Isolation of Dendritic Cells and Macrophages from the Murine Kidneys of Lupus by Cell Sorter

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[Abstract] Methods for the isolation and characterization of mononuclear phagocytes from the kidneys of mice with Systemic Lupus Erythematosus (SLE) are essential to understand the patho-physiology of the disease. Activation of these cells is associated with the onset of clinical disease in mice and infiltration with these cells is associated with poor prognosis in humans. An analysis of the function of these cells should lead to a better understanding of the inflammatory processes that lead to renal impairment in SLE and other renal inflammatory diseases.

Materials and Reagents

1. Fetal bovine serum (FBS)
2. Sterile PBS (Life Technologies, Invitrogen™, catalog number: 20012-027)
3. Ammonium chloride
4. Collagenase Type I (CLS I) (Worthington, catalog number: 4197, specific activity 230 U mg^{-1})
5. DMEM, High glucose (Life Technologies, Gibco®, catalog number: 10313)
6. Paraformaldehyde (Tousimis, catalog number: 1108A)
7. BSA (Fraction V) (Sigma-Aldrich, catalog number: A-7030)
8. FACS antibodies:
    a. FC block (BD Biosciences, Falcon®, catalog number: 553142)
    b. CD11B APC (BD Biosciences, Falcon®, catalog number: 553310)
    c. F/480 FITC (Serotech Laboratories, catalog number: MCA497FB)
    d. Streptavidin PERCP (BD Biosciences, Falcon®, catalog number: 554064)
    e. CD4, PE (BD Biosciences, Falcon®, catalog number: 553049)
    f. CD5, PE (eBioscience, catalog number: 12-0051-82)
    g. B220, PE (BD Biosciences, Falcon®, catalog number: 553090)
    h. CD49b, PE (BD Biosciences, Falcon®, catalog number: 558759)
    i. CD11C (Biotin, catalog number: 553800)
    j. Streptavidin PERCP (BD Biosciences, Falcon®, catalog number: 554064)

9. DAPI (MP Biomedicals, catalog number: 157574)
10. FACS staining buffer (see Recipes)

Equipment

1. FACS Aria or similar cell sorter
2. Bench-top refrigerated centrifuge
3. BD cell strainer (40 nm) (BD Biosciences, Falcon®, catalog number: 352340)
4. Conical tube
5. 30 ml syringe (BD Biosciences, Falcon®, catalog number: 309661)
6. 21G Needles (BD Biosciences, catalog number: 305165)
7. 26G needles (BD Biosciences, catalog number: 305111)
8. V bottom 96 well assay plate (Corning, Costar®, catalog number: 3897)
9. Glass slides frosted (Thermo Fisher Scientific, catalog number: 12-550-11)

Procedure

A. Procedure for harvesting the kidney from the nephritic mice for analysis of kidney infiltrates

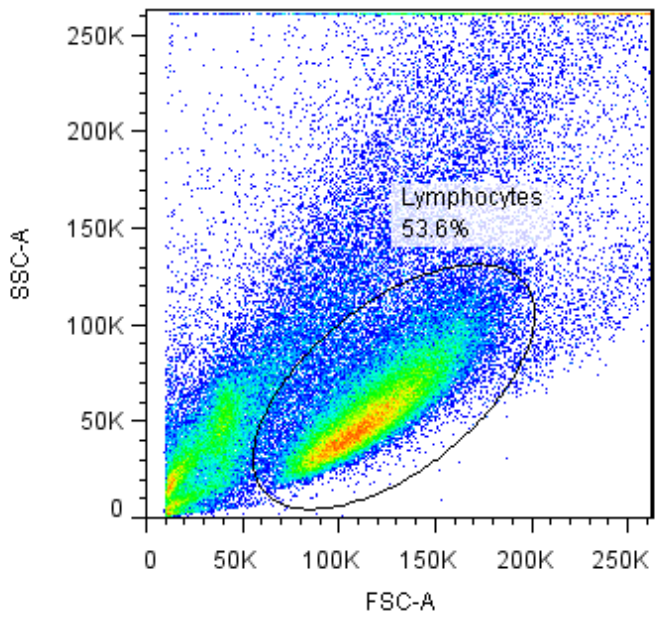
1. Anesthetize the mouse and perfuse with 60 ml of cold PBS over 3-5 min through the left ventricle after snipping the right atrium, and observe for pale white color change in liver and kidney. If needed, repeat perfusion with another 60 ml of cold PBS.
2. Carefully remove and cut the kidneys into 1 to 2 mm^3 pieces, excluding any adjoining renal fat.
3. Incubate the slices in DMEM containing 2 mg/ml Collagenase Type I (Worthington) for 30 min at 37 °C (use 10 ml per two kidneys).
4. Gently disrupt the tissue by pipetting up and down sequentially through 25 ml, 10 ml, and 5 ml pipettes to obtain a fine cell suspension.
5. Filter the cell suspension through a BD cell strainer (40 nm) into a conical tube.
6. Gently rub the remaining material between two glass slides, resuspend in 2 ml DMEM, filter and add to the suspension.
7. Allow the suspension to settle briefly (3-5 min) during which most of the larger fragments settle to the bottom. Harvest the suspension excluding the bottom 200 µl containing the fragments.
8. Examine the settled cells under microscope to see if any clumps are present. If so, resuspend the settled cells in fresh DMEM, filter and repeat step 6.
9. Pool the suspension(s) obtained and centrifuge at 1,200 rpm or 300 *x g* for 10 min.

10. Decant supernatant; resuspend the pellet in 5 ml of ice cold ammonium chloride (0.17 M, pH 7.2) for 5 min on ice.
11. Add 15 ml of DMEM. Count cells to estimate the number of total cells in the suspension. Spin at 1,200 rpm for 5 min.
12. Resuspend cells in 1 ml of FACS buffer (3% FCS in PBS). Cells are now ready for flow cytometric analysis or further isolation procedures.

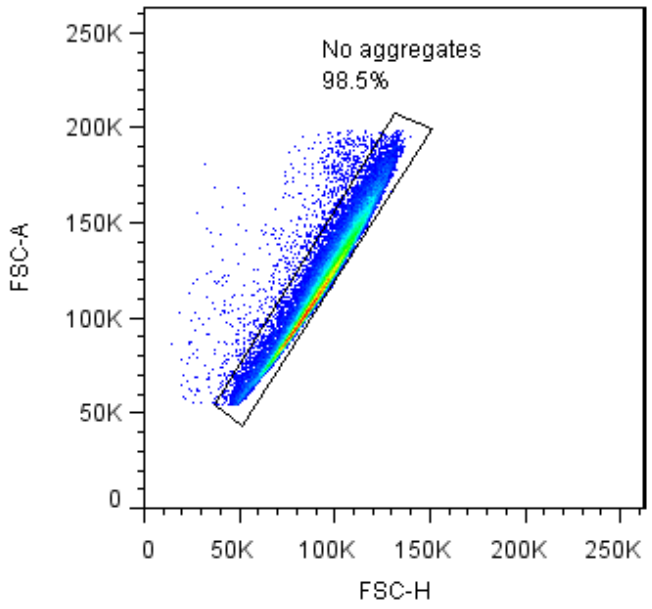
B. Procedure for sorting renal mononuclear phagocytes (macrophages and dendritic cells) by cell sorting.
   1. Suspend the cell suspensions from both kidneys from one mouse in 2 ml of FACS buffer with Fc block and incubate on ice for 20 min.
   2. Add 7 µl of CD11c-biotin antibody to the tube and incubate for 30 min on ice.
   3. Centrifuge the cells at 1,200 rpm for 5 min and decant the supernatant.
   4. Resuspend the cell pellet in 2ml of FACS buffer containing anti-CD11b APC, F4/80 FITC, and Streptavidin PERCP. The cocktail should also include a combination of antibodies to facilitate the exclusion of unwanted cells, such as PE anti-CD3, CD5, B220, CD138 (optional) and CD49b (5-7 µl each); incubate for another 30 min.
   5. Wash the cells with 2 ml of ice cold PBS and centrifuge at 1,200 rpm for 5 min.
   6. Resuspend the cell pellet in 500 µl of FACS buffer and filter the suspension using a strainer cap FACS tube. Later, adjust the volume for the cells based on the number of events acquired per second on the cell sorter (FACS Aria IIu).
   7. Just before sorting the cells, add 2 µl of DAPI (1 µg/ml) to exclude the dead cells.
   8. Pass the cells through the sorter with a flow rate optimized to 5,000 to 7,000 events per sec and maintain the efficiency of the sort at between 75-85%.

C. Sorting strategy

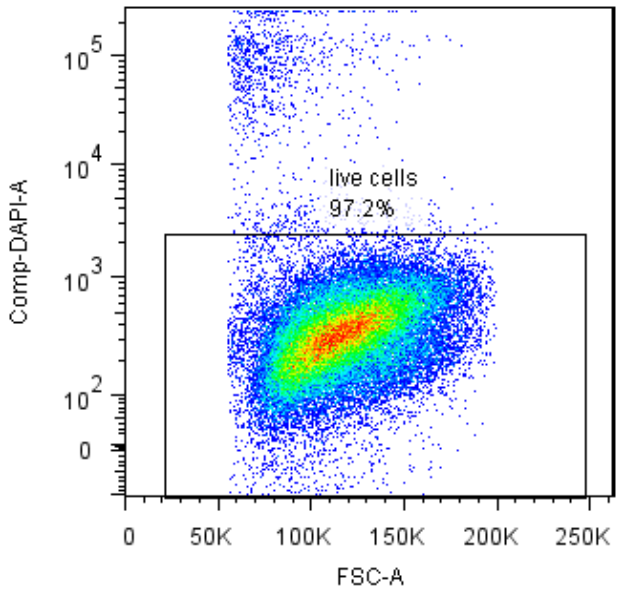
9. Gate on lymphocytes/mononuclear cells



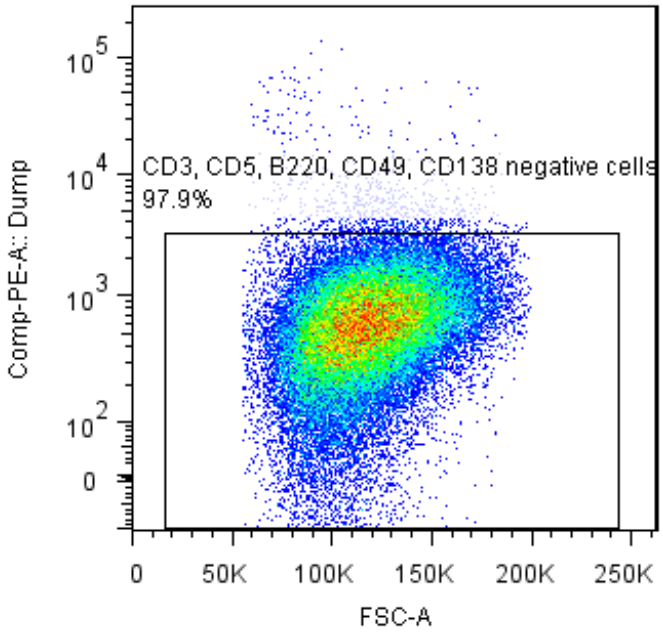
10. Gate on singlets



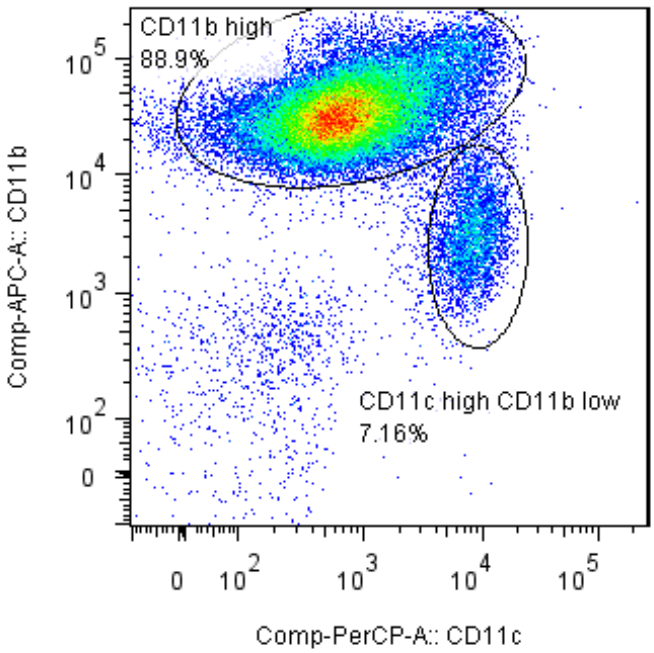
11. Gate on live cells (DAPI negative)



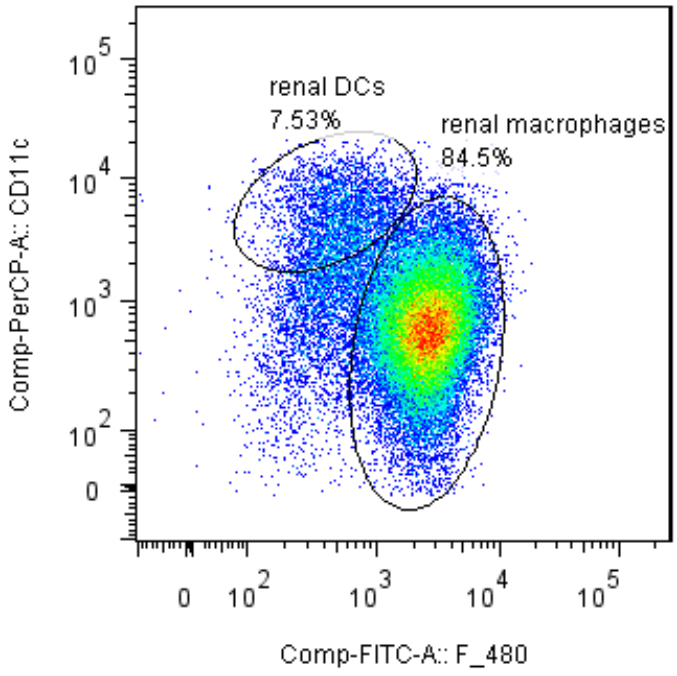
12. Gate out unwanted cell types (B cells, plasma cells, T cells, NK cells)



13. Separate CD11bhigh and CD11blowCD11chigh population (the former is the subject of the next step)



14. Sort renal dendritic cells and macrophages, respectively, into FACS buffer.



15. Process the sorted cells based on further study requirements (cell culture, RNA isolation, morphological and functional characterization, western blot analysis).

Recipes

1. 0.17 M ammonium chloride
2. FACS staining buffer
   PBS
   3% FBS

Acknowledgments

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References

1. Bethunaickan, R., Berthier, C. C., Ramanujam, M., Sahu, R., Zhang, W., Sun, Y., Bottinger, E. P., Ivashkiv, L., Kretzler, M. and Davidson, A. (2011). A unique hybrid renal mononuclear phagocyte activation phenotype in murine systemic lupus erythematosus nephritis. *J Immunol* 186(8): 4994-5003.

