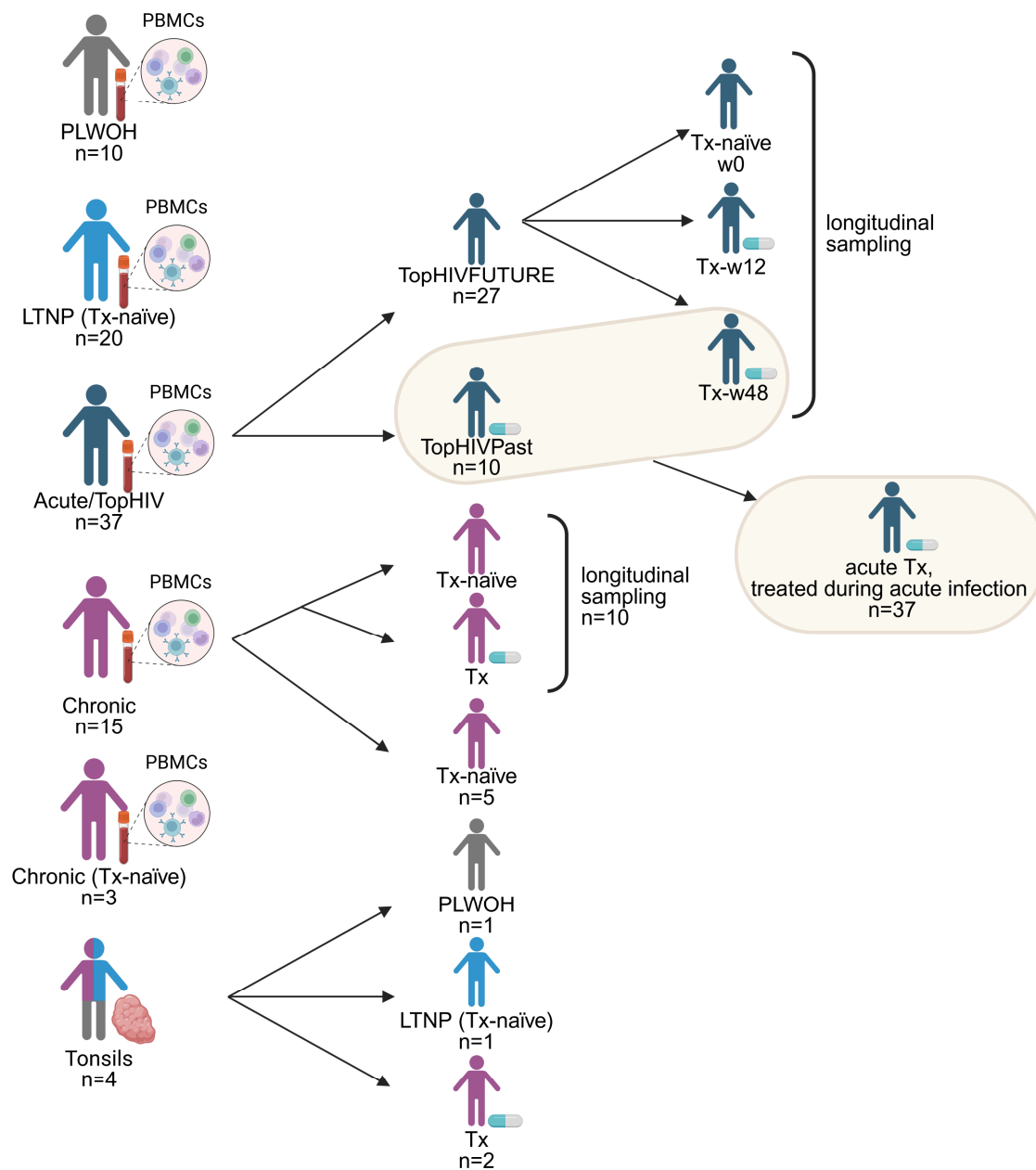
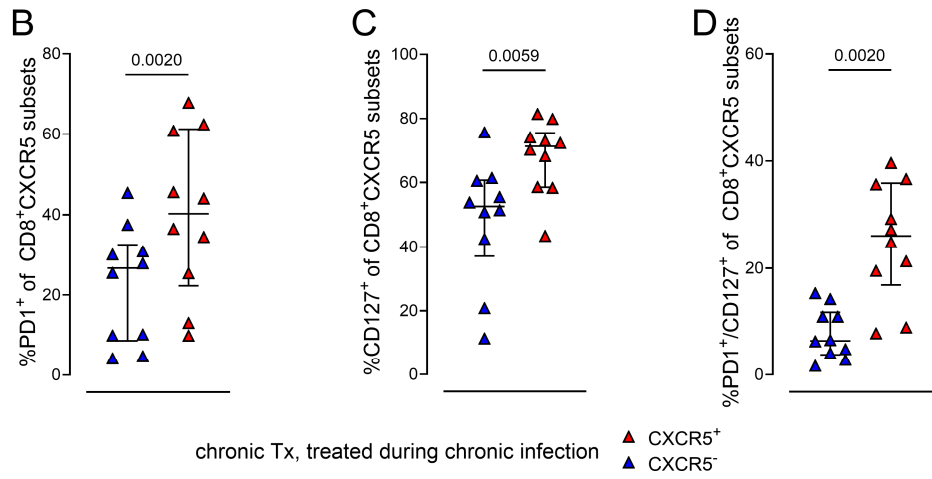
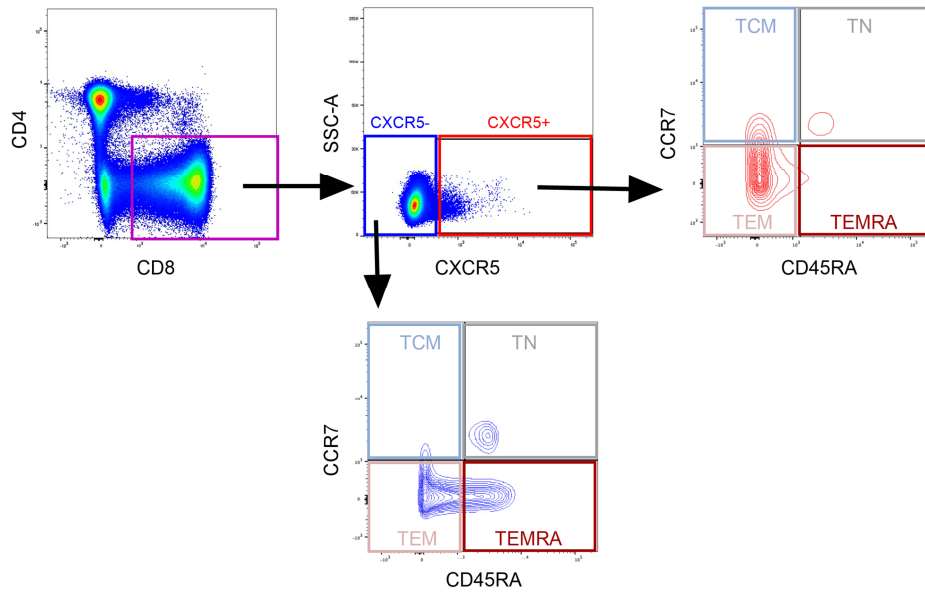


Supplemental Information



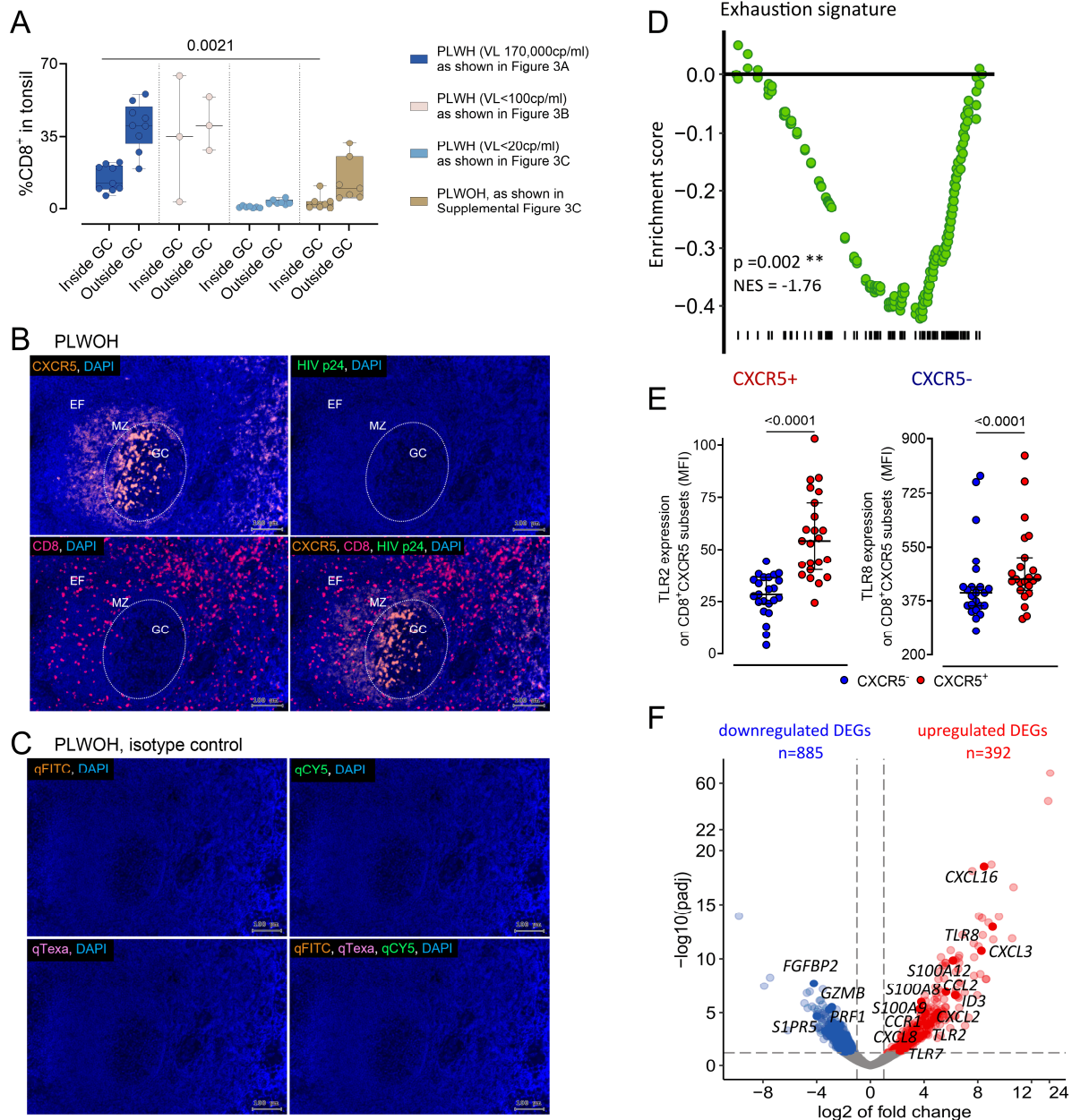
Supplemental Figure 1: Schematic representation of the cohort and sampling timepoints used for analyses.
Created in BioRender. Immunity, I. (2025) <https://BioRender.com/i33u513>.

A gated on live CD3⁺ cells



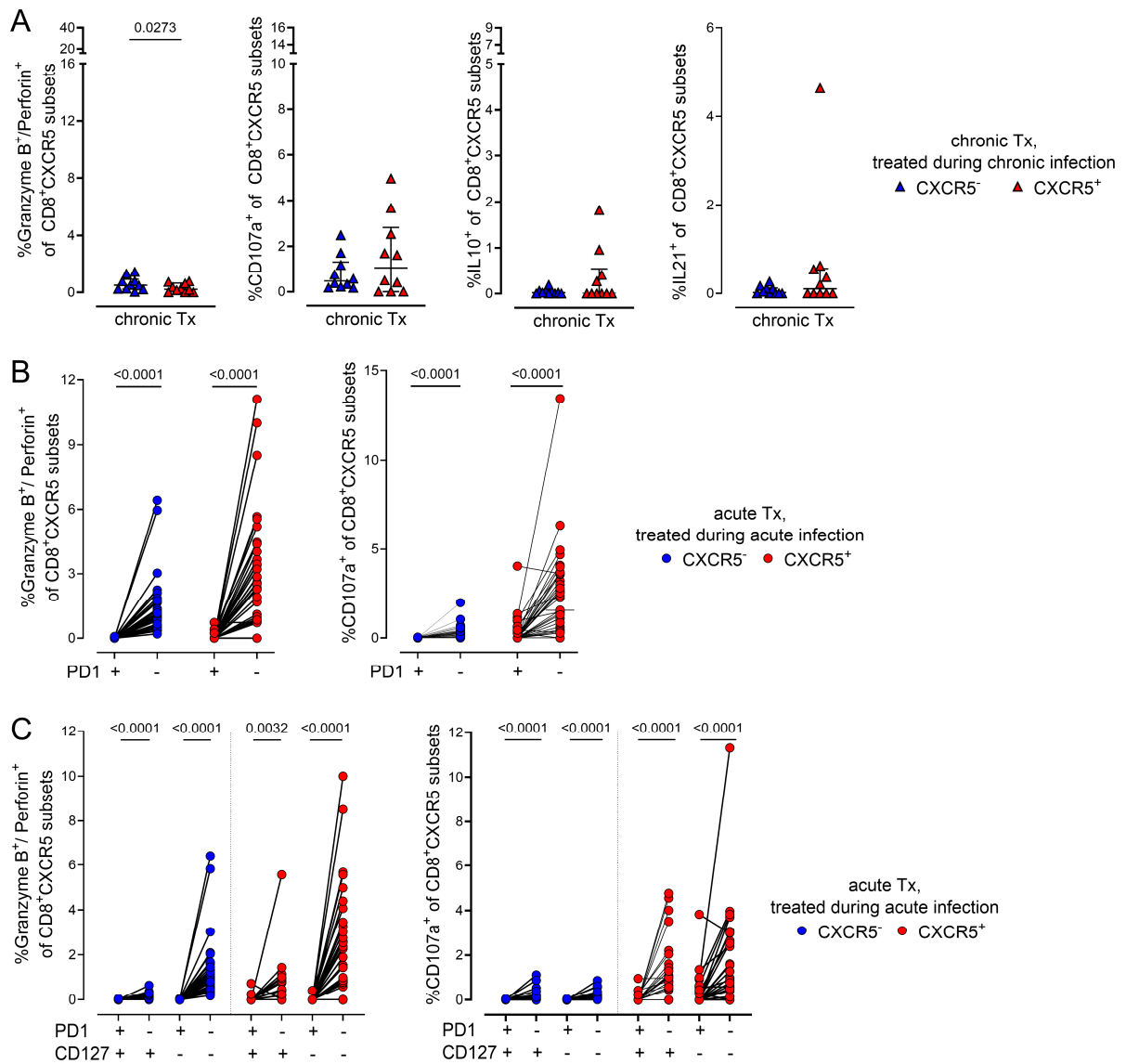
Supplemental Figure 2: Memory subset distribution in CXCR5+ vs CXCR5- CD8 T cells.

(A) Exemplary gating strategy for memory subset distribution in CXCR5+ vs CXCR5- CD8 T cells. TCM: central memory (CCR7+CD45RA-), TN: naïve (CCR7+CD45RA+), TEMRA: effector memory re-expressing CD45RA (CCR7-CD45RA+), TEM: effector memory (CCR7-CD45RA-). **(B-D)** Frequencies of CXCR5- (blue) and CXCR5+ (red) CD8 T cells expressing PD1 **(B)**, CD127 **(C)** and co-expressing PD1 and CD127 **(D)** in individuals diagnosed and treated during chronic infection (n=10). P-values by Wilcoxon matched-pairs signed rank test. Medians and IQRs are indicated.



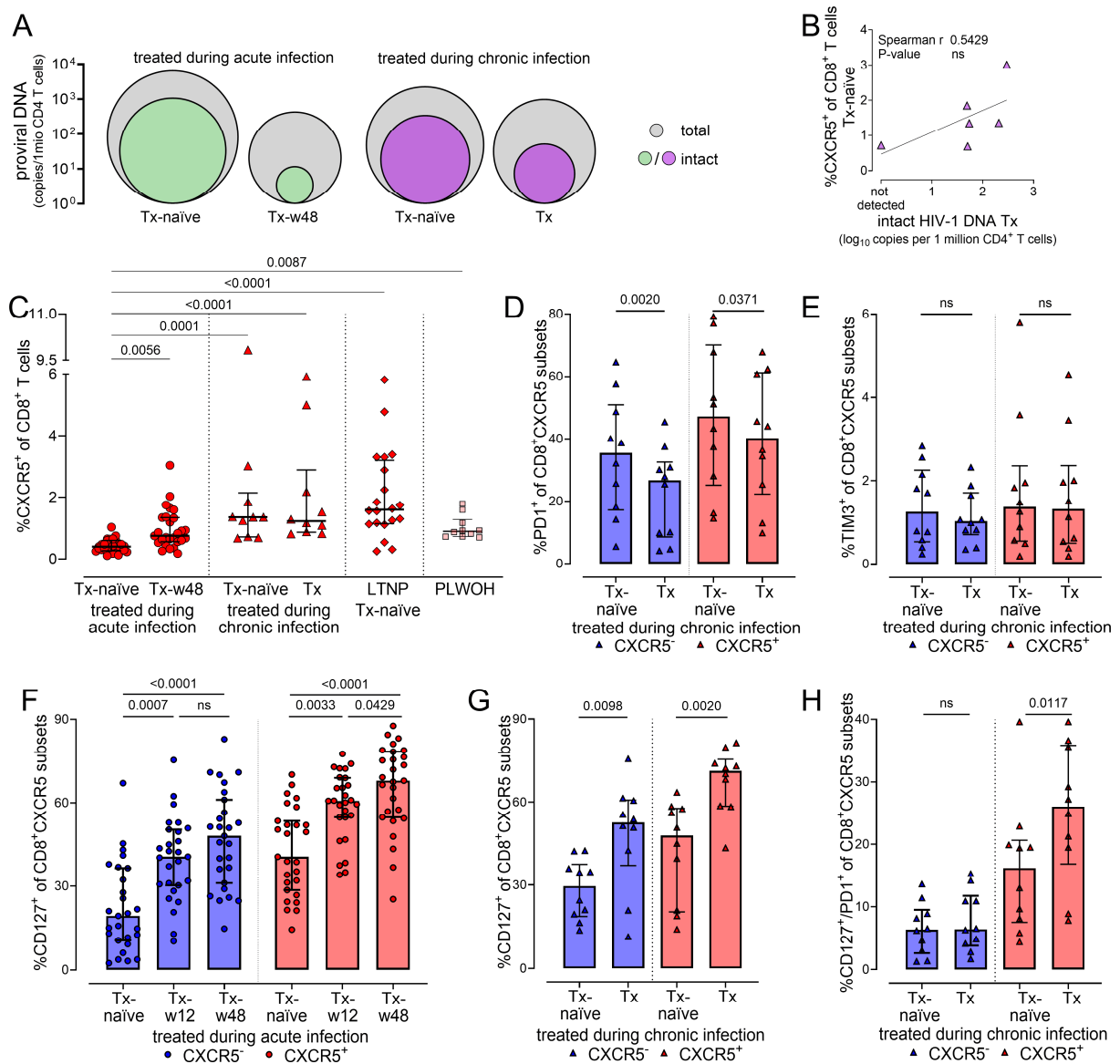
Supplemental Figure 3: Differing transcriptomic profile in CXCR5- and CXCR5+ CD8 T cells and confirmation of selected DEGs at a protein level.

(A) Quantification of CD8 T cells inside and outside the germinal center (GC) in tonsil stains from the same donors as shown in Figure 3, A-C and Supplemental Figure 3B. Percentage positivity is calculated with the following formula: %CD8 T cells=(area of positive region/total area)x100%. P-value was calculated with Mann-Whitney-test. **(B-C)** Immunofluorescence microscopy of tonsil tissue from a participant living without HIV: stained for CXCR5 (orange), CD8 (pink) and HIV p24 (green) **(B)** and mouse IgG isotype negative control which was used with each of the opal fluorophores **(C)** within germinal centers (GC), mantle zone (MZ) or extrafollicular (EF) regions. Spectral DAPI was used as a nuclear counterstain (blue). Imaging was performed with a Zeiss Axio Z2 microscope at 40x magnification and scale bars =100 μ m. **(D)** GSEA showing the enrichment of genes associated with exhaustion in CXCR5- but not CXCR5+ circulatory CD8 T cells. NES=normalised enrichment score. **(E)** Expression of selected proteins encoded by DEGs in people treated during acute HIV infection prior ART initiation (n=23) detected by flow cytometry in CXCR5+ and CXCR5- CD8 T cells. Median fluorescent intensity (MFI) of CXCR5 subsets and IQR are depicted. P-values were calculated with Wilcoxon matched-pairs signed rank test. **(F)** Volcano plot showing upregulated (red) and downregulated (blue) DEGs in CXCR5+ vs CXCR5- CD8 T cells in people living with HIV (n=13; max 4 weeks on ART). Cut-offs were set to adjusted p-value <0.05 and absolute log₂ fold-change >1. Selected immune-related genes are highlighted.



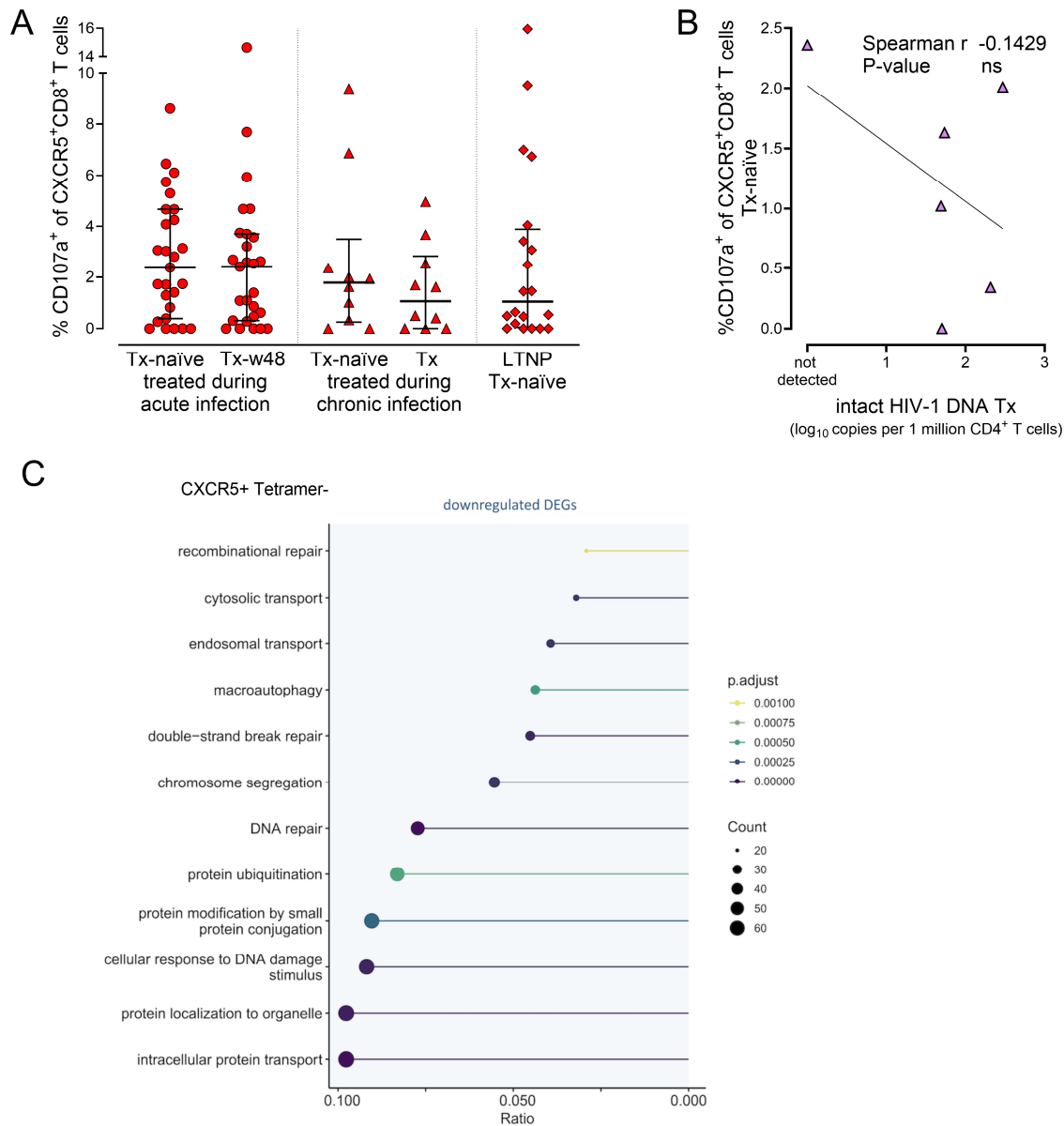
Supplemental Figure 4: Expression of several effector function markers following stimulation on CXCR5- vs CXCR5+ CD8 T cells.

(A) Frequency of CXCR5⁻ (blue) and CXCR5⁺ (red) CD8 T cells co-expressing Granzyme B and Perforin ex-vivo, and CD107a, IL10, or IL21 after stimulation with HIV-gag in individuals diagnosed and treated during chronic infection (n=10). P-values by Wilcoxon matched-pairs signed rank test. Medians and IQRs are depicted. **(B and C)** Frequency of CXCR5⁻ (blue) and CXCR5⁺ (red) CD8 T cells co-expressing Granzyme B and Perforin ex-vivo (left) or expressing CD107a (right) in response to overnight (O/N) stimulation with HIV-gag peptide pool in perspective to PD1 **(B)** or combined PD1 and CD127 **(C)** expression in individuals treated during acute infection (n=37; median time on treatment 52 weeks). P-values by Wilcoxon matched-pairs signed rank test.



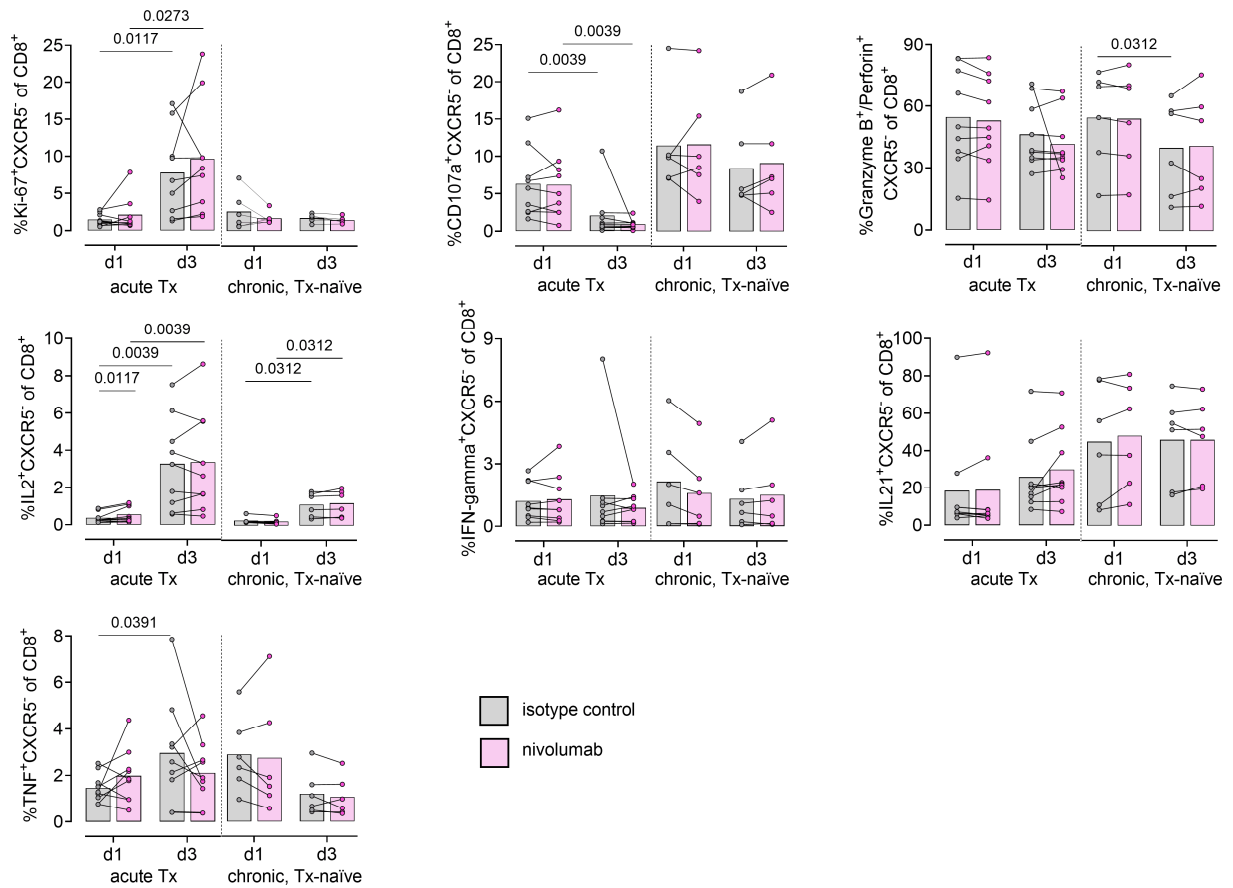
Supplemental Figure 5: Longitudinal dynamics of proviral HIV DNA and phenotypic markers in people treated during acute or chronic HIV infection.

(A) Median copies of intact (colored) or total (gray) proviral HIV DNA per one million CD4 T cells prior and during ART in individuals treated during acute (prior ART n=22; during ART n=25; green) and chronic (prior ART n=8; during ART n=6; purple) infection. **(B)** Association between the frequencies of CXCR5⁺ CD8 T cells prior ART and levels of intact proviral HIV-DNA in individuals treated during chronic infection (n=6; median time on ART 18 weeks). Spearman rank correlation was performed. **(C)** Frequencies of CXCR5⁺ CD8 T cells prior and during ART in individuals with acute (n=27; circles) and chronic (n=10; triangles) HIV infection, individuals naturally controlling HIV (n=20; diamonds) and people living without HIV (PLWOH; n=10; squares). P-values by Kruskal-Wallis-test with Dunn's correction for multiple comparisons. Medians and IQRs are depicted. **(D-H)** Effect of ART initiation on PD1 **(D)**, TIM3 **(E)**, CD127 **(F and G)** and CD127/PD1 **(H)** (co-)expression on CXCR5⁻ (blue) and CXCR5⁺ (red) CD8 T cells in individuals treated during chronic **(D, E, G and H)**; n=10; median time on ART 18 weeks) or acute **(F)**; TopHIVFUTURE; n=27) infection. P-values by Wilcoxon matched-pairs signed rank test (comparison of two groups) or Friedman test with Dunn's correction for multiple comparisons (comparison of >2 groups). Medians and IQRs are displayed.



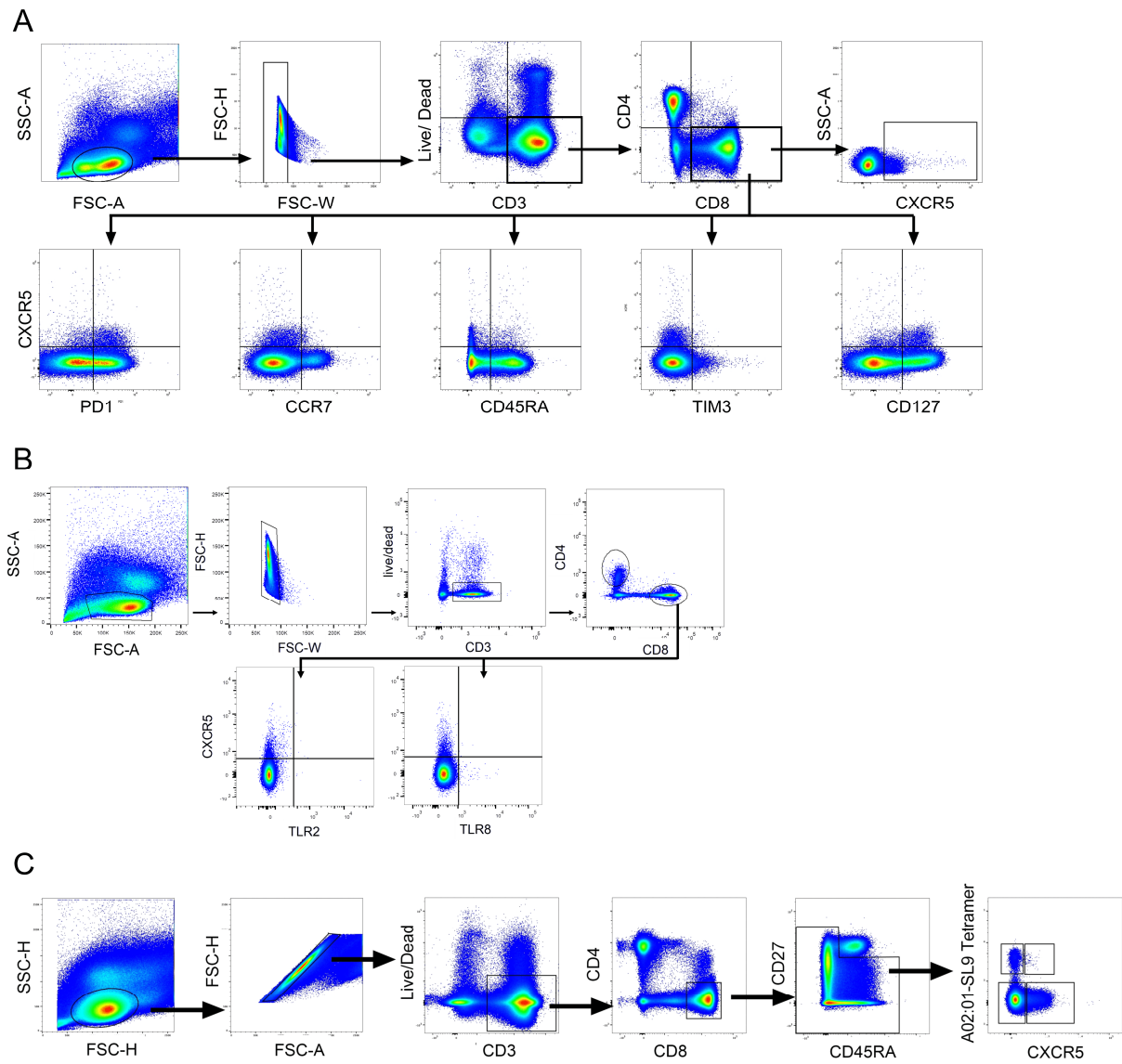
Supplemental Figure 6: Influence of ART initiation on CD107a expression and on downregulation of differentially expressed genes.

(A) Frequencies of CD107a⁺CXCR5⁺ CD8 T cells after O/N stimulation with HIV-gag peptide pool in individuals diagnosed and treated during acute (n=27; circles) or chronic infection (n=10; triangles) prior and during ART and in individuals naturally controlling HIV (LTNP; n=20; diamonds). Kruskal-Wallis-test corrected for multiple comparisons was performed. Medians and IQRs are displayed. **(B)** Association between HIV-specific CD107a-expressing CXCR5⁺ CD8 T cells responding to O/N stimulation with HIV-gag peptide pool at baseline and intact HIV DNA after median 18 weeks of ART in individuals treated during chronic infection (n=6). Spearman rank correlation was performed. **(C)** Enriched GO terms (biological process) for significantly differentially expressed genes that are downregulated in CXCR5⁺ CD8 T cells w4 vs w48 post ART initiation (BH adjusted p-values <0.1*10⁻²). Length of the line represents the ratio of the number of observed differentially expressed genes (DEGs) per pathway and total number of DEGs, dot size represents the absolute observed number of DEGs per pathway. Color code is according to the adjusted p-value.



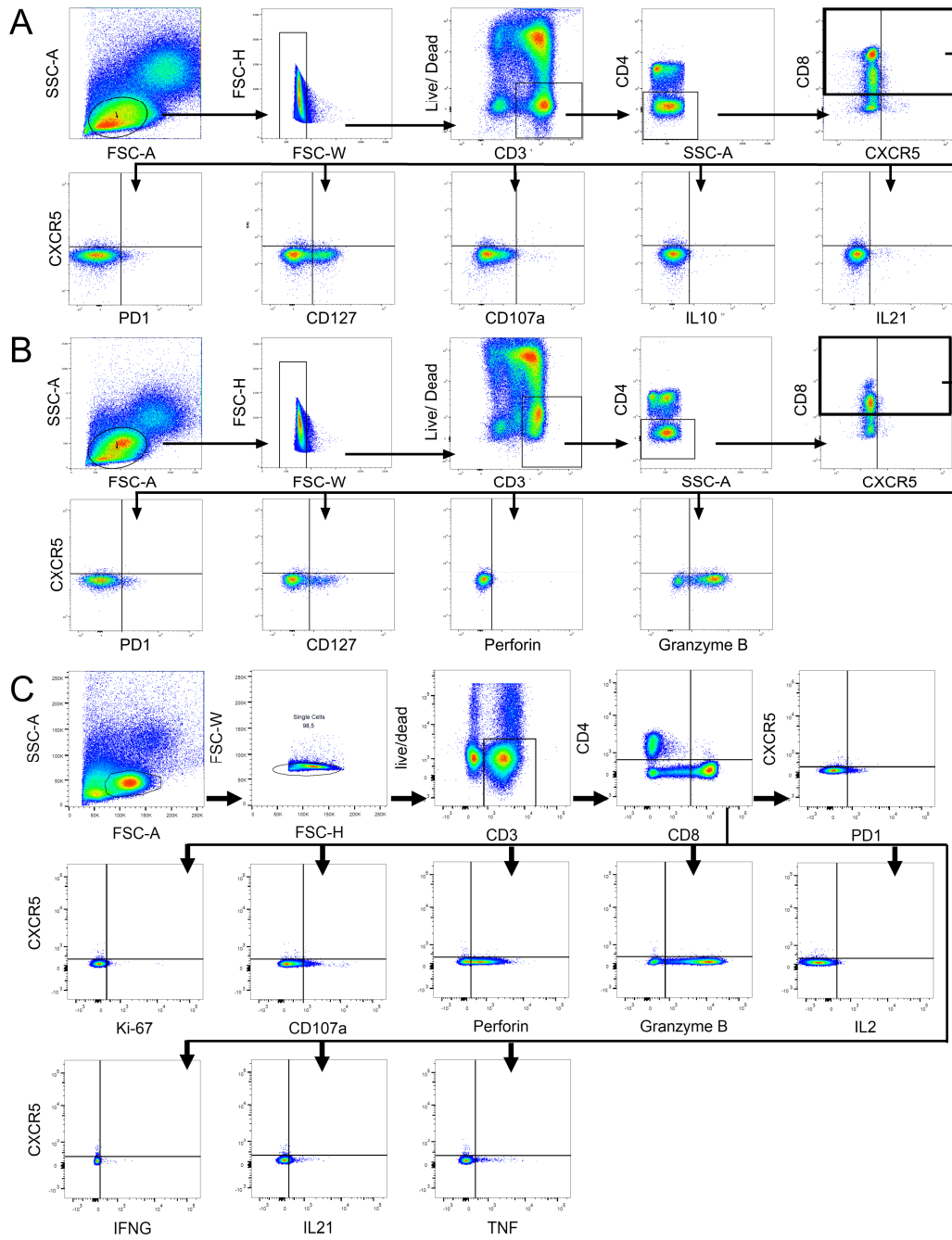
Supplemental Figure 7: Nearly absent influence of PD1-blockade on CXCR5- CD8 T cells.

PBMC were stimulated with HIV-gag peptide pool for 20h (d1) or 68h (d3) in presence of a PD1-inhibitor (nivolumab; pink) or isotype control (gray) as described for the corresponding Figure 8. Expression of markers for proliferation (Ki-67), cytotoxicity (CD107a, Granzyme B and Perforin), and non-cytotoxic effector functions (IL2, IFN-gamma, IL21, TNF) of CXCR5- CD8 T cells from individuals treated during acute infection (left; 12 weeks on ART; n=9) or living with chronic HIV infection (right; Tx-naïve; n=3 viremic and n=3 naturally controlling HIV). P-values are calculated with Wilcoxon matched-pairs signed rank test.



Supplemental Figure 8: Exemplary gating strategy for phenotypic markers.

(A) Extracellular markers detected by flow cytometry, **(B)** confirmation of the expression of selected DEGs by flow cytometry and **(C)** FACS gating strategy for tetramer sorting of HIV-specific CD8 T cells are shown.



Supplemental Figure 9: Exemplary gating strategy for intracellular cytokine staining assays measured by flow cytometry.

PBMCs were stimulated O/N with a gag peptide pool (**A**), kept unstimulated (**B**) or additionally treated with a PD1-blockade (sample without PD1 blockade is depicted) (**C**).

Supplemental Table 2: Characteristics of participants for viral inhibition assay.

| Participant | Sex | Age (years)^A | CD4-count (cells/μl)^B | Plasma viral load (copies/ml)^B | Time diagnosis to sampling (months)^A | Time between samples (months) |
|--------------------|------------|--------------------------------|--|--|--|--------------------------------------|
| 38 | m | 28 | 589 | 134,946 | 8 | 9 |
| 48 | m | 22 | 599 | 46,372 | 1 | 7 |
| 53 | m | 28 | 639 | 137,976 | 10 | 12 |

^Aas first blood sample was drawn; ^Bmean value of the blood samples

Supplemental Table 3: Summary of main reagents and their supplier.

| Reagent | Source | Concentration (µg/ml) | Used for... |
|---|-----------------------------------|-----------------------|--|
| Histopaque 1077 | Sigma Aldrich | n.a. | PBMC processing |
| SL9 monomers | Provided by David A. Price | n.a. | Low input RNA-sequencing |
| RM9 monomers | ImmuneAware | n.a. | Low input RNA-sequencing |
| KL9 monomers | MBL International | n.a. | Low input RNA-sequencing |
| Zombie NIR™ Fixable Viability Kit | BioLegend | n.a. | Flow cytometry/cell sorting for RNA-sequencing |
| FIX & PERM Medium A&B | Thermo Fisher Scientific | n.a. | Flow cytometry |
| Rainbow beads | BD Bioscience | n.a. | Flow cytometry |
| HIV gag and nef | NIBSC | 12.5 | ELISpot |
| IFNG monoclonal antibody 1-D1K (#3420-3-250) | Mabtech | n.a. | ELISpot |
| SEB | Sigma Aldrich | 12.5 | ELISpot |
| FEC-peptide pool (CMV, EBV, Influenza peptides) | NIBSC | 12.5 | ELISpot |
| IFNG detection antibody 7-B6-1 (#3420-6-250) | Mabteck | n.a. | ELISpot |
| AP Conjugated Substrate Kit | Biorad | n.a. | ELISpot |
| Opal™ 4-Color Manual IHC Kit | Ayoka Biosciences | n.a. | Multiplex immunofluorescence microscopy |
| SuperScript II Reverse Transcriptase | Thermo Fisher Scientific | n.a. | Low input RNA-sequencing |
| KAPA HiFi HotStart ReadyMix | Roche | n.a. | Low input RNA-sequencing |
| Nextera XT DNA Library Preparation Kit | Illumina | n.a. | Low input RNA-sequencing |
| Nextera XT Index Kit v2 | Illumina | n.a. | Low input RNA-sequencing |
| Agencourt AMPure XP beads | Beckman Coulter | n.a. | Low input RNA-sequencing |
| Qubit dsDNA HS Assay Kit | Thermo Fisher Scientific | n.a. | Low input RNA-sequencing |
| Agilent High Sensitivity DNA Kit | Aglient | n.a. | Low input RNA-sequencing |
| HIV SF2 overlapping peptide pools spanning gag proteins | NIBSC | 2 | Intracellular cytokine staining assays |
| SEB | Sigma Aldrich | 1 | Intracellular cytokine staining assays |

| | | | |
|---|--------------------------|------|---|
| Anti-CD28 antibody (#555726) | BD Bioscience | 0.5 | Intracellular cytokine staining assays |
| Anti-CD49d antibody (#555502) | BD Bioscience | 0.5 | Intracellular cytokine staining assays |
| Brefeldin A | Sigma Aldrich | 10 | Intracellular cytokine staining assays |
| GolgiPlug protein transport inhibitor | BD Bioscience | 0.75 | Intracellular cytokine staining assays |
| EasySep™ Human CD4 T Cell Isolation Kit | StemCell Technologies | n.a. | Intact proviral DNA assay; viral inhibition assay |
| DNAzoI™ | Invitrogen | n.a. | Intact proviral DNA assay |
| QiaCuity Probe PCR Kit | Qiagen | n.a. | Intact proviral DNA assay |
| HIV p24 ELISA | Perkin-Elmer | n.a. | Viral inhibition assay |
| Gag-peptide pool (ARP-12437) | NIH HIV reagents program | 0.5 | PD1 blockade |
| Nivolumab (BMS-936558) | Bristol-Myers Squibb | 10 | PD1 blockade |
| IgG4-isotype control (clone QA16A15) | BioLegend | 10 | PD1 blockade |

Supplemental Table 4: Composition of buffer and media prepared in the lab.

| Buffer/medium | Composed of... | Used for... |
|----------------------|---|--------------------------|
| Cryo medium | 90% FCS, 10% DMSO | PBMC processing/storage |
| R10 medium | RPMI-1640 supplemented with 10% FCS, 1% L-glutamine, 1% HEPES, 1% penicillin/streptomycin | Cell culture |
| FACS buffer | PBS containing 1mM EDTA, 0.5% FCS or BSA | Cell sorting |
| Lysis buffer | 0.2% Triton-X-100, 100 μ M Oligo dT primer, 10mM dNTP mix, 40U/ μ l recombinant Rnase Inhibitor (Takara Bio in molecular grad H ₂ O) | Low input RNA-sequencing |
| R10-50 | R10 with 50U/ml IL2 | Viral inhibition assay |
| R10 containing ART | R10 medium supplemented with 18 μ M Azidothymidin, 10 μ M Nevirapine, 20 μ M Raltegravir | PD1 blockade |

Supplemental Table 5: Devices.

| Device | Manufacturer | Used for... |
|--|----------------------------|---|
| Casy 1TT | SCHÄRFE SYSTEM | Cell counting |
| MoFlo Astrios Cell Sorter | Beckmann Coulter | Cell sorting |
| BD LSRFortessa™ | BD Bioscience | Flow cytometry |
| AID ELISPOT reader ELR04 | Autoimmun Diagnostika GmbH | ELISpot |
| Shandon Finesse 325 rotary microtome | Thermo Fisher Scientific | Multiplex immunofluorescence microscopy |
| Axio Z2 microscope | Zeiss | Multiplex immunofluorescence microscopy |
| HiSeq™ | Illumina | Low input RNA-sequencing |
| Novaseq™ | Illumina | Low input RNA-sequencing |
| Quantus™ Fluorometer | Promega | Intact proviral DNA assay |
| QiaCuity One 5-plex digital PCR instrument | Qiagen | Intact proviral DNA assay |

Supplemental Table 6: List of antibodies used for flow cytometry and multiplex immunofluorescence microscopy.

| Antigen | Fluorochrome | Clone | Source | Identifier | Pheno | ICS | Sorting for RNAseq | Sorting for viral inhibition assay | PD1 Block | Confirmation of selected DEGs | Immunofluorescence microscopy |
|------------|-------------------|----------|-----------------|---------------|-------|-----|--------------------|------------------------------------|-----------|-------------------------------|-------------------------------|
| CCR7 | BV 650 | G043H7 | BioLegend | 353233 353234 | x | | | | | | |
| CD107a | BV 785 | H4A3 | BioLegend | 328644 | | x | | | x | | |
| CD127 | PE/Cyanine 5 | R34.34 | Beckman Coulter | A64617 | x | x | | | x | | |
| CD161 | BV 421 | HP-3G10 | BioLegend | 339913 | x | | | | | | |
| CD27 | PE | M-T271 | BioLegend | 356406 | | | x | | | | |
| CD3 | BV 510 | OKT3 | BioLegend | 317332 | x | x | x | x | x | | |
| CD39 | BUV 737 | TU66 | BD Bioscience | 564726 | x | | | | | | |
| CD4 | PerCP/Cyanine 5.5 | RPA-T4 | BioLegend | 300530 | x | x | x | x | x | | |
| CD45RA | BUV 395 | HI100 | BD Bioscience | 740298 | x | | | | | | |
| CD45RA | BV 421 | HI100 | BioLegend | 304130 | | | x | | | | |
| CD57 | PE | NK-1 | BD Bioscience | 560844 | x | | | | | | |
| CD73 | PE/Dazzle 594 | AD2 | BioLegend | 344019 | x | | | | | | |
| CD8 | AlexaFluor 700 | HIT8a | BioLegend | 300920 | x | x | x | x | x | | |
| CXCR5 | AlexaFluor 488 | RF8B2 | BD Bioscience | 558112 | x | x | x | x | x | | |
| Granzyme B | Pacific blue | GB11 | BioLegend | 515408 | | x | | | x | | |
| HLA-DR | PE/Cyanine 7 | 3D12 | BioLegend | 342607 | x | | | | | | |
| IFNG | BUV 395 | B27 | BD Bioscience | 563563 | | x | | | x | | |
| IL10 | BV 650 | JES3-9D7 | BD Bioscience | 564051 | | x | | | | | |
| IL17A | APC | BL168 | BioLegend | 512334 | | x | | | | | |

| | | | | | | | | | | | |
|-----------|---------------|-------------|----------------------|-----------|---|---|--|--|---|---|---|
| IL21 | PE | 3A3-N2 | BioLegend | 513004 | | x | | | x | | |
| PD1 | BV 605 | EH12.2H7 | BioLegend | 329923 | x | x | | | x | | |
| Perforin | PE/Dazzle 594 | dG9 | BioLegend | 308132 | | x | | | x | | |
| TCR Vα7.2 | BV 785 | 3C10 | BioLegend | 351721 | x | | | | | | |
| | | | | 351722 | | | | | | | |
| TIM3 | BV 711 | F38-2E2 | BioLegend | 345024 | x | | | | | | |
| TNF | PE/Cyanine 7 | MAB11 | BioLegend | 502930 | | x | | | x | | |
| Ki-67 | BV711 | B56 | BD Bioscience | 563755 | | | | | x | | |
| IL2 | APC | MQ1-17H12 | BioLegend | 500309 | | | | | x | | |
| CTLA | BUV 737 | 14D3 | Invitrogen | 367152941 | | | | | x | | |
| CD4 | AF700 | RPA-T4 | BioLegend | 300526 | | | | | | x | |
| CD3 | BUV395 | SK7 | BD Bioscience | 564001 | | | | | | x | |
| CD69 | BUV737 | FN50 | BD Bioscience | 612817 | | | | | | x | |
| CD8 | BV480 | RPA-T8 | BD Bioscience | 566121 | | | | | | x | |
| TLR2 | FITC | TL2.1 | BioLegend | 309706 | | | | | | x | |
| HLADR | RB705 | G46-6 | BD Bioscience | 756955 | | | | | | x | |
| TLR5 | PE | S16021I | BioLegend | 394504 | | | | | | x | |
| CXCR5 | PE-Cy7 | J252D4 | BioLegend | 356924 | | | | | | x | |
| CD172a | APC | 15-414 | BioLegend | 372106 | | | | | | x | |
| CD38 | BUV496 | HIT2 | BioLegend | 612946 | | | | | | x | |
| TLR8 | BV421 | S16018A | BioLegend | 395510 | | | | | | x | |
| CXCR5 | / | EPR23463-30 | Abcam | ab254415 | | | | | | | x |
| CD8 | / | C8/144B | Agilent Technologies | M710301-2 | | | | | | | x |
| HIV-p24 | / | Kal-1 | Agilent Technologies | M085701-8 | | | | | | | x |

Supplemental Table 7: Primers used for intact proviral DNA assay.

| Primer/probe | Sequence |
|---------------------|---------------------------|
| Env-MA f | AGTGGTGSAGAGAGAAAAAAGAGC |
| Env-TV p V3 | AA+G C+AC T+A+T +GGG C |
| Env-TV p comp. | A+GC +ACT +AT+A +AGC GC |
| Env-MA r | CTGGCCTGTACCGTCAGCG |
| | |
| Psi-MA f | GCAGGACTCGGCTTGCTG |
| Psi-MA p | CGACTG+GTGA+GTACGCC |
| Psi-MA r | CACCCATCTCTCTCCTTCTAGCC |
| | |
| RPP30 fwd | GATTTGGACCTGCGAGCG |
| RPP30 probe | CTGACCTGAAGGCTCT |
| RPP30 rev | GCGGCTGTCTCCACAAGT |
| | |
| RPP30d1 fwd | AGAGAGCAACTTCTTCAAGGG |
| RPP30d1 probe | CCCGGCTCTATGATGTTGTTGCAGT |
| RPP30d1 rev | TCATCTACAAAGTCAGAACATCAGA |

“+” indicates usage of locked nucleic acids (LNAs)

Supplemental Table 8: Sex as biological variable.

| Group | Frequency of male participants^A |
|--------------|---|
| PLWOH | 40% (4/10) |
| LTNP | 60% (12/20) |
| TopHIVFUTURE | 96% (26/27) |
| TopHIVPAST | 100% (10/10) |
| Chronic | 90% (9/10) |
| Tonsils | 0% (0/4) |

^Ain brackets: ratio of male to total participants