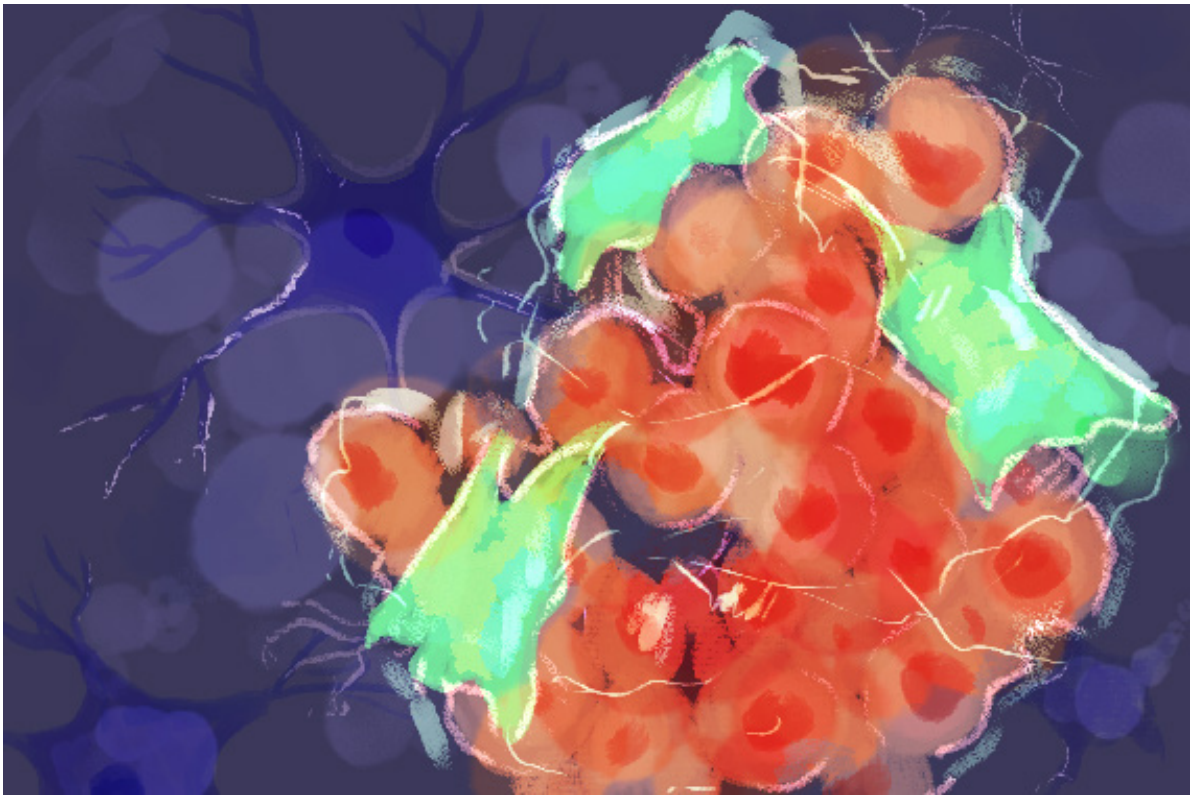
The Influence of Cancer Associated Fibroblasts on Glioblastoma Tumor Migration

Eugene Cho

Volume 3, Issue 2

Article Synopsis

Glioblastoma is a complex cancer that resides in an intricate tumor environment in the brain. Cancer associated fibroblasts have previously shown to contribute to tumor expansion, and similar cells have been found in the brain of Glioblastoma patients. This study demonstrates the influence of cancer associated fibroblasts on tumor cell migration which may contribute to patient outcomes.



Graphic by Amanda Li

The Influence of Cancer Associated Fibroblasts on Glioblastoma Tumor Migration

Eugene Cho

https://doi.org/10.55894/dv3.25

ABSTRACT

Glioblastoma (GBM) is a malignant central nervous system tumor with a low patient prognosis. Given this challenging outlook, there has been a great interest in the development of immunotherapies for patients with GBM. To enhance the efficacy of immunotherapy, researchers have studied the immunosuppressive nature of the GBM tumor microenvironment (TME). One element of the TME that is of particular interest is cancer associated fibroblasts (CAFs). Often found in carcinomas, previous studies of single cell RNA sequencing have revealed CAF-like cells in the GBM TME, which promotes angiogenesis and hinder immune cell infiltration. This study revealed that GBM tumor cells and GBM CAFs cannot be easily differentiated by the following surface markers: CD45, CD73, CD90, CD49e, CD105, CD140b, and CD29. Additionally, the presence of GBM CAFs was found to induce GBM tumor cell migration and the addition of IL-12 to the GBM CAFs was found to reduce GBM tumor cell migration. This project aimed to understand the differences in surface markers of GBM tumor cells and GBM CAFs, as well as to explore the influence of GBM CAFs and the cytokine IL-12 on GBM tumor cell migration. This research can supplement existing efforts to increase the efficacy of immunotherapies when trying to target GBM.

INTRODUCTION

Glioblastoma (GBM) is the most common malignant tumor of the central nervous system with a median prognosis of about 15 months (Weenink et al., 2020). Many efforts have been made to treat patients with GBM through chemotherapy and radiation; however, there is no standardized method of treatment proven to be effective for all GBM patients (Ostrom et al., 2017). The heterogeneity of GBM contributes to difficulties in treating patients at the population level. Therefore, immunotherapy has been widely explored as a way to treat GBM, by taking advantage of patient-specific characteristics and recruiting a patient's own immune cells to target the tumor (Weenink et al., 2020). By activating T-cells and other immune cells, their ability to infiltrate and target tumors can be further optimized to improve patient prognosis.

Tumor Microenvironment

To study the efficacy and optimization of immune cell infiltration in GBM, elements in the tumor's microenvironment must be considered. Although immune cells are able to cross the blood-brain barrier and target GBM tumor cells, its immunosuppressive tumor microenvironment (TME) reduces the efficacy of immune cell infiltration (Genoud & Migliorini, 2021). In other words, immune cells are easily exhausted in the GBM TME. The TME is made up of cancer cells, brain cells, and immue cells, including microglia, natural killer (NK) cells, and endothelial cells (Sharma et al., 2023). Elements in the tumor microenvironment communicate with other immune cells through the release of cytokines and chemokines. These soluble factors can be involved in both tumor progression and suppression (Abdul-Rahman et al., 2024). Specifically, the cytokine interleukin 12 (IL-12) has been essential in directing T-cells into the tumor microenvironment and exhibit antitumor properties (Abdul-Rahman et al., 2024). IL-12 has been of great interest in immunotherapies

with its abilities to increase the persistence of T-cells, despite its toxicity in clinical studies (Agliardi et al., 2021). It will be important to explore the additive effect of IL-12 on GBM tumor cell migration.

Cancer Associated Fibroblasts

One element of the TME that is of particular interest is cancer-associated fibroblasts (CAFs). CAFs have been detected in carcinomas, like breast cancer and lung cancer, where they influence the surrounding tumor environment. They contribute to angiogenesis, meaning the growth of new blood vessels, encourage tumor expansion, and reduce the efficacy of immunotherapies (Galbo et al., 2022; Zarodniuk et al., 2023). Additionally, CAFs are largely responsible for remodeling and altering the extracellular matrix (ECM) (Sahai et al., 2020). This remodeling can make it even more challenging for immune cell infiltration and the killing of tumor cells.

Although fibroblasts are not found in the brain, studies have shown the presence of CAF-like cells or mesenchymal stem cells (MSCs) that share characteristics with CAFs found in carcinomas (Jain et al., 2021). Using single-cell sequencing, researchers have confirmed the presence of CAF-like cells in the GBM TME. Furthermore, single cell RNA sequencing has revealed shared characteristics between CAFs and GBM CAFs, like shared surface markers and their role in tumor progression (Galbo et al., 2022; Jain et al., 2021). Some shared surface markers include fibroblast-specific protein 1 (FSP1), alpha-smooth muscle actin (αSMA), fibroblast activation protein (FAP), PDGFRA, and PDGFRB (Galbo et al., 2022; Zarodniuk et al., 2023). Further, studies have identified surface markers on CAFs, to differentiate it from other cell types in the TME. CAFs were found to express CD73, which is involved in tumor adhesion, proliferation, and chemoresistance and CD140b (PDGFRb), a CAF marker known to be important in CAF formation and promote angiogenesis (Jain et al., 2021; Yan et al., 2019). Additionally, CD29 is integrin B1 and is found to be characteristic of CAFs in breast cancer. Lastly CD49e is integrin a5 that binds to fibronectin, which mediates the relationship between CAFs and cancer cells (Galbo et al., 2022; Han et al., 2020; Pelon et al., 2020).

GBM CAFs are of interest due to their ability to promote angiogenesis, modify the GBM extracellular matrix (ECM), and create a physical barrier to infiltrating immune cells in the brain (Galbo et al., 2022). More specifically, the abundance of GBM CAFs can alter the ECM, promoting GBM migration (Zarodniuk et al., 2023). GBM patients were found to have greater tissue remodeling compared to patients with low-grade gliomas. Further, patients with a greater abundance of perivascular fibroblasts experienced poorer prognosis and survived for a shorter period of time (Zarodniuk et al., 2023). Consequently, this finding suggests that the presence of increased fibroblasts contributes to the immunosuppressive TME, resulting in a reduced immune response. Other studies have demonstrated that fibroblasts serve as a chemical and physical barrier against immune cells by secreting cytokines and exhausting and reducing the efficacy of immune cells that try to infiltrate (Hilmi et al., 2020).

Current Study

This project aims to explore the differences between tumor cells and GBM CAFs and to determine the influence of GBM CAFs on GBM tumor cell migration using flow cytometry and transwell migration assays. Additionally, the project aims to determine the role of IL-12 on GBM migration when added to GBM CAFs. It is hypothesized that GBM tumor cells and GBM CAFs can be differentiated by the following surface and intracellular markers: CD45, CD73, CD140b, GFAP, CD29, and CD49e. It is also hypothesized that the presence of GBM CAFs will promote GBM cell migration; however, the addition of IL-12 to GBM CAFs will reduce GBM tumor cell migration. This project will add to the existing knowledge about the effects of the immunosuppressive GBM TME by providing insight about CAF characteristics and its role on GBM tumor cells.

METHODS

Cell Culture

The U87 GBM mouse cell line was cultured in Dulbecco's Modified Eagle Media (DMEM) and 10% Fetal Bovine Serum (FBS). The U87 cells were thawed

into a T75 flask and once 80-90% confluent (about 2 days after), the cells were passaged with 0.05% Trypsin ethylenediaminetetractic acid (ETDA), centrifuged, counted, and split based on their cell counts. The cells were then frozen down at passage 8 when about 90 million cells were cultured.

The GBM CAFs (Vitro Biopharma, Cat. No. CAF03) were cultured in VitroPlusIII Low Serum, Complete Medium (Vitro Biopharma, Cat. No. PC00B1). The GBM CAFs were thawed in a T150 flask and split once 80-90% confluent (7 days) as per supplier protocol.

Identification of Surface Markers through Flow Cytometry

Flow cytometry is a method of identifying the shape and size of cells, as well as intracellular and extracellular markers present on the cells. The flow cytometry panel was developed using surface and intracellular markers found in the GBM CAFs based on existing literature. Additionally, the fluorescent colors of antibodies were determined by comparing the excitation wavelengths of the different antibodies of interest. When compared to the fluorescent expression of a one cell to that of a second cell, a shift in the expression of the second cell would indicate a difference in the expression of the surface marker of interest between the two cells. Whereas if there is not a difference in the shift in the two cells, that would indicate no difference. The GBM CAFs and U87 cells were stained for the antibodies and prepared for flow (Table 1).

Table 1: Flow panel for CAF markers of interest

	Marker	Laser Line	Color	Location
1	Live/Dead	UV6	Zombie UV	
2	CD45	UV16	Buv805	Extracellular
3	CD73	V3	PacBlue	Extracellular
4	CD90	V10	BV605	Extracellular
5	GFAP	B2	AF-488	Intracellular
6	CD49e	YG1	PE	Extracellular
6	CD105	YG9	PE-Cy7	Extracellular
8	CD140b	R1	APC	Extracellular
9	CD29	R7	APC-Cy7	Extracellular

Transwell Migration Assay

While GBM CAFs were initially cultured in VitroPlusIII Low Serum, Complete Medium, the GBM CAFs were cultured in DMEM with 10% FBS for the transwell migration assay. By splitting the GBM CAFs into three flasks containing VitroPlusIII Low Serum, Complete Medium, DMEM/10% FBS and Advanced DMEM/F12, it was determined that DMEM/10% FBS promotes the growth of the GBM CAFs and therefore can be used for the transwell migration assay. Transwell assays are important in assessing how cells respond to certain chemo-attractants (like cytokiines) of interest. The transwell plate (Thermo Scientific™, Cat. No. 141006) had an 8μM filter, with an approximate distance of 1.0mm from the insert to the bottom membrane.

GBM CAF-conditioned media (CM) was prepared by seeding 25,000 CAFs into the number of wells needed for transwell migration towards CAF CM in 10% FBS. Following 24 hours, the media was removed and the CAFs were washed with phosphate-buffered saline (PBS). Serum-free media was added to the wells and left to culture. After 72 hours, the media was collected and immediately used for transwell migration.

The transwell migration assay included 9 groups for testing, which were arranged onto three 24-well plates (Table 2 & Table 3). The top insert contained the GBM tumor cells, and the bottom insert contained GBM CAFs, GBM CAFs supplemented with IL-12, CAF CM, or CAF CM supplemented with IL-12. This transwell was designed to test the migration of GBM tumor cells downward towards the GBM CAFs and CAF CM.

Table 2 Transwell migration conditions

Well	Top Insert	Bottom of Well	Justification
1	Empty	CAF cells	CAF control for counting/flow
2	Empty	U87	Control for 100% migration
3	Empty	U87 & CAF CM	Control for 100% migration in soluble factors
4	U87	DMEM/2% FBS	Negative Control
5	U87	DMEM/30% FBS	Positive Control
6	U87	CAF cells	Migration towards CAF cells
7	U87	CAF CM	Migration toward CAF soluble factors
8	U87	CAF & IL-12	IL-12 and CAF induced migration
9	U87	CAF CM & IL-12	IL-12 and CAF soluble factors induced migration

Table 3: Layout of three 24 well plates used for transwell migration.

1A	1B	1C	1D	1E	1F	4A	4B	4C	4D	4E	4F	6A	6B	6C	6D	6E	6F
2A	2B	2C	2D	2E	2F	5A	5B	5C	5D	5E	5F	7A	7B	7C	7D	7E	7F
3A	3B	3C	3D	3E	3F							8A	8B	8C	8D	8E	8F
												9A	9B	9C	9D	9E	9F

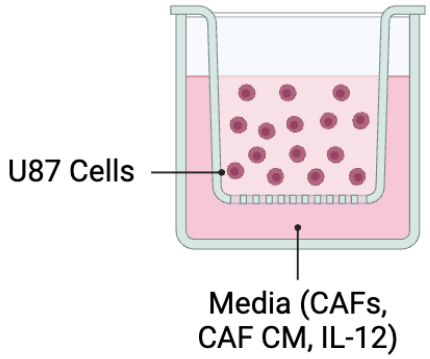


Figure 1: Layout of transwell migration insert with U87 tumor cells in the top insert and CAF media on the bottom.

Following the transwell assay, the U87 tumor cells and GBM CAFs were dyed using membrane dye to differentiate between the two cell types. GBM CAFs were thawed in a T150 flask and dyed with CellTracker Green CMFDA, then 25,000 GBM CAFs were seeded into the bottom chamber of the transwell. The following day, the U87 tumor cells were dyed using CellTrace Red and 100,000 U87 cells were seeded into the insert or bottom of the transwell. Then the U87 cells were left to migrate for 24 and 72 hours (Figure 1). Fluorescent images of the cells were taken (Figure 4). Afterwards, the cells were fixed and prepared for analysis using flow cytometry.

RESULTS

Differentiation of CAFs and GBM CAFs through Flow Cytometry

Upon running flow cytometry on the GBM CAFs and U87 cells, differentiation of the two cell types was not possible. Although there were differences in the expression of some surface markers, the expression of other surface markers was too similar between the U87 cells and GBM CAFs. There were differences in expressions of CD45, CD73, and CD140b, which were demonstrated by the shift in red peak (stained U87 cells) from the stained blue peak (stained GBM CAFs) (Figure 2). However, there were no differences in expression of GFAP (glial fibrillary acidic protein), CD29, and CD49e (Figure 3). The overlap in GBM CAF and U87 peaks demonstrate similar expression

of the surface markers. Therefore, the U87 cells and GBM CAFs could not be confidently differentiated by these surface markers.

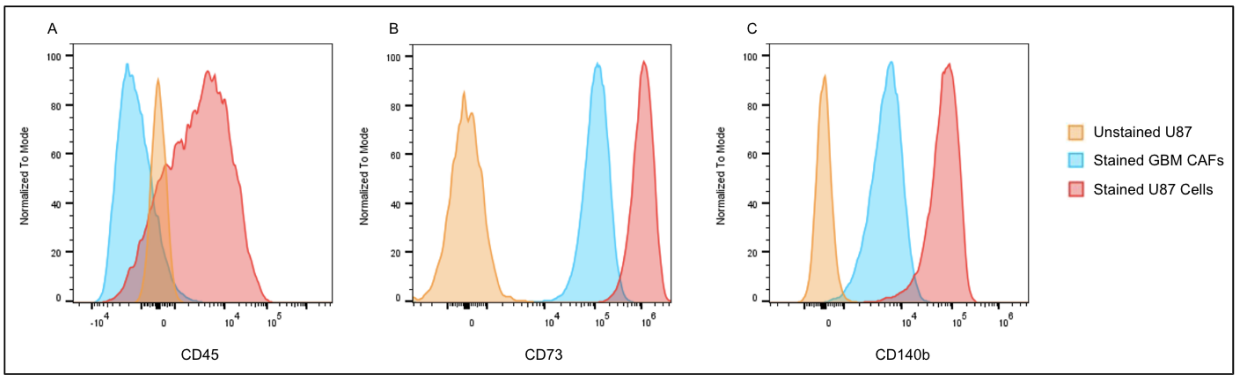


Figure 2: Flow histogram demonstrating that U87 cells and GBM CAFs can be differentiated by A) CD45 B) CD73 C) CD140b expression.

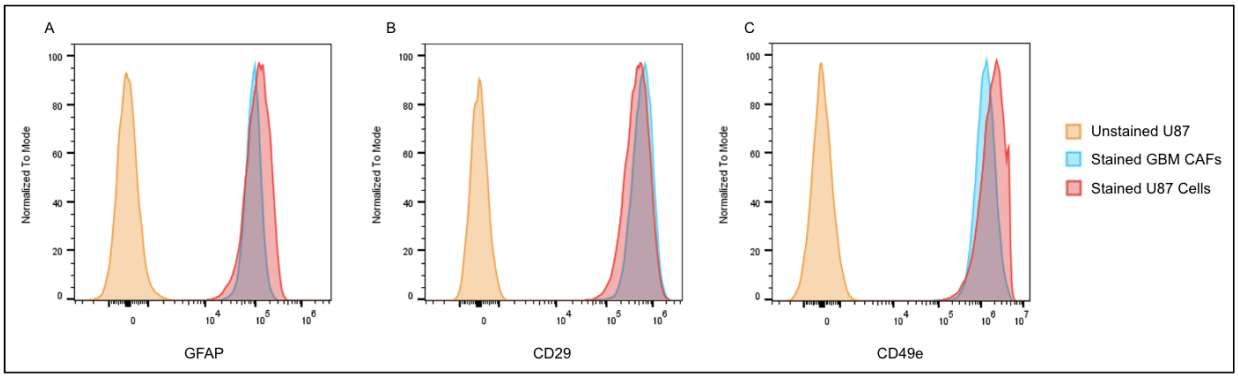


Figure 3: Flow histogram demonstrating that U87 cells and GBM CAFs cannot be differentiated by A) GFAP B) CD29 C) CD49e expression.

GBM CAF Transwell Migration Assay

Transwell migration assays allowed for the exploration of U87 tumor cell migration in response to the presence of CAFs. The U87 tumor cells were seeded in the transwell migration wells in different conditions. Figure 4 presents the CAF cells (Fig. 4A), U87 cells in DMEM (Fig. 4B), and U87 cells in CAF CM (Fig. 4C), seeded at the bottom of the inserts, showing the maximum number of cells that would be present given full migration.

While 100,000 U87 cells were seeded at the bottom of the wells prior to migration, the U87 cells were determined to have roughly doubled during the 72-hour migration period (Figure 5). In DMEM supplemented with 2% FBS, the U87 cell count increased, on average, to 220,220 cells, while in the CAF-conditioned media (CM), the U87 cell count increased to 226,480, on average. This difference was not statistically significant, highlighting the potential role that CAFs have in releasing cytokines that can be involved in CAF proliferation.

Additionally, migration of the U87 cells through the transwell inserts, with base media supplemented with varying amounts of FBS, was determined by the positive and negative controls (DMEM supplemented with 30% FBS and DMEM supplemented with 2% FBS respectively) (Figure 6). Based on the fluorescent image alone, differences in U87 migration in the two control groups were not identifiable. However, upon running the samples on flow, there were, on average, 23 and 61 cells for the negative and positive controls

respectively (Figure 6C).

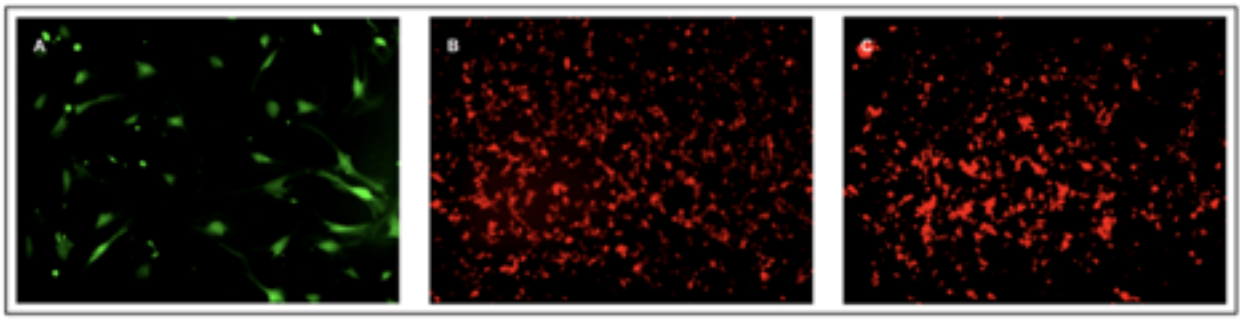


Figure 4: Fluorescent images of (A) CAF cells plated at the bottom of the well (B) U87 tumor cells plated at the bottom of the well in DMEM/2% FBS (C) U87 tumor cells plated at the bottom of the well in CAF-conditioned media.

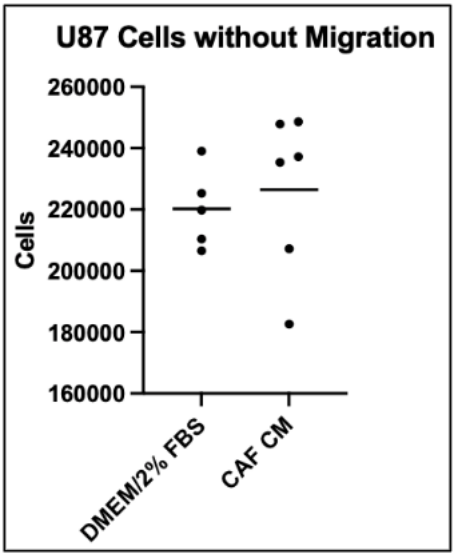
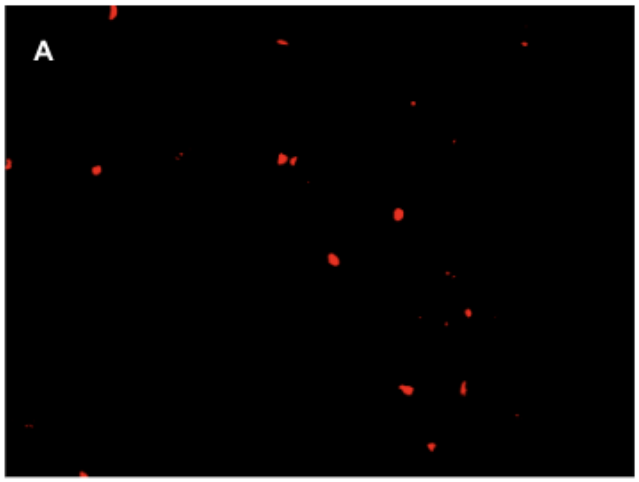
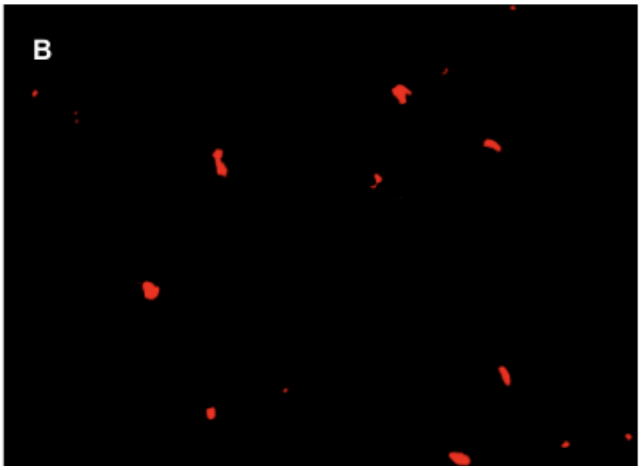


Figure 5: U87 cell count without migration following 72 hours.





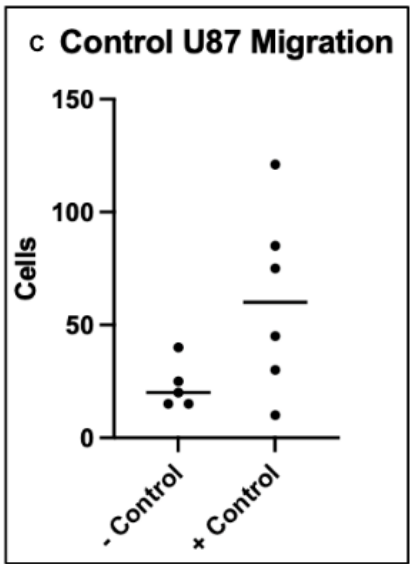


Figure 6: Fluorescent images of A) Negative control of U87 cell migration to DMEM/2% FBS (minimal growth factor) B) Positive control of U87 cell migration to DMEM/30% FBS. C) Cell counts of U87 cell migration in the positive and negative controls.

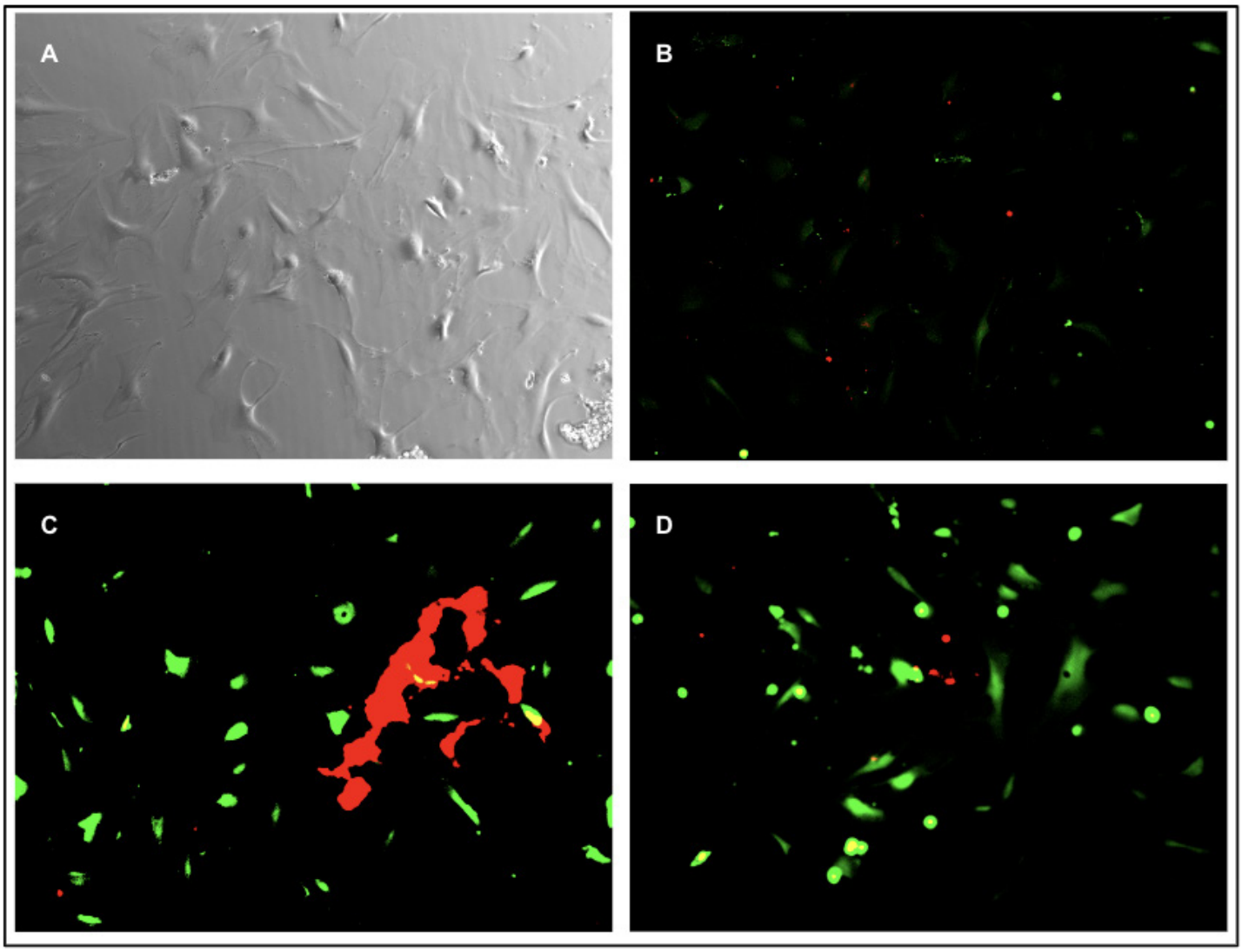


Figure 7: A) Brightfield image of U87 cell co-culture with CAF cells and B) Fluorescent image of U87 cells (red) that have migrated towards the CAF cells (green) C) U87 cells that have migrated towards CAF cells that appear to be clumped together C) U87 cells that have migrated towards the GBM CAFs supplemented with IL-12.

Fluorescent imaging shows U87 cells that have migrated towards the CAFs following a 72-hour migration. Visually, it is difficult to determine the extent to which the U87 cells have migrated towards the CAFs (Figure 7). In some instances, the U87 cells seem dead, forming clumps when co-cultured with the GBM CAFs (Figure 7c).

The number of U87 cells that have migrated towards the GBM CAFs was also quantified. More U87 cells migrated towards the GBM CAFs in 72 hours, than in 24 hours (Figure 8). On average, 23 U87 cells migrated towards GBM CAFs in 24 hours, while 121 U87 cells migrated towards GBM cells in 72 hours. 50,000 U87 cells were seeded in the top insert of the well, prior to the 24-hour migration period, while 100,000 U87 cells were seeded in the top insert prior to the 72-hour migration. Figure 8 demonstrates the percentage of U87 migration after 24 and 72 hours to demonstrate the percentage difference in migration. Following the 24-hour migration period, on average, 0.047% of U87 cells migrated towards the GBM CAFs, while following a 72-hour migration period, 0.121% of U87 cells migrated towards the GBM CAFs (Figures 8C and 8D).

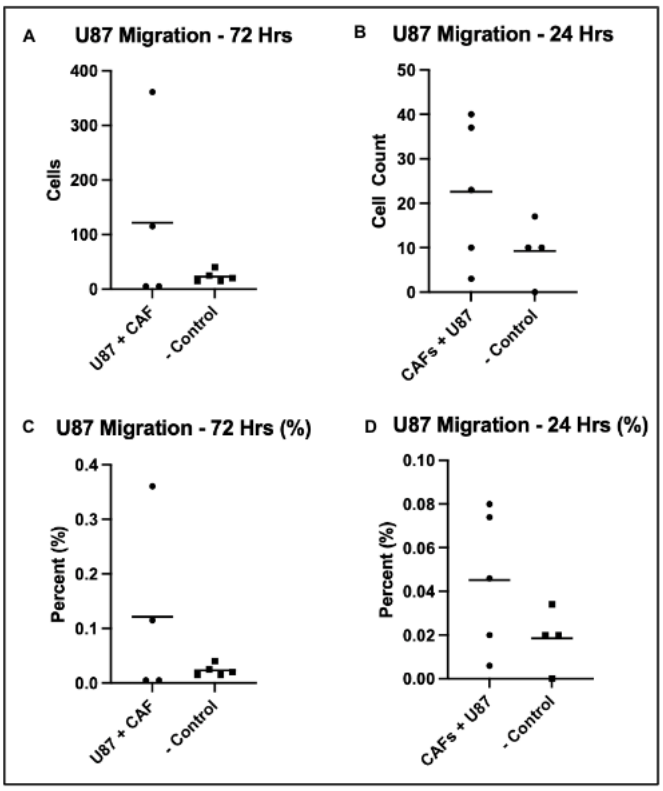


Figure 8: Number of U87 cells that have migrated following A) 72-hour migration period B) 24-hour migration period. Percentage of U87 cells that have migrated following C) 72-hour migration period D) 24-hour migration period.

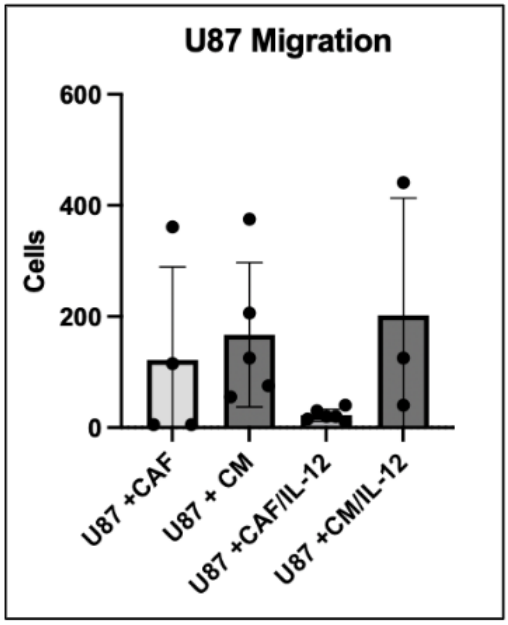
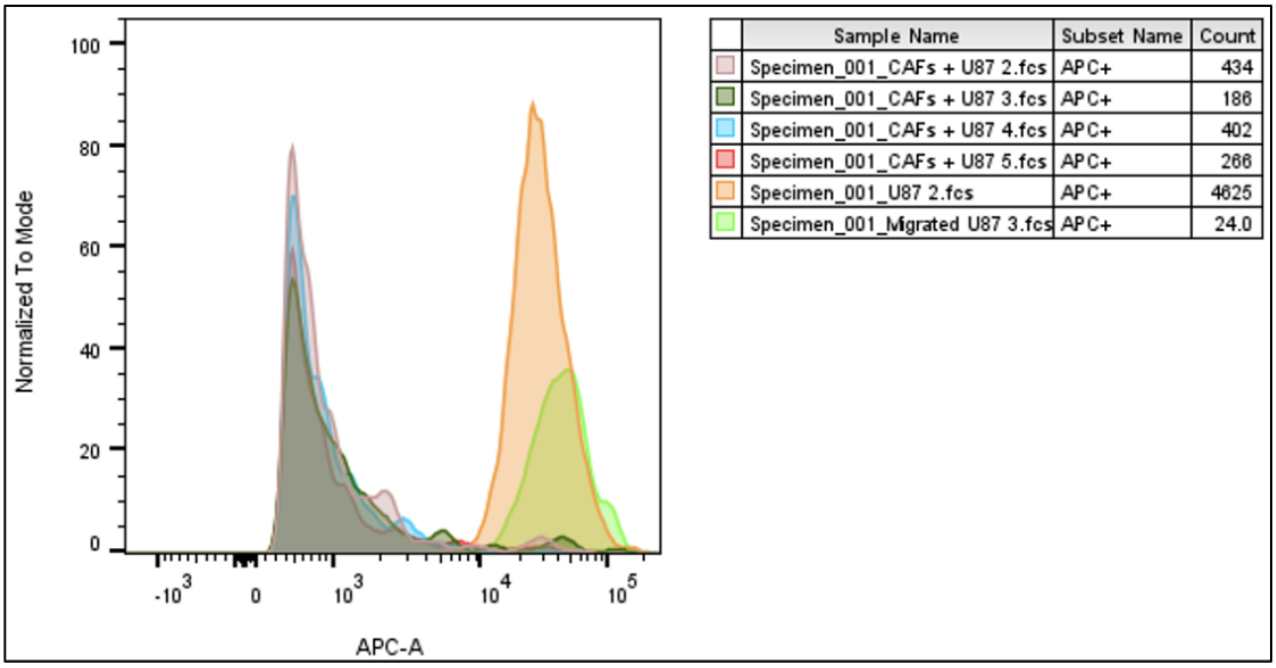


Figure 9: U87 cell migration towards GBM CAFs, GBM CAF Conditioned Media, GBM CAFs supplemented with IL-12 and GBM CAF Conditioned Media supplemented with IL-12.



	Sample Name	Subset Name	Count
	Specimen_001_CAFs + U87 2.fcs	APC+	434
	Specimen_001_CAFs + U87 3.fcs	APC+	186
	Specimen_001_CAFs + U87 4.fcs	APC+	402
	Specimen_001_CAFs + U87 5.fcs	APC+	266
	Specimen_001_U87 2.fcs	APC+	4625
	Specimen_001_Migrated U87 3.fcs	APC+	24.0

Figure 10: Proliferation of U87 cells can be seen by the dimming of the APC expression by cells on the left when co-cultured with the CAFs following migration.

Another observation was the proliferation of the U87 tumor cells when co-cultured with CAF cells. Following the initial 24-hour migration assay, the U87 cells exhibited diminishing expression of allophycocyanin (APC), a fluorescent protein that can stain cells of interest, which led to the hypothesis that the U87 could be proliferating upon interaction with the GBM CAF soluble factors. If the cells were proliferating, it would be expected that upon each divison, the cell would express reduced levels of APC as shown in figure 10. The U87 cells that migrated in the control conditions and that were cultured alone exhibited the strongest APC peak suggesting a lack of cell replication. However, when cultured with the CAF cells, the APC peak had shifted to the left, indicating reduced brightness and cell replication (Figure 10). Nevertheless, when repeated in the 72-hour migration assay, no reduced APC expression was detected, neither in the conditions with CAF cells nor with CAF CM.

DISCUSSION

Flow Cytometry

The flow cytometry data revealed that the U87 tumor cells and GBM CAFs cannot be differentiated. There were differences in GBM tumor cell and GBM CAF expression of the surface markers CD45, CD73, and CD140b, as hypothesized. It was also hypothesized that the GBM CAFs could be able to be differentiated from the U87 cells by the expression of the intracellular marker GFAP and the extracellular markers, CD29 and CD49e; however, there were no differences in expression of those markers. One interesting marker that was expressed by the GBM CAFs was GFAP, typically associated with astrocytes, which GBM CAFs do not express. GFAP supports the structures of astrocytes and the blood-brain barrier (Jurga et al., 2021). Nevertheless, it was listed as positive on the commercial data sheet for these primary human GBM CAFs and the positive expression of GFAP was confirmed in the flow panel.

Based on the conflicting flow cytometry data in CAF surface marker expression, differentiation between the U87 and GBM CAF cell types could not be made when co-cultured. Although there is no literature directly citing differences in the expression of these specific markers between GBM tumor cells and GBM CAFs, separate studies have identified CAFs using these surface markers. This suggests that CAFs could be identified by these markers, although this was not confirmed in the current study. Additional markers have been used to identify CAFs and CAF-like cells, specifically fibroblast associate protein (FAP) which could be assessed in the future. Although the difference in surface marker expression would have allowed for the ability to distinguish between cell types in a patient GBM sample, that was not possible due to the high similarity between the two cell types, that was not possible. Subsequently, the GBM CAFs and U87 tumor cells were dyed with a membrane dye to differentiate between the two cells during experiments to establish the role of GBM CAFs in GBM cell migration.

Transwell Migration

Following the 24-hour and 72-hour U87 migration, the assay demonstrated that the presence of the GBM CAFs did induce U87 cell migration, as hypothesized. Compared to the control conditions, there was an increase in U87 cell migration through the transwell insert. Further, similar amounts of the U87 cells migrated towards the GBM CAF cells and the GBM CAF CM. GBM CAF soluble factors, like IL-6, may induce U87 migration, yet further studies are necessary to confirm the CAF soluble factors (Kitzberger et al., 2023). Nevertheless, these values were not statistically significant and need further optimization to clarify the relationship between GBM CAFs in GBM cell migration.

After IL-12 was supplemented to the GBM CAFs, the number of U87 cells that migrated dropped considerably. This may be due to the antagonistic interactions between IL-12 and GBM CAF cells themselves, as hypothesized. However, CAF CM that was supplemented with IL-12 exhibited greater U87 cell migration. Although the mechanism for this induced migration is unclear, it is hypothesized that IL-12 may directly act on GBM CAFs to change the secretion of soluble factors and subsequent GBM migration.

Limitations & Future Directions

A limitation of the study is that there are other cells and factors in the TME that influence GBM tumor

migration, aside from just GBM CAFs. NK cells and macrophages are just a few examples of cells that can influence GBM tumor cell migration, and their roles cannot be excluded when exploring GBM tumor cell migration. Additionally, although flow cytometry is effective when a different cell type as the unstained control, the vast differences in size and characteristics between GBM tumor cells and GBM CAFs suggest it may be more effective to use an unstained GBM CAF sample for comparison. Had the unstained GBM control been included, changes in expression of the surface markers of the GBM CAF cells may have been more clearly noted. Further, running flow cytometry on the same surface markers for a CAF originally from another cancer type, like breast cancer, may have also been useful in distinguishing the differences between GBM CAF and carcinoma CAF surface markers. Lastly, due to the GBM CAFs being commercially purchased, they may not be the best representation of GBM CAFs taken directly from a patient's TME. However, there are challenges in culturing GBM CAFs, as they require more care and time.

For future study designs, rather than using the commercially available GBM CAF line, dissociating GBM CAFs from a patient tumor sample will result in GBM CAFs that are more representative of cells that are directly interacting with GBM tumor cells in the TME. The commercially available GBM CAF line may have caveats due to some of the surface markers identified (like GFAP) that would not otherwise be found in the GBM CAF cells. Additionally, there must be greater efforts to optimize the transwell migration assay protocols. The interest of this project was of U87 cell migration, which made the number of CAFs present in the transwell irrelevant. However, upon counting the cells present in the assay, no CAFs were counted. This may be due to an error in the harvesting of the cells. Following transwell migration, the U87 and CAF co-culture can be collected to further explore the CAF soluble factors that may influence U87 migration. An enzyme-linked immunosorbent assay (ELISA) or multiplex ELISA like a LegendPlex would be a good approach to gain a better understanding of the soluble factors influencing U87 migration. ELISAs can detect specific cytokines and other soluble factors that are released; therefore, it may be useful to understand what soluble factors CAFs are releasing which influence GBM tumor cell migration. Further, due to specific interests in the role of GBM tumor cell migration and growth due to the presence of CAFs in the brain, as well as the prevention of immune cell proliferation due to immune cell exhaustion markers and a physical barrier, a mouse study could be conducted. Injecting varying amounts of CAFs into the brain alongside the CT2A tumor cell line can help determine the effects of CAFs on tumor progression and prognosis.

CONCLUSION

With the pervasive interest in immunotherapies to treat patients with GBM, it is important to consider the GBM tumor microenvironment to optimize immune cell infiltration. GBM CAFs have been reported to exhibit similar characteristics as CAFs found in carcinomas and further have been determined to create an immunosuppressive environment surrounding the tumor and encourage angiogenesis and ECM modification in the GBM TME. The present study showed that some markers differentiated GBM cells from GBM CAFs, while others did not. GBM CAFs promoted GBM migration, with IL-12 reducing migration when applied to GBM CAFs. However, GBM migration was increased with added IL-12 in CAF-conditioned media. Refining assays and using patient-derived CAFs could identify key soluble factors, highlighting importance of the TME in optimizing GBM immunotherapy. Further studies are necessary to optimize transwell migration assay conditions and to understand the specific soluble factors through which the GBM CAFs are promoting U87 cell migration.

REFERENCES

1. Clavreul, A., & Menei, P. (2020). Mesenchymal Stromal-Like Cells in the Glioma Microenvironment: What Are These Cells? *Cancers*, *12*(9), 2628. https://doi.org/10.3390/cancers12092628

2. Galbo, P. M., Liu, Y., Peng, M., Wei, Y., Madsen, A. T., Graff, S., Montagna, C., Segall, J. E., Sidoli, S., Zang, X., & Zheng, D. (2022). *Functional Contribution of Cancer-Associated Fibroblasts in Glioblastoma*. https://doi.org/10.1101/2022.04.07.487495

3. Genoud, V., & Migliorini, D. (2021). Challenging Hurdles of Current Targeting in Glioblastoma: A Focus on Immunotherapeutic Strategies. *International Journal of Molecular Sciences*, *22*(7), 3493. https://doi.org/10.3390/ijms22073493

4. Han, C., Liu, T., & Yin, R. (2020). Biomarkers for cancer-associated fibroblasts. *Biomarker Research*, *8*(1), 64. https://doi.org/10.1186/s40364-020-00245-w

5. Jain, S., Rick, J. W., Joshi, R., Beniwal, A., Spatz, J., Chang, A. C.-C., Nguyen, A. T., Sudhir, S., Chandra, A., Haddad, A., Wadhwa, H., Shah, S. S., Choi, S., Hayes, J. L., Wang, L., Yagnik, G., Costello, J. F., Diaz, A., & Aghi, M. K. (2021). *Identification of Cancer-Associated Fibroblasts in Glioblastoma and Defining Their Pro-tumoral Effects*. https://doi.org/10.1101/2021.05.08.443250

6. Jain, S., Rick, J. W., Joshi, R. S., Beniwal, A., Spatz, J., Gill, S., Chang, A. C.-C., Choudhary, N., Nguyen, A. T., Sudhir, S., Chalif, E. J., Chen, J.-S., Chandra, A., Haddad, A. F., Wadhwa, H., Shah, S. S., Choi, S., Hayes, J. L., Wang, L., … Aghi, M. K. (2023). Single-cell RNA sequencing and spatial transcriptomics reveal cancer-associated fibroblasts in glioblastoma with protumoral effects. *Journal of Clinical Investigation*, *133*(5), e147087. https://doi.org/10.1172/JCI147087

7. Jurga, A. M., Paleczna, M., Kadluczka, J., & Kuter, K. Z. (2021). Beyond the GFAP-Astrocyte Protein Markers in the Brain. *Biomolecules*, *11*(9), 1361. https://doi.org/10.3390/biom11091361

8. Kitzberger, C., Shehzad, K., Morath, V., Spellerberg, R., Ranke, J., Steiger, K., Kälin, R. E., Multhoff, G., Eiber, M., Schilling, F., Glass, R., Weber, W. A., Wagner, E., Nelson, P. J., & Spitzweg, C. (2023). Interleukin-6-controlled, mesenchymal stem cell-based sodium/iodide symporter gene therapy improves survival of glioblastoma-bearing mice. *Molecular Therapy - Oncolytics*, *30*, 238–253. https://doi.org/10.1016/j.omto.2023.08.004

9. Ostrom, Q. T., Gittleman, H., Liao, P., Vecchione-Koval, T., Wolinsky, Y., Kruchko, C., & Barnholtz-Sloan, J. S. (2017). CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010–2014. *Neuro-Oncology*, *19*(suppl_5), v1–v88. https://doi.org/10.1093/neuonc/nox158

10. Pelon, F., Bourachot, B., Kieffer, Y., Magagna, I., Mermet-Meillon, F., Bonnet, I., Costa, A., Givel, A.-M., Attieh, Y., Barbazan, J., Bonneau, C., Fuhrmann, L., Descroix, S., Vignjevic, D., Silberzan, P., Parrini, M. C., Vincent-Salomon, A., & Mechta-Grigoriou, F. (2020). Cancer-associated fibroblast heterogeneity in axillary lymph nodes drives metastases in breast cancer through complementary mechanisms. *Nature Communications*, *11*(1), 404. https://doi.org/10.1038/s41467-019-14134-w

11. Sahai, E., Astsaturov, I., Cukierman, E., DeNardo, D. G., Egeblad, M., Evans, R. M., Fearon, D., Greten, F. R., Hingorani, S. R., Hunter, T., Hynes, R. O., Jain, R. K., Janowitz, T., Jorgensen, C., Kimmelman, A. C., Kolonin, M. G., Maki, R. G., Powers, R. S., Puré, E., … Werb, Z. (2020). A framework for advancing our understanding of cancer-associated fibroblasts. *Nature Reviews Cancer*, *20*(3), 174–186. https://doi.org/10.1038/s41568-019-0238-1

12. Trylcova, J., Busek, P., Smetana, K., Balaziova, E., Dvorankova, B., Mifkova, A., & Sedo, A. (2015). Effect of cancer-associated fibroblasts on the migration of glioma cells in vitro. *Tumor Biology*, *36*(8), 5873–5879. https://doi.org/10.1007/s13277-015-3259-8

13. Weenink, B., French, P. J., Sillevis Smitt, P. A. E., Debets, R., & Geurts, M. (2020). Immunotherapy in Glioblastoma: Current Shortcomings and Future Perspectives. *Cancers*, *12*(3), 751. https://doi.org/10.3390/cancers12030751

14. Yan, A., Joachims, M. L., Thompson, L. F., Miller, A. D., Canoll, P. D., & Bynoe, M. S. (2019). CD73 Promotes Glioblastoma Pathogenesis and Enhances Its Chemoresistance via A_{2B} Adenosine Receptor Signaling. *The Journal of Neuroscience*, *39*(22), 4387–4402. https://doi.org/10.1523/

JNEUROSCI.1118-18.2019

15. Zarodniuk, M., Steele, A., Lu, X., Li, J., & Datta, M. (2023). CNS tumor stroma transcriptomics identify perivascular fibroblasts as predictors of immunotherapy resistance in glioblastoma patients. *Npj Genomic Medicine*, *8*(1), 35. https://doi.org/10.1038/s41525-023-00381-w