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# HIV-1 Antibody Neutralization Breadth Is Associated with Enhanced HIV-Specific CD4$^+$ T Cell Responses

**Srinika Ranasinghe,[a,b] Damien Z. Soghoian,[a] Madelene Lindqvist,[a] Musie Ghebremichael,[a] Faith Donaghey,[a] Mary Carrington,[a,c] Michael S. Seaman,[d] Daniel E. Kaufmann,[a,b,e] Bruce D. Walker,[a,b,f] Filippos Porichis[a,b]**

Ragon Institute of MGH, MIT, and Harvard, Massachusetts General Hospital and Harvard Medical School, Cambridge, Massachusetts, USA[a]; Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery, The Scripps Research Institute, La Jolla, California, USA[b]; Cancer and Inflammation Program, HLA Immunogenetics Section, Leidos Biomedical Research, Inc., National Cancer Institute, Frederick, Maryland, USA[c]; Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA[d]; Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Montreal, Quebec, Canada[e]; Howard Hughes Medical Institute, Chevy Chase, Maryland, USA[f]

## ABSTRACT

**Antigen-specific CD4$^+$ T helper cell responses have long been recognized to be a critical component of effective vaccine immunity. CD4$^+$ T cells are necessary to generate and maintain humoral immune responses by providing help to antigen-specific B cells for the production of antibodies. In HIV infection, CD4$^+$ T cells are thought to be necessary for the induction of Env-specific broadly neutralizing antibodies. However, few studies have investigated the role of HIV-specific CD4$^+$ T cells in association with HIV neutralizing antibody activity in vaccination or natural infection settings. Here, we conducted a comprehensive analysis of HIV-specific CD4$^+$ T cell responses in a cohort of 34 untreated HIV-infected controllers matched for viral load, with and without neutralizing antibody breadth to a panel of viral strains. Our results show that the breadth and magnitude of Gag-specific CD4$^+$ T cell responses were significantly higher in individuals with neutralizing antibodies than in those without neutralizing antibodies. The breadth of Gag-specific CD4$^+$ T cell responses was positively correlated with the breadth of neutralizing antibody activity. Furthermore, the breadth and magnitude of gp41-specific, but not gp120-specific, CD4$^+$ T cell responses were significantly elevated in individuals with neutralizing antibodies. Together, these data suggest that robust Gag-specific CD4$^+$ T cells and, to a lesser extent, gp41-specific CD4$^+$ T cells may provide important intermolecular help to Env-specific B cells that promote the generation or maintenance of Env-specific neutralizing antibodies.**

## IMPORTANCE

**One of the earliest discoveries related to CD4$^+$ T cell function was their provision of help to B cells in the development of antibody responses. Yet little is known about the role of CD4$^+$ T helper responses in the setting of HIV infection, and no studies to date have evaluated the impact of HIV-specific CD4$^+$ T cells on the generation of antibodies that can neutralize multiple different strains of HIV. Here, we addressed this question by analyzing HIV-specific CD4$^+$ T cell responses in untreated HIV-infected persons with and without neutralizing antibodies. Our results indicate that HIV-infected persons with neutralizing antibodies have significantly more robust CD4$^+$ T cell responses targeting Gag and gp41 proteins than individuals who lack neutralizing antibodies. These associations suggest that Gag- and gp41-specific CD4$^+$ T cell responses may provide robust help to B cells for the generation or maintenance of neutralizing antibodies in natural HIV-infection.**

Evolving evidence suggests that an effective human immunodeficiency virus type 1 (HIV-1) vaccine will require a combination of high-quality cellular and humoral immune responses. Increasingly, the HIV vaccine community has focused on the elicitation of broadly neutralizing antibodies (bnAbs), which exhibit potent activity against many viral strains simultaneously, as a mechanism of protection against HIV acquisition (1). In natural infection, only a small number of HIV-infected subjects develop broadly neutralizing antibodies (5 to 30%), which are characterized by extensive somatic hypermutation with long heavy-chain CDR3 (HCDR3) regions and take several years to develop postinfection (2–5). The generation of broadly neutralizing antibodies likely requires functional germinal center formation and the provision of CD4$^+$ help to B cells. In nonhuman primates infected with simian-human immunodeficiency virus (SHIV), the quality and quantity of germinal center Env-specific CD4$^+$ T-follicular helper (Tfh) cells have been associated with the expansion of Env-specific B cells and broader antibody neutralization activity (6). In HIV-infected humans, studies have investigated the association between the frequencies of peripheral Tfh (pTfh) cells and the development of neutralizing antibodies (7–9). However, these and other studies in HIV-infected humans have examined only bulk CD4$^+$ subpopulations with unknown specificities and have

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Address correspondence to Srinika Ranasinghe, sranasinghe@mgh.harvard.edu, or Filippos Porichis, fporichis@mgh.harvard.edu.

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**TABLE 1** Clinical characteristics of our 34 HIV controller study participants

| HIV controller profile | | | |
|---|---|---|---|
| | Value for the group[b] | | |
| Parameter[a] | Neutralizers | Nonneutralizers | *P* value (neutralizers vs nonneutralizers) |
| No. of subjects | 14 | 20 | |
| Median (IQR) viral load (copies/ml) | 176 (79–809) | 209.5 (89–496) | 0.74 |
| Median (IQR) $CD4^+$ count (cells/μl) | 671.5 (494–790) | 813.5 (612–887) | 0.06 |
| Median (IQR) no. of yr since HIV diagnosis | 11 (4.75–22.5) | 8 (5.0–15.0) | 0.37 |
| Median (IQR) age at assay (yr) | 56 (43.75–64.50) | 51 (42.25–54.0) | 0.08 |
| Gender (% male) | 100 | 75 | 0.06 |
| Race (% Caucasian) | 71 | 65 | 1.00 |
| Risk (% MSM) | 57 | 51 | 0.74 |

[a] IQR, interquartile range; MSM, men having sex with men.
[b] Viremic controller neutralizers exhibit an antibody neutralization breadth of ≥2 tier 2/3 viruses; nonneutralizers exhibit an antibody neutralization breadth of 0 tier 2/3 viruses.

not investigated the quantity or quality of the actual HIV-specific $CD4^+$ T cell responses associated with antibody neutralization.

Although HIV-specific $CD4^+$ T cell responses have been shown to be an important feature of viral immunity (10–19), the specificity, function, and phenotypic characteristics of peripheral HIV-specific $CD4^+$ T cells associated with optimal B cell help in natural HIV infection are currently unknown. One of the critical aspects that needs to be addressed is whether subjects with neutralizing antibody responses have more robust HIV-specific $CD4^+$ T helper cells and if these $CD4^+$ T cell responses target specific HIV proteins or peptides that allow them to provide optimal help to B cells. Understanding the impact of HIV-specific $CD4^+$ T cells on the generation of neutralizing antibodies will remove a major obstacle toward the rational design of vaccine regimens (20–24).

Thus, in this study, we sought to characterize HIV-specific $CD4^+$ T cell targeting in the peripheral blood of chronically HIV-infected individuals with and without neutralizing antibody responses to a large panel of tier 2/3 Env-pseudotyped HIV strains. Our results demonstrate that the breadth and magnitude of Gag-specific and gp41-specific $CD4^+$ T cell responses, but not gp120-specific responses, are significantly associated with enhanced neutralization breadth in HIV controllers. These data are consistent with the hypothesis that robust Gag-specific $CD4^+$ T cells and, to a lesser extent, gp41-specific $CD4^+$ T cells are likely to provide important helper function to B cells that promote more potent antibody neutralization activity.

## MATERIALS AND METHODS

**Subjects.** A total of 67 HIV-infected subjects were recruited from the Massachusetts General Hospital. Thirty-four HIV controllers, defined as HIV-infected individuals who spontaneously control HIV infection (viral loads of >50 and <2,000 HIV RNA copies/ml for >1 year) in the absence of antiretroviral therapy, were recruited into our study (Table 1). Viremic controllers were primarily chosen for this study as they have detectable viremia in the absence of antiretroviral therapy and typically exhibit robust HIV-specific $CD4^+$ T cell responses. To supplement this analysis, we also enrolled 33 treatment-naive HIV progressors with high-level viremia who typically exhibit weaker HIV-specific $CD4^+$ T cell responses (Table 2). In both cohorts, we observed no significant difference in neutralizers versus nonneutralizers in viral loads, $CD4^+$ counts, or years since HIV diagnosis. The number of years since HIV diagnosis did not correlate with neutralizing antibody breadth in these cohorts. From each individual, fresh blood was obtained and processed, with CD8-depleted peripheral blood mononuclear cells (PBMC) used for enzyme-linked immunosorbent spot (ELISPOT) assay, and the corresponding plasma was frozen for an HIV-1 neutralization assay. Neutralizing antibody titers were revealed only at the end of the study (see Table S1 in the supplemental material). All study subjects gave informed consent and IRB approval was obtained from the Massachusetts General Hospital institutional regulatory board (IRB).

**HIV-1 neutralization assay in TZM.bl cells.** Neutralizing antibody titers were determined using a luciferase-based TZM.bl cell assay as previously described (25). This assay measures a decrease in luciferase reporter gene expression following single-round viral infection of TZM.bl cells. Briefly, 3-fold serial dilutions of heat-inactivated plasma samples (56°C for 1 h) were performed in duplicate and incubated with HIV-1 Env

**TABLE 2** Clinical characteristics of our 33 HIV progressor study participants

| HIV progressor profile | | | |
|---|---|---|---|
| | Value for the group[b] | | |
| Parameter[a] | Neutralizers | Nonneutralizers | *P* value (neutralizers vs nonneutralizers) |
| No. of subjects | 22 | 11 | |
| Median (IQR) viral load (copies/ml) | 26,051 (6,502–56,075) | 39,200 (11,200–57,061) | 0.48 |
| Median (IQR) $CD4^+$ count (cells/μl) | 452 (378–594) | 594 (469–709) | 0.08 |
| Median (IQR) no. of yr since HIV diagnosis | 7 (3–17.3) | 3 (2–8) | 0.08 |
| Median (IQR) age at assay (yr) | 41.5 (33.75–47.25) | 31 (27–45) | 0.05 |
| Gender (% male) | 86 | 91 | 1.00 |
| Race (% Caucasian) | 55 | 73 | 0.45 |
| Risk (% MSM) | 77 | 91 | 0.64 |

[a] IQR, interquartile range; MSM, men having sex with men.
[b] HIV progressor neutralizers exhibit an antibody neutralization breadth of ≥2 tier 2/3 viruses; nonneutralizers exhibit an antibody neutralization breadth of 0 tier 2/3 viruses.

pseudoviruses for 1 h at 37°C. TZM.bl cells were then added in growth medium containing DEAE-dextran at a final concentration of 11 μg/ml, and assay plates were incubated for 48 h at 37°C in 5% $CO_2$. Luciferase reporter gene expression was measured using Bright-Glo luciferase reagent (Promega) and a Victor 3 luminometer (PerkinElmer). Neutralization titers (50% inhibitory dose [$ID_{50}$]) were calculated as the serum dilution at which relative light units (RLU) were reduced by 50% compared to the number of RLU in virus control wells after subtraction of background RLU counts in cell control wells. The plasma from each subject was tested against a standardized panel of clade B tier 1 ($n = 4$) and tier 2/3 ($n = 11$) Env pseudoviruses. Neutralization was considered positive if the $ID_{50}$ titer against an HIV-1 isolate was >3-fold higher than that observed against a negative-control murine leukemia virus (MuLV), as shown in Table S1 in the supplemental material. All assays were performed in a laboratory meeting good clinical laboratory practice (GCLP) standards. In this study, subjects with an HIV-1 antibody neutralization breadth of ≥2 tier 2/3 viruses were classified as "neutralizers," and subjects that failed to show HIV-1 antibody neutralization of any of the 11 tier 2/3 viruses were classified as nonneutralizers, as described in Tables 1 and 2. For some analyses, the neutralizers were further stratified into low neutralizers, with a neutralization breadth of 2 to 4, and high neutralizers, with a neutralization breadth of ≥5 tier 2/3 viruses. Additionally, we calculated the antibody neutralization score to quantify the breadth and potency of plasma neutralization activity per patient. Points were assigned based on the $ID_{50}$ titers for each of the 11 tier 2/3 viruses: one point was awarded for titers up to 99, two points were given for titers of 100 to 999, and three points were awarded for titers greater than 1,000 $ID_{50}$s, as described previously (6). The points were then summed to provide the overall neutralization score per patient.

**CD8 depletion and modified IFN-γ CD4 ELISPOT assay.** A modified gamma interferon (IFN-γ) ELISPOT protocol was utilized, as described previously (15). In brief, peripheral blood mononuclear cells (PBMC) were freshly isolated from each of the 67 HIV-infected individuals. The PBMC were depleted of CD8 T cells prior to Ficoll-Hypaque density gradient centrifugation by incubation with anti-CD8 RosetteSep antibody (Stem Cell Technologies), and flow cytometry confirmed at least 98% CD8-negative PBMC. HIV-specific $CD4^+$ responses were then screened using a modified IFN-γ enzyme-linked immunospot (ELISPOT) assay utilizing a panel of 179 overlapping peptides (OLPs) spanning the clade B consensus 2001 proteome for Gag protein (66 OLPs), Env gp120 protein (66 OLPs), and gp41 protein (47 OLPs). The ELISPOT plates were incubated at 37°C with 5% $CO_2$ for 40 h to maximize IFN-γ release from $CD4^+$ T cells. The plates were then processed as previously described (15), with controls and threshold calculations to determine the number of spot-forming units (SFU). An HIV-specific $CD4^+$ T cell response was considered positive only if it met the following criteria: ≥55 SFU/$10^6$ CD8-depleted PBMC, ≥4 times the mean background, and also ≥4 times the standard deviation of the number of SFU/$10^6$ CD8-depleted PBMC compared to the count in the negative controls. In this study, the breadth of HIV-specific $CD4^+$ T cell responses is defined as the sum of IFN-γ-positive peptide responses within a given HIV-infected subject for a specified HIV protein. The magnitude of HIV-specific $CD4^+$ T cell responses is defined as the sum of individual peptide-specific responses (as measured by SFU/$10^6$ cells) within a given HIV-infected subject for a specified HIV protein. Due to limited sample availability of fresh blood per patient, for the majority of the subjects it was only possible to define HIV-specific CD4 T cell responses to Gag and Env at the single-peptide level. Where we had extra samples available ($n = 22$), we also tested Nef-specific $CD4^+$ T cell responses at the single-peptide level, but we did not detect any differences in epitope targeting between neutralizers and nonneutralizers (data not shown).

**HLA typing.** High-resolution four-digit HLA class I and II genotyping was performed by sequence-specific PCR in accordance with standard procedures. (HLA information was not acquired for two subjects). In brief, HLA class II DRB1 genes were identified by PCR amplification and sequencing of exon 2 (and HLA class I genes were PCR amplified with primers spanning exons 2 and 3). ASSIGN, version 3.5, software developed by Conexio Genomics was used to interpret the sequencing results.

**Statistical analysis.** Descriptive measures were used to summarize the data. Continuous variables were summarized using the median and interquartile range (IQR); categorical variables were summarized using frequency and percent. Spearman's rank correlations were used to examine bivariate associations between study variables. Fisher's exact test was used to compare categorical variables between the study groups. A Mann-Whitney test, Wilcoxon signed-rank test, and Kruskal-Wallis test with Dunn's *post hoc* analyses were used for comparing continuous variables. All *P* values are two-sided, and a *P* value of <0.05 was considered significant. Statistical analysis and graphing were performed using GraphPad Prism, version 5.0, and R.

## RESULTS

To evaluate the role of HIV-specific $CD4^+$ T cells in the development of antibody neutralization breadth, we comprehensively assessed a cohort of 34 chronically HIV-infected controllers for circulating *ex vivo* Gag- and Env-specific $CD4^+$ T cell responses at the single-peptide level in a modified IFN-γ ELISPOT assay (utilizing CD8-depleted PBMC incubated for 40 h) (15). Contemporaneously, patient plasma was used in a standardized TZM.bl neutralization assay against a panel of Env pseudoviruses to determine their HIV antibody neutralization status (25). Subjects with HIV antibody responses that neutralized two or more tier 2/3 viruses were defined as neutralizers, while subjects that showed no detectable HIV antibody neutralization of the 11 tier 2/3 viruses were classified as nonneutralizers. This definition is similar to other cohort studies that have differentiated HIV-infected individuals based on neutralization assays, where sera from neutralizers demonstrate monoclonal or polyclonal antibodies capable of neutralizing multiple HIV-1 isolates (7–9, 26). In order to control for ongoing exposure to virus, we selected viremic controllers, who maintain detectable viremia of less than 2,000 HIV RNA copies/ml plasma for greater than 1 year and typically demonstrate robust well-preserved HIV-specific $CD4^+$ T cell responses (15). This included 14 HIV controller neutralizers and 20 nonneutralizers. Importantly, no significant differences were observed between neutralizers and nonneutralizers in HIV viral loads, $CD4^+$ T cell counts, or years since HIV diagnosis (Fig. 1A to C; Table 1). Furthermore, the two groups also demonstrated no significant differences for gender, race, risk of HIV acquisition, or median age (Table 1), and there were no clear distinctions between the two groups in terms of their HLA class II DRB1 allele frequencies (Fig. 1D).

**The breadth and magnitude of Gag-specific $CD4^+$ T cell responses are significantly enhanced in neutralizers.** Our results showed that neutralizers have significantly greater Gag-specific $CD4^+$ T cell responses than nonneutralizers ($P = 0.0026$; median numbers of peptides targeted, 5.5 [IQR, 3 to 8.3] and 2.0 [IQR, 1 to 3], respectively) (Fig. 2A). In addition, neutralizers also demonstrated a significantly higher magnitude of responses to Gag than nonneutralizers ($P = 0.0043$; median magnitudes of 860.0 [IQR, 341 to 1,359] and 220.8 [IQR, 122 to 384] SFU per $10^6$ cells, respectively] (Fig. 2B). We next conducted a subgroup analysis in which we further divided the neutralizers into high neutralizers (who exhibited antibody neutralization of five or more tier 2/3 viruses) and low neutralizers (who demonstrated neutralization activity against two to four tier 2/3 viruses). The high neutralizers demonstrated significantly greater Gag-specific $CD4^+$ T cell re-

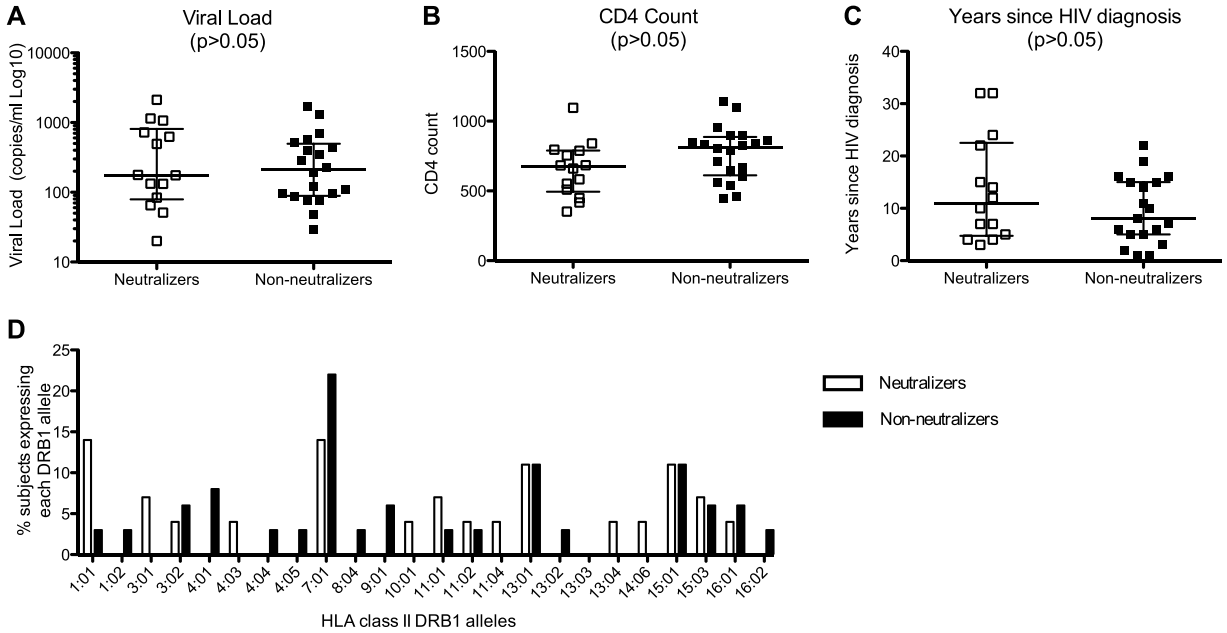


**FIG 1** No significant differences were observed between viremic controller neutralizers and nonneutralizers in HIV viral loads (A), $CD4^+$ T cell counts (B), years since HIV diagnosis (C), or HLA class II DRB1 allele frequencies (D). Scatter plots show the distribution of data for neutralizers and nonneutralizers for graphs in panels A to C, with box plots denoting the median values and bars denoting the interquartile ranges. Statistical significance was tested by a Mann-Whitney test. For the graph in panel D, the bars represent percentages of neutralizers and nonneutralizers expressing each HLA-DRB1 allele, with each of 32 subjects represented twice in the bar graph (as each individual expresses two DRB1 alleles). Statistical significance was not tested; much larger sample sizes would be necessary to determine whether there are significant DRB1 allele frequency differences between these groups at the population level.

sponses than nonneutralizers although no significance was detected between high neutralizers and low neutralizers or between low neutralizers and nonneutralizers (Fig. 2C and D).

Given these observations, we also interrogated whether responses to the three Gag subunits (p17, p24, and p15) were differentially targeted between neutralizers and nonneutralizers. We found that targeting of the p24 protein ($P = 0.0031$ for breadth data; $P = 0.0054$ for magnitude data) (Fig. 2E and F) and also p17 ($P = 0.039$ for breadth data; $P = 0.018$ for magnitude data) (Fig. 2E and F) was significantly enhanced in neutralizers. Collectively, these data indicate that neutralizers exhibit the most robust Gag-specific $CD4^+$ T cell responses, with strong responses to both the p24 and p17 subunits driving the overall association between Gag-specific $CD4^+$ T cell targeting and antibody neutralization breadth.

In addition, we expanded our analysis to HIV chronic progressors (Table 2) in order to understand whether the patterns of HIV-specific $CD4^+$ T cell targeting observed in viremic controllers is also shared with neutralizers who exhibit high-level viremia. Similar to our previous observations, we noted that the 11 HIV progressor neutralizers exhibited a higher median Gag-specific $CD4^+$ T cell response than 22 HIV progressor nonneutralizers although this response was just above the threshold of statistical significance ($P = 0.0521$ for magnitude data; $P = 0.0897$ for breadth data) (Fig. 3). These trends imply that Gag-specific $CD4^+$ T cell responses in HIV progressors may be linked with antibody neutralization, yet assessment of these responses is likely confounded by T cell dysfunction, altered regulatory pathways, and $CD4^+$ T cell depletion driven by prolonged high viremia.

**Env-specific $CD4^+$ T cell responses targeting gp41 are significantly enhanced in neutralizers.** We next analyzed the HIV-specific $CD4^+$ T cell responses to Env protein in our viremic controllers and found that the breadth of Env-specific $CD4^+$ T cell responses was significantly enhanced in our viremic controller neutralizers ($P = 0.0101$) (Fig. 4A). In particular, neutralizers targeted a median of 2 peptides (IQR, 1 to 4) while nonneutralizers targeted only 0.5 peptides (IQR, 0 to 2). Almost all neutralizers demonstrated a minimum of one detectable Env-specific $CD4^+$ T cell response, which was significantly different from that of nonneutralizers, of whom only half of the subjects made a detectable response (13/14 neutralizers and 10/20 nonneutralizers; $P = 0.011$). The magnitude of Env-specific $CD4^+$ T cell responses showed a trend toward higher median responses in neutralizers than in nonneutralizers ($P = 0.0568$) (Fig. 4B).

To further characterize responses to the Env protein, we also evaluated differential targeting of the gp120 versus gp41 subunits. The difference in median number of peptide epitopes targeted was minimal but significant: neutralizers targeted a median of only one gp41 peptide (IQR, 0 to 2.3), and nonneutralizers targeted a median of zero gp41 peptides ($P = 0.0162$) (Fig. 4C). In addition, the magnitude of gp41-specific $CD4^+$ T cell responses was significantly greater in neutralizers ($P = 0.0040$) (Fig. 4D). Further, while over half of the neutralizers targeted one or more gp41 peptides, just 20% of nonneutralizers made a detectable gp41-specific $CD4^+$ T cell response (8/14 neutralizers and 4/20 nonneutralizers; $P = 0.0356$). These data suggest that that $CD4^+$ targeting of Env is primarily driven by responses to gp41. In contrast to our gp41 results, we detected no difference in gp120 targeting between neutralizers and nonneutralizers (Fig. 4E and F).

**Gag- and Env-specific $CD4^+$ T cell responses are correlated with neutralizing antibody breadth.** To further understand the relationship between T helper responses and B cell-secreted anti-

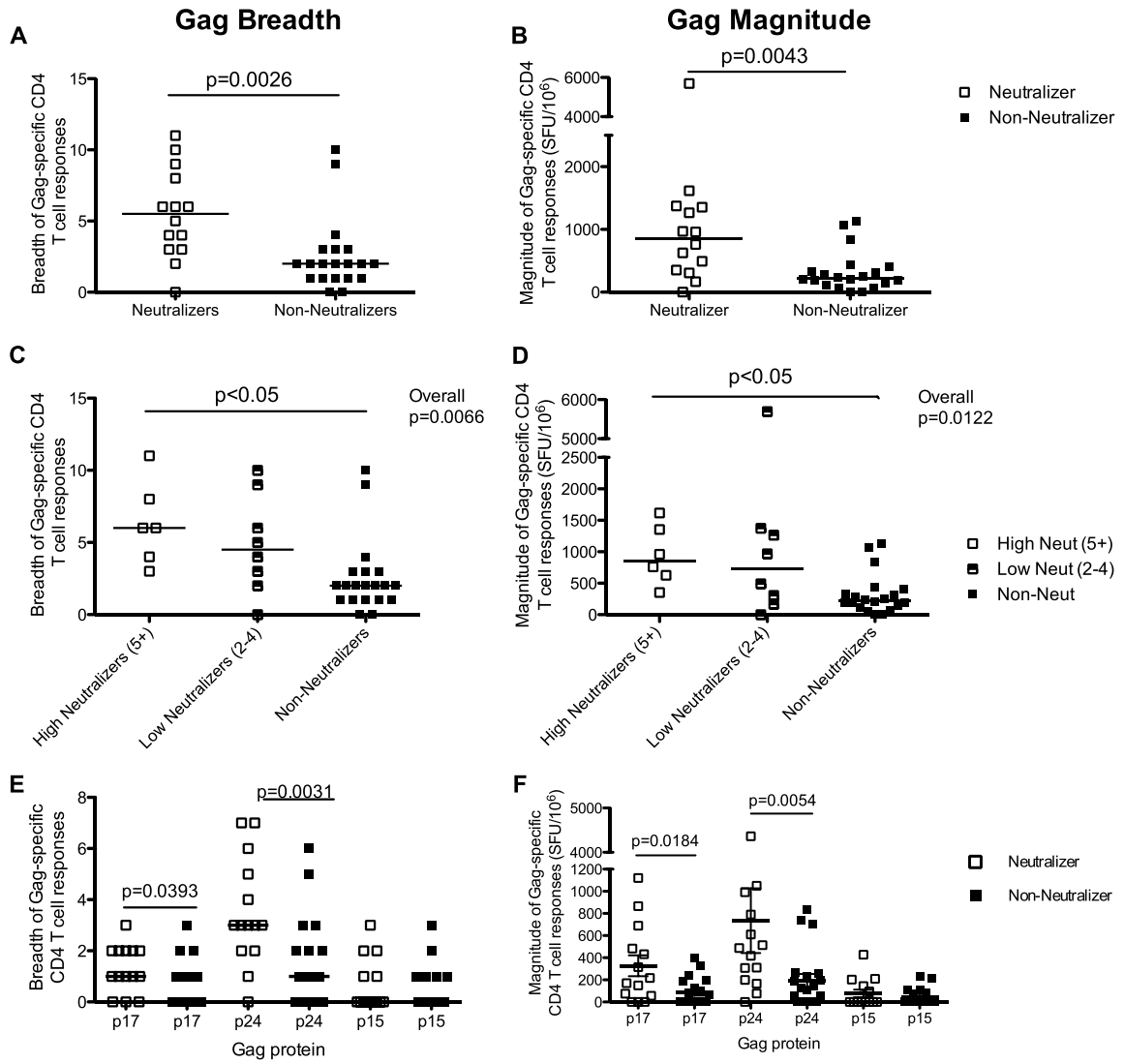


**FIG 2** Gag-specific CD4$^+$ T cell responses are associated with enhanced neutralizing antibody breadth in viremic controllers. The breadth (A) and magnitude (B) of Gag-specific CD4$^+$ T cell responses are shown for 34 viremic controllers stratified into neutralizers and nonneutralizers. Significant differences were observed based on a Mann-Whitney test. The breadth (C) and magnitude (D) of Gag-specific CD4$^+$ T cell responses are shown for subgroups consisting of high neutralizers, low neutralizers, and nonneutralizers. The overall *P* value is shown for the three groups based on Kruskal-Wallis, with Dunn's posttest used to compare values between each subgroup. In the posttest analysis, statistical significance at a *P* value of <0.05 was detected only between results for high neutralizers and nonneutralizers. No significance was detected between results for high neutralizers and low neutralizers or between results for low neutralizers and nonneutralizers. The breadth (E) and magnitude (F) of Gag-specific CD4$^+$ T cell responses for Gag subunits, p17, p24, and p15 are shown for neutralizers and nonneutralizers. Significance between clinical groups for each protein subunit was tested using a Mann-Whitney test. Values in all graphs are medians.

body responses within each viremic controller subject, we next assessed whether there is a correlation between antibody neutralization breadth and protein-specific CD4$^+$ T cell responses to Gag, Env, and each of the respective subunits (Fig. 5). We found that both the breadth and magnitude of Gag-specific CD4$^+$ T cell responses were positively correlated with antibody neutralization ($P = 0.0013$ and $r = 0.53$ for Gag breadth data and $P = 0.0016$ and $r = 0.52$ for magnitude data) (Fig. 6A and B). Moreover, the association between Gag-specific CD4$^+$ T cell responses and neutralization activity was primarily driven by responses to the p24 and p17 subunits (Fig. 5). These data indicate that HIV-specific CD4$^+$ T cell responses targeting these areas of the Gag protein are correlated with the breadth of antibody neutralization in our cohort of 34 viremic controllers. However, we were underpowered to evaluate whether a correlation exists between Gag-specific CD4$^+$ T cell responses and antibody neutralization breadth within the 14 neutralizers.

To better understand the potential relationship between Env-specific CD4$^+$ T cell responses and Env-specific antibody neutralization breadth, we also looked for a correlation between these two variables. We found that the breadth of Env-specific CD4$^+$ T cell responses was positively correlated to the breadth of antibody neutralization ($P = 0.0220$, $r = 0.39$) (Fig. 6C). However, no significant correlation was detected for the magnitude of Env-specific responses (Fig. 6D). In addition, there was no significant correlation between the breadth or magnitude of gp120- or gp41-specific CD4$^+$ T cell responses with antibody neutralization activity in our analysis (Fig. 5).

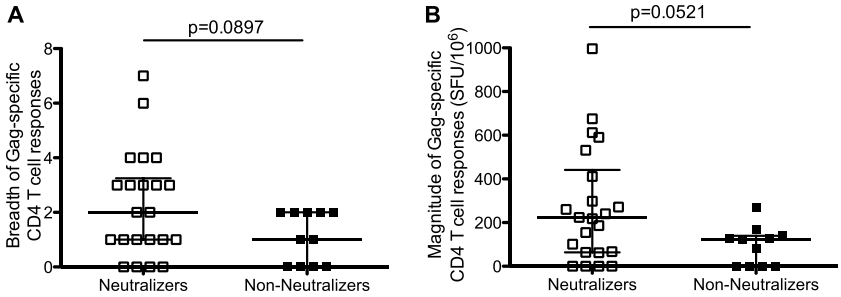


**FIG 3** HIV chronic progressors exhibit Gag-specific $CD4^+$ T cell responses that may be linked with antibody neutralization breadth. The breadth (A) and magnitude (B) of Gag-specific $CD4^+$ T cell responses are shown for 33 HIV progressors stratified into 22 neutralizers and 11 nonneutralizers. Differences between neutralizers and nonneutralizers were just above the threshold of significance based on a Mann-Whitney test. Medians and bars denoting IQRs are shown.

Since overall potency is another important aspect of the antibody response, we next investigated whether there are additional associations between HIV-specific $CD4^+$ T cell responses and HIV neutralization score. The neutralization score is used to quantify both the breadth and potency of serum neutralization activity (6), where points are assigned and summed based on the 50% inhibitory dose ($ID_{50}$) titers for each of the 11 tier 2/3 viruses (one point for titers up to 99, two points for titers of 100 to 999, and three points for titers greater than 1,000). In this analysis, we observed that the breadth and magnitude of Gag-specific $CD4^+$ T cell responses were positively correlated with neutralization score, and a positive correlation was also observed for the breadth of Env-specific $CD4^+$ T cell targeting (Fig. 6E and F). Overall, we saw strong concordance between the antibody neutralization breadth and neutralization score in our 14 HIV neutralizers.

Taking these results together, although it is not possible to determine cause and consequence from these cross-sectional analyses (Fig. 5 and 6), these data are nonetheless consistent with our hypothesis that strong and broad Gag-specific $CD4^+$ T cell responses may provide superior intermolecular helper activity to Env-specific B cells that promote the secretion of Env-specific neutralizing antibodies. In addition, broad Env-specific $CD4^+$ T cell responses may facilitate intramolecular help for the development of Env-specific neutralizing antibodies.

**Magnitudes of Gag-specific $CD4^+$ T cell responses were significantly higher than gp41-specific responses within the same subject.** Given the associations of Gag- and Env-specific $CD4^+$ T cell responses with neutralization status, we next assessed the relative contribution of these responses within the same subject. In our cohort of 34 viremic controllers, we observed that the intrapatient magnitude of Gag-specific $CD4^+$ T cell responses was significantly higher than that of gp41-specific responses ($P = 0.0002$) (Fig. 7A). A subanalysis of only the 11 viremic controllers that responded to both Gag and gp41 showed a median magnitude of 493 SFU/$10^6$ cells to Gag protein while that for gp41 was 176 SFU/$10^6$ cells, a difference which was just above the threshold of significance ($P = 0.0577$) (data not shown). Taking the results together, almost all of our 34 viremic controller subjects exhibited a stronger Gag-specific $CD4^+$ T cell response, with a median of 372.8 SFU/$10^6$ cells per individual (IQR, 184 to 962) than the gp41-specific response, with a median of 0 SFU/$10^6$ cells per individual (IQR, 0 to 123). In contrast, there was a trend but no significant difference detected between the magnitude of Gag-specific $CD4^+$ T cell responses and gp120-specific $CD4^+$ T cell responses intrapatient ($P = 0.058$) (Fig. 7B).

We next sought to understand whether there was any correlation between the size of Gag and gp41-specific responses within the same subject. We found that the magnitude of Gag-specific responses was not correlated with the magnitude of gp41-specific responses (Fig. 5). These data suggest that strong Gag-specific $CD4^+$ responses may exist in the absence of detectable gp41-specific responses, and vice versa, which implies that Gag- and gp41-specific $CD4^+$ T cell responses are likely to be independently associated with antibody neutralization breadth.

**$CD4^+$ T cell targeting of several Gag peptides may differ between neutralizers and nonneutralizers.** We next evaluated whether viremic controller neutralizers exhibit differential targeting of $CD4^+$ T cell peptides. We noted that the targeting of some individual peptides differed between neutralizers and nonneutralizers although this did not reach statistical significance. Our analysis found that 68% of Gag peptides (45/66 OLPs) were targeted by viremic controllers, with a high density of epitopes observed in p24 (Fig. 8A). Fourteen epitopes across Gag were targeted exclusively by neutralizers, albeit at low levels (7 to 14% of neutralizers responding). The most immunodominant peptide targeted by $CD4^+$ T cells was Gag-41 (YVDRFYKTLRAEQASQEV), with 50% of the cohort responding to this peptide, as observed previously, independent of neutralization status (13, 15). Although 71% of neutralizers targeted the Gag-41 peptide, only 35% of nonneutralizers responded to the same peptide (10/14 neutralizers and 7/20 nonneutralizers; $P = 0.0799$) (Fig. 8B and data not shown). The magnitudes of the Gag-41-specific $CD4^+$ T cell response were also not significantly different between the two groups ($P = 0.0697$) (data not shown). Similarly, the second most immunodominant peptide, Gag-6 in the p17 region, was targeted by 50% of neutralizers yet by only 30% of nonneutralizers. This trend of a higher percentage of epitope targeting in neutralizers was observed in over 48% of Gag (32/66 Gag peptides, with 21/66 Gag peptides targeted by neither group), in accordance with our data showing that the breadth of Gag-specific $CD4^+$ T cell responses is significantly higher in neutralizers (Fig. 2A).

**$CD4^+$ T cell targeting of some Env peptides may differ between neutralizers and nonneutralizers.** Our analysis also assessed Env-specific $CD4^+$ T cell responses at the peptide level. Only 33% of Env peptides were targeted by the cohort, which is less than half of that observed for Gag (68%). These Env-specific $CD4^+$ T cell responses were predominantly in the conserved (C) domains and to a lesser extent in the variable (V) loops (Fig. 8A), which may be due to superior $CD4^+$ targeting of conserved re-

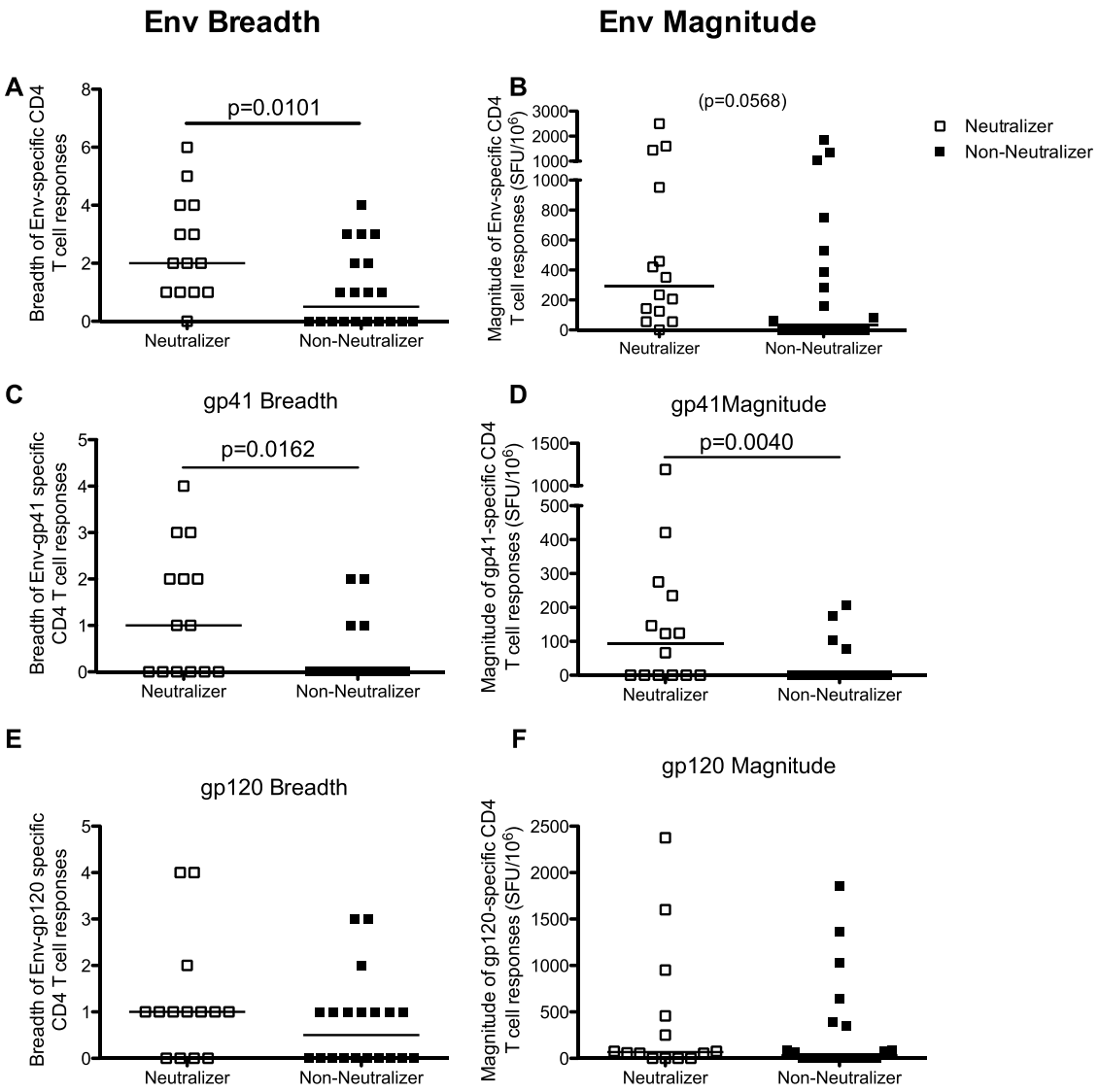


**FIG 4** Env-specific $CD4^+$ T cell responses, particularly those targeting gp41, are associated with enhanced neutralizing antibody breadth in viremic controllers. The breadth (A) and magnitude (B) of Env-specific $CD4^+$ T cell responses are shown for neutralizers and nonneutralizers. Significant differences were observed for Env breadth based on a Mann-Whitney test. The breadth (C) and magnitude (D) of gp41-specific $CD4^+$ T cell responses were significantly different between neutralizers and nonneutralizers by a Mann-Whitney test. However, the breadth (E) and magnitude (F) of gp120-specific $CD4^+$ T cell responses were not significantly different between the two groups (Mann-Whitney). Data shown are median values.

gions or limited detection of variable T cell epitopes by ELISPOT assay with consensus peptides. The most immunodominant peptide in gp120 was Env-293 (AAEQLWVTVYYGVPVWK) (6/34, 17.6%), which represents a highly conserved region within the C1 domain. We observed that 28.6% of neutralizers and only 10% of nonneutralizers targeted this peptide (Fig. 8B). In gp41, the most immunodominant peptide was Env-365 (GIKQLQARVLAVER YLK) (5/34, or 14.5%) (Fig. 8A), which was targeted by 28.5% of neutralizers and by only a single nonneutralizer. Neutralizers exclusively targeted nine peptides in gp41 (Fig. 8B), which was concordant with our earlier observation that neutralizers exhibited a significantly greater breadth of gp41-specific $CD4^+$ T cell responses (Fig. 4C). Three of the peptide-specific $CD4^+$ T cell responses made exclusively by neutralizers were either in or in close proximity to the highly conserved membrane-proximal external region (MPER), which is a structurally contiguous sequence of amino acids known to be targeted by well-defined bnAbs such as 4E10, 2F5, and 10E8 (27). However, since these potential MPER-specific $CD4^+$ T cell responses were observed in only a very small percentage of our neutralizers (7.1 to 14.2%), with no statistical significance observed between the two groups, it remains unclear whether there is any link between peptide-specific $CD4^+$ T cell responses and antibody neutralization breadth.

## DISCUSSION

Enhanced virus-specific $CD4^+$ T cell responses to HIV have been repeatedly linked to better clinical outcomes, suggesting that HIV-specific $CD4^+$ T helper cells contribute to efficient antiviral immunity (10–19). However, determining if and how HIV-specific $CD4^+$ T cell responses aid the generation of antibody breadth

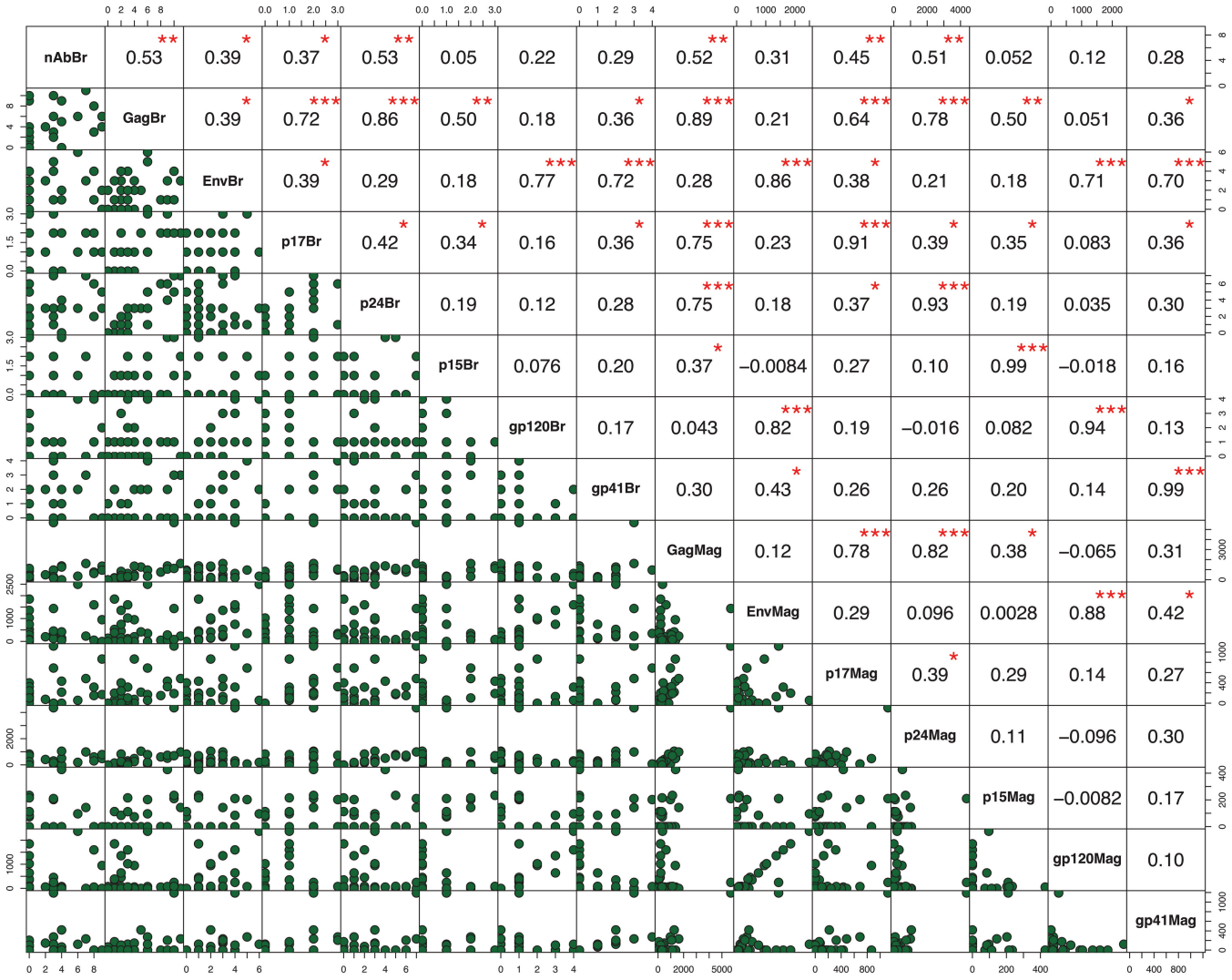


**FIG 5** Scatter plot matrices represent the relationship between breadth of neutralizing antibodies and CD4$^+$ T cell targeting of different HIV proteins (Gag and its p17, p24 and p15 components and Env and its gp120 and gp41 components) in 34 viremic controllers. The diagonal cells of the matrices contain the names of the variables, with Br and Mag denoting the breadth and magnitude of HIV-specific CD4$^+$ T cell targeting, respectively. The lower triangle contains all the pairwise scatter plots (with data for all 34 viremic controllers shown as green circles). The mirror upper triangle contains the Spearman rank correlation values (*r* values). Significant correlations are identified as follows: ***, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$. For example, the magnitude of the Gag-specific CD4 T cell responses is positively correlated with antibody neutralization breadth, with an *r* value of 0.52 and a *P* value of <0.01.

have remained unclear. Here, we show in a cohort of untreated HIV controllers matched for viral loads and CD4$^+$ T cell counts, but discordant for neutralizing antibodies, that HIV-specific CD4$^+$ T cell responses are associated with the generation of antibody neutralization breadth in chronic infection. Specifically, we find that Gag-specific CD4$^+$ T cell responses and, to a lesser extent, gp41-specific CD4$^+$ T cell responses are significantly enhanced in neutralizers. These data suggest that although vaccine-induced HIV-specific CD4$^+$ T cells could provide more targets for HIV infection (24), the induction of such responses by immunization will likely aid in the development of effective antiviral humoral immunity.

Our findings are consistent with the hypothesis that robust HIV-specific CD4$^+$ T cell responses may provide superior helper activity to Env-specific B cells, promoting their differentiation, maturation, and secretion of neutralizing antibodies. That we see the greatest differential CD4$^+$ targeting to Gag, an internal HIV protein unlinked to Env, is particularly intriguing. Larger viruses, such as vaccinia virus, which encodes hundreds of genes, elicit deterministically linked CD4$^+$ and B cell responses at the protein level in animal models (28). However, HIV is comparatively quite small, with only nine genes. Typically, smaller pathogens are more easily endocytosed as whole particles, enabling targeting of humoral and cellular immunity across separate proteins; indeed, studies of smaller pathogens like influenza virus and hepatitis B virus (HBV) indicate that protein specificity does not need to be shared by helper CD4$^+$ T cells and B cells (29–32). In addition, a recent study in vaccinia virus-vaccinated humans also advocates that there is no direct linkage between antibody and CD4$^+$ T cell targeting to individual proteins (33). Rather, the process of intermolecular help (also known as intrastructural help or heterotypic help) can enable T helper cells targeting internal proteins to pro-

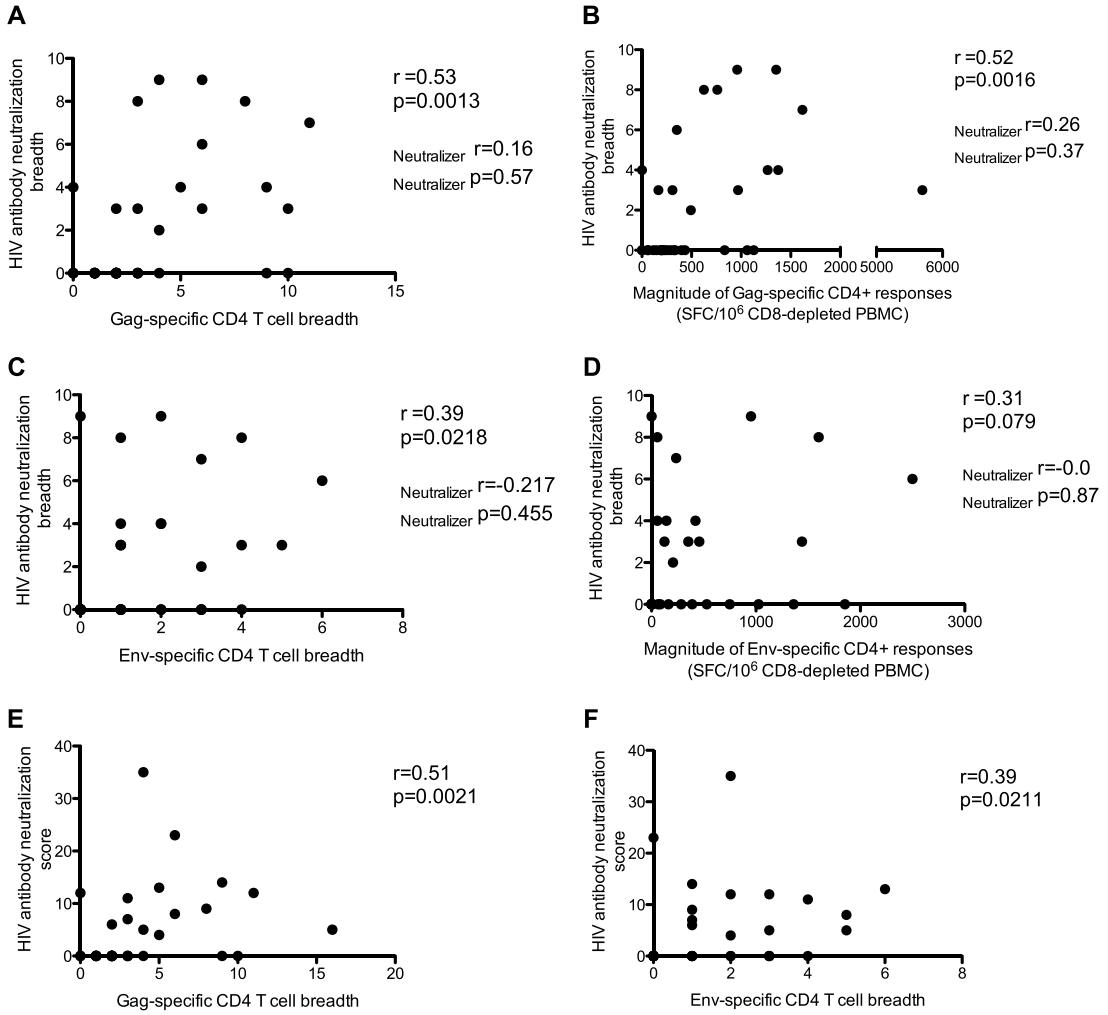


**FIG 6** Gag- and Env-specific CD4$^+$ T cell responses are correlated with neutralizing antibody breadth/score in 34 viremic controllers. A positive correlation was observed between the breadth (A) and magnitude (B) of Gag-specific CD4$^+$ T cell responses and antibody neutralization breadth. Similarly, a positive correlation was observed for the breadth of Env-specific CD4$^+$ T cell responses and antibody neutralization breadth (C) but not the magnitude (D). Lastly, associations with antibody neutralization scores were evaluated. A positive correlation was observed between the antibody neutralization scores and the breadth of Gag-specific CD4$^+$ T cell responses (E) and between the antibody neutralization scores and the breadth of Env-specific CD4 T cell responses (F). Spearman's rank was used to assess all correlations.

vide cognate help for surface protein-specific B cells to generate robust antibody responses.

A growing body of evidence has recently reinforced the idea that intermolecular help by Gag-specific CD4$^+$ T cells may enable Env-specific B cell responses in the setting of simian immunodeficiency virus (SIV)/HIV infection. Several studies have suggested that Gag-immunized macaques exhibit higher neutralizing antibody responses (or higher Env-specific antibodies after viral chal-

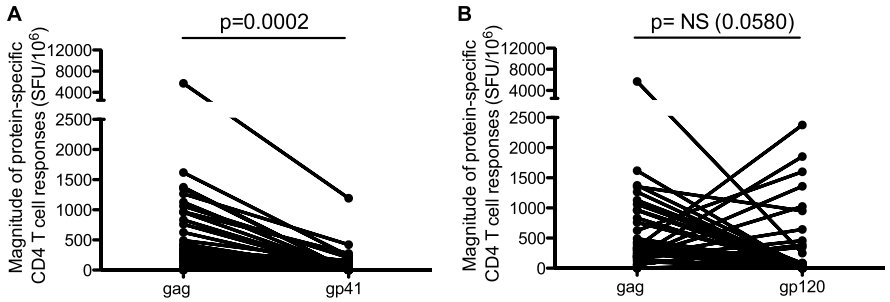


**FIG 7** The magnitude of Gag-specific CD4$^+$ T cell responses is significantly greater than that of gp41-specific CD4$^+$ T cell responses intrapatient in viremic controllers. The magnitudes of Gag- versus gp41-specific CD4$^+$ T cell responses (A) and Gag- versus gp120-specific CD4$^+$ T cell responses (B) are shown within each subject. Lines represent each subject. A paired *t* test was used to assess statistical significance.

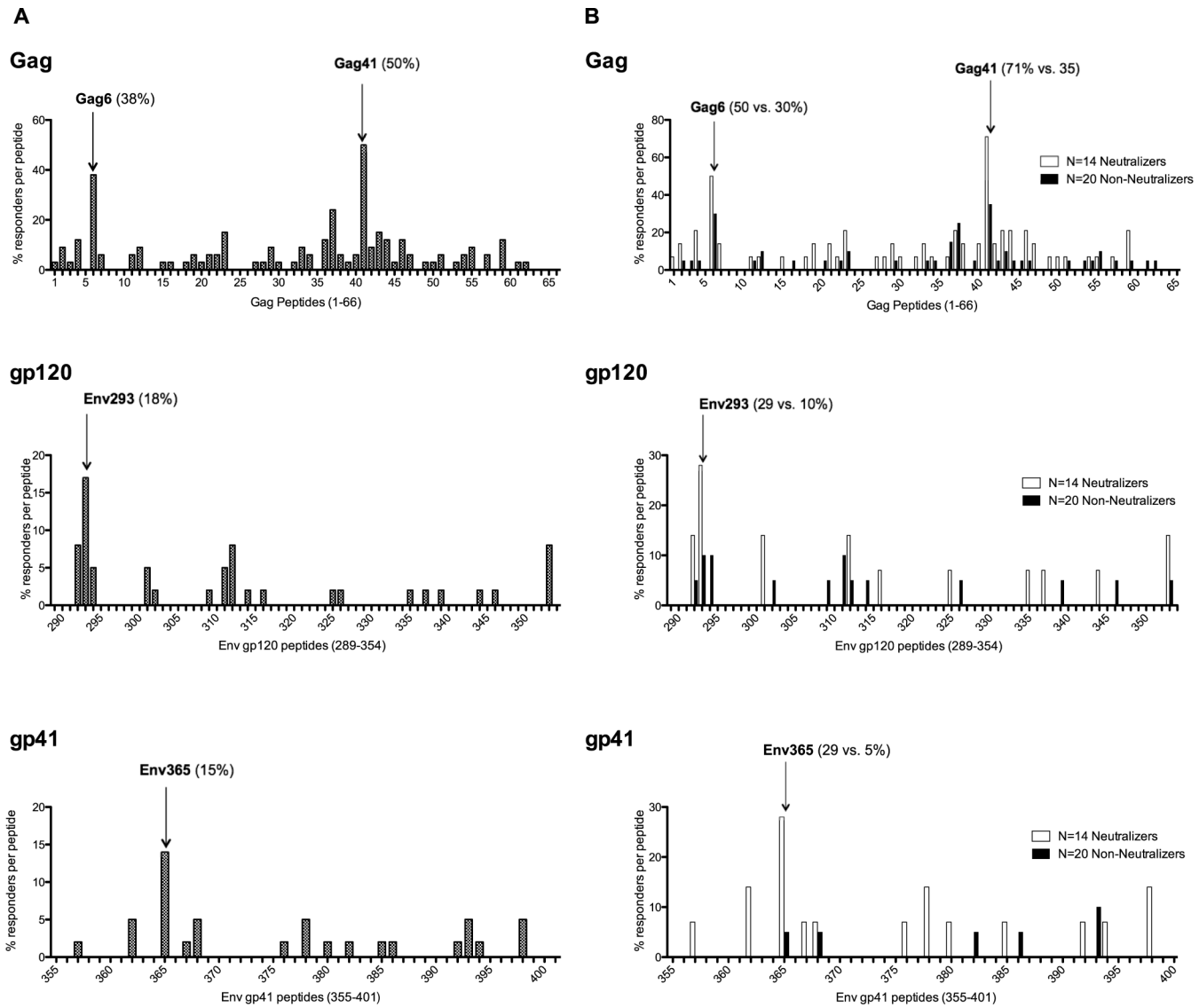


**FIG 8** HIV-specific CD4$^+$ T cell responses in viremic controller neutralizers may target distinct epitopes in Gag, gp120, and gp41. (A) The percentages of the 34 viremic controller subjects with detectable HIV-specific CD4$^+$ T cell responses to overlapping peptides (OLPs) spanning Gag, gp120, and gp41 are shown. (B) The percentages of neutralizers and nonneutralizers showing detectable HIV-specific CD4$^+$ T cell responses to each OLP spanning Gag, gp120, and gp41. For Gag, OLPs 1 to 66 were tested, with Gag p17 represented in linear amino acid sequence by OLPs 1 to 17, p24 represented by OLPs 18 to 47, and p15 represented by OLPs 48 to 66. For Env gp120, OLPs 289 to 354 were tested. Env gp120 compasses variable (V) loops V1 to V5 and the conserved (C) domains, with the V1-V2 loops represented in linear amino acid sequence by OLPs 307 to 313, the V3 loop represented by OLPs 328 to 331, the V4 loop represented by OLPs 340 to 342, and the V5 loop represented by OLP 349. Last, OLPs 355 to 401 were tested spanning Env gp41, with the membrane-proximal external regions (MPERs) represented in linear amino acid sequence by OLPs 376 to 379. For each of the three proteins, the most immunodominant OLPs are highlighted, with values for the percent targeting shown in parentheses (Gag41, Gag6, Env293, and Env365).

lenge) than control animals (34–37). In one study, three out of four Gag-immunized macaques challenged with SHIV exhibited neutralizing antibodies at day 40 postinfection, while the controls showed detectable neutralizing antibodies only at day 67 postinfection (35). Likewise, low neutralizing antibody titers were observed in Gag-immunized macaques at day 24 after SIV challenge while the controls developed neutralizing antibody titers only after 4 months (34). Importantly, a recent study of rhesus macaques immunized with a vector expressing SIV Gag/Pol and challenged with SHIV resulted in the rapid appearance of Env antibodies, despite the absence of Env in the vaccine (37). Nabi et al. showed (in subsequent mouse models) that Gag/Pol-specific CD4$^+$ T cells increase the antibody response to Env by providing intermolecular help. These studies therefore support the notion that robust Gag-specific CD4$^+$ T cells in HIV-infected humans may provide cognate help to aid in the development and preservation of Env-specific neutralizing antibodies. Indeed, our data imply that intermolecular Gag-specific CD4$^+$ help may act in concert with intramolecular Env-specific CD4$^+$ help to B cells for the generation of Env-specific antibodies. However, since it is not possible to determine cause and consequence in our analysis, an alternate hypothesis is that only Env-specific CD4$^+$ help is important *in vivo*, with

Gag-specific responses merely linked by their association with gp41. Moreover, although 68% of viremic controllers made one or more detectable responses to Env (23/34 subjects), the lack of an association between gp120-specific $CD4^+$ T cell responses and antibody neutralization could be influenced by the highly variable nature of gp120 and the use of peptides that do not represent the autologous virus.

When differences in $CD4^+$ T cell targeting between patient groups are analyzed, it is important to consider the influence of clinical parameters. Viremic controllers were selected for this study as they have detectable viral loads in the absence of antiretroviral therapy (50 to 2,000 HIV RNA copies/ml) and typically exhibit robust HIV-specific $CD4^+$ T cell responses. Our viremic controller cohort showed no significant difference between neutralizers and nonneutralizers in median viral loads, $CD4^+$ T cell counts, or years since HIV diagnosis. However, we did observe a trend toward lower median $CD4^+$ T cell counts in our viremic controller neutralizers than that in nonneutralizers ($P = 0.06$). The lower median $CD4^+$ T cell count was not correlated with any of the other clinical parameters studied. Although the $CD4^+$ T cell count in neutralizers trended lower than that in nonneutralizers, it is important to note that neutralizers demonstrated significantly enhanced HIV-specific $CD4^+$ T cell responses.

To further interrogate differences in $CD4^+$ T cell targeting between our patient groups, we also considered the influence of HLA DRB1 haplotypes, which have been reported to influence clinical outcome (14). Overall, the viremic controller neutralizers and nonneutralizers in our study showed comparable HLA-DRB1 allele distributions although much larger sample sizes would be necessary to determine whether there are significant DRB1 allele frequency differences between these groups at the population level. Despite some differences in HLA allele frequencies, such as DRB1*01:01 which was more common among the neutralizers and a higher proportion of DRB1*07:01 expression in nonneutralizers, it is not clear whether DRB1 variation has any impact on the immunodominance of $CD4^+$ peptides. Indeed, we found no apparent associations between commonly targeted $CD4^+$ peptides and a given DRB1 allele. Importantly, while CD8 peptides are typically presented by a single HLA class I molecule, a characteristic trait of $CD4^+$ peptides is their promiscuous presentation in the context of multiple diverse HLA class II molecules (38). This degeneracy in binding likely minimizes the impact of HLA variation and allows greater viral sequence diversity within the peptide. Thus, differential targeting of promiscuous $CD4^+$ epitopes by neutralizers and nonneutralizers is likely independent of variation in patient HLA haplotypes. Further work will be required to elucidate whether neutralizers exhibit any differences in their autologous viral sequences or modifications in envelope glycosylation that may influence the presentation and recognition of T cell epitopes.

Beyond targeting, the nature of $CD4^+$ T help provided to B cells *in vivo* remains poorly defined in humans but is thought to include signals primarily mediated by CD40 ligand interaction or by the release of cytokines such as interleukin-4 (IL-4) and IL-21 (39). In our study, IFN-γ was used to identify HIV-specific $CD4^+$ T cell responses. The IFN-γ ELISPOT assay is a powerful tool that is widely used and stringently validated for the quantification and epitope mapping of HIV-specific CD8 and $CD4^+$ T cell responses in natural infection studies and vaccine trials. Arguably, a limitation of this assay is that a readout of IFN-γ secretion by $CD4^+$ T cells may skew toward a Th1 phenotype and may miss the quantification of other $CD4^+$ T helper responses with alternative functions and phenotypes. However, it is now well established that the traditional dichotomy between Th1 cells and Th2 cells is insufficient to describe the complexity of $CD4^+$ T cells, which show great plasticity in their functional properties and cytokine profiles (40). Indeed, numerous studies have found that IFN-γ is secreted (at various levels and frequencies) by several human T helper subsets, including T follicular helper (Tfh) cells. Notably, Gag- and Env-specific Tfh cells can secrete IFN-γ in addition to IL-21 (41). This raises several possibilities for interpreting our data; peripheral Gag-specific Tfh cells could be driving the correlation between Gag-specific $CD4^+$ T cells and neutralizing antibody breadth or may act as a surrogate marker of Gag-specific $CD4^+$ T cells that traffic to and provide help for B cells localized within germinal centers. Locci et al. and Cohen et al. showed that higher proportions of bulk peripheral Tfh cells and Tfh-like cells are correlated with the development of broadly neutralizing antibodies in HIV-infected individuals assessed early postinfection (7, 9). Yet a different study in chronically HIV-infected individuals found no correlation between the frequencies of peripheral Tfh cells with neutralization activity (8). Notably, none of these studies examined HIV-specific $CD4^+$ T cells. However, an eloquent study by Yamamoto et al. recently reported that the quantity and quality of Env-specific germinal center (GC) Tfh cells were associated with broader antibody neutralization in SHIV-infected macaques (6). These authors found that IL-4-producing Env-specific GC Tfh cells were associated with an expansion of Env-specific immunoglobulin G-positive GC B cells, and using cluster analysis they identified 13 variables, including the percentage of Env- and Gag-specific $CD154^+$ GC Tfh cells that were associated with neutralization score, whereas this was not true for Gag-specific IFN-γ responses.

A focus of our study was to accurately define the specificity of $CD4^+$ T cell responses to individual HIV proteins and peptides in the peripheral blood of HIV-infected subjects. To achieve this, we utilized an IFN-γ secretion ELISPOT assay that we along with other investigators have used extensively to map T cell responses (13–16). Using this assay, we were able to show increased HIV-specific $CD4^+$ T cell targeting in subjects with neutralizing breadth, but the caveats of this approach are that we were not able to delineate peripheral Tfh cells, and we were not able to investigate the functional characteristics of $CD4^+$ T cells that may be associated with optimal $CD4^+$ help to B cells. Further work analyzing paired peripheral blood and lymph node biopsy specimens from HIV-infected neutralizers will be required to identify whether epitope targeting of peripheral HIV-specific $CD4^+$ T cells accurately represents the targeting of HIV-specific $CD4^+$ T cells in the germinal centers and whether the quantity, quality, or helper function of HIV-specific Tfh cells to distinct proteins is correlated with antibody neutralization.

In conclusion, while HIV-specific $CD4^+$ T cell responses were detected in persons with and without neutralization breadth, our results reveal that Gag-specific T helper responses and, to a lesser extent, gp41-specific T helper responses were significantly enhanced in viremic controller patients with Env-specific neutralizing antibodies. Moreover, the increasing breadth and magnitude of Gag-specific $CD4^+$ T cell responses were moderately correlated with enhanced antibody neutralization breadth. Our data also suggest that differences may exist in $CD4^+$ targeting at the pep-

tide-level among patients, with preferential targeting of discrete Gag, gp120, and gp41 epitopes by neutralizers. These patterns in HIV-specific $CD4^+$ targeting are not driven by differences in viral loads, number of years since HIV diagnosis, or HLA class II DRB1 allele expression levels. Together, these data suggest that robust Gag-specific $CD4^+$ T cell responses, in addition to gp41-specific $CD4^+$ T cell responses, may provide superior intermolecular help *in vivo* to Env-specific B cells necessary for the generation or maintenance of Env-specific neutralizing antibodies. The comprehensive identification of HIV-specific $CD4^+$ T cell responses associated with antibody neutralization during chronic HIV infection are also likely to have important implications for HIV vaccines. Our data suggest that HIV-specific $CD4^+$ T helper cells should be considered a component of an effective vaccine-induced response for the production of neutralizing antibodies.

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