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Human Immunodeficiency Virus-Specific Gamma Interferon Enzyme-Linked Immunospot Assay Responses Targeting Specific Regions of the Proteome during Primary Subtype C Infection Are Poor Predictors of the Course of Viremia and Set Point▽

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It is unknown whether patterns of human immunodeficiency virus (HIV)-specific T-cell responses during acute infection may influence the viral set point and the course of disease. We wished to establish whether the magnitude and breadth of HIV type 1 (HIV-1)-specific T-cell responses at 3 months postinfection were correlated with the viral-load set point at 12 months and hypothesized that the magnitude and breadth of HIV-specific T-cell responses during primary infection would predict the set point. Gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay responses across the complete proteome were measured in 47 subtype C HIV-1-infected participants at a median of 12 weeks postinfection. When corrected for amino acid length and individuals responding to each region, the order of recognition was as follows: Nef > Gag > Pol > Rev > Vpr > Env > Vpu > Vif > Tat. Nef responses were significantly ($P < 0.05$) dominant, targeted six epitopic regions, and were unrelated to the course of viremia. There was no significant difference in the magnitude and breadth of responses for each protein region with disease progression, although there was a trend of increased breadth (mean, four to seven pools) in rapid progressors. Correlation of the magnitude and breadth of IFN-γ responses with the viral set point at 12 months revealed almost zero association for each protein region. Taken together, these data demonstrate that the magnitude and breadth of IFN-γ ELISPOT assay responses at 3 months postinfection are unrelated to the course of disease in the first year of infection and are not associated with, and have low predictive power for, the viral set point at 12 months.

The identity of T-cell immune responses during early infection that would serve as a predictor of the viral set point, and hence disease progression, would be important not only for understanding pathogenesis, but also for providing insight into immunological markers that could be used in vaccine trials. Many studies have utilized the gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay for identifying T-cell immunity during chronic infection and for identifying T-cell epitopes (1, 13, 16, 28, 29), and it is in current use for measuring vaccine-induced immunogenicity. Whether IFN-γ ELISPOT assay responses, measured during acute infection, have utility for understanding the course of early disease in human immunodeficiency virus type 1 (HIV-1) infection remains open to question.

Acute infection is characterized by an explosion of viral replication, with antigen-specific memory $CD4^+$ T cells being preferentially infected (7, 12), most likely resulting in CD4 depletion mainly in gut-associated lymphoid tissue (8, 30, 39). Studies of simian immunodeficiency virus (SIV)-infected monkeys have shown that a massive loss of $CD4^+$ T cells in gut-associated lymphoid tissue occurs within days of infection (25, 40), and it is thought that immunity to HIV during the acute stage of infection determines the subsequent course of the disease. It is known that some individuals can successfully contain viral replication over years of infection (11), and the initial rise in HIV-specific $CD8^+$ T cells, imparting cytotoxic T-cell activity, is thought to be one important component in the control of viremia (6). It is also apparent that virus fitness interlinked with the host genetic background can contribute to virus control and the delay of HIV disease progression (2, 9, 17, 20).

The identity of important host immune responses has shown that $CD8^+$ T-cell recognition of Gag is associated with control of HIV replication (22, 28, 33, 43) and that a broad spread of epitopes recognized within Gag appear to be correlated with low viremia (22), which may impact viral fitness. Some studies, including our own, found correlations between preferential recognition of Gag and the viral load (13, 28), with other studies finding no associations (1). It is possible that these association differences are due to the types of cohorts investi-

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TABLE 1. Clinical details of the cohort at enrollment and 12 and 48 to 56 weeks postinfection

Parameter	Value at:								
	Enrollment			12 wk postinfection			48–56 wk postinfection		
	Wk	Log_{10} RNA copies/ml	No. of CD4 cells/μl	Wk	Log_{10} RNA copies/ml	No. of CD4 cells/μl	Wk	Log_{10} RNA copies/ml	No. of CD4 cells/μl
Mean	6.1	4.73[a]	546	12.3	4.46	559[b]	52	4.33[a]	471[b]
95% confidence interval limits		4.45–5.0	482–609		4.21–4.71	490–628		4.04–4.62	405–537
Range		2.73–6.17	197–989		2.6–5.79	255–1,358		2.6–6.22	174–1,093

[a] $P < 0.01$.
[b] $P < 0.05$.

gated and the time since infection, with most studies being performed at the chronic stage of infection.

While there have been many studies investigating the relationship between cytotoxic-T-lymphocyte (CTL) responses in chronic infection and disease progression, there have been few studies to show whether these associations hold true during the acute and primary stages of infection. Events in acute infection may be critical for governing the subsequent course of disease but are also pertinent for identifying clues to vaccine-induced immunity. This study investigates the relationship between the magnitude and breadth of T-cell responses in primary infection and the viral load at 12 months postinfection, with the latter measurement being used as a surrogate marker for disease progression. We hypothesized that the magnitude and breadth of HIV-specific T-cell responses during primary infection would be correlated with the viral set point.

MATERIALS AND METHODS

Study subjects. An ongoing longitudinal cohort of acutely HIV-1-infected individuals were enrolled as part of the Centre for AIDS Programme Research in South Africa (CAPRISA) 002 acute-infection study in Durban, South Africa (38). We report on 47 individuals in the cohort. The time postinfection was estimated by a prospective RNA-positive/antibody-negative measurement or was taken as the midpoint between the last antibody-negative test and the first antibody-positive enzyme-linked immunosorbent assay. The study cohort wasn described previously (38), when study participants were followed for up to 24 months, and the study is still ongoing; data from events within the first 3 to 12 months are reported here. The University of KwaZulu-Natal, University of Witwatersrand, and University of Cape Town Ethics Committees approved this study, and all the subjects provided written informed consent for participation in the study.

Plasma viral-load determination, CD4 T-cell counts, and HLA typing. Plasma HIV-1 RNA levels were quantified using the Cobas Amplicor HIV-1 monitor test version 1.5 (Roche Diagnostics, Branchburg, NJ). Absolute blood $CD4^+$ T-cell counts were determined using the FACSCalibur flow cytometer and were expressed as cells/mm^3. High-resolution HLA typing was performed as previously described (29).

Synthetic subtype C peptides. A set of 432 synthetic overlapping peptides spanning the entire expressed HIV-1 clade C proteome corresponding to gene products from the HIV-1 consensus C (Gag, Vif, Vpr, and Vpu), isolate Du151 (Pol, Nef, Tat, and Rev), and isolate Du179 (gp160 Env) were synthesized using 9-fluorenylmethoxy carbonyl chemistry and standard-based solid-phase techniques (Natural and Medical Sciences Institute, University of Tubingen, Tubingen, Germany). The nonconsensus synthesized peptides were based on sequences from isolates used for the manufacture of a clade C vaccine (42). The estimated purities of the peptides were >80% as measured by high-performance liquid chromatography and mass spectrometry. Individual peptides were diluted in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and prepared as previously described (28).

Cell preparation, generation of cell lines, and HLA restriction. Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-Hypaque density gradient centrifugation (Amershan Pharmacia, Uppsala, Sweden), cryopreserved in 90% heat-activated fetal bovine serum (Invitrogen, Paisley, United Kingdom) plus 10% dimethyl sulfoxide, and stored in liquid nitrogen until they were needed. The frozen PBMC were thawed and rested in RPMI 1640 (Invitrogen, Paisley, United Kingdom) containing 10% heat-inactivated fetal bovine serum and 50 U gentamicin 50 (Invitrogen, Paisley, United Kingdom) at 37°C and 5% CO_2 for 18 h prior to use in ELISPOT assays. Epstein-Barr virus-transformed B-cell lines for use as class I HLA-matched targets were established for each individual and maintained in RPMI 1640 supplemented with 20% fetal calf serum and 50 U of gentamicin (R20) at 37°C, 5% CO_2. PBMC (10^6) were stimulated with 10 μg peptide and 330 U/ml interleukin 7 (IL-7) (Roche), and the cells were transferred to a 24-well plate and incubated at 37°C, 5% CO_2. After 1 week in culture, 25 U/ml of IL-2 was added, and after 14 to 21 days in culture, PBMC (effectors) were harvested for use in a standard ^{51}Cr release assay. HLA-matched B cells (targets) were pulsed with 10 μg peptide and 100 to 150 μCi of ^{51}Cr for 1 h at 37°C, 5% CO_2. Following extensive washing with 1% fetal calf serum (FCS) in RPMI, the B cells were resuspended in 10% FCS in RPMI and mixed with effectors in triplicate at a 50:1 and 25:1 effector/target ratio in V-bottom 96-well plates, and the killing assay mixture was incubated for 4 to 5 h at 37°C, 5% CO_2. Twenty-five microliters of the supernatant was harvested onto LumaPlates-96 (Packard Bioscience, Billerica, MA) and allowed to dry overnight, and the radioactivity was detected using a gamma counter (TopCount-NXT; Packard Bioscience). Spontaneous release of ^{51}Cr was determined by incubating targets with R10, and the maximum release was determined from the total radioactivity released by the targets in the presence of 0.01% Triton X-100. Specific lysis (percent) was calculated as follows: $(\text{experimental}_{\text{average}} - \text{spontaneous}_{\text{average}})/(\text{maximum}_{\text{average}} - \text{spontaneous}_{\text{average}}) \times 100$.

IFN-γ ELISPOT assay. HIV-1-specific T-cell responses were quantified by the IFN-γ ELISPOT assay using the set of overlapping peptides arranged in a pool-matrix format as previously described. Briefly, PBMC were plated at 1×10^5 cells/ml with peptides at a concentration of 2 μg/ml in 96-well polyvinylidene difluoride plates (MAIP S45; Millipore, Johannesburg, South Africa) that had been coated with 5 μg/ml anti-IFN-γ monoclonal antibody 1-DIK (Mabtech, Stockholm, Sweden) overnight at 4°C. Phytohemagglutinin (Calbiochem, San Diego, CA) stimulation at 4 μg/ml was used as a positive control, and no peptide stimulation (medium alone) was used as a negative control. As part of an ongoing quality assurance program, thawed PBMC that had been tested previously for responses to a pool of optimal peptides corresponding to cytomegalovirus, Epstein-Barr virus, and influenza viruses were included on the same plate as a positive quality control sample for assay consistency. The plates were incubated overnight at 37°C, 5% CO_2, and developed as previously described. Individual spots were counted with an automated ImmunoSpot plate reader (Cellular Technology Ltd., Cleveland, OH) and expressed as spot-forming units (SFU) per million PBMC. Responses were initially evaluated by reacting PBMC with peptides arranged in a pool-matrix format and followed with a second ELISPOT assay on selected participants to confirm positive responses at the single-peptide level in triplicate. The following criteria were used to define positive responses: (i) reactivities to peptide pools of ≥67 SFU/10^6 PBMC after background subtraction and at least three times greater than the mean background activity and (ii) a matching peptide in the matrix pool array. Peptide truncations corresponding to immunodominant peptides in Nef were generated and used in an ELISPOT assay to identify the optimal epitope. A total of 100,000 PBMC per well were incubated with the truncated peptides at different concentrations from 0.2 to 2,000 ng/ml in an ELISPOT assay as described previously. The optimal epitope was defined as the peptide that induced the highest specific IFN-γ production at the lowest peptide concentration.

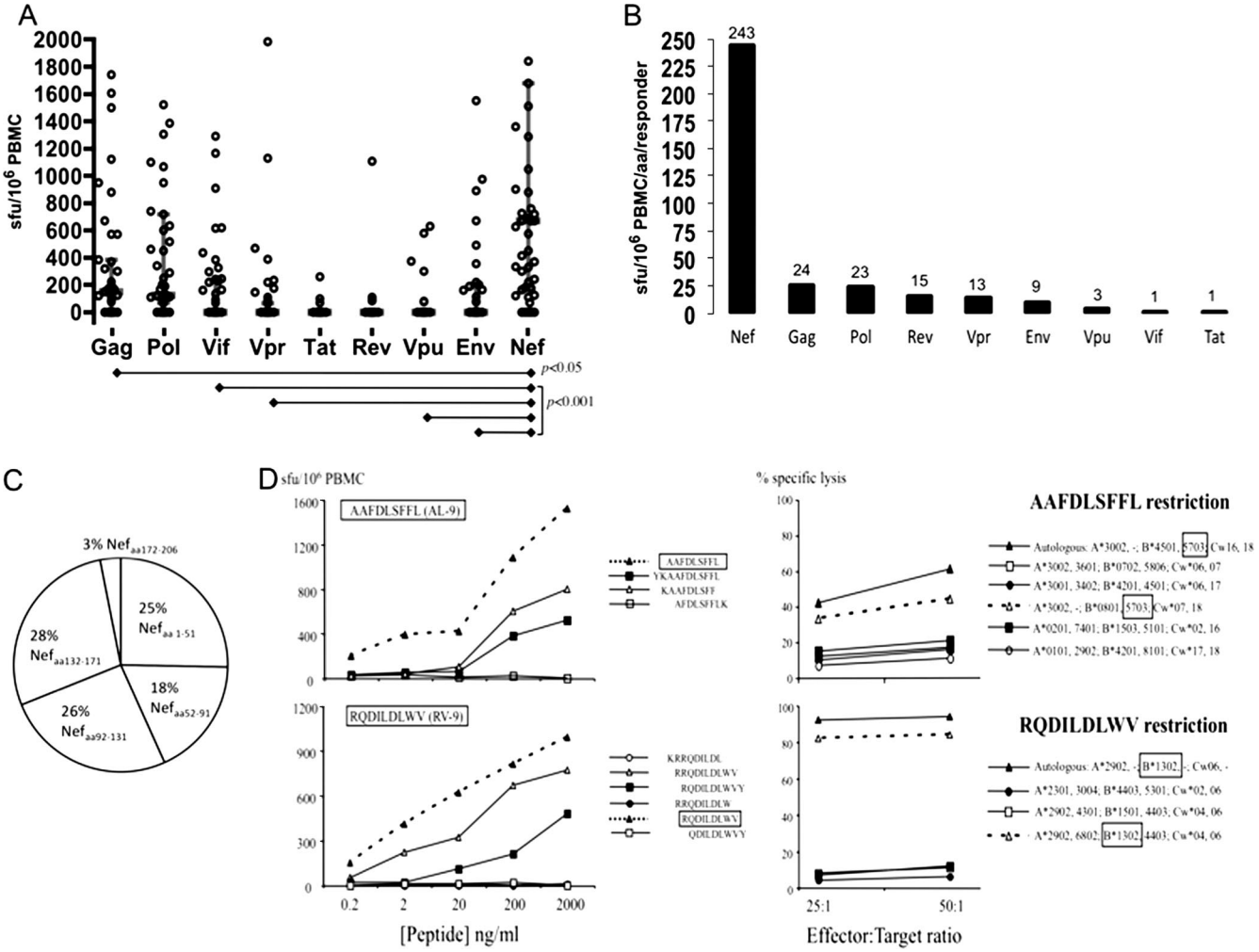

FIG. 1. Magnitude, frequency, and hierarchy of IFN-γ ELISPOT assay responses at 3 months postinfection. (A) Cumulative magnitude of IFN-γ ELISPOT assay responses across the expressed subtype C HIV-1 genome showing statistical significance between Gag, Pol, Vif, Vpu, Env, and Nef. The magnitude is shown as the total response of all pools for each region, and each point represents a response per participant measured as SFU/million PBMC. Vertical lines represent the interquartile ranges. (B) Order of the corrected magnitudes of IFN-γ ELISPOT assay responses across the genome, calculated by dividing the total magnitude (SFU/10^6 PBMC) of the response per protein by the amino acid length for each protein and expressing it as a proportion of responders for that region. (C) Pie chart showing the amino acid stretches (corresponding to peptide pools) recognized within Nef. (D) Identification of two optimal epitopes in Nef using truncated peptides and HLA restriction killing assays. The boxed sequences in the truncation experiments were the optimal epitopes giving the highest IFN-γ ELISPOT readout at the lowest peptide concentration. The optimal epitope was then used in HLA restriction experiments, where the partial mismatched B-lymphoblastoid target that gave the percent specific CTL lysis at 25:1 and 50:1 effector/target ratios closest to that of the autologous B-cell line provided the restricting allele. The boxed HLA types are the HLA alleles giving the highest CTL response that matched the autologous HLA background. The data were generated from an early infection cohort reported previously (4).

Statistical analysis. Statistical analysis and graphical presentation were performed using InStat and GraphPad Prism version 3.0 software. Data are expressed as median values with interquartile ranges and were analyzed by the use of nonparametric statistics. Statistical analysis of significance was performed with either Mann-Whitney or Kruskal-Wallis analysis of variance using Dunn's test for multiple comparisons. All tests were two tailed, and a P value of <0.05 was considered statistically significant. The relationships between the plasma viral load, CD4 T-cell counts, CD4 slopes, and the breadth or magnitude of HIV-specific T-cell responses were analyzed using Spearman rank correlations. Correlations with the viral load were made by transforming RNA copies/ml to $\log_{10}$ copies.

RESULTS

Study subjects. Forty-seven subtype C HIV-1-infected participants in the CAPRISA 002 acute-infection study were recruited within a mean of 6.1 weeks after infection (Table 1), and responses across the complete proteome were measured using the IFN-γ ELISPOT assay at a median of 12 weeks and a range of 10 to 17 weeks postinfection. Table 1 shows the mean $\log_{10}$ RNA copies and CD4 counts at three time points during the first year of infection (at enrollment and 12 and 48 to 56 weeks postinfection), showing an overall decline in viremia of 0.4 $\log_{10}$ copies across the cohort, with a significant parallel reduction of 75 CD4 cells/ml.

HIV-specific IFN-γ$^+$ T-cell responses at 3 months postinfection are dominated by recognition of Nef. Nef responses were found to be highly dominant at 12 weeks after infection. Figure 1A shows the cumulative magnitude of IFN-γ ELISPOT

TABLE 2. Reactive peptides in Nef pools 2 to 5, magnitudes of response, frequencies, and known epitopes within the sequence

Reactive-peptide sequence	% Responders (no./total)	Mean response ± SD (SFU/10^6 PBMC)	Range (SFU/10^6 PBMC)	Described epitopes[a]
EVGFPVRPQVPLRPMTYKA	16 (3/9)	554 ± 214	390–960	FPVRPQVPL (B7; B3501); RPQQVPLRPM (B4201; B8101); PQVPLRPMTY (B35); QVPLRPMYTK (A3; A11); VPLRPMTY (B35; B42); PLRPMTYK (A11)
RPMTYKAAFDLSFFLKEKG	36 (9/25)	382 ± 320	110–1,255	KAAFDLSFF (B5701; B5801); AAFDL SFFL (B5703); DLSFLKEK (A3; A11)
IHSKRRQDILDLWVYYHTQG	24 (6/25)	1,240 ± 1,113	150–3,915	RQDILDLWV (B1302); RQDILDLWVY (Cw07; B7); ILDLWVYHT (A2)
WVYHTQGYFPDWQNYTPGP	12 (3/25)	1,567 ± 1,578	145–3,710	YHTQGYFPDW (B17); HTQGYFPDW (B57; B5801); TQGYFPDWQNY (B15); GYFPDWQNY (A24); YFPDWQNY (A29; B57; B5801); YFPDWQNYT (A01)
PGPGVRYPLTFGWCFKLVP	80 (8/10)	2,005 ± 1,503	170–4,800	TPGPGVRYPL (B7;) GPGVRYPLTF (B35); VRYPLTFGW (B27); RYPL TFGW (A2301; A24); YPLTFGWCF (B18; B35; B5301); LTFGWCFKL (A2)
NNCLLHPMSQHGMEDADRE	14 (1/7)	570 ± 169		No previously defined epitopes

[a] From reference 23.

assay responses per expressed gene region, where the hierarchy of the total sum of responses was as follows: Nef (56,380 SFU/10^6 PMBC) > Pol (29,610 SFU/10^6 PMBC) > Gag (18,868 SFU/10^6 PMBC) > Env (16,995 SFU/10^6 PMBC) > Rev (12,550 SFU/10^6 PMBC) > Vpr (5,098 SFU/10^6 PMBC) > Vpu (2,038 SFU/10^6 PMBC) > Vif (508 SFU/10^6 PMBC) > Tat (430 SFU/10^6 PMBC). When corrected for responses per amino acid to account for the density of responses per expressed gene, and on a per responder basis, the order of recognized proteins became Nef > Gag > Pol > Rev > Vpr > Env > Vpu > Vif > Tat (Fig. 1B), with Nef being highly immunodominant. Figure 1C shows which regions of Nef were targeted, where there was a uniform distribution of responses between amino acids 1 and 171, and there was little recognition in the more hypervariable N terminus of Nef (amino acids 172 to 206).

Narrow and focused recognition of Nef. The majority of responses within Nef involved six recognized peptides, with multiple possible epitopes within each stretch. Table 2 shows the six peptides frequently recognized, where over 70% of responses were accounted for by three peptides in pool 3 (^{77}RPMTYKAAFDLSFFLKEKG^{95}, ^{101}IHSKRRQDILDLWVYYHTQG^{119}, and ^{113}WVYHTQGYFPDWQNYTPGP^{131}). The CTL epitopes that are embedded within each of these peptides have been described (23), except for the one found toward the N terminus of Nef (Table 2). The peptide corresponding to the most dominant response (80%; ^{129}PGPGVRYPLTFGWCFKLVP^{147}) possessed up to six previously described epitopes, and we were able to show in two additional peptide stretches, ^{77}RPMTYKAAFDLSFFLKEKG^{95} and ^{101}IHSKRRQDILDLWVYYHTQG^{119}, two unique HLA-restricted epitopes: B*5703, restricting AAFDLSFFL, and B*1302, restricting RQDILDLWIV (Fig. 1D and Table 2). Together, these data show that narrowly focused responses to selected epitopes in Nef during acute HIV-1 subtype C infection account for the majority of IFN-γ ELISPOT assay responses.

HIV-specific IFN-γ$^+$ T-cell responses at 3 months postinfection are not associated with the course of viremia in the first 12 months. To assess whether quantitative differences in HIV-1-specific cellular immune responses in the first few weeks of subtype C HIV-1 infection were related to the direction of viremia over the first 12 months of infection, we tested for an association between IFN-γ ELISPOT assay responses and changes in the viral load between 3 and 12 months. We based our analysis on the hypothesis that the magnitude of early HIV-specific T-cell responses at 3 months would determine the course of viremia. Teasing out individual viral-load changes within the cohort revealed positive and negative differences in viremia between 3 and 12 months. Figure 2A show the changes in $\log_{10}$ viremia, where some individuals showed a >1-$\log_{10}$ unit increase in viremia and some displayed <1-$\log_{10}$ unit-lower viremia. We used these differences to track the course of viremia for each participant and to relate these changes with the IFN-γ ELISPOT assay response profile for each participant. Figure 2B shows the total proportions of ELISPOT assay responses across the expressed genome for each participant as a heat map, where no discernible response profile was evident in relation to viral-load differences (Fig. 2A). Figure 2C quantifies this lack of association with the direction of viremia over the first year. As shown in Fig. 1A to C and Table 2, focused responses to Nef were omnipresent regardless of the negative or positive changes in the viral load at 12 months. We also examined whether responses at 6 months were associated with changes in the viral load over 12 months and identified a similar lack of association (data not shown). Also notable was the lack of association between the viral load and responses to Gag (Fig. 1C), which differs from our previous findings (28) in a cohort examined around 12 months after infection.

HIV-specific IFN-γ$^+$ T-cell responses at 3 months postinfection are not associated with early disease profiles. By additionally factoring in the course of absolute CD4 cell count changes, we were able to define disease profiles in the first year of infection according to rapid, intermediate, and slow progression. Rapid progressors were defined as those who had CD4 cell counts consistently below 350 cells/ml between 10 and 15 months postinfection. Slow progressors were defined as having viral loads consistently below 2,000 RNA copies/ml between 10 and 15 months postinfection, and intermediate

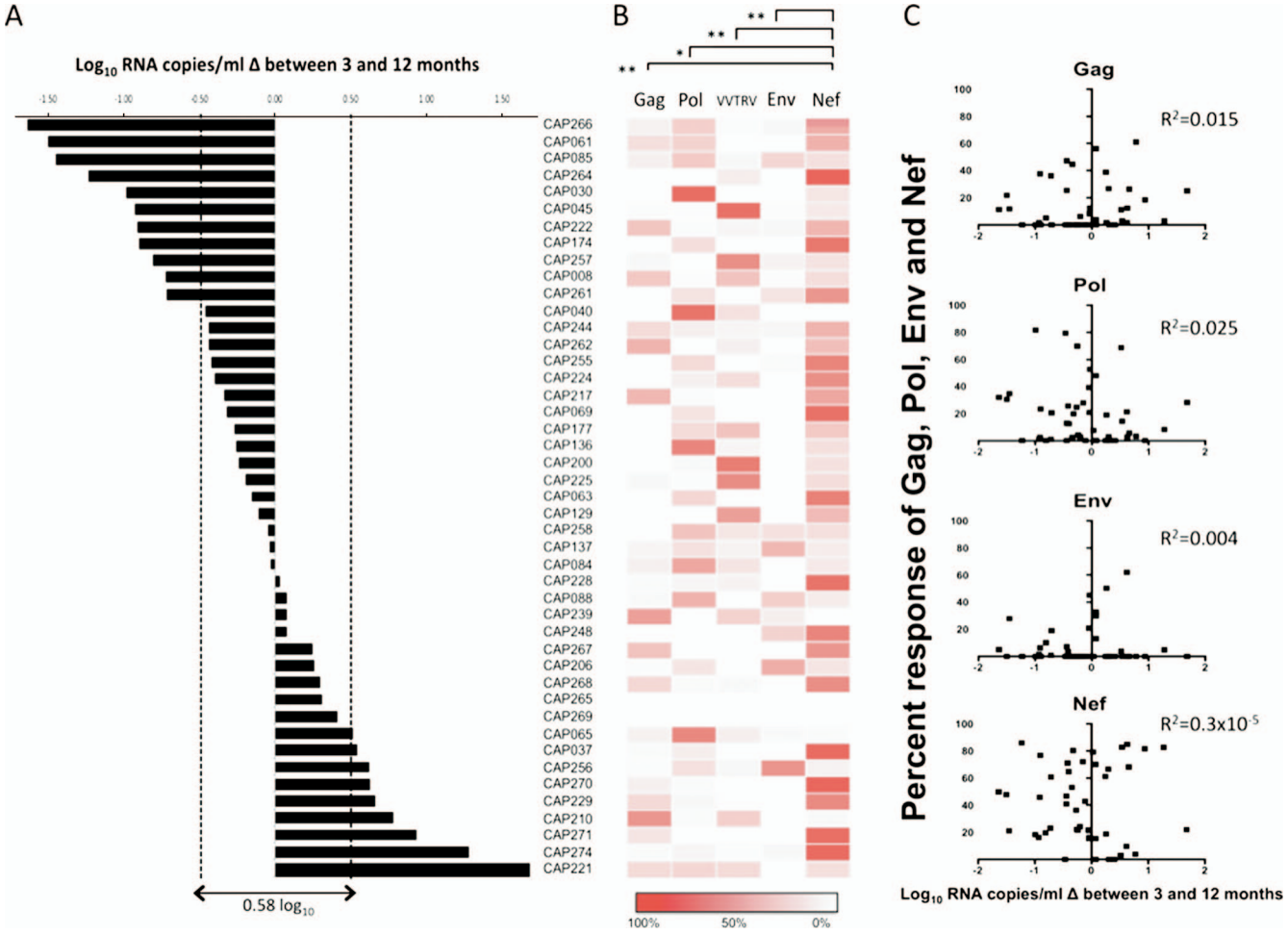

FIG. 2. Association of viral-load differences between 3 and 12 months with the magnitudes of ELISPOT assay responses at 3 months. (A) Tracking of $\log_{10}$ RNA copies/ml between 3 and 12 months for each of the 47 participants arranged from negative to positive differences between the two time points. The dotted line demarcates the calculated 95% confidence limits for the mean of three $\log_{10}$ viral-load measurements between 48 and 56 weeks postinfection (0.58 $\log_{10}$ RNA copies/ml) (Table 1). (B) Heat map reflecting the proportions of Gag; Pol; combined Vif, Vpr, Tat, Rev, and Vpu (VVTRV); and Env responses out of the total ELISPOT assay response across the complete proteome for each participant at 3 months. Statistical differences in the proportions of responses are shown (*, $P < 0.05$, and **, $P < 0.001$). (C) Spearman rank correlations between the percent responses for Gag, Pol, Env, and Nef and the difference in $\log_{10}$ RNA copies/ml between 3 and 12 months.

progressors were defined as those who fit in neither the rapid nor the slow category. We were able to validate the usefulness of using CD4 counts in our definition of slow and rapid progressors by associating CD4 slopes with the viral set point at 12 months (Fig. 3A). Across the complete group, there was a strong relationship between loss of CD4 cells and a high set point and between a positive CD4 slope and a low set point. Additionally, when we compared the subset of individuals who we classified as slow or rapid progressors ($n = 4$ and 8, respectively), we found that in those who were progressing slowly and controlling viremia, there was a median positive CD4 slope compared to the median negative slope found in the individuals classified as rapid progressors (Fig. 3A, inset). We then wished to identify whether the magnitudes of ELISPOT assay responses across the proteome had any relationship with CD4 slopes and hence with the clinical course of disease in the first year of infection. No association existed (r ranged from -0.018 to -0.002) between the magnitudes of ELISPOT assay responses and the CD4 slopes, suggesting that T-cell responses measured during primary infection had no bearing on the clinical measurement of disease progression.

The total magnitude and breadth (defined as the number of peptide pools recognized) of responses for each participant are shown in Table 3. Although there was no difference in the mean magnitude of response between slow and rapid progressors, there was a nonsignificant trend of increased breadth of response (mean, four to seven pools). It is possible that with a larger sample size, increased breadth might reveal a significant proportional response with the viral load and might indicate that an early broad response is not related to subsequent virus control. Interestingly, there was no response to any protein at 3 months in individual CAPRISA 269 (CAP269), despite virus control (Table 3). By mapping single peptide responses in the subset of four controllers and eight rapid progressors, we showed that rapid progressors possessed a diverse epitope recognition pattern, with Nef and Vif being dominant (8 and 6

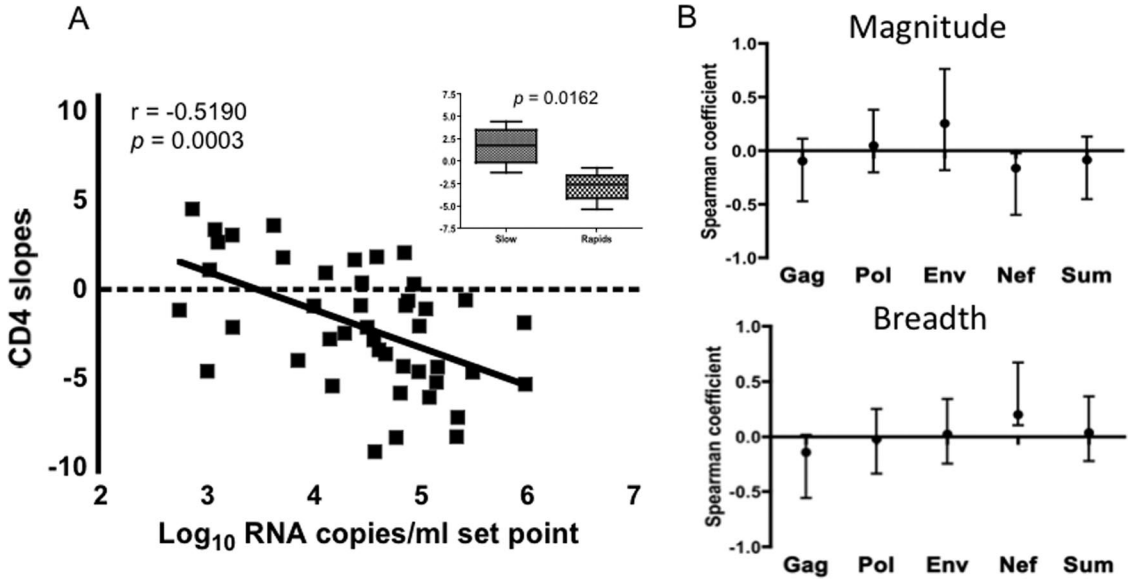

FIG. 3. Correlation of CD4 slopes with the viral set point and of the IFN-γ ELISPOT magnitude and breadth with the viral set point. (A) Correlation plot of $\log_{10}$ RNA copies/ml at the set point with CD4 slopes. CD4 slopes were calculated in Prism and utilized a mean of 15 ± 1.6 CD4 measurements over 52 weeks. The dashed line depicts zero slope. CD4 slope differences between slow ($n = 4$) and rapid ($n = 8$) progressors are shown in the inset. Box and whisker plots show median and interquartile range limits for CD4 slopes. The sloping line is the best-fit line. (B) Spearman correlation coefficients with 95% confidence intervals for the magnitudes and breadths of Gag-, Env-, Pol-, and Nef-specific T-cell responses with the viral set point at 12 months.

peptides, respectively, out of a total of 30 detected). Out of the eight rapid progressors, only two peptides in Gag could be identified (in individuals CAP008 and CAP210). As we had data for single-peptide responses from only two slow progressors, it was not possible to draw any conclusions or interpret differences between slow and rapid progression. What was notable, however, was that many of the peptide responses in both slow and rapid progressors matched previously described epitopes when the HLA allele background of each individual was taken into account (Table 3). Although fine mapping of epitopes and HLA restrictions was not performed, these data imply that globally common epitope responses are elicited during acute subtype C HIV-1 infection.

Lack of correlation between HIV-specific IFN-γ$^+$ T-cell responses and the viral load at 12 months postinfection. We wished to assess whether HIV-specific IFN-γ$^+$ T-cell responses measured at 3 months postinfection were correlated with the viral load at 12 months, with the aim of identifying if responses at 3 months could predict the set point. The set point was defined as the viral load at 12 months postinfection. To account for viral spikes, we calculated the mean $\log_{10}$ plasma RNA copies/ml at three time points around 52 weeks, ranging from 48 to 56 weeks. Correlating the magnitude and breadth of IFN-γ responses with the viral set point at 12 months revealed low (i.e., close to zero), nonsignificant associations for each protein region. Figure 3B shows plots of Spearman coefficients for the total magnitude and breadth of response for each region, depicting 95% confidence intervals. When the responses were normalized based on the number of peptides in the pool (i.e., response per peptide), there was similarly no significant association with the viral load at 12 months (data not shown). We also found that there were no associations with the set point when subregions within Gag, Pol, and Env were analyzed, namely, p17, p24, RT, integrase, gp41, and gp20 (data not shown). The breadth of response ranged from 0 to 11 peptide pools, with an average of 5.7 ± 3 pools. We initially hypothesized that early, broad recognition of epitopic regions would be important for virus control, as broader recognition of epitopes by HIV-1-specific T cells appears to be associated with virus control (22, 33). Depicting the 95% confidence intervals provided us with enough evidence to say that neither magnitude nor breadth, at the full protein level, is associated with the viral set point at 12 months, and together with the data shown in Fig. 2, we can conclude that IFN-γ ELISPOT assay responses at 3 months have no predictive power for the set point.

DISCUSSION

The ability to define immune responses early after HIV-1 infection that provide the foundation for controlling disease is a major focus of AIDS research. The role of CD8$^+$ T cells in the initial control of viremia is well known (6, 24), and there is evidence to show that in SIV-infected macaques, CD8 cells play a role in controlling viremia (19, 36), although this may not necessarily be the case in the natural host of SIV (4). Most studies of HIV-infected humans have examined antigen-specific CD8$^+$ T-cell responses during chronic disease (1, 10, 15), and fewer studies have been performed with those controlling (5) or progressing in (37) the disease. Immunological responses in acute infection are thought to be crucial for controlling initial viral replication and may predict the subsequent viral set point.

Our study is the first to identify HIV-specific T-cell responses during the first few weeks of HIV-1 infection and to correlate these responses with the subsequent viral load and

TABLE 3. Selected confirmed peptide responses identified in the first 12 weeks postinfection in early controllers and rapid progressors

Progressor	Total SFU/10^6 PBMC[a]	Breadth[b]	HLA background			Selected confirmed peptides	Previously published HLA restricted epitopes[c]
Early controllers							
CAP045	11,400	8	A*2301/2902	B*1510/4501	Cw*0602/1601	Vif p10, Rev p7-8, Nef p33-34	Vif, WHLGHGVSI (B*1510); Rev, IHSISERIL (B*1510); Nef, RYP LTFGW (A*2301)
CAP061	345	3	A*6602/6802	B*1401/4201	Cw*0802/1701	ND	
CAP222	2,970	5	A*3001/3303	B*5301/8101	Cw*0401/-	Gag p25, Nef p17-18	Gag, TPQDLNTML (A*4201, B*8101, B*0702); Nef, RPQVP LRPM (B*4201), QVPLRP MTYK (A*0301)
CAP269	0	0	A*0205/6802	B*0702/5802	Cw*0602/0611	Nil	
Rapid progressors							
CAP008	9,165	9	A*2301/-	B*0801/1510	Cw*0701/1601	Gag p4, Pol p64, Rev p7-8, Env p106-7, Nef p26-27	Gag, HYMLKHLVW (A*2301, A*2402); Pol, IYQEPFKNL (A*2301, A*2402); Rev, IHSIS ERIL (B*1510); Env, LQREWE VLKYLGSLVQYW; Nef, KRQ DILDLWIY (Cw*0701)
CAP037	5,130	4	ND[d]	ND	ND	Pol p51, Nef p2-3, Nef p33-34	Pol, TVQPIQLPEKDSWTVNDI; Nef, AVRERMRRT (B7, B8), RYPLTFGW (A*2301), YPLTF GWCF (B*5301, B*18, B*35)
CAP065	5,950	11	A*2301/6802	B*1510/5802	Cw*0511/0611	Pol p77-78, Vpr p4, Vif p2, Env p77	Pol, GAETFYVDGA (A*6802); Env, RYLKDQQLL (A*2301, A*2402)
CAP069	560	3	A*0301/2301	B*1503/5802	Cw*0210/0602	Vif p4, Vif p8-9, Nef p19-21	Vif, HIPLGEARLVIKTYWGL; Nef, QVPLRPMTYK (A*0301), KAAFDLSFF (B*57)
CAP206	8,040	10	A*3204/7412	B*0702/4403	Cw*0210/0702	Env p54, Nef p32-33	Env, RIKQIINMW (A*3201); Nef, TPGPGVRYPL (B*0702)
CAP210	2,850	6	A*6802/-	B*1510/-	Cw*0304/-	Gag p40-42, Vif p6, Vpr p6	Gag, YVDRFFKTL (Cw*0303); Vpr, ETYGDTWTGV (A*6802)
CAP224	1,985	6	ND	ND	ND	Vif p6, Vif p21, Vpr p4, Nef p17-19	Vif, HPKVSSEVHI (B*4201); Vpr, FPRPWLHGL (B*0702, B*4201, B*8101); Nef, RPQVPLRPM (B*4201), QVPLRPMTYK (A*0301)
CAP258	12,440	9	A*2301/2902	B*4101/4201	Cw*1701/-	Pol p54-55, Vif p6-7, Vpr p4, Vpu p7, Env p27, Nef p33-34	Pol, YPGIKVRQL (B*4201); Vif, HPKVSSEVHI (B*4201); Vpr, FPRPWLHGL (B*4201); Vpu, ERAEDSGNESEGDTEELSA; Env, SFDPIPIHY (A*29); Nef, RYPLTFGW (A*2301)

[a] Total ELISPOT assay response. Means: early controllers, 3,679 ± 5,315; rapid progressors, 5,765 ± 3,992.
[b] Breadth at 3 months postinfection. Means: early controllers, 4 ± 3.4; rapid progressors, 7 ± 3.
[c] Previously identified HLA restricted epitopes corresponding to participant HLA type (23).
[d] ND, not determined.

with disease progression in the first year. We defined primary infection as that within the first 3 months of infection, and we screened, in a comprehensive manner, IFN-γ-specific responses across the proteome using the ELISPOT assay. We found that responses to Nef were highly immunodominant, with recognition being focused on a few epitope regions, and that responses to other regions were less recognized. Overall, there was no significant relationship between the magnitude of IFN-γ ELISPOT assay responses and the course of viremia, early disease progression, or the set point.

Our study examined a subtype C HIV-1 acute-infection cohort recruited in Kwa-Zulu Natal, the province in South Africa worst hit by the HIV-1 epidemic, which we have followed for more than 2 years (38). We hypothesized that Gag-specific T-cell responses established early after infection would be associated with virus control and a low set point based on prior data showing that preferential targeting of Gag regions was associated with low viral loads (28). Similarly, others have shown the fundamental importance of responses to Gag (13, 33) in virus control, where transmission of an attenuated B*5801-restricted TW10 epitope in Gag resulted in persistently lower viremia in the new host (9). It would thus appear that recognition of key epitopes in Gag can attenuate viral replication and hence disease progression. In the present study, we found that only 60% of individuals at 3 months postinfection responded to p17, p24, or both regions in Gag and identified only minimal epitope responses among slow progressors who showed virus control within the first year of infection. It has been shown that some epitope responses in Gag that dominate chronic infection may not be present during initial infection (18), and we speculate from our data that epitopes in Gag have yet to emerge at 3 months postinfection and that the effect of Gag targeting impacts virus control only during later, more established infection.

The viral set point has been defined as stable viremia over time, and from subtype B infections, viremia has been shown to reach a peak following transmission and then to drop to a hypothesized set point, which has been shown to be inversely

correlated with the time to disease progression (31, 32, 35). It has been shown that a single measurement 12 to 18 months post-HIV infection was the most predictive of disease progression (27), although others have determined set point viral loads as a composite series of measurements starting earlier, around 6 months after infection (14). In this study, we defined the set point as a composite of three measurements around 12 months, although we realize that stable viremia may never be reached (41). We thus determined that neither the magnitude nor the breadth of responses to any region in the expressed subtype C genome, utilizing the IFN-γ ELISPOT assay, during early infection had any predictive power for the viral set point at 1 year. This implies that either the IFN-γ ELISPOT assay is not able to identify relevant early responses or the character of the initial immune response that may dictate the course of viremia is more complex than the magnitude and breadth of response at one time point. Another caveat to our findings is the possible viral sequence divergence from autologous epitopes and the peptide sets used in this study. It is possible that using peptides based on autologous infecting strains might have yielded stronger associations than our peptide sets, which were based on a mixture of consensus Gag and subtype C HIV vaccine-matched strains (42).

What is also clear from this study is the overwhelming and ubiquitous targeting of Nef that appears to be focused on six epitope regions. Nef dominance during primary infection has also been identified in subtype B infection (26), as well as in our previous studies of early subtype C infection (28), in which we showed that responses are positively correlated with the viral load. Additionally, variant Nef epitopes transmitted from donor to recipient in transmission studies showed no impact on viremia (17). Taken together, responses to Nef are unlikely to play a role in determining virus control or disease progression.

The lack of predictive power of IFN-γ-dependent responses at 3 months postinfection for subsequent viral loads and set point is likely due to a multitude of factors. There is no doubt that IFN-γ responses during chronic infection identify specific epitopes that impact virus control (22), but it remains to be seen whether other cytokine markers may predominate during acute infection. Our data showed that no association existed between the IFN-γ ELISPOT assay magnitude and breadth at 3 months and the viral load at any time, although it is possible that important responses during early infection may consist of other cytokines, such as IL-2 or tumor necrosis factor alpha. We additionally speculate that increasing viral diversity during the first year of infection would render an early static T-cell measurement redundant. It has recently been shown that in more than 75% of acutely subtype B-infected individuals a single virus is transmitted (21). The ensuing viral diversification that occurs after transmission, as the disease becomes established, is likely shaped by immune pressure and escape (3, 34), resulting in diverse and variant epitope changes over time. If it is likely that important "critical" responses to Gag emerge only later during infection (18), could it be that the highly focused and dominant response to Nef during acute infection is at the expense of other responses and thus allows viral propagation? Knowing that attenuating mutations in key epitopes, rather than the magnitude or breadth of T-cell responses, may be important contributors to establishing virus control (9), could it be that the set point has more to do with the accumulation of CTL mutations?

Our data thus bring into question the utility of using the IFN-γ ELISPOT assay for assessing the impact of T-cell immunity on the viral set point. It is possible that measuring more specific immune responses at the single-peptide level may provide greater insight into epitope changes that may be associated with a high or low set point. It is also possible that IFN-γ alone does not reflect the status of T cells that are associated with viremia, especially as it has been shown that polyfunctional $CD8^+$ T-cell responses may serve as markers of virus control (5). It is also possible that CD8 memory may play an important role and that the early status of memory differentiation could provide a more robust predictive marker for virus control. The current quest for identifying immune correlates of protection relies on studying acute HIV-1 infection, and the earliest responses may establish the foundation for subsequent virus control. We have shown here that defining these responses using the IFN-γ ELISPOT assay is unlikely to provide the necessary immunological insight.

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