

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
14 May 2010 (14.05.2010)

PCT

(10) International Publication Number
WO 2010/054141 A2

(51) International Patent Classification:
A61K 38/17 (2006.01)

(21) International Application Number:
PCT/US2009/063481

(22) International Filing Date:
6 November 2009 (06.11.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/112,013 6 November 2008 (06.11.2008) US
61/240,505 8 September 2009 (08.09.2009) US

(71) Applicant (for all designated States except US): **THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK** [US/US]; 412 Low Memorial Library, 535 West 116th Street, New York, NY 10027 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **LUBAN, Jeremy** [US/US]; 500 E. 63rd St., Apt. 15J, New York, NY 10021 (US). **NEAGU, Martha** [US/US]; 200 Meadowgate Terrace, Gaithersburg, MD 20887 (US).

(74) Agents: **LOVE, Jane, W.** et al.; Wilmer Cutler Pickering Hale And Dorr LLP, 399 Park Avenue, New York, NY 10022 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: POLYNUCLEOTIDES ENCODING A HUMAN TRIM-CYP FUSION POLYPEPTIDE, COMPOSITIONS THEREOF, AND METHODS OF USING SAME

(57) Abstract: A nucleic acid is provided which encodes a human TRIM-cyclophilin A fusion sequence encoding a human TRIM-CypA fusion protein which is active as an anti-viral agent, and in particular, as an anti-HIV-1 agent. Also provided is a nucleic acid encoding a polypeptide having both TRIM activity and cyclophilin activity. Also provided is an isolated polynucleotide encoding a human TRIM-CypA fusion protein, or variants thereof retaining the TRIM and cyclophilin A activities. Also provided are compositions thereof, antibodies that specifically bind thereto, and vectors and host cells comprising the nucleic acid or polypeptide. In addition, methods are provided for treating or preventing viral infection, or reducing viral load in a subject comprising administering the nucleic acid, polypeptide, vector, or composition to the subject in an amount effective to treat or prevent the viral infection. In some embodiments, the viral infection is HIV-I infection, hepatitis C infection, pox virus infection, vaccinia virus infection, or HTLV infection.



WO 2010/054141 A2

**POLYNUCLEOTIDES ENCODING A HUMAN TRIM-CYP FUSION
POLYPEPTIDE, COMPOSITIONS THEREOF, AND METHODS OF USING SAME**

[0001] This application claims priority to Application Ser. No.: 61/112013 filed November 6, 2008 and Application Ser. No.: 61/240505 filed September 8, 2009, the contents of which applications are hereby incorporated in their entirety.

[0002] The invention disclosed herein was made with Government support under NIH Grant Nos. RO1AI36199 and RO1AI59159 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

[0003] This patent disclosure contains material that is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure, as it appears in the U.S. Patent and Trademark Office patent file or records, but otherwise reserves any and all copyright rights.

[0004] Throughout this application, patent applications, published patent applications, issued and granted patents, texts, and literature references are cited. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

Background

[0005] Several million people die each year as a consequence of HIV-1 infection. Currently used antiviral therapies suppress HIV-1 replication and resultant disease but these antiviral therapies are plagued with complications and cannot eliminate the virus. Gene therapy is an alternative to life-long pharmacotherapy. Ideally, gene therapy should potentially suppress HIV-1 replication without eliciting viral resistance. While all steps of the viral life cycle are potential gene therapy targets, blocking the virus before reverse transcription (RT) would preclude the genetic diversity that permits emergence of viral resistance. Additionally, targeting the virus before HIV-1 cDNA is ligated into host chromosomal DNA would prevent the virus from becoming a heritable genetic element in that cellular lineage. The discovery that certain TRIM5 (T5) orthologues inhibit HIV-1 infection immediately after the virus enters otherwise susceptible cells raised the prospect that these host factors might be exploited in HIV-1 gene therapy.

[0006] HIV-1 infection is a serious problem throughout the world and there is a great need for a composition that will prevent infection of a subject by HIV-1 and for a composition that treats or ameliorates the effects of HIV-1 infection in humans. There is a need for life-long anti-HIV-1 pharmacotherapy, and therapies that treat or prevent HIV-1 associated morbidity and mortality.

Summary

[0007] Provided herein is a nucleic acid which comprises a sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and a variant having at least about 50% identity to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, wherein the nucleic acid encodes a polypeptide having both TRIM activity and cyclophilin activity. In one embodiment, the variant has at least about 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7. Another embodiment provides a nucleic acid which comprises a sequence complementary to a nucleic acid provided by an embodiment of the invention.

[0008] Provided herein is a nucleic acid encoding a polypeptide comprising SEQ ID NO:4 and SEQ ID NO:2, or a polypeptide comprising SEQ ID NO:10, 11, or 12.

[0009] Provided herein is a nucleic acid encoding a human TRIM5-cyclophilin A fusion protein. In one embodiment, the polynucleotide encodes a polypeptide comprising SEQ ID NO:2 and SEQ ID NO:4, wherein the last amino acid of SEQ ID NO:2 is serine at position 309, serine at position 322, or alanine at position 331. In another embodiment, the nucleic acid encodes a polypeptide consisting essentially of SEQ ID NO:4 and SEQ ID NO:2, wherein the last amino acid of SEQ ID NO:2 is serine at position 309, serine at position 322, or alanine at position 331. In one embodiment, the last amino acid of SEQ ID NO:2 is serine at position 309, serine at position 322, or alanine at position 331. In one embodiment, the last amino acid of SEQ ID NO:2 is any one amino acid residue from about residue 280 to about residue 400, or from about residue 280 to about residue 290, or from about residue 308 to about residue 311; or from about residue 321 to about residue 346; or from about residue 366 to about residue 371; or from about residue 381 to about residue 393. In one embodiment, the last residue of SEQ ID NO:2 is residue 493. The last residue of SEQ ID NO:2 can be fused to Cyp A (for example, at residue 2 of SEQ ID NO:4). In another embodiment, the nucleic acid encodes a polypeptide comprising a sequence that is at least 60%, 65%, 70%,

75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:4 and a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:2, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:10, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:11, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:12, and the polypeptide has the function of TRIM5 and specifically binds a capsid protein of HIV.

[0010] In another embodiment, the fusion protein comprises a polypeptide linker sequence, wherein (i) TRIM5 is located upstream of the linker sequence, so that the N-terminal amino acid of the linker sequence is attached to the C-terminal amino acid of TRIM5, and (ii) cyclophilin A is located downstream of the linker sequence, so that the C-terminal amino acid residue of the linker sequence is attached to the N-terminal amino acid residue of cyclophilin A. In one embodiment, the linker sequence comprises from about 10 to about 20 amino acids. In one embodiment, wherein the linker sequence comprises the consecutive amino acids SGGSGGSGGSGG.

[0011] Provided herein is a polypeptide encoded by a nucleic acid of the invention. Also provided is a polypeptide comprising SEQ ID NO:4 and SEQ ID NO:2, or a polypeptide comprising SEQ ID NO:10, 11, or 12. In one embodiment, the last (C-terminal) amino acid of SEQ ID NO:2, which can be fused to cyclophilin, is serine at position 309, serine at position 322, or alanine at position 331. In one embodiment, the last amino acid of SEQ ID NO:2 is any one amino acid residue from about residue 280 to about residue 400, or from about residue 280 to about residue 290, or from about residue 308 to about residue 311; or from about residue 321 to about residue 346; or from about residue 366 to about residue 371; or from about residue 381 to about residue 393. In one embodiment, the last residue of SEQ ID NO:2 is residue 493. In one embodiment, the last residue of SEQ ID NO:2 is 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 308, 309, 310, 311, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 366, 367, 368, 369, 370, 371, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, or 493. The last residue of SEQ ID NO:2 can be fused to Cyp A (for example, at residue 2 of SEQ ID NO:4). Also provided is a polypeptide comprising a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:4 and a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or

99% identical to SEQ ID NO:2, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:10, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:11, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:12.

[0012] Provided herein is an antibody that specifically binds to a polypeptide of the invention. Also provided is an antibody that specifically binds to a polypeptide encoded by a nucleic acid provided by the invention. In one embodiment, the antibody is a polyclonal antibody, a monoclonal antibody, or a chimeric antibody.

[0013] Provided herein is a method of producing a polypeptide of the invention, which comprises: (a) introducing a nucleic acid encoding the polypeptide into a host cell under conditions that are compatible with expression of the polypeptide by the host cell, and (b) recovering the polypeptide.

[0014] Provided herein is a nucleic acid vector, which comprises a nucleic acid of the invention. In one embodiment, the vector comprises a viral vector. In one embodiment, the vector is a lentiviral vector, an adenovirus vector, a retroviral vector, or an adeno-associated viral (AAV) vector. Also provided is a lentiviral vector comprising a nucleic acid of the invention.

[0015] Also provided is a lentiviral vector encoding a human TRIM5-cyclophilin A fusion protein, wherein the vector comprises a promoter from a spleen focus-forming virus (SFFV) long terminal repeat (LTR), a promoter from a human cyclophilin A gene, or both. Provided herein is a nucleic acid vector having SEQ ID NO:8 or SEQ ID NO:9.

[0016] Provided herein is a host organism comprising a vector of the invention. In one embodiment, the host is a prokaryote, a eukaryote or a fungus.

[0017] Provided herein is a method for preparing a pharmaceutical composition which comprises admixing a polypeptide of the invention or a fragment thereof, thereby preparing the pharmaceutical composition. Also provided is a pharmaceutical composition comprising a nucleic acid of the invention, a polypeptide of the invention, or a vector of the invention, and a carrier.

[0018] Provided herein is a method for treating a subject suffering from a disease or condition, the method comprising administering to the subject a nucleic acid of the invention, a polypeptide of the invention, or a vector of the invention. Also provided is a method for treating a subject suffering from a disease or a condition, the method comprising administering to the subject a polypeptide or a fragment thereof of the invention, so as to treat the subject.

[0019] Provided herein is a method for preventing retroviral infection of a subject, or for treating a subject with retroviral infection, the method comprising administering to the subject a pharmaceutical composition of the invention. Also provided is a method for treating or preventing a viral infection of a cell, the method comprising introducing a human TRIM5-Cyp A fusion polypeptide into the cell. In one embodiment, the introducing comprises transfection, transduction, viral-mediated introduction, or liposome-mediated introduction of a polynucleotide encoding a human TRIM5-Cyp A fusion polypeptide. In one embodiment, the viral infection is an HIV infection, an HTLV infection, a pox virus infection, a retrovirus infection, a malaria infection, a hepatitis C virus infection, a hepatitis B virus infection, or a vaccinia virus infection.

[0020] Provided herein is a method for treating HIV infection or preventing HIV infection of a subject which comprises introducing into cells of the subject a human TRIM5-Cyp A fusion polypeptide. In one embodiment, the human TRIM5-Cyp A fusion polypeptide binds to a capsid protein of the HIV and subsequently degrades the HIV, thereby treating or preventing HIV infection in the subject.

[0021] Provided herein is a peptidomimetic comprising an amino acid sequence substantially identical to the amino acid sequence of a polypeptide of the invention and wherein the peptidomimetic comprises TRIM5 function and cyclophilin A function.

[0022] Provided herein is a cell comprising a nucleic acid of the invention. In one embodiment, the cell is a stem cell. In another embodiment, the cell is a T cell.

[0023] Provided herein is a method for imparting resistance to HIV to a subject or to cells of a subject, the method comprising administering to the cells of the subject a nucleic acid of the invention, a vector of the invention, or a polypeptide of the invention. Provided herein is a method for reducing or inhibiting lentiviral replication, including but not limited to HIV replication, to a subject or to cells of a subject, the method comprising administering to the

cells of the subject a nucleic acid of the invention, a vector of the invention, or a polypeptide of the invention. Provided herein is a method for reducing viral load, including but not limited to HIV, to a subject or to cells of a subject, the method comprising administering to the cells of the subject a nucleic acid of the invention, a vector of the invention, or a polypeptide of the invention. Also provided is a therapeutic composition comprising a nucleic acid of the invention, a polypeptide of the invention, or a peptidomimetic of the invention, and a therapeutically acceptable carrier. In one embodiment, the carrier comprises a vector, a liposome, or a viral vector.

[0024] Provided herein is a method for *ex vivo* gene therapy, the method comprising: (a) removing bone marrow cells from a subject; (b) transfecting the removed bone marrow cells with a nucleic acid of the invention *in vitro*; and (c) transplanting the transfected bone marrow back into the subject. Also provided is a method for reducing viral burden or load in a subject infected by a virus, the method comprising administering to the subject a nucleic acid of the invention, a polypeptide of the invention, or a vector of the invention.

[0025] Provided herein is a topical composition for prevention or treatment of a viral infection which comprises a nucleic acid of the invention or a polypeptide of the invention, and a topical carrier. In one embodiment, the composition is for use on the skin, in the vagina, in the nose, in the mouth, or on any skin or mucosal surface.

[0026] Provided herein is a method for delivering a therapeutically effective amount of a human TRIM5-cyclophilin A fusion protein systemically to a subject, comprising administering to the subject a vector of the invention under conditions that are compatible with expression of the nucleic acid in the subject. In one embodiment, the vector comprises a nonviral vector. In another embodiment, the vector comprises a gold or tungsten particle and the coated particle is administered to the subject using a gene gun. In another embodiment, the vector comprises a liposome. In another embodiment, the vector comprises a viral vector. In another embodiment, the vector is an adenovirus vector, a lentiviral vector, a retroviral vector, or an adeno-associated viral (AAV) vector.

[0027] Provided herein is a method for delivering a therapeutically effective amount of a human TRIM5-cyclophilin A fusion protein systemically to a subject, comprising transfecting cells of said subject with a vector of the invention, under conditions that permit the expression of the nucleic acid and production of a human TRIM5-cyclophilin A fusion

protein. In one embodiment, the transfecting occurs *ex vivo* and the transfected cells are reintroduced into the subject.

[0028] In certain embodiments the invention provides a fusion polypeptide comprising the following consecutive amino acids in N-to C-terminus orientation: (i) SEQ ID NO: 13, which consists of amino acids at position 1-298 from hTRIM5 α (SEQ ID NO:2) or a variant of SEQ ID NO: 13; (ii) a linker of 12 to 33 amino acids linking SEQ ID NO: 13 and SEQ ID NO: 15 or the variants thereof; and (iii) SEQ ID NO:15, which consists of amino acid at positions 2 to 166 from hCypA (SEQ ID NO: 4) or a variant of SEQ ID NO: 15, wherein the polypeptide has lentiviral restriction activity.

[0029] In certain embodiments the invention provides a fusion polypeptide comprising the following consecutive amino acids in N-to C-terminus orientation: (i) SEQ ID NO: 13, which consists of amino acids at position 1-298 from hTRIM5 α (SEQ ID NO:2) or a variant of SEQ ID NO: 13; (ii) a linker of 11-33 amino acids linking SEQ ID NO: 13 and SEQ ID NO: 15 or the variants thereof; and (iii) SEQ ID NO:15, which consists of amino acid at positions 2 to 166 from hCypA (SEQ ID NO: 4) or a variant of SEQ ID NO: 15, wherein the polypeptide has lentiviral restriction activity.

[0030] In certain embodiments the invention provides a fusion polypeptide comprising the following consecutive amino acids in N-to C-terminus orientation: (i) SEQ ID NO: 13, which consists of amino acids at position 1-298 from hTRIM5 α (SEQ ID NO:2) or a variant of SEQ ID NO:13; (ii) a linker of 11, 12, 24 or 33 amino acids linking SEQ ID NO: 13 and SEQ ID NO: 15 or the variants thereof; and (iii) SEQ ID NO:15, which consists of amino acid at positions 2 to 166 from hCypA (SEQ ID NO: 4) or a variant of SEQ ID NO:15, wherein the polypeptide has lentiviral restriction activity. The linker of 11, 12, 24, or 33 amino acids may consist of any amino acid, or have the specific amino acid as described herein, including conservative variants of the specific linker as described herein.

[0031] In certain embodiments the invention provides a fusion polypeptide comprising the following consecutive amino acids in N-to C-terminus orientation: (i) SEQ ID NO: 13, which consists of amino acids at position 1-298 from hTRIM5 α (SEQ ID NO:2); (ii) a linker of 11, 12, 24 or 33 amino acids linking SEQ ID NO: 13 and SEQ ID NO: 15; and (iii) SEQ ID NO:15, which consists of amino acid at positions 2 to 166 from hCypA (SEQ ID NO: 4), wherein the polypeptide has lentiviral restriction activity.

[0032] In certain embodiments, the lentiviral restriction activity is HIV-1 restriction activity. In other embodiments, the lentiviral restriction activity includes TRIM5 and Cyclophilin activity as described herein and known in the art.

[0033] In certain embodiments, the fusion polypeptides of the invention are recombinant polypeptides. In certain embodiments, the fusion polypeptides are not naturally occurring. In certain embodiments, the fusion polypeptides of the invention are isolated polypeptides. In certain embodiments, the nucleic acids which encode the fusion polypeptide are isolated. In certain embodiments, the nucleic acids which encode the fusion polypeptides are not naturally occurring.

[0034] In certain embodiments the variants of SEQ ID NO: 13 or 15 have at least about 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 13 or 15. In certain embodiments, variants include any of the variants described herein. In certain embodiments the variants of SEQ ID NO: 13 or 15 have conservative substitutions, as described herein or known in the art. In certain embodiments, the conservative variants of SEQ ID NO: 13 or 15 have at least about 85%, 90%, 95% or 99% identity to SEQ ID NO: 13 or 15.

[0035] In certain embodiments the invention provides polypeptide comprising SEQ ID NO: 10, 11, 12, or 17 or a variant of SEQ ID NOS: 10, 11, 12, or 17 having about 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 10, 11, 12, or 17. In certain embodiments, the variant have conservative substations as described herein or known in the art. In certain embodiments the conservative variants have about 85%, 90%, 95% or 99% identity to SEQ ID NO: 10, 11, 12, or 17.

[0036] In certain embodiments, the linker linking the TRIM5 and Cyclophilin portions of the fusion polypeptide has SEQ ID NO: 20, 21, 22 or 23 or a variant of SEQ ID NO: 20, 21, 22 or 23 having about 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 20, 21, 22 or 23. In certain embodiments, the linker has SEQ ID NO: 20, 21, 22 or 23 or a conservative variant of SEQ ID NO: 20, 21, 22 or 23. In certain embodiments the conservative variants have about 85%, 90%, 95% or 99% identity to SEQ ID NO: 20, 21, 22 or 23.

[0037] In certain aspects, the invention provides an isolated nucleic acid encoding any of the polypeptides of the invention. In certain aspects, the invention provides a vector comprising any one of the nucleic acids of the invention. In certain aspects, the invention provides use of

any of the inventive nucleic acids or variants thereof, and/or protein encoded by these nucleic acids or variants thereof, in any of the methods of the invention.

[0038] In certain aspects the invention provides use of the nucleic acid and the polypeptides encoded by these nucleic acids in any of the methods of the invention, including but not limited to methods of treatment of lentiviral infection, methods of reducing viral loads, methods of restricting viral replication, and so forth.

Brief Description Of The Figures

[0039] Figures 1a – 1c. Identification of human T5Cyp proteins that potently restrict HIV-1.

(a) Amino acid sequence at the junction of the indicated T5Cyp fusion proteins and the corresponding anti-HIV-1 activity. **(b)** The sequence of hT5 α protein. Horizontal lines indicate the RING finger and B-box domains. The register of the predicted coiled-coils is indicated with lower case letters. PRYSPRY residues are boxed. Vertical lines indicate the site of fusion of CypA to T5. Numbers indicate the T5 amino acid to which CypA is fused and are color-coded for restriction phenotype: red, potently restrictive (S309, S322, A331); green, permissive (M244, W298, T302, S314, G357, G398); orange, variably restrictive (M284, T369). **(c)** Jurkat T cells (left panel) and THP-1 monocytes (right panel) 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 vector encoding GFP (left to right on X-axis). The percentage of GFP⁺ cells (Y-axis) was determined 48 hrs later.

[0040] Figures 2a – 2d. HIV-1 restriction activity correlates with CypA fusion to the T5 α specificity determinant and the ability to form cytoplasmic bodies. **(a)** Expression of hT5-Cyp fusion proteins. FLAG-hT5Cyp fusion proteins synthesized in 293T cells were immunoprecipitated and immunoblotted with anti-CypA antibodies. **(b)** HIV-1 CA binding activity of hT5Cyp fusion proteins. FLAG-hT5Cyp and GST-CA fusion proteins were co-expressed in 293T cells, pulled out on glutathione-sepharose beads in the presence or absence of 20 μ M CsA, and immunoblotted with anti-CypA and anti-p24-CA antibodies. **(c)** Model of the PRYSPRY domain of human T5 α based on crystal structures of PRYSPRY, GUSTAVUS and TRIM21. Two ribbon representations showing the position of hCypA fusions (spheres) to the hT5 α PRYSPRY domain. The grey transparent ribbon indicates regions of the model that would be replaced by CypA in hT5-S331-Cyp. **(d)** Restrictive

T5Cyp fusion proteins form discrete puncta in the cytoplasm. Indirect immunofluorescence images of CRFK cells stably expressing the indicated T5Cyp fusions. Fixed samples were stained with anti-T5 antibody (green) and anti-tubulin antibody (red), followed by counter-stain with DAPI (blue) to visualize the nuclear DNA. For each color, one individual stack of 20 0.35 μm optical sections was acquired and subjected to maximum intensity projection along the optical axis. Images represent three-color overlays. Bar: 5 μm . All panels are color-coded for restriction phenotype: red, restrictive; green, permissive; orange, variably restrictive.

[0041] Figures 3a – 3e. T5Cyp restricts lentiviruses that encode Capsid with CypA-binding activity. CRFK cells stably expressing the indicated T5 proteins were transduced with increasing amounts (X-axis) of GFP vectors derived from HIV-1_{NL4-3} (**a**), HIV-2_{ROD} (**b**), SIV_{AGMtan} (**c**), FIV_{PET} (**d**), or SIV_{MAC251} (**e**). The percentage of GFP⁺ (infected) cells was determined at 48 hrs (Y-axis).

[0042] Figures 4a – 4e. T5 and CypA components each make essential contributions to T5Cyp restriction activity. (**a-c**) Disruption of the CA-CypA interaction by the G89V mutation in HIV-1 CA (**a**), the H126Q mutation in CypA (**b**), or the competitive inhibitor cyclosporine (CsA) at 2.5 μM (**c**), blocks HIV-1 restriction activity in CRFK cells stably expressing the indicated T5 proteins or controls. Cells were challenged with increasing amounts (X-axis) of HIV-1 GFP vector bearing the G89V mutation in CA (**a**) or wild-type HIV-1 GFP vector (**b, c**). (**d**) Introduction of a naturally-occurring quadruple mutation flanking the CA-CypA binding site in HIV-1 CA does not abrogate hT5Cyp-mediated restriction of HIV-1. CRFK cells stably expressing T5Cyp or control were infected with increasing amounts (X-axis) of an HIV-1 GFP vector containing the V86P/H87Q/I91V/M96I mutation in CA. (**e**) Disruption of T5 by As₂O₃ blocks HIV-1 restriction activity by T5Cyp. CRFK cells stably expressing the indicated T5Cyp were infected with HIV-1 GFP vector in the presence of increasing amounts of As₂O₃ (X-axis). In each case (**a-e**) the percentage of GFP⁺ cells was measured by flow cytometry 48 hrs post-infection (Y-axis).

[0043] Figures 5a – 5g. hT5Cyp potently blocks replication-competent HIV-1. (**a, b**) Jurkat T cells expressing the indicated proteins were infected with HIV-1_{NL4-3}-GFP-IRES-Nef, 15 ng p24/10⁶ cells (**a**) or 1 ng p24/10⁶ cells (**b**) and the percentage of infected cells was recorded. (**c**) Design of bicistronic (AIG) and dual promoter (scALPS) lentiviral vectors. (**d**) GFP expression by flow cytometry 48 hrs after transduction of Jurkat T cells (left) or primary

CD4⁺ T cells (right) with the indicated vectors. (e) hT5Cyp inhibits HIV-1 in primary human CD4⁺ T cells. CD4⁺ T cells transduced with scALPS encoding the indicated hT5Cyp proteins were sorted for GFP, infected with HIV-1_{NL4-3}, and supernatant RT activity was measured. Triplicate spreading infection for one representative donor of four is shown. (f) hT5Cyp inhibits HIV-1 in monocyte-derived macrophages. CD14⁺ monocytes differentiated with GM-CSF were transduced, sorted for GFP, and challenged with HIV-1_{NL4-3} as in (e) except that the viral envelope was modified to be CCR5-tropic. (g) Selective advantage of hT5Cyp-bearing CD4⁺ T cells in the presence of HIV-1. CD4⁺ T cells were transduced with scALPS encoding the indicated T5Cyp proteins. Cultures containing 23% GFP⁺ cells were challenged with 15 ng/10⁶ cells HIV-1_{NL4-3} and monitored for percentage GFP⁺ cells (left Y-axis, open symbols). Supernatant RT activity was measured (right Y-axis, filled symbols). Cultures were re-stimulated using allogeneic-PBMC, IL-2, and PHA on day 26 post-infection (arrow).

[0044] Figures 6a – 6e. hT5Cyp protects CD4⁺ T cells from HIV-1 infection *in vivo*. (a) Experimental design for adoptive transfer of transduced CD4⁺ T cells and *in vivo* HIV-1 challenge in Rag2^{-/-}γc^{-/-} mice. Gy = Gray; i.p. = intraperitoneal injection. (b) hT5Cyp prolongs CD4⁺ T cell-survival *in vivo*. 6-10 week old Rag2^{-/-}γc^{-/-} mice were engrafted with GFP-sorted scALPS-transduced CD4⁺ T cells expressing either hT5Cyp or hT5CypH126Q. Mice were infected 5 days after adoptive CD4⁺ T cell transfer with CXCR4-tropic HIV-1_{NL4-3} and analyzed for presence of hCD45⁺ lymphocytes (Y-axis) at 4 weeks post-infection. Individual mice are shown and fold-difference in mean peripheral blood CD45⁺ lymphocyte engraftment (red bar) is indicated above the bracket. (* p = 0.0037, Mann-Whitney test) (c, d, e) hT5Cyp protects CD4⁺ from productive HIV-1 infection in lymphoid organs. Age-matched Rag2^{-/-}γc^{-/-} mice transplanted with CD4⁺ T cells expressing either hT5Cyp or hT5CypH126Q were infected with CXCR4-tropic HIV-1_{NL4-3} 2 weeks after adoptive transfer of CD4⁺ T cells. Single-cell suspensions of thymi were analyzed 14 days post-infection for p24⁺ (X-axis) CD3⁺ (Y-axis) T cells (c) or CD4-downregulation (Y-axis) (d). Representative CD3- and p24-stained paraffin-embedded tissue sections of thymus are shown. While engraftment of CD3⁺ human T cells is similar in thymi of mice transplanted with either hT5Cyp- (top panel) or hT5CypH126Q-expressing CD4⁺ T cells (bottom panel), rare and weaker p24 staining is observed in organs from mice transplanted with hT5Cyp-CD4⁺ T cells as compared to those transplanted with hT5CypH126Q-CD4⁺ T cells (e). (c,d,e) Representative results from three sets of experiments are shown.

[0045] Figures 7A – 7C. hTRIMCyp Fusion proteins and superimposition of site of fusion onto 3-D Model of TRIM5 PRYSPRY domain

[0046] Figures 8A – 8D. hTRIMCyp fusions and HIV-1 restriction in multiple cell lines

[0047] Figure 9A – 9C. hTRIMCyp fusions and restriction of multiple lentiviruses

[0048] Figures 10A – 10C. CA-binding by the CypA-moiety of hTRIMCyp is required for HIV-1 restriction

[0049] Figure 11. hTRIMCyp restriction of NL4-3 in spreading infection

[0050] Figures 12A – 12B. HIV-1 mouse model and transduction of hHSCs

[0051] Figure 13. Human TRIM5alpha mRNA (SEQ ID NO:1) (Homo sapiens tripartite motif-containing 5 (TRIM5) mRNA, transcript variant alpha). GenBank Accession No. NM_033034.

[0052] Figure 14. Human TRIM5alpha protein (SEQ ID NO:2) (Homo sapiens tripartite motif protein TRIM5 isoform alpha amino acid sequence). GenBank Accession No. NP_149023.

[0053] Figure 15. Human cyclophilin A mRNA (SEQ ID NO:3) (Homo sapiens peptidylprolyl isomerase A (PPIA; cyclophilin A) mRNA). GenBank Accession No. NM_021130.

[0054] Figure 16. Human cyclophilin A protein (SEQ ID NO:4) (Homo sapiens peptidylprolyl isomerase A). GenBank Accession No. NP_066953.

[0055] Figure 17. Nucleotide sequence (SEQ ID NO:5) encoding human hT5Cyp fusion polypeptide (SEQ ID NO:10) where Cyp is fused to TRIM5 at alanine 331 (underlined) of TRIM5 (hT5-A331-Cyp).

[0056] Figure 18. Nucleotide sequence (SEQ ID NO:6) encoding human hT5Cyp fusion polypeptide (SEQ ID NO:11) where Cyp is fused to TRIM5 at serine 309 (underlined) of TRIM5 (hT5-S309-Cyp).

[0057] **Figure 19.** Nucleotide sequence (SEQ ID NO:7) encoding human hT5Cyp fusion polypeptide (SEQ ID NO:12) where Cyp is fused to TRIM5 at serine 322 (underlined) of TRIM5 (hT5-S322-Cyp).

[0058] **Figures 20A - 20C.** Nucleotide sequence (SEQ ID NO:8) of vector scALPS-GFP.

[0059] **Figures 21A – 21D.** Nucleotide sequence (SEQ ID NO:9) of vector scALPS-GFP containing a nucleotide sequence encoding hT5Cyp.

[0060] **Figures 22A – 22B.** Comparison of anti-HIV-1 activity of proteins as determined by single cycle HIV-1 infection in CRFK cells. Experimental data is displayed in a linear scale (a) and logarithmic scale (b). Note that hT5Cyp has superior anti-HIV-1 activity compared to full-length rhesus monkey TRIM5 α (RhT5 α) and a human/rhesus monkey chimera TRIM5 α (hT5 α R323-332). *See Example 6.*

[0061] **Figures 23A – 23B.** Comparison the effect on spreading of HIV-1 infection in Jurkat cells (24 days). Experimental data is displayed in a linear scale (a) and logarithmic scale (b). Note that hT5Cyp has superior anti-HIV-1 activity compared to full-length rhesus monkey TRIM5 α (RhT5 α) and a human/rhesus monkey chimera TRIM5 α (hT5 α R323-332). *See Example 6.*

[0062] **Figure 24A-24B. Identification of human T5Cyp proteins that potently restrict HIV-1.** (A) The sequence of hT5 α protein. Horizontal lines indicate the RING finger and B-box domains. The register of the predicted coiled-coils is indicated with lower case letters. PRYSPRY residues are boxed. Vertical lines indicate the site of fusion of CypA to T5. Numbers indicate the T5 amino acid to which CypA is fused and are color-coded for restriction phenotype: red, potently restrictive; green, permissive; orange, variably restrictive. (B) Jurkat T cells (left panel) and THP-1 monocytes (right panel) were transduced with vectors encoding puromycin-resistance and either AoT5Cyp or hT5Cyp (hT5-S222-Cyp) fusion proteins. Pools of puromycin-resistant cells were infected with increasing amounts of a single-cycle, HIV-1 vector encoding GFP (left to right on X-axis). The percentage of GFP⁺ cells (Y-axis) was determined 48 hrs later.

[0063] **Figure 25A-25D.** HIV-1 restriction activity correlates with CypA fusion to the T5 α specificity determinant and the ability to form cytoplasmic bodies. (A) Expression of hT5-Cyp fusion proteins. FLAG-hT5Cyp fusion proteins synthesized in 293T cells were

immunoprecipitated and immunoblotted with anti-CypA antibodies. (B) HIV-1 CA binding activity of hT5Cyp fusion proteins. FLAG-hT5Cyp and GST-CA fusion proteins were co-expressed in 293T cells, pulled out on glutathione-sepharose beads in the presence or absence of 20 μ M CsA, and immunoblotted with anti-CypA and anti-p24-CA antibodies. (C) Model of the PRYSPRY domain of human T5 α based on crystal structures of PRYSPRY, GUSTAVUS and TRIM21. Two ribbon representations showing the position of hCypA fusions (spheres) to the hT5 α PRYSPRY domain. The grey transparent ribbon indicates regions of the model that would be replaced by CypA in hT5-S331-Cyp. (D) Restrictive T5Cyp fusion proteins form discrete puncta in the cytoplasm. Indirect immunofluorescence images of CRFK cells stably expressing the indicated T5Cyp fusions. Fixed samples were stained with anti-T5 antibody (green) and anti-tubulin antibody (red), followed by counter-stain with DAPI (blue) to visualize the nuclear DNA. For each color, one individual stack of 20 0.35 μ m optical sections was acquired and subjected to maximum intensity projection along the optical axis. Images represent three-color overlays. Bar: 5 μ m. All panels are color-coded for restriction phenotype: red, restrictive; green, permissive; orange, variably restrictive.

[0064] Figure 26A-26E. T5 and CypA components each make essential contributions to T5Cyp restriction activity. (A-C) Disruption of the CA-CypA interaction by the G89V mutation in HIV-1 CA (A), the H126Q mutation in CypA (B), or the competitive inhibitor cyclosporine (CsA) at 2.5 μ M (C), blocks HIV-1 restriction activity in CRFK cells stably expressing the indicated T5 proteins or controls. Cells were challenged with increasing amounts (X-axis) of HIV-1-GFP single-cycle vector bearing the G89V mutation in CA (A) or wild-type HIV-1 GFP vector (B, C). (D) Introduction of a naturally-occurring quadruple mutation flanking the CA-CypA binding site in HIV-1 CA does not abrogate hT5Cyp-mediated restriction of HIV-1. CRFK cells stably expressing T5Cyp or control were infected with increasing amounts (X-axis) of an HIV-1 GFP vector containing the V86P/H87Q/I91V/M96I mutation in CA. (E) Disruption of T5 by As₂O₃ blocks HIV-1 restriction activity by T5Cyp. CRFK cells stably expressing the indicated T5Cyp were infected with HIV-1 GFP vector in the presence of increasing amounts of As₂O₃ (X-axis). In each case (A-E) the percentage of GFP⁺ cells was measured by flow cytometry 48 hrs post-infection (Y-axis).

[0065] Figure 27A-27E. T5Cyp potently restricts lentiviruses that encode Capsid with CypA-binding activity. CRFK cells stably expressing the indicated T5 proteins were transduced with increasing amounts (X-axis) of single-cycle GFP vectors derived from HIV-1_{NL4-3} (A), HIV-2_{ROD} (B), SIV_{AGMtan} (C), FIV_{PET} (D), or SIV_{MAC251} (E). The percentage of GFP⁺ positive cells (Y-axis) was determined 48 hrs later.

[0066] Figure 28A-D. The anti-HIV-1 activity of hT5Cyp is superior to that of rhT5α. CRFK (A) and Jurkat T cells (B) were transduced with lentiviral vectors encoding the indicated T5 proteins and a puromycin-resistance cassette. Pools of puromycin-resistant cells were infected with increasing amounts of a single-cycle HIV-1-GFP vector (left to right on X-axis). The percentage of GFP⁺ positive cells (Y-axis) was determined 48 hrs later. (C and D) As in (A) and (B), Jurkat T cells were transduced with the indicated vectors and challenged with HIV-1_{NL4-3}-GFP-IRES-Nef. The percentage of infected cells was determined by flow cytometry.

[0067] Figure 29A-G. New lentiviral vectors bearing hT5Cyp potently block replication-competent HIV-1 in primary cells. (A) Design of conventional bicistronic (AIG) and new dual-promoter (scALPS) lentiviral vectors. (B) 48 hrs after transduction with the indicated vectors, Jurkat T cells (left) or primary hCD4⁺ T cells (right) were analyzed by flow cytometry for GFP. (C and D) Primary human CD4⁺ T cells transduced with scALPS encoding the indicated hT5Cyp proteins were sorted for GFP and infected with lab clone HIV-1_{NL4-3}, (C) or primary isolate HIV-1_{1DH12} (D) and RT activity in the supernatant was measured. Triplicate spreading infection for one representative donor of four is shown. (E and F) Human macrophages generated from GM-CSF-treated, CD14⁺ monocytes were transduced with the indicated vectors, sorted for GFP, and challenged with modified HIV-1_{NL4-3} bearing a CCR5-tropic Env (E) or primary isolate HIV-1_{132W} (F). Infection was monitored as in (C) and (D). Error bars in (C) and (E) represent +/- standard error of the mean, N = 3. (G) hT5Cyp-bearing CD4⁺ T cells exhibit a selective advantage after challenge with HIV-1. CD4⁺ T cells were transduced with scALPS encoding the indicated T5Cyp proteins. Cultures containing 23% GFP⁺ cells were challenged with 15 ng/10⁶ cells HIV-1_{NL4-3} and monitored for percentage GFP⁺ cells (left Y-axis, open symbols). Supernatant RT activity was measured (right Y-axis, filled symbols). Cultures were re-stimulated using allogeneic-PBMC, IL-2, and PHA on day 26 post-infection (arrow).

[0068] Figure 30A-30C. Adoptive transfer of hT5Cyp-transduced hCD4 cells into Rag2^{-/-}γc^{-/-} mice. Human peripheral blood CD4⁺ T cells transduced with lentiviral vector scALPS bearing either hT5Cyp or hT5CypH126Q were sorted for GFP and injected into 6-10 wk old Rag2^{-/-}γc^{-/-} mice. The experimental design is shown schematically in (A). (B) Flow cytometric analysis of the transduced, sorted cells prior to adoptive transfer. The left panel shows that nearly all cells are hCD4⁺. The x-axis of the right panel shows GFP fluorescence in transduced cells (open histograms, scALPS) or in untransduced, donor-identical CD4⁺ T cells (grey histogram, Mock). (C) 6 weeks post-transplant, single cell suspensions of thymus were analyzed for human cell engraftment and transgene expression. The left panel shows recipient-derived CD45⁺ mouse cells (mCD45.2, Y-axis) and donor derived hCD45⁺ cells (X-axis). The right panel shows CD4 and GFP signals for the hCD45⁺ cells (right panels). In (A) and (C), the numbers indicate the percentage of cells within the indicated gates.

[0069] Figure 31A-31E. hT5Cyp inhibits HIV-1 in a humanized mouse model. 6-10 week old Rag2^{-/-}γc^{-/-} mice were engrafted with GFP-sorted scALPS-transduced CD4⁺ T cells expressing either hT5Cyp or hT5CypH126Q. (A) 5 days after engraftment, mice were challenged with HIV-1_{NL4-3}. The percentage hCD4⁺ T cells among all nucleated cells in the peripheral blood of individual mice is shown at 2 weeks post-infection. The fold-difference for the mean is indicated (red bar, ***p = 0.0002, Mann-Whitney test). (B-E) 2 weeks post-engraftment, mice were infected with HIV-1_{NL4-3}. (B) Plasma viral load for individual mice was determined 3 wks post-infection. The fold-difference in the mean is indicated (red bar, **p = 0.005, Mann-Whitney test). (C) 3 wks post-infection, single-cell suspensions from thymus were analyzed by flow cytometry for p24 and hCD3 cells (C) or hCD45 and hCD4 (D). The numbers indicate the percentage of cells within the indicated gates. (E) Paraffin-embedded sections of thymus are shown, stained with hematoxylin/eosin, anti-human-CD3 or anti-p24, as indicated. (C, D, E) Representative results from three sets of experiments are shown. (E) all panels are at 40X magnification, except the two right panels which are at 80X.

[0070] Figure 32A-32E. HIV-1 restriction activity of different hT5Cyp 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.

[0071] Figure 33A-33D. 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.

[0072] Figure 34A-34B. Transgene expression in T cells transduced with dual promoter

Vectors. (A) Quantitative RT-PCR 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).

[0073] Figure 35A-C. 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 ³HThymidine incorporation. Corrected counts per minute (ccpm, Y-axis) were measured 24 hours after addition of ³HThymidine (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.

[0074] Figure 36A-36B. 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 29G**) 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).

[0075] Figure 37. In vivo GFP expression in hCD4⁺ T cells 6 weeks posttransplant. 6-10 week old Rag2^{-/-}γc^{-/-} 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.

[0076] Figure 38. hT5Cyp protects T cells from HIV-1 infection in vivo. 6-10 week old Rag2^{-/-}γc^{-/-} 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).

[0077] Figure 39A-39C. hT5Cyp reduces viral load in HIV-1 infected huRag2^{-/-}γc^{-/-} mice. (A) huRag2^{-/-}γc^{-/-} 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^{-/-}γc^{-/-} with HIV-

1. (left Y-axis, black symbols = % GFP⁺ of CD45⁺ cells; right Y-axis, grey symbols = % CD45⁺ of nucleated cells).

[0078] Figure 40A-40B. (A.) Organization of HIV-1 provirus genome (top panel), Gag polyprotein components (bottom panel). MA = matrix; CA = capsid; NC = nucleocapsid. **(B.)** Simplified schematic of retroviral life cycle and site of action of HIV-1 restriction factors.

[0079] Figure 41A-41B. (A.) Organization of functional domains along the linear TRIM5 sequence; BB = B box, RF = RING finger, E3 = E3 ubiquitin ligase activity, CA = capsid **(B.)** Alignment of amino acid sequences at the fusion junction; Ma = *Macacca* (sequence identical for *mulatta*, *nemestrina*, and *fascicularis*), Ao = *Aotus*, h = human, Rh = rhesus (*macacca mulatta*); * indicates engineered not naturally-occurring protein. Color coded for domains: Blue = RBCC, Green = PRYSPRY, Red = Cyclophilin A, underlined residues represent amino-acid changes between human and rhesus PRYSPRY domains, black = Rhesus-inspired amino-acid changes in the human T5 α PRYSPRY domain.

[0080] Figure 42. Alignment of AoT5Cyp and hT5Cyp amino-acid sequences.

[0081] Figures 43A - C. (A.) Amino acid sequence at the junction of the indicated T5Cyp fusion proteins and the corresponding anti-HIV-1 activity. **(B.)** Schematic of lentiviral vector used for generation of stable cell lines; Ubx = Ubiquitin promoter, PuroR = puromycin-N-acetyl-transferase, IRES = internal ribosome entry site from encephalomyocarditis virus (ECMV) **(C.)** Jurkat T 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 vector encoding GFP (left to right on X-axis). The percentage of GFP⁺ cells (Y-axis) was determined 48 hrs later.

[0082] Figures 44A - B. (A.) The sequence of hT5 α protein. Horizontal lines indicate the RING finger and B-box domains. The register of the predicted coiled-coils is indicated with lower case letters. PRYSPRY residues are boxed. Vertical lines indicate the site of fusion of CypA to T5. Numbers indicate the T5 amino acid to which CypA is fused and are color-coded for restriction phenotype: red, potently restrictive; green, permissive; orange, variably restrictive. **(B.)** Jurkat T cells (left panel) and THP-1 monocytes (right panel) 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 vector encoding GFP (left to right on X-axis). The percentage of GFP⁺ cells (Y-axis) was determined 48 hrs later.

[0083] Figures 45A - D. Comparison of restrictive T5Cyp fusions in CRFK (**A.**) and Jurkat (**B.**) cells. (**C.**) Comparison of all designed fusions with reduced anti-HIV-1 activity to restrictive hT5Cyp and AoT5Cyp in Jurkat T cells. 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 (left to right on X-axis). The percentage of GFP⁺ positive cells (Y-axis) was determined 48 hrs later. (**D.**) Total cellular DNA was purified from Jurkat cell lines and late RT products were quantified by PCR. Values were normalized to mitochondrial DNA content.

[0084] Figures 46A - C. (**A.**) Staining of indicated cell-surface markers in stable Jurkat T cell lines expressing the indicated T5. (**B.**) Anti-HIV-1 activity of hT5Cyp in Jurkat T cell lines with stable CypA knockdown. (**C.**) B- and N-MLV titration in Jurkat T cell lines stably expressing the indicated T5 or control. For B and C increasing viral doses were used (X-axis) and infected, GFP⁺ cells were measured (Y-axis) 48 hours post-infection.

[0085] Figure 47A-D. (**A.**) FLAG-hT5Cyp fusion proteins synthesized in 293T cells were immunoprecipitated and immunoblotted with anti-CypA antibodies. (**B.**) FLAG-hT5Cyp and GST-CA fusion proteins were co-expressed in 293T cells, pulled down with glutathione-sepharose beads in the presence or absence of 20 mM CsA, and immunoblotted. (**C.**) Model of the hT5 α PRYSPRY domain based on crystal structures of PYRIN, GUSTAVUS and TRIM21. Ribbon representations show the position of hCypA fusions (spheres) to the hT5 α PRYSPRY domain. The grey ribbon indicates regions of the model that would be replaced by CypA in hT5-A331-Cyp. (**D.**) Indirect immunofluorescence images of CRFK cells stably expressing the indicated T5Cyp fusions. Fixed samples were stained with anti-T5 antibody (green) and anti-tubulin antibody (red), followed by counter-stain with DAPI (blue) to visualize the nuclear DNA. Images represent three-color overlays. Bar: 5 μ m. All panels are color-coded for restriction phenotype: red, restrictive; green, permissive; orange, variably restrictive.

[0086] Figures 48A - E. T5Cyp restricts lentiviruses that encode Capsid with CypA-binding activity. CRFK cells stably expressing the indicated T5 proteins were transduced with increasing amounts (X-axis) of GFP vectors derived from HIV-1_{NL4-3} (**A.**), HIV-2_{ROD} (**B.**),

SIV_{AGMtan} (C), FIV_{PET} (D), or SIV_{MAC251} (E). The percentage of GFP⁺ (infected) cells was determined at 48 hrs (Y-axis).

[0087] Figures 49A - F. (A-C) Disruption of the CA-CypA interaction by the G89V mutation in HIV-1 CA (A), the H126Q mutation in CypA (B), or the competitive inhibitor cyclosporine (CsA) at 2.5 μ M (C), blocks HIV-1 restriction activity in CRFK cells stably expressing the indicated T5 proteins or controls. Cells were challenged with increasing amounts (X-axis) of HIV-1 GFP vector bearing the G89V mutation in CA (A) or wild-type HIV-1 GFP vector (B,C). (D) Introduction of a naturally-occurring quadruple mutation flanking the CA-CypA binding site in CA does not abrogate hT5Cyp-mediated restriction of HIV-1 in Jurkat T cells (E) Disruption of the CA-CypA interaction using the competitive, non-immunosuppressive inhibitor DEBIO-025 blocks HIV-1 restriction in Jurkat T cells. (F) Disruption of T5 by As₂O₃ blocks HIV-1 restriction activity by T5Cyp. CRFK cells stably expressing the indicated T5Cyp were infected with HIV-1 GFP vector in the presence of increasing amounts of As₂O₃ (X-axis). In each case (A-F) the percentage of GFP⁺ cells was measured by flow cytometry 48 hrs post-infection.

[0088] Figures 50A - C. Comparison of T5 restriction activity in CRFK cells, (A,) 293T cells (B,), and Jurkat T cells (C.) In A-C, cells were transduced with vectors encoding puromycin-resistance and the indicated T5 proteins. Pools of puromycin-resistant cells were infected with increasing amounts of an HIV-1 GFP (left to right on X-axis). The percentage of GFP⁺ positive cells (Y-axis) was determined 48 hrs later.

[0089] Figures 51A - E. hT5Cyp potently blocks replication-competent HIV-1. (A,B,C) Jurkat T cells expressing the indicated proteins were infected with cell-free HIV-1_{NL4-3-GFP-IRES-Nef}, 15 ng p24/10⁶ cells (A, C) or 1 ng p24/10⁶ cells (B) and the percentage of infected cells was recorded on indicated days. (D) Indicated DiD-labelled target T cell lines were infected with HIV-1_{NL4-3}-infected Jurkat donor cells. The percentages of infected target cells were analyzed at the 4, 16, and 24 hours post initiation of co-culture by intracellular p24 staining. (E) Jurkat T cell lines were electroporated with HIV-1_{NL4-3-GFP-IRES-Nef} and the percentage of infected cells was recorded on indicated days. A-E: time post-infection is depicted on the X-axis, and % infected cells on the Y-axis.

[0090] Figures 52A - E. Indicated DiD-labelled target T cell lines were infected with HIV-1_{NL4-3-GFP-IRES-Nef}-infected Jurkat donor cells. The percentages of infected target cells were

analyzed at the indicated times post initiation of co-culture by flow cytometry for GFP expression. Puromycin was added at day 4 of co-culture to eliminate donor cells. Donor (D) and target (T) cells were mixed at a D:T ratio of (A) 2:1, (B) 1:1, (C) 1:10, (D) 1:100, (E) 1:1000.

[0091] Figures 53A - E. (A) Schematic of experimental layout for analysis of cell-to-cell spread in a single cycle of infection. (B) Jurkat T cells (target, left panel) and 293T cells (donor, right panel) stably transduced with lentiviral vectors coding for indicated T5Cyps or controls were challenged with increasing doses of an HIV-1 GFP vectors (X-axis). The percentage of infected cells (Y-axis) was determined 48 hours later. (C) Donor 293T cell lines shown in **Fig. 53B** were transfected with HIV-1_{NL4-3}-GFP-IRES-Nef proviral DNA. 48 hours post-transfection cells were washed and target DiD-labelled Jurkat T control or hT5Cyp-expressing cell lines shown in **Fig. 53B** were co-cultured with donor 293T cell lines for 2 hours (left panel) or 12 hours. The percentage of infected target Jurkat cells (Y-axis) was assessed by flow cytometry 48 hours later. T5-expression in donor 293T cell lines is indicated on the X-axis. (D) Prior to addition of target cells, relative concentration of virus produced by donor 293T cells was determined by RT assay of the supernatant. (E) Release of ³⁵S-labelled Gag from indicated donor T cells (Y-axis) was measured at given time points (X-axis) following addition of pulse.

[0092] Figures 54A – D. (A) Design of bicistronic (AIG) and dual promoter (scALPS) lentiviral vectors. (B) GFP expression by flow cytometry 48 hrs after transduction of Jurkat T cells (left) or primary CD4⁺ T cells (right) with the indicated vectors. (C, D) Quantitative RT-PCR for GFP and hT5Cyp wt or H126Q mRNA expression in CD4⁺ T cells stably transduced with scAPLS vectors.

[0093] Figures 55A - B. (A) Schematic of dual-promoter lentiviral vectors (scALPS and scALPSdsRed) used to transduce Jurkat T cells. (B) Representative plots of GFP and dsRed expression in scALPS-transduced Jurkat T cells.

[0094] Figures 56A - D. hT5Cyp inhibits HIV-1 in primary human CD4⁺ T cells. CD4⁺ T cells transduced with scALPS encoding the indicated hT5Cyp proteins were sorted for GFP, infected with HIV-1_{NL4-3} (A) or the primary isolate HIV-1_{DH12} (B), and supernatant RT activity was measured. (C, D) hT5Cyp inhibits HIV-1 in monocyte-derived macrophages. (C) CD14⁺ monocytes differentiated with GM-CSF were transduced, sorted for GFP, and

challenged with HIV-1_{NL4-3} as in (A) except that the viral envelope was modified to be CCR5-tropic or with the primary isolate HIV-1_{132W} (D).

[0095] Figures 57A - C. Selective advantage of hT5Cyp-bearing CD4⁺ T cells in the presence of HIV-1. (A) CD4⁺ T cells were transduced with scALPS encoding the indicated T5Cyp proteins. Cultures containing 23% GFP⁺ cells were challenged with 15 ng/10⁶ cells HIV-1_{NL4-3} and monitored for percentage GFP⁺ cells (left Y-axis, open symbols). Supernatant RT activity was measured (right Y-axis, filled symbols). Cultures were re-stimulated using allogeneic-PBMC, IL-2, and PHA on day 26 post-infection (arrow). (B) % viable cells (Y-axis) was assessed as a function of time (X-axis) in the mixed cultures described in (A). (C) Representative FACS plots showing viability (left panels) and GFP expression (right panels) are shown for three time points plotted in (B).

[0096] Figures 58A - C. 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 ³HThymidine incorporation. Corrected counts per minute (ccpm, Y-axis) were measured 24 hours after addition of ³HThymidine (for each condition, n=4). (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.

[0097] Figures 59A - C. (A) *In vitro* differentiated Th2 memory cells from wildtype and CypA^{-/-} mice were restimulated using plate-bound αCD3 and αCD28 antibodies. Supernatants were collected at given time points and assayed for IL-2 by ELISA. (B) The same cells as in (A) were assayed for IL-2 mRNA content by quantitative RT-PCR at given time points. Results as shown as IL-2/HPRT mRNA ratios. (C) Mice were injected intraperitoneally with αCD3 antibody and biotinylated αIL-2 detection antibody. Serum IL-2 levels were assayed by ELISA at given time points.

[0098] Figures 60A - D. (A) Schematic of APM lentiviral vector used to deliver shRNAs targeting CypA and T5 to hCD4⁺ T cells. (B) CD4⁺ T cells were stably transduced with APM coding for CypA, T5, and luciferase (Luc) control shRNAs. Following puromycin selection, relative expression of CypA, T5, and T5 α in transduced CD4⁺ T cells was measured by quantitative RT-PCR. (C) CD4⁺CrTh2⁺ human memory Th2 cells were stably transduced and selected as in (A). CypA expression in transduced Th2 cells was assessed by western blot. (D) CypA, T5, and Luc KD CD4⁺ T cells were challenged with increasing doses (X-axis) of HIV-1, HIV-2, SIV_{mac}239 GFP vectors. The percentage of infected cells (Y-axis) was assessed 72 hours post-infection by flow cytometry.

[0099] Figures 61A - E. (A) Proliferation in CypA KD T cells. CD4⁺ T and CD4⁺CrTh2⁺ cells were stably transduced with CypA KD and Luc KD APM. Following puromycin selection, proliferation 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=4). (B) CypA (right panel) and Luc KD (left panel) CD4⁺ T cells proliferating at steady state were stained with Pyronin-Y (Y-axis) for RNA content and 7-AAD (X-axis) for DNA content and analyzed by flow cytometry. (C) CypA (open histogram) and Luc KD (grey histogram) CD4⁺ T cells proliferating at steady state were stained with CFSE and assayed by flow cytometry 48 hours later. (D) Steady state CypA, T5, and Luc KD memory Th2 cells were assessed for IL-2 production by ELISA. (E) IL-4, IFN γ , and IL-2 (bottom panel, X-axis) in proliferating CypA and Luc KD CD4⁺ T cells was analyzed by flow cytometry following stimulation with PMA and ionomycin.

[00100] Figures 62A - C. (A) Proliferation in T5 KD T cells. CD4⁺ T cells were stably transduced with APM coding for T5 and Luc shRNAs. Following puromycin selection, steady-state proliferation 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=4). (B) T5 (right panel) and Luc KD (left panel) CD4⁺ T cells proliferating at steady state were stained with Pyronin-Y (Y-axis) for RNA content and 7-AAD (X-axis) for DNA content and analyzed by flow cytometry. (C) T5 (open histogram) and Luc KD (grey histogram) CD4⁺ T cells proliferating at steady state were stained with CFSE and assayed by flow cytometry 48 hours later.

[00101] Figures 63A - B. (A) Schematic describing generation of HHLS- Rag2^{-/-} γ_c ^{-/-} mice. CD34⁺ cells are enriched from cord blood. Following optional transduction, > 5 x 10⁵

CD34⁺ cells are injected intrahepatically into newborn Rag2^{-/-}γc^{-/-} mice preconditioned with sublethal irradiation. Engraftment of hCD45⁺ cells in peripheral blood is assessed 2-4 months post-transplant, and if satisfactory, mice are infected with HIV-1 by intraperitoneal (i.p.) injection. **(B)** GFP expression (X-axis) in CD34⁺ (top panel) and CD34⁺CD38⁻ cells 48 hours following optimized transduction of freshly-thawed CD34⁺ cells with FCGW (hCypA promoter driving GFP expression).

[00102] Figures 64A - B. HIV-1 infection in HHLS- Rag2^{-/-}γc^{-/-} mice. **(A)** HHLS- Rag2^{-/-}γc^{-/-} mice were generated and mock-infected with HIV-1_{ΔNef} (top panel) or infected with HIV-1_{NL4-3} (bottom panel) 10 weeks post-transplant. Four weeks post-infection, mice were sacrificed, and single cell suspensions from indicated organs were analyzed for presence of CD4⁺ (Y-axis) and CD8⁺ (X-axis) T cells. Viral load in plasma from these mice was determined. **(B)** Representative CD3- and p24-stained paraffin-embedded tissue sections of thymus are shown. Engraftment of CD3⁺ human T cells in the thymus is robust and p24 staining is observed.

[00103] Figures 65A - C. **(A)** SIV-VLPs enhance CD34⁺ HSC transduction. Donor-identical CD34⁺ cells were transduced with FCGW following mock pretreatment (left panel) or pretreatment with SIV-VLPs (right panel). The percentage of GFP⁺ (X-axis) cells was determined 72 hours post-transduction. **(B)** HHLS- Rag2^{-/-}γc^{-/-} mice were generated using CD34⁺ cells transduced with BID, a dual-promoter vector in which the mCMV promoter drives GFP expression (see schematic). Mice were sacrificed 12 weeks post-transplant and the percentage of GFP⁺ cells in the thymus (left panel) and bone marrow (right panel) was determined by flow cytometry. **(C)** HHLS- Rag2^{-/-}γc^{-/-} mice were generated using CD34⁺ cells transduced with FCGW, a lentiviral vector in which the hCypA promoter drives GFP expression (see schematic). Mice were sacrificed after 12 weeks and analyzed as in **(B)**.

[00104] Figures 66A - C. hT5Cyp reduces viral load in HIV-1 infected HHLS-Rag2^{-/-}γc^{-/-} mice. **(A)** HHLS-Rag2^{-/-}γc^{-/-} were generated using CD34⁺ cells stably transduced with scAPLS-hT5Cyp or scAPLS-hT5CypH126Q. ~ 12% of CD34⁺ cells were GFP⁺ 72 hours post-transplant *in vitro* (left panels). Human cell engraftment in peripheral blood 8 weeks post-transplant and transgene expression in CD45⁺ human lymphocytes, CD3⁺ T cells, and CD19⁺ B cells was analyzed by flow cytometry. **(B)** Viral load was determined in plasma of

infected mice at 24 days post-infection. (C) Change in transgene (GFP) expression in CD45⁺ cells was compared pre- and post-infection by flow cytometry.

[00105] Figures 67A - D. Establishment of a hCD4-Rag2^{-/-}γc^{-/-} mouse model. (A) Experimental design for adoptive transfer of PBMC or CD4⁺ T cells into Rag2^{-/-}γc^{-/-} mice. Gy = Gray; i.p. = intraperitoneal injection. (B) Titration of human PBMC and CD4⁺ T cells adoptively transferred to Rag2^{-/-}γc^{-/-}. Engraftment of CD45⁺ cells (Y-axis) in the peripheral blood was assessed by flow cytometry on indicated days post-transfer (X-axis). (C) Survival of Rag2^{-/-}γc^{-/-} mice after transfer of human PBMC or CD4⁺ T cells. Mice were sacrificed upon the appearance of severe GvHD symptoms. (D) Left panel shows a representative plot of input CD4⁺ T cells following enrichment from PBMCs. Right panel shows engraftment of human lymphocytes in peripheral blood 21 days following transfer of PBMCs or CD4⁺ T cells to pre-conditioned mice.

[00106] Figures 68A - C. (A) Experimental design for adoptive transfer of transduced CD4⁺ T cells into Rag2^{-/-}γc^{-/-} mice. 6-10 week old Rag2^{-/-}γc^{-/-} mice were engrafted with GFP-sorted scALPS-transduced CD4⁺ T cells expressing either hT5Cyp or hT5CypH126Q. (B) A representative plot of input cells is shown (left panel). Grey histograms represent GFP-expression (X-axis) in un-transduced, donor identical CD4⁺ T cells, open histograms represent scALPS-transduced cells. (C) Mice were sacrificed 6 weeks post-transplant and analyzed for human cell engraftment and transgene expression. Single cell suspensions of thymus were analyzed for presence of recipient-derived CD45⁺ mouse cells (mCD45.2, x-axis) and donor derived hCD45⁺ lymphocytes (X-axis). GFP expression (X-axis) in mCD45.2⁺ cells (grey histogram) and hCD45⁺ (open histogram) cells is shown.

[00107] Figures 69A – E. hT5Cyp protects CD4⁺ T cells from HIV-1 infection *in vivo*. (A) hT5Cyp prolongs CD4⁺ T cell-survival *in vivo*. 6-10 week old Rag2^{-/-}γc^{-/-} mice were engrafted with GFP-sorted scALPS-transduced CD4⁺ T cells expressing either hT5Cyp or hT5CypH126Q. Mice were infected 5 days after adoptive CD4⁺ T cell transfer with CXCR4-tropic HIV-1_{NL4-3} and analyzed for presence of hCD4⁺ T cells (Y-axis) at 2 weeks post-infection. Individual mice are shown and fold-difference in mean peripheral blood CD4⁺ lymphocyte engraftment (red bar) is indicated above the bracket. (***)p = 0.0002, Mann-Whitney test). (B) hT5Cyp reduces viral burden *in vivo*. Rag2^{-/-}γc^{-/-} mice were engrafted with transduced CD4⁺ T cells were infected 2 weeks after adoptive CD4⁺ T cell transfer with

CXCR4-tropic HIV-1_{NL4-3}. Plasma viral load was determined at 4 weeks post-infection. Individual mice are shown and fold-difference in viral load (red bar) is indicated above the bracket. (**p = 0.005, Mann-Whitney test). **(C,D,E)** hT5Cyp protects CD4⁺ from productive HIV-1 infection in lymphoid organs. Age-matched Rag2^{-/-}γc^{-/-} mice transplanted with CD4⁺ T cells expressing either hT5Cyp or hT5CypH126Q were infected with CXCR4-tropic HIV-1_{NL4-3} 2 weeks after adoptive transfer of CD4⁺ T cells. Single-cell suspensions of thymi were analyzed 14 days post-infection for p24⁺ (X-axis) CD3⁺ (Y-axis) T cells **(C)** or CD4-downregulation (X-axis) **(D)**. Representative CD3- and p24-stained paraffin-embedded tissue sections of thymus are shown. While engraftment of CD3⁺ human T cells is similar in thymi of mice transplanted with either hT5Cyp- (top panel) or hT5CypH126Q-expressing CD4⁺ T cells (bottom panel), rare and weaker p24 staining is observed in organs from mice transplanted with hT5Cyp-CD4⁺ T cells as compared to those transplanted with hT5CypH126Q-CD4⁺ T cells **(E)**.

[00108] Figures 70A - D. hT5Cyp protects CD4⁺ T cells from HIV-1 infection *in vivo*. **(A)** Lymphoid organs from Rag2^{-/-}γc^{-/-} mice engrafted with transduced CD4⁺ T cells are shown 4 weeks after infection with CCR5-tropic HIV-1. T = thymus, S = spleen, MLN = mesenteric lymph node, PLN = peripheral lymph node, A = axillary, I = inguinal, C = cervical, P = popliteal, B = brachial **(B,C,D)** hT5Cyp protects CD4⁺ from productive HIV-1 infection in lymphoid organs. Single cell suspensions from MLN and PLN of mice infected with CCR5-tropic HIV-1 were analyzed for GFP transgene expression **(B, left panel, X-axis)**, CD4 downregulation **(B, right panel, Y-axis)**, and presence of p24⁺ (X-axis) CD3⁺ (Y-axis) cells **(C)**. Representative CD3- and p24-stained paraffin-embedded tissue sections of mesenteric lymph node are shown. While engraftment of CD3⁺ human T cells is similar in MLN of mice transplanted with either hT5Cyp- (top panel) or hT5CypH126Q-expressing CD4⁺ T cells (bottom panel), rare and weaker p24 staining is observed in organs from mice transplanted with hT5Cyp-CD4⁺ T cells as compared to those transplanted with hT5CypH126Q-CD4⁺ T cells **(D)**.

[00109] Figures 71A - D. **(A)** Experimental design for adoptive transfer of transduced CD4⁺ T cells and *in vivo* challenge with cell-associated HIV-1 in Rag2^{-/-}γc^{-/-} mice. Gy = Gray; i.p. = intraperitoneal injection. **(B)** hT5Cyp reduces viral burden *in vivo*. 6-10 week old Rag2^{-/-}γc^{-/-} mice were engrafted with GFP-sorted scALPS-transduced CD4⁺ T cells expressing either

hT5Cyp or hT5CypH126Q. Mice were infected 5 days after adoptive CD4⁺ T cell transfer with autologous, untransduced, HIV-1 infected cells. Plasma viral load (Y-axis) was assessed at indicated times post-infection. (C) hT5Cyp prolongs CD4⁺ T cell-survival *in vivo*. CD4⁺ T cell retention (Y-axis) was assessed as the percentage of CD4⁺ T cells in peripheral blood at 24 days compared to 11 days post-infection. (D) Single cell suspensions of thymuses from mice that received hT5Cyp- or hT5CypH126Q-expressing CD4⁺ T cells were analyzed for p24-, CD3-, and GFP-expression by flow cytometry.

Detailed Description

[00110] As used herein the term “lentiviral restriction activity” of a fusion protein or polypeptide is defined as binding and degradation of a lentivirus, wherein the cyclophilin A part of the fusion protein specifically binds to the lentiviral capsid protein, while the TRIM5 part of the fusion protein acts as TRIM5 alone has been characterized, causing the degradation of the substance bound to the cyclophilin A portion of the fusion protein. Thus, the fusion protein has coupled a specific binding tropism, i.e., the binding of cyclophilin portion to capsid of a lentivirus, with the degradation activity of TRIM5. The term “lentiviral restriction activity” of a fusion protein or polypeptide may be used interchangeably with the term “both TRIM activity and cyclophilin activity.”

[00111] In certain embodiments the term “lentiviral restriction activity” specifically includes the term “HIV-1 restriction activity” of a fusion protein or polypeptide, which is defined as binding and degradation of HIV-1, wherein the cyclophilin A part of the fusion protein specifically binds to the HIV-1 capsid protein once HIV-1 enters the cell, while the TRIM5 part of the fusion protein acts as TRIM5 alone has been characterized, causing the degradation of the substance bound to the cyclophilin A portion of the fusion protein. Thus, the fusion protein has coupled a specific binding tropism, i.e., the binding of cyclophilin portion to capsid of HIV-1, with the degradation activity of TRIM5.

[00112] As used herein, "administration" means any of the standard methods of administering a pharmaceutical composition known to those skilled in the art. Examples include, but are not limited to, intravenous, intraperitoneal or intramuscular administration, *in vitro* gene therapy via adenoviral vector or other vector (liposome), *ex vivo* gene therapy, oral, and inhalation. In another embodiment of the invention, the administering is carried out via injection, oral administration, or topical administration. In another embodiment, administration is intramuscularly, intramucosally, intranasally, subcutaneously,

intradermally, transdermally, intravaginally, intrarectally, orally or intravenously. In one embodiment of this invention, the subject is a mammal, e.g., a mouse or a human. Preferably, the mammal is a human. In another embodiment of the invention, the "introducing" or administering is carried out by a means selected from the group consisting of transduction, viral-mediated introduction, adenovirus infection, liposome-mediated transfer, topical application to the cell, and microinjection. In another embodiment of the invention, the carrier is an aqueous carrier, a liposome, a vector, a viral vector, or a lipid carrier.

[00113] As used herein "nucleic acid molecule" includes both DNA and RNA and, unless otherwise specified, includes both double-stranded and single-stranded nucleic acids. Also included are hybrids such as DNA-RNA hybrids. Reference to a nucleic acid sequence can also include modified bases as long as the modification does not significantly interfere either with binding of a ligand such as a protein by the nucleic acid or Watson-Crick base pairing.

[00114] Two DNA or polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. As used herein, "substantially homologous" also refers to sequences showing identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, 2nd ed., Cold Springs Harbor, New York (1989).

[00115] A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* or *in vitro* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5'-(amino) terminus and a translation stop codon at the 3'-(carboxy) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) sources, viral RNA or DNA, and even synthetic nucleotide sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

[00116] "Operably linked" refers to an arrangement of nucleotide sequence elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

[00117] A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. In eukaryotic cells, a stably transformed cell is generally one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication, or one which includes stably maintained extrachromosomal plasmids. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

[00118] As used herein, a "variant", refers to a nucleic acid or a protein of the invention differing in sequence from a "reference" molecule, for example a sequence specifically described herewith, but retaining function, activity and/or therapeutic property of the inventive fusion proteins or inventive nucleic acids which encode the inventive fusion proteins with lentiviral restriction activity, which activity is described elsewhere herein or otherwise known in the art. In certain embodiments, the lentiviral restriction activity is HIV-1 restriction activity. Generally, variants are overall very similar, and, in many regions, identical to the amino acid sequence of the inventive proteins or the nucleic acid sequences encoding the same.

[00119] As used herein, "mutation" with reference to a polynucleotide or polypeptide, refers to a naturally-occurring, synthetic, recombinant, or chemical change or difference to the primary, secondary, or tertiary structure of a polynucleotide or polypeptide, as compared to a reference polynucleotide or polypeptide, respectively (e.g., as compared to sequences of polynucleotide or polypeptide as described herein). Mutations include such changes as, for

example, deletions, insertions, or substitutions. Polynucleotides and polypeptides having such mutations can be isolated or generated using methods well known in the art.

[00120] As used herein, "deletion" is defined as a change in either polynucleotide or amino acid sequences in which one or more polynucleotides or amino acid residues, respectively, are absent.

[00121] As used herein, "insertion" or "addition" is that change in a polynucleotide or amino acid sequence which has resulted in the addition of one or more polynucleotides or amino acid residues, respectively, as compared to a reference polynucleotide or amino acid sequence.

[00122] As used herein, "substitution" results from the replacement of one or more polynucleotides or amino acids by different polynucleotides or amino acids, respectively.

[00123] The present invention is also directed to proteins which comprise, or consist of, or consist essentially of an amino acid sequence which is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100%, or which is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% identical to, for example, the amino acid and nucleic acids sequences of the invention.

[00124] Further polypeptides encompassed by the invention are polypeptides encoded by polynucleotides which hybridize to the complement of a nucleic acid molecule encoding a fusion protein of the invention under stringent hybridization conditions (e.g., hybridization to filter bound DNA in 6X Sodium chloride/Sodium citrate (SSC) at about 45 degrees Celsius, followed by one or more washes in 0.2X SSC, 0.1% SDS at about 50-65 degrees Celsius), under highly stringent conditions (e.g., hybridization to filter bound DNA in 6X sodium chloride/Sodium citrate (SSC) at about 45 degrees Celsius, followed by one or more washes in 0.1X SSC, 0.2% SDS at about 68 degrees Celsius), or under other stringent hybridization conditions which are known to those of skill in the art (see, for example, Ausubel, F. M. et al., eds., 1989 Current protocol in Molecular Biology, Green publishing associates, Inc., and John Wiley & Sons Inc., New York, at pages 6.3.1-6.3.6 and 2.10.3). Polynucleotides encoding these polypeptides are also encompassed by the invention.

[00125] By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject/reference polypeptide sequence may in a non-limiting example include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid. These alterations of the reference sequence may occur at the amino- or carboxy-terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

[00126] As a practical matter, whether any particular polypeptide is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% identical to, for instance, the amino acid sequence of a fusion protein of the invention or a fragment thereof, can be determined conventionally using known computer programs. A non-limiting example of a method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990)). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said global sequence alignment is expressed as percent identity. Parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

[00127] If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N-

and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

[00128] For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. In certain embodiments, no other manual corrections are to be made for the purposes of the present invention.

[00129] The variants of the invention will usually have at least 75% , 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% or 75% , 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100% sequence identity with any length or the full length of any of the inventive sequences provided herein. Homology or identity at the nucleotide or amino acid sequence level maybe determined by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp,

blastn, blastx, tblastn and tblastx (Karlin et al., Proc. Natl. Acad. Sci. USA 87: 2264-2268 (1990) and Altschul, J. Mol. Evol. 36: 290-300 (1993), fully incorporated by reference) which are tailored for sequence similarity searching.

[00130] The approach used by the BLAST program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al., (Nature Genetics 6: 119-129 (1994)) which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (i.e., the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff et al., Proc. Natl. Acad. Sci. USA 89: 10915-10919 (1992), fully incorporated by reference). For blastn, the scoring matrix is set by the ratios of M (i.e., the reward score for a pair of matching residues) to N (i.e., the penalty score for mismatching residues), wherein the default values for M and N are 5 and -4, respectively. Four blastn parameters may be adjusted as follows: Q=10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent Blastp parameter settings were Q=9; R=2; wink=1; and gapw=32. A Bestfit comparison between sequences, available in the GCG package version 10.0, uses DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty) and the equivalent settings in protein comparisons are GAP=8 and LEN=2. Other sequence analysis programs and algorithms are known and used in the art. Non-limiting examples of programs for sequence analysis include DNASTar®Lasergene® and MacVector.

[00131] The polynucleotide variants of the invention may contain alterations in the coding regions, non-coding regions, or both. The invention provides polynucleotide variants containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. The invention provides nucleotide variants produced by silent substitutions due to the degeneracy of the genetic code. Moreover, polypeptide variants in which less than 50, less than 40, less than 30, less than 20, less than 10, or 5-50, 5-25, 5-15, 5-10, 1-5, 1-3, 1-2 or 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino

acids are substituted, deleted, or added in any combination are also preferred. Polynucleotide variants can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host, for example but not limited to yeast or mammalian cells.

[00132] In certain embodiments, the invention provides naturally occurring variants which are called "allelic variants," and refer to one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. (Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985)). These allelic variants can vary at either the polynucleotide and/or polypeptide level and are included in the present invention. Alternatively, non-naturally occurring variants may be produced by mutagenesis techniques or by direct synthesis as described herein and known in the art. See Sambrook et al. In certain embodiments, the invention contemplates allelic variants of the TRIM5 and/or CyclophilinA portion of the nucleic acids which encode the fusion proteins of the invention. Using known methods of protein engineering and recombinant DNA technology, variants may be generated to improve or alter the characteristics of the polypeptides of the present invention. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of the polypeptide of the present invention without substantial loss of biological function. It is well known in the art that variants often retain a biological activity similar to that of the reference protein. In certain embodiments, variants of proteins of the invention or nucleic acids encoding proteins of the invention retain HIV-1 restriction activity as described herein. The HIV-1 restriction activity of variants may be comparable, increased or reduced compared to the HIV-1 restriction activity of the reference polypeptides. Furthermore, even if deleting one or more amino acids from the N-terminus or C-terminus of a polypeptide results in modification or loss of one or more biological activities, other biological activities may still be retained. For example, the ability of a variant to bind to HIV-1 may be reduced compared to a reference polypeptide of the invention, while the variant maintains the HIV-1 degradation activity, or vice versa, thus preserving the "HIV-1 restriction activity," which can readily be determined by methods described herein and otherwise known in the art.

[00133] In certain embodiments, the variants of the invention have conservative substitutions. The term "conservative substitutions" refers to substitution of an amino acid residue for a different amino acid residue that has similar chemical properties as known and understood by a person of ordinary skill in the art. Non-limiting examples are replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl

residues Ser and Thr; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly. The term "nonconservative" refers to substituting an amino acid residue for a different amino acid residue that has different chemical properties. In certain embodiments the invention provides non-conservative variants which have substantially the same HIV-1 restriction activity compared to the reference polypeptides. The invention contemplates conservative and/or nonconservative variants, so long as these variants retain HIV-1 restriction activity.

[00134] In certain embodiments the invention provides variants which modify the nucleic acids or amino acids of the linker between the TRIM5 and Cyclophilin portion of the fusion proteins of the invention. Without being bound by theory, the linker between the TRIM5 and Cyclophilin portions of the fusion proteins of the invention may tolerate a larger % variation in the number and/or types of modifications, including different length of the linker, while maintaining the HIV-1 restriction activity. In certain embodiments, the invention contemplates modifications of the linker which result in larger % variation in the linker sequence and thus lower % identity as compared to the % identity between the TRIM5 and/or Cyclophilin portions of the variants and the fusion protein of the invention. A non-limiting example includes variants wherein the identity between the TRIM5 and/or Cyclophilin portions of a variant and the fusion proteins of the invention is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100%, or 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100%, while the identity between the linker of the variant and the fusion proteins of the invention is at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100%, or 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 100%, or any combination thereof.

[00135] Guidance concerning how to make phenotypically silent amino acid substitutions is provided, for example, in Bowie et al., "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid sequence to change.

[00136] The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in different species, conserved amino acids can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions where substitutions have been tolerated by natural selection indicates that these positions are not critical for protein function. Thus, positions tolerating amino acid substitution could be modified while still maintaining biological activity of the protein. The second strategy uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene to identify regions critical for protein function. For example, site directed mutagenesis or alanine-scanning mutagenesis (introduction of single alanine mutations at every residue in the molecule) can be used. See Cunningham and Wells, *Science* 244:1081-1085 (1989). The resulting mutant molecules can then be tested for biological activity.

[00137] As the authors state, these two strategies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at certain amino acid positions in the protein. For example, most buried (within the tertiary structure of the protein) amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Moreover, tolerated conservative amino acid substitutions involve replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Thr; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly.

[00138] Besides conservative amino acid substitution, variants of the present invention include (i) polypeptides containing substitutions of one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) polypeptides containing substitutions of one or more of the amino

acid residues having a substituent group, or (iii) polypeptides which have been fused with or chemically conjugated to another compound, such as a compound to increase the stability and/or solubility of the polypeptide (for example, polyethylene glycol), (iv) polypeptide containing additional amino acids, such as, for example, an IgG Fc fusion region peptide. Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

[00139] For example, polypeptide variants containing amino acid substitutions of charged amino acids with other charged or neutral amino acids may produce proteins with improved characteristics, such as less aggregation. Aggregation of pharmaceutical formulations both reduces activity and increases clearance due to the aggregate's immunogenic activity. See Pinckard et al., *Clin. Exp. Immunol.* 2:331-340 (1967); Robbins et al., *Diabetes* 36: 838-845 (1987); Cleland et al., *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993).

[00140] In specific embodiments, the polypeptides of the invention comprise, or alternatively, consist of, or consist essentially of fragments or variants of the amino acid sequences of the invention wherein the fragments or variants have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 1-5, 5-10, 5-15, 5-25, 5-50, 10-50 or 50-150, amino acid residue additions, substitutions, and/or deletions when compared to the reference amino acid sequence. In preferred embodiments, the amino acid substitutions are conservative. Nucleic acids encoding these polypeptides are also encompassed by the invention.

[00141] The polypeptide of the present invention can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. It will be appreciated that polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids, and that many amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes such as glycosylation and other post-translational modifications, or by chemical modification techniques which are well known in the art. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains

and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, PROTEINS--STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W.H. Freeman and Company, New York (1993); POST-TRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., Meth. Enzymol. 182:626-646 (1990); Rattan et al., Ann. N.Y. Acad. Sci. 663:48-62 (1992)).

[00142] The invention further provides a stem cell comprising a nucleic acid of the invention, wherein the nucleic acid is part of the genome of the stem cell. As used herein a "stem cell" refers to an undifferentiated cell in the bone marrow that has the ability both to multiply and to differentiate into a specific, specialized cell, such as a blood cell. In one embodiment, human hematopoietic stem cells are mobilized from the bone marrow of an HIV-1 infected subject, the cells are transduced *in vitro* with vectors expressing human TRIMCyp, and the cells are injected back into the patient as an auto-transplant.

[00143] As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

[00144] Provided herein is a novel fusion between human TRIM5alpha (TRIM5 α ; T5; TRIM) and human cyclophilin A (CypA; Cyp; Cyp A). A fully human nucleic acid fusion sequence and polypeptide fusion sequence comprising a human TRIM5 sequence and a human CypA sequence are provided. Such fusion molecules are referred to as human

TRIM5-CypA (or hT5-CypA or TRIM-Cyp or hTRIMCyp, or similar variations). Also provided are methods of using the fusion molecules.

[00145] The discovery that certain TRIM5 (T5) orthologues inhibit HIV-1 infection immediately after the virus enters otherwise susceptible cells (Diaz-Griffero et al., 2006b; Perez-Caballero et al., 2005a; Sayah et al., 2004; Stremlau et al., 2004) raised the prospect that these host factors might be exploited in HIV-1 gene therapy. The α -isoform of TRIM5 (T5 α) contains a C-terminal PRYSPRY domain that is required for T5 α binding to the capsid (CA) of restriction-sensitive retroviruses (Sebastian and Luban, 2005; Stremlau et al., 2006). The specificity of the PRYSPRY-CA interaction determines which retrovirus a given T5 α orthologue inhibits (Luban, 2007). While human T5 α (hT5 α) weakly blocks HIV-1, it potently blocks N-tropic murine leukemia virus (N-MLV). In contrast, rhesus T5 α (RhT5 α) inhibits HIV-1 but N-MLV only weakly. The specificity of retroviral restriction and the modular nature of the T5 components are further demonstrated by the enhanced HIV-1 restriction activity that results when the hT5 α PRYSPRY domain is replaced with that from RhT5 α (Perez-Caballero et al., 2005a; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005).

[00146] The T5 gene in the New World owl monkey (genus *Aotus*) is unusual. It resulted from retrotransposition of the cyclophilin A (CypA) cDNA into intron 7 (Nisole et al., 2004; Sayah et al., 2004). CypA is a CA-binding protein (Luban et al., 1993) and the *Aotus* TRIM5Cyp fusion (AoT5Cyp) prevents HIV-1 infection (Nisole et al., 2004; Sayah et al., 2004). AoT5Cyp has several properties that make it appealing for gene therapy. It potently inhibits HIV-1 infection when expressed in human cells (Nisole et al., 2004; Sayah et al., 2004), acting on the virus within minutes of entry (Diaz-Griffero et al., 2006b; Perez-Caballero et al., 2005a). Furthermore, AoT5Cyp is the only TRIM5 allele in 10 *Aotus* species (Ribeiro et al., 2005), indicating that cells bearing AoT5Cyp retain functionality. One drawback of AoT5Cyp is that it is not a human protein and if employed as gene therapy it might elicit an immune response (Riddell et al., 1996). Provided herein is an engineered fully human TRIM5-cyclophilin A fusion proteins (hT5Cyp) exhibiting HIV-1 restriction activity comparable to AoT5Cyp and possessing all the properties desired of anti-HIV-1 gene therapy.

[00147] In Old World primates, TRIM5- α confers a potent block to HIV-1 infection that acts after virus entry into cells. Cyp A binding to viral capsid protects HIV-1 from a similar activity in human cells. Among New World primates, owl monkeys exhibit post-entry restriction of HIV-1. Paradoxically, the barrier to HIV-1 in owl monkey cells is released by capsid mutants or drugs that disrupt capsid interaction with CypA.

[00148] U.S. Patent Application Publication No. 2008/0045454, incorporated herein by reference, describes a fusion protein between TRIM5 and cyclophilin A which was discovered and isolated from Owl monkey, which is a species of primate from South America that are resistant to HIV-1 infection. The TRIM-cyclophilin fusion protein provides the Owl monkey with its resistance to HIV. The cyclophilin A part of the fusion protein specifically binds to the HIV-1 capsid protein once HIV-1 enters the cell. The TRIM5 part of the fusion protein acts as TRIM5 alone has been characterized, causing the degradation of the substance bound to the cyclophilin A portion of the fusion protein, namely the degradation of HIV-1. Thus, the fusion protein has coupled a specific binding tropism, i.e., the binding of cyclophilin to capsid of HIV-1, with the degradation activity of TRIM5. The cyclophilin part of the fusion protein is highly conserved among different species. Cyclophilin is a protein that is present in yeast and in humans. It plays a role in protein-folding and in the biological response to the immunosuppressive drug cyclosporine. The TRIM5 part of the fusion protein is highly variable from one species to another.

[00149] The α -isoform of TRIM5 (T5 α) contains a C-terminal PRYSPRY domain that is required for T5 α binding to the capsid (CA) of restriction-sensitive retroviruses^{6,7}. The specificity of the PRYSPRY-CA interaction determines which retrovirus a given T5 α orthologue inhibits⁸. While human T5 α (hT5 α) weakly blocks HIV-1, it potently blocks N-tropic murine leukemia virus (N-MLV). In contrast, rhesus T5 α (rhT5 α) inhibits HIV-1 but not N-MLV. Restriction specificity and the modular nature of the T5 components are further demonstrated by the enhanced HIV-1 restriction activity that results when the hT5 α PRYSPRY domain is replaced with that from rhT5 α ⁹⁻¹².

[00150] The potency of a human TRIM-Cyp fusion has been demonstrated in vitro and in vivo in a humanized mouse model of HIV-1. The use of TRIM-Cyp provides several benefits and improvements over current anti-HIV-1 technologies, such as recombinant RevM10 protein, RNAi approaches, and disruption of the CCR5 co-receptor. The high

efficacy of human TRIM-Cyp would enable its use as a monotherapy, which is a significant benefit over the use of combination therapies required by the current technologies. Also, the potency of human TRIM-Cyp minimizes the emergence of viral resistance, a recurrent problem with current technologies. Human TRIMCyp acts early in the viral life cycle conferring true protection and blocking the ligation of HIV-1 into host chromosomal DNA, thus minimizing viral persistence and potential mutagenic effects of HIV-1 infection.

[00151] Provided herein is an HIV-1 inhibitor modeled after AoT5Cyp generated by fusing human CypA to hT5 (hT5Cyp). As further described in Example 1, of 13 fusions, three were as potent as AoT5Cyp. Antiviral activity of the human fusions required capsid (CA)-binding by hCypA, linkage of hCypA to the capsid-specificity determinant in hT5, and the ability to form cytoplasmic bodies. CXCR4- and CCR5-tropic HIV-1 clones and primary isolates, a circulating HIV-1 isolate previously reported as AoT5Cyp-resistant, HIV-2, SIV_{AGM}tan, and FIV were all inhibited by hT5Cyp. hT5Cyp blocked HIV-1 infection of primary CD4⁺ T cells and macrophages, and conferred selective advantage after HIV-1 infection, without selection for resistant virus or disruption of host cell function. Moreover, hT5Cyp preserved hCD4⁺ T cells after adoptive transfer to Rag2^{-/-}γc^{-/-} mice and challenge with HIV-1; viremia and productive infection in lymphoid organs were markedly reduced. Thus, hT5Cyp is a robust candidate for HIV-1 gene therapy.

[00152] The more successful anti-HIV-1 gene therapy approaches to date have followed two main strategies: either modification of host-cell factors required for viral replication or inhibition of essential viral elements (Strayer et al., 2005). As described herein, a third approach was adopted: the exploitation of natural inhibitors that evolved over millions of years in primates in response to retroviral attack (Li, 2006b; Sawyer, 2005; Stremlau, 2005; Yap, 2005). The potent block to HIV-1 observed with AoT5Cyp inspired the design of a human equivalent, hT5Cyp, that robustly blocks HIV-1 *in vitro* and *in vivo*. The strict structural requirements for engineering a functional hT5Cyp emphasize the remarkable nature of the fusion gene that was generated by retrotransposition in New World owl monkeys (Sayah et al., 2004). A distinct T5Cyp fusion gene was generated by an independent retrotransposition event in Old World macaques. In this case, restriction activity was detected against HIV-2 and FIV, but not against HIV-1 (Stoye and Yap, 2008). The convergent evolution of T5Cyp fusion proteins with distinct retroviral specificities indicates a strong selection for these potent restriction factors. While the specific force behind selection remains

to be elucidated for individual T5 orthologues, in each case the selective pressure is likely to have been a retrovirus (Sawyer et al., 2005).

[00153] Since T5 α and T5Cyp are modular proteins, it was expected that the engineering of a restrictive hT5Cyp would be trivial. Surprisingly, only three of 13 hT5Cyp fusions inhibited HIV-1 comparably to AoT5Cyp. No correlation was observed between protein levels and antiviral activity, and CA-binding activity was not sufficient to restrict HIV-1 (*See Fig. 47*). Structural modeling showed that anti-HIV-1 activity was observed when hCypA was fused at the apex of the hT5 α PRYSPRY domain (*See Fig. 47C*). Analysis of nonsynonymous mutations indicates that this region undergoes some of the strongest selective pressure in the primate lineage and functional experiments pinpoint it as a specificity determinant for CA recognition (Sawyer et al., 2005; Song et al., 2005b; Yap et al., 2005). CypA fusion to this site may have antiviral activity because this apical loop is uniquely situated for coordinating CA recognition and effector domains of T5. Correlation between restriction activity and the ability of hT5Cyp fusion proteins to form cytoplasmic bodies suggests that activity requires particular spatial constraints or association with cofactors.

[00154] Optimal gene therapy candidates should provide high efficacy and low antigenicity. Hematopoietic progenitors transduced with rhesus macaque T5 α or human-rhesus T5 α chimeras differentiate into HIV-1-resistant macrophages and T cells (Anderson and Akkina, 2005a, 2008). These proteins, however, are potentially antigenic, which could lead to the elimination of modified cells *in vivo* (Riddell et al., 1996). By substituting only critical amino acids within the hT5 α PRYSPRY domain one can reduce the risk of antigenicity but this also decreases anti-HIV-1 efficacy (hT5 α R332P; *See Fig. 5, A and B*). hT5Cyp potently inhibits HIV-1 and, being composed of human components, is of low antigenic potential.

[00155] HIV-1 *rev*, *tat*, and *gag* (Boden et al., 2007; Strayer et al., 2005), as well as the HIV-1 co-receptor CCR5 (An et al., 2007; Perez et al., 2008), have been targeted by various means including siRNAs (Boden et al., 2007; Strayer et al., 2005) and zinc-finger endonucleases (Perez et al., 2008). With either approach there are concerns regarding toxicity³⁴, off-target effects³⁵, and viral resistance (Boden et al., 2007; Strayer et al., 2005). For siRNA approaches, escape mutants arise readily, since single point mutations are

sufficient to escape the siRNA-mediated blocks to HIV-1 replication (Boden et al., 2007). CCR5 disruption could select for CCR5-independent viruses (Taylor et al., 2008) and is not without consequence, as the *CCR5* Δ 32 allele is a risk factor for symptomatic West Nile Virus infection (Glass et al., 2006).

[00156] hT5Cyp is a broadly acting, anti-lentiviral agent that blocks CCR5- and CXCR4-tropic HIV-1 clones and primary isolates, as well as some HIV-2, SIV, and FIV clones (*See* Figs. 48, 49, 56). T5 isoforms form hetero-multimers and hT5Cyp might interfere with endogenous hT5 α function (Berthoux et al., 2005a). However, no significant decrease in hT5 α -mediated restriction of N-MLV, and no alteration in cell surface markers or cytokine secretion, was observed in hT5Cyp-transduced cells (*See* Fig.46). hT5Cyp-resistant viruses were not detected, perhaps due to the potency of the antiviral activity and the fact that hT5Cyp blocks HIV-1 prior to reverse transcription. hT5Cyp additionally blocked a natural HIV-1 CA variant reported to be resistant to AoT5Cyp (Chatterji et al., 2005) (*See* Fig.49D). These results suggest that, if hT5Cyp were utilized as anti-HIV-1 gene therapy in people, viral resistance would not emerge and host cell function would remain intact.

[00157] The evaluation of HIV-1 therapies is limited by a lack of adequate animal models for HIV-1 replication and pathogenesis. The hCD4⁺-Rag2^{-/-} γ c^{-/-} mouse described in Example 2 is a robust model for assessing inhibition of HIV-1 infection and CD4⁺ T cell protection by lentiviral transduction with hT5Cyp (*See* Fig. 69, 70, 71). Autotransfusion of *ex vivo* expanded CD4⁺ T cells is of clinical benefit to HIV-1-infected people (Levine et al., 2002). Thus, given that hT5Cyp-transduced CD4⁺ cells can be expanded *in vitro* and *in vivo* (*See* Fig. 54, 69), and that these cells exhibit a selective advantage during HIV-1 infection (*See* Fig. 57), an impressive effect of hT5Cyp-transduction on autologous CD4⁺ T cell gene therapy in the clinical setting is expected. CD34⁺ hematopoietic stem cells transduced with state-of-the-art lentiviral vectors (*see* Fig. 65,66 (Amendola et al., 2005)) achieve long-term engraftment of Rag2^{-/-} γ c^{-/-} mice, but transgene expression in the mature CD4⁺ T cells that develop within these animals has not yet been consistently detected. One could examine the effect of stem cell transduction with hT5Cyp on subsequent immune cell development and HIV-1 infection with a humanized mouse models currently under development (Goldstein, 2008).

[00158] Human TRIM5 and CypA sequences have been deposited in GenBank (accession numbers NM_033034 (human TRIM5alpha mRNA; Figure 13; SEQ ID NO:1), NP_149023 (human TRIM5alpha protein; Figure 14; SEQ ID NO:2), NM_021130 (human CypA mRNA; Figure 15; SEQ ID NO:3) and NP_066953 (human CypA protein; Figure 16; SEQ ID NO:4)).

[00159] The invention provides for a newly engineered nucleic acids and polynucleotides that comprise the nucleotide sequence of SEQ ID NO:1, 3, 5, 6 or 7. The encoded polypeptide is a fusion between human TRIM and cyclophilin, i.e., hTRIMCyp, which acts as an anti-HIV-1 factor.

[00160] The invention also provides for nucleic acid variants of any of SEQ ID NO: 1, 3, 5, 6 or 7 having at least about 50% identity to the SEQ ID NO, and encoding a polypeptide having both TRIM activity and cyclophilin activity. The variants may have at least about 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity to the SEQ ID NO. Techniques for determining sequence identity are well known to one skilled in the art, and include, for example, analysis with a sequence comparison algorithm or FASTA version 3.0t78 using default parameters.

[00161] The invention also provides for an isolated nucleic acid which comprises consecutive nucleotides having a sequence complementary to the nucleic acids comprising the nucleotide sequence of SEQ ID NO:1, 3, 5, 6 or 7 or variants of at least about 50% identity thereof. The invention also provides for an isolated nucleic acid encoding a polypeptide comprising SEQ ID NO:4 (human Cyp A), and SEQ ID NO:2 (human TRIM5), wherein the last amino acid of SEQ ID NO:2 is alanine 331 (SEQ ID NO:5; Figure 17), serine 309 (SEQ ID NO:6; Figure 18), or serine 322 (SEQ ID NO:7; Figure 19). Also provided by the invention is an isolated nucleic acid that hybridizes to a nucleic acid of the invention under conditions of high stringency, moderate stringency, or low stringency.

[00162] The invention further provides for an isolated polypeptide encoded by an isolated nucleic acid of the invention. Purified polypeptides substantially identical to the isolated polypeptide, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 using default parameters, are also included in the invention.

[00163] The invention also provides a human TRIM-cyclophilin fusion protein, and nucleic acids encoding such fusion proteins, where the N-terminus of human CypA (SEQ ID

NO:4) is fused to the C-terminus of human TRIM5 (SEQ ID NO:5). Fusion proteins with anti-HIV-1 activity include hT5-S309-Cyp (SEQ ID NO:11), hT5-S322-Cyp (SEQ ID NO:12), hT5-A331-Cyp (SEQ ID NO:10), hT5-M284-Cyp, hT5-T369-Cyp, where the indicated amino acid is the C-terminal residue of TRIM5 (SEQ ID NO:2) to which the N-terminal amino acid (for example, residue 2 of SEQ ID NO:4) of CypA is directly attached or fused. Embodiments of the invention include a polypeptide comprising SEQ ID NO:4 and SEQ ID NO:2, or a polypeptide comprising SEQ ID NO:10, 11, or 12. The invention provides a polypeptide comprising a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:4 and a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:2, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:10, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:11, or a sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to SEQ ID NO:12.

[00164] Figures 7A and 7C show amino acid residues depicted in red (308-311, 321-346, 366-371 and 381-393) indicating residues that comprise loops in the PRYSPRY domain that contain S309, S322 and A331. Thus fusion of CypA to any residue in these regions would be predicted to generate a hTRIM-Cyp fusion that has HIV-1 restrictive activity. Additionally, fusion of CypA at M285 generates a fusion protein that is variably restrictive, thus fusing to amino acid residues 280-290 would be predicted to have anti-HIV-1 activity. In one embodiment, the last (C-terminal) amino acid of SEQ ID NO:2, which is fused to CypA, is serine at position 309, serine at position 322, or alanine at position 331. In one embodiment, the last amino acid of SEQ ID NO:2 is any one amino acid residue from about residue 280 to about residue 435, or from about residue 280 to about residue 400, or from about residue 280 to about residue 290, or from about residue 308 to about residue 311; or from about residue 321 to about residue 346; or from about residue 366 to about residue 371; or from about residue 381 to about residue 393. In one embodiment, the last residue of SEQ ID NO:2 is residue 493, the end of the TRIM5 PRYSPRY domain, thus generating a fusion protein where a sequence encoding cyclophilin A replaces the stop codon of the PRYSPRY domain of TRIM5. In one embodiment, the last residue of SEQ ID NO:2 is 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 308, 309, 310, 311, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 366, 367, 368, 369, 370, 371, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390,

391, 392, 393, or 493. The last amino acid of SEQ ID NO:2 can be fused to Cyp A (for example, at residue 2 of SEQ ID NO:4) by experimental methods known in the art and described in the Examples.

[00165] The polynucleotides provided by the invention may further comprise a sequence encoding a linker located between the TRIM sequence and the Cyp sequence. In one embodiment, the linker comprises from about 10 to about 20 amino acids. In other embodiments the linker comprises, consists of or consists essentially of from about 11 to about 20, from about 12 to about 20, from about 11 to about 24, from about 12 to about 24, 11 to about 33, from about 12 to about 33, 11 to about 43, from about 12 to about 43, 11 to about 59, from about 12 to about 59 amino acids. In other embodiments the linker comprises, consists of or consists essentially of from 11 to 20, from 12 to 20, from 11 to 24, from 12 to 24, 11 to 33, from 12 to 33, 11 to 43, from 12 to 43, 11 to 59, from 12 to 59 amino acids. In other embodiments the linker comprises, consists of or consists essentially of 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 59, 71, or up to 100 amino acids. In another embodiment, the linker comprises the amino acid sequence SGGSGGSGGSGG (SEQ ID NO: 20) or a variant thereof. In one embodiment, the linker consists essentially of the amino acid sequence SGGSGGSGGSGG (SEQ ID NO: 20) or a variant thereof. In certain embodiments the linker comprises or consists of a synthetic sequence. In other embodiments the linker comprises, consists essentially of or consists of sequences from hTRIM5, for example as described herein. In certain embodiments the fusion proteins, which comprise linkers as described herein, are not naturally occurring. In certain embodiments, the fusion proteins of the invention do not encompass the naturally occurring protein AoT5Cyp.

[00166] It may also be desirable to produce mutants or analogs of the proteins of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, 2nd ed., Cold Springs Harbor, New York (1989).

[00167] The invention further provides for a purified antibody that specifically binds to a polypeptide of the invention or to a protein encoded by a polynucleotide of the invention.

The antibody may be a polyclonal antibody, a monoclonal antibody, or a chimeric antibody. Polyclonal antibodies may be obtained by procedures which are well known to the skilled artisan, including injecting purified fusion protein into various animals and isolating the antibodies produced in the blood serum. The antibodies may be monoclonal antibodies whose method of production is well known to the art, including, for example, injecting purified fusion protein into a mouse, isolating the spleen cells producing the anti-serum, fusing the cells with tumor cells to form hybridomas and screening the hybridomas.

[00168] Methods for producing the polypeptides of the invention include introducing a nucleic acid encoding the polypeptide into a host cell under conditions that permit expression of the polypeptide by the host cell, and recovering the peptide. A nucleic acid may be introduced into a host cell, for example, with a replicable nucleic acid vector, such as a viral vector, a retroviral vector, a lentiviral vector, an adenovirus vector, or an adeno-associated viral (AAV) vector. Provided for in the invention is a replicable nucleic acid vector comprising a nucleic acid of the invention, and a host organism comprising the replicable nucleic acid vector. Suitable host organisms include a prokaryote, a eukaryote, or a fungus.

[00169] The invention provides for a host cell comprising the recombinant expression construct encoding the human TRIMCyp fusion protein as described herein. In another embodiment of the invention, the host cell is stably transformed with the recombinant expression construct described herein. In one embodiment the host cell is a bone marrow cell of a subject. In another embodiment of the invention, the cell is an immortalized cell. In another embodiment, the cell is a stem cell, a blood cell, a bone marrow cell or an immune cell. In one embodiment, the cell is a T cell or a B cell. In one embodiment, the cell is a CD4⁺ T cell.

[00170] A nucleic acid, a polypeptide, host cell or a nucleic acid vector of the invention may be used to prepare pharmaceutical compositions of the invention. Methods for preparing a pharmaceutical composition include admixing a polypeptide of the invention or a fragment thereof.

[00171] The pharmaceutical composition further comprises a pharmaceutically acceptable carrier. The carrier comprises a diluent. The carrier may also comprise an appropriate adjuvant, a herpes virus, an adenovirus, a liposome, a microencapsule, a polymer

encapsulated cell or a retroviral vector. The pharmaceutically acceptable carrier may be an aerosol, intravenous, oral or topical carrier.

[00172] A nucleic acid, a polypeptide, a nucleic acid vector, or a pharmaceutical composition of the invention is suitable for treating a subject suffering from a disease or condition, such as a retroviral infection. In one embodiment, the invention provides for methods of preventing retroviral infection in a subject, or for treating a subject with a retroviral infection, by administering to the subject a pharmaceutical composition of the invention. In another embodiment, the invention provides for methods of reducing viral burden, or load, in a subject infected by a virus by administration of a nucleic acid, a polypeptide, or a nucleic acid vector of the invention, to the subject.

[00173] The invention also provides for a peptidomimetic comprising an amino acid sequence substantially identical to the amino acid sequence of a polypeptide of the invention. As used herein, a "peptidomimetic" refers to a chemical compound that mimics the biological activity of a peptide. In one embodiment of the invention, a peptidomimetic comprises TRIM function and cyclophilin function.

[00174] A peptidomimetic, a nucleic acid, or a polypeptide of the invention is suitable for preparing a therapeutic composition of the invention. The therapeutic composition further comprises a therapeutically acceptable carrier. The carrier may comprise a vector, a liposome, or a viral vector.

[00175] Provided by the invention is a topical composition for prevention or treatment of a viral infection which comprises a nucleic acid or a polypeptide of the invention. The topical composition further comprises a topical carrier, and may be used on the skin, in the vagina, in the nose, in the mouth, or on any skin or mucosal surface.

[00176] The present invention encompasses use of virus based vectors for transformation or transfection of the TRIMCyp coding region into a cell. In the case of eukaryotic cells, retrovirus, adenovirus or lentiviral based vectors are preferred. Such vectors contain all or a part of a viral genome, such as long term repeats ("LTRs"), promoters (e.g., CMV promoters, SV40 promoter, RSV promoter), enhancers, and other sequences known in the art. When the host cell is a prokaryote, bacterial viruses or phages may be used. Exemplary of such vectors are vectors based upon, e.g., lambda phage. The vector may comprise elements of more than one virus. The resulting vectors are transfected or

transformed into a host cell, which may be eukaryotic or prokaryotic. The gene transfer vector may additionally comprise a gene encoding a marker or reporter molecule to more easily trace expression of the vector. The gene transfer vector may contain more than one gene encoding the same or different foreign polypeptides or RNAs. The gene transfer vector may be any construct which is able to replicate within a host cell and includes plasmids, DNA viruses, retroviruses, as well as isolated nucleotide molecules. Liposome-mediated transfer of the gene transfer vector may also be carried out in the present invention.

[00177] Adenoviruses can be used for transformation or transfection of the nucleic acids of the present invention into cells. Examples of such adenovirus serotypes which can be employed in the present invention are well-known in the art and include more than 40 different human adenoviruses, e.g., Ad12 (subgenus A), Ad3 and Ad7 (Subgenus B), Ad2 and Ad5 (Subgenus C), Ad8 (Subgenus D), Ad4 (Subgenus E), Ad40 (Subgenus F) (Wigand et al, in: Adenovirus DNA, Doerfler, Ed., Martinus Nijhoff Publishing, Boston, pp. 408-441 (1986)). Ad5 of subgroup C is the preferred adenovirus employed in the present invention. This is because Ad5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector. Also, adenoviral vectors are commercially available, e.g., pCA3 (Microbix Biosystems Inc.). Methods for producing adenovirus vectors are well-known in the art (Berkner et al, Nucleic Acids Res., 11:6003-6020 (1983); van Doren et al, Mol. Cell. Biol., 4:1653-1656 (1984); Ghosh-Choudhury et al, Biochem. Biophys. Res. Commun., 147:964-973 (1987); McGrory et al, Virol., 163:614-617 (1988); and Gluzman et al, in: Eukaryotic Viral Vectors, Ed. Gluzman, Y. pages 187-192, Cold Spring Harbor Laboratory (1982)). Vectors which can be used in the methods of the present invention include adenoviruses, retroviral vectors, and adeno-associated viral (AAV) vectors. Other virus vectors that may be used for gene transfer into cells include retroviruses such as Moloney murine leukemia virus (MoMuLV); papovaviruses such as JC, SV40, polyoma, adenoviruses; Epstein-Barr Virus (EBV); papilloma viruses, e.g. bovine papilloma virus type I (BPV); vaccinia and poliovirus and other human and animal viruses. Expression can be amplified by placing an amplifiable gene, such as the mouse dihydrofolate reductase (dhfr) gene adjacent to the coding sequence. Cells can then be selected for methotrexate resistance in dhfr-deficient cells. See, e.g. Urlaub et al. (1980) Proc. Natl. Acad. Sci. USA 77:4216-4220; Rungold et al. (1981) J. Mol. and Appl. Genet. 1:165-175.

[00178] A novel vector is provided which can be used for expression of a human T5Cyp, for example, in primary T cells. The vector (SEQ ID NO:8; Figure 20), scALPS, was engineered using two promoters, one from the SFFV LTR and the other from the hCypA gene. As described in Example 2, CD4⁺ T cells were transduced with scALPS encoding either hT5Cyp (SEQ ID NO:9; Figure 21). The novel vector compares favorably to previously published dual-promoter lentiviral gene-delivery systems that fail to lead to efficient reporter gene expression in primary T cells. Indeed the engineering of the novel vector was an advance that enabled direct assessment of T5Cyp-mediated inhibition of HIV-1 spreading infection in primary human cells (see Example 2).

[00179] The invention provides for methods of gene therapy of subjects for the prevention or treatment of viral infection comprising administering to the subject an effective amount of a pharmaceutical composition comprising, or consisting essentially of, a polynucleotide encoding a fusion protein having cyclophilin fused to a TRIM polypeptide, wherein the subject's cells takes up the polynucleotide or polypeptide such that the fusion protein is present in cells of the subject, so as to prevent or treat viral infection in the subject. In one embodiment, the treated subject has a reduced viral load, or burden. In one embodiment of this invention, the administering is via an autologous bone marrow transplant to the subject, where the polynucleotides coding for the fusion protein is transfected into the bone marrow cells *ex vivo* and then replaced into the subject. In this case, the bone marrow cells returned to the subject will have taken up (been transduced) the nucleic acid encoding the fusion protein.

[00180] The constructs can also be used in gene therapy or nucleic acid immunization, to direct the production of the desired gene product *in vivo*, by administering the expression constructs directly to a subject for the *in vivo* translation thereof. See, e.g. EPA Publication No. 336,523 (Dreano et al., published Oct. 11, 1989). Alternatively, gene transfer can be accomplished by transfecting the subject's cells or tissues with the expression constructs *ex vivo* and reintroducing the transformed material into the host. The constructs can be directly introduced into the host organism, i.e., by injection (see International Publication No. WO 90/11092; and Wolff et al., (1990) Science 247:1465-1468). Liposome-mediated gene transfer can also be accomplished using known methods. See, e.g., Hazinski et al., (1991) Am. J. Respir. Cell Mol. Biol. 4:206-209; Brigham et al. (1989) Am. J. Med. Sci. 298:278-281; Canonico et al. (1991) Clin. Res. 39:219A; and Nabel et al. (1990) Science 249:1285-

1288. Targeting agents, such as antibodies directed against surface antigens expressed on specific cell types, can be covalently conjugated to the liposomal surface so that the nucleic acid can be delivered to specific tissues and cells for local administration.

[00181] There are several protocols for human gene therapy which have been approved for use by the Recombinant DNA Advisory Committee (RAC) which conform to a general protocol of target cell infection and administration of transfected cells (see for example, Blaese, R.M., et al., 1990; Anderson, W. F., 1992; Culver, K.W. et al., 1991). In addition, U.S. Patent No. 5,399,346 (Anderson, W. F. et al., March 21, 1995, U.S. Serial No. 220,175) describes procedures for retroviral gene transfer. The contents of these support references are incorporated in their entirety into the subject application. Retroviral-mediated gene transfer requires target cells which are undergoing cell division in order to achieve stable integration hence, cells are collected from a subject often by removing blood or bone marrow. It may be necessary to select for a particular subpopulation of the originally harvested cells for use in the infection protocol. Then, a retroviral vector containing the gene(s) of interest would be mixed into the culture medium. The vector binds to the surface of the subject's cells, enters the cells and inserts the gene of interest randomly into a chromosome. The gene of interest is now stably integrated and will remain in place and be passed to all of the daughter cells as the cells grow in number. The cells may be expanded in culture for a total of 9-10 days before reinfusion (Culver et al., 1991). As the length of time the target cells are left in culture increases, the possibility of contamination also increases, therefore a shorter protocol would be more beneficial.

[00182] One way to get DNA into a target cell is to put it inside a membrane bound sac or vesicle such as a spheroplast or liposome, or by calcium phosphate precipitation (CaPO_4) (Graham F. and Van der Eb, A., Virology 52:456 1973; Schaefer-Ridder M., et al., Liposomes as gene carriers: Efficient transduction of mouse L cells by thymidine kinase gene. Science 1982; 215:166; Stavridis J. C., et al., Construction of transferrin-coated liposomes for *in vivo* transport of exogenous DNA to bone marrow erythroblasts in rabbits. Exp Cell Res 1986; 164:568-572).

[00183] A vesicle can be constructed in such a way that its membrane will fuse with the outer membrane of a target cell. The vector of the invention in vesicles can home into the cancer cells. The spheroplasts are maintained in high ionic strength buffer until they can be fused through the mammalian target cell using fusogens such as polyethylene glycol.

[00184] Liposomes are artificial phospholipid vesicles. Vesicles range in size from 0.2 to 4.0 micrometers and can entrap 10% to 40% of an aqueous buffer containing macromolecules. The liposomes protect the DNA from nucleases and facilitate its introduction into target cells. Transfection can also occur through electroporation. Before administration, the modified vectors are suspended in complete PBS at a selected density for injection. In addition to PBS, any osmotically balanced solution which is physiologically compatible with the subject may be used to suspend and inject the modified vectors into the host.

[00185] For injection, the cell suspension is drawn up into the syringe and administered to anesthetized recipients. Multiple injections may be made using this procedure. The viral suspension procedure thus permits administration of genetically modified vectors to any predetermined site in the skin, is relatively non-traumatic, and allows multiple administrations simultaneously in several different sites or the same site using the same viral suspension. Multiple injections may consist of a mixture of therapeutic genes.

[00186] Viral infections that may be treated or prevented by introducing a human TRIM-Cyp fusion polypeptide into the subject's cells include an HIV infection, a human T-cell lymphotropic virus (HTLV) infection, a pox virus infection, a retrovirus infection, a malaria infection, a hepatitis C virus infection, a hepatitis B virus infection, or a vaccinia virus infection.

[00187] The present invention provides for a human TRIMCyp fusion protein which combines a specific binding activity (specific for HIV-1 capsid protein) and a killing activity (TRIM activity, like ubiquitin). In this way, the HIV-1 is degraded in the cell.

[00188] The invention provides methods for treating HIV infection or preventing HIV infection of a subject by introducing a human TRIM-Cyp fusion polypeptide into the subject's cells. The human TRIM-Cyp polypeptide binds to a capsid protein of the HIV and subsequently degrades the HIV, thereby treating or preventing the HIV infection in the subject. Resistance to HIV may also be imparted to a subject or to the cells of a subject by administering to the cells of the subject a nucleic acid, a polypeptide, or a nucleic acid vector of the invention.

[00189] Embodiments of the invention are illustrated in the Examples that follow. These sections are set forth to aid in an understanding of the invention but are not intended to,

and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Examples

EXAMPLE 1: DESIGN AND IN VITRO TESTING OF HUMAN TRIM5Cyp FUSION PROTEINS

The Antiviral Response and Restriction Factors

[00190] Mammalian cells have engaged in a host-pathogen arms race for millions of years and developed both broad and specific defense mechanisms. The most notable broad-range response in higher organisms is the type I interferon (IFN) response (Isaacs and Lindenmann, 1957). Type I IFNs are ubiquitous and most cells respond via the IFN alpha receptor, leading to both a broad antiviral state through effector molecules such as PKR, eIF2a kinases, and RNaseL, as well as to a specific antiviral state through the upregulation of specialized antiviral restriction factors (Pichlmair and Reis e Sousa, 2007). The IFN-induced antiviral state is characterized by translational shutdown, RNA degradation and de-amination with the potential for causing apoptosis of the responding cell. IFN-dependent effectors are not constitutively active and their expression is therefore tightly controlled (Samuel, 2001). To this end, cells distinguish between self and non-self through pattern recognition receptors (PRRs). These include Toll-like receptors (TLRs) (Takeda et al., 2003) located on the cell surface and within endosomes, and cytosolic PRRs which detect double-stranded DNA (e.g. DNA-dependent activator of IRFs; DAI, (Takaoka et al., 2007)), double stranded and 5' triphosphate single stranded RNA (e.g. melanoma differentiation factor 5 (MDA-5) and retinoic acid inducible gene (RIG-I))(Yoneyama et al., 2005), and unusual protein structures (e.g. TRIM5 family members recognizing in-coming retroviral capsid (CA))(Sayah et al., 2004; Stremlau et al., 2004)).

[00191] A virus not cleared by either the innate or adaptive immune response is the human immunodeficiency virus (HIV). HIV infects immune cells leading to fatal immune suppression (AIDS) in infected patients (Barre-Sinoussi et al., 1983; Popovic et al., 1984), with HIV-1 being responsible for the majority of worldwide lethality each year while the HIV-2 epidemic is concentrated in western Africa (Grant and De Cock, 2001). Zoonotic transmission of the chimpanzee simian immunodeficiency virus (SIV_{cpz}) to humans is the likely cause of the HIV-1 pandemic (Keele et al., 2006). Like its relative, SIV, HIV is a

complex retrovirus. In addition to the genes encoded by *gag*, *pol*, and *env* (**Fig. 40A**) common to all retroviruses, HIV-1 possesses several accessory genes: *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef* (**Fig. 40A**) (Muesing et al., 1985). The HIV life cycle begins with HIV binding to its receptor CD4 and its co-receptor CCR5 or CXCR4 via its envelope glycoprotein complex gp120 and gp41 encoded by *env* (Feng et al., 1996; Maddon et al., 1986). Recently, the mucosal homing receptor for peripheral T cells, α4β7 integrin, has been identified as another receptor for CCR5-tropic HIV-1 (Arthos et al., 2008). Following receptor and co-receptor binding, the viral envelope fuses with the cellular envelope and delivers the virion core (composed of a capsid (CA) encoded by *gag* protecting the viral double-stranded genome and associated proteins) to the cytoplasm (**Fig. 40A, B**). There, the virion core uncoats and the RNA genome is reverse-transcribed by the reverse transcriptase (RT) encoded by *pol* into cDNA. Viral cDNA present in a pre-integration complex (PIC) is imported into the nucleus and ligated into the host chromosomal DNA with the help of integrase (IN) encoded by *pol*. This renders the virus a stable heritable element in the infected cell. The viral promoter (the 5' long terminal repeat; LTR) then directs transcription of viral proteins and genomic RNA. Together these assemble into progeny virions in the cytoplasm that bud from the infected cell. Upon viral protease-mediated maturation, progeny virus is capable of infecting target cells in a fresh infection cycle (**Fig. 40B**) (Ho and Bieniasz, 2008).

[00192] Clearly, such a complex life-cycle is dependent on intricate interactions with host cells. The most obvious determinant of retroviral tropism is lack of host co-factors essential for infection and replication (HIV-1 dependency factors, HDFs). As discussed in Example 3, rodents, for instance lack the receptors required for HIV-1 entry. More than 250 candidate HDFs have been identified or confirmed recently in a large-scale functional siRNA-based genomic screen (Brass et al., 2008). More recently, a similar screen identifying 213 likely HDFs was combined with the 11 HIV-1 genes and data from the human interactome database to create a human-pathogen biochemical network (Konig et al., 2008). Aside from lack of HDFs, natural, dominantly-acting, restriction factors (**Fig. 40B**) determine retroviral tropism and protect the host from the deleterious effects of retroviral replication: the alteration of gene expression, the transduction of toxic genes, and the risk of insertional mutagenesis (Luban, 2007). Three HIV-1 restriction factors (APOBEC3G, tetherin, and TRIM5) will be described in further detail and one served as a model to design a human equivalent with therapeutic potential.

APOBEC3G (A3G)

[00193] Several observations led to the identification of APOBEC3G (A3G; apolipoprotein B mRNA-editing catalytic polypeptide) as an HIV-1 restriction factor. For one, production of infectious HIV-1 virions was dependent on the accessory protein Vif in a cell-type specific manner (Fisher et al., 1987; Strebel et al., 1987). Also, when fusion of permissive and non-permissive cells resulted in a heterokaryon non-permissive for *vif*-deleted HIV-1 (Madani and Kabat, 1998; Simon et al., 1998a); the search began for a dominant restriction factor counteracted by Vif. This factor was isolated by subtractive cloning using the nearly isogenic cell lines CEM-S (permissive) and CEM-SS (non-permissive) and identified as the cytidine deaminase A3G (Sheehy et al., 2002). Expression of A3G correlated with restrictive phenotype. Moreover, expression of A3G into permissive cells rendered them non-permissive and enforced Vif-dependency (Sheehy et al., 2002). A3G belongs to a large family of cytidine deaminases with a conserved zinc-coordinating catalytic motif (H-x-E-x₂₃₋₂₈-P-C-x₂₋₄-C) (Jarmuz et al., 2002), which bind to nucleic acid and hydrolytically deaminate (deoxy)-cytidines at the C4 position into (deoxy)-uridines (Chiu and Greene, 2008; Wolf and Goff, 2008). Two other famous members of the APOBEC family are APOBEC1 (the prototypical member) and AID. APOBEC1 is required to deaminate C6666 in the extensive apolipoprotein B mRNA. This leads to an in-frame stop codon and, upon translation, a truncated protein with cellular functions distinct from the full-length protein (Teng et al., 1993). AID (activation-induced cytidine deaminase) is expressed in germinal center B cells and is required for B cell maturation events such as class-switch recombination and somatic hypermutation (Goff, 2004; Muramatsu et al., 2000).

[00194] A3G selectively deaminates deoxycytidines into deoxyuridines on single-stranded (ss) DNA (Iwatani et al., 2006; Yu et al., 2004); for HIV-1 this is the minus strand of the viral genome. Other viruses that go through ssDNA intermediates during genome replication can also be attacked by A3G; examples include other retroviruses like MLV, SIV, EAV, foamy viruses, and HTLV (Delebecque et al., 2006; Harris et al., 2003; Mangeat et al., 2003; Sasada et al., 2005; Strebel, 2005), but also hepatitis B where A3G interferes with packaging of the pregenomic RNA into particles (Turelli et al., 2004a). In the case of HIV, minus strand deamination has two major effects: DNA base-repair enzymes degrade the destabilized minus strand resulting in reduction of nascent cDNA production (Schrofelbauer et al., 2005; Yang et al., 2007); the cDNA surviving this first attack is fatally hypermutated, leading to non-infectious progeny (Mangeat et al., 2003; Zhang et al., 2003).

[00195] A3G is a cytoplasmic protein and despite its selective ssDNA deamination activity, it also binds to single- and double-stranded DNA and RNA complexes, DNA:RNA hybrids, and thus to viral RNA (Iwatani et al., 2006; Yu et al., 2004). Consequently, A3G is packaged into nascent virions in the donor cell as part of a RNA-protein complex with the NC-portion of the Gag polyprotein awaiting attack during the next reverse transcription cycle in new target cells (Iwatani et al., 2006; Soros et al., 2007; Zennou et al., 2004). Vif binds to A3G and counteracts its antiviral effect by inhibiting its encapsidation into virions (Kao et al., 2003; Opi et al., 2007), potentially interfering with A3G translation (Stopak et al., 2003), and by targeting A3G for proteasomal degradation via the ElonginB/C, Cullin5, and Ring Box 1 E3 ubiquitin ligase complex (Goila-Gaur and Strebel, 2008; Sheehy et al., 2003; Yu et al., 2003). Vif inhibits A3G in a species-specific manner with narrow range limited to A3Gs from closely related primates (Mariani et al., 2003; Simon et al., 1998b; Simon et al., 1995). Mapping the Vif-A3G interaction identified one critical amino acid at position 128 in A3G required for the interaction and acting as the viral tropism determinant (Bogerd et al., 2004; Schrofelbauer et al., 2004).

[00196] Aside from driving species-specific retroviral tropism, A3G also determines cell-specific tropism in a permissive organism through a distinct anti-HIV-1 activity. A3G can recruit nascent, viral mRNA into intracellular low molecular mass (LMM) complexes; an effect that is not counteracted by Vif. This activity partly explains why monocytes and naïve or resting T cells are non-permissive for HIV-1 infection, while mitogen-activated CD4⁺ T cells and macrophages, in which A3G is predominantly found in catalytically inactive high molecular weight (HMM) complexes, are permissive (Chiu et al., 2005; Stopak et al., 2007). siRNA-based knockdown of LMM hA3G in non-permissive T cells renders them permissive (Chiu et al., 2005).

[00197] Cells of the immune system are exquisitely sensitive to outside cues expressed by cytokines. Upon stimulation with IL-2, -7, and 15, A3G shifts from the active LMM to the inactive HMM complexes, rendering resting T cells permissive for HIV-1 infection after cytokine priming (Stopak et al., 2007; Unutmaz et al., 1999). In contrast, type I IFN release during DC-maturation leads to an increase in A3G expression, with A3G localization to restrictive LMM complexes and loss of HIV-1 permissivity in mature DCs (Stopak et al., 2007). As seen with APOBEC1, expression level and intracellular localization may be of maximal importance for orchestrating the different aspects of A3G. Intriguingly, upon HIV-1

infection, Pin-1, a peptidyl-prolyl isomerase, has been shown to directly associate with A3G and reduce both its expression levels and its incorporation into virions in a Vif-independent manner (Watashi et al., 2008).

[00198] The host-retrovirus interactions observed in the lab now are the result of millions of years of co-evolution. The A3 gene cluster has been undergoing positive selection (Sawyer et al., 2004) long before the birth HIV-1 like viruses. Most likely, the force behind A3G-selection and evolution were ancient retroviruses. Their fossils now make up nearly 10% percent of our genomes as retroelements: endogenous retroviruses or non-LTR retrotransposons (Chiu and Greene, 2008; Kazazian, 2004). These are mobile DNA sequences that are characterized by the ability to insert into the genome of host cells and replicate first by transcription and then reverse transcription. The nascent cDNA then inserts itself back into the genome at novel locations (Chiu and Greene, 2008; Kazazian, 2004). Mobilization of retroelements is associated with the obvious risk of insertional mutagenesis, putting strong evolutionary pressure on the host to develop defenses. Recently, accumulation of extragenomic retroelement DNA due to disruption of the DNA nuclease Trex1, has been shown to increase the risk of fatal autoimmunity in mice (Stetson et al., 2008).

[00199] Indeed, both mouse and human A3 have been shown to inhibit retrotransposition of the murine retroelements MusD and IAP (intracisternal A particle) both by reducing the overall number of retrotransposition events as well as by editing inserted cDNA (Esnault et al., 2005). While replication-competent human endogenous retroviruses have not been derived to date, a reconstituted ancestral human retrovirus based on fossil records of HERV-K-family proviruses in the human genome was efficiently restricted by A3F (Lee and Bieniasz, 2007). Furthermore, analysis of the mutational footprint in two other HERV-K family members fixed in the human genome indicates that they have undergone A3G-mediated hypermutation (Lee et al., 2008). While A3G does not affect the retrotransposition of LINE1 (L1) retroelement (Turelli et al., 2004b), it strongly inhibits the retrotransposition of the non-autonomous L1-dependent Alu retroelements (Chiu et al., 2006). Alu inhibition does not depend on A3G catalytic activity. A3G sequesters Alu RNA in cytoplasmic HMM A3G-containing complexes and thus prohibits access to the nuclear retrotransposition machinery provided by L1 (Chiu et al., 2006). Unlike A3G, other A3 family members have access to the nucleus and there block L1 retrotransposition (Chiu and Greene, 2008).

[00200] Ultimately restriction factors are innate immune-system effectors involved in complex and finely-tuned, specific responses to retroviral attack. Indeed such an intriguing orchestration of the innate immune system, A3G, and the adaptive immune system was recently reported. Mouse A3 was identified as the susceptibility determinant encoded by *Rfv3* (*Recovery from friend virus* (FV) 3), an autosomal genetic locus known to regulate retroviral neutralizing antibody responses and viremia (Santiago et al., 2008). Genetic inactivation of mA3 in FV-resistant mice was linked to significantly enhanced viremia, reduced neutralizing antibody titers, and increased mortality. Furthermore, FV-susceptible strains showed alternative splicing of the mA3 RNA leading to excision of exon 2 correlating with reduced antiviral activity against FV in vitro (Santiago et al., 2008). While acting as a FV restriction factor, functional mA3 leads to reduced viremia *in vivo* and enhances production of neutralizing antibodies – effectors of the adaptive immune system – essential for viral control *in vivo* (Santiago et al., 2008).

Tetherin

[00201] In a success story reminiscent to that of A3G, Tetherin was identified based on the differential Vpu requirement for efficient release of HIV-1 particles from certain cell lines (Gottlinger et al., 1993; Klimkait et al., 1990; Neil et al., 2008; Strebel et al., 1989; Terwilliger et al., 1989). When HeLa cells (Vpu-dependent phenotype) were fused with COS-7 cells (Vpu-independent phenotype) the resulting heterokaryon had a Vpu-dependent phenotype. This experiment pointed to Vpu as a factor protecting HIV-1 from a dominant retroviral restriction factor (Geraghty et al., 1994; Neil et al., 2006; Varthakavi et al., 2003). In cells with a Vpu-dependent phenotype, *vpu*-deleted HIV-1 virions fail to be released from infected cells. Instead, they accumulate at the cell surface from where they are subsequently internalized to endosomes (Neil et al., 2006). The particles themselves are fully mature and infectious upon proteolytic release from the cell surface (Neil et al., 2006; Neil et al., 2007), prompting the search for a protein “tether” at the plasma membrane that inhibits particle release in the absence of Vpu (Neil et al., 2008; Nomaguchi et al., 2008). CD317, now renamed tetherin, has been recently shown to fulfill all the requirements for the dominant, particle-tethering restriction factor counteracted by Vpu (Neil et al., 2008; Van Damme et al., 2008).

[00202] Tetherin is highly expressed in cell lines with the Vpu-dependent phenotype as well as strongly induced in cells that acquire this phenotype upon IFN α treatment.

Overexpression of tetherin in cells with low endogenous levels and no Vpu-dependency, enforced a Vpu-requirement for HIV-1 particle release (Neil et al., 2008). Conversely, reducing endogenous mRNA levels via siRNA-targeting in cells with high endogenous levels of tetherin abolishes the Vpu-requirement for HIV-1 particle release (Neil et al., 2008; Van Damme et al., 2008). Moreover, in the absence of Vpu, Gag and tetherin co-localize both at the plasma membrane and in endosomes. When present, Vpu itself co-localizes with tetherin, an association that is required for efficient HIV-1 particle release (Neil et al., 2008; Van Damme et al., 2008). How Vpu interferes with tetherin-mediated HIV-1 restriction is unclear. One group did not observe a change in intracellular tetherin levels in the presence of Vpu, indicating that it sterically hinders a Gag:tetherin association, rather than causing a degradation of tetherin reminiscent of the effects of Vpu on CD4 (Neil et al., 2008). Another group showed that the KSHV protein K5 functions as an ubiquitin ligase and reduces levels of tetherin. They observed similar drops in tetherin concentrations in the presence of Vpu (Bartee et al., 2006; Gottlinger, 2008).

[00203] Tetherin has an N-terminal cytoplasmic domain, followed by a single transmembrane domain, a cytoplasmic coiled-coil domain and a C-terminal GPI-anchor implying that it directly traps the budding virions via the GPI-anchor (Gottlinger, 2008; Neil et al., 2006). Since tetherin tethers a budding membrane to the plasma membrane, it is a potent antiviral of fairly broad specificity targeting enveloped viruses (e.g. Ebola, HIV-1, MLV) that is strongly upregulated upon IFN α treatment (Neil et al., 2008; Van Damme et al., 2008).

[00204] Calcium modulating cyclophilin ligand (CAML) was identified as a Vpu interaction partner in a yeast two hybrid screen (Varthakavi et al., 2008). The interaction was confirmed by co-immunoprecipitation of endogenous CAML with Vpu in HIV-1 infected cells. Vpu and CAML were further seen to colocalize by immunofluorescence in HeLa cells. Like tetherin, expression of CAML in cells permissive to *vpu*-deleted HIV-1 enforced Vpu dependence. On the other hand, RNAi-mediated disruption of CAML expression in cells with a Vpu-dependent phenotype rescued particle release of *vpu*-deleted HIV-1. Like tetherin, CAML causes accumulation of mature particles at the plasma membrane. Given the strikingly similar Vpu-sensitive restriction observed with both CAML and tetherin, CAML may represent a required co-factor for tetherin-mediated HIV-1 restriction (Varthakavi et al., 2008).

Fv1, Lf1 and Cyp A

[00205] The field of retroviral restriction factors began, in 1957, with Charlotte Friend's description of a leukemia-like illness in mice whose causative agent was found to be the Friend murine leukemia virus (MLV) (Friend, 1957). Interestingly, two strains of mice showed different susceptibility (Friend, 1957; Lilly, 1967) to the etiologic agent, an observation upon which the on-going search for the host resistance genetic loci was based. The first such genetic locus recognized was named *Fv1* (Friend virus susceptibility 1). The inbred mouse strains NIH Swiss and Balb-c showed different susceptibilities to MLV isolates; one MLV isolate capable of infecting NIH Swiss but not Balb-c mice or cells derived from the mice was termed N-MLV and the predicted resistance locus in NIH Swiss mice *Fv1^b*; while a strain infecting Balb-c but not NIH Swiss mice and cells derived from them was termed B-MLV and the resistance locus in Balb-c mice *Fv1ⁿ* (Hartley et al., 1970; Pincus et al., 1971b). The offspring of NIH Swiss and Balb-c crosses, while susceptible to NB-tropic viruses, were not susceptible to either N- or B-MLV, implying that each parent contributed a dominant restriction factor to offspring (Pincus et al., 1971a).

[00206] A series of experiments demonstrated CA-specificity of *Fv1*. MLV containing CA from both N and B-tropic strains are restricted by both *Fv1ⁿ* and *Fv1^b* (Rein et al., 1976). Furthermore, a single amino acid at position 110 of CA is sufficient to modulate N- or B- tropism of MLV (Kozak and Chakraborti, 1996) showing the exquisite specificity of restriction. Another essential concept derived from the studies of *Fv1* is that of saturability: restriction of MLV by *Fv1* can be overcome either by high multiplicities of infection or by pre-treatment of cells with restricted virions or virus-like particles (protease-matured virions without genome) derived from restricted viruses (Bassin et al., 1978; Boone et al., 1990; Pincus et al., 1975). This shows that the restriction factor can be "titered out" and thus saturated. Expression of CA-protein alone in cells was unable to saturate restriction. Saturation of *Fv1*-mediated restriction consequently requires maintenance of the three-dimensional CA-structure. This points to a direct association of the incoming virion CA with the restriction machinery (Duran-Troise et al., 1981). After years of intense effort *Fv1* was cloned by a positional cloning strategy and found to encode a Gag protein closely related to those found in the HERV-L and Mu-ERV-L endogenous retroviruses (Benit et al., 1997; Best et al., 1996; Qi et al., 1998).

[00207] Expression of the appropriate Fv1 protein in otherwise susceptible cells is sufficient to block the corresponding MLV-strain (Bishop et al., 2001). Fv1 blocks MLV after entry into the cell and before integration of the viral cDNA into the host genome. Since isolation of the pre-integration complex (PIC) from restrictive but infected cells contains integration-competent virus when assayed *in vitro*, the restriction step can be narrowed down to a point after reverse transcription and before nuclear translocation of the PIC (Pryciak and Varmus, 1992). This type of restriction is termed “post-entry” restriction to differentiate it from the earlier “entry” restriction or later inhibition following integration into the host-genome, such as suppression of viral gene expression or particle release (Jolicoeur and Baltimore, 1976). Thus, Fv1 serves as the prototypical post-entry retroviral restriction factor and fulfills the classical requirements of this class of retroviral inhibitors: it provides a genetic host-determinant for retroviral susceptibility, it is CA-specific, it is dominant, and it is saturable.

TRIM5 α (T5) and TRIMCyp

[00208] Human and monkey cells have an activity to block N-MLV similar to the *Fv1^b*-encoded activity termed *ref1* activity (Towers et al., 2000). Ref-1 activity causes post-entry restriction at the level of reverse transcription (albeit at a slightly earlier time-point than Fv-1), is CA-dependent, saturable, and dominant (Besnier et al., 2003; Towers et al., 2000). Ref1 activity, however, had to be due to a factor distinct from Fv1, since the human genome does not encode the *Fv1* locus (Best et al., 1996). Finally, cells from both new world and old world monkeys exhibit a potent post-entry block to HIV-1 replication (Hofmann et al., 1999); which has hampered efforts of creating an adequate animal model for HIV-1. This restriction activity, termed *Lv-1*, shares striking similarities with Fv1 and Ref-1 restriction, blocking HIV-1 (but not SIVs) at the time of reverse transcription (Besnier et al., 2002; Cowan et al., 2002; Himathongkham and Luciw, 1996; Munk et al., 2002). Analysis of SIV-HIV-1 chimeras showed that viral Lv1 susceptibility determinates (as for Fv1) were found in CA. (Cowan et al., 2002; Kootstra et al., 2003; Owens et al., 2003). Like with Fv1, restriction was saturable (Besnier et al., 2002; Cowan et al., 2002) and cell-fusion experiments demonstrated that restriction was due to a dominant factor (Cowan et al., 2002; Munk et al., 2002).

[00209] Cells from the new world owl monkey (*Aotus ss*) and the old-world rhesus macaques *Macacca mulatta*) showed an especially strong HIV-1 restriction and were used in independent screens to clone the factors responsible for Lv1 activity (Besnier et al., 2002;

Hofmann et al., 1999). The factor responsible for Lv1 activity in rhesus macaques was cloned by introduction of a rhesus macaque cDNA library into HIV-1 susceptible HeLa cells. Subsequent screening for HIV-1-resistant cells using HIV-1 GFP vectors led to the cloning of rhesus macaque TRIM5 α (RhT5 α) as the factor responsible for Lv1 activity in rhesus macaque cells (Stremlau et al., 2004). Further investigations demonstrated that both Ref 1 and Lv-1 activity were encoded by different orthologs of primate T5 α (Hatzioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004).

[00210] The cloning of Lv1 from new world owl monkey cells was based on a different strategy involving cyclophilin A (CypA). Although much remains to be elucidated about CypA, it is functionally a peptidyl-prolyl isomerase (PPIase) which catalyses the rate-limiting *cis-trans* interconversion of peptide bonds N-terminal to proline (Fischer et al., 1984; Liu et al., 1991; Schmid, 1995). CypA is known for its interaction with cyclosporine (CsA), an immunosuppressive agent used to prevent allograft rejection, with which it forms a complex at subnanomolar affinity. This complex inhibits T cell function by binding to the serine/threonine phosphatase calcineurin and blocking downstream signaling events (Handschumacher et al., 1984; Liu et al., 1991). In one of the first yeast two-hybrid screens designed to isolate CA-specific retroviral restriction factors like Fv-1, CypA was found to bind to HIV-1 CA affecting HIV-1 replication and infectivity (Braaten and Luban, 2001; Franke et al., 1994; Luban et al., 1993). Furthermore, CypA has a paradoxical effect on HIV-1 replication in human and non-human primate cells.

[00211] CypA is incorporated into HIV-1 virions and binds to CA via a hydrophobic pocket associating with glycine 89 and proline 90 in the conserved cyclophilin-binding loop of CA, which can serve as the catalytic substrate for CypA (Bosco et al., 2002; Braaten et al., 1997; Franke et al., 1994). Disruption of the CypA:CA interaction through genetic (mutations in CypA or CA) or pharmacologic (uses of competitive inhibitors like CsA) means inhibits viral replication in human cells at the stage of viral cDNA synthesis; the same stage as Ref 1 and Lv1 activity leading to viral escape mutants in serial passage of HIV-1 in the presence of CsA (Braaten et al., 1996; Braaten and Luban, 2001; Dorfman and Gottlinger, 1996; Franke and Luban, 1996). Furthermore, it is neither the producer cell nor the virion-associated CypA but the target cell CypA that is responsible for this effect, again indicating an involvement of CypA early in the viral life cycle (Sokolskaja et al., 2004). Stunningly, exactly the opposite effect is observed in owl monkey cells.

[00212] In non-permissive owl monkey cells the disruption of the CA-CypA interaction increases HIV-1 titers over 100-fold (Towers et al., 2003). This observation together with the intriguing relationship of CypA, CA-binding, and HIV-1 tropism ultimately led to identification of the factor conferring Lv-1 activity to Owl monkey cells: a TRIM5-Cyp fusion protein (AoT5Cyp) (Sayah et al., 2004). It was noted, as before, that reducing the levels of CypA expression in Owl monkey cells by siRNA increased HIV-1 titers. Re-introduction of non-targetable CypA, however, did not restore the restrictive phenotype. This sparked the search for variant CypA mRNA sequences that could explain this phenotype (Sayah et al., 2004). Another group cloned the same factor by searching for owl monkey genes homologous to RhT5 α (Nisole et al., 2004). Like RhT5 α , AoT5Cyp is a member of the T5 family of tripartite motif proteins. In AoT5Cyp, however, the C-terminal PRYSPRY domain is replaced by the CA-binding protein CypA (**Fig 1-2A**); a fusion resulting from the retrotransposition of the entire owl monkey CypA cDNA into the owl monkey T5 locus (Sayah et al., 2004).

Mechanism of T5-mediated retroviral restriction

Structure and Tropism

[00213] RhT5 α as well as AoT5Cyp are both members of the TRIM (TRIPartite Motif) protein family. This large (over 68 members) and disparate family of proteins includes PML (TRIM19), TRIM1, TRIM28, TRIM21, and TRIM22, which all have unrelated antiviral activities against a broad range of viruses (Nisole et al., 2005; Yap et al., 2006). TRIM28, for instance, acts as a primer binding site-specific transcriptional repressor inhibiting MLV replication in ES and EC cells (Wolf et al., 2007). TRIM22 blocks HIV-1 LTR-driven transcription (Tissot and Mechti, 1995). TRIM1, on the other hand, exhibits a moderate post-entry restriction of N-MLV at a step before reverse transcription, reminiscent of the more potent T5 (Zhang et al., 2006). Over 20 TRIM family members with antiretroviral activities have recently been identified in a comprehensive functional screen of 55 candidate TRIMs (Uchil et al., 2008). Noteworthy among them are TRIM11 and 31 which affect HIV-1 entry, TRIM11 which interferes with ref 1 activity indicating that heterologous TRIMs act in concert to modulate and fine-tune an antiretroviral response, and TRIM 25, 31, and 62 that inhibit viral release (Uchil et al., 2008).

[00214] TRIM proteins possess a cysteine-rich RING finger zinc-coordinating domain, a B-box zinc-coordinating domain, and a coiled-coil domain (Nisole et al., 2005; Reymond et

al., 2001) and are also named RBCC proteins. The RING domain is often found in E3 ubiquitin ligases and T5 itself can act as an ubiquitin ligase (Xu et al., 2003). Since neither mutations in the RING domain nor the use of proteasome inhibitors fully abolish T5-mediated restriction activity, it is unclear whether ubiquitin ligase activity is required for retroviral restriction (Javanbakht et al., 2005; Perez-Caballero et al., 2005b). The B-Box domain may be a protein-protein interaction domain that regulates substrate specificity of the RING E3 ubiquitin ligase domain (Massiah et al., 2006). The T5 B-box is required for retroviral restriction and its deletion results in lack of antiviral activity (Javanbakht et al., 2005; Perez-Caballero et al., 2005a). Homo- and hetero-multimerization of T5 is achieved by the coiled-coil domain (Reymond et al., 2001) (**Fig. 41A**). Replacement of this domain with a heterologous trimeric coiled-coil in RhT5 α did not support anti-HIV-1 function, despite efficient trimer formation and CA-binding, indicating that the coiled-coil domain in T5 may accomplish more than multimerization (Li et al., 2006a).

[00215] The T5 gene in the New World owl monkey (genus *Aotus*) has an unusual structure that resulted from retrotransposition of the complete cyclophilin A (CypA) cDNA into intron 7 (Nisole et al., 2004; Sayah et al., 2004), thus replacing the PRYSPRY domain and fusing CypA C-terminal to RBCC. Since CypA is a CA-binding protein (Luban et al., 1993), the fusion protein AoT5Cyp binds directly to CA (Nisole et al., 2004; Sayah et al., 2004). This implied that T5 retroviral restriction factors from other species may function similarly by binding to CA. Indeed, the C-terminal PRYSPRY domain the T5 α is required for T5 α binding to the CA of restriction-sensitive retroviruses (Sebastian and Luban, 2005; Stremlau et al., 2006). Restriction activity requires recognition of CA by multimerized T5 (Javanbakht et al., 2006; Mische et al., 2005) in context of intact viral cores (Forshey et al., 2005; Shi and Aiken, 2006).

[00216] The specificity of the PRYSPRY-CA interaction determines which retrovirus a given T5 α orthologue inhibits. Human T5 α (hT5 α), for instance, weakly blocks HIV-1, but potently blocks N-tropic murine leukemia virus (N-MLV) (Hatzioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004). RhT5 α potently blocks HIV-1 as well as N-MLV weakly, but fails to restrict SIV (Newman and Johnson, 2007). The specificity of this restriction, as well as the modular nature of the T5 components, is further demonstrated by the HIV-1 restriction activity that results when the hT5 α PRYSPRY domain is replaced with that from RhT5 α (Perez-Caballero et al., 2005a; Sawyer et al., 2005;

Stremlau et al., 2005; Yap et al., 2005). Thus, T5, like A3G, is a major host determinant of retroviral tropism.

Restriction mechanism

[00217] T5-mediated retroviral restriction occurs within minutes of viral entry, attacking the incoming virus at a step post-entry but prior to or during reverse transcription (Diaz-Griffero et al., 2006b; Perez-Caballero et al., 2005a). While much about the restriction mechanism remains unknown, restriction occurs in two steps. One step targets reverse transcription itself and a second step targets nuclear import of the pre-integration complex (PIC) (Berthoux et al., 2004; Wu et al., 2006). These distinct steps are sensitive to inhibition by different methods: the inhibition of reverse transcription requires proteasome function and is blocked by proteasome inhibitors as well as As_2O_3 and other drugs affecting mitochondrial membrane potential. Proteasome inhibitors, rescue reverse transcription in the presence of a restrictive T5 but fail to rescue nuclear import of PICs (Anderson et al., 2006). It is noteworthy that proteasome inhibitors, even in non-restricting environments, can modestly increase viral titers (Schwartz et al., 1998). Furthermore, mutation of the RING-domain residues required for E3 ubiquitin ligase activity diminishes, but does not fully abolish restriction (Diaz-Griffero et al., 2006a). Most surprisingly, however, even ubiquitin itself may be dispensable for restriction activity (Perez-Caballero et al., 2005b).

[00218] The CA-binding requirement for retroviral restriction indicates a model in which T5 binds to CA and targets it for proteasomal degradation. Indeed, T5 promotes the premature uncoating of incoming viruses as has been shown in CA-sedimentation assays (Perron et al., 2007; Stremlau et al., 2006). This could disturb the optimal coordination of the poorly understood uncoating process and reverse transcription; a disturbance that has been observed with destabilizing CA-mutants as well (Forshey et al., 2002). Proteasome inhibitors prevented T5-mediated loss of particulate CA, indicating that T5-mediated uncoating is a proteasome dependent process (Diaz-Griffero et al., 2007). Intriguingly, restrictive T5 itself is polyubiquitinated and, in the presence of restriction-sensitive CAs, T5 is targeted for proteasomal degradation. Thus, an entire T5-CA protein complex may be targeted for proteasomal degradation (Diaz-Griffero et al., 2006a; Rold and Aiken, 2008).

[00219] Even before the discovery of T5, it was observed that reverse transcription could be stimulated with As_2O_3 and other drugs perturbing mitochondrial membrane potential

(Berthoux et al., 2004; Berthoux et al., 2003). By an unknown mechanism, As₂O₃ counteracts a HIV-1 restriction by all T5 orthologs tested in a cell-type specific manner (Sebastian et al., 2006). Nuclear import of PICs, on the other hand, can be inhibited by disruption of the CA-CypA interaction through genetic or pharmacologic means (Berthoux et al., 2004). While both As₂O₃ and proteasome inhibitors rescue reverse transcription, T5-mediated inhibition of PIC nuclear translocation can account for the proteasome-independent mechanism of T5-mediated CA degradation (Chatterji et al., 2006). Fusion of heterologous TRIM-family member RBCC domains with CypA leads to novel TRIMCyp fusion proteins with distinct blocks to HIV-1 replication depending on the RBCC domain used: either at reverse transcription or PIC nuclear import, or both (Yap et al., 2006). Aside from highlighting the modularity of T5 restriction factors (i.e. as long as the C-terminal PRYSPRY or Cyp domain target RBCC to CA, most RBCCs will be able to restrict retroviruses); these findings reaffirm the concept of a two-step restriction mechanism.

Evolution

[00220] In humans, T5 is located on chromosome 11 Between TRIM22, TRIM34, and TRIM6, in a cluster of SPRY domain containing, IFN-inducible TRIMs (Newman and Johnson, 2007). Since T5 α has been evolving under extreme positive selection in the primate lineage for the last 30 million years (Sawyer et al., 2005), T5 exhibits significant sequence diversity between primate species. The most significant finding in regard to co-evolution with viral pathogens is the detection of footprints of positive selection in which sequence diversity is concentrated. These are located mainly in the PRYSPRY domain, which by binding to retroviral CA, directs retroviral tropism (Sawyer et al., 2005). Evolutionary hot-spots occur in what have become termed variable regions V1-V4 of the PRYSPRY domain (Song et al., 2005b) and were further predicted to correspond to retroviral tropism determinants. The most impressive evidence of positive selection was found in a 14 amino acid patch in the PRYSPRY domain. Phylogenetic data were backed up beautifully in tissue culture experiments that analyzed anti-HIV 1 activity of different simian-human chimeras containing this 14 amino acid patch. Indeed, this patch proved to be the key determinant of retroviral tropism (Sawyer et al., 2005). Independent experimental mapping intended to reveal restriction specificity determinants reinforced this finding (Perez-Caballero et al., 2005a; Stremlau et al., 2005; Yap et al., 2005). The pattern of specific positive-selection hotspots forming variable regions anchored in more constant domains is reminiscent of the selection

of peptide binding regions in the major histocompatibility (MHC) loci (Hughes and Yeager, 1998).

[00221] Analysis of polymorphisms in the hT5 α gene revealed several with a high allelic frequency, mainly found in the RBCC domain. Strikingly, none have been reported for the variable regions of the PRYPSRY domain; those regions undergoing the strongest positive selection and comprising the greatest inter-species sequence diversity (Sawyer et al., 2006). This points to the possibility that hT5 α in its current form is the likely result of a selective sweep exerted on the human genome by an ancient retroviral epidemic (Newman and Johnson, 2007). One reported candidate retrovirus is the chimpanzee endogenous retrovirus ptERV (Kaiser et al., 2007). Our closest relative, the chimpanzee, contains about 150 copies of ptERV in its genome, that are completely absent from the human genome. The absence of any trace of ptERV in the human genome suggests active elimination of ptERV or its parent virus from the human species. hT5 α could indeed efficiently block an MLV-based vectors engineered to express the ptERV CA (Kaiser et al., 2007).

[00222] Another indication of the immense evolutionary pressure on the primate T5 locus is the discovery of additional T5Cyp fusion proteins. The generation of AoT5Cyp, the first such fusion protein discovered, seemed unique. It resulted from a Line-1 mediated retrotransposition of the CypA cDNA between Exon 7 and 8 of the aotus TRIM5 locus thus fully replacing the PRYPSRY domain encoded by exon 8 with CypA (Sayah et al., 2004). The fixation of the AoT5Cyp gene as the only T5 allele in more than 10 Aotus species speaks for an extremely strong positive selection force on this gene (Ribeiro et al., 2005). The discovery of a second TRIMCyp fusion protein (MaT5Cyp) in three species (*rhesus*, *nemestrina*, and *fascicularis*) of the macaque genus is a stunning example of convergent evolution (Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008; Wilson et al., 2008) (**Fig. 41B**). In MaT5Cyp the CypA insertion occurred within Exon 8 leading to a splicing-out of exon 7. Together with some amino-acid differences in the CypA moiety, this distinct retrotransposition event leads to a retroviral specificity different from that of AoT5yp. MaT5Cyp does not inhibit HIV-1 but potently blocks infection by HIV-2, SIV_{AGM}tan, and FIV (Virgen et al., 2008). The T5Cyp fusion was generated by Line-1 mediated retrotransposition on two separate occasions in old world and new world monkeys. Evolutionary pressure then drove fixation of these potent retroviral restriction factors in the owl monkey and macaque lineages. What the driving force in each case was remains to be

determined, but there is a high likelihood that the genesis and maintenance of these genes is a result of an ancient battle against retroviruses.

[00223] T5Cyp, a natural anti-retroviral created by evolution, has several properties that would make it appealing for anti-HIV-1 therapy. In the case of AoT5Cyp, it potentially blocks HIV-1 infection when expressed in human cells (Nisole et al., 2004; Sayah et al., 2004) and it acts on the virus within minutes of entry (Diaz-Griffero et al., 2006b; Perez-Caballero et al., 2005a) protecting the host from potentially deleterious insertional mutagenesis. Since AoT5Cyp is the single TRIM5 allele present in the *Aotus* genus (Ribeiro et al., 2005), cells bearing AoT5Cyp retain functionality. AoT5Cyp is not a human protein, and if expressed in patients may lead to an immune response. This Example focuses on generating a hT5Cyp fusion protein with reduced antigenic potential but equal anti-HIV-1 potency. T5 proteins are modular with the C-terminal PRYSPRY or CypA domain targeting the proteins to CA of restriction-sensitive viruses and RBCC acting as an effector domain. Fusion of the hT5a RBCC domain to AoCypA resulted in a fusion with anti-HIV-1 activity (Nisole et al., 2004). Similarly, addition of RBCC from different TRIM family members to the RhT5a PRYSPRY (Li et al., 2006a) domain or AoCypA (Yap et al., 2006) yielded chimeras with restriction activity. This Example will focus on design and *in vitro* testing of a hT5Cyp fusion protein with activity against HIV-1. Example 2 will discuss testing hT5Cyp in primary human cells and developing required gene therapy methodology. Example 3 will concentrate on testing the anti-HIV-1 activity of hT5Cyp *in vivo*, and on the establishment of humanized mouse models for HIV-1 infection necessary for this aim.

Design of hT5Cyp fusions with anti-HIV-1 activity

[00224] While hCypA and AoCypA are highly conserved, the RBCC domains of human and owl monkey T5 have a substantial number of amino acid changes (**Fig. 42**). Therefore, AoT5Cyp could generate an immune response if expressed in hCD4⁺ T *in vivo*, and experiments were designed to make a human T5Cyp with reduced antigenic potential. Given the significant sequence dissimilarities between the owl monkey and human T5 RBCC domains, the approach of “humanizing” AoT5Cyp by single amino acid substitution was not used here (**Fig. 42**). Instead, a human T5Cyp was generated *de novo* by fusion of hT5 to hCypA.

[00225] A cryptic splice acceptor at the AoT5Cyp fusion junction results in the synthesis of 12 amino acids derived from the CypA 5'UTR(Sayah et al., 2004). An equivalent human fusion protein cannot be engineered due to lack of sequence homology in the CypA 5'UTR and the fact that the human 5'UTR, most likely remains untranslated. hT5Cyp was generated using only components normally expressed in human cells. Consequently, hT5-direct-Cyp was engineered with hCypA fused to a position in the linear sequence of hT5 analogous to that of the AoT5Cyp retrotransposition event (**Fig. 43A**). Since the 12 amino acids encoded by the AoCypA 5'UTR might be an essential spacer between the AoT5 and AoCypA moieties, sequences from the hT5 α , γ , and δ isoforms, and a synthetic 12 amino acid spacer were used to generate linkers of similar length and character: SEQ ID NO:31 is hT5- α -Cyp (VDVTVAPNNISCAVIS), SEQ ID NO:32 is hT5- γ -Cyp (GKEKSHYHKPPCGLS), SEQ ID NO:33 is hT5- δ -Cyp (GWSAMARSRFTATSTS), and SEQ ID NO:17 is hT5(SGG)₄Cyp (SGGSGGSGGSGG) (**Fig. 43A**). Jurkat T cells were transduced with a bicistronic lentiviral vector coding for puromycin resistance and the indicated T5Cyp fusion protein (**Fig.43B**). Transduced Jurkat cells were then selected as pools using puromycin and challenged with serial dilutions of an HIV-1-GFP vector. hT5(SGG)₄Cyp (SEQ ID NO:17) blocked HIV-1 comparably to AoT5Cyp but none of the other constructs exhibited detectable HIV-1 restriction activity (**Fig. 43A,C**).

[00226] Since the objective was to engineer a fully human HIV-1 restriction factor, hCypA was fused at 10 different positions along the length of hT5 α (**Fig. 44A**). Of the hT5Cyp constructs tested for HIV-1 restriction activity in Jurkat T cells and THP-1 monocytes, three had activity comparable to AoT5Cyp (hT5-A331-Cyp (SEQ ID NO:5 and corresponding amino acid sequence of SEQ ID NO:10); hT5-S309-Cyp (SEQ ID NO:6 and corresponding amino acid sequence of SEQ ID NO:11); and hT5-S322-Cyp (SEQ ID NO:7 and corresponding amino acid sequence of SEQ ID NO:12)), one (hT5-T369-Cyp) with SEQ ID NO: 34 was moderately restrictive, while another (hT5-M284-Cyp) with SEQ ID NO:35 exhibited variable restriction activity based on cell-type and activation status (**Fig. 44B, Fig. 45, Table 1**). hT5-S322-Cyp with SEQ ID NO:12, consistently the strongest HIV-1-inhibitor, became the focus of subsequent experiments, and for simplicity will be referred to in the Examples as hT5Cyp (**Fig. 45, Table 1**). hT5Cyp also restricted HIV-1 in promonocytic U937 cells, the CEM-SS, SUP-T1, H9 T cell lines, HUH-7 human hepatoma cells, CRFK feline kidney cells, and the 293T embryonic kidney cell line (**Table 1**). Like AoT5Cyp(Diaz-

Griffero et al., 2006b), hT5Cyp blocked the accumulation of viral cDNA, as measured by quantitative PCR in stable Jurkat T cell lines (**Fig. 45D**).

[00227] When compared to Jurkat T cells transduced with control vectors, Jurkat T cells transduced with hT5Cyp or AoT5Cyp grew with the same kinetics and expressed similar levels of cell surface CD4, CXCR4, CD11a, CD18, and ICAM3; members of the virological synapse which includes the HIV-1 receptor and co-receptor (Jolly et al., 2004); as well as MHC-I (**Fig.46A**). Since CypA is highly abundant in the cytoplasm of most eukaryotic cells, endogenous CypA may inhibit hT5Cyp function by acting as a competitive inhibitor for CA-binding. hT5Cyp potency may be increased by modulating endogenous CypA levels. To investigate this, stable CypA KD Jurkat T cell lines were generated using a lentiviral vector with a CypA shRNA targeting the 3'UTR embedded in miR-30 downstream of the SFFV promoter driving hygromycin resistance. The control was a Luciferase shRNA (Luc KD). Hygromycin resistant cells were then stably transduced with bicistronic lentiviral vectors coding for the control or hT5Cyp and puromycin resistance. Cells resistant to both puromycin and hygromycin were then challenged with an HIV-1 GFP vector. The magnitude of hT5Cyp-mediated restriction was unexpectedly lower in the absence of CypA (**Fig.46B**). Equivalent results were obtained for AoT5Cyp in Jurkat T cells and for both T5Cyps in H9 cells. Since T5 proteins form homo- and heteromultimers (Mische et al., 2005), T5 γ , a splice-isoform of T5 α , which lacks the PRYPSRY domain, acts as a dominant negative inhibitor of T5 α antiretroviral activity (Stremlau et al., 2004). While gross defects were not observed in proliferation or morphology of Jurkat T cells expressing T5Cyp, it was tested whether expression of exogenous T5Cyp analogously affects N-MLV inhibition by endogenous hT5 α . Jurkat T cells stably expressing control vector or T5Cyps were challenged with B- and N-MLV. Cells expressing T5Cyp restricted N-MLV 4-10 fold less than cells transduced with empty vector, but continued to robustly restrict N-MLV 20-40 fold compared to B-MLV (**Fig. 46C**).

Table 1. HIV-1 inhibition by hT5Cyp

T5	HIV-1 Inhibition			
	Jurkat	THP-1	PMA treated THP-1	CRFK
hT5-M244-Cyp	-	-	-	-
hT5-M284-Cyp	+++	-	+	+++
hT5-T302-Cyp	-	-	-	-

hT5-S309-Cyp	+++	+++	+++	+++
hT5-S314(α)-Cyp	-	-	-	-
hT5-S322-Cyp	+++	++++	+++	+++
hT5-A331-Cyp	++	ND	ND	+++
hT5-G357-Cyp	-	ND	ND	ND
hT5-T369-Cyp	+	ND	ND	ND
hT5-G398-Cyp	-	ND	ND	-
hT5directCyp	-	ND	ND	ND
hT5(SGG ₄)Cyp	+++	+++	+++	+++
hT5 δ Cyp*	-	-	-	-*
hT5 γ Cyp*	-	-	-	-*
RhT5a	+	ND	ND	+++?
AoT5Cyp	+++	+++	+++	++++
Despite lacking anti-HIV-1 activity, both hT5 δ Cyp and hT5 γ Cyp* restrict SIV _{AGMtan} 6- and 15-fold in CRFK cells.				

Structural requirements for hT5Cyp-mediated HIV-1 restriction activity

[00228] Despite the apparent modularity of TRIM5(Perez-Caballero et al., 2005a; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005), most hT5Cyp fusions lacked anti-HIV-1 activity. Steady-state protein level in stably-transduced Jurkat T cells did not correlate with restriction activity: AoT5Cyp and restrictive hT5Cyp fusions were undetectable by immunoblot while inactive or variable hT5Cyps (hT5-M244-Cyp and hT5-M284-Cyp) were highly expressed. Treatment with sodium butyrate, which is known to increase T5 protein levels (Perez-Caballero et al., 2005b), did not reveal expression of additional hT5Cyp fusion proteins in stably transduced Jurkat cell lines by western blot. To increase the possibility of detection, hT5Cyp proteins were then expressed as triple-FLAG-tagged fusions by plasmid transfection of 293T cells. Anti-FLAG immunoprecipitates were probed in immunoblots with anti-CypA antibody. Two fusions with no detectable restriction activity, hT5-M244-Cyp and hT5-T302-Cyp, were expressed at high-level, whereas fusions with potent anti-HIV-1 activity, hT5-A331-Cyp and AoT5Cyp, were expressed at low-level (**Fig.47A**). Again, restriction activity did not correlate with protein level.

[00229] Lack of activity could result from failure to bind HIV-1 CA. Glutathione S-transferase (GST)-CA fusion protein was used to test CA-binding activity of hT5Cyp proteins produced in 293T cells. Among hT5Cyp fusions with no anti-HIV-1 activity, hT5-T302-Cyp bound HIV-1 CA as well as the potently restrictive hT5-S322-Cyp (**Fig. 47B**). Specificity of binding was demonstrated by blocking the CypA:CA interaction with the competitive

inhibitor cyclosporine A (CsA) (**Fig. 47B**). Thus, the ability to bind CA was required but not sufficient for restriction activity.

[00230] To visualize the sites of CypA linkage to hT5, a three-dimensional model of the T5 α PRYSPRY domain was generated using crystal structure coordinates from the C-terminal B30.2/SPRY domains of three homologues: GUSTAVUS, Pyrin, and TRIM21 (Grutter et al., 2006; James et al., 2007; Woo et al., 2006) (**Fig. 47C**). hT5Cyp fusions with potent anti-HIV-1 activity clustered near the main specificity determinant for HIV-1 restriction activity (Ohkura et al., 2006; Sawyer et al., 2005; Yap et al., 2005); a proposed protein interaction surface (Grutter et al., 2006; Woo et al., 2006) (**Fig. 47C**). No inactive hT5Cyp proteins mapped to this region (**Fig. 47C**). The inactive insertions, in fact, map on the “opposite” side of the proposed protein-protein interaction surface. One exception, hT5Cyp-M285-Cyp, is located in the linker region (linker 2) between the coiled-coil and the PRYSPRY domains of hT5 α (N-terminal α -helix), and shows variable restriction activity based on cell type (**Fig. 47C, Table 1**).

[00231] T5, like some other TRIM-family members, can form aggregates in the cytoplasm called cytoplasmic bodies (Reymond et al., 2001). The exact function and the requirement of cytoplasmic body formation for lentiviral restriction is unclear and under debate (Campbell et al., 2007; Perez-Caballero et al., 2005b; Song et al., 2005a; Wu et al., 2006). In stable CRFK cell lines, visualization of hT5Cyp fusions by static immunofluorescence showed that AoT5Cyp and all three hT5Cyps with potent anti-HIV-1 activity formed distinct cytoplasmic bodies (**Fig. 47D**). In contrast, diffuse cytoplasmic staining was observed for hT5Cyp fusions that lacked restriction activity. Taken together, these results demonstrate strict conformational requirements for antiviral activity that are determined by the site of CypA fusion and correlate with the ability of the fusion proteins to form cytoplasmic bodies.

hT5Cyp restricts multiple lentiviruses

[00232] AoT5Cyp restricts HIV-1_{NL4-3}, HIV-2_{ROD}, SIV_{AGMTan}, and FIV_{PET}, all viruses encoding a CA that binds CypA (Diaz-Griffero et al., 2006b; Lin and Emerman, 2006; Luban et al., 1993; Nisole et al., 2004; Saenz et al., 2005; Sayah et al., 2004; Zhang et al., 2006). To evaluate whether hT5Cyp blocked infection by these lentiviruses, stable cell lines were generated using CRFK cells. AoT5Cyp and hT5Cyp restricted all four lentiviruses (**Fig.**

48;A-D). in these cells, AoT5Cyp blocked HIV-1 infection about 10-fold more effectively at low multiplicities of infection (MOI) (**Fig. 48A; Table 1**); an effect that is lost in Jurkat T cell line and THP-1 monocytes where Ao- and hT5Cyp have comparable effects on HIV-1 infection (**Fig. 43B, Table 1**). In contrast, hT5Cyp inhibits SIV_{AGMtan} infection impressively, and significantly better than AoT5Cyp at low MOI (**Fig. 48C**). Interestingly, hT5 γ Cyp and hT5 δ Cyp, which lack detectable restriction activity against HIV-1 and FIV, show a maximal 15- and 6-fold restriction of SIV_{AGMtan}. HIV-2_{ROD} and FIV_{PET} are blocked comparably by AoT5Cyp and hT5Cyp (**Fig. 48; B, D**). Since CypA does not bind to SIV_{MAC251} CA, neither AoT5Cyp nor hT5Cyp can restrict this virus (**Fig. 48E**).

hT5Cyp restriction activity depends on both the hCypA and hT5 components

[00233] To confirm that hT5Cyps have the same CA-binding requirements as AoT5Cyp, the CRFK stable cell lines were challenged with HIV-1-G89V, a CA mutant that disrupts interaction with CypA (Sayah et al., 2004). Both AoT5Cyp and hT5Cyp failed to restrict HIV-1-G89V (**Fig. 49A**). The H126Q mutation in CypA also disrupts the CA-CypA interaction (Braaten et al., 1997) and hT5Cyp bearing the H126Q mutation in its CypA domain had no detectable anti-HIV-1 restriction activity (**Fig. 49B**). Since this single amino acid change in hT5Cyp leads to complete loss of HIV-1 restriction activity, hT5CypH126Q provides an attractive control for hT5Cyp and will be used in further studies. Finally, cyclosporine, a competitive inhibitor of the CypA-CA interaction (Luban et al., 1993), abrogated HIV-1 restriction by AoT5Cyp and hT5Cyp (**Fig. 49C**). Equivalent results were obtained when DEBIO-025, a non-immunosuppressive competitor of CypA, was used in place of CsA (**Fig. 49E**). A previous study searched the database for CA-variants circulating in HIV-1-infected people that are resistant to cyclosporine and to AoT5Cyp-mediated HIV-1-restriction (Chatterji et al., 2005). V86P/H87Q/I91V/M96I HIV-1, the CA-variant with the strongest cyclosporine resistance *in vitro* (Chatterji et al., 2005), an effect confirmed here (**Fig. 49D**). Both hT5Cyp and AoT5Cyp, however, blocked infection by viruses bearing this sequence as efficiently as they block standard HIV-1 clones (**Fig. 49D**).

[00234] To assess the contribution of the T5 domain to T5Cyp-mediated restriction, CRFK cell lines expressing AoT5Cyp or hT5Cyp were treated with arsenic trioxide (As₂O₃). This drug was previously shown to inhibit T5-mediated restriction (Sebastian et al., 2006). AoT5Cyp and hT5Cyp restriction activity was blocked to the same extent by As₂O₃ (**Fig.**

49F). These results show that, with respect to CA-recognition by the CypA domain, and effector function by the T5 domain, AoT5Cyp and hT5Cyp behave nearly identically.

hT5Cyp and AoT5Cyp have the highest anti-HIV activity among characterized T5 proteins

[00235] Since the discovery of T5, its potential as anti-HIV-1 therapy has been in sight. While a protein modeled after AoT5Cyp is described here, other groups are considering proteins based on RhT5 α . In contrast to hT5 α , rhT5 α restricts HIV-1 (Stremlau et al., 2004). Chimeric T5 proteins in which the PRYSPRY specificity determinant from the rhesus orthologue replaces the corresponding human residues (hT5 α _{R323-332} (Sawyer et al., 2005; Stremlau et al., 2005)), or even in which a single, critical amino acid was substituted (hT5 α _{R332P} (Li, et al. 2006b; Yap et al., 2005)), exhibit HIV-1 restriction activity approaching that of RhT5 α in HeLa and CRFK cells, respectively. Since these molecules are nearly identical to the human protein (**Fig. 41B**), it has been proposed that they may have therapeutic utility (Anderson et al, 2008; Li et al, 2006b; Stremlau et al, 2005; Yap, 2005).

[00236] Anti-HIV-1 potency is an essential consideration in any potential therapy, so a direct comparison of hT5Cyp and other candidates was undertaken. hT5 α _{R323-332} and hT5 α _{R332P} caused moderate inhibition of HIV-1 (10-fold) in single-cycle infection of CRFK cells approaching RhT5 α restriction activity. hT5Cyp, however, was ~ 10-fold more potent than either, with AoT5Cyp inhibiting HIV-1 infection the strongest by almost 1000-fold (**Fig. 50A**). CRFK, feline kidney fibroblast cells, lack endogenous T5 and are commonly used in the field as they have large T5-restriction phenotypes in single-cycle assays. In 293T human embryonic kidney cells, however, hT5Cyp and AoT5Cyp were equivalent in their anti-HIV 1 activity and over 10-fold more potent than RhT5 α (**Fig. 50B**). In human Jurkat T cell lines, hT5 α -R332P and hT5 α -R323-332 have a negligible (maximum two-fold) effect on HIV-1 infection and RhT5 α a modest one compared to AoT5Cyp and hT5Cyp (**Fig. 50C**).

DISCUSSION

[00237] The potent block to HIV-1 infection observed with AoT5Cyp inspired the design of a human equivalent: hT5Cyp. Since T5 α and T5Cyp are modular proteins, generation of an hT5Cyp fusion with anti-HIV-1 activity was expected to be trivial. Surprisingly, only three of 14 hT5Cyp fusions exhibited HIV-1 restriction activity

comparable to AoT5Cyp. No correlation was observed between protein levels and antiviral activity (Fig. 47A). In fact, in stable Jurkat T cell lines, restricting hT5Cyp fusion proteins could not be detected by western blot while the only two fusion proteins that were highly expressed both lacked anti-HIV activity. Since T5 itself is polyubiquitinated and rapidly degraded when it encounters a restriction-sensitive CA (Rold and Aiken, 2008), rapid turnover may in fact be of physiological relevance and correlate with restriction activity.

[00238] The unexpectedly rare frequency with which restrictive fusion proteins were obtained suggests that there are strict spatial requirements for anti-HIV-1 activity. Structural modeling showed that the best anti-HIV-1 activity was obtained when hCypA was fused at the apex of the hT5 α PRYSPRY domain. Interestingly, analysis of nonsynonymous mutations in T5 α indicates that this region undergoes some of the strongest selective pressure in the primate lineage and functional experiments pinpoint this hypervariable region as a specificity determinant for CA recognition (Sawyer et al., 2005; Song et al., 2005b; Yap et al., 2005). CypA fusions 309, 322, and 332 may have the greatest antiviral activity simply because this region is especially tolerant of sequence variation. More likely, this apical loop is uniquely situated for coordinating the CA recognition and effector domains of T5.

[00239] T5-mediated restriction occurs by a two-pronged mechanism leading to reduction of viral cDNA (Fig. 45D) as well as inhibition of its nuclear import (Anderson et al., 2006; Wu et al., 2006). The presence of a restrictive T5 orthologue results in reduction of particulate CA in the target cell cytosol by promoting the premature uncoating of retroviral capsids (Diaz-Griffero et al., 2007; Stremlau et al., 2006). Proteasome inhibitors restore retroviral cDNA synthesis and the amount of cytosolic particulate CA without eliminating restriction (Anderson et al., 2006; Berthoux et al., 2004; Diaz-Griffero et al., 2007; Sebastian and Luban, 2007). In contrast, a B-box mutation in T5Cyp restricts HIV-1 while not reducing amount of particulate cytoplasmic CA, indicating that restriction can be observed even in the presence of particulate CA (Diaz-Griffero et al., 2007). The results presented here add to these observations by identifying a hT5Cyp-fusion (hT5-T302-Cyp) that does not restrict HIV-1 despite its ability to bind to CA (Fig. 47B). This indicates that simple CA-binding is not sufficient for HIV-1 restriction, either due to particular spatial constraints or a requirement for restriction cofactors. Due to its unique attribute of CA-binding in the absence of restriction activity, hT5-T302-Cyp could be used in a proteomics approach side by side with a restrictive T5Cyp in an effort to isolate and verify potential co-factors for restriction.

Closer analysis of the level of reverse transcription, the amount of particulate CA, and the efficiency of nuclear import in the presence of the permissive hT5-T302-Cyp in comparison to restrictive hT5Cyp may lead to more detailed insights into the mechanism of T5-mediated restriction of HIV-1. Similarly, hT5- γ -Cyp and hT5- α -Cyp show no restriction activity against either HIV-1 or FIV, yet inhibit SIV_{AGM}tant in CRFK. They could also prove useful tools in further analysis of requirements for CA-interaction and effector functions.

[00240] While there seems to be no requirement for localization of restrictive T5 orthologues in pre-existing cytoplasmic bodies (Perez-Caballero et al., 2005b; Song et al., 2005a), T5 orthologs, much like their family member TRIM19, can form these highly dynamic structures (Campbell et al., 2007). Furthermore, T5-containing cytoplasmic bodies often form *de novo*, surrounding incoming viral complexes, and are better visualized by live rather than static immunofluorescence (Campbell et al., 2008). Proteasome inhibitors, which rescue cDNA synthesis and levels of particulate CA, induce the stable accumulation of viral complexes in cytoplasmic bodies. Thus cytoplasmic bodies could represent the visualization of a restriction-intermediate (Campbell et al., 2008). Static immunofluorescence of stable CRFK lines showed that the highly restrictive T5Cyp fusions 309, 322, and 332 (**Fig. 45**) all showed a punctate cytoplasmic staining also seen for AoT5Cyp, whereas non-restricting hT5Cyp fusions stained diffusely cytoplasmic (**Fig. 47**). Live imaging of hT5Cyp would be worth undertaking with a particular focus on whether permissive hT5-T302-Cyp which binds CA has the ability to form *de novo* cytoplasmic bodies surrounding HIV-1 complexes only to fail at a later point in the restriction pathway.

[00241] hT5Cyp fusion proteins with HIV-1 restriction activity behave comparably to AoT5Cyp. They are able to restrict the same range of lentiviruses (HIV-1, HIV-2, FIV, and SIV_{AGM}tant) at similar magnitudes of inhibition as AoT5Cyp (**Fig. 48**). Lentiviruses sensitive to T5Cyp-mediated restriction all have CA that bind CypA. Disruption of the CypA:CA interaction using pharmacologic or genetic means abolishes T5Cyp restriction activity, as does As₂O₃ (**Fig. 49**). This confirms for hT5Cyp that CA binding is necessary if not sufficient for restriction activity. There are few naturally occurring HIV-1 CA variants with amino acid substitutions surrounding the CypA binding loop that have lost CsA sensitivity in human cells (Chatterji et al., 2005). This may be due to reduced affinity of CypA to these CA variants. The same group reported a partial resistance of these variant viruses to T5Cyp restriction in owl monkey cells (Chatterji et al., 2005). T5Cyp-expressing Jurkat T cells lines

were challenged with such a CA variant, confirming the CsA insensitivity. T5Cyp, however, retained the ability to inhibit this CA variant. CypA is hypothesized to protect HIV-1 against an unknown restriction factor (“factor x”) in human cells. Treatment with CsA disrupts the CypA:CA interaction allowing for access of factor x to CA. One explanation for loss of CsA sensitivity together with retention of T5Cyp restriction sensitivity, is that CA-mutations affect the binding site for factor x to a greater extent than the CypA binding loop. Therefore, even when the CypA:CA interaction is blocked by CsA, factor x cannot access the variant CA. T5Cyp, however, retains the ability to bind the variant CA and restricts this virus (**Fig. 49D**).

[00242] Jurkat T cells expressing hT5Cyp proliferate at the same rate and express similar levels of cell surface markers involved in HIV-1 spread (**Fig. 46A**). T5 γ , a shorter isoform of T5 interferes with T5 α -mediated restriction in a dominant negative manner (Stremlau et al., 2004). Despite the apparent health of T5Cyp-transduced Jurkat T cells, T5Cyp could affect endogenous T5 α akin to T5 γ . To evaluate this possibility, T5Cyp was expressed in CypA KD and control cells and challenged with N- or B-MLV (**Fig. 46C**). In the presence of T5Cyp, there was a reduction in Ref-1 activity; which is still robust. While this is unlikely to cause wide-spread N-MLV infections in humans, the function of T5 α in human T cells is unknown. Furthermore, endogenous T5 α expression is upregulated upon IFN α treatment (Asaoka et al., 2005). One could investigate whether different T5 isoforms are differentially expressed upon IFN α stimulation and how constant expression of T5Cyp from a heterologous promoter may affect the immune function of T5 α in the face of viral infection.

[00243] Since CypA binds to CA, as does T5Cyp, high levels of endogenous CypA could compete with hT5Cyp for CA-binding, thus reducing restriction activity. Intriguingly, this was not the case, and endogenous CypA has no effect on restriction by hT5Cyp in T cell lines (**Fig. 46B**). This could be simply because low levels of T5Cyp suffice for potent restriction of HIV-1. Indeed, expression of restricting T5Cyp fusion proteins could not be detected in Jurkat cell lines. Alternatively, T5Cyp and cytoplasmic CypA may not encounter CA in the same compartments. For instance, CypA concentration in cytoplasmic bodies may be significantly lower than in the rest of the cytoplasm. A rapid association of HIV-1 with cytoplasmic bodies in which T5Cyp is concentrated is observed in live imaging of cells expressing fluorescently-labeled restriction-competent T5 (Campbell et al., 2008). One question is whether CypA affects the formation of cytoplasmic bodies, warranting live

imaging of T5Cyp-expressing CypA KD and control cells during HIV-1 infection. CypA is required for T5 α -mediated resistance to HIV-1 infection in old world monkey cells (Berthoux et al., 2005b). A further possibility is that CypA is, in fact, required for T5Cyp-mediated restriction as well. Consistent with this possibility, an actual reduction in magnitude of HIV-1 restriction is observed in CypA KD cells compared to Luc KD cells. CypA could be specifically knocked down in a context where T5Cyp is expressed at physiologic levels such as in owl monkey kidney cell (OMK) cells, without affecting expression of T5Cyp. This should enable a clear assessment whether HIV-1 infection in OMK cells is enhanced in the absence of CypA, implicating CypA as a co-factor for T5Cyp-mediated restriction.

[00244] One consistent finding has been that the magnitude of T5-mediated restriction depends on the cell-type in which exogenous T5Cyp is expressed. The most potent restriction is observed in CRFK cells, feline kidney fibroblasts that lack endogenous T5. Next, adherent cells such as 239T or TE671 have large T5-mediated HIV-1 restriction phenotypes. Lymphocytic cells in suspension such as Jurkat and THP-1 have consistently shown the smallest magnitude with 10-20 fold restriction of HIV-1 both by hT5Cyp and AoT5Cyp (**Fig. 43, 5, 6, 11**). Potent HIV-1 inhibitors lower the risk of emerging viral resistance. Consequently, efficiency of HIV-1 inhibition is an essential consideration for any candidate therapy. The efficiency of HIV-1 inhibition by hT5Cyp was compared to that of RhT5 α and modified hT5 α with substitutions in the PRYSPRY domain. In all cell lines, T5Cyp was consistently 10-fold more potent than RhT5 α . The largest, > 10-fold inhibition of HIV-1 by RhT5 α observed in CRFK and 293T cells (**Fig. 50**). In Jurkat T cells RhT5 α is more than 4-fold less potent than T5Cyp. The effect of modified hT5 α in T cell lines, however, is minimal (**Fig. 50**).

[00245] Most data published on potency of T5-based restriction factors were obtained in CRFK or HeLa cells that allow for large HIV-1 restriction phenotypes. Furthermore, in published reports, constructs are most commonly HA-tagged at the N-terminus; a modification that seems to enhance restriction activity (Stremlau et al., 2004). Published data are often represented on a linear scale that obscures effects on HIV-1 at low MOI, where T5 activity is the strongest (**Fig 50**). This is the optimal experimental system and data representation when analyzing T5 function and small modulations of T5 restriction. In CRFK and Cf2Th cells even multimerized CypA alone (Javanbakht et al., 2007) and fusions of CypA to Fv1 or afpartin, a protein known to form extended dimers, were reported to block

HIV-1 infection when visualized on a linear scale, albeit with lower potency than T5Cyp(Schaller et al., 2007; Yap et al., 2007). Notwithstanding, other models may be useful for the analysis of physiological potency. Here a first side-by-side comparison is shown of untagged T5-based restriction factors in T cell lines, a model system that is more physiologically relevant for treatment of HIV-1 infection than feline fibroblasts. T5Cyp is a significantly more potent HIV-1 inhibitor than RhT5 α in T cells, which may be due to the high CA-binding affinity of CypA combined with distinct interactions of the exogenous T5-restriction factor with endogenous hT5 α . To address the latter, one could assess the HIV-1 inhibition efficiency of various T5 proteins in T5 α KD T cells and see if the magnitude of HIV-1 inhibition now approaches that seen in CRFK cells.

[00246] The strict structural requirements for engineering a functional hT5Cyp fusion emphasize that the generation of the fusion gene by retrotransposition within New World owl monkeys (*Aotus* sp.) was truly a remarkable event. As it turns out, a TRIMCyp fusion gene was independently generated by retrotransposition in Old World macaques, though in the case of the latter, restriction activity is detected against HIV-2 and FIV, but not HIV-1(Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008; Wilson et al., 2008). The convergent evolution of TRIMCyp fusion proteins with distinct retroviral specificities indicates a strong selection for these potent restriction factors. While the force behind the selection remains to be elucidated for each T5 orthologue, it is likely to have been exerted by challenge with a retrovirus(Sawyer et al., 2005). Here a fully human restriction factor, hT5Cyp, was engineered that potently blocks HIV-1 *in vitro* and *in vivo*.

EXAMPLE 2: ASSESSMENT OF hT5Cyp EFFECTS ON HIV-1 SPREADING INFECTION IN PRIMARY CD4⁺ T CELLS AND MACROPHAGES

Current anti-HIV-1 gene therapy approaches

[00247] Recently, gene- and stem cell therapies in the treatment of HIV-1 have come into renewed focus. Furthermore, life-long pharmacotherapy is associated with serious long-term health risks and cannot lead to clearance of HIV-1 in infected patients due to persisting reservoirs of latently-infected cells (Han et al., 2007). The ideal gene therapy candidate should have high efficacy as well as low toxicity and antigenicity. The following anti-HIV-1 gene therapy approaches have been put forward: at the cellular level, modification of host-cell factors required for viral replication or inhibition of essential viral elements at all steps of

the viral life cycle; and systemically, accelerated destruction of infected cells (Strayer et al., 2005). To block HIV-1 entry, the HIV-1 co-receptor CCR5 has been targeted by various means including siRNA (An et al., 2007; Kumar et al., 2008) and zinc-finger endonucleases (Perez et al., 2008) with success *in vitro* and *in vivo*. In rhesus macaques, transplant of autologous CD34⁺ cells transduced with a lentiviral vector coding for GFP and CCR5-targeting shRNAs led to stable maintenance of transduced PBMCs for the 12-month duration of the experiment *in vivo*. Furthermore, transduced PBLs derived from monkeys were SIV-resistant upon *ex vivo* challenge in contrast to untransduced PBLs from the same animal. This approach revealed a dose-dependent toxicity of shRNA expression in T cells (An et al., 2007).

[00248] An elegant adaptation of the CCR5-knockdown approach is the targeting of CCR5-specific siRNAs in combination with siRNAs against tat and vif directly to T cells *in vivo* (Kumar et al., 2008). This was achieved by non-covalently linking siRNAs to a single-chain antibody against IL-7, which is rapidly internalized to deliver the siRNAs to T cells. The approach efficiently blocked established HIV-1 replication in a humanized NOD/SCIDIl2rg^{-/-} mouse model. More impressive results were obtained in a huPBL-NOD/SCIDIl2rg^{-/-} model with significant T cell protection and > 4-fold reduction in plasma viral load following HIV-1 infection. Transplant of HIV⁺ PBMCs into NOD/SCIDIl2rg^{-/-} mice leads to re-activation of viral replication due to T cell activation in the xenogeneic context. With triple siRNA-targeting to T cells *in vivo* reactivation fails to occur, viral loads remain below detection, and T cell destruction is not apparent. Notably, the triple-treatment was significantly more efficient than anti-CCR5 siRNA alone. Furthermore, since the siRNAs are transiently expressed, it is unclear how frequently treatment would be required (Kumar et al., 2008). Recently, a novel approach using a zinc-finger nuclease targeting CCR5 has been described that protects CD4⁺ T cells and reduces viral load in a hPBL-NOD/SCIDIl2rg^{-/-} mouse model for HIV-1 infection. While this approach was quite effective at blocking spread of CCR5-tropic HIV-1 strains, there was a concern of off-target effects specifically with CCR2 excision (Perez et al., 2008). For all strategies targeting CCR5, disruption could select for CCR5-independent viruses (Taylor et al., 2008) and is not without consequence, as the CCR5Δ32 allele is a risk factor for symptomatic West Nile Virus infection (Glass et al., 2006).

[00249] CD34⁺ human hematopoietic progenitor cells expressing RhT5 α can be differentiated into HIV-1-resistant macrophages(Anderson and Akkina, 2005b). RhT5 α , however, is a simian protein and potentially antigenic. This could lead to the elimination of modified cells *in vivo* as was observed for the introduction of the suicide gene thymidine kinase into gag-specific CD8⁺ T cells in clinical trials(Riddell et al., 1996). To minimize antigenic potential, a chimeric rhesus-human T5 α _{R323-332} with improved anti-HIV activity was introduced into hCD34⁺ HSCs. Upon maturation in SCID thy/liv mice, T cells showed reduced infectability with HIV-1 *ex vivo* (Anderson and Akkina, 2008). RhT5 α , however, is not as potent a restriction factor as hT5Cyp in T cells (**Fig. 50**), and hT5 α _{R323-332} is indeed quite weak in T cell lines, raising the risk of *in vivo* selection for T5-resistant viruses.

[00250] One protein-based gene therapy strategy is delivery of a transdominant rev (Malim et al., 1992). RevM10 contains point mutations in the transactivation domain and retains the ability to bind the RRE and form multimers with wildtype rev, but can no longer facilitate transfer of immature RNAs to the cytoplasm. RevM10 reduces viral load *in vitro* and is safe *in vivo*, providing cells with a selective advantage in HIV-1 infected patients without, however, reducing viral load (Ranga et al., 1998). The major difficulty in this study was efficient retroviral delivery of RevM10 to patient-autologous T cells *ex vivo* combined with their short life-span (~6 months, compared to 3 weeks for controls) upon re-infusion into patients(Ranga et al., 1998). Equally poor maintenance of transduced cells was observed upon transduction of autologous CD34⁺ cells derived from bone marrow with retroviral vectors directing the expression of a codon-optimized RevM10 in two HIV-1⁺ children. RevM10 expression was lost in PBMCs at three months post-infusion (Marathe and Wooley, 2007). Other transdominant proteins used are tat, tat-rev fusions, and transdominant gag (Strayer et al., 2005).

[00251] Essential viral factors, such as rev, tat, gag, and nef have also been targeted by siRNA(Boden et al., 2003; Boden et al., 2007). Escape mutants arise easily *in vitro*, as single point mutations suffice to escape the siRNA-mediated block to HIV-1 replication(Boden et al., 2007). Thus, few siRNA approaches have been clinically tested. Consequently, siRNAs are commonly used in combination with each other or other strategies to synergize in increasing the block to HIV-1 replication and to reduce the potential of viral escape mutants(Anderson et al., 2007). The retroviral delivery of transdominant rev combined with TAR antisense sequence, for example, was tested in 10 sets of twins with discordant HIV-1

status (Morgan et al., 2005). CD4⁺ cells from the uninfected twin were transduced with retroviruses coding for RevM10 and anti-sense elements directed against HIV-2 TAR. Transduction efficiency was dependent on envelope used and ranged between 9-20%. Soon after transfer into patients, increased survival of modified T cells was observed by PCR and cells persisted over two years.(Morgan et al., 2005) Although data are insufficient, combination therapies could be associated with increased risk of toxicity (off-target effects of siRNAs, induction of the type I interferon response, or global changes of mRNA expression profiles)(An et al., 2007).

[00252] The accelerated destruction of infected cells includes approaches such as the tat-dependent expression of herpes simplex thymidine kinase (HSV-TK) causing conditional suicide of HIV-1 infected cells treated with acyclovir (Marathe and Wooley, 2007). As pointed out previously, expression of foreign proteins can lead to elimination of transplanted cells *in vivo*. Similarly, direct transduction of Jurkat T cells with a HIV-1 protease-dependent caspase 3 causes efficient apoptosis of infected cells without harming non-infected cells(Vocero-Akbani et al., 1999). A “suicide”-strategy that has been tested in clinical trials is the use of a CD3/CD4 chimeric receptor termed CD4 ζ . CD4 ζ has the transmembrane and extracellular domains of CD4, enabling attachment of HIV-1, and the cytoplasmic domain of CD3 (ζ chain), enabling TCR-like signaling in transduced cells upon HIV-1 binding (Roberts et al., 1994). CD4 ζ has been expressed in autologous CD4⁺ and CD8⁺ T cells, generating HIV-1-specific T cells with the ability to enhance or execute killing of infected cells (Marathe and Wooley, 2007). Transfer of $\sim 10^{10}$ autologous CD4⁺ and CD8⁺ T cells expressing CD4 ζ is safe and leads to persistence of CD4 ζ ⁺ T cells for more than 1 year post-transfer in the blood. Additionally, there was early evidence of mucosal seeding following *ex vivo* stimulation of T cells with α CD3/ α CD28 beads *ex vivo*. Nevertheless, there was no significant change in viral burden and clear therapeutic effect was not obtained (Mitsuyasu et al., 2000). Similarly, assessment of CD4 ζ autologous T cell therapy in twins with discordant HIV-1 status (gene modified cells are HIV-1⁻ prior to infusion into the HIV-1⁺ twin), showed no reduction of viral load or significant therapeutic effect. Transferred T cells could be maintained at 0.1-1% of peripheral blood lymphocytes for more than 1 year, showing long-term maintenance of transferred cells in the host after appropriate prior stimulation (Walker et al., 2000). Overall, enhancing the destruction of infected cells is a strategy that has failed to produce a therapeutic benefit. Possibly, killing HIV-1 infected targets occurs too late in the

viral life cycle, thus allowing production of sufficient progeny virus for sustained viremia(Mitsuyasu et al., 2000; Walker et al., 2000).

[00253] Instead of accelerated destruction of infected cells, an actual cure of HIV-1 infection at the cellular level would be preferable. “Cure” can be achieved in tissue culture by the excision of integrated provirus from infected cells using a “molecular scissor”: *tre*, a *cre*-recombinases evolved *in vitro* to be LTR-specific (Sarkar et al., 2007). In general, gene therapies that act early and potently in the viral life cycle would block the virus from becoming a heritable genetic element and would protect cells from CTL-mediated killing. If these therapies additionally act before reverse transcription they preclude genetic diversity acquisition required for development of resistance. Mathematical modeling predicts a therapeutic benefit only for approaches targeting HIV-1 before integration in the context of autologous T cell therapy(von Laer et al., 2006b). Approaches targeting viral gene expression (most of the approaches tested to date) would require a much higher inhibitory activity to promote regeneration of T cells and viral load reduction, whereas approaches targeting viral assembly and release would not have a clinical benefit in isolation (von Laer et al., 2006b). According to this mathematical prediction, using *tre*-therapy alone would be unlikely to have much immediate therapeutic benefit. Given that it takes more than 3 months to cure tissue culture cells of provirus(Sarkar et al., 2007), curing latently-infected, resting primary cells *in vivo* will be a great challenge. Nonetheless, excision of integrated provirus, may cure previously infected memory T cells including those found in GALT. In combination with other early-acting therapies, *tre* could assist in T cell regeneration while maintaining the established memory T cell repertoire including HIV-1 specific T cells; an exciting prospect indeed.

Delivery of candidate gene therapy to target cells

[00254] Efficient and safe delivery of gene therapy vectors is crucial for any potential clinical treatment. The deleterious potential of insertional mutagenesis in humans was sadly highlighted following the activation of the LMO2 proto-oncogene by proximal insertion of gammaretroviral vectors carrying the IL-2 receptor gamma chain to treat X-linked SCID in patients (Hacein-Bey-Abina et al., 2008; Howe et al., 2008), marring an otherwise impressive success (Cavazzana-Calvo et al., 2000). Retroviruses are a preferred vector for gene delivery due to their ability to integrate into the host genome and provide heritable gene modification in progeny cells(Bushman, 2007). Regarding integration target site preference not all

retroviruses are created equal. Gammaretroviruses like MLV, which have been used in human trials, have a preference for the 5' ends of transcriptional units (TUs) and associated CpG islands, particularly near promoters of active genes (Bushman, 2007; Wu et al., 2003). Lentiviruses, of which HIV-based vectors are most frequently used, prefer to integrate within active transcription units (Mitchell et al., 2004). Integrase (IN) is the principle viral determinant of integration specificity as chimeric HIV-1 containing MLV-IN integrates at MLV-preferred sites (Lewinski et al., 2006).

[00255] Efforts are underway to modulate integration site specificity for increased safety of retroviral vectors. LEDGF/p75 is known to tightly bind HIV-1 IN and target provirus integration (Maertens et al., 2003). siRNA-mediated knockdown of LEDGF/p75 altered the insertion profile of HIV-1 indicating that LEDGF/p75 tethers IN to DNA and promotes HIV-1 insertion into active TUs (Ciuffi et al., 2006). While LEDGF/p75 binds DNA relatively non-specifically, a proof-of-principle *in vitro* experiment showed that a LEDGF/p75 fusion to the DNA-binding domain of the λ repressor directed integration of HIV-1 provirus close to λ repressor binding sites in target λ phage DNA (Ciuffi et al., 2006). Another approach to controlling integration target sites takes advantage of the homologous recombination pathway. Lentiviral vectors defective for IN are used to ensure nuclear import of a site-specific integration system bearing desired sequences (Lombardo et al., 2007). The benefit of such a strategy is both directed integration and physiologic expression control (Lombardo et al., 2007). By combining a zinc-finger-endonuclease and IL2R γ sequences flanked with homologous arms in an IN-defective lentiviral vector, removal of the endogenous IL2R γ segment and replacement with a trackable donor IL2R γ sequence in the IL2R γ locus (gene editing) was achieved (Lombardo et al., 2007). Both T cells and CD34⁺ HSCs are targets of candidate gene therapy. While only CD34-based therapy has the potential to regenerate the entire T cell repertoire and with it anti-HIV-1 immune responses, it clearly has greater therapeutic potential. At the same time, it is a difficult procedure prone to complications and high toxicity as patients need to be conditioned prior to transplant. Additionally, safe retroviral vectors for HSC-based therapy are still under development. Autologous T cell therapy is less toxic, easier to perform and administer, and may lead to improvement in disease progression upon repeated administration even in the absence of restoring the T cell repertoire (Strayer et al., 2005; von Laer et al., 2006a).

[00256] To date, therapeutic levels of transgene expression were not achieved in clinical gene therapy trials despite some encouraging effects on T cell survival (von Laer et al., 2006a). Problems arise at two levels: one is high-efficiency gene delivery to T cells or CD34⁺ hHSCs, and the other is design of early-acting HIV-1 inhibitors potent enough to allow for T cell regeneration in the context of viral load reduction. hT5Cyp has several properties that make it appealing for gene therapy. It inhibits HIV-1 infection even more potently than RhT5 α when expressed in human cells, acting early in the viral life cycle. Furthermore, AoT5Cyp, after which hT5Cyp is modeled, is the only TRIM5 allele in 10 *Aotus* species (Ribeiro et al., 2005), indicating that cells bearing AoT5Cyp retain functionality. Similarly, no deleterious effects of hT5Cyp-expression were observed in T cell lines. This Example analyzes the anti-HIV-1 activity of hT5Cyp in spreading infection of HIV-1 as well as primary human T cells and macrophages.

hT5Cyp eliminates spreading infection of HIV-1

[00257] While Example 1 dealt with the design and characterization of a hT5Cyp with potent anti-HIV-1 activity in single cycle assays, this Example assesses the effects of hT5Cyp on HIV-1 spreading infection. Jurkat T cells stably transduced with T5-expression vectors were infected with replication-competent HIV-1_{NL4-3-GFP-IRES-Nef}. No HIV-1 replication was detected in cells transduced with hT5Cyp or AoT5Cyp for the two-month observation period (**Fig. 51A,B**). HIV-1 replication in cells transduced with hT5Cyp bearing a mutation that blocks binding to HIV-1 CA (CypA-H126Q, see **Fig. 49B**), hT5CypH126Q, was as rapid as in cells transduced with empty vector (**Fig 51A,B**).

[00258] In contrast to hT5 α , RhT5 α restricts HIV-1 (Stremlau et al., 2004). Chimeric T5 protein in which the PRYSPRY specificity determinant from the rhesus orthologue replaces the corresponding human residues (hT5 α -R323-332 (Stremlau et al., 2005) (Sawyer et al., 2005)), or even in which a single, critical amino acid was substituted (R332P (Li et al., 2006b)), exhibit HIV-1 restriction activity approaching that of RhT5 α in HeLa and CRFK cell lines. Since these molecules are nearly identical to the human protein it has been proposed that they may have therapeutic utility (Anderson, 2008; Li, 2006; Stremlau, 2005; Yap, 2005). As reported in Example 1, hT5 α -R323-332 and hT5 α -R332P caused moderate inhibition of HIV-1 transduction in single-cycle infection, each behaving similarly to the other, though neither was as potent as hT5Cyp or AoT5Cyp (**Fig. 50**). When the effect of hT5 α -R332P was tested in a spreading infection, there was a delay in the peak of HIV-1 that

was inversely proportional to initial viral dose, but in contrast to hT5Cyp or AoT5Cyp, it failed to prevent HIV-1 infection (**Fig. 51A,B**). Similarly, when compared to T5Cyp, RhT5 α inhibited HIV-1 spread over 5-fold 12 days post-infection (**Fig. 51C**). Furthermore, peak viral replication was delayed by 24 days in Jurkat T cells transduced with lentiviral vectors coding for RhT5 α . In stark contrast to hT5cyp and AoT5Cyp, RhT5 α was unable to block spread of HIV-1 in these cells (**Fig. 51C**).

[00259] HIV-1 has the capacity to acquire resistance to many antivirals during the step of reverse transcription, which allows for accumulation of genetic diversity in progeny. One focus was to see whether a hT5Cyp-resistant virus could be generated, with the purpose of studying its pathogenicity further. hT5Cyp and AoT5Cyp-resistant HIV-1 could not be isolated on 10 separate occasions (representative data **Fig. 51, A-C**), despite initiation of infection with a wide-range of HIV-1 inocula. Similarly, addition of untransduced cells and their subsequent removal using puromycin (the Jurkat T cell lines are puromycin resistant) failed to yield resistant virus. Furthermore, T5-mediated restriction in our Jurkat T cell lines can be overcome by using cell-associated rather than cell-free virus. To this end Jurkat T cell lines expressing the indicated T5Cyp or control were labeled with the fluorescent dye DiD (target) and mixed with Jurkat T cells previously infected with HIV-1_{NL4-3} (donor) at a 1:1 ratio. All cell lines were equally infectible by cell-associated virus (**Fig. 51D**). Similar results were obtained by another group in primary CD4⁺ T cells expressing RhT5 α and in CD4⁺ T cells from rhesus macaques (Richardson et al., 2008).

[00260] Studies were designed to see whether a similar system could be exploited in order to select for a T5Cyp-resistant virus. One approach was to introduce HIV-1_{NL4-3}-GFP-IRES-Nef proviral DNA into Jurkat T cell lines expressing T5 or control by electroporation, to ensure that spreading infection was initiated by cell associated virus in the absence of free virus. The transfection efficiency was comparable for all cell lines analyzed and remained constant at about 1% of total cells in culture for the first 10 days post-electroporation (**Fig. 51E**). In Jurkat T cells expressing AoT5Cyp and hT5Cyp the infection was self-limiting and the percentages of infected cells had dropped to background levels by 34 days post-electroporation, without evidence for spread of HIV-1_{NL4-3}-GFP-IRES-Nef (**Fig. 51E**). hT5 α -R332P, in contrast, was able to delay peak viral replication by 10 days but failed to ultimately control spread (**Fig. 51E**). Again no T5Cyp-resistant HIV-1 could be derived in this experimental setting.

[00261] Regardless of how spreading infection is initiated, cell-associated virus is essential and mechanical disruption of cell-to-cell association blocks spread of virus *in vitro* (Sourisseau et al., 2007). Furthermore, it represents the main method of transmission *in vivo* (Haase, 1999). In an attempt to reconcile that T5Cyp-mediated HIV-1 restriction is overcome by cell-associated virus in the first 24 hours post-infection (**Fig. 51D**) but concomitantly T5Cyp blocks spread when a long-term spreading infection was initiated with cell-associated virus (**Fig. 51E**), further experiments were undertaken. Spreading infections were initiated by co-culturing HIV-1_{NL4-3-GFP-IRES-Nef}-infected Jurkat T cells (donors) with DiD-labeled Jurkat T cell lines stably transduced with bicistronic lentiviral vectors coding for T5Cyp and puromycin resistance (targets). Donors (D) and targets (T) were mixed at different ratios: 2D:1T, 1D:1T, 1D:10T, 1D:100T, and 1D:1000T (**Fig. 52, A-E**). Unlike the previous experiment (**Fig. 52D**), where infected target cells were monitored for only 24 hours, here infected target cells were monitored for 24 days (**Fig. 52, A-E**). Since DiD is a fluorescent membrane dye and is diluted out with cell division, puromycin was added on day 3 of the co-culture to ensure continued monitoring of target cells only.

[00262] For all donor to target ratios, initial infection of T5Cyp-expressing cells was comparable to control and proportional to infecting dose (**Fig. 52, A-E**). HIV-1 spread in T5Cyp-expressing cells is less efficient, however, resulting in control of infection over time (**Fig. 52, A-E**). The largest difference in levels of infection between control and T5Cyp-expressing Jurkat T cells were observed at the lowest D:T ratios of 1D:100T and 1D:1000T with a respective 400- and 100-fold difference at day 22 (**Fig. 52D,E**). At the highest D:T ratios of 2D:1T and 1D:1T, this difference was reduced to 20-fold at day 22 (**Fig. 52A,B**). Combined, these results indicate that while T5Cyp-transduced cells can be infected by cell-associated HIV-1, over time a T5Cyp-mediated inhibition of HIV-1 spread is apparent.

[00263] The mechanism underlying these observations has not yet been determined. Overexpression of RhT5 α reduced HIV-1 production without affecting infectivity (Sakuma et al., 2007; Zhang et al., 2008a). This would implicate T5-mediated restriction both at post-entry and at a much later step in the viral life cycle (Sakuma et al., 2007; Zhang et al., 2008a). These observations may be an artifact due to overexpression of an exogenous protein, while knockdown of endogenous RhT5 α seems to have no effect on HIV-1 production (Zhang et al., 2008a). To assess the effects of donor and target cell T5Cyp on cell-to-cell spread of HIV-1, a single-cycle cell-to-cell spreading assay for HIV-1 infection was designed. 293T

donor cell lines expressing control or indicated T5Cyp were transfected with HIV-1_{NL4-3-IRES-GFP-Nef} provirus and supernatant was harvested during the first 48 hours to measure production of virus by RT activity. 48 hours post-transfection, donor cells were washed three times with PBS and then DiD-labeled target Jurkat T cells transduced with control vector or vector coding for hT5Cyp were added to the 239T donor cells (**Fig. 53A**). At the indicated time points dextran sulfate was added to the co-culture to block further infection and cells were harvested. 48 hours later the percentage of infected target Jurkat T cells was measured by flow cytometry (**Fig. 53A**). The magnitude of HIV-1 restriction in single cycle assays using cell-free virus is shown for recipient Jurkat T cell lines and donor 293 T cell lines is shown (**Fig. 53B**).

[00264] In this single-cycle cell-to-cell infection it became clear that for short co-culture time (2 hours) both donor and target cell-T5Cyp cooperate to inhibit infection of donor cells (**Fig. 53C, left panel**). Infection of control target cells is 10-fold and 3-fold lower when the donor cells expressed hT5Cyp or AoT5Cyp, respectively, as compared to donor cells transduced with empty vector (**Fig. 53C, left panel**). Similarly, donor and target cell hT5Cyp cooperate to reduce infection of hT5Cyp-expressing targets further than when donor cells contain empty vector (**Fig. 53C, left panel**). With increased time of co-culture, the effects of donor T5Cyp are lost. When target cells transduced with empty vector were co-cultured with donor cells for 12 hours, the percentage of infected cells approached similar values regardless of donor cell phenotype (**Fig. 53C, right panel**). Similarly, after 12 hours of co-culture, target cells containing hT5Cyp inhibit infection to the same degree whether donor cells contained control vector or hT5Cyp (**Fig. 53C, right panel**). This effect is not due to an effect of T5Cyp on viral production in the 293T donor cells, as quantification of relative RT units 48 hours post-transfection showed comparable viral production in cells transduced with T5Cyp or control hT5Cyp^{H126Q} and higher viral production in cells transduced with empty vector alone (**Fig. 53D**). Similarly, virus release was similar in all target cells (**Fig. 53E**). The effects observed for T5Cyp in donor cells during short co-cultures could account for our observation that, once initiated, spreading infection of HIV-1 dwindles and is controlled over time.

hT5Cyp blocks HIV-1 infection of primary human CD4⁺ T cells and macrophages

[00265] To assess the feasibility of hT5Cyp as a viable HIV-1 therapy, hT5Cyp-mediated restriction of HIV-1 in primary human CD4⁺ T cells and macrophages was

assessed. To identify cells transduced with T5, a bicistronic lentiviral vector was used that directs synthesis of GFP from an internal ribosome entry site (**Fig. 54A**). These vectors failed to generate GFP in primary CD4⁺ T cells (**Fig. 54B**) although they functioned efficiently in Jurkat T cell lines. Next, hT5Cyp was cloned into a bidirectional dual-promoter vector (BiD) in which the PGK promoter and a minimal CMV promoter drive transcription of hT5Cyp and GFP, respectively (Amendola et al., 2005). GFP production and hT5Cyp function in a single cycle assay using an HIV dsRed vector were efficient in Jurkat T cell lines transduced with BiD encoding hT5Cyp or hT5CypH126Q. BiD, however, failed to yield GFP⁺ primary CD4⁺ T cells. Better results were obtained when a vector, scALPS, was engineered using two promoters, one from the SFFV LTR and the other from the hCypA gene (SEQ ID NO:8) (**Fig. 54A,B**). CD4⁺ T cells were transduced with scALPS encoding either hT5Cyp (SEQ ID NO:9) or the non-restrictive hT5CypH126Q. Cells were then sorted based on GFP-expression. Relative quantification of GFP, hT5Cyp, and hT5CypH126Q mRNA showed that all were efficiently transcribed at comparable levels in CD4⁺ T cells (**Fig. 54C, D**). Expression of hT5Cyp and hT5CypH126Q was not detectable in these cells by western blot, corresponding to our previous experience in Jurkat T cell lines. To test for equivalent protein synthesis from both promoters, dsRed was cloned into scAPLS, such that the SFFV and hCypA promoters drive transcription of dsRed and GFP, respectively (**Fig. 55A**). While dsRed fluorescence was too weak in CD4⁺ T cells to obtain reliable data, in transduced Jurkat T cells the majority of GFP⁺ cells also expressed dsRed efficiently compared to mock-transduced cells (**Fig. 55B**). Also, expression of dsRed did not interfere with GFP expression as GFP-expression was equally efficient when scALPS-GFP or scAPLS-GFPdsRED vectors were used to transduce Jurkat T cells (**Fig. 55B**).

[00266] CD4⁺ T cells were enriched from PBMCs using MACS, and activated using irradiated allo-PBMC and IL-2. Activated CD4⁺ T cells were then transduced with dual-promoter vectors encoding either hT5Cyp or the non-restrictive hT5CypH126Q. Cells were sorted based on GFP-expression and then challenged with replication-competent, CXCR4-tropic, HIV-1_{NL4-3}. Viral replication in hT5CypH126Q-transduced cells peaked on day 12 and infection was not established in hT5Cyp-transduced cells (**Fig. 56A**). The same hT5Cyp-mediated block to infection was observed with the CXCR4-tropic primary isolate HIV-1_{DH12} (**Fig. 56B**). To test hT5Cyp activity in human macrophages, CD14⁺ cells were enriched from PBMCs using MACS and differentiated for 10 days in the presence of GM-CSF. Monocyte-derived macrophages were pre-treated with SIV_{MAC251} virus-like particles (VLPs) (Goujon

et al., 2006) and transduced with vectors encoding hT5Cyp or hT5CypH126Q and sorted based on GFP-expression four days after transduction. Cells were infected with HIV-1_{NL4-3}, modified to be CCR5-tropic. While input virus decays in cells transduced with hT5Cyp and infection is not established, infection peaks on day 10 post-infection in cells transduced with the non-restrictive hT5CypH126Q (**Fig. 56C**). Similar results were obtained when transduced macrophages were challenged with the CCR5-tropic primary isolate HIV-1_{132W} (**Fig. 56D**). Both CD4⁺ T cells and monocyte-derived macrophages transduced with hT5Cyp were resistant to HIV-1 infection (**Fig. 56**).

hT5Cyp confers selective advantage to CD4⁺ T cells challenged with HIV-1

[00267] The previous experiments showed that HIV-1 infection is blocked when all cells in the culture have been transduced with the hT5Cyp vector. Next anti-HIV-1 efficacy was evaluated under suboptimal conditions, in which < 25% of cells were transduced with hT5Cyp. These conditions would be more realistic of hT5Cyp therapy in patients. In the absence of HIV-1 infection, the percent transduced cells persisting in the culture over the course of a month was equivalent for hT5Cyp and hT5CypH126Q. In the face of HIV-1 infection, the hT5CypH126Q cells declined to <10% of the cells in the culture. hT5Cyp cells, in contrast, had a distinct advantage, expanding to 75% of the cells in the population after re-stimulation of the CD4⁺ T cell cultures using irradiated allo-PBMC and IL-2 (**Fig. 57A**). Consistent with these data, viral replication as assessed by RT activity in the culture supernatant was significantly lower in cultures transduced with hT5Cyp than in those transduced with hT5CypH126Q (**Fig. 57A**). Furthermore, while high viral replication is already apparent on day 12 post-infection in CD4⁺ T cells transduced with hT5CypH126Q, viral replication does not peak until day 28 of the culture in CD4⁺ T cells transduced with hT5Cyp; the first time-point after re-stimulation of the culture (**Fig. 57A**). The survival advantage of CD4⁺ T cell becomes apparent when analyzing the viability of the cells by forward versus side-scatter and gating on live lymphocytes; a method that had to be adopted since the cells were fixed in paraformaldehyde for flow cytometry analysis. While transduced CD4⁺ T cells were equally viable at the beginning of the experiment, throughout the course of the experiment CD4⁺ T cells transduced with hT5Cyp had a viability advantage (**Fig. 57B**). After restimulation of the cultures, viable cells were rescued in CD4⁺ T cell cultures transduced with hT5Cyp while there was no rescue of cells transduced with hT5CypH126Q (**Fig. 57B, day 34**). Representative plots are shown in **Fig. 57C**. Both the GFP selection and survival advantage observed for CD4⁺ T cells transduced with hT5Cyp were most likely due

to the antiviral activity of hT5Cyp, since HIV-1 replication was reduced in cultures containing hT5Cyp cells (**Fig.57A**). Thus, even when a minority of cells are transduced in culture conditions where HIV-1 can spread directly from cell-to-cell, hT5Cyp is potent enough to block HIV-1 infection.

Effects of hT5Cyp on CD4⁺ T cell function

[00268] While hT5Cyp efficiently blocks HIV-1 infection, in view of a potential therapeutic use, is essential that this occurs without perturbing primary CD4⁺ T cell function. Primary CD4⁺ T cells transduced with scAPLS bearing hT5Cyp (SEQ ID NO:9) were sorted based on GFP-expression. Transduced cells proliferated at the same rate as untransduced cells as untransduced cells as seen by thymidine incorporation (**Fig.58A**). When stimulated with PMA and ionomycin, scAPLS-hT5Cyp transduced CD4⁺ T cells produced comparable amounts of IL-2 as untransduced cell (**Fig. 58B**). Similarly, untransduced and scAPLS-transduced CD4⁺ T cells expressed the same level of cell-surface CD4, CXCR4, and MHC I (**Fig.58C**) as well as CD11a, CD18, ICAM3, CCR5, CD25, CD69, and PD-1. The functions of endogenous CypA and T5 in hCD4⁺ T cells remain largely uncharacterized. Addressing these functions could improve predictions regarding the potential impact of introducing hT5Cyp into CD4⁺ T cells. CypA is known to interact at subnanomolar affinity with cyclosporine (CsA), an immunosuppressive agent used to prevent allograft rejection. This complex inhibits T cell function by binding to the serine/threonine phosphatase calcineurin and blocking downstream signaling events (Handschumacher et al., 1984; Liu et al., 1991). Functionally, CypA is a peptidyl-prolyl isomerase (PPIase) which catalyses the rate-limiting *cis-trans* interconversion of peptide bonds N-terminal to proline, an enzymatic activity not required for immunosuppression via CsA (Fischer et al., 1984; Liu et al., 1991; Schmid, 1995). The peptidyl-prolyl isomerases are represented by three structurally distinct families: cyclophilins, FKBP, and parvulins. Given their astounding evolutionary persistence, from some archeobacteria to all mammals, cyclophilins are not well understood (Colgan et al., 2000; Gothel and Marahiel, 1999).

[00269] Despite ubiquitous, high-level expression, CypA is not essential for viability in mice (Colgan et al., 2000; Ryffel et al., 1991). CypA^{-/-} mice show hallmarks of allergic disease, such as allergic belparitis, tissue infiltration by mast cells and eosinophils and elevation of serum IgE, IgG1, and IL-4 (Colgan, 2004). CypA is a negative regulator of the tec kinase itk which, primarily expressed in Th2 cells, potentiates signals downstream of the

T cell receptor. Disruption of CypA in mice thus results in a hypersensitive Th2 response (Colgan, 2004). Consistent with the allergic phenotype, *in vivo*- and *in vitro*-generated CypA^{-/-} effector and memory Th2 cells produced 2-4 fold higher IL-4 than wildtype cells accompanied by a comparable increase in IL-4 mRNA (Colgan, 2004).

[00270] IL-2 production, however, was also found to be consistently elevated in effector and memory CD4⁺ T cells derived from CypA^{-/-} mice, following stimulation with plate-bound α CD3 and α CD28 antibodies (Colgan et al., 2004). Similar results were obtained with *in vitro* differentiated Th2 effector or memory cells (**Fig. 59A**). The magnitude of this effect ranges from 5-30 fold (**Fig. 59A**). Surprisingly, unlike IL-4 elevation, increase in IL-2 production cannot be correlated with increased transcription of IL-2 mRNA (**Fig. 59B**), indicating that CypA regulates IL-2 expression post-transcriptionally. Furthermore, *in vivo* IL-2 capture shows that following TCR-stimulation, serum IL-2 levels are elevated more than 2-fold at time of peak production (**Fig. 59C**).

[00271] To evaluate if CypA had similar effects in human primary CD4⁺ T cells, a lentiviral vector was generated to efficiently knock down target mRNAs in primary human CD4⁺ T cells. APM contains a promoter derived from the SFFV LTR to drive puromycin-N-acetylase and sequence coding for the desired shRNA flanked on each side by miR-30 (**Fig. 60A**). CD4⁺ T cells were enriched from PBMCs of healthy donors using MACS, stimulated with irradiated allo-PBMC and IL-2, and transduced with APM containing shRNA targeting luciferase (Luc) as a control, or either CypA or T5 (see below). After puromycin selection, knockdown efficiency was assessed at the mRNA-level by quantitative RT-PCR (**Fig. 60B**) and at the protein level by Western blot (**Fig. 60B**). Given efficient knockdown of CypA in primary human CD4⁺ T cells, infectability of these cells was assessed by VSVG-pseudotyped HIV-1_{NL4-3} GFP, HIV-2_{ROD}-GFP, and SIV_{mac}239-GFP vectors, primarily to see whether phenotypes previously reported in human cell lines were preserved in primary CD4⁺ T cells (**Fig. 60D**).

[00272] Indeed, as observed in cell lines, CypA promotes HIV-1 infection in primary CD4⁺ T cells, presumably by protection of HIV-1 from an unknown restriction factor. In the absence of CypA, HIV-1 infection is reduced by more than 2-fold (**Fig. 60D**). CypA did not have an effect on HIV-2_{ROD} or SIV_{mac}239 infection. A more than 100-fold restriction of SIV_{mac}239 in CD4⁺ T cells that was previously noted in Jurkat T cells and PBMCs was confirmed (**Fig. 60D**). Furthermore, an association of either CypA or T5 with restriction of

SIV_{mac}239 in CD4⁺ T cells was ruled out (**Fig. 60D**). Knockdown of T5 in primary CD4⁺ T cells had no effect on HIV-1_{NL4-3}, HIV-2_{ROD}, and SIV_{mac}239 infection (**Fig. 60D**).

[00273] The most consistent and impressive phenotype observed in CypA KD CD4⁺ T cells was on proliferation. At steady state, 4 days after secondary stimulation with irradiated allo-PBMC and IL-2, CypA KD bulk, non-polarized CD4⁺ T cells incorporated 4-fold more thymidine than Luc KD control cells (**Fig. 61A**). This effect was even greater (close to 10-fold higher thymidine incorporation in CypA KD cells compared with Luc KD control) when proliferation was assessed in a Th2 memory T cell population isolated from a healthy donor based on CD4 and CrTh2 expression (Messi et al., 2003) (**Fig. 61A**). Consistent with increased cell cycling, staining CypA KD CD4⁺ T cells for RNA content by Pyronin Y and DNA content by 7-AAD showed the majority of cells in the G2/M/S phase in CypA KD cells while control Luc KD cells had the expected majority in the G1 phase of the cell cycles (**Fig. 61B**). Similarly, CypA KD CD4⁺ T cells are dividing faster than Luc KD control CD4⁺ T cells as visualized by CFSE dilution 48 hours after staining (**Fig. 61C**). These results are consistent with a T cell hyperactivation in the absence of CypA through loss of itk regulation as described for the CypA^{-/-} mouse (Colgan et al., 2004). As observed for murine Th2 cells, human CD4⁺ Th2 memory cells produce almost 10-fold more IL-2 in the absence of CypA compared to Luc KD cells; in this case at steady state without stimulation (**Fig. 61D**). By staining for intracellular cytokines, both IL-2 and IL-4 production are increased two-fold in CypA KD, non-polarized human CD4⁺ T cells compared to the Luc KD controls following PMA/ionomycin stimulation (**Fig. 61E**).

[00274] Since T5 has no mouse homolog, little is known about its functions in human T cells outside of modulation of HIV-1 infection than is the case for CypA. When T5 expression was knocked down as previously described (**Fig. 60A,B**), there was a strong reduction in thymidine incorporation of CD4⁺ T cells compared to control Luc KD CD4⁺ T cells (**Fig. 62A**). T5 seems required for sustained proliferation of CD4⁺ T cells. Consistent with cell cycle arrest, staining T5 KD CD4⁺ T cells for RNA content by Pyronin Y and DNA content by 7-AAD showed that only 16% of T5 KD cells were cycling (G1/S/G2/M phase) while 84% were quiescent, compared with 89% cycling LucKD and 98% cycling CypA KD CD4⁺ T cells (**Fig. 62A,B**). Similarly, T5 KD CD4⁺ T cells failed to divide in comparison to Luc KD CD4⁺ T cells as visualized by CFSE dilution 48 hours after staining (**Fig. 61C**). Interestingly, T5 affects steady-state proliferation but not an immediate response to

stimulation. When T5 KD and Luc KD cells are synchronized by serum starvation and stimulated either using plate-bound α -CD3 and α -CD28 antibodies or irradiated allo-PBMC with IL-2, proliferation in the first 48 hours is similar in T5 and Luc KD T cells.

[00275] Differences in proliferation become increasingly apparent by 10 days post-stimulation with T5 KD cells reaching a quiescent state earlier than Luc KD control cells (**Fig.61**) and dying in the absence of re-stimulation. The effects of T5 on steady-state proliferation posed some technical difficulties, and information could not be obtained on Th1 or Th2-skewed memory cells (Messi et al., 2003) that are significantly more difficult to handle than non-polarized bulk CD4⁺ T cells. Notwithstanding, intracellular cytokine staining for IL-2, IL-4, IFN γ , and TNF α did not reveal any significant differences between T5 KD and Luc KD CD4⁺ T cells despite stimulus titration. Similarly, neither CypA nor T5 KD cells showed any difference in steady-state expression of the cell-surface markers CD3, TCR $\alpha\beta$, CD4, CXCR4, CCR5, CD25, CD69, MHCI, and PD1 compared to Luc KD CD4⁺ T cells at steady state.

DISCUSSION

[00276] The more successful gene therapy approaches to date have followed two main strategies: either modification of host-cell factors required for viral replication or inhibition of essential viral elements (Strayer et al., 2005). Here, a third approach was adopted: the exploitation of natural inhibitors that evolved over millions of years in primates in response to retroviral attack (Li, 2006;Sawyer, 2005;Stremlau, 2005;Yap, 2005). The potent block to HIV-1 observed with AoT5Cyp inspired the design of a human equivalent, hT5Cyp, that robustly blocks HIV-1 in single cycle assay *in vitro*. This Example assesses the anti-HIV function of hT5Cyp under more physiological conditions, including spreading infection of HIV-1 in primary human cells. An optimal gene therapy candidate should provide a broad-range high efficacy combined with low antigenic potential. It should further preclude the emergence of viral resistance while not perturbing host cell function.

[00277] hT5Cyp is a potent HIV-1 inhibitor and acts early in the viral life cycle (**Fig. 44**). Since it targets reverse transcription it precludes the accumulation of genetic diversity during this stage of the viral life cycle. Therefore, viral escape mutants to T5Cyp are not expected to arise easily. Indeed, when stably expressed in Jurkat T cells, T5Cyp blocked spread of HIV-1 for the nearly two-month duration of the experiment, while the modified

hT5 α _{R332P} gene therapy candidate only delayed spread of HIV-1 (**Fig 51A, B**). Furthermore, a comparison of various T5s stably expressed in Jurkat T cells, showed that even while RhT5 α reduced the level of HIV-1 infection, it was not sufficient to block spread. In this assay, hT5 α _{R332P} and hT5 α _{R323-332} had little effect on magnitude of HIV-1 infection and kinetics of spread, while hT5Cyp completely blocked HIV-1 spread (**Fig. 51C**). This points to hT5Cyp as the most efficient HIV-1 inhibitor in this new class of candidates.

[00278] Viruses resistant to T5Cyp-mediated restriction could not be obtained in more than 10 separate long-term spreading infections initiated with a broad range of viral inocula. To allow for improved accumulation of genetic diversity and increase the probability of obtaining T5Cyp resistant HIV-1 in culture, untransduced Jurkat cells were co-cultured in spreading infection with T5Cyp-expressing Jurkat cells. Untransduced cells were efficiently infected, however, no hT5Cyp-resistant virus could be obtained upon their removal from co-culture using puromycin. The generation and characterization of a hT5Cyp resistant virus would have allowed its pathogenic potential to be gauged. Example 1, for instance, shows that HIV-1 bearing the G89V mutation in CA is resistant to T5Cyp-mediated restriction. This virus, however, replicates poorly in tissue culture (Sayah and Luban, 2004) as do other CA-mutants with reduced CypA binding affinity (Yin et al., 1998). Should a T5Cyp resistant virus arise *in vivo* it is likely to bear mutations in the CypA binding loop of CA. Given attenuated infectivity of such CA variants, a hT5Cyp resistant virus may act to boost an immune response to HIV-1 rather than cause HIV-1 related pathogenicity.

[00279] A naturally-occurring CA mutant, resistant to CsA treatment, was nonetheless susceptible to T5Cyp-mediated restriction (**Fig. 49D**)(Chatterji et al., 2005). This CA-variant is reportedly partially sensitive to AoT5Cyp-mediated restriction in OMK cells and bears 4 amino acid substitutions surrounding the CypA binding loop (Chatterji et al., 2005). Initiation of spreading infection using this CA-variant HIV-1 may allow for *in vitro* generation of hT5Cyp resistant HIV-1. Similarly, initiation of spreading infection in the presence of low levels of a pharmacologic inhibitor of the CA-CypA interaction may promote such emergence of T5Cyp-resistant viruses.

[00280] During co-culture of hT5Cyp-expressing cells with untransduced cells, it was noted that T5Cyp-bearing cells can be infected by cell-associated HIV-1, despite their resistance to cell-free virus. Especially at early time-points after initiation of co-culture, there was no difference in infectability of T5Cyp-expressing cells and controls (**Fig. 51D**). Since

T5-mediated restriction is saturable, and cell-to-cell transmission of HIV-1 is 2-3 orders of magnitude more efficient than infection with cell-free virions (Dimitrov et al., 1993), this is likely a saturation of T5-mediated restriction. Similar results were obtained by a group assessing the potential for RhT5 α and modified hT5 α as candidate HIV-1 gene therapy in primary human CD4⁺ T cells (Richardson et al., 2008). Given the resistance of cell-associated HIV-1 to T5 α -mediated restriction, modified hT5 $\alpha_{R323-332}$ was considered unlikely to have therapeutic benefit *in vivo* (Richardson et al., 2008). T5Cyp, however, is a significantly stronger HIV-1 inhibitor than hT5 $\alpha_{R323-332}$ and even RhT5 α (**Fig. 51C**). When the duration of spreading infections initiated with cell-associated HIV-1 was extended, a control of HIV-1 spread over time by hT5Cyp was observed (**Fig. 52,A-E**).

[00281] The mechanism underlying the gradual control of HIV-1 cell-to-cell transmission by hT5Cyp is unclear. A system was designed to clarify the effects of T5Cyp on cell-to-cell spread in a single cycle of infection (**Fig. 53**). During short time of co-culture both donor and target cell T5Cyp had an effect on HIV-1 infection of targets. Longer coculture times eliminated the effect of donor cell T5Cyp, while target cell T5Cyp still reduced level of HIV-1 infection. Both RT activity in donor cell supernatant as well and gag-production assayed by pulse-chase are similar in T5Cyp-expressing and control cells. Consequently, T5Cyp has no effect on viral production in donor cells (**Fig. 53D, E**). One possibility is that some T5Cyp is transferred from donor cell to the target cell either through filopodial bridges formed during cell to cell transfer (Sherer et al., 2007) or through encapsidation of T5Cyp into nascent virions (Sakuma et al., 2007). These possibilities could be distinguished by a thorough biochemical analysis as well as imaging the fate of T5Cyp during cell to cell transfer. Following prolonged cell-to-cell contact, the concentration of transferred virus may be high enough to saturate an effect of transferred T5Cyp. During each viral life cycle the effects of donor and target cell T5Cyp can accumulate, ultimately leading to T5Cyp-mediated control of spreading infection despite initial productive infection of T5Cyp-expressing cells.

[00282] We wished to assess hT5Cyp-mediated inhibition of HIV-1 in primary CD4⁺ T cells and macrophages. Ideally, it would be possible to co-express hT5Cyp and a reporter in primary human cells to eventually enable *in vivo* tracking of transduced cells. Since pre-existing gene-delivery vectors available failed to express GFP at satisfactory levels, a new dual-promoter lentiviral vector, scAPLS, was designed which routinely yielded 20-80% GFP⁺

CD4⁺ T cells post-transduction. While hT5Cyp in CD4⁺ T cells was undetectable by western blot in CD4⁺ T cells transduced with scAPLS-hT5Cyp, robust mRNA levels of both GFP and hT5Cyp could be detected in these cells indicating that both promoters were driving transgene transcription. scALPS compares favorably to previously published dual-promoter lentiviral gene-delivery systems that fail to lead to efficient reporter gene expression in primary T cells (Amendola et al., 2005). To assess whether the promoters chosen based on optimization experiments in CD34⁺ HSCs and CD4⁺ T led to similar levels of protein expression, Jurkat T cells were transduced with scAPLS coding for GFP and dsRed. The majority of transduced T cells expressed both GFP and dsRed (**Fig. 55**). Indeed the engineering of scAPLS was an advance that enabled direct assessment of T5Cyp-mediated inhibition of HIV-1 spreading infection in primary human cells. Another successful method to express T5 together with a GFP-reporter in primary human T cells is the use of multicistronic lentiviral vectors using the self-cleaving picorna-virus 2A peptides and 2A-like peptides to successfully translate up to four cistrons (Richardson et al., 2008; Szymczak et al., 2004).

[00283] Expression of hT5Cyp in hCD4⁺ T cells and monocyte-derived macrophages inhibited spread of CCR5- and CXCR4-tropic HIV-1 strains as well as primary isolates (**Fig. 56**). When hT5Cyp-scAPLS transduced cells were infected in a competition assay containing untransduced T cells, there was selection for GFP⁺ T cells previously transduced with hT5Cyp-scAPLS compared to a selection against GFP⁺ T cells previously transduced with the permissive scAPLS-hT5CypH126Q (**Fig. 57**). Furthermore, mixed cultures containing hT5Cyp-expressing CD4⁺ T cells showed a reduction in viral burden. This led to an overall protection of viability in these cultures (**Fig. 57**). As with spreading infection in T cell lines, infection of primary human cells failed to generate viral escape mutants resistant to T5Cyp-mediated restriction *in vitro*. T5Cyp could be considered as a candidate monotherapy in the autologous T cell or hematopoietic progenitor transplant context. Few other gene therapy approaches are as potent as T5Cyp, with the possible exception of CCR5 disruption. Notwithstanding, advances in gene delivery combined with intriguing new gene therapy approaches could yield highly effective future combination therapies. For instance, one could envision *in vivo* delivery of bicistronic P2A-based lentiviral vector (Szymczak et al., 2004) coding for hT5Cyp to block *de novo* HIV-1 infection and *tre* to “cure” infected cells (Sarkar et al., 2007), flanked by homologous sequences allowing for concomitant gene replacement

of CCR5 (Lombardo et al., 2007) targeted specifically to T cells (Kumar et al., 2008) or CD34⁺ HSCs (Yang et al., 2006).

[00284] So far, treatment with as much as 3×10^{10} transduced, *ex vivo* expanded autologous T cells has been proven safe in phase II clinical trials (Mitsuyasu et al., 2000; Walker et al., 2000). This number of scAPLS-transduced CD4⁺ T cells can be readily expanded *ex vivo*. Furthermore, autotransfusion of *ex vivo* expanded CD4⁺ T cells is of clinical benefit to HIV-1-infected people partly because these cells are CCR5^{lo} (Levine et al., 2002). This is consistent with the observation that in non-pathogenic SIV-infection of natural hosts T cells are predominantly CCR5^{lo}, especially in the gut, (Pandrea et al., 2007). Thus, given that hT5Cyp-transduced CD4⁺ cells can be expanded *in vitro* acquiring a CCR5^{lo} phenotype and exhibit a selective advantage during HIV-1 infection (**Fig.57**), an impressive therapeutic effect could potentially be obtained using hT5Cyp in autologous CD4⁺ T cell gene therapy.

[00285] It is essential that any gene therapy candidate is not toxic to cells and does not perturb host cell function. One potential mode of toxicity would be the elimination of transduced cells *in vivo* following expression of a foreign protein (Riddell et al., 1996). hT5 was fused to hCypA in order to minimize non-human sequences in the fusion protein (**Fig 1-3**). Since hT5Cyp is an extremely efficient HIV-1 inhibitor and acts at an early point in the viral life cycle, integration of viral cDNA into the host genome is diminished. With it, the production of viral proteins is reduced and could lead to improved survival of infected cells and maintenance of a more intact memory repertoire. Aside from antigenicity, hT5Cyp expression should not perturb host cell function. Transduction of scAPLS vectors coding for hT5Cyp did not affect proliferation, expression of cell surface markers, or cytokine production of transduced CD4⁺ T cells compared to untransduced cells (**Fig. 58**). At the same time, T5 isoforms form heteromultimers and hT5Cyp may thus affect endogenous T5 α function. Indeed, expression of hT5Cyp in Jurkat T cells moderately attenuated the N-MLV restriction activity of endogenous hT5 α (**Fig.46C**).

[00286] For the study of CypA in CD4⁺ T cells the CypA^{-/-} mouse was used (Colgan et al., 2000). One finding in memory Th2 cells was that CypA regulates IL-2 production post-transcriptionally. Thus CypA^{-/-} mTh2 cells overproduced IL-2 by ELISA without a proportional increase in IL-2 mRNA (**Fig. 59**). Moreover, CypA^{-/-} mice were confirmed to have elevated serum levels of IL-2 compared to wildtype littermates (**Fig. 59**). Whether

control of IL-2 production by the peptidyl prolyl isomerase CypA occurs at the level of translation or secretion remains to be elucidated. Since CypA controls the activity of the *tec* kinase *itk*, which is involved in activation-induced rearrangement of the actin cytoskeleton, CypA could control localization and amount of IL-2 secretion (Labno et al., 2003). Pulse-chase experiments as well as cytokine capture assays can provide insights into a potential control of IL-2 secretion by CypA. Alternatively, CypA could regulate translation of IL-2 mRNA. Polyribosome fractionation and assessment of IL-2 association with polyribosomes in the presence and absence of CypA should provide insights into a potential effect of CypA on polysome loading of IL-2 mRNA.

[00287] Another peptidyl-prolyl isomerase, Pin 1 is involved in post-transcriptional regulation of cytokine production by mRNA stabilization (Shen et al., 2005). GM-CSF, like many postranscriptionally regulated cytokine and proto-oncogene mRNAs is regulated through AU-rich elements (AREs) in the 3'UTR. AREs are responsible for both mRNA degradation through association with tristetraproline (TTP) as well as translational silencing through recruitment T-cell internal antigen (TIA) (Anderson et al., 2004). Pin-1 associates with an mRNA-regulating protein at the ARE and recruits GM-CSF mRNA, modulating mRNA levels dependent on signaling events (Shen et al., 2005). IL-2 also contains an ARE in its 3'-UTR and it is possible that CypA orchestrates ARE-dependent modulation of either IL-2 mRNA stability (which should have appeared in quantitative RT-PCR, **Fig. 59**) or translation.

[00288] To confirm whether CypA had similar effects in human CD4⁺ T cells, a lentiviral vector, APM, for the efficient delivery and processing of a shRNA targeting CypA, was designed. APM contains a pol II promoter driving expression puromycin-N-acetyltransferase followed by the target-specific shRNA embedded within a miR-30 sequence (Stegmeier et al., 2005). APM was used to knockdown expression of CypA and of T5 (see below) in side-by-side experiments. APM transduces primary CD4⁺ T cells efficiently and allows for puromycin selection of transduced cells resulting in effective knockdown of target genes by quantitative RT-PCR and Western blot (**Fig. 60, A-C**). To confirm functional CypA KD in human CD4⁺ T cells, HIV-1, SIV, and HIV-2 infection of these cells was assessed in the presence or absence of CypA KD. In human CD4⁺ T cells, analogous to previous observations in Jurkat T cell lines, disruption of CypA expression leads to a reduction in

HIV-1 infection without any effects on HIV-2 or SIV infection. Disruption of T5, in contrast had no effect on infection with HIV-1, HIV-2, or SIV (**Fig. 60D**).

[00289] The most impressive phenotype of CypA KD CD4⁺ T cells is enhanced proliferation compared to Luc KD control. Restimulated CypA KD Th2 memory cells (Messi et al., 2003), incorporate thymidine almost 10-fold more efficiently and lead to more rapid CFSE dilution in steady state than Luc KD controls (**Fig. 61**). The proliferation phenotype in CypA KD cells is more prominent in human cells than in mouse Th2 cells (Colgan et al., 2004). Enhanced proliferation is further confirmed by rapidly cycling CypA KD CD4⁺ T cells concentrated in the G2/M/S phase of the cell cycle, while control cells had an expected distribution for moderately fast cycling cells (**Fig. 61**). Whether the unchecked proliferation observed in CypA KD cells is due to loss of itk control (Colgan et al., 2004) could be assessed by expression of itk mutants unable to bind CypA (itk_{P287G}) or lacking kinase activity (itk_{K390R}) in CypA KD and Luc KD CD4⁺ T cells. Itk_{P287G}-expressing Luc KD CD4⁺ T cells should then have the same phenotype as CypA KD⁺ T cells. Following PMA/Ionomycin stimulation, CypA KD CD4⁺ T cells produce more than 2-fold higher levels of IL-2 and IL-4 by intracellular cytokine stain (**Fig. 61E**). Similarly, memory Th2 cells produce more than 10-fold higher levels of IL-2 by ELISA in the absence of stimulation (**Fig. 61D**). Whether CypA has effects on transcription or is regulating post-transcriptional control of cytokine production in human CD4⁺ T cells remains to be elucidated.

[00290] Since mice do not have T5, its function has to be directly assessed in human CD4⁺ T cells. T5α KD CD4⁺ T cells exhibited cell cycle arrest much earlier than their Luc KD counterparts. This is reflected in 5-fold lower steady-state proliferation by thymidine incorporation and failure of CFSE dilution. Upon cell cycle analysis, the majority of cells were either in G₀ or dead; technically complicating the characterization of T5α KD CD4⁺ T cells. A more thorough analysis has to be undertaken to distinguish cell cycle arrest and subsequent death by neglect from increased apoptosis or AICD in the absence of T5α. Upon restimulation of T5α KD CD4⁺ T cells, growth kinetics are at first similar to Luc KD T cells without evidence for an increased number of PI⁺ cells. One week later, however, the proliferation defect becomes apparent. There were no striking differences in cytokine production or activation marker expression between T5α KD and Luc KD CD4⁺ T cells.

[00291] The closest murine relative of T5 α is TRIM30 α (T30 α). By targeting TAB2,3 for degradation, T30 α disassembles the TAB2/TAB3/TAK1 complex required for TLR4 signaling to NF κ B (Shi et al., 2008). Whether T5 α KD has an analogous effect on TLR4 signaling in CD4⁺ T cells and what the exact protein expression profiles and functions of TLRs are in these cells, remains to be elucidated. T cell receptor triggering itself, however, activates NF κ B via the TAK1 complex (Wan et al., 2006). Should T5 α , akin to T30 α , disrupt this signaling downstream of the T cell receptor, a defect in T cell proliferation in the absence of T5 α is not surprising. Furthermore, the effect would be potentiated by the fact that TAK1 activates signaling to both MAPKs and NF κ B. TAK1 is essential for T cell development as well as for the survival and cytokine-driven proliferation of effector T cells (Wan et al., 2006). Intriguingly, peripheral T cells from TAK1^{-/-} mice have a similar reduced proliferation and survival phenotype as hT5 α KD CD4⁺ T cells. The data presented here indicate that T5 α coordinates essential T cell functions. Since hT5Cyp may have the potential to interfere with endogenous T5 α , a thorough understanding of its function is required.

EXAMPLE 3: ASSESSMENT OF hT5Cyp EFFECTS ON HIV-1 INFECTION IN VIVO

Monkey models for lentiviral infection

[00292] A major hurdle to the study of HIV-1 pathogenicity and therapy is the paucity of adequate animal models. HIV-1 infection is almost exclusively restricted to humans, where it is the primary cause of AIDS (Barre-Sinoussi et al., 1983). HIV-1 can also replicate in chimpanzees, the apes from which HIV-1 originated by zoonotic transmission of SIVcpz to humans (Gao et al., 1999), as well as gibbon apes (Ambrose et al., 2007). In chimpanzees, HIV-1 establishes a long-term chronic infection accompanied by seroconversion and establishment of cellular and humoral immune responses, but rarely causes an AIDS-like syndrome (Ambrose et al., 2007; Fultz et al., 1989; Novembre et al., 1997). Furthermore, the use of chimpanzees for experimental purposes has become increasingly undesirable first and foremost for ethical reasons (Goodall, 1995). Secondarily, from a practical standpoint, maintenance of chimpanzees is prohibitively expensive and data output will be limited by long experimental duration and small cohort sizes (Nath et al., 2000).

[00293] In most new world monkeys, the CD4 receptor and CCR5 co-receptor do not support HIV-1 entry and thus replication (Kunstman et al., 2003; LaBonte et al., 2002). Even in the case of the old world monkeys, however, whose CD4 and CCR5 surface receptors

allow for HIV-1 entry (Kunstman et al., 2003), viral replication is blocked by restriction factors such as TRIM5 (T5) and APOBEC3G (A3G). At the same time, most African monkey species harbor the HIV-related SIVs. While SIV-infection leads to high viral replication coupled with acute depletion of mucosal T cells, it seems to be a well-tolerated and non-progressive infection in natural hosts, in contrast to HIV-1 in humans (Pandrea et al., 2008). The major difference between non-pathogenic infection and pathogenic infection seems to be the degree of immune activation during the chronic phase of infection (Pandrea et al., 2008). Systemically, the chronic immune activation observed during HIV-1 infection results in part from destruction of the mucosal barrier allowing for translocation of microbial products to the peritoneum (Brenchley et al., 2006). On a cellular level, HIV-1 *Nef*, in contrast to SIV *Nef* fails to suppress T-cell activation, again leading to high-level immune activation associated with morbidity in infected individuals (Schindler et al., 2006).

[00294] While SIV-infection in its natural host is non-pathogenic, infection of Asian monkeys with SIVs derived from African hosts, leads to a rapid and fatal immune suppression with symptoms highly reminiscent of AIDS in humans (Letvin et al., 1985). To date, infection of rhesus macaques with SIV_{mac} or modified SIV_{mac} has thus become the most widespread animal model to approximate HIV-1 infection in the human host as well as for assessment of candidate antivirals (Ambrose et al., 2007). In this model, immunity against SIV-infection was obtained using vaccination with an attenuated *Nef*-deleted SIV prior to challenge with high-dose, pathogenic SIV_{mac}251 (Daniel et al., 1992). Notwithstanding failures in the vaccine field, this study is an essential reminder that protective immunity against a lentivirus is possible in primates and may encourage a new approach to vaccine development. Furthermore, in some long-term nonprogressors with low viral loads and stable CD4⁺ T cell counts, HIV strains with mutations and deletions in the *Nef* gene were detected (Kirchhoff et al., 1995).

[00295] Despite similarities in genomic organization and pathogenic potential between the closely-related HIV-1 and SIV_{mac}, they are distinct viruses with substantial differences in genomic sequence, accessory genes, and progression of infection. A similar distinction has to be made between immune responses in the closely-related *Macacca* genus and *Homo sapiens*. The sequence dissimilarities between HIV-1 and SIV partly limit the usefulness of the SIV animal model for study of antivirals. Neutralizing antibodies produced in humans and monkeys will differ as the *env* sequences governing these responses differ (Ambrose et al.,

2007). Sequence dissimilarities provide the same consideration for cytotoxic T cell responses. HIV-1 contains the accessory gene *vpu* which, absent in SIV, counteracts tetherin-mediated restriction of HIV-1 (Wolf and Goff, 2008). SIV, on the other hand contains the accessory protein *vpx*, which, while absent in HIV-1, increases HIV-1 infection in human DCs and macrophages in trans (Goujon et al., 2007). SIV_{mac} and HIV-1 also exhibit differences in the course of infection. The decline in immune function of SIV-infected macaques takes 1-3 years on average; a process requiring an average 10 years in HIV-1 infected individuals (Brown et al., 2007). Infected macaques can fail to mount immune responses to SIV_{mac}, leading to a severe AIDS-like disease and death within 6 months post-infection (Brown et al., 2007). This represents a reduction in the duration of the chronic phase of infection; a phase which deserves particular scrutiny since it is when most differences between a pathogenic and non-pathogenic SIV infection are apparent (Pandrea et al., 2008).

[00296] To minimize shortcomings based on genomic differences of HIV and SIV in the macaque animal model, SIV-HIV chimeric viruses were generated. The first SHIV was a SIV_{mac} containing *tat*, *rev*, *vpu*, and *env* from HIV-1 (Shibata et al., 1991). After SHIV was shown to be infectious in rhesus macaques ever more sophisticated CXCR4- and CCR5-tropic versions have emerged, culminating in simian-tropic HIV-1 (Ambrose et al., 2007; Hatzioannou et al., 2006). stHIV-1 incorporates the recent understanding that dominant intracellular restriction factors determine lentiviral tropism. RhT5 α targets HIV-1 but not SIV CA. Similarly, SIV but not HIV-1 *vif* blocks lentiviral restriction by A3G in simian cells. By substituting HIV-1 CA and *vif* with their SIV counterparts, a chimeric virus (stHIV-1) was generated that has the highest sequence homology with HIV-1 to date. After tissue-culture adaptation, stHIV-1 is able to replicate in rhesus macaque cells at similar levels as SIV. Once stHIV-1 infection is successfully established in monkeys, stHIV-1 could provide a significant improvement of the SIV animal model (Hatzioannou et al., 2006). Despite the unquestionable value of the SIV/SHIV monkey model coupled with recent advances like stHIV-1, CA-sequences differ between the two lentiviruses, and CA-variation governs the ability to infect non-dividing cells (Yamashita et al., 2007), with potential effects on immune responses to stHIV-1 in monkeys. An ideal model would allow for direct testing of HIV-1. The discovery of a macaque T5Cyp that does not block HIV-1 infection and is encoded for by the Mamu7 allele in rhesus macaques raises the possibility that Mamu7 homozygous animals may be infectible with HIV-1 (Wilson et al., 2008).

Mouse models for HIV-1 infection

[00297] A different approach has been to “humanize” small animal models, ideally providing high-throughput systems for the study of HIV-1 pathogenesis, immunity, and antivirals *in vivo*. Rodents do not allow for HIV-1 replication partly because mouse homologs of required cellular co-factors do not support HIV-1 replication. This provides blocks at multiple steps of the viral life cycle (Goldstein, 2008). A direct approach to overcome this problem in the context of a pre-existing functional immune system is the creation of rodents transgenic for the required human homologs. Thus, the introduction of the human CD4 receptor and CCR5 co-receptor for HIV-1 into rats (Keppler et al., 2002) and mice (Zhang et al., 2008b) allowed for efficient viral entry and reverse transcription. In rats, this approach led to low-level plasma viremia as well as evidence of viral cDNA in thymus and spleen up to 6 months post infection (Keppler et al., 2002). This model was further used in proof-of-principle experiments showing that antivirals known to affect entry (enfuvirtide) or RT (efavirenz) inhibited HIV-1 infection as measured by quantification of viral cDNA and 2-LTR circles in rat splenocytes 4 days post-infection, while a semen-derived amyloid fibril (SEVI) shown to enhance HIV-1 infection in culture, had the same effect *in vivo* (Goffinet et al., 2007; Goldstein, 2008; Munch et al., 2007).

[00298] Generation of hCD4/hCCR5 transgenic mice highlights a further block to HIV-1 replication: murine Cyclin T1 (CycT1) does not support efficient tat-mediated transactivation of HIV-1 transcription (Zhang et al., 2008b). To overcome this block, hCycT1/hCD4/hCCR5 mice were bred. In hCD4⁺ T cells from transgenic hCycT1/hCD4/hCCR5 mice, HIV-1 undergoes reverse transcription and nuclear import efficiently, but proviral integration is deficient. Intriguingly, secondary TCR-activation of primary murine T cells in culture during the first 12-24 hours post-infection relieved the block to proviral integration; an effect potentiated by treatment with cyclosporine A (Zhang et al., 2008b). Gag processing and virion budding remains defective in hCycT/hCD4/hCCR5 transgenic T cells, leading to significant reduction in the release of infectious progeny compared to infected human cells (Zhang et al., 1996). This model provides valuable insights both into the requirements for HIV-1 replication and reveals distinct steps at which species-specific restriction factors may act.

[00299] An alternative to mouse modification is modification of viral tropism. One group has attempted to adapt HIV-1 to mice by replacing HIV-1 *env* with the envelope

glycoprotein from ecotropic murine leukemia virus resulting in the chimeric virus EcoHIV-1 (Potash et al., 2005). Viral cDNA can be found in the spleen and brain of infected mice which can even mount an antibody response against tat and gag (Potash et al., 2005). In the absence of documented plasma viremia and CD4⁺ T cell destruction, however, it is unclear whether infection of mice with EcoHIV-1 is self-limiting or productive since this model does not address species-specific post-entry blocks to HIV-1 replication.

[00300] To circumvent the HIV-1 entry defect into murine cells, mice transgenic for infectious provirus under the control of a CD4 promoter/enhancer cassette were generated. Depending on level of transgene expression, these mice developed a rapidly progressing AIDS-like syndrome leading to very early death and thus limiting the utility of this model to study effects of chronic HIV-1 infection (Hanna et al., 1998a). Another approach was the generation of HIV-1 provirus (under the control of the HIV-1 LTR) and hCycT1 (under the control of a CD4 promoter/enhancer cassette) double transgenic mice (Sun et al., 2006). This method limits tat-expression to murine CD4⁺ T cells, dendritic cells, and myeloid cells (monocytes, macrophages, microglia); increasing HIV-1 transcription and production in these targets. There was evidence for production of infectious HIV-1 by CD4⁺ cells in all organs examined and a gradual depletion of peripheral CD4⁺ T cells over the course of one year (Goldstein, 2008; Sun et al., 2006). HIV-1 production in myeloid cells was more than eight-fold higher than in CD4⁺ T cells, indicating a T-cell specific block to viral replication. The HIV-1 provirus/hCyc T transgenic model for pathogenesis may be further improved once the T-cell specific block to HIV-1 replication is identified and alleviated (Sun et al., 2006).

[00301] Rather than studying the *in vivo* effects of an entire integrated HIV-1 provirus, some have focused on the pathogenic effects of single viral genes in transgenic mice. The discovery that *nef* is a major determinant for HIV-1 pathogenicity was made during the generation of mice transgenic for mutant HIV-1 proviruses. Severe AIDS-like disease affecting multiple organs and leading to early death occurred only in mice transgenic for HIV-1 proviruses with intact *nef* (Hanna et al., 1998b). Furthermore, mice transgenic for *nef* under the control of the CD3 ϵ promoter show aberrant T cell activation, impaired CD4⁺ T cell development in the thymus, and both central and peripheral CD4⁺ T cell depletion (Skowronski et al., 1993). A similar approach showed that gp120 in isolation has the capacity to cause CNS damage in transgenic mice (Toggas et al., 1994). When placed under the glial fibrillary acidic protein (GFAP) promoter, gp120 expression was limited to

astrocytes and targeted to the CNS of transgenic mice. Dependent on expression level, it caused reactive astrocytosis, dendrite damage, synapse destruction, and loss of neuronal subpopulations reminiscent of the neurological complications observed in HIV-1 infected patients (Toggas et al., 1994). These studies demonstrate how such a “reductionist” approach can lead to valuable insights regarding the pathogenic potential of single HIV-1 elements *in vivo*.

Humanized mouse models for HIV-1 infection

[00302] The ideal remains to study the interaction of HIV-1 with the human immune system *in vivo*. To this end, immuno-compromised mice have been reconstituted with human immune cells that support HIV-1 infection *in vivo*. The first such model took advantage of the CB17-*Prkdc*^{scid} (SCID) mouse. SCID mice contain a spontaneous autosomal mutation in the DNA-dependent protein kinase (DNA-PK) required for successful completion of VDJ recombination during the rearrangement of lymphocyte antigen receptors (Blunt et al., 1996; Bosma et al., 1983). This defect results in the absence of mature, functional B and T cells in SCID mice, allowing for their reconstitution with human PBMCs (huPBL-SCID mice) (Mosier et al., 1988); human fetal hematopoietic tissues (SCID-hu thy/liv mice) (McCune et al., 1988); and human bone marrow cells (Lapidot et al., 1992). It was not long before these models were tested for support of productive HIV-1 infection (Mosier et al., 1991; Namikawa et al., 1988). The huPBL-SCID model allows for evidence of infection using both cell-free HIV-1 as well as infected T cell blasts with apparent depletion of CD4⁺ T cells *in vivo*. Infectious virus could be recovered through culture from the spleen, peripheral blood, and lymph nodes of infected mice (Mosier et al., 1991). In the huPBL-SCID model, however, human T cells are CCR5⁺ memory cells aberrantly activated in the xenogeneic background and experimental duration is limited by the appearance of xGVHD symptoms within two-months post-transplant (Koyanagi et al., 2008). Recently, the SCIDhu Thy/Liv model has been validated as a high-fidelity model for preclinical drug-testing since HIV-1 infected SCIDhu Thy/Liv mice have comparable responses to four classes of licensed antiretrovirals at equivalent dosages used in humans (Stoddart et al., 2007). Notwithstanding such applications of humanized SCID mice, utility of these models is limited by low levels of human cell engraftment, especially in the T cell compartment in the huPBL-SCID mouse, a failure to mount primary immune responses, and limited distribution of human cells in the SCIDhu thy/liv mouse (Manz, 2007; Wege et al., 2008).

[00303] In the recipient mouse, the degree of both adaptive and innate immune deficiency correlates with the success of immune reconstitution using human cells. The SCID mouse is suboptimal in both categories. The *scid*-mutation is “leaky” in up to 20% of young mice, allowing for spontaneous development of mature B and T cells (Bosma et al., 1988). Additionally, mice have high numbers of host-derived NK cells combined with enhanced innate immune activation (Shultz et al., 2007). The *scid*-mutation also causes a defect in DNA repair leading to radiosensitive mice with short life spans (Fulop and Phillips, 1990). The generation of NOD/SCID mice significantly improved engraftment of human cells due to the synergistic effects of the *scid*-mutation on lymphoid development combined with NOD-specific defects in innate immune function, including defects in macrophage and NK cell activity (Shultz et al., 1995). Adoptive transfer of human PBMCs into NOD/SCID mice leads to 5-10 fold higher reconstitution than seen in SCID mice although reconstitution of the T cell compartment remained deficient in this model (Hesselton et al., 1995). HIV-1 replication in hPBL-NOD/SCID mice was observed in the spleens of > 80% infected mice at much higher levels of plasma viremia (> 1 ng p25/ml plasma) than in infected huPBL-SCID mice (Koyanagi et al., 2008). Unfortunately, expression of an endogenous retrovirus (Emv30) located on chromosome 11 leads to spontaneous development of thymomas in NOD/SCID mice and limits their life span (Shultz et al., 1995).

[00304] Despite this shortcoming, one NOD/SCID model deserves special mention as it supports high-level human cell engraftment and reconstitution of a functional human immune system. The bone marrow/liver/thymus (BLT) mouse is a NOD/SCID-based model in which tissue from fetal thymus and liver is transplanted beneath the kidney capsule of adult mice. Three weeks later mice are conditioned with sublethal irradiation and transplanted with autologous fetal CD34⁺ hematopoietic stem and progenitor cells (HSCs) (Merkus et al., 2006). High-level engraftment (20-40%) of functional human cells is observed in peripheral blood and reconstitution encompasses human B and T cells with appropriate subsets as well as monocytes and DCs, 4-8 weeks after CD34⁺ HSC transfer. Human T cells in the mouse have a broad V β repertoire and can mount MHC-restricted, antigen-specific immune responses to EBV infection (Merkus et al., 2006). Human DCs were also functional in BLT mice since inoculation of mice with the TSST-1 superantigen led to the selective expansion of V β 2⁺ T cells and elaboration of inflammatory cytokines as well as transient upregulation of activation/maturation markers in splenic DCs (Merkus et al., 2006).

[00305] The mucosa of BLT mice is successfully reconstituted with multi-subset human immune cells. Systemic infection could be established through intrarectal HIV-1 inoculation, and led to a depletion of CD4⁺ T cells in the mesenteric lymph node as well as depletion of T cells located in the lamia propria of the gut; analogous to the course of infection in humans. Moreover, three of four infected mice mounted antibody responses against gp120 and p24 (Denton et al., 2008). These studies were extended to show that systemic infection could be initiated through intravaginal HIV-1 inoculation, with resulting T cell depletion in the gut-associated lymphoid tissues. Furthermore, topical antiviral prophylaxis protected 5/5 mice from vaginal HIV-1 transmission in a proof-of-principle experiment showing the utility of the BLT model for pre-clinical evaluation of microbicides and antiretrovirals in HIV-1 prophylaxis (Sun et al., 2007). Despite these outstanding results, the surgical intervention required for generation of the BLT-model is not trivial. So far, this model is not a high-throughput solution for multi-center, preclinical drug testing.

[00306] The generation of recombination-activating gene (RAG1 and 2) knockout mice eliminated the problems of leakiness and radiosensitivity observed with SCID-mice, while retaining the drawbacks of host NK cell activity and innate immune activation (Mombaerts et al., 1992; Shinkai et al., 1992). While gradual improvements in humanized mouse models continued over the next decade, T cell development remained elusive. A major breakthrough came with inhibition of NK cell activity by disruption of IL-2 signaling. Thus, using anti-mIL-2R β antibodies in pretransplantation conditioning regimens increased *de novo* thymopoiesis (Kerre et al., 2002). A further improvement was the genetic disruption of NK cell activity through deletion or truncation of the mIL-2R γ chain (Manz, 2007). The mIL-2R γ chain is required for high affinity binding of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 to their receptor complexes and for subsequent signaling events. Mutations in the human IL-2R γ chain are responsible for X-linked SCID (Sugamura et al., 1996). When the NOD/SCID and Rag2^{-/-} were crossed to IL-2R γ ^{-/-} mice, yielding NOD/SCID/Il2rg^{-/-} and NOG mice (Ishikawa et al., 2005; Ito et al., 2002; Shultz et al., 2005) as well as Rag2^{-/-} γ_c ^{-/-} mice (Gimeno et al., 2004; Traggiai et al., 2004), respectively, a major breakthrough in the field of humanized mice was achieved.

[00307] Also, a hPBL-NOG mouse model was validated for pre-clinical drug testing in a proof-of-principle experiment where a CCR5 antagonist blocked protected CD4⁺ from depletion following HIV-1 infection and > 200-fold reduced viral load (Nakata et al., 2005).

Adoptive transfer of hPBMCs into the NOG mouse represents an improvement over the huPBL-SCID model. Human cell engraftment even in peripheral blood (<40% CD45⁺) is consistently higher and allows for a higher level of viremia (up to 10⁶ copies proviral DNA/10⁵ CD4⁺ T cells); which is both more similar to what is observed in patients during acute viremia and also allows for longitudinal monitoring of HIV-1 infection without the need for sacrificing mice (Nakata et al., 2005). As with the hPBL-SCID model, the experimental duration is limited due to the potential development of xGVHD.

[00308] The major benefit of these new mouse models is consistent and robust *de novo* T cell development following transplant of Rag2^{-/-}γc^{-/-} or NOD/SCID β 2rg^{-/-} and NOG mice with hHSCs. These strains lack B, T, and functional NK cells without any apparent tumor susceptibility or life-span reduction (Manz, 2007). Optimal results are achieved with transplantation of CD34⁺ HSCs into the liver of sublethally-irradiated newborn mice. Targeting human HSCs to the site of perinatal hematopoiesis in newborns leads to T cell development in mouse strains that do not support human T cell development well in adults (Wege et al., 2008). Both strains allow for differentiation and maintenance (> 6 months) of a human hemato-lymphoid system (HHLS) with robust reconstitution of the lymphoid compartment including multiple T cell subsets (CD4⁺ and CD8⁺ T cells with broad Vβ distribution, as well as Foxp3⁺CD25⁺ regulatory T cells), B cells (including CD5⁺ B cells), and NK cells; reconstitution of cDCs and IPC; and with some erythroid, and myeloid reconstitution (mono-myeloid cells, RBCs and platelets) (Ishikawa et al., 2005; Manz, 2007; Traggiai et al., 2004). Immune-reconstitution leads to formation and structural organization of the major primary and secondary lymphoid organs absent or hypotrophic in non-reconstituted mice (Manz, 2007). EBV-infected HHLS-Rag2^{-/-}γc^{-/-} mice showed evidence of EBV infection in B cells of spleen, lymph node, and bone marrow by PCR, and elaborated appropriate T cells responses with CD4:CD8 ratio inversion and control of LMP⁺ B cell proliferation *in vivo*. With high-dose EBV infection, however, human-hemato-lymphoid-system (HHLS)-Rag2^{-/-}γc^{-/-} mice failed to control proliferation of LMP⁺ B cells (Traggiai et al., 2004).

[00309] Upon HIV-1 infection with either CCR5- or CXCR4-tropic HIV-1, HHLS-Rag2^{-/-}γc^{-/-} mice peak viral loads (10⁵-10⁶ copies viral RNA/ml plasma) were observed 2-4 weeks post-infection. As in humans, viremia stabilized at lower levels and was detectable throughout the course of analysis (190 days) (Baenziger et al., 2006). Infectious virus could

be retrieved from mice by co-culture of cells from infected organs with human lymphocytes. CXCR4-tropic HIV-1 infected CD3⁺ T cells in thymus, spleen, lymph nodes, and peripheral blood while CCR5-tropic HIV-1 was excluded from the thymus as few thymocytes express CCR5. Consistent with this distribution, CD4 depletion was observed in infected organs and was particularly dramatic for CXCR4-tropic strains. Less than 10% of the mice were able to mount α -p24 antibody responses and HIV-1 specific T cell responses were absent as assayed by *in vitro* IFN γ production upon restimulation. This limits the utility of the HHLS-Rag2^{-/-} γ c^{-/-} model in HIV-1 immunity and vaccine study (Baenziger et al., 2006). Similar results were obtained for HIV-1 infection of NOG mice, with long-term viremia at levels comparable to infection in patients, gradual CD4:CD8 ratio reversal, and elaboration of α -p24 and α -gp120 antibodies in a minority of infected animals combined with a failure to demonstrate cellular immune responses to HIV-1 infection (Watanabe et al., 2007).

[00310] Most groups do not report significant restoration of GALT or consistent establishment of long-term, systemic HIV-1 infection via the rectal or vaginal routes in HHLS-Rag2^{-/-} γ c^{-/-} mice (Goldstein, 2008; Hofer et al., 2008), although better results may be obtained by reconstituting with CD34⁺ cells of fetal rather than cord blood origin (Berges et al., 2008). Rag2^{-/-} γ c^{-/-} mice breed well, with litter-sizes between 6-11 animals, and have long life-spans in specified pathogen-free environments. Generation of HHLS-Rag2^{-/-} γ c^{-/-} mice is straightforward with consistent engraftment of human cells (90% of mice have > 5-10% hCD45⁺ cells in PB) (Goldstein, 2008). The limitation on numbers of mice in a single study is the availability of hHSCs. Thus, the HHLS-Rag2^{-/-} γ c^{-/-} mouse is a valuable and straightforward model for the study of HIV-1 pathogenesis and antivirals.

Establishing the Rag2^{-/-} γ c^{-/-} mouse model for HIV-1 infection

[00311] HIV-1 infection spreads from cell to cell at high titer in complex lymphoid tissues with a specific hierarchy of T cell destruction and distinct kinetics *in vivo* (Douek et al., 2003). If considering hT5Cyp as potential HIV-1 therapy, *in vitro* assays remain incomplete surrogates to *in vivo* testing. Therefore, studies were designed to assess the ability of hT5Cyp to protect CD4⁺ T cells from HIV-1 infection in human hemato-lymphoid system (HHLS) mice. This mouse model takes advantage of the lack of functional B, T, and NK cells in Rag2^{-/-} γ c^{-/-} mice and allows for improved engraftment of xenografts as well *de novo* intrathymic generation of human T cells (Baenziger et al., 2008; Mazurier et al., 1999).

Briefly, CD34⁺ hematopoietic stem and progenitor cells are enriched from cord blood of healthy donors by MACS and transplanted intrahepatically into irradiated, newborn Rag2^{-/-}γc^{-/-} mice (**Fig. 63A**). 2-4 months post-transplant, reconstitution of peripheral blood is assessed by flow cytometry, and mice with more than 5% hCD45⁺ cells in peripheral blood are infected intraperitoneally (i.p.) with HIV-1 (**Fig. 63A**). In order to assess the anti-HIV-1 efficacy of hT5Cyp in this model, CD34⁺ cells are transduced with lentiviral vectors coding for hT5Cyp and ideally a reporter such as GFP prior to transplant into Rag2^{-/-}γc^{-/-} neonates.

[00312] An optimal transduction of CD34⁺ cells using a lentiviral vector in which the hCypA promoter drives GFP synthesis is shown in **Fig. 63B**. Transduction here was performed in media containing serum replacement in the absence of cytokines and GFP expression was monitored 48 hours later. 70% of CD34⁺ T cells were GFP⁺ and 40% of CD34⁺CD38⁻ cells believed to contain a higher proportion of hematopoietic stem cells are GFP⁺ *ex vivo* (**Fig. 63B**).

[00313] When untransduced CD34⁺ cells are used to reconstitute newborn Rag2^{-/-}γc^{-/-} mice and the resulting HHLS-mice are infected with HIV-1 eight weeks later, there is a striking depletion of CD4⁺ T cells four weeks post-infection (**Fig. 64A**). This is most visible in the mesenteric lymph node, with a 28-fold reduction in CD4⁺ T cells and a two-fold increase in CD8⁺ T cells in infected mice compared to mice infected with a *Nef*-deleted virus that fails to replicate *in vivo* (**Fig. 64A**). Similarly, both in the peripheral blood as well as in the spleen of infected mice, there is a 3- and 10-fold reduction in CD4⁺ T cells, respectively, although CD8 T cell levels remain constant (**Fig. 64A**). In the infected mouse, it is of note that in organs consisting almost entirely of lymphocytes such as the thymus and lymph node, a consistent 2-fold increase in CD8⁺ T cells is observed, potentially as a reaction to infection (**Fig. 64A**). As can be seen by p24-staining of tissue sections from the infected thymus, infection of T cells was clearly established and is confirmed by a viral load of 45,000 copies viral RNA/ml plasma (**Fig. 64A, B**).

Optimizing transduction of CD34⁺ cells

[00314] Once the HHLS-mouse model for HIV-1 infection was established, the next step was high-level transduction of CD34⁺ cells with lentiviral vectors carrying hT5Cyp and a GFP reporter, coupled with efficient reconstitution of the T cell compartment in mice transplanted with transduced cells. While a number of groups report transduction efficiencies

equivalent to ours in cultured CD34⁺ cells (**Fig. 63B**), often in the presence of the early-acting cytokines TPO, SCF, and Flt3L, little is published on the reconstitution of Rag2^{-/-}γ_c^{-/-} mice with transduced CD34⁺ cells. One group has successfully knocked down the tumor suppressor p53 as well as the transcription factor SpiB in HHLS-mice (Gimeno et al., 2004; Schotte et al., 2004). Here, lentiviral vectors were used in which a pol III promoter drives expression of the shRNA and a pol II promoter the GFP reporter to transduce CD34⁺ cells of fetal origin, which show both superior transduction efficiencies and reconstitution capacities compared to CD34⁺ cells derived from cord blood.

[00315] Experiments were designed to express an exogenous protein with an unknown effect on hematopoiesis and immune system development in CD34⁺ cells and to track transduced cells and their progeny *in vivo*. Transduction optimization experiments were carried out. As can be seen in **Table 2** many factors affect transduction efficiency in CD34⁺ cells including promoter choice, insert size, and envelope glycoprotein used for pseudotyping. The highest GFP-expression in transduced CD34⁺ cells is consistently observed with a lentiviral vector in which the hCypA promoter drives GFP expression (FCGW) with up to 70% GFP⁺ cells at 48 hours post-transduction (**Table 2, Fig 63B**).

Table 2. Transduction of CD34⁺ cells						
Vector	Envelope	Promoter	Insert Size (bp)	IRES-GFP	% GFP ⁺ (Average)	Repeats
FCGW ^a	VSVG	hCypA	700	-	49.4	N=6
FSGW ^a	VSVG	SFFV	700	-	15.2	N=1
FCGW	VSVG	hCypA	2200	-	13.9 ^b	N=3
FCGW	VSVG	hCypA	2200	-	14.2 ^b	N=3
cAPLS	VSVG	hCypA	700	-	11.6	N=2
scALPS ^{3c}	VSVG	SFFV/hCypA	1500/700	-	8.9 ^c	N=2
scAPLS ^c	VSVG	SFFV/hCypA	1500/700	-	9.0 ^c	N=2
FCGW ^d	RD114/TR ¹	hCypA	700	-	8.5 ^d	N=1
BiD ²	VSVG	PGK/mCMV	0/700	-	6.2	N=2
sAPLS	VSVG	SFFV	700	-	6.1	N=2
BiD ²	VSVG	PGK/mCMV	3000/700	-	3.1	N=2
APLS-IRES	VSVG	SFFV	1500	+	1.9 ^e	N=1
APLS-IRES	RD114/TR	SFFV	1500	+	1.2 ^e	N=1
^a FCGW and FSGW are FUGW ⁴ with the ubiquitin promoter replaced by the hCypA and SFFV promoters, respectively ^b GFP-hT5Cyp fusion proteins were cloned into FCW; fluorescence intensity was low ^c scAPLS was used for further studies ^d FCGW was pseudotyped with RD114/TR ¹ ; when VSVG was used side-by-side, 74% of CD34 ⁺ cells were GFP ⁺ at 72 hours post-transduction ^e GFP fluorescence intensity was low						
1. Sandrin, V., <i>et al.</i> Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34 ⁺ cells derived from human and nonhuman primates. <i>Blood</i> 100 , 823-832 (2002). 2. Amendola, M., Venneri, M.A., Biffi, A., Vigna, E. & Naldini, L. Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. <i>Nature biotechnology</i> 23 , 108-116 (2005). 3. Pertel, C. & Luban, J. Unpublished. (2008). 4. Lois, C., Hong, E.J., Pease, S., Brown, E.J. & Baltimore, D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. <i>Science (New York, N.Y)</i> 295 , 868-872 (2002).						

[00316] Three approaches were compared for the ability to track transduced cells bearing hT5Cyp *in vivo*: using lentiviral vectors with an IRES driving GFP-synthesis, using hT5Cyp proteins with GFP fused at the N-terminus, and using scAPLS, a dual-promoter vector described previously (**Table 2**). While lentiviral vectors coding for GFP-hT5Cyp fusion proteins allow for adequate transduction efficiencies, GFP fluorescence intensity is low in this context, precluding efficient tracking *in vivo* (**Table 2**). As previously seen with transduction of primary CD4⁺ T cells, lentiviral vectors coding for an IRES to drive GFP

synthesis lead to inefficient GFP synthesis and extremely weak fluorescence intensity post-transduction (**Table 2**). The best combination of percentage of GFP⁺ cells and GFP fluorescence intensity in CD34⁺ cells was obtained using scAPLS, which also efficiently transduces hCD4⁺ T cells (**Table 2, Fig. 66A**; left, pre-transplant panels). scAPLS was therefore chosen to test hT5Cyp in HHLS-mice. Additionally, BiD, another dual-promoter lentiviral vector employing the mCMV and PGK promoters (Amendola et al., 2005) was tested. Since the percentage of GFP⁺ cells was lower post-transduction than with scAPLS and since transduction of hCD4⁺ T cells using BiD was inefficient (Example 2), this vector was not used in experiments requiring target gene expression in T cells (**Table 2**).

[00317] Since high transduction efficiencies should lead to improved *in vivo* models, efforts were undertaken to optimize CD34⁺ transduction. The application of spin-infection, the coating of plates with retronectin prior to transduction, or the use of polybrene during transduction had little and inconsistent effects on the percentage of GFP⁺ cells in transduced CD34⁺ cells. The pre-incubation of monocyte-derived DCs cells with SIV virus like particles (VLPs) has been shown to increase their transduction by more than 10-fold; an effect dependent on the SIV accessory protein *Vpx* (Goujon et al., 2006; Goujon et al., 2007). Similarly, pre-incubation of CD34⁺ cells with SIV VLPs consistently led to a 2-fold increase in transduction efficiency (**Fig. 65A**).

[00318] The HHLS-mouse can be used as a model for evaluation of candidate gene-therapies, if development and maturation of transduced T cell progeny derived from transduced CD34⁺ precursors is successful *in vivo*. BiD-transduced or FCGW-transduced CD34⁺ cells from donor-identical cord blood were used to transplant newborn Rag2^{-/-}γc^{-/-} littermates (**Fig. 65B,C**). Mice were analyzed 12 weeks later, with 6% and 8.9% of total CD45⁺ lymphocytes in the bone marrow GFP⁺ for BiD and FCGW-transduced CD34⁺ input cells, respectively; a proportion equivalent to initial transduction efficiency (**Fig. 65B,C**). Since the majority of human CD45⁺ lymphocytes in the HHLS-mouse bone marrow are CD19⁺ B cells, similar results are obtained for B-cells with 6.6 % and 9.2% of CD19⁺ B cells GFP⁺ for BiD and FCGW transduction, respectively (**Fig. 65B,C**; right panels). In the thymus, however, where the majority of CD45⁺ human lymphocytes are T cells, there is no evidence for GFP⁺ T cells, even when FCGW was used to transduce input CD34⁺ cells (**Fig. 65B,C**; left panels). Similarly, GFP⁺ T cells were not detected in the peripheral blood of these mice or in spleen, bone marrow, or lymph nodes.

hT5Cyp reduces viral load in HHLS-mice challenged with HIV-1

[00319] Despite the results obtained for T cells in HHLS-mice transplanted with transduced CD34⁺ cells, hT5Cyp was tested in the HHSL-mouse model. Donor-identical CD34⁺ cells were transduced with scAPLS coding for hT5Cyp (**Fig. 66A**, top panel, pre-transplant) or the non-restricting hT5CypH126Q (**Fig. 66**, bottom panel, pre-transplant) and transplanted into newborn Rag2^{-/-}γ_c^{-/-} littermates. An aliquot of cells was maintained in culture with the subsequent addition of SCF, TPO, and Flt3L, and assessed for GFP-expression 72 hours post-transduction. Transduction efficiency for hT5Cyp- and hT5CypH126Q-scALPS as measured by GFP-expression was equivalent at 11.4% and 12.4% GFP⁺, respectively (**Fig. 66A**). Eight weeks post-transplant mice were analyzed by flow cytometry for peripheral blood engraftment, and mice containing between 1.3% and 24% hCD45⁺ of nucleated cells in the peripheral blood were infected i.p. with HIV-1 (**Table 3**). As observed for mice reconstituted with BiD or FCGW-transduced CD34⁺ cells, GFP⁺ cells were found almost exclusively in the B-cell compartment with a somewhat lower proportion of GFP⁺ cells than the original transduction efficiency reported for the CD34⁺ cells. Again, CD3⁺ T cells were not GFP⁺ (**Table 3**, representative plots **Fig. 66A**). At 25 days post-infection there was a 3.3-fold reduction in plasma viral load when mice were reconstituted with hT5Cyp-transduced CD34⁺ cells (**Table 3**, **Fig. 66B**). Similarly, there seemed to be a selection for GFP⁺ CD45⁺ cells in the peripheral blood of mice reconstituted with hT5Cyp-transduced CD34⁺ cells (**Table 3**, **Fig. 65C**), although no increase in GFP⁺ CD3⁺ T cells was observed either in the peripheral blood or in the thymus post-infection (**Table 3**).

Table 3: hT5Cyp effects in HIV-1 infected HHLS-Rag2^{-/-}γ_c^{-/-} mice

Mouse	Pre-infection Peripheral Blood (% nuc. cells)							Post-infection Peripheral Blood (% nuc. cells)							Viral load (copies RNA/ml plasma)
	CD45			GFP ⁻		GFP ⁺		CD45			GFP ⁻		GFP ⁺		
	45	GFP ⁻	GFP ⁺	CD19	CD3	CD19	CD3	45	GFP ⁻	GFP ⁺	CD19	CD3	CD19	CD3	
hT5Cyp-1	1.3	100	0	41.8	0	0	0	0.81	94.3	5.71	13.6	8.51	100	LC ^b	660
hT5Cyp-2 ^a	13.9	97	2.97	89.1	1.79	96.9	0	6.07	94.7	5.34	96.1	0.19	96.7	0	1062
hT5Cyp-3	5.8	99.5	0.52	64	1.05	80	0	1.06	96.1	3.95	78.1	12	88.9	LC ^b	8612
hT5Cyp-4	24.3	98.1	1.85	87.7	3.11	95.2	0	11.2	96.6	3.3	88.1	7.13	90.9	0	8620
H126Q-1	28.9	99.7	0.35	88	2.69	100	LC ^b	27.3	99.6	0.41	83.2	10.6	70	33.3	15966
H126Q-2	10.1	100	0	86.4	1.84	0	0	5.28	99	1	87.8	6.34	20	20	4353

H126Q-3 ^a	9.04	92.6	7.41	89.3	1.57	98	1.96	1.88	94.7	5.26	86.9	14.5	100	0	27789
H126Q-4	23.7	98.1	1.9	83.2	4.32	100	0	10.6	99.8	0.23	88.4	8.55	100	0	ND ^c

CD45 values are given as percent of nucleated cells. GFP⁺ and GFP⁻ are determined as percent CD45⁺ cells.
 CD19 and CD3 are described as percentage of CD45⁺GFP⁻ or CD45⁺GFP⁺ cells.
^aPre-infection cytometry profiles are shown in Fig. 66A
^bLC indicates low cell count precluding reliable determination of percentage
^cND = not determined

Establishment of a hCD4⁺-Rag2^{-/-}γ_c^{-/-} mouse model for HIV-1 infection

[00320] To test the ability of hT5Cyp to protect CD4⁺ T cells against HIV-1 infection *in vivo*, an experimental model was established in which human CD4⁺ T cells were adoptively transferred into Rag2^{-/-}γ_c^{-/-} mice. Clodronate liposomes were used to deplete macrophages and 6-10 week-old mice were conditioned with sublethal irradiation prior to transplant of human cells (van Rijn et al., 2003). Since activated human T cells will eventually cause xGVHD in this mouse model, resting and activated PBMC as well activated, purified CD4⁺ T cells were titrated at 1, 5, and 10 x 10⁶ cells/mouse (**Fig. 67A**). While as little as 1 x 10⁶ resting PBMC lead to more than 60% reconstitution of peripheral blood with human CD45⁺ lymphocytes, a similar result was obtained with 5 x 10⁶ CD4⁺ T cells (**Fig. 67B,D**). Peripheral blood engraftment was low at 6 days post-transplant and peaked by 21 days, at which point the percentage of human cells in the peripheral blood seemed to plateau (**Fig. 67B**). Despite the unexplained death of one mouse three days after transplant of 1 x 10⁶ activated CD4⁺ T cells, transplant of higher cell numbers generally seemed to lead to earlier development of GVHD-related symptoms and death (**Fig. 67C**). The longest survival observed in this setting was between 54 and 60 days post-transplant (**Fig. 67C**). When purified CD4⁺ T cells were used to reconstitute Rag2^{-/-}γ_c^{-/-} mice, the very low levels of CD8⁺ and CD4⁺CD8⁺ cells in the input seem to have an expansion advantage. The appearance of CD8⁺ and CD4⁺CD8⁺ cells was repeatedly observed in mice reconstituted with CD4⁺ T cells enriched from PBMC, even when CD8⁺ cells were specifically sorted out by FACS (**Fig. 67D**).

hT5Cyp protects against HIV-1 infection *in vivo*

[00321] To evaluate the anti-HIV-1 activity of hT5Cyp in this model, CD4⁺ T cells were transduced with the hT5Cyp or hT5CypH126Q vectors and sorted for GFP-expression (**Fig. 68C**). Age-matched, 6-10 week-old Rag2^{-/-}γ_c^{-/-} mice were conditioned with clodronate

and sublethal irradiation (van Rijn et al., 2003), and injected intraperitoneally with 2.5×10^6 transduced $CD4^+$ T cells (**Fig. 68A,B**). In uninfected mice, engraftment efficiency was not altered by hT5Cyp or hT5CypH126Q (**Fig. 68D**). While over 90% of input cells were GFP^+ , 6 weeks post-transplant it was apparent that GFP expression was down-modulated in a proportion of cells, consistently yielding a GFP^{hi} and a GFP^{lo} population of human $CD4^+$ T cells in mice (**Fig. 68D**).

[00322] To determine if hT5Cyp protected $hCD4^+$ T cells against HIV-1 *in vivo*, mice were infected with HIV-1 four days after adoptive transfer of the transduced $CD4^+$ T cells (**Fig. 68A**). When mice received hT5Cyp-transduced $CD4^+$ T cells, mean peripheral blood $CD4^+$ cells constituted 6% (+/- 2.87%) (**Fig. 69B, Table 4**) two weeks post-infection. This was 34-fold higher than the percentage $CD4^+$ cells (mean 0.18%, +/- 0.04%) observed in mice transplanted with hT5CypH126Q-transduced $CD4^+$ T cells, a difference that was statistically significant ($p=0.0002$, $n=10$ hT5Cyp, $n=8$ hT5CypH126Q) (**Fig. 69B, Table 4**). In this model, mice were infected with HIV-1 as cells are still expanding rapidly in the mouse (**Fig. 65B**). HIV-1 infection thus precludes T cell expansion in mice engrafted with hT5CypH126Q-expressing $CD4^+$ T cells. While this model reveals a 34-fold protective effect of hT5Cyp on $CD4^+$ T cell survival, some cells are still productively infected with HIV-1 in the context of hT5Cyp. Given the large difference in overall $CD4^+$ numbers between mice transplanted with hT5Cyp- and those transplanted with hT5CypH126Q-expressing $CD4^+$ T cells, an effect of hT5Cyp on absolute viral load was not observed in the absence of correction for $CD4^+$ T cell numbers (**Table 4**).

Table 4: hT5Cyp protects T cells from HIV-1 infection *in vivo*

Mouse		hT5Cyp	Peripheral Blood (2 wks. postinfection)		Viral load (copies RNA/ml)	Viral load (Corrected for CD4 count)
#	Donor (A or B)		CD45 ^a	CD4 ^{a,b}		
1A		wt	2.12	1.89	492800	260305
2A		wt	1.16	1.1	27826	25410
3A		wt	1.08	1	103208	101231
4A		wt	0.47	0.44	46007	104916
5A		wt	0.24	0.33	43551	192227
6B		wt	1.64	1.56	251320	159325
7B		wt	2.75	2.7	66416	24321
8B		wt	7.86	7.7	358800	47206
9B		wt	15.6	15.1	566835	37382
10B		wt	28.9	28.3	3811676	134446

11A	H126Q	0.17	0.15	13228	29396
12A ^c	H126Q	0.09 ^c	0.09	LE ^c	LE ^c
13A	H126Q	0.1	0.1	64465	644650
14A	H126Q	0.17	0.14	92330	659500
15B	H126Q	0.22	0.20	4755	23775
16B	H126Q	0.12	0.12	17811	148425
17B	H126Q	0.54	0.46	26520	57652
18B	H126Q	0.19	0.16	15314	95713

^aCD45 values were determined as percentage of nucleated cells. CD4 was calculated to represent percentage of total nucleated cells. T cell protection is plotted in **Fig. 69A**

^aAll cells were GFP⁺; with a GFP^{hi} and GFP^{lo} population. Representative profiles are in **Fig. 68D**.

^c Mouse 12A had low engraftment (LE) and viral load below detection (<40) at week 2; at week 4 the viral load was at 7604 copies viral RNA/ml plasma.

[00323] To determine if hT5Cyp reduced absolute viral load *in vivo*, mice were infected with HIV-1 two weeks after adoptive transfer of the transduced CD4⁺ T cells into Rag2^{-/-}γc^{-/-} mice (**Fig. 68B**). The timing of HIV-1 infection was changed to allow for a stabilization of engraftment prior to assessment of viral replication. Mice transplanted with hT5Cyp-expressing CD4⁺ T cells had a mean viral load of 1.6 x 10⁵ (+/- 1.1 x 10⁵) compared to 1.5 x 10⁶ (+/- 6.9 x 10⁵) copies viral RNA/ml plasma in mice transplanted with hT5CypH126Q-expressing CD4⁺ T cells (**Fig. 69B, Table 5**). This 9-fold reduction in mean viral load in mice engrafted with hT5Cyp-expressing CD4⁺ T cells was statistically significant (p=0.005; n=8 hT5Cyp, n=8 hT5CypH126Q) (**Fig. 69B, Table 5**). Taken together, these results from the peripheral blood indicate that hT5Cyp inhibits HIV-1 replication and protects against HIV-1-associated destruction of CD4⁺ T cells *in vivo* (**Fig. 69A,B; Tables 4 and 5**).

Table 5. hT5Cyp reduces viral loads *in vivo*

Mouse #, donor	T5Cyp	Viral tropism	Peripheral Blood (pre-infection)		Peripheral Blood (post-infection)		Viral load (copies RNA/ml plasma) ^d
			CD45	CD4	CD45	CD4	
1C	wt	X4	2.02	1.9	0.67	0.66	40 ^c
2C ^b	wt	X4 ^b	3.64	3.3	1.93	1.90	938000
3D ^a	wt	X4 ^a	1.22	1.2	NE	NE	42402
4C	wt	R5	0.58	0.5	1.11	0.86	5824
5C	wt	R5	1.64	1.5	0.65	0.61	18900
6E	wt	R5	3.83	3.8	0.28	0.26	173067
7E	wt	R5	0.88	0.7	0.05	0.04	52436
8E	wt	R5	1.69	1.6	0.23	0.22	72067
9C	H126Q	X4	5.38	5.2	1.14	1.08	10000000 ^c

10C ^b	H126Q	X4 ^b	6.62	6.0	3.21	3.14	5160000
11D ^a	H126Q	X4 ^a	1.2	1.1	NE	NE	297500
12C	H126Q	R5	3.9	2.8	11	10.32	3906000
12C	H126Q	R5	0.2	0.2	1	0.95	95077
14E	H126Q	R5	9.58	8.6	0.02	0.02	251458
15E	H126Q	R5	4.34	2.0	0.18	0.16	244916
16E	H126Q	R5	16.8	8.0	0.11	0.10	782286
CD45 values are given as percent of nucleated cells, CD4 values are calculated as percent of nucleated cells ^aPathology and intracellular p24 stain of thymus in Fig. 69C,E ^bCD4-downregulation shown in Fig. 69D ^c40 is below and 10000000 is above range of the assay ^dViral loads shown in Fig. 69B; ^eWhen CD45-count was not determined (ND), human CD4 counts are of total lymphocytes (by FSC and SSC in lymph node and Thymus). NE = no engraftment, ND = not determined, NL= no lymph node, LE = low engraftment							

[00324] Further evidence for the *in vivo* effects of hT5Cyp was sought by examining lymphoid organs of HIV-1-infected mice. Single-cell suspensions were analyzed for the human T cell marker CD3 (which, unlike CD4, is not downregulated by HIV-1), the hT5Cyp vector reporter GFP, and HIV-1 p24 antigen. Few if any of the hT5Cyp-transduced human T cells in thymi or mesenteric lymph nodes of HIV-1-infected mice were p24⁺, as compared with 1.5-5% of the cells transduced with hT5CypH126Q (**Fig. 69C**). HIV-1 downregulates cell-surface expression of CD4 (Chen et al., 1996). Consistent with inhibition of productive infection in T cells, hT5Cyp prevented the down-regulation of cell surface CD4 in thymi and lymph nodes of mice infected with CXCR4-tropic or CCR5-tropic HIV-1, respectively (**Fig. 69D, Fig. 70B**). Finally, tissue sections of thymi were directly examined for human T cells (using anti-CD3 antibody) and HIV-1 p24 antigen. The engraftment of CD4⁺ T cells transduced with hT5Cyp and hT5CypH126Q was similar (**Fig. 69E**). Intense p24 staining was observed in tissue sections from animals engrafted with hT5CypH126Q-transduced CD4⁺ T cells but not in tissues from mice engrafted with CD4⁺ T cells transduced with hT5Cyp (**Fig. 69E, α-p24 stain**). These experiments show that hT5Cyp prevented productive HIV-1 infection *in vivo*.

[00325] Due to lack of B, T, and NK cells, Rag2^{-/-}γc^{-/-} do not have peripheral lymph nodes (Goldman et al., 1998). Lack of lymphocytes in these mice precludes migration of lymph node inducer cells into the lymph node anlage, leading to the degradation of the anlage within the first week of life (Coles et al., 2006). Transfer of mouse peripheral lymph node

cells into 4 day old Rag2^{-/-}γc^{-/-} leads to complete recovery of inguinal lymph nodes. The equivalent experiment performed in 4-week old mice fails to recover peripheral lymph nodes (Coles et al., 2006). When transferring transduced hCD4⁺ T cells into 6-10 week old Rag2^{-/-}γc^{-/-} the presence of peripheral lymph nodes was occasionally observed at 4-6 weeks post-transplant. An example is shown in **Fig. 70A**. These results are consistent with the lymph node anlage remaining intact well into adulthood.

[00326] Mice were reconstituted with hT5Cyp- or hT5CypH126Q-expressing CD4⁺ T cells and infected two weeks later with CCR5-tropic HIV-1. When mice were reconstituted with hT5Cyp-expressing CD4⁺ T cells, both mesenteric (MLN) and peripheral (PLN) lymph nodes showed GFP^{hi} and GFP^{lo} populations of hCD4⁺ cells (**Fig. 70B**). Additionally, there was little evidence of CD4 downregulation in either PLN or MLN upon HIV-1 infection (**Fig. 70B**). In contrast, MLN and PLN of HIV-1 infected mice reconstituted with hT5CypH126Q-expressing CD4⁺ T cells showed CD4 downregulation (**Fig. 70B**). In the MLN of mice reconstituted with hT5CypH126Q only the GFP^{lo} CD45⁺ lymphocyte population persists in contrast to mice reconstituted with hT5Cyp. This is consistent with a selection for GFP⁺ cells during HIV-1 infection in the context of restrictive hT5Cyp but not of the permissive hT5CypH126Q (**Fig. 70B**). The MLNs are closest to the site of HIV-1 infection (i.p), while the PLNs are further away. In the same mice where a selection against GFP⁺ hT5CypH126Q-expressing cells is observed in the MLN, the PLN contains both GFP^{hi} and GFP^{lo} human lymphocytes at comparable proportions to the MLN and PLN of mice transplanted with hT5Cyp-expressing cells (**Fig. 70B**). These data are consistent with delayed kinetics of HIV-1 infection in PLNs.

[00327] Few if any of the hT5Cyp-transduced human T cells in MLN or PLN of HIV-1-infected mice were p24⁺, as compared with 1.7-3.6% of the cells transduced with hT5CypH126Q (**Fig. 70C**). CD3 downregulation is observed in p24⁺ cells here as HIV-1_{NL4-3} was modified to express the SIV_{mac239} *Nef* which inhibits T cell activation (**Fig. 70C**) in contrast to the isogenic virus containing HIV-1 *Nef* (**Fig. 69C**) (Schindler et al., 2006). Tissue sections of MLNs were directly examined for human T cells (using anti-CD3 antibody) and HIV-1 p24 antigen. The engraftment of CD4⁺ T cells transduced with hT5Cyp and hT5CypH126Q was similar (**Fig. 70D**). Intense p24 staining was observed in tissue sections from animals engrafted with hT5CypH126Q-transduced CD4⁺ T cells but not in

tissues from mice engrafted with CD4⁺ T cells transduced with hT5Cyp (**Fig. 6D**, α -p24 stain). Again, hT5Cyp prevented productive HIV-1 infection *in vivo*.

hT5Cyp protects CD4⁺ T upon infection with cell-associated virus *in vivo*

[00328] The previous experiments showed that CD4⁺ T cells were protected from HIV-1 infection *in vivo* when mice were reconstituted with cells that had all been transduced with the hT5Cyp vector. Furthermore, the previous infections were initiated with cell-free HIV-1. Example 2 shows that cell-associated virus initially overcomes hT5Cyp-mediated restriction. Therefore, hT5Cyp anti-HIV-1 efficacy was evaluated in a more complex model. Mice were transplanted with 5×10^6 CD4⁺ T cells previously transduced with scALPS-hT5Cyp or hT5CypH126Q. Cells were over 80% transduced as measured by GFP expression at the time of transplant and were not sorted further. Untransduced (GFP⁻) CD4⁺ cells from the same donor were cultured side by side and infected with CXCR4-tropic HIV-1 prior to infection of reconstituted mice. 4 days after transplant, mice were infected with 2.5×10^6 CD4⁺ T cells that were <1% p24⁺ (**Fig. 71A**). Peripheral blood was analyzed one, two, and three weeks after infection and mice were sacrificed three weeks after infection (**Fig. 71A**). Mice that had been transplanted with hT5Cyp-expressing cells had a 9.7-fold reduction in mean viral load one week post-infection (peak level of infection) and a 4.3-fold reduction at two weeks post-infection compared to mice transplanted with hT5CypH126Q-expressing cells (**Fig. 71B**).

[00329] There was a 3.7-fold protection of total CD3⁺ T cell numbers in the thymus in the context of hT5Cyp (2×10^7 CD3⁺ T cells hT5Cyp thymus, n=2; 5.4×10^6 CD3⁺ T cells hT5CypH126Q thymus, n=2). T cell protection was also apparent in the peripheral blood, where a higher degree of CD4⁺ T cell depletion occurred in mice transplanted with hT5CypH126Q-expressing cells compared to hT5Cyp-expressing cells (**Fig. 71C**). For hT5Cyp-expressing cells, CD4⁺ T cell numbers did not decline beyond 40% of initial levels three weeks post-infection, while hT5CypH126Q-expressing cells declined as low as 1% of initial levels (**Fig. 71C**). In mice that had received hT5Cyp-transduced CD4⁺ T cells, few (0.9%) of the total human T cells in the infected thymus were p24⁺, as compared with 6.5% of the cells in mice that received hT5CypH126Q-transduced cells (**Fig. 71D**). Next GFP^{hi} and GFP^{neg/lo} CD3⁺ cell populations were analyzed in infected thymi for presence of p24. These populations represent transduced cells (GFP^{hi}) and untransduced cells or transduced cells that downmodulated GFP-expression (GFP^{neg/lo}). In thymi of animals reconstituted with hT5CypH126Q-transduced CD4⁺ T cells both GFP populations showed significant p24

staining (15.2% for GFP^{neg/lo} and 4.4% for GFP^{hi}) cells (**Fig. 71D**). The reduced percentage of p24⁺ cells observed here is likely due to reduced infectability of previously transduced cells. For hT5Cyp-transduced CD4⁺ T cells, few of the GFP^{hi} cells are p24⁺, while 3.2% of the GFP^{neg/lo} cells are p24⁺, indicating a preferential HIV-1 infection of cells lacking hT5Cyp (**Fig. 71D**). Data shown are representative of two thymi in each group.

DISCUSSION

[00330] The hCD4⁺-Rag2 γ_c ^{-/-} mouse developed here proved a robust model for assessing inhibition of HIV-1 infection and CD4⁺ T cell protection by lentiviral transduction with hT5Cyp (**Fig. 69, 70, 71**). Due to aberrant T cell activation in the xenogeneic context and eventual development of xGVHD symptoms in hCD4⁺-Rag2 γ_c ^{-/-} mice, this model is limited to assessment of hT5Cyp efficacy in the context of acute HIV-1 infection. Furthermore, in the absence of an intact innate and adaptive immune system, immune responses to HIV-1 cannot be assessed in hCD4⁺-Rag2 γ_c ^{-/-} mice. Similarly, this model will not reveal any antigenic potential hT5Cyp itself may have. Humanized mouse models to date do not elicit sufficiently robust and consistent primary immune responses to address this question. One pre-clinical approach could be screening the frequency of hT5Cyp-reactive cells in the average human T cell repertoire.

[00331] A new model with 100% infection rate and greatly improved T cell reconstitution has been developed allowing for excellent viral replication compared to the preceding huPBL-SCID models for HIV-1 infection (Mosier et al., 1991). Given that these observations are some of the highest viral loads reported, this model may prove robust enough to study infection with primary isolates and mutant viruses which replicate less efficiently in tissue culture than the lab-adapted, wildtype HIV-1 strains.

[00332] Cell-to-cell spread of HIV-1 in lymphoid organs is a major method of HIV-1 transmission in hCD4⁺-Rag2 γ_c ^{-/-} mice (Haase, 1999). Nonetheless, T5Cyp leads to an impressive T cell protection and a reduction in viral loads in these animals. These data support the validity of a T5-based HIV-1 therapy approach. While it takes a much more potent T5 than hT5 $\alpha_{R323-332}$ to control cell-to-cell spread of HIV-1 (Richardson et al., 2008), hT5Cyp not only accomplishes this *in vitro* (**Fig. 52**) but also in an *in vivo* model (**Fig. 69,70**).

[00333] To render the model more physiologically relevant, infection with cell-associated virus was initiated in mice transplanted with transduced CD4⁺ T cells. This leads to HIV-1 infection in mice that now have mixed transduced and untransduced T cell populations, establishing a constant reservoir of CD4⁺ cells permissive to HIV-1 infection. Despite this rigorous set-up, mice containing hT5Cyp-expressing T cells showed protection of CD4⁺ T cells with a concomitant reduction in viral load (**Fig. 71**). Analysis of infected cells in the thymus showed a significant overall reduction in p24⁺ T cells in mice that received hT5Cyp-transduced T cells. This reduction in p24⁺ cells was not only apparent in GFP⁺, transduced T cells but also in GFP⁻, untransduced cells, implying a potential effect of hT5Cyp on bystander cells. Consistent with bystander protection, while mice that received hT5Cyp-transplanted cells, had a three-fold increase in total numbers of CD3⁺ T cells in the thymus, they had a > 3.3-fold reduction in total numbers of GFP⁻ p24⁺ cells compared to mice that received hT5CypH126Q-transplanted cells; likely due simply to lower levels of circulating virus (**Fig. 71**). This *in vivo* “competition” model can be expanded to allow for the assessment of CD4⁺ and CD8⁺ T cell engraftment as well as some limited B cell engraftment (van Rijn et al., 2003), by infecting mice transplanted with transduced CD4⁺ T cells with HIV-1 infected PBMCs from the same donor.

[00334] Since mouse availability is the only limiting factor, the hCD4⁺-Rag2 γ_c ^{-/-} model for HIV-1 infection is straightforward and allows for initiation of infection using cell-free and cell-associated virus. The model could easily be adapted to a hPBL- Rag2 γ_c ^{-/-} model, as total PBMCs engraft even more efficiently in Rag2 γ_c ^{-/-} mice than transduced CD4⁺ T cells (**Fig. 67**). Furthermore, analysis points to presence of human T cells in the gut of hCD4⁺-Rag2 γ_c ^{-/-} mice, potentially allowing for mucosal initiation of systemic HIV-1 infection. hCD4⁺-Rag2 γ_c ^{-/-} mice were invaluable for our preclinical assessment of hT5Cyp as a candidate anti-HIV-1 gene therapy.

[00335] While, hT5Cyp had no effect on proliferation, cell-surface-marker expression, and IL-2 production of transduced CD4⁺ T cells in culture (**Fig. 46A, 58**), endogenous T5 α functions may be interfered with by formation of heteromultimers (**Fig. 46B, 62**). hT5Cyp is a new protein with unknown potential effects on hematopoiesis, immune cell development, and effector functions. hT5Cyp effects on T cell differentiation could be directly assessed *in vitro* by culturing CD34⁺ cells transduced with scAPLS-hT5Cyp and scAPLS-hT5CypH126 with OP-9 stromal cells expressing delta-like ligand 1 (OP9-DL-1) in the presence of Flt3L and

IL-7 (La Motte-Mohs et al., 2005). This co-culture system allows for the gradual accumulation first of CD4⁺ single- positive T cells followed by CD4⁺CD8⁺ double-positive T cells by 24 days of co-culture. *In vitro*-differentiated T cells have some effector function as they upregulate activation markers following stimulation with α CD3 and α CD28 antibodies. Since scAPLS allows tracking of transduced cells by GFP expression, this system would allow for detecting possible effects of hT5Cyp on T cell development. Furthermore, addressing whether HIV-1 resistant T cells can be generated from hT5Cyp-transduced CD34⁺ HSCs would be a valuable *in vitro* proof of principle experiment.

[00336] To study T5Cyp-mediated inhibition of HIV-1 in the HHLS-Rag2 $\gamma_c^{-/-}$ mouse model directly, two obstacles were overcome: efficient transduction of CD34⁺ HSCs and efficient differentiation of transduced CD34⁺ cells into T cells *in vivo*. CD34⁺ HSCs are naturally resistant to HIV-1 infection despite expression of CD4 and CXCR4 due to restriction by p21^{Waf/Cip1/Sdfl}, a cyclin-dependent-kinase inhibitor that complexes with HIV-1 integrase and inhibits proviral integration (Zhang et al., 2007). With entry into the cell-cycle and subsequent expansion and differentiation, p21-expression is downregulated allowing for improved lentiviral transduction. Furthermore, high proteasome activity in HSCs restricts HIV-1 post-entry (Santoni de Sio et al., 2006). Early-acting cytokines (SCF, Flt3L, TPO) are often used in short-term CD34⁺ cell culture to increase transduction efficiency partly by reducing proteasome activity. This effect that can be further enhanced by the addition of proteasome inhibitors to yield transgene expression in ~ 75% of cells (Santoni de Sio et al., 2006). It is unclear what effect such *ex vivo* HSC manipulation has on multilineage engraftment potential, specifically regarding T cell development, as cells were only assayed in the adult NOD/SCID repopulation assay (Santoni de Sio et al., 2006). While many groups add polybrene or pre-coat plates with retronectin to enhance CD34⁺ HSC transduction efficiency, no consistent improvement in transduction efficiency was observed using these methods. However, a > 2-fold enhancement of transduction efficiency was consistently observed by treating target cells with SIV viral-like particles (VLPs) prior to transduction. SIV VLPs have been shown to enhance transduction of DCs more than 10-fold due to the presence of the SIV accessory protein Vpx (Goujon et al., 2006; Goujon et al., 2007). Vpx may affect HIV-1 restriction mechanisms found in CD34⁺ HSCs such as p21 and proteasome activation without a requirement for cytokine stimulation and entry into cell cycle. The semen-derived enhancer of HIV-1 infection (SEVI) has also shown up to 10-fold enhancement of CD34⁺ HSC transduction efficiency in preliminary experiments.

[00337] In the absence of a proliferation advantage such as disruption of p53 (Gimeno et al., 2004), reliable transgene expression in T cells derived from transduced CD34⁺ *in vivo* is rare. This is independent of hT5Cyp expression as three different lentiviral vectors coding only for GFP were used to transduce CD34⁺ HSCs and all failed to yield GFP⁺ thymocytes despite generation of GFP⁺ B cells and DCs (Fig. 65). Notwithstanding, hT5Cyp reduced viral load more than 3-fold in HIV-infected HHLS-Rag2 $\gamma_c^{-/-}$ mice transplanted with hT5Cyp-transduced CD34⁺ cells (Fig. 66). Based on T cell depletion kinetics observed in prior experiments, the mice were sacrificed 24 days post-infection. On the other hand, a longer duration of infection may have allowed for visible selection of GFP⁺ T cells.

[00338] T cell fate and differentiation in HHLS-Rag2 $\gamma_c^{-/-}$ mice may be easily perturbed by any form of *ex vivo* HSC-manipulation, despite the unquestionably superior *de novo* thymopoiesis compared to previous humanized mouse models (Manz, 2007). Sorting cells based on transgene expression prior to transplant would ensure that the majority of immune-reconstituting cells are transduced and may thus enforce development of transduced T cells. A problem arising with use of CD34⁺ HSCs derived from cord blood is a small sample size with $< 1 \times 10^6$ cells enriched from one donor on average. Transplantation of less than 1×10^5 cells/animal is undesirable, and reconstitution efficiency is proportional to the dose of transplanted CD34⁺ HSCs. This precludes *ex vivo* selection of cells based on transgene expression.

[00339] An alternative is *in vivo* selection using lentiviral vectors expressing the MGMT_{P140K} selection marker or safer adaptations thereof targeted for clinical applications. MGMT is a methyltransferase that repairs DNA damage caused by DNA alkylating agents (e.g. BCNU, used clinically as anti-cancer chemotherapy). Since HSCs contain little endogenous MGMT, they are exceptionally susceptible to elimination by alkylating agents. The MGMT_{P140K}-variant is particularly useful, as it is resistant to small molecule-inhibitors (e.g. BG) of endogenous MGMT while still protecting from DNA damage caused by BCNU (Schambach et al., 2006). Transduction of hHSCs with lentiviral vectors coding for MGMT_{P140K} allowed for efficient reconstitution of BG/BCNU-conditioned NOD/SCID mice observed after two rounds of subsequent selection. Human cells comprised $> 20\%$ of cells in the bone marrow and over 99% of CFCs contained provirus as compared to 11% in controls without BG/BCNU treatment (Zielske et al., 2003). This system has not been tested in newborn HHLS-Rag2 $\gamma_c^{-/-}$ mice. *In vivo* selection of MGMT_{P140K}-transduced cells in HHLS-

Rag2^{-/-}γ_c may allow for multilineage engraftment of transduced immune cells, including high level of transgene expression in the T cell compartment, as selection results in a pure population of transduced HSCs *in vivo*.

[00340] A stable and inexhaustible source of isogenic CD34⁺ cells would allow for *in vitro* manipulation prior to transplant, larger experimental cohorts, and increased experimental consistency. Directing *in vitro* differentiation of genetically modified human embryonic stem cells (hESCs) to hematopoietic precursors (HSCs) would revolutionize gene therapy approaches. hESCs are immortal, pluripotent cells with considerable genetic manipulability in stark contrast to HSCs. Limited hematopoietic potential *in vivo* has been demonstrated for hESC-derived HSCs upon direct injection into mouse bone marrow, but engraftment into adult NOD/SCID mice was very low, engrafted cells failed to migrate to target tissue, and T cell development was deficient (Wang et al., 2005). T cell development is rescued by priming hESC-derived HSCs transduced with GFP-lentivectors on OP9-DL1 before injection into the SCID-hu thy/liv. Over 90% of ES-derived thymocytes obtained by this method were GFP⁺ (Galic et al., 2006). Given the SCID-hu model used here, it is unclear whether these cells would have the capability for multilineage reconstitution of immunocompromised mice, in addition to reconstitution of the T cell compartment. To address this, OP9-DL1-primed hESC-derived HSCs should be used to reconstitute newborn Rag2^{-/-}γ_c or NOG mice.

[00341] In a proof of principle experiment, sickle-cell anemia was cured in mice by reprogramming autologous tail fibroblasts into induced pluripotent cells (iPSs), genetic modification through homologs recombination of iPSs, and immune-reconstitution of lethally irradiated mice with “cured” iPSs (Hanna et al., 2007). *Ex vivo* culture of CD34⁺ HSCs combined with OP9-DL1 priming towards T cells would allow for repeated transduction opportunities and higher efficiencies, provided culture conditions allowing for maintenance of multilineage engraftment potential are established. If conditions allowing for true hematopoietic stem cell-expansion combined with T cell priming could be established, transduced cells may even be sorted prior to transgene expression.

[00342] While reconstitution of a functional human T cell compartment in prior humanized mouse models is yet unsuccessful, *de novo* thymopoiesis, the ability to generate specific primary T cell responses to EBV infection, as well as the elaboration of T-cell dependent antibody responses to tetanus toxoid observed in HHLS-Rag2^{-/-}γ_c^{-/-} mice have

been major advances (Traggiai et al., 2004). Given the lack of human MHC in the HHLS-Rag2^{-/-}γ_c^{-/-} thymus, how does positive and negative T cell selection enabling these immune responses occur? Under normal developmental conditions positive selection is preferentially driven by cortical thymic epithelial cells (Palmer, 2003) while both epithelial cells and HSC-derived DCs are involved in negative selection. Since no human epithelial cells, but both murine and human DCs were found in the HHLS-Rag2^{-/-}γ_c^{-/-} thymus, positive selection should occur on mouse epithelial cells, while negative selection can occur on both mouse and human cells (Baenziger et al., 2008). This would limit positive selection to mouse MHC only, and negative selection to both human and mouse MHC. Under certain circumstances, however, positive selection can also take place on cells of hematopoietic origin (here human DCs) (Shizuru et al., 2000; Zinkernagel and Althage, 1999). The empirical situation is the following: in mixed lymphocyte reactions, human T cells from HHLS-Rag2^{-/-}γ_c^{-/-} lymph nodes and spleen react most strongly to allogeneic hDCs and weakly to allogeneic mDCs (Baenziger et al., 2008; Traggiai et al., 2004). Cytotoxic activity against allogeneic human cells is blocked by anti-human MHC antibodies (Ishikawa et al., 2005; Manz, 2007). At the same time, specific T cell responses to influenza infection are only observed in the context of mouse MHC (Legrand et al., 2006; Manz, 2007). In the midst of this complicated situation, HHLS-Rag2^{-/-}γ_c^{-/-} mice do not mount robust, specific T cell responses to HIV-1 infection (Baenziger et al., 2006). A specific concern for analysis of HIV-1 infection in HHLS-Rag2^{-/-}γ_c^{-/-} mice is that peripheral T cells isolated from HHLS-Rag2^{-/-}γ_c^{-/-} mice and transferred into “empty” Rag2^{-/-}γ_c^{-/-} mice do not undergo homeostatic expansion (Legrand et al., 2006). Combined with high peripheral T cell turnover and a lack of long-term T cell maintenance in HHLS-Rag2^{-/-}γ_c^{-/-} mice, disturbed T cell homeostasis may potentially mask immune responses to HIV-1.

[00343] Given improvements, the HHLS-Rag2^{-/-}γ_c^{-/-} mouse holds great promise as a straightforward and efficient small animal model for multiple human plagues including HIV, HTLV, EBV, Hepatitis C, and Malaria (Goldstein, 2008). Concerted multicenter efforts funded by the Bill and Melinda Gates foundation are focused on improving reconstitution and functional immunity in HHLS-Rag2^{-/-}γ_c^{-/-} mice as well as standardizing model generation and evaluation with the ultimate goal of creating a standard, pre-clinical, HIV-1 vaccine model. The approach is to further humanize the Rag2^{-/-}γ_c^{-/-} mouse through introduction of human MHCs, and human cytokines relevant to human hemato-lymphoid development (both cross-

species reactive and species-restricted cytokines) (Manz, 2007). It is believed that these modifications will lead to better engraftment, development, homing, and effector function of immune cells in second generation HHLS-Rag2^{-/-}γ_c^{-/-} mice.

[00344] An improved HHLS-Rag2^{-/-}γ_c^{-/-} mouse model would allow assessment long-term hT5Cyp-mediated protection from HIV-1 infection and the potential for viral resistance emerging *in vivo*. Furthermore, it could be used to assess if hT5Cyp would enable an improved anti-HIV immune response and may help establish what characterizes a successful anti-HIV-1 response in human cells. If second-generation HHLS-Rag2^{-/-}γ_c^{-/-} mice develop peripheral lymph nodes in a more consistent manner than observed, cell-to-cell spread of HIV-1 in the presence or absence of restriction factors like hT5Cyp could be visualized by live imaging *in vivo*.

EXAMPLE 4: HIV-1 INHIBITION BY TRIM5-CYCLOPHILIN FUSION PROTEINS ENGINEERED OF HUMAN COMPONENTS

Design of hT5Cyp fusions with potent anti-HIV-1 activity

[00345] A cryptic splice acceptor at the AoT5Cyp fusion junction results in the synthesis of 12 amino acids derived from the CypA 5'UTR³. An equivalent human fusion protein cannot be engineered due to lack of sequence homology in the CypA 5'UTR. The goal was to generate hT5Cyp using components normally expressed in human cells. Consequently, hT5-direct-Cyp was engineered with hCypA fused to a position in the linear sequence of hT5 analogous to that of the AoT5Cyp retrotransposition event (Fig. 1a). Since the 12 amino acids encoded by the AoCypA 5'UTR might be an essential spacer between the AoT5 and AoCypA moieties, sequences from the hT5α, γ, and δ isoforms, and a synthetic 12 amino acid spacer were used to generate linkers of similar length and character: hT5-α-Cyp, hT5-γ-Cyp, hT5-δ-Cyp, and hT5(SGG)₄Cyp (Fig. 1a). Jurkat T cells stably expressing the indicated hT5Cyp fusions were challenged with serial dilutions of an HIV-1-GFP vector. hT5(SGG)₄Cyp restricted HIV-1 comparably to AoT5Cyp but none of the other constructs exhibited detectable HIV-1 restriction activity (Fig. 1a).

[00346] Next, hCypA was fused at 10 different positions along the length of hT5α (Fig. 1b). Of the hT5Cyp constructs tested for HIV-1 restriction activity in Jurkat T cells and THP-1 monocytes, three had activity comparable to AoT5Cyp, one (hT5-T369-Cyp) was

moderately restrictive, while another (hT5-M284-Cyp) exhibited variable restriction activity based on cell-type (Fig. 1c). hT5-S322-Cyp, consistently the strongest HIV-1-inhibitor, became the focus of subsequent experiments, and is referred to herein as hT5Cyp. hT5Cyps also restricted HIV-1 in promonocytic U937 cells, the CEM-SS, SUP-T1, and H9 T cell lines, and the 293T embryonic kidney cell line. Like AoT5Cyp⁵, hT5Cyp blocked the accumulation of viral cDNA after acute infection, as measured by quantitative PCR.

Structural requirements for hT5Cyp-mediated HIV-1 restriction activity

[00347] Despite the apparent modularity of TRIM5⁹⁻¹², most hT5Cyp fusions lacked anti-HIV-1 activity. Steady-state protein level in stably-transduced Jurkat T cells did not correlate with restriction activity: AoT5Cyp and restrictive hT5Cyp fusions were undetectable by immunoblot while some inactive hT5Cyps were highly expressed. To increase the possibility of detection, hT5Cyp proteins were expressed as triple-FLAG-tagged fusions by plasmid transfection of 293T cells. Anti-FLAG immunoprecipitates were probed in immunoblots with anti-CypA antibody. Two fusions with no detectable restriction activity, hT5-M244-Cyp and hT5-T302-Cyp, were expressed at high-level, whereas fusions with potent anti-HIV-1 activity, hT5-A331-Cyp and AoT5Cyp, were expressed at low-level (Fig. 2a). Again, restriction activity did not correlate with protein level.

[00348] Lack of activity could result from failure to bind HIV-1 CA. Glutathione S-transferase (GST)-CA fusion protein was used to test CA-binding activity of hT5Cyp proteins produced in 293T cells. Among hT5Cyp fusions with no anti-HIV-1 activity, hT5-T302-Cyp bound HIV-1 CA as well as the potently restrictive hT5-S322-Cyp (Fig. 2b). Specificity of binding was demonstrated by blocking interaction with the competitive inhibitor cyclosporine A (CsA) (Fig. 2b). Thus, the ability to bind CA was required but not sufficient for restriction activity.

[00349] To visualize the sites of CypA linkage to hT5, a three-dimensional model of the T5 α PRYSPRY domain was generated using coordinates from three homologues (Fig. 2c). hT5Cyp fusions with anti-HIV-1 activity clustered near the main specificity determinant for HIV-1 restriction activity^{11,12,17}; a proposed protein interaction surface^{18,19} (Fig. 2c). No inactive hT5Cyp proteins mapped to this region (Fig. 2c). Visualization of hT5Cyp fusions by immunofluorescence showed that AoT5Cyp and all three hT5Cyps with potent anti-HIV-1 activity formed distinct cytoplasmic bodies (Fig. 2d). Diffuse cytoplasmic staining was

observed for hT5Cyp fusions that lacked restriction activity. Taken together, these results demonstrate strict conformational requirements for antiviral activity that are determined by the site of CypA fusion and revealed by the ability of the fusion proteins to form cytoplasmic bodies.

hT5Cyp restriction activity depends on both the hCypA and hT5 components

[00350] AoT5Cyp restricts HIV-1_{NL4-3}, HIV-2_{ROD}, SIV_{AGMTan}, and FIV_{PET}, all viruses encoding a CA that binds CypA^{3,5,13,14,20-22}. To evaluate whether hT5Cyp blocked infection by these lentiviruses, stable cell lines were generated using CRFK cells, feline fibroblasts that lack endogenous T5. AoT5Cyp and hT5Cyp restricted all four lentiviruses (Fig. 3a-d). CypA does not bind to SIV_{MAC251} CA and neither AoT5Cyp nor hT5Cyp restrict this virus (Fig. 3e).

[00351] To confirm that hT5Cyps have the same CA-binding requirements as AoT5Cyp, the CRFK cell lines were challenged with HIV-1-G89V, a CA mutant that disrupts interaction with CypA³. Both AoT5Cyp and hT5Cyp failed to restrict HIV-1-G89V (Fig. 4a). The H126Q mutation in CypA also disrupts the CA-CypA interaction²³ and hT5Cyp bearing the H126Q mutation in its CypA domain had no detectable anti-HIV-1 restriction activity (Fig. 4b). Finally, cyclosporine, a competitive inhibitor of the CypA-CA interaction¹⁴, abrogated HIV-1 restriction by AoT5Cyp and hT5Cyp (Fig. 4c). A previous study searched the database for CA-variants circulating in HIV-1-infected people that are resistant to cyclosporine and to AoT5Cyp-mediated HIV-1-restriction²⁴. Though V86P/H87Q/I91V/M96I HIV-1 was indeed resistant to cyclosporine, both hT5Cyp and AoT5Cyp blocked infection by viruses bearing this sequence as efficiently as standard HIV-1 clones (Fig. 4d).

[00352] To assess the contribution of the T5 domain to T5Cyp-mediated restriction, CRFK cell lines expressing AoT5Cyp or hT5Cyp were treated with arsenic trioxide (As₂O₃). This drug was previously shown to inhibit T5-mediated restriction²⁵. AoT5Cyp and hT5Cyp restriction activity was blocked to the same extent by As₂O₃ (Fig. 4e). These results show that, with respect to CA-recognition by the CypA domain, and effector function by the T5 domain, AoT5Cyp and hT5Cyp behave nearly identically.

hT5Cyp eliminates spreading infection of HIV-1

[00353] Jurkat T cells stably transduced with T5-expression vectors were infected with replication-competent HIV-1. No HIV-1 replication was detected in cells transduced with hT5Cyp or AoT5Cyp for the two-month observation period (Fig. 5a, b). The same result was obtained 8 separate times, despite initiation of infection with a wide-range of HIV-1 inocula. HIV-1 replication in cells transduced with hT5Cyp bearing a mutation that blocks binding to HIV-1 CA (CypA-H126Q, see Fig. 4c and ref. 23), was as rapid as in cells transduced with empty vector (Fig. 5a, b).

[00354] In contrast to hT5 α , rhT5 α potently restricts HIV-1². Chimeric T5 protein in which the PRYSPRY specificity determinant from the rhesus orthologue replaces the corresponding human residues (hT5 α -R323-332^{10,11}), or even in which a single, critical amino acid was substituted (R332P^{12,26}), exhibit HIV-1 restriction activity approaching that of rhT5 α . Since these molecules are nearly identical to the human protein it has been proposed that they may have therapeutic utility^{10,12,26,27}. As previously reported, hT5 α -R323-332 and hT5 α -R332P caused moderate inhibition of HIV-1 transduction in single-cycle infection, each behaving similarly to the other, though neither was as potent as hT5Cyp or AoT5Cyp. When the effect of hT5 α R332P was tested in a spreading infection, there was a delay in the peak of HIV-1 that was inversely proportional to initial viral dose, but in contrast to hT5Cyp or AoT5Cyp, it failed to prevent HIV-1 infection (Fig. 5a, b).

[00355] hT5Cyp-mediated restriction of HIV-1 in primary human CD4⁺ T cells and macrophages was assessed next. To identify cells transduced with T5, a bicistronic lentiviral vector was used that directs synthesis of GFP from an internal ribosome entry site. These vectors failed to generate GFP in primary CD4⁺ T cells (Fig. 5d). Better results were obtained when a vector was engineered using two promoters, one from the SFFV LTR and the other from the hCypA gene (Fig. 5c, d). Primary CD4⁺ T cells transduced with dual-promoter vectors bearing hT5Cyp were sorted based on GFP-expression. Transduced cells proliferated at the same rate as untransduced cells, produced comparable amounts of IL-2 upon stimulation, and expressed the same level of cell-surface CD4, CXCR4, and MHC I.

[00356] CD4⁺ T cells were transduced with dual-promoter vectors encoding either hT5Cyp or the non-restrictive hT5CypH126Q. Cells were sorted based on GFP-expression and then challenged with replication-competent, CXCR4-tropic, HIV-1_{NL4-3}. Viral replication in hT5CypH126Q-transduced cells peaked on day 12 and infection was not established in

hT5Cyp-transduced cells (Fig. 5e). The same hT5Cyp-mediated block to infection was observed with primary isolate HIV-1_{DH12}. Monocyte-derived macrophages were also transduced with vectors encoding hT5Cyp or hT5CypH126Q and sorted based on GFP-expression. Cells were infected with HIV-1_{NL4-3}, modified to be CCR5-tropic, or with primary isolate HIV-1_{132W}. As with the CD4⁺ T cells, macrophages transduced with hT5Cyp were resistant to HIV-1 infection (Fig. 5f).

hT5Cyp confers selective advantage to CD4⁺ T cells challenged with HIV-1

[00357] The previous experiments showed that HIV-1 infection is blocked when all cells in the culture have been transduced with the hT5Cyp vector. Next, anti-HIV-1 efficacy was evaluated under suboptimal conditions, in which < 25% of cells were transduced with hT5Cyp. In the absence of HIV-1 infection, the percent transduced cells persisting in the culture over the course of a month was equivalent for hT5Cyp and hT5CypH126Q. In the face of HIV-1 infection, the hT5CypH126Q cells declined to <10% of the cells in the culture. hT5Cyp cells, in contrast, had a distinct advantage, expanding to 75% of the cells in the population (Fig. 5g). The survival advantage was most likely due to the antiviral activity of hT5Cyp since HIV-1 replication was also reduced in cultures containing hT5Cyp cells. Thus, even when a minority of cells are transduced in culture conditions where HIV-1 can spread directly from cell-to-cell, hT5Cyp is potent enough to block HIV-1 infection.

hT5Cyp protects against HIV-1 infection *in vivo*

[00358] To test the ability of hT5Cyp to protect against HIV-1 infection *in vivo*, an experimental model was established in which human CD4⁺ T cells were adoptively transferred into Rag2^{-/-}γc^{-/-} mice. Due to a lack of functional B, T, and NK cells, this mouse strain does not reject xenografts²⁸. CD4⁺ T cells were transduced with the hT5Cyp or hT5CypH126Q vectors and sorted for GFP-expression. Age-matched, 6-10 week-old Rag2^{-/-}γc^{-/-} mice were conditioned with clodronate and sublethal irradiation²⁹, and injected intraperitoneally with 2.5 x 10⁶ transduced CD4⁺ T cells (Fig. 6a). One week post-transplant, peripheral blood engraftment was assessed by measuring the percentages of CD45, CD4, and GFP-positive cells. Engraftment efficiency was not altered by hT5Cyp or hT5CypH126Q.

[00359] To determine if hT5Cyp protected hCD4⁺ T cells against HIV-1 *in vivo*, mice were infected with HIV-1 after adoptive transfer of the transduced CD4⁺ T cells. Since HIV-1 downregulates cell-surface expression of CD4³⁰, CD45 was used to assess the percentage of

human cells in the peripheral blood four weeks post-infection with HIV-1. When mice received hT5Cyp-transduced CD4⁺ T cells, mean peripheral blood CD45⁺ cells constituted 6.7% (Fig. 6b). This was 15-fold higher than the percentage CD45⁺ cells (mean 0.45%) observed in mice transplanted with hT5CypH126Q-transduced CD4⁺ T cells, a difference that was statistically significant ($p=0.0037$, $n=9$ hT5Cyp, $n=8$ hT5CypH126Q) (Fig. 6b). Mice engrafted with hT5Cyp-expressing CD4⁺ T cells had a 22-fold reduction in mean viral load compared to mice engrafted with hT5CypH126Q-expressing CD4⁺ T cells ($p=0.03$; $n=5$ hT5Cyp, $n=4$ hT5CypH126Q). Taken together, these results from the peripheral blood indicate that hT5Cyp inhibits HIV-1 replication and protects against HIV-1-associated destruction of CD4⁺ T cells *in vivo*.

[00360] Further evidence for the *in vivo* effects of hT5Cyp was sought by examining lymphoid organs of HIV-1-infected mice. Single-cell suspensions were analyzed for the human T cell marker CD3 (which, unlike CD4, is not downregulated by HIV-1), the hT5Cyp vector reporter GFP, and HIV-1 p24 antigen. Few if any of the hT5Cyp-transduced human T cells in thymi or mesenteric lymph nodes of HIV-1-infected mice were p24⁺, as compared with 3-5% of the cells transduced with hT5CypH126Q (Fig. 6c). Consistent with this inhibition of productively infected T cells, hT5Cyp prevented the down-regulation of cell surface CD4, both in thymi and in mesenteric lymph nodes of mice infected with CXCR4-tropic or CCR5-tropic HIV-1, respectively (Fig. 6d).

[00361] Finally, tissue sections of thymi and mesenteric lymph nodes were directly examined for human T cells (using anti-CD3 antibody) and HIV-1 p24 antigen. The engraftment of CD4⁺ T cells transduced with hT5Cyp and hT5CypH126Q was similar (Fig. 6e). Intense p24 staining was observed in tissue sections from animals engrafted with hT5CypH126Q-transduced CD4⁺ T cells but not in tissues from mice engrafted with CD4⁺ T cells transduced with hT5Cyp (Fig. 6e, α -p24 stain). These experiments show that hT5Cyp prevented productive HIV-1 infection *in vivo*.

DISCUSSION

[00362] Anti-HIV-1 gene therapy approaches have followed two main strategies: either modification of host-cell factors required for viral replication or inhibition of essential viral elements¹. Here, a third approach was adopted: the exploitation of natural inhibitors that evolved over millions of years in primates in response to retroviral attack^{10-12,26}. The potent

block to HIV-1 observed with AoT5Cyp inspired the design of a human equivalent, hT5Cyp, that robustly blocks HIV-1 *in vitro* and *in vivo*. The strict structural requirements for engineering a functional hT5Cyp emphasize the remarkable nature of the fusion gene that was generated by retrotransposition in New World owl monkeys³. As it turns out, a distinct T5Cyp fusion gene was generated by an independent retrotransposition event in Old World macaques. In this case, restriction activity was detected against HIV-2 and FIV, but not against HIV-1³¹. The convergent evolution of T5Cyp fusion proteins with distinct retroviral specificities indicates a strong selection for these potent restriction factors. While the specific force behind selection remains to be elucidated for individual T5 orthologues, in each case the selective pressure is likely to have been a retrovirus¹¹.

[00363] Since T5 α and T5Cyp are modular proteins, it was expected that the engineering of a restrictive hT5Cyp would be trivial. Surprisingly, only three of 13 hT5Cyp fusions inhibited HIV-1 comparably to AoT5Cyp. No correlation was observed between protein levels and antiviral activity, and CA-binding activity was not sufficient to restrict HIV-1 (Fig. 2a,b). Structural modeling showed that anti-HIV-1 activity was observed when hCypA was fused at the apex of the hT5 α PRYSPRY domain (Fig. 2c). Analysis of nonsynonymous mutations indicates that this region undergoes some of the strongest selective pressure in the primate lineage and functional experiments pinpoint it as a specificity determinant for CA recognition^{11,12,32}. CypA fusion to this site may have antiviral activity because this apical loop is uniquely situated for coordinating CA recognition and effector domains of T5. Correlation between restriction activity and the ability of hT5Cyp fusion proteins to form cytoplasmic bodies suggests that activity requires particular spatial constraints or association with unknown cofactors.

[00364] Optimal gene therapy candidates should provide high efficacy and low antigenicity. Hematopoietic progenitors transduced with rhesus macaque T5 α or human-rhesus T5 α chimeras differentiate into HIV-1-resistant macrophages and T cells^{27,33}. These proteins, however, are potentially antigenic, which could lead to the elimination of modified cells *in vivo*¹⁶. By substituting only critical amino acids within the hT5 α PRYSPRY domain one can reduce the risk of antigenicity but this also decreases anti-HIV-1 efficacy (hT5 α R332P; Fig. 5a,b). hT5Cyp potently inhibits HIV-1 and, being composed of human components, is of low antigenic potential.

[00365] HIV-1 *rev*, *tat*, and *gag*^{1,34}, as well as the HIV-1 co-receptor CCR5^{35,36}, have been targeted by various means including siRNAs^{1,34} and zinc-finger endonucleases³⁶. With either approach there are concerns regarding toxicity³⁴, off-target effects³⁵, and viral resistance^{1,34}. For siRNA approaches, escape mutants arise readily, since single point mutations are sufficient to escape the siRNA-mediated blocks to HIV-1 replication³⁴. CCR5 disruption could select for CCR5-independent viruses³⁷ and is not without consequence, as the CCR5Δ32 allele is a risk factor for symptomatic West Nile Virus infection³⁸.

[00366] hT5Cyp is a broadly acting, anti-lentiviral agent that blocks CCR5- and CXCR4-tropic HIV-1 clones and primary isolates, as well as some HIV-2, SIV, and FIV clones (Figs. 4 and 5). T5 isoforms form hetero-multimers and hT5Cyp might interfere with endogenous hT5α function³⁹. However, no significant decrease in hT5α-mediated restriction of N-MLV, and no alteration in cell surface markers or cytokine secretion, was observed in hT5Cyp-transduced cells. hT5Cyp-resistant viruses were not detected, perhaps due to the potency of the antiviral activity and the fact that hT5Cyp blocks HIV-1 prior to reverse transcription. hT5Cyp additionally blocked a natural HIV-1 CA variant reported to be resistant to AoT5Cyp²⁴ (Fig. 4d). These results suggest that, if hT5Cyp were utilized as anti-HIV-1 gene therapy in people, viral resistance would not emerge and host cell function would remain intact.

[00367] The evaluation of HIV-1 therapies is limited by a lack of adequate animal models for HIV-1 replication and pathogenesis. The hCD4⁺-Rag2^{-/-}γc^{-/-} mouse developed here proved to be a robust model for assessing inhibition of HIV-1 infection and CD4⁺ T cell protection by lentiviral transduction with hT5Cyp (Fig. 6). Autotransfusion of *ex vivo* expanded CD4⁺ T cells is of clinical benefit to HIV-1-infected people⁴⁰. Thus, given that hT5Cyp-transduced CD4⁺ cells can be expanded *in vitro* and *in vivo* (Fig. 5 and 6), and that these cells exhibit a selective advantage during HIV-1 infection (Fig. 5g), one would expect an impressive effect of hT5Cyp-transduction on autologous CD4⁺ T cell gene therapy in the clinical setting. CD34⁺ hematopoietic stem cells transduced with state-of-the-art lentiviral vectors (see Fig. 5c and ref⁴¹) achieve long-term engraftment of Rag2^{-/-}γc^{-/-} mice, but transgene expression in the mature CD4⁺ T cells that develop within these animals has not been consistently detected. With improved transduction methods it will be exciting to examine the effect of stem cell transduction with hT5Cyp on subsequent immune cell

development and HIV-1 infection within one of the humanized mouse models currently under development⁴².

EXAMPLE 5: HIV-1 INHIBITION BY HUMANIZED TRIMCyp

[00368] TRIM5 α is a host factor in humans and other primates that blocks retroviral infection in a capsid (CA)-specific manner. Binding to CA requires the C-terminal PRYSPRY domain. Uniquely in owl monkeys, the PRYSPRY domain of TRIM5 is replaced by the HIV-1 CA-binding protein cyclophilin A (CypA). The resulting TRIMCyp fusion protein potently blocks HIV-1 infection (Nature 430:569). Experiments were designed to engineer a potent anti-HIV-1 fusion protein in which human CypA was fused to human TRIM5 (hTRIMCyp). Of 14 fusion proteins, five had HIV-1 restriction activity comparable to that of owl monkey TRIMCyp; the other 9 lacked detectable HIV-1 restriction activity. The site of the CypA fusions was visualized on a three-dimensional model of the TRIM5 PRYSPRY domain. hTRIMCyps with restriction activity were found to have the CypA moiety fused to the putative CA interaction face of the protein. Functional hTRIMCyps exhibited strong restriction activity in multiple cell types, and against FIV and SIVAGMtan, two other viruses known to be restricted by owl monkey TRIMCyp. hTRIMCyp restriction activity was disrupted by As2O3 and by factors that disrupt the hTRIMCyp-CA interaction, including cyclosporine, CA mutant G89V, or CypA mutant H126Q. In ongoing experiments, viruses capable of escaping hTRIMCyp-mediated restriction are being sought, and hTRIMCyp is being tested for restriction activity in primary human cells and in a humanized mouse model for HIV-1 infection.

[00369] Figure 7A shows the site of fusion of human CypA to human TRIM5 generating hTRIMCyps. Fusions restricting HIV-1 are depicted in red, those without restriction activity are in green. As shown in Figure 7B, hTRIMCyps were fused to an N-terminal 3xFLAG tag, transfected into HEK 293T cells, immuno-precipitated with an anti-FLAG antibody, and probed in western blot with an anti-CypA antibody. All nomenclature is TRIM5 AA to which CypA is fused as described for Figure 7. Figure 7C shows superimposition of site of hTRIMCyp fusion onto a 3-D model of the TRIM5 PRYSPRY domain. Red = restricts HIV-1, green = does not restrict HIV-1.

[00370] Cells (Jurkat T cells (Figure 8A), THP-1 cells (Figure 8B), PMA-matured THP-1 cells (Figure 8C), and CRFK cells (Figure 8D) were transduced with indicated

TRIMCyp fusion proteins, selected with puromycin, and challenged after 48 hrs. with an HIV-1 vector carrying the GFP gene. Infected cells were counted by flow cytometry. All data presented are representative of three experiments.

[00371] CRFK cell lines expressing TRIMCyp fusion proteins were challenged with FIV (Figure 9A), SIVAGMtant (Figure 9B), and SIVmac239 (Figure 9C) vectors carrying the GFP gene. Infected cells were counted by flow cytometry. Lentiviruses restricted by Ao TRIMCyp are also restricted by hTRIMCyp.

[00372] As shown in Figure 10A, G89V mutation in CA abrogates HIV-1 restriction by TRIMCyp in CRFK cell lines. Figure 10B shows disruption of the CA-TRIMCyp interaction with Cyclosporine abrogates HIV-1 restriction in CRFK cell lines. Figure 10C shows the H126Q mutation in the CypA moiety of TRIMCyp abrogates the CA-TRIMCyp interaction and HIV-1 restriction in CRFK cell lines.

[00373] Jurkat T cell lines expressing the indicated proteins were infected with NL4-3 GFP IRES nef virus. Infection was monitored at given time points using flow cytometry, and results are presented in Figure 11.

[00374] A description of a humanized mouse model for HIV-1 infection is shown in Figure 12A. hHSC-transduction efficiency using a SIN HIV-1 vector carrying the GFP gene is shown in Figure 12B.

EXAMPLE 6: HUMAN T5CYP IS SUPERIOR TO TRIM5 α HUMAN/RHESUS CHIMERA AND NON-CHIMERIC RHESUS TRIM5 α

[00375] The anti-HIV-1 activity of human T5Cyp was directly compared to other TRIM5 α proteins known in the art. Stremlau et al. (J Virol 79:3139-3145 (2005)) describe anti-HIV-1 activity of chimeric TRIM5 α proteins. The chimerae were generated by inserting selected amino acid sequences of rhesus monkey TRIM5 α into the sequence of human TRIM5 α or vice versa. Stremlau et al. (Nature 427:848-53 (2004)) describe cloning of full length rhesus monkey TRIM5 α and its anti-HIV-1 activity. Sayah et al. (Nature 430:569-73 (2004)) describe cloning of the Aotus (owl monkey) T5Cyp protein and its anti-HIV-1 activity.

[00376] Anti-HIV-1 activity was assessed by single cycle HIV-1 infection of CRFK feline kidney cells expressing anti-HIV-1 proteins known in the art, the human T5Cyp fusion, and controls (Figure 22). Anti-HIV-1 activity was also assessed by determining the effect on spreading HIV-1 infection in Jurkat cells (Figure 23). Notably, in both assays, hT5Cyp has significantly greater anti-HIV-1 activity than a chimeric human/rhesus monkey TRIM5 α (hT5 α R323-332) and greater activity than full length rhesus monkey TRIM5 α . Indeed, the anti-HIV-1 activity of hT5Cyp is comparable to the activity of owl monkey T5Cyp (AoT5Cyp). The comparisons show that the human T5Cyp fusion protein has superior anti-HIV-1 activity compared to the TRIM5 α chimera and the nonchimeric full length rhesus monkey TRIM5 α .

EXAMPLE 7: METHODS

[00377] The following are examples of methods that can be used in the described embodiments:

[00378] **Plasmids.** Most HIV-1 plasmids and vectors have been described previously (Berthou et al., 2004). pNL4-3 contains a complete infectious HIV-1 provirus. pNL4-3-R5 bears the V3 loop of the CCR5-tropic 92TH014-2 HIV-1 strain (Papkalla et al., 2002). To generate pNL4-3-GFP-IRES-Nef, enhanced green fluorescent protein (eGFP) and the ECMV (encephalomyocarditis virus) IRES (internal ribosome entry site) were inserted upstream of *nef*. pNL Δ envGFP is pNL4-3 with an *env*-inactivating mutation and eGFP in place of *nef*. CSGW is an HIV-1 vector expressing eGFP under the control of the spleen focus forming virus (SFFV) promoter. pFUPI, was engineered from pFUW (Lois et al., 2002) and uses the ubiquitin promoter to drive expression of a puromycin resistance cassette followed by ECMV IRES-T5. pAIG uses the SFFV promoter to drive expression of T5 followed by IRES-eGFP. In pscALPS, the SFFV promoter drives expression of T5 and the human CypA promoter drives expression of eGFP. For three-component vector production, HIV-1 *gag* and *pol* were expressed using either psPAX2, p8.9 Δ SB, p8.9 Δ SB-G89V, or p8.9 Δ SB-V86P/H87Q/I91V/M96I. p8.9 Δ SB-V86P/H87Q/I91V/M96I is p8.9 Δ SB with the V86P/H87Q/I91V/M96I mutation (Chatterji et al., 2005) introduced in CA. SIV_{mac}251 vectors were produced using GAE1.0 and the packaging vector SIV3+ (Mangeot et al., 2002). SIV_{AGM}tan and HIV-2_{ROD} vectors each have an *env*-deletion and eGFP in place of *nef* (Diaz-Griffero et al., 2006b). FIV vectors were produced using the genomic vector pGINSIN and

the packaging vector pFP93(Saenz et al., 2005). All lentiviral vectors were pseudotyped using the vesicular stomatitis virus glycoprotein (VSV-G). For biochemistry, hT5Cyps were cloned into the p3xFLAG-CMV-7.1 expression vector (Sigma) and HIV-1 CA was expressed as a GST-fusion using the EF1 α promoter.

[00379] Cells. 293T human embryonic kidney cells and CRFK feline kidney cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and glutamax (Invitrogen). Jurkat E6-1, H9, CEM-SS, and SUP-T1 T cell leukemia/lymphoma cell lines, U937 promonocytic lymphoma, and THP-1 monocytic leukemia cells were grown in RPMI-1640 with 10% FBS and glutamax (Invitrogen). CD4⁺ T cells were enriched from PBMCs (>99% CD4⁺) using positive selection magnetic beads (Miltenyi Biotec) and stimulated for 24 hrs on multisorb 96-well plates (Nunc) coated with 2 μ g/ml anti-CD3 and 2 μ g/ml anti-CD28 antibodies (BD Biosciences) in RPMI with 10% FBS, glutamax (Invitrogen) and 20 IU/ml hIL-2. When indicated and in preparation for adoptive transfer to mice, CD4⁺ T cells were stimulated using allogeneic-PBMCs irradiated at 50 Gy in the presence of 20 IU/ml IL-2 and phytohemagglutinin (PHA) at 1 μ g/ml. Cells were washed and replated 24 hrs prior to viral transduction. CD14⁺ monocytes were enriched from PBMCs (>99% CD14⁺) using positive selection magnetic beads (Miltenyi Biotec). CD14⁺ monocytes were resuspended in complete RPMI supplemented with 50 ng/ml recombinant human GM-CSF (Leukomax, Novartis) at 1 x 10⁶ cells/ml. 2 x 10⁶ were plated in each well of a 6-well plate (Nunc) and allowed to differentiate into macrophages for 10 days before transduction. Transduction of human macrophages 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. For both CD4⁺ T cells and monocyte-derived macrophages, cells were sorted for GFP expression by FACS 72 hours post-transduction.

[00380] Drugs. All chemicals were obtained from Sigma unless otherwise noted. Puromycin was used at 1 μ M. Cyclosporine (CsA) (Bedford Laboratories) was diluted to 10 mM in dimethyl sulfoxide (DMSO) and then further to 2.5 μ M in tissue culture media. Arsenic trioxide (As₂O₃) was made at 100 mM in 1N sodium hydroxide (NaOH) and then further diluted to 1 mM in phosphate buffered saline (PBS). pH was adjusted to 7.5 with hydrochloric acid and stored at 4°C.

[00381] Virus production. Vectors and viruses were produced by transfection of 293T cells using Lipofectamine 2000 (Invitrogen). Two-part vectors were produced by co-

transfection of viral genome and VSV-G plasmids at a ratio of 7:1. Three-part vectors were produced by co-transfection of viral genome, *gag-pol*, and VSV-G plasmids at a ratio of 4:3:1. Infectious viruses were produced by transfection of 90% confluent 293T cells in a T75 flask (Nunc) with 40 µg of pNL4-3, pNL4-3-R5, or pNL4-3-GFP-IRES-Nef. Supernatants containing viruses and vectors were cleared by 400 x g centrifugation, filtered (0.45 µm; Pall Acrodisc), tested for exogenous RT activity (Sayah et al., 2004) titrated on Jurkat E6 cells in single cycle assay (Sayah et al., 2004) and stored at -80°C. When indicated, the content of viral p24 antigen was quantified by an HIV-1 p24 enzyme-linked immunoabsorbent assay kit (NIH ARRRP).

[00382] Infections. 2×10^4 adherent cells/well or 8×10^4 suspension cells/well were plated in 48-well plates (Nunc) for challenge with single-cycle GFP reporter vectors that were titrated as indicated. GFP synthesis was assessed by flow cytometry 48 hrs post-infection. For spreading infection, 10^6 Jurkat cells were infected with 1 to 15 ng p24/ 10^6 cells of HIV-1_{NL4-3-GFP-IRES-Nef}. Cells were split every 3 days, fixed in 0.5% paraformaldehyde/PBS, and assayed for GFP synthesis by flow cytometry. For infection of 10^6 primary CD4⁺ T cells or macrophages, 15 ng p24/ 10^6 cells of a CXCR4- or CCR5-tropic HIV-1_{NL4-3} stock was used, and infection was monitored by assessing the accumulation of RT activity in the supernatant (Sayah et al., 2004). FACSCalibur, Cellquest Pro (Becton Dickinson), and FlowJo software (Treestar, Inc.) were used to record and analyze fluorescence. $1-5 \times 10^5$ events were acquired for analysis.

[00383] Western Blotting and Immunoprecipitation Assays. To detect hT5Cyp, 5×10^6 293T cells were transfected with 20 µg of each 3xFLAG-hT5Cyp plasmid using Lipofectamine 2000 (Invitrogen). Cells were lysed at 48 hrs in Triton lysis buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCL, 1% Triton X-100, with Complete Mini protease inhibitor cocktail (Roche)), cleared at 15,000 x g for 5 minutes, incubated 4 hours at 4°C with 2 µg/ml anti-FLAG M2 monoclonal antibody (Sigma), and then incubated 1 hour at 4°C with 20 µl of protein G-sepharose beads (GE-Amersham). The beads were washed 3 times with 1 mL Triton Lysis Buffer, and 20 µl immunoprecipitate was subjected to SDS-PAGE on a 12% gel, transferred to a PVDF membrane, and immunoblotted with rabbit polyclonal antibody to CypA (Biomol).

[00384] For co-immunoprecipitation of hT5Cyp with HIV-1 CA, 5×10^6 293T cells were co-transfected as above with 20 μ g p3xFLAG hT5Cyp and 20 μ g of pEF1-GST-CA. At 48 hrs cells were lysed with RIPA Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate, with Complete Mini protease inhibitor cocktail (Roche)) and accelerated at 15,000 x g for 5 min. Lysate was divided into two equal parts and incubated on ice for 30 minutes with 20 μ M CsA/DMSO or an equivalent volume of DMSO for 30 minutes. Lysates were incubated with 30 μ l glutathione-sepharose beads (GE-Amersham) for 1 hour at 4°C. Beads were washed three times with RIPA buffer. Proteins were subjected to SDS-PAGE and immunoblotting with rabbit polyclonal anti-CypA antibody (Biomol) and goat polyclonal anti-HIV-1 CA (NCI).

[00385] Structural model of the hT5 α PRYSPRY domain. StruPro was used to superimpose and align the crystal structures for PRYSPRY-19q13.4.1 (Protein Data Bank (PDB) entry 2FBE; residue range 11 to 184 of chain A), GUSTAVUS (PDB entry 2FNJ; residue range 35 to 83 and 88 to 233 of chain A), and TRIM21 (PDB entry 2IWG; residue range 2 to 182 of chain B), using an alpha carbon cutoff distance of 3.5 Å. ClustalX was used to combine the hT5 α sequence with the structural alignments. A ClustalW sequence-based multiple alignment of 10 characteristic psiBLAST hits of all three aforementioned sequences was used to localize insertions. A secondary structure prediction of hT5 α obtained with NPS@ was used to manually correct the model. The final alignment was then fed into the model and loop optimization procedures of Modeller 8v2, using 2FBE, 2FNJ, and 2IWG as templates. 100 models were built and evaluated for stereochemical quality with PROCHECK. Pictures of the single best model were generated with PyMol v0.98.

[00386] Immunofluorescence microscopy. CRFK cells stably expressing wild-type AoT5Cyp or hT5Cyp fusion proteins were grown on glass coverslips. Cells were fixed at 25°C for 10 min with 3.7% formaldehyde in PBS and permeabilized on ice for 2 min with 2% Triton-X100 in 0.1% sodium citrate. After quenching with 0.1 M glycine in PBS at 25°C for 10 min, cells were blocked for 30 min at 25°C with 10% fetal bovine serum and 0.1% Tween-20 in PBS, before overnight incubation with goat polyclonal anti-TRIM5 (AbCam) and mouse monoclonal anti-tubulin (SIGMA) antibodies. After extensive washing in PBS, cells were sequentially incubated with donkey-Alexa Fluor 488-anti-goat and goat-Alexa Fluor 594 anti-mouse secondary antibodies (Invitrogen) for 1 hr at 25°C in the dark. Finally coverslips were mounted in Vectashield with DAPI (Vector laboratories). Cells were

visualized with a 63x 1.4 NA Leica HCX Planapochromat oil immersion objective using an inverted Leica DMI 6000 CS microscope fitted with a TCS SP5 laser scanning confocal microscope system. Images from individual 3-dimensional stacks were subjected to noise reduction using the Leica LCS software (Leica Microsystems) and subjected to maximum intensity projection using the MetaMorph software (Universal Imaging Corp).

[00387] Mice. Rag2^{-/-}γc^{-/-} mice on a BALB/c background (provided by M. Ito; Central Institute for Experimental Animals, Kawasaki, Japan) were bred and maintained under specific pathogen-free conditions in accordance with the guidelines of the animal facility at the Institute for Research in Biomedicine. CL2MBP (clodronate) was from Roche Diagnostics (Mannheim, Germany). Preparation of liposomes containing CL2MBP was done as described previously (Van Rooijen and Sanders, 1994). 6-10 week-old mice received 100 μl of clodronate liposomes intraperitoneally (i.p.) 24 hours prior to irradiation using a single sublethal dose of 4.0 Gray (Gy) from a Cesium 137 source (Biobeam 8000, STS GmbH, Braunschweig, Germany) at 3.75 Gy/min. 4 hrs post-irradiation, mice were transplanted with 2.5 x 10⁵ hCD4⁺ T cells expressing hT5Cyp or control hT5CypH126Q in 100 μl PBS i.p.. Mice were infected with CXCR4- or CCR5-tropic HIV-1_{NL4-3}, as previously described (Baenziger et al., 2006), either 5 or 14 days post-transplant and analyzed 2 or 4 weeks post-infection. CD4⁺ T cell engraftment and maintenance in mice was measured by flow cytometry. To obtain peripheral blood cells and plasma, mice were bled from the retro-orbital venous sinus under anesthesia and red blood cells were lysed. When sacrificed, single cell suspensions from organs were prepared. For FACS analysis monoclonal antibodies against the following antigens were used: CD3 (UCHT1), CD4 (13B8.2), CD8 (B9.11), p24 (clone KC57) (all Immunotech/Beckman Coulter, Marseille, France), CD45 (HI30) (Caltag, Burlingame, CA). Intracellular staining for p24 was performed on single cell suspensions from mesenteric lymph nodes and thymus following staining with anti-CD3 antibody. Cells were washed, permeabilized, and fixed by treatment with BD Cytotfix/Cytoperm (BD Pharmingen) and stained with anti-p24 antibody in BD Permwash solution (BD Pharmingen) following the manufacturer's instructions. Plasma HIV RNA concentrations were determined by Cobas Amplicor RT-PCR assay (Roche Diagnostics, Basel, Switzerland). Immunohistochemical stainings were performed as previously described (Baenziger et al., 2006). To assess nonspecific binding, tissue from untransplanted and uninfected transplanted mice was stained as controls.

[00388] Proliferation of hT5Cyp-expressing primary human CD4 T cells.

Activated, scAPLS-transduced primary human CD4⁺ T cells were maintained in culture for 6 days. 1×10^5 cells were transferred in triplicate into 96-well flat-bottom microplates (Falcon) and labeled 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.

[00389] 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 Permashield solution following the manufacturer's instructions (all reagents BD/Pharmingen).

[00390] 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).

[00391] Infections. CD4⁺ T cells and monocyte-derived macrophages were infected with low passage derivatives of primary isolates HIV-1_{DH12} and HIV-1_{132W}, respectively. CD4⁺ T cells and monocyte-derived macrophages had been previously transduced with T5Cyp- and T5CypH126Q scALPS and sorted for GFP expression. Infection was monitored by assessing the accumulation of RT activity in the supernatant at the indicated day post-infection, at which point CD4⁺ cells were split and macrophages were fed as required.

[00392] Real-Time RT-PCR. Total RNA was extracted from 5×10^6 CD4⁺ T cells using the RNeasy Plus Mini kit (Qiagen). RNA was treated with RNase-free DNase I (Ambion) and reverse transcribed using the SuperScriptTM 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).

[00393] qPCR for HIV-1 DNA. Total cellular DNA was extracted from 2×10^6 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).

[00394] Statistical Analysis. Significant differences between groups was calculated using the Mann-Whitney U test . *P*-values < 0.05 were considered significant.

[00395] Primers and Sequences:

Human TRIM5 α (AY625000)

Primers used to clone hT5 α , hT5 α _{R332P}, hT5 α _{R322-332}:

hTRIM5a XbaI 5'

ccctctagagccaccATGGCTTCTGGAATCCTGGTTA (SEQ ID NO:36)

hTRIM5a NotI 3'

aaaagcggccgcTCAAGAGCTTGGTGAGCACAGAGT (SEQ ID NO:37)

Cloned into pcDNA3.1 (-), sequenced, sub-cloned into scALPS-puro Xba-Pme/Pme.

RhTRIM5 α (NM 001032910)

Primers used to clone RhTRIM5 α :

RhTRIM5a XbaI 5'

ccctctagagccaccATGGCTTCTGGAATCCTGCTTA (SEQ ID NO:38)

RhTRIM5a HpaI 3'

aaaagttaacTCAAGAGCTTGGTGAGCACA (SEQ ID NO:39)

Cloned into pcDNA3.1 (-) Xba-Hpa/EcoRV, sequenced, sub-cloned into scALPS-puro Xba-Pme/Pme

AoTRIMCyp (AY646198):

AoTRIM5Cyp XbaI 5'

ccctctagagccaccATGGTCAACCCACCGTGTT (SEQ ID NO:40)

AoTRIM5Cyp BamHI 3'

AaaaggatccTTATTAGAGTTGTCCACAGTCAGC (SEQ ID NO:41)

hTRIMCyp:

External primers:

hTRIM5Cyp XbaI 5'

ccctctagagccaccATGGCTTCTGGAATCCTGGTTA (SEQ ID NO:42)

hTRIM5Cyp BamHI 3'

aaaaggatccTTATTCGAGTTGTCCACAGTC (SEQ ID NO:43)

*Internal primers (nested PCR):*M244:

CGGCTGCAGGGGTCAGTGATGGTCAACCCACCGTGTTT (SEQ ID NO:44)
 GAACACGGTGGGGTTGACCATCACTGACCCCTGCAGCCG (SEQ ID NO:45)

M284:

GCTCCTGATCTGAAAGGAATGGTCAACCCACCGTGTTT (SEQ ID NO:46)
 GAACACGGTGGGGTTGACCATCCTTTTCTGATCAGGAGC (SEQ ID NO:47)

T302:

CGCTACTGGGTTGATGTGACAGTCAACCCACCGTGTTT (SEQ ID NO:48)
 GAACACGGTGGGGTTGACTGTCACATCAACCCAGTAGC (SEQ ID NO:49)

S309:

GTGGCTCCAAACAACATTTCTGATCAACCCACCGTGTTT (SEQ ID NO:50)
 GAACACGGTGGGGTTGACTGAAATGTTGTTTGGAGCCAC (SEQ ID NO:51)

S322 (= hT5Cyp):

CAAGTGAGCTCTGTCAACCCACCGTGTTT (SEQ ID NO:52)
 GGGGTTGACAGAGCTCACTTGTCTCTTATCTTCAG (SEQ ID NO:53)

A331:

CCACAGATAATATATGGGGCAGTCAACCCACCGTGTTCTTC (SEQ ID NO:54)
 CACGGTGGGGTTGACTGCCCCATATATTATCTGTGGTTTTCG (SEQ ID NO:55)

G357:

GCTCTCAAAGTATCACATCAGGGGTCAACCCACCGTGTTCTTC (SEQ ID NO:56)
 GAAGAACACGGTGGGGTTGACCCCTGATGTGATACTTTGAGAGC (SEQ ID NO:57)

T369:

GAGGTAGACGTGTCCAAGAAACTGTCAACCCACCGTGTTCTTC (SEQ ID NO:58)
 GAAGAACACGGTGGGGTTGACAGTTTTCTTGGACACGTCTACCTC (SEQ ID NO:59)

G398:

GAAAATTATCAACCTAAATACGGCGTCAACCCACCGTGTTCTTC (SEQ ID NO:60)
 GAAGAACACGGTGGGGTTGACGCCGTATTTAGGTTGATAATTTTC (SEQ ID NO:61)

AoT5Cyp, hT5Cyp, and hT5CypH126Q were cloned into FUPI Xba/Bam and sequenced;
 then subcloned from FUPI into scALPS-puro Xba-Hpa/Pme

Target sequences for APM KD vector:**CypA (3' UTR)**

Ctggattgcagagttaagtta (SEQ ID NO:62)

TRIM5 (CDS) "T2"

tgccaagcatgcctcactgcaa (SEQ ID NO:63)

TRIM5alpha (3' UTR) "T8"

tcgtgtgataattgttcaccaa (SEQ ID NO:64)

Primers used to clone V86P/H87Q/I91V/M96I CA into p8.9ΔNS:**Spe R:**

gggtactagtagttcctg (SEQ ID NO:65)

NotI F:

ggggcgccgctggtagagatgggtgagagcgctc (SEQ ID NO:66)

Sequence of hT5Cyp:

ATGGCTTCTGGAATCCTGGTTAATGTAAAGGAGGAGGTGACCTGCCCCATCTGCCTGGAACCTCTGACACAACCCC
 TGAGCCTGGACTGCGGCCACAGCTTCTGCCAAGCATGCCTCACTGCAAACCACAAGAAGTCCATGCTAGACAAAGG
 AGAGAGTAGCTGCCCTGTGTGCCGGATCAGTTACCAGCCTGAGAACATACGGCCTAATCGGCATGTAGCCAACATA
 GTGGAGAAAGCTCAGGGAGGTCAAGTTGAGCCCAGAGGGGAGAGAAAGTTGATCATTGTGCACGCCATGGAGAGAAAC
 TTCTACTCTTCTGTGTCAGGAGGACGGGAAGGTCATTTGCTGGCTTTGTGAGCGGTCTCAGGAGCACCGTGGTCACCA
 CACGTTCTCTACAGAGGAGGTTGCCCCGGGAGTACCAAGTGAAGCTCCAGGCAGCTCTGGAGATGCTGAGGCAGAAG
 CAGCAGGAAGCTGAAGAGTTGGAAGCTGACATCAGAGAAGAGAAAGCTTCTTGGAAGACTCAAATACAGTATGACA
 AAACCAACGTCTTGGCAGATTTTGAGCAACTGAGAGACATCCTGGACTGGGAGGAGAGCAATGAGCTGCAAAACCT
 GGAGAAGGAGGAGGAAGACATTCTGAAAAGCCTTACGAACTCTGAACTGAGATGGTGCAGCAGACCCAGTCCCTG
 AGAGAGCTCATCTCAGATCTGGAGCATCGGTGCAGGGGTCAGTGATGGAGCTGCTTCAGGGTGTGGATGGCGTCA
 TAAAAAGGACGGAGAACGTGACCTTGAAGAAGCCAGAAACTTTTCCAAAAAATCAAAGGAGAGTGTTCGAGCTCC
 TGATCTGAAAGGAATGCTAGAAGTGTTCAGAGAGCTGACAGATGTCCGACGCTACTGGGTTGATGTGACAGTGGCT
 CCAAACAACATTTTCATGTGCTGTCTTTGAAAGATAAGAGACAAGTGAGCTCTGTCAACCCACCGTGTCTTCG
 ACATTGCCGTCGACGGCGAGCCCTTGGGCGCGTCTCCTTTGAGCTGTTTGACAGCAAGGTCCCAAAGACAGCAGA
 AAATTTTCGTGCTCTGAGCACTGGAGAGAAAGGATTTGGTTATAAGGGTTCCTGCTTTCACAGAATTATTCAGGG
 TTTATGTGTGTCAGGGTGGTGACTTCACACGCCATAATGGCACTGGTGGCAAGTCCATCTATGGGGAGAAATTTGAAG
 ATGAGAACTTCATCCTAAAGCATACGGGTCTGGCATCTTATCGATGGCAAATGCTGGACCCAACACAAATGGTTC
 CCAGTTTTTCATCTGCACTGCCAAGACTGAGTGGTTGGATGGCAAGCATGTGGTGTTCGCAAAAGTGAAGAAGGC
 ATGAATATTGTGGAGGCGATGGAGCGCTTTGGGTCCAGGAATGGCAAGACCAGCAAGAAGATCACCATTGCTGACT
 GTGGACAACCTCGAATAA (SEQ ID NO:7)

SYBR Green qRT-PCR primers**18s rRNA RT 5'**

CGGCTACCATCCAAGGAA (SEQ ID NO:67)

18s rRNA RT 3'

GCTGGAATTACCGCGGCT (SEQ ID NO:68)

ZsGreen1 RT 5'

CCCCGTGATGAAGAAGATGA (SEQ ID NO:69)

ZsGreen1 RT 3'

GTCAGCTTGTGCTGGATGAA (SEQ ID NO:70)

hTC-L RT 5'

CTGGGTTGATGTGACAGTGG (SEQ ID NO:71)

hTC-L RT 3'

TCTGCTGTCTTTGGGACCTT (SEQ ID NO:72)

Taqman probes18s rRNA fwd

CGGCTACCACATCCAAGGAA (SEQ ID NO:73)

18s rRNA rev

GCTGGAATTACCGCGGCT (SEQ ID NO:74)

18s rRNA probe

5-(FAM)-TGCTGGCACCAGACTTGCCCTC-(TAMRA)-3' (SEQ ID NO:75)

CypA

Assay ID: Hs99999904_m1 (Applied Biosystems)

TRIM5 (all isoforms)

Assay ID: Hs01552558_m1 (Applied Biosystems)

TRIM5alpha (specific)

Assay ID: Hs01552552_g1 (Applied Biosystems)

HIV-1 products qPCRMitochondrial forward primer (MH533)

ACCCACTCCCTCTTAGCCAATATT (SEQ ID NO:76)

Mitochondrial reverse primer (MH534)

GTAGGGCTAGGCCACCG (SEQ ID NO:77)

Mitochondrial probe (mito-probe)

5'-(TET)-CTAGTCTTTGCCGCCTGCGAAGCA-(TAMRA)-3' (SEQ ID NO:78)

J1 RT forward

acaagctagtaccagttgagccagataag (SEQ ID NO:79)

J2 RT reverse

gccgtgcgcgcttcagcaagc (SEQ ID NO:80)

RT probe (LRT-P)

5'-(FAM)-CAGTGCGCGCCGAACAGGGA-(TAMRA)-3' (SEQ ID NO:81)

2-LTR forward (MH535)

AACTAGGGAACCCACTGCTTAAG (SEQ ID NO:82)

2-LTR reverse (MH536)

TCCACAGATCAAGGATATCTTGTC (SEQ ID NO:83)

2-LTR probe (MH603)

5'-(FAM)-ACACTACTTGAAGCACTCAAGGCAAGCTTT-(TAMRA)-3' (SEQ ID NO:84)

Alu forward (MH535)

AACTAGGGAACCCACTGCTTAAG (SEQ ID NO:85)

Alu reverse (SB704)

TGCTGGGATTACAGGCGTGAG (SEQ ID NO:86)

Alu probe (MH603)

5'-(FAM)-ACACTACTTGAAGCACTCAAGGCAAGCTTT-(TAMRA)-3' (SEQ ID NO:87).

EXAMPLE 8: Potent inhibition of HIV-1 by TRIM5-cyclophilin fusion proteins engineered from human components

[00396] Abstract: New World monkeys of the genus *Aotus* synthesize a TRIM5-cyclophilin A fusion protein (AoT5Cyp) that potently blocks HIV-1 infection. We attempted to generate an HIV-1 inhibitor modeled after AoT5Cyp by fusing human CypA to hT5 (hT5Cyp). Of 13 constructs, only three had significant HIV-1-inhibitory activity. Activity required capsid-binding by hCypA and correlated with hCypA linkage to the hT5 α capsid-specificity determinant and the ability to form cytoplasmic bodies. CXCR4- and CCR5-tropic HIV-1 clones and primary isolates were inhibited by hT5Cyp, as were HIV-2_{ROD}, SIV_{AGMtan}, FIV_{PET}, and a circulating HIV-1 isolate previously reported as AoT5Cyp-resistant. Anti-HIV-1 activity of hT5Cyp was surprisingly better than that of the well-characterized rhesus T5 α , especially in T cells. hT5Cyp blocked HIV-1 infection of primary CD4⁺ T cells and macrophages, and conferred a survival advantage to these cells without disruption of host cell function. Extensive attempts to elicit HIV-1 resistant to hT5Cyp were unsuccessful. Finally, Rag2^{-/-} γ_c ^{-/-} mice were engrafted with hCD4⁺ T cells that had been transduced by optimized lentiviral vectors bearing hT5Cyp; upon challenge with HIV-1, hT5Cyp decreased viremia and productive infection in lymphoid organs and preserved hCD4⁺ T cells. Thus, hT5Cyp is an extraordinarily robust inhibitor of HIV-1 replication and a promising anti-HIV-1 gene therapy candidate.

[00397] **Introduction:** Over 60 million people have been infected with HIV-1 and nearly half of these people have died as a consequence of this infection (1). Despite the discovery of HIV-1 25 years ago, it continues to kill several million people each year. In theory, the clearest path to the elimination of the HIV-1 pandemic would be an effective, anti-HIV-1 vaccine. In practice, the virus has proven to be an elusive target for the immune system (2, 3). Combination antiviral therapy potently suppresses HIV-1 replication and resultant disease, but these treatments are plagued with complications and they do not eliminate the virus.

[00398] The difficulties of controlling HIV-1 have stimulated thinking about alternatives to conventional vaccination or life-long pharmacotherapy. Among these creative possibilities are anti-HIV-1 gene therapy (4, 5). Ideally, gene therapy should potently suppress HIV-1 replication without eliciting viral resistance. While all steps of the viral life cycle are potential gene therapy targets, blocking the virus before reverse transcription (RT) would preclude the genetic diversity that permits emergence of viral resistance. Additionally, targeting the virus before HIV-1 cDNA is ligated into host chromosomal DNA would prevent the virus from becoming a heritable genetic element in that cellular lineage. The discovery that certain TRIM5 (T5) orthologues inhibit HIV-1 infection immediately after the virus enters otherwise susceptible cells (6-9) raised the prospect that these host factors might be exploited in HIV-1 gene therapy.

[00399] The α -isoform of TRIM5 (T5 α) contains a C-terminal PRYSPRY domain that is required for T5 α binding to the capsid (CA) of restriction-sensitive retroviruses (10, 11). The specificity of the PRYSPRY-CA interaction determines which retrovirus a given T5 α orthologue inhibits (12). While human T5 α (hT5 α) weakly blocks HIV-1, it potently blocks N-tropic murine leukemia virus (N-MLV). In contrast, rhesus T5 α (rhT5 α) inhibits HIV-1, but N-MLV only weakly. The specificity of retroviral restriction and the modular nature of the T5 components are further demonstrated by the enhanced HIV-1 restriction activity that results when the hT5 α PRYSPRY domain is replaced with that from rhT5 α (13-16). Furthermore, anti-HIV-1 activity is observed when other TRIM family members (17) or even the murine anti-retroviral factor Fv1 (18) are fused to an HIV-1 CA-binding protein.

[00400] The T5 gene in the New World owl monkey (genus *Aotus*) is unusual. It resulted from retrotransposition of the cyclophilin A (CypA) cDNA into intron 7 (7, 19).

CypA is a CA-binding protein (20) and the *Aotus* TRIM5Cyp fusion (AoT5Cyp) prevents HIV-1 infection (7, 19). AoT5Cyp has several properties that make it appealing for gene therapy. It potently inhibits HIV-1 infection when expressed in human cells (7, 19), acting on the virus within minutes of entry (8, 9). Furthermore, AoT5Cyp is the only TRIM5 allele in 10 *Aotus* species (21), indicating that cells bearing AoT5Cyp retain functionality. One drawback of AoT5Cyp is that it is not a human protein and if employed as gene therapy it might elicit an immune response (22). Here we engineered fully human TRIM5-cyclophilin A fusion proteins (hT5Cyp) exhibiting HIV-1 restriction activity comparable to AoT5Cyp and possessing all the properties desired of anti-HIV-1 gene therapy.

[00401] Results: *Design of hT5Cyp fusions with potent anti-HIV-1 activity.* Our goal was to generate a hT5Cyp fusion modeled after AoT5Cyp using only human components. A cryptic splice acceptor at the AoT5Cyp fusion junction results in the synthesis of 12 amino acids derived from the CypA 5'UTR (7). An equivalent human fusion cannot be engineered due to lack of sequence homology in the CypA 5'UTR. Sequences from T5 γ and T5 δ , of similar length and character to the AoT5Cyp 5'UTR, were included at the T5-CypA junction, but these failed to result in proteins with anti-HIV-1 activity. hCypA was then fused directly to hT5 α at 11 different positions along its linear sequence, eliminating subsequent hT5 α sequence and resulting in hT5Cyp fusions consisting entirely of components expressed in human cells (Figure 24A). Each hT5Cyp fusion was engineered into a bicistronic lentiviral vector (FUPI, Figure 32A). Vector stocks generated for each construct were shown to have equivalent titers and then used to generate stable cell lines.

[00402] Pools of Jurkat or THP-1 cells, each bearing a different hT5Cyp construct, were tested for restriction activity against a single-cycle, HIV-1-GFP vector. Among the hT5Cyp constructs, three had activity comparable to AoT5Cyp. See representative data in Figure 24B for hT5-S322-Cyp. This fusion was used in all subsequent experiments and will be referred to as simply hT5Cyp. Data for all of the fusion constructs is shown in Figure 32. hT5Cyp also restricted HIV-1 in promonocytic U937 cells, the CEM-SS, SUP-T1, and H9 T cell lines, and the 293T embryonic kidney cell line. Like AoT5Cyp (9), hT5Cyp blocked viral cDNA accumulation after acute infection, as measured by quantitative PCR (Figure 32B).

[00403] *Structural requirements for hT5Cyp-mediated HIV-1 restriction activity.* Despite the apparent modularity of TRIM5 (13-16), most hT5Cyp fusions lacked anti-HIV-1 activity. Steady-state protein level in stably-transduced Jurkat T cells did not correlate with

restriction activity: AoT5Cyp and restrictive hT5Cyp fusions were undetectable by immunoblot while some inactive mutants were highly expressed. To increase the possibility of detection, hT5Cyp proteins were expressed as triple-FLAG-tagged fusions by plasmid transfection of 293T cells. Anti-FLAG immunoprecipitates were probed in immunoblots with anti-CypA antibody. Two fusions with no detectable restriction activity, hT5-M244-Cyp and hT5-T302-Cyp, were expressed at high-level, whereas fusions with potent anti-HIV-1 activity, were variably expressed at moderate to high-level (hT5-S309-Cyp and hT5-S322-Cyp) or at low-level (hT5-A331-Cyp and AoT5Cyp) (Figure 25A). Again, restriction activity did not correlate with protein level.

[00404] Lack of activity could result from failure to bind HIV-1 CA. Glutathione S-transferase (GST)-CA fusion protein was used to test CA-binding activity of hT5Cyp proteins produced in 293T cells. Among hT5Cyp fusions with no anti-HIV-1 activity, hT5-T302-Cyp bound HIV-1 CA as well as the potentially restrictive hT5-S322-Cyp (Figure 25B). As described for the HIV-1 CA-CypA interaction (20), specificity of binding was demonstrated by blocking the interaction using the competitive inhibitor cyclosporine A (Figure 25B). Thus, CA-binding was necessary but not sufficient for restriction activity.

[00405] To visualize the sites of CypA linkage to hT5, a three-dimensional model of the T5 α PRYSPRY domain was generated using coordinates from three homologues (Figure 25C). hT5Cyp fusions with anti-HIV-1 activity clustered near a putative, protein interaction surface (23, 24) (Figure 25C, red spheres), the main specificity determinant for HIV-1 restriction activity (15, 16, 25). No inactive hT5Cyp proteins mapped to this region (Figure 25C, green spheres). Indirect immunofluorescence showed that AoT5Cyp and all three hT5Cyps with potent anti-HIV-1 activity formed distinct cytoplasmic bodies (Figure 25D). In contrast, diffuse cytoplasmic staining was observed for hT5Cyp fusions that lacked restriction activity. These results suggest that there are conformational requirements for antiviral activity, determined by the site of CypA fusion and revealed by the ability to form cytoplasmic bodies.

[00406] *hT5Cyp restriction activity depends on both the hCypA and hT5 components.* CypA is an abundant protein with potential to inhibit hT5Cyp activity by competing for CA-binding. The magnitude restriction of a single-cycle HIV-1-GFP vector by hT5Cyp was unaffected by CypA (Figure 33D), as determined in Jurkat T cells with a stable knockdown of endogenous CypA (26). To confirm that hT5Cyps have the same CA-binding requirements

as AoT5Cyp, stable hT5Cyp lines were generated using CRFK, a feline fibroblast that lacks endogenous T5. These cells were challenged with a single-cycle, HIV-1-GFP vector, containing a mutation in CA (G89V) that disrupts interaction with CypA (7). As expected, HIV-1-G89V was less fit than wild-type HIV-1, but neither AoT5Cyp nor hT5Cyp restricted this virus (Figure 26A). The CA-CypA interaction is also disrupted by the CypA-H126Q mutation (27) and hT5Cyp bearing the H126Q mutation in its CypA domain had no detectable anti-HIV-1 restriction activity (Figure 26B). Cyclosporine, a competitive inhibitor of the CypA-CA interaction (20), abrogated HIV-1 restriction by AoT5Cyp and hT5Cyp (Figure 26C). HIV-1-V86P/H87Q/I91V/M96I, a CA-variant circulating in HIV-1-infected people, is reported to be resistant to cyclosporine and to AoT5Cyp-mediated HIV-1-restriction (28). Though HIV-1-V86P/H87Q/I91V/M96I was indeed resistant to cyclosporine, hT5Cyp blocked infection by single-cycle vectors bearing this CA variation (Figure 26D).

[00407] To assess the contribution of the T5 domain to T5Cyp-mediated restriction, CRFK cell lines expressing AoT5Cyp or hT5Cyp were treated with arsenic trioxide (As₂O₃). This drug was previously shown to inhibit T5-mediated restriction (29). AoT5Cyp and hT5Cyp restriction activity was blocked to the same extent by As₂O₃ (Figure 26E).

[00408] AoT5Cyp restricts HIV-1_{NL4-3}, HIV-2_{ROD}, SIV_{AGMTan}, and FIV_{PET}, all viruses encoding a CA that binds CypA (7, 9, 19, 20, 30-32). AoT5Cyp and hT5Cyp restricted single-cycle GFP-vectors derived from all four of these lentiviruses (Figure 27A-D). CypA does not bind to SIV_{MAC251} CA and neither AoT5Cyp nor hT5Cyp restricted a SIV_{MAC251} GFP vector (Figure 27E). These results show that, with respect to CA-recognition by the CypA domain, and effector function by the T5 domain, AoT5Cyp and hT5Cyp behave similarly.

[00409] *hT5Cyp is more potent than rhT5α.* In contrast to hT5α, the T5α orthologue from rhesus monkeys (rhT5α) potently restricts HIV-1 (6). Chimeric proteins in which critical residues from the rhT5α-PRYSPRY domain replace corresponding residues in the human orthologue exhibit HIV-1 restriction activity approaching that of rhT5α (14-16, 33, 34). These human-rhesus chimeras have been proposed as anti-HIV-1 gene-therapy candidates. Since potency is an essential consideration for any anti-HIV-1 therapy, hT5Cyp was compared side-by-side with the most promising human-rhesus chimeras. hT5α_{R332P} (16, 33) and hT5α_{rh323-332} (14, 15) transgenes, stably expressed in CRFK cells, inhibited single-

cycle HIV-1 vectors ~10-fold, approaching the anti-HIV-1 activity of rhT5 α (Figure 28A and Figure 33B). hT5Cyp, however, restricted HIV-1 almost 10-fold more potently than even rhT5 α (Figure 28A and Figure 33B). In stable Jurkat T cell lines, the relative potency of hT5Cyp, and AoT5Cyp, was even more impressive (Figure 28B and Figure 33A). Similar results were obtained in 293T cells stably expressing the indicated T5 proteins (Figure 33C).

[00410] *hT5Cyp eliminates HIV-1 spreading infection.* Jurkat T cells stably transduced with T5-expression vectors were infected with replication-competent HIV-1. hT5 α_{R332P} and hT5 $\alpha_{rh323-332}$ had minimal effect on the kinetics of HIV-1 infection (Figure 28C). rhT5 α delayed the HIV-1 replication peak by 8 days (Figure 28C). In contrast, HIV-1 replication was not detected in cells transduced with hT5Cyp or AoT5Cyp for the two-month observation period (Figure 28C and D). HIV-1 replication in cells transduced with hT5Cyp bearing a mutation that blocks binding to HIV-1 CA (CypA-H126Q, see ref. (27)), was as rapid as in cells transduced with empty vector (Figure 28C and D). In the interest of identifying HIV-1 mutations that confer resistance to hT5Cyp, HIV-1 infection was initiated on 10 separate occasions, over a range of infectious doses, in co-culture of hT5Cyp-expressing and untransduced cells, and by direct transfection of infectious HIV-1 proviral DNA into hT5Cyp-expressing Jurkat cells; no evidence of HIV-1 spreading infection was detected in the presence of hT5Cyp.

[00411] *New lentiviral vectors bearing hT5Cyp block HIV-1 infection of primary cells.* hT5Cyp-mediated restriction of HIV-1 in primary human CD4⁺ T cells and macrophages was assessed next. To identify cells transduced with hT5Cyp, attempts were made to use a bicistronic lentiviral vector that directs synthesis of GFP from an internal ribosome entry site (AIG in Figure 29A). These vectors failed to generate GFP in primary CD4⁺ T cells (Figure 29B). Better results were obtained when a vector was engineered that bears two promoters, one from the SFFV LTR and the other from the hCypA gene (scALPS in Figure 29A and B). Co-synthesis of two reporter genes, GFP and dsRED, within primary CD4⁺ T cells transduced with scALPS, was confirmed by flow cytometry (Figure 34).

[00412] Primary CD4⁺ T cells transduced with dual-promoter vectors bearing hT5Cyp or the non-restrictive hT5CypH126Q were sorted based on GFP-expression and expression of the transgenes was confirmed by real-time RT-PCR (Figure 34). Transduced cells proliferated at the same rate as untransduced cells, produced comparable amounts of IL-2 upon stimulation, and expressed the same level of cell-surface CD4, CXCR4, and MHC I

(Figure 35). Upon challenge with replication-competent, CXCR4-tropic HIV-1_{NL4-3}, viral replication in hT5CypH126Q-transduced cells peaked on day 12 and infection was not established in hT5Cyp-transduced cells (Figure 29C). The same hT5Cyp-mediated block to infection was observed with a primary isolate of HIV-1 (Figure 29D). Monocyte-derived macrophages were also transduced with vectors encoding hT5Cyp or hT5CypH126Q and sorted based on GFP-expression. Cells were infected with HIV-1_{NL4-3}, modified to be CCR5-tropic, at a high multiplicity of infection (MOI = 2). Upon decay of input virus, macrophages transduced with hT5Cyp were clearly resistant to HIV-1 infection (Figure 29E). Furthermore, macrophages transduced with hT5Cyp-encoding vectors were resistant to a primary isolate of HIV-1 (Figure 29F).

[00413] *hT5Cyp confers selective advantage to CD4⁺ T cells challenged with HIV-1.*

The previous experiments showed that HIV-1 infection is blocked when all cells in the culture have been transduced with the hT5Cyp vector. Next we evaluated anti-HIV-1 efficacy under suboptimal conditions, in which < 25% of cells were transduced with hT5Cyp. In the absence of HIV-1 infection, the percent transduced cells persisting in the culture over the course of a month was equivalent for hT5Cyp and hT5CypH126Q; in the face of HIV-1 infection, the hT5CypH126Q cells declined to <10% of the cells in the culture (Figure 29G). hT5Cyp cells, in contrast, had a distinct advantage, expanding to 75% of the cells in the population (Figure 29G). This selective advantage was mirrored in prolonged cell viability within the cultures transduced with hT5Cyp-coding vector compared to those transduced with hT5CypH126Q-expressing vector (Figure 36). The survival advantage was most likely due to the antiviral activity of hT5Cyp, since HIV-1 replication was reduced in cultures containing hT5Cyp cells (Figure 29G). Thus, even when a minority of cells are transduced in culture conditions where HIV-1 can spread directly from cell-to-cell, hT5Cyp is potent enough to block HIV-1 infection.

[00414] *hT5Cyp protects against HIV-1 infection in vivo.* To test the ability of hT5Cyp to protect against HIV-1 infection *in vivo*, two approaches analogous to those used in human gene therapy strategies were taken. In one experimental model, human CD4⁺ T cells were adoptively transferred into Rag2^{-/-}γc^{-/-} mice. This mouse strain lacks B, T, and NK cells and does not reject xenografts (35). CD4⁺ T cells were transduced with the hT5Cyp or hT5CypH126Q vectors and sorted for GFP-expression, leading to a >93% purity of GFP⁺ input cells (Figure 30A). Age-matched, 6-10 week-old Rag2^{-/-}γc^{-/-} mice were conditioned with

clodronate and sublethal irradiation (36), and injected intraperitoneally with 2.5×10^6 transduced $CD4^+$ T cells (Figure 30B). Two and six weeks post-transplant, peripheral blood engraftment was assessed by measuring the percentages of human $CD45^+CD4^+GFP^+$ cells. 50 to 90% were GFP^+ at both time points (2 weeks mean $\pm$ SEM: hT5Cyp 81.9% $\pm$ 4.5, n=8, hT5CypH126Q 73.4% $\pm$ 4.4, n=8; six weeks mean $\pm$ SEM: hT5Cyp 76.1 $\pm$ 6.0, n=8, hT5CypH126Q 76.8 $\pm$ 1.56, n=8), indicating that GFP-percentages remained stable and engraftment efficiency was not altered by hT5Cyp or hT5CypH126Q. Similar to peripheral blood, percentages of engraftment and GFP^+ cells did not differ in lymphoid organs between the two constructs used (Figure 30C). However, we consistently observed that GFP expression was higher in peripheral blood, bone marrow and spleen, compared to somewhat lower levels of GFP expression in thymus and mesenteric lymph nodes (Figure 37). The reasons for this are unclear. One can speculate that a) the minor fraction of T cells that were GFP-low/negative and thus non-transduced at transfer ($<10\%$, Figure 30A) have different biologic properties (e.g. better homing capacity to lymphoid organs as thymus and lymph nodes) compared to GFP^+ cells; or that b) GFP expression correlates with activation status of transduced, initially GFP^+ cells, with metabolically less active cells expressing lower levels or lacking GFP expression in thymus and mesenteric lymph nodes, an issue that needs to be clarified in further studies.

[00415] To assess the effect of hT5Cyp *in vivo*, mice were infected with HIV-1 after adoptive transfer of transduced $CD4^+$ T cells. When mice received hT5Cyp-transduced $CD4^+$ T cells, two weeks after HIV-1 infection the $CD4^+$ cells constituted 6% ($\pm$ 2.87%) of mean nucleated peripheral blood cells (Figure 31A). This was 34-fold higher than the percentage of $CD4^+$ cells (mean 0.18% $\pm$ 0.04%) observed in mice transplanted with hT5CypH126Q-transduced $CD4^+$ T cells, a difference that was statistically significant ($p=0.0002$, n=10 hT5Cyp, n=8 hT5CypH126Q; Figure 31A). A nine-fold reduction in mean plasma viral load was observed when mice engrafted with hT5Cyp-expressing $CD4^+$ cells were compared to mice engrafted with hT5CypH126Q-expressing $CD4^+$ T cells ($p=0.005$; n=8 hT5Cyp, n=8 hT5CypH126Q) (Figure 31B). These results indicate that hT5Cyp inhibits HIV-1 replication and protects against HIV-1-associated destruction of $CD4^+$ T cells *in vivo*.

[00416] Further evidence for the *in vivo* effects of hT5Cyp was sought by examining lymphoid organs of mice infected with HIV-1. Three weeks post-transplant, single-cell suspensions were analyzed for the human T cell marker CD3, the hT5Cyp vector reporter

GFP, and HIV-1 p24 antigen. Few if any hT5Cyp-transduced human T cells isolated from thymus or lymph node of HIV-1-infected mice were p24⁺, as compared with 1.5-6.5% of the cells transduced with hT5CypH126Q (Figure 31C). Consistent with fewer productively infected T cells, down-regulation of cell-surface CD4 was not observed with hT5Cyp-bearing cells (37), either in thymus or mesenteric lymph node of mice infected with CXCR4-tropic or CCR5-tropic HIV-1 (Figure 31D).

[00417] Tissue sections of thymus and mesenteric lymph node were directly examined for human CD3⁺ T cells and HIV-1 p24 antigen. The engraftment of the CD4⁺ T cells transduced with hT5Cyp and hT5CypH126Q was similar (Figure 31E, α -CD3). Following HIV-1 infection, intense p24 staining was observed in thymic tissue sections from animals engrafted with hT5CypH126Q-transduced CD4⁺ T cells but not in tissues from mice engrafted with CD4⁺ T cells transduced with hT5Cyp (Figure 31E, α -p24). Similar results were obtained for tissue sections from mesenteric lymph nodes (Figure 38). These experiments show that hT5Cyp significantly reduced or prevented productive HIV-1 infection *in vivo*. Given these findings, we speculate that most of the human T cells in the thymus and lymph node were indeed transduced, although levels of GFP expression were lower compared to blood, bone marrow, and spleen CD4⁺ cells.

[00418] In the second *in vivo* approach to study the effects of hT5Cyp on HIV-1 replication, CD34⁺ hematopoietic progenitor cells transduced with vectors bearing hT5Cyp or hT5CypH126Q were transplanted into pre-conditioned neonatal Rag2^{-/-} γ c^{-/-} mice (38) leading to B and T cell reconstitution in peripheral blood at 8 weeks post-transplant. The percentage of GFP⁺ cells among the CD45⁺ lymphocytes and CD19⁺ B cells that developed in the mice was similar to the percent GFP⁺ cells among the input CD34⁺ cells, but development of GFP⁺ CD4⁺ T cells was rarely observed (Figure 39A). Nonetheless, mice with hCD45⁺ cells in peripheral blood were challenged with HIV-1. 25 days post-infection, mean viremia was reduced >4-fold in mice reconstituted with CD34⁺ cells bearing hT5Cyp compared to the hT5CypH126Q control (Figure 39B; n=4 hT5Cyp, n=3 hT5CypH126Q). Moreover, while peripheral blood engraftment of hCD45⁺ remained unchanged or declined over the 25 days of HIV-1 infection, GFP⁺CD45⁺ cells selectively expanded in the context of hT5Cyp but not of hT5CypH126Q (Figure 39C).

[00419] **Discussion:** Anti-HIV-1 gene therapies have generally modified host-cell factors required for viral replication or inhibited essential viral elements (4). Here, a third

approach was adopted: the exploitation of natural inhibitors that evolved over millions of years in primates in response to retroviral attack (14-16, 33). The potent block to HIV-1 observed with AoT5Cyp inspired the design of a human equivalent, hT5Cyp, that robustly blocks HIV-1 *in vitro* and *in vivo*. As it turns out, a distinct T5Cyp fusion gene was generated by an independent retrotransposition event in Old World macaques. In this case, restriction activity was detected against HIV-2 and FIV, but not against HIV-1 (39-43). The convergent evolution of T5Cyp fusion proteins with distinct retroviral specificities indicates a strong selection for these potent restriction factors. While the specific force behind selection remains to be elucidated for individual T5 orthologues, in each case the selective pressure is likely to have been a retrovirus (15).

[00420] Since T5 α and T5Cyp are modular proteins, it was expected that the engineering of a restrictive hT5Cyp would not be difficult. Surprisingly, only three of 13 hT5Cyp fusions inhibited HIV-1 comparably to AoT5Cyp. No correlation was observed between protein levels and antiviral activity, and CA-binding activity was not sufficient to restrict HIV-1 (Figure 25A and B). Anti-HIV-1 activity was observed when hCypA was fused at the apex of the hT5 α PRYSPRY domain in the structural model (Figure 25C). Analysis of nonsynonymous mutations indicates that this region undergoes some of the strongest selective pressure in the primate lineage and functional experiments pinpoint it as a specificity determinant for CA recognition (15, 16, 44). CypA fusion to this site may have antiviral activity because this apical loop is uniquely situated for coordinating CA recognition and effector domains of T5. Correlation between restriction activity and the ability of hT5Cyp fusion proteins to form cytoplasmic bodies suggests that activity requires particular spatial constraints or association with unknown cofactors. The strict structural requirements for engineering a functional hT5Cyp emphasize the remarkable nature of the fusion gene that was generated by retrotransposition in New World owl monkeys (7).

[00421] Optimal gene therapy candidates should provide high efficacy and low antigenicity. Hematopoietic progenitors transduced with rhesus macaque T5 α or human-rhesus T5 α chimeras differentiate into HIV-1-resistant macrophages and T cells (34, 45). These proteins, however, are potentially antigenic, which could lead to the elimination of modified cells *in vivo* (22). Nonetheless, efforts have been taken to minimize potential sources of antigenicity. By substituting only critical amino acids within the hT5 α PRYSPRY domain (hT5 α _{R332P}, hT5 α _{rh332-332}) one can reduce the risk of antigenicity, but this also

decreases anti-HIV-1 efficacy (Figure 28). hT5Cyp is the most potent HIV-1 inhibitor of the T5 proteins we compared, including rhT5 α , in both single-cycle and spreading infections of human T cells with HIV-1 (Figure 28). This difference in activity might be due to the apparently higher CA-affinity of the Cyp domain of hT5Cyp (46). In addition to its superior potency, hT5Cyp is composed of human components with no amino acids derived from non-human orthologues. Despite having a novel fusion junction, hT5Cyp is likely to have the lowest antigenic potential among these T5-based gene therapies.

[00422] HIV-1 *rev*, *tat*, and *gag* (4, 47, 51), as well as the HIV-1 co-receptor CCR5 (48-50), have been targeted by various means including siRNAs (4, 50, 51) and zinc-finger endonucleases (49). With either approach there are concerns regarding toxicity (51), off-target effects (48), and viral resistance (4, 51). Transdominant revM10 proteins, have been tested in patients without reducing viremia (47). Furthermore, revM10-resistant HIV-1 strains were observed *in vitro* (52). Similarly, for siRNA approaches, escape mutants arise readily, since single nucleotide changes are sufficient to escape the siRNA-mediated blocks to HIV-1 replication (51). CCR5 disruption, one of the most promising approaches to date, could select for CCR5-independent viruses (53) and is not without consequence, as the *CCR5* Δ 32 allele is a risk factor for fatal West Nile Virus infection (54).

[00423] hT5Cyp is a broadly acting, anti-lentiviral agent that blocks CCR5- and CXCR4-tropic HIV-1 clones and primary isolates, as well as some HIV-2, SIV, and FIV clones (Figures 27 and 29). T5 isoforms form hetero-multimers and hT5Cyp might interfere with endogenous hT5 α function (55). However, no significant decrease in hT5 α -mediated restriction of N-MLV, and no alteration in cell surface markers or cytokine secretion, was observed in hT5Cyp-transduced cells (Figure 35). Despite extensive efforts to identify hT5Cyp-resistant viruses none were detected, perhaps due to the potency of the antiviral activity and the fact that hT5Cyp blocks HIV-1 prior to reverse transcription, thus precluding the generation of the genetic diversity required for emergence of resistance. hT5Cyp additionally blocked a natural HIV-1 CA variant reported to be resistant to AoT5Cyp(28) (Figure 26D). These results suggest that, if hT5Cyp were utilized as anti-HIV-1 gene therapy in people, viral resistance would not emerge readily and host cell function would remain intact.

[00424] The evaluation of HIV-1 therapies is limited by a lack of adequate animal models for HIV-1 replication and pathogenesis. The hCD4⁺-Rag2^{-/-} γ c^{-/-} mouse developed here

is a straight-forward alternative to the HIV-1 mouse models used to date (56-59). It permitted high-level HIV-1 viremia and assessment of the effect of hT5Cyp transduction on HIV-1 infection and CD4⁺ T cell protection (Figure 31). Autotransfusion of *ex vivo* expanded CD4⁺ T cells is of clinical benefit to HIV-1-infected people (60). Human CD4⁺ T cells can be transduced at high efficiency and expanded to >10⁹ cells *ex vivo*. Given that hT5Cyp-transduced CD4⁺ cells can be expanded *in vitro* and *in vivo* (Figures 29 and 30), and that these cells exhibit a selective advantage during HIV-1 infection (Figures 29G and 31A), there might be an impressive effect of hT5Cyp-transduction on autologous CD4⁺ T cell gene therapy in the clinical setting.

[00425] CD34⁺ hematopoietic stem cells transduced with the state-of-the-art lentiviral vectors described here and elsewhere (61) achieve long-term engraftment of Rag2^{-/-}γc^{-/-} mice, but transgene expression in the mature CD4⁺ T cells that develop within these animals has not been consistently detected. Nonetheless, hT5Cyp modestly reduced HIV-1 viremia in humanized Rag2^{-/-}γc^{-/-} mice (Figure 39). With improved transduction methods, it will be important to examine the effect of stem cell transduction with hT5Cyp on immune cell development and HIV-1 infection within one of the humanized mouse models currently under development aimed at enhancing *de novo* T cell development and *in vivo* immune function (62, 63).

[00426] **Methods: Plasmids and cloning.** Most HIV-1 plasmids and vectors have been described previously (64). pNL4-3 contains a complete infectious HIV-1 provirus. pNL4-3-R5 bears the V3 loop of the CCR5-tropic 92TH014-2 HIV-1 strain (65). To generate pNL4-3-GFP-IRES-Nef, enhanced green fluorescent protein (eGFP) and the ECMV (encephalomyocarditis virus) IRES (internal ribosome entry site) were inserted upstream of *nef*. pNLΔenvGFP is pNL4-3 with an *env*-inactivating mutation and eGFP in place of *nef*. CSGW is an HIV-1 vector expressing eGFP under the control of the spleen focus forming virus (SFFV) promoter. pFUPI, engineered from pFUW (66), uses the ubiquitin promoter to drive expression of a puromycin resistance cassette followed by the ECMV IRES (Figure 32A).

[00427] hT5Cyp variants were cloned by overlapping PCR using the oligonucleotides shown in Table 6. rhT5α, hT5α_{R332P} (16, 33) and hT5α_{rh323-332} (14, 15) were also obtained. All T5 constructs were cloned downstream of the IRES in FUPI. All PCR products were sequenced to eliminate unwanted mutations.

[00428] pAIG uses the SFFV promoter to drive expression of T5 followed by IRES-eGFP. In pscALPS, the SFFV promoter drives expression of T5 and the human CypA promoter drives expression of eGFP. For three-component vector production, HIV-1 *gag* and *pol* were expressed using either psPAX2, p8.9 Δ SB, p8.9 Δ SB-G89V, or p8.9 Δ SB-V86P/H87Q/I91V/M96I. The V86P/H87Q/I91V/M96I mutation in CA (28) was obtained. SIV_{mac}251 vectors were produced using GAE1.0 and the packaging vector SIV3+ (67). SIV_{AGM}tan and HIV-2_{ROD} vectors each have an *env*-deletion and eGFP in place of *nef* (9). FIV vectors were produced using the genomic vector pGINSIN and the packaging vector pFP93 (30). All lentiviral vectors were pseudotyped using the vesicular stomatitis virus glycoprotein (VSV-G). For biochemistry, hT5Cyps were cloned into the p3xFLAG-CMV-7.1 expression vector (Sigma) and HIV-1 CA was expressed as a GST-fusion using the EF1 α promoter.

[00429] *Cells.* 293T human embryonic kidney cells and CRFK feline kidney cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and glutamax (Invitrogen). Jurkat E6-1, H9, CEM-SS, and SUP-T1 T cell leukemia/lymphoma cell lines, U937 promonocytic lymphoma, and THP-1 monocytic leukemia cells were grown in RPMI-1640 with 10% FBS and glutamax (Invitrogen). For creation of T5-expressing cell lines, suspension cells were transduced with 2×10^5 Jurkat infectious units/ml and adherent cells were transduced with 2×10^4 Jurkat infectious units/ml of FUPI containing the indicated T5. Jurkat infectious units were determined by serial dilutions of concomitantly-produced FUPI-GFP on Jurkat E6 cells. 48 hrs. post-transduction, cells were selected with puromycin. The different constructs were normalized to each other by comparing RT activity of the supernatants and assessing the puromycin-resistant colonies after serial dilution on HeLa cells. CD4⁺ T cells were enriched from PBMCs (>99% CD4⁺) using positive selection magnetic beads (Miltenyi Biotec) and stimulated for 24 hrs on multisorb 96-well plates (Nunc) coated with 2 μ g/ml anti-CD3 and 2 μ g/ml anti-CD28 antibodies (BD Biosciences) in RPMI with 10% FBS, glutamax (Invitrogen) and 20 IU/ml hIL-2. When indicated and in preparation for adoptive transfer to mice, CD4⁺ T cells were stimulated using allogeneic-PBMCs irradiated at 50 Gy in the presence of 20 IU/ml IL-2 and phytohemagglutinin (PHA) at 1 μ g/ml. Cells were washed and replated 24 hrs prior to viral transduction. CD14⁺ monocytes were enriched from PBMCs (>99% CD14⁺) using positive selection magnetic beads (Miltenyi Biotec). CD14⁺ monocytes were resuspended in complete RPMI supplemented with 50 ng/ml recombinant human GM-CSF (Leukomax, Novartis) at 1×10^6

cells/ml. 2×10^6 were plated in each well of a 6-well plate (Nunc) and allowed to differentiate into macrophages for 10 days before transduction. Transduction of human macrophages 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. For both $CD4^+$ T cells and monocyte-derived macrophages, cells were sorted for GFP expression by FACS 72 hours post-transduction.

[00430] *Drugs.* All chemicals were obtained from Sigma unless otherwise noted. Puromycin was used at 1 μ M. Cyclosporine (CsA) (Bedford Laboratories) was diluted to 10 mM in dimethyl sulfoxide (DMSO) and then further to 2.5 μ M in tissue culture media. Arsenic trioxide (As_2O_3) was made at 100 mM in 1N sodium hydroxide (NaOH) and then further diluted to 1 mM in phosphate buffered saline (PBS). pH was adjusted to 7.5 with hydrochloric acid and stored at 4°C.

[00431] *Virus production.* Vectors and viruses were produced by transfection of 293T cells using Lipofectamine 2000 (Invitrogen). Two-part vectors were produced by co-transfection of viral genome and VSV-G plasmids at a ratio of 7:1. Three-part vectors were produced by co-transfection of viral genome, *gag-pol*, and VSV-G plasmids at a ratio of 4:3:1. Infectious viruses were produced by transfection of 90% confluent 293T cells in a T75 flask (Nunc) with 40 μ g of pNL4-3, pNL4-3-R5, or pNL4-3-GFP-IRES-Nef. Supernatants containing viruses and vectors were cleared by 400 x g centrifugation, filtered (0.45 μ m; Pall Acrodisc), tested for exogenous RT activity (7) titrated on Jurkat E6 cells in single cycle assay (7) and stored at -80°C. When indicated, the content of viral p24 antigen was quantified by an HIV-1 p24 enzyme-linked immunoabsorbent assay kit (NIH AIDS Research and Reference Reagent Program, Germantown, MD).

[00432] *Infections.* 2×10^4 adherent cells/well or 8×10^4 suspension cells/well were plated in 48-well plates (Nunc) for challenge with single-cycle GFP reporter vectors that were titrated as indicated. GFP synthesis was assessed by flow cytometry 48 hrs post-infection. For spreading infection, 10^6 Jurkat cells were infected with 1 to 15 ng p24/ 10^6 cells of HIV-1_{NL4-3-GFP-IRES-Nef}. Cells were split every 3 days, fixed in 0.5% paraformaldehyde/PBS, and assayed for GFP synthesis by flow cytometry. 10^6 primary $CD4^+$ T cells or macrophages were infected with 15 ng p24/ 10^6 cells of CXCR4- or CCR5-tropic HIV-1_{NL4-3} stocks, or with low passage, primary isolates HIV-1_{DH12} and HIV-1_{132W}, (Olivier Schwartz, Pasteur Institute, Paris). Infection was monitored by assessing the accumulation of RT activity in the

supernatant (7). FACSCalibur, Cellquest Pro (Becton Dickinson), and FlowJo software (Treestar, Inc.) were used to record and analyze fluorescence. $1-5 \times 10^5$ events were acquired for analysis.

[00433] *Western Blotting and Immunoprecipitation.* To detect hT5Cyp, 5×10^6 293T cells were transfected with 20 μ g of each 3xFLAG-hT5Cyp plasmid using Lipofectamine 2000 (Invitrogen). Cells were lysed at 48 hrs in Triton lysis buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCL, 1% Triton X-100, with Complete Mini protease inhibitor cocktail (Roche)), cleared at 15,000 x g for 5 minutes, incubated 4 hours at 4°C with 2 μ g/ml anti-FLAG M2 monoclonal antibody (Sigma), and then incubated 1 hour at 4°C with 20 μ l of protein G-sepharose beads (GE-Amersham). The beads were washed 3 times with 1 mL Triton Lysis Buffer, and 20 μ l immunoprecipitate was subjected to SDS-PAGE on a 12% gel, transferred to a PVDF membrane, and immunoblotted with rabbit polyclonal antibody to CypA (Biomol).

[00434] For co-immunoprecipitation of hT5Cyp with HIV-1 CA, 5×10^6 293T cells were co-transfected as above with 20 μ g p3xFLAG hT5Cyp and 20 μ g of pEF1-GST-CA. At 48 hrs cells were lysed with RIPA Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate, with Complete Mini protease inhibitor cocktail (Roche)) and accelerated at 15,000 x g for 5 min. Lysate was divided into two equal parts and incubated on ice for 30 minutes with 20 μ M CsA/DMSO or an equivalent volume of DMSO for 30 minutes. Lysates were incubated with 30 μ l glutathione-sepharose beads (GE-Amersham) for 1 hour at 4°C. Beads were washed three times with RIPA buffer. Proteins were subjected to SDS-PAGE and immunoblotting with rabbit polyclonal anti-CypA antibody (Biomol) and goat polyclonal anti-HIV-1 CA (NCI).

[00435] *Structural model of the hT5 α PRYSPRY domain.* StruPro was used to superimpose and align the crystal structures for PRYSPRY-19q13.4.1 (Protein Data Bank (PDB) entry 2FBE; residue range 11 to 184 of chain A), GUSTAVUS (PDB entry 2FNJ; residue range 35 to 83 and 88 to 233 of chain A), and TRIM21 (PDB entry 2IWG; residue range 2 to 182 of chain B), using an alpha carbon cutoff distance of 3.5 Å. ClustalX was used to combine the hT5 α sequence with the structural alignments. A ClustalW sequence-based multiple alignment of 10 characteristic psiBLAST hits of all three aforementioned sequences was used to localize insertions. A secondary structure prediction of hT5 α obtained with

NPS@ was used to manually correct the model. The final alignment was then fed into the model and loop optimization procedures of Modeller 8v2 (<http://salilab.org/modeller>), using 2FBE, 2FNJ, and 2IWG as templates. 100 models were built and evaluated for stereochemical quality with PROCHECK. Pictures of the single best model were generated with PyMol v0.98.

[00436] *Immunofluorescence microscopy.* CRFK cells stably expressing wild-type AoT5Cyp or hT5Cyp fusion proteins were grown on glass coverslips. Cells were fixed at 25° C for 10 min with 3.7% formaldehyde in PBS and permeabilized on ice for 2 min with 2% Triton-X100 in 0.1% sodium citrate. After quenching with 0.1 M glycine in PBS at 25° C for 10 min, cells were blocked for 30 min at 25° C with 10% fetal bovine serum and 0.1% Tween-20 in PBS, before overnight incubation with goat polyclonal anti-TRIM5 (AbCam) and mouse monoclonal anti-tubulin (SIGMA) antibodies. After extensive washing in PBS, cells were sequentially incubated with donkey-Alexa Fluor 488-anti-goat and goat-Alexa Fluor 594 anti-mouse secondary antibodies (Invitrogen) for 1 hr at 25°C in the dark. Finally coverslips were mounted in Vectashield with DAPI (Vector laboratories). Cells were visualized with a 63x 1.4 NA Leica HCX Planapochromat oil immersion objective using an inverted Leica DMI 6000 CS microscope fitted with a TCS SP5 laser scanning confocal microscope system. Images from individual 3-dimensional stacks were subjected to noise reduction using the Leica LCS software (Leica Microsystems) and subjected to maximum intensity projection using the MetaMorph software (Universal Imaging Corp).

[00437] *Mice.* Rag2^{-/-}γc^{-/-} mice on a BALB/c background (provided by M. Ito; Central Institute for Experimental Animals, Kawasaki, Japan) were bred and maintained under specific pathogen-free conditions in accordance with the guidelines of the animal facility at the Institute for Research in Biomedicine (Bellinzona) and the Swiss Federal Veterinary Office (Bern). Mouse experiments were reviewed and approved by the Veterinary Commission of the State of Ticino (Bellinzona, Switzerland). CL2MBP (clodronate) was from Roche Diagnostics (Mannheim, Germany). Preparation of liposomes containing CL2MBP was done as described previously (68). 6-10 week-old mice received 100 ul of clodronate liposomes intraperitoneally (i.p.) 24 hours prior to irradiation using a single sublethal dose of 4.0 Gray (Gy) from a Cesium 137 source (Biobeam 8000, STS GmbH, Braunschweig, Germany) at 3.75 Gy/min. 4 hrs post-irradiation, mice were transplanted with 2.5 x 10⁵ hCD4⁺ T cells expressing hT5Cyp or control hT5CypH126Q in 100 μl PBS i.p..

Mice were infected with HIV-1_{NL4-3}, as previously described (58), either 5 or 14 days post-transplant and analyzed 2, 3, or 4 weeks post-infection. CD4⁺ T cell engraftment and maintenance in mice was measured by flow cytometry. To obtain peripheral blood cells and plasma, mice were bled from the retro-orbital venous sinus under anesthesia and red blood cells were lysed. When sacrificed, single cell suspensions from organs were prepared. For FACS analysis monoclonal antibodies against the following antigens were used: CD3 (UCHT1), CD4 (13B8.2), CD8 (B9.11), p24 (clone KC57) (all Immunotech/Beckman Coulter, Marseille, France), CD45 (HI30) (Caltag, Burlingame, CA). Intracellular staining for p24 was performed on single cell suspensions from mesenteric lymph nodes and thymus following staining with anti-CD3 antibody. Cells were washed, permeabilized, and fixed by treatment with BD Cytofix/Cytoperm (BD Pharmingen) and stained with anti-p24 antibody in BD Permashield solution (BD Pharmingen) following the manufacturer's instructions. Plasma HIV RNA concentrations were determined by Cobas Amplicor RT-PCR assay (Roche Diagnostics, Basel, Switzerland). Immunohistochemical stainings were performed as previously described (58). To assess nonspecific binding, tissue from untransplanted and uninfected transplanted mice was stained as controls.

[00438] *Statistical Analysis.* Significant differences between groups were calculated using the Mann-Whitney U test. *P*-values < 0.05 were considered significant.

[00439] References for Example 8:

1. Fauci, A.S. 2008. 25 years of HIV. *Nature* **453**:289-290.
2. Barouch, D.H. 2008. Challenges in the development of an HIV-1 vaccine. *Nature* **455**:613-619.
3. Medzhitov, R., and Littman, D. 2008. HIV immunology needs a new direction. *Nature* **455**:591.
4. Strayer, D.S., et al. 2005. Current status of gene therapy strategies to treat HIV/AIDS. *Mol Ther* **11**:823-842.
5. Baltimore, D. 2008. Presidential address. A global perspective on science and technology. *Science* **322**:544-551.
6. Stremlau, M., et al. 2004. The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys. *Nature* **427**:848-853.

7. Sayah, D.M., Sokolskaja, E., Berthoux, L., and Luban, J. 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* **430**:569-573.
8. Perez-Caballero, D., Hatzioannou, T., Zhang, F., Cowan, S., and Bieniasz, P.D. 2005. Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. *J Virol* **79**:15567-15572.
9. Diaz-Griffero, F., et al. 2006. Requirements for capsid-binding and an effector function in TRIMCyp-mediated restriction of HIV-1. *Virology* **351**:404-419.
10. Sebastian, S., and Luban, J. 2005. TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. *Retrovirology* **2**:40.
11. Stremlau, M., et al. 2006. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc Natl Acad Sci U S A* **103**:5514-5519.
12. Luban, J. 2007. Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. *J Virol* **81**:1054-1061.
13. Perez-Caballero, D., Hatzioannou, T., Yang, A., Cowan, S., and Bieniasz, P.D. 2005. Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. *J Virol* **79**:8969-8978.
14. Stremlau, M., Perron, M., Welikala, S., and Sodroski, J. 2005. Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. *J Virol* **79**:3139-3145.
15. Sawyer, S.L., Wu, L.I., Emerman, M., and Malik, H.S. 2005. Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* **102**:2832-2837.
16. Yap, M.W., Nisole, S., and Stoye, J.P. 2005. A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Curr Biol* **15**:73-78.
17. Yap, M.W., Dodding, M.P., and Stoye, J.P. 2006. Trim-cyclophilin A fusion proteins can restrict human immunodeficiency virus type 1 infection at two distinct phases in the viral life cycle. *J Virol* **80**:4061-4067.
18. Schaller, T., Ylinen, L.M., Webb, B.L., Singh, S., and Towers, G.J. 2007. Fusion of cyclophilin a to fv1 enables cyclosporine-sensitive restriction of human and feline immunodeficiency viruses. *J Virol* **81**:10055-10063.

19. Nisole, S., Lynch, C., Stoye, J.P., and Yap, M.W. 2004. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. *Proc Natl Acad Sci U S A* **101**:13324-13328.
20. Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V., and Goff, S.P. 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73**:1067-1078.
21. Ribeiro, I.P., et al. 2005. Evolution of cyclophilin A and TRIMCyp retrotransposition in New World primates. *J Virol* **79**:14998-15003.
22. Riddell, S.R., et al. 1996. T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. *Nat Med* **2**:216-223.
23. Grutter, C., et al. 2006. Structure of the PRYSPRY-domain: implications for autoinflammatory diseases. *FEBS Lett* **580**:99-106.
24. Woo, J.S., et al. 2006. Structural and functional insights into the B30.2/SPRY domain. *EMBO J* **25**:1353-1363.
25. Ohkura, S., Yap, M.W., Sheldon, T., and Stoye, J.P. 2006. All three variable regions of the TRIM5alpha B30.2 domain can contribute to the specificity of retrovirus restriction. *J Virol* **80**:8554-8565.
26. Sokolskaja, E., Berthoux, L., and Luban, J. 2006. Cyclophilin A and TRIM5alpha independently regulate human immunodeficiency virus type 1 infectivity in human cells. *J Virol* **80**:2855-2862.
27. Braaten, D., Ansari, H., and Luban, J. 1997. The hydrophobic pocket of cyclophilin is the binding site for the human immunodeficiency virus type 1 Gag polyprotein. *J Virol* **71**:2107-2113.
28. Chatterji, U., et al. 2005. Naturally occurring capsid substitutions render HIV-1 cyclophilin A independent in human cells and TRIM-cyclophilin-resistant in Owl monkey cells. *J Biol Chem* **280**:40293-40300.
29. Sebastian, S., Sokolskaja, E., and Luban, J. 2006. Arsenic counteracts human immunodeficiency virus type 1 restriction by various TRIM5 orthologues in a cell type-dependent manner. *J Virol* **80**:2051-2054.
30. Saenz, D.T., Teo, W., Olsen, J.C., and Poeschla, E.M. 2005. Restriction of feline immunodeficiency virus by Ref1, Lv1, and primate TRIM5alpha proteins. *J Virol* **79**:15175-15188.

31. Zhang, F., Hatzioannou, T., Perez-Caballero, D., Derse, D., and Bieniasz, P.D. 2006. Antiretroviral potential of human tripartite motif-5 and related proteins. *Virology* **353**:396-409.
32. Lin, T.Y., and Emerman, M. 2006. Cyclophilin A interacts with diverse lentiviral capsids. *Retrovirology* **3**:70.
33. Li, Y., Li, X., Stremlau, M., Lee, M., and Sodroski, J. 2006. Removal of arginine 332 allows human TRIM5alpha to bind human immunodeficiency virus capsids and to restrict infection. *J Virol* **80**:6738-6744.
34. Anderson, J., and Akkina, R. 2008. Human immunodeficiency virus type 1 restriction by human-rhesus chimeric tripartite motif 5alpha (TRIM 5alpha) in CD34(+) cell-derived macrophages in vitro and in T cells in vivo in severe combined immunodeficient (SCID-hu) mice transplanted with human fetal tissue. *Hum Gene Ther* **19**:217-228.
35. Mazurier, F., et al. 1999. A novel immunodeficient mouse model--RAG2 x common cytokine receptor gamma chain double mutants--requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. *J Interferon Cytokine Res* **19**:533-541.
36. van Rijn, R.S., et al. 2003. A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/- gammac-/- double-mutant mice. *Blood* **102**:2522-2531.
37. Chen, B.K., Gandhi, R.T., and Baltimore, D. 1996. CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef. *J Virol* **70**:6044-6053.
38. Traggiai, E., et al. 2004. Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* **304**:104-107.
39. Liao, C.H., Kuang, Y.Q., Liu, H.L., Zheng, Y.T., and Su, B. 2007. A novel fusion gene, TRIM5-Cyclophilin A in the pig-tailed macaque determines its susceptibility to HIV-1 infection. *Aids* **21 Suppl 8**:S19-26.
40. Virgen, C.A., Kratovac, Z., Bieniasz, P.D., and Hatzioannou, T. 2008. Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species. *Proc Natl Acad Sci U S A* **105**:3563-3568.
41. Wilson, S.J., et al. 2008. Independent evolution of an antiviral TRIMCyp in rhesus macaques. *Proc Natl Acad Sci U S A* **105**:3557-3562.

42. Brennan, G., Kozyrev, Y., and Hu, S.L. 2008. TRIMCyp expression in Old World primates *Macaca nemestrina* and *Macaca fascicularis*. *Proc Natl Acad Sci U S A* **105**:3569-3574.
43. Newman, R.M., et al. 2008. Evolution of a TRIM5-CypA splice isoform in old world monkeys. *PLoS Pathog* **4**:e1000003.
44. Song, B., et al. 2005. The B30.2(SPRY) domain of the retroviral restriction factor TRIM5alpha exhibits lineage-specific length and sequence variation in primates. *J Virol* **79**:6111-6121.
45. Anderson, J., and Akkina, R. 2005. TRIM5alpharh expression restricts HIV-1 infection in lentiviral vector-transduced CD34+-cell-derived macrophages. *Mol Ther* **12**:687-696.
46. Li, X., and Sodroski, J. 2008. The TRIM5alpha B-box 2 domain promotes cooperative binding to the retroviral capsid by mediating higher-order self-association. *J Virol* **82**:11495-11502.
47. Ranga, U., et al. 1998. Enhanced T cell engraftment after retroviral delivery of an antiviral gene in HIV-infected individuals. *Proc Natl Acad Sci U S A* **95**:1201-1206.
48. An, D.S., et al. 2007. Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. *Proc Natl Acad Sci U S A* **104**:13110-13115.
49. Perez, E.E., et al. 2008. Establishment of HIV-1 resistance in CD4(+) T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol* **26**:808-816.
50. Kumar, P., et al. 2008. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* **134**:577-586.
51. Boden, D., Pusch, O., and Ramratnam, B. 2007. Overcoming HIV-1 resistance to RNA interference. *Front Biosci* **12**:3104-3116.
52. Hamm, T.E., Rekosh, D., and Hammariskjold, M.L. 1999. Selection and characterization of human immunodeficiency virus type 1 mutants that are resistant to inhibition by the transdominant negative RevM10 protein. *J Virol* **73**:5741-5747.
53. Taylor, B.M., et al. 2008. An alteration of human immunodeficiency virus gp41 leads to reduced CCR5 dependence and CD4 independence. *J Virol* **82**:5460-5471.
54. Glass, W.G., et al. 2006. CCR5 deficiency increases risk of symptomatic West Nile virus infection. *J Exp Med* **203**:35-40.
55. Berthoux, L., Sebastian, S., Sayah, D.M., and Luban, J. 2005. Disruption of human TRIM5alpha antiviral activity by nonhuman primate orthologues. *J Virol* **79**:7883-7888.

56. Mosier, D.E., et al. 1991. Human immunodeficiency virus infection of human-PBL-SCID mice. *Science* **251**:791-794.
57. Namikawa, R., Kaneshima, H., Lieberman, M., Weissman, I.L., and McCune, J.M. 1988. Infection of the SCID-hu mouse by HIV-1. *Science* **242**:1684-1686.
58. Baenziger, S., et al. 2006. Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2^{-/-}-gamma c^{-/-} mice. *Proc Natl Acad Sci U S A* **103**:15951-15956.
59. Nakata, H., et al. 2005. Potent anti-R5 human immunodeficiency virus type 1 effects of a CCR5 antagonist, AK602/ONO4128/GW873140, in a novel human peripheral blood mononuclear cell nonobese diabetic-SCID, interleukin-2 receptor gamma-chain-knocked-out AIDS mouse model. *J Virol* **79**:2087-2096.
60. Levine, B.L., et al. 2002. Adoptive transfer of costimulated CD4+ T cells induces expansion of peripheral T cells and decreased CCR5 expression in HIV infection. *Nat Med* **8**:47-53.
61. Amendola, M., Venneri, M.A., Biffi, A., Vigna, E., and Naldini, L. 2005. Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. *Nat Biotechnol* **23**:108-116.
62. Goldstein, H. 2008. Summary of presentations at the NIH/NIAID New Humanized Rodent Models 2007 Workshop. *AIDS Res Ther* **5**:3.
63. Manz, M.G. 2007. Human-hemato-lymphoid-system mice: opportunities and challenges. *Immunity* **26**:537-541.
64. Berthoux, L., Sebastian, S., Sokolskaja, E., and Luban, J. 2004. Lvl inhibition of human immunodeficiency virus type 1 is counteracted by factors that stimulate synthesis or nuclear translocation of viral cDNA. *J Virol* **78**:11739-11750.
65. Papkalla, A., Munch, J., Otto, C., and Kirchhoff, F. 2002. Nef enhances human immunodeficiency virus type 1 infectivity and replication independently of viral coreceptor tropism. *J Virol* **76**:8455-8459.
66. Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**:868-872.
67. Mangeot, P.E., et al. 2002. High levels of transduction of human dendritic cells with optimized SIV vectors. *Mol Ther* **5**:283-290.

68. Van Rooijen, N., and Sanders, A. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* **174**:83-93.

EXAMPLE 9: Methods describing Figures 32-39

[00440] 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 10⁵ 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.

[00441] **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 Permash solution following the manufacturer's instructions (all reagents BD/Pharmingen).

[00442] **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).

[00443] **Real-Time RT-PCR.** Total RNA was extracted from 5x10⁶ CD4⁺ T cells using the RNeasy Plus Mini kit (Qiagen). RNA was treated with RNase-free DNase I (Ambion) and reverse transcribed using the SuperscriptTM 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 (SEQ ID NO:88), 18S RNA 3': GCTGGAATTACCGCGGCT (SEQ ID NO:89), GFP 5': CCCCGTGATGAAGAAGATGA (SEQ ID NO:90), GFP 3': GTCAGCTTGTGCTGGATGAA (SEQ ID NO:91), hT5Cyp 5':

CTGGGTTGATGTGACAGTGG (SEQ ID NO:92), hT5Cyp 3':
TCTGCTGTCTTTGGGACCTT (SEQ ID NO:93).

[00444] qPCR for HIV-1 DNA. Total cellular DNA was extracted from 2×10^6 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).

[00445] Following primers were used:

Mitochondrial forward primer (MH533): ACCCACTCCCTCTTAGCCAATATT (SEQ ID NO:94),

Mitochondrial reverse primer (MH534): GTAGGGCTAGGCCACCG (SEQ ID NO:95),

Mitochondrial probe (mito-probe): 5'-(TET)-CTAGTCTTTGCCGCCTGCGAAGCA-(TAMRA)-3' (SEQ ID NO:96),

J1 RT forward: ACAAGCTAGTACCAGTTGAGCCAGATAAG (SEQ ID NO:97),

J2 RT reverse: GCCGTGCGCGCTTCAGCAAGC (SEQ ID NO:98),

RT probe (LRT-P): 5'-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3' (SEQ ID NO:99).

[00446] huRag2^{-/-}γ_c^{-/-} mice. huRag2^{-/-}γ_c^{-/-} mice were generated as previously described¹. 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. 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). 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^{-/-}γ_c^{-/-} mice as previously

described. Traggiai, E., *et al.* Mice were infected with HIV-1 by intraperitoneal injection as previously described. 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).

[00447] Non-limiting examples of sequences of the invention are provided below. Certain sequences not provided herein are available in the public domain. There are methods known in the art to reverse translate and obtain a nucleic acid sequence based on a known polypeptide sequence, non-limiting example is provided by the ExPASy proteomics tools available from the Swiss Institute for Bioinformatics.

[00448] DNA Sequence of a portion of hTRIM5 encoding polypeptide ending with amino acids RWY:

```
ATGGCTTCTGGAATCCTGGTTAATGTAAAGGAGGAGGTGACCTGCCCCATCTGCCTGGAAC
TCTGACACAACCCCTGAGCCTGGACTGCGGCCACAGCTTCTGCCAAGCATGCCTCACTGCAA
ACCACAAGAAGTCCATGCTAGACAAAGGAGAGAGTAGCTGCCCTGTGTGCCGGATCAGTTAC
CAGCCTGAGAACATACGGCCTAATCGGCATGTAGCCAACTTAGTGGAGAAGCTCAGGGAGGT
CAAGTTGAGCCCAGAGGGGCAGAAAGTTGATCATTGTGCACGCCATGGAGAGAACTTCTAC
TCTTCTGTTCAGGAGGACGGGAAGGTCATTTGCTGGCTTTGTGAGCGGTCTCAGGAGCACCGT
GGTCACCCACACGTTCCCCACAGAGGAGGTTGCCAGGAGTACCAAGTGAAGCTCCAGGCAGC
TCTGGAGATGCTGAGGCAGAAGCAGCAGGAAGCTGAAGAGTTGGAAGCTGACATCAGAGAAG
AGAAAGCTTCCTGGAAGACTCAAATACAGTATGACAAAACCAACGTCTTGGCAGATTTTGAG
CAACTGAGAGACATCCTGGACTGGGAGGAGAGCAATGAGCTGCAAAACCTGGAGAAGGAGGA
GGAAGACATTCTGAAAAGCCTTACGAACTCTGAAACTGAGATGGTGCAGCAGACCCAGTCCC
TGAGAGAGCTCATCTCAGATCTGGAGCATCGGCTGCAGGGGTCAGTGATGGAGCTGCTTCAG
GGTGTGGATGGCGTCATAAAAAGGACGGAGAACGTGACCTTGAAGAAGCCAGAACTTTTCC
AAAAAATCAAAGGAGAGTGTTTCGAGCTCCTGATCTGAAAGGAATGCTAGAAGTGTTTAGAG
AGCTGACAGATGTCCGACGCTACTGG (SEQ ID NO:18).
```

[00449] Amino acid sequence of a portion of hTRIM5 ending with amino acids RWY:

```
1 masgilvnvk eevtcpicl lltqplslldc ghsfcqaclt anhkksmldk gesscpvcrl
61 syqpenirpn rhvanlvekl revklspegq kvdhcarhge klllfcqedg kvicwlcers
121 gehrhhtfp teevaqeyqv klqaalemlr qkqqaeele adireekasw ktqiyydktl
181 vladfeqlrd ildweesnel qnlekeeedi lksltlnsete mvqqtqslre lisdlehlrlq
241 gsvmellqgv dgvikrtenv tlkkpetfpk nqrrvfrapd lkgmlevfre ltdvrryw
```

(SEQ ID NO: 13).

[00450] Linkers of fusion proteins:

Linker X_n, where X is any amino acid, n=12-33 (SEQ ID NO:14).

Linker X_n, where X is any amino acid, n=12 (SEQ ID NO:24),
n=24 (SEQ ID NO:25), n=33 (SEQ ID NO:26).

Linker SGGSGGSGGSGG (SEQ ID NO:20).

Linker V D V T V A P N N I S C A V I S E D K R Q V S S P K P Q I I Y G A (SEQ ID NO:21).

Linker V D V T V A P N N I S S (SEQ ID NO:22).

Linker V D V T V A P N N I S C A V I S E D K R Q V S S (SEQ ID NO:23).

[00451] DNA Sequence of CypA encoding polypeptide from position 2 to the end
(missing the starting Met):

GTCAACCCACCGTGTTCTTCGACATTGCCGTCGACGGCGAGCCCTTGGGCGCGTCTCCTT
TGAGCTGTTTTCGACAAGGTCCCAAAGACAGCAGAAAATTTTCGTGCTCTGAGCACTGGAGA
GAAAGGATTTGGTTATAAGGGTTCCTGCTTTCACAGAATTATTCCAGGGTTTATGTGTCAGG
GTGGTGACTTCACACGCCATAATGGCACTGGTGGCAAGTCCATCTATGGGGAGAAATTTGAA
GATGAGAACTTCATCCTAAAGCATACGGGTCCTGGCATCTTGTCCATGGCAAATGCTGGACC
CAACACAAATGGTTCCCAGTTTTTCATCTGCACTGCCAAGACTGAGTGGTTGGATGGCAAGC
ATGTGGTGTGGCAAAGTGAAAGAAGGCATGAATATTGTGGAGGCCATGGAGCGCTTTGGG
TCCAGGAATGGCAAGACCAGCAAGAAGATCACCATTGCTGACTGTGGACAACTCGAATAA
(SEQ ID NO:19).

[00452] Amino acid sequence of CypA from position 2 to the end (missing the starting
Met):

vnptvffdi avdgeplgrv sfelfadkvp ktaenfrals tgekgfgykg scfhriipgf
61 mcqggdftrh ngtggksiyg ekfedenfil khtgpgilsm anagpntngs qffictakte
121 wldgkhvvfg kvkegmive amerfgsrng ktskkitiad cggle (SEQ ID NO:15).

[00453] Fusion protein between hTRIM5(aa position 1 to 298)-linker(SGG)4-
hCypA(aa position 2 to 166):

M A S G I L V N V K E E V T C P I C L E L L T Q P L S L D C G H S F C Q A C L T A N
H K K S M L D K G E S S C P V C R I S Y Q P E N I R P N R H V A N I V E K L R E V K
L S P E G Q K V D H C A R H G E K L L L F C Q E D G K V I C W L C E R S Q E H R G
H H T F L T E E V A R E Y Q V K L Q A A L E M L R Q K Q Q E A E E L E A D I R E E
K A S W K T Q I Q Y D K T N V L A D F E Q L R D I L D W E E S N E L Q N L E K E E
E D I L K S L T N S E T E M V Q Q T Q S L R E L I S D L E H R L Q G S V M E L L Q G
V D G V I K R T E N V T L K K P E T F P K N Q R R V F R A P D L K G M L E V F R E
L T D V R R Y W S G G S G G S G G S G G V N P T V F F D I A V D G E P L G R V S F E L F A D
K V P K T A E N F R A L S T G E K G F G Y K G S C F H R I I P G F M C Q G G D F T R
H N G T G G K S I Y G E K F E D E N F I L K H T G P G I L S M A N A G P N T N G S Q
F F I C T A K T E W L D G K H V V F G K V K E G M N I V E A M E R F G S R N G K T
S K K I T I A D C G Q L E (SEQ ID NO:17).

[00454] Additional amino acid sequences provided by the invention:

masgilvnmk eevtpicle lltqplslde ghsfcqactl anhkksmldk gesscpvcri
syqpenirpn rhvanlvekl revklspgk qvdhcarhge klillfcqedg kvicwlcers
qehrghtfp teeveyqyv klqaalemlr qkqaeaele adireekasw ktqiyydkt
vladfeqlrd ildweesnel qnlekeeedi lksltsete mvqqtslre lisdlehlq
gsvmellqgv dgvikrtenv tlkkpetfpk nqrrvfrapd lkgmlevfre ltdvrryw[Xn]
vnptvffdiavdgeplgrv sfelfadkvp ktaenfrals tgekgfgykg scfhriipgf
mcqggdfrh ngtggksiyg ekfedentil khtgpgilsm anagpntngs qffictakte
wldgkhvvg kvkegmive amerfgsrng ktskkitiad cgql

where X is any amino acid, n=12-33 (SEQ ID NO: 16); n=12 (SEQ ID NO: 27); n=24 (SEQ ID NO:28); n=33 (SEQ ID NO:29); n=11 (SEQ ID NO:30).

Table 6: Cloning Primers

Name	Primer Sequence
<i>External Primers hT5Cyp</i>	
hT5Cyp XbaI 5'	5'- CCCTCTAGAGCCACCATGGCTTCTGGAATCCTGGTTA-3' (SEQ ID NO:100)
hT5Cyp BamHI 3'	5'- AAAAGGATCCTTATTCGAGTTGTCCACAGTC-3' (SEQ ID NO:101)
<i>Internal Primers hT5Cyp</i>	
M244F	5'- CGGCTGCAGGGGTCAGTGATGGTCAACCCACCGTGTTC-3' (SEQ ID NO:102)
M244R	5'- GAACACGGTGGGGTTGACCATCACTGACCCCTGCAGCCG-3' (SEQ ID NO:103)
M284F	5'- GCTCCTGATCTGAAAGGAATGGTCAACCCACCGTGTTC-3' (SEQ ID NO:104)
M284R	5'- GAACACGGTGGGGTTGACCATTCCTTTCAGATCAGGAGC-3' (SEQ ID NO:105)
W298F	5'- GGTGGGGTTGACCCAGTAGCGTCGGACATCTGTC-3' (SEQ ID NO:106)
W298R	5'- CGACGCTACTGGGTCAACCCACCGTGTTC-3' (SEQ ID NO:107)
T302F	5'- CGCTACTGGGTGATGTGACAGTCAACCCACCGTGTTC-3' (SEQ ID NO:108)
T302R	5'- GAACACGGTGGGGTTGACTGTCACATCAACCCAGTAGC-3' (SEQ ID NO:109)
S309F	5'- GTGGCTCCAAACAACATTTTCAGTCAACCCACCGTGTTC-3' (SEQ ID NO:110)
S309R	5'- GAACACGGTGGGGTTGACTGAAATGTTGTTTGGAGCCAC-3' (SEQ ID NO:111)
S314F	5'- TGGGTTGATGTGACAGTGGCTCCAAACAACATTTTCATGTGC TGTCATTTTCGTCAACCCACCGTG-3' (SEQ ID NO:112)
S314R	5'- GTTGACAGAAATGACAGCACATGAAATGTTGTTTGGAGCC ACTGTCACATCAACCCAGTAGCGTCGGAC-3' (SEQ ID NO:113)
S322F	5'- CAAGTGAGCTCTGTCAACCCACCGTGTTC-3' (SEQ ID NO:114)
S322R	5'- GGGGTTGACAGAGCTCACTTGTCTCTTATCTTCAG-3' (SEQ ID NO:115)
A331F	5'- CCACAGATAATATATGGGGCAGTCAACCCACCGTGTTCCTTC-3' (SEQ ID NO:116)
A331R	5'- CACGGTGGGGTTGACTGCCCCATATATTATCTGTGGTTTCG-3' (SEQ ID NO:117)
G357F	5'- GCTCTCAAAGTATCACATCAGGGGTCAACCCACCGTGTTCCTTC-3' (SEQ ID NO:118)
G357R	5'- GAAGAACACGGTGGGGTTGACCCCTGATGTGATACTTTGAGAGC-3' (SEQ ID NO:119)
T369F	5'- GAGGTAGACGTGTCCAAGAAAAGTGTCAACCCACCGTGTTCCTTC-3' (SEQ ID NO:120)
T369R	5'- GAAGAACACGGTGGGGTTGACAGTTTTCTTGGACACGTCTACCTC-3' (SEQ ID NO:121)
G398F	5'- GAAAATTATCAACCTAAATACGGCGTCAACCCACCGTGTTCCTTC-3' (SEQ ID NO:122)
G398R	5'- GAAGAACACGGTGGGGTTGACGCCGTATTTAGGTTGATAATTTTC-3' (SEQ ID NO:123)
<i>T5a cloning primers</i>	
RhT5a XbaI 5'	5'-CCCTCTAGAGCCACCATGGCTTCTGGAATCCTGCTTA-3' (SEQ ID

	NO:124)
RhT5 α HpaI 3'	5'-AAAAGTTAACTCAAGAGCTTGGTGAGCACA-3' (SEQ ID NO:125)
hT5 α XbaI 5'	5'-CCCTCTAGAGCCACCATGGCTTCTGGAATCCTGGTTA-3' (SEQ ID NO:126)
hhT5 α HpaI 3'	5'-AAAAGTTAACTCAAGAGCTTGGTGAGCACAGAGT-3' (SEQ ID NO:127)

[00455] As various changes can be made in the above methods and compositions without departing from the scope and spirit of the invention as described, it is intended that all subject matter contained in the above description, shown in the accompanying drawings, or defined in the appended claims be interpreted as illustrative, and not in a limiting sense.

References

1. Strayer, D.S., *et al.* Current status of gene therapy strategies to treat HIV/AIDS. *Mol Ther* **11**, 823-842 (2005).
2. Stremlau, M., *et al.* The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys. *Nature* **427**, 848-853 (2004).
3. Sayah, D.M., Sokolskaja, E., Berthou, L. & Luban, J. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* **430**, 569-573 (2004).
4. Perez-Caballero, D., Hatzioannou, T., Zhang, F., Cowan, S. & Bieniasz, P.D. Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. *Journal of Virology* **79**, 15567-15572 (2005).
5. Diaz-Griffero, F., *et al.* Requirements for capsid-binding and an effector function in TRIMCyp-mediated restriction of HIV-1. *Virology* **351**, 404-419 (2006).
6. Sebastian, S. & Luban, J. TRIM5 α selectively binds a restriction-sensitive retroviral capsid. *Retrovirology* **2**, 40 (2005).
7. Stremlau, M., *et al.* Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5 α restriction factor. *Proc Natl Acad Sci* **103**, 5514-5519 (2006).
8. Luban, J. Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. *Journal of Virology* **81**, 1054-1061 (2007).
9. Perez-Caballero, D., Hatzioannou, T., Yang, A., Cowan, S. & Bieniasz, P.D. Human tripartite motif 5 α domains responsible for retrovirus restriction activity and specificity. *Journal of Virology* **79**, 8969-8978 (2005).
10. Stremlau, M., Perron, M., Welikala, S. & Sodroski, J. Species-specific variation in the B30.2(SPRY) domain of TRIM5 α determines the potency of human immunodeficiency virus restriction. *Journal of Virology* **79**, 3139-3145 (2005).
11. Sawyer, S.L., Wu, L.I., Emerman, M. & Malik, H.S. Positive selection of primate TRIM5 α identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci* **102**, 2832-2837 (2005).
12. Yap, M.W., Nisole, S. & Stoye, J.P. A single amino acid change in the SPRY domain of human Trim5 α leads to HIV-1 restriction. *Curr Biol* **15**, 73-78 (2005).
13. Nisole, S., Lynch, C., Stoye, J.P. & Yap, M.W. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. *Proc Natl Acad Sci* **101**, 13324-13328 (2004).

14. Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V. & Goff, S.P. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73**, 1067-1078 (1993).
15. Ribeiro, I.P., *et al.* Evolution of cyclophilin A and TRIMCyp retrotransposition in New World primates. *Journal of Virology* **79**, 14998-15003 (2005).
16. Riddell, S.R., *et al.* T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. *Nature Medicine* **2**, 216-223 (1996).
17. Ohkura, S., Yap, M.W., Sheldon, T. & Stoye, J.P. All three variable regions of the TRIM5alpha B30.2 domain can contribute to the specificity of retrovirus restriction. *Journal of Virology* **80**, 8554-8565 (2006).
18. Grutter, C., *et al.* Structure of the PRYSPRY-domain: implications for autoinflammatory diseases. *FEBS Lett* **580**, 99-106 (2006).
19. Woo, J.S., *et al.* Structural and functional insights into the B30.2/SPRY domain. *EMBO J* **25**, 1353-1363 (2006).
20. Saenz, D.T., Teo, W., Olsen, J.C. & Poeschla, E.M. Restriction of feline immunodeficiency virus by Ref1, Lv1, and primate TRIM5alpha proteins. *Journal of Virology* **79**, 15175-15188 (2005).
21. Zhang, F., Hatzioannou, T., Perez-Caballero, D., Derse, D. & Bieniasz, P.D. Antiretroviral potential of human tripartite motif-5 and related proteins. *Virology* **353**, 396-409 (2006).
22. Lin, T.Y. & Emerman, M. Cyclophilin A interacts with diverse lentiviral capsids. *Retrovirology* **3**, 70 (2006).
23. Braaten, D., Ansari, H. & Luban, J. The hydrophobic pocket of cyclophilin is the binding site for the human immunodeficiency virus type 1 Gag polyprotein. *Journal of Virology* **71**, 2107-2113 (1997).
24. Chatterji, U., *et al.* Naturally occurring capsid substitutions render HIV-1 cyclophilin A independent in human cells and TRIM-cyclophilin-resistant in Owl monkey cells. *J Biol Chem* **280**, 40293-40300 (2005).
25. Sebastian, S., Sokolskaja, E. & Luban, J. Arsenic counteracts human immunodeficiency virus type 1 restriction by various TRIM5 orthologues in a cell type-dependent manner. *Journal of Virology* **80**, 2051-2054 (2006).
26. Li, Y., Li, X., Stremlau, M., Lee, M. & Sodroski, J. Removal of arginine 332 allows human TRIM5alpha to bind human immunodeficiency virus capsids and to restrict infection. *Journal of Virology* **80**, 6738-6744 (2006).
27. Anderson, J. & Akkina, R. Human immunodeficiency virus type 1 restriction by human-rhesus chimeric tripartite motif 5alpha (TRIM 5alpha) in CD34(+) cell-derived macrophages in vitro and in T cells in vivo in severe combined immunodeficient (SCID-hu) mice transplanted with human fetal tissue. *Human Gene Therapy* **19**, 217-228 (2008).
28. Mazurier, F., *et al.* A novel immunodeficient mouse model--RAG2 x common cytokine receptor gamma chain double mutants--requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. *J Interferon Cytokine Res* **19**, 533-541 (1999).
29. van Rijn, R.S., *et al.* A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/- gammac-/- double-mutant mice. *Blood* **102**, 2522-2531 (2003).
30. Chen, B.K., Gandhi, R.T. & Baltimore, D. CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef. *Journal of Virology* **70**, 6044-6053 (1996).

31. Stoye, J.P. & Yap, M.W. Chance favors a prepared genome. *Proc Natl Acad Sci* **105**, 3177-3178 (2008).
32. Song, B., *et al.* The B30.2(SPRY) domain of the retroviral restriction factor TRIM5alpha exhibits lineage-specific length and sequence variation in primates. *Journal of Virology* **79**, 6111-6121 (2005).
33. Anderson, J. & Akkina, R. TRIM5alpharh expression restricts HIV-1 infection in lentiviral vector-transduced CD34+-cell-derived macrophages. *Mol Ther* **12**, 687-696 (2005).
34. Boden, D., Pusch, O. & Ramratnam, B. Overcoming HIV-1 resistance to RNA interference. *Front Biosci* **12**, 3104-3116 (2007).
35. An, D.S., *et al.* Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. *Proc Natl Acad Sci* **104**, 13110-13115 (2007).
36. Perez, E.E., *et al.* Establishment of HIV-1 resistance in CD4(+) T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol* (2008).
37. Taylor, B.M., *et al.* An alteration of human immunodeficiency virus gp41 leads to reduced CCR5 dependence and CD4 independence. *Journal of Virology* **82**, 5460-5471 (2008).
38. Glass, W.G., *et al.* CCR5 deficiency increases risk of symptomatic West Nile virus infection. *The Journal of Experimental Medicine* **203**, 35-40 (2006).
39. Berthoux, L., Sebastian, S., Sayah, D.M. & Luban, J. Disruption of human TRIM5alpha antiviral activity by nonhuman primate orthologues. *Journal of Virology* **79**, 7883-7888 (2005).
40. Levine, B.L., *et al.* Adoptive transfer of costimulated CD4+ T cells induces expansion of peripheral T cells and decreased CCR5 expression in HIV infection. *Nature Medicine* **8**, 47-53 (2002).
41. Amendola, M., Venneri, M.A., Biffi, A., Vigna, E. & Naldini, L. Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. *Nat Biotechnol* **23**, 108-116 (2005).
42. Goldstein, H. Summary of presentations at the NIH/NIAID New Humanized Rodent Models 2007 Workshop. *AIDS Research and Therapy* **5**, 3 (2008).
43. Berthoux, L., Sebastian, S., Sokolskaja, E. & Luban, J. Lvl inhibition of human immunodeficiency virus type 1 is counteracted by factors that stimulate synthesis or nuclear translocation of viral cDNA. *Journal of Virology* **78**, 11739-11750 (2004).
44. Papkalla, A., Munch, J., Otto, C. & Kirchhoff, F. Nef enhances human immunodeficiency virus type 1 infectivity and replication independently of viral coreceptor tropism. *Journal of Virology* **76**, 8455-8459 (2002).
45. Lois, C., Hong, E.J., Pease, S., Brown, E.J. & Baltimore, D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868-872 (2002).
46. Mangeot, P.E., *et al.* High levels of transduction of human dendritic cells with optimized SIV vectors. *Mol Ther* **5**, 283-290 (2002).
47. Van Rooijen, N. & Sanders, A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* **174**, 83-93 (1994).
48. Baenziger, S., *et al.* Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2-/-gamma c-/- mice. *Proc Natl Acad Sci* **103**, 15951-15956 (2006).

Ambrose, Z., KewalRamani, V.N., Bieniasz, P.D., and Hatziioannou, T. (2007). HIV/AIDS: in search of an animal model. *Trends in biotechnology* **25**, 333-337.

- Amendola, M., Venneri, M.A., Biffi, A., Vigna, E., and Naldini, L. (2005). Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. *Nature biotechnology* 23, 108-116.
- An, D.S., Donahue, R.E., Kamata, M., Poon, B., Metzger, M., Mao, S.H., Bonifacino, A., Krouse, A.E., Darlix, J.L., Baltimore, D., *et al.* (2007). Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. *Proc Natl Acad Sci U S A* 104, 13110-13115.
- Anderson, J., and Akkina, R. (2005a). CXCR4 and CCR5 shRNA transgenic CD34⁺ cell derived macrophages are functionally normal and resist HIV-1 infection. *Retrovirology* 2, 53.
- Anderson, J., and Akkina, R. (2005b). TRIM5 α expression restricts HIV-1 infection in lentiviral vector-transduced CD34⁺-cell-derived macrophages. *Mol Ther* 12, 687-696.
- Anderson, J., and Akkina, R. (2008). Human immunodeficiency virus type 1 restriction by human-rhesus chimeric tripartite motif 5 α (TRIM 5 α) in CD34(+) cell-derived macrophages in vitro and in T cells in vivo in severe combined immunodeficient (SCID-hu) mice transplanted with human fetal tissue. *Human gene therapy* 19, 217-228.
- Anderson, J., Li, M.J., Palmer, B., Remling, L., Li, S., Yam, P., Yee, J.K., Rossi, J., Zaia, J., and Akkina, R. (2007). Safety and efficacy of a lentiviral vector containing three anti-HIV genes--CCR5 ribozyme, tat-rev siRNA, and TAR decoy--in SCID-hu mouse-derived T cells. *Mol Ther* 15, 1182-1188.
- Anderson, J.L., Campbell, E.M., Wu, X., Vandegraaff, N., Engelman, A., and Hope, T.J. (2006). Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. *J Virol* 80, 9754-9760.
- Anderson, P., Phillips, K., Stoecklin, G., and Kedersha, N. (2004). Post-transcriptional regulation of proinflammatory proteins. *J Leukoc Biol* 76, 42-47.
- Arthos, J., Cicala, C., Martinelli, E., Macleod, K., Van Ryk, D., Wei, D., Xiao, Z., Veenstra, T.D., Conrad, T.P., Lempicki, R.A., *et al.* (2008). HIV-1 envelope protein binds to and signals through integrin $\alpha 4 \beta 7$, the gut mucosal homing receptor for peripheral T cells. *Nature immunology* 9, 301-309.
- Asaoka, K., Ikeda, K., Hishinuma, T., Horie-Inoue, K., Takeda, S., and Inoue, S. (2005). A retrovirus restriction factor TRIM5 α is transcriptionally regulated by interferons. *Biochemical and biophysical research communications* 338, 1950-1956.
- Baenziger, S., Tussiwand, R., Schlaepfer, E., Mazzucchelli, L., Heikenwalder, M., Kurrer, M.O., Behnke, S., Frey, J., Oxenius, A., Joller, H., *et al.* (2006). Disseminated and sustained HIV infection in CD34⁺ cord blood cell-transplanted Rag2^{-/-}-gamma c^{-/-} mice. *Proc Natl Acad Sci U S A* 103, 15951-15956.
- Baenziger, S., Ziegler, P., Mazzucchelli, L., Bronz, L., Speck, R.F., and Manz, M.G. (2008). Human T cell development and HIV infection in human hemato-lymphoid system mice. *Current topics in microbiology and immunology* 324, 125-131.
- Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., *et al.* (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 220, 868-871.
- Bartee, E., McCormack, A., and Fruh, K. (2006). Quantitative membrane proteomics reveals new cellular targets of viral immune modulators. *PLoS pathogens* 2, e107.
- Bassin, R.H., Duran-Troise, G., Gerwin, B.I., and Rein, A. (1978). Abrogation of Fv-1b restriction with murine leukemia viruses inactivated by heat or by gamma irradiation. *J Virol* 26, 306-315.

- Benit, L., De Parseval, N., Casella, J.F., Callebaut, I., Cordonnier, A., and Heidmann, T. (1997). Cloning of a new murine endogenous retrovirus, MuERV-L, with strong similarity to the human HERV-L element and with a gag coding sequence closely related to the Fv1 restriction gene. *J Virol* 71, 5652-5657.
- Berges, B.K., Akkina, S.R., Folkvord, J.M., Connick, E., and Akkina, R. (2008). Mucosal transmission of R5 and X4 tropic HIV-1 via vaginal and rectal routes in humanized Rag2^{-/-} gamma^{mac} ^{-/-} (RAG-hu) mice. *Virology* 373, 342-351.
- Berthoux, L., Sebastian, S., Sayah, D.M., and Luban, J. (2005a). Disruption of human TRIM5 α antiviral activity by nonhuman primate orthologues. *J Virol* 79, 7883-7888.
- Berthoux, L., Sebastian, S., Sokolskaja, E., and Luban, J. (2004). Lv1 inhibition of human immunodeficiency virus type 1 is counteracted by factors that stimulate synthesis or nuclear translocation of viral cDNA. *J Virol* 78, 11739-11750.
- Berthoux, L., Sebastian, S., Sokolskaja, E., and Luban, J. (2005b). Cyclophilin A is required for TRIM5 α -mediated resistance to HIV-1 in Old World monkey cells. *Proc Natl Acad Sci U S A* 102, 14849-14853.
- Berthoux, L., Towers, G.J., Gurer, C., Salomoni, P., Pandolfi, P.P., and Luban, J. (2003). As(2)O(3) enhances retroviral reverse transcription and counteracts Ref1 antiviral activity. *J Virol* 77, 3167-3180.
- Besnier, C., Takeuchi, Y., and Towers, G. (2002). Restriction of lentivirus in monkeys. *Proc Natl Acad Sci U S A* 99, 11920-11925.
- Besnier, C., Ylinen, L., Strange, B., Lister, A., Takeuchi, Y., Goff, S.P., and Towers, G.J. (2003). Characterization of murine leukemia virus restriction in mammals. *J Virol* 77, 13403-13406.
- Best, S., Le Tissier, P., Towers, G., and Stoye, J.P. (1996). Positional cloning of the mouse retrovirus restriction gene Fv1. *Nature* 382, 826-829.
- Bishop, K.N., Bock, M., Towers, G., and Stoye, J.P. (2001). Identification of the regions of Fv1 necessary for murine leukemia virus restriction. *J Virol* 75, 5182-5188.
- Blunt, T., Gell, D., Fox, M., Taccioli, G.E., Lehmann, A.R., Jackson, S.P., and Jeggo, P.A. (1996). Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the scid mouse. *Proc Natl Acad Sci U S A* 93, 10285-10290.
- Boden, D., Pusch, O., Lee, F., Tucker, L., and Ramratnam, B. (2003). Human immunodeficiency virus type 1 escape from RNA interference. *J Virol* 77, 11531-11535.
- Boden, D., Pusch, O., and Ramratnam, B. (2007). Overcoming HIV-1 resistance to RNA interference. *Front Biosci* 12, 3104-3116.
- Bogerd, H.P., Doehle, B.P., Wiegand, H.L., and Cullen, B.R. (2004). A single amino acid difference in the host APOBEC3G protein controls the primate species specificity of HIV type 1 virion infectivity factor. *Proc Natl Acad Sci U S A* 101, 3770-3774.
- Boone, L.R., Innes, C.L., and Heitman, C.K. (1990). Abrogation of Fv-1 restriction by genome-deficient virions produced by a retrovirus packaging cell line. *J Virol* 64, 3376-3381.
- Bosco, D.A., Eisenmesser, E.Z., Pochapsky, S., Sundquist, W.I., and Kern, D. (2002). Catalysis of cis/trans isomerization in native HIV-1 capsid by human cyclophilin A. *Proc Natl Acad Sci U S A* 99, 5247-5252.
- Bosma, G.C., Custer, R.P., and Bosma, M.J. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature* 301, 527-530.

- Bosma, G.C., Fried, M., Custer, R.P., Carroll, A., Gibson, D.M., and Bosma, M.J. (1988). Evidence of functional lymphocytes in some (leaky) scid mice. *The Journal of experimental medicine* 167, 1016-1033.
- Braaten, D., Aberham, C., Franke, E.K., Yin, L., Phares, W., and Luban, J. (1996). Cyclosporine A-resistant human immunodeficiency virus type 1 mutants demonstrate that Gag encodes the functional target of cyclophilin A. *J Virol* 70, 5170-5176.
- Braaten, D., Ansari, H., and Luban, J. (1997). The hydrophobic pocket of cyclophilin is the binding site for the human immunodeficiency virus type 1 Gag polyprotein. *J Virol* 71, 2107-2113.
- Braaten, D., and Luban, J. (2001). Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. *The EMBO journal* 20, 1300-1309.
- Brass, A.L., Dykxhoorn, D.M., Benita, Y., Yan, N., Engelman, A., Xavier, R.J., Lieberman, J., and Elledge, S.J. (2008). Identification of host proteins required for HIV infection through a functional genomic screen. *Science* 319, 921-926.
- Brenchley, J.M., Price, D.A., Schacker, T.W., Asher, T.E., Silvestri, G., Rao, S., Kazzaz, Z., Bornstein, E., Lambotte, O., Altmann, D., *et al.* (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nature medicine* 12, 1365-1371.
- Brennan, G., Kozyrev, Y., and Hu, S.L. (2008). TRIMCyp expression in Old World primates *Macaca nemestrina* and *Macaca fascicularis*. *Proc Natl Acad Sci U S A* 105, 3569-3574.
- Brown, C.R., Czapiga, M., Kabat, J., Dang, Q., Ourmanov, I., Nishimura, Y., Martin, M.A., and Hirsch, V.M. (2007). Unique pathology in simian immunodeficiency virus-infected rapid progressor macaques is consistent with a pathogenesis distinct from that of classical AIDS. *J Virol* 81, 5594-5606.
- Bushman, F.D. (2007). Retroviral integration and human gene therapy. *The Journal of clinical investigation* 117, 2083-2086.
- Campbell, E.M., Dodding, M.P., Yap, M.W., Wu, X., Gallois-Montbrun, S., Malim, M.H., Stoye, J.P., and Hope, T.J. (2007). TRIM5 alpha cytoplasmic bodies are highly dynamic structures. *Mol Biol Cell* 18, 2102-2111.
- Campbell, E.M., Perez, O., Anderson, J.L., and Hope, T.J. (2008). Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5alpha. *The Journal of cell biology* 180, 549-561.
- Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.L., *et al.* (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 288, 669-672.
- Chatterji, U., Bobardt, M.D., Gaskill, P., Sheeter, D., Fox, H., and Gallay, P.A. (2006). Trim5alpha accelerates degradation of cytosolic capsid associated with productive HIV-1 entry. *The Journal of biological chemistry* 281, 37025-37033.
- Chatterji, U., Bobardt, M.D., Stanfield, R., Ptak, R.G., Pallansch, L.A., Ward, P.A., Jones, M.J., Stoddart, C.A., Scalfaro, P., Dumont, J.M., *et al.* (2005). Naturally occurring capsid substitutions render HIV-1 cyclophilin A independent in human cells and TRIM-cyclophilin-resistant in Owl monkey cells. *The Journal of biological chemistry* 280, 40293-40300.
- Chen, B.K., Gandhi, R.T., and Baltimore, D. (1996). CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef. *J Virol* 70, 6044-6053.
- Chiu, Y.L., and Greene, W.C. (2008). The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. *Annual review of immunology* 26, 317-353.

- Chiu, Y.L., Soros, V.B., Kreisberg, J.F., Stopak, K., Yonemoto, W., and Greene, W.C. (2005). Cellular APOBEC3G restricts HIV-1 infection in resting CD4⁺ T cells. *Nature* 435, 108-114.
- Chiu, Y.L., Witkowska, H.E., Hall, S.C., Santiago, M., Soros, V.B., Esnault, C., Heidmann, T., and Greene, W.C. (2006). High-molecular-mass APOBEC3G complexes restrict Alu retrotransposition. *Proc Natl Acad Sci U S A* 103, 15588-15593.
- Ciuffi, A., Diamond, T.L., Hwang, Y., Marshall, H.M., and Bushman, F.D. (2006). Modulating target site selection during human immunodeficiency virus DNA integration in vitro with an engineered tethering factor. *Human gene therapy* 17, 960-967.
- Coles, M.C., Veiga-Fernandes, H., Foster, K.E., Norton, T., Pagakis, S.N., Seddon, B., and Kioussis, D. (2006). Role of T and NK cells and IL7/IL7r interactions during neonatal maturation of lymph nodes. *Proc Natl Acad Sci U S A* 103, 13457-13462.
- Colgan, J., Asmal, M., and Luban, J. (2000). Isolation, characterization and targeted disruption of mouse ppia: cyclophilin A is not essential for mammalian cell viability. *Genomics* 68, 167-178.
- Colgan, J., Asmal, M., Neagu, M., Yu, B., Schneidkraut, J., Lee, Y., Sokolskaja, E., Andreotti, A., and Luban, J. (2004). Cyclophilin A regulates TCR signal strength in CD4⁺ T cells via a proline-directed conformational switch in Itk. *Immunity* 21, 189-201.
- Colgan, J., Asmal, M., Yu, B., and Luban, J. (2004). Cyclophilin A-deficient Mice are Resistant to Immunosuppression by Cyclosporine.
- Cowan, S., Hatzioannou, T., Cunningham, T., Muesing, M.A., Gottlinger, H.G., and Bieniasz, P.D. (2002). Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. *Proc Natl Acad Sci U S A* 99, 11914-11919.
- Daniel, M.D., Kirchhoff, F., Czajak, S.C., Sehgal, P.K., and Desrosiers, R.C. (1992). Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 258, 1938-1941.
- Delebecque, F., Suspene, R., Calattini, S., Casartelli, N., Saib, A., Froment, A., Wain-Hobson, S., Gessain, A., Vartanian, J.P., and Schwartz, O. (2006). Restriction of foamy viruses by APOBEC cytidine deaminases. *J Virol* 80, 605-614.
- Denton, P.W., Estes, J.D., Sun, Z., Othieno, F.A., Wei, B.L., Wege, A.K., Powell, D.A., Payne, D., Haase, A.T., and Garcia, J.V. (2008). Antiretroviral Pre-exposure Prophylaxis Prevents Vaginal Transmission of HIV-1 in Humanized BLT Mice. *PLoS Med* 5, e16.
- Diaz-Griffero, F., Kar, A., Lee, M., Stremlau, M., Poeschla, E., and Sodroski, J. (2007). Comparative requirements for the restriction of retrovirus infection by TRIM5alpha and TRIMCyp. *Virology* 369, 400-410.
- Diaz-Griffero, F., Li, X., Javanbakht, H., Song, B., Welikala, S., Stremlau, M., and Sodroski, J. (2006a). Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5. *Virology* 349, 300-315.
- Diaz-Griffero, F., Vandegraaff, N., Li, Y., McGee-Estrada, K., Stremlau, M., Welikala, S., Si, Z., Engelman, A., and Sodroski, J. (2006b). Requirements for capsid-binding and an effector function in TRIMCyp-mediated restriction of HIV-1. *Virology* 351, 404-419.
- Dimitrov, D.S., Willey, R.L., Sato, H., Chang, L.J., Blumenthal, R., and Martin, M.A. (1993). Quantitation of human immunodeficiency virus type 1 infection kinetics. *J Virol* 67, 2182-2190.
- Dorfman, T., and Gottlinger, H.G. (1996). The human immunodeficiency virus type 1 capsid p2 domain confers sensitivity to the cyclophilin-binding drug SDZ NIM 811. *J Virol* 70, 5751-5757.

- Douek, D.C., Picker, L.J., and Koup, R.A. (2003). T cell dynamics in HIV-1 infection. *Annual review of immunology* 21, 265-304.
- Duran-Troise, G., Bassin, R.H., Wallace, B.F., and Rein, A. (1981). Balb/3T3 cells chronically infected with N-tropic murine leukemia virus continue to express Fv-1b restriction. *Virology* 112, 795-799.
- Esnault, C., Heidmann, O., Delebecque, F., Dewannieux, M., Ribet, D., Hance, A.J., Heidmann, T., and Schwartz, O. (2005). APOBEC3G cytidine deaminase inhibits retrotransposition of endogenous retroviruses. *Nature* 433, 430-433.
- Feng, Y., Broder, C.C., Kennedy, P.E., and Berger, E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272, 872-877.
- Fischer, G., Bang, H., and Mech, C. (1984). [Determination of enzymatic catalysis for the cis-trans-isomerization of peptide binding in proline-containing peptides]. *Biomed Biochim Acta* 43, 1101-1111.
- Fisher, A.G., Ensoli, B., Ivanoff, L., Chamberlain, M., Petteway, S., Ratner, L., Gallo, R.C., and Wong-Staal, F. (1987). The sor gene of HIV-1 is required for efficient virus transmission in vitro. *Science* 237, 888-893.
- Forshey, B.M., Shi, J., and Aiken, C. (2005). Structural requirements for recognition of the human immunodeficiency virus type 1 core during host restriction in owl monkey cells. *J Virol* 79, 869-875.
- Forshey, B.M., von Schwedler, U., Sundquist, W.I., and Aiken, C. (2002). Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. *J Virol* 76, 5667-5677.
- Franke, E.K., and Luban, J. (1996). Inhibition of HIV-1 replication by cyclosporine A or related compounds correlates with the ability to disrupt the Gag-cyclophilin A interaction. *Virology* 222, 279-282.
- Franke, E.K., Yuan, H.E., and Luban, J. (1994). Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* 372, 359-362.
- Friend, C. (1957). Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. *The Journal of experimental medicine* 105, 307-318.
- Fulop, G.M., and Phillips, R.A. (1990). The scid mutation in mice causes a general defect in DNA repair. *Nature* 347, 479-482.
- Fultz, P.N., McClure, H.M., Swenson, R.B., and Anderson, D.C. (1989). HIV infection of chimpanzees as a model for testing chemotherapeutics. *Intervirology* 30 Suppl 1, 51-58.
- Galic, Z., Kitchen, S.G., Kacena, A., Subramanian, A., Burke, B., Cortado, R., and Zack, J.A. (2006). T lineage differentiation from human embryonic stem cells. *Proc Natl Acad Sci U S A* 103, 11742-11747.
- Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M., *et al.* (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* 397, 436-441.
- Geraghty, R.J., Talbot, K.J., Callahan, M., Harper, W., and Panganiban, A.T. (1994). Cell type-dependence for Vpu function. *Journal of medical primatology* 23, 146-150.
- Gimeno, R., Weijer, K., Voordouw, A., Uittenbogaart, C.H., Legrand, N., Alves, N.L., Wijnands, E., Blom, B., and Spits, H. (2004). Monitoring the effect of gene silencing by RNA interference in human CD34+ cells injected into newborn RAG2-/- gammac-/- mice: functional inactivation of p53 in developing T cells. *Blood* 104, 3886-3893.
- Glass, W.G., McDermott, D.H., Lim, J.K., Lekhong, S., Yu, S.F., Frank, W.A., Pape, J., Cheshier, R.C., and Murphy, P.M. (2006). CCR5 deficiency increases risk of symptomatic West Nile virus infection. *The Journal of experimental medicine* 203, 35-40.

- Goff, S.P. (2004). Retrovirus restriction factors. *Molecular cell* 16, 849-859.
- Goffinet, C., Allespach, I., and Keppler, O.T. (2007). HIV-susceptible transgenic rats allow rapid preclinical testing of antiviral compounds targeting virus entry or reverse transcription. *Proc Natl Acad Sci U S A* 104, 1015-1020.
- Goila-Gaur, R., and Strebel, K. (2008). HIV-1 Vif, APOBEC, and intrinsic immunity. *Retrovirology* 5, 51.
- Goldman, J.P., Blundell, M.P., Lopes, L., Kinnon, C., Di Santo, J.P., and Thrasher, A.J. (1998). Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. *British journal of haematology* 103, 335-342.
- Goldstein, H. (2008). Summary of presentations at the NIH/NIAID New Humanized Rodent Models 2007 Workshop. *AIDS Res Ther* 5, 3.
- Goodall, J. (1995). Why is it unethical to use chimpanzees in the laboratory? *Altern Lab Anim* 23, 615-620.
- Gothel, S.F., and Marahiel, M.A. (1999). Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cell Mol Life Sci* 55, 423-436.
- Gottlinger, H.G. (2008). HIV/AIDS: virus kept on a leash. *Nature* 451, 406-408.
- Gottlinger, H.G., Dorfman, T., Cohen, E.A., and Haseltine, W.A. (1993). Vpu protein of human immunodeficiency virus type 1 enhances the release of capsids produced by gag gene constructs of widely divergent retroviruses. *Proc Natl Acad Sci U S A* 90, 7381-7385.
- Goujon, C., Jarrosson-Wuilleme, L., Bernaud, J., Rigal, D., Darlix, J.L., and Cimarelli, A. (2006). With a little help from a friend: increasing HIV transduction of monocyte-derived dendritic cells with virion-like particles of SIV(MAC). *Gene therapy* 13, 991-994.
- Goujon, C., Riviere, L., Jarrosson-Wuilleme, L., Bernaud, J., Rigal, D., Darlix, J.L., and Cimarelli, A. (2007). SIVSM/HIV-2 Vpx proteins promote retroviral escape from a proteasome-dependent restriction pathway present in human dendritic cells. *Retrovirology* 4, 2.
- Grant, A.D., and De Cock, K.M. (2001). ABC of AIDS. HIV infection and AIDS in the developing world. *BMJ (Clinical research ed)* 322, 1475-1478.
- Grutter, C., Briand, C., Capitani, G., Mittl, P.R., Papin, S., Tschopp, J., and Grutter, M.G. (2006). Structure of the PRYSPRY-domain: implications for autoinflammatory diseases. *FEBS Lett* 580, 99-106.
- Haase, A.T. (1999). Population biology of HIV-1 infection: viral and CD4+ T cell demographics and dynamics in lymphatic tissues. *Annual review of immunology* 17, 625-656.
- Hacein-Bey-Abina, S., Garrigue, A., Wang, G.P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., *et al.* (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *The Journal of clinical investigation* 118, 3132-3142.
- Han, Y., Wind-Rotolo, M., Yang, H.C., Siliciano, J.D., and Siliciano, R.F. (2007). Experimental approaches to the study of HIV-1 latency. *Nature reviews* 5, 95-106.
- Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J., and Speicher, D.W. (1984). Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* 226, 544-547.
- Hanna, J., Wernig, M., Markoulaki, S., Sun, C.W., Meissner, A., Cassady, J.P., Beard, C., Brambrink, T., Wu, L.C., Townes, T.M., *et al.* (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* 318, 1920-1923.

- Hanna, Z., Kay, D.G., Cool, M., Jothy, S., Rebai, N., and Jolicoeur, P. (1998a). Transgenic mice expressing human immunodeficiency virus type 1 in immune cells develop a severe AIDS-like disease. *J Virol* 72, 121-132.
- Hanna, Z., Kay, D.G., Rebai, N., Guimond, A., Jothy, S., and Jolicoeur, P. (1998b). Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. *Cell* 95, 163-175.
- Harris, R.S., Bishop, K.N., Sheehy, A.M., Craig, H.M., Petersen-Mahrt, S.K., Watt, I.N., Neuberger, M.S., and Malim, M.H. (2003). DNA deamination mediates innate immunity to retroviral infection. *Cell* 113, 803-809.
- Hartley, J.W., Rowe, W.P., and Huebner, R.J. (1970). Host-range restrictions of murine leukemia viruses in mouse embryo cell cultures. *J Virol* 5, 221-225.
- Hatzioannou, T., Perez-Caballero, D., Yang, A., Cowan, S., and Bieniasz, P.D. (2004). Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha. *Proc Natl Acad Sci U S A* 101, 10774-10779.
- Hatzioannou, T., Princiotta, M., Piatak, M., Jr., Yuan, F., Zhang, F., Lifson, J.D., and Bieniasz, P.D. (2006). Generation of simian-tropic HIV-1 by restriction factor evasion. *Science* 314, 95.
- Hesselton, R.M., Greiner, D.L., Mordes, J.P., Rajan, T.V., Sullivan, J.L., and Shultz, L.D. (1995). High levels of human peripheral blood mononuclear cell engraftment and enhanced susceptibility to human immunodeficiency virus type 1 infection in NOD/LtSz-scid/scid mice. *The Journal of infectious diseases* 172, 974-982.
- Himathongkham, S., and Luciw, P.A. (1996). Restriction of HIV-1 (subtype B) replication at the entry step in rhesus macaque cells. *Virology* 219, 485-488.
- Ho, D.D., and Bieniasz, P.D. (2008). HIV-1 at 25. *Cell* 133, 561-565.
- Hofer, U., Baenziger, S., Heikenwalder, M., Schlaepfer, E., Gehre, N., Regenass, S., Brunner, T., and Speck, R.F. (2008). RAG2-/-{gamma}c-/- mice transplanted with human cord blood CD34+ cells show low intestinal engraftment and are resistant to rectal HIV transmission. *J Virol*.
- Hofmann, W., Schubert, D., LaBonte, J., Munson, L., Gibson, S., Scammell, J., Ferrigno, P., and Sodroski, J. (1999). Species-specific, postentry barriers to primate immunodeficiency virus infection. *J Virol* 73, 10020-10028.
- Howe, S.J., Mansour, M.R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempinski, H., Brugman, M.H., Pike-Overzet, K., Chatters, S.J., de Ridder, D., *et al.* (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *The Journal of clinical investigation* 118, 3143-3150.
- Hughes, A.L., and Yeager, M. (1998). Natural selection at major histocompatibility complex loci of vertebrates. *Annual review of genetics* 32, 415-435.
- Isaacs, A., and Lindenmann, J. (1957). Virus interference. I. The interferon. *Proceedings of the Royal Society of London Series B, Containing papers of a Biological character* 147, 258-267.
- Ishikawa, F., Yasukawa, M., Lyons, B., Yoshida, S., Miyamoto, T., Yoshimoto, G., Watanabe, T., Akashi, K., Shultz, L.D., and Harada, M. (2005). Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood* 106, 1565-1573.
- Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K., Ueyama, Y., Koyanagi, Y., Sugamura, K., Tsuji, K., *et al.* (2002). NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 100, 3175-3182.

- Iwatani, Y., Takeuchi, H., Strebel, K., and Levin, J.G. (2006). Biochemical activities of highly purified, catalytically active human APOBEC3G: correlation with antiviral effect. *J Virol* 80, 5992-6002.
- James, L.C., Keeble, A.H., Khan, Z., Rhodes, D.A., and Trowsdale, J. (2007). Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proc Natl Acad Sci U S A* 104, 6200-6205.
- Jarmuz, A., Chester, A., Bayliss, J., Gisbourne, J., Dunham, I., Scott, J., and Navaratnam, N. (2002). An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. *Genomics* 79, 285-296.
- Javanbakht, H., Diaz-Griffero, F., Stremlau, M., Si, Z., and Sodroski, J. (2005). The contribution of RING and B-box 2 domains to retroviral restriction mediated by monkey TRIM5alpha. *The Journal of biological chemistry* 280, 26933-26940.
- Javanbakht, H., Diaz-Griffero, F., Yuan, W., Yeung, D.F., Li, X., Song, B., and Sodroski, J. (2007). The ability of multimerized cyclophilin A to restrict retrovirus infection. *Virology*.
- Javanbakht, H., Yuan, W., Yeung, D.F., Song, B., Diaz-Griffero, F., Li, Y., Li, X., Stremlau, M., and Sodroski, J. (2006). Characterization of TRIM5alpha trimerization and its contribution to human immunodeficiency virus capsid binding. *Virology* 353, 234-246.
- Jolicoeur, P., and Baltimore, D. (1976). Effect of Fv-1 gene product on proviral DNA formation and integration in cells infected with murine leukemia viruses. *Proc Natl Acad Sci U S A* 73, 2236-2240.
- Jolly, C., Kashefi, K., Hollinshead, M., and Sattentau, Q.J. (2004). HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. *The Journal of experimental medicine* 199, 283-293.
- Kaiser, S.M., Malik, H.S., and Emerman, M. (2007). Restriction of an extinct retrovirus by the human TRIM5alpha antiviral protein. *Science* 316, 1756-1758.
- Kao, S., Khan, M.A., Miyagi, E., Plishka, R., Buckler-White, A., and Strebel, K. (2003). The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. *J Virol* 77, 11398-11407.
- Kazazian, H.H., Jr. (2004). Mobile elements: drivers of genome evolution. *Science* 303, 1626-1632.
- Keckesova, Z., Ylinen, L.M., and Towers, G.J. (2004). The human and African green monkey TRIM5alpha genes encode Ref1 and Lv1 retroviral restriction factor activities. *Proc Natl Acad Sci U S A* 101, 10780-10785.
- Keele, B.F., Van Heuverswyn, F., Li, Y., Bailes, E., Takehisa, J., Santiago, M.L., Bibollet-Ruche, F., Chen, Y., Wain, L.V., Liegeois, F., *et al.* (2006). Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. *Science* 313, 523-526.
- Keppler, O.T., Welte, F.J., Ngo, T.A., Chin, P.S., Patton, K.S., Tsou, C.L., Abbey, N.W., Sharkey, M.E., Grant, R.M., You, Y., *et al.* (2002). Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. *The Journal of experimental medicine* 195, 719-736.
- Kerre, T.C., De Smet, G., De Smedt, M., Zippelius, A., Pittet, M.J., Langerak, A.W., De Bosscher, J., Offner, F., Vandekerckhove, B., and Plum, J. (2002). Adapted NOD/SCID model supports development of phenotypically and functionally mature T cells from human umbilical cord blood CD34(+) cells. *Blood* 99, 1620-1626.
- Kirchhoff, F., Greenough, T.C., Brettler, D.B., Sullivan, J.L., and Desrosiers, R.C. (1995). Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *The New England journal of medicine* 332, 228-232.

- Klimkait, T., Strebel, K., Hoggan, M.D., Martin, M.A., and Orenstein, J.M. (1990). The human immunodeficiency virus type -specific protein Vpu is required for efficient virus maturation and release. *J Virol* 64, 621-629.
- Konig, R., Zhou, Y., Elleder, D., Diamond, T.L., Bonamy, G.M., Irelan, J.T., Chiang, C.Y., Tu, B.P., De Jesus, P.D., Lilley, C.E., *et al.* (2008). Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. *Cell* 135, 49-60.
- Kootstra, N.A., Munk, C., Tonnu, N., Landau, N.R., and Verma, I.M. (2003). Abrogation of postentry restriction of HIV-1-based lentiviral vector transduction in simian cells. *Proc Natl Acad Sci U S A* 100, 1298-1303.
- Koyanagi, Y., Tanaka, Y., Ito, M., and Yamamoto, N. (2008). Humanized mice for human retrovirus infection. *Current topics in microbiology and immunology* 324, 133-148.
- Kozak, C.A., and Chakraborti, A. (1996). Single amino acid changes in the murine leukemia virus capsid protein gene define the target of Fv1 resistance. *Virology* 225, 300-305.
- Kumar, P., Ban, H.S., Kim, S.S., Wu, H., Pearson, T., Greiner, D.L., Laouar, A., Yao, J., Haridas, V., Habiro, K., *et al.* (2008). T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* 134, 577-586.
- Kunstman, K.J., Puffer, B., Korber, B.T., Kuiken, C., Smith, U.R., Kunstman, J., Stanton, J., Agy, M., Shibata, R., Yoder, A.D., *et al.* (2003). Structure and function of CC-chemokine receptor 5 homologues derived from representative primate species and subspecies of the taxonomic suborders Prosimii and Anthroidea. *J Virol* 77, 12310-12318.
- La Motte-Mohs, R.N., Herer, E., and Zuniga-Pflucker, J.C. (2005). Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. *Blood* 105, 1431-1439.
- Labno, C.M., Lewis, C.M., You, D., Leung, D.W., Takesono, A., Kamberos, N., Seth, A., Finkelstein, L.D., Rosen, M.K., Schwartzberg, P.L., *et al.* (2003). Itk functions to control actin polymerization at the immune synapse through localized activation of Cdc42 and WASP. *Curr Biol* 13, 1619-1624.
- LaBonte, J.A., Babcock, G.J., Patel, T., and Sodroski, J. (2002). Blockade of HIV-1 infection of New World monkey cells occurs primarily at the stage of virus entry. *The Journal of experimental medicine* 196, 431-445.
- Lapidot, T., Pflumio, F., Doedens, M., Murdoch, B., Williams, D.E., and Dick, J.E. (1992). Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* 255, 1137-1141.
- Lee, Y.N., and Bieniasz, P.D. (2007). Reconstitution of an infectious human endogenous retrovirus. *PLoS pathogens* 3, e10.
- Lee, Y.N., Malim, M.H., and Bieniasz, P.D. (2008). Hypermutation of an ancient human retrovirus by APOBEC3G. *J Virol* 82, 8762-8770.
- Legrand, N., Weijer, K., and Spits, H. (2006). Experimental models to study development and function of the human immune system in vivo. *J Immunol* 176, 2053-2058.
- Letvin, N.L., Daniel, M.D., Sehgal, P.K., Desrosiers, R.C., Hunt, R.D., Waldron, L.M., MacKey, J.J., Schmidt, D.K., Chalifoux, L.V., and King, N.W. (1985). Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLIV-III. *Science* 230, 71-73.
- Levine, B.L., Bernstein, W.B., Aronson, N.E., Schlienger, K., Cotte, J., Perfetto, S., Humphries, M.J., Ratto-Kim, S., Birx, D.L., Steffens, C., *et al.* (2002). Adoptive transfer of costimulated CD4+ T cells induces expansion of peripheral T cells and decreased CCR5 expression in HIV infection. *Nature medicine* 8, 47-53.
- Lewinski, M.K., Yamashita, M., Emerman, M., Ciuffi, A., Marshall, H., Crawford, G., Collins, F., Shinn, P., Leipzig, J., Hannenhalli, S., *et al.* (2006). Retroviral DNA

- integration: viral and cellular determinants of target-site selection. *PLoS pathogens* 2, e60.
- Li, X., Li, Y., Stremlau, M., Yuan, W., Song, B., Perron, M., and Sodroski, J. (2006a). Functional replacement of the RING, B-box 2, and coiled-coil domains of tripartite motif 5alpha (TRIM5alpha) by heterologous TRIM domains. *J Virol* 80, 6198-6206.
- Li, Y., Li, X., Stremlau, M., Lee, M., and Sodroski, J. (2006b). Removal of arginine 332 allows human TRIM5alpha to bind human immunodeficiency virus capsids and to restrict infection. *J Virol* 80, 6738-6744.
- Lilly, F. (1967). Susceptibility to two strains of Friend leukemia virus in mice. *Science* 155, 461-462.
- Lin, T.Y., and Emerman, M. (2006). Cyclophilin A interacts with diverse lentiviral capsids. *Retrovirology* 3, 70.
- Liu, J., Farmer, J.D., Jr., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66, 807-815.
- Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295, 868-872.
- Lombardo, A., Genovese, P., Beausejour, C.M., Colleoni, S., Lee, Y.L., Kim, K.A., Ando, D., Urnov, F.D., Galli, C., Gregory, P.D., *et al.* (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nature biotechnology* 25, 1298-1306.
- Luban, J. (2007). Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. *J Virol* 81, 1054-1061.
- Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V., and Goff, S.P. (1993). Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* 73, 1067-1078.
- Madani, N., and Kabat, D. (1998). An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. *J Virol* 72, 10251-10255.
- Maddon, P.J., Dagleish, A.G., McDougal, J.S., Clapham, P.R., Weiss, R.A., and Axel, R. (1986). The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* 47, 333-348.
- Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., and Engelborghs, Y. (2003). LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. *The Journal of biological chemistry* 278, 33528-33539.
- Malim, M.H., Freimuth, W.W., Liu, J., Boyle, T.J., Lyster, H.K., Cullen, B.R., and Nabel, G.J. (1992). Stable expression of transdominant Rev protein in human T cells inhibits human immunodeficiency virus replication. *The Journal of experimental medicine* 176, 1197-1201.
- Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003). Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* 424, 99-103.
- Mangeot, P.E., Duperrier, K., Negre, D., Boson, B., Rigal, D., Cosset, F.L., and Darlix, J.L. (2002). High levels of transduction of human dendritic cells with optimized SIV vectors. *Mol Ther* 5, 283-290.
- Manz, M.G. (2007). Human-hemato-lymphoid-system mice: opportunities and challenges. *Immunity* 26, 537-541.

- Marathe, J.G., and Wooley, D.P. (2007). Is gene therapy a good therapeutic approach for HIV-positive patients? *Genet Vaccines Ther* 5, 5.
- Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H., and Landau, N.R. (2003). Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 114, 21-31.
- Massiah, M.A., Simmons, B.N., Short, K.M., and Cox, T.C. (2006). Solution structure of the RBCC/TRIM B-box1 domain of human MID1: B-box with a RING. *Journal of molecular biology* 358, 532-545.
- Mazurier, F., Fontanellas, A., Salesse, S., Taine, L., Landriau, S., Moreau-Gaudry, F., Reiffers, J., Peault, B., Di Santo, J.P., and de Verneuil, H. (1999). A novel immunodeficient mouse model--RAG2 x common cytokine receptor gamma chain double mutants--requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. *J Interferon Cytokine Res* 19, 533-541.
- McCune, J.M., Namikawa, R., Kaneshima, H., Shultz, L.D., Lieberman, M., and Weissman, I.L. (1988). The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science* 241, 1632-1639.
- Melkus, M.W., Estes, J.D., Padgett-Thomas, A., Gatlin, J., Denton, P.W., Othieno, F.A., Wege, A.K., Haase, A.T., and Garcia, J.V. (2006). Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nature medicine* 12, 1316-1322.
- Messi, M., Giacchetto, I., Nagata, K., Lanzavecchia, A., Natoli, G., and Sallusto, F. (2003). Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nature immunology* 4, 78-86.
- Mische, C.C., Javanbakht, H., Song, B., Diaz-Griffero, F., Stremlau, M., Strack, B., Si, Z., and Sodroski, J. (2005). Retroviral restriction factor TRIM5alpha is a trimer. *J Virol* 79, 14446-14450.
- Mitchell, R.S., Beitzel, B.F., Schroder, A.R., Shinn, P., Chen, H., Berry, C.C., Ecker, J.R., and Bushman, F.D. (2004). Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS biology* 2, E234.
- Mitsuyasu, R.T., Anton, P.A., Deeks, S.G., Scadden, D.T., Connick, E., Downs, M.T., Bakker, A., Roberts, M.R., June, C.H., Jalali, S., *et al.* (2000). Prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous CD4(+) and CD8(+) T cells in human immunodeficiency virus-infected subjects. *Blood* 96, 785-793.
- Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68, 869-877.
- Morgan, R.A., Walker, R., Carter, C.S., Natarajan, V., Tavel, J.A., Bechtel, C., Herpin, B., Muul, L., Zheng, Z., Jagannatha, S., *et al.* (2005). Preferential survival of CD4+ T lymphocytes engineered with anti-human immunodeficiency virus (HIV) genes in HIV-infected individuals. *Human gene therapy* 16, 1065-1074.
- Mosier, D.E., Gulizia, R.J., Baird, S.M., and Wilson, D.B. (1988). Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* 335, 256-259.
- Mosier, D.E., Gulizia, R.J., Baird, S.M., Wilson, D.B., Spector, D.H., and Spector, S.A. (1991). Human immunodeficiency virus infection of human-PBL-SCID mice. *Science* 251, 791-794.
- Muesing, M.A., Smith, D.H., Cabradilla, C.D., Benton, C.V., Lasky, L.A., and Capon, D.J. (1985). Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. *Nature* 313, 450-458.

- Munch, J., Rucker, E., Standker, L., Adermann, K., Goffinet, C., Schindler, M., Wildum, S., Chinnadurai, R., Rajan, D., Specht, A., *et al.* (2007). Semen-derived amyloid fibrils drastically enhance HIV infection. *Cell* 131, 1059-1071.
- Munk, C., Brandt, S.M., Lucero, G., and Landau, N.R. (2002). A dominant block to HIV-1 replication at reverse transcription in simian cells. *Proc Natl Acad Sci U S A* 99, 13843-13848.
- Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102, 553-563.
- Nakata, H., Maeda, K., Miyakawa, T., Shibayama, S., Matsuo, M., Takaoka, Y., Ito, M., Koyanagi, Y., and Mitsuya, H. (2005). Potent anti-R5 human immunodeficiency virus type 1 effects of a CCR5 antagonist, AK602/ONO4128/GW873140, in a novel human peripheral blood mononuclear cell nonobese diabetic-SCID, interleukin-2 receptor gamma-chain-knocked-out AIDS mouse model. *J Virol* 79, 2087-2096.
- Namikawa, R., Kaneshima, H., Lieberman, M., Weissman, I.L., and McCune, J.M. (1988). Infection of the SCID-hu mouse by HIV-1. *Science* 242, 1684-1686.
- Nath, B.M., Schumann, K.E., and Boyer, J.D. (2000). The chimpanzee and other non-human-primate models in HIV-1 vaccine research. *Trends in microbiology* 8, 426-431.
- Neil, S.J., Eastman, S.W., Jouvenet, N., and Bieniasz, P.D. (2006). HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. *PLoS pathogens* 2, e39.
- Neil, S.J., Sandrin, V., Sundquist, W.I., and Bieniasz, P.D. (2007). An interferon-alpha-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein. *Cell host & microbe* 2, 193-203.
- Neil, S.J., Zang, T., and Bieniasz, P.D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451, 425-430.
- Newman, R.M., Hall, L., Kirmaier, A., Pozzi, L.A., Pery, E., Farzan, M., O'Neil, S.P., and Johnson, W. (2008). Evolution of a TRIM5-CypA splice isoform in old world monkeys. *PLoS pathogens* 4, e1000003.
- Newman, R.M., and Johnson, W.E. (2007). A brief history of TRIM5alpha. *AIDS Rev* 9, 114-125.
- Nisole, S., Lynch, C., Stoye, J.P., and Yap, M.W. (2004). A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. *Proc Natl Acad Sci U S A* 101, 13324-13328.
- Nisole, S., Stoye, J.P., and Saib, A. (2005). TRIM family proteins: retroviral restriction and antiviral defence. *Nature reviews* 3, 799-808.
- Nomaguchi, M., Fujita, M., and Adachi, A. (2008). Role of HIV-1 Vpu protein for virus spread and pathogenesis. *Microbes Infect.*
- Novembre, F.J., Saucier, M., Anderson, D.C., Klumpp, S.A., O'Neil, S.P., Brown, C.R., 2nd, Hart, C.E., Guenther, P.C., Swenson, R.B., and McClure, H.M. (1997). Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. *J Virol* 71, 4086-4091.
- Ohkura, S., Yap, M.W., Sheldon, T., and Stoye, J.P. (2006). All three variable regions of the TRIM5alpha B30.2 domain can contribute to the specificity of retrovirus restriction. *J Virol* 80, 8554-8565.
- Opi, S., Kao, S., Goila-Gaur, R., Khan, M.A., Miyagi, E., Takeuchi, H., and Strebel, K. (2007). Human immunodeficiency virus type 1 Vif inhibits packaging and antiviral activity of a degradation-resistant APOBEC3G variant. *J Virol* 81, 8236-8246.

- Owens, C.M., Yang, P.C., Gottlinger, H., and Sodroski, J. (2003). Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. *J Virol* 77, 726-731.
- Palmer, E. (2003). Negative selection--clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol* 3, 383-391.
- Pandrea, I., Apetrei, C., Gordon, S., Barbercheck, J., Dufour, J., Bohm, R., Sumpter, B., Roques, P., Marx, P.A., Hirsch, V.M., *et al.* (2007). Paucity of CD4+CCR5+ T cells is a typical feature of natural SIV hosts. *Blood* 109, 1069-1076.
- Pandrea, I., Sodora, D.L., Silvestri, G., and Apetrei, C. (2008). Into the wild: simian immunodeficiency virus (SIV) infection in natural hosts. *Trends in immunology* 29, 419-428.
- Papkalla, A., Munch, J., Otto, C., and Kirchhoff, F. (2002). Nef enhances human immunodeficiency virus type 1 infectivity and replication independently of viral coreceptor tropism. *J Virol* 76, 8455-8459.
- Perez, E.E., Wang, J., Miller, J.C., Jouvenot, Y., Kim, K.A., Liu, O., Wang, N., Lee, G., Bartsevich, V.V., Lee, Y.L., *et al.* (2008). Establishment of HIV-1 resistance in CD4(+) T cells by genome editing using zinc-finger nucleases. *Nature biotechnology*.
- Perez-Caballero, D., Hatzioannou, T., Yang, A., Cowan, S., and Bieniasz, P.D. (2005a). Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. *J Virol* 79, 8969-8978.
- Perez-Caballero, D., Hatzioannou, T., Zhang, F., Cowan, S., and Bieniasz, P.D. (2005b). Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. *J Virol* 79, 15567-15572.
- Perron, M.J., Stremlau, M., Lee, M., Javanbakht, H., Song, B., and Sodroski, J. (2007). The human TRIM5alpha restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid. *J Virol* 81, 2138-2148.
- Perron, M.J., Stremlau, M., Song, B., Ulm, W., Mulligan, R.C., and Sodroski, J. (2004). TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells. *Proc Natl Acad Sci U S A* 101, 11827-11832.
- Pichlmair, A., and Reis e Sousa, C. (2007). Innate recognition of viruses. *Immunity* 27, 370-383.
- Pincus, T., Hartley, J.W., and Rowe, W.P. (1971a). A major genetic locus affecting resistance to infection with murine leukemia viruses. I. Tissue culture studies of naturally occurring viruses. *The Journal of experimental medicine* 133, 1219-1233.
- Pincus, T., Hartley, J.W., and Rowe, W.P. (1975). A major genetic locus affecting resistance to infection with murine leukemia viruses. IV. Dose-response relationships in Fv-1-sensitive and resistant cell cultures. *Virology* 65, 333-342.
- Pincus, T., Rowe, W.P., and Lilly, F. (1971b). A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to friend murine leukemia virus. *The Journal of experimental medicine* 133, 1234-1241.
- Popovic, M., Sarngadharan, M.G., Read, E., and Gallo, R.C. (1984). Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* 224, 497-500.
- Potash, M.J., Chao, W., Bentsman, G., Paris, N., Saini, M., Nitkiewicz, J., Belem, P., Sharer, L., Brooks, A.I., and Volsky, D.J. (2005). A mouse model for study of systemic HIV-1 infection, antiviral immune responses, and neuroinvasiveness. *Proc Natl Acad Sci U S A* 102, 3760-3765.

- Pryciak, P.M., and Varmus, H.E. (1992). Fv-1 restriction and its effects on murine leukemia virus integration in vivo and in vitro. *J Virol* 66, 5959-5966.
- Qi, C.F., Bonhomme, F., Buckler-White, A., Buckler, C., Orth, A., Lander, M.R., Chattopadhyay, S.K., and Morse, H.C., 3rd (1998). Molecular phylogeny of Fv1. *Mamm Genome* 9, 1049-1055.
- Ranga, U., Woffendin, C., Verma, S., Xu, L., June, C.H., Bishop, D.K., and Nabel, G.J. (1998). Enhanced T cell engraftment after retroviral delivery of an antiviral gene in HIV-infected individuals. *Proc Natl Acad Sci U S A* 95, 1201-1206.
- Rein, A., Kashmiri, S.V., Bassin, R.H., Gerwin, B.L., and Duran-Troise, G. (1976). Phenotypic mixing between N- and B-tropic murine leukemia viruses: infectious particles with dual sensitivity to Fv-1 restriction. *Cell* 7, 373-379.
- Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S., *et al.* (2001). The tripartite motif family identifies cell compartments. *The EMBO journal* 20, 2140-2151.
- Ribeiro, I.P., Menezes, A.N., Moreira, M.A., Bonvicino, C.R., Seuanez, H.N., and Soares, M.A. (2005). Evolution of cyclophilin A and TRIMCyp retrotransposition in New World primates. *J Virol* 79, 14998-15003.
- Richardson, M.W., Carroll, R.G., Stremlau, M., Korokhov, N., Humeau, L.M., Silvestri, G., Sodroski, J., and Riley, J.L. (2008). Mode of Transmission Affects the Sensitivity of HIV-1 to Restriction by Rhesus TRIM5{alpha}. *J Virol*.
- Riddell, S.R., Elliott, M., Lewinsohn, D.A., Gilbert, M.J., Wilson, L., Manley, S.A., Lupton, S.D., Overell, R.W., Reynolds, T.C., Corey, L., *et al.* (1996). T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. *Nature medicine* 2, 216-223.
- Roberts, M.R., Qin, L., Zhang, D., Smith, D.H., Tran, A.C., Dull, T.J., Groopman, J.E., Capon, D.J., Byrn, R.A., and Finer, M.H. (1994). Targeting of human immunodeficiency virus-infected cells by CD8+ T lymphocytes armed with universal T-cell receptors. *Blood* 84, 2878-2889.
- Rold, C.J., and Aiken, C. (2008). Proteasomal degradation of TRIM5alpha during retrovirus restriction. *PLoS pathogens* 4, e1000074.
- Ryffel, B., Woerly, G., Greiner, B., Haendler, B., Mihatsch, M.J., and Foxwell, B.M. (1991). Distribution of the cyclosporine binding protein cyclophilin in human tissues. *Immunology* 72, 399-404.
- Saenz, D.T., Teo, W., Olsen, J.C., and Poeschla, E.M. (2005). Restriction of feline immunodeficiency virus by Ref1, Lv1, and primate TRIM5alpha proteins. *J Virol* 79, 15175-15188.
- Sakuma, R., Noser, J.A., Ohmine, S., and Ikeda, Y. (2007). Rhesus monkey TRIM5alpha restricts HIV-1 production through rapid degradation of viral Gag polyproteins. *Nature medicine* 13, 631-635.
- Samuel, C.E. (2001). Antiviral actions of interferons. *Clinical microbiology reviews* 14, 778-809, table of contents.
- Santiago, M.L., Montano, M., Benitez, R., Messer, R.J., Yonemoto, W., Chesebro, B., Hasenkrug, K.J., and Greene, W.C. (2008). Apobec3 encodes Rfv3, a gene influencing neutralizing antibody control of retrovirus infection. *Science* 321, 1343-1346.
- Santoni de Sio, F.R., Cascio, P., Zingale, A., Gasparini, M., and Naldini, L. (2006). Proteasome activity restricts lentiviral gene transfer into hematopoietic stem cells and is down-regulated by cytokines that enhance transduction. *Blood* 107, 4257-4265.
- Sarkar, I., Hauber, I., Hauber, J., and Buchholz, F. (2007). HIV-1 proviral DNA excision using an evolved recombinase. *Science* 316, 1912-1915.

- Sasada, A., Takaori-Kondo, A., Shirakawa, K., Kobayashi, M., Abudu, A., Hishizawa, M., Imada, K., Tanaka, Y., and Uchiyama, T. (2005). APOBEC3G targets human T-cell leukemia virus type 1. *Retrovirology* 2, 32.
- Sawyer, S.L., Emerman, M., and Malik, H.S. (2004). Ancient adaptive evolution of the primate antiviral DNA-editing enzyme APOBEC3G. *PLoS biology* 2, E275.
- Sawyer, S.L., Wu, L.I., Akey, J.M., Emerman, M., and Malik, H.S. (2006). High-frequency persistence of an impaired allele of the retroviral defense gene TRIM5alpha in humans. *Curr Biol* 16, 95-100.
- Sawyer, S.L., Wu, L.I., Emerman, M., and Malik, H.S. (2005). Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* 102, 2832-2837.
- Sayah, D.M., and Luban, J. (2004). Selection for loss of Ref1 activity in human cells releases human immunodeficiency virus type 1 from cyclophilin A dependence during infection. *J Virol* 78, 12066-12070.
- Sayah, D.M., Sokolskaja, E., Berthouix, L., and Luban, J. (2004). Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* 430, 569-573.
- Schaller, T., Ylinen, L.M., Webb, B.L., Singh, S., and Towers, G.J. (2007). Fusion of cyclophilin a to fv1 enables cyclosporine-sensitive restriction of human and feline immunodeficiency viruses. *J Virol* 81, 10055-10063.
- Schambach, A., Schiedlmeier, B., Kuhlcke, K., Verstegen, M., Margison, G.P., Li, Z., Kamino, K., Bohne, J., Alexandrov, A., Hermann, F.G., *et al.* (2006). Towards hematopoietic stem cell-mediated protection against infection with human immunodeficiency virus. *Gene therapy* 13, 1037-1047.
- Schindler, M., Munch, J., Kutsch, O., Li, H., Santiago, M.L., Bibollet-Ruche, F., Muller-Trutwin, M.C., Novembre, F.J., Peeters, M., Courgnaud, V., *et al.* (2006). Nef-mediated suppression of T cell activation was lost in a lentiviral lineage that gave rise to HIV-1. *Cell* 125, 1055-1067.
- Schmid, F.X. (1995). Protein folding. Prolyl isomerases join the fold. *Curr Biol* 5, 993-994.
- Schotte, R., Nagasawa, M., Weijer, K., Spits, H., and Blom, B. (2004). The ETS transcription factor Spi-B is required for human plasmacytoid dendritic cell development. *The Journal of experimental medicine* 200, 1503-1509.
- Schrofelbauer, B., Chen, D., and Landau, N.R. (2004). A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif). *Proc Natl Acad Sci U S A* 101, 3927-3932.
- Schrofelbauer, B., Yu, Q., Zeitlin, S.G., and Landau, N.R. (2005). Human immunodeficiency virus type 1 Vpr induces the degradation of the UNG and SMUG uracil-DNA glycosylases. *J Virol* 79, 10978-10987.
- Schwartz, O., Marechal, V., Friguet, B., Arenzana-Seisdedos, F., and Heard, J.M. (1998). Antiviral activity of the proteasome on incoming human immunodeficiency virus type 1. *J Virol* 72, 3845-3850.
- Sebastian, S., and Luban, J. (2005). TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. *Retrovirology* 2, 40.
- Sebastian, S., and Luban, J. (2007). The Retroviral Restriction Factor TRIM5alpha. *Curr Infect Dis Rep* 9, 167-173.
- Sebastian, S., Sokolskaja, E., and Luban, J. (2006). Arsenic counteracts human immunodeficiency virus type 1 restriction by various TRIM5 orthologues in a cell type-dependent manner. *J Virol* 80, 2051-2054.

- Sheehy, A.M., Gaddis, N.C., Choi, J.D., and Malim, M.H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 418, 646-650.
- Sheehy, A.M., Gaddis, N.C., and Malim, M.H. (2003). The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nature medicine* 9, 1404-1407.
- Shen, Z.J., Esnault, S., and Malter, J.S. (2005). The peptidyl-prolyl isomerase Pin1 regulates the stability of granulocyte-macrophage colony-stimulating factor mRNA in activated eosinophils. *Nature immunology* 6, 1280-1287.
- Sherer, N.M., Lehmann, M.J., Jimenez-Soto, L.F., Horensavitz, C., Pypaert, M., and Mothes, W. (2007). Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission. *Nature cell biology* 9, 310-315.
- Shi, J., and Aiken, C. (2006). Saturation of TRIM5 alpha-mediated restriction of HIV-1 infection depends on the stability of the incoming viral capsid. *Virology* 350, 493-500.
- Shi, M., Deng, W., Bi, E., Mao, K., Ji, Y., Lin, G., Wu, X., Tao, Z., Li, Z., Cai, X., *et al.* (2008). TRIM30 alpha negatively regulates TLR-mediated NF-kappa B activation by targeting TAB2 and TAB3 for degradation. *Nature immunology* 9, 369-377.
- Shibata, R., Kawamura, M., Sakai, H., Hayami, M., Ishimoto, A., and Adachi, A. (1991). Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J Virol* 65, 3514-3520.
- Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., *et al.* (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68, 855-867.
- Shizuru, J.A., Weissman, I.L., Kernoff, R., Masek, M., and Scheffold, Y.C. (2000). Purified hematopoietic stem cell grafts induce tolerance to alloantigens and can mediate positive and negative T cell selection. *Proc Natl Acad Sci U S A* 97, 9555-9560.
- Shultz, L.D., Ishikawa, F., and Greiner, D.L. (2007). Humanized mice in translational biomedical research. *Nat Rev Immunol* 7, 118-130.
- Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., *et al.* (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* 174, 6477-6489.
- Shultz, L.D., Schweitzer, P.A., Christianson, S.W., Gott, B., Schweitzer, I.B., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T.V., Greiner, D.L., *et al.* (1995). Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol* 154, 180-191.
- Simon, J.H., Gaddis, N.C., Fouchier, R.A., and Malim, M.H. (1998a). Evidence for a newly discovered cellular anti-HIV-1 phenotype. *Nature medicine* 4, 1397-1400.
- Simon, J.H., Miller, D.L., Fouchier, R.A., Soares, M.A., Peden, K.W., and Malim, M.H. (1998b). The regulation of primate immunodeficiency virus infectivity by Vif is cell species restricted: a role for Vif in determining virus host range and cross-species transmission. *The EMBO journal* 17, 1259-1267.
- Simon, J.H., Southerling, T.E., Peterson, J.C., Meyer, B.E., and Malim, M.H. (1995). Complementation of vif-defective human immunodeficiency virus type 1 by primate, but not nonprimate, lentivirus vif genes. *J Virol* 69, 4166-4172.
- Skowronski, J., Parks, D., and Mariani, R. (1993). Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. *The EMBO journal* 12, 703-713.
- Sokolskaja, E., Sayah, D.M., and Luban, J. (2004). Target cell cyclophilin A modulates human immunodeficiency virus type 1 infectivity. *J Virol* 78, 12800-12808.

- Song, B., Diaz-Griffero, F., Park, D.H., Rogers, T., Stremlau, M., and Sodroski, J. (2005a). TRIM5alpha association with cytoplasmic bodies is not required for antiretroviral activity. *Virology* 343, 201-211.
- Song, B., Gold, B., O'Huigin, C., Javanbakht, H., Li, X., Stremlau, M., Winkler, C., Dean, M., and Sodroski, J. (2005b). The B30.2(SPRY) domain of the retroviral restriction factor TRIM5alpha exhibits lineage-specific length and sequence variation in primates. *J Virol* 79, 6111-6121.
- Soros, V.B., Yonemoto, W., and Greene, W.C. (2007). Newly synthesized APOBEC3G is incorporated into HIV virions, inhibited by HIV RNA, and subsequently activated by RNase H. *PLoS pathogens* 3, e15.
- Sourisseau, M., Sol-Foulon, N., Porrot, F., Blanchet, F., and Schwartz, O. (2007). Inefficient human immunodeficiency virus replication in mobile lymphocytes. *J Virol* 81, 1000-1012.
- Stegmeier, F., Hu, G., Rickles, R.J., Hannon, G.J., and Elledge, S.J. (2005). A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci U S A* 102, 13212-13217.
- Stetson, D.B., Ko, J.S., Heidmann, T., and Medzhitov, R. (2008). Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* 134, 587-598.
- Stoddart, C.A., Bales, C.A., Bare, J.C., Chkhenkeli, G., Galkina, S.A., Kinkade, A.N., Moreno, M.E., Rivera, J.M., Ronquillo, R.E., Sloan, B., *et al.* (2007). Validation of the SCID-hu Thy/Liv mouse model with four classes of licensed antiretrovirals. *PLoS ONE* 2, e655.
- Stopak, K., de Noronha, C., Yonemoto, W., and Greene, W.C. (2003). HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. *Molecular cell* 12, 591-601.
- Stopak, K.S., Chiu, Y.L., Kropp, J., Grant, R.M., and Greene, W.C. (2007). Distinct patterns of cytokine regulation of APOBEC3G expression and activity in primary lymphocytes, macrophages, and dendritic cells. *The Journal of biological chemistry* 282, 3539-3546.
- Stoye, J.P., and Yap, M.W. (2008). Chance favors a prepared genome. *Proc Natl Acad Sci U S A* 105, 3177-3178.
- Strayer, D.S., Akkina, R., Bunnell, B.A., Dropulic, B., Planelles, V., Pomerantz, R.J., Rossi, J.J., and Zaia, J.A. (2005). Current status of gene therapy strategies to treat HIV/AIDS. *Mol Ther* 11, 823-842.
- Strebel, K. (2005). APOBEC3G & HTLV-1: inhibition without deamination. *Retrovirology* 2, 37.
- Strebel, K., Daugherty, D., Clouse, K., Cohen, D., Folks, T., and Martin, M.A. (1987). The HIV 'A' (sor) gene product is essential for virus infectivity. *Nature* 328, 728-730.
- Strebel, K., Klimkait, T., Maldarelli, F., and Martin, M.A. (1989). Molecular and biochemical analysis of human immunodeficiency virus type 1 *vpu* protein. *J Virol* 63, 3784-3791.
- Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427, 848-853.
- Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D.J., Sundquist, W.I., and Sodroski, J. (2006). Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc Natl Acad Sci U S A* 103, 5514-5519.
- Stremlau, M., Perron, M., Welikala, S., and Sodroski, J. (2005). Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. *J Virol* 79, 3139-3145.

- Sugamura, K., Asao, H., Kondo, M., Tanaka, N., Ishii, N., Ohbo, K., Nakamura, M., and Takeshita, T. (1996). The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annual review of immunology* 14, 179-205.
- Sun, J., Soos, T., Kewalramani, V.N., Osiecki, K., Zheng, J.H., Falkin, L., Santambrogio, L., Littman, D.R., and Goldstein, H. (2006). CD4-specific transgenic expression of human cyclin T1 markedly increases human immunodeficiency virus type 1 (HIV-1) production by CD4⁺ T lymphocytes and myeloid cells in mice transgenic for a provirus encoding a monocyte-tropic HIV-1 isolate. *J Virol* 80, 1850-1862.
- Sun, Z., Denton, P.W., Estes, J.D., Othieno, F.A., Wei, B.L., Wege, A.K., Melkus, M.W., Padgett-Thomas, A., Zupancic, M., Haase, A.T., *et al.* (2007). Intrarectal transmission, systemic infection, and CD4⁺ T cell depletion in humanized mice infected with HIV-1. *The Journal of experimental medicine* 204, 705-714.
- Szymczak, A.L., Workman, C.J., Wang, Y., Vignali, K.M., Dilioglou, S., Vanin, E.F., and Vignali, D.A. (2004). Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nature biotechnology* 22, 589-594.
- Takaoka, A., Wang, Z., Choi, M.K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., *et al.* (2007). DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 448, 501-505.
- Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. *Annual review of immunology* 21, 335-376.
- Taylor, B.M., Foulke, J.S., Flinko, R., Heredia, A., DeVico, A., and Reitz, M. (2008). An alteration of human immunodeficiency virus gp41 leads to reduced CCR5 dependence and CD4 independence. *J Virol* 82, 5460-5471.
- Teng, B., Burant, C.F., and Davidson, N.O. (1993). Molecular cloning of an apolipoprotein B messenger RNA editing protein. *Science* 260, 1816-1819.
- Terwilliger, E.F., Cohen, E.A., Lu, Y.C., Sodroski, J.G., and Haseltine, W.A. (1989). Functional role of human immunodeficiency virus type 1 vpu. *Proc Natl Acad Sci U S A* 86, 5163-5167.
- Tissot, C., and Mechti, N. (1995). Molecular cloning of a new interferon-induced factor that represses human immunodeficiency virus type 1 long terminal repeat expression. *The Journal of biological chemistry* 270, 14891-14898.
- Toggas, S.M., Masliah, E., Rockenstein, E.M., Rall, G.F., Abraham, C.R., and Mucke, L. (1994). Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice. *Nature* 367, 188-193.
- Towers, G., Bock, M., Martin, S., Takeuchi, Y., Stoye, J.P., and Danos, O. (2000). A conserved mechanism of retrovirus restriction in mammals. *Proc Natl Acad Sci U S A* 97, 12295-12299.
- Towers, G.J., Hatzioannou, T., Cowan, S., Goff, S.P., Luban, J., and Bieniasz, P.D. (2003). Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. *Nature medicine* 9, 1138-1143.
- Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J.C., Lanzavecchia, A., and Manz, M.G. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* 304, 104-107.
- Turelli, P., Mangeat, B., Jost, S., Vianin, S., and Trono, D. (2004a). Inhibition of hepatitis B virus replication by APOBEC3G. *Science* 303, 1829.
- Turelli, P., Vianin, S., and Trono, D. (2004b). The innate antiretroviral factor APOBEC3G does not affect human LINE-1 retrotransposition in a cell culture assay. *The Journal of biological chemistry* 279, 43371-43373.

- Uchil, P.D., Quinlan, B.D., Chan, W.T., Luna, J.M., and Mothes, W. (2008). TRIM E3 ligases interfere with early and late stages of the retroviral life cycle. *PLoS pathogens* 4, e16.
- Unutmaz, D., KewalRamani, V.N., Marmon, S., and Littman, D.R. (1999). Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *The Journal of experimental medicine* 189, 1735-1746.
- Van Damme, N., Goff, D., Katsura, C., Jorgenson, R.L., Mitchell, R., Johnson, M.C., Stephens, E.B., and Guatelli, J. (2008). The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell host & microbe* 3, 245-252.
- van Rijn, R.S., Simonetti, E.R., Hagenbeek, A., Hogenes, M.C., de Weger, R.A., Canninga-van Dijk, M.R., Weijer, K., Spits, H., Storm, G., van Bloois, L., *et al.* (2003). A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/- gammac-/- double-mutant mice. *Blood* 102, 2522-2531.
- Van Rooijen, N., and Sanders, A. (1994). Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 174, 83-93.
- Varthakavi, V., Heimann-Nichols, E., Smith, R.M., Sun, Y., Bram, R.J., Ali, S., Rose, J., Ding, L., and Spearman, P. (2008). Identification of calcium-modulating cyclophilin ligand as a human host restriction to HIV-1 release overcome by Vpu. *Nature medicine* 14, 641-647.
- Varthakavi, V., Smith, R.M., Bour, S.P., Strebel, K., and Spearman, P. (2003). Viral protein U counteracts a human host cell restriction that inhibits HIV-1 particle production. *Proc Natl Acad Sci U S A* 100, 15154-15159.
- Virgen, C.A., Kratovac, Z., Bieniasz, P.D., and Hatzioannou, T. (2008). Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species. *Proc Natl Acad Sci U S A* 105, 3563-3568.
- Vocero-Akbani, A.M., Heyden, N.V., Lissy, N.A., Ratner, L., and Dowdy, S.F. (1999). Killing HIV-infected cells by transduction with an HIV protease-activated caspase-3 protein. *Nature medicine* 5, 29-33.
- von Laer, D., Hasselmann, S., and Hasselmann, K. (2006a). Gene therapy for HIV infection: what does it need to make it work? *J Gene Med* 8, 658-667.
- von Laer, D., Hasselmann, S., and Hasselmann, K. (2006b). Impact of gene-modified T cells on HIV infection dynamics. *J Theor Biol* 238, 60-77.
- Walker, R.E., Bechtel, C.M., Natarajan, V., Baseler, M., Hege, K.M., Metcalf, J.A., Stevens, R., Hazen, A., Blaese, R.M., Chen, C.C., *et al.* (2000). Long-term in vivo survival of receptor-modified syngeneic T cells in patients with human immunodeficiency virus infection. *Blood* 96, 467-474.
- Wan, Y.Y., Chi, H., Xie, M., Schneider, M.D., and Flavell, R.A. (2006). The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. *Nature immunology* 7, 851-858.
- Wang, L., Menendez, P., Shojaei, F., Li, L., Mazurier, F., Dick, J.E., Cerdan, C., Levac, K., and Bhatia, M. (2005). Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. *The Journal of experimental medicine* 201, 1603-1614.
- Watanabe, S., Terashima, K., Ohta, S., Horibata, S., Yajima, M., Shiozawa, Y., Dewan, M.Z., Yu, Z., Ito, M., Morio, T., *et al.* (2007). Hematopoietic stem cell-engrafted NOD/SCID/IL2Rgamma null mice develop human lymphoid systems and induce long-lasting HIV-1 infection with specific humoral immune responses. *Blood* 109, 212-218.

- Watashi, K., Khan, M., Yedavalli, V.R., Yeung, M.L., Strebel, K., and Jeang, K.T. (2008). Human immunodeficiency virus-1 replication and regulation of APOBEC3G by peptidyl prolyl isomerase Pin1. *J Virol*.
- Wege, A.K., Melkus, M.W., Denton, P.W., Estes, J.D., and Garcia, J.V. (2008). Functional and phenotypic characterization of the humanized BLT mouse model. *Current topics in microbiology and immunology* 324, 149-165.
- Wilson, S.J., Webb, B.L., Ylinen, L.M., Verschoor, E., Heeney, J.L., and Towers, G.J. (2008). Independent evolution of an antiviral TRIMCyp in rhesus macaques. *Proc Natl Acad Sci U S A* 105, 3557-3562.
- Wolf, D., and Goff, S.P. (2008). Host Restriction Factors Blocking Retroviral Replication. *Annual review of genetics*.
- Woo, J.S., Imm, J.H., Min, C.K., Kim, K.J., Cha, S.S., and Oh, B.H. (2006). Structural and functional insights into the B30.2/SPRY domain. *The EMBO journal* 25, 1353-1363.
- Wu, X., Anderson, J.L., Campbell, E.M., Joseph, A.M., and Hope, T.J. (2006). Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection. *Proc Natl Acad Sci U S A* 103, 7465-7470.
- Wu, X., Li, Y., Crise, B., and Burgess, S.M. (2003). Transcription start regions in the human genome are favored targets for MLV integration. *Science* 300, 1749-1751.
- Xu, L., Yang, L., Moitra, P.K., Hashimoto, K., Rallabhandi, P., Kaul, S., Meroni, G., Jensen, J.P., Weissman, A.M., and D'Arpa, P. (2003). BTBD1 and BTBD2 colocalize to cytoplasmic bodies with the RBCC/tripartite motif protein, TRIM5delta. *Exp Cell Res* 288, 84-93.
- Yamashita, M., Perez, O., Hope, T.J., and Emerman, M. (2007). Evidence for direct involvement of the capsid protein in HIV infection of nondividing cells. *PLoS pathogens* 3, 1502-1510.
- Yang, B., Chen, K., Zhang, C., Huang, S., and Zhang, H. (2007). Virion-associated uracil DNA glycosylase-2 and apurinic/apyrimidinic endonuclease are involved in the degradation of APOBEC3G-edited nascent HIV-1 DNA. *The Journal of biological chemistry* 282, 11667-11675.
- Yang, L., Bailey, L., Baltimore, D., and Wang, P. (2006). Targeting lentiviral vectors to specific cell types in vivo. *Proc Natl Acad Sci U S A* 103, 11479-11484.
- Yap, M.W., Dodding, M.P., and Stoye, J.P. (2006). Trim-cyclophilin A fusion proteins can restrict human immunodeficiency virus type 1 infection at two distinct phases in the viral life cycle. *J Virol* 80, 4061-4067.
- Yap, M.W., Mortuza, G.B., Taylor, I.A., and Stoye, J.P. (2007). The design of artificial retroviral restriction factors. *Virology* 365, 302-314.
- Yap, M.W., Nisole, S., Lynch, C., and Stoye, J.P. (2004). Trim5alpha protein restricts both HIV-1 and murine leukemia virus. *Proc Natl Acad Sci U S A* 101, 10786-10791.
- Yap, M.W., Nisole, S., and Stoye, J.P. (2005). A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Curr Biol* 15, 73-78.
- Yin, L., Braaten, D., and Luban, J. (1998). Human immunodeficiency virus type 1 replication is modulated by host cyclophilin A expression levels. *J Virol* 72, 6430-6436.
- Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y.M., Gale, M., Jr., Akira, S., *et al.* (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175, 2851-2858.
- Yu, Q., Konig, R., Pillai, S., Chiles, K., Kearney, M., Palmer, S., Richman, D., Coffin, J.M., and Landau, N.R. (2004). Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nature structural & molecular biology* 11, 435-442.

- Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X.F. (2003). Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* 302, 1056-1060.
- Zennou, V., Perez-Caballero, D., Gottlinger, H., and Bieniasz, P.D. (2004). APOBEC3G incorporation into human immunodeficiency virus type 1 particles. *J Virol* 78, 12058-12061.
- Zhang, B.W., Zimmer, G., Chen, J., Ladd, D., Li, E., Alt, F.W., Wiederrecht, G., Cryan, J., O'Neill, E.A., Seidman, C.E., *et al.* (1996). T cell responses in calcineurin A alpha-deficient mice. *The Journal of experimental medicine* 183, 413-420.
- Zhang, F., Hatzioannou, T., Perez-Caballero, D., Derse, D., and Bieniasz, P.D. (2006). Antiretroviral potential of human tripartite motif-5 and related proteins. *Virology* 353, 396-409.
- Zhang, F., Perez-Caballero, D., Hatzioannou, T., and Bieniasz, P.D. (2008a). No effect of endogenous TRIM5alpha on HIV-1 production. *Nature medicine* 14, 235-236; author reply 236-238.
- Zhang, H., Yang, B., Pomerantz, R.J., Zhang, C., Arunachalam, S.C., and Gao, L. (2003). The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* 424, 94-98.
- Zhang, J., Scadden, D.T., and Crumpacker, C.S. (2007). Primitive hematopoietic cells resist HIV-1 infection via p21. *The Journal of clinical investigation* 117, 473-481.
- Zhang, J.X., Diehl, G.E., and Littman, D.R. (2008b). Relief of preintegration inhibition and characterization of additional blocks for HIV replication in primary mouse T cells. *PLoS ONE* 3, e2035.
- Zielske, S.P., Reese, J.S., Lingas, K.T., Donze, J.R., and Gerson, S.L. (2003). In vivo selection of MGMT(P140K) lentivirus-transduced human NOD/SCID repopulating cells without pretransplant irradiation conditioning. *The Journal of clinical investigation* 112, 1561-1570.
- Zinkernagel, R.M., and Althage, A. (1999). On the role of thymic epithelium vs. bone marrow-derived cells in repertoire selection of T cells. *Proc Natl Acad Sci U S A* 96, 8092-8097.

What is claimed is:

1. A nucleic acid which comprises a sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and a variant having at least about 85% identity to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, wherein the nucleic acid encodes a polypeptide having both TRIM activity and cyclophilin activity.
2. A nucleic acid which comprises a sequence complementary to the nucleic acid of claim 1.
3. The nucleic acid of claim 1, wherein the variant has at least about 85%, 90%, 95% or 99% identity to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7.
4. A nucleic acid encoding a polypeptide comprising SEQ ID NO:4 and SEQ ID NO:2, or a polypeptide comprising SEQ ID:10, 11 or 12.
5. The nucleic acid of claim 4, wherein the last amino acid of SEQ ID NO:2 is serine at position 309, serine at position 322, or alanine at position 331.
6. The nucleic acid of claim 4, wherein the last amino acid of SEQ ID NO:2 is any one residue from about 280 to about 290, about 308 to about 311, about 321 to about 346, about 366 to about 371, about 381 to about 393.
7. A nucleic acid encoding a human TRIM5-cyclophilin A fusion protein.
8. The nucleic acid of claim 7, wherein the fusion protein comprises SEQ ID NO:10, 11 or 12.
9. The nucleic acid of claim 7, wherein the nucleic acid encodes a polypeptide consisting essentially of SEQ ID NO:4 and SEQ ID NO:2, wherein the last amino acid of SEQ ID NO:2 is serine at position 309, serine at position 322, or alanine at position 331.
10. The nucleic acid of claim 7, wherein the nucleic acid encodes a polypeptide comprising a sequence that is at least 85%, 90%, 95%, or 99% identical to SEQ ID NO:4 and a sequence that is at least 85%, 90%, 95%, or 99% identical to SEQ ID NO:2 and the polypeptide has the function of TRIM5 and specifically binds a capsid protein of HIV.

11. The nucleic acid of claim 7, wherein the fusion protein comprises a polypeptide linker sequence, wherein (i) TRIM5 is located upstream of the linker sequence, so that the N-terminal amino acid of the linker sequence is attached to the C-terminal amino acid of TRIM5, and (ii) cyclophilin A is located downstream of the linker sequence, so that the C-terminal amino acid residue of the linker sequence is attached to the N-terminal amino acid residue of cyclophilin A.
12. The nucleic acid of claim 11, wherein the linker sequence comprises from about 10 to about 20 amino acids.
13. The nucleic acid of claim 11, wherein the linker sequence comprises the consecutive amino acids SGGSGGSGGSGG.
14. The nucleic acid of claim 11, wherein the C-terminal amino acid of TRIM5 is methionine at position 284 of SEQ ID NO:2 or threonine at position 369 of SEQ ID NO:2.
15. A polypeptide encoded by the nucleic acid of any of claims 1 – 14.
16. A polypeptide comprising SEQ ID NO:4 and SEQ ID NO:2, or comprising SEQ ID NO:10, 11 or 12.
17. The polypeptide of claim 16, wherein the last amino acid of SEQ ID NO:2 is serine at position 309, serine at position 322, or alanine at position 331.
18. An antibody that specifically binds to the polypeptide of claim 15 or 16.
19. The antibody of claim 18, wherein the antibody is a polyclonal antibody, a monoclonal antibody, or a chimeric antibody.
20. An antibody that specifically binds to the polypeptide encoded by the nucleic acid of any one of claims 1 – 14.
21. A method of producing the polypeptide of claim 15 or 16, which comprises:
 - (a) introducing a nucleic acid encoding the polypeptide into a host cell under conditions that are compatible with expression of the polypeptide by the host cell, and
 - (b) recovering the polypeptide.

22. A nucleic acid vector, which comprises the nucleic acid of any one of claims 1 – 14.
23. The nucleic acid vector of claim 22, wherein the vector comprises a viral vector.
24. The nucleic acid vector of claim 23, wherein the vector is a lentiviral vector, an adenovirus vector, a retroviral vector, or an adeno-associated viral (AAV) vector.
25. A lentiviral vector comprising the nucleic acid of any one of claims 1 – 14.
26. A lentiviral vector encoding a human TRIM5-cyclophilin A fusion protein, wherein the vector comprises a promoter from a spleen focus-forming virus (SFFV) long terminal repeat (LTR), a promoter from a human cyclophilin A gene, or both.
27. A nucleic acid vector having SEQ ID NO:8.
28. A nucleic acid vector having SEQ ID NO:9.
29. A host organism comprising the vector of any one of claims 21 – 28.
30. The host organism of claim 29, wherein the host is a prokaryote, a eukaryote or a fungus.
31. A method for preparing a pharmaceutical composition which comprises admixing the polypeptide or a fragment thereof claim 15 or 16, thereby preparing the pharmaceutical composition.
32. A pharmaceutical composition comprising the nucleic acid of any of claims 1 – 14, the polypeptide of claim 15 or 16, or the vector of any of claims 21 – 28, and a carrier.
33. A method for treating a subject suffering from a disease or condition, the method comprising administering to the subject the nucleic acid of any of claims 1 – 14, the polypeptide of claim 15 or 16, or the vector of any of claims 21 – 28.
34. A method for treating a subject suffering from a disease or a condition, the method comprising administering to the subject a polypeptide or a fragment thereof of claim 15 or 16, so as to treat the subject.
35. A method for preventing retroviral infection of a subject, or for treating a subject with retroviral infection, the method comprising administering to the subject the pharmaceutical composition of claim 32.

36. A method for treating or preventing a viral infection of a cell, the method comprising introducing a human TRIM5-Cyp A fusion polypeptide into the cell.
37. The method of claim 36, wherein the introducing comprises transfection, transduction, viral-mediated introduction, or liposome-mediated introduction of a polynucleotide encoding a human TRIM5-Cyp A fusion polypeptide.
38. The method of claim 36, wherein the viral infection is an HIV infection, an HTLV infection, a pox virus infection, a retrovirus infection, a malaria infection, a hepatitis C virus infection, a hepatitis B virus infection, or a vaccinia virus infection.
39. A method for treating HIV infection or preventing HIV infection of a subject which comprises introducing into cells of the subject a human TRIM5-Cyp A fusion polypeptide.
40. The method of claim 39, wherein the human TRIM5-Cyp A fusion polypeptide binds to a capsid protein of the HIV and subsequently degrades the HIV, thereby treating or preventing HIV infection in the subject.
41. A peptidomimetic comprising an amino acid sequence substantially identical to the amino acid sequence of the polypeptide of claim 15 or 16 and wherein the peptidomimetic comprises TRIM5 function and cyclophilin A function.
42. A cell comprising the nucleic acid of any one of claims 1 – 14.
43. The cell of claim 42, wherein the cell is a stem cell or a T cell.
44. A method for imparting resistance to HIV to a subject or to cells of a subject, the method comprising administering to the cells of the subject the nucleic acid of any of claims 1 – 14, the vector of any one of claims 21 – 28, or the polypeptide of claim 15 or 16.
45. A therapeutic composition comprising the nucleic acid of any of claims 1 – 14, the polypeptide of claim 15 or 16, or a peptidomimetic of claim 41, and a therapeutically acceptable carrier.
46. The therapeutic composition of claim 45, wherein the carrier comprises a vector, a liposome, or a viral vector.

47. A method for *ex vivo* gene therapy, the method comprising: (a) removing bone marrow cells from a subject; (b) transfecting the removed bone marrow cells with a nucleic acid of any one of claims 1 – 14 *in vitro*; and (c) transplanting the transfected bone marrow back into the subject.
48. A method for reducing viral burden or load in a subject infected by a virus, the method comprising administering to the subject the nucleic acid of any one of claims 1 – 14, the polypeptide of claim 15 or 16, or the vector of any of claims 21 – 28.
49. A topical composition for prevention or treatment of a viral infection which comprises the nucleic acid of any of claims 1 – 14 or the polypeptide of claim 15 or 16, and a topical carrier.
50. The topical composition of claim 49, wherein the composition is for use on the skin, in the vagina, in the nose, in the mouth, or on any skin or mucosal surface.
51. A method for delivering a therapeutically effective amount of a human TRIM5-cyclophilin A fusion protein systemically to a subject, comprising administering to the subject a vector of any one of claims 21 – 28 under conditions that are compatible with expression of the nucleic acid in the subject.
52. The method of claim 51, wherein the vector comprises a nonviral vector.
53. The method of claim 51, wherein the vector comprises a gold or tungsten particle and the coated particle is administered to the subject using a gene gun.
54. The method of claim 52, wherein the vector comprises a liposome.
55. The method of claim 51, wherein the vector comprises a viral vector.
56. The method of claim 50, the vector is an adenovirus vector, a lentiviral vector, a retroviral vector, or an adeno-associated viral (AAV) vector.
57. A method for delivering a therapeutically effective amount of a human TRIM5-cyclophilin A fusion protein systemically to a subject, comprising transfecting cells of said subject with a vector of claim 21 – 24, under conditions that permit the expression of the nucleic acid and production of a human TRIM5-cyclophilin A fusion protein.

58. The method of claim 52, wherein the transfecting occurs *ex vivo* and the transfected cells are reintroduced into the subject.
59. A polypeptide comprising the following consecutive amino acids in N-to C-terminus orientation: (i) SEQ ID NO: 13, which consists of amino acids at position 1-298 from hTRIM5 α (SEQ ID NO:2) or a variant of SEQ ID NO: 13; (ii) a linker of 12 to 33 amino acids linking SEQ ID NO: 13 and SEQ ID NO: 15 or the variants thereof; and (iii) SEQ ID NO:15, which consists of amino acid at positions 2 to 166 from hCypA (SEQ ID NO: 4) or a variant of SEQ ID NO: 15, wherein the polypeptide has lentiviral restriction activity.
60. A polypeptide comprising the following consecutive amino acids in N-to C-terminus orientation: (i) SEQ ID NO: 13, which consists of amino acids at position 1-298 from hTRIM5 α (SEQ ID NO:2) or a variant of SEQ ID NO: 13; (ii) a linker of 11-33 amino acids linking SEQ ID NO: 13 and SEQ ID NO: 15 or the variants thereof; and (iii) SEQ ID NO:15, which consists of amino acid at positions 2 to 166 from hCypA (SEQ ID NO: 4) or a variant of SEQ ID NO: 15, wherein the polypeptide has lentiviral restriction activity.
61. A polypeptide comprising the following consecutive amino acids in N-to C-terminus orientation: (i) SEQ ID NO: 13, which consists of amino acids at position 1-298 from hTRIM5 α (SEQ ID NO:2) or a variant of SEQ ID NO:13; (ii) a linker of 11, 12, 24 or 33 amino acids linking SEQ ID NO: 13 and SEQ ID NO: 15 or the variants thereof; and (iii) SEQ ID NO:15, which consists of amino acid at positions 2 to 166 from hCypA (SEQ ID NO: 4) or a variant of SEQ ID NO:15, wherein the polypeptide has lentiviral restriction activity.
62. A polypeptide comprising the following consecutive amino acids in N-to C-terminus orientation: (i) SEQ ID NO: 13, which consists of amino acids at position 1-298 from hTRIM5 α (SEQ ID NO:2); (ii) a linker of 11, 12, 24 or 33 amino acids linking SEQ ID NO: 13 and SEQ ID NO: 15; and (iii) SEQ ID NO:15, which consists of amino acid at positions 2 to 166 from hCypA (SEQ ID NO: 4), wherein the polypeptide has lentiviral restriction activity.

63. The polypeptide of claim 59-62, wherein the lentiviral restriction activity is HIV-1 restriction activity.
64. The polypeptide of claim 59, 60 or 61, wherein the variants of SEQ ID NO: 13 or 15 have at least about 85%, 90%, 95% or 99% identity to SEQ ID NO: 13 or 15.
65. The polypeptide of claim 59, 60 or 61, wherein the variants of SEQ ID NO: 13 or 15 have conservative substitutions.
66. A polypeptide comprising SEQ ID NO: 10, 11, 12, or 17 or a variant of SEQ ID NOS: 10, 11, 12, or 17 having about 85%, 90%, 95% or 99% identity to SEQ ID NO: 10, 11, 12, or 17.
67. The polypeptide of claim 66, comprising a conservative variant of SEQ ID NO: 10, 11, 12, or 17.
68. The polypeptide of claim 59-62, wherein the linker has SEQ ID NO: 20, 21, 22 or 23 or a variant of SEQ ID NO: 20, 21, 22 or 23 having about 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 20, 21, 22 or 23.
69. The polypeptide of claim 59-62, wherein the linker has SEQ ID NO: 20, 21, 22 or 23 or a conservative variant of SEQ ID NO: 20, 21, 22 or 23.
70. An isolated nucleic acid sequence encoding a polypeptide of any of claims 59-69.
71. A vector comprising a nucleic acid sequence encoding a polypeptide of any of claims 59-69.

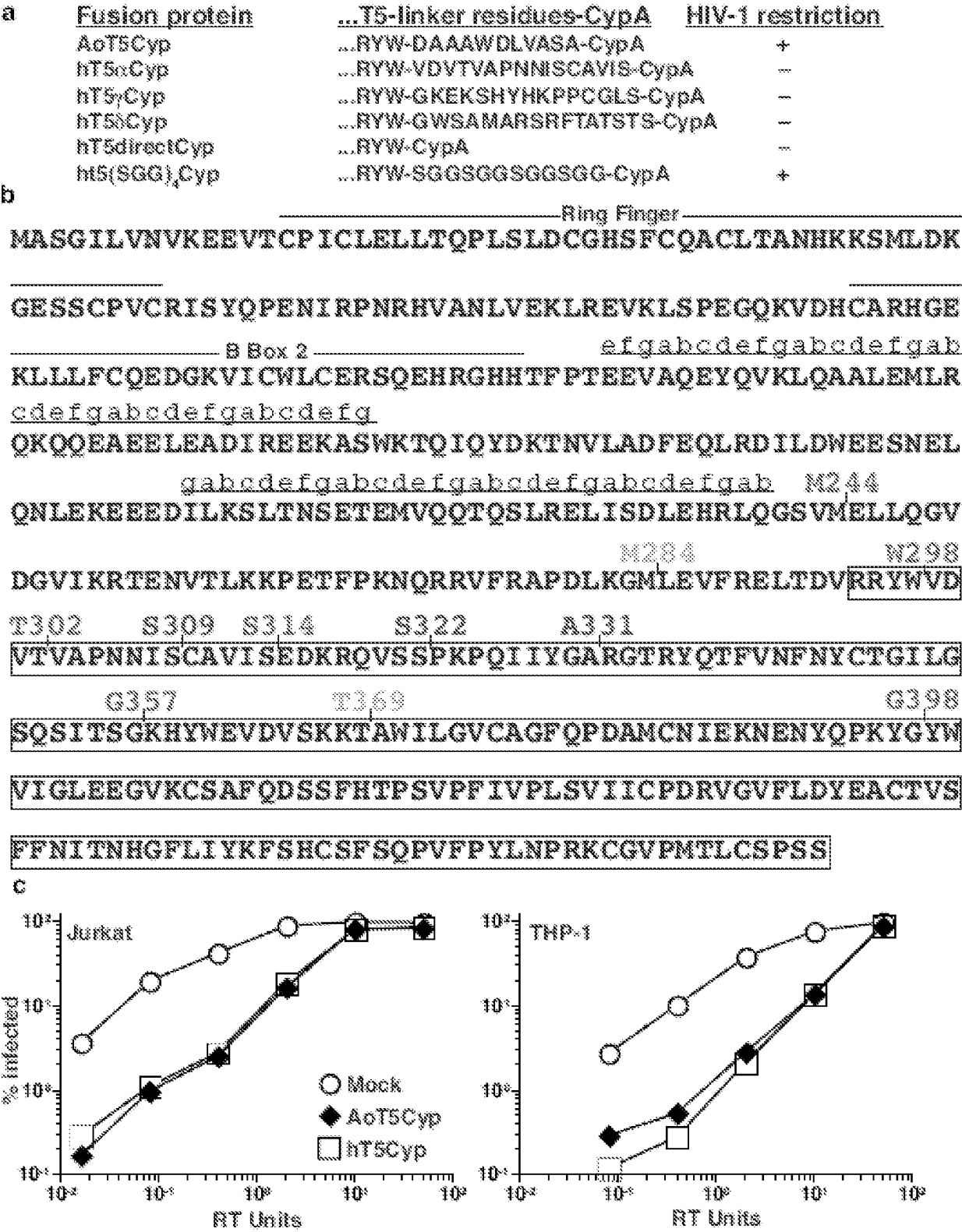


FIG. 1

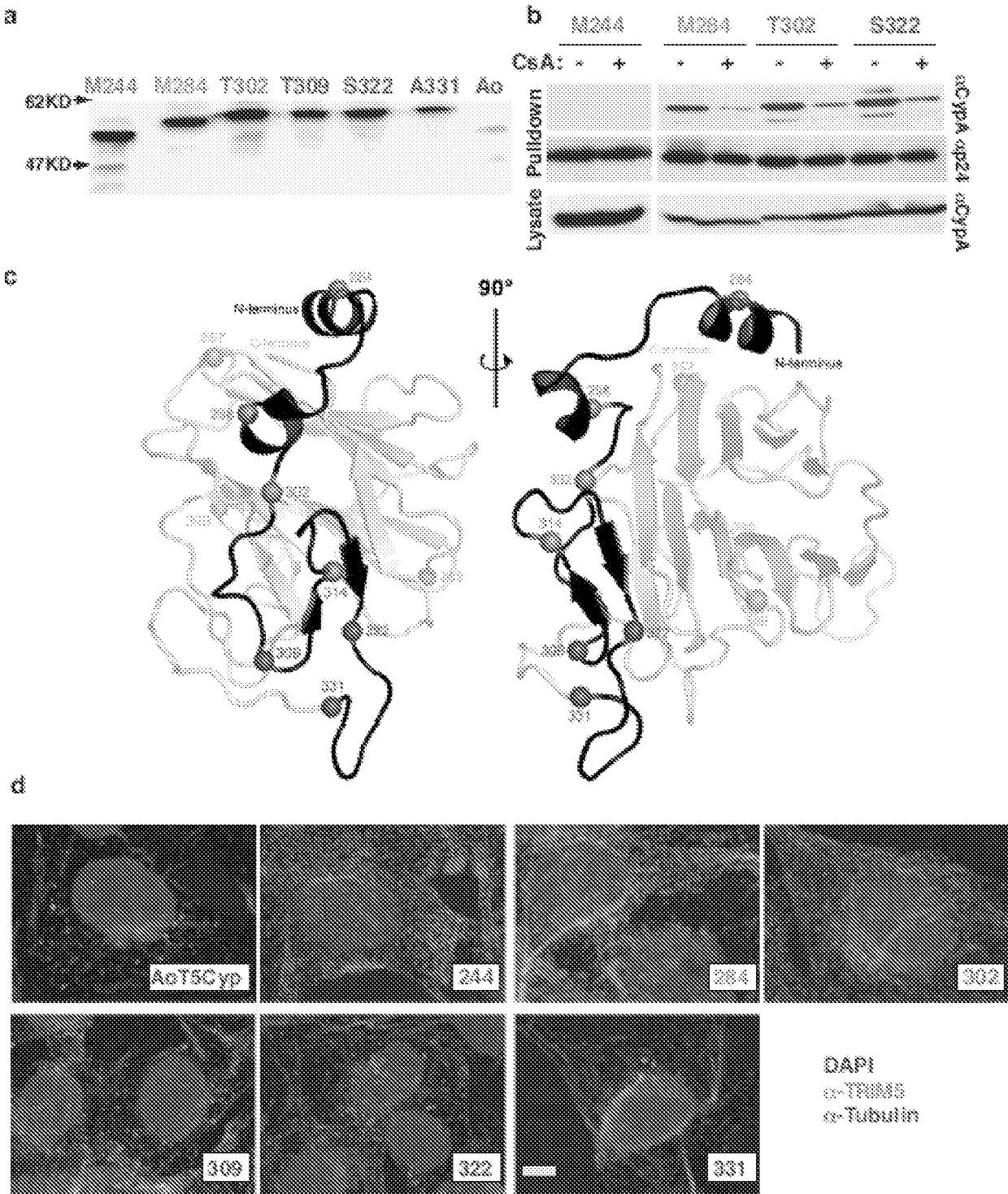


FIG. 2

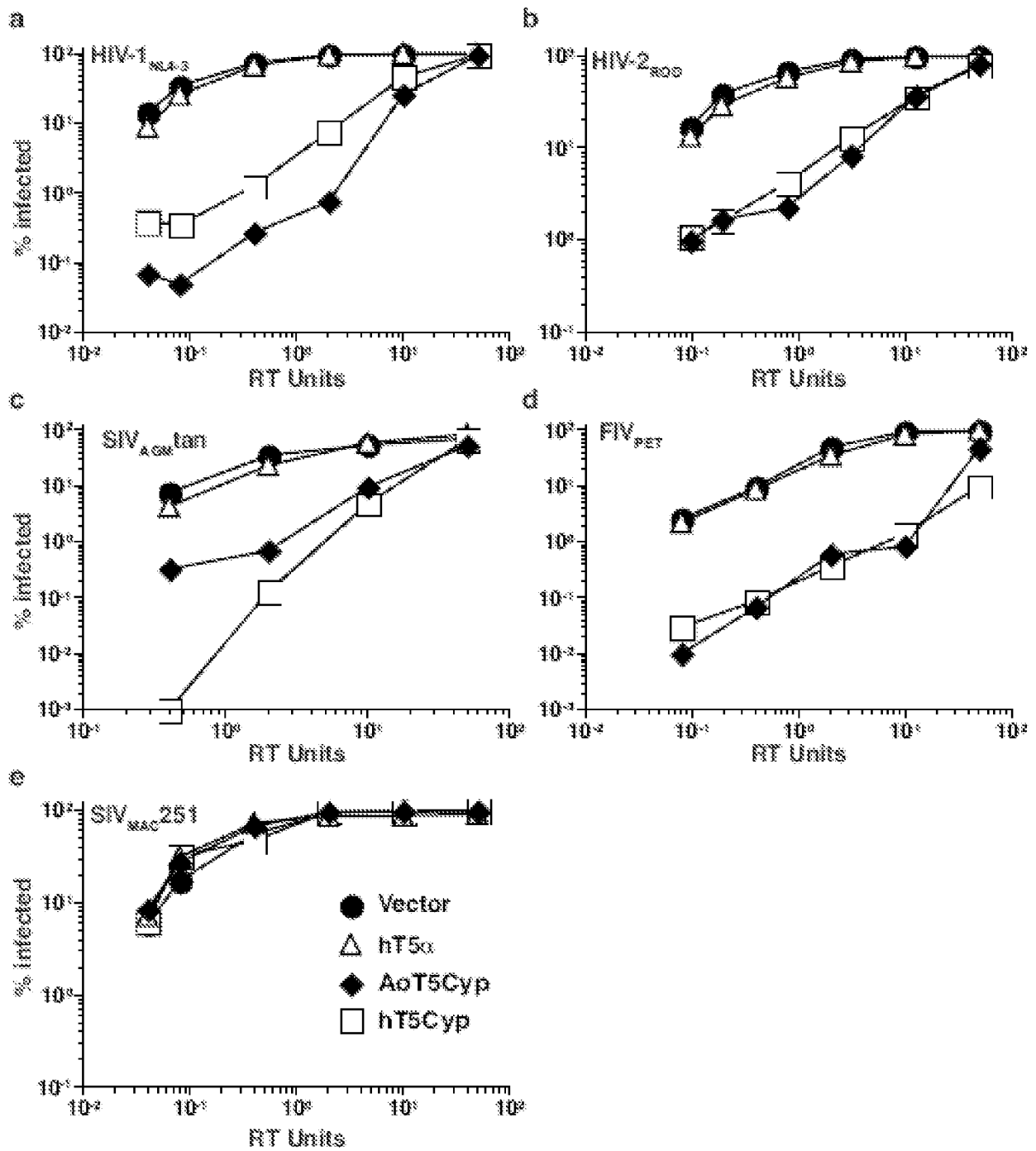


FIG. 3

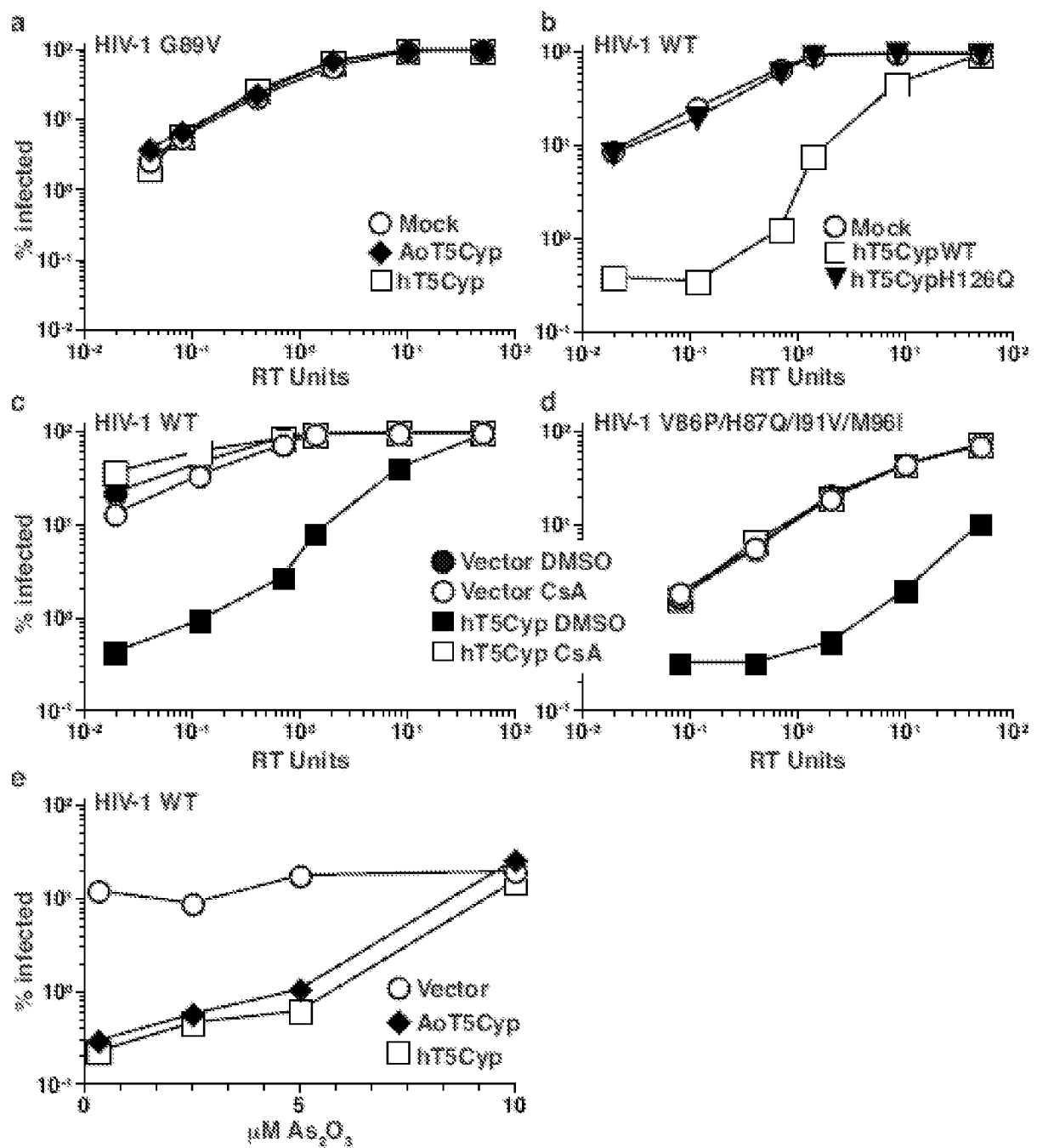


FIG. 4

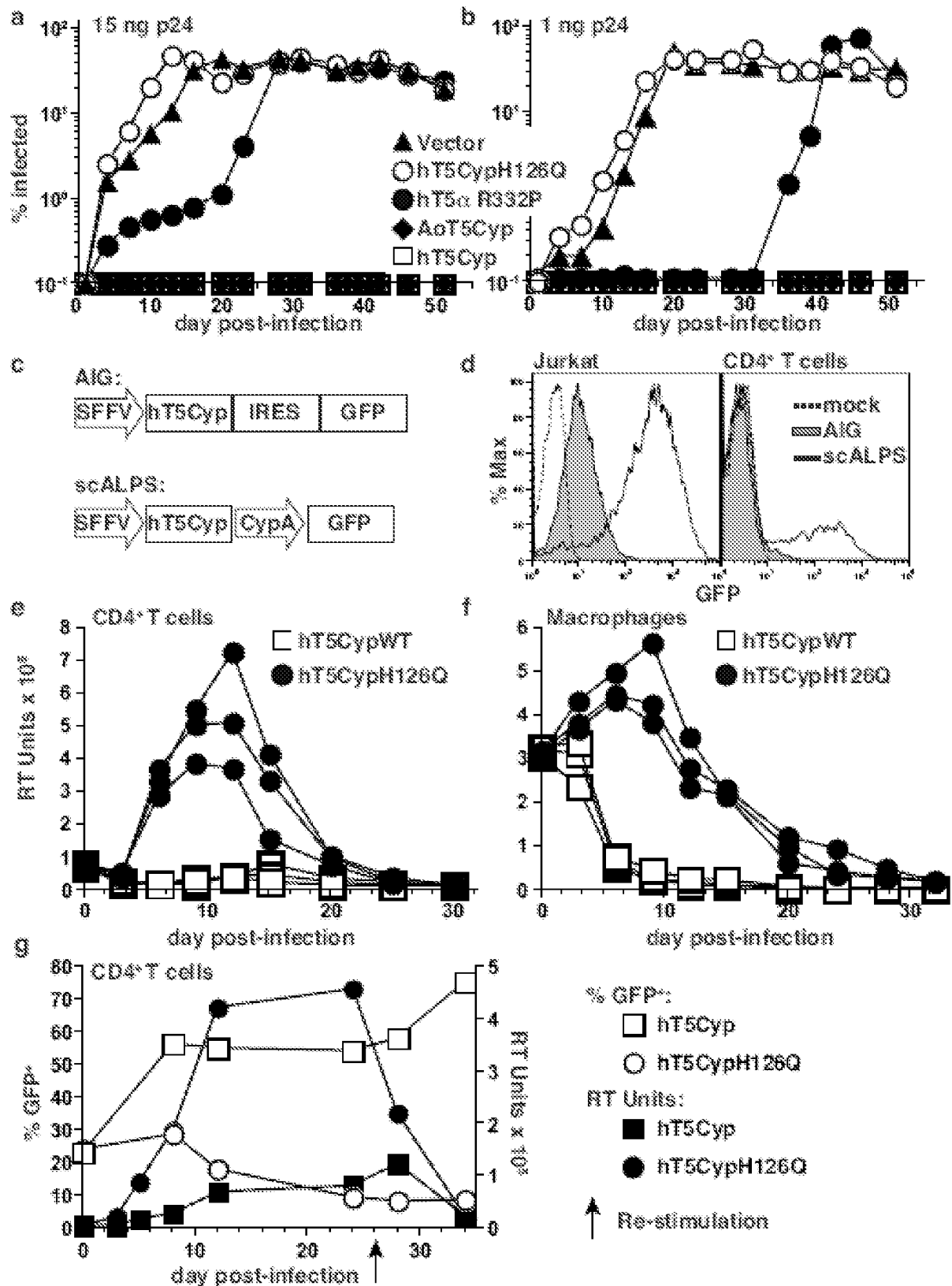


FIG. 5

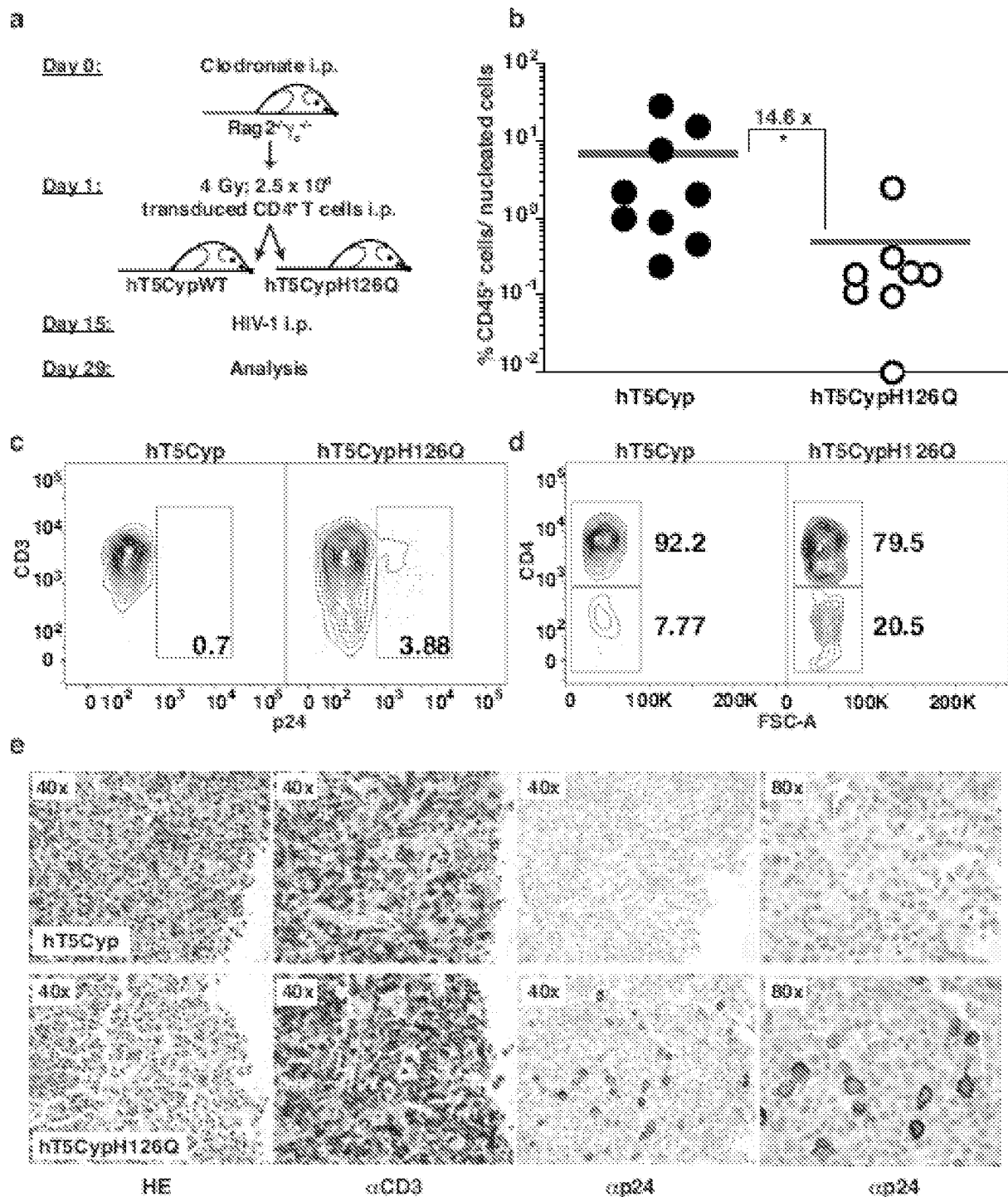


FIG. 6

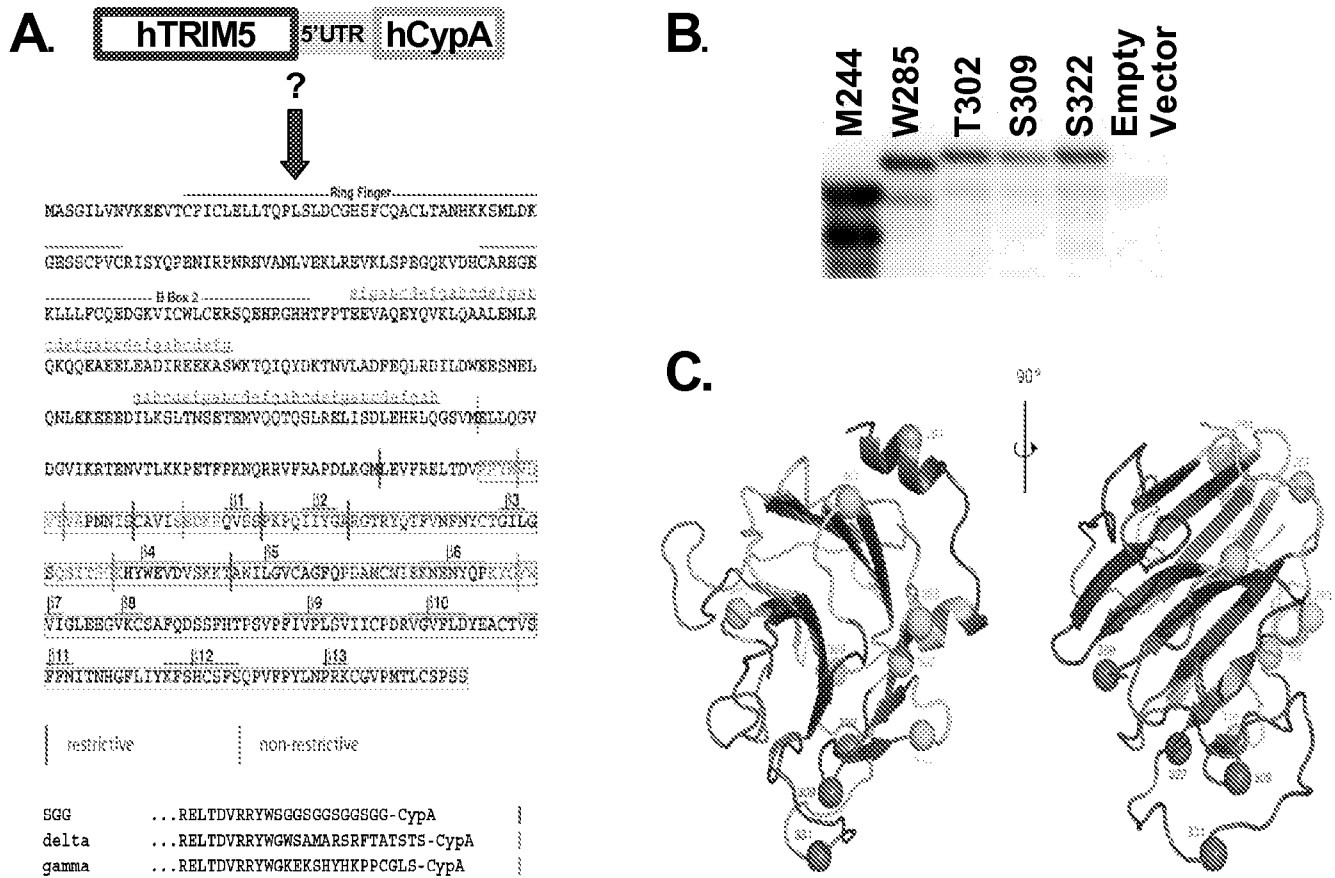


FIG. 7

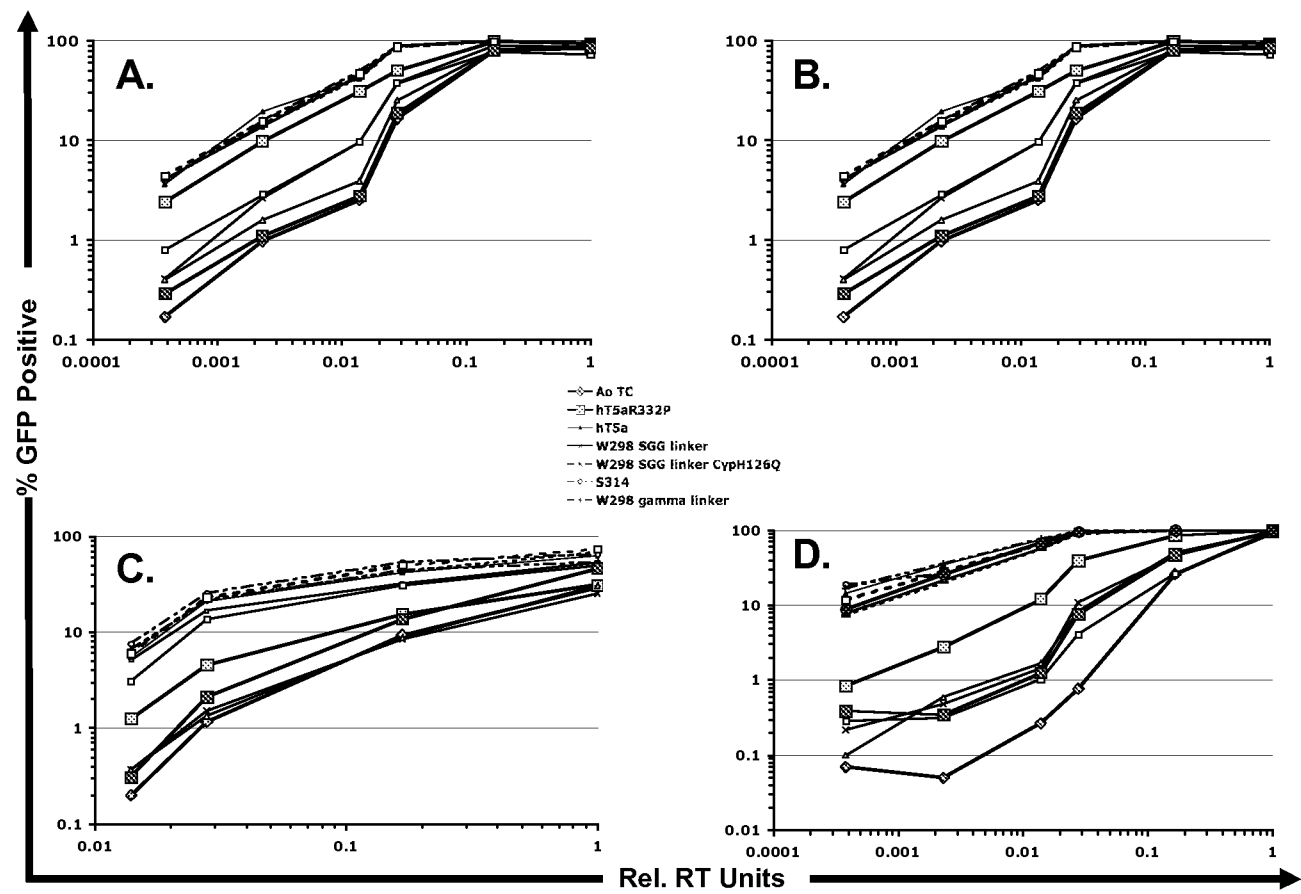


FIG. 8

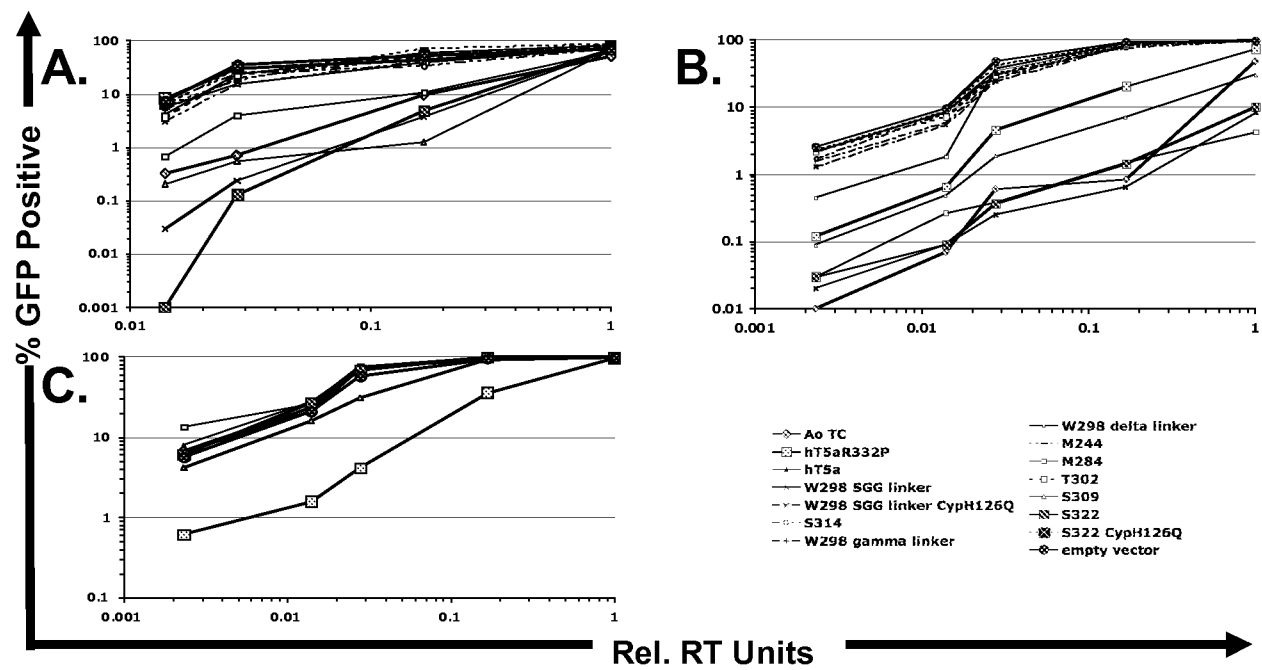


FIG. 9

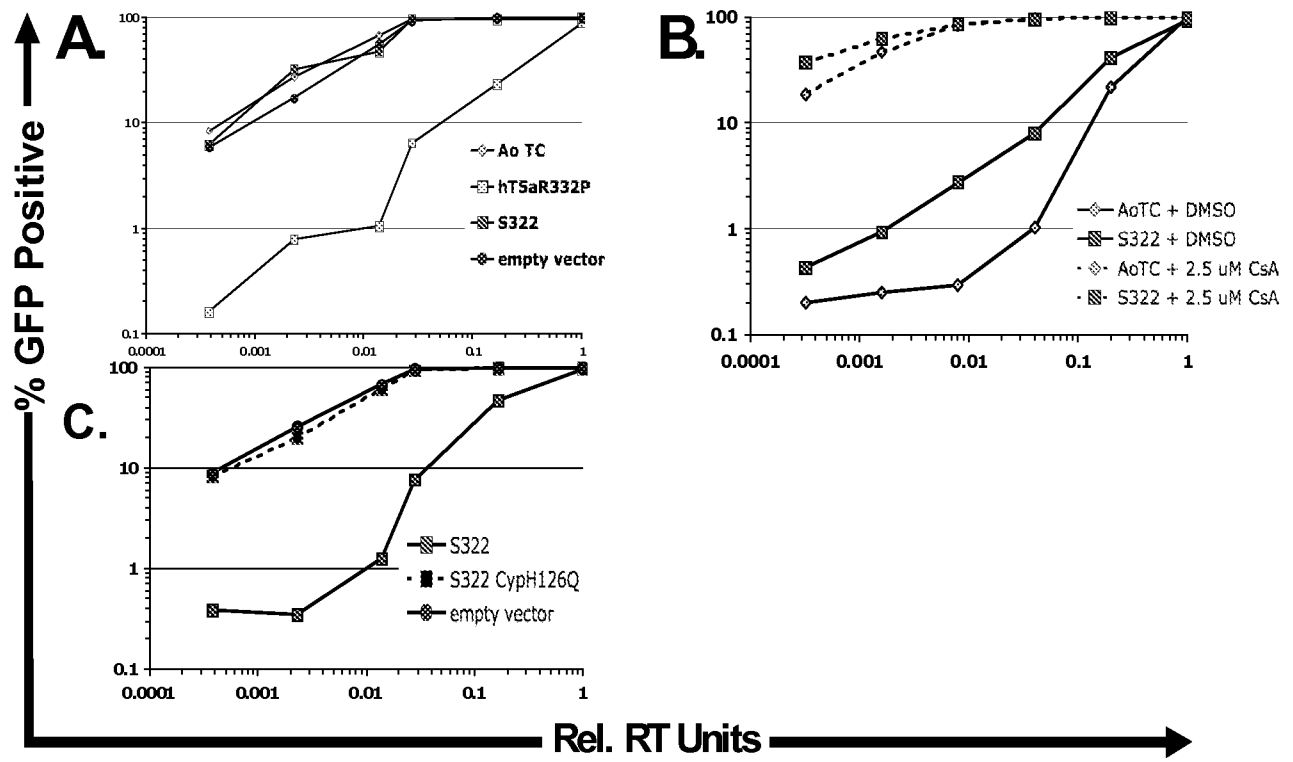


FIG. 10

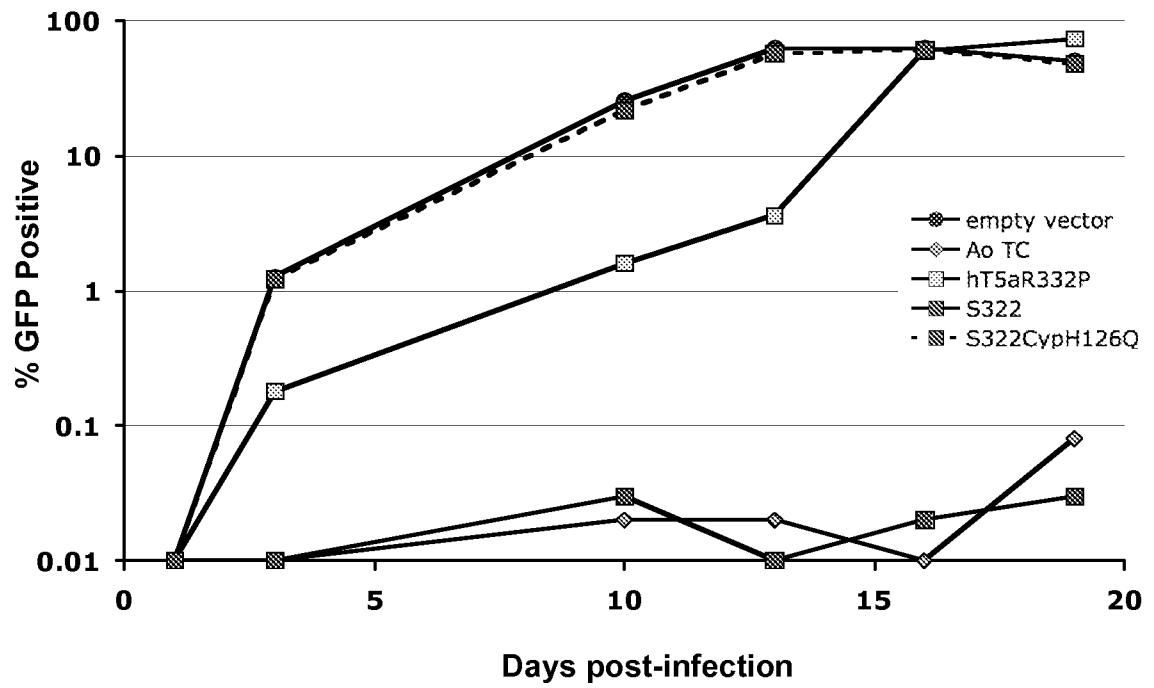


FIG. 11

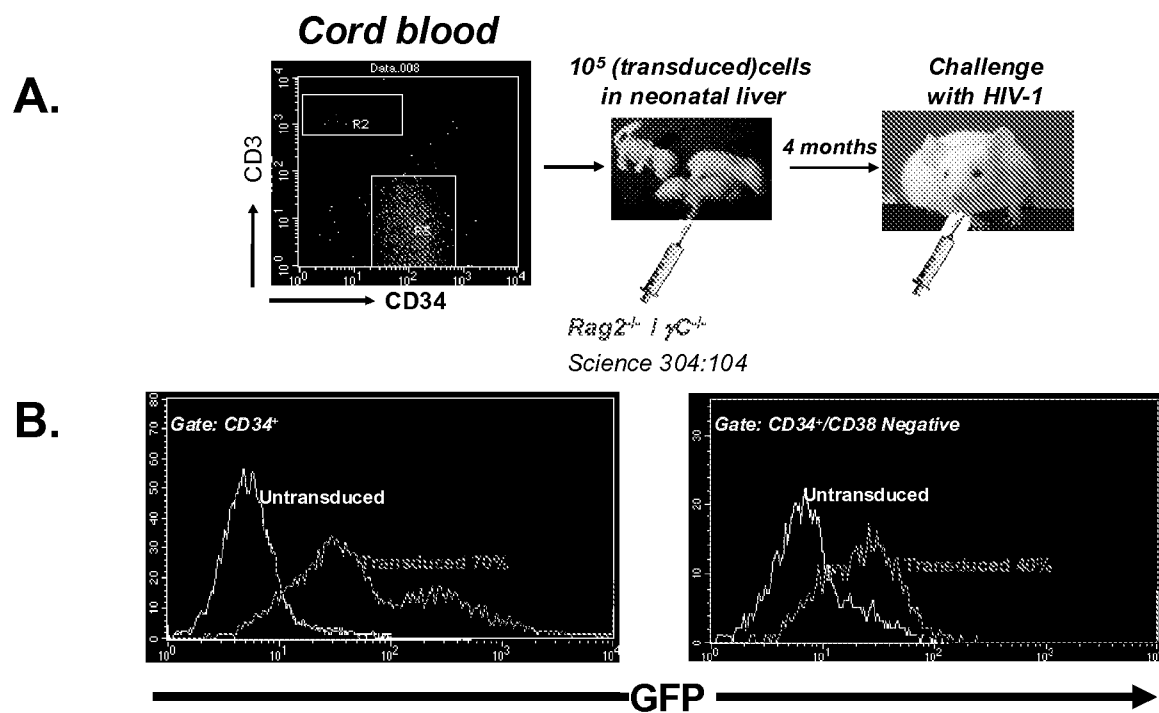


FIG. 12

```

1  gcgggttcctc taggaaaatt cctttgtgca gatcaggccc gtggattggt gagtgaatcc
61  taaccacgtc ttccctggcc tgtcttcact cttctcccca gaatcaccac ttctgcactg
121 gtgtctgaag gtgtattgag tgattttgtg gagggcagaa gtaggaagtc tttgggacaa
181 aactgtattt accttgggat ctgtgaacaa gaggaacctc agcagccagg acaggcagga
241 gcagtggaat agctactatg gcttctggaa tcctgggttaa tgtaaaggag gaggtgacct
301 gccccatctg cctggaactc ctgacacaac ccctgagcct ggactgcggc cacagcttct
361 gccaaagcatg cctcactgca aaccacaaga agtccatgct agacaaagga gagagtagct
421 gccctgtgtg ccggatcagt taccagcctg agaacatacg gcctaatcgg catgtagcca
481 acttagtgga gaagctcagg gaggtcaagt tgagcccaga ggggcagaaa gttgatcatt
541 gtgcacgcca tggagagaaa cttctactct tctgtcagga ggacgggaag gtcatttgcct
601 ggctttgtga gcggtctcag gagcaccgtg gtcaccacac gttccccaca gaggaggttg
661 ccaggagta ccaagtgaag ctccaggcag ctctggagat gctgaggcag aagcagcagg
721 aagctgaaga gttggaagct gacatcagag aagagaaaagc ttcttggaag actcaaatac
781 agtatgacaa aaccaacgtc ttggcagatt ttgagcaact gagagacatc ctggactggg
841 aggagagcaa tgagctgcaa aacctggaga aggaggagga agacattctg aaaagcctta
901 cgaactctga aactgagatg gtgcagcaga ccagtcacct gagagagctc atctcagatc
961 tggagcatcg gctgcagggg tcagtgtatg agctgcttca ggggtgtggat ggcgtcataa
1021 aaaggacgga gaacgtgacc ttgaagaagc cagaaaacttt tccaaaaaat caaaggagag
1081 tgtttcgagc tcctgatctg aaaggaatgc tagaagtgtt tagagagctg acagatgtcc
1141 gacgctactg ggttgatgtg acagtggctc caaacaacat ttcatgtgct gtcatttctg
1201 aagataagag acaagtgagc tctccgaaac cacagataat atatggggca cgagggacaa
1261 gataccagac atttgtgaat ttcaattatt gtactggcat cctgggctct caaagtatca
1321 catcagggaa acattactgg gaggtagacg tgtccaagaa aactgcttgg atcctggggg
1381 tatgtgctgg cttccaacct gatgcaatgt gtaatatga aaaaaatgaa aattatcaac
1441 ctaaatacgg ctactgggtt ataggggttag aggaaggagt taaatgtagt gctttccagg
1501 atagttcctt ccatactcct tctgttcctt tcattgtgcc cctctctgtg attatttgtc
1561 ctgatcgtgt tggagttttc ctagactatg aggcttgcac tgtctcattc ttcaatatca
1621 caaaccatgg atttctcatc tataagtttt ctcactgttc tttttctcag cctgtatttc
1681 catatttaaa tcctagaaaa tgtggagtcc ccatgactct gtgctcacca agctcttgaa
1741 ccttcttaca cactcagccc cttctgtaca gcacctcttg tccagtgcac tctcatcac
1801 ctgaactcat ttgcatcatt ttaacctctt ttctcttctg gtctcccttc tttctatttg
1861 aacgtccttc actcatcagt aaaatgtaat aattgccttg tgccatattg tcccaatat
1921 tttattgaca tttgatagca atttttttca tcattttccg tactcctaag gaaaactgac
1981 ctatacctca taaaatgaga ccgctattta ggtattactt ctgccagata tttatcacc
2041 aattgcctct gacactgact aagaagatga agaaaagctt ttcaacagcc tttctatatc
2101 atcgtgtgat aattgttcac caatgaatga gtccttagcc ctgtgtcagt ttaccctcga
2161 tgcccttatt tgtgagttaa agagaaaata tcataaatgg tatactctta agtatagagg
2221 ttttgtatct agaggatctc agttcaactc ctgtctctcc atataccagc agtgtaaactg
2281 tgaataacat acttaaatgg ctgtgcttat ttctttttct tttctttttt cttttttttt
2341 ttttttgaga tgaagttttg ctcttggtcc ccaggctgga gtgcaatggc acgatctcgg
2401 ttcactgcaa cctccacctc tcagattcaa gcaattctcc tgcctcagcc tcccaagtag
2461 ctgggattac aggtgcccac caccacccct ggctaaattt gtattttcag tagagacggg
2521 gtttcccat gttggttagg ctcgctctaga acctctgacc tcaggtgatc caccgcctc
2581 ggccctccaa agtgctggga ttacaggcgt gagccacggc gccagcctg tgcttatttt
2641 cttaaaataa tttttgtatt aaaaacttca cattaaataa gtgctaattg tttattgcat
2701 agtagggtag cttaggttaa caataaccta ttgcatatat tttgaaatag ctagaagaga
2761 ggattttgaa agttctcaac acaaagaaac gacacatat ttgagtgatg gatagctaa
2821 ttaccctggg tcggttatta cgcaatgtat acatgtatca aaacatcaca ctgtaccaca
2881 taaatatgta tttttattat ttgtcaatta aaagcaaaat aaaacaaaaa accttcattc
2941 aatactttgg atcattgtga aaaaataaat tcctgaagta taaagcatct (SEQ ID NO:1)

```

FIG. 13

```
1 masgilvnvk eevtcpicle lltqpplslde ghsfcqaclt anhkksmldk gesscpvcric
61 syqpenirpn rhvanlvekl revklspegg kvdhcarhge klillfcqedg kvicwlcers
121 qehrghhtfp teevaheyqv klqaalemlr qkqgeaele adireekasw ktqiqydktn
181 vladfeqlrd ildweesnel qnlekeeedi lksltnsete mvqqtqslre lisdlehlrlq
241 gsvmellqgv dgvikrtenv tlkkpetfpk nqrrvfrapd lkgmlevfre ltdvrrywvd
301 vtvapnnisc avisedkrqv sspkpqiyy argtryqtfv nfnyctgilg sqsitsgkhy
361 wevdvskkta wilgvcagfq pdamcniekn enyqpygyw vicleegvkc safqdssfht
421 psvpfivpls viicpdrvqv fldyeactvs ffnitnhgfl iykfshcsfs qpvpfpylnpr
481 kcgvpmtlcs pss (SEQ ID NO:2)
```

FIG. 14

```

1  gaacgtggta  taaaaggggc  gggaggccag  gctcgtgccg  ttttgcagac  gccaccgccg
61  agggaaaaccg  tgtactatta  gccatggtca  accccaccgt  gttcttcgac  attgccgtcg
121  acggcgagcc  cttgggcccgc  gtctcctttg  agctgtttgc  agacaaggtc  ccaaagacag
181  cagaaaattt  tcgtgctctg  agcactggag  agaaaggatt  tggttataag  ggttcctgct
241  ttcacagaat  tattccaggg  tttatgtgtc  aggggtgtga  cttcacacgc  cataatggca
301  ctgggtggcaa  gtccatctat  ggggagaaat  ttgaagatga  gaacttcata  ctaaagcata
361  cgggtcctgg  catcttgtcc  atggcaaatg  ctggacccaa  cacaaatggg  tcccagtttt
421  tcatctgcac  tgccaagact  gagtggttgg  atggcaagca  tgtgggtgtt  ggcaaagtga
481  aagaaggcat  gaatatgtg  gaggccatgg  agcgctttgg  gtccaggaat  ggcaagacca
541  gcaagaagat  caccattgct  gactgtggac  aactcgaata  agtttgactt  gtgttttata
601  ttaaccacca  gatcattcct  tctgtagctc  aggagagcac  ccctccaccc  catttgctcg
661  cagttaccta  gaatctttgt  gctctcgctg  cagttccctt  tgggttccat  gttttccttg
721  ttcctccca  tgcctagctg  gattgcagag  ttaagtattat  gattatgaaa  taaaaactaa
781  ataacaattg  tcctcgtttg  agttaagagt  gttgatgtag  gctttatttt  aagcagtaat
841  gggttacttc  tgaaacatca  cttgtttgct  taattctaca  cagtacttag  atttttttta
901  ctttccagtc  ccaggaagtg  tcaatgtttg  ttgagtggaa  tattgaaaat  gtaggcagca
961  actgggcatg  gtggctcact  gtctgtaatg  tattacctga  ggcagaagac  cacctgaggg
1021  taggagtcaa  gatcagcctg  ggcaacatag  tgagacgctg  tctctacaaa  aaataattag
1081  cctggcctgg  tgggtgatgc  ctagtcctag  ctgatctgga  ggctgacgtg  ggaggattgc
1141  ttgagcctag  agtgagctat  tatcatgcca  ctgtacagcc  tgggtgttca  cagatcttgt
1201  gtctcaaagg  taggcagagg  caggaaaagc  aaggagccag  aattaagagg  ttgggtcagt
1261  ctgcagttag  ttcattgcatt  tagaggtgtt  cttcaagatg  actaatgtca  aaaattgaga
1321  catctgttgc  gggttttttt  tttttttttt  cccctggaat  gcagtggcgt  gatctcagct
1381  cactgcagcc  tccgcctcct  gggttcaagt  gattctagt  cctcagcctc  ctgagtagct
1441  gggataatgg  gcgtgtgcca  ccatgccag  ctaatttttg  tatttttagt  atagatgggg
1501  tttcatcatt  ttgaccaggc  tgggtctaaa  ctcttgacct  cagctgatgc  gcctgccttg
1561  gcctcccaaa  ctgctgagat  tacagatgtg  agccaccgca  ccctacctca  ttttctgtaa
1621  caaagctaag  cttgaacact  gttgatgttc  ttgagggaag  catattgggc  tttaggctgt
1681  aggtcaagtt  tatacatctt  aattatgggt  gaattcctat  gtagagtcta  aaaagccagg
1741  tacttgggtg  tacagttagt  ctccctgcag  aggggttaag  cgagactac  ctgcagttag
1801  gaggtactgc  ttgtagcata  tagagcctct  ccctagcttt  ggttatggag  gctttgaggt
1861  tttgcaaacc  tgaccaatth  aagccataag  atctggtcaa  agggataccc  ttcccactaa
1921  ggacttgggt  tctcaggaaa  ttatatgtac  agtgcttgct  ggcagttaga  tgtcaggaca
1981  atctaagctg  agaaaacccc  ttctctgccc  accttaacag  acctctaggg  ttcttaaccc
2041  agcaatcaag  tttgcctatc  ctgagaggtg  cggatttgat  catttggtgt  gttgggcaat
2101  ttttgtttta  ctgtctgggt  ccttctgcgt  gaattaccac  caccaccact  tgtgcatctc
2161  agtcttgtgt  gttgtctggg  tacgtattcc  ctgggtgata  ccattcaatg  tcttaatgta
2221  cttgtggctc  agacctgagt  gcaagggtgga  aataaacatc  aaacatcttt  tcatta (SEQ
ID NO:3)

```

FIG. 15

```

1  mvnptvffdi  avdgeplgrv  sfelfadkvp  ktaenfrals  tgekgfgykg  scfhriipgf
61  mcqggdftrh  ngtggksiyg  ekfedenfil  khtgpgilsm  anagpntngs  qffictakte
121  wldgkhvvfg  kvkegmhive  amerfgsrng  ktskkitiad  cgqle (SEQ ID NO:4)

```

FIG. 16

ht5-A331-Cyp-

ATGGCTTCTGGAATCCTGGTTAATGTAAAGGAGGAGGTGACCTGCCCCATCTGCCTGGAAC
CCTGACACAACCCCTGAGCCTGGACTGCGGCCACAGCTTCTGCCAAGCATGCCTCACTGCAA
ACCACAAGAAGTCCATGCTAGACAAAGGAGAGAGTAGCTGCCCTGTGTGCCGGATCAGTTAC
CAGCCTGAGAACATACGGCCTAATCGGCATGTAGCCAACTTAGTGGAGAAGCTCAGGGAGGT
CAAGTTGAGCCCAGAGGGGCAGAAAGTTGATCATTGTGCACGCCATGGAGAGAACTTCTAC
TCTTCTGTCAGGAGGACGGGAAGGTCATTTGCTGGCTTTGTGAGCGGTCTCAGGAGCACCGT
GGTCACCACACGTTCCCCACAGAGGAGGTTGCCCAGGAGTACCAAGTGAAGCTCCAGGCAGC
TCTGGAGATGCTGAGGCAGAAGCAGCAGGAAGCTGAAGAGTTGGAAGCTGACATCAGAGAAG
AGAAAGCTTCCTGGAAGACTCAAATACAGTATGACAAAACCAACGTCTTGGCAGATTTTGAG
CAACTGAGAGACATCCTGGACTGGGAGGAGAGCAATGAGCTGCAAAACCTGGAGAAGGAGGA
GGAAGACATTCTGAAAAGCCTTACGAACTCTGAAACTGAGATGGTGCAGCAGACCCAGTCCC
TGAGAGAGCTCATCTCAGATCTGGAGCATCGGCTGCAGGGGTGAGTGATGGAGCTGCTTCAG
GGTGTGGATGGCGTCATAAAAAGGACGGGAGAACGTGACCTTGAAGAAGCCAGAACTTTTCC
AAAAAATCAAAGGAGAGTGTTTCGAGCTCCTGATCTGAAAGGAATGCTAGAAGTGTTTAGAG
AGCTGACAGATGTCCGACGCTACTGGGTTGATGTGACAGTGGCTCCAAACAACATTTTCATGT
GCTGTCAATTTCTGAAGATAAGAGACAAGTGAGCTCTCCGAAACCACAGATAATATATGGGGC
AGTCAACCCACACCGTGTTCTTCGACATTGCCGTCGACGGCGAGCCCTTGGGCCGCGTCTCCT
TTGAGCTGTTTGCGACAAGGTCCCAAAGACAGCAGAAAATTTTCGTGCTCTGAGCACTGGAG
AGAAAGGATTTGGTTATAAGGGTTTCCTGCTTTCACAGAATTATTCCAGGGTTTATGTGTCAG
GGTGGTGACTTCACACGCCATAATGGCACTGGTGGCAAGTCCATCTATGGGGAGAAATTTGA
AGATGAGAACTTCATCCTAAAGCATAACGGGTCCTGGCATCTTGTCCATGGCAAATGCTGGAC
CCAACACAAATGGTTCCCAGTTTTTCATCTGCACTGCCAAGACTGAGTGGTTGGATGGCAAG
CATGTGGTGTGTTGGCAAAGTGAAAGAAGGCATGAATATTGTGGAGGCCATGGAGCGCTTTGG
GTCCAGGAATGGCAAGACCAGCAAGAAGATCACCATTGCTGACTGTGGACAACCTCGAATAA
(SEQ ID NO:5)

MASGILVNVKEEVTCPICLELLTQPLSLDCGHSFCQACLTAN
HKKSMLDKGESSCPVCRISYQPENIRPNRHVANIVEKLREVK
LSPEGQKVDHCHARHGEKLLLFQEDGKVICWLCERSQEHRG
HHTFLTEEVAREYQVKLQAALEMLRQKQQEAEELEADIREE
KASWKTQIQYDKTNVLADFEQLRDILDWEESNELQNLEKEE
EDILKSLTNSETEMVQQTQSLRELISDLEHRLQGSMELLQG
VDGVIKRTENVTLKKPETFPKNQRRVFRAPDLKGMLEVFRE
LTDVRRYWVDVTVAPNNISCAVISEDKRQVSSPKPQIIYGAV
NPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEK
GFGYKGSFCFHRIIPGFMCGGGDFTRHNGTGGKSIYGEKFED
NFILKHTGPGILSMANAGPNTNGSQFFICTAKTEWLDGKHV
VFGKVKEGMNIVEAMERFGSRNGKTSKKITIADCGQLE (SEQ
ID NO:10)

FIG. 17

>hTC_S309.fa -

ATGGCTTCTGGAATCCTGGTTAATGTAAAGGAGGAGGTGACCTGCCCCATCTGCCTGGAAC
CCTGACACAACCCCTGAGCCTGGACTGCGGCCACAGCTTCTGCCAAGCATGCCTCACTGCAA
ACCACAAGAAGTCCATGCTAGACAAAGGAGAGAGTAGCTGCCCTGTGTGCCGGATCAGTTAC
CAGCCTGAGAACATACGGCCTAATCGGCATGTAGCCAACATAGTGGAGAAGCTCAGGGAGGT
CAAGTTGAGCCCAGAGGGGCGAGAAAGTTGATCATTGTGCACGCCATGGAGAGAACTTCTAC
TCTTCTGTGAGGAGGACGGGAAGGTCATTTGCTGGCTTTGTGAGCGGTCTCAGGAGCACCGT
GGTCACCACACGTTCCCTCACAGAGGAGGTTGCCCAGGAGTACCAAGTGAAGCTCCAGGCAGC
TCTGGAGATGCTGAGGCAGAAGCAGCAGGAAGCTGAAGAGTTGGAAGCTGACATCAGAGAAG
AGAAAGCTTCCTGGAAGACTCAAATACAGTATGACAAAACCAACGTCTTGGCAGATTTTGAG
CAACTGAGAGACATCCTGGACTGGGAGGAGAGCAATGAGCTGCAAAACCTGGAGAAGGAGGA
GGAAGACATTCTGAAAAGCCTTACGAACTCTGAAACTGAGATGGTGCAGCAGACCCAGTCCC
TGAGAGAGCTCATCTCAGATCTGGAGCATCGGCTGCAGGGGTGAGTGTGAGCTGCTTCAG
GGTGTGGATGGCGTCATAAAAAGGACGGGAGAACGTGACCTTGAAGAAGCCAGAACTTTTCC
AAAAAATCAAAGGAGAGTGTTTCGAGCTCCTGATCTGAAAGGAATGCTAGAAGTGTTTAGAG
AGCTGACAGATGTCCGACGCTACTGGGTTGATGTGACAGTGGCTCCAAACAACATTTTCAGTC
AACCCACCGTGTTCTTCGACATTGCCGTCGACGGCGAGCCCTTGGGCCGCGTCTCCTTTGA
GCTGTTTGCAGACAAGGTCCCAAAGACAGCAGAAAATTTTCGTGCTCTGAGCACTGGAGAGA
AAGGATTTGGTTATAAGGGTTCCTGCTTTCACAGAATTATTCAGGGTTTATGTGTCAGGGT
GGTGAATTCACACGCCATAATGGCACTGGTGGCAAGTCCATCTATGGGGAGAAATTTGAAGA
TGAGAACTTCATCCTAAAGCATAACGGGTCTGGCATCTTGTCCATGGCAAATGCTGGACCCA
ACACAAATGGTTCCCAGTTTTTCATCTGCACTGCCAAGACTGAGTGGTTGGATGGCAAGCAT
GTGGTGTGGTGGCAAAGTGAAAGAAGGCATGAATATTGTGGAGGCCATGGAGCGCTTTGGGTC
CAGGAATGGCAAGACCAGCAAGAAGATCACCATTGCTGACTGTGGACAACTCGAATAA

(SEQ ID NO:6)

MASGILVNVKEEVTCPICLELLTQPLSLDCGHSFCQACLTAN
HKKSMLDKGESSCPVCRISYQOPENIRPNRHVANIVEKLREVK
LSPEGQKVDHCCARHGEKLLLFQEDGKVICWLCERSQEHRG
HHTFLTEEVAREYQVKLQAALEMLRQKQQEAEELEADIREE
KASWKTQIQYDKTNVLADFEQLRDILDWEESNELQNLEKEE
EDILKSLTNSETEMVQQTQSLRELISDLEHRLQGSVMELLQG
VDGVIKRTENVTLKKPETFPKNQRRVFRAFDLKGMLEVFRE
LTDVRRYWVDVTVAPNNISVNPTVFFDIAVDGEPLGRVSFE
LFADKVPKTAENFRALSTGEKGFYKGSFHHRIIPGFMCGG
DFTRHNGTGGKSIYGEKFEDENFILKHTGPGILSMANAGPNT
NGSQFFICTAKTEWLDGKHVVFGKVKEGMNIVEAMERFGSR
NGKTSKKITIA DCGQLE (SEQ ID NO:11)

FIG. 18

>hT-S322-Cyp.fa -

ATGGCTTCTGGAATCCTGGTTAATGTAAAGGAGGAGGTGACCTGCCCCATCTGCCTGGAAC
CCTGACACAACCCCTGAGCCTGGACTGCGGCCACAGCTTCTGCCAAGCATGCCTCACTGCAA
ACCACAAGAAGTCCATGCTAGACAAAGGAGAGAGTAGCTGCCCTGTGTGCCGGATCAGTTAC
CAGCCTGAGAACATACGGCCTAATCGGCATGTAGCCAACATAGTGGAGAAGCTCAGGGAGGT
CAAGTTGAGCCCAGAGGGGCGAGAAAGTTGATCATTGTGCACGCCATGGAGAGAACTTCTAC
TCTTCTGTGAGGAGGACGGGAAGGTCATTTGCTGGCTTTGTGAGCGGTCTCAGGAGCACCGT
GGTCACCACACGTTCCCTCACAGAGGAGGTTGCCCGGGAGTACCAAGTGAAGCTCCAGGCAGC
TCTGGAGATGCTGAGGCAGAAGCAGCAGGAAGCTGAAGAGTTGGAAGCTGACATCAGAGAAG
AGAAAGCTTCCTGGAAGACTCAAATACAGTATGACAAAACCAACGTCTTGGCAGATTTTGAG
CAACTGAGAGACATCCTGGACTGGGAGGAGAGCAATGAGCTGCAAAACCTGGAGAAGGAGGA
GGAAGACATTCTGAAAAGCCTTACGAACTCTGAAACTGAGATGGTGCAGCAGACCCAGTCCC
TGAGAGAGCTCATCTCAGATCTGGAGCATCGGCTGCAGGGGTGAGTGATGGAGCTGCTTCAG
GGTGTGGATGGCGTCATAAAAAGGACGGGAGAACGTGACCTTGAAGAAGCCAGAACTTTTCC
AAAAAATCAAAGGAGAGTGTTTCGAGCTCCTGATCTGAAAGGAATGCTAGAAGTGTTTAGAG
AGCTGACAGATGTCCGACGCTACTGGGTTGATGTGACAGTGGCTCCAAACAACATTTTCATGT
GCTGTCATTTCTGAAGATAAGAGACAAGTGAGCTCTGTCAACCCACCGTGTTCTTCGACAT
TGCCGTCGACGGCGAGCCCTTGGGCGCGTCTCCTTTGAGCTGTTTGCAGACAAGGTCCCAA
AGACAGCAGAAAATTTTCGTGCTCTGAGCACTGGAGAGAAAGGATTTGGTTATAAGGGTCC
TGCTTTCACAGAATTATTCAGGGTTTATGTGTGAGGGTGGTGAAGTTCACACGCCATAATGG
CACTGGTGGCAAGTCCATCTATGGGGAGAAATTTGAAGATGAGAACTTCATCCTAAAGCATA
CGGGTCTTGGCATCTTATCGATGGCAAATGCTGGACCCAAACACAAATGGTTCCAGTTTTTC
ATCTGCACTGCCAAGACTGAGTGGTTGGATGGCAAGCATGTGGTGTGGTGGCAAGTGAAAGA
AGGCATGAATATTGTGGAGGCGATGGAGCGCTTTGGGTCCAGGAATGGCAAGACCAGCAAGA
AGATCACCATTGCTGACTGTGGACAACTCGAATAA (SEQ ID NO:7)

MASGILVNVKEEVTCPICLELLTQPLSLDCGHSFCQACLTAN
HKKSMLDKGESSCPVCRISYQOPENIRPNRHVANIVEKLREVK
LSPEGQKVDHCCARHGEKLLLFQEDGKVICWLCERSQEHRG
HHTFLTEEVAREYQVKLQAALEMLRQKQQEAEELEADIREE
KASWKTQIQYDKTNVLADFEQLRDILDWEESNELQNLEKEE
EDILKSLTNSETEMVQQTQSLRELISDLEHRLQGSVMELLQG
VDGVIKRTENVTLKKPETFPKNQRRVFRAFDLKGMLEVFRE
LTDVRRYWVDVTVAPNNISC ADVISEDKRQVSSVNPTVFFDIA
VDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFYKGCSC
FHRIIPGFMCQGGDFTRHNGTGGKSIYGEKFEDENFILKHTG
PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKVKEG
MNIVEAMERFGSRNGKTSKKITIADCGQLE (SEQ ID NO:12)

FIG. 19

scALPS-GFP vector

GTCGACGGATCGGGAGATCTCCCGATCCCCCTATGGTGCACCTCTCAGTACAATCTGCTCTGAT
 GCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGC
 GAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTTA
 GGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGCTGTGGAATGTGT
 GTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAAGTATGCAAAGCATGCAT
 CTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAAGTATGCA
 AAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCCTAACTCCGCCCATCCCGCCCC
 TAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTTTATTTATGCA
 GAGGCCGAGGCCGCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTTGGAGG
 CCTAGGCTTTTGC AAAAAGCTTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTA
 CGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
 CCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCAT
 AGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCC
 ACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGT
 AAATGGCCCCGCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCTACTTGGCAGTA
 CATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGC
 GTGGATAGCGGTTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGT
 TTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACCTCCGCCCCATTGAC
 GCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGCGCGTTTTGCCTGTACT
 GGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACT
 GCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCCCCGTCTGTTGTGTG
 ACTCTGGTAACTAGAGATCCCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGC
 GCCCGAACAGGGACTTGAAAGCGAAAGGGAAACCAGAGGAGCTCTCTCGACGCAGGACTCGG
 CTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGTACGCCAAAAATTTT
 GACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAA
 TTAGATCGCGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAA
 CATATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAAC
 ATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAG
 AACTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATA
 AAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGACCACCGC
 ACAGCAAGCg_gccGGCCGCTGATCTTCAGACCTGGAGGAGGAGATATGAGGGACAATTGGAG
 AAGTGAATTATATAAATATAAAGTAGTAAAAAATTGAACCATTAGGAGTAGCACCCACCAAGG
 CAAAGAGAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAATAGGAGCTTTGTTCCCTGGG
 TTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACGCTGACGGTACAGGCCAG
 ACAATTATTGTCTGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAAC
 AGCATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAATCCTGGCTGTG
 GAAAGATACCTAAAGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACCTCATTTG
 CACCACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATTTGGAATC
 ACACGACCTGGATGGAGTGGGACAGAGAAATTAACAATTACACAAGCTTAATACACTCCTTA
 ATTGAAGAATCGCAAAACCAGCAAGAAAAGAATGAACAAGAATTATTGGAATTAGATAAATG
 GGCAAGTTTGTGGAATTGGTTTAACATAACAAATTGGCTGTGGTATATAAAATTATTCATAA
 TGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGTGTACTTTCTATAGTGAATAGA
 GTTAGGCAGGGATATTCACCATTATCGTTTTAGACCCACCTCCCAACCCCGAGGGGACCCGA
 CAGGCCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTTCGATTAG
 TGAACGGATCGGCACTGCGTGCGCCAATTCTGCAGACAAATGGCAGTATTCATCCACAATTT
 TAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAA

Fig. 20A

CAGACATACAAACTAAAGAATTACAAAAACAAATTACAAAAATTCAAAATTTTCGGGTTTAT
 TACAGGGACAGCAGAGATCCAGTTTGGTTAATt aaCTGCAGCCCCGATAAAATAAAAGATTT
 TATTTAGTCTCCAGAAAAAGGGGGGAATGAAAGACCCACCTGTAGGTTTGGCAAGCTAGCT
 GCAGTAACGCCATTTTGAAGGCATGGAAAAATACCAACCAAGAATAGAGAAGTTCAGATC
 AAGGGCGGGTACATGAAAATAGCTAACGTTGGGCCAAACAGGATATCTGCGGTGAGCAGTTT
 CGGCCCCGGCCCCGGGCCAAGAACAGATGGTCACCGCAGTTTTCGGCCCCGGCCGAGGCCAA
 GAACAGATGGTCCCCAGATATGGCCCAACCCTCAGCAGTTTCTTAAGACCCATCAGATGTTT
 CCAGGCTCCCCCAAGGACCTGAAATGACCCTGCGCCTTATTTGAATTAACCAATCAGCCTGC
 TTCTCGCTTCTGTTTCGCGCGCTTCTGCTTCCCGAGCTCTATAAAAGAGCTCACAACCCCTCA
 CTCGGCGCGCCAGTCCTCCGACAGACTGAGTCGCCCGGGGGtCTAGAAGCGCTGGATCCGTT
 TAAACGCGGCCGCCAGCACAGTGGCTCGAGCCGCGGGTTAACTGGCCAGaattctcgacct
 tgactggcgggcgcgaccttgaggcctgcgttcgcctcagttgccccctctgtgcaatgggga
 gacgcgcctcatcgcttgacaacggccgaagagccgcgcgcttcggtctcccgcggtgcgcg
 cgccatgctgcccacccccgttcgcgactgacctcccccggtgccccgcgtcccgctactgcc
 gccccgccccgagtgccatgcccgcagccaccgcgcagggagcccgaggcgggaaacctgcctc
 cgcgcggttagcgcgcaacgcgcgcctcatgtgtcgtccccatcagcgccggcttcggtctata
 ggccagatgcactgtcactctggcgaaagtcgcgagaccgattggccgggagcgaggcgcgag
 accgggttgcgggcgggggccGAACGTGGTATAAAAGGGGCGGGAGGCCAGGCTCGTGCCGTT
 TTGCAGACGCCACCGCCGAGGAAAACCGTGTACTATTAGCCacgcgtgccaccATGGCCCAG
 TCCAAGCACGGCCTGACCAAGGAGATGACCATGAAGTACCGCATGGAGGGCTGCGTGGACGG
 CCACAAGTTTCGTGATCACCGGCGAGGGCATCGGCTACCCCTTCAAGGGCAAGCAGGCCATCA
 ACCTGTGCGTGGTGGAGGGCGGCCCTTGCCTTTCGCCGAGGACATCTTGTCCGCCGCCTTC
 ATGTACGGCAACCGCGTGTTCACCGAGTACCCCCAGGACATCGTCGACTACTTCAAGAACTC
 CTGCCCCGCCGGCTACACCTGGGACCGCTCCTTCTGTTCGAGGACGGCGCCGTGTGCATCT
 GCAACGCCGACATCACCGTGAGCGTGGAGGAGAACTGCATGTACCACGAGTCCAAGTTCTAC
 GGCGTGAACCTTCCCCGCCGACGGCCCCGTGATGAAGAAGATGACCGACAACCTGGGAGCCCTC
 CTGCGAGAAGATCATCCCCGTGCCCAAGCAGGGGCATCTTGAAGGGCGACGTGAGCATGTACC
 TGCTGCTGAAGGACGGTGGCCGCTTGCCTGCGCTGCCAGTTCGACACCGTGTACAAGGCCAAGTCC
 GTGCCCCGCAAGATGCCCGACTGGCACTTCATCCAGCACAAAGCTGACCCGCGAGGACCGCAG
 CGACGCCAAGAACCAGAAGTGGCACCTGACCGAGCACGCCATCGCCTCCGGCTCCGCCTTGC
 CCTGAgcgatcgcTAATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTT
 AACTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTAT
 TGCTTCCCGTATGGCTTTTCATTTTCTCCTCCTTGTATAAATCCTGGTTGCTGTCTCTTTATG
 AGGAGTTGTGGCCCGTTGTACGGCAACGTGGCGTGGTGTGCACTGTGTTTGTGACGCAACC
 CCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTTCGCTTTCCCCCT
 CCCTATTGCCACGGCGGAACATCATCGCCGCCTGCCTTGCCCGCTGCTGGACAGGGGCTCGGC
 TGTTGGGCACTGACAATTCCTGTTGTGTCGGGGAAGctgaCGTCCCTTTCCaTGGCTGCTC
 GCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTACGTCCCTTCGGCCCTCAA
 TCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGGCTCTGCGGCCCTTCCGCGTCTTCGCC
 TTCGCCCTCAGACGAGTCGGATCTCCctttgggcgcctccccgcTTAATCGCGTCGAGACC
 TAGAAAAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGATTGTGCCTGG
 CTAGAAGCACAAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACC
 AATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAG
 GGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGC
 TACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGATCAGATATCCACTGACCTTTGG
 ATGGTGCTACAAGCTAGTACCAGTTGAGCAAGAGAAGGTAGAAGAAGCCAATGAAGGAGAGA
 ACACCCGCTTGTTACACCCTGTGAGCCTGCATGGGATGGATGACCCGGAGAGAGAAGTATTA

Fig. 20B

GAGTGGAGGTTTGACAGCCGCCTAGCATTTTCATCACATGGCCCCGAGAGCTGCATCCGGACTG
TACTGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACC
CACTGCTTAAGCCTCAATAAAGCTTGCCCTTGAGTGCTTCAAGTAGTGTGTGCCCGTCTGTTG
TGTGACTCTGGTAACTAGAGATCCCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAG
GGCCCCGTTTCATGTGAGCAAAAAGGCCAGCAAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG
AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGT
GCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTTCTCCCTTCGGGAA
GCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCC
AAGCTGGGCTGTGTGCACGAACCCCCCGTTTCAGCCCGACCGCTGCGCCTTATCCGGTAACATA
TCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACA
GGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTAC
GGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAA
AAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTT
GCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACG
GGGTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGGATTTTGGTCATGAGATTATCAAA
AAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATAT
ATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATC
TGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGA
GGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACC GGCTCCAG
ATTTATCAGCAATAAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTA
TCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTTCGCCAGTTAA
TAGTTTTCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTA
TGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGC
AAAAAAGCGGTTAGCTCCTTCGGTCCCTCCGATCGTTGTGAGAAGTAAGTTGGCCGCAGTGTT
ATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTGTCATGCCATCCGTAAGATGCT
TTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGT
TGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCT
CATCATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCA
GTTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTT
TCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAA
ATGTTGAATACTCATACTCTTCCTTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTC
TCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACA
TTTCCCCGAAAAGTGCCACCTGAC (SEQ ID NO:8)

Fig. 20C

scALPS-hT5Cyp-GFP

GTCGACGGATCGGGAGATCTCCCGATCCCCCTATGGTGCACCTCTCAGTACAATCTGCTCTGAT
GCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGC
GAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTTA
GGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGCTGTGGAATGTGT
GTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAAGTATGCAAAGCATGCAT
CTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAAGTATGCA
AAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCCTAACTCCGCCCCATCCCGCCCC
TAACTCCGCCCCAGTTCCGCCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTTTATTTATGCA
GAGGCCGAGGCCGCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGG
CCTAGGCTTTTGC AAAAAGCTTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTA
CGGGGTCATTAGTTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
CCGCTGGCTGACCGCCCAACGACCCCCGCCCATTTGACGTCAATAATGACGTATGTTCCCAT
AGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCC
ACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGT
AAATGGCCCCGCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTA
CATCTACGTATTAGTCATCGCTATTACCATGGTGTATGCGGTTTTTGGCAGTACATCAATGGGC
GTGGATAGCGGTTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTTGACGTCAATGGGAGT
TTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGAC
GCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGCGCGTTTTTGCCTGTACT
GGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACT
GCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCCCGTCTGTTGTGTG
ACTCTGGTAACTAGAGATCCCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGC
GCCCCAACAGGGACTTGAAAGCGAAAGGGAAACCAGAGGAGCTCTCTCGACGCAGGACTCGG
CTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGTACGCCAAAAATTTT
GACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAA
TTAGATCGCGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAGAAAAAATATAAATTAAAA
CATATAGTATGGGCAAGCAGGGAGCTAGAACGATTTCGAGTTAATCCTGGCCTGTTAGAAAC
ATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAG
AACTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATA
AAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGACCACCGC
ACAGCAAGCg g c c G G C C G C T G A T C T T C A G A C C T G G A G G A G A G A T A T G A G G G A C A A T T G G A G
A A G T G A A T T A T A T A A A T A T A A A G T A G T A A A A A T T G A A C C A T T A G G A G T A G C A C C C A C C A A G G
C A A A G A G A A G A G T G G T G C A G A G A G A A A A A A G A G C A G T G G G A A T A G G A G C T T T G T T C C T T G G G
T T C T T G G G A G C A G C A G G A A G C A C T A T G G G C G C A G C G T C A A T G A C G C T G A C G G T A C A G G C C A G
A C A A T T A T T G T C T G G T A T A G T G C A G C A G C A G A A C A A T T T G C T G A G G G C T A T T G A G G C G C A A C
A G C A T C T G T T G C A A C T C A C A G T C T G G G G C A T C A A G C A G C T C C A G G C A A G A A T C C T G G C T G T G
G A A A G A T A C C T A A A G G A T C A A C A G C T C C T G G G G A T T T G G G G T T G C T C T G G A A A C T C A T T T G
C A C C A C T G C T G T G C C T T G G A A T G C T A G T T G G A G T A A T A A A T C T C T G G A A C A G A T T T G G A A T C
A C A C G A C C T G G A T G G A G T G G G A C A G A G A A T T A A C A A T T A C A C A A G C T T A A T A C A C T C C T T A
A T T G A A G A A T C G C A A A C C A G C A A G A A A G A A T G A A C A A G A A T T A T T G G A A T T A G A T A A A T G
G G C A A G T T T G T G G A A T T G G T T T A A C A T A A C A A A T T G G C T G T G G T A T A T A A A A T T A T T C A T A A
T G A T A G T A G G A G G C T T G G T A G G T T T A A G A A T A G T T T T T G C T G T A C T T T C T A T A G T G A A T A G A
G T T A G G C A G G G A T A T T C A C C A T T A T C G T T T C A G A C C C A C C T C C C A A C C C C G A G G G G A C C C G A

Fig. 21A

CAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTTCGATTAG
 TGAACGGATCGGCACTGCGTGCGCCAATTCTGCAGACAAATGGCAGTATTCATCCACAATTT
 TAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAA
 CAGACATACAACTAAAGAATTACAAAAACAAATTACAAAAATTCAAAATTTTCGGGTTTAT
 TACAGGGACAGCAGAGATCCAGTTTGGTTAATtaaCTGCAGCCCCGATAAAATAAAAGATTT
 TATTTAGTCTCCAGAAAAAGGGGGGAATGAAAGACCCACCTGTAGGTTTGGCAAGCTAGCT
 GCAGTAACGCCATTTTGAAGGCATGGAAAAATACCAAACCAAGAATAGAGAAGTTCAGATC
 AAGGGCGGGTACATGAAAATAGCTAACGTTGGGCCAAACAGGATATCTGCGGTGAGCAGTTT
 CGGCCCCGGCCCCGGGGCCAAGAACAGATGGTCACCGCAGTTTTCGGCCCCGGCCCGAGGCCAA
 GAACAGATGGTCCCCAGATATGGCCCAACCCTCAGCAGTTTCTTAAGACCCATCAGATGTTT
 CCAGGCTCCCCCAAGGACCTGAAATGACCCTGCGCCTTATTTGAATTAACCAATCAGCCTGC
 TTCTCGCTTCTGTTTCGCGCGCTTCTGCTTCCCGAGCTCTATAAAAGAGCTCACAAACCCCTCA
 CTCGGCGCGCCAGTCCTCCGACAGACTGAGTCGCCCGGGGGTctagagccaccATGGCTTCT
 GGAATCCTGGTTAATGTAAAGGAGGAGGTGACCTGCCCCATCTGCCTGGAACCTCCTGACACA
 ACCCCTGAGCCTGGACTGCGGCCACAGCTTCTGCCAAGCATGCCTCACTGCAAACCACAAGA
 AGTCCATGCTAGACAAAGGAGAGAGTAGCTGCCCTGTGTGCCGGATCAGTTACCAGCCTGAG
 AACATACGGCCTAATCGGCATGTAGCCAACATAGTGGAGAAGCTCAGGGAGGTCAAGTTGAG
 CCCAGAGGGGCAGAAAGTTGATCATTGTGCACGCCATGGAGAGAACTTCTACTCTTCTGTC
 AGGAGGACGGGAAGGTCATTTGCTGGCTTTGTGAGCGGTCTCAGGAGCACCGTGGTCACCAC
 ACGTTCCCTCACAGAGGAGGTTGCCCGGGAGTACCAAGTGAAGCTCCAGGCAGCTCTGGAGAT
 GCTGAGGCAGAAGCAGCAGGAAGCTGAAGAGTTGGAAGCTGACATCAGAGAAGAGAAAGCTT
 CCTGGAAGACTCAAATACAGTATGACAAAACCAACGTCTTGGCAGATTTTGAGCAACTGAGA
 GACATCCTGGACTGGGAGGAGAGCAATGAGCTGCAAAACCTGGAGAAGGAGGAGGAAGACAT
 TCTGAAAAGCCTTACGAACCTCTGAAACTGAGATGGTGCAGCAGACCCAGTCCCTGAGAGAGC
 TCATCTCAGATCTGGAGCATCGGCTGCAGGGGTCAGTGATGGAGCTGCTTCAGGGTGTGGAT
 GGCGTCATAAAAAGGACGGAGAACGTGACCTTGAAGAAGCCAGAACTTTTCCAAAAAATCA
 AAGGAGAGTGTTTCGAGCTCCTGATCTGAAAGGAATGCTAGAAGTGTTTAGAGAGCTGACAG
 ATGTCCGACGCTACTGGGTGTGATGTGACAGTGGCTCCAAACAACATTTTCATGTGCTGTCATT
 TCTGAAGATAAGAGACAAGTGAGCTCTGTCAACCCACCGTGTTCTTCGACATTGCCGTCGA
 CGGCGAGCCCTTGGGCCGCGTCTCCTTTGAGCTGTTTGCAGACAAGGTCCCAAAGACAGCAG
 AAAATTTTCGTGCTCTGAGCACTGGAGAGAAAGGATTTGGTTATAAGGGTTCCCTGCTTTTAC
 AGAATTATTCCAGGGTTTATGTGTCAGGGTGGTGACTTCACACGCCATAATGGCACTGGTGG
 CAAGTCCATCTATGGGGAGAAATTTGAAGATGAGAACTTCATCCTAAAGCATAACGGGTCTTG
 GCATCTTATCGATGGCAAATGCTGGACCCAACACAAATGGTTCCCAGTTTTTTCATCTGCACT
 GCCAAGACTGAGTGGTTGGATGGCAAGCATGTGGTGTTTGGCAAAGTGAAAGAAGGCATGAA
 TATTGTGGAGGCGATGGAGCGCTTTGGGTCCAGGAATGGCAAGACCAGCAAGAAGATCACCA
 TTGCTGACTGTGGACAACTCGAATAA_gGATCCGTTTAAACGCGGCCGCCAGCACAGTGGCT
 CGAGCCGCGGGTTAACTGGCCAGAattctcgaccttgactggcggcgcgaccttgaggcctg
 cgttcgcctcagttgccccctctgtgcaatggggagacgcgcctcatcgcttgacaacggcc
 gaagagccgcgcgcgttccgtctcccgcgtgcgcgcgccatgctgcccacccccgttccgca
 ctgaccttcccccggtgccccgcgtcccgtactgccgccccgccccgagtcccatgccgcagc
 caccgcgacggagcccgagggcggaacctgcctccgcgcgttagcgcgacgcgcgcctca
 tgtgtcgtccccatcagcgccggttccgtctataggccagatgcactgtcactctgpgcga
 gtcgcagacccgattggccgggacggaggcgcgagaccgggttgcgggcgggggccGAACGTG
 GTATAAAAGGGGCGGGAGGCCAGGCTCGTGCCGTTTTGCAGACGCCACCGCCGAGGAAAACC
 GTGTACTATTAGCCacgcgtgccaccATGGCCCAGTCCAAGCACGGCCTGACCAAGGAGATG

Fig. 21B

ACCATGAAGTACCGCATGGAGGGCTGCGTGGACGGCCACAAGTTTCGTGATCACCGGCGAGGG
CATCGGCTACCCCTTCAAGGGCAAGCAGGCCATCAACCTGTGCGTGGTGGAGGGCGGCCCT
TGCCCTTCGCCGAGGACATCTTGTCCGCCGCCTTCATGTACGGCAACCGCGTGTTCACCGAG
TACCCCCAGGACATCGTTCGACTACTTCAAGAACTCCTGCCCCGCCGGCTACACCTGGGACCG
CTCCTTTCCTGTTTCGAGGACGGCGCCGTGTGCATCTGCAACGCCGACATCACCGTGAGCGTGG
AGGAGAACTGCATGTACCACGAGTCCAAGTTCTACGGCGTGAACCTCCCCGCCGACGGCCCC
GTGATGAAGAAGATGACCGACAACCTGGGAGCCCTCCTGCGAGAAGATCATCCCCGTGCCCAA
GCAGGGCATCTTGAAGGGCGACGTGAGCATGTACCTGCTGCTGAAGGACGGTGGCCGCTTGC
GCTGCCAGTTCGACACCGTGTACAAGGCCAAGTCCGTGCCCCGCAAGATGCCCGACTGGCAC
TTCATCCAGCACAAAGCTGACCCGCGAGGACCGCAGCGACGCCAAGAACCAGAAGTGGCACCT
GACCGAGCACGCCATCGCCTCCGGCTCCGCCTTGCCCTGA_gcga_tcgcTAATCAACCTCTGG
ATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAACCTATGTTGCTCCTTTTACGCTATGT
GGATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTTCATTTTCTC
CTCCTTGTATAAATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTGTCAGGCAAC
GTGGCGTGGTGTGCACTGTGTTTGTGACGCAACCCCCACTGGTTGGGGCATTGCCACCACC
TGTCAGCTCCTTTCCGGGACTTTTCGCTTTCCCCCTCCCTATTGCCACGGCGGAACCTCATCGC
CGCCTGCCTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAATTCCGTGGTGT
TGTCGGGGAA_gctgaCGTCCTTTCCaTGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGC
GGGACGTCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCT
GCTGCCGGCTCTGCGGCCTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGGATCTCCc
tttggggccgcctccccgcTTAATCGCGTCGAGACCTAGAAAAACATGGAGCAATCACAAGTA
GCAATACAGCAGCTACCAATGCTGATTGTGCCTGGCTAGAAGCACAAAGAGGAGGAGGAGTG
GGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCT
TAGCCACTTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAG
ATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACA
CCAGGGCCAGGGATCAGATATCCACTGACCTTTGGATGGTGTACAAGCTAGTACCAGTTGA
GCAAGAGAAGGTAGAAGAAGCCAATGAAGGAGAGAACACCCGCTTGTTACACCCTGTGAGCC
TGCATGGGATGGATGACCCGGAGAGAGAAGTATTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACATGGCCCGAGAGCTGCATCCGGACTGTACTGGGTCTCTCTGGTTAGACCAGAT
CTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGC
CTTGAGTGCTTCAAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCTC
AGACCCTTTTAGTCAGTGTGGAATCTCTAGCAGGGCCCGTTTCATGTGAGCAAAAGGCCA
GCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCC
CTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAA
AGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCT
TACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCT
GTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCC
GTTTCAGCCCCGACCGCTGCGCCTTATCCGGTAACCTATCGTCTTGAGTCCAACCCGGTAAGACA
CGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCG
GTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGT
ATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAA
ACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAA
AAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAAC
TCACGTAAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAA

Fig. 21C

TTAAAAATGAAGTTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACC
AATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCC
TGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGC
AATGATACCGCGAGACCCACGCTCACC GGCTCCAGATTTATCAGCAATAAACCCAGCCAGCCG
GAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGT
TGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGC
TACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAAC
GATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCT
CCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCA
TAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCA
AGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGAT
AATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCG
AAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGACCCCA
ACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAA
AATGCCGCAAAAAAGGGAATAAGGGCGACACGGAATGTTGAATACTCATACTCTTCCTTTT
TCAATATTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTA
TTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGAC
(SEQ ID NO:9)

Fig. 21D

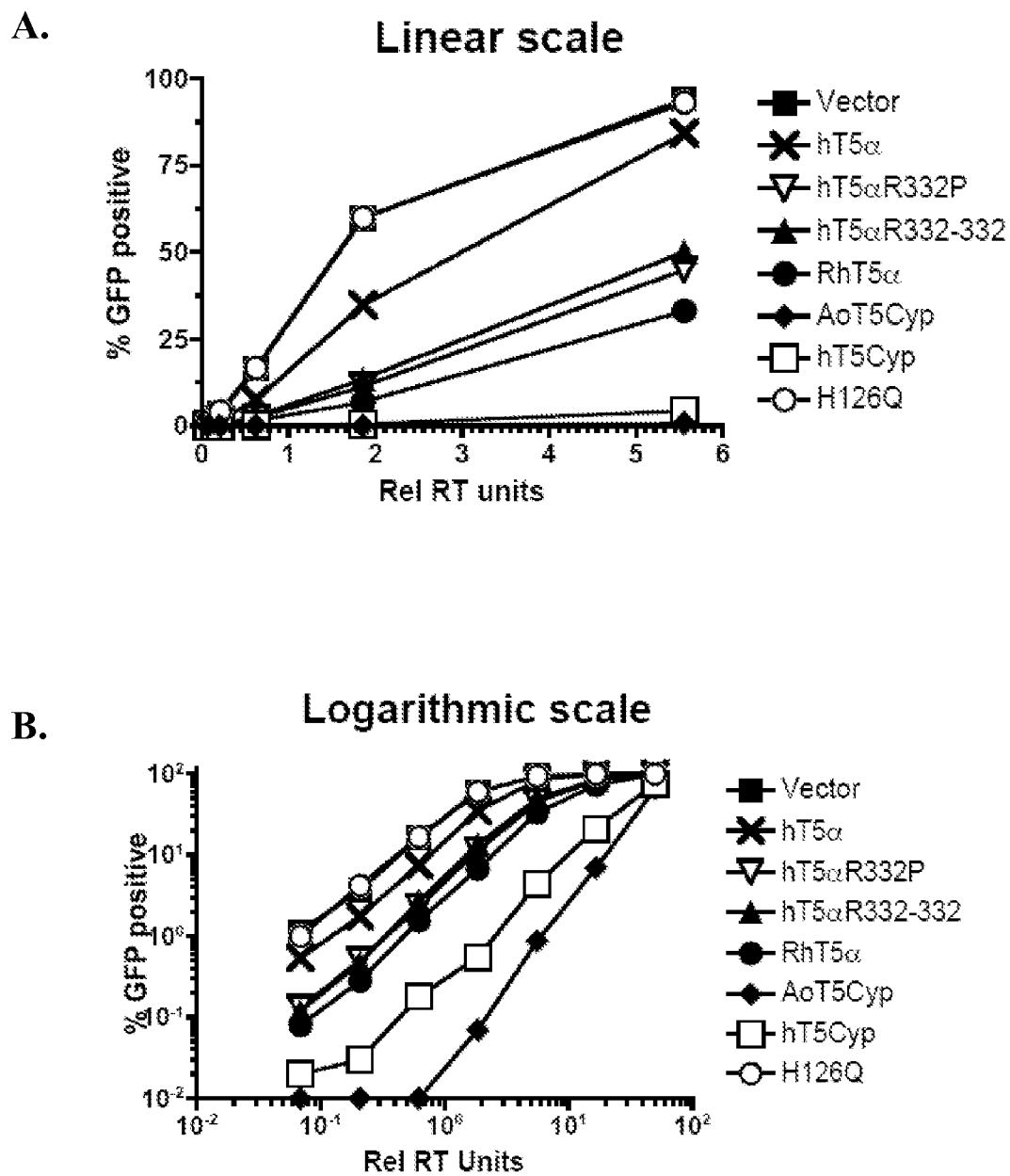
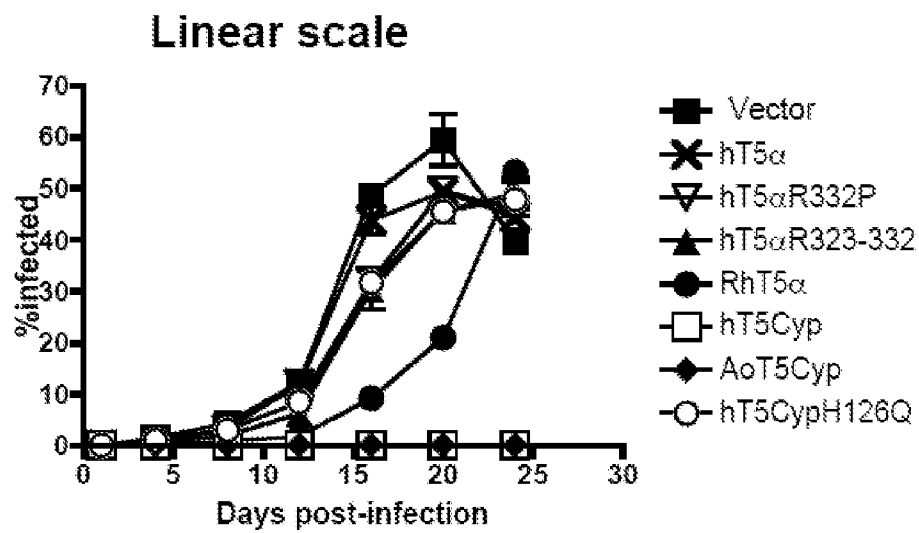


FIG. 22

A.



B.

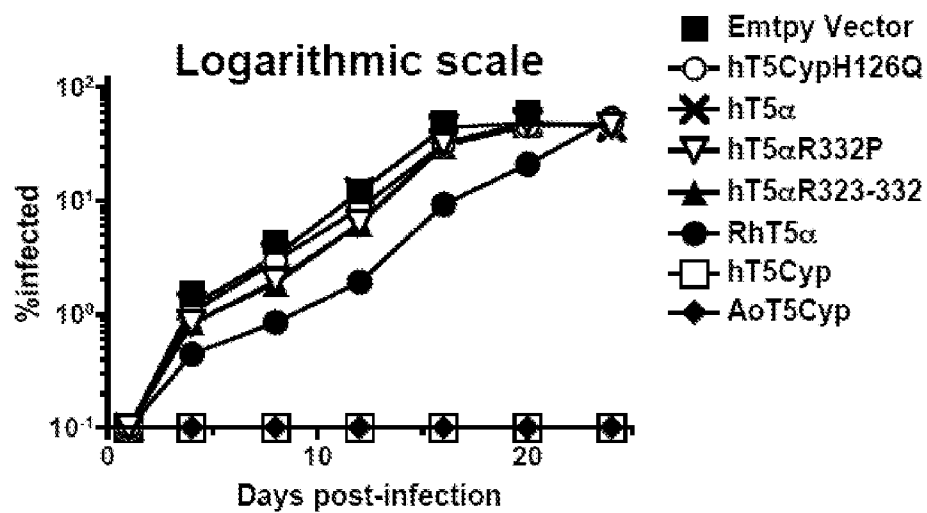


FIG. 23

B



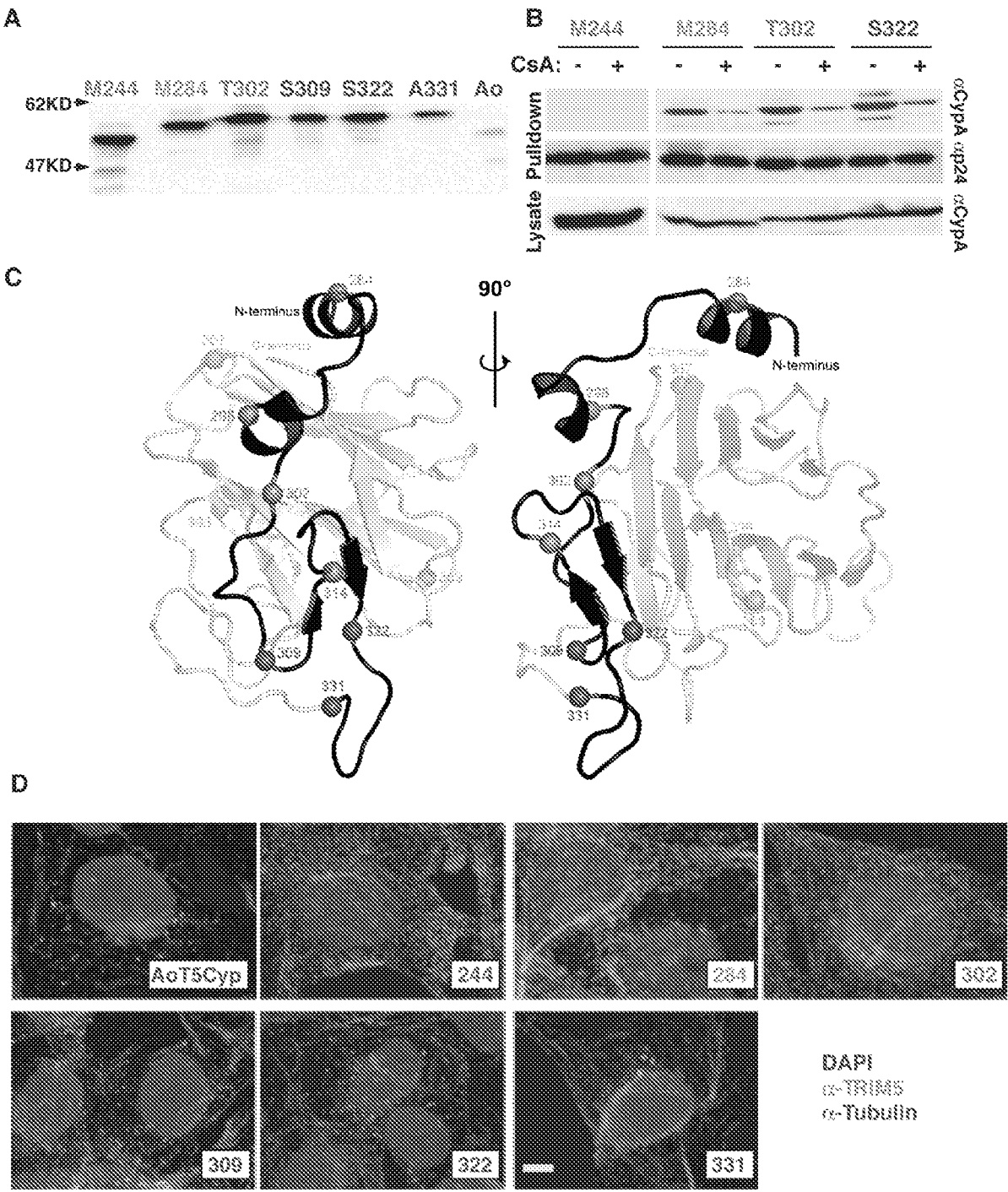


FIG. 25

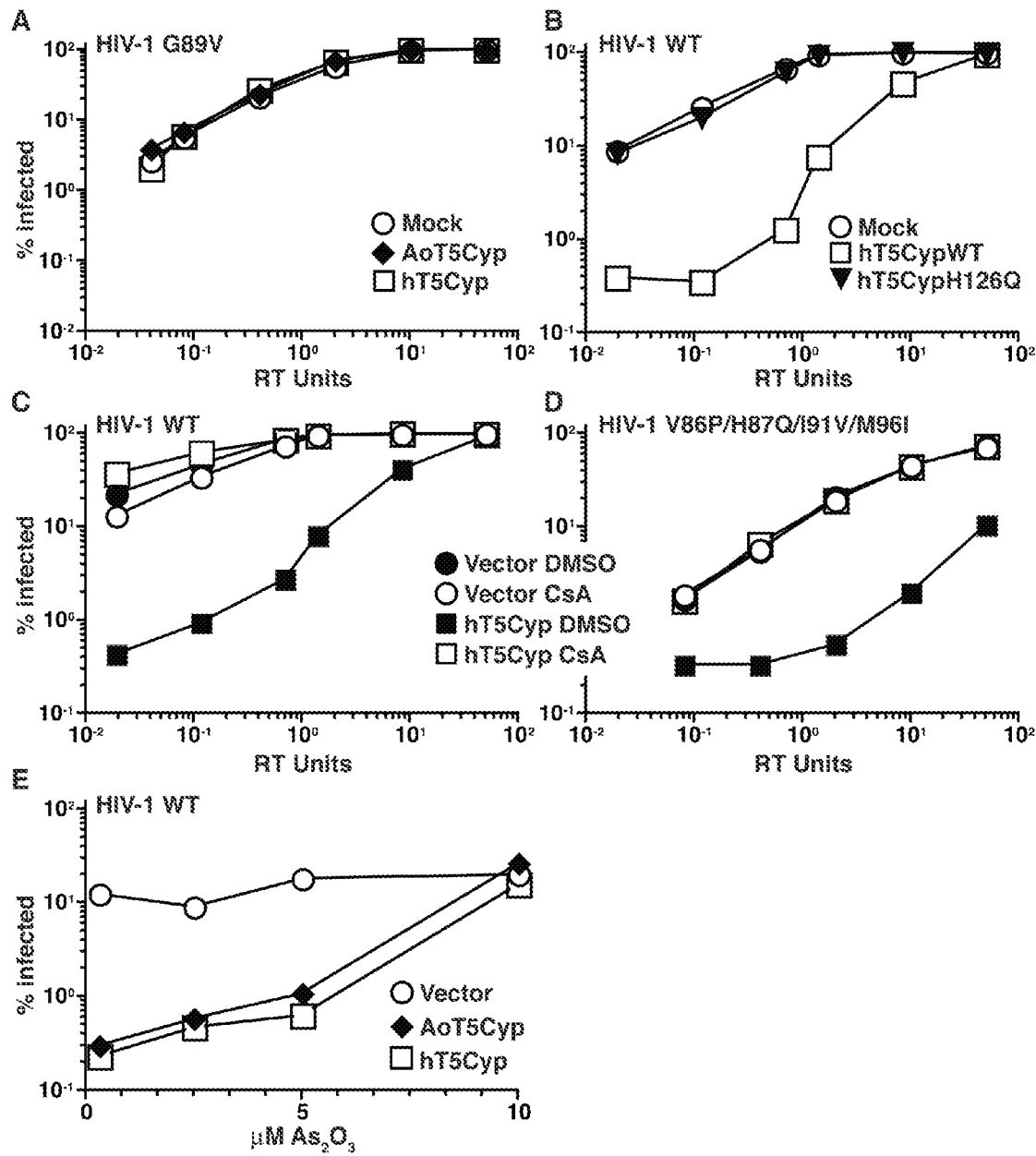


FIG. 26

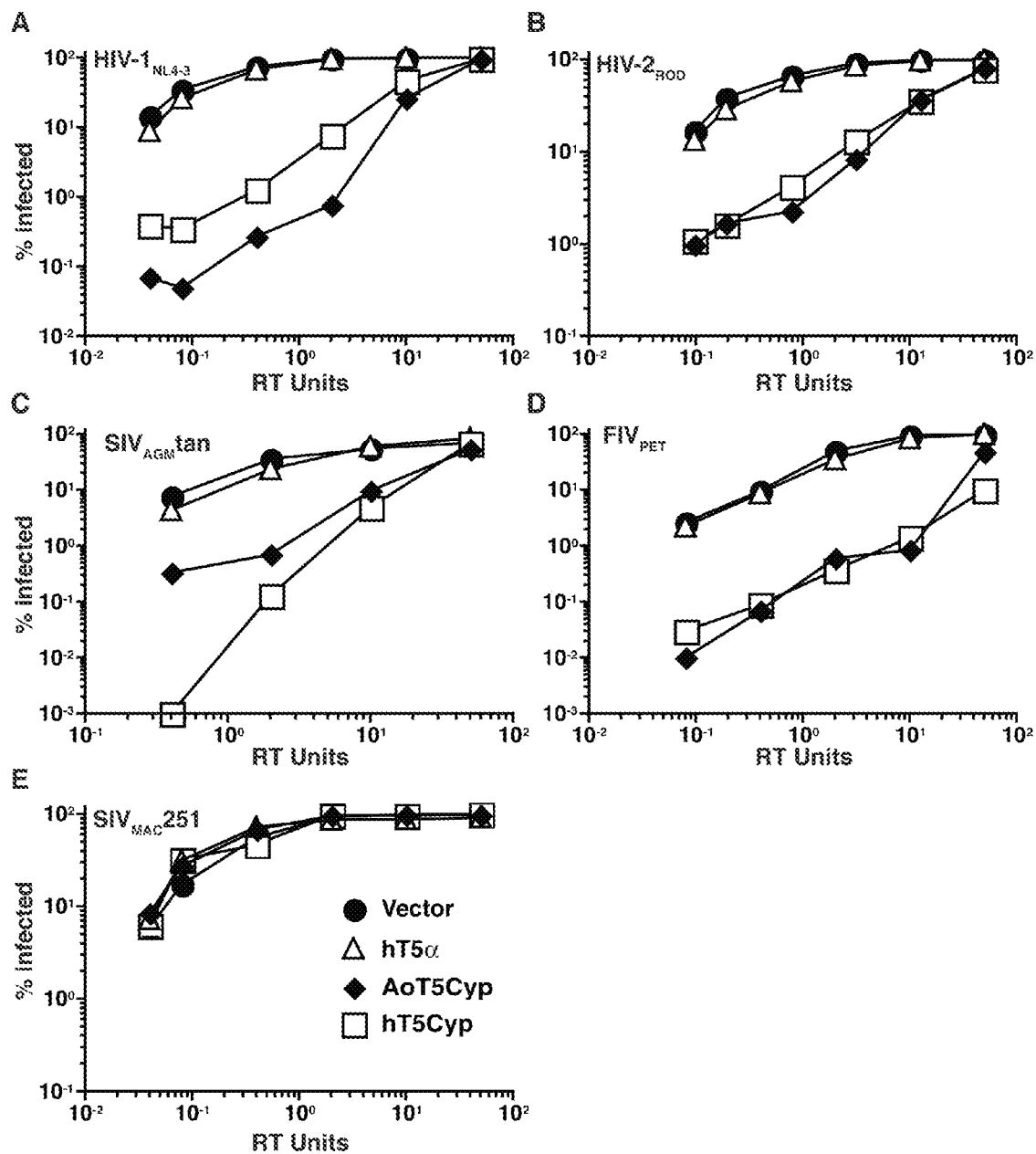


FIG. 27

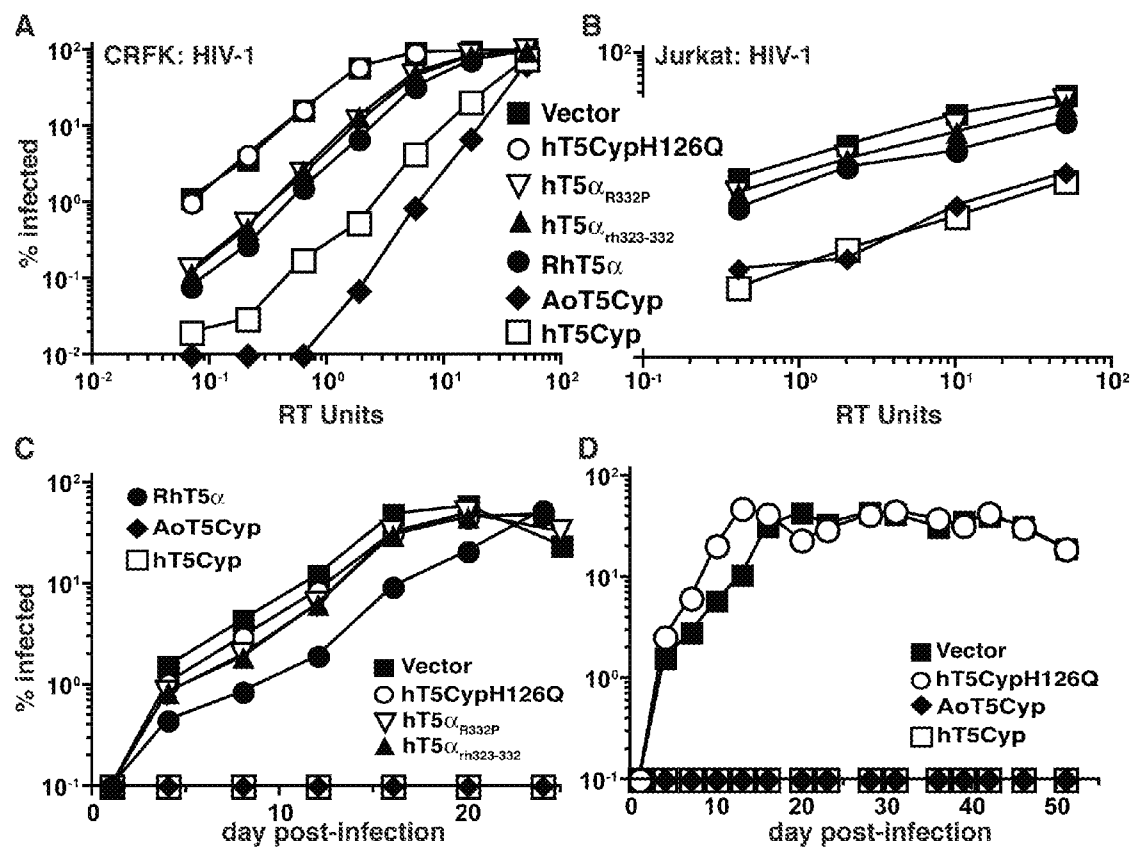


FIG. 28

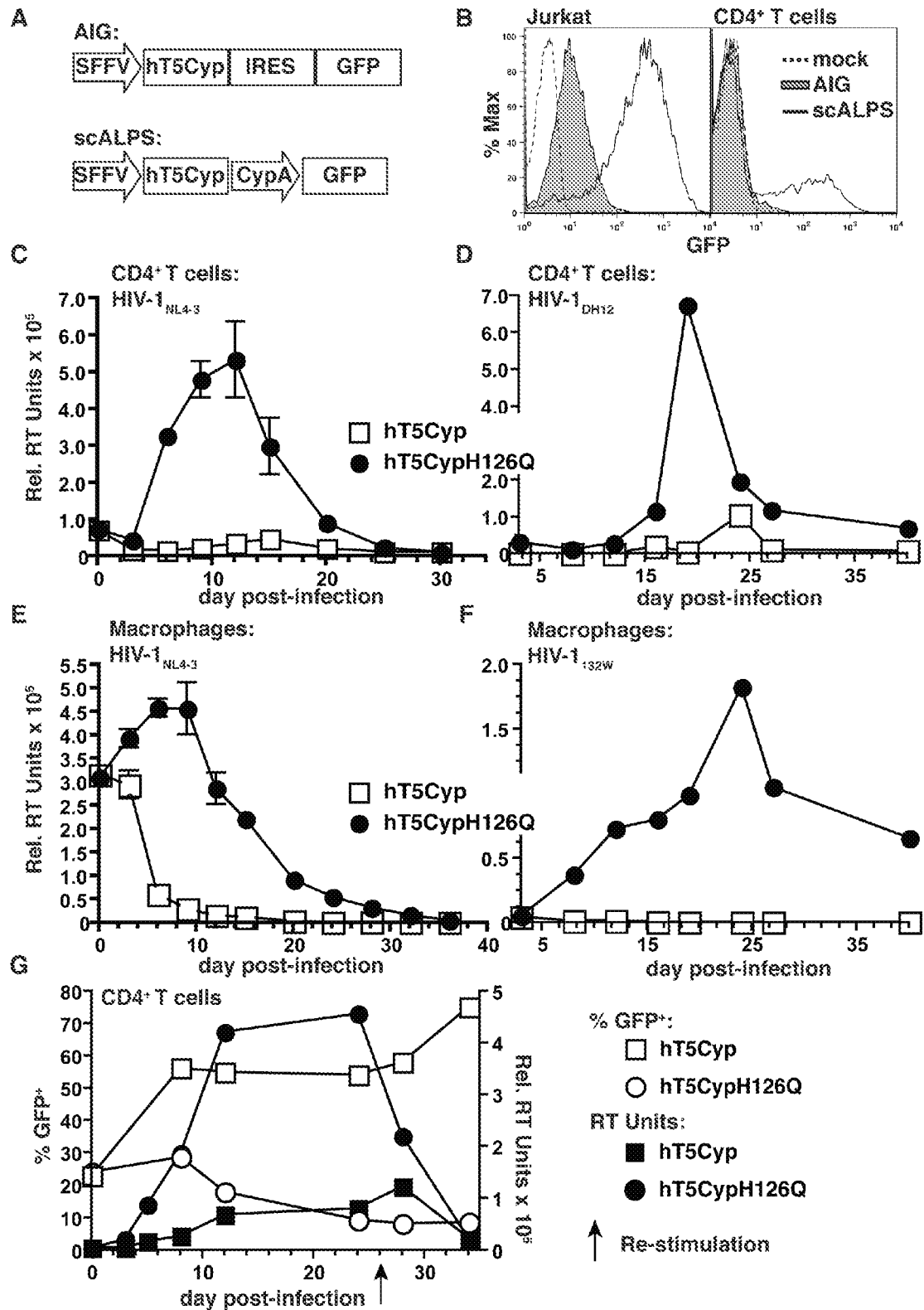


FIG. 29

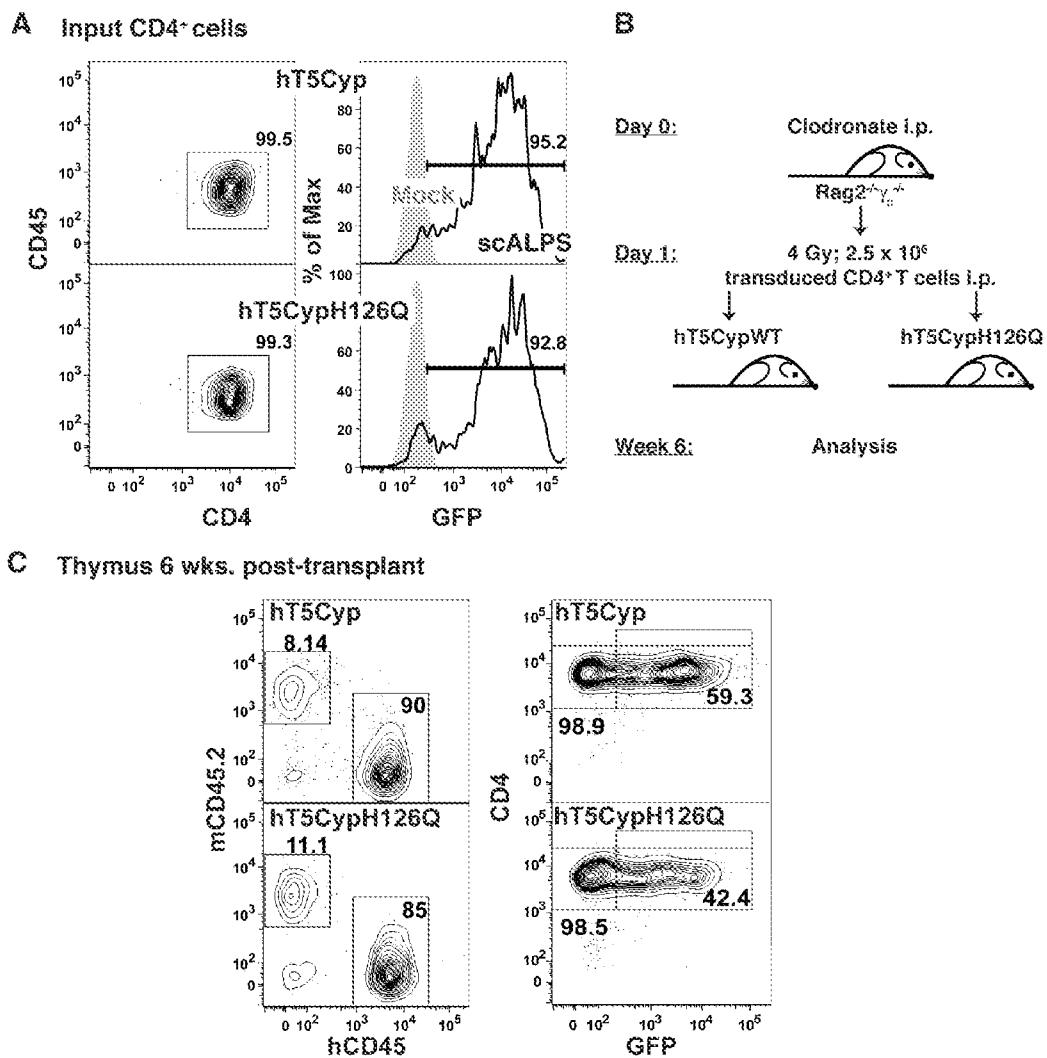


FIG. 30

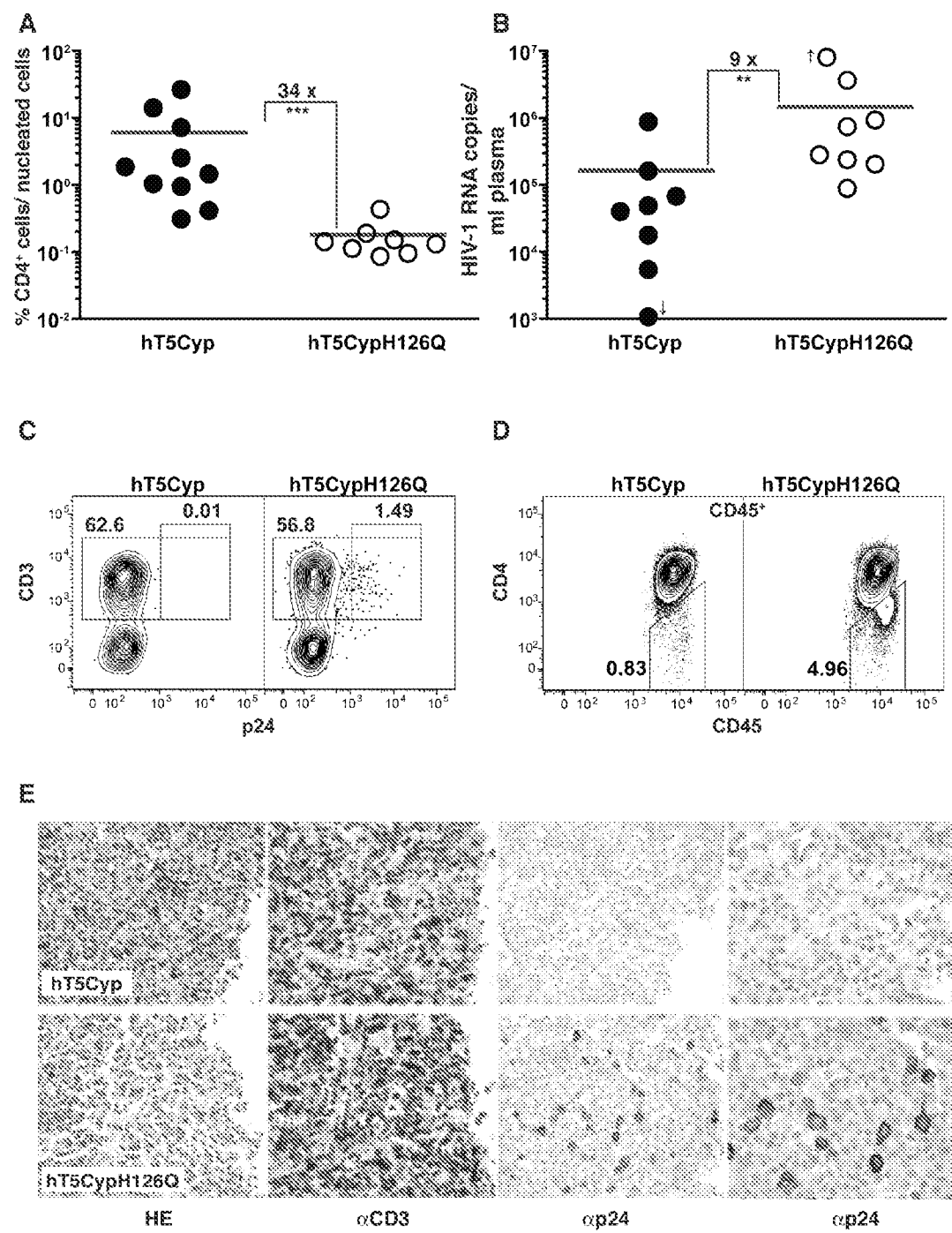


FIG. 31

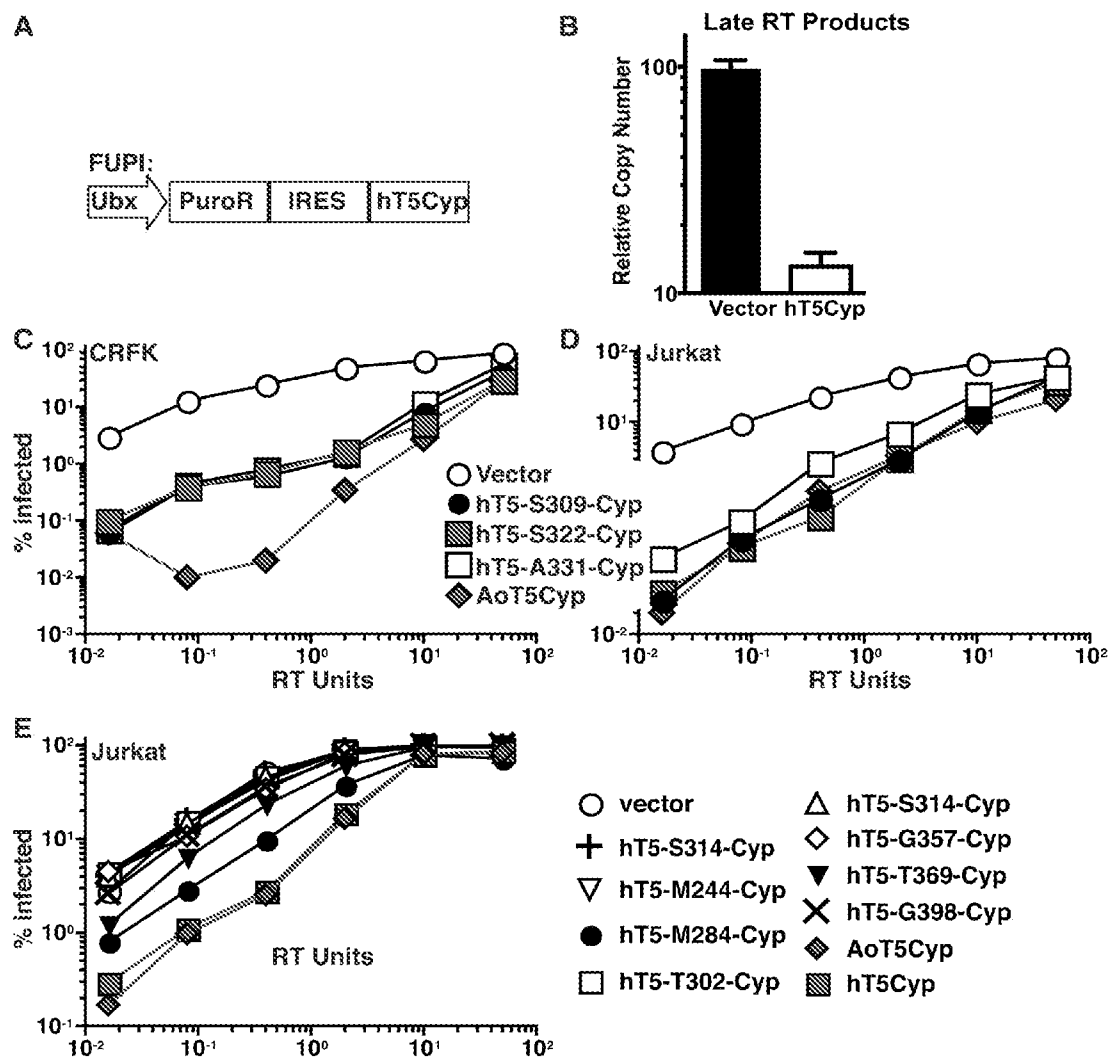


FIG. 32

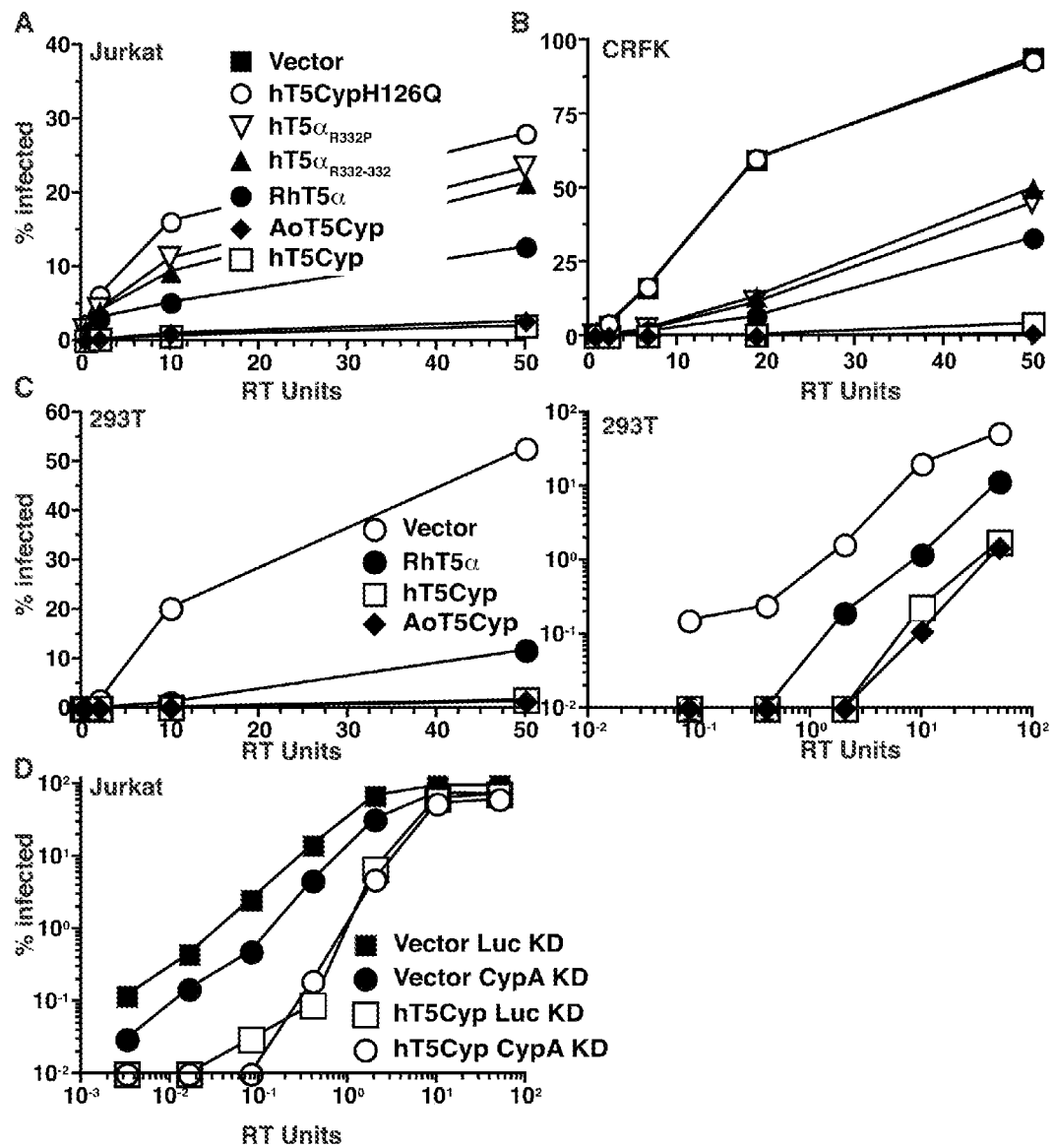


FIG. 33

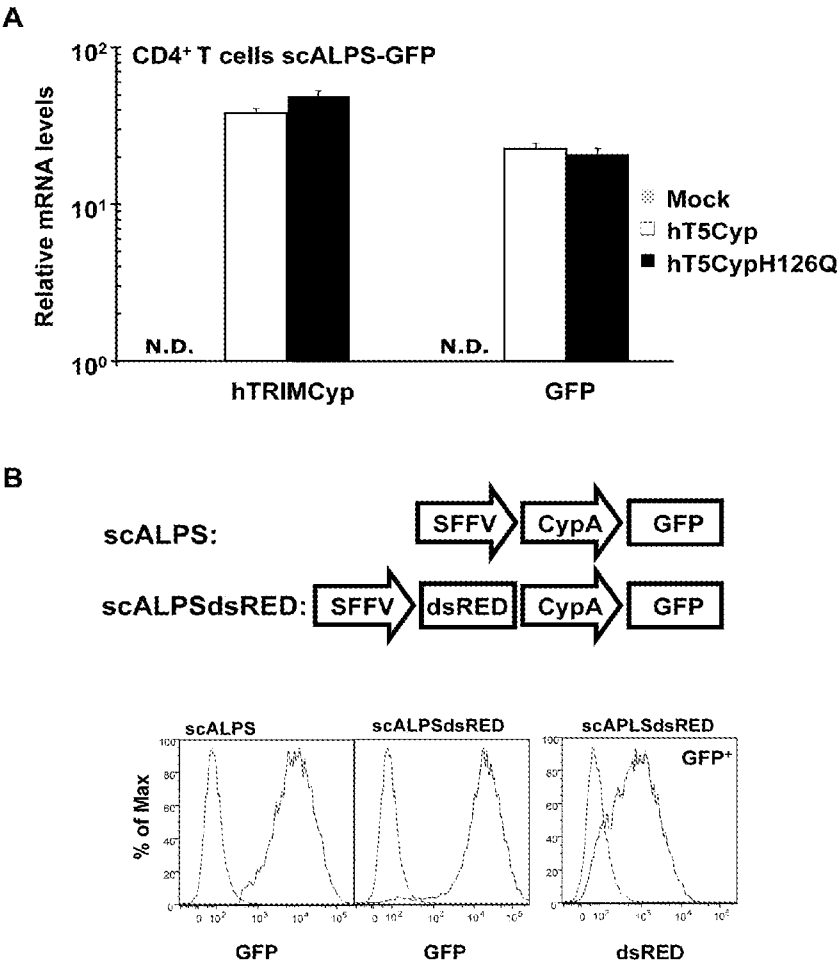


FIG. 34

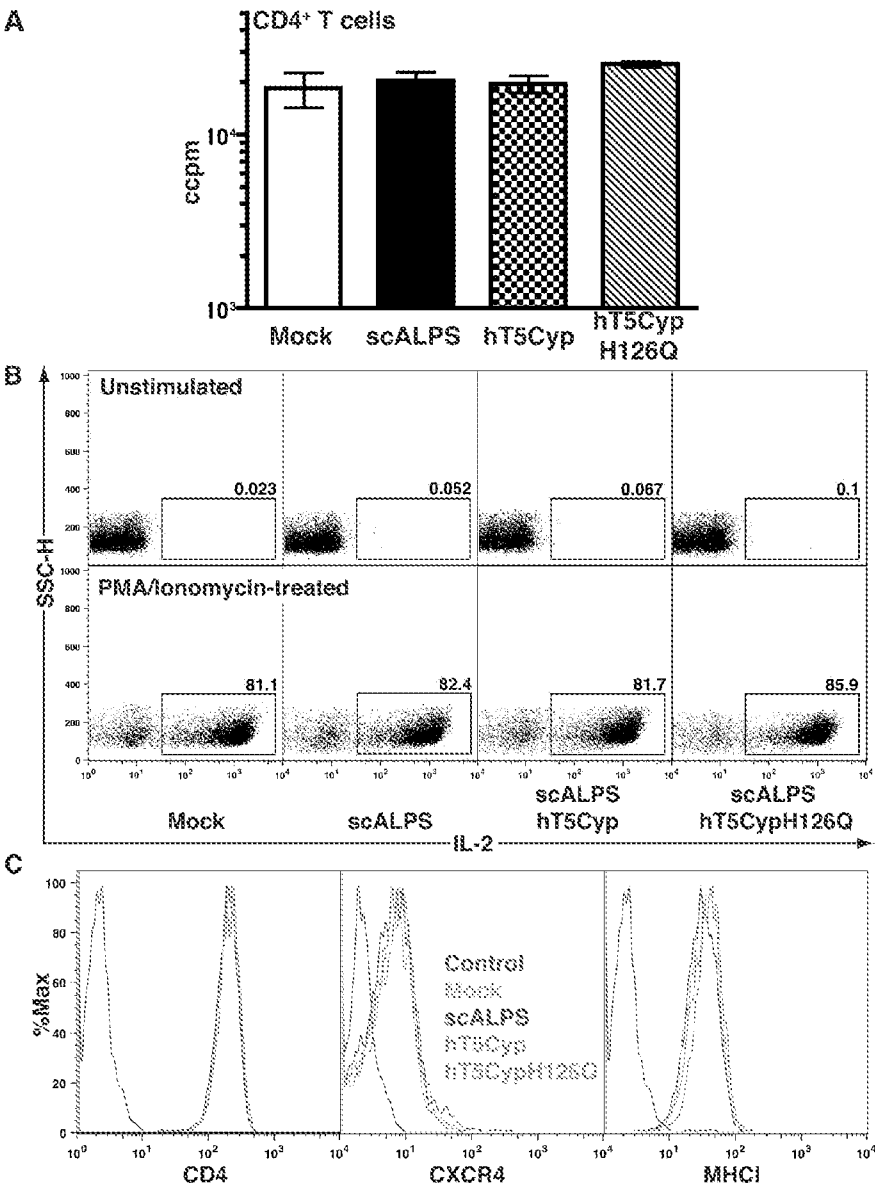


FIG. 35

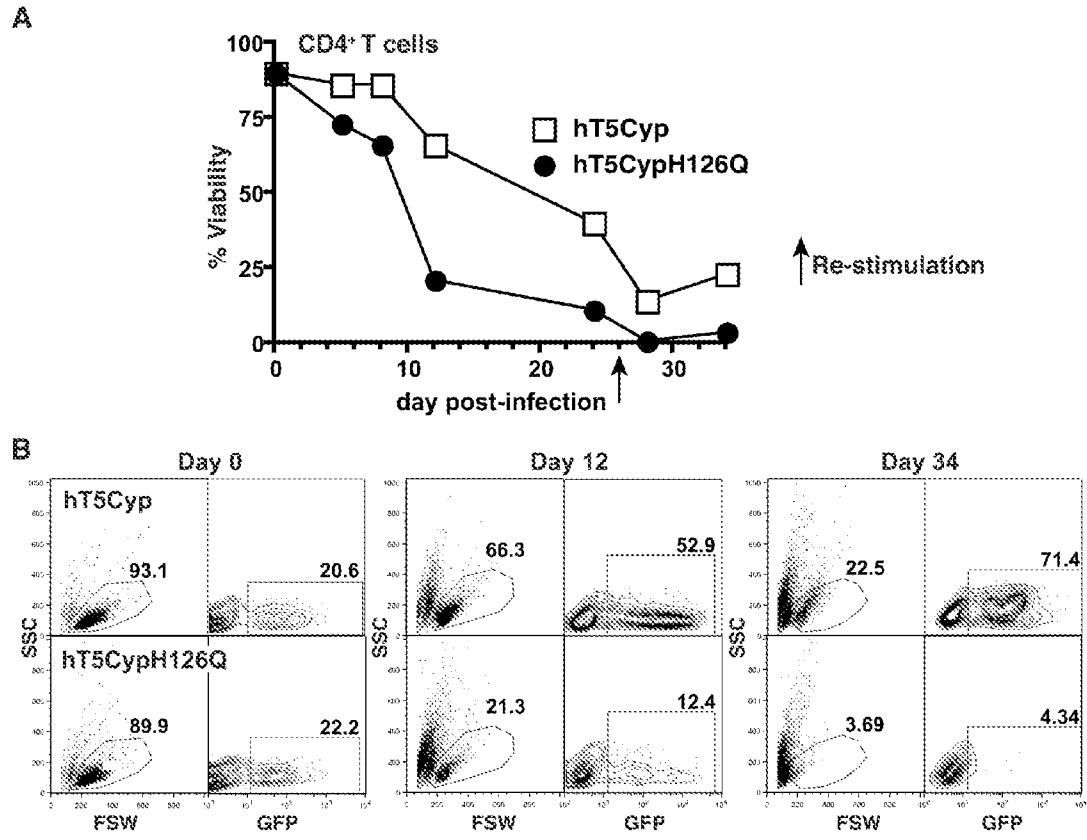


FIG. 36

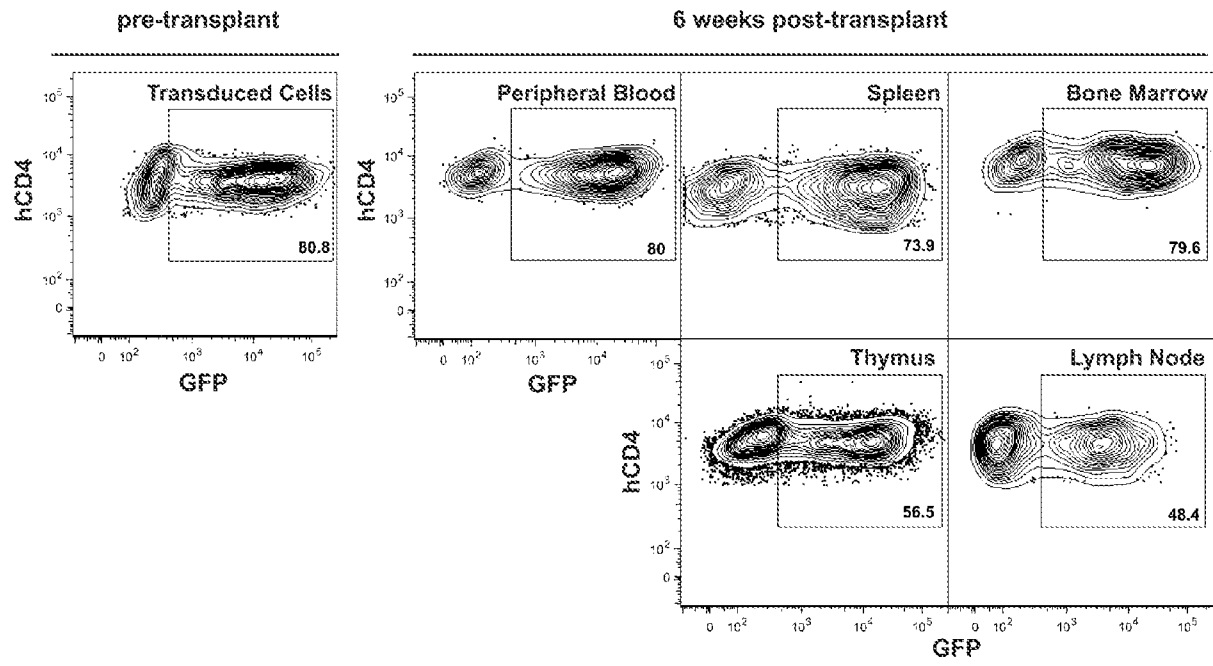


FIG. 37

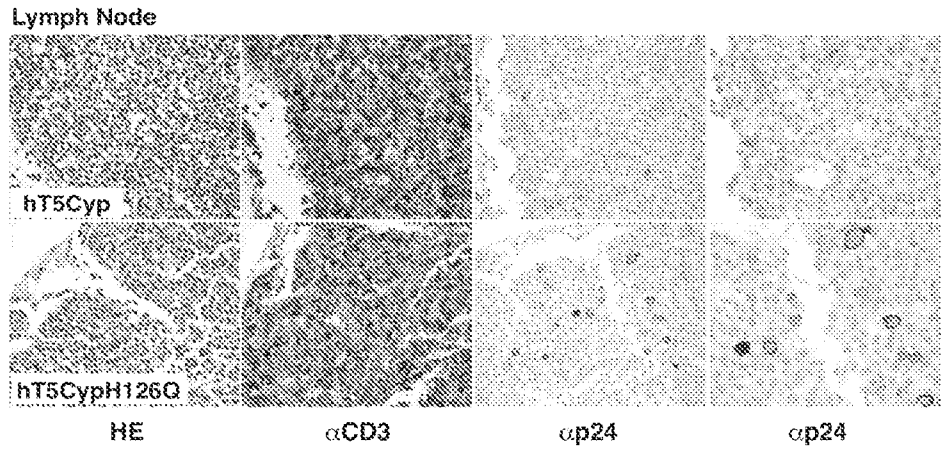


FIG. 38

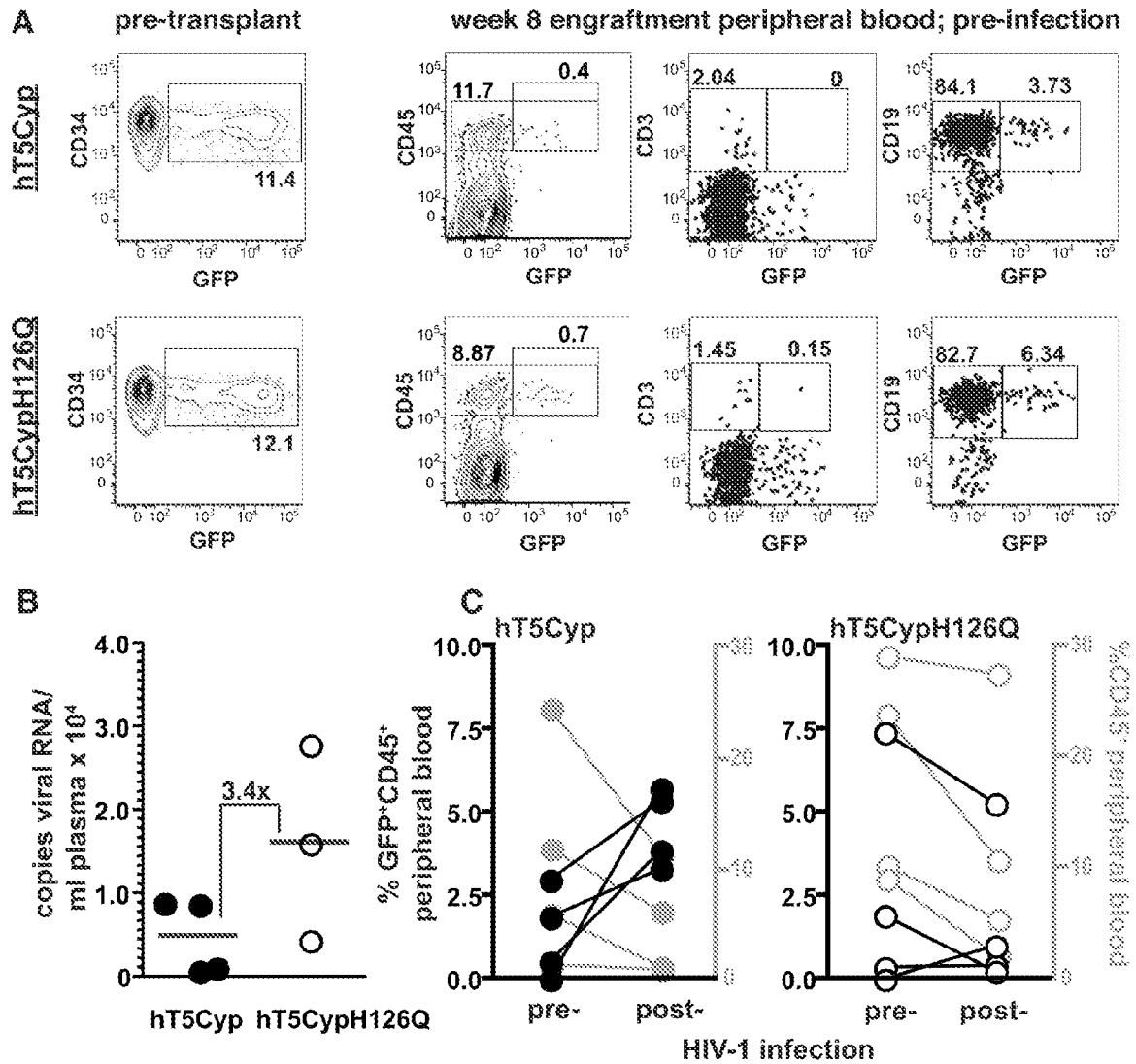


FIG. 39

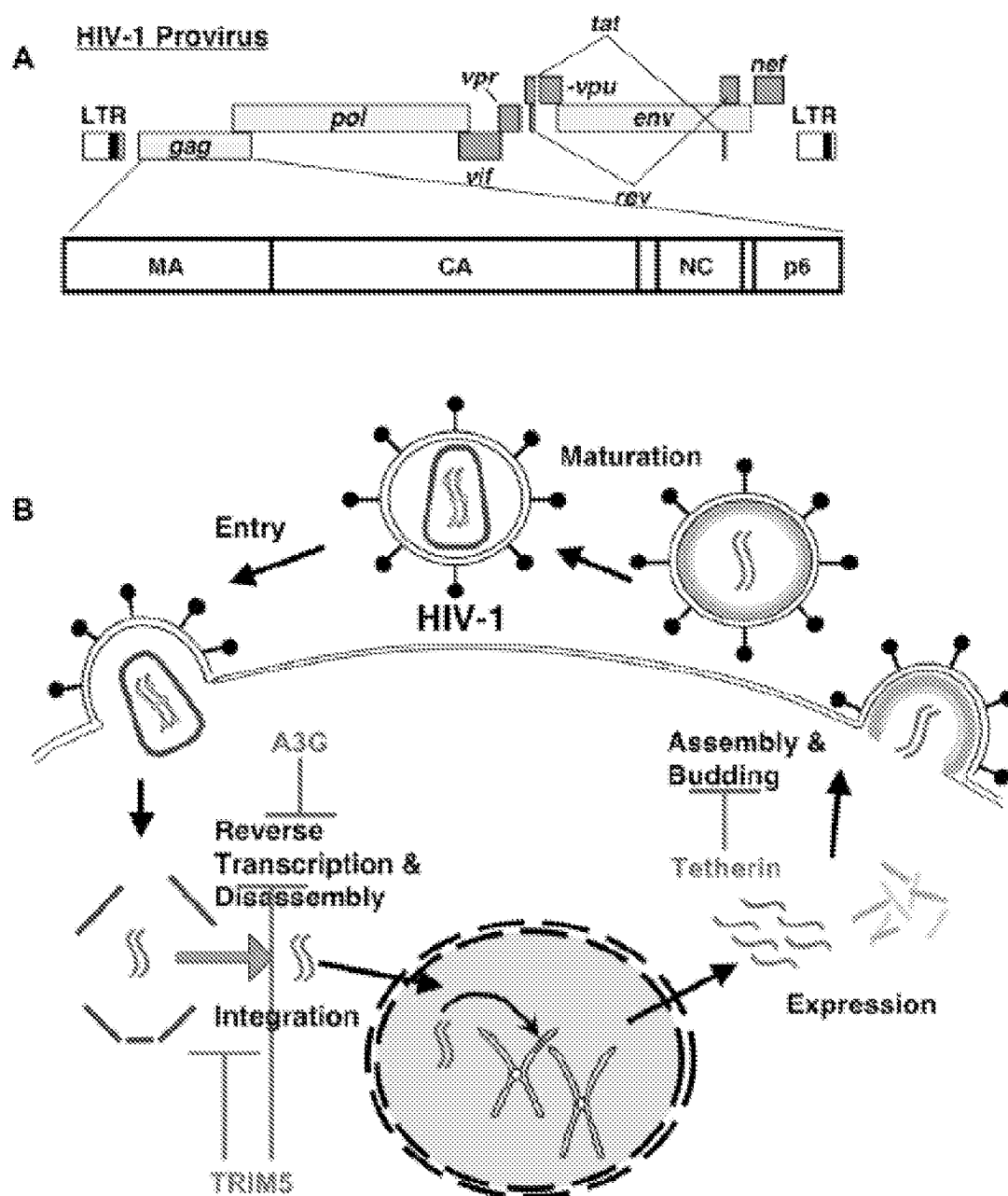


FIG. 40

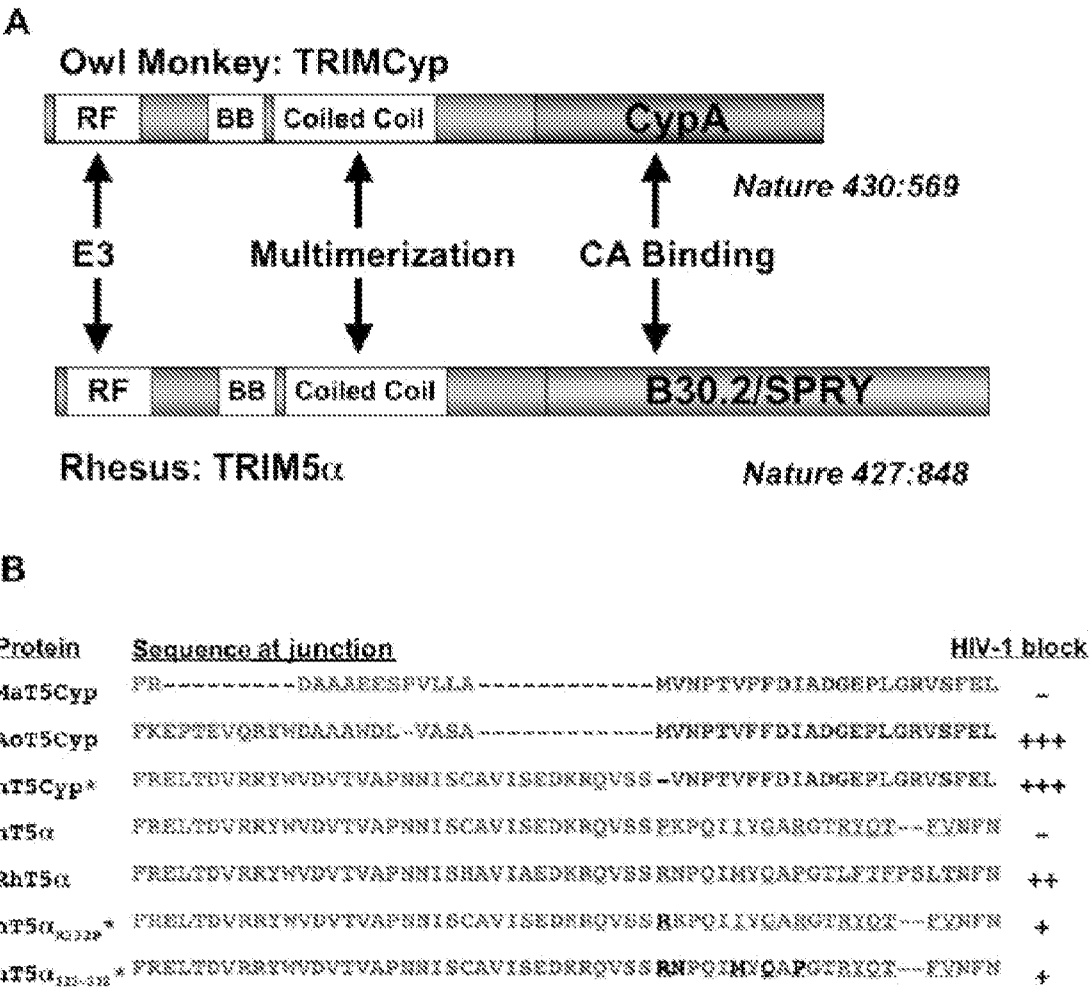


FIG. 41

79.6% identity in 486 residues overlap; Score: 2002.0; Gap frequency: 2.7%

```

HsTRIMCyp,      1 MASGILVNVKEEVTCPICLELLTQPLSLDCCGHSFCQACLTANHHKSMLEKGESSCPVCRI
AoTRIMCyp,      1 MASRILVNIKEEVTCPICLELLTSEPLSLDCCGHSFCQACITANHHKSMPEHQGERSCPLCRI
    ***  ****  *****  *****  *****  *****  *****  **  ***  ***

HsTRIMCyp,     61 SYQPENIRPNRRNVANIVEKLREVKLSPE-CQKVDHCAHGEKLLLFQSEDGKVICWLCER
AoTRIMCyp,     61 SYSSENLRPNRNLVNIVERLREVMLSPEEGQKVDHCAHGEKLVLFQQDGVVICWLCER
    **  **  *****  ****  ****  ****  *****  *****  ****  *****

HsTRIMCyp,    120 SQEHRCHHTFLTEEVAREYQVKLQAALEMLRQKQGEASELEADYREEKASWKTQIQYDKT
AoTRIMCyp,    121 SQEHRCHQTFLVEEVAQKYREKLQVALEMMRQKQKDAEKLEADVREEQASWKIQIQNDKT
    *****  ***  ****  *  ***  ****  ****  **  ****  ***  ****  ****

HsTRIMCyp,    180 NVLADFEQLRDILDWEESNELQNLKEESEDILKSLTNSETEMVQQTQSLRELISDLEHRL
AoTRIMCyp,    181 NIMAEFKKRRDILDCEESKELQNLKEEKNILKRLVQSENDMVLQTSVVRVLISDLEHRL
    *  *  *  *****  ***  *****  ****  *  **  **  ****  *  *****

HsTRIMCyp,    240 QGSVMELLQGVDCGVIKRTENVTLKKPETTPKNQRRVFPAPDLKCMLEVFRELTEVRRYWV
AoTRIMCyp,    241 QGSVMELLQGVDCGVIKRIEKVTLQNPKTFLNEKRRIPOTPDLKCTLQVFEKPTVQRVW...
    *****  *****  *  ***  *  **  **  *  *****  *  **  *  ****

HsTRIMCyp,    300 DVTVAPNNISCAVISEKHKQVSSVNPVTVPFDIAVDGEPLGRVSEFELFADKVPKTAENFRA
AoTRIMCyp,    300 -----DAAANDLVASAHVNPTVPFDIAVDGEPLGRVSEFELFADKVPKTAENFRA
    *  *****

HsTRIMCyp,    360 LSTGEKGFQYKGSCEFHRIPCFMQCGGDFTRHNGTGCKSIYGEKFEDENFILKHTCPGIL
AoTRIMCyp,    349 LSTGEKGFQYKGSCEFHRIPCFMQCGGDFTRHNGTGCKSIYGEKFDDENFILKHTCPGIL
    *****  *****

HsTRIMCyp,    420 SMANACPNTEGSGQFFICTAKTEWLDGKHVVFGKVKEGMNIVEAMERFGSRNCKTSKKITI
AoTRIMCyp,    409 SMANACPNTEGSGQFFICTAKTEWLDGKHVVFGKVKEGMNVEAMERFGCRYGKTSKKITI
    *****  *****  *****  *  *****

HsTRIMCyp,    480 ADCCQL
AoTRIMCyp,    469 ADCCQL
    *****

```

Fig. 42

A

Fusion protein	...T5-linker residues-CypA	HIV-1 restriction
AoT5Cyp	...RYW-DAAANDLVASA-CypA	+
hT5 α Cyp	...RYW-VDVTVAPNNISCAVIS-CypA	-
hT5 γ Cyp	...RYW-GKEKSHYHKPPCGLS-CypA	-
hT5 δ Cyp	...RYW-GWSAMARSRFTATSTS-CypA	-
hT5directCyp	...RYW-CypA	-
hT5(SGG) ₄ Cyp	...RYW-SGGSGGSGGSGG-CypA	+

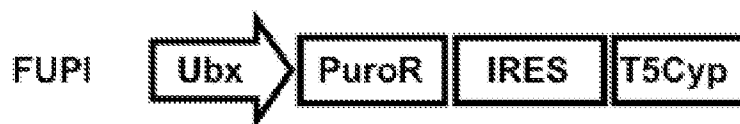
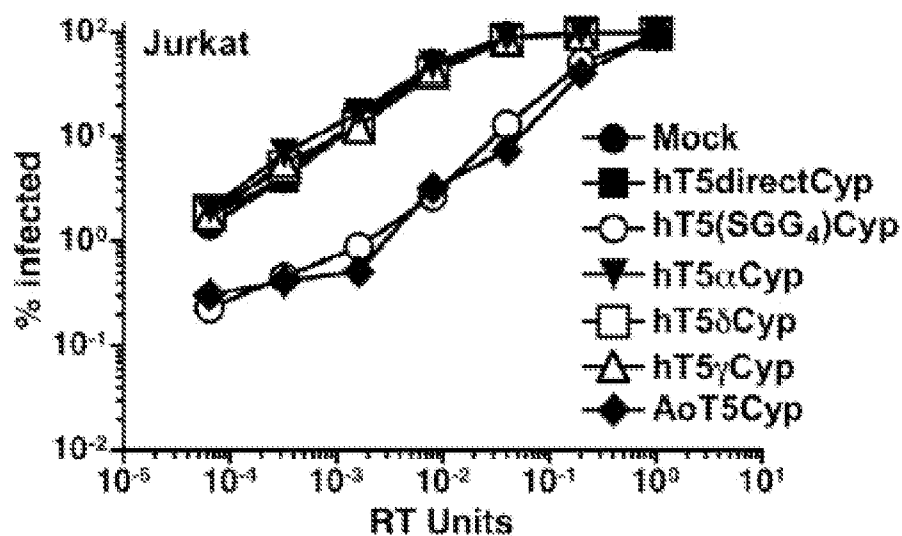
B**C**

FIG. 43

A

..... Ring Finger
 MASGILVNVKEEVTCPICLELLTQPLSLDCGHSFCQACLTANHKKSM LDK

 GESSCPVCRI SYQPENIRPNRHVANLVEKLREVKLSPEGQKVDH CARHGE
 S Box 2 efgabdefgfabdefgab
 KLLFCQEDGKVICWLCERSQEH RGHHTFPTEEVAQEYQVKLQA ALEMLR
 cdefgabdefgfabdefg
 OKQQAEELEADIREEKASWKTQIQYDKTNVLADFEQLRDILDWEESNEL
 gabdefgfabdefgfabdefgfabdefgab M244
 QNLEKEEDILKSLTNSETEMVQQTQSLRELISDLEHRLQGSVMELLQGV
 M284 W298
 DGVIKRTENVTLKKPETFPKNQRRVFRAPDLKGMLEVFRELTDVRRYWVD
 T302 S309 S314 S322 A331
 VTVAPNNISCAVISEDKRQVSSPKPQIIYGARGTRYQTFVNFNYCTGILG
 G357 T369 G398
 SQSITSGKHYWEVDVSKKTAWILGVCAGFPDAMCNIEKNENYQPKYGYW
 VIGLEEGVKCSAFQDSSPHTPSVPPFIVPLSVIICPDRVGVFLDYEA CTVS
 FFNITNHGFLIYKFSSHCSFSQPVPFPYLNPRKCGVPMTLCSPSS

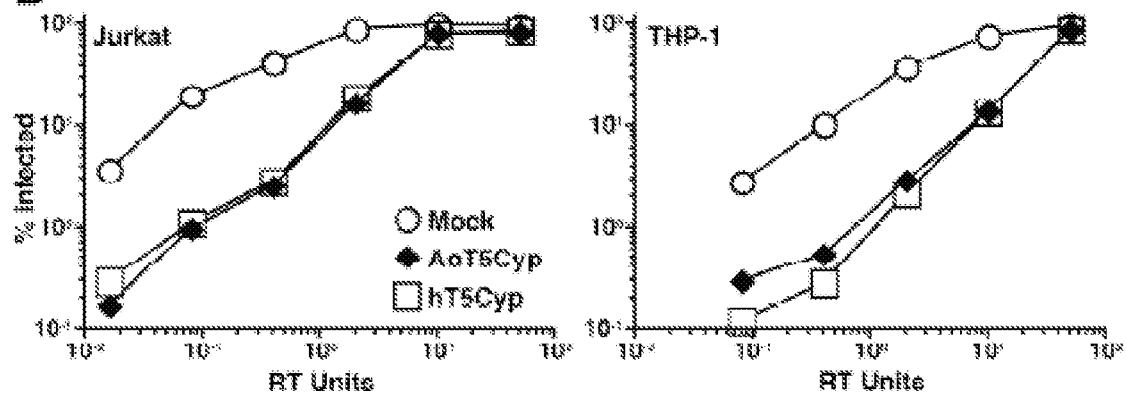
B

FIG. 44

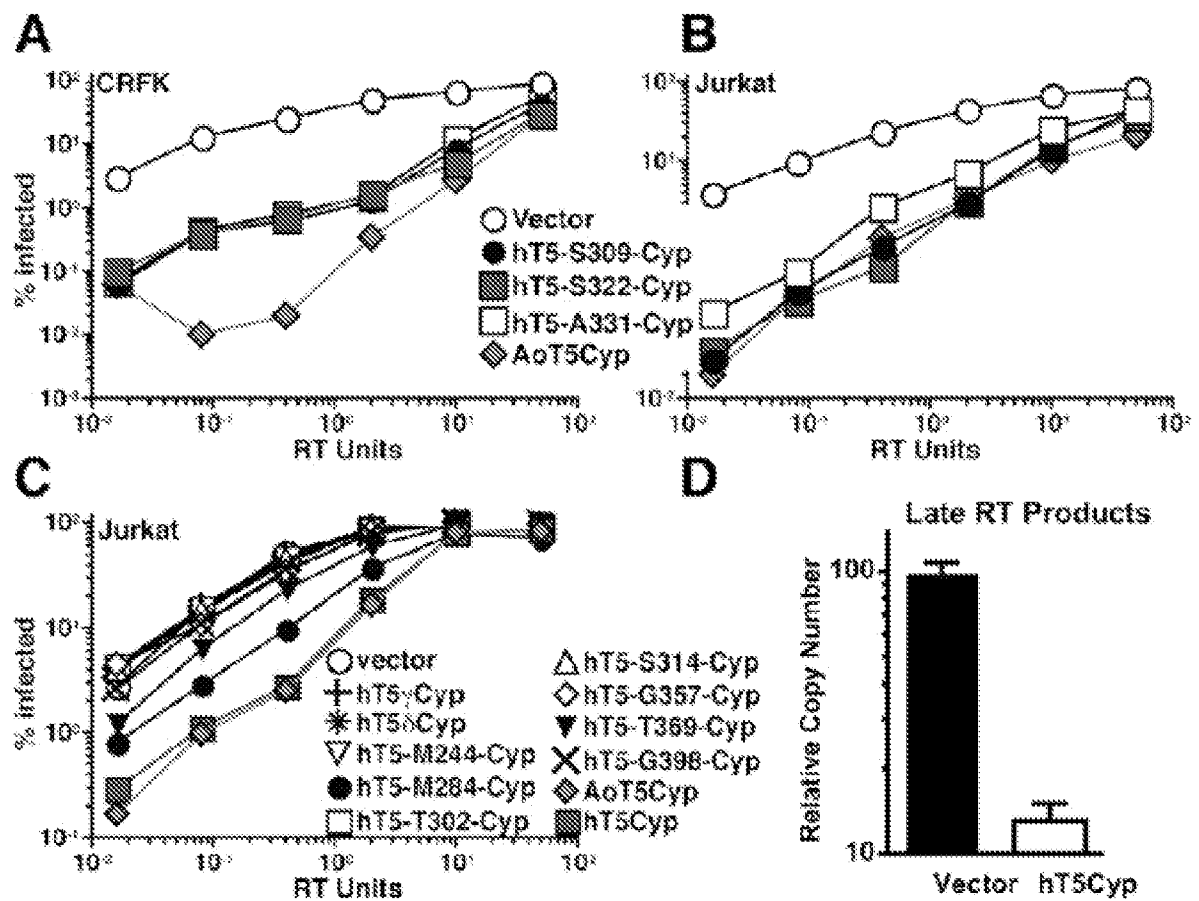


FIG. 45

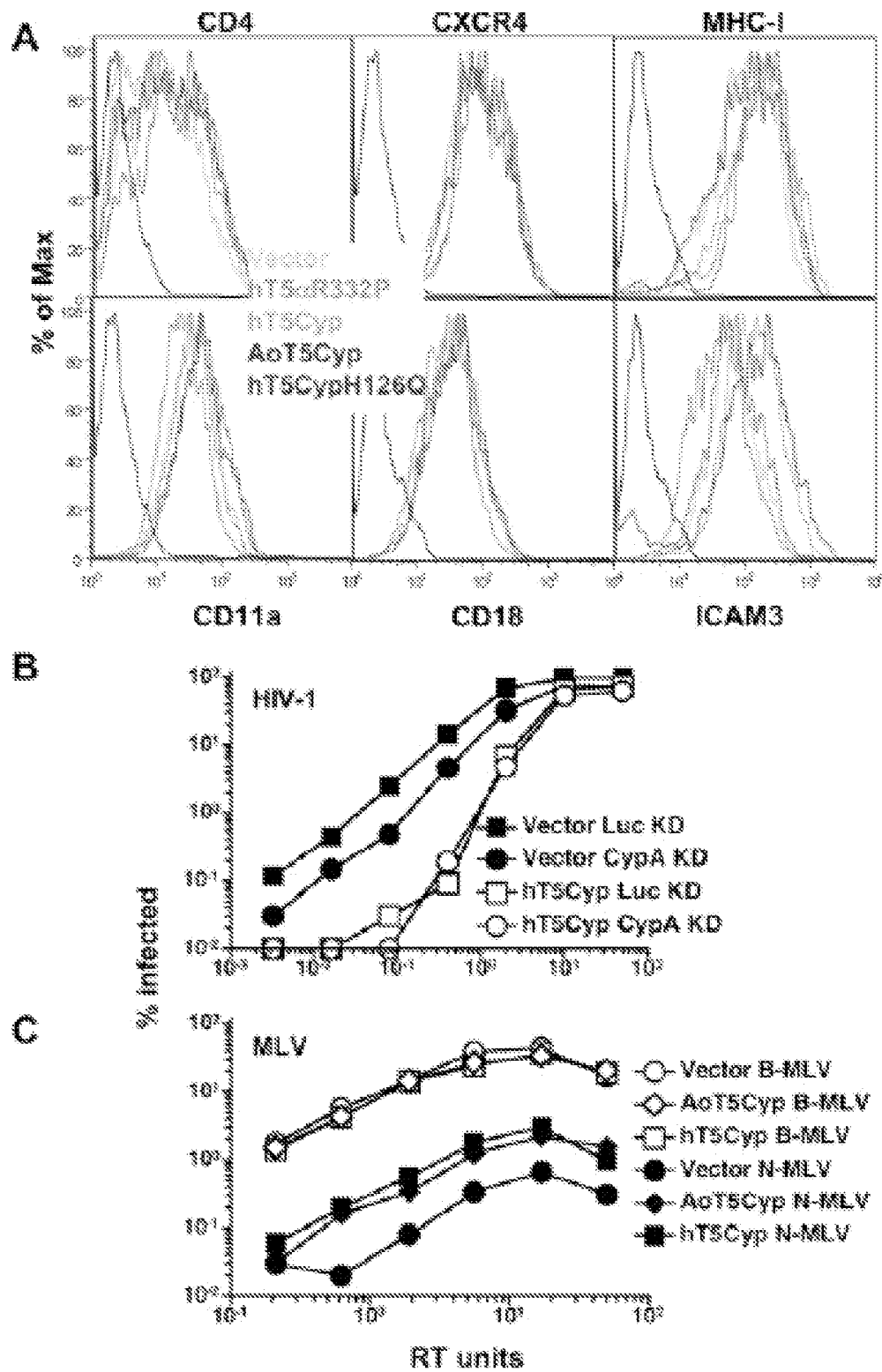


FIG. 46

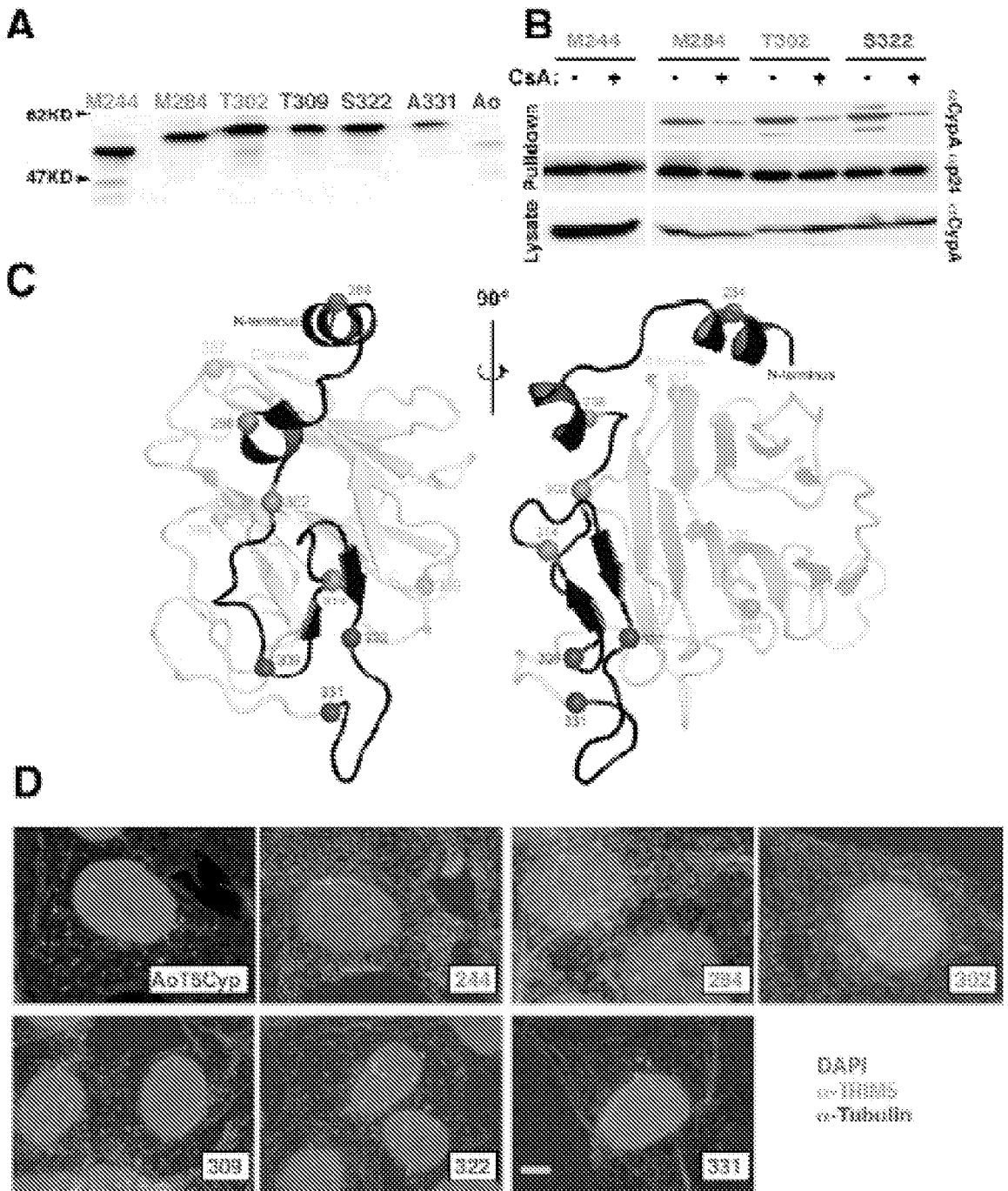


FIG. 47

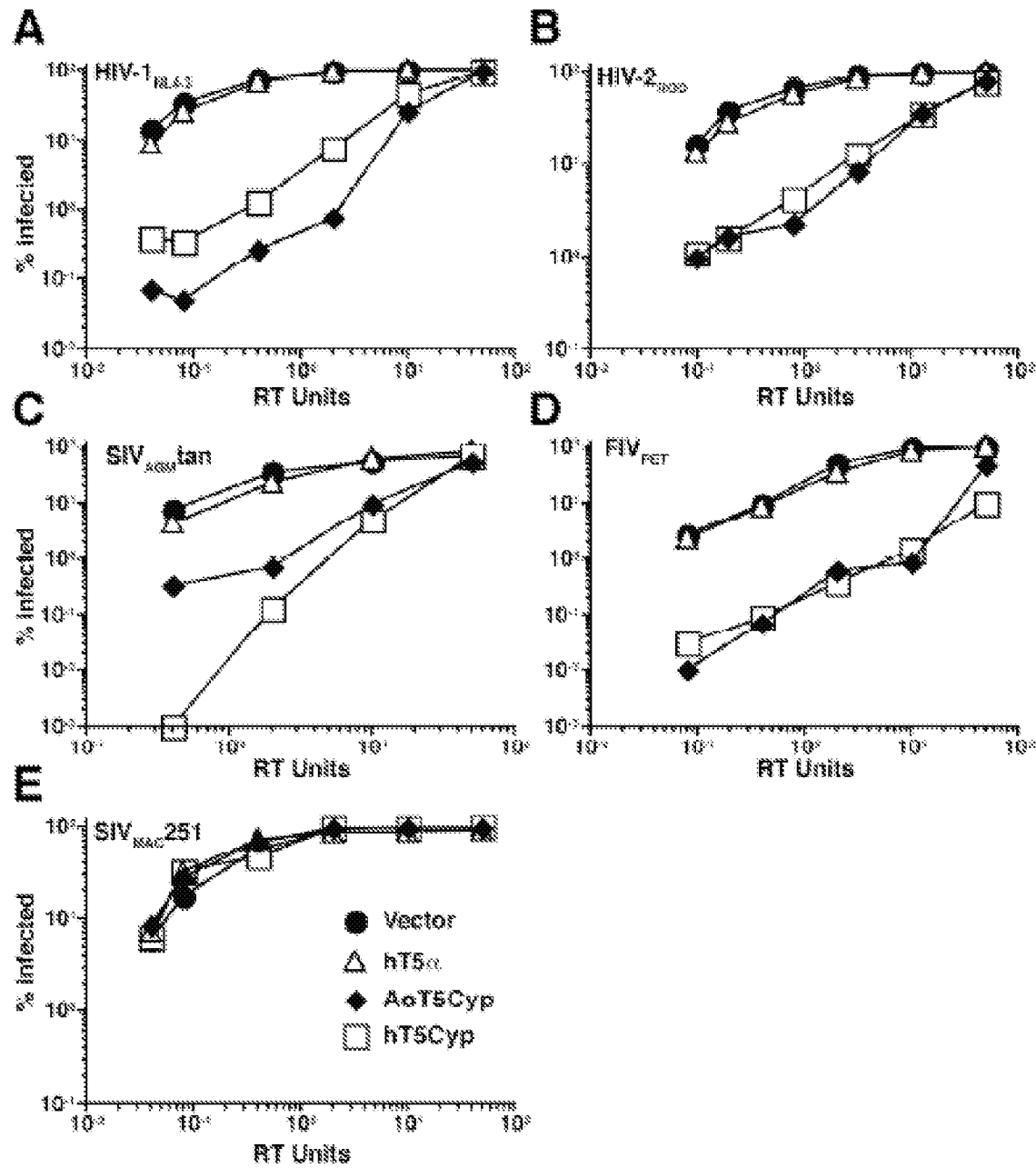


FIG. 48

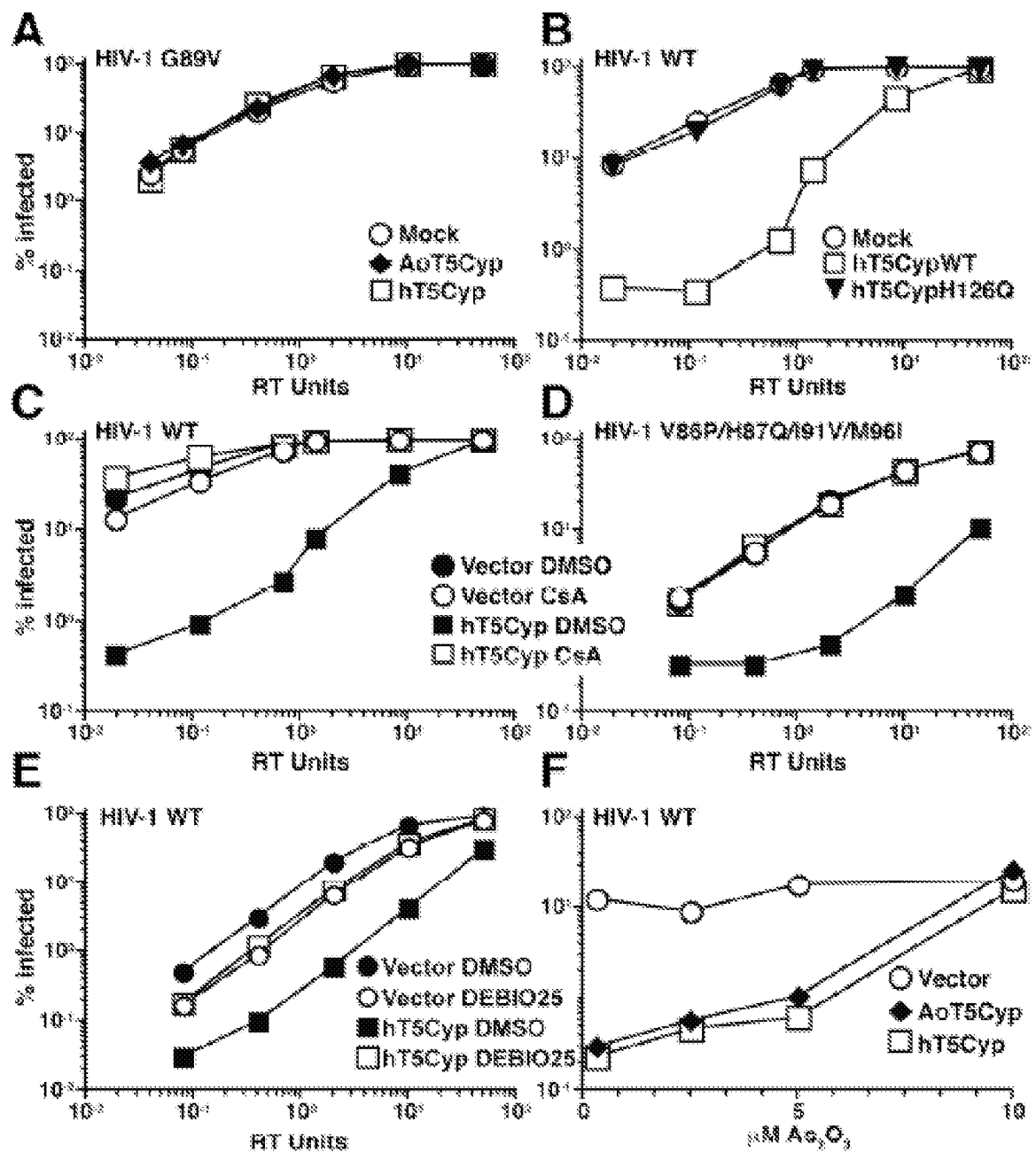


FIG. 49

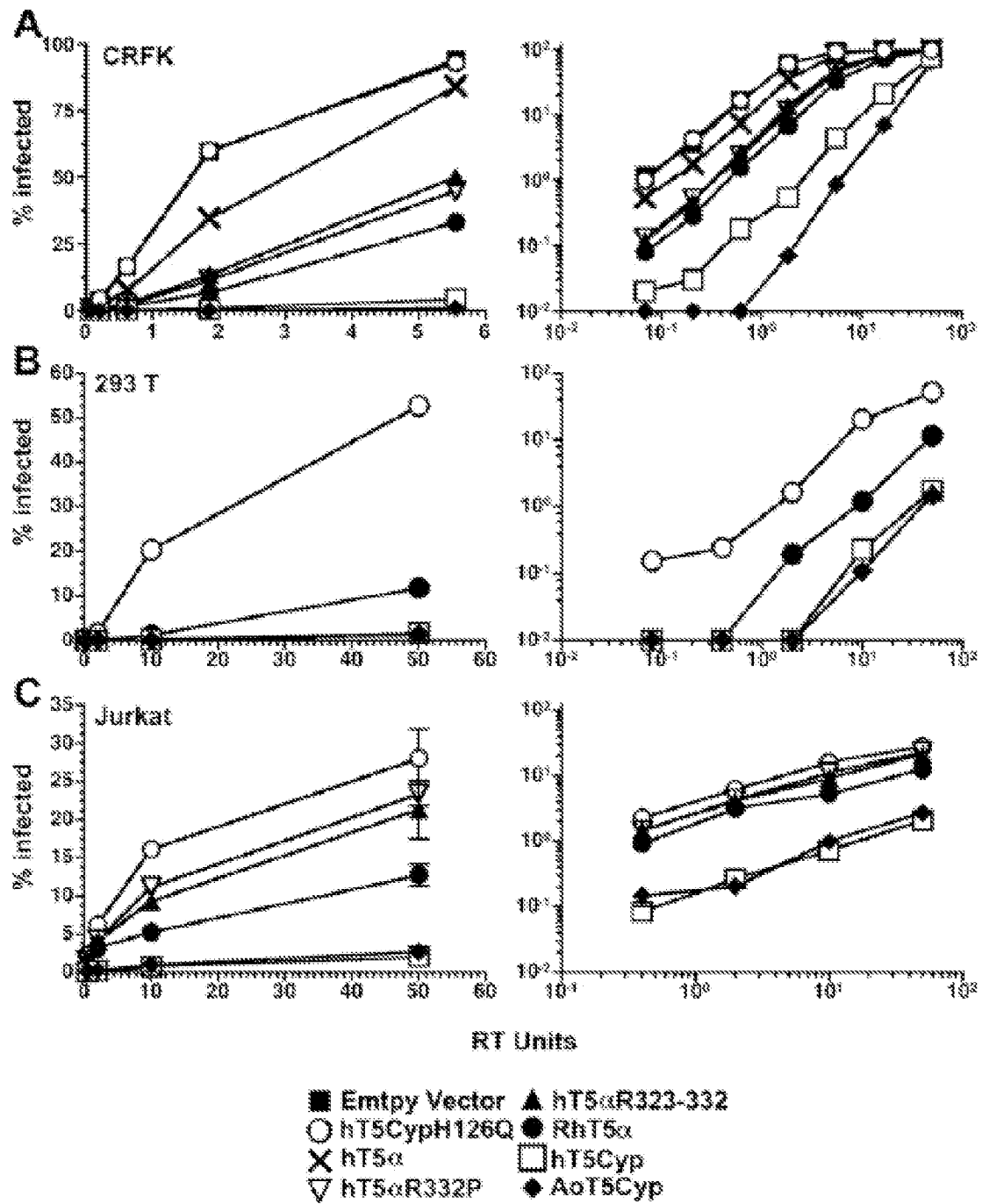


FIG. 50

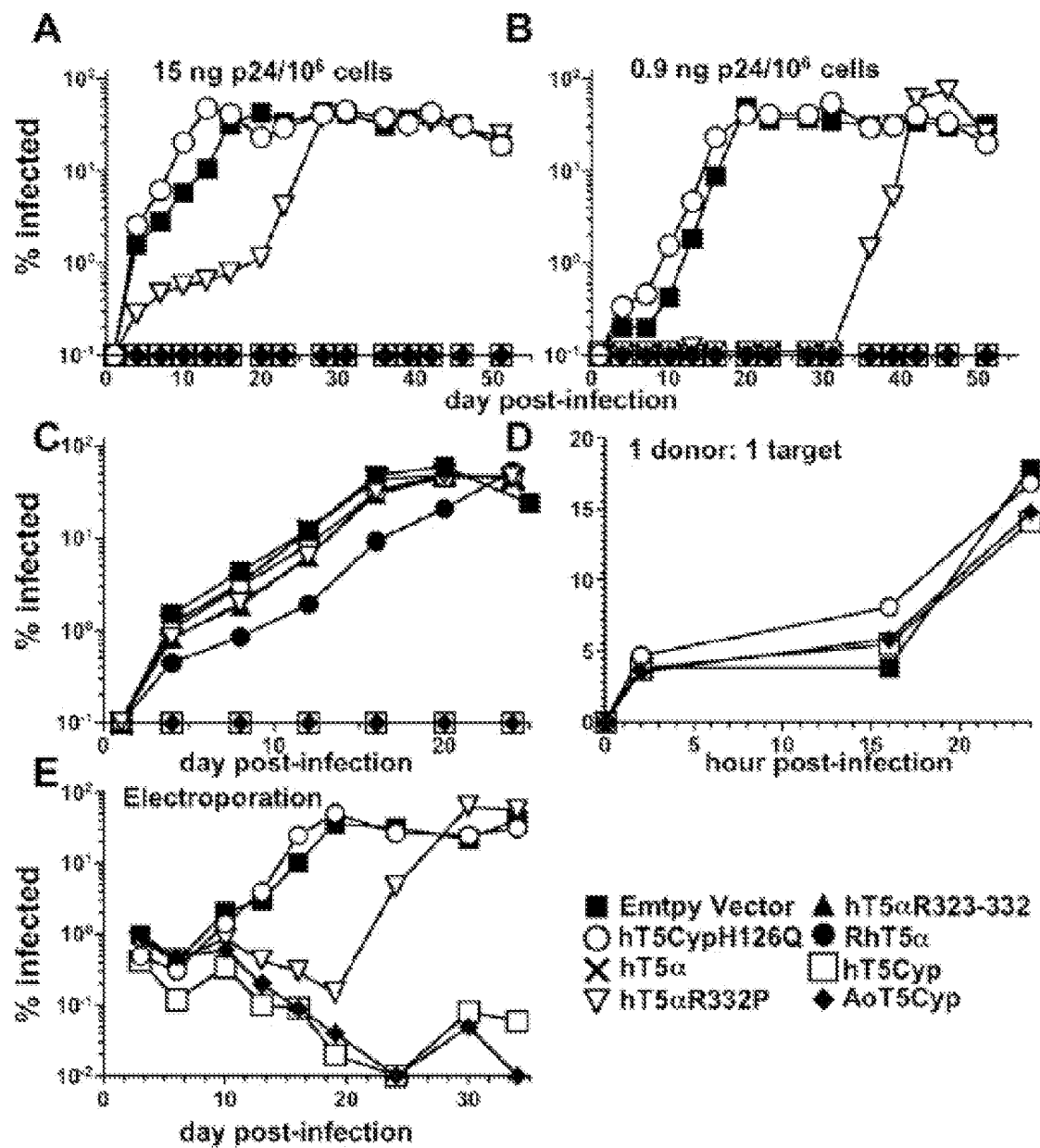


FIG. 51

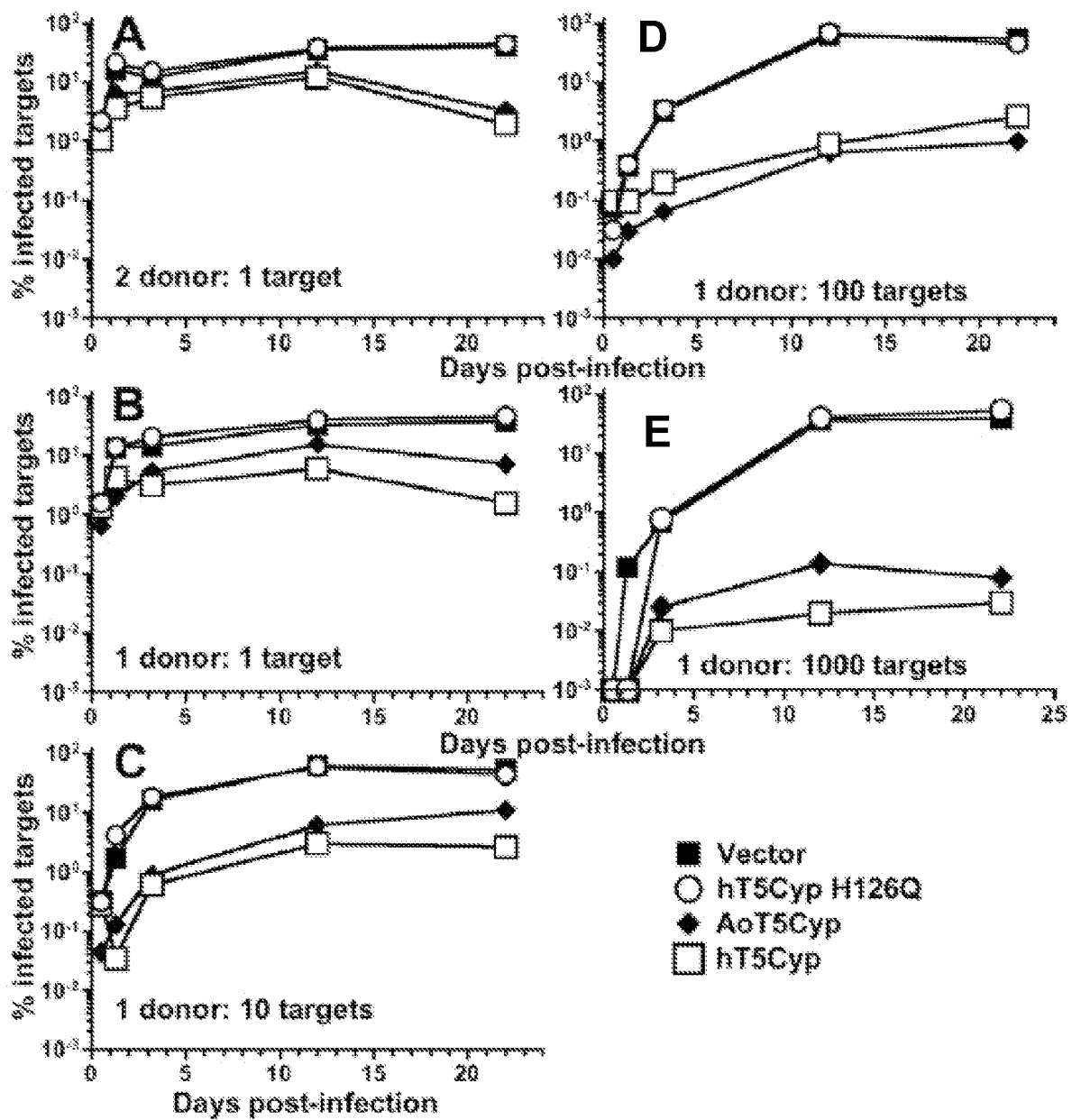


FIG. 52

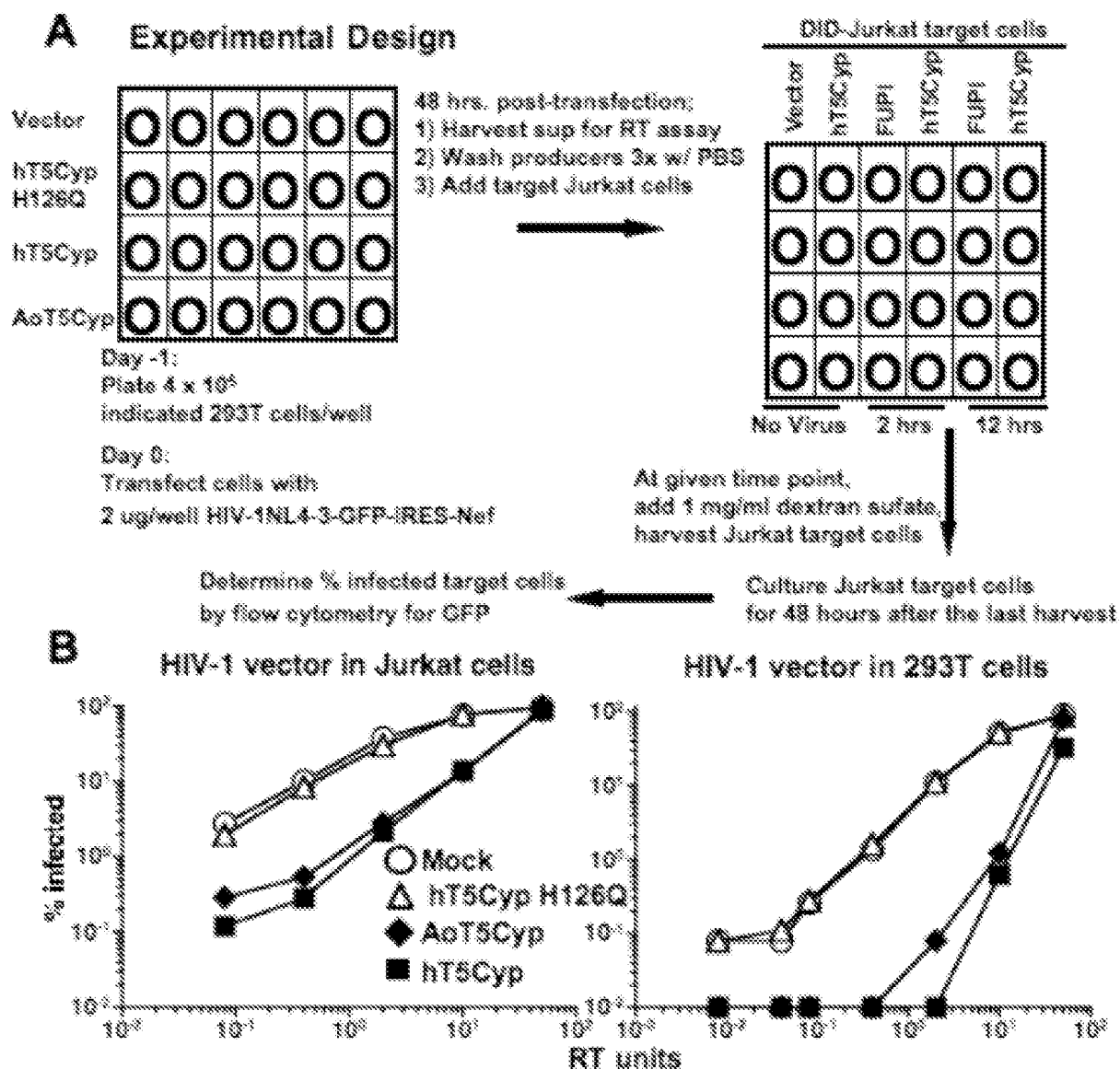


FIG. 53A-B

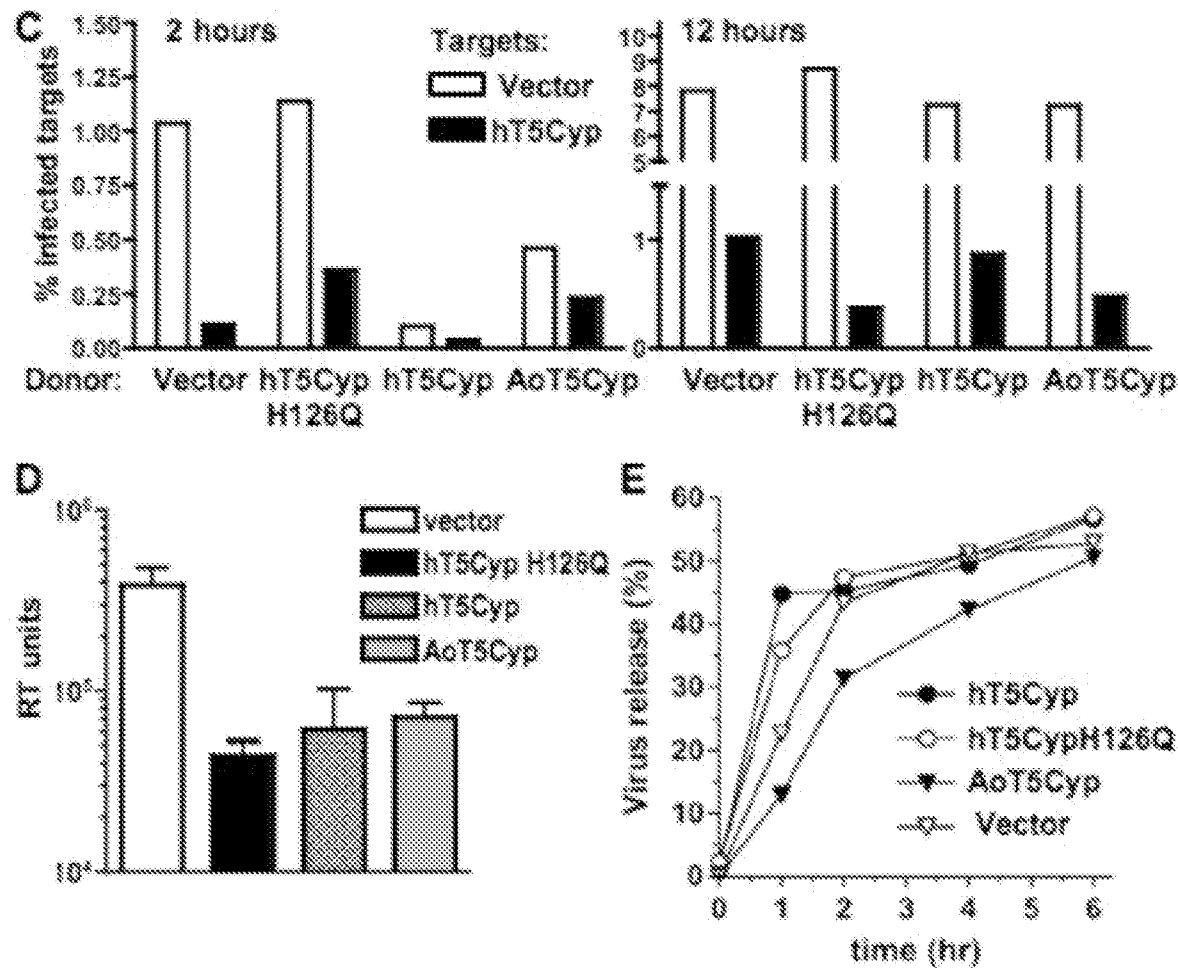


FIG. 53C-E

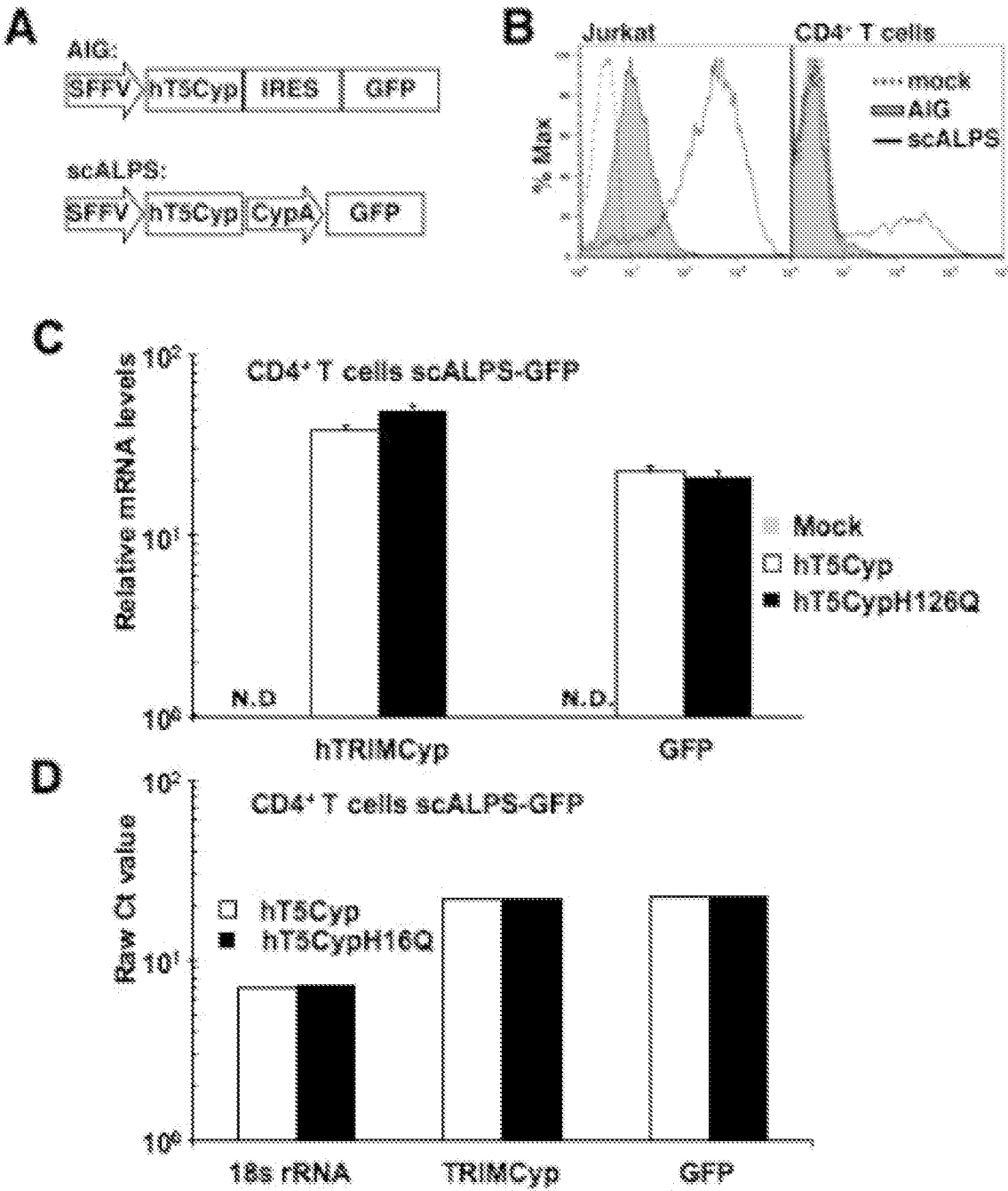


FIG. 54

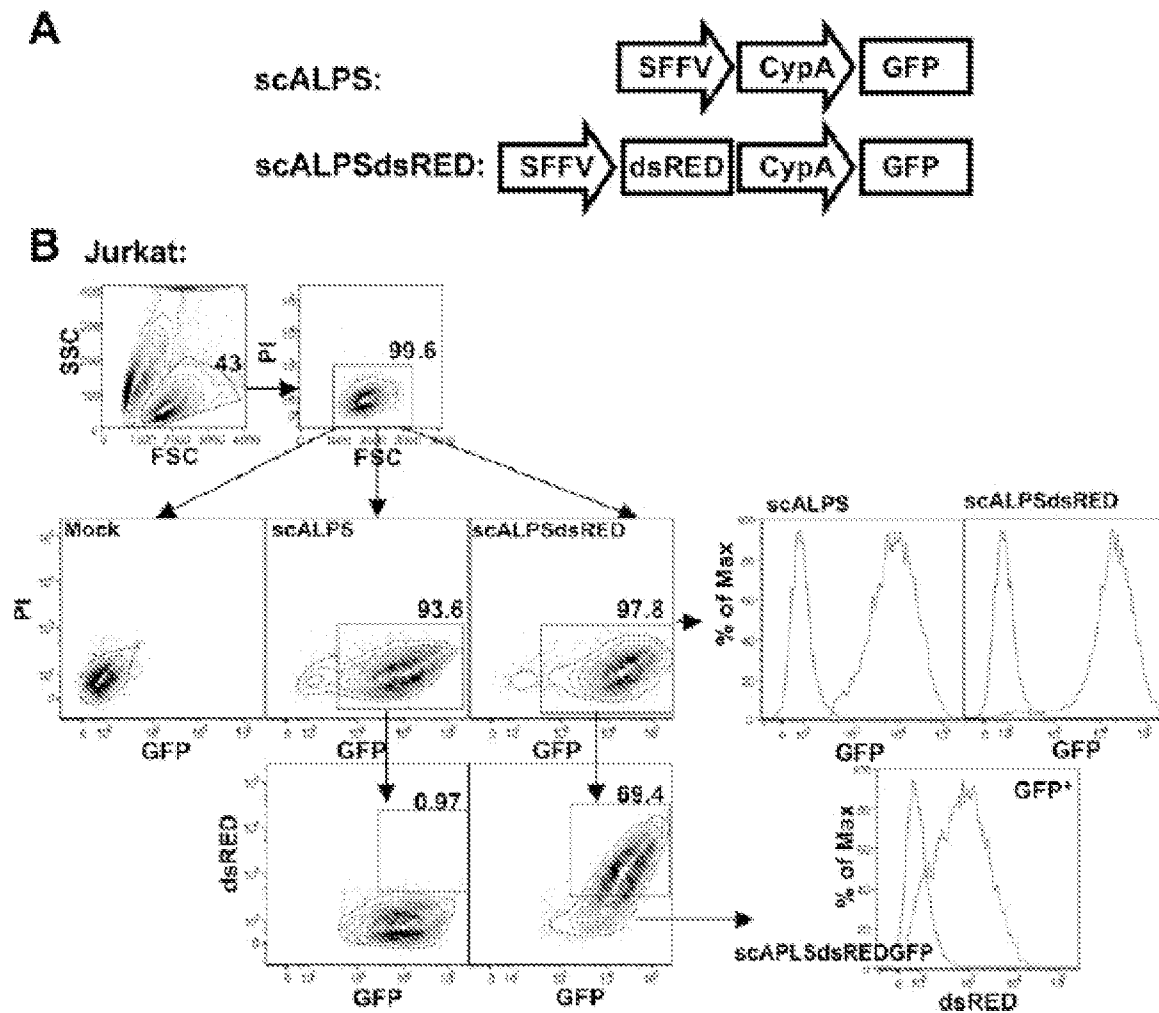


FIG. 55

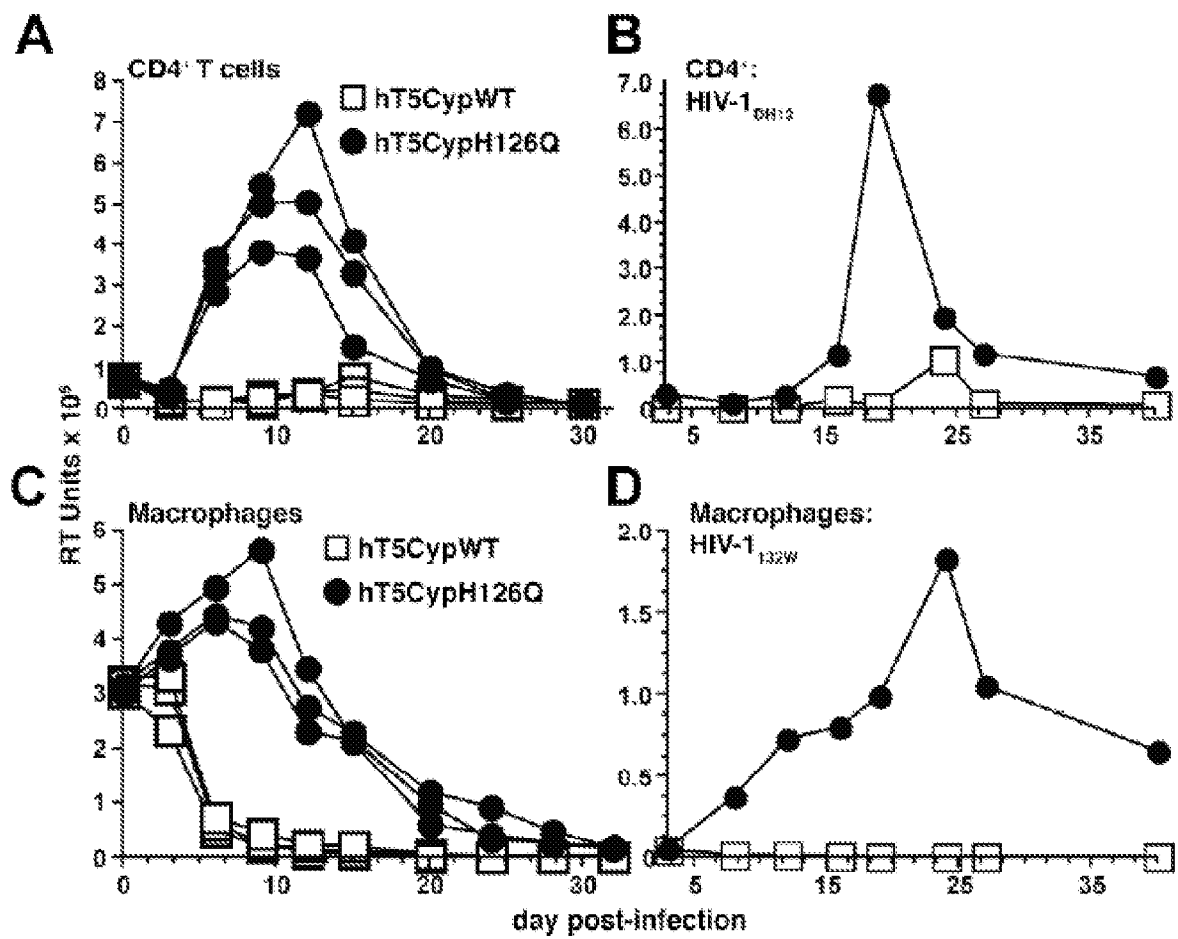


FIG. 56

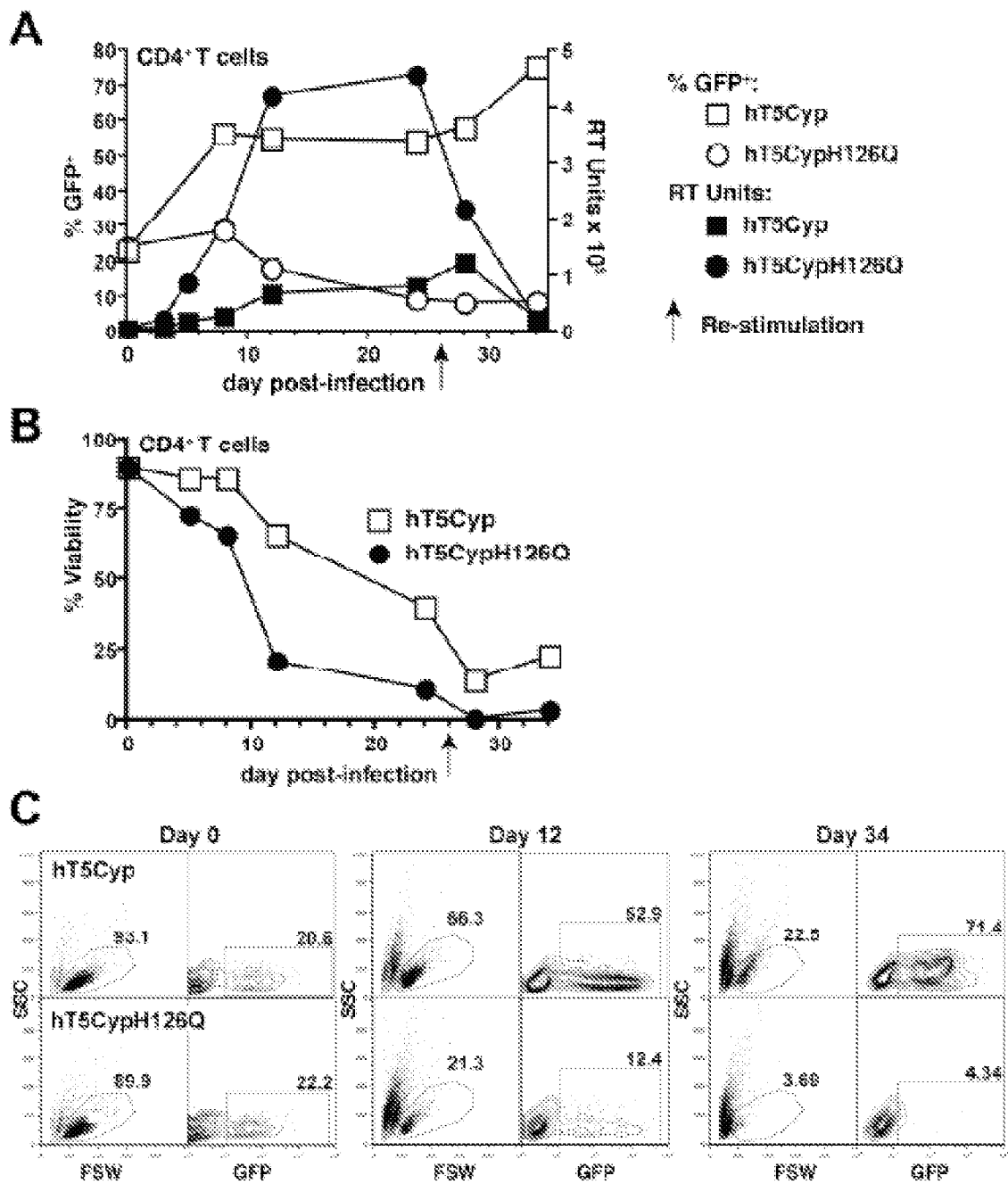


FIG. 57

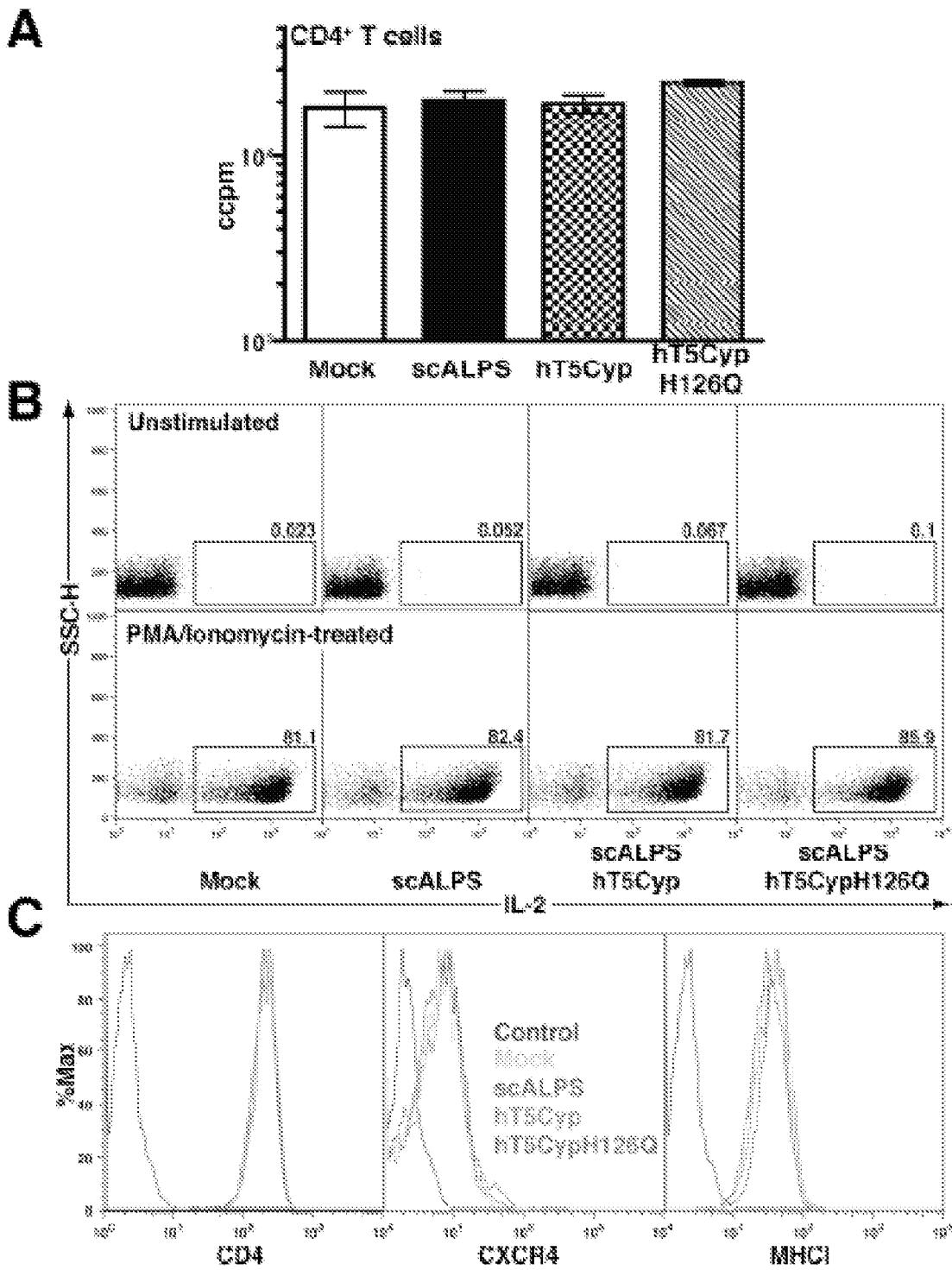


FIG. 58

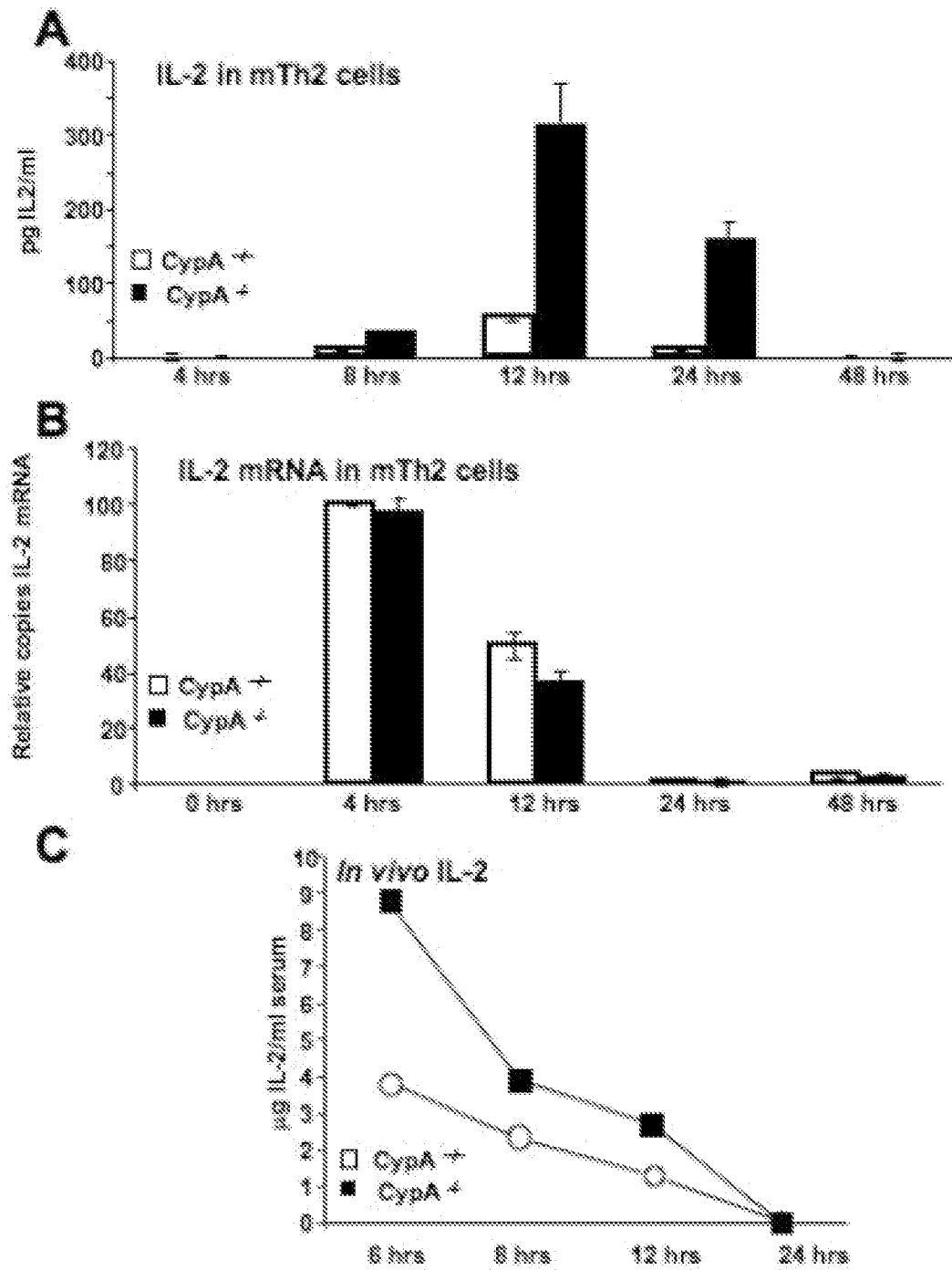


FIG. 59

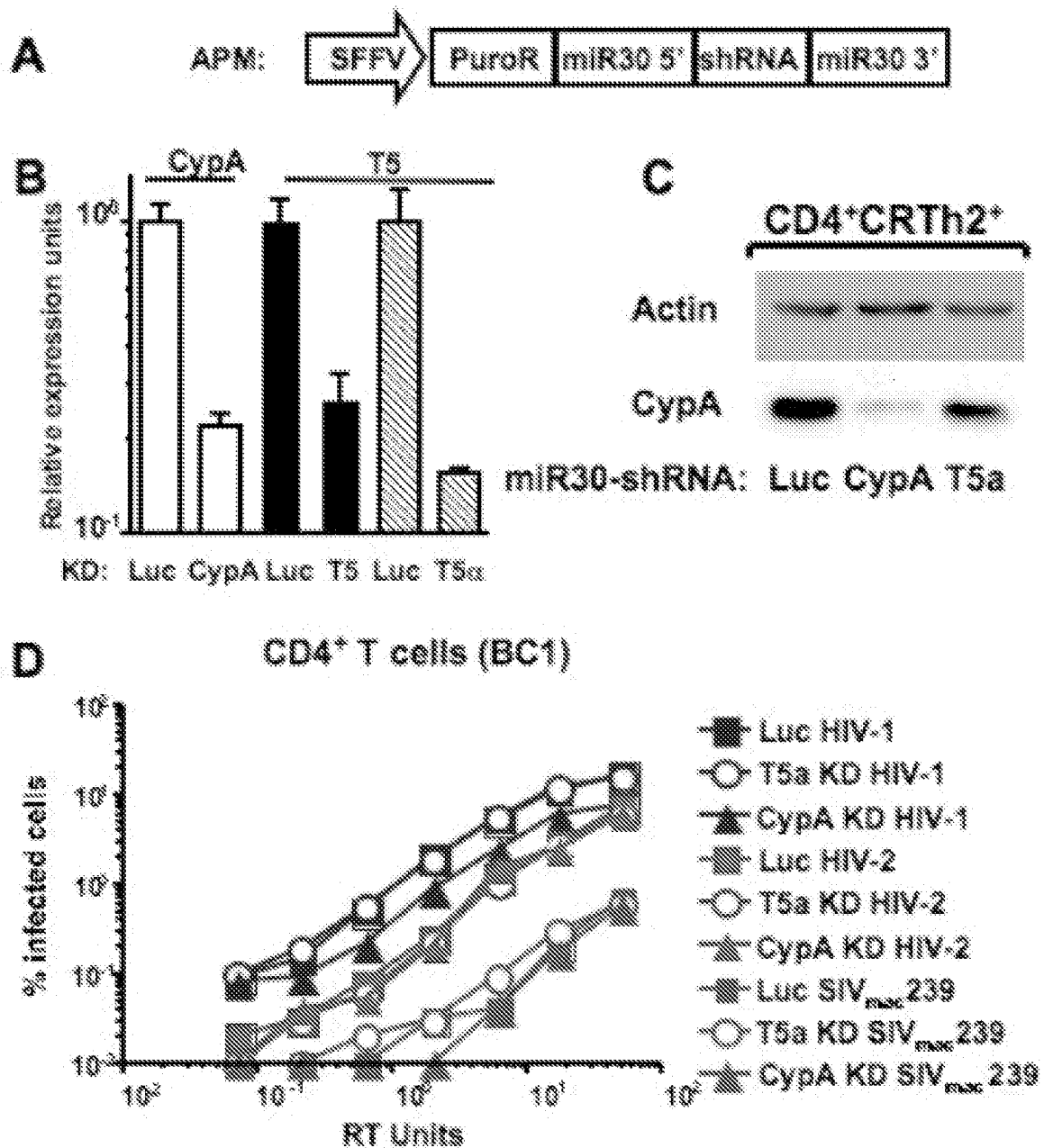


FIG. 60

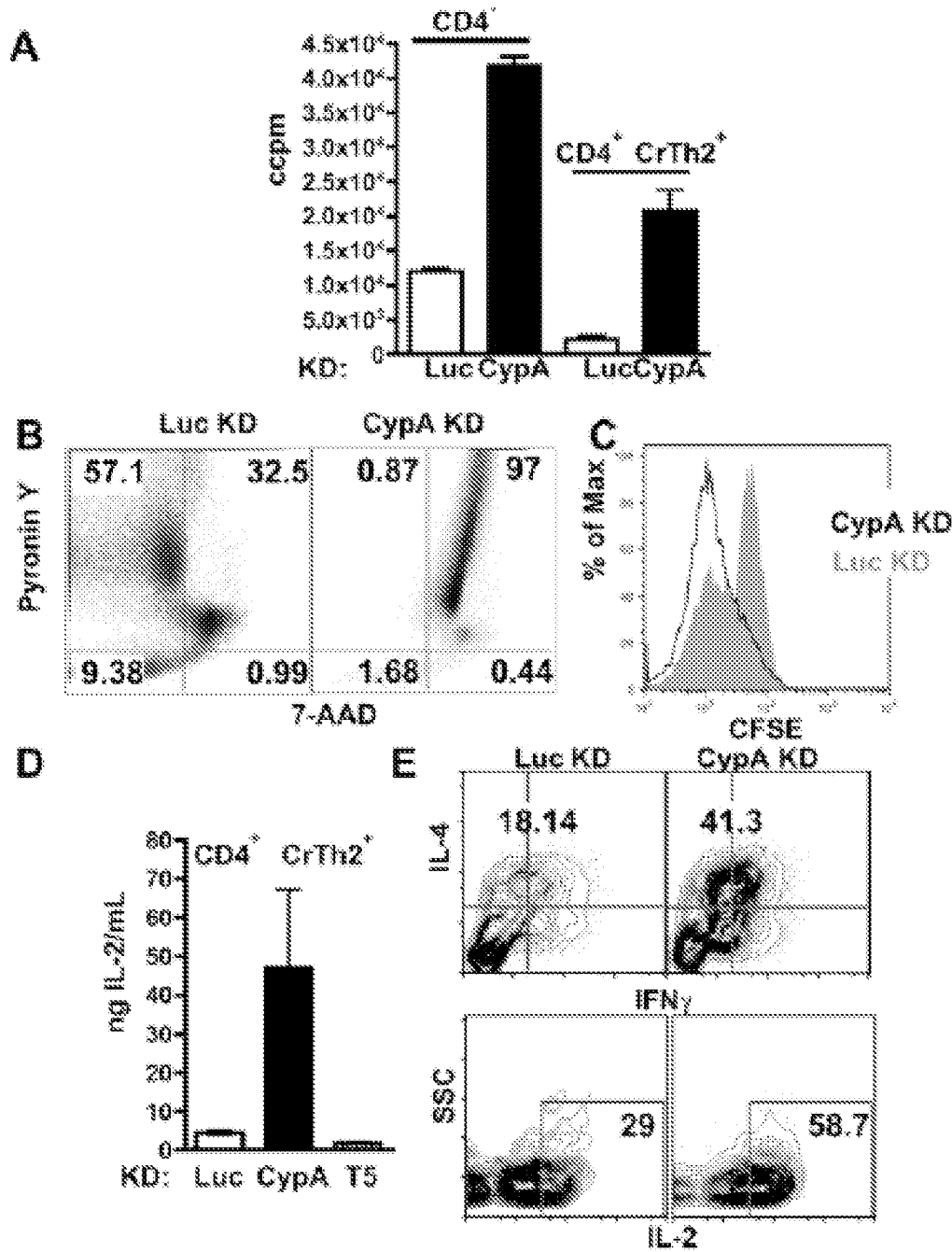


FIG. 61

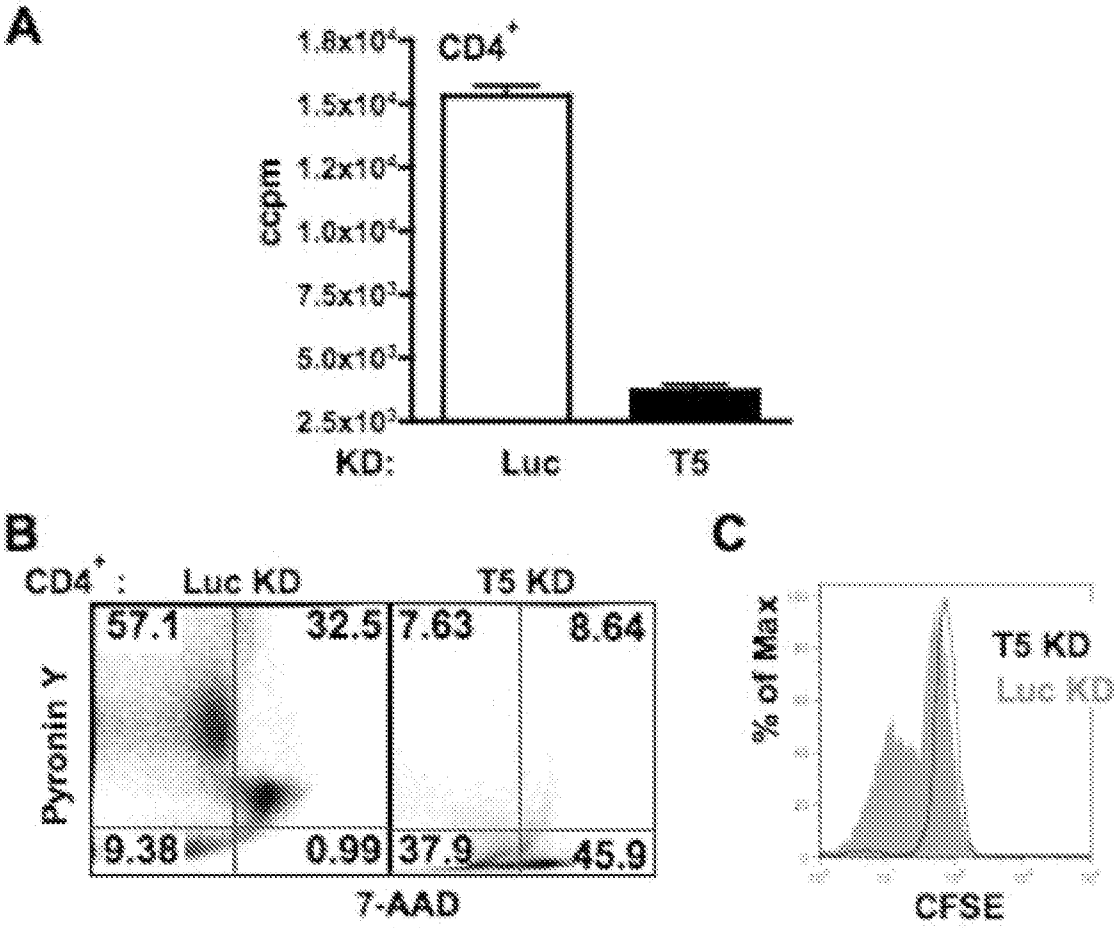


FIG. 62

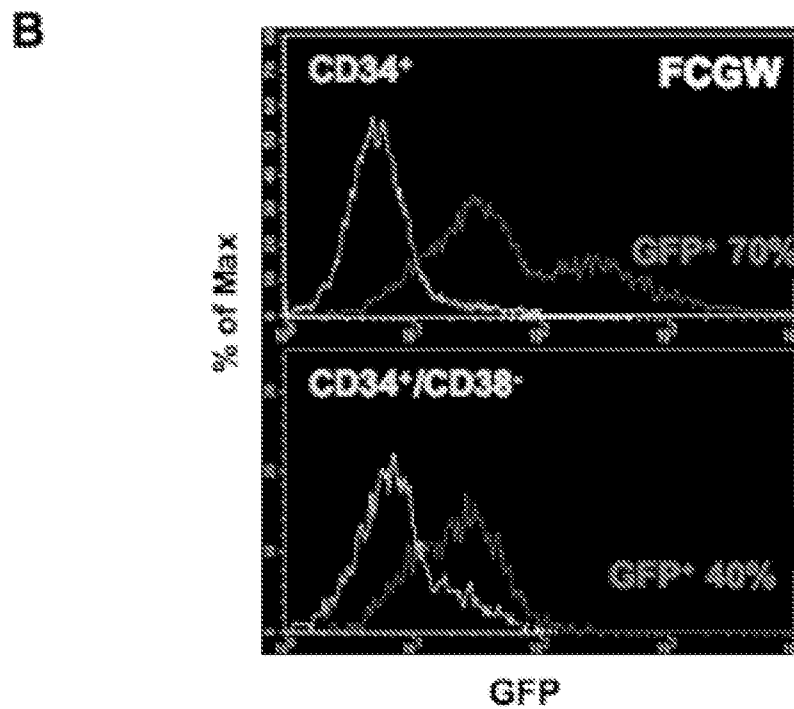
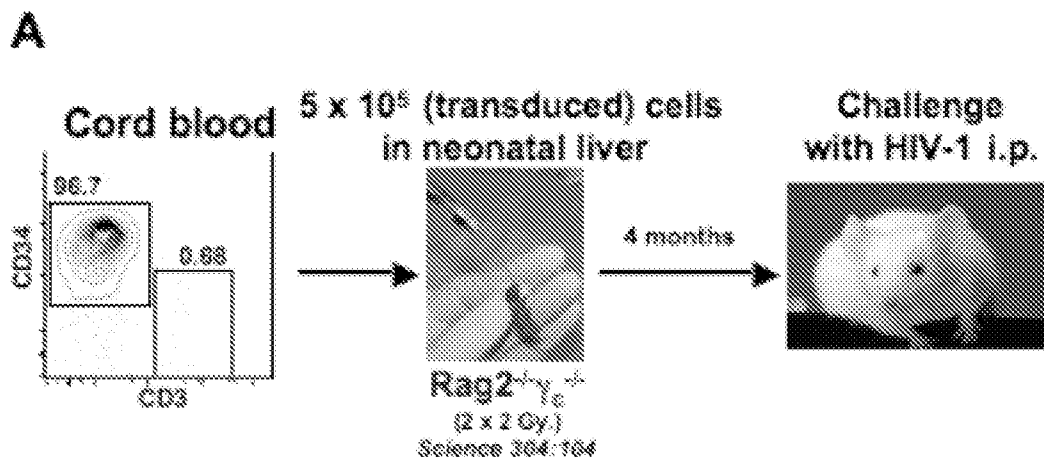


FIG. 63

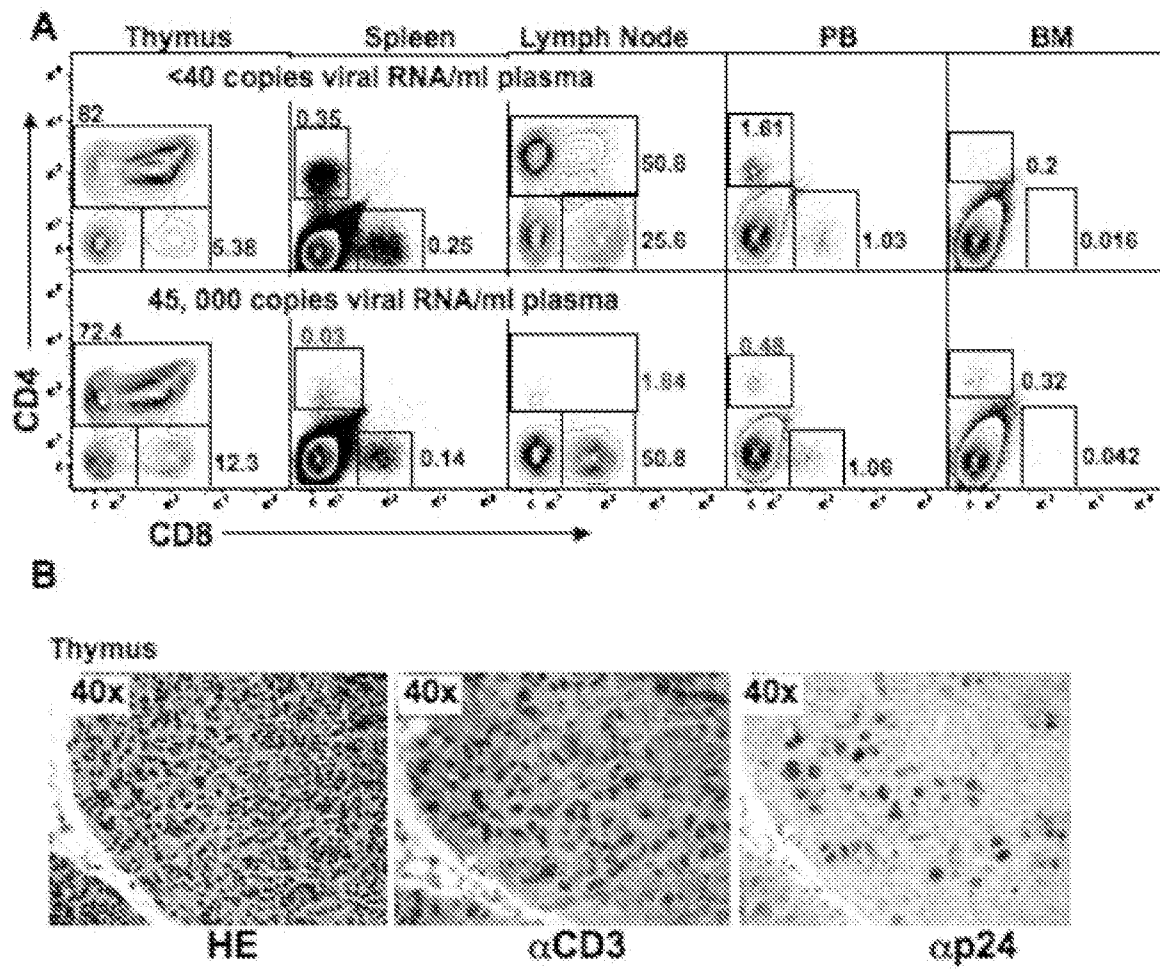


FIG. 64

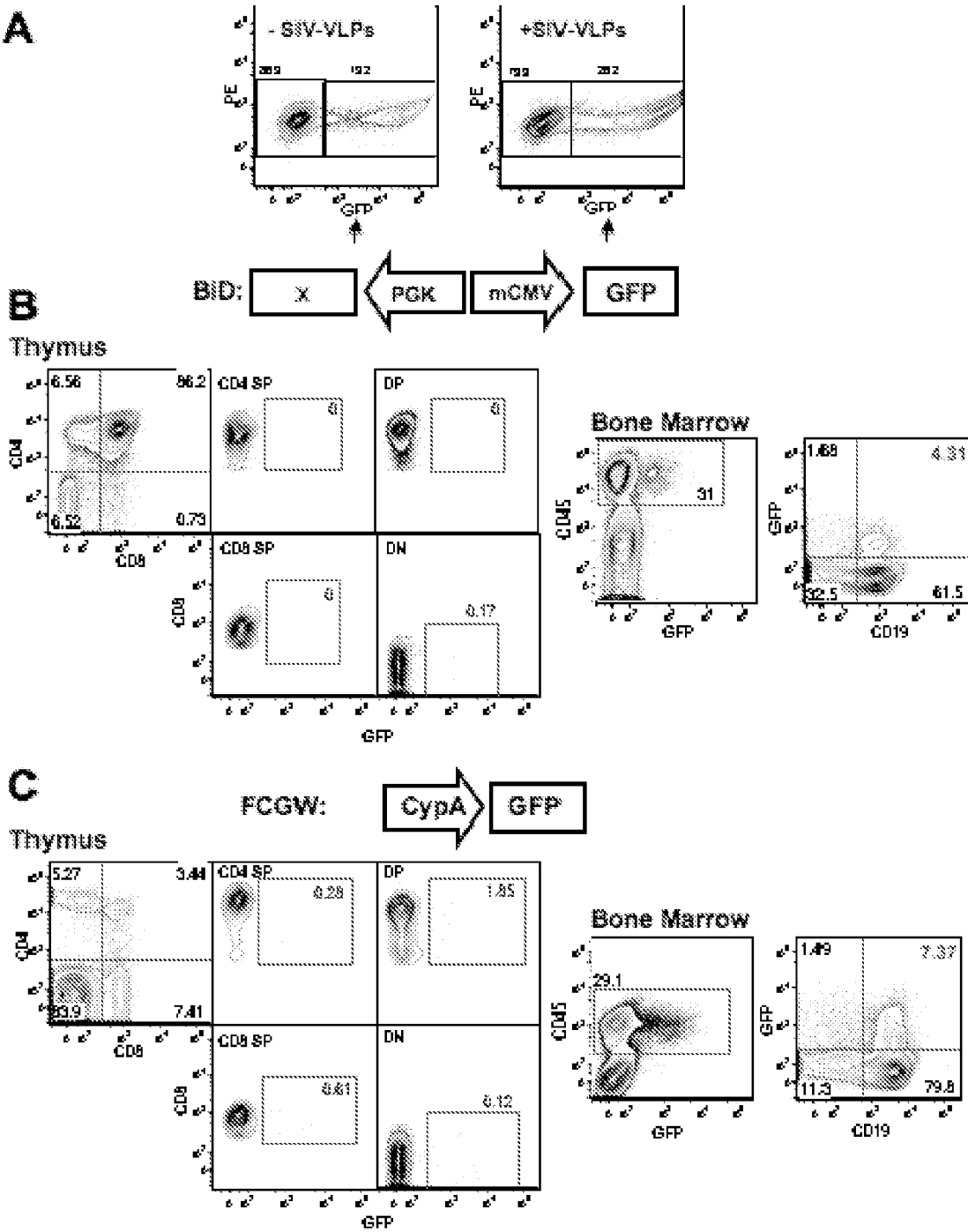


FIG. 65

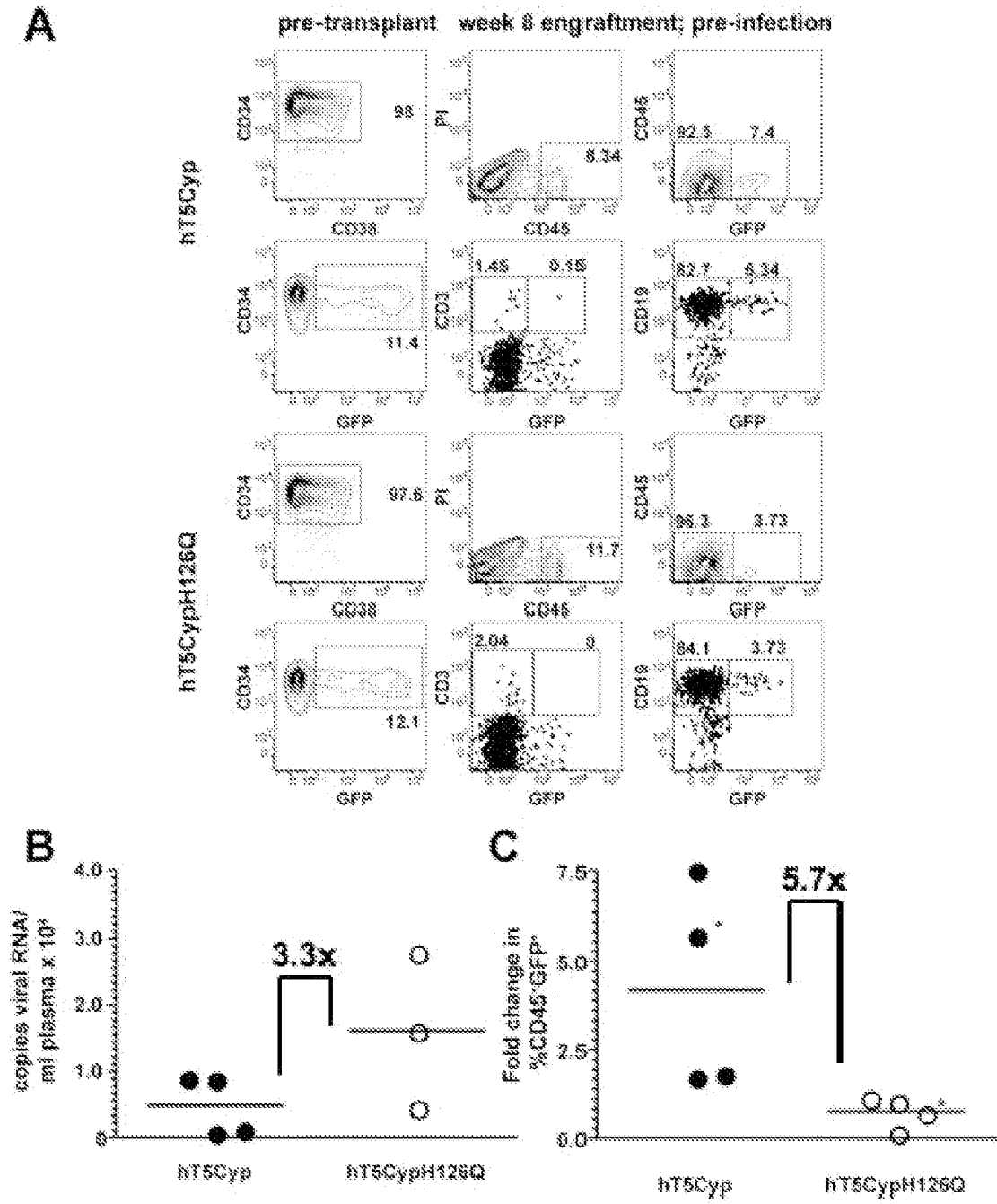


FIG. 66

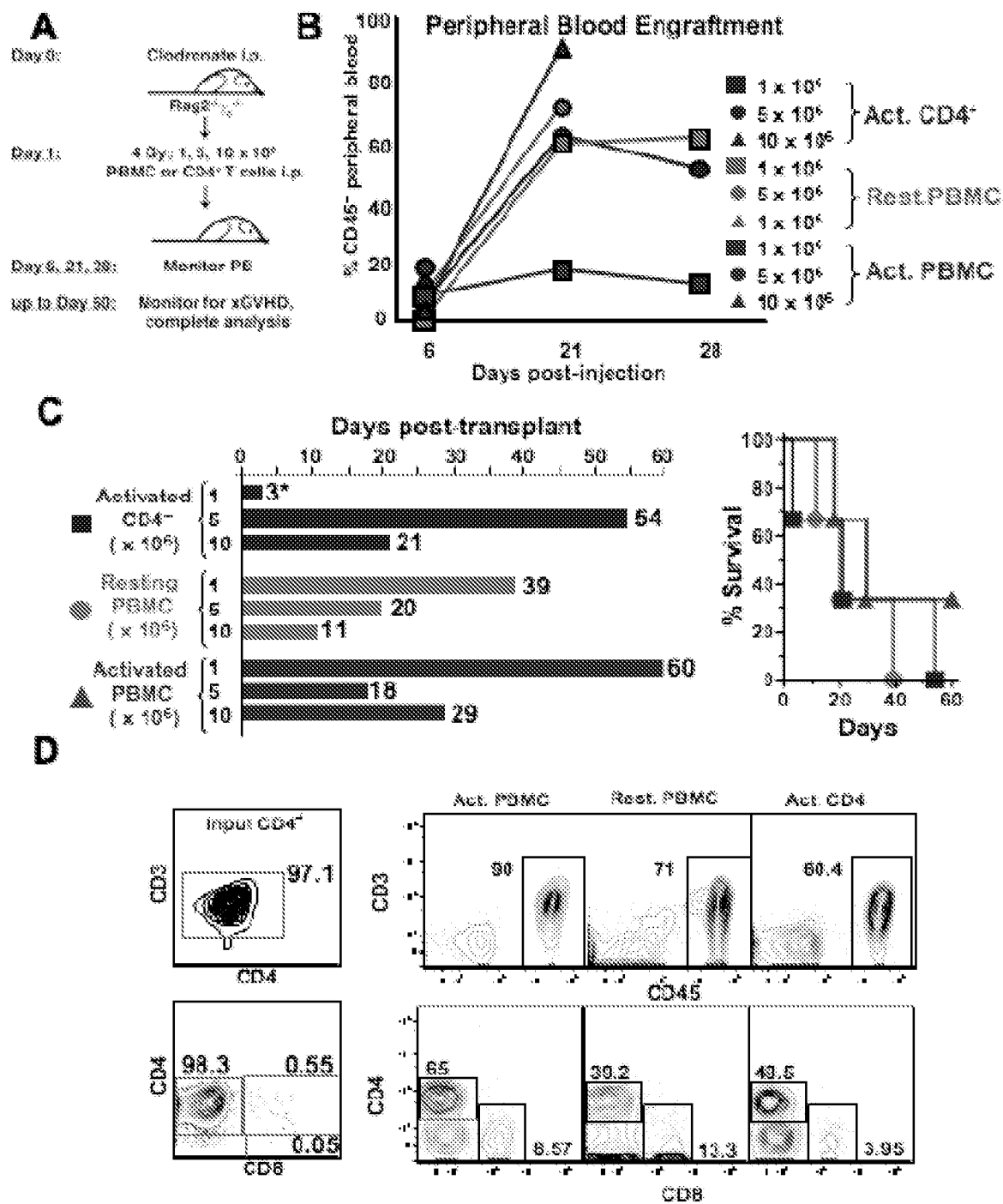


FIG. 67

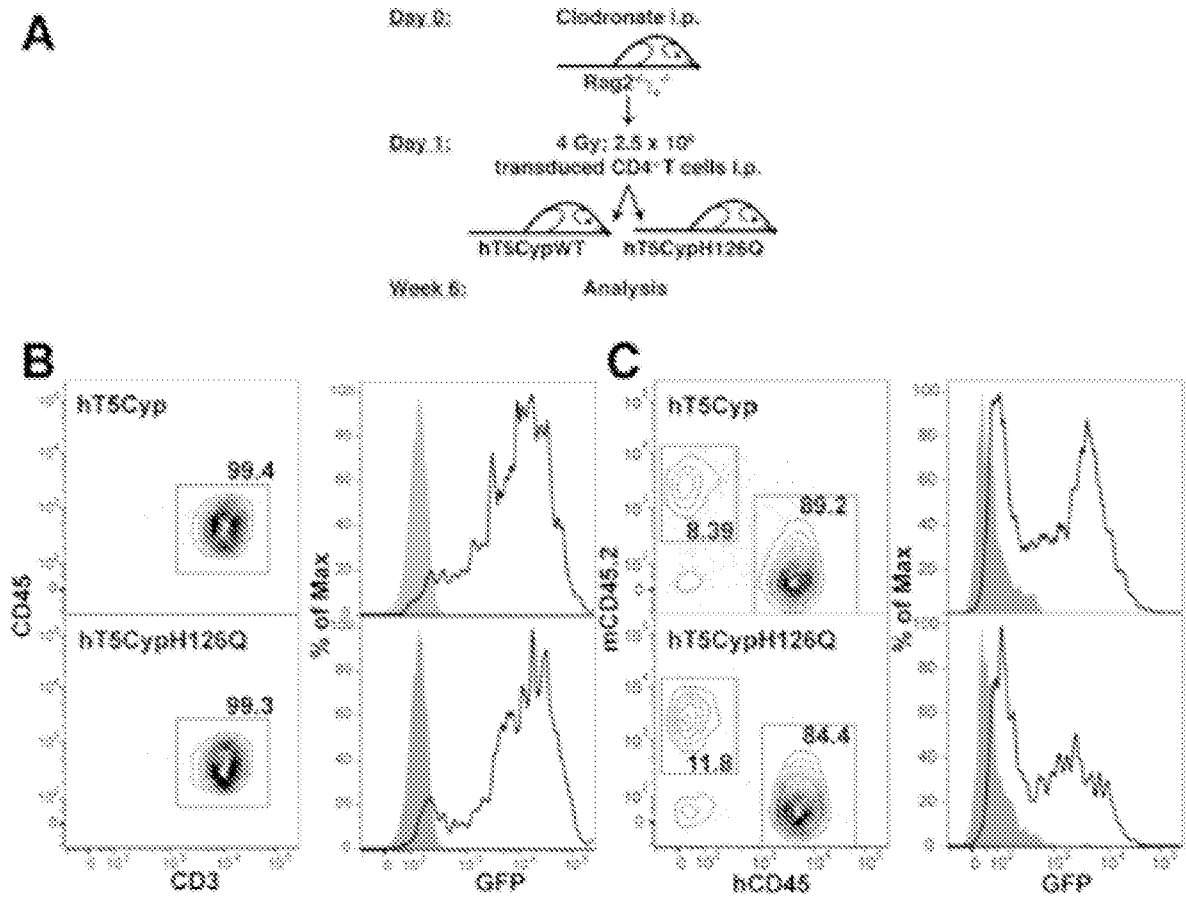


FIG. 68

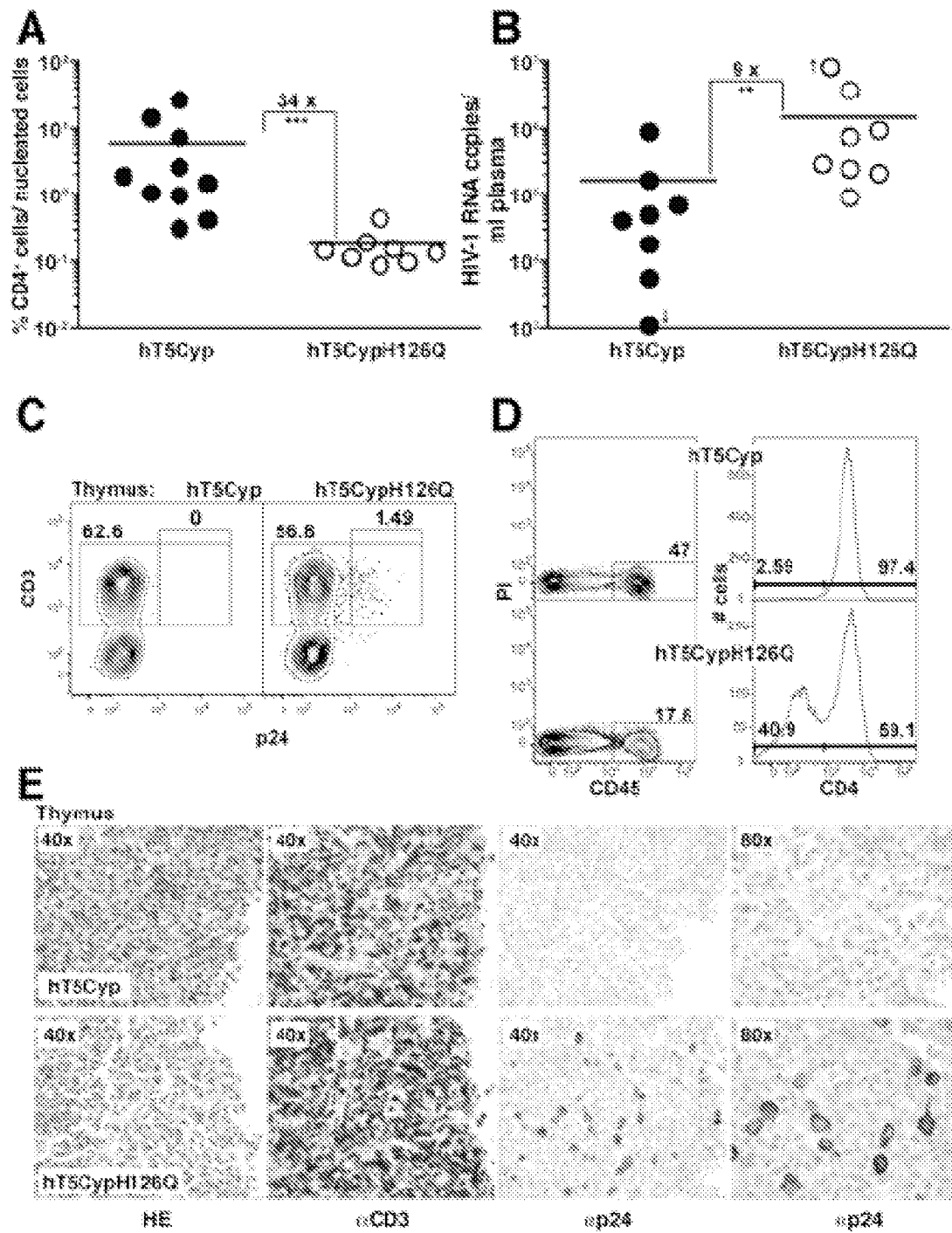


FIG. 69

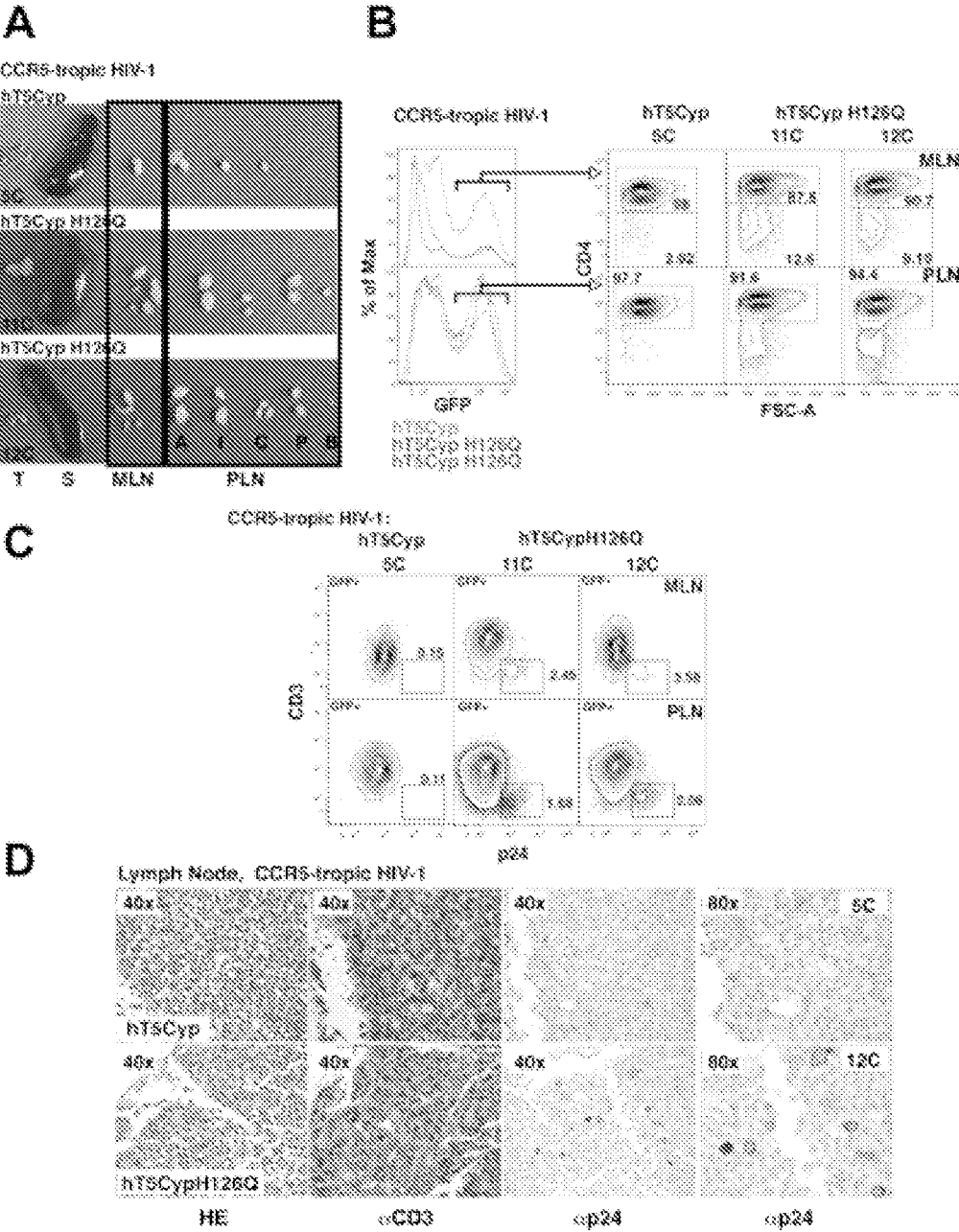


FIG. 70

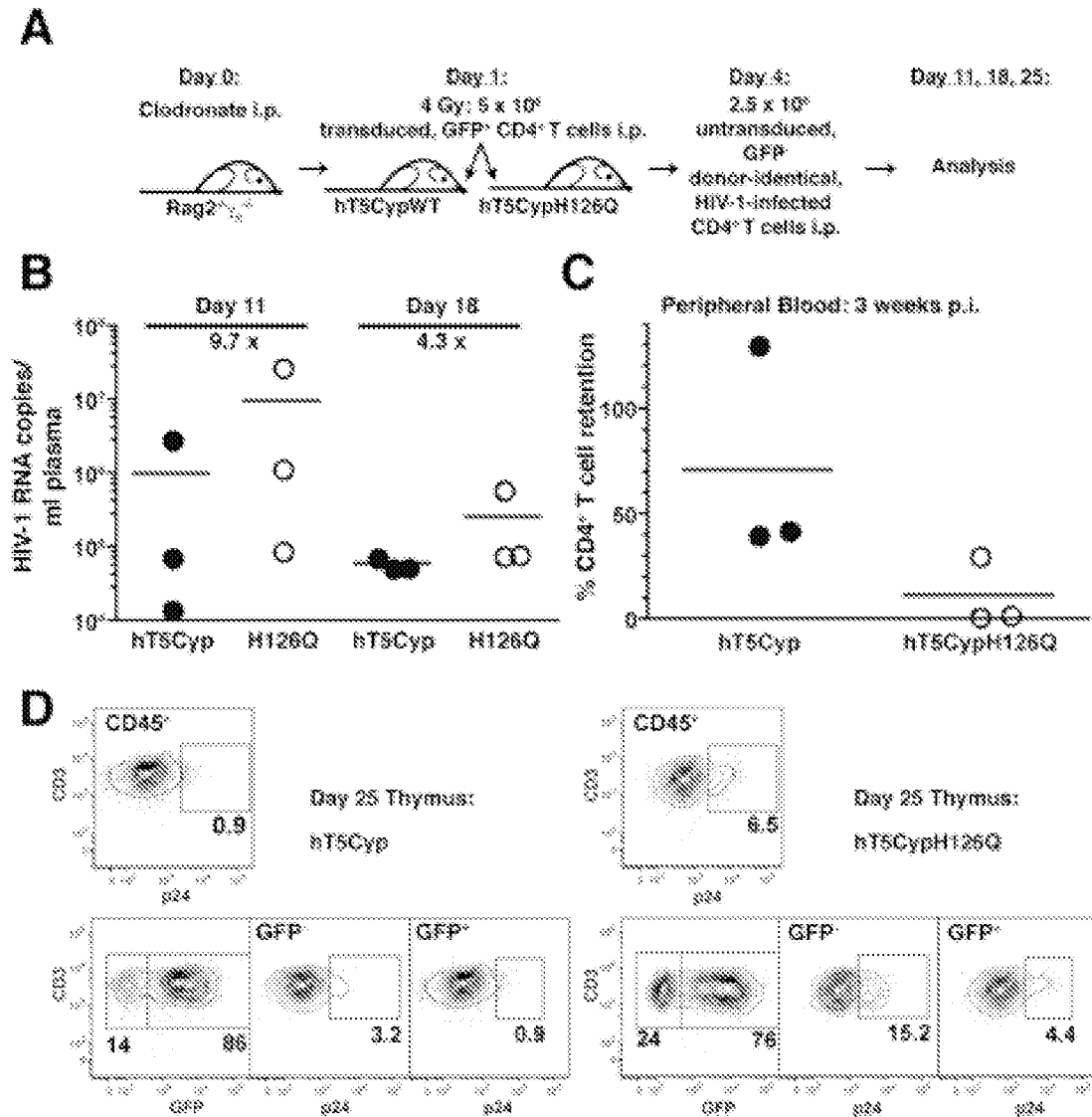


FIG. 71