In Chronic Infection, HIV Gag-Specific CD4⁺ T Cell Receptor Diversity Is Higher than CD8⁺ T Cell Receptor Diversity and Is Associated with Less HIV Quasispecies Diversity

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ABSTRACT Cellular immune responses to Gag correlate with improved HIV control. The full extent of cellular immune responses comprises both the number of epitopes recognized by CD4⁺ and CD8⁺ T cells and the diversity of the T cell receptor (TCR) repertoire directed against each epitope. The optimal diversity of the responsive TCR repertoire is unclear. Therefore, we evaluated the TCR diversity of CD4⁺ and CD8⁺ T cells responding to HIV-1 Gag to determine if TCR diversity correlates with clinical or virologic metrics. Previous studies of TCR repertoires have been limited primarily to CD8⁺ T cell responses directed against a small number of well-characterized T cell epitopes restricted by specific human leukocyte antigens. We stimulated peripheral blood mononuclear cells from 21 chronic HIV-infected individuals overnight with a pool of HIV-1 Gag peptides, followed by sorting of activated CD4⁺ and CD8⁺ T cells and TCR deep sequencing. We found Gag-reactive CD8⁺ T cells to be more oligoclonal, with a few dominant TCRs comprising the bulk of the repertoire, compared with the highly diverse TCR repertoires of Gag-reactive CD4⁺ T cells. HIV sequencing of the same donors revealed that high CD4⁺ T cell TCR diversity was strongly associated with lower HIV Gag genetic diversity. We conclude that the TCR repertoire of Gag-reactive CD4⁺ T helper cells displays substantial diversity without a clearly dominant circulating TCR clonotype, in contrast to a hierarchy of dominant TCR clonotypes in the Gag-reactive CD8⁺ T cells, and may serve to limit HIV diversity during chronic infection.

IMPORTANCE Human T cells recognize portions of viral proteins bound to host molecules (human leukocyte antigens) on the surface of infected cells. T cells recognize these foreign proteins through their T cell receptors (TCRs), which are formed by the assortment of several available V, D, and J genes to create millions of combinations of unique TCRs. We measured the diversity of T cells responding to the HIV Gag protein. We found that the CD8⁺ T cell response is primarily made up of a few dominant unique TCRs, whereas the CD4⁺ T cell subset has a much more diverse repertoire of TCRs. We also found there was less change in the virus sequences in subjects with more diverse TCR repertoires. HIV has a high mutation rate, which allows it to evade the immune response. Our findings describe the characteristics of a virus-specific T cell response that may allow it to limit viral evolution.

KEYWORDS T cell diversity, T cells, human immunodeficiency virus

T cell responses to HIV are important for bringing acute viremia under control and keeping it suppressed, in rare cases indefinitely (1–3). Immune control of various

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chronic pathogens is improved with diverse T cell receptor (TCR) repertoires (4, 5). Conversely, limited TCR diversity may be a marker of disease severity (6). HIV can rapidly adapt to immune pressure exerted by T cells resulting in epitope modifications that evade or subvert T cell recognition or cytotoxicity (2, 7–10). However, T cell coverage against a broad number of HIV epitopes correlates with lower viral load. Our current understanding of the full range of the antigen-specific TCR repertoire targeting HIV and its relationship to viral diversity is limited.

A more complete picture of HIV-specific TCR diversity will be important to evaluate the role of the TCR repertoire in the response to natural infection or vaccination. Tetramers have facilitated studies of the diversity of T cell clonotypes targeting specific human leukocyte antigen (HLA)-restricted T cell epitopes. While this technology opened the field of antigen-specific TCR diversity, it is reliant on having well-characterized HLA-epitope combinations and limits studies to only subjects with the corresponding HLA alleles. These studies have primarily used HLA class I tetramers to determine CD8⁺ TCR diversity, as HLA class II tetramers are more challenging to produce and the responses tend to be of a lower magnitude (11). Methods to isolate T cells relying on the production of a specific cytokine after antigen stimulation assume all the antigen-specific cells make the cytokine the assay is directed against. Other studies have isolated antigen-specific T cells after proliferation measured by dye dilution in response to an antigen (12). However, cells that have gone through multiple cell divisions *in vitro* often have highly skewed clonotype frequencies compared with those in the peripheral circulation, depending on their *in vitro* replicative capacity and concentration of antigen. Identification of antigen-specific T cells can be achieved by staining for key activation markers upregulated following stimulation with cognate antigen. Several recent studies have taken advantage of these characteristics to evaluate antigen-specific T cells (13–18).

Deep sequencing of the TCR repertoire has tremendously expanded our knowledge of the diversity of T cell responses. This technique has predominantly been applied to TCR repertoire analysis of total T cell populations or large T cell subsets, such as memory or naive populations, and has led to significant gains in our understanding of TCR repertoire diversity in health and disease (19–23). However, few studies have evaluated the TCR repertoires of antigen-specific T cells by deep sequencing. Postvaccine changes in TCR repertoires have been evaluated after inferring antigen specificity by upregulation of cellular activation markers on CD8⁺ T cells 2 weeks postvaccination (24). Other studies have evaluated herpesvirus-specific immune responses to single epitopes or peptide pools in healthy individuals (16, 25) or in transplant recipients (26).

We determined the relationship between HIV-specific TCR repertoire diversity and HIV sequence variation in chronically HIV-infected antiretroviral (ART)-naïve individuals. We sorted cells expressing activation markers in response to HIV Gag peptide stimulation and determined the TCR repertoires of Gag-reactive CD4⁺ and CD8⁺ T cells. We found significantly higher TCR diversity of Gag-reactive CD4⁺ T cells than that of CD8⁺ T cells. We also found that increased TCR repertoire diversity correlates with reduced viral diversity, suggesting this feature of the immune response may help maintain control of infection by limiting HIV adaptation.

RESULTS

Identification of HIV Gag-specific T cells and evaluation of stability of TCR repertoire analysis. We evaluated the HIV-1 Gag-reactive CD4⁺ and CD8⁺ T cell responses of 21 ART-naïve individuals (Table 1). We evaluated expression of several activation markers after *in vitro* stimulation and, similar to findings from other groups, found several marker combinations able to detect antigen-reactive T cells (14–18, 27). We used a combination of CD69 and CD25 expression, which provided consistent identification of stimulated CD4⁺ and CD8⁺ T cells after overnight stimulation with Gag peptides (Fig. 1A). The frequency of Gag-reactive CD4⁺ T cells directly correlates with the frequency of CD8⁺ Gag-reactive cells (Fig. 1B; $P = 0.01$, $r = 0.66$, $n = 21$). Higher frequencies of CD8⁺ Gag-reactive cells inversely correlated with viral load (Fig. 1C); albeit

TABLE 1 CD4⁺ T cell count and viral load for cohort of subjects^a

Subject	Yrs of infection	VL	CD4 ⁺ T cell count	Allele names for:							
				HLA-A	HLA-A	HLA-B	HLA-B	HLA-C	HLA-C	HLA-DRB1	HLA-DRB1
10004 ^b	21	50	140	03:01	30:02	07:02	57:01	06:02	7:02	03:01:01	07:01:01
10031	12	50	612	03:01	03:01	14:01	57:04	02:02	18:01	11:01:02	15:03:01
10071	14	50	959	01:01	66:02	08:01	57:01	07:01	13:01	13:01:01	15:03:01
10067	14	215	661	30:02	33:01	13:02	57:03	07:01	13:02	13:02:01	15:03:01
10002	21	221	819	03:01	31:01	27:05 ^c	57:01	02:02	6:02	04:03:01	07:01:01
10061 ^b	5	432	1,026	01:01	24:02	08:01	44:02	07:01	16:01	03:01:01	07:01:01
10024	8	465	794	02	30:02	57:03	58:02	05:02	7:01	12:01:01	15:03:01
10022	12	518	713	01	31	08:01	27:03	02:02	7:01	04:01:01	13:01:01
10060	4	722	688	01:01	33:01	42:01	57:01	06:02	17:01	04:05:01	13:01:01
10055	7	900	650	36:01	68:01	27:05 ^c	53:01	01:02	04:01	11:01:02	13:02:01
10070	9	1,407	1,176	23:01	74:01	57:03	58:02	04:01	7:01	11:02:01	13:03:01
20018	2	1,886	504	02:01	26:01	40:01	57:01	03:04	18:01	03:01:01	16:02:01
10069	7	2,400	900	30:02	30:07	45:01	58:01	07:01	16:01	N/A	N/A
20010	2	3,740	1,124	11:01	32:01	27:05 ^c	51:01	02:02	14:02	N/A	N/A
10027	12	4,827	775	01:01	02:01	08:01	57:01	06:02	7:01	03:01:01	07:01:01
10007	13	7,525	500	03:01	33:01	15:16	42:02	14:02	17:01	01:01	03:01:01
10028	14	7,783	900	33:01	34:02	44:03	53:01	04:01	4:01	N/A	N/A
10076	6	21,339	700	02:01	30:02	35:01	57:01	04:01	7:01	N/A	N/A
10006 ^b	2	21,670	543	02:01	03:02	07:02	44:02	05:01	07:02	11:01:01	12:01:01
20021	1	26,000	518	23:01	23:01	44:03	58:01	04:01	07:01	07:01:01	15:03:01
10097	3	36,810	580	29	66:01	41:02	49:01	07:01	17:01	07:01:01	11:01:02

^aVL, viral load; N/A, not available.^bTCR deep sequencing not performed for CD4⁺ and/or CD8⁺ T cells.^cHLA-B*27:05 could not be distinguished from HLA-B*27:03/4/13.

this relationship did not reach statistical significance ($P=0.07$, $r=-0.4$, $n=21$). There was no trend between the frequency of CD4⁺ Gag-reactive T cells and viral load (Fig. 1D).

Deep sequencing can yield large complex data sets and is prone to sample selection bias, so we first quantified the stability of the Gag-reactive TCR repertoire using deep sequencing. For three subjects, namely, 10060, 10067, and 20018, Gag-reactive CD4⁺ and CD8⁺ T cells from the same leukapheresis time point were stimulated, sorted, and sequenced on three independent occasions. The number of unique CD4⁺ TCRs specific to Gag in each sample was consistently much greater than that of Gag-reactive CD8⁺ T cells, even when normalized by the total number of TCR reads detected (Table 2). For example, the number and percent unique CD8⁺ Gag-reactive TCRs for subject 10060 was 2,823 (22%) in replicate 1, 5,757 (18%) in replicate 2, and 2,880 (22%) in replicate 3. There were 775 unique TCRs shared by all 3 replicates. The corresponding numbers of unique CD4⁺ TCR sequences were 15,407 (71%), 26,411 (67%), and 9,411 (88%), with 1,294 in all 3 replicates.

We plotted the frequency of unique TCRs that appeared in two replicates and then color coded in red identical TCRs that were also in a third replicate (Fig. 2A to F). This demonstrates the reproducibility of our technique, as the most frequent TCRs repeatedly appeared in all three replicates. Additionally, we show that the most abundant Gag-specific CD4⁺ T cells are present at a much lower frequency than the most abundant Gag-specific CD8⁺ T cells.

To further strengthen the specificity of our results, we compared the frequency of Gag-reactive TCRs to the frequencies of these TCRs in unstimulated *ex vivo*-sorted memory T cells in 3 individuals (subjects 10060, 10067, and 20018). The frequencies of dominant Gag-reactive CD4⁺ and CD8⁺ TCRs were enriched severalfold after a brief stimulation followed by AIM sorting compared with the circulating frequencies within memory T cells. In subject 10060, we also evaluated the frequencies of Gag-reactive TCR within individual memory subsets and demonstrate that while antigen-reactive cells are detectable within memory T cell populations, they are rarely detectable or are present at extremely low frequencies within CD4⁺ and CD8⁺ T naive populations

FIG 1 Activation-induced marker sorting and frequency of activated Gag-reactive T cells. (A) T cells from HIV+ subjects were stimulated overnight with Gag peptides and gated on memory T cells coexpressing CD69 and CD25. (B) Correlation between the frequencies of CD69⁺ and CD25⁺ CD8⁺ and CD4⁺ Gag-reactive T cells. Correlation between HIV viremia and frequency of CD8⁺ (C) or CD4⁺ activated T cells (D) (Spearman's rank order correlation).

(Fig. 3). These data confirm that sorting activated HIV Gag-specific T cells enriches for antigen-specific TCR sequences. These sequences are detectable within unstimulated circulating T cell memory populations by deep sequencing, albeit at much lower frequencies.

Known epitope-specific TCRs identified within the peptide stimulated TCR repertoire. We next determined whether sorting cells based on expression of activation markers following stimulation with the pool of HIV Gag peptides would identify TCRs previously detected by tetramer binding for specific Gag epitope-HLA combinations. We previously used tetramers of HLA-B*57:01 complexed with the Gag epitopes KF11 (KAFSPEVIPMF), IW9 (ISPRTLNAW), QW9 (QATQDVKNW), or HLA-B*27:05 complexed with KK10 (KIRLRPGGKK) to determine epitope-specific T cell frequencies and TCR repertoires that included 8 individuals from this current cohort of HIV+ individuals (28, 29). Responses to KF11 and IW9 are particularly dominant in several of these individuals, representing 1% to 10% of the CD8⁺ T cell populations. The original study identified TCR clonotypes by isolating RNA from tetramer-positive T cells, followed by cDNA cloning and Sanger sequencing of 50 to 90 colonies. The frequency of a specific clonotype is reported as the number of colonies with that unique TCR divided by the total number of colonies sequenced (Fig. 4). We compared TCR frequencies derived from our current activation-induced memory sorting approach to frequencies identified by tetramer binding. The dominant KF11- or IW9-specific TCRs tended to also be dominant in the bulk HIV Gag-reactive TCR repertoires, despite the presence of many more potential epitopes in the peptide pool. We were also able to identify several

TABLE 2 TCR alpha and beta pairing from single-cell sorting^a

Study subject	Frequency (%)	TRBV	TCRb CDR3	TRBJ	TRAV	TCRa CDR3	TRAJ
10060 gag TCRB and matched TCRA	4.7	12	CASSLGLNTIYF	1-3	26-1	CIVRAPNNAGNMLTF	39
	16.5	7-8	CASSQDRIHTEAFF	1-1	6	CALGENFNKFYF	21
	5.7	19-1	CATTDSYGYTF	1-2	5	CAASGGYQKVTF	13
	0.9	7-3	CASRLTGGSEKLF	1-4	14/DV4	CAMRYNFNKFYF	20
	0.5	5-4	CASSFRGQGNIGYTF	12	29/DV5	CAAINQGGKLI	23
	0.9	28-1	CASSSTVLSSGNTIYF	1-3		ND	
	0.5	13-1	CASRELSLNTGELFF	2-2		ND	
	0.9	7-9	CASSPRLAGGPDYEQYF	2-7		ND	
	0.5	17-1	CASSLSWTGGSYEQYF	2-7	26-2	CILRDRDDKIIF	30
	0.9	7-3	CASSLLGGRLNGNEQFF	2-1		ND	
	0.9	27-1	CASSLWGQGPDTQYF	2-3	17	CVRGGSNYKLT	53
	0.5	7-9	CASSLEAIAF	1-2	13-1	CAARGNQFYF	49
	0.5	19-1	CASTDRDRGRQPQHF	1-5		ND	
	1.9	11-2	CASSFGETAHGYTF	1-2		ND	
	0.9	4-1	CASSQKAGTAYEQYF	2-7		ND	
10067 gag TCRB and matched TCRA	16.34	7-6	CASSFWGQGTDTQYF	2-3	14/DV4	CAMRGSSSGSARQLTF	22
	2.9	4-1	CASSQEGNSPSSYNEQFF	2-1	21	CAVHNARLMF	31
	0.15	18	CASSPLGDQAFF	1-1		ND	
	0.61	5-1	CASSLFGSGEQYV	2-7	12-3	CAMSGVGGGYNKLI	4
	1.37	9	CASSESSGRAILDTQYF	2-3	27	CAGANAGNMLTF	39
	1.53	7-6	CASSPGGVGNQPQHF	2-7		ND	
	0.31	7-9	CASSLQGIYGYTF	1-2		ND	
	0.92	5-1	CASSALAGVDTGELFF	2-2	2-1	CVVKPRPGGYQKVTF	13
	1.22	7-9	CASSLKETSGRAETGELFF	2-2	14/DV4	CAPLIGAGSYQLTF	28
	0.31	29-1	CSVEFSGQGNIEQYF	2-7		ND	
	0.15	4-1	CASSQVLSLVESGYTF	1-2			
	0.15	6-6	CASSYSRYEQYF	2-7		ND	
	0.15	7-9	CASSFDSEAFF	1-1		ND	
	1.22	7-8	CASSLYGLAYDKNIQYF	2-4	26-2	CIRGGGQGGSEKLVF	57
	0.15	7-9	CASSLDAGLTLYTF	1-2		ND	
	0.31	6-1	CASIPGSGGGLTTGELFF	2-2		ND	

^aTRBV, TCR beta variable gene; TRBJ, TCR beta joining gene; TRAV, TCR alpha variable gene; TRAJ, TCR alpha joining gene.

tetramer-specific subdominant KF11-specific TCRs within the bulk TCR repertoire down to a frequency of 0.01%. This finding further confirms the specificity of detecting Gag-reactive TCRs based on activation markers and the power of deep sequencing techniques to accurately describe diverse TCR repertoires. In one subject, namely, 10002, for whom four tetramer-specific TCR repertoires were previously determined, 60% of the Gag-reactive TCR repertoire matched TCRs recognizing four epitopes (HLA-B*57:01-restricted KF11, IW9, and QW9 epitopes and the HLA-B*27:05-restricted KK10 epitope).

To address potential PCR amplification bias in the bulk TCR sequencing approach, we performed single-cell sorting of our activated CD8⁺ T cells and used cDNA as the input for sequencing. Using only a few hundred sorted cells (212 TCRβ sequences from subject 10060 and 655 TCRβ sequences from 10067), we again identified the same highly frequent T cell clonotypes (Fig. 5A and B). Single-cell sequencing has the additional advantage of being able to identify paired TCRα and TCRβ subunits (Table 3). We found that each unique TCRβ subunit consistently paired with the same unique TCRα subunit. When we coexpressed the known tetramer-specific TCRβ with its paired TCRα subunit in a reporter assay, they responded to the optimal peptides down to 10 ng/ml (Fig. 5C and D). The TCRs did not respond to alternative peptides or stimulation with antigen-presenting cells that did not have the appropriate HLA allele (in this case HLA-B*57:01; data not shown).

Antigen-specific TCR repertoire diversity is greater in CD4⁺ than in CD8⁺ T cell populations. We next evaluated the differences between CD4⁺ and CD8⁺ TCR repertoire diversity for 15 individuals in Table 1 with chronic untreated HIV infection and with both CD4⁺ and CD8⁺ Gag-reactive T cell responses. In all cases, there were far more unique sequences within the TCR repertoires of sorted CD4⁺ Gag-specific T cells

FIG 2 Overlapping TCR β repertoires of independently sorted samples. HIV Gag-reactive cells from three subjects were sorted in triplicate; subjects 10060 (A, B), 10067 (C, D), and 20018 (E, F). For each individual, the TCR frequency of each unique TCR for each of 2 replicates for CD4 $^{+}$ (A, C, E) and CD8 $^{+}$ T cells (B, D, F) are plotted on the x and y axes and colored in red if that sequence was also present in the 3rd replicate. Subject 20018 had duplicate analysis only of CD4 $^{+}$ T cells.

10060 CD4									
	TRBV	CDR3	TRBJ	Gag AVG	Total mem	Tcm	Tnaive	Fold Enrichment	
1	18-1	CASSPLSAANTGELFF	2-2	0.80%	0.059%	0.02%	0.0000%	14.65	
2	5-1	CASSLSQGDYGYTF	1-2	0.19%	0.039%	0.02	*	4.76	
3	20	CSARDPLQGNTTEAFF	1-1	0.19%	0.029%	0.03	0.0006	6.46	
4	12-2	CASSRPEQYTF	2-7	0.12%	0.010%	*	*	12.56	
5	4-1	CASSLGGRRDQYTF	1-2	0.07%	*	0.003	*	NA	
6	3	CASSPRYSGSEKLF	1-4	0.10%	0.015%	0.007	*	6.85	
7	20	CSARESSGRGAYNEQFF	2-1	0.10%	0.010%	0.007	*	9.87	
8	21-1	CASSKKTGASGYEQYF	2-7	0.08%	0.005%	0.02	*	16.27	
9	7-3	CASSPLSGRSRQYF	2-7	0.08%	0.005%	0.001	*	16.54	
10	7-9	CASSLGTEINKLFF	1-4	0.10%	0.005%	0.007	*	19.64	
11	20	CSARAYRGWTEAFF	1-1	0.14%	0.029%	0.01	0.002	4.91	
12	5-4	CASSLQGVYEQPHF	1-5	0.10%	0.029%	0.02	*	3.55	
13	3	CASSVSGSYGYTF	1-2	0.07%	0.020%	0.0009	*	3.81	
14	27-1	CASVYRGGASPLHF	1-6	0.07%	*	0.006	*	NA	
15	10-3	CAISESWDRGTGYTF	1-2	0.06%	0.010%	0.004	*	5.89	
16	3	CASSRSGGEQYTF	2-5	0.06%	0.005%	0.005	0.002	12.51	
17	14-1	CATMVLAGNTTYF	1-3	0.07%	0.039%	0.04	*	1.67	
18	201	CAIEASATSYEQYF	2-7	0.07%	*	0.006	0.004	NA	
19	12	CASSRVPEYQYF	2-7	0.08%	0.005%	0.01	0.001	16.38	
20	4-3	CASSQDEGWKFGKGGELFF	2-2	0.07%	0.010%	0.003	0.002	6.74	

10067 CD4									
	TRBV	CDR3	TRBJ	Gag AVG	Total mem	Fold Enrichment			
1	20	CSARDQGTGYTEAFF	2-3	0.27%	0.027%	10.02			
2	2-1	CASQGLSGLSYEQYF	2-1	0.27%	0.019%	14.24			
3	24	CATSSSGQGISGSEYQYF	2-7	0.27%	0.018%	14.59			
4	7-2	CASSPRLGAGNANTGYTF	1-1	0.24%	0.025%	7.69			
5	2-1	CASKEEKLF	2-2	0.18%	0.016%	10.91			
6	2-1	CRAFMTTEAFF	2-3	0.18%	0.044%	4.08			
7	9-1	CASSVAGASGYGYTF	2-7	0.17%	0.042%	3.95			
8	7-9	CASSRGGESITDQYF	1-2	0.14%	0.019%	7.66			
9	25-1	CASSDYGDTQYF	2-2	0.13%	0.047%	2.83			
10	5-1	CASSDRVGNNTTYF	2-7	0.13%	0.007%	18.42			
11	7-9	CASSSRITGITDQYF	2-2	0.12%	0.034%	3.61			
12	19-1	CASATLNLQPKHF	2-4	0.12%	0.014%	8.70			
13	19-1	CASITGDRGPQHF	1-2	0.11%	0.028%	4.03			
14	20	CSAVSDRGVDYQYF	2-2	0.11%	0.012%	9.08			
15	5-4	CASSVDRGDSYTF	2-2	0.11%	0.013%	8.45			
16	29-1	CSVALGASGNTTYF	2-7	0.11%	0.028%	3.87			
17	2-1	CASNEQGTNEKLF	2-7	0.11%	0.013%	7.86			
18	20	CSARVGSSTTEAFF	1-2	0.10%	0.025%	4.18			
19	4-2	CASSQDAGGQYEQYF	2-2	0.10%	0.009%	10.64			
20	20	CSARRNLKLF	2-1	0.09%	0.013%	7.06			

20018 CD4									
	TRBV	CDR3	TRBJ	Gag AVG	Total mem	Fold Enrichment			
1	20	CSASISGALDEKLF	1-04	0.34%	1.176%	0.29			
2	10-03	CATQGLGYGYTF	1-02	0.25%	0.062%	3.99			
3	2-01	CASSGQGLNLYGYTF	1-02	0.20%	0.062%	3.31			
4	11-03	CASGRDYNPLHF	1-06	0.13%	0.062%	2.13			
5	7-02	CASGMQGRGSPPLHF	1-06	0.12%	0.062%	1.86			
6	18-01	CASSPSPHMYF	1-03	0.11%	0.031%	3.61			
7	6	CASSIFGYIPYEQYF	2-07	0.11%	*	NA			
8	6	CASSSQSVTGRESGNTTYF	1-03	0.11%	0.062%	1.77			
9	11-02	CASSRSEYQYF	2-07	0.11%	*	NA			
10	2-01	CASSKRTGSSNQPHF	1-05	0.10%	0.031%	3.31			
11	12	CASSSGVNDYGYTF	1-02	0.09%	0.960%	0.09			
12	19-01	CASSRQGLTANYGYTF	1-02	0.07%	0.031%	2.40			
13	6-01	CASSESQDEQYF	2-07	0.07%	0.062%	1.17			
14	12	CASSAPGQDTQYF	2-07	0.07%	0.062%	1.09			
15	2-01	CASSVGEQYF	2-07	0.06%	*	NA			
16	5-01	CASSLGGGRTGYTF	2-03	0.05%	0.062%	0.89			
17	5-01	CASSVLGERGEQYF	2-07	0.05%	*	NA			
18	6-5	CASSRDSSEYQYF	2-07	0.05%	0.031%	1.72			
19	25-1	CASSRGTDQYF	2-07	0.05%	0.031%	1.56			
20	4-3	CASSHTFWGSSGNTTYF	1-03	0.05%	0.093%	0.52			

10060 CD8										
	TRBV	CDR3	TRBJ	Gag AVG	Total mem	Tem	TemRA	Tcm	Tnaive	Fold Enrichment
1	7-8	CASSQQRHTEAFF	1-1	11.19%	1.390%	2.90%	0.0400%	0.009	0.0001	8.02
2	12	CASSLGLNTTYF	1-3	10.64%	0.619%	0.024	0.00003	0.010	*	17.20
3	19-1	CATIDSYGYTF	1-2	5.62%	0.454%	0.0127	0.0003	0.006	0.00002	12.38
4	6-4	CASSDGLDNLPLHF	1-4	5.38%	0.429%	0.008	0.00008	0.004	*	12.55
5	7-3	CASRLTGSGNEKLF	1-4	2.17%	0.219%	0.0054	*	0.002	*	9.94
6	5-4	CASSFRGGQNGYGYTF	1-2	1.12%	3.460%	0.0073	0.0408	0.004	0.01	0.32
7	11-2	CASSFGETAHGYTF	1-2	1.11%	0.161%	0.0012	0.00009	0.001	*	6.90
8	13-1	CASRELSLNTGELFF	2-2	1.10%	0.109%	0.0007	0.0002	0.000	0.00006	10.04
9	28-1	CASSVILSGNTTYF	1-3	0.86%	0.136%	0.001	0.00006	0.003	0.00004	6.29
10	7-3	CASSLGLRLNGNEQFF	2-1	0.73%	0.087%	0.0015	0.00002	0.001	*	8.39
11	27-1	CASRLMGRHEQYF	2-7	0.55%	0.064%	0.001	*	0.000	*	8.60
12	7-9	CASSPRLAGDPYEQYF	2-7	0.53%	1.905%	0.01	0.0214	0.006	0.004	0.28
13	27-1	CASSLWGTGSEYQYF	2-7	0.51%	1.153%	0.0012	*	0.001	0.003	0.45
14	27-1	CASSLWGGQAPDTQYF	2-3	0.51%	1.031%	0.0018	0.018	0.001	0.003	0.50
15	5-6	CASSMQQDYGYTF	1-2	0.41%	0.499%	0.0016	0.0057	0.001	0.001	0.81
16	7-9	CASSGLAGSTGELFF	2-2	0.38%	0.031%	0.0002	*	0.001	*	12.29
17	19-1	CATSTRDRGRQPHF	1-5	0.32%	0.740%	0.0016	0.0113	0.001	0.002	0.44
18	4-1	CASSQKAGTAYEQYF	2-7	0.32%	2.650%	0.0353	0.0112	0.016	0.001	0.12
19	28-1	CASSILSGSNTTYF	1-3	0.31%	0.019%	0.00007	0.00009	0.0002	0.0001	16.53
20	7-9	CASSLEAIF	1-2	0.30%	0.355%	0.0007	0.0047	0.0004	0.001	0.84

10067 CD8									
	TRBV	CDR3	TRBJ	Gag AVG	Total mem	Fold Enrichment			
1	7-6	CASSFWGGQDITQYF	2-3	4.01%	1.543%	2.60			
2	4-1	CASSQEGNSPSSYNEQFF	2-1	2.40%	0.063%	37.98			
3	5-1	CASSLFGSGEQYV	2-7	1.39%	0.287%	4.84			
4	18-1	CASSLGGQAPDTQYF	1-1	1.24%	0.042%	29.25			
5	5-1	CASSALAGVDTGELFF	2-2	1.13%	0.475%	2.37			
6	9-1	CASSSSGRAILDTQYF	2-3	1.06%	0.040%	26.60			
7	23-1	CASSVAGDLQYV	2-7	0.95%	0.035%	27.12			
8	7-9	CASSLQGYGYTF	1-2	0.75%	0.032%	23.02			
9	7-9	CASSLFGSGRAETGELFF	2-2	0.71%	0.139%	5.10			
10	29-1	CSVYFSGGQYGYTF	2-7	0.53%	0.036%	14.99			
11	24	CATWQNTGELFF	2-2	0.53%	0.020%	26.73			
12	7-8	CASSLYGLADKNQYF	2-4	0.51%	0.042%	12.27			
13	19-1	CASSQQPYGYTF	1-2	0.51%	0.019%	27.36			
14	13-1	CASSSRWGGVSTGELFF	2-2	0.47%	0.609%	0.76			
15	6	CASIPSGGGLITGELFF	2-2	0.46%	0.011%	41.12			
16	6-6	CASSYRSEYQYF	2-7	0.45%	0.043%	10.41			
17	2-1	CASSFQGPYEQYV	2-7	0.43%	0.034%	12.76			
18	4-1	CASSQVLSVSGYTF	1-2	0.39%	0.010%	37.09			
19	19-1	CASSISGINTGELFF	2-2	0.39%	0.016%	23.65			
20	7-8	CASSAPGIDAFITYNEQFF	2-1	0.25%	0.014%	18.04			

20018 CD8									
	TRBV	CDR3	TRBJ	Gag AVG	Total mem	Fold Enrichment			
1	7-9	CASELSGNTTYF	1-03	25.13%	4.95%	5.07			
2	19-1	CASSPGNTYATYGYTF	1-02	8.17%	1.74%	4.69			
3	7-8	CASSASTGFRVGYTF	1-02	4.74%	1.38%	3.43			
4	24	CATSELMQNEQFF	2-01	3.54%	0.63%	5.64			
5	7-9	CASEAGNTTYF	1-03	3.40%	0.61%	5.56			
6	7-9	CASEGAGKGYTYF	1-03	2.57%	0.74%	3.49			
7	7-9	CATEASGNTTYF	1-03	2.29%	0.47%	4.90			
8	30-1	CAWSYSGSEYQYF	2-07	1.57%	0.25%	6.25			
9	20	CSARTGYGYTF	1-02	1.55%	0.34%	4.54			
10	6-5	CASRTGGTLGYGYTF	1-02	1.50%	0.41%	3.63			
11	7-9	CASESNTTYF	1-03	0.81%	0.14%	5.62			
12	20	CSARDWGGDNQPHF	1-05	0.73%	0.11%	6.75			
13	7-9	CASEQNTTYF	1-03	0.60%	0.11%	5.58			
14	20	CSARDYGGHNPQPHF	1-05	0.58%	0.16%	3.48			
15	20	CSATHGTGLYEQYF	2-07	0.55%	1.76%	0.31			
16	20	CSARGGAGNTTYF	1-03	0.54%	0.18%	3.01			
17	7-9	CASSYNTTYF	1-03	0.50%	0.09%	5.59			
18	7-9	CASRPAGAYSEYQYF	2-07	0.43%	0.50%	0.86			
19	7-8	CASSLTGLEKLF	1-04	0.43%	0.09%	4.74			
20	20-1	CSAKDGYGYTF	1-02	0.38%	0.14%	2.67			

FIG 3 Frequency of Gag-reactive TCR in memory T cell populations. The average frequency of Gag-reactive TCRs and frequency of the same TCR in total memory or, for subject 10060, total memory and memory subsets (Tcm and Tnaive). Fold enrichment is the frequency of TCR in sorted activated (CD69⁺CD25⁺) population compared with total nonactivated T cell memory population.

than those of CD8⁺ Gag-specific T cell populations. The clonality score provides a measure of the diversity ranging from 0, which is infinitely diverse to 1, representing a monoclonal population (30). The clonality score of the CD4⁺ Gag-specific TCR repertoire ranged from 0.006 to 0.08 with a median of 0.03, while the values for the CD8⁺ Gag-specific repertoire ranged from 0.04 to 0.6 with a median of 0.38 ($P < 0.001$) (Fig. 6A). Correspondingly, the frequency of the most abundant single clonotype in the CD4⁺ TCR repertoire across subjects was only a median of 0.6% but for the CD8⁺ TCR repertoire, it was 12% ($P < 0.001$) (Fig. 6B). CD8⁺ TCR clonality positively correlated with the magnitude of the activated CD8⁺ T cells ($r = 0.6$, $P = 0.03$), suggesting the greater frequency of activated CD8⁺ T cells was due to an expansion of dominant clones in circulation and not the result of a higher number of activated clonotypes (Fig. 6C).

Higher diversity of the HIV Gag-specific TCR repertoire is associated with lower HIV genetic diversity. The relationship between HIV-specific TCR diversity and viral diversity is complex. We hypothesized that TCR diversity might limit the ability of the HIV genome to diversify and adapt to immune recognition. Alternatively, viral diversity might drive TCR diversity as the immune system attempts to respond to the replication of variants. We successfully sequenced virus from the plasma in 18 of the 21 individuals and assessed the mean genetic distance within the Gag sequences. Of these 18 individuals, we were able to sort and sequence HIV-reactive CD4⁺ and/or CD8⁺ TCRs from 17

10002			10024			10071		
TCRs specific for KF11	tet+ clones screened =31	Gag 15mer repertoire	TCRs specific for KF11	tet+ clones screened =50	Gag 15mer repertoire	TCRs specific for KF11	tet+ clones screened =52	Gag 15mer repertoire
CASGGANYGYTF	74%	16.1%	CASDTDTGYGYT	64%	16.6%	CASYNFGQYGYT	75%	30.3%
CASSLGLDETQYF	3%	9.1%	CASSQGVTTGGFLNYGYT	32%	9.5	CASSPMDLLDEQY	25%	13.2%
CASSLNSKVSSEYQYF	3%	ND	CASMGLAQETQY	2%	N.D.			
CASSLIAGGGEDTQYF	13%	ND	CASDTDTGYGYT	2%	N.D.			
TCRs specific for KK10			TCRs specific for KF11			20018		
tet+ clones screened =38	Gag 15mer repertoire		tet+ clones screened =88	Gag 15mer repertoire		TCRs specific for KF11	tet+ clones screened n=51	Gag 15mer repertoire
CASSLYGEYEQYF	79%	18.1%	CAIGGHDYGYTF	45%	2.0%	CASELSGNTIY	69%	16.5%
CASSQGTDTQYF	21%	0.8%	CALTGGDYGYTF	7%	1.1%	CATSDLMNDNEQF	2%	2.1%
			CSAREKGSQETQY	8%	0.014	CATSDLMNDNEQF	4%	2.0%
TCRs specific for IW9			CASSSLVNTGELF	23%	N.D.	CATEASGNTIY	12%	1.7%
CASRPQGQGYEQY	64%	5.8%	CSARGVWSNNRETQY	14%	N.D.	CASSYLNTIY	2%	0.4%
CASRTQRWETQY	2%	3.8%	CAASTSAVLGKKGQSQETQY	2%	N.D.	CASSGFTGFANEAF	2%	0.3%
CASSLAQGWKTQY	20%	1.6%	CASSGPGGEQY	1%	N.D.	CASSSWTQDQEQF	2%	0.2%
CASSSTGQQPQH	4%	0.4%				CASILTSGRNEQF	2%	0.12%
CASSIQGLRATNEKLF	2%	0.3%	10060			CASEITDRRNTIY	2%	0.05%
CASSIQGLRATNEKLF	2%	0.3%	TCRs specific for IW9	tet+ clones screened =54	Gag 15mer repertoire	CASEGNTIY	2%	0.02%
CASRSPLYEQY	2%	ND	CASSQDRIHTEAF	96%	10.3%			
CASSSGTSGSAGYNEQF	2%	ND	CASSLGLDETQYF	4%	N.D.			
CASSTNNEQF	2%	ND	10067					
CASRSPLYEQY	2%	ND	TCR specific for KF11	tet+ clones screened = 54	Gag 15mer repertoire			
CASSGTSGSAGYNEQF	2%	ND	CASSFWGQGTDTQY	100%	3.5%			
CASSTNNEQF	2%	ND	10070					
TCRs specific for QW9			TCRs specific for KF11	tet+ clones screened =44	Gag 15mer repertoire			
CASRTQRWETQY	100%	3.80%	CASSLGGGYT	52.3%	3.4%			
10022			CASSDGGQRLGYT	2.3%	0.9%			
TCRs specific for KK10	tet+ clones screened =84	Gag 15mer repertoire	CASSPGQTNQYGYT	13.6%	0.8%			
CASRGQGDWEAF	5%	22.6%	CASSLAGGYT	2.3%	0.8%			
CSARGVWSNNQETQY	57%	3.2%	CASEDFKNIQY	15.9%	0.45%			
CASSIAGGGEDTQY	31%	2.2%	CATPGEVLSPNYGYT	2.3%	0.45%			
CSARDPLPEASGGAGTDTQY	1%	ND	CASSLGGGQNGYT	2.3%	0.45%			
CASSTPPGF	2%	ND	CASSSMGGGDTQY	2.3%	0.34%			
CASSQWTGELF	1%	ND	CASSPRDSQETQY	2.3%	0.2%			
			CASSDGTGVLGYT	4.5%	N.D.			

FIG 4 TCR β s identified after stimulation with Gag putative T cell epitope (PTE) peptides are in tetramer-sorted T cell populations. For each tetramer-specific (tet+) T cell population, the percentage of colonies screened as having a specific TCR is shown. For the Gag peptide TCR repertoire, the frequency of the identical TCR identified in the entire TCR repertoire is shown. For subject 10002, there were four different tetramer-specific TCR repertoires to compare. ND, not detected.

individuals. There was a strong relationship between the HIV Gag-specific CD4⁺ TCR clonality score and HIV Gag mean genetic distance ($r=0.74$; $P=0.002$) (Fig. 7A), but there was no such relationship with CD8⁺ T cell clonality ($r=0.2$, P value was not significant [ns]) (Fig. 7B). Overall, greater Gag-specific CD4⁺ TCR diversity (i.e., lower clonality scores) is associated with lower HIV Gag mean genetic distance. This finding suggests that a diverse clonal response might limit viral diversity. Time since infection may also be a contributing factor influencing the genetic diversity of HIV within an individual. Although we did not have seroconversion dates from the subjects in this study, we used estimates of time since infection based on clinical presentation and prior screening outcomes (if known) and did not find a correlation between genetic diversity and estimated duration of infection ($r=0.37$, $P=0.12$) (Fig. 7C). We also did not find significant relationships between HIV Gag-specific TCR repertoire diversity and viral load in this cohort.

DISCUSSION

In this study, we simultaneously sequenced the Gag-specific CD4⁺ and CD8⁺ TCR repertoires in HIV-infected ART-naïve individuals and discovered that the CD4⁺ TCR repertoires were highly diverse, while the CD8⁺ Gag-specific TCR repertoires were oligoclonal and dominated by a few highly expanded clonotypes. We were able to simultaneously evaluate both cell types by sorting on the surface activation markers CD69 and CD25. CD69 is a well-established marker of activation for CD4⁺ and CD8⁺ T cells (31). CD25 has been previously paired with OX40 to evaluate CD4⁺ T cell responses (15, 18). Alternatively, antigen-specific cells have been identified based on acute upregulation of activation markers, such as CD154 or OX40, to identify antigen-specific CD4⁺ T cells and CD137 to identify antigen-specific CD8⁺ T

FIG 5 Single-cell sorting and TCR sequencing correlate with bulk TCR sequencing. Correlation of single-cell (sc) TCR frequency with replicate from bulk sorting indicated for subjects 10060 (A) and 10067 (B). TCR α and TCR β heterodimers corresponding to the TCR β identified with the IW9 HLA-B57 tetramer (10060; C) or KF11 HLA-B57 tetramer (10067; D) were exogenously expressed in Jurkat cells coelectroporated with CD8 α and a nuclear factor of activated T cells (NFAT) luciferase reporter. Epstein Barr Virus (EBV)-transformed autologous B cells were used as the antigen-presenting cell and loaded with decreasing concentrations of the optimal peptide. After 6 hours of coincubation the luciferase output was read and expressed as relative light units (RLUs).

cell responses (13–18). We found the combination of CD69 and CD25 to be consistently upregulated on both CD4 $^{+}$ and CD8 $^{+}$ T cells after overnight peptide stimulation with a high signal-to-noise ratio.

Establishing the stability of the TCR repertoire using activation markers was important for analyzing these responses. While bystander activation and upregulation of surface markers are potential concerns, Reiss et al. demonstrated bystander activation to be minimal over a short-term stimulation (18). Using the same leukapheresis peripheral blood mononuclear cells (PBMCs) and sorting and sequencing at different times, we demonstrated strong correlation among replicates, especially for TCR clonotypes at a frequency of greater than or equal to 0.05%. Furthermore, the TCR clonotypes within the activated TCR repertoires were enriched relative to the unstimulated memory compartments (Fig. 3). Specificity was confirmed by comparing our results with tetramer-specific TCR repertoires previously sequenced from the same individuals. There was more background activation in the CD4 $^{+}$ T cell samples than in the CD8 $^{+}$ T cell samples, which may increase the chance of a nonspecific TCR clonotype appearing in the CD4 $^{+}$ TCR repertoires relative to the CD8 $^{+}$ TCR repertoires and may explain some of the variability in Fig. 2.

Functional assays using the paired TCR α and TCR β subunit can further characterize the TCR heterodimer. We performed single-cell sorting and sequencing to obtain

TABLE 3 Sequencing results for subjects whose Gag-specific TCR repertoire was performed in triplicate

Sample	Total no. of productive CD8 TCR sequences	Total no. of unique CD8 clonotypes	% of unique CD8 clonotypes	No. of unique CD8 clonotypes in triplicates	Total no. of productive CD4 TCR sequences	Total no. of unique CD4 clonotypes	% of unique CD4 clonotypes	No. of unique CD4 clonotypes in triplicates
10060 Gag replicate 1	12,710	2,823	22		21,621	15,407	71	
10060 Gag replicate 2	31,556	5,757	18		39,589	26,411	67	
10060 Gag replicate 3	13,107	2,880	22	775	10,685	9,411	88	1,294
10067 Gag replicate 1	4,119	2,078	50		11,671	7,562	65	
10067 Gag replicate 2	4,622	1,707	37		11,961	8,243	69	
10067 Gag replicate 3	6,207	2,359	38	325	22,442	13,815	62	1,918
20018 Gag replicate 1	3,414	1,157	34		12,989	10,183	78	
20018 Gag replicate 2	15,728	1,492	9		25,265	17,111	68	
20018 Gag replicate 3 ^a	1,676	217	13	102	319	N/A ^b		2,504

^a20018 Gag replicate 3 for CD4⁺ T cells had too few productive TCR sequences for further calculations.^bN/A, not applicable.

FIG 6 Comparison between HIV Gag-reactive CD4⁺ and CD8⁺ TCR repertoire diversity. TCR clonality (A) and the frequency of the most abundant TCR clone (B) for CD4⁺ and CD8⁺ Gag-reactive T cells (Wilcoxon signed-rank test). (C) Relationship between HIV Gag-reactive clonality and the frequency of HIV Gag-reactive T cells (Spearman's rank order correlation).

paired beta and alpha TCR subunits. Using only a few hundred sorted cells, we again identified the same highly abundant TCR clonotypes. While some TCR clonotypes identified in the bulk sequencing were not found in the single-cell work, this is most likely related to sample size and comparing a few hundred individually sorted cells to thousands of pooled cells. This approach is essential for further analysis of peptide-responsive T cells at single-cell resolution (Fig. 3C and D) (32–37).

FIG 7 Mean genetic distance for Gag correlated with TCR repertoire clonality. Correlation between mean genetic distance (substitutions per site on y axis) and (A) HIV Gag CD4 clonality, (B) HIV Gag CD8 clonality, and (C) infection duration (Spearman's rank order correlation).

There were clear differences in the repertoire diversity and clonality of the CD4⁺ and CD8⁺ Gag-specific TCR repertoires. Because we did not have to expand cells, we were able to draw conclusions about their *in vivo* TCR frequency. The most abundant CD8⁺ TCR clonotypes were far more expanded than the most abundant CD4⁺ TCR clonotypes. While our group has described inflation of cytomegalovirus (CMV) epitope-specific CD4⁺ TCR clonotypes (36), we saw no similarly expanded HIV-specific CD4⁺ clonotypes across the Gag-specific TCR repertoires of these individuals. While this study applied deep sequencing to the antigen-specific population of CD4⁺ and CD8⁺ T cells, these results are consistent with recent studies using deep sequencing to analyze the TCR diversity of nonantigen-specific CD4⁺ and CD8⁺ TCR repertoires. In healthy adults, the richness, or unique number, of CDR3s is five times higher for the CD4⁺ TCR repertoire than for the CD8⁺ TCR repertoire (37). This relationship between CD4⁺ and CD8⁺ TCR diversity is consistent across young and old individuals (22). The CD4⁺ TCR diversity of hematopoietic stem cell transplant recipients has been calculated as 50 times greater than that of the CD8⁺ TCR repertoire (38). While these studies potentially demonstrate an intrinsically increased diversity of CD4⁺ T cell populations, here, we applied deep sequencing to a focused population of antigen-responsive T cells and demonstrate a higher degree of TCR repertoire diversity among antigen-specific CD4⁺ T cells than that among CD8⁺ T cells.

Human leukocyte antigen class II molecules have an open-ended structure and can accommodate longer peptides than HLA class I molecules. This may allow class II-bound peptides to fit into the HLA binding pocket in different registers (39), leading to a greater variety of uniquely presented epitopes to be interrogated by CD4⁺ T cells interacting with HLA class II. The biologic implications of these multiple potential interactions are unclear, but given the various roles of CD4⁺ T helper cells in influencing the immune response, including several subpopulations of CD4⁺ T cells with distinct cytokine profiles (e.g., Th1, Th2, Th17, and regulatory T cell [Treg], among others), it may be important for these populations of cells to “see” a greater array of potential immunogens. In these experiments, we did not attempt to further define the CD4⁺ T cell subgroups. Future studies should examine if particular functional classes of Gag-specific CD4⁺ T cells are more diverse than others.

The amount of TCR diversity necessary to exert optimal control of HIV is not known. Having an immune system that recognizes multiple T cell epitopes, particularly directed against Gag, correlates with decreased viral load (40–44). Unlike the work we presented here, prior studies evaluated the number of epitopes eliciting a T cell response rather than the number of unique responding TCR clonotypes. Here, we measured the broader TCR repertoires of CD4⁺ and CD8⁺ Gag-reactive T cells. The apparent lack of relationship between TCR diversity and clinical parameters may be because this is a relatively immunologically intact group of individuals with CD4⁺ T cell counts greater than 500. We included a significant number of individuals with HLA-B*57 in this study to enrich for individuals whose TCR repertoires would presumably exert greater control. However, not all of these individuals were elite controllers, and there was no relationship between Gag-specific CD4⁺ or CD8⁺ TCR repertoire diversity and the presence of the HLA-B*57 allele. Importantly, though, we did find that more TCR diversity correlated with less viral diversity. While this cross-sectional analysis is limited by not having the transmitted founder virus to assess the degree of change to the current circulating variants, it is reasonable to hypothesize that a diverse T cell response may quickly eliminate viral variants before they expand. Data are accumulating that show both dominant and subdominant TCR clonotypes have an important functional role in immune control, likely due to recognition of variant epitopes (4, 28, 29, 44). Although CD4⁺ T cells can be cytotoxic and may serve to eliminate virus-infected cells, it is also possible that a diverse pathogen-specific CD4⁺ T cell repertoire is a marker of a relatively successful cellular immune response (4, 45). Further studies will be required to see whether populations of HIV-specific CD4⁺ T cells directly limit HIV-1 evolution. Ultimately, we found greater HIV Gag-specific CD4⁺ T cell TCR

TABLE 4 Primer combinations for sequencing the HIV Gag region

Primer name	Direction	PCR type	Sequence (5'-3')	Length (kb)
G00	Forward	First round	GACTAGCGGAGGCTAGAAG	1.5
G01	Reverse		AGGGGTCGTTGCCAAGA	
G20	Forward	Nested	GTATGGCAAGCAGGGAGCTAGAA	1.1
R3	Reverse		TGTCCTTCCTTCCACATTCC	

repertoire diversity correlated with diminished viral diversity. TCR clonality should be evaluated in vaccine studies designed to elicit cellular immune responses to determine whether broadening the diversity of the HIV-specific TCR repertoire protects from infection or limits viral diversity and affords better control of viremia after breakthrough infection.

MATERIALS AND METHODS

Clinical samples. Samples were from HIV-1-infected ART-naïve individuals who provided informed written consent under institutional review board (IRB) approval by Vanderbilt University Medical Center (IRB030005). PBMCs were purified from whole blood or leukapheresed samples by Ficoll density gradient separation and cryopreserved.

Stimulation. Gag PTE peptides from the AIDS Reagent and Repository were used as stimulants at a final concentration of 1 μ g/ml per peptide. Staphylococcal enterotoxin B (SEB) (Millipore) was used at 1 μ g/ml as a positive control, and PBMCs were cultured in RPMI supplemented with 10% human AB serum, 10 mM glutamine, and 10 mM HEPES as a negative control.

Flow cytometry. Cryopreserved cells were thawed and stimulated overnight with HIV Gag peptides and appropriate controls. Cells were washed twice with phosphate-buffered saline (PBS) and stained with fluorescently labeled antibodies. Live/Dead fixable aqua (ThermoFisher) was used as a viability marker. The following antibodies were used for flow cytometry: anti-CD14-V500, CD19-V500, CD3-Alexa Fluor 700, CD4-PerCP, CD8 APC-Alexa Fluor 750, CD69-APC, CD25-FITC, CCR7-BV421 (Becton, Dickinson; BD), and anti-CD45RO-PE-Texas Red (Beckman Coulter). CD4⁺ or CD8⁺ activated T cells were defined as CD69 and CD25 dual positive. T cells were first gated to exclude naïve cells (defined as CD45RO-CCR7⁺), and then CD69⁺CD25⁺-positive CD4⁺ or CD8⁺ memory T cells were sorted with a BD fluorescence-activated cell sorter (FACS) Aria III instrument. Data were analyzed with FlowJo 10.1 (TreeStar).

Bulk TCR repertoire sequencing. Sorted cells were centrifuged, and the supernatant was removed. The cell pellet was frozen and stored at -80°C until extraction of genomic DNA. Genomic DNA was extracted from purified cells using the DNA IQ system (Promega) with the number of beads adjusted to the number of sorted cells. Sequencing of the TCR from genomic DNA was performed at the Vanderbilt VANTAGE DNA sequencing core using the hTCR β kit from Adaptive Biotechnologies (Seattle, WA). Sequences were analyzed using the ImmunoSEQ platform (Adaptive Biotechnologies). Sequencing of tetramer-specific TCRs has been previously described (28, 29).

Single-cell TCR sequencing. Single-cell sequencing was performed as described in Han et al. (32). Individual cells were sorted into a 96-well PCR plate containing 5 μ l of 2 $\times$ reverse transcriptase PCR (RT-PCR) buffer (Qiagen) followed by one-step RT-PCR (Qiagen) using gene-specific primers to the TCR α and TCR β variable genes and the TCR α and TCR β constant regions. Product was amplified with a nested PCR followed by addition of library adapter and well-specific barcodes. They were sequenced on a MiSeq instrument (Illumina, San Diego, CA, USA) and deconvoluted using a modified VDJfast platform.

Clonality. Clonality is calculated on the ImmunoSEQ platform by normalizing productive entropy using the total number of unique productive rearrangements and subtracting the result from 1. The normalized clonality provides a measure of the diversity ranging from 0 (infinitely diverse) to 1 (representing a monoclonal population).

Viral sequencing. Contemporaneous plasma samples or samples within 6 months of time points tested above were used to obtain HIV quasi-species sequences for each subject. Viral RNA was isolated from approximately 200 μ l of plasma using the MagMAX-96 viral RNA isolation kit (Life Technologies, Carlsbad, CA, USA) as per the manufacturer's instructions. Following RT-PCR of the viral RNA, the Gag region was amplified by nested PCR. RT-PCR conditions were 30 minutes at 55 $^{\circ}\text{C}$; 2 minutes at 94 $^{\circ}\text{C}$; and then 40 cycles of 15 seconds at 94 $^{\circ}\text{C}$, 30 seconds at 60 $^{\circ}\text{C}$, and 1.5 minutes at 68 $^{\circ}\text{C}$. Nested PCR conditions were 1 minute at 94 $^{\circ}\text{C}$; 20 seconds at 60 $^{\circ}\text{C}$; 2 minutes at 72 $^{\circ}\text{C}$; followed by 14 cycles of 15 seconds at 94 $^{\circ}\text{C}$, 20 seconds at 60 $^{\circ}\text{C}$, and 5 minutes at 72 $^{\circ}\text{C}$; and finally 20 cycles of 15 seconds at 94 $^{\circ}\text{C}$, 20 seconds at 65 $^{\circ}\text{C}$, and 1.5 minutes at 68 $^{\circ}\text{C}$. Table 4 lists primer combinations. PCR products were sequenced on the Illumina MiSeq sequencer, and sequence reads were analyzed following application of quality-control filters.

Mean genetic distance. Sequence reads were aligned to the reference HXB2 sequence using a best-fit approach. Specifically, individual aligned files were constructed by reiteratively mapping reads back onto the reference sequence such that each file contained reads with no overlap. Mean genetic distance was calculated using MEGA version 7 (46), as previously described (47). Aligned concatenated files were constructed by reiteratively mapping reads to the reference sequence HXB2 using an in-house program,

visual genomics analysis studio (VGAS; <http://www.iiid.com.au/software/vgas>). Each resultant file contained concatenated sequence reads spanning the specific HIV protein with no overlap between reads. The first 5,000 concatenated files were used in the final data set. The bestmodel function was used to identify the model Tamura-Nei as the best-fit model for the data.

Statistical analysis. The Pearson correlation was used to compare single-cell TCR frequencies. The difference in clonality between CD4⁺ and CD8⁺ Gag-specific TCR repertoires was determined with the Wilcoxon signed-rank test. Spearman's rank-order correlation was used to assess the relationship between clonality score and HIV Gag mean genetic distance (GraphPad Prism 9).

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