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In vivo DCs Depletion with Diphtheria Toxin and MARCO$^+$/MOMA1$^+$ Cells Depletion with Clodronate Liposomes in B6.CD11c-DTR Mice

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[Abstract] To evaluate precisely the relative roles of different splenic phagocytic cells during an immune response, efficient methods for the depletion of specific populations are needed. Here, we describe the protocols for the depletion of splenic dendritic cells (DCs) by human diphtheria toxin (DTx) treatment in target mice (which express the human DTx receptor in all CD11c$^+$ DCs) and for the specific depletion of MARCO$^+$/MOMA-1$^+$ marginal zone macrophages (MZMΦs) with clodronate liposomes (ClLip) treatment (when a small dose of ClLip is ministered, MZMΦs preferentially uptake ClLip, and clodronate is released inside those cells causing apoptosis-mediated cell death). These protocols are adaptations from previous works (Jung *et al*., 2002; McGaha *et al*., 2011), and were used to evaluate the respective roles of DCs and of MZMΦs during the acute phase of experimental blood-stage malaria infection (Borges da Silva *et al*., 2015).

Materials and Reagents

1. 25 gauge needle (BD Biosciences, catalog number: 305122)
2. Cell strainer (100 µm pore size) (Corning Incorporated, catalog number: 352360)
3. 15 ml tubes (TPP, catalog number: 91015)
4. 1 ml syringes (BD Biosciences, catalog number: 309659)
5. 1 ml syringes with 30 gauge needle (BD Biosciences, catalog number: 328278)
6. B6 mice (Jackson Laboratories, model: B6)
7. B6.CD11c-DTR mice (Jackson Laboratories, model: C57BL/6)
8. Diphtheria toxin (Sigma-Aldrich, catalog number: D0564)
9. Sodium clodronate (Melone Pharmaceutical, catalog number: 22560-50-5)
10. RPMI 1640 (Thermo Fisher Scientific, catalog number: 11875093)
11. Fetal bovine serum heat inactivated (Thermo Fisher Scientific, catalog number: 10437028)
12. Penicillin-Streptomycin (Thermo Fisher Scientific, catalog number: 15140122)
13. L-glutamine (Thermo Fisher Scientific, catalog number: 25030081)
14. Sodium pyruvate (Thermo Fisher Scientific, catalog number: 11360070)
15. 2-mercaptoethanol Thermo Fisher Scientific, catalog number: 21985023)
16. Halothane (Sigma-Aldrich, catalog number: H0150000)
17. Monoclonal antibody (mAb) to MARCO (R&D Systems, catalog number: FAB2956P)

18. mAb to MOMA-1 (Abcam, catalog number: ab51814)
19. mAb to F4/80 (eBioscience, catalog number: 47-4801)
20. mAb to CD11b (BD Biosciences, catalog number: 562950)
21. mAb to CD11c (eBiosciences, catalog number: 17011482)
22. mAb to I-Ab (eBiosciences, catalog number: 46532082)
23. NaCl
24. KCl
25. Na_2HPO_4
26. KH_2PO_4
27. 1x phosphate buffered saline (PBS) (see Recipes)
28. Staining Buffer (see Recipes)
29. Supplemented RPMI 1640 (see Recipes)
30. ClLip (see Recipes)

Equipment

1. Centrifuge (Eppendorf, model: 5804)
2. Laminar flow hood (AirClean Systems, catalog number: AC8000HLF)
3. FACSCanto II Flow Cytometer, 8-color, lasers blue/red/violet (BD Biosciences, catalog number: 338962)
4. Push-Pull syringe pump (KD Scientific, Model: KDS120)

Procedure

A. DCs depletion in B6.CD11c-DTR mice
   1. Inject B6.CD11c-DTR mice (6-8 weeks age) *i.p.* with 2 ng/g of body weight of DTx (200 µl volume per mouse), or with PBS as depletion control (200 µl volume per mouse), using a syringe with 25 gauge needle.
   2. 24 h after injection, mice are euthanized with halothane by inhalation (or other approved euthanasia protocol).
   3. The spleens are removed and processed in a cell strainer inside a sterile culture hood (with 5 ml of supplemented RPMI 1640), followed by two washes with RPMI (at 300 *x g*, 5 min, 4 °C).
   4. Splenocytes are then stained with mAbs (0.5 µl per 10^6 cells – each mAb at initial 0.5 mg/ml concentration, diluted in 25 µl of Staining Buffer) to CD11c and I-Ab (1 incubation period of 30 min), re-suspended in staining buffer (200 µl) and analyzed in a FACSCanto device, to evaluate depletion efficiency.

B. MZMΦs depletion in B6 mice with ClLip
   1. Inject B6 mice (6-8 weeks age) *i.v.* with 8.5 µg/g of body weight of ClLip (200 µl per mice)

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prepared as described in (van Rooijen *et al*., 1993), or with PBS-loaded liposomes (200 µl per mice, 8.5 µg/g of body weight) as depletion control, using a syringe with 30 gauge needle.

2. 24 h to seven days after injection, mice are euthanized with halothane by inhalation (or other approved euthanasia protocol) and bled by cardiac puncture with a 1 ml syringe with a 25 gauge needle.

   Note: In both time points only MZMΦs are depleted in our protocol (this was done as a control to ensure only MZMΦs are depleted in our protocol, opposite to injection of higher concentrations of ClLip).

3. The spleens are removed and processed in a cell strainer inside a sterile culture hood (with 5 ml of supplemented RPMI 1640), followed by two washes with RPMI (at 300 *x g*, 5 min, 4 °C).

4. Splenocytes are then stained with mAbs (0.5 µl per 10^6 cells – each mAb at initial 0.5 mg/ml concentration, diluted in 25 µl of Staining Buffer) to MARCO, MOMA-1, CD11b, CD11c and I-Ab (1 incubation period of 30 min), re-suspended in Staining Buffer (200 µl) and analyzed in a FACSCanto device, to evaluate depletion efficiency. An example of results obtained with this experiment is shown in Figure 1A and 1B, respectively.

   Note: Different DC subsets might have different sensitivity to DTx treatment, with different repopulation rates following initial depletion. Thus, for prolonged experiments, an important step would be to perform repopulation rate assays for the DCs subsets of interest.

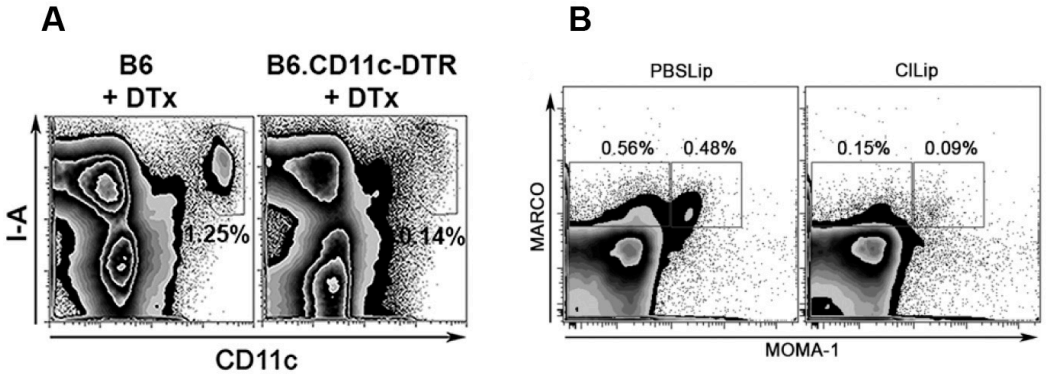

Figure 1. *In vivo* depletion of splenic DCs and MZMΦs. A. Representative plots of mice depleted of DCs using as controls of depletion B6 mice treated with DTX (here, CD11c-DTR mice treated with DTx present with a lower percentage of CD11c^{+}I-A^{+} DCs, as showed in the gate in the upper right of each histogram (this decrease in percentage is an indicative of cell depletion). Adapted from Borges da Silva *et al*. (2015). B. Representative plots of mice depleted of MZMΦs using as controls B6 mice treated with ClLip (here, ClLip-treated mice present with a lower percentage of MARCO^{+}MOMA-1^{+} MZMΦs, as showed in the gates represented in each histogram (this decrease in percentage is an indicative of cell depletion). Adapted from Borges da Silva *et al*. (2015).

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Recipes

1. 1x phosphate buffered saline (PBS)
   Dissolve the following in 800 ml distilled H_2O
   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   Adjust pH to 7.4
   Adjust volume to 1 L with additional distilled H_2O
   Sterilize the solution
   Note: Adjust the pH using HCl and NaOH.
2. Staining Buffer
   Dissolve the following in 200 ml PBS
   1 ml 10% azide
   2 ml fetal bovine serum heat inactivated
3. Supplemented RPMI 1640
   Dissolve the following in 200 ml RPMI 1640 medium
   2 ml L-glutamine
   20 ml fetal bovine serum heat inactivated
   2 ml sodium pyruvate
   2 ml Penicillin-Streptomycin
   200 µl 2-mercaptoethanol
4. ClLip
   a. Inject (0.2 ml/min) an ethereal solution of 50 mg phosphatidylcholine and 8 mg cholesterol into 5 ml of a 50 mM/L clodronate aqueous solution maintained at 42 °C, by using a syringe adapted in a Push-Pull syringe pump, equipped with a fine-gauge needle (Nº 3D).
   b. During injection, a nitrogen stream will be infused into the clodronate solution, up to liposome formation and removal of residual solvent.
   c. Centrifuge liposome suspension (22,800 *x g*, 30 min, 25 °C). Wash twice with PBS, and resuspend in 2 ml PBS.
   d. Filter through a 0.8 Am polycarbonate membrane.

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adapted from Jung *et al*. (2002), and the ClLip-mediated depletion of MZMΦs was adapted from McGaha *et al*. (2011).

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