BONE MARROW CHIMERA GENERATION

Written by: Kevin Zhao and Erica DeJong
Date: July 2023
Bowdish Lab, McMaster University
Hamilton, ON, Canada
www.bowdish.ca

BACKGROUND

This protocol describes the process by which bone marrow chimera mouse models are generated in the Bowdish research group from spinal bone marrow (BM) donors. This procedure can result in the death of mice, therefore endpoint monitoring forms will need to be filed with the animal facilities. Busulfan is a hazardous chemical, thus cages housing mice that have been injected with busulfan must be labelled with chemical hazard labels. Busulfan depletes the immune system of mice it is administered to; therefore, mice must be handled sterilely after injections are started.

NOTE

This protocol has been successfully used for isolating and preparing BM cells from up to 5 donor mice. Typically, the cell yield per mouse is approximately 30-40 million BM cells, which is sufficient to transplant 12-16 recipient mice. If more donor mice are needed, the protocol may need to be adjusted accordingly. It is recommended that you use a minimum of 2 mice as donors per group of recipients to avoid any individual mouse issues with engraftment. Please refer to Montecino-Rodriguez, E. et al. https://doi.org/10.1016/j.xpro.2020.100159 for troubleshooting suggestions and more details.

MATERIALS

CONDITIONING

- Scale and container for weighing mice.
- 70% ethanol
- Marker and pen and endpoint monitoring sheets
- Busulfan (stored as a powder at -20°C)
- Sterile PBS
- Hot water bath (set to 37°C)
- Vortex
- Ensure + small paper cups
- Serological pipette gun
- 10mL serological pipettes
- 5mL Luer-Lok syringes + 0.2µM syringe filters
- 0.5mL 28G insulin syringes

BONE MARROW TRANSPLANTATION

Bone Marrow Extraction

- Isoflurane
- Gauze
- Absorbent pads
- 50 mL Falcon tubes
- 4°C PBS
- Ice
- Sterile mortar & pestle
- Sterile scissors & tweezers
- Petri dishes
- 40µM cell strainers
- Serological pipette gun
- 10mL serological pipettes
- 1.5mL Eppendorf tube
- Trypan blue
- Timer
- Sterile FACS tubes with 40µM cell strainer caps

Bone Marrow Injection

- Isoflurane
- 0.5mL 28G insulin syringe
- Recovery cage
- Heating pad
- Ensure + small paper cups
- Eye lubricant
- Alcaine

PROTOCOL

CONDITIONING

DAY 0

1. Prepare aliquots of busulfan for all days
    a. These aliquots can be stored at -20°C until use
    b. Plan for 1.5mg per mouse
    c. Prepare multiple aliquots (min 2) per day, and prepare one extra aliquot
    d. example: For 30 mice – 3 aliquots of 9mg of Busulfan will be enough (unless you have very large mice)

DAY 1-4

1. Set the hot water bath to 37°C, keep the sterile PBS warmed until use
2. Weigh all mice, noting their weights down on the endpoint monitoring form
    e. If a mouse loses 20% of its starting weight over the conditioning period, it has reached endpoint and must be euthanized
    f. Be sure to spray down the weighing container with ethanol between cages to minimize residual scent and keep reduce between cage contamination.

2. Resuspend the aliquoted busulfan in DMSO at 10% of your final volume. Ie. If using 9mg aliquot, final volume is 3mls. 10% of that is 300uL. Resuspend in 300uL DMSO then dilute with sterile PBS to 3mg/mL and vortex well.
    a. You must resuspend in DMSO first before adding PBS otherwise the solution will precipitate immediately.
    b. It also precipitates with time – move quickly.
    c. Powder busulfan may not resuspend properly or may precipitate out of solution if left alone for too long. If this happens, resuspend the extra aliquot of busulfan (step 1c)
3. Filter sterilize the resuspended and diluted busulfan solution using a 0.2um syringe filter on a 5ml syringe. Pour or pipette solution into syringe then filter.
4. Administer 20 mg/kg of diluted busulfan to recipient mice via intraperitoneal injection
    a. A chart is available at the end of the protocol to help calculate the volume of diluted busulfan to administer
5. Repeat steps 1-3 ever day for 4 consecutive days
6. Give each cage of busulfan-conditioned mice a small paper cup ¾ filled with ensure

BONE MARROW TRANSPLANTATION

DAY 5

1. Following the last day of busulfan conditioning, euthanize donor mice by abdominal aorta bleed + diaphragm puncture
2. Collect the spines and place in chilled PBS in 50mL Falcon tubes on ice
3. Bring spines up to the tissue culture room on ice, prepare BSCs
4. Transfer spines from their Falcon tube to a Petri dish containing chilled PBS
5. Clean tissues off the spine using scissors, being careful not to cut bone
    a. Bones from old mice tend to be much more fragile
6. When the spine is cleaned, cut off the edges (approx. 0.25cm) near the tail and brainstem
7. Cut spine using bone scissors into approximately 3 equal pieces
8. Holding a piece of the spine with tweezers, cut through the spine, holding the scissors vertically (as if cutting open a lobster tail) to avoid hitting bone marrow
9. Once open, spread apart the sides of the spine using scissors or tweezers to expose the spinal cord. Remove the spinal cord with tweezers
    a. The spinal cord will appear white and gooey
    b. Repeat for all 3 spine pieces
10. Place the cleaned spine pieces into the mortar and add 5-10mL chilled PBS
11. Crush the spine pieces until the PBS turns pink
    a. Red-pink tint indicates bone marrow is being expelled into the PBS
12. Pipette the supernatant PBS onto a 40µM cell strainer placed in a 50mL Falcon tube using a pipette gun
13. Repeat steps 10-12 until the PBS is clear after crushing
    a. Clear PBS indicates that all bone marrow has been extracted
14. Centrifuge the bone marrow for 5min at 500g at 4°C, discarding the supernatant
15. Resuspend the pellet in 3mL of 1x RBC lysis buffer. Incubate on ice for 8.5 minutes
    a. When the timer hits 7.5-8mins, uncap all Falcon tubes inside the hood to allow for rapid quenching in the next step
16. Add ~30mL of sterile PBS to quench the RBC lysis buffer
    a. It is important to add PBS in the same order as you added RBC lysis buffer, so as to ensure all samples sit in RBC lysis buffer for similar amounts of time
17. Centrifuge the bone marrow for 5min at 500g at 4°C, discarding the supernatant
18. Resuspend the pellet in 1mL of sterile PBS and keep on ice

    a. Bone marrow from multiple animals can be pooled at this point
19. Take a small aliquot of the cell suspension and quantify cell concentration using a hemocytometer (example below)
    a. Take 10µL of the cell suspension and add to a 1.5mL Eppendorf with 10µL Trypan blue and 80µL cold PBS, mixing well
    b. Count the number of cells in each of the 4 corners, and take an average of the 4 counts (this number being x)
    c. Your concentration will be as follows: $x*10^5$ cells/mL
20. Dilute the cell suspension to $1x10^7$ cells/mL, keep on ice until injections
21. Optional - If you need to verify what you are putting into the mice – you could save an aliquot of cells for flow cytometry at this point and stain as usual.
22. Optional - If you would like to test for the efficacy of Busulfan – you will need to euthanize an animal and count bone marrow cells as Busulfan acts on HSCs. Peripheral blood will look normal at this point – takes 10 days for peripheral cells to reflect the effects on bone marrow.
23. Filter the diluted cell suspension through the cap of a sterile FACS tube (used for flow cytometry) shortly before use – This is a 40um filter. We typically do 2 filters. One after diluting to the correct concentration and one right before taking 200uL to inject into the mouse.
24. Fill a 0.5mL syringe with 200µL of filtered, diluted cell suspension for each mouse to be injected
    a. Be sure to remove any air bubbles present in the syringe before injection
25. Anesthetize mice, setting the isoflurane vaporizer to ~3.5
    a. Err on the side of lower isoflurane vaporization so as to minimize anesthesia-related mortality
26. Place a drop of Alcaine on the eye you will inject and put back into chamber. Allow Alcaine to sit on eye for a minute or 2 before injecting.
27. Slowly inject each mouse with the prepared 200µL cell suspension 0.5mL syringe retro-orbitally
    a. The bevel of the needle should be facing towards you. Inject into the corner of the eye.
    b. Hold the needle in place for few seconds before removing needle.
    c. Force the eye closed like is done for Retro-orbital bleeds.
    d. Apply some eye ointment to the injected eye.
28. Place the mouse in the recovery cage, with half the mouse on the heating pad and the other half off
    a. The heating pad should be placed such that it only overlaps with half the recovery cage
    b. Visually confirm that all injected mice have recovered and return to home cage.
29. Repeat steps 23-26 for all mice
30. 6-8 weeks following transplant mice should be engrafted. This can be tested using peripheral immunophenotyping. See figure 1 for an example.

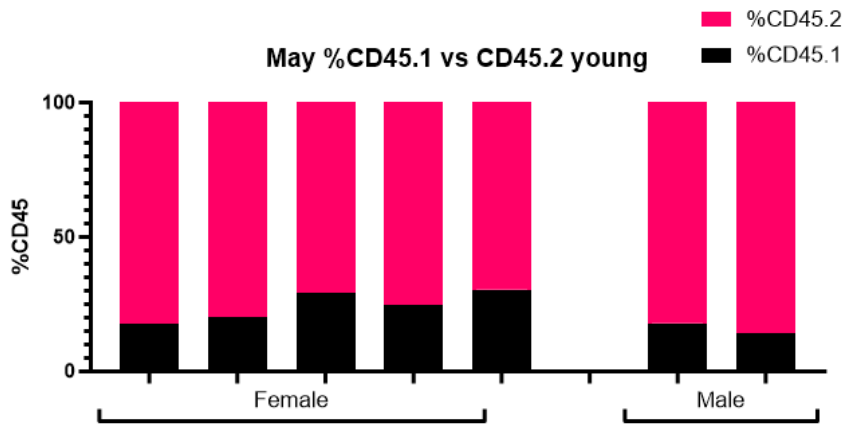

Figure 1 Recipient mice (CD45.1 background) were given CD45.2 donor bone marrow. Average: 22% CD45.1 vs 78% CD45.2.

Mouse weight (grams)	Volume of 3mg/mL busulfan to inject (mLs)
10	0.066667
10.5	0.07
11	0.073333
11.5	0.076667
12	0.08
12.5	0.083333
13	0.086667
13.5	0.09
14	0.093333
14.5	0.096667
15	0.1
15.5	0.103333
16	0.106667
16.5	0.11
17	0.113333
17.5	0.116667
18	0.12
18.5	0.123333
19	0.126667
19.5	0.13
20	0.133333
20.5	0.136667
21	0.14
21.5	0.143333
22	0.146667
22.5	0.15
23	0.153333
23.5	0.156667
24	0.16
24.5	0.163333
25	0.166667
25.5	0.17
26	0.173333
26.5	0.176667
27	0.18
27.5	0.183333
28	0.186667
28.5	0.19
29	0.193333
29.5	0.196667
30	0.2
Mouse weight (grams)	Volume of 3mg/mL busulfan to inject (mLs)
30.5	0.203333
31	0.206667
31.5	0.21
32	0.213333
32.5	0.216667
33	0.22
33.5	0.223333
34	0.226667
34.5	0.23
35	0.233333
35.5	0.236667
36	0.24
36.5	0.243333
37	0.246667
37.5	0.25

38	0.253333
38.5	0.256667
39	0.26
39.5	0.263333
40	0.266667
40.5	0.27
41	0.273333
41.5	0.276667
42	0.28
42.5	0.283333
43	0.286667
43.5	0.29
44	0.293333
44.5	0.296667
45	0.3
45.5	0.303333
46	0.306667
46.5	0.31
47	0.313333
47.5	0.316667
48	0.32
48.5	0.323333
49	0.326667
49.5	0.33
50	0.333333