CXCR4-STAT3 Axis Plays a Role in Tumor Cell Infiltration in an Orthotopic Mouse Glioblastoma Model

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INTRODUCTION

Glioblastoma multiforme (GBM) is a fatal malignant tumor that is characterized by diffusive growth of tumor cells into the surrounding brain parenchyma. However, the diffusive nature of GBM and its relationship with the tumor microenvironment (TME) is still unknown. Here, we investigated the interactions of GBM with the surrounding microenvironment in orthotopic xenograft animal models using two human glioma cell lines, U87 and LN229. The GBM cells in our model showed different features on the aspects of cell growth rate during their development, dispersive nature of glioma tumor cells along blood vessels, and invasion into the brain parenchyma. Our results indicated that these differences in the two models are in part due to differences in the expression of CXCR4 and STAT3, both of which play an important role in tumor progression. In addition, the GBM shows considerable accumulation of resident microglia and peripheral macrophages, but polarizes differently into tumorsupporting cells. These results suggest that the intrinsic factors of GBM and their interaction with the TME determine the diffusive nature and probably the responsiveness to non-cancer cells in the TME.

Keywords: glioblastoma, invasion, LN229, tumor microenvironment, U87
 deposited to the American Type Culture Collection (ATCC). In the past couple of decades, tremendous preclinical studies aiming to develop GBM therapy have utilized brain orthotopic models that contain U87 cells as human glioma cells. However, U87 cells can represent diffusive human glioma cells because of their rapid growth and demarcated, circumscript tumor margins, which may be a concern in some studies (Allen et al., 2016; Zeng et al., 2018).

The LN229 cell line was isolated from human patients with GBM (Van Meir et al., 1990). These cells have an epithelial morphology, grow with diffusive margins, and has been regarded to represent human GBM more closely than U87 cells.

In this study, we investigate the diffuse nature of GBM and its microenvironment in cellular and animal levels using two human glioma cell lines: U87 and LN229. We described the differences in growth rate, the mediators promoting the migration, the infiltrative nature of glioma cells along blood vessels, and the interaction with surrounding microglia and macrophages. Our data suggest that either the human LN229 or U87 cell line in mouse xenograft models of glioma will be useful for future research to develop drugs through molecular regulation of tumor cell infiltration.

**MATERIALS AND METHODS**

**Study approval**

All experimental protocols were approved by the Institutional Review Board of the Ajou University Hospital (No. AJIRB-GN-GEN-10-175) and all animal protocols were approved by the Institutional Animal Care and Use Committee of the Ajou University School of Medicine (No. 2017-0052).

**Cell culture and transduction**

The LN229 and U87MG human glioma cell line were obtained from ATCC (USA). U87MG and LN229 were maintained in Dulbecco’s modified Eagle medium (DMEM; Welgene, Korea) supplemented with 5% fetal bovine serum (FBS; GE Healthcare Life Sciences, Australia), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, USA) in a 37°C incubator. The lentiviral vector LL3.7 encoding green fluorescence protein (GFP) (Addgene, USA) was used to transduce U87 or LN229 cell lines in mouse xenograft models of glioma.

In two days later, transduced cells were sorted by fluorescence-activated cell sorting (FACS; BD Biosciences, USA). The resulting U87/GFP and LN229/GFP cells were expanded in growth medium (GM).

**In vivo xenograft mouse model**

Briefly, adult BALB/C nude mice (~20 g; Nara Biotech, Korea) were anesthetized and 1 × 10⁶ cells each of U87/GFP or LN229/GFP cells in 3 μl of phosphate-buffered saline (PBS) were sequentially transplanted into the striatum (AP, +0.05 cm; ML, −0.18 cm; DV, −0.3 cm) using a stereotaxic device (Stoelting, USA). To assess the tumor volumes, the animals were subject to magnetic resonance imaging (MRI) analysis or sacrificed for H&E staining at the indicated time.

**Measurement of tumor volume**

MRI was performed with a 9.4-T animal MRI scanner (Biospec 94/30: Bruker BioSpin MRI GmbH, Germany). T2-weighted images were acquired with the following parameters: repetition time = 3,000 ms, echo time = 32 ms, average = 2, acquisition matrix = 256 × 256; 10 slices with 1-mm slice thickness, and flip angle = 90°. A total of 10 slices were scanned to cover the whole brain. Reconstruction of MRI images was performed with the Digital Imaging and Communications in Medicine software (DICOM Viewer R3.0 SP3; Philips Healthcare, Netherlands). Immediately after the MRI experiments, 2-mm-thick coronal brain slices were prepared using a mouse brain mold (CellPoint Scientific, USA) and processed for H&E staining or immunohistochemical analysis.

The tumor volume was calculated using the following formula: \( V = \frac{L \times W \times W}{2} \), where \( V \) is the tumor volume, \( W \) is the tumor width, \( L \) is the tumor length.

**Tissue preparation**

At the indicated time, animals were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) (Yuhan, Korea), perfused transcardially with ice-cold saline, and then fixed with 10% neutral buffered formalin (NBF; BBC Biochemical, USA). The brains were post-fixed in 10% NBF, and dehydrated by serially soaking in 70%, 80%, 90%, 95%, 100% ethanol, and xylene, and eventually embedded in paraffin using a Leica TP1020 (Leica Microsystems, Germany).

**Immunohistochemistry**

Paraffin sections were prepared with a thickness of 5 μm and deparaffinized by reversing the dehydration process. The sections were placed in boiled citrate buffer (pH 6.0) for 10 min and incubated with 0.3% H₂O₂ for 30 min to inhibit endogenous peroxidase activity. After blocking nonspecific interactions via an incubation with 10% of the host serum at 24°C for 1 h, the samples were incubated at 4°C overnight with primary antibodies against STAT3 (1:300; Cell Signaling, USA), pSTAT3 (1:200; Cell Signaling), Iba1 (1:3,000; Wako, USA), IL-1β (1:200; Abcam, USA), and CD206 (1:1,000; Abcam). After washing in PBS-T (0.1% Tween X-100), the sections were treated with biotinylated goat anti-rabbit IgG (Vector Lab, USA) or biotinylated horse anti-mouse IgG (Vector Lab) in the blocking buffer. The reaction was amplified for 30 min with an avidin-biotin complex, using an ABC kit (Vector Lab) and visualized with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, USA). After dehydration through a graded series of alcohol, the sections were mounted in Shandon synthetic mountant (Thermo Scientific, UK) and cover-slipped.

For immunofluorescence staining, sections were incubated overnight at 4°C with antibodies against CD31 (1:200; Abcam), GFP (1:500; Abcam), and STAT3 (1:300; Cell Signaling). After washing 3 times for 5 min each in PBS containing 0.1% Tween-20, the immunoreactivity was visualized using Alexa Fluor 488- or 568-conjugated goat anti-mouse or rabbit IgG. After washing, the slides were air-dried and mounted with aqueous mounting media (SouthernBiotech, USA).

Sections without primary antibodies were processed in parallel as a negative control to monitor the nonspecific peroxidase staining of secondary antibodies in the damaged tissue.
The brain sections were scanned under high power magnification (40×) on the Axio Scan.Z1 (Carl Zeiss, Germany) and the digital images were acquired.

The number of immunoreactive cells per mm² were manually counted in each field of interest by an investigator blinded to the groups. The number of GFP-positive cells around the blood vessels were counted in divided regions from the adjacent area to the remote areas. The number of Iba1-, IL-1β-, and CD206-positive cells were counted in the intra-tumoral and peri-tumoral areas of the striatum. The results are presented as mean counts per mm² ± SE.

**In vitro cell migration and invasion assay**

A scratch wound assay with LN229/GFP and U87/GFP cultures was performed to determine the migration potential of the cells. GBM cells were plated onto 24-well plates at a density of 5 × 10⁴ cells/cm² and were allowed to grow for a 24-h period in serum-free media to reach a con uent monolayer. The serum-free media was removed and the con uent cell sheet was wounded by scratching the culture well surface with a 1 ml pipette tip. Fluorescence microscopic imaging was used to record a frame every 12 h for 24 h with an AxioCam single-channel camera and AxioVision software (Carl Zeiss). The GFP intensity was also measured in the wounded area at indicated times. The results from three independent experiments are presented as mean ± SE.

The migration/invasion assays were performed in a 24-well transwell cell culture chamber with an 8-μm pore polycarbonate membrane insert (Corning Costar, USA). GBM cells were plated at a density of 5 × 10⁴ cells/cm² with or without coated Matrigel (Corning Costar) in the upper compartment of the chamber. The lower compartment was loaded with bEND.3 cells at a density of 5 × 10⁶ cells/cm² or conditioned medium (CM) of bEND.3 cells, and the GBM cells were then incubated for 24 h at 37°C. The insert was washed with PBS and fixed with 10% NBF (BBC Biochemical). The cells at the upper surface of the membrane in the insert were removed with a cotton swab, and the fluorescence images of the invaded cells in the bottom surface were acquired with a fluorescence IX71 microscope (Olympus, Japan). The GFP levels remaining on the bottom surface of the entire membrane was presented as mean ± SE from three independent experiments.

**In vitro experiment to mimic the interaction of GBM cells with TME**

GBM cells were co-cultured with BV2 cells in a 6-well transwell cell culture chamber with a 0.4-μm pore polycarbonate membrane insert (Corning, USA). GBM cells were plated at a density of 2 × 10⁵ cells/cm² in the upper compartment of the chamber. The lower compartment was loaded with BV2 cells at a density of 5 × 10⁴ cells/cm². After co-culturing for 24 h, total RNA was extracted from BV2 cells, and the amount of NOS2 mRNA and ARG1 mRNA were quantified by quantitative real-time polymerase chain reaction (qRT-PCR).

**RT-PCR analysis**

Total RNA was extracted through Hybrid-R (GeneAll, Korea). One microgram of RNA template was reverse-transcribed using a SuperScript III First-Strand Synthesis System (Invitrogen). The qRT-PCR was performed using 100 ng of cDNA solution in a 20 μl reaction mixture containing 10 μl of power SYBR green PCR Master mix (Applied Biosystems, USA), and 1 μl of the forward primer, and 1 μl of the reverse primer. Relative mRNA expression was assessed using the comparative ΔΔCt method. GAPDH was used as an internal standard. The qRT-PCR primers are shown in Table 1.

**Western blot**

GBM cells were homogenized in a buffer consisting of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, and protease inhibitors. The homogenates were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected, and the total protein contents were determined using the BCA protein assay kit (Pierce, USA). Equal amounts of total protein were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, USA). The membranes were blocked in 5% nonfat milk for 1 h at room temperature followed by an overnight incubation at 4°C with primary antibodies for anti-STAT3 (1:1,000; Cell Signaling) and anti-pSTAT3 (1:500; Cell Signaling). Actin was used as a loading control for protein quantification. Membranes were then washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized using the ChemiDoc image system (Bio-Rad). Quantitative results are presented as the ratio of the band intensity of the protein of interest to the band intensity of actin.

**Statistical analysis**

Statistical analyses were carried out using Sigmaplot (Systat Software, USA). Data were analyzed by Student’s t-test or one-way ANOVA. Significant differences were further evaluated using Tukey’s honest significant difference post-hoc test. A P value < 0.05 was considered statistically significant. All data are presented as mean ± SE.

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**Table 1. Primers for qRT-PCR**

| Gene  | Forward (5′-3′)                   | Reverse (5′-3′)                   | Accession No.          |
|-------|----------------------------------|----------------------------------|------------------------|
| CXCR4 | ACTACACCGAGGAAATGGGCT            | CCCACAATGCCAGTTAAGAAGA          | NM_001008540.2         |
| CXCL12| CGCCAGGCCCCAACGTCAAG            | CTTGTAAAGCTCTTCTCAGGTAC         | NM_013655.4            |
| hGAPDH| GTTCCTGCTGACCCTTCCAGGAAG         | ACCACCTTGTGCGTAGCGCAA           | NM_002046.7            |
| NOS2  | GTTCTGACGCTAACACTACACAGA        | GTGGACGGGTGCGATTGAC             | NM_019274.4            |
| ARG1  | CTCAGGACAAAAGTCTCAGAG           | AGGAGCTGTGATTAGGGACATC          | NM_007482.3            |
| mGAPDH| AAATTTGCCATGTTGAAGGCC           | GGATGCAAGGATGATGTTCT            | NM_001289726.1         |
Fig. 1. Validation of GFP-expressing GBM cells. (A) Phase contrast and fluorescence images of U87 and LN229 cells grown in monolayers. Note that the cell morphology is more uniform in LN229 cells (magnification, ×200). (B) Fluorescence-activated cell sorting analysis showing the U87/GFP and LN229/GFP cells had a > 95%. (C) Comparison of doubling times of U87 and LN229 cells before and after transduction with a GFP-expressing lentiviral vector.

Fig. 2. Tumor growth in two GBM cell lines in orthotopic xenograft mouse models. (A) T2-weighted MRI scans of mouse brains at the indicated time points after tumor transplantation. (B) H&E staining of brain sections showing the tumor xenografts of U87/GFP and LN229/GFP cells. Note the ectopic location of LN229/GFP cells (arrows). Scale bar = 1 mm. (C) Quantification of histological images at the indicated time showing the tumor volume as mean ± SE (3 representative sections per animal and 3 animals per group).
RESULTS

Comparison of characteristics between LN229/GFP cells and U87/GFP cells
To better monitor the migration/invasion in vivo, the LN229 and U87 cells were labeled with GFP using a lentiviral vector pLL3.7 encoding GFP (Fig. 1A). Transduced cells were sorted using FACS to obtain LN229/GFP and U87/GFP cells, which were then expanded in GM. Both U87 and LN229 cells were labeled with GFP to a similar level (98% vs 96%) (Fig. 1B). Importantly the doubling time of both GBM cell lines was not altered by transduction and FACS sorting (Fig. 1C).

Characterization of tumor growth in orthotopic xenograft mouse models
Next, we compared the tumor growth of U87/GFP and LN229/GFP cells in orthotopic GBM models. An MRI analysis of the GBM models revealed that U87/GFP cells grew rapidly and the growth peaked at 3 weeks. By comparison, LN229/GFP cells grew at a slower rate than U87/GFP cells and reached a peak at 8 weeks (Figs. 2A and 2B). We verified the MRI results with classical histological analysis. Simple H&E staining revealed the densely stained basophilic tumor mass in both GBM models. As shown by MRI, U87/GFP cells grew more compactly with demarcated margins. By comparison, LN229/GFP cells grew in a disperse manner from the main tumor and sometimes out of the meninges, which was easily lost during preparation of paraffin samples (arrows, Figs. 2B and 3B).

Tumor cell migration along blood vessels
Perivascular growth is the most common characteristics of GBM (Burden-Gulley et al., 2011; Cheng et al., 2013), which is achieved by migration and distribution of GBM cells to/near endothelial cells. The brain regions were divided into 1 to 4 zones from the tumor inoculation site to theventral surface of the brain intratumoral area (zone 1), tumor-associated boundary (zone 2), and remote areas (zone 3 and zone 4) (Fig. 3A). H&E staining revealed the demarcating boundaries between zones 2 and 3 for U87/GFP cells but diffuse boundaries for LN229/GFP cells. To determine whether the demarcating margin of U87/GFP cells and the diffused nature of LN229/GFP cells were due to the intrinsic tropism toward/along blood vessels, the brain sections from U87/GFP and LN229/GFP xenografts were stained with an antibody specific for the endothelial cell marker CD-31. U87/GFP cells were found together with blood vessels within zones 1 and 2 but scarcely found in zones 3 and 4. It was evident that U87/GFP cells were found closely associated with CD-31-positive vasculature in zone 3. By comparison, LN229/GFP cells were found not only in zone 1 and 2 but also abundantly in zones 3 and 4 (Figs. 3B and 3C). LN229/GFP cells were found in association with blood vessels in all zones (arrowheads in Fig. 3D). The results indicated that LN229 cells grow at a lower rate than U87 but infiltrate diffusively to disperse in a wide range.

Comparison of GBM cells migration and invasion activity
To test the intrinsic migration activity of GBM cells, a scratch assay using a pipette tip was performed to investigate whether the dispersal difference between the two GBM cells along the blood vessel was due to differences in intrinsic migratory activity. For U87/GFP cells, the GFP intensity in the scratched area increased significantly in a time-dependent manner, whereas it remained minimally for LN229/GFP cells (Figs. 4A and 4B). This result indicates that U87 cells had higher intrinsic migratory activity than LN229 cells.

To assess the invasiveness of GBM cells, a transwell invasion assay was performed with or without Matrigel as an extracellular matrix (ECM) to better mimic in vivo conditions. The GFP-labeled GBM cells were plated in the upper chamber with a brain endothelial cell line while, bEND.3 cells or the CM of bEND.3 cells were added in the lower chamber (Fig. 4C). In the absence of Matrigel, co-culture with bEND.3 cells or its CM in the lower chamber enhanced migration of LN229/GFP cells compared to U87/GFP cells. The enhanced activity was specific to bEND.3 cells since the GM was less effective. The GFP levels decreased significantly by addition of Matrigel in the upper chamber for both GFP-labeled GBM cells (Figs. 4C and 4D). Importantly, co-culture with bEND.3 cells or its CM markedly increased the LN229/GFP cells, whereas they did not affect migration of U87/GFP (Figs. 4C and 4E). The results suggest that although intrinsic migratory activity was higher in U87 cells than LN229 cells in the absence of any stimuli, the invasion activity through ECM particularly toward the endothelial cells was much higher in LN229. The result also implies the presence of chemoattractants in the CM that interact with LN229 cells and promote its migration.

CXCL12 from bEND.3 cells promote migration of GBM cells
Recently, it was reported that endothelial cells promote migration of human GBM tumor cells by direct interaction through the chemokine CXCL12 (also known as stromal cell-derived factor 1), and its receptor CXCR4 (Rao et al., 2012; Yadav et al., 2016). CXCL12 was expressed to a level comparable to a housekeeping GAPDH in bEND.3 cells since the Ct value of CXCL12 was 2.5-fold higher than that of GAPDH in a RT-PCR analysis.

To assess whether the CXCL12-CXCR4 signaling differ in the two GBM cell lines, we investigated the expression levels of CXCR4 mRNA in LN229 and U87 cells. CXCR4 mRNA expression was higher in LN229 cells compared to U87 cells (Fig. 5A). It has been well established that the JAK2/STAT3 pathway initiates transcription of multiple cancer-associated genes promoting cell proliferation, migration, and invasion of neoplastic cells (Luwor et al., 2013).

STAT3 can be activated by CXCL12-CXCR4 (Ahr et al., 2005; Vila-Coro et al., 1999), we assessed STAT3 expression in two GBM cell lines by western blot analysis. Without activation in vitro, the expression levels of STAT3 and pSTAT3 were slightly higher in LN229 cells than U87 cell; however, the difference was insignificant (Figs. 5B and 5C). To assess STAT3 expression in vivo in GBM models, the brain tumor sections bearing U87/GFP and LN229/GFP cells were analyzed using anti-STAT3 and anti-pSTAT3 antibodies. The immunohistochemistry analysis revealed that level of both
STAT3 and pSTAT3 were higher in the tumor bearing LN229/GFP cells than the tumor bearing U87/GFP cells (Fig. 5D). Double immunohistochemistry analysis indicated that high STAT3 immunoreactivity was colocalized with GFP signals in LN229/GFP-bearing tumors (Fig. 5E). By comparison, the STAT3 expression was weak in most U87/GFP-bearing tumors (Fig. 5E). The results suggest that LN229 cells respond to interact with the brain microenvironment in response to CXCL12.

Functional analysis of microglia responses to the GBM cells
In the TME, GBM shows considerable accumulation of resident microglia and peripheral macrophages, and their
polarization into tumor-supporting cells (Dello et al., 2017; Lisi et al., 2017). Recent evidence suggests that exposure to glioma-derived chemokines induce constitutive STAT3 activation in microglia, with consequent suppression of antitumor mechanisms or even tolerance to tumor antigens (Hussain et al., 2006; Kostianovsky et al., 2008; Zhang et al., 2009). Conversely, STAT3 has been shown to be constitutively active in a large percentage of GBM tissue and inactivation of STAT3 in GBM cell line causes microglia suppression (Luwor et al., 2013).

To assess whether different levels of STAT3 in U87 and LN229 cells in the brain tumors influences microglia in the TME, we evaluated M1- and M2-type microglia. Iba1-positive microglia was found in the intra-tumoral area (zone 2) for both U87 and LN229-bearing tumors whereas more microglia were found in the peri-tumoral region (boundary between zones 2 and 3) for LN229 than U87-bearing tumors (Figs. 6A and 6B). Interestingly, higher IL-1β-positive immunoreactivity was observed in the peritumoral region of U87-bearing tumors whereas more CD206-positive immunoreactivity was high in LN229-bearing tumors (Fig. 6A). Quantitative analysis indicated that the number of IL-1β-positive M1 types was higher in U87-bearing tumors whereas the number of CD206-positive M2 type was higher in LN229-bearing tumors (Fig. 6C). To determine the interaction of GBM cells and microglia, we co-cultured each of GBM cells in a transwell chamber with the BV2 microglial cell line, and performed a RT-PCR analysis. The mRNA level of NOS2, a M1-type marker, was dramatically increased in BV2 cells by 58- and 25-fold following co-culturing with U87 and LN229, respectively (Fig. 6D). By comparison, the mRNA level of ARG1, a M2-type marker, was significantly upregulated in BV2 cells profoundly after co-culturing with LN229 (10-fold) but to a lesser degree with U87 cells (4.2-fold) (Fig. 6E). These results suggest that LN229 cells, unlike U87 cells, may produce a polarization factor that induces conversion of microglia and macrophage to M2 type in the invasion process.

**DISCUSSION**

In this study, two types of GBM cell lines (U87 and LN229) that grow distinctively in the brain were utilized to delineate the cellular interactions of neoplastic cancers with surrounding TME using orthotopic xenograft mouse models.

The U87MG cell line that can be obtained from ATCC was derived from a male patient of unknown age. During the past few decades, tremendous preclinical studies aiming to develop GBM therapy have utilized brain orthotopic models that contain U87 cells as human glioma cells. U87 glioma cells grow with demarcated circumspect tumor margins. Therefore, there have been several concerns with respect to U87 cells because it can represent diffusive human glioma cells. U87 glioma cells grow rapidly to form the tumor mass compared with U87 and LN229-bearing tumors whereas more microglia were found in the peri-tumoral region (boundary between zones 2 and 3) for LN229 than U87-bearing tumors (Figs. 6A and 6B). Interestingly, higher IL-1β-positive immunoreactivity was observed in the peritumoral region of U87-bearing tumors whereas more CD206-positive immunoreactivity was high in LN229-bearing tumors (Fig. 6A). Quantitative analysis indicated that the number of IL-1β-positive M1 types was higher in U87-bearing tumors whereas the number of CD206-positive M2 type was higher in LN229-bearing tumors (Fig. 6C). To determine the interaction of GBM cells and microglia, we co-cultured each of GBM cells in a transwell chamber with the BV2 microglial cell line, and performed a RT-PCR analysis. The mRNA level of NOS2, a M1-type marker, was dramatically increased in BV2 cells by 58- and 25-fold following co-culturing with U87 and LN229, respectively (Fig. 6D). By comparison, the mRNA level of ARG1, a M2-type marker, was significantly upregulated in BV2 cells profoundly after co-culturing with LN229 (10-fold) but to a lesser degree with U87 cells (4.2-fold) (Fig. 6E). These results suggest that LN229 cells, unlike U87 cells, may produce a polarization factor that induces conversion of microglia and macrophage to M2 type in the invasion process.
**Fig. 4. In vitro migration and invasion assays.** (A) Fluorescence microscopic images of wound closure of U87/GFP and LN229/GFP cells cultured to a monolayer at the indicated time after scratching with a pipette. Scale bar = 400 μm. (B) Quantification of GFP intensity in the scratched area at the indicated time. Results from three independent experiments are presented as mean ± SE. *P < 0.05 compared to LN229/GFP at indicated time. (C) Schematic illustration of the transwell invasion assays. Representative fluorescence images of U87/GFP and LN229/GFP cells with (w/) or without (w/o) Matrigel in the upper chamber and bEND.3 cells or CM in the lower chamber. (D and E) The intensity of GFP levels measured at the bottom surface of membrane of the upper chamber. The data are presented as mean ± SE from three independent experiments. *P < 0.05, **P < 0.01.
Fig. 5. Comparison of expression of genes and proteins related to the interaction between GBM cells and brain-endothelial cells. (A) High expression of CXCR4 in LN229/GFP cells. The data is presented as mean ± SE from three independent experiments. *P < 0.05. (B) Representative western blot analysis showing the endogenous expression of STAT3 and p-STAT3 in U87 and LN229 cells. (C) Densitometry analysis of STAT3 and p-STAT3 immunoblots showing no significant difference between GBM cell lines. (D) The STAT3 and p-STAT3 immunoreactivity in the zones 2 and 3 of GBM-bearing tumors. Scale bar = 100 μm. (E) Double immunostaining for the presence of STAT3 (red) and GFP (green) showing co-localization (or proximity) of GFP and STAT3 immunoreactivity in the LN229/GFP-bearing tumors. Scale bar = 500 μm.
between two glioma cells but was significantly different in the peri-tumoral area (Fig. 6). Accordingly, the STAT3 expression was higher in the peri-tumoral area of LN229-bearing tumors (Fig. 5). STAT3 in GBM cells can induce microglia suppression (Luwor et al., 2013). Indeed, the M2 polarization of microglia/macrophage was much higher at the peri-tumoral region with LN229 cells compared to the U87 cells (Fig. 6). These results suggest that LN229 cells, unlike U87 cells, may promote the polarization of microglia and macrophage to the M2 type during invasion process. The M2-polarization by LN229 was verified by reduced expression of IL-1β and increased expression of Arg-1 in co-culture assays with BV2, a
In summary, we investigated the distinct nature of tumor growth as well as, diffusive nature, migration, invasion, and polarization of microglia/macrophages at the cellular and animal levels using two distinct GBM cell lines. The studies including our results provide a better understanding of the interactions of the GBM with the microenvironment and can eventually lead to the development of therapeutics by that regulate the dispersive nature and tumor suppressive environment of the GBM.

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AUTHOR CONTRIBUTIONS
J.H. performed experiments. J.H., J.S.Y., D.Y.C., K.G.C., J.L., and S.S.K. analyzed the data. S.S.K. and H.S.K. supervised the research. J.H., J.S.Y., and H.S.K. wrote the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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