

Research Paper

Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system

Shane Crotty^{a,b,1}, Rachael D. Aubert^{a,1}, John Glidewell^a, Rafi Ahmed^{a,*}

^aEmory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, 1510 Clifton Road, Rm G-211, Atlanta, GA 30322, USA

^bLa Jolla Institute for Allergy and Immunology, San Diego, CA 92121, USA

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Abstract

In the interest of better understanding the role of human memory B cells in protection against disease, we developed an assay to quantitate antigen-specific memory B cells in human blood. This assay utilizes a 6-day polyclonal stimulation of PBMC followed by an antigen-specific ELISPOT for the detection of memory B cells that have differentiated into antibody secreting cells (ASC) *in vitro*. We have used this assay to demonstrate that the anthrax vaccine (AVA; BioThrax) elicits a substantial population of protective-antigen (PA) specific memory B cells, and these B cells satisfy the canonical surface phenotype of human memory B cells: CD19⁺CD20⁺Ig⁺CD27⁺. These anti-PA antigen-specific memory B cells are IgG⁺ and represent up to 2% of circulating IgG⁺ B cells. Furthermore, these results confirm that vaccine-elicited memory B cells reside in the CD27⁺ B cell population. This ELISPOT-based system has been designed in a generalized manner, such that the assay can be rapidly adapted to detect human antigen-specific memory B cells of any given specificity. This method should be useful for quantitatively assessing the potency of vaccines and the longevity of B cell immunological memory to various vaccines or infectious diseases.

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1. Introduction

Memory B cells and long-lived plasma cells are responsible for the long-term humoral immunity elicited by most vaccines (Crotty and Ahmed, *in press*). Long-lived plasma cells are responsible for the con-

tinuous maintenance of serum antibody levels (Slifka et al., 1998; Manz et al., 2002; Crotty et al., 2003b). Memory B cells are responsible for driving the rapid anamnestic antibody response that occurs after re-exposure to antigen, which is important for eliminating the pathogen and toxic antigens not cleared by pre-existing circulating antibodies. Memory B cells may also play a role in replenishing the pool of long-lived plasma cells to maintain long-term antibody levels in the absence of pathogen (Slifka et al., 1998; Bernasconi et al., 2002). Though memory B cells are important for long-term humoral immunity, it

* Corresponding author. Tel.: +1-404-727-4700; fax: +1-404-727-3722.

E-mail addresses: shane@liai.org (S. Crotty), ra@microbio.emory.edu (R. Ahmed).

¹ These authors contributed equally to this work.

is unclear how well memory B cells are generated after different vaccinations, how large antigen-specific pools of memory B cells are, and how long they are maintained in the absence of re-exposure to antigen. It is also unclear whether memory B cell and serum antibody levels correlate well for most antigens, and it is therefore important to be able to track memory B cells as an independent parameter of antigen-specific immune memory.

There are two immune memory patterns that have been observed for the majority of licensed human vaccines: (1) the vaccine elicits long-term stable antibody production, or (2) the vaccine elicits antibody levels that decline over a period of a few years. Several human vaccines elicit stable long-term (>40 years) antibody responses: the smallpox vaccine (el-Ad et al., 1990; Crotty et al., 2003a; Hammarlund et al., 2003; Manischewitz et al., 2003), the yellow fever vaccine (Plotkin and Orenstein, 1999), and both the live (Plotkin and Orenstein, 1999) and inactivated (Bottiger et al., 1998) poliovirus vaccines. For those exceptionally potent vaccines, it would be quite valuable to know whether they elicit stable long-term memory B cell responses. And if they do, it would be valuable to characterize and understand the role of memory B cells in the protective immunity provided by these vaccines. Antibodies are usually the measured correlate of protection for a vaccine, but the actual protection may be mediated by circulating antibodies in combination with other arms of the adaptive immune system, such as memory B cells and memory T cells. Therefore, it is important to quantify memory B cell responses to fully assess the armamentarium of protective immune responses elicited by these very effective vaccines. In addition, memory B cells may be central players in the long-term maintenance of antibody levels and it is crucial to understand the mechanism of this process in the context of vaccines that elicit lifelong antibody production, so that we can design better vaccines in the future.

The second pattern mentioned above is observed in numerous other vaccine situations, including AVA (Anthrax Vaccine Adsorbed, now being marketed as BioThrax): circulating antibody levels drop to low levels within a few years post-vaccination. The hepatitis B (HBV) vaccine is a well-characterized example known for antibody titers that drop

over several years. Antibodies against HBsAg are the defined correlate of protection for the HBV vaccine, and the minimum protective level has been established as 10 mIU/ml (Plotkin and Orenstein, 1999). When serum anti-HBsAg antibody levels drop below that level, booster immunization is recommended. However, many individuals with low or undetectable levels of HBsAg fail to obtain booster immunizations, and some fraction of that population subsequently becomes exposed to HBV. Interestingly, many of those individuals are still protected from HBV infection. Why? It has been proposed that this protection could be due to either memory T cells or memory B cells. Memory B cells are an appealing explanation since HBV surface antigen-specific antibodies are the correlate of protection for the vaccines, and memory B cells will rapidly differentiate into anti-HBsAg antibody secreting cells upon virus exposure (West and Calandra, 1996). Of course, it is entirely reasonable to expect that memory T cells would also play a role in protection from HBV.

As mentioned above, antibodies are frequently correlates of protective immunity. Indeed, for all human vaccines for which the correlate of protection is known, that correlate is antigen-specific antibody. This is particularly true of bacterial vaccines, and in this regard the AVA anthrax vaccine functions like a classic bacterial toxin vaccine. Anthrax protective antigen (PA) is the shared protein component of both anthrax toxins (lethal toxin and edema toxin). Passive immunization with anti-PA antibodies is sufficient to protect guinea pigs from a lethal anthrax challenge (Reuveny et al., 2001; Welkos et al., 2001; Kobilier et al., 2002). This demonstrates the clear protective value of anti-PA antibodies. PA protein is a major component of the AVA vaccine. High levels of anti-PA antibodies elicited by vaccination are correlated with protection in primates given a lethal inhalation challenge with anthrax (Ivins et al., 1996, 1998). However, in some experiments some vaccinated monkeys did not have significant levels of anti-PA antibodies at the time of challenge (>1 year post-vaccination), but yet were still protected (Ivins et al., 1996). This indicates that circulating anti-PA neutralizing antibodies are sufficient, but not necessary, for protection. What is this alternative source of protection? Memory B cells are an excellent candidate for this alternative source of

protection. Memory B cells may be a particularly crucial component of protective immunity after levels of circulating anti-anthrax antibodies wane at some point post-vaccination. The memory B cells are programmed to rapidly respond to antigen exposure, proliferating and differentiating into antibody secreting cells quickly after detecting the infectious agent. This would result in a rapid replenishment of the toxin neutralizing antibody levels post-exposure. If this memory B cell response occurs rapidly enough (and it may occur within the first 3–5 days of exposure), the vaccinated individual should be protected from the anthrax toxins by the newly synthesized toxin-neutralizing antibodies. Therefore, one of our goals is to understand memory B cell responses after anthrax vaccination.

In the interest of better understanding human memory B cells, we developed an ELISPOT-based assay to quantitate antigen-specific memory B cells in human blood. Here we report the characteristics of this technique and demonstrate the ability of this technique to detect antigen-specific memory B cells after anthrax vaccination and other antigen exposures. We have also demonstrated that the detected B cells satisfy the canonical surface phenotype of human memory B cells: CD19⁺CD20⁺Ig⁺CD27⁺. Importantly, this human memory B cell assay can be readily adapted to almost any antigen of interest and should be a valuable tool for numerous investigators.

2. Materials and methods

2.1. Study participants

All AVA-vaccinated individuals were normal healthy volunteers employed at the Centers for Disease Control (CDC) and vaccinated due to potential occupational exposure. Informed consent was obtained. All AVA-vaccinated individuals had received the full schedule of five subcutaneous immunizations prior to inclusion in this study. Initial date of vaccination for the individuals ranged from 1971 to 1999, and all had received a booster immunization within the past 2 years. None had been boosted within 2 months of the dates the blood samples were obtained. Note that these individuals were not part of the AVRVP human clinical trial. Smallpox vaccine-

immunized individuals were normal healthy adult volunteers.

Anthrax-exposed individuals were part of a CDC study tracking immune responses in the individuals exposed to anthrax in the 2001 bioterrorism attacks (Quinn et al., manuscript submitted).

2.2. Cell and serum isolation

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood using 10-ml Vacutainer cell preparation tubes (CPT) (Becton Dickinson, San Diego, CA) with sodium citrate, following the instructions of the manufacturer, except washes were done using PBS+2% FCS. PBMC were resuspended in R-10: RPMI-1640+10% fetal calf serum (FCS, heat inactivated) (HyClone, Logan, Utah) and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (200 mM), and freshly added B-mercaptoethanol (β-me)(50 mM). Fresh cells were used in all assays. The memory B cell assay has since been validated on frozen cells, with similar results (Crotty et al., unpublished data).

2.3. Antibodies and reagents

Concanavalin A, phytohemagglutinin (PHA), and *Staphylococcus aureus*, Cowan (SAC) were purchased from Sigma (St. Louis, MO). CD20 FITC (clone 2H7), CD27 PE (clone M-T271), and CD3 APC were all purchased from PharMingen (San Diego, CA). Recombinant PA was provided via the AVRVP. Vaccinia virus (VV) antigen was produced by first growing VV_{WR} stocks on HeLa cells in T175 flasks, infecting at a multiplicity of infection of 0.5 (MOI=0.5). Cells were harvested at ~ 60 h, when the cells were not yet floating, and virus was isolated by rapid freeze-thawing the cell pellet 3 × in a volume of 2.3-ml RPMI+1% FCS. Cell debris was removed by centrifugation. Clarified supernatant was frozen at – 80 °C as virus stock. VV_{WR} stocks were titered on Vero cells (~ 2 × 10⁸ PFU/ml). VV antigen preparation for antibody ELISA and B cell ELISPOT use was done by UV inactivating stock VV_{WR} with trioxsalen/psoralen (4' aminomethyl-trioxsalen HCl; Calbiochem)(Tsung et al., 1996). 1 × 10⁸ PFU/ml VV_{WR} in 0.1% BSA and 10 µg/ml trioxsalen were incubated for 10 min at room temperature and then

UV-inactivated with 2.25 J/cm^2 (10 min in a Fisher UV Crosslinker UVXL-1000). This resulted in a $>10^8$ -fold reduction in PFU. UV-inactivated virus was then used at a 1:5 (ELISPOT) or 1:25 (ELISA) dilution in PBS with BSA supplemented to a final concentration of 0.1%.

2.4. Memory B cell assay

PBMC were plated in 24-well dishes at 5×10^5 cells/well in R-10 supplemented with an optimized mix of polyclonal mitogens: 1/100,000 pokeweed mitogen extract (PWM) (made at Emory University, Atlanta, GA), 6 $\mu\text{g/ml}$ fully phosphothioated CpG ODN-2006 (Hartmann et al., 2000), and 1/10,000 fixed *S. aureus*, Cowan (SAC) (Sigma). Six to twenty-four wells were cultured per individual. A negative control well was cultured in R-10 alone. Cells were cultured for 6 days at 37°C , 6–8% CO_2 . Substantial lymphocyte blasting and clustering could be observed in the culture wells from days 3 to 6. Note that experiments done for Figs. 2 and 3 were done without CpG oligonucleotide supplement. Addition of CpG improves the overall culture growth and ASC numbers by $\sim 1.5\text{--}2.0 \times$.

In preparation for the ELISPOT, 96-well filter plates (Millipore, MAHA N4510) were coated with recombinant anthrax protective antigen (PA) at a concentration of 1 $\mu\text{g/ml}$. Keyhole limpet hemocyanin (KLH, 2.5 $\mu\text{g/ml}$) (Pierce Biochemicals) was used as a non-anthrax antigen control. To detect all IgG secreting cells, a separate plate was coated with 10 $\mu\text{g/ml}$ goat anti-human Ig (or 4 $\mu\text{l/ml}$) (Caltag Laboratories, Burlingame, CA). Plates were washed and blocked with RPMI-1640 plus 1% bovine serum albumin (BSA, fraction V; Sigma) for 2–4 h at 37°C prior to use.

The cultured PBMC were washed thoroughly, plated onto the ELISPOT plates, and incubated at 37°C for 5 h. Plates were then washed with phosphate buffered saline (PBS, pH 7.2) followed by PBS containing 0.05% Tween-20 (PBST). Plates were then incubated overnight in 1 $\mu\text{g/ml}$ mouse anti-human pan IgG Fc biotin conjugated antibody (Hybridoma Reagent Laboratory #HP6043B, Baldwin, MD) in PBST+1% FCS. Plates were washed and then incubated with 5 $\mu\text{g/ml}$ HRP-conjugated avidin-D (Vector Laboratories, Burlingame, CA) in PBST–1% FCS. Plates were again washed and then developed using 3

amino-9 ethyl-carbazole (AEC, Sigma). Developed plates were counted by inspection or using a plate reader (Cellular Technology, Cleveland, OH). Data are best represented as the frequency of antigen-specific B cells as a percentage of the total IgG^+ memory B cells per million PBMC. Standard growth, as determined by total IgG^+ spots per 5×10^5 cells cultured, was 5–15,000 IgG^+ spots. A Good Laboratory Practices-like (GLP-like) standard operating procedure (SOP) of this assay is available upon request.

2.5. Pokeweed mitogen (PWM)

PWM was first identified as a mitogenic lectin for lymphocytes in 1964 (Farnes et al., 1964) and can be isolated as a crude saline extract from the roots of *Phytolacca americana* (common names: pokeweed, pokesalad, or pigeonberry), generally harvested during the summer months (Borjeson et al., 1966). Pokeweed mitogen was produced at Emory according to classic techniques. Two kilograms of *P. americana* roots were harvested in August 2002. The roots were washed extensively, chopped into small pieces, and puréed in a counter top blender. Roots were then solubilized in 2 l of sterile PBS. Cold saline extraction was performed by leaving the solution overnight at 4°C . The liquid phase was then siphoned off as the middle phase of the separated slurries. Approximately 1 liter of extract was obtained in this manner. The extract was purified and sterilized by ultracentrifugation at $27,000 \times g$ for 1.5 h. The clarified extract was then aliquoted and frozen at -80°C . $\text{PWM}_{\text{emory}}$ was compared to an old stock of PWM from Gibco (no longer available) and four ICN lots (ICN, Irvine, CA) (PWM available via Sigma is produced by ICN). $\text{PWM}_{\text{emory}}$ (hereafter simply referred to as PWM) exhibited activity greater than or equal to that of commercially available PWM, and we found enormous variability in the commercially available material, leading us to produce and use our own PWM for consistency. We recommend that individual lots of PWM from ICN be titrated for activity across a dilution range from 1:1000–1:1,000,000 (ICN, Irvine, CA).

2.6. ELISA

Direct ELISA was done using Linbro/Titertek flat-bottomed 96-well plates (ICN, Costa Mesa, CA)

coated overnight with 0.1 $\mu\text{g/ml}$ PA. Plates were washed and culture supernatant samples were added to the plate and serially diluted (twofold dilutions) in PBS + 0.2% Tween-20 + 1% FCS. Plates were incubated 90 min at room temperature. Plates were washed and then incubated with 0.25 $\mu\text{g/ml}$ mouse anti-human pan IgG Fc biotin conjugated antibody (Hybridoma Reagent Laboratory #HP6043B) in PBS + 0.2% Tween-20 + 1% FCS for 90 min at room temperature. Plates were washed and then incubated for 60 min with 1.0 $\mu\text{g/ml}$ HRP-conjugated avidin-D (Vector Laboratories) in PBS + 0.2% Tween-20 + 1% FCS. Plates were again washed and then developed using 0.4 mg/ml *O*-phenyleneamine (OPD) in citrate buffer (pH 5.0) with 0.01% H_2O_2 . Reaction was quenched with 1 M HCl after 15 min. Plates were read immediately at OD₄₉₀, with an OD₅₉₅ baseline, in a BioRad 3550 plate reader.

2.7. Purification and culture of CD27⁺ and CD27⁻ B cell populations

For the B cell sorting experiments, approximately 250×10^6 PBMC were isolated from 11 CPT blood tubes drawn from a single AVA-vaccinated donor. These PBMC were initially enriched for B cells by positive selection using anti-CD19 paramagnetic beads and MACS columns (MiltenyBiotech, Germany), using the manufacturer's recommended protocol, except the buffer used was PBS plus 0.5% FCS plus 2 mM EDTA. Both the positive selected cell fraction and the flowthrough (non-binding) cells were retained for further use. The B cell depleted fraction (CD19⁻) was run over a second positive selection column and the resulting flow through was <0.5% B cells. Following the MACS purification, there were approximately $6-7 \times 10^6$ CD19⁺ B cells eluted from the column and 120×10^6 CD19⁻ cells in the flow through fraction. The CD19 positively selected cell fraction was further fractionated by flow cytometric cell sorting (FACSVantage; Becton Dickinson) to obtain CD27⁺ vs. CD27⁻ B cell populations (all CD19⁺CD20⁺). Following cell sorting, approximately 1×10^6 CD27⁺ B cells and 1.5×10^6 CD27⁻ B cells were recovered.

Unsorted PBMCs (1×10^5) were cultured in 1 ml of R-10 containing PWM, SAC and β -me as described in the previous experiments. To mimic the

natural percentage of approximately 5% B cells in the unmanipulated PBMCs and to provide T cell help to sorted B cells, 10,000 of either the CD27⁺ or CD27⁻ B cell fraction were remixed with 450,000 of the B cell depleted fraction. The "reconstituted" well contained 10,000 each of the CD27⁺ and CD27⁻ B cell fraction. For the "T cell only" well, 450,000 of the B cell depleted fraction were cultured without any added B cells.

3. Human memory B cell assay development

Several years ago, our laboratory developed an assay to track the development and maintenance of antigen-specific memory B cells in a murine model (Slifka and Ahmed, 1996; Crotty et al., 2003b). This assay utilizes an ex vivo 6-day stimulation of splenocytes to induce quiescent memory B cells to differentiate into plasma cells/antibody secreting cells (ASC) which can then be readily measured by ELISPOT analysis, using plates coated with the viral antigen of interest (Crotty et al., 2003b). The mouse memory B cell assay was initially done using specific antigen as part of the ex vivo stimulation (Slifka and Ahmed, 1996), but was later modified to utilize a polyclonal stimulation to make the assay easier to use and standardized (Crotty et al., 2003b). Using techniques based on our mouse memory B cell assay, we have now developed a human memory B cell assay. To develop the human memory B cell assay, we screened a variety of mitogens for maximal activity inducing polyclonal proliferation of human IgG⁺ memory B cells and their differentiation into antibody secreting cells (Fig. 1). PBMC were isolated from normal human volunteers, and total PBMC were stimulated in culture for 5–7 days. After culture, cells were transferred to ELISPOT plates coated with anti-IgG antibody, and IgG secreting cells were detected in a short-term (5 h) assay. Pokeweed mitogen (PWM) combined with fixed *S. aureus* (Cowan strain, SAC) and a CpG oligonucleotide (ODN-2006) was the best combination identified (Fig. 1A). Analysis of cultures harvested at various time points indicated that maximal numbers of ASC were present at day 6 of stimulation (Fig. 1B), though robust results were also observed at day 5.

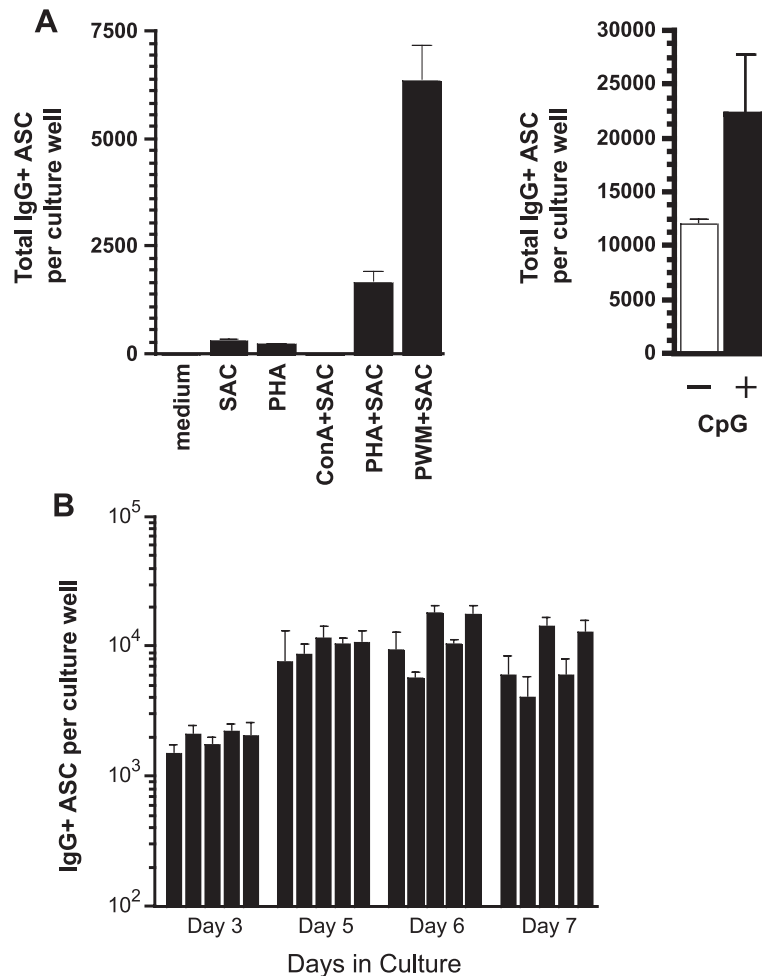


Fig. 1. Human memory B cell assay. (A) Memory B cell growth and differentiation under varied stimulation conditions. PBMC from healthy donors were cultured for 5 days with varying concentrations of polyclonal mitogens. After 5 days, these cells were washed and plated onto ELISPOT plates coated with α -human Ig antibodies, and antibody secreting cells (ASC) were detected using an α -IgG γ secondary antibody. The left graph shows a comparison of several mitogens. "Medium", medium alone. SAC, 1/10,000. Phytohemagglutinin (PHA), 2.0 μ g/ml. Concanavalin A (ConA), 3 μ g/ml. PWM, 1/100,000. Of the mitogens tested, PWM and SAC provided the best proliferation and differentiation of memory B cells into ASC. Error bars were calculated as the S.E.M. of three samples. On the right is a comparison of ASC production in PWM+SAC supplemented (+) or not supplemented (-) with phosphothiolated CpG oligonucleotide. Addition of CpG resulted in a ~ 1.5 – $2.0 \times$ increase in the total IgG⁺ ASC observed. (B) Kinetics of memory B cell expansion and differentiation into antibody secreting cells (ASC). PBMC were stimulated with PWM and SAC and assayed for IgG ASC by ELISPOT on days 3, 5, 6, and 7. Five individuals are shown. For each individual, six replicate wells were stimulated. Error bars show the full growth range among the six replicates. Maximal IgG⁺ ASC were detected on days 5 and 6 post-stimulation.

We then adapted the assay to function in an antigen-specific manner by utilizing ELISPOT plates coated with recombinant Protective Antigen (PA) of anthrax, and we used the assay to test for the presence of anti-anthrax memory B cells in the blood of individuals who received the licensed anthrax

vaccine, AVA. Memory B cell responses to the anthrax PA protein were detected in AVA-vaccinated individuals (Fig. 2A–B). PA-specific responses were not seen in unvaccinated individuals (Fig. 2A–B). The absence of responses to an irrelevant protein (KLH) is shown as a negative control for all subjects

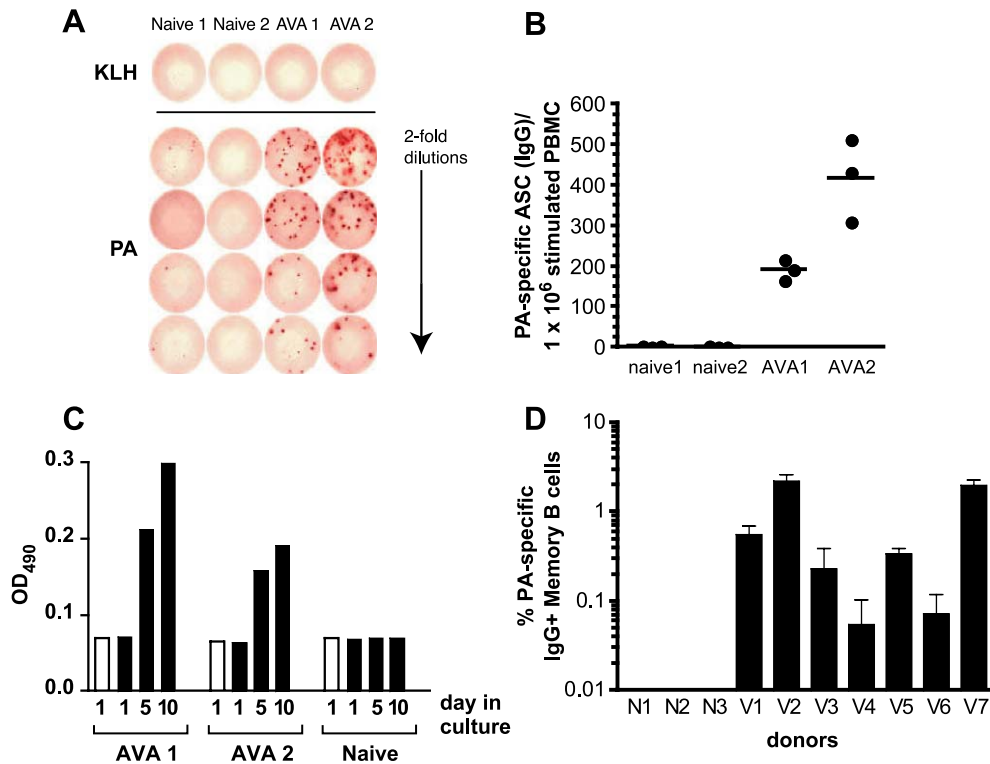


Fig. 2. Anthrax-specific memory B cells in vaccinated individuals. AVA-vaccinated volunteers were tested for the presence of PA-specific memory B cells. PBMCs from two vaccinated (AVA1, AVA2) and unvaccinated (naive1, naive2) volunteers were isolated and stimulated in vitro for 5 days with PWM and SAC. After 5 days in culture, these cells were plated onto ELISPOT plates coated with PA. Individual antibody secreting cells were detected through the use of α -IgG γ antibodies. Wells coated with KLH were used as a specificity control. (A) ELISPOT. Half of a culture well (seeded with 5×10^5 PBMC) was put into the first well of an ELISPOT plate coated with PA or KLH. Twofold serial dilutions of cells were done down the PA-coated wells. (B) Antigen-specific B cells in PBMC. PA-specific ASC detected per million cultured PBMC. Each data point represents results from a single culture well, and three replicates for each donor are shown. The bar represents the arithmetic mean of the three samples. (C) Antibody production in memory B cell cultures. To determine the level of antigen-specific antibody generated by memory B cells in vitro, culture supernatant was collected on days 1, 5, and 10 after start of culture stimulation and PA-specific ELISA was done. Black bars indicate supernatants from mitogen-stimulated cultures, white bars indicate supernatants from medium alone negative control cultures. Two representative AVA-vaccinated individuals are shown along with one unvaccinated individual. (D) Percentage of antigen-specific IgG $^+$ memory B cells. Data are represented as the percentage PA-specific B cells of the total IgG $^+$ memory B cells in PBMC. Three unvaccinated (N1–N3) and seven fully vaccinated (V1–V7) individuals are shown. Each vaccinated donor had received a full AVA vaccination schedule and each had received a booster immunization within 2 years prior to the time of experiment. PA-specific IgG $^+$ memory B cells range from 0.05% to 2.0% of the total IgG $^+$ memory B cells detected. The limit of detection of these samples ranges from 0.004 to 0.025, with the majority being <0.01 . Error bars result from the S.E.M. of six replicate ELISPOT wells per donor.

(Fig. 2A). Supernatants from the memory B cell cultures were tested for anti-anthrax PA antibodies by ELISA. Supernatants of culture wells from AVA-vaccinated individuals tested positive for anti-PA antibodies (Fig. 2C).

The number of spots observed in the ELISPOT assay is not a direct reflection of the number of antigen-specific memory B cells seeded in a culture well, as proliferation occurs during the 6-day culture.

Therefore, all PBMC samples are assayed both for total IgG memory B cells (spots observed in anti-IgG-coated ELISPOT wells) and antigen-specific memory B cells (spots observed in antigen-coated ELISPOT wells), thereby allowing quantitation of the antigen-specific memory B cells as a percentage of total IgG $^+$ memory B cells. Seven AVA-vaccinated individuals were tested and all were positive for PA-specific memory B cells (Fig. 2D). Up to 2% of circulating

IgG⁺ B cells were PA-specific in these individuals (median=0.3% of IgG⁺ B cells). Donors were at varying time points post-vaccination, and received different numbers of booster immunizations and therefore responses were expected to vary in magnitude.

Since this human memory B cell ELISPOT assay is novel, we performed a series of experiments designed to demonstrate the specificity of this assay for memory B cells. Three of these controls were shown in Fig. 2: (1) antigen-specific memory B cells are not detected in naive individuals, only vaccinated individuals; (2) memory B cells are not detected against an irrelevant protein; and (3) anti-PA antibody was only detected (by ELISA) in cultures wells where PA-

specific ASC were detected by ELISPOT. Additional sets of control experiments demonstrated that memory B cell differentiation into ASC in vitro was only detected in the presence of mitogenic stimulation, and no antigen-specific ASC were detectable in peripheral blood directly ex vivo, as expected (data not shown).

4. Vaccine-elicited memory B cells are CD27⁺

We next wanted to demonstrate that the cells identified as memory B cells in this functional assay possessed canonical characteristics of human memory

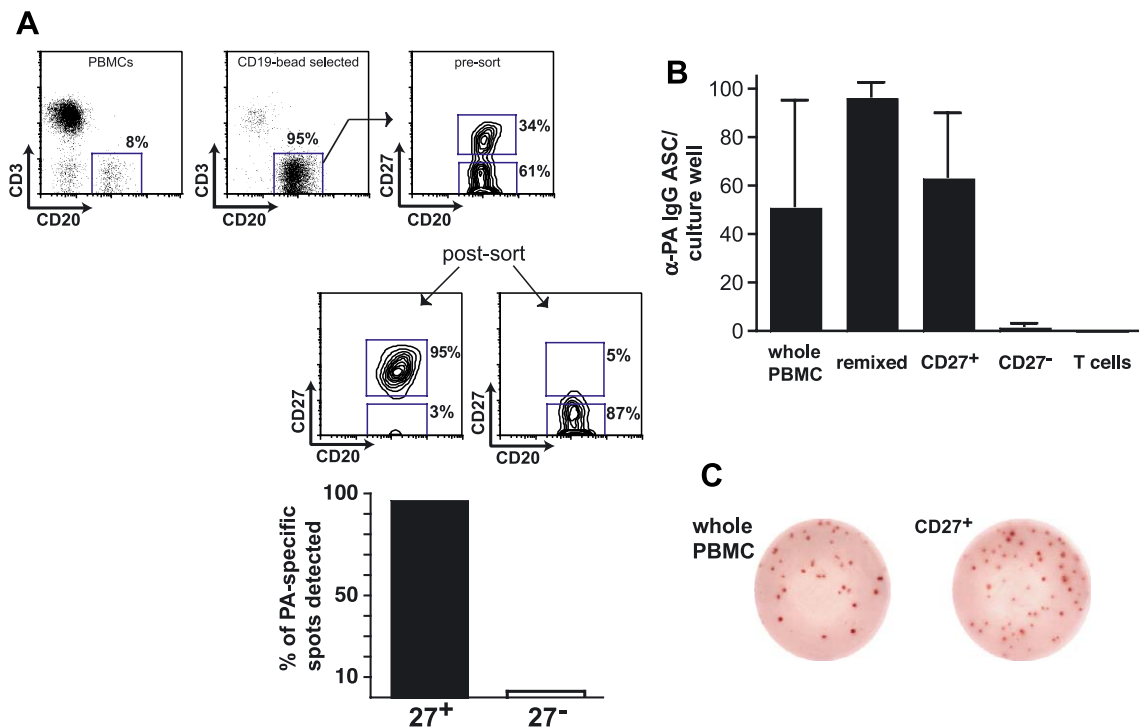


Fig. 3. Identification of anthrax-specific memory B cells as CD27⁺ B cells. (A) Purification of CD27⁺ and CD27⁻ B cells. PBMCs isolated from an AVA-vaccinated individual were enriched for B cells by anti-CD19 antibody-linked paramagnetic bead selection. The B cell enriched fraction was FACS-sorted based on CD27 expression to give 90–95% pure populations of CD27⁺ and CD27⁻ B cells. The sorted CD27⁺ and CD27⁻ fractions were cultured with B cell-depleted PBMC (<0.5% CD20⁺). Cells were cultured for 6 days with PWM and SAC and total numbers of IgG secreting and PA-specific IgG secreting cells were then measured by ELISPOT. Graph shows the percentage of PA-specific IgG ASC that were detected in each fraction; 95% of the ASC were observed in the CD27⁺ fraction. (B) Antigen-specific B cells are CD27⁺. The number of PA-specific B cells detected per culture well is shown for each combination of B and T cells. “T cells”: wells containing only B cell-depleted PBMC. “Remixed”: wells containing cells that were fractionated into non-B cells, CD19⁺CD27⁺, and CD19⁺CD27⁻ populations and then reconstituted in appropriate ratios. The sorted CD27⁺ and CD27⁻ fractions were each separately cultured with B cell-depleted PBMC (<0.5% CD20⁺) (“CD27⁺” and “CD27⁻”). Data shown are from one experiment, and are representative of the two experiments done. (C) ELISPOTS. There is no qualitative difference in ASC spots produced from sorted and unsorted B cell populations.

B cells. CD27 has recently been identified as a good surface marker for human memory B cells, as determined by identification of somatic hypermutations of heavy- and light-chain loci in CD27⁺ B cells and not in CD27⁻ B cells (Agematsu et al., 1998, 2000; Klein et al., 1998; Tangye et al., 1998). Therefore, we performed cell sorting analysis to determine if the anthrax-specific memory B cells we describe were CD27⁺ memory B cells.

B cells comprise ~5–20% of human peripheral blood lymphocytes. Approximately 30–50% of the B cells are CD27⁺ (Fig. 3A). Human peripheral blood from an AVA-vaccinated individual was isolated and then separated into B cell and non-B cell populations using anti-CD19 magnetic bead purification. The CD19⁺CD20⁺ cells were then further purified by flow cytometric cell sorting to separate CD27⁻ (naive) and CD27⁺ (memory) B cells (Fig. 3A). Purity of the populations was checked by flow cytometry. CD27⁻ naive B cells and CD27⁺ memory B cells were then separately cultured with autologous non-B cells for 6 days in vitro using our standard memory B cell culture conditions. On day 6, the cultures were assessed for both total memory B cells and anthrax-specific memory B cells by ELISPOT. The majority of the total IgG⁺ B cells (data not shown) and, importantly, the anthrax-specific memory B cells were found in the CD27⁺ B cell population (Fig. 3A–C). This demonstrates that our assay accurately detects antigen-specific memory B cells elicited by a human vaccine and that these cells reside in the CD27⁺ B cell compartment.

5. Detecting other responses: smallpox vaccine- or anthrax infection-specific memory B cells

To demonstrate that this assay is useful for the detection of other antigen-specific B cell responses, we explored antigen-specific memory B cell responses in smallpox vaccine (vaccinia virus; DryVax) recipients. Immune memory after smallpox vaccination (DryVax) is a valuable benchmark for understanding the kinetics and longevity of B cell memory in the absence of re-exposure to antigen, since immunization of the US population was stopped in 1972 and smallpox disease was declared eradicated worldwide in 1980 (Fenner et al., 1988). In addition,

there is currently great public health interest in smallpox immunity due to the possible threat of bioterrorism (Fauci, 2002). We used vaccinia virus-infected

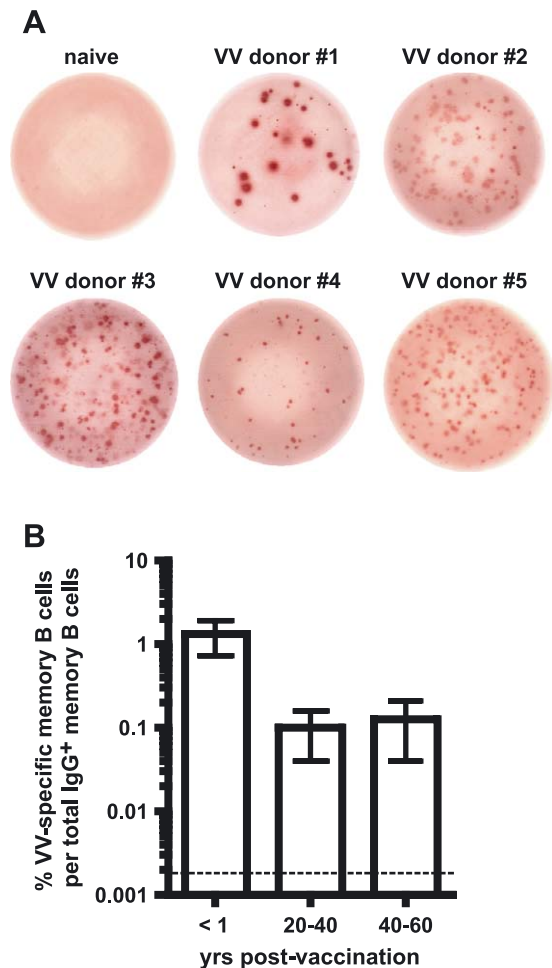


Fig. 4. Anti-vaccinia virus memory B cells in DryVax (smallpox vaccine) immunized individuals. (A) Individuals were tested for vaccinia virus-specific memory B cells using VV-infected cell lysate as the ELISPOT coating antigen. HeLa cell lysate was used as a control antigen. One non-vaccinated (“naive”) individual and several vaccinated (“VV donor”) individuals are shown. One of many replicate wells is shown for each. (B) Vaccinees were divided into three group (<1, 20–40, and 40–60 years post-vaccination) and mean VV-specific memory B cell levels were compared. Error bars represent 95% confidence interval. There is a statistically significant difference between <1 vs. 20–40 years ($P < 0.001$, two-tailed, one-way ANOVA) and <1 vs. 40–60 years ($P < 0.001$). No statistically significant difference is observed between 20 and 40 years vs. 40–60 years ($P \gg 0.05$). Limit of detection is indicated by dashed line. (Data from Crotty et al., 2003a).

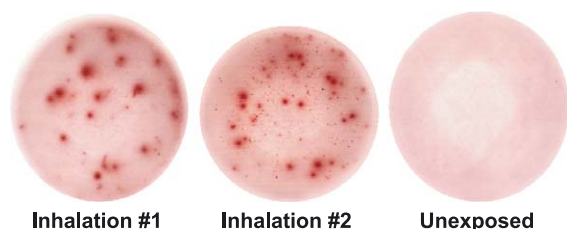


Fig. 5. Anti-anthrax memory B cells in anthrax-exposed individuals. Shown in the left and center wells are PA-specific IgG⁺ spots from two individuals who developed inhalational anthrax from the Fall 2001 bioterrorist attacks. Samples are approximately 1 year post-exposure. Shown in the right well is an example of an unvaccinated/unexposed donor.

cell lysate as the antigen, and uninfected HeLa cells were used as the appropriate negative control antigen. Vaccinia virus (VV)-specific memory B cells were detected in vaccinated individuals (Fig. 4). In contrast to the anti-anthrax PA responses (which were relatively uniform), a variety of VV-specific spot sizes and spot densities were observed in VV-immunized individuals (Fig. 4A), perhaps reflective of responses directed against a variety of VV protein antigens. Peak anti-VV responses of 1–3% of circulating IgG⁺ memory B cells were observed, similar to what we have observed with anthrax (Fig. 4B). Impressively, responses were detectable for up to 60 years post-vaccination (Fig. 4B) (Crotty et al., 2003a).

In addition, PA-specific memory B cells were detected in individuals afflicted by inhalation anthrax after exposure to anthrax in the terrorism incidents of the Fall of 2001 (Fig. 5, and Quinn et al., submitted). These individuals were tested for PA-specific memory B cells at approximately 1 year post-exposure. These results demonstrate that PA-specific memory B cells are elicited by anthrax infection as well as by AVA vaccination.

6. Discussion

Efficient techniques are available for detecting human antigen-specific memory CD8⁺ T cells, memory CD4⁺ T cells, and antibodies, but there is a dearth of techniques for detecting human memory B cells. It is unclear whether memory B cell and serum antibody levels correlate well for most antigens, and it is therefore important to be able to track memory B

cells as an independent parameter of antigen-specific immune memory.

The strengths of this ELISPOT memory B cell assay are: (1) that it is readily generalizable to virtually any antigen (since the stimulation conditions are nonspecific, and the only antigen-specific step is the coating of the ELISPOT plate); (2) it provides an accessible quantitation of memory B cell numbers (percent of circulating IgG⁺ memory B cells that are antigen-specific); and (3) it uses relatively inexpensive reagents (pokeweed mitogen (PWM), SAC, and CpG) and a modest amount of human blood (5 ml). Additionally, we have demonstrated that the vaccine-elicited antigen-specific memory B cells do indeed reside in the CD27⁺ compartment previously identified as the compartment containing the majority of B cells possessing hypermutated Ig heavy and light chains.

Two previously available techniques for tracking human memory B cells involve either direct detection of antigen-binding cells by flow cytometry (Leyendeckers et al., 2002), or long-term culture (14–28 days) followed by ELISA (Bernasconi et al., 2002). Direct detection of memory B cells by antigen-binding is an appealing technique, and is of great value because it does not involve any stimulation, and allows for further phenotypic analysis of individual cells (Leyendeckers et al., 1999, 2002; Maruyama et al., 2000; Gonzalez et al., 2003; Weitkamp et al., 2003). However, development of antigen-binding flow cytometry assays can be tricky (Bell and Gray, 2003), and the low frequency of most antigen-specific B cells causes detection problems. Detecting memory B cells on the supernatants is a strategy similar to ours (Bernasconi et al., 2002). We prefer the ELISPOT assay because it gives an estimate of the actual frequency of the antigen-specific memory B cells, and is more rapid (5–6 days instead of 14+).

One to two percent of IgG⁺ memory B cells in PBMC are smallpox or anthrax vaccine-specific after recent immunization, as determined using our memory B cell ELISPOT assay. By using best estimates of the relevant parameters (1.25 × 10⁶ PBMC recovered per milliliter of blood collected into CPT; B cells are 10% of PBMC; memory B cells are 30% of B cells; IgG⁺ memory B cells are 50% of memory B cells), we can calculate that there are approximately 200–400 anthrax or smallpox vaccine-specific IgG⁺ memory B

cells per milliliter of blood (of the ~ 125,000 total B cells/ml blood). This translates to 1–2 million anthrax or smallpox-specific memory B cells circulating in the blood of a recently immunized person.

This assay can be readily adapted for use in detecting virtually any antigen recognized by human B cells (we have also recently validated this memory B cell assay in non-human primates, with minor modifications (unpublished data)). Here we have demonstrated the ability to detect memory B cells specific for two different antigens of interest—anthrax PA protein, and vaccinia virus—and we are in the process of expanding that repertoire. We have recently completed studies determining the magnitude and longevity of memory B cell responses after vaccination against smallpox (Crotty et al., 2003a) and the magnitude of memory B cell responses after bioterrorism-related anthrax (Quinn et al., submitted). This assay is also being implemented as part of a large (1500 volunteers) multi-center vaccine trial (the Anthrax Vaccine Research Program, AVRVP) to enable us to track humoral immune responses longitudinally over a 4-year time course. In addition, this assay is being used to determine the development of immunological memory in individuals infected with or exposed to monkeypox in the recent Midwest outbreak (I. Damon et al., unpublished data). As such, this assay makes a variety of questions accessible regarding human memory B cells and the longevity of human immune memory.

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