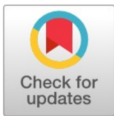

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Crispr/Cas: An Emerging Genome Editing Tool To Combat Viral Infections In Humans

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Abstract: Viral infection is one of the major health concerns of humans and animals around the globe. Only a few selected treatments are available to treat viral infections/diseases. Even though vaccination is one of the preventive measures to combat viral infections, it is not available or possible for most of the viruses we know today. "Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is an RNA-mediated adaptive immune system of bacteria and archaea" that provides immunity against viruses, and humans can exploit this biological mechanism to combat viral infections in humans and animals. The "CRISPR technology," a gene editing tool, unlike Zinc Finger Nucleases and Transcription Activator-Like Effector Nucleases, does not require the engineering of any kind of protein to work. As a result, CRISPR technology can be an effective tool to combat human viral infections. This review focuses on the outcome of various researchers' attempts to edit genes in humans capable of providing resistance against many deadly human pathogenic viruses such as "Human Immunodeficiency Virus (HIV)," "Coronavirus disease," and "Herpesvirus." Although CRISPR/Cas technology is in the developmental stage, it looks like a promising emerging technology in the present scenario.

Keywords: *CRISPR, zinc finger nucleases, human immunodeficiency virus, coronavirus disease, herpesvirus*

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I. INTRODUCTION

The life cycle of a virus involves delivering its viral genome into the host cell. The host's cellular machinery is invaded by viruses, which utilize it for their replication. Viruses with a lipid envelope complete their life cycle first by binding to the surface-specific receptor and then get themselves fused with the cell membranes [1]. Among the various viruses present, RNA (Ribonucleic Acid) viruses pose a huge threat to all living organisms around the globe. Vaccination, above all, is the most widely adopted strategy to prevent the infection of viruses [2]. Unlike DNA (Deoxyribonucleic Acid) viruses, RNA viruses possess high evolution rates, resulting in their variant strains. This often results in the failure of vaccines. The invasion of viruses into immune cells and subsequent integration into the host genome in lentiviral infections, such as HIV, has provided a significant obstacle to creating effective vaccinations. Antiviral drug treatments are used to combat viruses. Still, only a few are accessible for treating viruses such as Hepatitis B Virus, Herpes virus, HIV, Hepatitis C Virus, and influenza virus, which present a grave danger to the human population [2, 3]. Some strategies that target the host factors necessary for the virus for its replication have been identified to inhibit its replication in the host cell. However, such strategies are limited to a group of viruses. Therefore, there is a need to implement new and improved strategies, and "CRISPR technology" is one such strategy [4] for limiting the viral pathogen

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in the host and thereby acting as an effective technique to combat viruses and foreign DNA. Its ability to alter the genome has brought about a new revolution in gene therapies [5].

CRISPR is an adaptive immune system mediated by RNA that is found in bacteria and archaea [6]. The repeats in CRISPR are made up of 21-48 bp (base pairs) and are interspaced with variable sequences of 20-58 bp. The length of the spacers varies and is determined by the CRISPR locus and species of bacteria [7]. CRISPR was first recognized in the genome of Escherichia coli (E coli) in the year 1987, which had repeats made up of 29 bp interspaced by a 32 bp variable sequence. Later, similar short repeated palindromic sequences of 24-40 nucleotides were discovered in a few groups of archaea and bacteria [8]. This system present in bacteria and archaea prevents virus and plasmid invasions [6].

This article discusses the classification and mechanism of action of CRISPR-Cas-based gene editing. It gives a brief description of the delivery and history of CRISPR-Cas systems. The applications of the CRISPR-Cas system for therapeutic and diagnostic purposes are explained by taking a few viruses as examples. Also, a few of the limitations of this system are discussed.

II. CLASSIFICATION

The CRISPR-Cas system is comprised of "CRISPR-associated (Cas)" genes and a "CRISPR array." These Cas genes code for functional proteins called effector complexes [6]. These genes are linked to the CRISPR loci, both evolutionarily and physically. Although 45 families of Cas genes have been identified, an organism can possess only a subset of these genes. Cas1-6 are the core Cas genes present in various organisms, but most organisms have only some of these six genes, of which only Cas1 and Cas2 are universal. Apart from the core Cas genes, nine sub-type-specific Cas genes are set that supplement an organism's core Cas genes [9]. The CRISPR-Cas system is classified as Class 1 (present in both archaea and bacteria), includes multi-subunit-protein, and Class 2 (found only in bacteria), comprising single effector proteins, which are further divided into several types and sub-types (Figure 1). The sub-types II, V, and VI are the most used tools for various applications. The sub-type II consists of "Cas9 effector nuclease," "dual RNA guide (sgRNA)," "crRNA (CRISPR RNA)," and a "trans-activating crRNA." Subtype V is characterized by the presence of Cas12, and subtype VI consists of Cas13 (13a, 13b, 13c, 13d) [6, 10]. "CRISPR-Cas system is found in 90% of archaea and 50% of bacteria". Unlike other defensive cellular mechanisms, CRISPR-Cas works analogously to the vertebrate immune system by storing the history of past infections, which helps in a quick and robust response upon reinfection [11].

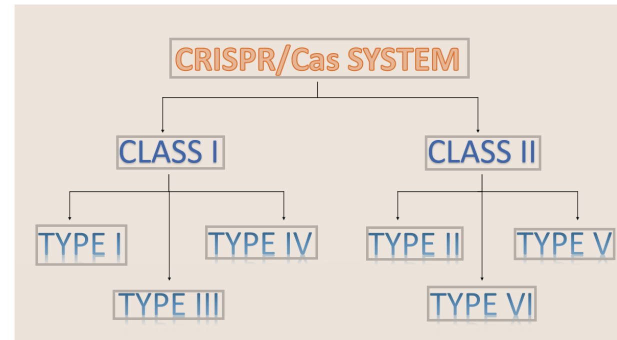

Fig. 1. Classification of CRISPR/Cas system

III. MECHANISM

The invasion of foreign DNA activates the Cas effector proteins, which help insert novel spacers into the CRISPR array, which in turn helps identify and destroy the infectious elements upon their entry [12]. The CRISPR-Cas system targets the invading DNA and eliminates it in a specific manner. This process takes place in three steps: Adaptation, RNA processing, and Interference. The adaptation step is initiated by the recognition of the foreign DNA, part of which is cleaved by the Cas proteins and integrates this sequence in the bacterial DNA genome between two adjacent repeats of the CRISPR locus. Next, the CRISPR matrix is transcribed into a primary sequence known as pre-CRISPR RNA (pre-crRNA), which is further transcribed from the locus and cleaved into active CRISPR RNAs (crRNAs). In the interference step, the active crRNAs guide the Cas proteins to hydrolyze the invading DNA and maintain phage immunity [7, 13] (Figure 2). The effector complexes, which make use of guide RNAs for the recognition and destruction of foreign DNA or RNA, require a guide RNA to identify a novel target and can adapt rapidly to diverse targets. This attribute makes CRISPR-Cas effector complexes an exceptional tool for applications in genome editing and modulation of gene expression [13]. Spacer acquisition occurs when a novel virus is encountered, which can be considered a naive adaption. Previous studies on E coli by researchers have shown that adaptation involves two steps: naive acquisition (acquisition of new spacer from the invading foreign DNA) and priming integration [14]. The priming process enables multiple spacers acquisitions against the invaders. Therefore, the presence of multiple spacers can increase the resistance to the virus [15].

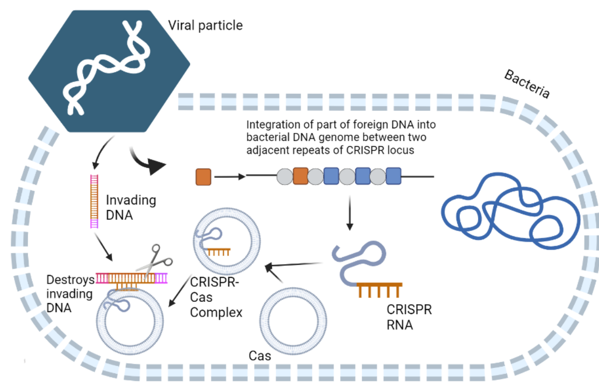

Fig. 2. Mechanism of CRISPR/Cas system (Created with BioRender)

Human Papilloma Virus (HPV), a DNA virus containing E6 and E7 proteins, causes cervical cancer. The E6 and E7 proteins are responsible for the malfunctioning of the p53 protein, which is a tumor suppressor. By targeting the HPV genome with Cas9, E6, and E7, proteins can be disrupted, and the p53 activities can be restored, thereby inducing the death of infected cells [4]. DNA viruses can also be obstructed by CRISPR/Cas system by targeting certain factors in the host necessary for virus replication. Unlike DNA viruses, the CRISPR/Sp Cas9 (Cas9 obtained from Streptococcus pyogene) system cannot target RNA viruses where DNA intermediates are not found. However, Fn Cas9 (Cas nuclease obtained from Francisella novicida) has been used to target such RNA viruses [4].

Cas nucleases are made pathogen-specific by a unique property of the enzyme (i.e., the requirement of an RNA guide sequence capable of both activating the enzyme and targeting the complementary DNA sequence with the nuclease). This unique property makes Cas nucleases useful in generating DNA breaks or nicks at any desired location. Cas9, among other Cas nucleases obtained from Escherichia coli, is the most commonly used nuclease [16]. The SpCas9, Cas9 protein with 1368 residue and a PAM (Protospacer adjacent motif) sequence of 5'-NGG-3', is the most used CRISPR-associated protein. The variants of SpCas9 are obtained by mutating three to four base pairs which can target different PAMs such as NGA, NGAG, and NGCG. Cleavage of the targeted DNA varies depending on PAM sequences [17, 18].

With the emergence of "Zinc Finger Nucleases (ZFNs)," "Transcription Activator-Like Effector Nucleases (TALENs)," and "CRISPR-Cas9" systems, the fundamental limitations of conventional methods for gene addition can be overcome very easily [19]. Unlike ZFNs and TALENs, which require the engineering of novel proteins for targeting different genome regions, CRISPR-Cas9 does not require any engineering of proteins and can introduce double-stranded breaks (DSBs) efficiently [19].

IV. DELIVERY AND HISTORY OF CRISPR/CAS SYSTEM

Implementing the CRISPR-Cas system as a gene editing tool has greatly impacted the research field. The ease and flexibility with which this system can be applied in various fields have generated immense excitement about its potential in the clinical field. With the rapid improvement in the delivery, functionality, and specificity, the CRISPR-Cas system will help develop various necessary treatment options [20]. Various delivery vehicles can be used to deliver the CRISPR-Cas gene, of which viral delivery and non-viral delivery are the major types. Viral delivery engineers the viral particles by removing rep genes, which help in replication, and being replaced by a therapeutic gene of interest. However, without rep genes, the viral particles can still enter the host cell and introduce the therapeutic gene of interest without replicating itself. The most commonly used vector for this type of delivery is the "Adeno-Associated Virus (AAV)." Non-viral delivery methods do not involve the viral vectors for transgene transfer. This includes physical and chemical methods, such as disrupting cellular barriers [21].

The CRISPR system was first used in genetic engineering in 2013. As the method is simple, precise, fast, accurate, specific, and cheap compared to other methods, it is widely adopted in genome editing. This method was used to edit human embryos in 2015 [7]. A few years later, transgenic animals were generated using CRISPR-Cas technology to diagnose COVID-19. Emmanuelle Charpentier and Jennifer Doudna, the two researchers responsible for discovering "the CRISPR gene editing method," received the 2020 Nobel Prize in Chemistry [7].

V. CLINICAL APPLICATIONS OF THE CRISPR/CAS SYSTEM

CRISPR/Cas system can be utilized as a therapeutic tool against chronic and acute diseases. In patients, who require lifelong treatment, CRISPR/Cas can be a promising technology to cure them. They have been used to generate disease models by identifying potential antiviral agents [10]. Cas9 has been the most focused protein by researchers. However, recent advancements have also taken place in the potential of Cas12 and Cas13 proteins, of which, Cas13 targets RNA viruses directly, while Cas12a and Cas9 target DNA viruses mainly and RNA viruses with dsDNA intermediaries. These nucleases can also be incorporated into antiviral drugs and biomolecules [10].

CRISPR/Cas can detect infections and can be a diagnostic tool. Barnes et al., 2020 developed a CRISPR-based SHERLOCK platform for diagnostic purposes. Cas13a can detect a viral nucleic acid. It involves pairing isothermal RPA (Recombinase Polymerase Amplification) with crRNA-guided Cas13a detection. The Cas13a specifically pairs with the target sequence, and the signal is amplified [22]. The diagnostic technologies based on CRISPR are highly sensitive and specific, and there is no need for expensive laboratory equipment. The CRISPR-based technologies have specificity like that of PCR (Polymerase Chain Reaction) [23]. CRISPR/Cas can be used to detect the Coronavirus in human beings [24, 25]

VI. LIMITATIONS

The generation of variants due to the escape of the virus is one of the few drawbacks of the CRISPR/Cas9 technology. Induction of indels occurs as the result of delivery of the CRISPR/Cas9 system, and mutation occurs, leading to variants that are resistant to CRISPR/Cas9, thereby rendering CRISPR/Cas9 cleavage ineffective. It also leads to the development of strains having higher pathogenicity. The problem of spawning these escape strains can be overcome by disintegrating the viral genome by simultaneously targeting multiple sites [12]. The precise delivery of CRISPR/Cas9 to the infected in vivo cells is the major obstacle faced by this technology. The clearance of the virus in the circulating cells require systematic delivery to different body organs, which might increase the risk of off-target effects in healthy uninfected tissues [12].

One of the main issues with CRISPR implementation in humans is the off-target cleavage of DNA. "Transcriptome-wide off-target RNA editing" caused by "CBEs (Cytidine Base Editors)" and "ABEs (Adenine Base Editors)" leads to unwanted off-target effects. Selecting the sgRNA (single guide RNA) and its optimization might help in reducing off-target effects to an extent [26]. In addition to these, the immunogenicity of the Cas9 protein is also a major issue arising from the application of the CRISPR/Cas system. CRISPR/Cas-based gene editing employing "NHEJ (Non-homologous end joining)" and "HDR (Homology-directed repair)" generates "DSBs (Double strand breaks)," bringing about a temporary cell cycle arrest by activating a p-53 dependent DNA damage response [26]. Although no direct evidence is available, the activation of the p-53 signaling pathway by DSBs suggests that CRISPR/Cas-edited cells might have the potential to become cancer-inducing cells [27]. The requirement of the PAM (Protospacer adjacent motif) near the target site is another limitation of the CRISPR/Cas application [28].

VII. EXAMPLE

A. *Human Immunodeficiency Virus (HIV)*

HIV is a single-stranded RNA (ssRNA) virus belonging to the lentivirus genus. The virus genome is flanked by two long Terminal Repeats (LTR) structures. HIV causes AIDS (Acquired Immune Deficiency Syndrome) by targeting CD4+ human immune cells. It also infects human T cells within the CNS (Central Nervous System) [29, 30]. According to the World Health Organization (WHO), AIDS is one of the deadliest pandemic diseases in the world, with about 36.7 million people having AIDS and a death toll of around 2.2 million people approximately [29].

HIV infects the target cells in two ways; active infection, where the pro-virus is active and replicates actively, or latent infection, where the pro-virus is silenced transcriptionally, and hence no progeny is produced [29]. However, these latently infected cells are reactivated under various conditions, such as induction of cytokines, or immunological responses to various recall antigens or routine vaccinations, to produce the virus and thereby hinder its eradication [29].

The "shock and kill" method, where shock refers to the reactivation of latent reservoirs and kill refers to the killing of those reactivated reservoirs, has been employed to eliminate viral reservoirs with the help of Anti Retroviral Therapy (ART) and host immune responses. But ART is proven ineffective as it only limits viral replication and does not interfere with latent infection in CD4+ memory T-cells. So, to completely eradicate HIV reservoirs, the reservoir cells' provirus must be removed or disrupted. Several studies have shown this to be possible by targeting the LTR of the pro-viral region with CRISPR/Cas9. CRISPR/Cas9 successfully removed the provirus from the infected cells by impairing the gene expression of HIV [29, 30].

Several researchers have used the CRISPR/Cas9-based approach to treat HIV-1. Ebina et al. (2013) "targeted the NF-KB binding sites by CRISPR/Cas9 and proved the method effective in inhibiting the replication and transcription of HIV-1 provirus in Jurkat cell lines. This method also showed the potential of CRISPR/Cas9 to eliminate the viral genes integrated internally into the chromosome of infected cells" [31, 32]. Later Hu et al. inactivated the viral gene expression by targeting conserved sites in the HIV-1 LTR U3 region with Cas9/gRNA and thereby restricted the replication of the virus in the latently infected T cell line, microglial cell line, and pro-monocytic cell line [32, 33]. Liao et al. showed that

the non-integrated viral genome could be more effectively disrupted by targeting multiple sites of the viral genome [32]. HIV-1 provirus can also be inactivated by mutating the target site while targeting the viral replication by a single sgRNA [32].

Apart from targeting the viral genome, the entry of HIV-1 can be blocked by editing the co-receptors via CRISPR/Cas9 technology. The fact that the virus binds to the CD4 receptor and CCR5 or CXCR4 co-receptors for their entry can be exploited to prevent the entry of the HIV-1 virus. The co-receptors CCR5 and CXCR4 are the potential targets to prevent HIV-1 infection because the CD4 receptors cannot be disrupted as they play a major role in the functioning of the immune system. It has also been shown that a person with homozygous 32-bp deletion of the CCR5 gene (CCR532) remains unaffected and healthy and capable of preventing the replication of the HIV-1 virus. Some researchers have used CRISPR/Cas9 technology to disrupt these co-receptors. Li et al. disrupted the CCR5 expression by targeting the fourth exon of the co-receptor CCR5 by combining CRISPR/Cas9 with sgRNA and adenovirus as the vector, thereby preventing the infection of the HIV-1 [32, 34]. The CXCR4 gene in human and rhesus macaque CD4 + T cells were effectively disrupted by Hou et al. by using CRISPR/Cas9 and lentivirus as the vector. They also used two sgRNA to specifically target the conserved sequence of the CXCR4, and the modified CXCR4 cells showed resistance to the infection of HIV-1 [32, 35] (Figure 3).

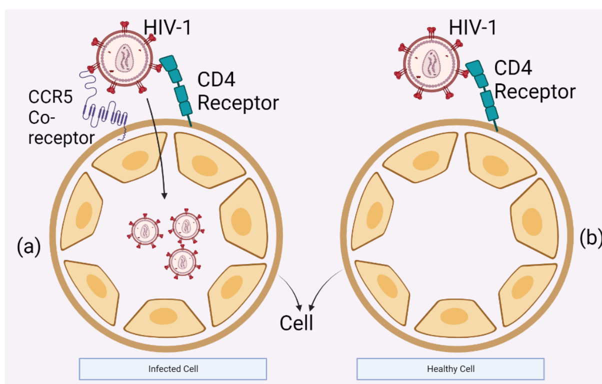

Fig. 3. Molecular mechanism of CCR5 (a) HIV enters the cell by binding to the CD4 Receptor and CCR5 Co-Receptor and thereby infects the cell. (b) In a person with homozygous 32-bp deletion of the CCR5 gene (CCR5Δ32), the virus cannot enter the cell, and thereby the cell remains uninfected and healthy. (Created with BioRender)

[36] experimented with evaluating the efficiency of CRISPR/Cas9 on HIV-1 variants. Although the dual gRNA/Cas9 strategy used did not prevent replication of all tested isolates, it showed that by at least one of the combinations of dual-gRNA, the replication of nearly all the isolates could be blocked permanently, and the replication-competent viruses cells cannot be revived from the infected cells culture [36]. Yin et al. showed that Cas9 not only causes the indels by NHEJ repair but also reduces the viral DNA product levels and provides higher DNA product levels, and provides higher anti-HIV-1 activity [37].

Liang et al. have mentioned some of the limitations of CRISPR/Cas in HIV treatment, such as the escape of the HIV-1 from a CRISPR/Cas9 attack. Apart from this, there are several other limitations mentioned by them, such as the fact that CRISPR/Cas9, which is effective in HIV-1 treatment by several researchers, has not discussed the ability of CRISPR/Cas9 in terms of resting CD4 T cells from infected individuals. Liang et al. believe that recognizing these limitations could be useful in developing CRISPR/Cas9 in the future, which could be a potential treatment strategy [38].

B. COVID-19

The emergence of the novel coronavirus disease (COVID-19) in Wuhan city of China, in late 2019 led to a pandemic worldwide. COVID-19, accompanied by pneumonia, is a respiratory condition caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-Cov-2) and spread via respiratory means by coughing and sneezing [25]. Coronaviruses belong to the Coronavirinae family. SARS-Cov-2 is a -coronavirus that is pathogenic to humans [39]. It is a positive-sense RNA virus, and its lifecycle resembles that of SARS and infects both lower and upper respiratory tracts. COVID-19 has no specific treatment, and there is a need to find effective therapies. CRISPR technology could be one such way that could lead to the development of diagnostic and therapeutic methods for the novel coronavirus disease [25].

Unlike traditional vaccines, the CRISPR system identifies the intracellular viral genome and degrades its mRNA [25]. [40] used the PAC-MAN strategy to show that the Cas13d system targets the RNA sequence of SARS-Cov-2 in the epithelial cells of the lungs and cleaves it with the help of crRNAs. Their result showed that 91% of 3051 sequenced coronaviruses could be targeted by six crRNAs [40]. As many as 10333 guide RNAs were designed by Nguyen et al. to target the peptide-coding region of the virus genome [25, 41].

CRISPR/Cas13 can inactivate the SARS-Cov-2 virus in mammalian cells by cleaving the specific RNA sequence at the post-transcriptional level. It consists of a 64-66-nt CRISPR RNA and a programmable single-effector RNA-guided RNase Cas13, which only detects a 24-30-nt sequence on the target RNA via the protospacer-flanking site. Cas13 cleaves and knocks down the desired

RNA targets once the gRNA binds to the complementary target RNA sequence [25]. If the targeted components are present in the right cellular compartment at the right concentration, it can decrease gene expression post-transcriptionally in less than 24 hours. The Cas13a enzyme can be used for detective purposes as well. The latest technique, SHERLOCK v2, employs various Cas13 enzymes and enzymes exhibiting RNase activity activated by Cas13 nuclease products to detect the virus. The first FDA-approved detective kit for COVID-19 based on CRISPR can provide results in an hour [25].

The delivery of CRISPR/Cas13 for therapeutic action against COVID-19 is a major obstacle [25]. One of the strategies by which CRISPR/Cas13 can be delivered efficiently is with the help of the virus itself. The Cas13 nucleases are engineered to cling to the surface of the viral particle so that the CRISPR/Cas13 is delivered to the host by the virus itself. After entering the host cell, the CRISPR system accesses the released viral RNA and cleaves it before its replication and translation into the host cell. This is achieved by tethering the Cas13 onto the spike(S) protein, which is a vital structural protein responsible for viral infection in the host cell, by an approach known as AntiBody and CAS fusion (ABACAS), where the Cas13 nuclease is fused with the S protein-specific antibody fragment. This ABACAS approach ensures that Cas13 is delivered selectively into the infected cell alongside the virus and thereby cleaves the viral RNA after its delivery [39]. Hence, several scientists have concluded that the CRISPR/Cas13 approach can potentially prevent and treat RNA viruses such as COVID-19 [25].

C. *Herpesvirus*

The herpes viruses are DNA viruses belonging to the Herpesviridae family. The capsid of the herpesvirus virions, which surrounds a linear dsDNA genome of 125-295 kbp, consists of tegument and viral glycoprotein containing lipid bilayer envelope [42]. There are 3 subfamilies of herpesvirus: alpha-, beta-, and gamma-herpesviruses. The herpes simplex virus type 1 (HSV-1), which causes cold sores, and the herpes simplex virus type 2 (HSV-2), which is the cause of genital herpes, belong to the -herpesvirus subfamily. The β-herpesvirus subfamily includes the "human cytomegalovirus (HCMV)," and the "γ-herpesvirus comprises Kaposi's sarcoma-associated herpesvirus (KSHV)" and "Epstein-Barr Virus (EBV)" [43]. Herpesviruses latently infect the host cells, and protein expression of the virus is restricted during latency, helping them to evade the host immune system. Mostly unknown triggers lead to the reactivation of the virus, helping its production and spread. The current treatment strategies that limit the productive phase show no efficiency in the latent stage of the infection [42]. Hence, CRISPR/Cas9 system that can selectively modify the dsDNA virus can be a powerful strategy to combat herpesvirus [43].

Van Diemen et al. edited the EBV genomes directly using the CRISPR/Cas9 system and showed that by targeting essential genetic elements of the EBV, the virus could be eliminated from the latently infected lymphoma cells. They also impaired the HSV-1 replication by targeting essential protein-encoding genes of the HSV-1 via CRISPR/Cas9 system [43]. Roehm et al. demonstrated that the anti-ICPO gRNAs reduced the virus production by $\pm$ 10 folds by interfering in the replication of HSV-1 in TC620 human oligodendroglioma cells [42, 44]. Bi et al. showed that during the virus replication, the presence of anti-HSV-1 gRNAs and Cas9 resulted in DSBs of the viral genome and site-specific indels being introduced by the NHEJ in the target site. They also showed that with simultaneous delivery of a donor template with shared sequence homology to the target region, the heterologous genes could be inserted into the viral genome [42, 45].

VIII. CONCLUSION

The emergence of the coronavirus disease in 2019 has shown the world how deadly a virus can be and the problems it can cause to humans [25]. Although there are some strategies, like vaccination, to combat viruses, these strategies are limited to only a group of viruses [2]. The CRISPR/Cas system has enormous potential for future human viral infection treatment or cure. So far, a significant proportion of research has concentrated on cell-culture-based systems. However, several issues must be resolved before the antiviral therapeutic potential of CRISPR-Cas9 can be validated in vivo [12]. CRISPR/Cas technology has been employed in human cells to target the invading foreign DNA selectively and efficiently. The use of CRISPR technology in treating numerous viral and microbial infectious diseases is currently being intensively discussed. Targeting host pathogens that might prevent treatments for various illnesses is a cost-effective strategy. Despite several setbacks, it is frequently employed because it has the potential to be effective. CRISPR technology has become a very demanding therapeutic tool nowadays as it is easy to correct genetic defects and translate the genetic code of causative agents [5]. However, there are some limitations of this technology, such as the generation of variants due to the escape of the virus [12], the precise delivery of CRISPR/Cas to infected in vivo cells [12], the off-target cleavage of DNA [26], the requirement of PAM near the target site [28], etc. Thus, this

article notes that further studies are needed to understand the limitations and ways to overcome them and ensure that these technologies can be used safely and ethically by all.

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