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Functional Assay to Assess T-cell Inhibitory Properties of Myeloid Derived Suppressor Cells (MDSCs) Isolated from the Tumor Microenvironment of murine Glioma Models

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Abstract

Despite advances in uncovering the molecular mechanisms that mediate glioma progression and the implementation of novel therapeutic modalities, patients' prognosis remains dismal. This is due to both systemic and local tumor induced immune suppression. We are particularly interested in the role played by infiltrating immunosuppressive myeloid derived suppressor cells (MDSCs) in the glioma tumor microenvironment (TME). This immunosuppressive TME also interferes with the effectiveness of immunotherapies against glioma. Development of multipronged treatment approaches is imperative when aiming to generate a robust anti-glioma immune response. Evaluating the inhibitory potential of MDSCs within the TME is an important aspect for developing effective treatments for glioma. Herein, we discuss methodology to assess the inhibitory effects of MDSCs isolated from the TME using a mouse glioma model.

Keywords

MDSCs; T-cell inhibition; immunosuppression; Tumor microenvironment

1. INTRODUCTION

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells that give rise to immature macrophages, dendritic cells, granulocytes, and monocytes (Khaled, Ammori, & Elkord, 2013; Marvel & Gabrilovich, 2015; Parker, Beury, & Ostrand-Rosenberg, 2015). They mainly originate in pathological conditions such as persistent infections, chronic inflammation, or cancer, which alter normal hematopoiesis and block the myeloid cells' differentiation pathway. Immature MDSCs are highly immune suppressive; large numbers of MDSCs are recruited to the tumor microenvironment (TME) which hinder immunosurveillance and aid tumor immune evasion (Angell et al., 2016; Arihara et al.,

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2013; Diaz-Montero et al., 2009; A. Huang et al., 2013; Jordan et al., 2013; Sun et al., 2012; Yang et al., 2017; Zhang et al., 2016). Such scenario could impact survival and adversely affect prognosis.

MDSCs are classified into two subtypes; granulocytic MDSCs (also known as PMN-MDSCs) and monocytic MDSCs (M-MDSCs) (Youn, 2008). In mice PMN-MDSCs are identified as CD11b⁺Ly6G⁺Ly6C^{low} (Gr-1^{high}) whereas, the M-MDSCs are identified as CD11b⁺Ly6G⁻Ly6C⁺ (Gr-1^{low}). In humans, PMN-MDSCs are identified as CD11b⁺CD14⁻CD15⁺ or CD11b⁺CD14⁻CD66b⁺, and M-MDSCs are identified as CD11b⁺CD14⁺HLA-DR^{-/low} CD15⁻ (Youn, 2008). Both MDSCs subtypes express immune suppressive molecules such as arginase (ARG1), iNOS, TGFβ, IL-10, COX2, indoleamine 2,3-dioxygenase (IDO), and PD-L1 (Gabrilovich, 2017; Kamran, Chandran, Lowenstein, & Castro, 2016; Kamran et al., 2017). Many studies have shown that the mechanisms of suppression also appear to be specific to the MDSCs subtype. For example, M-MDSC suppress T-cell responses both in antigen-specific and non-specific manners utilizing mechanisms associated with production of Nitric Oxide (NO), whereas PMN-MDSCs exert their function in an antigen-specific manner and require production of Reactive Oxygen Species (ROS) (Movahedi et al., 2008; Youn, 2008). Direct contact of MDSCs with T cells results in the nitration of the T cell receptor and the CD8 molecule, which renders T cells' unresponsive to specific stimuli (Nagaraj et al., 2007). MDSCs have also been shown to support the development of FoxP3⁺ regulatory T cells (Tregs) through several mechanisms (B. Huang et al., 2006).

Several reports have shown the relation between immunosuppressive myeloid cells and tumor progression in a variety of tumors (Almand et al., 2001; Filipazzi et al., 2007; Hoechst et al., 2008; Iclozan, Antonia, Chiappori, Chen, & Gabrilovich, 2013; Kusmartsev et al., 2008; Poschke, Mougiakakos, Hansson, Masucci, & Kiessling, 2010; Romano et al., 2015; Wang et al., 2013). MDSCs from cancer patients have been shown to express elevated levels of PDL1, iNOS, arginase, ROS and IDO (Dubinski et al., 2016; Zea et al., 2005). MDSCs isolated from melanoma, glioma, squamous cell carcinoma, non-small cell lung cancer, breast cancer, hepatocellular carcinoma, gastric cancer, colorectal and chronic myeloid leukemia cancer patients have been shown to suppress antigen-specific and CD3-ligation induced T cell proliferation (Filipazzi et al., 2007; Hoechst et al., 2008; Iclozan et al., 2013; Kusmartsev et al., 2008; Poschke et al., 2010; Romano et al., 2015; Wang et al., 2013). MDSC expansion has been negatively associated with tumor progression, metastatic burden, response to immunotherapies and progression-free survival (Almand et al., 2001; Kamran et al., 2017; Romano et al., 2015).

The exact phenotypic, and molecular characteristics of MDSCs have not been identified to date. Recently, an algorithm defining the true immunosuppressive MDSCs characteristics based on phenotypic, biochemical, and functional characteristic has been proposed (Bronte et al., 2016). The algorithm classifies MDSCs mainly based on their T-cell inhibitory potential. Therefore, it is crucial to perform reproducible functional assays to uncover the functional properties of any MDSCs population. Here, we detail an *in vitro* reproducible methodology to test the T-cell inhibitory potential of MDSCs isolated from mouse brain tumors.

2. PRINCIPLE OF THE ASSAY:

The assay utilizes OT-1 Rag2 Knockout mice that carry a transgene encoding for T-cell receptor specific for a chicken ovalbumin peptide antigenic fragment (257–264 (SIINFEKL)), which is presented by the MHC class I molecule H2-Kb. When splenocytes from OT-1 mice are cultured *in vitro* in the presence of SIINFEKL peptide, T-cells will proliferate. Splenocytes from OT-1 mice are labelled with Carboxyfluorescein succinimidyl ester (CFSE) (Cell permeable and stable intracellular dye) prior to *in vitro* culturing. As the OT-1 T-cells proliferate in response to SIINFEKL stimulation, the resulting daughter cells incorporate half the number of CFSE-tagged molecules (Figure 1). By measuring the intensity of CFSE in Naïve T-cells vs stimulated T-cells, we can quantify T cells proliferation. If SIINFEKL stimulated OT-1 T-cells are cultured with inhibitory immune cells (such as MDSCs) T-cells' proliferation will diminish. Please note that this protocol has been optimized only in OT-1 Rag2 Knockout mice. A list of reagents and the sources are listed in table 1

3. ISOLATION OF OT-1 SPLEENOCYTES FROM RAG 2 KNOCKOUT MICE

Note: Six to eight-week old female OT-1 Rag 2 knockout mice were purchased from Taconic Farm (Cat number 2334-F, Rensselaer, NY) and were housed in pathogen free conditions at the University of Michigan. All experimental studies were performed in compliance with Institutional Animal Care & Use Committee (IACUC).

1. Sacrifice an OT-1 Rag2 knockout mouse by injecting intraperitoneally (i.p.) 400uL of anesthetics containing Ketamine (75mg/Kg, Pfizer, NY) and dexmedetomidine (1 mg/kg) to induce deep anesthesia.
2. Place the anesthetized mouse on a clean dissection board and check for pedal and tail reflexes. When no responses are observed in both tests, the mouse is considered to be in a deep anesthetized state.
3. Pin the mouse down ventral side up using 26G needles and disinfect the abdomen with 70% Ethanol.
4. Hold the skin above the peritoneal cavity using surgical forceps and with scissors make two slits on either side of the cavity.
5. Carefully extract the spleen that is located to the left side of the abdomen, inferior to the stomach.
6. Place the spleen in a 1.5mL eppendorf tube containing 1mL of Dulbecco's modified eagle media (DMEM, Gibco, NH) supplemented with 10% fetal bovine serum (FBS, Gibco, NH) 100 unit/mL penicillin, and 0.3mg/mL L-glutamine (Gibco, NH) (i.e, solution 1).
7. To prepare the splenocytes' cell suspension, place the spleen onto a 70um (Alkali Inc, FL) strainer attached to a 50mL conical tube.
8. Using the plunger end of a syringe, carefully mash the spleen through the strainer.

9. Wash the cells through the strainer with 20mL of solution 1.
10. Centrifuge the cell suspension at 500 xg for 5minutes and aspirate the supernatant.
11. Resuspend the cell pellet in 2mL of red blood cells (RBCs) lysis buffer (Biolegend) and incubate the cells at 4°C for 2minutes.
12. Add at least 20mL of solution 1 and centrifuge the cells at 500 xg for 5 minutes at 4 °C.
13. Discard the supernatant and resuspend the cells in 2mL of solution 1 and place the tube on ice.
14. To check cell viability, perform a cell count using trypan blue and a hemocytometer.
15. After counting, wash the splenocytes twice with Dulbecco's phosphate-buffered saline (DPBS, Gibco, NH) and resuspend them in DBPS at a concentration of 1.0×10^6 cells/ml.

4. LABELING OT-1 SPLENOCYTES WITH CARBOXYFLUORESCEIN SUCCINIMIDYL ESTER

1. Prepare 10mM stock solution of carboxyfluorescein succinimidyl ester (CFSE, Invitrogen,CA) by adding 9uL DMSO to a vial of Cell Trace CFSE staining solution. Then dilute the stock solution in 20mL of DPBS for a 5μM staining solution.
2. Also, prepare Roswell Park Memorial Institute (RPMI, Gibco, NH) media supplemented with 10% FBS, 100unit/mL penicillin, and 55μM 2-mercaptoethanol (i.e., solution 2).
3. Resuspend the 1mL of cells in DPBS from part 3 in 1mL of 5μM CSFE solution prepared in step 1 (final concentration of CFSE will be 2.5uM).
4. Incubate the cells in the dark for 5 minutes at 37°C with occasional shaking.
5. At the end of the incubation, add 2mL of pure FBS to quench the staining reaction.
6. Pellet the splenocytes at 500 xg for 5minutes at 4 °C (the cell pellet will appear yellow at this point).
7. Wash the pellet with solution 2 check cell viability by performing a cell count using trypan blue (Invitrogen) and a hemocytometer.
8. After counting, resuspend the splenocytes at a concentration of 100,000 cells/ 50μL in solution 2.

5. MDSCS ISOLATION FROM TUMOR

We have established protocol to isolate and maintain brain tumor neurospheres (NS) generated using the sleeping beauty (SB) transposase system (Calinescu et al., 2015; Koschmann et al., 2016; Núñez et al., 2019). Tumor implantation was preformed using the SB generated-NS as previously described (Baker, Castro, & Lowenstein, 2015). Briefly, 6–8 weeks old C57BL6 female mice were anesthetized using ketamine (75mg/kg) and dexmedetomidine (1mg/kg) prior to stereotactic implantation with 50,000 NS in the right striatum. The coordinates for NS implantation are 0.5mm anterior and 2.0mm lateral from the bregma and 3.0 mm ventral from the dura (Figure 1A). NS were injected at a rate 1µl/min (Baker et al., 2015). At moribund stage, tumor infiltrating immune cells were purified from the tumor tissue by percoll™ plus lymphocyte purification kit as described before (Kamran et al., 2017). A detailed procedure for percoll purification is detailed in the next section. After purification, immune cells were stained with Alexa fluor 700- CD45 (Biolegend), APC-CD11b (Biolegend), PE-Texas Red-Gr-1 (Biolegend). Granulocytic and monocytic MDSCs were sorted based on the expression of CD45^{high} Gr-1^{high} and CD45^{high} Gr-1^{low}, respectively (Figure 2). Purified Gr-1^{high} and Gr-1^{low} MDSCs were counted and diluted to 100,000 cells/50µl solution 2 in preparation for co-culturing with OT-1 splenocytes.

6. ISOLATION OF TUMOR INFILTRATING LYMPHOCYTES BY DENSITY GRADIENT

1. Prepare stock isotonic percoll™ (SIP, GE Healthcare life sciences), 4 ml per brain, by mixing 9 parts of percoll with one part of 10X HBSS (Gibco) without Ca⁺⁺ and Mg⁺⁺.
2. Prepare SIP at 70% in 1X HBSS (Gibco) without Ca⁺⁺ and Mg⁺⁺, 3 ml per brain dispensed into a 15 ml polypropylene conical tube
3. Resuspend the pellet with 7 ml of DMEM 10%FCS media
4. Add 3 ml of SIP to the cell suspension pellet to make a final 30% SIP
5. Slowly layer the 10 mL cell suspension on top of the 1 ml of 70% SIP (pipet slowly and carefully). This is the most critical step of the procedure, use a pipette-aid set in the gravity mode avoiding mixing of the 70% and 30% solutions. A very clear flat line should be visible at the 70%–30% interface.
6. Centrifuge 20 min at 800 xg 18°C. Make sure centrifuge will stop with minimal or no brake so that the interphase is not disturbed.
7. Using a transfer pipet, gently remove the layer of debris from the top of the tube and collect 2.0–3.0 ml of the 70%–30% interphase into a clean conical tube containing 8 ml of 1X HBSS. Ensure that the percoll containing the interphase is diluted about three fold, mix a few times by inversion and centrifuge 7 min at 500xg at 18°C.

8. Resuspend pellet in 1 ml of blocking buffer and proceed to the cell staining procedure for MDSC cells sorting as described in section 5.

7. COCULTURING MDSCS WITH OT-1 SPLENOCYTES

1. Plate the CFSE labelled splenocytes at a density of 100,000 cells in 50µl in a 96 well round bottom plate (Thermo-fisher, MA) for the following conditions:
 - a. Condition 1: Unstimulated splenocytes (No SIINFEKL)
 - b. Condition 2: Stimulated OT-1 splenocytes (+ SIINFEKL)
 - c. Condition 3: Stimulated OT-1 splenocytes + 50µl Gr-1^{high} MDSCs
 - d. Condition 4: Stimulated OT-1 splenocyte+ 50µl Gr-1^{low} MDSCs
2. Prepare 1mL of 200nM SIINFEKL peptide (AnaSpec Inc, CA) in solution 2 to stimulate the OT-1 splenocytes.
3. For condition 1, add 150uL of solution 2 (NO SIINFEKL should be added).
4. For condition 2, add 100uL of 200nM SIINFEKL peptide to the wells from step 1 + 50uL of solution 2 (final SIINFEKL concentration in each well will be 100nM).
5. For condition 3 and 4, add 100uL of 200nM SIINFEKL peptide to the wells from step 1 + 50uL (100,000 cells) of sorted Gr-1^{high} or Gr-1^{low} MDSCs, respectively. (final SIINFEKL concentration in each well will be 100nM).
6. Incubate the cells at 37°C, 5% CO2 humidified incubator for 4 days before flow staining for PE-Texas red-CD45 (Biolegend), Percp cy5.5 -CD3 (Biolegend), PB-CD8 (Biolegend), and APC-Cy7 live/dead staining (ebioscience).
7. Proceed with Flow cytometry analysis to determine the percentage of T-cells proliferation based on CFSE expression as illustrated in Figure 2.

8. NOTES:

1. It is important to stain the splenocytes with CFSE in FBS free media. FBS quenches the CFSE staining, therefore avoid using FBS containing media to label the OT-1 splenocytes (see section 4, step 1–4)
2. CFSE overlaps with the FITC channel, therefore do not use FITC conjugated antibody when staining for CD3, CD8, or when MDSCs are being sorted by flow cytometry.
3. We notice that using a round bottom 96 well plate for plating the cells has led to optimal T cell proliferation compared to plating the cells in flat bottom plate. This is probably due to the enhanced antigen presentation, which leads to a greater cell proliferation when the OT-1 splenocytes are stimulated by SIINFEKL.

4. When plating the splenocytes in a 96 wells plate, avoid plating at the edge of the plate, instead, add PBS to all wells at the edges of the plate.
5. SIINFEKL is a peptide that can be degraded by freeze-thawing. Therefore, it is important to prepare small aliquots of the peptide upon receiving from the manufacture to prevent freeze-thawing
6. To get enough MDSCs for performing splenocyte: MDSCs co-culturing, you may need to pool together cells from more than one animal's tumor. In this protocol, we plated 100,000 MDSCs/ well.
7. As a positive control condition, we recommend to co-culture CD11b⁺ Gr-1⁺ cells isolated from BM of WT mice (tumor free animal) with OT-1 splenocytes. We have previously observed that CD11b⁺ Gr-1⁺ cells isolated from BM of WT mice do not inhibit T-cell proliferation.
8. We also recommend co-culturing CFSE labelled MDSCs splenocytes in a dose dependent manner, for example; the ratio of splenocyte: MDSCs could be 1:1, 1:2, or 1:4.
9. It is important to add the β -ME to the splenocytes culture media to reduce the oxidative stress on the splenocytes cultured in the 96-well plate.
10. Use appropriate antibody isotype to gate for positive population of CD45, CD3, CD8.

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Figure 1. Schematic illustrating NS implantation and the *in vitro* T-cell proliferation assay to analyze immune suppressive potential of MDSCs.

(A) Schematic shows stereotactic coordinates for injection of syngeneic mouse glioma NS and GL26 cell line as well as the cell number, and expected median survival. AP: Anteroposterior; ML: Mediolateral; DV: Dorsoventral; MS: Median Survival. (B) OT-1 splenocytes are labelled with the CFSE dye and co-cultured with Gr-1^{high} or Gr-1^{low} MDSCs sorted from tumor. When OT-1 splenocytes divide due to SIINFEKL stimulation, the resulting daughter cells incorporate half the number of cytoplasmic carboxyfluorescein- (CFSE) fluorescent molecules. As cells T cells divide in response to SIINFEKL stimulation, fluorescence intensity decreases exponentially. T cell proliferation can be assessed by measuring the corresponding decrease in the dye intensity via flow cytometry.

Figure 2. Gating strategy to detect percentage of proliferating T cells.

Splenocytes from OT-1 mice were extracted, labelled with CFSE dye, and stimulated *in vitro* with SIINFEKL peptide in the absence or presence of MDSCs for 4 days. Cells were stained with efluor 780 viability dye, Alexa fluor 700-CD45, Percp cy5.5-CD3, and Pacific Blue-CD8. Percentage of proliferating T cells were gated based on control condition (no SIINFEKL stimulation).

Table 1.

Reagents/Materials used in the protocol

Reagent	Source	Catalog Number
Dulbecco's Modified Eagle Medium (DMEM)	Thermofisher	Cat #: 12430054
heat-inactivated fetal bovine serum (FBS)	Atlanta Biologicals	Cat #: S11150
Penstrep (10,000U/mL)	Thermofisher	Cat #:15140122
G418	Thermofisher	Cat #: 10131035
Trypsin-EDTA (0.05%), phenol red	Thermofisher	Cat #:25300054
DPBS, no calcium and no magnesium	Thermofisher	Cat #: 14190144
SIHFEKL (OVA)	Anaspec	Cat #: 60193-1
RBC Lysis Buffer	Biolegend	Cat #: 420301
β -Mercaptoethanol	Sigma-Aldrich	Cat #: MS620-10ML
Percoll	Sigma-Aldrich	Cat #: P4937-500ML
Roswell Park Memorial Institute Medium (RPMI)	Thermofisher	Cat #: 12633012
10X Hanks' Balanced Salt Solution (HBSS)	Thermofisher	Cat #: 14185052
1X Hanks' Balanced Salt Solution (HBSS)	Thermofisher	Cat #: 14175095
Ethyl alcohol (Ethanol)	Sigma-Aldrich	Cat #: E7023-1L
Corning 70 μ m cell strainer	Sigma-Aldrich	Cat #: CLS431751-50EA
Ketamine	Zoetis	Cat #: 000680
Dexmedetomidine	Precedex	Cat #: 122692-5
Covidine Monoject 1mL tuberculin Syringes	Thermofisher	Cat #: 22-257-154
26-gauge precision glide needles	BD Bioscience	Cat #: 30511