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The isolation, differentiation and quantification of human antibody-secreting B cells from blood: ELISpot as a functional readout of humoral immunity

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Abstract:	The hallmark of humoral immunity is to generate functional ASCs, which synthesize and secrete Abs specific to an antigen (Ag), such as a pathogen, used for host defense. In the determination of the functional status of humoral immune response of an individual in a quantitative manner, both serum Abs and circulating ASCs are commonly measured as functional readouts. In humans, peripheral blood is the most convenient and readily accessible sample that can be used in the determination of humoral immune response elicited by host B cells. Distinct B-cell subsets, including ASCs, can be isolated directly from peripheral blood via selection with lineage-specific Ab-conjugated microbeads or via cell sorting with flow cytometry. Moreover, purified naïve and memory B cells can be activated and differentiated into ASCs in culture. The functional activities of ASCs in the contribution of Ab secretion can be quantified by ELISpot, which is an assay that converges enzyme-linked immunoabsorbance assay (ELISA) and western blotting technologies to enable the enumeration of individual ASCs at the single-cell level. In practice, the ELISpot assay has been increasingly used to evaluate vaccine efficacy because the handling of a large number of blood samples is amenable. The methods of isolating human B cells from peripheral blood, the differentiation of B cells into ASCs in vitro, and the employment of ELISpot for the quantification of total IgM- and IgG-ASCs will be described.
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TITLE:
The isolation, differentiation, and quantification of human antibody-secreting B cells from blood: ELISpot as a functional readout of humoral immunity

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SHORT ABSTRACT:
Human peripheral blood is commonly used for the assessment of the humoral immune response. Here, the methods for isolating human B cells from peripheral blood, differentiating human B cells into antibody (Ab)-secreting B cells (ASCs) in culture, and enumerating the total IgM- and IgG-ASCs via an ELISpot assay are described.

LONG ABSTRACT:
The hallmark of humoral immunity is to generate functional ASCs, which synthesize and secrete Abs specific to an antigen (Ag), such as a pathogen, and are used for host defense. For the quantitative determination of the functional status of the humoral immune response of an individual, both serum Abs and circulating ASCs are commonly measured as functional readouts. In humans, peripheral blood is the most convenient and readily-accessible sample that can be used for the determination of the humoral immune response elicited by host B cells. Distinct B-cell subsets, including ASCs, can be isolated directly from peripheral blood via selection with lineage-specific Ab-conjugated microbeads or via cell sorting with flow cytometry. Moreover, purified naïve and memory B cells can be activated and differentiated into ASCs in culture. The functional activities of ASCs to contribute to Ab secretion can be quantified by ELISpot, which is an assay that converges enzyme-linked immunoabsorbance assay (ELISA) and western blotting technologies to enable the enumeration of individual ASCs at the single-cell level. In practice, the ELISpot assay has been increasingly used to evaluate vaccine efficacy because of the ease of handling of a large number of blood samples. The methods of isolating human B cells from peripheral blood, the differentiation of B cells into ASCs *in vitro*, and the employment of ELISpot for the quantification of total IgM- and IgG-ASCs will be described here.

INTRODUCTION:

B cells play a central role in the development of humoral immunity. They initially develop in the bone marrow and enter the blood stream as naïve B cells, which can migrate into the lymphoid tissues, such as the spleen, lymph nodes, and tonsils, for further development. Upon Ag encounter, some naïve B cells migrate into lymphoid follicles, where germinal center B cells can differentiate into memory B cells and plasmablasts (PBs)/plasma cells (PCs). While most PBs/PCs egress into the blood stream, a few eventually reside in the bone marrow to undergo terminal differentiation into long-lived PCs[1]. B cells in circulation are heterogeneous, and at steady state, PBs/PCs are rare in peripheral blood[2]. As a result of the availability of lineage-specific surface markers, flow cytometry has become a popular method for the identification and characterization of the B-cell subsets in peripheral blood. An extended application of flow cytometry is the addition of a cell sorter function, which permits the separation and isolation of individual subsets of B cells with high purity. Based on the expression of specific surface receptors at different developmental stages, human circulating B cells are generally classified into three main subpopulations: naïve B cells ($CD19^+CD27^-CD38^-$), memory B cells ($CD19^+CD27^+CD38^-$), and PBs/PCs ($CD19^+CD27^+CD38^+$)[3-4] (**Figure 1**). Naïve B cells by nature have not encountered Ags. However, they can be differentiated into IgM^+CD27^+ memory B cells. Although naïve B cells are homogeneous in expressing B-cell antigen receptor (BCR)-associated molecules (*e.g.*, CD19, CD20 and CD22) they are heterogeneous in their immunoglobulin repertoire[5]. The majority of $CD27^+$ memory B cells can be differentiated into $CD27^{+/hi}CD38^+$ PBs/PCs[6]. In addition, memory B cells and PBs/PCs are polyclonal and exhibit developmental and functional heterogeneity[4-7]. PBs/PCs in circulation are normally short-lived and do not express CD138, but those made to settle down in the bone marrow will terminally differentiate and become long-lived. Terminally differentiated PCs express CD138 and down-regulate CD27 molecules on their surfaces[8]. Since both PBs and PCs are capable of secreting Abs, in many occasions they are collectively denoted as ASCs. In contrast, neither naïve B cells nor memory B cells can produce appreciable amounts of Abs[9-10]. Nevertheless, when isolated, both naïve and memory B cells can be differentiated into ASCs in 3-10 days when placed in the proper culture conditions[6, 11-15]. In fact, ASCs derived from *in vitro* differentiation share similar surface expressions of CD27 and CD38 with those directly isolated from peripheral blood[6]. In addition, the ASCs differentiated *in vitro* express a low level of surface CD20, similar that of circulating PBs/PCs[6]. Although the culture-derived ASCs are all short-lived, they can secrete Abs, indicating that they are functionally competent and able to contribute to the humoral immunity.

Both ELISA and ELISpot are by far the most commonly-applied methods with which to obtain functional information on the humoral immune response. ELISA is a 96-well plate-based assay, and it is frequently used to measure the titers of serum Ag-specific Abs and other analytes (*e.g.*, cytokines). It is convenient and scalable. ELISA is designed to use a solid-phase enzyme assay to detect the presence of Abs or other substances, such as serum, in a liquid sample[16]. The readouts from serum ELISAs have been widely-used to represent the immune response of the body. A tool necessary for the acquisition of readouts from ELISA assays is a spectrophotometric microplate reader. The reader can determine the optical density (O.D.) of the end products typically resulting from the reaction of horseradish peroxidase (HRP)-conjugated detection Abs and their specific substrates[17]. With regard to reporting the humoral immune response, serum Ab levels determined by ELISA denote the collective, but not individual, performance of ASCs in

the body. In addition, ELISA fails to take into account the participation by memory B cells, which do not secrete Abs.

Like ELISA, ELISpot is a widely-used method for detecting and monitoring the immune response in peripheral blood samples[17-18]. ELISpot is a technique related to a sandwich ELISA. In it, cells are placed into the polyvinylidene difluoride (PVDF) membrane-backed wells of 96-well microplates for a short-term culture. The ELISpot assay is analogous to performing western blotting on a microplate and developing the spots on the PVDF membrane in each well. An automated ELISpot reader system or a stereomicroscope for manual counting is required. The main advantage of ELISpot in detecting an immune response is its superb sensitivity in the quantification of ASCs and cytokine-secreting cells. It reports their functional activities in humoral and cellular immunity, respectively. In the measurement of humoral immune function, serum Ab levels determined by ELISA and the number of ASCs enumerated by ELISpot are often correlated, but the data readouts from these two assays have some differences in functional implications[19-20]. The main advantage of ELISpot is its sensitivity of method. The level of serum Ab titers as reported by ELISA is presented semi-quantitatively as O.D. readouts, denoting the relative Ab level, or more quantitatively, as concentration readouts when a known amount of the proper isotypes of Abs is included for reference. In contrast, the results of ELISpot are presented as the absolute number of ASCs in a cell pool of interest (*e.g.*, unfractionated peripheral blood mononuclear cells (PBMCs) and purified B cells from PBMCs). ELISpot can detect a single ASC, but ELISA requires Ab amounts from ASCs to reach optimized assay-dependent concentrations prior to measurement. Hence, ELISpot is obviously superior to ELISA in sensitivity of quantification. Moreover, ELISpot is also suitable for quantifying the *in vitro* differentiated ASCs from activated memory B cells. Memory B cells do not secrete Abs but can differentiate into ASCs upon activation; they therefore have no contribution to serum Abs detected by ELISA. Thus, ELISpot is the method of choice in the measurement of the immune response of circulating memory B cells after activation in culture. It allows for the monitoring of the maintenance of long-term humoral immunity.

PROTOCOL:

Human peripheral blood must be obtained from healthy donors under informed consent, and the use of blood samples must conform to the approved guidelines established by individual institutional review boards. In this study, the protocol to use human blood in a demonstration of the results of flow cytometry (**Figure 1**) and ELISpot assays (**Figure 3**) was approved by the Internal Review Board of National Taiwan University Hospital (protocol number 201307019RINB).

1. Isolation and purification of human peripheral blood B cells

1.1 Draw ~10 mL of blood from the median cubital vein (in the cubital fossa anterior to the elbow) into a 15-mL tube containing K_2EDTA (1.5 to 2.0 mg/mL blood) and immediately invert the tube several times to prevent clot formation.

1.2 Add 35 mL of autoclaved (121 ˚C, 15 min) red blood cell (RBC) lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 1 mM EDTA; pH 7.4) to the tube containing the fresh blood sample (≥ 3:1 vol/vol) and incubate at room temperature (RT) for no longer than 5 min.

NOTE: The appearance of light transmission through the tube indicates the completion of RBC lysis.

1.3 Centrifuge at 600 x g at RT for 5 min. Ensure that the pellet is white in color.

1.4 Resuspend the pellet with 10 mL of autoclaved phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4; pH 7.4) and centrifuge as in step 1.3.

1.5 Discard the supernatant, resuspend the pellet with 10 mL of RPMI 1640 medium (supplements: 10% fetal calf serum, 100 U/mL penicillin/streptomycin, 0.25 µg/mL amphotericin B, and 2 mM L-glutamine), and then plate the cells into a 10-cm culture dish (10 mL of blood per dish). Place the dish in a 37 °C incubator with 5% CO_2 for 30 min.

1.6 Gently swirl the culture dish a few times and place the culture medium (suspension cells) into 15-mL plastic conical tubes. Discard the adherent cells (mostly macrophages) on the culture dishes.

1.7 Centrifuge at 600 x g at RT for 5 min. Discard the supernatant.

1.8 Resuspend the pellet with 1 mL of RPMI 1640 medium. Count the cell number with a hemocytometer or an automated cell counter.

NOTE: The viability of isolated leukocytes is normally greater than 90% by trypan blue exclusion.

1.9 Centrifuge as in step 1.7 and resuspend the cells in ~200 µL of cold PBS buffer (0.5% bovine serum albumin (BSA) and 2 mM EDTA) at the concentration of 5-10x10^6 cells/mL.

1.10 Add 5 µL of biotinylated anti-human Ab cocktail specific to blood cells (for the negative selection of B-cells) per 10^6 cells and incubate on ice for 30 min[21].

NOTE: The anti-human Ab cocktail should at least include Abs specific to CD2 (or CD3), CD14, and CD16.

1.11 Add a 10-fold excess volume of sterile PBS to the cells, centrifuge at 600 x g for 5 min, and discard the supernatant.

1.12 Add equal amounts of streptavidin-conjugated microbeads (5 µL per 10^6 cells) to the pellet and mix thoroughly.

1.13 Incubate on ice for 30 min in a 15-mL plastic conical tube.

1.14 Add 2 mL of PBS buffer into the tube.

1.15 Place the tube into a magnetic stand and incubate at RT for 8 min. The brown microbeads will gradually attach to the tube wall next to the magnet[22].

1.16 With the tube remaining in the magnetic stand, carefully transfer the supernatant into a new sterile 15-mL tube[22].

1.17 Repeat steps 1.14 to 1.16, and combine the two supernatants that contain the untouched B cells. Discard the microbeads.

1.18 Centrifuge at 600 x g at RT for 5 min. Discard the supernatant.

1.19 Resuspend the pellet in RPMI 1640 medium for downstream experiments.

NOTE: Typically, 1-5x10^5 B cells with a purity greater than 95% from 10 mL of peripheral blood can be isolated[23].

2. Purification and separation of memory and naïve B cells from isolated B cells

2.1 Use cells purified from step 1 and determine the cell number using a hemocytometer or an automatic cell counter.

2.2 Resuspend the cells in 100 µL of cold PBS buffer after centrifugation at 600 x g and RT for 5 min.

2.3 Add 1-2 µg of biotinylated CD27 mAb per 10^6 cells and incubate on ice for 30 min.

2.4 Add 10 mL of PBS buffer to the tube and centrifuge at 600 x g for 5 min.

2.5 Discard the supernatant and resuspend the cells in 50 µL of PBS buffer.

2.6 Add equal amounts of streptavidin magnetic microbeads (1-2 µg/10^6 cells) to cells in a 15-mL plastic conical tube.

2.7 Gently mix well and incubate on ice for 30 min.

2.8 Add 2-3 mL of PBS buffer to the tube.

2.9 Place the tube into a magnetic stand and incubate at RT for 8 min to allow the brown microbeads to attach to the side closest to the magnet.

2.10 With the tube in the magnetic stand, carefully transfer the supernatant fraction into a new sterile tube. This fraction contains the enriched $CD27^-$ naïve B cells.

2.11 Add 5 mL to the tube containing the microbeads and gently resuspend the microbeads (*i.e.*, the enriched fraction of $CD27^+$ memory B cells)[24-25].

2.12 Centrifuge at 600 x g at RT for 5 min. Resuspend the pellets with RPMI 1640 medium for the downstream experiments.

NOTE: Typically, ~30-60% of B cells can be purified as $CD27^+$ memory cells from the PBMCs of a healthy donor[7, 25-26].

3. Cell sorting for the collection of naïve B cells, memory B cells, and PBs/PCs

3.1 Using the cells purified from step 1, determine the cell number using a hemocytometer or an automatic cell counter.

3.2 Resuspend the cells in cold PBS buffer at the concentration of 10^7 per mL in a 5-mL polystyrene tube.

3.3 Add 1-2 µg of human IgG per 10^6 cells and incubate on ice for 10 min for the Fc block.

3.4 Add 1 µg each of anti-CD19-APC (clone: HIB19), anti-CD27-eFluor450 (clone: O323), and anti-CD38-PE (clone: HIT2) per 10^6 cells; mix well and incubate on ice for 30 min[4, 27].

3.5 In the last 5 min in step 3.4, add 5 µL of the commercial 7-aminoactinomycin D (7-AAD).

3.6 Add 2 mL of PBS to the tube, vortex, and centrifuge at 600 x g for 5 min.

3.7 Resuspend the cells in sorting buffer (sterile PBS with 2% BSA and 2 mM EDTA) at a concentration of 1-5x10^7 cells per mL in a 15-mL tube.

3.8 Filter the cells through a nylon mesh cell strainer (40 µm pore size) to eliminate cell clumps.

3.9 Separate the cells with a flow cytometric sorter equipped with three lasers: violet (405 nm), blue (488 nm), and red (640 nm).

NOTE: The blue laser alone is sufficient for 3-color flow cytometry[27-28].

3.10 Sort the cells into three 15-mL tubes (containing 5 mL of RPMI medium) for the simultaneous collection of naïve B cells ($CD19^+CD27^-$), memory B cells ($CD19^+CD27^+$), and PBs/PCs ($CD19^+CD27^{+/hi}CD38^+$)[3-4, 28].

NOTE: Sorted naïve and memory B cells can be cultured as described in step 4.

4. *In vitro* differentiation of isolated human $CD19^+$ B cells, $CD19^+CD27^+$ memory B cells, and $CD19^+CD27^-$ naïve B cells

4.1 Using the cells purified in steps 1.19, 2.10, 2.11 and 3.10, determine the cell number with a hemocytometer or an automatic cell counter.

4.2 Resuspend the cells with RPMI 1640 medium at a concentration of 1-10x10^5 per mL and aliquot them into the wells of a 12-well plate.

4.3 Add CpG (ODN 2006) at 5 µg/10^6 cells/mL[18, 29].

4.4 Culture the cells in a 37 °C incubator with 5% CO_2 for 5 days.

4.5 Harvest the cells from each well, place them separately into 15-mL tubes, add 5 mL of PBS to each tube, and centrifuge them at 600 x g and RT for 5 min.

4.6 Count the cells using a hemocytometer or an automatic cell counter. Resuspend the cells at a concentration of 1-10x10^5 per mL with RPMI 1640 medium.

5. ELISpot assay

5.1 Add 30 µL of 35% ethanol in distilled water to each well of the ELISpot plates for 30 s.

NOTE: When pipetting, avoid touching the membrane in the wells at all times.

5.2 Invert the ELISpot plates to remove the ethanol.

5.3 Put 150 µL of autoclaved ddH2O into each well and incubate them at RT for 5 min to flush off the residual ethanol; follow with a wash of sterile PBS at RT for 3 min.

NOTE: Steps 5.1 to 5.3 may be optional, depending on the manufacture of the plates.

5.4 Put 50 µL of 5 µg/mL polyclonal F(ab')$_2$ fragment of anti-human Ig (IgG+IgM+IgA) (in PBS) into each well of the ELISpot plates and incubate them at 4 °C overnight (preferred) or 37 °C for 2 h[11].

NOTE: Seal the edges of plates with parafilm until use.

5.5 Invert the plates to remove unbound Abs, add 200 µL of PBS to each well, and incubate them twice at RT for 3 min each time.

5.6 Add 200 µL of PBS with 5% BSA (or RPMI 1640 medium) to each well for blocking, and incubate them at RT for 2 h.

5.7 Invert the plates to remove the blocking buffer. Wash each well twice with 200 µL of PBS, as in step 5.5.

5.8 Add 100 µL of RPMI 1640 medium to each well and incubate them at 37 °C.

5.9 When ready to seed the cells, invert the plates to remove the RPMI 1640 medium.

5.10 Seed 100 µL (5x10^4), 50 µL (2.5x10^4), and 25 µL (1.25x10^4) of the cells (from steps 1.19, 2.10, 2.11, 3.10, and 4.6) into the wells of an ELISpot plate. With RPMI 1640 medium, bring the volume to 150 µL/well.

NOTE: A minimum of one or two 2-fold serial dilutions for plating the cells is recommended.

5.11 Incubate the plates in a 37 °C incubator with 5% CO_2 for 8-14 h[18]. Avoid moving the plates during incubation.

5.12 Invert the plates to remove the cells and RPMI 1640 medium.

5.13 Add 200 µL/well of PBS-T (PBS with 0.05% Tween 20) and incubate them 5 times at RT, each time for 3 min. Invert the plate to remove the wash buffer between each of the 5 washes.

5.14 Add either goat anti-human IgG-alkaline phosphatase (AP), Fcγ-specific Abs (for IgG detection, 1:5,000 in PBS-T) or goat anti-human IgM-AP, Fcµ fragment-specific Abs (for IgM detection, 1:5,000 in PBS-T) into the designated wells and incubate at RT for 2 h in the dark.

5.15 Wash each well twice with 200 µL of PBS, as in step 5.5.

5.16 Add 50 µL/well of bromochloroindolyl phosphate-nitro blue tetrazolium (BCIP/NBT) substrate solution. Purple-colored spots normally appear in 5-15 min.

5.17 Add 100 µL/well of ddH_2O to prevent the over-development of spots.

5.18 Rinse the plates with running tap water after the complete development of all spots.

5.19 Remove the underdrain of the plates and allow them to air dry in the dark.

5.20 Count the spots using an automated plate reader with an image acquisition/analysis unit (*e.g.*, an automatic scanner or manually via a dissecting microscope).

NOTE: The plates can be stored at RT in the dark and analyzed later.

REPRESENTATIVE RESULTS

PBMCs were depleted of RBCs and adherent cells (steps 1.2 to 1.7). An aliquot ($2x10^6$) of cells were subjected to a flow cytometric analysis to illustrate the populations of naïve B cells, memory B cells, and PBs/PCs in peripheral blood (**Figure 1**). In this donor's PBMCs, about 10% of the lymphocytes were $CD19^+$ B cells. In the B-cell compartment, the percentage of $CD19^+CD27^-$ naïve B cells was around 50%. On the other hand, about 50% of the $CD19^+$ B cells were $CD27^+$ memory B cells[7, 25-26]. Of note, $CD27^+$ memory B cells can be further separated with the inclusion of IgD[4]. The phenotype of circulating PBs can be better defined with low or no surface expression of CD20 ($CD19^+CD27^{+/hi}CD38^+CD20^{lo/-}$)[2, 30-31]. To exclude the dead cells, the 7-AAD can be included in cell staining (step 3.5), and a plot for FSC versus 7-AAD allows for gating the population of live cells ($7\text{-}AAD^-$).

The key steps of the ELISpot assay are depicted in **Figure 2**. Both purified human B cells and $CD19^+CD27^+$ memory B cells were cultured in the presence of CpG with RPMI 1640 medium for 5 days. Cells were harvested for the ELISpot assay for quantifying the newly-emerged ASCs. If present, the pre-existing PBs isolated from the peripheral blood will die out in 48 h[11]. The ELISpot assay was performed, and the spot images acquired by an automatic plate scanner are

demonstrated in **Figure 3**. The results of ELISpot typically will show 50-200 IgM-ASCs from 10^4 B cells treated with CpG for 5 days, whereas the number of IgG-ASCs is 10-50 in 10^4 cells[11].

[Place Figure 1 here]

Figure 1. **Illustration of the gating strategy to separate the naïve B cells, memory B cells, and PBs in the PBMCs.**
(A) Cells ($2x10^6$) were stained with 2 μg each of CD19-APC, CD27-eFluor450, and CD38-PE mAbs (step 3.4). The lymphocyte compartment was gated (G1) on the dot plot for forward scattering (FSC) versus side scattering (SSC). **(B)** Cell doublets, which are formed by two cells stuck together, were excluded (those outside G2) on the plot for FSC-W (width) versus FSC-H (height). **(C)** $CD19^+$ B cells were gated as G3 on the plot for SSC-A (area) and CD19-APC. **(D)** Of the $CD19^+$ cells, the dot plot of CD27 and CD38 parameters showed naïve B cells ($CD19^+CD27^-$; Q1 and Q4), memory B cells ($CD19^+CD27^+$; Q2 and Q3), and PBs ($CD19^+CD27^{+/hi}CD38^+$; G4, 0.6% of Q2).

[Place Figure 2 here]

Figure 2. Illustrations of the key steps of the ELISpot assay.
(A) Put 50 μL/well (5 μg/mL) of $F(ab')_2$ fragment of anti-human Ig (IgG+IgM+IgA) into an ELISpot plate for 2 h at 37 °C or overnight at 4 °C (preferred) (step 5.4). Seed the cells into the wells of the ELISpot plate. Allow the ASCs to attach to the PVDF membrane and to secrete Abs for 8-14 h (step 5.11**)**. **(B)** Wash off the cells with PBS (with 0.05% Tween 20). **(C)** Add the AP- or HRP-conjugated detection Abs specific to either IgG or IgM. **(D)** Wash off the detection Abs that are unbound. **(E)** Develop the spots with a substrate solution of AP or HRP to match the detection Abs.

[Place Figure 3 here]

Figure 3. ELISpot results from a representative plate.
(A) Purified $CD19^+$ B cells were placed into three adjacent wells (50,000 cells in well #1, 25,000 cells in well #2, and 12,500 cells in well #3, respectively) of the ELISpot plate. Cells were then cultured in RPMI 1640 medium overnight (~14 h). The ELISpot assay was performed after completing the culture (steps 5.12 to 5.18). To analyze the results, the ELISpot plate was scanned to acquire images via an automatic analyzer equipped with the scanner and software. **(B)** Purified $CD19^+CD27^+$ memory cells (10^6/mL) were cultured in the presence of 5 μg/mL of CpG for 5 days. The cells were treated as in **(A)** for the ELISpot assay.

DISCUSSION:

Isolation and purification of human peripheral blood B cells

Normally, RBCs can be efficiently ruptured and cleared by lysis buffer (step 1.2). It is important not to incubate PBMCs with the RBC lysis buffer longer than 5 min, as cell viability might be affected by the ammonium chloride. Alternatively, RBCs and platelets can be simultaneously removed by the following protocol.

Mix fresh whole blood with acid-citrate-dextrose (ACD) buffer (39 mM citric acid, 75 mM sodium citrate, and 135 mM dextrose; pH 7.4) in a volume ratio of 9:1, blood-to-buffer. Centrifuge at 250 x g for 10 min at RT. Pay attention to the formation of layers, indicative of separation. Aspirate and discard the upper layer, which contains platelets and platelet-rich plasma (yellow color). Remove the thin middle layer at the interface, which contains PBMCs (*i*.e., the buffy coat). Avoid the contamination of RBCs in the bottom layer (dark red color). Add lysis buffer to the PBMCs to remove the residual RBCs, and then follow steps 1.2 to 1.19.

Two approaches can be used to isolate B cells from PBMCs: positive and negative selection[21]. The method of negative selection (steps 1.1 to 1.19) is used to obtain untouched, functional B cells, with no bound Abs or microbeads, for downstream experiments. It is crucial to take this into account when activation and/or differentiation of purified B cells occurs in culture. The concern is that cells purified by positive selection might be inadvertently influenced by the mAb-conjugated microbeads (0.2 to 5 µm in diameter). mAbs can bind to specific receptors on B cells and therefore might crosslink receptors for activation. Moreover, microbeads can be endocytosed by B cells through bound receptors. Although biodegradable, the microbeads can stay inside cells for longer than a week. Whether the retention of microbeads will influence experimental results needs to be empirically determined. In the purification of human B cells via positive selection, anti-CD19 (clones: 4G7 or HIB19) microbeads are commonly used because CD19 is widely expressed throughout the developmental stages of B cells. The purity of B cells after separation can be determined by flow cytometry with anti-CD19 mAbs (clones: SJ25C1 or LT19), which recognize distinct epitopes from anti-CD19 microbeads.

Purification and separation of memory and naïve B cells from isolated B cells
CD27 is a widely-accepted surface marker for human memory and naïve B cell distinction[24]. The CD27 molecule belongs to the tumor necrosis factor receptor (TNFR) family. Because CD27 is expressed on most human memory B cells, it is commonly used to discriminate them from $CD27^-$ naïve B cells. However, because CD27 is also expressed on T cells and NK cells, the use of anti-CD27 microbeads (clone: O323) in positively selecting memory B cells requires prior purification of B cells (steps 1.1 to 1.19). For a purity check after separation, mAbs specific for human CD27 (clones: L128 or LG.3A10) are recommended (step 2.12). Because $CD27^+$ memory B cells are heterogeneous, additional surface markers, such as IgD and FCRL4, can be considered for further separation in cell sorting[4, 7].

Cell sorting to obtain naïve B cells, memory B cells, and PBs/PCs
Because B cells express FcγRIIB, an Fc block (step 3.3) is recommended prior to staining the cells with mAbs for flow cytometry. The Fc fragments of the human IgG alone can block equally well as the whole human IgG at 1 µg/10^6 cells. Anti-human CD32 mAb (clones: 3D3 or AT10) is another option for the Fc block. Of note, if FcγRIIB is a surface marker to be stained in purified B cells, the fluorophore-conjugated anti-CD32 mAb should be added to waive the Fc block, followed by staining with other mAbs without a PBS wash.

For three-color flow cytometry, CD38-FITC (Fluorescein), CD27-PE (Phycoerythrin), and CD19-APC (Allophycocyanin) are a typical combination of fluorophores. Nevertheless, if the flow cytometric sorter is equipped with three lasers, such as those of 405 nm, 488 nm and 640 nm, the researchers then have the luxury to choose fluorophores excited by different lasers for

the cell sorting. As illustrated in **Figure 1**, cells were stained with CD19-APC (clone: HIB19), CD27-eFluor450 (equivalent to pacific blue, clone: O323), and CD38-PE (clone: HB7) to separate the naïve B cells, memory B cells, and PBs. Of note, some researchers prefer the inclusion of CD45 (clone: HI30) as a leukocyte lineage marker and gate B cells as a $CD45^+CD19^+$ population. Because the detection of PBs in circulation at steady state is a rare event in healthy individuals, low or even no surface expression of CD20 on PBs ($CD19^+CD27^{+/hi}CD38^+CD20^{lo/-}$) substantiates their bona fide existence[2, 30-31]. This is useful when the quantification results of PBs by surface phenotypes and ASCs by ELISpot assays are being compared. During the period prior to cell-sorting, 7-AAD is preferred to propidium iodide for the exclusion of dead cells, due to its narrower emission spectrum and lower spillover in multicolor flow cytometry.

***In vitro* stimulation and differentiation of isolated human B cells**

The type B CpG (ODN 2006) alone can induce limited differentiation of both IgM- and IgG-ASCs from memory B cells. Moreover, naïve B cells are only weakly responsive to CpG and mainly give rise to IgM-ASCs[6, 11]. The concentration of CpG in culture is in the range of 2.5-5 μg/mL per 10^6 B cells or PBMCs[11, 18]. Other than CpG, purified B cells and PBMCs can be cultured in the presence of combinations of CpG with mitogens, cytokines, and BCR agonists, to differentiate human B cells into ASCs in culture (step 4.3). For instance, commonly-used recipes for 10^6 cells per mL include (a) CpG (5 μg/mL), recombinant human IL-2 (10 ng/mL), and recombinant human IL-10 (10 ng/mL)[15, 34]; (b) CpG (5 μg/mL) and $F(ab')_2$ fragments of anti-human Ig (IgG+IgM+IgA) (20 μg/mL)[11]; (c) CpG (5 μg/mL), protein A from *S. aureus* Cowan (SAC) (1:10,000 dilution), and Pokeweed mitogen (PWM) (1:100,000 dilution)[18]; (d) recombinant human IL-21 (100 ng/mL) and recombinant sCD40L (1 μg/mL)[12, 15, 33]; and (e) recombinant human IL-2 (10 ng/mL) and R848 (1 μg/mL), a TLR7 agonist[33-34]. Although B cells can be differentiated into ASCs in three days[11], cells are generally pre-stimulated for 5 to 6 days prior to being added to the ELISpot plate for the quantification of ASCs[11, 18]. During the culture period, there is usually no replenishment of the starting differentiation agents.

The addition of IL-2 and IL-10 to CpG-containing cultures can significantly increase the number of differentiated IgM- and IgG-ASCs[15, 35]. The presence of IL-2 in culture can provide mitogenic stimulation to facilitate the expansion of differentiated B cells by CpG. IL-10 in the range of 10-25 ng/mL can greatly increase the production of ASCs (~3- to 10-fold) in the presence of CpG[35]. BCR agonists, including anti-BCR and SAC, and PWM can also stimulate proliferation of primary human B cells[18]. Incubating memory B cells with CpG and polyclonal anti-BCR mainly results in an increase in IgM-ASCs but not in IgG-ASCs[28]. For the prevention of the inhibition of signaling through BCR by FcγRIIB, a $F(ab')_2$ fragment of anti-Ig (10-20 μg/mL per 10^6 cells) is recommended when crosslinking BCR[11]. Instead of polyclonal anti-Ig, anti-BCR mAbs specific to either IgM^+ or IgG^+ B cells should be considered to target isotype-specific B-cell subsets for differentiation into ASCs. A combination of CpG, SAC (1:10,000 dilution), and PWM (1:100,000 dilution) can efficiently activate memory B cells to differentiate into IgM- and IgG-ASCs[18, 24]. Moreover, CpG can moderately induce class switching of Ig *in vitro*[28-29]. In contrast, IL-21 (100 ng/mL) and sCD40L (1 μg/mL) effectively induce memory B cell differentiation exclusively to switched IgG-ASCs[12, 30]. The sCD40L can activate B cells by mimicking T-cell help through CD40 on B cells, influencing their differentiation into PCs *in vivo*. Of note, anti-CD40 (clones: 89 or G28.5) can substitute for sCD40L in culture[15]. However, neither sCD40L

nor anti-CD40 alone is able to induce the differentiation of memory B cells. Thus, the CD40 activation of cultured B cells is often combined with a differentiation agent, such as IL-4, CpG, R848, or IL-21, any of which can promote Ig class switching. IL-4 plays a key role in the differentiation of Th2 CD4 cells, allowing the induction of most IgM^+ B cells to switch to $IgG1^+$ B cells in culture. Similarly, in the presence of IL-21, both memory B cells and naïve B cells differentiate exclusively into class-switched ASCs[12, 32]. Like CpG, R848 can induce a marginal class switch of B cells[34]. Finally, it is worth mentioning that although lipopolysaccharide (LPS) can effectively induce differentiation of mouse B cells into ASCs, human B cells express low levels of TLR4 and CD14[29]. Because the addition of a differentiation agent in culture promotes the generation of switched B cells, this addition should be avoided when pre-existing PBs/PCs or unswitched memory B cells are to be measured.

ELISpot assay

The ELISpot assay combines plate-based ELISAs with membrane-based western blotting technologies, allowing for the detection of Abs secreted by a single B cell[18]. The ELISpot has also been adapted to quantify Ag-specific T cells that secrete cytokines[17, 36]. In an ELISpot assay, either purified cells or unfractionated PBMCs (free of RBCs) can be used. However, ~10-fold more PBMCs are required than purified cells to obtain consistent results in ELISpot assays. A good starting point is $1\text{-}2x10^5$ cells per well for detecting the circulating ASCs in PBMCs. In the detection of Ag-specific ASCs, anti-Ig is usually replaced by Ag (5-10 µg/mL in PBS) to capture Ag-specific ACSs (step 5.4). The use of isotype-specific detection Abs permits the distinction between Ag-specific IgM-ASCs and IgG isotype-ASCs (step 5.14). Of note, regardless of whether anti-Ig or Ag are used to capture ASCs, the detection Abs used for ELISA might not always be suitable for ELISpot. Similarly, not all color substrates for ELISA function properly in ELISpot. AP- and HRP-conjugated Abs are most commonly used for spot detection in ELISpot. The BCIP/NBT substrate solution is a popular choice for AP-based detection, whereas 3,3',5,5'-tetramethyl-benzidine (TMB) and 3-amino-9-ethylcarbazole (AEC) solutions are common substrates for HRP in ELISpot. If the AP or HRP is directly conjugated to the detection Abs, only one step is needed for detection, as described in the protocol (step 5.14). In contrast, the detection Abs are biotinylated and require ensuing incubation with AP- or HRP-conjugated strepavidins before reacting with the substrates in a two-step method. Both one-step and two-step methods work efficiently to detect spots in ELISpot.

The ELISpot assay can quantify not only circulating but also differentiated ASCs in culture (step 1.19 versus 4.6). If present, circulating ASCs, which normally live no longer than two days in culture, can be directly cultured in the ELISpot plate, with or without purification from PBMCs[11]. In contrast, the differentiation of memory B cells requires 5 days. Hence, direct culturing in ELISpot plates after cell isolation (step 1.19) is not recommended; the dead cell debris resulting from the extended culture period can increase the background in spot detection. Instead, both purified B cells and total PBMCs can first be cultured in a 6-well or 12-well plate in the presence of mitogens and differentiation agents (step 4.3). During the culture process, the repeated addition of stimulation agents is unnecessary (step 4.3). While plates are in the incubator, the door of the incubator should be gently opened and closed to prevent seeded ASCs from moving, for they can diffuse secreted Abs along the moving tracks, generating spots with tails (the so-called "comet-like" spots) (step 5.11). The incubation period in ELISpot plates is determined by the time needed for the cells to secrete detectable amounts of Abs without allowing the cells to

divide, which would create difficulties in spot counting. Thus, cells are usually cultured in the range of 8 h to overnight (step 5.11).

The ELISpot assay is the most widely-used method for quantifying memory B cell responses. It has become a popular, independent readout of humoral immune response and immunological memory. In practice, the preceding mitogenic culture is required to activate memory B cells for differentiation into ASCs (step 4.3). However, the frequency at which memory B cells expand and differentiate into ASCs varies in different culture milieu, as described above. Although without consensus, most researchers adopt the induction condition that can maximize the production of ASCs. In terms of the agent used for spot detection, a soluble biotinylated Ag can replace the unsatisfactory detection Ab without compromising the sensitivity[37]. This is of particular importance when the availability of Ag is limited because a much lower quantity of Ag is required for detection than for coating the plates. Lastly, ELISpot does not allow phenotypic analysis of cellular heterogeneity, and thereby requires prior fractionation of cell subpopulations. Recently, a multicolor ELISpot assay, in which detection Abs are labeled with different fluorophores, has been developed to detect multiple types of cytokine-secreting cells in a single well[38-39]. This new technology will be of particular interest for the detection of low-frequency cells in PBMCs and for a large-scale analysis of vaccine-induced B-cell responses.

Because the ELISpot assay is fast and efficient in quantifying ASCs and cytokine-secreting cells, it has been extended to measure the functionality of not only circulating lymphocytes, but also tissue immune cells such as intrahepatic leukocytes[40]. Because of its sensitivity reaching the level of detecting even a single ASC, the ELISpot assay has been increasingly used to measure human immune responses to vaccine efficacies against existing and emerging pathogens. Although the high-throughput performance and robustness of ELISpot assay are superb for large-scale assessments of immune responses using PBMCs, the results of the assays can be influenced by various factors, such as the processing delay of samples, whether cells are fresh or frozen, serum components in the culture medium, and the number of cells added to the ELISpot plate[41]. Thus, normalization procedures should be included in the ELISpot protocol to minimize intra- and inter-assay variations in large-scale and longitudinal follow-up studies (*e.g.*, vaccine trials). The normalization method of plate reading is also critical for linearity, accuracy, and consistency in the assay results[41-42]. One way to perform plate reading normalization is to extend the procedure of the serial dilution of cells seeded to ELISpot plates (step 5.10). By creating a reference plate, more serial dilutions should demonstrate a linear relationship between the cell numbers plated per well and the spot counts per well. Repeated scans of the ELISpot plate can be used to validate the template of spot counting, both automatically and manually. Minimal intra-well variability in spot counts is anticipated after these normalization procedures[43]. Lastly, the application of ELISpot can be extended in many ways, such as to the monitoring of the treatment responses of patients with infections and taking cancer immunotherapies, as well as to the evaluation of immunotoxicity (*e.g.,* immunosuppression and drug-induced autoimmunity).

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Figure

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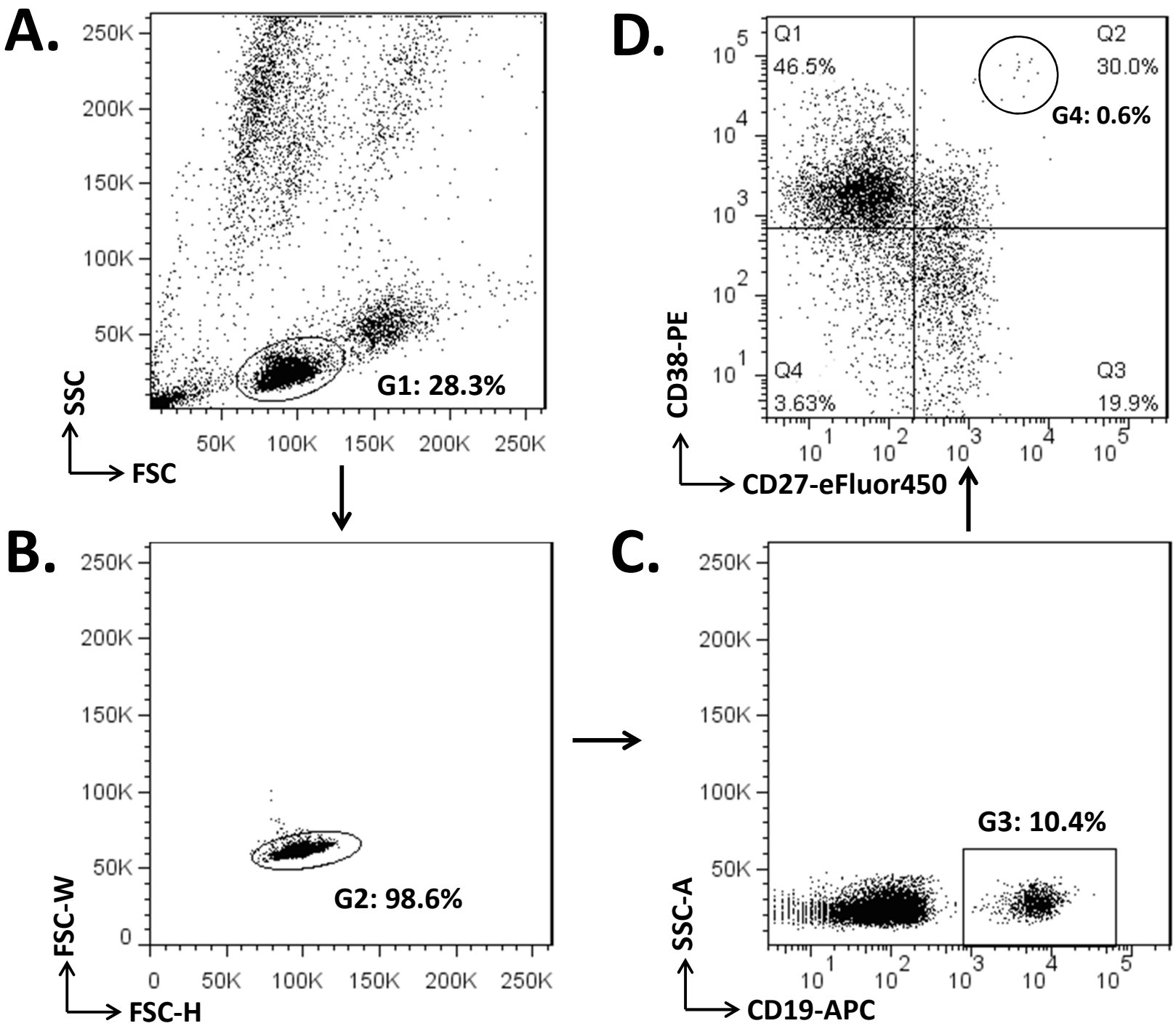

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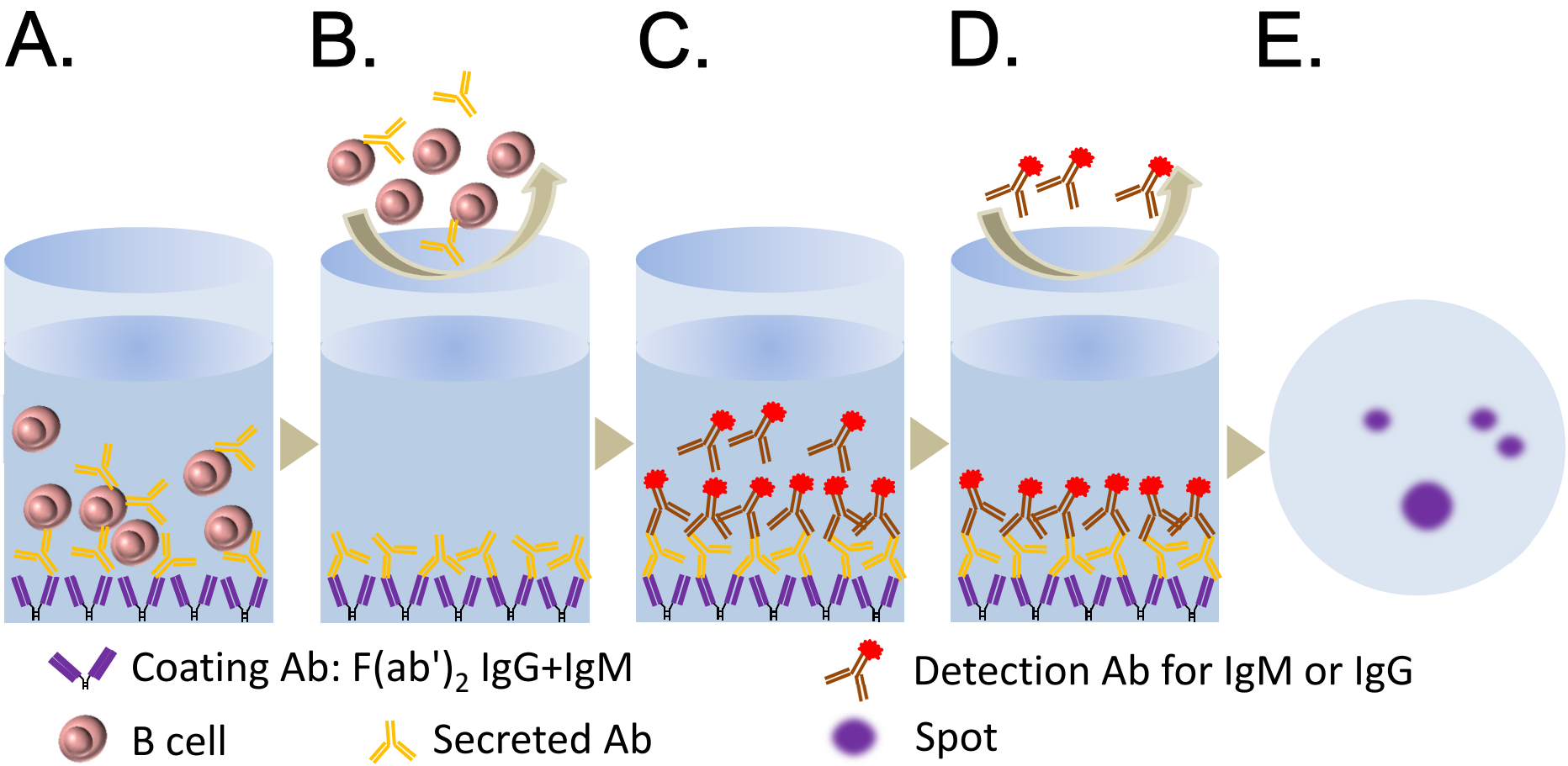

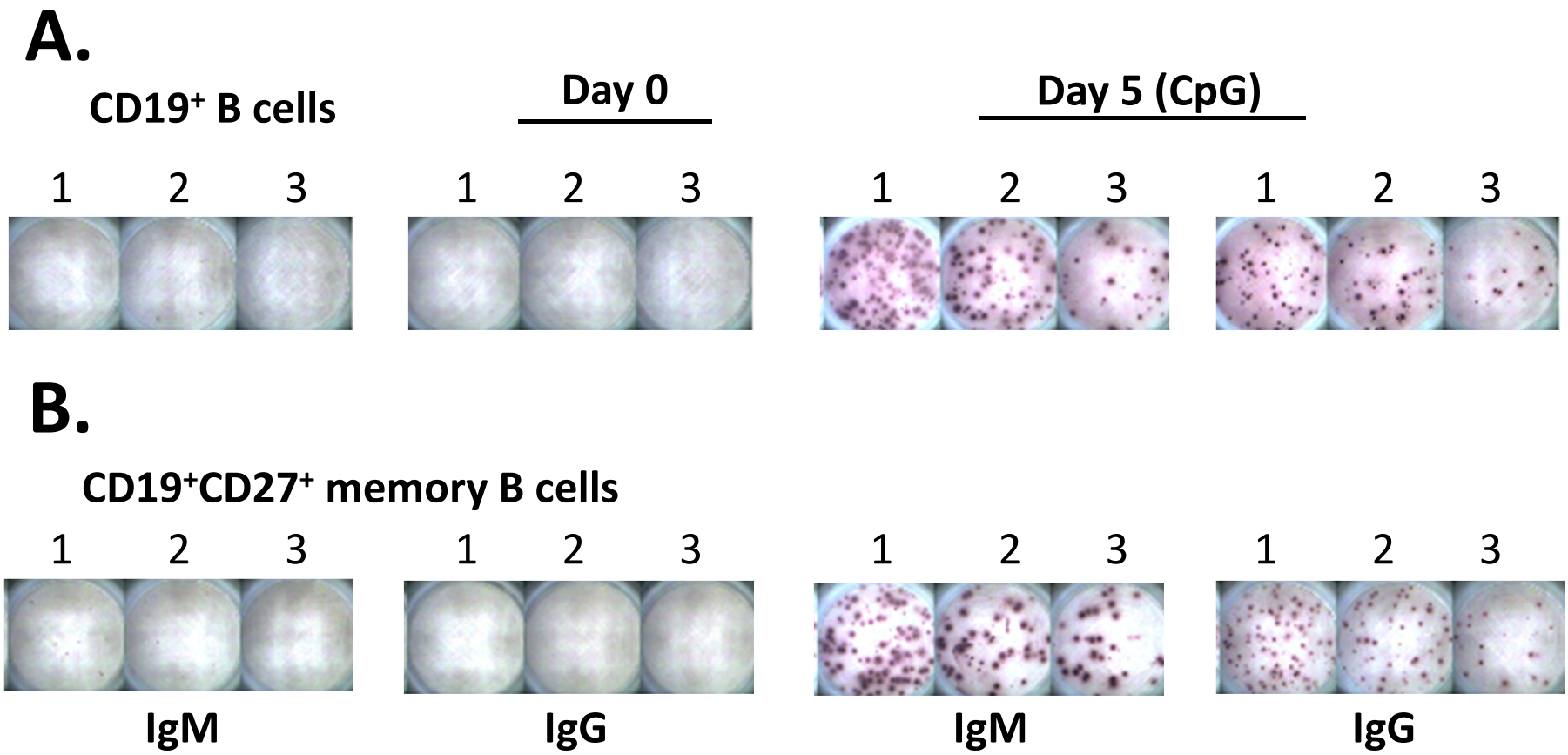

Excel Spreadsheet- Table of Materials/Equipment

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Name of the Material/Equipment	Company
BD Vacutainer K2E	BD Biosciences
Ficoll-Paque Plus	GE Healthcare
Trypan blue 0.5% solution	Biological Industries
IMag Human B lymphocyte enrichment set	BD Biosciences
Biotinylated CD27 mAb	Biolegend
Streptavidin magnetic microbeads	BD Biosciences
15 ml Falcon tubes	BD Falcon
Blue nylon mesh cell strainer, 40 µm	BD Falcon
Anti-human CD19-APC	Biolegend
Anti-human CD27-eFluor 450	eBioscience
Anti-human CD38-PE-Cy7	Biolegend
Anti-human CD38-PE-Cy7	BD Biosciences
Anti-human CD45-FITC	Biolegend
Anti-human CD45-FITC	BD Biosciences
Anti-mouse/rat/human CD27-PerCP Cy5.5	Biolegend
Anti-human CD27-PerCP Cy5.5	BD Biosciences
Anti-human CD19-FITC	Miltenyi Biotec
Anti-human CD19-FITC	GeneTex
Anti-human CD20-FITC	BD Biosciences
biotinylated anti-human CD27	Biolegend
biotinylated anti-human CD27	eBioscience
7-aminoactinomycin D (7-AAD)	BD Biosciences
CpG (ODN 2006)	InvivoGen
Recombinant human IL-2	PeproTech
Recombinant human IL-10	PeproTech
Recombinant human IL-21	PeproTech
Recombinant human sCD40L	PeproTech
Protein A of *S. aureus* Cowan (SAC)	Sigma-Aldrich
Pokeweed mitogen (PWM)	Sigma-Aldrich
MultiScreen filter plates, 0.45 µm pore size	Merck Millipore
BCIP/NBT solution	Sigma-Aldrich
BCIP/NBT single reagent, alkaline phosphatase substrate	Merck Millipore
Human IgG	Jackson ImmunoResearch
Human IgG, Fc fragment	Jackson ImmunoResearch
$F(ab')_2$ fragment of goat anti-human Ig (IgG+IgM+IgA)	Jackson ImmunoResearch
Goat anti-human IgG-alkaline phosphatase, Fcγ fragment specific	Jackson ImmunoResearch
Goat anti-human IgM-alkaline phosphatase, Fcµ fragment specifi	Jackson ImmunoResearch
Goat anti-human IgG-peroxidase, Fcγ fragment specific	Jackson ImmunoResearch
Goat anti-human IgM-peroxidase, Fcµ fragment specific	Jackson ImmunoResearch
BD ELISPOT AEC substrate kit	BD Biosciences
C.T.L. ImmunoSpot analyzer	C.T.L.

Catalog Number	Comments/ Description
367525	10 ml tube
17-1440-02	endotoxin-free
03-102-1B	
558007	
302804	clone O323
9000810	
352196	
352340	
302212	clone HIB19
48-0279-42	clone O323
303516	clone HIT2
560677	clone HIT2
304006	clone HI30
555482	clone HI30
124213	clone LG.3A10
65429	clone L128
130-098-064	clone LT19
GTX75599	clone LT19
555622	clone 2H7
302804	clone O323
13-0279-80	clone O323
559925	
tlrl-2006	type B CpG
200-02	
200-10	
200-21	
310-02	
82526	
L9379	
MSIPS4510	sterile, clear 96-well filter plate with hydrophobic PVDF membrane
B6404	
ES006	
009-000-003	
009-000-008	
109-006-127	
109-055-008	
109-055-095	
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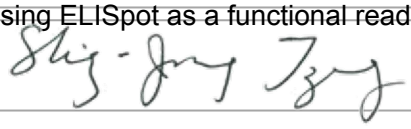
Signature:	
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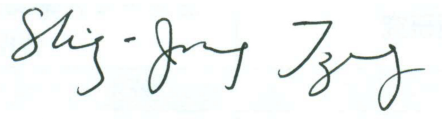
July 19, 2016

Dear Editors,

I am submitting a revised manuscript (JoVE54582R1) entitled: “The isolation, differentiation and quantification of human antibody-secreting B cells from blood: ELISpot as a functional readout of humoral immunity” by Tzeng S-J for revision. Dr. Nam Nguyen has assisted me in the submission process.

I have addressed the editorial and reviewers’ comments point by point (please see below). With the reviewers’ comments, I believe the contents in this manuscript will be very useful for researchers, who work in the fields of human B-cell biology and who would like to measure vaccine-induced B cell response.

Best Regards,



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Editorial comments:

1. Grammar:

-Please copyedit the manuscript for numerous grammatical errors. This editing is required prior to acceptance and should be performed by a native English speaker. In particular, many articles (a, an, the) are either misused or missing.

Reply: In addition to previous two Americans, the manuscript has been edited by a

native English teacher in my school.

-1.2.1 – Please correct the grammar in this step. The step should begin “To determine whether RBCs have been lysed…”
Reply: I have deleted the sentence and rephrased it, **“**NOTE: The appearance of light transmission through tube indicates completion of RBC lysis.”(line 139)

-1.15 – Should be “to the tube wall next to the magnet”.
Reply: I have corrected it (line 181).

-1.19 note – “Typically, can isolate”
Reply: I have corrected it, “Typically, “we” can isolate …”. (line 193)

-5.18 – Should be “to air dry”
Reply: I have corrected it (line 334).

-5.17 – “after completion of developing all spots”
Reply: I have rephrased it, “after completing the development of all spots.” (line 332).

2. Formatting: Please define all abbreviations at first occurrence (Abs, Ab, ELISpot, etc.). This should not be done in the keywords section, but rather in the main text.
Reply: I have corrected it.

3. Additional detail is required:
-1.2.1 – Please clarify “place the tube toward a bench daylight lamp.”
Reply: I have deleted the sentence (line 139).

-5.2 – Please clarify “Avoid touching the membrane in wells any time.”
Reply: I rephrase it as “When pipetting, avoid touching the membrane in wells at all times (line 283)”.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this manuscript, authors try to describe a method for total IgG/IgM/IgA ELISPOT following polyclonal activation of peripheral blood B cells. They describe a negative

selection method for B cells, mention several different polyclonal activation cocktails and then provide only a couple of representative ELISPOT pictures for some Ig isotypes. They do not give any information on how many samples they have tested with which culture condition and what results they have obtained upon different stimuli for total Ig spot production.

Major Concerns:

The journal clearly states that their main interest is not the novelty of a method but more sharing and teaching highly reproducible techniques. To achieve that even for a very straightforward method such as total Ig ELISPOT assay, the manuscript needs to be very much improved.

General remarks:

-Punctuation and grammar mistakes need to be corrected.

-References should be in order of appearance.

-In introduction, authors do not do not mention what type of an ELISPOT assay protocol (total Ig ELISPOT or antigen-specific ELISPOT) they will explain in the following sections.

-At least a model antigen should be used to show the applicability of the ELISPOT assay (such as tetanus toxoid). A total Ig ELISPOT is quite useless in a clinical setting. Furthermore, the authors use so many isolation steps of cells, how representative are their results for the in vivo situation?

Reply: This scope of this work was to describe how to perform the ELISpot assay. In the Discussion section, I have mentioned the application of antigen-specific ELISpot (lines 519 and 555) and its clinical applications.

-An overview of the method before getting into the protocol section should be given. Each step regarding incubation times, number of cells, type of cells, culture cocktail used should be written precisely and remain consistent throughout the whole manuscript.

Reply: An overview on the basics and advantages of the ELISpot was described in Introduction (lines 95-119).

-A detailed result section is missing. How many samples are tested, what are the overall findings for all the samples tested?

Reply: Since I only showed ELISpot data of CpG differentiated PCs in Figure 1, I moved the entire non-CpG parts (line 270) in the original Protocol section to the Discussion section (lines 467-481).

-Informed consent information should be complete. Just mentioning one reference number of the review board approval is not sufficient.
Reply: The reference number was actually an “approved protocol number” (lines 126-128).

Other remarks:
-Line 50: It is important to mention in introduction that memory B cells/plasma cells develop after germinal center reaction. Furthermore, short-lived plasma blasts are not PB precursors, as written in the introduction
Reply: I have deleted the sentence (line 51).

-Line 78-79: What do the authors want to tell about ELISA in this sentence? It needs rephrasing.
Reply: It was a general description about ELISA. To be more specific, I add “the titers of” serum Ag-specific Abs… (line 83).

-Line 98-99-100: Authors mention that there is a positive correlation between serum ab levels by ELISA and ASC numbers by ELISPOT. Is this always the case? References for this and contra statements should be added.
Reply: I have rephrased the sentence (lines 105-106). Consistent with our experiences, references 19 and 20 show a correlation between ELISA and ELISpot.

-Line 161: Authors isolate B cells from peripheral blood by negative selection. Do they use a commercial kit, if yes they should specify, or if they make their own anti-human Ab cocktail specific for blood cells what are these antibodies directed at?
Reply: We did use a commercial kit. Since JoVE does not allow commercial trademark or language in the text, I put the kit info in the Reagent list.

-Line 167: Which microbeads are used?
Reply: JoVE does not allow commercial trademark or language in the text. The info is in the Reagent list.

-Line 187: The authors state that typically, 1-3x10^6 B cells can be isolated from 10 mL of peripheral blood. This is impossible. Even in their own example from flow cytometry only 6.14% of lymphoctes are B cells
Reply: I have made a correction, “1-5x10^5 B cells” can be isolated (line 194). Based on the reference 23, it is estimated 1.6x10^6 B cells in 10 ml blood.

-Line 209: Brownish microbeads? Beads are either brown or they are not
Reply: At line 180, “brownish” has been corrected to “brown”.

-Line 216 and 222: references are jumping from 10 to 21 and then to 15.
Reply: I have arranged the references in order.

-Line 215: add ''what?'' to the tube?
Reply: transfer the supernatant “fraction” into a new sterile tube (line 219).

-Line 250-251-252: What do authors do with these cells? Do they culture them like the other cells or not?
Reply: I added “Note: Sorted naïve and memory B cells can be cultured as described in section 4.” at line 261.

-Line 270: Authors mention a culture frame of 3-6 days. How many days of culture exactly do they recommend for each activation condition they used? What condition should be used when? What is the most optimal condition? Why is R848 not mentioned? Any differences in memory vs. naive B cells activation?
Reply: We cultured cells in the presence of CpG for 5 days (line 271). I have added R848 in Discussion section (lines 478 and 501) and added references 32-33.

-Line 272: It would be better to use the term ''harvest the cells' instead of ''take the culture medium".
Reply: I have corrected it (line 275).

-Line 291: Do authors have any data comparing the spot count and intensity between previously coated (1week old) and freshly coated (overnight) ELISPOT plates?
Reply: I deleted the sentence.

-Line 307: Authors mention cells from section 4.7, however, there is no section number like that. Are cells from section 3.10 added to ELISPOT plates without being activated? How many cells do they exactly plate in ELISPOT wells and what is the range for cell titration they make?
Reply: I have made a correction; section 4.7 should be section 4.6 (line 310). I added a note, “NOTE: Sorted naïve and memory B cells can be cultured as described in section 4.” (line 260). Step 5.10 describes the cell number put into the ELISpot plate (line 309).

-Line 333: Which of these tools did authors use to count the spots?
Reply: We used CTL ImmunoSpot analyzer and this info is in the reagent list. JoVE does not allow commercial language in the text.

-Line 343**:** Authors mention a very high percentage of memory B cells in PBMC? Which part of figure 1 shows that 83% of CD19+ B cells is CD27+?
Reply: Base on references 25 and 26, the percentage of CD27+ memory B cells in PBMCs is 30-60% (line 228). The new Figure 1 shows about 50% of CD27+ memory B cells (line 351).

-Line 368: Here, authors mention a 6 hr to overnight incubation whereas in line 312, 8-14hr incubation is mentioned? For how long exactly, do they incubate the cells in ELISPOT plates?
Reply: Cells were cultured overnight (~14 hr, line 395)". Cells were counted in the end of CpG stimulation (step 4.6) before culturing in the ELISpot plate.

-Line 375: Where do these cell numbers come from? These cell counts seem to very high to plate for a total Ig ELISPOT assay? How do authors explain the presence of IgG spots at day 0 for CD19+ B cells and why is there no spots in well #2, if the cells plated here are half of the well# 1? Authors coat and detect the plates also with IgA abs however, do not show any representative pictures for IgA.
Reply: Yes, circulating PBs are rare (line 458). We use a new Figure 3 (line 392). Thank you for your comments. We don't routinely do IgA ELISpot though and it is not the scope of this work.

-Line 388: This paragraph does not belong to discussion since it is an alternative method to remove RBC and platelets. It should be integrated into the protocol section.
Reply: It was in the Protocol section, but I was suggested to move it to the Discussion section because it was too long as a note.

-Line 396: in this and the next paragraph, the authors discuss how to best isolate (memory/naive) B cells. They have not considered activating whole PBMC with a R848 based strategy, and optionally perform isotype ELISPOT to determine memory vs. naive.
Reply: I have added R848 (line 478) as a differentiation agent and described the use of PBMCs for the ELISpot assay (lines 470 and 539).

-Line 440: In this paragraph of the discussion, authors give an overview of the literature on different polyclonal activation cocktails for B cells. However, they do not comment on their own results about several different cocktails they mention in their protocol section. In fact, they do not show their own results regarding these cocktails.

Reply: Except for the R848, our experiences on different polyclonal activation cocktails for B cells can be found in the reference 11. Other researchers' experiences, including on the R848, can be found in the references 12-15, 18, 31-33.

-Line 464: Agents to promote Ig class switching are mentioned: this you don't want in an ELISPOT pre-culture system. In fact, the authors should rule out class switching in their pre-culture.

Reply: In vitro differentiated PCs, whether class switched or not, can be used to study the physiology of PCs. It is then up to the researchers to decide the use of agents to promote Ig class switching. Regarding your concern in vaccine studies, I have added a comment, "Since the addition of a differentiation agent in culture promotes the generation of switched B cells, it is not advised when pre-existing PBs/PCs or unswitched memory B cells are to be measured." (lines 507-510)

-Line 481: Antigen can also be used a detection agent, with the benefit of requiring less antigen. This should be mentioned (Karahan et al., Hum Immunol 2015)

Reply: Thank you for the great suggestion. I have described this at lines 555-558 and added Karahan's paper as the reference 36.

-Line 502: Authors recommend shaking of ELISPOT plates when cells are added before placing in the incubator. Doesn't this act also cause moving of the cells and produce spots with tails since these cells are activated and already producing antibodies?

Reply: I have deleted the sentence.

-Line 508: The ballpark of 6hr to overnight? Please. Keep this scientific.

Reply: I have corrected it to "the range of 8 hr to overnight" (line 547).

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

This protocol focuses on the ELISpot technique applied to the analysis of human humoral immune response. The author also describes how to purify and stimulate subsets of human B cells from peripheral blood.

The manuscript suffers from several approximations and mistakes that preclude its publication as it stands.

Two PhD students from my lab who performed this experiment routinely helped for the reviewing of this manuscript.

Our comments are listed below.

General comment:

The English shall be corrected throughout the manuscript.

There are several occasions when a range of volume or concentration is given without clear indication of how/when to choose between them.

Reply: In the Protocol section, I have minimized the description of “a range of volume and concentration”.

Specific comments:

Introduction:

- It is unclear for us what the author means by "Since naïve B cells by nature do not experience Ags, they tend to be homogeneous. In contrast, memory B cells and PBs/PCs are polyclonal and heterogeneous". The naïve B cells are even more heterogeneous than activated B cells in terms of immunoglobulin repertoire.

Reply: Thank you for the comment and I have rephrased it at lines 62-65.

- page 2 line 91: the sentence "ELISpot is a technique that employs a sandwich ELISA…" is not appropriate and could be replaced by "ELISpot is a technique related to a sandwich ELISA..".

Reply: I have corrected it as you suggested (line 96).

Protocol:

- 1.1: Blood is almost never draw from radial artery. Most blood samples are collected by venous puncture.

Reply: Thanks for the suggestion. Indeed, most people draw blood from the median

cubital vein now. I have made correction at lines 131-132 (step 1.1).

-1.3: Two rounds of RBC lysis may affect surface epitopes and lead to a very high mortality and shall be avoided
Reply: I have deleted the sentence (step 1.2).

-1.5: The macrophage elimination does not seem necessary if B cells are then purified. This part of the protocol shall be optional.
Reply: We routinely perform steps 1.5 to 1.7 to enhance the efficiency of microbead-based B cell isolation.

-1.6: The centrifuge step is detailed in 1.7 and shall then be removed from 1.6.
Reply: I have deleted the sentence (line 152).

-1.9: "centrifuge as step 1.6" shall be replaced by "centrifuge as step 1.7"
Reply: I have corrected it (line 162).

-1.10: The composition of the anti-human Ab cocktail should be given as Ab from other companies could be used instead of this kit.
Reply: I have added “NOTE: Anti-human Ab cocktail should at least include Abs specific to CD2 (or CD3), CD14 and CD16 at line 168.

-1.15: The reference of the magnetic stand should be given.
Reply: In steps 1.15 and 1.16, I have added reference 22 of the magnetic stand.

-1.17: "repeat step 1.13 to 1.15" shall be replaced by "repeat step 1.12 to 1.16".
Reply: I have made correction. It should be “steps 1.14 to 1.16. (line 187)

-1.19: It is unclear in which conditions the cells shall be resuspended in PBS rather than RPMI medium. Moreover, keeping the cells in PBS only for some period of time could lead to increased cell mortality. The same applies to 2.12.
Reply: I have deleted “PBS”.

-2.1: A centrifugation step seems to be missing between 2.1 and 2.2.
Reply: I have added the centrifugation step (step 2.2, line 201).

-2.2: In which case shall the cells be resuspended in 50ul rather than 100ul of cold PBS buffer?

Reply: The 100 ul has been deleted (line 201).

-2.3: The composition of the PBS Buffer has already been given and does not need to be repeated. The same applies to 3.2.
Reply: I have deleted them at steps 2.4 and 3.2 (lines 205 and 235).

-4.2: Which volume is added per well depending on the type of plates used?
Reply: The sentence is rephrased; “Resuspend cells with RPMI 1640 medium at a concentration of 1-10 x 10^5 per mL and aliquot them into wells of a 12-well plate.” (lines 268-269).

-4.5: This sentence is unclear and should be rephrased.
Reply: I have rephrased the sentence; “Harvest the cells from each well, place them into 15 mL tubes separately, add 5 mL of PBS to each tube and centrifuge at 600 x g at RT for 5 min.” (lines 275-276)

-5.1: Ethanol treatment is not necessary for all ELISpot plates. This should be optional.
Reply: We follow the manufacturer’s instructions. Nevertheless, I have added a note; “NOTE: Steps 5.1 to 5.3 may be optional, depending on the manufacture of plates.” (line 291)

-5.6: 0.5% of BSA is not enough to saturate the wells. It is more efficient to saturate the wells with RPMI medium supplemented with FCS at 37°C for 2 hours. Thus step 5.6 could be suppressed providing that step 5.8 (incubation with RPMI 1640 at 37°C) is performed for 2 hours.
Reply: It should be 5% of BSA and it works fine in our hands. I added RPMI medium as an alternative (line 302)

-5.10: "4.7" is actually "4.6".
Reply: I have corrected it.

-5.10: The number of cells indicated here does not correspond to what is indicated in the legend of figure 3.
Reply: They are corresponded.

-5.10: It is good practice to do triplicates or at minima duplicates of all conditions.
Reply: Thank you for your suggestion, but this requires enough cells to do so. I have

described the method of normalization to enhance accuracy in the Discussion section (lines 570-582).

-5.15: A wash step is missing between 5.14 and 5.15.
Reply: I have added the wash step (line 330).

Representative results and figure legends:
There are some issues with figure 1 and figure 3.
Figure 1: the B cell subsets from peripheral blood are not gated properly. Naïve B cells are CD19+ CD27-CD38+, plasma cells are CD19lowCD27highCD38high.
Usually, all CD19+ B cells are first gated from the lymphocyte gate. On the B cell gate, naïve B cells are gated as CD27-IgD+, memory B cells are gated as CD27+ (IgD-: switched memory, IgD+:IgM memory) and plasma cells are gated as CD27highCD38high.
Accordingly, the % for the different subsets given in "representative results" are not right.
Reply: The percentage of CD27+ memory B cells is 30-60% (lines 228, ref. 7, 25 and 26). The new Figure 1 showed ~50% of B cells are CD27+.

Figure 3: It is highly surprising to observe IgG+ spots at day 0 with both total CD19+ cells and memory B cells. One possibility could be that some plasma cells are still present in the purified populations. However, based on the literature, the frequency of IgG+ and IgM+ plasma cells in the peripheral blood is equivalent. It is thus unexpected to detect spots only for the IgG ELISpot. That should be discussed.
Another surprising point is the absence of clear differences between the conditions 1 and 2 considering that twice as many cells are plated in condition 1 compared to condition 2. Altogether this suggests that the wells depicted are not representative of the experiment described.
Reply: Thank you for the comment. Please see the Figure 3 (lines 392-399).

Discussion:
In the second paragraph of the discussion, the author seems to describe the realization of a Ficoll gradient. However, a sentence must be missing as Ficoll is never mentioned. If it is indeed the description of PBMCs enrichment by Ficoll gradient, a subsequent red blood cell lysis step is normally not required.
Reply: I have deleted the Ficoll gradient.

List of Material/Equipment:

It is not clear at which step of the protocol the Biotinylated CD27 Ab-conjugated microbeads are used. The anti-human CD19-APC from Biolegend is indicated twice.
Reply: I have deleted the biotinylated CD27 Ab-conjugated microbeads and the duplicated anti human CD19-APC.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The submitted MS describes a useful and adaptable method for the isolation, differentiation and quantification of human ASC cells using ELISpot. Acceptance for publication is recommended with the following changes.

Major Concerns:
Flow cytometry gating strategy for memory, plasmablasts, and naïve B cells:

a. An excellent example of plasmablast gating exists in both, Smith, K. et al, Nat Protoc. 2009; 4(3): 372-384 and Caraux, A. et al, Haematologica, 2010;95:1016-1020. The former also describes another important utilization of the author's proposed protocol - following ASC responses to vaccines. Please include these references in the appropriate sections of the paper.

Reply: I have added these two references (ref. 30 and 31).

b. Figure 1 comments: There are several problems with the flow cytometric gating and labels. First, please add instructions for a doublet discrimination gating.

Reply: I have made correction and added a new Figure 1 (lines 368-377).

c. The protocol indicates that the cells to be sorted by flow cytometry were from the "…cells purified from section 1." The cells purified in section 1 are untouched, negatively selected B cells. Therefore, most of these cells will be CD19+ (low to high). The second flow diagram (CD27 x CD19) should therefore

represent a gradient of CD19+ cells, and not a positive and negative population of CD19. It appears possible that the authors have gated on CD19hi cells and gated out many CD19low/int cells, which should have been included in the analysis. It is hard to tell without seeing the flow gating controls that were used. See your reference #2: Jackson, S.J., et al (2008), Table 5.2.

Reply: I used unpurified PMBCs in Figure 1 (lines 368-377).

d. More terminally differentiated "plasma cells" express CD138 and are generally located in the bone marrow. The flow diagram indicating, Q2 - "Plasma cells" should be labled as "plasmablasts" without this discriminating marker. Please correct this in the manuscript as well.

Reply: I have deleted plasma cells (PCs) and used PBs (lines 369 and 68).

e. Furthermore, circulating plasmablasts will be rare in healthy individuals (2/ul blood, see Caraux, A. et al, Haematologica, 2010;95:1016-1020) and are normally able to be sorted only after an immunization (or during infection or disease state), which gives rise to a flush of newly-generated plasmablasts. While the plasmablast population can be found increased without immunization in some disease states, such as SLE (Potter, K.N., et al Lupus, 2002;11, 872-877), it is very unlikely that sorting this population would be possible in the peripheral blood of healthy, unimmunized subjects - especially at the numbers needed for sorting. This should be mentioned.

Reply: I have mentioned this at lines 55 and 458.

f. The gating for the Q2 plasmablast population shown in the flow diagram of this manuscript is most certainly incorrect at 64%. This is a result of incorrect gating.

Reply: The new Figure 1 gated the CD19+CD27+/hiCD38+ as the PBs (lines 368-377).

g. Assuming plasmablasts are present in the blood of represented sample, they will have begun to downregulate CD19 (see Jackson SJ, et al,2008, your ref #2). Therefore, be sure to include the entire CD19+ subset in analysis so the cells nearing plasma cell stage will be included. It is suggested that CD20 could be added to help discriminate the plasmablast population. Also, the plasmablast gate should not include all positive CD27 and CD38 cells, but be limited to the CD27+/high/CD38high cells (CD19+CD20-/loCD27+/hiCD38hi).

Reply: I have gated the CD19+CD27+/hiCD38+ as the PBs in Figure 1 and comment on the use of CD20 (lines 368-377, 460).

g. Naïve cells: In a study of 106 healthy donors, Caraux, A, et al, 2010, demonstrated that their naïve cell population represented 60-70% of the peripheral CD19+ population. The author's gating scheme results in a naïve population of 17% (Q4). It is unclear if this is a percentage of the parent, CD19+, or FSC/SSC gate. Regardless, excluding CD38+ cells from a CD19+CD20+CD27- naïve population may result in the exclusion of a substantial population of activated naïve B cells (see Potter, K.N., et al Lupus, 2002;11, 872-877).

Reply: The percentage of CD27- naïve B cells in the new Figure 1 is ~50% (lines 368-377).

Figure 2 Comments:

i. It appears that D (top panel) is color-coded incorrectly for the secreted antibody (should be yellow, but was changed to purple). Actually, the entire top-down view (A-E, top panel) is confusing and unnecessary. Please remove.

Reply: I have removed the top-down view in the Figure 2.

ELISpot dilutions and normalization.

j. Figure 3, panels A & B, Day 5 (CpG): The 1:2 dilutions of IgM and IgG CD19+ and memory B cells are not apparent. It is also unlikely at even the lower dilution that these could be quantified consistently and accurately at this density. To these figures, please include the ELISpot image from (at least) the third dilution described in section 5.10. If the dilution is still not apparent at the third dilution, include additional dilutions. See Smith, et al, 2009, fig. 1 for example of proper titrations for quantitation.

Reply: A new Figure 3 is presented.

k. In discussion section, please include discussion on ELISpot normalization procedures for inter-assay and longitudinal studies including commentary on plate reading linearity and accuracy. Please reference: Smith, S.G., et al, (2009) Identification of Major Factors Influencing ELISpot-Based Monitoring of Cellular Responses to Antigens from Mycobacterium tuberculosis. PLoS One 4(11):e7972. Doi:10.1371/journal.pone.0007972.

Reply: The ELISpot normalization procedures have been discussed at lines 570-582. Reference 41 is Smith SG's.

Minor Concerns:

1) Page 2, lines 60-62: The sentence, "Since naïve B cells by nature….." is unclear. Please expand on what is meant by "homogeneous" and "heterogenous."

Reply: I have rephrased it," Although naïve B cells are homogeneous in expressing BCR (B-cell antigen receptor)-associated molecules - e.g., CD19, CD20 and CD22 - they are heterogeneous in immunoglobulin repertoire (ref. 5)". (line 63).

2) Please provide reference for Page 2, line 69-71, "As a matter of fact, ASCs derived from in vitro……"

Reply: I added the Ref. 6 (line 76).

3) Page 2, line 85-86; Remove statement, "Moreover, the amount of Abs secreting into culture medium by ASCs is far below the sensitivity of detection by ELISA." This is not supported by many studies. Three are cited here for example: Immunology and Cell Biology (2003) 81, 305-310; doi:10.1046/j.1440-1711.2003.t01-1-01173.x AND: Clin Vaccine Immunol, May 2001 vol. 8 no. 3 482-488 doi: 10.1128/CDLI.8.3.482-488.2001 AND: Infect. Immun. January 2010 vol. 78 no. 1 253-259 doi: 10.1128/IAI.00868-09.

Reply: Thank you for the comment. I have removed the statement.

4) Page 2, line 91: (grammar) Change "sample" to "samples."

Reply: I have corrected it (line 96).

5) Please include reference for Page 3, lines 98-100, "In the measurement of humoral immune function…"

Reply: I have added references 19-20 (lines 105-106).

5) Page 3, line 93: (grammar) Remove "In a way" and start the sentence with "The ELISpot assay…."

Reply: I have corrected it (line 96).

6) Page 3, line 96: (grammar) Insert "an" before "immune response."

Reply: I have corrected it (line 101).

7) Page 3, line 101: (grammar) Replace "out of" with "from."

Reply: I have corrected it (line 105).

8) Page 3, line 107: Remove "ng/mL range" and replace with language such as, "optimized assay dependent concentrations," as many commercial ELISA kits

can detect in the pg/mL range.

Reply: I have corrected it (line 112).

9) Page 3, line 117: Please replace "was obtained" with "must be obtained" for consistency in present tense use in Protocol section.

Reply: I have corrected it (line 122).

10) Page 4, line 146: (grammar) Remove "in" before "into."

Reply: I have corrected it (line 151).

11) Page 4, line 161: Replace "(exclude B-cell specific Abs)" with "(for negative selection of B-cells).

Reply: I have corrected it (line 165).

12) Page 4, line 162: Should this read "….per 1 x 10^6 cells…," instead of "…to 10^6 cells…" What if the starting number of cells exceeded 1 x 10^6? Could the volume be increased in the same incubation tube?

Reply: Yes, “per 1 x 10^6 cells” was what I meant (line 166). Thank you.

13) Page 4, line 180: The authors instruct reader to Repeat steps 1.13 to 1.15, and combine supernatants. It is unclear what fractions are being combined. Please clarify.

Reply: I have rephrased it as **“**Repeat steps 1.13 to 1.16, and combine the two supernatants” **(**line 187).

14) Page 6, Sec. 4.1, line 256: Instructions for counting isolated cell fractions by various purification steps. Please include "or section 3.10 isolated fractions."

Reply: I have corrected it (line 265).

15) Page 7, Sec. 5.2, line 281: (grammar) insert "at" between "wells" and "any" in sentence.

Reply: I have corrected it (line 284).

16) Page 7, Sec 5.10, line 307: reference to section 4.7 should be replaced with "section 4.6."

Reply: I have corrected it (line 313).

17) Page 7, Sec 5.13, line 317: Reword this sentence to make clear that it is a "wash

step." Add instructions to invert plate to empty wash buffer between each of the 5 washes. This should be evident to one that does this assay regularly, but should be written more precisely for a reader that has no experience with the assay.

Reply: Thank you for the suggestion. I have added it at line 324.

18) Page 10, line 424: (grammar) Change "do an equally good block" to "block equally well."

Reply: I have corrected it (line 445).

19) Page 10, line 454: Change "…million" to scientific notation, as used in the rest of the MS ("1 x 10^6").

Reply: I have corrected it (lines 445 and 490).

20) Page 11, line 464: (grammar) Insert "is" between "cells" and "often."

Reply: I have corrected it (line 500).

22) Page 11, line 465: (grammar) Change "combines" to "combined."

Reply: I have corrected it (line 501).

23) Page 11, line 469: (grammar) Change "mention" to "mentioning."

Reply: I have corrected it (line 506).

24) Page 11, line 474: (grammar) Insert "The" before "ELISpot assay."

Reply: I have corrected it (line 514).

25) Page 11, line 476: Also reference Leehan, KM, Methods Mol Biol. 2015;1312:427-34. doi: 10.1007/978-1-4939-2694-7_43.

Reply: Thank you for the great suggestion. I have added it as Ref. 36 (line 515).

26) Page 11, line 478: (grammar) Change from "…of ELISpot…" to "…in ELISpot…"

Reply: I have corrected it (line 515).

27) Page 11, line 495: Remove reference to step 4.7 and replace with step 4.6 (step 4.7 does not exist).

Reply: I have corrected it (line 534).

28) Page 12, line 508: (grammar) The use of idioms in scientific writing should be

avoided, as they are phrases that have a figurative meaning that may not be understood by all readers. Please remove the idiom, "in the ballpark," and replace with more formal language.
Reply: I have corrected it to “in the range” (line 547).

29) Page 12, line 527: Include reference, Smith, K. et al, Nat Protoc. 2009; 4(3): 372-384, along with your reference #13.
Reply: I have added the reference as ref. 30 (line 539).

30) Reference number 1 is incorrectly shown as "Benmark." Please correct to "Bemark."
Reply: I have corrected it (line 597).

Additional Comments to Authors:
N/A