicates the potential functional significance of these two chemokine receptors in the progression of human GBM. In this study we have demonstrated that CXCL12 induces GBM cell migration *in vitro*, which may contribute to the infiltrative characteristic of GBM cells. In addition, CXCL11 and CXCL12 both promote GBM cell growth. Therefore, targeting CXCL11/CXCL12/CXCR4/CXCR7 could be a possible therapeutic approach to

treat GBM patients. Our future studies will focus on the interaction between these chemokine receptors.

Does CXCR7 Impact Cell Migration in GBM?

In this research we determined that CXCL12 is able to induce cell migration in GBM cells. However, we did not address the question of whether CXCR4 or CXCR7 is responsible for CXCL12-regulated cell migration of GBM cells. Therefore, CXCL12-dependent migration of GBM cells will need to be determined in the presence of either AMD3100, a CXCR4-specific antagonist, or CCX733, a CXCR7-specific inhibitor, to elucidate whether CXCL12 evokes the migration response through CXCR4 or CXCR7. If the cell migration is regulated by CXCR4, as most published information suggests, the next question to be addressed is whether CXCR7 is involved in CXCR4-mediated cell migration. To investigate this question, migration of GBM cells in the presence of CXCL11, CXCL12, and NBI-74330 (CXCR3 antagonist) will determine the impact of CXCR7 activation on CXCL12-CXCR4 induced migration.

Do CXCR4 and CXCR7 Play Critical Roles in GBM Stem-Like Cells?

In this study we have shown that CXCL11 and CXCL12 have no effect on primary sphere formation of GBM L0 and L1 cells. However, since CXCL11 and CXCL12 promote GBM cell growth, which may include symmetric and asymmetric cell division of stem-like cells, the stem-like cell frequency could be altered by CXCL11 and CXCL12 stimulation, which would not be reflected in the primary sphere formation assay. A previous study has suggested that the frequency of stem cells, which should exhibit long-term proliferation ability, could be reflected in the slope of the growth curve[195]. Therefore, GBM cells will be cultured in CXCL11 or CXCL12 combined with different antagonists, and the long-term growth curve analysis as well as the sphere formation

ability at each passage will be measured. Results from these experiments will determine if CXCR4 and/or CXCR7 activation impacts stem-like cell frequency in GBM cells.

Will CXCR4 or CXCR7 Antagonism Inhibit Tumor Growth of GBM Cells *In Vitro* and *In Vivo*?

Our results indicate that CXCL11 and CXCL12 promote GBM cell growth *in vitro*. Further study using CXCR4 and CXCR7 antagonists to inhibit GBM cell growth *in vitro* and also *in vivo* would be beneficial in the search of new therapeutic targets for GBM patients. The *in vitro* effect would be evaluated by incubating GBM cells with the combination of chemokines and antagonists. Cell growth will be determined by counting total cell numbers. For the *in vivo* studies, GBM tumors will be established in immune-compromised NSG mice and the animals will be subsequently treated with either CXCR4 or CXCR7 inhibitors. The survival rate of tumor-bearing animals will be documented.

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BIOGRAPHICAL SKETCH

Che Liu was born in 1978 at Taipei, Taiwan. He developed a great interest in nature and ecology in his junior high school. He graduated from National Taiwan University in 2000, where he received a Bachelor of Science degree in zoology. He came to the United States in 2005 to pursue his dream of doing biological research. Che Liu enrolled the University of Florida in 2006 and jointed the lab of Dr. Jeffrey K. Harrison in the summer of 2007. During his PhD he was focused on studying the functions of chemokines and receptors in cancers, and eventually dedicated himself into looking for potential cures for human glioblastoma. Che Liu was active in his academic career. He had participated in several national conferences and received awards from the McKnight Brain Institute and from the International Student Center at UF. His scientific discoveries had been published in two biomedical journals. In the future he will devote himself to helping the community and human society as a scientist by his research.