# Applications of CRISPR-Cas9 in the Treatment of Herpes Simplex Keratitis

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**Abstract.** Herpes simplex keratitis (HSK) is a leading cause of infectious corneal blindness. Herpes simplex virus type 1 (HSV-1) establishes lifelong latency in trigeminal ganglion neurons and reactivates in the cornea. While antivirals such as acyclovir (ACV) suppress viral replication, they do not eradicate the infection, and drug resistance is increasing. Genome editing offers a direct strategy to disrupt essential viral functions. CRISPR-Cas9, guided by single guide RNA (sgRNA), has been shown to reduce HSV-1 replication in cell cultures, organoids, and animal models, with multi-target designs limiting viral escape. Recent delivery advancements include HSV-1-erasing lentiviral particles (HELP), which package spCas9 mRNA with guides targeting UL8 and UL29, enabling transient editing in the cornea and retrograde transport to the trigeminal ganglion. Complementary approaches utilize adeno-associated virus serotype 2 (AAV2) to deliver SaCas9 targeting ICP0 and ICP27 in human induced pluripotent stem cell-derived cerebral organoids. Sensor-effector platforms such as autonomous virus-inducible immune-like cells (ALICE) integrate CRISPR editing with neutralizing antibodies, demonstrating antiviral activity comparable to high-dose ACV in certain contexts. Key challenges include off-target effects, access to latent chromatin, immunogenicity, and scalable ocular delivery. This review aims to synthesize current evidence, compare delivery platforms including HELP, AAV2, and ALICE, and identify priorities for clinical translation.

**Keywords:** HSK, HSV-1, HELP, CRISPR-Cas9, antiviral therapy.

## 1. Introduction

Herpes simplex keratitis (HSK) is a common cause of blindness in developed nations and one of the primary reasons for corneal scar formation. This disease, caused by herpes simplex virus type 1 (HSV-1), is characterized by symptoms including photophobia, itching, and severe ocular pain. It exhibits a high recurrence rate and is challenging to cure radically [1]. Following primary infection, HSV-1 establishes latent infections within the neurons of the trigeminal ganglion. Upon reactivation, progeny viral particles are transported along axonal microtubules to the nerve terminals, where they infect the peripheral corneal epithelium, triggering inflammation and tissue damage [2]. Acyclovir, which inhibits viral replication, is one of the most commonly used drugs for treating HSK. However, latent virus does not express DNA polymerase, making long-term use prone to inducing drug resistance and ineffective at eliminating the latent viral reservoir [3]. Therefore, exploring and developing more innovative and effective therapeutic approaches is crucial.

Current treatments for HSV primarily focus on inhibiting viral replication. However, during latency, HSV does not express all viral genes, allowing the virus to persist within the host, evading clearance by vaccines and the immune system. Since HSV does not actively replicate its genome in this state, traditional drugs targeting DNA polymerase, which is largely inactive during latency, have limited efficacy. With the advancement of CRISPR-Cas9 technology, it has become feasible to design specific guide RNAs (gRNAs) targeting single or dual sites, potentially enabling the complete knockout of viral genes. Specifically, these targets are often located within essential viral genes such as the DNA polymerase (UL54), polymerase processivity factor (UL44), single-stranded DNA-binding protein (UL57), primase (UL70), DNA helicase (UL105), major capsid protein (UL86), and UL84. Targeting a single essential gene for viral replication can effectively suppress up to

approximately 95% of viral replication, potentially leading to viral clearance over time. Dual targeting strategies are more effective than single targeting, can help avoid off-target effects, and may lead to the complete eradication of the virus [4].

CRISPR-Cas9 offers the potential to eradicate the HSV-1 latent reservoir due to its programmable nuclease activity. First, specific guide RNAs (gRNAs) direct Cas9 precisely to the target sequences within essential viral genes via base complementarity pairing. Subsequently, Cas9 induces a double-strand break (DSB) at the targeted site, activating the cell's DNA repair mechanisms. One such pathway, non-homologous end joining (NHEJ), directly ligates the broken DNA ends. This error-prone process often results in small insertions or deletions (indels), leading to viral gene knockout. Consequently, CRISPR technology can precisely inhibit viral gene replication.

Current anti-HSV drugs primarily aim to enhance the host's intrinsic ability to eliminate the virus, thereby preventing viral entry or interfering with replication within human cells. These drugs function by penetrating cells and interfering with stages of the viral replication cycle (e.g., S phase, G1 phase, G2 phase). Based on their mechanisms of action, antiviral drugs can be classified into: DNA polymerase inhibitors, reverse transcriptase inhibitors, protease inhibitors, neuraminidase inhibitors, entry and uncoating inhibitors, and broad-spectrum antiviral agents. While these drugs can alleviate acute HSV infections, their long-term efficacy is limited due to the emergence of drug-resistant mutations and their inactivity against latent virus. In contrast, CRISPR-Cas9 can achieve precise cleavage of the HSV-1 genome, enabling fundamental knockout of critical viral genes, thus offering a more specific and potentially durable therapeutic strategy [3].

Delivery efficiency and tissue specificity represent core challenges for in vivo applications. Modified lentiviral particles (mLPs) capable of concurrently delivering spCas9 mRNA and dual gRNAs targeting essential HSV-1 genes (UL8/UL29) via intrastromal injection have been developed to target and clear HSV-1, effectively treating herpes stromal keratitis. In mouse models, this strategy effectively controlled acute infection; for latent infection, viral load was significantly reduced, remained localized to the eye, and showed no systemic dissemination. Replication of HSV-1 was also effectively suppressed in ex vivo human corneal models. This therapeutic approach overcomes the limitation of existing drugs being unable to clear latent virus and establishes a safety foundation for clinical translation. This review aims to outline the principles and design strategies of CRISPR-Cas9 gene editing, discuss the clinical value of locally delivered vectors for targeted clearance of HSV-1 latent in ganglia, and analyze challenges such as off-target effect safety concerns, immune response risks, and editing efficiency, thereby providing reference and research ideas for subsequent studies and clinical translation.

## 2. Clinical Burden and Virological Characteristics of HSK

Herpes simplex keratitis (HSK) is a common disease with a globally high blinding rate and is a leading cause of corneal ulceration and blindness. HSK typically presents unilaterally, with clinical manifestations encompassing herpetic dendritic keratitis, geographic ulceration, stromal keratitis, disciform keratitis, and neurotrophic keratitis [5], among which stromal keratitis is the most common presentation [6]. Epidemiological investigations indicate that over 1.8 million cases of herpetic eye disease may occur annually, posing a significant threat to ocular health. Estimates suggest that approximately 4.85 billion people globally are infected with the lifelong and periodically recurrent herpes simplex virus type 1 (HSV-1), with significant regional variation; the African region has a far higher prevalence compared to other regions, while the Americas have the lowest [7]. This substantial burden highlights the urgent need for more effective intervention strategies capable of preventing recurrence and potentially offering a cure.

HSK is primarily caused by HSV-1. HSV-1 is an enveloped double-stranded DNA virus, protected by an icosahedral capsid, with a complex structure and the inherent ability to replicate within human neurons and epithelial cells. HSV-1 has a greater impact on humans than on other animals. Following initial infection, the virus establishes a lifelong latent state within the host. During infection and

transmission, HSV-1 initially becomes latent within the nuclei of trigeminal ganglion (TG) neuronal cells, establishing a long-term latent state within the neuronal nucleus. Upon host immune suppression or exposure to stimuli, HSV-1 reactivates and travels along neuronal axon microtubules (MTs) to nerve endings, infecting adjacent peripheral epithelial tissues (such as the human eye) [2].

Currently, as there are no specific drugs that effectively cure HSK, antiviral therapy remains the mainstay treatment. In clinical antiviral practice, nucleoside analogues, including acyclovir (ACV) and ganciclovir eye drops or gel, are the most commonly used agents. Acyclovir is a synthetic acyclic analogue of guanosine, available in oral, topical, and intravenous formulations, with a good safety and tolerability profile [8]. These antiviral drugs function by interfering with the viral replication cycle (G1, S, and G2 phases). ACV acts by interfering with the DNA replication of HSV. Although ACV is effective in the short-term treatment of HSV infections, there is a persistent risk of drug-resistant variants or mutations emerging over time. Consequently, these drugs cannot completely suppress viral replication, posing a threat to long-term public health. Therefore, genome-level intervention strategies such as CRISPR-Cas9 and miRNAs, which hold promise for precisely knocking out or regulating key viral genes, are becoming focal points in animal experiments and early clinical research, offering potential value for improving recurrence control and achieving more durable efficacy [9, 10]. With the advancement of CRISPR technology, which has the potential to precisely edit viral genes, understanding the mechanisms of CRISPR editing and experimentally validating the efficacy of this technology is crucial.

## 3. Application of CRISPR-Cas9 in Combating HSV-1 Infection

CRISPR-Cas9 originates from the adaptive immune system of bacteria. When bacteria detect the presence of viral DNA, they record the invading sequence at their CRISPR locus and transcribe it to produce complementary guide RNA (gRNA). The gRNA binds to the Cas9 protein to form a complex that can specifically recognize and cleave target DNA. When the gRNA finds its target within the viral genome, Cas9 cleaves the target DNA, rendering the virus non-functional. CRISPR can not only cleave viral DNA but can also be programmed to precisely cut any type of DNA by altering the target sequence. Furthermore, this mechanism can be applied to eukaryotes. Within living cells, Cas9 can unwind DNA and match it to the target DNA. If a match is completed, Cas9 cleaves the DNA. To repair Cas9-induced double-strand breaks (DSBs), cells employ two primary DNA repair pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR). The former pathway rejoins the broken ends, often introducing errors, while the latter involves resection of the DNA strands flanking the break for repair using a template. Both pathways can introduce errors or mutations into the cell, leading to deletions or insertions. Alternatively, if an identical DNA sequence is present near the break site, it can serve as a template for precise repair via the HDR pathway. In this manner, researchers can design template DNA to introduce specific edits or large fragment insertions or deletions.

Typically, the replication of human cytomegalovirus (HCMV) relies on various viral replication factors. Based on this, one study selected seven genes potentially affecting viral replication and then examined whether CRISPRs targeting these genes could influence viral gene expression. This study tested 58 putative gRNAs to assess their ability to control the in vitro cleavage of HSV-1 target DNA. The experiment initially incubated individual nuclease/gRNA complexes with several DNA substrates containing different target sequences and evaluated cleavage efficiency using gel electrophoresis. After identifying gRNAs with the highest cleavage efficiency, their effectiveness in preventing HSV-1 lytic replication in human fibroblasts was further examined. Cells were infected with HSV-1 and transduced with different nuclease/gRNA complexes, followed by plaque assays to assess viral (lytic) replication. Results showed that targeting the UL30 region, which encodes the viral DNA polymerase, significantly reduced viral replication (>50%). Control experiments included targeting several non-essential genes to evaluate specificity and efficacy. Targeting essential genes like US7 and US11 eliminated up to 95% of the virus, whereas targeting non-essential genes like US6,

US7, and US11 had no significant impact on HCMV infection. Thus, CRISPR gene editing technology exerts a potent inhibitory effect on viral gene replication. Besides its significant role in restricting the replication of slow-replicating viruses like HCMV, CRISPR also shows efficacy against fast-replicating viruses like HSV. A similarity between these viruses is that most gRNAs targeting essential HSV genes significantly reduce viral replication. However, unlike HCMV findings, targeting non-essential HSV genes also reduced HSV replication. To facilitate quantification and tracking, one study utilized Burkitt's lymphoma Akata-Bx1 cells carrying recombinant HSV and expressing eGFP under the control of a CMV promoter as a model system. Consequently, viral presence in these cells was indicated by eGFP production. Due to the risk of single-target site editing being "escaped" by host repair mechanisms, losing long-term efficacy, researchers subsequently evaluated the efficiency of dual or multi-target strategies. As controls, researchers used single gRNAs targeting essential genes and included cells transduced with empty vectors [4].

Building upon the aforementioned in vitro screening and dual-target strategies, researchers advanced candidate gRNAs to more complex tissue models to evaluate their potency and controllability within intricate neural structures. Specifically, based on the Doench et al. scoring system, four high-scoring gRNAs (two targeting ICP0, two targeting ICP27) were selected, balancing targeting efficiency and off-target risk. These were packaged into expression cassettes, constructing AAV2-SaCas9-ICP0 and AAV2-SaCas9-ICP27 vectors for subsequent validation. At the cellular level, co-expression of SaCas9 with multiple ICP0/ICP27 gRNAs in HSV-1 infected Vero cells induced specific deletion of the target genes and significantly reduced viral replication. Compared to single gRNAs, the multi-gRNA strategy achieved larger fragment deletions through parallel cleavage, significantly reducing escape mutations caused by NHEJ repair. Considering neurotropism and clinical accessibility, AAV2 was chosen as the delivery vector. In Vero cells, AAV2-mediated SaCas9/gRNA expression achieved efficient target cleavage and markedly inhibited HSV-1 replication, providing a feasible delivery foundation for progression to three-dimensional tissue models.

To better approximate the in vivo environment and simultaneously assess acute infection and latency/reactivation phases, one study differentiated cerebral organoids (COs) from human induced pluripotent stem cells (hiPSCs) derived from adult dermal fibroblasts. Prior to infection, immunohistochemistry (MAP2/TUJ-1, IBA1, GFAP, Olig2) confirmed the presence of neurons, astrocytes, microglia, and oligodendrocytes within the organoids, which became abundant 60-80 days post embryoid body induction. Within 48 hours of acute infection, Western blot detected viral proteins such as ICP0, gC, and ICP27, while RT-qPCR confirmed the expression of ICP27, gB, and LAT. The higher levels of LAT might reflect the accumulation of primary transcripts across cell types during late productive infection. Co-staining revealed that viral proteins (ICP0, gC, HSV-1 GFP) were primarily localized at the organoid periphery 48 hours post-infection, while neuronal markers (MAP2/TUJ-1) were concentrated in the core. In the latency model, ICP0 spread from the surface towards the core, suggesting that COs effectively recapitulate the spatiotemporal migration characteristics of HSV-1 within complex neural tissue. Within the established CO system, it was further validated as a platform for studying latency and reactivation and as an endpoint for therapy screening. Approximately 12.5% of organoids exhibited spontaneous reactivation by day 11, comparable to the 17% spontaneous activation rate observed by D'Aiuto et al. 8-11 days after antiviral drug withdrawal. Using induction methods, the reactivation rate could be increased to 62.5%, thereby providing an operable, quantifiable endpoint for evaluating the efficacy of CRISPR-Cas9 and other candidate strategies in suppressing reactivation. Synthesizing these results, the multi-site CRISPR design, delivered via neurotropic AAV2, significantly inhibited replication at the cellular level and reduced reactivation at the 3D tissue scale, establishing a methodological and technical foundation for in vivo validation and subsequent translation [11].

Multiple studies have utilized CRISPR-Cas9 to edit HSV-1 genes (e.g., gE, UL23, ICP0) in cell lines and primary cultures. Early work, using the replication-defective HSV-1 d109 genome (heterochromatic state, used to mimic latency), demonstrated the feasibility of CRISPR-Cas9

cleavage. In quiescent infection models, gRNAs jointly targeting multiple essential genes reduced the probability of reactivation but also exposed limitations in the editing tools and delivery strategies. However, these studies have limitations, including issues with the gene editing systems and delivery. Targeting two viral DNA regions is more effective than targeting one, as multiple cleavages can more efficiently delete or mutate the gene. The chromatin structure of latent HSV-1 DNA is more condensed than that of replicative DNA. CRISPR-Cas9 can edit both genomic forms but is more effective on replicative genomes. Therefore, improving CRISPR's editing capability for latent genomes could enhance editing and reduce latent infection.

Delivery remains crucial for translational applications. Adeno-associated virus (AAV) and lentivirus are common vectors. Lentiviruses can integrate into host DNA, posing a risk of off-target effects, whereas AAVs persist for shorter durations in dividing cells but longer in non-dividing cells (e.g., neurons), and may require strong promoters for efficient nuclease expression. Research using 3D CO models addresses these limitations, testing the effect of CRISPR-SaCas9 on both quiescent and active HSV-1 genomes. Compared with controls, organoids treated with AAV2_CRISPR_ICP0 or AAV2_CRISPR_ICP27 exhibited lower reactivation rates. Compared to two-dimensional cultures, 3D COs better recapitulate the tissue complexity found *in vivo*. Their origin from reprogrammed primary fibroblasts and relatively stable genetic background also make them suitable as evaluation platforms for anti-HSV-1 intervention strategies. In summary, using CRISPR-Cas9 to target multiple sites within early HSV-1 genes can induce editing and may serve as a key therapeutic solution for HSV infection [11].

## 4. HELP Delivery System and Neural Targeting for Clearance

To address the dual challenges of local control and latent reservoir clearance, a study developed an innovative CRISPR/Cas9 delivery system named HELP (HSV-1-erasing lentiviral particles). This system achieves layered intervention from the lesion site to the trigeminal ganglion via intrastromal corneal injection. It demonstrated for the first time in an *in vivo* model that CRISPR gene editing can effectively block HSV-1 acute infection, prevent HSK pathology development, and significantly reduce the latent viral load in the trigeminal ganglion (TG), offering a breakthrough strategy for curing herpetic eye disease. Existing therapies (e.g., acyclovir), while able to suppress viral replication, have three major limitations: inability to clear the latent viral reservoir in the TG (leading to patient recurrence), poor penetration into deep corneal tissues, and the potential to induce drug resistance and nephrotoxicity. In contrast, the HELP system utilizes an engineered integrase-defective lentiviral vector (IDLV) to co-deliver spCas9 mRNA and dual guide RNAs (gRNAs) targeting two essential HSV-1 genes (the helicase-encoding gene UL8 and the single-stranded DNA-binding protein-encoding gene UL29). Its innovative design includes: inserting the UL8 gRNA into the ΔU3 region of the lentiviral LTR, leveraging the reverse transcription mechanism for gRNA self-replication; expressing the Cas9 enzyme in mRNA form to achieve "Hit-and-Run" transient editing (action time $< 72$ hours), significantly reducing long-term off-target risks; and utilizing the anatomical connection between corneal nerve endings and the TG, confirming that the vector can undergo retrograde transport to the latent lesion site, laying the foundation for reservoir clearance.

Mechanistically, the study rigorously excluded interference from type I interferons through experiments. In IFNAR2-knockout HaCaT cells, HELP still effectively suppressed HSV-1 replication, and the vector itself did not activate innate immune pathways like TLR/STING. Notably, although the editing rate at the UL29 site was relatively low, even minor mutations in the essential ICP8 protein, due to its core role in viral DNA replication, could completely disrupt the viral life cycle. In vivo experiments using a C57BL/6J mouse model revealed that in acute infection, a single corneal injection of HELP reduced ocular viral load by 1000-fold, nearly cleared virus from the TG and brain, and completely prevented typical HSK pathology, including corneal stromal thickening, $CD4^+$ or $CD8^+$ T cell infiltration, and PD-L1-mediated immunosuppression. The survival rate in the treated group reached 100%, whereas all control animals died. More importantly, in the latent infection

model, HELP treatment reduced TG viral DNA by 70% following UV-B-induced reactivation, providing the first direct evidence for CRISPR-mediated regulation of the herpesvirus latent reservoir.

The study further validated clinical translation potential in four human donor corneas. HELP distributed evenly within the corneal stroma, significantly reducing viral protein VP5 expression, viral genome copy number, and infectious virus titer. Compared to existing therapies, HELP exhibits three advantages: it targets the viral genome (not human genes), offering higher safety; local injection did not elicit systemic anti-Cas9 antibodies; and it overcomes the bottleneck of acyclovir's inability to clear the latent reservoir. Future directions could involve enhancing retrograde delivery efficiency using rabies virus glycoprotein (RVG)-pseudotyped vectors and conducting long-term toxicological assessments. This study not only provides the first CRISPR-mediated curative (solution/approach) for HSK but also establishes a generalizable "local injection-neural targeting" paradigm for antiviral gene therapy [10].

## 5. ALICE: Autonomous Antiviral System

By integrating viral sensing and therapeutic components such as STING protein, antiviral cytokines, CRISPR-Cas9, and neutralizing antibodies, ALICE combines pathogen detection and antiviral functions, enabling immune cells to autonomously detect viral presence and deliver targeted antiviral agents. The system primarily consists of two parts: an unstable STING-based sensor (ALICEsen) and an effector module (ALICECas9+Ab) that incorporates multiple antiviral molecules and their subsequent derivatives. After multiple iterations and refinements, proof-of-concept experiments using the ALICE system in HSK mouse models demonstrated that the dual-output ALICESaCas9+Ab system delivered via AAV vectors exhibited consistent antiviral activity [1]. Regardless of further upgrades, the ALICECas9+Ab module remains central to viral clearance. In the context of HSV-1 treatment, the virus-inducible promoter PALICE6 , activated by ALICEsen, precisely drives Cas9 expression. Guided by specific sgRNAs, it targets and deletes highly conserved HSV-1 replication-related genes (such as US8/UL29) to inhibit viral replication. Combined with the neutralizing function of the known human monoclonal neutralizing antibody E317 (mAb E317Ab) against HSV-1 glycoprotein D, ALICECas9+Ab delivers a dual attack on HSV-1 under the control of the viral-inducible promoter. While Cas9 disrupts highly conserved gene loci, E317Ab blocks viral infection by targeting the HSV-1 glycoprotein D epitope. Compared to conventional ACV treatment, this system performed significantly better than low-dose ACV (10 μM) and showed antiviral efficacy comparable to high-dose ACV (50 μM) under specific conditions. Thus, in antiviral trials for HSK and in comparisons with ACV, multi-output immune cells have demonstrated remarkable potential, which may alleviate concerns regarding ACV resistance [12].

## 6. Conclusion

In summary, against the challenging persistence of HSV-1, advanced gene editing technology such as CRISPR-Cas9 has been proven through continuous research and experimentation to effectively and precisely delete viral genes and reduce HSV-1 replication rates, potentially preventing viral recurrence. Studies using 3D cerebral organoid models have investigated the effects of CRISPR-SaCas9 on both latent and active HSV-1 genomes, demonstrating that targeting multiple sites within early HSV-1 genes can induce editing and serve as a key therapeutic strategy for HSV infection. Furthermore, innovative antiviral and delivery systems (such as HELP) developed in various studies are expected to enable multi-level viral clearance in clinical applications, enhancing treatment efficiency and effectiveness through the integration of diverse technological advantages.

Current limitations are mainly reflected in the following aspects. Firstly, there are safety concerns related to off-target effects; despite high specificity, non-specific cleavage remains a risk, potentially leading to unintended consequences such as cellular transformation. Second, editing efficiency varies significantly across cell types and models, necessitating optimization of sequence design and delivery

intensity. Thirdly, although local injection in the HELP system did not elicit significant immune responses to Cas9, the potential risk of immune reaction to this exogenous protein in humans remains. Fourth, ethical and regulatory requirements demand that gene editing interventions comply with stricter clinical and social standards. In the case of ALICE, the current design is more suitable for double-stranded DNA viruses; extending its applicability to highly pathogenic RNA viruses will require the development of compatible sensing and effector modules. Looking forward, progress can be pursued along three pathways. Firstly, delivery optimization, such as pseudotyping with rabies virus glycoprotein (e.g., RVG-Lamp2B) to enhance retrograde neural transport efficiency, coupled with long-term large animal studies and toxicological assessments, may accelerate clinical translation. Secondly, broadening the scope of antiviral CRISPR therapy: this strategy is not only applicable to HSV-1 but also provides proof-of-concept and a technical framework for treating other DNA viruses. Finally, the potential for combination therapy should be explored, including the integration of this technology with existing antiviral drugs or immunomodulators to achieve more thorough therapeutic outcomes, particularly for recurrent infections.

## Authors Contribution

All the authors contributed equally to this study.

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