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Detection and Enrichment of Rare Antigen-specific B Cells for Analysis of Phenotype and Function

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Abstract

B cells reactive with a specific antigen usually occur at a frequency of <0.05% of lymphocytes. For decades researchers have sought methods to isolate and enrich these rare cells for studies of their phenotype and biology. Approaches are inevitably based on the principle that B cells recognize native antigen by virtue of cell surface receptors that are representative in specificity of antibodies that will eventually be secreted by their differentiated daughters. Perhaps the most obvious approach to the problem involves use of fluorochrome-conjugated antigens in conjunction with fluorescence-activated cell sorting (FACS). However, the utility of these methods is limited by cell frequency and the achievable rate of analysis and isolation by electronic sorting. A novel method to enrich rare antigen-specific B cells using magnetic nanoparticles that results in high yield enrichment of antigen-reactive B cells from large starting cell populations is described. This method enables improved monitoring of the phenotype and biology of antigen reactive cells before and following *in vivo* antigen encounter, such as after immunization or during development of autoimmunity.

Keywords

Immunology; Issue 120; B cells; flow cytometry; antigen-specific; enrichment; vaccination; autoimmunity

Introduction

Limiting dilution analyses of antibody-secreting cell precursor frequency have suggested that B cells reactive to a particular antigen typically occur at a frequency of 0.05 to 0.005%

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Disclosures

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in the normal repertoire, depending on vaccination status and size/number of epitopes present on the antigen. The low frequency of these cells has made it difficult to study changes in their status during development of immune responses, such as following vaccination or exposure to a foreign antigen, or during development of autoimmunity. Previously, researchers have undertaken isolation of antigen-reactive B cells using techniques ranging from antigen coated plates or column adsorbents, to antigen-coated red blood cell resetting, to fluorescence-activated cell sorting[1,2,3,4,5,6]. Though these techniques have been successful in identifying and isolating antigen-reactive B cells, the results have varied in terms of yield, purity and scalability. Recently we developed a novel method to both detect and enrich rare B lymphocyte subpopulations using magnetic nanoparticles. The method enables enrichment with relatively high yield and purity from large starting populations, and is compatible with analysis of responses to antigen. By enriching from populations of cells in suspension, the method eliminates constraints that are associated with the geometry of antigen-coated plates or columns, and limit throughput. Finally, since enriched cells remain associated with antigen and a fluorescent reporter, they can be further purified by FACS sorting. As described herein we have used this approach for study of peripheral blood tetanus toxoid-reactive B cells before and following immunization of human subjects, as well as autoantigen-reactive B cells from subjects with various autoimmune disorders, including type 1 diabetes, Graves' disease, and Hashimoto's disease[7]. The method works equally well in mouse and human, and is compatible with analysis of antigen-reactive B cells from a variety of tissues (manuscript in preparation).

In its basic format, peripheral blood mononuclear cells are first incubated with biotinylated antigen along with antibodies to cell surface antigens required for phenotypic analysis. This labeling step is followed by washing and fixation, and addition of streptavidin coupled to far-red-fluorescent dye for detection of the biotinylated-antigen binding cells (Figure 1). Previous studies have identified antigen-specific B cells in a similar manner but using antigens directly conjugated to a fluorochrome[8,9,10,11]. Although this is a worthy approach, use of biotinylated antigens in conjunction with streptavidin enables greater signal amplification (hence better differentiation of binding and non-binding cells), particularly when antigens are small[12,13,14]. An additional consideration is the use of streptavidin instead of avidin because streptavidin is deglycosylated, decreasing non-specific binding. Further, we use far-red-fluorescent dye as the fluorochrome due to its photostability, quantum yield (brightness), and its small size (~1.3 kD). Protein fluorochromes such as phycoerythrin (~250 kD) and allophycocyanin (~105 kD)[15] are not optimal because they potentially contain many antigenic epitopes. Use of a small organic fluorescent dye composed of a single epitope, such as far-red-fluorescent dye, reduces complexity of the isolated cell population.

Once cells are biotinylated-antigen and far-red-fluorescent dye-streptavidin adsorbed, they are enriched using anti-far-red-fluorescent dye-conjugated magnetic nanoparticles. Single nanoparticles are not detected by most flow cytometers and therefore need not be removed prior to purification by FACS sorting and downstream assays[16]. Magnetic selection for antigen-specific B cells enriches the population of interest, eliminating the time and cost of sorting rare events using a flow cytometer.

Below we show representative results from enrichment of tetanus-toxoid-specific B cells from a subject before and seven days after tetanus toxoid booster immunization. We chose this particular application as an example in order to demonstrate the ability of this method to enrich antigen-specific B cells following acute *in vivo* stimulation. When coupled with flow cytometry, this method is capable of enriching and differentiating antigen-specific naïve, memory, and plasmablast B cells and allows the researcher to follow changes in their frequency over time. In addition, we include another possible downstream assay, *e.g.* an ELISPOT assay, which demonstrates that cells retain the ability to secrete antibody following enrichment. Another application of this method could involve adoptive transfer of enriched cells into a host. We have previously shown cells maintain the ability to act as antigen presenting cells to antigen-specific T cells following isolation and transfer (data not shown). Hence, there are a number of possible downstream assays that could be coupled to the method, which together informs the understanding of the antigen-specific immune response. We have described the method below, including controls to determine overall yield, purity, cell specificity, and fold-enrichment.

Protocol

1. Isolation of Human PBMCs

1. Collect 30–50 ml of blood using heparinized blood collection tubes.

   NOTE: The amount of blood used depends on the particular experimental question and frequency in blood of antigen-specific B cells of interest. Heparinized blood can be processed immediately or rocked gently overnight at room temperature for processing the following day. The delay in processing has very little effect on the efficiency of enrichment and is associated with minimal loss of viability.

2. Mix whole blood 1:1 with sterile, room temperature magnesium and calcium free phosphate-buffered saline (PBS).

3. In a 50 ml sterile conical tube, add 10 ml of room temperature density gradient solution. Store the opened density gradient bottles in the dark at 4 °C, but warm to room temperature before use.

4. If possible set the pipette gun to the slow setting and/or apply very little, yet consistent, trigger pressure to slowly layer 30–35 ml of the diluted blood on top of the density gradient, being careful not to mix the two.

   NOTE: Placing the tip of the pipette against the edge of the 50 ml conical while adding the diluted blood will help ensure a consistent steady flow.

5. Centrifuge at 400 × g for 30 min at 20 °C with the brake turned off.

6. Remove the upper layer, which contains plasma and platelets, being careful not to disturb the mononuclear cell layer below.

7. Using a sterile pipette, collect the mononuclear cell layer (buffy coat) and place into a separate sterile 50 ml conical tube.

8. Add PBS to the cell suspension to a volume of 50 ml. Centrifuge at 400 × g for 10 min at 20 °C with brake.

9. Remove supernatant and resuspend pelleted cells in 10 ml of PBS. Count cells and determine viability using a hemocytometer or automated cell counter.

10. Resuspend cells at 10^7 cells/ml and place on ice until ready for further processing.

2. Staining of Cells

1. Remove 1–2 × 10^6 cells for each fluorescence compensation/FMO control needed, if applicable. Centrifuge remainder of cells at 400 × g for 10 min at 4 °C with brake.

2. Resuspend cells in sterile filtered, cold FACS buffer (PBS + 1% bovine serum albumin (BSA) + 0.01% sodium azide) at 3–6 × 10^7 cells/ml (remove in a 1.5 ml centrifuge tube). Ensure that the FACS buffer is cold and keep it on ice throughout the staining procedure.

    1. Divide the cell population into fourths (A–D) when optimizing the assay. Use fraction A to enrich antigen-specific B cells of interest, use fractions B and C to determine the specificity of enrichment/staining (see 2.5 and 2.6 below), and use fraction D to determine the frequency and number of antigen-specific cells in the unenriched population to enable calculation of yield and fold-enrichment.

3. Add human FcγR blocking reagent to fractions A–D on ice to prevent binding of antibodies to Fc receptors.

    NOTE: Follow manufacturer's instructions for appropriate conditions.

4. Add fluorochrome-conjugated antibodies to cell surface antigens of interest to fractions A–D for 30 min on ice in the dark, being careful not to include fluorochromes that may cross react with the anti-far-red-fluorescent dye antibodies coupled to magnetic nanoparticles.

    NOTE: These include antibody conjugates to near-IR fluorescent dye, Cy5, Cy5.5, and Cy7. Any viability stains that are added should be compatible with the use of a fixative. If one does not intend to analyze the cells using FACS, but determine the antibody-secreting frequency, for example, by ELISPOT, surface staining with antibodies is unnecessary.

5. To test the specificity of the assay, incubate Fraction B with sufficient amount of unlabeled antigen for 30 min on ice, such that the majority of the receptors with an affinity of at least 10^{-6} M are blocked.

    NOTE: Typically, a good starting concentration is a 50–100 fold excess of the amount of antigen deemed sufficient in 2.6 below (*e.g.* 50–100 μM). This should block any B cells with a high affinity for the unlabeled antigen to bind to the biotinylated antigen, which is added in step 2.6 below, thus allowing

confirmation of the specificity of the cells enriched. This step can be completed in conjunction with step 2.4 above.

6. Add biotinylated antigen to Fractions A, B, and D. Incubate on ice for 30 min.

   NOTE: Concentration of biotinylated antigen should be titrated to determine the optimal amount needed to ensure adequate detection and separation of binding cells from non-specific bystander cells. Typically a good starting concentration to test is 1–5 μM.

   NOTE: For Fractions A and D, this step can be done concurrently with step 2.4 above. For Fraction B, this step should be done after step 2.5 (washing in between steps is unnecessary).

7. Omit biotinylated antigen from Fraction C in order to determine any binding of B cells to other reagents in the protocol, such as streptavidin-far-red-fluorescent dye and the magnetic beads.

8. Wash cells twice by suspension of cells in 1 ml of cold FACS buffer per 5×10^7 cells and centrifugation at 400 × g for 5 min at 4 °C.

9. After the final wash, dilute concentrated cell suspension in 1 ml 2% formaldehyde per 5×10^7 cells and let sit in dark on ice for 5 min.

   NOTE: Fixation of the cells at this point ensures that the biotinylated antigen remains bound to the BCR for the remaining of the procedure. For viable cells – for downstream analysis, such as in ELISPOTs or adoptive transfers, omit the fixation step.

10. Wash cells twice with 1 ml of cold FACS buffer per 5×10^7 cells and centrifuge at 400 × g for 5 min at 4 °C. Resuspend in 1 ml cold FACS buffer per 5×10^7 cells.

11. Add 1–2 μg streptavidin-far-red-fluorescent dye/ml and incubate on ice for 20 min in the dark. Titrate the amount of streptavidin for each application.

12. Wash cells twice with 1 ml cold FACS buffer per 5×10^7 cells and centrifuge at 400 × g for 5 min at 4 °C. Fraction D preparation is completed at this point as it will not undergo enrichment using the magnetic beads.

    NOTE: This sample will be used to determine the frequency and number of antigen-specific B cells in the sample. This will be used to determine the yield and fold-enrichment achieved by enrichment.

3. Magnetic Nanoparticle-based Enrichment

1. Resuspend cells from samples A–C in 1 ml of cold separation buffer (PBS + 0.5% BSA + 2mM EDTA) per 5×10^7 cells and pass through a 40 μm filter to eliminate any clumps that could clog the column.

2. Add Anti-Cy5/Anti-far-red-fluorescent dye nanoparticles. For optimal results, the concentration of nanoparticles should be titrated for each use, but a good

starting point is 50 µl suspension per 5×10^7 cells/ml. Rotate in the dark at 4 °C for 10 min.

3. Wash twice with 1 ml cold separation buffer at 400 × g for 5 min at 4 °C. Resuspend cells in 1 ml of cold separation buffer per 5×10^7 cells.

4. Magnetic Enrichment Using LS or LD Columns

1. Place three LS or LD columns in the magnetic field of a magnetic separator and add 3 ml of separation buffer to wet the columns (see product description to determine which type of column is best suited). Allow all 3 ml to pass through the column and discard after collection.

   NOTE: Although separation of magnetic nanoparticle labeled cells can be accomplished using any one of the magnetic separation devices that are commercially available, the laboratory has had the most success with the use of LS columns, in terms of yield, purity, and efficiency of enrichment.

2. Place three 15 ml conical tubes labeled “Fraction A/(B)/(C) negative”, for negatively selected cells, under the column. Add the magnetic particle labeled cells to their respective column and allow the entire volume to pass through.

3. Add 3 ml of cold separation buffer on top, and allow this to pass through the column and repeat with 2 ml. Collect the negatively selected cells and set aside on ice.

4. Remove the column from the magnetic field and place on top of a 15 ml conical tube labeled “Fraction A/(B)/(C) positive”, for positively selected cells.

5. Fill the column to the top with approximately 6 ml of separation buffer and immediately plunge this volume through the column to collect cells that had bound to the magnetic field using the provided plunger.

6. To increase purity, the positively selected cells can be further enriched using a second clean column, repeating the procedure.

7. Spin both the negatively and positively selected fractions at 400 × g for 10 min at 4 °C and suspend in desired final volume. Proceed with downstream analysis, such as FACS, ELISPOT or adoptive transfer.

Representative Results

Analysis of purity, yield, and fold-enrichment using flow cytometry

Populations enriched as described above inevitably contain contaminating cells that have not bound streptavidin- far-red-fluorescent dye but are trapped in the matrix. These impurities can be removed from enriched populations by FACS sorting. To estimate purity of enriched populations, gate on live cells based on forward and side scatter and/or live/dead stain and analyze far-red-fluorescent dye + cell frequency. The percentage within this latter gate is indicative of purity. Further analysis of the specificity and antigen affinity of these cells

requires FACS sorting of single cells, followed by cloning and sequencing of expressed immunoglobulin genes and recombinant antibody production and analysis.

Yield refers to the number of isolated antigen-binding B cells recovered in Fraction A relative to the number in the starting population (Fraction D). Since the same number of cells were in Fraction D as Fraction A, one can directly determine the yield (# of antigen-binding B cells in Fraction A ÷ # of antigen-binding B cells in Fraction D).

Fold-enrichment (E) is reflected by the percentage of far-red-fluorescent dye + B cells in the "positively" enriched Fraction A relative to that in Fraction D. This value indicates the proportional increase in antigen-specific B cells achieved by nanoparticle enrichment. The value can be found using the following equation:

$$\mathrm{E}=\frac{\%\,\text{antigen}-\text{specific in enriched}}{\%\,\text{antigen}-\text{specific in unenriched}}$$

To demonstrate this technique, we show representative results obtained using tetanus-toxoid (Tet-Tox) as a model antigen to detect and enrich for Tet-Tox-reactive B cells in the peripheral blood of subjects who have been vaccinated against tetanus at varying time points. Figure 1 is a schematic of the method and the antigen adsorbent. Figure 2a shows the frequency of Tet-Tox-reactive B cells from an individual who was last vaccinated against tetanus more than a decade ago. Shown are the enriched ("positive") and depleted ("negative") populations from Fraction A and the baseline frequency of Tet-Tox-specific B cells from Fraction D. Hence, in this example we are able to enrich binding cells by around 7-fold and achieved a purity of 4% in the enriched fraction.

Figure 2b shows the specificity of the reaction using blood from a subject who had been vaccinated ~3 years earlier. When we added a 50-fold excess (50 μM) of unlabeled tetanus-toxoid to the cells before addition of the biotinylated antigen (Fraction B) we are able to block binding by 83%, demonstrating the specificity of antigen binding. When we omitted biotinylated antigen from the procedure (Fraction C), a small number (~ 0.2%) of B cells still bound; this small fraction of B cells presumably reflects binding to some non-antigen in the adsorbant, such as the streptavidin far-red-fluorescent dye, the anti far-red-fluorescent dye antibody or the magnetic beads.

To demonstrate use of the method to analyze changes in antigen-binding cell phenotype following immunization we show in Figure 3 the frequency of Tet-Tox-specific B cells in the naïve, memory, and plasmablast populations in blood drawn before and 7 days after booster vaccination of a healthy subject. While the percentage of naïve B cells among total Tet-Tox-binding B cells decreased from 84% to 64% after boost, the percentage of memory and plasmablast populations among total Tet Tox-reactive cells increased from 16% to 36% and 0% to 40%, respectively. Figure 3b shows representative ELISPOT data from peripheral blood from the 7 days post-boost healthy subject. Isolation of the Tet-Tox-binding B cells led to 4-fold enrichment in tetanus toxoid antibody secreting-cell frequency.

Discussion

Here we describe a novel method to accomplish isolation and enrichment of antigen-binding B cells from human peripheral blood. The method is readily applicable to mice and to other tissues, such as the spleen and lymph nodes, and is compatible with post-enrichment analysis of cell phenotype and function (manuscript in preparation).

The user should be cognizant of a number of variables that can affect success of this procedure. From experience dead cells tend to stick to the magnetic beads and “take up” fluorescent dyes, such as the Streptavidin-far-red-fluorescent dye, leading to non-specific increased background. It is critical to remove as many dead cells from samples as possible before beginning this procedure. Use of stains that enable live/dead discrimination to gate out dead cells from the analysis may be helpful, but presence of dead cells may compromise interpretation of assays of antibody secreting cell frequency and function following adoptive transfer.

In addition, we have found that careful titration of the biotinylated antigen added is essential to distinguishing antigen-specific and non-specific binding. Use of too little antigen will lead to underestimation of the frequency of antigen-binding cells, while too much antigen can cause false positive detection/enrichment. Use of antigen concentrations that are too high may can be indicated by apparent binding to non-B cells in the samples. Further, apparent detection of an excessively high frequency (>1%) of antigen reactive cells may be an indication of binding specificity issue. The ultimate goal when determining the optimal amount of antigen to use is to add a little bit more antigen than is present at physiological concentrations, such that the majority of the B cell receptors with an affinity of $<10^{-6}$ M, for example, for the antigen are saturated. This ensures that antigen-specific B cells are being detected with an affinity that has pathogenic potential. Addition of too little antigen, *i.e.* at or below physiological concentrations, may lead to detection of very few antigen-specific B cells due to decreased antigen occupation of the BCRs. On the other hand, addition of too much antigen, far exceeding physiological concentrations, can lead to increased non-specific binding which will include B cells with a low affinity for the antigen ($>10^{-6}$ M) and are actually ignorant of the antigen *in vivo*. Hence, it is vital to first titrate the biotinylated antigen across a few logs in order to be confident that only true antigen-reactive B cells are being detected. Similarly, we have found that use of too much streptavidin-far-red-fluorescent dye can increase background.

It is also important to note that biotinylation of antigen can affect availability of antigenic epitopes. Hence, optimization using different biotinylation methods may be important. Alternatively biotin can be coupled to primary amines, tertiary amines, sulfhydryls, or carboxyl groups. Use of extended spacer arm lengths can improve epitope availability by reducing steric hindrance.

One of the limitations to this technique is its reduced effectiveness for enrichment of antigen-binding B cells from cryopreserved compared to fresh cells. This could relate to greater cell death and associated “stickiness” in frozen samples. Nonetheless, the method can be applied to frozen samples but it is probably important not to directly compare results

from enrichment and analysis of fresh and frozen cells. Another limitation is the purity of antigen-specific B cells in the enriched sample. From experience, there are always some cells that “tag along”, which includes both non-specific B cells and T cells. This is to be expected given the rarity of the cell population on is trying to enrich. However, the purity of the cells can be increase by ensuring that a single cell suspension is obtained before adding the cells to the column, enriching a second time on a new column, or by FACS sorting the antigen-specific B cells. Depending on the type of study, the researcher can decide which option is best and whether it is necessary.

As we optimized this technique we envisioned that it would be amenable to discrimination of high affinity versus low affinity antigen-reactive cells based on antigen titration or staining intensity. However, in a previous study, we showed antigen-reactive B cells with up to a 1,000-fold difference in affinity to their antigen were equally enriched using this method[7]. Hence, formal measurement of the affinity of enriched cells using surface plasmon resonance analysis of expressed recombinant antigen receptors is required if knowledge of the affinity is pertinent to the study.

Inherent in this technique is the limitation that addition of the biotinylated antigen and streptavidin can activate the cells by cross-linking the BCR. This could pose a problem for particular downstream functional assays, such as those that seek to compare stimulated cells to a resting control. This problem may be mitigated by keeping control samples cold to prevent transduction of signals. It is also possible that BCR signals could affect an ongoing biological response. From experience antigen-enriched cells maintain their ability to secrete antibody, as indicated by ELISPOT analysis (Figure 3b) and function as antigen presenting cells to T cells *in vitro* and after adoptive transfer into mice (data not shown). A final limitation to this technique is the use of anti-fluorochome antibodies to accomplish nanoparticle binding to antigen adsorbed cells. Anti- farred-fluorescent dye cross-reacts with certain structurally related fluorochromes, including Cy7 and Cy5.5. This can limit the options of antibody conjugates available for measurement of phenotypic markers. However, with increased availability of new fluorochromes, this is becoming less of an issue.

Using this detection and enrichment method, it is possible to easily identify antigen-binding B cells in *ex vivo* peripheral blood, spleen, or other tissues. This method is particularly useful in studying the immune response of antigen reactive cells following immunization or exposure to foreign antigens and in the setting of autoimmune disease. Although no method to detect antigen-reactive B cells has proven to be without its faults, this method is fast, simple, and produces a relatively high purity, yield, and enrichment of antigen-reactive cells that can be coupled to a variety of downstream assays. In addition, due to the basic principles of the method, this procedure could easily be tailored to a variety of experimental needs.

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Figure 1. Method for detection and enrichment of tetanus toxoid (Tet Tox)-binding B cells

(**A**) Protocol for enrichment and isolation of Tet-Tox-binding B cells. (**B**) Diagram of adsorbent employed to identify and enrich Tet-Tox-binding B cells.

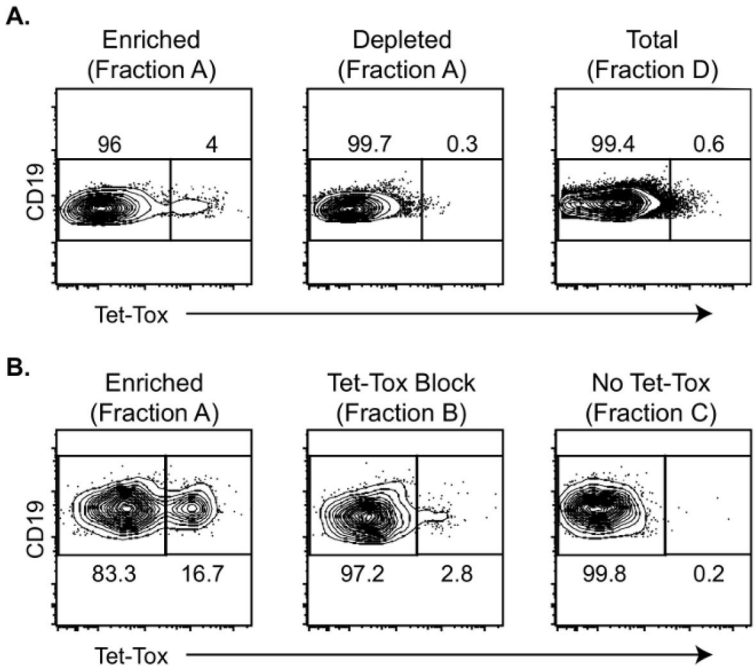

Figure 2. Representative cytograms demonstrating enrichment of Tet Tox-binding B cells from human peripheral blood

(**A**) Cytograms of enriched and depleted Tet Tox-reactive B cells from Fraction A and total Tet-Tox-reactive B cells from Fraction D from a normal subject last vaccinated >10 years previously. (**B**) Cytograms of PBMCs from the "positive" populations from a subject vaccinated ~3 years previously and enriched as in A (Fraction A), after pre-incubation with excess of 50 uM unlabeled Tet-tox (Fraction B), or enriched with Tet-Tox-biotin omitted during the procedure (Fraction C). All analyses were gated on $CD19^+$ lymphocytes. Percentages represent the percent of Tet-Tox-binding cells of all B cells ($CD19^+$) in the final fraction.

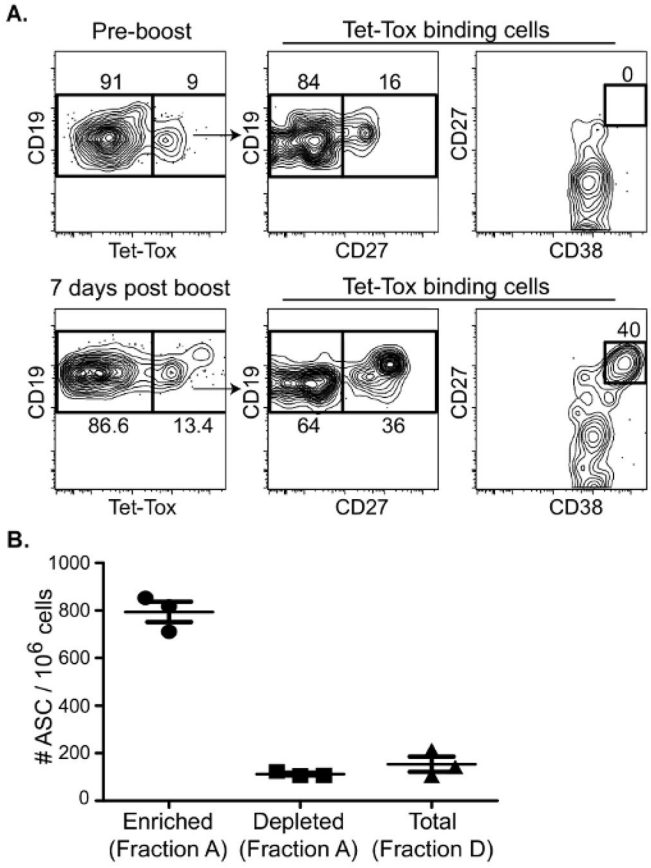

Figure 3. Examples of post-enrichment analysis of antigen-binding cells

(**A**) Representative flow cytometric analysis of cell phenotype following enrichment of Tet-Tox-binding B cells. Cytograms depict identification and analysis of Tet-Tox-reactive naïve ($CD27^-$), memory ($CD27^+$), and plasmablasts ($CD27^{++}$ $CD38^{++}$) in B cell subpopulations of a subject before and 7 days after booster vaccination against tetanus. All cells were gated on $CD19^+$ lymphocytes from the enriched "positive" populations. (**B**) Representative ELISPOT analysis of antibody secreting cells in enriched, depleted, and total unenriched B cell fractions from peripheral blood of a healthy subject 7 days after boost. For the ELISPOT assay, wells of a 96 well plate were first coated with a 10 μg/ml solution of tetanus-toxoid. Two-fold serial dilutions of cell suspensions were made in wells (across the plate) starting with cells at equal concentration from the enriched, depleted, and total unenriched fractions. Anti-tetanus antibody secreting cells were detected with biotinylated anti-human IgG (H&L), followed by Streptavidin-AP. The plate was developed with ELISPOT development buffer and the reaction was stopped by washing the plate three times with double-distilled H_2O. The number of spots at a cell dilution in the linear range was determined, and the number of ASCs was calculated.