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The Breadth of Expandable Memory CD8$^+$ T Cells Inversely Correlates with Residual Viral Loads in HIV Elite Controllers

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ABSTRACT

Previous studies have shown that elite controllers with minimal effector T cell responses harbor a low-frequency, readily expandable, highly functional, and broadly directed memory population. Here, we interrogated the *in vivo* relevance of this cell population by investigating whether the breadth of expandable memory responses is associated with the magnitude of residual viremia in individuals achieving durable suppression of HIV infection. HIV-specific memory CD8$^+$ T cells were expanded by using autologous epitopic and variant peptides. Viral load was measured by an ultrasensitive single-copy PCR assay. Following expansion, controllers showed a greater increase in the overall breadth of Gag responses than did untreated progressors ($P = 0.01$) as well as treated progressors ($P = 0.0003$). Nef- and Env-specific memory cells expanded poorly for all groups, and their expanded breadths were indistinguishable among groups ($P = 0.9$ for Nef as determined by a Kruskal-Wallis test; $P = 0.6$ for Env as determined by a Kruskal-Wallis test). More importantly, we show that the breadth of expandable, previously undetectable Gag-specific responses was inversely correlated with residual viral load ($r = -0.6$; $P = 0.009$). Together, these data reveal a direct link between the abundance of Gag-specific expandable memory responses and prolonged maintenance of low-level viremia. Our studies highlight a CD8$^+$ T cell feature that would be desirable in a vaccine-induced T cell response.

IMPORTANCE

Many studies have shown that the rare ability of some individuals to control HIV infection in the absence of antiretroviral therapy appears to be heavily dependent upon special HIV-specific killer T lymphocytes that are able to inhibit viral replication. The identification of key features of these immune cells has the potential to inform rational HIV vaccine design. This study shows that a special subset of killer lymphocytes, known as central memory CD8$^+$ T lymphocytes, is at least partially involved in the durable control of HIV replication. HIV controllers maintain a large proportion of Gag-specific expandable memory CD8$^+$ T cells involved in ongoing viral suppression. These data suggest that induction of this cell subset by future HIV vaccines may be important for narrowing possible routes of rapid escape from vaccine-induced CD8$^+$ T cell responses.

Most human immunodeficiency virus (HIV)-infected individuals have continuous viral replication and, if left untreated, eventually progress to AIDS (1–4). Only a very small group of infected individuals, referred to as elite controllers (EC) or elite suppressors, achieves spontaneous control of viral replication for prolonged periods in the absence of treatment (5–8). This remarkable control of viral replication among elite controllers is believed to be mediated largely by major histocompatibility complex (MHC) class I-restricted CD8$^+$ T cell responses (9–13). These individuals therefore present a unique model for understanding the *in vivo* mechanisms of T cell-mediated immune control (14).

Most studies examining the relationship between CD8$^+$ T cell responses and viral load in elite controllers have focused on assays that measure effector memory rather than central memory responses. Using gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assays, the most frequent and robust CD8$^+$ T cell responses in elite controllers are directed toward the HIV Gag protein; particularly, p24 capsid protein targeting has been repeatedly shown to be associated with enhanced control of viremia *in vivo* (15) and *in vitro* (16). In contrast, preferential targeting of the HIV envelope (Env) protein has been associated with higher viral loads in both human and monkey studies (17–20). Although the precise mechanisms responsible for the enhanced antiviral function associated with Gag-specific responses are not fully understood, fitness costs associated with escape mutations from CD8$^+$ T cell responses directed at the highly conserved Gag protein have been implicated in both humans infected with HIV and primates infected with simian immunodeficiency virus (SIV) (21–27).

Despite extensive evidence supporting a specific role for CD8$^+$ T cells in immune-mediated control of HIV, not all elite controllers exhibit readily detectable CD8$^+$ T cell responses. Measure-

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ments of $CD8^+$ T cell responses by *ex vivo* cytokine secretion assays fail to accurately measure central memory responses (18). Consequently, the role of this cell subset in the immune-mediated control of HIV remains ill defined. A recent analysis of HIV-specific $CD8^+$ T cells following *ex vivo* enrichment and after expansion in culture defined the phenotype and functional features of HIV-specific central memory $CD8^+$ T cells (28). These studies show that in addition to the readily detectable responses, most elite controllers harbor a wide range of low-frequency but highly functional and readily expandable Gag-specific memory cells, which are able to inhibit virus replication *in vitro* (28). However, it is not known whether this population contributes to durable viral suppression. Moreover, it is not known if this is a property that is limited to responses targeting the Gag protein or whether expandable central memory responses to other HIV protein targets are also involved.

In this study, we interrogated the *in vivo* relevance of the expandable memory population based on the premise that there are two possibilities for the role of this population in HIV elite controllers. They may be directly responsible for ongoing active suppression of the virus, or they could be footprints of immune responses to epitopes that have escaped or were completely suppressed. We measured the breadth, specificity, and functional characteristics of expandable memory cells in elite controllers and chronic progressors (CP). We also investigated whether there was a relationship between the breadth of the expandable responses and viral load. Our data demonstrate that expandable responses in HIV controllers are directed predominantly against the HIV Gag protein and show a link between the abundance of expandable responses to epitope variants and durable virus suppression. Importantly, we show an inverse relationship between the breadth of Gag-specific expandable responses and the level of plasma viremia. These data shed light on immune responses that are most associated with sustained viral control and therefore desirable to induce through HIV vaccination.

MATERIALS AND METHODS

Study subjects. HIV-infected individuals were recruited from outpatient clinics at Massachusetts General Hospital and affiliated Boston-area hospitals. The respective institutional review boards approved this study, and all subjects gave written informed consent. A total of 16 EC, 2 viremic controllers (VC), 13 untreated CP, and 15 treated CP were studied. Detailed definitions of controllers and chronic progressors were described previously (29). In brief, EC were defined as having plasma HIV RNA levels of <50 copies/ml in the absence of antiretroviral therapy, on at least three determinations over at least a year of follow-up. VC had detectable HIV-1 RNA levels of <2,000 copies/ml. CP were defined as subjects having untreated HIV infection for >1 year with plasma viral loads of >2,000 copies/ml for at least 1 year of follow-up; treated (antiretroviral therapy [ART]) chronic progressors had HIV RNA levels below the limit of detection for the respective available standard assays (e.g., <75 RNA copies/ml by branched DNA assay or <50 copies by PCR). All subjects selected for this study had absolute $CD4^+$ T cell counts of >400 cells/mm^3. All experiments were performed on cryopreserved peripheral blood mononuclear cells (PBMCs).

IFN-γ ELISPOT assays. Gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays were performed as described previously (28), using a final concentration of 100 ng/ml (20 ng of the peptide mix in a 200-μl volume) of overlapping peptides (OLPs) and incubation overnight. We used OLPs corresponding to the HIV clade B sequence from 2001 (17). OLPs averaged 18 amino acids in length, overlapped by 10 amino acids, and spanned the entire HIV Gag, Nef, and Env proteins. The number of input cells ranged from 50,000 to 100,000 cells per well, depending on cell availability. The number of specific spot-forming cells (SFC) was calculated by subtracting the number of spots in the negative-control wells from the number of spots in each experimental well. A positive response was defined as a well having at least three times the mean number of SFC in the three negative-control wells. Wells with positive responses also had to have at least 50 SFC/10^6 PBMCs (28). The magnitude of the epitope-specific response was reported as the number of SFC per million PBMCs.

Cultured IFN-γ ELISPOT assays. Peptide-stimulated cells were cultured at 37°C with 5% CO_2 for 12 days in RPMI medium containing 10% heat-inactivated fetal calf serum (R10 medium) and in R10 medium supplemented with 50 U/ml of recombinant human interleukin-2 (IL-2) (R10/50 medium). A 100-ng/ml final peptide concentration was used for cultured stimulation because it was determined to be the optimal concentration at which pooled overlapping HIV peptides stimulated the greatest number of responses (28). Cultures were supplemented with fresh R10/50 medium at 3-day intervals or as needed. On day 12, cells were washed three times with fresh R10 medium and rested at 37°C with 5% CO_2 overnight in fresh R10 medium. The cells were then retested against OLPs spanning HIV Gag, Nef, and Env proteins in an overnight ELISPOT assay, as described above.

Flow cytometry. After 12 days of culture stimulation, cells were rested overnight in R10 medium and then restimulated with Gag OLPs at a 20-ng/ml final concentration or optimal peptides for 1 h at 37°C with 5% CO_2. After 1 h, 10 ng/ml of brefeldin A (Sigma-Aldrich, St. Louis MO, USA) was added, and the cells were incubated for another 5 h. At the end of the stimulation period, intracellular cytokine staining (ICS) was performed according to the BD Biosciences ICS protocol. Briefly, cells were first stained with dead cell dye for 10 min and then washed and surface stained with anti-CD3, -CD4, and -CD8 and exclusion channel antibodies (CD14, CD19, and CD56). The cells were then fixed and permeabilized with Cytofix/Cytoperm solution and stained with anti-IFN-γ antibody (BD Biosciences, San Jose, CA, USA). Following staining, the cells were resuspended in phosphate-buffered saline (PBS) containing 2% paraformaldehyde. The cells were acquired on a BD LSRFortessa cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed with the FlowJo software package (Treestar, Ashland, OR).

Virus inhibition assay. The ability of $CD8^+$ T cells to inhibit virus replication in autologous primary $CD4^+$ T cells was assessed by measuring p24 antigen production, as described previously, with modifications (16). Briefly, primary $CD4^+$ and $CD8^+$ T cells were isolated by using CD4 MicroBeads and CD8 MicroBeads, respectively (Miltenyi Biotech, San Diego, CA, USA). Enriched $CD8^+$ T cells were cultured for 9 days with Gag, Nef, or Env OLP pools. IFN-γ ICS was used to assess the frequency of expandable cells specific for each of the three HIV genes. $CD4^+$ T cell targets were prepared by stimulating the magnetic bead-enriched $CD4^+$ T cells with a CD3/CD8-bispecific antibody and infecting the cells on day 3 with the NL4-3 laboratory-adapted HIV strain at a multiplicity of infection (MOI) of 0.001 for 4 h at 37°C (30). The virus-infected $CD4^+$ T cells were then incubated in the presence or absence of expanded $CD8^+$ T cells at an adjusted effector-to-target-cell ratio of 1:1. The number of input $CD8^+$ T cells for each cell culture was adjusted based on the frequency of IFN-γ-secreting cells, such that equivalent numbers of HIV-specific $CD8^+$ T cells were added to each culture. The cultures were fed at regular intervals by removing and replacing one half of the culture supernatant with fresh medium. Supernatants harvested at days 3, 5, and 7 were cryopreserved for later p24 antigen quantification by an enzyme-linked immunosorbent assay (ELISA) (PerkinElmer, Boston, MA). Log inhibition values were calculated by subtracting $\log_{10}$ p24 values for cultures with $CD8^+$ T cells from $\log_{10}$ p24 values for cultures without $CD8^+$ T cells at day 7.

Determination of HIV-1 RNA levels by single-copy assays. To accurately quantify low-level viremia in elite controllers and in patients receiving antiretroviral therapy, we used a highly sensitive quantitative real-time

reverse transcriptase (RT)-initiated PCR (RT-PCR) assay that detects and quantifies HIV-1 RNA levels down to 1 copy/ml. The assay was performed as described previously (31). Briefly, the assay was performed in three steps: RNA extraction, reverse transcription, and quantitative real-time PCR. A key internal control aimed at monitoring the recovery of HIV-1 from plasma samples involved the spiking of plasma samples with a known transcript copy of RNA isolated from an avian sarcoma leukosis retroviral vector (RCAS). Seven milliliters of plasma from each subject was mixed with 200 μl of the RCAS stock (300,000 copies of RNA) before RNA extraction and quantification by RT-PCR. PCR amplification of the HIV Gag region was performed on either plasma or PBMC samples from each subject, as previously described (31).

Real-time PCR plate setup. For each real-time PCR run, two standard curves were generated, one for HIV and the other for RCAS. To generate the standard curves, a prequantified HIV RNA stock serially diluted to between 1 million copies and 0.3 copies/10 ml was used. Similarly, RCAS standards were generated in duplicate by using a serially diluted RCAS RNA stock. To ensure that there was no contamination of the PCR reagents, a template control was tested in duplicate for both RCAS and HIV primers/probes with no plasma samples added (31). For each specimen, three replicate reactions were performed for HIV quantification. The number of copies of HIV was derived from the calculated number of copy equivalents per reaction mixture and was expressed as the number of copies per milliliter of the starting plasma sample.

Assay reproducibility and limit of quantitation. The assay was validated by running 6 independent real-time PCRs in duplicate, using previously determined HIV-1 RNA amounts ranging from 100,000 to 0.4 copies per reaction mixture. Linear regression analyses revealed that the assay was sensitive enough to detect as little as a single copy of HIV-1 RNA in the linear dynamic range of between 0.4 copies and 100,000 HIV RNA copies per reaction mixture ($R^2 = 0.94$). The assay also showed excellent agreement between the expected values and the measured values for 6 independent serial dilutions (run in duplicate) of the control HIV RNA transcript ($R^2 = 0.96$).

Statistical analyses. Spearman rank correlation simple linear regression and Mann-Whitney tests were performed by using GraphPad Prism version 5.0b. All tests were two tailed, and P values of <0.05 were considered significant.

RESULTS

We studied 46 subjects chronically infected with HIV, including 16 HIV EC, 2 VC, 13 CP, and 15 individuals treated with ART. Table 1 provides information on age, sex, absolute CD4 cell counts, and virus loads at the time when samples were tested for HIV-specific immune responses. Table 2 shows HLA class I alleles of the study participants and indicates the experiments performed on samples from each subject.

Expandable memory CD8$^+$ T cells from elite controllers preferentially target Gag rather than Env and Nef. Given that EC maintain prolonged control of HIV viremia, it is important to understand the immune responses that are associated with this equilibrium. To determine the potential contribution of expandable responses to various epitopic regions in chronically infected individuals, we examined HIV protein targeting and the breadth of the expandable responses. Our analysis focused on memory responses to three HIV proteins: Gag, Nef, and Env. We chose these three HIV proteins for the following reasons: (i) T cell responses directed to the HIV Gag protein have consistently been associated with lower-level viremia (17, 32–35); (ii) Nef responses are immunodominant early during acute HIV infection and may be involved in the initial control of viremia, and therefore, their persistence may be relevant to long-term viral control (36–39); and (iii) responses targeting the Env region have been shown to be important for both T cell and B cell immune responses (40–42), and the breadth of Env-specific responses has been positively associated with viral load (17, 43). Thus, elucidating the role of long-lived responses to these HIV proteins may be relevant for vaccines.

TABLE 1 Baseline characteristics of study participants

Patient characteristic[a]	Value for group ($n = 46$)		
	Controllers (EC, $n = 16$; VC, $n = 2$)	Progressors (CP, $n = 13$)	Treated subjects (ART, $n = 15$)
No. (%) of patients of gender			
Male	16 (88.9)	12 (92.3)	12 (80)
Female	4 (11.1)	1 (7.7)	3 (20)
Age (yr)			
Median	56	50	50.5
Q1–Q3	46–73	38–64	38–64
No. of CD4$^+$ T cells/mm^3			
Median	733	559	508.5
Q1–Q3	486–1,786	436–1,320	443.3–1,028
Log_{10} HIV copies/ml determined by a standard assay			
Median	1.7	3.8	1.7
Q1–Q3	1.7–2.4	3.6–4.6	1.7–3.5

[a] Q1–Q3 denotes interquartile ranges for the parameters indicated.

We first carried out a comparative analysis of expandable responses in three subject groups using cultured ELISPOTs. Overlapping peptides (OLPs) were used to screen for HIV-specific T cell responses. The peptides spanned HIV Gag, Nef, and Env proteins and were based on the consensus clade B sequence. The peptides were 15 to 20 amino acids in length, overlapping by 10 amino acids. PBMCs were initially cultured for 12 days in the presence of OLPs. An IFN-γ ELISPOT assay was then performed on the cultured cells. To investigate whether there were quantitative differences in the frequencies of expandable responses directed against the three HIV proteins, we compared the breadths of Gag, Nef, and Env responses in cultures. Breadth of response in this study is defined as the sum of IFN-γ-positive OLP responses for the entire HIV protein. Although the breadths of *ex vivo* Gag responses determined by IFN-γ ELISPOT assays were comparable between HIV controllers and progressors ($P = 0.28$, as determined by a Kruskal-Wallis test) (Fig. 1A), upon peptide stimulation, controllers showed a greater breadth of Gag responses than did untreated progressors ($P = 0.01$) as well as treated progressors ($P = 0.0003$) (Fig. 1B). Furthermore, stimulated expansion of controller PBMCs with Gag peptides resulted in a significantly larger number of responses that were not detectable before expansion (new responses) than those of untreated progressors ($P = 0.03$, as determined by a Kruskal-Wallis test) (Fig. 1C). The breadths of *ex vivo* Nef responses were indistinguishable among the groups ($P = 0.1$, as determined by a Kruskal-Wallis test) (Fig. 1D), and the responses expanded poorly upon cul-

TABLE 2 Study participants, class I HLA alleles, and experiments[a]

Subject	HLA class I allele						Subject used for expt[b]			
	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2	Cultured ELISPOT	SCA	VIA	TW10, T3N, G9D responses
Elite controllers										
586181	0201	0302	0801	5701	0602	0701	✓			
108896	0201	2402	0801	4001	0304	0304	✓		✓	
835698	0201	3001	1302	5701	0602	0602	✓	✓	✓	✓
255675	0201	0201	2705	5701	0102	0602	✓	✓	✓	✓
540772	0101	6801	5501	5701	0303	0602	✓	✓	✓	✓
731849	0101	7400	3701	8100	0602	1800	✓	✓		
701554	1101	3201	3501	5001	0401	0602	✓	✓		
194133	0201	3004	3901	4101	1203	1700	✓	✓		
382086	3001	3201	1302	4001	0304	0602	✓	✓	✓	
849151	0101	2601	2705	5701	0202	0602	✓	✓		✓
473516	0101	3402	0801	5701	0701	0602	✓	✓		✓
595424	0201	1101	1402	5101	0802	1402	✓	✓		
555477	0301	2601	1401	5701	0602	0802	✓	✓	✓	✓
275432	0103	3201	3508	7301	0401	1505	✓	✓		
553064	0201	0301	1501	2705	0102	0304	✓	✓		
847733	0201	2301	4403	5106	1601	1601	✓	✓		
164007	0101	0301	5701	4102	0602	1202	✓	✓		
831969	0301	2601	4501	5701	0602	0602	✓	✓		
Chronic progressors										
387879	2902	3002	0702	3501	0401	1505	✓			
950005	0101	3101	4001	5801	0302	0304	✓			
690641	0201	3303	1302	2705	0401	0202	✓			
185075	0101	0201	4402	5701	0102	0602	✓		✓	✓
805181	0201	0201	0702	4403	0401	0702	✓			
667335	0201	2902	4403	4403	1601	1601	✓			
156261	0101	0206	1501	5701	0304	0602	✓			✓
703459	2902	2902	4403	4501	0602	1601	✓			
743887	0201	0301	1801	3501	0401	0701	✓		✓	
930133	0101	2402	1302	5701	0602	0602	✓	✓		✓
705357	3301	3601	1402	5301	0401	0802	✓		✓	
930024	0101	6801	0801	5701	0602	0701				✓
388555	0101	3201	4402	5701	0501	0602				✓
ARV treated										
930213	0201	0201	4001	4001	0304	0501	✓	✓		
453230	0301	3601	3502	5703	0401	0701	✓	✓		
508473	0217	2902	4403	5101	1502	1601	✓	✓		
540319	0101	6801	1517	2705	0102	0702	✓	✓	✓	
289151	0101	0201	2705	5101	0102	1502	✓	✓		
985170	3001	3303	4201	4201	1700	1700	✓	✓		
516917	0101	0101	0801	1501	0401	0701	✓	✓	✓	
672068	0301	1101	4001	4402	0304	0501	✓	✓		
409231	0101	0201	0801	5101	0701	1502	✓	✓	✓	
672068	0301	0110	4001	4402	0304	0501	✓	✓		
608520	3101	7400	3501	5701	0602	1601	✓	✓		✓
350103	0201	0301	1501	5701	0401	0602	✓	✓		✓
898049	0201	0301	4102	5701	0602	1700	✓	✓		✓
922403	0101	0301	0702	5701	0602	0702	✓	✓		✓
403998	0101	0201	4402	5701	0602	1203	✓	✓		✓

[a] Shown is a list of all subjects studied, including class I HLA information and the all the experiments performed. SCA, single copy PCR assay; VIA, virus inhibition assay.
[b] ✓ indicates that the subject was used for the indicated experiment.

ture stimulation in all groups ($P = 0.9$ for Nef, as determined by a Kruskal-Wallis test) (Fig. 1E). Similarly, *ex vivo* Env responses (Fig. 1G) and expanded Env responses (Fig. 1H) were indistinguishable among the groups ($P = 0.07$ for *ex vivo* Env, as determined by a Kruskal-Wallis test; $P = 0.6$ for expanded Env, as determined by a Kruskal-Wallis test). The breadth of expanded responses that were not detectable *ex vivo* (new responses) was small for both Nef and Env stimulations (Fig. 1F and I) for all groups. Together, these data demonstrate that the majority of the HIV-specific expandable memory pool

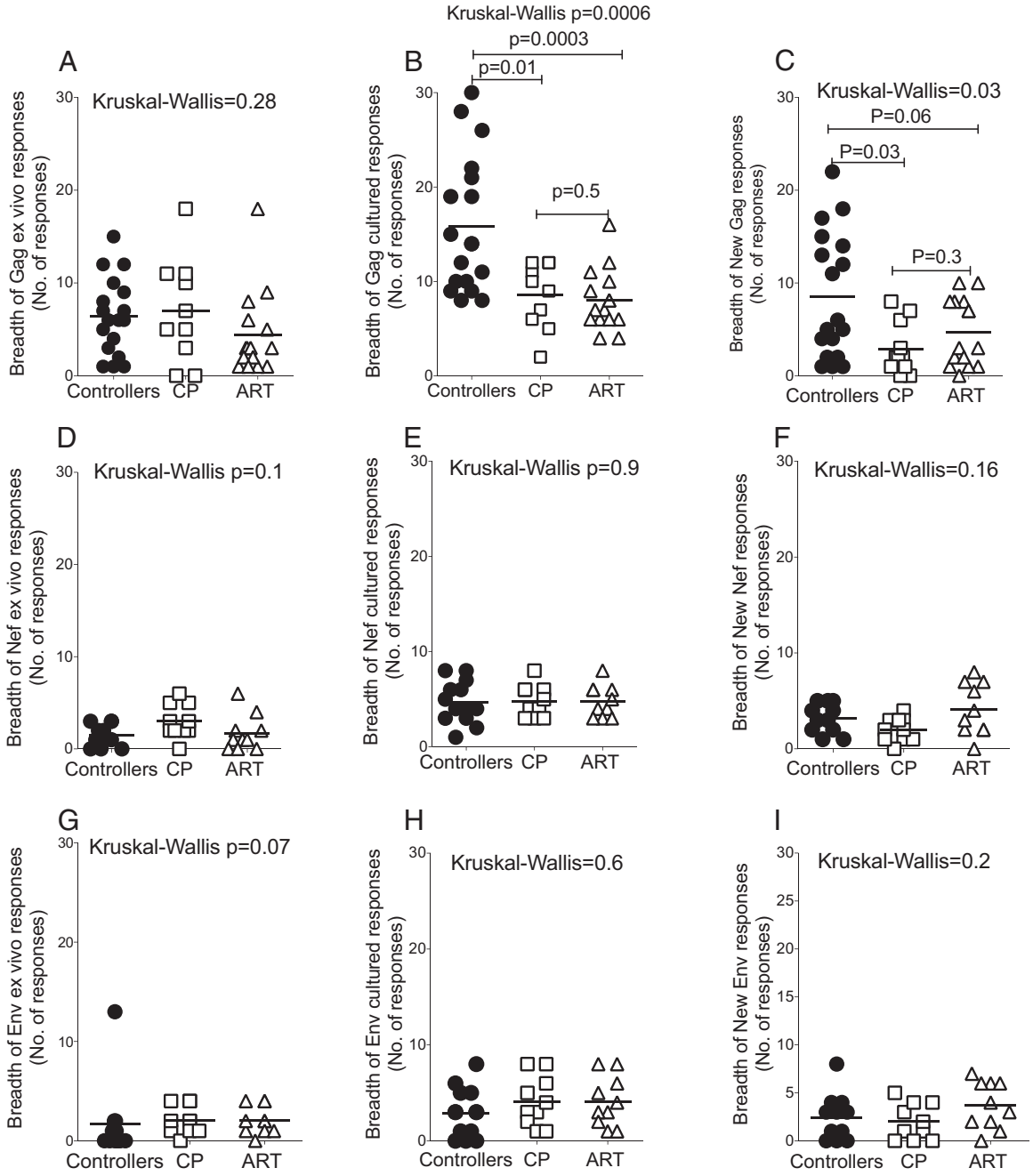

FIG 1 Expandable memory responses in elite controllers are directed predominantly toward the HIV Gag protein. (A and B) Gag-specific responses of controllers and treated and untreated progressors measured *ex vivo* (A) and in cultures (B). (C) Gag-specific responses, undetectable at baseline, become detectable following stimulation of cultures. (D and E) Nef-specific responses in controllers and treated and untreated progressors measured *ex vivo* (D) and in cultures (E). (F) Nef-specific responses are undetectable at baseline but become detectable following stimulation of cultures. (G and H) Env-specific responses in controllers and treated and untreated progressors measured *ex vivo* (G) and in cultures (H). (I) Env responses are low at baseline but increase following stimulation of cultures. For all panels, horizontal bars denote mean values. *P* values were calculated by using the two-tailed Mann-Whitney test and the Kruskal-Wallis one-way analysis of variance.

maintained by HIV controllers is directed predominantly against Gag.

Expandable responses of HIV controllers to Gag retain a superior virus-inhibitory capacity compared to those for Env and Nef. Previous studies have shown that qualitative features of host CD8$^+$ T cell responses are more strongly associated with immune-mediated control of HIV replication than quantitative parameters (10, 44–47). We therefore compared the virus inhibition capacities of memory CD8$^+$ T cells directed against the three HIV proteins. We chose to examine this function because we had previously shown that Gag-specific central memory cells preferentially retain the capacity to inhibit virus replication upon stimulated

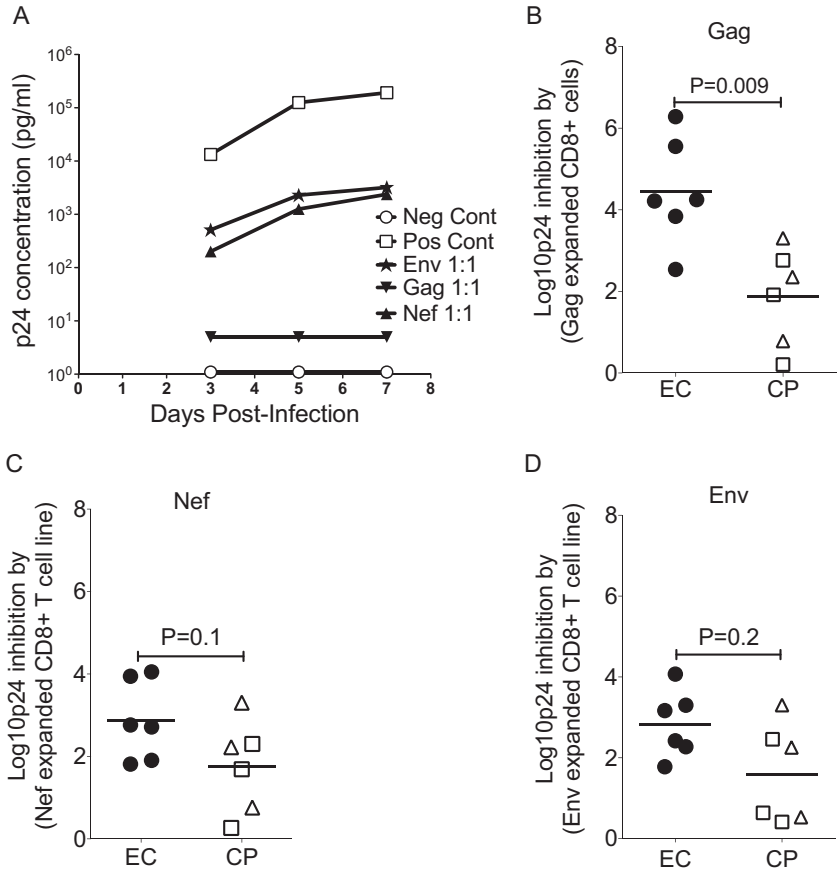

FIG 2 Virus inhibition of NL4-3-infected autologous $CD4^+$ T cells by memory responses. (A) NL4-3-infected $CD4^+$ T cells were cultured at an adjusted ratio of 1:1 with unstimulated PBMCs or PBMCs stimulated with HIV Gag, Nef, or Env peptide pools. (The adjusted ratio is described in Materials and Methods.) Control conditions included infected $CD4^+$ T cells alone (positive control) and uninfected $CD4^+$ T cells (negative control). Log_{10} differences in p24 values between $CD4^+$ T cells alone and those cocultured with $CD8^+$ T cells at days 3, 5, and 7 for a representative elite controller are displayed. (B to D) Virus inhibition data for 6 elite controllers and 6 progressors, reported as fold reductions in log_{10} p24 antigen concentrations in supernatants from Gag (B)-, Nef (D)-, and Env (E)-expanded $CD8^+$ T cells cocultured with NL4-3-infected autologous $CD4^+$ T cells. Data are calculated as fold reductions in log_{10} p24 antigen concentrations at day 7.

expansion with overlapping Gag peptides (28). We directly compared the inhibitory capacities of Gag-, Nef-, and Env-specific expanded $CD8^+$ T cells by measuring their ability to inhibit HIV replication in autologous CD4 T cells infected *in vitro*. We selected donors with no detectable inhibitory activity before culture (data not shown). Representative data for one elite controller (Fig. 2A) show that $CD8^+$ T cells that expanded following HIV Gag, Nef, and Env stimulations resulted in a reduction in p24 antigen production over a 7-day culture period, with the most effective suppression being shown by Gag-specific $CD8^+$ T cells. We next compared the inhibitory capacities of expandable responses in 6 elite controllers and 3 treated and 3 untreated chronic progressors. Subjects with at least five expandable responses to Gag, Nef, and Env were selected for these studies. On a per-cell basis, Gag responses of HIV controllers had a significantly greater inhibition capacity than did those of progressors ($P = 0.009$) (Fig. 2B), whereas no significant differences were observed for Nef responses ($P = 0.1$) (Fig. 2C) or Env responses ($P = 0.2$) (Fig. 2D) among groups. The expansion data and the virus inhibition data collectively suggest that HIV controllers have a greater capacity to maintain a larger breadth of functionally superior expandable responses directed against HIV Gag.

Correlation between the breadth of HIV-specific memory $CD8^+$ T cell responses and HIV plasma viral load. In order to ascertain the *in vivo* relevance of expandable memory responses, we next set out to examine the relationship between the breadth of this cell population and viral load, which is a well-defined prognostic marker of HIV disease progression and has been associated with $CD4^+$ T cell loss in elite controllers (48–50). We used an internally controlled real-time reverse transcriptase PCR assay that quantifies the HIV RNA concentration down to 1 copy per ml of plasma to accurately quantify viral loads in elite controllers and in progressors whose plasma HIV RNA levels are suppressed to below 75 copies/ml. We first used the single-copy assay to measure HIV RNA levels in plasma samples obtained from elite controllers and ART-suppressed HIV-infected patients. Our analysis revealed comparable viral load levels between elite controllers and ART-suppressed subjects ($P = 0.11$, as determined by a Mann-Whitney test) (Fig. 3A). Two treated progressors had 100 copies/ml of HIV RNA as determined by the single-copy assay, even though they had levels that were below the limit of detection according to standard viral load measurements, highlighting the greater sensitivity of this assay. We next investigated if residual viremia influences HIV disease progression. Here, residual viremia refers to

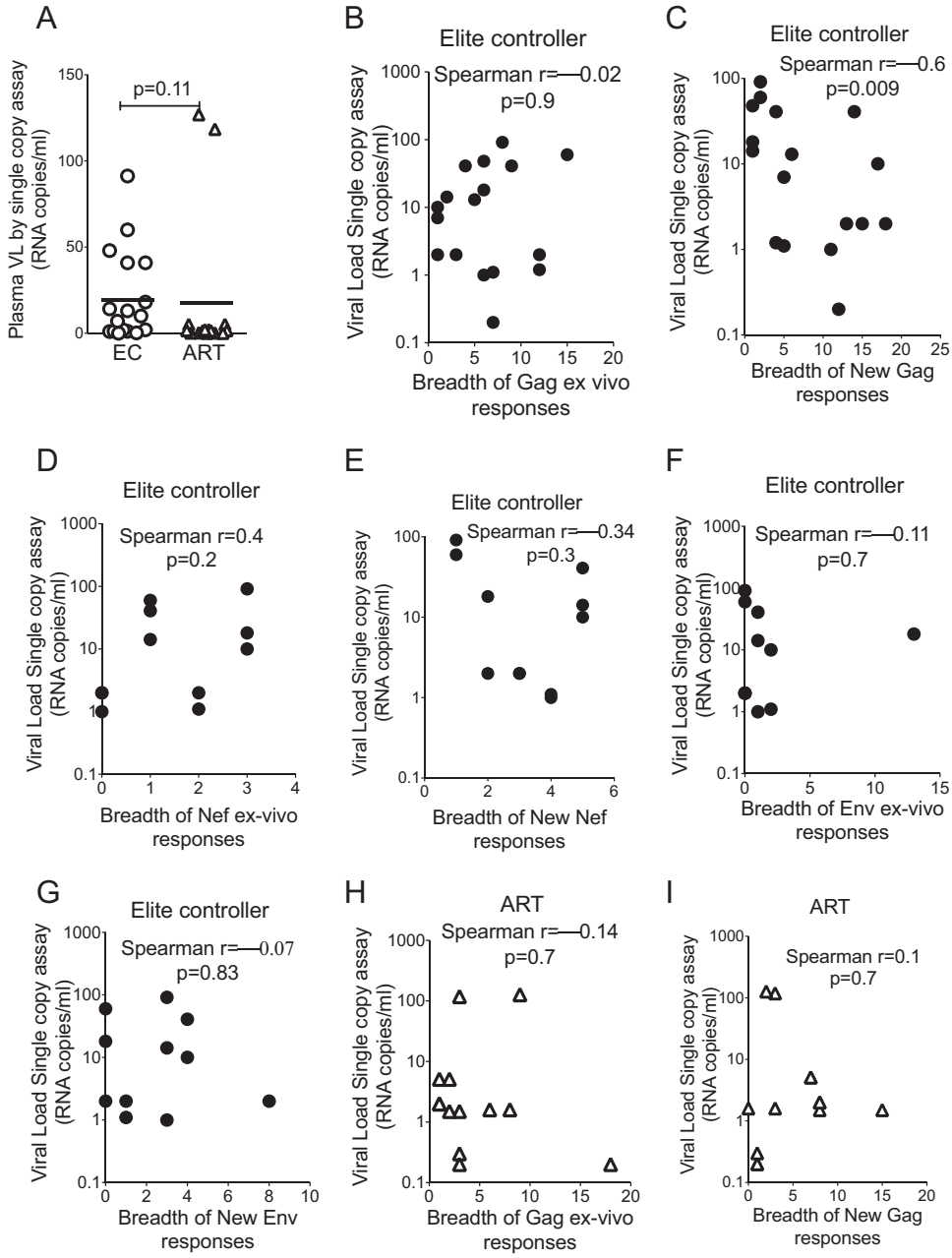

FIG 3 The breadth of Gag-specific memory responses is inversely correlated with residual plasma viral load in HIV elite controllers. (A) Plasma viral RNA copy numbers measured by a single-copy PCR assay for elite controllers and ART-suppressed chronic progressors whose HIV RNA levels were below the limit of detection by a standard viral load (VL) assay. (B and C) Elite controller plasma viral loads measured by a single-copy assay, plotted against the breadth of *ex vivo* Gag-specific responses (B) or expandable, previously undetectable (new) Gag-specific responses (C). (D and E) Elite controller plasma viral loads measured by a single-copy assay, plotted against the breadth of *ex vivo* Nef-specific responses (D) or new Nef-specific responses in cultures (E). (F and G) Elite controller plasma viral loads measured by a single-copy assay, plotted against the breadth of *ex vivo* Env-specific responses (F) or (new) Env-specific responses in cultures (G). (H and I) Plasma viral loads of ART-suppressed subjects measured by a single-copy assay, plotted against the breadth of *ex vivo* Gag-specific responses (H) or new Gag-specific responses in cultures (I). The correlation coefficient (r) values and P values were determined by the Spearman rank correlation test.

low-level viremia that can be detected by the ultrasensitive single-copy viral load assay but not standard viral load assays. Interestingly, the residual plasma viral load in controllers was inversely correlated with the breadth of new (absent before expansion) Gag memory responses (Spearmen's $r = -0.6$; $P = 0.009$) (Fig. 3C), whereas *ex vivo* responses (Spearman's $r = -0.02$; $P = 0.9$) (Fig. 3B) and total expanded responses (data not shown) did not. Neither *ex vivo* nor expanded (new) Nef responses (Spearman's $r = 0.4$ and $P = 0.2$ for *ex vivo* responses; Spearman's $r = 0.3$ and $P = 0.3$ for expanded responses) correlated with viral load (Fig. 3D and E). Similarly, Env responses (Spearman's $r = -0.1$ and $P = 0.7$ for *ex vivo* responses; Spearman's $r = -0.07$ and $P = 0.8$ for expanded responses) did not correlate with residual viral load (Fig. 3F and G). These data show an association between the

breadth of new Gag-specific (previously undetectable) responses and viral load. We next carried out similar analyses for ART-suppressed subjects, but we were unable to detect significant correlations for either *ex vivo* responses (Spearman's $r = -0.1$; $P = 0.7$) (Fig. 3H) or expanded (new) Env responses (Spearman's $r = 0.1$; $P = 0.7$) (Fig. 3I). Collectively, our data demonstrate a strong association between a larger breadth of the Gag-specific memory pool and effective viral suppression in the absence of antiretroviral therapy. These data also support a model in which very-low-frequency but highly expandable HIV-specific memory cells may be directly involved in sustained viral suppression *in vivo* and that broad Gag-specific memory responses are important for sustained HIV suppression.

HIV controllers maintain a large pool of epitope variant (type)-specific expandable memory CD8$^+$ T cells directed against *in vivo*-occurring viral variants. Previous studies have demonstrated that accumulation of viral escape mutants contributes to higher viral loads during chronic AIDS virus infection (51–55). In addition, the ability to mount *de novo* CD8$^+$ T cell responses with sufficient cross-reactivity to potential escape variants may be one mechanism by which elite controllers maintain lower viral loads despite ongoing viral evolution (47, 56). We hypothesized that maintenance of a greater breadth of memory responses against escape variants might also contribute to sustained suppression of HIV. We addressed this issue by determining whether EC maintain immune responses to a wide range of epitope variants known to arise *in vivo*. To simplify the analysis, we focused on a well-characterized protective $Gag_{240–249}$ TW10 epitope and two of its *in vivo* variants (57). We chose the TW10 epitope because the T242N (T3N) mutation in this epitope is the most frequently detected mutation, which occurs rapidly after acute infection in HLA-B*57/B*58-positive persons (58). The T3N mutation is also associated with viral replicative fitness cost, and the arising variants can be targeted by specific immune responses (26, 59).

We performed cultured expansions of HIV-specific CD8$^+$ T cells using the wild-type (WT) TW10 epitope as well as the T3N and G248D (G9D) variant epitopes. The no-peptide-stimulation culture served as the negative control. Sixteen HLA-B*5701 donors, comprised of 6 elite controllers, 5 chronic progressors, and 5 treated progressors, were used for these studies (Table 2). To determine the specificity of the expanded memory CD8$^+$ T cells, we tested the reactivity of each expanded population against the peptide used to generate the cell line as well as against the other two TW10 variant peptides. Culture stimulation of PBMCs from one elite controller (known to have the G9D mutation in plasma viruses) in the presence of TW10 peptide variants resulted in a significant expansion of the G9D-, WT-, and T3N-specific cells populations (Fig. 4A). In contrast, a chronically infected untreated B*5701 donor with the T3N mutation in plasma viruses expanded only T3N-reactive cells (Fig. 4B). Interestingly, each expanded memory cell population was more strongly reactive to the peptide used for its expansion (Fig. 4A and B). Figure 4C to E show summary data for all 16 donors studied. Figure 4C shows data for 6 elite controllers. PBMCs were expanded with the TW10 (Fig. 4C, left), T3N (middle), and G9D (right) epitopes and tested for reactivity to the three variant peptides. The data show that elite controllers maintained significantly higher frequencies of memory CD8$^+$ T cells with the wild-type TW10 and G9D variant epitopes. Figure 4D shows data from a similar analysis for untreated chronic progressors. The data reveal that memory responses of chronic progressors were narrowly directed to T3N (Fig. 4D, middle). Figure 4E shows that ART-suppressed subjects also maintained only T3N memory responses. Overall, these data show that elite controllers maintain larger pools of expandable memory responses to epitope variants, at least in the context of HLA-B*5701, than do chronic progressors.

DISCUSSION

This study confirms and extends data from a previous study showing that maintenance of a greater breadth of central memory T cell (Tcm) responses targeting the HIV Gag protein is associated with durable immune-mediated control in the setting of natural HIV infection. These data support the hypothesis that non-Gag-specific CD8$^+$ T cell responses, particularly Nef- and Env-specific responses, contribute less to durable HIV suppression. Although the importance of Gag-specific CD8$^+$ T cell responses for the elite control of HIV has been demonstrated in many studies, we now demonstrate that Gag-specific new (absent before expansion) memory responses are associated with sustained control of plasma HIV viremia. More importantly, the observed effect of expandable responses on residual viremia strongly suggests that immune control in chronic HIV infection is an active and ongoing process.

In this study, we further elucidated the superiority of Gag responses by showing that under conditions of very low antigen loads, HIV controllers preserve larger breadths of Gag responses to viral variants. We also show that on a per-cell basis, the expandable Gag responses retain superior antiviral qualities compared to the Nef and Env responses, even within individual subjects. Furthermore, the breadth of Gag memory responses was associated with lower residual viral loads, whereas no association was observed for either Nef or Env responses. We also showed that the superiority of Gag memory responses in terms of virus-inhibitory capacity is lost during progressive HIV infection, and complete suppression of viral replication with combination ART did not restore this. More importantly, in ART-treated individuals, the breadth of memory responses (including Gag responses) did not correlate with viral load, although there was a trend toward a negative association. Even though cross-sectional analyses cannot unequivocally provide a direct proof of causality, these data strongly suggest that low-frequency but readily expandable memory responses play an active role in sustained suppression of HIV. These data also indicate that there are qualitative differences in memory responses in controllers compared to those in progressors in terms of antiviral function, and a lack of effective expandable memory responses in progressors might allow rapid viral rebound upon cessation of antiviral therapy.

Other features associated with the superiority of Gag responses in HIV controllers are the capacity to cross-recognize viral variants and the ability to induce *de novo* responses to mutant viruses (9, 47, 56). Both HIV and SIV infections exhibit selection of escape variants during both primary and chronic infections. It has also been shown that the selection of viral escape variants during chronic SIV and HIV infections can result in a loss of immune control and disease progression (53, 60–62). Thus, the ability of HIV to escape from virus-specific CD8$^+$ T cell responses has been proposed to be an important obstacle for the maintenance of protective immune responses, and likewise, the viral diversity resulting from CD8$^+$ T cell-driven viral evolution represents a major hurdle for HIV vaccine design (63). Interestingly, elite controllers

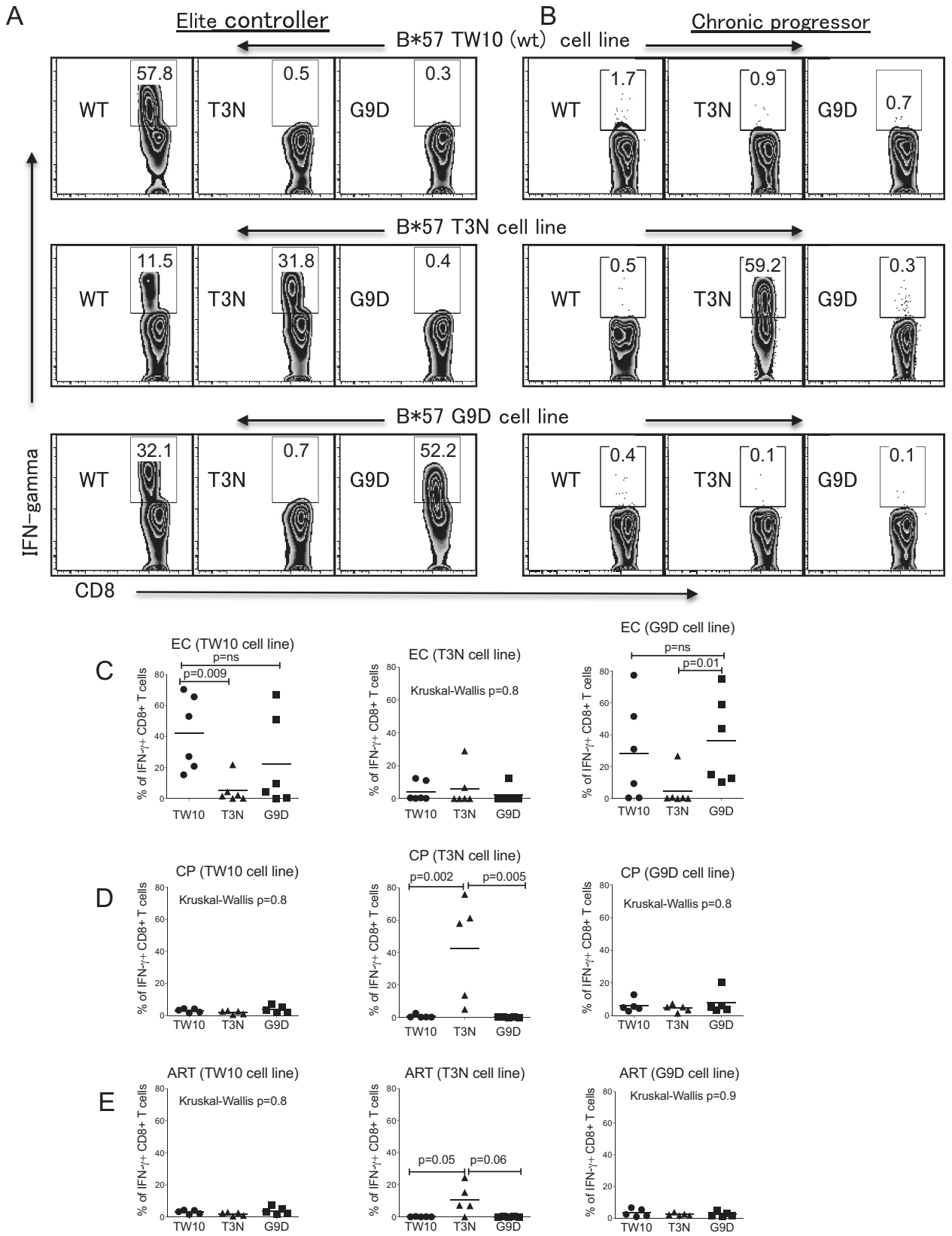

FIG 4 Elite controllers maintain a large pool of expandable memory responses specific for the TW10 epitope and its *in vivo*-occurring variants. PBMCs from HLA-B*57 donors were stimulated with either wild-type TW10 or variant peptides. Each expanded population was then tested for reactivity to other variants by using IFN-γ ICS. (A) Representative flow cytometry plot for one elite controller. Numbers in the gates represent the proportions of IFN-γ-secreting $CD8^+$ T cells. (B) Representative flow cytometry plot for a chronic progressor. (C to E) Summary data for 6 controllers (C), 5 progressors (D), and 5 ART-suppressed subjects (E). Data show how each population expands when a specific epitope cross-reacts with variant epitopes. ns, not significant.

appear to be able to control the virus despite ongoing evolution and the development of escape mutations in key $CD8^+$ T cell epitopes. Two main mechanisms of control of virus replication by $CD8^+$ T cells in the face of viral variation include the generation of a repertoire of effective T cell clonotypes and the ability to recognize individual viral epitope variants (47, 57). In this study, recognition of variant epitopes was measured by an IFN-γ ICS assay, which was shown in a previous SIV study to not necessarily correlate with the ability to suppress escape mutants (64). However, consistent with our findings, a recent study used $CD4^+$ T cells infected with mutant viruses as target cells in virus inhibition assays and also showed that $CD8^+$ T cells of elite controllers can suppress mutant viruses (65). Our data suggest that the capacity to maintain highly functional expandable memory responses with antiviral activity against viral variants is one possible mechanism contributing to durable virus suppression in HIV controllers.

The precise mechanisms that render Gag responses more effective in controlling HIV are unclear. Some proposed mechanisms include a rapid expression of epitopes derived from the Gag protein contained in the infecting viral particles and structural constraints of the Gag protein that impede $CD8^+$ T cell escape (66–70). However, these suggestions are inconsistent with the fact that beneficial effects of Gag-specific responses are not universal among elite controllers, and indeed, most progressors also mount detectable responses against Gag (71, 72). This discrepancy can partly be explained by our recent data showing that only a subset of Gag responses, particularly those restricted by protective alleles, exhibits superior antiviral function (10).

It is important to note that although we show strong evidence for the role of expandable memory responses in durable virus suppression, other cell subsets may play equally important roles in different stages of HIV infection. Indeed, several studies have shown that a wide range of phenotypically distinct memory subsets possess virus-inhibitory potential. In these *in vitro* studies, effector populations demonstrate an inhibitory capacity in short-term stimulations, whereas long-term stimulation results in increased inhibitory activity by central memory cells (73, 74). Effector memory T cells (Tem) and Tcm play complementary roles in the immune-mediated suppression of HIV replication. Tem are much more effective at controlling new infections because they are endowed with more immediate effector functions and can populate lymphoid and extralymphoid sites, which are the initial sites of HIV exposure (75). A case in point is a recent study in which a Tem-based vaccine blocked the establishment of chronic infection following challenge with a highly pathogenic SIV strain (76–78). However, Tem have a limited proliferative capacity, are dependent on persistent antigens, and therefore may be less effective at controlling a fully established systemic infection. On the other hand, central memory cells are much more effective at suppressing systemic infections because they provide a readily expandable reserve force of HIV-specific $CD8^+$ T cells with diverse specificities. Additionally, because central memory cells have a high proliferative potential, a relatively small population of pathogen-specific central memory cells can quickly mount greater anamnestic effector responses than those of Tem.

Several limitations of this study should be noted. First, this work focused on three HIV proteins, Gag, Nef, and Env. While we believe that the approach used provides a robust assessment of relevant expandable memory populations, we did not assess the potential roles of this population in responses against other HIV proteins. Second, the use of consensus peptides for our screening assays may have reduced our ability to detect some memory responses directed against autologous viruses. However, this should have affected the different cohorts equally, and yet we saw significant differences only in comparisons of the Gag-specific responses of EC to those of the other groups. Despite these limitations, our data suggest that in-depth interrogation of this previously underappreciated cell population can lead to a clearer understanding of the role that $CD8^+$ T cell responses play during successful containment of HIV replication.

In conclusion, our data support a superior role for Gag-specific $CD8^+$ T cell responses in immune-mediated control of HIV infection compared to those directed at Env and Nef. Furthermore, our data reinforce the concept that not all $CD8^+$ T cell responses are equally beneficial. Together with data from previous reports, the results here link expandable memory responses to sustained virus suppression in the elite control of HIV. The demonstration of a negative correlation between expandable Gag-specific memory T cell responses and residual viremia strongly suggests that this cell population is not a mere footprint of escaped viruses but rather actively contributes to the sustained suppression of virus replication. Importantly, these data also show that HIV controllers possess a greater ability to mount responses to mutant viruses. Finally, the presence among elite controllers of large proportions of Gag-specific memory cells that are more reactive to epitope variants suggests that their induction by future HIV vaccines may be important for narrowing possible routes of rapid escape from vaccine-induced $CD8^+$ T cell responses.

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