

Purging viral latency by a bifunctional HSV-vectored therapeutic vaccine in chronically SIV-infected macaques

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eLife Assessment

In this **useful** study, the authors tested a novel approach to eradicate the HIV reservoir by constructing a herpes simplex virus (HSV)-based therapeutic vaccine designed to reactivate HIV from latently infected cells and induce an immune response to kill such infected cells. Testing this approach with SIV in a primate model, the authors report that the SIV reservoir was reduced. However, the evidence presented appears to be **incomplete** because the animal group size was small and the SIV reservoir size highly variable.

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Abstract

The persistence of latent viral reservoirs remains the major obstacle to eradicating human immunodeficiency virus (HIV). We herein found that ICP34.5 can act as an antagonistic factor for the reactivation of HIV latency by herpes simplex virus type I (HSV-1), and thus recombinant HSV-1 with ICP34.5 deletion could more effectively reactivate HIV latency than its wild-type counterpart. Mechanistically, HSV-ΔICP34.5 promoted the phosphorylation of HSF1 by decreasing the recruitment of protein phosphatase 1 (PP1α), thus effectively binding to the HIV LTR to reactivate the latent reservoirs. In addition, HSV-ΔICP34.5 enhanced the phosphorylation of IKKα/β through the degradation of IκBα, leading to p65 accumulation in the nucleus to elicit NF-κB pathway-dependent reactivation of HIV latency. Then, we constructed the recombinant HSV-ΔICP34.5 expressing simian immunodeficiency virus (SIV) env, gag, or the fusion antigen sPD1-SIVgag as a therapeutic vaccine, aiming to achieve a functional cure by simultaneously reactivating viral latency and eliciting antigen-specific immune responses. Results showed that these constructs effectively elicited SIV-specific immune responses, reactivated SIV latency, and delayed viral rebound after the interruption of antiretroviral therapy (ART) in chronically SIV-infected rhesus macaques. Collectively,

these findings provide insights into the rational design of HSV-vectored therapeutic strategies for pursuing an HIV functional cure.

Introduction

The epidemic of acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus type I (HIV-1), is still a huge challenge for global public health, with approximately 39 million people living with HIV-1 as of 2022. To date, there is neither a curable drug nor a prophylactic vaccine for clinical use against HIV-1 infections (Sun et al., 2023b [↗](#), Sun et al., 2023a [↗](#)). Antiretroviral therapy (ART) can effectively control HIV-1 replication to an undetectable level, but the termination of ART usually results in prompt viral rebound from latent viral reservoirs (Archin et al., 2014 [↗](#), Looker et al., 2017 [↗](#), Calistri et al., 2003 [↗](#), Heng et al., 1994 [↗](#)). Thus, it is of great priority to explore novel strategies for curing HIV latency. The “Shock and Kill” strategy is considered a promising approach for purging HIV-1 reservoirs, involving the activation of latently infected cells to express viral products (Shock), followed by viral cytopathic effects or specific cytolytic T lymphocytes (CTLs) to eliminate the activated cells (Kill) (Wu et al., 2022 [↗](#), Yang et al., 2019 [↗](#), Kim et al., 2018 [↗](#)). Numerous latency-reversing agents (LRAs) (Yang et al., 2019 [↗](#), Wu et al., 2022 [↗](#)), including methylation inhibitors, histone deacetylase (HDAC) inhibitors (Archin et al., 2017 [↗](#), Lehrman et al., 2005 [↗](#)), and bromodomain and extra terminal domain (BET) protein inhibitors (Li et al., 2013 [↗](#), Bisgrove et al., 2007 [↗](#)), have been identified to reactivate latent HIV-1 in preclinical studies, but there is no ideal LRA available for clinical patients yet.

Herpes simplex virus (HSV), a human herpesvirus, features a 152-kb double-stranded DNA genome encoding over 80 proteins (Poh, 2016 [↗](#)). Owing to its distinctive genetic background, high capacity, broad tropism, thermostability, and excellent safety profile, the modified HSV constructs have extensive applications in gene therapy and oncolytic virotherapy. For example, talimogene laherparepvec (T-VEC), an HSV-1 variant with ICP34.5 deletion and GM-CSF insertion, received FDA approval in 2015 for treating malignant melanoma, showcasing notable safety and efficacy in clinical practice. Recombinant HSV-based constructs have also emerged as efficacious gene delivery vectors against infectious diseases. Early studies indicated that prophylactic vaccines based on HSV, expressing simian immunodeficiency virus (SIV) antigens, could elicit robust antigen-specific CTL responses in mice and monkeys, providing enduring and partial protection against pathogenic SIVmac239 challenges (Kaur et al., 2007 [↗](#), Murphy et al., 2000 [↗](#)). Moreover, increasing data suggest the crucial role of HIV-specific CTL in controlling viral replication and eliminating potential HIV reservoirs (Collins et al., 2020 [↗](#), Leitman et al., 2017 [↗](#)). Significantly, epidemiological research suggests a synergistic effect between HSV and HIV infections, with HSV infection in HIV patients being associated with increased HIV-1 viral load and disease progression (Looker et al., 2017 [↗](#), Calistri et al., 2003 [↗](#), Heng et al., 1994 [↗](#)). Some studies have further unveiled the ability of HSV to activate HIV latent reservoirs (Amici et al., 2004 [↗](#), Amici et al., 2001 [↗](#), Pierce et al., 2023 [↗](#)). Given the potential of HSV to simultaneously induce antigen-specific immune responses and reactivate latent viral reservoirs, we propose a proof-of-concept strategy to achieve an HIV functional cure using a modified bifunctional HSV-vectored therapeutic vaccine.

Results

The modified HSV-ΔICP34.5-based constructs reactivated HIV latency more efficiently than wild-type HSV counterparts

The J-Lat 10.6 cell line, derived from Jurkat T cells containing latent HIV-1 provirus, was infected with wild-type HSV-1 Mckrae strain at different multiplicities of infection (MOI) to assess its capability to reactivate HIV latency. Flow cytometry analysis showed a dose-dependent increase in green fluorescent protein (GFP) expression (**Figure 1A**), indicating that HSV-1 can reactivate latent HIV. Subsequently, we infected J-Lat 10.6 cells with an HSV-1 17 strain containing GFP (HSV-GFP) and observed a significant upregulation in the mRNA levels of HIV-1 driven transcripts, including Tat, Gag, Vif, and Vpr, along with 5'-LTR-containing RNA. This demonstrates that the attenuated HSV-1 strain can reactivate latent HIV at different MOIs (**Figure 1B**). Using the bacterial artificial chromosome (BAC)/galactokinase (galK) system, we then constructed the recombinant HSV deleted with ICP34.5 (HSV-ΔICP34.5) based on HSV-GFP. Notably, we discovered for the first time that the HSV-ΔICP34.5 reactivated HIV latency more efficiently than its parental strain (HSV-GFP). Specifically, the mRNA levels of HIV-1 transcripts, including those driven by the 5' LTR, as well as Tat, Gag, Vpr, Vif were significantly higher in J-Lat 10.6 cells infected with HSV-ΔICP34.5 compared to those treated with HSV-GFP (**Figure 1C**), despite the weaker replication ability of HSV-ΔICP34.5 in these cells, as indicated by the mRNA level of HSV UL27 (**Figure 1D**, *Figure S1*).

Furthermore, this finding was validated in ACH-2 cells, which are derived from T cells latently infected with replication-competent HIV-1. A significantly higher level of p24 protein, a key indicator of HIV replication, was found in the HSV-ΔICP34.5-infected ACH-2 cells compared to HSV-GFP-treated cells (**Figure 1E**). Additionally, the mRNA levels of HIV-related transcripts, including those driven by the 5' LTR, as well as Tat, Gag, Vpr, Vif, were also significantly increased (**Figure 1F**). Subsequently, we generated J-Lat 10.6 cells stably expressing ICP34.5-Flag-Tag (J-Lat 10.6-ICP34.5) using a recombinant lentivirus system and confirmed the expression of the ICP34.5 protein. Our research revealed that HSV-ΔICP34.5 displayed a reduced capacity to reverse HIV latency in J-Lat 10.6-ICP34.5 cells compared to J-Lat 10.6 cells (**Figure 1G**). Moreover, in J-Lat 10.6-ICP34.5 cells, the potency of latent reversal agents such as phorbol 12-myristate 13-acetate (PMA) and TNF-α was notably reduced when contrasted with J-Lat 10.6 cells (**Figure 1H**). We also confirmed the enhanced reactivation of HIV latency by HSV-ΔICP34.5 in primary CD4⁺ T cells from people living with HIV (PLWH) (*Figure S2*). In addition, HSV-ΔICP34.5 induced a lower level of inflammatory cytokines (including IL-6, IL-1β, and TNF-α) in primary CD4⁺ T cells from PLWH compared to HSV-GFP stimulation, likely due to its lower virulence and replication ability (**Figure 1I-K**). Thus, these data suggested that the safety of HSV-ΔICP34.5 in PLWH might be tolerable, at least *in vitro*. In addition, neither adenovirus nor vaccinia virus was able to reactivate HIV latency in this study (*Figure S3*), and the deletion of ICP0 gene from HSV-1 diminished its reactivation effect on HIV latency (*Figure S4*). Importantly, overexpressing ICP0 greatly reactivates latent HIV (*Figure S5*), implying that this reactivation is virus-specific and that ICP0 plays an important role in reversing HIV latency. Overall, these findings indicated that the recombinant HSV-ΔICP34.5 construct specifically reactivated HIV latency with high efficiency in the presence of ICP0.

The modified HSV-based constructs effectively reactivated HIV latency by modulating the IKKα/β-NF-κB pathway and PP1-HSF1 pathway

Next, based on RNA-seq analysis for HSV-ΔICP34.5-induced signaling pathways (*Figure S6*) and previous literature, we explored the mechanism of reactivating viral latency by HSV-ΔICP34.5-based constructs. J-Lat10.6 cells were infected with HSV-GFP or HSV-ΔICP34.5, and results showed that HSV-ΔICP34.5 significantly enhanced the phosphorylation of IKKα/β, promoted the degradation of IκBα, and thus led to the accumulation of p65 in the nucleus (**Figure 2A**). NF-κB is a well-known host transcription factor that exists in the form of the NF-κB-IκB complex in

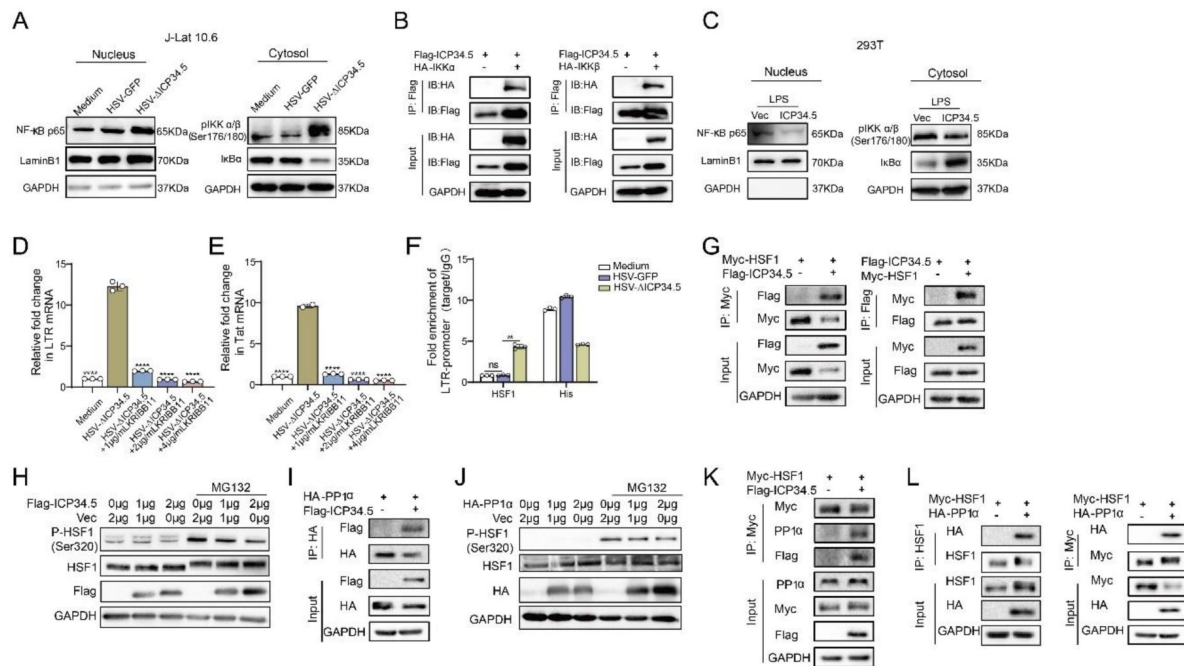


Figure 1.

The modified HSV-ΔICP34.5-based constructs reactivated HIV latency more efficiently than wild-type HSV counterparts.

(A) J-Lat 10.6 cells (1×10^6) were infected with varying MOIs of wild-type HSV-1 Mckrae strain for 30 h. The proportion of GFP+ cells, indicating activated latent cells, is shown in the pseudocolor plot (left) and the corresponding bar chart (right). (B) J-Lat 10.6 cells (1×10^6) were infected with varying MOIs of HSV-1 17 strain containing GFP (HSV-GFP) for 30 h, and the mRNA levels of HIV-1 transcripts driven by the 5' LTR, as well as Tat, Gag, Vpr, and Vif, are presented in the histogram. (C) J-Lat 10.6 cells (1×10^6) were infected with HSV-GFP or HSV-ΔICP34.5 at an MOI of 0.1 for 30 h. The mRNA levels of HIV-1 transcripts driven by the 5' LTR, as well as Tat, Gag, Vpr, Vif, and (D) HSV-1 UL27 are presented in the histogram. (E) ACH-2 cells (1×10^6) were infected with HSV-GFP or HSV-ΔICP34.5 at an MOI of 0.1 for 30 h. The p24 protein level was detected using an HIV-1 p24 ELISA kit, and the mRNA levels of HIV-1 transcripts driven by the 5' LTR, as well as Tat, Gag, Vpr, and Vif, are shown in the histogram (F). (G) J-Lat 10.6 and J-Lat 10.6-ICP34.5 cells were infected with HSV-GFP or HSV-ΔICP34.5, and the mRNA levels of HIV-1 Tat were shown with the histogram (left). Blotting showed that J-Lat 10.6 cells stably expressing HSV ICP34.5 (J-Lat 10.6-ICP34.5) can appropriately express ICP34.5 protein using Flag-tag antibodies (right). (H) J-Lat 10.6 and J-Lat 10.6-ICP34.5 cells were respectively stimulated with PMA (10 ng/mL) and TNF-α (10 ng/mL), and the expression level of GFP+ cells is displayed with the corresponding bar chart. (I-K) Primary CD4+ T cells from people living with HIV (PLWH) were infected with HSV-GFP or HSV-ΔICP34.5. The inflammatory response was assessed by evaluating mRNA levels of IL-6, IL-1β and TNF-α using qPCR. Data shown are mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns: no significance.

resting cells, but I κ B can degrade and release the NF- κ B dimer to enter the nucleus and promote gene transcription in response to external stimulation. Using the coimmunoprecipitation (Co-IP) assay, we verified that the ICP34.5 protein had a specific interaction with IKK α / β , and then ICP34.5 could dephosphorylate IKK α / β . Moreover, the overexpression of ICP34.5 effectively inhibited lipopolysaccharide (LPS)-induced NF- κ B pathway activation by inhibiting p65 entry into the nucleus (**Figure 2B-C**).

To further clarify the underlying mechanism, we performed the immunoprecipitation-mass spectrometry (IP-MS) analysis in ICP34.5 overexpressing cells to identify other potential molecules contributing to this reactivation, and we found that ICP34.5 can also interact with heat shock 1 protein (HSF1) (*Table S1*). HSF1 has been reported as a transcription factor correlated with the reactivation of HIV latency (Xu et al., 2022; Lin et al., 2018; Zeng et al., 2017). To test whether HSF1 contributes to the reactivation of HIV latency by HSV- Δ ICP34.5-based constructs, KRIBB11, an inhibitor of HSF1, was administered to HSV- Δ ICP34.5-infected J-Lat 10.6 cells. The results indicated that the reactivation ability of HSV- Δ ICP34.5 was significantly inhibited by KRIBB11 treatment in a dose-dependent manner (**Figure 2D-E**). Furthermore, a significant enhancement of the binding of HSF1 to the HIV LTR was observed upon HSV- Δ ICP34.5 infection, leading to an increase in the reactivation of HIV latency (**Figure 2F**). The direct interaction between ICP34.5 and HSF1 was also identified by Co-IP assay. Importantly, HSF1 was effectively dephosphorylated at Ser320 as a result of the overexpression of ICP34.5, while no influence on the level of HSF1 expression was observed (**Figure 2G-H**, *Figure S7*). Considering that protein phosphatase 1 (PP1) can interact with ICP34.5 and dephosphorylate eIF2 α (Li et al., 2011), we then investigated the interaction between PP1 α and ICP34.5 (**Figure 2I**). Additionally, a direct interaction between PP1 α and HSF1 was found, allowing for the dephosphorylation of HSF1 and then affecting its ability to reactivate HIV latency (**Figure 2J-L**). Collectively, these findings demonstrated that our modified HSV- Δ ICP34.5-based constructs effectively reactivated HIV latency by modulating the IKK α / β -NF- κ B pathway and PP1-HSF1 pathway.

Recombinant HSV- Δ ICP34.5 constructs expressing SIV antigens elicited robust immune responses in mice

Subsequently, we explored the potential of HSV- Δ ICP34.5 as a bifunctional therapeutic vector to not only reactivate latent viral reservoirs but also induce antigen-specific immune responses against viral replication. To achieve this, SIV antigen genes were introduced into HSV- Δ ICP34.5 vector based on BAC/ galK system. Additionally, the ICP47 gene was ablated to augment the immunogenicity of the HSV vector (**Figure 3A**, *Figure S8*). A series of recombinant HSV- Δ ICP34.5 Δ ICP47-based vectors expressing SIV gag and env antigen were constructed, and the antigen expression of these constructs was confirmed by Western blotting assay (**Figure 3B-C**). Furthermore, to improve the immunogenicity of the targeted antigen, we fused the SIV gag with soluble PD1 (sPD1), enabling it to competitively bind with PD-L1 and thereby block the PD1/PD-L1 immune inhibitory pathway (**Figure 3D**). Consistent with the above findings, these HSV- Δ ICP34.5 Δ ICP47-based SIV vaccines also efficiently reactivated HIV latency (*Figure S9*). Then, the immunogenicity of the above modified HSV- Δ ICP34.5 Δ ICP47-based SIV vaccine was assessed in mice (**Figure 3E**). Our results showed that these constructs effectively elicited SIV antigen-specific T cell immune responses using the interferon γ (IFN- γ) ELISpot assay and the intracellular cytokine staining (ICS) assay. Of note, the frequency of SIV Gag-specific IFN- γ -secreting spot-forming cells (SFCs) in the HSV-sPD1-SIVgag group (1350 SFCs per 10⁶ splenocytes) was significantly higher than that in the HSV-SIVgag group (498 SFCs per 10⁶ splenocytes) (**Figure 3F**). The frequency of SIV Env2-specific IFN- γ -secreting SFCs in the HSV-SIVenv group was significantly higher than the HSV-empty group (**Figure 3G**). Furthermore, the polyfunctionality of antigen-specific T cell subsets in response to SIV antigen stimulation was confirmed using the ICS assay (**Figure 3H-M**). Consistently, the HSV-sPD1-SIVgag group showed a significantly higher frequency of SIV-specific CD3⁺ T, CD4⁺ T, and CD8⁺ T cell subsets secreting IFN- γ , IL-2, and TNF- α cytokines compared to the HSV-SIVgag group (**Figure 3I-K**). Notably, a heightened proportion of

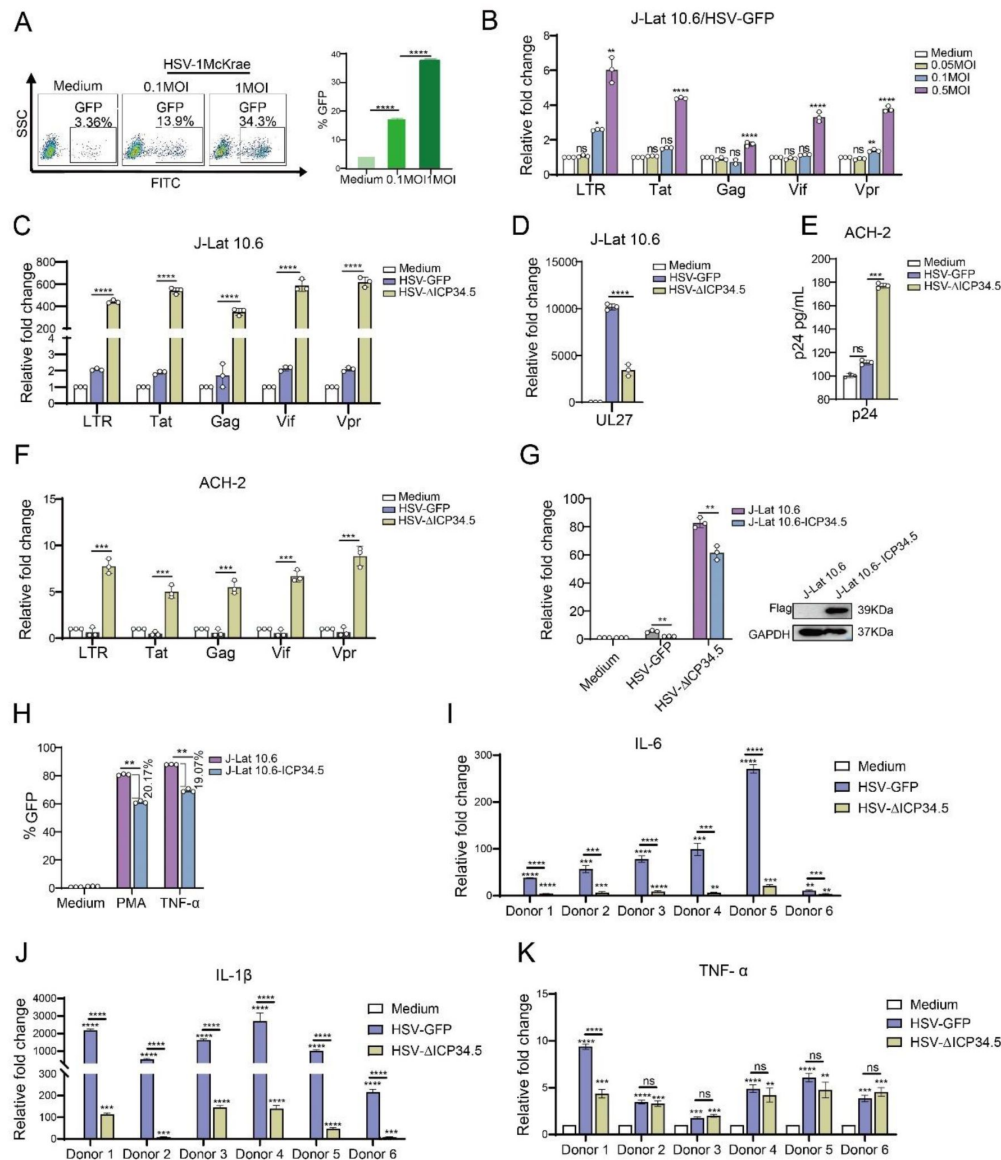


Figure 2.

The modified HSV-based constructs effectively reactivated HIV latency by modulating the NF-κB pathway and HSF1 pathway.

(A) J-Lat 10.6 cells infected with HSV-GFP and HSV-ΔICP34.5 at MOI 0.1. Cytoplasmic and nuclear proteins were analyzed for p65, p-IKKα/β and IκBα levels. GAPDH and Lamin B1 served as loading controls for cytoplasmic and nuclear proteins, respectively. (B) 293T cells transfected with Flag-ICP34.5, IKKα (left), or IKKβ (right) analyzed through Co-IP assays. (C) 293T cells were transfected with Flag-ICP34.5 or empty vector (Vec) for 24h, treated with LPS (1μg/mL) for 8h. Cytoplasmic and nuclear proteins analyzed by Western blot (WB). (D-E) J-Lat 10.6 cells infected with HSV-ΔICP34.5 treated with various KRIBB11 concentrations. Levels of mRNA for Tat and HIV-1 transcripts driven by the 5' LTR were analyzed by qPCR. (F) J-Lat 10.6 cells infected with HSV-wt or HSV-ΔICP34.5 at MOI 0.1 for 36h. ChIP-qPCR assessed HSF1 binding to LTR. IgG and Histone antibody (His) served as negative and positive controls. (G) 293T cellstransfected with Flag-ICP34.5 and Myc-HSF1, analyzed by Co-IP assays. (H) The immunoblot depicts the alterations in protein levels in 293T cells transfected with either empty vector or ICP34.5 for 6 h, followed by 24 h of treatment with 10 μM MG132. (I) 293T cells transfected with Flag-ICP34.5 and HA-PP1α, analyzed by Co-IP assays. (J) The immunoblot depicts the alterations in protein levels in 293T cells transfected with either empty vector or HA-PP1α for 6 h, followed by 24 h of treatment with 10 μM MG132. (K) 293T cells transfected with Myc-HSF1 with or without Flag-ICP34.5, analyzed by Co-IP assays. (L) 293T cells transfected with Myc-HSF1 and HA-PP1α, analyzed through Co-IP assays. Data shown are mean ± SD. ** $P < 0.01$, **** $P < 0.0001$. ns: no significance.

Gag-specific effector memory T cells (Tem) of CD8⁺ T cell subset in HSV-ΔICP34.5ΔICP47-sPD1-SIVgag group was observed in comparison to the HSV-Gag group (**Figure 3L** [↗](#)). In addition, a higher frequency of SIV-Env2-specific CD4⁺ T cells secreting IFN-γ was observed in the HSV-ΔICP34.5ΔICP47-SIVenv group than in the HSV-ΔICP34.5ΔICP47-empty group (**Figure 3M** [↗](#)). These data indicated that the vaccines constructed in this study elicited a robust T cell immune response in mice. Moreover, the blockade of PD1/PDL1 signaling pathway effectively enhanced vaccine-induced T cell immune responses, which was consistent with our and other previous studies (Zhou et al., 2013 [↗](#), Wu et al., 2022 [↗](#), Pan et al., 2018 [↗](#), Xiao et al., 2014 [↗](#)).

The modified HSV-based constructs efficiently elicited SIV-specific immune responses in chronically SIV-infected macaques

Next, the immunogenicity of these HSV-ΔICP34.5ΔICP47-vectored SIV vaccines was further investigated in chronically SIV_{mac239}-infected rhesus macaques (RMs). To mimic chronically infected HIV patients in clinic practice, all RMs used in this study were chronically infected with SIV and received ART treatment for several years, as reported in our previous studies (Pan et al., 2018 [↗](#), Yang et al., 2019 [↗](#), Wu et al., 2021 [↗](#), Wu et al., 2022 [↗](#), He et al., 2023 [↗](#)). Based on sex, age, viral load, and CD4 count, nine RMs were assigned into three groups: ART+saline group (n=3), ART+HSV-ΔICP34.5ΔICP47-empty group (n=3), and ART+HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv group (n=3) (*Table S2*). All RMs received ART (FTC/6.7 mg/kg/once daily, PMPA/10 mg/kg/once daily) treatment to avoid the interference of free SIV particles. On day 33 and day 52, these RMs were immunized with saline, HSV-ΔICP34.5ΔICP47-empty, or HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv respectively. On day 70, ART treatment in all RMs was discontinued to evaluate the time interval of viral rebound. Samples were collected at different time points to monitor virological and immunological parameters (**Figure 4A** [↗](#)). To reduce the impact of individual RM variations, the difference in SIV-specific IFN-γ-secreting SFCs between post-immunization and pre-immunization (ΔSFCs) was used to evaluate the immune response induced by HSV-ΔICP34.5ΔICP47-vectored SIV vaccines. The results showed that SIV Gag-specific ΔSFCs in the ART+HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv group were greatly increased when compared with those in the ART+HSV-ΔICP34.5ΔICP47-empty group and ART+saline group (**Figure 4B-D** [↗](#)). A similar enhancement of SIV Gag-specific TNF-α-secreting CD4⁺ T and CD8⁺ T subsets was also verified by ICS assay (**Figure 4E** [↗](#)). Collectively, these data demonstrated that the HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv construct elicited robust SIV-specific T cell immune responses in ART-treated, SIV-infected RMs.

The modified HSV-based constructs partly reactivated SIV latency *in vivo* and delayed viral rebound in chronically SIV-infected, ART-treated macaques

Finally, we investigated the therapeutic efficacy of HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv in chronically SIV-infected, ART-treated RMs. Consistent with our previous studies, the plasma viral load (VL) in these RMs was effectively suppressed during ART treatment, but rebounded after ART discontinuation. The VL in the ART+saline group promptly rebounded after ART discontinuation, and one of three RMs showed a significantly increased peak VL compared with the pre-ART VL (**Figure 5A** [↗](#) and **5D** [↗](#)). However, plasma VL in the ART+HSV-ΔICP34.5ΔICP47-empty group and the ART+HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv group exhibited a delayed rebound interval (**Figure 5B-C** [↗](#)). Remarkably, there was a lower rebounded peak VL (two of three RMs) than pre-ART VL in the ART+HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv group, while two of three RMs had a higher rebounded peak VL than pre-ART VL in the ART+HSV-ΔICP34.5ΔICP47-empty group (**Figure 5D** [↗](#)). Then, we assessed the potential effect on the latent SIV reservoirs *in vivo* by administering our modified HSV-based SIV therapeutic constructs in these RMs. Although there were no obvious viral blips observed in these RMs, we found significant suppression of total SIV DNA and integrated SIV DNA provirus in the ART+HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv group. However,

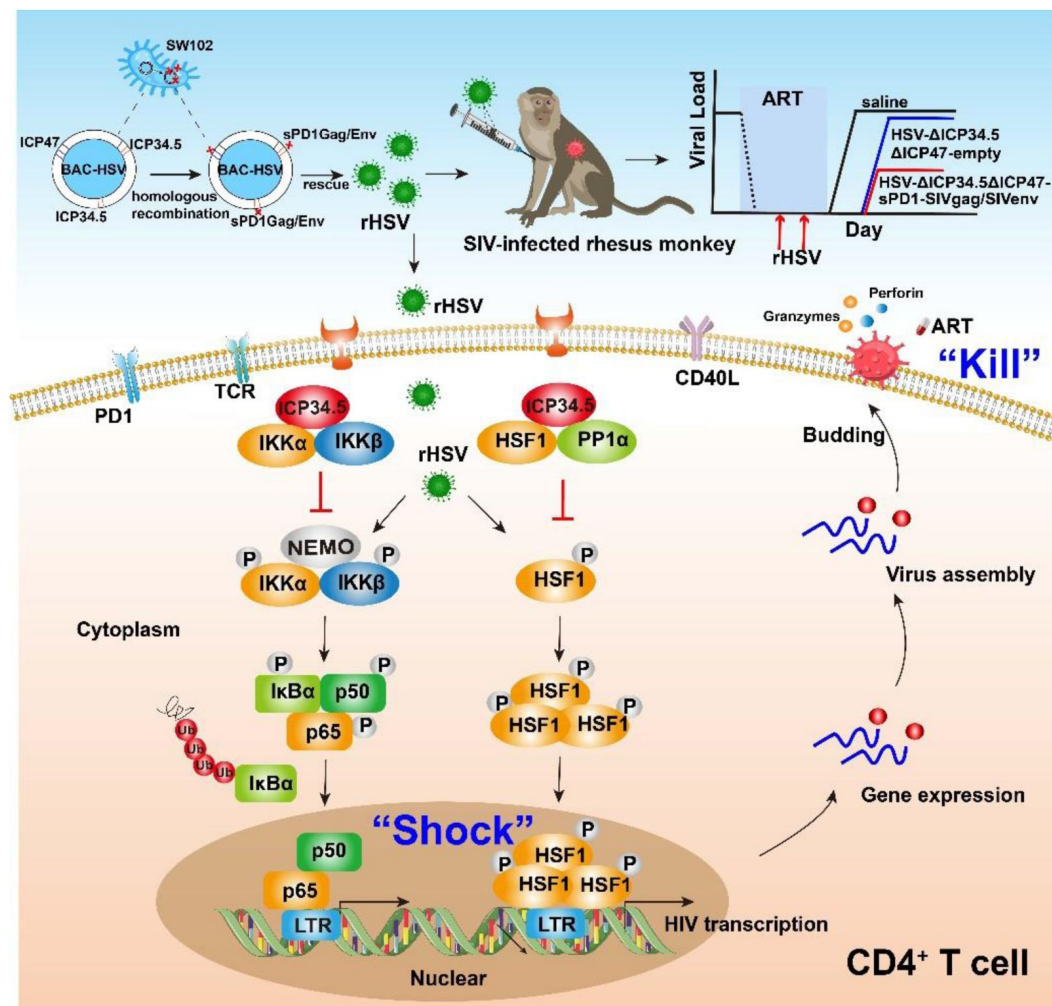


Figure 3.

Recombinant HSV-1 vector-based SIV vaccines induce specific T cell immune responses in mice.

(A) Schematic diagram illustrating the construction of recombinant HSV through the BAC/galK selection system. The ICP34.5 gene was replaced with the galK gene via homologous recombination, followed by substituting galK with a target gene expression cassette containing the hCMV promoter and BGH terminator. Finally, the ICP47 gene was deleted. (B) Brightfield (top) and fluorescence (bottom) images of a clone of the rescued recombinant HSV. (C) Vero cells infected with recombinant HSV constructs, with protein expression of targeted genes detected using SIV-infected monkey serum. (D) HeLa cells transfected with Myc-PDL1 and then infected with HSV-ΔICP34.5ΔICP47-empty, HSV-ΔICP34.5ΔICP47-SIVgag, or HSV-ΔICP34.5ΔICP47-sPD1-SIVgag at an MOI of 0.1 for 24 h. Cell lysates were subjected to Co-IP analysis. (E) Schematic schedule of mouse vaccination. Twenty-five mice were randomly allocated to five groups: HSV-ΔICP34.5ΔICP47-empty, HSV-ΔICP34.5ΔICP47-sPD1, HSV-ΔICP34.5ΔICP47-SIVgag, HSV-ΔICP34.5ΔICP47-sPD1-SIVgag, and HSV-ΔICP34.5ΔICP47-SIVenv. Mice were injected with the corresponding vaccines at weeks 0 and 2. At week 4, mice were sacrificed, and spleen lymphocytes were collected to evaluate immune response. (F-G) Column graphs showing the number of Gag or Env1, Env2-specific spot-forming cells (SFCs) per 10^6 spleen lymphocytes, as measured by IFN- γ ELISpot assay. (H) Pseudocolor plot of flow cytometry illustrating the gating strategy. Column graphs showing the frequencies of IFN- γ , IL-2, and TNF- α production from gag-specific CD3 $^{+}$ T (I), CD4 $^{+}$ T (J), and CD8 $^{+}$ T cells (K). (L) Bar chart showing the proportion of Tem (effector memory T cells) among CD4 $^{+}$ T and CD8 $^{+}$ T cells upon stimulation with the SIV Gag peptide pools. (M) Bar chart showing the frequencies of Env2-specific IFN- γ^{+} CD4 $^{+}$ T cells. Data were expressed as mean $\pm$ SD from five mice samples. Three independent experiments for the animal immunization were repeated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns: no significance.

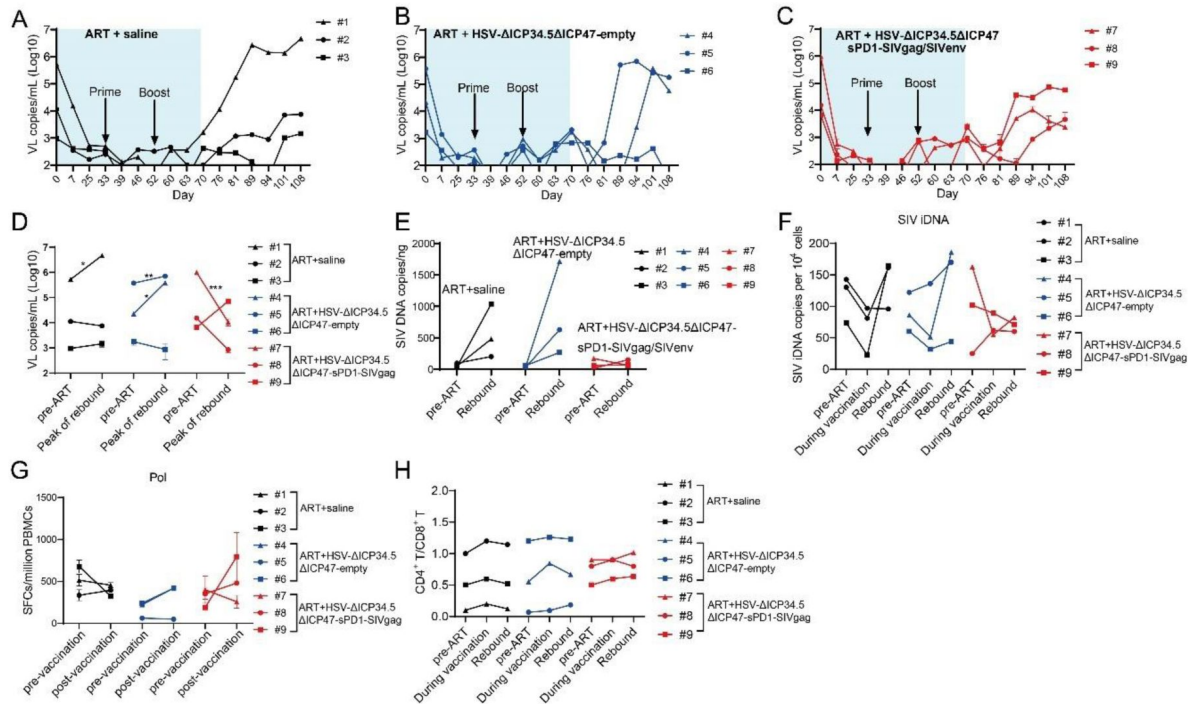


Figure 4.

The modified HSV-based constructs efficiently elicited SIV-specific immune responses in chronically SIV-infected macaques.

(A) Schematic schedule of the macaque experiment. Nine chronically SIV-infected macaques were assigned into three groups: ART+saline group (n=3), ART+HSV-ΔICP34.5ΔICP47-empty group (n=3), and ART+HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv group (n=3). All SIV-infected macaques received ART treatment (FTC/6.7 mg/kg/once daily, PMPA/10 mg/kg/once daily) for 33 days. On days 33 and 52, macaques were immunized with saline, HSV-ΔICP34.5ΔICP47-empty, and HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv respectively. ART treatment, was interrupted in all macaques on day 70 after the second vaccination. Samples were collected at various time points to monitor virological and immunological parameters. (B) Representative images of Gag or Env-specific spots (2.5×10^5 cells per well) from each macaque pre-vaccination (before, day 33) and post-vaccination (after, day 70) by ELISpot assay. (C-D) Difference in SIV-specific IFN- γ -secreting cells (ΔSFCs) between pre- and post-immunization, assessing the immune response induced by HSV-ΔICP34.5ΔICP47-vectored SIV vaccines. (E) Difference in SIV-specific TNF- α /IFN- γ /IL-2-secreting CD4 $^+$ T and CD8 $^+$ T subsets between pre- and post-immunization, detected by ICS assay.

the copies of the SIV DNA provirus were significantly improved in the ART+HSV- Δ ICP34.5 Δ ICP47-empty group and ART+saline group (**Figure 5E-F**). More interestingly, we assessed the magnitude of SIV Pol antigen-specific immune responses, which could represent to some extent the level of newly generated virions from the reactivated SIV reservoirs, because SIV Pol antigen was not included in our designed vaccine constructs. Specifically, the Pol-specific SFCs amount on Day 70 (511 SFCs/ 10^6 PBMCs, post-vaccination) was higher than that on Day 33 (315 SFCs/ 10^6 PBMCs, pre-vaccination) in the ART+HSV- Δ ICP34.5 Δ ICP47-sPD1-SIVgag/SIVenv group. In addition, there was a similar observation in the ART+HSV- Δ ICP34.5 Δ ICP47-empty group. In contrast, the Pol-specific SFCs gradually decreased with the duration of ART treatment in the ART+saline group (**Figure 5G**). In addition, the CD4⁺/CD8⁺ T cell ratio (**Figure 5H**) and body weight (*Figure S10*) after treatment were effectively ameliorated in the RMs of the ART+HSV- Δ ICP34.5 Δ ICP47-sPD1-SIVgag/SIVenv group, but not in the ART+HSV- Δ ICP34.5 Δ ICP47-empty group and ART+saline group. Our data also demonstrated that there was no significant effect on the cell composition of peripheral blood in the macaques of ART+HSV- Δ ICP34.5 Δ ICP47-sPD1-SIVgag/SIVenv group (*Figure S11*). Taken together, these findings suggested that the latent SIV reservoirs might be effectively purged because of the effect of simultaneously reactivating viral latency and eliciting SIV-specific immune responses by our modified HSV-based SIV therapeutic constructs, thus resulting in a delayed viral rebound in chronically SIV-infected, ART-treated macaques.

Discussion

To conquer the continuous epidemic of AIDS, exploring novel strategies to render and eliminate HIV latency should stand as a pivotal pursuit. Currently, numerous strategies, including shock and kill, block and lock, chimeric antigen receptor T-cell therapy, therapeutic vaccination, and gene editing, have been extensively investigated to target the latent HIV reservoirs for an HIV functional cure (Deeks, 2012, Yeh and Ho, 2021, Maldini et al., 2020, Herzig et al., 2019, Dash et al., 2023, Dashti et al., 2023, Walker-Sperling et al., 2022). However, there is no safe and effective approach for clinical use in HIV patients yet. In the present study, we occasionally found that the modified HSV- Δ ICP34.5-based constructs could reactivate HIV latency more efficiently than wild-type HSV counterpart, which inspired us to develop a proof-of-concept strategy based on a bifunctional HSV-vectored therapeutic vaccine, aiming to simultaneously reactivate viral latency and elicit HIV/SIV-specific immune responses for HIV functional cure. Our results indicated that these modified HSV-based constructs efficiently elicited antigen-specific immune responses in mice and chronically SIV-infected macaques, and further therapeutic efficacy experiments showed that this strategy partly reactivated SIV latency *in vivo* and delayed viral rebound in chronically SIV-infected, ART-treated macaques (**Figure 6**).

The latent HIV proviruses can harbor it into the host genome with a quiescent transcription state, and thus cannot be recognized by immune surveillance or drug killing (Churchill et al., 2016, Pierson et al., 2000). Therefore, it is critical to disrupt viral latency for developing an HIV cure strategy. Based on our experimental data, the mechanism for efficiently reactivating viral latency by the modified HSV- Δ ICP34.5-based constructs may involve regulating the IKK α / β -NF- κ B pathway and PP1-HSF1 pathway. Indeed, during its replication, HSV can activate the double-stranded RNA-dependent protein kinase (PKR) pathway, and thus phosphorylate the protein translation initiation regulator eIF2 α , resulting in the initiation of protein translation (Farassati et al., 2001). Previous studies have shown that the reactivation potential of HSV might be intertwined with NF- κ B, Sp1, and other unknown transcription factors by ICP0, ICP4, and ICP27 (Chapman et al., 1991, Amici et al., 2004, Vlach and Pitha, 1992, Mosca et al., 1987b, Golden et al., 1992, Vlach and Pitha, 1993, Mosca et al., 1987a). However, the underlying mechanism by which the ICP34.5-deleted HSV construct can greatly improve the reactivation efficacy of HIV latency remains elusive.

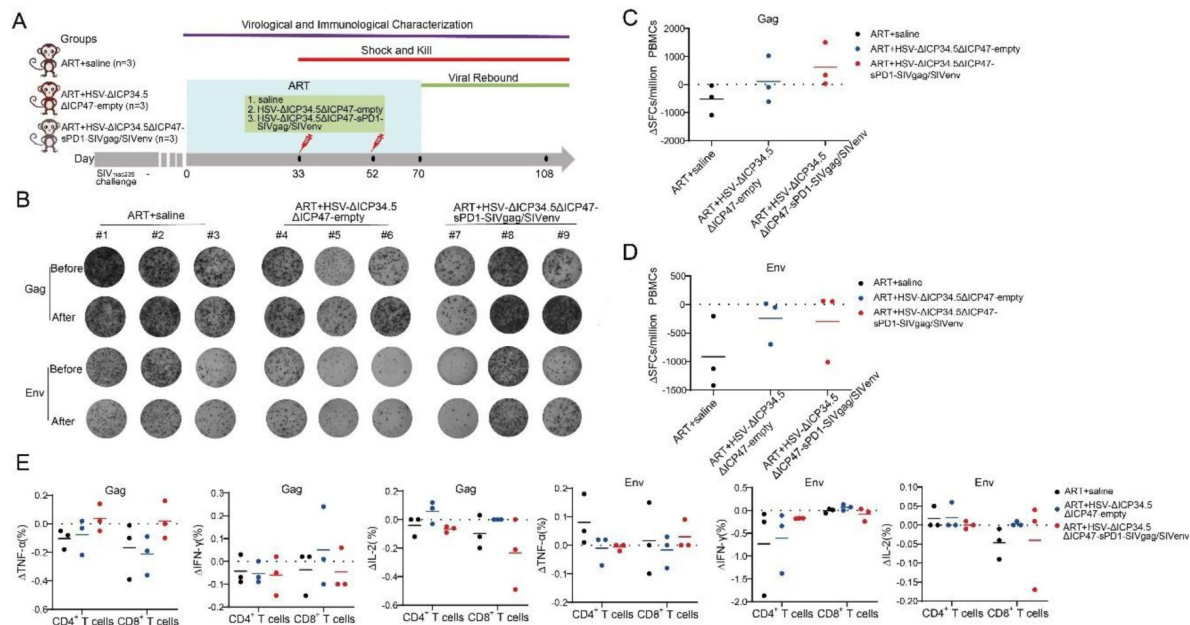


Figure 5.

The modified HSV-based constructs effectively reactivated SIV latency in vivo in chronically SIV-infected, ART-treated macaques.

(A-C) Viral load (VL) changes in plasma for each animal were monitored throughout the experiment using real-time PCR. The detection limit is 100 copies per mL plasma. The shaded area represented the duration of ART administration. (D) The VL change in plasma between pre-ART and the peak value in the rebound stage after ART discontinuation. (E) Change in total SIV DNA copies between pre-ART and ART rebound after ART discontinuation. (F) SIV integrated DNA (iDNA) copy numbers detected by Alu-PCR at different time points. (G) Change in the number of SIV Pol-specific IFN- γ -secreting cells between pre-immunization (day 33) and post-immunization (day 70), as detected by ELISpot assay. (H) Change in the CD4⁺ T/CD8⁺ T ratio. Data shown are mean $\pm$ SD. ** P <0.01, *** P <0.0001, **** P <0.0001.

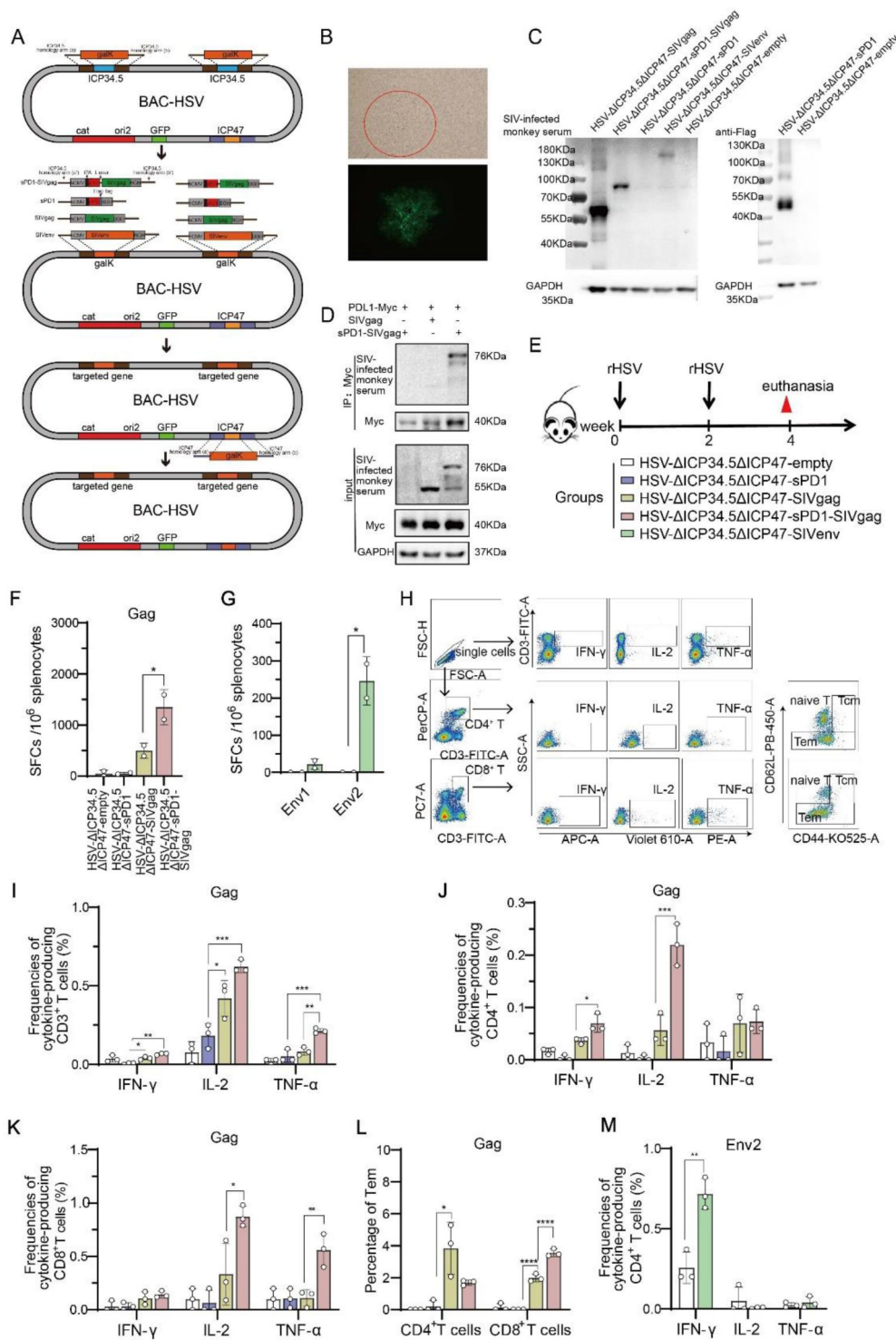


Figure 6.

Pattern to illustrate the proof-of-concept strategy based on a bifunctional HSV-ΔICP34.5-vectored therapeutic vaccine for HIV functional cure.

In the present study, the modified HSV-ΔICP34.5-based constructs effectively reactivated HIV/SIV latency by modulating the IKKα/β-NF-κB pathway and PP1-HSF1 pathway (Shock) and simultaneously elicited antigen-specific polyfunctional CD8⁺ T cells to eliminate cells infected with the reactivated virion (Kill). BAC: bacterial artificial chromosome; rHSV: recombinant HSV; TCR: T-cell receptor; PD1: Programmed Cell Death Protein 1; CD40L: CD40 Ligand.

ICP34.5 is a neurotoxicity factor that can antagonize innate immune responses, including PKR, TANK binding kinase (TBK1) signaling, and Beclin1-mediated apoptosis (He et al., 1997 [\[1\]](#), Manivanh et al., 2017 [\[2\]](#), Orvedahl et al., 2007 [\[3\]](#)). Thus, ICP34.5 deletion is beneficial to improve the safety of HSV-based constructs. As expected, our data have demonstrated experimentally that HSV-ΔICP34.5 exhibited lower virulence and replication ability than wild-type HSV-1 (Figure S1). Importantly, we also observed a significant decrease in the expression of inflammatory factors in PWLH when compared to wild-type HSV-1 (Figure 1I-K [\[4\]](#)). These data suggested that the safety of HSV-ΔICP34.5 should be more tolerable than wild-type HSV-1. ICP34.5 binds to host PP1 and mediates the dephosphorylation of eIF2α, thus allowing protein synthesis and reversing the effects of PKR and host antiviral functions (He et al., 1997 [\[1\]](#), He et al., 1998 [\[5\]](#)). In this study, our findings further unveiled an interaction between ICP34.5 and HSF1, resulting in reduced HSF1 phosphorylation via recruitment of PP1α. Interestingly, previous reports indicated that HSF1 could positively regulate HIV gene transcription (Rawat and Mitra, 2011 [\[6\]](#)), which is facilitated by its nuclear entry post-phosphorylation and subsequent recruitment of p300 for self-acetylation, along with binding to the HIV-1 LTR. Studies have also shown that HSF1 could further orchestrate p-TEFb recruitment to promote HIV-1 transcriptional elongation (Peng et al., 2020 [\[7\]](#), Lin et al., 2018 [\[8\]](#), Pan et al., 2016b [\[9\]](#), Pan et al., 2016a [\[10\]](#)). Under stress-induced conditions, phosphorylation triggers the formation of the HSF1 trimer, thus facilitating its nuclear entry to bind to heat shock elements (HSEs) for gene transcription regulation (Bonner et al., 2000 [\[11\]](#), Timmons et al., 2020 [\[12\]](#)). Additionally, we also demonstrated that ICP34.5 interacted with IKKα and IKKβ, thereby impeding NF-κB nuclear entry and further curbing HIV latency. Consistently, previous studies have also suggested that ICP34.5 could disrupt the NF-κB pathway and possibly affect the maturation of dendritic cells (Jin et al., 2011 [\[13\]](#)). Intriguingly, these findings collectively indicated that ICP34.5 might play an antagonistic role in the reactivation of HIV by HSV-1, and thus our modified HSV-ΔICP34.5 constructs can effectively reactivate HIV/SIV latency through the release of imprisonment from ICP34.5. However, ICP34.5 overexpression had only a partial effect on the reduction of the HIV latency reactivation, indicating that HSV-ΔICP34.5-based constructs can also reactivate HIV latency through other yet-to-be-determined mechanisms.

Another observation in this study is that the HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv construct elicited robust and persistent SIV-specific T cell immune responses in ART-treated, chronically SIV-infected macaques. Increasing evidence has indicated that HIV-specific cytolytic T lymphocytes (CTLs) can facilitate the suppression of latent viral reservoirs, and thus, the induction of robust and persistent HIV-specific CTL responses is essential for achieving long-term disease-free and transmission-free HIV control (Collins et al., 2020 [\[14\]](#)). Featured polyfunctional CD8⁺ T cells may contribute to HIV elite controllers or long-term non-progressors, a rare proportion of HIV-infected individuals who can spontaneously control viral replication even without ART treatment (Owen et al., 2010 [\[15\]](#), Ferre et al., 2009 [\[16\]](#), O'Connell et al., 2009 [\[17\]](#)). In addition, both our previous study and others have demonstrated that strong antigen-specific CD8⁺ T cell immune responses, especially effector memory CD8⁺ T cells, were associated with a lower viral load, and *in vivo* CD8⁺ lymphocyte depletion with intravenous infusion of anti-CD8 monoclonal antibody could lead to dramatic viral rebound in these vaccinated elite macaques (Perdomo-Celis et al., 2022 [\[18\]](#), Pandrea et al., 2011 [\[19\]](#), Sun et al., 2013 [\[20\]](#), Sun et al., 2010 [\[21\]](#), Pan et al., 2018 [\[22\]](#), Sun et al., 2012 [\[23\]](#)). Notably, previous studies have shown that HSV-based vaccines expressing HIV/SIV antigen did elicit specific CD8⁺ T cell immune responses in mice and macaques, but the magnitude was not as strong as other viral vectors (Parker et al., 2007 [\[24\]](#), Murphy et al., 2000 [\[25\]](#), Kaur et al., 2007 [\[26\]](#)). Therefore, to further improve its immunogenicity *in vivo*, some modifications were adopted to optimize the HSV-vectored vaccine. 1) The ICP47 protein, encoded by the US12 gene, can bind to the transporter associated with antigen presentation (TAP) 1/2, inhibiting the transport of viral peptides into the endoplasmic reticulum and the loading of peptides onto nascent major histocompatibility complex (MHC) class I molecules to activate CD8⁺ T cells (Orr et al., 2005 [\[27\]](#)). Therefore, the ICP47 gene was deleted from our developed HSV vector. 2) Negatively immunoregulatory molecules, including PD1, TIM-3, and LAG-3, are usually involved in the pathogenesis of HIV infection, as well as in chronically SIV-infected macaques. Among them, PD1

upregulation can result in the exhaustion and dysfunction of CD8⁺ T cells (Trautmann et al., 2006 [DOI](#)). In addition, PD1 expression on memory CD4⁺ T cells might be linked with HIV latency. In this study, we modified the SIV antigen by fusing to soluble PD1 (sPD1), which can block the PD1/PDL1 pathway by competitively binding with PDL1 and thus improve HIV/SIV vaccine-induced CD8⁺ T cell immune responses (Zhou et al., 2013 [DOI](#), Wu et al., 2022 [DOI](#), Pan et al., 2018 [DOI](#), Xiao et al., 2014 [DOI](#)).

Although promising, there are still some limitations to our study. Firstly, this is a pilot study with a relatively small numbers of RMs, and future studies with a larger number of animals can be conducted to better verify our strategy. Secondly, the HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv vaccine resulted in delayed viral rebound and a lower peak viral load post-rebound than pre-ART treatment but did not completely suppress SIV virus rebound in this study, which may be attributed to suboptimal doses and treatment, implying that we should further optimize this regimen for eventually achieving an HIV functional cure in future studies. The current consensus on HIV/AIDS vaccines emphasizes the importance of simultaneously inducing broadly neutralizing antibodies and cellular immune responses. Therefore, we believe that incorporating the induction of broadly neutralizing antibodies into our future optimizing approaches may lead to better therapeutic outcomes. Taken together, these findings demonstrated that our modified HSV-ΔICP34.5-based constructs potentially reactivated HIV/SIV latency by modulating the IKKα/β-NF-κB pathway and PP1-HSF1 pathway, and thereby we developed a proof-of-concept strategy based on a bifunctional HSV-vectored therapeutic vaccine, which can provide insights into the rational design of novel strategies for pursuing an HIV functional cure.

Methods

Mouse ethics statement and vaccination

Female BALB/c mice, aged six to eight weeks, were procured from the Experimental Animal Center of Sun Yat-sen University. A total of twenty-five mice were randomly divided into five groups to evaluate the immunogenicity of the recombinant HSV-vector-based SIV vaccine. During the initial week, each mouse was subcutaneously administered a vaccination of 1×10^6 PFU of the respective recombinant HSV-vector vaccines (HSV-ΔICP34.5ΔICP47-empty, HSV-ΔICP34.5ΔICP47-sPD1, HSV-ΔICP34.5ΔICP47-SIVgag, HSV-ΔICP34.5ΔICP47-sPD1-SIVgag, HSV-ΔICP34.5ΔICP47-SIVenv). Following a two-week interval, a booster vaccination was administered via the subcutaneous route using 2×10^6 PFU of recombinant HSV-vector vaccines. The subsequent assessment of the immune response involved ELISpot and intracellular cytokine staining (ICS) assays in accordance with the vaccination schedule.

Ethics statement and vaccination of macaques

A total of 9 chronically SIV_{mac239}-infected rhesus macaques (RMs) were included in this study. All RMs received ART treatment (FTC/6.7 mg/kg/once daily, PMPA/10 mg/kg/once daily) for a duration of 33 days. The RMs were allocated into three groups based on their baseline plasma SIV viral loads (VL): ART + saline (n=3) as the control group, ART+ HSV-ΔICP34.5ΔICP47-empty (n=3) as the sham group, and ART+ HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv (n=3) as the vaccinated group. On day 0, all RMs received ART treatment. Once the VL dropped below the limit of detection (1×10^2 copies/mL), the vaccinated group and sham group were primed with a subcutaneous vaccination of 1×10^7 PFU HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv or HSV-ΔICP34.5ΔICP47-empty, respectively, while the control group received an injection of 0.9% saline. After three weeks of the prime vaccination, the vaccinated group and sham group RMs received a subcutaneous booster vaccination of 5×10^7 PFU HSV-ΔICP34.5ΔICP47-sPD1-SIVgag/SIVenv or HSV-ΔICP34.5ΔICP47-empty, respectively. After two weeks of booster vaccination, the ART treatment for all RMs was interrupted. The immune response was evaluated by IFN-γ ELISpot and ICS assays. Plasma viral load was regularly monitored throughout the studied period.

Peptide pools

The peptide pools, encompassing the complete sequences of SIV Gag, Env, and Pol proteins, comprising 15 amino acids with 11 overlaps, were generously provided by the HIV Reagent Program, National Institutes of Health (NIH), USA. Gag pools comprise 125 peptides, while Env and Pol pools are subdivided into Env1 (109 peptides) and Env2 (109 peptides) pools, as well as Pol1 (131 peptides) and Pol2 (132 peptides) pools. These peptide pools were dissolved in dimethyl sulfoxide (DMSO, Sigma) at a concentration of 0.4 mg/ peptide/mL for subsequent immunological assays.

Construction of recombinant HSV

For the generation of recombinant HSV-vector-based vaccines, we implemented modifications at the ICP34.5 loci through homologous recombination within the BAC-HSV-1 system. Specifically, double copies of the ICP34.5 and ICP47 genes were either deleted or inserted into the respective counterparts: sPD1, SIVgag, sPD1-SIVgag, and SIVenv genes. The sPD1-SIVgag gene was created by fusing the N-terminal region of mouse soluble PD1 (sPD1) with the C-terminal segment of SIVgag, connected by a GGGSGGG linker, which was engineered through overlapping PCR.

Plaque Assay

Vero cells were seeded at a density of 2×10^5 cells per well in a 12-well plate and cultured for 24 h. Virus samples were serially diluted and then added to the wells. After 2 h of incubation at 37°C with 5% CO₂, the supernatant was aspirated, and the cells were washed with PBS. Then, a medium containing 1% FBS, 1% low-melting-point agarose, and 1% penicillin-streptomycin was added to each well. After 3 days of incubation, cells were fixed with 4% paraformaldehyde at room temperature for 2 h and stained with 1% crystal violet for 10 min. Plaques were visualized and counted, and virus titers were calculated as plaque-forming units per milliliter (PFU/mL).

IFN-γ ELISpot assay

The IFN-γ-ELISpot assay was conducted in accordance with our previous study (Sun et al., 2010 [\[10\]](#)). In the mouse experiment, 2.5×10^5 freshly isolated mouse spleen lymphocytes were stimulated with SIV Gag, Env1, and Env2 peptide pools. In the monkey experiment, 2×10^5 peripheral blood mononuclear cells (PBMCs) were seeded and stimulated with the SIV Gag, Env, and Pol peptide pools. DMSO (Sigma) was utilized as Mock stimulation, while concanavalin A (ConA, 10 μg/mL) was employed as a positive control. Spot quantification was performed using an ELISpot reader (Mabtech), and peptide-specific spot counts were determined by subtracting the spots from mock stimulation.

Intracellular cytokine staining (ICS)

The ICS assay was performed as described previously (Wu et al., 2022 [\[11\]](#)). In the mouse experiment, 2×10^6 freshly isolated mouse spleen lymphocytes were stimulated with SIV Gag, Env1, and Env2 peptide pools. For the monkey experiment, 2×10^6 PBMCs were seeded and stimulated with the SIV Gag, Env, and Pol peptide pools. DMSO and PMA (Thermo Scientific) plus ionomycin were used as the mock and positive controls, respectively. Data analysis was performed using FlowJo software (version 10.8.1), and the antibodies used are listed in Table S3. The frequencies of cytokines produced from peptide-specific cells were analyzed by subtracting mock stimulation.

SIV viral RNA and DNA copy assays and absolute T cell count

Absolute T cell count and the levels of plasma mRNA and SIV total DNA were quantified as described previously (Wu et al., 2022 [\[11\]](#)). Plasma viral RNA copy numbers were determined via SYBR green-based real-time quantitative PCR (Takara), using SIV gag-specific primers (Table S4). Viral RNA copy numbers were calculated based on the standard curve established using SIV_{mac239}

gag standards. The lower limit of detection for this assay was 100 copies/mL of plasma. Total cellular DNA was extracted from approximately 0.5 to 5 million cells using a QIAamp DNA Blood Minikit (Qiagen). PCR assays were performed with 200 ng samples of DNA, and SIV viral DNA was quantified using a pair of primers targeting a conserved region of the SIV gag gene, as previously described. Quantitation was performed by comparing the results to the standard curve of SIV gag copies. Integrated SIVmac239 DNA was measured using nested PCR with two sets of primers. The first round used SIVgag-reverse and Alu-forward primers, while the second round used primers specific to a conserved SIVgag region. Quantification was compared to a standard of CEMss/pWPXLD-rc cell genome as our previously described (Yang et al., 2019 [DOI](#)).

Plasmids, cells, and viruses

pVAX-Myc-PDL1, pVAX-Flag-ICP34.5, pVAX-Myc-HSF1, pVAX-HA-PP1α: full-length mouse PDL1, full-length HSV-1 ICP34.5, and full-length human HSF1 and human PP1α with N or C-terminal Tag were cloned into the pVAX vector. pVAX-empty, pcDNA3.1-IKKα, and pcDNA3.1-IKKβ plasmids were stored in our laboratory. pVAX-empty was used as a mock transfection in our study.

293T cells (from the embryonic kidney of a female human fetus), Vero cells (from the kidney of a female normal adult African green monkey), and Hela cells (from the cervical cancer cells of an African American woman) were cultured in complete Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in an atmosphere of 5% CO₂. The J-Lat 10.6 cells (Jurkat cells contain the HIV-1 full-length genome whose Env was frameshifted and inserted with GFP in place of Nef) and the HIV-1 latently infected CD4⁺ CEM cells ACH-2 (A3.01 cell integrated HIV-1 proviral DNA) were cultured in complete RPMI640 (Gibco) containing 10% FBS and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂. J-Lat 10.6-ICP34.5 cells were constructed in our laboratory.

The HSV-1 (McKrae) strains were stored in our laboratory. HSV-GFP (17 strain) was rescued from pBAC-GFP-HSV in Vero cells. HSV-ΔICP34.5 (17 strain) was rescued from pBAC-GFP-HSV-ΔICP34.5 (with deletion of double copies of ICP34.5 gene) in Vero cells.

RT-qPCR

RT-qPCR was performed as described in our previous study (Zhao et al., 2022 [DOI](#)). Data were normalized to β-actin. The primer sequences are listed in Supplemental Table 4. Fold changes in the threshold cycle (Ct) values were calculated using the 2^{-ΔΔCt} method.

Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was conducted using the SimpleChIP Enzymatic Chromatin IP kit (Agarose Beads) (CST), following the manufacturer's instructions. Quantitative real-time PCR was employed for detecting the LTR sequence. The sequences of primers used in the LTR ChIP are listed in Supplemental Table 4. % Input = $2\% \times 2^{[(ct)2\% \text{ input sample} (ct) \text{ IP sample}]}$.

Co-immunoprecipitation (Co-IP)

Cells were harvested and subjected to Co-IP assay, following the protocol outlined in our previous study (Zhao et al., 2022 [DOI](#)).

Protein extraction and western blot

Nuclear and cytoplasmic proteins were extracted by kits following the manufacturer's instructions (Beyotime). The Western blotting assay was performed as previously described (Zhao et al., 2022 [DOI](#)).

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, San Diego, California) was used for statistical analysis. For intragroup direct comparisons, Student's unpaired two-tailed *t* test was performed to analyze significant differences. For comparisons of multiple groups, one-way ANOVAs were performed. Significance levels are indicated as **P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001.

Study approval

Mice experiment was approved by the Laboratory Animal Ethics Committee guidelines at Sun Yat-sen University (approval number: SYSU-IACUC-2021-000185). Chinese rhesus macaques (*Macaca mulatta*) were housed at the Landau Animal Experimental Center, Guangdong Landau Biotechnology Co., Ltd. (approval number: N2021101). The primary PBMC samples were isolated from the chronically HIV-1-infected participants who were recruited from Guangzhou Eighth People's Hospital (Guangzhou, Guangdong, China), which was approved by the Medical Ethics Review Board of the School of Public Health (Shenzhen), Sun Yat-sen University (2022-037). All participants provided written informed consent, agreeing to participate in this study. The enrollment criteria for HIV-1-infected individuals included substained suppression of plasma HIV-1 viremia under ART, with undetectable plasma HIV-1 RNA levels (less than 50 copies/mL) for at least 12 months and a CD4⁺ T cell count more than 350 cells/μL).

Additional information

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Author Contributions

All authors were involved in drafting or critically revising the manuscript for important intellectual content, and all authors approved the final version for publication. CS conceived the project and provided the main funding. CS and LC designed the experiments and reviewed the manuscript. ZW, PL, YY, and CW performed most of the experiments and analyzed the data. ZW, PL, YY, CW, ML, HW, MS, YH, and MC established the animal models and performed animal experiments. ZW and CS interpreted the data and wrote the manuscript.

Additional files

Data Availability Statement

Source data contain the numerical data used to generate the figures.

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Additional files

Supplemental figures [↗](#)

supplemental figures and tables [↗](#)

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Reviewer #1 (Public review):

Summary:

Authors constructed a novel HSV-based therapeutic vaccine to cure SIV in a primate model. The novel HSV vector is deleted for ICP34.5. Evidence is given that this protein blocks HIV reactivation by interference with the NF κ B pathway. The deleted construct supposedly would reactivate SIV from latency. The SIV genes carried by the vector ought to elicit a strong immune response. Together the HSV vector would elicit a shock and kill effect. This is tested in a primate model.

Strengths and weaknesses:

(1) Deleting ICP34.5 from the HSV construct has a very strong effect on HIV reactivation. The mechanism underlying increased activation by deleting ICP34.5 is only partially explored. Overexpression of ICP34.5 has a much smaller effect (reduction in reactivation) than deletion of ICP34.5 (strong activation); this is acknowledged by the authors that no full mechanistic explanation can be given at this moment.

(2) No toxicity data are given for deleting ICP34.5. How specific is the effect for HIV reactivation? A RNA seq analysis is required to show the effect on cellular genes.

A RNA seq analysis was done in the revised manuscript comparing the effect of HSV-1 and deleted vector in J-LAT cells (Fig S5). More than 2000 genes are upregulated after transduction

with the modified vector in comparison with the WT vector. Hence, the specificity of upregulation of SIV genes is questioned. Authors do NOT comment on these findings. In my view it questions the utility of this approach.

(3) The primate groups are too small and the results too variable to make averages. In Fig 5, the group with ART and saline has two slow rebounders. It is not correct to average those with the single quick rebounder. Here the interpretation is NOT supported by the data.

Although authors provided some promising SIV DNA data, no additional animals were added. Groups of 3 animals are too small to make any conclusion, especially since the huge variability in response. The average numbers out of 3 are still presented in the paper, which is not proper science.

No data are given of the effect of the deletion in primates. Now the deleted construct is compared with an empty vector containing no SIV genes. Authors provide new data in Fig S2 on the comparison of WT and modified vector in cells from PLWH, but data are not that convincing. A significant difference in reactivation is seen for LTR in only 2/4 donors and in Gag in 3/4 donors. (Additional question what is meaning of LTR mRNA, do authors relate to genomic RNA??)

Discussion

HSV vectors are mainly used in cancer treatment partially due to induced inflammation. Whether these are suitable to cure PLWH without major symptoms is a bit questionable to me and should at least be argued for.

The RNA seq data add on to this worry and should at least be discussed.

Comments on revisions:

The authors accept the limitations of the primate study (too small for strong conclusions). The new way of presenting the data clearly shows these limitations.

<https://doi.org/10.7554/eLife.95964.3.sa1>

Author response:

The following is the authors' response to the previous reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

The authors constructed a novel HSV-based therapeutic vaccine to cure SIV in a primate model. The novel HSV vector is deleted for ICP34.5. Evidence is given that this protein blocks HIV reactivation by interference with the NFkappaB pathway. The deleted construct supposedly would reactivate SIV from latency. The SIV genes carried by the vector ought to elicit a strong immune response. Together the HSV vector would elicit a shock and kill effect. This is tested in a primate model.

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than deletion of ICP34.5 (strong activation); this is acknowledged by the authors that no full mechanistic explanation can be given at this moment.

Thank you for your comments. We agree with you that the mechanism underlying increased reactivation by deleting ICP34.5 is only partially explored. As you pointed out, the deletion of ICP34.5 leads to a significant reactivation, while the overexpression of ICP34.5 has a relatively weak inhibitory effect on reactivation. This difference prompts us to further contemplate the role of HSV-1 in regulating HIV latency and reactivation. Our data (Figure S4), along with previous literature (Mosca et al., 1987, Nabel et al., 1988), have indicated that the ICP0 protein might play a crucial role in the reactivation of HIV latency. However, we found for the first time that ICP34.5 can play an antagonistic role with this reactivation. This is a very interesting topic for understanding the complicated interactions between host cells and different viruses. We will investigate the deeper insights in future studies, and we have mentioned this limitation in the revised Discussion Section. Thank you!

(2) No toxicity data are given for deleting ICP34.5. How specific is the effect for HIV reactivation? A RNA seq analysis is required to show the effect on cellular genes.

A RNA seq analysis was done in the revised manuscript comparing the effect of HSV-1 and deleted vector in J-LAT cells (Fig S5). More than 2000 genes are upregulated after transduction with the modified vector in comparison with the WT vector. Hence, the specificity of upregulation of SIV genes is questioned. Authors do NOT comment on these findings. In my view it questions the utility of this approach.

Thank you for your mentions.

(1) As for the toxicity of HSV-ΔICP34.5, it is well known that ICP34.5 is a neurotoxicity factor that can antagonize host immune responses, and thus deleting ICP34.5 is beneficial to improve the safety of HSV-based constructs. As expected, we have demonstrated experimentally that HSV-DICP34.5 exhibited lower virulence and replication ability than wild-type HSV-1 (Figure S1). Importantly, we also observed a significant decrease in the expression of inflammatory factors in PWLH when compared to wild-type HSV-1 (Figure 1I-K). These data suggested that the safety of HSV-DICP34.5 should be more tolerable than wild-type HSV vector.

(2) The RNASeq analysis is aimed to explore the HSV-ΔICP34.5-induced signaling pathways, but it is not suitable to use this data for assessing the toxicity of HSV-ΔICP34.5 constructs. As for the RNASeq data, we think it is reasonable to observe many upregulated genes (which are involved in a variety of signaling pathways), since HSV-DICP34.5 constructs reactivated HIV latency more effectively than wild-type HSV by modulating the IKKα/β-NF-κB pathway and PP1-HSF1 pathway.

(3) To further validate whether HSV-ΔICP34.5 can specifically activate the HIV latent reservoir, we conducted additional experiments using vaccinia virus and adenovirus as controls, and results showed that both vaccinia virus and adenovirus cannot effectively reactivate HIV latency (Figure S3). Moreover, the deletion of ICP0 gene from HSV-1 diminished the reactivation effect of HIV latency by HSV-1, and overexpressing ICP0 greatly reactivate the latent HIV (Figure S4, Figure S5), implying that this reactivation should be virus-specific and ICP0 plays an important factor on reversing HIV latency. Interestingly, we herein found that ICP34.5 can act as an antagonistic factor for this reactivation of HIV latency by HSV-1. Thus, after the deletion of ICP34.5, the ability of HSV to reverse HIV latency was significantly enhanced. Our research group will investigate the underlying mechanism in future studies. Thank you for your insightful mention.

(3) The primate groups are too small and the results too variable to make averages. In Fig 5, the group with ART and saline has two slow rebounders. It is not correct to average those with the single quick rebounder. Here the interpretation is NOT supported by the data.

Although authors provided some promising SIV DNA data, no additional animals were added. Groups of 3 animals are too small to make any conclusion, especially since the huge variability in response. The average numbers out of 3 are still presented in the paper, which is not proper science.

No data are given of the effect of the deletion in primates. Now the deleted construct is compared with an empty vector containing no SIV genes. Authors provide new data in Fig S2 on the comparison of WT and modified vector in cells from PLWH, but data are not that convincing. A significant difference in reactivation is seen for LTR in only 2/4 donors and in Gag in 3/4 donors. (Additional question what is meaning of LTR mRNA, do authors relate to genomic RNA??)

Thank you for your serious review and kind reminder.

(1) We agree with you that it is not appropriate to use averages for this pilot study with limited numbers of macaques. We are currently unable to conduct another experiment with a larger number of macaques, but we think the results of this pilot study were very promising for further studies. Now, following your kind suggestions, we have removed the averages and now presented the data for each monkey individually in the revised manuscript. We have also modified the corresponding description accordingly (Line 254 to 262). Thank you for your understanding.

(2) Regarding your comment about the lack of data on the deletion of ICP34.5 from HSV-1, we are sorry for previously unclear description. In fact, the empty vector used in our animal experiments not only does not contain SIV antigens but also has the ICP34.5 deletion. We have revised the corresponding description accordingly (For example, we use HSV-DICP34.5DICP47-empty, HSV-DICP34.5DICP47-sPD1-SIVgag/SIVenv instead of HSV-empty, HSV-sPD1-SIVgag/SIVenv). We hope this revision will address your question.

(3) As for the reactivation effects observed in PLWH samples, the data may be not perfect, but we think this result (a significant difference in reactivation is seen for LTR in 2/4 donors and for Gag in 3/4 donors, and the purpose of detecting LTR RNA is to evaluate the level of virus replication) is promising to support our conclusion (The enhanced reactivation effect in primary CD4⁺ T cells by HSV-ΔICP34.5 than wild-type HSV). Of course, we recognize the need for more samples to gain a comprehensive understanding of reactivation effect in different individuals in future study. In addition, we corrected the description of LTR RNA (Lines 99-106 and 115-116). Thank you for the reminder!

Discussion

HSV vectors are mainly used in cancer treatment partially due to induced inflammation. Whether these are suitable to cure PLWH without major symptoms is a bit questionable to me and should at least be argued for.

The RNA seq data add on to this worry and should at least be discussed.

Thank you for your mention. As mentioned above, the RNASeq analysis is aimed to explore the HSV-ΔICP34.5-induced signaling pathways, but it is not suitable to use this data for assessing the toxicity of HSV-ΔICP34.5 constructs. Actually, ICP34.5 is a neurotoxicity factor that can antagonize innate immune responses, and thus ICP34.5 deletion is beneficial to

improve the safety of HSV-based constructs. As expected, our data have demonstrated experimentally that HSV-DICP34.5 exhibited lower virulence and replication ability than wild-type HSV-1 (Figure S1). Importantly, HSV-DICP34.5 induced a lower level of inflammatory cytokines (including IL-6, IL-1 β , and TNF- α) in primary CD4⁺ T cells from PLWH compared to HSV stimulation, likely due to its lower virulence and replication ability (Figure 1I-K). In addition, the CD4⁺ /CD8⁺ T cell ratio (Figure 5H) and body weight (Figure S10) after treatment were effectively ameliorated in the SIV-infected macaques of the ART+HSV-DICP34.5DICP47-sPD1-SIVgag/SIVenv group. Our data also demonstrated that there was no significant effect on the cell composition of peripheral blood in the SIV-infected macaques of ART+HSV-DICP34.5DICP47-sPD1-SIVgag/SIVenv group (Figure S11). These data suggested that the safety of HSV-DICP34.5 should be more tolerable than wild-type HSV vector. We have added a more comprehensive description in the revised Discussion (Lines 328-334). Thank you again for all of your kind comments and suggestions.

Reviewer #2 (Public review):

Summary:

In this article Wen et. al., describe the development of a 'proof-of-concept' bi-functional vector based out of HSV-deltaICP-34.5's ability to purge latent HIV-1 and SIV genomes from cells. They show that co-infection of latent J-lat T-cell lines with a HSV-deltaICP-34.5 vector can reactivate HIV-1 from a latent state. Over- or stable expression of ICP 34.5 ORF in these cells can arrest latent HIV-1 genomes from transcription, even in the presence of latency reversal agents. ICP34.5 can co-IP with- and de-phosphorylate IKK α /b to block its interaction with NF-k/B transcription factor. Additionally, ICP34.5 can interact with HSF1 which was identified by mass-spec. Thus, the authors propose that the latency reversal effect of HSV-deltaICP-34.5 in co-infected JLat cells is due to modulatory effects on the IKK α /b-NF-kB and PP1-HSF-1 pathway.

Next the authors cleverly construct a bifunctional HSV based vector with deleted ICP34.5 and 47 ORFs to purge latency and avoid immunological reflexes, and additionally expand the application of this construct as a vaccine by introducing SIV genes. They use this 'vaccine' in mouse models and show the expected SIV-immune responses. Experiments in rhesus macaques (RM), further elicit potential for their approach to reactivate SIV genomes and at the same time block their replication by antibodies. What was interesting in the SIV experiments is that the dual-functional vector vaccine containing sPD1- and SIV Gag/Env ORFs effectively delayed SIV rebound in RMs and in some cases almost neutralized viral DNA copy detection in serum. Very promising indeed, however there are some questions I wish the authors explored to answer, detailed below.

Overall, this is an elegant and timely work demonstrating the feasibility of reducing virus rebound in animals, and potentially expand to clinical studies. The work was well written, and sections were clearly discussed.

Strengths:

The work is well designed, rationale explained and written very clearly for lay readers.

Claims are adequately supported by evidence and well designed experiments including controls.

We appreciate your positive comment for our work.

Weaknesses:

(1) It looks like ICP0 is also involved in latency reversal effects. More follow-up work will be required to test if this is in fact true.

Both our data (Figure S4, Figure S5) and previous literature (Nabel et al., 1988, Mosca et al., 1987) have reported that HSV ICP0 may play a role in reversing HIV latency. However, the exact mechanisms behind this effect have not yet been fully elucidated. Of note, we herein reported for the first time that ICP34.5 can act as an antagonistic factor for this reactivation of HIV latency by HSV-1. Thus, after the deletion of ICP34.5, the ability of HSV to reverse HIV latency was significantly enhanced. Our research group will investigate the underlying mechanism in future studies. Thank you for your insightful mention.

(2) It is difficult to estimate the depletion of the latent viral reservoir. The authors have tried to address this issue. A more convincing argument to this reviewer will be data to demonstrate that after the bi-functional vaccine, the animals show overall reduction in the number of circulating latent cells. The feasibility to obtain such a result is not clearly demonstrated.

Thank you for your comment. As you mentioned, we have indeed measured both total DNA and integrated DNA (iDNA) in blood cells (see Figure 5E-F), which can provide support for the reduction of the latent viral reservoir. Thank you for your kind reminder.

(3) The authors state that the reduced virus rebound detected following bi-functional vaccine delivery is due to latent genomes becoming activated and steady-state neutralization of these viruses by antibody response. This needs to be demonstrated. Perhaps cell-culture experiments from specimen taken from animals might help address this issue. In lab cultures one could create environments without antibody responses, under these conditions one would expect higher level of viral loads being released in response to the vaccine in question.

Thank you for your valuable suggestion. We believe that the reduced virus rebound observed may be influenced by immune responses from T cells and antibodies induced by both ART and the vaccine. We appreciate your insight and agree that future studies should focus on investigating the activation effects of the vaccine under controlled conditions that simulate the absence of immune responses in primary animal cells. This will help us better understand the mechanisms involved and address your concerns more comprehensively.

Reviewer #2 (Recommendations for the authors):

The Authors have sufficiently addressed my comments. Below are a few minor changes that can help with clarity.

Lines 126-127: This sentence should be changed. Perhaps, "these data suggests that Safety of... in PLWH might be tolerable, at least in vitro."

Thanks for your suggestion. We have revised it accordingly. (Line 130).

Lines 128-132: Would this not mean that reactivation is due to ICP0 gene? Have the authors tried to express ICP0-gene into J-Lat cells and see if that is the reason for reactivation? This seems somewhat incomplete. At the end of 132, please add ", in the presence of ICP0". Also a sentence describing this effect is warranted.

Thank you for your insightful suggestion. Yes, both our data and previous literature supported that the ICP0 gene can play a significant role in the reactivation of HIV latency (Figure S4, Figure S5). Of note, we herein reported for the first time that ICP34.5 can act as an antagonistic factor for this reactivation of HIV latency by HSV-1. Thus, after the deletion of ICP34.5, the ability of HSV to reverse HIV latency was significantly enhanced. We have

described this effect in the revised version accordingly. Additionally, we have added the phrase “in the presence of ICP0” to the results section (Lines 137) to clarify this point.

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