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Analyses of Microglia Effector Function Using CX3CR1-GFP Knock In Mice

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Summary

The generation of bone marrow radiation chimeric mice is a beneficial tool to utilize when studying inflammation of the central nervous system (CNS). It is widely accepted that blood-derived progenitors are capable of populating the CNS during chronic diseases and severe injuries (1) however, they are neither consistent nor efficient in doing so. The lack of the appropriate recruitment could explain delays in recovery and repair after an increase of toxic proteins in chronic neurodegenerative diseases. With the ingenious development of bone marrow chimeric mice, some of these concerns can be addressed and allow us to hypothesize about further implications and possible mechanisms that may lead to medicinal applications. Bone marrow chimeric mice are often used to distinguish the intrinsic versus extrinsic effects of specific mutations. In our case, chimeras help us to better understand the role of CX3CR1 in microglia and peripheral myeloid cells. To detect cell autonomous effects on myeloid cell differentiation, CX3CR1-deficient mice are used as donors and wild type mice are used as recipients. In order to detect effects on the “immune cell environment,” wild type donors are used for the transfer into *Cx3cr1*^{-/-} recipients. The resulting chimeric mice are often used for the analysis of microglial motility, regulation of neuroinflammation and persistence. This technique can be applied to a broad spectrum of research ranging from neurodegenerative diseases to viral and parasitic pathogenicity and everything in between. This protocol describes the approach to generate chimeric mice and analyze the role of CX3CR1 in CNS inflammation in bone marrow radiation chimeras.

Keywords

CX3CR1; bone marrow; chimeras; microglia; trafficking; chemokines; radiation

1. Introduction

Microglia are specialized, surveillant phagocytic cells (2, 3) that colonize CNS during embryonic development and are best known for their ability to scan the environment with their extensive, motile processes. The knowledge of responses and pathogenic functions of microglia can be useful in developing inhibition and therapeutic targets for disease modulation (3). In the CNS, microglia are the only cells that express CX3CR1, also known as the fractalkine receptor (4). It has been shown via *in vivo* models of autoimmune diseases that in the absence of CX3CR1 dysregulation of microglial responses occur, leading to neurotoxicity. CX3CR1 deficiency has been associated with neuronal cell death subsequent to lipopolysaccharide (LPS) challenge (4). To monitor the role of CX3CR1 in the CNS, we

utilized mice in which the CX3CR1 gene was disrupted by the insertion of DNA encoding green fluorescent protein (GFP), allowing for the direct visualization of microglial cells (4–8). The generation of bone marrow chimeric mice allows us to investigate the effects of infiltration and adaptive immunity triggered by extraneous bone marrow cells on their endogenous counterparts. Considerable speculation of the chimeric model is focused on the ability of radiation to break down the blood brain barrier (BBB). This issue can be addressed by shielding the brain from irradiation while knocking out the rest of the immune system. The only downfall is that the efficiency of chimerism is sacrificed due to the fact that skull bone marrow can contribute to hematopoiesis (9). To further investigate roles of adaptive immunity when exploring the pathogenicity of multiple sclerosis (MS), we utilize the animal model experimental autoimmune encephalomyelitis in conjunction with the chimeric model (2, 10). In such an experiment, C57BL6 recipient mice are challenged with MOG₃₅₋₅₅ peptide 6 weeks post chimerism via active or passive immunization. A variety of combinations can be investigated when using this approach. For instance, CD45.1 or Ly5.1 donor cells deficient of CX3CR1 can be tracked in recipient CD45.2 or Ly5.2 WT mice via flow cytometry analysis by the detection of the CD45.1 marker in addition to the microglia expressing GFP. Alternatively, CX3CR1 deficient mice can serve as recipients for CD45.2 *Cx3cr1* WT donor cells. Overall, this protocol can be applied to any genetic models of interest with potential implications in translational and medicinal research in the development of systems to moderate microglial function.

2. Materials

Prepare solutions and handle all tools under sterile conditions to prevent any source of contamination.

2.1 Radiation

1. RS 2000 Rad Source Biological Irradiator with radiation output of 160kV, 25mA 0.3mm Cu filter (Rad Source Technologies, Alpharetta, GA, USA). Or any x-ray machine or cesium source of gamma irradiation as available to your institution.
2. 1/16" lead helmets (New Shield Inc., Pomona, NY, USA)(*see* Note 1).
3. Anesthesia cocktail: Mix Ketaset and Xylazine in 1× HBSS for a final concentration of 10mg/ml Ketaset and 1mg/ml Xylazine, and inject 100mg/kg Ketaset and 10mg/kg Xylazine.

2.2 Bone Marrow Isolation

1. Mice: B6.SJL-*Ptprc*^a *Pep3*^b/BoyJ (CD45.1 WT) #002014, B6.129P (Cg)-*Ptprc*^a *Cx3cr1*^{tm1Litt}/LittJ (CD45.1 KO) and C57BL/6J (CD45.2 WT) #000664 (The Jackson Laboratory, Bar Harbor, MA, USA).
2. 6-well plate (BD Falcon).
3. Kim Wipes (Kimberly-Clark, Neenah, WI, USA) or autoclaved paper towels
4. 70% ethanol
5. 70 µM cell strainer (Fisher Brand #22363548 Fisher Scientific).

¹Select donor and recipient mice properly, ensuring the differentiation of peripheral cells from injected cells by using different strains of mice that you can identify by flow cytometry staining. For example, in the figures shown, we used CD45.1 KO donor mice and CD45.2 WT recipients. Anti-CD45.1 and anti-CD45.2 specific antibodies are then used to determine the percentage of chimerism of the recipient mice. Our *Cx3cr1*^{-/-} mice contain a knock-in of GFP within CX3CR1 making it possible to determine KO from WT cells.

6. 2–200 ml beakers for holding tools in 1× PBS and 1 sterile with 70% ethanol for submerging mouse to disinfect skin.
7. Sterile 1× PBS
8. Absorbent pads, paper towels, plastic bags to collect carcass.
9. Scissors and forceps
10. 50 ml polypropylene conical tubes
11. 1.5 ml graduated micro centrifuge tubes
12. 3 ml Luer-lock tip syringes (BD #309657).
13. 0.4% Trypan Blue diluted to 0.05% in HBSS (Gibco).
14. Iscove's/10% FBS/0.5% Gentamicin Reagent Solution (Fetal Bovine Serum heat inactivated) (Sigma Aldrich).
15. Needles: Precision Glide 25G ⁵/₈ and 23G ³/₄ (BD #305122 and #305143 respectively).
16. Hemocytometer
17. Bright field microscope
18. Carbon dioxide asphyxiation chamber

2.3 Injection of bone marrow cells

1. Iscove's Modified Dulbecco's Medium supplemented with L-Glutamine, 25mM HEPES (Gibco #12440) and 50 µg/ml Gentamicin Reagent Solution (Gibco).
2. 1 ml tuberculin slip tip syringe with Precision Glide 27G ¹/₂ needle (BD #309623)
3. 1.5 ml graduated micro centrifuge tubes
4. Isoflurane and vaporizer with oxygen chamber

2.4 Flow Cytometry analysis of peripheral blood

1. Double distilled sterile water
2. 1×HBSS free of calcium, magnesium chloride and magnesium sulfate (Gibco)
3. 10 mM HEPES (Gibco)
4. 10× HBSS (Gibco)
5. 15 ml conical tubes
6. 1.5 ml micro centrifuge tubes
7. Sterile 5 mm Goldenrod Animal Lancet 5mm
8. FC block: rat anti-mouse CD16/CD32 clone 2.4G2 in cell staining buffer (optimal concentration should be determined by the investigator) (BD Pharmingen)
9. Cell staining buffer (Biolegend)
10. Fluorescent antibodies: CD45.1 anti-mouse PE-Cy7 clone A20 in cell staining buffer (eBioscience), CD45.2 anti-mouse APC clone 104 in cell staining buffer (eBioscience) (Note: optimal concentration should be determined by the investigator)

11. Prepare Sorenson's Buffer by adding 3.59g sodium phosphate monobasic and 24.7g sodium phosphate dibasic heptahydrate to 400 ml ddH₂O. Ensure that pH is between 7.0–7.3. Adjust final volume to 500 ml with ddH₂O and store at room temperature.
12. 4% Paraformaldehyde in phosphate buffer (Sigma): First prepare 8% PFA by dissolving 40g paraformaldehyde to 400 ml ddH₂O and stir for 15 minutes at 55–65°C. Add 100 µl 5M NaOH for 100 ml of 8% PFA solution. Stir at 30°C and add ddH₂O for a final volume of 500 mL. Add 200 mL of Sorenson's Buffer to 500 ml of 8% PFA and 300 ml ddH₂O. Filter sterilize and store at –20°C.
13. Heparin sodium salt 5,000 units/ml (Sigma Aldrich).

2.5 Flow Cytometry analysis of microglia

1. Isotonic Percoll (GE Healthcare) buffered with 10× HBSS. Prepare 100% ISP by adding 45 ml of Isotonic Percoll to 5 ml 10× HBSS. Prepare 70% Percoll by adding 21 ml of 100% ISP to 9 ml 1× HBSS. This is enough for 9 samples. Scale up volumes as needed.
2. RPMI 1640 supplemented with L-glutamine without phenol red (Gibco).
3. Dounce homogenizers for 7 and 15 ml volume with A (loose) and B (tight) pestles (Pyrex, Wheaton).
4. S1 pipet filler (Thermo Scientific #9531) or pipet aid.
5. 1×HBSS without calcium, magnesium chloride and magnesium sulfate (Gibco) with 10mM HEPES (Gibco)
6. FC block, antibodies, reagents as in Materials 2.4 in addition to any other antibodies of interest.

3. Methods

3.1 Radiation Conditioning of Recipient Mice

1. Subject the recipient mice (*see* Note 1) to radiation to deplete the immune system. This will also help prevent any rejection of injected cells. Determine if your experiment requires total body or brain protected radiation.
2. For total body radiation, subject the recipient mice to 900 rads, a sub lethal dose of radiation. Place all mice in an animal cage that does not contain any metal and place cage into radiation chamber with lid secured. Deliver the required amount of radiation and remove cage from radiation chamber.
3. If you decide to protect the head from radiation treatment anesthetize the mice to ensure that they will not move around in the cage potentially causing their protective helmet to fall off (*see* Note 2). Once the mice have been anesthetized, roll them on to their back and place the helmet onto the face of the mouse. The idea is to protect the brain while keeping the thymus exposed to radiation treatment. Once the helmets are in place, replace the cage lid and place the cage into the radiation chamber. For protected head recipients it is important to note that 2 consecutive doses of 600 rads are required.

²To protect the mouse brain from radiation you must first anesthetize it using 100 mg/kg Ketaset/10 mg/kg Xylazine. For this procedure, inject 100µL of the solution for every 10 grams of body weight via intra-peritoneal (i.p.) route. Secure the helmet as discussed in the procedure and treat with correct dosage of radiation. Be sure to monitor the mice until they regain righting reflex and normal activity which takes approximately 1–2 hours after injection of cocktail.

4. To ensure a good percentage of chimerism, it is recommended to treat recipient mice with radiation the same day just prior to injecting bone marrow isolated cells as seen in the next step.
5. If anesthesia was used, it is imperative to monitor the mice until they regain consciousness.

3.2 Isolation of Bone Marrow Cells

1. Euthanize donor mice by CO₂ asphyxiation and submerge them in 70% ethanol to disinfect fur and skin. Place disinfected mouse onto absorbent pads or paper towels to collect any bodily fluids or tissues that may be extracted.
2. Remove the skin from the lower limbs and cut away muscles revealing the femur and tibia bones.
3. After removing as much muscle as possible, cut the entire leg off with scissors by cutting above the femoral head making sure to keep the femur intact (*see* Note 3).
4. Once the hind limb is removed from the body, you can carefully cut off the foot making sure to keep the tibia bones intact. Clean the bones with Kim Wipes or sterile paper towels of any excess muscle and collect bones in a 6 well plate with Iscove's media containing 10% FBS and 50 µg/ml Gentamicin and keep on ice.
5. After collecting all hind limbs, separate the femur and tibia bones by cutting at the patella, area between the femur and tibia, making sure not to fracture the bones (*see* Note 4).
6. Using Kim Wipes or autoclaved paper towels, carefully remove any excess tissue from the bones until you can clearly see the epiphyses and entire shaft of the bone. This is important so that you can see when the red bone marrow flushes out.
7. Fill a 3 ml syringe with Iscove's/10%FBS/50 µg/ml Gentamicin. Cut off the epiphyses at both ends of the femur and use filled syringe with 25^{5/8} needle to flush out the bone marrow collecting the flow through in a 50 ml conical tube. While holding the bone tightly with forceps, insert the needle inside the bone in an up and down motion while pressing the plunger. The bone will turn white as the cells are flushed out. Flip the bone over and repeat flushing from the opposite end (*see* Note 5). Follow the same procedure for the tibia.
8. After you have collect all marrow cells, pour them through a 70 µM mesh filter and collect in a clean 50 ml conical tube. If there are clumps on the filter dissociate them with a syringe plunger and wash with Iscove's/10% FBS/50 µg/ml Gentamicin to ensure that you recover as many cells as possible.
9. Centrifuge the cells suspension at 2200 rpm for 7 minutes at 4°C. Aspirate supernatant and suspend pellet in 1 ml Iscove's with 0.5% Gentamicin.
10. Count cells and adjust the final volume with Iscove's with 50 µg/ml Gentamicin to prepare cells at 130–140 ×10⁶ cells/ml.

³It can be helpful to cut into the spine just above the femoral head when detaching the limb. This will ensure that you will not cut the femoral bone resulting in a loss of cells. After removing skin and muscle, locate the femoral epiphysis and cut into the spine just above it. Once you have removed the limb, you can cut off any excess tissue and spinal bones with greater ease.

⁴You can also separate the bones by bending the knee backwards with a twisting motion until the bones detach. This aids in keeping the femur and tibia bones intact.

⁵As you flush out cells from either end of the bone shaft, you might find a population of cells located in the middle portion of the shaft. Once you see that the epiphyseal end of the bone is white, you can trim the bone to better reach the middle of the shaft. Take care when cutting the bone as splitting can occur.

3.3 Injection of Bone Marrow Cells

1. Anesthetize recipient mice with an Isoflurane vaporizer by placing the mice in the gas filled chamber.
2. Remove mouse from the chamber and inject 150–200 μ L of your cell suspension to deliver $10\text{--}20 \times 10^6$ cells per mouse via retro orbital injection using a 1 ml syringe with 27G needle (*see* Note 6).
3. Monitor recipient mice until they are fully awake.
4. Treat recipient mice with 50 μ g/ml Gentamicin in drinking water for 2 weeks to avoid infection of immune-compromised mice.
5. Four weeks later, evaluate percentage of chimerism by flow cytometry analysis of peripheral blood.

3.4 Flow Cytometry Analysis of Peripheral Blood

1. Using a 5 mm lancet, draw 100–150 μ L of blood from cheek pouch and collect in 60 μ L heparin sodium salt solution (*see* Note 7). Be sure to mix well to prevent coagulation. Transfer blood to 15 ml conical tube and measure with a pipette to determine the volume of water (ddH_2O) needed to lyse red blood cells. Determine the appropriate amount of ddH_2O and 10X HBSS buffer needed to lyse your sample on a 20 $\times$ final volume. For example to lyse 0.1 ml of blood, you will need 1.7 ml of water and 0.2 ml of 10X HBSS, so the final volume of the lysed suspension is 2 ml (20 $\times$ excess of the initial 0.1 ml of blood) and in 1 $\times$ buffer.
2. Have the appropriate volumes of water, 10 $\times$ HBSS and 1 $\times$ HBSS/10mM HEPES measured out and ready as overtreatment with water can lead to lysis of white blood cells which are needed for staining. Add the correct amount of water, close cap tightly and mix by inversion for 20 seconds. Immediately add 10 $\times$ HBSS and invert tube twice to mix. Then add 5 ml 1 $\times$ HBSS/10mM HEPES to ensure complete buffering of your cell suspension.
3. Centrifuge for 7 minutes at 2200 rpm at 4°C. Aspirate supernatant and suspend pellet in 1 ml cell staining buffer and transfer cells to 1.5 ml micro centrifuge tube.
4. Centrifuge for 1 minute at 10,000 rpm at room temperature. Aspirate supernatant and suspend in 50 μ L of FC block (CD16/32) to prevent non-specific binding of antibodies (*see* Note 8). Incubate on ice for 10 minutes.
5. Prepare antibody mix at appropriate dilutions for all samples. Be sure to choose antibodies that will allow you to stain for donor and recipient cells. (i.e. if you are using CD45.1 donor mice and CD45.2 recipients, be sure that you are using antibodies that can identify those markers). Add 50 μ L antibody mix to all samples and incubate for 30 minutes on ice protected from light.

⁶Retro orbital injections take some practice. It is recommended to practice by injecting 150 μ L of saline or PBS into other mice prior to your experiment. After you have anesthetized the mice as listed in the procedure, roll the mouse on its side. Secure the head with your index finger behind the skull and thumb under the chin. Insert the needle at a 45° angle into the conjunctiva, inner corner of the eye, until you feel a soft spot about a quarter inch deep. Begin to inject saline/cells. If you see the fluid coming out of the eye, you are not deep enough. Insert the needle deeper and continue injecting fluid. After injecting the correct amount of saline/cells, remove the needle and blot any excess fluid from the eye. The mice should wake within 2 minutes. Be sure to monitor the mice until they regain consciousness.

⁷First locate the submandibular vein. This is usually found behind the cheek below the ear and above the jaw bone. Make a deep puncture with an animal lancet and collect up to 200 μ L of blood in a tube with 60 μ L Heparin. Mix blood with heparin instantly to prevent coagulation.

⁸It is recommended to suspend sample pellets in 50 μ L of FC block solution for incubation. After blocking the cells, add 50 μ L of the antibody mix to the cells in FC block for a final volume of 100 μ L.

6. Add 1 ml of cell staining buffer to wash any excess antibodies from the cell suspension and centrifuge at 10,000 rpm for 1 minute at room temperature.
7. Aspirate supernatant and suspend pellet in 150 μ L cell staining buffer, then transfer to equal volume of 4% paraformaldehyde (PFA) making a final concentration of 2% PFA. The final volume of the cells depends on the volume needed to acquire by your flow cytometer. Cells can be kept at 4°C protected from light until ready to acquire, but no longer than 36 hours.
8. Analyze results by arranging markers for recipient versus donor mice on the X and Y axis respectively. The resulting populations will show the percentage of chimerism (Figure 1).

3.5 Flow Cytometric Analysis of Microglia

1. Anesthetize mouse with Ketaset/Xylazine cocktail at 100 mg/kg Ketaset and 10 mg/kg Xylazine and perfuse with 1 $\times$ HBSS by cutting the thoracic cavity open and cut the right atrium of the heart allowing blood to flow out. Inject 10 ml of ice cold 1 $\times$ HBSS into the left ventricle to completely remove blood from tissues.
2. Remove brain from skull and obtain spinal cord by flushing with 1 $\times$ HBSS (*see* Note 9). Transfer tissues to 6-well plate containing RPMI and keep on ice until all tissues are collected.
3. Fill Dounce homogenizers with 3.5 ml RPMI without phenol red, label them accordingly, and keep on ice. It is possible to homogenize up to 2 brains in one 7 ml homogenizer. When combining more than 2 brains, use a 15 ml homogenizer.
4. Transfer tissues to their respective homogenizer and grind the tissue using the “A” or “Loose” plunger until the suspension has reached confluency, then use the “B” or “Tight” plunger. Once the suspension is homogeneous, transfer the suspension to a 15 ml conical tube and top off with RPMI to 7 ml. Continue this step with all samples and keep on ice before moving to the next step.
5. Add 3 ml of 100% Percoll solution to the cell suspension and mix well to gain confluency. Slowly layer the homogenized suspension onto a new 15 ml conical tube containing 2 ml of 70% Percoll. The idea is to set up a density gradient in which the brain mononuclear cells including microglia will layer atop the 70% Percoll (*see* Note 10).
6. Centrifuge at 500–600 $\times$ g for 30 minutes at 18°C.
7. Locate the interphase which contains the microglia just above the 2 ml graduation on the 15 ml tube. It is easily seen as a cloudy halo when held up to the light. Using a transfer pipette, carefully remove the interphase, approximately 2 ml, and transfer to a 15 ml tube containing 8 ml of 1 $\times$ HBSS/10mM HEPES to wash cells of Percoll.
8. Centrifuge for 7 minutes at 2,200 rpm at 4°C. Aspirate supernatant and suspend pellet in 1 ml cell staining buffer and transfer to 1.5 ml tubes.

⁹To flush out the spinal cord you will need to remove the entire spinal cord from the carcass. With a 10 ml syringe and blunt 16 gauge needle, find the spinal cord within the column; hold the column tight while you press the plunger with great force. It is important to hold tight to the spinal column as the suction is required to expel the spinal cord. The spinal cord should shoot out in one piece.

¹⁰If cells are pipetted too fast or into the 70% Percoll instead of on top, the cells will be lost indefinitely. It is helpful to use the gravity or lowest setting on the pipette if possible.

9. Centrifuge cells for 1 minute at 10,000 rpm, aspirate supernatant and suspend pellet in volume necessary for blocking step (*see* Note 8). Incubate cells in FC block solution for 10 minutes on ice.
10. Without washing, add equal volume of antibody mix to the cells and incubate for 30 minutes on ice protected from light.
11. Wash cells of any excess antibody by adding 1 ml cell staining buffer to each tube and centrifuge for 1 minute at 8,000 G.
12. Aspirate supernatant and resuspend pellet in cell staining buffer, then transfer to equal volume of 4% paraformaldehyde (PFA) for a final concentration of 2% PFA. The final volume of the cells depends on the volume needed to acquire by your flow cytometer. Cells can be kept at 4°C protected from light until ready to acquire.
13. To analyze microglia, you must first distinguish your donor cells from your recipients based on the expression of CD45 and the presence or absence of GFP (*see* Note 11). With CD45 on the Y axis and SSC on the X axis, locate the microglial population expressed as CD45^{lo}. This population denotes both donor and recipient microglia. By changing the x axis to FITC to observe the presence of GFP, it is now possible to differentiate donor from recipient microglia. (Note: other antibodies can be included to compare effector function or proliferation) (Figure 2).

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¹¹As previously stated, we use CD45.1 donor mice and use the CD45.1 anti-mouse PE-Cy7 to identify those cells. We use CD45.2 anti-mouse APC to label our recipient endogenous cells. The *Cx3cr1*^{-/-} mice contain GFP which allows us to visualize donor microglia. The CD45^{lo} GFP⁻ population represents the resident microglia. The CD45^{lo} GFP⁺ population of cells is representative of CD45.1 donor microglia. CD45^{hi} populations are indicative of infiltrating monocytes and can also be segregated by the presence or absence of GFP.

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Figure 1.

Peripheral blood analysis from a CD45.2 WT mouse reconstituted with CD45.1 KO bone marrow cells. CD45.1 cells are stained with anti-mouse CD45.1 Phycoerythrin –Cy7 (PE-Cy7). CD45.2 (recipient) cells are stained with anti-mouse CD45.2 Allophycocyanin (APC). Data shows 96.6% CD45.1 reconstitution of the CD45.2 recipient indicating a successful chimerism.

Figure 2.

(A) Cell homogenates were prepared from the brain of EAE affected *Cx3cr1^{gfp/gfp}* → wild type chimeric mice and separated over density percoll gradients. CD45 (APC) antibody was used to locate the CD45^{hi} population of hematogenous cells and CD45^{lo} or resident microglia. (B) From the same population of cells seen in (A), we can determine that 18.7% of the population is GFP positive (FITC) indicating donor microglia. The other population (18.4%) is FITC negative representative of resident microglia. Additional markers can be incorporated to determine differences in effector function between the different experimental groups.