HIV-1 Envelope Induces Memory B Cell Responses That Correlate with Plasma Antibody Levels after Envelope gp120 Protein Vaccination or HIV-1 Infection

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HIV-1 Envelope Induces Memory B Cell Responses That Correlate with Plasma Antibody Levels after Envelope gp120 Protein Vaccination or HIV-1 Infection

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Successful vaccines (i.e., tetanus and diphtheria) can induce long-lived Ab levels that are maintained by bone marrow plasma cells and plasma Ab levels do not correlate with numbers of blood memory B cells. Destruction of CD4+ T cells early in HIV-1 acute infection may result in insufficient induction of neutralizing Ab responses; thus, an HIV-1 vaccine should elicit high levels of durable Abs by long-lived plasma cells to be protective. We asked if HIV-1 envelope-specific memory responses were sustained by memory B cells in the settings of HIV-1 gp120 envelope vaccination and chronic HIV-1 infection. Levels of anti-HIV-1 envelope plasma Abs and memory B cells were found to correlate in both settings. Moreover, whereas the expected half-life of plasma Ab levels to protein vaccines was >10 years when maintained by long-lived plasma cells, anti-envelope Ab level half-lives were ~33–81 wk in plasma from antiretroviral drug-treated HIV-1+ subjects. In contrast, anti-p55 Gag Ab level half-life was 648 wk, and Ab titers against influenza did not decay in-between yearly or biennial influenza vaccine boosts in the same patients. These data demonstrated that HIV-1 envelope induces predominantly short-lived memory B cell-dependent plasma Abs in the settings of envelope vaccination and HIV-1 infection. The inability to generate high titers of long-lived anti-envelope Abs is a major hurdle to overcome for the development of a successful HIV-1 vaccine. The Journal of Immunology, 2009, 183: 0000 – 0000.

Despite an improvement in effectiveness and utilization of antiretroviral therapies worldwide and improved prevention efforts, HIV-1 infection continues to spread, with an estimated 2.7 million new infections worldwide in 2007 and 56,300 new HIV cases in the United States in 2006 (1, 2). Thus, the development of a safe and effective HIV-1 vaccine remains a global priority (1, 3). The recent lack of efficacy of T cell-based and gp120 envelope-based vaccine phase III clinical trials has dramatically emphasized that two major and central goals of HIV-1 vaccine design are the induction of both broadly neutralizing Abs and long-lived protective Ab responses (1, 3). Therefore, understanding B cell responses to HIV-1 in the settings of infection and vaccination has become a key aim of research toward design of a preventive HIV-1 vaccine (1, 3–6).

In many viral infections or vaccinations, the initial burst of Ab after reexposure is dependent on the presence of Ag and is mediated by differentiation of memory B cells into short-lived plasma cells (5, 7, 8). In contrast, long-lived Ab responses generated by natural infections or vaccines are not dependent on the continuous presence of memory B cells but are rather produced by long-lived plasma cells that reside in the bone marrow and do not require Ag for continued production of Ab (5, 7, 9, 10).

Amanna et al. have recently described that the Ab responses to tetanus toxoid and diphtheria vaccines have half-lives of 11 and 19 years, respectively, and that the serum Ab levels present long-term in these successful vaccinations did not correlate with blood memory B cell levels (11). This lack of correlation of memory B cell levels with serum Ab implied that, with these vaccines, serum Ab was maintained by long-lived plasma cells in the bone marrow and not by memory B cells (11, 12).

In this paper, we have studied the plasma Ab and memory B cell responses to HIV-1 envelope in the context of chronic HIV-1 infection and in the setting of immunization of HIV-1-negative subjects with recombinant gp120 (rgp120). We found a significant correlation between blood anti-Env memory B cell levels and plasma anti-Env Ab titers in both chronic HIV-1 infection and after vaccination with rgp120, suggesting that in these settings plasma Ab was maintained predominantly by short-lived memory B cells. Ab response duration was measured over the course of ~380 wk of antiretroviral therapy (ART) in HIV-1-infected subjects in whom plasma viremia was completely suppressed. We found an apparent half-life of anti-Env Ab plasma levels between 33 (gp41) and 81 (gp120) wk following initiation of ART leading to viral load control, compared with longer (i.e., years) plasma level half-lives for HIV-1 p55 Gag, tetanus toxoid, and influenza Abs. Taken together, our observations demonstrate that anti-Env Ab titers are shorter lived than p55 Gag, influenza, and tetanus
The PBMCs were transferred to 50-ml conical tubes, washed twice with HBSS without calcium and magnesium, and resuspended in 10 ml of RPMI 1640 (Invitrogen) plus 10% heat-inactivated FBS (Gemini Bio-Products), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich). FBS had been previously tested for lack of mitogenic activity by [3H]thymidine incorporation assay and the same lot has been used throughout the experiments. Viability was checked using the Guava ViaCount kit (Guava Technologies) according to the manufacturer’s instructions.

**PBMC thawing**

Cryopreserved PBMCs were rapidly thawed, treated with Benzonase (Novagen), washed twice using warm media, resuspended in RPMI 1640 plus 20% FBS, and incubated overnight at 37°C in 5% CO2 humidified atmosphere before proceeding with experiments.

**Memory B cell stimulation**

Memory B cells were driven into Ab-secreting cells (ASCs) by culturing 2 × 10^6 PBMCs/ml in RPMI 1640 plus 10% human AB serum (Gemini Bio-Products), 100 U/ml penicillin, 100 µg/ml streptomycin, pokeweed mitogen (1/1000) prepared as previously described (17), 24 µg/ml CpG-oligodeoxynucleotide 10103 (Coley Pharmaceuticals), and 5 ng/ml IL-15 (R&D Systems) for 6 days at 37°C, 5% CO2 humidified atmosphere on a rocking platform. Optimal culture conditions were previously established. Human AB serum was previously tested for lack of mitogenic activity by [3H]thymidine incorporation assay and the same lot has been used throughout the experiments. Unstimulated cells were cultured simultaneously. Cells were then washed twice and resuspended in RPMI 1640 plus 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Viability was determined using the Guava ViaCount kit, and cells were resuspended at proper concentrations before being used in the ELISPOT assay.

**Evaluation of survival of HIV+ and HIV- PBMCs upon stimulation**

To evaluate the extent of PBMC loss after in vitro stimulation, we compared the recovery ratio, defined as the ratio of viable PBMCs at the end of culture to the number of PBMCs at culture initiation, of stimulated and unstimulated PBMCs from chronically HIV-1-infected and uninfected subjects after a 6-day incubation. The recovery ratio of HIV-1-positive and HIV-1-negative unstimulated PBMCs was comparable (0.35 ± 0.15 and 0.37 ± 0.21, respectively; p = 0.74). On the contrary, stimulated PBMCs from chronically HIV-1-infected subjects underwent significantly higher levels of cell death compared with stimulated uninfected PBMCs (recovery ratio of 0.25 ± 0.10 and 0.41 ± 0.14, respectively; p = 0.0002). Thus, all data are expressed as ASC/10^6 viable PBMCs at the end of culture period.

**HIV envelope peptides**

To detect epitope-specific ASCs in the ELISPOT assay we conjugated biotinylated peptides of the desired specificity to streptavidin to generate tetramerized peptides for increased sensitivity, to be used as capture reagents in plate-based assays. Diamond and colleagues previously reported that, in a murine model, monomeric peptides are not suitable in B cell ELISPOT assays to detect epitope-specific responses (18). We compared monomeric and tetramerized MN V3 gp120 Env (MN V3) peptide (TRPNYNKRKRIHIGPGRAFYTTKIHQGRFORQ) and membrane-proximal external region (MPER) gp41 cluster II 2F5-epitope (SP62) peptide (QQEKNEQELLELDKWASLWN), as well as tetramerized peptides with the same amino acid content but with scrambled sequences (MN V3scr peptide, AIKRRKHINTQRPKPGYTHRRNKGTQANYF, SP62scr peptide, NKEQDDQAEESLQWEKLNWL) for their ability to detect ASCs using mAb-producing cell lines. We confirmed that spots were detectable with tetramers but not with the respective monomers and that the spots obtained using tetramers where Ag-specific, as the scrambled versions of the tetramers failed to capture the secreted Abs and hence did not form spots. All the tetramerized peptides used in this study were routinely controlled for their ability to block binding of fluorochrome-conjugated homologous tetramerized peptide to Ab-coated beads by flow cytometry. The fluorochrome-conjugated tetramerized peptides had been previously tested for specific binding to both mAb-producing cell lines and Ab-coated beads. Additionally, we performed a checkerboard ELISA on cell lines producing mAbs of known specificity and confirmed that the tetramers only bound to the appropriate mAbs when used as capture reagents in plate-based assays.

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**Table I. Clinical data of HIV-1-positive subjects at enrollment**

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* CD4 indicates CD4 counts (cells/mm³); VL, viral load (copies/ml) determined at the time of enrollment; YOD, year of diagnosis; n/a, data not available; M, male; F, female.

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Materials and Methods

**Memory B cell study population**

Twenty-six HIV-1 chronically infected patients were enrolled at the Duke Infectious Diseases Clinic for this study. Of them, 18 were on therapy and 8 were off therapy at the time of enrollment (Table I). Nineteen HIV-negative individuals were enrolled as controls.

An additional eight HIV-1 chronically infected subjects who were treated with ART for both 125 wk with low expression of plasma viremia, and for whom only retrospective plasma samples were available, were studied for levels of plasma anti-gp120, -gp41, -p55, -tetanus toxoid, and -influenza (2007 Fluzone vaccine; Sanofi Pasteur) IgG Abs over time of ART using a sensitive Lumienx assay as previously described (13) and direct-binding ELISAs. The relative Ab level half-lives were determined over time as described below under statistical methods.

Aliquots of frozen PBMCs from 25 vaccinated volunteers and 5 placebo recipients were obtained from the VaxGen clinical trial (VAX004). Vaccines were administered HIV MN and GNE8 rgp120 proteins in alum (14). It has been previously reported that in animal models (15) and in humans (16) Ag-specific B cells peak as early as 1 wk after immunization. Therefore, to get as close as possible to this time point, we obtained VAX004 patient and control peripheral blood samples (plasma and cells) 2 wk after the fourth immunization. VAX004 specimens were obtained from the Division of AIDS (DAIDS), a division of the National Institute of Allergy and Infectious Diseases (National Institutes of Health, Department of Health and Human Services), and from Global Solutions for Infectious Diseases. All human studies were approved by the Institutional Review Board of the Duke University Medical Center.

**PBMC isolation**

PBMCs were isolated from acid citrate dextrose-treated blood using Acuspin tubes (Sigma-Aldrich) according to the manufacturer’s instructions. Briefly, 25 ml of whole blood was loaded onto the frit of each 50-ml tube and centrifuged for 20 min at 800 × g at 22°C. Plasma was removed and the PBMCs were transferred to 50-ml conical tubes, washed twice with...
Memory B cell ELISPOT assay

We performed a modified version of the memory B cell ELISPOT assay (17) in two ways. First, 96-well Immobilon P flat-bottom plates (Millipore), pretreated with 70% ethanol for 30 s and washed twice with PBS, were coated overnight at 4°C with 100 μg of goat polyclonal anti-human Ig (Invitrogen) (H17000), Immunoprecipitation keyhole limpet hemocyanin (Pierce Biotechnology; catalog no. PI77600), HIV-1 consensus gp140CFL envelope, HIV-1 MN-V3 tetrameric peptides, and HIV-1 2FS epitope tetrameric peptides at 10 μg/mL in PBS. The gp140 consensus B gp140CFL envelope protein was produced and purified as previously described (19). The plates were washed twice with PBS and blocked for 2 h at 37°C with PBS/15% BSA. Cells were added at 4 × 10^6 viable PBMCs/ml in a volume of 100 μL for Ag-specific tests and at 4 × 10^4 and 4 × 10^5 viable PBMCs/well for total IgG detection. Plates were loosely wrapped in aluminum foil (20) and incubated for 3 h at 37°C in a humidified atmosphere at 5% CO2. The plates were then washed four times with PBS/0.05% Tween 20 using the ELx405 automated plate washer (BioTek Instruments), and 100 μL of mouse biotin-conjugated anti-human IgG Ab (HybriDyn Reagent Laboratory; catalog no. HP6043B) diluted 1/4000 in PBS/1% BSA/0.05% Tween 20 was added for 1 h at room temperature. The detection Ab had been previously checked for isotype specificity and optimal concentration. Plates were washed four times with PBS/0.05% Tween 20, and 100 μL of HRP (Vector laboratories; catalog no. A2004) diluted 1/10000 in PBS/1% BSA/0.05% Tween 20 was added to each well for 1 h at room temperature. Plates were washed twice with PBS/0.05% Tween 20 before being washed again. Spots were developed adding 100 μL well of 3-amino-9-ethylcarbazole at 0.3 mg/ml diluted in 0.1 M sodium acetate (pH 5.0) and 0.035% hydrogen peroxide for 5 min in the dark. Plates were extensively washed with distilled water and let dry overnight. Spots were counted with the ImmunoSpot series 3B analyzer and ImmunoSpot 4.0 software (Cellular Technology). Spot counts were then reported as ASCs ± SEM/10^5 viable PBMCs at the end of culture. In the present report, the term ASC refers to memory B cells detected with the ELISPOT assay. Results were background subtracted as follows: total IgG counts from unstimulated cells were subtracted from total IgG counts of stimulated cells, and responses obtained against the KLH protein were subtracted from Ag-specific tests. IgG spots against KLH were rare events: among the 26 stimulated cells were subtracted from total IgG counts of stimulated cells, 10^6 viable PBMCs, respectively.

To determine how our memory B cell assay performed compared with previous reports with regard to total and tetanus toxoid-specific memory IgG detection, we assayed total and tetanus toxoid-specific IgG ASC frequencies and found in uninfected and chronically HIV-1-infected subjects frequencies that were similar in magnitude to recent reports by others (21, 22). We found that 12–15% of PBMCs from nine HIV-1-negative donors were tested for tetanus toxoid-specific memory B cells. All but two individuals had detectable tetanus toxoid-specific ASCs with a frequency over the total IgG ASCs ranging from 0.02% to 0.37% and an average of 0.11%. Tetanus toxoid-specific memory B cells have been previously shown to have a frequency of ~0.1% of total IgG memory B cells in adults vaccinated long (>12 years) (21). Also, this is in accordance with other reports focusing on circulating memory B cells specific for hepatitis B virus and smallpox (23, 24). Finally, we tested a further subset of PBMC samples from chronically HIV-1-infected subjects for reactivity against the bare tetramer (tetramerized biotin with streptavidin with no peptide) and no spots were formed (data not shown).

Second, we extended our observations on MPER-specific memory B cells to an additional 13 chronically HIV-1-infected subjects using a reversed format of ELISPOT assay with anti-IgG1 as capture reagent (HybriDyn Reagent Laboratory; catalog no. HP6046P) and the biotinylated SP62 gp41 MPER peptide (2.5 μM) as detector. Total IgG ASCs from stimulated and unstimulated cells and KLH-specific ASCs were enumerated and used as controls as described above. Data obtained were identical to the data obtained with the direct ELISPOT assay using peptide tetramers.

Direct binding ELISA

Ninety-six-well ELISA plates (Corning; catalog no. 3569) were coated with 0.2 μg/well Ag in 0.1 M sodium bicarbonate and blocked with assay diluent (PBS containing 4% (w/v) whey protein/15% normal goat serum/0.5% Tween 20/0.05% sodium azide). Sera were incubated for 1 h in 2-fold serial dilutions beginning at 1/400 followed by washing with PBS/0.1% Tween 20 and blocked with assay diluent (PBS containing 4% (w/v) whey protein/15% normal goat serum/0.5% Tween 20/0.05% sodium azide). All assay steps were conducted in assay diluent (except substrate step) and incubated for 1 h at room temperature (for 2FS) or at 37°C (for 13H11) followed by washing with PBS/0.05% Tween 20. Sera were diluted 1/50 and incubated in triplicate wells. Fifty microliters of biotinylated target mAb was added at the EC50 (determined by direct binding curve of biotinylated mAb to JRFL). The extent of biotin-mAb binding was detected with streptavidin-alkaline phosphatase at 1/1000 (Promega; catalog no. V595) followed by substrate (CBC buffer pH 9.8 containing 1 mM MgCl2, plus 1 mg/ml p-nitrophenyl (4-nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol) salt)). Plates were developed for 45 min and read with a plate reader at 405 nm. Triplicate wells were background subtracted and averaged. Percentage inhibition was calculated as follows: 100 − (sera triplicate mean/no inhibition control mean)100.

Luminex assay

A sensitive Luminex assay was performed as previously described (13). Briefly, carboxylated fluorescent beads (Luminex) covalently coupled to p55 Gag, gp140 Env, or gp120 Env proteins were incubated with plasma serial dilutions ranging from 1/50 to 1/10240. Beads were then washed and acquired on a Bio-Plex instrument (Bio-Rad). Purified IgG protein (Sigma-Aldrich) and a constant HIV-positive serum titration were utilized as positive controls in every assay. Background values (beads in the absence of detection Ab) and normal human plasma were utilized as the negative controls. Results were background subtracted and expressed as mean fluorescence intensity.

Statistical analysis

Statistical tests and the apparent half-life models were performed using SAS v9.1 (SAS Institute). Midpoint titers were determined with a 4-point fit using both SoftMax Pro 5 ( Molecular Devices) and GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). To address possible biases due to therapeutic failures affecting the HIV-1-specific Ab titers and the estimated Ab level half-lives, we monitored the viral load throughout the follow-up period for each patient and considered the removal of a specific time point and those from the next 3 years in case a 10-fold increment between two contiguous time points was observed. No significant changes in viral load were observed over time, and therefore all the data were included in the analysis. One subject (PID 835) received a tetanus toxoid boost early before initiation of ART. To ensure that the resulting spike in Ab titers for this patient did not affect the estimate of the overall duration of the Ab level half-life, we excluded from the analysis of the tetanus toxoid-specific Ab titer half-life all of the data points before week 105 from initiation of ART. Additionally, we repeated the analysis just excluding this subject and obtained comparable results (data not shown). The apparent Ab half-lives of gp120 and gp41 were estimated using a single exponential decay model (y = (β0 − β) e^−t/β0 + β), where β0 is the level at time 0, β is the distance of the lower asymptote, y, is time, β is the exponential rate of decay such that ln(2)/β is the computed half-life). This model was chosen based on its a priori compatibility to the data collected (i.e., true 0) as well as better fit than other two- and three-phase exponential decay models. Apparent half-lives for p55 Gag, anti-tetanus toxoid, and anti-influenza Abs were estimated using the mixed model method described by Amanna et al. (11).

Results

Detection of levels of memory B cells producing anti-whole Env and anti-gp120 V3 loop IgG Abs in vaccinated subjects and correlation with plasma Ab levels

We first evaluated the levels of HIV-1 Ag- and epitope-specific circulating memory B cells in a setting in which we could identify 2-ethyl-1,3-propanediol) salt)). Plates were developed for 45 min and read with a plate reader at 405 nm. Alternatively, binding to tetanus toxoid (EMD/Calbiochem; catalog no. 5882231) and 2007 Fluzone vaccine were tested in 384-well ELISA plates (Corning; catalog no. 3700) using 3-fold sera dilutions starting at 1/25 and developed with 30 μl/well of both secondary Ab and substrate. Results were reported as OD450 values at the same plasma dilution across patients. In every case the OD450 reading at the dilution used was within the linear range of the assay. The results shown did not change when other plasma dilutions within the linear range of the assay were used for analysis (data not shown).

Ab binding inhibition assay

Ninety-six-well ELISA plates were coated with 0.2 μg/well HIV-1 gp140 JRFL envelope in 0.1 M sodium bicarbonate and blocked with assay diluent (PBS containing 4% (w/v) whey protein/15% normal goat serum/0.5% Tween 20/0.05% sodium azide). All assay steps were conducted in assay diluent (except substrate step) and incubated for 1 h at room temperature (for 2FS) or at 37°C (for 13H11) followed by washing with PBS/0.05% Tween 20. Sera were diluted 1/50 and incubated in triplicate wells. Fifty microliters of biotinylated target mAb was added at the EC50 (determined by direct binding curve of biotinylated mAb to JRFL). The extent of biotin-mAb binding was detected with streptavidin-alkaline phosphatase at 1/1000 (Promega; catalog no. V595) followed by substrate (CBC buffer pH 9.8 containing 1 mM MgCl2, plus 1 mg/ml p-nitrophenyl (4-nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol) salt)). Plates were developed for 45 min and read with a plate reader at 405 nm. Triplicate wells were background subtracted and averaged. Percentage inhibition was calculated as follows: 100 − (sera triplicate mean/no inhibition control mean)100.
B cells producing Abs commonly made, such as anti-gp140 Env and anti-V3 loop, and where we could match the V3 sequences in our indicator peptides with an immunogen in a human vaccine trial. We therefore analyzed samples from the VaxGen VAX004 gp120 trial for detection of gp120 and V3 peptide-specific memory B cells using consensus B gp140 oligomer envelope and HIV MN V3 peptides, respectively. PBMC samples obtained 2 wk after the fourth immunization were stimulated in vitro for 6 days with pokeweed mitogen, CpG-oligodeoxynucleotides, and IL-15 to induce differentiation of memory B cells into ASCs (21, 25–27).

Total IgG-producing B cells, consensus B whole Env- and MN V3-specific IgG-secreting cells were measured in a B cell ELISPOT assay. IgG ASCs were detected in all the samples (9140 ± 1997 ASCs/10^6 viable stimulated PBMCs) (Fig. 1A). Consensus B Env-specific IgG memory B cells were detected in 80% of the subjects. Among these positive subjects, the number of spots ranged from 1 to 228 ASCs/10^6 viable stimulated PBMCs, with a mean of 70 ± 15 ASCs/10^6 viable stimulated cells. Overall, consensus B Env-specific responses represented 1.79% of the total IgG ASCs (Fig. 1A).

HIV MN V3-specific ASCs were detected in 60% of the gp120 vaccine recipients, with V3-specific ASC frequency an order of magnitude lower than whole Env ASC frequency (V3 = 0.13% of total IgG spots) and a mean of 9 ± 2 ASCs/10^6 viable stimulated PBMCs (Fig. 1A). No HIV-1 envelope-specific and HIV MN V3-specific ASCs were detected in placebo recipients (Fig. 1B). These data demonstrated that our assay was adequately sensitive and specific to detect HIV whole Env and epitope-specific ASCs in the context of vaccinated individuals.

Plasma Abs may be derived from short-lived plasma cells, either matured from the naive compartment or differentiated by resting memory B cells upon reencounter with Ag, or derived from long-lived plasma cells that reside in the bone marrow (5). When plasma Ab levels are predominantly maintained by long-lived plasma cells, then the plasma levels of Ab do not correlate with the levels of memory B cells (11). Thus, we asked if there was a correlation between the frequency of circulating HIV-specific gp140 or Env V3 memory B cells and levels of plasma anti-Env Abs of the same specificity in the VaxGen gp120 recipients. Fig. 1C shows the analysis of ratios of anti-gp140 Env ASCs vs total IgG memory B cells and levels of anti-gp140 Env plasma Abs, performed as previously reported (11, 22, 27), in 18 samples for which paired observations were available. We found a significant correlation between gp140 Env ASC ratios and anti-gp140 Env plasma Ab levels (r = 0.66, p = 0.003). Similarly, a strong correlation between HIV MN V3-specific circulating memory B cell ratios of total ASCs and anti-HIV MN V3 Ab levels was also present (r = 0.75, p = 0.0003; Fig. 1D). The direct correlation between anti-gp140 and anti-Env V3 memory B cell ratios and plasma Ab levels implied that the contribution of long-lived plasma cells to plasma Ab levels was not predominant in this vaccination setting and that most of the detectable Ab response was maintained by shorter lived memory B cells. However, note that some patients had no detectable Ag-specific circulating memory B cells, yet they did have detectable plasma Ab titers. We cannot rule out the notion that in some patients the plasma Ab levels are indeed maintained by cells other than memory B cells, that is, bone marrow plasma cells.

Importantly, when analyses were performed using absolute numbers of Ag-specific ASCs and not the ratio of Ag-specific ASCs to total ASCs, no correlation with Ab levels was found (data not shown), most likely due to the wide variability (>2 logs) in frequencies of memory B cells among patients. Thus, as others have suggested (11, 22, 27), it appears that the biologically important comparison of ASCs is the ratio of Ag-specific to total ASCs vs plasma Ab levels. Ab level half-lives could not be calculated from VaxGen plasma because of the frequent dosing regimen in the trial (14).

Detection of IgG-producing total and Env-specific memory B cells in chronically HIV-1-infected subjects

We next determined anti-gp140 Env and anti-HIV MN V3 IgG memory B cell levels in 26 chronically HIV-1-infected and 23 uninfected subjects from the United States. Fig. 2A shows that, upon stimulation of HIV-1-infected PBMCs, the total memory B cell level increased from a mean of 239 ± 70 ASCs/10^6 viable PBMCs with no stimulation to 7963 ± 1702 ASCs/10^6 viable PBMCs after 6 days in culture (p = 0.0001). Similarly, memory B cells from HIV-1 uninfected subjects increased from 256 ± 86 ASCs/10^6 viable PBMCs with no stimulation to 6457 ± 1426 ASCs/10^6 cultured PBMCs (p = 0.0003; Fig. 2B). We next assayed for anti-gp140 consensus B Env memory B cells and found
memory B cells in the gp120 Env vaccination trial subjects since neutralizing Abs (28, 29). We could not assay for HIV-1 Env gp41 lived plasma cell pool (11).

The plasma Env Ab was maintained predominantly by the plasma Env V3 Ab levels (Fig. 3A). The positive correlation of gp140 Env- and HIV MN V3 epitope-specific memory B cells and plasma Env V3 Ab levels (Fig. 3C). Overall, the levels of gp140 Env- and HIV MN V3 epitope-specific memory B cells in chronically infected subjects were comparable with observations in vaccinated individuals (Fig. 1A), being −2 (p = 0.0001) and 3 (p = 0.016) orders of magnitude lower than total IgG, respectively (Fig. 3A). No HIV-1 envelope-specific or HIV MN V3-specific ASCs were detected in HIV-1-negative subjects (Fig. 3B).

The analysis of memory B cells and plasma Ab levels in HIV-1 chronically infected subjects also showed a correlation between gp140 memory B cell levels and plasma gp140 Env Ab levels (r = 0.50, p = 0.031; Fig. 3C). In contrast, no significant correlation was found between the memory Env V3-positive B cells and plasma Env V3 Ab levels (Fig. 3D), possibly relating to the heterogeneity of the Env V3 loop in chronically infected subjects and/or the inability of the V3 peptide to capture all Env V3-reactive memory B cells. The positive correlation of gp140 memory B cell ratio and plasma gp140 Ab, however, is similar to data obtained in gp120-immunized subjects, suggesting that the plasma Env Ab was maintained predominantly by the shorter lived memory B cell response to Env and not by a long-lived plasma cell pool (11).

The ability of the MPER of gp41 to induce Abs is a key question for the development of an HIV-1 envelope-based vaccine (28), since a cluster of epitopes in the MPER is the target for broadly neutralizing Abs (28, 29). We could not assay for HIV-1 Env gp41 memory B cells in the gp120 Env vaccination trial subjects since they were immunized with only gp120. However, as another example of HIV-1 epitope-specific memory B cell response, it was of interest to determine whether we could identify any gp41 MPER memory B cells in chronically infected and uninfected subjects.

We first assayed for gp41 MPER cluster II 2F5-related epitope Abs in two ways: direct binding ELISA to the nominal epitope, and competitive inhibition of patient plasma of biotinylated 2F5 mAb (Fig. 4A). On the contrary, no correlation was found in the same subjects between MN V3-specific frequencies of IgG ASCs shows a pattern that is similar to vaccinated individuals. Statistical differences were calculated with paired Student’s t tests. B, The frequency of total IgG in 19 HIV-negative individuals was comparable to that in HIV-positive samples. No HIV-specific responses were detected. C, The plasma Ab levels detected by ELISA (OD$_{abs}$ at 1/800 dilution; x-axis) of 19 HIV-positive samples were correlated to the percentage of consensus B gp140-specific ASCs over total IgG ASCs detected by ELISPOT assay (y-axis) for which paired observations were available. A significant linear correlation was detected (r = 0.5, p = 0.031). All plasmas were assayed at the same time and the same dilution was used for each OD reading across patients; in every case, the OD at the dilution used was within the linear range of the ELISA. D, On the contrary, no correlation was found in the same subjects between MN V3-specific ASCs and plasma Ab levels.
13H11 (Fig. 4B) (30). Both 2F5 neutralizing and 13H11 non-neutralizing MPER mAbs bind to the SP62 peptide (30). However, while the 2F5 epitope represented in the SP62 peptide was highly conserved among clade B HIV-1 isolates (28) and 87% of the subjects studied had Abs that could competitively block the binding of mAb 13H11, only 16.7% of chronically HIV-1-infected subjects had circulating memory B spot-forming cells that recognized the SP62 peptide (Fig. 4C). The magnitude of the MPER 2F5 epitope-specific memory response was 1–3 ASCs/10⁶ viable stimulated PBMCs (see Fig. 4C and supplemental Table S2). Interestingly, all four MPER ASC responders were on ART at the time of study, and only one subject, 8982-135, had both detectable MPER 2F5 epitope-specific memory B cells and plasma Abs (see supplemental Table S2). No ASCs specific for the 2F5 epitope were detected in HIV-1-negative subjects (Fig. 4C).

To confirm these findings, we tested an additional 13 chronically HIV-1-infected subjects using the reverse ELISpot assay format for the detection of epitope-specific ASCs (see Materials and Methods). In this cohort, the frequency and numbers of IgG ASCs were comparable to those of the 26 chronically HIV-1-infected subjects previously studied: 11 of 13 samples had circulating IgG memory B cells with an average of 6309 ± 2372 ASCs/10⁶ viable PBMCs, but only 3 of 13 subjects (23%) had MPER cluster II gp41 epitope-specific memory responses with 10, 13, and 41 ASCs/10⁶ viable PBMCs, respectively (data not shown).

Taken together, these results demonstrated that most chronically HIV-1-infected patients did not have detectable levels of circulating IgG memory B cells against the MPER cluster II gp41 peptide containing the 2F5-related neutralizing or the 13H11-related non-neutralizing B cell epitopes. Since a subset of anti-gp41 MPER cluster II-specific Abs bind to conformational epitopes (30), the magnitude of the response against the 2F5/13H11-related epitope defined by the SP62 peptide is likely to represent only a fraction of the overall responses. Nonetheless, memory B cells capable of making 13H11-related MPER non-neutralizing Ab (defined by Abs that block the binding of non-neutralizing mAb 13H11 that are commonly made in chronic HIV-1 infection) should have been more readily detected by the SP62 peptide ELISpot assay, yet they were not. These data implied that MPER specificities of memory B cells were rare in blood.

**Half-life of anti-gp120 and anti-gp41 Ab titers in chronically HIV-1-infected subjects with ART-suppressed viremia**

To further characterize the duration of anti-HIV-1-specific responses during chronic HIV-1 infection, we determined the time of decay of anti-gp120 and anti-gp41 Env IgG plasma Ab levels in eight chronically HIV-1-infected subjects followed for up to 387 wk (>7 years) after successful suppression of viremia upon initiation of ART. Both anti-gp120 (Fig. 5A) and anti-gp41 (Fig. 5B) Abs declined rapidly during the time of study on ART (4.5-fold and 5.5-fold decline in anti-gp120 and anti-gp41 titers, respectively) and then stabilized to a lower baseline level. We estimated the apparent half-life of Ab titers for each Ag and compared them to that for HIV-1 p55 Gag. Additionally, we also determined the plasma Ab titer for two non-HIV-1-related Ags: tetanus toxoid and 2007 influenza vaccine Fluzone. Exposure to tetanus toxoid occurred years before the period of observation, whereas repeated boost vaccinations were documented for influenza. As mentioned, a short estimated Ab level half-life implies a greater dependency of the Ab titer to active viral replication and persistent antigenic stimulation, and thus indicates a higher contribution of short-lived memory B cell responses to the Ag-specific Ab pool. Anti-gp120 Ab level half-life was ~81 wk (95% confidence interval, 30–377 wk; Fig. 5A) and anti-gp41 Env IgG Ab level half-life was similarly short at 33 wk (95% confidence interval, 23–58 wk; Fig. 5B). Similar findings for rapid decline of gp120 plasma Abs on ART have been reported previously (33). Conversely, overall anti-p55 Ab titers did not consistently decline over time (Fig. 5C); the variable patterns of the eight subjects precluded a precise definition of a half-life of p55 Ab levels, although an apparent titer half-life of 648 wk was estimated by a special linear mixed model (see Materials and Methods). This finding provides direct evidence that the rapid decay observed in the anti-gp120 and anti-gp41 Ab titers is not consistently observed, under the same conditions, for other non-HIV-1 envelope-related Ags, such as p55 Gag.

Our chronic HIV-1 subjects received annual or biennial influenza vaccinations, and Ab titers spiked in response to these immunizations (Fig. 5E). As a consequence, the overall Ab levels to the 2007 influenza vaccine Fluzone rose over time, as expected (6). To better characterize the decay of the anti-influenza Ab levels...
after vaccination, we estimated the anti-influenza titer half-life of each period in-between documented influenza vaccine boosts as independent longitudinal series. Again, as opposed to anti-envelope titers and similarly to p55 titers, anti-influenza titers did not fall after each vaccination and no significant decay was observed (Fig. 5). Finally, the Ab level half-life against tetanus toxoid, an Ag that has been shown to elicit predominantly long-lived plasma cell responses (11), was estimated at 543 wk (11 years; Fig. 5D), consistent with prior observations of Amanna et al. (11) in HIV-negative individuals.

Taken together, these data provided evidence for the inability of HIV-1 Env molecule to induce long-lived duration of Ab in chronic HIV-1 infection at levels comparable to those induced by other HIV-1 molecules (i.e., p55 Gag) or vaccines (tetanus toxoid or influenza).

Discussion
HIV-1 Env is the target for all known neutralizing epitopes of HIV-1 (29, 34). A critical goal of HIV-1 vaccine development is to understand how to design and formulate HIV-1 Env to induce broadly reactive, protective Ab responses that are durable and of sufficient titer. In this study we show that in both Env gp120 vaccination and HIV-1 chronic infection, plasma levels of induced Abs correlated with the levels of blood memory B cells, directly implying that short-lived, Ag-driven B cells are primarily responsible for anti-Env plasma Ab levels. We were able to confirm this notion in ART-treated chronically HIV-1-infected subjects with near complete suppression of plasma viremia by showing that the half-life of anti-Env Abs was relatively short (33 wk for gp41 and 81 wk for gp120) compared with the half-life of anti-p55 Gag (648 wk) and postvaccination anti-influenza Ab levels. Most if not all successful anti-infectious agent vaccines have neutralizing Abs as a correlate of immunity and, in contrast to HIV-1 Env, the apparent half-lives of vaccine-induced Ab levels range from 10 to 19 years for protein-induced Abs (e.g., tetanus, diphtheria) to 30 or more years for live vector-induced Abs (e.g., polio, measles) (11). We showed that the tetanus toxoid-specific Ab titer half-life, which has been previously described to be predominantly sustained in HIV-1-negative subjects by long-lived plasma cells rather than memory B cells (11), was maintained at ~11 years in chronically HIV-1-infected subjects.

Our study is important in that it directly demonstrates that, in addition to the inability of HIV-1 Env to routinely induce broadly neutralizing protective Abs (29), HIV-1 Env is also limited in its ability to induce long-lived B cell responses by persistent plasma cells. Moreover, this is the first study to our knowledge that examines the frequency of epitope-specific memory B cell levels in HIV-1-infected or vaccinated subjects.

The generation of immunological memory is a hallmark of successful vaccination (5, 6). Such protection is generated and exerted by two independently regulated mechanisms that lead to the development of reservoirs of long-lived plasma cells and memory B cells (5, 8, 12, 15, 35). Long-lived plasma cells are terminally differentiated, nondividing cells generated upon first encounter with an Ag or derived from previously generated memory B cells that reside in the bone marrow for many years and constantly secrete low levels of highly specific Abs without the need of further contact with the same Ag (5, 7, 9). Since Ag-specific Abs are already circulating by the time of reexposure, long-lived plasma cells represent the very first line of defense exerted by humoral memory (5).

As opposed to long-lived plasma cells, memory B cells do not prevent infection per se, but they proliferate and differentiate into ASCs upon Ag reexposure, resulting in a rise of high-affinity Ag-specific plasma Ab levels (7). Moreover, memory B cell levels in peripheral blood have been suggested to represent the overall pool of memory B cells since they constantly recirculate through the blood and secondary lymphoid organs, such as tonsils, spleen, peripheral lymph nodes, and Peyer’s patches (11, 15, 35). Therefore,
the detection of circulating memory B cells with a sensitive ELISPOT assay upon polyclonal in vitro stimulation allows the detection of epitope-specific memory B cells representative of the overall population of memory B cells produced upon vaccination or during chronic infection that are functionally able to differentiate into ASCs and are therefore capable of providing protection against reexposure to the cognate Ag.

The correlation between the ratio of HIV-1 Env-specific vs total memory B ASCs and plasma Ab levels describes the relative contribution of memory B cells to the maintenance of immunological memory after vaccination or during chronic infection (11, 22, 27). Whenever immunological memory is sustained by memory B cells, a correlation with plasma Ab levels is predicted to be maintained after reexposure to the same Ag (11, 21, 35).

In the present report, a correlation between ratios of circulating memory B cells and plasma Ab levels against gp140 as well as to the Env gp120 V3 region was detected in vaccinees and for gp140 Abs in chronically infected individuals. These findings imply that the contribution of long-lived plasma cells to the maintenance of immunological memory against the HIV-1 envelope after vaccination with rG120 and during chronic infection may be limited, and that HIV-1 envelope-specific plasma Abs are more short- than long-lived in both settings. However, several subjects with no anti-gp140 or anti-V3 region memory B cells still had detectable Ab titers, implying that these specificities were maintained by cells other than memory B cells. Therefore, it is entirely likely that Env in some patients is able to induce a degree of long-lived plasma cells to Env. It will be of interest to determine any correlation of long-lived Ab responses with maintenance of T follicular helper T cells in peripheral generative B cell microenvironments such as Peyer’s patches in gut.

The direct analysis of anti-gp41 and gp120 Ab level half-lives in chronically HIV-1-infected subjects in which viremia was suppressed by ART confirmed the hypothesis that HIV-1 envelope-specific plasma Abs are more short- than long-lived. Additionally, the lack of a consistent rapid decay in HIV-1 p55 Ab titers after initiation of ART and influenza after booster vaccinations suggested that, in the setting of chronic HIV-1 infection, the regulation of anti-p55 Gag Abs may differ from that of anti-Env Abs. In particular, it is possible that ART might have different effects on envelope Abs, which are HIV-1 protease-processed Abs, and p55 Gag, which is a polypeptide precursor, that would lead to differential regulation of these B cell responses.

Our half-life estimate of anti-gp120 Ab level is longer than that previously reported by Morris and colleagues (33), most likely due to the prolonged length of our observations (up to 7 years vs 1 year), allowing the Ab titers to reach a plateau at a lower level in most cases, such that the relative contribution of sustained Ab responses was reflected in the estimated Ab half-life. Note that the estimate of the plasma Ab level half-lives reflects the balance between at least two distinct components: sustained production of Abs by long-lived plasma cells, with very slow decline over several years, and the faster Ab level decay shortly after antigenic exposure (11). Additionally, other elements can contribute to extending the predicted 3-wk IgG half-life (36) and prolong plasma Ab levels even in absence of long-lived plasma cells: (1) Ag-specific IgG production is not immediately stopped at initiation of therapy when the viral load is still high; (2) residual Ag produced during antiretroviral therapy could circulate and continue to stimulate Ab production; (3) Ags could be trapped on follicular dendritic cells and in other compartments that are not in equilibrium with peripheral blood; and (4) other infection-related abnormalities might alter the normal clearance of IgGs from plasma (33).

The drop of anti-gp120 and anti-gp41 IgG Ab titers was rapid and dramatic, implying direct dependence of plasma anti-gp120 and anti-gp41 Env IgG Ab levels on antigenic stimulation. In contrast, anti-p55 Gag Ab responses did not decay consistently over time nor did anti-influenza Ab titers after each documented influenza vaccine boost. Since the specific contribution of long-lived plasma cells to plasma Ab levels can only be directly studied by analyzing bone marrow samples, our data imply, but do not prove, that the pool of circulating anti-gp120 and anti-gp41 IgG Abs was predominantly supported by short-lived plasma cells.

Previous work has suggested a selectively short duration of various HIV-1 Env Abs after vaccination (14, 37–39). A number of studies have also suggested a decrease in Env Abs in subjects followed for short periods on ART (33, 40–43). Recently, evidence for short-lived envelope responses were seen in an HIV-1-positive individual with acute leukemia transplanted with a CCR5Δ32 bone marrow stem cell transplant (44). Both his leukemia and HIV-1 were controlled posttransplant, and with a fall in plasma HIV-1 viral load to undetectable, his anti-Env Ab level continually fell over the ensuing 1.5 years (44). However, follow-up of Ab levels over years is needed for accurate determinations of duration of plasma Abs (33).

The present study is novel in that the levels of HIV-1 Env-specific memory B cells vs plasma Abs were analyzed in both the settings of chronic infection and vaccination, and in that it demonstrates that the mechanism of short duration of plasma anti-Env Abs in chronic HIV-1 infection and upon vaccination is dependent on memory B cell Ab production.

Finally, it was surprising that gp41 cluster II MPER 2FS/13H11-related epitope-specific memory B cells were present in only a small fraction of chronically HIV-1-infected patients and did not correlate with plasma Ab levels. These observations warrant additional studies of the persistence of 13H11-related epitope Abs, as this non-neutralizing MPER gp41 epitope, against which Abs are commonly made in HIV-1-infected subjects (30), may induce long-lived plasma cells.

The different biologies of memory B cells and long-lived plasma cells should be taken in account in the design of an AIDS vaccine (4). A preventive HIV-1 vaccine needs to extinguish infection before viral latency is established in CD4+ T cells (4, 13, 45), and to do this, high levels of long-lived protective Ab needs to be present before HIV-1 infection. In fact, while viral latency is certainly established by the time of seroconversion (46), several studies suggest that the window of opportunity to prevent the establishment of a reservoir of latent viruses may be as short as few days after transmission (4, 13, 47). Therefore, memory B cells may not have the time to differentiate into ASCs within the window of opportunity, and neutralizing Abs may need to be available at the time of transmission (4), implying that the induction of long-lived plasma cells is required for an AIDS vaccine to be effective. Previous studies correlating Ag-specific memory B cell frequencies in peripheral blood with plasma Ab levels after booster vaccination suggest that circulating memory B cells peak at 1–2 wk postre-vaccination and drive the Ag-specific effector response for only 2–4 wk after revaccination (8, 35, 48).

A number of recent observations provide strong indications regarding why HIV-1 Envelope does not routinely induce longer lived Ab responses and why memory B cell function is disrupted upon infection (6): we have recently demonstrated very early damage to B cell germinal centers in Peyer’s patches early on in acute HIV-1 infection (49); HIV patients contain a subset of “exhausted”-like FCRH4+ memory B cells enriched for HIV specificities (50); and, certainly, the early loss of robust CD4+ T cell help in early HIV-1
infection is an obvious mechanism for lack of induction of long-lived B cell responses (5, 6, 51). However, in the context of vaccination, in which the immune system of the recipients is not impaired by HIV infection, potential factors responsible for the lack of long-term responses should be focused on the characteristics of the immunogen: the biological activity of HIV-1 Env gp160 (52). T cell suppression by gp120 binding to CD4 (53), and the ability of the carbohydrates of gp120, which comprise 50% of the Env mass, to bind to mannose receptors on both dendritic cells (54) and B cells (55), hence suppressing Ag-specific immune responses.

One mechanism for overcoming the short duration of induced HIV-1 envelope Abs is the formulation of HIV-1 Env in potent adjuvants. In this regard, Goepfert and colleagues have reported immunization of humans with the adjuvant AS02A (QS-21 and 3-deacetylated monophosphoryl lipid A in an oil-in-water emulsion), which induced anti-gp120 Env Abs lasting up to 9 mo after final boost, although a half-life of Ab levels was not determined (56).

In conclusion, we have shown that anti-HIV-1 envelope plasma levels are correlated with envelope blood memory B cell levels both in the setting of vaccination and during chronic infection. With the rapid induction of immune suppression and CD4+ T cell loss as well as germinal center by transmitted/founder HIV-1 (47, 49, 57–60), it will be critical to be able to induce long-lived plasma and mucosal Abs against protective epitopes of HIV-1 Env that are present before contact with HIV-1. Even if the loss as well as germinal center damage by transmitted/founder HIV infection, potential factors responsible for the lack of memory B cells but also long-lived plasma cells will be critical for the development of a successful HIV-1 vaccine.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Table 1 – Frequency of anti-HIV-1 gp140 Env-specific antibody-secreting cells over total IgG memory B cells in chronically HIV-1 infected subjects.

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| Mean ASC/10^6 PBMC | 7,724 | 40 | 0.54 |
| Mean ASC/10^6 PBMC of gp140 responders | 8,016 | 49 | 0.64 |
Supplemental Table 2 – MPER gp41 2F5-related epitope-specific plasma antibody levels, reported both as µg/ml 2F5 equivalents and as end-point titers, and memory IgG responses in chronically HIV-1 infected subjects.

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<th>Patient no.</th>
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Results were considered positive when above 3 times + 3SD the average OD₄₀₅ of HIV negative subjects (representative sample in the lower right panel). Five of 21 subjects had detectable levels of MPER gp41 2F5 epitope-specific antibodies.