Video Article

Coculture Assays to Study Macrophage and Microglia Stimulation of Glioblastoma Invasion

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URL: http://www.jove.com/video/53990
DOI: doi:10.3791/53990

Keywords: Cancer Research, Issue 116, invasion, glioma, glioblastoma, microglia, microenvironment, malignancy

Date Published: 10/20/2016

Citation: Coniglio, S., Miller, I., Symons, M., Segall, J.E. Coculture Assays to Study Macrophage and Microglia Stimulation of Glioblastoma Invasion. *J. Vis. Exp.* (116), e53990, doi:10.3791/53990 (2016).

Abstract

Glioblastoma multiforme (grade IV glioma) is a very aggressive human cancer with a median survival of 1 year post diagnosis. Despite the increased understanding of the molecular events that give rise to glioblastomas, this cancer still remains highly refractory to conventional treatment. Surgical resection of high grade brain tumors is rarely complete due to the highly infiltrative nature of glioblastoma cells. Therapeutic approaches which attenuate glioblastoma cell invasion therefore is an attractive option. Our laboratory and others have shown that tumor associated macrophages and microglia (resident brain macrophages) strongly stimulate glioblastoma invasion. The protocol described in this paper is used to model glioblastoma-macrophage/microglia interaction using *in vitro* culture assays. This approach can greatly facilitate the development and/or discovery of drugs that disrupt the communication with the macrophages that enables this malignant behavior. We have established two robust coculture invasion assays where microglia/macrophages stimulate glioma cell invasion by 5 - 10 fold. Glioblastoma cells labelled with a fluorescent marker or constitutively expressing a fluorescent protein are plated without and with macrophages/microglia on matrix-coated polycarbonate chamber inserts or embedded in a three dimensional matrix. Cell invasion is assessed by using fluorescent microscopy to image and count only invasive cells on the underside of the filter. Using these assays, several pharmacological inhibitors (JNJ-28312141, PLX3397, Gefitinib, and Semapimod), have been identified which block macrophage/microglia stimulated glioblastoma invasion.

Video Link

The video component of this article can be found at http://www.jove.com/video/53990/

Introduction

Glioblastoma multiforme is an aggressive human brain cancer with a median survival of approximately 12 months from the time of diagnosis [1,2]. Glioblastoma is one of the most deadly and clinically challenging cancers as it is refractory to standard chemotherapy and surgical resection. The diffuse nature of glioblastoma enables tumor cells to spread throughout the normal brain making the advanced tumor practically impossible to surgically resect completely. This highly invasive aspect is a hallmark feature of glioblastoma and other advanced astrocytomas. Therefore, the focus of much research has been on the molecular mechanism of glioblastoma cell invasion. The glioblastoma tumor microenvironment plays vital roles in establishing malignancy [3-6]. Tumor associated macrophages/microglia were shown to be responsible for promoting glioblastoma invasion [7,8]. Most of these studies however measured the effect of macrophages/microglia using assays which physically separate them from the glioblastoma cells. Our laboratory has set out to generate improved assays which allow us to study how glioblastoma invasion is dependent on macrophages/microglia in cocultures and enable us to image the physical interaction between them during invasion.

Classic assays to measure cell invasion include the "standard" Boyden chamber chemotaxis and chemoinvasion formats. Here the cells to be studied are plated in a plastic chamber which contains a polycarbonate filter on the bottom that has pores of a specified size (generally between 0.4 and 8 µM in diameter). The process of cell invasion involves a physical barrier, usually composed of extracellular matrix protein. In the chemoinvasion assay, the preferred matrix used is Matrigel (hereafter referred to as "matrix"), an extracellular matrix protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells and consists largely of collagen type IV and laminin. The chambers are then placed in a tissue culture well which contains cell culture media with or without growth factors that are suspected to stimulate invasion. Cells which have a higher invasive capacity will invade through the extracellular matrix coated filter at a higher frequency and adhere to the underside of the filter. We have modified this assay in order to assess the role of microglia and tumor associated macrophages on glioblastoma cell invasion.

We have been able to determine using coculture assays described within this paper that microglia can stimulate the invasion of two glioblastoma cell lines by 5 - 10 fold [9,10]. This reflects what is observed in animal models of glioblastoma. Furthermore, we developed a three dimensional invasion assay where the interactions between glioblastoma cells and macrophages/microglia can be examined more directly. The extent of glioblastoma cell invasion stimulated by macrophages/microglia in the 3D assay is comparable to what is seen using the matrix coated chamber

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approach. Similar assays were previously developed to study breast carcinoma interactions with macrophages during invasion [11-13]. Both methods described in this paper should aid in the ability to dissect the molecular mechanism(s) of macrophage/microglia-stimulated invasion of glioblastoma cells.

Protocol

1. Fluorescent Labeling of Cells

NOTE: Label glioblastoma cell lines and microglia with fluorescent dyes [9]. Alternatively, generate cell lines that constitutively express fluorescent proteins such as GFP/RFP as described in [14].

1. Plate cells on a 6 well plate such that they will be 70 - 80% confluent on day of staining. For the murine glioblastoma cell line GL261 and human glioblastoma cell line U87, plate 1×10^6 and 1.5×10^6 cells, respectively on a 6 cm dish 24 hr before staining.
2. Prepare fluorescent cell stain dye solution in DMSO and add 5 µM dye to media, either Roswell Park Memorial Institute medium (RPMI) or Macrophage Serum Free Medium (MSFM), vortex well.
3. Incubate cells with dye for 30 min at 37 °C and 5% CO_2.
4. Remove dye containing media and add fresh media (RPMI or MSFM) to the cells.
5. Incubate cells for 30 min. Note: Cells are now stained and ready to be used in experiment.

2. Pre-coated Matrix Chamber Coculture Invasion Assay

1. Differentiate THP-1 cells using phorbol myristate acetate (PMA)[15].
    1. Plate 2 - 3 x 10^5 THP-1 cells in 1.5 ml of RPMI/10% FBS in a 6 well culture plate. Immediately after plating add 100 nM PMA and incubate at 37 °C, 5% CO_2 for 48 hr.
    NOTE: After 48 hr, THP-1 cells that have successfully undergone differentiation will be adherent and spread to the bottom of the well taking on a round morphology.
    2. Remove PMA by washing once with 1x PBS and replace with fresh RPMI/10% FBS. Wait another 48 hr until cells are ready to use in assay.

2. Equilibrate matrix pre-coated chambers by placing them in a well containing 500 µl of media alone (no serum) and adding 500 µl of media alone to the top. Incubate chambers for 2 hr at 37 °C and 5% CO_2.
NOTE: Pre-coated chambers were purchased from the manufacturer; see **Table of Materials/Equipment**.
3. To gently detach cells (fluorescently labeled glioblastoma cell lines and the microglia/macrophages), remove media from cells by aspiration. Wash cells on dish with 2 mM EDTA/PBS. Add 500 µl of 2 mM EDTA/PBS and incubate for 5 - 10 min in a cell incubator at 37 °C and 5% CO_2.
4. Resuspend cells in 5 ml of media containing 0.3% Bovine Serum Albumin (BSA).
5. Centrifuge for 5 min at 120 x g.
6. Aspirate supernatant and resuspend pellet in MSFM/0.3% BSA media (for GL261 cells and microglia) or RPMI/0.3% BSA (for U87 and THP-1 cells) such that the concentration of cells is equal to 1×10^6 cells/ml. Use a hemocytometer to count the cells.
7. Add 500 µl media/0.3% BSA per well of 24 well plate.
8. Remove media from chambers that have been equilibrated for at least 2 hr (step 2.2). Place chambers in well containing 500 µl media/0.3% BSA (step 2.7).
9. If inhibitors are being used to study their effect on macrophage/microglia stimulated glioma invasion, add the appropriate concentration to both the bottom and top portions of the chamber.
NOTE: The concentrations of CSF-1R inhibitor used to fully inhibit microglia stimulated GL261 glioblastoma invasion can be found in reference [9].
10. Depending on the cell lines used, add cells to the top chamber as described in the next two steps: mouse glioblastoma (2.10.1) and human glioblastoma (2.10.2).
    1. Mix 1.5 x 10^5 GL261 (glioblastoma) cells (150 µl) and 5 x 10^4 mouse microglia (50 µl) (see step 2.6 for media details). Bring volume to 500 µl by adding 300 µl MSFM/0.3% BSA. Add cell mixture to the top chamber and incubate at 37 °C , 5% CO_2 for 48 hr.
    NOTE: Murine microglia are isolated from neonatal mice as described in [16].

Mix 7.5 x 10^4 U87 (glioblastoma) cells (75 µl) and 2.5 x 10^4 THP1 (macrophage) cells (25 µl). Bring volume in 500 µl by adding 400 µl RPMI/0.3% BSA. Add the cell mixture to the top chamber. Incubate cells at 37 °C, 5% CO_2 for 24 hr.

1. After incubation, remove cells from top of chamber by gentle aspiration and fix chambers by placing in 3.7% formaldehyde in PBS. Allow chambers to remain in fixative for 15 min at room temperature (or overnight at 4 °C). Remove fixative by gentle aspiration and replace with 500 µl 1x PBS. Proceed to section 4 for image analysis.
NOTE: Experiment can be paused at this moment and saved for imaging in 1x PBS. Fluorescent dye signals can be detected for up to 2 weeks after fixation.

3. Invasion Assay "3D"-embedding Glioma and Macrophages/Microglia in Matrix

1. Thaw matrix mix at 4 °C overnight. Perform all further manipulations using matrix on ice in the hood.
2. Prepare 10 mg/ml concentration of matrix in media (MSFM or RPMI) with 0.3% BSA on ice.
NOTE: Stock concentration of matrix is typically around 15 mg/ml.

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3. To prepare cells (labelled in step 1) for suspension in matrix, remove media from cells by aspiration. Wash cells on dish with 2 mM EDTA/PBS. Add 500 µl of 2 mM EDTA/PBS to cells and incubate for 5 - 10 min in incubator at 37 °C and 5% CO_2.
4. Resuspend cells in 5 ml of media containing 0.3% BSA.
5. Centrifuge for 5 min at 120 x g.
6. Aspirate supernatant from pellet. Resuspend pellet in media/0.3% BSA such that the concentration of cells is equal to 1 x 10^6 cells/ml. Use a hemocytometer to count the cells.
7. Add 1.5 x 10^5 GL261 cells (in 150 µl) + 5 x 10^4 microglia cells (in 50 µl) into a 1.5 ml microfuge tube. Add 2 x 10^5 GL261 cells (200 µl) into a separate 1.5 ml microfuge tube.
   NOTE: GL261 cells alone without microglia serves as a control.
8. Spin cells in microfuge for 5 min at 120 x g.
9. Resuspend cells in 200 µl cold matrix on ice.
10. Plate 50 µl (50,000 cells) onto the center of the top compartment of 8 µM pore size chamber insert.
11. Incubate at 37 °C for 30 min for polymerization to occur.
12. Add 200 µl serum-free medium to the upper chamber and 700 µl serum containing cell growth medium to the lower well.
13. Incubate at 37 °C, 5% CO_2 for 48 hr.
14. After incubation, remove cells from top of chamber by gentle aspiration and fix chambers by placing in 3.7% formaldehyde in PBS. Allow chambers to remain in fixative for 15 min at room temperature (or overnight at 4 °C). Remove fixative by gentle aspiration and replace with 500 µl 1x PBS. Proceed to section 4 for image analysis.

4. Imaging Assays

1. Remove chambers from 3.7% formaldehyde and wash in well containing fresh 500 µl 1x PBS.
   NOTE: Do not allow chambers to dry.
2. Using a cotton tipped applicator, gently clean the non-invasive cells from the top portion of the filter (side facing the inside of the chamber) by scraping the filter surface. Start gently and increase pressure gradually. Do this at least 3 times per chamber.
3. Place chambers in either a glass-bottomed dish or leave in a 24 well plate.
4. Place sample on the stage of an epifluorescent or laser confocal fluorescent microscope equipped with camera and image capture software [9,10].
5. Take several 10X or 20X images of representative fields.
6. Using an image analysis software, such as ImageJ, count the number of glioblastoma cells that have crossed the filter to the underside [9,10].
7. If imaging the "3D" invasion assay (described in section 3), generate a Z stack series using a fluorescent laser confocal microscope [5-6]. To image the invasive cell population on the underside of the filter, follow the protocol steps 4.2 - 4.6 described above.

Representative Results

Using the methods outlined here, we have shown that microglia and macrophages can substantially stimulate glioblastoma cell invasion. Two different invasion assays are employed and are depicted in **Figure 1**. In **Figure 2**, GL261 cells that constitutively express the fluorescent protein mCherry were plated on pre-coated chambers with and without microglia for 48 hr. GL261 cells were minimally invasive on their own however when cultured with microglia the invasive capacity increased by approximately 10 fold.

We observe a similar effect using human cell lines. In **Figure 3**, U87 cells (stained with 5-chloromethylfluorescein diacetate (CMFDA) green) shows 5-6 fold higher invasion when cocultured with differentiated THP-1 cells. THP-1 is a human monocytic cell line (derived from an acute monocytic leukemia patient) that can be differentiated into a macrophage-like state using phorbol myristate acetate (PMA) [15]. Differentiated THP-1 cells display many of the characteristics of human macrophages such as cytokine secretion and the ability to carry out phagocytosis.

Alternatively, cell invasion can be measured by embedding them in matrix and plating this directly onto the filter in a chamber insert. Laser confocal microscopy can be used to produce a series of images that produces a three dimensional representation (Z-stack). Similar to what is observed in the matrix-coated chamber assay (shown in **Figures 2 - 3**), the coculture of microglia (stained with CMPTX red) stimulates GL261 cells (CMFDA green) to invade as measured by the number of cells that have crossed to the underside of the filter (**Figure 4**). This format has the added advantage of being able to visualize potential cell-cell interactions between glioblastoma cells and microglia during invasion. Indeed, microglia seem to closely associate with the glioma cells suspended in 3D matrix (**Figure 5**). If only endpoint analysis is required and a laser confocal is not available for use, cell chambers can be cleaned as described for the matrix coated chambers. Then the number of the remaining (invasive) cells can be determined by counting using images obtained from a standard epifluorescent microscope. **Movie 1** shows the three dimensional assembly of such an assay.

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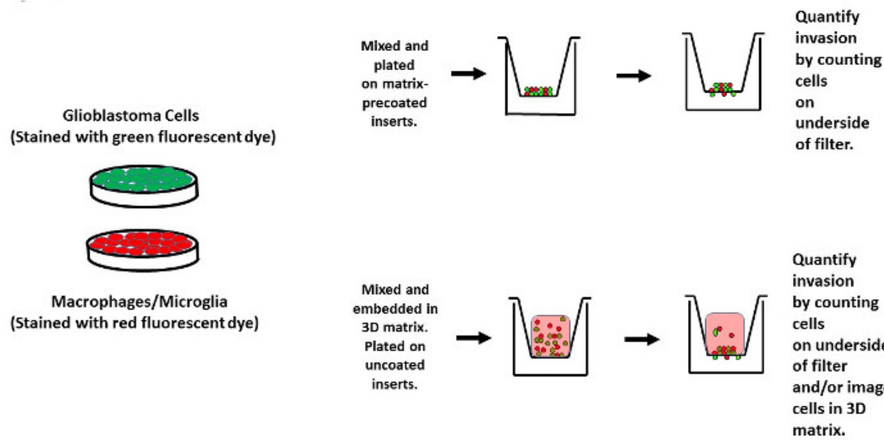

Figure 1: Schematic of Two Different Protocols for Assaying Microglia/Macrophage-stimulated Glioblastoma Cell Invasion. Top panel illustrates pre-coated matrix chamber invasion assay (Section 2). Bottom panel illustrates 3D matrix invasion assay (Section 3). Figure adapted from [9]. Please click here to view a larger version of this figure.

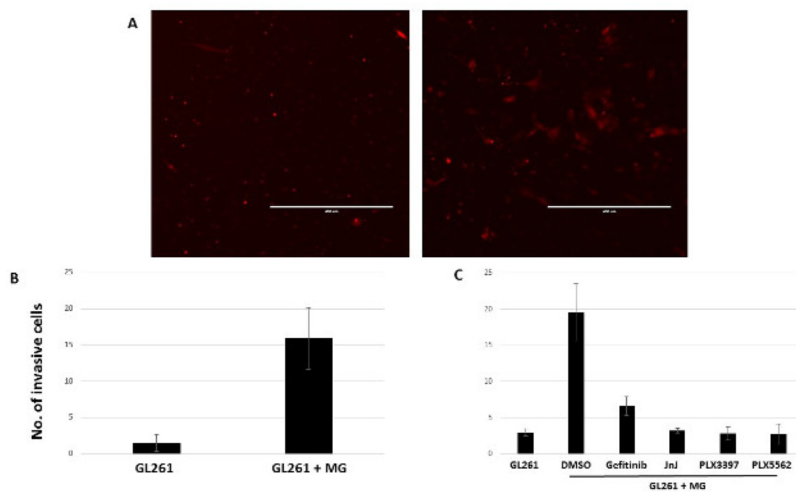

Figure 2. Microglia (MG) Enhance GL261 Glioblastoma Cell Invasion. (A) Representative images of invading GL261 cells (expressing mCherry) in the absence (left panel; GL261 alone) or presence of microglia (right panel; GL261+MG) were combined and plated on matrix-coated chambers, followed by incubation for 48 hr and then evaluation of the number of cells that have crossed the filter. Images taken using 10X objective. Scale bar = 400 µM. **(B)** Quantitation of assay counting only invasive GL261 cells (red). Data shown are Mean ± SEM from three independent experiments. **(C)** Quantitation of microglia-stimulated GL261 invasion in the presence of pharmacological inhibitors gefitinib (5 µM), JNJ-28312141 (10 µM), PLX3397 (1 µM), PLX5562 (1 µM). Data shown are Mean ± SEM from six independent experiments. Please click here to view a larger version of this figure.

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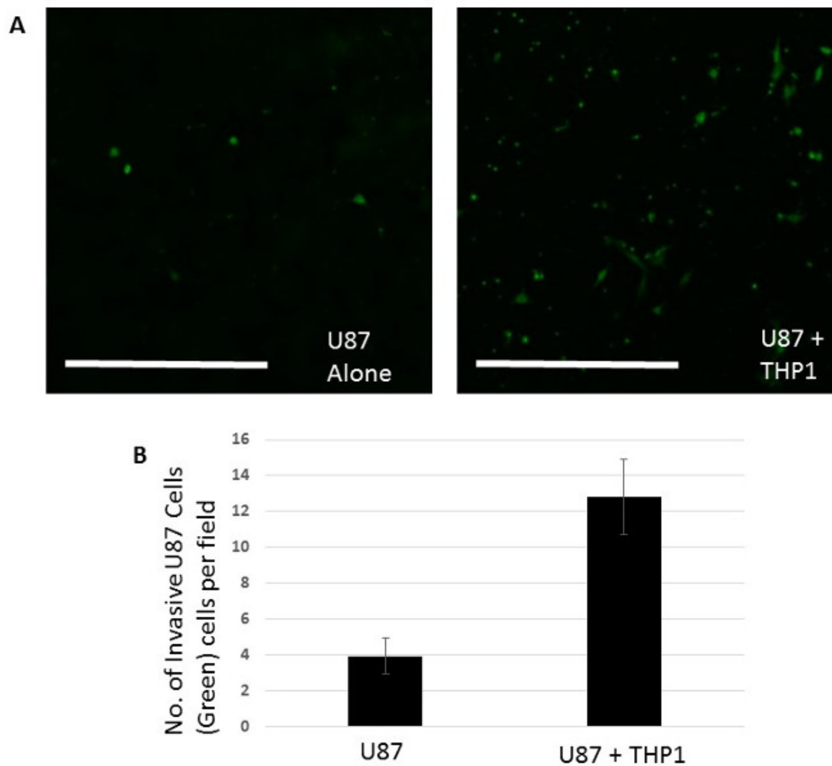


Figure 3. THP1 Macrophages Enhance U87 Glioblastoma Cell Invasion. (A) Representative images of invading U87 cells stained with CMFDA green plated on matrix-coated chambers alone (left panel; U87) or with differentiated THP1 macrophages (right panel; U87 + THP1), followed by incubation for 24 hr and then evaluation of the number of cells that have crossed the filter. Images taken using a 10X objective. Scale bar = 400 µM. **(B)** Quantitation of assay counting only invasive U87 cells (green). Data shown are Mean ± SEM from ten independent experiments.

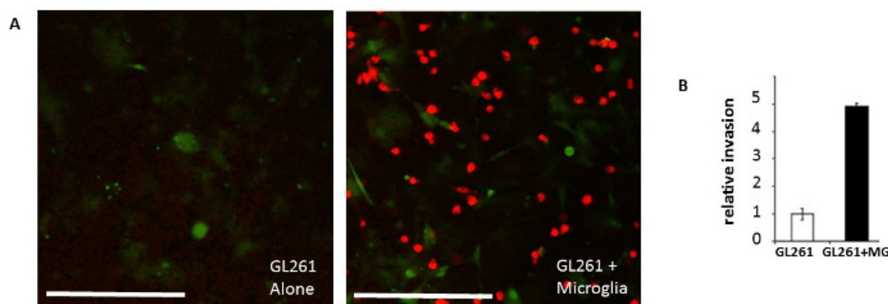


Figure 4. 3D Invasion Assay of GL261 and Microglial Coculture. (A) Images shown are projections from a Z-series of images of GL261 cells (stained with CMFDA green) embedded in matrix alone (left panel; GL261) or with murine microglia (labeled with CMPTX red; right panel; GL261+MG). The Z-projections were of the bottom 4 slices of the image stack which encompasses the bottom of the chamber filter and therefore represents invasive cells. Scale bar = 200 µM. **(B)** Quantitation of GL261 cells (green) determined to be on the underside of the filter as described above. The total number of invading GL261 cells was determined and normalized to that of GL261 cells in monoculture. Data shown represent the Mean ± SEM of 3 independent experiments, performed in duplicate (figure adapted from [10]). Please click here to view a larger version of this figure.

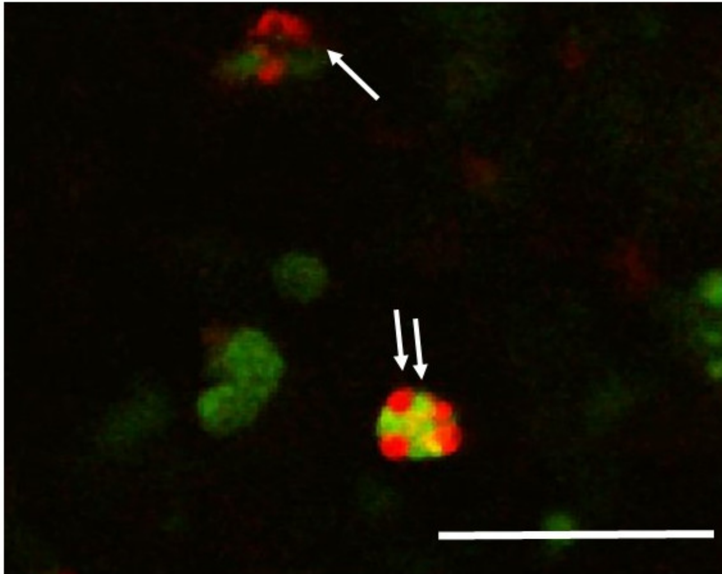
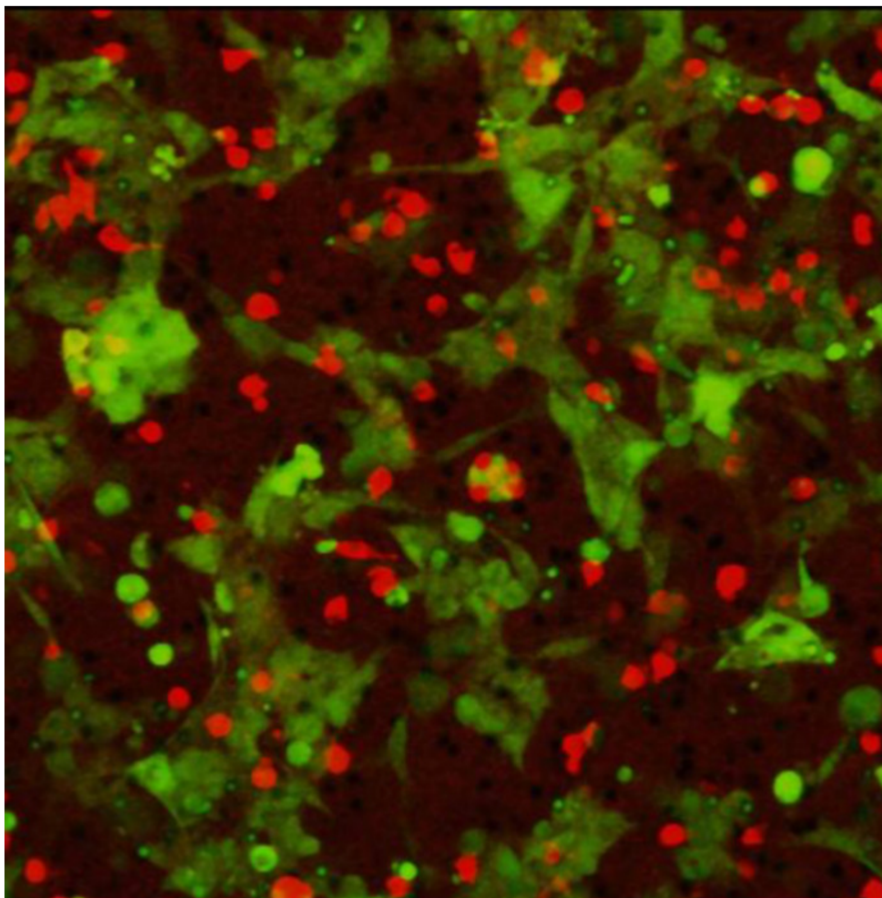


Figure 5. Image from a Slice within the 3D Stack of GL261 (green) and Microglia (red) Coculture Embedded in Matrix. GL261 and microglia cell interactions are highlighted with white arrows. Scale bar = 100 µM.



Movie 1: Glioblastoma and Microglia Interaction in 3D. Rotation around the X axis of a 3D projection of entire Z series of images taken from GL261 (green) and microglia (red) coculture embedded in matrix. Slices are in 5 µM increments. Please click here to view this video.

Discussion

The highly invasive nature of high grade astrocytomas and glioblastoma make these brain cancers very deadly. It is therefore of paramount importance to understand the molecular and cellular mechanisms of glioblastoma invasion. Much has been learned about the process of

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glioblastoma invasion already [17]. Using the assay formats detailed in this paper, our laboratory has shown in both mouse and human models that tumor associated macrophages can stimulate glioma cell invasion by 5 - 10 fold. This coculture model faithfully replicates the ability of glioblastoma cells to physically interact with macrophages/microglia which would allow for paracrine and juxtacrine interactions (via ligand-receptor systems such as Notch-Delta and Ephrin-Ephs) that are potentially involved in bidirectional communication between the tumor cells and macrophages. Other assay formats which seek to discover the effect of macrophages on glioma cell invasion typically have the cells physically separated as the macrophages are plated on the bottom of the well and the glioblastoma cells are on the chamber [3,4]. Consequently, these studies show a more modest increase in macrophage-stimulated glioma invasion (2 fold). This coculture assay improves on this effect (5 - 10 fold) likely indicating the importance of juxtacrine interactions during macrophage/microglia stimulated glioblastoma invasion.

The protocol outlined in this paper involves several steps which must be performed carefully to achieve satisfactory results. If the experiment is carried out as described here and no invading cells are seen, the fluorescent dye may have expired which will lead to very weak or non-existent staining. For the invasion assays using the pre-coated chambers, it is essential to use fresh invasion chambers which are not past the expiration date and have been kept frozen the entire time up until performing the experiment. Chambers which were left out at room temperature for over 12 hr were found to be unsatisfactory in that the matrix barrier was no longer intact and cells invaded at a much higher frequency than normal. For the 3D assay, it is absolutely crucial to keep all pipets and tubes on ice. If at room temperature for a significant length of time, the matrix will polymerize and cannot be used to resuspend the cells in it. It is recommended that a small tray filled with ice is used in the hood to hold all materials which will come into contact with the matrix. The protocol can be modified to include a higher cell density, however this may affect the length of time required to see the optimal effect on glioma invasion. It would be of interest to see if other cell types can be added in the coculture assay and what potential effects they may have on invasion. Finally, we acknowledge that the brain microenvironment is unique and difficult to replicate *in vitro.* One limitation of this protocol is that the invasion chambers used here lack the typical components of normal brain which glioblastoma cells will encounter during invasion (*i.e.*, densely packed neurons and astrocytes). Although with that caveat, the stimulatory effect of macrophages/microglia in this assay is consistent with what is observed *in vivo* and can be used to predict which inhibitors and/or proteins may interfere with this process.

Mimicking the tumor microenvironment in culture is a daunting task given how many varied cell types and matrix proteins are present in tumors at various stages of malignancy. Yet reasonably accurate *in vitro* models of tumor interaction with components of the microenvironment would greatly facilitate the discovery of new therapeutic avenues. It is now appreciated that cells such as macrophages play a critical role in several aspects associated with progression to malignancy including angiogenesis, invasion, immune evasion and drug resistance. The coculture invasion assay described here uses the ratio of tumor cells to macrophages that is observed in patients with high grade glioblastomas (approximately 3:1; [5]). It has been shown that the ability of macrophages/microglia to stimulate glioblastoma invasion is dependent on CSF-1R and EGFR signaling as pharmacological inhibition of these pathways using JNJ-28312141, PLX3397 (CSF-1R) and gefitinib (EGFR) strongly attenuates the increase in invasion [9]. Furthermore, using the embedded matrix assay, it was demonstrated that Semapimod, a small molecule known to target macrophages and microglia, can also block microglia stimulated glioblastoma invasion [10]. Results from these coculture experiments provided the impetus to validate these compounds using *in vivo* models. Consistent with data from the *in vitro* assays described in this paper, blockade of CSF-1R with the inhibitor PLX3397 (which crosses the blood brain barrier) largely blocked the ability of GL261 glioma cells to invade in the brains of C57BL/6 mice [9]. Similarly, Semapimod delivered into the brain was shown to inhibit invasion and to synergize with standard radiation treatment in greatly prolonging the survival of mice harboring GL261 tumors [10]. Both of these approaches may prove effective in the clinic.

It is now well appreciated that the macrophage system is quite important for progression of various cancers to malignancy [3-6, 11-13]. It has been clearly established in breast and brain cancer models that tumor-associated macrophages are critical for tumor cell invasion and metastasis. In addition, macrophages are likely to be very important for mediating immune escape as macrophages (and cells of related lineage such as dendritic cells) function as professional antigen presenting cells which orchestrate adaptive immunity in response to infection [18]. It is now clear that one of the normal physiological functions of the adaptive immune system is to prevent uncontrolled proliferation by destroying aberrant cells. Immunity towards the tumor cells is likely due to the fact that the highly mutagenic nature of the cancer cell generates neo-antigens that are recognized as foreign by the adaptive immune system. Indeed the process of "immune evasion" is postulated to be an important part of malignancy as cancer cells need to prevent the immune system (in particular cytotoxic lymphocytes, natural killer cells and inflammatory macrophages) from destroying the tumor. It is hoped the assay described in this paper will facilitates the study of glioblastoma interaction with macrophages and potentially allow the expansion of the types of questions we can address about malignant cancer using *in vitro* culture assays.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We would like to thank Dr. Konstantin Dobrenis for providing murine microglia for these studies.

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