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Optimization and qualification of a memory B-cell ELISpot for the detection of vaccine-induced memory responses in HIV vaccine trials

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Abstract

Various aspects of the human immune system can be analyzed to determine the efficacy of a vaccine. We have developed a B-cell ELISpot to measure HIV-specific antibody-secreting B cells in the peripheral blood as a result of vaccination or natural infection. Our method includes stimulating peripheral blood mononuclear cells with interleukin-2 and a polyclonal activator, R848, to induce memory B cells to differentiate into antibody-secreting cells. Total immunoglobulin-secreting as well as antigen-specific B cells are then quantified. We have tested several HIV Env gp120 and gp140 proteins from different HIV subtypes, as well as a sensitive consensus group M Env gp140. Our findings indicate that the B-cell ELISpot provides a sensitive and specific tool to detect antigen-specific memory B-cell responses, and it is equally suited to detect antibody-secreting plasmablasts present in the circulation shortly after infection or vaccination.

Keywords

B-cell ELISpot; Antibody-secreting cells; Memory B-cell responses; Vaccine; HIV; Mucosal responses

1. Introduction

Antibody responses are crucial for prevention of many infections, and represent a correlate of protection for almost all effective vaccines (Plotkin, 2010). Their titers are therefore the

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predominant measure in most vaccine trials. Memory B cells, along with terminally differentiated plasma cells, are responsible for the long-term persistence of the humoral immunity elicited by most vaccinations and some infections (West and Calandra, 1996; Crotty et al., 2003), so the assessment memory B-cell responses may provide additional information on vaccine take and longevity of vaccine-induced protection. Immunoglobulin-G (IgG) titers and memory B-cell responses can correlate (Crotty et al., 2003), but can also be distinct (Leyendeckers et al., 1999; Amanna et al., 2007), suggesting that the measurement of memory B cells is not redundant with that of circulating antibodies. The detection of antigen-specific B cells rather than circulating antibodies can also be helpful in differentiating maternal antibodies from *de novo* responses induced in infants upon infection or vaccination; as well as to distinguish local IgG production from transudation of serum IgG in mucosal samples.

The generation of high-affinity antibody-secreting plasma cells and memory B cells occurs within the germinal center of lymphoid tissues through somatic hypermutation and selective expansion. These memory B cells can persist independently of the immunizing antigen (Ag) and are capable of mounting a rapid anamnestic secondary response upon re-exposure to the antigen, during which some of the memory B cells terminally differentiate into antibody-secreting plasma cells (Crotty et al., 2004; LeBien and Tedder, 2008). Different pools of long-lived plasma cells are generated after the primary and secondary exposures, which migrate to the bone marrow from the spleen and can survive for the life of the host without expanding (Manz et al., 2002; McHeyzer-Williams and McHeyzer-Williams, 2005; Radbruch et al., 2006).

In this paper, we describe the optimization of a previously published memory B-cell ELISpot assay specific for HIV-1 surface proteins in order to determine the immune stimulating effects of HIV vaccines (Crotty et al., 2004; Bonsignori et al., 2009; Dosenovic et al., 2009). This optimization focused on the stimulation conditions that result in the most robust and consistent detection of vaccine-induced memory B-cell responses, resulting in a reliable qualified assay ready to be applied in clinical trials. This assay is equally well suited to identify *ex vivo* antibody-secreting cells (ASCs) circulating in the blood shortly after vaccination and resident mucosal ASCs, as well as memory B cells in the periphery and mucosal tissues.

2. Material and methods

2.1 Study participants

Samples were obtained from four HIV subtype B-infected and twenty uninfected individuals enrolled in the study “Establishing Immunologic Assays for Determining HIV-1 Prevention and Control,” also referred to as Seattle Area Controls or SACs. HIV-infected subjects were chronically infected and on antiretroviral treatment. In addition, we tested samples from 19 individuals enrolled in HVTN 204 (Churchyard et al., 2011). HVTN 204 is a phase II clinical trial to test the immunogenicity of a multiclade HIV-1 DNA plasmid vaccine (subtype B Gag, Pol, and Nef; subtypes A, B, and C Env) followed by a multiclade recombinant adenovirus serotype 5 vector HIV-1 vaccine boost (subtype B Gag-Pol fusion; subtypes A, B, C Env) in HIV-1 uninfected adult participants. Samples tested were taken at baseline and one month post final vaccination.

All volunteers provided informed written consent before participating in the studies, and all studies were approved by the Institutional Review Boards of the Fred Hutchinson Cancer Research Center and other participating institutions for HVTN 204.

2.2 Sample processing

Cryopreserved peripheral blood mononuclear cells (PBMC) were used for assay development, but other cell sources can equally be used in this assay (e.g., gut mucosa mononuclear cells [GMMC] obtained through flexible sigmoidoscopy). PBMC were isolated from whole blood treated with acid citrate dextrose or sodium heparin using Leucosep tubes (Greiner Bio-One, Monroe, NC) according to the manufacturer's instructions. PBMC were counted using a Coulter counter and frozen at 15 million cells/vial in cryopreservation solution (90% fetal bovine serum [FBS] with 10% DMSO). GMMC from biopsies obtained by flexible sigmoidoscopy were isolated by two rounds of digestion with collagenase II (Sigma-Aldrich, St. Louis, MO) followed by gradient centrifugation using Histopaque (Sigma, St. Louis, MO). GMMC were counted on a Guava Counter using α CD45-FITC, α CD19-PE and 7AAD (BD Biosciences, San Jose, CA) for enumeration of live B cells, and were used after overnight rest at 37°C.

2.3 PBMC thawing

Cryopreserved PBMC were rapidly thawed in a 37°C water bath and then slowly added to 10 ml of warmed R10 (RPMI 1640 [GibcoBRL, Carlsbad, CA], 10% FBS [Gemini Bioproducts, West Sacramento, CA], 2 mM L-glutamine [Gibco], 100 μ g/ml streptomycin sulfate [Gibco], 100 U/ml penicillin G [Gibco]) containing 20 μ l Benzonase (25 U/ μ l; Novagen, Madison, WI). The cells were then counted using the Guava ViaCount Kit (Millipore, Bedford, MA) according to manufacturer's instructions and stimulated as described in section 2.4.

2.4 Memory B-cell stimulation

PBMC were resuspended at 1×10^6 PBMC/ml in stimulation media and incubated at 37°C in 5% CO₂. Stimulation media and conditions were optimized as described in section 3.3; best results were obtained using stimulation media composed of R10, 5 ng/ml interleukin 2 (IL-2, Mabtech, Mariemont, OH) and 0.5 μ g/ml imidazoquinoline resiquimod (R848, Mabtech), and stimulating cells for 5 days. Stimulated cells were washed in R10 prior to plating (see section 2.6).

2.5 HIV Envelope proteins

We tested numerous HIV Env gp140 and gp120 proteins to identify ones that were readily available and consistently detected a response with clear, defined spots in HIV-infected subjects or vaccinees. We selected two HIV-1 envelope (Env) proteins for general use: an HIV-1 Subtype B gp140, BaL (GenBank accession number ABC55874, Immune Tech Corporation, New York City, NY); and a consensus group M gp140, ConS (kindly provided by Dr. Georgia Tomaras, Duke University). We also tested another HIV-1 Subtype B gp120 protein, SF162 (GenBank accession number AAT67508, Immune Tech Corporation, New York, NY); however, it produced very high background staining and was therefore used to demonstrate the effects of biotinylation in background reduction and spot definition. In addition, we used the two HIV-1 proteins in AIDSVAX B/E (Billich, 2001) for testing vaccine-specific responses in clinical trials: subtype AE gp120 A244 and subtype B gp120 MN. We used EZ-Link Micro Sulfo-NHS-Biotinylation kit (Thermo Scientific, Pittsburgh, PA) to chemically bind biotin to primary amines, such as in the side-chain of lysine residues and the N-terminus of polypeptides. Because random biotinylation by chemical linking of the side-chain of lysine that is often critically important for HIV-1 antibody binding can potentially negatively impact some important epitopes such as K169 for V2 antibody binding (Rolland et al., 2012; Liao et al., 2013), alternatively, the addition of an AVI-tag (GLNDIFEAQKIEWHE) (Kay et al., 2009) to the antigen of choice allows for specific biotinylation at the C-terminus of the protein that eliminates the possibility of obscuring

critical residues for antibody binding. The biotinylated HIV proteins are then titrated to find the optimal working concentration.

2.6 B-cell ELISpot assay

Sterile 96-well Multiscreen-IP filter plates with a PVDF membrane (Millipore) were pretreated with 50 μ l 70% ethanol and then washed five times with sterile water before being coated with 100 μ l of 15 μ g/ml capture anti-human IgG (Mabtech, Cincinnati, OH). Coated plates were incubated overnight at 4°C, and were kept at 4°C for up to one week as long as the liquid has not evaporated from the wells.

On the day of the assay, plates were washed five times with sterile phosphate buffered saline (PBS) and blocked with R10 for at least 30 minutes at room temperature. The blocking solution was then removed, and cells were added at 5,000 to 500,000 per well in R10 media, depending on the optimal cell number defined empirically for each antigen. Each condition was tested in triplicate and the plates were incubated for 16-24 hours at 37°C in 5% CO₂.

In order to develop the spots, the plates were first washed five times with sterile PBS. To measure total IgG, a biotinylated anti-IgG antibody (Mabtech) was added at 1 μ g/ml diluted in PBS/0.5% FBS. To measure antigen-specific responses, biotinylated HIV gp120 and gp140 proteins were added at 0.1 μ g/ml. As a negative control, biotinylated Imject® Mariculture Keyhole Limpet Hemocyanin (KLH, Pierce, Rockford, IL) was added at 2.5 μ g/ml. The plates were then incubated at room temperature for two hours, washed five times with PBS before the addition of streptavidin-horseradish peroxidase (Mabtech) diluted 1:100 with PBS/0.5% FBS. The plates were incubated for one hour at room temperature, and then washed five times with PBS. Finally, 100 μ l tetramethylbenzidine (TMB) substrate was added to each well and allowed to develop until distinct spots formed. The reaction was stopped by washing the wells under running water eight times. The plates were allowed to air dry before being counted on the CTL ImmunoSpot Analyzer (Cellular Technologies, Shaker Heights, OH).

Experiments using non-biotinylated antigens were conducted in a similar manner, except plates were coated with 10 μ g/ml of antigen, and biotinylated anti-IgG antibody was added at a concentration of 1 μ g/ml prior to development.

After development with the TMB reagent, spots appear blue to blue-green in color. Supplementary Figure 1 shows anticipated and some false positive results. The KLH (negative control) wells should not contain any spots (Supplementary Figure 1A), while the total IgG (positive control) wells should have spots covering the entire surface of the membrane (Supplementary Figure 1B). Precise appearance may vary depending on the sample, number of cells plated and viability of the cells. The spots should be uniform in shape and slightly darker in color at the center than on the outer edges. The antigen-specific spots using non-biotinylated antigens are generally larger and more diffuse than the total IgG spots, however this effect is minimized when the antigen is biotinylated, as in our optimized method (Supplementary Figure 1C; see section 3.3). False positive responses may occur due to substrate or cytokine aggregates within the well (Supplementary Figure 1D), hair or other foreign bodies. This method has also been optimized to detect IgA using anti-IgA antibodies (Mabtech) rather than anti-IgG (Supplementary Figure 1E).

2.7 B-cell FluoroSpot

B-cells that produce multiple antibody types are stimulated by vaccination or natural infection in the blood and mucosal tissue. The IgG-IgA FluoroSpot (Mabtech) allows for the simultaneous detection and enumeration of IgG and IgA producing ASCs using fluorophore-labelled detection reagents. The cells are stimulated exactly as described above and the rest

of the assay is performed according to the manufacturer's instructions. The ASCs were detected by exposing the plate to pre-defined wavelengths that visualize the different fluorophores. The iSpot FluoroSpot reader system (AID Diagnostika GmbH, Strassberg, Germany) was used to read and analyze the plates after development. Positivity calls were identical to those of the colorimetric B-cell ELISpot.

2.8 B-cell phenotyping

Phenotyping of PBMCs as well as of the cultures after stimulation was performed using antibodies to CD3-Alexa700, CD20-PE-Cy7, CD38-PerCP-Cy5.5, IgD-FITC, IgG-APC, IgM-V450 (all BD Biosciences, San Jose, CA); CD27-PE-Cy5, CD19-ECD (Beckman Coulter, Brea, CA), and HLA-DR-Qdot605 (Invitrogen, Grand Island, NY). After incubation with Aqua Live/Dead Fixable Dead Cell Stain (Invitrogen, Grand Island, NY), cells were surface-labeled with antibodies for 20 min at room temperature, fixed and acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA). Analysis was performed using FlowJo (Treestar, Inc., Ashland, OR).

3. Results and discussion

3.1 Experimental Design

This 7-day assay is used to detect memory B-cell responses by stimulating PBMC for five days with the polyclonal activators R848 and IL-2, which induce memory B cells to differentiate into antibody secreting cells. Although the stimulation period can be adjusted from 3-8 days, five days was optimal for our testing as it allowed the B cells to mature and expand sufficiently to detect lower level responses while minimizing the die-off associated with extended incubation.

Following stimulation, PBMC are plated in ELISpot plates that can be prepared in two ways: i) wells are coated with anti-human IgG, which captures all IgG produced by the stimulated B cells regardless of antigen specificity, and biotinylated HIV proteins and negative control antigens are then added to the wells and bound by the captured IgG that is specific for that antigen; ii) wells are coated with the HIV antigen, which will only capture secreted antibodies that are specific to that antigen, followed by addition of a secondary, biotinylated anti-IgG antibody. In both cases, the reaction is visualized by the addition of streptavidin-horseradish peroxidase, which binds to the biotin moiety and reacts with a substrate, TMB, to produce a blue spot. Total secreted IgG is also measured as a positive control to confirm that the stimulation step was successful, and in order to calculate the HIV-specific B cells as a percentage of the total ASCs. For a negative control, we used the neo-antigen KLH to assess whether the assay detects any nonspecific reactivity to an irrelevant protein.

3.2 Data analysis

Responses in the B-cell ELISpot can be reported in two ways: as spot-forming cells (SFC) per million input cells, or as percent antigen-specific cells of total IgG or IgA-secreting cells. Positivity is established as at least 20 spot-forming cells (SFC) per million input cells and at least 5 spots/well. In addition, a positive response must be 3 times greater than the average background measured by KLH. To determine the proportion of antigen-specific memory B cells, the antigen-specific readout is divided by the total IgG-producing cells and multiplied by 100 to be reported as percentage. This magnitude adjusts for the varying proportion of B cells present in human PBMC.

3.3 Biotinylation of protein antigens leads to reduced background and increased definition of spots

Certain HIV proteins produced higher background staining or diffuse spots regardless of development time, protein size (gp120, gp140) or cell number. Supplementary Figure 2A shows an example of memory B-cell responses to several HIV Env proteins in an HIV-infected subject when the assay was performed by coating the plate directly with non-biotinylated protein antigens. The biotinylated antigen assay produces more-defined spots and greatly reduces background staining (Supplementary Figure 2B). As an additional benefit, reproducible results can be obtained using much lower concentrations of biotinylated protein (0.1µg/ml instead of 10µg/ml for the non-biotinylated Ag assay), thereby greatly reducing the cost of the assay. As with the HIV antigens, KLH can be biotinylated or directly coated to the plate to match the chosen assay platform.

3.4 Optimization of the stimulation conditions to induce antibody secretion in memory B cells

We tested several stimulation agents to determine which induced the most robust transition from memory B cells to ASCs while minimizing cytotoxicity. We initially tested a combination of pokeweed mitogen, β-mercaptoethanol and *Staphylococcus aureus* Cowan (Crotty et al., 2004). This combination caused high levels of cell death and minimal stimulation (data not shown). We next tested two kits from Mabtech: one using pokeweed mitogen and IL-2 as stimulants, the other using IL-2 in combination with the TLR7 agonist imidazoquinoline resiquimod (R848). R848/IL-2 provided much stronger stimulation than pokeweed mitogen (Supplementary Figure 3) without being more cytotoxic to the cells (Bishop et al., 2000; Tomai et al., 2000). We then tested various concentrations of R848 and varied the number of cells per well to identify the smallest amount of each that still gave consistent results (Figure 1). We determined that using half the manufacturer's recommended concentration of R848 (0.5µg/ml instead of 1µg/ml, Figure 1A) and IL-2 (5ng/ml instead of 10ng/ml, Figure 1B) gave results comparable to those obtained when using the higher concentrations. We also found that 5×10^3 PBMC/well were sufficient to test for total IgG (Figure 1C).

Several studies have suggested that the synthetic oligonucleotide CpG-C is a potent stimulant to induce antibody secretion (Marshall et al., 2003; Poeck et al., 2004). We therefore compared our optimized R848/IL-2 stimulation to that using 2µg/ml CpG-C with the same 5ng/ml IL-2 in samples from five HIV-infected subjects (Figure 2). In our hands, stimulation using R848/IL-2 compared to CpG-C/IL-2 led to both increased numbers of B cells obtained after the 5 day incubation (Figure 2A) as well as increased magnitude of the antigen-specific response without increased background staining to KLH (Figure 2B). Phenotyping of the B-cell cultures both prior to and after stimulation showed that R848 leads to increased percentage of total B cells in the culture and drives the B cells further down the path of maturation than CpG-C, as more plasmablasts [defined as $CD19^+ CD20^{-/low} CD27^+ CD38^+$ B cells (Caraux et al., 2010)] were generated at day 5 with the R848 stimulation (representative example in Figure 2C).

We tested the length of stimulation and determined a 5-day incubation was optimal; there was a marked drop in cell viability with longer stimulations (data not shown), and a reduced number of antigen-specific spots when cells were stimulated for shorter times (Supplementary Figure 4). We also determined that the type of tissue culture container used for stimulation, such as a 24-well-plate or a 25cm² flask, did not impact the results of this assay, but that the use of 25cm² flasks reduced the setup time when dealing with a larger number of samples.

3.5 Optimization of protein concentration and input cell number

Depending on the number of cells available and the level of staining for each protein, PBMC input may be decreased for some antigen-specific readouts. We suggest setting up a cell number titration for each new protein ranging from 5×10^5 to $\sim 5 \times 10^4$ PBMC/well (Figure 3A), and titrating the concentration of the antigen as well (Figure 3B). These titrations should be tested on a set of known responders, alongside a known antigen such as gp140 BaL. We recommend testing whether the biotinylation was successful by coating the well with the biotinylated protein and developing the well with S-HRP and TMB in the absence of cells. If the well is entirely blackened out, this indicates that biotinylation was successful.

3.6 Assay qualification

Once the assay procedure was finalized, its reproducibility was determined by measuring the intra-assay, inter-day and inter-technician variability. We first determined assay specificity by testing twenty HIV-negative and four HIV-positive samples. As shown in Figure 4A, none of the HIV-negative samples showed a response that was considered positive using our positivity criteria: 20 spot-forming cells (SFC) per million input cells, 3 times the average background and 5 spots per well. In contrast, all four HIV-infected participants had a positive response for at least one of the tested proteins. Precision was determined by running all tests in triplicate in order to calculate the intra-assay variability. One technician tested three of the HIV-positive samples on three different days to determine the inter-day variability; and three technicians tested the same three samples on the same day to determine the inter-technician variability. Each sample was run in triplicate to test the intra-assay variability (Figure 4 B and C). All coefficients of variation remained under 20%; intra-sample variation is slightly higher for BaL than for ConS since the magnitude of the response was lower.

3.7 Assessment of HIV vaccine-induced memory B-cell responses

To address whether the optimized B-cell ELISpot would be suitable for the detection of vaccine-induced HIV-specific memory B-cell responses, we tested 19 samples from HVTN 204, a DNA-prime, Ad5-boost vaccine trial that includes HIV-Env from subtypes A, B and C and induced binding antibody responses to ConS in 95% of vaccine recipients (Churchyard et al., 2011). Responses were tested at baseline and 4 weeks after the Ad5 boost using ConS and BaL gp140. As shown in Figure 5, no responses were seen at baseline, while all vaccine recipients showed responses to BaL, and all but one responded to ConS.

3.8 FluoroSpot

The correlates of risk assessment for the recent RV144 HIV-1 vaccine trial found that vaccinees who elicited high V1V2-specific IgG antibodies had a reduced risk of infection compared to those with low level V1V2-specific IgG, but other immune responses (such as antibody-dependent cell-mediated cytotoxicity [ADCC] and HIV-specific CD4⁺ T cells) were only protective in the absence of high vaccine-induced Env-specific IgA antibodies (Haynes et al., 2012). Therefore we tested a dual color IgG-IgA FluoroSpot (Mabtech) to simultaneously measure both IgG and IgA responses. Using PBMC samples from HIV-positive participants and from vaccine recipients, we were able to detect total and HIV-specific IgG and total IgA, but we were unable to detect HIV-specific IgA memory B cells in the blood (Figure 6). We were not able to find a concentration of PBMC high enough to detect the HIV-specific IgA response without saturating the signal of the HIV-specific IgG response, suggesting that this assay is not ideal for use in PBMC unless a high frequency of antigen-specific IgA production is expected. We instead developed a colorimetric IgA ELISpot that can accurately detect B cells producing IgA in HIV-positive samples after the 5-day stimulation period (Supplementary Figure 1E).

We are currently using the FluoroSpot assay to detect IgG and IgA in mucosal samples, where IgA is more abundantly produced. To this end, biopsies obtained by flexible sigmoidoscopy are collagenase-treated to isolate discrete gut mucosa mononuclear cells (GMMC), which are either plated directly *ex vivo* (Supplementary Figure 5A) to detect resident antibody secreting cells, or stimulated for 5 days with R848/IL-2 to assess the presence of memory B cells (Supplementary Figure 5B). As expected, the proportion of IgA-secreting B cells is much higher in the mucosa than in the blood, and most B cells secreting IgA are doing so constitutively, while actively IgG secreting cells are rare in GMMC and require stimulation for their detection. Using the B-cell ELISpot in this context also allows the differentiation of locally produced and transudated Ig; this assay therefore provides complementary information to the measurement of mucosal antibodies in secretions.

4. Conclusions

We have optimized a B-cell ELISpot assay that provides robust detection of antibody secreting cells such as plasmablasts as well as memory B cells, both in circulation and in mucosal biopsies. Our assay is highly reproducible and highly specific, and it is sensitive enough for the detection of vaccine-induced B-cell responses. The B-cell ELISpot provides a readout on pathogen-specific B-cell responses that is not redundant with antibody titers, and that may identify a correlate of protection for HIV vaccines (Haynes et al., 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PBMC	peripheral blood mononuclear cells
Ag	antigen
Ig	immunoglobulin
ASC	antibody-secreting cell
TMB	tetramethylbenzidine
KLH	Keyhole Limpet Hemocyanin
HBSS	Hanks balanced salt solution
PVDF	polyvinylidene fluoride
SFC	spot-forming cells

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Figure 1. Titration of R848 concentration, R848/IL-2 and cell number per well in an HIV-infected subject

A) R848 was titrated in duplicate in twofold dilutions from 2 µg/ml to 0.015 µg/ml to find the optimal concentration for B-cell stimulation using 5,000 cells/well and total IgG as a readout. B) R848 and IL-2 were tested in duplicate at 1 µg/ml / 10 ng/ml or 0.5 µg/ml / 5 ng/ml for total IgG, ConS and BaL gp140 using 5,000 cells/well for total IgG and 500,000 cells/well for ConS and BaL. C) The number of PBMC added per well for total IgG was titrated in duplicate in twofold dilutions from 10,000 to 312 cells/well to determine the optimal number that would give a readable result on the AID plate reader while still being able to detect low level responders.

Figure 2. R848/IL-2 provides superior stimulation than CpG-C in five HIV-infected subjects

A) The total number of CD19⁺ B cells prior to stimulation (open bars) as well as after 5-day stimulation with CpG-C (light grey bars) or R848 (dark grey bars) was assessed on a Guava Counter. B) R848/IL-2 leads to higher magnitude of HIV-specific memory B-cell responses (dark grey bars) than CpG-C (light grey bars) without increasing background (open bars) using 200,000 cells/well for each stimulation. The dotted grey line represents the positivity threshold of 20 SFC/million. C) Phenotyping of PBMC prior to stimulation (top row) and after 5 days of stimulation with CpG-C/IL-2 (middle row) or R848/IL-2 (bottom row). Not shown is the gating tree for singlets, live cells (AViD-negative) and lymphocytes (FSC/SSC). R848 stimulation leads to a higher percentage of total B cells and higher proportion of CD20^{lo/-} CD27^{hi} CD38^{hi} plasmablasts that are class-switched (IgD⁻) than CpG-C.

Figure 3. Optimal readout depends on PBMC and antigen concentration used in the B-cell ELISpot

A) PBMCs from an HIV-infected subject were titrated by two-fold dilutions in duplicate using wells coated with 10µg/ml (top rows) or 5µg/ml (bottom rows) SF162 gp140. Input cell numbers ranged from 500,000 to 31,250 cells/well. B) The concentration of AVI-tagged ConS was titrated by two-fold dilutions in duplicate from 4µg/ml to 0.5µg/ml using 250,000 cells/well.

Figure 4. B-cell ELISpot qualification

A) Assay specificity was determined by testing triplicate samples from 20 HIV-negative and 4 HIV subtype B-infected subjects enrolled in the Seattle Area Control (SAC) cohort with the negative control (KLH, open circles) and two HIV antigens (gp140 ConS, black squares; and gp140 BaL, grey diamonds). The dotted line at 20 SFC/million PBMC represents the cut-off under which responses are considered negative. PBMC were plated at 500,000 cell/well. B and C) The variability of the assay was determined by calculating the coefficient of variation (%CV) for the intra-assay, inter-technician and inter-day variables for gp140 ConS (B) and gp140 BaL (C). Only data from positive responses were included. The cut-off was set at 20% (dotted line). None of the samples reached this level.

Figure 5. Vaccine-induced memory B-cell responses

Vaccine recipients from HVTN 204 were tested at baseline (left columns) and four weeks after the Ad5 boost (right columns) with ConS (diamonds) and BaL (circles) gp140 proteins. The dotted line at 20 SFC/million PBMC represents the cut-off under which responses are considered negative. Open symbols represent negative responses, closed symbols represent positive responses.

Figure 6. IgG/IgA FluoroSpot for PBMC

PBMC from two vaccine recipients after vaccination (Participant 1 and 2) and one at baseline (Participant 3) were added to a FluoroSpot plate to determine the reactivity towards different HIV proteins and the negative control (KLH), and to measure total IgG and total IgA. IgA was detected using FITC-labeled secondary antibodies and develops as green spots. IgG was detected using Streptavidin-Red-labeled secondary antibodies and develops as yellow spots. 5×10^5 stimulated PBMC were added to wells coated with KLH, gp140 ConS and gp140 BaL. 2.5×10^5 stimulated PBMC were added to wells coated with gp140 MN and gp120 A244. 1×10^5 stimulated PBMC were added to wells coated with gp140 ConS. 5×10^3