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# Distinct Kinetics of Gag-Specific CD4<sup>+</sup> and CD8<sup>+</sup> T Cell Responses during Acute HIV-1 Infection

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HIV infection is characterized by a gradual deterioration of immune function, mainly in the CD4 compartment. To better understand the dynamics of HIV-specific T cells, we analyzed the kinetics and polyfunctional profiles of Gag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in 12 subtype C-infected individuals with different disease-progression profiles, ranging from acute to chronic HIV infection. The frequencies of Gag-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells showed distinct temporal kinetics. The peak frequency of Gag-responsive IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells was observed at a median of 28 d (interquartile range: 21–81 d) post-Fiebig I/II staging, whereas Gag-specific IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cell responses peaked at a median of 253 d (interquartile range: 136–401 d) and showed a significant biphasic expansion. The proportion of TNF- $\alpha$ -expressing cells within the IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cell population increased ( $p = 0.001$ ) over time, whereas TNF- $\alpha$ -expressing cells within IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells declined ( $p = 0.005$ ). Both Gag-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells showed decreased Ki67 expression within the first 120 d post-Fiebig I/II staging. Prior to the disappearance of Gag-responsive Ki67<sup>+</sup>CD4<sup>+</sup> T cells, these cells positively correlated ( $p = 0.00038$ ) with viremia, indicating that early Gag-responsive CD4 events are shaped by viral burden. No such associations were observed in the Gag-specific CD8<sup>+</sup> T cell compartment. Overall, these observations indicated that circulating Gag-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequencies and functions are not synchronous, and properties change rapidly at different tempos during early HIV infection. *The Journal of Immunology*, 2012, 188: 2198–2206.

In many viral infections, functional Ag-specific CD4<sup>+</sup> T cells play a critical role in orchestrating immune responses. In animal models, the absence or disruption of CD4<sup>+</sup> T cell responses can impair immune protection (1, 2) by limiting Ab production (3, 4), CD8<sup>+</sup> T cell activation (5–7), or the maintenance

of CTL responses (8–10). Maintenance of Ag-specific CD4<sup>+</sup> T cell responses during HIV infection presents a particular challenge, because these cells are targets for the virus (11, 12). During acute HIV-1 infection, there is an ordered burst of inflammatory cytokines (13) promoting cell activation and conceivably increasing the pool of CD4<sup>+</sup> T cell targets, which would fuel viral replication. Moreover, as a result of preferential targeting of HIV-specific CD4<sup>+</sup> T cells (11), it could be hypothesized that these cells are rendered dysfunctional or eliminated early during infection. The timing of HIV-specific CD4<sup>+</sup> T cell impairment appears to occur early postinfection (14), and it is of importance to understand more precisely the dynamics and functional characteristics of virus-specific CD4<sup>+</sup> T during acute HIV infection. We previously showed that Gag-responsive CD4<sup>+</sup> T cell differentiation and activation profiles at 3 mo postseroconversion associate with those observed at 12 mo, suggesting that a steady state of activation is reached in the early phase of HIV infection (15). In this study, we examined more closely the evolution of CD4<sup>+</sup> T cell responses during acute HIV-1 infection and describe the distinct kinetics of Gag-responsive CD4 and CD8<sup>+</sup> T cell frequencies, function, and activation. Our results showed that major changes occur in peripheral blood Gag-responsive CD4<sup>+</sup> T cells within the first few months of infection and that acute viral burden likely plays a dominant role in driving these dynamics.

## Materials and Methods

### Study participants

Twelve HIV-1 clade C-infected subjects were enrolled as part of the CHAVI 001 acute infection cohort. Five individuals (CHA198, CHA067, CHA164, CHA162, and CHA696) were recruited from Durban, South

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N.G., P.B., G.F., M.R.B., B.F.H., A.J.M., and C.M.G. conceived and designed the experiments; C.R., M.M., S.C., M.K.P.L., and V.E.W. performed the experiments; C.R. and V.V.G. analyzed the data; S.C., M.K.P.L., and V.E.W. contributed reagents/materials/analysis tools; and C.R., G.F., P.B., M.R.B., and C.M.G. wrote the manuscript.

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Abbreviation used in this article: IQR, interquartile range.

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Africa; six (CHA010, CHA228, CHA813, CHA1280, CH895, and CH976) were recruited from Lilongwe, Malawi; and one (CHA470) was recruited from Durham, NC. Fiebig staging was used to characterize the timing of infection after enrollment, and the stage was classified by measuring the presence/absence of plasma HIV RNA content and HIV-specific Abs using ELISA and Western blot. Participants were enrolled, the first blood samples were drawn between 2 and 24 d postscreening, and all participants were antiretroviral therapy-naïve throughout the study. Viral load was measured with the COBAS AMPLICOR HIV-1 monitor test, version 1.5 (Roche Diagnostics, Branchburg, NJ), and the CD4 count was measured by flow cytometry. Six of the participants presented as Fiebig stage I/II at screening, with detectable plasma HIV mRNA and no detectable HIV-1 serum Abs. Three individuals were classified as Fiebig stage III at screening, with HIV Abs detectable by ELISA (HIV-1/HIV-2 PLUS O EIA third generation; Bio-Rad, Hercules, CA) but negative by Western blot (Bio-Rad). The other three participants had detectable HIV Abs by ELISA but indeterminate using Western blot and were considered Fiebig stage IV. The time post-Fiebig stage I/II (when unavailable) was calculated by adjusting the time at screening by 3 d for subjects in stage III at screening and by 6 d for subjects in stage IV at screening (3 d of phase III and 3 d to the midpoint of phase IV) (16). We also used a secondary classification of early and chronic HIV infection, using the arbitrary cut-off of 120 d before (early) and after (chronic) Fiebig stage I/II. Samples analyzed in this study were taken from individuals who were not undergoing antiretroviral therapy. According to the CHAVI 001 protocol, voluntary HIV counseling and testing and risk-reduction and safe-sex counseling were offered and were done so in accordance with local standards and regulations. The participants requiring clinical care for the management of symptomatic sexually transmitted infections were either received at the clinical site or referred. Enrolled participants were referred for HIV care following each country's national guidelines for management and treatment of people with HIV. If referral for antiretroviral treatment was made, this included possible enrollment into other available HIV treatment resources, such as the Global Fund, President's Emergency Plan for AIDS Relief, or national roll-out programs. The protocol for CHAVI 001 acute HIV-1 infection prospective cohort study can be found at [https://chavi.org/wysiwyg/downloads/CHAVI\\_001\\_protocol\\_v2.pdf](https://chavi.org/wysiwyg/downloads/CHAVI_001_protocol_v2.pdf). Duke University, University of North Carolina, University of Witwatersrand, and University of Cape Town institutional review boards approved this study, and all the subjects provided written informed consent for participation in this study.

### Cell preparation

Blood samples were collected at different time points postinfection. PBMCs were isolated by a standard Ficoll-Hypaque density-gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden), cryopreserved in 90% heat-activated FBS (Invitrogen, Paisley, U.K.) plus 10% DMSO, and stored in liquid nitrogen until needed. Thawed PBMCs were washed twice with RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 1.7 mM sodium glutamate (R10). The cells were then rested in R10 at 37°C and 5% CO<sub>2</sub> for 2 h in the presence of 10 U/ml DNase I (Roche Diagnostics) prior to use in intracellular cytokine staining assays.

### Synthetic subtype C peptides

Peptides were synthesized by Sigma-Aldrich (St Louis, MO) and/or the Medical Research Council Human Immunology Unit (Weatherall Institute of Molecular Medicine) as 18mers overlapping by 10 aa. Consensus subtype C Gag peptides were used for seven study participants (CHA010, CHA228, CHA696, CHA813, CHA895, CHA976, and CHA1280), and peptides matching the founder autologous virus were used in five participants (CHA067, CHA162, CHA164, CHA198, and CHA470). Peptides were pooled by protein and used at a final concentration of 2 µg/ml in a maximum of 0.45% DMSO.

### Cell stimulation and intracellular staining

Thawed PBMCs were washed, resuspended at  $2 \times 10^6$  cells/ml with RPMI 1640 supplemented with 10% heat-inactivated FCS, and rested at 37°C and 5% CO<sub>2</sub> overnight. Cells were then stimulated for 6 h with autologous or consensus HIV-1 C Gag pools (2 µg/ml) in the presence of 1 µg/ml anti-CD28 and anti-CD49d costimulatory Abs (BD Biosciences, San Diego, CA) and 10 µg/ml brefeldin A (Sigma-Aldrich). A negative control containing PBMCs and costimulatory Abs from the same subject, but without the peptide mix, was also included for each assay. Following stimulation, cells were washed with PBS and surface stained with red-amine LIVE/DEAD reagent (Molecular Probes/Invitrogen, Paisley, U.K.) and a mixture of mAbs containing CD14 PE-Texas Red (Beckman Coulter, Brea, CA),

CD19 PE-Texas Red (Beckman Coulter), CD3 QD655 (Invitrogen, Paisley, U.K.), CD4 PEcy5.5 (BD Pharmingen, San Diego, CA), CD8 QD705 (Invitrogen), CD45RA QD605 (Invitrogen), CD27 PEcy5 (BD Pharmingen), and CD38 allophycocyanin (BD Pharmingen) for 30 min in the dark at 4°C. The cells were then washed with PBS containing 1% FBS and permeabilized according to the manufacturer's instructions using a Cytofix/Cytoperm buffer kit (BD Biosciences) and stained with IFN-γ Alexa Fluor 700 (BD Pharmingen), IL-2 PE (BD Pharmingen), TNF-α Pacific Blue (eBioscience, San Diego, CA), and Ki-67 FITC (BD Pharmingen). After labeling, cells were washed and fixed in PBS containing 1% paraformaldehyde and stored at 4°C prior to flow cytometry acquisition within 24 h.

### IFN-γ-ELISPOT assay

Briefly, cryopreserved PBMCs were thawed and rested for 2 h before being placed in the ELISPOT plates at  $1 \times 10^5$  cells/well. PBMCs were then stimulated with 9- to 18-mer autologous single peptides (2 µg/ml, based on transmitted viral sequence) and incubated for 20 h at 37°C, 5% CO<sub>2</sub>. Coating, development, and reading of ELISPOT plates were described previously (17). The peptides used for each donor are listed here: CH162: Nef KRQEILDWVYHTQGYFP, Vif HMHYFDCFAGS, Gag HKGKIK-VKDTKEALDKIEE; CH470: Nef FDSRLAYQHIAKEKHPEF, Pol QIY-PGIKVK, Env RQGLERALI, Gag DIKDTKEAL; CH164: Nef EEVG-FPVRPQV, Pol KGEAIHGQVDCSPGIWQL, Env CPKISFDPIPIHYCAPAG, and Gag PGHKARVLAEAMSQANNA. Positive responses were defined as  $\geq 50$  spot forming units/million, greater than four times background. For all assays, six negative control wells (media only) and at least one positive control well (10 µg/ml PHA; Sigma-Aldrich) were used.

### Flow cytometry analysis

Approximately 700,000–1,200,000 events were collected per sample on an LSRII flow cytometer (BD Biosciences). Electronic compensation was conducted with Ab capture beads (BD Biosciences) stained separately with individual mAbs used in test samples. Data were analyzed with FlowJo, version 8.8.6 (Tree Star, Ashland, OR). Dead cells (live/dead<sup>+</sup>), monocytes (CD14<sup>+</sup>), and B cells (CD19<sup>+</sup>) were removed from the analysis. Cells were then gated on singlets, live CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, memory cells and then on combinations of maturation and activation markers. Gating strategy is depicted in Supplemental Fig. 1. For cytokine expression, values  $>0.04\%$  (after background subtraction) and higher than twice the background were considered positive. Evaluation of all nonstimulated samples tested ( $n = 69$ , four to nine time-points in 12 donors) showed that the average ( $\pm$  SD) frequencies for IFN-γ<sup>+</sup> events was  $0.0115 \pm 0.0103\%$  for CD4<sup>+</sup> T cells and  $0.0125 \pm 0.019\%$  for CD8<sup>+</sup> T cells. Maturation and activation phenotype were analyzed only on HIV-specific IFN-γ<sup>+</sup> cells presenting the following criteria: frequency of HIV-specific CD4<sup>+</sup> T cells  $>0.04\%$  of total memory T cells, cytokine responses more than two-fold that in the corresponding nonstimulated sample, and number of positive events in HIV-stimulated samples  $>30$ .

### Statistical analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism software, version 4.0 (GraphPad Software, La Jolla, CA) and Mathematica 8.0 (Wolfram Research, Champaign, IL). For correlations, data were analyzed by the use of nonparametric statistics. Statistical significance was determined using either the Mann-Whitney or Wilcoxon paired *t* test. All tests were two-tailed, and a value of  $p < 0.05$  was considered statistically significant. The relationship between the HIV-specific CD4<sup>+</sup> T cell phenotype and plasma viral loads was assessed by Spearman rank correlations. We used the following procedure to investigate whether changes in a particular cell phenotype followed change determined by a single slope or biphasic change (determined by two different slopes). We fitted the data with a model allowing biphasic change to the data to determine the slopes and the time when the slopes changed. The resulting model was then compared with the model with a single slope change; using an *F* test for nested models, we determined whether the biphasic model described the data significantly better (18). For significance, we report the *p* value from an *F* test when comparing the best model for the data against the model with no change of phenotype over time. We log-transformed the data to normalize the behavior of residuals in the regressions.

## Results

### Cohort characteristics

We longitudinally analyzed 12 subtype C HIV-infected subjects enrolled into the CHAVI 001 acute-infection cohort. Ten of twelve

were male, and the median age of subjects was 27 y (range, 17–42 y). Longitudinal characteristics of absolute CD4<sup>+</sup> T cell counts and viral loads are shown in Fig. 1. Time postinfection was adjusted according to Fiebig staging (16) at screening, as discussed in *Materials and Methods*. Subjects with a viral set point > 100,000 RNA copies/ml, persisting beyond the first year of infection, were classified as rapid progressors ( $n = 5$ ); subjects whose set point fell between 10,000 and 100,000 RNA copies/ml were classified as intermediate progressors ( $n = 5$ ), and individuals with a viral set point consistently <10,000 RNA copies/ml were classified as slow progressors ( $n = 2$ ) (Fig. 1A). As expected, CD4<sup>+</sup> T cell counts <350 CD4 cells/ $\mu$ l were observed in the rapid progressors (Fig. 1B), whereas intermediate and slow progressors were characterized by higher CD4 counts. Of note, two individuals (CH813 and CH696) maintained CD4 counts (745 and 440 CD4<sup>+</sup> T cells/ $\mu$ l, respectively) over a 2-y follow-up, despite high viremia. Viral load was negatively associated with absolute CD4<sup>+</sup> T cell count at set point ( $r = -0.48$ ,  $p = 0.0013$ , data not shown).

#### Differential kinetics of Gag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses

The dynamics of Gag-specific T cell responses were evaluated by analyzing the changes in the frequency of Gag-responsive IFN- $\gamma$ <sup>+</sup> cells within total memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells (excluding naive cells) from early to chronic stages of infection. The gating strategy consistently used for each sample is shown in Supplemental Fig. 1.

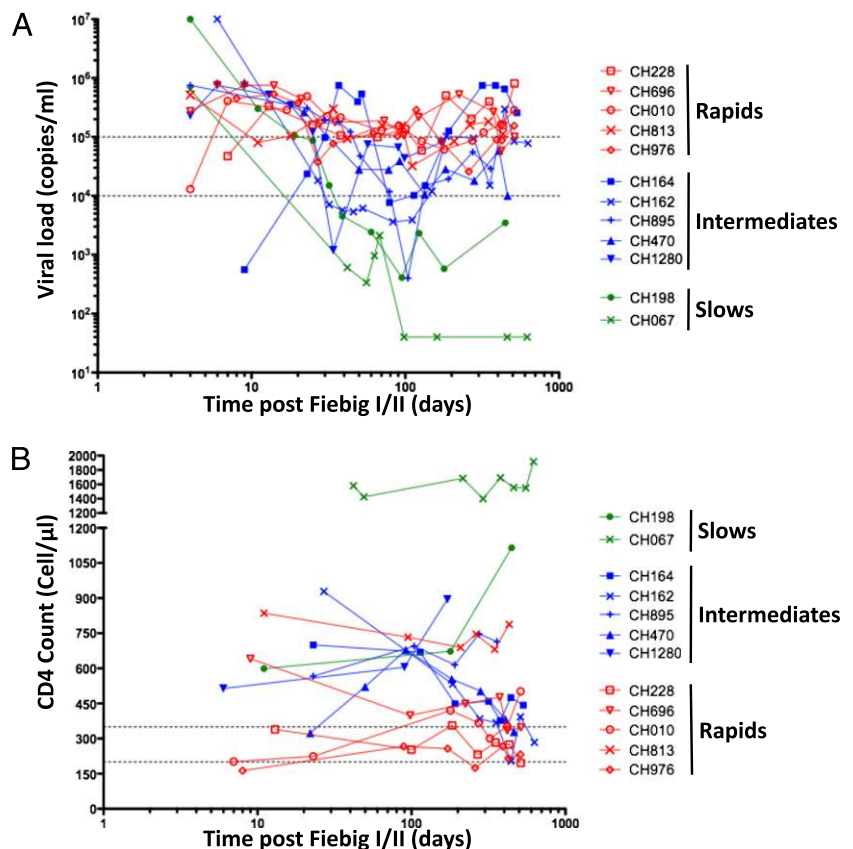
In the CD4 compartment, the pattern of Gag-specific IFN- $\gamma$ <sup>+</sup> responses showed three profiles: Gag-specific CD4<sup>+</sup> T cell responses decreasing over time in 7/12 (58%) individuals (CH10, CH976, CH162, CH164, CH198, CH1280, and CH470), in which a 1.6–4.4-fold decrease in responses was observed between early and later samples; Gag-specific CD4<sup>+</sup> T cell responses being

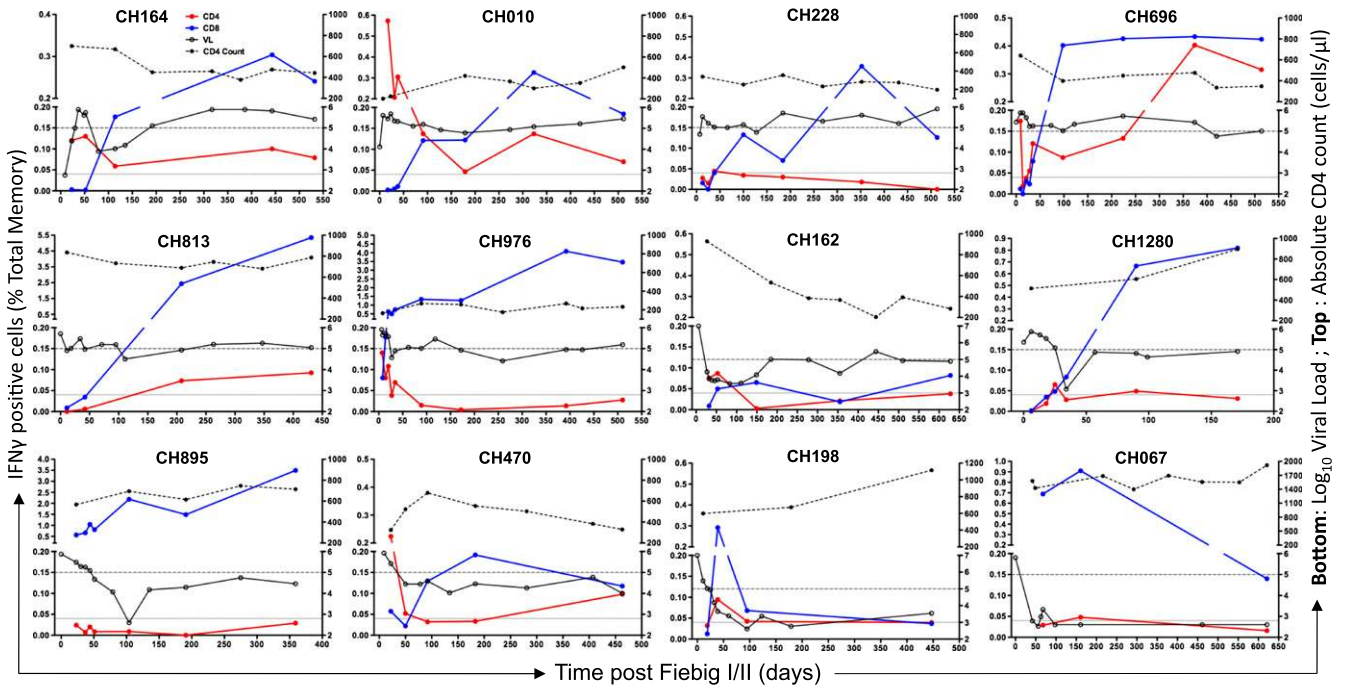
barely detectable and consistently <0.05% at any time point analyzed in 3/12 (25%) individuals (CH228, CH895, and CH067); and Gag-specific CD4<sup>+</sup> T cell responses increasing over time in 2/12 (17%) individuals (CH696 and CH813) (Fig. 2). In most subjects with detectable CD4 responses (7/9, 78%), the maximal frequency of Gag-responsive CD4<sup>+</sup> T cells was observed at a median of 28 d (interquartile range [IQR]: 21–81 d) post-Fiebig I/II staging. Of note, the three patterns of response were unrelated to disease progression or the magnitude of viremia. To address the possibility that the apparent decrease in Gag-responsive CD4<sup>+</sup> T cell frequencies was a reflection of changes within absolute CD4<sup>+</sup> T cells, we correlated the number of Gag-responsive CD4<sup>+</sup> T cells/ $\mu$ l blood with the frequency of Gag-specific CD4<sup>+</sup> T cell responses and observed a positive association ( $p < 0.0001$ ) (Fig. 3A). This would indicate that there are selective changes in the absolute numbers of circulating Gag-responsive CD4<sup>+</sup> T cells. This is further supported by the finding that no association existed between the rate of decrease in Gag-responsive CD4<sup>+</sup> T cell frequencies and the rate of decrease in absolute CD4<sup>+</sup> T cell counts (data not shown).

In the CD8 compartment, the dynamics of Gag-specific responses showed a similar tempo over time in most of the studied individuals; the first detectable responses occurred at a median of 72 d (IQR: 34–103 d) post-Fiebig I/II staging (Fig. 3B). The maximal frequency of Gag-responsive CD8<sup>+</sup> T cells was reached at a median of 253 d (IQR: 136–401 d) post-Fiebig I/II staging, and regression analysis showed a significant ( $p = 1.6 \times 10^{-6}$ ) biphasic expansion of these cells over time (Fig. 3B), reaching a plateau at ~120 d post-Fiebig I/II staging.

The asynchronous nature of Gag-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cell kinetics was further confirmed by comparing the time to maximal frequency of Gag-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The median peak for Gag-specific CD4<sup>+</sup> T cell responses (at

**FIGURE 1.** Clinical characteristics of study subjects ( $n = 12$ ). The course of viral loads over time (A) and absolute CD4 counts (B) in each individual. Time is defined as days post-Fiebig I/II staging for each donor. Dotted horizontal lines identify 10,000 and 100,000 RNA copies/ml in (A) and 200–350 CD4<sup>+</sup> T cells/ $\mu$ l in (B). Individuals displaying viral loads consistently >100,000 RNA copies/ml were classified as rapid progressors (red,  $n = 5$ ). Individuals with viral load <1000 RNA copies/ml were considered slow progressors (green,  $n = 2$ ). All other subjects were defined as intermediate progressors (blue,  $n = 5$ ).



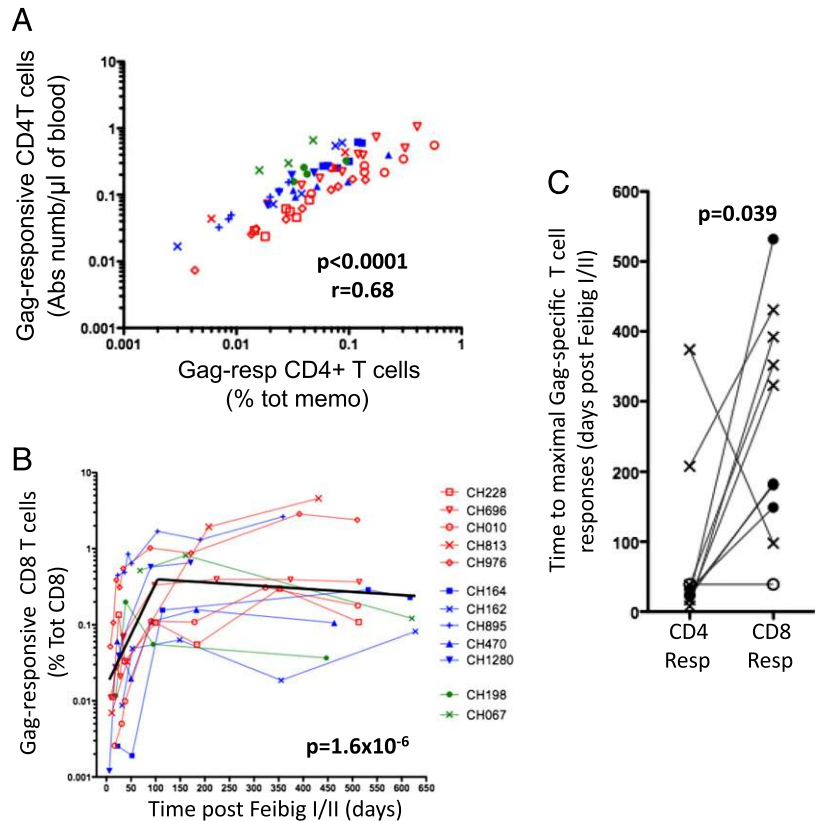


**FIGURE 2.** Kinetics of Gag-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Profiles of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> (blue lines) and CD4<sup>+</sup> T cell responses (red lines) over time. IFN- $\gamma$  responses (left y-axis), expressed as the percentage of total CD4<sup>+</sup> or CD8<sup>+</sup> memory T cells, were analyzed by flow cytometry. The lower dotted line was set at 0.04% and considered a positive cut-off for IFN- $\gamma$  responses. The upper dashed line identifies log<sub>10</sub> viral load = 5 (i.e., 100,000 RNA copies/ml). VL, viral load.

28 d [IQR: 21–81 d] post-Fiebig I/II staging) was significantly earlier ( $p = 0.039$ ) compared with the median peak response for CD8<sup>+</sup> T cells (at 253 d [IQR: 136–401 d]) (Fig. 3C). Notably, in five of nine individuals (CH164, CH10, CH696, CH162, and

CH470), the first detectable Gag-specific CD4<sup>+</sup> T cells responses were observed either in the absence or with very low frequencies of Gag-specific IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells responses. Furthermore, discordant dynamics were also confirmed by calculating the rates

**FIGURE 3.** Profiles of Gag-responsive T cells. (A) Correlation between absolute numbers of Gag-responsive IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells/ $\mu$ l of blood (calculated based on CD4 count) and the frequency of Gag-responsive IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells expressed as the percentage of total memory CD4<sup>+</sup> T cells. Each symbol represents one studied individual, with red denoting rapid progressors, blue denoting intermediate progressors, and green denoting the two controllers. (B) Longitudinal assessment of Gag-responsive IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells expressed as a percentage of total CD8<sup>+</sup> T cells (including naive CD8<sup>+</sup> T cells). Each line represents one studied individual; nonlinear regression analysis is depicted as the black line. (C) Comparison of time post-Fiebig I/II (days) to maximal frequencies of IFN- $\gamma$ <sup>+</sup> Gag-specific T cell responses in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Each symbol represents one individual ( $\times$ , rapid progressors;  $\circ$ , slow progressors;  $\bullet$ , intermediate progressors). Statistical comparisons were determined using the nonparametric Wilcoxon matched-pair  $t$  test. Abs numb, absolute number; Gag-resp, Gag-responsive cells; tot memo, total memory cells.



of overall change in the frequency of Gag-responsive IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells for which a significant difference in the slopes for the change in CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequencies was observed ( $p = 0.024$ , data not shown).

Importantly, because Gag-specific CD8<sup>+</sup> T cell responses were shown to emerge at a later time compared with other HIV proteins (19–21), we wished to identify whether other regions of the proteome were targeted prior to Gag during acute infection. Supplemental Fig. 2 shows that in three of the participants, using previously derived ELISPOT epitope-mapping data using autologous peptides, responses in Nef and/or Pol were identified prior to Gag: as early as 22 d post-Fiebig I/II staging. However, it was not possible to delineate whether CD4<sup>+</sup> or CD8<sup>+</sup> T cells imparted these responses.

#### *Divergent cytokine profiles between Gag-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells*

To assess whether the distinct frequencies of Gag-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells over time were also accompanied by poly-functional changes, IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 expression levels were measured. Because of the inherent high backgrounds of TNF- $\alpha$  and IL-2 observed in nonstimulated samples [a phenomena previously described in African cohorts (22, 23)], we focused on IFN- $\gamma$ -producing cells and measured the proportional expression of TNF- $\alpha$  and IL-2 within IFN- $\gamma$ <sup>+</sup> T cells. Representative dot plots of TNF- $\alpha$ , IL-2, and IFN- $\gamma$  expression by total memory CD4<sup>+</sup> and

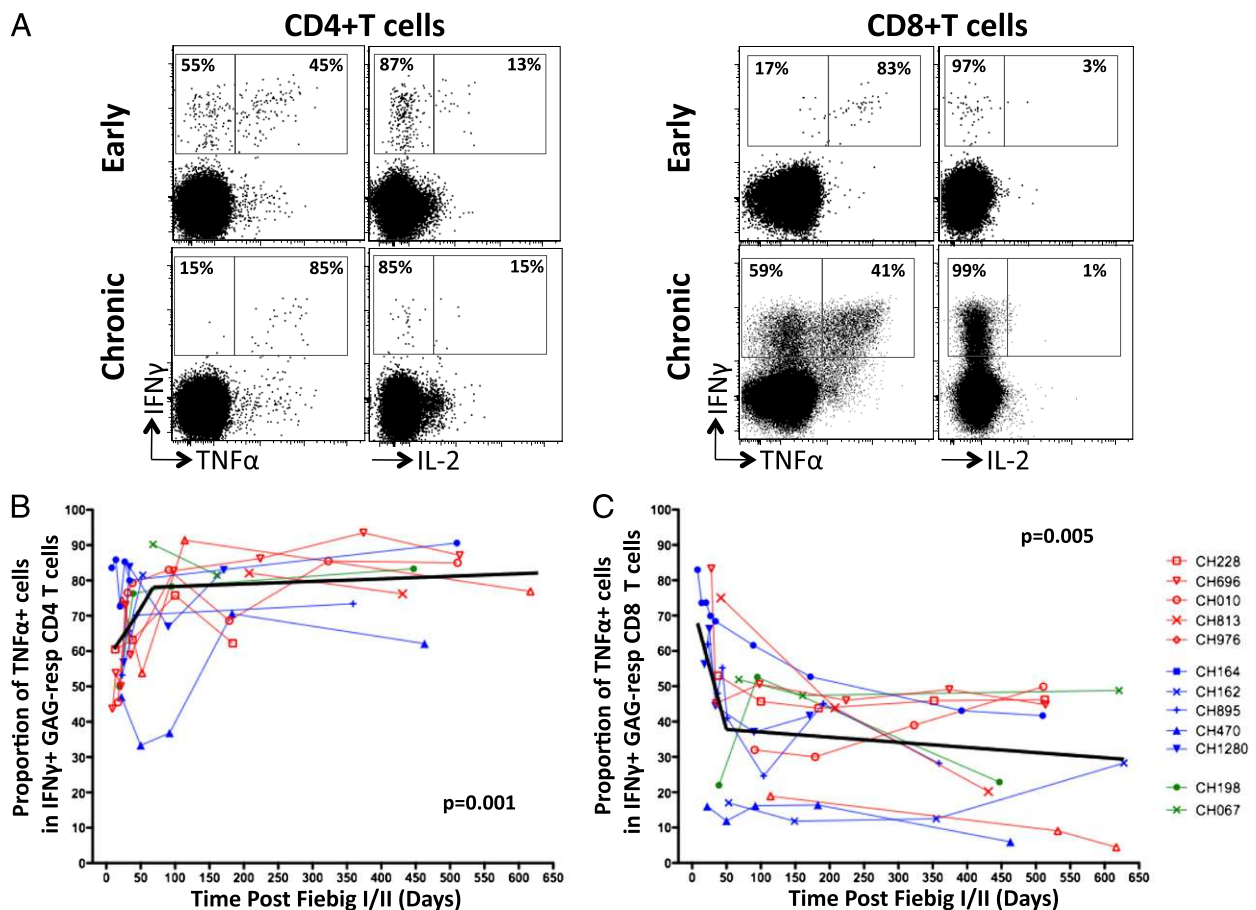
CD8<sup>+</sup> T cells at <120 d post-Fiebig I/II staging (early) and >120 d post-Fiebig I/II staging (chronic) are shown in Fig. 4A.

In the CD4 compartment, there was a significant biphasic increase in the proportion of Gag-responsive dual-functional CD4<sup>+</sup> T cells (IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>) over time ( $p = 0.001$ , Fig. 4B); IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells coexpressing TNF- $\alpha$  increased from a median of 55% (IQR: 46–67%) to 83% (IQR: 76–88%) from early to chronic infection. Of note, the median IL-2 expression within IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells was 15% (IQR: 11–22%) at the earliest time-point analyzed and remained constant over time (data not shown).

In the CD8 compartment, a contrasting pattern was observed (Fig. 4C). Expression levels of TNF- $\alpha$  within IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells significantly decreased over time ( $p = 0.004$ ), from a median of 51% (IQR: 19–72%) to 33% (IQR: 21–44%). Of note, changes in TNF- $\alpha$  expression occurred predominantly during early infection (within the first 120 d post-Fiebig I/II staging). Collectively, these data showed that Gag-specific CD8<sup>+</sup> T cell responses, which increase in magnitude over time (Fig. 3B), progressively lose their ability to express TNF- $\alpha$ , as previously described (24).

#### *Gag-responsive CD4<sup>+</sup> T cells lose Ki67 and CD38 expression*

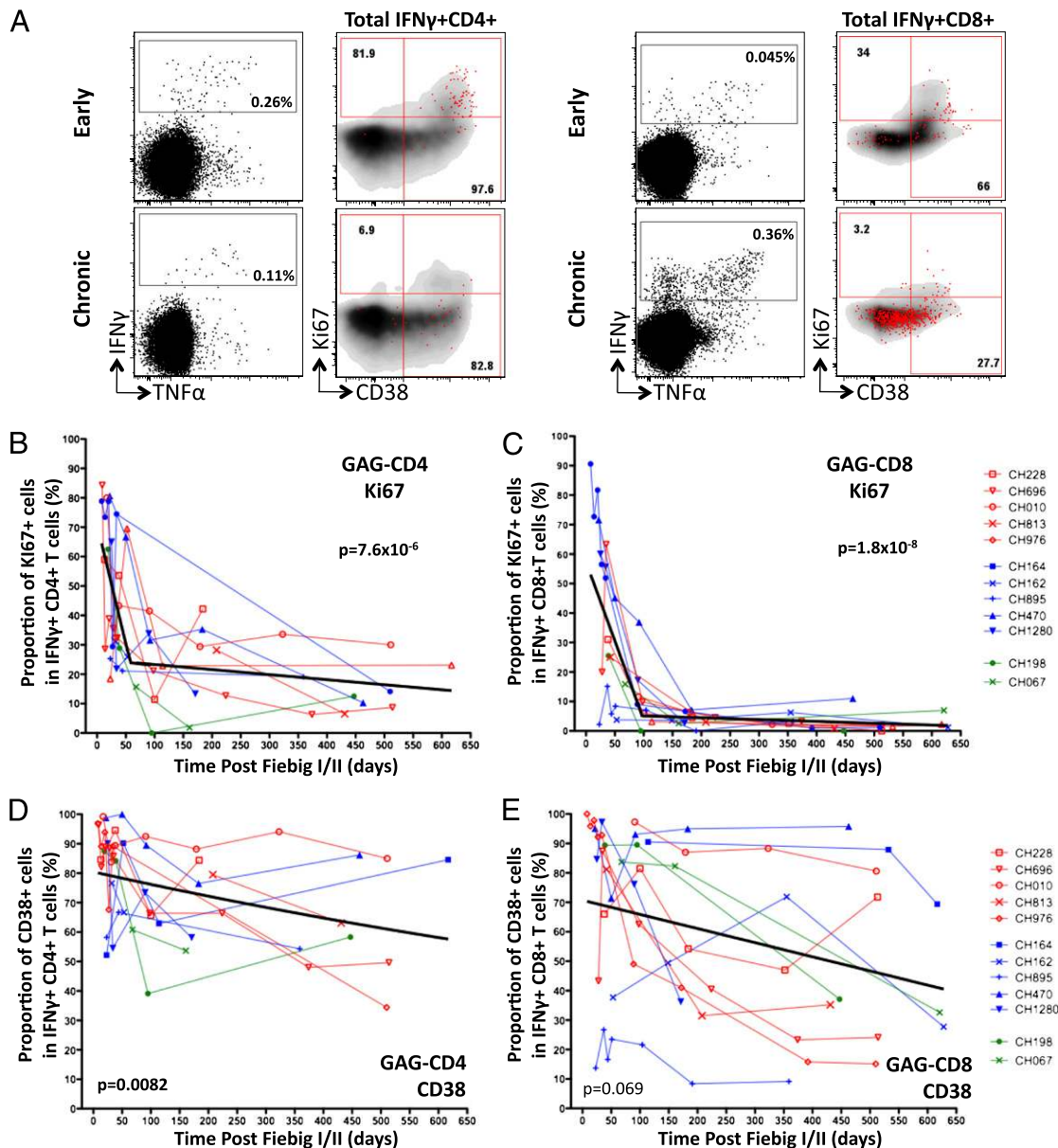
We previously showed that, at 3 mo postseroconversion, Gag-responsive CD4<sup>+</sup> T cells were highly activated and that this state persisted over the first year of infection (15). In the current study, we had the opportunity to examine activation and proliferation potential much closer to the time of infection. Represent-



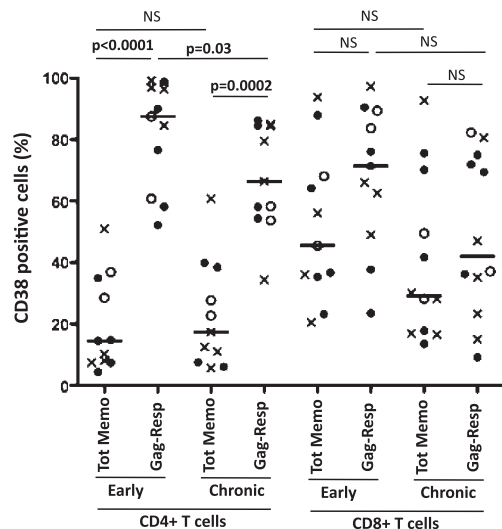
**FIGURE 4.** Evolution of TNF- $\alpha$  expression profiles of Gag-responsive IFN- $\gamma$ <sup>+</sup> T cells over time. (A) Representative dot plots of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 expression profiles in Gag-responsive CD4<sup>+</sup> T cells at <120 d after Fiebig I/II staging (early) or >120 d after Fiebig I/II staging (chronic). Percentages correspond to the proportion of TNF- $\alpha$  expressing cells within the total IFN- $\gamma$ <sup>+</sup> population. Longitudinal assessment of the proportion of TNF- $\alpha$ -expressing cells within IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells (B) and IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells (C). Nonlinear regression analyses are depicted by black lines.

tative expression levels of CD38 and Ki67 in Gag-responsive IFN- $\gamma^+$ CD4 $^+$  and CD8 $^+$  T cells are shown in Fig. 5A. There was a significant decline in the proportion of Gag-responsive CD4 $^+$ Ki67 $^+$  cells ( $p = 7.6 \times 10^{-6}$ ) and Gag-responsive CD4 $^+$ CD38 $^+$  cells ( $p = 0.0082$ ) over time (Fig. 5B, 5D). A similar significant decline in Gag-responsive CD8 $^+$  Ki67 $^+$  T cells was observed (Fig. 5C), whereas the profiles of CD38 $^+$  Gag-responsive CD8 $^+$  T cells were more heterogeneous among different individuals and did not show any significant changes over time (Fig. 5E). The decrease in Ki67 expression in both Gag-responsive CD4 $^+$  and CD8 $^+$  T cells was biphasic, with a rapid loss of replicating cells within approximately the first 120 d. In contrast, the decrease in CD38 expression levels on Gag-responsive CD4 $^+$  T cells was monophasic.

When we compared Gag-responsive CD4 $^+$  T cells with total memory CD4 $^+$  T cells, it was evident that Ag-responsive CD4 $^+$  T cells were significantly more activated, as measured by CD38 expression, in both early and chronic infection (Fig. 6). The median frequency of CD38 $^+$  cells during early infection was 14% (IQR: 7–34%) of total CD4 $^+$  T cells and 87% (IQR: 61–95%) of Gag-responsive CD4 $^+$  T cells ( $p < 0.0001$ ). During chronic infection, the median frequencies of CD38 $^+$  cells were 19% (IQR: 8–35%) and 62% (IQR: 54–84%) of total and Gag-responsive memory CD4 $^+$  T cells, respectively ( $p = 0.0002$ , Fig. 6). In the CD8 compartment, activation of total and Gag-responsive CD8 $^+$  T cells was similar during both early and chronic infection. These data suggested that Gag-responsive CD4 $^+$  T cells are preferentially activated within the overall CD4 compartment and specifically



**FIGURE 5.** Evolution of proliferation and activation profiles of Gag-responsive IFN- $\gamma^+$  T cells. **(A)** Representative dot plots of IFN- $\gamma$ , TNF- $\alpha$ , and CD38, Ki67 expression profiles in Gag-responsive CD4 $^+$  (left panels) and CD8 $^+$  (right panels) T cells at <120 d after Fiebig I/II staging (early) or >120 d after Fiebig I/II staging (chronic). The gray density plots represent total memory T cell population staining for CD38/Ki67 cells, and the red dots represent IFN- $\gamma^+$  Gag-responsive T cells. Longitudinal assessments of the proportion of Ki67 $^+$ IFN- $\gamma^+$ CD4 $^+$  T cells **(B)** and Ki67 $^+$ IFN- $\gamma^+$ CD8 $^+$  T cells **(C)**. Longitudinal assessments of the proportion of CD38 $^+$ IFN- $\gamma^+$ CD4 $^+$  T cells **(D)** and CD38 $^+$ IFN- $\gamma^+$ CD8 $^+$  T cells **(E)**. Linear and nonlinear regression analyses are depicted by black lines.



**FIGURE 6.** Comparison of CD38 expression levels in Gag-responsive IFN- $\gamma^+$  T cells and total memory T cells in early (<120 d post-Fiebig I/II staging) and chronic (>120 d post-Fiebig I/II staging) phases of infection. Each symbol represents one individual ( $\times$ , rapid progressors;  $\circ$ , slow progressors;  $\bullet$ , intermediate progressors), in whom CD38 expression was evaluated at the maximal IFN- $\gamma^+$  responses measured. Statistical significance was determined using a two-tailed nonpaired Mann-Whitney  $t$  test. Value of  $p < 0.05$  was considered statistically significant. Gag-Resp, Gag-responsive cells; Tot Memo, total memory cells.

during early infection. Activation of CD8<sup>+</sup> T cells appears more uniform both in specificity and over time postinfection.

Collectively, these data showed that Gag-responsive CD4<sup>+</sup> T cells show high expression of CD38 and Ki67 at the onset of infection, with Ki67<sup>+</sup> cells being rapidly lost within the first 120 d after Fiebig I/II staging. Although the residual circulating Gag-responsive CD4<sup>+</sup> T cells remain activated, these data suggested that these cells are not replenished.

#### Impact of early viremia on Gag-responsive CD4<sup>+</sup> T cells

We hypothesized that the magnitude of viremia occurring during acute HIV infection may influence the dynamics and function of T cells. When we investigated the association between viral load and the proportion of TNF- $\alpha^+$  cells expressed in IFN- $\gamma^+$  cells in early infection (<120 d post-Fiebig I/II staging) or in chronic infection (>120 d post-Fiebig I/II staging), there was a negative correlation with the Gag-responsive CD4 compartment during early infection ( $p = 0.03$ ) but no association with Gag-responsive CD8<sup>+</sup> T cells at any period postinfection (Fig. 7A). Thus, it appears that elevated viremia is associated with IFN- $\gamma^+$  Gag-responsive CD4<sup>+</sup> T cells expressing low levels of TNF- $\alpha$  during the early stage of infection. However, there was a loss of CD4<sup>+</sup> T cell associations over time, probably reflecting the almost complete absence of circulating Gag-responsive IFN- $\gamma^+$ TNF- $\alpha^-$  CD4<sup>+</sup> T cells.

When viral load was correlated with the proportion of Ki67<sup>+</sup> Gag-responsive CD4<sup>+</sup> T cells, there was a highly significant positive correlation but only during the early stage of infection ( $p = 0.00038$ ,  $r = +0.8$ , Fig. 7B). At the chronic stage, Gag-responsive CD4<sup>+</sup> T cells were characterized by low expression of Ki67 (Fig. 7B), despite the maintenance of relatively high viral loads. There was no correlation between the proportions of Ki67<sup>+</sup> Gag-responsive CD8<sup>+</sup> T cells and viral load at either the early or chronic stage of infection (Fig. 7B). Similarly, CD38 expression by Gag-responsive CD4<sup>+</sup> T cells was positively associated with viral load during early infection ( $p = 0.018$ ), whereas no associ-

ations were found at the chronic stage or for Gag-responsive CD8<sup>+</sup> T cells at any time point (Fig. 7C). It is also worth noting that the magnitude, activation level, TNF- $\alpha$  proportion, and proliferation status of Gag-responsive IFN- $\gamma^+$ CD4<sup>+</sup> T cells during acute infection bore no relationship to viral set point, calculated as the average viral load of the last three data points spanning 6–18 mo postinfection (data not shown). This suggested that the poly-functionality and activation status of circulating Gag-responsive CD4<sup>+</sup> T cells, as measured during early infection, is short-term and that viral set point is independent of these early systemic events.

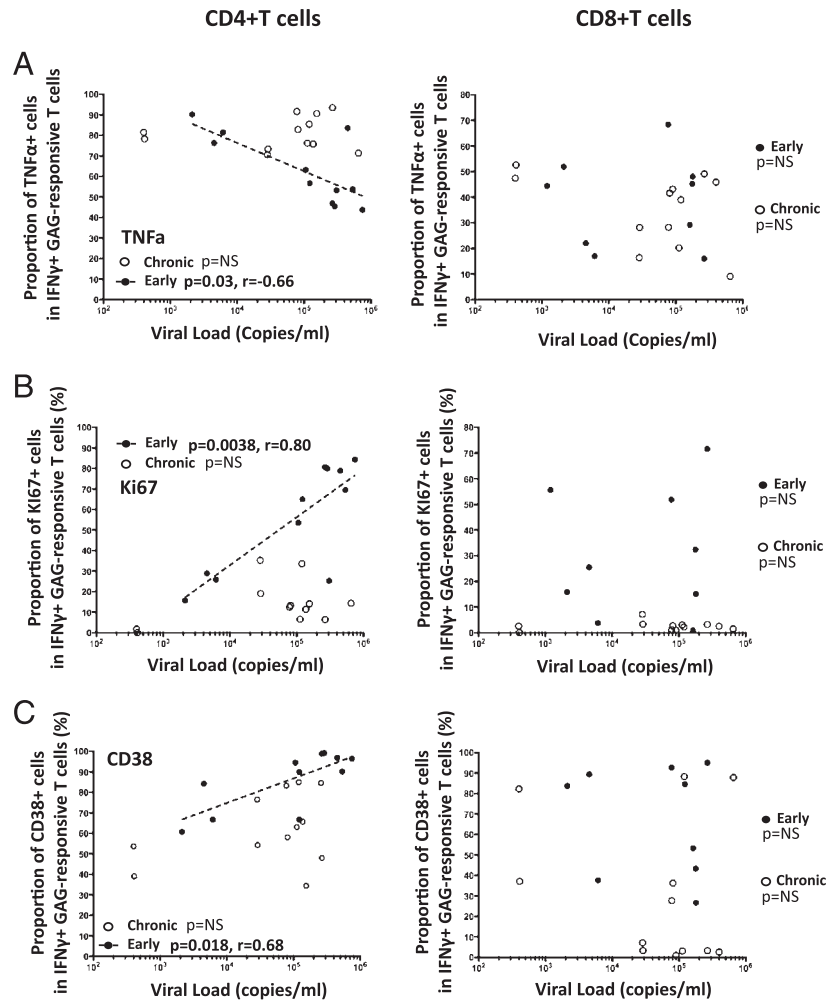
Together, these data suggested that during the first few months of HIV infection, antigenemia most likely shapes these early measured events and is unique to the CD4 compartment.

## Discussion

This article described the frequencies, activation profile, and functional and proliferative potential of Gag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses from acute to chronic infection, in 12 untreated subtype C HIV-1-infected individuals. We revealed that these cells display distinct kinetics and no longer have proliferative potential by 3 mo postinfection.

We showed that in 68% (6/9) of individuals, in whom CD4 responses were detectable, the maximal frequencies of Gag-specific IFN- $\gamma^+$ CD4<sup>+</sup> T cell responses were observed at the earliest time point studied. This suggested that Gag-specific CD4<sup>+</sup> T cell responses are high in the peripheral blood during acute infection, after which the magnitude of these cells declines rapidly. The lack of detectable Gag-specific CD4<sup>+</sup> T cell responses in the remaining three individuals could be due to missing early kinetics, where a more rapid elimination of these cells may have occurred prior to measurement. Several mechanisms may account for the disappearance of these cells, including HIV-specific CD4<sup>+</sup> T cell contraction (25), preferential elimination (as the result of being in vivo targets for HIV) (11), and/or cell exhaustion (12). Support for the last possibility is that residual Gag-responsive CD4<sup>+</sup> T cells measured during chronic infection do not actively proliferate (26). By the time we first measured Gag-responsive IFN- $\gamma^+$ CD4<sup>+</sup> T cells, they were expressing high levels of Ki67, were activated, and coexpressed moderate levels of TNF- $\alpha$ . By 3 mo postinfection, these cells had lost Ki67 expression and showed reduced CD38 expression, and there was an accumulation of TNF- $\alpha$  in the residual cells. Loss of HIV-specific CD4<sup>+</sup> T cell proliferative ability has been considered a hallmark of HIV infection (27–29), and our data showed that this likely occurs rapidly after HIV infection. We hypothesized that Ag-triggered activation of CD4<sup>+</sup> T cells is proportional to Ag concentration during early infection, after which there is a deleterious effect on CD4<sup>+</sup> T cell maintenance and possible renewal capacities (30). Although several publications reported on the functional profile of HIV-specific CD4<sup>+</sup> T cells in chronic infection (22, 31, 32), less is known about the evolution of HIV-specific CD4<sup>+</sup> T cell function over time from the acute infection. The importance of CD4 cells during acute infection was highlighted recently in SIV-infected rhesus macaques, in whom depletion of total CD4<sup>+</sup> T cells abrogated post-peak decline of viremia (33). Although there was a decline in total Gag-responsive IFN- $\gamma^+$ CD4<sup>+</sup> T cells, we observed the seeming paradox of increased expression of TNF- $\alpha^+$  within these cells. This likely reflects the selective elimination of IFN- $\gamma^+$ TNF- $\alpha^-$  CD4<sup>+</sup> T cells. The resulting negative association of TNF- $\alpha^-$  expression profiles in Gag-responsive CD4<sup>+</sup> T cells with viremia during early infection further confirmed that high antigenic burden drives functional and phenotype changes in Gag-responsive CD4<sup>+</sup> T cells. Of note, no differences in CD38 and Ki67 expression





**FIGURE 7.** Correlations between the proportion of TNF- $\alpha$ + cells (**A**), Ki67+ cells (**B**), and CD38+ cells (**C**) within IFN- $\gamma$ + Gag-responsive T cells and viral load in early and chronic infection. Each symbol represents one individual, in whom the studied markers were quantified at the maximal IFN- $\gamma$ + responses measured during early (<120 d after Fiebig I/II staging) or chronic (>120 d after Fiebig I/II staging) infection. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation.

levels were observed between IFN- $\gamma$ +TNF- $\alpha$ - and IFN- $\gamma$ +TNF- $\alpha$ + Gag-responsive CD4+ T cells (data not shown).

Gag-specific CD8+ T cell responses showed different kinetics in comparison with Gag-specific CD4+ T cell responses; the magnitude of Gag-specific CD8+ T cell responses reached maximal frequencies significantly later than did Gag-specific CD4+ T cell responses. Throughout the course of infection, Gag-responsive IFN- $\gamma$ +CD8+ T cells accumulated but remained highly activated and lost the ability to coexpress TNF- $\alpha$  at ~120 d post-Fiebig I/II staging. These data corroborated reports showing progressive impairment of multicytokine production observed in chronically infected individuals (24). Unusual kinetics of T cell responses, where Ag-specific CD4 responses also occur prior to CD8 responses, have been observed in other viral infections, such as hepatitis C virus in chimpanzees (34) and acute EBV infection in humans (35), as well as in response to live antigenic cells in mice (36). One potential explanation for the failure to detect a high frequency of Gag-responsive CD8+ T cells in peripheral blood of some patients is that cells may accumulate in other compartments, such as lymph nodes or in tissues at the site of infection (37–39). It is also plausible that the delayed detection of Gag protein by CD8+ T cells could be inherent to the tempo of Gag processing and presentation (40, 41) during HIV replication. MHC class I tetramers or a more extended cytokine intracellular staining panel would be necessary to define the exact timing of the appearance of Gag-responsive CD8+ T cell responses during HIV infection. The lack of samples precluded us from measuring either of these

options. Further experiments would also be required to determine separately the precise kinetics of CD4+ and CD8+ T cell responses toward other immunodominant HIV regions (such as Nef and Pol) to define whether distinct CD4+ and CD8+ T cell kinetics observed in peripheral blood are merely specific to Gag or are a general hallmark of HIV-specific responses.

We showed, by using markers of replication, cytokine function, and activation levels, that circulating Gag-responsive CD4+ T cells generated at the onset of HIV infection are not sustainable. The rapid loss of these cells in the blood occurs, regardless of the course of viremia or categories of disease progression. By the time that these cells disappear, there is a significant expansion in the magnitude of Gag-responsive IFN- $\gamma$ +CD8+ T cells, which infers that there is a limited window of time postinfection when systemic Gag-responsive CD4+ T cells are potentially functional. We speculate that preventing high levels of viral replication, with either an effective vaccine or with other early interventions, would mitigate the early loss of peripheral CD4+ T cell responses upon HIV infection.

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### Disclosures

The authors have no financial conflicts of interest.

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