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Lentiviral Gene Therapy Against Human Immunodeficiency Virus Type 1, Using a Novel Human TRIM21-Cyclophilin A Restriction Factor

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Abstract

TRIM5α (*tri*partite *m*otif-containing protein-5, isoform α)–cyclophilin A fusion proteins are anti-human immunodeficiency virus (HIV) restriction factors that have evolved in certain nonhuman primates over millions of years and protect against HIV and related viruses. Restriction by TRIM5αCypA is potent and highly resistant to viral escape by mutation and, in combination with a suitable gene delivery platform, offers the possibility of novel therapeutic approaches against HIV. Here we report that lentiviral vector delivery of human mimics of TRIM5α-cyclophilin A (TRIM5CypA) fusion proteins afforded robust and durable protection against HIV-1, but resulted in downregulation of host cell antiviral responses mediated by endogenous TRIM5α. We found that substitution of TRIM5α RING, B-box, and coiled-coil domains with similar domains from a related TRIM protein, TRIM21, produced a novel and equally potent inhibitor of HIV-1. Both TRIM5CypA and TRIM21CypA inhibited transduction by HIV-1-derived viral vectors and prevented propagation of replication-competent HIV-1 in human cell lines and in primary human T cells. Restriction factor-modified T cells exhibited preferential survival in the presence of wild-type HIV. Restriction was dependent on proteasomal degradation and was reversed in the presence of the cyclophilin inhibitor cyclosporin. Importantly, TRIM21CypA did not disturb endogenous TRIM5α-mediated restriction of gammaretroviral infection. Furthermore, endogenous TRIM21 antiviral activity was assessed by measuring inhibition of adenovirus–antibody complexes and was found to be preserved in all TRIMCypA-modified groups. We conclude that lentivirus-mediated expression of the novel chimeric restriction factor TRIM21CypA provides highly potent protection against HIV-1 without loss of normal innate immune TRIM activity.

Introduction

Therapeutic strategies using human immunodeficiency virus (HIV)-1-derived lentiviral vectors to target HIV-1 infection are conceptually attractive and have been investigated in phase 1 clinical trials for years, with no reports of vector-mediated adverse effects (Levine *et al*., 2006). To be efficacious, strategies must disrupt the HIV life cycle and inhibit the virus so that it is not prone to escape through viral mutation. During primate evolution, in both Old and New World monkeys, retrotransposition of the gene encoding the HIV-binding enzyme cyclophilin A (CypA) into the TRIM5 (*tri*partite *m*otif-containing protein-5) gene locus has independently resulted in the production of novel fusion proteins that are powerful inhibitors of primate lentiviruses (Nisole *et al*., 2004; Sayah *et al*., 2004; Liao *et al*., 2007; Brennan *et al*., 2008; Newman *et al*., 2008; Virgen *et al*., 2008; Wilson *et al*., 2008a). TRIM5α comprises a tripartite motif consisting of RING, B-box2, and coiled-coil (RBCC) domains. It targets the incoming viral capsid and blocks infectivity by inhibiting reverse transcription through proteasome recruitment (Wu *et al*., 2006) as well as by promoting innate immune signaling through ubiquitin mediated stimulation of TAK1 phosphorylation and NF-κb activation (Pertel *et al*., 2011). CypA is a

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peptidylprolyl isomerase that binds an exposed loop of the HIV-1 capsid protein, an interaction that can be disrupted by the immunosuppressive drug cyclosporin (Towers, 2007). Oligomerization of CypA by fusion to the murine restriction factor Fv1 creates a potent HIV-1 restriction factor, and the restrictive potential can be increased by fusing CypA to domains that, like TRIM5, can recruit the proteasome (Schaller *et al*., 2007). The fusion protein TRIM5CypA, in which the TRIM5 PRYSPRY domain has been replaced by CypA, is a powerful inhibitor of lentiviral infection (Sayah *et al*., 2004; Wilson *et al*., 2008b; Price *et al*., 2009). Importantly, human TRIM5CypA variants have been shown to mediate robust inhibition of HIV-1 *in vitro* and in human–murine chimeric models of T cell engraftment, with no evidence of virus escape from restrictive effects (Neagu *et al*., 2009). However, short forms of TRIM5, TRIM5γ and TRIM5δ, that do not encode a PRYSPRY domain can titrate natively dimeric TRIM5α proteins and inhibit restriction in a dominant negative way, thereby rescuing retroviral infectivity (Stremlau *et al*., 2004). Whether the short forms are expressed naturally remains unclear, as does the importance of this dominant negative effect in TRIM5 biology. Data suggest that TRIM5α participates in regulation of normal innate immune signaling events (Pertel *et al*., 2011), suggesting that disruption of these pathways by ectopic expression of TRIM5CypA could have unanticipated consequences. We postulated that substitution of the TRIM5α RING, B-box2, coiled-coil, and PRYSPRY domains with structurally related domains from a phylogenetically related TRIM molecule would obviate this effect. It has been reported that heterologous RING, B-box2, and coiled-coil domains from related TRIM proteins, including TRIM21, can functionally substitute for rhesus TRIM5α domains (Li *et al*., 2006), but studies in human proteins with fusion to cyclophilin A have not been previously described. Here we report that TRIM21CypA is a potent inhibitor of HIV-1 and has favorable characteristics compared with TRIM5CypA through preservation of endogenous antiviral activity of both TRIM5α and TRIM21.

Materials and Methods

Lentiviral vectors

Human TRIM5αCypA and TRIM21CypA fusion cassettes were generated by PCR amplification and cloned into a previously described self-inactivating (SIN) HIV-1-derived lentiviral vector encoding a spleen focus-forming virus (SFFV) promoter and woodchuck posttranscriptional regulatory element (WPRE) (Demaison *et al*., 2002). Expression was linked to enhanced green fluorescent protein (EGFP) by an internal ribosomal entry sequence (IRES) as previously described (Di *et al*., 2011). HIV-1 and murine leukemia viral (MLV) vectors encoding EGFP alone or yellow fluorescent protein (YFP) were also produced. In some experiments modified constructs devoid of IRES-EGFP were used where indicated.

Cells

Human embryonic kidney (HEK293T), Crandell-Reese feline kidney (CRFK), TE671, HeLa, and GFP-encoding human osteosarcoma (GHOST) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (Sigma, Gillingham, UK) and penicillin–streptomycin (Invitrogen, Paisley, UK). Jurkat T cells and SupT1 cells were maintained in RPMI with 10% fetal calf serum. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers (with institutional ethics committee approval) by Ficoll gradient centrifugation and were cultured in X-VIVO 10 (Lonza, Walkersville, MD) supplemented with human AB serum (Lonza).

Vector production

Lentiviral vector stocks were produced by transient transfection of subconfluent HEK293T cells with vector plasmid, vesicular stomatitis virus (VSV) envelope plasmid (pMDG), and *gag/pol* packaging plasmid (pCMVΔ8.74) as previously described (Qasim *et al*., 2007). Viral supernatant was harvested 48 and 72 hr later, filtered (pore size, 0.22 μm), and concentrated by ultracentrifugation before cryopreservation.

B- and N-tropic MLV retroviral stocks expressing YFP were produced by transient transfection of HEK293T cells with pCNCY, pMDG2, and pCIG3 (B- or N-tropic), using Lipofectamine 2000 (Invitrogen, Paisley, UK). TRIMCypA gammaretroviral vector stocks was produced by transient transfection of HEK293T cells with pEXN-TRIMCyp, pMDG, and Moloney murine leukemia viral (MoMLV) *gag/pol* packaging plasmid (pCMVi), using FuGENE 6 (Roche, Burgess Hill, UK). Retrovirus-containing supernatant was harvested 48 and 72 hr later before filtration (pore size, 0.45 μm) and cryopreservation. Viral titers were quantified by flow cytometry and/or quantitative PCR as previously described (Vink *et al*., 2009).

HIV-1 viral stocks

NL4-3 encoding Ba-L envelope and HIV-1 R9 plasmids (Centre for AIDS Reagents, National Institute for Biological Standards and Control [NIBSC], Potters Bar, UK) were transfected into HEK293T cells, using FuGENE 6 (Roche), and viral supernatant was harvested 48 and 72 later, filtered (pore size, 0.45 μm), and cryopreserved. Viral titer was quantified by p24 ELISA as described below.

Quantitative PCR

HEK293T cells (1×10^5) were exposed to serial dilutions of lentiviral vector, and 72 hr later the genomic DNA was extracted. A primer–probe set targeting the WPRE sequence was used to detect integrated lentiviral vector copies, and the data were normalized against β-actin gene copy and titer calculated as previously described (Vink *et al*., 2009).

ELISA

p24 levels in HIV-1 viral stocks were quantified by p24 ELISA (ZeptoMetrix, Buffalo, NY) according to manufacturer's guidelines. Absorbance was read with a FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany).

Flow cytometry

GFP and YFP were measured by flow cytometry, using a BD LSR II analyzer (BD Biosciences, San Jose, CA) and

FACSDiva software. Cells were sorted on a MoFlo XDP sorter (Beckman Coulter, Miami, FL). A set of dichroic mirrors and optical filters was used to separate GFP and YFP on the LSR II analyzer. A 525LP dichroic mirror was used to separate GFP and YFP signals. Cells were washed in phosphate-buffered saline (PBS) before fixation in 4% paraformaldehyde. Viable cells negative for LIVE/DEAD stain (Invitrogen, Paisley, UK) were back-gated on a forward/side scatter plot to confirm that they were included in the original viability gate.

Western blot

Transduced CRFK cells were suspended in lysis buffer (60 m*M* Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and boiled at 100°C for 10 min. Protein was resolved on NuPAGE Novex 4–12% Bis-Tris gels with 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (Invitrogen, Carlsbad, CA) and transferred onto polyvinylidene difluoride (PVDF) membrane. Blots were probed with anti-cyclophilin antibody (SA296; Enzo Life Sciences, Exeter, UK). Anti-rabbit horseradish peroxidase-conjugated secondary antibody (NA934; GE Healthcare, Buckinghamshire, UK) and a Pierce enhanced chemiluminescence (ECL) detection kit (Thermo Scientific, Rockford, IL) were used to visualize bands.

Restriction assays

CRFK cells were transduced with lentiviral vector encoding TRIM5CypA or TRIM21CypA. Transduction was confirmed by flow cytometry for EGFP before challenge with HIV-YFP vector at a multiplicity of infection (MOI) of 3 in the presence or absence of 5 μM cyclosporin (Sandoz Pharmaceuticals, Camberley, UK), a competitive inhibitor of cyclophilin A binding. Flow cytometry was used to quantify HIV-YFP infection 72 hr later. Culture in the presence of the proteasome inhibitor MG132 was used to determine whether restriction by TRIM21CypA was mediated by proteasomal degradation as previously reported for TRIM5CypA. In these experiments, viral DNA was assessed by quantitative PCR in order to provide a measure of HIV genomes, which had undergone reverse transcription. Human PBMCs were activated with anti-CD3/anti-CD28 beads at a 1:1 ratio (Invitrogen Dynal, Oslo, Norway) in the presence of interleukin (IL)-2 (Proleukin, 100 U/ml; Chiron/Novartis Diagnostics, Emeryville, CA) for 48 hr before lentiviral transduction as previously described (Qasim *et al*., 2007). HIV-YFP challenge at an MOI of 35 after 72 hr was assessed by flow cytometry to determine levels of HIV restriction. In experiments testing challenge by replication-competent HIV-1, GHOST cells were transduced with TRIMCyp vectors and purified by flow cytometric sorting for GFP and CCR5 expression. Sorted cells were seeded at 5×10^4 in triplicate and subjected to a single round of infection with 2 ng of HIV-1 NL4-3 encoding a Ba-L envelope gene (NL4-3 Ba-L) and cultured over 28 days. Culture medium was harvested at 1-week intervals and p24 levels were measured by ELISA. For primary cell experiments, transduced human T cells were enriched by flow cytometric cell sorting, gating on $CD4^+$/GFP double-positive cells after lentiviral transduction and compared with nontransduced enriched $CD4^+$ T cells. Purified cells were seeded in triplicate in 96-well U-bottom plates and challenged with NL4-3 Ba-L, and viral propagation was assessed by measurement of p24 in the culture supernatant by ELISA 5 days later.

Endogenous TRIM response assays

Endogenous TRIM5α activity was assessed in TE671 cells, transduced with lentiviral TRIMCypA vectors, and cultured in the presence of 1000 U of interferon (IFN)-β. Cells were challenged with either HIV-YFP or with B- or N-MLV-YFP vectors and then assessed for YFP expression by flow cytometry, gating on the EGFP-positive fraction. Endogenous TRIM21 activity was assessed by determination of adenovirus-GFP infection inhibition as described (Mallery *et al*., 2010). Briefly, TRIM21 restricts adenoviral infection of HeLa cells after incubation with adenovirus-specific serum, and this effect is augmented in the presence of IFN-α. HeLa cells were transduced to express TRIMCyp (using vectors devoid of EGFP) and were exposed to adenovirus-GFP opsonized with a monoclonal antibody against adenovirus (9C12; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and examined by flow cytometry for EGFP expression.

Statistics

Where shown, error bars represent the standard error of the mean of the replicates indicated, and differences were found to be significant by Student *t* test at $p < 0.05$.

Results

Lentivirus-mediated TRIMCypA restriction of HIV

A fragment encoding the first 284 amino acids of TRIM21, encoding its RBCC motif, and a fragment encoding the first 306 amino acids of the TRIM5α RBCC motif were fused in-frame to a human CypA cDNA and cloned into an SIN HIV-1 construct encoding a strong retroviral promoter (from SFFV) and linked to an EGFP reporter gene (Fig. 1A) (Qasim *et al*., 2007; Di *et al*., 2011). To test for restriction activity TRIMCypA/EGFP was expressed in permissive CRFK cells. These cells were then challenged with a VSV-G-pseudotyped replication-defective HIV-1-based vector encoding YFP. When TRIMCyp expression restricted infection, cells coexpressing EGFP were protected from infection by incoming HIV-YFP. In the absence of restriction both the green and nongreen populations became equally infected by HIV-YFP. These experiments were performed in the presence or absence of cyclosporin, a reversible inhibitor of CypA binding and thus an inhibitor of TRIMCyp restriction. In cell lines transduced with control vectors expressing EGFP alone, high levels of YFP and EGFP coexpression were seen after HIV-1-YFP infection, as expected. In contrast, when cells expressed EGFP in combination with TRIM5CypA or TRIM21CypA, the modified cells were protected from infection and YFP coexpression was not observed after HIV-1-YFP infection (Fig. 1B). Both TRIM5CypA and TRIM21CypA expression protected the green cells from infection by HIV-1 encoding YFP. In the presence of cyclosporin, the restrictive effects of both TRIM5CypA and TRIM21CypA were abrogated, allowing the expression of YFP in the GFP-positive cells. In repeat experiments, 100-fold HIV-1 restriction was

consistently afforded by both TRIM5CypA and TRIM21CypA expression.

It is possible to saturate restriction effects mediated by TRIM5CypA, and similar effects have been reported for TRIM5α (Towers *et al.*, 2002). We compared restriction mediated by TRIM5CypA and TRIM21CypA in transduced CRFK cells exposed to HIV-YFP up to an MOI of 1000. Flow cytometry for YFP expression was undertaken 72 hr after challenge with HIV-1-YFP (Fig. 1C). Control populations (nontransduced or expressing GFP alone) were all transduced by HIV-YFP at an MOI of 10. In contrast, both TRIM5CypA- and TRIM21CypA-modified cells exhibited similar saturation effects, and required exposure to an MOI of 1000 to achieve complete transduction, providing further evidence for a similar mechanism of action.

Western blot analysis confirmed expression of the appropriate-sized fusion proteins, with TRIM5CypA at 53 kDa, TRIM21CypA at 51 kDa, and endogenous cyclophilin A at 18 kDa (Fig. 1D). Retroviral restriction mediated by TRIM5CypA has previously been shown to involve the

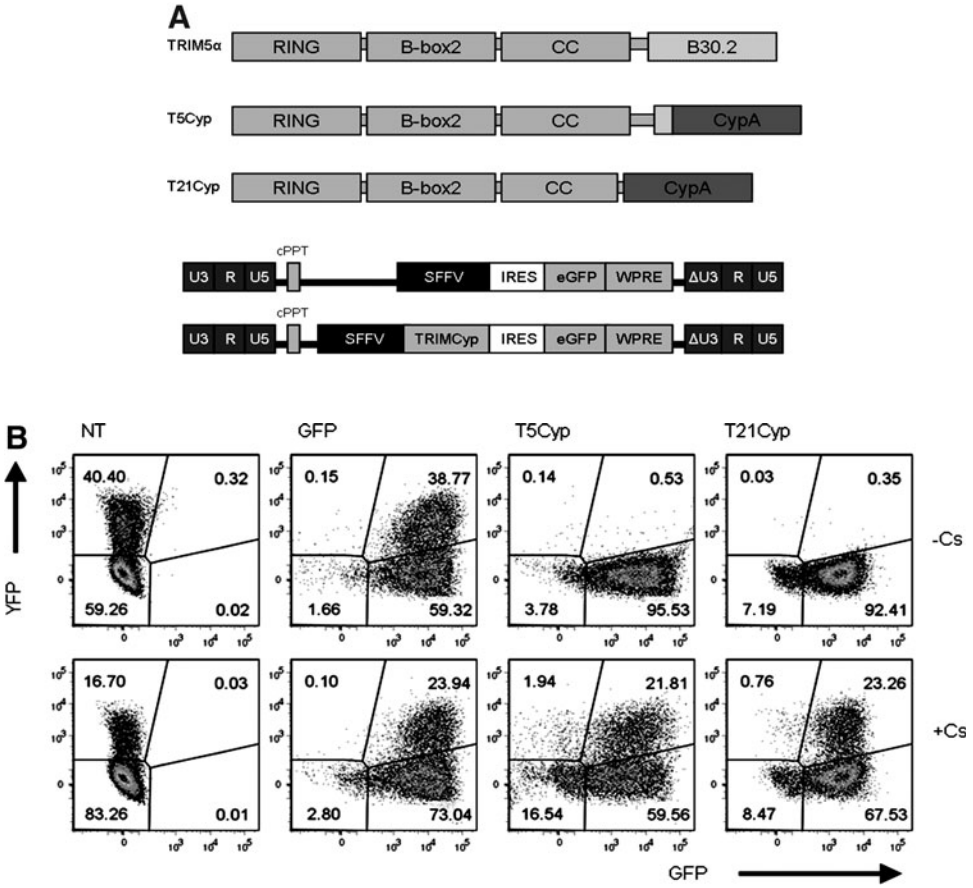

FIG. 1. Lentivirus-mediated expression of TRIMCypA restriction factors. **(A)** Schematic representation of native TRIM5α showing RING, B-box2, coiled-coil, and B30.2 (PRYSPRY) domains. Substitution of the B30.2 domain with human cyclophilin A (CypA) produced TRIM5CypA. An analogous construct was derived from TRIM21 to produce TRIM21CypA. The transgene cassettes were cloned into a self-inactivating lentiviral vector under the control of the spleen focus-forming virus long terminal repeat (SFFV LTR) and linked to expression of EGFP by an internal ribosomal entry sequence (IRES). **(B)** Challenge of transduced CRFK cells with HIV-YFP in the presence or absence of cyclosporin (Cs). Flow cytometry of cells revealed high levels of YFP and EGFP coexpression in cells transduced with control vectors expressing EGFP alone, but cells transduced to express TRIM5CypA or TRIM21CypA, did not coexpress YFP (*top*), indicating highly specific restriction. Cs inhibited these restrictive effects (*bottom*). **(C)** Transduced CRFK cells expressing GFP alone or in combination with TRIM5CypA and TRIM21CypA were exposed to increasing MOIs of HIV-YFP. Flow cytometry for YFP expression was undertaken 72 hr later and showed efficient transduction of control populations at MOIs less than 10. Both TRIM5CypA and TRIM21CypA exhibited similar saturation effects, and required exposure to an MOI of 1000 to achieve complete transduction. **(D)** Western blotting of lentivirally transduced CRFK cells using anti-CypA antibody detected the presence of TRIMCyp species (TRIM5CypA 53kDa and TRIM21CypA 51kDa) in transduced populations (lanes 2 and 3) as well as native cyclophilin A (18 kDa) in control vector transduced cells (lane 1) and β-actin (*bottom*). **(E)** TRIM21CypA restriction of HIV reverse transcription was rescued by proteasomal inhibition by MG132, manifested as increased copies of GFP DNA. Rescue at this level was only partial, as GFP expression was still reduced compared with control (vector only) modified cells. Restriction was fully abrogated by treatment with Cs. **(F)** Flow cytometry showing inhibition of HIV-YFP infectivity in primary T cells transduced to express TRIM5CypA or TRIM21CypA, manifested as an absence of EGFP–YFP coexpression. NT, nontransduced control cells.

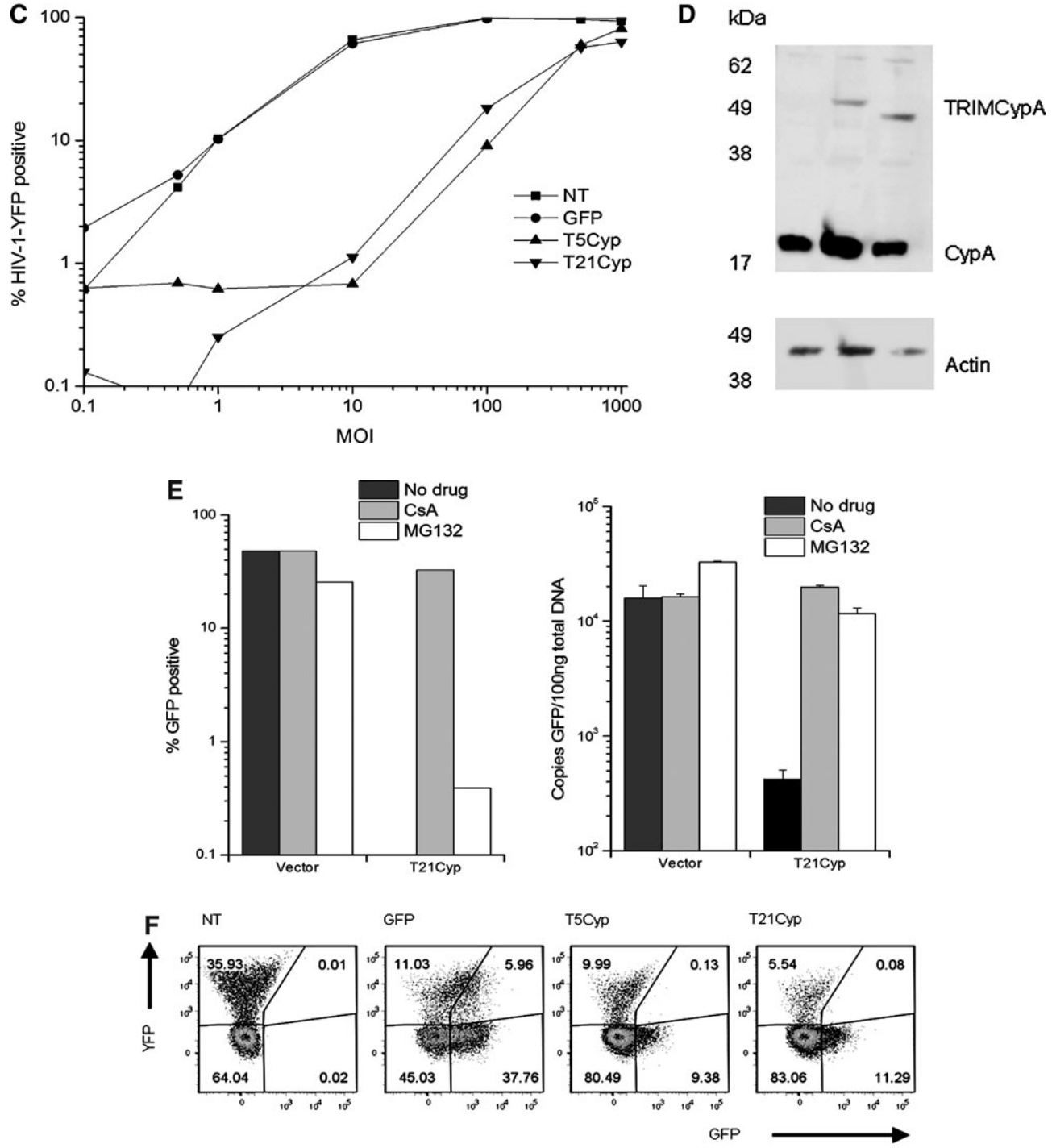

FIG. 1. (Continued).

proteasome (Anderson *et al.*, 2006). Accordingly, the proteasome inhibitor MG132 inhibited restriction of viral DNA synthesis mediated by TRIM21CypA, indicating that TRIM21CypA is likely to restrict infection in a mechanistically similar way to TRIM5CypA (Fig. 1E).

We next assessed the ability of TRIM21CypA and TRIM5CypA proteins to restrict HIV-1 infection in primary human T cells. TRIMCypA-expressing cells were again identified by coexpression of EGFP, and these cells clearly excluded YFP, whereas control cells exhibited good levels of YFP expression as expected (Fig. 1F). Our results demonstrate that lentiviral vectors encoding either TRIM21CypA or TRIM5CypA can transduce human primary T cells and can restrict subsequent infection with HIV-1.

TRIMCypA proteins inhibit HIV replication and confer a survival advantage

The HIV-1-permissive GHOST cell line was transduced with lentiviral constructs expressing TRIMCypA genes, and

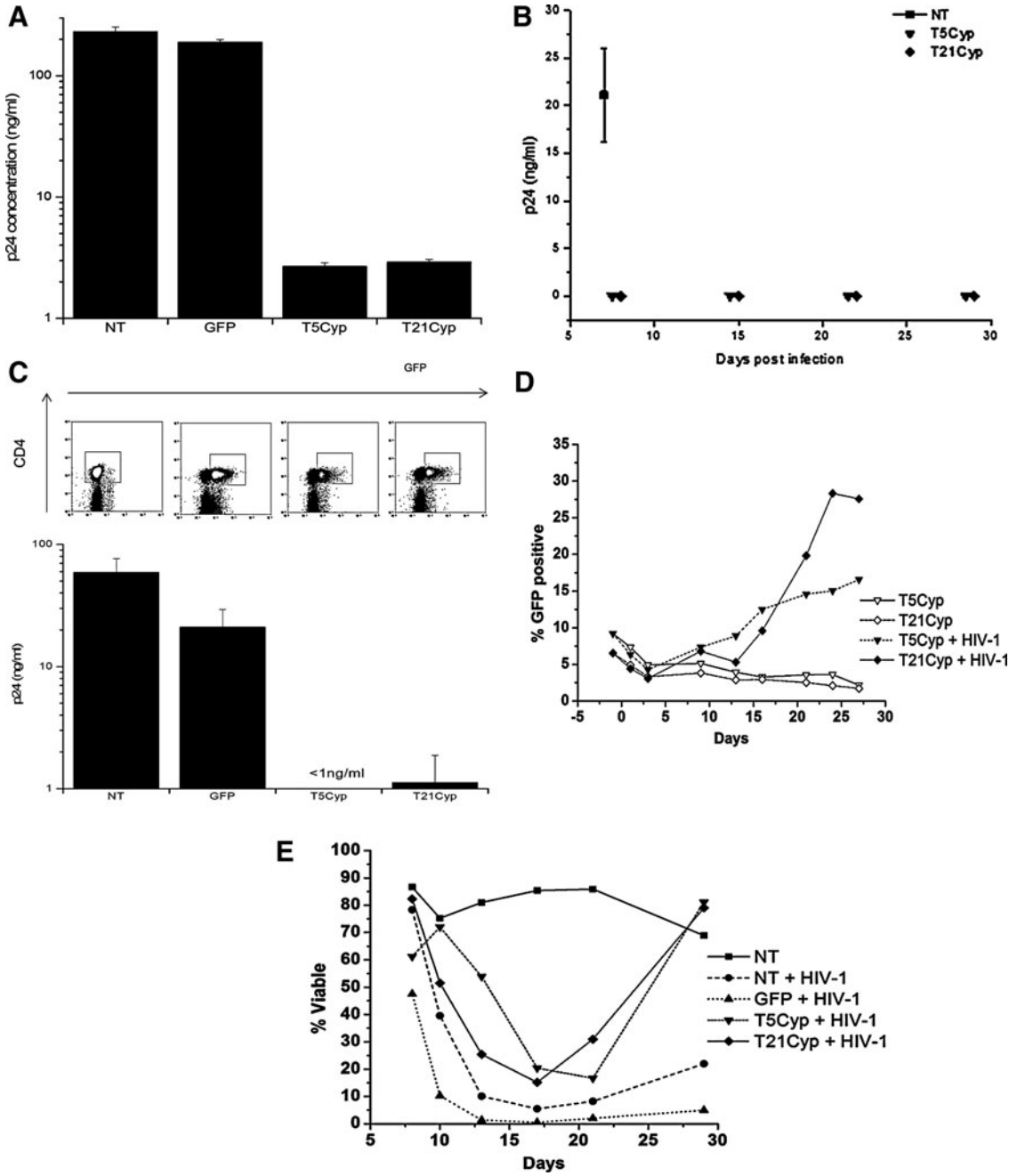

FIG. 2. **(A)** TRIM5CypA and TRIM21CyA inhibit HIV replication. GHOST cells transduced and enriched for EGFP/TRIMCypA expression were infected with HIV-1 NL4-3 (Ba-L Env) and virus propagation was quantified by ELISA for p24 after 7 days. TRIM5CypA and TRIM21CypA groups had low levels of detectable p24 whereas in control cultures, HIV-1 had propagated as anticipated. NT, nontransduced control cells ($n=3$). **(B)** In cultures tracked for 28 days, we found that GHOST cells fully modified to express TRIM5CypA or TRIM12CypA did not support HIV propagation, and p24 levels were barely detectable by ELISA in these cultures. In contrast, nontransduced control populations had high p24 levels within 7 days of exposure to HIV, and cultures did not survive beyond this period, precluding further assessments ($n=3$). **(C)** Transduced primary T cells were enriched on the basis of CD4 and GFP coexpression and seeded in triplicate before challenge with HIV. Nontransduced T cells, or cells modified to express GFP alone, supported HIV replication and p24 was detectable within 5 days. In contrast, p24 levels in supernatant collected from TRIM5CypA- or TRIM21CypA-modified T cells were barely detectable, with no significant difference between TRIMCypA-modified populations ($n=6$, from two independent experiments). **(D)** SupT1 cells transduced to express TRIM5CypA or TRIM21CypA (linked to EGFP) were exposed to replication-competent HIV-1 in culture. Over a period of 14–28 days cells expressing HIV restriction factors increased in frequency, indicating preferential survival in the presence of replicating virus. **(E)** Survival advantage of TRIMCypA-modified populations was investigated in cultures in which approximately 20% of lentivirus-transduced cells were expressing GFP. Jurkat T cell viability was monitored by serial flow cytometry (with viability gating validated by LIVE/DEAD dye staining) and was found to be markedly reduced within 10 days of culture with HIV-1-nontransduced (NT) and GFP-transduced control populations. In cultures of TRIM5CypA- and TRIM21CypA-modified cells, the lowest viability mirrored the 20% proportion of cells expressing restriction factor and thus protected against HIV, and this rapidly improved over a further 7- to 14-day period, suggesting a strong survival advantage in the presence of replicating HIV.

the modified cells were enriched to high purity by flow cytometric cell sorting. Cells were then infected with a high multiplicity of replication-competent HIV-1 NL4-3 (Ba-L) and cultured for 28 days (Fig. 2A and B). Quantification of HIV-1 p24 in the culture supernatant by ELISA found undetectable levels of HIV-1 in either the TRIM5CypA- or TRIM21CypA-expressing cells for up to 4 weeks. In contrast, control cultures supported HIV-1 propagation as anticipated, with high levels of p24 detected by 7 days, and cell death thereafter. These data indicate robust protection over prolonged culture times and suggest that in cell cultures highly purified for TRIMCyp expression, HIV-1 is unable to escape restriction and thus the life cycle of the virus effectively terminates. We also investigated the ability of TRIMCypA to prevent HIV-1 infection and propagation in primary human T cells. Human PBMCs were activated for 48 hr with anti-CD3/anti-CD28 beads and transduced with lentiviral vectors expressing either GFP alone or in combination with TRIMCypA. Five days later, highly purified populations of $CD4^+$/GFP^+ T cells were challenged with HIV-1 NL4-3 and cultured for a further 5 days before ELISA-based quantification of p24 in the culture supernatant. Whereas p24 was detected in nontransduced cells as well as cells expressing EGFP alone, p24 levels in supernatant collected from TRIMCypA-modified cells were barely detectable. This indicates that both TRIM5CypA and TRIM21CypA had restricted replication-competent HIV in primary T cells (Fig. 2C).

We next prepared populations of SupT1 cells coexpressing EGFP and TRIMCypA fusion proteins, with about 10% of the cells expressing the restriction factor. We then infected these cultures with replication-competent HIV-1 clone R9. Flow cytometry quantified EGFP expression and cell viability over a period of 4 weeks, and revealed an increasing proportion of TRIMCypA-expressing cells, indicating a survival advantage in the presence of replication-competent HIV-1 (Fig. 2D). The survival advantage conferred by TRIM5CypA and TRIM21CypA was further demonstrated in Jurkat T cells by measuring viability of transduced populations over time. Whereas viability of control populations exposed to HIV deteriorated over 7–14 days, cultures in which about 20% of cells were modified to express TRIMCypA proteins recovered viability after an initial decline to about 20%, again supporting the notion of a powerful survival advantage for cells expression TRIMCyp chimeras (Fig. 2E).

TRIM5CypA but not TRIM21CypA disrupts endogenous TRIM5 activity

For therapeutic purposes it would be advantageous to select chimeric TRIMCypA restriction factors that do not disturb the endogenous innate functions of the parental TRIM proteins. The ability of TRIM5CypA-expressing cells to restrict gammaretrovirus through endogenous TRIM5α was therefore investigated. We used TRIM5α-sensitive MLV-N and TRIM5α-insensitive MLV-B and HIV-1 (all expressing YFP) as controls. As expected, unmodified cells strongly restricted MLV-N but not MLV-B, or HIV-1, and this effect was augmented in the presence of IFN-β, which is known to stimulate TRIM5α expression (Fig. 3). However, cells expressing TRIM5CypA were unable to restrict MLV-N, even in the presence of IFN-β. This observation confirms that TRIM5CypA chimeras have dominant negative activity

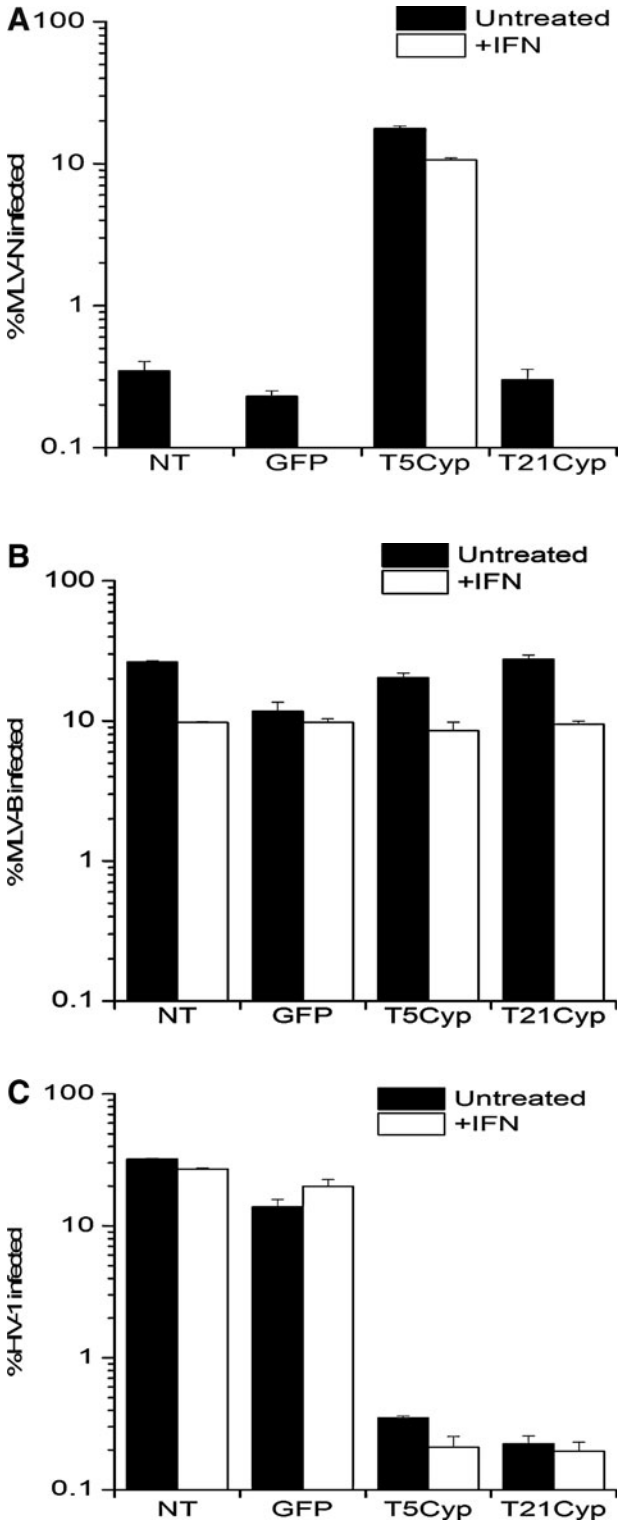

FIG. 3. TRIM5CypA but not TRIM21CypA inhibits endogenous TRIM5 function. **(A)** Loss of TRIM5-mediated restriction of the gammaretrovirus N-MLV was seen in cells modified to express TRIM5CypA, and this effect could not be rescued with interferon (IFN). TRIM21CypA-transduced cells retain their ability to restrict N-MLV-YFP, and as in the control groups, IFN-β augmented this effect, reducing YFP to background levels. **(B)** B-MLV was not restricted by either endogenous TRIM5α or the TRIMCyp proteins whereas **(C)** HIV was restricted by both TRIM5CypA and TRIM21CypA ($n=3$).

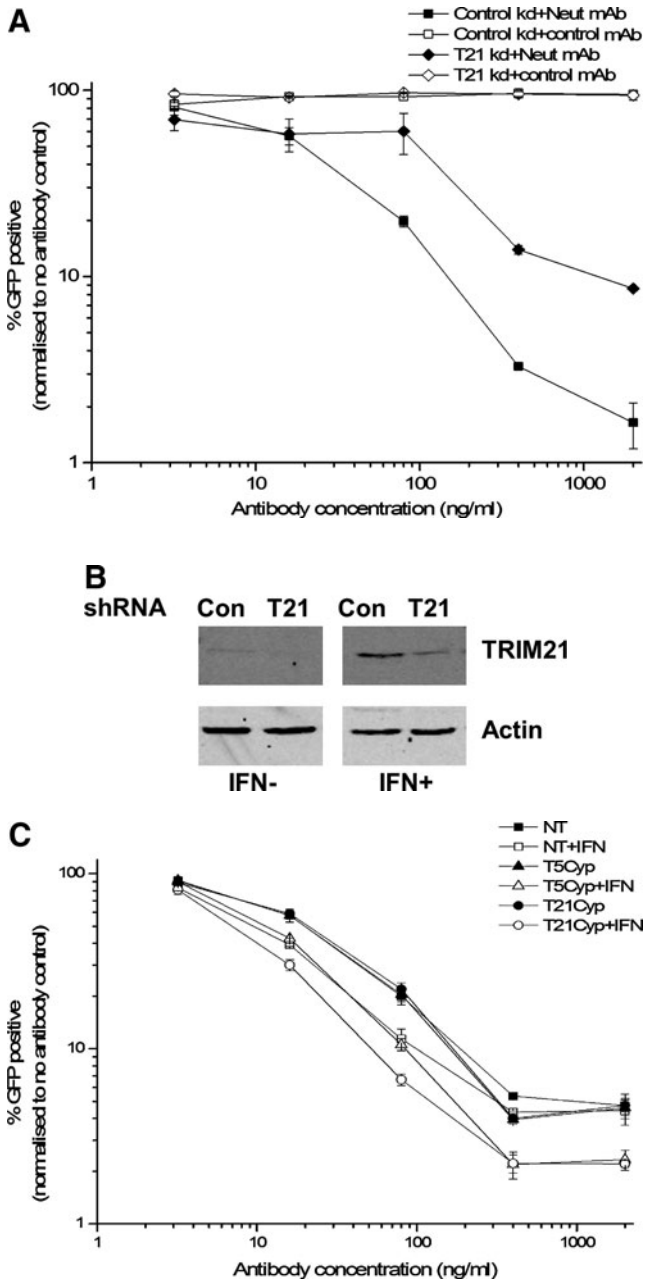

FIG. 4. Endogenous TRIM21 restricts adenovirus in the presence of neutralizing antibody, and functions in TRIM5-CypA- or TRIM21CypA-expressing cells. **(A)** HeLa cells stably knocked down for TRIM21 (T21 kd) or control cells expressing nontargeting shRNA (Control kd) were infected with adenovirus-GFP incubated with a neutralizing (Neut mAb) or isotype-matched control (Control mAb) antibody. The control shRNA-expressing cells efficiently neutralized adenovirus infection when neutralizing antibody was present. In contrast, HeLa cells depleted for TRIM21 were less efficient in neutralizing adenovirus infection in the presence of neutralizing antibody. **(B)** Confirmation that interferon-α induced TRIM21 expression, and this could be depleted by shRNAi targeting TRIM21 but not by control shRNAi. **(C)** After lentiviral transduction, TRIM5CypA- and TRIM21CypA-modified cells retained their ability to restrict adenovirus treated with neutralizing antibody, in the presence or absence of interferon-α, indicating intact endogenous TRIM21 activity in all populations. NT, nontransduced control cells ($n=3$).

against endogenous TRIM5 and even treatment with IFN-β, which increases production of endogenous TRIM5α, cannot overcome the dominant negative effect. In sharp contrast, TRIM21CypA expression did not inhibit endogenous TRIM5α activity in these experiments, and N-MLV-YFP infectivity was reduced to below the limits of detection (Fig. 3A). As predicted, both TRIM5CypA and TRIM21CypA did not restrict B-MLV, even in the presence of IFN (Fig. 3B), but did notably inhibit HIV-1 (Fig. 3C).

TRIM21 function is preserved in TRIMCypA-modified cells

TRIM21 binds intracellular antibody via its PRYSPRY domain. This activity is conserved across mammals, and provides a novel interface between humoral and innate immunity (Mallery *et al*., 2010). Transduction of HeLa cells by an adenoviral vector expressing EGFP (ADV-GFP) can be prevented when the virus is preincubated with monoclonal neutralizing antibody, and we first confirmed that this effect is dependent on endogenous TRIM21 activity. Briefly, cells in which TRIM21 was knocked down by short hairpin RNA interference (shRNAi) became susceptible to ADV-GFP, whereas control populations resisted infection in a manner dependent on the concentration of neutralizing antibody (Fig. 4A and B). We next undertook similar experiments in cells transduced with TRIM5CypA or TRIM21CypA, and found that these populations retained their ability to block ADV-GFP infection, both in the presence or absence of IFN-α (Fig. 4C), indicating that there was no disruption of endogenous TRIM21 antiviral function.

Discussion

Highly active antiretroviral therapy (HAART) against HIV is effective in controlling HIV-1 replication, and reduces morbidity and mortality (Palella *et al*., 1998), but there are widespread issues relating to cost, compliance, and side effects. Alternative therapies based on the use of gammaretroviral, lentiviral, and adenoviral vectors are in early-phase clinical studies, testing a variety of anti-HIV strategies including disruption of CCR5 (Ledford, 2011), expression of anti-Env sequences (Levine *et al*., 2006), anti-HIV ribozymes (Mitsuyasu *et al*., 2009), fusion inhibitors (van Lunzen *et al*., 2007), and other approaches (Rossi *et al*., 2007). Here we have investigated the feasibility of developing a gene therapy strategy against HIV-1 that is based on lentivirus-mediated expression of human TRIM5CypA, reasoning that similar molecules appear to have evolved for this purpose in certain nonhuman primates. In human cells, Neagu and colleagues (2009) found that human mimics of simian TRIM5CypA potently inhibited HIV-1 and they reported durable, escape-resistant restriction *in vitro* and *in vivo* in a human–murine chimeric animal model. This strongly suggests that any mutation of the HIV-1 capsid sequence that leads to viral escape from TRIM5CypA-mediated restriction would have detrimental consequences for viral fitness. It has been shown that HIV-1 mutants that do not recruit cyclophilins integrate into different regions of chromatin and replicate poorly in cell lines and primary cells, in part due to an inability to interact with the nuclear pore cyclophilin Nup358 (Schaller *et al*., 2011). For this reason we believe that the emergence of TRIMCypA-resistant mutants is unlikely to occur and further that cyclophilin-binding mutants are likely to have severe replication defects.

We have independently confirmed the potency of human TRIM5CypA against HIV but also found that domains from a related TRIM protein, TRIM21, can substitute for TRIM5α in the fusion construct. Both TRIM5CypA and TRIM21CypA mediated equally potent anti-HIV effects in human cell lines and in primary human T cells. However, TRIM21CypA had the advantage of not disrupting the known antiviral activities of endogenous TRIM5α or TRIM21. Overexpression of TRIM5CypA compromised endogenous TRIM5α function, probably through direct interaction between the two TRIM moieties. Although data suggest that simian TRIM5CypA activates the TAK1 and NF-κB pathways (Pertel *et al*., 2011) and in turn may mediate inhibitory feedback on TRIM5α transcription, TRIM5α mRNA levels in transduced cells were comparable to those in nonmodified populations. Notably, cells expressing TRIM21CypA not only retain IFN-mediated endogenous TRIM5α responses but also exhibited intact TRIM21 function. Until more recently, little was known about the antiviral activity of TRIM21, and here we have used an assay (Mallery *et al*., 2010) to show that the molecule mediates restriction of antibody–virion complexes and inhibition of adenovirus-mediated GFP expression. This effect is thought to be mediated by the PRYSPRY domain of the TRIM21 molecule through a stoichiometric complex with one antibody molecule. We speculate that an excess of TRIM21-CypA (which is devoid of the PRYSPRY domain) does not interfere with the endogenous antiviral pathway of TRIM21. Thus as a therapeutic antiviral approach, TRIM21CypA vectors could have important advantages over TRIM5CypA-based strategies. One potential problem with a TRIM21-based expression strategy is the connection between TRIM21 and the rare chronic autoimmune diseases systemic lupus erythematosus (SLE) and Sjögren syndrome. TRIM21 is the target of autoantibodies (anti-Ro52) present in some of these individuals, although the role of these antibodies in the disease remains unclear. Anti-Ro52 antibodies are polyclonal but the most immunogenic epitopes implicated in autoimmune disease reside within the B30.2 domain and carboxy terminus (Burbelo *et al*., 2010). Importantly, these regions are not present in the TRIM21CypA restriction factor described here, reducing the risk of autoimmune rejection of TRIM21-CypA-modified cells by typical anti-Ro52 antibodies.

In the first instance, the safety and efficacy of anti-HIV gene therapy approaches using TRIM21CypA will be investigated by *ex vivo* modification and reinfusion of T cells or T cell precursor stem cells. If such modification confers a survival advantage *in vivo* through sustained protection against HIV, a relatively small number of engineered cells could expand and reconstitute cellular immunity within a period of a few months, offering a valuable alternative to conventional antiretroviral drug therapy.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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