MAJOR ARTICLE

Magnitude and Breadth of a Nonprotective Neutralizing Antibody Response in an Efficacy Trial of a Candidate HIV-1 gp120 Vaccine

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Background. A candidate vaccine consisting of human immunodeficiency virus type 1 (HIV-1) subunit gp120 protein was found previously to be nonprotective in an efficacy trial (Vax004) despite strong antibody responses against the vaccine antigens. Here we assessed the magnitude and breadth of neutralizing antibody responses in Vax004.

Methods. Neutralizing antibodies were measured against highly sensitive (tier 1) and moderately sensitive (tier 2) strains of HIV-1 subtype B in 2 independent assays. Vaccine recipients were stratified by sex, race, and high versus low behavioral risk of HIV-1 acquisition.

Results. Most vaccine recipients mounted potent neutralizing antibody responses against HIV-1_{MN} and other tier 1 viruses. Occasional weak neutralizing activity was detected against tier 2 viruses. The response against tier 1 and tier 2 viruses was significantly stronger in women than in men. Race and behavioral risk of HIV-1 acquisition had no significant effect on the response. Prior vaccination had little effect on the neutralizing antibody response that arose after infection.

Conclusions. Weak overall neutralizing antibody responses against tier 2 viruses is consistent with a lack of protection in this trial. The magnitude and breadth of neutralization reported here should be useful for identifying improved vaccines.

Efforts to develop an effective human immunodeficiency virus type 1 (HIV-1) vaccine have emphasized an ability to elicit virus-specific $CD8^+$ T cells and neutralizing antibodies (NAbs) [1–3]. Genetic variability has given rise to multiple genetic subtypes of HIV-1 that exhibit a wide spectrum of antigenic diversity within and between subtypes [4–8] and pose major obstacles for vaccine development. Most variability occurs in the surface gp120 and transmembrane gp41 envelope (Env) glycoproteins that mediate virus entry and serve as the sole targets for NAbs [9–13]. HIV-1 evades many NAbs by altering primary recognition sequences and by masking epitopes with N-linked glycans and other conformational and steric constraints that limit antibody access [10, 14, 15]. An ideal vaccine may need to overcome these evasion strategies and elicit NAbs against a wide range of circulating variants. Although it is not clear how to achieve this goal, evidence suggests that the virus has vulnerabilities, and that broadly cross-reactive NAb induction is indeed possible [16, 17].

Various Env-containing vaccine candidates have elic-

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ited NAbs in phase 1 and phase 2 human clinical trials [3]. The antibodies often neutralize T cell line adapted strains and other strains highly sensitive to neutralization, but they do not neutralize most circulating strains of HIV-1 [18–20]. T cell line adapted strains and a subset of circulating strains that exhibit a high level of neutralization susceptibility are classified as tier 1 viruses [21]. The tier 1 phenotype is associated with spontaneous epitope exposure in the sequence-variable cysteine-cysteine loops and in the conserved coreceptor binding domain of gp120 [22–24]. Most circulating strains have evolved under immune pressure to conceal these epitopes, resulting in an overall lower level of neutralization susceptibility that is classified as a tier 2 phenotype [21]. Whether a certain level of neutralizing activity against tier 1 and tier 2 viruses will predict protection against HIV-1 is not known; however, it is generally agreed that neutralization of tier 2 viruses should be a priority for vaccines [25–28].

Many previous evaluations of vaccine-elicited NAb responses against tier 2 viruses used poorly defined virologic reagents and substandard assay methodologies. New high throughput assay technologies are now available that use engineered cell lines and reporter genes for highly sensitive, quantitative, and reproducible results [14, 29]. These new assays have been optimized and validated and use well-characterized Env-pseudotyped viruses, including transmitted and/or founder viruses from sexually acquired infections that are thought to be important targets for vaccination [30–35].

Here we assessed the NAb response in the Vax004 efficacy trial of a candidate HIV-1 gp120 vaccine (AIDSVAX B/B; VaxGen) that was evaluated on the basis of eliciting NAbs [36, 37]. Strong antibody responses were detected by enzyme-linked immunosorbent assay and by neutralization of HIV-1_{MN} [38]; however, the vaccine did not prevent the acquisition of infection, nor did it impact viral loads in participants who acquired infection after vaccination [39, 40]. A similar bivalent gp120 vaccine (AIDSVAX B/E; VaxGen) [41] was ineffective in an efficacy trial in Bangkok intravenous drug users despite comparable antibody responses [42]. Lack of efficacy in both trials precluded an assessment of NAbs as a correlate of protection. However, a recent trial of a prime-boost regimen that included AIDSVAX B/E provided modest evidence for a reduced rate of HIV-1 infection [43], which in the future may afford such assessments. The Vax004 trial is the first opportunity to quantify the magnitude and breadth of a nonprotective NAb response in human efficacy trials of HIV-1 vaccines, providing a useful reference for future vaccine evaluations.

VOLUNTEERS, MATERIALS, AND METHODS

Clinical trial design. The Vax004 trial design was described elsewhere [38–40]. The vaccine consisted of 2 gp120 proteins derived from HIV-1 subtype B strains MN and GNE8. Vax004 and the present study were conducted in accordance with the Declaration of Helsinki and local institutional review board requirements. Written informed consent was obtained from all subjects.

Serologic specimens and stratification. Peripheral blood for plasma was collected in Vacutainer CPT tubes containing sodium citrate as anticoagulant (Becton-Dickinson). Peripheral blood for serum was collected without an anticoagulant. Plasma and serum samples were stored at −80°C, thawed, and heat-inactivated at 56°C for 1 h prior to assay. Vaccine recipients were stratified by sex, race, and high versus low risk of acquiring HIV-1 infection, selected randomly within each group. Low and higher risk groups were defined on the basis of a behavioral risk score variable constructed from baseline questionnaire data, which was used in the primary analyses of Vax004 [38, 39]. The low-risk group consists of participants with lowest risk score 0, and the higher risk group were those with risk score ⩾4. For the nonwhite and female strata, there were not enough available participants with risk score ⩾4, and in these cases the higher risk group includes some participants with risk scores 1–3.

Viruses. HIV-1 subtype B reference strains 6535.3, QH0692.42, SC422661.8, PVO.4, TRO.11, AC10.0.29, RHPA4259.7, THRO4156.18, REJO4541.67, TRJO4551.58, WITO4160.33, and CAAN5342.A2 closely approximate transmitted/founder viruses from sexually acquired infections [30]. Additional subtype B viruses from sexually acquired infections included WEAU-d15.410.787, BB1006–11.C3.1601, BB1054–07.TC4.1499, BB1056–10.TA11.1826, BB1012–11.TC21, 6240.08.TA.4622, 6244.13.B5.4576, and 62357.14.D3.4589, which are considered authentic transmitted/early founder viruses [34]. Tier 1 viruses included HIV-1_{MN}, SF162.LS, Bal.26, BZ167.12, Bx08.16, SS1196.1, MW965.26, and 92BR025.9. All tier 1 viruses are subtype B except MW965.26 and 92BR025.9, which are subtype C. HIV-1_{MN} was used as an uncloned stock. All other viruses were used as Env-pseudotyped viruses containing a single full-length gp160 clone of the designated strain.

Additional viruses were derived by random sampling from 13 vaccine recipients and 14 placebo recipients within 6 months of infection from Vax004 subjects who received at least 4 inoculations before infection. These viruses were used as cloned quasispecies of plasma-derived Env-pseudotyped viruses [29].

Neutralization assays. Neutralization was measured with blinded samples in 96-well culture plates by using firefly luciferase (Luc) reporter gene expression to quantify infection. One assay [30, 31] was performed in a HeLa cell line (TZM-bl, also known as JC53-BL) that expresses CD4, CCR5, and CXCR4 [44] and contains a Luc reporter gene [45]. Unless otherwise specified, plasma samples were assayed at 8 dilutions starting at 1:10. Nab titers were calculated as the sample dilution conferring a 50% reduction in relative luminescence

units (RLU) relative to virus control wells after subtraction of background RLU in cell control wells. An additional set of assays tested a 1:10 dilution of serum rather than plasma to avoid the mild toxicity of anticoagulant. Results in these latter assays were calculated as the percentage of reduction in RLU in wells containing postimmunization serum relative to the RLU in wells containing corresponding preimmune serum from the same subject. HIV-1_{MN} was prepared in H9 cells. Env-pseudotyped viruses were prepared by cotransfecting 293T/17 cells (American Type Culture Collection) with an Env-expressing plasmid plus an Env-defective backbone plasmid (pSG3Δenv) as described elsewhere [30, 31].

A second assay [29, 35] used an astroglioma cell line engineered to express viral fusion receptors (U87.CD4.CCR5.CXCR4). Plasma samples were assayed at 8 dilutions starting at 1:10. Nab titers were calculated as the sample dilution conferring a 50% reduction in RLU relative to virus control wells after subtraction of background RLU in cell control wells. Env plasmid libraries were cloned from either infected cell cultures, *env* expression vectors (tier 1 and 2 reference panels), or plasma from HIV-infected trial participants. Viral stocks were prepared by cotransfecting HEK293 cells with *env* plasmid libraries along with an HIV genomic vector containing a Luc indicator gene in place of *env*.

Statistical methods. Box plots were used to graphically display distributions of log10 NAb titers to individual isolates. NAb responses to an individual isolate were summarized by the percentage of subjects who had a positive response ("positive response rate"), and by the geometric mean titer (GMT) of NAbs ("titers of NAbs") within the subgroup of subjects with a positive response (responders). Positive response rates were compared between groups by 95% confidence intervals (CIs) about the difference in positive response rates, and by a Fisher exact test for different rates. Titers of NAbs among responders were compared between groups by 95% CIs about the ratio of GMTs. Equality of the overall distribution of log10 NAb titers between 2 groups was tested as described elsewhere [46], using 10,000 permutated data sets to compute a *P* value. The false discovery rate (FDR) was used to determine tests that remained statistically significant after adjustment for the multiple hypothesis tests. The FDR method was performed at level 0.05.

Assessment of magnitude and breadth of neutralization of a panel of isolates. A magnitude-breadth (M-B) curve was used to describe the magnitude (NAb titer) and breadth (number of isolates neutralized) of an individual plasma sample assayed against a panel of tier 2 HIV-1 isolates [47]. On the basis of NAb titers to *m* isolates, the *x*-axis of an M-B plot is the threshold of neutralization that is considered positive, whereas the *y*-axis is the percentage of the *m* targets neutralized. The area under the curve (AUC) of a M-B curve provides an overall summary of the M-B profile and equals the average log10 NAb titer over the *m* targets. The Mann-Whitney test was used to compare the AUC of M-B curve between groups, which provides an overall test for different aggregate NAb responses. Wilcoxon signed rank tests were used to compare within-subject differences in the AUC of M-B plots between 2 distinct panels of HIV-1 isolates, which determined whether one panel was more easily neutralized than the other. All *P* values are 2-sided.

RESULTS

Preinfection NAb responses. Plasma samples obtained 2 weeks after fourth inoculation (90 vaccine recipients and 30 placebo recipients who were uninfected at the time of blood draw) were assessed in 2 independent assays; this time point corresponds to peak vaccine-elicited antibody responses [38]. High-titer NAbs were detected against HIV-1_{MN} and SF162.LS in most vaccine recipients in both assays (Figure 1*A* and 1*B*). Sporadic weak neutralizing activity was detected against tier 2 reference strains in both assays (Figure 1*A* and 1*B*). Positive response rates (frequency of results $\geqslant$1:10 plasma dilution) and titers of NAbs against the tier 2 reference viruses were significantly higher for vaccine than placebo recipients for 9 of 12 viruses in the TZM-bl assay and for 6 of 12 viruses in the U87.CD4.CCR5.CXCR4 assay. False positive results (ie, higher responses in placebo recipients than in vaccine recipients) were obtained with RHPA4259.7 in the TZM-bl assay and with PVO.4 in the U87.CD4.CCR5.CXCR4 assay. Because of the low plasma dilutions tested, occasional false positive neutralization was not unexpected. Overall positive response rates against any tier 2 viruses were 47% (range, 17%–92%) and 23% (range, 0–57%) for vaccine and placebo recipients, respectively, in the TZM-bl assay. Corresponding positive response rates in the U87.CD4.CCR5.CXCR4 assay were 44% (range, 12%–72%) and 32% (range, 0–60%), respectively. Therefore, net positive response rates for vaccine recipients (subtracting positive response rates for placebo recipients) were 24% in the TZM-bl assay and 12% in the U87.CD4.CCR5.CXCR4 assay. Neutralization of tier 2 reference strains was significantly greater for vaccine recipients than for placebo recipients in both assays when magnitude and breadth of neutralization were considered in aggregate.

Preinfection plasma from vaccine recipients exhibited weak neutralizing activity against early viruses from 13 vaccine and 14 placebo recipients (Figure 2). Pooling over the 27 isolates, overall positive response rates were 5% for vaccine and 0% for placebo recipients (Mann-Whitney test, $P = .05$). When M-B curves were compared, vaccine-elicited antibodies were more likely to neutralize viruses from placebo recipients than viruses from vaccine recipients ($P = .004$; Figure 2*B*). The magnitude of this latter difference was small, with 54 vaccine recipients

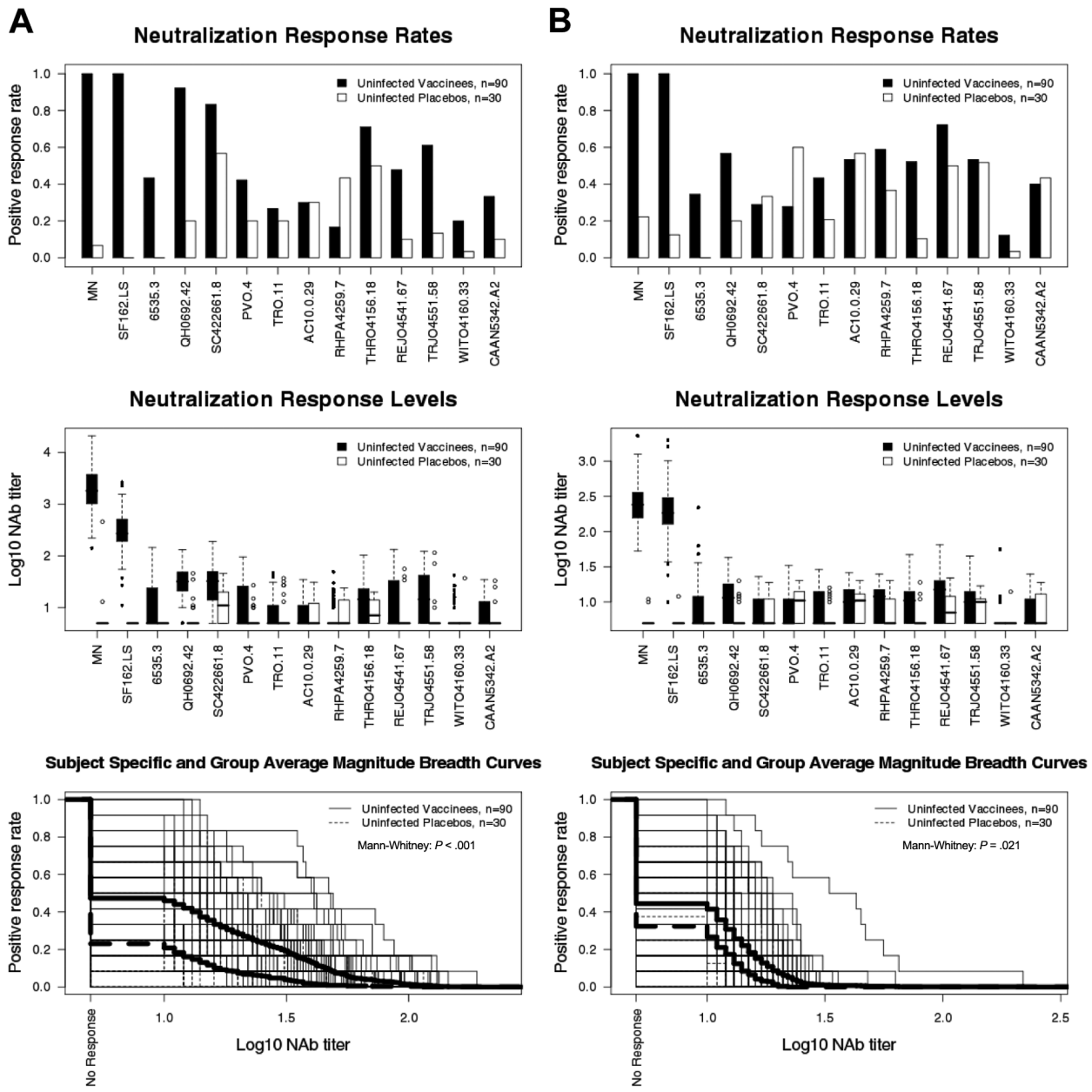

Figure 1. Comparison of preinfection neutralizing antibody (NAb) responses among vaccine and placebo recipients as measured with tier 1 and tier 2 reference strains. NAbs in plasma samples from 90 randomly selected vaccine recipients and 30 randomly selected placebo recipients, all of whom who were uninfected at the time of blood draw (2 weeks after the fourth inoculation), were assessed against HIV-1_{MN}, SF162.LS and a panel of 12 subtype B tier 2 reference strains. Positive response rates (frequency of positive results at ⩾1:10 plasma dilution), titers of NAbs and magnitude-breadth (M-B) curves were derived from results obtained in the TZM-bl (*A*) and U87.CD4.CCR5.CXCR4 (*B*) assays. For the box plots of NAb titers (*middle panel*), 25% of values lie below the box, 25% lie above the box, and 50% lie below the horizontal line (the median) inside the box. Vertical lines above the box extend to a distance 50% greater than the height of the box; points beyond this are unusually high values (*outliers*). Subject-specific and group averages in M-B plots are shown as light and heavy lines, respectively, and are for the tier 2 viruses only.

having equal AUC for the 2 sets of viruses, 23 having greater AUC for placebo viruses, and 8 having smaller AUC for placebo viruses; thus, the result may be of little biological importance. Results with postinfection plasma from placebo recipients (ie, natural NAb response to infection) showed that viruses from infected placebo recipients were intrinsically slightly more sensitive to neutralization (data not shown; $P = .013$).

Plasma from a subset of vaccine and placebo recipients in Figure 1 were assessed for neutralization breadth against a larger panel of tier 1 viruses and one additional prototypic tier 2 virus

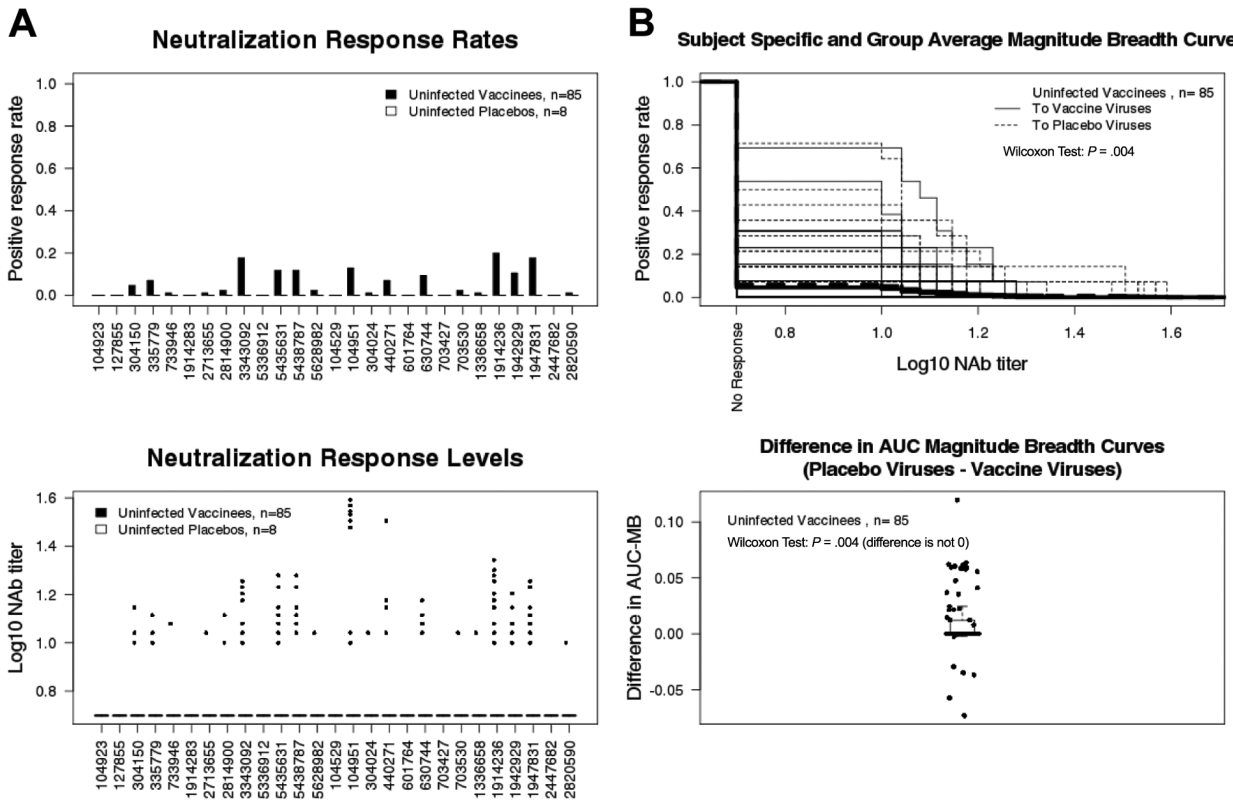

Figure 2. Comparison of preinfection neutralizing antibody (NAb) responses among vaccine and placebo recipients as measured with viruses from trial participants. Plasma samples in Figure 1 were assessed for neutralizing activity against viruses from 27 trial participants obtained at the earliest available postinfection time point. *A,* Neutralization response rates and the titers of NAbs. The first 13 viruses from the left are from vaccine recipients and the second 14 viruses are from placebo recipients. *B,* Magnitude-breadth (M-B) curves to the vaccine recipient isolate panel and to the placebo recipient isolate panel (*top*) and differences in AUC of M-B curves for the placebo and vaccine isolate panels (*bottom*). Subject-specific and group averages in M-B plots are shown as light and heavy lines, respectively. All results in *A* and *B* were obtained in the U87.CD4.CCR5.CXCR4 assay. Parallel assessments in the TZM-bl assay were not performed.

(JR-FL) in the TZM-bl assay (Figure 3*A*). Plasma from placebo recipients were mostly negative. Plasma obtained from all vaccine recipients neutralized HIV-1_{MN} and SF162.LS, with GMTs of 4931 and 1431, respectively. Moderate to low levels of NAbs were detected against tier 1 viruses MW965.26, SS1196.1, Bal.26, Bx08.16, 92BR025.9 and BZ167.12, with GMTs of 263, 134, 48, 44, 34 and 17, respectively. Plasma obtained from a single vaccine recipient neutralized JR-FL (titer, 24).

Additional assays were performed with serum rather than with plasma and compared a 1:10 dilution of postimmune serum (2 weeks after 4th inoculation) to a 1:10 dilution of corresponding preimmune serum from additional randomly sampled vaccine and placebo recipients who were uninfected at the time of blood draw. This method automatically adjusts for nonspecific activity in corresponding preimmune serum and thus may be a more stringent measure of true neutralization. Serum samples were assayed against the 12 tier 2 reference strains and 8 authentic tier 2 transmitted/founder viruses. Subjects were randomly sampled to comprise an equal distribution of blacks and whites of both sexes; this number was not adequate for statistical comparisons between races and sexes. Vaccine recipients exhibited weak but statistically significant neutralizing activity against both sets of viruses (Figure 3*B*). Transmitted and early founder viruses were slightly less sensitive to neutralization than the tier 2 reference viruses, but this difference was not significant ($P = .09$ for vaccine recipients and $P = .53$ for placebo recipients). Overall positive response rates (≥50% neutralization) against the tier 2 reference viruses were 8.3% for vaccine recipients and 0% for placebo recipients. The positive response rate against transmitted/founder viruses was 0% for both the vaccine and placebo groups.

Association between neutralization of HIV-1_{MN} and of tier 2 viruses. Titers of NAbs against HIV-1_{MN} in vaccine recipients were positively correlated with titers against 4 tier 2 strains in the U87.CD4.CCR5.CXCR4 assay (6535.3, THRO4165.18, REJO4541.67, PVO.4; Spearman rank test, $r > 0.20$), one of which was significant after FDR adjustment (6535.3; $r = 0.40$). HIV-1_{MN} NAb titers were weakly positively correlated with AUC

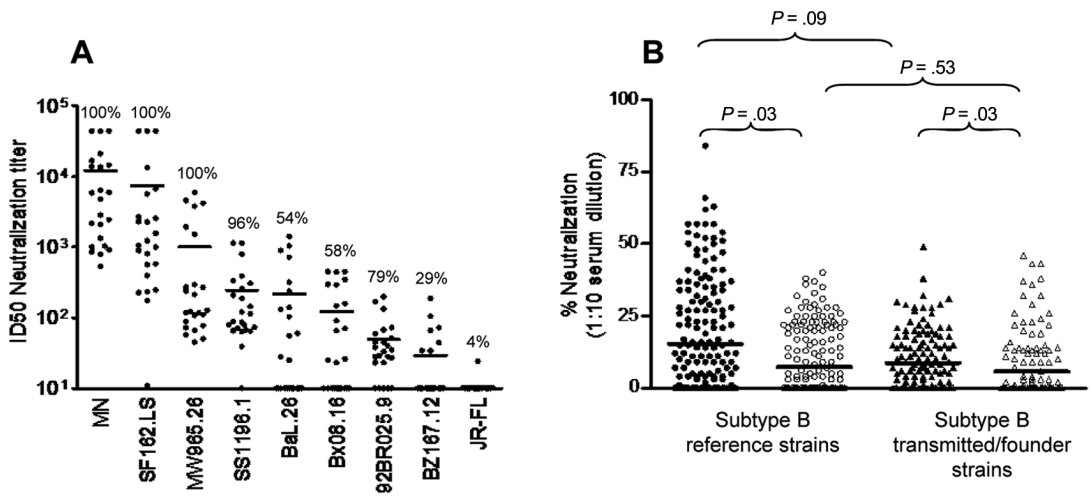

Figure 3. Breadth of preinfection neutralizing antibodies (NAbs) against tier 1 and tier 2 viruses among vaccine and placebo recipients. *A*, Plasma samples from 24 randomly selected vaccine recipients and 5 placebo recipients (2 weeks after the fourth immunization, before infection) among the same 120 trial participants in Figure 1 were assayed against HIV-1MN, SF162.LS, 6 additional tier 1 viruses and 1 prototypic tier 2 virus (JR-FL) in the TZM-bl assay. Plasma samples were assayed at 8 dilutions starting at 1:20. NAb titers <20 were assigned a value of 10. Results are shown for vaccine recipients only. Results with placebo recipient plasma were low (SS1196.1, 4 samples with NAb titers of 29–59; MW965.26, 1 sample with a NAb titer of 31) or negative (all remaining tests). Positive response rate (% of values ⩾50 neutralization) is shown above each scatter plot. *B*, Serum samples from additional vaccine and placebo recipients ($n = 20$ each) were tested for neutralizing activity at a 1:10 dilution in the TZM-bl assay against the 12 subtype B tier 2 reference strains (same as Figure 1*A*, excluding tier 1 viruses MN and SF162.LS). Many of these same samples (16 vaccine and 17 placebo recipients) were also assayed against 8 tier 2 transmitted/founder clade B strains (WEAU-d15.410.787, BB1006–11.C3.1601, BB1054–07.TC4.1499, BB1056–10.TA11.1826, BB1012–11.TC21, 6240.08.TA.4622, 6244.13.B5.4576, 62357.14.D3.4589); sufficient quantities were not available for all samples to be assayed against this latter panel of viruses. Serum samples before the first inoculation (preimmune) and 2 weeks after fourth inoculation (before infection) were assayed in triplicate on the same assay plate. Percentage of neutralization was calculated by dividing the average RLU of preimmune serum by the average RLU of postimmune serum, subtracting this result from 1 and multiplying by 100. For each subject and each tier 2 panel (12 reference viruses and 8 transmitted/founder viruses), the average of the percent neutralization values across the isolates in the panel was computed. These averages were compared between the vaccine and placebo groups for each panel with Mann-Whitney tests, and were compared between the 2 panels with a paired data Wilcoxon signed-rank test. Filled symbols, vaccine recipients; open symbols, placebo recipients.

of M-B curves against the 12 tier 2 reference viruses ($r = 0.24$ and $P = .025$ TZM-bl assay; $r = 0.15$ and $P = .16$ U87.CD4.CCR5.CXCR4 assay). No significant correlation was seen between HIV-1_{MN} NAb titers and neutralization of viruses from trial participants.

Comparison of postinfection NAb responses among vaccine and placebo recipients. NAbs were assessed in plasma from 14 vaccine recipients and 14 placebo recipients 12–24 months after diagnosis of infection (prior to antiretroviral therapy). All subjects received 4 inoculations of either the vaccine or placebo prior to diagnosis. Results in the TZM-bl assay were published elsewhere [47]. Results in the U87.CD4.CCR5.CXCR4 assay are shown in Figure 4. Titers of postinfection NAbs against HIV-1_{MN} were significantly higher for vaccine recipients than placebo recipients in both assays, suggesting the vaccine augmented the response to HIV-1_{MN}. No significant difference was seen between vaccine and placebo recipients for NAbs against SF162.LS, the 12 tier 2 reference strains and the 27 viruses from trial subjects. Assays with viruses from infected trial participants included autologous plasma and virus combinations from 2 vaccine and 8 placebo recipients that yielded considerably stronger neutralization than heterologous combinations. Differences among vaccine and placebo recipients were nonsignificant regardless of whether autologous combinations were included in the statistical analysis.

Comparison of NAb responses among preinfection vaccine recipients and postinfection placebo recipients. Peak vaccine-elicited NAb responses in 90 trial participants (2 weeks after fourth inoculation) were compared with the early response that arose after infection in 14 placebo recipients (1–2 years after diagnosis). Results are shown in Figure 5. Titers of NAbs against HIV-1_{MN} were similar in both cases, whereas titers against SF162.LS were significantly elevated in infected placebo recipients (GMT 2451 vs 288, $P < .001$ in TZM-bl assay; GMT 1006 vs 184, $P < .001$ in U87.CD4.CCR5.CXCR4 assay). M-B curves in the TZM-bl assay showed that responses against the tier 2 reference strains were similar among the 2 groups ($P = .24$), whereas a small but significantly elevated response was seen in infected placebo recipients using the U87.CD4.CCR5.CXCR4 assay: $P < .001$ for all 39 tier 2 viruses (data not shown); $P =$

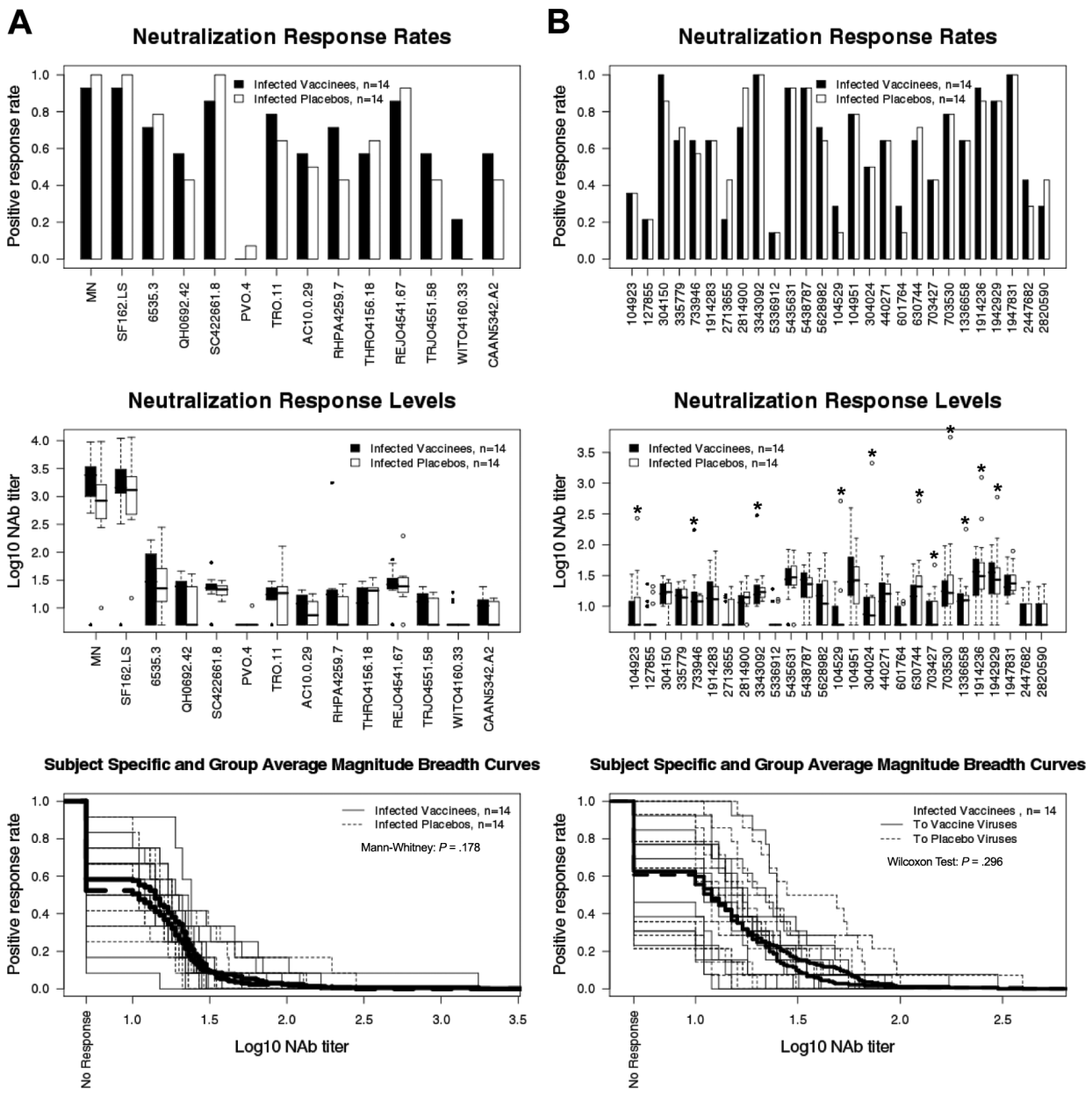

Figure 4. Comparison of postinfection neutralizing antibody (NAb) responses among vaccine and placebo recipients. NAbs were assessed in plasma samples from 14 vaccine recipients and 14 placebo recipients 12–24 months after diagnosis of infection. All subjects were antiretroviral therapy naïve at the time of plasma collection. *A*, Assays with MN, SF162.LS and the subtype B reference panel of tier 2 viruses. *B*, Assays with viruses from trial participants. In the top 2 diagrams, the first 13 viruses from the left are from vaccine recipients and the second 14 viruses are from placebo recipients. Autologous virus/plasma combinations in the middle diagram (neutralization response levels) are indicated by an asterisk. All results in panels *A* and *B* were obtained in the U87.CD4.CCR5.CXCR4 assay. Subject-specific and group averages in magnitude-breadth plots are shown as light and heavy lines, respectively, and are for the tier 2 viruses only.

.008 for the tier 2 reference strains shown in Figure 5. Thus, the vaccine-elicited response did not exceed the response that arose after 1–2 years of infection in the absence of vaccination.

Influence of demographic factors on the preinfection neutralizing antibody response in vaccine recipients. NAbs in the 90 vaccine recipients (2 weeks after fourth inoculation, before infection) were compared among sex, race (blacks and whites), and low versus high risk behavior groups. Results in both assays demonstrated higher titers of NAb against HIV-1_{MN} and SF162.LS in women than in men (~2-fold increase in GMT; $P < .008$). Additionally, M-B curves showed higher aggregate responses to the 12 tier 2 reference viruses in

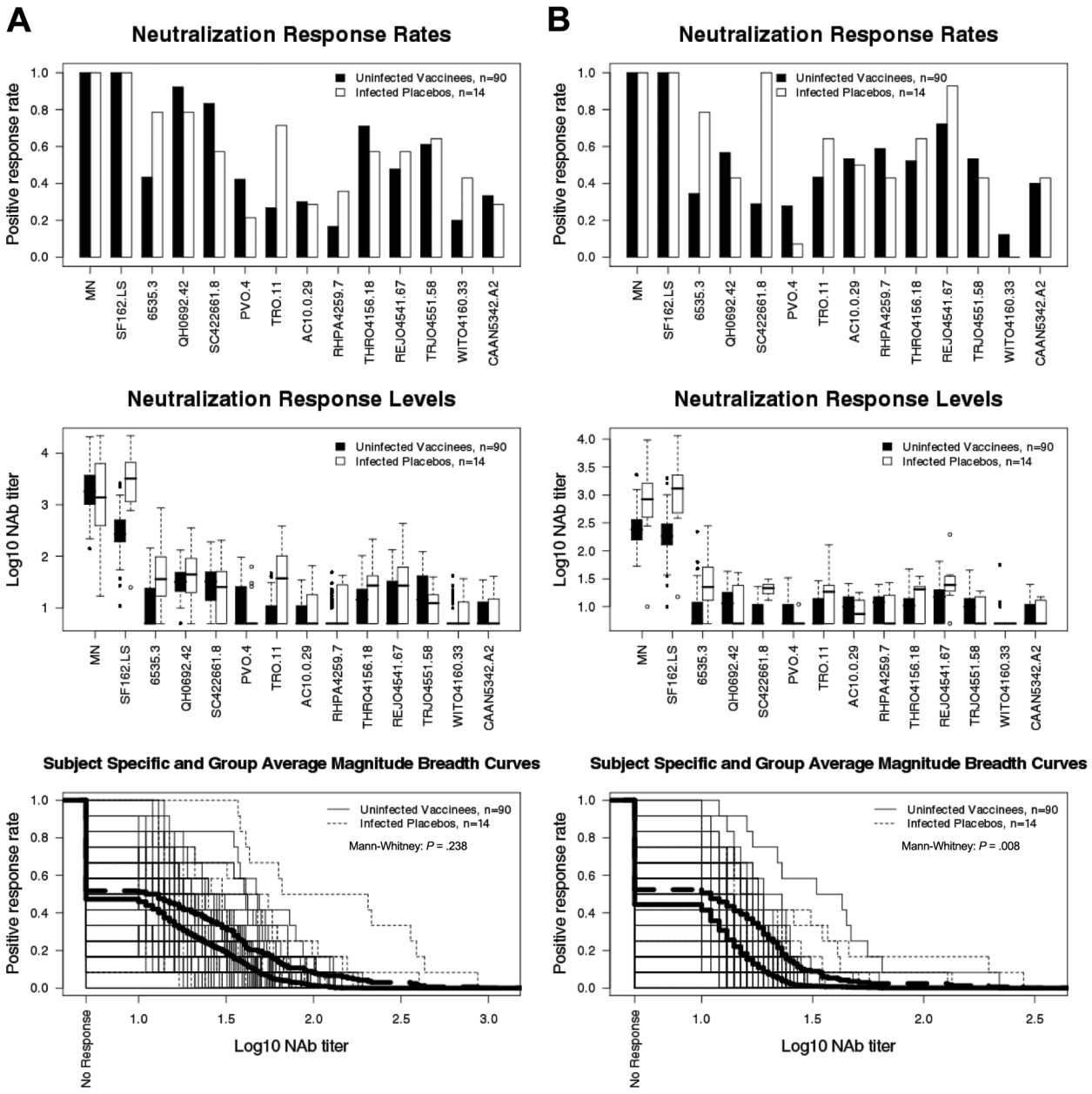

Figure 5. Comparison of preinfection neutralizing antibody (NAb) responses in vaccine recipients to postinfection NAb responses in placebo recipients. Plasma obtained from 90 vaccine recipients (2 weeks after the fourth inoculation) and 14 placebo recipients (1–2 years after diagnosis) were assayed against MN, SF162.LS, and the subtype B reference panel of tier 2 viruses. *A*, TZM-bl assay. *B*, U87.CD4.CCR5.CXCR4 assay. Subject-specific and group averages in magnitude-breadth plots are shown as light and heavy lines, respectively, and are for the tier 2 viruses only.

women than in men ($P < .001$, TZM-bl assay; $P = .034$, U87.CD4.CCR5.CXCR4 assay) (Figure 6). A nonsignificant trend toward higher M-B curves was also seen for women when all 39 tier 2 viruses were considered in aggregate ($P = .073$; U87.CD4.CCR5.CXCR4 assay). Race and risk behavior level had no significant effect.

Comparison of tier 2 reference strains and viruses from trial participants. The tier 2 reference strains were more susceptible to nonspecific neutralization ($P = .004$ for preinfection placebo samples) and to specific neutralization ($P < .001$ for preinfection vaccine samples) (Figure 2*B*) than viruses from trial participants. Having a positive response to the reference

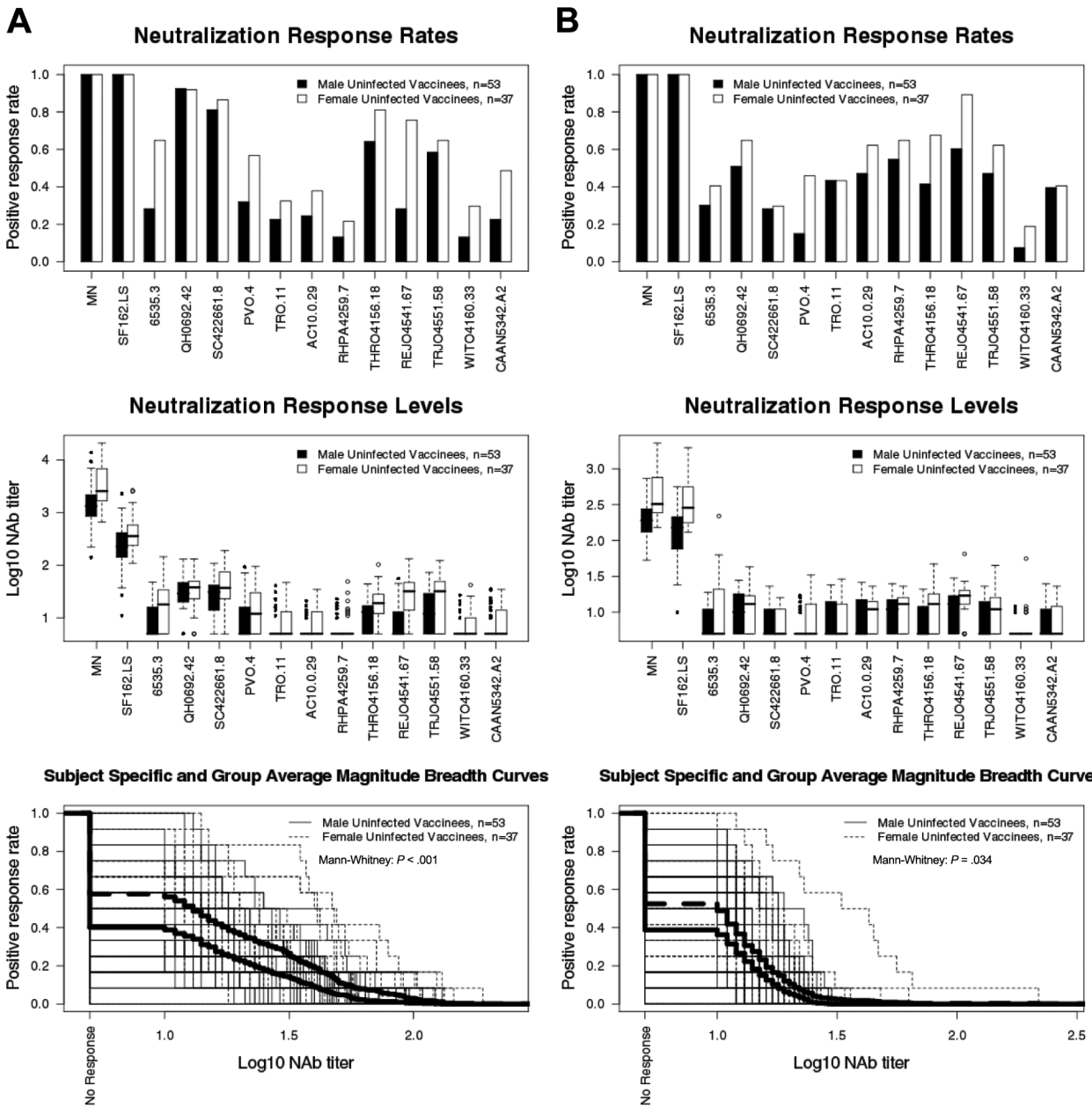

Figure 6. Comparison of preinfection neutralizing antibody (NAb) responses among men and women vaccine recipients ($n = 90$, evaluated in Figure 1), as measured with the tier 1 and tier 2 reference strains evaluated in Figure 1. Positive response rates (frequency of positive results at ⩾1:10 plasma dilution), titers of NAbs and magnitude-breadth (M-B) curves were derived from results obtained in the TZM-bl (*A*) and U87.CD4.CCR5.CXCR4 (*B*) assays. Subject-specific and group averages in M-B plots are shown as light and heavy lines, respectively, and are for the tier 2 viruses only.

panel was predictive of having a positive response to the trial participant panel for postinfection vaccine and placebo samples (odds ratio, 8.17; $P = .019$) but not for preinfection vaccine samples. Because viruses from trial participants were only assayed in U87.CD4.CCR5.CXCR4 cells, where slightly elevated NAb responses were detected after infection, the magnitude of vaccine-elicited NAb response against tier 2 viruses might border the magnitude required to achieve reproducible results in the 2 independent assays.

DISCUSSION

We confirm that most vaccine recipients in Vax004 possessed moderate to high titers of NAbs against HIV-1_{MN}. Moderate

neutralizing activity was often detected against other tier 1 strains, but only occasional weak neutralizing activity was detected against tier 2 strains. Prior vaccination augmented the NAb response against HIV-1_{MN} after infection but had little measurable effect on the postinfection NAb response against tier 2 viruses. Overall, the vaccine-elicited NAb response was no better than the relatively weak response that arose after 1–2 years of infection in the absence of vaccination. Relatively weak NAb responses against tier 2 strains is consistent with the lack of protection in this trial.

Vaccine-elicited NAb responses against tier 2 viruses, albeit weak, were statistically significant (compared to placebo) against tier 2 Env-pseudotyped reference strains and against pseudoviruses containing a more recent set of authentic transmitted/founder Envs, suggesting that the reference panel detects NAbs of interest for vaccines. Both sets of pseudoviruses contained single Env clones, whereas pseudoviruses containing Env from trial participants were a quasispecies. Greater genetic complexity of the Env quasispecies might account for observed differences in nonspecific activity and neutralization-sensitivity when assayed in U87.CD4.CCR5.CXCR4 cells. In both cases, neutralization of tier 2 viruses was poorly predicted by NAbs against the HIV-1_{MN}, thus reinforcing the importance of including tier 2 viruses when assessing vaccine-elicited NAbs. Additionally, vaccine recipient plasma appeared more likely to neutralize Env quasispecies from infected placebo recipients than from infected vaccine recipients ($P = .004$), although the small magnitude of this possible effect suggests little if any biological significance. Beyond Vax004, for efficacy trials with evidence for positive vaccine efficacy, a larger effect of this kind could indicate that some circulating viruses are more sensitive to vaccine-elicited NAbs that blocked their transmission to exposed vaccine recipients. We encourage similar assessments of NAbs, combined with complementary genetic analyses of the viruses [48], in RV144 and future trials where measurable protection is achieved.

Our results are consistent with a previous report [38] showing significantly more elevated titers of NAbs against HIV-1_{MN} in women than in men (2 times higher GMT in both assays) and no significant difference in the response between high and low behavioral risk groups in Vax004. We also observed significantly stronger responses in women than in men for NAbs against SF162.LS and tier 2 reference strains. Contrary to previous reports [38, 49, 50], we found no significant difference in NAb responses between blacks and whites. Our results lend support to a possible effect of sex on the NAb response to certain HIV-1 vaccines. Additional studies are needed to delineate the nature of this effect.

Modest protection in the recent efficacy trial in Thailand (RV144) will provide additional opportunities to learn more about the requirements for effective vaccination against HIV-1. One way to improve the efficacy of current HIV-1 vaccines may be to elicit stronger NAb responses against tier 2 strains of the virus. The magnitude and breadth of neutralization reported here for a nonprotective vaccine should serve as a useful reference to identify improved vaccine designs.

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