Isolation of Phagosomes from Dendritic Cells by Using Magnetic Beads

Bénédicte Manoury*

INSERM, Unité 1013, Paris, France

*For correspondence: benedicte.manoury@inserm.fr

[Abstract] Phagosomes are intracellular organelles in dendritic cells in which pathogens such as viruses, bacteria and parasites are internalised to be proteolysed and killed. Phagosomes are formed by fusion with the plasma membrane, some area of the endoplasmic reticulum as well as the lysosome. This protocol described the purification of phagosomal compartments at different stages of their maturation using magnetic beads.

Materials and Reagents

1. CO_2 independent medium (Life Technologies, Invitrogen™, catalog number: 18045054)
2. Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich, catalog number: I3390)
3. Fetal calf serum (FBS) (Hyclone/PAA, catalog number: sv-143-03)
4. Penicillin streptomycin (Sigma-Aldrich, catalog number: P11-010)
5. Glutamine (Sigma-Aldrich, catalog number: G7513)
6. GM-CSF (10 ng/ml) (Peprotech, catalog number: 315-03)
1. EDTA (Life Technologies, Invitrogen™, catalog number: 15575-038)
7. BSA (Sigma-Aldrich, catalog number: A2153)
8. DTT (Sigma-Aldrich, catalog number: 43813)
9. ULTRA tablets protease inhibitor cocktail solution (Roche, catalog number: 05892791001)
10. NaCl (Sigma-Aldrich, catalog number: S-7653)
11. Tris pH 7 (Sigma-Aldrich, catalog number: T-1503)
12. NP40 (Sigma-Aldrich, catalog number: I8896)
13. $MgCl_2$ (Sigma-Aldrich, catalog number: M8266)
14. EGTA (Sigma-Aldrich, catalog number: E3889)
15. Imidazole (Sigma-Aldrich, catalog number: I-5513)
16. Sucrose (Sigma-Aldrich, catalog number: S79-03)
17. RIPA (Pierce Antibodies, catalog number: 89900)
18. Antibodies:
    EEA1 (Abcam, catalog number: ab2900)
    TfR rabbit serum (Home made)
    LAMP1 (Abcam, catalog number: ab62562)

Rab7 (Abcam, catalog number: ab126712)

19. 1x PBS (see Recipes)
20. RIPA buffer (see Recipes) supplemented with 2 mM DTT and 1x complete ULTRA Protease inhibitor cocktail tablets
21. Bone marrow derived dendritic cells medium (BMDCs) (see Recipes)
22. Homogenisation buffer (HB) (see Recipes)
23. Lysis buffer (see Recipes)

Equipment

1. Water bath
2. Magnetic beads (Dynal, Dynabeads M-280 streptavidine)
3. Magnetic stand for Eppendorf (Dynal, catalog number: R670001)
4. 22 g needle (Terumo, catalog number: NN-2238R)
5. 1 ml syringe (Terumo, catalog number: S*025E1)
6. Centrifuge

Procedure

1. Detach bone marrow-derived dendritic cells (BMDCs) with 1x PBS-5 mM EDTA (10 min at 37 °C).
2. Wash the cells with PBS twice by centrifugation at 367 *x g* for 10 min.
3. Resuspend the cells in 15 ml falcon tube (7.5 x 10^7 cells/750 µl) in CO_2 independent medium.
4. Add the magnetic beads (ratio: 6.5 x 10^5 beads/µl) to the cells.
5. Incubate 20 min in the water bath at 30 °C.
6. Raise the temperature of the water bath to 37 °C and leave the cells for 20 min (pulse).
7. Stop the reaction by adding 10 ml cold-ice 1x PBS-0.1% BSA and centrifuge for 10 min at 340 *x g* at 4 °C.
8. Repeat step 7 3 times.
9. Resuspend the cells in 3 ml of BMDCs medium and split the cells in 3 falcon tubes of 15 ml (1 ml of cells each).
10. Add 10 ml cold PBS-0.1% BSA fort = 0 (pulse) and leave on ice, put the other tubes at 37 °C for different times (chase, 40 min and 100 min).
11. T = 0 corresponds to early phagosomes (20 min of pulse); t = 60 min correspond to intermediate phagosomes (20 min of pulse + 40 min of chase), t = 120 min correspond to phagolysosomes (20 min of pulse + 100 min of chase).

12. At the end of the chase, centrifuge the cells at 463 *x g* for 10 min.
13. Now, perform the following steps on ice.
14. Wash the cells in ice-cold HB buffer.
15. Resuspend the cells in 1 ml of HB buffer.
16. Break the cells using a 1 ml syringe and a 22 g needle. You need about 30 flushes to break the cells. Check the broken the cells under a microscope (about 70% of the cells should be dead).
17. Transfer the broken cells into cold eppendorf tubes and place the eppendorf tubes on the magnetic stand on ice.
18. Leave 5 min; aspirate the supernatant with a thin tip. Keep the supernatant aside (on ice).
19. Wash the beads carefully with 1 ml PBS-0.1% BSA and repeat 8x step 16.
20. Resuspend the beads in lysis buffer or RIPA buffer (50 µl) and leave on ice for 15 min.
21. Centrifuge at 13, 400 *x g* for 15 min and freeze the supernatant.
22. Phagosomes are ready to use and can be checked for their purity using a panel of antibodies (EEA1 or TfR for early markers, LAMP1 or Rab7 for late markers, see Figure 1).

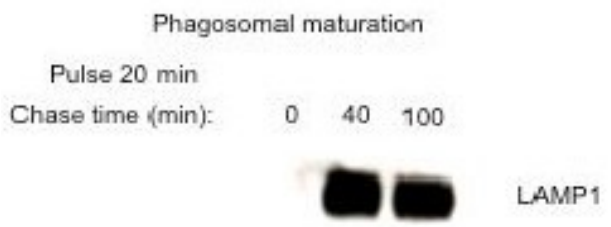

Figure 1. Phagosomes from BMDCs are purified with magnetic beads after 20, 60 and 120 min. 5 µg of proteins are resolved by SDS-PAGE. Late marker (LAMP1) is visualized by immunoblot.

Recipes

1. 1x PBS
   137 mM NaCl
   2.7 mM KCl
   8 mM Na_2HPO_4
   1.46 mM KH_2PO_4
2. RIPA buffer
   50 mM Tris pH 7.5
   150 mM NaCl

1% NP40
0.5% desoxycholate de sodium
20 mM EGTA

3. Bone marrow derived dendritic cells medium (BMDCs)
   IMDM supplemented with
   10% fetal calf serum
   1% penicillin streptomycin
   1% glutamine
   10 ng/ml GM-CSF
4. Homogenisation buffer (HB)
   8% sucrose
   3 mM Imidazole
   2 mM DTT
   A complete 1x ULTRA tablets protease inhibitor cocktail solution (dissolve 1 tablet in 2 ml of H_2O, 25x)
5. Lysis buffer
   150 mM NaCl
   50 mM Tris pH 7
   A complete 1x ULTRA tablets protease inhibitor cocktail solution (1 tablet for 10 ml)
   0.5% NP40
   2 mM $MgCl_2$

Acknowledgments

This protocol is adapted from Cebrian *et al*. (2011); Maschalidi *et al*. (2012) and Mantegazza *et al*. (2012).

References

1. Cebrian, I., Visentin, G., Blanchard, N., Jouve, M., Bobard, A., Moita, C., Enninga, J., Moita, L. F., Amigorena, S. and Savina, A. (2011). Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. *Cell* 147(6): 1355-1368.
2. Maschalidi, S., Hassler, S., Blanc, F., Sepulveda, F. E., Tohme, M., Chignard, M., van Endert, P., Si-Tahar, M., Descamps, D. and Manoury, B. (2012). Asparagine endopeptidase controls anti-influenza virus immune responses through TLR7 activation. *PLoS Pathog* 8(8): e1002841.

3. Mantegazza, A. R., Guttentag, S. H., El-Benna, J., Sasai, M., Iwasaki, A., Shen, H., Laufer, T. M. and Marks, M. S. (2012). Adaptor protein-3 in dendritic cells facilitates phagosomal toll-like receptor signaling and antigen presentation to CD4(+) T cells. *Immunity* 36(5): 782-794.