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Functional characterization of tumor antigen-specific T-cells isolated from the Tumor microenvironment of Sleeping Beauty induced murine glioma models

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Abstract

Glioma is one of the most aggressive tumors. The median survival of the most aggressive form (Glioblastoma (GBM)) is approximately 14–20 months. The current standard of care includes tumor resection, chemotherapy and radiation, nevertheless, the incidence of recurrence remains high and there is a critical need for developing new therapeutic strategies. T-cell mediated immunotherapy that triggers an anti-tumor T cell-mediated memory response is a promising approach since it will not only attack the primary tumor but also prevent recurrence. Multiple immunotherapeutic strategies against glioma are currently being tested in clinical trials. We have developed an immune-mediated gene therapy (Thymidine kinase plus Fms-like tyrosine kinase 3 ligand: TK/Flt3L) which induces a robust anti-tumor T cell response leading to tumor regression, long-term survival and immunological memory in glioblastoma models. Efficacy of the anti-glioma T cell therapy is determined by anti-tumor specific effector T cells (Han et al., 2014). Therefore, assessing effector T cell activation status and function are critical readouts for assessing the effectiveness of the therapy. Here, we detail methodologies to evaluate tumor specific T-cell responses using a genetically engineered Sleeping Beauty transposase-mediated glioma model. We first describe the glioma model and the generation of neurospheres (NS) that express the surrogate antigen cOVA. Then, we describe functional assays to determine anti-tumor T-cell response.

Keywords

Glioma; sleeping beauty; Neurospheres; T_{cell} activation; immunotherapy

1. INTRODUCTION

Gliomas are the most common primary central nervous system neoplasm in adults (Acs, 2013). The current classification of gliomas (reviewed in 2016) are based not only on

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histopathologic appearance but also on well-established molecular parameters which identified multiple glioma subtypes (Ceccarelli et al., 2016; Louis et al., 2016; Reifenberger, Wirsching, Knobbe-Thomsen, & Weller, 2016). The most common glioma subtype is the most aggressive grade IV astrocytoma, known as glioblastoma (GBM). GBM carries a poor prognosis with a median survival of approximately 15 months (Ostrom et al., 2018; Reifenberger et al., 2016). Lower grade gliomas include grade II and III oligodendrogliomas and astrocytomas, which are now classified as diffuse gliomas. Patients with grade II and grade III oligodendroglioma have 10-year survival rates of 63 and 39% respectively, while those with grade II and grade III astrocytomas have 5-year survival rates of 47 and 27% respectively (Ostrom et al., 2018). The median age at diagnosis ranges from 43 years for oligodendroglioma, 48 years for low grade astrocytoma, to 65 years for GBM (Ostrom et al., 2018).

Treatment for GBM is relatively standardized and includes maximal surgical resection, when possible and dependent on the location of the tumor, followed by concomitant radiotherapy and temozolomide (Stupp et al., 2005). Surgical resection is often offered if the tumor can be safely removed without risking neurologic injury (Buckner et al., 2016). Ionizing radiation, however, can result in severe cognitive dysfunction and this must be given appropriate consideration particularly for patients with low grade tumors who have relatively favorable prognosis (Racine, Li, Molinaro, Butowski, & Berger, 2015).

In the mid-2000s, large initiatives such as The Cancer Genome Atlas (TCGA) Consortium were established in order to comprehensively define the molecular characteristics of gliomas in a large number of samples (Cancer Genome Atlas Research et al., 2015; R. G. W. Verhaak, 2016). These efforts revealed the high heterogeneity of gliomas (Bai et al., 2016; Brat et al., 2015; Michele Ceccarelli, 2016). These molecular studies identified a distinct genetic signature found within low grade gliomas defined by mutations in the isocitrate dehydrogenase gene 1 (IDH1) TP53 and ATRX mutations in astrocytomas as well as chromosomal deletions involving 1p and 19q in oligodendrogliomas (Ceccarelli et al., 2016). Molecular analysis is necessary for the discovery of novel treatments, effective clinical trial design, and clinical decision making (Ceccarelli et al., 2016; R. G. Verhaak et al., 2010).

Immunotherapy for glioma is a promising area of active investigation (Dunn-Pirio & Vlahovic, 2017; Kamran et al., 2018). There are a variety of approaches under investigation in ongoing clinical trials including passive immunotherapy with antibodies, autologous activated lymphocytes, immune-mediated gene therapy, oncolytic viral therapy or active immunotherapy with tumor cell based vaccines, peptides, or dendritic cells (Polivka Jr et al., 2017). Some examples of these therapies include Rindopepimut, a peptide based vaccine that targets the mutant variant of the epidermal growth factor receptor (EGFRvIII) that is present in more than 40% of glioblastoma (Sampson et al., 2011). Dendritic cell (DC) vaccines loaded with autologous tumor lysate are another example of immunotherapy approaches being studied (Garg et al., 2016). We have developed an immune-mediated gene therapy strategy, which involves two adenoviral vectors (Ad) encoding, Herpes Simplex Type 1-Thymidine kinase (TK) used in combinations with a second Ad encoding Fms-like tyrosine kinase 3 ligand (TK/Flt3L). This gene therapy approach induces a robust anti-tumor

T cell response leading to tumor regression, long-term survival and immunological memory in glioblastoma models (Ali et al., 2005; Candolfi et al., 2009; Candolfi et al., 2014; Curtin et al., 2009; Kamran et al., 2017; Larocque et al., 2010; Yang et al., 2010) that is currently in being tested in a Phase I clinical trial at our institution (). Other dendritic cell-based vaccine includes the DCVax-L phase III study () which is currently ongoing (Dunn-Pirio & Vlahovic, 2017). Given the success for immune checkpoint inhibitors for other advanced solid tumor types such as melanoma (Gide, Wilmott, Scolyer, & Long, 2018), there is significant interest in this class of immune-based therapy for use in patients with glioma. Current studies involve the CTLA-4 inhibitor, ipilimumab as well as PD-1 inhibitors, nivolumab and pembrolizumab for patients with GBM. For instance, the phase III CheckMate 143 trial () is evaluating nivolumab alone and nivolumab plus ipilimumab versus the anti-vascular endothelial growth factor antibody, bevacizumab in patients with recurrent GBM.

The generation of robust animal models for glioma is critical to expand our knowledge of the biology of these tumors and also to develop new therapeutic approaches that could improve patient outcomes in combination with standard treatments. One of the main goals in the generation of animal models is to reproduce the genetics and phenotypic features found in glioma patients, including molecular composition, tumor heterogeneity and tumor microenvironment. We and others have established the Sleeping Beauty (SB)-transposon system (Calinescu et al., 2015; Koschmann et al., 2016; Núñez et al., 2019; Wiesner et al., 2009) which represents a powerful tool to generate genetically engineered glioma models. This system is based on the SB-transposase activity (Paschka et al., 2010), which allows the generation of mouse brain tumors through the insertion of specific genetic lesions into the genome of stem cells present along the lateral ventricle of neonate mice (Calinescu et al., 2015). Therefore, this system efficiently captures different glioma subtypes, maintaining the genetic make-up and neuropathological features encountered in human gliomas (Koschmann et al., 2016; Mendez et al., 2018; Núñez et al., 2019). Here, we describe the SB glioma model and the method used to generate SB-derived NS. Then, we detail the expression of the surrogate antigen cOVA which will be used to determine antigen specific T cell responses against glioma. Finally, we describe functional assays determine tumor specific T cell activation and proliferation against glioma.

2. GENERATION OF GLIOMA NEUROSPHERES DERIVED FROM SLEEPING BEAUTY TRANSPOSON MOUSE GLIOMA MODEL

Glioma NS represent a valuable source for *in vitro* studies of tumor biology, as well as, to generate orthotopic glioma models by intracranial implantation. Glioma NS can be generated from glioma tissue obtained from patient's biopsies and also from genetically engineered mouse glioma models. Here, we described a protocol to generate NS derived from SB transposase mouse glioma models.

1. Neonatal pups are injected with the plasmid mix, that will induce the genetic lesions of interest, delivered into the lateral ventricle, as described before (Calinescu et al., 2015).

2. The tumor must grow to ($>10^6$ photons/s/cm²/sr of luminescence) for an optimal yield of cells. The mice glioma obtained using the SB transposon system express luciferase and they are monitored by bioluminescence, whose signal level is proportional to the tumor size. A bioluminescence signal of $> 10^6$ photons/s/cm²/sr is considered enough to proceed with the protocol of glioma NS generation.
3. Euthanize the mouse harboring the selected tumor using an overdose of isoflurane anesthetic. After 5–10 min, check absence of pedal reflex of the mouse, decapitate the mouse and dissect the brain as previously described (Calinescu et al., 2015).
4. Once the brain is removed, the tumor mass can be dissected by GFP expression, using a dissecting microscope (Olympus SZX16) equipped with a fluorescent lamp in a sterile environment.
5. For tumor homogenization, place the dissected tissue in a 1.5 mL tube containing 300 μ L of NS media (DMEM/F12 with 1x B27 supplement, 1x N2 supplement, 1x Normocin, and 1x Antibiotic/Antimycotic supplemented with human recombinant EGF and basic-FGF at a concentration of 20 ng/mL each). Continue with the homogenization, in the same 1.5 mL tube, using a sterile pestle and moving it in up and down motion 20 times under laminar flow hood.
6. The cell clumps must be dissociated by adding 1 mL of enzyme-free dissociation media (Accutase, BioLegend) then incubate for 5 min at 37 °C. Subsequently, filter the cell suspension through a 70 μ m cell strainer and wash the strainer with 10 mL of NS media.
7. To collect the glioma NS, centrifuge for 5 min at $300 \times g$. Discard the supernatant, resuspend the cells into 6 mL of NS media and plate into a T-25 culture flask and maintain the cells at 37 °C (5% CO₂).
8. After 3 days in culture a mixed population of cells can be identified: i) adherent cells; ii) dead cells and iii) glioma NS. Continue collecting the NS population (floating cell clusters), and plate in a new T-25 culture flask in the same conditions as in step 7.
9. After 3 more days, centrifuge glioma NS for 5 min at $300 \times g$ and dissociate as step 8. Split the NS in T-25 culture flasks with 6 mL of NS media at a density of 500,000 cells per flask.
10. Freeze aliquots of glioma NS after dissociation (at least one million per aliquot) by resuspending the pellet in Fetal Bovine Serum + 10% DMSO and store at -80 for 48 hours, then transfer to liquid nitrogen for long term storage.

3. STABLE TRANSFECTION OF NEUROSPHERES WITH A SURROGATE TUMOR ANTIGEN

The plasmid encoding cytoplasmic chicken ovalbumin, pCI-neo-cOVA, was developed in our lab (Addgene ID:25097). NS were transfected with pCI-neo-cOva, pCI-neo, or pMax-GFPTM vector using the P3 primary cell 4D-NucleofectorTM X kit (Lonza, Allendale, NJ). The following procedure was followed for NS transfection:

1. Collect the NS from an 80–90% confluent T75 flask by centrifugation at 1500 rpm, and discard the supernatant by decantation.
2. Dissociate the cells using 1ml of Accutase® cell detachment solution (Biolegend). Incubate for 90sec at 37 °C, neutralize with 9ml HBSS, and centrifuge the NS again at 1500rpm to separate the supernatant.
3. Resuspend the detached NS in 10ml HBSS. Take 10µl to count, then split the NS to have 200,000 cell/ tube.
4. Spin the tubes at 90G (Note: any higher centrifugation speed would impair viability), for 10 min, at room temp.
5. Resuspend the NS with 20µl of transfection solution (4D-neuclofector solution)
6. Add 500ng of pCI-neo-cOva, pCI-neo, or pMax-GFPTM (to determine the transfection efficacy) to each tube (duplicate each condition). Mix gently by pipetting up and down. Keep the cells at room temperature, allow to settle down for 5 min.
7. Transfer the suspended NS in each well to the NucleocuvetteTM Vessel (note: insert the pipet tip all the way to the bottom of the vessel to avoid any air bubbles)
8. Tap the NucleocuvetteTM Vessel gently to get rid of any air bubbles.
9. Place the NucleocuvetteTM Vessels with closed lid into the retainer of the 4D-NucleofectorTM X Unit in the right orientation.
10. Enter the right information regarding the protocol number (Pulse code EN 138) and hit enter to pulse the NS with the plasmid mix. If the pulse was successful you will see a green mark on the screen, otherwise, red mark means either air bubbles interference or failure to pulse due to other reasons.
11. Combine NS from two wells and culture them in one well from a 24-well plate containing 400–600µg/ml G418 selection antibiotic.
12. Remove dead cells and supplement with 20ng/ml of EGF, FGF growth factors as well as 400–600µg/ml G418 every other day.
13. Cells are cultured until stable cOva expression is established which can be confirm by western blot.

4. ASSESSMENT OF T-CELL FUNCTION

4.1. TK-Flt3L gene therapy

Tumor implantation is done as previously described (Baker, Castro, & Lowenstein, 2015). Briefly, 6–8 weeks old female mice are anesthetized using ketamine and dexmedetomidine prior to stereotactic implantation with 50,000 of cOVA expressing NS in the right striatum. The coordinates for implantation are 0.5mm anterior and 2.0mm lateral from the bregma and 3.0 mm ventral from the dura. NS were injected at a rate 1µl/min. The therapeutic mechanism of the T cell mediated immunotherapy is described in Figure 1. The procedure for Ad-TK and Ad-Flt3L treatment is as follows:

1. First-generation replication-defective, recombinant adenovirus type 5 (Ad) vectors expressing transgenes (TK or Flt3L) under transcriptional control of the human CMV major intermediate early promoter within the E1 region were developed, purified, characterized, and scaled up using methods described previously, as described previously (Ali et al., 2005; Dewey et al., 1999; Southgate, Kroeger, Liu, Lowenstein, & Castro, 2008).
2. Twelve days after tumor implantation, each mouse received an intratumoral injection of either saline or the combination of the two viruses: AdV 5×10^8 plaque-forming units (pfu) of Ad-Flt3L and 2×10^8 pfu of Ad-TK in 1.5 µL volume in three locations at 3.5 mm, 3.0 mm, and 2.5 mm ventral from the dura
3. Ganciclovir (GCV; TSZ Chemicals) was administered 200µl of 25mg/kg i.p twice a day for 10 days starting immediately the day after viral injection
4. By the end of the tenth day of GCV treatment, tumor was harvested and infiltrating immune cells were purified using percoll™ plus lymphocyte purification kit as described before (Kamran et al., 2017)

4.2. Isolation of tumor infiltrating lymphocytes (TILs)

1. Prepare the following reagents: Complete media: RPMI/DMEM medium supplemented with 10% FBS, 50 unit/mL penicillin and 50 µg/mL streptomycin. 90% stock isotonic percoll (SIP)-prepared by mixing 9 parts of percoll and 1 part of 10X HBSS. 70% percoll™-prepared by mixing 7 parts of 90% SIP and 3 parts of 1X HBSS.
2. Dissect the tumor carefully avoiding the normal areas of the brain.
3. Place the tumor onto a 70 µm strainer attached to a 50mL conical tube, filled with 10 ml of complete media (Dulbecco's modified eagle (DMEM) media supplemented with 10% fetal bovine serum (FBS))
4. Using the plunger end of a syringe, carefully mash the tumor through the 70µm strained (Alkali Scientific Inc.,) on ice. Wash the strained two times with 10 ml complete media for force cell through the strainer and to minimize cell loss.
5. Spin the suspension at 1500rpm (Allegra™ 6R centrifuge, Beckman Coulter) for 5 min at room temperature. Discard the supernatant and resuspend the cells in

7ml of complete media (in 15ml Falcon tube). To this, add 3ml of the 90% SIP (GE Healthcare) and mix well by pipetting up and down 3–5 times. The 30% gradient is now ready.

6. To layer the 70% percoll™ under the 30% percoll™ gradient, fill 1ml serological pipette with 1ml of 70% percoll™ and push it to the bottom of the 15ml Falcon tube containing the homogenized brain tissue. Now slowly release the 1ml of the 70% percoll™, making sure a clear interface is formed.
7. Repeat steps 2–5 for each mouse in the study.
8. Spin the falcon tubes at 2200 rpm for 20 min at room temperature.
9. After centrifugation, a white band will have formed at the interface containing the immune cells. Discard the supernatant (by pipetting) until about 1 ml from the interface. Collect the PBMCs that have accumulated at the interface between the two density centrifugation media layers using a 1ml pipette and transfer to a fresh falcon tube. Add an additional 12ml 1X HBSS to the isolated cells and spin at 1500 rpm for 5 min to wash out the percoll™.
10. Discard the supernatant and resuspend the cells in an appropriate amount of complete media for counting using Trypan blue. After the percoll™ separation, the cells can be stored on ice.
11. After counting, 50,000 cells can be plated per well of a 96 well V-bottom plate for staining for flow cytometry analysis. Cells can be stained with a variety of markers to distinguish the various immune cells as indicated in Table.1. Prior to staining cells should be labeled with viability dye and Fc receptor block (Biolegend) according to the manufacturer's instructions. Antibody staining is performed in Fluorescence-activated cell sorting (FACS) buffer on ice in the dark for 30min. Post staining the cells are spun down at 1500rpm for 5min at 4°C. Supernatant is discarded and cells are washed with 200µl of FACS buffer.

4.3. Intracellular staining of T cell activation cytokines IFN- γ and GzB:

Activated CD8+ T cells lyse cancer cells by secreting Granzyme B (GzB) and interferon gamma (IFN- γ). Methodologies for assessment of GzB and IFN- γ in activated CD8+ T cell are shown in Figure 2. The detailed procedure is as follows:

1. For intracellular GzB and IFN- γ level, purified tumor infiltrating lymphocytes (TILs) were counted, stimulated *in vitro* with 200 mg/mL lysate from cOVA expressing NS for 24 hr in 10% FCS-containing media supplemented with IL-2. (Note: The lysate from cOVA expressing NS is prepared by resuspending 1×10^7 NS in 1ml RIPA lysis buffer (ThermoFisher Scientific) followed by 3 cycles of freeze-thawing, 30sec each).
2. Six hours before harvesting the TILs, cells were treated with 4nM Brefeldin and 1nM monensin to accumulate the cytokines and other secreted proteins, intracellularly.

3. Cells were stained with CD3 (Biolegend) and CD8 (Biorad) antibodies, followed by intracellular stains for granzyme B (eBioscience) and IFN γ (BD Biosciences)
4. For the tumor specific CD8 T cells, TILs were stained with Phycoerythrin (PE) conjugated H2Kb- OVA tetramer (MBL international). Cells were first stained with CD3 (Biolegend) and CD8 (Biorad) antibodies.
5. Exhausted T-cells can also be examined by analyzing the expression level of exhausted Tcell markers such as; PD-1, TIM-3, CTLA-4.
6. Antigen-induced T cell proliferation is used to validate the T cell response upon antigen (SIINFEKL) stimulation:
7. Splenocytes from tumor bearing animals were isolated in a single cell suspension.
8. They were then labeled with CFSE dye (fisher scientific) using manufacturer's protocol.
9. Labelled splenocytes were cultured in vitro in the presence or absence of the cOVA peptide (SIINFEKL) for 4–5 days.
10. Cells were harvested, gated on live/dead, CD3, CD8. The percentage of proliferating T cells were analyzed based on the intensity of CFSE and compared to control (Figure 2).

5. SUMMARY

Choosing a robust animal model as well as evaluating the immune response in glioma is critical for future implementation of immune-mediated therapeutic strategies in clinical trials. The methods presented here describe in detail the generation of NS-expressing the surrogate tumor antigen, cOVA from SB glioma models. This model can be used to determine and evaluate the efficacy of T cell-mediated anti-glioma immunotherapy induced by a variety of strategies, such as DC mediated immunotherapy. This in turn will have a strong impact on the preclinical evaluation of many immunotherapies in glioma. We also explain detailed methodology to analyze the functional T cell response against glioma. Such methodologies can be applied to other T cell mediated immunotherapy that target a variety of cancers in preclinical animal models.

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Figure 1: Depiction of T cell response to tumor specific antigens following TK/Flt3 mediated immunotherapy.

Neurospheres derived from sleeping beauty induced tumors are implanted intracranially into immunocompetent mice [1]. These neurospheres express cytoplasmic ovalbumin (cOVA) to assess antigen specificity. Two weeks later, animals are treated with adenoviral vectors delivered directly within the tumor mass [2]. The adenoviral vector encodes thymidine kinase (TK) and Fms-like tyrosine kinase 3 ligand (Flt3L). The infected tumor cells secrete the immunostimulatory cytokine Flt3L into circulation, which recruits dendritic cells to the tumor microenvironment. Intraperitoneal injection of ganciclovir (GCV) is administered the day after immunotherapy [3]. Ganciclovir is an inactive prodrug, but can become active by TK. TK initiates the first phosphorylation step followed by a cascade of cellular kinases Guanylate Kinase (GK) and Nucleoside Diphosphokinase (NDK) that further phosphorylate

the drug. Activated GCV mimics guanosine and is incorporated into DNA where it induces replication stalling and apoptosis. Tumor cells that undergo apoptosis release damage associated molecular patterns (DAMPs) and cOVA. This stimulates dendritic cell expansion and phagocytosis of cOVA. Dendritic cells migrate to the nearby lymph nodes and present cOVA peptide on major histocompatibility complex (MHC) to naïve T cells. The primed T cells express T cell receptor (TCR) against cOVA peptide and migrate from the lymph node into the bloodstream. The activated T cells then infiltrate the tumor microenvironment and identify cOVA-expressing tumors cells by the formation of immunological synapses. The release of cytolytic (perforin, and granzyme B) and inflammatory (interferon-gamma) molecules by T cells lyses tumor cells.

Figure 2: Functional Assay to evaluate the activation of T cells in response to tumor lysate and stimulation with cOVA peptide *ex vivo*.

Following immunotherapy, tumor bearing animals are euthanized to extract CD45⁺ cells from the tumor mass and leukocytes from the spleen. Leukocytes from the tumor microenvironment (TME) are co-cultured with tumor lysate to stimulate T cell activation *in vitro*. Additionally, interleukin-2 (IL-2) is added to the media to enhance this activation. Monensin and Brefeldin inhibit ER secretory pathway which caused accumulation of intracellular cytokines in order to be measured by flow cytometry. The degree of T cell activation is evaluated based on the levels of activation markers such as INF- γ and GzB. Unstimulated T cells isolated from the spleen are labelled with carboxyfluorescein succinimidyl ester (CFSE). With each cell division, the CFSE dye is diluted signifying the proliferation of the T cells in response to SIINFEKL. Proliferating and non-dividing cells can be assessed by gating CD3⁺/CD8⁺ against CFSE fluorescence. Non-dividing cells will retain high intensity of CFSE, and proliferating T cells will have lower intensity.

Table 1.

Immune cell markers use to characterize lymphocytes in mice

IMMUNE CELLS	MARKERS
CD4 T cells	CD45+, CD3+, CD4+
CD8 T cells	CD45+, CD3+, CD8+
Tregs	CD45+, CD3+, CD4+, FoxP3+, CD25+
Monocytes	CD45+, CD14+
Macrophages	CD45+, CD11b+, F4/80+
M1 Macrophages	CD45+, CD11b+, CD206–
M2 Macrophages	CD45+, CD11b+, CD206+, Arginase+
PMN MDSCs	CD45+, CD11b+, Ly6G+, Ly6Clow
Monocytic MDSCs	CD45+, CD11b+, Ly6G–, Ly6C+
MDSCs	CD45+, CD11b+, Gr-1+
DCs	CD45+, CD11c+, MHCII+
B cells	CD45+, CD19+
NK cells	CD45+, CD11b ^{int} , NK1.1+