Isolation and FACS Analysis on Mononuclear Cells from CNS Tissue

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[Abstract] Immune cells, such as microglia are resident in the brain and spinal cord of normal mice and humans. Furthermore, macrophages, dendritic cells, T cells, B cells and NK cells infiltrate the CNS during certain infections or in neurodegenerative/neuroinflammatory diseases, such as experimental autoimmune encephalomyelitis (EAE) (a model for multiple sclerosis) or Alzheimer's disease (Sutton *et al*., 2009; Browne *et al*., 2013). Infiltrating cells can be identified using immunohistological staining of sections from brain or spinal cords. However, more detailed phenotypic and functional analysis is possible following isolation of the immune cells from the CNS of normal or diseased mice. Purification of mononuclear cells from brain or spinal cord is dependent on perfusing the mouse to ensure removal of the blood from the CNS tissue, prior to dissociating the tissue and purification of the mononuclear cells on a percoll gradient. The technique provides single cell suspensions with cells of high viability that are suitable for FACS analysis or limited functional studies. The yields are usually low from the normal mouse brain or spinal cord, but higher from mice with EAE or CNS infection. When combined with intracellular cytokine staining and FACS, this technique is particularly useful for analysis of the pathogenic T cells (Th17 and Th1 cells) and their regulation/modulation in EAE.

Materials and Reagents

1. Mice (adult >6 weeks, any strain, *e.g.* C57BL/6 used for MOG-induced EAE)
2. Sodium pentobarbital (euthatal) (Merial)
3. Phosphate buffered saline (PBS) (Sigma-Aldrich, catalog number: D8537)
4. 10x PBS (Sigma-Aldrich, catalog number: D1408)
5. RPMI solution(Sigma-Aldrich, catalog number: R0883)
6. Penicillin-streptomycin (Sigma-Aldrich, catalog number: P4333)
7. L-glutamine (Sigma-Aldrich, catalog number: G7513)
8. FBS (Sigma-Aldrich, catalog number: F9665)
9. Hank's balanced salt solution (HBSS) (Sigma-Aldrich, catalog number: H9394) supplemented with 3% FBS
10. Collagenase D (Roche, catalog number: 11088858001)
11. DNase I (Sigma-Aldrich, catalog number: D4263)

12. Percoll Plus (Sigma-Aldrich, catalog number: 17-5445-01)
13. Cell permeabilisation kit (contains IntraStain Reagent A and B) (Dako, Denmark, catalog number: K2311)
14. Phorbol myristate acetate (PMA) (Sigma-Aldrich, catalog number: P1585)
15. Ionomycin (Sigma-Aldrich, catalog number: I0634)
16. Brefeldin A (BFA) (Sigma-Aldrich, catalog number: B7651)
17. LIVE/DEAD® Fixable Aqua Dead Cell Stain kit (Life Technologies, catalog number: L34957)
18. CD16/CD32 FcγRIII (BD Biosciences, catalog number: 553141)
19. FACS antibodies (as appropriate)
20. Propidium iodide (PI) (Sigma-Aldrich, catalog number: P4864)
21. Complete RPMI solution (see Recipes)
22. FACS buffer (see Recipes)
23. Stock isotonic percoll (SIP) (see Recipes)

Equipment

1. 70 µm nylon mesh filter (Corning, catalog number: 352350)
2. Shaker capable of 200 rpm at 37 °C
3. Tissue culture facilities including class II laminar flow hood
4. Bench-top centrifuge, preferably refrigerated (any model)
5. Flow cytometer

Software

1. Summit software (Dako)
2. FlowJo software (Tree Star)

Procedure

1. Anaesthetise mice with sodium pentobarbital (40 µl i.p.) and perfuse intracardially with sterile ice-cold PBS (20 ml). This is achieved by slowly and steadily injecting the ice-cold PBS into the left ventricle of the heart using a 20 ml syringe.
2. Dissect out brains by cutting away the skull with a sharp scissors, gently removing the brain with a forceps and place in 1 ml complete RPMI solution (or HBSS supplemented with 3% FBS can be used).

3. Prepare a single cell suspension by forcing the tissue through a sterile 70 µm nylon mesh filter using the plunger of a 5 ml syringe, wash with complete RPMI solution and centrifuge at 280 *x g* for 5 min.
4. Remove the supernatant and the remaining pellet and resuspend in complete RPMI (2 ml) containing collagenase D (1 mg/ml) and DNAse I (10 µg/ml), and incubate for 1 h at 37 °C with agitation.
5. Wash cells with complete RPMI and centrifuge at 280 *x g* for 5 min. Discard supernatants and resuspend cells in 40% Percoll [5 ml; 40% stock isotonic percoll (SIP) in PBS; 1.052 g/ml].
6. Carefully layer cell suspension on top of 70% Percoll (5 ml; 70% SIP in PBS; 1.088 g/ml).
7. Centrifuged at 600 *x g* for 20 min with the brake of the centrifuge switched off.
8. Remove mononuclear cells from the 1.088: 1.052 g/ml interface by aspiration with sterile plastic pipette, wash twice in complete RPMI and count.
9. Samples are centrifuged at 280 *x g* for 5 min and cells are incubated in sterile FACS tubes at 37 °C in complete RPMI in the presence of PMA (10 ng/ml), ionomycin (1 µg/ml) and BFA (5 µg/ml) for 5 h.
10. After 5 h wash the cells by centrifuging the cells at 280 *x g* for 5 min, ready for intracellular staining using a cell permeabilisation kit.
11. Resuspend in 50 µl PBS with 1:1,000 LIVE/DEAD® Fixable Aqua Dead Cell Stain kit for 20 min at 4 °C.
12. Wash cells in FACS buffer and resuspend in 50 µl FACS buffer containing CD16/CD32 FcγRIII (1:100) and incubate at 4 °C for 10 min to block low-affinity IgG receptors.
13. Incubate cells in 50 µl/sample FACS buffer containing the appropriate FACS antibodies for 15 min at 4 °C.
14. Fix cells using IntraStain Reagent A (50 µl/sample) for 15 min at RT, wash twice with FACS buffer and centrifuge at 280 *x g* for 5 min.
15. Permeabilise cells with IntraStain Reagent B (50 µl/sample) including intracellular antibodies for 15 min at room temperature in the dark.
16. Wash cells twice in FACS buffer and centrifuge at 280 *x g* for 5 min.
17. Mononuclear cells which were surface stained only are blocked for 10 min, incubated with the appropriate antibodies for 15 min at 4 °C as described above, washed twice in FACS buffer and centrifuged at 280 *x g* for 5 min. PI can be used as a live/dead stain (with surface staining only) by adding 1:100 immediately before reading the samples on a flow cytometer.
18. Perform flow cytometric analysis on a flow cytometer and acquire data using Summit software. Analyse results using FlowJo software. Representative data can be viewed in References 1 and 2 below.

Recipes

1. Complete RPMI solution
   RPMI solution supplemented with 1% penicillin-streptomycin, 1% L-glutamine and 10% FBS
   Note: HBSS supplemented with 3% FBS can be used as a substitute for complete RPMI solution.
2. FACS buffer
   PBS supplemented with 2% FBS
3. Stock isotonic Percoll (SIP)
   9 parts Percoll plus (from the bottle), 1 part 10x PBS

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References

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