Soluble HIV-1 Env trimers in adjuvant elicit potent and diverse functional B cell responses in primates

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Broadly neutralizing antibodies (bNAbs) against the HIV-1 envelope glycoproteins (Envs) have proven difficult to elicit by immunization. Therefore, to identify effective Env neutralization targets, efforts are underway to define the specificities of bNAbs in chronically infected individuals. For a prophylactic vaccine, it is equally important to define the immunogenic properties of the heavily glycosylated Env in healthy primates devoid of confounding HIV-induced pathogenic factors. We used rhesus macaques to investigate the magnitude and kinetics of B cell responses stimulated by Env trimers in adjuvant. Robust Env-specific memory B cell responses and high titers of circulating antibodies developed after trimer inoculation. Subsequent immunizations resulted in significant expansion of Env-specific IgG-producing plasma cell populations and circulating Abs that displayed increasing avidity and neutralization capacity. The neutralizing activity elicited with the regimen used was, in most aspects, superior to that elicited by a regimen based on monomeric Env immunization in humans. Despite the potency and breadth of the trimer-elicited response, protection against heterologous rectal simian-HIV (SHIV) challenge was modest, illustrating the challenge of eliciting sufficient titers of cross-reactive protective NAbs in mucosal sites. These data provide important information for the design and evaluation of vaccines aimed at stimulating protective HIV-1 immune responses in humans.

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Abbreviations used: ASC, antibody-secreting cell; bNAb, broadly neutralizing antibody; Env, envelope glycoprotein; NHP, nonhuman primate; PC, plasma cell; PWM, pokeweed mitogen; SAC, *Staphylococcus aureus Cowan*; SHIV, simian-HIV.

An increasing number of licensed human vaccines against infectious agents are based on recombinant proteins, including the hepatitis B virus (HBV) and the recently developed human papilloma virus (HPV) vaccines (McAleer et al., 1984; Harper et al., 2004; Joura et al., 2007). These successful vaccines demonstrate the principle that an effective antibody response can provide protection against real world challenges, providing encouragement for ongoing attempts to develop a vaccine against human immunodeficiency virus type 1 (HIV-1). However, unlike the HBV and HPV vaccines, which are produced as virus-like particles, most recombinant envelope glycoproteins (Envs) tested in immunogenicity studies so far are soluble and heavily glycosylated proteins, two properties which may have an impact on the elicited humoral response. Early attempts to stimulate immune responses against HIV-1 using monomeric Env protein administered with Alum failed to demonstrate protection (VAX04). In contrast, recent results from the Thai phase III clinical trial (RV144) suggest that immunization regimens that include Env protein as a boost, after priming with a recombinant viral vector, decreases the risk of HIV-1 acquisition (Rerks-Ngarm et al., 2009). However, the protective effect appeared to be transient and the mechanisms mediating this, including potential antibody-mediated effects, are not yet determined.

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Despite the lack of protective correlates for HIV-1 infection, a vaccine that elicits broadly neutralizing antibodies (bNAbs) remains a high priority as this type of B cell response is likely to be most protective (Burton et al., 2004; Pantophlet and Burton, 2006; Karlsson Hedestam et al., 2008). Most antiviral vaccines do protect via NAb, and several studies demonstrate that passively administered NAbs can protect against challenge with simian-HIV (SHIV) in nonhuman primate (NHP) models (Baba et al., 2000; Mascola et al., 2000; Parren et al., 2001). A major limitation for current attempts to design an Env immunogen capable of eliciting bNAbs is the lack of a high resolution structure of the native glycan-shrouded HIV-1 Env spike. Most recombinant trimers tested so far are empirical in their design and elicit Abs possessing relatively limited breadth of neutralization, perhaps as a result of their failure to faithfully mimic the functional Env spike (for review see Forsell et al., 2009).

During chronic HIV-1 infection, bNAbs develop, but only in a subset of individuals, and these responses do not usually appear until several years after establishment of chronic viral infection (for review see Stamatatos et al., 2009). Approximately 25% of infected individuals develop Ab responses capable of neutralizing a diverse set of primary viruses and a small percentage of this select group develops very broad and potent neutralizing responses (Doria-Rose et al., 2009; Sather et al., 2009; Simek et al., 2009). Studies aimed at defining the Ab specificities present in individuals harboring broad plasma neutralization has intensified over the last few years as new methods to facilitate these analyses were described (Dhillon et al., 2007; Li et al., 2007; Binley et al., 2008; Moore et al., 2008; Sather et al., 2009; Scheid et al., 2009a,b). Recently, new broadly neutralizing mAbs were isolated and characterized (Walker et al., 2009; Corti et al., 2010; Wu et al., 2010). These mAbs will provide valuable information for immunogen design, especially once their cognate target epitopes are defined at the atomic level of resolution.

In addition to the need to design more effective Env immunogens, an improved basic understanding of vaccine-induced B cell responses in primates may be required to advance the development of an effective prophylactic HIV-1 vaccine. To date, most HIV-1 Env-based vaccine studies examined the humoral immune responses at the serologic level, whereas only a few studies investigated anti-Env responses at the B cell level (Bonsignori et al., 2009; Dosenovic et al., 2009). An increased focus on the cells that produce vaccine-elicited antibodies is therefore needed to guide efforts to better replicate the successful generation of bNAbs that is seen in some infected humans. Examination of neutralizing mAbs isolated from chronically HIV-1–infected individuals suggests that extensive Ab affinity maturation is required to achieve efficient neutralization (Burton et al., 1994; Scheid et al., 2009b; Walker et al., 2009). However, little is known about how B cell selection in the germinal center reaction can be stimulated most effectively with nonreplicating subunit vaccines to drive somatic mutation of Ab genes. Additionally, we lack knowledge regarding how different immunization regimens affect the development of Env-specific memory B cell and plasma cell (PC) populations in healthy primate subjects.

In this paper, we used rhesus macaques to assess the B cell response in a detailed and comprehensive manner after inoculation with soluble Env protein in adjuvant over a relatively long period of antigen exposure. Initially, we evaluated a panel of stimulation conditions to allow efficient in vitro expansion and differentiation of macaque memory B cells into antibody-secreting cells (ASCs). This allowed us to enumerate the frequency of memory B cells and PCs from PBMC cultures and bone marrow after sequential immunization with soluble HIV-1 Env trimers. To our knowledge, this is the first time a comprehensive analysis of the stimulation conditions for HIV-1 Env-specific rhesus memory B cells is reported. Using these protocols, we demonstrate that each Env trimer boost resulted in a significant increase in peripheral Env-specific PCs, peaking at ∼20% of total IgG^+ PCs before declining to baseline levels within 1 wk. This is similar to the response kinetics reported for other protein-based antigens in humans (Wrammert et al., 2008; Blanchard-Rohner et al., 2009). Env-specific IgG^+ memory B cells constituted between 10 and 20% of total IgG^+ memory B cells in peripheral blood after two trimer inoculations and these levels remained stable between the inoculations. These results suggest that, when administered at high doses and in a potent adjuvant, the soluble HIV-1 Env immunogen used in this study does not appear to be a weak antigen in terms of the overall magnitude and kinetics of the elicited response. Furthermore, circulating Abs were generated that were capable of in vitro neutralizing a diverse panel of viruses. The response was considerably broader than that measured in humans inoculated with monomeric Env in Alum in the VAX04 phase III clinical trial, with the caveat that several variables need to be taken into account when making this comparison.

After our extensive immunogenicity analysis, we subjected the animals to rectal challenge with a heterologous SF162 Env-based SHIV, SHIV-SF162P4. We observed a modest, but nonsterilizing, impact on acquisition of infection. The limitation of the protective effect may be a result of the low levels of NAbs available in the rectal mucosa because the circulating Abs in plasma neutralized SHIV-SF162P4 with moderate potency in vitro. The results presented in this paper highlight the challenge of achieving sterilizing protection against heterologous HIV-1 challenge. This study also provides benchmark immunogenicity results, which can be used to guide the design of Env-based immunization regimens that are under advancement into human clinical trials.

RESULTS

Pokeweed mitogen (PWM) + *Staphylococcus aureus Cowan* (SAC) + CpG stimulation of PBMCs yields efficient differentiation of macaque memory B cells into PCs in vitro

Memory B cells do not produce Abs unless they are reactivated by antigen or polyclonal stimulation to differentiate into ASC. Enumeration of total and antigen-specific memory B cells is greatly facilitated by methods to differentiate memory B cells into ASC to allow their detection by B cell ELISpot analysis. Several cytokines, mitogens, and toll-like receptor

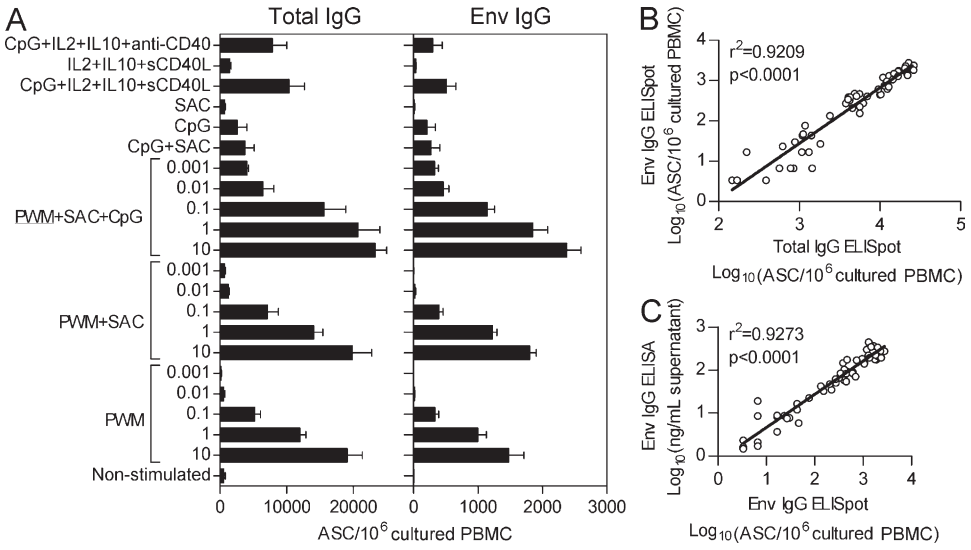

Figure 1. Differentiation of macaque memory B cells into ASC in vitro. (A) PBMCs isolated from rhesus macaques inoculated with Env trimers in adjuvant were stimulated 4 d in vitro for differentiation of memory B cells into ASC. Stimulations were performed in 48-well plates with combinations of CpG, IL-2, IL-10, CD40L, anti-CD40, SAC, and PWM as indicated. PWM (underlined) was tested at concentrations ranging from 10 µg/ml to 1 ng/ml. Stimulated cells were added to ELISpot plates for detection of total and Env-specific IgG-producing cells. Bar graphs represent mean values + SEM of two to three monkeys normalized to 10^6 cultured PBMCs. (B) The correlation between Env-specific and total IgG-producing ASCs was determined for each stimulation after $\log_{10}$ transforming the values. (C) The correlation between Env-specific ASC quantified by B cell ELISpot assay and secreted Env-specific Abs quantified by ELISA for each stimulation condition was determined. Pearson's correlation (r^2) and the p-value for the correlation efficiency are shown. The experiment was repeated three times.

ligands were described as important factors for the proliferation and differentiation of human memory B cells into ASC (Arpin et al., 1995; Krieg et al., 1995; Crotty et al., 2004; Amanna and Slifka, 2006; Huggins et al., 2007). To establish protocols for efficient macaque memory B cell differentiation, we used rhesus and cynomolgus macaque PBMCs collected 14 d after Env immunization. At this time, circulating PCs are not detected by direct B cell ELISpot analysis, whereas memory B cells require a period of in vitro expansion and differentiation into ASC before they are detected (Fig. S1). The ELISpot assay used in this study is based upon a recently described protocol where total IgG is captured on the plate and antigen-specific IgG is detected with biotinylated Env probes, generating high definition spots with low background (Dosenovic et al., 2009).

We evaluated a panel of stimuli for in vitro differentiation of macaque memory B cells. The efficiency of the stimulations varied considerably, with a combination consisting of PWM, SAC, and CpG (PWM + SAC + CpG) being the most potent under the conditions tested in this study (Fig. 1 B). There was a strong correlation between the number of Env-specific and total IgG^+ memory B cells per million cultured PBMCs, indicating nonbiased polyclonal stimulation ($r^2 = 0.9209$; Fig. 1 B). A strong correlation between the number of Env-specific memory B cells detected by ELISpot assay and secreted Ab measured by ELISA was also observed ($r^2 = 0.9273$; Fig. 1 C). To compare the quality of the response stimulated by PWM + SAC + CpG or IL-2 + IL-10 + CpG + CD40L, we also measured the kinetics of the response. For both stimulation conditions, the highest number of ASC (total and Env-specific) was present in the culture after 4 d and declined thereafter (Fig. S2 A), whereas maximal accumulation of Abs in the supernatant required an additional 4 d of culture (Fig. S2 B). The frequencies of Env-specific ASC and secreted IgG in the supernatant were similar at all time points measured for both stimulation conditions (Fig. S2, A and B).

The phenotype of the B cells cultured in the presence of PWM + SAC + CpG or IL-2 + IL-10 + CpG + CD40L was also investigated. As shown in Fig. 2 A, the largest fraction of IgG-expressing cells (20%) of the CD4/CD8-negative population was found in the PWM + SAC + CpG–stimulated cultures. The corresponding population in the IL-2 + IL-10 + CpG + CD40L–stimulated culture was 7.8%. As expected, only cells displaying low CD20 expression were IgG producers and no CD20 down-regulation was observed in nonstimulated control cultures (Fig. 2 A). To assess the proliferative capacity of lymphocytes in response to the different stimuli, PBMCs were CFSE stained and incubated with PWM + SAC + CpG, IL-2 + IL-10 + CpG + CD40L, or CpG alone. The proliferation of T cells ($CD4^+$, $CD8^+$, and $CD20^-$) and B cells ($CD4^-$, $CD8^-$, and $CD20^{+/low}$) was evaluated after 6 d of culture. Consistent with the B cell ELISpot results, the PWM + SAC + CpG cocktail stimulated B cell expansion and differentiation most efficiently. This cocktail also stimulated T cell proliferation (Fig. 2 B). In contrast, IL-2 + IL-10 + CpG + CD40L and, to a lesser extent CpG alone, stimulated B cell proliferation and down-regulation of CD20 but not T cell proliferation. To further elucidate the role of T cells for the responsiveness of human and rhesus PBMCs to

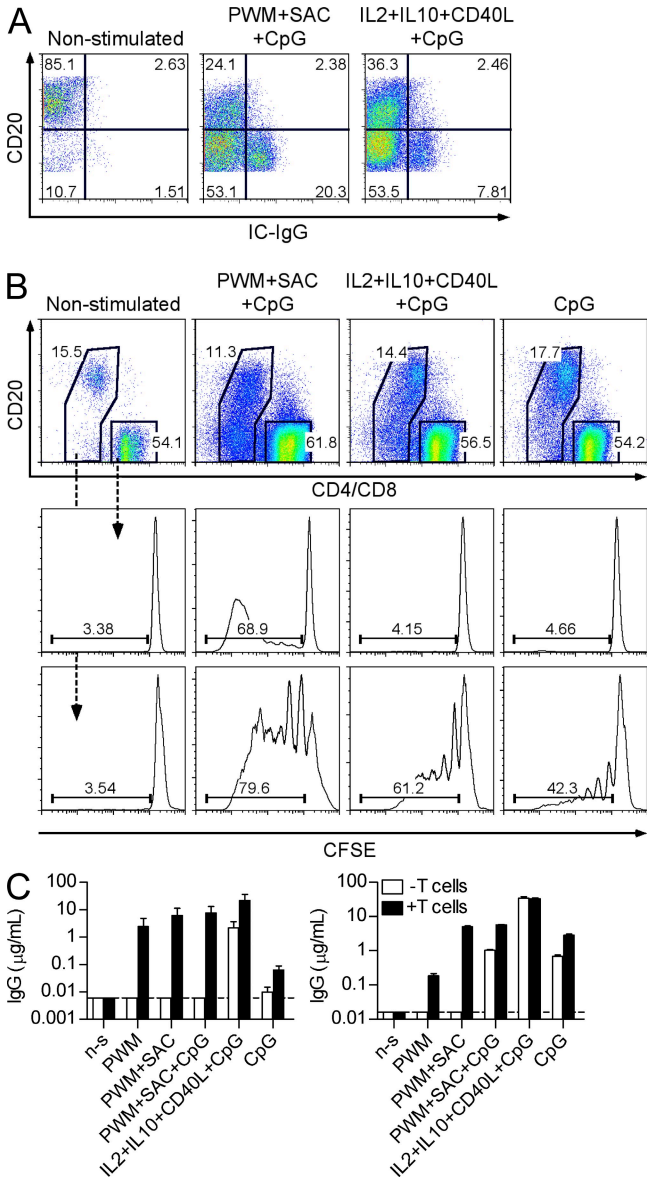

Figure 2. Phenotypic characterization of rhesus macaque lymphocytes after in vitro stimulation. (A) Rhesus macaque PBMCs were stimulated with PWM + SAC + CpG, IL-2 + IL-10 + CpG + CD40L, or were nonstimulated (n-s) for 4 d and then stained for CD20, CD4, CD8, and intracellular IgG (IC-IgG). Nonviable cells were excluded via forward scatter and side scatter gating and B cells were defined as $CD4^-CD8^-CD20^{+/low}$ cells. The numbers in the quadrants represent the percent cells of the total $CD4^-CD8^-CD20^{+/low}$ cell population. The dot blots are representative of two donors. (B) Rhesus PBMCs were stained with CFSE and stimulated with PWM + SAC + CpG, IL-2 + IL-10 + CpG + CD40L, or CpG or nonstimulated for 6 d. Proliferation of $CD20^{+/low}$ $CD4^-CD8^-$ B cells (bottom) and $CD20^{low}CD4^+CD8^+$ T cells (middle) was analyzed. The numbers represent the percentage of cells in each indicated gate of total cells gated on side scatter/forward scatter. The dot blots and histograms are representative of two donors. (C) $CD19^+$ or $CD20^+$ B cells and $CD4^+$ T cells were isolated from human (right) and rhesus macaque (left) PBMCs. The B cells were stimulated alone (white) or in combination with T cells (black) for 7 d and IgG production was evaluated by ELISA. The bar graph describing human data represents mean and standard deviation of triplicate samples. Stimulations on sorted human B cells were independently repeated three times with similar results. Data from rhesus macaque PBMCs represent mean and SEM of three donors.

stimulation by PWM, PWM + SAC, PWM + SAC + CpG, IL-2 + IL-10 + CpG + CD40L, or CpG alone, B cells were sorted and cultured in the presence or absence of autologous T cells and the different stimuli. The results demonstrate that the stimulatory effect of PWM on both human and rhesus memory B cells was dependent on the presence of T cells. Only the IL-2 + IL-10 + CpG + CD40L cocktail induced differentiation of human and rhesus memory B cells in the absence of T cells (Fig. 2 C). The data also show that human memory B cells were more responsive to CpG than rhesus memory B cells under the assay conditions used in this study (Fig. 2 C).

Boosting results in high HIV-1 Env-binding Ab titers and increased Ab avidity

To examine the kinetics by which antigen-specific Abs, memory B cells, and PCs develop in response to Env immunizations, rhesus macaques were inoculated with HIV-1 Env trimers in adjuvant (immunized) or adjuvant alone (controls) via the i.m. route. We used cleavage-defective soluble gp140 trimers (gp140-F) derived from the primary clade B isolate YU2. The trimers possess a heterologous trimerization motif from fibritin (F), as previously described (Yang et al., 2002). The schedule for inoculations and sample collections is shown in Fig. 3 A. Plasma and PBMCs were collected before the first protein inoculation and 1 and 2 wk after each inoculation. Bone marrow samples were collected 3 wk after the second and fourth inoculations. 1 mo after the last inoculation, all animals were challenged with SHIV-SF162P4 to assess the protective capacity of the immune response.

Plasma from Env trimer-inoculated animals were tested for binding Abs to YU2 Env by ELISA (gp120; Fig. 3 B). The data are shown as half-maximum binding titers of Env-specific IgG and IgA (Log10 optical density [OD] 50 titers). Env-specific IgG responses were detected 2 wk after the first inoculation and they were boosted by the second inoculation, resulting in OD50 titers of $\sim 10^5$, which were maintained after the subsequent inoculations. Env-specific IgA responses were ~1.5 log lower but followed the same kinetics. The response was comparable between individual animals for both IgG and IgA. When the plasmas were examined for increases in functional affinity, often referred to as avidity, using a more stringent binding assay involving incubation in 1.5 M NaSCN (see Materials and methods), we recorded an enhancement in avidity with repeated boosting (Fig. 3 C). These data demonstrate that i.m. immunizations with Env trimers administered in Abisco-100 and CpG ODN 2395 elicit potent plasma Ab responses, which evolve with repeated antigen exposure. To investigate if this increase was selective for subspecificities of Env, we performed the avidity assay with three trimeric YU2-based Env probes: gp140-F, gp120-F, and gp120ΔV1/2/3-F (Dosenovic et al., 2009). We observed a significant increase in

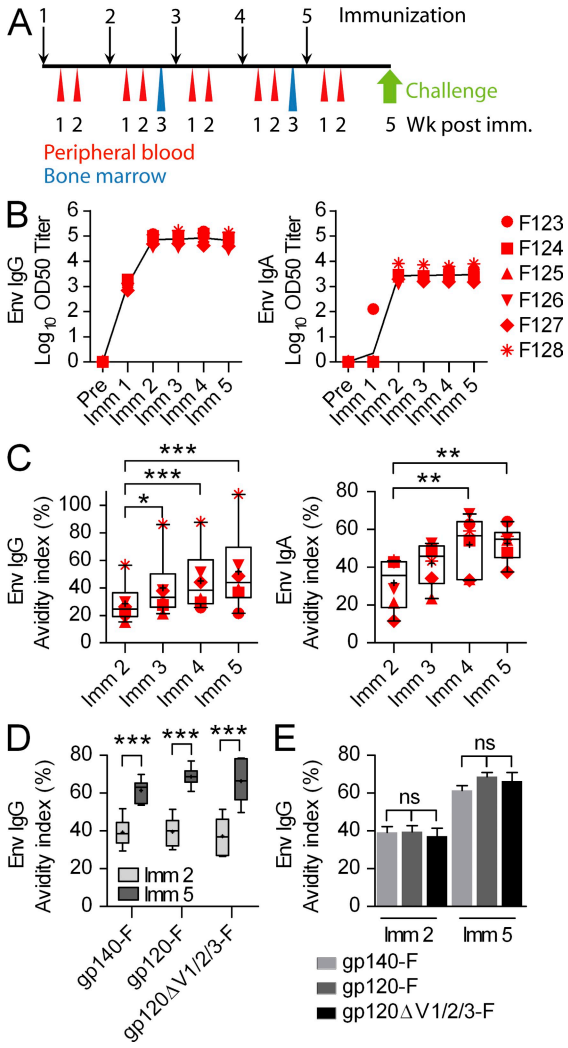

Figure 3. Development of anti-Env binding Abs in Env trimer–inoculated macaques. (A) Schematic representation of the study design. Inoculations (black arrows) were given monthly for 5 mo with Env trimers in Abisco-100 + CpG to six monkeys (F123-F128). Six other monkeys were inoculated with Abisco-100 + CpG alone. Blood (red arrowheads) was collected 1 and 2 wk after each inoculation. Bone marrow (blue arrowheads) was sampled 3 wk after the second and fourth protein inoculation. The animals were challenged (green arrow) with SHIV-SF162P4 virus 5 wk after the last vaccination. (B) Env-binding IgG and IgA Abs were measured 2 wk after each inoculation (Pre, preimmunization; Imm 1, first immunization). Binding titers are presented as Log_{10} OD50 titer, and the black line corresponds to the mean of six monkeys. (C) Avidity index was measured 2 wk after each inoculation with the individual monkeys ($n = 6$) shown. The experiment was repeated at two independent occasions with similar results for the IgG measurements and once for the IgA measurements. (D) Avidity index against gp140-F, gp120-F, and gp120ΔV1/2/3-F in samples collected 2 wk after immunizations two and five ($n = 6$). The experiment was performed once. (E) Comparison of avidity index against the different Env antigens in the same samples as in D. The experiment was performed once. The box plots illustrate the following: horizontal line, median; plus, mean; box, interquartile range; whiskers, min/max and error bars, +SEM. Significance was evaluated using ANOVA. ns, nonsignificant. *, $P \leq 0.05$; **, for $P \leq 0.01$; ***, for $P \leq 0.001$.

the avidity index between the samples after immunization two and after immunization five for all three probes (Fig. 3 D). Furthermore, there was no significant difference between the avidity indexes for the three probes at either time point (Fig. 3 E). These results indicate that the increase in avidity between the second and fifth immunization was not selective for variable region 1–3 determinants (which are absent from gp120ΔV1/2/3-F) or for gp41 determinants (which are absent from gp120-F and gp120ΔV1/2/3-F) but probably reflect overall avidity increases for several determinants displayed by these complex glycoprotein immunogens.

Boosting results in transient peaks of Env-specific PCs and high levels of memory B cells

To examine the dynamics of Env-specific memory B cells and PCs in the Env trimer-inoculated animals over the prime/boost regimen, we collected PBMCs 1 and 2 wk after each inoculation. The cells were either plated directly in ELISpot plates for detection of PCs or stimulated in vitro for 4 d with PWM, SAC, and CpG for detection of memory B cell–derived ASC. Circulating PCs are presented as ASC per 10^6 PBMCs (Fig. 4 A, top) and as the percent Env-specific of total IgG-producing cells (Fig. 4 A, bottom). Circulating memory B cells are presented as ASC per 10^6 cultured PBMCs (Fig. 4 B, top) and as the percent Env-specific of total IgG-producing cells (Fig. 4 B, bottom). The results demonstrate that the frequencies of total IgG^+ PCs and memory B cells were stable during the course of the immunization schedule in both control and Env trimer-inoculated animals. In contrast, Env-specific PCs peaked 1 wk after each Env inoculation and then declined to near baseline levels. Env-specific memory B cells were detectable 2 wk after the first inoculation and achieved peak levels 1 wk after the second inoculation. Thereafter, the frequency of Env-specific memory B cells was maintained at stable levels at the time points measured. Though not shown in these animals, a decline in specific memory B cells at later time points after protein inoculation is expected based on data from another set of animals where we analyzed additional time points after immunization (Fig. S3). No Env-specific PC or memory B cells were detected in the adjuvant-only inoculated control animals.

We also examined the frequency of Env-specific PCs in the bone marrow after both the second and the fourth trimer inoculations. Approximately 3% of total IgG^+ PCs were Env specific at both time points (Fig. 4 C). We did not detect any memory B cells in the bone marrow using either PWM + SAC + CpG stimulations or IL-2 + IL-10 + CpG + CD40L, which stimulates memory B cells in the absence of T cells (Fig. 4 D). These data suggest that memory B cells are rare in the bone marrow of rhesus macaques.

An important issue to address is if Env selectively boosts B cells that recognize specific regions of the trimers during the immunization regimen. To determine the subspecificities of circulating Abs and memory B cells, and to ask if these specificities shift during the course of the immunization schedule, we used the same series of trimeric Env probes as described in the previous section (gp140-F, gp120-F, and gp120ΔV1/2/3-F).

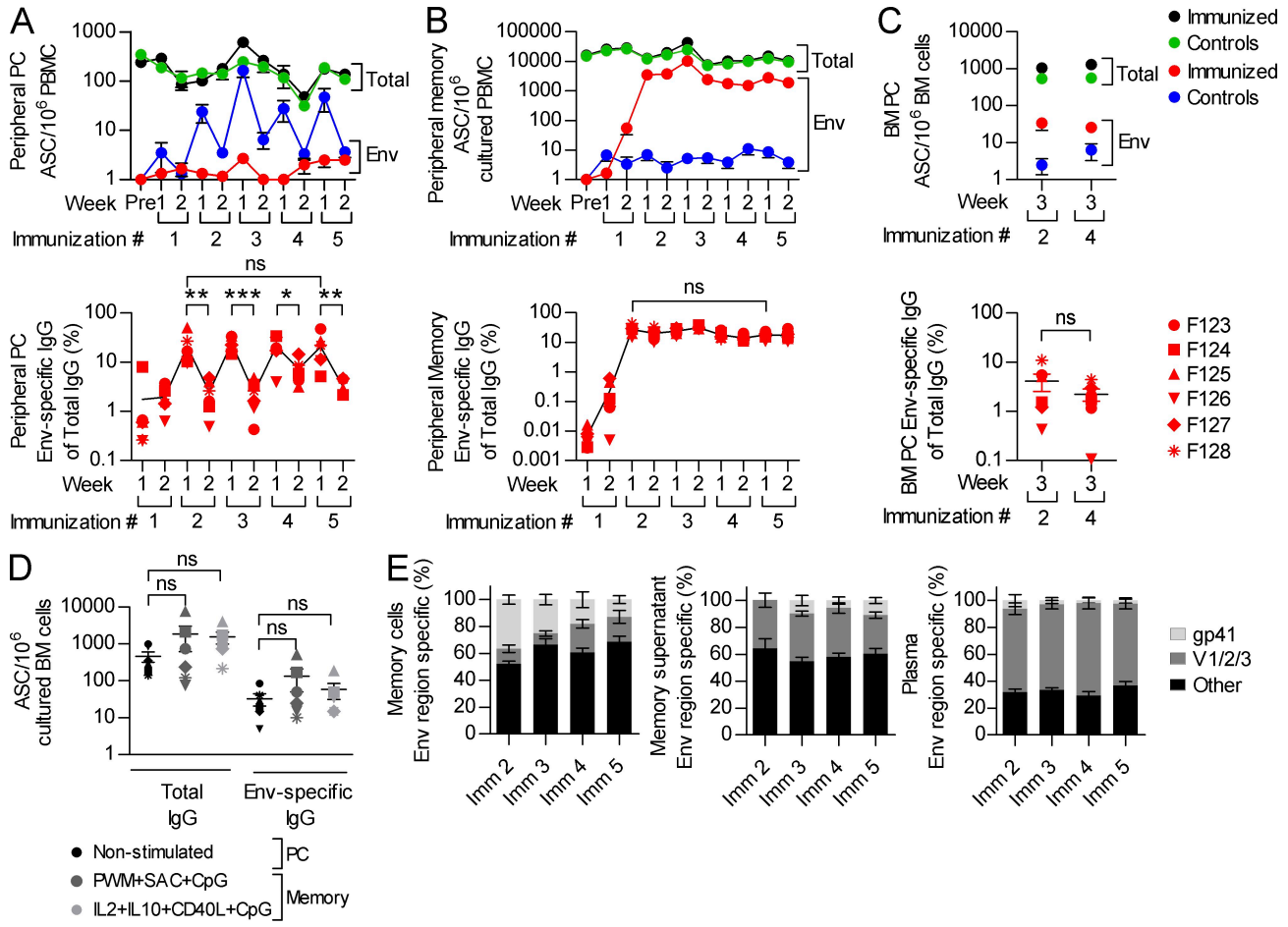

Figure 4. Dynamics of total and Env-specific B cell subsets in peripheral blood and bone marrow. The frequencies of total and Env-specific PCs (directly plated) and memory B cells (4 d in vitro stimulation with PWM + SAC + CpG before plating) were determined by B cell ELISpot analysis in animals inoculated with Env trimers in Abisco-100 + CpG (immunized) or Abisco-100 + CpG alone (controls). (A) The top shows total PC from immunized (black) and control (green) animals and Env-specific PCs from immunized (red) and controls (blue) in blood, plotted as ASC/10^6 PBMC. Circles indicate group mean ± SEM ($n = 6$). The bottom shows the percentage of Env-specific of total PCs, with individual animals (F123-F128) shown as different symbols. (B) The top shows frequencies of total and Env-specific IgG^+ memory B cells as group means, plotted as ASC/10^6 cultured PBMC. The bottom shows the percentage of Env-specific of total IgG^+ memory B cells for individual animals. (C) The top shows total and Env-specific PC in bone marrow samples (BM PC) collected 3 wk after inoculations two and four, plotted as ASC/10^6 BM cells. The bottom shows the percent Env-specific of total PC in bone marrow for individual animals. The error bars represent means ± SEM. (D) Bone marrow cells from individual animals were cultured in vitro with PWM + SAC + CpG or IL-2 + IL-10 + CpG + CD40L or were left non-stimulated for 4 d. Data are shown as ASCs per 10^6 cultured bone marrow cells and significance was determined using ANOVA. The experiment was performed at two independent occasions with similar results. (E) Subspecificities of the B cell responses 2 wk after each inoculation were measured using a differential ELISpot assay (left) and differential ELISA (middle and right). Bars represent fractions of subspecificities of total gp140-specific responses, presented as the percentage of region-specific of total Env responses ± SEM ($n = 6$). Differential ELISA experiments were performed on three separate occasions with similar results and the differential B cell ELISpot was performed once for each time point. ns = nonsignificant. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

We determined the frequency of memory B cells reactive to the three probes using subtractive analysis to calculate the percentage of gp41-reactive, V1/2/3-reactive, and non-gp41/non-V1/2/3 (termed "other") fractions. By plotting the data as the percent region specific of total Env-specific memory B cells, the results reveal that a considerable fraction (~30% of memory B cells) was directed against gp41 after two immunizations, but this fraction diminished during the course of the vaccination schedule to ~10% of the total Env-specific response (Fig. 4 E). In contrast, the fraction directed against the gp120 variable regions expanded over time and especially after the third immunization. The results were different when the supernatants from PWM + SAC + CpG–stimulated PBMCs were analyzed in ELISA coated with the different Env probes. After converting the data to the percent region specific of total Env-specific reactivity, there was nearly no detectable gp41-specific response. Also, there was no difference in the relative reactivity against variable region versus other during the course of the immunizations. A similar pattern was observed when the plasma was analyzed by differential ELISA (Fig. 4 E). These results suggest that analyses based on ELISA, which measures secreted Ab in solution, are not sufficiently sensitive to measure subtle shifts

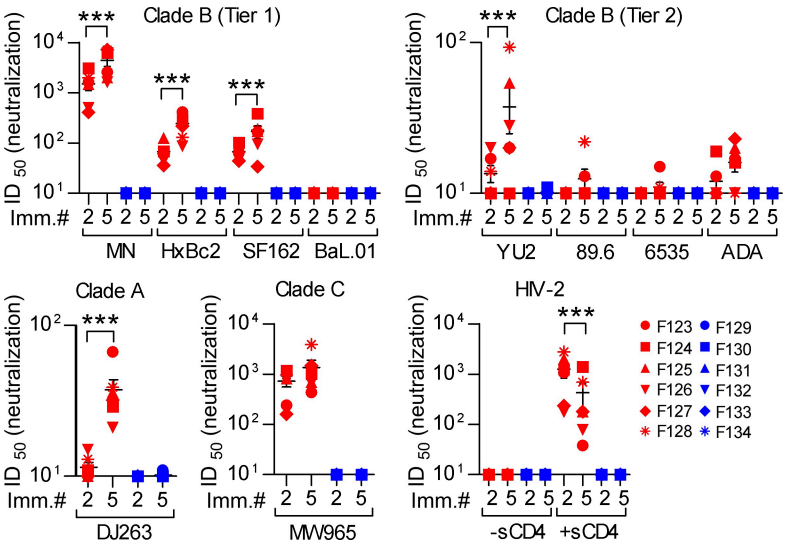

Figure 5. Maturation of neutralizing Ab responses with repeated immunizations. Neutralizing activity in plasma collected 2 wk after the second and fifth inoculations of Env-immunized macaques (red; $n = 6$) and controls (blue; $n = 6$) using the TZM-bl pseudovirus assay. The analysis was performed once, at multiple dilutions of each plasma sample. Data from individual animals are shown as the reciprocal dilution giving 50% neutralization (ID_{50}) with group means ± SEM indicated. Clade B HIV-1 isolates that are sensitive (tier 1) or resistant (tier 2) to neutralization were examined. The viruses tested were: tier 1 clade B viruses MN, HxBc2, SF162, and BaL.01 (top left); and Tier 2 clade B viruses YU2, 89.6, 6535, and ADA (top right). The clade A virus DJ263 (bottom left) and the clade C virus MW965 (bottom middle) were also examined. Plasma was also tested for the presence of coreceptor binding site-directed Abs (CoRbs Abs) that neutralize virus pseudotyped with HIV-2 Env in the presence of subinhibitory concentration of soluble CD4 (sCD4; bottom right). ID_{50} values between inoculations two and five were compared with repeated measures ANOVA. ***, $P < 0.001$.

in Ab subspecificities, likely as a result of competition between Abs of different specificities in this assay format.

The YU2 trimers elicit NAbs capable of neutralizing clade A, B, and C viruses, and the response is broader than that elicited in the VAX04 study

To investigate the NAb activity elicited by the Env trimers, we examined the capacity of the plasma collected after two and five immunizations to neutralize a panel of HIV-1 isolates. The selected isolates are known to display varying degrees of neutralization sensitivity (Li et al., 2005). The panel included clade B isolates representing neutralization-sensitive viruses (tier 1) and isolates representing more neutralization-resistant primary circulating variants (tier 2). Recombinant viruses were pseudotyped with the following clade B Envs: MN, HxBc2, SF162, and BaL.01; and the following tier 2 clade B Envs: YU2, 89.6, 6535, and ADA. In addition, viruses were pseudotyped with one clade A (DJ263) and one clade C (MW965) Envs. The data are presented as 50% plasma inhibitory dilution (ID_{50}) values. In agreement with previous studies in rhesus macaques (Mörner et al., 2009; Douagi et al., 2010), we observed potent ID_{50} neutralizing titers against several Tier 1 viruses and detectable, but less potent, neutralization against some Tier 2 viruses (Fig. 5 A). Mean ID_{50} neutralization titers against MN were ∼1,000 after two immunization and these responses were significantly increased after five immunizations. High ID_{50} titers against HxBc2 and SF162 were also measured after two immunization and these responses were boosted by the subsequent immunizations. Neutralization of viruses pseudotyped with the neutralization-resistant homologous YU2 Env was observed in three animals after two immunizations and in five animals after five immunizations. A similar pattern was observed for ADA. There was no or only sporadic neutralization of 89.6, 6535, TRO.11, JRCSF, or REJO (Fig. 5 A and not depicted). In contrast, neutralizing activity against Tier 1 viruses DJ263 and MW965 was obtained, suggesting some breadth of neutralization across HIV-1 clades. Furthermore, we also observed neutralization of viruses pseudotyped with the HIV-2 Env in the presence of subinhibitory concentrations of soluble CD4. This assay detects the presence of coreceptor binding site–directed Abs (Decker et al., 2005), which are elicited in NHPs inoculated with soluble HIV-1 Env immunogens (Forsell et al., 2008; Douagi et al., 2010).

To benchmark the soluble YU2 trimers used in the immunization regimen in this study against the recombinant gp120 protein regimen used in the VAX04 trial, we obtained 20 clinical serum samples from VAX04 collected 2 wk after four inoculations with MN and GNE8-derived gp120 protein in Alum. The samples were analyzed for neutralizing activity using the same panel of viruses and the same assay conditions as used for the NHP samples described in the previous paragraph. To strengthen the comparison, we included data from 10 animals described in our previous studies: five rhesus macaques inoculated with YU2 trimers in Abisco-100 + CpG (Douagi et al., 2010) and five cynomolgus macaques inoculated with YU2 trimers in GSK Adjuvant system AS01B (Mörner et al., 2009). The results demonstrate that the neutralizing activity elicited by the monomers (VAX04) and the trimers (NHP) was comparable against MN, whereas the trimers elicited superior neutralizing activity against all other viruses tested except ADA (Fig. 6). With the caveat that somewhat higher anti-Env binding Ab titers were achieved in the NHP study than in the VAX04 study (unpublished data), perhaps as a result of the use of different adjuvants, these data demonstrate that improved NAb responses can be generated by optimized subunit Env protein immunization regimens.

SHIV challenge and analysis of Ab binding titers in mucosal washes

Although the major and primary goal of this study was to define the kinetics and magnitude of the B cell response elicited

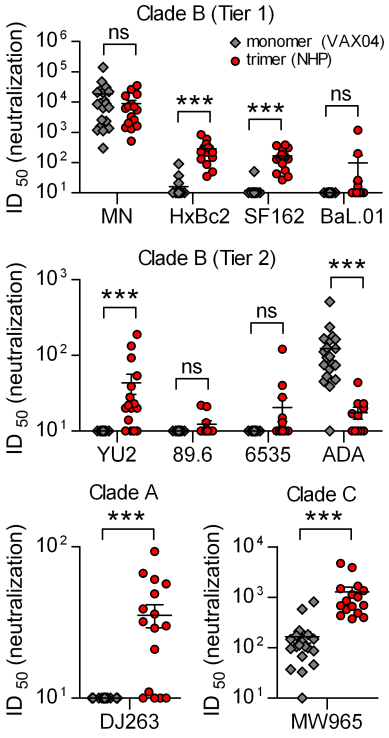

Figure 6. Comparison of neutralizing activity in trimer-immunized macaques and monomer-immunized humans. The ID_{50} NAb titers in Env trimer-immunized macaques (NHPs; $n = 16$) and gp120 monomer-immunized humans participating in the VAX04 study ($n = 20$) were compared. The analysis was performed once, at multiple dilutions of each plasma sample. Plasmas were assayed against clade B Tier 1 viruses MN, HxBc2, SF162, and BaL.01 (top) and Tier 2 viruses YU2, 89.6, 6535, and ADA (middle), and against the clade A virus DJ263 (bottom left) and the clade C virus MW965 (bottom right). Significance was determined using ANOVA. ns = nonsignificant. ***, $P \leq 0.001$.

by the soluble HIV-1 Env trimers in healthy primates, the potent response observed in this study led us to ask if this could mediate protection against heterologous SHIV challenge. We used the SHIV-SF162P4 challenge virus, which induces high but transient viremia in rhesus macaques (Harouse et al., 1999; Barnett et al., 2008; Polacino et al., 2008). To model one route of human mucosal transmission via sexual exposure, we subjected the animals to repeated rectal exposures, two per week, using a moderate dose of virus (50 $TCID_{50}$). This dose corresponds to $\sim 3 \times 10^6$ virus RNA copies, which is within the physiological range of one transmission event (Chakraborty et al., 2001; Pilcher et al., 2004). From previous in vivo titration results, we predicted that this dose would infect the Env-naive control animals in a few exposures, thus representing a higher dose than that estimated in humans under average transmission conditions but a lower dose than in some other SHIV studies (Barnett et al., 2008, 2010; Bogers et al., 2008). Blood was collected at each challenge occasion for detection of plasma viremia.

The Kaplan-Meier graph in Fig. 7 A (left) shows the percentage of uninfected animals after each challenge. After one challenge, one macaque in the control group became infected but no animal in the vaccine group. After the second challenge, four control animals were infected compared with two in the Env-immunized group. The remaining animals in the control group were infected after the third exposure, whereas six exposures were required to infect all animals in the Env-immunized group. This result suggests that there is a trend toward a modest degree of protection in the vaccine group; however, it is not statistically significant. Further studies involving larger groups of animals are needed to confirm this trend. We also asked if the high plasma Ab titers measured in the immunized animals (Fig. 3 B) provided a benefit in terms of control of viremia once the animals were infected. We measured this by cumulative viral loads during the viremic period (area under the curve calculated from viral load curves shown in Fig. S6; Fig. 7 A, middle) and by peak viral load (Fig. 7 A, right panel). By both measures, there was a trend for the immunized animals to better control the infection, but the difference between the groups was not statistically significant.

The modest, but suggestive, effect observed in the challenge experiment led us to analyze additional parameters of the prechallenge immune response. We first asked if the prechallenge plasma contained Abs capable of neutralizing either the SHIV-SF162P4 stock or recombinant virus pseudotyped with an Env clone derived from the SHIV-SF162P4 stock, clone 41.1. Moderate ID_{50} neutralization titers ranging between 200 and 300 were measured against both the stock and the cloned virus (Fig. 7 B). Some background activity against the stock was observed in the plasma from the control immunized animals, but not against the pseudotyped cloned virus, suggesting that the latter assay is less prone to nonspecific serum effects.

To further address why more efficient protection against SHIV-SF162P4 challenge was not achieved despite detectable plasma neutralizing activity against the challenge virus in the immunized animals, we measured Env-specific and total IgG and IgA titers in mucosal lavages collected 2 wk after each immunization (Fig. 7 C, top). Env-specific IgG was detected in both rectal lavages and vaginal lavages and these responses correlated weakly (rectal lavages) or strongly (vaginal lavages) with the plasma Ab titers measured in the corresponding animal (Fig. 7 C, bottom). Env-specific IgA in vaginal lavages and rectal lavages were below detection (unpublished data). By comparing all data points from the vaginal and rectal samples collected between the second and the fifth immunization with the corresponding IgG concentrations measured in plasma, we conclude that the IgG concentrations in vaginal lavages are ~1,000-fold lower than in plasma and the IgG concentrations in the rectal lavages are ~10,000-fold lower than in plasma (Fig. 7 D). These lower levels of antibody just outside the mucosa, presumably as a result of inefficient exudation, may not be sufficient to neutralize all incoming virus before interaction with $CD4^+/CCR5^+$ target cells in this challenge model.

DISCUSSION

Vaccine-induced neutralizing antibody and memory B cell recall responses play critical roles in preventing infection and limiting virus dissemination. Although there is a pressing need to

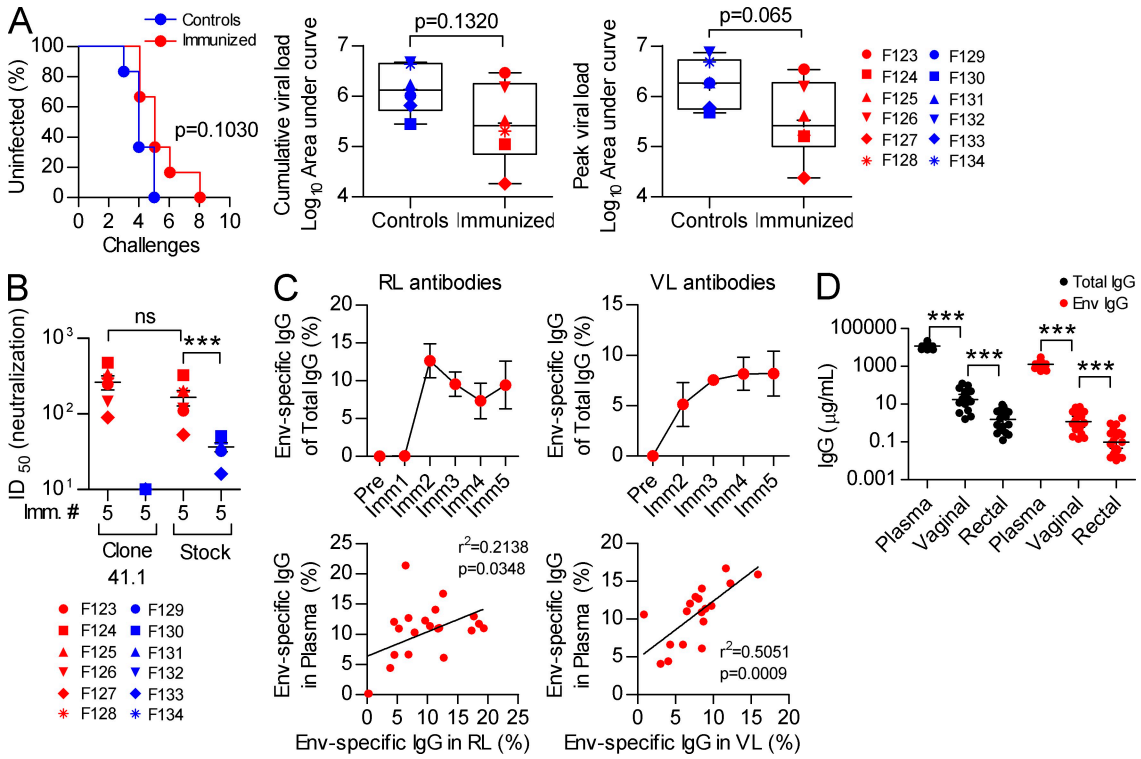

Figure 7. SHIV-SF162P4 rectal challenge and mucosal Env-specific Ab responses. Macaques immunized with YU2 trimers in adjuvant (F123-F128, red symbols; $n = 6$) or adjuvant alone (F129-F134, blue symbols; $n = 6$) were challenged with 50 $TCID_{50}$ SHIV-SF162P4 twice a week via the rectal route until confirmed infected by Q-RT-PCR. (A) The Kaplan-Meier graph illustrates percentage of uninfected animals after each challenge occasion (left). Difference in number of challenges needed to achieve infection was determined by Log-rank (Mantel-Cox) test where $P < 0.05$ was considered significant. The cumulative viral load (middle) is shown as log_{10} area under curve where each symbol represents an individual animal. Immunized and controls were compared with the Mann-Whitney test. Peak viral load (right panel) was measured at time of peak viremia for each individual animal and groups were compared with the Mann-Whitney test. Box plots show interquartile range and whiskers min-max values. (B) Plasma collected 2 wk after inoculation five from immunized (red, $n = 6$) and control (blue, $n = 6$) animals were assayed for neutralization against the SHIV-SF162P4 clone (41.1) and stock. Evaluation of neutralization was done with ANOVA. (C) Env-specific and total IgG in rectal lavages (RL, left) and vaginal lavages (VL, right) were measured 2 wk after each immunization. Circles represent group means ($n = 3–6$) ± SEM. Correlates between plasma and mucosal Env-specific IgG (of total IgG) for rectal samples (bottom left) or vaginal samples (bottom right) with linear regression shown. Correlations (r^2) were determined through Pearson's correlation analysis where $P < 0.05$ was considered significant. (D) Total levels of IgG (black, $n = 21–24$) and Env-specific IgG (red, $n = 21–24$) was quantified in plasma, rectal, and vaginal washes. Concentrations from all data points after reaching peak levels (immunization two to five) are shown. For comparison between compartments data were evaluated for significance via ANOVA. Lines and error bars indicate group mean ± SEM. ns, nonsignificant. ***, for $P \leq 0.001$.

understand relevant B cell responses an effective HIV-1 vaccine should elicit, a detailed analysis of the magnitude and kinetics of Env-specific memory B cells and PCs generated in response to Env protein immunizations in humans or NHPs is currently lacking. Accordingly, we analyzed the generation of Env-specific B cell populations in rhesus macaques inoculated with recombinant Env trimers in adjuvant. We assessed the functionality of the response at several levels, including requirements for activation of resting macaque memory B cells, elicitation of Env-specific binding Abs and increases in avidity after prolonged antigen exposure, neutralization capacity of Env-elicited antibodies in vitro, and protection against in vivo SHIV challenge.

We demonstrate that Env-specific IgG-producing cells peaked 7 d after each boost and then declined sharply. This is similar to the kinetics reported for other vaccine antigens where boosting results in a transient burst of PCs (Blink et al., 2005; Wrammert et al., 2008; Blanchard-Rohner et al., 2009). The rapidity of the recall response suggests that it originates from circulating memory B cells that differentiate into PCs in the periphery or that it arises from circulating memory B cells that enter already existing germinal center reactions, as shown in a recent study (Schwickert et al., 2009). We also demonstrate that Env-specific IgG^+ memory B cells peaked 2 wk after the second immunization and remained at stable levels between immunizations, comprising 10–20% of total IgG^+ memory B cells. This pool is likely maintained by the frequent boosting regimen and would be expected to wane with time in the absence of antigen. We did not investigate the sustainability of the Env-specific memory B cell and PC pool over time once inoculation of antigen was terminated, but this is an important issue to address in future studies. Previous studies showed that the Ab response to gp120 immunization was poorly sustained after clearance of antigen (Anderson et al., 1989; Graham et al., 1996), suggesting a lack of long-lived PCs.

Evidence for short half-lives of Env-specific Abs was also presented in a recent study based on gp120-vaccinated and HIV-1–infected individuals (Bonsignori et al., 2009).

Although the frequency of Env-specific memory B cells in PBMCs was high at the time points measured in our study, we were unable to detect total IgG^+ or Env-specific memory B cells in the bone marrow. This is consistent with a recent study reporting a lack of detectable memory B cells in the human bone marrow. Instead, the spleen was shown to be the major reservoir for human memory B cells (Mamani-Matsuda et al., 2008). In this regard, we note that many basic aspects of rhesus macaque B cell biology remain uncharacterized despite their frequent use as a nonhuman model. We suggest that the systems of analysis described in the current study, including the protocols for in vitro expansion and differentiation of memory B cells, have broad applications for studies aimed at understanding the distribution and frequency of functionally distinct B cell subsets in NHPs and to assess them relative to human biology and vaccine-elicited immune responses.

High titers of circulating Abs capable of neutralizing a panel of sensitive viruses spanning clades A, B, and C were shown here, but improvements in Env immunogen design and/or mode of administration are needed to achieve neutralization of primary viruses represented in the Tier 2 panel used here. The relatively short immunization schedule used here and in many other preclinical immunogenicity studies may not allow optimal maturation of the B cell response. With the intervals of antigen exposure used in this study, we observed no improvement in the breadth of neutralization with boosting despite an increase in neutralizing activity against sensitive Tier 1 viruses. Early HIV-1 Env studies in baboons suggest that longer intervals between immunizations may provide some benefit (Anderson et al., 1989). Further analyses are needed to address this question in a comprehensive and systematic manner.

In light of the renewed interest in the responses elicited by the monomeric gp120 proteins used in the VAX04 and RV144 phase III clinical trials, we sought to directly compare the neutralizing Ab activity elicited by the monomeric gp120 used in VAX04 with that elicited by the trimers in NHPs. Previous evaluation of the antigenic properties of the YU2 trimers show that they possess several of the features desired of an immunogen aimed to elicit bNAbs, such as occlusion of nonneutralizing epitopes in the C- and N-terminal domains of gp120 and recognition by a set of broadly neutralizing mAbs (Yang et al., 2000). Consistent with this, the YU2 trimers are superior to monomeric YU2 gp120 for elicitation of neutralizing Abs in small animals (Li et al., 2006). However, a direct comparison between the VAX04 monomeric gp120 immunogen and the YU2 trimeric immunogens was not previously performed. In our in vitro neutralization comparison, we observed a broader response in the trimer-inoculated macaques than in the monomer-inoculated humans. Although there are several caveats to this comparison, this analysis provides initial benchmark information about the gp140 trimer-elicited response in NHPs relative to the response elicited by monomeric gp120 in humans.

The recent results from RV144 indicate that boosting with purified Env protein in adjuvant after priming with a recombinant canary pox vector provided a modest but transient protective effect in low risk populations (Rerks-Ngarm et al., 2009). Several preclinical studies also demonstrate that boosting with recombinant Env protein elicits high Ab responses after priming with recombinant viral vectors or DNA (Montefiori et al., 1992; Lubeck et al., 1997; Forsell et al., 2005; Shu et al., 2007; Mörner et al., 2009). Both VAX04 and RV144 used gp120 administered in Alum, an adjuvant known to stimulate Th2-biased immune responses (Marrack et al., 2009). However, because RV144 used a viral vector to prime the response, this might lead to a different spectrum of Ab isotypes than those elicited in VAX04, which used protein in Alum adjuvant alone. A difference in Ab isotypes could affect Ab-mediated activities such as ADCC. Consistent with shifts in Ab isotypes, we demonstrated previously that priming with an Env-expressing recombinant Semliki Forest virus vector resulted in an Ab response that remained Th1 biased even after boosting with Env protein in a Th2-biasing adjuvant (Forsell et al., 2005). In the current study, we used an adjuvant consisting of Abisco-100 and CpG, which stimulates Th1-biased responses including generation of IFN-γ–producing $CD4^+$ and $CD8^+$ T cells (Douagi et al., 2010). A better understanding of the role for Ab isotypes relative to antibody neutralization and protection against infection is an important issue yet to be clarified (Liu et al., 2003; Huber and Trkola, 2007; Nimmerjahn and Ravetch, 2008).

Previous studies established that protection against SHIV challenge via the mucosal route was achievable by either active immunization with a homologous Env-based immunogen (Barnett et al., 2008, 2010; Bogers et al., 2008) or by passive infusion of Env-directed neutralizing Abs (Mascola et al., 2000; Hessell et al., 2009a,b). However, heterologous challenge to Env vaccine-elicited B cell responses is rarely attempted even though it is likely a better model to mimic real-world human-to-human transmission events. Although the circulating Abs elicited by the regimen described in this paper neutralized SHIV-SF162P4 in vitro with reasonable potency, only a modest vaccine effect was observed upon in vivo rectal challenge. Upon closer examination, this outcome is perhaps not surprising. In the Barnett studies, an effective impact on acquisition was observed with homologous vaginal (Barnett et al., 2008) or rectal (Barnett et al., 2010) SHIV-SF162P4 challenge. The plasma neutralizing titers observed in the Barnett studies were generally higher against the homologous SHIV-SF162P4 than those elicited by the heterologous YU2 trimers here. To mediate protection, some circulating IgG induced by systemic immunizations would be expected to enter the mucosa by transcytosis (Rojas and Apodaca, 2002), which is consistent with the detection of mAbs in mucosal washes after intravenous infusion of mAbs (Mascola et al., 2000; Parren et al., 2001; Hessell et al., 2009b). In our measurements, the Ab titers in the rectal mucosa were approximately four orders of magnitude lower than those in plasma, likely bringing the neutralization titers below effectiveness with regard to viral

challenge via this route of exposure. Further improvements in the specificity and affinity of vaccine-elicited neutralizing Abs may be needed for this process to be efficient enough to achieve protection against heterologous rectal challenge. For an antibody-dependent HIV-1 vaccine, the rectal route of challenge may be more difficult to protect against because of lower levels of exudates and more readily available $CD4^+$/$CCR5^+$ gut-associated target cells. Regimens designed to stimulate mucosal immune responses, including the production of antigen-specific secretory IgA, may provide benefit (Kozlowski and Neutra, 2003; Sundling et al., 2008), even though mucosal immunization was not required for protection against homologous infection via the vaginal route in a previous study (Barnett et al., 2008).

In conclusion, the extreme genetic diversity of circulating HIV-1 variants worldwide remains the main challenge for the development of an effective HIV-1 vaccine. Because of the difficulties in eliciting bNAbs through vaccination, the field has been spurred to dissect B cell responses in chronically infected individuals who display such activities. In parallel with these efforts, we contend that it is important to characterize B cell responses elicited by Env vaccination in healthy primates where potential pathogenic effects caused by chronic HIV-1 infection are not operational. In this regard, macaques offer several advantages as a result of their close genetic relationship with humans, their susceptibility to infection with SIV/SHIV, and because a more limited number of vaccine candidates can be tested in human clinical trials compared with in NHPs. The data presented here provide substantial insight into many issues relative to Env-specific primate B cell responses. Additional well designed HIV-1 vaccine and SHIV challenge studies, in coordination with selected human immunogenicity studies, are likely required to fully dissect the protective requirements at relevant portals of viral entry.

MATERIALS AND METHODS

Expression and purification of Env immunogens. Soluble gp140 trimers (gp140-F) were produced by transient transfection of Freestyle 293F suspension cells (Invitrogen) as previously described (Forsell et al., 2008). In brief, cells were transfected at a density of 1×10^6/ml in GIBCO Freestyle293 expression media using 293Fectin, according to manufacturer's instructions (Invitrogen). Supernatants were collected 4 d after transfection. After collection, all supernatants were centrifuged at 3,500 *g* to remove cells or cell debris, filtered through a 0.22-µm filter, supplemented with Complete EDTA-free protease inhibitor cocktail (Roche) and penicillin-streptomycin (Invitrogen), and stored at 4°C until further purification. First, the proteins were captured via glycans by lentil-lectin affinity chromatography (GE Healthcare). After extensive washing with PBS, supplemented with 0.5 M NaCl, the proteins were eluted with 1 M methyl-α-D-mannopyranoside and captured in the second step via the His-tag by nickel-chelation chromatography (GE Healthcare). After a wash with 40 mM imidazole (IM) and 0.5M NaCl in PBS, proteins were eluted with 300 mM IM in PBS. Trimers were then separated from lower molecular weight forms by gel filtration chromatography using a superdex200 26/60 prep grade column by the ÄKTA Fast protein liquid chromatography system (GE Healthcare). Biotinylated Env probes used in the B cell ELISPOT assay were purified by lentil-lectin and nickel-chelation chromatography but not subjected to subsequent gel filtration chromatography. The biotinylation was performed using site-specific biotinylation of an Avitag sequence inserted at the C terminus of each Env probe, allowing for covalent linkage of biotin to the lysine residue in the motif distal to the Env antigenic surface.

Animals. 12 rhesus macaques (Macaca Mulatta) of Chinese origin, ∼3 yr old, and four cynomolgus macaques of Chinese origin, ∼5 yr old, were housed at the animal facility Astrid Fagraeus Laboratory at the Swedish Institute for Infectious Disease Control. Housing and care procedures were in compliance with the provisions and general guidelines of the Swedish Board of Agriculture, and the facility has been assigned an Animal Welfare Assurance number by the Office of Laboratory Animal Welfare (OLAW) at National Institutes of Health. All procedures were approved by the Local Ethical Committee on Animal Experiments (Stockholms Norra Djurförsöksetiska Nämnd). The animals were housed in pairs in 4-m^3 cages, enriched to give them possibility to express their physiological and behavioral needs. They were habituated to the housing conditions for >6 wk before the start of the experiment and subjected to positive reinforcement training to reduce the stress associated with experimental procedures. All immunizations and blood samplings were performed under sedation with 10 mg/kg ketamine i.m. (100 mg/ml Ketaminol; Intervet), and when sampled for rectal or vaginal lavages or bone marrow the macaques were given an additional 0.5 mg/kg i.m. Xylazine (Rompun; Bayer) to induce total muscle relaxation and analgesia. The macaques were weighed at each immunization or sampling occasion. Before entering the study, all animals were confirmed negative for SIV, simian T cell lymphotropic virus, and simian retrovirus type D.

Immunizations and sampling. The 12 rhesus macaques (two groups of six animals) were inoculated five times with the immunogens described in the Expression and purification of Env immunogens section. Immunizations were performed at weeks 0, 4, 8, 12, and 18 by the i.m. route of injection. All protein immunizations were administered in 75 µg ABISCO-100 (Isconova AB) and 500 µg CpG ODN2395 (Coley Pharmaceutical Group) as adjuvant (Abisco-100 + CpG). Protein doses were 200 µg per animal for the first inoculation and 100 µg for the subsequent injections. The vaccine was given in a total volume of 1 ml, divided equally between the left and right hind leg. Blood and rectal lavage samples were taken before and 1 and 2 wk after each immunization. The rectal mucosa was sampled through insertion of a syringe and rinsing with 4 ml PBS. The lavage was then added to a concentrated cocktail of protease inhibitors, spun, and the liquid phase frozen at −80°C. Vaginal lavage was taken 2 wk after each immunization through insertion of a pediatric feeding tube and rinsing with 2 ml PBS. The vaginal lavage was then treated as for rectal samples. 3 wk after immunizations two and four, the macaques were sampled for bone marrow through strictly aseptic insertion of a pediatric spinal needle between the greater and lesser tuberosity of humerus. A maximum of 5 ml was aspirated from each animal into a heparinized syringe and transferred to an EDTA tube.

PBMC isolation and in vitro stimulation. Bleeds were taken in EDTA-coated tubes and spun at 1,200 *g* for 15 min for separation of plasma. The mononuclear cells were purified through density gradient centrifugation with Ficoll-Paque (GE Healthcare) according to the manufacturer's instructions and then counted and frozen in 90% heat-inactivated FBS and 10% DMSO (Sigma-Aldrich). Experiments were performed on both frozen and fresh cells. PBMCs were thawed in a 37°C water bath followed by two washes in RPMI 1640 media supplemented with 10% FBS, 2 mM L-Glutamine, 100 U/ml penicillin, 100 µM streptomycin, 2% Hepes (all from Sigma-Aldrich), and 50 mM 2-ME (Invitrogen) before resting at 37°C supplemented with 5% CO_2 overnight (ON). The PBMCs were cultured at 2×10^6 cells/ml in a total of 500 µl per well in 48-well flat bottom plates (Thermo Fisher Scientific). The stimulation mix was composed of different combinations of 5 µg/ml CpG ODN 10103 (TCGTCGTTTCGTCGTTTTGTCGTT; Coley Pharmaceuticals), PWM (Sigma-Aldrich), SAC strain 1/10,000 (Sigma-Aldrich), 100 ng/ml interleukin 2, 100 ng/ml interleukin 10, and 1 µg/ml of soluble CD40 ligand (Peprotech).

To evaluate the stimulatory effect on B cell and T cell proliferation through FACS analysis, PBMCs were first labeled with 0.5 µM CFSE for 7 min at 37°C and then washed with complete media. The cells were then incubated with the indicated stimulation for 6 d, after which the cells were stained with Abs for CD20 (clone 2H7), CD4 (clone L200), CD8 (clone SK1), and CD27 (clone M-T271). All Abs (BD) were previously titrated for optimal staining using rhesus macaque PBMCs. Samples were collected on a FACSCalibur (BD) and analyzed using FlowJo software (Tree Star, Inc.). For experiments using sorted macaque cells, PBMCs were incubated with CD20- or CD4-conjugated magnetic beads (Miltenyi Biotec) and positive fractions were isolated using autoMACS (Miltenyi Biotec) separation according to the manufacturer's instructions. The purity of sorted cells was evaluated through FACS analysis and was >90% for the $CD20^+$ fraction and between 50 and 90% for the $CD4^+$ fraction. Contaminating cells in the $CD4^+$ fraction were mainly $CD20^+$ B cells. Human B cells were sorted by either CD20 or CD19 positive selection with magnetic beads as described according to the manufacturer's instructions and were >90% $CD20^+$ B cells. Human $CD4^+$ T cells were sorted from buffy coats by negative selection using $CD4^+$ T cell enrichment cocktail (STEMCELL Technologies Inc.) and were >95% $CD3^+$ and $CD4^+$, as determined by flow cytometry.

Analysis of Env-binding Abs. HIV-1 gp120-specific plasma, rectal or vaginal IgG, and IgA was measured by ELISA as previously described (Mörner et al., 2009) with modifications. In brief, insect cell–produced YU2 gp120 protein was coated onto MaxiSorp (Thermo Fisher Scientific) microtiter plates at 100 ng/well in 50 mM carbonate buffer, pH 9.6, ON at 4°C. After blocking in PBS containing 2% nonfat dry milk, samples were added and incubated for 1.5 h at 37°C. The gp120-specific IgG or IgA was detected by adding secondary HRP-conjugated anti–monkey IgG (Nordic Immunology) or anti–monkey IgA (KPL). The signal was developed by addition of *O*-phenylenediamine (OPD; Sigma-Aldrich) or TMB^+ (Invitrogen). Reactions were terminated by adding 1 M HCl and the OD was read at 492 (for OPD) or 450 (for TMB^+) and 620 nm. Between each incubation step, the plates were washed six times with PBS 0.05% Tween 20. The half-max binding titers (OD50) for each sample was calculated by interpolation from mean OD50 values calculated from an immunized control plasma using the formula ([(ODmax-ODmin)/2] + ODmin). As a result of dilution of the vaginal and rectal lavages upon sample collection, data were set in relation to the total amount of IgG or IgA in the sample. Total IgG and IgA was measured by coating plates with 200 ng/well of goat anti–human IgG (Jackson ImmunoResearch Laboratories) or 100 ng/well of goat anti–monkey IgA (KPL).

When ELISA was used to measure Env region-specific Abs, the plates were coated with the same molar ratios of gp140-F, gp120-F, or gp120ΔV1/2/3-F as in the previous paragraph. Percentages of gp41 and variable region 1/2/3–specific Abs were calculated using the formulas (gp140-gp120)/gp140 × 100 and (gp120-gp120ΔV1/2/3)/gp140 × 100. To calculate absolute concentrations of gp120-specific Abs in samples, a gp140-F–immunized rhesus control sera was quantified for IgG gp120-specific Abs via a rhesus IgG standard (Southern-Biotech) and subsequently used as standard for unknowns.

Avidity index was evaluated as described previously (Vermont et al., 2002; Lai et al., 2007) with some modifications. ELISA plates were coated overnight at 4°C with 100 ng/well *Galanthus nivalis* lectin (Sigma-Aldrich) in PBS. S2-produced gp120 or 293F-produced gp140-F, gp120-F, or gp120Δ1/2/3-F was added to the wells and incubated for 2 h at 37°C. Plasma was added in dilution series and incubated for 1.5 h before exposure to 1.5 M NaSCN or PBS for 10 min. The bound Abs were detected as in the ELISA described in the previous paragraph. Titers for each sample were calculated from the OD50 value of an in-house standard plasma sample. The avidity index was calculated by dividing the titer for NaSCN-treated and PBS-treated samples and multiplying by 100. To determine if treatment with 1.5 M NaSCN disrupted the integrity of Env, we performed a dose titration experiment with increasing concentrations of NaSCN and assessed binding by a panel of mAbs directed against distinct gp120 epitopes (Fig. S4). The results demonstrate that recognition by several conformation-sensitive Abs (17b, b6, and F91) but not all (E51, 2.1C, b12, and F105) was retained after the 1.5-M NaSCN treatment, suggesting that Abs recognizing both linear and conformational epitopes are detected under the assay conditions used in the present study.

B cell ELISpot assay. The frequency of antigen-specific memory B cells was determined as previously described (Douagi et al., 2010). In brief, total PBMCs were cultured at 2×10^6 cells/ml and stimulated with 5 µg/ml CpG-B (ODN-10103; Coley), 10 µg/ml PWM (Sigma-Aldrich), and SAC (1:10,000; Sigma-Aldrich) for 4 d in 48-well plates. To detect Ab-secreting cells, MAIPSWU10 96-well plates (Millipore) were coated with 10 µg/ml of anti–human IgG (Fcγ; Jackson ImmunoResearch Laboratories) and PBMCs were transferred to the plates in dilution series and incubated ON at 37°C in 5% CO_2. The plates were then washed with PBS containing 0.05% Tween and incubated with biotinylated gp140-F, gp120-F, or gp120ΔV1/2/3-F (Dosenovic et al., 2009) followed by washing and incubation with streptavidin-AP (Mabtech). The reactions were developed using BCIP/NBT substrate (Sigma-Aldrich) and stopped by washing in water. Spots corresponding to ASC were counted using an Immunospot analyzer (Cellular Technology Ltd.). The results were converted to Ab-secreting cells per million cultured PBMCs (ASCs/10^6 PBMCs cells). Percentages of gp41 and variable region 1/2/3-specific memory B cells were calculated using the formulas (gp140-gp120)/gp140 × 100 and (gp120-gp120ΔV1/2/3)/gp140 × 100, respectively.

Virus neutralization assays. Plasmas from immunized animals were tested for virus neutralization capacity against a panel of diverse HIV-1 isolates. Neutralization assays were performed using a single round of infection HIV-1 Env pseudovirus assay and TZM-bl target cells as previously described (Li et al., 2005; Shu et al., 2007). Env pseudoviruses were prepared by co-transfecting 293T cells with an Env expression plasmid containing a full gp160 *env* gene and an *env*-deficient HIV-1 backbone vector (pSG3ΔEnv). To determine the serum dilution that resulted in a 50% reduction in RLU, serial dilutions of sera were performed and the neutralization dose–response curves were fit by nonlinear regression using a four-parameter hill slope equation programmed into JMP statistical software (JMP 5.1; SAS Institute Inc.). The results are reported as the serum neutralization ID_{50}, which is the reciprocal of the serum dilution producing 50% virus neutralization. Diverse HIV-1 virus isolates, including viruses from clades A, B, and C, were used in the neutralization assays. Clade B viruses included a panel of Env pseudoviruses that were recently characterized and recommended for use in assessing neutralization by HIV-1 immune sera (Li et al., 2005). Several investigators also provided replication-competent viruses or functional Env plasmids for pseudoviruses. D. Gabuzda (Dana Farber Cancer Institute, Boston, MA) provided the Env plasmids for YU2 and MuLV. Env plasmids for SF162 and JRFL were provided by L. Stamatatos (Seattle Biomedical Research Institute, Seattle, WA) and J. Binley (Torrey Pines Institute, La Jolla, CA), respectively. The clade A DJ263.8 sequence was cloned from the original PBMC-derived virus (provided by F. McCutchan and V. Polonis, U.S. Military HIV Research Program, Rockville, MD) and the clade C MW965 Env plasmid was obtained from the AIDS Research and Reagent Repository. Isolation of the Env plasmids BaL.01 were recently described by our laboratory (Shu et al., 2007) and the SS1196.1 Env was previously described (Li et al., 2005). Neutralization was also measured against the SHI-SF162P4 stock and against a cloned Env from this stock, Env clone 41.1.

Human serum samples. 20 randomly chosen serum samples were obtained via a Materials Transfer Agreement with the Global Solutions for Infectious Diseases. These sera were derived from volunteers from the VaxGen Inc phase III clinical trial. At the time of sampling (month 12.5), the participants had received four injections (months 0, 1, 6, and 12) of the AIDSVAX B/B vaccine containing 300 µg each of recombinant HIV-1MN– and HIV-1GNE8–derived gp120 in Alum adjuvant (Pitisuttithum et al., 2006).

SHIV challenge. Plasma from an acutely SHIV-SF162P4 (Kraft et al., 2007)–infected rhesus macaque was originally provided by L. Stamatatos and was first inoculated i.v. into a naive rhesus macaque. During peak viremia, blood and bone marrow from this animal was injected into a second

macaque (32M). Virus was reisolated from 32M at peak viremia by co-culture with naive rhesus PBMC, and a virus stock was prepared. This virus was used to prepare a new virus stock by i.v. inoculation into a naive rhesus macaque of Indian origin and subsequent reisolation as described in Virus neutralization assays. The resulting virus was further expanded in vitro on C8166-CCR5 cells to produce the challenge stock used in the present study. The stock was titrated on rhesus PBMC cells in vitro and in vivo in six monkeys via the rectal route. 5 wk after the last immunization, 3 ml, corresponding to 50 $TCID_{50}$ (tissue culture infectious dose, 50), was inoculated rectally in 12 macaques. The macaques were then challenged with this dose twice a week until confirmed positive for viral load as was monitored by quantitative (Q) RT-PCR with a cutoff of 214 copies per ml. Anamnestic Ab responses were evaluated up to 7 wk after confirmed infection by ELISA.

Q-RT-PCR. A DNA template was created by inserting a 458-bp segment of p27 from SHIV-SF162P4, using the forward primer SIVMac239Gag5 (5′-GTGCATTCACGCAGAAGAGA-3′) and reverse primer SIVMac-239Gag.r.out (5′-TTCCTGCAATATCTGATCCTGA-3′), into the pJET1.2-blunt vector (Fermentas; designated pJET1.2-gag458). The insert was verified through sequencing (GATC Biotech AG) and alignment with SIVMac239. RNA standard was produced by in vitro transcription of NcoI-linearized pJET1.2-gag458 with T7 polymerase followed by DNase I digestion (Fermentas). The RNA was then purified using the RNeasy mini kit (QIAGEN) and frozen in aliquots, corresponding to 10-fold dilution series ranging from 10^6 to 10^1 RNA copies, supplemented with 30 µg/ml *S. cerevisiae* transfer RNA (Sigma-Aldrich).

Viral RNA was extracted from 280 µl EDTA-plasma using the QIAamp Viral RNA mini kit (QIAGEN) and eluted in 60 µl RNase-free water containing 0.04% sodium azide according to the manufacturer's instructions. The eluted RNA was assayed immediately after recovery using a One-Step Q-RT-PCR protocol. The primers and probe chosen for amplification of the SIVMac239 p27 were published previously (Leutenegger et al., 2001) and worked with high efficiency for detection of SHIV-SF162P4 (Fig. S4). In brief, the forward primer SIV.510f (5′-GCCAGGATTTCAGGCAC-TGT-3′) and reverse primer SIV.591r (5′-GCTTGATGGTCTCCCA-CACA-3′) amplified an 81-bp segment, where the probe SIV.535p (5′-AAGGTTGCACCCCCTATGACATTAATCAGATGTTA-3′) bound. The one-step Q-RT-PCR was run using the qScript One-Step Fast qRT-PCR Low ROX kit (Quanta BioSciences Inc.) according to the manufacturer's instructions under the following conditions: 20 µl total reaction containing 80-nM probes, 400 nM of each primer, and 10 µl RNA. Cycling conditions were 5 min at 50°C for initial cDNA synthesis and 95°C at 30 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s The reactions were performed on an ABI PRISM 7500 Fast cycler (Applied Biosystems). A standard curve and variability coefficients are shown in Fig. S4.

Statistical analysis. In all group comparisons including three or more than three groups, statistical significances were determined by one-way ANOVA with Bonferroni's posttest for individual comparisons on log-transformed data using Prism software (version 5; GraphPad Software, Inc.) and considered significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$. When comparing three or less than three groups the Mann-Whitney test was used.

Online supplemental material. Fig. S1 shows a schematic representation of the experimental procedure for quantifying PCs and memory B cells. Fig. S2 demonstrates the kinetics of memory B cell differentiation into ASC under different in vitro stimulations conditions. Fig. S3 describes the frequency of PC and memory B cell populations at multiple time points after Env trimer inoculation. Fig. S4 shows a standard curve and the variability of the Q-RT-PCR assay used to evaluate virus load after in vivo SHIV challenge. Fig. S5 shows how the binding of anti-Env mAbs is affected by SCN treatment. Fig. S6 shows the plasma viral loads in individual animals challenged with SHIV-SF162P4. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100025/DC1.

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