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CRISPR-Cas Based Targeting of Host and Viral Genes as an Antiviral Strategy

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Abstract

Viral infections in human are leading cause of mortality and morbidity across the globe. Several viruses (including HIV and Herpesvirus), have evolved ingenious strategies to evade host-immune system and persist life-long. *C*lustered *R*egularly *I*nterspaced *S*hort *P*alindromic *R*epeats (CRISPR) and CRISPR-associated (Cas) is an ancient antiviral system recently discovered in bacteria that has shown tremendous potential as a precise, invariant genome editing tool. Using CRISPR-Cas based system to activate host defenses or genetic modification of viral genome can provide novel, exciting and successful antiviral mechanisms and treatment modalities. In this review, we will provide progress on the CRISPR-Cas based antiviral approaches that facilitate clearance of virus-infected cells and/or prohibit virus infection or replication. We will discuss on the possibilities of CRIPSR-Cas as prophylaxis and therapy in viral infections and review the challenges of this potent gene editing technology.

Keywords

CRISPR-Cas; Viruses; Antiviral immunity; Gene editing; Restriction factors

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Author Contributions

LK, DS and ARN conceptualized the review; LK and ARN prepared all the figures and compiled the tables; LK. DS and ARN wrote the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1. Introduction

1.1 Genome Engineering

Genetic engineering is a powerful technology that has resulted in the advancements of many fields such as medicine, industrial biotechnology and agriculture [1,2]. Specifically, the ability to alter genetic makeup of an organism has revolutionized medical research and expanded our ability to understand disease development and prevention. The application of various genome engineering tools such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and, most recently, CRISPR-Cas systems have advanced biological research and allowed for novel discoveries to be made.

ZFN and TALEN are both synthetic proteins with nucleus activity that achieve specific DNA binding via protein-DNA interactions. However, retargeting the specific activity of these nucleases is achieved through protein engineering or cloning which limits its versatility, feasibility and application. Recently, harnessing the CRISPR-Cas system, initially observed in prokaryotes, as a gene editing tool in eukaryotic cells has revolutionized the field as a robust and efficient tool for precise genome editing [3-5]. CRISPR/Cas system is a prokaryotic adaptive immune system that confers resistance to foreign genetic elements and has been repurposed by researchers as an invariant genome editing tool that can be more easily redirected to a new target [6].

The use of genome editing technology, primarily CRISPR, is reaching exciting stages and surpassing milestones in basic science research with applications extending beyond genome editing. More recently, CRISPR is being used as an antiviral by targeting genes in both the human genome as well as the invading viral genome. Both in vitro and in vivo studies have shown effective targeting of key viral genes important for the virus life cycle as well as host genes involved in viral entry, replication and persistence [7].

1.2 CRISPR/Cas9: Precise genome editing

CRISPR is an abbreviation for clustered regularly interspaced short palindromic repeats, which were initially observed in Escherichia coli, and found to be separated by non-repeated DNA sequences called spacers. These spacers, known as CRISPR arrays, where later identified as acquired copies of previously encountered foreign DNA and provides the organism with memory of the plasmid and phage genomes [8-10]. Upon encountering the foreign genetic element again, the bacteria produce RNA segments from the CRISPR arrays to target the pathogen directly and employ an adaptive immune response. Adjacent to CRISPR arrays are well conserved CRISPR-associated (Cas) genes that have been grouped into families and subtypes based on sequence similarity of encoded proteins [11]. There are six types of CRISPR/Cas system that have been divided into two classes: class 1 CRISPR-Cas systems (types I, III, and IV) employ multi-Cas protein complexes for interference, whereas in class 2 systems (types II, V, and VI), interference is accomplished by a single effector protein [12].

Type II CRISPR/Cas9 system of Streptococcus pyogenes (Sp) is the most studied and used system. Figure 1 describes generalized biogenesis and function of CRISPR/Cas9 system. The CRISPR array, which contains unique protospacer sequences that have homology to

foreign DNA, is transcribed to make long precursor CRISPR RNA (pre-crRNA). Pre-crRNA are then processed to individual crRNA which guide the interference machinery to cleave complementary sequences or protospacers and ultimately eliminate the foreign DNA [13]. An invariant trans-activating CRISPR RNA (tracrRNA) is required to bind crRNA in a sequence complementary manner and recruit RNAse III and CRISPR-associated 9 (Cas9) enzymes [14]. Together, tracrRNA, RNAse III and Cas9 break up and form a complex with each individual unique crRNAs [15]. Protospacer adjacent motifs (PAMs), located directly after where the crRNA would bind, are critical to this system and contribute to the CRISPR targeting specificity [16]. Cas protein motifs have helicase and nuclease activity that are guided by crRNA resulting in DNA cleavage upon target binding, which stimulates non-homologous end joining (NHEJ) or homology-directed repair (HDR)-mediated genome editing, resulting in the elimination of invading foreign DNA [17-19].

Cas9 effector endonuclease have been repurposed by researchers as a tool for precise gene editing to eliminate or integrate target genes in the human genome [6,20]. Specifically, the tracrRNA:crRNA complex have been engineered into a single guide RNA (sgRNA), guiding CRISPR-Cas9 to desired targets in the genome [21]. The use of this system and its gene editing potential in eukaryotic cells proved to be successful and the application of CRIPSR/Cas9 is now widely adapted in multiple research areas [5,6,20,22].

1.3 The need for a new antiviral: CRISPR/Cas9

Multiple viruses still persist and infect a large number of the human population while still without a cure [23-26]. Current antiviral treatments are able to block active viral replication or subside symptoms of infection; however, they lack the ability to completely abolish the virus from the host, primarily due to escape mutants or establishment of latent infection [27-29]. CRISPR/Cas system has been adapted from its natural function as an immune response in prokaryotes against phage infection to studying its gene editing potential in eukaryotes. This application has extended to the development of a novel antiviral; where CRISPR/Cas systems are directed to target viral genome or host genes that aide in the persistence of the virus [30,31]. In this review we will discuss these approaches that have been studied as well as other potential applications against viruses including Herpesvirus, Human immunodeficiency virus (HIV), Hepatitis B Virus (HBV) and Influenza A Virus (IAV). Different CRISPR/Cas based antiviral strategies targeting host and viral genomes are illustrated in Figure 2.

Of the viruses listed, herpesvirus has been recognized as the most adapted human pathogen resulting in lifelong infections and the development of a wide range of innocuous diseases [32-34]. The Herpesviridae family is divided into three subfamilies of double stranded DNA viruses: Alpha-, Beta- and Gammaherpesvirinae [35]. Herpes simplex virus-1 and 2 (HSV-1 and HSV-2) are neurotropic viruses belonging to the Alphaherpesvirinae family and are capable of establishing lifelong latency in the central nervous system. HSV-2 is responsible for genital herpes resulting in painful ulcers while HSV-1 primarily infects the corneal epithelium resulting in lesions and the development herpes simplex keratitis as well as other less frequent diseases such as encephalitis [36-39]. Human Cytomegalovirus (HCMV), belongs to the Betaherpesvirinae family, is capable of infecting multiple cell types including

epithelial, endothelial, myeloid, neuronal and fibroblast cells [40]. Epstein–Barr virus (EBV) belongs to the Gammaherpesvirinae family, primarily infecting B cells and epithelial cells causing infectious mononucleosis [41]. Acyclovir and Ganciclovir, nucleoside analogs that inhibit viral replication upon incorporation into viral DNA, are the current drug choice for treatment of HSV and CMV disease respectively [42,43]. While these drugs are highly effective in most cases, drug resistant viral strains have evolved [44-46]. On the other hand, EBV is reported to infect 95% of the population and no current treatment is available [47].

Another prominent virus, Hepatitis B Virus (HBV), remains a global health threat as infection results in life-threatening complications including elevated risks for liver damage and liver cancer [48]. HBV belongs to the family of *Hepadnaviridae* and replicates in infected hepatocytes in the liver via reverse transcription. HBV virions contain a partial dsDNA genome which is converted to double-stranded covalently closed circular (ccc)DNA in the nucleus shortly after infection [49]. The cccDNA is maintained as a 'minichromosome' and serves as a template for transcription of viral pregenomic RNA and mRNAs [50]. Current antivirals against HBV include IFN-α, which bolsters the host antiviral immune response, and nucleoside/nucleotide analogues to inhibit reverse transcriptase activity. However, due to persistence of cccDNA, long-term treatment is required is not a feasible option for some patients and has resulted in drug resistance among some strains [51].

Human Immunodeficiency virus (HIV) is also among the viruses that are highly prevalent worldwide and still remain without a cure. HIV is a single-stranded enveloped RNA virus that predominately infects human T cells and results in the development of acquired immune deficiency syndrome (AIDS). An estimated number of 35 million individuals suffer from HIV infection worldwide and require life-long treatment, which has resulted in many side effects and the emergence of escape mutants [52,53]. Antiretroviral therapy is the current mode of treatment and while it does efficiently limit virus replication to undetectable levels in HIV patients, it suffers from serious drawbacks [54].

Influenza A virus (IAV) is considered the most serious influenza capable of causing widespread outbreaks and disease [55]. IAV infection attacks the respiratory system and is able to rapidly evolve, resulting in seasonal epidemics and the constant emergence of variant- and drug-resistant strains [56]. The genome of IAV, which consists of multiple segments of single-stranded negative-sense RNA, contributes to its susceptibility to mutations which allow for evasion of the immune system and resistance to antiviral strategies [57]. Current vaccinations are limited in their efficacy and therapies only alleviate the symptoms and manage disease development to prevent hospitalization and death in patients [58].

The conventional antiviral drugs pose many concerning disadvantages. First of all, resistance among infected patients for all current FDA approved antiviral drugs, especially in the case of IAV, have been repeatedly reported [27,58-60]. Also, while current antivirals are effective in clearing productive viral replication in most cases, they are unable to eradicate the virus from latently infected cells [61,62]. This causes the infection to persist throughout the lifetime of the host with the potential of reactivation and recurrence. For these cases, life-

long treatments are prescribed to patients in order to suppress viral reactivation, which results in side effects and escape mutants in many cases.

The viruses mentioned here remain a global health threat and continued efforts are aimed towards developing an effective antiviral or vaccination that eliminates viral genomes from the host. Most efforts to develop antivirals in the past have been directed towards targeting the viral genome in order to dismantle the virus' ability to infect. However, with the emergence of escape mutants and drug resistance, efforts are now also being directed towards targeting the host cell machinery [31,63]. To prevent viral reactivation, drug resistance and protein expression, new antiviral strategies are needed that provide a durable and feasible antiviral effect. Ideally, eradication of the integrated proviral DNA in all infected cells, including the latent reservoir is required to effectively combat viral infection.

2. Targeting Host Factors Using CRISPR/Cas9

While targeting viral genomes with CRISPR/Cas9 may be subjected to the same disadvantages as current therapeutics, targeting host factors provides a more promising solution to abolish the virus from the host. Clinical advantages of host-based interventions using CRISPR/Cas9 include lower chances of drug resistances due to genetic stability of host factors, higher efficacy due to larger therapeutic timeframe and reduced side effects due to minimal dosing requirements.

Upon infection, the virus exploits host factors to complete its life cycle [64]. While the host's innate immune system works to clear viral infection, the virus hijacks cell signaling pathways to escape immune surveillance and persist. Exploiting the dynamic virus-host interactions is a strategy used to block virus infection and make the virus less susceptible to survival in the cellular environment [65]. CRISPR/Cas systems have been used in multiple studies for genome editing of dispensable target host genes involved in viral replication and persistence. Recent advancements in CRISPR/Cas9 technology has also allowed for genome-wide CRISPR knockout (GeCKO) screening, providing a powerful tool to discover host factors and to highlight biological pathways essential for the replication of intracellular pathogens [66].

2.1 Host factors involved in viral entry

While current drug targets include viral proteins required for cell entry, most of the developed drugs have been regarded as ineffective over long term control of infection. In an effort to produce antivirals with long-lasting effects, reduced risks of developed resistance and toxicity, new mechanisms of action need to be developed. With the recent advancements made in gene editing, CRISPR/Cas9 and other tools have been used to target both the viral and host genome for factors involved in viral entry (Table 1). Recent studies have focused on inhibiting viral entry by targeting host factors such as cell surface receptors. Results from these studies have shown been high antiviral efficacy against HIV and IAV.

2.1.1 Targeting HIV cell-surface entry receptors—CC chemokine receptor 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4) are cell surface receptors involved in viral entry during primary infection [67]. Individuals homozygous for a naturally

occurring deletion of 32 nt in the CCR5 gene prevents the expression of CCR5 on the cell surface and deems them highly resistant to HIV infection [68]. This discovery proposed a new antiviral approach to combat HIV: targeting cell surface entry receptors in order to disrupt membrane fusion between the virus and cell [69]. A competitive inhibitor of CCR5, maraviroc, was clinically approved and showed affective anti-HIV activity. However, development of resistance by selection for escape mutants was later observed by viral adaptations to the drug [70]. While CCR5 is the predominant receptor involved in HIV entry and primary infection, viral tropism towards CXCR4 usage occurs at later stages of infection and maraviroc was also dispensable in the presence of CXCR4-tropic HIV strains [71]. To combat CCR5-tropic HIV strains, gene therapy approaches to block CCR5 expression were evaluated and found to be promising both *in vitro* and *in vivo* [72]. Perez and colleagues published a study using ZFN-guided genomic editing of CCR5 in primary human CD4+ T lymphocytes showing high specificity, tolerance and robust protection against HIV-1 and soon underwent clinical trials targeting mature CD4+ T cells [73,74].

More interest has been developed in the past decade following a report that an acute myeloid leukemia patient who received a hematopoietic stem/progenitor cell (HSC) transplant from a CCR5Δ32 donor was also cured from HIV-1 [75,76]. Subsequent studies in mice transplanted with CCR5-disrupted HSCs showed significant reduction in viral load when challenged with CCR5-tropic HIV-1 strain, as well as protection of human T cell populations in key tissues that HIV-1 infects. Long-term control of HIV-1 replication was observed for the first time using this technique [77]. This study suggested that modification of autologous HSCs by CCR5-ZFNs could provide a permanent supply of HIV-resistant progeny, leading to immune reconstitution and long-term control of viral replication [78]. While ZFN and TALEN are effective in enabling mutagenesis in a variety of cells, they both exhibit challenges including variability in mutation frequencies, lack of specificity, feasibility and off-target effects. The most important off-target sites associated with CCR5-specific ZFNs and TALENs reside in CCR2, a close homolog of CCR5, located 15kpb upstream of CCR5.

In order to increase specificity and reduce undesired off-target effects, CRISPR/Cas9 has been employed to induce a site-specific genome modification in human cells and in vivo using mouse model of infection [79-82]. Mulitiple groups successfully transduced primary CD4+ T-lymphocytes and disrupted CCR5 by gene editing using adenovirus-delivered CRISPR/Cas9 targeting the open reading frames (ORF) of CCR5. This resulted in inhibition of HIV-1 infection with no significant off-target effects [81]. Targeting CCR5 in both HSC and CD4+ T cells has shown promise as a gene-editing strategy for persistent resistance to HIV. However, its ineffectiveness towards CXCR4-tropic HIV strains and therefore lacks conclusive evidence for its use as a cure. Studies have also directed Cas9-mediated gene editing of CXCR4 with high precision and efficacy, showing negligible off-target effects and resistance to HIV infection [79,83]. Targeting both coreceptors CCR5 and CXCR4 may provide more breadth in protection and use in clinical settings.

2.1.2 Targeting host factors involved in IAV entry—CRISPR/Cas9 has also been used to limit IAV infection by targeting host factors involved in viral entry. IAV enters host cells after attachment of its hemagglutinin (HA) to the cell surface receptor sialic acid. Entry

inhibitors have been developed in the case of IAV that either bind to the HA globular head to prevent interaction with the receptor or by acting on the sialic acid receptor itself [84,85]. However, currently available drugs are ineffective against newly emerging viruses with developed resistance. Recent studies using a genome-wide CRISPR knockout (GeCKO) screen demonstrated that a CMP-sialic acid transporter (SLC35A1) is an essential host factor for IAV entry [86]. Validation studies revealed that loss of SLC35A1 resulted in resistance against IAV infection due to the absence of cell-surface sialic acid. Clonal knockouts infected with H5N1, H1N1 and H3N2 observed a significant reduction in viral titers, confirming the role of SLC35A1 in replication of multiple IAV strains. SLC35A1 knockout cell lines also showed inability to support recombinant HA binding and therefore resulting in a robust reduction of IAV entry.

Genome wide overexpression screen using CRISPR/dCas9 was recently used as a tool to uncover IAV restriction factors that, when overexpressed, are sufficient to prevent infection. A glycosyltransferase (B4GALNT2) that modifies sialic acid-containing glycans, and thus prevents IAV infection, was identified as a top hit, leading to greater than 100-fold reduction in IAV infection. The inhibitory activity of B4GALNT2 was restricted to viruses with avian a2,3-linked receptor which include avian influenza viruses H7N2, H9N2, H5N9, H10N4, some of which infect humans as well [87].

2.2 Antiviral Host Effectors (Cell-intrinsic immunity)

Upon viral infection, the host triggers a robust innate immune response as a defense mechanism to clear the pathogen [64]. The dynamic virus-host interaction that occurs at early stages of infection determines whether the cell will overcome the viral infection or succumb to it. While the host cells have evolved to express restriction factors that block virus infection, the virus in turn exploits host factors and uses specific strategies to escape immune surveillance by manipulating its environment. Studies of this dynamic interaction has allowed for the identification of host effectors that aid or suppress the cell-intrinsic immunity. These host effectors are now key targets for developing novel antiviral strategies through the use of CRISPR/Cas9.

In regard to HIV, APOBEC3G (A3G) and APOBEC3B (A3B) are restriction factors in human cells that have recently been targeted. A3G is expressed in human T cells and macrophages, which are usually the target cells of HIV-1, and is capable of restricting the virus. However, HIV-1 Vif protein is able to target and degrade this host effector. A3B on the other hand is restricted to expression in pluripotent cell types and is not targeted for degradation by Vif. In a recent study, a gain-of-function approach using a cleavage defective Cas9 (dCas9) allowed for specific and effective induction of A3G and A3B genes in cell culture [88]. Potent inhibition of HIV-1ΔVif infectivity was achieved by induced endogenous A3B and A3G in 293 T cells, which typically lack both proteins, and a decrease in HIV-1WT infectivity upon A3B expression. This study shows that CRISPR/Cas gene editing technology can be used as a novel platform to combat infection by efficiently targeting human genes that regulate virus replication.

Using a genome-wide CRISPR/Cas9 screen, key regulators of cell-intrinsic immunity upon IAV infection have been identified and serve as targets for further investigation [86]. In this

study, capicua (CIC) was shown to play a role in suppressing antiviral gene expression by its transcriptional repressor activity. Confirming this, knocking down CIC using CRISPR/Cas9 resulted in decreased replication of both IAV and VSV by the observed heightened antiviral responses. Similarly, JAK2 was also identified as a host effector involved in negatively regulating cell-intrinsic immunity by lowering antiviral gene expression. While more studies are required to elucidate the exact role JAK2 plays at different stages of IAV infection, using CRISPR/Cas9 this group was able to identify key factors that contribute to virus survival against the innate immune system.

Similarly, a genome-wide CRISPR/Cas9 loss-of-function screen was conducted to identify host effectors critical for EBV induced B cell-transformation leading to B cell malignancies and how oncoproteins evade tumor suppressor responses [89]. The screen identified BATF, IRF4, IRF2, CDK6 and cyclin D2 as lymphoblastoid cell lines (LCLs) dependency factors that are highly upregulated in EBV infection of primary human B cells. Another gene, cFLIP, was highlighted as a potential EBV therapeutic target for its role as a survival factor in LCLs by blocking TNFα-mediated apoptosis and necroptosis.

Other host factors recently identified as having an integral role in viral infection are interferon-induced transmembrane proteins (IFITM) which restrict the entry of diverse enveloped viruses through unknown mechanism [90,91]. In the case of IAV, initial infection upregulates p53, a protein involved in key cellular stress mechanisms, which in turn downregulates IFITMs and therefore promotes virus survival in the host. Since IFITMs were detected as downstream targets that are negatively regulated by p53, CRISPR/Cas9 was used to generate p53null cells to investigate its role in influenza infectivity. While p53 was not shown to affect initial IAV entry, overexpression of IFITMs reduced cell susceptibility to IAV infection by blocking viral entry in A549 cells [92]. The role of IFITM on restricting HIV-1 infection was also investigated using CRISPR/Cas9 by directly targeting IFITMs in U87 CD4+ CCR5+ cells. While similar findings as those regarding IAV were concluded from the study, the clear mechanism of IFITM upon viral infection is still poorly understood and lacks *in vivo* relevance [93].

2.3 Host Factors involved in viral transcription and replication

Infectious particles take over various cellular pathways and processes in order to ensure its survival and persistence in the host. Certain cellular genes are upregulated in the presence of viral particles and aid in viral transcription and replication. For example, genes known to support HBV replication include sodium-taurocholate cotransporting polypeptide (NTCP), heat stress cognate 70 (Hsc70), heat shock protein Hsp90 and tyrosyl-DNA-phosphodiesterase 2 (TDP2) [31]. While studies have not shown the antiviral effect of knocking down these genes using CRISPR/Cas9, these are still potential targets to be considered.

Various host factors identified as restricting HIV replication include APOBEC3C, TRIM5α, SAMHD1, and tetherin, and also serve as potential CRISPR/Cas targets in developing therapeutics [31,94,95]. Many of these are involved in eliciting robust immune responses upon detection of HIV, and ultimately acting as restriction factors to terminate HIV replication by blocking viral entry or release from the host cell. TRIM5α is associated with

antiviral activity by recognizing retroviral capsid proteins and, in turn, transducing diverse cellular signaling pathways, including triggering innate immunity [95]. Studies showed efficient antiviral activity using TRIM-cyclophilin fusion protein both *in vitro* and *in vivo* with robust inhibition of HIV-1 replication [96]. Similarly, SAMHD1 is a restriction factor that prevents HIV replication. SAMHD1 is a deoxynucleotide triphosphate (dNTP) hydrolase that is activated by the binding of GTP to its allosteric site which consequently reduces the intracellular dNTP pool below levels that support HIV-1 reverse transcription [97]. This results in the blockage of HIV-1 replication and prevents the induction of IFN responses. However, transducing the activity of SAMHD1 is counteracted by Viral Protein X (VpX) which targets and degrades the protein, leading to the restoration of dNTP levels and allowing for HIV-1 replication [98,99]. Tetherin is another restriction factor of HIV induced by interferon and prevents viral replication by restricting viral particle release [100]. While HIV-1 Viral Protein U (Vpu) counteracts tetherin activity, a single amino acid substitution in tetherin results in Vpu-resistance [101]. These restriction factors serve as promising targets for the use of CRIPSR/Cas9 in either activating these restriction factors or inducing the mutations that make the proteins resistant to inhibition by viral proteins.

Similarly, various studies have investigated the host-pathogen interaction that occurs upon IAV infection in order to identify key host genes involved. A genome-wide RNAi study identified hundreds of host genes involved in restricting or promoting cellular infection by RNA viruses, including IAV [102]. Of the genes identified, COPI-protein families, fibroblast growth factor receptor (FGFR) proteins, glycogen synthase kinase 3 (GSK3)-beta and calcium/calmodulin-dependent protein kinase (CaM kinase) II beta (CAMK2B) were significantly enriched and found to be involved in early steps of influenza virus replication. This study also showed that inhibition of these identified targets using small molecules resulted in attenuated viral replication; further proving that these may be suitable targets using CRISPR/Cas9.

A study conducting a genetic screening using CRISPR/Cas9 identified host factor dependencies of flavivirus replication including proteins involved in endocytosis, heparin sulfation and endoplasmic reticulum membrane complex (EMC) [103]. This study serves as a resource and paves the way for drug targeting and development. More recently, CIRPSR/Cas9 knock-out of viperin, an interferon stimulated gene (ISG) involved in innate immunity, further elucidated its role in Zika virus (ZIKV) infection [104]. ZIKV was originally isolated from a sentinel monkey in Uganda and belongs to the Flaviviridae family of positive-sense RNA viruses that includes dengue (DENV), West Nile (WNV) and Japanese encephalitis (JEV) [105]. Vanwalscappel and colleagues identified a TLR agonists with potent inhibition of ZIKV infection and identified enrichment of viperin upon treatment with the TLR agonist. Gene editing tools such as CRISPR/Cas9 may be used to target and induce antiviral host machinery to combat the infection.

2.4 Boosting Host Immunity with CRISPR/Cas9

Dysregulated immune responses are central to pathogenesis of various diseases and autoimmune disorders. Dysregulation could be a result of genetic mutation, pathogen-mediated suppression of effector immune responses or altered phenotype of cells in diseased

microenvironment. Devising CRISPR/Cas system to correct these genetic errors or enhancement of immune cell function by targeted modification can benefit humans against numerous diseases. Multiple studies have shown positive outcomes with CRISPR/Cas-mediated improvement of T cells that are central to adaptive immune response. While specific role in boosting antiviral T cell immunity has not been studied, evidences support CRISPR/Cas-based enhancement of T cell-mediated immunity in cancer immunotherapy.

To test CRISPR/Cas system-mediated enhancement of T cell function, Rupp et al. examined disruption of programmed cell death protein 1 (PD-1) in chimeric antigen receptor (CAR) T cells which are modified to detect specific tumor antigens [145]. Recognition of tumor cells by CAR T cells alone is not sufficient to clear cancerous cells due to the immunosuppressive tumor microenvironment and expression of inhibitory ligands, such as PD-L1, on both tumor cells and surrounding tissues. PD-L1 can interact with its ligand PD-1 on T cells and trigger immunosuppressive signals leading to T cell exhaustion and hypo-function. This scenario is frequently observed in chronic viral infections and tumor infiltrating lymphocytes in patients with advanced malignancy. Using a Cas9 RNP gene editing (targeting PDCD-1) and lentiviral transduction (encoding the anti-CD19 CAR) of primary human T cells, PDCD-1 gene was ablated while CD19 CAR expression was simultaneously achieved in primary human T cells. The genetically modified T cells (PD-1^{-}: CD19 CAR^{+}) when cultured with K562 tumor cells (CD19^{+}; PD-L1^{+}) displayed efficient at killing PD-L1^{+} tumor cells. The efficacy of PD-1^{-}: CD19 CAR^{+} T cells was assessed in vivo in NOD scid gamma (NSG) mice. Mice with adoptively transferred PD-1^{-}: CD19 CAR^{+} T cells, following subcutaneous injection of CD19^{+} PD-L1^{+} K562 cells and establishment of tumors, showed reduced tumor growth, increased survival and accelerated tumor clearance compared with controls. Overall, CRISPR-mediated targeted disruption of the Pdcd1 locus in human CAR T cells strongly enhance in vivo anti-tumor efficacy.

Eyquem *et al.* employed a different approach of targeted delivery of CD19-specific CAR to the T-cell receptor α constant (TRAC) locus using CRISPR/Cas system [146]. They demonstrated that CAR expression is uniform in T cells and these genetically modified cells are more potent as in their antitumor activity and outperform CAR T cell generated using conventional approach in a mouse model of acute lymphoblastic leukaemia (AML). For instance, markers of T cell exhaustion such as PD1, LAG3 and TIM3 were expressed at higher levels in conventional (retrovirally delivered) CAR compared to TRAC-CAR T cells and the former showed accelerated terminal differentiation further correlating with higher antitumor activity of TRAC-CAR T cells. It can be noted that *in vitro* functional studies did not reveal any notable differences between TRAC-encoded and randomly integrated 1928z, in terms of both cytotoxicity and T-cell proliferation in response to weekly stimulation with CD19^{+} antigen-presenting cells strongly suggesting that readouts of *in vitro* and *in vivo* studies may marked vary thereby signifying the need to perform in vivo experiments. These findings clearly show that targeted delivery of CAR using CRISPR/Cas can reduces tonic signalling, averts accelerated T-cell differentiation and exhaustion, and increases the therapeutic potency of engineered T cells in vivo.

In another study, a different strategy was employed to evaluate CRISPR/Cas-mediated genomic editing to reprogram human T cells by correcting a gene locus in a genetic disease

as well as selective targeting of cancer cell [147]. An electroporation was used to deliver crRNA and tracrRNA into T cells in order to generate genetically modified, highly viable, therapeutic grade T cells with no significant evidence of off-target integrations. The efficacy and targeted editing of the approach was tested on individuals (three siblings) with monogenic primary immune deficiency, an autoimmune disease caused by recessive loss-of-function mutations in the gene encoding the IL-2 alpha receptor (IL2RA), required for healthy regulatory T cells [148-149]. Two different mutations were identified on the ILRA of the subjects (1) c.530A>G, creates a premature stop codon and (2) c.800delA, causes a frameshift in the reading frame. Following restoration of genetic mutation, T cells were functionally assessed and active IL2 signaling was demonstrated by increased STAT5 phosphorylation upon IL-2 treatment, a hallmark of productive signaling. Furthermore, this CRISPR/Cas strategy was employed to assess in vivo anti-tumor function of T cells. A pair of T cell receptor (TCR) genes viz., TCR-β and TCR-α which recognizes the NY-ESO-1 tumor antigen was incorporated into the TRAC locus of polyclonal T cells isolated from healthy human donors [150]. Interestingly, it was observed that NY-ESO-1 TCR knock-in T cells preferentially localized to, persisted at, and proliferated in the tumor rather than the spleen. Adoptive transfer of sorted NY-ESO-1 TCR T cells also reduced the tumor burden in treated animals. These findings clearly support successful use of non-viral templates to harness potency of CRISPR/Cas genome editing capability in human health and disease. Most importantly, this methodology is cost-effective, absence of viral templates poses low risk of adverse impact on human health and is easy to adapt good manufacturing practices (GMP) for clinical use. However, a key limitation of using non-viral templates is low efficiency of genome editing. All of these studies strongly support that CRISPR/Cas can be utilized to boost T cell immunity and selectively targeting of cancer cells by T cells in vivo. Further investigations on long-term impact on the functions of genetically-modified T cells and how their phenotype is impacted in different individuals should be thoroughly examined. It will be important to assess CRISPR/Cas technology in boosting antiviral immunity with specific focus on CD8+ T cell and natural killer (NK) cell which are central to antiviral immunity and actively participate in clearing virus infected cells.

3. Targeting Viral Genomes

CRISPR/Cas9 has also been used to directly target and edit viral genome to impair its activity and eradicate it from the host (Table 2). While drugs targeting the virus, such as inhibitors that neutralize viral particles and nucleoside analogues that terminate viral replication have been developed, most have resulted in development of escape mutants from repeated use or ineffective against some viral strains. Using CRISPR/Cas9 to target one or multiple parts of the viral genome may be more effective in abolishing the virus from the host.

The use of CRISPR/Cas9 to directly mutate viral genomes and impair viral replication was demonstrated by multiple groups that targeted viral genetic elements important for both productive and latent herpesvirus infection [106-108]. Diemen el al. designed gRNAs targeting viral microRNAs BART5, BART6, and BART15 in latently infected gastric carcinoma cell line NSU-719 and showed direct editing of the EBV genome [106]. This study, along with others, showed clearance of latent viral infection in cell culture by

effectively targeting EBNA1, which is crucial for EBV functions and latent genome replication, with CRISPR/Cas9. Direct editing of viral genome was proved to be possible, allowing for continued efforts to be made in this direction.

Along the same lines, multiple gRNAs targeting essential genes of HSV-1 and HCMV also showed high efficacy in abrogating virus replication in vitro. Specifically, the use of double gRNAs showed complete blocking of HSV-1 replication and was suggested to be more effective in preventing the outgrowth of resistant strains. Other studies have shown efficient use of CRISPR/Cas9 to edit both essential and nonessential genes in the HSV genome and this in turn has accelerated our understanding of HSV [106,109]. gRNAs targeting UL7 gene, a tegument protein of HSV, were designed by Xu et al. in 2016 using CRISPR/Cas9 and found that the replication capacity of this mutant strain was 10-fold lower than wild-type HSV-1 [109]. Similarly, a different group showed high efficacy in directly targeting HSV-1 genome by modifying ICP0, an immediate early gene involved in HSV-1 gene expression and replication, with no off-target modifications [107]. In 2018, a group designed a multiplex antiviral CRISPR/Cas9 system targeting UL122/123 gene in the HCMV genome which are important for regulating lytic replication and reactivation from latency. This study showed effective targeting of the viral genome and abrogation of viral replication in primary fibroblasts and U-251 MG cells [110]. The authors also suspect reduction in viral reactivation from latency following the destruction of the IL122/123 HCMV gene.

Multiple studies have shown effective targeting of cccDNA genome of HBV by Cas9-induced double-stranded DNA break [111,112]. Seeger et al. designed several sgRNAs spanning the ENII/CP region and the PC of the HBV genome and showed effective CRISPR/Cas9 targeting of HBV cccDNA in HEPG2 cells expressing HBV receptor sodium taurocholate cotransporting polypeptide (NTCP). A different group showed the eradication of persistent HBV infection both in vitro, using Huh-7 cells transfected with an HBV-expression vector, and in vivo, using a hydrodynamics-HBV persistence mouse model, using CRISPR/Cas9 [113]. In vitro, reduced production of HBV core and surface proteins was observed using two out of the eight designed HBV-specific gRNAs. Reduced serum surface antigen levels were observed in vivo, further suggesting a robust role of CRISPR/Cas9 -mediated disruption of HBV infection. Soon after, multiple studies showed the complete eradication of HBV infection and elimination of persistent HBV genome, including full-length integrated HBV DNA and HBV cccDNA [113-121].

CRISPR/Cas9 was also used to directly target HIV-1 proviral genome and showed elimination from latently infected human T-cells, and therefore inhibition of HIV-1 reactivation, without off-target effects [122]. Whole genome sequencing verified CRISPR/Cas9 mediated-excision of provirus of two copies of HIV-1 by designing sgRNAs with specific DNA sequences positioned within the HIV-1 promoter spanning the 5’ long terminal sequence (LTR). In another approach to target latent HIV-1 provirus, viral reactivation was achieved using Cas9 -synergistic activation mediators (dCas9-SAM) targeting the enhancer of the HIV-1 LTR promoter. This was done in an effort to develop an additional “shock and kill” therapeutic strategy where following viral reaction, the clearing of the viral genomes is achieved by mechanisms of viral cytotoxicity or host immune defense. While this approach

suffers from limitations and requires more investigation, it proposes a unique therapeutic strategy with clinical significance [123].

In a different study, 10 sgRNAs were designed targeting different regions of the HIV-1 genome: LTR, *pol* gene, and the second exon of tat/rev [124]. The site in the second exon of Rev exhibited the highest level of mutation by Cas9 and as a result showed diminished levels of HIV-1 gene expression and virus production. Combined use of different gRNAs, which is predicted to diminish the change of HIV-1 escape mutants arising, targeting the viral genome at once showed reduced viral yields by more than 24-fold. Similarly, Wang and collogues further elucidate the impact of using combinatorial CRISPR/Cas9 as an antiviral to extinguish HIV-1 DNA without consequences such as escape mutants and resistance [125]. Most recently, Ophinni et al. demonstrated CRISPR/Cas9 transduction in 293T and HeLa cells using tat and rev-targeting regions of HIV-1 genome [126]. This study confirms no off-target effects as well as no effects on cell viability, further showing the therapeutic potential of CRISPR/Cas9 as a single-time intervention to clear viral genomic persistence and thus become a new cure for HIV-1.

Systemic removal of HIV from various cellular reservoirs is important for complete cure, however, during latency, viruses may reside in more than one cell or tissue. CRISPR/Cas technology has also been employed, both *in vivo* and *ex vivo*, to remove latent viruses (EBV, HSV-1, HCMV, etc.) including viruses that incorporate into host genome like HIV-1 [108]. While various studies have demonstrated successful use of CRISPR/Cas system to inhibit virus replication by targeting critical virus-encoded, efficient delivery of CRISPR/Cas components may vary *in vivo* and *ex vivo/in vitro* and this may impact the excision of desired pathogenic sequences hidden inside host cells. Kaminski *et al.* demonstrated CRISPR/Cas-mediated excision of HIV sequence in mice and rats infected with HIV-1 [143]. They utilized a recombinant adeno-associated virus (AAV) to express modified version of *Staphylococcusaureus* Cas9 (saCas9) and guide RNAs, targeting viral DNA sequences within the 5'-LTR and the Gag gene, for systemic delivery using tail vein (mice) or retroorbital inoculation (rats) in HIV infected animals. Deletion of target viral DNA region was noticed as well as reduced expression of viral transcripts indicating efficient systemic knockout of integrated viral genomes. The same research group further examined the feasibility and efficiency of HIV-1 excision using previously tested recombinant AAV-mediated delivery of saCas9 and a multiplex of sgRNA targeting various regions of HIV-genome editing in humanized bone marrow/liver/thymus (BLT) mice inoculated with HIV-1 and in mice during an acute infection of EcoHIV [144]. While targeting of LTR was sufficient to induce HIV genome excision, a combination of LTR and gal or pol targeting induces potent excision of viral genome suggesting multiple targeting is more efficient. However, further studies are required on possible off-target effects of CRISPR/Cas system in vivo and how this system can be effectively used in patients with other systemic illnesses. Additionally, the functional impact of CRISPR/Cas9 knockout on host cells should be thoroughly investigated. Whether the effector functions of these cells are similar to normal cells also demand a comprehensive analysis.

CRISPR/Cas9 has also successfully been used to edit the viral genomes of other viruses such as Vaccinia virus (VACV). JC polyomavirus (JCPyV) and human papillomavirus

(HPV). In VACV, CRISPR/Cas9 has been used to generate efficient site-specific mutations in the genome of the virus that can be employed as vaccine vectors against infectious diseases. Specifically, sgRNAs targeting N1L and A46R genes were constructed due to their role in virulence and regulating host immune response to VACV [127, 128]. Homologous recombination was efficiently induced (62.5% and 85% respectively) by the gRNA-guided Cas9, showing direct mutation of the viral genome. Another study utilized CRISPR/Cas9 to inhibit virus replication and viral protein expression of JCPyV, a member of the *Polyomaviridae* family, by targeting the noncoding control region and the late gene open reading frame of the JCPyV genome [129]. Similarly, a study using CRISPR/Cas9 to eliminate the oncogenic function of HPV constructed gRNAs targeting E6 and E7 genes which are essential for malignant phenotype of cervical cancer by inactivating tumor suppressor proteins p53 and pRb [130]. CRISPR/Cas9 has been applied to a variety of viruses and shown to be both versatile and effective in most cases.

4. Current limitations to the use of CRISPR/Cas9 as an antiviral

While the use of CRISPR/Cas9 as an antiviral has shown to be both effective and precise in various studies, limitations to its use still exist. Most of the published studies discussed in this review have been performed in cell-culture-based systems and, while showing high antiviral efficacy, lack convincing in vivo data from animal model studies. Although these studies give an indication regarding the application of the system, they lack various considerations and therefore fail to elucidate its therapeutic potential in humans. This includes ensuring safe and efficient delivery of Cas9 and gRNA.

The time of addition, dosage and delivery vehicle of the CRISPR materials are all critical to ensure proper use and exert the desired therapeutic benefits [131, 132]. Although various studies suggest the activity of Cas9 is highly precise and specific, studies have indicated unintended mutations resulting from the repair process of DNA breaks generated by CRISPR/Cas [133, 134]. Additionally, the development of CRISPR/Cas9-induced virus escape mutants and, as a result, the development of more pathogenic virus strains has been reported in recent studies. This has been resolved by the use of multiple gRNAs directed towards various sites of the genome but still presents itself as an issue in some cases [110, 136].

The consideration of viral vectors used for therapeutic delivery of Cas9:gRNA is also a key component to ensure success and efficiency [30, 137]. The large size of Cas9 proteins and delivery to specific cell types and tissues limits the vehicles that can be used. Lentiviruses induce long-term expression by integration into the target cell genome, exhibit high infection efficacy and have demonstrated safe delivery in vivo [138]. However; lentiviruses yield low virus titers and makes them ineffective in cases where large numbers of virally infected cells need to be targeted. Additionally, a high potential for insertional mutagenesis due to long-term and systematic expression has been observed [139]. In contrast, adeno-associated virus (AAV) is another common option that does not integrate into the genome, yields high viral titers and is capable of infecting various cell types. AAV is suitable in many cases, however, the relatively small viral genome size limits the DNA cargo that can be delivered to target cells [140]. The development of a safe and efficient delivery system is crucial for the success

of CRIPSR/Cas9 in clinics and targeting tissues or cells in human body still remains to be a challenge.

As we inch towards personalized gene editing and medicine, it is of utmost importance to identify and evaluate adverse effect of antiviral sgRNAs on host physiology and absolutely assuring that they do not impair genome integrity as well as transcriptome profiles. With these challenges resolved, CRISPR/Cas will be an attractive tool to eradicate numerous viral infections most of which remain without cure.

5. Conclusion

The recent advancements regarding gene editing technology has achieved broad application and progressed our knowledge in multiple research areas, including microbiology and immunology. Achieving precise genome editing in eukaryotic cells has long been studied with programmable endonucleases, such as ZFNs and TALENs, to interfere with certain genes. However, the feasibility and precision of these tools has been at question. CRISPR/Cas9 presents a more powerful platform with wide application, easier retargeting ability and reduced off-target events.

CRISPR/Cas9 has been widely used to target human genomic DNA and recently has been directed towards viral DNA in hopes of finding a cure for viruses such as herpesvirus, HBV, HIV and IAV. This review summarizes the application of CRISPR/Cas9 in abolishing these viruses from the host by directly targeting viral genomes or particular host genes that aid in viral replication and persistence. First and foremost, the goal is to identify host and virus genes that are indispensable for virus infection. Once these targets are ascertained, a major challenge will be to develop sgRNA that can specifically target gene-of-interest with no off-target effect. Another challenge is to thoroughly characterize sgRNA impact on cellular physiology both in vitro and in vivo. Using CRISPR/Cas9 in human as antiviral may be a near future reality, however, researchers must proceed with extreme caution regarding potential biological and ethical consequences.

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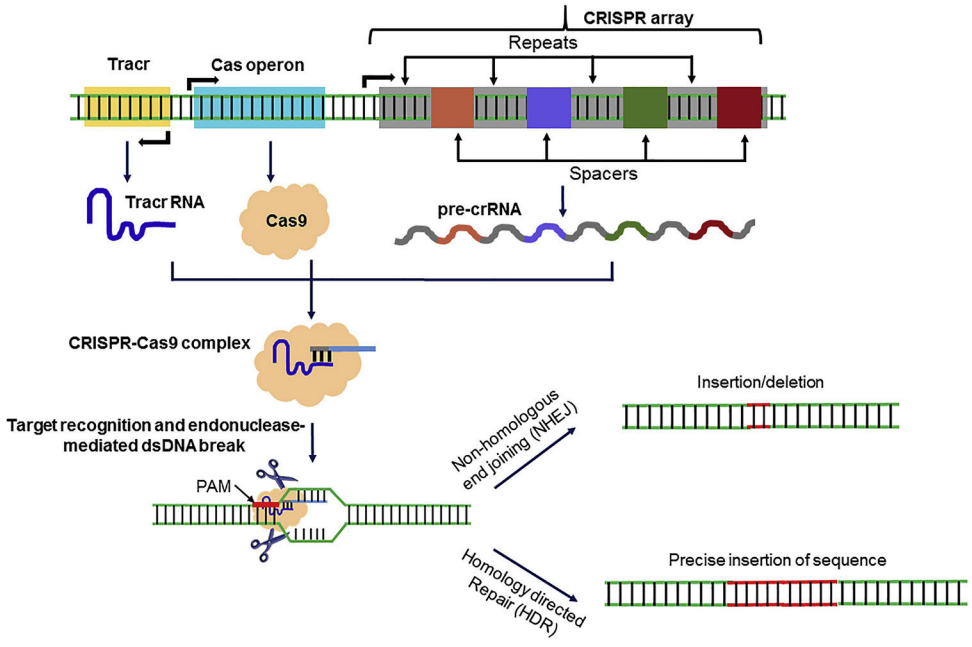

Figure 1.

Overview of guide RNA biogenesis and genomic targeting by CRISPR-Cas9. Key components of *Streptococcus pyogenes* CRISPR-Cas locus are shown. Tracr, Cas operon and CRISPR locus (containing clustered repeats and spacers) are transcribed to tracr RNA with stem loop structure, Cas9 endonuclease and pre-crRNA, respectively. RNase III then generate mature crRNA which is eventually incorporated into Cas9 along with tracr RNA. Guide RNA (gRNA) along with RNA-protein complex binds to the specific genomic locus with complementary sequence. Binding of guide RNA and target sequence is facilitated by protospacer adjacent motif (PAM). DNA is cleaved by Cas9 and other accompanying endonucleases thus generating double strand DNA breaks which are eventually repaired by non-homologous end joining (NHEJ) or by homology directed repair (HDR).

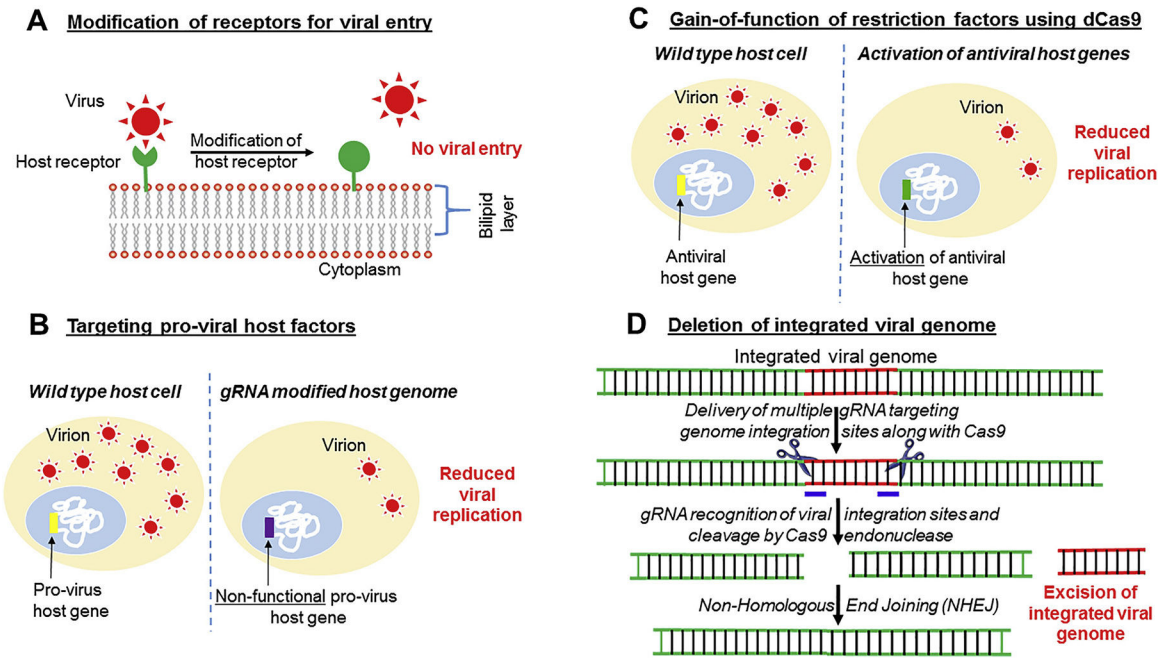

Figure 2. Schematic illustration of different antiviral strategies targeting host and viral genomes using CRISPR/Cas.

(A) Viral entry into host cell is generally mediated through direct interaction of viral and host surface proteins. Targeting host entry receptors that are required for virus to gain access to host cellular machinery can hamper viral spread and also interfere with the viral tropism. Editing host entry receptor motifs required for virus-host interaction or utilizing naturally selected mutations for genomic modification using CRISPR can prevent entry of virus into host cells.

Viral replication and virion packaging requires numerous host factors. Targeting pro-viral and anti-viral host factors can restrict viral spread. (B) Dependency on host factors is a key characteristic of viruses. Some host proteins facilitate virus survival and spread. Targeting such pro-virus host genes may prevent viral replication and render virus more susceptible to host antiviral responses. CRISPR/Cas mediated functional knock-down of such genes will inhibit viral replication or virion production. (C) Induced expression of host restriction factors. Transcriptional activation of host factors that can block viral replication is achieved by coupling gRNA to a catalytically inactive Cas9(dCas9). Upon targeting, host restriction factor expression is activated leading to reduced viral replication and/or viral RNA transcription.

(D) Targeting viruses that integrate their DNA in the host's genome. Integrated viral genome can be inactivated either by disruption of the essential viral genes or multiple sgRNAs can be targeted to the termini of the integrated viral DNA. Both strategies will lead to the reduced viral replication. Similar strategy can also be used to disrupt viral genes or fragmentation of viral genome of dsDNA genome of DNA viruses or RNA viruses with a non-integrated DNA intermediate.

Table 1:

List of **host** genes targeted by CRISPR(/Cas9) strategy and their impact on viral replication and persistence.

Virus	Viral infection model	Target Gene(s)	Functional impact	Reference
HIV	Various cell lines and primary CD4+ T cells	CCR5	Restriction of HIV-1 infection	[79,80,82]
	Mice transplanted with human $CD34^+$ HSPCs with *CCR5*-ablation treatment			
	Human CD4+ T cells	CXCR4	Restriction of HIV-1 infection	[83]
	HeLa and 293T cells	APOBEC3B and 3G	Increase expression of antiviral effectors	[88]
IAV	human lung carcinoma cell line A549; H1N1 (A/Puerto Rico/8/1934; PR8) and H3N2 (A/Hong Kong/1/1968; HK68)	SLC35A1	resistance against IAV infection due to inefficient binding of IAV particles to cell surface	[86]
		CIC, JAK2, PIAS2	CIC-deficient cells demonstrated upregulated antiviral gene expression and decreased replication of multiple viruses.	[86]
	293T and human lung carcinoma cell line A549; human IAV strain (A/Puerto Rico/8/1934 H1N1)	B4GALNT2	Prevented infection by multiple avian IAV strain	[87]
	human lung carcinoma cell line A549; human IAV strain (A/Puerto Rico/8/1934 H1N1)	p53	significantly reduced viral propagation	[92]

Table 2:

List of **viral** genes targeted by CRISPR/Cas9 strategy and their impact on viral replication and persistence.

Virus	Viral infection model	Target Gene(s)	Functional impact	Reference
HSV-1	Vero cells (4 gRNAs per target site); HSV-1-eGFP	essential viral genes (UL15; UL27; UL29; UL30; UL36; UL37; UL42; UL5; UL52; UL8; UL54; UL9)	Impaired HSV-1 replication	[106]
		nonessential viral genes (US3; US8)	Impaired HSV-1 replication to a lesser extent	
	Vero cells, HEK293T cells, and intranasal infection of BALB/b mice	UL7	Impaired HSV-1 replication	[109]
	HEK293 T and U-2 OS Cells; HSV-1 KOS and GFP strain	ICP0	Modification of ICP0 gene with no off target effects	[107]
HCMV	MRC5 cells (4 gRNAs per target site);	essential viral genes (UL54; UL44; UL57; UL70; UL105; UL86; UL84)	Impaired HCMV replication	[106]
	HCMV strain TB40/E or AD169	nonessential viral genes (US6; US7; US11)	No impact on HCMV replication	
	MRC5 and U-251 MG cells; HCMV strains: TB40GFP, Toledo and VR1814	UL122/123	Impaired HCMV replication	[110]
EBV	Latently infected SNU-719 tumor cells	miRNA BART5, BART6 or BART 16	Enhanced expression of miRNA target reporter	[106]
	Akata-Bx1 cells carrying recombinant EBV expressing eGFP (2 gRNAs per target site)	EBNA1	~50% loss in GFP expressing cells with single gRNA and ~90% loss with two gRNAs targeting different sites on the viral genome	
		OriP (multiple sites)		
		EBNA1+ OriP		
	Raji cells: Burkitt's lymphoma cell line with latent EBV infection	EBNA1	EBV genome content was absent or reduced, cell proliferation arrest and apoptosis	[108]
		OriP LMP1		
HBV	NTCP expressing HepG2 cells	Targeting cccDNA via: ENII/CP and Pre-C ORFs	Inhibition of HBV infection by 8-fold	[112]
	Huh-7 cells	HBV-specific gRNAs P1, S1, XCp, PS2,	reduced HBV protein production	[113]
	HBV hydrodynamics-mouse model	HBV- specific gRNAs P1 and XCp	reduced serum levels of HBsAg	
	Huh-7 and HepG2.2.15 cells	X/L and X ORFs	Reduced HBsAg and HBeAg level in cell culture medium and intracellular cccDNA Reduced levels of HBsAg and HBeAg level in serum and intrahepatic cccDNA	[121,141]
	Hydrodynamic-mouse model			
	Huh-7, HepAD38, HepaRG, HepG2 cells	P, S, X and C ORFs	Reduced levels of HBV antigens	[114-117,119,141]
	Hydrodynamic-mouse model		Reduced serum levels of HBsAg and viral DNA	[117,119,141]
HIV	Primary CD4+ T cells, Jurkat cells, SupT1 T cells, HEK 293T cells	long terminal repeat (LTR) promoter region	Disruption and excision of the latent provirus from human cells	[122,125,142]
	JLat10.6 HIV-latent cell line, HEK293T cells, HeLa cells	LTR, *pol* gene and second exon of *tat/rev*	*Tat/rev* target gene mutated with high frequency and no off target effects	[124,126]
VACV		N1L and A46R genes	Efficient targeting of viral genes	[128]

Virus	Viral infection model	Target Gene(s)	Functional impact	Reference
JCPyV	SVG-A cells; JCPyV-MAD1 virus	non-coding control region (NCCR), Capsid proteins VP1 and VP2	Inhibition of viral replication	[129]
HPV	Cervical HPV-16-positive cell line SiHa	E6, E7 genes, promoter of E6/E7	Reduction in proliferation of cervical cancer cells in vitro and inhibition of tumorigenicity in xenograft studies	(130)