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Glycosyl Phosphatidylinositol-Anchored C34 Peptide Derived From Human Immunodeficiency Virus Type 1 Gp41 Is a Potent Entry Inhibitor

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Abstract

Lipid rafts of the plasma membrane have been shown to be gateways for HIV-1 budding and entry. In nature, many glycosyl-phosphatidylinositol (GPI) anchored proteins are targeted to the lipid rafts. In the present study we constructed two fusion genes, in which C34 peptide or AVF peptide control was genetically linked with a GPI-attachment signal. Recombinant lentiviruses expressing the fusion genes were used to transduce TZM.bl and CEMss-CCR5 cells. Here, we show that with a GPI attachment signal both C34 and AVF are targeted to the lipid rafts through a GPI anchor. GPI-C34, but not GPI-AVF, in transduced TZM.bl cells efficiently blocks the infection of diverse HIV-1 strains of various subtypes. GPI-C34-transduced CEMss-CCR5 cells are totally resistant to HIV-1 infection. Importantly, maximum percentage of inhibition (MPI) by GPI-C34 is comparable to, if not higher than, a very high concentration of soluble C34. Potent blocking by GPI-C34 is likely due to its high local concentration, which allows GPI-C34 to efficiently bind to the prehairpin intermediate and prevent its transition to six helical bundle, thereby interfering with membrane fusion and virus entry. Our findings should have important implications in GPI-anchor-based therapy against HIV-1.

Keywords

HIV-1; lipid rafts; GPI anchor; C34 peptide; gene delivery

Human Immunodeficiency virus type 1 (HIV-1) envelope spike is a trimeric form of the gp120/gp41 heterodimer. Gp120 interacts with the CD4 receptor and CCR5 or CXCR4 co-receptor on the surface of target cells. HIV-1 gp120/gp41 then goes through a series of

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Compliance with Ethical Standards

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

conformational changes that leads to the insertion of fusion peptide of gp41 into target cell membrane and ultimately viral and cellular membrane fusion (Eckert & Kim, 2001). The ectodomain of gp41 in its fusogenic and post-fusogenic states consists of a hairpin trimer. Each subunit of the trimer comprises N- and C-terminal helices connected by a 25 to 30 amino acid residue loop. The N-terminal helices form a parallel, trimeric coiled-coil in the interior of the complex surrounded by C-terminal helices oriented antiparallel to the N-terminal helices (Chan, Fass et al., 1997, Weissenhorn, Dessen et al., 1997). Peptides derived from N- or C-terminal helices inhibit envelope-mediated fusion at micromolar or nanomolar concentration (Bewley, Louis et al., 2002, Chan, Chutkowski et al., 1998, Chong, Yao et al., 2012, Egelhofer, Brandenburg et al., 2004, Ingallinella, Bianchi et al., 2009, Lalezari, Henry et al., 2003, Liu, Lu et al., 2005, Malashkevich, Chan et al., 1998, Melikyan, Egelhofer et al., 2006, Stoddart, Nault et al., 2012, Wexler-Cohen & Shai, 2009). One such peptides, enfuvirtide, has been used for treatment of therapy-experienced AIDS patients (Lalezari et al., 2003). Another such peptides, C34, corresponding to amino acid residues 628–661 of C-terminal helices, is also a potent fusion inhibitor (Chan et al., 1998, Malashkevich et al., 1998). However, due to its very short half-life C34 is not suitable as a therapeutic. Because of this various modifications of C34 such as sequence variants, albumin-conjugation, and lipopeptides have been synthesized in order to further improve potency and breadth of the peptide (Chong et al., 2012, Ingallinella et al., 2009, Liu et al., 2005, Stoddart et al., 2012). For example, Ingallinella *et al.* chemically linked C34 with a cholesterol group (C34-Chol). When C34-Chol is incubated with target cells, cholesterol anchors C34 to the plasma membrane. They demonstrated that membrane-anchored C34 dramatically increases antiviral potency and more favorable pharmacokinetics (Ingallinella et al., 2009).

Lipid rafts are specialized dynamic microdomains of the plasma membrane that have been shown to be gateways for HIV-1 budding (Liao, Cimasky et al., 2001) as well as for HIV-1 entry into T cells and macrophages (Carter, Bernstone et al., 2009, Chazal & Gerlier, 2003, Liao et al., 2001). CD4 molecules are located in the lipid rafts of the plasma membrane (Platt, Wehrly et al., 1998, Popik, Alce et al., 2002). In nature, glycosyl-phosphatidylinositol (GPI) anchor is a post-translation modification and many GPI-anchored proteins are targeted to the lipid rafts. The GPI anchor attachment signals in some GPI-anchored proteins are well characterized (Medof, Kinoshita et al., 1984). Previously, we showed that by genetically linking single-chain Fv (scFv) or third-heavy-chain complementarity-determining region (HCDR3) of human anti-HIV-1 envelope antibodies with a glycosyl-phosphatidylinositol (GPI) attachment signal from decay accelerating factor (DAF) (Medof et al., 1984), scFvs and HCDR3s are targeted to lipid rafts of the plasma membrane. GPI-scFv X5 and 48d and GPI-HCDR3 PG9 and PG16 on the surface of transduced human CD4⁺ cell lines exhibit potent neutralization of diverse HIV-1 strains (Liu, Wen et al., 2011, Wen, Arora et al., 2010). Recently we showed that trimerization of GPI-HCDR3 PG9 and PG16 further improves anti-HIV-1 neutralizing activity (Liu, Wang et al., 2013).

In the present study, we constructed fusion genes in which sequences encoding the C34 or control peptide AVF and an IgG3 hinge region and histidine (his) tag were genetically linked with the sequence encoding the GPI attachment signal of DAF. The peptide AVF was derived from third-heavy-chain complementarity-determining region (HCDR3) of antibody

AVF, which recognizes the influenza virus hemagglutinin (Hu, Voss et al., 2012). The fusion genes were inserted into the third generation lentiviral vector pRRLsin-18.PPT.hPGK.Wpre (Follenzi, Ailles et al., 2000). Recombinant viruses were produced and used to transduce human CD4⁺ cell lines TZM.bl and CEMss-CCR5. Anti-HIV-1 activity of GPI-C34 on the surface of transduced human CD4⁺ cell lines was tested with diverse HIV-1 strains of various subtypes.

Materials and Methods

Cell lines

The packaging cell line 293FT was purchased from Invitrogen Life Technologies and was maintained in complete Dulbecco's modified Eagle's medium (DMEM) (i.e., high-glucose DMEM supplemented with 10% fetal bovine serum [FBS], 2mM L-glutamine, 1 mM sodium pyruvate, penicillin [100 U/ml], and streptomycin [100 µg/ml] plus G418 (500 µg/ml) (Invitrogen Life Technologies). The human CD4 T cell line CEMss-CCR5 was generated as described previously (21). TZM.bl cells were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP; Germantown, MD), contributed by J. Kappes and X. Wu (Derdeyn, Decker et al., 2000, Platt, Bilska et al., 2009, Platt et al., 1998, Takeuchi, McClure et al., 2008, Wei, Decker et al., 2002). CEMss-CCR5 and TZM.bl cells were maintained in complete DMEM.

Gene constructs

Fusion genes encoding C34 or AVF peptide, an IgG3 hinge, a his tag, with or without a GPI attachment signal (a C-terminus 34 amino acid residues of DAF) were generated by overlapping PCR, ligated into the TA vector system (Invitrogen Life Technologies, San Diego, CA), and sequenced as described previously (Liu et al., 2011). The fusion genes with the correct sequences were ligated between the Bam HI and Sal I sites of a third-generation lentiviral transfer vector, pRRLsin-18.PPT.hPGK.Wpre (Follenzi et al., 2000). The resulting lentiviral transfer constructs were designated pRRL-C34 or AVF/hinge/His tag/DAF (Fig 1A).

Generation of recombinant lentiviral viruses

Recombinant lentiviral viruses expressing fusion genes were generated as described previously (Liu et al., 2011). Briefly, 4 X 10⁶ 293FT cells were seeded onto a P-100 dish in 10 ml complete DMEM. After culturing overnight, cells were cotransfected with 20 µg transfer construct the pRRL-C34 or AVF/hinge/his-tag/DAF, 10 µg packaging construct encoding HIV-1 Gag/Pol (pLP1), 7.5 µg plasmids encoding the VSV-G protein envelope (pLP/VSV-G), and 7.5 µg HIV-1 Rev protein (pLP2) (Invitrogen), using a calcium phosphate precipitation method. Sixteen hours later, culture supernatants were removed and replaced with fresh complete DMEM plus 1 mM sodium butyrate (Sigma). Eight hours later, supernatants were again removed and replaced with fresh DMEM plus 4% FBS. After another 20 hours, the culture supernatants were harvested and concentrated by ultracentrifugation as described previously (Liu et al., 2011). The vector pellets were resuspended in a small volume of DMEM and stored in aliquots in a -80°C freezer. Vector titers were determined as previously described (Liu et al., 2011). The amount of HIV-1 gag

p24 in concentrated vector stocks was determined by an enzyme-linked immunosorbent assay (ELISA) (see below).

Generation of stably transduced TZM.bl and CEMss-CCR5 cells

To generate stably transduced TZM.bl cell lines, 5×10^4 TZM.bl cells per well were seeded onto a 24-well plate. After overnight culture, 2×10^6 TU of one of the recombinant lentiviral viruses expressing C34 or AVF/hinge/his-tag/DAF was added onto a 24-well tissue culture plate in the presence of 8 $\mu\text{g/ml}$ of polybrene. Twenty-four hours later cells were extensively washed and cultured in complete DMEM. The expression of pRRL-C34 or AVF/hinge/his-tag/DAF construct were measured by FACS analysis and (see below). We usually found that after a single round of transduction, over 98% of cells express transgenes (data not shown). After transduced cells were generated, cells were continuously cultured in complete DMEM and split every 3 or 4 days. Periodically, the expression of transgenes was measured. We found that the level of transgene expression was very stable in the stably transduced cell lines (Liu et al. data not shown).

Fluorescence-activated cell sorter (FACS) analysis

To measure the cell surface expression of GPI-C34 or AVF, 2×10^5 mock- and C34 or AVF/hinge/his-tag/DAF-transduced TZM.bl or CEMss-CCR5 cells were incubated with a mouse anti-his tag antibody for 45 min on ice. Cells were then washed twice with FACS buffer and stained with phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody (Sigma) for another 45 min on ice. Cells then were washed twice with FACS buffer and fixed with 1% formaldehyde in 0.5 ml of FACS buffer. FACS analysis was performed with a FACScan instrument (Becton Dickinson, Mountain View, CA).

To determine whether the expression of fusion genes is truly targeted through a GPI anchor, 8×10^5 mock- and C34 or AVF/hinge/his-tag/DAF-transduced TZM.bl cells were first incubated with or without 6 U/ml phosphatidylinositol-specific phospholipase C (PI-PLC) (Invitrogen) in 0.5 ml 1 x PBS and rocked at 4°C for 20 min. After the incubation, cells were washed twice to remove the remaining PI-PLC and then stained with a mouse anti-his tag antibody as described above.

To determine whether the expression of GPI-C34 or AVF altered the cell surface expression of CD4, CCR5 and CXCR4, 2×10^5 mock- and GPI-C34 or AVF-transduced TZM.bl or CEMss-CCR5 cells were incubated with PE-conjugated anti-human CD4 (BD Science), PE-conjugated anti-human CCR5 (BD Science), or PE-conjugated anti-human CXCR4 (BD Science) antibodies for 45 min on ice. Cells were then washed twice with FACS buffer and fixed with 1% formaldehyde in 0.5 ml of FACS buffer. FACS analysis was performed with a FACScan instrument (Becton Dickinson, Mountain View, CA).

To measure GFP expression, 1×10^5 VSV-G pseudotype-transduced CEMss-CCR5-GPI-C34 or AVF cells were collected 4 days after the transduction (see below), washed twice with FACS buffer (phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA] and 0.02% NaN₃) and fixed with 1% formaldehyde in 0.5 ml of FACS buffer. GFP expression was analyzed using the FACScan (Becton Dickinson, Mountain View, CA).

Generation of pseudotypes of HIV-1 vector and a single-cycle infectivity assay

To generate pseudotypes with HIV-1 vector, 4×10^6 293T packaging cells were cotransfected with 10 μ g of an HIV-1-luciferase transfer vector (He, Choe et al., 1995) and 1 μ g of a DNA plasmid encoding several HIV-1 envelopes Q168, Yu2, AD8, JRFL, ADA, HXBc2, Mj4, Consensus C, CNE2, CNE5, CNE 6, CNE8, CNE11, CNE15, CNE17, CNE30, CNE50, CNE55, CH091.9, CH110.2, CH114.8, CH119.10, and CH181.12 or control retroviral envelope 10A1 (Miller & Chen, 1996) or VSV-G (Liu et al., 2011) by using a calcium phosphate precipitation method (Tsai, Caillet et al., 2009). DNA plasmids encoding HIV-1 envelopes Q168, Yu2, AD8, JRFL, ADA, HXBc2 and Mj4 were obtained from the ARRRP. The DNA plasmid encoding consensus C were a generous gift of B. H. Hahn at the University of Pennsylvania. The DNA plasmid encoding CNE2, CNE5, CNE 6, CNE8, CNE11, CNE15, CNE17, CNE30, CNE50, and CNE55 were a generous gift of Linqi Zhang at the Tsinghua University. The DNA plasmid encoding CH091.9, CH110.2, CH114.8, CH119.10, and CH181.12 were a generous gift of Yiming Shao at China CDC. The pseudotype-containing supernatants were harvested and stored in aliquots in a freezer at -80°C . Titers of pseudotypes were determined as previously described (Tsai et al., 2009).

In a single-cycle assay, 1×10^4 mock-, GPI-C34 or AVF-transduced TZM.bl cells were transduced with HIV-1 or 10A1 pseudotype-containing supernatants equivalent to a relative luciferase activity (RLA) of 100,000 to 500,000 or VSV-G pseudotype expressing GFP overnight or infected with HIV-1 strains Bru-3, Yu2 and AD8 as well as a simian immunodeficiency virus (SIV) strain SIVMne027 (Kimata, Kuller et al., 1999) as a control at a multiplicity of infection (MOI) of 2 in a final volume of 0.2 ml overnight. Cells were then washed twice with PBS and cultured in complete DMEM for 2 days. Cells transduced with HIV-1 or 10A1 pseudotypes or infected with replication competent HIV-1 and SIV strains were then washed once with PBS and lysed in 100 μ l of lysis buffer. The luciferase activity in 50 μ l cell suspensions was measured by a BrightGlo luciferase assay according to the manufacturer's instructions (Promega). Cells transduced with VSV-G pseudotypes expressing GFP were analyzed by FACS for GFP expression (see above).

HIV-1 infection and p24 assay

To test the effect of GPI-C34 on HIV-1 infection in a transduced human CD4^+ T cell line, 1×10^6 CEMss-CCR5-GPI-C34 or AVF cells were infected with HIV-1 strains Bru-3, Yu2 and AD8 at multiple of infection of 0.01 in a final volume of 0.5 ml overnight. Cells were then extensively washed with HBSS, resuspended in 6 ml of complete DMEM, and cultured for 21 days. Every 3 days, 4.5 ml of cell suspensions was harvested and replaced with fresh medium. The supernatants were then collected. HIV-1 Gag p24 in the supernatants was measured by ELISA (Beckman Coulter), according to the manufacturer's instructions.

Immunofluorescent staining and confocal analysis

Mock-, GPI-C34 or AVF-transduced TZM.bl cells were seeded (5,000 cells per well) onto a tissue culture-treated glass slide (BD Biosciences) and incubated at 37°C in 5% CO_2 for 2 days. Cells were then washed twice with 500 μ l PBS and fixed by fixation buffer (4% formaldehyde in PBS plus 1% BSA) for 15 minutes. Cells were washed twice with 500 μ l PBS and blocked with blockage buffer (5% goat serum in PBS plus 1% BSA) for 1 hour.

Cells were stained with Alexa 555-conjugated cholera toxin subunit B (CtxB) (Invitrogen Life Technologies) at 4°C for 45 min. After washing 3 times with PBS, cells were stained with mouse anti-his tag antibody at 4°C for 45 minutes and then stained with Alexa 488-conjugated goat anti-mouse IgG antibody (Invitrogen) at 4°C. Subsequently, the cells were washed 3 times with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) in permeabilization buffer (blockage buffer plus 0.5% saponin) for 5 minutes. The slides were mounted before being analyzed under a confocal fluorescence microscope (Zeiss model LSM 510).

Results

Expression of C34 or AVF peptide in the lipid raft of the plasma membrane through a GPI anchor

To generate GPI-anchored C34 or AVF, the sequences encoding C34 or AVF control were genetically linked with sequences encoding IgG3 hinge region, his-tag and the GPI-attachment signal of DAF. Both fusion genes were inserted into a third generation lentiviral vector pRRLsin-18.PPT.hPGK.Wpre (Follenzi et al., 2000). The recombinant lentiviruses were used to transduce human CD4⁺ cell line TZM.bl or CEMss-CCR5 cells (see below). Fig. 1B and C show that C34 or AVF/hinge/his-tag/DAF fusion genes were expressed at high levels on the cell surface of transduced TZM.bl cells. The expression of these two fusion genes, as measured by the mean values of fluorescent intensity, was significantly reduced from 15,972 to 4,773 and from 16,699 to 4,584, respectively, after PI-PLC treatment, indicating that the expression of fusion genes on the cell surface is indeed through a GPI anchor. Thus, we refer C34 or AVF/hinge/his-tag/DAF as GPI-C34 and GPI-AVF, respectively.

To localize GPI-C34 or AVF on the cell surface, mock, GPI-C34 or AVF-transduced TZM.bl cells were seeded onto a tissue culture-treated glass slide (BD Biosciences) and incubated at 37°C in 5% CO₂ for 2 days. Cells were co-stained with 1) anti-his-tag antibody followed by Alexa 488-conjugated anti-mouse IgG antibody; 2) Alexa 555-conjugated cholera toxin subunit B (CtxB); and 3) DAPI. CtxB interacts with GM1 (a lipid raft marker). Fig 1D shows that the GPI-C3 or AVF is co-localized with GM1 on cell surface, indicating that it is located in the lipid rafts of the plasma membrane.

GPI-C34 exhibits a remarkable degree of breadth and potency against HIV-1 pseudotypes

We next tested whether the expression GPI-C34 or AVF alters the expression of CD4, CCR5 and CXCR4. We stained mock-, GPI-C34 or AVF-transduced TZM.bl cells with anti-human CD4, CCR5 and CXCR4 antibodies followed by FACS analysis. Fig. 2A shows that the fluorescent intensity of CD4, CCR5 and CXCR4 of GPI-C34 or AVF-transduced TZM.bl cells are very similar. Similar cell growth curves were also observed between mock-GPI-C34 or AVF-transduced TZM.bl cells (Liu *et al.* Data not shown).

To test anti-HIV-1 activity of GPI-C34, mock-, GPI-C34 or AVF-transduced TZM.bl cells were transduced with a panel of 24 HIV-1 pseudotyped virions, representing different subtypes, or control virus pseudotyped with 10A1 in a single round infectivity assay.

Pseudotype Q168 is subtype A. Pseudotypes Yu2, AD8, JRFL, ADA and HXBc2 are subtype B. Pseudotypes CNE 6 and CNE11 are subtype B'. Pseudotypes CNE2, CNE15, CNE17, CNE30, CNE50, CH091.9, CH110.2, CH114.8, CH119.10 and CH181.12 are derived from B/C recombinants. Pseudotypes consensus C and Mj4 are subtype C. Pseudotypes CNE3, CNE5, CNE8 and CNE55 are derived from A/E recombinants. Of note, among them Q168, JRFL, CNE11, CH110.2 and CH119.10 have been classified as tier 2 and CH114.8 and CH181.12 tier 3 envelope proteins (Seaman, Janes et al., 2010). Fig. 2B shows that compared to mock-transduced cells, GPI-AVF did not have any inhibitory activity as measured by relative luciferase activity (RLA). In contrast, GPI-C34 significantly inhibited all HIV-1 pseudotypes tested. The maximum percentage of inhibition (MPI) ranged from 94% to 100%. As expected, no neutralization was detected in GPI-C34-transduced TZM.bl against 10A1 control. Thus, we conclude that GPI-C34 in transduced TZM.bl cells potently and broadly inhibits diverse HIV-1 pseudotypes.

Peptides C34 and AVF were also synthesized by GL Biochem Ltd (Shanghai). In parallel experiments, inhibition of infection by peptides C34 and AVF was measured against HIV-1 AD8 strain. Fig. 2C shows that while peptide AVF did not have any neutralization activity, peptide C34 at 0.1, 1 and 10 μ M inhibited HIV-1 AD8 strain 36, 85 and 98%, respectively. Taken together, these results indicate that when targeted to the lipid rafts of plasma membrane the inhibition by GPI-C34 is comparable to, if not higher than, inhibition by a very high concentration of soluble C34.

GPI-C34 exhibits a remarkable degree of potency against replication competent HIV-1

We next tested inhibitory activity of GPI-C34 against replication competent HIV-1. Table 1 shows that GPI-C34 in transduced TZM.bl cells almost completely (100, 99, or 99%) inhibited HIV-1 Bru-3, Yu2 and AD8, respectively, but had no neutralization against the control virus, SIVmne027.

GPI-C34 renders human CD4⁺ T cells resistant to HIV-1 but not to transduction of VSV-G-pseudotyped lentivirus

While TZM.bl cells, which were engineered with a HIV-1 LTR-driven reporter gene, have been widely used in antibody neutralization assay (Derdeyn et al., 2000, Platt et al., 2009, Platt et al., 1998, Takeuchi et al., 2008, Wei et al., 2002), they are of epithelial origin (Platt et al., 1998). Therefore, we transduced human CD4⁺ T cell line CEMss-CCR5 cells with the lentiviral vectors expressing GPI-C34 or AVF as described before (Liu et al., 2013, Liu et al., 2011). Importantly, both GPI-C34 or AVF are expressed at high levels on transduced CEMss-CCR5 cells (Fig. 3A and B), and no significant difference in CD4, CCR5 and CXCR4 expression was observed in GPI-C34 or AVF-transduced CEMss-CCR5 cells as compared to mock-transduced CEMss-CCR5 cells (Fig 3C).

GPI-C34 or AVF-transduced CEMss-CCR5 cells were then infected with HIV-1 strains Bru-3, Yu2 or AD8 at a multiple of infection of 0.01 as described before (Liu et al., 2011) and cultured in the complete DMEM for 21 days. Fig 3D shows that robust replication of all three HIV-1 strains was observed in GPI-AVF-transduced CEMss-CCR5 cells with peaks at

15 or 18 days post infection. In contrast, no HIV-1 replication, thereby no break-through of C34-resistant mutants, was observed in GPI-C34-transduced CEMss-CCR5 cells.

Finally, GPI-C34 or AVF-transduced CEMss-CCR5 cells were transduced with various doses of VSV-G-pseudotyped lentiviruses expressing green fluorescent protein (GFP). The VSV-G envelope interacts with the lipid moiety in the lipid bilayer of the plasma membrane. Because of this, the VSV-G-pseudotyped lentiviral vector bypasses the requirement for the interaction between the HIV-1 envelope and its receptor and coreceptor to enter cells. Fig. 4A and B show that at all doses tested, no difference in the percentage of GFP⁺ cells and fluorescent intensity of GFP, indicating that the potent blockage of HIV-1 infection by GPI-C34 is HIV-1 envelope-specific and at the level of viral entry.

Discussion

In this study, we demonstrated that by genetically linking C34 or AVF peptide with a GPI attachment signal, peptides are targeted to the lipid rafts of the plasma membrane through a GPI anchor (Fig. 1). GPI-C34, but not GPI-AVF, effectively blocks HIV-1 infection in transduced TZM.bl (Fig. 2 and Table 1) and CEMss-CCR5 cells (Fig 3). HIV-1 viruses inhibited by GPI-C34 include diverse strains of various subtypes and tier 2 or 3 strains (Fig. 2B). Importantly, 99% inhibition potency was observed in GPI-C34-transduced TZM.bl cells; whereas soluble peptide C34 at very high concentration (10 μ M) achieves 98% inhibition potency (Fig. 2C). Thus, the inhibition by GPI-C34 is comparable to, if not higher than, that by soluble C34 peptide. Potent neutralization by GPI-C34 is likely due to its high local concentration, which allows GPI-C34 to efficiently bind to the prehairpin intermediate to prevent its transition to six helical bundle, thereby membrane fusion and virus entry. Although in the present study, we did not observe any break-through of GPI-C34-resistant mutants in challenge experiments with replication competent HIV-1 (Fig. 3), it will be interesting to test in future whether GPI-C34 could effectively inhibit some HIV-1 mutants that are partially resistant to soluble C34 (He, Cheng et al., 2008).

Our findings in this study are consistent with what was reported by Ingallinella *et al.* (Ingallinella et al., 2009). In their study they showed that chemically linking C34 peptide with a cholesterol group (C34-Chol) lead to anchor C34 into the plasma membrane and membrane-anchored C34 dramatically increases antiviral potency and more favorable pharmacokinetics (Ingallinella et al., 2009). We envision that although both C34-Chol and GPI-C34 utilize similar membrane-anchored mechanism to enhance their antiviral activity, their applications may be quite different due to the different ways to anchor C34 onto the plasma membrane. As a lipopeptide, C34-Chol could be developed into a microbicide to anchor C34 peptide into the apical membrane of mucosal epithelia for HIV-1 prevention. On the other hand, as a fusion gene construct, GPI-C34 could be delivered into autologous human primary CD4⁺ T cells or hematopoietic stem cells *ex vivo* and GPI-C34-modified autologous cells could then be transfused into the patients as recently described by DiGiusto *et al* (DiGiusto, Krishnan et al., 2010) or by Tebas *et al* (Tebas, Stein et al., 2014). The latter study shows that the infusion of autologous CD4 T cells in which CCR5 gene was permanently disrupted by a zinc-finger nuclease (ZFN) is safe and modified cells have a half-life of 48 weeks. Importantly during treatment interruption, the decline in CCR5-

modified cells was significantly less than the decline in unmodified cells. The blood level of HIV-1 DNA decreased in most patients (Tebas et al., 2014).

While the CCR5 knockout strategy is promising, it too has potential drawbacks. One drawback is that besides R5-tropic HIV-1 that use CD4 and CCR5 co-receptor to enter cells, X4-tropic and dual-tropic HIV-1 could use CD4 and CXCR4 coreceptor to enter cells. Therefore it is necessary to simultaneously knockout both CCR5 and CXCR4 genes in human CD4 T cells (Didigu, Wilen et al., 2014). Another drawback with the CCR5 knockout strategy is the potential for disease exacerbation. For example, it has been shown that trafficking of CCR5 knockout leukocytes to the CNS after West Nile virus infection is reduced, leading to more severe disease (Durrant, Daniels et al., 2015, Glass, McDermott et al., 2006). In the present study we show that GPI-C34 efficiently blocks both R5- and X4-tropic HIV-1 (Fig. 2 and 3). Therefore, when used in HIV-1 treatment, the GPI-C34-transduced autologous CD4 T cells could resist both R5- and X4-tropic HIV-1. Repopulating patients with HIV-1 resistant GPI-C34-transduced autologous CD4 T cells would in turn decrease the viral reservoir, improve immune function and viral clearance. Presumably, this would reduce HIV neurological disease as well.

Finally, because C34 is a virus-derived peptide, expression of this peptide on cell surface may lead to immune-mediated rejection of cells expressing the GPI-C34 peptide. However, such immune-mediated rejection did not occur in a membrane-bound version of C46 (mC46), another HIV-1 gp41-derived peptide. In both immune competent pigtailed macaque model as well as in a phase I human trial, mC46-modified macaque hematopoietic stem cells or mC46-modified autologous human CD4 T cells expand in the host without attracting host immune mediated rejection (van Lunzen, Glaunsinger et al., 2007, Younan, Polacino et al., 2013). Maybe GPI-C34, like mC46, is not very immunogenic. But it will be important to test whether this occurs in vivo.

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Fig. 1. Expression of GPI-C34 or AVF in transduced TZM.bl cells
(A) Schematic diagram of the lentiviral vectors pRRL-C34 or AVF/hinge/his-tag/DAF. C34 or AVF amino acid sequences are shown; hinge: a human IgG3 hinge region; his-tag: a 6 histidine residue tag; DAF: the C-terminal 34 amino acid residues of decay accelerating factor. (B and C) FACS analysis of cell surface expression as well as the mean and the median values of fluorescent intensity of GPI-C34 or AVF in mock, C34 or AVF/hinge/his-tag/DAF-transduced TZM.bl cells with or without PI-PLC treatment detected by an anti-his-tag antibody. (D). Confocal analysis of mock or pRRL-C34 or AVF/hinge/his-tag/DAF-transduced TZM.bl cells. CtxB: cells were stained with Alexa 555-conjugated cholera toxin B subunit; anti-his: cells were stained with mouse anti-his-tag antibody followed by Alexa 488-conjugated goat anti-mouse IgG antibody. Scale bar: 10 μm.

Fig. 2. The inhibition by GPI-C34 or AVF in transduced TZM.bl cells against HIV-1 using a single cycle infectivity assay

(A) The fluorescent intensity of CD4, CCR5 and CXCR4 expression in GPI-C34 or AVF-transduced TZM.bl cells. (B) The inhibition by GPI-C34 or AVF against a panel of 24 HIV-1 pseudotypes along with 10A1 control. The numbers represent the % of inhibition, which were calculated as follows: (RLA in virus alone to a given transduced cell–RLA in no virus to the same transduced cell)/(RLA in virus alone to parental cells–RLA in no virus to parental cell). (C) The inhibition activity of various indicated concentrations of soluble synthetic peptides C34 and AVF against wild type HIV-1 AD8. The numbers represent the % of inhibition, which were calculated the same as above.

Fig. 3. The inhibition activity of GPI-C34 or AVF in transduced CEMss-CCR5 cells to replication competent HIV-1
 (A and B) The cell surface expression of GPI-AVF (A) or C34 (B) on the surface of transduced CEMss-CCR5 cells stained with anti-his-tag antibody followed by FACS analysis. (C) Summary of mean and median fluorescent intensity of CD4, CCR5 and CXCR4 expression in mock, GPI-C34 or AVF-transduced CEMss-CCR5 cells. (D) GPI-C34 confers long-term resistance to HIV-1 Bru3, Yu2 and AD8 in transduced CEMss-CCR5 cells. GPI-AVF: GPI-AVF-transduced CEMss-CCR5 cells; GPI-C34: GPI-C34-transduced CEMss-CCR5 cells.

Fig. 4. Comparison of susceptibility of CEMss-CCR5-GPI-C34 or AVF to VSV-G-pseudotyped lentiviral vector expressing GFP

A. Mean fluorescent intensity (MFI). B. % of GFP positive cells.

Table 1

GPI-C34 potently inhibits replication competent HIV-1 infection.

Wild Type Viruses	GPI-AVF	GPI-C34
SIVmne027	0	0
Bru3(B,X4)	0	100
YU2(B,R5)	2	99
AD8(B,R5)	1	99

*
The numbers stand for percentages of inhibition.