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Supplemental information

KLRG1 expression on natural killer cells is associated with HIV persistence, and its targeting promotes the reduction of the viral reservoir

Antonio Astorga-Gamaza, David Perea, Nerea Sanchez-Gaona, Marta Calvet-Mirabent, Ana Gallego-Cortés, Judith Grau-Expósito, Ildefonso Sanchez-Cerrillo, Joan Rey, Josep Castellví, Adrian Curran, Joaquin Burgos, Jordi Navarro, Paula Suanzes, Vicenç Falcó, Meritxell Genescà, Enrique Martín-Gayo, and Maria J. Buzon

Supplementary Figure 1

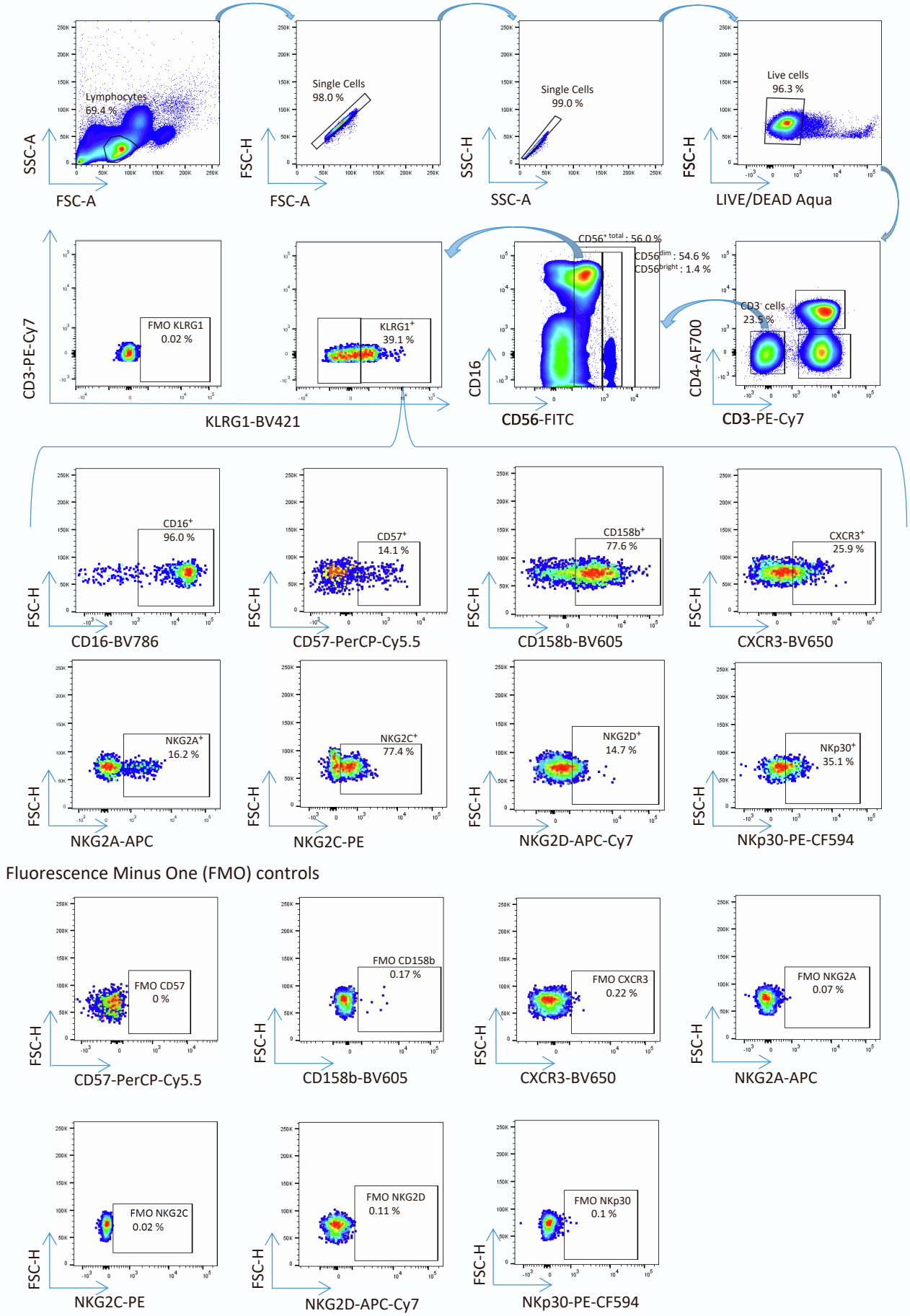

Figure S1. **Gating strategy used for the quantification of the different NK receptors**. FMOs are shown for markers with continuous expression. Related to Figures 1 and 2.

Supplementary Figure 2

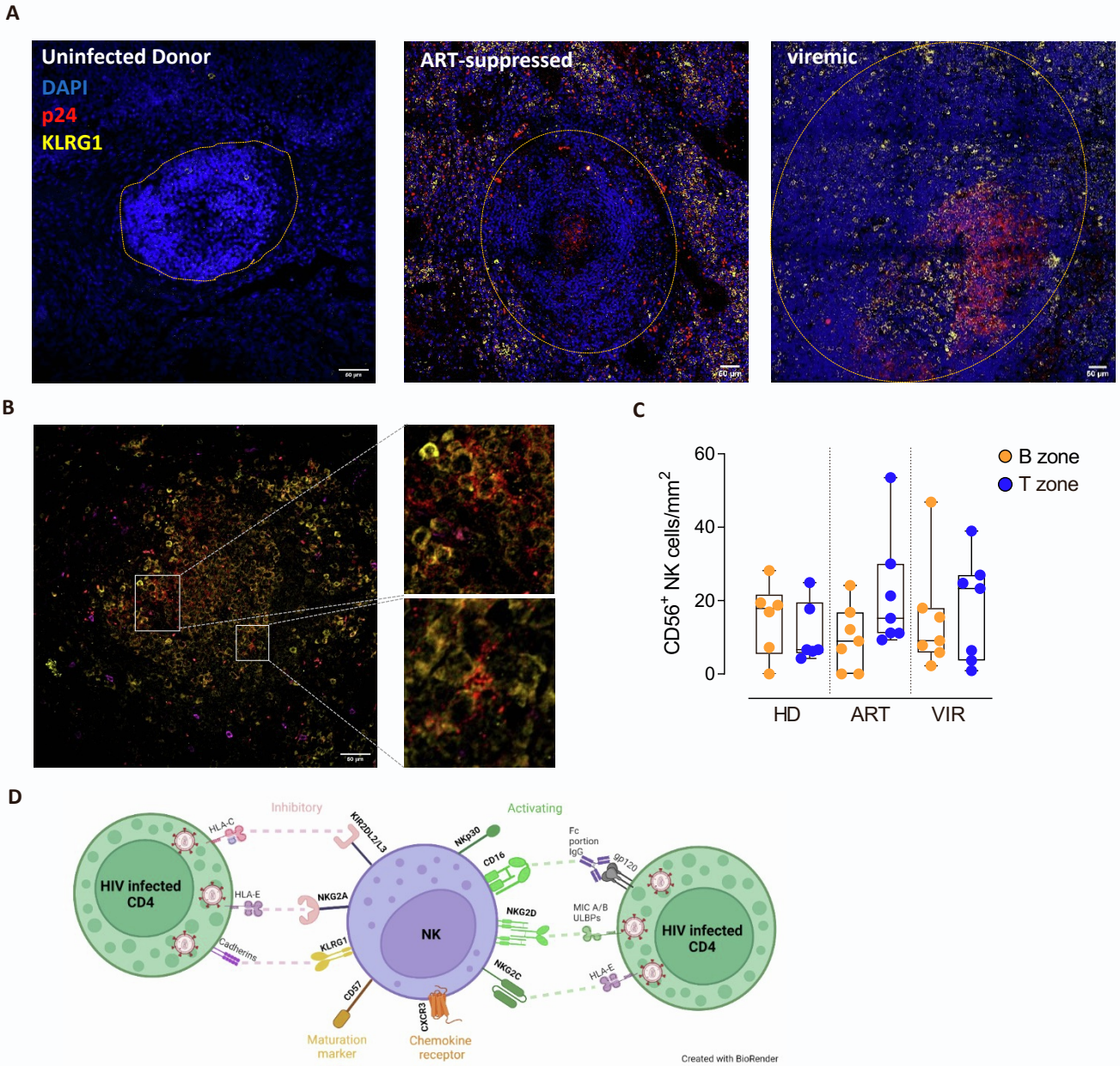

Figure S2. Visualization of KLRG1 expression and quantification of CD56$^+$ cells in lymph nodes. A) Representative images of a lymph node corresponding to an uninfected donor (HD), one ART-suppressed (#41), and one VIR (#42) PWH. P24$^+$ cells are visualized in red and KLRG1$^+$ in yellow. Cell nuclei are stained with DAPI and is shown in blue. Orange dashed lines indicate the limit between the intrafollicular (B zone) and extrafollicular (T zone) zones. B and T cell zones were identified based on the intensity of the DAPI staining and the morphology of the cell nuclei. **B)** Representative micrograph of a lymph node section from one HIV$^+$ VIR individual stained with anti-CD56 (purple), anti-KLRG1 (yellow), and anti-p24 (red) antibodies. White boxes indicate regions with different p24 pattern staining. The right panels correspond to zoomed views of network-like staining, probably corresponding to virions captured by follicular dendritic cells (upper panel), and cells with a more dense spherical signal indicating productive HIV infection (lower panel). **C)** Number of CD56$^+$ NK cells per mm^2 in lymph node samples from an HD, one ART, and one VIR PWH (each dot corresponds to one follicle). Comparisons intra-sample between values in the B or T-zones were performed using the Wilcoxon test, and comparisons inter-sample between PWH and HD were performed using the Mann–Whitney test. **D)** NK receptors included in the study. NK cells express an array of receptors with activating or inhibitory potential upon interaction with ligands found on HIV-infected cells. For this study, we have included the following receptors; the natural cytotoxicity receptor NKp30, the maturation marker CD57, the chemokine receptor CXCR3, the activating receptor CD16 which mediates ADCC, the activating receptors NKG2D and NKG2C, which are able to interact with the major histocompatibility complex (MHC) class I-related molecules MIC A/B and different UL16-binding proteins (ULBPs), or HLA-E, respectively. Moreover, we included the NK inhibitory receptor NKG2A, a C-type lectin-like inhibitory receptor that influences the NK effector responses through the interaction with HLA-E ligand, and killer-cell immunoglobulin-like receptor KIR2DL2/L3, which is able to interact with HLA-C ligands. All potential interactions are depicted in this figure. Related to Figures 1 and 2.

Supplementary Figure 3

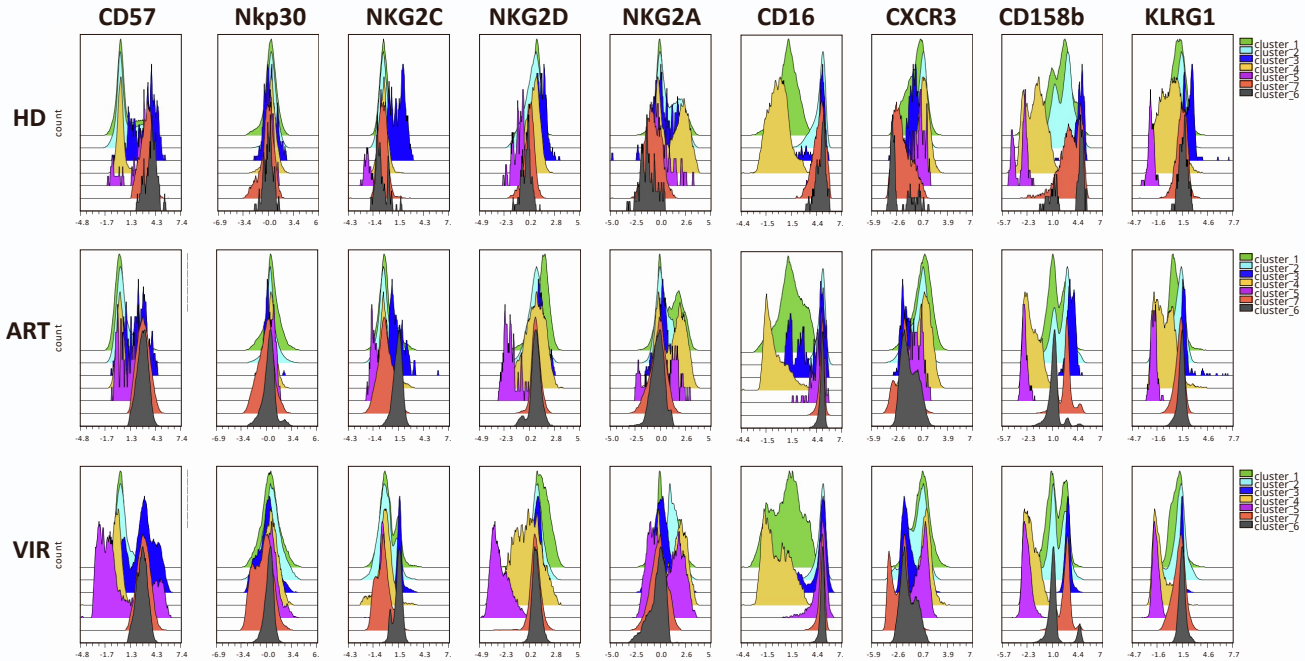

Figure S3. Expression of NK receptors in the different NK clusters. Histograms showing the intensity of expression of the different markers on the NK clusters represented in different colors. Cohorts (HD, ART, and VIR) are shown in different lines. Related to Figure 2.

Supplementary Figure 4

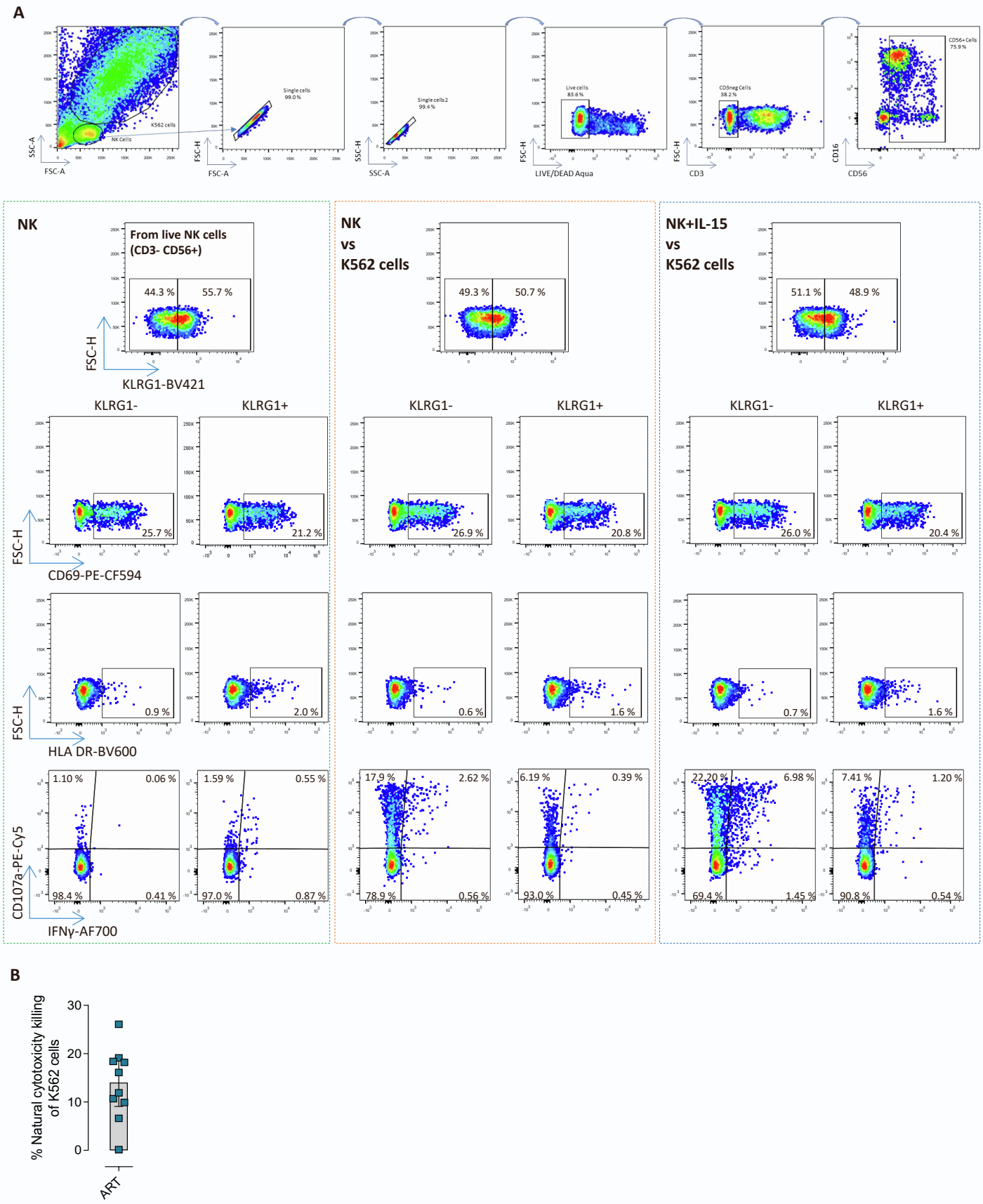

Figure S4. Gating Strategy. A) Gating strategy used for the analyses of the functional NK assays using the K562 MHC-devoid cell line, with and without the stimulation with IL-15. **B)** Natural cytotoxicity killing assay of the K562 cell line after co-culturing with NK cells isolated from different ART PWH. Related to Figure 3.

Supplementary Figure 5

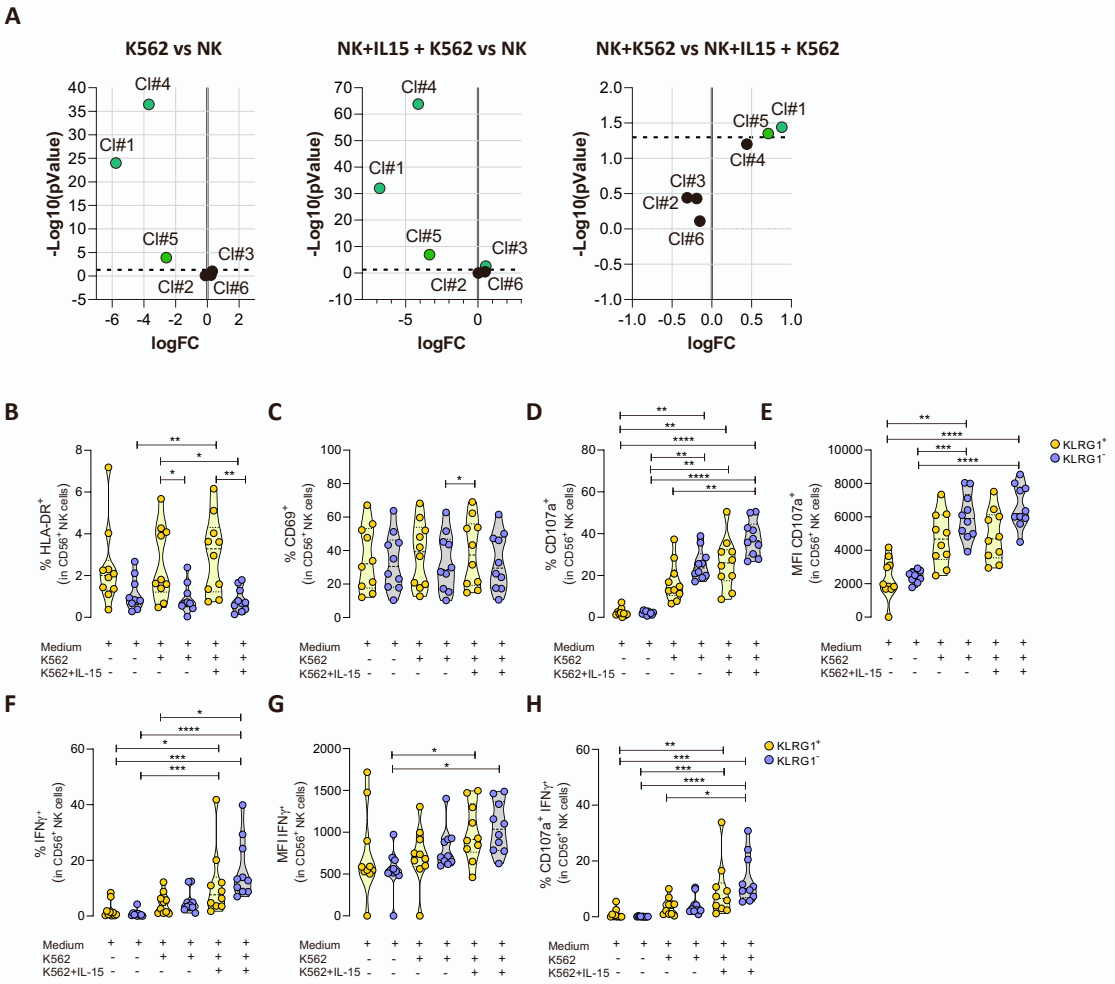

Figure S5. Functional assays. A) Volcano plots showing the statistically significant differences in the cluster composition between conditions; NK alone, NK+K562, and NK (IL15)+K562. **B)** Percentage of HLA-DR$^+$ cells in total CD56$^+$ KLRG1$^+$ (yellow) or KLRG1$^-$ (blue) NK cells in basal conditions, after co-culturing with the K562 target cells and with the additional IL-15 stimulus. Similarly, the values of other parameters are represented in **C)** frequency of CD69$^+$ cells, **D)** percentage of cells expressing the CD107a degranulation marker, **E)** Mean Fluorescence Intensity (MFI intensity) signal for CD107a expression, **F)** frequency of cells producing IFN-γ, **G)** MFI for IFN-γ, and **H)** percentage of polyfunctional, double positive CD107a$^+$IFN-γ$^+$ cells. All graphs represent the median and ranges. Statistical comparisons were performed using the Friedman test or Kruskall-Wallis, when required. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Related to Figure 3.

Supplementary Figure 6

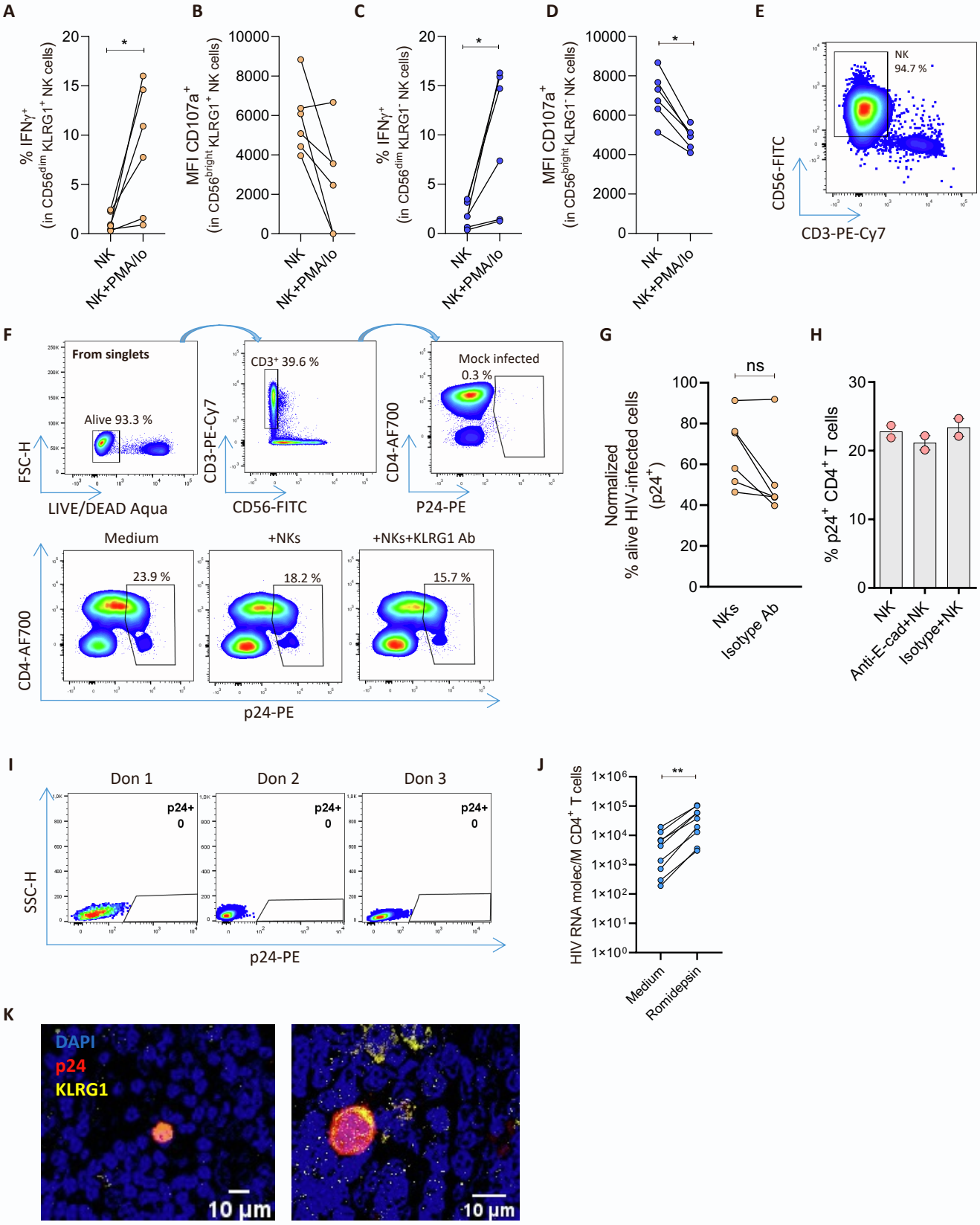

Figure S6. Expression of functional molecules after NK stimulation and NK killing assays. NK cells were cultured with *ex vivo* infected CD4$^+$ T cells alone or after stimulation with the positive control PMA/Ionomycin. Functional parameters were quantified by flow cytometry. **A)** Frequency of IFN-γ$^+$ cells in NK KLRG1$^+$ cells, **B)** Mean Fluorescence Intensity (MFI) of CD107a in KLRG1$^+$ cells, **C)** frequency of IFN-γ$^+$ cells in NK KLRG1$^-$ cells, **D)** MFI of CD107a in KLRG1$^-$ cells. Statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank test. *p<0.05. **E)** Purity of the NK cells used in the NK-killing assays after ex vivo infection, and after viral reactivation

of the natural viral reservoir from ART PWH samples. **F)** Gating strategy used for the identification of ex vivo HIV-infected cells in the NK-killing assays, with and without the addition of the anti-KLRG1 antibody. **G)** Reduction in $p24^+$ after co-culturing *ex vivo* HIV-infected $CD4^+$ T cells with NK cells alone or previously stimulated with an isotype control. **H)** NK-killing assays after blocking the E-cadherin protein with blocking antibodies. **I)** p24 staining in samples from 3 healthy donors after cell activation with PMA + Ionomycin. **J)** Intracellular HIV-RNA levels measured by qPCR in $CD4^+$ T cells from ART PWH after stimulation with romidepsin as a positive control (n=10). **K)** Two images of productively HIV-infected cells expressing the receptor KLRG1 in lymph nodes. p24 HIV protein is shown in red and KLRG1 in yellow. Related to Figures 4 and 5.

Supplementary Figure 7

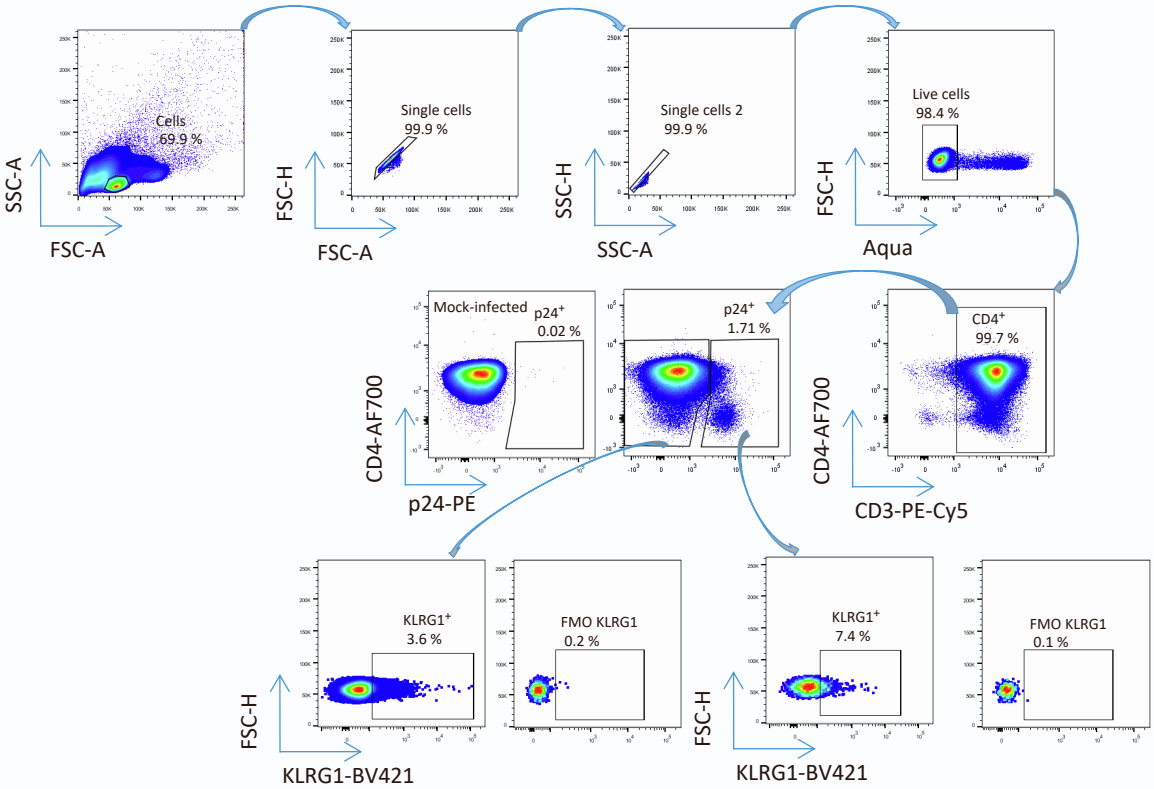

Figure S7. Gating strategy used for the detection of $CD4^+$ T cells expressing the KLRG1 receptor after ex vivo viral infection. Related to Figure 5.

Table S1. Clinical data of PWH included in the study. Related to Figures 1, 2, 3, 4 and 5.

#PWH ID	Age (years)	Gender	Time since HIV diagnosis (months)	$CD4^+$ T Cell Count (cells/µl)	% CD4	Viral Load (copies/ml)	Time on ART with VL suppressed (months)	ART regimen
1	26	F	50	740	29.1	<50	47	ABC+3TC+DTG
2	44	M	76	1280	40.6	<50	60	ABC+3TC+DTG
3	70	F	74	280	11.2	<50	68	ABC+3TC+RTG
4	39	M	78	430	20.4	<50	72	TDF+FTC+RPV
5	52	M	150	780	39.5	<50	82	ABC+3TC+DTG
6	43	F	38	430	17.9	<50	31	ABC+3TC+DTG
7	44	M	46	770	24.0	<50	34	ABC+3TC+DTG
8	47	F	294	250	20.0	<50	45	MRV+DRV+RTV+DTG
9	38	M	178	850	39.5	<50	81	TDF+FTC+EFV
10	49	M	106	750	29	<50	53	ABC+3TC+DTG
11	28	M	41	580	33.7	<50	34	TAF+FTC+BIC
12	62	M	74	590	22.0	<50	52	TDF+FTC+RPV
13	68	M	403	650	27.8	<50	98	DRV+RTV+RTG
14	46	M	226	510	34.6	<50	123	TDF+FTC+RPV
15	41	M	143	380	23.5	<50	123	ATRIPLA
16	38	M	70	1100	43.5	<50	47	TDF+FTC+DRV+COBI
17	59	F	43	1120	52.0	<50	35	TDF+FTC+RPV
18	32	M	55	770	34.1	<50	53	DTG+RPV
19	52	M	44	750	35.1	<50	37	TAF+FTC+EVG+COBI
20	35	M	125	900	37.6	<50	59	ABC+3TC+DTG
21	58	F	453	1070	44.5	<50	48	DRV+RPV+COBI
22	36	F	57	800	36.7	<50	54	TAF+FTC+BIC
23	23	F	30	830	43.1	<50	24	ABC+3TC+DTG
24	32	M	81	730	30.0	4100	NA	UNT
25	47	M	73	320	22.1	47300	NA	UNT
26	25	F	303	150	13.0	31200	NA	UNT
27	42	M	65	830	30.0	57900	NA	UNT
28	41	M	22	470	27.3	5680	NA	UNT
29	44	M	2	NA	NA	NA	NA	UNT
30	28	M	1	80	14.9	148000	NA	UNT
31	53	F	210	240	10.2	124	NA	UNT
32	28	F	335	230	28.3	78600	NA	UNT
33	55	M	85	240	11.0	834000	NA	UNT
34	46	M	45	760	21.1	11000	NA	UNT
35	27	M	2	710	32.7	69000	NA	UNT
36	24	F	* 12 days	470	35.1	54800	NA	UNT
37	37	M	* 7 days	410	21.4	333000	NA	UNT
38	52	M	2	840	47.0	855000	NA	UNT
39	23	M	8	420	30.5	74500	NA	UNT
40	19	M	*4 days	460	32.0	52900	NA	UNT
41	55	M	94	600	10.3	<50	40	DRV+RTV
42	N/A	N/A	*3 days	150	7.5	5000000	NA	UNT
43	61	M	74	590	25.1	<50	52	TDF+FTC+RPV
44	43	M	46	770	23.9	<50	35	ABC+3TC+DTG
45	26	F	43	1120	52.0	<50	35	TDF+FTC+RPV
46	32	M	118	750	32.6	<50	114	TAF+FTC+EVG+COBI
47	64	F	63	230	12.9	<50	61	TAF+FTC+BIC
48	46	M	65	580	30.0	<50	52	ABC+3TC+DTG
49	62	M	276	530	31.0	<50	208	ABC+3TC+DTG
50	31	M	73	480	29.0	<50	65	TAF+FTC+BIC
51	54	M	374	840	27.6	<50	56	ABC+3TC+EFV
52	43	M	156	1110	48.5	<50	119	TAF+FTC+RTV
53	53	M	22	920	36.0	<50	4	ABC+3TC+DTG
54	54	F	381	620	37.3	<50	30	TAF+FTC+RPV

Table S1. (*continuation*)

#PWH ID	Age (years)	Gender	Time since HIV diagnosis (months)	$CD4^+$ T Cell Count (cells/µl)	% CD4	Viral Load (copies/ml)	Time on ART with VL suppressed (months)	ART regimen
55	31	M	22	530	40.7	<50	6	ABC+3TC+DTG
56	50	F	265	1610	53.4	<50	109	TAF+FTC+NVP
57	79	F	263	420	23.5	<50	81	ABC+3TC+DTG
58	84	M	159	730	26.4	<50	87	TAF+FTC+BIC
59	50	M	156	580	28.0	<50	93	TAF+FTC+BIC
60	61	M	390	380	12.2	<50	69	TDF+FTC+DRV+COBI
61	48	M	239	530	31.4	<50	221	TAF+FTC+BIC
62	48	F	300	310	23.0	<50	51	MRV+DRV+RTV+DTG
63	29	F	100	1300	40.7	<50	50	TAF+FTC+EVG+COBI
64	61	M	275	490	33.9	<50	86	TAF+FTC+NVP
65	38	M	42	730	40.3	<50	20	TDF+FTC+RPV
66	30	F	33	950	32.1	<50	12	ABC+3TC+DTG
67	37	M	66	1430	38.0	<50	43	ABC+3TC+RPV
68	57	M	381	870	31.9	<50	51	TDF+FTC+EFV
69	34	F	18	1520	46.4	<50	12	TDF+FTC+EFV
70	64	M	121	370	23.5	<50	100	RPV+FTC+ TDF
71	55	M	189	880	36.2	<50	112	3TC+ABV+DTG
72	32	M	142	770	46.3	<50	116	FTC+TDF+ ETV
73	30	F	101	590	28.8	<50	98	3TC+ABV+ RTG
74	56	M	52	1470	50.6	<50	34	DRV+COBI+FTC+TAF
75	52	M	170	1280	40.8	<50	157	3TC+DTG
76	63	M	205	1190	41.7	<50	137	3TC+ABV+ RPV

PWH: people with HIV; FTC: emtricitabine; TDF: tenofovir; NVP: nevirapine; ATV: atazanavir; 3TC: lamivudine; EFV: efavirenz; ABC: abacavir; RAL: raltegravir; EVG: elvitegravir; DTG: dolutegravir; DRV: darunavir; RPV: Rilpivirine; TAF: tenofovir alafenamida; BIC: Bictegravir; LPV: Lopinavir; COBI: boosted with cobicistat; ATRIPLA: TDF+FTC+EFV; UNT: untreated; NA: Not Available; VL: viral load; ART: antiretroviral therapy; M: male; F: female.