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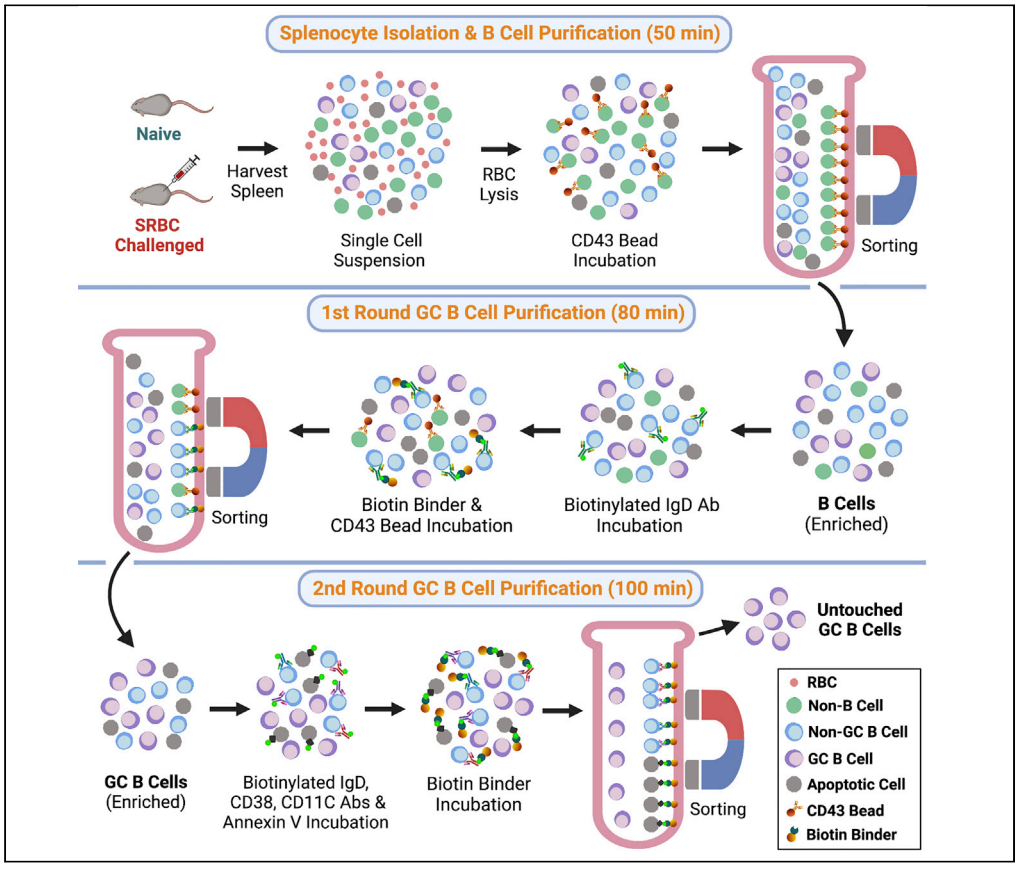
Protocol

A MACS protocol for purification of untouched germinal center B cells from unimmunized or germinal center-induced mice



Highly enriched germinal center (GC) B cell populations are essential for studying humoral immunity. Current MACS protocols that isolate untouched GC B cells require GC induction and typically require further FACS purification with direct antibody labeling to achieve sufficiently high purities. We present a MACS protocol with progressive and repeated negative selections that yields highly purified untouched GC B cells from both unimmunized and GC-induced mice and allows further FACS isolation of unlabeled GC B cells from remaining debris by scatter.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Shreya A. Desikan, Sailee Chavan, Peiqi Ou, Christopher A.J. Roman, Chongmin Huan

chongmin.huan@downstate.edu

Highlights

Isolates highly enriched untouched GC B cells from both naive and immunized mice

Increases GC B cell purities by progressive and repeated negative selection steps

Minimizes cell loss by diminishing DNA-mediated nonspecific adherence to beads

Enables FACS isolation of unlabeled GC B cells by scatter for removing cell debris

Desikan et al., STAR Protocols 3, 101388
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101388

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A MACS protocol for purification of untouched germinal center B cells from unimmunized or germinal center-induced mice

Shreya A. Desikan,[1] Sailee Chavan,[1] Peiqi Ou,[1,2] Christopher A.J. Roman,[3] and Chongmin Huan[3,4,5,6,*]

[1]Program in Molecular and Cellular Biology, The School of Graduate Studies, State University of New York (SUNY) Downstate Health Sciences University, Brooklyn, NY 11203, USA

[2]Department of Oncology, R & D, Amgen Research, Amgen Inc., South San Francisco, CA 94080, USA

[3]Department of Cell Biology, State University of New York (SUNY) Downstate Health Sciences University, Brooklyn, NY 11203, USA

[4]Department of Surgery, State University of New York (SUNY) Downstate Health Sciences University, Brooklyn, NY 11203, USA

[5]Technical contact

[6]Lead contact

*Correspondence: chongmin.huan@downstate.edu
https://doi.org/10.1016/j.xpro.2022.101388

SUMMARY

Highly enriched germinal center (GC) B cell populations are essential for studying humoral immunity. Current MACS protocols that isolate untouched GC B cells require GC induction and typically require further FACS purification with direct antibody labeling to achieve sufficiently high purities. We present a MACS protocol with progressive and repeated negative selections that yields highly purified untouched GC B cells from both unimmunized and GC-induced mice and allows further FACS isolation of unlabeled GC B cells from remaining debris by scatter.

BEFORE YOU BEGIN

Fluorescence-activated cell sorting (FACS) remains a standard method for isolating highly enriched mouse GC B cells. However, using FACS to isolate large numbers of GC B cells is time-consuming and causes cell stress. Furthermore, while forward and side scatter can identify cells by cell size and granularity respectively, scatter cannot be used to discriminate GC B cells from other cell types. For FACS, GC B cells need to be labeled by fluorescent conjugated antibodies, such as anti-Fas antibody, which may impair cell activity and viability (Goillot et al., 1997; Wise et al., 2013).

Unlike FACS, magnetic-activated cell sorting (MACS) provides a means for fast bulk isolation of unlabeled GC B cells from mouse spleens by negative selection of antibody-labeled non-GC splenocytes. However, current MACS protocols cannot be used for mice that have limited GC formation because they require sheep red blood cell (SRBC) challenge to induce robust GC responses. Therefore, the use of current MACS protocols is limited if GC formation is irresponsive to SRBC challenge due to gene mutations, or SRBC challenge affects the intended downstream studies. In addition, compared to FACS-purified GC B cells, the purities of MACS-isolated untouched GC B cells from immunized mice are relatively low (Ramezani-Rad and Rickert, 2021), which often necessitates further enrichment by FACS via antibody labeling of MACS-enriched GC B cells, depending on the application (Ou et al., 2021).

Built upon published MACS protocols (Cato et al., 2011; Ramezani-Rad and Rickert, 2021), we have developed a MACS protocol with innovative changes to enable isolation of GC B cells of high purity from both naive and immunized mice. More specifically, we first eliminated the need for a separation column in the existing protocols by using the Dynabeads system (Invitrogen) to increase ease and scalability. Secondly, we added DNase to the incubation buffers to prevent non-specific cell adherence to magnetic beads,

making the isolation of GC B cells more reproducible. Thirdly, we maximized the effectiveness of MACS by splitting the isolation process into 3 negative selection steps that progressively deplete non-B cells and non-GC B cells at each step, including repeated selection steps against CD43 and IgD. Finally, we introduced a step of Annexin V-mediated negative selection to enhance viability of isolated GC B cells. We found that this protocol can reliably isolate a 96%–98% pure B cell population that contains $\geq$ 98% GC B cells from both naive and immunized mice (Figure 1). Average numbers of GC B cells isolated from 2.5 × 10^7 splenocytes are about 9,000 in naive mice and 15,000 in immunized mice. If higher yield is desired, a 4–7-fold increase of GC B cell numbers can be achieved by skipping Annexin V treatment (Figure 2). In addition, it is possible to exclude debris by fast FACS isolation of unlabeled GC B cells, because following MACS purification, the remaining population in the forward and side scatter lymphocyte gate is almost exclusively GC B cells. For example, further enrichment by FACS may reduce debris made up of cell membranes, which otherwise may interfere with the analysis of cell membrane proteins and lipids in the isolated GC B cells. On the other hand, further FACS may not be required for applications such as cell culture or cell activity assay.

Institutional permissions

All animal experiments in this protocol were approved by the Institutional Animal Care and Use Committee of SUNY Downstate Health Sciences University. Please note that an institutional approval of animal use must be acquired before starting to use this protocol.

GC-induction

This step details the stimulation of GC formation in mice before in preparation for isolation of GC B cells.

> *Note:* This step applies only to immunized mice. If using this protocol for unchallenged (naive) mice, proceed to the next step and harvest spleens from mice on the day of the experiment.

1. Preparation and intraperitoneal injection of SRBCs have been performed as described in literature (Cato et al., 2011; Ramezani-Rad and Rickert, 2021).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotin Rat Anti-Mouse CD43 (S7) (1:50 dilution)	BD Pharmingen	Cat# 553269 RRID: AB_2255226
Anti-Mouse IgD (11-26c) Biotin (1:25 and 1:50 dilution)	Thermo Fisher Scientific	Cat# 13-5993-82, RRID: AB_466860
Anti-Mouse CD38 (90) Biotin (1:50 dilution)	Thermo Fisher Scientific	Cat# 13-0381-82 RRID: AB_466428
Anti-Mouse CD11c (N418) Biotin (1:50 dilution)	Thermo Fisher Scientific	Cat# 13-0114-82 RRID: AB_466363
Anti-Mouse TER119 (TER-119) Biotin (1:60 dilution)	Thermo Fisher Scientific	Cat# 13-5921-82 RRID: AB_466797
Anti-Mouse Ly-6G/Ly-6C (Gr-1) (RB6-8C5) Biotin (1:60 dilution)	Thermo Fisher Scientific	Cat#: 13-5931-82 RRID: AB_466800
Anti-Hu/Mo GL7 (GL-7) AF488 (1:100 dilution)	Thermo Fisher Scientific	Cat# 53-5902-82 RRID: AB_2016717
PE Hamster Anti-Mouse CD95 (Jo2) (1:100 dilution)	BD Pharmingen	Cat#: 554258 RRID: AB_395330
FITC Rat Anti-Mouse T- and B- Cell Activation Antigen (GL7) (1:100 dilution)	BD Pharmingen	Cat# 562080 RRID: AB_394981
PE-Cy5 Rat Anti-Mouse CD45R/B220 (RA3-6B2) (1:100 dilution)	BD Pharmingen	Cat# 553091 RRID: AB_394621
Biological samples		
Citrated Sheep Red Blood Cells	Colorado Serum Company	Cat# 31102

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Biotin Annexin V (1:50 dilution)	BD Pharmingen	Cat# 556417 RRID: AB_2869070
FITC Annexin V (1:100 dilution)	BD Pharmingen	Cat# BDB556420
PE Annexin V (1:100 dilution)	BioLegend	Cat# 640947
APC Annexin V (1:100 dilution)	BD Pharmingen	Cat# 561012 RRID: AB_2868885
Propidium Iodide Staining Solution (1:100 dilution)	BD Pharmingen	Cat# 556463 RRID: AB_2869075
7-AAD Viability Staining Solution (1:100 dilution)	eBioscience	Cat# 00-6993-50
Annexin V Binding Buffer, 10× concentrate	BD Pharmingen	Cat# 556454 RRID: AB_2869074
Dynabeads Mouse CD43 (Untouched B cells)	Invitrogen	Cat# 11422D
Dynabeads Biotin Binder	Invitrogen	Cat# 11047
DNase I	Roche	Cat# 10104159001
Bovine Serum Albumin (BSA)	Fisher Scientific	Cat# BP1605-100
Fetal Bovine Serum (Heat Inactivated)	Gibco	Ref# 10082-147
PBS pH 7.4 (1×) (-$CaCl_2$) (-$MgCl_2$)	Gibco	Ref# 10010-031
eBioscience 1× RBC Lysis Buffer	Invitrogen	Ref# 00-4333-57
HEPES Buffer, 1 M Solution	Fisher	Cat# BP299-100
Ammonium Chloride	Fisher	Cat# A661-500
Potassium Bicarbonate	Fisher	Cat# P235-500
Sodium Chloride	Fisher	Cat# BP358-10
Magnesium Chloride	Fisher	Cat# M33-500
Calcium Chloride Dihydrate	Sigma	Cat# C5080
Critical commercial assays		
Debris Removal Solution	Miltenyi Biotec	Cat# 130-109-398
Experimental models: Organisms/strains		
Mouse: 3 to 6-month-old male and female wild type C57BL/6	In house breeding, originally purchased from JAX lab	N/A
Mouse: 4-6-month-old female NZBWF1	JAX lab	Strain#: 100008 RRID: IMSR_JAX:100008
Software and algorithms		
NovoExpress	Agilent	N/A
Excel	Microsoft	N/A
PowerPoint	Microsoft	N/A
Illustration	BioRender	N/A
Other		
Magnetic Particle Concentrator (Dynal MPC-L)	Dynal	Product# 120.21
Novocyte	Agilent	N/A
Eppendorf Centrifuge 5417R	Eppendorf	N/A
Eppendorf Centrifuge 5430R	Eppendorf	N/A
Sorvall ST 16R Centrifuge	Thermo Scientific	N/A
Frosted Microscope Slides	Fisher Scientific	Cat# 12-550-343
Cell Strainer 70 μm Nylon	Corning Incorporated	Ref# 352350
Labquake Rotator/Shaker	Barnstead Thermolyne	Model 40011

MATERIALS AND EQUIPMENT

10× Annexin V Stock Solution

Reagent	Final concentration	Amount
HEPES 1 M pH 7.3	0.1 M	5 mL
NaCl	1.4 M	4.09 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
$CaCl_2.H_2O$	25 mM	183.75 mg
ddH_20	n/a	45 mL
Total		**50 mL**

Storage at –4°C, discard if solution begins to precipitate.

Alternatives: Commercial alternatives for 10× Annexin V Buffer, such as 10× Annexin V Binding Buffer, BD Bioscience, Cat#: 556454, can be used.

Note: The following quantities are for isolating GC B cells from approximate 2.5 × 10^7 splenocytes, which can be harvested from $^1/_2$ of the spleen (≈38 mg) of a naive mouse or $^1/_4$ of the spleen (≈21 mg) of an immunized mouse (3 to 6-month-old male and female C57BL/6); adjust volumes accordingly when working with different spleen sizes. Make the buffers fresh on experiment day and keep them on ice for the duration of the experiment.

CD43 Beads Buffer		
Reagent	Final concentration	Amount
PBS (-Ca, -Mg) 1×	n/a	1,960 µL
BSA (20%)	0.1%	10 µL
$MgCl_2$ (25 mM)	0.25 mM	20 µL
DNase I (10 mg/mL)	0.05 mg/mL	10 µL
Total		**2,000 µL**

1× Annexin V Buffer		
Reagent	Final concentration	Amount
10× Annexin V Stock Solution	1×	120 µL
ddH_20	n/a	1,080 µL
Total		**1,200 µL**

Annexin V Beads Buffer		
Reagent	Final concentration	Amount
10× Annexin V Buffer	1×	90 µL
ddH_20	n/a	792 µL
BSA (20%)	0.1%	4.5 µL
$MgCl_2$ (25 mM)	0.25 mM	9 µL
DNase I (10 mg/mL)	0.05 mg/mL	4.5 µL
Total		**900 µL**

STEP-BY-STEP METHOD DETAILS

Note: This protocol is optimized for isolating GC B cells from 2.5 × 10^7 splenocytes (approximate $^1/_2$ splenocytes of a naive mouse or $^1/_4$ splenocytes of an immunized mouse). The timing is calculated based on the simultaneous processing of two samples. To enhance cell survival, all buffers and reagents should be kept on ice, and all centrifugations and beads incubations should be carried out at 4°C.

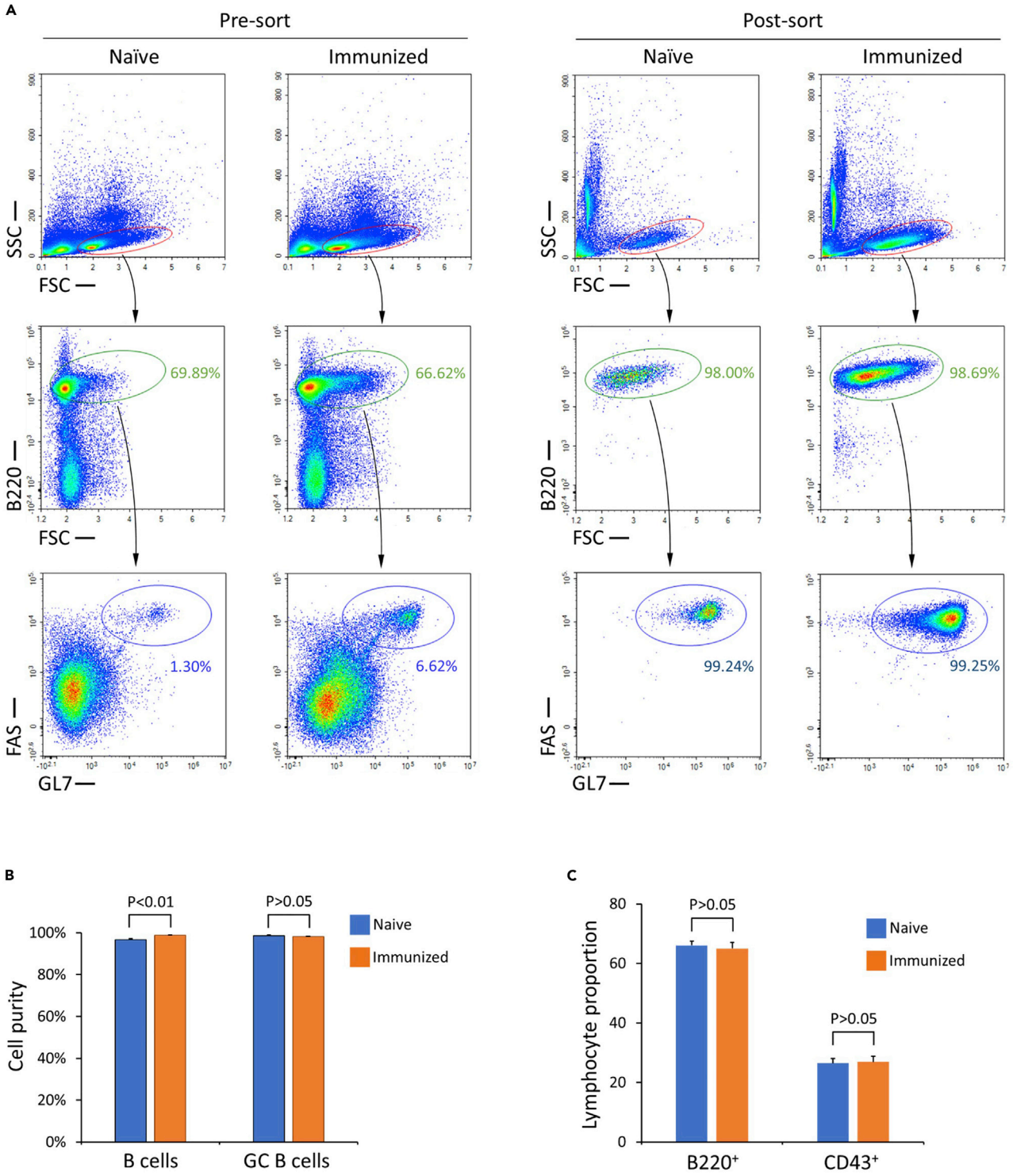
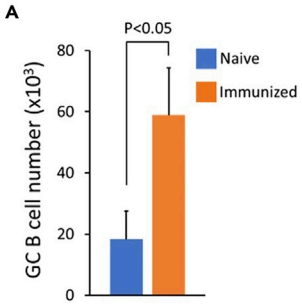


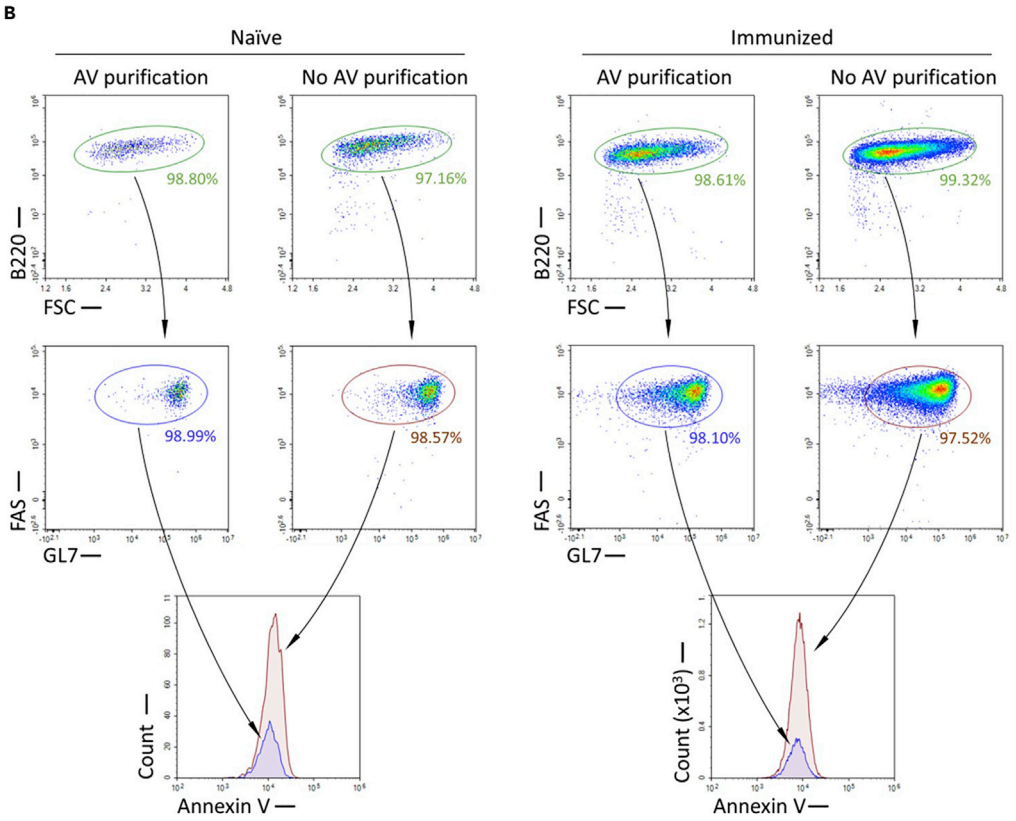
Figure 1. Analysis of GC B cells isolated from naive and immunized mice

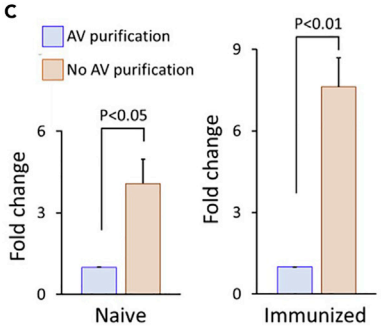
(A) Pre and Post Sort flow cytometry analysis of purified GC B cells from naive mice (left) and immunized mice (right). B cells are identified as $B220^+$, and GC B cells are identified as $B220^+FAS^+GL7^+$.

(B) Bar graph of compiled flow cytometry data depicting means with SEM error bars; 2-tailed T test; n = 8 biological replicates of naive and immunized mice.

(C) Bar graph showing the proportions of $B220^+$ and $CD43^+$ cells prior to sorting in naive and immunized mice, depicting means with SEM error bars; 2-tailed T test; n= 12 biological replicates for immunized mice; n = 13 biological replicates for naive mice.


A
P<0.05
Naive
Immunized
GC B cell number (x10³)
80
60
40
20
0


B
Naïve
Immunized
AV purification
No AV purification
AV purification
No AV purification
98.80%
97.16%
98.61%
99.32%
B220
FSC
98.99%
98.57%
98.10%
97.52%
FAS
GL7
Count
Annexin V
Count (x10³)
Annexin V


C
AV purification
No AV purification
P<0.05
P<0.01
Fold change
Fold change
Naive
Immunized

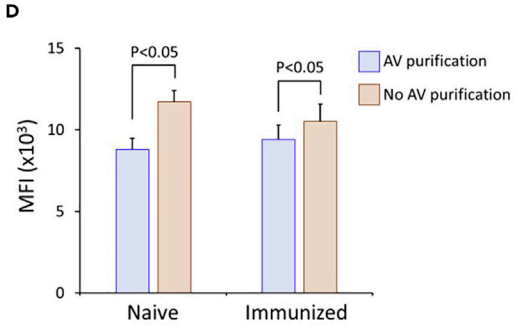

D
P<0.05
P<0.05
AV purification
No AV purification
MFI (x10³)
Naive
Immunized

Figure 2. Yield of GC B cells isolated from naive and immunized mice, and the effect of Annexin V-mediated negative selection on the yield

(A) Bar graph of normalized numbers of isolated GC B cells from one spleen of naive and immunized mice, depicting means with SEM error bars; 2-tailed T test; n= 8 biological replicates for immunized mice; n = 10 biological replicates for naive mice.

(B) Flow cytometry analysis of Annexin V staining intensity of purified GC B cells from naive mice (left) and immunized mice (right). The cells are separated into two halves for the treatment with and without Annexin V right before the step of Annexin V incubation to avoid the variances caused by the isolation steps prior to the Annexin V incubation. B cells are identified as $B220^+$, and GC B cells are identified as $B220^+FAS^+GL7^+$.

(C) Bar graph showing fold changes of isolated GC B cell numbers without Annexin V-mediated negative selection in naive and immunized mice, depicting means with SEM error bars; 2-tailed T test; n = 12 biological replicates for immunized mice; n = 4 biological replicates for naive mice.

(D) Bar graph of median fluorescence intensity (MFI) of Annexin V staining of GC B cells isolated from naive and immunized mice with and without Annexin V-mediated negative selection, depicting means with SEM error bars; 1-tailed T test; n = 10 biological replicates for immunized mice; n = 5 biological replicates for naive mice.

Harvesting splenocytes and removing red blood cells

Timing: 15–20 min

This step includes spleen removal from euthanized mice, splenocyte isolation and RBC lysis.

1. Harvest mouse spleens as reported (Cato et al., 2011; Ramezani-Rad and Rickert, 2021).
2. Scissor the spleen into 4–5 small segments in a 60 mm culture dish containing 1 mL of PBS.

Note: Using FBS supplemented cell culture media may enhance cell survival.

3. Using minimal pressure, gently disassociate splenic tissues between the frosted ends of two glass slides to release splenocytes.

⚠ CRITICAL: Gentle release of splenocytes from fragmented splenic tissues is essential for reducing cell injury and assuring the high purity and yield of isolated GC B cells.

4. Make single-cell suspension by gently pipetting up and down >10 times with a 1 mL pipette.
5. Filter the cells through a 70 μm cell straining and transfer approximate 2.5×10^7 splenocytes (Equivalent to $^1/_4$ of splenocytes harvested from an immunized mouse or $^1/_2$ of splenocytes harvested from a naive mouse) to an Eppendorf tube.

Note: Using $< 2 \times 10^7$ splenocytes may reduce the yield and purity of isolated GC B cell. However, if using $\geq 4 \times 10^7$ splenocytes, we recommend splitting the cells into halves and isolating in two 1.5 mL tubes to avoid the decrease of the yield and purity. Alternatively, the protocol may be scaled up to isolate GC B cells from up to 3×10^8 splenocytes by using 15 mL conical tubes with proportionally increased amounts of antibodies and beads. We also suggest that a new user of this protocol should not handle more than 2 samples simultaneously.

6. Pellet cells by centrifugation at 300 × *g* for 3 min and remove supernatant.
7. Resuspend the pellet in 300 μL 1× RBC Lysis Buffer (eBioscience) and incubate on ice for 60–90 s to remove erythrocytes.

⚠ CRITICAL: Incubation with RBC lysis buffer for over 3 min may increase cell death of lymphocytes.

8. Stop the lysis reaction by washing the splenocytes twice in 1 mL PBS with centrifugation at 300 × *g* for 3 min.

At the same time, wash CD43 Dynabeads (5 min).

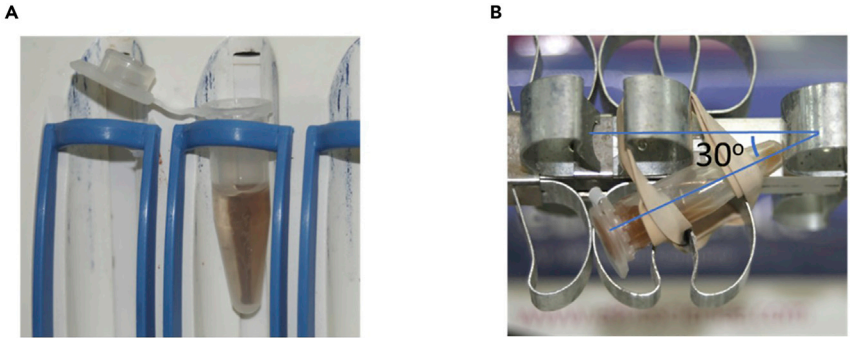


Figure 3. Placement of the Eppendorf tube on the magnet and the rotator for cell sorting

(A) When place the Eppendorf on the magnet, ensure that the tube is closely contacted with the magnet wall. The tube can be kept open for convenience. Keep the tube on the magnet when collecting beads free supernatant.

(B) When place the Eppendorf tube on the rotator for beads incubation, the tube should be positioned with an approximate 30-degree angle to the axis of the rotator to allow smooth and constant movement of the beads in the tube.

a. Transfer 100 μL beads per sample into an Eppendorf tube.
b. Add 1 mL of PBS and resuspend.
c. Place the tube on the magnet for 1 min as shown in Figure 3A.
d. Discard the supernatant.
e. Repeat wash steps b–d.
f. Resuspend in 200 μL **CD43 Beads Buffer.**

9. Resuspend the pellet in 600 μL of **CD43 Beads Buffer.**

⚠ CRITICAL: Without addition of DNase or $MgCl_2$ in the beads buffer, B cells may clump/have nonspecific binding to beads due to genomic DNA present from cell injury, resulting in the loss of cells.

B cell purification by negative selection of $CD43^+$ cells

Timing: 30–35 min

This step details the enrichment of the B cell population by removal of $CD43^+$ cells.

10. Add 160 μL of pre-washed CD43 beads to the cell suspension and gently mix.
11. Place the tube on the rotator as shown in Figure 3B, rotate and tilt the tube at 4°C for 20 min.
12. Remove the tube and resuspend the bead-bound cells by pipetting 10 times to limit trapping of unbound B cells.
13. Place the tube on the magnet for 2 min as shown in Figure 3A.
14. Transfer the supernatant containing the unbound B cells to a new tube.
15. Pellet cells by centrifugation at 300 × *g* for 3 min and resuspend in 50 μL **CD43 Beads Buffer** containing 3% FBS.

1st round GC B cell purification by negative selection of IgD^+ cells and $CD43^+$ cells

Timing: 75–80 min

This step details the first round of enrichment of the GC B cell population by removal of IgD^+ cells and additional removal step of $CD43^+$ cells.

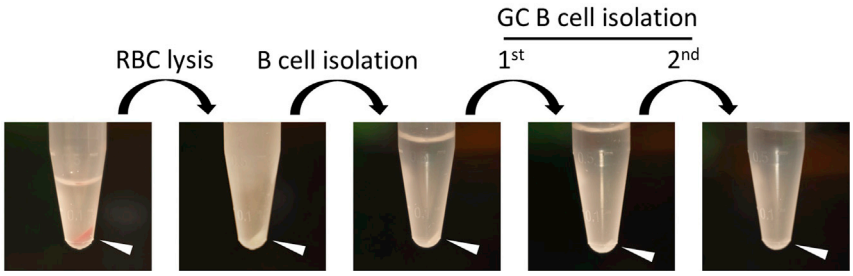


Figure 4. Changes of cell pellet during GC B cell purification

Representative cell pellets at each step of the isolation of GC B cells from 1/4 spleen of SRBC-challenged mice. Cell pellets are indicated by white arrows. Please note that when purifying GC B cells from 1/2 spleen of a naive mouse, cell pellets may become invisible at the stages of GC B cell isolation.

16. Add 2 μL biotinylated anti-IgD antibody, mix and incubate on ice for 20 min.

At the same time, prepare Biotin Binder beads (5 min).

a. Transfer 150 μL beads into an Eppendorf tube.
b. Add 1 mL of PBS and resuspend.
c. Place tube on magnet for 1 min.
d. Remove supernatant.
e. Repeat wash steps b–d.
f. Resuspend the beads in 150 μL **CD43 Beads Buffer.**

17. Wash cells twice in 600 μL PBS by centrifugation at 300 × *g* for 3 min and resuspend cells in 600 μL **CD43 Beads Buffer.**
18. Add 150 μL pre-washed Biotin Binder beads and 40 μL pre-washed CD43 beads, gently mix.
19. Place the tube on the rotator as shown in Figure 3B, rotate and tilt the tube at 4°C for 20 min.
20. Remove the tube and resuspend the bead-bound cells by pipetting 10 times to limit trapping of unbound GC B cells.
21. Place the tube on magnet for 2 min as shown in Figure 3A.
22. Transfer the supernatant containing unbound GC B cells to a new tube.

Note: Wash steps after this point in the protocol are performed at 350 × *g* for 5 min to minimize cell loss, as the pellet size is now significantly reduced as shown in Figure 4.

23. Pellet cells at 350 × *g* for 5 min and resuspend in 50 μL **CD43 Beads Buffer.**

2nd round GC B cell purification by negative selection of IgD$^+$, CD11c$^+$, CD38$^+$ cells

Timing: 90–100 min

This step details the second round of GC B cell purification by negative selection of IgD$^+$, CD11c$^+$, CD38$^+$, and Annexin V$^+$ cells.

24. Add 1 μL each of biotinylated IgD, CD11c, and CD38 antibodies, mix and incubate on ice for 15–20 min.

Optional: 0.8 μL of Ter119 and Gr1 antibodies may be added at this step to remove additional non-B cells and enhance purity further.

Note: Skipping the following steps 25 and 26 of Annexin V incubation and proceed to step 27 may dramatically increase yield of isolated GC B cells without decreasing the cell purity but

may increase Annexin V$^+$ GC B cells (Figure 2). This approach can be used if a higher yield is desired, and when the cells undergoing apoptosis do not significantly affect the downstream experiments.

25. Wash with 600 μL PBS by centrifugation at 350 × *g* for 5 min and resuspend in 50 μL **Annexin V Beads Buffer**.

⚠ CRITICAL: All following steps should be conducted in Annexin V Beads Buffer or Annexin V Buffer to ensure optimal binding of biotinylated Annexin V with cells undergoing apoptosis.

26. Add 1 μL biotinylated Annexin V, mix and incubate on ice for 15 min.

⚠ CRITICAL: Incubation with biotinylated Annexin V for an extended period (beyond 15 min) may result in a lower yield of GC B cells.

Note: If skipping the Annexin V incubation, use **CD43 Beads Buffer** and **PBS** to replace **Annexin V Beads Buffer** and **Annexin V Buffer** respectively in the following steps.

At the same time, prepare Biotin Binder beads (5 min):

a. Transfer 100 μL beads into an Eppendorf tube.
b. Add 1 mL of PBS and resuspend.
c. Place the tube on magnet for 1 min.
d. Remove supernatant.
e. Repeat wash steps b–d.
f. Resuspend in 100 μL **Annexin V Beads Buffer** (Use **CD43 Beads Buffer** instead if skipping Annexin V incubation).

27. Wash cells twice with 600 μL 1× **Annexin V Buffer** (Use **PBS** instead if skipping Annexin V incubation) by centrifugation at 350 × *g* for 5 min and resuspend in 600 μL **Annexin V Beads Buffer** (Use **CD43 Beads Buffer** instead if skipping Annexin V incubation).
28. Add 100 μL pre-washed Biotin Binder beads, gently mix.
29. Place the tube on the rotator, rotate and tilt the tube at 4°C for 20 min.
30. Remove the tube and resuspend the bead-bound cells by pipetting 10 times to limit trapping of unbound GC B cells.
31. Place the tube on magnet for 2 min and transfer unbound GC B cells to a new tube.

Note: Purified GC B cells are now ready for downstream applications including flow cytometry.

EXPECTED OUTCOMES

As shown in Figure 1, this protocol can yield highly enriched, viable and untouched GC B cells from both naive and immunized mice. More than 96% cells isolated from naive mice are B cells, in which ≥ 98% are untouched GC B cells. Similarly, more than 98% cells isolated from immunized mice are B cells, in which ≥ 98% are untouched GC B cells. Therefore, the purities of untouched GC B cells isolated by this protocol are comparable to the purities of FACS-isolated antibody-labeled GC B cells. Please note that the gating sequence used in the flow cytometry analysis here parallels the steps of GC B cell isolation and is different from that used in the published protocol (Cato et al., 2011; Ramezani-Rad and Rickert, 2021). However, this does not affect the results of purity assay (Figure S1). Interestingly, although the proportions of GC B cells in total B cells isolated from naive and immunized mice are almost identical, we noticed that the purities of B cells isolated from naive mice are slightly lower than that from immunized mice with a statistical significance ($p = 0.003$) (Figure 1B), despite that the

proportions of $B220^+$ B cells and $CD43^+$ non-B cells in the splenocytes appear to be comparable between naive mice and immunized mice (Figure 1C).

As shown in Figure 2A, expected average numbers of GC B cells isolated from 2.5×10^7 splenocytes are about 9,000 in naive mice and 15,000 in immunized mice. As described in the step-by-step methods, yield can be increased 4-fold in naive mice and 7-fold in immunized mice by omission of the Annexin V incubation step (Figures 2B and 2C). While omission of this step will not affect cell purities, it can result in increased staining intensity of Annexin V in the isolated GC B cell sample, as shown in Figures 2B and 2D. However, even without the negative selection by Annexin V incubation, non-viable cells (PI^+ or $7\text{-}AAD^+$) are nearly absent in isolated GC B cells (Figure S2). This approach should be considered when the effects of GC B cells undergoing apoptosis on the downstream experiments are negligible or can be excluded by designed control samples.

In addition to C57BL/6 mice, this protocol can be used to isolate GC B cells from other strains. As shown in Figure S2, B cells and GC B cells isolated from NZBWF1 mice without SRBC challenge have similar purities to those isolated from C57BL/6 mice (Figure 1).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details of experiments can be found in the figure legend. Data were analyzed using Excel. One- or Two-tailed T test was used to compare the two groups. Results were presented as mean + SEM and statistical significance was accepted at $p < 0.05$.

LIMITATIONS

Due to the nature of negative selection, the enrichment process itself cannot remove the cell debris generated during the procedure. If cell debris could affect downstream molecular and biochemical assays, such as those analyzing cell membranes, we recommend excluding the debris from untouched GC B cells by FACS, but without antibody labeling. This is feasible because following this MACS purification, the dominant cell population resolved by forward and side scatter is composed of nearly 100% GC B cells (Figure 1A), which can be cleanly gated for FACS isolation from debris with different scatter properties. Alternatively, density gradient centrifugation, such as the Debris Removal Solution (Miltenyi Biotec), can be used to partially remove cell debris, but may cause some loss of yield and death of purified GC B cells.

In addition, this protocol uses three rounds of negative selection to progressively and repeatedly remove different types of unwanted cells, which takes more time to complete compared to the existing MACS protocols (Cato et al., 2011; Ramezani-Rad and Rickert, 2021). We believe that progressive and repeated depletions are essential for increasing the efficiency of GC B cell purification in this protocol. Progressive depletion can increase bead-to-cell ratio at the following depletion steps, which can more effectively remove unwanted cells. Repeated depletion is necessary for isolating cells of higher purities. This is because once the maximum depletion efficiency is reached with a certain concentration of Dynabeads, it cannot be further increased to remove residual unwanted cells by using more beads (Dyer et al., 1998). However, in contrast to these existing protocols that use column-based MACS separation, the Dynabeads system used in this protocol does not require column purification, which makes it convenient and straightforward to scale up all reagents and volumes when working with a whole spleen or multiple mouse spleens. For example, Eppendorf tubes can be replaced by 15 mL conical tubes based on the size of samples being used, a method that has been used by members of our research group. In addition, MACS allows for the processing of multiple samples simultaneously. Therefore, this protocol can be easily adapted for the efficient isolation of large numbers of untouched GC B cells from multiple samples.

Finally, despite the effectiveness of this protocol for GC B cell isolation from naive mice, low GC B cell abundance in naive mice may affect the purity and recovery. For GC B cell isolation from mice

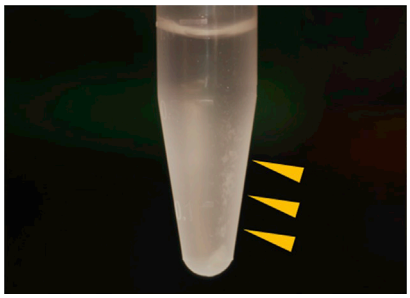


Figure 5. An example of cells attached on the tube wall
Cells on the wall are indicated by yellow arrows. Please note that the cells attached on the wall become invisible at the stages of GC B cell isolation. Always collect cells attached on the tube wall by gently washing even they are invisible.

with less than 0.5% of GC B cells in total splenic B cells, it may be necessary to scale up the sample size, pool cells from the same or different mice following the first round of GC B cell purification, and/or skip the step of Annexin V removal.

TROUBLESHOOTING

Problem 1

Low yield of GC B cells. This can be due to very low proportions of GC B cells in the starting splenocytes, harsh dissociation of splenic tissues and RBC lysis, DNA contamination, the trap of unbound B cells and GC B cells in the bead aggregates when using the magnet to sort cells, and failure to collect the cells attached to the inner wall of the Eppendorf tube (Figure 5).

Potential solution

Skip Annexin V negative selection step (steps 25 and 26). Scale up the starting splenocytes, reagents and volumes (step 5). Keep all buffers and reagents cold. Always process the samples on ice or at 4°C. Cut the spleen into 4–5 pieces before gently dissociating splenic tissues (steps 2 and 3). Gently resuspend splenocytes to ensure single-cell suspension (step 4). Do not incubate with RBC lysis buffer longer than 3 min (step 7). Add sufficient DNase and $MgCl_2$ to digest genomic DNA released from injured cells. Pipet up and down at least ten times following the beads incubation and before sorting with the magnet. This may prevent unbound GC B cells from being trapped in the fraction of bead aggregates (steps 12, 20, and 30). Gently wash the inner wall of the tube after centrifugation to collect the cells attached to the wall (steps 15, 23, and 25).

Problem 2

High contamination of $B220^-$ or $B220^{low}$ cells in isolated B cell population as shown in Figure 6. This can be due to the insufficient removal of $CD43^+$ splenocytes because the number of splenocytes exceeded bead capacity.

Potential solution

Sufficient RBC lysis can avoid the interference of MACS isolation by RBCs (step 7). Deplete $CD43^+$ cells twice by repeatedly using CD43 beads or by CD43 beads and CD43 antibodies (steps 10 and 18). Use CD38, CD11c, Ter119, and Gr-1 antibodies to further remove non-B cells in 2^{nd} round GC B cell isolation (step 24).

Problem 3

High contamination of non-GC B cells in isolated GC B cells as shown in Figure 6. This can result from inadequate removal of IgD^+ and IgD^{low} B cells, plasmablasts, and plasma cells.

Potential solution

Ensure to remove IgD^+ B cells after the depletion of non-B cells (step 16). Reinforce the removal of IgD^+ B cells by repeatedly using IgD antibody in 2^{nd} round GC B cell isolation (step 24). CD38 and CD11C antibodies may help deplete IgD^{low} B cells, plasmablasts, and other non-GC B cells (step 24).

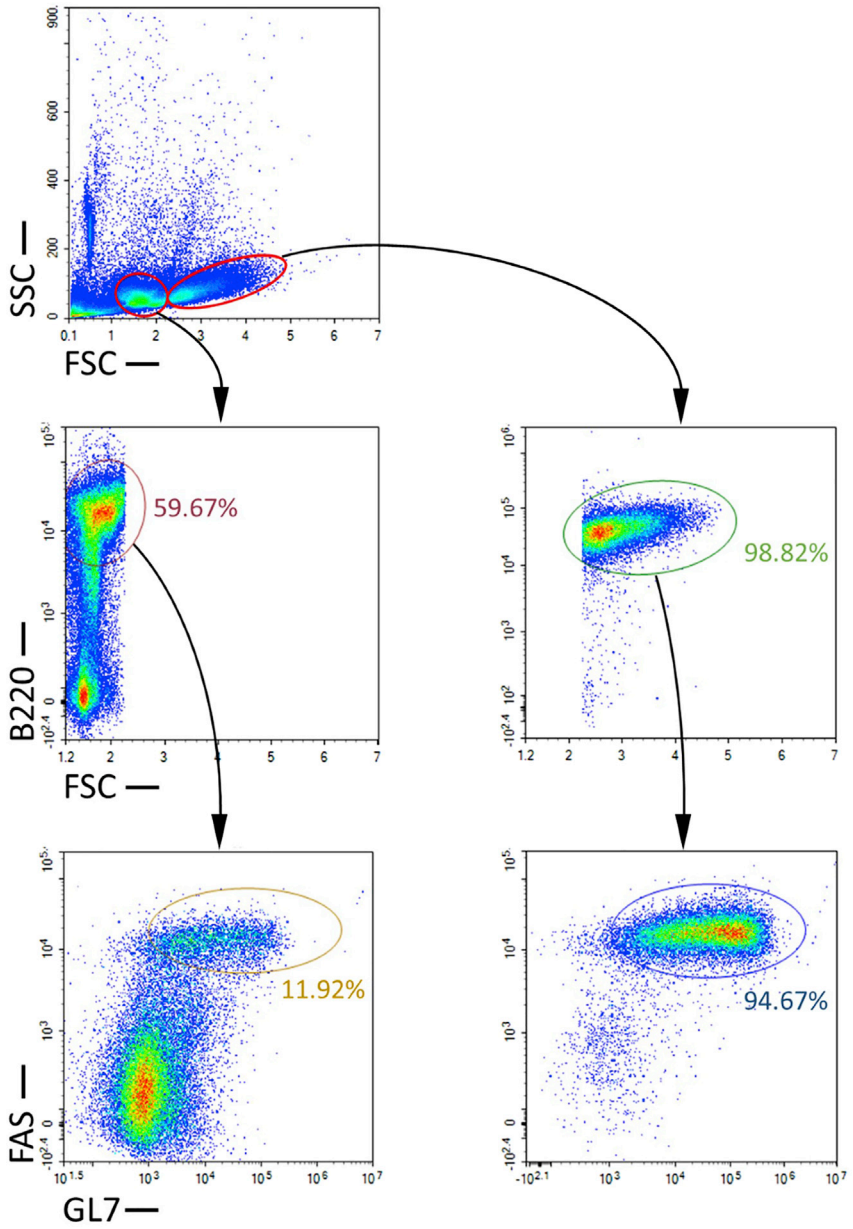


Figure 6. An example of insufficient depletion of non-B cells and non-GC B cells
In flow cytometry analysis, B cells are identified as $B220^{+}$, and GC B cell are identified as $B220^{+}FAS^{+}GL7^{+}$.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Chongmin Huan (chongmin.huan@downstate.edu).

Materials availability

This study did not generate new reagents.

Data and code availability

This study did not generate or analyze any datasets. No code is available.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101388.

ACKNOWLEDGMENTS

We thank A. Schwartzman, L. Dresner, and the Department of Surgery and Cell Biology (SUNY Downstate) for the support; J.A. Edwards for the discussion; and C. Mueller for technical support. This study was supported by DoD Lupus Research Program (Concept Award, log no. LR190106) to C.H. Institutional permissions: All animal experiments in this protocol were approved by the Institutional Animal Care and Use Committee of SUNY Downstate Health Sciences University. Please note that an institutional approval of animal use must be acquired before starting to use this protocol.

AUTHOR CONTRIBUTIONS

C.H. conceptualized research with input from S.A.D.; C.H. designed the experiments in conjunction with S.A.D., S.C., and P.O.; S.A.D. and C.H. implemented and conducted most of the experimental work in conjunction with S.C. and P.O.; S.A.D. analyzed the data with input from C.H.; S.A.D., C.H., and C.A.J.R. wrote the manuscript with input from S.C. and P.O.; and C.H. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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