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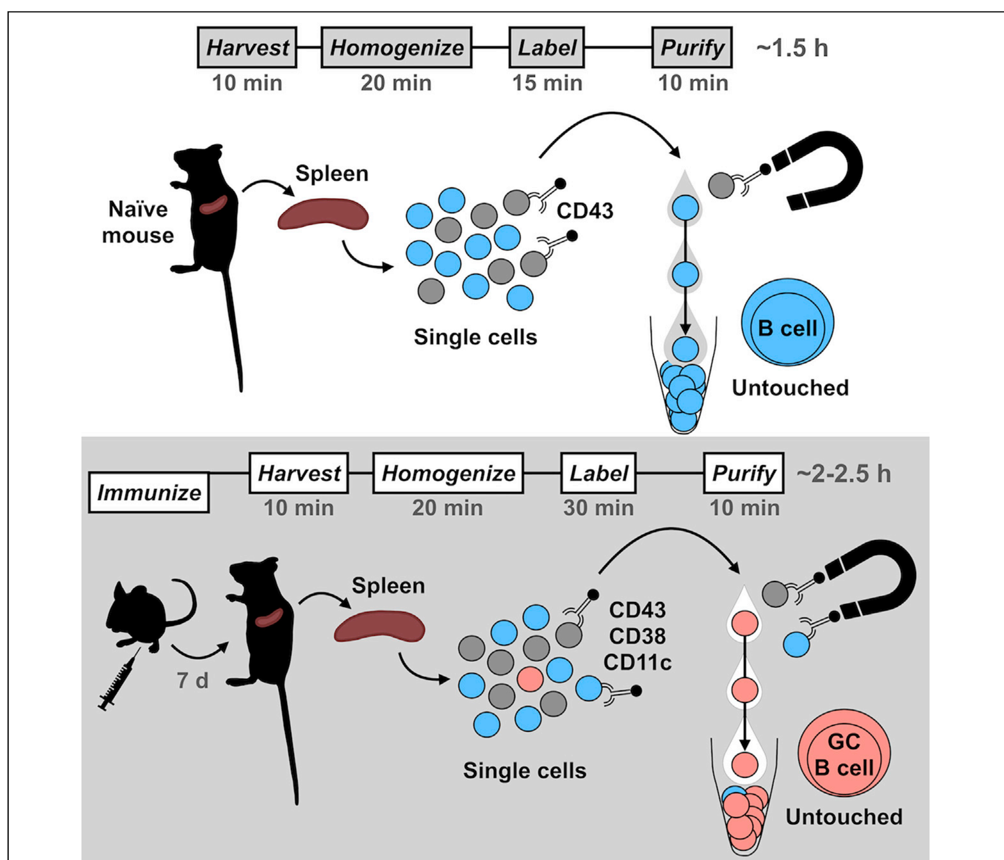
Quick and easy purification of murine untouched naive B cells or germinal center B cells by MACS



Humoral immune responses depend on the generation of high-affinity antigen-specific antibodies. Germinal center (GC) B cells are the cornerstone of this response in peripheral lymphoid organs. High purities of GC B cells, and also naive B cells, are required for accurate analysis in downstream assays to yield essential knowledge on immunity. This protocol lays out quick and easy steps to purify GC B cells from spleens of immunized mice or B cells from naive animals by negative selection using MACS.

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HIGHLIGHTS

The murine spleen is a major source of naive mature B cells

Untouched naive B cells are purified by depleting CD43^{+} cells

Upon immunization, the spleen is a large source of germinal center (GC) B cells

Untouched GC B cells are purified by depleting CD43^{+} CD38^{+} CD11c^{+} cell populations

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Quick and easy purification of murine untouched naive B cells or germinal center B cells by MACS

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SUMMARY

Humoral immune responses depend on the generation of high-affinity antigen-specific antibodies. Germinal center (GC) B cells are the cornerstone of this response in peripheral lymphoid organs. High purities of GC B cells, and also naive B cells, are required for accurate analysis in downstream assays to yield essential knowledge on immunity. This protocol lays out quick and easy steps to purify GC B cells from spleens of immunized mice or B cells from naive animals by negative selection using MACS.

For complete details on the use and execution of this protocol, please refer to Ramezani-Rad et al. (2020).

BEFORE YOU BEGIN

This protocol below describes the specific steps for purifying untouched (=unlabeled) B cells or germinal center (GC) B cells from mouse spleens using Magnetic-Activated Cell Sorting (MACS). Here, B cells are robustly enriched by depleting CD43-expressing cell populations, which is not expressed on conventional mature B cells. GC B cells are enriched by depleting cell populations that express CD43, CD38, and CD11c. GCs form in peripheral lymphoid organs during an immune response and the formation of mature GCs takes several days post antigen exposure. In the mammalian experimental model *Mus musculus* (mouse) this response can be elicited by injection of a T cell-dependent antigen. In this protocol the steps are optimized for the immunogen sheep red blood cells (SRBCs), however this protocol is generally applicable to other T cell-dependent antigens. Furthermore, this protocol was established utilizing C57BL/6 mice and may be applicable to other mouse strains. Female and male mice over 6 weeks of age are suitable for this protocol. Please note that this purification method applies specifically to murine GC B cells, as human GC B cells express CD38 and cannot be purified using this method.

> *Note:* This protocol has been optimized for speed and simplicity. Certain steps may be different from the manufacturer's recommendation.

> ⚠ CRITICAL: The purification of naive B cells is included in the workflow of GC B cell purification. If only naive B cells are desired, omit immunization of animals and start directly from the spleen harvest (from naive animals) until completion of the B cell purification step (CD43-depletion) as outlined in the Step-by-step method details.

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Preparation of immunogen (SRBCs)

Timing: 20 min

1. Prepare 1–2 × 10^8 SRBCs in 100 μL PBS per injection into each animal.
   a. In a laminar flow hood under aseptic conditions, aspirate 1 mL of citrated SRBCs (18-gauge needle with 1-mL syringe) into a 50-mL conical tube. Invert bottle several times before aspiration.
   b. Fill tube to 50 mL with cold PBS and spin at 800 × *g* for 6 min at 4°C to wash the cells.
      i. Aspirate supernatant without disturbing the pellet.
      ii. Repeat wash as in step 1b.
      iii. Supernatant should be relatively clear after two washes. However, an additional wash may be required.
      iv. Resuspend SRBC pellet with cold PBS to 4 mL total volume (add for this ~3.5 mL PBS) and count this solution to ensure cell number is around 1–2 × 10^9 per mL (=1–2 × 10^8 in a 100 μL injection dose).

Note: SRBCs should be prepared just before the injections and used right away. Citrated SRBC batches should be used within a month post draw date. Utilization at later time points may require additional washes and can lead to lower immune responses.

Immunization of mice

Timing: ~1 min per mouse (7 days for germinal center reaction)

2. Load the washed and counted SRBCs into an insulin syringe (or a 1-mL syringe with a 26–28 gauge needle)
   a. Load the syringe(s) accordingly with the volume necessary for the total number of mice (100 μL per mouse).
3. Restrain the mouse and inject 100 μL SRBCs intraperitoneally.
   b. Alternatively, inject 100 μL intravenously into the tail vein using a device to safely secure the mouse, which will require additional handling time.

CRITICAL: Animal procedures need to be in accordance with IACUC regulations and guidelines. Only trained and experienced personnel should perform animal handling to ensure the least stressful procedures to the animals.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-CD16/CD32 (clone: 2.4G2)	BD Biosciences	Cat# 553142; RRID:AB_394657
Rat anti-B220 (clone: RA3-6B2) APC-eFluor780	Thermo Fisher Scientific	Cat# 47-0452-82; RRID:AB_1518810
Armenian hamster anti-FAS (clone: Jo2) PE-Cy7	BD Biosciences	Cat# 557653; RRID:AB_396768
Rat anti-mouse T and B cell activation antigen (clone: GL7) FITC	BD Biosciences	Cat# 553666; RRID:AB_394981
Rat anti-CD43 MicroBeads	Miltenyi Biotec	Cat# 130-049-801; RRID:AB_2861373
Rat anti-CD38 (clone: 90) biotin	Thermo Fisher Scientific	Cat# 13-0381-81; RRID:AB_466427
Armenian hamster anti-CD11c (clone: N418) biotin	Thermo Fisher Scientific	Cat# 13-0114-85; RRID:AB_466364

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse anti-biotin MicroBeads	Miltenyi Biotec	Cat# 130-090-485; RRID:AB_244365
Biological samples		
Citrated sheep red blood cells	Colorado Serum Company	Cat# 31102
Chemicals, peptides, and recombinant proteins		
Ammonium chloride (NH_4Cl)	Sigma	Cat# A0171
Potassium bicarbonate ($KHCO_3$)	Macron Chemicals	Cat# 6748-03
Ethylenediaminetetraacetic acid (EDTA), disodium salt ($C_{10}H_{18}N_2Na_2O_{10}$)	Thermo Fisher Scientific	Cat# S25687
Bovine serum albumin (BSA)	RPI	Cat# A30075
Sodium azide (NaN_3)	RPI	Cat# S24080
1× DPBS	Corning	Cat# 21-031-CV
10× DPBS	Corning	Cat# 20-031-CV
Fetal bovine serum (FBS)	Thermo Fisher Scientific	Cat# 26140079
Ethanol (200 proof)	Decon Labs	Cat# 2105
Trypan blue solution 0.4% (w/v)	Corning	Cat# 25-900-CI
7-AAD	BD Biosciences	Cat# 559925
Critical commercial assays		
UltraComp eBeads Compensation Beads	Thermo Fisher Scientific	Cat# 01-2222-42
Experimental models: organisms/strains		
Mouse: wild-type (C57BL/6)	The Jackson Laboratory	Cat# JAX:000664; RRID:IMSR_JAX:000664
Software and algorithms		
FlowJo 10	Becton, Dickinson and Company	https://www.flowjo.com/
FACSDiva	BD Biosciences	https://www.bdbiosciences.com/
Spectrum Viewer	BD Biosciences	https://www.bdbiosciences.com/en-us/applications/research-applications/multicolor-flow-cytometry/product-selection-tools/spectrum-viewer
Other		
Pipettes (P2, P20, P200, P1000)	Major Supplier	n/a
Pipette tips (10 μL, 20 μL, 200 μL, 1,000 μL)	Major Supplier	n/a
Pipet controller	Major Supplier	n/a
10-mL serological pipet	Major Supplier	n/a
15-mL conical tube	Sarstedt	Cat# 62.554.205
50-mL conical tube	Sarstedt	Cat# 62.547.205
1.5 mL microcentrifuge tubes	Major Supplier	n/a
Micro dissecting scissors	Roboz	Cat# RS-5990
Micro dissecting forceps	Roboz	Cat# RS-5137
Frosted microscope slides	Thermo Fisher Scientific	Cat# 12-550-343
70 μm Nylon mesh or cell strainer	Major Supplier	n/a
60-mm cell culture dish	Sarstedt	Cat# 83.3901.500
5-mL round bottom tube	Corning	Cat# 352008
1.1-mL mini tubes	Neptune Scientific	Cat# 89092-226
0.22 μm filter	MilliporeSigma	Cat# S2GPU05RE
Insulin syringe	Becton, Dickinson and Company	Cat# 329420
1-mL syringe	Becton, Dickinson and Company	Cat# 309659
18-gauge needle	Becton, Dickinson and Company	Cat# 305195
Hemocytometer	Hausser Scientific	Cat# 3200
LS columns	Miltenyi Biotec	Cat# 130-042-401
QuadroMACS separator (magnet)	Miltenyi Biotec	Cat# 130-090-976
Vortex mixer	Scientific Industries	n/a

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Allegra X-15R (refrigerated swing bucket centrifuge)	Beckman Coulter	n/a
Laminar flow hood	Major Supplier	n/a
Magnetic stirrer w/ magnetic stir bar	Major Supplier	n/a
pH meter	Major Supplier	n/a
Scale	Major Supplier	n/a
FACSCanto (flow cytometer)	BD Biosciences	n/a

MATERIALS AND EQUIPMENT

ACK (ammonium-chloride-potassium) buffer for red blood cell lysis		
Reagent	Final concentration	Amount
Ammonium chloride	150 mM	8.02 g
Potassium bicarbonate	10 mM	1 g
EDTA, disodium salt	0.1 mM	37.2 mg
ddH$_2$O	n/a	see below
Total	n/a	1,000 mL

Dissolve ammonium chloride, potassium bicarbonate, and disodium EDTA in 800 mL ddH$_2$O in a 1 L screw cap glass bottle on a magnetic stirrer. Adjust the pH to 7.2–7.4 and q.s. to 1,000 mL with ddH$_2$O. Sterilize solution by autoclave or filtration (0.22 µm). Store ACK buffer at 20°C–25°C (room temperature), which can be used for at least 6 months.

⚠ CRITICAL: Adjusting the pH with strong acids or bases should be performed in a fume hood with extra caution and chemical-resistant gear.

Alternatives: Commercial buffers for red blood cell lysis (such as 1× RBC Lysis Buffer from Thermo Fisher Scientific Cat# 00-4333-57) may be used and should be followed per manufacturer's recommendation.

MACS buffer for cell labeling and purification		
Reagent	Final concentration	Amount
BSA	0.5%	5 g
EDTA (0.5 M)	2 mM	4 mL
PBS (10×)	1×	100 mL
ddH$_2$O	n/a	896 mL
Total	n/a	1,000 mL

For a 0.5 M EDTA stock solution, dissolve 18.61 g of Disodium EDTA in 80 mL ddH$_2$O on a magnetic stirrer while adjusting the pH to 8 and then q.s. to 100 mL with ddH$_2$O (store at 20°C–25°C (room temperature)). Alternatively, 0.5 M EDTA (pH 8) is available commercially (for example from Thermo Fisher Scientific Cat# AM9260G).

For the MACS buffer, dissolve 5 g BSA in the 1 L solution (containing 2 mM EDTA and 1× PBS) then sterilize solution by filtration (0.22 µm). Ensure final buffer pH is 7.2–7.4 and degas buffer for best performance. Final MACS buffer is stored at 4°C and can be used for at least 3 months.

Staining buffer for purity analysis by flow cytometry		
Reagent	Final concentration	Amount
FBS	1%	10 mL
Sodium azide (5%)	0.01%	2 mL
PBS (10×)	1×	100 mL
ddH_2O	n/a	888 mL
Total	n/a	1,000 mL

For a 5% (w/v) Sodium azide stock solution, dissolve 5 g of Sodium azide in 100 mL ddH_2O (store at 20°C–25°C (room temperature) in a safety cabinet). Final Staining buffer is stored at 4°C and can be used for at least 6 months.

> ⚠ CRITICAL: Sodium azide is toxic! All handling steps including preparation and use of the 5% solution should be performed in a fume hood.

STEP-BY-STEP METHOD DETAILS

Harvest the spleen, homogenize and lyse red blood cells

⏲ Timing: ∼30 min

These steps lay out how to obtain single-cell suspension of white blood cells from mouse spleens.

1. On the day of the harvest (7 days after immunization for GC B cells or same day for B cell purification from naive animals), euthanize mice by CO_2 asphyxiation according to institutional guidelines.

   Note: If downstream applications require sterile conditions, please perform all steps in a laminar flow hood. Sterilize all equipment with 70% ethanol or by autoclave (before utilization in the hood).

2. Lay mouse on the side with the left side upwards on a paper towel. Spray mouse with 70% ethanol for easier incision. With dissecting scissors (and the aid of dissecting forceps) cut away skin and open the body cavity around the midline of the mouse. Carefully lift the spleen up with the dissecting forceps and cut the vessels and other attachments at the hilum of the spleen with the dissecting scissors (Figure 1; numbers 1–4).
   a. Place isolated spleen in a 15-mL conical tube filled with 4 mL PBS on ice. Collect additional spleens if applicable.
   b. Pour the collected spleen together with the PBS into a 60-mm cell culture dish on a stable surface. Save the 15-mL conical tube for the homogenized cell solution below.
3. Dissociate the spleen mechanically using gentle force between the rough ends of frosted microscope slides (Figure 1; numbers 5–9).
   a. Work through the spleen in smaller segments and dip ends in the PBS of the dish for cells to flush off before moving onto the next segment of the spleen until the whole spleen is homogenized.
4. Pipette carefully (with a P1000) the homogenized cell solution (∼4 mL) back into the 15-mL conical tube. Layer the tube with a precut 70 μm Nylon mesh (or a cell strainer) to filter the solution.
   a. Rinse remaining cells in the dish with 1–2 mL cold PBS and pipette the remainder of the cells (through the Nylon mesh) into the conical tube.
5. Spin down the conical tube at 400 × *g* for 3 min at 4°C to pellet the homogenized cells.
6. Aspirate the supernatant and resuspend the pellet (Figure 1; number 10) in 3 mL ACK buffer for 3 min at 20°C–25°C (room temperature) to lyse red blood cells.
   a. Add 7 mL cold PBS to dilute ACK buffer and quench the lysis.

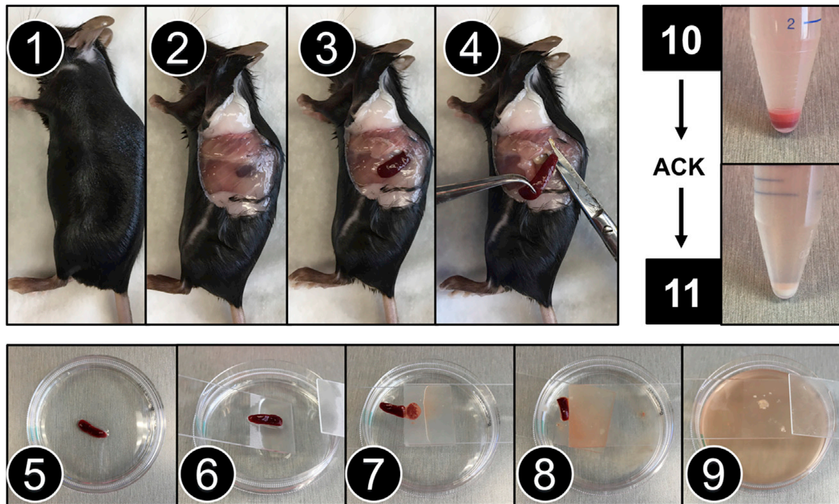

Figure 1. Spleen harvest, homogenization, and red blood cell lysis for single-cell suspension of white blood cells

7. Spin down the conical tube at 400 × *g* for 3 min at 4°C to pellet the white blood cells (Figure 1; number 11).

   ⚠ CRITICAL: Ensure red blood cells have been efficiently removed from the pellet, which should appear white. Do not leave cells > 4 min in ACK buffer (step 6) as this can affect the cell viability of white blood cells. Generally, one round of lysis is enough to remove most red blood cells, however when combining spleens more volume or an additional round might be required.

8. Aspirate the supernatant and resuspend white blood cells in 10 mL cold PBS.
   a. Determine the total cell number by a hemocytometer or an automated cell counter.
   b. Save a small aliquot of the cell solution for flow cytometry pre-purification analysis (for step 31) of B cells and/or GC B cells on ice.
9. Spin down the conical tube at 400 × *g* for 3 min at 4°C.

   Note: Cell clumps may form during this process and can be removed with a pipette tip or through additional filtering (70 μm Nylon mesh or Cell strainer).

MACS purification of B cells or GC B cells

⏲ Timing: ~45 min (B cell purification), ~1 or 2 h (GC B cell purification 1-step or 2-step)

These steps lay out how to purify B cells or GC B cells from the single-cell suspension using depletion of other cell populations by magnetic separation.

10. Aspirate the supernatant from step 9 in the tube with the enumerated white blood cell pellet.
11. Resuspend 1 × 10^7 cells in 100 μL MACS buffer.
    a. Adjust total volume accordingly to total cell number. (Example: 45 × 10^6 cells are resuspended in 450 μL MACS buffer).
12. Add 10 μL anti-CD43 MicroBeads per 1 × 10^7 cells.
    a. Adjust to the total cell number of white blood cells. (Example: Add 45 μL anti-CD43 MicroBeads to 45 × 10^6 cells).
    b. Gently shake or flick the tube.

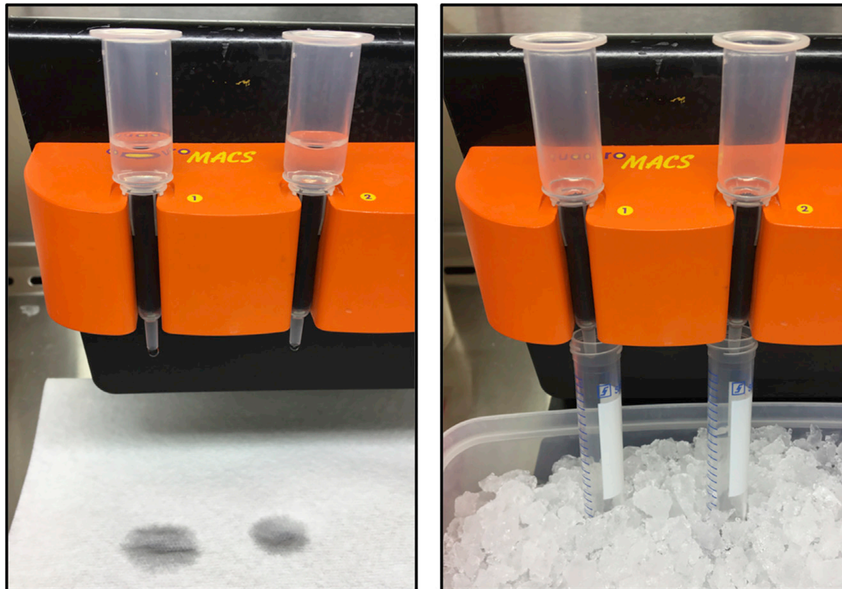

Figure 2. MACS magnet setup for column equilibration and cell collection

13. Label cells for 10–15 min on ice.
14. Add 1 mL MACS buffer for each 1×10^7 cells and spin tube at 400 × *g* for 3 min at 4°C.
    a. (Example: add 4.5 mL MACS buffer onto 45×10^6 cells)
15. While spinning, place LS column in the magnet and equilibrate each column with 3 mL MACS buffer (see Figure 2 example; left).
    a. Ensure buffer has completely passed column.
    b. Place new uncapped 15-mL conical tube under the column (see Figure 2 example; right).

Note: Load up to 1×10^8 cells per LS column to ensure the efficient binding of labeled cells to the column. Spleens can be combined if the total cell number does not exceed this value.

Optional: Placing the collection tubes on ice can decrease cell death.

16. Remove supernatant from anti-CD43 MicroBeads-labeled cell pellet and resuspend the pellet in 500 μL MACS buffer (for up to 1×10^8 cells) and load onto equilibrated column.
    a. Once cells passed the column reservoir, add 6 mL of MACS buffer.
    b. Once buffer passed the column reservoir, add additional 3 mL of MACS buffer.
    c. Solution captured (~9.5 mL) in the 15-mL conical tube contains unlabeled B cells. Labeled non-B cells ($CD43^+$) are bound onto the column in the magnet.

⚠ CRITICAL: Stop here if naive B cells were purified from (unimmunized) naive mice. Determine naive B cell number, wash cells, and use in the desired downstream assay. Also, save a small aliquot for flow cytometry post-purification analysis (for step 31) on ice. Continue with steps below if GC B cell purification from immunized mice is used.

17. Determine purified B cell number and spin tube at 400 × *g* for 3 min at 4°C.
18. Remove supernatant and resuspend 1×10^7 cells in 100 μL MACS buffer (as described in step 11).
19. Add the following biotin-conjugated antibodies per 1×10^7 cells and adjust the concentration/volumes to the total B cell number accordingly:
    a. 4 μL (0.2 μg) of diluted anti-CD38-biotin (dilute stock at 0.5 μg/μL **1:10** in PBS to 0.05 μg/μL).

   b. 2 μL (0.01 μg) of diluted anti-CD11c-biotin (dilute stock at 0.5 μg/μL **1:100** in PBS to 0.005 μg/μL).
   c. Gently shake or flick the tube.
20. Label cells for 10–15 min on ice.
21. Add 1 mL MACS buffer for each 1×10^7 cells and spin tube at 400 × *g* for 3 min at 4°C.
22. Remove supernatant and resuspend each 1×10^7 cells in 100 μL MACS buffer.
23. Add 20 μL of anti-biotin MicroBeads for each 1×10^7 cells (adjust total volume to the total B cell number accordingly).
   a. Gently shake or flick the tube.
24. Label cells for 10 min on ice.
25. Add 1 mL MACS buffer for each 1×10^7 cells and spin tube at 400 × *g* for 3 min at 4°C.
26. While spinning, place a new LS column in the magnet and equilibrate MACS LS column with 3 mL MACS buffer (as described in step 15).
27. Remove supernatant from anti-biotin MicroBeads-labeled cell pellet and resuspend pellet in 500 μL MACS buffer and load onto the newly equilibrated column (as described in step 16).

Note: Alternatively, GC B cell purification steps can be combined into a 1-step protocol loaded onto a single column. For this, white blood cells are resuspended (step 11) and are labeled together with anti-CD43 MicroBeads (as used in step 12), anti-CD38-biotin and anti-CD11c-biotin (as used in step 19) for 15 min on ice. After this, cells are washed (step 21). The cells are then labeled with anti-biotin MicroBeads, washed and loaded onto a single column (steps 22–27). This leads to the purification of GC B cells as described below.

28. Once the buffer has completely passed the column, place screwcap onto the 15-mL conical tube containing the purified GC B cells and spin at 400 × *g* for 3–5 min at 4°C.
29. Remove supernatant leaving a small residual volume of ~50 μL to not disturb the pellet, resuspend GC B cell pellet in 1 mL cold PBS and transfer the GC B cell suspension into a 1.5-mL Microcentrifuge tube (prechilled on ice).

Note: GC B cell pellet may be barely visible.

30. Determine the cell number of GC B cells.
   a. Save a small aliquot for flow cytometry post-purification analysis (for step 31) on ice.

Purity analysis by flow cytometry

Timing: ~30 min

These steps lay out how to analyze pre-sort total splenocytes and the purity of purified B cells and/or GC B cells by flow cytometry.

31. Aliquots of total splenocytes, purified B cells and/or GC B cells (step 8 and 16 and/or 30) are transferred to 1.1-mL Mini tubes (or 5-mL Round bottom tubes).
   a. Use up to 1×10^6 cells per stain. For GC B cells, low input may be used but ideally a minimum of 5×10^4 cells should be used.
   b. Add 1 mL of Staining buffer to the sample tubes.
32. Spin at 400 × *g* for 3–5 min at 4°C.
33. While spinning, make an antibody staining master mix for 50 μL per stain with the following antibody dilutions:
   a. 0.5 μL (0.25 μg) of anti-CD16/CD32 Fc Block (0.5 μg/μL; final dilution 1:100).
   b. 0.5 μL (0.1 μg) of anti-B220-APC-eFluor780 (0.2 μg/μL; final dilution 1:100).
   c. 0.1 μL (0.05 μg) of anti-GL7-FITC (0.5 μg/μL; final dilution 1:500).
   d. 0.5 μL (0.1 μg) of anti-FAS-PE-Cy7 (0.2 μg/μL; final dilution 1:100).

e. (Example: For 6 stains, in a 1.5-mL tube add 300 μL Staining buffer, 3 μL anti-CD16/CD32, 3 μL anti-B220-APC-eFluor780, 0.6 μL anti-GL7-FITC and 3 μL anti-FAS-PE-Cy7.)

Optional: Different antibody conjugations or combinations of (GC) B cell markers may be suitable based on the specification of the flow cytometer used. Please refer to a spectral viewer tool (such as BD Spectrum Viewer) for easy setup and compatibility of the flow panel. The settings in this protocol are based on a BD FACSCanto flow cytometer equipped with 488 nm blue and 633 nm red lasers, which allows for multicolor analysis of up to six fluorescent markers and two scatter parameters. For B cell purity analysis from naive animals, markers of GC B cells (GL7 and FAS) are not required.

34. Carefully aspirate the supernatant and scratch the 1.1-mL Mini tubes over the rack to disturb the pellet. Add ~50 μL of the staining solution made in step 33 to each sample tube. Vortex quickly.
35. Incubate cells for 15 min on ice in the dark.

Note: While staining the cells, make single compensation controls using Compensation Beads with the following combinations 1) unstained, 2) anti-B220-APC-eFluor780, 3) anti-GL7-FITC and 4) anti-FAS-PE-Cy7. For this, add one small drop of Compensation Beads into 1.1-mL tubes and add ~1 μL of indicated antibodies to each compensation control. Stain for 5–10 min in the dark and wash with 1 mL staining buffer once staining of the cells (above) is completed.

36. Add 1 mL Staining buffer and spin cells at 400 × *g* for 3–5 min at 4°C.
37. Carefully aspirate supernatant of tubes and resuspend in Staining buffer corresponding to the cell number (~500 μL for 1 × 10^6 cells; add 50 μL to the compensation controls).
    a. 1.1-mL Mini tubes are placed in a 5-mL Round bottom tube to properly seal and be placed in position for the Sample Injection Port (SIP) of the flow cytometer. Remove 1.1-mL Mini tube between samples from the 5-mL Round bottom tube.

Note: A viability dye such as 7-AAD allows for efficient exclusion of dead cells in addition to FSC/SSC gating. For this, resuspend the stained cell sample (step 37) in 100 μL Staining buffer and add 5 μL 7-AAD. Incubate cells 5–10 min on ice in the dark and then add ~400 μL Staining buffer and continue with the steps below. (Please note that unstained cells incubated with 7-AAD are required as a compensation control).

38. For first time acquisition of this stain on a flow cytometer:
    a. Adjust FSC and SSC (and other channels) with unstained cell sample.
    b. Set compensation based on single compensation controls.
    c. FMO (Fluorescence Minus One) controls can help with gating strategies.
39. Acquire samples on flow cytometer and analyze purities in FlowJo.

EXPECTED OUTCOMES

Total B cell purification (CD43-depletion) should result in purities > 95% (95%–98%) yielding ~15–35 × 10^6 cells per spleen. The purified B cell number is roughly half of the initial total white blood cell number. After immunization (7 days), the expected range of purity for GC B cells is 85%–91% (Figure 3) and the expected yield is ~0.5 × 10^6 cells per spleen.

LIMITATIONS

This protocol works best when robust GC responses are induced. Generally, best results are achieved at the peak of the GC response. Early or late GC B cells may not be as efficiently purified using this protocol. Relatedly, when using experimental animals where GC responses are compromised (such as knockout animals in essential genes of the GC response), the purity and yield may be reduced.

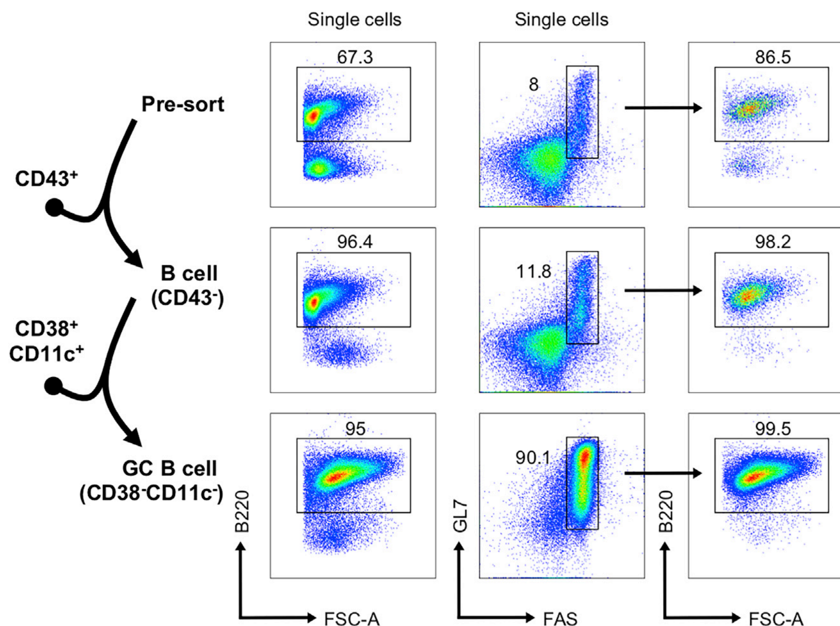

Figure 3. Purity analysis of B cell and GC B cell enriched cell populations by flow cytometry

The use of MACS in this protocol can achieve high purities for naive B cells comparable to purification by FACS. The GC B cell purities in this protocol are high, albeit they are lower than FACS purities. Purification by FACS is required in situations where the highest purities of GC B cells or sorting of GC B cell subpopulations (i.e., light zone or dark zone GC B cells) are desired. However, in some instances the enrichment by MACS can complement FACS to aid in faster sorting times.

For simplicity and efficiency, untouched naive B cells or GC B cells are purified from naive or immunized mice, respectively. If untouched B cells are required from the same mouse from which untouched GC B cells are purified, please refer to (Cato et al., 2011) for details.

The spleen is the largest peripheral lymphoid organ hosting a high quantity of B cells including GC B cells upon immunization. Therefore, the spleen is an ideal source for the purification of these cell populations. Lymph nodes also host B cells and GC B cells, but are smaller in size and thus can be more challenging to efficiently purify GC B cells for a wide array of downstream assays.

This protocol can yield sufficient GC B cell numbers for many downstream assays including genomic, epigenomic, and transcriptomic analysis. The purified GC B cell numbers would also allow for certain proteomic analyses and some functional assays. The need of high GC B cell numbers for certain experimental assays (such as immunoblotting) can require large numbers of mice. However, experimental animals need to be used ethically and animal numbers should be reduced wherever possible. A reasonable alternative can be *in vitro*-derived GC B cells (originally described by (Nojima et al., 2011)). These cells recapitulate some major functions of *in vivo*-derived GC B cells and can be investigated without the need of large animal numbers. Starting with 0.5×10^6 naive B cells can yield > 50×10^6 *in vitro*-derived GC B cells after a few days. New observations tested in these cells can then be validated in purified *in vivo*-derived GC B cells as previously outlined (Ramezani-Rad et al., 2020).

TROUBLESHOOTING

Problem 1

No GC/immune reaction is observed

Potential solution

The GC B cell percentage after SRBC immunization in wild-type mice should be > 4% (within the B cell gate) on day 7 post-immunization. SRBCs should be used as close as possible to the draw date. Renew SRBC batch if response is low. Intravenous injection can lead to more robust responses in the spleen compared to intraperitoneal injection.

Problem 2

Low cell viability

Potential solution

The following aspects are critical to ensure high cell viability:

- GC B cells are more fragile than naive B cell population. On the day of the spleen harvest, work fast and keep cells cold throughout the procedures.
- Gently dissociate spleen, do not use excessive force during dissociation, which can lead to cell death.
  - This protocol uses mechanical dissociation due to the advantages over enzymatic dissociation associated specifically for this protocol. Mechanical dissociation of spleens can sufficiently liberate lymphocytes, avoids enzymatic reactions (i.e., collagenase), which can affect surface molecules, and most importantly is much quicker and therefore easier to utilize.
- During red blood cell lysis, do not incubate cells in ACK buffer longer than indicated as this can decrease the viability of B cells.

Problem 3

Cells do not pass the column/column is clogged

Potential solution

Columns can clog due to the following reasons:

- Overloading the column with too many cells. LS columns allow for up to 1×10^8 labeled cells to bind to the column.
- Cell clumps obstruct the column. Ensure cell suspensions are filtered properly and no clumps are visible.
- Air bubbles in the column. Carefully load the cells and the MACS buffer onto the column avoiding the introduction of air bubbles into the column. Degas MACS buffer before use.

Problem 4

Low GC B cell purity

Potential solution

There are potentially several underlying problems for this to occur:

- Make sure the immunogen induces robust GC responses (see also Problem 1).
- Ensure red blood cell lysis works efficiently to avoid carryover of red blood cell impurities.
- For novice users, ensure the B cell purification step works efficiently and thus all other MACS steps including reagents, work flawlessly. B cell enrichment (CD43-depletion) purity needs to be at least 95% to fall within the acceptable GC B cell purity range (see Expected outcomes). Lower purities of total B cell purification will carryover the impurities and affect GC B cell purity.
- Make sure correct antibody clones and concentrations are used.
- The use of CD43, CD38, and CD11c antibodies should be sufficient for high GC B cell purities. However, impurities may be (further) decreased by addition of low concentrations (0.01 µg per 1×10^7 cells) of anti-Ter119-biotin, anti-Gr1-biotin, and/or anti-CD138-biotin. Additional use of antibodies can however affect the yield.

Problem 5

Low GC B cell yield

Potential solution

Proper (yet gentle) dissociation of spleens is paramount for maximum cell recovery. Optionally, combine spleens up to 1×10^8 cells for greater GC B cell number. Generally, no more than two spleens should be combined for GC B cell purification. Alternatively, samples can be pooled after GC B cell purification.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Parham Ramezani-Rad (prad@sbp.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

P.R.R. designed, performed, optimized, and wrote the protocol. R.C.R. secured funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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