C1q binding to and uptake of apoptotic lymphocytes by human monocyte-derived macrophages (1)

**Marie E. Benoit and Andrea J. Tenner**

Department of Molecular Biology and Biochemistry, Institute for Immunology, University of California Irvine, Irvine, CA 92697, USA.

**Material and Reagents**

* RPMI1640+L-Glutamine+Hepes (Life Technologies cat. 22400-105)
* FBS (Hyclone defined FBS cat. SH30070.03, inactivate for 30 min at 56˚C)
* Penicillin/Streptomycin (Life Technologies cat. 15070-063)
* L-Glutamine (200 mM, Life Technologies cat. 25030-081)
* 0.4% Trypan blue Solution (Sigma cat. T8154)
* HBSS (Corning/Cellgro cat. MT-21-023-CV)
* PBS
* 25% Human Serum Albumin (Talecris Biotherapeutics)
* Recombinant human IL-2 (Peprotech cat. 200-02)
* Recombinant human M-CSF (Peprotech cat. 300-25)
* Bovine Serum Albumin (BSA) (albumin from bovine serum, lyophilized powder, ≥96%, Sigma cat.# A2153-100G)
* C1q – Comptech (Tyler, Tx)
* PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma cat. PKH26GL)
* CellStripper Dissociation Reagent (Fisher cat. 25-056-CI)
* Apoptosis detection kit (BioVision cat. K101-100)
* Anti-human C1q (Quidel cat. A201)
* FITC conjugated anti-mouse IgG (Jackson Immunoresearch cat. 115-096-006)
* FcR blocking reagent human (Miltenyi cat. 130-059-901)
* FITC conjugated anti-human CD11b (Life Technologies cat. CD11B01)
* FITC mouse IgG1 isotypes (Life Technologies cat. MG101)
* Fluorescein phalloidin (Life Technologies cat. F432)
* Stericup (Fisher cat. SCGVU11RE)
* Microtubes
* 50 ml conical tubes
* 12 x 75 mm polypropylene round bottom sterile tube (Fisher cat. 14-956-1D)
* 12 x 75 mm polystyrene round bottom tubes (Fisher cat. 14-961-13)
* Tissue culture plates and vented flasks (any size)
* 12 mm cover slips (Fisher cat. GG12), sterilize by soaking in 100%? Ethanol for 2 x 5 min
* Non tissue culture treated 100 mm petri dish (Fisher cat. 0875712) – referred to as petri dishes
* Prolong gold antifade reagent (Life Technologies cat. P36930)

**Equipment**

* Tissue culture hood
* Gamma-irradiator
* Humidified incubator 5% CO2, 37°C
* Centrifuge for 50 ml conical tubes and 5 ml bottom round tubes
* Centrifuge rotor for plates
* Optic and fluorescent microscopes
* Hemocytometer
* Flow cytometer
* Automatic pipettes (full range volumes)
* Tips (full range volumes)

**Recipes**

* + - 1. Complete media
* RPMI1640+L-Glutamine+Hepes (500 ml)
* 50 ml (10%) heat-inactivated FBS
* 5 ml (1%) Penicillin/Streptomycin
* 5 ml (1%) L-Glutamine 200 mM
  + - 1. Phagocytosis buffer
* RPMI1640+L-Glutamine+Hepes
* 25 mM Hepes
* 5 mM MgCl2
  + - 1. ACK buffer
* To 450 ml milliQ water add
  + 4.145 g Ammonium Chloride (anhydrous)
  + 0.5 g potassium bicarbonate
  + 18.6 mg disodium EDTA
* Adjust pH to 7.4
* Bring final volume to 500 ml with milliQ water
* Filter sterilize using stericup
  + - 1. FACS buffer

500 ml HBSS (no phenol red)

0.2% NaN3

0.2% BSA

1. **Human peripheral blood lymphocytes and monocytes are isolated by counterflow elutriation using a modification of the technique of Lionetti et al. (2) as described previously (3).**
2. **Lymphocyte isolation, staining and apoptosis induction** 
   1. During elutriation collect the lymphocyte fraction in 2 x 50 ml tubes
   2. Centrifuge cell suspension 10 min at 700 rpm, RT to remove majority of platelets
   3. Discard supernatant and pool cell pellets in 10 ml ACK buffer. Incubate 2-5 min RT
   4. Add 20 ml complete media
   5. Centrifuge cell suspension 10 min at 700 rpm, RT
   6. Add 20 ml HBSS. Count viable cell number using 0.4% Trypan blue solution, a hemocytometer chamber and an optic microscope
   7. Centrifuge cell suspension 10 min at 700 rpm, RT
   8. Resuspend the lymphocytes at 1 million/ml in complete media in a vented tissue culture flask
   9. Add 100 U/ml recombinant human IL-2
   10. Incubate at 37˚C, 5% CO2 for 7 days
   11. Centrifuge lymphocyte cell suspension 5 min 1200 rpm
   12. Discard supernatant and wash cell pellet with 10 ml HBSS. Count viable cell number using 0.4% Trypan blue solution, a hemocytometer chamber and an optic microscope
   13. Centrifuge cell suspension 5 min 1200 rpm
   14. Discard supernatant and resuspend 20 million lymphocytes in 1 ml diluent C of PKH26 Red Fluorescent Cell Linker Kit
   15. Dilute 4 μl PKH26 dye in 1 ml diluent C (4 μM)
   16. Mix dye and cells (PKH26 at 2 μM final) , invert the tube gently and incubate for 5 min RT
   17. Add 2 ml FBS. Mix well. Incubate 1 min RT
   18. Add 16 ml complete media. Mix well by inversion. Centrifuge cell suspension 10 min 1200 rpm
   19. Discard supernatant and resuspend cell pellet in 10 ml complete media. Centrifuge cell suspension 10 min 1200 rpm
   20. Repeat step “s” twice for a total of 3 washes.
   21. Count viable cell number using 0.4% Trypan blue solution, a hemocytometer chamber and an optic microscope
   22. Resuspend PKH26-labeled lymphocytes at 2 million/ml (up to 50 million in 25 ml) in RPMI1640 media without FBS in a T25 vented tissue culture flask
   23. Induce apoptosis by exposing lymphocytes to γ-irradiation (10 Gy)
   24. Incubate lymphocytes overnight 5%CO2, 37°C in either complete media (for early apoptotic cells) RPMI without serum for late apoptotic cells at 2 million/ml
3. **Isolation and culture of monocytes**
4. After recovering monocytes from elutiation chamber, wash in HBSS, count, and resuspend at 0.5 million/ml in complete media (Day 0)
5. Add 10 ml per 100 mm petri dish
6. Add recombinant human M-CSF to a final concentration of 25 ng/ml
7. Place at 37˚C, 5% CO2
8. After 3-4 days, add 5 ml fresh complete media (containing 25 ng/ml M-CSF) per plate
9. On day 6-8, discard media from plates and wash adherent cells twice with 5 ml HBSS
10. Discard last HBSS and add 5 ml CellStripper to the plates, incubate 20 min RT
11. Pipet up and down to detach the cells and transfer to 50 ml conical tube containing 25 ml prewarmed complete media (one tube = 5 plates, final volume 50 ml)
12. Centrifuge cell suspension 1200rpm, 5 min RT
13. Wash cell pellet twice with 10 ml HBSS, centrifuge cell suspension 5 min 1200 rpm
14. Count viable cell number using 0.4% Trypan blue solution, a hemocytometer chamber and an optic microscope
15. Plate monocytes at 0.25 million/ml in complete media, 500 cells/mm2. For immunocytochemistry (ICC), plate cells in 24-well plates containing 12 mm coverslips (0.5 ml)
16. Incubate at 37˚C, 5% CO2 overnight
17. **C1q binding to apoptotic lymphocytes**
18. Transfer apoptotic lymphocytes to conical tube. Assess apoptosis by flow cytometry using the apoptosis detection kit from Biovision
19. Centrifuge cell suspension 5 min 1200 rpm
20. Discard supernatant and carefully resuspend lymphocytes in 10 ml prewarmed HBSS. Count ALL cells (viable and permeable) using 0.4% Trypan blue solution, a hemocytometer chamber and an optic microscope
21. Centrifuge cell suspension 5 min, 1200 rpm
22. Resuspend cell pellet at 5 x 106 cells/ml in HBSS/1%HSA in sterile 12x75 mm round bottom tube
23. Depending on the number of C1q coated apoptotic cells desired, add human purified C1q to a final concentration of 150 μg/ml. Pipet gently up and down or invert to mix.
24. Incubate for 1 h at 37°C, gently shake tubes every 15 min
25. Add 2 ml HBSS per tube and centrifuge cell suspension 5 min, 1200 rpm
26. Discard supernatant, add 2 ml HBSS per tube and centrifuge cell suspension 5 min, 1200 rpm
27. Resuspend cell pellet at desired concentration in phagocytosis buffer for uptake assay. Set aside 2 x 105 apoptotic lymphocytes +/- C1q to assess C1q binding efficiency as described below
28. **C1q binding efficiency**
29. Resuspend 2 x 105 apoptotic lymphocytes +/- C1q in 100 l FACS buffer
30. Add 1-2 l murine anti-human C1q and incubate for 30 min on ice
31. Add 2 ml FACS buffer, centrifuge cell suspension 5 min 1200 rpm, 4°C
32. Discard supernatant and resuspend cell pellet in 100 l FACS buffer
33. Add 1 l FITC conjugated anti-mouse IgG and incubate for 30 min on ice in the dark
34. Add 2 ml FACS buffer, centrifuge cell suspension 5 min 1200 rpm, 4°C
35. Discard supernatant and resuspend cell pellet in 200 l FACS buffer
36. Analyze by flow cytometry to determine C1q binding efficiency to apoptotic lymphocytes. Percentage of apoptotic cells binding C1q should be greater than 50%.
37. **Uptake assay**
38. Recover HMDMs plate from step 3. Discard media and wash adherent cells twice with HBSS
39. Recover apoptotic lymphocytes from step 4j. Add apoptotic lymphocytes +/- C1q at a 5:1 ratio (for example 5 x 105 apoptotic lymphocytes for 1 x 105 HMDMs in phagocytosis buffer in a final total volume of 2 ml per a 6-well plate well)
40. Centrifuge the plate 3 min at 700 rpm
41. Incubate for 1 h at 37°C
42. To assess uptake by flow cytometry:
43. Discard media and wash adherent cells twice with HBSS
44. Add 0.5 ml 0.05% trypsin and incubate 2 min 37 °C. Pipet cells up and down and transfer into 12 x 75 mm tubes. (Microscopically check that all macrophages have been recovered.)
45. Centrifuge cells 5 min 1200 rpm, RT
46. Discard supernatant and resuspend cell pellet in 100 ml FACS buffer
47. Add 5 μl CD11b-FITC antibodies per 1 x 106 cells , incubate 45 min in the dark on ice
48. Add 2 ml FACS buffer, spin down 5 min 1200 rpm 4°C
49. Centrifuge cells 5 min 1200 rpm, RT
50. Discard supernatant and resuspend in 300 µl FACS buffer to read
51. Analyze by flow cytometry
52. To assess uptake by ICC (using 12 mm coverslips placed in a 24-well plate):
53. After step 6d, discard media and wash adherent cells twice with 0.5 ml HBSS
54. Fix cells with 3.7% formaldehyde (300 l/well), 10 min RT. *Do not use methanol or acetone as it can disrupt the PKH26 membrane labeling of apoptotic lymphocytes.*
55. Wash cells twice with PBS
56. Stain cells with 4U FITC-phalloidin per well diluted in 250 l PBS for 20 min RT.
57. Wash cells twice with PBS
58. Mount coverslips with a drop of prolong gold antifade reagent
59. Analyze by fluorescence or confocal microscopy

Reference List

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3. Bobak, D. A., M. M. Frank, and A. J. Tenner. 1986. Characterization of C1q receptor expression on human phagocytic cells: Effects of PDBu and FMLP. *Journal of immunology (Baltimore, Md. : 1950)* 136: 4604-4610.