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van den Kerkhof, T.L.G.M.

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Chapter 5

The neutralizing antibody response in an individual with triple HIV-1 infection remains directed at the first infecting subtype

Marion Cornelissen*, Zelda Euler*, Tom L.G.M. van den Kerkhof, Marit J. van Gils, Brigitte D. Boeser-Nunnink, Neeltje A. Kootstra, Fokla Zorgdrager, Hanneke Schuitemaker, Jan M. Prins, Rogier W. Sanders, Antoinette C. Van der Kuyl

* Equal contribution

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Abstract

The effect of serial HIV-1 infection on the development of the broadly neutralizing antibody (bNAb) response was studied in an individual, H01-10366, with a serial HIV-1 superinfection (SI), hence triple infection, and compared with the bNAb response in 3 superinfected, as well as 11 mono-infected men who have sex with men (MSM) from Amsterdam, the Netherlands.

Neutralization assays measuring heterologous NAb titers on a panel of 6 representative viruses from different HIV-1 subtypes were performed on blood serum samples obtained approximately 3 years after primary HIV-1 infection (PHI), and longitudinally for H01-10366. A bNAb response was defined as having a geometric mean neutralization titer (the reciprocal serum dilution giving 50% inhibition of virus infection, ID\textsubscript{50}) $\geq$100, and neutralizing >50% of viruses in the panel with an ID\textsubscript{50} titer $\geq$100. H01-10366 quickly developed a potent NAb response against subtype B viruses before subtype B SI, but no broadening of the response occurred after the second subtype B infection or the third infection with CRF01_AE. When comparing H01-10366 with matched mono- (N=11) and superinfected (N=3) individuals analysed 3 years after PHI, we found that 5 of the 15 individuals (4/11 mono-infected, 1/4 SI) developed a bNAb response. However, there was no statistically discernible difference between the bNAb response and HIV-1 SI. Thus, HIV-1 SI was not associated with the bNAb response in these Dutch MSM with superinfection, including a triple HIV-1 infected individual, as there was no statistically significant difference in neutralization breadth or potency of the antibody response between mono- or serial HIV-1 infected individuals.
Introduction

The neutralizing antibody response (NAb) against the HIV-1 envelope glycoprotein (Env) has been the topic of many studies ever since it was appreciated that such a response could be protective against infection, and thus should be the focus of vaccine development (reviewed by ([1, 2]; see also [3]). As HIV-1 is a highly variable virus, comprising multiple clades, strains and recombinant forms, ideally the neutralizing antibody response should be cross-reactive to anticipate this variety. The natural development of such an effective broad neutralizing antibody (bNAb) response is a relatively slow process that besides time, requires adequate viral replication and ample env gene variation ([4-6], reviewed by ([7, 8])). It is estimated that around 15 - 30% of infected individuals develop such a bNAb response, generally 2-4 years after primary HIV-1 infection (reviewed in [9] and [8]), although examples exist in which bNAb responses developed quickly [10]. In most cases, multiple distinct NAb lineages were found that together constitute the breadth of the response [11-14]. However, a potent lineage may sometimes dominate the serum neutralizing activity. Molecular analysis of isolated potent monoclonal anti-Env bNAbs showed that they were all the result of long-term evolution and adaptation as exemplified by extensive somatic hypermutation and selection, explaining why the bNAb response takes a relatively long time to mature and often fails to develop in the first place. In mice engineered to express human immunoglobulin heavy chain genes, immunization with a series of related Env immunogens was used to recreate the maturation of the bNAb response [15]. The limited cross-clade breadth observed here, suggested again that a repeated stimulation of the immune system is in all likelihood a necessity for the generation of a potent bNAb response.

Although a HIV-1 superinfection (SI) actually represents a failure of the immune system to protect from a second infection, there has been much interest in HIV-1 SI in relation to the development of bNAb. These serial infections can be seen as a natural booster of the immune response, where the already-infected patient is challenged with a second virus strain carrying a divergent env gene. Indeed, broadening and strengthening of the NAb response was found in Kenyan and Cameroonian cohorts of heterosexually infected women after intra- or intersubtype SI [16-18]. In addition, a potent, high titer bNAb response peaking 3 years post-infection was described in a South African female who had experienced intrasubtype C SI 15 weeks after primary HIV infection (PHI) [19]. However, SI was not associated with the development of bNAbs in a Tanzanian mixed subtype cohort [20]. Also, two men with intra-subtype B dual infection from the San Diego Primary Infection Cohort that contains mainly, but not exclusively, white men who have sex with men (MSM) as participants, were reported not to differ from mono-infected controls in the NAb response [21]. Not only the influence of SI, but also the effects of gender and ethnicity upon the generation of NAb responses in HIV-1 infection has not yet been decisively resolved. In HIV-1 vaccine studies with healthy volunteers, it was reported that
the NAb response is significantly stronger in women compared to men [22] and in African Americans compared to whites [23]. However, the findings might be different in HIV-1 infected persons, as HIV-1 infection severely affects the humoral immunity (reviewed by [24]). Indeed, one study of mainly African-American HIV-1 infected individuals showed that men had higher bNAb activity compared to women [25]. In contrast, in another study breadth of neutralization was not associated with gender or ethnicity [26], while a third found that NAb responses were significantly associated with site of origin of the tested sample, whereby European and Australian samples more often showed lack of neutralization and also a decreased breadth compared with African samples [27]. To complicate matters even more, differences in neutralization breadth were also found between two distinct African female cohorts from South Africa and Tanzania [20]. It is thus possible that the effects of HIV-1 SI upon the neutralizing activity are dependent upon gender and/or geographic origin of the patients or on the HIV-1 subtype that also often has a relation with geography.

Although HIV-1 SI could be an adequate booster of the humoral immune system, it is likely that a single stimulation will not be sufficient in vaccine trials, and animal studies already suggest that repeated stimuli are needed [15]. Thus, individuals with serial SI could be more informative regarding the development of the NAb response. Although HIV-1 SI is not rare, and is estimated to occur at rates that can be similar to incidence rates of primary infection [28-30], very few cases of serial SI are known. In 2005, we described a patient, H01-10366, with serial SI resulting in a triple HIV-1 infection [31]. We reasoned that it might be valuable to investigate the development of the NAb response in this individual in relation to the occurrence of SI. Therefore, we investigated the effect of serial HIV-1 infections on the breadth and potency (as indicated by serum NAb titers) of the heterologous NAb response in patient H01-10366. In addition, we incorporated 11 HIV-1 mono-infected individuals and 3 superinfected individuals from the same cohort of Dutch MSM as matched controls in the study. All mono-infections were with subtype B strains, while the HIV-1 SI cases consisted of one case with an intrasubtype B SI and two with intersubtype SI. The triple infected individual had been serially infected with two subtype B strains, followed by an infection with CRF01_AE.

Materials and methods

Patient characteristics
The triple HIV-1 infected MSM H01-10366 (B/B/CRF01_AE) has been described before [31]. H01-10366 carries no protective HLA alleles [32], nor the delta-32 deletion variant of the CCR5 gene (result not shown). The first SI occurred around 22 months after the estimated month of PHI, the second around 31 months after this moment. CD19+ B cell counts were available for this individual from entry into care until month 37 after PHI. Three HIV-1
superinfected MSM with a PHI with subtype B and subsequent SI were identified in an earlier study [33]. Two had experienced intersubtype SI (SI2 B/CRF02_AG; SI3 B/CRF01_AE), while SI1 was superinfected with a second subtype B strain (B/B). SI occurred 6 to 12 months after PHI in these three individuals, and in all cases the initial and

<table>
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<th>HIV-1 subtype</th>
<th>Time to SI (months after SC)</th>
<th>Sampling time (months after SC)</th>
<th>pVL at baseline (cps/ml)</th>
<th>pVL at sampling (cps/ml)</th>
<th>CD4+ T cell count at baseline (10^6 cells/l)</th>
<th>CD4+ T cell count at sampling (10^6 cells/l)</th>
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<td>22/31</td>
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<td>1,66E+03</td>
<td>3,16E+04</td>
<td>480</td>
<td>270</td>
</tr>
</tbody>
</table>

Table 1. Patient characteristics of HIV-1 mono-infected (MI), superinfected (SI) and triple infected (H01-10366) individuals

^ NA= not available
^ The time point of 37 months was used in the group analysis, associated values are shown in bold
superinfecting virus strains persisted throughout the study period. NAb responses were measured at the latest time point after SI but before the initiation of antiretroviral therapy. Patient characteristics are summarized in Table 1.

A total of 11 HIV-1 subtype B mono-infected MSM with PHI were selected from the Amsterdam Cohort Studies on HIV infection and AIDS (www.amsterdamcohortstudies.org) based upon year of infection, time since seroconversion, CD4+ T cell counts and plasma viral load (pVL) to match the superinfected individuals. CD4+ T cell counts of the control individuals at baseline (median 535 cells/μl, range 160-700) or at sampling (median 360 cells/μl, range 180-700) were similar to SI cases (median 605 cells/μl, range 480-660 at baseline; median 285 cells/μl, range 270-610, p-value for comparison = 0.39 at baseline and p = 0.47 at sampling) as was the pVL at baseline (median 2.57E+05 copies/ml in control cases, range 2.51E+04-5.01E+06; median 9.77E+04 copies/ml in SI cases, range 1.66E+03-7.76E+05, p-value for comparison = 0.16) or at sampling (median 7.59E+04 copies/ml in control cases, range 1.29E+04-2.00E+05; median 5.13E+04 copies/ml in SI cases, range 1.07E+04-1.15E+05, p-value for comparison = 0.31).

Sequencing of approximately 300 nucleotide fragments encompassing the env V3-V4 region from the first and the follow-up sample used in this study of each control patient as described previously [34], revealed no HIV-1 SI had occurred during this period. The research protocol was approved by the medical ethical committee of the Academic Medical Center, all participants gave written informed consent.

All individuals were white, except one SI case with Asian ancestry. HIV-1 infections in the selected individuals occurred during 2001-2009, and all individuals were antiretroviral therapy naïve during the study period.

**Estimation of timing of the first HIV-1 infection in the triple infected patient**

The triple HIV-1 infected patient H01-10366 tested HIV-1 antibody positive in March 2001, was superinfected with a second subtype B strain in mid-2002 and with CRFO1_AE in mid-2003 [31]. To estimate the date of the first subtype B HIV-1 infection, approximately 630 nucleotide env-gene fragments were amplified, cloned and sequenced as described [35] from serial plasma samples obtained in 2001-2002 (antedating the first SI). Time to most recent common ancestor (tMRCA) analysis was done with BEAST v1.6.2 [36] using the GTR model with an invariant gamma-shape substitution model and empirical nucleotide frequencies under an uncorrelated relaxed clock and a coalescent tree prior assuming a constant population size as the pVL remained low and unchanged at around 2000 copies/ml. The Markov Chain Monte Carlo (MCMC) chain was ran for 100,000,000 replications; the first 2000 replications were discarded from the analysis (burn-in). From these data, it was estimated that PHI had occurred ±3 months before the first available sample (April 6, 2001), e.g. in or around January 2001. Using a non-Bayesian method implemented in Path-O-Gen (tree.bio.ed.ac.uk/software/pathogen/) [37], to estimate the “best fitting root”, the position that is most compatible when assuming a molecular clock
for trees that have been inferred without a molecular clock assumption, on the tip-dated maximum-likelihood tree obtained with MEGA5 (megasoftware.net) on the same sequence set, it was estimated that PHI occurred in or around November 2000, antedating the estimation with BEAST by approximately 2 months. For clarity, we will use the PHI date of January 2001 in this paper, but it is possible that H01-10366 was already HIV-infected shortly before that date.

**Statistical analysis**
Statistical analysis were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

**U87/Pseudovirus assay for testing of HIV-1 heterologous NAb in serum**
Data on bNAb in serum for MSM were either available from our previous studies [38] or newly generated. In short; sera were tested in duplicate for bNAb in a single round viral infection assay involving six tier two pseudoviruses as developed by Monogram Biosciences [27]. Previously we have shown that classification of bNAbs in patient serum samples as determined on an independent 23 viral panel or on the smaller six viral panel used in this study, was highly correlated [39]. Reactivity was measured at the latest time point after SI and before the initiation of antiretroviral therapy (median 36 months after PHI (range 28-44 months)) to exclude time of infection as factor for the development of a broad neutralizing response. For the triple infected patient, the time point estimated at 37 months post PHI was used in the group analysis. Longitudinal samples from this patient were also tested with this assay. The geometric mean ID₅₀ titer was calculated per patient across the 6 viral panel. Neutralization titers were considered positive if they were 3 times greater than the negative aMLV control. The lowest serum dilution used in the assay was 1:40. For calculation of ID₅₀ values for viruses that were not inhibited by the 1:40 serum dilution, we assumed that 50% inhibition would have occurred at a 1:20 serum dilution. A broad neutralizing response was defined as having a GM ID₅₀ titer ≥100 with neutralization of >50% of viruses in the panel, e.g. neutralizing at least 4 out of 6 viruses with an ID₅₀ titer ≥100 [27].

**Results**

**Longitudinal heterologous NAb responses in the triple infected patient**
To investigate the effect of serial SI on the development of the NAb response, the triple infected individual was followed longitudinally. Relatively high NAb titers against the subtype B viral strains in the panel (JRCSF and 92BR020) were detected throughout follow-up, consistent with clade B infection (Fig. 1A). Moderately high NAb titers (ID₅₀>500) against strain JRCSF were already present at an early time point, e.g. at approximately 3 months after PHI, and were increased (ID₅₀>1000) 6 months later. Consistent, low level A
neutralization of one of the subtype C viruses (93IN905) from the panel, next to subtype B neutralization, was seen throughout follow-up, corresponding with an earlier observation.

Figure 1. Broad neutralizing activity between individuals with mono or serial HIV-1 infection. (A) ID_{50} neutralizing titers across a six viral panel containing isolates belonging to subtypes A, B, and C, and CRF01_AE. NL43, a tier 1 virus served as a positive control, aMLV (Abelson murine leukaemia virus) as a negative control. Numbers indicate the reciprocal dilution of serum at the median inhibitory dilution (ID_{50}): red, >1,000; orange, 301–1000; yellow, 100-300; grey, 40-99; and white, <40. The duration of the HIV-1 (super-)infection (months after SC and/or SI where applicable) and the geometric mean of the ID_{50} titers are also presented. (B) Geometric mean (GM) ID_{50} titers across a 6-viral panel in HIV-1 infected MSM without SI (non-SI) or with SI (SI) including patient H01-10366 with two serial HIV-1 infections (black triangle). Control individuals and SI cases were tested for broad neutralizing activity (bNAb) at approximately 36 months after PHI. Prior to SI (tested 3-8 months after PHI), all GM ID_{50} titers were <40 in the three SI cases (result not shown). Horizontal bars indicate mean value per group, which was not significantly different between non-SI or SI groups (Mann-Whitney test, p > 0.05).

<table>
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<tr>
<th>No./subtype</th>
<th>Months after SC</th>
<th>Months after SI</th>
<th>94UG103B</th>
<th>92TH021</th>
<th>MGRM-C-026</th>
<th>93IN905</th>
<th>92R62D0</th>
<th>JRCSF</th>
<th>NL43</th>
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<td>87</td>
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Chapter 5
that 93IN905 is relatively easy to neutralize [27]. Surprisingly, after SI with recombinant CRF01_AE, a small, transient increase in the NAb response with a virus from the same subclass was observed (Fig. 1A, virus 92TH021). A similar weak response was seen against the reference subtype A strain in the panel, with a transient increase after the third infection, while the second subtype C virus was not neutralized at all.

So, after triple HIV-1 infection, no broadening of the NAb response was seen after the first SI with a virus from the same subtype, while intersubtype SI did lead to a minor increase in overall NAb titers. A strong neutralizing response against the first infecting subtype B is already present within one year after PHI, and this anti-subtype B response is stimulated but not significantly broadened after each SI, even after superinfection with a distinct subtype, but did lead to a small increase in NAb response to other subtypes.

**Longitudinal B-cell dynamics and NAb titers in the triple infected patient**

B-cells, the cell type producing antibodies, are known to be severely affected by HIV-1 infection (for a review see: [24]). Elevated levels of B-cell apoptosis, and thus decreased B-cell counts, are seen in all HIV-1 infected individuals [40, 41]. Higher rates of B-cell depletion in patients correlate with poor neutralizing activity against HIV-1 in both an European and an African cohort [40, 41]. Longitudinal CD19+ B-cell counts of patient H01-10366 were plotted in relation to the GM ID50 titers to investigate their behaviour in this specific individual (Fig. 2). GM ID50 titers increase after each SI, but finally decrease below 100 at 50 months. The CD19+ B cell counts show a delayed but sharp increase followed by a steep decline after each SI. B cell numbers first return to the normal range (between 100-500 cells/ml for healthy Dutch adults [42] after the first SI, but remain below normal after the second SI. For healthy white males from Germany, a neighbouring country, an age-related decline in CD19+ B cell counts has been reported, whereby the typical range for males <50 years of age would be 140-600 cells/ml blood [43]. Using such a modified age-dependent range would imply that patient H01-10366, who was well below 40 years of age at the study endpoint, has significantly decreased peripheral B-cell numbers at almost every time point. Irrespective of the reference range, however, it is clear that circulating CD19+ B cell numbers decline after each HIV-1 infection, while the specific anti-HIV response lags behind and is increased possibly through stimulation and differentiating of resting memory cells to antibody secreting plasma cells residing mostly in the bone marrow.
Heterologous NAb responses in Dutch MSM with and without SI

To compare the heterologous NAb response seen in patient H01-10366 with the development of the NAb response in a larger group of Dutch MSM, single time-point samples from 3 individuals with HIV-1 SI and 11 time-matched individuals with mono-HIV-1 infection from contemporary Dutch MSM infected with mainly subtype B viruses, were also tested for bNAbs across the six-virus panel. The 37-month follow-up sample from H01-10366 was used in the comparison (Fig.1A). There were no statistical differences in the number of viruses neutralized (Fig. 1A) or the GM ID_{50} titers (Fig. 1B) between the mono-infected and SI cases, including the triple infected case (Mann-Whitney test, p > 0.05). More specifically, 4 of the 11 (36%) mono-infected (MI-14, MI-15, MI-16 and MI-17) and 1 of 4 (25%) superinfected (SI-2) individuals, but not H01-10366, developed a bNAb response after 3 years. Further analysis showed that no single parameter such as age at infection, pVL at baseline, CD4+ T cell counts at baseline or sampling were significantly different between the 5 individuals that developed bNAb and the group that did not show such a response (result not shown). Only pVL at sampling was borderline significant (Student t-test, p < 0.049) between the groups, but the implication of this finding is unclear and a larger group should be analysed to confirm such an association. An earlier study in this MSM cohort indicated a significant association between CD4+ T-cell counts at baseline with the development of bNAb [27, 39], but this finding could not be
Neutralizing antibody response in superinfected MSM

corroborated in the present study, possibly because our patients were selected based upon pVL and CD4+ T-cell counts. Interestingly, an enhanced bNAb response was seen in the two individuals with an intersubtype SI compared with the patient with an intrasubtype SI (Fig. 1A). However, in the triple infected individual, an intersubtype SI only marginally and transiently increased the bNAb response.

Discussion

The NAb response against HIV-1 is highly variable, but most individuals appear capable of generating at least low to moderate NAb levels during chronic infection [44]. Time since PHI is an important parameter in predicting NAb levels, as the NAb response is slow to mature; viral replication and evolution appear to be essential during the process (see: [1, 7]). Boosting the humoral immune system once or serially with a divergent HIV-1, as has been proposed for future vaccination schemes, could likely increase and broaden the NAb response [15]. As HIV-1 SI constitutes a natural booster, and a second SI would likely enhance the response even more, we examined the breadth and potency of the NAb response in an HIV-1 infected individual with two subsequent HIV-1 superinfections and 3 HIV-1 infected MSM with a single SI and compared the response to 11 matched HIV-1-infected MSM in whom superinfection was not detected. As the time since infection is a major contributor to the development of the NAb response, we only examined samples from individuals that were either seroconverters or presented with PHI. For the triple infected patient, we estimated the time of PHI through sequence analysis and demonstrated that he was in all probability infected with the first virus shortly before entering the study.

When the triple infected patient H01-10366 was tested for heterologous neutralizing activity, a very fast and potent response against the subtype B virus JRCSF and a somewhat lesser response to another subtype B virus, 92BR020, was seen. Cross-neutralization of other subtypes in the test panel, e.g. subtypes A, C or CRF01_AE, was lower during the whole study than to subtype B strains, and did not increase after an intra-subtype B SI. However, there was a small but transient increase to cross-subtypes after the intrasubtype SI, implying that the neutralizing response was at best marginally and transiently broadened after stimulation of the immune system with a third, divergent virus. This subdued response is surprising because infection with CRF01_AE in 2003 was accompanied by a sharp increase in the pVL to $10^6$ copies/ml that could be attributed to the incoming virus [31] and CRF01_AE persisted and replicated to intermediate levels for almost 2 years [32]. The weak heterologous reaction to CRF01_AE could be due to the chronic immunodeficiency caused by more than thirty months of HIV-1 infection, with CD4+ T-cells being relatively low at 310 cells/μl at the moment of the second SI [31].
However, there was no general deficiency in humoral activity, as NAb titers to subtype B viruses remained high over time and were even increased after the second SI. Total B-cell counts in blood increased sharply after every SI, then decreased to or below threshold value. A reduction in peripheral total B-cells is more pronounced during PHI than during chronic HIV-1 infection [45], suggesting that SI is similar here to PHI in reducing B-cell numbers. The initial rise in peripheral blood B-cell numbers could likely be associated with a rise in plasmablasts that itself is related to the SI-induced HIV-1 viremia [46]. There is no obvious correlation between the occurrence of SI, peripheral B-cell counts and the concentration of bNAb in serum in patient H01-10366, although after each SI the GM ID_{50} values peak, albeit on a different timescale. Recently, not peripheral plasmablasts but HIV-1 Env-specific plasma cells in the bone marrow have been associated with HIV-specific circulating antibodies concentrations [46], which may explain why no clear relation between B-cell counts and bNAb titers is seen in H01-10366. An inverse relationship between declining CD19+ peripheral B cells and elevated mean NAb titers against the infecting subtype was observed in an African HIV-1 infected cohort [41], suggesting that low circulating B cell numbers do not preclude a neutralizing antibody response. Indeed, low B-cell numbers did not prohibit an NAb response in H01-10366. Here, the highest titers were always directed against the initial infecting subtype. There was no significant broadening of the NAb response according to our criteria, suggesting that ongoing stimulation of the originally induced B cell lineage(s) probably resulted in enhancement of the initial antibody response. An inability to induce new B cell lineages would be suggestive of ‘repertoire freeze’, clonal dominance after a restricted B cell response, a common occurrence in HIV-1 infection [47]. This concept, termed “original antigenic sin” was first coined in 1953 by Francis et al. [48], and has subsequently been postulated for many different pathogens, including HIV-1 [49]. Relatively high titer NAb against subtype B viruses were elicited by H01-10366 at the early time point of approximately 9 months post PHI, but this response did not protect from intrasubtype SI. It has been reported that the quality of the heterologous NAb response is not correlated with the occurrence of HIV-1 SI [50], although often an absent or narrow autologous or heterologous response is seen prior to SI [51-53]. When analysing all MSM in this study together, no significant differences in NAb breadth and potency associated with serial HIV-1 infection were observed, in contrast to a report on African women that also studied individuals with PHI before and after SI [16]. It is possible that there is a dissimilarity between men and women in the robustness of the NAb response, but there is also some other variation between the studies apart from gender, ethnicity, subtype and mode of infection. In the study by Cortez et al [16], the post-SI time point analysed was much later (approx. 5 years after PHI) than in our study (≤ 3 years after PHI). NAb responses have been shown to increase at least up to four years after PHI [6, 54], although we have reported early bNAb responses in individuals with elite neutralizing ability [10, 55]. Increased time of follow-up could possibly enable the
amplification and detection of initially subtle differences. On the other hand, the observation that there are no significant differences in bNAb levels even after 3 years of infection suggests that SI does not in general boost or quicken the broadly neutralizing response. Although the incidence of SI can be as high as that of PHI (reviewed in [56]), suggesting that host factors besides exposure are not important in acquiring SI, it has been noted that in SI cases the primary virus is often replicating at a low level [33]. Consequently, this virus might not induce an adequate cross-reactive NAb response thereby facilitating the SI with a different, better replicating strain. In line with our findings, two individuals with intra-subtype B dual infection from the San Diego Primary Infection Cohort that contains mainly, but not exclusively, white MSM as participants, were reported not to differ from mono-infected controls in the NAb response [21].

Besides increasing with declining B-cell counts, the NAb response has been shown to increase also with declining CD4+ T cell counts [6, 39]. As the CD4+ T cell count was similar here at baseline and at sampling in the single and SI infected individuals, the absence of an SI effect on the NAb response is probably not due to a difference in the number of CD4+ T cells. As CD4+ T-cell counts and pVL are correlated, pVL could also be an important factor inducing the NAb response. Indeed, in a recent study in two African HIV-1 infected female cohorts analysed two years after PHI, the strongest predictor of the development of a NAb response was found to be the initial pVL, and not dual infection among other factors, although the only elite neutralizer identified in this study was again a patient with HIV-1 SI [20]. However, in our study, both the baseline pVL and that at sampling also did not differ between the mono- and superinfected groups, and also did not differ retrospectively between those that did or did not develop an bNAb response. But of course the numbers analysed in this study are low. The pVLat sampling was marginally associated with the development of an bNAb response, in line with many earlier studies that find such an association [4-6, 26], however, low numbers preclude the detection of a robust association in the present study.

Another explanation for the failure of SI to stimulate the NAb response is that the SI virus is too divergent from the original infecting virus, and therefore not naturally guiding the B-cell response to a matured bNAb response. Viral evolution through escape from NAb pressure has been postulated to be the most important mechanism in shaping the development of the HIV-1 neutralizing breadth (reviewed in [8]). Therefore, in future vaccine schemes, it is probably essential to vaccinate serially with more “advanced” viral Env proteins, e.g. a protein with longer variable regions and increased numbers of potential asparagine (N)-linked glycosylation sites, to guide B-cell reactivity [1]. In SI cases, the second infecting virus is again a primary variant, albeit different in sequence, and it might not be able to trigger the right B-cell lineages. A recent analysis of the characteristics of the superinfecting virus in three HIV-1 subtype C infected women showed that SI was established by a “single CCR5-using founder virus with shorter, less glycosylated variable regions than matched chronic viruses” [57]. SI then indeed occurs
with a virus type similar to PHI, which might explain its perceived failure to broaden and stimulate bNAb responses.

In conclusion, HIV-1 SI does not significantly enhance the heterologous NAb response in a Dutch cohort of MSM that included an individual with triple HIV-1 infection.

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