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A novel gene therapy strategy using secreted multifunctional anti-HIV proteins to confer protection to gene-modified and unmodified target cells

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Current HIV gene therapy strategies focus on rendering HIV target cells non-permissive to viral replication. However, gene-modified cells fail to accumulate in patients and the virus continues to replicate in the unmodified target cell population. We have designed lentiviral vectors encoding secreted anti-HIV proteins to protect both gene-modified and unmodified cells from infection. Soluble CD4 (sCD4), a secreted single chain variable fragment (sscFv$_{17b}$), and a secreted fusion inhibitor (sFI$_{T45}$) were used to target receptor binding, co-receptor binding, and membrane fusion, respectively. Additionally, we designed bi- and tri-functional fusion proteins to exploit the multistep nature of HIV entry. Of the seven antiviral proteins tested, sCD4, sCD4-scFv$_{17b}$, sCD4-FI$_{T45}$, and sCD4-scFv$_{17b}$-FI$_{T45}$ efficiently inhibited HIV entry. The neutralization potency of the bi-functional fusion proteins sCD4-scFv$_{17b}$ and sCD4-FI$_{T45}$ was superior to that of sCD4 and the Food and Drug Administration-approved fusion inhibitor T-20. In co-culture experiments, sCD4, sCD4-scFv$_{17b}$ and sCD4-FI$_{T45}$ secreted from gene-modified producer cells conferred substantial protection to unmodified peripheral blood mononucleocytes. In conclusion, continuous delivery of secreted anti-HIV proteins *via* gene therapy may be a promising strategy to overcome the limitations of the current treatment.

Keywords: bi-functional fusion proteins, fusion inhibitor, gene therapy, HIV entry, secreted antiviral proteins

BACKGROUND

The human immunodeficiency virus type I (HIV), a member of the *retroviridae* family, is the causative agent of acquired immunodeficiency syndrome (AIDS). HIV-positive individuals are commonly treated with a cocktail of antiretroviral drugs known as combination antiretroviral therapy (cART), which drastically increases survival of patients with access to the treatment. While cART suppresses viral replication, HIV persists in latent reservoirs. Treatment interruptions result in re-emergence of the virus,[1] which necessitates strict daily administration of potentially complicated drug regimens. Life-long patient adherence to cART is further impaired by the accumulating costs, short-term side effects, and long-term toxicities.[2] Consequently, patients need to be continuously monitored for drug tolerance and emergence of drug-resistant viruses. In the absence of a cure, vaccines, or reliable microbicides, there is a strong need for the development of alternative well-tolerated and long-lasting therapies.

Gene therapy has successfully been used to treat a variety of diseases, including adenosine deaminase-deficient (ADA) severe combined immunodeficiency (SCID), X-linked SCID, X-linked adrenoleukodystrophy, Leber's congenital amaurosis, and hemophilia B.[3-7] A genetic HIV treatment strategy may also be a promising alternative to cART, since life-long expression of a therapeutic gene could be achieved after a single treatment and may advantageously replace continuous drug administration. Proof of principle has been demonstrated in a HIV-positive patient with acute myeloid leukemia, who received allogeneic hematopoietic stem cells that were resistant to HIV infection due to a naturally occurring genetic mutation.[8] The treatment resulted in the long-term suppression of viral replication after cessation of cART.[9]

Conventional anti-HIV gene therapeutics render HIV target cells non-permissive to virus replication by targeting viral and cellular factors that are dispensable for the host. The gene products used to date can generally be classified into RNA- and protein-based therapeutics. Examples of RNA molecules used in HIV gene therapy include antisense RNAs, short hairpin or small interfering RNAs, decoy RNAs, ribozymes, and group II introns.[10-18] Examples of protein-based molecules include *trans*-dominant negative mutants, targeted or packageable RNases, intrabodies, intrakines, membrane-bound fusion inhibitors, and Zinc-finger nucleases.[19-25] Ideally, gene-modified CD4$^+$ T cells would have a survival advantage over unmodified cells and autologous transplantation would eventually lead to the reconstitution of a HIV-resistant immune system. Clinical trials have shown the safety of genetic approaches.[26,27] Long-term persistence of gene-modified CD4$^+$ T cells expressing anti-HIV genes has been demonstrated in a recent follow up of three clinical trials, where the modified cells were detected in 98% of all samples tested for at least 11 years after infusion.[28] However, the therapeutic benefit of all tested strategies has been limited due to low-level engraftment of gene-modified cells in patients undergoing therapy.[28,29]

In contrast, using secreted antiviral proteins that inhibit HIV entry would protect both gene-modified and unmodified HIV target cells and may address the issue of poor gene marking. Injections of recombinant protein-based entry inhibitors are currently explored for the treatment of HIV-positive individuals. Continuous secretion of such antiviral proteins from gene-modified cells may lead to the accumulation of therapeutic concentrations *in vivo* and bypass many problems associated with the administration

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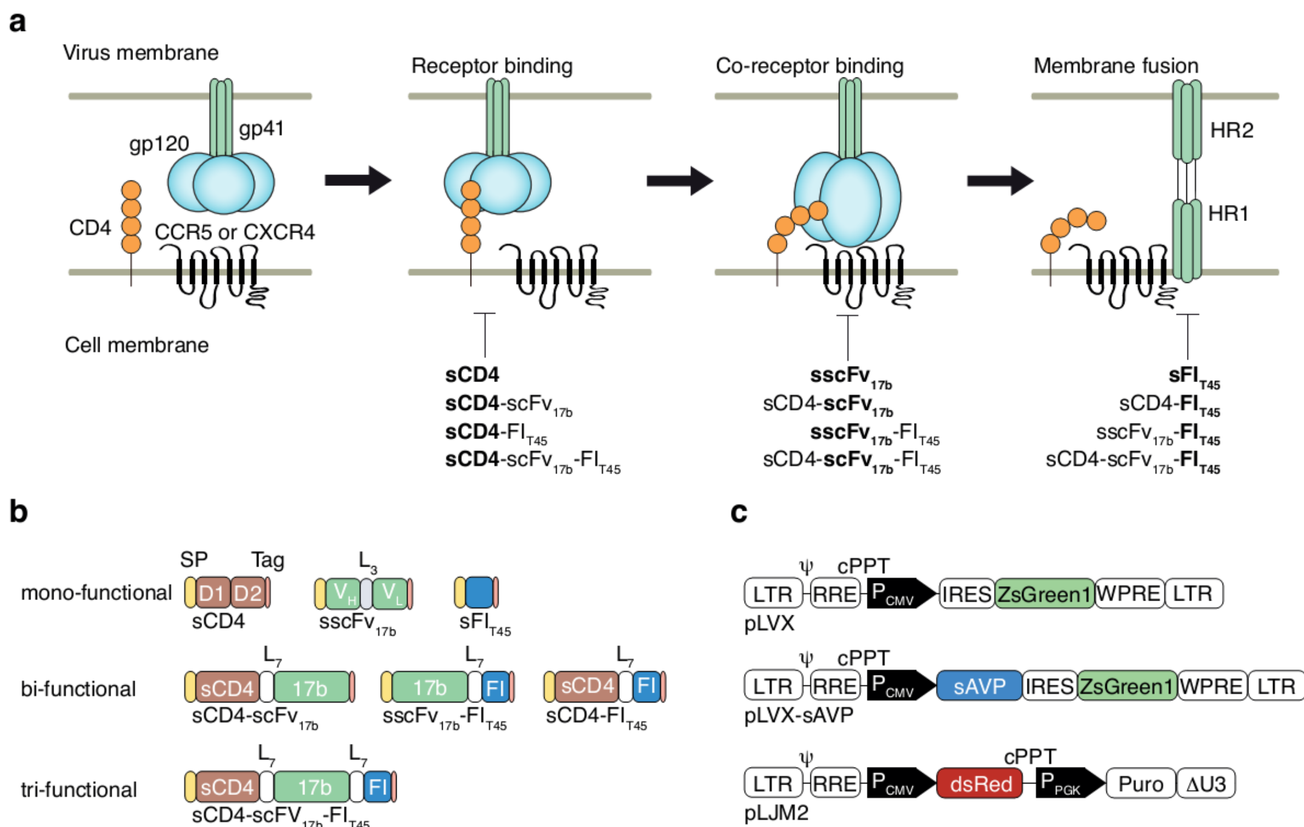

Figure 1. Model of HIV inhibition mediated by secreted mono-, bi- and tri-functional antiviral proteins, design of secreted antiviral proteins, and lentiviral vector constructs used for the expression and testing of antiviral proteins. **(a)** The three major steps of HIV entry are depicted. The antiviral proteins employed in this study are listed according to the steps they are expected to inhibit. Bold characters indicate the moiety that inhibits the respective step. **(b)** Schematic overview of secreted antiviral proteins. Each protein contained the signal peptide of CD4 (SP) at the N-terminus and a C-terminal 6xHis-tag (Tag). sCD4 consists of domains 1 and 2 (D1 and D2) of CD4. $sscFv_{17b}$ was designed by connecting the mAb variable heavy chain (V_H) to the variable light chain (V_L) by a $(GGGGS)_3$ linker (L_3). sFI_{T45} is based on an improved fusion inhibitor derived from the HR2 region of gp41. Bi-functional proteins were designed by connecting the different antiviral moieties *via* a $(GGGGS)_7$ linker (L_7). **(c)** Schematic diagram of lentiviral vectors. The genes encoding the secreted antiviral proteins (sAVP) were cloned into the multiple cloning site of pLVX. These vectors express a bicistronic mRNA encoding the secreted proteins and the green fluorescent protein ZsGreen1 under the control of the cytomegalovirus immediate-early promoter (P_{CMV}). The *cis*-acting Rev-response element (RRE), central polypurine tract (cPPT), and woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) improve vector particle production, transduction, and transgene expression. ψ indicates the packaging signal, and IRES indicates the internal ribosome entry site. All vector elements were flanked by HIV long terminal repeats (LTRs). pLJM2 was used to generate HIV Env-pseudotyped reporter vector particles that express dsRed from P_{CMV} and the puromycin N-acetyl-transferase (Pac) from the human phosphoglycerate kinase promoter (P_{PGK}). Deletion in the U3 region (ΔU3) of the 3′LTR would prevent 5′ LTR-promoter-driven expression from the provirus DNA.

of highly purified proteins, such as the high production cost and the need for frequent injections to maintain therapeutic concentrations. To date, very few secreted antiviral proteins were investigated for delivery *via* gene therapy. Expression of soluble CD4 (sCD4), a truncated version of CD4 that interferes with receptor binding, reduced infection of neighboring cells,[30,31] but the secretion levels were too low to efficiently inhibit primary isolates of HIV. Recently, promising results were obtained with the fusion inhibitor C46 (FI_{C46}), which is a peptide derived from the HIV envelope (Env) glycoprotein gp41 that prevents membrane fusion.[32] Secreted neutralizing antibodies were also tested,[33,34] but their neutralization breadth was limited.[35] Clinical trials using passive transfer of these antibodies have shown emergence of resistant viruses.[36,37]

Bi-functional fusion proteins that contain different antiviral moieties connected with a linker were shown to have broader and more potent HIV neutralization activity than neutralizing antibodies.[38,39] In one example, the antiviral potency of recombinant sCD4 increased significantly, when it was fused to the single chain variable fragment (scFv) of the monoclonal antibody 17b, which targets the CD4-inducible co-receptor binding site of HIV Env gp120.[40] Recombinant $sCD4\text{-}scFv_{17b}$ was superior to a panel of neutralizing antibodies and neutralized over 40 primary isolates of HIV from diverse genetic subtypes.[41] Very recently, sCD4 was also linked to a fusion inhibitor, FI_{T1144}.[42] Binding of sCD4 to gp120 exposed gp41 and allowed binding of FI_{T1144} , which resulted in cell-free inactivation of HIV in the absence of target cells.[42]

Although these recombinant bi-functional proteins were shown to be effective, their use in gene therapy was not tested.

In the present report, we investigated the feasibility of developing a HIV gene therapy strategy based on secreted antiviral proteins. Of various proteins that were designed to target one or multiple steps of HIV entry, we show that sCD4 and sCD4-derived fusion proteins confer substantial protection to both gene-modified and unmodified HIV target cells, and thus are promising candidates for use in HIV gene therapy.

RESULTS

Design of secreted antiviral proteins

HIV entry is a highly sequential and time-sensitive process,[43] which can be divided into CD4 receptor binding, CCR5 (R5 HIV) or CXCR4 (X4 HIV) co-receptor binding, and membrane fusion (Figure 1a). The first step during entry is the binding of HIV Env gp120 to the cellular CD4 receptor. Upon CD4 interaction, gp120 undergoes conformational changes resulting in the exposure of the highly conserved CCR5 or CXCR4 co-receptor binding site. Co-receptor engagement results in a conformational change in HIV Env gp41, whereby the N-terminal and C-terminal heptad repeats (HR1 and HR2, respectively) are exposed. Interaction of HR1 and HR2 subsequently enables fusion between viral and cellular membranes.

We have designed secreted antiviral proteins that target the three major steps of HIV entry (Figure 1a). sCD4 is comprised of the extracellular CD4 domains D1 and D2 and is used to prevent receptor binding. Its interaction with gp120 has been shown to cause shedding and premature activation of gp120.[44] To inhibit co-receptor engagement, we used sscFv$_{17b}$, which contains the variable heavy chain (V$_H$) of mAb 17b connected to the variable light chain (V$_L$) by a flexible three-repeat GGGGS linker (L$_3$). It binds to a CD4/sCD4-inducible epitope on gp120.[45] The sFI$_{T45}$ is based on the third generation fusion inhibitor T-45, which has been shown to potently neutralize several primary HIV isolates.[46] It consists of 45 amino acid residues derived from the HR2 of gp41. Upon binding to the exposed HR1, sFI$_{T45}$ is expected to prevent membrane fusion.[46]

Additionally, we designed bi- and tri-functional fusion proteins to exploit the multi-step nature of the entry process. The bi-functional sCD4-scFv$_{17b}$ is a well-characterized inhibitor of HIV entry. Both neutralization breadth and antiviral potency of this protein have been studied,[40,41] but its administration *via* gene therapy has not been investigated. We have chosen to connect sCD4 to scFv$_{17b}$ by a seven-repeat GGGGS linker (L$_7$), because shorter linkers were shown to abrogate the function of sCD4-scFv$_{17b}$, while longer linkers did not increase the potency of the fusion protein.[41] Binding of sCD4 to gp120 is expected to expose the otherwise masked scFv$_{17b}$-binding site on gp120, and should thus enable inhibition of receptor and co-receptor binding. Binding of sCD4 to gp120 has also been shown to activate a gp41 pre-hairpin fusion-intermediate, which fusion inhibitors can bind to.[47] Therefore, we chose L$_7$ to link FI$_{T45}$ to the C-terminus of sCD4 and sCD4-scFv$_{17b}$. sCD4-FI$_{T45}$ is expected to inhibit receptor binding and fusion, while sCD4-scFv$_{17b}$-FI$_{T45}$ was engineered to inhibit all steps of viral entry *via* its three different antiviral moieties. sscFv$_{17b}$-FI$_{T45}$ was designed as a control since both moieties can only bind HIV after the virus binds to the target cells.

Secreted proteins contain a signal peptide sequence at their N-terminus, which marks them for the secretory pathway. We utilized the signal peptide of CD4 comprised of 25 amino acids to ensure secretion of all antiviral proteins because it is the natural choice for secretion of sCD4[31] and yielded a high score for cleavage when analyzed with the SignalP 4.0 software.[48] In addition to the CD4 signal peptide, all antiviral proteins contained a C-terminal 6xHis-tag for detection and purification purposes. Furthermore, all proteins were designed to meet the previously reported minimum length requirement of 50-80 amino acids to enter the secretory pathway.[49,50] The smallest protein used in this study was sFI$_{T45}$, which consisted of 90 amino acids

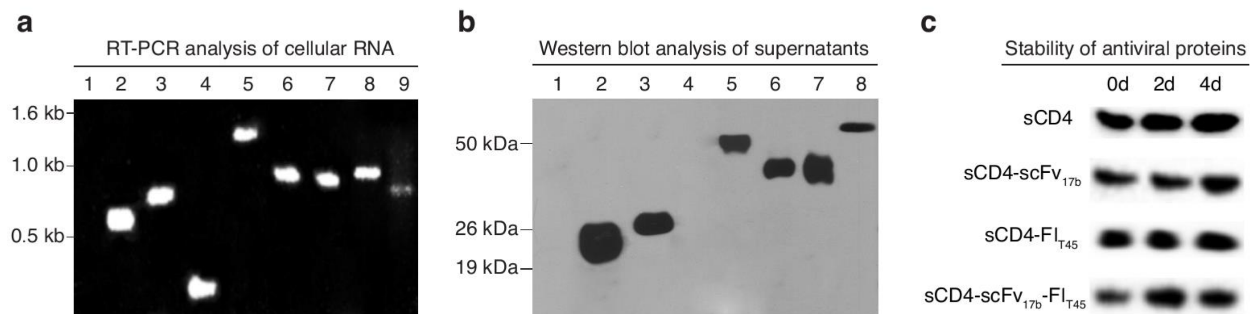

Figure 2. Expression, secretion, and stability of secreted antiviral proteins. 293T cells were transduced with lentiviral vector particles and cultured as described under Materials and Methods. **(a)** Cellular RNA was isolated from the gene-modified 293T cells. Primer pairs flanking the genes encoding the secreted proteins were used for reverse transcriptase (RT)-PCR analysis. Two primer pairs were used for the amplification sCD4-scFv$_{17b}$-FI$_{T45}$ because the gene was too long to be amplified with the primer pair flanking the gene alone. RT-PCR products are analyzed from cells transduced with the control vector particles (lane 1) or with vector particles encoding sCD4 (lane 2), sscFv$_{17b}$ (lane 3), sFI$_{T45}$ (lane 4), sCD4-scFv$_{17b}$ (lane 5), sscFv$_{17b}$-FI$_{T45}$ (lane 6), sCD4-FI$_{T45}$ (lane 7), or sCD4-scFv$_{17b}$-FI$_{T45}$ (lanes 8 and 9). **(b)** Equal amounts of culture supernatants from gene-modified 293T cells were analyzed by 12% SDS-PAGE, followed by Western blot analysis using an anti-6xHis-tag antibody. Lanes: 1, control vector; 2, sCD4; 3, sscFv$_{17b}$; 4, sFI$_{T45}$; 5, sCD4-scFv$_{17b}$; 6, sscFv$_{17b}$-FI$_{T45}$; 7, sCD4-FI$_{T45}$; and 8, sCD4-scFv$_{17b}$-FI$_{T45}$. **(c)** Culture supernatants from gene-modified 293T cells expressing antiviral proteins were incubated at 37°C. Samples were collected at the indicated time points and were analyzed by Western blot using an anti-6xHis-tag antibody.

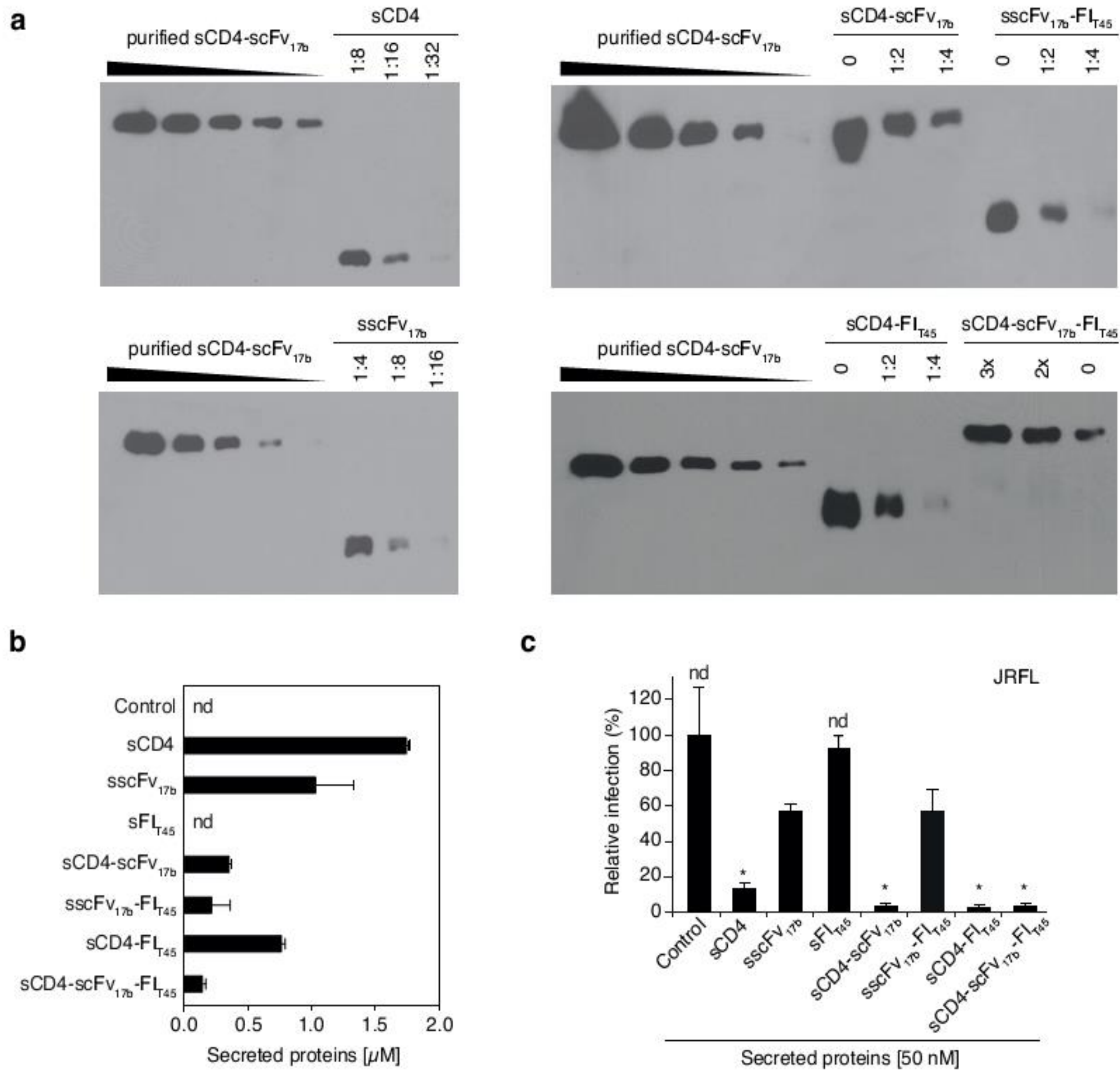

Figure 3. Quantification of secreted antiviral proteins in the culture supernatants of gene-modified 293T cells and inhibition of HIV entry. **(a)** Purified sCD4-scFv$_{17b}$ (0.8, 0.4, 0.2, 0.1, and 0.05 μM) and serial dilutions of culture supernatants from gene-modified cells were analyzed in parallel by Western blot to determine the molar concentration of secreted antiviral proteins. The fold dilutions for each protein are as indicated. 0 refers to undiluted samples, and 2x or 3x indicates that double or triple the volume of undiluted sample was loaded. To determine protein concentrations, the signal intensities of the purified sCD4-scFv$_{17b}$ standard were compared to the signal intensities of the secreted proteins using the software ImageJ. **(b)** Comparison of the secreted antiviral protein concentrations in the culture supernatants of gene-modified 293T cells. Culture supernatants from cells transduced with the control vector and sFI$_{T45}$ showed no detectable (nd) band; n = 2. **(c)** Culture supernatants from gene-modified 293T cells were diluted to contain the secreted antiviral proteins at a final concentration of 50 nM and were incubated with replication-incompetent HIV$_{JRFL}$ Env-pseudotyped reporter vector particles expressing dsRed. Supernatants from cells transduced with control or sFI$_{T45}$ vector particles, showing undetectable levels of antiviral proteins, served as controls. The reporter vector particles were then added to unmodified U373-MAGI-CCR5E target cells. The number of dsRed^{+} cells was determined by fluorescence microscopy on day 5 post-infection; n = 2; $^{*}p<0.05$.

including the signal peptide and 6xHis-tag. The design of all antiviral proteins is outlined in Figure 1b. The genes encoding the proteins were codon-optimized for optimal expression in human cells and were cloned into the lentiviral vector pLVX-IRES-ZsGreen1 (hereafter referred to as pLVX; Figure 1c). Expression of a bicistronic transcript encoding the secreted antiviral proteins and the green fluorescent protein ZsGreen1 was driven by the constitutively active human cytomegalovirus immediate-early promoter.

Secretion of antiviral proteins from gene-modified producer cells
Lentiviral control vector particles and vector particles encoding the secreted antiviral proteins were used to transduce 293T cells to generate producer cells. 90% of the transduced cells were positive for ZsGreen1 and PCR analysis of genomic DNA revealed that all genes encoding the secreted antiviral proteins integrated into the chromosome (results not shown). RT-PCR analysis of cellular RNA showed that full-length transcripts were generated (Figure 2a). Equal volumes of cell culture supernatants were analyzed by Western blot using an anti-6xHis-tag antibody (Figure 2b). Culture supernatants from gene-modified 293T cells expressing sCD4, $sscFv_{17b}$, $sCD4-scFv_{17b}$, $sscFv_{17b}-FI_{T45}$, $sCD4-FI_{T45}$, and $sCD4-scFv_{17b}-FI_{T45}$ displayed distinct protein bands corresponding to the predicted sizes of the secreted antiviral proteins, while sFI_{T45} could not be detected.

To assess the stability of secreted antiviral proteins, we incubated the supernatants containing sCD4, $sCD4-scFv_{17b}$, $sCD4-FI_{T45}$, and $sCD4-scFv_{17b}-FI_{T45}$ at 37°C and analyzed aliquots at different time points by Western blot (Figure 2c). No significant differences in the band intensities were observed for up to 4 days, indicating that the secreted proteins were stable under the tested conditions.

To determine the concentration of secreted antiviral proteins, serial dilutions of supernatants and purified $sCD4-scFv_{17b}$ were analyzed in parallel by Western blot (Figures 3a). A comparison of the concentration of the secreted antiviral proteins present in the supernatant is shown in Figure 3b. sCD4 was present at the highest concentration (1.75 μM), followed by $sscFv_{17b}$ (1.04 μM), $sCD4-FI_{T45}$ (0.76 μM), $sCD4-scFv_{17b}$ (0.35 μM), $sscFv_{17b}-FI_{T45}$ (0.22 μM), and $sCD4-scFv_{17b}-FI_{T45}$ (0.15 μM). The tri-functional fusion protein $sCD4-scFv_{17b}-FI_{T45}$ was present at a ~20 times lower concentration than sCD4. The maximum concentration of secreted sCD4 reported in a previous study was 0.6 nM,[31] which is ~2900 times lower than the maximum concentration of sCD4 secreted from our gene-modified cells.

Antiviral potency of secreted proteins
To determine inhibition of HIV entry by the secreted antiviral proteins, we performed single-round infection assays with replication-incompetent lentiviral reporter vector particles that were pseudotyped with R5 HIV_{JRFL} Env and expressed the red fluorescent protein dsRed as a marker for infection. The culture supernatants from gene-modified 293T cells were diluted to contain the secreted antiviral proteins at a concentration of 50 nM. Culture supernatants from cells transduced with the control vector or sFI_{T45}, which did not contain detectable levels of the secreted protein, were also included. The supernatants were incubated with reporter vector particles and added to U373-MAGI-CCR5E cells (Figure 3c). Supernatant containing sCD4 reduced HIV entry by 87% ($p<0.05$), while those containing $sCD4-scFv_{17b}$, $sCD4-FI_{T45}$, and $sCD4-scFv_{17b}-FI_{T45}$ inhibited viral entry by over 95% ($p<0.05$).

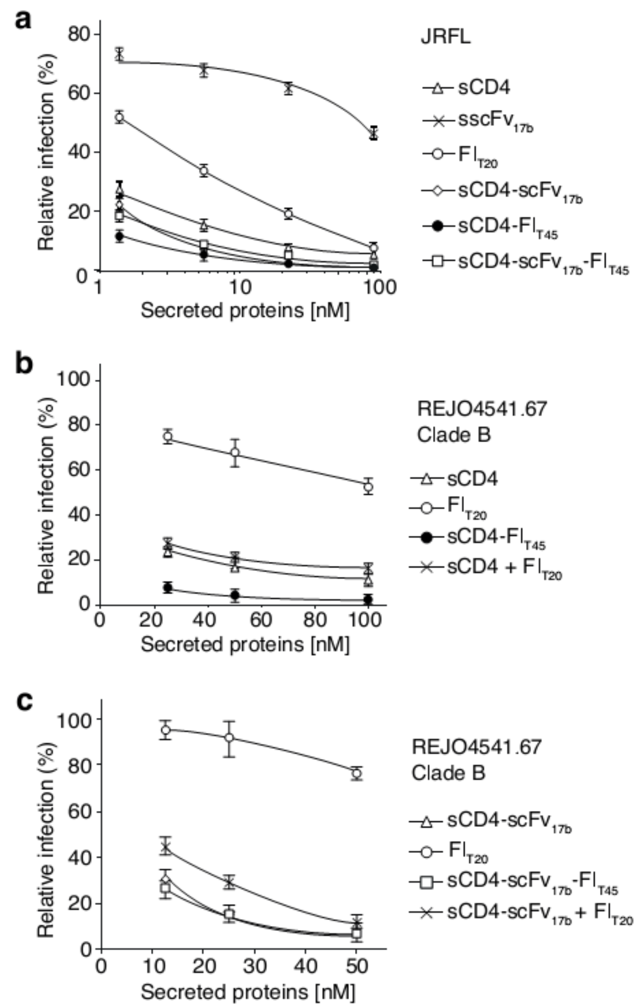

Figure 4. Antiviral effect of secreted proteins compared to synthetic FI_{T20}. (**a)** Culture supernatants from gene-modified 293T cells containing the antiviral proteins or synthetic FI_{T20} were incubated with replication-incompetent HIV_{JRFL} Env-pseudotyped reporter vector particles at the indicated concentrations and added to unmodified U373-MAGI-CCR5E target cells. Infected cells were analyzed by flow cytometry on day 5 post-infection; n = 2. **(b** and **c)** Single-round infections were performed with reporter vector particles pseudotyped with Env from a primary HIV isolate, REJO4541.78. The number of $dsRed^+$ cells was analyzed by flow cytometry on day 5 post-infection**;** n =2. **(b)** Antiviral effect of sCD4 in combination with FI_{T20} compared to $sCD4-FI_{T45}$ fusion protein. The ratio of sCD4 to FI_{T20} when mixed together was 1:1. **(c)** Antiviral effect of $sCD4-scFv_{17b}$ in conjunction with FI_{T20} compared to $sCD4-scFv_{17b}-FI_{T45}$ fusion protein. The ratio of $sCD4-scFv_{17b}$ to FI_{T20} was 1:1.

The $sscFv_{17b}$ only mediated a 50% reduction of entry, which was expected as the co-receptor-binding site is only exposed after binding of gp120 to surface CD4. In the absence of sCD4, the short time window for binding to the co-receptor-binding site on gp120 and steric hindrance when gp120 is bound to surface CD4 most likely diminished the activity of $sscFv_{17b}$.[43] Inclusion of FI_{T45} did not increase the potency of $sscFv_{17b}$, consistent with reports that fusion inhibitors target a transient conformation of gp41, which is only accessible upon gp120 interaction with the receptor and the co-receptor on the target cells.[51] As sFI_{T45} was

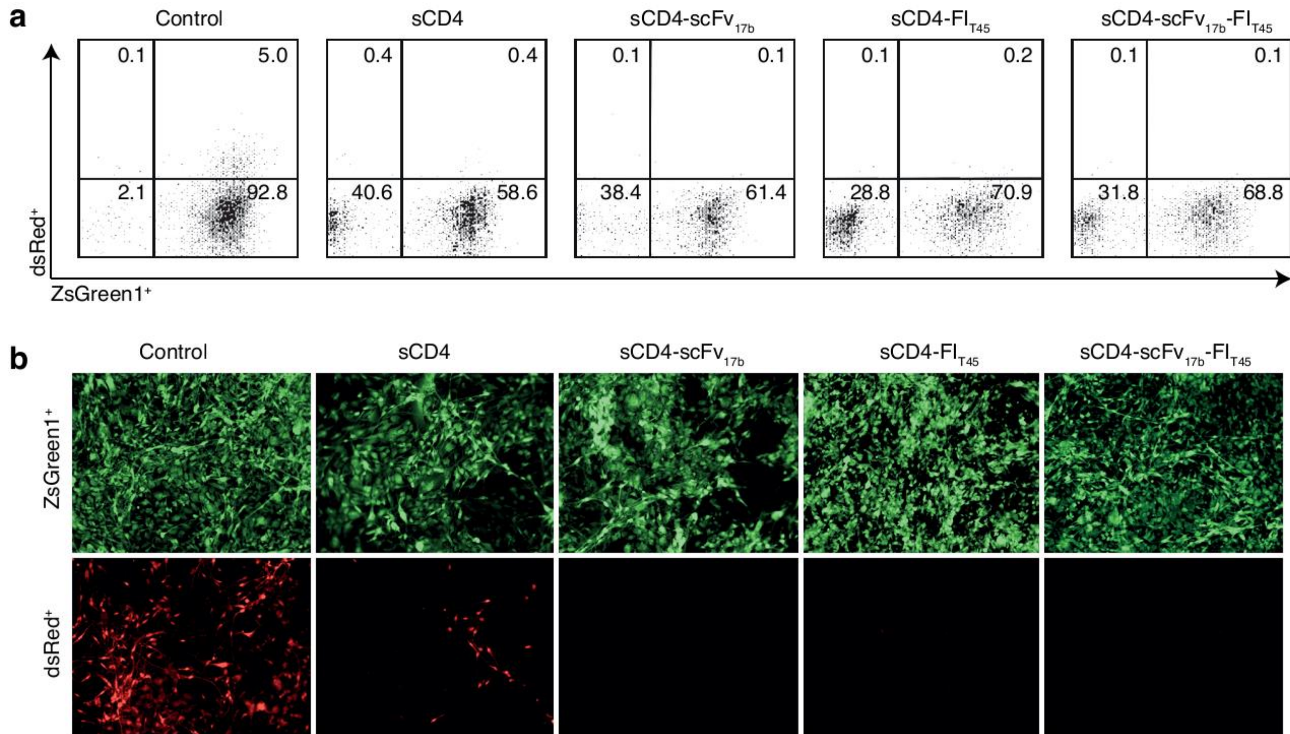

Figure 5. Gene-modified HIV target cells secreting antiviral proteins are protected from infection. U373-MAGI-CCR5E cells were transduced with the lentiviral vector particles encoding sCD4, sCD4-scFv$_{17b}$, sCD4-FI$_{T45}$, or sCD4-scFv$_{17b}$-FI$_{T45}$. Cells transduced with the control vector served as control. Upon transduction, gene-modified cells co-expressed ZsGreen1. The gene-modified cells were infected with HIV$_{JRFL}$ Env-pseudotyped reporter vector particles encoding dsRed. Infected gene-modified cells were dsRed$^+$ZsGreen1$^+$. **(a)** The number of gene-modified (ZsGreen1$^+$) and infected (dsRed$^+$) cells was determined by flow cytometry. (**b**) Infected (dsRed$^+$) gene-modified (ZsGreen1$^+$) cells were analyzed by fluorescence microscopy; scale bar = 100 nm.

present at undetectable levels, supernatant from cells secreting this protein did not affect viral entry.

To further investigate the antiviral activity of sCD4, sCD4-scFv$_{17b}$, sCD4-FI$_{T45}$, and sCD4-scFv$_{17b}$-FI$_{T45}$, we performed single-round infection assays with supernatants adjusted to contain increasing concentration of these proteins (Figure 4a). sscFv$_{17b}$ was included as a control. Since sFI$_{T45}$ was not secreted at detectable levels, we also included the Food and Drug Administration (FDA)-approved synthetic fusion inhibitor T-20 (FI$_{T20}$) in our assays.[52] At a concentration of 90 nM, sCD4-scFv$_{17b}$ and sCD4-FI$_{T45}$ mediated the strongest inhibition and reduced HIV$_{JRFL}$ entry by >99% ($p<0.05$). sCD4, FI$_{T20}$, and sCD4-scFv$_{17b}$-FI$_{T45}$ mediated >90% inhibition, whereas sscFv$_{17b}$ conferred ~50% inhibition. At a 2 nM concentration, FI$_{T20}$ inhibited HIV$_{JRFL}$ entry by 50% in our assays, which is consistent with the half maximal inhibitory concentration of FI$_{T20}$ for HIV$_{JRFL}$ in comparable assays (1.9 nM) reported by others.[53]

To assess whether addition of FI$_{T45}$ to sCD4 or sCD4-scFv$_{17b}$ further increases the neutralization potency of the respective molecules, equimolar concentrations of the secreted antiviral proteins and the synthetic FI$_{T20}$ were mixed, and tested along with sCD4-FI$_{T45}$ or sCD4-scFv$_{17b}$-FI$_{T45}$. Single-round infection assays were performed with reporter vector particles pseudotyped with the Env from a primary clade B isolate, HIV$_{REJO4541.67}$. Both sCD4-FI$_{T45}$ (Figure 4b) and sCD4-scFv$_{17b}$-FI$_{T45}$ (Figure 4c) fusion proteins were more potent than when sCD4 or sCD4-scFv$_{17b}$ was mixed with FI$_{T20}$. The antiviral activity of sCD4-FI$_{T45}$ was superior to that of sCD4, FI$_{T20}$, or non-covalent combinations thereof (Figure 4b), whereas sCD4-scFv$_{17b}$-FI$_{T45}$ and sCD4-scFv$_{17b}$ displayed comparable activities (Figure 4c). Inhibition of viral entry by synthetic FI$_{T20}$ has been reported to vary greatly among different primary isolates.[54,55] FI$_{T20}$ was relatively ineffective against the tested HIV isolate (50% inhibition at 100 nM in our assay), which is consistent with the previous data for the same HIV isolate (50% inhibition at 80 nM).[54]

Antiviral effect of the secreted proteins on gene-modified HIV target cells

To determine whether gene-modified HIV target cells are also protected from infection, the U373-MAGI-CCR5E cell line, which expresses CD4 and CCR5, was transduced with the lentiviral control vector particles and vector particles encoding sCD4, sCD4-scFv$_{17b}$, sCD4-FI$_{T45}$, or sCD4-scFv$_{17b}$-FI$_{T45}$. The gene-modified cells were infected with HIV$_{JRFL}$ Env-pseudotyped reporter vector particles expressing dsRed. Since gene-modified cells expressed ZsGreen1 and infected cells expressed dsRed, we analyzed the cells by flow cytometry to distinguish between gene-modified and unmodified cells that became infected (Figure 5a). Entry was reduced by 75% in cells expressing sCD4 and by >94% in cells expressing the sCD4-fusion proteins. The result was confirmed by fluorescence microscopy (Figure 5b).

Antiviral potency of purified proteins

sCD4, sCD4-scFv$_{17b}$, sCD4-FI$_{T45}$, and sCD4-scFv$_{17b}$-FI$_{T45}$ were purified from culture supernatants of gene-modified 293T cells *via* native affinity chromatography based on the C-terminal 6xHis-tag. Equal volumes of culture supernatant,

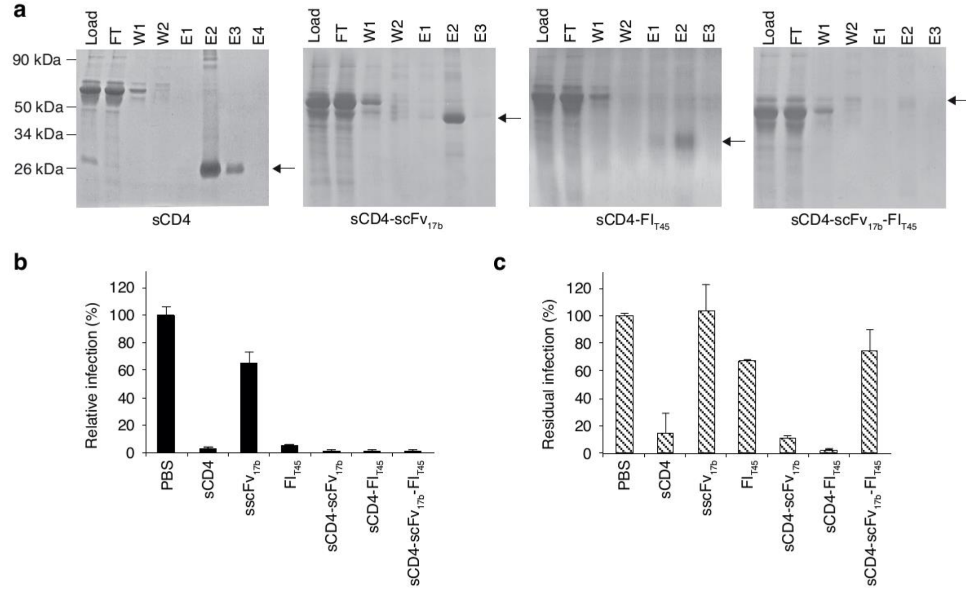

Figure 6. Purification of secreted antiviral proteins and inactivation of cell-free HIV. **(a)** sCD4, sCD4-FI_{T45}, sCD4-$sscFv_{17b}$, and sCD4-$scFv_{17b}$-FI_{T45} were enriched from culture supernatants of gene-modified 293T cells by native affinity chromatography based on the 6xHis-tag. The culture supernatant (load), flow through (FT), washes (W), and elution fractions (E) were separated by SDS-PAGE followed by Coomassie Blue staining. The name of the analyzed antiviral protein is indicated at the bottom of each gel. Arrows indicate the expected size of the antiviral protein. **(b** and **c)** HIV_{JRFL} Env-pseudotyped reporter vector particles were incubated in the presence of 90 nM purified antiviral proteins; n =2. **(b)** The mixture was added to unmodified U373-MAGI-CCR5E cells and $dsRed^+$ cells were quantified by flow cytometry 5 days post-infection; n = 2. **(c)** The reporter vector particles were separated from unbound proteins prior to addition to target cells by precipitation with PEG. The number of $dsRed^+$ cells was determined by flow cytometry 5 days post-infection; n = 2.

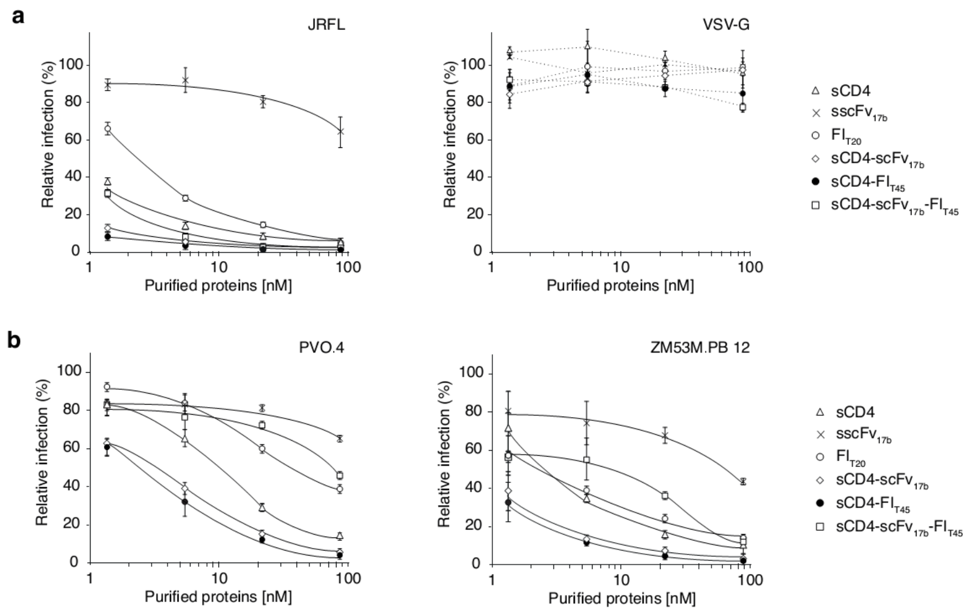

Figure 7. Antiviral effect of purified proteins. **(a)** Single-round infection assays were performed with HIV Env- or VSV-G-pseudotyped reporter vector particles in the presence of purified antiviral proteins. The number of $dsRed^+$ U373-MAGI-CCR5E cells was determined by flow cytometry on day 5 post-infection; n = 2. **(b)** Purified antiviral proteins were incubated with reporter vector particles pseudotyped with HIV Env glycoproteins from primary isolates PVO.4 (Clade B) or ZM53M.PB12 (Clade C) and added to U373-MAGI-CCR5E cells. The number of $dsRed^+$ cells was measured by flow cytometry on day 5 post-infection; n = 2.

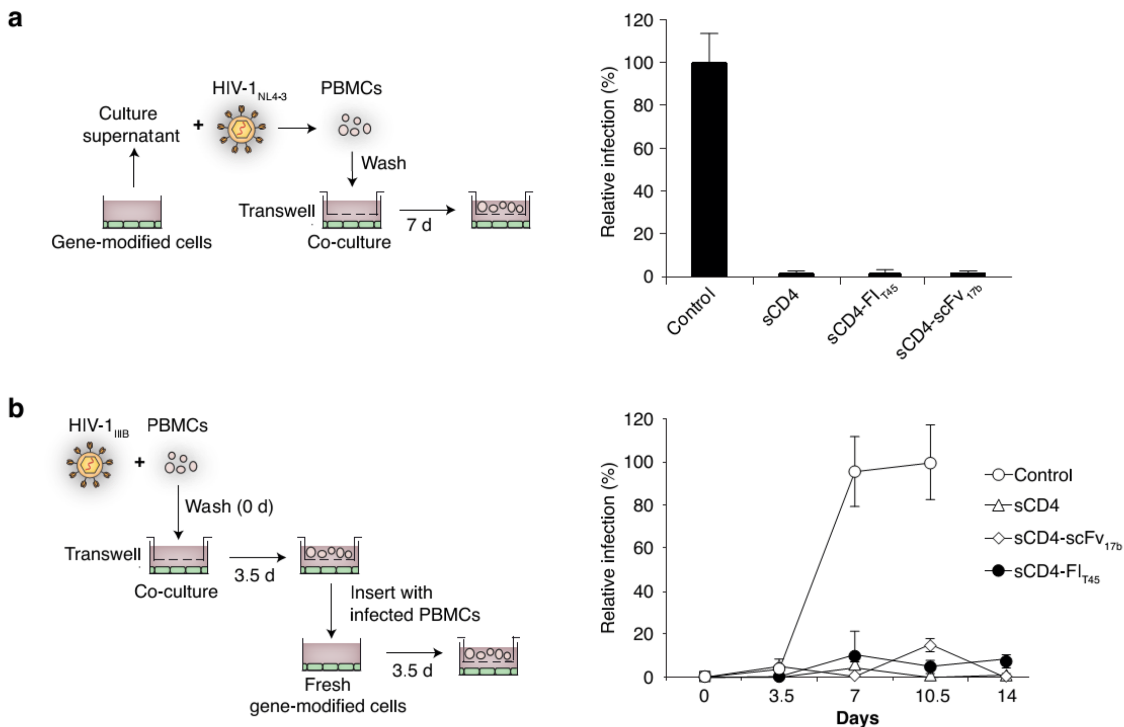

Figure 8. Unmodified PBMCs co-cultured with gene-modified 293T cells are protected from infection. **(a)** Culture supernatants from gene-modified 293T cells transduced with the control vector particles or vector particles encoding sCD4, sCD4-scFv$_{17b}$ or sCD4-FI$_{T45}$ were incubated with replication-competent HIV$_{NL4-3}$ and added to PBMCs. The virus was washed out and the infected PBMCs were co-cultured with the gene-modified 293T cells in transwell dishes. HIV p24 antigen levels were determined by ELISA on day 7 post-infection; n = 2. **(b)** PBMCs were infected with replication-competent HIV$_{IIIB}$ in the absence of antiviral proteins. The infected PBMCs were washed to remove the unbound virus and were co-cultured with the gene-modified 293T cells in transwell dishes. Every 3.5 days, the inserts containing the infected PBMCs were transferred to new dishes containing fresh gene-modified 293T cells and HIV p24 antigen levels were determined. p24 values in this experiment are representative of virus produced from the infected cells within the 3.5 days. The experiment was stopped at day 14 days post-infection; n = 2.

the column flow through, column washes, and eluted fractions from the same column were analyzed by SDS-PAGE, followed by Coomassie Blue staining (Figure 6a). Distinct bands corresponding to the expected sizes of antiviral proteins were detected in the second and third elution fractions. Diffuse bands corresponding to higher molecular weight proteins, possibly representing a minor contamination of the purified samples with serum proteins, were also present in these fractions.

The concentration of purified sCD4-scFv$_{17b}$ was determined by the Bradford assay. The concentration of the other purified antiviral proteins was determined by Western blot analysis and compared to serial dilutions of purified sCD4-scFv$_{17b}$. Compared to the concentrations of unpurified proteins present in the culture supernatants, the concentrations of the purified proteins were consistently lower than theoretically expected. It has previously been reported that affinity chromatography enrichment of secreted proteins from human cell culture supernatants is less efficient than enrichment of recombinant proteins expressed in bacteria.[56,57] The major obstacle seems to be the excess of serum proteins in the culture medium, which negatively affects the purification of proteins that are present at relatively low concentrations.[56,57] Accordingly, we observed the highest enrichment for sCD4 (8-9 fold), which was present at the highest concentration in the unpurified supernatant, followed by sCD4-FI$_{T45}$ (5-6 fold), sCD4-scFv$_{17b}$ (3-4 fold), and sCD4-scFv$_{17b}$-FI$_{T45}$ (~3 fold).

Purified sCD4, sCD4-scFv$_{17b}$, sCD4-FI$_{T45}$ and sCD4-scFv$_{17b}$-FI$_{T45}$ (90 nM) mediated a strong antiviral effect (Figure 6b). To determine whether the purified antiviral proteins can inactivate cell-free HIV, we incubated HIV$_{JRFL}$ Env-pseudotyped reporter vector particles with 90 nM purified proteins followed by precipitation with polyethylene glycol (PEG)[42] to separate the reporter vector particles from the unbound antiviral proteins. The precipitated reporter vector particles were then used to infect unmodified U373-MAGI-CCR5E cells. As shown in Figure 6c, sCD4, sCD4-scFv$_{17b}$, and sCD4-FI$_{T45}$ inactivated cell-free reporter vector particles. In contrast, sscFv$_{17b}$, FI$_{T20}$, and sCD4-scFv$_{17b}$-FI$_{T45}$ lost the majority of their antiviral activity in comparison to the unprecipitated vector particles, indicating that these proteins are not capable of inactivating cell-free virus.

Single-round infection assays were also performed in the presence of different concentrations of purified antiviral proteins. Inhibition of HIV$_{JRFL}$ Env-pseudotyped reporter vector particles with the purified proteins (Figure 7a) was comparable to the inhibition mediated by the unpurified proteins (Figure 4a), while entry of reporter vector particles pseudotyped with the Env of an unrelated virus, vesicular stomatitis virus (VSV)-G, was not affected by the proteins (Figure 7a). Two reporter vector particles pseudotyped with Envs from a clade B or clade C primary isolate were also strongly inhibited by sCD4-scFv$_{17b}$ and sCD4-FI$_{T45}$ (Figure 7b).

Protection of unmodified primary peripheral blood mononucleocytes (PBMCs)

To assess the protective effect of sCD4, sCD4-scFv$_{17b}$ and sCD4-FI$_{T45}$ in a more relevant cell type, gene-modified 293T producer cells that are not susceptible to HIV infection were co-cultured with unmodified PBMCs in transwell dishes, in which the two cell types are physically separated by a 0.45 µm filter that is permeable to macromolecules, such as proteins, but not cells (Figure 8a). Unadjusted culture supernatants from gene-modified 293T cells transduced with the control vector particles or vector particles encoding sCD4, sCD4-scFv$_{17b}$, or sCD4-FI$_{T45}$ were incubated with replication-competent X4 HIV$_{NL4-3}$ and the mixture was added to PBMCs. The cells were then washed to remove the free virus and co-cultured with producer cells in the transwell dishes. Producer to target cell ratio was 1:4 at the time of infection. All four secreted antiviral proteins reduced HIV$_{NL4-3}$ replication by >99% on day 7 post-infection (Figure 8a). The strong inhibition by sCD4 was most likely due to high concentration of this protein in the culture supernatant. Consistent with previous reports, it is clear that HIV can be inactivated if the concentration of sCD4 is high.[35,44,56] No cytotoxic effects were detected in gene-modified 293T cells or in the co-cultured PBMCs, as determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (results not shown).

In another experiment, we infected unmodified PBMCs with replication-competent X4 HIV$_{IIIB}$ in the absence of secreted antiviral proteins (Figure 8b). The infected PBMCs were washed to remove the free virus and were added to the inserts of transwell dishes that contained gene-modified producer cells in the lower compartment. Every 3.5 days, the producer cells needed replacement and the inserts containing the infected PBMCs were transferred to new dishes containing fresh producer cells. The remaining supernatants were used to determine the HIV p24 antigen levels. Because the majority of the old culture medium containing newly produced virus had to be removed during each PBMC transfer, the p24 values are representative of virus produced from the infected cells every 3.5 days. Progeny virus production at day 3.5 post-infection was at the limit of detection in the control and test samples. The control cells produced detectable amount of progeny virus on day 7 and 10.5 post-infection. At day 14, HIV p24 production was reduced in the control cells, potentially because infected cells started dying. sCD4, sCD4-scFv$_{17b}$ and sCD4-FI$_{T45}$ suppressed viral replication for the duration of the experiment. The secreted antiviral proteins were not expected to inhibit progeny virus production from the infected cells. However, our results indicate that they could prevent the newly produced virus from infecting unmodified cells, and thus contain viral spread.

DISCUSSION

In this study, we have assessed the feasibility of developing a HIV gene therapy approach using secreted antiviral proteins to confer protection to gene-modified and unmodified target cells. We have shown that sCD4, sCD4-scFv$_{17b}$, sCD4-FI$_{T45}$, and sCD4-scFv$_{17b}$-FI$_{T45}$ were secreted in significant quantities from gene-modified cells and protected both gene-modified and unmodified HIV target cells from infection.

Fusion proteins containing FI$_{T45}$ were efficiently secreted. However, sFI$_{T45}$ was not and culture supernatant from gene-modified cells transduced with the vector particles encoding sFI$_{T45}$ did not inhibit viral entry. We confirmed that the gene encoding sFI$_{T45}$ integrated into the genome of 293T cells and that it was transcribed. The absence of sFI$_{T45}$ in the supernatant could be due to protein instability or inefficient secretion despite meeting the theoretical minimum length requirement for secretion. Secreted proteins can enter the rough endoplasmic reticulum either co-translationally or post-translationally.[57] The majority of secreted proteins is over 100 amino acids in length and enters the endoplasmic reticulum co-translationally.[58,59] Proteins smaller than 100 amino acids seem to enter the endoplasmic reticulum post-translationally, because co-translational translocation may be inefficient due to the small time window for the recognition of the signal peptide sequence.[59] However, it has been suggested that the signal peptide sequences specify the targeting route to the endoplasmic reticulum membrane.[60] Pairing of the CD4 signal peptide with a short protein, such as a fusion inhibitor, may have induced co-translational translocation and caused inefficient secretion. Pairing a signal peptide from a small protein with fusion inhibitors should result in post-translational translocation and overcome this issue in the future.

The highest antiviral protein concentration that we detected in the culture supernatants of gene-modified 293T cells was for sCD4. Retroviral vectors have previously been used to express and secrete sCD4 from mammalian cells, but the reported sCD4 concentration in the culture supernatant was ~2900 times less than what we observed.[31] The increased secretion level from our gene-modified cells could be due to the use of a codon-optimized gene[61] and advanced vector design. The pLVX vector used in our study contains the cytomegalovirus immediate early promoter that has a strong transcription activity in the tested cell types.[62] This vector also contains the *cis*-acting woodchuck hepatitis virus post-transcriptional regulatory element, which facilitates transgene expression,[62] and a central polypurine tract that enhances transduction of target cells.[63] In comparison to sCD4, we observed a decrease in the level of secretion of fusion proteins. Since a relatively long linker (L_7) was used to connect the different antiviral moieties in the fusion proteins, improper protein folding and/or protein instability could have caused this decrease. Our results indicate that the secreted proteins were stable at 37°C for several days with no significant difference between sCD4 and the sCD4-derived fusion proteins. However, protein misfolding in the rough endoplasmic reticulum followed by degradation could have reduced the amount of secreted fusion proteins. Signal peptides from the tissue plasminogen activator and *Gaussia princeps* luciferase were shown to increase secretion of hepatitis C virus glycoproteins.[63] These signal peptides may be used in the future to test whether they can enhance secretion of sCD4-derived fusion proteins.

sCD4 and sCD4-derived bi- and tri-functional fusion proteins significantly inhibited viral entry. The weak antiviral activity observed for sscFv$_{17b}$ and sscFv$_{17b}$-FI$_{T45}$ may be explained by the fact that both of these proteins target transient epitopes on the virus that only become accessible upon viral binding to the target cells.[45,64] Consistent with the finding that the sequential HIV entry process leaves the virus vulnerable to cooperative inhibition, sCD4-FI$_{T45}$ exhibited a strong antiviral activity. sCD4 has been shown to expose gp41 in surface-expressed HIV Env proteins in the absence of any interaction with cellular CD4 or CCR5/CXCR4.[65] The sCD4 moiety has a high affinity for gp120 and binds Envs from laboratory-adapted and primary isolates with equal potency.[66] Linking FI$_{T45}$ to sCD4 increased the size of the protein, which in turn may have further enhanced inhibition due to steric hindrance. However, as the larger four-domain sCD4 has a very similar anti-HIV activity compared to the two-domain sCD4 used in

our study,[67] the enhanced inhibition by sCD4-FI_{T45} cannot be entirely due to increased steric hindrance. It is more likely that the sCD4 moiety in sCD4-FI_{T45} brings FI_{T45} into proximity of gp41. Since sCD4-gp120 interaction also exposes binding sites for fusion inhibitors on gp41,[47] FI_{T45} could then bind to the HIV Env in the absence of target cells and inactivate the virus. sCD4-$scFv_{17b}$-FI_{T45} also inhibited viral entry. However, the antiviral effect was not increased in comparison to sCD4-$scFv_{17b}$. A possible explanation for this result could be that the different antiviral moieties in the triple fusion protein interfere with each other, resulting in a lower binding affinity for the virus. Indeed, our results indicate that sCD4-$scFv_{17b}$-FI_{T45} is less efficient than sCD4 at inactivating cell-free HIV.

We did not observe any cytotoxicity for any of the secreted antiviral proteins. Administration of purified sCD4 or FI_{T20} was found to be safe in clinical trials.[36,56,68] The safety of expressing membrane-bound FI_{C46} has also been demonstrated in clinical trials.[24] The bi-functional proteins sCD4-$scFv_{17b}$ and sCD4-FI_{T45} tested in our study inhibit two major steps of viral entry. Targeting multiple, sequential steps of entry with a single antiviral protein could surpass results obtained with inhibitors that only target a single step. Even though the individual proteins are well tolerated, bi-functional proteins can be immunogenic due to the presence of additional epitopes. Identifying and removing such epitopes by *in silico* screening and altering amino acid residues that do not affect the antiviral potency could lead to less immunogenic variants.[69] Additionally, *in vivo* expression and secretion from gene-modified human cells yields antiviral proteins with a human glycosylation pattern, which should reduce potential immunogenicity, increase stability, and reduce the clearance rate of antiviral proteins from the blood.[70]

An advantage of secreted antiviral proteins over conventional HIV gene therapy strategies is that *in vivo* production of these proteins is not limited to HIV target cells and could be achieved by either *ex vivo* or direct *in vivo* modification of various cell types. Hematopoietic stem/progenitor (HSP) cells have successfully been transduced with lentiviral vectors *ex vivo*[5,71] and are attractive targets for the delivery of genes encoding secreted antiviral proteins. Our preliminary results indicate that gene-modified HSP cells expressing sCD4 and sCD4-derived fusion proteins develop normally into progenitor T cells (unpublished data). Continuous secretion of antiviral proteins from all hematopoietic cell lineages would drastically increase the ratio of gene-modified producer cells to unmodified HIV target cells, since there are usually twice as many B cells and five times more neutrophils than T cells in the blood.[72] Gene-modified macrophages, B cells, and T cells would also be present directly at the sites of HIV replication. Secretion by these cell types may lead to high local antiviral protein concentrations in tissues and organs that antiretroviral drugs and recombinant proteins fail to reach. Ideally, expression of secreted antiviral proteins from the progeny of gene-modified HSP cells should also lead to a state of immune tolerance upon reconstitution of the immune system.

While HSP cell gene therapy holds great potential for the treatment of HIV-infected individuals, it requires *ex vivo* gene delivery, which is not the preferred treatment option in the developing world. Hence, a direct *in vivo* gene delivery into organs that are more accessible may also be envisaged for systemic (*e.g.* muscle for treatment) or local (*e.g.* vaginal epithelial cells for prevention) production and secretion of antiviral proteins. Secretion of therapeutic proteins from gene-modified skin and muscle cells has been evaluated for the treatment of other diseases, such as erythropoietin deficiency, α-1 antitrypsin deficiency, hemophilia A, and Duchenne muscular dystrophy.[73-76] Skin and muscle tissues are readily accessible and are highly vascularized, making them suitable for systemic delivery of therapeutic proteins *via* circulation. Indeed, serum levels of therapeutic proteins reached 1000 μg/ml *in vivo*.[74] This is well above the sCD4 concentration required for neutralization of primary HIV strains.[56,77,78] Based on these and our findings, continuous delivery of secreted bi-functional antiviral proteins *via* gene therapy may lead to therapeutic concentrations of these proteins *in vivo*. Systemic and local inhibition of HIV infections by these proteins could lead to long-term control of HIV infection.

MATERIALS AND METHODS

Cells, plasmids, and peptides

Human embryonic kidney 293T cells[79] were obtained from Dr. Jason Moffat (University of Toronto, Toronto, ON) and were cultured in complete DMEM (Life Technologies, Carlsbad, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, USA) and 1% Antibiotic-Antimycotic (Life Technologies). $CD4^+CCR5^+$ U373-MAGI-CCR5E cells[80] were obtained from Dr. Michael Emerman (AIDS Research and Reference Reagent Program). These cells were cultured in complete DMEM supplemented with 0.2 mg/ml G418 (Sigma-Aldrich, St. Louis, USA), 0.1 mg/ml hygromycin B (Life Technologies), and 1 µg/ml puromycin (Bioshop, Burlington, ON). PBMCs isolated from the blood of healthy volunteers were obtained from Dr. Mario Ostrowski (University of Toronto) and were activated for 2 days in RPMI 1640 (Life Technologies) supplemented with 20% FBS, 4 mM L-glutamine (Life Technologies), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Life Technologies), 1% phytohemagglutinin-M (PHA-M, Life Technologies), and 10 units/ml interleukin-2 (Life Technologies). PBMCs were propagated in the same medium without PHA-M, hereafter referred to as PBMC culture medium. The lentiviral transfer vector pLVX was obtained from Clontech (Mountain View California, USA). pLJM2 (a lentiviral transfer vector expressing dsRed), psPAX2 (a lentiviral packaging construct expressing HIV Gag, Gag/Pol, Tat, and Rev proteins), and pMD2.G (an Env-expressing plasmid encoding VSV-G) were obtained from Dr. Moffat. The pJRFL-env plasmid was a kind gift from Dr. Donald Branch (University of Toronto). Plasmids expressing Env from primary HIV isolates were obtained through the AIDS Research and Reference Reagent Program as part of the HIV clade B and C reference panels.[81-85] Synthetic FI_{T20} was also obtained from the AIDS Research and Reference Reagent Program.

Lentiviral vectors expressing secreted antiviral proteins

The genes encoding various secreted antiviral proteins were codon-optimized for expression in human cells. These genes were synthesized, cloned in pUC57, and sequenced by Genscript (Piscataway, USA). pLVX-sCD4-FI_{T45} was generated by inserting the sCD4-FI_{T45} gene as an *Eco*RI-*Bgl*II fragment into the *Eco*RI and *Bam*HI sites of pLVX. pLVX-sCD4 was obtained by *Ngo*MIV deletion of pLVX-sCD4-FI_{T45}. $scFv_{17b}$ was inserted as a *Bam*HI fragment into pLVX-sCD4-FI_{T45} to obtain pLVX-sCD4-$scFv_{17b}$-FI_{T45}. An *Ngo*MIV deletion of pLVX-sCD4-$scFv_{17b}$-FI_{T45} generated pLVX-sCD4-$scFv_{17b}$. pLVX-sFI_{T45} was constructed by deleting the sCD4 domain from pLVX-sCD4-FI_{T45} by *Eco*RI and *Spe*I digestion and inserting the signal peptide from pUC57-SP as an *Eco*RI and *Spe*I fragment. To construct pLVX-$sscFv_{17b}$ and pLVX-$sscFv_{17b}$-FI_{T45}, the linker region preceding the

$scFv_{17b}$-coding region had to be removed. Therefore, pUC57-$scFv_{17b}$ was digested with *Bsp*EI and *Pst*I. An oligonucleotide containing an additional *Bam*HI site, Bam-IVM, was inserted between the linker and $scFv_{17b}$ sequence. The resulting *Bam*HI fragment was cloned into the *Bam*HI site of pLVX-sFI_{T45} to generate pLVX-$sscFv_{17b}$-FI_{T45}. pLVX-$sscFv_{17b}$ was constructed by an *Ngo*MIV deletion of pLVX-$sscFv_{17b}$-FI_{T45}.

Generation of VSV-G Env-pseudotyped lentiviral vector particles and transductions

Amphotropic lentiviral vector particles were generated by transient calcium phosphate transfection of 293T cells as described previously.[86] Briefly, 293T cells were grown to 90% confluence in a 10 cm dish and co-transfected with each of the lentiviral transfer vector constructs (10.5 µg), psPAX (7.0 µg), and pMD2.G (3.4 µg). 16 hours later, the culture supernatants were exchanged with 8 ml of fresh medium. The supernatants containing the vector particles were harvested every 12 hours for a total of 4 times. Subsequently, the supernatants were clarified using filters with a 0.45 µm pore size and concentrated by PEG precipitation as follows.[87] PEG 8000 (8.5%, Sigma-Aldrich) and NaCl (0.25%) were added to the vector particles and after a 1.5 hour incubation at 4°C, the mixture was centrifuged at 3600x*g* for 10 minutes at 4°C. The pellet was resuspended in complete DMEM. 293T cells were transduced with serial dilutions of vector particle stocks and the number of $ZsGreen1^+$ cells was counted to determine the transducing units (TU) per ml. To generate producer cells secreting antiviral proteins, $2x10^5$ HIV non-target (293T) or target (U373-MAGI-CCR5E) cells were transduced with vector particles (1-2 TU/cell) in the presence of 8 µg/ml polybrene (Sigma-Aldrich).

PCR and RT-PCR

Genomic DNA from gene-modified cells was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. The primers used to amplify the genes encoding the antiviral proteins were designed to hybridize to the N-terminal signal peptide (SP-F primer) and C-terminal 6xHis-tag (Tag-R primer) sequences. Two additional primers were designed to hybridize to the upper (17b-F primer) and lower (17b-R primer) DNA strands within the *scFv17b*-coding region. For amplification of the large gene encoding the triple fusion protein, SP-F/17b-R and 17b-F/Tag-R primer pairs were used. The primer sequences were as follows: SP-F, 5′-CGT-GCC-TTT-CAG-ACA-CCT-G-3′; Tag-R, 5′-GAT-GGT-GAT-GGT-GAC-AGC-AG-3′; 17b-F, 5′-GAA-CTG-CGG-AAC-CTG-AGA-AG-3′; and 17b-R, 5′-CTT-CTC-AGG-TTC-CGC-AGT-TC-3′. The primers were synthesized by ACGT DNA Technologies Corporation (Toronto, ON). PCR conditions were 0.5 µg gDNA, 250 µM dNTPs, 50 mM KCl, 10 mM Tris-Cl pH 8.8, 1.5 mM $MgCl_2$, 3 mM DTT, 100 µg/µl BSA, 1.5 µM each forward and reverse primers, and 2.5 units taq polymerase. The cycles were: 1) 94°C for 5 minutes; 2) 94°C for 1 minute; 3) 60°C for 1 minute; 4) 72°C for 2 minutes, and 5) 72°C for 10 minutes. Cycles 2-4 were repeated 30 times. For RT-PCR analyses, total RNA from the gene-modified cells was extracted using the PureLink RNA mini kit (Invitrogen) according to the protocol provided by the manufacturer. The cDNA synthesis was performed using the First Strand synthesis kit (Promega) as per manufacturer's instructions. PCR on the cDNA was performed using the conditions described above.

Detection, quantification, and purification of antiviral proteins

High concentration of serum proteins present in the culture supernatants interfered with the detection and purification of secreted antiviral proteins. Therefore, gene-modified 293T cells were grown to 95% confluence and the medium was replaced with DMEM containing a reduced concentration of FBS (1% instead of 10%). The cells were incubated for 5 days before the culture supernatants were harvested and filtered through a 0.45 µm filter. For Western blot analyses, culture supernatants were analyzed by SDS-PAGE. The proteins were transferred to BioTrace 0.45 µm polyvinylidene difluoride transfer membranes (Port Washington, USA) using the Trans-Blot Semi-Dry Transfer system (Bio-Rad, Hercules, USA). The membranes were blocked with 5% skim milk in PBST (phosphate buffered saline with 0.5% Tween-20, Sigma-Aldrich) for 1 hour. Once blocked, the membranes were incubated for 1.5 hours with anti-6xHis mouse mAb conjugated to horseradish peroxidase (Clontech) at a 1:1000 dilution in PBS. The membranes were washed three times with PBST for 10 min each. 1 ml of enhanced chemiluminescent solution (FroggaBio, North York, ON) was added to each membrane for 1 minute. The membranes were then exposed to CL-Xposure Films (Pierce Biotechnology, Rockford, USA) or scanned using the ChemiDoc MP System (Bio-Rad). For affinity chromatography enrichment, 40 ml of culture supernatants containing the antiviral proteins were loaded onto HisTALON cobalt columns (Clontech) under native conditions according to the manufacturer's instructions. The purified proteins were applied to Zeba Spin columns (Thermo Fisher Scientific) to remove salts and small molecules from the samples and exchange the buffer to PBS according to the instructions from the manufacturer. The concentration of purified sCD4-$scFv_{17b}$ was determined using the Bradford protein assay (Bio-Rad) according to the manufacturer's instructions. To determine the concentration of purified proteins as well as of unpurified proteins present in the culture supernatants, serial dilutions of these proteins were analyzed by Western blot along with known amounts of purified sCD4-$scFv_{17b}$. The band intensities were compared using the software program ImageJ.[88]

Single-round infection assays

Replication-incompetent HIV Env-pseudotyped reporter vector particles expressing dsRed were generated by calcium phosphate co-transfection of 293T cells using pLJM2 (9 µg), psPAX (6 µg), and an HIV Env-encoding plasmid (6 µg). 16 hours after addition of the plasmids, the supernatants were exchanged with 8 ml of fresh medium. 30 hours later, the supernatants were filtered through 0.45 µm filters, aliquoted, and stored at -80°C. For single-round infection assays, all infections were performed at a multiplicity of infection (moi) of 0.05-0.10 except for the experiments shown in Figure 3c (moi of 0.27) and Figure 4c (moi of 0.20). Data are presented as the mean with error bars representing the standard deviation. Data were analyzed by Student's t-test for significance and considered significantly different if $p < 0.05$. For infections of unmodified cells, $1.2x10^5$ U373-MAGI-CCR5E cells were seeded into 12-well plates for 24 hours. On the day of infection, HIV Env-pseudotyped reporter vector particles were incubated with the antiviral proteins for 30 minutes, mixed with 8 µg/ml polybrene, and then added to the cells. For infection of gene-modified U373-MAGI-CCR5 cells, $1x10^5$ cells were seeded in a 12-well plate and cultured for 2 days. The old medium was replaced with medium containing HIV Env-pseudotyped vector particles with 8 µg/ml polybrene. For cell-free binding assays, the reporter vector particles were pre-incubated with the antiviral proteins for 30 minutes at 37°C. The reporter vector particles were precipitated with PEG 8000 (3%) and NaCl (0.25%) by incubating the mixture for 1.5 hours at 4°C, followed by a 30 minute centrifugation at 14000xg at 4°C.

The vector particles were then washed with 3% PEG 8000 in PBS supplemented with 1 mg/ml BSA. The pellets were resuspended in complete DMEM and the vector particles were used to infect unmodified U373-MAGI-CCR5E cells cultured as described above. In all single-round infection assays, the media were changed 24 hours post-infection and the cells were cultured for an additional 4 days. The number of dsRed$^+$ cells was determined by flow cytometry or fluorescence microscopy.

Co-culture experiments
2x10^5 gene-modified 293T cells were seeded in the lower compartment of 24-well transwell dishes (Corning Inc., Corning, USA) in 600 µl of complete DMEM and grown for 2 days. The culture supernatants containing the antiviral proteins were removed and PBMC culture medium was added. 250 µl of culture supernatants from gene-modified 293T producer cells were each incubated with 250 µl of the virus stock containing 325 pg of replication-competent HIV_{NL4-3} strain for 30 minutes. Subsequently, the mixture was added to 1x10^6 activated PBMCs in 500 µl PBMC culture medium. After a 4 hour-incubation at 37°C, the cells were washed four times with RPMI 1640, resuspended in 100 µl PBMC culture medium, and transferred to the inserts of the 24-well transwell dishes. On day 7 days post-infection, the HIV p24 antigen concentration in the culture supernatants was analyzed with a p24 ELISA kit (Beckman Coulter, Brea, USA) according to the manufacturer's instructions. A relative infection of 100% corresponded to 190 ng/ml of HIV p24 antigen in the control cells. Data are presented as the mean with error bars representing the standard error. To determine whether antiviral proteins can contain infection, 2-2.5x10^5 gene-modified 293T cells were plated in the lower compartment of 24-well transwell dishes in 600 µl of PBMC culture medium without interleukin-2 and cultured for 3 days. On the day of infection, interleukin-2 was added to the gene-modified 293T cells at a final concentration of 20 units/ml. In parallel, 1x10^6 activated PBMCs in 1 ml of PBMC culture medium were infected with 5 µl of the virus stock containing 5 ng of replication-competent HIV_{IIIB} for 4 hours at 37°C without antiviral proteins. The cells were washed to remove the unbound virus as described above and the infected PBMCs were added to the inserts of the transwell dishes that contained the gene-modified producer cells (total culture volume ~700 µl). Every 3.5 days, the inserts containing the infected PBMCs (insert culture volume ~100 µl) were transferred to 24-well dishes containing fresh gene-modified producer cells cultured as described above. The remaining PBMC culture medium from the lower compartment was analyzed using a p24 ELISA kit (ZeptoMetrix, Buffalo, NY, USA). A relative infection of 100% corresponded to 13 pg/ml of HIV p24 antigen in the control cells at day 10.5 post-infection. Data are presented as the mean with error bars representing the standard error.

Cytotoxicity of secreted antiviral proteins
2x10^5 gene-modified 293T cells expressing antiviral proteins were seeded in 600 µl complete DMEM in 24-well dishes and grown for 2 days. The supernatants were then replaced with 600 µl PBMC culture medium. In parallel, PBMCs were treated as described above for infection with HIV_{NL4-3}, except that no virus was added. The mock-infected PBMCs were added to the gene-modified 293T cells in transwell dishes, separating the two cell types. The cells were grown for additional 7 days. MTT assays (Life Technologies) were performed on 293T cells and on PBMCs according to the instructions from the manufacturer.

COMPETING INTERESTS

The authors declare no competing interest.

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