Supplementary Information	
Article Title	Potent inhibition of HIV-1 by TRIM5-cyclophilin fusion proteins engineered from human components
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SUPPLEMENTARY METHODS

Proliferation of hT5Cyp-expressing primary human CD4 T cells. Activated, scAPLS-transduced primary human $CD4^+$ T cells were maintained in culture for 6 days. 1 x 10^5 cells were transferred in triplicate into 96-well flat-bottom microplates (Falcon) and labelled for 18 h with 1 Ci of $^{[3H]}$thymidine (GE-Amersham). The DNA-incorporated radioactivity was measured by liquid scintillation counting. Data were expressed as mean corrected counts per minute (ccpm) of quadruplicate cultures.

Staining for intracellular IL-2. IL-2 producing capacity of hT5Cyp-expressing primary human T cells was assessed after stimulation for 6 h with 100 nM PMA and 1 μg/ml Ionomycin (Sigma-Aldrich). Brefeldin A (Sigma-Aldrich) was added at 10 μg/ml during the last 4 h of stimulation. Cells were washed, permeabilized, and fixed by treatment with BD Cytofix/Cytoperm and stained with anti-IL-2 antibody (5344.111) in BD Permwash solution following the manufacturer's instructions (all reagents BD/Pharmingen).

Staining for cell surface markers. The following antibodies were used for cell surface stains of primary $CD4^+$ T cells: α-CD4 (RPA-T4), α-CXCR4 (12G5), and α-MHC I (HLA A, B, C) (G46-2.6) (all from BD-Pharmingen).

Real-Time RT-PCR. Total RNA was extracted from 5x10^6 $CD4^+$ T cells using the RNeasy Plus Mini kit (Qiagen). RNA was treated with RNase-free DNase I (Ambion) and reverse transcribed using the SuperScript™ III First-Strand Synthesis System

(Invitrogen). qPCR was performed with the Applied Biosystems 7900HT system, using Taqman Gene Expression or Power SYBR green PCR master mixes (Applied Biosystems). Each experimental condition was performed in triplicate and data analyzed using the SDS software, version 2.2.2 (Applied Biosystems). Following primers were used: for 18S RNA 5': CGGCTACCACATCCAAGGAA, 18S RNA 3': GCTGGAATTACCGCGGCT, GFP 5': CCCCGTGATGAAGAAGATGA, GFP 3': GTCAGCTTGTGCTGGATGAA, hT5Cyp 5': CTGGGTTGATGTGACAGTGG, hT5Cyp 3': TCTGCTGTCTTTGGGACCTT.

qPCR for HIV-1 DNA. Total cellular DNA was extracted from $2x10^6$ Jurkat T cells using the DNeasy Blood and Tissue kit (Qiagen). qPCR was performed with the Applied Biosystems 7900HT system, using 250 ng total DNA and Taqman Gene Expression master mix (Applied Biosystems). Each experimental condition was performed in triplicate and data analyzed using the SDS software, version 2.2.2 (Applied Biosystems). Following primers were used:

Mitochondrial forward primer (MH533): ACCCACTCCCTCTTAGCCAATATT,

Mitochondrial reverse primer (MH534): GTAGGGCTAGGCCCACCG,

Mitochondrial probe (mito-probe): 5'-(TET)-CTAGTCTTTGCCGCCTGCGAAGCA-(TAMRA)-3',

J1 RT forward: ACAAGCTAGTACCAGTTGAGCCAGATAAG,

J2 RT reverse: GCCGTGCGCGCTTCAGCAAGC,

RT probe (LRT-P): 5'-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3'

huRag2$^{-/-}\gamma_c^{-/-}$ mice. huRag2$^{-/-}\gamma_c^{-/-}$ mice were generated as previously described[1]. Briefly, fresh human cord blood was obtained with parent written informed consent from healthy full-term newborns (Department of Gynecology and Obstetrics, Ospedale San Giovanni, Bellinzona). CD34$^+$ cells were enriched from fresh cord blood to > 95 % purity (with less than 0.1% CD3$^+$ T cells) and frozen until newborn mice were available for transplant[1]. Prior to transplant, CD34$^+$ cells were thawed and resuspended in RPMI with serum replacement (StemCell Technologies). Transduction of human CD34$^+$ cells was performed as follows: cells were pretreated for three hours with SIV VLPs (50% per volume, made with SIV3+ vector and pMD2.G at 7:1 ratio) prior to viral transduction of three hours duration using concentrated scAPLS vectors coding for either hT5Cyp or hT5CypH126Q. Cells were then washed in PBS and injected into newborn, conditioned Rag2$^{-/-}\gamma_c^{-/-}$ mice as previously described[1]. Mice were infected with HIV-1 by intraperitoneal injection as previously described[2].

1. Traggiai, E.*, et al.* Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science (New York, N.Y* **304**, 104-107 (2004).
2. Baenziger, S.*, et al.* Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2-/-gamma c-/- mice. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 15951-15956 (2006).

Supplementary Table S1: Cloning Primers

Name	Primer Sequence
External Primers hT5Cyp	
hT5Cyp XbaI 5’	5’- CCCTCTAGAGCCACCATGGCTTCTGGAATCCTGGTTA-3’
hT5Cyp BamH1 3’	5’- AAAAGGATCCTTATTCGAGTTGTCCACAGTC-3’
Internal Primers hT5Ccyp	
M244F	5’- CGGCTGCAGGGGTCAGTGATGGTCAACCCCACCGTGTTC-3’
M244R	5’- GAACACGGTGGGGTTGACCATCACTGACCCCTGCAGCCG-3’
M284F	5’- GCTCCTGATCTGAAAGGAATGGTCAACCCCACCGTGTTC-3’
M284R	5’- GAACACGGTGGGGTTGACCATTCCTTTCAGATCAGGAGC-3’
W298F	5’- GGTGGGGTTGACCCAGTAGCGTCGGACATCTGTC-3’
W298R	5’- CGACGCTACTGGGTCAACCCCACCGTGTTC-3’
T302F	5’-CGCTACTGGGTTGATGTGACAGTCAACCCCACCGTGTTC-3’
T302R	5’- GAACACGGTGGGGTTGACTGTCACATCAACCCAGTAGC-3’
S309F	5’- GTGGCTCCAAACAACATTTCAGTCAACCCCACCGTGTTC-3’
S309R	5’-GAACACGGTGGGGTTGACTGAAATGTTGTTTGGAGCCAC-3’
S314F	5’- TGGGTTGATGTGACAGTGGCTCCAAACAACATTTCATGTGC TGTCATTTCGTCAACCCCACCGTG-3’
S314R	5’-GTTGACAGAAATGACAGCACATGAAATGTTGTTTGGAGCC ACTGTCACATCAACCCAGTAGCGTCGGAC-3’
S322F	5’- CAAGTGAGCTCTGTCAACCCCACCGTGTTC-3’
S322R	5’- GGGGTTGACAGAGCTCACTTGTCTCTTATCTTCAG-3’
A331F	5’- CCACAGATAATATATGGGGCAGTCAACCCCACCGTGTTCTTC-3’
A331R	5’- CACGGTGGGGTTGACTGCCCCATATATTATCTGTGGTTTCG-3’
G357F	5’- GCTCTCAAAGTATCACATCAGGGGTCAACCCCACCGTGTTCTTC-3’
G357R	5’- GAAGAACACGGTGGGGTTGACCCCTGATGTGATACTTTGAGAGC-3’
T369F	5’- GAGGTAGACGTGTCCAAGAAAACTGTCAACCCCACCGTGTTCTTC-3’
T369R	5’- GAAGAACACGGTGGGGTTGACAGTTTTCTTGGACACGTCTACCTC-3’
G398F	5’- GAAAATTATCAACCTAAATACGGCGTCAACCCCACCGTGTTCTTC-3’
G398R	5’- GAAGAACACGGTGGGGTTGACGCCGTATTTAGGTTGATAATTTTC-3’
T5α cloning primers	
RhT5α Xba1 5’	5’-CCCTCTAGAGCCACCATGGCTTCTGGAATCCTGCTTA-3’
RhT5α Hpa1 3’	5’-AAAAGTTAACTCAAGAGCTTGGTGAGCACA-3’
hT5α Xba1 5’	5’-CCCTCTAGAGCCACCATGGCTTCTGGAATCCTGGTTA-3’
hhT5α Hpa1 3’	5’-AAAAGTTAACTCAAGAGCTTGGTGAGCACAGAGT-3’

Supplementary Figure S1

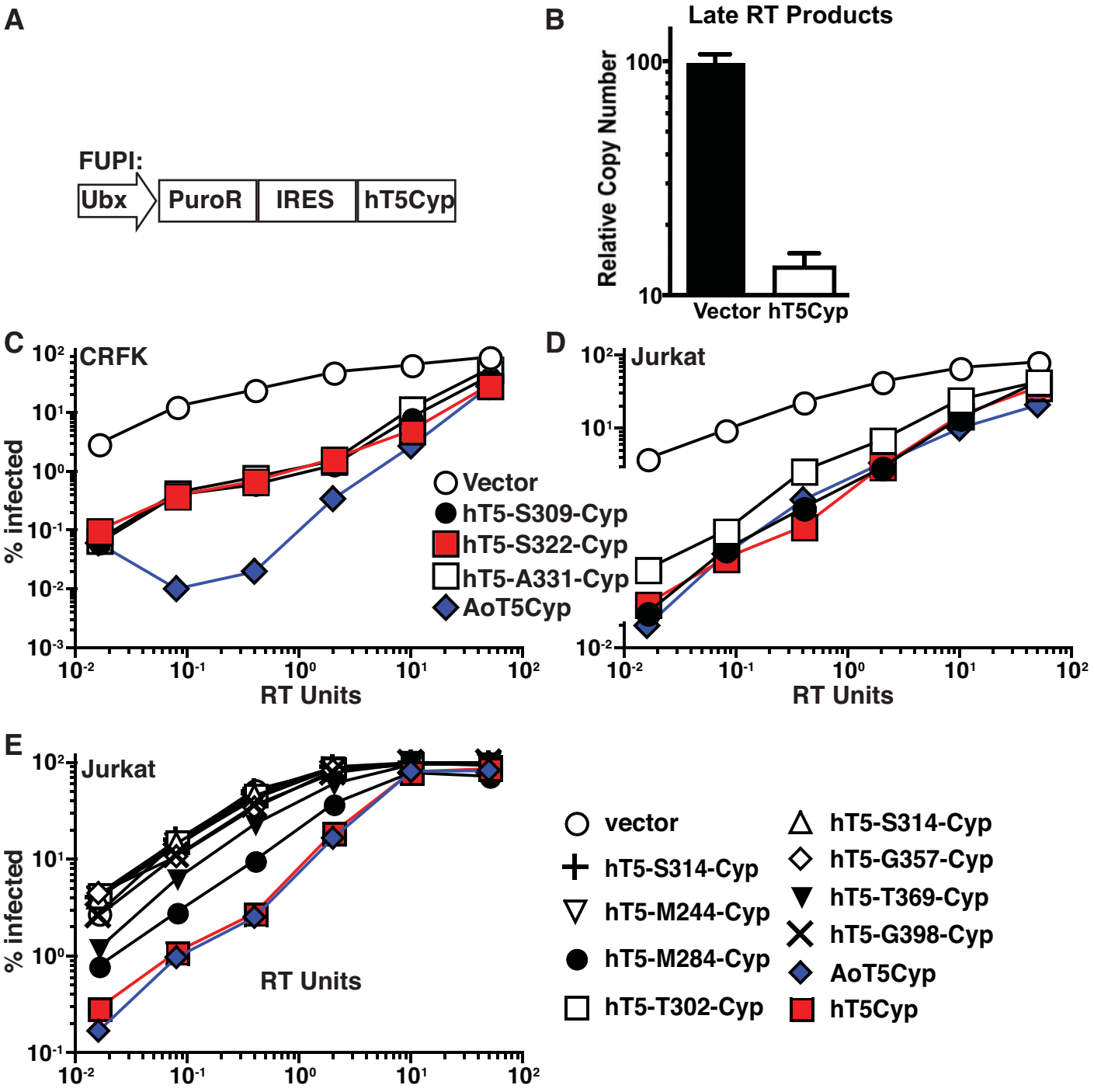

Figure S1. **HIV-1 restriction activity of different T5Cyp fusion proteins.** (**A**) Design of a bicistronic lentiviral vector, FUPI, used to establish T5-expressing cell lines. Ubx = ubiquitin promoter, PuroR = puromycin N-acetyltransferase, IRES = internal ribosome entry site. (**B**) Total cellular DNA was purified from HIV-1 vector-infected Jurkat T cell lines. Late RT products were quantified by PCR. DNA was normalized to mitochondrial DNA content. (**C, D**) Comparison of restrictive T5Cyp fusions in CRFK (**C**) and Jurkat (**D**) cells. (**E**) Comparison of all designed fusions with reduced anti-HIV-1 activity to restrictive T5Cyp and AoT5Cyp in Jurkat T cells. In all cases, cells were transduced with FUPI encoding puromycin-resistance and the indicated T5Cyp fusion proteins. Pools of puromycin-resistant cells were infected with increasing amounts of an HIV-1 GFP vector (left to right on X-axis). The percentage of GFP+ positive cells (Y-axis) was determined 48 hrs later.

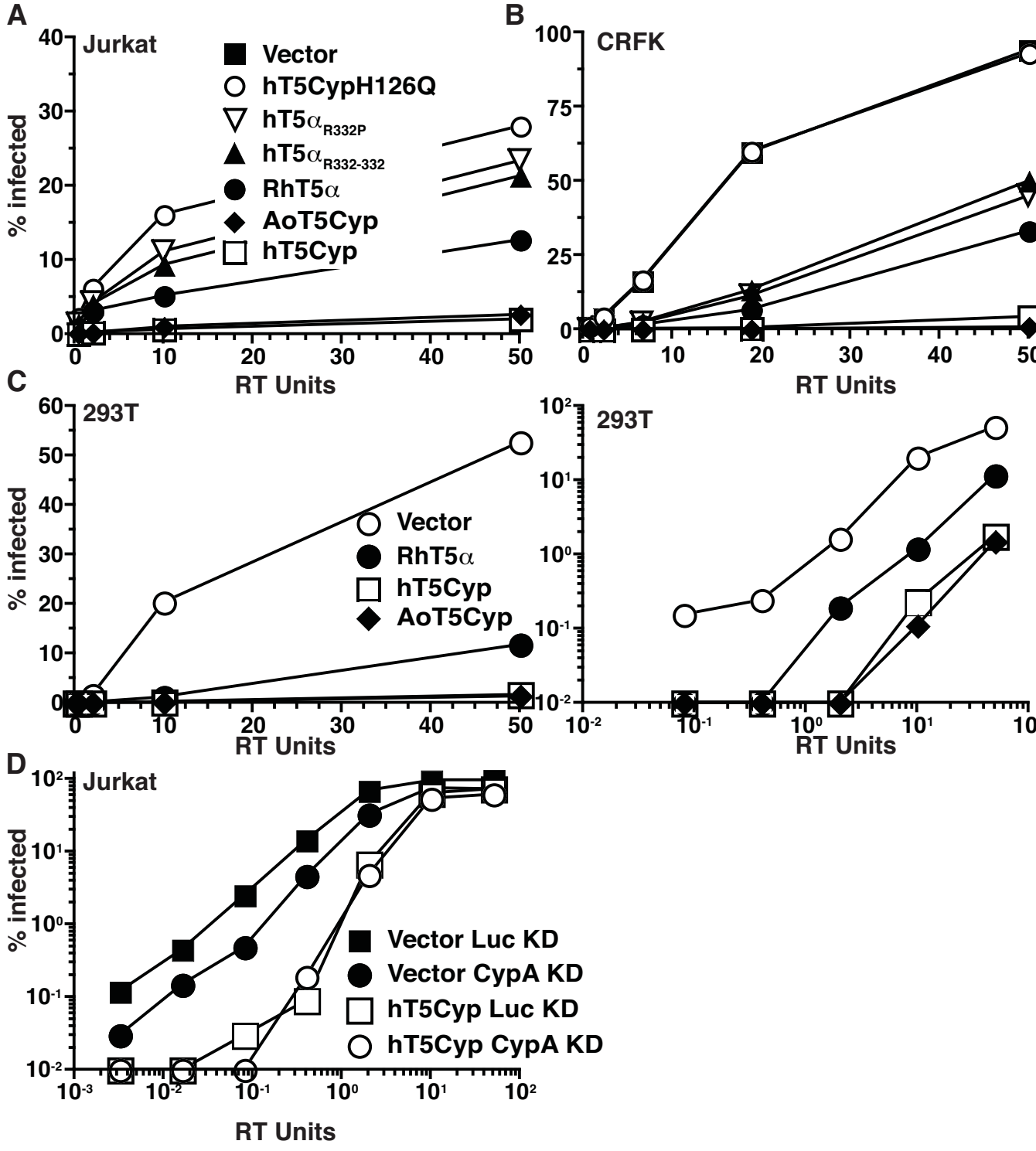

Figure S2. HIV-1 restriction activity of different T5 proteins. (A, B) Comparison of T5-mediated restriction of HIV-1 vectors in Jurkat **(A)**, CRFK **(B),** and 293T (**C;**left panel linear scale, right panel logarithmic scale) cells. **(D)** hT5Cyp restriction activity in Jurkat T cells stably transduced with lentiviral vectors coding for shRNAs targeting either cyclophilin A (CypA) or luciferase (Luc). In all cases, cells were transduced with vectors encoding puromycin-resistance and the indicated T5Cyp fusion proteins. Pools of puromycin-resistant cells were infected with increasing amounts of an HIV-1 GFP vector (left to right on X-axis). The percentage of GFP^+ positive cells (Y-axis) was determined 48 hrs later.

Supplementary Figure S3

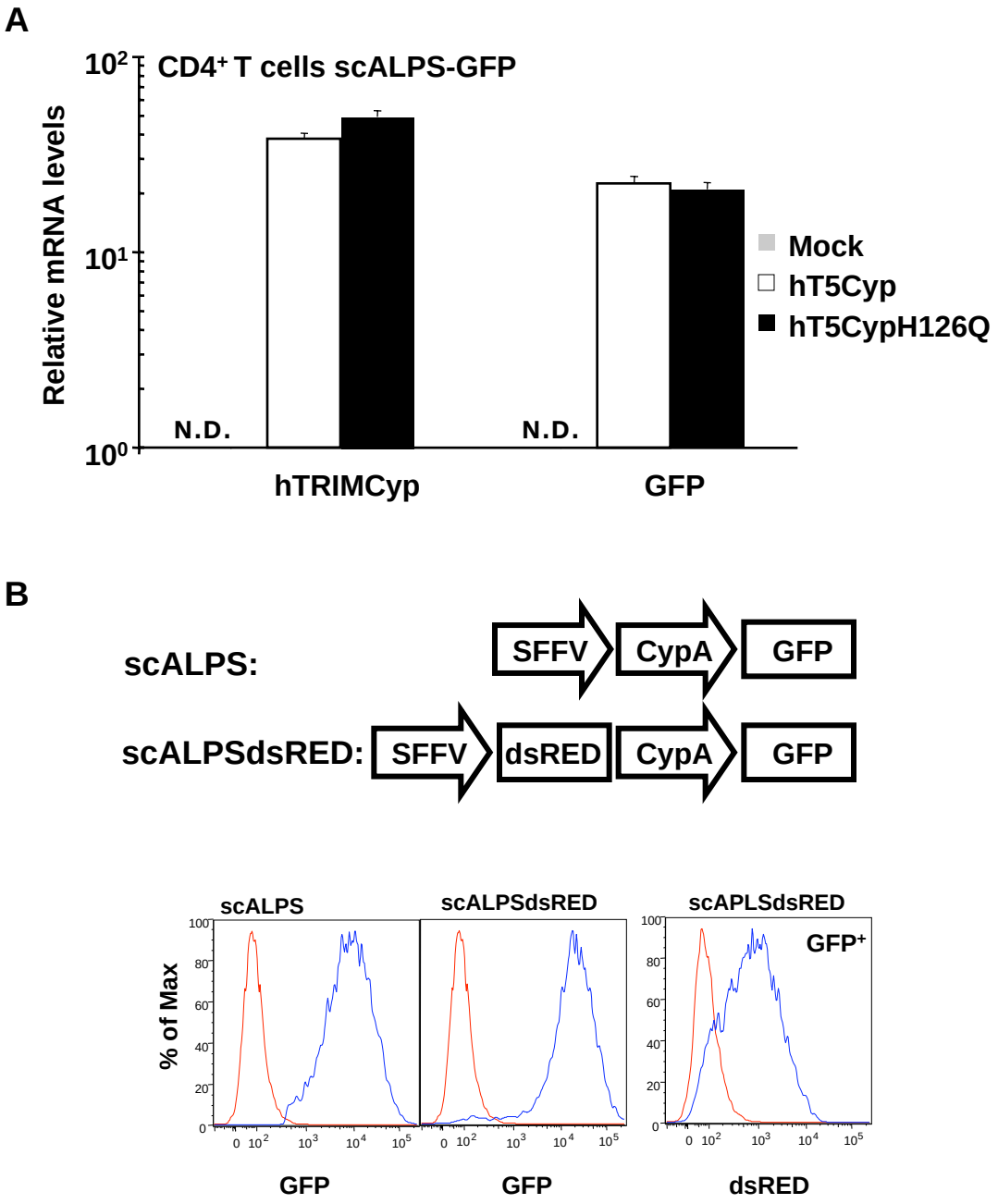

Figure S3. Transgene expression in T cells transduced with dual-promoter vectors. (**A**) Quantitative RT-PCR for for GFP and hT5Cyp wt or hT5CypH126Q mRNA expression normalized by 18S RNA expression 48 hours after transduction of primary CD4+ T cells with scAPLS dual promoter vectors. N.D. = not detected (**B**) Schematic of dual promoter vectors (scAPLS and scAPLSdsRed) used to transduce Jurkat T cells (top panel). Transgene expression in transduced Jurkat T cells (bottom panel).

Supplementary Figure S4

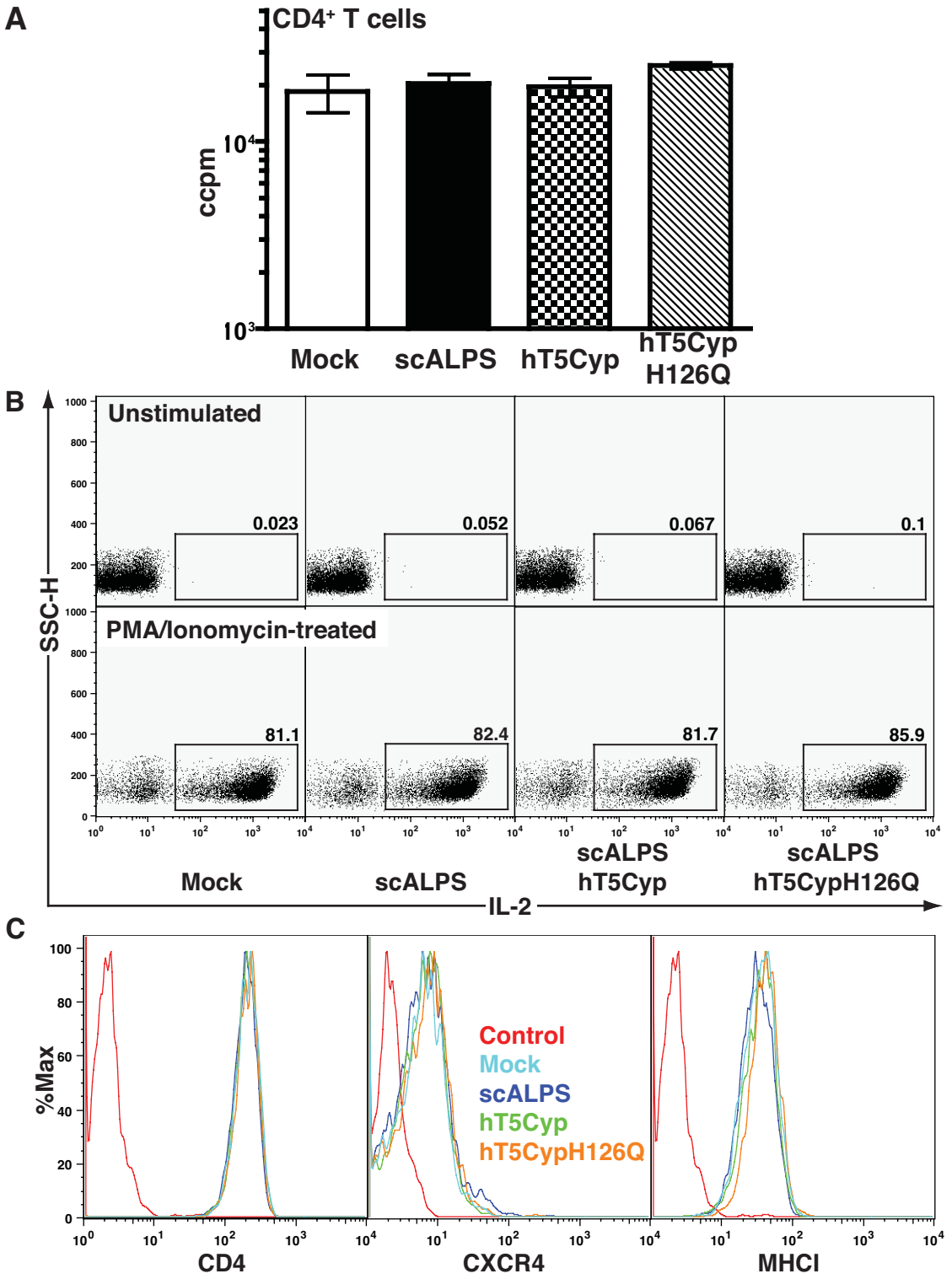

Figure S4. Transduction of primary human CD4⁺ T cells using a dual-promoter, lentiviral vector encoding T5Cyp and GFP does not affect basic T cell functions. (**A**) T5Cyp does not affect steady-state proliferation of CD4⁺ T cells. CD4⁺ T cells transduced with the indicated vector were sorted for GFP expression and steady-state proliferation as compared to un-transduced cells was measured using ^{3H}Thymidine incorporation. Corrected counts per minute (ccpm, Y-axis) were measured 24 hours after addition of ^{3H}Thymidine (for each condition, n=3). (**B**) IL-2 production in scALPS-transduced CD4⁺ T cells. IL-2 production (X-axis) in proliferating un-transduced CD4⁺ T cells and CD4⁺ T cells transduced with indicated scALPS vectors was analyzed by flow cytometry following stimulation with PMA and ionomycin. (**C**) Cell-surface marker expression on scALPS-transduced CD4⁺ T cells. The expression of the indicated cell surface markers (X-axis) was assessed in proliferating un-transduced CD4⁺ T cells and CD4⁺ T cells transduced with indicated scALPS vectors by flow cytometry.

Supplementary Figure S5

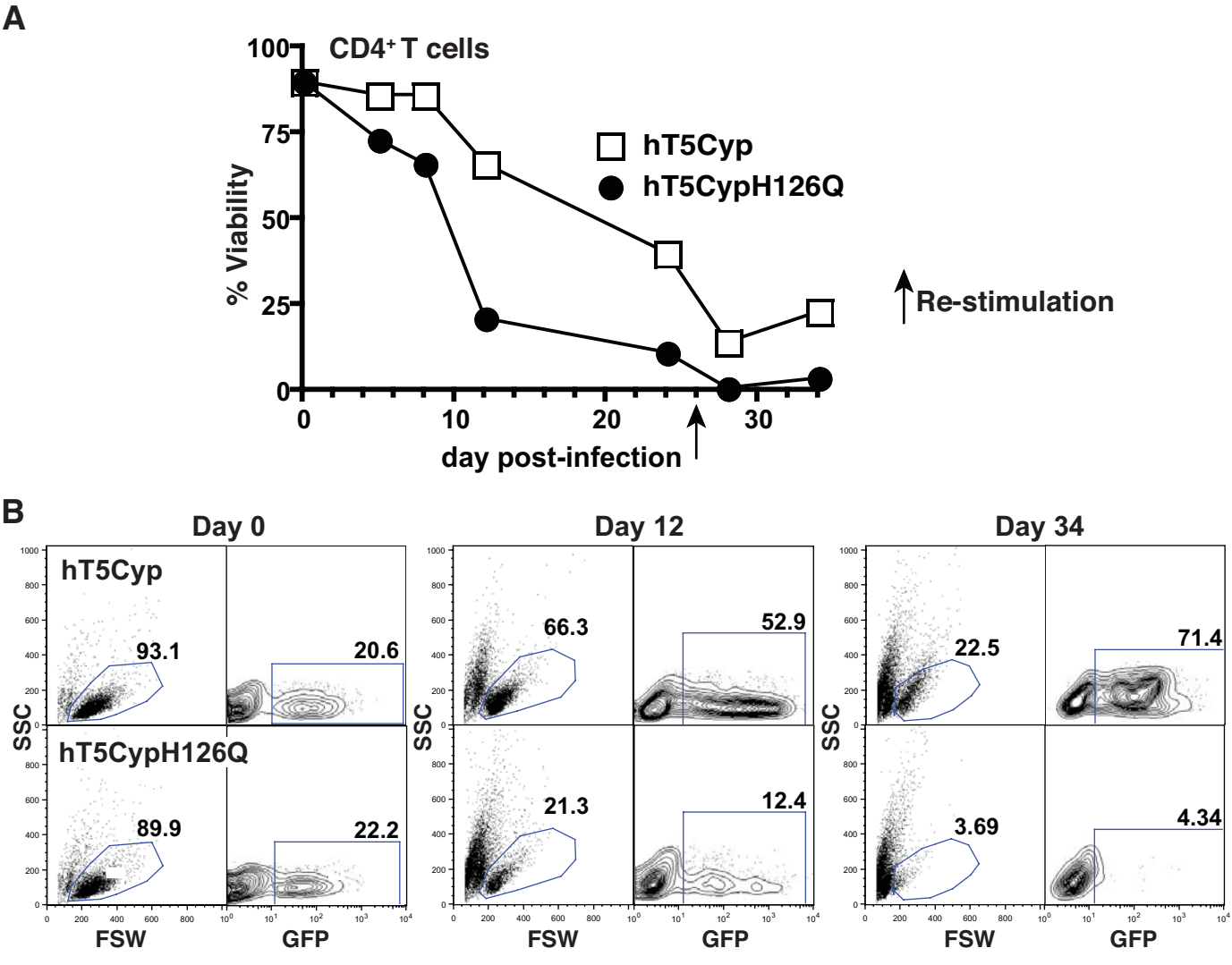

Figure S5. hT5Cyp enhances viability of CD4+ T cells in mixed cultures infected with HIV-1. **(A)** CD4+ T cells were transduced with scAPLS encoding the indicated hT5Cyp proteins. Cultures containing 23% GFP+ cells **(same as in Figure 6G)** were challenged with 15 ng p24/106 cells HIV-1NL4-3 and monitored for % viable cells by forward and side scatter profiles (Y-axis) on the indicated day post-infection (X-axis). Representative flow cytometry plots showing viability (left panels) and % GFP+ cells (right panels) at three time points are shown in **(B).**

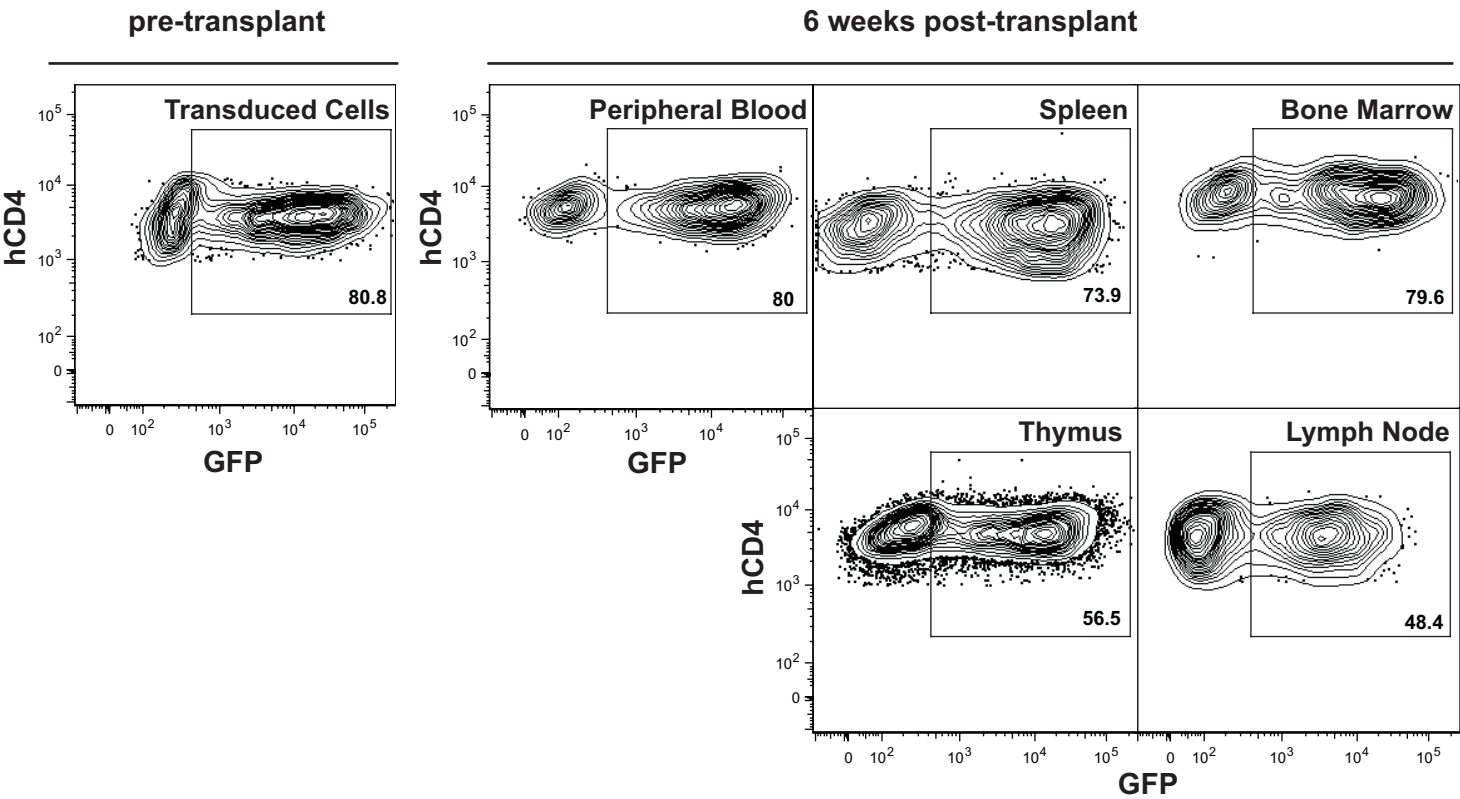

Figure S6. In vivo GFP expression in hCD4$^+$ T cells 6 weeks post-transplant. 6-10 week old Rag2$^{-/-}\gamma_c^{-/-}$ mice were engrafted with GFP-sorted scAPLS-transduced CD4$^+$ T cells (left panel). Indicated organs were analyzed for GFP expression 6 weeks post-transplant (right panel). Gates denote the percentage of GFP$^+$ cells in the hCD4$^+$ population. Data from one representative mouse are shown.

Supplementary Figure S7

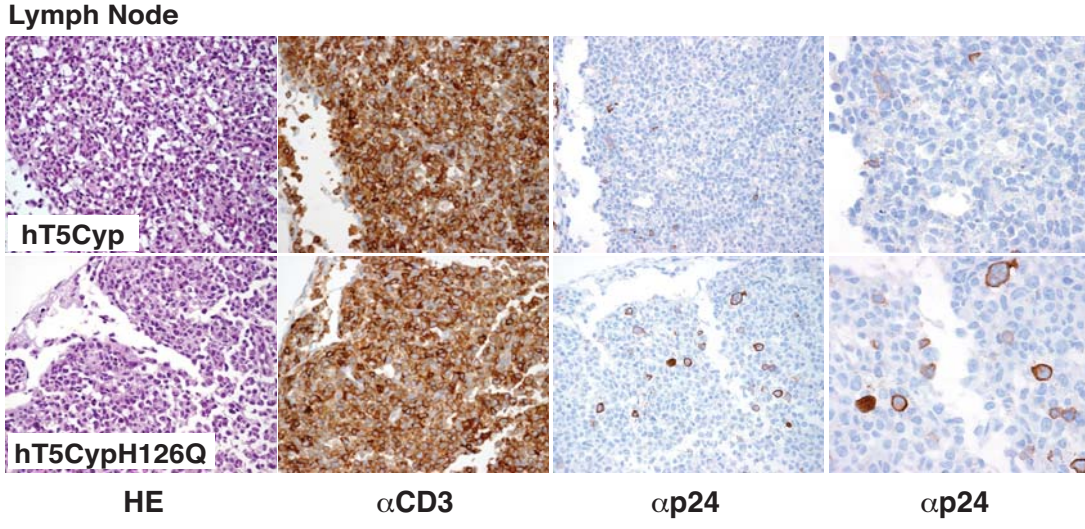

Figure S7. hT5Cyp protects T cells from HIV-1 infection in vivo. 6-10 week old Rag2$^{-/-}\gamma_c^{-/-}$ mice were engrafted with GFP-sorted scAPLS-transduced CD4$^+$ T cells expressing either hT5Cyp or hT5CypH126Q. Mice were infected with HIV-1NL4-3 two weeks after transplant. Representative CD3- and p24-stained paraffin-embedded tissue sections of mesenteric lymph nodes are shown. While engraftment of CD3$^+$ human T cells is similar in lymph nodes of mice transplanted with either hT5Cyp- (top panel) or hT5CypH126Q-expressing (bottom panel) T cells, rare and weaker p24 staining is observed in lymph nodes from mice transplanted with hT5Cyp-CD4$^+$ T cells (top panel) as compared to those transplanted with hT5CypH126Q-CD4$^+$ T cells (bottom panel).

Supplementary Figure S8

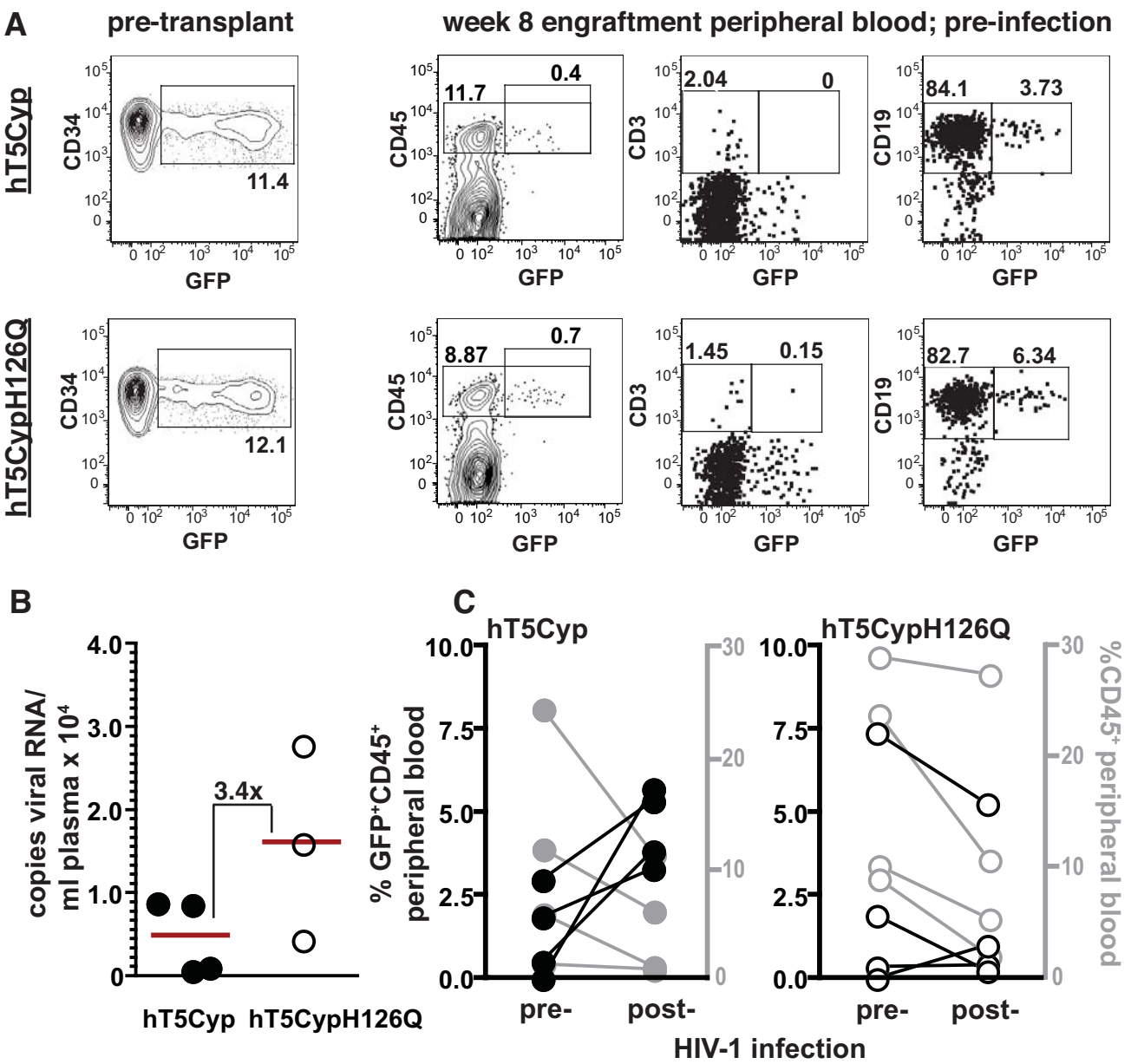

Figure S8. hT5Cyp reduces viral load in HIV-1 infected huRag2$^{-/-}\gamma_c^{-/-}$ mice.
(**A**) huRag2$^{-/-}\gamma_c^{-/-}$ mice were generated using CD34$^+$ cells stably transduced with scAPLS-hT5Cyp or scAPLS-hT5CypH126Q. ~ 12% of CD34$^+$ cells were GFP$^+$ 72 hours post-transduction *in vitro* (left panels). Human cell engraftment in peripheral blood and transgene expression in CD45$^+$ human lymphocytes, CD3$^+$ T cells, and CD19$^+$ B cells was analyzed by flow cytometry at eight weeks post transplant. (**B**) Viral load was determined in plasma of infected mice at 24 days post-infection. (**C**) GFP expression of CD45$^+$ cells is shown in peripheral blood pre-infection and 24 days post-infection of huRag2$^{-/-}\gamma_c^{-/-}$ with HIV-1. (left Y-axis, black symbols = % GFP$^+$ of CD45$^+$ cells; right Y-axis, grey symbols = % CD45$^+$ of nucleated cells)