Supplementary Methods

Antibodies and reagents for flow cytometry

BV480-labeled anti-CD3, BUV496-labeled anti-CD8, BUV563-labeled anti-CD4, BUV615P-labeled anti-Perforin, BUV737-labeled anti-PD-1, BUV805-labeled anti-CD45RA, FITC-labeled anti-CD8, Brilliant Stain Buffer, and Brilliant Stain Buffer Plus were obtained from BD Biosciences. APC eF750-labeled anti-Granzyme B, BV421-labeled anti-CD95, BV421-labeled Mouse IgG1κ Isotype Control, BV605-labeled anti-CD4, BV711-labeled anti-T-bet, BV750-labeled anti-CD27, PE-labeled anti-TCF-1, PE Cy5-labeled anti-CD127, PE Cy7-labeled anti-CCR7, PE Cy7-labeled anti-CD3, and PerCP Cy5.5-labeled anti-CD8 came from BioLegend. Alexa Fluor 488-labeled anti-TOX obtained from Cell Signaling Technology. PE-eFluor 610-labeled anti-EOMES, LIVE/DEAD Fixable Dead Cell Stain Kits (Aqua, Near-IR, and Yellow), CellTrace Cell Proliferation Kits (Violet and CFSE), Foxp3/ Transcription Factor Staining Buffer Set, and CountBright Absolute Counting Beads were from Thermo Fisher Scientific. HLA-A*1101 HIV-1 NEF (GAFDLSFFLK and QVPLRPMTYK), HLA-A*1101 HIV-1 POL (AIFQSSMTKIL), HLA-A*1101 EBV EBNA 3B (AVFDRKSDAK), HLA-Cw*0102 HIV-1 GAG (YSPVSILDI), and HLA-Cw*0102 HIV-1 ENV (CTPAGYAIL) soluble biotinylated monomers were produced at NIH Tetramer Core Facility as previously described[53] and tetramerized with BV650-labeled Streptavidin (BioLegend) or PE-conjugated ExtrAvidin (Sigma-Aldrich).

Flow cytometry analysis

For phenotypic analysis of antigen-specific $CD8^+$ T cells, thawed (*ex vivo*) or peptide stimulated PBMCs were first stained with BV650-labeled pMHC tetramer and LIVE/DEAD Yellow at

37°C for 15 minutes. Cells were stained for other cell surface markers in Brilliant Stain Buffer at 4°C for 20 minutes followed by 2 times wash with PBS containing 2% FBS (washing buffer). Cells were fixed/permeabilized and stained for c-Myc, Eomes, Granzyme B, Perforin, T-bet, and TCF-1 in Foxp3 permeabilization buffer supplemented with Brilliant Stain Buffer Plus at room temperature for 30 minutes followed by 2 times wash with Foxp3 permeabilization buffer and washing buffer, respectively. Stained cells were fixed in PBS containing 2% formaldehyde, CountBright beads were added to all the samples before flow cytometry analysis, and the tetramer$^+$ cell number was normalized based on the number of the CountBright beads recorded using a FACSymphony A5 (BD Biosciences).

For the cytotoxic killing sassy, co-cultured cells were first stained with BV650-labeled tetramer and LIVE/DEAD Near-IR at 37°C for 15 minutes followed by staining of the other cell surface markers as described above. The stained cells were fixed in PBS containing 2% formaldehyde and analyzed on a LSRII (BD Biosciences).

For single cell RNA sequencing, thawed PBMCs were first stained with PE-labeled HLA-A*1101 HIV-1 NEF (GAFDLSFFLK) tetramer and LIVE/DEAD aqua followed by staining of CD8 as described above. Individual live CD8$^+$tetramer$^+$ cells were directly sorted into wells of a PCR 96 well plate pre-loaded with 5µl of Buffer TCL (Qiagen) containing 1% 2-mercaptoethanol using a FACS Aria II (BD Biosciences).

All flow cytometry data were analyzed with FlowJo v10 (FlowJo, LLC).

Single cell RNA sequencing

A total of 405 single HIV-specific CD8$^+$ T cells from 6 participants (3 each from AHI Stage 1 and 3) were sorted directly into 5 µl of Buffer TCL (Qiagen, Germantown, MD) + 1% 2-

Mercaptoethanol (Sigma-Aldrich, St. Louis, MO) in individual wells of skirted MicroAmp 96-well plates (Applied Biosystems) and immediately snap-frozen (see Flow cytometry analysis in Supplemental Materials and Methods). After thawing, 10 µl of exogenous ERCC RNA Spike-In Mix 1 (Life Technologies Corp, Carlsbad, CA) was added to each lysed cell to a final dilution of ~1:110,000,000. Total RNA from these cells was then purified, and full-length cDNA prepared using SmartSeq technology [1,2]. Libraries were made using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA). Briefly, 300 pg of cDNA from each cell were fragmented, uniquely dual-indexed, amplified, and purified. The resulting libraries were quantitated with the Quant-iT HS DNA kit (ThermoFisher Scientific) on the FilterMax 3 (Molecular Devices, San Jose, CA) with random samples also run on the BioAnalyzer using High Sensitivity DNA chips (both Agilent, Santa Clara, CA) for QC and fragment size determinations. Each cell library was diluted to 2 nM with Resuspension Buffer (Illumina). Libraries were pooled and denatureded prior to loading on 3 NextSeq 500/550 Hi Output 300-cycle v2.5 reagent cartridges and sequenced on the NextSeq (all Illumina) sequencing instrument per manufacturer's recommendations. We targeted ~1.5 million reads per sample.

Single cell sequence analysis

Paired-end sequencing data was converted to fastq, and adapters were masked using bcl2fastq v2.17.1.14 software (Illumina). The raw fastq files were examined for quality and reads were trimmed using fastp v0.19.7 [3] when the average quality score of a 15-base pair sliding window fell below Q20. Trimmed reads with a minimum length of 50 base pairs were retained. A median of 10% raw reads was discarded when using these parameters. Trimmed paired-end reads were aligned with HISAT2 v2.1.0 [4] to the human genome GRCh38 with Ensemble gene annotation

v97 and ERCC sequences. Gene expression quantification was performed using Subread featureCounts v1.6.2 [5].

TCR repertoire analysis

MiXCR v2.1.5 was used to identify the CDR3 region of the TRA and TRB from the scRNA-seq reads. TCR repertoire similarity for α and β chains was calculated between 2 visits more than 1 year apart during ART using the Morisita-Horn index in the vegan R package. Analysis was done for the two participants that had at least 20 cells with one functional TRA or TRB CDR3 (MixCR cloneCount >1) at both visits.

Supplementary References:

1. Trombetta JJ, Gennert D, Lu D, Satija R, Shalek AK, Regev A. Preparation of Single-Cell RNA-Seq Libraries for Next Generation Sequencing. *Curr Protoc Mol Biol* 2014; **107**: 4 22 1-17.
2. Picelli S, Bjorklund AK, Faridani OR, Sagasser S, Winberg G, Sandberg R. Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat Methods* 2013; **10**(11): 1096-8.
3. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 2018; **34**(17): i884-i90.
4. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* 2019; **37**(8): 907-15.
5. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 2014; **30**(7): 923-30.

Supplemental Table

Table S1. Epitope peptides and pMHC tetramers used.

Epitope AA Seq	Symbol	Antigen	Location	HLA restriction
GAFDLSFFLK	GK10	HIV	Nef 83-92	HLA-A*11:01
QVPLRPMTYK	QK10	HIV	Nef 73-82	HLA-A*11:01
AIFQSSMTK	AK9	HIV	Pol 313-321	HLA-A*11:01
YSPVSILDI	YI9	HIV	Gag 277-285	HLA-C*01:02
CTPAGYAIL	CL9	HIV	Env 218-226	HLA-C*01:02
AVFDRKSDAK	EBV	EBV	EBNA3B 399-408	HLA-A*11:01

Table S2. Epitope in autologous sequences in participants treated from AHI

PID	Epitope designation	Epitope (Tested)	Epitope (Participant sequence)	n_mismatch (tested - participant)
P0006	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0022	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:13	0
P0024	Cw01 CL9	CTPAGYAIL	CTPAGYAIL:10	0
P0027	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0028	A11 GK10	GAFDLSFFLK	AAFDLSFFLK:9, EAFDLSFFLK:1	1, 1
P0029	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0030	A11 GK10	GAFDLSFFLK	AAFDLSFFLK:10	1
P0033	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:1	0
P0050	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0055	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0059	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:1	0
P0062	Cw01 YI9	YSPVSILDI	YSPVSILDI:9	0
P0070	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0076	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0078	Cw01 YI9	YSPVSILDI	YSPTSILDI:10	1
P0088	A11 QK10	QVPLRPMTYK	QVPLRPMTYK:10	0
P0091	Cw01 YI9	YSPVSILDI	YSPVSILDI:10	0
P0100	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0101	A11 GK10	GAFDLSFFLK	AAFDLSFFLK:10	1
P0105	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:9	0
P0110	Cw01 CL9	CTPAGYAIL	CTPAGYALL:10	1
P0105	A11 AK9	AIFQSSMTK	AIFQSSMTK:10	0

"Epitope (Participant sequence)": predicted epitope in the HIV-1 sequences from a participant, with the number of sequences obtained. *"n_mismatch (tested - participant)"*: number of mismatches between the epitopes tested and in the participant's sequences.

Table S3. Epitope in autologous sequences in participants treated from CHI

PID	Epitope designation	Epitope (Tested)	Epitope (Participant sequence)	n_mismatch (tested - participant)
P0018	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:11	0
P0048B	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:1	0
P0050B	Cw01 YI9	YSPVSILDI	YSPVSILDI:1	0
P0051B	A11 QK10	QVPLRPMTYK	QVPLRPMTFK:1	1
P0054B	Cw01 YI9	YSPVSILDI	YSPVSILDI:1	0
P0004	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0029	A11 GK10	GAFDLSFFLK	GAFDLSFFLK:10	0
P0034	Cw01 CL9	CTPAGYAIL	CTPAGYAIL:28, CTPAGYTIL:1	0, 1

"Epitope (Participant sequence)": predicted epitope in the HIV-1 sequences from a participant, with the number of sequences obtained. "n_mismatch (tested - participant)": number of mismatches between the epitopes tested and in the participant's sequences.

Table S4. CD8$^+$ T cell epitope prediction in sequences from participants.

HLA-A*11:01 restricted GK10 Nef peptides

Peptide	Score EL	Rank EL (%)	Score BA	Rank BA (%)	Affinity (nM)	Bind Level
GAFDLSFFLK	0.79	0.11	0.82	0.02	6.72	Strong
AAFDLSFFLK	0.89	0.04	0.86	0.01	4.75	Strong
EAFDLSFFLK	0.21	1.05	0.58	0.54	93.78	Weak

HLA-A*11:01 restricted QK10 Nef peptides

Peptide	Score EL	Rank EL (%)	Score BA	Rank BA (%)	Affinity (nM)	Bind Level
QVPLRPMTYK	0.64	0.21	0.72	0.11	19.8	Strong
QVPLRPMTFK	0.57	0.29	0.71	0.13	22.4	Strong

HLA-A*11:01restricted AK9 Pol peptides

Peptide	Score EL	Rank EL (%)	Score BA	Rank BA (%)	Affinity (nM)	Bind Level
AIFQSSMTK	0.98	0.01	0.83	0.02	6.64	Strong
AIFQCSMTK	0.91	0.03	0.82	0.02	7.04	Strong

HLA- C*01:02 restricted YI9 Gag peptides

Peptide	Score EL	Rank EL (%)	Score BA	Rank BA (%)	Affinity (nM)	Bind Level
YSPVSILDI	0.45	0.16	0.55	0.06	132.70	Strong
YSPTSILDI	0.57	0.10	0.58	0.05	96.34	Strong

HLA- C*01:02 restricted CL9 Env peptides

Peptide	Score EL	Rank EL (%)	Score BA	Rank BA (%)	Affinity (nM)	Bind Level
CTPAGYAIL	0.41	0.19	0.61	0.03	64.91	Strong
CTPAGYALL	0.38	0.21	0.59	0.04	82.40	Strong
CTPAGYTIL	0.52	0.13	0.61	0.03	71.08	Strong

Table S5. TCR repertoire similarity of memory HIV-specific CD8$^+$ T cells during long term suppressive ART in people treated from AHI.

PID	AHI stage started ART	ART duration at first visit (yrs)	ART duration at second visit (yrs)	Morisita-Horn similarity index	
				TCRα	TCRβ
P0006	Stage 3	2.3	4.4	0.77	0.65
P0070	Stage 3	1.6	3.0	0.84	0.64

The CDR3 region of the TRA and TRB was sequenced by single cell RNA sequencing, and MiXCR was used to identify individual TCRs. TCR repertoire similarity was calculated as the Morisita-Horn index between 2 visits more than 1 year apart during suppressive ART. Analysis was done for the two participants that had at least 20 cells with one functional TRA or TRB CDR3 (MixCR cloneCount >1) at both visits.

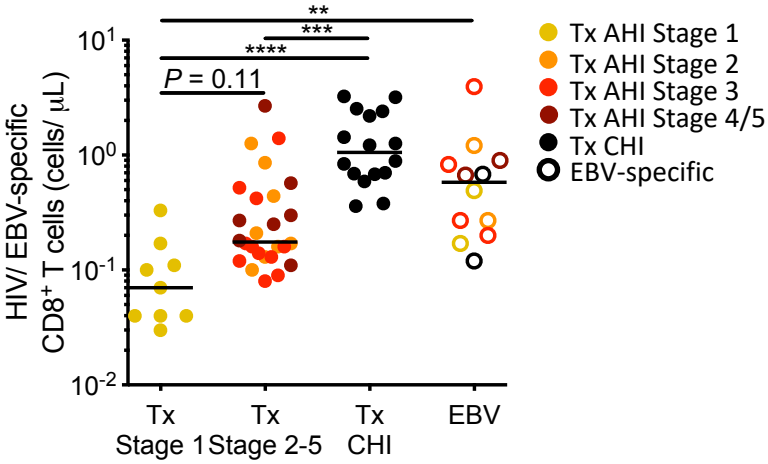

Figure S1. HIV-specific CD8⁺ T cells in people treated from different stages of acute and chronic HIV infection. Number of HIV- and EBV-specific tetramer⁺ CD8⁺ T cells per μL of peripheral blood in individuals who initiated treatment in AHI or in CHI and were on ART for more than 1 year. The fourth-generation immunoassay (4G IA) staging system was used to group RV254 participants who initiated ART at the earliest stages of acute infection, AHI 4G stage 1 (NAT+, 4G IA-), and AHI Stage 2 (NAT+4G IA+). Participants who started ART at peak viremia or later in AHI (NAT+4G IA+3G IA+) were further classified into Stage 3 (WB-) and Stage 4/5 (WB+/- and WB+, respectively). Differences among groups were analyzed by Kruskal-Wallis test (**P< 0.01; ***P< 0.001; ****P< 0.0001).

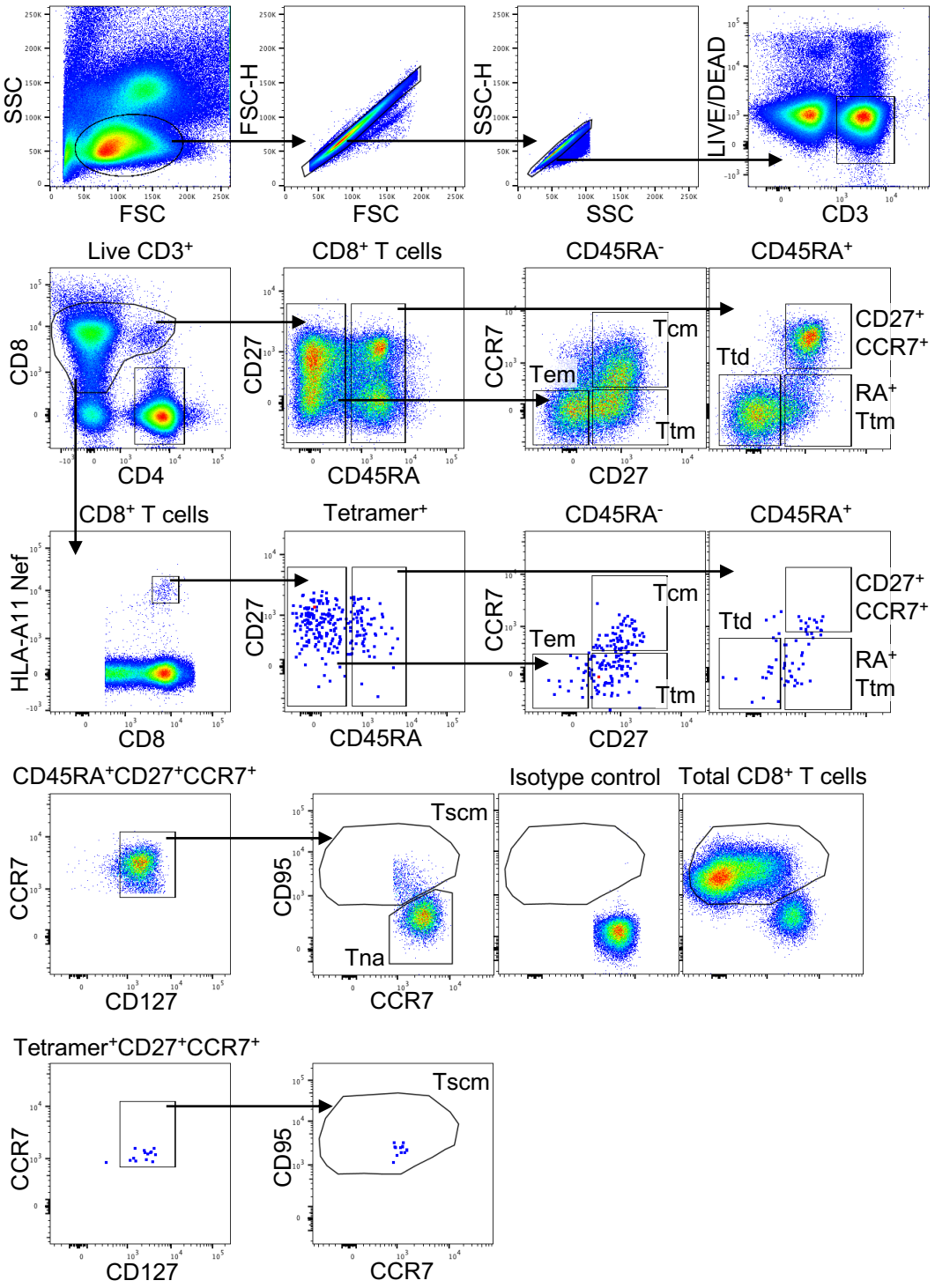

Figure S2. Gating strategy for flow cytometry analysis of tetramer⁺ HIV- or EBV-specific CD8⁺ T cells. Each CD8 T cell subset was classified as follow; Tna: $CD45RA^{+}CCR7^{+}CD27^{+}$, Tscm: $CD45RA^{+}CCR7^{+}CD27^{+}$ $CD127^{+}CD95^{+}$, Tcm: $CD45RA^{-}CCR7^{+}CD27^{+}$, Ttm: $CD45RA^{-}CCR7^{-}CD27^{+}$, RA^{+}Ttm: $CD45RA^{+}CCR7^{-}CD27^{+}$, Tem: $CD45RA^{-}CCR7^{-}CD27^{-}$, Ttd: $CD45RA^{+}CCR7^{-}CD27^{-}$. To confirm $CD95^{+}$ Tscm, isotype matched control antibody was used.

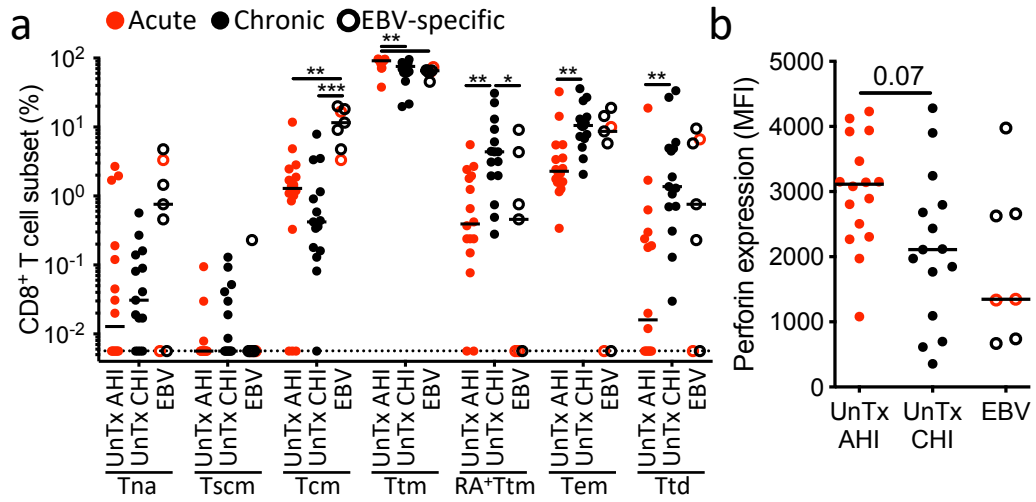

Figure S3. Phenotypic change of HIV-specific CD8⁺ T cells before and during ART initiated in chronic and acute HIV infection. (a) Frequencies of naïve-like (Tna), stem cell memory (Tscm), central memory (Tcm), transitional memory (Ttm), CD45RA⁺ transitional memory (RA⁺ Ttm), effector memory (Tem), and terminally differentiated cells (Ttd) in HIV/EBV-specific CD8⁺ T cells prior to ART initiation in AHI (n=19) or CHI (n=15). (b) Expression of Perforin in HIV/EBV-specific CD8⁺ T cells. Differences between groups were analyzed by Kruskal-Wallis test. *P< 0.05; **P< 0.01; ***P< 0.001.

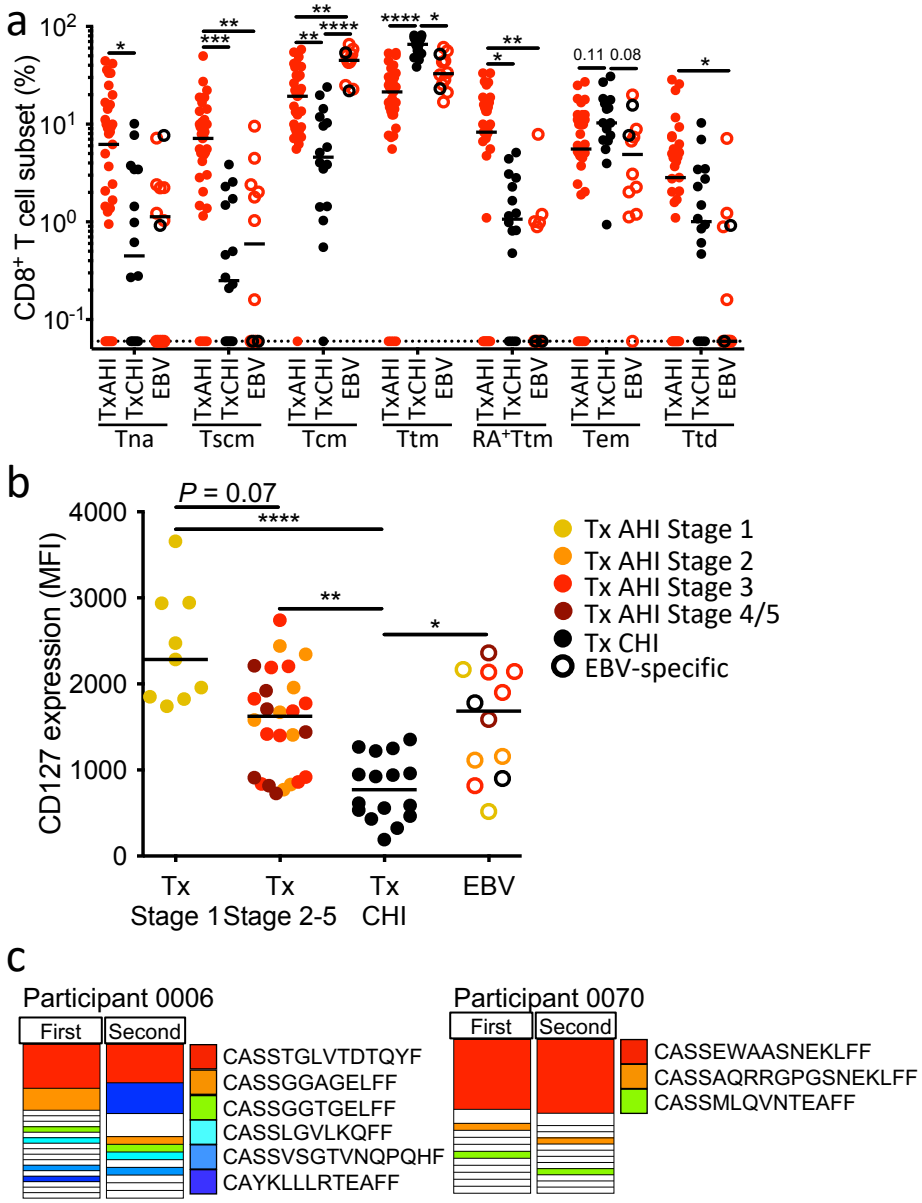

Figure S4. HIV-specific memory CD8⁺ T cells in people treated from different stages of acute and chronic HIV infection. (a) Frequencies of Tna, Tscm, Tcm, Ttm, RA⁺ Ttm, Tem, and Ttd in HIV/EBV-specific CD8⁺ T cells from individuals who initiated treatment from AHI or CHI and were on ART for more than 1 year. (b) Expression of CD127 on HIV/EBV-specific CD8⁺ T cells. (c) Repertoire clonality of memory HIV-specific CD8⁺ T cells during long-term ART was determined by comparing the CDR3 region of the TRB using MiXCR after single cell RNA sequencing. Each segmented bar represents the frequency of cells within singly sorted HIV Nef epitope-specific CD8⁺ T cells carrying each unique TCR clone. TCR clones shared between 2 visits more than 1 year apart during long-term ART within each participant are color-coded and their amino acid sequences are shown. Differences among groups were analyzed by Kruskal-Wallis test (*P< 0.05; **P< 0.01; ***P< 0.001; ****P< 0.0001).

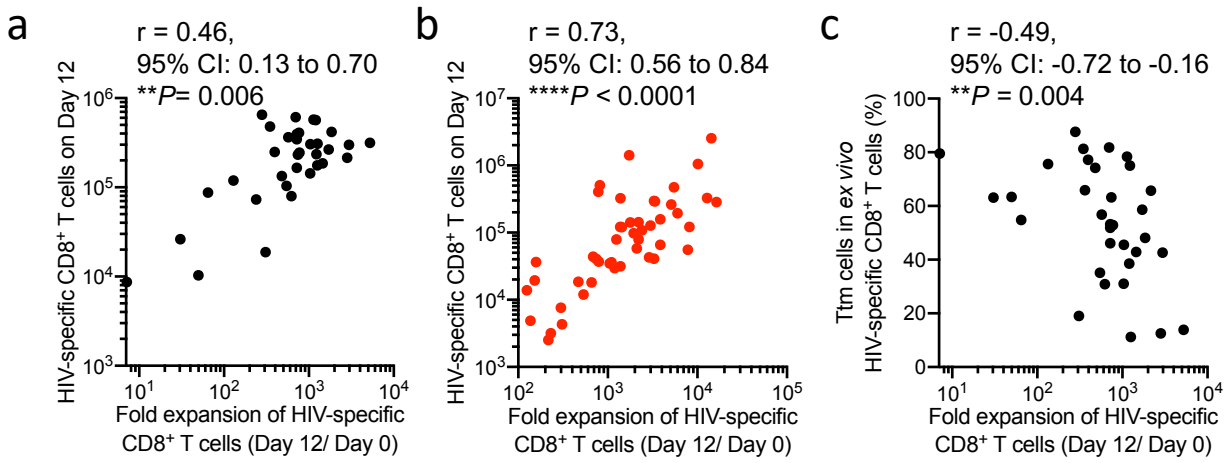

Figure S5. Correlations with the magnitude of recalled memory CD8⁺ T cell expansion. (a, b) Correlation between the fold expansion of HIV-specific CD8⁺ T cells from people treated from CHI (a) and AHI (b) and the cell number 12 days after peptide stimulation. (c) Correlation between the fold expansion of HIV-specific CD8⁺ T cells and *ex vivo* frequency of Ttm in HIV-specific CD8⁺ T cells from people treated from CHI. Correlations were analyzed by Spearman correlation with the Benjamini-Hochberg procedure for multiple comparisons (FDR < 0.1).

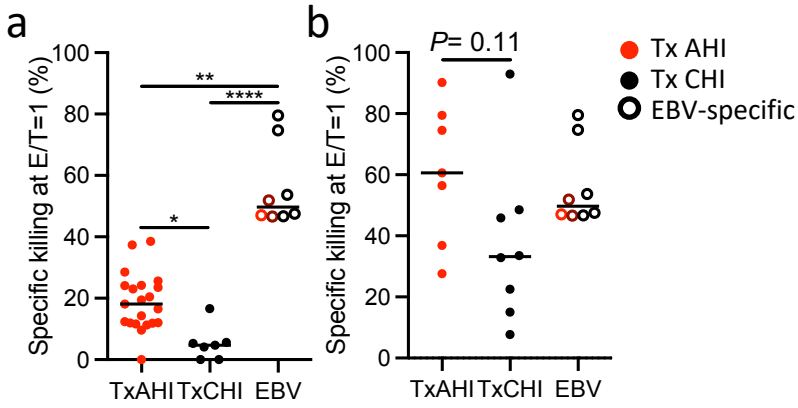

Figure S6. Cytotoxic capacity of recalled HIV-specific and EBV-specific CD8⁺ T cells. (a) Cytotoxic capacity of day 13 recalled HIV Nef GK10- or EBV- specific CD8⁺ T cells cultured with autologous CD4⁺ T cells at an effector/ target ratio of 1. (b) Cytotoxic capacity of recalled non GK10-specifc CD8⁺ T cells, HLA-A11 Nef (QK10, n= 5) and Pol (AK9, n= 1), HLA-Cw01 Gag (YI9, n= 6) and Env (CL9, n= 3), or EBV- specific CD8⁺ T cells cultured at an effector/target ratio of 1. Differences were analyzed by Kruskal-Wallis test (*P< 0.05; **P< 0.01; ****P< 0.0001).

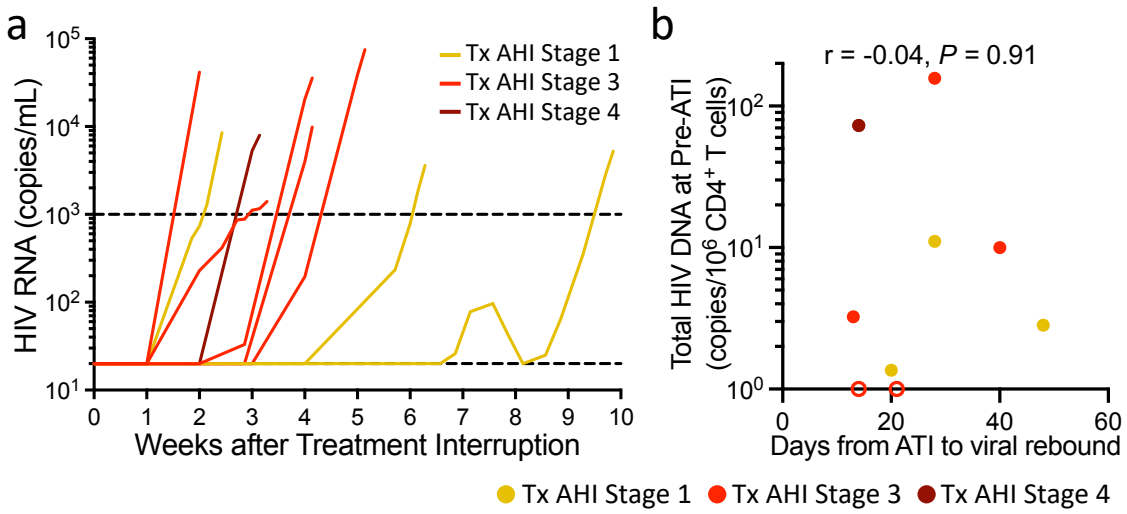

Figure S7. Time to viral load rebound does not correlate with HIV reservoir size in the participants underwent ATI. (a) Plasma HIV-1 RNA after ART interruption. The detection limit 20 HIV-1 RNA copies per mL and ART resumption criteria 1000 or more copies per mL are shown as dotted lines. (b) Correlation between days to viral load rebound (VL>20 copies/mL) after ATI and total HIV DNA copies per 10^6 $CD4^+$ T cells at baseline ATI. For samples in which no positive cells were detected, the limit of detection based on cell input is plotted as an open symbol. Correlations were analyzed by Spearman correlation.

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