Purification of Tumor-Associated Macrophages (TAM) and Tumor-Associated Dendritic Cells (TADC)

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[Abstract] Tumors are heterogeneous microenvironments where complex interactions take place between neoplastic cells and infiltrating inflammatory cells, such as tumor-associated macrophages (TAM) and tumor-associated dendritic cells (TADC). The relevance of tumor-infiltrating mononuclear myeloid cells is underscored by clinical studies showing a correlation between their abundance and poor prognosis (Laoui *et al.*, 2011). These cells are able to promote tumor progression via several mechanisms, including induction of angiogenesis, remodeling of the extracellular matrix, stimulation of cancer cell proliferation and metastasis and the inhibition of adaptive immunity (Laoui *et al.*, 2011). Moreover, mononuclear myeloid cells are characterized by plasticity and versatility in response to microenvironmental signals, resulting in different activation states, as illustrated by the presence of distinct functional TAM subsets in tumors (Movahedi *et al.*, 2010; Laoui *et al.*, 2014). Here, we describe a valuable isolation technique for TAM and TADC permitting their molecular and functional characterization.

Materials and Reagents

1. Mouse
2. RPMI-1640 medium (RPMI) (Life Technologies, catalog number: 52400-041)
3. Collagenase I (Worthington Biochemical, catalog number: LS004214)
4. Collagenase IV (Worthington Biochemical, catalog number: LS004209)
5. DNase I (Worthington Biochemical, catalog number: LS002060)
6. Hank's buffered salt solution (HBSS) (Life Technologies, Gibco®)
7. NH_4Cl (Merck KGaA)
8. $KHCO_3$ (Merck KGaA)
9. EDTA (Duchefa Biochemie)
10. Fetal calf serum (FCS) (Life Technologies, Gibco®)
11. Lymphoprep (Axis-shield, catalog number: 1114547)
12. LS colums (Miltenyi Biotec, catalog number: 130-042-401)

13. Anti-CD11b microbeads (Miltenyi Biotec, catalog number: 130-049-601)
14. Anti-CD11c microbeads (Miltenyi Biotec, catalog number: 130-052-001)
15. Purified CD16/CD32 (FcBlock) (clone 2.4G2) (BD Biosciences, catalog number: 553142)
16. PE-Cy7-conjugated anti-CD11b antibody (clone M1/70) (BD Biosciences, catalog number: 552850)
17. AF647-conjugated anti-Ly6C antibody (clone ER-MP20) (Bio-Rad Laboratories, AbD Serotec®, catalog number: MCA2389A647)
18. PerCP-Cy5.5-conjugated anti-I-A/I-E (MHC-II) antibody (clone M5/114.15.2) (BioLegend, catalog number: 107626)
19. FITC-conjugated anti-Ly6G antibody (clone 1A8) (BD Biosciences, catalog number: 551460)
20. PE-conjugated anti-SiglecF antibody (clone E50-2440) (BD Biosciences, catalog number: 552126)
21. PE-conjugated anti-CD11c antibody (clone HL3) (BD Biosciences, catalog number: 553802)
22. Tumor digestion medium (see Recipes)
23. Erythrocyte lysis buffer (see Recipes)
24. MACS buffer (see Recipes)
25. Sorting buffer (see Recipes)
26. Complete medium (see Recipes)

Equipment

1. Polyester filters cut in 10 x 10 cm squares (thread diameter 70 µm) (Spectrum® Laboratories, catalog number: 146490)
2. 6-well plates (Greiner Bio-One GmbH, catalog number: 657185)
3. 10 ml syringes (Omnifix, catalog number: 473203)
4. BD Falcon 50 ml polypropylene tubes (BD Biosciences, catalog number: 2070)
5. BD Falcon 15 ml polypropylene tubes (BD Biosciences, catalog number: 2096)
6. BD Falcon 5 ml polypropylene round-bottom tube (BD Biosciences, catalog number: 352063)
7. Sterile culture hood
8. Surgical scissors and forceps
9. 37 °C, 5% CO_2 cell culture incubator
10. Pipettes
11. Centrifuges
12. Shaker

13. Microscope
14. MidiMACS™ Separator and MultiStand (Miltenyi Biotec, catalog number: 130-042-301)
15. Multicolour FACS sorter (BD Biosciences, Aria flow cytometer)

Procedure

A. Preparation of a tumor single cell suspension

1. Sacrifice the mouse when the tumor has reached the desired diameter (as from the moment a tumor becomes palpable, it can be analyzed. The maximal tumor diameter depends on the limits set by the ethical commission and may vary in different countries) and restrain it by pinning its paws into a foam surface using syringe needles. Make a parallel incision from the base of the tail up to the neck along the mouse's abdomen and to the paws without puncturing the peritoneum. Gently pull back the skin and pin it to the foam surface to reveal the tumor. Here, LLC lung carcinoma was used as an example, however the protocol is applicable for all solid tumors (Figure 1A).
2. Cut the tumor free from the skin and the body. Try to remove as much excess tissue surrounding the tumor as possible and take care to exclude the draining lymph node (Figure 1A).
3. Store the harvested tumors in 2 ml RMPI medium in a 6-well plate on ice until the digestion procedure (Figure 1B).
4. Cut the tumors in small pieces (1-1.5 mm) using scissors or a scalpel, subsequently add 1 ml digestion medium in the same well and incubate subsequently at 37 °C for 25 min (Figure 1C).
5. Crush the tumors with the plunger of 10 ml syringe, add 5 ml RPMI medium and homogenize by thoroughly pipetting with a 10 ml pipet.
6. Filter the tumor suspensions through a 70 µm sterile nylon gauze into a sterile 50 ml conical tube and wash the gauze with 10 ml RPMI medium.
7. Centrifuge the 50 ml tubes at 450 *x g* for 6 min at 4 °C and discard the supernatants.
8. Remove the red blood cells by resuspending the pellet in 4 ml erythrocyte lysis buffer and leave at room temperature for 2 min.
9. Neutralize by adding 12 ml RPMI medium, and transfer the suspension to a new 50 ml tube through a 70 µm sterile nylon gauze.
10. Centrifuge the 50 ml tubes at 450 *x g* for 6 min at 4 °C and discard the supernatants.
11. Count the living cells using trypan bleu and resuspend the cell pellet in Lymphoprep at a concentration of approximately 1-2 x 10^7 cells/ml and transfer the suspension to sterile 15 ml tube(s) (6 ml/tube). Cover this cell suspension slowly and cautiously with 6 ml RPMI to obtain a two-phase gradient (Figure 1D).

12. Centrifuge the gradients at 800 *x g* for 30 min at room temperature without acceleration or break.
13. Carefully collect the interphase and the upper phase containing the RPMI medium and as less Lymphoprep contamination as possible (Figure 1E). The interphase contains the living cells and is enriched in myeloid cells, and transfer to a new sterile 15 ml tube. Wash by filling the 15 ml tube unto the top with MACS buffer, centrifuge at 800 *x g* for 5 min at 4 °C and discard the supernatants.
14. Resuspend the cells in MACS buffer at a concentration of 10^8 cells/ml.

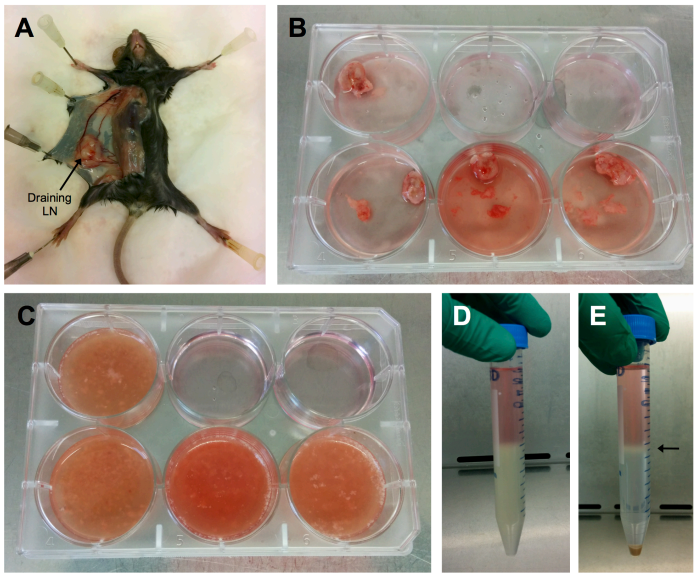

Figure 1. Tumor single-cell preparation. A. LLC tumor-bearing mouse. The arrow points to the draining lymph node, in this case the inguinal lymph node. B. Tumors in a 6 well plate in 2 ml RPMI medium. C. Tumors after digestion. D. Prepared gradient. E. Gradient after centrifugation with a clearly visible interphase.

B. Purification of tumor-associated macrophages (TAM)

1. Add a 5 µl aliquot of anti-CD11b magnetic microbeads per 10^7 cells and incubate for 20 min at 4 °C on an orbital shaker at 50 rpm.
2. Wash by adding 10 ml MACS buffer, centrifuge at 450 *x g* for 6 min at 4 °C and discard the supernatants.
3. Place an LS column in a MidiMACS Separator attached to a magnetic MultiStand and wash it by putting 3 ml MACS buffer on the top. The liquid passes the column by gravity.
4. Resuspend the pelleted cells in 1 ml MACS buffer and pipette the labeled cell suspension on top of the LS separation column. Wash the column by adding 3 x 3 ml MACS buffer.
5. Remove the LS column from the separator and wash the magnetically labeled cells out with 5 ml MACS buffer using a plunger in a sterile 15 ml tube.

6. Incubate the $CD11b^+$ cell suspension with rat anti-mouse CD16/CD32 (10 µg per 10^7 cells) on ice water for 20 min, in order to block the Fc receptors present on the cells' surface.
7. Incubate the cell suspension with fluorescently labeled antibodies (1 µg per 10^7 cells) for another 20 min on ice water, protected from exposure to light. TAM can be sorted on a BD FACS Aria as $CD11b^{pos}$ $Ly6G^{neg}$ $SiglecF^{neg}$ $Ly6C^{low}$ cells and can be separated into MHC-II^{high} and MHC-II^{low} TAM subsets (Laoui *et al.*, 2014) (Figure 2A).
8. Wash by adding 10 ml MACS buffer, centrifuge at 450 *x g* for 6 min at 4 °C and discard the supernatants.
9. Meanwhile precoat 5 ml polypropylene round-bottom tubes and 15 ml tubes with heat-inactivated fetal calf serum, add respectively 1 ml or 2 ml heat-inactivated fetal calf serum. Shake the tubes gently by hand so that the heat-inactivated fetal calf serum covers the whole surface of the tube, and discard the excess of heat-inactivated fetal calf serum. This will prevent the cells to stick to the tubes and hence enhance the recovery of cells.
10. Resuspend the pellet in 1 ml sorting buffer per 10^7 cells and transfer into a sterile 5 ml polypropylene round-bottom tube precoated with heat-inactivated fetal calf serum.
11. Collect the sorted TAM in 15 ml tubes precoated with heat-inactivated fetal calf serum containing 3 ml complete medium.

C. Purification tumor-associated dendritic cells (TADC)

1. Add a 5 µl aliquot of anti-CD11c microbeads per 10^7 cells and incubate for 20 min at 4 °C on an orbital shaker at 50 rpm.
2. Wash by adding 10 ml MACS buffer, centrifuge at 450 *x g* for 6 min at 4 °C and discard the supernatants.
3. Place an LS column in a MidiMACS Separator attached to a magnetic MultiStand and wash it by putting 3 ml MACS buffer on the top. The liquid passes the column by gravity.
4. Resuspend the pelleted cells in 1 ml MACS buffer and pipette the labeled cell suspension on top of the LS separation column. Wash the column by adding 3 x 3 ml MACS buffer.
5. Remove the LS column from the separator and wash the magnetically labeled cells out with 5 ml MACS buffer using a plunger in a sterile 15 ml tube.
6. Incubate the $CD11c^+$ cell suspension with rat anti-mouse CD16/CD32 (10 µg per 10^7 cells) on ice water for 20 min, in order to block the Fc receptors present on the cells' surface.
7. Incubate the cell suspension with fluorescently labeled antibodies (1 µg per 10^7 cells) for another 20 min on ice water, protected from exposure to light. TADC can be sorted on a BD FACS Aria as $Ly6G^{neg}$ $SiglecF^{neg}$ $CD11c^{pos}$ MHC-II^{pos} cells. Take care to gate only on

the MHC-II^{highest} cells and to exclude the MHC-II^{high} TAM, which also express CD11c, albeit at a lower level than TADC (Figure 2B).

8. Wash by adding 10 ml MACS buffer, centrifuge at 450 *x g* for 6 min at 4 °C and discard the supernatants.
9. Meanwhile precoat 5 ml polypropylene round-bottom tubes and 15 ml tubes with heat-inactivated fetal calf serum, add respectively 1 ml or 2 ml heat-inactivated fetal calf serum. Shake the tubes gently by hand so that the heat-inactivated fetal calf serum covers the whole surface of the tube, and discard the excess of heat-inactivated fetal calf serum. This will prevent the cells to stick to the tubes and hence enhance the recovery of cells.
10. Resuspend the pellet in 1 ml sorting buffer per 10^7 cells and transfer into a sterile 5 ml polypropylene round-bottom tube precoated with heat-inactivated fetal calf serum.
11. Collect the sorted TADC in 15 ml tubes precoated with heat-inactivated fetal calf serum containing 3 ml complete medium.

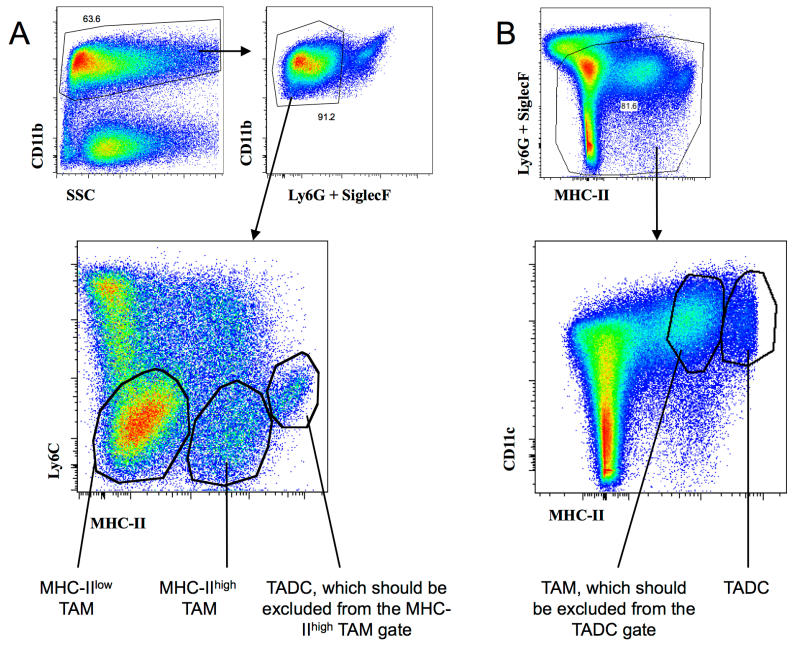

Figure 2. TAM and TADC gating. A. TAM subsets can be gated using MHC-II and Ly6C in CD11b^{pos} Ly6G^{neg} SiglecF^{neg} tumor single-cell suspensions. B. TADC can be gated using MHC-II and CD11c in Ly6G^{neg} SiglecF^{neg} tumor single-cell suspensions.

Recipes

1. Tumor digestion medium
   10 U/ml Collagenase I
   400 U/ml Collagenase IV

30 U/ml DNAse I
All diluted in HBSS
Aliquoted and frozen at -20 °C

2. Erythrocyte lysis buffer
   8.29 g/L NH_4Cl
   1 g/L $KHCO_3$
   37.2 mg/L EDTA
   Bring at pH 7.2
3. MACS buffer
   Hank's buffered salt solution
   0.5% (v/v) heat-inactivated fetal calf serum
   2 mM EDTA
4. Sorting buffer
   Hank's buffered salt solution
   0.5% (v/v) heat-inactivated FCS
   5 mM EDTA
5. Complete medium
   Roswell Park Memorial Institute (RPMI)-1640
   10% (v/v) heat-inactivated fetal calf serum (FCS)
   300 µg/ml L-glutamine
   100 U/ml penicillin
   100 µg/ml streptomycin
   0.02 mM β-mercaptoethanol
   1 mM sodium pyruvate
   1 mM non-essential amino acids

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