SUPPLEMENTARY ANALYSES

Virologic outcomes

The median (range) VL levels at first detection was 122 (26-7784) copies/ml and the maximum level prior to ART resumption was 5,211 (2,005-13,462) copies/ml within a median (range) of 4 (1-21) days (**Fig. 1**). Unlike other studies, pre-ART VL, CD4+ T, CD4+ T/CD8, duration of infection and duration of ART were not associated with time to VL rebound[1-3].

HIV genotyping

Pre-ART genotypes showed only two mutations in two different participants. One participant (6579) had the M41L thymidine analog mutation (TAM), conferring low-level resistance to the nucleoside reverse transcriptase inhibitor (NRTI) drugs stavudine and zidovudine. This same mutation was also found in the post-ATI sample at time of ART resumption. Another participant (4320) had the I47T mutation in the protease gene, which does not confer resistance to the protease inhibitor drugs but is considered an accessory mutation. No new major or minor resistance mutations were found after ATI in any participant.

HIV sequence analysis

To characterize rebound viruses, we compared HIV-1 sequences amplified at a median of 6 days (range: 1-15) after VL rebound > 20 copies/ml to those obtained a median of 3 days (range: 1-5) after acute HIV infection diagnosis. We obtained 15 HIV-1 sequences at HIV-1 diagnosis from all eight participants and following HIV-1 rebound (prior to ART resumption), *pro-rt* sequences from seven participants (n = 15) and *env* sequences from three participants (n = 9, 13 or 15 sequences per participant). HIV-1 amplification following VL rebound may have been hampered by the lower VLs (median = 3,204 copies/mL, range: 196-18,953) than at the beginning of infection (median = 16,316 copies/mL, range: 2,063-85,265). Sequences from acute HIV infection (n = 15) were used to infer a consensus sequence referred to as the founder sequence.

The analysis of *pro-rt* sequences showed that most sequences (71%) following HIV-1 rebound were identical to the founder sequence: a median of 11 out of 15 (range: 9-13) sequences were identical and 91% of sequences (a median of 14 out of 15; range: 12-15) had at most 1 mutation against the founder sequence. Across all participants, mutations were found as singletons unique to a given sequence except for one G-to-A transition that was shared across 3 sequences in one participant (P7010) following HIV- 1 rebound. The lack of notable

diversification seen in RV411 HIV-1 sequences post- rebound was consistent with a period of HIV replication of about a month, corresponding to the time between treatment interruption and viral rebound (**Supplementary Fig. 5a to 5c**). There was no evidence of drug resistance mutations upon HIV-1 rebound. Finally, we compared the number of mutations away from the founder sequence observed in sampled sequences for each participant to expected numbers calculated based on known substitution rates in an independent cohort of Thai individuals infected with HIV-1 CRF01_AE (sequence data sampled at multiple time points during natural infection in twelve individuals). The number of mutations that we observed across sequences in seven participants (median range: 0-2) matched estimates expected during the duration of treatment interruption and prior to ART resumption (median range: 0.09-1.63) and was considerably lower than numbers expected under the assumption that replication would have proceeded since HIV-1 diagnosis (median range: 3.39-58.89). The analysis of *env* sequences, which were only available from three participants post-rebound, revealed similar patterns.

HIV DNA in mucosal mononuclear cells (MMCs) from sigmoid tissue

Pre-ATI sigmoid colon biopsy was performed in three participants, and total HIV DNA was 2.5, <2 and <4 copies/10^6 MMCs in participants 4878, 6579 and 4320, respectively. Integrated HIV DNA and 2LTR circles were <8 copies/10^6 MMCs in all three. At ART resumption post-rebound, total/integrated HIV DNA levels remained low at 3/3 (4878), <6/<7 (6579) and <22/< 20 (4320) copies/10^6 MMCs. Additionally 6054 had pre- ART sigmoid colon biopsy that showed total/integrated HIV DNA to be <51/<29 copies/10^6 MMCs.

HIV DNA and Ki67+CD45RA-CD8+ T cells from lymph node mononuclear cells (LNMCs)

Total/integrated HIV DNA levels in total LNMCs from participant 4878 were below the detection limit at pre–ATI (<12/<12 copies/10^6 LNMCs, respectively). Isolation of CD4+ T cells from this pre-ATI lymph node resulted in low but positive measures both by the integrated HIV DNA assay and TILDA (1.45 copies/10^6 CD4+ T cells and 1.4 cells/10^6 CD4+ T cells, respectively). At ART resumption, the total HIV DNA level was 75.8 copies/10^6 LNMCs while the integrated HIV DNA was below 88 copies/10^6 LNMCs).

Ki67+CD45RA-CD8+ T cells at pre-ATI were detected at low frequencies in both peripheral blood mononuclear cells (PBMCs) (5.1%) and LN
MCs (7.2%). At ART resumption, there was substantial expansion of these cells in the PBMCs (frequency 9.4%) but not in LNMCs (frequency 8.1%) (**Supplementary Fig. 9a and 9b**).

SUPPLEMENTARY ANALYSES REFERENCES

1 Steingrover, R. *et al.* HIV-1 viral rebound dynamics after a single treatment interruption depends on time of initiation of highly active antiretroviral therapy. *AIDS (London, England)* **22**, 1583-1588, doi:10.1097/QAD.0b013e328305bd77 (2008).

2 Fidler, S. *et al.* Virological Blips and Predictors of Post Treatment Viral Control After Stopping ART Started in Primary HIV Infection. *Journal of acquired immune deficiency syndromes (1999)* **74**, 126-133, doi:10.1097/qai.0000000000001220 (2017).

3 Treasure, G. C. *et al.* Brief Report: Relationship Among Viral Load Outcomes in HIV Treatment Interruption Trials. *Journal of acquired immune deficiency syndromes (1999)* **72**, 310-313, doi:10.1097/qai.0000000000000964 (2016).

SUPPLEMENTARY TABLES

Supplementary Table 1 Baseline characteristics prior to analytical treatment interruption of eight Fiebig I treated participants.

ID	Time to VL rebound (days)[1]	Age (years)	Sex	HIV Risk	HIV Clade	Initial ART regimen[2]	ART regimen prior to enrollment[3]	Duration of ART (years)	Pre-ART				Pre-ATI			
									HIV RNA (log_{10}c/ml)	Total/ Integrated HIV DNA (c/10^6CD4)	CD4 (cells/ mm^3)	CD4 /CD8	HIV RNA (log_{10}c/ml)	Total/ Integrated HIV DNA (c/10^6CD4)	CD4 (cells/ mm^3)	CD4 /CD8
4617	40	34	Female	Heterosexual	CRF01_AE	TDF/FTC/EFV	TDF/3TC/EFV	5.5	4.4	51.7/1.2	454	1.1		1.4/0	570	1.1
7010	13	29	Male	MSM	CRF01_AE	TDF/FTC/EFV/MVC/RAL	TDF/3TC/EFV	4.5	4.1	483.2/38.5	565	0.6	<1.3	11.1/0	538	0.5
4878	21	26	Male	MSM	CRF01_AE/B	TDF/3TC/EFV	TDF/3TC/EFV	2.7	4.9	414.2/31.1	261	0.6	<1.3	3.7/0.7	671	0.9
6054	15	29	Male	MSM	CRF01_AE	TDF/3TC/EFV/MVC/RAL	TDF/3TC/EFV	2.8	4.2	81.1/2.2	481	0.7	<1.3	1.5/0.6	584	0.5
6579	23	22	Male	MSM	CRF01_AE	TDF/3TC/EFV/MVC/RAL	TDF/3TC/EFV	2.6	4.1	43.7/0.7	332	0.7	<1.3	0.9/0	601	1.4
6345	34	24	Male	MSM	CRF01_AE	TDF/3TC/EFV/MVC/RAL	TDF/3TC/EFV	2.5	4.3	0/0	371	1.6	<1.3	0/0	472	1.2
4320	48	33	Male	Heterosexual	CRF01_AE/B	TDF/3TC/EFV/MVC/RAL	TDF/FTC/EFV	2.6	4.6	147.8/12.3	227	0.8	<1.3	2.8/0	391	1.3
8534	28	31	Male	MSM	CRF01_AE	TDF/FTC/EFV	AZT/3TC/EFV	4.7	3.3	0/0	534	1.1	<1.3	0.1/0	703	1.4
Median	**26**	**29**						**2.8**	**4.3**	**66/2**	**413**	**0.8**		**1/0**	**577**	**1.2**
Min	**13**	**22**						**2.5**	**3.3**	**0/0**	**227**	**0.6**		**0/0**	**391**	**0.5**
Max	**48**	**34**						**5.5**	**4.9**	**483/39**	**565**	**1.6**		**11/0.7**	**703**	**1.4**

VL: viral load, MSM: men who have sex with men, ART: antiretroviral therapy, TDF: tenofovir, FTC: emtricitabine, 3TC: lamivudine, EFV: efavirenz, MVC: maraviroc, RAL: raltegravir.

[1]VL rebound is defined as VL > 20 copies/ml after analytical treatment interruption (ATI)

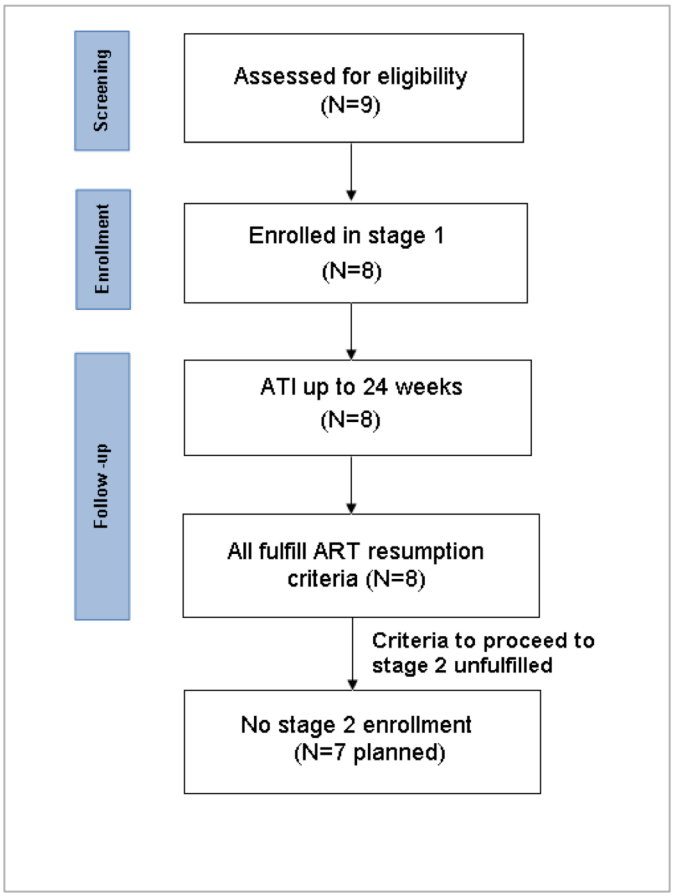
[2]MVC and RAL were given during the first 24 weeks of treatment in some participants.

[3]Efavirenz was replaced with darunavir/ritonavir 4 weeks prior to ATI in all participants to limit the risk for development of genotypic resistance to the non- nucleoside reverse transcriptase inhibitor drug class.

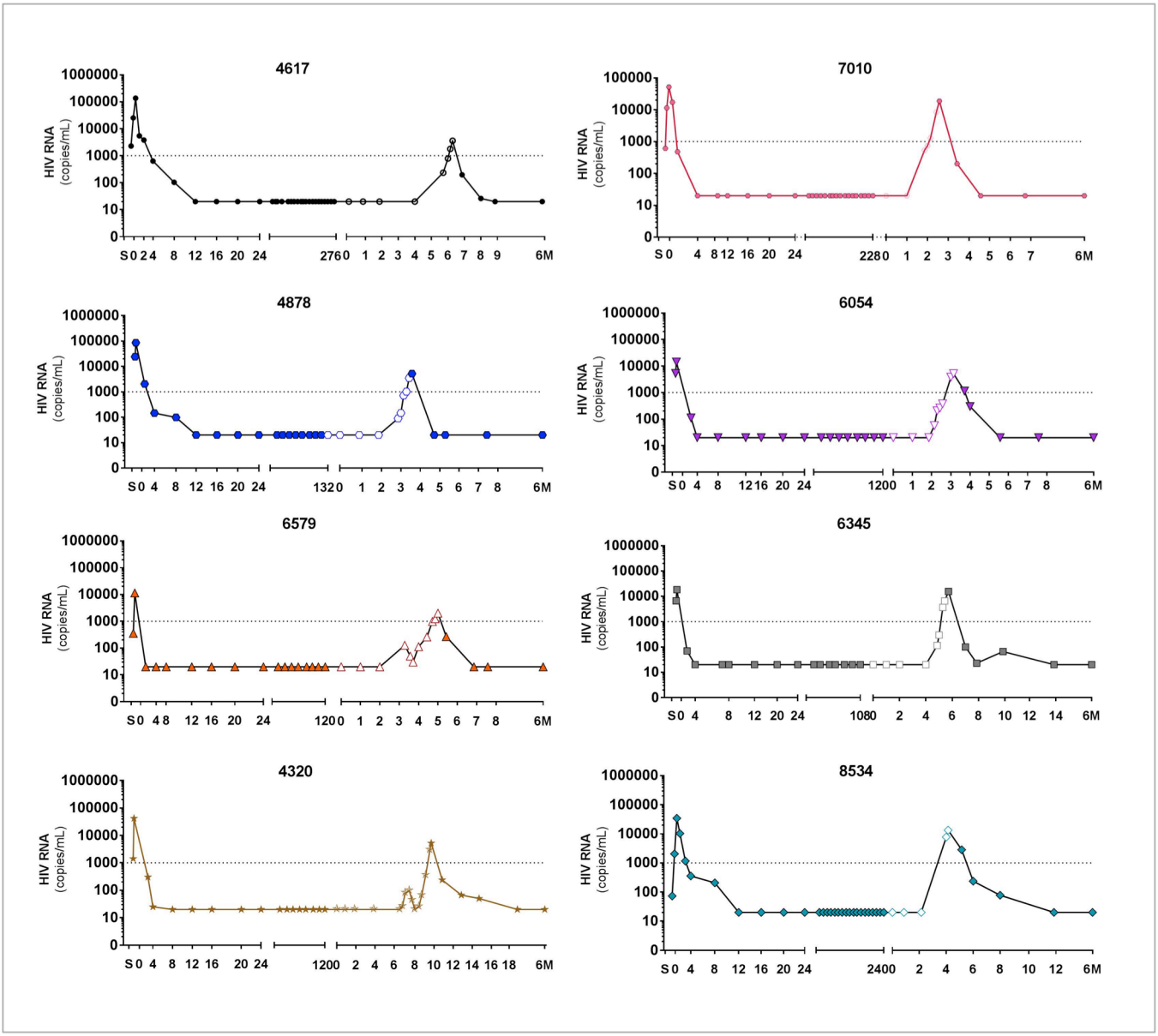
Supplementary Table 2 Genotypic drug resistance mutations before antiretroviral therapy and after analytical treatment interruption.

ID	RT NRTI		RT NNRTI		PR	
	Pre-ART	Post-ATI	Pre-ART	Post-ATI	Pre-ART	Post-ATI
4617	None	None	None	None	None	None
7010	None	None	None	None	None	None
4878	None	None	None	None	None	None
6054	None	None	None	None	None	None
6579	M41L	M41L	None	None	None	None
6345	None	None	None	None	None	None
4320	None	None	None	None	I47T	None
8534	None	None	None	None	None	None

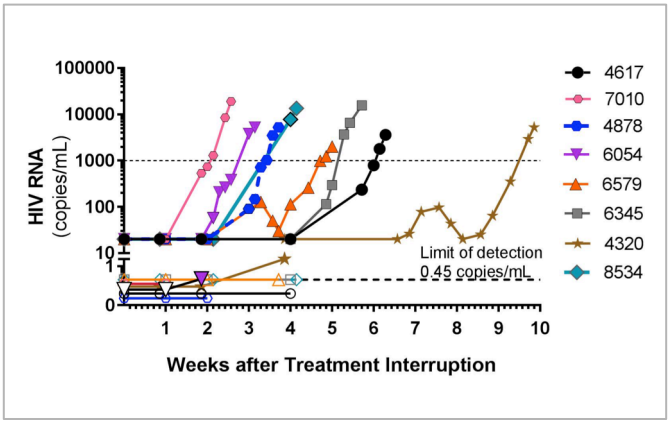
SUPPLEMENTARY FIGURES



Supplementary Figure 1 ATI: analytical treatment interruption, ART: antiretroviral therapy ART resumption criteria included confirmed VL > 1,000 copies/ml, VL rise of ≥ 0.5 $\log_{10}$copies/ml per day provided that the last VL was > 1000 copies/ml, a single VL > 10,000 copies/ml, confirmed CD4+ T cells < 350 cells/mm^3, CD4+ T cell count decline > 50% from baseline ATI, acute retroviral syndrome, clinical progression to CDC category B or C disease, pregnancy or request by participants. Criterion proceed to stage 2 was that at least 1 of 8 participants in stage 1 achieved viral load < 50 copies/ml at week 12 post-ATI.

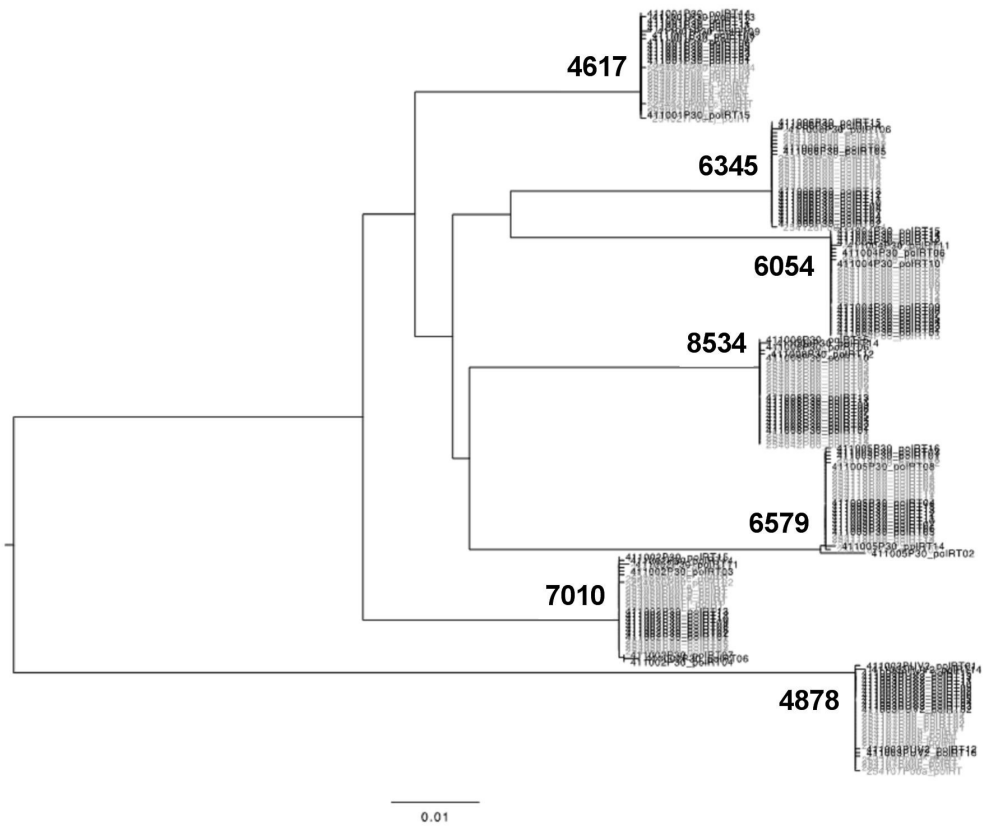


Supplementary Figure 2 Plasma VL on individual participants from acute HIV infection to ATI and resumption of ART. On the X axis, “s” represents the screening of acute infection into the RV254 study that is followed by week 0 (start of ART) and weeks on ART. The interruption in the X axis is followed by week 0 that represents the start of ATI in the RV411 study. Following confirmed VL above 1000 copies/ml (corresponding to the highest observed VL level in the graph), ART is resumed, and the levels up to 6 months post ART resumption are shown. The close symbol represents the period “on ART” and the open symbol represents the period “off ART”.



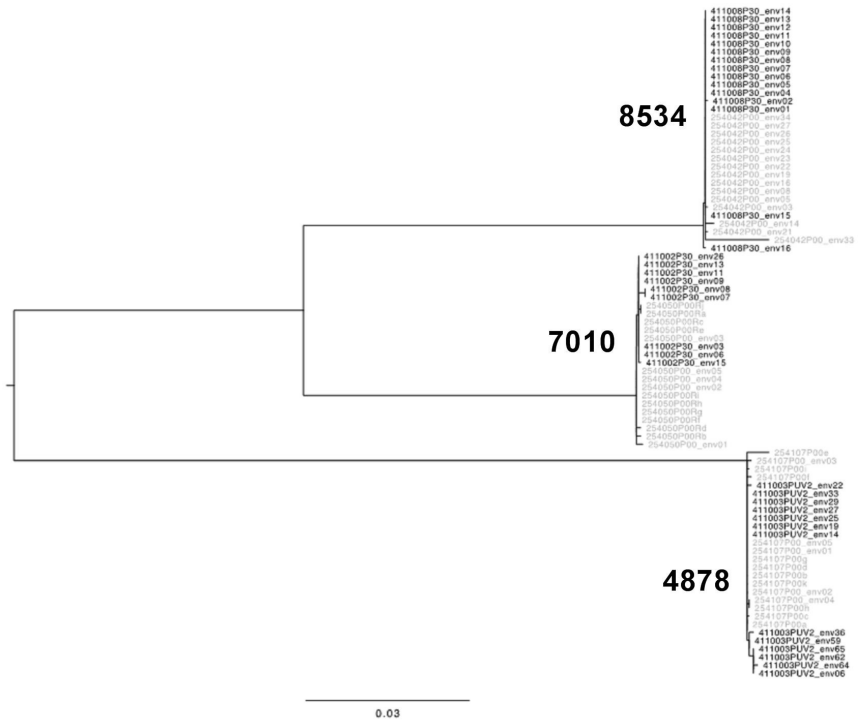
Supplementary Figure 3 HIV VL following ATI. The lower panel displays results of the single copy VL assay (SCA) performed on samples with VL < 20 copies/ml by the standard method. All had undetectable SCA at baseline ATI. Two participants had detectable SCA during ATI: 6054 (0.47 copies/ml) and 4320 (1.5 copies/ml) at 2 and 21 days prior to detection by the standard assay.

a



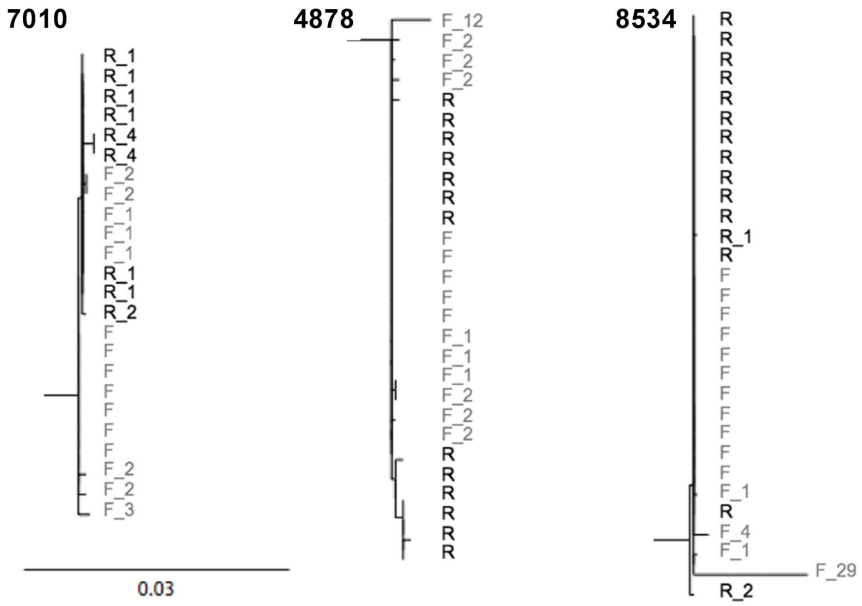
Supplementary Figure 4 HIV-1 phylogenetic tree at acute HIV infection (and following HIV viral rebound. (**a**) Phylogenetic tree based on 210 *pro-rt* sequences derived in acute infection (in gray) and following viral rebound (in black). All participants were infected with CRF01_AE viruses except for participant 4878 who was infected by HIV-1 subtype CRF01_AE/B recombinant.

b

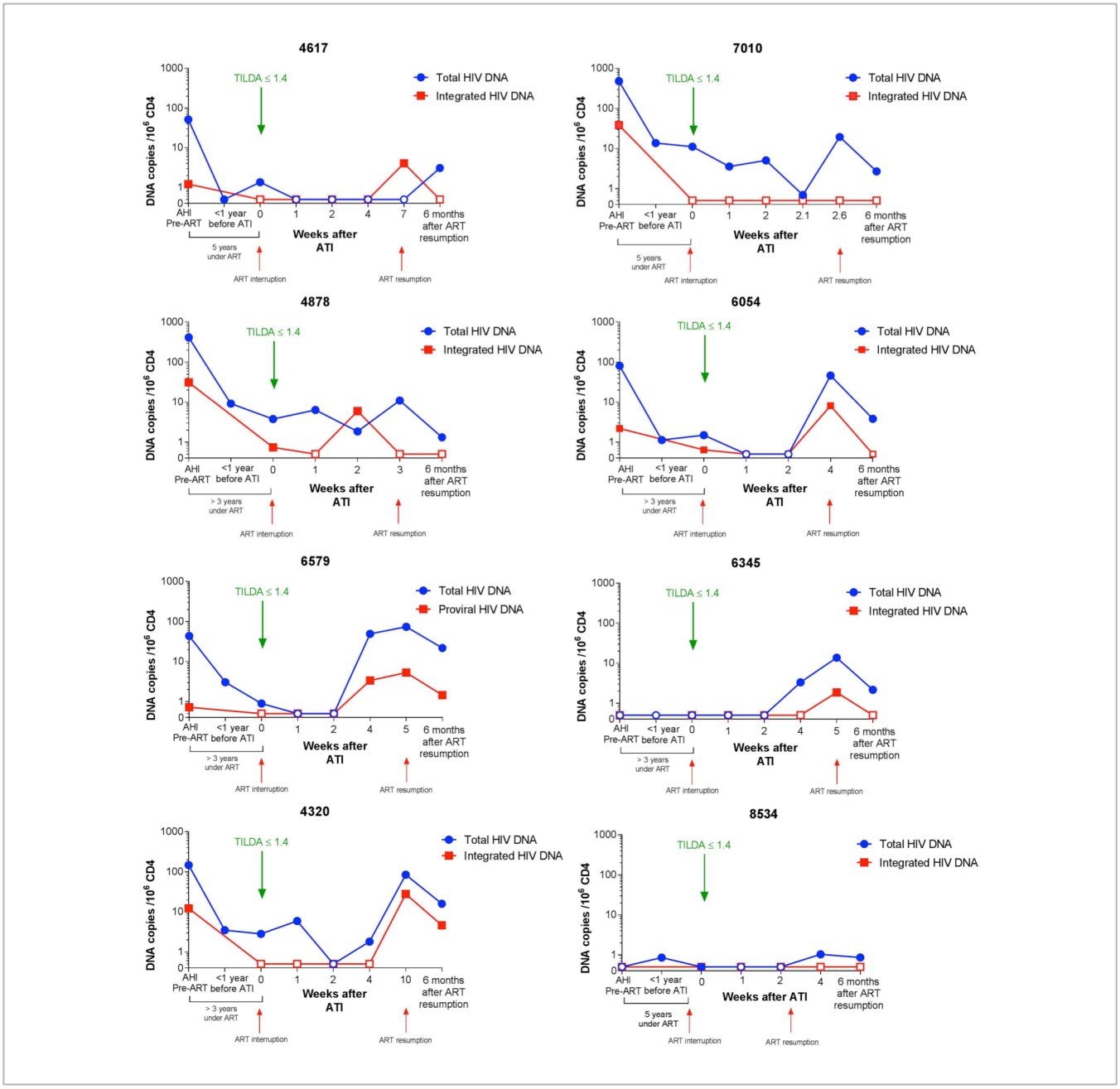


(**b**) Phylogenetic tree based on 82 *env-gp160* sequences derived in AHI (in gray) and following viral rebound (in black).

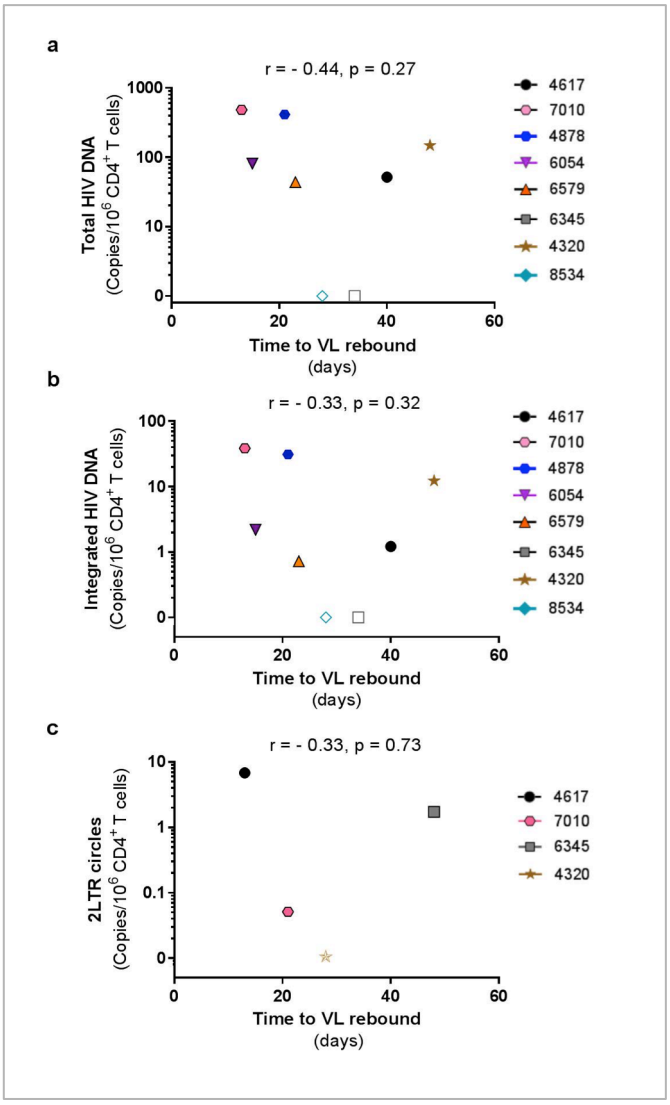
C



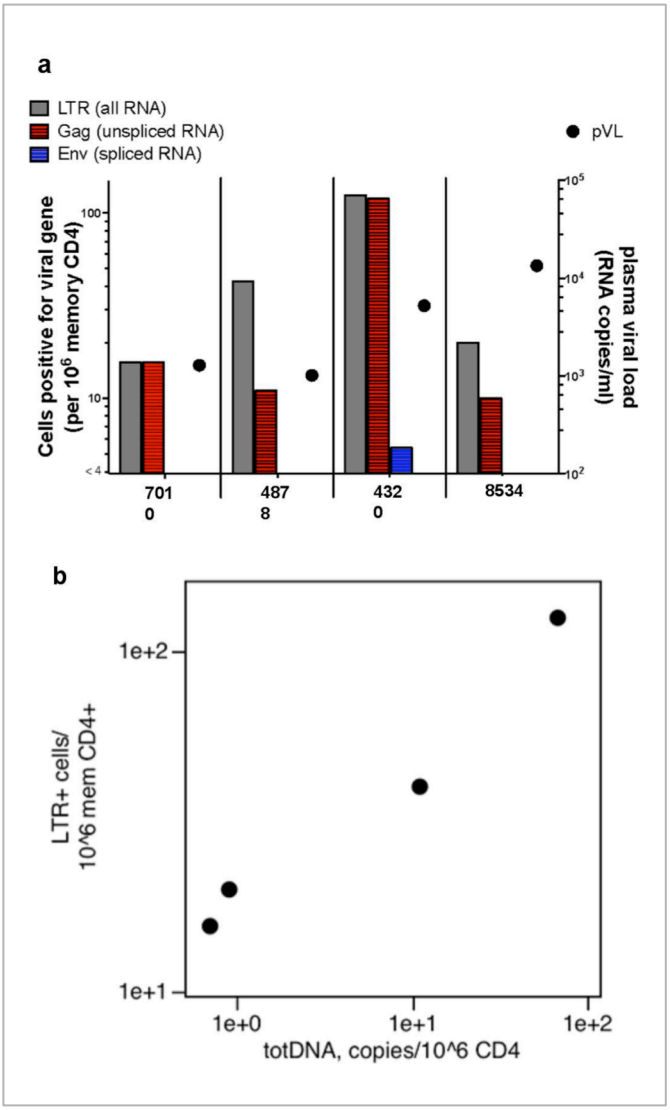
(**c**) HIV-1 phylogenetic trees from 3 participants. Maximum likelihood trees based on *env-gp160* sequences represent sequences amplified at rebound (R) in black and those sampled in AHI (F) in gray. When sequences differed from the founder sequence (consensus of all sequences at HIV-1 diagnosis), the number of mutations away from the founder sequence is figured as a suffix at the tips of each individual cluster.



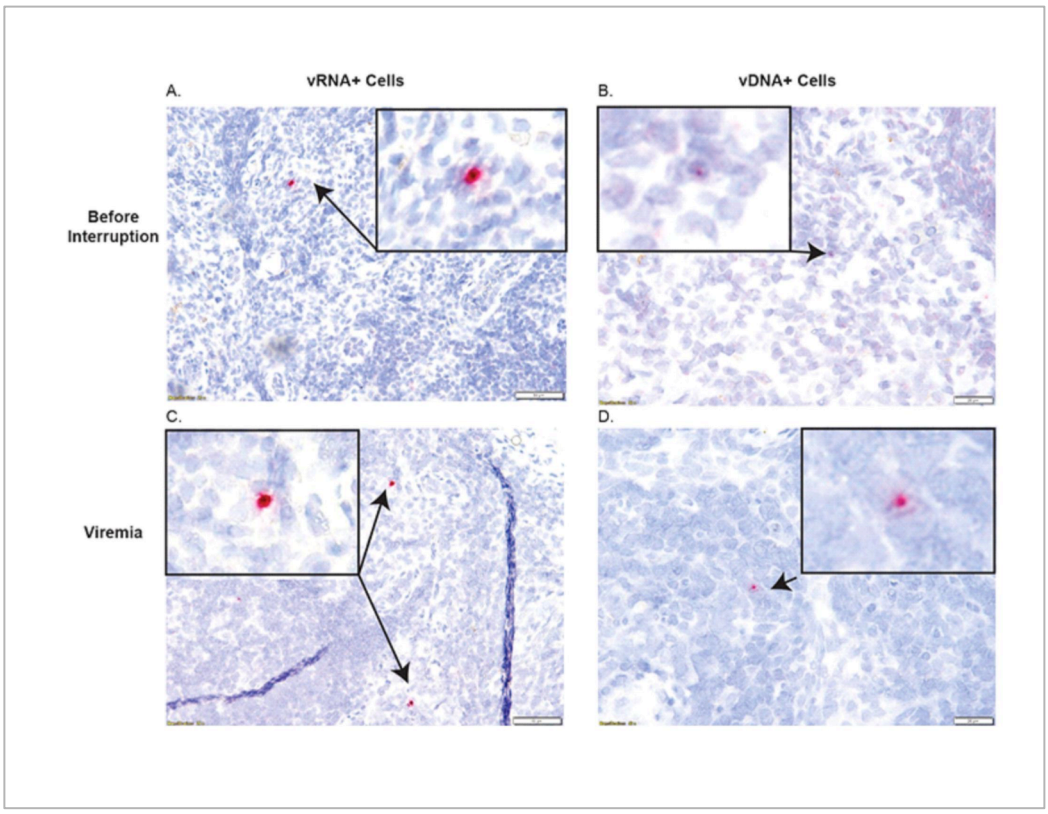
Supplementary Figure 5 TILDA: Tat/rev Induced Limiting Dilution Assay. Frequencies of peripheral blood CD4+ T cells harboring total (blue) and integrated HIV DNA (red) from acute HIV infection to ATI and 6 months after resumption of ART. Open symbols depict levels below the limit of detection of the assay and were plotted at 0 copy/10^6CD4+ T cells.



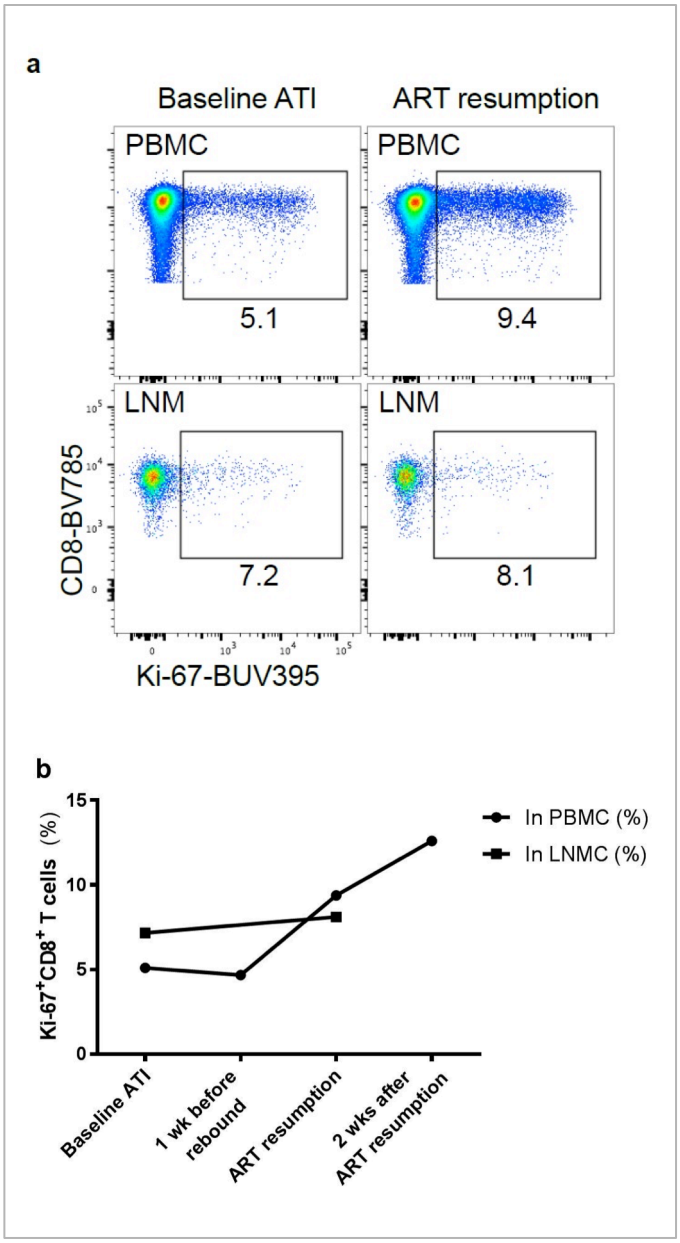
Supplementary Figure 6 HIV reservoir markers before ART did not predict time to viral load rebound >20 copies/ml post-ATI. Open symbols depict levels below the limit of detection of the assay and were plotted at 0 copy/10^6CD4+ T cells. (**a**) (Pre-ART total HIV DNA. (**b**) Pre-ART integrated HIV DNA. (**c**) Pre-ART 2LTR circles.



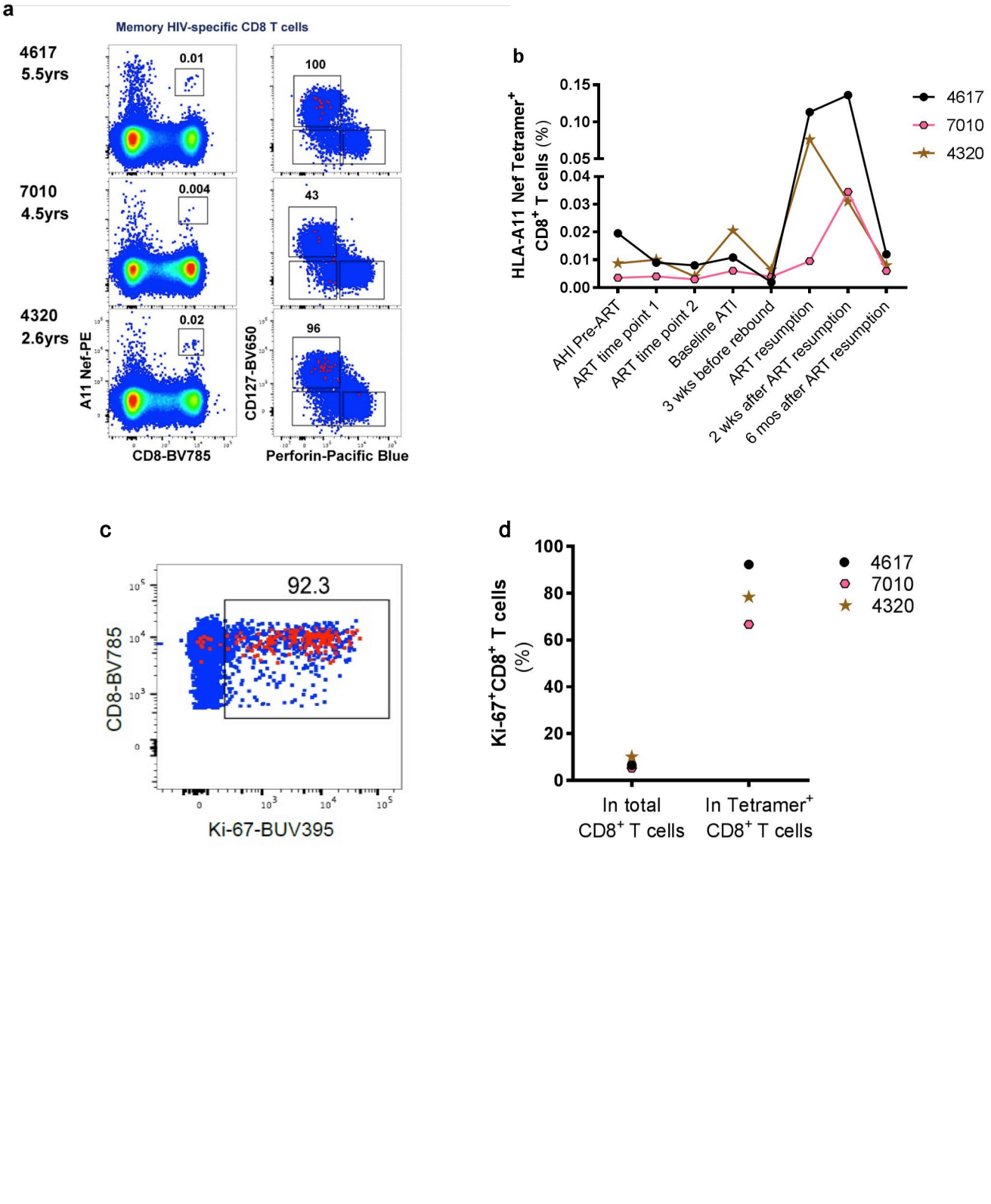
Supplementary Figure 7 Frequencies of HIV-infected memory CD4+ T cells at ART resumption following treatment interruption. (**a**) Infected cells, as defined by cell-associated *LTR* or *gag*, were present in PBMC of all four individuals at a frequency of 0.001-0.01% of memory CD4+ T cells. Productively infected cells, as defined by expression of spliced HIV-1 RNA, were detected in one participant (4320, 10 weeks post treatment interruption) at ~0.0005% of memory CD4+ T cells. The frequency of productively infected cells in the other three participants was less than the assay's detection threshold, as determined by the cellular input (0.0004%). (**b**) The frequencies of RNA+ memory CD4+ T cells (%LTR+) at ART resumption were associated with frequencies of CD4+ T cells harboring total HIV DNA at the same time-point.



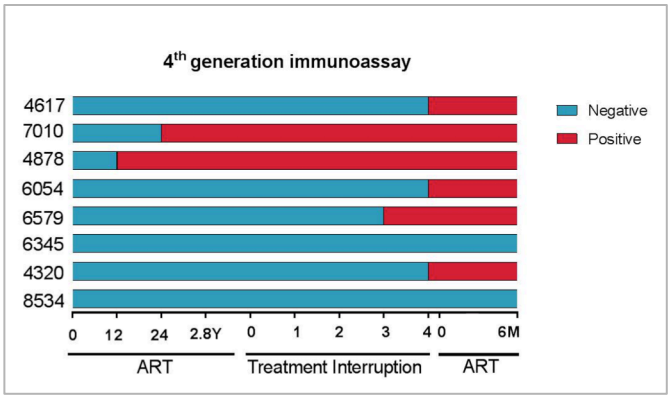
Supplementary Figure 8 In situ hybridization of HIV RNA+ and HIV DNA+ cells in the inguinal lymph node tissue from participant 4878.



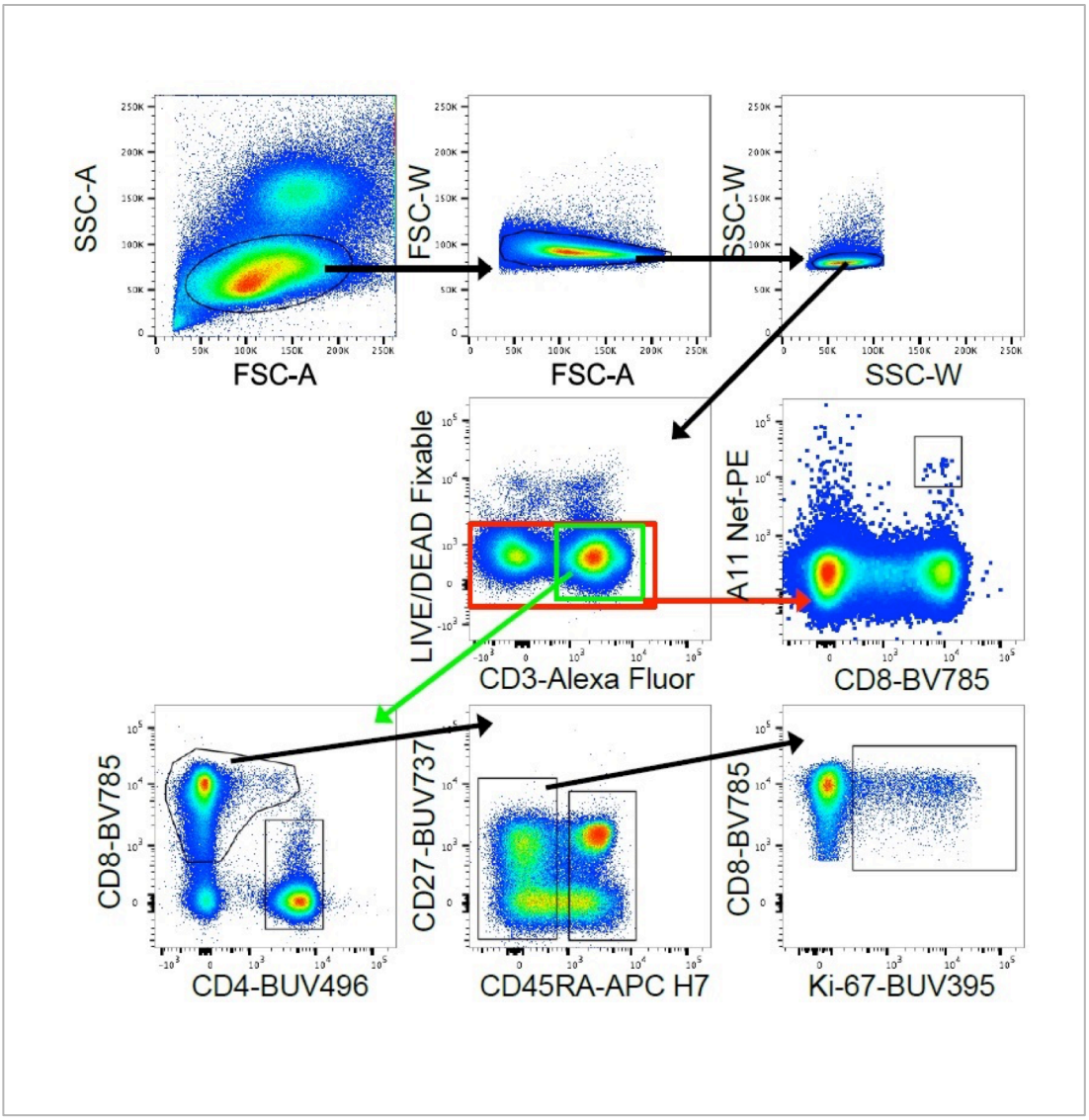
Supplementary Figure 9 Ki-67 expression in CD45RA-CD8+ T cells from peripheral blood mononuclear cells (PBMCs) and lymph node mononuclear cells (LNMCs) in participant 4878 at baseline analytical treatment interruption (ATI) and virus rebound (ART resumption). (**a**) The percentages of Ki-67+ cells in CD45RA-CD8+ T cells are shown. (**b**) Longitudinal analysis of Ki-67+CD8+ T cells in PBMCs and LNMCs after ATI.


a
Memory HIV-specific CD8 T cells
4617
5.5yrs
7010
4.5yrs
4320
2.6yrs
0.01
100
0.004
43
0.02
96
A11 Nef-PE
CD8-BV785
CD127-BV650
Perforin-Pacific Blue
b
HLA-A11 Nef Tetramer+ CD8+ T cells (%)
0.15
0.10
0.05
0.04
0.03
0.02
0.01
0.00
4617
7010
4320
AHI Pre-ART
ART time point 1
ART time point 2
Baseline ATI
3 wks before rebound
ART resumption
2 wks after ART resumption
6 mos after ART resumption
c
92.3
CD8-BV785
Ki-67-BUV395
d
Ki-67+CD8+ T cells (%)
100
80
60
40
20
0
4617
7010
4320
In total CD8+ T cells
In Tetramer+ CD8+ T cells

Supplementary Figure 10 Memory HIV-specific CD8+T cells persisted under early ART and rapidly expanded after viral detection (at time of ART resumption) of three participants with HLA-A11. (**a**) Tetramer staining was performed on pre-ATI samples after 5.5, 4.5 and 2.6 years under ART. Percentage of HLA-A*1101 NEF tetramer+ CD8+ T cells in total CD8+ T cells and percentage of perforin-CD127+ cells within tetramer+ cells are shown. (**b**) Percentage of HLA-A*1101 Nef tetramer+ CD8+ T cells in total CD8+ T cells. The ART time points 1 and 2 after ART initiation are 144 and 267 weeks for 4617, 144 weeks and 228 weeks for 7010, and 60 weeks and 120 weeks for 4320, respectively. (**c**) Ki-67 expression in HIV-specific CD8+ T cells (total and A11 Nef-tetramer) at VL rebound. Dot plot shows overlay of total CD8+ T cells (Blue) and tetramer+ cells (Red) for co-expression of CD8 and Ki-67. (**d**) The percentages of Ki-67+ cells in total CD8+ T cells and tetramer+ cells.



Supplementary Figure 11 Serostatus of individual participant from initiation of antiretroviral therapy following acute HIV infection, to treatment interruption and 6 months after ART resumption.



Supplementary Figure 12 Gating strategy for flow cytometry analysis on Ki-67+CD8+ T cells and HLA-A11 Nef tetramer+CD8+ T cells.