

## UJEMI PEARLS

# Human Cytomegalovirus: The Implications of MicroRNAs in Latency and Therapeutics

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**SUMMARY** Human cytomegalovirus (HCMV) infects the majority of the world's population. Primary infection is generally asymptomatic in healthy individuals and as with all herpesviruses, a lifelong latency associated with periodical reactivations is established. It is a common congenitally acquired infection and cause of congenital neurological disease. In immunocompromised individuals, such as HIV/AIDS patients and organ transplant recipients, HCMV poses a high risk of severe disease and death. A major cause of opportunistic infections in these individuals, HCMV is able to spread and damage multiple organs by direct cytopathic effects. Due to limitations associated with current therapeutics there is a need for the development of novel therapeutics, particularly for these individuals. MicroRNAs (miRNAs) act as important post-transcriptional regulators for many normal cellular processes in many organisms. In recent years miRNAs have also been identified in a number of double-stranded DNA viruses, including herpesviruses, and have been implicated in the regulation of viral and host genes. As has been observed for a number of other herpesviruses, viral miRNAs are expressed during both lytic infection and latency for HCMV. How HCMV establishes and maintains latency is not fully understood. While studies have clarified some of the roles of miRNAs for lytic HCMV infection, their roles during latent infection are not as defined. This article will provide an overview of what is currently known about HCMV latency, the possible roles of some key host and viral miRNAs in HCMV latency, and a novel therapeutic approach for targeting HCMV latency along with current obstacles to this approach. Understanding how miRNAs contribute to the lifecycle of HCMV and its pathogenesis may change how we approach therapeutics, particularly for organ transplant recipients at risk for severe HCMV-mediated disease.

## INTRODUCTION

Herpesviruses are enveloped DNA viruses that are divided into three subfamilies, with HCMV belonging to the  $\beta$ -herpesviruses and being one of nine herpesviruses having been identified to infect humans (Fig. 1) [1, 2]. The ability to establish lifelong latent infections is a key feature of herpesviruses [1]. HCMV infects greater than 50% of the human population, with seroprevalence increasing with age [3, 4]. Transmission of HCMV can occur through bodily fluids such as saliva, sperm, and urine, breastfeeding, hematopoietic stem cell transplantation, blood transfusion, solid-organ transplantation, or through placental transmission [5]. Primary infection and reactivation from latency are usually not problematic for healthy individuals (Fig. 2). For neonates, HCMV is a major cause of birth defects and is the leading infectious cause of congenital neurological disease [6]. Both primary infection and reactivation from latency pose high risk for severe disease in the immunocompromised, particularly in organ transplant recipients (Fig. 2) [7, 8]. Graft-versus-host-disease (GvHD), the use of immunosuppressants such as lymphocyte-depleting antibodies, and the serostatus of the organ donor and recipient are some of the main risk factors for organ transplant recipients [8]. Transmission of HCMV from the transplanted organ into a seronegative recipient, reactivation from latency in a seropositive recipient, or primary infection in a seronegative recipient can all result in severe invasive disease [7]. Viral syndrome characterized by fever, leukopenia, thrombocytopenia, and elevated liver enzymes is the most common clinical presentation [7]. Respiratory symptoms, clinical

**Published Online:** September 2020

**Citation:** Harvir Grewal. 2019. Human Cytomegalovirus: The Implications of MicroRNAs in Latency and Therapeutics. UJEMI PEARLS 4:1-9

**Editor:** François Jean (Ph.D.), University of British Columbia

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hepatitis, meningoencephalitis, and other severe presentations can also occur due to widespread organ damage, including death [7].

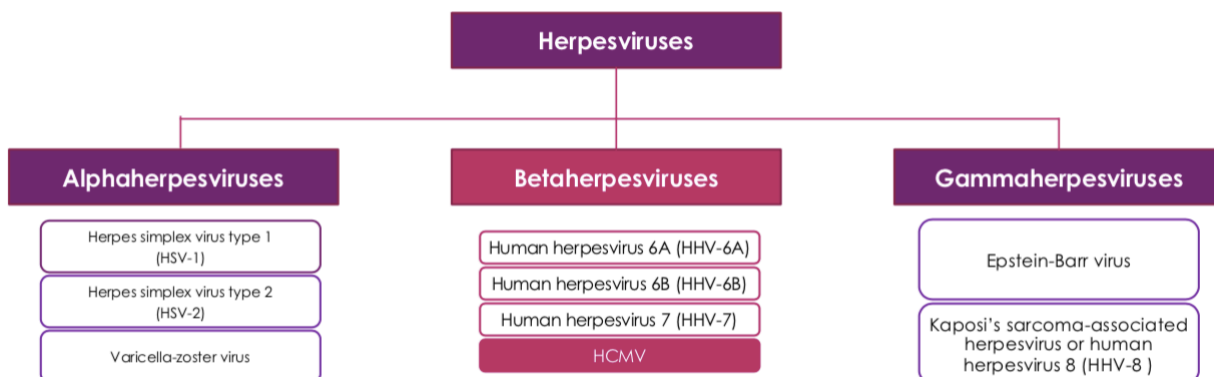
Both  $\alpha$  and  $\gamma$  herpesviruses have a highly restricted tropism [9]. HCMV, on the other hand, is able to infect and replicate in a wide variety of cells [10]. Primary infection typically starts with replication in the mucosal epithelium [11]. Lytic infection involves a temporally-controlled cascade of gene expression divided into three phases: immediate early (IE), early (E), and late (L) gene expression [12]. IE genes are involved in the evasion of intrinsic and innate immune responses, viral replication, and expression of transcription factors necessary for E gene expression [12, 13]. E gene expression initiates viral genome replication and L gene expression initiates capsid assembly in the nucleus, after which egress to the cytosol occurs [14]. Virion release involves exocytosis at the plasma membrane [11]. Viral dissemination to cells of the myeloid lineage ultimately occurs, which is believed to be the primary site for HCMV latency [4, 15].

miRNAs are small non-coding RNAs that bind messenger RNAs (mRNAs) to regulate their activity as important post-transcriptional regulators for many normal cellular processes in many organisms, including several double-stranded viruses that replicate in the nucleus [16]. Regulation is accomplished through translational inhibition or degradation, with binding of miRNAs usually occurring at sites within the 3' untranslated regions (UTRs) of mRNA [16, 17]. The majority of viral miRNAs identified to date have been in herpesviruses, with latently-expressed miRNAs involved in latency for a number of herpesviruses [16, 18]. The role of miRNAs in the lifecycle of HCMV is less understood than the role of HCMV-encoded proteins, particularly for latency. HCMV miRNAs are differentially expressed during lytic and latent infection, with only a subset of the 26 mature HCMV miRNAs expressed during latency [19, 20]. HCMV miRNAs have been found to have key roles in active infection, including roles in immune evasion, cell cycle regulation, and viral replication [17, 21]. Although less understood, viral and host miRNAs have also been implicated in HCMV latency and have been suggested to be important in the establishment and maintenance of HCMV latency [18].

## RESEARCH QUESTIONS

The eradication of HCMV in seropositive organ transplant recipients and seropositive donors prior to the procedure and the associated immunosuppression would significantly reduce the risk of severe HCMV-mediated disease in organ transplant recipients (Fig. 3). There is currently no cure or vaccine for HCMV. While antivirals are given as prophylaxis to organ transplant recipients in order to try and prevent symptomatic HCMV infections, there is a need for novel therapeutics due to the emergence of resistance to these antivirals as well as associated toxicities [8, 22]. With the increasing understanding of viral and host miRNAs in viral pathogenesis, there has also been an increased interest towards the development of therapeutics using or targeting miRNAs [23]. HCMV poses a challenge for the development of therapeutics due to its widespread tropism, questions about the exact

**FIG. 1 Human-infecting herpesviruses.** The herpesviruses are divided into the alpha, beta, and gamma subfamilies with 9 herpesviruses that are known to infect humans [1, 2].



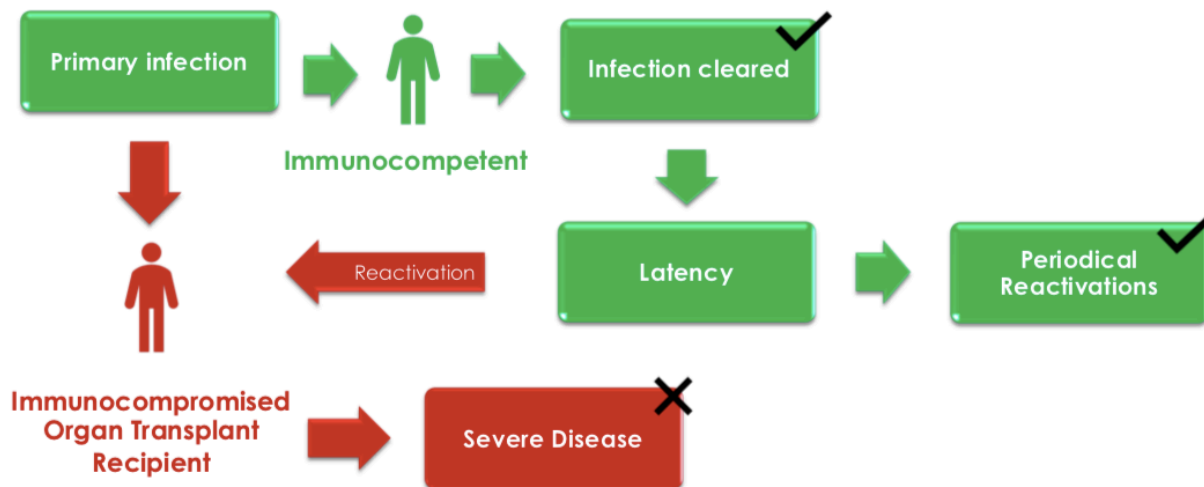
location of latency, and incomplete understanding of how latency is established and maintained. This article will underline the importance of some key miRNAs in HCMV latency based on what is currently known. First, the current understanding of HCMV latency will be reviewed. Second, recent findings of miRNAs and their possible importance in HCMV latency will be presented. Lastly, the implications of miRNAs for novel therapeutics targeting HCMV latency will be discussed. miRNAs may be crucial pieces in the puzzle that is HCMV latency.

## PROJECT NARRATIVE

**How is HCMV Latency Established and Maintained?** Research to answer these questions remains ongoing, although many key features of HCMV latency have been identified over the years. HCMV latency involves restricted viral gene expression, which also limits detection by effector cells of the immune system [11]. Latency has been documented most convincingly in cells of the myeloid lineage, to which HCMV ultimately disseminates [11]. Less-differentiated CD34<sup>+</sup> hematopoietic progenitor cells, CD14<sup>+</sup> monocytes, and immature myeloid dendritic cells are all believed to be the primary sites of latency, as indicated by the presence of viral genome in the absence of viral replication [24-26]. Further studies are required to clearly define the sites of HCMV latency [9]. Differentiation of infected cells to macrophages or mature myeloid dendritic cells results in reactivation of the lytic cycle (Fig. 4) [27].

IE genes are transcribed from the major immediate-early promoter (MIEP). The expression of IE genes is a crucial step in initiating the lytic gene cascade. During latency, IE gene expression is suppressed due to the repression of the MIEP [27, 28]. Transcriptional silencing of this promoter is a key step in establishing HCMV latency. This is achieved through the combined actions of cellular transcriptional suppressor proteins and viral regulators of IE expression [27-30]. The MIEP is associated with repressive chromatin in naturally latently infected cells [27]. Lytic genes are heterochromatinized during latency, resulting in a largely inactive viral genome, while genes expressed during latency have euchromatic histone modifications [9]. Only a subset of the more than 200 genes encoded by the HCMV genome is expressed during latency [31-35]. Reactivation involves differentiation-dependent chromatin remodelling of the MIEP [27, 36]. Heterochromatinized genes obtain euchromatic histone modifications to allow their expression, starting with the IE genes to initiate the lytic cascade [9].

The MIEP directs the expression of a number of regulatory genes encoding for proteins required for both efficient viral replication as well as the coordination of viral gene expression [16]. The major trans-activators IE1 (also known as IE72) and IE2 (also known as IE86) are the most abundant of the proteins produced [16, 37]. Histone deacetylases



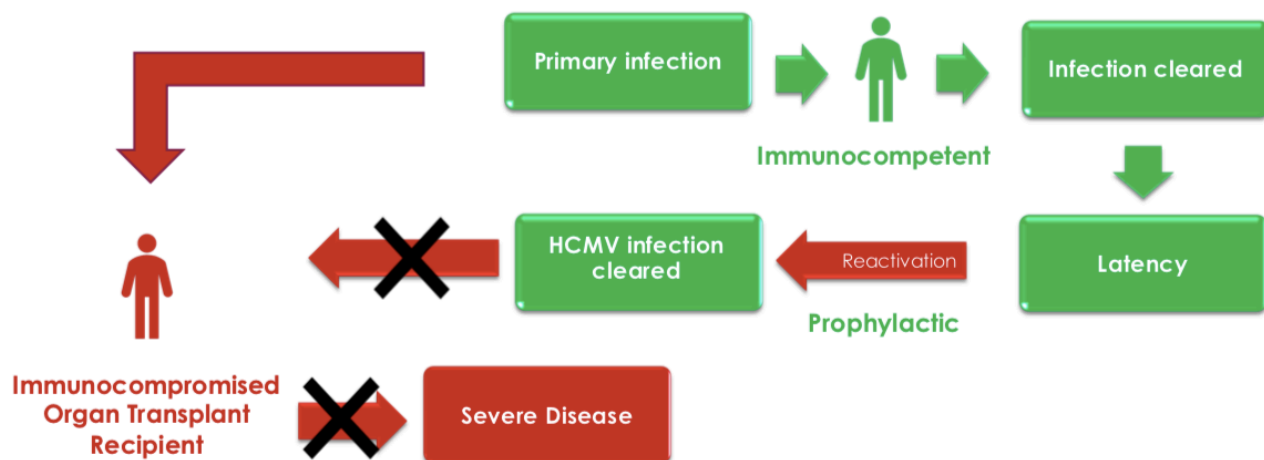
**FIG. 2 Usual outcomes of HCMV infection.** The immune response of a healthy individual is usually adequate in controlling primary infection and periodical reactivations. Primary infection or reactivation from latency can result in severe disease in an immunocompromised individual [7, 8].

(HDACs) limit viral transcription through the formation of inhibitory chromatin structures on the viral genome [38]. IE1 promotes transcription by inhibiting HDACs [38, 39]. IE2 transactivates viral E and L genes as well as host genes in order to facilitate viral replication [38]. While only IE2 is essential for virus replication, disturbances in IE1 expression significantly attenuate viral replication following low multiplicity infections [37, 40]. Although reactivation from latency is not yet fully understood, expression of both of IE1 and IE2 is thought to be crucial in this [38, 40]. Elucidation of the regulatory mechanisms controlling the expression of IE1 and IE2 would also further our understanding of HCMV latency.

**How may miRNAs Contribute to HCMV Latency?** HCMV encodes 26 mature miRNAs, with only a subset of miRNAs expressed during latency [20]. Unlike viral proteins which elicit strong immune responses, viral miRNAs are not immunogenic and are therefore ideal agents for the suppression of antiviral responses and modification of functions in latently infected cells [17, 37]. HCMV miR-UL112-1 is one such viral miRNA and is expressed during lytic infection as well as throughout latency, being one of the most abundant viral miRNAs during latent infection [19, 41]. miR-UL112-1 has numerous targets, both host and viral. MHC I polypeptide related sequence B (MICB) is a stress-induced ligand for NKG2D, the activating receptor for natural killer (NK) cells. The expression of MICB is repressed by miR-UL112-1 to facilitate immune evasion [21]. Other validated host targets of this miRNA include the cellular transcription factor BcIAF1 and multiple components of the secretory pathway [42, 43].

This miRNA may also have a role during latency in limiting immune detection. miR-UL112-1 targets the 3' untranslated region (UTR) of IE1 and expression of this miRNA is also observed in primary myeloid cells, the documented site of HCMV latency [19, 44]. During lytic infection, miR-UL112-1 attenuates replication significantly through repression of IE1 translation [37]. During latency, studies suggest that miR-UL112-1 functions to minimize the translation of any IE1 transcript that escapes MIEP transcriptional repression (Fig. 5A) [41, 44]. IE1 translation would lead to detection of latently infected cells by the antiviral response against IE1, as is observed in healthy HCMV carriers [44]. Repression of IE1 translation during latency is likely essential for latency to be maintained through the avoidance of detection by this IE1-specific antiviral T cell response [44].

The repression of IE2 translation is also important during latency. This is accomplished by members of the hsa-miR-200 miRNA family, which target the 3'UTR of IE2 transcripts (Fig. 5B) [18]. These cellular miRNAs have been found to be crucial in tumorigenesis, and are downregulated in aggressive tumours [45]. For HCMV, targeting of IE2 transcript by these miRNAs ensures the silencing of lytic infection and therefore the maintenance of



**FIG. 3 Potential therapeutic strategy for clearing HCMV infections.** A therapeutic that targets latent HCMV to ultimately result in the clearance of HCMV would benefit individuals such as organ transplant recipients if done prior to the procedure and the associated immunocompromised status. This would reduce the risk of severe disease.

**FIG. 4 Differentiation-dependent reactivation from latency.** Differentiation of latently infected immature myeloid cells results in reactivation of the lytic cascade [27].

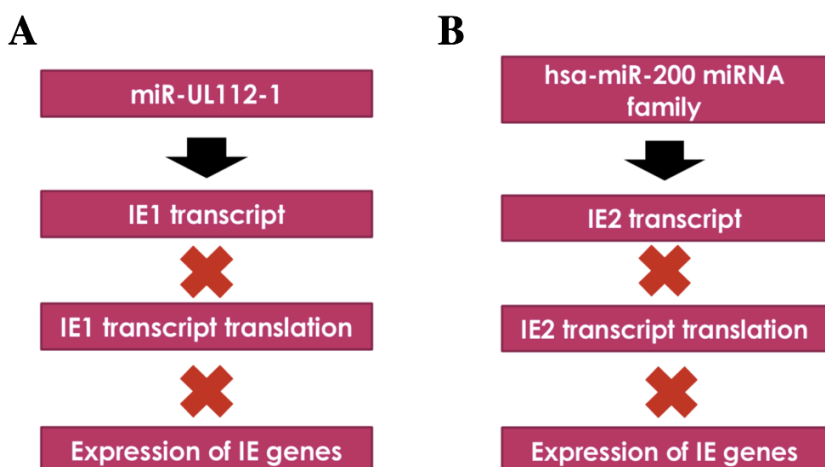


latency as the expression of IE2 alone has been observed to be sufficient for turning on IE gene expression [18]. These miRNAs were found to be expressed at high levels in primary human umbilical cord CD34+ cells and primary human monocytes, cells that support latent infection, while expression was lower in primary human monocyte-derived macrophages [18]. Expression of these miRNAs was found to decrease as cells differentiated into cells permissive for lytic infection [18]. This supports their role in maintaining HCMV latency, as lower levels of these miRNAs in differentiated cells would remove the translational block on IE2, thereby allowing IE2 translation and ultimately lytic reactivation through the expression of IE genes.

It is believed that the expression of the IE genes initiates the lytic cascade [39]. The above viral and host miRNAs have been observed to inhibit the production of proteins that are important for IE gene expression. As such, miRNAs appear to have important roles in maintaining HCMV latency.

**Can we target miRNAs as a therapeutic strategy against HCMV latency?** As miRNAs may be crucial in the maintenance of latency for HCMV, miRNAs may be potential targets for therapeutics aiming to eliminate latent HCMV. In some HCMV seropositive carriers, IE-specific cytotoxic T cells (CTLs) may make up to 10% of the CTL component in peripheral blood [44]. If the induction of IE protein expression in the absence of full virus replication in latently infected cells could be achieved, these cells could be targeted by the IE-specific CTLs to result in the elimination of latent HCMV (Fig. 6B) [41]. This would be especially beneficial if done for organ transplant recipients and donors prior to the procedure (Fig. 3).

In latently infected myeloid cells, the removal of the miR-UL112-1 target site in the IE1 transcript results in increased levels of IE1 in experimentally-infected primary monocytes and THP-1 cells [44]. This results in the induction of low-level IE gene expression that was found to be detectable by IE-specific CTLs, and full reactivation of the lytic cycle resulting in virus production does not occur [44]. Targeting miR-UL112-1 by anti-miRNA oligonucleotides (AMOs) as prophylaxis for organ transplant recipients may therefore be a potential therapeutic technique (Fig. 6A). This would prevent IE1 translational inhibition by miR-UL112-1, thereby allowing latently infected cells to be eliminated by endogenous IE-



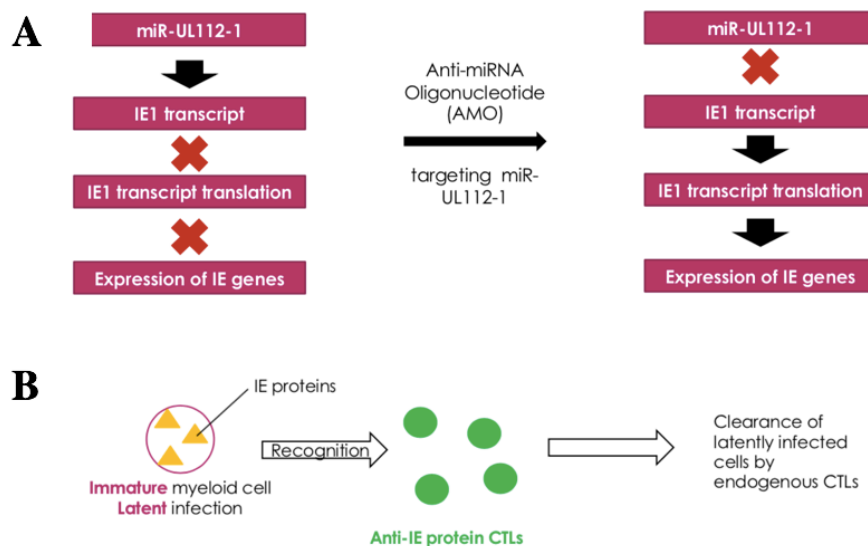
**FIG. 5 miRNA control of IE1 and IE2 translation.** A. HCMV miR-UL112-1 prevents the production of IE1, a protein involved in the expression of IE genes [41, 44]. B. Host miRNAs from the hsa-miR-200 miRNA family function to prevent the production of IE2 in latently infected immature myeloid cells, which is another protein involved in the expression of IE genes [18].

specific CTLs and ultimately resulting in the elimination of HCMV from the individual (Fig. 6) [41, 44].

While this may sound straightforward, this is currently quite difficult in practice. As of 2017, approximately 20 clinical trials using miRNA- and siRNA-based therapeutics had been initiated [23]. One of these, miravirsin, is the first miRNA-targeting therapeutic. It is a locked nucleic acid (LNA)-modified oligonucleotide that targets miR-122, a hepatocyte-expressed host factor essential for hepatitis C virus (HCV) replication [46]. Phase IIA clinical trials in 2013 found this drug to be effective in reducing HCV RNA levels in a dose-dependent manner [46]. This drug is currently in phase II clinical trials [23]. In the case of miravirsin the target is localized and defined, simplifying the development and administration of such a therapeutic.

For HCMV, targeting of the therapeutic AMOs would be difficult. While cells of the myeloid lineage are believed to be the primary sites of HCMV latency, there may be other sites as well [9]. Targeting of the therapeutic drug would also require the development of an efficient delivery system, such as nanoparticles. Nanoparticles can deliver the oligonucleotides, protecting them from degradation by serum nucleases and allowing them to overcome the poor cell-permeability of naked oligonucleotides [47, 48]. For miravirsin, these obstacles were overcome through LNA-modification [49]. Nanoparticles can also be conjugated to specific molecules to confer target-specificity [47]. As myeloid progenitor cells reside in the bone marrow, higher doses would likely be required for a therapeutic effect [50]. This, as well as the targets being myeloid progenitor cells, raises the question of potential adverse effects.

The development of such a therapeutic would also require the development of an appropriate companion diagnostic (CDx), a diagnostic test that would provide information about the safety and effectiveness of the corresponding therapeutic [51]. A potential CDx could be exosome-based. Herpesviruses exploit exosomes, extracellular vesicles that function in intercellular communication, and affect processes in recipient cells to aid in their survival and persistence [52, 53]. This is done through the incorporation of viral products, including viral miRNAs and glycoproteins [52-55]. The enrichment of miRNAs in exosomes makes them a promising biomarker to detect the presence of a specific herpesvirus [52]. If HCMV was successfully eradicated we would expect to see the absence of HCMV miRNAs in exosomes. Future studies should first aim to verify whether



**FIG. 6 Potential anti-miRNA oligonucleotide (AMO) therapeutic.** A. An oligonucleotide targeting miR-UL112-1 would allow the expression of IE1 in latently infected cells, which would result in low-level IE gene expression without full virus replication [44]. B. The low-level expression of IE genes without full virus replication would result in products that could be recognized by endogenous CTLs, allowing for the clearance of latently infected cells [41, 44].

exosomes released from HCMV-infected cells, which have been found to be able to stimulate memory CD4<sup>+</sup> T cells isolated from HCMV-infected donors, also carry HCMV miRNAs [52, 56]. Looking for the absence of other viral products in exosomes may also be promising.

## CONCLUSIONS

HCMV continues to pose a severe risk of morbidity and mortality for immunocompromised individuals [7, 8]. Preventative measures for organ transplant recipients, one of the most high-risk groups, include prophylaxis and pre-emptive treatment with antivirals [7]. With the increasing emergence of resistance to these agents as well as associated toxicities, there is a need for novel therapeutics [8]. An optimal therapeutic would allow for the eradication of HCMV from individuals most at risk for severe HCMV-mediated disease. This would require the targeting of latent HCMV, a task made more difficult due to our currently incomplete understanding of HCMV latency. One contributing factor to this is the widespread tropism of HCMV [10]. Whether HCMV undergoes latency or a lytic replication cycle differs depending on the host cell type [10]. This adds to the challenge due to the need to develop a therapeutic that targets multiple, specific cell types. Along with this, there has been evidence of HCMV latency in cell types other than cells of the myeloid lineage [9]. Eradication of this virus may not be possible unless all locations of latency are known and can be targeted. Lastly, the exact mechanisms that allow the establishment and maintenance of HCMV latency are not fully understood as of yet.

The cytomegaloviruses are unique due to their single-species specificity. There is little evidence of single cytomegaloviruses infecting multiple species [57]. This trait has contributed to long-term co-evolution of cytomegaloviruses to their respective hosts [57]. This poses a unique challenge for studying HCMV, as humans are its only host. One way to overcome this is through the use of animal models. The relatedness of the host species is indicative of the relatedness of the respective CMV genomes [58]. As humans are closely related to rhesus monkeys, the rhesus CMV (rhCMV) genome is very similar to that of HCMV [59]. Although miRNAs have been identified for rhCMV, more work needs to be done to identify their functions and see if they regulate the same functions as HCMV [59]. While rhesus monkeys would be an optimal animal model for the study of HCMV therapeutics, access and cost are some of the key obstacles. An effective alternative is murine cytomegalovirus (MCMV), which has many similarities to HCMV [9]. MCMV also encodes for miRNAs, and the overexpression of some of these viral miRNAs was found to reduce viral replication [60]. This supports the development of a novel miRNA-based therapeutic to target HCMV, although further research into the roles of MCMV miRNAs is also required [60].

The evolutionary history and origin of miRNAs are important questions yet to be answered. In recent years, it has become increasingly clear that miRNAs play key roles in the regulation of viral and host functions for many viruses. Further research into how miRNAs, including exosome-mediated transmission of miRNAs, may contribute to the lifecycle of HCMV both during lytic and latent infection will provide valuable knowledge about the complex lifecycle of HCMV and possibly aid in the development of novel therapeutics and diagnostics for HCMV.

## ACKNOWLEDGEMENTS

I would like to thank Dr. François Jean for his invaluable guidance throughout the research process. I would also like to thank my MICB 406 peers for their helpful feedback during group discussions.

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