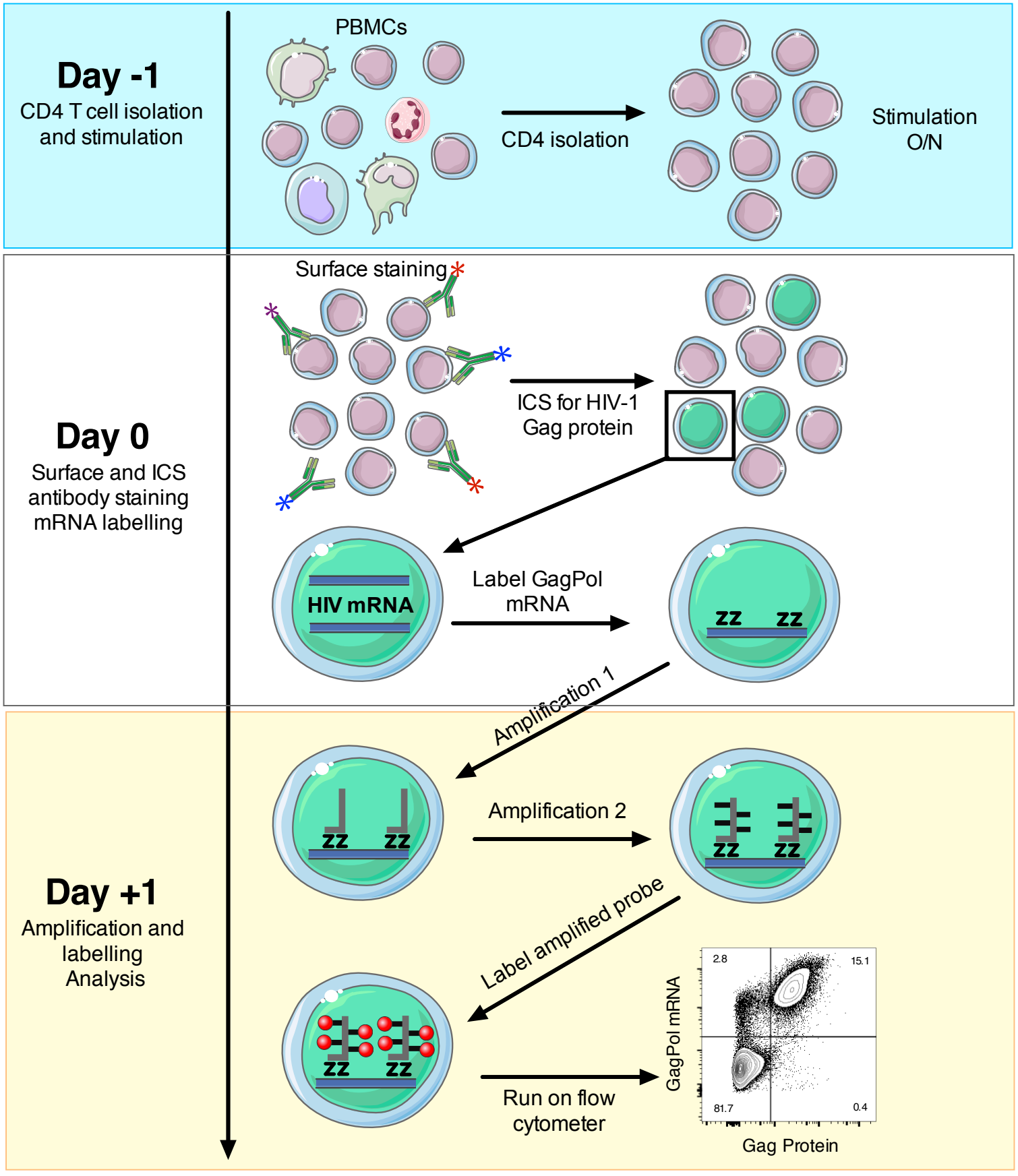



**Figure S1, related to Figure 1.** Schematic overview of timeline and experimental protocol for dual HIV mRNA and protein stain.

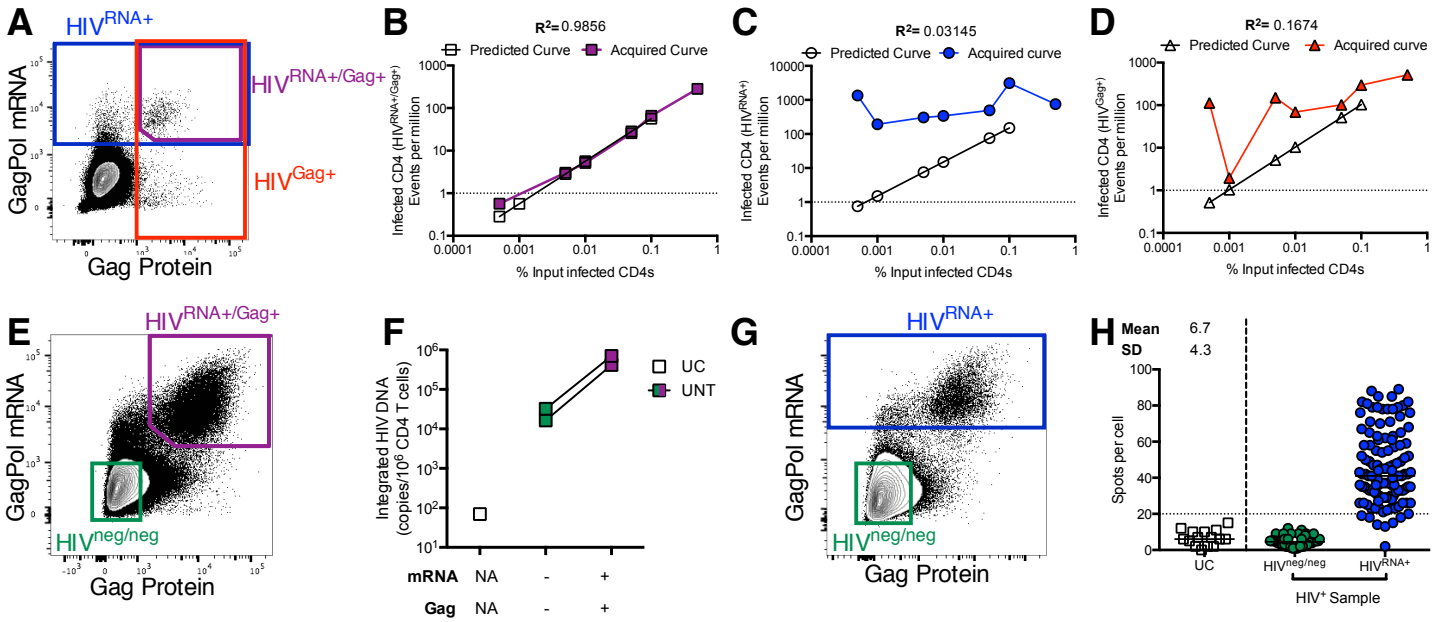
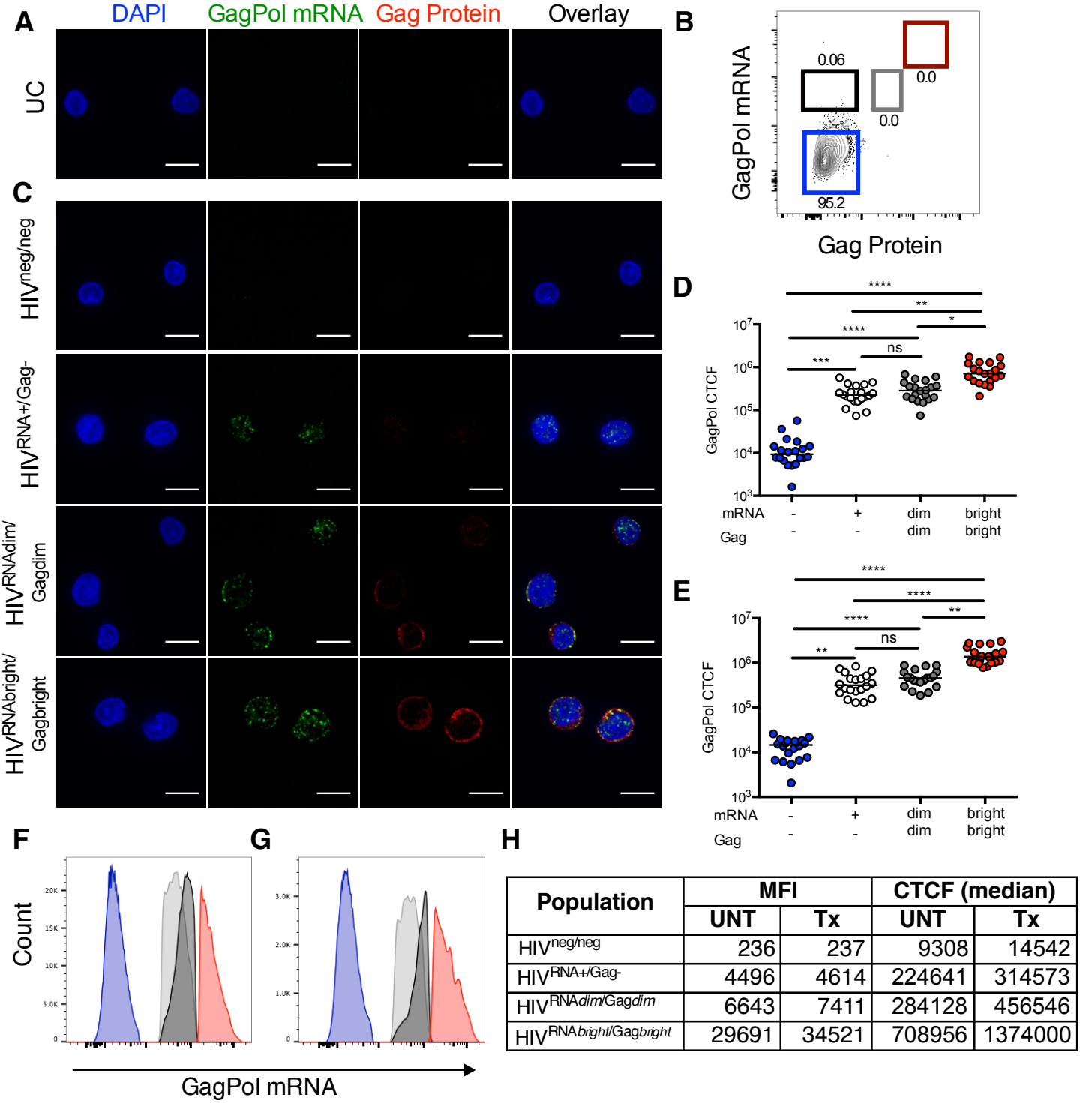



**Figure S2, related to Figure 1. Linearity and specificity of HIV$^{RNA/Gag}$ assay.** (**A-D**) HIV-infected CD4 expanded *ex vivo* from a viremic, UNT patient were "spiked" into CD4 T cells from an uninfected control (UC) at different ratios to determine the linearity and sensitivity of the assay. (A) Example plot showing gating of cells expressing Gag RNA and protein (purple), Gag RNA only (blue) or Gag protein only (red). Quantification of predicted (clear symbols) vs acquired result (colored symbols) using (B) double mRNA and protein expression, (C) mRNA only or (D) protein only. $R^2$ was calculated on log-transformed data. Data are representative from n=2. (**E** and **F**) HIV-infected CD4 T cells were expanded *ex vivo* from two UNT patients. HIV$^{neg/neg}$ and HIV$^{RNA+/Gag+}$ populations were sorted as in (E) and the number of integrated HIV DNA copies determined (F). (**G** and **H**) CD4 T cells were expanded *ex vivo* from one UNT and one aviremic, Tx patient or an UC. Cells were sorted based on mRNA GagPol stain into HIV$^{neg/neg}$ or HIV$^{RNA+}$ populations (G) and analyzed by microscopy (H).




| Population | MFI | | CTCF (median) | |
|---|---|---|---|---|
| | UNT | Tx | UNT | Tx |
| HIV$^{neg/neg}$ | 236 | 237 | 9308 | 14542 |
| HIV$^{RNA+/Gag-}$ | 4496 | 4614 | 224641 | 314573 |
| HIV$^{RNA\,dim/Gag\,dim}$ | 6643 | 7411 | 284128 | 456546 |
| HIV$^{RNA\,bright/Gag\,bright}$ | 29691 | 34521 | 708956 | 1374000 |

**Figure S3, related to Figure 1. Confocal microscopy confirms GagPol mRNA and Gag protein expression patterns observed by flow cytomtery.** CD4 T cells from HIV-infected patients were reactivated with PHA and a spreading infection of endogenous virus established. (**A** and **B**) Example images (A) and plot (B) from a sorting experiment on expanded CD4 T cells from an uninfected donor (UC). Scale bars are 10μm. (**C**) Split panel view of images shown in Figure 1G. Blue = DAPI, Green = GagPol mRNA and red = Gag protein. Scale bars are 10μm (**D** and **E**) Corrected total cell fluoresence (CTCF) for sorted populations described in Figure 1G from a viremic (D) or treated (E) patient. n=20 cells. Statistics shown are Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison post-test. ns signifies p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (**F** and **G**) Histograms showing GagPol mRNA MFI determined by flow cytometry in sorted populations from a viremic (F) or treated (G) patient CD4 T cells. In histogram plots, blue = HIV$^{neg/neg}$, light grey = HIV$^{RNA+/Gag-}$, dark grey = HIV$^{RNAdim/Gagdim}$, red = HIV$^{RNAbright/Gagbright}$. (H) Summary table of data in D-G. Median shown for CTCF. In all cases, data is shown for one individual.

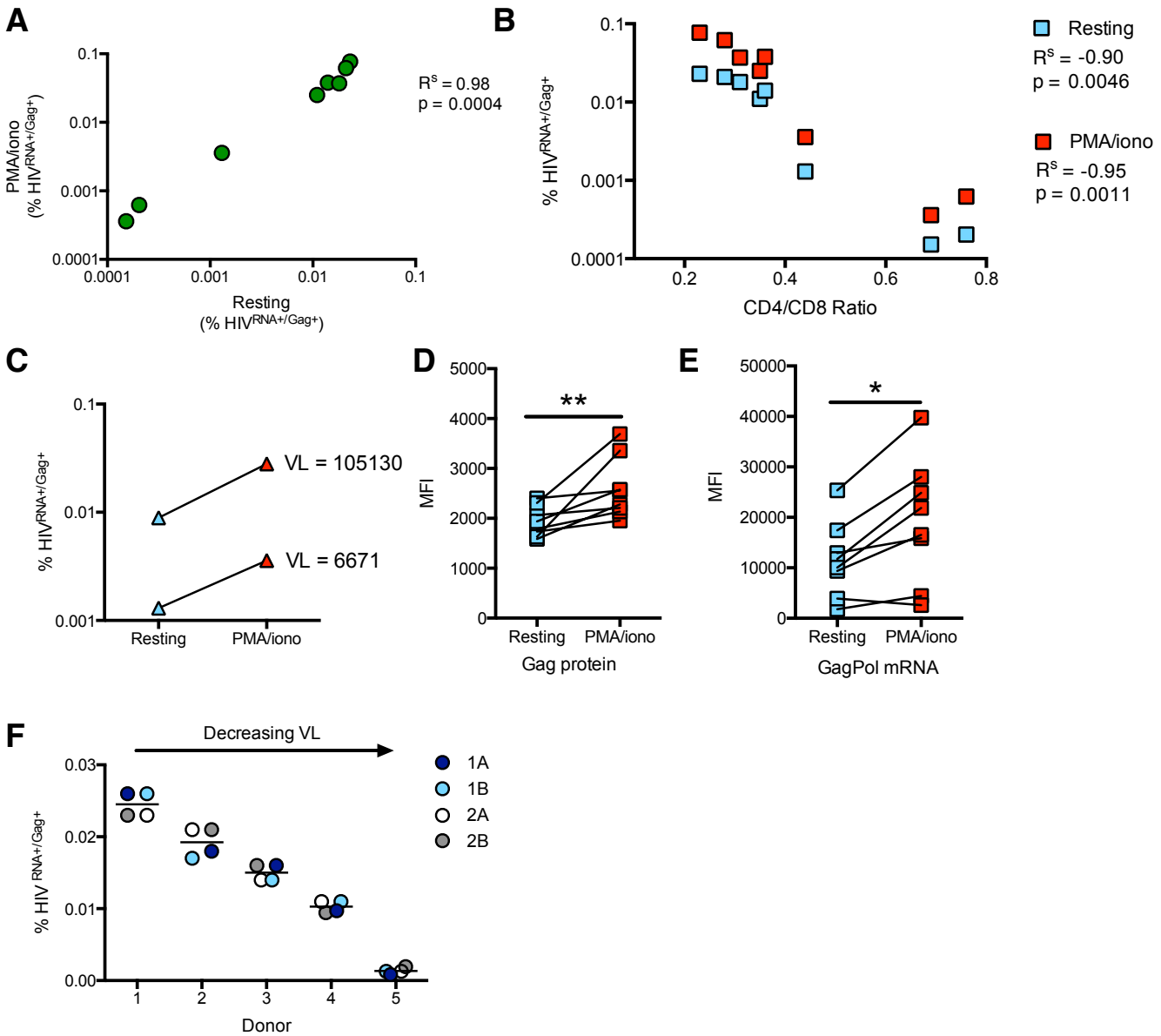



**Figure S4, related to Figure 2. Detection and characterization of CD4 T cells supporting ongoing and activation-inducible infection in viremic patients.** Detection of HIV$^{RNA+/Gag+}$ T cells directly *ex vivo* from viremic (UNT) patients. Data from ongoing infection is shown with light blue symbols, while data from reactivated infection is shown with red symbols. (**A**) Correlation between frequency of HIV$^{RNA+/Gag+}$ CD4 T cells in ongoing infection (Resting) compared to reactivated with PMA/ionomycin (PMA/iono). $R^s$ is Spearman's rank correlation coefficient with associated p value. (**B**) Correlation between ongoing and reactivated infection and CD4/CD8 ratio. (**C**) Long term analysis of a viremic patient at two time points with different viral loads. Respective viral loads (VL) are indicated. (**D** and **E**) Gemometric mean (MFI) of Gag protein (D) and GagPol mRNA (E) within HIV$^{RNA+/Gag+}$ CD4 T cells during ongoing (resting) or reactivated (PMA/iono) infection. (**F**) Reproducibility of HIV$^{RNA+/Gag+}$ cell detection in ongoing infection, in four experiments. Each dot represents an independent experiment (A-B) and experimenter (1-2), n=5 UNT. *p<0.05, **p<0.01 by Wilcoxon rank test. n= 8 UNT unless stated.

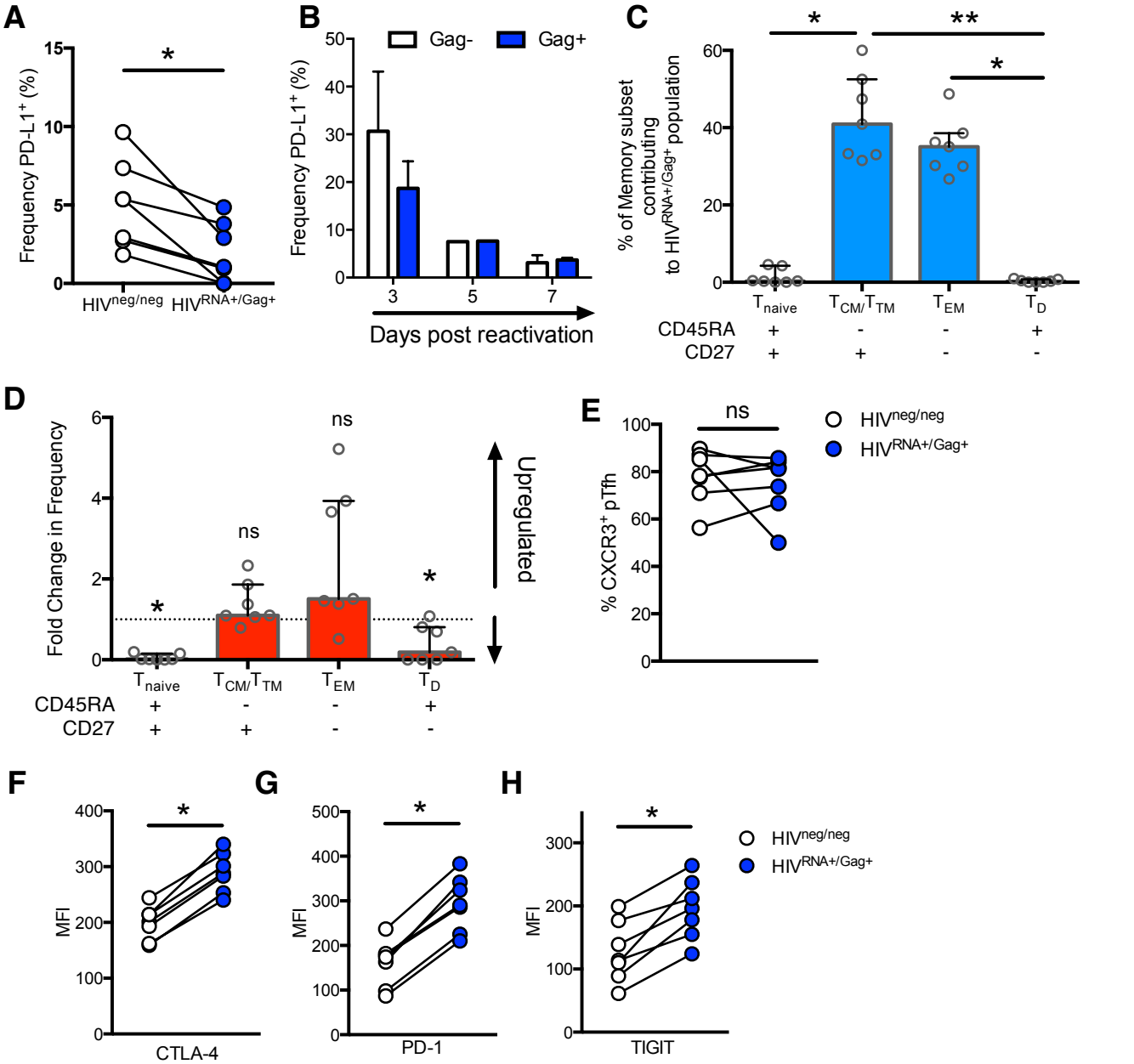



**Figure S5, related to Figures 3 and 4. CD4 T cells maintaining ongoing replication during viremia have a memory phenotype and express markers of activation and exhaustion.** $HIV^{RNA+/Gag+}$ T cells are indicated with blue symbols, while $HIV^{neg/neg}$ T cells are represented by clear symbols. (**A**) PD-L1 expression (%) on $HIV^{RNA+/Gag+}$ compared to $HIV^{neg/neg}$ T cells. *p<0.05 by Wilcoxon signed rank test. (**B**) PD-L1 expression on in vitro expanded CD4 (as in Fig.1) from untreated patients at 3, 5 and 7 days following reactivation. Infected cells identified by Gag protein staining only. n=2 UNT. (**C**) Contribution of different memory subsets to $HIV^{RNA+/Gag+}$ pool. Bars represent median, error bars show interquartile range.*p<0.05, **p<0.01 by Friedman one-way ANOVA with Dunn's multiple comparison post-test. (**D**) Fold change in frequency of memory subsets in uninfected compared to HIV-infected CD4. *p<0.05 by Wilcoxon signed rank test comparing each column to a theoretical median of 1. ns signifies p>0.05. Bars represent median, error bars show interquartile range. (**E**) Comparison of frequency of $CXCR3^+$ pTfh ($CD45RA^-CXCR5^+PD\text{-}1^+$) in $HIV^{RNA+/Gag+}$ compared to HIV-$1^{neg/neg}$ CD4. (**F-H**) Single inhibitory coreceptor expression (geometric mean, MFI) on $HIV^{RNA+/Gag+}$ compared to HIV-$1^{neg/neg}$ T cells. *p<0.05 by Wilcoxon signed rank test.

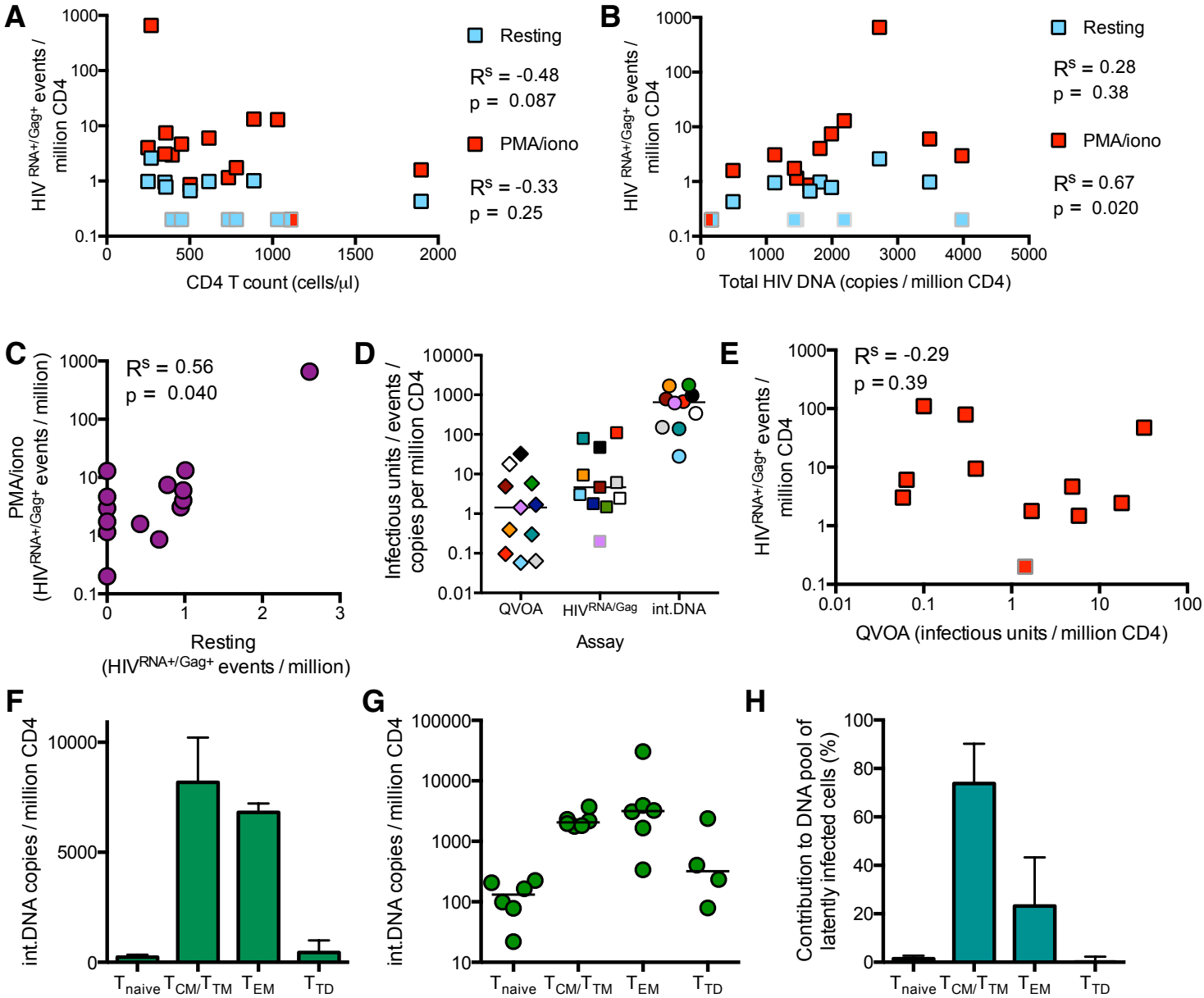



**Figure S6, related to Figure 5 and 6. Detection of latently HIV-infected CD4 T cells from ART-treated patients.** Data from ongoing infection (resting) is shown in light blue, while data from reactivated patient samples is shown in red. (**A** and **B**) Correlation between latent reservoir size measured by HIV$^{RNA/Gag}$ assay and CD4 count (A) or total HIV DNA content (B). Gray bordered symbols are below the LOD. Where values are the same, split box is shown. (**C**) Correlation between limited ongoing (resting) and reactivated HIV$^{RNA+/Gag+}$ (PMA/iono) CD4. n=14 Tx donors. (**D**) Comparison of reservoir measured by different techniques; Quantitative Viral Outgrowth Assay (QVOA), HIV$^{RNA/Gag}$ assay (with PMA/iono stimulation) and integrated HIV DNA (int.DNA). n=11 Tx donors, independent donors are illustrated by color. Line shown at median. (**E**) Correlation analysis for data shown in (D) between QVOA and HIV$^{RNA/Gag}$ assays. n=11 Tx donors. Grey bordered symbols are below the LOD. (**F**) Quantification of integrated HIV DNA levels in CD4 T cell subsets in viremic (UNT) patients, sorted based on CD45RA and CD27 expression. n=2 UNT with mean +/- SEM shown. (**G**) Quantification of integrated HIV DNA levels in CD4 T cell subsets from Tx patients, sorted based on CD45RA and CD27 expression. (**H**) Data as in (**G**) expressed as contribution of each subset to pool of HIV DNA-containing cells, based on relative frequencies at sort. Bars = median +/- interquartile range. For G+H n=6 Tx, except for $T_{TD}$ subset where data was only available for 4 Tx patients. For correlations, statistics shown are Spearman's rank correlation coefficient ($R^S$) with associated p values. $p<0.05$ considered significant.

**Table S1, related to Figure 1. Sequences used for design of GagPol probes.** Sequences from JR-CSF complete genome.

| | Target Sequence |
|---|---|
| **Gag Probe Pairs** | aaggccaggaggaaagaaaaaatatagattaaaacatatagtatgggcaagcagggagctagaacgtttcgcagtcaatcctggcct<br>gttagaatcatcagaaggctgtagacaaatactgggacaactacaaccatcccttaagacaggatcagaagaacttacatcattatataa<br>tacagtagcaaccctctattgtgtacatcaaaggatagagataaaagacaccaaggaagctttagaaaagatagaggaagagcaaacc<br>aaaagtatgaaaaaggcacagcaagcagcagctgacacaggaaacagcagccaggtcagccaaaattaccctatagtgcagaacct<br>gcaggggcaaatggtacatcaggccatatcacctagaactttaaatgcatgggtaaaagtaatagaagagaaggctttcagccccgaa<br>gtaatacccatgttttcagcattatcagaaggagccaccccacaagatttaaacaccatgctaaacacagtgggggacatcaagcagc<br>tatgcaaatgctaaaagaaaccatcaatgaggaagctgcagaatgggatagattgcatccagtgcatgcagggcctattgcaccaggc<br>cagatgagagaaccaaggggaagtgacatagcagggactactagtacccttcaggaacaaataggatggatgacaaataatccacct<br>atcccagtaggagaaatctataaaagatggataatcctggggttaaataaaatagtaaggatgtatagccctgtcagcattctggacataa<br>gacaaggaccaaaggaacccctttagagactatgtagaccggttctataaaaccctaagagccgagcaagctacacaggaggtaaaaa<br>attggatgacagaaaccttgttggtccaaaatgcgaacccagattgtaaaactatttttaaaagcattgggaccagcagctacactagaag<br>aaatgatgacagcatgtcagggagtgggaggacccggccataaagcaagagttttggctgaa |
| **Pol Probe Pairs** | aaagcatagtaatatggggaaagattcctaaatttaaattacccatacaaaaagaaacatgggaaacatggtggacagagtattggcaa<br>gccacctggattcctgagtgggagtttgtcaataccctccttagtgaaattatggtaccagttagaaaaagaacccatagtaggagca<br>gaaactttctatgtagatggggcagctaacagggagactaaattaggaaaagcaggatatgttactagcagaggaagacaaaaagttg<br>tctccctaacagacacaacaaatcagaaaactgagttacaagcaattcacctagctttgcaggattcaggattagaagtaaacatagtaa<br>cagactcacaatatgcattaggaatcattcaagcacaaccagataaaagtgaatcagagttagtcagtcaaataatagaacagctaataa<br>aaaaggaaaaagtctacctggcatgggtaccagcacacaaaggaattggaggaaatgaacaggtagataaattagtcagtgctggaa<br>tcaggaaagtgctatttttagatggaatagataaggcccaagaagatcatgaaaaatatcacagtaattggagagcaatggctagtgattt<br>taacctgccacctatagtagcaaaagaaatagtagccagctgtgataaatgtcagctaaaaggagaagccatgcatggacaagtagac<br>tgtagtccaggaatatggcaactagattgtacacatttagaaggaaaaattatcctggtagcagttcatgtagccagtggatatatagaag<br>cagaagttattccagcagaaacagggcaggaaacagcatactttctcttaaaattagcaggcagatggccagtaacaacaatacataca<br>gacaatggcagcaatttcaccagtactacagttaaggccgcctgttggtgggctgggatcaagcaggaatttggcattccc |

**Table S2, related to Figure 2. Phenotypic data and raw reactivation results for viremic patients (UNT) and associated uninfected control donors (UC).**

| | | | HIV$^{RNA+/Gag+}$ | | Total Cell Count | Patient Phenotype | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | % | Cell count | | Gender | Age (years) | VL (copies/ml) | CD4/CD8 | CD4 count (cells/µl) |
| UC | UC 1 | Resting | 0 % | 0 | 1550000 | M | 55 | NA[a] | 1.77 | 562 |
| | | PMA/Iono | 0 % | 0 | 1390000 | | | | | |
| | UC 2 | Resting | 0 % | 0 | 995000 | M | 43 | NA[a] | 2.98 | 1053 |
| | | PMA/Iono | 0 % | 0 | 1110000 | | | | | |
| | UC 3 | Resting | 1.45E-4 % | 1 | 692000 | M | 60 | NA[a] | 3.55 | 854 |
| | | PMA/Iono | 0 % | 0 | 834000 | | | | | |
| | UC 4 | Resting | 0 % | 0 | 428000 | M | 62 | NA[a] | 1.07 | 482 |
| | | PMA/Iono | 0 % | 0 | 698000 | | | | | |
| UNT | UNT 1 | Resting | 2.04E-4 % | 3 | 1470000 | M | 22 | 286 | 0.76 | 750 |
| | | PMA/Iono | 6.22E-4 % | 9 | 1450000 | | | | | |
| | UNT 2 | Resting | 1.52E-4 % | 2 | 1320000 | M | 46 | 667 | 0.69 | 504 |
| | | PMA/Iono | 3.59E-4 % | 5 | 1390000 | | | | | |
| | UNT 3 | Resting | 1.30E-3 % | 8 | 616000 | M | 41 | 6671 | 0.44 | 406 |
| | | PMA/Iono | 3.58E-3 % | 19 | 531000 | | | | | |
| | UNT 4 | Resting | 0.011 % | 24 | 222052 | M | 38 | 14614 | 0.35 | 138 |
| | | PMA/Iono | 0.025 % | 198 | 805000 | | | | | |
| | UNT 5 | Resting | 0.014 % | 55 | 393484 | M | 49 | 44848 | 0.36 | 281 |
| | | PMA/Iono | 0.038 % | 70 | 185417 | | | | | |
| | UNT 6 | Resting | 0.021 % | 78 | 363672 | M | 45 | 73109 | 0.28 | 228 |
| | | PMA/Iono | 0.062 % | 511 | 822000 | | | | | |
| | | Resting | 0.018 % | 235 | 1300000 | | | | | |
| | | Bryostatin | 0.042 % | 561 | 1330000 | | | | | |
| | UNT 7 | Resting | 0.023 % | 157 | 681000 | M | 38 | 132886 | 0.23 | 320 |
| | | PMA/Iono | 0.077 % | 634 | 826000 | | | | | |
| | | Resting | 0.026 % | 393 | 1530000 | | | | | |
| | | Bryostatin | 0.061 % | 1309 | 2160000 | | | | | |
| | UNT 8 | Resting | 0.018 % | 207 | 1140000 | M | 43 | 193437 | 0.31 | 356 |
| | | PMA/Iono | 0.037 % | 384 | 1020000 | | | | | |

[a] NA=not applicable

**Table S3, related to Figure 5 and 6. Phenotypic data and raw reactivation results for treated, aviremic patients (Tx) and associated uninfected control donors (UC).**

| | | | HIV$^{RNA+/Gag+}$ | | Total Cell Count | Patient Phenotype | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | % | Cell count | | Gender | Age (years) | VL (copies/ml) | CD4/CD8 | CD4 count (cells/µl) |
| UC | UC 5 | Resting | 0 % | 0 | 447000 | M | 62 | NA[a] | 2.41 | 789 |
| | | PMA/iono | 0 % | 0 | 1000000 | | | | | |
| | UC 6 | Resting | 0 % | 0 | 994000 | M | 43 | NA[a] | 2.98 | 1053 |
| | | PMA/iono | 0 % | 0 | 1110000 | | | | | |
| | UC 7 | Resting | 0 % | 0 | 1790000 | F | 44 | NA[a] | 2.36 | 754 |
| | | PMA/iono | 5.96E-5 % | 1 | 1680000 | | | | | |
| | UC 8 | Resting | 0 % | 0 | 2580000 | M | 45 | NA[a] | 4.87 | 550 |
| | | PMA/iono | 0 % | 0 | 2170000 | | | | | |
| | UC 9 | Resting | 0 % | 0 | 1740000 | M | 27 | NA[a] | 2.02 | 1368 |
| | | Bryostatin | 0 % | 0 | 1420000 | | | | | |
| | UC 10 | Resting | 0 % | 0 | 2070000 | F | 39 | NA[a] | 2.92 | 1488 |
| | | PMA/iono | 0 % | 0 | 1950000 | | | | | |
| | | Resting | 0 % | 0 | 2040000 | | | | | |
| | | Bryostatin | 5.42E-5 % | 1 | 1850000 | | | | | |
| | UC 11 | Resting | 0 % | 0 | 3310000 | F | NP[b] | NA[a] | 1.60 | 705 |
| | | Ingenol | 0 % | 0 | 2690000 | | | | | |
| Tx | Tx 1 | Resting | 0 % | 0 | 1390000 | M | 31 | 49 | 0.53 | 394 |
| | | PMA/iono | 2.97E-6 % | 4 | 1350000 | | | | | |
| | Tx 2 | Resting | 1.01E-4 % | 1 | 992000 | M | 46 | < 48 | 0.78 | 887 |
| | | PMA/iono | 1.32E-3 % | 13 | 981000 | | | | | |
| | | Resting | 0 % | 0 | 689000 | | | | | |
| | | Bryostatin | 9.11E-5 % | 1 | 1100000 | | | | | |
| | Tx 3 | Resting | 0 % | 0 | 998000 | F | 46 | < 20 | 0.31 | 541 |
| | | PMA/iono | 4.65E-4 % | 7 | 1500000 | | | | | |
| | Tx 4 | Resting | 2.61E-4 % | 3 | 1150000 | M | 39 | < 20 | 0.52 | 267 |
| | | PMA/iono | 0.066 % | 651 | 988000 | | | | | |
| | | Resting | 4.33E-4 % | 2 | 462000 | | | | | |
| | | Bryostatin | 3.79E-3 % | 23 | 608000 | | | | | |
| | Tx 5 | Resting | 0 % | 0 | 1730000 | F | 46 | < 20 | 0.86 | 1030 |
| | | PMA/iono | 1.29E-3 % | 15 | 1160000 | | | | | |
| | | Resting | 0 % | 0 | 2210000 | | | | | |
| | | Bryostatin | 4.29E-4 % | 11 | 2560000 | | | | | |
| | Tx 6 | Resting | 4.27E-5 % | 1 | 2340000 | M | 46 | < 20 | 2.29 | 1897 |
| | | PMA/iono | 1.59E-4 % | 3 | 1880000 | | | | | |
| | Tx 7 | Resting | 9.48E-5 % | 1 | 1050000 | F | 38 | < 41 | 1.02 | 351 |
| | | PMA/iono | 3.09E-4 % | 3 | 970000 | | | | | |
| | Tx 8 | Resting | 0 % | 0 | 1000000 | M | 32 | < 20 | 1.04 | 734 |
| | | PMA/iono | 1.15E-4 % | 2 | 1740000 | | | | | |
| | Tx 9 | Resting | 0 % | 0 | 2610000 | M | 50 | < 20 | 2.76 | 1110 |
| | | PMA/iono | 0 % | 0 | 2110000 | | | | | |
| | Tx 10 | Resting | 9.81E-5 % | 2 | 2040000 | M | 32 | < 20 | 0.66 | 248 |
| | | PMA/iono | 4.02E-4 % | 8 | 1990000 | | | | | |
| | Tx 11 | Resting | 0 % | 0 | 1170000 | M | 63 | < 40 | 0.52 | 782 |
| | | PMA/iono | 1.75E-4 % | 3 | 1710000 | | | | | |
| | | Resting | 5.26E-5 % | 1 | 1900000 | | | | | |
| | | Bryostatin | 1.14E-4 % | 2 | 1750000 | | | | | |
| | | Resting | 0 % | 0 | 2460000 | | | | | |
| | | Ingenol | 1.01E-4 % | 3 | 2980000 | | | | | |
| | Tx 12 | Resting | 7.76E-5 % | 1 | 1290000 | M | 47 | < 40 | 0.44 | 356 |
| | | PMA/iono | 7.46E-4 % | 16 | 2150000 | | | | | |
| | | Resting | 0 % | 0 | 2370000 | | | | | |
| | | Bryostatin | 1.21E-4 % | 4 | 3310000 | | | | | |
| | | Resting | 0 % | 0 | 4130000 | | | | | |
| | | Ingenol | 3.63E-4 % | 14 | 3850000 | | | | | |
| | Tx 13 | Resting | 9.83E-5 % | 1 | 1020000 | M | 39 | < 40 | 0.49 | 616 |
| | | PMA/iono | 6.00E-4 % | 13 | 2170000 | | | | | |
| | | Resting | 0 % | 0 | 2110000 | | | | | |
| | | Bryostatin | 1.65E-4 % | 4 | 2430000 | | | | | |
| | | Resting | 0 % | 0 | 3240000 | | | | | |
| | | Ingenol | 1.38E-4 % | 4 | 2900000 | | | | | |
| | Tx 14 | Resting | 6.69E-5 % | 1 | 1500000 | M | 46 | < 40 | 0.74 | 503 |
| | | PMA/iono | 8.57E-5 % | 2 | 3650000 | | | | | |
| | | Resting | 0 % | 0 | 2420000 | | | | | |
| | | Bryostatin | 1.10E-4 % | 4 | 2330000 | | | | | |

[a] NA = not applicable, [b] NP = not provided

**Table S4, related to Figure 5 and S6. Phenotypic data and reactivation results for HIV$^{RNA/Gag}$ assay and QVOA for treated, aviremic patients (Tx).**

| | HIV$^{RNA/Gag}$ assay (HIV$^{RNA+/Gag+}$ events/million) | QVOA (IU/million) | Patient Phenotype | | | | |
|---|---|---|---|---|---|---|---|
| | | | Gender | Age (years) | VL (copies/ml) | CD4/CD8 | CD4 count (cells/µl) |
| **Tx 3**[b] | 4.65 | 4.90 | F | 45 | <20 | 0.31 | 541 |
| **Tx 15** | 6.11 | 0.064 | M | 59 | <40 | 1.87 | 1112 |
| **Tx 16** | 1.79 | 1.69 | M | 43 | <40 | 0.76 | 1065 |
| **Tx 17** | 9.42 | 0.39 | M | 40 | <40 | 0.43 | 503 |
| **Tx 18** | 47.14 | 32.23 | M | 63 | <40 | 0.92 | 715 |
| **Tx 19** | 0.00* | 1.43 | F | 20 | <40 | 1.21 | 297 |
| **Tx 20**[b] | 110.4 | 0.10 | M | 58 | <50 | 0.62 | 309 |
| **Tx 21** | 79.16 | 0.29 | NP[a] | NP[a] | <50 | 1.26 | 1177 |
| **Tx 22**[b] | 2.45 | 17.89 | M | 50 | <40 | 0.32 | 309 |
| **Tx 23** | 3.04 | 0.058 | M | 40 | <20 | 0.81 | 1145 |
| **Tx 24** | 1.49 | 5.82 | M | 52 | <40 | 0.71 | 815 |

* No HIV$^{RNA+/Gag+}$ events detected in 2.1 million CD4 T cells analyzed

[a] NP = not provided

[b] QVOA and HIV$^{RNA/GAG}$ assay performed on sequential leukapheresis samples. Patient characteristics (including reservoir size measured by integrated HIV DNA) were stable.

## SUPPLEMENTAL MOVIE LEGENDS

**Movies S1 and S2, related to Figure 1.** Sorted HIV-infected CD4 T cells from Figures 1G-I were analysed by confocal microscopy as described and Z stacks collected. **(Movie S1)** Example Z stack for $HIV^{RNA\textit{bright}/Gag\textit{bright}}$ CD4. **(Movie S2)** Example Z stack for $HIV^{RNA+/Gag-}$ CD4. Scale bars represent 10μm.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Study Approval

Leukapheresis samples obtained at McGill University Hospital, Montréal, QC, Canada were approved by the ethics committee of the McGill University Health Centre (reference BMB-01-028). For samples processed at the VGTI, Florida, USA approval was granted by the Martin Memorial Health Systems institutional review board (IRB reference HIV-002) and Canada approval by the Centre Hospitalier de l'Université de Montréal (CHUM) (IRB reference number 14.372).

### Infection with exogenous virus

CD4+ T cells infected with exogenous wild type NL4.3 (NIH AIDS reagent program, (Adachi et al., 1986)) were used for fluorescence-minus-one controls only. CD4+ T cells were isolated, resuspended at 2 x $10^6$/ml in complete media and stimulated for 72 hr with PHA-L (2µg/ml, Sigma) in the presence of IL-2 (50U/ml). Following stimulation, cells were spinoculated (800 x g, 25 $^oC$, 1.5 hr) with NL4.3 (MOI 0.01) and incubated for a further 48 hr in complete media plus IL-2 (50U/ml).

### Combined HIV mRNA Flow FISH/Gag protein assay

All buffers and fixation reagents were provided with the kit, with the exception of flow cytometry staining buffer (2% FBS/PBS). Briefly, samples were collected, stained with a viability dye (20 min, 4$^oC$, Fixable LiveDead, eBioscience) and then surface markers were labelled (30 min 4$^oC$). Samples were fixed, permeabilized and labeled intracellularly for HIV-1 p24 (KC57 RD1, Beckman Coulter, 30 min RT, 30 min 4 oC), followed by a further fixation step. HIV-1 GagPol mRNA was labelled with a set of 40 probe pairs (20 targeting Gag and 20 targeting Pol; sequences are listed in Table S1. The probes were diluted 1:5 in diluent and hybridized to the target mRNA for 2 hr at 40 $^oC$. Samples were washed to remove excess probes and stored overnight in the presence of RNAsin. Signal amplification was achieved as described in Porichis *et al*. 2014 by sequential 1.5 hr, 40 $^oC$ incubations with the pre-amplification and amplification mix. Amplified mRNA was labelled with fluorescently-tagged probes for 1 hr at 40 $^oC$. A negative control probe and a positive control probe (against house-keeping gene RPL13A) were included in each experiment. Gates were set on the HIV-uninfected donor control, or unstimulated control where appropriate.

### Antibody panels

In reactivation and spiking experiments, isolated CD4+ T cells were stained with a basic phenotyping panel. Dead cells were excluded by staining with Fixable Live/Dead (eF780, eBioscience). Cells were surface stained with CD14/CD19 (BUV737 or V500), CD8 (BV711), CD3 (BUV395) and CD4 (PE-Cy7 or BV605). In all experiments investigating the phenotype of CD4+ T cells harboring ongoing replication, the same basic antibody panel was used. Dead cells were excluded by staining with Fixable Live/Dead (eF506, eBioscience). Cells were surface stained with directly conjugated antibodies against CD3 (UCHT1, BUV395), CD4 (RPA-T4, BUV496), CD8 (SK1, BUV737, BD), CD14 (M5E2, BUV737, BD) and CD19 (H1B19, BUV737, BD). For analysis of pTfh phenotype, CD4 T cells were stained with CXCR5 (RF8B2, BB515, BD) and CXCR3 (G025H7, BV605, BioLegend) during overnight rest (12 hr, 37oC), and surface stained the following day for CD45RA (HI100, BV711, BioLegend), PD-1 (EH12.2H7, BV421, BioLegend) and ICOS (ISA-3, PECy7, eBioscience). For analysis of memory and activation phenotype, cells were stained after rest with HLA-ABC (W6/32, PE-Cy7, BioLegend), HLA-DR (G46-4, BV711, BD), CD45RA (HI100, BB515, BD), CD27 (L128, BV605, BD) and PDL-1 (29A.3E, BV421, BioLegend). For analysis of inhibitory receptors, cells were surfaced stained after rest with PD-1 (EH12.2H7, BV421, BioLegend), TIGIT (MBSA43, PE-Cy7,

eBioscience). At the same time as staining for HIV-1 Gag by intracellular staining, samples were also stained with CTLA-4 (BNI3, APC, BD). For analysis of phenotypes following bryostatin or ingenol stimulation, a modified pTfh panel was used, where CD27 (L128, BV605, BD) replaced CXCR3. We confirmed that bryostatin or ingenol treatment did not alter the distribution of the memory subsets by comparing the unstimulated to bryostatin/ingenol-treated for each donor. In the majority of experiments, Type 1 mRNA probes were used (AF647). However, when investigating the role of CTLA-4 Type 4 mRNA probes were used (AF488).

**Linearity and specificity experiment**

For experiments shown in Figure 1, CD4+ T cells from an untreated patient were reactivated with PHA-L (10µg/ml) for 72 hrs. Reactivated cells were split into two groups; one was maintained in complete media + IL-2 ("infected") and the second in the presence of antiretroviral drugs ((T20 (7.5µg/ml) + AZT (1µM)) to limit the spreading infection ("uninfected"). For experiments shown in Figure S2, CD4+ T cells from an untreated patient and an uninfected control patient were reactivated with PHA-L (10µg/ml) for 72 hrs. Cells from the untreated patient were used as the "infected" population while cells from an uninfected control were used as "uninfected". In both experiments, following a further 7 days of culture, the cells were collected, washed to remove free virus, counted and resuspended with both the "infected" and "uninfected" at the same concentration. The "infected" culture was then spiked into the "uninfected" culture at different ratios to set up a dilution series. Samples were then analysed using the RNA flow-FISH assay. Using the % $HIV^{RNA+/Gag+}$ (or $HIV^{RNA+}$ or $HIV^{protein+}$) as determined for the starting "infected" sample, the infection rate for each sample in the dilution series was predicted. The actual result was then compared to the predicted to determine the linearity and sensitivity of the system.

**Microscopy on sorted HIV-infected cells following mRNA Flow-FISH**

CD4+ T cells from one uninfected, one aviremic, treated and one viremic, untreated patient were isolated and reactivated, and a spreading infection established over 7 days as described above. Cells were collected and stained with a viability dye (Fixable Live/Dead (eF780, eBioscience)) and with antibodies against surface CD8, CD14 and CD19 (BV510). mRNA Flow FISH was performed as described above. Samples were stored overnight in Storage Buffer and sorted with a BD FACS Aria. Live, single, $CD8/14/19^-$ T cells were sorted into four populations based on KC57-RD1 (Gag protein) and GagPol mRNA-AF647 staining and stored in Storage buffer. Prior to microscopy analysis, samples were collected, quenched (100mM glycine, 10 min, RT) and the nucleus stained (DAPI, 1ng/ml, 2 min RT). Samples were imaged in ibidi µ-Slide VI 0.4 microscopy chambers using a Zeiss Observer inverted spinning disk confocal microscope with Evolve camera and ZEN blue software. Fiji was used for all image analysis. DAPI staining was used to define the nuclear compartment and the "Find Maxima" command was used to identify and count GagPol mRNA spots within and outside of the defined compartment (nuclear spots/ cytoplasmic spots). To determine the Corrected Total Cell Fluorescence (CTCF), a region of interest (ROI) was drawn around each cell. The integrated density, area and mean of the ROI was measured in the GagPol channel. Background fluorescence was also measured. The CTCF for each cell was then determined using the formula = Integrated density - (area of selected cell * background mean).

**Detection of integrated DNA in sorted HIV-infected cells following mRNA Flow-FISH**

CD4+ T cells from one uninfected, two viremic, untreated patients were isolated and reactivated, and a spreading infection established over 7 days as described above. Cells were collected and stained as for microscopy above. Samples were sorted into double negative ($HIV^{neg/neg}$) and double positive ($HIV^{RNA+/Gag+)}$ populations based on KC57-RD1 (Gag protein) and GagPol mRNA-AF647 staining. Dry pellets of sorted cells were stored at -80$^o$C and analyzed for integrated DNA by *alu* PCR as described in Experimental Procedures.

## SUPPLEMENTAL REFERENCE

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M.A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. Journal of Virology *59*, 284–291.