Chapter 4

Evaluating B-Cells: From Bone Marrow Precursors to Antibody-Producing Cells

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Abstract

Lymphocyte characterization is primarily based on the differential expression of surface markers. In this context, flow-cytometry analysis (FACS) is an exceptional technique that not only allows the identification of B-cell subsets, but can also be used to evaluate cell function, activation, and division. Here, we will combine the use of FACS analysis and ELISA techniques to identify murine bone marrow and peripheral B-cell subsets. The main function of B cells, derived through a multistage differentiation process from precursor cells, is to produce antibodies. This task is performed by terminally differentiated B cells called antibody-secreting cells (ASC) present at mucosal sites, in the bone marrow and in the spleen. The number and specificity of ASC can be measured by Enzyme-linked immunosorbent spot (ELISPOT) assay, a variation of the enzyme-linked immunosorbent assay (ELISA) used to quantify serum immunoglobulins.

Key words Flow-cytometry, Mouse B cell subsets, Antibodies

1 Introduction

B-lymphocytes can be classified in different subsets according to their origin, function, and localization. Each B-cell subset expresses a combination of cell surface markers that allows for their identification/purification using FACS analysis and cell sorting. The B-cell identity is given by the B-cell receptor (BCR). In the mouse, the first BCR expressing cells appear as early as at the embryonic day 16 (ED16) of gestation and are generated from fetal liver haematopoietic stem cells (HSCs) [1]. Although HSCs start to colonize the embryonic spleen at ED12 and bone marrow at ED15-16, the fetal liver retains haematopoietic functions until birth [2, 3]. At birth, the main districts responsible for B cell production is the bone marrow and, to a lesser extent, the spleen. In the bone marrow, commitment to the B cell lineage starts at the pro-B cell stage, when cells start to rearrange BCR genes. If the

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gene rearrangement is productive, pro-B cells differentiate into pre-B-cells. Surface expression of the BCR identifies immature B cells (Fig. 1). Immature/transitional B cells exit the bone marrow and migrate to the peripheral organs [4] where they differentiate into mature, memory, and antibody-secreting cells (ASC). Bone marrow-derived B cells preferentially replenish B cell pools in charge of the acquired immune responses, mainly follicular B cells and B2 cells (Fig. 2a, b) [5]. Although B cell turnover at the periphery is low, the daily B-cell out-put from the bone marrow allows a continuous "refreshment" of antigenic specificities. Moreover, B cell precursors present in the spleen can generate B cells "on demand." These precursors sustain the production of the so-called innate B cells, specifically B-1a B cells, in the body cavities (Fig. 3) and marginal zone B cells in the spleen. B-1a B cells produce antibodies through a T cell-independent mechanism and are responsible for generating the majority of the IgM natural antibody compartment. B-1a B cells are generated at a very low rate from fetal-derived precursor cells residing in the adult spleen. The B-1a B cells also supply the gut with IgA plasma cell precursors and are responsible for maintaining the secretory IgA compartment at mucosal sites [3].

In spite of the lack of consensus on the origin of certain B cell subsets, such as the dichotomy of B-1 versus B2 B cells, characterization of the mouse B cell compartments are well established. Indeed, through multiparametric FACS analyses it is now possible to identify almost all of the B cell subsets. Table 1 shows a combination of useful markers for each subset with some suggested bibliography.

2 Materials

2.1 Solutions for Cell Preparation

1. PBS (10×): PBS (l0×) washing buffer pH = 7.2, indicated amounts for 10 L: dissolve 43 g of $NaH_2PO_4 \cdot 2H_2O$; 258 g of $Na_2HPO_4 \cdot 12H_2O$ and 850 g of NaCl in 500 ml of distilled H_2O. Adjust the volume by adding distilled H_2O, check the pH, and store at 4 °C.
2. Incomplete medium: RPMI 1640 supplemented with heat inactivated 2 % FCS (*see* **Note 1**).
3. Culture medium B cell stimulation: RPMI 1640 supplemented with heat inactivated 10 % FCS, 2 %L-glutamine, 5×10^{-5} M 2-β mercaptoethanol and antibiotics (either gentamicin or penicillin/streptomycin).
4. Culture medium for antibody secretion: RPMI 1640 supplemented with heat inactivated 2 % FCS, 2 %L-glutamine, 5×10^{-5} M 2-β mercaptoethanol and antibiotics (either gentamicin or penicillin/streptomycin).

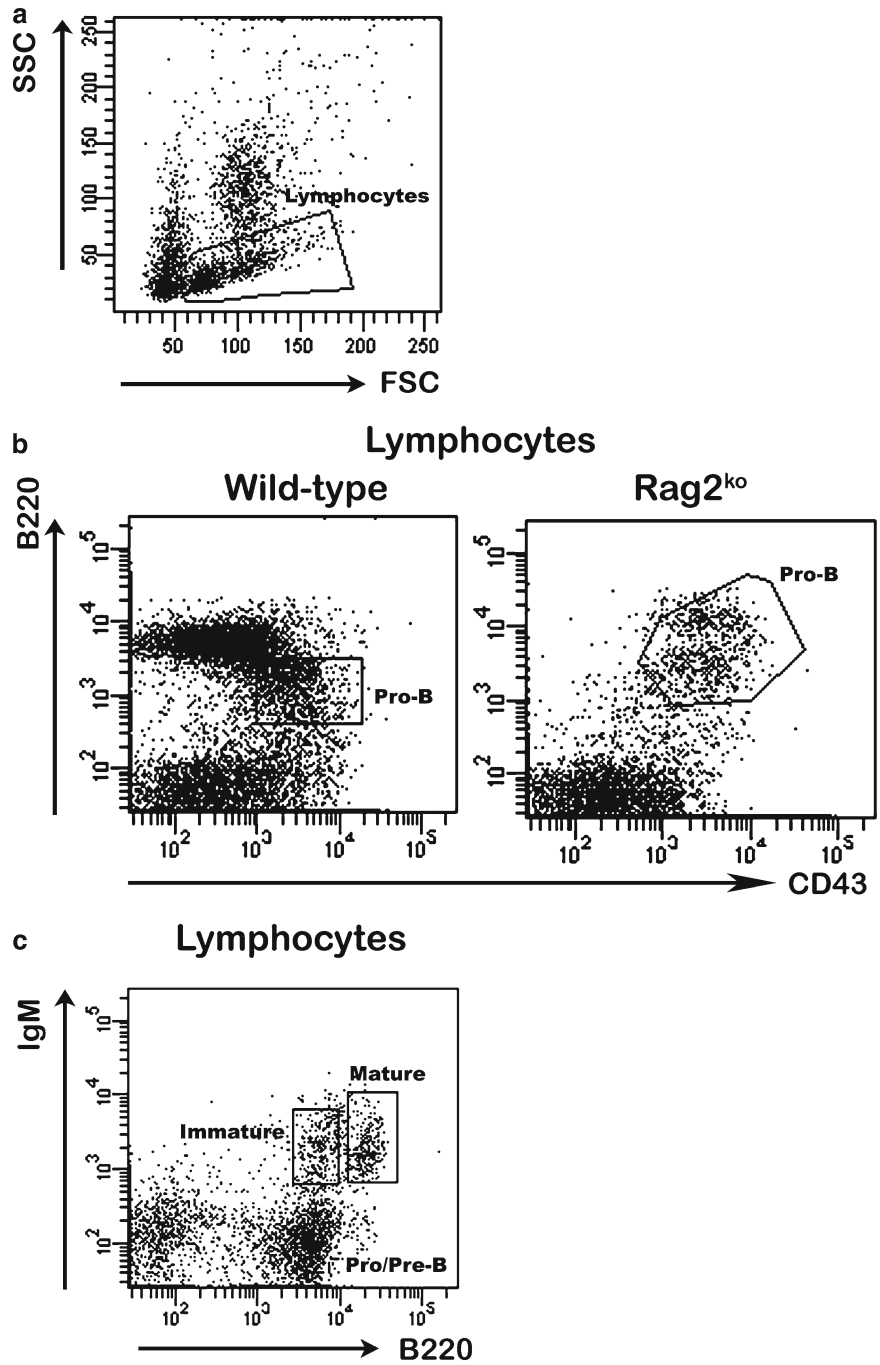

Fig. 1 Representative flow-cytometry dot plots showing the expression of B220 (formally CD45), CD43, and IgM in the bone marrow. (**a**) Shows a forward (FSC)/side scatter (SSC) plot. FSC correlates to cell volume and SSC to cell complexity, i.e., shape of nucleus, amount and type of cytoplasmic granules, or membrane roughness. FSC/SSC plotting allows the exclusion of dead cells and debris from the analysis. It is also helpful to define and select the lymphocyte gate used later for analysis of the different fluorescent cell markers. (**b**) Plot shows the expression of B220 and CD43 in the bone marrow of wild-type (*left*) and Rag2-deficient mice (*right*) inside the lymphocyte gate. Pro-B cells on the way to rearrange Ig genes are identified as $B220^{low}CD43^{low}$. This is the only B cell population present in Rag2-deficient mice, because lack of *Rag* genes impairs BCR and TCR gene rearrangement, blocking the development before the pre-B or pre-T cell stage. (**c**) Plots show the expression of B220 and IgM: $B220^{pos}IgM^{neg}$ correspond to pro/pre-B cells, $B220^{pos}IgM^{pos}$ are immature B cells and $B220^{bright}IgM^{pos}$ represent mature B cells that recirculate with the blood

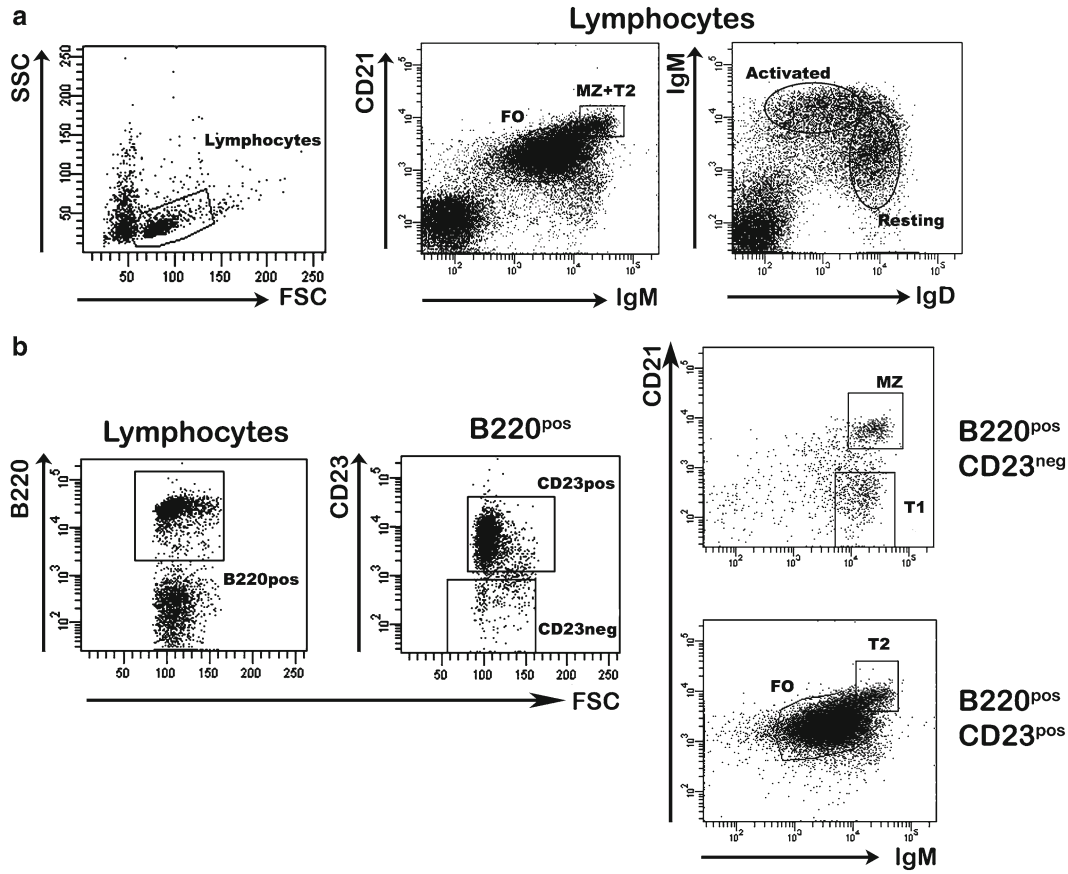

Fig. 2 Representative flow-cytometry plots showing spleen cell suspensions analyzed for the expression of CD21, CD23, B220, IgM, and IgD. (**a**) *First plot* shows that the majority of splenic lymphocytes are small. Spleen B cells can be classified in various subsets according to the expression of CD21, CD23, IgD, and IgM. Briefly, transitional type 1 B cells are $CD23^{neg}CD21^{neg}IgM^{bright}$ (T1), transitional type 2 are $CD23^{pos}CD21^{bright}IgM^{bright}$ (T2), marginal zone B cells are $CD23^{neg}CD21^{bright}IgM^{bright}IgD^{low}$ (MZ/activated), and follicular B cells are $CD23^{pos}CD21^{pos}IgM^{pos}IgD^{pos}$ (in other districts are also named B2 cells, FO/resting). The *middle plot* shows FO, MZ, and transitional B cells analyzed inside the lymphocyte gate using anti-CD21 and anti-IgM antibodies. *Right plot* exemplifies another type of analysis using IgM and IgD: activated B cells are $IgM^{bright}IgD^{dull}$, contain MZ and transitional B cells and B-1 B cells; resting B cells are $IgM^{dull}IgD^{bright}$, that correspond to FO or B2 B cells. (**b**) Analysis strategy used to distinguish transitional 2 cells from marginal zone B cells that share the CD21 markers and are both bright for IgM: first plot B220 versus FSC and gate on $B220^{pos}$, inside the $B220^{pos}$ check the expression of CD23 and define two new gates for $CD23^{neg}$ and $CD23^{pos}$. $CD23^{neg}CD21^{high}IgM^{bright}$ identifies marginal zone B cells (MZ) and $CD23^{neg}CD21^{neg}IgM^{bright}$ transitional type 1 B cells (T1). The majority of the $CD23^{pos}$ cells are $CD21^{pos}IgM^{pos}$ follicular B cells (FO) and a small population of transitional type 2 cells (T2) that are $CD21^{high}IgM^{bright}$

5. Gey's solution: It is useful to deplete erythrocytes from the peripheral blood and spleen. Gey's solution destroys erythrocytes while maintaining membrane integrity of mononuclear cells. Solutions should be prepared fresh each time by mixing 14 ml of sterile H_2O + 4 ml of solution A + 1 ml of solution B + 1 ml of solution C.

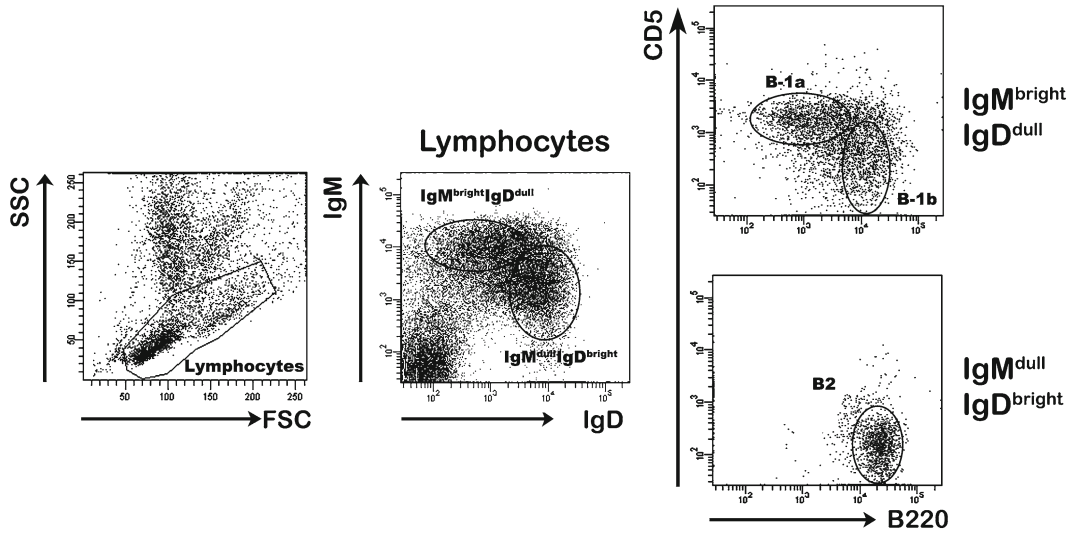

Fig. 3 Representative example of flow-cytometry analysis showing B cell subsets present in the peritoneal cavity. Cell suspensions were stained for CD5, B220, IgM, and IgD. SSS/FSC plot, mouse peritoneal cavity is composed of a large fraction of macrophages and large lymphocytes corresponding to activated cells. *Middle plot* shows the expression of IgM and IgD in total lymphocytes, activated B cells are indicated as $IgM^{bright}IgD^{dull}$ and resting B cells as $IgM^{dull}IgD^{bright}$. Expression of CD5 surface markers and the amount of B220 allows the separation of the B-1a and B1b B cells contained in the activated pool. B-1a cells are $CD5^{pos}B220^{dull}$ whereas B-1b cells are $CD5^{neg}B220^{pos}$. $IgM^{dull}IgD^{bright}$ B cells correspond to B2 cells that are $B220^{bright}$ and do not express CD5 molecule

Solution A (for 1 L of water): Dissolve 35 g of NH_4Cl + 1.85 g of KCl + 1.5 g of $Na_2HPO_4{\cdot}12H_2O$ + 0.119 g of KH_2PO_4 + 5.0 g of glucose + 25 g of gelatine in distilled H_2O. Add 0.05 g of phenol red to the solution. Adjust the volume to 1 L, autoclave at 120 °C and store at 4 °C.

Solution B (for 100 ml water): Dissolve 0.14 g of $MgSO_4{\cdot}7H_2O$ + 0.42 g of $MgCl_2{\cdot}6H_2O$ + 0.34 g of $Ca\,Cl_2{\cdot}2H_2O$ in distilled H_2O. Adjust the volume to 100 ml with distilled H_2O, autoclave at 120 °C and store in dark at 4 °C.

Solution C (for 100 ml of water): Dissolve 2.25 g of $NaHCO_3$ in distilled H_2O. Adjust the volume to 100 ml with distilled H_2O, autoclave at 120 °C and store in dark at 4 °C.

2.2 Flow Cytometry Analysis

1. FACS buffer: PBS 1×, 2 % FCS, and 0.01 % Sodium azide (*see* **Note 2**).

2.3 Solutions for ELISA and ELISA Spot Assay

1. K_2HPO_4 (0.5 M solution): Dissolve 87.05 g of K_2HPO_4 in 500 ml distilled H_2O and adjust the volume to 1 L with dH_2O.
2. KH_2PO_4 (0.5 M solution): Dissolve 68.045 g of KH_2PO_4 in 500 ml of distilled H_2O and adjust the volume to 1 L with dH_2O.

Table 1
B cell subsets in central and peripheral lymphoid organs

	Main surface markers	Main localization	References
Pro-B[a]	$B220^{pos}CD43^{pos}IgM^{neg}$	Bone marrow	[7, 8]
Pre-B[a]	$B220^{pos}CD43^{neg}IgM^{neg}$	Bone marrow	[9, 10]
Immature B	$B220^{pos}IgM^{pos}$	Bone marrow	
Re-circulating B	$B220^{bright}IgM^{pos}IgD^{pos}$	Bone marrow/blood	
Transitional 1	$B220^{pos}IgM^{bright}CD21^{neg}CD23^{neg}$	Spleen	[4]
Transitional 2	$B220^{pos}IgM^{bright}CD21^{bright}CD23^{pos}$	Spleen	[4]
Follicular	$B220^{pos}IgD^{pos}IgM^{pos}CD21^{pos}CD23^{pos}$	Spleen/lymph node	[11]
Marginal zone	$B220^{pos}IgM^{bright}CD21^{bright}CD23^{neg}$	Spleen	[12]
B-1a	$B220^{low}IgM^{bright}IgD^{dull}CD5^{pos}CD11b^{pos}$	Peritoneal cavity	[13]
B-1b	$B220^{low}IgM^{bright}IgD^{dull}CD5^{neg}CD11b^{pos}$	Peritoneal cavity	[14]
B2	$B220^{pos}IgM^{dull}IgD^{bright}CD5^{pos}$	Peritoneal cavity	
ASC	$B220^{low}CD138^{pos}PNA^{low}Ig^{low}$	Spleen, bone marrow mucosal sites	

[a]pro-B/pre-B cells can be subdivided in fractions according to the expression of BP-1, CD24, CD25 surface markers, the state of Ig gene rearrangement and expression of the recombination enzymes. For further details *see* refs. 15, 16
Because we still lack a unique phenotypic marker able to distinguish memory B cells from naïve B cells in the mouse, memory B cell subset was not included in the table

3. Coating buffer: 0.5 M K_2HPO_4 pH = 8.0 (stock solution, 10×). Add the KH_2PO_4 solution to the K_2HPO_4 solution until pH 8.0. Autoclave and filter the solution or store at 4 °C. Always visually inspect each solution to ensure that no contamination is present. The working concentration of the coating buffer should be 0.05 M.
4. ELISA blocking solution: PBS (1×) + 1 % gelatin (100 ml). In a microwave oven, dissolve 1 g of gelatin in an Erlenmeyer flask with 50 ml of dH_2O. Place the flask on ice until the solution reaches room temperature. Add 10 ml of PBS (10×) and adjust to the final volume (100 ml) with dH_2O. This buffer cannot be stored for more than overnight at 4 °C.
5. ELISpot blocking solution: PBS (1×) + 1 % gelatin + 0.05–0.1 % Tween-20 (100 ml) (*see* **Note 3**).
6. ELISA substrate buffer:

   Solution A: Dissolve 38.82 g of $Na_2HPO_4 \cdot 12H_2O$ in dH_2O and adjust the volume to 500 ml.

   Solution B: Dissolve 10.53 g of citric acid in dH_2O and adjust the volume to 500 ml.

   Substrate buffer: Add buffer solution B to solution A until pH = 5.6. Autoclave the solution and store at 4 °C. Prepare

0.5 mg/ml of OPD (*ortho*-phenylenediamine) in substrate and add 1 µl/ml of H_2O_2 (30 %) (*see* **Note 4**).

7. ELISpot substrate buffer: *AMP l0× buffer* (2-amino-2-methyl-L-propanol, pH 10.25). Add to 100 ml AMP (1.5 M, pH 10.3) + 752 µl of $MgCl_2$ (1 M) + 152 µl of Triton X-405 + 1.5 ml of NaN_3 (10 %) + 47.6 ml of dH_2O and adjust the pH to 10.25 with HCl. Store the solution at 4 °C. Dissolve 500 mg of BCIP in 50 ml of AMP l0× buffer + 450 ml of dH_2O. Stir the solution at room temperature for 1 h, protected from the light, and filter through a 0.45 µm filter. The solution should be stored in the dark at 4 °C.
8. SDS (10 %) stop solution: Add 50 g of Dodecylsulfate–Na salt in 350 ml of dH_2O. Stir the solution overnight and adjust the volume to 500 ml with dH_2O.

3 Methods

3.1 Cell Preparation

1. Collect peripheral blood from the retro-orbital sinus using a Pasteur pipette (*see* **Note 5**) and transfer into 1.5 ml eppendorf tubes containing 50 µl of heparin. Deplete erythrocytes by incubating the blood sample with 1 ml of Gey's solution for 1 min. Add 1 ml of incomplete RPMI and spin at 250 × *g* for 5 min at 4 °C. Discard the liquid by inverting the tube or by aspiration and resuspend the pellet in 300 µl of FACS medium. Leave the tubes on ice.
2. To obtain serum, collect the blood into 1.5 ml eppendorf tubes, leave overnight at 4 °C. The following day, remove and discard the clot with the help of a needle or a toothpick. Spin the tubes at 700 × *g* for 2 min and transfer the serum to new 1.5 ml eppendorf tubes. Store the serum at –20 °C until analysis.
3. Sacrifice the animals after blood withdrawal by cervical dislocation (*see* **Note 5**).
4. Collect peritoneal cavity cells by injecting 5 ml cold PBS (1×) into the peritoneum (10 ml syringe with a 21 G × 1.5″ needle). Allow the PBS to move in the cavity by gentle shaking and recover it in 15 ml tubes prefilled with 5 ml of cold PBS (1×) 2 % FCS. Spin tubes at 250 × *g* for 10 min at 4 °C. Discard the liquid by inverting the tube or by aspiration and resuspend the pellet in 1 ml of FACS medium. Leave the tubes on ice.
5. Collect femurs and place into Petri dishes containing 5 ml of RPMI supplemented with 2 % FCS. Prepare single cell suspensions by flushing the bones with incomplete culture medium into 15 ml tubes using a 1 ml syringe with 26 G × 0.5″ needle.

6. Prepare spleen and lymph node cell suspensions by smashing the organ between two frosted slides in 5 ml of incomplete culture medium. Leave the spleen cells on ice for 5 min to allow debris to sediment and transfer cells into new tubes. Wash the cells by centrifuging the tubes at 250×*g* for 10 min at 4 °C. Resuspend the pellets in 5 ml incomplete culture medium and keep the tubes on ice (*see* **Note 6**).
7. Count the nucleated cells. For each tissue take 10 µl of the cell suspension and mix it with 90 µl of Trypan blue (stock solution should be diluted 1:1 in PBS (1×)). Count living cells using the Burker counting chamber.

3.2 Cell Cultures

1. Prepare duplicates for each culture condition in order to evaluate cell proliferation at day 3 and Ig secretion at day 7.
2. Take 4×10^6 spleen cells spin and centrifuge at 250×*g* for 7 min at 4 °C.
3. Resuspend the cell pellet in 4 ml of 5-chloromethylfluorescein diacetate (CellTracker CMFDA) [6] at a final concentration of 2.5 µg/ml in PBS (1×). Incubate the sample for 30 min at 37°C in the water bath. Protect the sample from the light.
4. Add 10 ml of PBS (1×) and centrifuge the sample at 250×*g* for 5 min at 4 °C.
5. Discard the supernatant, resuspend the pellets in 800 µl of complete culture medium and distribute 200 µl (1×10^6 cells)/well into 96-well flat-bottom plates. Add 10 µl of LPS (10 µg/ml) in two wells.
6. Incubate the plates at 37 °C and 5 % CO_2 for 3 or 7 days.
7. After 3 days of stimulation, collect the LPS-stimulated and nonstimulated cells from the two wells by gently pipetting up and down. Transfer the cells into 15 ml tubes and add 10 ml of FACS medium. Centrifuge the tubes at 250×*g* for 7 min at 4 °C. Resuspend the cells in 1 ml of FACS medium and follow the staining procedures indicated below.
8. After 7 days of stimulation, centrifuge the plate at 250×*g* for 5 min at 4 °C. Collect supernatants into 1.5 ml eppendorf tubes and store at –20 °C until analysis by ELISA.

3.3 Staining Procedures for Flow-Cytometry Analysis

1. Carry out all procedures on ice and protect the samples from light.
2. Collect 1×10^6 cells/staining in a round-bottom 96-well plate (*see* **Note 7**).
3. Centrifuge plate at 250×*g* for 5 min at 4 °C and remove the supernatant by inverting the plate (*see* **Note 8**).
4. Add 10 µl of each antibody diluted in FACS medium to the cell pellet (Table 2) and shake gently. Make sure that the cell pellet is resuspended.

Table 2
Monoclonal antibodies used to characterize B cell subsets

	Clone
CD5	53-7.3
CD16 (FcγIII/IIR)	2.4G2
CD21	7G6
CD23	B3B4
CD43	S7
CD45R (B220)	RA3-6B2
IgD	11.26c
IgM	2911

5. Incubate the cells on ice for 20 min and protect from light. At the end of the incubation, add 200 µl of FACS medium and spin as described under Protocol 3.3.
6. Remove supernatant and resuspend the pellet in 200 µl of FACS medium. Transfer the cells into FACS tubes. Wash the wells with an additional 200 µl of FACS medium to recover all the cells and add to the tubes. Analyze using a flow-cytometer (*see* **Note 9**).
7. Dead cells can be excluded from the analysis by side/forward scatter gating.

3.4 ELISpot

1. Coating: Distribute 50 µl per well of antibody or antigen diluted in coating buffer in a flat-bottom 96-well plate. Vortex the plate and seal it to avoid evaporation. Incubate at 4 °C overnight (or 37 °C for 1 h). The typical protein concentration for coating is 1–10 µg/ml
2. Washing: Submerge the plate in a container with PBS (1×). Empty the plate by inversion over a sink. Tap the inverted plate against some layers of soft paper tissue to remove residual liquid. There is no need to change PBS between washes, just add more to the container. Repeat the washing procedure three times.
3. Blocking agent: Add 200 µl of PBS (1×) + 1 % gelatin/well and incubate the plates at 37 °C for 1 h.
4. Washing: Remove the plates from the incubator and wash three times as described above in **step 2**.
5. Incubation with cells: Prefill the plate with 100 µl/well of RPMI containing 2 % FCS. Adjust the cell concentration to 2×10^6 cells/ml in incomplete medium. Add 100 µl of cells to

the first row and mix gently by pipetting up and down. Collect 100 µl from the first row of the plate and mix in the second row. Repeat the 1:2 dilution series until the end of the rows. Discard the last 100 µl. Incubate plates at 37 ° C, 5 % CO_2 for 4–6 h. The plate should not move during the incubation.

6. Washing: Remove plates from the incubator and submerge them in a container filled with water + 0.05 % Tween20. Empty the plate by inversion over a sink. Submerge the plate in a container with PBS-0.05 % Tween20 and leave the plate with washing solution on the bench for 10 min. Repeat the washes three times with PBS + 0.05 % Tween20 (total washing time 30 min). Tap the inverted plate against some layers of soft paper tissue to remove residual liquid.
7. Incubate with biotin or alkaline phosphatase-labeled antibody: Prepare the antibody dilution in PBS + 1 % gelatin + 0.05 %Tween20 and distribute 50 µl/well. Seal the plates and incubate overnight at 4 °C.
8. Remove the plates from 4 °C and leave them at room temperature for 10 min. Wash the plates three times with PBS + 0.05 %Tween20. Allow the plates to sit on the bench for 10 min between each wash with PBS-Tween20 in each well (the total washing time is 30 min).
9. Add 8 ml of ESA substrate solution to 3 ml of dH_2O. Distribute 50 µl/well of the diluted substrate solution and incubate overnight at 4 °C or 2 h at 37 °C (*see* **Note 10**).
10. Wash the plates three times with dH_2O as described above in **step 2**.
11. Air-dry the plates and count spots using a dissection microscope (Fig. 4a).

3.5 Quantification of Serum Igs by ELISA

1. Coating: Distribute 50 µl of antibody or antigen diluted in coating buffer into each well of a flat-bottom 96-well plate. Vortex and seal the plate to avoid evaporation. Incubate the plate at 4 °C overnight (or 37 °C for 1 h). The typical protein concentration for coating is 1–10 µg/ml.
2. Washing: Wash the plate three times with PBS (1×) as described for ELISpot under Protocol 3.4, **step 2**.
3. Blocking agent: Add 200 µl of PBS + 1 % gelatin/well and incubate the plates at 37 °C for 1 h.
4. Washing: Remove the plates from the 37 °C incubator and wash three times as described above under **step 2**.
5. Incubation with serum or culture supernatant: Generate a "masterplate" for dilutions using a round-bottomed 96-well plate. Fill all of the wells with 100 µl of PBS + 1 % gelatin + 0.05 % Tween20 and add 10 µl of serum to the

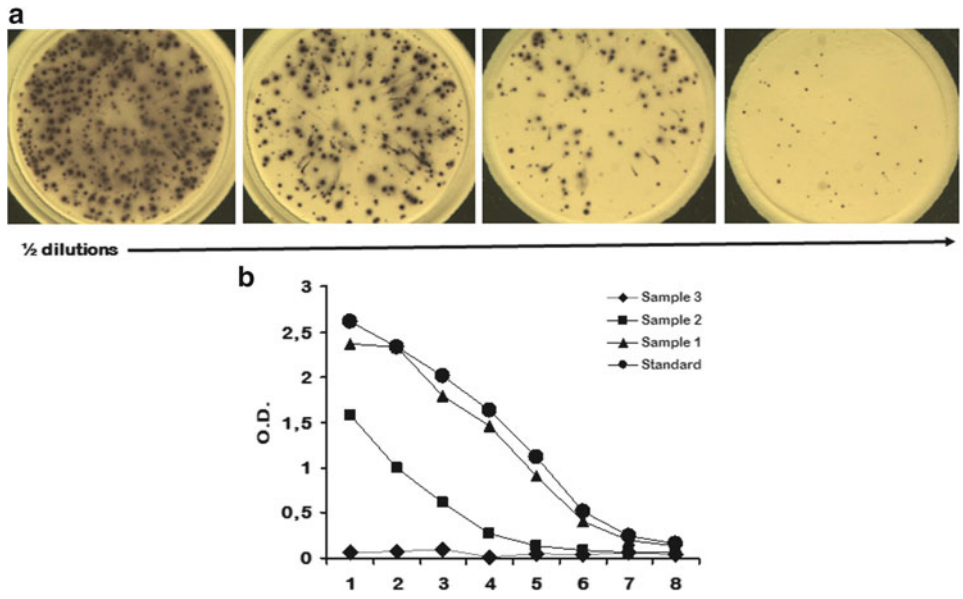

Fig. 4 Antibody-secreting cells (ASC) and serum/supernatant antibodies. (**a**) Images represent 1:2 serial dilutions of IgM-producing cells present in the spleen of an adult mouse. Each *dark spot* corresponds to a single cell that secreted IgM. The number of spots can be reported to the number of cells seeded in order to have the number of ASC/10^6. (**b**) Graph represents the optical density (OD) measured at 450 nm at the end of the ELISA. To calculate serum or culture supernatant Ig concentration is sufficient to extrapolate OD values of the exponential phase curve from the standard curve

first row (for the supernatant, *see* **Note 11**). Mix by pipetting up and down. Collect 50 µl from the first row and mix in the second row. Repeat the 1:3 dilution series for each row on the plate. Discard the last 50 µl. Transfer 50 µl of the dilutions to the coated, blocked, and washed ELISA plate. Incubate the plates at 37 °C for 1 h.

6. Washing: Remove the plates from the 37 °C incubator and wash three times as described above in **step 2**.
7. Incubate with biotin- or peroxidase-conjugated antibody: Prepare the antibody dilution in PBS + 1 % gelatin + 0.05 % Tween20 and distribute 50 µl/well. Incubate the plates at 37 °C for 1 h.
8. Washing: Remove the plates from the 37 °C incubator and wash three times as described above under **step 2**.
9. Substrate reaction: Add 100 µl/well of substrate solution (*see* **Note 11**). Incubate at room temperature for 10–45 min and protect the plates from light using aluminum foil or a box.
10. Stop the enzymatic reaction: Add 50 µl/well of 10 % SDS.
11. Read plates using a spectrophotometer at 450 nm (Fig. 4b).

4 Notes

1. Fetal calf serum should be heat inactivated by incubating for 30 min in a water bath at 56 °C.
2. Handle sodium azide with care.
3. When pipetting very dense solutions, such as Tween-20, use a pipette tip that has been trimmed 1–3 cm with a razor blade and aspirate slowly. Ensure that all of the volume is added.
4. OPD (*ortho*-phenylenediamine) is toxic and carcinogenic. Therefore it should be handled with gloves and other appropriate protective equipment. The ELISA substrate buffer with OPD and H_2O_2 should be used immediately after mixing the H_2O_2. The mix should be prepared fresh each time.
5. All the procedures involving animals have to be performed in compliance with national and international laws on the ethical use of the animals.
6. Quality of the FACS staining may be improved with the depletion of erythrocytes, in particular for peripheral blood and spleen samples. The use of Gey's solution is also recommended when there is a need to analyze or quantify minute cell subsets.
7. Before the use of labeled or unlabeled antibodies, make sure they specifically bind to the receptor of interest and that the antibodies are appropriately diluted. Antibodies to be used in the FACS analysis should be diluted in FACS medium each time. To remove fluorescent precipitates we recommend centrifuging the antibody dilution for 10 min at 9,600 × *g* before use.
8. Quality of the FACS staining can be improved by preincubating the cells with CD16-FcγIII/IIR (Fc-block reagent).
9. If cell acquisition is not possible shortly after the staining procedure, consider fixing the cells. After the surface staining and the last wash, add 200 μl of 1 % paraformaldehyde in PBS (1×) to the cell pellet and incubate at 4 °C for 20 min in the dark. Transfer cells from the plate into FACS tubes and add 200 μl of FACS medium.
10. Check the quality of ESA substrate solution before use. The solution should be transparent and without precipitates. Precipitates can be removed by filtering the stock solution with a 0.45 μm filter before diluting in dH_2O.
11. As Ig concentration in culture supernatants is expected to be lower than Ig concentration in sera from healthy subjects, the starting dilution in the ELISA plate should be lower. Fill all wells with 100 μl of PBS + 1 % gelatine + 0.05 %Tween20, except the first row of wells that should get only 50 μl of PBS + 1 % gelatine + 0.05 % Tween20. Take 50 μl of culture

supernatant and add to the first row. Proceed in the following steps as discussed for the serum.

12. Make sure the substrate buffer is at room temperature before adding the OPD and H_2O_2. ELISA substrate is unstable and should be used immediately.

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