We are using a purification of mouse splenic B-cells based on the finding that half of spleen cells are B220 positive, and the other half are either CD43 or Mac-1 positive. Thus doing a CD43 / Mac-1 depletion using magnetic beads results in a over 95% pure population of B-cells, and as all they have been subjected to is passing through a column, they are not activated.

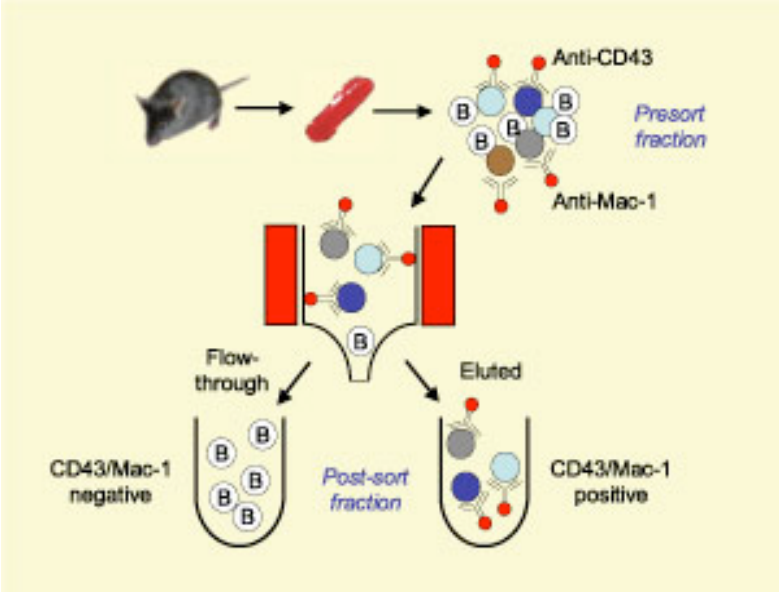
I have been using a standardized protocol from “the Signaling Gateway”, a collaboration of UCSD and Nature publishing. The detailed protocol is posted, as well as a PDF with a description and their yields and quality assessments.

We have been working on optimizing the pre-column manipulations as we find we get a similar yield of cells from spleen, but about 30% their final yield (15-20 million cells per spleen at over 95% purity and 90-95% viability).

Bender

11/2010

Isolation of Splenic B Cells

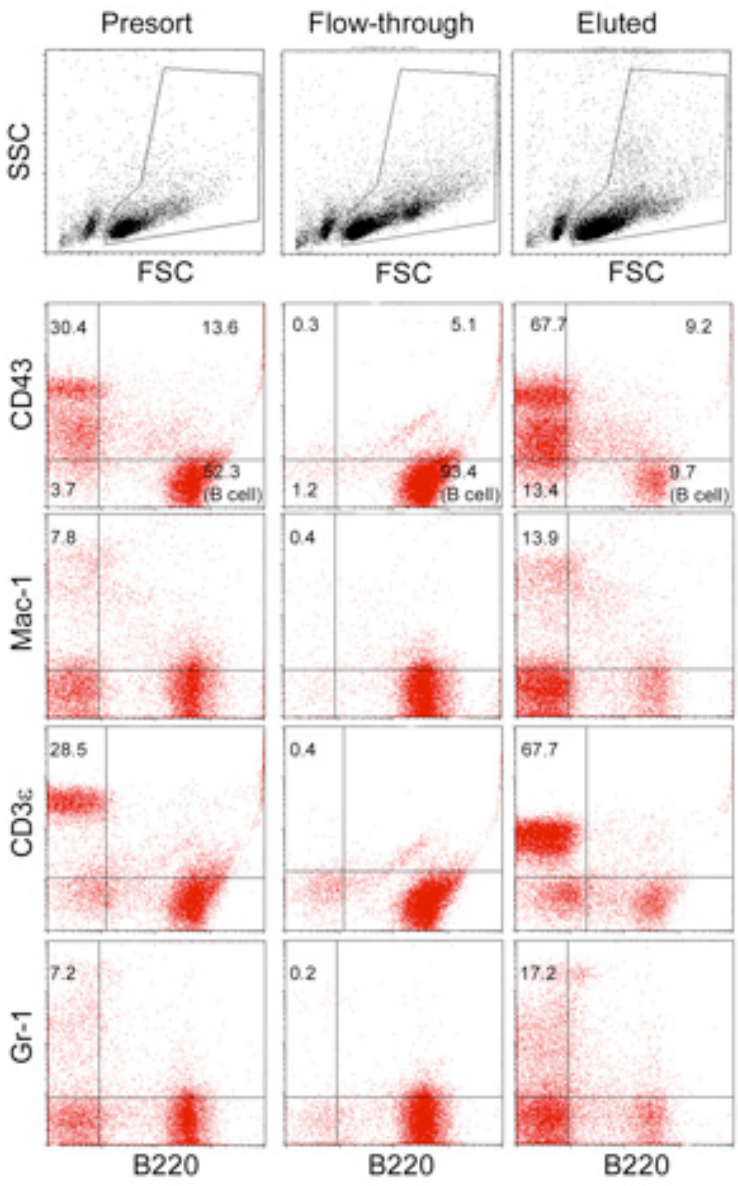


(View larger image)

Fig. 1. **Schematic representation of B-cell purification by negative selection.** Spleens are removed from anesthetized mice and dispersed through a nylon mesh to generate a single-cell suspension. The splenocytes are washed (following removal of erythrocytes by osmotic shock) and incubated with anti-CD43 and anti-Mac-1 antibody-conjugated microbeads (Miltenyi Biotec). The bead-bound cells (positive fraction) are separated from unbound cells (negative fraction) using an AutoMacs magnetic cell sorter. The magnetized column retains the positive fraction while the negative fraction containing resting B cells is collected in the flow-through. The positive fraction is later eluted for examination.

A negative-selection procedure developed by Miltenyi and colleagues (7) was chosen for purification of splenic B cells (Fig. 1). This method relies on the fact that most leukocytes express CD43; resting mature B cells are exceptions. Expression of CD43 has been demonstrated on immature B cells, plasma cells, and some mature B1 cells, in addition to granulocytes, monocytes, macrophages, platelets, natural killer (NK) cells, thymocytes, and peripheral $CD8^{pos}$ and most $CD4^{pos}$ T cells (8, 9, 10, 11, 12). The heterogeneous cell population isolated from spleen was incubated with paramagnetic microbeads coupled to an anti-CD43 monoclonal antibody. To improve the removal of non-B cells (specifically myeloid contaminants), anti-Mac-1 (CD11b) beads were also included during the negative-selection procedure. The beads with unwanted cells attached were then removed using an AutoMACS computer-controlled

magnetic cell sorter (Miltenyi Biotec, Auburn, CA). Resting, mature B cells remain relatively unperturbed during this procedure, and cell activation associated with positive selection is avoided.



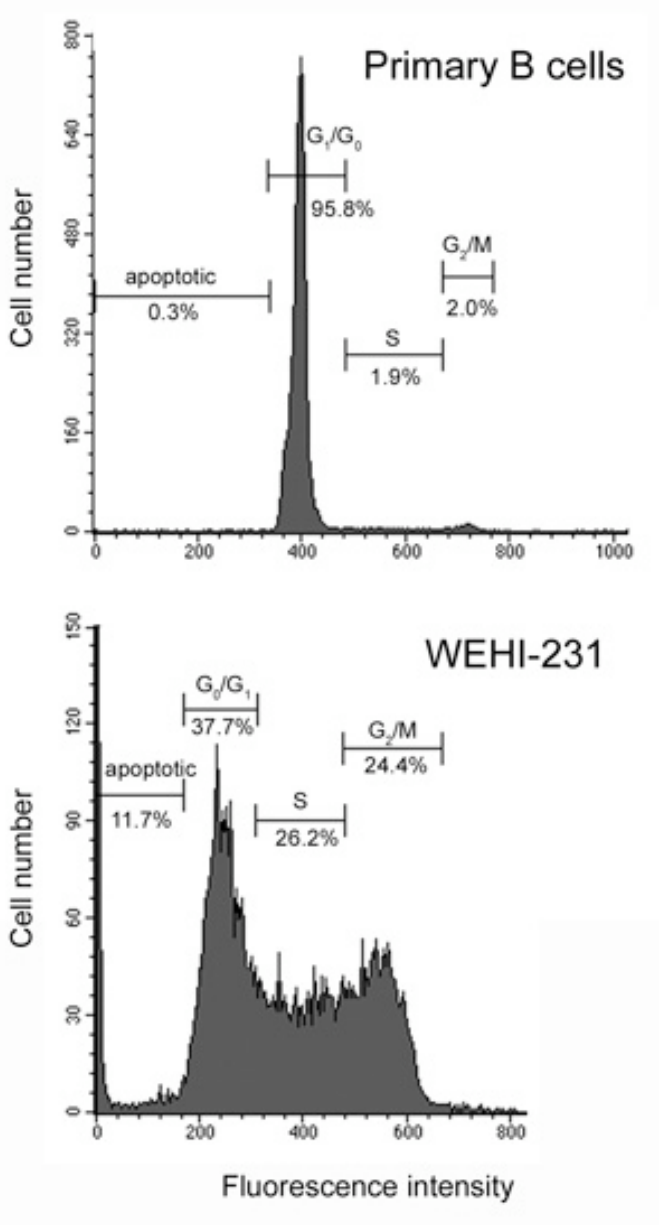
(View larger image)

Fig. 2. **Composition of the cell pools before and after sorting.** The effectiveness of the negative selection was evaluated by four-color FACS analysis using antibodies to CD43, Mac-1, CD3e, Gr-1, and B220. Cells were examined by forward scatter (FSC) and side scatter (SSC) to identify the viable cell fraction (first row). Each plot represents a typical analysis from 20 experiments; the percentage of cells expressing Mac-1 (macrophages), CD3e (T cells), Gr-1 (granulocytes), or B220 (B cells) is shown in the appropriate quadrants. Positioning of the cross-hair gates was done on control plots derived from cells stained only for B220.

The enriched cell population was subjected to multiparameter fluorescence-activated cell sorting (FACS) analysis to estimate the percentage of cells expressing B220, a marker present on cells committed to the B lineage (Fig. 2) (13,

14, 15). Contaminating cell populations that remained after enrichment were examined by staining the leukocyte markers Mac-1, CD43, Gr-1, and CD3 (Fig. 2). The enriched cell fraction contained approximately 47 x 10^6 cells/spleen, of which 96% expressed B220 ($B220^{pos}$) (Table 1). Only small numbers of $CD43^{pos}$/$Mac\text{-}1^{pos}$ cells were present. Blockade of the Fc receptor on splenocytes prior to incubation with magnetic beads did not improve yields significantly (not shown). Further characterization of the isolated cells is described below (see *Cell Characterization*).

To evaluate the proliferative status of the isolated B cells, cellular DNA content was determined by FACS analysis after staining with propidium iodide (Fig. 3)  . More than 95% of the cells resided within the G_0/G_1 phases of the cell cycle. Propidium iodide staining of a proliferating B lymphoma cell line, WEHI-231, is shown for comparison; only 38% of these cells were in G_0/G_1 phases of the cell cycle.



(View larger image)

Fig. 3. **Cell cycle distribution of isolated splenic B cells and a cultured, mouse B-cell lymphoma, WEHI-231**. Freshly isolated splenic B cells and WEHI-231 cells were stained with 1 mg/ml of propidium iodide and examined by FACS. The distribution of cells throughout the cell cycle was estimated from their total DNA content (measured by relative fluorescence intensity). Representative histograms are shown.

The Laboratory for Development of Signaling Assays (Veterans Administration Medical Center, San Francisco) and the Cell Preparation and Analysis Laboratory (University of Texas Southwestern Medical Center, Dallas) both isolate and culture B cells for AfCS experiments. It is essential that we are able to replicate these procedures at these sites. This will ensure that others in the research community can reproduce and extend our observations. The data presented in Table 1 demonstrate that the cell populations isolated in our two laboratories appear very

similar. However, results from the Cell Preparation and Analysis Laboratory demonstrate a consistent trend of obtaining a greater number of starting cells per spleen and consequently greater yields of $B220^{pos}$ cells. We cannot currently offer a clear explanation for this difference.

Table 1. ***Summary of primary B-cell preparations.*** Cells were counted with a hemocytometer under a phase-contrast microscope. Viability was determined by trypan blue exclusion. $B220^{pos}$ cells were quantified by FACS analysis. Data are mean ± SD. The numbers in parentheses are the number of experiments in which the information was quantified. Calculated values are the average of specified determinations. ND=Not determined.

	Cell Prep and Analysis Lab[1]		Assay Development Lab	
Body Weight (g)	ND		25.8 + 2.2	(190)
Spleen Weight (mg)	ND		84.1 + 12.8	(192)
Spleen Weight/Body Weight ($x10^3$)	ND		3.3 + 0.5	(190)
Presort				
Viable (%)	81.9 + 3.6	(19)	79.2 + 5.4	(19)
Cells/Spleen ($x10^6$)	112.0 + 14.0	(19)	98.7 + 16.0	(20)
$B220^{pos}$ (%)	56.1 + 4.5	(19)	62.2 + 5.9	(16)
$B220^{pos}$ Cells/Spleen ($x10^6$)	62.5 + 9.2	(19)	63.3 + 13.9	(16)
Post-Sort Flow-through (B-Cell)		(19)		
Viable (%)	90.4 + 4.0	(19)	95.1 + 1.9	(18)
Cells/Spleen ($x10^6$)	53.7 + 8.2	(19)	41.4 + 6.6	(20)
$B220^{pos}$ (%)	96.2 + 1.6	(19)	96.3 + 2.7	(20)
$B220^{pos}$ Cells/Spleen ($x10^6$)	52.2 + 7.7	(19)	39.8 + 6.4	(20)
Yield (%)*	82.8 + 8.2	(19)	65.4 + 16.8	(18)
Post-Sort Eluted		(19)		
Viable (%)	87.7 + 5.5	(19)	87.7 + 4.5	(7)
Cells/Spleen ($x10^6$)	36.7 + 11.2	(19)	29.7 + 5.9	(8)
$B220^{pos}$ (%)	15.0 + 3.0	(19)	26.6 + 1.7	(17)
$B220^{pos}$ Cells/Spleen ($x10^6$)	5.5 + 2.0	(19)	7.5 + 2.3	(8)

*Yield = (number of $B220^{pos}$ cells in the flow-through)/(number of $B220^{pos}$ cells in the total pool).

[1] All preparations were from 16 mice each.

Isolation of Resting B Lymphocytes from One or More Groups of Four Mouse Spleens

AfCS Procedure Protocol PP00000001
Version 2, 03/24/03

This procedure describes the isolation of resting B lymphocytes (B cells) from mouse spleens by using negative selection with anti-CD43 and anti-Mac-1/CD11b monoclonal antibodies coupled to magnetic microbeads. This strategy depletes non-B cells from a mixed population of splenocytes and relies on the fact that most mature leukocytes, with the exception of resting splenic B cells, express CD43. Anti-Mac-1/CD11b microbeads are included in the negative selection to improve the removal of myeloid cells. The B-cell isolation is automated by using the autoMACS automatic magnetic bead cell sorter (Miltenyi Biotec). As assessed by fluorescence analysis of B220+ cells, the isolation routinely yields approximately 4×10^7 B cells per spleen that are >95% pure. This method is also used for the routine preparation of B cells from sixteen spleens (see AfCS Protocol *Isolation of Resting B Lymphocytes from Sixteen Mouse Spleens*, PP00000016).

Splenectomy

Note: all experiments use male C57BL/6 mice at age 6 to 8 weeks.

1. Sterilize the surgical instruments in a hot bead sterilizer or autoclave.
2. For every group of one to four mice, add 5 ml of chilled (4 °C) magnetic cell sorting buffer (MACS buffer) to a 35 x 10 mm or 100 x 15 mm petri dish. Place a sterile 70-µm nylon mesh cell cup-shaped strainer (35-mm diameter) in each petri dish.
3. Anesthetize the mice (100% CO_2 for 45 to 60 sec in a container containing up to four mice), then sacrifice by cervical dislocation.
4. Place mice on a dissecting board on their right sides and douse the chest and abdominal fur with 70% ethanol.
5. Using surgical scissors, create an incision on the left side of each animal about 2.5 cm in length between the last rib and the hip joint, cutting the skin but not the peritoneal wall. Pull back the fur, exposing the peritoneal wall.
6. With a fresh pair of sterilized surgical scissors, create an incision approximately 2-cm long in the exposed peritoneal wall, in the same orientation as the skin incision. Grasp the spleen using sterilized medium forceps and pull it through the incision in the peritoneal wall. While holding the spleen with the medium forceps, separate the spleen from connective tissue by using fine forceps or scissors.
7. Place no more than four excised spleens on each 70-µm nylon mesh cell strainer immersed in 5 ml of MACS buffer in a 35 x 10 mm petri dish. With sterile sharp-end scissors, cut each spleen into three to six parts.

Preparation of the Splenocyte Cell Suspension

8. Transfer the petri dishes to a tissue culture hood. Squeeze the splenocytes from the splenic capsule through the 70-µm nylon mesh of the cell strainer into the MACS buffer to create a single cell suspension by gently mashing spleen pieces with the rubber end of a plunger from a 1-cc tuberculin syringe.
9. Pipette the 5 ml cell suspension from the petri dish (leaving the sterile mesh for a second processing, step 10). Perform a second filtration by pipetting the 5 ml suspensions of dispersed cells through a fresh 70-µm nylon mesh cell strainer into

50-ml conical tubes (on ice). Cell suspensions from two to eight spleens (in 5 to 10 ml) can be filtered through one nylon mesh cell strainer into a single tube.

10. Add 2.5 ml of chilled MACS buffer to the cell strainers in the petri dishes and further mash the remains of the splenic capsules. Filter and pool the second dispersions of 2.5 ml into the 50-ml conical tubes. Repeat this procedure with additional washes of 2.5 ml until the splenic capsules are white (approximately 3 to 4 more times). Make total volume of cell suspension with washes for first spin up to 20 (1 spleen) to 45 ml (4 spleens) per tube by adding additional MACS buffer.
11. Pellet the cells at 400 x g for 5 min at 4 °C (Eppendorf centrifuge 5804R with a type A-4-44 swinging bucket rotor or J6 centrifuge with a JS 4.2 swinging bucket rotor).
12. Aspirate the supernatant; this leaves about 0.5 ml of cells in MACS buffer in each tube (for 4 spleens). Loosen the cell pellets by stroking the tubes against a rack or by flicking the ends, then add 1 ml/spleen of red blood cell lysis buffer (RBC lysis buffer) at room temperature and gently resuspend the cells. By hand, gently roll the tubes for 2 min at room temperature and then quickly add 30 ml of chilled MACS buffer. Pass the cell suspension through a 70-µm nylon mesh strainer into a new 50-ml conical tube, then use 10 ml of the buffer to rinse the old tube and mesh. The final volume for the wash is 41 to 45 ml/tube for one to four spleens.
13. Collect the cells by centrifugation at 400 x g for 5 min at 4 °C.
14. Aspirate the supernatant entirely, then loosen the cell pellets as in step 12. Gently resuspend the pellet from each tube with iced MACS buffer (**0.2 ml for each spleen** included in the tube).
15. Use a 1-ml pipette to transfer the cell suspensions through a 70-µm nylon mesh strainer into a new 50-ml conical tube. At this point, all cells can be combined into the same tube. Wash the old tubes twice with **0.2 ml MACS buffer/spleen**, and transfer any remaining cells through the strainer into the same conical tube. Final volume is approximately 0.6 ml/spleen (this leaves room for magnetic beads and MACS buffer additions to adjust cells to the correct labeling density, i.e., 10^7/100 µl). Check the final volume of the combined/filtered cell suspension with a P1000 pipette. For multiple spleens, transfer measured portions to a new tube.

Separation of Resting B Cells

16. For counting cells in approximately 0.6 ml MACS buffer/spleen, make a 1/100 final dilution. For example, for four spleens/2.4 ml of buffer, mix cells well (vortex 3 sec), then take a 10-µl aliquot of cells and dilute to 0.5 ml in MACS buffer. Mix thoroughly (vortex 3 sec), immediately take 25 µl of the diluted sample, and further dilute with 25 µl trypan blue solution (1:1). Count cells under a phase contrast microscope, using a 40x objective, recording live (exclude trypan blue) and dead cell numbers. Count each sample twice by using both sides of the hemacytometer chamber. Calculate the total cells in the preparation. This presort count is used to calculate cell concentration and viability.
17. Remove and set on ice an aliquot of presorted cells (about 3 million) for subsequent analysis of cell surface proteins by flow cytometry (see AfCS Protocol *Characterization of Cells by Flow Cytometry*, PP00000018).
18. Adjust the cell concentration to 10^7 cells/85 µl with MACS buffer before the addition of microbeads. It is important to obtain a single cell suspension for

magnetic bead labeling. Using refrigerated Miltenyi beads (the 50-nM beads are in a colloidal suspension that does not require shaking), add beads to the single-cell suspensions as follows:

i) 10 µl anti-CD43 beads/10^7 cells;

ii) 5 µl anti-Mac-1/CD11b beads/10^7 cells.

Thus, the final labeling volume is 10^7 cells/100 µl.

Note: anti-CD16/CD32 (immunoglobulin FcR) blocking antibodies can be included during bead labeling as recommended by Miltenyi. The AfCS did not find a significant increase in yield or purity of B cells separated when FcR blocking antibodies were included at the bead labeling stage.

19. Mix the cells and beads on ice for 20 min, with inversion of the tubes at 10 min (or label at 6 to 12 °C for 15 min, with inversion at 7.5 min).
20. After the incubation of cells with beads, split the bead/cell mixture evenly into new, iced 50-ml conical tubes, using a maximum of 4 ml (cells from approximately four spleens)/tube.
21 Add 10 to 20 times the volume of iced MACS buffer and mix by inversion.
22. Pellet the cells by centrifuging at 400 x g for 5 min at 4 °C.
23. Aspirate the supernatant, then loosen the cell pellets. Gently resuspend loosened cell pellets in 1 ml of MACS buffer. Filter cells through a 70-µm strainer into new 50-ml conical tubes prior to loading on the autoMACS. Rinse each tube used to pellet the cells with 1-ml aliquots of MACS buffer and pass through the 70-µm strainer to add to the filtrates.
24. Increase the final volume of suspended cells in each tube (representing cells from one to four spleens) with MACS buffer to a total of 5 ml. Keep cells at 4 °C until loaded on the autoMACS.

    Note: it is important to obtain a single cell suspension for loading cells onto the magnetic columns. A larger loading volume/lower cell density helps minimize clumping, which leads to mechanical trapping of the antigen-negative population, together with antigen-positive cells, on the columns. The autoMACS separation columns have a reported loading capacity of approximately 2 x 10^8 magnetically labeled cells. Thus, cells from four spleens constitute the maximum number of cells that should be loaded on a column in a single run, since approximately 50% of splenocytes express CD43 and/or Mac-1/CD11b. Miltenyi Biotec suggests that the separation column in the autoMACS should be changed every 2 weeks or after passing 4 x 10^9 cells through the column, whichever limit is reached first.
25. Cell separation on the autoMACS is done at room temperature and follows the manufacturer's protocol for the autoMACS machine. The autoMACS is cleaned and primed (i.e., rinsed with 70% ethanol, then purged and filled with MACS buffer) to prepare for sorting. Place a tube containing labeled cells on the uptake port and choose the "Deplete S" program (flow rate 1 ml/min).
26. For each cell separation, when the sample has mostly been taken up through the uptake port, and only approximately 100 µl remains, add another 4.5 ml of MACS buffer. This will rinse the tube and ensure delivery of all of the cells to the column.

    Note: when processing multiple batches of cell/bead preparations, run a "Rinse" program after each sort (Deplete S) program. Failure to do so may result in clogging of the machine. Maintenance of the autoMACS tubing also includes running through the "SAFE" cleaning program using Coulter Clenz once per month, or <u>every week</u> if large numbers of cells are being processed.

27. Each sort yields two fractions:
    i) from the autoMACS “NEG 1” port, nonadherent CD43(-)/Mac-1(-) flow-through cells; the volume of this sample equals the loading volume (5 + 4.5 ml) plus 2 ml, for a total of 11.5 ml.
    ii) from the autoMACS “POS 1” port, the CD43(+)/Mac-1(+) cells labeled with magnetic beads, retained on the column then eluted; the volume of this sample equals 2 ml.
    Fractions from multiple sorts of spleens processed at the same time are combined into separate pools of CD43(-)/Mac-1(-) cells and CD43(+)/Mac-1(+) cells and stored on ice.
28. Remove an aliquot of cells from each fraction to determine the total number of viable cells by trypan blue dye exclusion. Dilute the cells sufficiently to obtain between 100 to 200 cells/quadrant of a hemacytometer grid (for 1 spleen, processed as above, a 1/2 to 1/3 final dilution is appropriate for counting the negative fraction, and a 1/10 to 1/20 final dilution for the positive fraction). Count duplicate samples.
    Remove an aliquot containing about 1 x 10^6 cells from both the CD43(-)/Mac-1(-) cells and the CD43(+)/Mac-1(+) cells to use for analysis of cell surface proteins by flow cytometry (see AfCS Protocol *Characterization of Cells by Flow Cytometry,* PP00000018). If preparing cells from more than four spleens (i.e., multiple separations through the autoMACS), the negative fraction samples for experimentation can now be combined.
29. Pellet the cells at 400 x g for 5 min at 4 °C. Gently resuspend the cells in culture medium for use in experiments.

Reagents and Materials

Mice (6- to 8-week-old males): Charles River Lab; catalog no. C57BL/6 (C57 Black)

Hot bead sterilizer: Fine Science Tools; catalog no. 18000-45

Magnetic cell sorting buffer (MACS buffer): AfCS Solution Protocol ID PS00000001

Petri dish (polystyrene), 35 x 10 mm: Fisher Scientific; catalog no. 08-757-11YZ

Petri dish (polystyrene), 100 x 15 mm: Fisher Scientific; catalog no. 08-757-103A

Nylon mesh cell strainer, 70 µm: Falcon; catalog no. 352350

Ethanol, 70%: AfCS Solution Protocol ID PS00000011

Surgical scissors: Fine Science Tools; catalog no. 14060-11

Medium forceps (straight): Fine Science Tools; catalog no. 13008-12

Fine forceps (straight): Fine Science Tools; catalog no. 11253-20

Plunger from 1-cc tuberculin syringe: Monoject; catalog no. 501400

Conical tubes, 50 ml: Greiner; catalog no. 4943

Eppendorf centrifuge model 5804R with type A-4-44 swinging bucket rotor: Brinkmann Instruments; catalog no. 2262350-8

J6 centrifuge with a JS 4.2 swinging bucket rotor: Beckman Coulter; catalog no. 360271

Red blood cell lysis buffer (RBC lysis buffer): AfCS Solution Protocol ID PS00000002

Trypan blue solution: Sigma-Aldrich; catalog no. T8154

Phase contrast microscope, Zeiss Axiovert 25: Brinkmann Instruments; catalog no. 517046

Hemacytometer: Fisher Scientific; catalog no. 02-671-5

Anti-CD43 (Ly-48) microbeads: Miltenyi Biotec; catalog no. 130-049-801

Anti-Mac-1/CD11b microbeads: Miltenyi Biotec; catalog no. 130-049-601

AutoMACS: Miltenyi Biotec; catalog no. 201-01

AutoMACS separation columns: Miltenyi Biotec; catalog no. 130-021-101

Coulter Clenz: Beckman Coulter; catalog no. 8546930

Author: Robert Hsueh/Tamara Roach

Date: 03/24/03

Approved: Paul Sternweis/Bill Seaman