Video Article

Stereotactic Adoptive Transfer of Cytotoxic Immune Cells in Murine Models of Orthotopic Human Glioblastoma Multiforme Xenografts

Ulrich Jarry*[1,2], Noémie Joalland*[1,2], Cynthia Chauvin*[1,2], Béatrice Clemenceau[1,2,3], Claire Pecqueur[1,2], Emmanuel Scotet[1,2]

[1]INSERM, CNRS, Université d'Angers, Université de Nantes, CRCINA

[2]Immunotherapy, Graft, Oncology, LabEx IGO

[3]Hopital de Nantes, Hotel Dieu

*These authors contributed equally

Correspondence to: Emmanuel Scotet at Emmanuel.Scotet@inserm.fr

URL: https://www.jove.com/video/57870
DOI: doi:10.3791/57870

Keywords: Immunology and Infection, Issue 139, Cancer, glioblastoma, immunotherapy, adoptive transfer, stereotaxy, lymphocytes

Date Published: 9/1/2018

Citation: Jarry, U., Joalland, N., Chauvin, C., Clemenceau, B., Pecqueur, C., Scotet, E. Stereotactic Adoptive Transfer of Cytotoxic Immune Cells in Murine Models of Orthotopic Human Glioblastoma Multiforme Xenografts. *J. Vis. Exp.* (139), e57870, doi:10.3791/57870 (2018).

Abstract

Glioblastoma multiforme (GBM), the most frequent and aggressive primary brain cancer in adults, is generally associated with a poor prognosis, and scarce efficient therapies have been proposed over the last decade. Among the promising candidates for designing novel therapeutic strategies, cellular immunotherapies have been targeted to eliminate highly invasive and chemo-radioresistant tumor cells, likely involved in a rapid and fatal relapse of this cancer. Thus, administration(s) of allogeneic GBM-reactive immune cell effectors, such as human VY9Vδ2 T lymphocytes, in the vicinity of the tumor would represents a unique opportunity to deliver efficient and highly concentrated therapeutic agents directly into the site of brain malignancies. Here, we present a protocol for the preparation and the stereotaxic administration of allogeneic human lymphocytes in immunodeficient mice carrying orthotopic human primary brain tumors. This study provides a preclinical proof-of-concept for both the feasibility and the antitumor efficacy of these cellular immunotherapies that rely on stereotactic injections of allogeneic human lymphocytes within intrabrain tumor beds.

Video Link

The video component of this article can be found at https://www.jove.com/video/57870/

Introduction

GBM (WHO grade IV astrocytoma), is the most frequent and aggressive primary brain cancer in adults. In spite of aggressive treatments that combine surgery and radio-chemotherapy, GBM remains associated with an extremely poor prognosis (median survival of 14.6 months and a 2-year-mortality > 73%)[1]. This evidences that few efficient therapeutic advances have been validated over the last decade[2]. Among candidates for the design of more effective therapeutic strategies[3,4,5], immunotherapies[6] are currently explored to track and eliminate highly invasive and radio/chemo-resistant tumor cells, suspected for their key contribution to rapid and fatal tumor relapse[7]. Various potential immunological targets were identified and proposed for immunotherapies, involving natural or modified αβ or Yδ T lymphocytes such as GBM-specific tumor antigens or stress-induced molecules[8,9,10]. The possibility to administrate selected GBM-reactive immune cell effectors represents a unique opportunity to deliver elevated amounts of effector lymphocytes directly into the site of residual malignancy. To support this strategy, we have recently shown that models based on immunodeficient mice carrying orthotopic primary human GBM xenografts faithfully recapitulate the development of brain tumors in GBM patients[9,11]. Moreover, these models were used to demonstrate the strong antitumor efficiency of adoptively transferred allogeneic human VY9Vδ2T lymphocytes.

This protocol describes the critical experimental steps for achieving stereotactic immunotherapies of brain tumors, such as GBM, based on the adoptive transfer of allogeneic T lymphocytes. The article shows: (i) the amplification of therapeutic allogeneic immune effector T lymphocytes, such as human VY9Vδ2T lymphocytes; (ii) the preparation of these effector T lymphocytes for injection; (iii) the procedure for stereotactic administration within the brain, near the tumor. This article also provides insight into the behavior of these cellular effectors after stereotactic injection.

The therapeutic approach presented here is based on the injection of 20 x 10^6 effector cells *per* dose for each brain tumor-bearing immunodeficient mouse. An initial *in vitro* expansion step is required to produce large quantities of immune cells. Therefore, non-specific cell expansions are performed using phytohemagglutinin (PHA-L) and irradiated allogeneic feeder cells: peripheral-blood mononuclear cells (PBMCs) from healthy donors and Epstein Barr Virus (EBV)-transformed B-lymphoblastoid cell lines (BLCLs), derived from PBMCs by *in vitro* infection with EBV-containing culture supernatant from the Marmoset B95-8 cell line, in the presence of 1 µg/mL cyclosporin-A.

GBM-reactive effector immune cells are compared and selected from *in vitro* assays[9]. These effector cells are activated and amplified using standard protocols, according to their nature (*e.g.*, human Vγ9Vδ2 T lymphocytes[9] or human anti-herpes virus αβ T lymphocytes[12]) with a

Copyright © 2018 Journal of Visualized Experiments

minimum purity of > 80%, as routinely checked by cytometric analysis. The cell expansion procedure detailed below applies to various human lymphocyte subsets.

Protocol

The following procedure involving animal subjects was performed according to institutional guidelines (Agreement #00186.02; regional ethics committee of the Pays de la Loire [France]). Human PBMCs were isolated from the blood collected from informed healthy donors (Etablissement Français du Sang Nantes, France). All steps are performed under sterile conditions.

1. Non-specific Expansion of Cytotoxic Effector T Lymphocytes

1. Prepare and irradiate feeder cells at 35 Gy. For the stimulation of 2 x 10^5 - 4 x 10^5 effector cells, prepare 10 x 10^6 PBMCs and 1 x 10^6 BLCLs from three distinct, healthy donors.
2. Resuspend both feeder cells and effector cells in 15 mL of RPMI supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, penicillin (100 IU/mL), and streptomycin (0.1 µg/mL) and 300 IU/mL recombinant IL-2.
3. Add PHA-L at a final concentration of 1 µg/mL, carefully mix, and distribute 150 µL of the cell suspension per well in 96-well U-bottomed plates.
4. Incubate at 37 °C and with 5% CO_2 in a humidified atmosphere.
5. Daily check the plate until large cell clumps form in the culture wells (~day 7).
6. Transfer the cells into a culture flask at 1 x 10^6 cells/mL in fresh culture medium.
7. Determine the total cell number by counting (2x a week) and maintain them at 1 x 10^6 cells/mL in fresh culture medium.
   NOTE: Effector immune cells should be ready for the therapeutic administration at a resting state (usually 3 weeks after initial amplification stimulus). The purity and the reactivity of these effector cells should be checked prior to *in vivo* injections (*e.g.*, with *in vitro* assays).

2. Pre-operative Effector Cells Preparation

1. After checking the effector cell count, collect the effector cells in a 50-mL tube by centrifugation (300 x *g* for 5 min).
   NOTE: To compensate for any loss, prepare an excess of cells (*e.g.*, 50 x 10^6).
2. Carefully remove the supernatant and resuspend the cells in 15 mL of sterile PBS and centrifuge for 5 min at 300 x *g* to perform the wash.
3. Carefully and completely remove the supernatant and then resuspend the cell pellet in 1 mL of sterile PBS.
4. Transfer the resuspended cells in a 1.5-mL microtube for centrifugation at 300 x *g* for 5 min.
5. Carefully and completely remove the supernatant by pipetting slowly.
6. Resuspend the cell pellet in 8 µL of sterile PBS per mouse.
   NOTE: This is a critical step.
7. Measure the volume of the cell suspension by using a micropipette. If necessary, add sterile PBS (20 x 10^6 cells in 15 µL of PBS per dose) and mix carefully.
8. Check, by using a micropipette, that the final volume per mouse is between 15 and 20 µL (imperatively < 20 µL).
9. Keep the cells on ice until stereotactic injection.
   NOTE: More than 3-h timepoints were not tested.

3. Stereotactic Injection

1. **Equipment set-up**
   1. Assemble and calibrate a small animal stereotactic frame according to the manufacturer's instructions to ensure the accuracy of intracranial injections (*e.g.*, syringe size, desired volume, and rate of injection).
      NOTE: A slow infusion rate is recommended (*i.e.*, 2 - 3 µL/min).
   2. Install the material under a microbial safety cabinet (MSC) to maintain the sterility of the instruments and to protect the mice from infections.
      NOTE: Place" isothermal blocs" in a water bath at 37 °C. This system limits the hypothermia of mice during the surgery. Heating pads, which are necessary for post-procedural care, must be used during the continuous temperature monitoring.

2. **Pre-operative animal preparation**
   1. Anesthetize a mouse with an intraperitoneal injection of ketamine (10 mg/mL) and xylazine (0.1 mg/mL) at 10 µL/g of body weight of the mouse.
   2. Perform a toe pinch test to ensure the complete anesthesia and analgesia of the animal.
      NOTE: Any movement is an indication of non-deep analgesia and, if that occurs, a few more minutes are required before repeating the operation.
   3. Once the mouse is properly anesthetized, use scissors to remove the fur from the surgical site (between the two ears, up to the nose).

3. **Pre-operative cell preparation**
   1. Carefully resuspend the cells with a pipette (several times) prior to each injection to prevent any cell clumping.
   2. Carefully draw the required cell suspension volume (15 - 20 µL) into the 22-G microsyringe to avoid the aspiration of bubbles.
      NOTE: This cell-loading step into the microsyringe is important to minimize variances in injected volumes. Reload cells for each individual injection between procedures to prevent any cell clumping and to ensure an even number of effector cells administration in the cohort.
   3. Then, place the syringe into the adapted syringe pump.

4. **Procedural care**

1. Disinfect the surgical site with swabs soaked in povidone-iodine 5% solution at least 3x.
2. Place a lubricating ophthalmic ointment in the mouse's eyes to prevent any drying of the corneas.
3. Position the anesthetized mouse on the stereotactic frame, on a warm isothermal block covered with a sterile plastic wrap to maintain the mouse's temperature during surgery and limit the mortality.
   NOTE: The mouse's nose and teeth should be appropriately positioned above the tooth bar, to ensure adequate respiratory flow during the procedure.
4. Once the mouse is positioned above the tooth bar, tighten the ear bars firmly under the mouse's ears to immobilize the head.
   NOTE: Be careful to not damage the eardrums or to compromise the respiration.
5. Make a 1 - 2 cm midline sagittal skin incision with sterile scissors along the upper part of the cranium from anterior to posterior to expose the skull.
6. Identify the intersection of the sagittal and coronal sutures (Bregma) to serve as landmarks for stereotactic localization prior to the injection (shown in **Figure 1**).
7. Place the microsyringe above this point.
8. Move the microsyringe 2 mm right lateral and 0.5 mm anterior of the Bregma.
9. Using a microdrill, make a small hole in the skull with a sterile drill bit at predetermined coordinates. Be careful to remain superficial in order to avoid any traumatic injury of the brain.
   NOTE: In this protocol, immune cells were injected within an established tumor (one week after tumor cell injection). The skin should be reopened (scar) and the injection is performed at the same coordinates used for the tumor cell implantation (the hole is generally still present up to 2 weeks after the injection). Coordinates were selected for injecting tumor and effector cells in the brain parenchyma[13,14].

5. **Injection of immune effector cells**
   1. Carefully insert the microsyringe into the drilled hole and, moving slowly, forward the needle 3 mm down in the dura and then backward 0.5 mm to a final depth of 2.5 mm prior to injecting the effector cells.
   2. Run the effector cell injection at 2 - 3 µL/min and monitor the mice all along the injection time.
   3. Once the injection is complete, withdraw the needle for only 1 mm and keep the microsyringe in place for one additional minute before slowly withdrawing completely the microsyringe, to prevent any leakage from the infusion site.
      NOTE: Following the removal of the animal from the stereotactic device, immediately clean the injection equipment for upcoming experiments.

6. **Post-operative care and follow-up of mouse**
   1. Remove the animal from the stereotactic frame and immediately apply povidone-iodine 5% solution on the incision site and close the skin with an appropriate surgical suture.
   2. Apply 2% lidocaine gel directly on the wound and administer 0.15 µg/g of buprenorphine by a subcutaneous injection for post-procedural analgesia.
   3. Place back the anesthetized mouse to its cage above a heating pad set to 37 °C to maintain an appropriate mouse body temperature and to avoid any hypothermia.
      NOTE: Separate housing is not necessary.
   4. Monitor the mouse until it is fully recovered from anesthesia and transfer it to a housing room.
      NOTE: To date, this protocol is well supported as few unforeseen complications have occurred (< 5% of injected mice).
   5. Daily monitor the mice and euthanize them when any declining health signs are observed (*e.g.*, hunched posture, reduced mobility, prostration, or significant body weight loss [≥ 15%]).

Representative Results

This study describes the strategy of adoptive transfers of cellular immune effector cells within the brain of tumor-carrying mice, based on stereotactic injections performed directly within the tumor bed.

To minimize any risk of brain injury associated with a large injection volume, the effector cell suspension needs to be concentrated (20 x 10^6 cells in 15 - 20 µL of PBS). To check whether this cell concentration step might affect the viability of the effector cells, these cells were prepared according to the described protocol and loaded into the microsyringe. The effector cells were collected immediately, or 10 min after loading them into the microsyringe. The cells were stained with propidium iodide (PI), a fluorescent DNA intercalating agent that is not permeant to live cells and analyzed by flow cytometry at different timepoints (0, 24, and 72 hours). The results show that the preparation and the loading into the microsyringe do not significantly affect the viability of effector cells for at least 24 hours (**Figure 2A** and **2B**). At 72 hours, a slight, but non-significant, increase of $PI^{positive}$ cells was observed (14% compared to 11% for unloaded cells). In a similar way, the antitumor reactivity of effector cells that were prepared and maintained on ice for 3 hours was analyzed. Effector cells were cocultured with brain tumor cells for 4 hours in presence of an anti-CD107a mAb. The upregulation of the activation marker CD107a, similar to the value obtained in the control conditions, indicates that the reactivity of effector cells is not affected by the preparation and the loading into the microsyringe (**Figure 2C**).

To evaluate whether effector cells survive and move within the brain parenchyma following their intra-tumoral implantation, 20 x 10^6 effector cells were injected into the tumor site of mice carrying a brain tumor (GBM-1[11]). One week later, the brains were collected, fixed, sectioned, and stained for hematoxylin, eosin, and safran coloration (HES) and anti-human CD3 mAb (IHC). HES coloration identified the structure of the brain tumors (**Figure 3**, *left panel*). The CD3 staining identified and localized the effector immune T lymphocytes (here, human VY9Vδ2 T cells) (**Figure 3**, *right panel*). Interestingly, effector T lymphocytes were detected around the tumor (**Figure 3**, *upper right panel*), in the tumor core (**Figure 3**, *middle right panel*), but also in the contralateral hemisphere (**Figure 3**, *bottom right panel*). Moreover, the function of human T lymphocytes isolated from the mouse brain 48 hours after their injection (4x10^6 αβ T cells), which represent 2% of the brain cells, was analyzed. The results indicate that collected brain-injected effector allogeneic αβ T cells expand and proliferate upon a non-specific PHA-feeder cells activation performed in the presence of IL-2 (**Figure 4**).

Copyright © 2018 Journal of Visualized Experiments

Together, these results show that effector T cells prepared and administrated under these procedures survive for hours in the brain and can patrol within the tumor and healthy brain tissues. These procedures have been used for assessing the antitumor efficiency of therapeutic stereotactic administrations of allogeneic human resting VY9Vδ2 T cells to control the development of human GBM brain tumors[9,11]. These studies evidence that injections of allogeneic human effector T lymphocytes in the tumor bed significantly improve the survival of mice carrying brain tumors. Interestingly, surviving mice did not carry detectable tumor cells, thus indicating a complete tumor rejection.

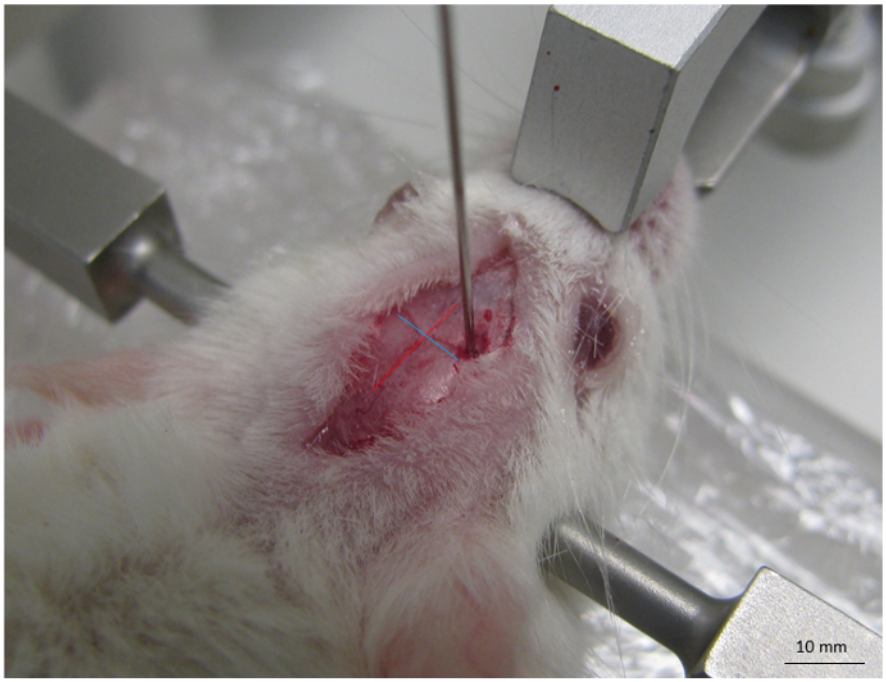

Figure 1: Picture of the main anatomical landmarks on the mouse skull. This includes sagittal (red line) and coronal (blue line) sutures and their intersection (Bregma) used to orient the site of injection. The scale bar is indicated. Please click here to view a larger version of this figure.

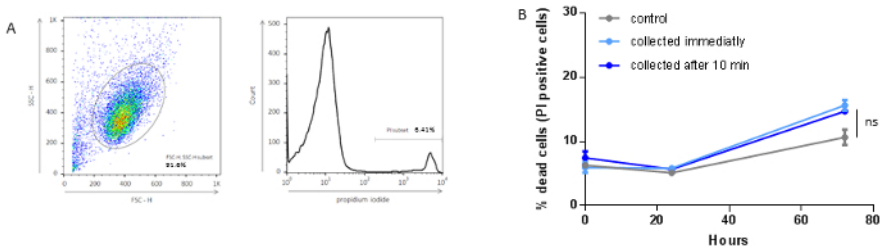

Figure 2: Survival and activation of prepared effector T lymphocytes. Effector cells (here, resting human peripheral VY9Vδ2 T cells) were amplified and prepared according to the described procedure. Staining of lymphocytes with propidium iodide (PI), a DNA intercalating fluorescent compound that is not permeant to live cells, and functional activation were performed. (**A**) This panel shows a representative plot of forward (FSC-H) *versus* side (SSC-H) scatter gating of effector lymphocytes (*left panel*). The histogram shows the PI staining of gated control lymphocytes (*right panel*). The percentage of $PI^{positive}$ lymphocytes is indicated. (**B**) This panel shows the percentage of dead lymphocytes ($PI^{positive}$) collected immediately (light blue line) or after 10 min (dark blue line) in the microsyringe, measured at the indicated timepoints. As control, unloaded prepared cells were used (grey line). (*n* = 3; mean ± SD; ns = not significant.) (**C**) CD107a surface mobilization was measured by flow cytometry on either $V\delta2^{positive}$ control cells (unprepared) or prepared lymphocytes maintained on ice (prepared + 3 h) following a coculture with target human primary brain tumor cells. The percentage of lymphocytes is indicated in each cytometric quadrant. Please click here to view a larger version of this figure.

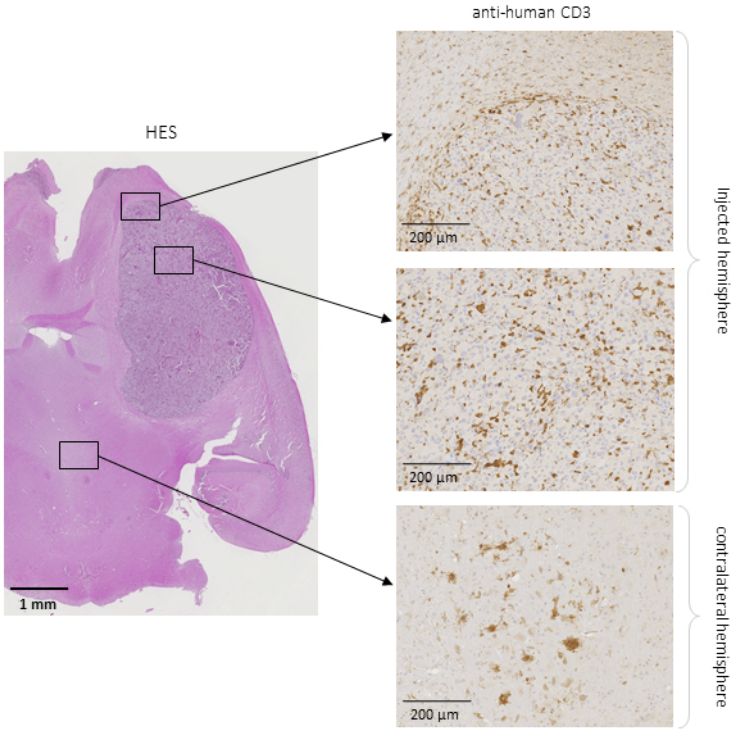

Figure 3: Detection and localization of effector T lymphocytes within the brain of tumor-bearing mice. One week after glioblastoma brain tumor implantation, effector immune cells (here, resting human peripheral VY9Vδ2 T cells) were injected into the brains of mice. One week after the immunotherapeutic treatment, the brains were collected, fixed, and sectioned. Brain sections were colored for immunohistochemistry analysis (hematoxylin, eosin, and safran [HES] coloration) (*left panel*) or stained with anti-human CD3 antibody (*right panel*). The results shown are representative of three independent experiments. The scale bar is indicated.

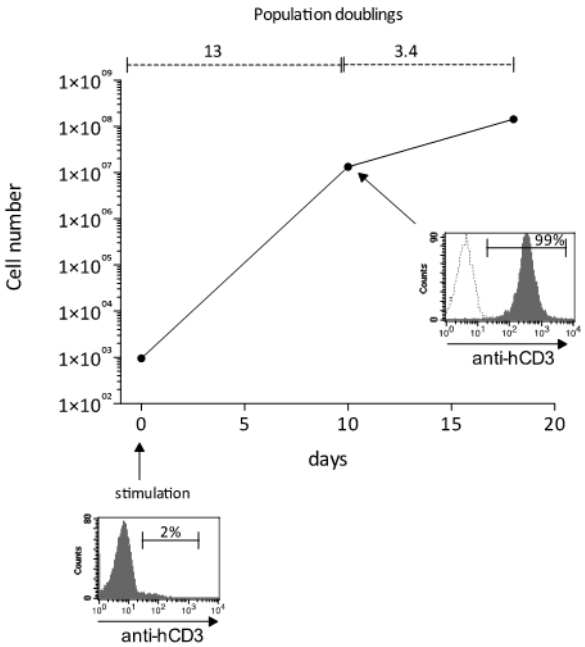

Figure 4: Activation of effector T lymphocytes collected from the brain of treated mice. Resting human T lymphocytes (here, 4 x 10^6 human αβ T lymphocytes) were injected into the brains of NSG mice. After 48 hours, the brains were collected and dissociated. The percentage of human brain-infiltrating T lymphocytes was measured by flow cytometry using an anti-human CD3 antibody (*bottom panel*). The collected cells were seeded (10 cells/well) in 96-well U-bottomed plates and stimulated (PHA-feeder cells and IL-2) for 20 days (16 doubling cycles). Note, on day 10, 13.3 x 10^6 of human T lymphocytes were obtained (*right panel*).

Discussion

An adoptive transfer of selected native or engineered immune effector cells represents a promising approach to efficiently treat tumors, such as infiltrative brain cancers, taking care of limiting reactivities against non-transformed cells[15,16,17,18]. However, the central nervous system, which comprises the brain, has a particular immune status, notably due to the existence of the blood-brain barrier and the lack of a classical lymphatic drainage system[19,20]. These physiological features affect tissue trafficking and might compromise systemic injections of immune cells. To overcome these hindrances, intraparenchymal injections have been explored on the principle that antitumor cells are rather locally delivered, closely to the tumor site, as for microspheres that contain pharmacological compounds[21,22]. On one hand, the limited dilution of lymphocytes within the organ might improve their antitumor efficiency, but it can also amplify deleterious mechanical or tumor adaptation effects, such as tissular compression, which is developing along brain tumor growth. This implies that this procedure requires small volumes of immunotherapeutic injections. This issue is even more critical in animal experimental models in which brain tumors cannot be surgically excised. This article describes a therapeutic approach for the preparation and the local delivery of brain tumor-specific cellular effectors, based on stereotactic injection(s) of allogeneic human T lymphocytes.

This study shows the preparation and the stereotaxic delivery of allogeneic human VY9Vδ2 T lymphocytes in immunodeficient NSG mice carrying human GBM xenografts. The first stage of this protocol describes a simple procedure for amplifying allogeneic T lymphocytes from PBMCs of healthy donors, using a standard nonspecific PHA-feeders-IL2 stimulation that produces large quantities of pure effector T lymphocytes, allowing their therapeutical utilization[23,24]. The second stage of this article focuses on the preparation of resting T lymphocyte suspensions on the day of the stereotaxic administration. A particular focus was placed on this important step that requires a high cellular density that should not affect the viability and the function of the selected effector T lymphocytes. Finally, regarding *in vivo* experiments, the preparation of effector T lymphocytes and their injection within the tumor core is associated with their dissemination, not only within the tumor but also in the surrounding brain tissues, highlighting their particular ability to patrol and to track invasive tumor cells. Importantly, these preparation and injection methods retain the ability of these T lymphocytes to be activated within the brain upon a specific recognition of brain tumor cells. Altogether, these compelling characteristics ensure the ability of T lymphocytes to specifically and efficiently target and eliminate deep infiltrative brain tumor cells which are a hallmark of GBM[25]. Of note, a special care has to be taken during the injection and the removal of the microsyringe to minimize any brain lesion or effector cell leaks.

In conclusion, this article describes an efficient procedure for delivering large amounts of allogeneic human anti-tumor lymphocytes, such as resting human VY9Vδ2T lymphocytes, within the vicinity of brain tumors. Importantly, this therapeutical procedure is not accompanied with adverse effects either on the transferred T lymphocytes (*e.g.*, viability, reactivity) or on the brain tissues. Recent studies, based on murine orthotopic models of primary human GBM, have demonstrated that VY9Vδ2T lymphocytes efficiently target GBM cells, including tumor cells which have deeply infiltrated the brain parenchyma[9,11]. These elements open opportunities for the development of novel adoptive T lymphocytes transfer procedures that could be applied in the first instance in mice carrying orthotopic brain tumors and, then, in clinical studies in GBM patients.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors thank the staff of the University Hospital animal facility (UTE) of Nantes for animal husbandry and care, the cellular and tissular imaging core facility of Nantes University (MicroPICell) for imaging, and the Cytometry facility (Cytocell) from Nantes for their expert technical assistance. This work was funded by INSERM, CNRS, Université de Nantes, Institut National du Cancer (INCa#PLBio2014-155), Ligue Nationale contre le Cancer (AO InterRegional 2017), and the European consortium ERA-Net Transcan2 (Immunoglio). The team is funded by the Fondation pour la Recherche Medicale (DEQ20170839118). This work was realized in the context of the LabEX IGO and the IHU-Cesti programs, supported by the National Research Agency Investissements d'Avenir *via* the programs ANR-11-LABX-0016-01 and ANR-10-IBHU-005, respectively. The IHU-Cesti project is also supported by Nantes Metropole and the Pays de la Loire Region. The authors thank Chirine Rafia for providing help in correcting the manuscript.

References

1. Stupp, R., Roila, F. Malignant glioma: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Annals of Oncology.* **20 Suppl 4**, 126-128 (2009).
2. Weller, M., Cloughesy, T., Perry, J. R., Wick, W. Standards of care for treatment of recurrent glioblastoma--are we there yet? *Neuro-Oncology.* **15** (1), 4-27 (2013).
3. Chen, S. H., Shine, H. D., Goodman, J. C., Grossman, R. G., Woo, S. L. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America.* **91** (8), 3054-3057 (1994).
4. Choi, P. J., Tubbs, R. S., Oskouian, R. J. Emerging Cellular Therapies for Glioblastoma Multiforme. *Cureus.* **10** (3), e2305 (2018).
5. Zhu, L. *et al.* Targeting and Therapy of Glioblastoma in a Mouse Model Using Exosomes Derived From Natural Killer Cells. *Frontiers in Immunology.* **9**, 824 (2018).
6. Chung, D. S., Shin, H. J., Hong, Y. K. A new hope in immunotherapy for malignant gliomas: adoptive T cell transfer therapy. *Journal of Immunology Research.* **2014**, 326545 (2014).

Copyright © 2018 Journal of Visualized Experiments

7. Vauleon, E., Avril, T., Collet, B., Mosser, J., Quillien, V. Overview of cellular immunotherapy for patients with glioblastoma. *Clinical and Developmental Immunology.* **2010** (2010).
8. Brown, C. E. *et al.* Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy. *The New England Journal of Medicine.* **375** (26), 2561-2569 (2016).
9. Jarry, U. *et al.* Stereotaxic administrations of allogeneic human Vgamma9Vdelta2 T cells efficiently control the development of human glioblastoma brain tumors. *Oncoimmunology.* **5** (6), e1168554 (2016).
10. Dutoit, V. *et al.* Exploiting the glioblastoma peptidome to discover novel tumour-associated antigens for immunotherapy. *Brain.* **135** (Pt 4), 1042-1054 (2012).
11. Joalland, N. *et al.* IL-21 Increases the Reactivity of Allogeneic Human Vgamma9Vdelta2 T Cells Against Primary Glioblastoma Tumors. *Journal of Immunotherapy.* **41** (5), 224-231 (2018).
12. Clemenceau, B. *et al.* Effector memory alphabeta T lymphocytes can express FcgammaRIIIa and mediate antibody-dependent cellular cytotoxicity. *The Journal of Immunology.* **180** (8), 5327-5334 (2008).
13. Abdelwahab, M. G., Sankar, T., Preul, M. C., Scheck, A. C. Intracranial implantation with subsequent 3D *in vivo* bioluminescent imaging of murine gliomas. *Journal of Visualized Experiments.* (57), e3403 (2011).
14. Jarry, U. *et al.* Treg depletion followed by intracerebral CpG-ODN injection induce brain tumor rejection. *Journal of Neuroimmunology.* **267** (1-2), 35-42 (2014).
15. June, C. H., O'Connor, R. S., Kawalekar, O. U., Ghassemi, S., Milone, M. C. CAR T cell immunotherapy for human cancer. *Science.* **359** (6382), 1361-1365 (2018).
16. Baruch, E. N., Berg, A. L., Besser, M. J., Schachter, J., Markel, G. Adoptive T cell therapy: An overview of obstacles and opportunities. *Cancer.* **123** (S11), 2154-2162 (2017).
17. Bryant, N. L. *et al.* Characterization and immunotherapeutic potential of gammadelta T-cells in patients with glioblastoma. *Neuro-Oncology.* **11** (4), 357-367 (2009).
18. Pereboeva, L., Harkins, L., Wong, S., Lamb, L. S. The safety of allogeneic innate lymphocyte therapy for glioma patients with prior cranial irradiation. *Cancer Immunology, Immunotherapy.* **64** (5), 551-562 (2015).
19. Bailey, S. L., Carpentier, P. A., McMahon, E. J., Begolka, W. S., Miller, S. D. Innate and adaptive immune responses of the central nervous system. *Critical Reviews in Immunology.* **26** (2), 149-188 (2006).
20. Louveau, A. *et al.* Structural and functional features of central nervous system lymphatic vessels. *Nature.* **523** (7560), 337-341 (2015).
21. Menei, P. *et al.* Local and sustained delivery of 5-fluorouracil from biodegradable microspheres for the radiosensitization of glioblastoma: a pilot study. *Cancer.* **86** (2), 325-330 (1999).
22. Menei, P. *et al.* Stereotaxic implantation of 5-fluorouracil-releasing microspheres in malignant glioma. *Cancer.* **100** (2), 405-410 (2004).
23. Salot, S. *et al.* Large scale expansion of gamma 9 delta 2 T lymphocytes: Innacell gamma delta cell therapy product. *Journal of Immunological Methods.* **326** (1-2), 63-75 (2007).
24. Bennouna, J. *et al.* Phase-I study of Innacell gammadelta, an autologous cell-therapy product highly enriched in gamma9delta2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. *Cancer Immunology, Immunotherapy.* **57** (11), 1599-1609 (2008).
25. Singh, S. K. *et al.* Identification of human brain tumour initiating cells. *Nature.* **432** (7015), 396-401 (2004).

Copyright © 2018 Journal of Visualized Experiments