Effects of Malignant Melanoma Initiating Cells on T-Cell Activation

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Abstract

Although human malignant melanoma is a highly immunogenic cancer, both the endogenous antitumor immune response and melanoma immunotherapy often fail to control neoplastic progression. Accordingly, characterizing melanoma cell subsets capable of evading antitumor immunity could unravel optimized treatment strategies that might reduce morbidity and mortality from melanoma. By virtue of their preferential capacity to modulate antitumor immune responses and drive inexorable tumor growth and progression, malignant melanoma-initiating cells (MMICs) warrant closer investigation to further elucidate the cellular and molecular mechanisms underlying melanoma immune evasion and immunotherapy resistance. Here we describe methodologies that enable the characterization of immunoregulatory effects of purified MMICs versus melanoma bulk populations in coculture with syngeneic or allogeneic lymphocytes, using [³H] thymidine incorporation, enzyme-linked immunosorbent spot (ELISPOT), or ELISA assays. These assays were traditionally developed to analyze alloimmune processes and we successfully adapted them for the study of tumor-mediated immunomodulatory functions.

Keywords

Cancer stem cell; Melanoma; Immunology; Tumor initiating cell; Antitumor immunity; Immune escape; Immunomodulation; Immunoregulatory; T cell activation; T effector cells; Cytokines; Proliferation; PBMC; [³H]thymidine incorporation; ELISA; ELISPOT

1 Introduction

The ability to purify defined cell subsets based on expression of a prospective marker or marker combination [1, 2] has made a tremendous impact on numerous scientific disciplines, including immunology and tumor biology. For example, cell separation techniques have enabled researchers to functionally characterize previously unknown immune cell populations [3]. In the cancer biology field, advances in cell sorting methodologies have led to the initial identification and prospective isolation of cancer stem cells (CSCs, also referred to as tumor-initiating cells (TICs)) in hematologic malignancies, by the pioneering work of Dick and colleagues [4, 5]. According to a consensus definition [6], CSCs are operationally defined as cancer cell fractions that harbor the preferential ability to propagate tumor growth through self-renewal and by giving rise to less tumorigenic, non-stem cancer cell progeny through a process termed differentiation. We and others have successfully used such sorting techniques to prospectively isolate MMICs, a type of CSC capable of initiating robust experimental tumor growth, from clinical melanoma biopsy specimens and xenograft tumors based on expression of the ABCB5 or CD271 surface epitopes [7–10].

Here we describe protocols employing these cell purification methods to study the immunoregulatory effects of ABCB5-positive MMICs vis-à-vis ABCB5-negative melanoma bulk populations on T-cell activation. Specifically, we harness well-established immunologic assays traditionally developed to characterize alloimmune responses [11, 12], including [³H]thymidine incorporation assays, to assess T-cell proliferation in the presence or absence of MMICs versus non-stem melanoma cells. Furthermore, we utilize ELISA and ELISPOT methodologies to determine cytokine profiles and thus the quality of the T-cell response in peripheral blood mononuclear cell (PBMC)–MMIC versus PBMC–melanoma bulk population cocultures. Importantly, our approach enables the characterization of ex vivo syngeneic immune responses using patient-matched melanoma–PBMC cocultures. Additionally, the herein described methods provide useful tools for the analysis of MMIC-specific immunoregulatory effects in allogeneic culture settings, using either tumor xenograft- or cell line-derived melanoma cells in the presence or absence of mitogen-stimulated PBMCs isolated from healthy donors. We were able to define novel immunoevasive and T-cell-modulatory functions of melanoma cells in general and particularly MMICs using these protocols [13]. Subsequently, several investigators have employed similar methodologies to define immunoregulatory properties of tumorigenic cancer cell fractions [14, 15]. In addition to yielding basic insights into the immunologic processes underlying CSC-driven tumor immune evasion, the protocols described herein permit preclinical assessment of human immune cell–melanoma target cell interactions that could critically inform the development and/or refinement of melanoma immunotherapies, with special attention to the MMIC component that drives tumor virulence.

2 Materials

2.1 Generation of Single Cell Suspensions from Surgical Tumor Specimen

1. Phosphate buffered saline (PBS): sterile, without calcium or magnesium, room temperature.
2. Preparation buffer: Sterile PBS containing 0.1 g/L calcium chloride. Solution has to be sterile filtrated (0.2 µm pore size) and prepared freshly.
3. Tumor digest buffer: Sterile PBS containing 0.1 g/L calcium chloride and 50 µg/mL collagenase (Serva NB6, SERVA Electrophoresis GmbH, Heidelberg, Germany) (see ^{Note 1}). Solution has to be sterile filtrated (0.2 µm pore size) and prepared freshly.
4. Sterile petri dishes, 5 cm in diameter.
5. Sterilized scalpel, surgical scissors, forceps.
6. 50 mL conical tubes, sterile.
7. Orbital shaker.
8. Cell strainers, 70 µm mesh size.
9. Trypan blue solution, 0.4 %.
10. Hemacytometer.

2.2 Purification of Malignant Melanoma Initiating Cells (MMICs) from Single Cell Suspensions

1. Staining Buffer (StB): PBS without calcium or magnesium supplemented with 2 % (v/v) fetal bovine serum (FBS).
2. Magnetic separation buffer (MSB): PBS without calcium or magnesium supplemented with 0.5 % (m/v) bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA).
3. Mouse anti-human ABCB5 monoclonal antibody (mAb, clone 3C2-1D12 [7, 13, 16]).
4. Anti-Mouse IgG MicroBeads.
5. Magnetic separation positive selection columns (MACS™ LS columns, Miltenyi Biotec, Bergisch Gladbach, Germany).
6. Magnetic separator (MidiMACS™ Separator, Miltenyi Biotec, Bergisch Gladbach, Germany).
7. RPMI full medium: RPMI 1640 medium supplemented with 10 % fetal bovine serum and penicillin (100 U/mL)–streptomycin (100 µg/mL).

¹Make sure to utilize collagenase type with low to absent content of trypsin or other surface epitope-cleaving enzymes to permit marker-defined isolation of MMC subpopulations.

2.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

1. Sterile 60 mL syringes.
2. Sterile butterfly needles.
3. Sterile luer stoppers.
4. Dextran 70 6 % in 0.9 % sodium chloride, sterile.
5. Sodium citrate solution 4 % (w/v), sterile.
6. Sterile 15 and 50 mL conical tubes.
7. Conical tube racks.
8. Hanks Balanced Salt Solution.
9. Ficoll-Paque Plus.
10. Sterile Pasteur pipettes.
11. RPMI full medium: RPMI 1640 medium supplemented with 10 % fetal bovine serum and penicillin (100 U/mL)–streptomycin (100 µg/mL).

2.4 Basis for Functional Analysis: PBMC–MMC Coculture

1. Facility with radiation source to irradiate tumor cells (performance needed: 7000 rad).
2. Buffer reservoirs.
3. Multichannel pipette (2–200 µL).
4. RPMI full medium: RPMI 1640 medium supplemented with 10 % fetal bovine serum and penicillin (100 U/mL)–streptomycin (100 µg/mL).
5. Phytohemagglutinin (PHA).

2.4.1 Effect on T Cell Proliferation: [³H]thymidine Incorporation

1. Sterile round bottom 96-well cell culture plates, with lid.
2. [³H]thymidine 1.0 mCi/mL.
3. Automated cell harvester.
4. 96-well filter mats.
5. Sealing pouches.
6. Hot sealer.
7. Liquid scintillation fluid.
8. Beckman Betamax counter.

2.4.2 Effect on Cytokine Production: ELISPOT

1. 96-well Multiscreen IP Plates.

2. PBS: sterile, without calcium or magnesium, room temperature.
3. PBS–Tween: 0.05 % (w/v) Tween 20 in PBS.
4. PBS–Tween–BSA: 0.05 % Tween 20 (w/v) and 1 % BSA (v/w) in PBS.
5. Elispot blocking solution: 1 % BSA (w/v) in PBS, sterile filtrated.
6. AEC (3-amino-9-ethylcarbazole) staining kit.
7. Streptavidin–horseradish peroxidase conjugate.
8. Primary antibodies against IL-2, IL-4, IL-5, IL-10, and IFN- γ .
9. Secondary, biotinylated antibodies against IL-2, IL-4, IL-5, IL-10, and IFN- γ .
10. ELISPOT image analyzer (CellularTechnology, Cleveland, OH).

2.4.3 Effect on Cytokine Production: ELISA

1. Sterile round bottom 96-well cell culture plates, with lid.
2. Human IL-2 Elisa Kit.
3. Human IL-10 Elisa Kit.
4. Spectrophotometric microplate reader.

3 Methods

Here we describe protocols enabling detailed ex vivo or in vitro analysis of immunomodulatory functions of MMIC populations isolated from primary surgical tumor specimens or xenograft melanomas. The functional assays can also be carried out using MMICs purified from malignant melanoma cell lines. In this case harvest cells as per general cell culture protocol and proceed from Section 3.2.

3.1 Generation of Single Cell Suspensions from Surgical Tumor Specimen

1. Obtain surgical specimen from primary clinical or xenograft malignant melanoma tissue according to experimental protocols approved by your institutional review board (IRB) and submerge in preparation buffer. Proceed immediately to step 2.
2. Dissect tissue into small pieces of approximately 1 mm³ using scalpel, surgical scissors, and/or forceps working in a sterile petri dish. Keep fragments submerged in 5 mL sterile preparation buffer.
3. Transfer fragments into 10 mL of sterile tumor digest buffer in a 50 mL conical tube. Incubate for 2–3 h at 37 °C with gentle agitation (200 rpm, orbital shaker) to obtain single cell suspensions (*see* ^{Note 2}).

²Make sure final collagenase concentration in tumor digest buffer is 50 µg/mL. Speed of orbital shaker should be increased if tumor fragments sediment at suggested speed of 200 rpm. During the digestion step, all tissue fragments have to float in suspension for optimal results. Adjust speed of orbital shaker, if tumor fragments sediment at suggested speed of 200 rpm.

4. After digestion is complete (*see* ^{Note 3}), strain cell suspension by passing them over a 70 μm mesh into a fresh 50 mL conical tube containing sterile PBS without calcium or magnesium. Wet mesh with PBS before use.
5. Pellet cell suspension by centrifugation at $300 \times g$ and 4 °C for 7 min. Wash cell suspension once with 50 mL of sterile PBS (without calcium and magnesium) to remove excess collagenase.
6. Perform cell count (*see* ^{Note 4}) and determine cell viability using Trypan Blue stain or alternative method (*see* ^{Note 5}).

3.2 Purification of Malignant Melanoma Initiating Cells (MMICs) from Single Cell Suspensions

1. Collect cells by centrifugation (settings: *see* **step 4**, Section 3.1) and resuspend pellet in 200 μL of StB per 10^7 total cells in 15 mL conical tube. Add 20 $\mu\text{g}/\text{mL}$ of anti-ABCB5 antibody and incubate for 30 min at 4 °C.
2. Add 10 mL StB and collect cells by centrifugation. Repeat washing step.
3. Resuspend pellet in 80 μL of MSB per 10^7 total cells. Add 20 μL of secondary anti-mouse IgG mAb-coated magnetic MicroBeads per 10^7 total cells, mix well, and incubate at 4 °C for 20 min.
4. Add 10 mL MSB and collect cells by centrifugation. Repeat washing step.
5. In the meantime place columns on magnet and rinse with 3 mL of MSB, each.
6. Resuspend pellet in 1 mL of MSB per 10^7 total cells. Add sample to the magnetic columns in 1 mL increments and collect flow-through containing the ABCB5-negative population in sterile 15 mL conical tubes. Do not exceed 5 mL of cell suspension per column. Constantly resuspend cell suspension to avoid formation of cell-aggregates (*see* ^{Note 6}).
7. Rinse columns with 3 mL of MSB, each.
8. For obtaining the ABCB5-positive MMIC population remove column from magnet and plunge cells into a sterile 15 mL conical tube after addition of 3 mL of MSB.
9. Repeat **steps 5–8** in order to increase purity of ABCB5-sorted melanoma cell fractions (*see* ^{Note 7}).

³Incubation times may vary depending on extracellular matrix composition of tumor tissue. It is therefore recommended that you visually inspect digestive progress every 15–30 min. Terminate digestion once tumor chunks are indiscernible.

⁴Cellular yield varies significantly, depending on tumor site, extracellular matrix composition, enzymatic activity etc. A tumor biopsy of ~1³ cm typically yields $>1 \times 10^8$ melanoma cells.

⁵Alternative viability assays include Calcein-AM or Annex-in-V-PE/7-AAD staining followed by flow cytometric analysis, as described [7, 13].

⁶Incubation of samples in a refrigerator (~6–12 °C) as opposed to incubation on ice (4 °C) may require alternative incubation times for MACSTM MicroBeads. Please see manufacturer's instructions.

⁷The loading capacity of the MACSTM LS columns should not exceed 5×10^7 melanoma cells. To avoid overloading, prepare enough columns according to the number of cells you purified via collagenase digestion. You can pool the MMC subsets from the respective

10. Pellet cells by centrifugation and wash twice with 10 mL of RPMI full medium. Resuspend cell pellets of MMC subsets in 10 mL of RPMI full medium each and determine number of viable cells using Trypan Blue. Keep cell suspensions on ice until ready to seed according to experimental layout.

3.3 Isolation of Peripheral Blood Mononuclear Cells

1. Prepare sterile 60 mL syringes for blood drawing by filling them with 8 mL of Dextran and 6 mL of sodium citrate solution.
2. Draw blood from healthy donor (allogeneic approach) or donor-identical melanoma patient (syngeneic approach). 50 mL blood will yield about 5×10^7 PBMCs. Close syringes with luer stopper and mix by gently inverting the syringes a couple of times.
3. Place syringes upright (luer stopper facing upwards) into the Styrofoam holder and transfer to incubator. Incubate at 37 °C, 5 % CO₂ for 60 min to allow for sedimentation of red blood cells.
4. Transfer the supernatant into separate 50 mL conical tubes by means of a butterfly needle. Work in a sterile environment. Discard syringes.
5. Fill the 50 mL tubes containing the supernatants up to the 35 mL mark with HBSS.
6. Slowly add 13 mL of Ficoll-Paque solution to the bottom of each tube. You will observe the formation of a second phase under the serum layer. You are ready for Ficoll-Paque density gradient centrifugation.
7. Centrifuge tubes for 30 min at $400 \times g$ at room temperature. Make sure to disable the brake in your centrifuge settings.
8. Post centrifugation you will find that several layers have developed. The PBMCs are located in the cloudy white interphase between the upper red plasma layer and the lower clear ficoll/granulocytes layer. Transfer tubes to sterile environment and harvest PBMCs into one sterile 50 mL conical tube using a Pasteur pipette.
9. Fill tube up to the 50 mL mark with HBSS and collect cells by centrifugation ($300 \times g$, 4 °C, 7 min).
10. Resuspend PBMCs in 10 mL RPMI full media and determine number of viable cells using Trypan Blue. Keep cell suspensions on ice until ready to seed according to experimental layout.

columns after purification. If columns clog extensively during the processing of a particular tumor sample, try increasing the EDTA concentration to maximally 4 mM and/or add DNase I enzyme to MSB.

3.4 Basis for Functional Analysis: PBMC–MMC Coculture

To analyze the immunomodulatory functions of malignant melanoma cell (MMC) subsets, we use PBMC–MMC cocultures and combine them with different readouts according to the effector function to be addressed (see below). The experimental setup and groups are the same for all endpoint analyses and are therefore outlined in general below. For all assays, purified MMC subsets are irradiated and used as non-proliferative stimulators for major histocompatibility complex (MHC)-mismatched (healthy donors) or syngeneic (patient-derived) PBMCs. Allogeneic, MHC-mismatched PBMCs require mitogen stimulation via phytohemagglutinin (PHA) and are seeded with or without irradiated MMC subsets at a 1:10 ratio. Donor-identical, syngeneic patient-derived PBMCs do not require the addition of PHA and are seeded with the irradiated MMC subsets at a 1:1 ratio.

Experimental groups for MHC-mismatched setups:

- Irradiated ABCB5-positive MMCs, 2.5×10^6 cells per well (negative control).
- Irradiated ABCB5-negative MMCs, 2.5×10^6 cells per well (negative control).
- Irradiated unsegregated MMCs, 2.5×10^6 cells per well (negative control).
- PBMCs, 2.5×10^5 cells per well (baseline subtraction).
- PBMCs, 2.5×10^5 cells per well + PHA (1 $\mu\text{g/mL}$) (positive control).
- PBMCs, 2.5×10^5 cells per well + PHA (1 $\mu\text{g/mL}$) + irradiated ABCB5-positive MMCs, 2.5×10^6 cells per well.
- PBMCs, 2.5×10^5 cells per well + PHA (1 $\mu\text{g/mL}$) + irradiated ABCB5-negative MMCs, 2.5×10^6 cells per well.
- PBMCs, 2.5×10^5 cells per well + PHA (1 $\mu\text{g/mL}$) + irradiated unsegregated MMCs, 2.5×10^6 cells per well.

Experimental groups for syngeneic setups:

- Irradiated ABCB5-positive MMCs, 2.5×10^5 cells per well (negative control).
- Irradiated ABCB5-negative MMCs, 2.5×10^5 cells per well (negative control).
- Irradiated unsegregated MMCs, 2.5×10^5 cells per well (negative control).
- PBMCs, 2.5×10^5 cells per well (positive control, basic proliferation).
- PBMCs, 2.5×10^5 cells per well + irradiated ABCB5-positive MMCs, 2.5×10^5 cells per well.
- PBMCs, 2.5×10^5 cells per well + irradiated ABCB5-negative MMCs, 2.5×10^5 cells per well.
- PBMCs, 2.5×10^5 cells per well + irradiated unsegregated MMCs, 2.5×10^5 cells per well.

Tumor cell suspensions should be diluted in RPMI full medium to a concentration of 5.0×10^7 cells/mL (MHC-mismatched setup) or 5.0×10^6 cells/mL (syngeneic setup), respectively. After irradiation with 7000 rad, tumor cells are seeded according to

experimental layout (50 μL /well). PBMCs (2.5×10^6 cells/mL in RPMI full medium with or without 2 $\mu\text{g/mL}$ PHA) are subsequently added (100 μL /well). All wells are filled to 200 μL total volume by addition of RPMI full medium (*see* ^{Note 8}).

3.4.1 Effect on T Cell Proliferation: [³H]thymidine Incorporation

1. Seed PBMC–MMC cocultures as per desired layout into 96-well round-bottom plates and incubate at 37 °C, 5 % CO₂ for 48 h.
2. Add 1 μL (i.e., 1 μCi) [³H]thymidine per well and return to incubator for 18 h (*see* ^{Note 9}). Observe radiation safety regulations.
3. Harvest cells using onto a 96-well filter mat an automated cell harvester. Dry filter mat by microwaving it briefly and hot-seal dry filter in sealing pouch with one dose of liquid scintillation fluid. Observe radiation safety regulations.
4. Determine differential proliferation by analyzing counts per minute using a beta scintillation counter. Observe radiation safety regulations.

3.4.2 Effect on Cytokine Production: ELISPOT

1. Dilute desired primary antibody in PBS according to manufacturer's specifications and coat Multiscreen IP plates by pipetting 100 μL of antibody solution to each well according to your experimental layout. Work in a sterile environment. Incubate plates at 4 °C in a humidified container overnight (*see* ^{Note 10}).
2. Wash plates twice with 200 μL PBS per well, rotating the plate by 180° after each washing step. Add 200 μL ELISPOT blocking solution to each well and incubate plate for 1 h at room temperature. Wash plates three times with 200 μL PBS per well, rotating the plate by 180° after each washing step. Work in a sterile environment.
3. Seed PBMC–MMC cocultures as per desired layout and incubate at 37 °C, 5 % CO₂ for the indicated time. We incubate plates for 12 h to determine IFN- γ production, 24 h to assess IL-2 secretion, 36 h to assess IL-4 and IL-10, and 48 h to determine IL-5 production
4. Wash plates three times with 200 μL PBS per well, rotating the plate by 180° after each washing step. Working in a sterile environment is not necessary from this point onward.
5. Wash plates three times with 200 μL PBS-Tween per well, rotating the plate by 180° after each washing step.

⁸Be sure to resuspend cell suspensions properly to ensure even distribution. Keep cell suspensions on ice at all times and work quickly, seeding the cells on ice using a multichannel pipette and buffer reservoirs.

⁹Upon 18 h incubation with [³H]thymidine, radioactive plates can be stored at –20 °C until further use. Seal microplates with Parafilm and freeze plates until ready to quantitate [³H]thymidine incorporation. Observe radiation safety regulations.

¹⁰We line a styrofoam box (with lid) with a humid paper towel and place the plates in it. Subsequently, we place the styrofoam box containing the ELISPOT plates into the cold room at 4 °C.

6. Dilute desired secondary antibody in PBS–Tween–1 % BSA according to the manufacturer's specifications and add 100 μ L per well. Incubate plates at 4 °C in a humidified container overnight.
7. Wash plates three times with 200 μ L PBS–Tween per well, rotating the plate by 180° after each washing step.
8. Dilute streptavidin–horseradish peroxidase conjugate 1:2000 in PBS containing 1 % BSA and add 200 μ L per well. Incubate plates for 2 h at room temperature.
9. Wash plates three times with 200 μ L PBS–Tween per well, and subsequently twice with PBS, rotating the plate by 180° after each washing step.
10. Prepare AEC visualization solution using the AEC Staining Kit according to the manufacturer's instructions. Add 200 μ L per well and observe development of spots (15–60 min). Upon sufficient development of spots, rinse with distilled water to terminate the reaction.
11. Determine number of spots per well using an ELISPOT image analyzer and calculate fold-differences between experimental groups [cytokine secretion (fold-difference) = spots per well (PBMC + PHA + ABCB5-positive MMC)/spots per well (PBMC + PHA + ABCB5-negative MMC)].

3.4.3 Effect on Cytokine Production: ELISA

1. Seed PBMC–MMC cocultures as per desired layout into 96-well round-bottom plates and incubate at 37 °C, 5 % CO₂ for 24 h (IL-2) or 36 h (IL-10).
2. After incubation harvest supernatants containing cytokines by brief centrifugation of the microplates to precipitate floating cells and subsequent careful removal of 100–150 μ L supernatant using a multichannel pipette. Transfer supernatants into separate microplate until further analysis (*see* Note 11).
3. Perform ELISA using the Human IL-2 or Human IL-10 ELISA Kits according to the manufacturer's instructions. Analyze differential cytokine production spectrophotometrically using a microplate reader.

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¹¹Supernatants can be stored at –20 °C until further use. Seal microplates with Parafilm and freeze plates until ready to run ELISA.

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