REVIEW

National Science Open
2: 20220061, 2023
https://doi.org/10.1360/nso/20220061

Life Sciences and Medicine

Special Topic: Gene Editing towards Translation

Treatment of infectious diseases by *in vivo* gene editing

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Received 25 November 2022; Revised 5 January 2023; Accepted 17 January 2023; Published online 7 August 2023

Abstract: Gene editing is the specific modification of genome sequences at desired sites using technologies derived from zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) nuclease systems. It is a promising tool for the development of new treatment strategies for infectious diseases. Due to its higher editing efficiency and lower off-target effect, gene editing therapy mainly uses CRISPR Cas-derived tools to resist viral and non-viral infections. Here, we reviewed the recent research progress of gene editing in antiviral therapy (human immunodeficiency virus, hepatitis B virus, severe acute respiratory syndrome coronavirus 2, and human papillomavirus) and inhibition of infectious diseases that involve bacteria, fungi and parasites.

Keywords: gene editing, *in vivo* therapy, CRISPR-Cas, infectious diseases, viral infection

Global combat of infectious diseases

Throughout traceable history, infectious diseases have caused major global health and economic burdens, particularly to underdeveloped and low-income societies [1]. Pathogenic infections are diseases caused by specific microorganisms, such as viruses, bacteria, fungi and parasites [2,3]. If the immune mechanism is fully functioning, these microorganisms will not easily invade and cause diseases [2,3]. However, when these microorganisms flood and the immune system is impaired, they may lead to infection [2,3]. With the progress of medicine and the technological progress in immunology and microbiology, the number of global and regional infectious diseases has decreased remarkably since World War II, with the eradication of smallpox [4] and the control of several preadolescent diseases, such as polio [5], leprosy [6], and rubella [7].

However, according to the World Health Organization's global health estimates [8], the harsh reality is that infectious diseases are still the most significant threat faced by residents in underdeveloped and low-income countries. In these regions, pathogenic infections rank sixth among the top ten most deadly diseases and they are more likely to be lethal than noninfectious diseases.

Moreover, the constant emergence of organisms and pathogens that cause infection has aggravated the great challenges of public health epidemics. In 2020, COVID-19 killed more than one million people and also

had a severe impact on the global economy [9]. According to the Organisation for Economic Co-operation and Development data, the actual global GDP growth rate in 2020 was −3.4%. Therefore, 2020 has become the only year with negative global economic growth, after 2000, and the year with the worst recession since World War II. Hence, the development of new methods for effectively treating infectious diseases is of great significance for improving people's well-being and ensuring social and economic development.

Gene editing in infectious diseases

Gene therapy is divided into *in vivo* and *in vitro* strategies (Figure 1A). The *in vivo* strategy works by loading gene editing agents into viral or non-viral vectors and injecting them into the body [10]. After *in vivo* gene modification, the therapeutic process is complete. The *in vitro* strategy edits desired genes in patient-derived cells, *in vitro*, then reinfuses the edited cells back into the patient, after cell expansion [10]. *In vitro* gene editing does not need a complicated delivery system and can usually be achieved with a more straightforward and safer electroporation method. However, the indications for this strategy are limited and most efforts focused on blood diseases due to the pathogenic mechanism of infectious diseases caused by parasites and transmission of viral and non-viral pathogens (Figure 1B) in the hosts. The pathogen often lives in specific tissues or cells of the host body, which are unsuitable for treatment using the *in vitro* strategy. Current treatment methods and research progress limit the application of *in vitro* gene editing for the treatment of infectious diseases. Hence, *in vivo* strategies are usually adopted for gene therapy for infectious diseases [1,10].

At present, three endonuclease-mediated gene editing tools have been extensively utilized in gene therapy research. These are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) nuclease systems [10]. The ZFNs and TALENs are endonucleases that specifically recognize genome editing sites through the *Fok* I domain-DNA interaction [11–18]. However, ZFNs and TALENs have limitations, such as low gene editing efficiency, extensive off-target edits, and high vector construction costs [11]. In contrast, the CRISPR-Cas system binds to the target nucleic acid strand, guided by the guide RNA (gRNA) sequence, and induces DNA/RNA strand breaks (SB) through endonucleases [19–21]. As double-strand breaks (DSBs) are introduced into the DNA targets, non-homologous end joining (NHEJ) or homologous directed repair (HDR) occurs simultaneously, which leads to the desired gene modifications at the target gene sites [19–22]. Due to its easy operation, high editing efficiency and low off-target effect, CRISPR-Cas has developed into a powerful gene editing tool in mammalian cells and has rapidly replaced ZFNs and TALENs, since 2013 [11,19,21,22]. To date, there are two types of gene editing tools (Figure 1C). The first type is called gene scissors, such as ZFNs, TALENs, CRISPR-Cas, and other nucleases (e.g., the engineered ARCUS nuclease). These interact with the target and induce nucleic acid strand breaks [11, 23]. The second type is called gene wrenches and includes CRISPR-Cas derived interference (CRISPRi) [24] or activation (CRISPRa) [25], base editing [26,27] and prime editing [28]. These gene editing tools induce the desired edits through inactive Cas proteins (e.g., dCas9 and SpCas9 D10A/H840A), without SBs [22].

Gene editing and virus infection

Here, we discuss the research progress of gene editing therapy for four viruses: human immunodeficiency

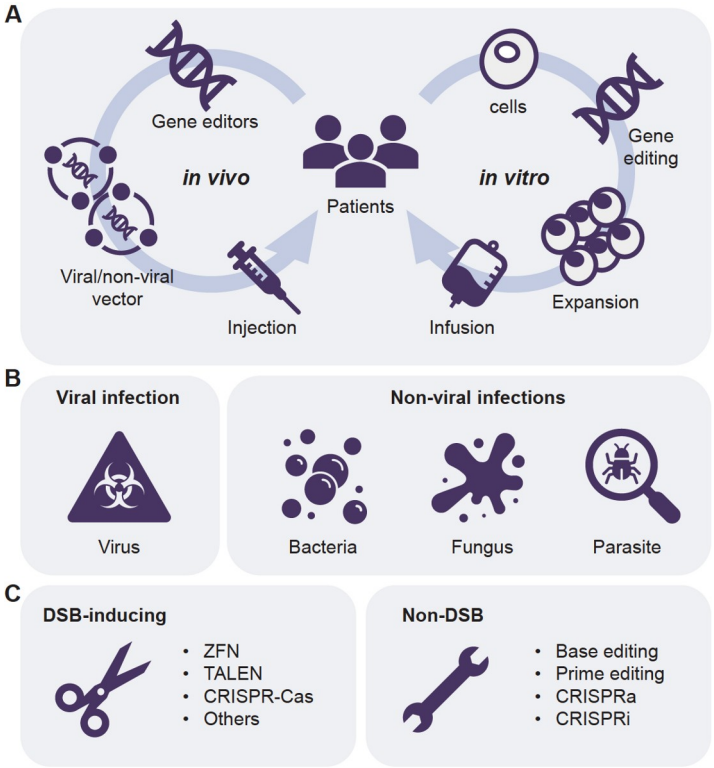

Figure 1 Gene therapy for infectious diseases. (A) Gene therapy is divided into two treatment strategies: *in vivo* and *in vitro*. The *in vivo* strategy (the left half) completes the treatment process by loading gene editing tools into viral or non-viral vectors and then injecting them into the body. The *in vitro* strategy (the right half) is to edit the patient's primary cells, expand the edited cells, *in vitro*, and finally transfer them back to the patients. (B) Currently, gene therapy is aimed at two types of infectious diseases: viral (the primary pathogens are RNA or DNA viruses) and non-viral (the primary pathogens are pathogenic bacteria, fungi, and parasites) infection. (C) There are two types of gene editing tools: the DNA double-strand break (DSB)-inducing type and the non-DSB-inducing type. The former type acts like scissors. Under the guidance of gRNA, a DSB is introduced at a targeted location, where NHEJ or HDR is simultaneously induced to conduct the desired gene modification. These tools include ZFNs and TALEN, various CRISPR-Cas systems, and other nucleases (e.g., engineered ARCUS nuclease). The latter type, such as base editing, leader editing, gene activation (CRISPRa) and gene silencing (CRISPRi), completes the desired gene modification at targeted sites without DSBs. Such gene editing tools mainly derive from the CRISPR-Cas system, with fully inactivated dCas or partly inactivated nCas. Therefore, these gene editing tools are like wrenches, which repair or modify target sites, rather than destroying them.

virus (HIV, leading to acquired immune deficiency syndrome (AIDS)), hepatitis B virus (HBV, which causes hepatitis B), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, which caused the global epidemic from the end of 2019), and human papillomavirus (HPV, which leads to cancer).

HIV

Human immunodeficiency virus-1 (HIV-1) causes the AIDS that severely threatens global human health [29]. An estimated 38.6 million people across the world are living with HIV-1. It is a retrovirus that targets human cells using the envelope surface protein, gp120. The virus attaches to the cell membrane, by binding to the $CD4^+$ receptor, and interacts with co-receptor chemokine receptor 5 (CCR5) or C-X-C motif chemokine receptor 4 (CXCR4). After entering the cell, HIV-1 converts the viral genome into double-stranded DNA using RNA reverse transcriptase. The viral DNA integrates into the host genome. Subsequently, it forms a provirus, which can actively transcribe RNA to generate a descendant virus or enter a latent state

without producing any virus. We review two therapeutic strategies for HIV, targeting viral or host genes.

Targeting viral genes

In 2013, Ebina *et al*. [30] performed CRISPR-Cas9-mediated anti-HIV-1 inhibition by editing integrated provirus DNA. The engineered CRISPR-Cas9 targeted the critical regions of the HIV-1 long terminal repeat (LTR), which involved the NF-κB binding site and trans-activation response (TAR). The results showed that CRISPR-Cas9 significantly impeded LTR-driven expression, which illustrated that CRISPR-Cas9 can inhibit a transcriptionally active provirus and simultaneously block the expression of the latently integrated provirus. In 2015, Zhu *et al*. [31] used CRISPR-Cas9 to disrupt ten sites within the HIV-1 genome in JLat10.6 cells that were latently infected with HIV-1. The CRISPR-Cas9 system efficiently introduced mutagenesis into all target sites. The second exon of *Rev* (named T10) exhibited the highest degree of disruption. The expressions of HIV-1 and virus production were significantly diminished. In addition, Liao *et al*. [32] screened and identified the gRNAs for effective and long-term protection against HIV-1 infection in primary human T cells and human pluripotent stem cell (hPSC)-derived HIV reservoir cell types. Their results showed that CRISPR-Cas9-mediated mutagenesis at the target sites within the LTR sequence, particularly in the R region, showed significant suppression of viral genes.

In addition to the above studies, using the CRISPR-Cas9 system from *Streptococcus pyogenes* (Sp), Wang *et al*. [33] utilized a smaller Cas9 from *Staphylococcus aureus* (Sa) and designed gRNAs aimed at the HIV-1 genome. The *in vitro* tests showed that the gRNAs/SaCas9 can efficiently diminish provirus gene expression in infected Jurkat C11 cells and reduce virus production in TZM-bl and Jurkat T cells. To test the *in vivo* SaCas9-mediated anti-HIV-1 effect, within three mouse models, Yin *et al*. [34] used an all-in-one adeno-associated virus (AAV) vector to deliver the gRNAs/SaCas9. In the HIV-1 transgenic mice (Tg26), intravenously injected quadruplex gRNAs/SaCas9 AAV-DJ/8 disrupted viral DNA and significantly reduced HIV-1 RNA expression. In acutely infected EcoHIV mice, a convenient mouse model of biosafety level 2 for animal testing of HIV vaccines and anti-retroviral drugs, the same method reduced systemic EcoHIV infection and induced efficient provirus excision in several tissues. In addition, the same method induced significant provirus DNA excision in humanized bone marrow/liver/thymus mice with chronic HIV-1 infection. Mancuso and colleagues [35] used *Rhesus macaques* that had been infected with simian immunodeficiency virus (SIV) as an HIV infection model in primate species. They demonstrated that intravenously injected AAV9-CRISPR-SaCas9 can eliminate the integrated SIV DNA within viral reservoir tissues, such as lymph nodes, spleen, bone marrow, and brain. These studies illustrate that AAV-mediated CRISPR-Cas9 treatment significantly suppresses provirus DNA expression, *in vivo*, which offers a promising clinical provirus elimination method for anti-HIV-1 researchers.

In contrast, an antiviral strategy known as *shock-and-kill* aims to eliminate HIV reservoirs by reactivating latent virus (shock) and removing the pool of latently infected cells (kill) with combined antiretroviral therapies (cARTs) [36]. Saayman *et al*. [37] fused a Cas9 mutant with nuclease-deficiency (dCas9) to the VP64 transactivation domain. This created dCas9-VP64, which was able to activate HIV-specific transcription. The results showed that dCas9-VP64 screened and targeted an activation hotspot within the enhancer sequence of the HIV-1 LTR promoter region. In several *in vitro* latency cell models, the dCas9-VP64-sgRNAs showed consistent and effective activation of the latent virus, with high specificity. Based on

the study by Saayman *et al*., Limsirichai *et al*. [38] employed the synergistic activation mediator (SAM) complex and fused dCas9-VP64 with a transactivation domain. This was used to recruit a complementary suite of transcription and chromatin remodeling factors, to target the HIV-1 LTR promoter region. Transient reporter assays of the LTR region showed that expression levels of SAM-activated genes exceeded the expression levels of genes activated by dCas9-VP64. This indicated that this second-generation dCas9-based gene activation is more suitable for the *shock-and-kill* treatment strategy for HIV-1. Similarly, in 2020, Olson *et al*. [39] created a dCas9 with a Kruppel-associated box (KRAB)-derived transcriptional repressor domain, for epigenetic silencing of the HIV-1 provirus DNA. The gRNAs/dCas9-KRAB specifically reactivates latent HIV-1 provirus and represses HIV-1 transcription using chromatin changes, such as decreased H3 histone acetylation and increased histone H3 lysine 9 trimethylation. This increases the anti-virus application of the *shock-and-kill* strategy for HIV-1.

Targeting host genes

To inhibit HIV-1 infection, *CCR5* and *CXCR4* are ideal targets for gene editing-mediated therapies [10]. The ZFN method has been applied to gene editing of *CCR5* in $CD4^+$ T cells and hematopoietic stem and progenitor cells (HSPCs), to treat patients with HIV infection. In 2008, Perez *et al*. [40] utilized engineered ZFNs to excise endogenous *CCR5*. The *in vitro* results showed that transient expression of ZFNs significantly disrupted approximately 50% of *CCR5* alleles in a pool of primary human $CD4^+$ T cells. In addition, the mutagenesis of *CCR5* has been shown to contribute to robust, stable and heritable protection against HIV-1 infection in an HIV-1-infected immunodeficient NOG mouse model. This suggested a promising anti-HIV-1 strategy by ZFN-mediated endogenous *CCR5* editing in human cells. Similarly, Holt *et al*. [41] used ZFNs to disrupt approximately 17% of *CCR5* alleles in human $CD34^+$ HSPCs. The HSPCs with the intended edits retained the ability to engraft mouse models infected by CCR5-tropic HIV-1 and exhibited significantly lower HIV-1 levels than engrafted untreated HSPCs. The results showed that $CCR5^{-/-}$ HSPCs provide HIV-1 resistance, *in vivo*, which supports the use of ZFN-modified autologous HSPCs as a therapeutic approach to treating HIV-1 infection. DiGiusto *et al*. [42] also developed a ZFN-mediated strategy to disrupt *CCR5* genomic sequences in HSPCs. They nucleofected *CCR5*-specific ZFN mRNA into HSPCs, which led to 72.9% biallelic *CCR5* excision. The *in vivo* test showed that the ZFN-treated $CCR5^{-/-}$ HSPCs maintained lineage potential in immunodeficient NSG mice. This demonstrates that a transplant of virus-resistant HSPCs could significantly improve the clinical management of HIV-1 infection.

However, due to the limitations of ZFNs, such as low gene editing efficiency, high rate of deviation from target, plus high cost, and labor of vector construction, CRISPR-Cas has rapidly replaced ZFNs and has been widely used for gene therapies [11]. In 2013, Cho *et al*. [21] used a recombinant Cas9 protein to diminish *CCR5* in human cells. The results showed that Cas9 efficiently excised *CCR5* at the intended sites and did not induce off-target edits.

In 2014, Ye *et al*. [43] performed genome editing of wild-type induced pluripotent stem cells (iPSCs) using a combination of CRISPR-Cas9 and the piggyBac technology. They efficiently introduced a naturally occurring $CCR5^{\Delta 32}$ mutation into iPSCs and seamlessly excised piggyBac using transposase, without detectable exogenous sequences. Li *et al*. [44] performed CRISPR-Cas9-mediated gene editing of the *CCR5* locus and showed that the identified RNAs induced undetectable off-target effects, with a high score. They

also constructed a chimeric Ad5F35 adenovirus vector for CRISPR-Cas9-mediated *CCR5* reduction in primary $CD4^+$ T-lymphocytes. The results showed that $CCR5^{-/-}$ $CD4^+$ T-lymphocytes exhibit significant HIV-1 resistance. Similarly, using the electroporation method, Xu *et al*. [45] efficiently performed CRISPR-Cas9-based *CCR5* gene knockout in human $CD34^+$ HSPCs. The *in vivo* results showed robust *CCR5* excision in immunodeficient NPG mice and a significant resistance against HIV-1. In 2020, Liu *et al*. [46] employed the CRISPR/AsCpf1 system to efficiently disrupt the endogenous *CCR5* gene, *in vitro*, using viral vectors. The identified sgRNAs for CRISPR/AsCpf1-mediated *CCR5*-targeting excision showed minimal off-target effects at the predicted sites, to give an improvement over the sgRNAs used with CRISPR-Cas9. Against CCR5-tropic HIV-1 infection, the $CCR5^{-/-}$ cells showed significant resistance and displayed a selective advantage over the wild-type. They also showed that the CRISPR/AsCpf1 system rarely affected the proliferation and apoptosis of $CCR5^{-/-}$ cells.

In contrast, Hou *et al*. [47] disrupted the endogenous *CXCR4* gene using CRISPR-Cas9 in multiple cells, which included primary human $CD4^+$ T cells. The lentiviral vector-delivered CRISPR-Cas9 generated biallelic mutagenesis within the *CXCR4* genomic region and thus rendered the modified cells resistant to HIV-1 infection. Sequence analysis also revealed a low off-target effect at predicted sites. In 2015, Hultquist *et al*. [48] employed electroporation to introduce CRISPR-Cas9 ribonucleoproteins (RNPs) into primary $CD4^+$ T cells for intended gene editing. They disrupted the *CXCR4* and *CCR5* HIV co-receptors, in multiple donors, to render the cells resistant to HIV-1 infection. The results also showed that targeting additional endogenous genes, such as *LEDGF* and *TNPO3*, can significantly block HIV-1 infection. They employed electroporation to screen endogenous HIV-1 integrase-interacting targets and identified novel dependency factors for further investigation of anti-HIV-1 infection. In addition, Wang *et al*. [49] targeted *CXCR4* by employing CRISPR/SaCas9 in human $CD4^+$ T cell lines to make these cells resistant to X4-tropic HIV-1 infection. They used the AAV-SaCas9/sgRNA system to generate efficient *CXCR4* excision in the primary $CD4^+$ T cells and create resistance to HIV-1 infection, without affecting cell proliferation and viability. A study has shown that a $CXCR4^{P191A}$ mutant can effectively block X4-tropic HIV-1 infection, without damaging hematopoietic differentiation [50]. Liu *et al*. [51] utilized a combination of CRISPR-Cas9 and the piggyBac transposon technology, in an HIV-1 infected cell line, to efficiently express the $CXCR4^{P191A}$ mutant. The results showed that the biallelic *CXCR4* gene-edited cells significantly inhibited viral gene expression, which suggested that introducing *CXCR4* missense mutations may be a promising treatment strategy for preventing or reducing HIV-1 infection.

HBV

Most hepatitis cases involve chronic liver inflammations that can lead to lethal cirrhosis or liver cancer [52]. They are mainly caused by infection with the HBV. Approximately two billion people worldwide are infected with HBV and 1.4 million people die annually of hepatitis complications [52]. The HBV virus is a circular DNA virus whose genome encodes the hepatitis B core antigen (HBcAg), e antigen (HBeAg), DNA polymerase, surface antigen (HBsAg), and a transcriptional transactivating protein, HBx [53]. It enters the hepatocytes by binding to the HBV receptor sodium taurocholate co-transport polypeptide (NTCP). After entering the nucleus, the viral DNA transforms into closed covalent circular DNA (cccDNA) that can integrate into the host genome and transcribe RNA molecules to express viral proteins. One of the RNA

molecules is pregenomic RNA (pgRNA), which plays an essential role in viral particle assembly [53]. The HBV cccDNA has become the primary candidate for gene editing because it provides templates for virus replication and pgRNA. It also has an important relationship with persistent HBV infection in hepatocytes and recurrence after antiviral treatment [53]. Therefore, the current treatment focuses on reducing the HBV cccDNA level in the liver, to inhibit chronic HBV infection [52,53].

The ZFN and TALEN methods have already been used to reduce viral cccDNA levels. In 2010, Cradick *et al*. [54] used ZFNs to target HBV DNA. The results showed that engineered ZFNs were able to specifically recognize and excise HBV genomic DNA, *in vitro*, which led to approximately 10% cleaving and misjoining, tail-to-tail. Three years later, Bloom *et al*. [55] employed TALENs to target HBV open-reading frames of the S or C genes. The TALENs method efficiently generated mutagenesis within the intended DNA sequences and significantly suppressed HBV DNA replication, *in vitro* and *in vivo*. The safety test also showed that the S/C-TALEN effectively cleaved at the desired sites without detectable cytotoxicity. In 2014, Weber *et al*. [56] designed three ZFNs to target the HBV P, C, and X genes. They employed self-complementary AAV (scAAV) vectors to deliver ZFN constructs and they tested ZFN-mediated anti-HBV *in vitro* activity. The scAAV-HBV-ZFNs efficiently and specifically disrupted the intended targets at the HBV genome sites. Moreover, scAAV-P-ZFNs effectively inhibited DNA replication and infectious virion production in the HBV-infected cell model, for at least two weeks. This indicated that the P gene is a promising target site for HBV treatment. Chen *et al*. [57] generated TALENs to target viral genomes with high conservation scores among different HBV genotypes. In the HBV-infected Huh7 cells, the TALENs method significantly diminished the expression of HBeAg, HBsAg, HbcAg, and pgRNA. It also efficiently decreased viral cccDNA levels and excised the cccDNAs with undetectable toxicity. A hydrodynamic injection-based mouse model further demonstrated the anti-HBV effect of TALENs. Chen *et al*. also showed that combinations of TALENs and interferon-α (IFN-α) treatment contribute to an enhanced antiviral effect.

The CRISPR-Cas9 technology has been applied to anti-HBV research. Ramanan *et al*. [58] utilized CRISPR-Cas9 to target and induce specific mutagenesis in the conserved sequences within the HBV genome. This led to robust *in vitro* and *in vivo* viral gene expression and replication inhibition. Similarly, to disrupt the HBV DNA sequences and inhibit viral replication with CRISPR-Cas9, Liu *et al*. [59] designed gRNAs against the conserved DNA sequences of different HBV genotypes. This leads to the effective inhibition of HBV replication and significant elimination of viral DNA, in *vitro*. Li *et al*. [60] developed a CRISPR-Cas9 system (gRNA-S4) that targeted the region encoding HBsAg and suppressed viral replication with minimal off-target effects and impact on cell viability. In a murine HBV-infected model, the gRNA-S4 system reduced serum HBsAg levels by 99.91%±0.05% and diminished serum HBV DNA levels to below the negative threshold. Wang *et al*. [61] developed a novel gRNA-microRNA (miRNA)-gRNA ternary cassette to inhibit cccDNA expression. This ternary construct was able to efficiently express two gRNAs and miR-HBV, thus, efficiently inhibiting HBV DNA replication and destroying the HBV genome sequences. To search for gene editing targets for gene therapy, Seeger *et al*. [62] employed next-generation sequencing technology and CRISPR-Cas9 to determine the entire spectrum of mutations within the HBV cccDNAs. The results showed that over 90% of cccDNA sequences could be excised by Cas9 and the Cas9-mediated editing of HBV DNA was over 15000 times more efficient than APOBEC-mediated cytosine deamination. Zhu *et al*. [63] targeted the conserved regions of the S and X genes within the HBV genome and performed CRISPR-Cas9-mediated gene disruption. The results showed a significant anti-HBV effect by Cas9-2 in the cultured

cell models. In the transgenic mouse model of HBV infection, S/C-Cas9 showed significant viral resistance by decreasing serum HBsAg and liver HBcAg. Song *et al*. [64] developed specific gRNAs to target the open reading frames of preS1/preS2/S, within the HBV genome, and established HBsAg knockout hepatocellular carcinoma (HCC) cell lines using CRISPR-Cas9. The results showed that diminishing HBsAg, in HCC cell lines, significantly attenuated *in vitro* HCC proliferation and *in vivo* tumorigenicity. Moreover, knockout of HBsAg in HCC cells decreased interleukin (IL)-6 production and inhibited STAT3 signaling. In contrast, overexpression of HBsAg caused intracellular accumulation of pY-STAT3, which revealed the tumorigenic role of HBsAg in HBV-associated HCC.

Recent studies have also shown that, in addition to SpCas9, the Cas9 systems from Sa, *Streptococcus thermophilus* (St) and specifically engineered ARCUS nuclease contribute to anti-HBV effects. Liu *et al*. [65] utilized CRISPR-SaCas9 to disrupt the HBV genome and designed specific gRNAs to target different HBV genotypes. The gRNA/SaCas9 efficiently excised the HBV genome sequences and significantly lowered HBV antigen production and pgRNA/cccDNA levels in multiple cell models. The *in vivo* tests showed that the AAV-RNA/SaCas9 significantly diminished HBV protein levels and persistently inhibited HBV replication. Kostyushev *et al*. [66] used SpCas9 and StCas9 systems to target conserved regions of the HBV genome. The results showed that HBV replication was blocked and viral cccDNA was degraded by over 90% at six days post-transfection. Deep sequencing revealed the presence of SpCas9-induced off-target mutagenesis, whilst StCas9 did not affect the host genome. This suggests that StCas9 is a safer system, with higher anti-HBV activity than SpCas9. Furthermore, Gorsuch *et al*. [67] described a potential therapeutic method using highly specific, engineered ARCUS nuclease (ARCUS-POL) to target the HBV genome. They achieved transient expression of ARCUS-POL in primary human hepatocytes with HBV infection and detected a significant decrease in viral cccDNA and HBsAg. To evaluate the antiviral effect of ARCUS-POL, *in vivo*, Gorsuch *et al*. developed HBV mouse and non-human primate (NHP) models that were infected by adjunct AAV, which contained partial HBV genome as a substitute for HBV cccDNA. They also performed clinically relevant delivery using systemic administration of lipid nanoparticles (LNPs) that contained ARCUS-POL mRNA. The results showed that the desired indels were effectively introduced into the intended site, which significantly reduced the copy numbers of AAV-HBV in mice and NHPs. In addition, the *in vivo* results showed that the level of circulating HBsAg was decreased by 96% in the mouse model.

The DSB mediated by CRISPR-Cas9 causes host genome instability and shows low efficiency in genome editing, which limits its application [11]. The CRISPR cytidine base editors (CBEs) can silence genes by producing premature termination codons [26]. Zhou *et al*. [68] designed a CBE approach to impair HBV gene expression by substituting a single nucleotide within the viral genome. The gRNA/CBE targeted the 30th codon of the S gene and mediated the substitution of the original CAG to a premature TAG stop codon. This led to approximately 71% of cultured cells generating premature stop codons at the intended site. As expected, HBV mRNA levels were significantly decreased, whilst secreted HBsAg decreased by 92% and intracellular HBsAg was reduced by 84% in cultured cells. Moreover, off-target effects were rarely detected within predicted off-target loci within the HBV genome.

SARS CoV-2

The SARS-CoV-2 virus is a linear, single-stranded RNA virus [69]. After binding to the receptor, SARS-

CoV-2 fuses to the cell membrane and transfers its genome into the cytoplasm, to assemble viral proteins. After the virus particles are assembled, SARS-CoV-2 is moved to the cell surface by vesicles and is released by exocytosis [69]. Since SARS-CoV-2 is an RNA virus, the RNA-targeting CRISPR-Cas13 shows excellent potential for treating COVID-19. The Cas13 protein employs a CRISPR RNA (crRNA) with an engineerable spacer sequence that can lead the Cas13 protein to target RNA molecules for precise excision [70].

In 2020, Abbott *et al*. [71] developed a treatment strategy called prophylactic antiviral CRISPR in human cells (PAC-MAN). This strategy is used to resist SARS-CoV-2 infection by targeting highly conserved regions of viral genomes and excising these sequences using CRISPR-Cas13d. In human lung epithelial cells, the crRNA directs the Cas13d to degrade the synthesized fragments of SARS-CoV-2 and efficiently reduces viral infection. The bioinformatics analysis demonstrated that the designed crRNAs can target over 91% of the sequenced regions of SARS-CoV-2, which indicates that the CRISPR-Cas13d-mediated viral sequence excision may be a promising antiviral method for SARS-CoV-2 treatment. Similarly, Blanchard *et al*. [72] designed Cas13a-derived crRNAs to target the essential genome sequences that encode the replicase and nucleocapsid of SARS-CoV-2. The engineered CRISPR-Cas13a gave a significant reduction in SARS-CoV-2 RNA levels in the cultured cells and inhibited viral replication, *in vivo*. This alleviated the hamster model's respiratory symptoms caused by SARS-CoV-2 infection. In addition, Fareh *et al*. [73] employed a reprogrammed CRISPR-Cas13b to efficiently repress the replicase gene of various SARS-CoV-2 genotypes in cultured cell models derived from monkeys and humans. Their results also showed that Cas13 can relate single-nucleotide mismatches to the designed crRNA and maintain catalytic activity. This indicates that CRISPR-Cas13 is a promising gene editing tool for SARS-CoV-2 treatment.

HPV

The HPV virus is a double-stranded DNA papillomavirus, with approximately 150 known subtypes. It can be divided into low-risk groups that cause genital warts and high-risk groups that cause various cancers (e.g., cervical cancer) [74]. Among the most investigated subtypes, HPV-16 and HPV-18 are known to be highly infectious and cause sexually transmitted infections related to cervical cancer. The HPV *E6* and *E7* genes are oncogenes that are essential in converting derived malignant cells. Hence, gene knockout that is aimed at *E6* and *E7* is a promising strategy for treating HPV infection-derived cervical cancers [74].

In 2015, Hu *et al*. [75] utilized TALENs to target *E6* and *E7*. The TALEN-mediated disruption of these genes lowered the viral DNA load. It restored the function of the tumor suppressors, p53 and retinoblastoma 1 (RB1), thereby recovering the malignant symptoms caused by HPV-16 infection in a transgenic murine model. Similarly, Kennedy *et al*. [76] demonstrated that CRISPR-Cas9 effectively induces cleavage of the HPV-16 genome and results in mutagenesis of the *E6* and *E7* genes. The chemotherapy agent, CDDP, is a first-line cancer treatment that is used for cancers such as metastatic cervical cancer [77]. Zhen *et al*. [78] utilized CRISPR-Cas9 to target the *E6* and *E7* genes of HPV-16 and sensitize cultured HPV-16-positive cervical cancer cells to CDDP. The results showed that combinatory exposure to CRISPR-Cas9 and CDDP exerts a synergistic cytotoxicity and antitumor effects in the HPV-16-derived cervical cancer models in cultured cells and xenograft mice. Jubair *et al*. [79] generated an *in vivo* cervical cancer model that carries HPV E6 and E7 proteins, and they employed CRISPR-Cas9-based gene therapy through PEGylated liposomes. This led to significantly diminished tumors. In addition, Inturi and Jemth [80] reported that CRISPR-

Cas9-induced elimination of *E6* and *E7* genes activates cellular senescence in immortalized HPV-18 infected cells. They revealed that the specific suppression of HPV-18 *E*6 expression activates the tumor-suppressing pathways of p53/p21 and pRb/p21. Furthermore, elimination of the *E7* gene lowers *E6* expression and triggers the pRb/p21 pathway. In another CRISPR-Cas9-mediated gene editing study, Gao *et al*. [81] targeted the *E7* gene in the HPV-driven spontaneous cervical carcinogenesis models of cultured cells and transgenic mice. The results showed that specific disruption of the *E7* gene restores the tumor-suppressing protein, retinoblastoma, and its downstream targets, E2F1 and CDK2. This rescues the pathological symptoms caused by K14-HPV16-derived cervical carcinogenesis.

Gene editing has made gratifying progress in clinical research into treating viral infections. However, the efficiency of *in vivo* editing and the delivery efficiency of editing tools still need to be improved. Future clinical research may achieve a better therapeutic effect by combining drugs.

Gene editing and non-viral infection

In addition to viral infections, gene editing can also be used to treat non-viral infections, such as those caused by bacteria, fungi and parasites.

Bacteria

Recent studies have shown that CRISPR-Cas9 has good prospects for the treatment of a broad range of infections caused by pathogenic bacteria, as it can be used to target antibiotic resistance and virulence genes. Due to the lack of DNA repair mechanisms, bacteria show specific vulnerability to genomic DNA impairment and cell death [82,83]. The antibacterial effects of CRISPR-Cas9 occur through targeting and impairing the essential cellular pathways and selectively eliminating specific bacteria subtypes. Bikard *et al*. [84] used bacteriophage-mediated CRISPR-Cas9 gene editing to develop programmable antimicrobials against *S. aureus*. Re-programming CRISPR-Cas9 disrupted the antibiotic-resistance genes and plasmids and immunized the avirulent clones by preventing the spread of plasmid-borne resistance genes. Further *in vivo* tests revealed that CRISPR-Cas9 efficiently inhibits *S. aureus* infection in the skin of a murine model.

Clostridioides difficile is a nosocomial pathogen that annually induces approximately half a million *C. difficile* infection (CDI) cases and nearly thirty thousand casualties in the United States. Abuse of antibiotics is a crucial risk factor for CDI due to broad-spectrum antimicrobials disrupting the indigenous gut microbiota and diminishing colonization resistance against *C. difficile*. Hence, there is an urgent need to develop a novel treatment strategy that manages CDI and precisely eliminates *C. difficile*, without impairing the gut microbiota. Selle *et al*. [85] employed a self-targeting system as an anti-*C. difficile* agent and subsequently induced the CRISPR-Cas3 expression that targets the bacterial chromosome. The results demonstrated that the bacteriophage-based CRISPR-Cas system effectively inhibits the replication of *C. difficile* in the mouse model. This indicates that CRISPR-Cas-mediated treatment is a promising antimicrobial strategy for CDI, *in vivo*.

Fungi

The CRISPR-Cas9 system can be optimized and adjusted for fungi by employing fungal gene elements (e.g.,

fungal promoters) to give highly efficient expression of CRISPR-Cas9. *Candida albicans* is a diploid pathogen that causes most fungal infection cases. However, there are genetic manipulation obstacles to Cas9-mediated gene editing in *C. albicans*, as discussed below. However, Shapiro *et al*. [86] developed a gene drive array (GDA) strategy to track genome manipulation and effectively introduce biallelic mutations into *C. albicans*. Using the GDA technology, they explored some promising antifungal targets for *C. albicans* treatment, such as drug pumps and biofilm adhesins.

Various barriers to genetic manipulation exist in *Candida* species. These include the inability to preserve engineered plasmids, the unique codon preference, and ineffective homologous recombination. Halder *et al*. [87] presented a fast CRISPR-Cas9-based protocol for the *C. albicans* genome to overcome the barriers and achieve genome manipulation, within approximately one month. They provided a practical approach for transformation via fungal haploids and gave a helpful strategy for crossing edited *Candida* to obtain biallelic mutant fungi. Researchers can use this protocol to progress genetic manipulation into other mating-competent, haploid, infectious fungi.

The use of editing technology to treat fungal infections still needs to be improved, in terms of editing efficiency and delivery methods. In the future, researchers should address these issues to allow gene editing therapy for fungal infections to enter clinical research as soon as possible.

Parasites

Malaria affects over 200 million people worldwide. *Plasmodium falciparum* is the most ferocious etiologic agent and is developing resistance to the latest generation of treatments [88]. Straimer *et al*. [89] targeted the *P. falciparum* genome, using ZFNs, and induced a double strand break within the *pfcrt* locus, which is responsible for resistance to chloroquine treatment. Ghorbal *et al*. [90] used the CRISPR-Cas9 system to disrupt *P. falciparum* genomic DNA sequences and efficiently induced single-nucleotide substitutions. Similarly, Wagner *et al*. [91] used CRISPR-Cas9 to target the genes that encode the native knob-associated histidine-rich protein (KAHRP) and erythrocyte binding antigen 175 (EBA-175). The results showed high gene disruption frequencies (⩾ 50%–100%).

Toxoplasma gondii is a diet-borne pathogen that results in toxoplasmosis, which is a potentially severe disease in immunocompromised or congenitally infected humans [92]. To perform gene therapy for toxoplasmosis, Shen *et al*. [93] employed CRISPR-Cas9 to disrupt the serine threonine kinase *rop18* gene, which is implicated in the virulence of *T. gondii*. The *in vivo* test revealed that *rop18*$^{-/-}$ mutants significantly decreased virulence in the highly virulent *T. gondii* strain. This indicated that this gene is a promising therapeutic target for gene editing-based treatment. Sidik *et al*. [94] also employed Cas9 to perform a genome-wide CRISPR examination of *Toxoplasma*, to find potential gene-editing targets. They identified the claudin-like apicomplexan microneme protein (CLAMP) as an essential factor for *P. falciparum* infection. This protein plays a critical role in the asexual stages of the parasite. Specific inhibition of the *CLAMP* gene significantly impairs the asexual cycle of *P. falciparum*. Furthermore, as almost all apicomplexan genomes contain *CLAMP* homologs, *CLAMP* could be a promising gene editing target for toxoplasmosis treatment. Palencia *et al*. [95] performed CRISPR-Cas9-mediated gene editing on the *TgCPSF3* gene. This gene encodes an endonuclease that is essential for mRNA processing in eukaryotes and is an ideal target for the development of anti-*T. gondii* drugs. The *in vivo* test revealed that murine models infected by edited *T.*

gondii, combined with oral treatment with AN3661 (a commonly used benzoxaborole agent for inhibition of *Toxoplasma* growth), had no detectable illness, whereas the untreated groups had fatal infections.

Trypanosoma cruzi is a parasite of humans and animals that affects over ten million people and numerous animals in America [96]. Lander *et al*. [97] used CRISPR-Cas9 to disrupt the *TcGP72* and *paraflagellar rod proteins 1* and *2* genes. These play essential roles in flagellar attachment and flagellum formation. The results indicated that CRISPR-Cas9 can efficiently edit the *T. cruzi* genes, with undetectable toxicity to the host. Furthermore, mutagenesis of *PFR1*, *PFR2*, and *GP72* significantly disrupts flagellar attachment and increases the motility of the parasites.

In another case, Sollelis *et al*. [98,99] employed CRISPR-Cas9 to eliminate genes of the *Leishmania* parasite, which induces lethal leishmaniasis in humans. They used a dihydrofolate reductase-thymidylate synthase (DHFR-TS) promoter to control the expression of the Cas9 protein and they utilized a U6 promoter for gRNA expression. Using this method, they succeeded in the specific knockout of the paraflagellar rod-2 locus.

Hence, CRISPR-Cas9 is an effective method to treat parasitic infection but its delivery in parasites is still one of the problems that needs to be solved to allow this method to be used for clinical treatment.

Outlook for *in vivo* gene therapeutics in infectious disease

Gene editing technology has broad application potential, particularly in treating many diseases caused by gene mutation or pathogen infection [100]. The latest studies will promote a new era of gene editing, in which the CRISPR-Cas and Cas-derived systems are applied to the treatment of infectious diseases. The rapid development of the CRISPR-Cas9 technology, as a genome editing method, will contribute to systematic studies on infectious diseases [3]. Therefore, the CRISPR-Cas9 system may be a weapon that is urgently needed by humans in the fight against a variety of drug-resistant pathogens and an epidemic outbreak that caused a quarter of all worldwide deaths.

This review showed that current gene editing methods and their efficiencies against bacteria, fungi, and parasite infections still need work to reach the same degree of effectiveness as that reached in the fight against tumors or viral diseases. An obstacle to CRISPR-Cas-based therapeutic application is the difficulty in editing pathological genomes. New technological advances are also required for novel delivery systems. The current delivery methods used to transfect gene editing agents into bacteria, fungi, and parasites are based on bacteriophage [84], plasmids [87], and electroporation [93], respectively. Although these methods are specifically designed to target the pathogen's DNA, it is hard to ensure the *in vivo* therapeutic effect, which is strongly affected by the accuracy of delivery and gene editing efficiency. However, the emergence of CRISPR-Cas systems has provided the ability to perform large-scale genetic analyses [101]. Therefore, CRISPR-Cas provides a straightforward method for analysis that may enable further exploration of the molecular biology, virulence factors, drug resistance, infection mechanisms, and host-pathogen interactions, which is essential for the development of novel approaches to combat bacteria, fungi, and parasite pathogens.

Viral and non-viral-based delivery of CRISPR-Cas, into the intended genomes, is essential for the clinical application of gene editing-based therapeutics. Lentiviral and AAV vectors have been developed for gene editing tool delivery. Lentiviral vectors derive from HIV-1 and are modified to be replication-defective

[102,103]. They can integrate into the host genome, possess broad cellular tropism, have a loading size of up to 8 kb, allow easy assembly and modification and ensure payload stability [104,105]. The AAV vectors have the advantages of impacting living cells and low pathogenicity [106]. The loading size of AAV vectors is approximately 5 kb and is known to significantly limit the encapsulation of SpCas9. However, novel Cas variants [107,108] that are remarkably smaller than SpCas9 have recently been discovered and can be used for loading within viral vectors. The clinical application of viral vectors is currently limited by several inherent obstacles, such as potential carcinogenicity, induction of immunogenicity, limited packaging sizes, toxic side effects, and high preparation and scale-up costs [109,110]. Non-viral vectors, such as LNPs, are highly efficient in delivering CRISPR-Cas tools into the organism, with six characteristics: (1) prevention of gene editing agent degradation, (2) maximization of target cell integration, (3) capability of endosomal escape, (4) effective cytosolic release, (5) minimal immunological effects, and (6) nonpersistent administration with high liver-targeted preference. Despite these advantages and disadvantages, both viral and non-viral-based vectors have been utilized for therapeutic delivery of gene editing agents, *in vivo*. They show significant potential for clinical application for inherent and viral infectious diseases.

Finally, future studies may combine CRISPR-Cas9 technology with synthetic biology technology to reduce off-target effects and undesirable edits, *in vivo*. Another focus of further development in this field will be the improvement of specificity of CRISPR gene editing and the standardization of CRISPR-Cas9 gene editing evaluation methods. Although researchers have made significant progress in understanding CRISPR-Cas9 functions, many core issues still need to be clarified. Interestingly, CRISPR-Cas9 not only plays a role in the adaptive immune system in bacteria but also seems essential for the occurrence of diseases. This provides us with opportunities to explore the unknown physiological functions of CRISPR-Cas9, to further our knowledge on its role in combating pathogens.

Funding

This work was supported by the National Key R&D Program of China (2019YFA0109900, 2019YFA0109901, 2019YFA0802800, 2019YFA0110803), the Shanghai Municipal Commission for Science and Technology (19PJ1403500), and the National Natural Science Foundation of China (82270125).

Author contributions

Y.W. and H.Z. jointly completed the writing and revision of this review.

Conflict of interest

The authors declare no conflict of interest.

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