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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.
Protocol
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.
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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### Reagents or Resources

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse anti-biotin MicroBeads | Miltenyi Biotec | Cat# 130-090-485, RRID:AB_244365 |

### Biological Samples

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Citrated sheep red blood cells | Colorado Serum Company | Cat# 31102 |

### Chemicals, Peptides, and Recombinant Proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Ammonium chloride (NH₄Cl) | Sigma | Cat# A0171 |
| Potassium bicarbonate (KHCO₃) | Macron Chemicals | Cat# 6748-03 |
| Ethylenediaminetetraacetic acid (EDTA), disodium salt (C₁₀H₁₈N₂Na₂O₁₀) | Thermo Fisher Scientific | Cat# S25687 |
| Bovine serum albumin (BSA) | RPI | Cat# A30075 |
| Sodium azide (NaN₃) | RPI | Cat# S24080 |
| 1x DPBS | Coming | Cat# 21-031-CV |
| 10x DPBS | Coming | 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) | Coming | Cat# 25-900-Ci |
| 7-AAD | BD Biosciences | Cat# 559925 |

### Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| UltraComp eBeads Compensation Beads | Thermo Fisher Scientific | Cat# 01-2222-42 |

### Experimental Models: Organisms/Strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: wild-type (C57BL/6) | The Jackson Laboratory | Cat# JAX:000664; RRID:IMSR_JAX:000664 |

### Software and Algorithms

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| FlowJo T10 | Becton, Dickinson and Company | https://www.flowjo.com/ |
| FACS Diva | 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

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 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 | Coming | 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 | Hauser 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 |

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**MATERIALS AND EQUIPMENT**

| 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 |

**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 |
| ddH₂O | n/a | see below |
| **Total** | n/a | 1,000 mL |

Dissolve ammonium chloride, potassium bicarbonate, and disodium EDTA in 800 mL ddH₂O 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 ddH₂O. 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 |
| ddH₂O | 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 ddH₂O on a magnetic stirrer while adjusting the pH to 8 and then q.s. to 100 mL with ddH₂O (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 |
| ddH₂O                    | 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 ddH₂O (store at 20°–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 CO₂ 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°–25°C (room temperature) to lyse red blood cells.
   a. Add 7 mL cold PBS to dilute ACK buffer and quench the lysis.
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.
13. Label cells for 10–15 min on ice.
14. Add 1 mL MACS buffer for each $1 \times 10^7$ cells and spin tube at $400 \times g$ for 3 min at 4°C.
   a. (Example: add 4.5 mL MACS buffer onto $45 \times 10^6$ 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 $1 \times 10^8$ 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 $1 \times 10^8$ 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 $400 \times g$ for 3 min at 4°C.
18. Remove supernatant and resuspend $1 \times 10^7$ cells in 100 µL MACS buffer (as described in step 11).
19. Add the following biotin-conjugated antibodies per $1 \times 10^7$ 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 × 10⁷ cells and spin tube at 400 × g for 3 min at 4°C.

22. Remove supernatant and resuspend each 1 × 10⁷ cells in 100 µL MACS buffer.

23. Add 20 µL of anti-biotin MicroBeads for each 1 × 10⁷ cells (adjust total volume to the total B cell number accordingly).

a. Gently shake or flick the tube.

24. Label cells for 10–15 min on ice.

25. Add 1 mL MACS buffer for each 1 × 10⁷ 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 Micro-centrifuge 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 × 10⁶ cells per stain. For GC B cells, low input may be used but ideally a minimum of 5 × 10⁴ 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 × 10⁶ cells; add 50 μL to the compensation controls).
   a. 1.1-mL Mini tubes are placed in a 5-mL Roundbottom 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 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 × 10⁶ 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 × 10⁶ 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.
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 x 10^6 naive B cells can yield > 5 x 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

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

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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 x 10^6 naive B cells can yield > 5 x 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).
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 $1 \times 10^8$ 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 $1 \times 10^7$ 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 $1 \times 10^8$ 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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