New World monkeys of the genus *Aotus* synthesize a fusion protein (AoT5Cyp) containing tripartite motif-containing 5 (TRIM5) and cyclophilin A (CypA) that potently blocks HIV-1 infection. We attempted to generate a human HIV-1 inhibitor modeled after AoT5Cyp, by fusing human CypA to human TRIM5 (hT5Cyp). Of 13 constructs, 3 showed substantial HIV-1–inhibitory activity when expressed in human cell lines. This activity required capsid binding by CypA and correlated with CypA linkage to the TRIM5a capsid-specificity determinant and the ability to form cytoplasmic bodies. CXCR4- and CCR5-tropic HIV-1 clones and primary isolates were inhibited from infecting multiple human macrophage and T cell lines and primary cells by hT5Cyp, as were HIV-2<sub>ROD</sub>, SIV<sub>AGM</sub><sub>tan</sub>, FIV<sub>PET</sub>, and a circulating HIV-1 isolate previously reported to be AoT5Cyp resistant. The anti–HIV-1 activity of hT5Cyp was surprisingly more effective than that of the well-characterized rhesus TRIM5α, especially in T cells. hT5Cyp also blocked HIV-1 infection of primary CD4<sup>+</sup> T cells and macrophages and conferred a survival advantage to these cells without disrupting their function. Extensive attempts to elicit HIV-1 resistance to hT5Cyp were unsuccessful. Finally, Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice were engrafted with human CD4<sup>+</sup> T cells that had been transduced by optimized lentiviral vectors bearing hT5Cyp. Upon challenge with HIV-1, these mice showed decreased viremia and productive infection in lymphoid organs and preserved numbers of human CD4<sup>+</sup> T cells. [...]

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

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New World monkeys of the genus Aotus synthesize a fusion protein (AoT5Cyp) containing tripartite motif-containing 5 (TRIM5) and cyclophilin A (CypA) that potently blocks HIV-1 infection. We attempted to generate a human HIV-1 inhibitor modeled after AoT5Cyp, by fusing human CypA to human TRIM5 (hT5Cyp). Of 13 constructs, 3 showed substantial HIV-1-inhibitory activity when expressed in human cell lines. This activity required capsid binding by CypA and correlated with CypA linkage to the TRIM5a capsid-specificity determinant and the ability to form cytoplasmic bodies. CXCR4- and CCR5-tropic HIV-1 clones and primary isolates were inhibited from infecting multiple human macrophage and T cell lines and primary cells by hT5Cyp, as were 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. 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 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 tripartite motif-containing 5 (TRIM5) orthologs 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.

The α isoform of TRIM5 (TRIM5α) contains a C-terminal PRYS RPRY domain that is required for TRIM5α binding to the capsid (CA) of restriction-sensitive retroviruses (10, 11). The specificity of the PRYS RPRY–CA interaction determines which retrovirus a given TRIM5α ortholog inhibits (12). While human TRIM5α (hTRIM5α) weakly blocks HIV-1, it potently blocks N-tropic murine leukemia virus (N-MLV). In contrast, rhesus TRIM5α (rhTRIM5α) inhibits HIV-1, but only weakly inhibits N-MLV. The specificity of retroviral restriction and the modular nature of the TRIM5 components are further demonstrated by the enhanced HIV-1 restriction activity that results when the hTRIM5α PRYS RPRY domain is replaced with that from rhTRIM5α (13–16). Furthermore, anti–HIV-1 activity is observed when other TRIM family members (17) or even the murine antiretroviral factor Fv1 (18) are fused to an HIV-1 CA-binding protein.

The TRIM5 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 T5Cyp fusion (AoS T5Cyp) 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

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 119:3035–3047 (2009). doi:10.1172/JCI39354.
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 hT5Cyp fusion proteins exhibiting HIV-1 restriction activity comparable to AoT5Cyp and possessing all the properties desired of anti–HIV-1 gene therapy.

**Results**

**Design of hT5Cyp fusions with potent anti–HIV-1 activity.** Our goal was to generate a hT5Cyp fusion protein 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 TRIM5γ and TRIM5δ, of similar length and character to the AoT5Cyp 5′ UTR, were included at the TRIM5-CypA junction, but these failed to result in proteins with anti–HIV-1 activity. hCypA was then fused directly to hTRIM5α at 11 different positions along its linear sequence, eliminating subsequent hTRIM5α sequences and resulting in hT5Cyp fusions consisting entirely of components expressed in human cells (Figure 1A). Each hT5Cyp fusion was engineered into a bicistronic lentiviral vector (FUPI; Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI39354DS1). Vector stocks generated for each construct were shown to have equivalent titers and then used to generate stable cell lines.

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, 3 had activity comparable to AoT5Cyp (see representative data in Figure 1B for hT5-S322-Cyp). This fusion was used in all subsequent experiments and will be referred to as “hT5Cyp”. Data for all of the fusion constructs is shown in Supplemental Figure 1. 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 (data not shown). Like AoT5Cyp (9), hT5Cyp blocked viral cDNA accumulation after acute infection, as measured by quantitative PCR (Supplemental Figure 1B).

**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 levels 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 levels, whereas fusions with potent anti–HIV-1 activity were variably expressed at moderate to high levels (hT5-S309-Cyp and hT5-S222-Cyp) (Figure 2A). Again, restriction activity did not correlate with protein levels.

Lack of activity could result from failure to bind HIV-1 CA. Glutathione S-transferase–CA (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 1B).
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 (CsA; Figure 2B). Thus, CA binding was necessary but not sufficient for restriction activity.

To visualize the sites of CypA linkage to hTRIM5α, a 3-dimensional model of the TRIM5α PRYSPRY domain was generated using coordinates from 3 homologs (Figure 2C). hT5Cyp fusions with anti–HIV-1 activity clustered near a putative protein interac-
tion surface (23, 24) (Figure 2C), the main specificity determinant for HIV-1 restriction activity (15, 16, 25). No inactive hT5Cyp proteins mapped to this region (Figure 2C). Indirect immunofluorescence showed that AoT5Cyp and all 3 hT5Cyps with potent anti–HIV-1 activity formed distinct cytoplasmic bodies (Figure 2D). 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.

hT5Cyp restriction activity depends on both the hCypA and hTRIM5 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 (Supplemental Figure 2D), as determined in Jurkat T cells with a stable knockdown of endogenous CypA (26). To confirm that hT5Cyp proteins have the same CA-binding requirements as AoT5Cyp, stable hT5Cyp lines were generated using CRFK, a feline fibroblast that lacks endogenous TRIM5. 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 3A). 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 3B). CsA, a competitive inhibitor of the CypA-CA interaction (20), abrogated HIV-1 restriction by AoT5Cyp and hT5Cyp (Figure 3C). HIV-1–V86P/H87Q/I91V/M96I, a CA variant circulating in humans infected with HIV-1, is reported to be resistant to CsA and to AoT5Cyp-mediated HIV-1 restriction (28). Though HIV-1–V86P/H87Q/I91V/M96I was indeed resistant to CsA, hT5Cyp blocked infection by single-cycle vectors bearing this CA variation (Figure 3D).

To assess the contribution of the TRIM5 domain to T5Cyp-mediated restriction, CRFK cell lines expressing AoT5Cyp or hT5Cyp were treated with arsenic trioxide (As$_2$O$_3$). AoT5Cyp and hT5Cyp restriction activity was blocked to the same extent by As$_2$O$_3$ (Figure 3E).

AoT5Cyp restricts HIV-1$_{NL43}$, HIV-2$_{BROD}$, SIV$_{mac}$, and FIV$_{FIV}$, 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 4 of these lentiviruses (Figure 4, A–D). CypA does not bind to SIV$_{mac}$251...
CA, and neither AoT5Cyp nor hT5Cyp restricted a SIVMAC251 GFP vector (Figure 4E). These results show that, with respect to CA recognition by the CypA domain, and effector function by the TRIM5 domain, AoT5Cyp and hT5Cyp behave similarly.

**hT5Cyp is more potent than rhTRIM5α.** In contrast to hTRIM5α, the TRIM5α ortholog from rhesus monkeys (rhTRIM5α) potently restricts HIV-1 (6). Chimeric proteins in which critical residues from the rhTRIM5α PRYSPRY domain replace corresponding residues in the human ortholog exhibit HIV-1 restriction activity approaching that of rhTRIM5α (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. hTRIM5αR332P (16, 33) and hTRIM5αRh323-332 (14, 15) transgenes, stably expressed in CRFK cells, inhibited single-cycle HIV-1 vectors by about 10-fold, approaching the anti–HIV-1 restriction activity of hTRIM5α (Figure 5A). hT5Cyp, however, restricted HIV-1 almost 10-fold more potently than even rhTRIM5α (Figure 5A and Supplemental Figure 2B). In stable Jurkat T cell lines, the relative potency of hT5Cyp, and AoT5Cyp, was even more impressive (Figure 5B and Supplemental Figure 2A). Similar results were obtained in 293T cells stably expressing the indicated TRIM5 proteins (Supplemental Figure 2C).

**hT5Cyp eliminates the spread of HIV-1 in tissue culture.** Jurkat T cells stably transduced with TRIM5 expression vectors were infected with replication-competent HIV-1. hTRIM5αR332P and hTRIM5αRh323-332 had minimal effect on the kinetics of HIV-1 infection (Figure 5C). rhTRIM5α delayed the HIV-1 replication peak by 8 days (Figure 5C). In contrast, HIV-1 replication was not detected in cells transduced with hT5Cyp or AoT5Cyp for the 2-month observation period (Figure 5, C and D). HIV-1 replication in cells transduced with hT5Cyp bearing a mutation that blocks binding to HIV-1 CA (CypA-H126Q; ref. 27) was as rapid as in cells transduced with empty vector (Figure 5, C 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 coculture of hT5Cyp-expressing and untransduced cells, and by direct transfection of infectious HIV-1 proviral DNA into hT5Cyp-expressing Jurkat cells. No evidence of the spread of HIV-1 was detected in the presence of hT5Cyp.

**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 trans-
duced with hT5Cyp, attempts were made to use a bicistronic lentiviral vector that directs synthesis of GFP from an internal ribosome entry site (IRES; Figure 6A). These vectors failed to generate GFP in primary CD4+ T cells (Figure 6B). Better results were obtained when a vector was engineered that bore 2 promoters, one from the spleen focus forming virus (SFFV) LTR and the other from the hCyPA gene (Figure 6, A and B). Cosynthesis of 2 reporter genes, GFP and dsRED, within primary CD4+ T cells transduced with scALPS, was confirmed by flow cytometry (Supplemental Figure 3).

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 flow cytometry (Supplemental Figure 3). The percentage of infected cells was determined by flow cytometry. The percentage of infected cells was determined by flow cytometry.

Figure 5

The anti–HIV-1 activity of hT5Cyp is superior to that of rhTRIM5α. (A and B) CRFK (A) and Jurkat T cells (B) were transduced with lentiviral vectors encoding the indicated TRIM5 proteins and a puromycin resistance cassette. Pools of puromycin-resistant cells were infected with increasing amounts of a single-cycle HIV-1–GFP vector. The percentage of GFP+ cells was determined 48 hours 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.

hT5Cyp protects against HIV-1 infection in vivo. To test the ability of hT5Cyp to protect against HIV-1 infection in vivo, 2 approaches analogous to those used in human gene therapy 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 greater than 93% purity of GFP+ input cells (Figure 7A). Age-matched 6- to 10-week-old Rag2-/-γc-/- mice were conditioned with clodronate and sublethal irradiation (36) and injected intraperitoneally with 2.5 × 106 transduced CD4+ T cells (Figure 7B). Two and 6 weeks after transplant, peripheral blood engraftment was assessed by measuring the percentages of human CD45+CD4+GFP+ cells. We found that 50% to 90% were GFP+ at both time points (2-week mean ± SEM: hT5Cyp, 81.9% ± 4.5%, n = 8; hT5CypH126Q, 73.4% ± 4.4%, n = 8; 6-week mean ± SEM: 75.1% ± 3.4%, n = 8).
hT5Cyp 76.1% ± 6.0%, n = 8; hT5CypH126Q, 76.8% ± 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 2 constructs used (Figure 7C). However, we consistently observed that GFP expression was higher in peripheral blood, bone marrow, and spleen compared with somewhat lower levels of GFP expression in thymus and mesenteric lymph nodes (Supplemental Figure 6). The reasons for this are unclear. One can speculate that (a) the minor fraction of T cells that were GFP low or GFP negative, the latter not transduced at transfer (<7%, Figure 7A), had different biologic properties (e.g., better homing capacity to lymphoid organs as thymus and lymph nodes) compared with GFP+ cells; (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.

Figure 6
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) Forty-eight hours after transduction with AIG and scALPS vectors, Jurkat T cells and primary hCD4+ T cells 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-1NL4-3 (C) or primary isolate HIV-1DH12 (D), and RT activity in the supernatant was measured. Triplicate spreading infection for 1 representative donor of 4 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-1NL4-3 bearing a CCR5-tropic Env (E) or primary isolate HIV-1132w (F). Infection was monitored as described in C and D. Error bars in C and E represent SEM. 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 HIV-1NL4-3 (15 ng p24/10^6 cells), and the percentage of GFP+ cells was monitored (left y axis, open symbols). Supernatant RT activity was measured (right y axis, filled symbols). Cultures were restimulated using allogeneic PBMCs, IL-2, and PHA 26 days following infection (arrow).
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, 2 weeks after HIV-1 infection the CD4+ cells constituted 6% (± 2.87%) of mean nucleated peripheral blood cells (Figure 8A). This was 34-fold higher than the percentage of CD4+ cells observed in mice transplanted with hT5CypH126Q-transduced CD4+ T cells (mean, 0.18% ± 0.04%), a difference that was statistically significant (P = 0.0002, n = 8 hT5Cyp, n = 8 hT5CypH126Q) (Figure 8A). A 9-fold reduction in mean plasma viral load was observed when mice engrafted with hT5Cyp-expressing CD4+ cells were compared with mice engrafted with hT5CypH126Q-expressing CD4+ T cells (P = 0.005; n = 8 hT5Cyp, n = 8 hT5CypH126Q) (Figure 8B). These results indicate that hT5Cyp inhibits HIV-1 replication and protects against HIV-1–associated destruction of CD4+ T cells in vivo.

Further evidence for the in vivo effects of hT5Cyp was sought by examining lymphoid organs of mice infected with HIV-1. Three weeks after 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-bearing cells were observed in tissues from mice infected with hT5Cyp (Figure 8E). Similar results were obtained for tissue sections from mesenteric lymph node from mice infected with CXCR4-tropic or CCR5-tropic HIV-1 (Figure 8D).

The percentage of GFP+ cells among the input CD4+ T cells transduced with hT5Cyp and hT5CypH126Q was similar (Figure 8E). 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 8E). Similar results were obtained for tissue sections from mesenteric lymph nodes (Supplemental Figure 7). These experiments show that hT5Cyp significantly reduced or prevented productive HIV-1 infection in vivo.

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 preconditioned neonatal Rag2γc–/– mice (38), leading to B and T cell reconstitution in peripheral blood 8 weeks after transplant. The percentage of GFP+ cells among the CD45– lymphocytes and CD19+ B cells that developed in the mice was similar to the percentage of GFP+ cells among the input CD34+ cells, but development of GFP+ CD4+ T cells was rarely observed (Supplemental Figure 8A). Nonetheless, mice with hCD45+ cells in peripheral blood were challenged with HIV-1. At 25 days following infection, mean viremia in mice reconstituted with CD34+ cells bearing hT5Cyp was less than 30% of the mean viremia of the hT5CypH126Q control mice (Supplemental Figure 8B;
The Journal of Clinical Investigation

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 (Supplemental Figure 8C).

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. 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 TRIM5 orthologs, in each case the selective pressure is likely to have been a retrovirus (15).

Because TRIM5α and T5Cyp are modular proteins, it was expected that the engineering of a restrictive hT5Cyp would not be difficult. Surprisingly, only 3 of 13 hT5Cyp fusions inhibited HIV-1 with results comparable to AoT5Cyp. No correlation was observed between protein levels and antiviral activity, and CA-binding activity was not sufficient to restrict HIV-1 (Figure 2, A and B). Anti-HIV-1 activity was observed when hCypA was fused at the apex of the hT5Cyp PRYSPRY domain in the structural model (Figure 2C). Analysis of non synonymous 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 TRIM5. 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).

Optimal gene therapy candidates provide high efficacy and low antigenicity. Hematopoietic progenitors transduced with rhesus macaque TRIM5α or human-rhesus TRIM5α chimera 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 hTRIM5α PRYSPRY domain (hTRIM5αR332P, hTRIM5αA332S) one can reduce the risk of antigenicity, but this also decreases anti-HIV-1 efficacy (Figure 5). hT5Cyp is the most potent HIV-1 inhibitor of the TRIM5 proteins we compared, including rhTRIM5α, in both single-cycle and spreading infections of human T cells with HIV-1 (Figure 5). This difference in activity may 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 orthologs. Despite having what we believe is a novel fusion junction, hT5Cyp is likely to have the lowest antigenic potential among these TRIM5-based gene therapies.

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 CCR5Δ32 allele is a risk factor for fatal West Nile Virus infection (54).

hT5Cyp is a broadly acting, antiviral agent that blocks CCR5- and CXCR4-tropic HIV-1 clones and primary isolates as well as some HIV-2, SIV, and FIV clones (Figures 4 and 6). TRIM5 isoforms form hetero-multimers, and hT5Cyp might interfere with endogenous hTRIM5α function (55). However, no significant decrease in hTRIM5α-mediated restriction of N-MLV, and no alteration in cell surface markers or cytokine secretion, was observed in hT5Cyp-transduced cells (Supplemental Figure 4). 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 RT, 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 3D). 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.

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 is a straightforward 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 8). 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 more than 106 cells ex vivo. Given that hT5Cyp-transduced CD4+ cells can be expanded in vitro and in vivo (Figures 6 and 7), and that these cells exhibit a selective advantage during HIV-1 infection (Figure 6G and Figure 8A), there might be an impressive effect of hT5Cyp transduction on autologous CD4+ T cell gene therapy in the clinical setting.

CD4+ 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 (Supplemental Figure 8). 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).

**Methods**

**Plasmids and cloning.** Most HIV-1 plasmids and vectors have been described previously (64). pNL4-3R5 contains a complete infectious HIV-1 provirus. pNL4-3-R5, a gift from F. Kirchhoff (University of Ulm, Ulm, Germany), bears the V3 loop of the CCR5-tropic 92TH014-2 HIV-1 strain (65). To generate pNL4-3-GFP-INES-Nef, EFGP and the encephalomyocarditis virus (ECMV) IRES were inserted upstream of nef. pNLΔenvGFP is pNL4-3 with an env-inactivating mutation and EFGP in place of nef. CSGW is an HIV-1 vector expressing EFGP under the control of the SFFV promoter. pFUPI, engineered from pFUW (66), uses the ubiquitin promoter to drive expression of a puromycin resistance cassette, followed by the ECMV IRES (Supplemental Figure 1A).

hT5Cyp variants were cloned by overlapping PCR using the oligonucleotides shown in Supplemental Table 1. hTRIM5α, hTRIM5αR332P (16, 33) and hTRIM5αA332S (14, 15) were gifts from J. Sodroski and F. Diaz-Griffero (Harvard University, Boston, Massachusetts, USA). All TRIM5 constructs were cloned downstream of the IRES in FUPI. All PCR products were sequenced to eliminate unwanted mutations.
pAIG uses the SFFV promoter to drive expression of TRIM5, followed by IRES EGFP. In pscALPS, the SFFV promoter drives expression of TRIM5 and the human CypA promoter drives expression of EGFP. For 3-component vector production, HIV-1 gag and pol were expressed using psPAX2, p8.9NAsB, p8.9NAsB-G89V or p8.9NAsB-V86P/H87Q/I91V/M96I. The V86P/H87Q/I91V/M96I mutation in CA (28) was provided by P. Gallay (The Scripps Research Institute, La Jolla, California, USA). SIVmac251 vectors were produced using GAE1.0 and the packaging vector SIV3 (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-Aldrich), and HIV-1 CA was expressed as a GST fusion using the EF1α promoter.

Cells. Human embryonic kidney cells (293T) and CRFK feline kidney cells were maintained in DMEM supplemented with 10% FBS and glutamax (Invitrogen). Jurkat E6-1, H9, CEM-SS, and SUP-T1 T cell leukemia/lymphoma cell lines and U937 monocytic lymphoma, and THP-1 monocytic leukemia cells were grown in RPMI-1640 with 10% FBS and glutamax (Invitrogen). For creation of TRIM5-expressing cell lines, suspension cells were transduced with 2 × 10⁶ Jurkat infectious units/ml and adherent cells were transduced with 2 × 10⁵ Jurkat infectious units/ml of FUPI containing TRIM5. Jurkat infectious units were determined by serial dilutions of concomitantly produced FUPI-GFP on Jurkat E6 cells. At 48 hours after transduction, cells were selected with puromycin. The different constructs were normalized to each other by comparing RT activity of the supernatant. CD14⁺ monocytes were enriched from PBMCs (>99% CD14⁺) using positive selection magnetic beads (Miltenyi Biotec) and stimulated for 24 hours on multisorb 96-well plates (Nunc) coated with 2 μg/ml anti-CD28 antibodies (BD Biosciences) in RPMI with 10% FBS, glutamax, and 2 μg/ml anti-CD28 antibodies (BD Biosciences) in RPMI with 10% FBS, glutamax (Invitrogen), and 20 μM anti-CD28 antibodies (GE Healthcare). The beads were washed 3 times with 1 ml Triton Lysis Buffer, and 20 μl immunoprecipitate was used to detect CD4⁺ T cells in the supernatant (7). CD8⁺ T cells were stained with an allophycocyanin-labeled antibody. The supernatants containing viruses and vectors were cleared by centrifugation at 400 × g, filtered (0.45 μm; Pall Acrodisc), and tested for exogenous RT activity (7), titered on Jurkat E6 cells in single-cycle assay (7), and stored at –80°C. When indicated in the figure legends, the content of viral p24 antigen was quantified by an HIV-1 p24 ELISA kit (NIH AIDS Research and Reference Reagent Program).

Infections. Adherent cells (2 × 10⁵/ml) or suspension cells (8 × 10⁶/ml) were plated in 48-well plates (Nunc) for challenge with single-cycle GFP reporter vectors that were tittered as indicated. GFP synthesis was assessed by flow cytometry 48 hours after infection. For spreading infection, 10⁴ Jurkat cells were infected with 1–15 ng p24/10⁶ cells of HIV-1NL4-3, HIV-1NL4-3-ΔGag, or HIV-1NL4-3ΔGagR5 stocks, or with low-passage primary isolates HIV-1HIV122 and HIV-1LAI (gift from Olivier Schwartz, Pasteur Institute, Paris, France). Infection was monitored by assessing the accumulation of RT activity in the supernatant (7). FACS Calibur, CellQuest Pro (BD), and FlowJo software (Treestar Inc.) were used to record and analyze fluorescence. We acquired between 10⁵ and 10⁶ events for analysis.

Western blotting and immunoprecipitation. To detect hT5Cyp, 5 × 10⁶ 293T cells were transfected with 20 μg of each triple-FLAG-H1T5Cyp plasmid using Lipofectamine 2000 (Invitrogen). Cells were lysed at 48 hours 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 g for 5 minutes, incubated for 4 hours at 4°C with 2 μg/ml anti-M2 monoclonal antibody (Sigma-Aldrich), and then incubated 1 hour at 4°C with 20 μl of protein G-Sepharose beads (GE Healthcare). The beads were washed 3 times with 1 ml Triton Lysis Buffer, and 20 μl immunoprecipitate was used for SDS-PAGE on a 12% gel, transferred to a PVDF membrane, and immunoblotted with rabbit polyclonal antibody to CypA (Biomol).

For communoprecipitation of hT5Cyp with HIV-1 CA, 5 × 10⁶ 293T cells were cotransfected as described above with 20 μg p3xFLAG-H1T5Cyp and 20 μg pEFi-GST-CA. At 48 hours, cells were lysed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGEPA CAL-630, 0.1% SDS, 0.5% sodium deoxycholate, with Complete Mini protease inhibitor cocktail [Roche]) and accelerated at 15,000 g for 5 minutes. Lysate was divided into 2 equal parts and incubated on ice for 30 minutes with 20 μg Ca/pDMSO or an equivalent volume of DMSO for 30 minutes. Lysates were incubated with 30 μl glutathione-Sepharose beads (GE Healthcare) for 1 hour at 4°C. Beads were washed 3 times with RIPA buffer. Proteins were subjected to SDS-PAGE and immunoblotted with rabbit polyclonal anti-CypA antibody (Biomol) and goat polyclonal anti-HIV-1 CA (NCl).

Structural model of the hTRIM5α 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, 11–184 of chain A), GUSTAVUS (PDB entry 2FNJ; residue range, 35–83 and 88–233 of chain A), and TRIM21 (PDB entry 2IWW; residue range, 2–182 of chain B), using an α carbon cutoff distance of 3.5 Å. ClustalX was used to combine the hTRIM5α sequence with the structural alignments. A ClustalW sequence-based multiple alignment of 10 characteristic psiBLAST hits of all 3 sequences was used to localize insertions. A secondary structure prediction of hTRIM5α obtained with Network Protein Sequence Analysis (NPS@; http://npsa-phil.ibcp.fr) 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 2IWW 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.
Immunofluorescence microscopy. CRFK cells stably expressing wild-type AoTSCyp or hTSCyp fusion proteins were grown on glass coverslips. Cells were fixed at 25°C for 10 min with 3.7% paraformaldehyde in PBS and permeabilized on ice for 2 min with 2% Triton X-100 in 0.1% sodium citrate. After quenching with 0.1 M glycine in PBS at 25°C for 10 minutes, cells were blocked for 30 minutes at 25°C with 10% FBS and 0.1% Tween-20 in PBS before overnight incubation with goat polyclonal anti-TRIM5 (AbCam) and mouse monoclonal anti-tubulin (Sigma-Aldrich) 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 hour at 25°C in the dark. Finally, coverslips were mounted in Vectashield with DAPI (Vector Laboratories). Cells were visualized with a 63× NA 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 2-dimensional stacks were subjected to noise reduction using the Leica TCS SP5 software and projection using the MetaMorph software (Universal Imaging Corp.).

Mice. Rag2–/– 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 and the Swiss Federal Veterinary Office (Bern, Switzerland). Mouse experiments were reviewed and approved by the Veterinary Commission of the State of Ticino (Bellinzona, Switzerland). CL2MBP (clopidronate) was a gift from Roche Diagnostics. Preparation of liposomes containing CL2MBP was done as described previously (68). Mice 6–10 weeks old received 100 μl of clopidronate liposomes i.p. 24 hours prior to irradiation using a single sublethal dose of 4.0 Gy from a Cesium 137 source (Biobeam 8000;STS GmbH) at 3.75 Gy/min. Four hours after irradiation, mice were transplanted with 2.5×10⁶ CD4+ T cells expressing hTSCyp or control hTSCyp-H162Q in 100 μl PBS i.p. Mice were infected with HIV-1Lai (as previously described (58), 5 or 14 days after transplantation and analyzed 2, 3, or 4 weeks following 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 mice were sacrificed, single-cell suspensions from organs were prepared for FACSc analysis and/or organs were assayed by immunohistochemistry. For FACSc analysis, monoclonal antibodies against the following antigens were used: CD3 (UCHT1), CD4 (13B8.2), CD8 (B9.11), and p24 (clone KC57) (all Beckman Coulter) and CD45 (H130) (CalTag). 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 Biosciences — Pharmingen) and stained with anti-p24 antibody in BD PermWash solution (BD Biosciences — Pharmingen) following the manufacturer’s instructions. Plasma HIV RNA concentrations were determined by Cobas Amplicor RT-PCR assay (Roche Diagnostics). Immunohistochemical stainings were performed as previously described (58). To assess nonspecific binding, tissue from untransplanted and uninfected transplanted mice was stained as a control.

Statistics. Significant differences between groups were calculated using the Mann-Whitney U test. P values less than 0.05 were considered significant.

Acknowledgments

We thank David Jarrossay for cell sorting. This work was supported by NIH grants RO1AI36199 and RO1AI59159 to J. Luban, Swiss National Science Foundation grants 3100A0-113558 and 3100A0-102218 to J. Luban and M. Grütter, and funding from the Bill and Melinda Gates Foundation Grand Challenges in Global Health initiative, to M.G. Manz. M.R. Neagu was supported by the Columbia University College of Physicians and Surgeons Medical Scientist Training Program and the Roche Research Foundation.

Received for publication March 27, 2009, and accepted in revised form July 8, 2009.

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