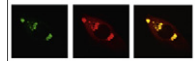


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## Research Report

# Stabilin-1 expression in tumor associated macrophages

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### ABSTRACT

Glioblastoma multiforme is a very aggressive and common form of brain tumor. Current therapies consist of a combination of surgical removal, chemotherapy and radiation therapy. These drastic treatments still leave a current prognosis of median survival of less than 1 year. Lack of effectiveness of these treatments has left researchers looking for alternative forms of treatment. A significant alternative currently being investigated is the use of the immune system to potentially target and eliminate tumor cells directly. Stabilin-1, a scavenger receptor expressed by macrophages, has the potential in inhibiting tumor growth by binding and internalizing secreted protein acidic and rich in cysteine (SPARC). SPARC is known to be upregulated in the tumor microenvironment and is involved in extracellular matrix remodeling, cell proliferation and migration. Decreasing SPARC expression using siRNA has been shown to decrease tumor invasiveness and survival. We investigated the phenotype of stabilin-1 expressing immune cells in the tumor environment and demonstrated a transient population of alternatively activated macrophages expressing stabilin-1 in the tumor environment and the disappearance of that population as the tumor progresses.

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## 1. Introduction

Glioblastoma multiforme is the most common and aggressive form of CNS tumors. Though current therapies including surgical removal, chemotherapy and radiation seem to extend the survival of the affected patients, the current prognosis still has a median survival of less than 1 year. Surgical removal and radiation therapy crudely target large regions of the brain while chemotherapy targets all dividing cells of the body, thus these methods tend to leave the patient with considerable side effects (Sughrue et al., 2009). Moreover, the lack of effectiveness of these treatments has left researchers looking for alternative forms of treatment. One significant alternative is the use of the immune system,

with its high specificity, it has the potential to target and eliminate the tumor cells directly; though knowledge of the immune environment within and around gliomas is lacking. Furthermore, the markers unique to glioma cells and the extent of immunosuppression in the glioma milieu remain largely unknown.

Matricellular proteins are secreted into the extracellular space and interact with cell surface receptors, proteases and structural proteins such as collagen (Bornstein and Sage, 2002). Secreted protein acidic and rich in cysteine, osteonectin (SPARC) is a matricellular protein which promotes cell migration as it facilitates an intermediate stage of adhesion as opposed to the strong adhesion of most matricellular proteins (Bornstein and Sage, 2002). The role of SPARC in cell

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migration makes it an important factor during normal development, wound healing and tissue remodeling (Workman and Sage, 2011; Framson and Sage, 2004; Brekken and Sage, 2001). SPARC is upregulated by glioma cells, and is associated with increased tumor metastasis and proliferation (Golembieski et al., 1999; Schultz et al., 2002; Rich et al., 2003; Schittenhelm et al., 2006). This may be partially due to the increase of several proteases, which leads to tissue degradation and allows room for infiltrative tumor cells to migrate (Golembieski et al., 2008). Targeting and decreasing SPARC expression with siRNA has proven to decrease tumor invasiveness and tumor cell survival (Seno et al., 2009; Shi et al., 2007).

Recently, the scavenger receptor stabilin-1 was identified as the first known receptor for SPARC. When bound, SPARC is internalized by stabilin-1 and rendered to the endosomal pathway where it is subsequently degraded (Kzhyshkowska, 2006). Stabilin-1 is expressed on the surface of alternatively activated macrophages (AAM $\emptyset$ ) which participate in wound healing and in the anti-inflammatory process (Kzhyshkowska et al., 2004; Park et al., 2009; Palani et al., 2011; Mosser and Edwards, 2008). Although SPARC is known to exist in the tumor environment, the presence or location of its receptors are not yet known. The capacity of stabilin-1 expressing macrophages to clear SPARC has the potential to be significant in the control of glioma growth and proliferation.

Macrophages display a wide spectrum of activation states which are generalized as either classically activated (M1) or alternatively activated (M2) (Mosser and Edwards, 2008). In contrast to the microbicidal, inflammatory classically activated macrophages; M2 macrophages are anti-inflammatory and are involved in tissue repair through upregulation of CXCR3, IL-10, arginase-1, the mannose receptor MMR and the scavenger receptor stabilin-1 (Mosser and Edwards, 2008; Kzhyshkowska, 2006; Kzhyshkowska et al., 2004). During glioblastomas, a population of macrophages associates with the tumor: tumor associated macrophages (TAMs). This population of macrophages comprises a significant proportion of the tumor, accounting for up to 50% of the tumor mass (Solinas et al., 2009). The phenotype of TAMs is not yet well defined, though it is generally accepted that TAMs are primarily M2 activated. Furthermore, TAMs display pro-tumor functions: promoting tumor cell survival, proliferation and metastasis (Mantovani et al., 2002; Luo et al., 2006; Solinas et al., 2009; Gordon and Martinez, 2010; Vasievich and Huang, 2011; Zhang et al., 2011). Indeed, high levels of TAMs are often associated with a worse prognosis (Bingle et al., 2002; Zhang et al., 2011).

In this study, we investigated the phenotype of tumor associated macrophages in an attempt to clarify their activation status and whether or not they expressed stabilin-1. Here we demonstrate a significant increase of total lymphocyte and macrophage infiltration in the glioma injected hemisphere (GL) 2 weeks post-injection, a two fold increase in stabilin-1 and MMR expression compared to the naïve hemisphere at day 7 post-injection and reduction of stabilin-1 expression in the glioma hemisphere by day 14 post-injection.

These data demonstrate the presence of stabilin-1 at the site of initial glioma growth but then a downregulation of

stabilin-1 as the tumor progresses. It is well known that tumors can cause phenotypic changes in infiltrating immune cells and thus may be responsible for the change in stabilin-1 expression. Increasing this receptor expression on infiltrating macrophages may help in the control of glioma growth.

## 2. Results

### 2.1. Scavenger receptor stabilin-1 is expressed in early glioma

A model of glioma was used in this study where GL-26 cells were injected intra-cranially and allowed to grow for either 7, 14 or 21 days (Fig. 1A, B). Injection of 90,000 GL-26 cells in the caudate putamen (CPU) leads to a stable tumor which initially grows in the needle tract before expanding in the border between the striatum and the external capsule (ec). By day 14, the needle tract is mostly closed and the tumor expands from the dorsal portion of the caudate putamen to the exterior of the cortex (Fig. 1A) and by day 21, the tumor has expanded to both hemispheres (Fig. 1B).

To determine if stabilin-1 is present in the tumor environment, expression of stabilin-1 was measured using RT-qPCR at day 7 post-injection in the right striatum. Furthermore, as stabilin-1 has been shown to be predominantly expressed on AAM $\emptyset$ , expression of MMR was also measured to test if AAM $\emptyset$  were present in our tumor model. Both stabilin-1 and MMR transcripts are up-regulated  $1.912 \pm 0.6391$  and  $1.881 \pm 0.5023$  times (Fig. 1C) respectively in the injected hemisphere (GL) when compared to the non-injected hemisphere (NI).

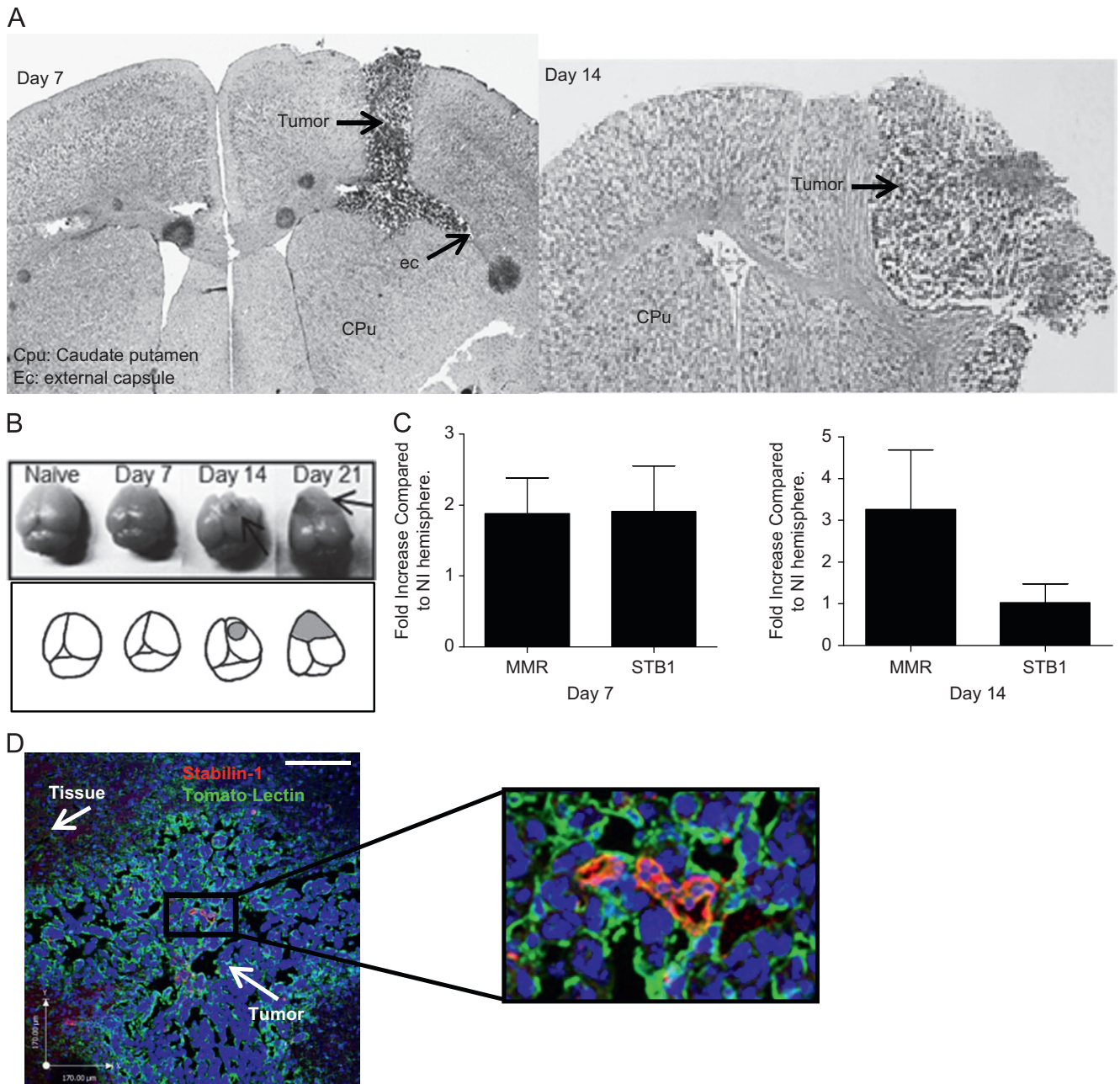
At day 14, although high levels of MMR are maintained on the injected hemisphere, the expression of stabilin-1 was reduced (Fig. 1C). Indeed, at day 14 post-glioma injection, MMR expression increased  $3.265 \pm 1.423$  fold higher in the injected hemisphere whereas stabilin-1 expression was reduced to equal levels in both hemispheres ( $1.031 \pm 0.4456$  fold higher in the injected hemisphere compared to non-injected hemisphere).

Immunofluorescent staining at day 7 post-injection for stabilin-1 and tomato lectin at the site of the tumor reveals both membrane and cytoplasmic stabilin-1 within the tumor tissue (Fig. 1D). Tomato lectin is known to stain for macrophages, blood vessels and microglia. These images suggest a small population of infiltrating macrophages which express stabilin-1 within gliomas.

These data demonstrate the presence of a growing glioma at days 7 and 14 post-injection which is associated with increased MMR expression and early (day 7) stabilin-1 upregulation in the injected hemisphere.

### 2.2. Stabilin-1 expression on macrophages decreases with tumor growth

To determine the cell types which express stabilin-1 in the tumor environment, infiltrating macrophages, lymphocytes and local microglial populations were analyzed at days 7 and 14 post-glioma injection by flow cytometry. At day 7 post-injection, there were no differences in total mononuclear cell

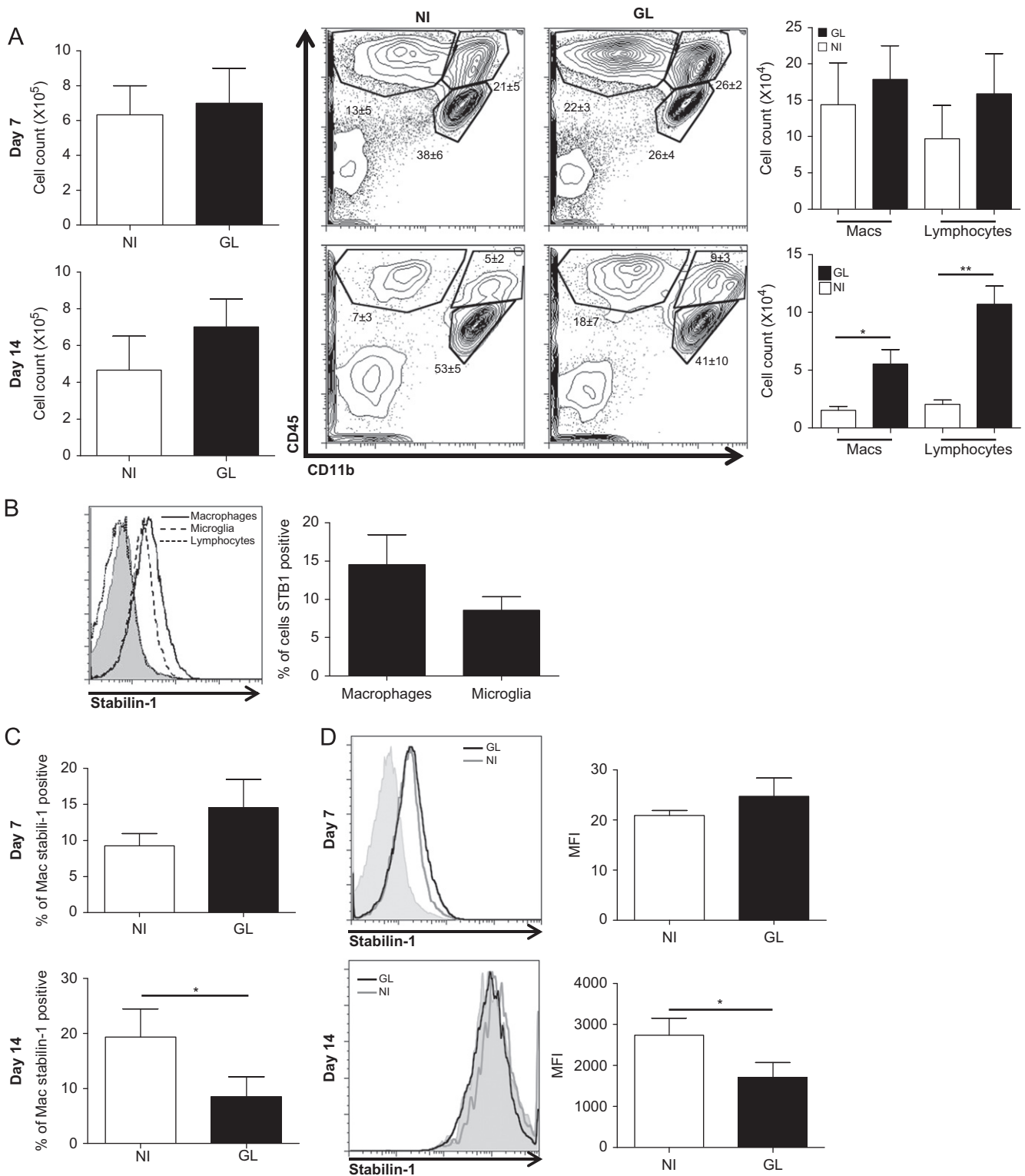


**Fig. 1** – Stabilin-1 expression in the tumor environment. 12 µm brain coronal sections of day 7 or 14 post-injection with GL-26 were stained with haematoxylin followed by eosin to confirm tumor presence and growth (A). Naïve, days 7, 14 and 21 post-GL-26 injected brains are photographed prior to sectioning, shading and arrows represents tumor area (B). Brains were taken at day 7 or 14 post-injection with GL-26 and separated by hemisphere (GL-26 injected and non-injected). RNA was extracted for real time PCR analysis and the differential CT method was used to plot stabilin-1 (STB1) and MMR expression in the injected hemisphere relative to the naïve hemisphere (NI) (C). Immunofluorescent staining for tomato lectin (green) and stabilin-1 (red) was performed on 18 µm thick section from brains extracted 7 days post-glioma injection (D). Scale bar is 170 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

numbers between the non-injected and injected hemisphere (Fig. 2A). Furthermore, no differences in macrophage, lymphocyte or microglial populations either in composition or cell numbers between the non-injected hemisphere and the injected hemisphere were apparent (Fig. 2A). Of the cells analyzed, stabilin-1 expression was detected on macrophages and microglia but not lymphocytes (Fig. 2B). The proportion

of stabilin-1 expressing macrophages (Fig. 2C) and the levels of expression (Fig. 2D) were not significantly different between the injected and the non-injected hemisphere at day 7 post-injection.

By day 14, although there were no significant differences observed in the total number of mononuclear cells recovered from the injected and non-injected hemisphere, the increase



**Fig. 2 – Stabilin-1 expression on macrophages. Brain mononuclear cells were extracted, counted and stained for CD45 and CD11b allowing for differentiation of lymphocyte, macrophage and microglial populations (A). Cells were also stained for stabilin-1, and cells expressing stabilin-1 were identified (B). Proportion of total macrophages at days 7 and 14 post-injection which are positive for stabilin-1(C), and expression levels measured with mean fluorescent intensity for stabilin-1 at days 7 and 14 post-injection (D) were determined. NI: non-injected hemisphere, GL: glioma injected hemisphere.**

in glioma size was accompanied by an increase in infiltrating macrophage ( $p=0.0371$ ) and lymphocyte ( $p=0.0056$ ) numbers in the injected hemisphere (Fig. 2A). This indicates a growing

adaptive T-cell associated response. The proportion of stabilin-1 expressing macrophages were significantly lower ( $p=0.0322$ ) in the injected compared to the non-injected

hemisphere (Fig. 2C). Experiments were conducted on separate days and thus a direct comparison cannot be made between expression levels (MFI) between days 7 and 14. However, the level of stabilin-1 expression on infiltrating macrophages at day 14 was significantly lower ( $p=0.0329$ ) in the tumor associated hemisphere when compared to the non-injected hemisphere (Fig. 2D).

These data suggest that stabilin-1 is expressed on AAM $\emptyset$  and that this expression is less likely to be associated with the tumor environment as the glioma progresses.

### 2.3. Stabilin-1 expressing macrophages also express CD40, MMR

In order to define the phenotype of stabilin-1 expressing macrophages, CD40 and MMR expression was measured as representation of classical and alternative activation respectively. At day 7, stabilin-1 was present on both alternative and CD40+ macrophages (Fig. 3A). As the tumor progresses, there is a loss of stabilin-1 expression on AAM $\frac{1}{2}$  in the injected compared to the non-injected hemisphere by day 14 ( $p=0.0443$ ) (Fig. 3B). Proportions of CD40 expressing macrophages also expressing stabilin-1 are not significantly different between hemispheres.

A group analysis at day 7 post-injection reveals no differences in stabilin-1 expression on AAM $\emptyset$  population and MMR-macrophages. As the tumor progresses, stabilin-1 is preferentially expressed on AAM $\emptyset$  by day 14 (Fig. 3C) (two-way ANOVA  $p=0.0192$ ). Furthermore, there is a significantly larger population of CD40+ macrophages expressing stabilin-1 at day 7 (two-way ANOVA  $p=0.0312$ ) compared to CD40-macrophages. This expression dramatically shifts at day 14 resulting in preferential stabilin-1 expression on CD40-macrophage populations as opposed to M1 (Fig. 3D) (two-way ANOVA  $p=0.0279$ ).

This data suggests a shift in the cell phenotype which expresses stabilin-1 between days 7 and 14 post-injection. Initially, stabilin-1 is expressed on both CD40+ and MMR+ macrophage phenotypes. However, as the tumor progresses and stabilin-1 is downregulated, it is mostly expressed on the MMR+ macrophages and is dramatically reduced on CD40+ macrophages.

## 3. Discussion

In this report, we stereotactically injected GL-26 tumor cells in the right hemisphere of mice brains to determine the phenotype and progression of the macrophage response during the first two weeks of tumor growth. GL-26 cells are very invasive, proliferate fast and form a glioblastoma in the mouse with similar properties to the glioblastomas seen in humans.

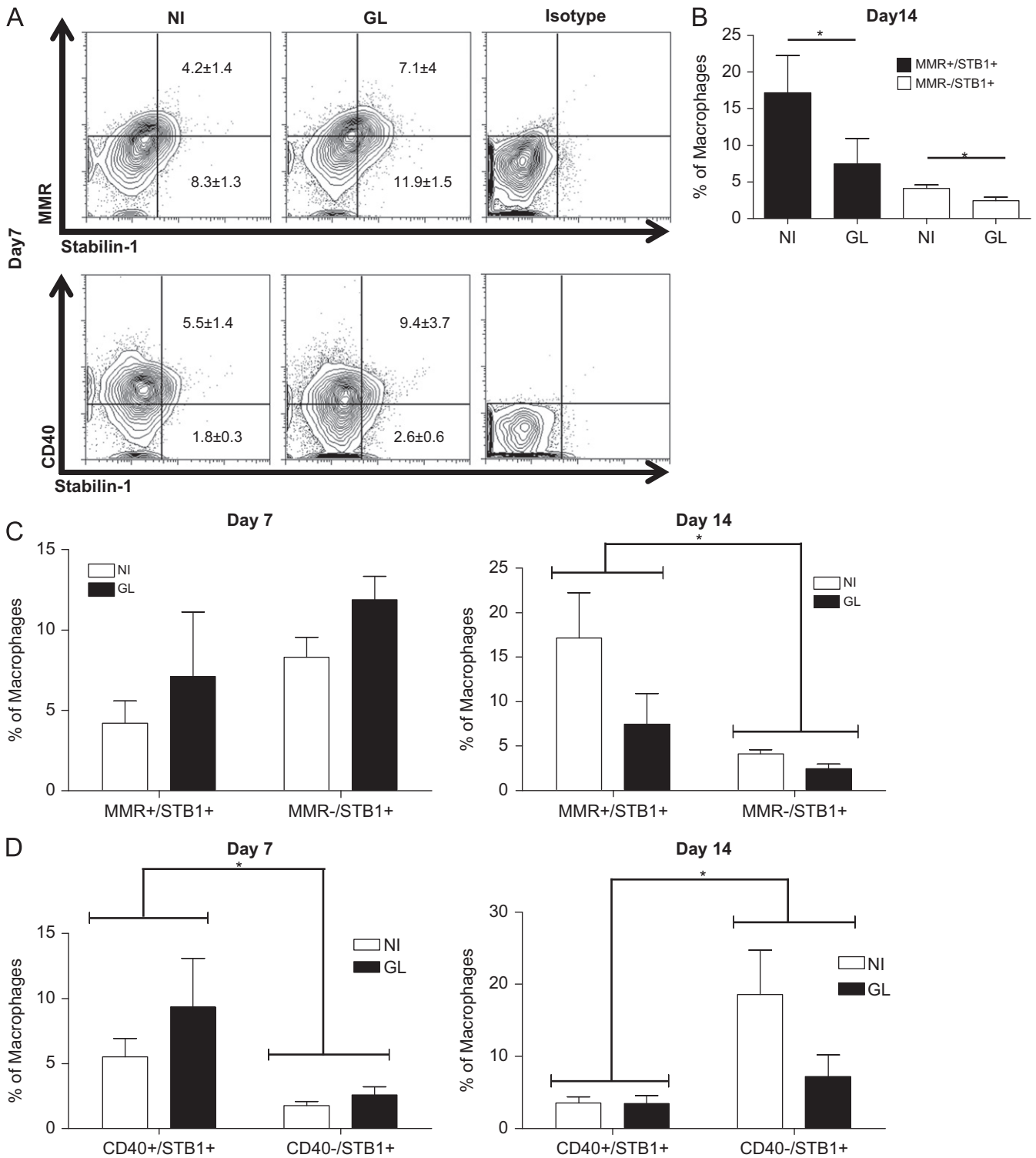
The role of SPARC in tumor proliferation and metastasis has been described since the late 1990s, but besides recent siRNA gene silencing of SPARC, no study has addressed a mechanism for blocking or degrading SPARC as a potentially therapeutic target in the control of gliomas (Rempel, 1998; Golembieski et al., 1999; Schultz et al., 2002; Rich et al., 2003; Seno et al., 2009; Shi et al., 2007). Stabilin-1 is the only known

receptor of SPARC and works as a scavenger receptor, internalizing and degrading bound SPARC (Kzhyshkowska, 2006; Kzhyshkowska et al., 2006). Being expressed on macrophages, stabilin-1 has the potential of being expressed in the tumor microenvironment and slow the tumor proliferation by decreasing local SPARC levels.

It is well known, however, that TAMs change phenotypes with the progression of the tumor (Ruffell et al., 2012; Mantovani et al., 2002; Luo et al., 2006; Talmadge, 2007; Solinas et al., 2009; Gordon and Martinez, 2010; Zhang et al., 2011). Indeed, as the tumor progresses, factors that promote the pro-tumorigenic polarization of TAMs can be divided into those derived from the immune system, from the tumor cells or from tissue stress. TAMs receive IL-10, IL-4 and IL-13 signaling from T-regs and Th2 cells, IL-10 and CCL2 signals from the tumor cells and sense hypoxia and ECM degradation in the tumor microenvironment. These signals cause the TAMs to in turn suppress the immune system with CCL22 and ROS secretion, promote tumor invasion through ECM remodeling and EGF secretion and finally, promote angiogenesis at the tumor site via VEGFA secretion (Ruffell et al., 2012; Mantovani et al., 2011). Though macrophages have the potential of expressing the SPARC scavenger receptor stabilin-1, it is unknown whether TAMs express stabilin-1 and whether this expression is maintained when TAMs become pro-tumorigenic. It should be noted that TAMs are known to also secrete SPARC. Whether SPARC expressing TAMs are also expressing stabilin-1 in a self-regulatory process or whether these are two distinct macrophage populations is not known and warrants further investigation. Our studies show a time dependent shift in the stabilin-1 expressing population. The disappearance of this phenotype as the tumor progresses may be due to tumor derived signals or intrinsic to the function of these macrophages leading to the dominant presence of SPARC.

In this report, we examined the macrophages in the tumor microenvironment for stabilin-1 expression during the initial progression of the tumor. We show an initial upregulation of stabilin-1 transcripts in the hemisphere injected with GL-26 cells which is not maintained 2 weeks post-injection. Furthermore, we show an initial population of stabilin-1 expressing macrophages which is significantly reduced at day 14 post-injection. At day 7, the stabilin-1 expressing macrophages are activated as demonstrated by their high co-expression of CD40. At day 14 however, the macrophages still expressing stabilin-1 are more polarized towards the M2 phenotype, as seen by the co-expression of MMR. These results suggest an initial anti-tumor attempt by the activated macrophage to express stabilin-1 in the tumor environment. Possibly due to signals from the tumor cells and tissue disruption, TAMs stop expressing stabilin-1 as they switch phenotypes to one that promotes tissue remodeling and angiogenesis.

The question that remains is obviously whether or not stabilin-1 expressing TAMs could possibly slow tumor progression or change the tumor microenvironment. Though we have not conducted these studies, overexpressing stabilin-1 in macrophages in glioma bearing mice would be a crucial next step in characterizing the role of this subset of macrophages in the tumor environment. In addition, although our model uses



**Fig. 3** – Stabilin-1 expressing macrophages change as the tumor progresses. **Stabilin-1 expressing macrophage populations were analyzed for co-expression of MMR and CD40 at day 7 post-injection (A).** Analysis of proportions of stabilin-1 (STB1+) macrophages for co-expression of MMR at day 14 (B). Macrophage populations in glioma brains at days 7 and 14 post-injection were analyzed for M1 and M2 phenotypes using MMR+/STB1+ or MMR-/STB1+ (C) and CD40+/STB1+ or CD40-/STB1+ (D).

the GL-26 line due to its similarity in aggressiveness and proliferative capabilities to human glioblastomas, the tumor in human glioblastomas differs from the mouse GL-26 line.

Indeed, human glioblastomas arise from several diverse cell types which can originate from neural, myeloid and glial origins (Seyfried, 2001; Yuan et al., 2004; Huysentruyt et al., 2011).

What role stabilin-1 could play in this situation remains to be investigated.

It has been shown through siRNA targeting of SPARC that depletion of SPARC slows tumor progression and metastasis (Seno et al., 2009; Shi et al., 2007). Though these results are exciting, they are not therapeutically relevant to humans. However, degradation of SPARC through stabilin-1 in our own macrophages deserves more attention as a potential target for glioblastoma therapy.

## 4. Experimental procedures

### 4.1. Mice and injections

Female C57BL/6 were obtained from Jackson Laboratories and maintained in a pathogen free environment under IACUC established protocols at the University of California Riverside. The murine (C57BL/6) glioma cell line, GL-26, which is highly tumorigenic in the C57BL/6 mice, was obtained as a generous gift from Dr. Pedro Lowenstein. GL26 cells were cultured in DMEM/F12 supplemented with 10% FCS, 1% penicillin/ streptomycin, 1% L-glutamine and 1% non-essential amino acids. Cultured GL-26 cells were then harvested by trypsinization and 90,000 GL-26 cells in 3  $\mu$ l of sterile PBS was injected intracranially 1 mm anterior and 2 mm lateral to the junction of the coronal and sagittal sutures (bregma) at a depth of 2 mm using a stereotactic mouse frame. Mice were anesthetized with ketamine (1:5) and xylazine (1:50) in sterile PBS with volume injected determined by individual mouse weight.

### 4.2. Histology

Mice were perfused intracardially with 4% paraformaldehyde (PFA) in PBS and brains were extracted, incubated in 4% PFA overnight and then 30% sucrose in PBS until brains equalized. Next, brains were flash frozen in isopentane, embedded in OCT and cryosectioned in coronal sections (12  $\mu$ m). Sections were stained with eosin and haematoxylin.

### 4.3. Real time PCR

Stabilin-1 specific primers (forward 5'-GCTTTGAACCTCAGC-CACTC-3' and reverse 5'-GGCAATTACACTGCCCACTT-3') and macrophage mannose receptor specific primers (forward 5'-CTGTTCAGCTATTGGACGC-3' and reverse 5'-TGGCACTCC-CAAACATAATTT-3') for real time PCR were purchased from IDT's primer Quest (<http://www.idtdna.com/Scitools/Applications/Primerquest/>). cDNA synthesis and real-time PCR were performed using the Bioline One-step Kit with the CFX-96 real-time PCR Detection System (Bio-Rad). The reaction total was a 20  $\mu$ l mixture with 10  $\mu$ l SYBR Green/SensiFAST qPCR Master Mix (2x) and 400 nM primer. The reaction conditions were as follows: 10 min at 45  $^{\circ}$ C, followed by 2 min at 95  $^{\circ}$ C and then 40 cycles of 5 s at 95  $^{\circ}$ C and 20 s at 60  $^{\circ}$ C. The HPRT (Hypoxine Phosphoribosyl-Transferase) forward primer (5'-CCCTCTGGTAGATTGTGCGTTA-3') and reverse primer (5'AGATGCTGTTACTGATAGGAAATTGA-3') were used as an endogenous control. Quantified results represent the fold

induction of target gene expression using the differential CT method. NTC, no-template control (reagent alone without template) was included in each assay to detect any possible contamination of the PCR reagents.

### 4.4. Preparation of brain mononuclear cells (BMNCs) suspension

For BMNCs, harvested brain tissues were minced with a razor blade and passed multiple times through an 18-gauge needle. The suspension was then incubated with 100  $\mu$ l of collagenase/dispase (1 mg/ml) (Roche Diagnostics, Indianapolis, IN) for 45 min at 37  $^{\circ}$ C and then a further 45 min at 37  $^{\circ}$ C with 300  $\mu$ l DNase (10 mg/ml) (Sigma). Following enzymatic digestion, the cell suspension was passed through a 70  $\mu$ m cell strainer, resuspended in 40 ml of RPMI complete and centrifuged at 1200 rpm for 10 min at 4  $^{\circ}$ C. The pellet was resuspended in 60% isotonic Percoll (GE Healthcare Bioscience, Uppsala, Sweden) solution (in RPMI complete) and overlaid with 30% percoll solution made in 1  $\times$  sterile PBS. The Percoll gradient was centrifuged at 2000 rpm for 25 min at 25  $^{\circ}$ C without brakes. After centrifugation, the myelin layer on top of the gradient was removed. BMNCs (lymphocytes, macrophages, dendritic cells and microglia) were harvested from the 30% to 60% percoll interphase and washed twice in complete RPMI medium for further analysis.

### 4.5. Immunofluorescent staining

Brains from C57BL/6 mice injected with gliomas were extracted 7 days post-injection and frozen in OCT. Serial section (18  $\mu$ m) were cut and incubated with primary antibodies for stabilin-1 (Santa Cruz antibodies) and biotinylated tomato lectin for 3 h at room temperature. Secondary antibodies (streptavidin 488 and donkey anti goat 568) were incubated for 2 h in room temperature. Slides were then mounted with ProLong Gold anti fade reagent with DAPI (Life Technologies).

### 4.6. Flow cytometry

1  $\times$  10<sup>6</sup> BMNCs were placed in FACS tubes (BD Falcon<sup>TM</sup>, MA, USA), centrifuged at 1200 rpm for 5 min at 4  $^{\circ}$ C, and resuspended in FACS buffer (1X PBS containing 4% BSA, 0.01% EDTA). After a further round of centrifugation the cells were pre-incubated with a saturating solution of FC block (eBioscience, San Diego, USA) for 5 min on ice and stained with various conjugated antibodies against CD11b, CD45, and CD40 (purchased from eBioscience), MMR (biolegend, San Diego, CA) as well as a non-conjugated antibody against rabbit anti stabilin-1 (Santa Cruz antibodies, Santa Cruz, CA) for 30 min on ice. Samples stained with stabilin-1 went through a secondary incubation with anti-rabbit Alexa Fluor 647 for 30 min on ice. Cells were washed with FACS buffer and analyzed using the BD FACSCanto<sup>TM</sup> II flowcytometer and FlowJo analysis software v.8.7.3.

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