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Article

Using CRISPR technology to inhibit the replication of human cytomegalovirus by deletion of a gene promoter

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SUMMARY

Human cytomegalovirus (HCMV) causes serious infections in immunocompromised patients. It is ubiquitous in nature and has the potential to be transmitted via non-sexual contact (saliva, breast-feeding, placental transfer, blood transfusion, organ transplantation) or sexual contact. Although HCMV infection resolves spontaneously in immunocompetent hosts, it causes serious life-threatening disease in immunocompromised patients and can reactivate from latency at any time during life when the host's immune system is compromised. Management of these infections is complex as currently available antiviral agents have several limitations, and thus mortality of these infections approaches 50%. Reactivation of latent HCMV remains a major concern and is associated with a high morbidity and mortality in high-risk immunocompromised adults. Although great progress has been made in the management of HCMV disease over the last several decades, management options are still limited due to major issues with dosing and side effects. Also, none of these medications inhibit latent HCMV lying dormant in cells. Gene-based targeting mechanisms have the added advantages of inhibiting not only the reactivated HCMV but also the latent HCMV in cells, as well as stopping the dysregulation of innate immunity occurring during the early stages of HCMV infection. Using CRISPR/Cas9 technology/synthetic single gRNA, site-specific cleavage was carried out with successful deletion of a major intermediate early gene promoter/enhancer (MIEP/E) of HCMV. This study demonstrated the efficacy of the sgRNA-CRISPR/Cas9 strategy to delete the MIEP/E of HCMV. Deletion of this promoter is expected to block the expression of early and late phase genes, inhibit HCMV replication, prevent latency, and protect the cellular immune system, in immunocompromised patients.

INTRODUCTION

Human cytomegalovirus (HCMV), also known as human herpesvirus 5, belongs to the family of *Beta-Herpesviridae*, a subfamily of the *Herpesviridae* family. Management of these infections is complex as currently available antiviral agents have several limitations, and mortality of these infections approaches 50% (1-3). HCMV is ubiquitous and infects most people prior to their adulthood. However, it can reactivate from latency at any time during life when the host's immunity is compromised. As shown in **Figure 1**, the HCMV virion is composed of a lipid envelope containing viral glycoproteins, a tegument layer consisting mainly of viral phosphoproteins, and an icosahedral protein capsid that encloses the HCMV viral genome (1).

The HCMV genome is comprised of ~235,000 base pairs making up a double-stranded DNA strand in a single chromosome. As shown in **Figure 1**, the HCMV virion is composed of a lipid envelope containing viral glycoproteins, a tegument layer consisting mainly of viral phosphoproteins, and an icosahedral protein capsid that encloses the HCMV viral genome (1). The HCMV genome is comprised of ~235,000 base pairs making up a double-stranded DNA strand in a single chromosome. Being a virus, HCMV needs a host for cellular replication and survival (3). Most HCMV infections occur in childhood, with a seroprevalence rate of 60% in developed countries and close to 90% in third world countries. HCMV is ubiquitous in nature and has the potential to be transmitted via non-sexual contact (saliva, breast-feeding, placental transfer, blood transfusion, organ transplantation) or sexual contact. Although HCMV infection resolves spontaneously in immunocompetent hosts, it causes serious life-threatening disease in immunocompromised patients (4). High risk patients include those on long-term corticosteroids, immune-modulating agents, cancer chemotherapy, those who are positive for the human immunodeficiency virus, hematopoietic stem cell transplant/solid organ transplant recipients, and neonates (5).

Gene based targeting mechanisms have an advantage of inhibiting not only the reactivated HCMV but also latent HCMV dormant in cells. Targeting early HCMV proteins is a viable option to target both reactivated and latent HCMV by blocking viral replication. However, these early phase genes have multiple open reading frames (ORFs). The early

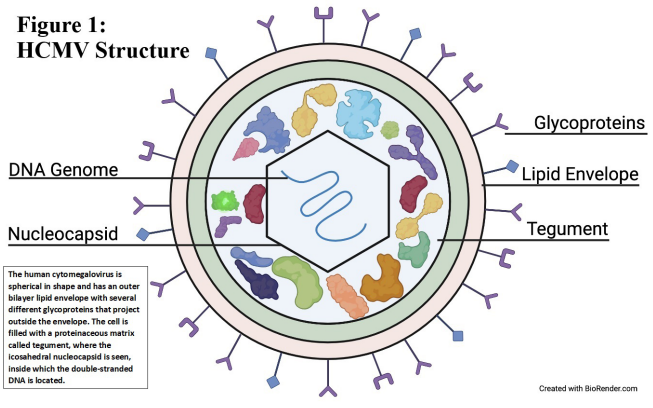

Figure 1: HCMV Structure. Created with BioRender.com.

genes involved in replication (IE 1 and 2, E and L) are under the control of a promoter and enhancer called the major immediate early promoter/enhancer (MIEP/E).

This promoter is downregulated during establishment of latency and is derepressed during reactivation of HCMV (6, 7). Hence, our hypothesis is that silencing the major immediate early gene promoter/enhancer (MIEP/E) would prevent HCMV replication by targeting the transcription and translation of early genes involved in HCMV replication. As immune suppressive effects of HCMV happen during early stages of infection, deletion of early promoter could prevent the suppressive effects of HCMV on the host innate immunity. Most importantly, it could eradicate latency/dormancy of HCMV within cells (8, 9).

A novel technology in gene deletion and transfer called "Clustered Regularly Interspaced Short Palindromic Repeats" (CRISPR) is a major breakthrough in the field of molecular biology and genetics (10-12). As the name indicates, CRISPR refers to small sequences in the genome of an organism (human, bacteria, fungi, or virus) that can be targets of bacterial nuclease enzymes to create DNA breaks. This system operates routinely in organisms to protect against invasion from foreign organisms. One of the CRISPR associated proteins called Cas9 is an endonuclease, with the ability to cut both strands of DNA. This endonuclease is directed to its host target by a segment of a synthesized single strand RNA called a single guide RNA (sgRNA). The segment of RNA that binds to the host genomic DNA is usually 18–20 nucleotides long. To ensure that the double stranded DNA is cut at the correct spot, a specific sequence of DNA called the protospacer adjacent motif (PAM) (2 to 5 nucleotides long and specific to the organism that produces the Cas9) must lie at the 3' end of the sgRNA. Once the DNA cut is completed, repair may occur either by non-homologous end joining (NHEJ), or homology directed repair (homologous piece of DNA is used as a repair template). Homology directed repair is preferred as it allows for precise genome editing. This technology has been used previously to target IE of HCMV, but required targeting multiple open reading frames (ORFs) and hence was complicated by the need for a complex multiplex strategy to make the targeting gene deletion more efficient (10). In our pilot study, a single plex CRISPR/Cas9 sgRNA technique was used as shown in **Figure 2**.

Targeting HCMV is not the first medical use of CRISPR technology. There are several other uses of CRISPR technology currently being explored, such as deletion of genes in cancers (acute myeloid leukemia; *BCR-ABL* gene), retinoblastoma (*RB1* gene), and multidrug resistant bacterial infections (*Mdr* genes). Using this technology, lentiviral vectors or plasmids could induce dominant negative mutations, premature stop codons, or delete genes in bacteria, fungi, and viruses (11-13).

Our hypothesis was tested by utilizing this CRISPR technology. An anti-HCMV replication CRISPR/Cas9 strategy was utilized to target the MIEP/E promoter/enhancer gene, which is known to control all the early genes involved in HCMV replication and reactivation from latency. Using CRISPR technology, site-specific cleavage was carried out using sgRNA and a 930bp segment of MIEP/E was targeted for deletion from HCMV. The nucleotide, RNA and protein sequences of the targeted MIEP/E shown below as sequences 1, 2 and 3.

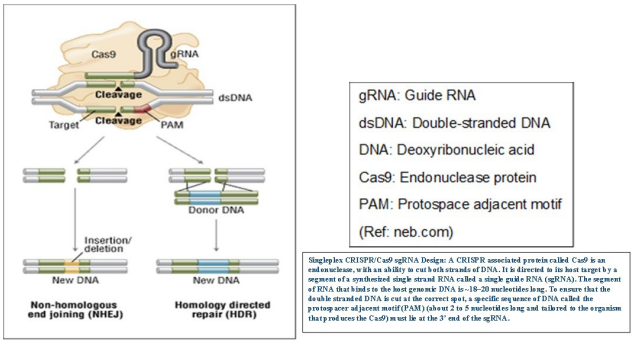

Figure 2: A Model of HCMV CRISPR-Cas9 System.

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   1 aatcaatatt ggccattagc catattattc attggttata tagcataaat caatattggc
  61 tattggccat tgcatacgtt gtatccatat cataatatgt acatttatat tggctcatgt
 121 ccaacattac cgccatgttg acattgatta ttgactagtt attaatagta atcaattacg
 181 gggtcattag ttcatagccc atatatggag ttccgcgtta cataacttac ggtaaatggc
 241 ccgcctggct gaccgcccaa cgacccccgc ccattgacgt caataatgac gtatgttccc
 301 atagtaacgc caatagggac tttccattga cgtcaatggg tggagtattt acggtaaact
 361 gcccacttgg cagtacatca agtgtatcat atgccaagta cgcccccctat tgacgtcaat
 421 gacggtaaat ggcccgcctg gcattatgcc cagtacatga ccttatggga ctttcctact
 481 tggcagtaca tctacgtatt agtcatcgct attaccatgg tgatgcggtt ttggcagtac
 541 atcaatgggc gtggatagcg gtttgactca cggggatttc caagtctcca ccccattgac
 601 gtcaatggga gtttgttttg gcaccaaaat caacgggact ttccaaaatg tcgtaacaac
 661 tccgccccat tgacgcaaat gggcggtagg cgtgtacggt gggaggtcta tataagcaga
 721 gctcgtttag tgaaccgtca gatcgcctgg agacgccatc cacgctgttt tgacctccat
 781 agaagacacc gggaccgatc cagcctccgc ggccgggaac ggtgcattgg aacgcggatt
 841 ccccgtgcca agagtgacgt aagtaccgcc tatagagtct ataggcccac cccttggct
 901 tcttatgcat gctatactgt ttttggcttg
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Sequence 1: Human cytomegalovirus major immediate-early gene, enhancer/promoter sequence. The sequence shown above is a 930bp nucleotide sequence (GenBank: K03104.1) of the MIEP/E gene from HCMV-HHV5 strain AD169. Blue shaded area depicts the highly conserved regulatory region of MIE/P. Yellow shaded area depicts the splice donor signal area of MIE/P.

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AAUCAAUAUUGGCCAUUAGCCAUAUUAUUCAUUGGUUAUAUAGCAUAAAUCAAUAUUGGCUAUUGGCCAUUG
CAUACGUUGUAUCCAUAUCAUAAUAUGUACAUUUAUAUUGGCUCAUGUCCAACAUUACCGCCAUGUUGACAUU
GAUUAUUGACUAGUUAUUAAUAGUAAUCAAUUACGGGGUCAUUAGUUCAUAGCCCAUAUAUGGAGUUCCGCG
UUACAUAACUUACGGUAAAUGGCCCGCCUGGCUGACCGCCCAACGACCCCCGCCCAUUGACGUCAAUAAUGAC
GUAUGUUCCCAUAGUAACGCCAAUAGGGACUUUCCAUUGACGUCAAUGGGUGGAGUAUUUACGGUAAACUGCC
CACUUGGCAGUACAUCAAGUGUAUCAUAUGCCAAGUACGCCCCCUAUUGACGUCAAUGACGGUAAAUGGCCCG
CCUGGCAUUAUGCCCAGUACAUGACCUUAUGGGACUUUCCUACUUGGCAGUACAUCUACGUAUUAGUCAUCGC
UAUUACCAUGGUGAUGCGGUUUUGGCAGUACAUCAAUGGGCGUGGAUAGCGGUUUGACUCACGGGGAUUUCC
AAGUCUCCACCCCAUUGACGUCAAUGGGAGUUUGUUUUGGCACCAAAAUCAACGGGACUUUCCAAAAUGUCGU
AACAACUCCGCCCCAUUGACGCAAAUGGGCGGUAGGCGUGUACGGUGGGAGGUCUAUAUAAGCAGAGCUCGUU
UAGUGAACCGUCAGAUCCGCCUGGAGACGCCAUCCACGCUGUUUUGACCUCCAUAGAAGACACCGGGACCGAUC
CAGCCUCCGCGGCCGGGAACGGUGCAUUGGAACGCGGAUUCCCCGUGCCAAGAGUGACGUAAGUACCGCCUAU
AGAGUCUAUAGGCCCACCCCCUUGGCUUCUUAUGCAUGCUAUACUGUUUUUGGCUUG
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Sequence 2: MIEP/E RNA sequence of HCMV. The RNA sequence above has been shown to encode several early proteins during HCMV replication in host. These proteins are involved in HCMV infection related production of proviral and antiviral inflammatory cytokines. RNA sequence of the highly conserved region of MIEP/E is highlighted in yellow above.

```
NQYWPLAILFIGYIA.INIGYWPLHTLYPYHNMYIYIGSCPTLPPC.H.LLTSY...SITGSLVHSPYMEFRVT.LTVNGPPG.PPN
DPRPLTSIMTYVPIVTPIGTFH.RQWVEYLR.TAHLAVHQVYHMPSTPPIDVNDGKWPAWHYAQYMTLWDFPTWQYIYV
LVIAITMVMRFWQYINGRG.RFDSRGFPSLHPIDVNGSLFWHQNQRDFPKCRNNSAPLTQMGGRRVRWEVYISRARLVN
RQIAWRRHPRCFDLHRRHRDRSSLRGRERCIGTRIPRAKSDVSTAYRVYRPTPLASYACYTVFGL
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Sequence 3: MIEP/E protein sequence of HCMV. Protein coding sequence above has been shown to encode several early proteins during HCMV replication in host. These proteins are involved in HCMV infection related production of proviral and antiviral inflammatory cytokines.

RESULTS

We performed PCR studies to confirm the presence of MIEP/E in HCMV-infected MRC5 primary human fibroblast cells as shown in **Figure 3**. We then designed a CRISPR/Cas9 system to target the most highly conserved region of the HCMV MIEP/E gene as shown above as sequences 1, 2 and 3. We transduced HCMV-infected human MRC5 fibroblasts with a lentiviral vector containing sgRNA targeting the MIEP/E gene. We performed PCR studies to assess the success of MIEP/E gene deletion using primers seen in **Table 1**. The single plex CRISPR/Cas9 system could be utilized to delete HCMV MIEP/E efficiently. We confirmed deletion of the target using standard PCR amplification as shown in **Figure 4**. Also, ~ 100 upstream and downstream sequences were included for PCR amplification that demonstrated that there were no off-target deletions, although it does not confirm the absence of off-target indels (insertions-deletions) beyond those sequences. A total of 6 samples were tested (one control and 5 test samples) following single plex CRISPR-Cas9 sgRNA incubation with MRC5 primary human fibroblast cells. Our experiments were performed using a NHEJ technique, and deletion was confirmed in 5 out of 6 (80%) of samples tested.

DISCUSSION

This study demonstrated the efficacy of the sgRNA-CRISPR/Cas9 strategy to delete the MIEP/E of HCMV. MIEP/E is the promoter/enhancer for early and late phase genes involved in HCMV replication, for maintaining HCMV latency in cells and regulates proteins involved in suppression of intrinsic and innate cell mediated immunity. Hence, deletion of this promoter is expected to block the expression of early and late phase genes, inhibit HCMV replication, prevent latency, and protect the cellular immune system.

Our experiment is an in vitro proof of concept pilot study

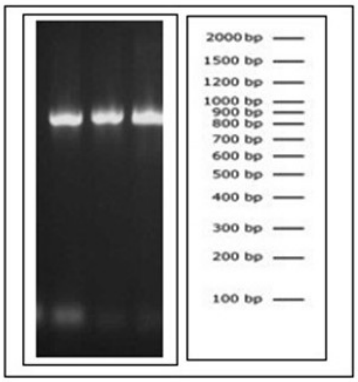

Figure 3: Agarose Gel electrophoresis of PCR products (n=3): MIEP/E (800-900 base pair segment). PCR amplification of HCMV-infected MRC5 primary human fibroblast cells confirmed the presence of MIEP/E, shown (3 samples were tested).

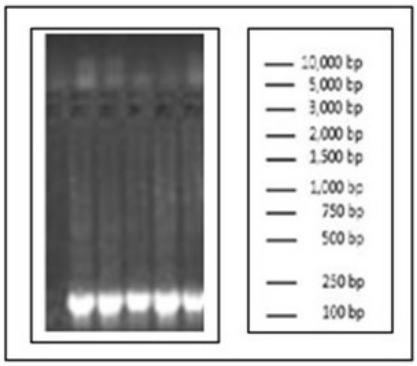

Figure 4: Agarose Gel electrophoresis of PCR products (n=5): post deletion of MIEP/I using the CRISPR-Cas9 technology. Please note absence of the 800-900 base pair segment of the MIEP/E.

that confirms our hypothesis. Our findings need to be further confirmed by experiments to study the growth and replication capacity of the transformed HCMV using standard culture and plaque reduction methods. The level of expression of early and late genes also needs evaluation using qRT-PCR experiments (for upregulation and/or quantification of genes) and western blot technique for protein identification.

Although great progress has been made in the management of HCMV disease over the last several decades, management options are still limited. Currently available therapeutic agents are DNA polymerase inhibitors namely ganciclovir, valganciclovir, foscarnet, and cidofovir. These agents are associated with severe toxicities such as bone marrow suppression, renal failure, drug to drug interactions, poor oral absorption and suboptimal pharmacokinetics and pharmacodynamics (14-16).

Agents recently added to the anti HCMV drug armamentarium are maribavir and letermovir. These drugs act on terminal DNA processing and inhibit DNA synthesis. Despite timely diagnosis and treatment, the management of HCMV disease continues to be a challenge and is associated with a high mortality rate (3, 4). The main reasons behind a poor outcome are attributed to emergence of cross-resistance to these agents, inadequate drug levels at target site, ineffective killing of latent HCMV in host cells, and poor host immunity. In fact, HCMV further decreases cell-mediated immunity in patients via direct effects on human T cells. Emerging drug resistance due to mutations in their target genes has emerged resulting in cross-resistance among drugs with similar mechanism of action or from the same class Another significant fact is that none of these agents inhibit latent HCMV that is dormant in cells. Importantly, these agents are currently available only in first world countries and are out of reach for third world countries (17).

Ongoing research to find better therapeutic agents for

Name	Sequence	Start Position	Strand	Length	Primer Tm	Purity	Modification	Scale
Primer Set: Amplicon Size = 274								
Forward	TGACGTCAATGGGAGTTTGT	597	forward	20	54.42	PAGE		0.05 µmol
Reverse	GGCGGTACTTACGTCACTCT	851	reverse	20	56.29	PAGE		0.05 µmol

Table 1: Primers used for amplification of MIEP/E by PCR.

HCMV and screening of a large library of bioactive molecules have discovered drugs that target different pathways. Some of these drugs had already been FDA approved but were repurposed and tested for anti-HCMV activity. Most importantly, agents that block immediate early gene expression appeared promising. These include nitazoxanide (an antiparasitic agent currently in antiviral clinical trials), deguelin, thioguanosine, and alexidine dihydrochloride that demonstrated good efficacy against HCMV with a low toxicity profile. Interestingly these repurposed agents did demonstrate inhibition of the early gene (E) of HCMV, but lack experimental data (18).

The unique feature of HCMV is its ability to establish latent infection in the host cells. The exact mechanism behind its dormancy and reactivation in the host continues to be an enigma. Review of literature on HCMV pathophysiology reveals that once HCMV invades the host cell, it harnesses RNA polymerase II, resulting in a highly complex transcriptome with mRNAs that have more than 700 open reading frames (ORF). There are 3 major genes that are expressed during the early replication stages of HCMV within the cell. These are the IE, E and L genes. The resulting proteins released during this early phase inhibit cell-mediated immunity causing further immunosuppression in the host (18). Deletion of the E gene using siRNA technology prevented DNA damage response signaling early after HCMV infection in vitro (19).

The question that follows gene deletion is the need for either homology-directed repair versus NHEJ in INDELmutants. It has already been demonstrated that NHEJ-mediated INDEL mutation of the HCMV genome occurs at very high efficiency and could therefore be a helpful tool for INDEL-mediated targeting of the HCMV genome (20). Deletion or replacement of one of these genes failed to completely block HCMV replication as there was overexpression of other 2 genes that permitted continuous HCMV replication *in vitro*. However, deletion or replacement of all three early genes was shown to block HCMV replication. A recent study using CRISPR technology showed that deletion of all the 3 genes required a multiplex strategy, was a complicated process, and associated with poor efficacy and accuracy (21).

Some of the strengths of this study include the use of MIEP/E as the target for the single plex CRISPR/Cas9 strategy, to silence several virulent genes, proper identification of the PAM region that results in high precision gene deletion without deleting the off-target genes, the short regulatory nucleotide/RNA sequence of MIEP/E that ensures enhanced deletion efficiency and the single plex strategy which is relatively simple using single stranded guided RNA. There are several limitations of this study. A confirmation by testing growth and replication capacity of HCMV under culture conditions using plaque reduction studies was not performed. Also, only 5 samples were tested to confirm deletion of MIE/P. More samples need to be tested to ensure the efficiency of gene deletion and check for potential upstream and downstream off-target gene deletions. There remains a possibility of other promoters being upregulated during deletion experiments in culture systems. Most importantly, the efficiency of this strategy needs to be determined with a large inoculum of cells to determine the appropriate dosage of donor sgRNA needed to achieve maximum efficiency and accuracy. There is a likelihood that other unstudied genes may result in HCMV reactivation despite deletion of MIEP/E gene.

The next step would be to perform *in vivo* experiments in a mouse model for which the appropriate dose of the CRISPR genes (Cas9, sgRNA) need to be validated. Site specific recognition of the cleavage site on DNA and efficient deletion or insertion need to be precise and efficient. Most importantly, off target mutations must be avoided as they may affect other proteins and cellular functions with deleterious effects. Nevertheless, deletion of this promoter is expected to block the expression of early and late phase genes, inhibit HCMV replication, prevent latency, protect the cellular immune system, and improve morbidity and mortality in immunocompromised patients.

MATERIALS AND METHODS

sgRNA Selection and Design

The CRISPR/Cas9 system used in our study was designed by our team based on above knowledge of its structure and function. The sgRNA was designed using the single plex strategy which ensured that it included the highly conserved regulatory region was included in the 930bp MIEP/E segment to be edited in this experiment. The single gRNA was designed to target the regulatory region of MIEP/E. The RNA sequence of the MIEPE/I was entered in the CRISPR design software and all possible off-site targets were noted. Alignment of highly conserved domains of several HCMV MIEP/E sequences was done using the NCBI database. Gene sequences of different strains of HCMV that shared more than 99% homology were further evaluated by the software. The HCMV strain that had the gene sequence with the highest selectivity score and lowest off-site target score was selected for sgRNA synthesis. Homologous HCMV strains' highly conserved region of the MIEP/E and their alignment sequences were obtained through nucleotide and BLAST search programs (BLASTx, BLASTn, BLASTp), by accessing the NLM-NCBI-Genbank database, that are presented as sequences 1, 2 and 3. Specific donor HCMV target sequences of the regulatory region, and the splice donor enhancer region were provided. All genetic data pertaining to the MIEP/E of HCMV-HH5, including gene sequences of nucleotide, RNA, and protein, as shown as sequences 1, 2 and 3, were provided to the Sigma Aldrich CRISPR laboratory. Instructions regarding methodology and single plex strategy were provided to the Sigma Aldrich CRISPR laboratory where sgRNA was designed using their standard protocol as follows.

MRC5 Cell Culture and Transduction

MRC5 cells (fibroblasts) were grown in DMEM (Dulbecco's Modified Eagle Medium) with 2% Penicillin-streptomycin antibiotics and 2mM L-glutathione. These cells were then cultured with a lentiviral type I vector with the sgRNA and Cas9 to allow for transduction to occur. This was carried out in DMEM medium at a 1:100 concentration of MRC5 cells and lentiviral vector, followed by a 2-step selection process with 2µg/ml and 0.5µg/ml of puromycin. Stock solutions were prepared using supernatant for further use.

Gene deletion and PCR

For the MIEP/E deletion procedure, HCMV-infected MRC5 primary human fibroblast cells (MIEP/E confirmed by PCR analysis as shown in **Figure 3**; three samples were tested), were transduced with sgRNA targeting MIEP/E ,

forming the CRISPR/Cas9 system (obtained from Sigma-Aldrich). These cells (6 samples) were then suspended in DMEM at 1 x 10^5 cells/ml and were cultured in a microtiter plate, with one well as a control with only MRC5 cells. The plate was then incubated at 33°C for 2 hours to allow for the CRISPR-based gene deletion to occur allowing for NHEJ. Cells from the plates were picked with a toothpick and re-suspended in DMEM. A total of five samples were selected. This was followed by HCMV genome sequencing of MIEP/E using standard PCR techniques using primers shown in table 1. [The PCR cycle was as follows: 1 cycle of denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min 30 s; and 1 cycle of elongation at 72°C for 7 min].

No ethics or IRB approval was required for this study.

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