Implantable tumor models to study tumor-infiltrating T cell function

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This protocol will detail two major types of experiment: tumor growth/survival and TIL analysis. Tumors are an incredibly immunosuppressive environment and are a major source of (1) regulatory T cells, (2) suppressive myeloid lineages, (3) exhausted effector T cells, (4) plasmacytoid DCs.

The highlighted portions are additions to this protocol that result in dramatically higher cellular yields. This was determined (painstakingly) empirically. They include a “marination” step in which intact tumors are injected with a protease mixture and a heavy vortex post processing. This vortex step (presumably) liberates additional T cells trapped between tumor cells after collagen and hyaluronic acid digestion.

Time for experiment: survival (25-30 days post implantation), TIL analysis (12-15 days)

Reagents and equipment needed

- Cell culture
  - B16-F10 melanoma cells, EL4 thymoma cells, MC38 adenocarcinoma, LL carcinoma
  - D10 media
  - Trypsin-Versene (or Trypsin EDTA)
  - 1X PBS
  - T25, T75 flasks
- Injection
  - Isofluorane and chamber
  - Nair or a razor
  - 28 ½ or 30 G needles
- Monitoring
  - Digital calipers
- Harvest
  - Sharp scissors/forceps
  - 5 cm plastic TC plates
  - Glass slides
  - 70 um filters
  - Type IV Collagenase
  - Dispase
  - Percoll

Cell Culture

1. Break out 1 vial of tumor cell line directly into 30 mL D10 and seed into a T75 flask.
2. Incubate at 37 deg overnight.
3. Change media to remove DMSO from cell culture and culture until at 60-75% confluence.
   ****This is a critical point. Passage number is less important than confluence at time of injection. Injecting cells at the same point of confluence ensures consistent tumor growth between replicates.*

Preparation of Tumor Cells for Injection (day 0)

1. Examine cells under the microscope. Make sure there are no floating particulates, contamination, etc.
2. Remove media by decanting.
3. Wash cells with 5-10 mL of 1X PBS (they are very adherent, so you can pipet directly in).
4. Remove 1X PBS by decanting.
5. Add 5-10 mL of cold trypsin versene and rock the flask back and forth to coat the whole surface of cells.

6. Incubate at 37 degrees for 3-5 minutes.
7. Remove from incubator and tap/slap/cajole flask until cells begin to detach.
8. Add 10-20 mL D10 to quench the protease. Wash vigorously with a strippetor and remove to a conical tube.
9. Spin 1500 rpm for 5 minutes, wash with 1X PBS, removing a 10uL sample to count.
10. Resuspend in hypersterile 1X PBS at 2.5 x 10^6 cells/mL and place on ice.
    ****I do not typically preload syringes, as the tumor cells, being larger, tend to settle in the syringe*.

Tumor cell injection (day 0)

1. In the mouse room, prepare workstation with electric razor, anesthesia chamber, acceptor cages.
2. Anesthetize mice with isofluorane and shave a wide swath of the back skin. I aim for a 1-1.5 inch square two-thirds of the way down the back (this ensures that the mice cannot reach around to chew on the growing tumor and does not impede their ability to ambulate).
   ****You can use Nair to remove the hair, which takes longer but results in a much barer skin surface. However, Nair has been shown to have inflammatory properties, so I generally only use Nair to remove hair prior to harvest.*
3. Re-anesthetize the mice, as they generally wake up after the shaving procedure.
4. While they're going back to sleep, resuspend tumor cells in the injection solution and draw up 50 uL per mouse plus extra. I never take more than 5 mice worth at a time so the cells don't have enough time to fall out of solution.
5. Inoculate the mice with 50 uL intradermally, in the back.
   ****This is very similar to a subcutaneous injection. While the mouse is asleep, pinch a fold of skin around the target area with the nondominant thumb and forefinger. Slip the 30-G needle into the skin just parallel to the fold and inject 50 uL. This should result in a visible BUBBLE that is in the skin, not a DISC that is under the skin.*
6. Place the mice in the acceptor cage and continue injections. Assure the mice reawaken before returning to the rack.

Tumor growth/survival (days 5-endpoint)

1. By day 5, a tiny mass should be palpable with the thumb and forefinger. This is a "1x1" tumor.
2. At least every 3 days, take a measurement with digital calipers. Measure in two axes, taking care not to "pinch" the tumor too much. These measurements should be done under anesthesia if at all possible.
3. Tumor size is an area (length x width). Tumor volume is calculated as ½ * (length x $width^2$), where length is the longer of the two measurements.
4. When a tumor reaches 15 mm in any direction, the mouse is to be anesthetized by CO_2 narcosis followed by cervical dislocation. This is marked as clinical death for survival studies.
   ****Death is NEVER an endpoint for these studies, due to the intradermal nature of the injection.*

Tumor-infiltrating lymphocytes analysis (day 12-day 15)

1. Euthanize mice by CO_2 narcosis followed by cervical dislocation.
2. Apply Nair to the tumor area and the surrounding skin for 3-5 minutes.
3. Remove dissolved hair with a EtOH-wetted napkin.
4. Spray with EtOH and remove the tumor to a 6 well dish or small culture dish with 2-3 mL R10 media. Take care to not include too much skin in the explant.
5. Remove nondraining lymph nodes (brachial/axial; I usually grab 4) and draining lymph nodes (inguinal) separately to individual 15 mL conical tubes with 2 mL R10.
6. For some analyses I include skin-infiltrating lymphocytes. For these experiments, I nair a much larger area and then remove the nontumoral skin to a separate 6-well with 2 mL R10.
7. Remove tissues to laboratory.

8. Make up a solution (2X) of 4 mg/mL collagenase IV, 2 U/mL dispase, and 2 U/mL DNase I (DNase is optional, but helps with “gooeyness”), enough for 2-3mL per tumor.
9. Load a syringe with the 2X protease mixture and a 25G needle. Keeping the tumor intact, inject the tumor with 2-3mL protease mixture; poking it in many different areas.
10. Incubate at RT for 5 min; the tumor will swell and then return to normalish size.
11. In a biosafety cabinet, cut the tumor (and skin if included) into small chunks; you do not want to mince the tumor here, as it will make the pieces hard to mash later.
12. Incubate at 37 deg for 30 min.
13. During the incubation, process the lymph nodes via standard method. I prefer the following:
    Per organ type in a group of mice, I reuse filters, transfer pipets, and plates to cut usage.
    a. Place 70 uM filter on empty 50 mL conical tube.
    b. Decant from the 15 mL conical the 2 mL R10 containing lymph node into 5 cm dish. (Keep the 15 mL conical tube)
    c. Mechanically disrupt the tissue between two frosted glass slides, taking care to remove any tissue stuck to the slides.
    d. Remove single cell suspension with a transfer pipet and filter through 70 uM filter.
    e. With a second transfer pipet, remove the filtered solution back to the original 15 mL harvest tube.
    f. Continue for the rest of the organ type (nondraining versus draining versus spleen, etc.) for that experimental group.
14. Remove the tumor samples from the incubator and mechanically disrupt between two frosted glass slides. Take your time here until all tumor pieces are fully disrupted into a cell suspension. It is not uncommon to spend 3-5 minutes per tumor. However, your patience and persistence will be rewarded.
15. Using a transfer pipet, filter through 70 uM filters into 50 mL conical tubes.
16. With a vortex set to its highest setting, vortex the TIL samples for 120-180 seconds. The sample should be agitating heavily, going up in down in the tube the entire time.
    THIS SINGLE, SIMPLE STEP RESULTS IN ALMOST 200% GREATER YIELD.
17. Spin LN and tissue samples 1500 rpm for 10 minutes
18. Resuspend LN in 1 or 2 mL R10. These are ready for analysis or sorting
19. If tumors are very large or the model is poorly infiltrated (MC38), an additional enrichment step is suggested to decrease time on flow cytometer/sorter.
    a. The tumors will still have a bit of “goo” in them. When removing the supernatant after the harvest, be careful not to suck up the pellet of cells, as it will sort of gel with the debris. I generally remove using a transfer pipet or strippetor so samples can be respun if necessary.
    b. RBC lyse the tumor homoegenate to remove red cell contamination.
    c. Make up a 90% Percoll stock solution with 10X PBS (2 mL 10X PBS into 18 mL Percoll).
    d. Using that 90% Percoll stock, make up a “80%” and a “40%” solution as if it were 100%, using HBSS. I like to use phenol-red treated HBSS for 80% and clear HBSS for the 40% so the interface is readily visible.
    e. Resuspend the tumor lysate in the “80%” Percoll solution.
    f. With a Strippetor set on LOW, gently layer the “40%” Percoll solution onto the 80% suspension, akin to making a layered shot.
    g. Carefully load a TC centrifuge and spin at 250 x g for 30 minutes with no brake.
    h. Carefully aspirate the top “tumor sludge” consisting of tumor cells.
    i. Harvest the interface cells, containing an enriched proportion of lymphocytes.
    j. Wash with high volume 1X PBS, resuspend in R10, count, and analyze or sort.