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Improved flow-based method for HIV/SIV envelope-specific memory B-cell evaluation in rhesus macaques

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Abstract

The ability to elicit potent and long-lasting broadly neutralizing HIV Envelope (Env)-specific antibodies has become a key goal for HIV vaccine development. Consequently, the ability to rapidly and efficiently monitor development of memory B cells in pre-clinical and clinical vaccine trials is critical for continued progress in vaccine design. We have developed an improved flow cytometry-based method for the rapid and efficient identification of gp120-specific memory B cells in peripheral blood, bone marrow, and mucosal tissues which allows their direct staining without the need for prior cell sorting or enrichment. We demonstrate staining of both HIV and SIV Env-specific memory B cells in PBMC, bone marrow, and rectal tissue of vaccinated and infected rhesus macaques. Validation of the method is illustrated by statistically significant correlations with memory B cell levels quantified by ELISPOT assay and with serum binding antibody titers determined by ELISA. In addition to quantification, this method will bring the power of flow cytometry to the study of homing and trafficking of Env-specific memory B cells.

Keywords

HIV; SIV; envelope-specific memory B cells

1. INTRODUCTION

Results of the promising HIV clinical vaccine trial in Thailand (RV144) highlighted the potential contribution of Env-specific antibody to protection against HIV infection (Rerks-

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Ngarm et al. 2009). Much of the field is now focused on the development of vaccines that can induce B cell maturation and elicit such antibodies. Many methods are available for characterizing binding and functional antibodies, however, until recently, investigations of memory B cells were limited to B cell ELISPOT assays. A few groups have used flow cytometry to evaluate HIV and SIV Env-specific memory B cells either by direct staining of PBMC (Doria-Rose et al., 2009) or following enrichment of the B cell population (Scheid et al., 2009; Fofana et al., 2011). Others have used a variety of methods for Env-specific staining of B cells in order to obtain antibody clones following single cell flow cytometry sorting either from HIV Env-vaccinated macaque or HIV-infected human PBMC (Gray et al., 2011; Morris et al., 2011; Mouquet et al., 2011; Moody et al., 2012; Klein et al., 2012; Li, O'Dell, et al., 2012; Li, Wang, et al., 2012; Sundling et al., 2012; Sundling et al., 2014). Flow cytometry-based assays are advantageous as they provide the flexibility to study different B cell subsets and their homing simultaneously. Moreover, in large pre-clinical or clinical vaccine studies, such assays could provide more efficient monitoring of B cell memory development and assessment of potential correlations with protective efficacy. Nevertheless, direct staining of memory B cells with labeled HIV or SIV envelope protein is problematic due to the envelope's high affinity for CD4 and other host cell surface molecules. Here we have improved the flow cytometry staining method using biotinylated Env protein together with stringent staining conditions, anti-CD4 antibodies to block non-specific gp120 binding, and a specific gating strategy for PBMC and bone marrow, as well as mucosal tissue. The method achieves no or minimal background and eliminates sorting of B cells which is tedious and expensive. Levels of Env-specific memory B cells in rhesus macaques vaccinated with HIV or SIV Env regimens and challenged with SIV or SHIV isolates are shown to correlate significantly with the frequency of antigen specific IgG/IgA antibody secreting cells (ASC) quantified by ELISPOT and with serum Env-specific IgG antibody titers quantified by ELISA.

2. MATERIALS AND METHODS

2.1. Animals and immunization

Indian origin rhesus macaques were housed and maintained at Advanced BioScience Laboratories, Inc. (ABL, Rockville, MD) according to the standards of the American Association for Accreditation of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals of the NIH. Animal protocols were reviewed and approved by the ABL Animal Care and Use Committee prior to implementation. Blood and tissue samples were obtained from macaques in 3 studies as follows.

Study 1—Viably frozen PBMC were obtained from twelve macaques, vaccinated as previously described (Vargas-Inchaustegui et al., 2014). Briefly, animals in the RepAd/Env protocol (n = 4), were given 2 doses of Ad5hr-SIV_{env/rev} and Ad5hr-SIV_{gag} by mucosal routes at weeks 0 and 12, and were subsequently boosted intramuscularly with adjuvanted Env protein at weeks 24 and 36. Animals in the DNA and DNA & Env protocols (n = 4 each) received the same DNA inoculations administered intramuscularly followed by *in vivo* electroporation (EP) at weeks 0, 9, 17 and 25. The DNA vaccine mixture contained SIVM766 gp160 DNA (EP1); SIV CG7V gp160 DNA (EP2) and both M766 gp160 and

gp140 and CG7V gp160 and gp140 for EP3 and EP4. Animals in the DNA & Env protocol additionally received adjuvanted gp140 Env proteins in the same muscle immediately following the DNA.

Study 2—Vially frozen PBMC from fourteen vaccinated macaques from a previously described pre-clinical trial were studied (Demberg et al. 2013). Briefly, the animals were vaccinated mucosally at weeks 0 and 12 with replication-competent Ad5hr- recombinants encoding HIV_{IIIB} Tat, HIV_{TV-1} gp160, or both Tat and Env. At weeks 24 and 36 the appropriate groups were boosted with HIV_{IIIB} Tat protein, oligomeric HIV_{TV-1} gp140 V2 envelope, or a combination of the two proteins formulated in Alum. The animals were challenged at week 50 intrarectally with the tier 2 clade C SHIV_{1157ipd3N4} (Song et al. 2006).

Study 3—Macaques were vaccinated mucosally with replicating Ad5hr-recombinants expressing *SIV_{smH4env/rev}*, *SIV_{239gag}* and *SIV_{nef}* followed by boosting with either monomeric *SIV_{mac251}* gp120 or oligomeric gp140 (Tuero et al., in preparation). The animals were subsequently challenged intrarectally with repeated low doses of *SIV_{mac251}*. Fresh rectal biopsies were obtained from 36 post-immunization and 48 post-*SIV_{mac251}* challenge vaccinated macaques and from 9 post-immunization and 12 post-challenge vector/adjuvant controls. Bone marrow was obtained from 33 macaques, pre- and post-immunization.

2.2. Sample collection

Blood and bone marrow samples were centrifuged over Ficoll gradients to obtain single cell suspensions (Demberg et al., 2012). After washing and lysis of contaminating red blood cells, PBMCs and bone marrow cells were stored in FBS/10% DMSO in liquid nitrogen until used. Serum was stored at -70°C . Rectal biopsies were rinsed with pre-warmed RPMI1640 (Invitrogen) containing 2 $\times$ antibiotic–antimycotic solution, 2-mM L-glutamine (Invitrogen) and 2 mg/ml Collagenase (Sigma–Aldrich). Prior to incubation (25 min at 37°C) the pinches were minced using a scalpel and a 19G needle, transferred in 10 ml of the same media to a 50 ml tube and pulse vortexed every 5 min. The digested tissue was passed 5 times through a blunt end cannula. The liberated cells and tissue debris were passed through a 70 μm cell strainer, and the cells were washed in R10 (RPMI1640 containing 2 $\times$ antibiotic–antimycotic solution, L-glutamine and 10% FBS) prior to staining.

2.3. Detection of Env specific memory B cells

Env-specific memory B cells in PBMC, bone marrow and rectal pinches were identified using *SIV_{mac251}* gp120 or HIV_{CZM} gp120, biotinylated using the Biotin-XX Microscale Protein Labeling Kit (Life Technologies). Cells (2×10^6) were surface stained with fluorescent antibodies and unconjugated anti-CD4 antibodies (2.5 μg) (OKT4 and T4/19Thy5D7 from the NIH Nonhuman Primate Reagent Resource) at room temperature for 25 minutes to block non-specific binding of gp120 to CD4. Surface staining antibodies included: PE-Cy5-anti-CD19 (J3–119, Beckman Coulter, Fullerton, CA); PerCP-eFluor710-anti-CD27 (0323) and eFluor650NC-anti-CD20 (2H7, both from eBioscience, San Diego, CA); PE-Cy7-anti-CD21 (B-ly4, BD Biosciences, San Jose, CA); PE-Texas Red-anti-IgD (polyclonal, Southern Biotech, Birmingham, AL); QDot605-anti-CD2 (S5.5), QDot800-anti-

CD14 (Tuk4), and Aqua Live/Dead viability Dye (all from Life Technologies, Grand Island, NY). The cells were then washed with 2% FBS in PBS and incubated with biotinylated gp120 (2 µg) at 4°C for 20 minutes. After washing with 2% FBS in PBS the cells were stained with APC-conjugated Streptavidin (Life Technologies) at 4°C for 20 minutes, washed with 2% FBS in PBS and fixed with 2% formaldehyde. Before acquisition, cells were passed through 0.35µm strainer to remove any clumps. At least 50,000 B cell events (Live CD2⁻CD14⁻CD20⁺CD19⁺ lymphocytes) were acquired on a LSRII (BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc. Ashland, OR). gp120-specific B cells in PBMC and bone marrow were detected within the memory (CD27⁺IgD⁻) B cell subpopulation. gp120-specific B cells in mucosal tissues were detected within the CD21⁺/IgD⁻ B cell subpopulation.

2.4. Evaluation of gp120-specific antigen secreting bone marrow cells

gp120-specific antibody secreting cells (ASC) were assayed in bone marrow by ELISpot as described (Brocca-Cofano et al., 2011) and previously reported (Vargas-Inchaustegui, et al. 2014; Demberg et al., 2013). Briefly, bone marrow cells were thawed and polyclonally-stimulated for three days in the presence of CpG (ODN-2006), IL-21, and human sCD40L. Env-specific IgA and IgG ASC were normalized to the total number of IgG and IgA ASC and reported as the percentage/frequency of IgA or IgG Env-specific ASC.

2.5. Evaluation of serum binding antibodies

Antibodies specific for HIV_{CZM} gp120 were evaluated by ELISA as described (Demberg et al., 2012) and previously reported (Demberg et al., 2013). Antibody titer was defined as the reciprocal of the serum dilution at which the optical density of the test serum was at least twice that of a negative rhesus macaque serum diluted 1:50.

2.6. Statistical Analysis

Data were analyzed using Prism (v5.03, GraphPad Software). A *p* value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Flow cytometry detection of SIV and HIV gp120 specific memory B cells in PBMC, bone marrow and rectal pinches

Viably frozen PBMC, bone marrow samples and fresh rectal pinch biopsies obtained from previous and on-going non-human primate studies were used to develop a direct staining method for Env-specific memory B cells in unfractionated PBMC, bone marrow or mucosal tissue. During surface staining with fluorescent antibodies, incubation with unconjugated anti-CD4 antibodies blocked gp120-CD4 interaction, enhancing the true signal and reducing the background signal resulting from non-specific binding of biotinylated gp120 to CD4 molecules on the cell surface. To further enhance specificity, staining with biotinylated gp120 and the APC-Streptavidin conjugate were performed separately at 4°C in the presence of 2% fetal bovine serum.

A stringent gating strategy was used for memory B cells. T cells and monocytes were excluded using anti-CD2 and anti-CD14 antibodies and dead cells using a live/dead marker. The memory B cell population in PBMC and bone marrow was defined as CD19⁺ CD20⁺ CD27⁺ IgD⁻ B cells, and gp120-specific cells were subsequently identified (Fig. 1A). Mucosal memory B cells which lack CD27 expression (Titanji et al., 2010; Thomas et al., 2014) were defined as CD19⁺ CD20⁺ CD21^{+/-} IgD⁻ B cells (Fig. 1C). Additional illustrations of B cell staining in macaque PBMC, bone marrow, and mucosal tissues have been published (Demberg et al., 2012; Demberg et al., 2014; Thomas et al., 2014). Representative staining of gp120-specific memory B cells in matched pre- and post-vaccination PBMC and a rectal pinch biopsy are shown in Fig. 1B and D, respectively, illustrating negligible staining of the pre-immunized control sample and positive staining of the post-vaccination samples.

To evaluate the reproducibility of this method, we analyzed SIV gp120-specific memory B cells in PBMC from Study 1 animals obtained prior to immunization (Pre), after the first two immunizations (post-prime), and at necropsy (two weeks after the last immunization). As shown in Fig. 2A, pre-vaccinated samples showed negligible gp120-specific staining, whereas levels following both the second (post-prime) and fourth immunizations (post-necropsy) were significantly elevated ($p < 0.0001$ and $p = 0.0057$, respectively). To evaluate direct staining of HIV gp120-specific memory B cells we used viably frozen PBMC from macaques of Study 2, obtained prior to immunization (Pre), two weeks after the last immunization (Post-protein boost) and two weeks post-SHIV challenge (post-challenge) (Fig. 2B). Background staining for HIV gp120-specific memory B cells in Pre samples was somewhat higher compared to Pre samples stained for SIV gp120-specific memory B cells (Fig. 2A). Nevertheless, HIV gp120-specific memory B cell levels were again significantly elevated at the conclusion of the vaccine regimen (Post-protein boost) and post-challenge ($p = 0.0007$ and $p = 0.0006$, respectively).

To evaluate memory B cells in another tissue compartment, we analyzed SIV gp120-specific memory B cells in bone marrow from Study 3 animals obtained prior to immunization and two weeks after the last immunization (Fig. 2C). SIV gp120-specific memory B cell levels were again significantly elevated following vaccination (Post-2nd boost; $p = 0.0060$).

Staining of SIV gp120-specific memory B cells in rectal tissue was evaluated using fresh tissue biopsies of Study 3 macaques. Pre-vaccination samples were not available. However, we assayed biopsies of 9 control macaques after 4 sham vaccinations, as well as 36 biopsies from vaccinated macaques at the same post-2nd protein boost time point (Fig. 2C). The control animals displayed no positive SIV gp120-specific staining after the sham vaccinations, while a small percentage of the vaccinated macaques were positive for SIV gp120-specific memory B cells at the same time point. Following SIV_{mac251} challenge, both control ($n=12$) and all but one of the vaccinated ($n=48$) macaques became infected over the course of repeated low-dose exposures (Tuero et al. in preparation). Two weeks post-challenge, SIV gp120-specific memory B cells were elevated in both controls and vaccinated animals, reflecting an expanded response to viral exposure. However, by 8 weeks post-infection memory B cells had declined in the rectal mucosa reflecting their expected loss post-SIV infection (Kuhrt et al., 2010; Peruchon et al., 2009).

3.2. Comparison of direct SIV gp120-specific memory B cell quantification with B cell ELISPOT frequencies and ELISA antibody titers

B cell ELISPOT is a standard method used to quantify antigen-specific ASC. A three-day polyclonal stimulation of B lymphocytes has been shown to induce B cell proliferation and differentiation, leading to conversion of memory B cells to ASC (Brocca-Cofano et al., 2011). In contrast, unstimulated B lymphocytes exhibit a sustained low level of ASC attributed to non-proliferating plasma cells. Therefore, we analyzed the percentage of SIV gp120-specific memory B cells detected in Study 1 PBMC by flow cytometry versus the frequency of IgG ASC obtained by ELISPOT following polyclonal stimulation of matched bone marrow samples from study 1 (Vargas-Inchaustegui et al., 2014). A highly significant correlation was observed at the Post-prime time point (Fig 3A, $p = 0.0069$), validating the direct staining method for SIV-gp120 memory B cell quantification. At the necropsy time point, a non-significant positive trend was observed (Fig. 3B, $p=0.075$).

We similarly validated the quantification of HIV gp120-specific memory B cells by flow cytometry in PBMC of Study 2 macaques post-SHIV challenge with the frequency of Env-specific ASC obtained by ELISpot in matched bone marrow samples. Statistically significant positive correlations were observed for both IgG and IgA memory B cells obtained by ELISpot (Fig. 3C, D; $p = 0.026$ and $p = 0.0063$, respectively). In samples with low frequencies of Env-specific memory B cells, positive results were more easily seen by direct staining than by ELISPOT (Figs 3A, C, D). This likely reflects some loss of bone marrow memory B cells which were polyclonally stimulated in culture for 3 days prior to assay of ASC by ELISPOT.

To further validate our method, we correlated the serum gp120-specific IgG titer quantified by ELISA in Study 2 (Demberg et al., 2013) with the SHIV gp120-specific memory B cells quantified by flow cytometry pre- and post-challenge. A significant correlation was seen post-protein boost (Fig 3E; $p = 0.038$) but not post-challenge (Fig. 3F). Complexing of serum antibody with virus or virus-infected cells could have impacted the assessment of binding antibody titer post-challenge. It has been established that HIV envelope-specific plasma antibody titers and memory B cell levels do not always correlate (Bonsignori et al. 2009).

To validate memory B cell quantitation by flow cytometry with that determined by ELISPOT in the same tissue compartment we used bone marrow cells. The percentage of SIV gp120-specific memory B cells detected in Study 3 bone marrow by flow cytometry versus the frequency of IgG ASC obtained by ELISPOT in matched bone marrow samples from study 3 was analyzed. A highly significant correlation was observed at the post-2nd boost time point (Fig 3G, $p = 0.0004$).

4. DISCUSSION

We have developed an improved flow cytometry-based method to identify and quantify Env-specific memory B cells in blood, bone marrow, and mucosal tissues without the need for prior cell enrichment or sorting. The method is specific, exhibiting low background levels on control and pre-immunization samples, and sensitive, correlating significantly with

both ELISPOT for quantification of B cells and in some cases ELISA titers for circulating antibody levels. The method will allow simultaneous monitoring of additional B cell parameters, including homing and trafficking.

The correlations of memory B cells post-vaccination and prior to challenge as assessed by flow cytometry with ASC in the bone marrow and serum antibody binding titers in the same macaques clearly validates the direct staining method. The lack of a significant correlation between Env-specific memory B cells and serum antibody binding titers in SHIV-infected macaques two weeks post-challenge in contrast reflects a complex of variables. Antibody levels at this time likely increase due to exposure to virus and expansion of the memory B cell pool. Some short-term plasma blasts will have matured to plasma cells and become long-term ASC, contributing to the levels of circulating Env-specific antibody. At the same time, some antibody may become complexed with viral particles or virus-infected cells, decreasing what is measured in a standard ELISA. Furthermore, memory B cells decline in numbers following SHIV infection and continued B cell dysfunction. Nevertheless, even in this situation, direct memory B cell monitoring will contribute to immune correlates analysis in vaccine studies, and enhance our understanding of B cell dynamics in HIV and SIV pathogenesis.

Overall the improved method described here provides a rapid and efficient means of monitoring Env-specific memory B cells over the course of large-scale investigations, and will help elucidate B cell maturation in response to different vaccine and adjuvant modalities.

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Highlights

Direct staining of un-enriched HIV/SIV Env-specific memory B cells is described.

The memory B cells were quantified in both SIV-vaccinated and SHIV-infected macaques.

The method is applicable to cells in mucosal tissues, bone marrow, and PBMC.

Env-specific memory B cell frequencies correlated with ASC assessed by ELISPOT.

Env-specific memory B cell frequencies correlated with serum antibody titers.

Figure 1. Gating strategy for identification of gp120-specific memory B cells in PBMC and rectal tissue

A) PBMC from a SHIV-challenged macaque were initially gated on a live/dead gate and CD2⁻, CD14⁻ cells. This population was further gated for CD19⁺ and CD20⁺ cells to obtain a pure B cell population. IgD⁻, CD27⁺, gp120⁺ cells were considered as Env-specific memory B cells. The numbers in the plot indicate the frequency of the gated population. B) A representative plot comparing gp120-specific memory B cell staining in PBMC obtained at pre- and post-vaccinated time points from the same macaque. C) A single cell suspension from rectal tissue from a SHIV-challenged macaque was initially gated as in panel A. Following gating on CD19⁺CD20⁺ B cells, Env-specific memory B cells were identified as CD21⁺IgD⁻gp120⁺. D) A representative plot comparing gp120-specific memory B cell staining in rectal pinches obtained at pre- and post-vaccination time points from the same macaque.

Figure 2. Evaluation of gp120-specific memory B cells in SIV Env-vaccinated and HIV Env-vaccinated and SHIV-infected macaques

A) PBMC from Study 1 animals were assayed for SIV gp120-specific memory B cells prior to vaccination (Pre), two weeks after 2 vaccinations (Post-Prime), and two weeks after 4 vaccinations (Necropsy). B) PBMC from Study 2 animals were assayed for HIV gp120-specific memory B cells prior to vaccination (Pre), two weeks after the last immunization (Post-Protein boost), and two weeks after SHIV challenge (Post-Challenge). C) Bone marrow samples from Study 3 animals were assayed for SIV gp120-specific memory B cells prior to vaccination (Pre) and two weeks after the last immunization (Post-2nd boost). D) Rectal pinches from Study 3 macaques were quantified for SIV gp120-specific memory B cells two weeks after the last immunization (Post-protein boost), and 2 and 8 weeks Post-SIV_{mac251} challenge. Results for vector/adjuvant controls and vaccinated macaques (n = 36) are shown separately. Data presented are means $\pm$ SEM. $p < 0.05$ indicates statistically significant differences between the indicated groups by the Mann-Whitney test.

Figure 3. gp120-specific memory B cells quantified by flow cytometry correlate with B cell ELISPOT data and antibody binding titers

(A, B) Correlation of SIV gp120-specific memory B cells in PBMC of Study 1 vaccinated macaques with bone marrow Env-specific memory B cells determined by ELISPOT, 2 weeks after two (A) and four (B) immunizations, respectively. (C, D) Correlation of HIV gp120-specific memory B cells in PBMC of Study 2 macaques with (C) Env-specific IgG- and (D) IgA-memory B cells in bone marrow determined by ELISPOT 2 weeks post-SHIV challenge. (E) Correlation of HIV gp120-specific memory B cells in PBMC of Study 2 vaccinated macaques with serum antibody titers determined by ELISA 2 weeks after the last immunization. (F) Lack of significant correlation of HIV gp120-specific memory B cells in PBMC of Study 2 SHIV-infected macaques 2 weeks post-infection with serum antibody titers obtained at the same time point. (G) Correlation of SIV gp120-specific memory B cells in bone marrow of Study 3 vaccinated macaques with matched bone marrow Env-specific memory B cells determined by ELISPOT, post-immunization. Correlation r and p values are from Spearman's rank analysis. $p < 0.05$ indicates statistically significant differences.