# Magnetic-based purification of untouched mouse germinal center B cells for *ex vivo* manipulation and biochemical analysis

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**Detailed biochemical analysis of unmanipulated germinal center (GC) B cells has not been achieved. Previously, we designed and used a simple, economical and new magnetic bead separation scheme for the purification of 'untouched' mature GC and non-GC B cells from the spleens of immunized mice and reported the first biochemical assessment of the signaling cascades that contribute to cyclin D stability and GC B cell proliferation. Here we provide a detailed protocol for the method we used, which involves preparing single-cell suspension from the spleens of immunized mice, followed by labeling of nontarget cells with biotinylated antibodies specific for CD43, CD11c and IgD (for GC enrichment) or GL7 (for non-GC enrichment); these steps are followed by cell depletion using standard magnetic bead technology. This protocol can yield GC and non-GC B cells with purities exceeding 90%. The sorting process can be carried out in ~1 h and provides a population of GC B cells of sufficient purity and quantity to allow *ex vivo* manipulation, including biochemical and genetic analysis as well as cell culture.**



## INTRODUCTION

A potent adaptive immune response requires the differentiation of B cells into Ig class-switched memory B cells bearing high-affinity antigen (Ag) receptors and plasma cells (PCs) secreting high-affinity antibody (Ab). The generation of these cells occurs in secondary lymphoid tissues within transient structures known as germinal centers (GCs). In addition to its role in normal humoral immunity, the GC has a critical role in lymphomagenesis, with the majority of B cell lymphomas thought to be GC or post-GC derived. As such, understanding how cellular signal-transduction pathways and genetic programs regulate GC B cell differentiation is of great importance not only to our understanding of adaptive immunity but also as a basis for understanding B cell lymphoma. Although our understanding of GC function has been greatly expanded through classic histological, flow cytometric and, more recently, advanced *in vivo* imaging approaches, a detailed understanding of the molecular cues directing GC B cell fate can only be obtained through biochemical analyses of these cells *ex vivo*[1].

Although several cell-sorting methodologies are currently available, and mouse models for the induction and identification of GCs are well established, two main factors have contributed to the difficulty in GC B cell isolation: a relatively low frequency *in vivo* and poor viability during and after sorting. Following immunization with a T cell–dependent Ag, such as a hapten-carrier with an adjuvant or heterologous erythrocytes, GC structures begin to form in as few as 3 d and continue to expand over the next several days as additional B cells enter the response and undergo significant bursts of proliferative expansion. Depending on the immunogen, with heterologous erythrocytes yielding the strongest response, the peak of the splenic GC response occurs 6–12 d after immunization[2,3]. During this time, GC B cells account for approximately 5–15% of the B cell pool, which translates to 2–10% of splenic lymphocytes and, typically, to <1% of the total splenocytes[4,5]. Although GC structures may persist for several weeks, the number of GC B cells decreases rapidly, nearly to preimmunization levels, within 1 week after the peak[5]. Further limiting *ex vivo* manipulation and interrogation of regulatory cascades in GC B cells is their poor survival after purification. Previous attempts at *ex vivo* manipulation of GC cells have revealed that a majority of positively sorted mouse GC B cells die in culture within 4 h of isolation[6].

### Development of the protocol

Several positive and negative cell-sorting methodologies have been used over the past several decades. These include panning (positive selection of target, or depletion of nontarget, cells based on binding to Ab- or lectin-coated polystyrene plates), complement-mediated lysis (removal of Ab-labeled nontarget cells by lysis mediated by purified complement proteins), fluorescence-activated cell sorting, commonly known as FACS (flow cytometry–based sorting of cells based on binding, or lack thereof, of fluorescently labeled Ab/Ag) and magnetic-activated cell sorting, commonly known as MACS (positive selection of target cells, or negative selection of target cells by depletion of nontarget cells, on the basis of binding to metal-containing beads and subsequent magnet-based removal). Identification of the GC B cell population requires flow cytometric and/or histological analysis that relies mainly on the identification of the induced surface markers GL7 and FAS and binding to peanut agglutinin, as well as on the lack of surface IgD[7–10]. As a result, most of the previous methods for sorting these rare cells have relied on positive selection of cells or on a combination of depletion and positive selection[11–13]. Inherent in any positive-selection approach is the possibility of altering the normal *in vivo* signaling cascades and gene expression profiles (by ligation of surface Ags during sorting), which may cloud the interpretation of results. Therefore, a reliable negative-selection method is preferable to ensure accurate experimentation and interpretation of results. Several crucial factors affect the success of cell sorting, including purity, yield and maintenance of cell phenotype (including viability). With the intention of maximizing these factors, while using readily available reagents and

equipment, we developed the procedure detailed herein and applied in our recent report[14]. The method is a simple and fast yet reliable magnetic bead–based negative-selection scheme that allows the purification of untouched mature GC and non-GC B cells from the spleens of sheep red blood cell (SRBC)-immunized mice (**Fig. 1**). This protocol yields GC and non-GC B cells of 85–90% purity or greater. The sorting process can be carried out in ~1 h and yields a population of pure GC B cells in numbers large enough to allow *ex vivo* manipulation, including biochemical analysis. Using this process, we were able to conduct the first biochemical (western blot) analysis of GC B cells, thereby leading to our discovery that the cAMP-PKA-GSK3 pathway is a critical regulator of cyclin D3 stability in GC B cells[14].

### Application of the method

The sorting protocol detailed here is optimized for magnetic sorting of GC and non-GC B cells from the spleen of SRBC-immunized mice for the study of intracellular signal cascades *ex vivo*, using classical biochemical approaches following receptor-mediated stimulation and use of pharmacological inhibitors. Though this protocol utilizes the Miltenyi quadroMACS magnet and LS columns, it is likely to be adaptable to other magnetic technologies. In addition, the protocol should be adaptable to sorting GC B cells induced by a variety of other Ags from the spleen or lymph node. GC B cells purified using this protocol could also be used for additional experimentation including, but not limited to, adoptive transfer (transplantation) into syngeneic hosts, cell culture–based studies including migration and differentiation, gene expression profiling and possibly advanced proteomics.

### Comparison with other methods

**Panning.** The use of panning to purify lymphocytes was first developed more than three decades ago[15]. Although this method has the potential to yield lymphocyte population of 98% purity, adaptation of this protocol to GC purification has proved less useful. Use of peanut agglutinin (PNA) binding to positively select human GC B cells resulted in just 50–80% purity even when a pre-enrichment step involving depletion of T cells and/or $IgD^+$ B cells (by panning or complement-mediated lysis) has been used to improve purification[11,12]. In addition to poor purity, a drawback of this method is low yield, which results from inefficient elution of bound cells from the plate. Therefore, this method does not yield cells of adequate purity or number for subsequent biochemical analysis.

**Complement-mediated lysis.** Complement-mediated lysis is an effective method for depletion of nontarget cells and has the ability to yield populations of cells exceeding 90% purity. It is also a good choice for depleting cells from multiple samples simultaneously or from large volume samples, as the only equipment required are a heated water bath and a centrifuge. However, the process of complement lysis involves incubation of cell samples for several hours at 37 °C. This is not favorable for GC B cells, which have poor viability *ex vivo* when not kept on ice or provided with extracellular stimuli.

**FACS.** The first fluorescence-based sorting of GCs was conducted nearly 30 years ago when a single-color sort was performed to isolate cells based on relative binding of fluorescently labeled PNA[13]. Despite being at the forefront of technology at the time, the experimental yield of just 1,000 GC B cells was certainly inadequate for extensive experimentation. FACS technology has made great advances since its advent in the 1970s, with the most advanced modern sorters capable of measuring 24 fluorescent parameters simultaneously, processing more than 200,000 cellular events per second, and sorting six populations exceeding 95% purity from the input sample (see http://www.bdbiosciences.com/instruments/). Despite this precision and resultant purity, two major drawbacks still exist. The first is availability. Limited access to instrumentation, highly trained operators capable of sorting rare populations and the cost associated with this technology prevent its use by most investigators. The second crucial problem, sorting time, is problematic because of the nature of the GC B cell. As stated, GC B cells have poor viability and rapidly undergo apoptosis *ex vivo*, including during sorting. As a result of the infrequency of GC B cells, long sort times are required to achieve high yield. Although the most advanced modern cell sorters boast sorting rates of 200,000 events per second, it is our experience that increased sorting speeds, especially with rare populations, necessarily result in either lower purity or increased abort rates, thereby resulting in lower yield, increased sort times and the need to use increased numbers of input cells. Therefore, sorting rates of <10,000 events per second are commonly used for sorting rare populations from relatively small starting samples. Despite the use of refrigerated chambers, aseptic collection and high sorting speeds, ~8 h of sorting time would be required to sort the $1 \times 10^7$ GC B cells (our average yield from eight SRBC-immunized mice) typically required for an inhibitor study, even when assuming an acquisition rate of 7,000 events per second and a GC B cell population accounting for 10% of the splenic B cells. The use of older and more commonly available sorters, which typically sort just 1,000 events per second for rare populations requiring high purity, in an experiment in which GC B cells account for 5% of the total B cells, would take nearly 100 h of sorting to obtain $1 \times 10^7$ GC B cells. In addition, sorting non-GC B cells from the same sample would either require the undesirable positive sorting of cells or would require sorting a second independently stained sample. This is in stark contrast to the speed at which GC B cells can be sorted using MACS technology. If a

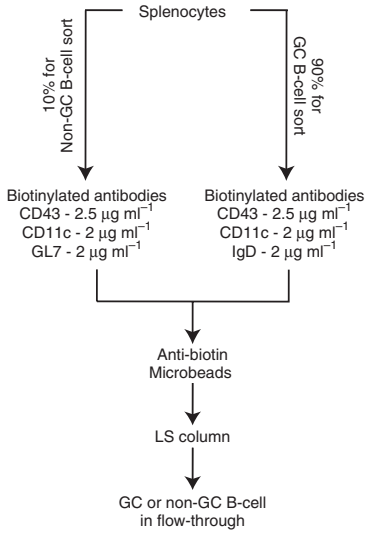


**Figure 1** | Flowchart of Ab labeling and magnetic sorting steps for sorting non-GC and GC B cells.

quadroMACS magnet with LS columns is used, ~$5 \times 10^6$ GC B cells can be sorted every 30 min. Therefore, the decision to use FACS to purify GC B cells depends on the instrumentation available, the number of cells required and the level of purity desired. For these reasons, FACS-based isolation of GC B cells is most appropriate for gene expression profiling, whereas the MACS scheme we developed is better suited for *ex vivo* manipulation and biochemical analysis, which require greater cell numbers.

**Experimental design**

Despite the simplicity of our approach, several considerations must be made during the sorting process. Several critical details including splenic disruption technique, specific Ab-labeling time and concentration and sample handling can have significant effects on the purity and yield of the cell sorting.

The protocol can be divided into five main steps: immunization (GC induction), isolation of tissue and preparation of cell suspension (cell preparation), labeling of cells with Ab-depletion cocktail (Ab labeling), sorting of labeled cells using MACS separator (magnetic sorting) and purity analysis.

**GC induction.** This protocol was optimized using intraperitoneal injection of SRBCs in 129svj and outbred (129svj × C57BL/6) animals. We have found that a fresh preparation of 10% (vol/vol) washed SRBCs in PBS yields the best induction. The quantity of injection should be based on animal-use guidelines; typically 100 µl should be injected. Intravenous injection may induce a more robust splenic GC response and can also be used if desired. We have not evaluated the effect of adjuvants on this protocol. If the use of other immunogens, such as hapten-carrier or protein Ag, which require adjuvant, is desired, then the protocol may require additional optimization. We have not evaluated possible strain-specific variation in the protocol. Similarly, we have not evaluated the protocol for isolation of GC B cells from lymph nodes after subcutaneous or intramuscular injection.

**Cell preparation.** The time of tissue harvest after immunization can have a substantial effect on yield and purity. We have found that harvesting 5–6 d after immunization with SRBC results in the best yield and purity. Tissues should be isolated immediately after euthanizing, placed in medium on ice and kept cold for the remainder of the procedure. Earlier isolation may result in poor yield of GC and poor purity of non-GC B cells, as a large fraction of $IgD^+GL7^-PNA^{hi}$ early GC B cells may be present. Later isolation may also result in low yield or altered proportions of IgG class-switched cells in the preparation, which must be considered depending upon downstream application. As other Ags and adjuvants induce GC response of different kinetics, the harvest time will require optimization if other immunogens are used. We speculate that harvesting on day 8–10 post-immunization with hapten-carrier in alum would yield similar results as those described in this protocol, with the exception of decreased yield. In addition to the time of harvest, the method of tissue dissociation can markedly affect purity. We have found that physical dissociation of spleens between the frosted ends of glass slides yields the best purity. Although dissociation with mesh or filters and/or the use of dissociation agents such as collagenase or pronase may increase yield, we have observed reduced purity with these methods. It is likely that these preparation methods free additional unidentified cell types from the tissue that is not depleted by the Ab cocktail used in this protocol. Addition of Abs specific for these cell types could theoretically be used to improve purity should these cell preparation methods be used. Red blood cells must be removed before magnetic sorting or poor yield will result. RBC lysis should be carried out on ice. This protocol calls for ammonium chloride potassium lysis buffer (ACK) solution; however, commercially available RBC lysis solution may be used according to the manufacturer's guidelines. Centrifugation and density-based removal of RBC (with Ficoll, for example) can be used, but we generally find that these methods reduce yield.

**Ab labeling.** Cells should be kept on ice during staining. The use of biotinylated Abs against CD43, CD11c and IgD should yield a GC B cell population of ~90% purity. The use of biotinylated Abs against CD43, CD11c and GL7 should yield a non-GC B-cell population exceeding 90% purity. We use 90–95% of splenocytes for the GC sort and 5–10% of splenocytes for the non-GC sort, on the basis of the expectation that ~10% of B cells will be of the GC phenotype at the time of isolation. This can be altered depending on the immunogen used. The purity and yield are greatly affected by the Ab-labeling process. The use of incorrect concentration of the Ab in the staining cocktail or inadequate washing following staining may result in poor yield and/or purity. Inadequate washing or the use of too little Ab will result in poor purity, whereas use of too much Ab will result in poor yield. The concentrations stated in the procedure have been empirically determined. Although we utilize biotinylated Abs and anti-biotin microbeads according to the manufacturer's protocols, we have found that the quantity of microbeads used can be reduced with minimal effect on purity and yield. We have used as little as 10 µl of microbeads per $10^7$ splenocytes in 100-µl final volume, with comparable yield and only slight reductions in purity compared with the recommended use of 20 µl. In addition, other methodologies such as the use of fluorescently labeled Ab and anti-fluorophore beads may be used with further optimization. In other sorting trials, we have found the use of streptavidin beads to result in lower purity and yields compared with anti-biotin microbeads and have thus avoided their use here. If isolation of non-GC B cells from the same mouse is not required, the non-GC sorting can be omitted.

**Magnetic sorting.** Although our protocol has been optimized using the Miltenyi midiMACS system with LS columns, we speculate that similar results should be attainable with magnetic depletion systems from other manufacturers. Our protocol is based on the manufacturer's suggested protocol. We load a total of $1 \times 10^8$ splenocytes per column for GC sorting. This is the maximum number of bound cells suggested for LS columns and we assume that greater than 90% of loaded cells will be retained in the column. We have successfully loaded as many as $1.2 \times 10^8$ splenocytes for GC B cell sorting without loss of yield or purity. However, care must be taken to avoid overloading the columns. Exceeding the maximum binding capacity of the column will result in poor purity as a result of bead-labeled cells flowing through the column without being bound, and also poor yield, and the possibility of clogging the column is increased. To minimize loss of cells due to cell death, it is important that MACS buffer be prechilled to 4 °C and kept cold during sorting and also that cells be collected on ice. Cells should be used for downstream application immediately following sorting.

## MATERIALS

### REAGENTS

- Mice, 8 to 12 weeks old (129SvJ/X1 and outbred 129svj × C57BL/6; The Jackson Laboratory, preferred research animal supplier or institutional animal facility) ! CAUTION Approval must be obtained in accordance with the Institutional Animal Care and Use Committee (IACUC).
- Citrated sheep blood (Colorado Serum Company, cat. no. 1102)
- Ethanol (70% (vol/vol))! CAUTION Flammable. Keep away from flame and sources of ignition. Store in dry and well-ventilated areas.
- RPMI 1640 (Mediatech, cat. no. 10-040-CV)
- Penicillin/streptomycin (Mediatech, cat. no. 30-002-CI)
- FBS (HyClone, cat. no. SH30396.03)
- BSA (MP Biomedicals, cat. no. 810531)
- Non-essential amino acids (Mediatech, cat. no. 25-025-CI)
- L-glutamine (Mediatech, cat. no. 25-015-CI)
- 2-mercaptoethanol (Gibco, cat. no. 0927)
- PBS (See REAGENT SETUP)
- NaCl (Fisher Scientific, cat. no. 7647-14-5)
- KCl (Fisher Scientific, cat. no. 7447-40-7)
- $Na_2HPO_4$ (Fisher Scientific, cat. no. 7778-77-0)
- $KHCO_3$ (Fisher Scientific, cat. no. 298-14-6)
- $NaN_3$ (Fisher Scientific, cat. no. 26628-22-8) ! CAUTION Avoid contact and inhalation. Wear gloves, safety glasses and lab coat.
- $Na_2EDTA$ (Fisher Scientific, cat. no. 6381-92-6) ! CAUTION Skin, eye and respiratory irritant. Avoid contact and inhalation. Wear gloves, safety glasses and lab coat.
- $NH_4Cl$ (Sigma, cat. no. 12125-02-9) ! CAUTION Skin, eye and respiratory irritant. Avoid contact and inhalation. Wear gloves, safety glasses and lab coat.
- Anti-biotin MicroBeads (Miltenyi Biotech, cat. no. 130-090-485)
- Biotinylated Abs—CD43 (clone S7—critical for removal of B1 cells; BD Bioscience, cat. no. 553–269), CD11c (eBioscience, cat. no. 13-0114-85), IgD (eBioscience, cat. no. 13-5993-85), GL7 (eBioscience, cat. no. 13-5902)
- Fluorescent Abs—GL7 FITC (BD Bioscience, cat. no. 553-666), Fas/CD95 PE-Cy7 (BD Bioscience, cat. no. 557–653), B220 APC-eFluor780 (eBioscience, cat. no. 47-0452-82), CD3e Allophycocyanin (APC; eBioscience, cat. no. 17-0031-83), Streptavidin PerCP-Cy5.5 (eBioscience, cat. no. 45-4317-82), CD138 Phycoerythrin (BD Bioscience, cat. no. 553-714))
- MACS buffer (See REAGENT SETUP)
- FACS buffer (See REAGENT SETUP)

### EQUIPMENT

- Insulin Syringe (Micro-Fine IV, Becton Dickinson, cat. no. 329-420)
- Dissection tools (surgical scissors, microforceps; Roboz)
- Filter, bottle top (0.2-μm pore size; Fisher Scientific, cat. no. 595-4520)
- Frosted microscope slides (Fisher Scientific, cat. no. 12-550-343)
- Swinging-bucket centrifuge −4 °C (e.g., Allegra X-15 R, Beckman Coulter)
- Hemocytometer/counting chamber (e.g., Hausser Scientific Partnership, cat. no. 3200)
- Microtubes (1.5 ml; Biopioneer, cat. no. CNT-1.5F)
- Falcon tubes (15 ml, Corning, cat. no. 430791)
- Tissue culture 6-well plates (Corning, cat. no. 3506)
- LS columns (Miltenyi Biotech, cat. no. 130-042-401)
- QuadroMACS separator and stand (Miltenyi Biotech, cat. no. 130-090-976)
- Flow cytometer (to analyze purity of sorted cells; any flow cytometer with at least three detectors can be used to analyze purity of sorted cells.)
- Vacuum source

### REAGENT SETUP

**RPMI 1641 medium** RPMI 1641 medium is prepared using 10% (vol/vol) FBS, 1% (vol/vol) Sodium pyruvate, 1% (vol/vol) non-essential amino acids, 1% (vol/vol) penicillin/streptomycin, 1% (vol/vol) L-glutamine and 0.1% (vol/vol) 2-mercaptoethanol. Prepare in advance and store at 4 °C for up to 1 month.

**PBS (1×)** 1× PBS is prepared using 80 g NaCl, 2.0 g KCl, 14.4 g $Na_2HPO_4$, 2.4 g $KH_2PO_4$ and 800 ml $H_2O$. Adjust pH to 7.4 and add $H_2O$ to adjust the volume to 1 liter. Prepare in advance and store at room temperature (21–24 °C) for up to 6 months.

**ACK (ammonium chloride potassium lysis buffer)** Mix 8.29 g $NH_4Cl$ (150 mM), 1.0 g $KHCO_3$ (1 mM), 37.2 mg $Na_2EDTA$ (0.1 mM) and 800 ml $H_2O$. Adjust pH to 7.2–7.4 and add $H_2O$ to adjust the volume to 1 liter. Prepare in advance and store at room temperature for up to 6 months. Use 0.5 ml per spleen.

**MACS buffer** To prepare MACS buffer, mix 5 ml of 0.5 M EDTA stock (5 mM) and 2.5 g BSA, add PBS to adjust the volume to 500 ml, and filter-sterilize and degas the solution. Prepare in advance and store at 4 °C for up to 1 month.

**FACS buffer** Mix 5 ml of FBS and 1 ml of 10% (vol/vol) $NaN_3$; add PBS to adjust the volume to 500 ml. Prepare in advance and store at 4 °C for up to 3 months.

## PROCEDURE

### GC Induction ● TIMING 30 min for animal handling, 5–6 d for GC induction

**1|** Draw up the desired amount of citrated sheep blood into a 15-ml conical tube. Blood typically contains ~50% (vol/vol) cells. A volume of 1 ml of citrated sheep blood yields enough SRBC suspension to immunize at least eight mice.

**2|** Wash by adding 10 volumes of PBS to the tube containing sheep blood, followed by centrifugation at 930*g* at 4 °C for 10 min.

**3|** Remove the supernatant and repeat the wash with PBS (for a total of two washes) to ensure removal of citrate and serum.

**4|** Resuspend the packed SRBC pellet to prepare a 10% (vol/vol) SRBC suspension by adding nine volumes of PBS and pipetting gently or inverting repeatedly until the pellet is completely dispersed.
▲ CRITICAL STEP The SRBC suspension must be used immediately for immunization (Step 5).

**5|** Inject 0.1–0.2 ml (according to IACUC guidelines) of SRBC suspension intraperitoneally into each mouse with an insulin syringe. After injection, house each injected mouse in accordance with IACUC guidelines.

### Cell preparation ● TIMING 30 min

**6|** Kill mice 5–6 d after immunization by $CO_2$ asphyxiation or other approved method.
! CAUTION All animal procedures must be carried out in accordance with IACUC guidelines.

**7|** Harvest spleens using standard dissection techniques in accordance with IACUC guidelines and place them in tissue culture–grade six-well plates in 2 ml of cold RPMI medium and place plates on ice.
▲ **CRITICAL STEP** All buffers, centrifuges and cell suspensions should be kept cold or on ice. This will increase the viability of GC B cells.

**8|** Isolate splenocytes by physically dissociating spleens between the frosted ends of two glass slides that have been previously sterilized by washing in 70% (vol/vol) ethanol and allowed to dry. Rotate the glass slides and gently apply pressure to a section of the spleen until the cells are released from the spleen and repeat on another section of the spleen until all cells have been dissociated from the splenic capsule. Any remaining cells in the splenic capsule can be removed by blasting the capsule with 1 ml of RPMI medium with a pipette until the splenic capsule is no longer red.
▲ **CRITICAL STEP** Physical dissociation of spleens between glass slides yields higher purity than using cell strainers, mesh filters or other methods of preparing single-cell suspensions.

**9|** Wash the cells off the glass slides by pipetting 1–2 ml of RPMI medium onto the slides and allowing the cells to wash into the wells.

**10|** Pipette cells into a 15-ml conical tube, taking care to avoid splenic capsule, fat and clustered debris (optional: cells can be filtered through a mesh or a cell strainer cap at this stage to remove debris).

**11|** Centrifuge at 430*g* at 4 °C for 5 min to pellet cells. Remove the supernatant and resuspend the cells in residual supernatant by gently flicking or raking across a test-tube rack. Avoid vortexing as excessive force may reduce cell viability.

**12|** Incubate cells with ACK (0.5 ml per spleen) on ice for 5 min to remove red blood cells.

**13|** Stop the lysis reaction by addition of cold PBS. Remove settled dead-cell debris with a pipette, or transfer the supernatant that contains splenocytes to a new tube, taking care to avoid transfer of dead-cell debris. Centrifuge at 430*g* at 4 °C for 5 min. If red blood cells remain, repeat Step 12 (optional: cells can be filtered through a mesh or a cell strainer cap at this stage to remove debris).

**14|** Count cells using a hemocytometer counting chamber (or automated counting instrumentation if available) and resuspend in MACs buffer at a final concentration of $10^8$ cells $ml^{-1}$.
▲ **CRITICAL STEP** To ensure high purity, it is important to determine an accurate cell count so that the appropriate quantities of Abs, MACS beads and MACS columns will be used in subsequent steps. Typically, 8 to 10 × $10^7$ total splenocytes are isolated per wild-type mouse.



**Ab labeling** ● **TIMING 1 h**
**15|** (Optional) Set aside 1 to 2 × $10^5$ cells in a microtube on ice for use as the 'presort' group for later flow cytometric assessment of enrichment. This step is optional but may prove useful in troubleshooting in the case of poor yield.

**16|** Allocate, into two separate tubes, 5–10% of total cells for non-GC B cell isolation and 90–95% of total cells for GC B cell isolation.

**17|** Incubate each group of cells with the following biotinylated Abs at 4 °C or on ice for 25 min:

Non-GC B cell—2.5 μg $ml^{-1}$ CD43, 2 μg $ml^{-1}$ GL7 and 2 μg $ml^{-1}$ CD11c
GC B cell—2.5 μg $ml^{-1}$ CD43, 2 μg $ml^{-1}$ IgD and 2 μg $ml^{-1}$ CD11c

**18|** Wash the cell suspensions with 10 volumes of MACS buffer and centrifuge at 430*g* at 4 °C for 5 min. Repeat the wash step again.
▲ **CRITICAL STEP** It is important to wash cells at least twice following Ab labeling. Failure to thoroughly wash away unbound Ab will result in low purity.

**19|** Resuspend the cells in 0.8 ml (or 0.9 ml, if using reduced bead option in Step 20) of MACS buffer for every $10^8$ cells.

**20|** Add 20 µl of anti-biotin microbeads for every $10^7$ cells and incubate for 20 min at 4 °C in the refrigerator (optional: a reduced bead volume of 10 µl may be used to reduce consumption of reagent, although it may slightly reduce purity).

**21|** Wash the cell suspensions with 10 volumes of MACS buffer and centrifuge at 430*g* at 4 °C for 5 min.

**Magnetic sorting ● TIMING 30 min to 1 h**

**22|** Calculate the number of LS columns that should be used, such that only $10^8$ cells are loaded per column. This usually totals ~1 column per mouse.

**23|** While cells are subjected to centrifugation (Step 21), place LS columns on the magnet and prepare LS columns by washing with 3 ml of cold, degassed MACS buffer.

**24|** Remove supernatant from cells in Step 21 and resuspend cells in 1 ml of MACS buffer per $10^8$ cells.

**25|** Load 1 ml of cell suspension per LS column and collect the flow-through in 15-ml conical tubes on ice. The flow-through population will be either the unlabeled non-GC B or GC B fraction.
**▲ CRITICAL STEP** Ensure that only $10^8$ cells per column are loaded. Overloading the column will lead to decreased purity and yield because nontarget cells will not be properly depleted and clogging may occur.

**26|** Wash each column three times with an additional 3 ml of MACS buffer for each wash and collect the flow-through into the same collection tube. Keep the collection tubes on ice while collecting the flow-through population.

**27|** Remove the columns from the MACS separator magnet.

**28|** (Optional) Collect the column-bound fraction into a separate 15-ml conical tube by adding 3 ml of MACS buffer onto the column and flushing by applying the supplied plunger. This step is optional, but it may prove useful for troubleshooting in the case of poor yield or purity, and it can be compared with the 'presort' population for measurement of recovery if desired.

**29|** Count isolated GC and non-GC B-cell fractions, using a hemocytometer counting chamber (or automated counting instrumentation if available). Set aside $10^5$ cells in a microtube on ice for purity analysis.

**30|** Centrifuge isolated cells at 430*g* at 4 °C for 5 min to pellet cells, and remove the supernatant. Resuspend the cell pellet in desired medium at the desired cell concentrations depending on downstream application (typically 1–2 × $10^6$ cells $ml^{-1}$ for short-term cell culture and stimulation).

**Purity analysis ● TIMING 45 min**

**31|** Ensure the purity of sorted cells by flow cytometric analysis. Following the manufacturer's recommended protocol, stain sorted cells with Abs specific to GL7, FAS and B220 in FACS buffer and analyze them by flow cytometry according to the operating guidelines of the instrument used. Fluorescent conjugates of each Ab should be selected on the basis of the instrumentation available for analysis. Additional Abs may be included for troubleshooting purposes if proper instrumentation is available. These include sAV, CD3, CD11c and CD138. However, this is not required for strict assessment of purity. The following staining set is an example of one that may be used, followed by acquisition on a BD FACSCanto (or other similar) instrument: GL7-specific FITC, FAS-specific Pe-Cy7, B220-specific APC-780, CD3-specific APC and streptavidin PerCP-Cy5 (to analyze if any biotinylated cells did not bind anti-biotin MicroBeads). CD11c-specific and CD138-specific Abs may prove useful in troubleshooting, as $CD11c^+$ dendritic cells and $CD138^+$ PCs are the most common contaminants when low purity is observed.
**? TROUBLESHOOTING**

**? TROUBLESHOOTING**
Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

| Problem | Possible reason | Solution |
|---|---|---|
| Low purity | Not enough biotinylated Abs were added during labeling | Ensure that the cell count is accurate, add more biotinylated Abs and repeat Steps 16–30 |
| | Too few anti-biotin MicroBeads were used | Add more anti-biotin MicroBeads and repeat Steps 19–30 |
| | Cells did not bind to LS column | Increase the amount of anti-biotin MACS beads and reload fraction onto a new column |
| | Numbers of cells loaded exceeded LS column binding capacity | Ensure that the cell count is accurate and reload the fraction onto a new column |
| | Washing of unbound Ab was insufficient | Make sure to wash cell suspensions twice with 10 volumes of MACS buffer |
| Low yield | Immunization was suboptimal or cells were harvested at the incorrect time after immunization | Make the SRBC solution fresh<br>Ensure injection of 100 µl<br>Harvest spleens at days 5–6 |
| | Cells were incubated with biotinylated Abs for too long, leading to nonspecific binding | Shorten the Ab incubation time for the next sort |
| | Abs were used at too high a concentration, leading to nonspecific binding | Ensure that the cell count is accurate |
| | Many dead cells | Carry out all procedures with cold reagents and keep cell suspensions in a cool environment at all times |



## ● TIMING

Steps 1–5, GC induction: ~30 min for animal handling, 5–6 d for GC induction
Steps 6–14, Cell preparation: ~30 min
Steps 15–21, Ab labeling (including washing): ~1 h
Steps 22–30, Magnetic sorting: ~30 min to 1 h, depending on starting cell number
Step 31, Purity analysis: ~45 min

## ANTICIPATED RESULTS

Approximately 1 to 2 × $10^6$ GC and non-GC B cells are expected from the spleen of an SRBC immunized mouse after sorting. A total of 90% of the cells collected during the GC sort are expected to be GL7$^+$Fas$^+$ GC B cells (**Fig. 2a**). A total of 95% of the cells collected during the non-GC sort are expected to be GL7$^-$Fas$^-$ non-GC cells, of which >98% are expected to be IgD$^+$ B cells (**Fig. 2a**). In our experience, the majority of contaminants in the GC fraction of a successful sort are non-lymphocytes. In our experiments, unsuccessful GC sorting (i.e., desired purity is not attained) typically results from the use of insufficient Ab concentration during labeling. If CD11c-specific Ab is omitted or used at an incorrect concentration, CD11c$^+$ dendritic cells will typically account for ~80% of contaminating cells in the GC sort (**Fig. 2b**). If CD43-specific Ab is used at the incorrect concentration, CD138$^+$ PCs, which express low levels of CD43 relative to T cells and myeloid cells, may be observed (**Fig. 2b**).

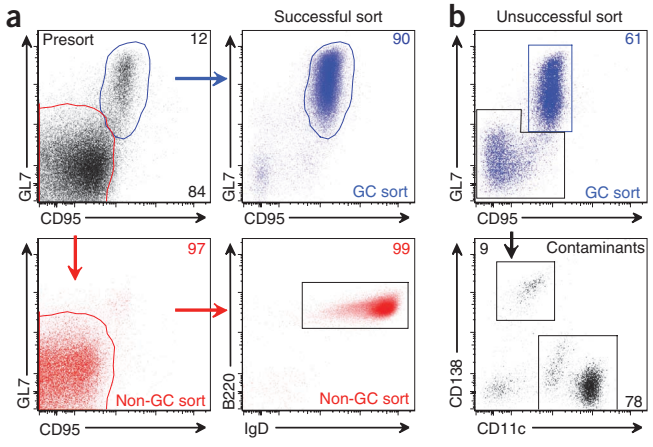


**Figure 2** | Representative flow cytometry plots. (**a**–**b**) A successful sort (**a**), and an unsuccessful sort (**b**). Presorted total splenocytes are shown in black (**a**, upper left). Blue and red gates indicate GC and non-GC populations, respectively, as indicated by GL7 and Fas (CD95) staining (**a**, upper left). Post-sort purity for GCs (**a**, upper right) and non-GCs (**a**, lower left) are indicated as the percentage of total events in the single-cell gate. For non-GCs, B220 and IgD staining confirm that GL7$^-$Fas$^-$ cells are mature B2 cells (**a**, lower right). Frequency of CD11c$^+$ dendritic cells and CD138$^+$ plasma cells are indicated as percentage of GL7$^-$Fas$^-$ non-GCs (**b**, top) from an unsuccessful GC sort (**b**, bottom).

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1. Allen, C.D., Okada, T. & Cyster, J.G. Germinal-center organization and cellular dynamics. *Immunity* **27**, 190–202 (2007).
2. Hanna, M.G. Jr. Nettesheim, P. & Francis, M.W. Requirement for continuous antigenic stimulation in the development and differentiation of antibody-forming cells. The effect of passive antibody on the primary and secondary response. *J. Exp. Med.* **129**, 953–971 (1969).
3. Olson, I.A. The kinetics of germinal center formation in lymph nodes responding to keyhole limpet hemocyanin. *Exp. Mol. Pathol.* **14**, 139–150 (1971).
4. Kraal, G., Hardy, R.R., Gallatin, W.M., Weissman, I.L. & Butcher, E.C. Antigen-induced changes in B cell subsets in lymph nodes: analysis by dual fluorescence flow cytofluorometry. *Eur. J. Immunol.* **16**, 829–834 (1986).
5. Shinall, S.M., Gonzalez-Fernandez, M., Noelle, R.J. & Waldschmidt, T.J. Identification of murine germinal center B cell subsets defined by the expression of surface isotypes and differentiation antigens. *J. Immunol.* **164**, 5729–5738 (2000).
6. Allen, C.D. *et al.* Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat. Immunol.* **5**, 943–952 (2004).
7. Rose, M.L. *et al.* Binding of peanut lectin to germinal-centre cells: a marker for B-cell subsets of follicular lymphoma? *Br. J. Cancer* **44**, 68–74 (1981).
8. Bhan, A.K., Nadler, L.M., Stashenko, P., McCluskey, R.T. & Schlossman, S.F. Stages of B cell differentiation in human lymphoid tissue. *J. Exp. Med.* **154**, 737–749 (1981).
9. Cervenak, L., Magyar, A., Boja, R. & Laszlo, G. Differential expression of GL7 activation antigen on bone marrow B cell subpopulations and peripheral B cells. *Immunol. Lett.* **78**, 89–96 (2001).
10. Watanabe, D., Suda, T. & Nagata, S. Expression of Fas in B cells of the mouse germinal center and Fas-dependent killing of activated B cells. *Int. Immunol.* **7**, 1949–1956 (1995).
11. Coico, R.F., Bhogal, B.S. & Thorbecke, G.J. Relationship of germinal centers in lymphoid tissue to immunologic memory. VI. Transfer of B cell memory with lymph node cells fractionated according to their receptors for peanut agglutinin. *J. Immunol.* **131**, 2254–2257 (1983).
12. Kosco, M.H., Szakal, A.K. & Tew, J.G. In vivo obtained antigen presented by germinal center B cells to T cells in vitro. *J. Immunol.* **140**, 354–360 (1988).
13. Kraal, G., Weissman, I.L. & Butcher, E.C. Germinal centre B cells: antigen specificity and changes in heavy chain class expression. *Nature* **298**, 377–379 (1982).
14. Cato, M.H., Chintalapati, S.K., Yau, I.W., Omori, S.A. & Rickert, R.C. Cyclin D3 is selectively required for proliferative expansion of germinal center B cells. *Mol. Cell Biol.* **31**, 127–37 (2011).
15. Wysocki, L.J. & Sato, V.L. 'Panning' for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA* **75**, 2844–2848 (1978).