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No SEVI-mediated enhancement of rectal HIV-1 transmission of HIV-1 in two humanized mouse cohorts

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Abstract

Amyloid fibrils from semen-derived peptide (SEVI) enhance HIV-1 infectivity *in vitro* but the ability of SEVI to mediate enhancement of HIV infection *in vivo* has not been tested. In this study we used immunodeficient mice reconstituted with human immune systems to test for *in vivo* enhancement of HIV-1 transmission. This mouse model supports mucosal transmission of HIV-1 via the intrarectal route leading to productive infection. In separate experiments with humanized mouse cohorts reconstituted with two different donor immune systems, high dose HIV-1_{JR-CSF} that had been incubated with SEVI amyloid fibrils at physiologically relevant concentrations did not show an increased incidence of infection compared to controls. In addition, SEVI failed to enhance rectal transmission with a reduced concentration of HIV-1. Although we confirmed potent SEVI-mediated enhancement of HIV infectivity *in vitro*, this model showed no evidence that it plays a role in the much more complex situation of *in vivo* transmission.

Keywords

HIV; Transmission; Semen-derived enhancer of viral infection; SEVI; Amyloid; Humanized mice

Introduction

In the three decades since the start of the AIDS pandemic HIV has killed more than 39 million people, with an additional 35 million people currently infected. The most common mode of transmission is via the sexual route and the risk of sexual transmission increases

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during the acute phase of infection when viral titers are at their peak (Dimitrov et al., 1993). The presence of human semen has been shown to augment HIV infectivity *in vitro* (Arnold et al., 2012; Kim et al., 2010; Roan et al., 2011) and in 2007, Münch and colleagues described an amyloidogenic peptide derived from the proteolytic cleavage of a human seminal fluid protein (PAP) that significantly enhanced HIV infectivity *in vitro* (Munch et al., 2007). Specifically, the infectivity enhancing activity was attributed to PAP peptide 248–286, which was shown to readily aggregate into amyloid fibrils. Formation of amyloid fibrils was integral to enhancement of infectivity and the peptide fibrils were termed SEVI for Semen-derived Enhancer of Viral Infection (Munch et al., 2007). Evidence indicates that SEVI enhances viral infectivity by facilitating virion and target cell interactions, likely by acting as a cationic bridge between negatively charged virions and cells (Roan et al., 2009; Roan et al., 2010), similar to other polycationic enhancers such as polybrene or protamine sulfate (Cornetta and Anderson, 1989; Manning et al., 1971).

The ability of SEVI to enhance not only viral infectivity *in vitro* but also viral transmission *in vivo* could have broad implications for HIV prophylaxis. For example, as semen is the major vector for sexual transmission of HIV (Royce et al., 1997), the presence of SEVI in seminal fluid could increase the rate of transmission and thus offer a target for intervention. In fact, several SEVI antagonists have already been described (Capule et al., 2012; Hauber et al., 2009; Olsen et al., 2010; Roan et al., 2010). However, transmission across the mucosal barrier *in vivo* is much more complex than infection of cell cultures and cationic bridging might have little impact. Thus, it is important to test the ability of SEVI to augment viral transmission *in vivo* before large investments in developing blocking strategies are made.

Because of the highly selective tropism of HIV for human cells it has not been possible to directly test the ability of SEVI to enhance transmission of HIV *in vivo*. The effect of SEVI on vaginal transmission of simian immunodeficiency virus (SIV) has been tested in the SIV/rhesus macaque model, albeit with no evidence of enhancement (Munch et al., 2013). It was suggested in that study that SEVI might show better enhancement in intrarectal rather than intravaginal transmission. In the current study we use C57BL/6 $Rag2^{-/-} \gamma_c^{-/-} CD47^{-/-}$ BLT-humanized (TKO-BLT) mice to analyze the impact of SEVI on rectal transmission of HIV-1. TKO-BLT mice are highly immunodeficient and irradiated **Triple Knock Out** mice were transplanted with: 1. human fetal CD34⁺ hematopoietic stem cells to reconstitute the **B**one marrow; 2. **L**iver as a source of additional CD34⁺ hematopoietic stem cells; and 3. **T**hymus for maturation and development of human T cells (Lavender et al., 2014). TKO-BLT mice develop high levels of human immune system reconstitution, are susceptible to HIV-1 infection via intrarectal inoculation and exhibit hallmarks of human HIV infection such as CD4⁺ T cell depletion and general immune activation (Lavender et al., 2013). The most dramatic enhancing effects of SEVI and semen *in vitro* have been observed with low doses of HIV-1 irrespective of the virus strain and producer or target cell type (Kim et al., 2010; Munch et al., 2007). Thus for the *in vivo* experiments we tested both a high dose of HIV-1 that typically infects approximately half of our mice in the absence of SEVI, and also a lower dose of HIV-1 that generally infects only one out of four or five mice. SEVI-mediated enhancement of HIV infection is evident *in vitro* starting at a concentration of 2µg/mL and increases with concentration up to at least 50µg/mL (Munch et al., 2007). The

amount of SEVI reported by Munch et al. to be present in semen was approximately 35µg/mL (Munch et al., 2007). To maximize our chances of observing an enhancing effect *in vivo*, most of our experiments were done with SEVI at a concentration of 50µg/mL, which is a physiologically relevant concentration that strongly enhances infectivity *in vitro*.

Results

In vitro enhancement of HIV infection by SEVI

Before testing SEVI in live animals we first confirmed the potency of our preparation of SEVI fibrils on HIV-1 infection *in vitro*. CD4⁺ T cells from human peripheral blood mononuclear cells (PBMCs) were stimulated for 3 days in culture and then infected with HIV-1 viruses alone, or HIV-1 pre-incubated with either SEVI (50µg/mL) or a non-amyloidogenic peptide (negative control). At two days post-infection infectivity enhancement was measured for three virus strains: HIV-1_{JR-CSF}, an R5-tropic molecular clone from a primary isolate from the cerebral spinal fluid of an AIDS patient (Cann et al., 1990; Koyanagi et al., 1987; Moore et al., 1995) that was tested by intracellular staining for p24, and two GFP-expressing HIV clones, one an R5-tropic strain and the other an X4-tropic strain. The flow cytometry gating strategy and representative data for each strain are shown in Figure 1A. The mean fold-enhancement of infection compared to HIV-1 infection in the absence of peptide was calculated and the compiled results are shown in Figure 1B. SEVI at 10 µg/mL produced approximately 20-fold enhancement of HIV-1 infectivity and approximately 24-fold enhancement at 50 µg/mL, which is consistent with previous reports (Hauber et al., 2009; Munch et al., 2007; Roan et al., 2009; Roan et al., 2010). These results indicated that SEVI peptide enhanced HIV-1 infectivity *in vitro* as expected.

Description of *in vivo* test groups

Next, TKO-BLT humanized mice were produced to test whether SEVI increased HIV-1 transmission across the mucosal barrier of the rectum *in vivo*. Previous studies on these mice showed that three intrarectal inoculations of 9×10^4 TCIUs of HIV-1_{JR-CSF} over the course of a week resulted in ~50% of the mice becoming infected, which was evident between 2 and 4 weeks post-infection (Lavender et al., 2013). HIV-1_{JR-CSF} was incubated with SEVI or a negative control peptide prior to inoculation and mice were inoculated once daily for three days with mixtures containing 9×10^4 tissue-culture infectious units (TCIU) (high dose) HIV-1 incubated with either SEVI or the negative control peptide. Humanized mice are relatively resistant to intrarectal inoculation of HIV at low doses (Hofer et al., 2008), but the enhancing effect of SEVI might be most evident at low virus concentrations as has been demonstrated *in vitro* (Munch et al., 2007). To test whether SEVI would enhance infection with lower doses of HIV-1, one group was also infected with a lower dose (1×10^4 TCIU) of HIV-1_{JR-CSF} that had been incubated with SEVI.

Human lymphocyte reconstitution levels and test group distribution

Due to variability in levels of human cell reconstitution of humanized mice it was important to analyze reconstitution levels prior to infection and distribute the mice across the experimental groups as evenly as possible. Human lymphocytes in peripheral blood were detected by flow cytometric staining of human-specific CD45⁺ cells, and the proportions of

T cells (CD3⁺) and CD4⁺ and CD8⁺ T cell subsets were examined. Mice were distributed across the groups to contain roughly equivalent absolute numbers of hCD45⁺ cells/mL of blood (Fig. 2A). Only mice with >10⁵ human CD45⁺ cells/mL of blood were included in the study. At this high level of human cell reconstitution, there was no association between whether a mouse eventually became HIV infected and either the absolute number (Fig. 2A,C) or percentages (Fig. 2B,D) of either total human blood cell numbers or CD4⁺ T cells (mice that eventually became infected in each group are depicted by stars, Fig. 2).

Effect of SEVI on rectal transmission of HIV-1

Blood samples were withdrawn from all mice at 2 weeks post-infection (wpi), a time point when HIV p24 antigen usually becomes detectable in the blood of most infected animals. The plasma was tested for HIV p24 by ELISA and the results are shown in Figure 3. At this time point half of the control mice and half of the SEVI test mice were positive for HIV p24. The infections were allowed to progress for an additional two weeks and the animals were then euthanized at 4wpi, the usual peak of infection and a time point after which we've not observed conversion to an HIV positive status in 3 different cohorts of mice containing a total of 37 mice (19 HIV⁺ and 18 HIV⁻) that were followed to 6 and 9 weeks post infection (data not shown). One mouse in the SEVI-treated group converted to HIV positive between 2 and 4 weeks, but the difference from the untreated controls (4/8 vs 5/8) was not statistically significantly. In mice inoculated with low dose HIV-1/SEVI, only one of five mice tested positive at either time point.

To exclude the possibility that some HIV p24 antigen negative mice had low-level infections not detectable by p24 ELISA we used a highly sensitive, quantitative RNA PCR assay to further probe for evidence of HIV infection. All of the plasma samples from the 2wpi time point were consumed for the p24 assays and there was not sufficient plasma from the 4 wpi terminal blood withdrawals to test all the mice individually. Therefore we pooled the samples from the p24 positive animals and the p24 negative animals from each group. Whereas the p24 positive pools had very high titers of HIV RNA, all of the p24 negative pools tested below the limit of detection (Table 1). Thus there was no evidence that SEVI increased either the intrarectal transmissibility or the antigenemia of HIV-1_{JR-CSF} in TKO-BLT mice, certainly not at levels approaching the 20-fold enhancement observed *in vitro* (Figure 1B).

The primary manifestations of HIV-1 infections in both humans (Day et al., 2006; Deeks et al., 2004; Giorgi et al., 1999; Margolick et al., 2006; Orendi et al., 1998) and TKO BLT mice (Lavender et al., 2013) include depletion of CD4⁺ T cells, increases in CD8⁺ T cell proportions, and broad activation of T cells as indicated by expression of CD38 and PD-1. To determine if SEVI-treated mice differed from controls in these characteristics, peripheral blood cells from all animals at the 4 wpi time point were assessed by flow cytometry for HIV-associated phenotypes. Across all groups the phenotypic changes including depletion of CD4⁺ T cells and increased proportions of CD8⁺ T cells (Figure 4A) as well as the activation levels of the T cells (Figure 4B) were associated with each animal's HIV status, not with whether SEVI was incubated with the virus. Taken as a whole, the results showed

no evidence that SEVI had increased the incidence of HIV transmission, the levels of HIV infection or the pathological changes associated HIV infections.

Second *in vivo* test group

To assure that these results were not peculiar to a single virus preparation or a specific tissue donor we prepared a new virus stock and inoculated a second cohort of mice that had been reconstituted with tissues from a separate donor. The human blood cell reconstitution levels between the SEVI-treated and untreated groups were equivalent for total human cells (hCD45⁺) and also CD3⁺ and CD4⁺ subsets (data not shown). Mice were inoculated intrarectally with a high dose of virus (90,000 TCIU) either with or without SEVI, and two weeks later animals were tested for plasma HIV p24 antigen. In the control group one of seven mice tested positive while three of eight mice in the SEVI group tested positive (Fig. 5A). At the two week time point the negative animals were again inoculated and 4 weeks later the mice were euthanized and re-tested for HIV. At that time four more controls and four more SEVI test mice were positive for HIV leaving two controls and one SEVI test mouse remaining negative (Fig. 5B). The difference between groups was not statistically significant at either time point ($P = 1.000$ and 0.5692 , respectively).

Discussion

The *in vitro* effects of SEVI on HIV infection have been well documented, and this study confirmed potent *in vitro* enhancement of HIV infection. However, the impact of SEVI amyloid fibrils on the *in vivo* transmission of HIV has not previously been investigated. An *in vivo* study on vaginal transmission of SIV in macaques revealed no significant enhancement and some conjectures regarding the lack of enhancement were forwarded (Munch et al., 2013). For example it was speculated that the effect of SEVI on virus transmission across the mucosal barrier might be dependent on low dose virus challenge and that SEVI might be more effective in rectal rather than vaginal transmission (Munch et al., 2013). In the current study we used physiologically relevant infectious doses of both SEVI and HIV (Dyer et al., 1996; Munch et al., 2007) as well as one experiment with low dose HIV. In our first experiment challenging with high dose HIV we achieved infection in about 50% of the mice, so there was plenty of room to observe SEVI-mediated enhancement of transmission. Reducing the virus dose only further reduced transmission. Transmission was higher in the second high dose experiment so the interpretation is not as clear-cut, but still there was no statistically significant enhancement. The result of the low dose challenge experiment, which produced only one infection out of four mice is consistent with a lack of SEVI-mediated enhancement of rectal transmission in this model.

Regarding aspects of the model that may have affected this outcome, the levels of human CD45 and CD4⁺ T cell reconstitution before infection were roughly equivalent in all groups and were not limiting factors determining whether any given mouse became infected or not (Fig. 2). It must be noted that although TKO BLT mice contain a human immune system including gut-associated lymphoid tissue, the GI tract itself is of mouse origin, which could potentially affect transmission enhancement. Mucosal infection by HIV in humans is not fully understood and appears able to proceed either directly or indirectly through different

cell types including CD4⁺ T cells, macrophages and dendritic cells (reviewed in (Wu, 2008)). While HIV infection obviously occurs in this mouse model, it may not proceed through quite the same mechanisms as in humans. That said, the current results are consistent with the previous failure to observe enhancement of infection in the SIV/maquette experiments (Munch et al., 2013).

There are a number of differences between *in vitro* infection of cells and *in vivo* transmission of HIV-1 that could explain the lack of *in vivo* enhancement. For instance, an important mechanism of mucosal transmission may involve the transfer of HIV from DC's to CD4⁺ T cells rather than direct infection of the CD4⁺ T cells (Wang et al., 2007). There could be differences in target cell populations between *in vitro* cultures and the genital tract or rectal mucosa. For example, one study found the enhancing effect of SEVI to be diminished in CD4⁺ T cells derived from the female genital tract compared to CD4⁺ T cells derived from PBMCs (Roan et al., 2009). Additionally, elements in the *in vivo* environment could be inhibiting the effect of SEVI on HIV-1 virions. Various polyanionic compounds in the *in vivo* environment have been shown to neutralize the ability of SEVI to enhance infectivity by disrupting the cationic bridge formed between virions and the cell surface (Roan et al., 2009). Furthermore, physiological concentrations of zinc and copper have been shown to inhibit fibrilization of SEVI *in vitro* (Sheftic et al., 2012). While not directly applicable to our experiments, one study found the enhancing activity of SEVI to be neutralized in the presence of human seminal fluid through the action of enzymes that degrade SEVI into non-enhancing fragments (Martellini et al., 2011). Another factor that might affect SEVI-mediated enhancement of HIV transmission is whether transmission occurs predominantly through free virus or cell-associated virus, a subject not clearly understood at this time. However, the enhancement of SEVI has not yet been studied in the context of cell-associated virus. Finally, a major factor may be differences in accessibility of target cells to SEVI between *in vitro* cultures and the mucosa. SEVI might not even penetrate the mucosal layer or the epithelium to reach the cellular targets for infection. It is possible that sexual activity that causes bleeding such that HIV could directly enter the blood stream might produce very different results and that SEVI enhancement might be important in that situation. Likewise, the presence of sexually transmitted diseases that cause lesions in the mucosal epithelium could also affect the ability of SEVI to cause enhancement.

While the current results do not exclude the possibility of SEVI having an enhancing effect during normal human sexual transmission of HIV they certainly cast some doubt on the issue. Given the lack of *in vivo* enhancement in either SIV-infected macaques or HIV-infected humanized mice it would seem prudent to reconsider whether further development of therapeutics targeting SEVI is warranted until this issue is resolved.

Materials and Methods

Peptides

SEVI and a non-amyloidogenic peptide (negative control), without chemical N- or C-terminal modifications, were purchased from Eurogentec/Anaspec. The sequence of synthetic semen-derived peptide PAP248–286

(GIHKQKEKSRLQGGVLVNEILNHMKRATQIPSYKKLIMY) (SEVI) has been described previously (Munch et al., 2007). Purity as assessed by HPLC was >97% for SEVI, and >99% for control peptide, respectively. Quality control was performed by means of mass spectrometry. Lyophilized synthetic peptides were resuspended in PBS at final concentrations of 2.5 mg/mL (SEVI) and 10 mg/mL (control peptide). The peptide solutions were agitated at 37°C for 36 hrs until SEVI was turbid indicative for fibril formation and then aliquoted and stored at -20°C until use.

Virus stocks

Virus stocks of HIV-1 were generated in human embryonic kidney 293FT cells (Invitrogen, Grand Island, NY). The cells were grown in DMEM containing 10% (v/v) Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodiumpyruvate, and non-essential amino acids, and maintained at 37°C and 5% CO₂. When cultures reached ~80% confluence, the cells were transiently transfected with the infectious molecular clones pYK-JRCSF (an infectious molecular clone of HIV-1_{JR-CSF}, a primary isolate from cerebral spinal fluid obtained from an AIDS patient (https://www.aidsreagent.org/reagentdetail.cfm?t=molecular_clones&id=851) obtained from Drs Irvin S. Y. Chen and Yoshio Koyanagi through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID), pBRNL43_005pf135(R5)nef+_IRES_eGFP clone 1872 to express R5-tropic GFP-HIV-1 (Munch et al., 2002), or NLENG1-IRES to express X4-tropic GFP-HIV-1 (Kutsch et al., 2002; Levy et al., 2004). Briefly, 60 µg plasmid DNA in Opti-Mem I medium was mixed with 180 µL Lipofectamine-2000 in Opti-Mem I medium and incubated at room temperature for 20 minutes. Trypsinized 293FT cells were added to the DNA/Lipofectamine mixture and incubated for 16 h at 37°C and 5% CO₂ for transfection. Transfected cells were then washed once with PBS and incubated for an additional 48 hours in supplemented DMEM medium. HIV-1 stock from cell culture supernatant was concentrated on Amicon Ultra-15 centrifugal filter units (EMD Millipore, Darmstadt, Germany) and frozen at -70°C. Infectious titer of stock concentrations was determined using TZM-B1 (JC53-bl) reporter cells (NIH AIDS Research and Reference Reagent Program from Drs John Kappes and Xiaoyun Wu and Tranzyme, Inc.) maintained in DMEM supplemented with 5% (v/v) FBS.

Effect of amyloid fibrils on HIV-1 infection *in vitro*

To purify CD4⁺ T cells, mononuclear cells derived from whole human blood by Ficoll-Paque PLUS centrifugal elutriation (GE Healthcare Life Sciences, Little Chalfont, UK) were depleted of CD8⁺ and CD56⁺ cells using MACS Microbeads and LD columns (Miltenyi Biotec Inc., Cologne, Germany) following the manufacturers instructions. Cells were stained with anti-human CD8-APC-eFluor780 (eBioscience, San Diego, CA) and anti-human CD56-A700 (Biolegend, San Diego, CA) to ensure <0.5% of depleted cell types. Purified CD4⁺ T cells were then stimulated with 500 ng/mL anti-CD3, 100 ng/mL anti-CD28, and 20 U/mL IL-2 for 3 days at 37°C and 5% CO₂. After 3 days, virus was incubated with 10 µg/mL or 50 µg/mL of SEVI, 50 µg/mL of the negative control peptide, or virus alone in 10µL PBS for 10 minutes at 37°C and transferred to cells for infection. Cells were incubated for 4 hours at 37°C and 5% CO₂ with 5 U/mL IL-2, washed once with PBS, and the virus/peptide mixture was replaced with 200 µL fresh RPMI +10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 25 U/mL IL-2. After 2 days, cells were

harvested and analyzed for infection. For analysis of infection with GFP-expressing virus, cells were washed once with 100 μ L PBS + 2% FBS, resuspended in 150 μ L 2% PFA, and analyzed for GFP expression by flow cytometry. For cells infected with HIV-1_{JR-CSF}, infection was determined by staining intracellular p24. Infected cells were fixed for 90 minutes using 4% PFA, permeabilized using BD Permeabilization buffer (Becton Dickinson, East Rutherford, NJ) for 20 minutes at 25°C, and intracellular p24 was stained using KC57-RD1 (Beckman Coulter, Pasadena, CA) (1:100) in 50 μ L BD Permeabilization buffer for 30 minutes at 4°C. Cells were washed once with BD Permeabilization buffer, resuspended in 150 μ L PBS and analyzed for p24 expression by flow cytometry.

Mice

BLT-humanized C57BL/6 Rag2^{-/-} γ c^{-/-} CD47^{-/-} (TKO) mice were generated by engrafting human fetal thymus and liver tissues and fetal liver-derived CD34⁺ hematopoietic progenitor cells (HPC) as previously described (Lavender et al., 2014; Lavender et al., 2013). Both male and female mice were used for experiments and no differences in susceptibility were observed. Animals were housed in filter-cover cages under specific pathogen-free conditions, and all experiments were performed in accordance with the regulations and guidelines of the Animal Care and Use Committee of Rocky Mountain Laboratories, National Institute of Allergy and Infectious Disease, National Institutes of Health (ASP #2014-33).

Effect of amyloid fibrils on transmission of HIV in humanized mice

Twenty-one mice were humanized from the same donor as previously described (Lavender et al., 2013; Sun et al., 2007) and infected starting 16 weeks post-transplantation. Only mice with sufficient levels of human reconstitution, as determined by number of hCD45⁺ cells/mL of peripheral blood, were chosen for the study. Mucosal infection was achieved by rectal inoculation. Prior to infection, 9.0 x 10⁴ TCIUs HIV-1_{JR-CSF} was incubated with 50 ug/ml SEVI or the negative control peptide and 1.0 x 10⁴ TCIUs HIV-1_{JR-CSF} was incubated with 50 ug/ml SEVI for 10 minutes at 37°C in a final volume of 20 uL per dose. For the first experiment, rectal inoculations were performed as previously described (Lavender et al., 2013) daily over a 3-day period. In the second experiment, the mice only received 2 inoculations, the second 2 weeks after the first. Mice were bled at 2 and 4 wpi for immunophenotyping and to determine HIV-1 viral load in plasma by p24 enzyme-linked immunosorbent assay (ELISA; Advanced Bioscience Laboratories, Rockville, MD). One mouse was found dead from unknown reasons on day 18 in the low dose HIV plus SEVI group.

Isolation and phenotypic analysis of human blood leukocytes

Blood leukocytes were purified as previously described (Lavender et al., 2013). Briefly, the blood volume was measured and the red blood cells were lysed with RBC Lysis Buffer (BioLegend). Following lysis, the leukocytes were counted using a hemocytometer with trypan blue exclusion. The number of leukocytes per mL of blood was calculated by dividing the total number of leukocytes by the bleed volume. Human leukocytes were distinguished within samples using a directly conjugated anti-human CD45-V500 antibody (clone HI30; BD Biosciences, San Jose, CA). Additional immunophenotyping used anti-

human CD4-PE-Cy7 and CD8-APC-eFluor780 (eBioscience, San Diego, CA); CD3-V450 and CD38-PE (Becton Dickinson); and PD-1-FITC (BioLegend, San Diego, CA). Isolated leukocytes were stained for 15 minutes at 4°C, washed once with 100 uL PBS + 2% FBS, fixed in 2% PFA for at least 20 minutes, and analyzed by flow cytometry using a BD LSR II flow cytometer system (Becton Dickinson). Lymphocytes from peripheral blood were stained with anti-hCD45, -CD3, -CD4 and -CD8 fluorescent antibodies and analyzed after gating on physical parameters (forward and side scatters). CD3⁺ cells were analyzed after gating on hCD45⁺. Likewise, CD4⁺ and CD8⁺ cells were analyzed after gating on CD3⁺ cells. Absolute counts of CD45⁺ and CD4⁺ cells were determined by multiplying the percentages of these populations among total lymphocytes by the number of Lymphocytes/mL blood (as described above).

HIV RNA detection

Plasma HIV-1 RNA in peripheral blood was measured by using the Abbott RealTime HIV-1 assay on the m2000 system. In short, viral RNA was isolated from 200µl plasma applying the manual sample preparation protocol from the manufacturer followed by RT-PCR in the m2000 system. The HIV-RNA detection limit for an input volume of 200µl plasma was 600 copies/mL.

Data analysis

All statistical calculations were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Analysis of flow cytometry data was performed using FlowJo X software (Treestar, Inc., Ashland, OR).

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Highlights

- semen-derived peptide (SEVI) enhances HIV-1 infectivity *in vitro*
- SEVI failed to enhance rectal transmission in two separate humanized mouse cohorts
- differences between *in vitro* infection of cells and *in vivo* transmission of HIV-1 likely explain the lack of *in vivo* enhancement

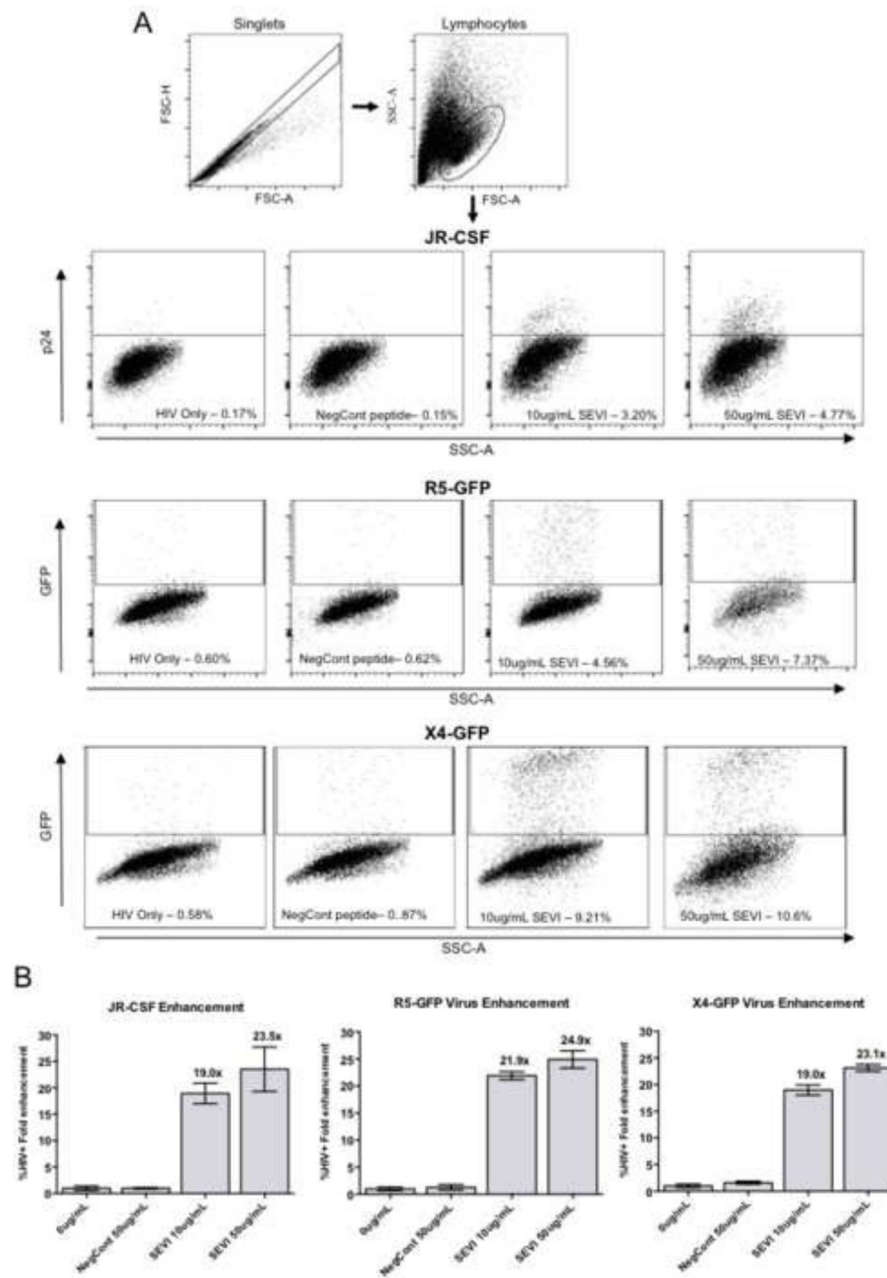


Figure 1. SEVI enhances HIV-1 infectivity of activated CD4 T cells *in vitro*

(A) Gating strategy for flow cytometric analysis of HIV-1_{JR-CSF} infectivity. Activated CD4⁺ T cells infected with HIV-1_{JR-CSF} were analyzed for p24 expression by flow cytometry at two days post-infection. Cells were first gated for singlets (FSC-A vs. FSC-H) and live lymphocytes (FSC-A vs. SSC-A). Infectivity was then measured on the gated population by staining for HIV p24 (JR-CSF) or GFP expression (R5-GFP, X4-GFP). Cell populations shown are representative of replicates. The peptide treatment and percentage of HIV-1+ cells are indicated. (B) The presence of SEVI resulted in enhancement of infectivity as determined by the percentage of HIV-1+ cells. Results were consistent between R5-tropic HIV-1_{JR-CSF} (MOI 0.001), X4-tropic GFP-HIV-1 (MOI 0.01), and R5-tropic GFP-HIV-1

(MOI 0.01). Numbers above bars indicate the fold enhancement of infectivity in the presence of peptide compared to virus alone. Data represent the average values \pm SD of triplicate infections. NegCont = the negative control peptide.

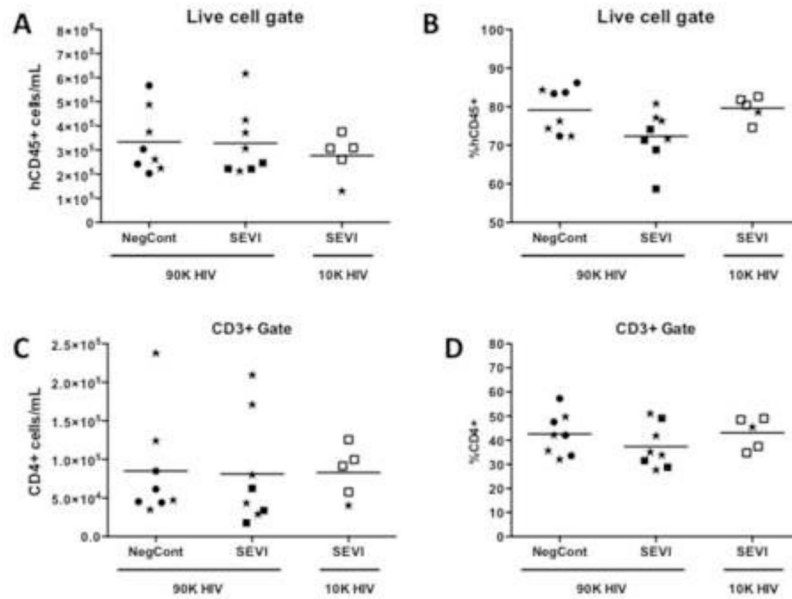


Figure 2. Pre-infection reconstitution levels of humanized mice

At 16 weeks post-transplant surgery humanized mice were analyzed for human immune cell reconstitution (hCD45+) and were distributed across the groups as indicated so that each group had mice with approximately equivalent numbers of CD45+ (human) cells. Since CD4+ T cells are the predominant targets for HIV infection, the numbers and proportions of those cells are also shown. Animals that tested positive for HIV at 4wpi are indicated by stars. Mean values are indicated by horizontal bars. NegCont = negative control peptide; 90K and 10K refer to the HIV_{JR-CSF} challenge dose of either 9 x 10⁴ TCIU s or 1 x 10⁴ TCIU.

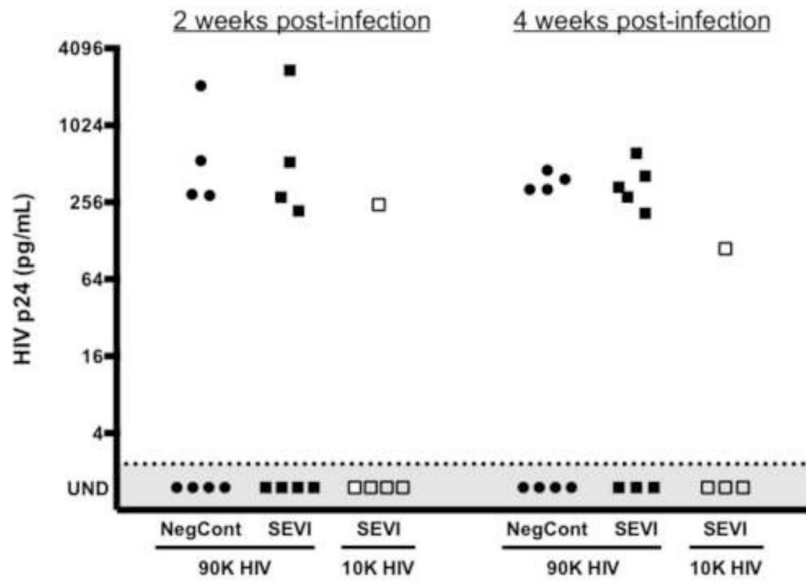


Figure 3. SEVI does not increase viral load or the rate of mucosal transmission of HIV-1 in TKO-BLT mice

Levels of p24 in plasma of infected humanized mice were determined at 2 and 4 weeks post-infection by p24 ELISA after generating a standard curve for each assay and diluting plasma samples appropriately. The dotted line denotes the limit of detection (2.32 pg/mL for 2wpi and 2.27 pg/mL for 4wpi). The line drawn here is the average of these two detection limits, 2.30 pg/mL, but the limit of detection was defined for each separate ELISA.

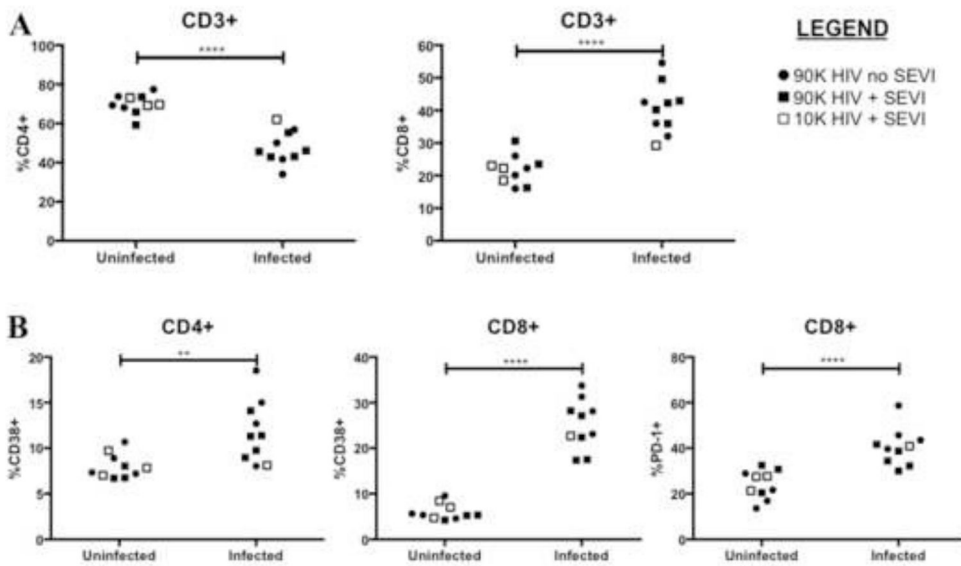


Figure 4. Immunophenotyping of peripheral blood lymphocytes at 4 weeks post-infection
Mice were bled at 4wpi for analysis of HIV-associated pathogenic changes. Each point represents one mouse and the points are grouped according to HIV status. (A) Infected mice in all groups had significant decreases in CD4⁺ T cell proportions and increases in CD8⁺ T cell proportions compared to uninfected animals. (B) Infected mice had significant increases in proportions of activated CD4⁺ and CD8⁺ T cells as measured by CD38 expression. PD-1 is another measure of activation on CD8⁺ T cells and becomes chronically expressed in exhausted T cells. **P<0.01, ****P<0.0001, ns = not significant by unpaired t test.

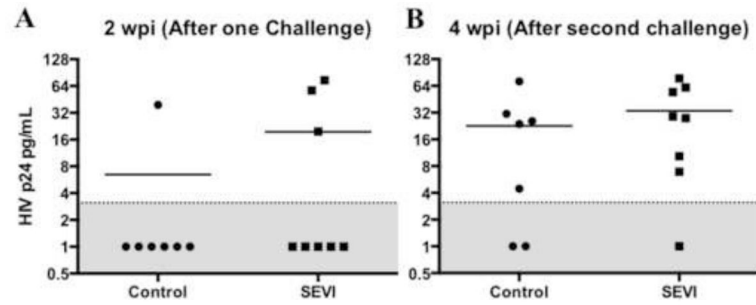


Figure 5. SEVI does not increase viral load or the rate of mucosal transmission of HIV-1 in TKO-BLT mice

Levels of p24 in plasma of infected humanized mice were determined at 2 weeks after the first infection (A) and 4 weeks after the second infection (B) by p24 ELISA as described in Figure 3 and the methods section. The dotted line denotes the limit of detection (3.125 pg/mL). Contingency table analysis by Fisher's exact test showed no difference between the two groups at either 2wpi ($P= 1.0000$) or 4wpi ($P= 0.5692$). The mean levels of human CD45+ cells in the blood before infections exceeded 5×10^5 /ml blood for both groups and mean CD4+ T cell levels exceeded 2×10^5 /ml blood (data not shown).

Table 1
HIV RNA qPCR from pooled plasma samples at 4 weeks post-infection

Plasma samples from the HIV p24 negative mice and the p24 positive mice from the groups indicated above were pooled and HIV-1 RNA levels were measured using the Abbott RealTime HIV-1 assay on the m2000 system. The limit of detection was 600 copies/mL.

| Pooled Samples | HIV dose | RNA copies/mL |
|--------------------------|-------------|--------------------------|
| No SEVI p24 ⁺ | 90,000 TCIU | 8.3 x 10 ⁶ |
| No SEVI p24 ⁻ | 90,000 TCIU | Assay inhibited, no data |
| SEVI p24 ⁺ | 90,000 TCIU | 7.0 x 10 ⁶ |
| SEVI p24 ⁻ | 90,000 TCIU | <600 |
| SEVI p24 ⁺ | 10,000 TCIU | 9.7 x 10 ⁵ |
| SEVI p24 ⁻ | 10,000 TCIU | <600 |

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