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The Initial Antibody Response to HIV-1: Induction of Ineffective Early B Cell Responses Against gp41 by the Transmitted/Founder Virus

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ABSTRACT

Background

A window of opportunity for immune responses to extinguish HIV-1 exists from the moment of transmission through establishment of the latent pool of HIV-1-infected cells. A critical time to study the initial immune responses to the transmitted/founder virus is the eclipse phase of HIV-1 infection (time from transmission to the first appearance of plasma virus) but, to date, this period has been logistically difficult to analyze. Studies in non-human primates challenged with chimeric simian-human immunodeficiency virus have shown that neutralizing antibodies, when present at the time of infection, can prevent virus infection.

Methods and Findings

To probe B cell responses immediately following HIV-1 transmission, we have determined envelope-specific antibody responses to autologous and consensus Envs in plasma donors for whom frequent plasma samples were available at time points before (eclipse phase), during, and after plasma viral load (VL) ramp-up, and modeled antibody effect on the kinetics of plasma viremia. The first detectable B cell response was in the form of immune complexes 8 days after plasma virus detection, whereas the first free plasma anti-HIV-1 antibody was to gp41 and appeared 13 days after appearance of plasma virus. In contrast, envelope gp120-specific antibodies were delayed an additional 14 days. Mathematical modeling of early viral dynamics was performed to determine the impact of antibody on HIV replication *in vivo* as assessed by plasma VL. Including the initial anti-gp41 IgG, IgM or both in the model did not significantly impact the early dynamics of plasma VL.

Conclusion

These results suggest that during the critical window of time in which antibody could plausibly extinguish the transmitted virus, HIV-1 selectively induces non-neutralizing antibodies capable of binding virions but that have little impact on viral control. Thus, for antibodies to play a role in prevention or control of HIV-1 infection, vaccines must be designed to induce pre-existing broadly neutralizing antibodies that are functionally different than the first antibody responses elicited in natural infection and/or induce an extremely rapid secondary neutralizing antibody response directed against an accessible epitope on the Env trimer.

Introduction

The development of a preventive HIV-1 vaccine is a global priority [1]. A major roadblock in development of a preventive HIV-1 vaccine is the inability to induce protective antibodies by vaccines or natural infection. Studies in non-human primates have demonstrated that passive infusion of broadly neutralizing anti-HIV-1 monoclonal antibodies prevents infection by simian-human immunodeficiency viruses (SHIVs) [2-4]. Thus, if sufficiently high levels of broadly neutralizing antibodies were present at the time of transmission, protection from HIV-1 infection may be possible. However, to date there is no immunogen formulation that consistently induces broadly neutralizing anti-Env antibodies. Moreover, autologous neutralizing antibody responses do not occur until months after transmission [5-8]. The window of opportunity during which a protective antibody might extinguish HIV-1 after the initial transmission event is uncertain, but is likely to be limited to the period of time prior to establishment of the latent pool of HIV-1 infected $CD4^+$ T cells [9,10]Although viral latency is certainly established at the time of seroconversion [11], it may be as early as a few days after transmission.

An important obstacle to the development of an effective HIV vaccine is the inability to induce antibodies that neutralize primary HIV-1 strains across all genetic subtypes [12,13]. While multiple forms of HIV-1 envelope-based vaccines express epitopes to which rare broadly neutralizing human mAbs bind (*i.e.* Envs are *antigenic)*, these vaccines have not been *immunogenic* and have failed to induce broadly neutralizing antibodies against the gp120 CD4 binding site, the membrane proximal external region (MPER) of gp41, or against gp120 carbohydrate Env antigens in animals or humans [12,14].

HIV-1 seroconversion has been reported to occur over a wide range of time, when estimated from the onset of clinical acute HIV-1 infection (AHI) [15-17]; however, the timing of seroconversion of HIV antibodies of particular specificities and isotypes has not been precisely quantified relative to the first time of detectable plasma viremia. Anti-HIV-1 IgM reactive with virus-infected cells has been detected during the course of AHI [18,19], but the timing of these antibodies and the presence of IgM-virion immune complexes relative to the first detection of viral RNA in AHI have yet to be defined. It is known that autologous neutralizing antibodies only arise months after the first appearance of HIV-specific antibodies [5-8]. Critical questions

for understanding the role of early HIV-1 antibodies in the control of HIV-1 are, first, what is the nature and timing of the earliest anti-HIV-1 antibodies and second, what are the contributions of these antibodies in the control of viral replication after transmission?

In this study, we have investigated the timing of specific anti-envelope (Env) antibody responses from the eclipse phase (time between transmission and detectable viremia) [20] through 6-12 months of established infection, and modeled the effect of the B cell response on control of initial plasma viremia. We show that the earliest detectable antibodies to HIV-1 are in the form of virion-antibody immune complexes followed 4 days later by free anti-gp41 IgM plasma antibodies. Mathematical modeling of viral dynamics suggested that the initial Env gp41 antibody responses had little effect on control of viral replication.

Results

Plasma Donor Cohort. HIV-1 seroconversion plasma donors from the US (Clade B) were studied for the earliest antibody events in HIV-1 infection. These subjects donated plasma every three days and the plasma units were held for weeks until tests for HIV-1, hepatitis B or hepatitis C were completed [20]. Once positive for an infectious disease, the plasma sample donations were stopped; therefore, neither cells nor long-term follow-up of these plasma donors were available. Analysis of the US Plasma Donor cohort provided for calculation of the earliest HIV-1 antibody response in relation to a defined point where viral RNA was first detected at transmission. A time zero (T_0) that represented the initial time at which the VL trajectory crossed the assay lower limit of detection (100 HIV-1 copies/ml) was established to align each donor panel to a single reference time (Figure 1A). The start of detectable plasma viremia, T_0, is approximately 10 days (range 7-21) [18,21-25] after virus transmission and represents the end of the eclipse phase of HIV-infection. The distribution of time intervals between subject samples that were studied here was a median of 4 days for plasma donors (Figure 1B).

The initial IgG anti-Env responses following transmission. To ensure that the earliest antibodies were detected, both standardized ELISAs and more sensitive Luminex quantitative antibody assays were utilized (SOM Figure S1). The lower level of sensitivity for the ELISAs for measurement of anti-HIV-1 Env IgG was 2.2 ng/ml and the lower level of sensitivity of Luminex assays for measurement of anti-HIV-1 IgG was 0.2 ng/ml (SOM Table S1). For

identification of initial antibody responses, we tested autologous, consensus B and clade B wildtype Envs as antigens. For non-Env antigens, all antigens were wildtype clade B. To validate that consensus B Env antigens detected the earliest antibodies, and to determine if earlier antibody responses could be detected using autologous Env antigens; gp140, and Env gp120 V3 peptides from 4 plasma donor subjects were produced and compared with consensus B Envs or V3 peptides for ability to detect plasma Env antibodies.

First, using consensus or wildtype clade B Envs we found the earliest detectable anti-Env IgG plasma antibody responses following HIV-1 transmission were to envelope gp41 and occurred at a median of 13.0 days after T_0. Figure 2 illustrates the earlier timing of the anti-gp41 antibody responses compared to the later and more variable timing of the antibody responses against gp120 ($p < .01$). Antibodies to gp41 developed in 100% of subjects by 18 days in contrast to gp120 antibody responses which came up in 37% of subjects during the 41 days post T_0 studied here (Table 1.) There was no significant difference in the timing of the anti-gp120 antibody responses when two additional wildtype clade B gp120 envelope proteins: JRFL and 89.6 gp120 Envs were examined (not shown). Figure 2B shows the relative time of appearance of different anti-Env antibodies compared to time of appearance of antibodies to HIV-1 p24, p55, p66, p17 and p31 HIV-1 proteins. Pair-wise comparison of the timing of each specificity of antibody demonstrated that HIV-1 structural component antibody timing (anti-Gag) was significantly later from that of HIV-1 gp41 antibodies.

A summary of the timing of various anti-Env antibody responses detected in US plasma donors is shown in Table 1. For the first antibody elicited against gp41, 13/19 (68%) of gp41 responses included the immunodominant region of gp41, and 7/7 of the plasma donor initial gp120 included responses that could be mapped to the V3 loop (i.e. 100% of the plasma donors that had gp120 antibodies within the first 40 days of transmission also had V3 antibodies) Antibodies that did not appear at all in US plasma donor subjects (within 40 days post T_0) were anti-MPER (neutralizing and non-neutralizing) antibodies, CD4 binding site antibodies (CD4bs) and CD4-induced antibodies (CD4i). Neutralizing antibodies to the easily neutralized Tier 1 [26] HIV-1 Env pseudoviruses such as B.SF162 and antibody dependent cell-mediated virus inhibition (ADCVI) activity [4] also did not appear within the first 40 days after T_0 as well (data not shown). ADCVI has previously been reported to be present during the later stages of acute infection, so the time points examined here during acute viremia in the plasma donor subjects are

likely just before the development of ADCVI [27]. As expected, broadly neutralizing antibodies with specificities similar to the broadly neutralizing antibodies 2F5, 4E10, 1b12 and 2G12 also did not appear during the first 40 days after T_0 (not shown).

Analysis of isotype-specific gp41 antibody responses. IgG antibodies are produced after immunoglobulin (Ig) class switching and are classically produced after IgM antibodies. We assayed for HIV Env specific IgM using Luminex assays with recombinant gp41, gp120 and consensus B and group M consensus gp140 protein antigens. As with IgG responses, the first IgM antibody against Env also only targeted gp41. The median time of rise in HIV-1-specific IgM antibodies was 13 days post T_0 (range 5-18 days).

We expressed 4 autologous Envs , from 4 different plasma donor subjects (Pts. 6246, 6240, 9021, 63521) representing the transmitted or founder virus [28] as gp140C protein oligomers and as well studied 4 subjects with autologous Env V3 loop peptides as targets for plasma antibody binding assays. Three of the gp140 Env were chosen from subjects in whom an antibody response was detected with consensus Env, while gp140 was expressed from one subject who did not have a detectable anti-gp41 response. A representative example from a single donor against the autologous and Consensus Clade B Env (ConB) for IgM, IgG and IgA is shown (Figures 3A-C, and SOM Figure S3.). We found that using both autologous Envs and autologous V3 peptides (not shown) we could not detect any earlier IgM, IgG and IgA antibody responses compared to those detected using group M consensus Envs or consensus B V3 peptides (Figures 3A-C). Interestingly, the gp41 antibody responses were greater in magnitude when tested with consensus Envs vs. autologous envelopes.

We next examined Ig class switching patterns in plasma donors (15 subjects), four clade B CHAVI 001 subjects, and in 3 clade B AHI subjects from the Trinidad/Tobago cohort where the plasma sampling was sufficiently early to determine the first appearances of anti-gp41 IgM and IgG (See methods and supporting online materials for viral load and timing of samples from the CHAVI 001 and Trinidad/Tobago acute HIV-1 infection cohorts; SOM Figure S2). Figure 4 shows representative subjects with either sequential class-switch kinetics (Figure 4A) or simulataneous class-switch kinetics (Figure 4B) in the plasma donor cohort. In both subjects, IgM responses were transient and decayed over a period of 20-40 days, while IgG responses rose over the same period. Anti-gp41 IgM responses appeared earlier than IgG responses in 9/22

(41%) of subjects; however, in 13/22 (59%) of subjects, IgM anti-gp41 was detected at the same time of IgG and IgA anti-gp41 antibodies (Figure 4C). We also tested anti-IgM, IgG and IgA responses to an gp41 immunodominant peptide in subject 6246 with simultaneous appearance of anti-gp41 IgM, IgG and IgA to determine if the simultaneous appearance of the three isotypes could potentially be due to responses to different gp41 epitopes. We found anti-immunodominant IgM, IgG and IgA were simultaneously detected at 10 days post T_0 demonstrating in this subject that the simultaneous appearance of antibodies could not solely be attributable to the development of antibody responses to multiple gp41 epitopes (not shown). To determine if simultaneous IgM, IgA and IgG antibody responses were unique to envelope or rather occurred in the response to other HIV-1 proteins, we also determined the pattern of isotype antibody responses to p55 Gag. We found that IgM, IgG and IgA antibodies to p55 Gag were also detected simultaneously in subjects that had simultaneous appearance of IgM, IgG and IgA antibodies to gp41 Env (Figure 4D).

Detection of immune complexes in AHI. We next assayed for earlier antibody responses in the form of antibody bound to virions to determine if earlier antibody was being made but was not in plasma but rather was only present in the form of virion-IgM or IgG antibody complexes in US plasma donors. In 6/6 of subjects tested with sufficiently high plasma viral RNA levels, immune complexes containing either IgG or IgM antibodies bound to virions were detected at a median time of 8 days post T_0 (Figure 5, SOM Figure S4). In 5 subjects the complexes became undetectable within a span of 10-15 days. Since virion-antibody complexes were detected both in patients with simultaneous and sequential Ig isotype kinetics, the presence of early immune complexes could not explain the simultaneous detection of IgM, IgG and IgA anti-gp41 isotypes. Plasma donor subjects 12008, 9015 and 9077 had detectable immune complexes before the detection of antibody responses, while plasma donor subjects 9079, 9076 and 9021 had detectable immune complexes coincident with antibody responses. Subjects in both groups (12008, 9015, 9076) all demonstrated simultaneous antibody isotype kinetics. Thus, there was no correlation with panels having simultaneous antibody isotype kinetics and immune complex detection.

The results for the IgG immune complexes were confirmed with a second method of detection (not shown) using a Protein G column to capture antibodies bound to virions followed by lysis of virions to measure viral RNA. Identical kinetics of the appearance and decline of

immune complexes were observed using both methods for measurement of IgG immune complexes (not shown). Taken together, these data suggest earlier production of anti-virion IgM and IgG on day 8 after T_0 and before the appearance of free plasma anti-HIV IgM and IgG. The simultaneous appearance of both IgM and IgG virion immune complexes either suggests simultaneous induction of anti-virion IgM and IgG in these subjects or indicates yet earlier induction of IgM and IgG antibodies to HIV virion components with specificities that are not detectable with our current assays. The decline in detection of immune complexes may be due to clearance by the reticuloendothelial cell system. It is of interest that the detection of these antibody-virion complexes declines while virus (antigen) and antibody are still present. Further study of the specificities of the antibodies bound in immune complexes and whether they are able to alter infectivity by enabling binding to antigen presenting cells is under investigation.

AHI anti-gp41 Env antibodies activate complement. A potentially important functional component of antibodies in AHI is their ability to fix complement. We determined if early anti-gp41 antibodies were capable of binding serum complement. Plasmas from 6 US plasma donors were examined for complement activation/ binding to CR2 using hPBMC co-cultured HIV-1 virions. Complement-activating binding antibodies were present in all panels at every time point that plasma antibodies were detected as shown in Figure 3. Moreover, the kinetics of appearance of complement-activating antibodies followed the same kinetics as gp41 binding antibodies. Both laboratory-adapted HIV-1 strain (B.SF162) and an early transmitted virus strain (B.QH0692) were examined as targets of antibody and complement with similar results obtained with each virus (Figure 6).

Polyclonal B cell activation following HIV-1 transmission in US plasma donors. HIV-1 Env gp120 has been suggested to be a polyclonal B cell activator [29], to bind to Ig VH3 as a superantigen [30], and to induce polyclonal Ig class switching [31]. Patients with chronic HIV-1 infection have polyclonal hypergammaglobulinemia [32], and a number of studies have reported hypergammaglobulinemia in early HIV-1 infection [33,34]. To examine whether polyclonal B cell activation occurs during the initial antibody response to HIV-1 transmitted/founder Env, we measured quantitative IgM, IgG and IgA levels on the initial and last plasma samples in US plasma donors. There was no significant elevation of IgM, IgG or IgA during AHI in plasma donor panels (Figure 7A). Similarly, the last plasma sample in each donor panel (range of 25-41 days after T_0) was analyzed for the following autoantibodies:

cardiolipin, SSA/Ro, SSB/La, Sm, RNP, Scl-70, Jo-1, double stranded DNA, centromere B, and histones and all were negative for all these specificities (not shown). However, in a screen for rheumatoid factor autoantibodies (IgM antibodies that react with IgG) of 19 plasma donor samples and 10 CHAVI 001 acute infection cohort subjects studied, 8/29 (28%) tested positive for rheumatoid factor following HIV-1 transmission (Figures 7B, 7C). Thus, in some subjects, B cell activation during acute HIV-1 infection can result in production of the autoantibody, rheumatoid factor [35].

Modeling of the initial gp41 antibody response with acute viral load kinetics and assessment of antibody pressure on viral sequence evolution. To determine the effects of initial anti-gp41 antibody responses on control of HIV-1 VL, we used mathematical modeling of the early viral dynamics. We first used the target cell-limited model [36], which does not include any effect of antibody, to analyze the plasma donor viral load data obtained over the first 40 days after T_0 for the six donors (6240, 6246, 9032, 9077, 9079 and 12008) for which both complete VL and antibody data were available over this time period. We found that for all donors except 9032 the target cell limited model gave good agreement with the experimentally determined VL data (Figure 8). We then fit to the same data three variants of this model that included enhanced virion clearance due to antibody opsonization, antibody-mediated viral neutralization, or antibody-dependent loss of HIV-1 infected cells. In these models (see methods) we included the measured levels of anti-gp41 IgM, IgG or the sum of IgM and IgG in mediating these effects. We found in all cases, except for donor 9032, that including antibody-mediated effects did not improve the model fit (Table 2). Thus, it appears that for the majority of patients the basic target cell-limited model is the most compatible with the data, i.e., including the humoral immune response involving either anti-IgG, IgM or both does not improve the model fit to the viral kinetics observed for the first 40 days after T_0. This suggests that early in AHI either target-cell limitation or cell-mediated responses play the predominant role in controlling viral load. In support of this notion, no statistically significant association was identified between the magnitude of anti-gp41 IgG and the viral load decay rate. There was also no statistically significant association identified between the viral load decay rate and the time to the first elevation of antibody.

Ontogeny of CD4 inducible (CD4i) antibodies, CD4 binding site antibodies, and non-neutralizing cluster II (MPER) gp41 antibodies in plasma donors and in three additional AHI

cohorts followed 6-12 months after transmission. For analysis of antibody responses during the down-slope of VL after transmission, we studied 12 Clade B subjects from Trinidad/Tobago with heterosexual transmission, 14 acute Clade C subjects from South Africa (the CAPRISA cohort) [8] , and 10 Clade B US AHI subjects (3 untreated, 7 on anti-retroviral treatment, CHAVI 001 cohort). The acute phase viral kinetics and distribution of samples used in this study from a subset of subjects in the Trinidad and Tobago and the CHAVI 001 Cohort are shown in SOM Figure S2. Subsequent to the initial B cell response to HIV-1 infection that produces anti-gp41 antibodies, the anti-HIV-1 Env B cell response eventually broadened to include other Env specificities. Antibodies that bind to the MPER gp41 (cluster II antibodies) [37] can either be neutralizing (e.g. Mabs 2F5, Z13, 4E10) or non-neutralizing (e.g. Mabs 267D, 126-6, 13H11) (reviewed in [38]). Whereas non-neutralizing anti-gp41 MPER antibodies are commonly made in ~80% of infected subjects [38], broadly neutralizing MPER antibodies are rarely made [12]. CD4i antibodies bind at or near the co-receptor binding site and potently neutralize HIV-1 generally only after sCD4 is added to the *in vitro* neutralizing assay [39], due to inability of a bivalent antibody to fit into the coreceptor binding site. Broadly neutralizing CD4BS antibodies are also rarely made [40]. Previously described assays for these three anti-Env specificities were used to probe plasma donor panels (followed up to 40 days after T_0) as well as to probe serial plasma samples from selected subjects in the clade C CAPRISA [8] and clade B Trinidad/Tobago [41] acute HIV-1 infection cohorts (both followed 6-12 months after transmission). As mentioned, CD4i, CD4BS, and non-neutralizing cluster II gp41 antibodies were not made during the first 40 days after T_0 (Table 1). In the CAPRISA and Trinidad/Tobago AHI cohorts, CD4i antibodies, CD4 binding site antibodies and non-neutralizing cluster II MPER gp41 antibodies arose at approximately the same time, from 5 to 10 weeks post-enrollment into the acute infection study (SOM, Figure S5) [38].

Evaluation of anti-HIV-1 heterologous Tier 1 and autologous neutralizing antibody responses in plasma donor, CAPRISA and Trinidad AHI cohorts. As previously mentioned, using the highly neutralization-sensitive tier 1 clade B virus, SF162.LS, no neutralizing antibody responses were detected in plasma donors for up to 40 days after T_0. Heterologous tier 1 neutralizing antibodies against HIV-1 MN, were present in the Trinidad/Tobago cohort as early as 8 weeks after infection (SOM Table S1) and were likely primarily V3-directed since autologous V3 peptides competed for heterologous HIV-1MN neutralization (Greenberg, M.L., unpublished). Autologous neutralizing antibodies arose after a median of 32 weeks from the time of

transmission in the Trinidad/Tobago clade B cohort (Tomaras, G.D., Greenberg, M.L., unpublished) and at a mean of 19 weeks following transmission in the CAPRISA clade C cohort [8].

Discussion

In this study we show that the initial B cell response to the transmitted/founder virus is to HIV-1 gp41 Env, with responses to gp120 delayed by an additional 14 days. Mathematical modeling of the effects of initial IgM and IgG gp41 antibodies on viral kinetics in the plasma donor cohort revealed little if any effect of the initial antibody response on control of acute phase plasma viremia.

The timing and specificity of the initial antibody response to HIV-1 Env was of interest for several reasons. First, the window of opportunity for a vaccine to extinguish the transmitted or founder strain of HIV-1 is likely quite short, and the timing of this window will vary from subject to subject depending on the time of establishment of the latent pool of $CD4^+$ T cells. That post-exposure prophylaxis does not protect beyond 24 hours after SIV challenge in rhesus macaques [42], and systemic dissemination of SIV occurs from 2-7 days in macaques [43] implies that the window of opportunity may be 10 days or less in humans [44]. Moreover, early appearance of evidence of systemic inflammation and acute phase reactants in plasma at 5 to 7 days *before* T_0 (Kessler, B, McMichael, A, and Borrow, P, personal communication), as well as the appearance of plasma analytes of apoptosis 7 days after T_0 [45] add support to this short estimated window of opportunity for vaccine efficacy. Given that a narrow window of time might exist for antibodies to be protective and given that immune complexes only arise ~18 days after transmission (8 days after T_0 with an estimate of time from transmission to T_0 of a mean of 10 days, range 7-21 [18,21-25]) , then the first antibody response to HIV-1 is quite delayed relative to when it optimally should occur to either extinguish or control transmitted/founder HIV-1 strains.

Our study is the first demonstration of virion–antibody complexes during the initial phase of viremia in acute HIV-1 infection. Previous work, looking at later times in acute infection, did not find immune complexes early in HIV-1 infection, but rather found immune complexes only in chronic infection [46]. The presence of these early immune complexes during acute infection

raises the question of whether antibody-coated virus is infectious; work is ongoing to determine the infectivity of opsonized virus (Montefiori, D.C., Tomaras, G.D., Haynes, B.F., unpublished). Overall, these data suggest an HIV-1 evasion strategy wherein the transmitted/founder virus initially induces antibodies that bind virions yet are non-neutralizing.

That the initial B cell response to Env selectively recognized gp41 also was of interest. Li *et al.* recently demonstrated that when broadly neutralizing antibodies do appear, they appear late and include antibodies against the CD4 binding site on gp120 [40]. While there are broadly neutralizing epitopes on gp41 in the MPER, like broadly neutralizing CD4 binding site antibodies, neutralizing antibodies to the MPER are rarely made, and when they are made, require > 2 years after transmission to arise (Shen., X, Tomaras, G.D., unpublished observations). Thus, the host-pathogen interactions occurring during and immediately after transmission results in a delay in recognition of gp120 by host B cells until after the latent pool of infected cells is likely established.

Polyclonal activation of B cells occurs in chronic HIV-1 infection and as well has been reported in early HIV-1 infection [34]. We found no polyclonal hypergammaglobulinemia in plasma donors, but did find plasma rheumatoid factor in ~30% of subjects. Thus, polyclonal B cell activation does occur early on as signaled by the detection of this autoantibody, likely indicating triggering of $CD5^+$ B cells that are the producers of rheumatoid factor autoantibodies [35].

Also of interest is that there was heterogeneity in the pattern of Ig class-switching seen following HIV-1 transmission. We have shown that the simultaneous appearance of anti-HIV IgM, IgG, and IgA are unlikely to be due to the presence of immune complexes that mask detection of antibodies, because immune complexes appeared at the same time as free antibody in half of the subjects. Other potential explanations for simultaneous appearance of IgM, IgG, and IgA anti-HIV-1 antibodies in plasma include: prior exposure to HIV-1, and primary T cell independent B1 and marginal zone B cell responses to HIV-1 following transmission [47].

If the simultaneous appearance of IgM, IgG and IgA to Env and Gag represent prior exposure of ~60% of subjects to HIV-1 antigens and represents a rapid secondary response to HIV-1 full infection, then an atypical aspect of the response is that the putative "secondary"

response occurs at exactly the same time as the primary IgM response (day 13.0 after T_0) occurs in those with sequential appearance of anti-Env and Gag IgM, IgG and IgA. If the simultaneous response is indeed secondary from prior HIV-1 exposure, then it should occur approximately 7 days earlier than observed. Thus, we believe it is unlikely that simultaneous appearance of IgM and class-switched antibodies in plasma in over 2/3 of AHI studied indicate prior exposure to HIV-1.

Soon after transmission in both humans and non-human primates when infection is established, there is a severe depletion of CD4 T lymphocytes [48,49] that could lead to a lack of sufficient CD4 help for stimulation of B cell responses. The early depletion of $CD4^+CCR5^+$ T lymphocytes with massive apoptosis could in addition to altering T cell homeostasis lead as well to profound dysregulation of the B cell response (Levesque, M., Ki-Hwang, K., Haynes, B.F. unpublished and [45,50]. Thus, elicitation of initial T independent antibody responses, in the setting of T cell depletion, could be responsible for simultaneous appearance of IgM, IgG and IgA anti-HIV-1 antibodies. A similar T independent pattern with simultaneous appearance of IgM, IgG and IgA anti-pneumococcal antibody occurs following pneumococcal vaccine [47].

It was important to model both the antibody timing and the viral load dynamics to determine any salutary or detrimental effects of early antibody responses on control of plasma viremia. As a null model we used a simple target-cell limited model that includes no effect of humoral or cellular immune responses [36]. We found that for 5 of the 6 plasma donors for which we had VL data that extended past the viral load peak and out to day 40 past T_0 this model gave good agreement with the VL data. Nonetheless, we asked if the fit could be improved by using a model that incorporated any of a variety of known functional effects of antibody. Not surprisingly, it was only for the one plasma donor, 9032, for which the target cell limited model did not give good agreement with the VL data that an improvement was seen when including antibody in the model. Interestingly, this donor was unusual in that the peak VL was significantly lower than in the other donors (3.4×10^4 copies/ml). Taken together, these analyses suggested that for most donors early antibody had little functional consequence for the control of viremia.

If early antibody induced by the transmitted virus had any antiviral effects, then antibody-induced viral escape should be detected after appearance of complexed or free antibody in plasma. In this regard, Keele *et al. [28]* have recently sequenced the transmitted founder virus

for the plasma donors studied in this report and found that virus sequences at 14 days after T_0 conformed to a model of random viral evolution thus showing no evidence of early antibody induced selection.

For HIV-1, functional consequences of antibody binding could include virus neutralization on T lymphocytes or macrophages [51,52], antibody-dependent cellular cytotoxicity (ADCVI/ADCC), complement-mediated neutralization, antibody Fc-mediated effector functions, virolysis and/or inhibition of transcytosis. A recent study [4] suggested that the concentrations of antibodies mediating the different anti-viral functions may be an important consideration for complete virus elimination, since Fcγ- receptor-binding function requires higher antibody concentrations than are required for virus neutralization. In addition, antibody and complement-mediated virion lysis can develop in acute infection and can correlate with plasma virus load during the acute stage of infection [53]. This antiviral activity did not correlate with neutralizing antibodies and is thought to be an antiviral component of non-neutralizing antibodies. Dendritic cells (DCs) are positioned in mucosae where they are thought to be one of the first cell types to help establish infection (reviewed in [54]). It will be important to determine whether the very earliest antibodies elicited in acute HIV-1 infection can block HIV-1 infection in dendritic cells at mucosal surfaces.

From the present study, it is clear that the initial B cell response to the transmitted/founder virus does not control initial virus levels during the first 40 days of infection. A critical question is whether these types of non-neutralizing antibodies could be protective, if present before infection or rather, if a completely different type of inhibitory antibodies will need to be induced by future HIV-1 vaccines. However, that the earliest antibody responses appeared to have little effect on initial plasma viremia suggests that the initial antibody response has no antiviral effect. Autologous neutralizing antibodies target envelope variable loops [6,7,55,56], that can arise long after any window of opportunity to extinguish HIV-1 has passed. Thus, an effective HIV-1 vaccine will need to induce antibodies that bind native virion envelope molecules that are functionally different from those first induced in natural infection and will lead to the maturation of a rapid secondary neutralizing antibody response within the first week after transmission.

Materials and Methods

Subjects Studied

A subset of subjects from four different acute infection cohorts were examined: 21 plasma donors, 12 AHI subjects from the Trinidad cohort (clade B), 14 AHI subjects from the CAPRISA (clade C) cohort and 10 AHI subjects from the CHAVI001 acute infection cohort.

Viral Load Testing

Plasma viral RNA was measured by Quest Diagnostics (Lyndhurst, NJ) (HIV-1 RNA PCR Ultra).

Antigens Used in Antibody Binding Assays.

The antigens used for direct antibody binding assays are: group M consensus Env CON-S gp140, consensus B gp140, clade B wildtype Env gp120s (produced by either recombinant vaccinia [57] or 293T transfection (see SOM methods). IIIB, JRFL, 89.6, as well as the following peptides (Primm Biotech Inc, Cambridge, MA) and their sequences. SP400 (gp41 immunodominant region, RVLAVERYLRDQQLLGIWGCSGKLICTTAVPW NASWSNKSLNKI), SP62, gp41 MPER, (QQEKNEQELLELDKWASLWN), 4E10 P, (SLWNWFNITNWLWYIK), Consensus B V3 gp120 region, (TRPNNNTRKSIHIGPG RAFYTTGEIIGDIRQAH), Consensus M V3 CON-S gp120 region, TRPNNN TRKSIRIGPGQAFYATGDIIGDIRQAH. Acute HIV-1 envelope gene sequences were derived from of 4 subtype B acute HIV-1 infected individuals (Subjects 6246, 6240, and 9021 by single genome amplification (SGA) [28]. To produce recombinant soluble gp140 proteins, a gp140 *env* gene construct named gp140C was designed, in which the transmembrane and cytoplasmic domains of HIV-1 Env were deleted and 2 critical Arg residues in the gp120-gp41 cleavage site were replaced with 2 Glu residues. All four gp140C env genes were codon-optimized by employing the codon usage of highly expressed human housekeeping genes, *de novo* synthesized (Blue Heron Biotechnology, Bothell, WA) and cloned into pcDNA3.1/Hygro expression plasmid (Invitrogen, Carlsbad, CA) using the standard molecular technology. Recombinant HIV-1 gp140C Env proteins were produced in 293T cells by transient transfection with the resulting plasmids and purified by *Galanthus nivalis* lectin-agarose (Vector Labs, Burlingame, Calif.) column chromatography [57] . Autologous V3 peptides were made from these same Envs.

Antibody Assay Criteria.

The positivity criterion per antigen per antibody isotype was determined by screening ≥30 seronegatives. A standardized HIV+ positive control is titrated on each assay (tracked with a Levy-Jennings plot with acceptance of titer only within 3 STDEV of the mean) and the average O.D. is plotted as a function of serum dilution to determine antibody titer using a four-parameter logistic equation (SoftMaxPro, Molecular Devices). The coefficient of variation (CV) per sample is ≤ 15%. Two negative sera and two HIV+ control sera are included in each assay to ensure specificity and for maintaining consistency and reproducibility of between assays. The integrity of raw data acquisition, data analyses are electronically tracked (21CFR part11 compliant). Supporting online material Table 3 shows the lower limit of detection of each assay employed in this study and the coefficient of variation of the assay.

Direct ELISAs

Direct ELISAs were performed using consensus clade B or envelope glycoproteins, gp41 proteins, consensus V3 peptides, gp41 immunodominant, and MPER epitopes, as well as autologous V3 and gp140 Env oligomers. ELISAs were conducted in 96 well ELISA plates (Costar #3369) coated with 0.2 μg/well antigen in 0.1M sodium bicarbonate and blocked with assay diluent (PBS containing 4% (w/v) whey protein/ 15% Normal Goat Serum/ 0.5% Tween20/ 0.05% Sodium Azide). Sera were incubated for 1 hour in two fold serial dilutions beginning at 1:25 followed by washing with PBS/0.1% Tween-20. 100 μl Alkaline phosphatase conjugated goat anti-human secondary antibody (Sigma A9544) was incubated at 1:4000 for 1 hour, washed and detected with 100μl substrate (CBC buffer + 2mM $MgCl_2$ +1mg/ml p-npp [4-Nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol) salt]). Plates were read at 405nm, 45 minutes.

Competitive inhibition studies (Antibody blocking assays)

Competitive inhibition studies (Antibody blocking assays) were performed with 1b12 (CD4BS), 2G12 (anti-CHO), and the MPER mAbs, 2F5 and 3H11. 96 well ELISA plates (Costar #3369) coated with 0.2 μg/well JRFL in 0.1M sodium bicarbonate and blocked with assay diluent (PBS containing 4% (w/v) whey protein/ 15% Normal Goat Serum/ 0.5% Tween20/ 0.05% Sodium Azide). All assay steps were conducted in assay diluent (except

substrate step) and incubated for 1 hour at room temp (13H11 assay at 37°) followed by washing with PBS/0.1% Tween-20. Sera were diluted 1:50 and incubated in triplicate wells. 50 µl biotinylated target Mab was added at the EC_{50} (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 V5591) followed by substrate (CBC buffer + 2mM $MgCl_2$ +1mg/ml p-npp [4-Nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol) salt]). Plates were read with a plate reader at 405 nm, 45 minutes. Triplicate wells were background subtracted and averaged. Percent inhibition was calculated as follows: 100-(sera triplicate mean/no inhibition control mean)*100. CD4 binding site blocking assays were conducted as above, except that 100 µl of a saturating concentration soluble CD4 (Progenics Pharm Inc.) was incubated between serum and biotin-Mab incubation steps

AtheNA Assay

Antibody binding to proteins 160, 120, 66, 55, 41, 31, 24 and 17 was measured on the Luminex platform (Luminex Corporation) using the AtheNA Multilyte HIV-1 Bead Blot kit (Zeus Scientific cat # A71001G) following the kit manufacturer's protocol.

Cardiolipin and Rheumatoid Factor Assays

Anti-cardiolipin antibody assays were performed as described [38]. Assays to measure IgM rheumatoid factor using IgG antigen were standardized using rheumatoid factor controls (kindly provided by Judy Fleming, Clinical Immunology Laboratory, Duke University Medical Center).

Isotype Binding antibodies

HIV antigens or purified IgM, IgG, IgA proteins (used as controls) were pre-coated overnight onto the wells of microtiter plates (NUNC), washed with an automated and calibrated plate washer (Bio-Tek). The serum/plasma test samples were diluted and incubated with the antigens bound to the plate. The plates were then washed and the antigen-antibody complexes were incubated with isotype specific anti-human IgG , IgA, IgM conjugated to alkaline phosphatase. Optical density readings are measured using a VersaMax plate reader (Molecular Devices) and an average O.D. reading for each pair of replicates, with the background subtracted, was calculated. For each test sample, duplicate antigen-containing and non-antigen-containing wells of a microtiter plate were examined. A score (i.e., O.D. antigen – O.D. non-

antigen) is regarded as positive if it is greater than or equal to an optical density of 0.2 for gp140 antigens and 0.4 for gp41 based. As another level of validation, in the plasma donor samples, we compared the HIV gp41 specific IgM binding antibody test with that of the third generation EIA (Abbott Diagnostics, Abbott Park, IL, USA) and found equal sensitivity to the commercially available kit for the first detection of any antibody response.

Specimen Prep MultiTrap IgG Removal.

For detection of IgA and IgM antibodies, IgG was removed using Protein G columns. Briefly, plasma was centrifuged (10,000 x g) for 10 minutes, diluted 2-fold in dilution buffer, and filtered in a 1.2 um filter plate (Pall AcroPrep). The filtered and diluted samples were depleted of IgG using a Protein G HP MultiTrap Plates (GE, Inc.) according to manufacturer's instructions with minor modifications. IgG removal in the specimens was greater than 90% as assayed by HIV specific binding assays.

Customized Luminex Assay

$5X10^6$ carboxylated fluorescent beads (Luminex Corp, Austin, TX) were covalently coupled to 25 μg of one of the purified HIV antigens used in ELISA assays and incubated with patient samples at a 1:10 dilution. HIV-specific Ab isotypes were detected with goat anti-human IgA (Jackson Immunoresearch, West Grove, PA), mouse-anti human IgG (Southern Biotech, Birmingham, AL), or goat-anti human IgM (Southern Biotech, Birmingham, AL), each conjugated to phycoerythrin, at 4 μg/ml. Beads were then washed and acquired on a Bio-Plex instrument (Bio-Rad, Hercules, CA). Purified IgM, IgG, IgA proteins (Sigma) and a constant HIV + sera 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. A control for Rheumatoid Factor for IgM detection was internal IgG protein standard. The limit of detection (LOD) for the customized luminex was determined for the detection of HIV specific IgM, IgG and IgA antibodies (SOM Figure S1.) and was compared to four other binding assay methods (SOM Table 1S).

HIV-1 Immune complex capture assays

ELISA plates (NUNC) were coated overnight at 4 °C with anti-human IgM or IgG at a concentration of 1 μg/ml diluted in phosphate-buffered saline (PBS). All subsequent steps were

performed at room temperature. After incubation and washing, coated plates were blocked for 2 h with PBS supplemented with 5 % FBS, 5% milk, 0.05% Tween. After blocking and washing, 90 μl undiluted plasma was added to each well and incubated for 90 min, followed by 4 washes with PBS supplemented with 0.05% Tween. 200 μl AVL lysis buffer with carrier RNA was added and shaken for 15 minutes and viral RNA in the lysis was extract by QIAGEN viral mini kit. HIV-1 RNA from the virion-antibody complexes were measured by gag real-time RT-PCR (See SOM Methods). The detection of immune complexes by the ELISA capture assay was confirmed using Protein G column absorption (Protein G HP, Pierce, Inc) to deplete IgG- virion immune complexes. IgG absorption was performed according the manufacturer's instructions. 90μl plasma was added to the Protein G column. After mix and incubation of 10 minutes, the column was centrifuged 1 minute at 5000Xg. The presence of immune complexes was calculated by the percentage of viral RNA input divided by viral RNA flow through similar to the method by Baron *et al.* [46]. HIVIG (NIH, DAIDS Reagent) plus HIV-1 NL4-3 pseudotyped virus was the positive control for immune complex capture (81 ± 4%), and normal human serum (Sigma) or RPMI-1640 plus HIV-1 NL4-3 was the negative control. The cutoff of non HIV-1 specific capture (normal human serum plus NL43) was 16.2 ± 0.8%, the background of virus only control was 6.5 ± 4.6%.

Complement Binding Assays

Virus and diluted plasma samples (1:40) were incubated at 37° C in the presence of 10 % Normal Human serum (Sigma, St Louis, MO) as a source of complement or with 10 % heat inactivated NHS. MT-2 cells which express high levels of CR2 were then added and the Virus/cell suspensions were incubated for 2 hours. Unbound virions were removed by successive washes. Bound virions were disrupted by treatment with 0.5% triton X and the released P24 was measured by ELISA. To determine % binding, the P24 obtained was divided to the P24 of original virus after correcting for complement non specific binding (hi NHS).

Neutralizing Antibody Assays

Antibody mediated neutralization in the plasma donor cohort was measured as a function of reductions in luciferase reporter gene expression after a single round of infection in TZM-bl cells as described [26]. For assay of plasma for 2F- and 4E10-specific neutralizing antibodies,

HIV-2 pseudoviruses expressing HIV-1 2F5 or 4E10 epitopes were used as described previously [8].

Statistical Analyses and Methods to classify Simultaneous and Sequential Kinetics

Statistical analyses were conducted using methods including mixed effects models [58-60], nonparametric regression [61], binomial test, Kaplan-Meier curve and accelerated failure time (AFT) model [62]. For all four cohorts, smoothing spline based nonparametric regression [61] was performed to obtain estimates of the viral load and antibody curves. For the plasma donor cohort where the acute burst of viremia was recorded, we define an accurate time origin (T_0) to align different study panels for the joint analysis. For each patient, the T_0 was estimated as a model parameter in the nonlinear mixed effects model of the upswing viral loads, accounting for censoring at the assay limits of detection [59]. For each analyte (e.g. anti IgA/IgG/IgM gp41 antibody response), data recorded prior to T_0 were fit to a linear mixed effects model [58] to determine the background level for that analyte, where the upper 95% prediction limit of a future response [60] was used as a positivity threshold to define the last negative observation and the first positive observation. The statistical method of classifying simultaneous and sequential kinetics verified the results obtained from ELISA calculations based on the positivity critierion.

Kaplan-Meier estimate was used to describe the distribution of the initial elevation timing. A two-sided Binomial test of relative ranking in elevation timing between pairs of analytes was performed with a positive difference in timing as a success and the number of non-zero differences as the number of trials. Adjusted p-values (q-values) were computed to control for the false discovery rate (FDR) of multiple testing [63]. To assess association between the viral loads and antibody markers in both the plasma donors and CHAVI 001 cohorts, accelerated failure time (AFT) models [62] were used to correlate the expansion or decay of the viral loads and the time to initial elevation (subject to censoring), and linear mixed effects models [58] were used to correlate the downswing viral loads and antibody response magnitudes over time. Additionally, statistical correlation and linear regression analysis were performed to identify the plausible association between different inhibition assays in the Trinidad and CAPRISA cohorts.

Modeling

The target-cell limited model used to mathematically model the plasma donor VL data is

$$\begin{aligned} \frac{dT}{dt} &= \lambda - dT - kVT \\ \frac{dI}{dt} &= kVT - \delta I \\ \frac{dV}{dt} &= pI - cV \end{aligned} \qquad (1)$$

Cells that are susceptible to HIV infection are termed target cells, T. The model assumes that target cells are produced at a constant rate λ and die at rate d per cell. Upon interaction with HIV these cells become productively infected cells, I, with infection rate constant k. Infected cells die at per cell rate δ and produce viral particles (virions), V, at rate p per infected cell. Virions are assumed to be cleared at a constant rate c per virion.

To incorporate enhanced virion clearance due to opsonization, we replaced the equation for $V(t)$ in the model above with the equation

$$\frac{dV}{dt} = pI - c(1+\alpha Ig(t))V \qquad (2)$$

Here virion clearance is enhanced by a factor $(1+\alpha Ig(t))$, where $Ig(t)$ is either the measured concentration of anti-gp41 IgM, anti-gp41 IgG or the total of both immunoglobulin concentrations in plasma. If $\alpha = 0$ then there is no opsonization effect.

To model the effects of antibody in neutralizing virus, we reduced the infectivity constant k in Eq. (1) by the factor $(1+\beta\ Ig(t))$. Here if $\beta=0$ there is no antibody mediated viral neutralization. Lastly, to incorporate the possibility of antibody enhancing the rate of infected cell loss through antibody-dependent cellular cytoxicity or complemented mediated lysis, we increased δ by the factor $(1+\gamma\ Ig(t))$. If $\gamma=0$ there is no enhanced death.

At T_0, which we chose as time $t=0$,, the plasma VL by definition is 100 copies/ml. While some CD4+ depletion could have occurred by T_0 for simplicity we assume the uninfected cell level is still 10^6 cells/ml. We estimated the number of infected cells at T_0 as either 1 or 10 cells/ml based on preliminary fits. This low number of infected cells supports our assumption of little T cell depletion by T_0.

The target-cell limited model as well as the three variants of it that included antibody effects were fit to VL data of each plasma donor using a spline fit to the measured anti-gp41 concentrations for Ig(t). Fitting to the VL data was done using a nonlinear least-squares method

where log V from the model was fit to the log of the measured VL. An F-test was used to determine whether the target-cell limited model or one of the three variants fit the data best. For donor 9032 the best-fit target-cell limited model gave an extremely poor fit to the data unless we added to the sum of squared residuals a penalty function for not attaining a maximum at the time the VL was maximum. That is, we add an additional term to the function to be minimized equal to the square of the difference between the time the VL was maximum in the data and in the model.

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Figure Legends

Fig.1. (A) Viral load kinetics of 21 HIV+ seroconversion plasma donor panels (eclipse phase clade B infection) were determined. The alignment of the subjects was by T_0 , the first day that VL reached 100 copies/ml. (B) The distribution of samples relative to time T_0 (Plasma donor cohort T_0= first day the viral RNA copies/ml = 100).

Fig. 2. (A) Kaplan Meier plot of anti-gp41 and anti-gp120 antibody responses in the Eclipse Phase Clade B plasma donor cohort. The solid line shows the increasing percentage of the population that develops HIV specific antibody responses each time interval following the calculated T_0. The dashed lines indicate the upper and lower point-wise confidence intervals respectively. (B) Pairwise comparison of the timing of anti-Env antibody responses compared anti-Gag (p24, p17, p55) and anti-Pol (p31) responses in the Eclipse Phase Clade B plasma donor cohort. The solid line (from left to right) indicates the median day of antibody elevation from T_0 and the gaps in the line indicate the HIV specific antibody responses that group together relative to their time of elevation from T_0. The median time for appearance of IgG anti-gp41 antibody was 13.5 days (panel A), while the median time for appearance of IgG gp120 antibody was 28 days (panel B).

Fig. 3. Anti-gp41 IgM antibodies are the first detectable HIV antibodies and autologous gp140 transmitted Env or consensus Env gp140 proteins are equally sensitive for the detection of the first antibody isotypes in HIV infection. ((A) IgM antibodies (B) IgG antibodies (C) IgA antibodies were detected using either consensus gp140 (ConB) or autologous Env (6246 Env). The asterisk indicates the plasma sample from which the autologous gp140 Env was derived. Consensus gp160 oligomer detects anti-gp41 antibodies at the same time as autologous gp140 Env oligomers.

Fig. 4. Kinetics of anti-gp41 specific antibody isotypes in acute HIV infection. Representative examples of (A) sequential development and (B) simultaneous development of early HIV specific antibody responses are shown. (C) The percentage of patients in each of the three cohorts that displayed different kinetic patterns. (D) Simultaneous Development of Gag Specific Antibody Responses. Anti-p55 antibodies of the IgM, IgG and IgA isotypes were measured for all subjects in the Eclipse Phase Clade B Cohort. Pt. 12007 could not be aligned to T_0 due to the large interval between the first RNA positive sample and the last RNA negative sample.

However, the short interval between antibody positive and antibody negative enabled measurement of antibody isotype kinetics, so the panel was aligned to T_0 as the first RNA positive sample.

Fig. 5. HIV immune complexes produced at a median time of 8.0 days post T_0. (A) The detection of immune complexes for Pt. 12008 and Pt. 9076 are shown in comparison to their viral RNA measurements and aligned to T_0 and (B) in comparison to the detection of free antibody responses.

Fig. 6. Ontogeny of complement binding antibodies during acute HIV-1 infection in times post T_0. Two representative patients from the eclipse phase cohort (6240, 6246) that had detectable HIV specific antibodies were assessed for complement activation with an early virus isolate, HIV QH0692, and a lab adapted isolate, HIV SF162.

Fig. 7. (A) No hypergammaglobulinemia observed within the first 40 days of acute infection. Total antibody levels were measured at the first HIV(-) sample and the last sample in the panel (HIV+). The median concentration across panels is indicated. (B.) Detection of rheumatoid factor during HIV acute phase viremia. IgM rheumatoid factor was measuring using standard ELISA detection with positive rheumatoid factor controls. An O.D. greater than 0.2 with background subtracted is positive. These patterns are representative of 4/19 IgM antibody positive Plasma Donor Panels and 7/10 CHAVI 001 subjects.

Fig. 8. Modeling the effect of antibody on plasma viremia in AHI with the target cell limited model. The target cell limited model (black line) is the best-fitting model for the plasma donors studied except 9032. For 9032, a model with virion clearance enhanced by the sum of anti-gp41 IgM and IgG (blue line) provides the best fit (red line).

Table 1. Ontogeny: Env Specific IgG in Eclipse Phase Clade B Cohort

Antigen	N [a]	Median Time [b]	0.95 LCL	0.95 UCL	Range
gp41	19	**13**	12	14	(9-18)
gp140	19	**13**	12	15	(6-17)
ID [c] gp41 peptide	13	**18**	16	- [d]	(13-34)
gp120	7	**28**	26	-	(13-41)
V3	7	**34**	19	-	(13-36)
MPER, CD4BS, CD4i	19	**>41**	-	-	-

[a] N = 19 plasma donor subjects that were not censored (out of 21 total subjects studied).

[b] Median time from T0, first day viral load reaches 100 copies/ml.

[c] ID= immunodominant

[d] Upper confidence limit is infinite (Elevations can occur greater than >41 days from T_0)

Subject	[a]CE IgG	CE, IgM	CE IgG+ IgM	[b]ID IgG	ID IgM	ID IgG+ IgM	[c]CDE IgG	CDE IgM	CDE IgG+ IgM	Best Fitting Model*
Pt. 6240	0.320	0.326	0.266	0.975	0.977	0.975	0.975	0.975	1.000	Target-cell limited
Pt. 6246	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	Target-cell limited
Pt. 9032	0.004	0.006	0.001	0.005	0.006	0.006	0.89	0.011	0.923	NAb enhanced or virion clearance
Pt. 9077	0.567	0.998	0.687	0.604	0.998	0.623	0.722	0.998	0.991	Target-cell limited
Pt. 9079	0.992	0.994	0.960	0.998	0.649	0.992	0.964	0.070	0.598	Target-cell limited
Pt. 12008	0.566	0.607	0.590	0.648	0.546	0.657	1.000	1.000	1.000	Target-cell limited

Table 2. Comparison of the ability of the target-cell limited model and variants including anti-gp41 antibodies to fit early plasma VL kinetic data. The viral load data from each donor was fit using the target cell-limited model and models that incorporated the effects of anti-gp41 IgG, IgM and IgG + IgM. [a]CE = Clearance Enhanced, [b]ID = Infectivity Diminished, [c]CDE = Cell Death Enhanced. The models with antibody effects include one additional parameter, which when set to zero reduces the model to the target-cell limited model. An F-test was used to determine if any of the models including antibody fit the VL data significantly better than the target-cell model. The Table gives the p-values computed from the F-test. A p-value <0.05 indicates a model including antibody fit better than the target-cell limited model.

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FIG. 1

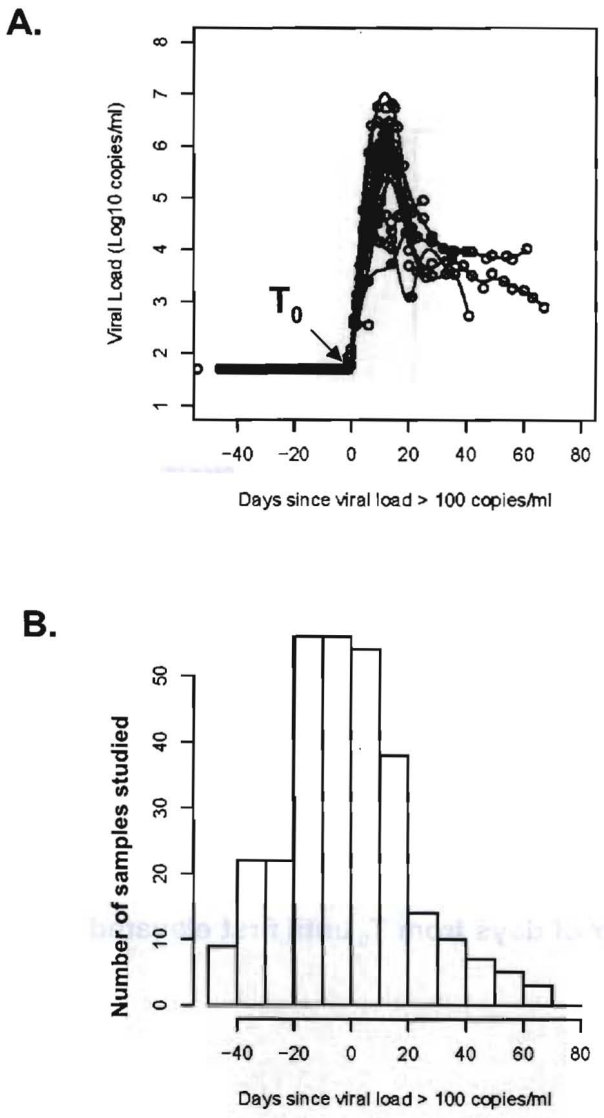

A.
Viral Load (Log10 copies/ml)
1
2
3
4
5
6
7
8
T_0
−40
−20
0
20
40
60
80
Days since viral load > 100 copies/ml
B.
Number of samples studied
0
10
20
30
40
50
−40
−20
0
20
40
60
80
Days since viral load > 100 copies/ml

FIG. 2 (A)

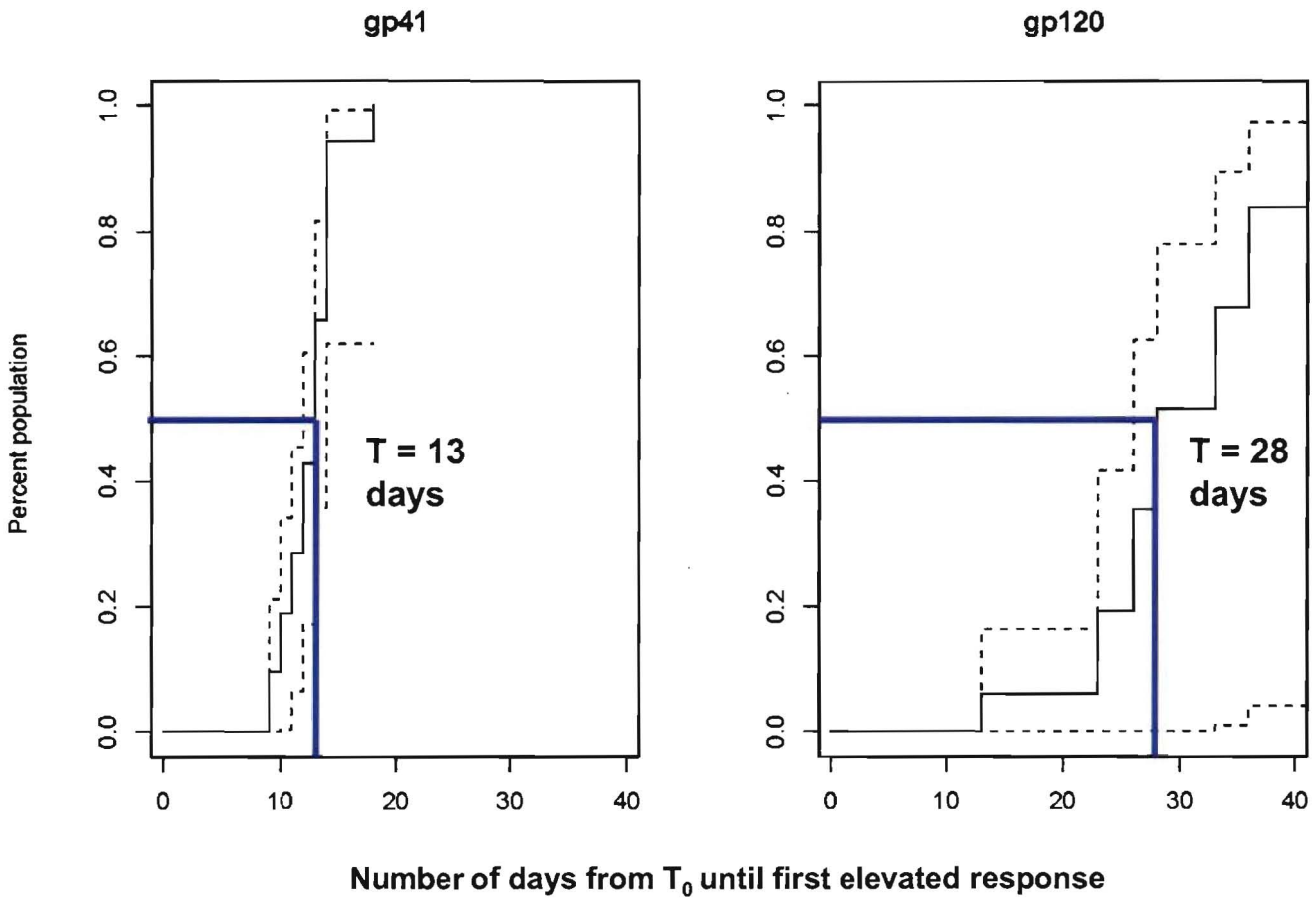
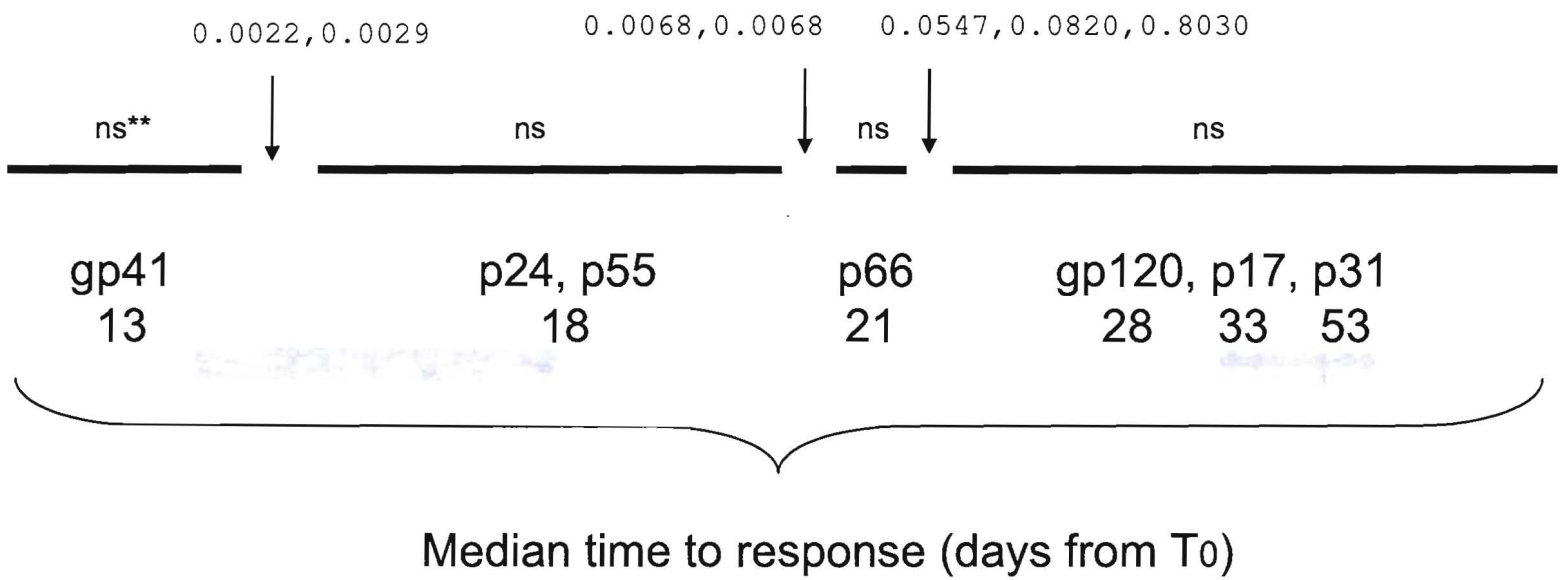

gp41
gp120
Percent population
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
T = 13 days
T = 28 days
Number of days from T_0 until first elevated response

FIG. 2 (B)



* List of q-values for pairwise tests for difference between adjacent groups of Ab specificities
** No statistically significant pairwise difference within group of Ab specificity

FIG. 3

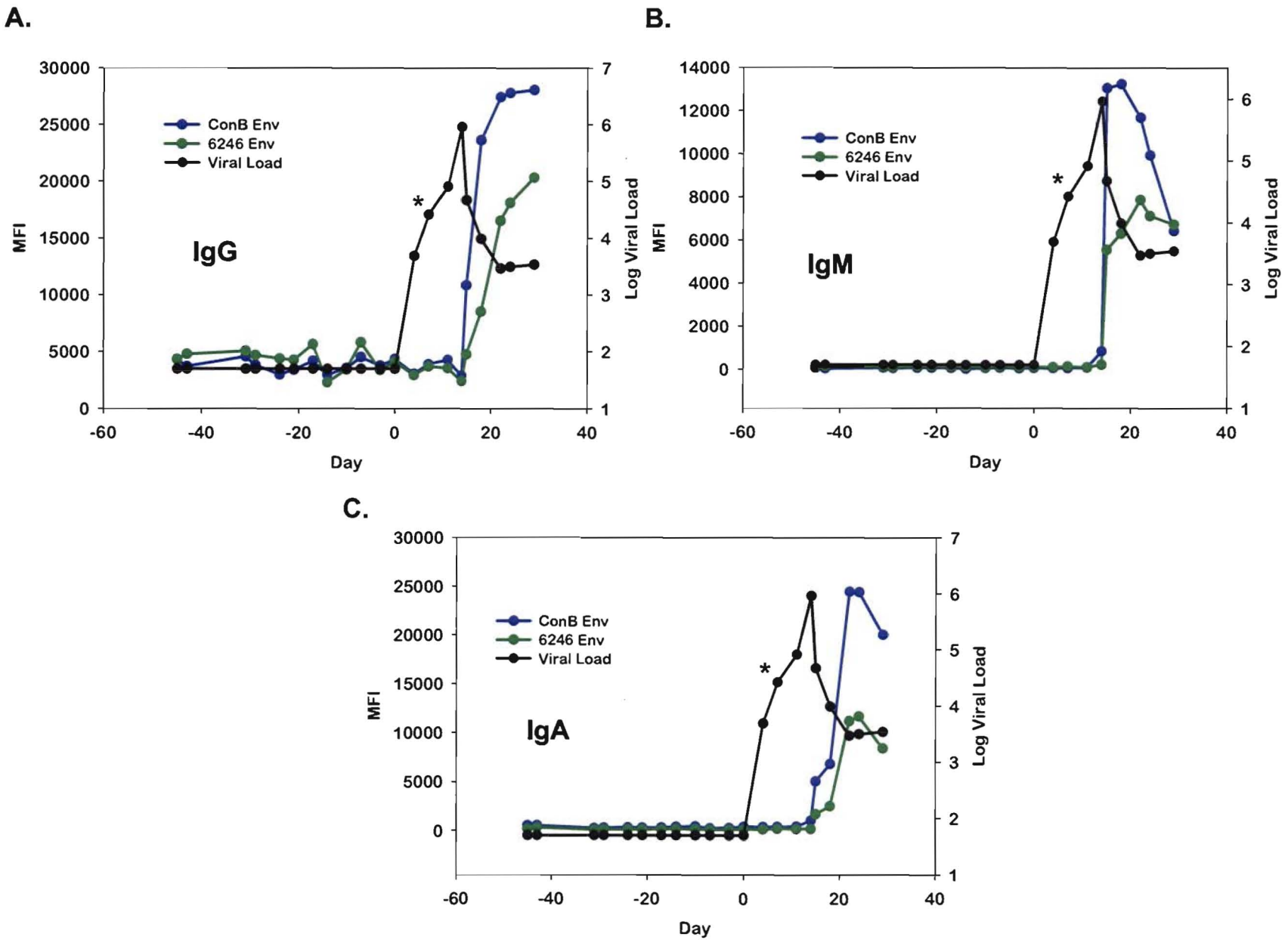
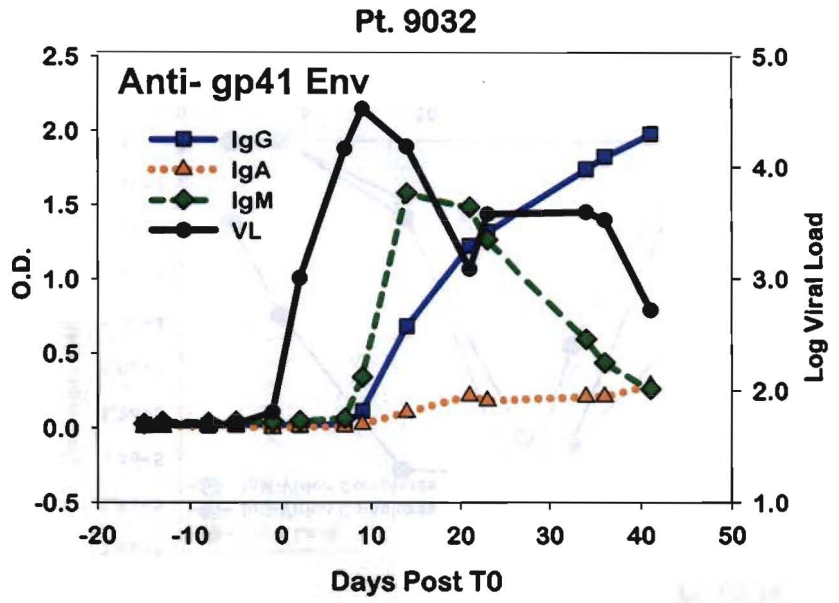
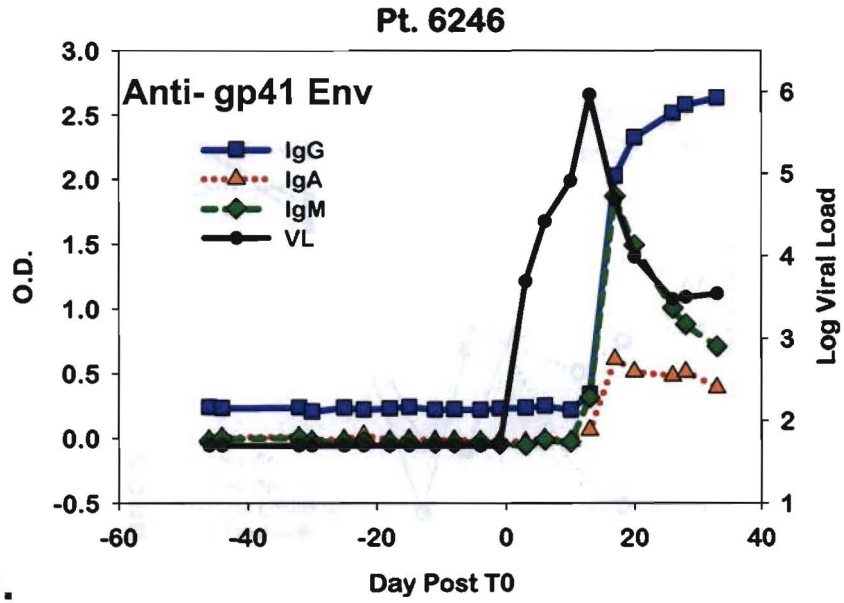

A.
B.
C.
ConB Env
6246 Env
Viral Load
IgG
IgM
IgA
MFI
Log Viral Load
Day
*
30000
25000
20000
15000
10000
5000
0
14000
12000
8000
6000
4000
2000
7
6
5
4
3
2
1
-60
-40
-20
0
20
40

FIG. 4

A.



B.



C.

Subjects[a]	Sequential Class Switch	Simultaneous Class Switch
Plasma Donor	5/15 (33%)	10/15 (67%)
CHAVI 001	3/4 (75%)	1/4 (25%)
Trinidad	1/3 (33%)	2/3 (67%)
Total	**9/22 (41%)**	**13/22 (59%)**

a Antibody Kinetics were determined only on the subjects whose first sample was IgG negative or low in that the ordering of IgM could be determined.

D.

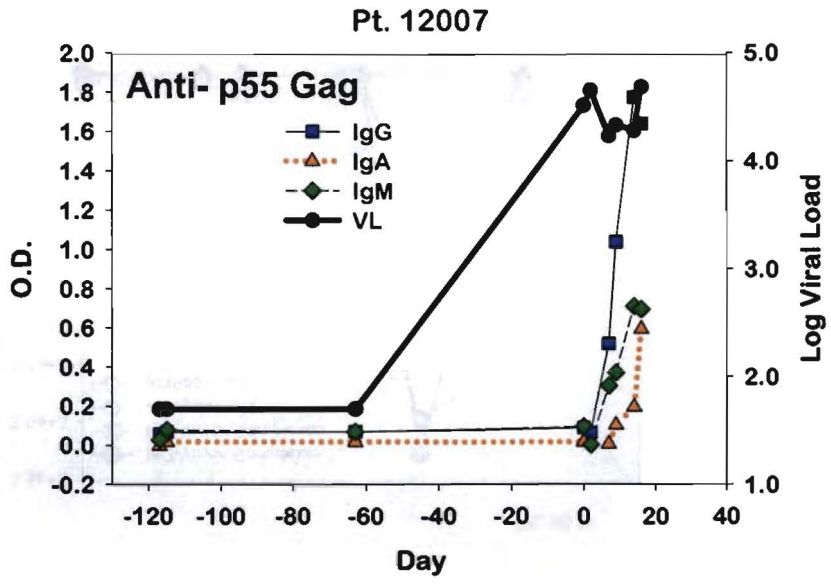


FIG. 5

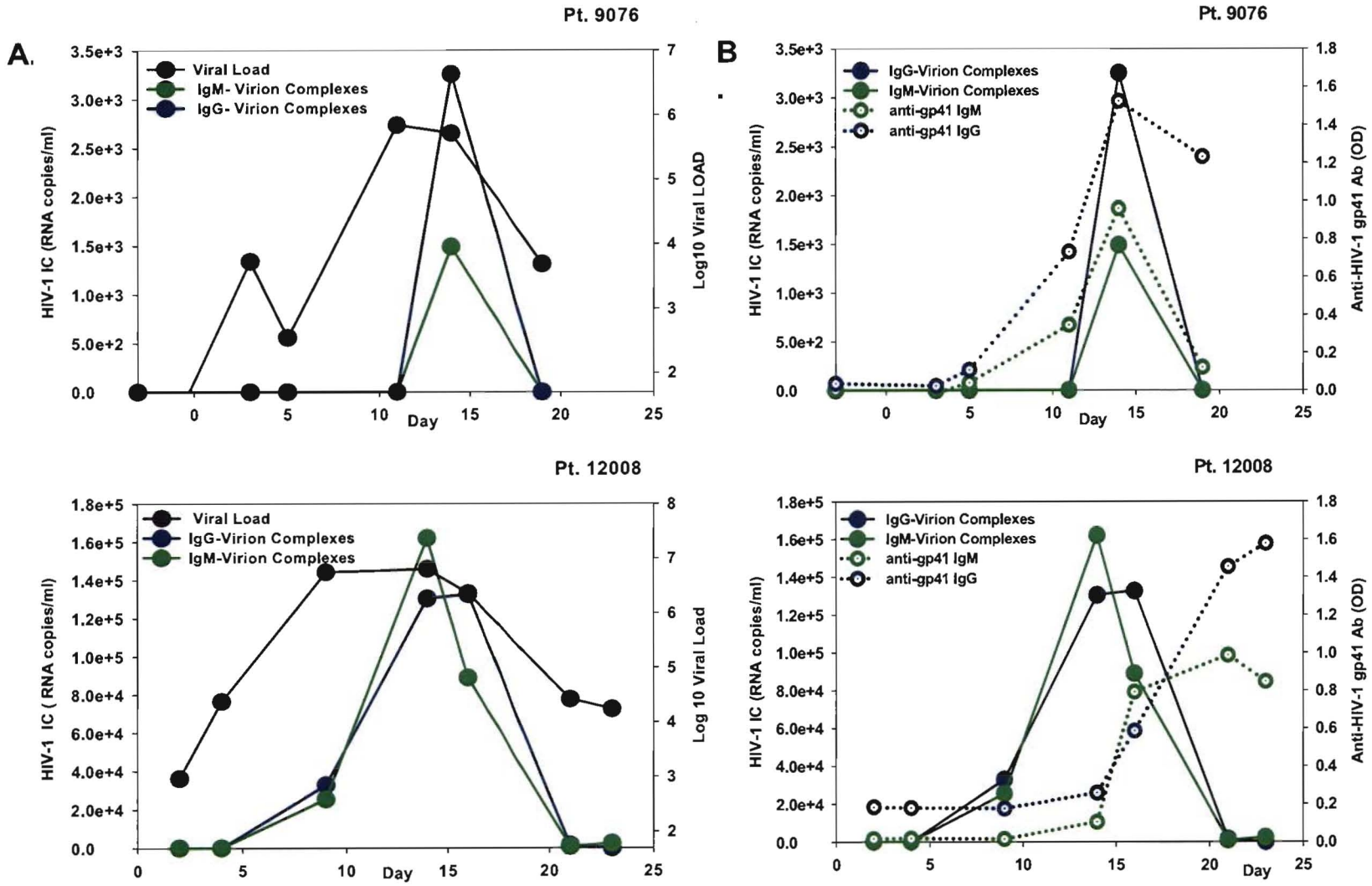

A.
Pt. 9076
Viral Load
IgM- Virion Complexes
IgG- Virion Complexes
HIV-1 IC (RNA copies/ml)
Log10 Viral LOAD
Day
B
Pt. 9076
IgG-Virion Complexes
IgM-Virion Complexes
anti-gp41 IgM
anti-gp41 IgG
HIV-1 IC (RNA copies/ml)
Anti-HIV-1 gp41 Ab (OD)
Day
Pt. 12008
Viral Load
IgG-Virion Complexes
IgM-Virion Complexes
HIV-1 IC (RNA copies/ml)
Log 10 Viral Load
Day
Pt. 12008
IgG-Virion Complexes
IgM-Virion Complexes
anti-gp41 IgM
anti-gp41 IgG
HIV-1 IC (RNA copies/ml)
Anti-HIV-1 gp41 Ab (OD)
Day

FIG. 6

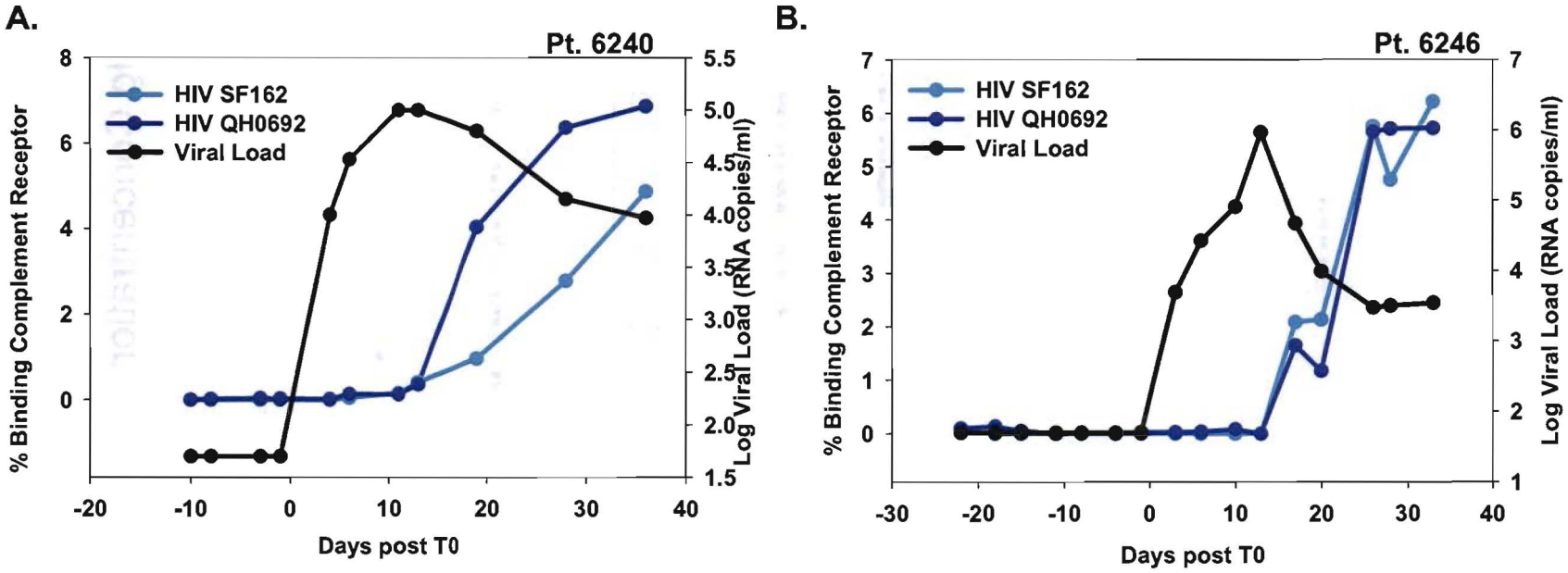

A.
Pt. 6240
HIV SF162
HIV QH0692
Viral Load
% Binding Complement Receptor
Log Viral Load (RNA copies/ml)
Days post T0
B.
Pt. 6246
HIV SF162
HIV QH0692
Viral Load
% Binding Complement Receptor
Log Viral Load (RNA copies/ml)
Days post T0

FIG. 7 (A)

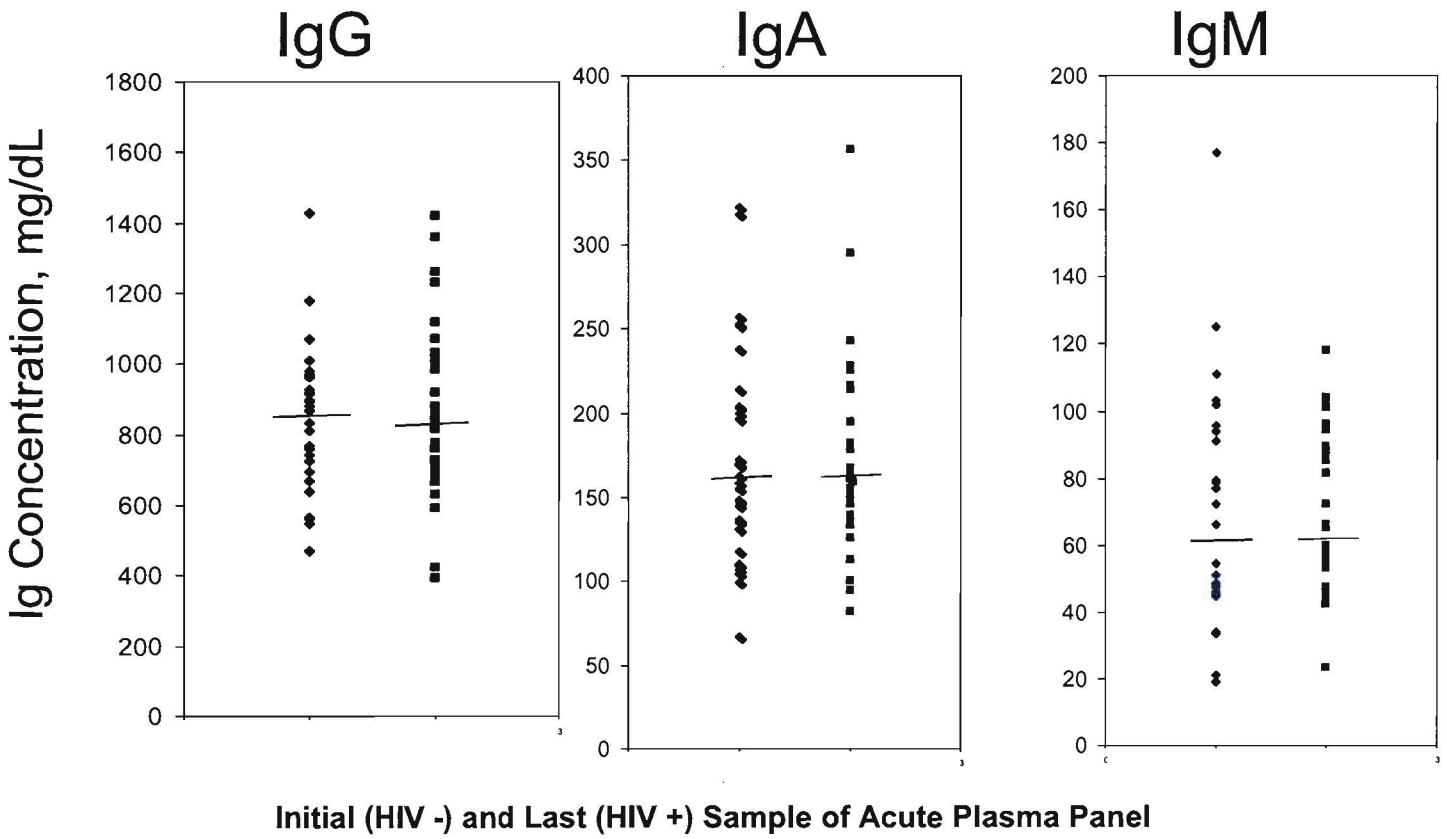

IgG
IgA
IgM
Ig Concentration, mg/dL
1800
1600
1400
1200
1000
800
600
400
200
0
400
350
300
250
200
150
100
50
0
200
180
160
140
120
100
80
60
40
20
0
Initial (HIV -) and Last (HIV +) Sample of Acute Plasma Panel

FIG. 7

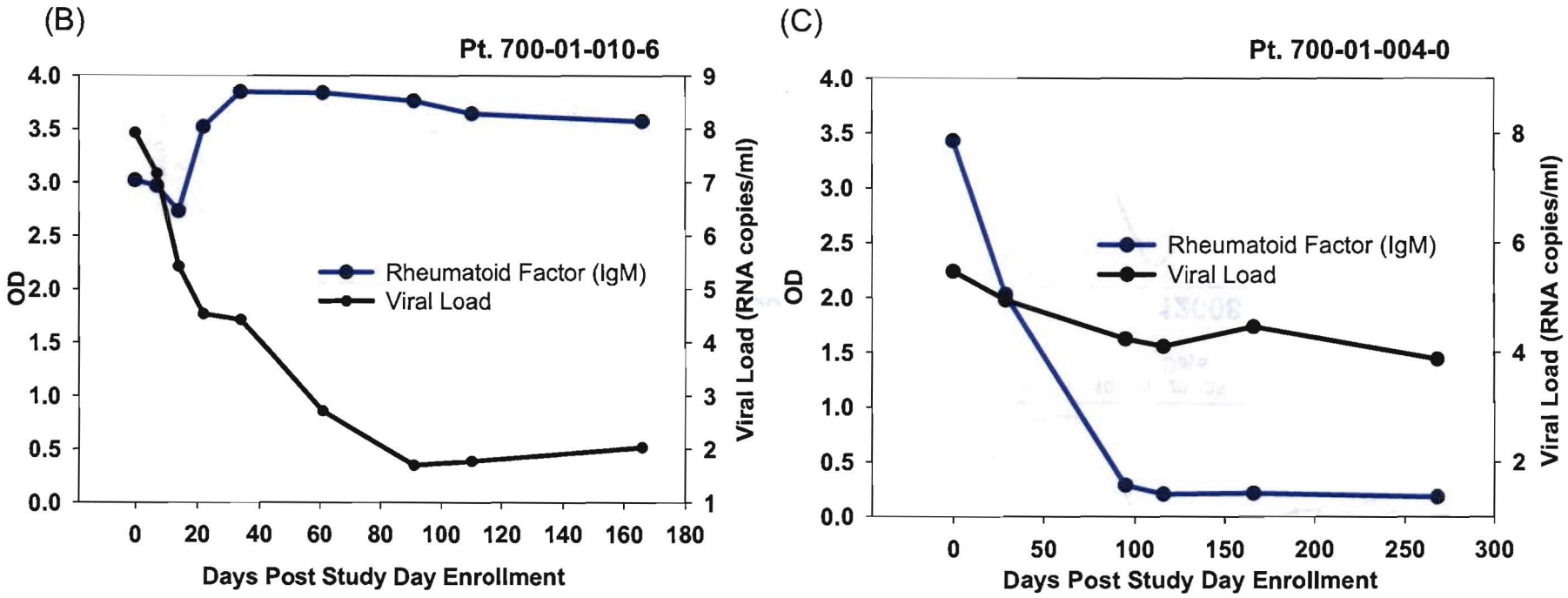
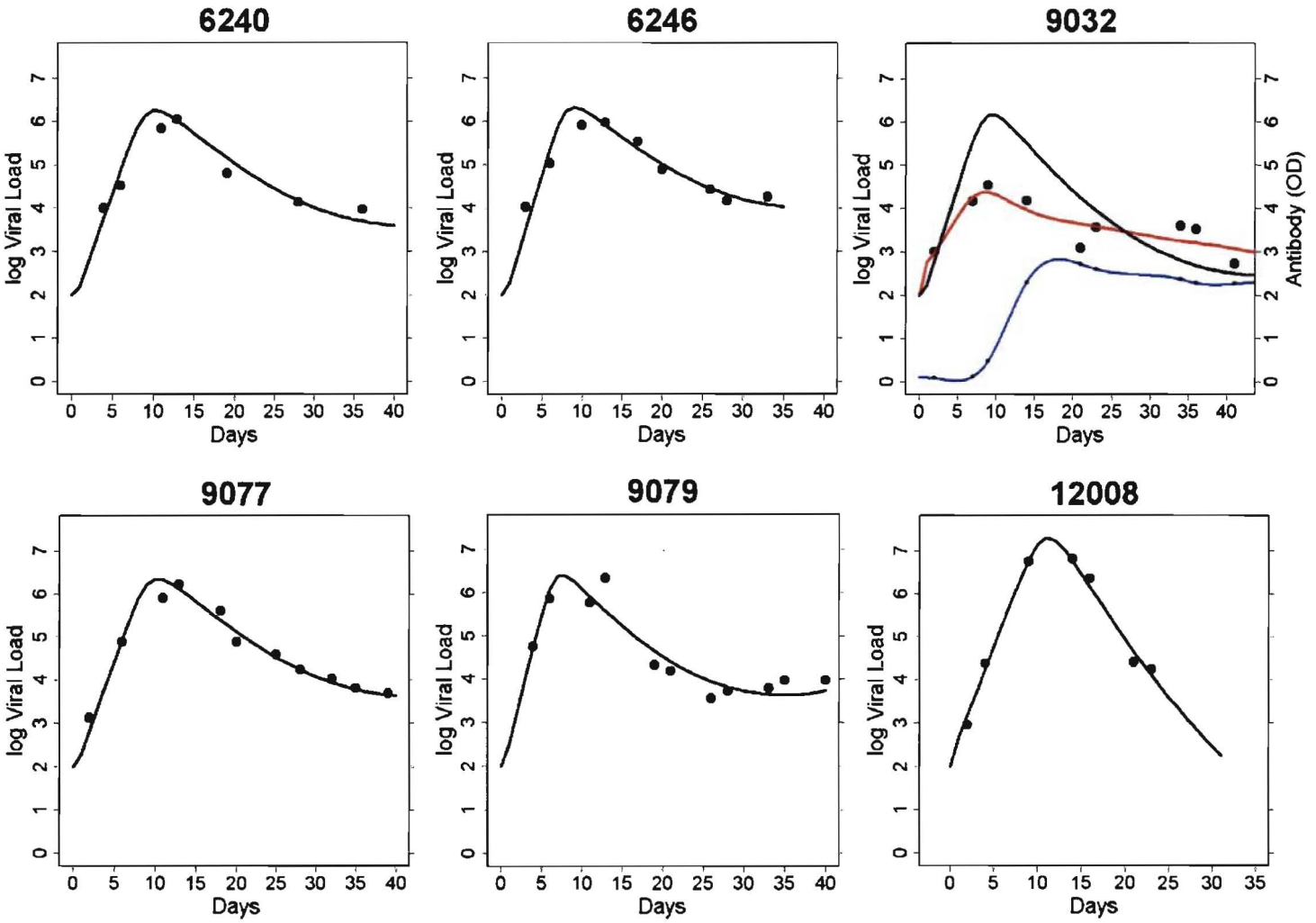

(B)
Pt. 700-01-010-6
OD
Viral Load (RNA copies/ml)
Rheumatoid Factor (IgM)
Viral Load
Days Post Study Day Enrollment
(C)
Pt. 700-01-004-0
OD
Viral Load (RNA copies/ml)
Rheumatoid Factor (IgM)
Viral Load
Days Post Study Day Enrollment

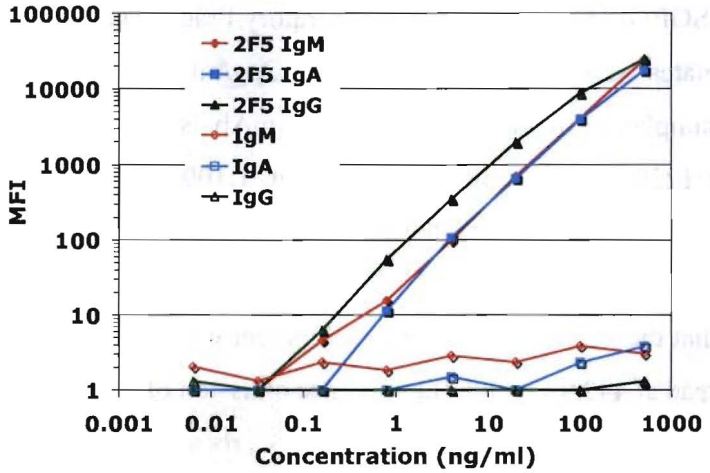
FIG. 8


6240
6246
9032
9077
9079
12008
log Viral Load
Antibody (OD)
Days

Supporting Online Materials

Binding Antibody Limit of Detection (LOD)

For the following 5 assays, HIV 2F5 mAb (IgG, IgA, and IgM) (Polymun, Inc. Vienna, Austria) were assayed throughout the range of 1000 ng/ml to 0.001 ng/ml (shown in Figure 1). Purified Ig isotypes were used as isotype controls (IC). Table 1 shows the calculated values for the limit of detection. The summary of the sensitivity testing is that the assays utilized to measure the earliest antibody responses after HIV transmission can conservatively detect antibody responses to 0.2 ng/ml, which is well in the physiologically relevant concentration range.



SOM Figure S1. The detection limit of the customized HIV-1 multiplex assay was determined using 2F5 monoclonal antibodies with recombinant gp41 antigen coupled to microspheres. Mean fluorescence intensity (MFI) was measured with the Bioplex Luminex reader and background was determined using isotype controls (IgM, IgG, IgA).

Assay	2F5 IgG	2F5 IgA	2F5 IgM
Colorimetric ELISA	2.2	11.5	8.9
Fluorescent ELISA	0.1	2.4	12.7
Luminex	0.2*	0.8*	4.5
(SPR) Biacore#	3.5	24.8	9.6
MSD	0.3	1.2	2.5

Table S1. Limits of detection for binding Ab assays in ng/ml. LOD calculated by 3xAVG isotype controls.

As an additional measure of assay sensitivity, it is noted that a high level of binding antibodies to gp120 in the Luminex assay positively correlated with ability of plasma antibodies to inhibit sCD4 binding to gp120, suggesting either a threshold level of anti-gp120 antibodies was required for CD4 inhibition, or alternatively suggesting that as the

anti-gp120 response affinity matured, antibodies progressively developed that could inhibit CD4 binding.

Methods for Limit of Detection (LOD) determination

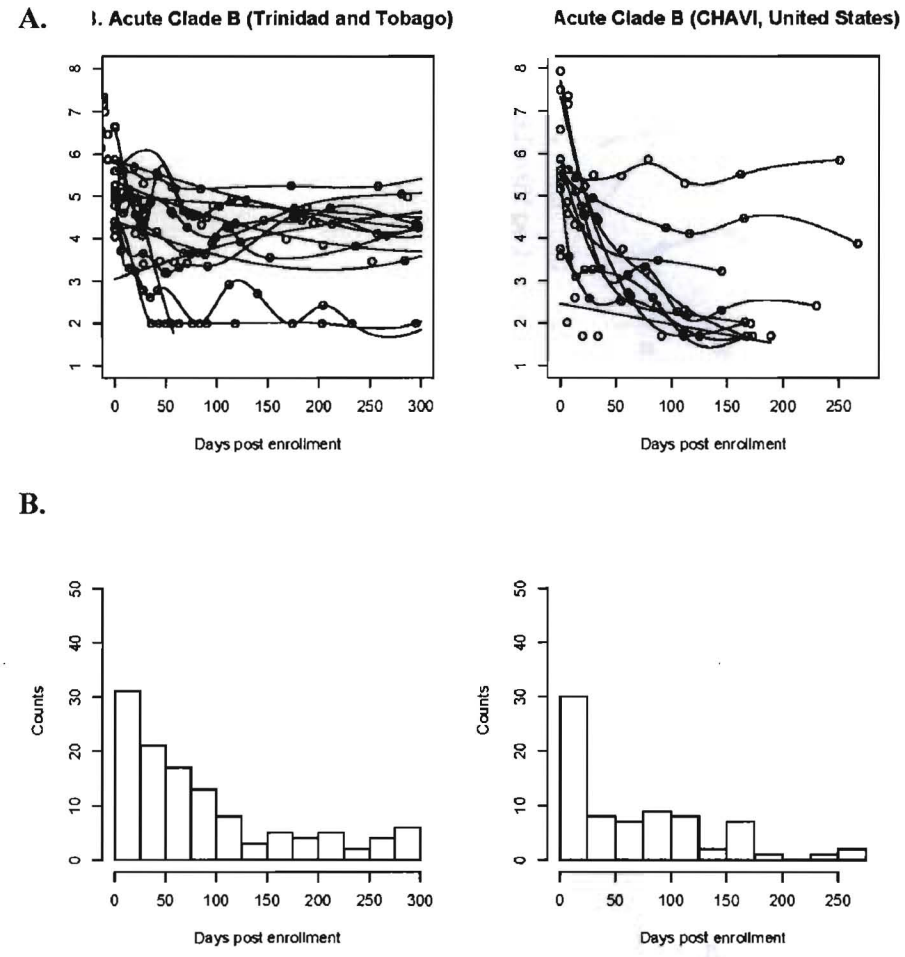
Colorimetric ELISA An optimized and validated ELISA Standard Operating Procedure (SOP) using Good Clinical Laboratory Practice (GCLP) Guidelines was used. Briefly, microtiter plates were coated overnight with 2μg/ml of HIV gp41. After washing, HIV 2F5 mAb-spiked samples or IC were added. 2F5 mAb isotypes were detected with goat anti-human IgA at 1:1250, mouse-anti human IgG at 1:1000, or goat-anti human IgM at 1:500. Plates were developed using a p-nitrophenyl phosphate substrate.

Fluorescence ELISA This assay was set up similarly to the colorimetric ELISA except that the substrate used for development was an Attophos substrate buffer (Promega). Plates were read at 440nm with a fluorescence emission of 550 nm.

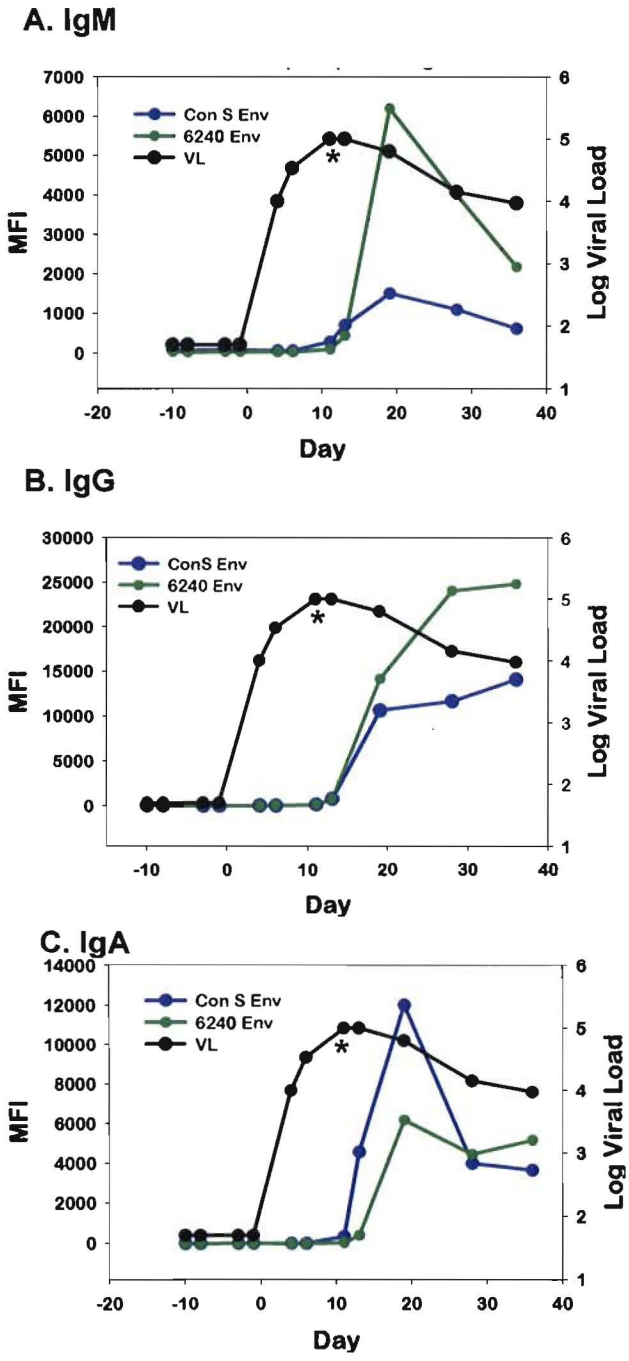
HIV Customized Luminex Carboxylated fluorescent beads were covalently coupled to HIV gp41 and incubated with HIV 2F5 mAb spiked samples or IC. 2F5 mAb isotypes were detected with goat anti-human IgA, mouse-anti human IgG, or goat-anti human IgM, each conjugated to phycoerythrin, at 4μg/ml. Beads were then washed and acquired on a Bio-Plex instrument. Background values (beads in the absence of detection Ab) were subtracted.

Electrochemiluminescence (MSD) The electrode surface of specialized microtiter plates (provided by Meso Scale Discovery (MSD) was coated with HIV gp41, washed, and incubated with HIV 2F5 mAb spiked samples or IC. 2F5 mAb isotypes were detected with SULFO-TAGTM-labeled Ab, goat anti-human IgA, mouse-anti human IgG, or goat-anti human IgM each at 1μg/ml. Plates were read in a Sector Imager 2400 (provided by Meso Scale Discovery).

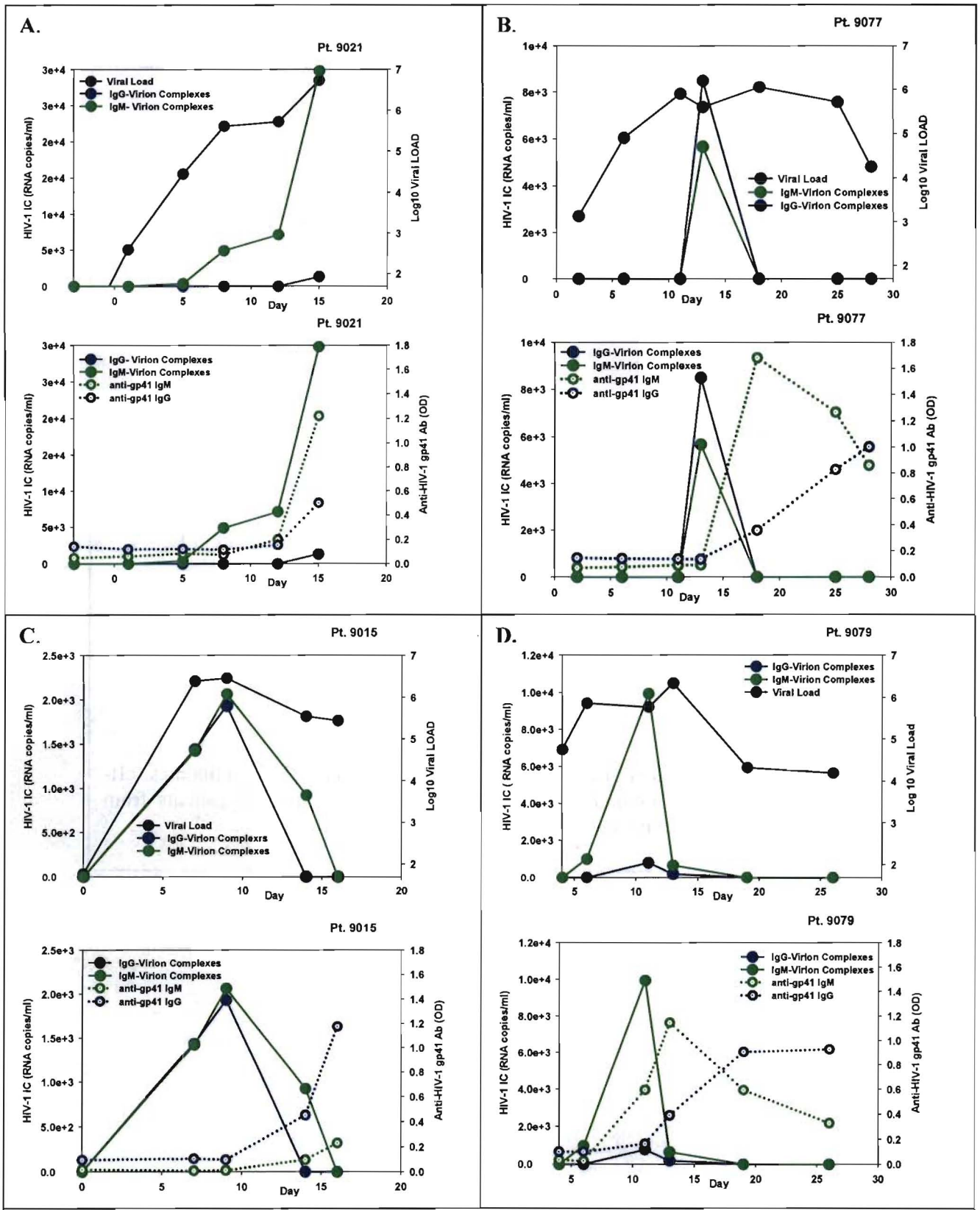
Biacore This system is specialized in the characterization of binding antibodies in terms of their specificity of interaction, binding kinetics, and binding affinity by visualizing the antigen/antibody interaction in real time [1].



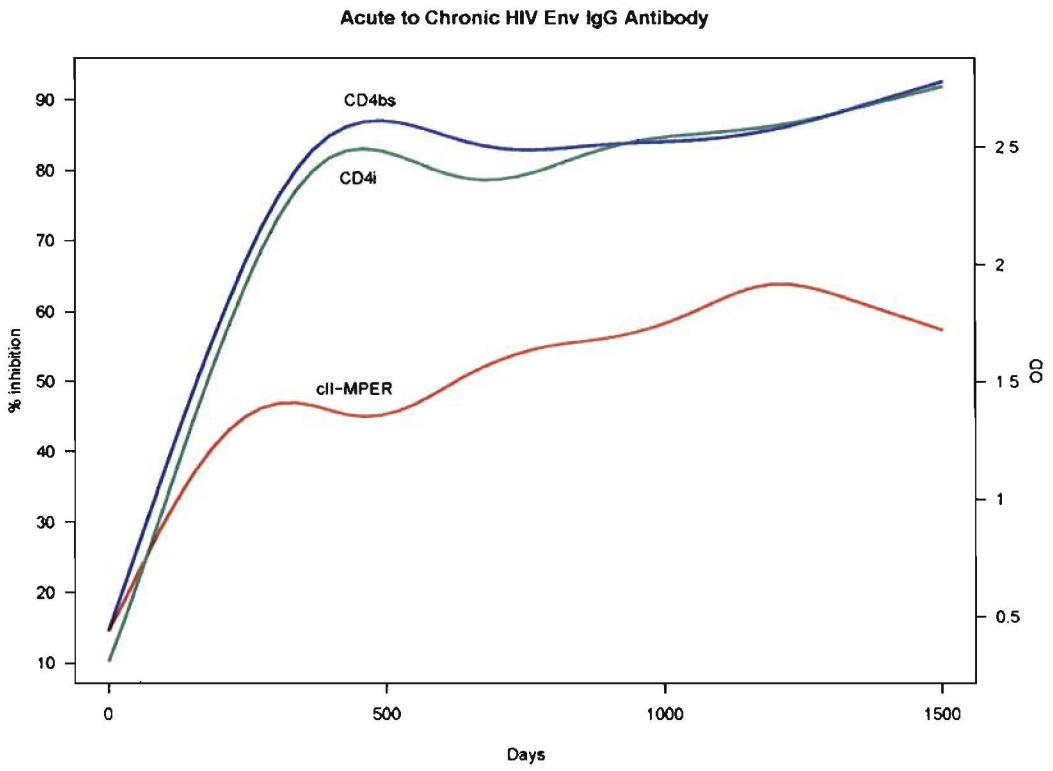
SOM Figure S2. (A) Viral load kinetics of 14 subjects from the Trinidad and Tobago Cohort and 10 CHAVI 001 Cohort that were utilized to characterize HIV-1 specific antibodies. The alignment of the subjects was by the first day of enrollment in the study. (B) The distribution of samples relative to first day of enrollment.



SOM Figure S3. Autologous gp140 transmitted Env from Subject 6240 or consensus Env gp140 proteins are equally sensitive for the detection of the first antibody isotypes in HIV infection. (A) IgM antibodies (B) IgG antibodies (C) IgA antibodies. The asterisk indicates the plasma sample from which the autologous gp140 Env was amplifed, sequenced and cloned for the expression of gp140 oligomers.



SOM Figure S4. HIV immune complexes in plasma donor subjects (A) 9021, (B) 9077, (C) 9015, and (D) 9079, appears at a median time of 8 days after the first detection of plasma viremia (T_0). The top panel of each subject shows the detection of immune complexes (IC) in comparison to viral RNA. The bottom panel shows the comparison of IC to the detection of free anti-gp41 antibody responses.



SOM Figure S5. Development of non-neutralizing cluster II anti-MPER antibodies (cII-MPER), CD4i and CD4bs antibodies in 14 CAPRISA and 12 Trinidad patients from enrollment in the acute infection study.

Development of MN Neutralizing Antibodies in select Subjects from the Trinidad Cohort

PtID	Time (Months)	Peak Titer IC50
SC03	10	812
SC05	10	813
SC11	3	1552
SC24	6	1907
SC25	13	361
SC41	9	364
SC42	2	8603
SC44	4	609
SC46	9	127
SC51	6	385

Table S2. Neutralizing Antibodies to MN Trinidad Cohort

Neutralization assays: cMAGI assay. Assays were peformed as previously described [2] with minor modifications. Briefly, serial dilutions of sera were incubated with virus for 1 hour and then added to duplicate wells of cMAGI cells. Infectivity was assessed after 2 days, cells by measuring ß-galactosidase expression in fixed and stained cells. Percent neutralization was calculated by [(Vo – Vn)/Vo] x 100, where Vn is virus infectivity in the presence of antibodies and Vo is virus infectivity in the absence of antibody. Virus HIV-1_{MN} was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

References:

1. Alam SM, McAdams M, Boren D, Rak M, Scearce RM, et al. (2007) The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes. J Immunol 178: 4424-4435.
2. Doria-Rose NA, Pierce CC, Hensel MT, Sutton WF, Sheikh N, et al. (2003) Multigene DNA prime-boost vaccines for SHIV89.6P. J Med Primatol 32: 218-228.

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