Supplemental Figure Legends
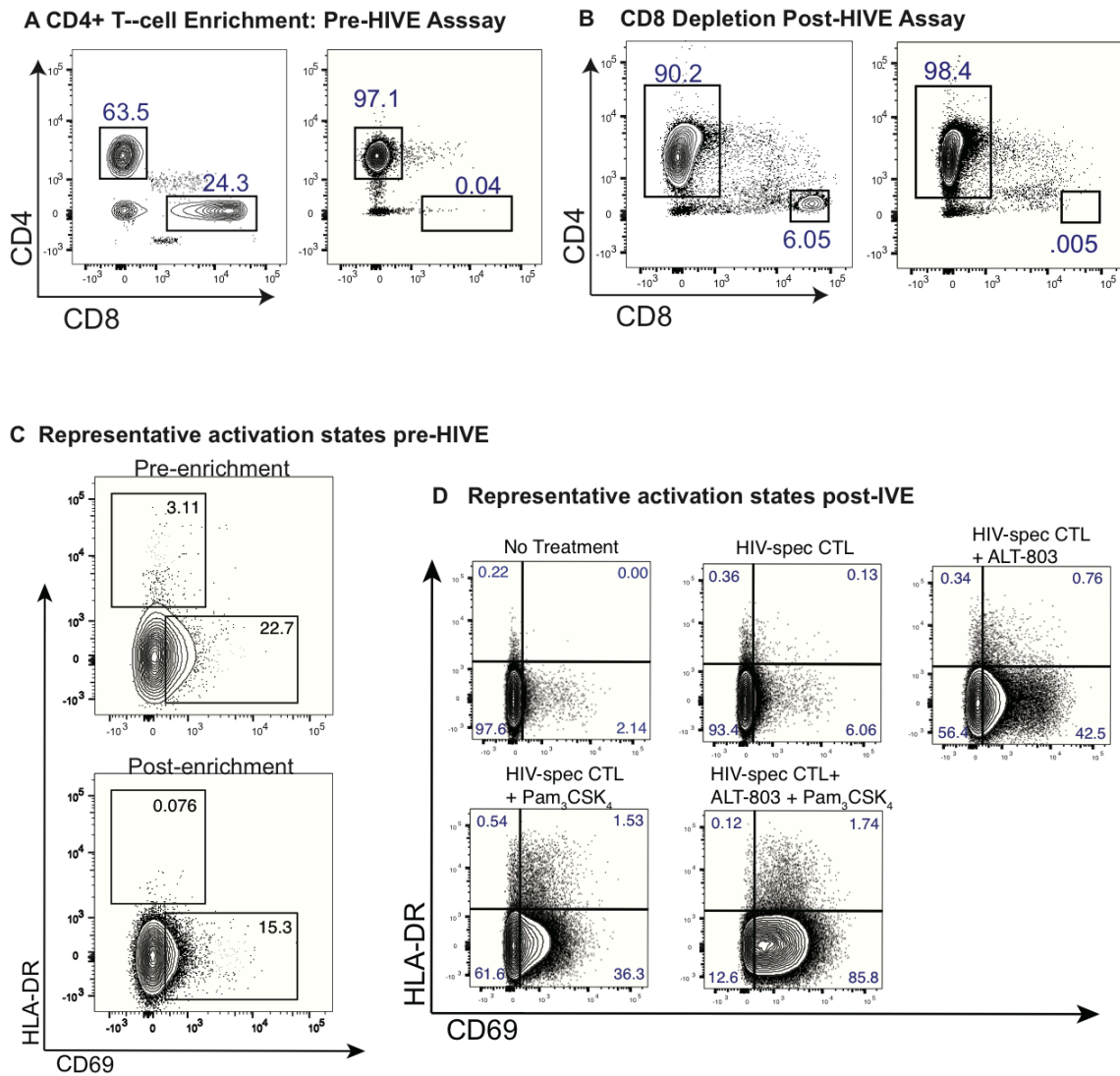
Supplementary Table 1

Table of Representative CD4+ T-cell Activation States Post-HIVE Assay

CD4+ T-cells Post-HIVE Assay	CD69+	HLA-DR+
No Treatment	2.18	0.21
HIV-Gag-HA9-spec CD8s	6.28	0.45
ALT-803	28.20	0.69
Bryostatin-1	61.70	2.45
Bryostatin-1+HIV-Gag-HA9-spec CD8s	83.80	2.28
Bryostatin-1+HIV-Nef-RA9-spec CD8s	61.30	9.57
Bryostatin-1+HIV-Nef-AL9-spec CD8s	74.50	7.57
Bryostatin-1+HIV-Gag-IK9-spec CD8s	45.90	8.79
Vorinostat	2.72	0.61
Vorinostat+HIV-Gag-HA9-spec CD8s	4.45	1.40
Romidepsin	32.7	0.00
Romidepsin+HIV-Gag-HA9-Spec CD8s	32.5	0.00
PMA/Ionomycin	87.3	73.6
PMA/Ionomycin+HIV-Gag-HA9-spec CD8s	90.9	61.8
LRA Cocktail	56.8	3.86
LRA Cocktail+HIV-Gag-HA9-spec CD8s	77.8	36.7
Pam3	25.00	1.11
Pam3 + HIV-Gag-HA9-spec CD8s	43.40	1.04
Pam3 + CMV-spec CD8s	36.10	0.75
ALT-803+ HIV-Gag-HA9-spec CD8s	38.00	1.98
ALT-803 + CMV-spec CD8s	34.70	1.78
Pam3+ALT+HIV-Gag-HA9-spec CD8s	87.20	1.74
Pam3+ALT+CMV-spec CD8s	92.30	1.89
CMV-spec CD8s	9.30	0.40

Supplemental Table 1: Activation States of CD4^{+} T-cells Post-HIVE assays. Table showing representative activation phenotypes after various treatment conditions, post-HIVE assays, as measured by %CD3^{+}, CD4^{+} cells expressing CD69 or HLA-DR. Abbreviations: ALT = ALT-803, Pam3 = Pam$_3$CSK$_4$.
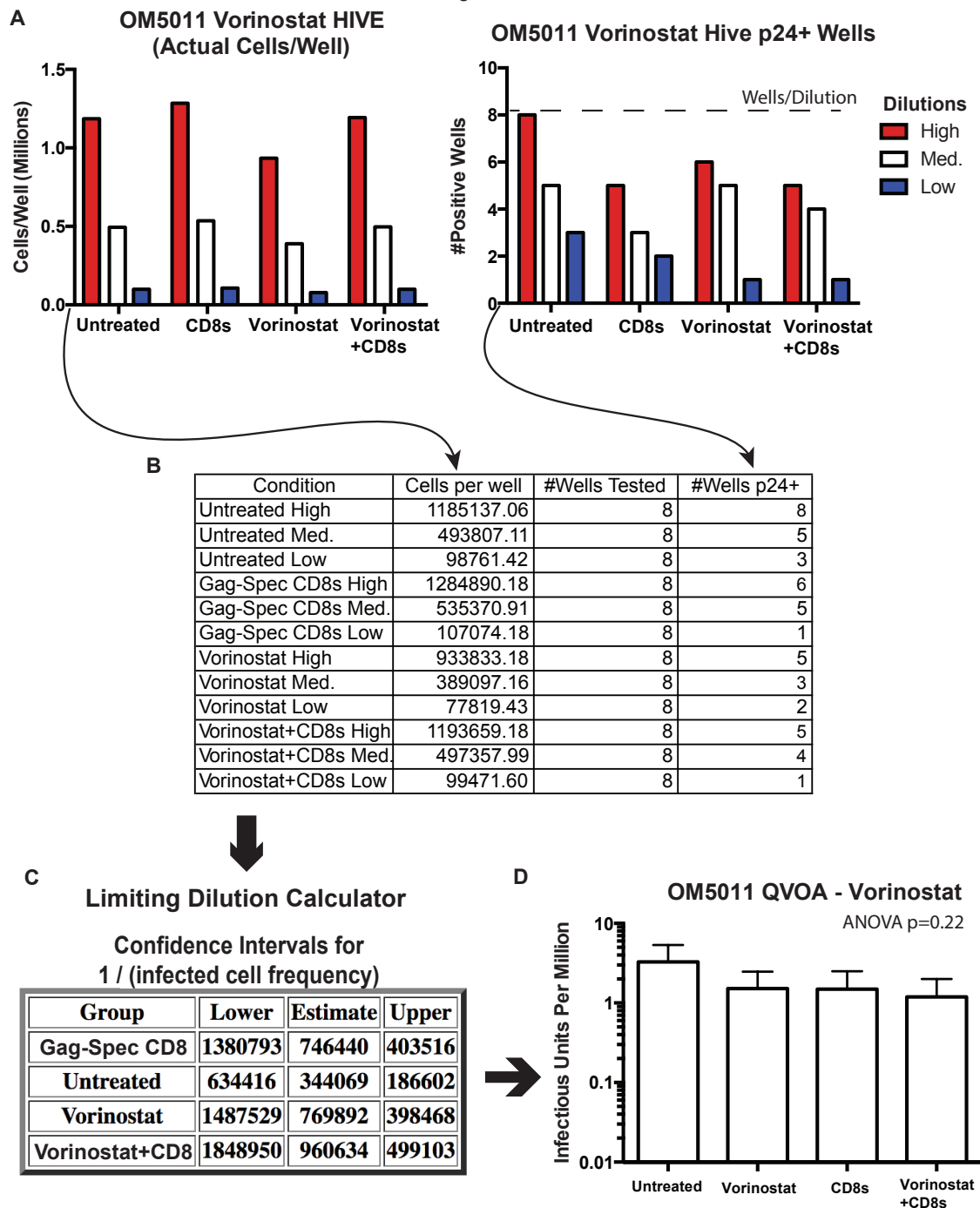
Figure S1

A CD4+ T--cell Enrichment: Pre-HIVE Asssay

B CD8 Depletion Post-HIVE Assay

C Representative activation states pre-HIVE

Pre-enrichment

Post-enrichment

D Representative activation states post-IVE

Supplemental Figure 1: Enrichment of CD4^{+} and CD8^{+} T-cells, Pre- and Post-HIVE

assays, and maturational phenotypes. **A.** Representative flow charts showing pre-

and post-enrichment of CD4^{+} T-cells from total PBMCs. Left: CD4^{+} and CD8^{+} T-cell

populations pre-enrichment. Right: purified CD4$^+$ T-cells depleted of CD8$^+$ T-cells, post-enrichment. **B.** Representative flow charts showing pre- and post-enrichment of CD4$^+$ T-cells after a HIVE assay. Left: CD4$^+$ and CD8$^+$ T-cell populations pre-enrichment. Right: enriched CD4$^+$ T-cells and depleted CD8$^+$ T-cells post-enrichment. Enriched CD4+ T cell populations contained a median of 0.01% CD8 T cells (range 0.00% to 0.06%). **C.** Representative flow charts showing proportion of cells expressing CD69 and HLA-DR, pre- and post-depletion of activated CD4$^+$ T-cells from total PBMCs prior to initiation of a HIVE assay. Top: CD4$^+$ T-cell activation states pre-enrichment. Bottom: CD4$^+$ T-cell activation states post HLA-DR depletion, showing decreased levels of cells expressing HLA-DR and CD69. **D.** Example flow charts of activation states in CD4$^+$ T-cells post-HIVE assay, after various treatment conditions. See **Table S1** for a full list of activation phenotypes post-HIVE assay.

A

OM5011 Vorinostat HIVE (Actual Cells/Well)

OM5011 Vorinostat Hive p24+ Wells

B

Condition	Cells per well	#Wells Tested	#Wells p24+
Untreated High	1185137.06	8	8
Untreated Med.	493807.11	8	5
Untreated Low	98761.42	8	3
Gag-Spec CD8s High	1284890.18	8	6
Gag-Spec CD8s Med.	535370.91	8	5
Gag-Spec CD8s Low	107074.18	8	1
Vorinostat High	933833.18	8	5
Vorinostat Med.	389097.16	8	3
Vorinostat Low	77819.43	8	2
Vorinostat+CD8s High	1193659.18	8	5
Vorinostat+CD8s Med.	497357.99	8	4
Vorinostat+CD8s Low	99471.60	8	1

C

Limiting Dilution Calculator

Confidence Intervals for 1 / (infected cell frequency)

Group	Lower	Estimate	Upper
Gag-Spec CD8	1380793	746440	403516
Untreated	634416	344069	186602
Vorinostat	1487529	769892	398468
Vorinostat+CD8	1848950	960634	499103

D

OM5011 QVOA - Vorinostat

ANOVA p=0.22

Supplemental Figure 2: Schematic for calculation of IUPM from QVOA. Sample schematic and calculations are based on the HIVE assay with vorinostat shown in **Figure S5** below. **A.** CD4^{+} T-cells enriched from HIVE assays are plated out at three dilutions (~1M, ~0.5M, and ~0.1M cells/well) with 8 replicates per dilution. Dilutions are
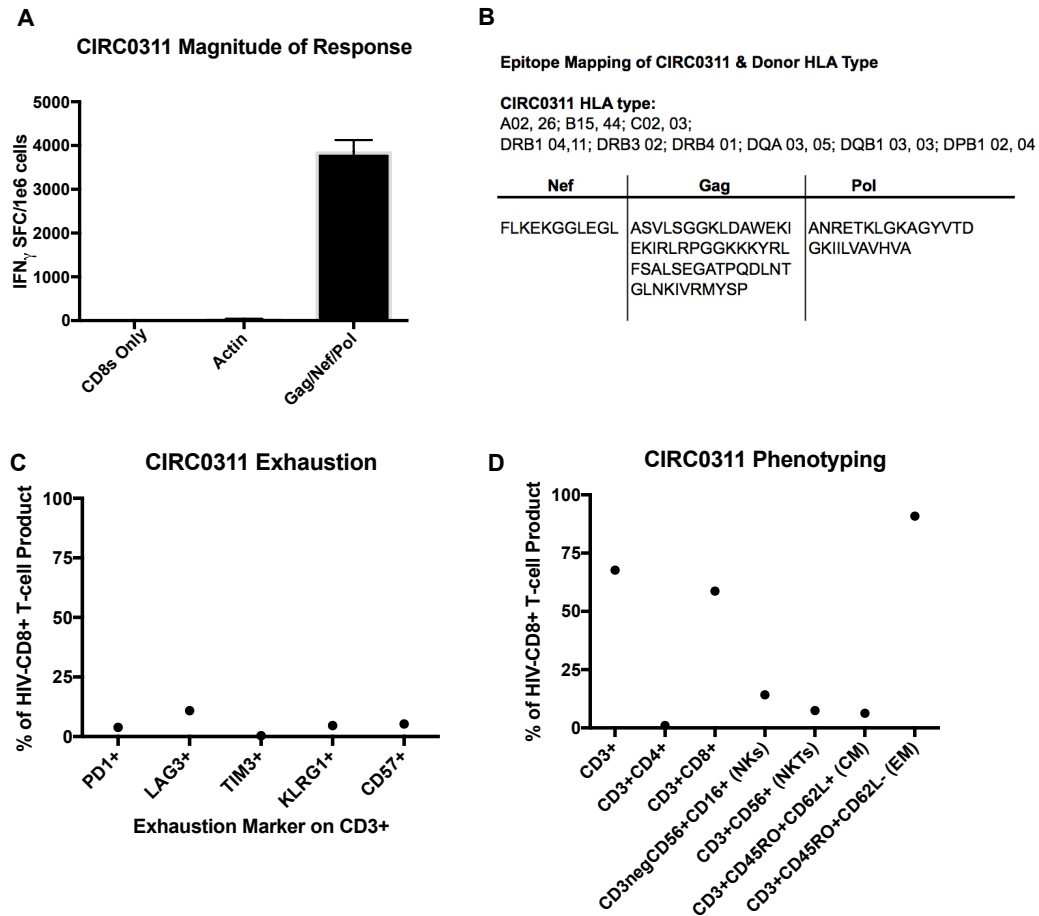
chosen to minimize the probability that any given dilution will have all positive or all negative wells. **B.** The number of p24 positive wells per dilution and the cells/well at the same dilution are used to calculate the 95% confidence intervals for the number of HIV⁻ cells per HIV⁺ cell, using a limiting dilution calculator (http://bioinf.wehi.edu.au/software/elda/ or http://silicianolab.johnshopkins.edu/). **C.** These values are divided into 10^6 to obtain the estimated IUPM with upper and lower limits of the 95% confidence interval, and depicted as median with range **D**. The limiting dilution calculator also tests for inequality in frequency between groups using a Chi-squared test, determining if there is an overall significance between means of different groups, as well as individual significances between treatment conditions. This method was used for all QVOAs in this study, with the exception of **Figure 3D**. For the experiment shown in **Figure 3D** we plated cells at 12 replicates per condition at a single cell number dilution. This cell number was selected based on prior knowledge of this individual's IUPM with the aim of obtaining ~50% p24⁺ wells in the no treatment condition.
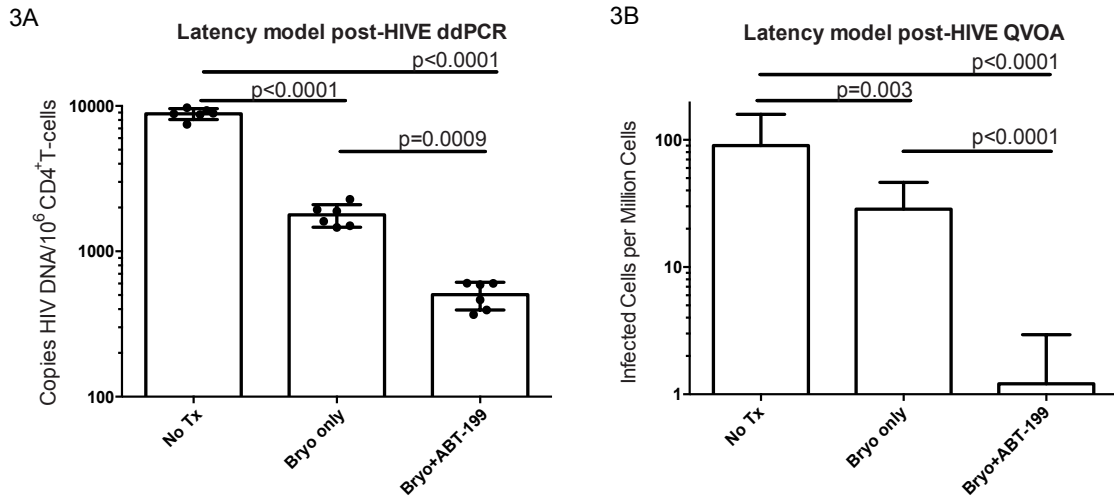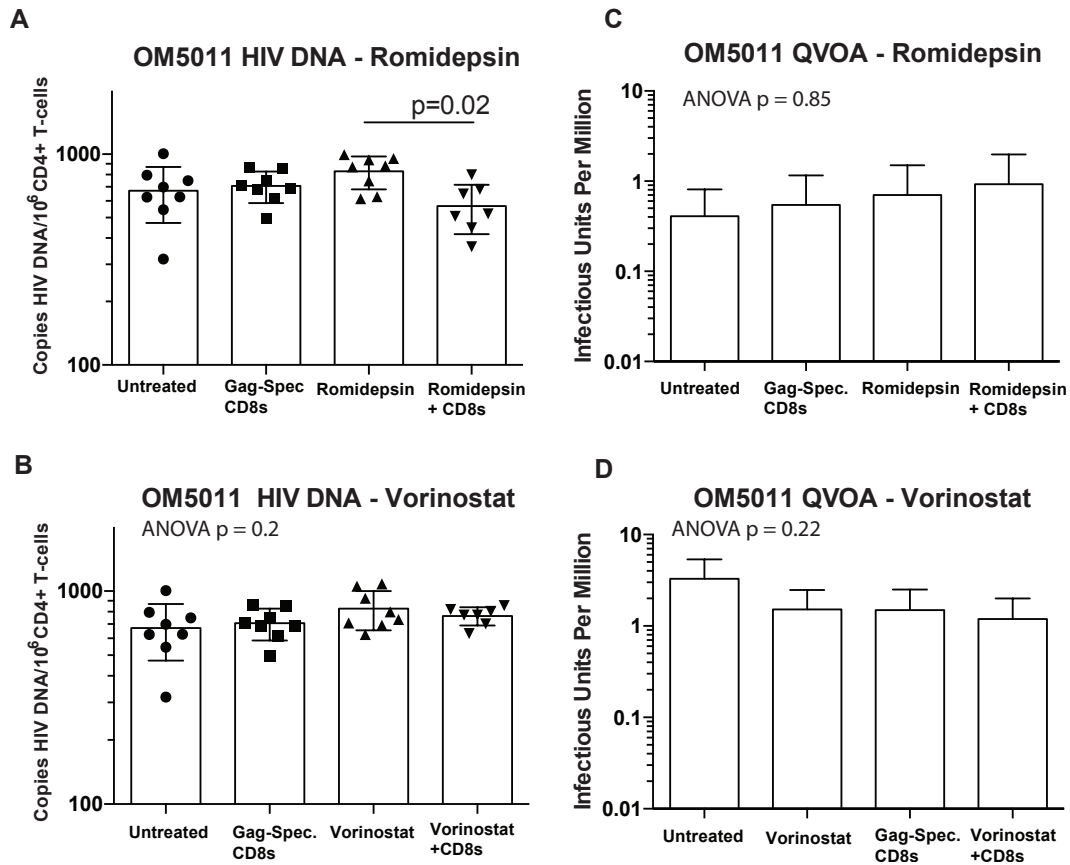
Supplemental Figure 3: Validation of the HIVE assay using a primary cell model of latency. Latently infected cells from study participant OM5203 were generated using a primary T_{CM} cell model of latency, and treated with either bryostatin alone, or bryostatin combined with ABT-199. **A.** ddPCR results from the HIVE assay show mean ± SD. P values were calculated by one-way ANOVA with Tukey's multiple comparison test. Significant decreases in HIV DNA were observed when cells were treated with bryostatin, and further significant decreases were observed with bryostatin + ABT-199. **B.** QVOA results from the same HIVE assay show median IUPM with 95% confidence intervals, and significant decreases in IUPM were observed when cells were treated with bryostatin, with further reductions achieved with bryostatin + ABT-199.

A. CIRC0311 Magnitude of Response

B. Epitope Mapping of CIRC0311 & Donor HLA Type

CIRC0311 HLA type:
A02, 26; B15, 44; C02, 03;
DRB1 04,11; DRB3 02; DRB4 01; DQA 03, 05; DQB1 03, 03; DPB1 02, 04

Nef	Gag	Pol
FLKEKGGLEGL	ASVLSGGKLDAWEKI EKIRLRPGGKKKYRL FSALSEGATPQDLNT GLNKIVRMYSP	ANRETKLGKAGYVTD GKIILVAVHVA

C. CIRC0311 Exhaustion

D. CIRC0311 Phenotyping

Supplemental Figure 4: Characterization of HIV-specific T-cells expanded from participant CIRC0311. **A.** Magnitude and target of T-cell responses in the HIV-expanded cell line, as measured by spot forming cells. Unstimulated cells and cells stimulated with actin serve as negative controls. **B.** Donor HLA types and specific epitope targets of the expanded cell line. **C.** Percent of HIV-specific T-cells expressing exhaustion markers PD1, LAG3, TIM3, KLRG1, or CD57. **D.** Composition of the HIV-specific expanded T-cell product, broken down into total %T-cells, CD4^{+} T-cells, CD8^{+} T-cells, NK cells, NKT cells, and central or effector memory cells.
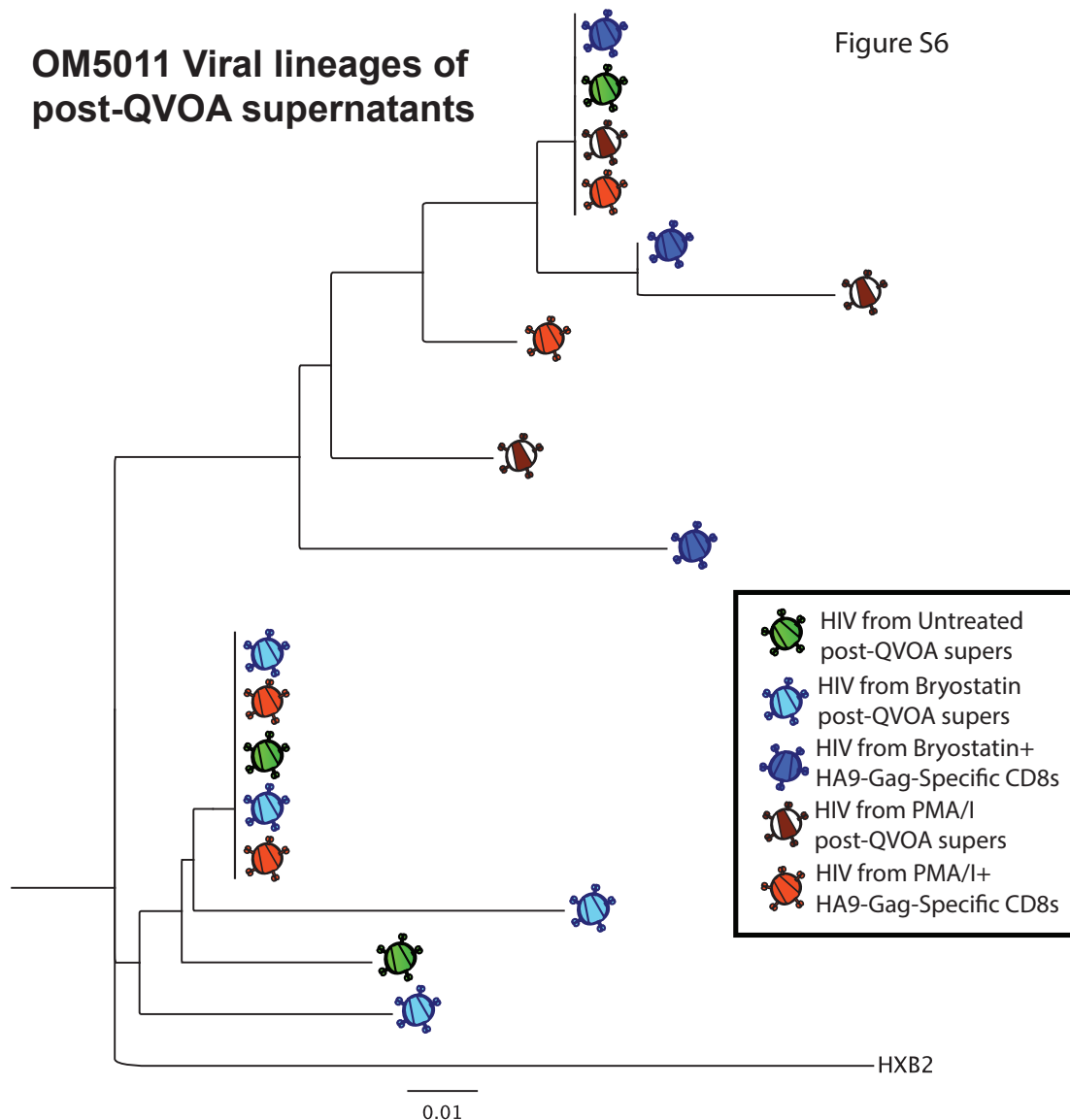
A

OM5011 HIV DNA - Romidepsin

p=0.02

C

OM5011 QVOA - Romidepsin

ANOVA p = 0.85

B

OM5011 HIV DNA - Vorinostat

ANOVA p = 0.2

D

OM5011 QVOA - Vorinostat

ANOVA p = 0.22

Supplemental Figure 5: Romidepsin or vorinostat in combination with HIV-Gag-HA9-specific CD8^{+} T-cells deplete cell-associated HIV DNA but not the latent reservoir in participant OM5011. **A & B.** ddPCR results from HIVE assays show mean ± SD. p values were calculated by one-way ANOVA with Tukey's multiple comparison test. No significant decreases in cell-associated HIV DNA was observed when CD4+ T-cells are treated with either romidepsin or vorinostat combined with HIV-Gag-HA9-specific CD8^{+} T-cells clones, compared to the "Untreated" condition. A slight, significant decrease in HIV DNA was observed when comparing "romidepsin" to "romidepsin+ CD8s" (p = 0.02) **C & D.** QVOA results from the same HIVE show mean IUPM with 95% confidence intervals. No significant changes in IUPM between any of the treatment conditions were observed (Overall ANOVA, p = 0.22).

OM5011 Viral lineages of post-QVOA supernatants

Figure S6

Legend:
- HIV from Untreated post-QVOA supers
- HIV from Bryostatin post-QVOA supers
- HIV from Bryostatin+ HA9-Gag-Specific CD8s
- HIV from PMA/I post-QVOA supers
- HIV from PMA/I+ HA9-Gag-Specific CD8s

HXB2

0.01

Supplemental Figure 6: Neighbor-joining distance trees of sequences from p24^{+} wells of QVOAs post-HIVE assays (participant OM5011). Phylogenetic trees were constructed using the neighbor joining method and Tamura-Nei genetic distance model, and rooted to a consensus sequence HIV strain HXB2. Env-V3V4 sequences were obtained by RT-PCR of viral RNA from p24^{+} wells of post-HIVE QVOAs, followed by nested PCR. While full-length sequences were not obtained to assess the presence of deleterious mutations, only replication competent virus are amplified in QVOAs. Different colored viruses represent virus from different HIVE assay conditions. Here, ~53% of viruses that grew out in QVOAs involving OM5011 fall into clonal families, which are populated by HIV from a variety of treatment conditions and/or HIVEs.

Figure S7
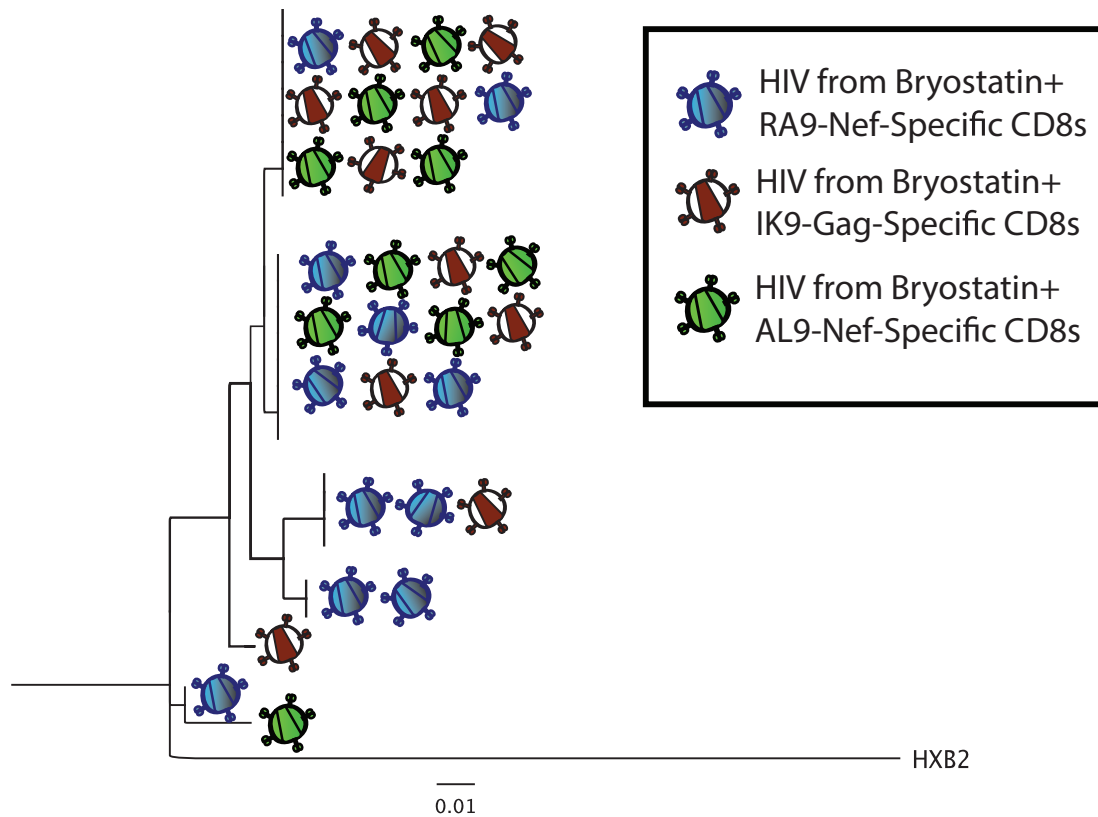
Supplemental Figure 7: Neighbor-joining distance trees of sequences from p24$^+$ wells of QVOAs post-HIVE assays (participant OM5267). Genetic sequences and tree construction were done as above. Full-length sequences were not obtained to assess the presence of deleterious mutations, as only replication competent virus are amplified in QVOAs. Different colored viruses represent virus from QVOAs of various HIVE assay conditions. Here, ~90% of viruses that grew out in QVOAs involving OM5011 fall into clonal families, which are populated by HIV from a variety of treatment conditions and/or HIVEs.

Supplemental Methods

Statistics

Statistical analyses were performed using Prism 6 (GraphPad) and methods used are reported within each figure legend. Overall experiments are judged to be significant when the differences by ANOVA reach $p < 0.05$. Comparisons between treatment conditions are judged to be significant when $p < 0.05$ compared to the no treatment condition, or LRA only condition, after adjusting for multiple comparison testing. For ddPCR assays, each sample was run with at least eight technical replicates, and the mean of these replicates was graphed with SD error bars. Outliers, deemed to be technical replicates more than two standard deviations outside the mean, were not included in the calculation of the mean. This exclusion criterion was defined before experiments were initiated and consistently applied to all samples. All ddPCR data were analyzed by one-way ANOVA with Tukey's multiple comparison test to determine significance. QVOAs were run with up to 3 dilutions of cells, with 8-12 replicates per dilution, at the end of the HIVE assay. IUPM and a 95% confidence interval were calculated using limiting dilution analysis software (http://bioinf.wehi.edu.au/software/elda/ or http://silicianolab.johnshopkins.edu): these algorithms use the inputs: number of cells/well, number of wells tested, and number of p24$^+$ positive wells, to calculate the probable frequency and range of infectious units. Statistical tests to check for overall differences between frequencies in any of the groups, as well as pairwise tests for differences between any two individual groups were also calculated using the ELDA software. We depicted these data graphically (QVOA figures) by plotting the IUPM with error bars representing the IUPM range within a 95% confidence interval.

As advised, we have consulted with a statistician (Daniel Rosenbloom) regarding our analysis methods, and have added him to the acknowledgments.

Viral outgrowth assays

Purified CD4$^+$ T-cells were washed 3x and plated at three 3-fold dilutions of 8 replicates each. In most experiments, cell numbers in the first dilution were matched to IUPM values for each individual (established prior to HIVE assays) – i.e: for an individual with an IUPM = 1, cells would be plated at 1x10^6, 0.3x10^6, and 0.1x10^6 cells/well. Cells were then stimulated with 2 x 10^6/well irradiated feeder cells (HIV-negative donor) and 2 ï g/ml of PHA (Sigma). Following 24 hours of co-culture, 2 x 10^6 MOLT-4 CCR5 cells were added to each well along with a ~50% media change. Cultures were then incubated for 14 days, with partial media changes every 3-4 days. On day 14, we quantified p24 in supernatant by ELISA (Perkin Elmer), following the manufacturer's instructions. For each treatment condition, values for cells/well, number of positive wells, and total wells plated were entered into a limiting dilution analyzer (either http://bioinf.wehi.edu.au/software/elda/ or http://silicianolab.johnshopkins.edu) to calculate the IUPM and 95% confidence intervals (see Supplemental Figure 2). Note that prior to plating QVOA assays, precise cell numbers were established for each condition by flow cytometry, gating on viable CD4$^+$ T-cells in comparison to counting beads (CountBright, Thermo Fisher).

Generation of HIV-specific T-cells

Briefly, dendritic cells were matured, pulsed with Gag, Pol, and Nef pepmixes (0.2 mg/mL) (JPT, Berlin), and then used to stimulate T-cells. After 7-10 days, T-cells underwent a second round of stimulation with peptide-mix-pulsed autologous irradiated PHA blasts. Cells then received a third and final round of stimulation with PHA blasts as

above, then allowed to rest before use in HIVE assays.

HIV infection of CD4$^+$ T-cells

CD4$^+$ T-cells were enriched as above and then activated for 48 hours at 37°C in RPMI-10 media with 1 μg/ml each of anti-CD3 (clone OKT3, BioLegend), anti-CD28 (clone CD28.2, BioLegend), and 50 U/ml IL-2 (NIH AIDS reagent program). Supernatants from positive wells of viral outgrowth assays or viral stocks were added to activated cells, which were centrifuged in plates at 1,200 x g for 60 minutes at room temperature. Cells were then washed with RPMI-10, re-suspended in 96-well plates, and then incubated at 37°C. A small portion of cells was stained with antibodies to intracellular Gag (KC57-RD1, Beckman Coulter), and analyzed by flow cytometry to check infection progression (see CD8$^+$ T-cell Biosensor/HIV killing assays below) starting at 48 hours post-spinoculation.

HIVE assay

Target cell preparation: 20 x 10^6 resting CD4$^+$ T-cells were isolated from PBMCs for each experimental condition. Resting CD4$^+$ T-cells were enriched from these PBMC by negative selection (Easysep CD4$^+$ T-cell enrichment kit supplemented with HLA-DR TAC, Stemcell Technologies) following the manufacturer's instructions. Cells were washed and re-suspended in 1 ml of 2% FBS PBS, and pre- and post-enrichment samples were stained with antibodies to CD3, CD4, CD8, HLA-DR (clone L243, BioLegend), and CD69 (clone H1.2F3, BioLegend) for flow cytometry analysis. We required >95% pure resting CD4$^+$ T-cells in the negative fraction to proceed. Bulk CD8$^+$ T-cell preparation: CD8$^+$ T-cells were isolated by negative selection from PBMCs autologous to the CD4$^+$ targets (Easysep CD8 T-cell enrichment kit, Stemcell Technologies). Clone preparation: CD8$^+$ T-cell clone specificity was confirmed by

CD107a degranulation assays, as well as by HIV killing assays the day before assay setup. Co-culture: CD8$^+$ T-cell clones were added with the LRAs indicated in the corresponding figures. Co-cultures were performed at 2x10^6 cells/ml in XVIVO-15 serum free medium (Lonza) supplemented with penicillin-streptomycin, L-glutamine, 0.1 nM IL-7, 1 µM tenofovir, 1 µM nevirapine, 1 µM emtricitabine, 10 µM T20, 10 U/ml DNAse I (ProSpec) (XVIVO-10+7+ARV). LRA concentrations are given below. For LRAs that have been previously associated with impairing CD8$^+$ T-cell function (e.g. bryostatin, PMA/I, HDAC inhibitors), CD4$^+$ T-cells were cultured with LRAs for 2 hours, then washed 3 times to prevent LRA carryover. CD4$^+$ T-cells were then transferred to XVIVO-10+7+ARV media and co-cultured with CD8$^+$ T-cell effectors. Harvest: Co-cultures were harvested at 4 days post-initiation of co-culture. For cell-free viral RNA, supernatants were ultracentrifuged at 143,821x g for 1 hour and discarded, then pelleted material was resuspended in 200 µl of PBS; viral RNA was extracted using the Qiagen viral RNA mini kit, and quantified by qRT-PCR. CD4$^+$ T-cell from each condition were pelleted at 1,200x g, then re-suspended and isolated twice by negative selection, as above. Cells were then pelleted and re-suspended in RPMI supplemented with 10% FBS, penicillin/streptomycin, and 50 U/mL IL-2 (R10-50). Aliquots of pre- and post- CD4 enrichment were stained with the antibodies (CD3, CD4, CD8, CD45RA (clone HI100, BD), CD27 (clone O323, BioLegend), CCR7 (clone G043H7, BioLegend)) to check purity and memory phenotype, and CD3, CD4, CD8, CD69, HLA-DR, and amine aqua viability dye with counting beads to check activation phenotypes and obtain an accurate cell count. CD4$^+$ T-cells were then fixed in 2% paraformaldehyde, and analyzed by flow cytometry on an LSR-II instrument and with FlowJo software. Quantifying remaining reservoir: 2 x 10^6 cells/condition were centrifuged and DNA, for ddPCR, was extracted from cell pellets using the Gentra Puregene kit (Qiagen), following the manufacturer's instructions. The remaining cells, to be used for QVOAs, were incubated overnight in

R10-50 + 4 nM IL-15SA (ALT-803) at a concentration of 2 x 10^6 cells/ml. These cells were then plated out in quantitative viral outgrowth assays.

Digital droplet polymerase chain reaction

Genomic DNA was extracted using the Gentra Puregene kit (Qiagen) following the manufacturer's instructions. For each PCR reaction, 5 units of restriction enzyme BsaJI (NEB) was directly mixed with 300ng of DNA, ddPCR Supermix for Probes (Bio-Rad), and final concentrations of 900nM primers and 250nM probe. Primers/Probes were: RPP30 – fprimer GATTTGGACCTGCGAGCG, rprimer GCGGCTGTCTCCACAAGT, probe VIC-CTGAACTGAAGGCTCT-MGBNFQ; HIV-gag – fprimer TACTGACGCTCTCGCACC, rprimer TCTCGACGCAGGACTCG, probe FAM-CTCTCTCCTTCTAGCCTC-MGBNFQ. Droplets were prepared using the QX100 Droplet Generator (Bio-Rad) following the manufacturer's instructions. Sealed plates were cycled using the following program: 95°C for 10 min; 40 cycles of 94°C for 30 s, 60°C for 1 min; and 98°C for 10 min, with 2°C/sec ramping speeds. Reactions were analyzed using the QX100 Droplet Reader.

Real-time RT-PCR

Reactions were performed with AgPath-ID one-step RT-PCR mastermix (Life Technologies) following the manufacturer's instructions in a final volume of 20 µL per reaction, using 400 nM primers (forward primer: 5'-TTTGGAAAGGACCAGCAAA-3'; reverse primer: 5'-CCTGCCATCTGTTTTCCA-3') and 200 nM dual labeled probe (probe: 5'-6FAM-AAAGGTGAAGGGGCAGTAGTAATACA-BHQ1-3') targeting a 127 base pair region of HIV integrase. This primer and probe was developed for the integrase single-copy assay (iSCA) (Cillo *et al*, 2014). Absolute quantifications were established by comparison to a standard curve of in vitro transcribed HIV RNA. These

standards were calibrated in comparison to iSCA assay standards provided by the laboratory of John Mellors.

Supplemental Text: Characteristics of participants for bulk *ex vivo* CD8$^+$ T-cell HIVE Assays

Characteristics of participants for bulk *ex vivo* CD8$^+$ T-cell HIVE Assays

<u>OM5334:</u>

OM5334 had a documented HIV negative test 5 months prior to diagnosis, and presented with a prolonged flu like illness and a baseline HIV viral load of 911,164, suggesting acute HIV infection. ART was initiated within weeks of diagnosis. HIV-specific T-cell responses in *ex vivo* PBMCs were measured by IFN-γ ELISPOT using pools of 15mer peptides overlapping by 11 and found to be: Gag – 125 spot forming units (SFU)/10^6 PBMCs, Env – 70 SFU/10^6 PBMCs, Pol - 218 SFU/10^6 PBMCs.

<u>OM5011:</u>

Participant 'OM5011 initiated therapy during chronic infection, and had *ex vivo* T-cell responses to Gag- (965 spot forming units (SFU)/10^6 PBMCs), Nef (280 SFU/10^6 PBMCs), and Vpr (100 SFU/10^6 PBMCs) as measured by IFN-γ ELISPOT using pools of overlapping 15mer peptides. Using a pool of 270 previously defined HIV optimal epitopes we also observed responses to HIV-Gag-ACQGVGGPGHK (AK11) – 680 SFU/10^6 PBMCs, HIV-Gag HPVHAGPIA (HA9) – 1,120 SFU/10^6 PBMCs, HIV-Gag-KRWIILGLNK (KK10) – 320 SFU/10^6 PBMCs.