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In Vitro Assay to Study Tumor-Macrophage Interaction

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Abstract

Tumor Associated Macrophages (TAMs) account for a large percentage of cells in the tumor mass for different types of cancers. Glioblastoma (GBM), a malignant brain tumor with no cure, has up to a half the tumor mass TAMs. TAMs can be pro-tumoral or anti-tumoral, depending on the activation of specific genes in the cells. Genetic mutations in the tumors, through regulating cytokine expression, can affect recruitment of TAMs to the tumor microenvironment. Here, we describe a quantitative cell-based assay to assess macrophage recruitment by the conditioned medium from the tumor cells. This assay uses the human macrophage cell line MV-4-11 to study macrophage attraction by the conditioned medium from glioblastoma, allows for high reproducibility and low variability. Data generated with this assay can contribute to a better understanding of the interaction between the tumor and the tumor microenvironment. Similar assay can be used to assess interaction between the tumor cells and other immune cells, including T cells and natural killer (NK) cells.

SUMMARY:

This article represents a useful in vitro assay to evaluate the capability of conditioned medium from tumor cells to attract macrophages.

Keywords

Macrophage; tumor; conditioned medium; the tumor microenvironment; glioblastoma; the epidermal growth factor receptor

INTRODUCTION:

Macrophages are immune cells with high phenotypic and functional heterogeneity[1]. They play important roles in the host defense systems, tissue repair, development and tumor

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DISCLOSURES:
William A. Weiss is a co-founder of StemSynergy Therapeutics. Zhenyi An has nothing to disclose.

progression[1]. TAMs are macrophages in the microenvironment of solid tumors. Certain TAMs can promote tumor growth through inhibiting T cell-mediated cytotoxic activity, modulating the tumor microenvironment (TME), promoting angiogenesis, invasion and metastasis[2-5]. TAMs are among the most abundant cell types in the TME and higher number of TAMs generally correlates with worse patient survival in many types of solid tumors[6]. The distinct genetic signatures of the tumor cells affect their ability to recruit macrophages. In glioblastoma (GBM), an aggressive brain tumor with no cure, macrophages can represent up to a half of the tumor mass[7]. Co-amplification of the epidermal growth factor receptor (*EGFR*) and its truncation mutant *EGFRvIII* is frequently observed in GBM, which confers tumor growth advantages[8]. Cells co-expressing EGFR and EGFRvIII attract more macrophages compared to cells expressing EGFR or EGFRvIII singly[7].

Chemokines are a family of small cytokines that play significant roles in regulating immune composition in the TME[6,9]. Human cells express more than 50 cytokines[10]. Immune infiltration in the tumors is largely realized by the interaction between cytokines and cytokine receptors[11]. Each type of immune cells expresses distinct chemokine receptors/chemokines and can be recruited by cells secreting specific chemokines/chemokine receptors[12]. Cancer cells can increase expression of certain chemokines to recruit immune cells such as TAMs, regulatory T cells and myeloid-derived suppressor cells (MDSCs)[6]. Blockade of specific chemokine secreted by the tumors can be a promising way in inhibiting infiltration of immune cells into the tumor mass.

Here, we describe a protocol that allows in vitro evaluation of tumor-macrophage interaction, using conditioned media from the tumor cells containing chemokines and macrophage cell lines.

PROTOCOL:

1. Medium Preparation

1.1. Prepare the serum-free stem cell medium: Thaw the 50 x B27 supplement, the epidermal growth factor (EGF, 20 μg/ml in 10 mM acetic acid with 0.1% BSA), and the fibroblast growth factor (FGF, 20 μg/ml in 10mM acetic acid with 0.1% BSA). Add 500 μL EGF (final concentration 20 ng/ml), 500μL FGF (final concentration 20 ng/ml), 10 ml 50 x B27 to 500 ml Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) and 5 ml 100 x penicillin streptomycin (Pen Strep). Invert the bottle 4-6 times to mix, filter sterilize through a 500 ml 0.1 μM filtration cup. Mark the bottle and store at 4 °C.

Note: The medium is good to use for up to a month at 4 °C.

1.2. Prepare the MV-4-11 culture medium: Add 50 ml fetal bovine serum (FBS) to 500 ml Iscove's Modified Dulbecco's Medium (IMDM). Invert the bottle 4-6 times to mix, filter sterilize through a 500 ml 0.1 μM filtration cup. Mark the bottle and store at 4 °C.

Note: The medium is good to use for up to a month at 4 °C.

1.3. Prepare the tumor cell maintenance medium: Add 50 ml FBS to 500 ml Dulbecco's Modified Eagle Medium (DMEM). Invert the bottle 4-6 times to mix, filter sterilize through a 500 ml 0.1 μM filtration cup. Mark the bottle and store at 4 °C.

Note: The medium is good to use for up to a month at 4 °C.

2. Cell preparation

Day 1:

2.1. Thaw tumor cells

2.1.1. Warm up the tumor cell maintenance medium (as described in 1.3) at a 37 °C water bath for 20 min.

2.1.2. Take the frozen U87, U87:EGFR, U87:EGFRvIII and U87:EGFR+EGFRvIII cells from the liquid nitrogen tank. Thaw the cells at 37 °C water bath for 2 minutes.

Caution: Be careful when taking cells from the liquid nitrogen tank. Wear gloves with thermal protection and safety goggles as needed.

2.1.3. Spray the tissue culture hood surface with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels.

2.1.4. Spray the cryogenic vials containing cells and the medium bottle with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels, bring them into the tissue culture hood.

2.1.5. Pipette 5 ml tumor cell maintenance medium into a 15 ml sterile centrifuge tube. Invert the tube containing tumor cells 4-6 times to mix. Pipette all the cells into the 15 ml centrifuge tube. Invert the centrifuge tube 4-6 times to mix.

2.1.6. Centrifuge the cells at 200 g for 5min at 4 °C. Aspirate the supernatant.

2.1.7. Wash the cells with 10 ml PBS, Centrifuge the cells at 200 g for 5min at 4 °C. Aspirate the supernatant.

2.1.8. Resuspend the cells in 12 ml tumor cell maintenance medium (as described in 1.3). Pipette up and down 3-5 times to mix. Transfer the cells into a T75 cell culture flask.

2.1.9. Grow the cells in a 37°C incubator with 5 % CO_2. Put the tumor cell maintenance medium back to the 4 °C fridge.

Day 2:

2.2. Check the cells under the microscope. Change medium if needed. Cells should grow in a monolayer in culture.

Day 3:

2. 3. Thaw MV-4-11 cells

2.3.1. Warm up the MV-4-11 culture medium (as described in 1.2) at a 37 °C water bath for 20 min.

NOTE: MV-4-11 can be changed to primary macrophages or other macrophage cell lines, depending on the need of the specific study.

2.3.2. Take the frozen MV-4-11 cells from the liquid nitrogen tank. Thaw the cells at 37 °C water bath for 2 min.

Caution: Be careful when taking cells from the liquid nitrogen tank. Wear gloves with thermal protection and safety goggles as needed.

2.3.3. Spray the tissue culture hood surface with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels.

2.3.4. Spray the cryogenic vials containing cells and the medium bottle with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels, bring them into the tissue culture hood.

2.3.5. Pipette 5 ml MV-4-11 culture medium (as described in 1.2) into a 15 ml sterile centrifuge tube. Invert the tube containing tumor cells 4-6 times to mix. Pipette all the cells into the 15ml centrifuge tube. Invert the centrifuge tube 4-6 times to mix.

2.3.6. Centrifuge the cells at 200 g for 5 min at 4 °C. Aspirate the supernatant.

2.3.7. Wash the cells in 10 ml phosphate buffered saline (PBS), centrifuge the cells at 200 g for 5 min at 4 °C. Aspirate the supernatant.

2.3.8. Resuspend the cells in 12 ml MV-4-11 culture medium (as described in 1.2). Gently pipette up and down 3-5 times to mix. Transfer the cells into a T75 cell culture flask.

2.3.9. Grow the cells in a 37 °C incubator with 5 % CO_2.

2.4. Split tumor cells

2.4.1. Warm up the serum-free stem cell medium (as described in 1.1) at a 37 °C water bath for 20 min.

2.4.2. Spray the tissue culture hood surface with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels. Take the accutase cell detachment solution from the fridge. Spray the medium and accutase cell detachment solution bottles with 70 % ethanol, wipe off 70% ethanol on the surface with paper towels, bring them into the tissue culture hood.

NOTE: Don’t pre-warm the accutase cell detachment solution.

2.4.3. Transfer the tumor cell culture from the incubator into the tissue culture hood. Aspirate the medium, add 2 ml accutase into the flask.

NOTE: Rinsing with PBS before adding accutase is optional here.

2.4.4. Set the flask in the hood. Check if the tumor cells round up every minute. Once the tumor cells round up, gently tap the side of the flask to help the cells detach from the flask.

2.4.5. Pipette 8 ml serum-free stem cell medium into the flask, pipette up and down 3 times to mix, transfer the cells into a 15 ml sterile centrifuge tube. Centrifuge the cells at 200 g for 5 min at 4 °C. Aspirate the supernatant.

2.4.6. Wash the cells in 10 ml PBS. Pipette up and down 3-5 times to mix. Centrifuge the cells at 200 g for 5 min at 4 °C. Aspirate the supernatant.

NOTE: This step is optional.

2.4.7. Resuspend the cells in 10 ml serum-free stem cell medium (as described in 1.1). Pipette up and down 3-5 times to mix. Take 10 μL cells and mix with 10 μL Trypan Blue solution. Pipette 10 μL of the mixture into the counting slide, quantify living cell number using an automatic cell counter.

2.4.8. Adjust the cell density to $2.5x10^5$ /ml with serum-free stem cell medium (as described in 1.1), seed 2 ml of the cells into each well of a 6-well cell-culture plate. For each type of cells, seed 3 wells (triplicate sample). At the same time, prepare 6 wells with 2 ml serum-free stem cell medium only (no cells). These samples will be used: 1) as negative controls. 2) for adjusting conditioned medium volume based on cell numbers.

2.4.9. Put the 6-well plates in a 37 °C incubator with 5 % CO_2. Allow the cells to grow for 24 h.

3. In vitro macrophage attraction assay

3.1. Prepare conditioned media

3.1.1. Warm up the tumor cell maintenance medium (as described in 1.3) at a 37 °C water bath for 20 min.

3.1.2. Spray the tissue culture hood surface with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels. Take the accutase cell detachment solution from the fridge. Spray the bottles of the medium and the accutase with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels, bring them into the tissue culture hood.

3.1.3. Take the 6-well plates out from the incubator. Carefully pipette the conditioned media into 15 ml sterile centrifuge tubes. Put the tubes on ice. Pipette the media in the “media only” well into a separate 15 ml sterile centrifuge tube.

3.1.4. Add 0.5 ml accutase into each well of the 6-well plate. Check if the tumor cells round up every minute. Once the tumor cells round up, gently tap the side of the plate to help the cells detach.

3.1.5. Pipette 2.5 ml tumor cell maintenance medium into the well, pipette up and down 3 times to mix, transfer the cells into a 15 ml sterile centrifuge tube. Take 10 μL cells and mix with 10 μL Trypan Blue solution. Pipette 10 μL of the mixture into the counting slide, quantify the number of living cells using an automatic cell counter.

3.1.6. Adjust the volume of the conditioned media according to cell number using the serum free stem cell medium (37 °C incubation overnight). For example, if Well A has 3x as many live cells as the well with the least number of cells, dilute its conditioned media 1:3. This step is used to control difference caused by cell proliferation rate.

NOTE: The reason to use serum free stem cell medium with 37 °C incubation overnight is to control the possible change in the media with prolonged 37 °C exposure.

3.1.7. Filter the conditioned media through 0.45 μM filters. Put the conditioned media on ice. NOTE: This step removes cells and cell debris in the conditioned media.

3.2. Prepare MV-4-11 cells

3.2.1. Warm up the IMDM medium (without FBS) at a 37 °C water bath for 20 min.

3.2.2. Spray the tissue culture hood surface with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels. Spray the medium bottle with 70 % ethanol, wipe off 70 % ethanol on the surface with paper towels, bring them into the tissue culture hood.

3.2.3. Take the flask of MV-4-11 cells into the tissue culture hood. Pipette 10 ml cells into a 15 ml sterile centrifuge tube. Take 10 μL cells and mix with 10 μL Trypan Blue solution. Pipette 10 μL of the mixture into the counting slide, quantify the number of living cells using an automatic cell counter. Centrifuge the cells at 200 g for 5min at 4 °C. Aspirate the supernatant.

3.2.4. Wash the cells with 10 ml PBS, centrifuge the cells at 200 g for 5 min at 4 °C. Aspirate the supernatant.

3.2.5. Resuspend the cells in IMDM medium (without FBS) to make the final cell concentration $1x10^6$/ml.

NOTE: The cell number of macrophages used here can be adjusted.

3.3. Transwell assay. For this step, one can use a cell migration assay kit or purchase the reagents and insert separately. For MV-4-11 cells, choose transwell inserts with 5μM pore size. To detect macrophage migration, one can directly quantify the number of macrophages in the lower chamber or use colorimetric/florimetric methods. Here we demonstrate the assay using the colorimetric method.

3.3.1. Bring the 24-well plate, the insert and the reagents to room temperature.

3.3.2. Add 250 μL of MV-4-11 cells prepared above into each insert.

3.3.3. Add 400 μL conditioned medium/medium only (with overnight incubation at 37 °C, these samples serve as the negative control) to the lower chambers of the 24-well plate. For each tumor line or control, use triplicate samples.

NOTE: Avoid bubbles between the insert and the conditioned medium. Bubbles will inhibit macrophages from migrating to the bottom wells.

3.3.4. Incubate the plates in a 37 °C incubator with 5 % CO_2 for 4 h.

NOTE: The incubation time can be adjusted. Cells migrating into the lower chambers can be seen under the microscope.

3.3.5. Gently tap the insert on the inner wall of the same well, discard the insert. As MV-4-11 cells grow in suspension, accutase or trypsin treatment is not needed here.

3.3.6. Gently pipette the cells in the wells up and down 3 times to mix. Transfer 225 μL cell suspension to a black-walled 96-well plate suitable for fluorescent measurement.

3.3.7. Dilute the CyQuant dye 1:75 with 4 x lysis buffer. Vortex briefly and spin down the solution. Add 75 μL solution to each well of the 96-well plate. Incubate 15 min at room temperature.

3.3.8. Read fluorescence using the 480/520 nm filter set with a fluorescence plate reader.

3.3.9. Analyze data by subtracting the blank and normalizing to the control tumor cell line.

REPRESENTATIVE RESULTS:

The results are usually showed via bar graphs (example shown in Figure 1). Samples with high 480/520 values indicate that the conditioned media has high capacity to recruit macrophages. Depending on experimental need, additional controls can be included. For example, one can use neutralizing antibodies to treat the conditioned media to abolish the macrophage chemotaxis, and perform the same assay. One can also add extra chemokines (i.e. CCL2) to the conditioned media, which serves as a positive control.

DISCUSSION:

In this protocol, there are several key steps: 1) selection of the transwell insert. For the MV-4-11 cell line, 5 μM transwell inserts work well. However, for other cell lines such as the commonly used monocyte cell line THP-1, a different pore size might work better. 2) As different cell lines grow at different speeds, it is important to adjust the volume of the conditioned media according to cell numbers. For this purpose, cell-free media incubated under the same experimental conditions can be used to dilute the conditioned media. 3) FBS contains cytokines. It is important to use serum-free medium to seed MV-4-11 cells in the upper chamber. If FBS is used here, sometimes cell migration cannot be observed.

Researchers can modify this assay for different applications. For example, other tumor cells, either primary patient-derived xenograft culture, or mouse cells can substitute the brain

tumor cell line used in the above-mentioned example. The macrophage cell line can be substituted by primary macrophages or monocytes from patients or mice depending on the specific need. One important point is that the researchers need to select cells from the same species to ensure best result. Although the macrophage chemotaxis pathway is highly conserved, the variation of protein sequence of structure between different species may add layers of complication to interpret experimental results.

TAMs are attracting increasingly more attention in cancer immunology[13,14]. How different genetic changes in the tumors affect recruitment of TAMs and other immune cells in the tumor microenvironment still awaits further studies. By changing pore sizes of the transwell inserts, this assay can be used to study interaction between tumor and other immune cells such as T cells and NK cells. One advantage of using the transwell assay to evaluate tumor-immune interaction is that it is easy to demonstrate how specific oncogenes/mutations affect recruitment of a certain type of immune cells. Moreover, this assay is easy to scale-up for genome-wide screens. This assay opens up additional possibilities for researchers to study tumor-immune cell interactions. Additionally, this assay can be used to study how the conditioned media from tumor cells affect the transcription profile of the macrophages/other immune cells.

All assays have their limitations. For this assay, the conditioned medium is from tumor cells cultured in vitro. The secreted chemokines here might be different from those secreted by tumors grown in vivo. Additionally, the macrophage cell line is different from tumor infiltrating macrophages, which are more phenotypically and transcriptionally diverse. Therefore, further confirmation of the in vitro findings using other in vitro and in vivo methods is necessary.

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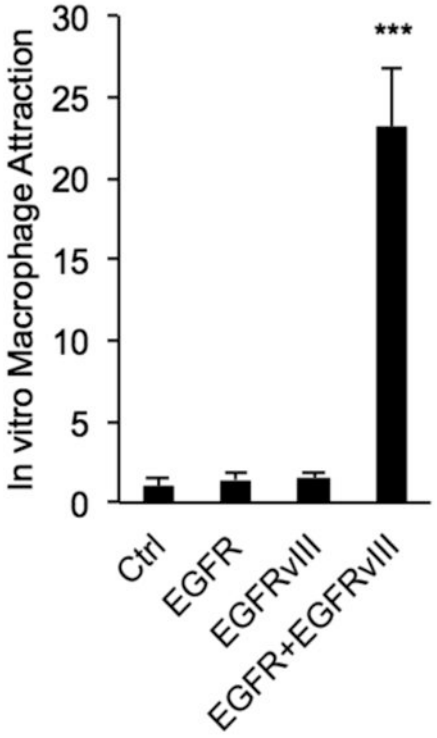

Figure 1.

Conditioned media from U87 cells co-expressing EGFR and EGFRvIII attract more macrophages than U87 cells expressing a control vector, or EGFR/EGFRvIII singly. This figure has been modified from An et al[7].

Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.1um filtration cup	Thermo fisher	566-0010	
0.45uM filter unit	Millipore	SLHA033SS	
10ml serological pipettes	Olympus plastics	12-104	
15ml sterile centrifuge tubes	Olympus plastics	28-103	
1ml pipette tip	ART molecular bioproducts	2779-RI	
2ml aspirating pipet	Falcon	357558	
24-well plate	Millipore	ECM507	Part of ECM507, or can be purchased separately
4x lysis buffer	Millipore	ECM507	Part of ECM507, or can be purchased separately
5μM Transwell insert	Millipore	ECM507	Part of ECM507, or can be purchased separately
75cm2 flask	Corning	430641U	
Accutase	Innovative cell technologies	AT-104	
B27	Gibco	12587-010	
CyQuant Dye	Millipore	ECM507	Part of ECM507, or can be purchased separately
DMEM	Gibco	11965-092	
DMEM:F12	Gibco	10565-018	
EGF	Peprotech	AF-100-15	
FBS	Gibco	26140	
FGF	Peprotech	100-18B	
IMDM	Gibco	12440-053	
PBS	Gibco	14190-144	
Pen Strep	Gibco	15140-122	
Trypan blue	Biorad	1450021	