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Recent advances in CMV tropism, latency, and diagnosis during aging

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Abstract Human cytomegalovirus (CMV) is one of the largest viruses known to cause human diseases. Chronic CMV infection, as defined by anti-CMV IgG serology, increases with age and is highly prevalent in older adults. It has complex biology with significant immunologic and health consequences. This article aims to summarize research findings presented at the 6th International Workshop on CMV and Immunosenescence that relate to advances in the areas of CMV tropism, latency, CMV manipulation of cell metabolism, and T cell memory inflation, as well as novel diagnostic evaluation and translational research of chronic CMV infection in older adults. Information summarized here represents the current state of knowledge in these important fields. Investigators have also identified a number of areas that deserve further and more in-depth investigation, including building more precise parallels between mouse CMV (mCMV) and human CMV (HCMV) research. It is hoped that this article will also stimulate engaging discussion on strategies and direction to advance the science to the next level.

Keywords CMV tropism · Latency · Diagnosis · Aging

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Cytomegalovirus (CMV) is one of the largest viruses known to cause human diseases, including life-threatening infections in immunocompromised patients. In immunocompetent individuals, primary HCMV infection is typically mild or asymptomatic. However, the virus can persist with its DNA genome harbored in myeloid lineage cells including CD34 progenitor cells and peripheral blood monocytes. This latent persistent (or in part smoldering chronic) CMV infection is highly prevalent in older adults with seroprevalence as high as 99% by some studies (Staras et al. 2006; Wang G and Walston J 2009; Wreghitt et al. 2003). While the possibility exists that CMV persistence could afford benefits, such as enhanced responses to immunization, to younger people (Furman et al. 2015), there are clear consequences for CMV persistence in older adults. This latent viral infection is considered a driving force for immunosenescence (Pawelec et al. 2005; Koch et al. 2007). Studies have shown expansion of CMV-specific T cells and related alteration of T cell repertoire in CMV-seropositive older adults (Khan et al. 2002; Vescovini et al. 2007; Wertheimer et al. 2014; Li et al. 2015), which is addressed in depth in the accompanying article by Jackson et al. Epidemiological data indicate that CMV seropositivity is associated with chronic conditions and adverse health outcomes, including cardiovascular diseases, frailty, disability, and mortality in older adults (Schmaltz et al. 2005; Wang et al. 2010; Aiello et al. 2008; Roberts et al. 2010; Strandberg et al. 2009), which is addressed in depth in the accompanying article by Aiello et al.

Much remains to be learned about the complex biology of chronic CMV infection, particularly when

recognizing its significant immunologic and health consequences. This article will summarize research findings presented at the 6th International Workshop on CMV and Immunosenescence in the session on virus biology. While details can be found in individual presentation summaries provided below, Dr. Jeremy Kamil presented his work on molecular mechanisms for regulation of CMV tropism, particularly the role of CMV-encoded viral protein UL148. Dr. John Purdy presented insights gained from lytic CMV infection into core metabolism, such as carbon and lipid mechanism pathways required for CMV infection. Much less is known about metabolic requirement for chronic/latent CMV infection or how the virus hijacks these pathways. Dr. Felicia Goodrum presented the work on molecular mechanisms for regulation of CMV latency, particularly the role of viral proteins UL138 and UL135 and their regulation of epidermal growth factor receptor (EGFR). Drs. Niels Lemmermann and Matthias Reddehase presented their findings on T cell "memory inflation" as a result of latency-associated gene expression, primarily from studies of mouse CMV (mCMV) infection. Immune recognition of CMV as well as CMV manipulation of the immune system are addressed in depth in the accompanying article by Souquette et al. and that by Jackson et al., respectively. Data on broad and massive T cell responses to human CMV peptide pools among HIV-infected and HIV-uninfected men who have sex with men (MSM) in the Multicenter AIDS Cohort Study (MACS) presented by Dr. Sean Leng could represent as a clue suggesting such T cell memory inflation secondary to latent/chronic human CMV infection in humans, at least in the MACS setting. Dr. Leng also presented the line of translational research being pursued in his laboratory on chronic CMV infection in older adults including the development of a novel nested PCR-based tool for diagnostic evaluation, evaluation of HCMV impact on T cell immunity in HIV and aging, and exploration of sirtinol, a sirtuin antagonist, and other compounds with potential as potent pharmacological therapy for HCMV infection.

Regulation of viral tropism

UL148 is a viral endoplasmic reticulum (ER)-resident glycoprotein that appears to influence human cytomegalovirus (HCMV) cell tropism by regulating the composition of alternative gH/gL complexes on progeny virions (Li and Kamil 2015). One key question is whether UL148 forms part of an in vivo tropism switch for HCMV. gH/gL complexes are thought to regulate the membrane fusion machinery that drives herpesvirus cell entry (reviewed in Vanarsdall and Johnson 2012; Heldwein 2016). Interestingly, a number of herpesviruses in the gamma- and beta-subfamilies, including HCMV, encode two different gH/gL complexes. In the case of Epstein-Barr virus, cell type-specific differences in expression of alternative gH/gL complexes in virions drive a tropism switch between epithelial cells and B-lymphocytes (Borza and Hutt-Fletcher 2002), but whether an analogous phenomenon occurs in HCMV is still unclear. The first HCMV gH/gL complex to be described was the trimer, gH/gL/gO; of the two HCMV gH/gL complexes, trimer is sufficient for fibroblast entry but also appears to be broadly required for entry of cell-free virions into all cell types (Zhou et al. 2015). The second gH/gL complex to be identified was the pentamer, gH/gL/UL128–131, which is necessary for infection of epithelial and endothelial cells. AD169 and Towne HCMV isolates lack a large portion of the viral genome, termed the "UL*b′*," which encodes factors important for cell tropism and entry, immune modulation, and latency. The *UL148* gene is located within the UL*b′*, and is absent from AD169. We find that in several strains that preserve a full-length UL*b′* region (e.g., TR, TB40/E, FIX), UL148 is required for high-level expression of the gH/gL/gO trimer in infected cells, and hence for production of gH/gL/gO-rich virions [(Li et al. 2015), and Kamil laboratory, unpublished results). Such trimer-rich virions infect fibroblasts efficiently, but at least in the case of strain TB40/E do not favor efficient infection of epithelial cells. *UL148*-null mutants of strain TB40/E show markedly improved replication in epithelial cells that correlates with decreased expression of gH/gL/gO. In infected cells, UL148 is found to co-localize with calnexin, an ER marker, and also shows endoglycosidase H sensitivity consistent with ER localization (Li et al. 2015). Thus, we are confident that UL148 is an ER-resident glycoprotein. However, why does UL148 exist? Several lines of evidence indicate that virion cell tropism is forged within the ER. gO and UL128–131 compete within the ER for assembly onto gH/gL; exemplifying this competition, a single cysteine position gL has been identified to form a mutually exclusive disulfide bond with cysteines from either gO or UL128 (Ciferri et al. 2015). Interestingly, UL116, a glycoprotein encoded by the gene neighboring *gL*

(*UL115*), was recently shown to form a complex with gH that lacks gL, gH/UL116, in which UL116 essentially substitutes for gL (Calo et al. 2016). Although its function remains unknown, the gH/UL116 complex was found to be expressed on HCMV virions. We have examined the composition of virions obtained from strain TB40/E viruses null for both *UL148* and *UL128–131*, and we have observed that these virions show wild-type levels of gH but reduced levels of gL, implying that UL148 might also influence the levels of the gH/UL116 complex in virions (Kamil J.P. and Siddiquey, M.N.A., unpublished results). In other recent experiments, we have sought to glean insights into the mechanism by which UL148 might influence the composition of virion glycoprotein complexes. One approach we have taken is to use mass spectrometry to identify proteins that co-immunoprecipitate from infected cells with UL148. Our results suggest that UL148 interacts with SEL1L, an adaptor for the E3 ubiquitin ligase Hrd1, which plays crucial roles in ER-associated degradation (ERAD) (Kamil J.P. and Siddiquey, M.N.A., unpublished results). The process of ERAD plays key roles in glycoprotein quality control and prevents the accumulation of misfolded proteins within the ER, which otherwise can trigger an unfolded protein response (reviewed in Brodsky 2012). Hence, maintenance of ERAD is crucial for the function of the secretory pathway and, more broadly, for the health of the cell. During ERAD, ER lectins deliver the misfolded glycoproteins to SEL1L, and the SEL1L/Hrd1 complex is involved in dislocating ERAD substrates across the ER membrane for proteasomal degradation (Christianson et al. 2008). Interestingly, HCMV is known to encode immune-evasins such as US11 and US2 that exploit ERAD at immediate-early and early times post infection to prevent antigen presentation (Schuren et al. 2016). Although the mechanistic details remain unclear, it is intriguing to consider that UL148 may represent an example wherein ERAD is exploited to modulate the cell tropism of progeny virions, with potential implications for horizontal spread and viral navigation of host tissues.

Manipulation of host metabolism

Host metabolism plays an essential role in the replication of HCMV (Rabinowitz et al. 2011; Goodwin et al. 2015; Sanchez and Lagunoff 2015). The products of metabolic pathways provide all of the energy and building materials (e.g., nucleotides for the virus genome, lipids for the envelope membrane) required for productive infection. Previous system-level examination of metabolites and their fluxes found that HCMV infection resulted in remodeling of carbon metabolism (Chambers et al. 2010; Munger et al. 2006; Purdy et al. 2015; Vastag et al. 2011; Hwang et al. 2014). The flow of carbons from glucose to fatty acid metabolism is increased, possibly involving the non-enzymatic conversation of a pyruvate to acetate to generate the substrates required for fatty acid synthesis and elongation (Vysochan et al. 2017). Likewise, carbons from glutamine are redirected for anaplerotic usage to replenish the TCA cycle (Munger et al. 2008; Chambers et al. 2010). HCMV infection in human fibroblast dramatically increases the elongation of fatty acids to generate very long chain fatty acid tails (VLCFAs) (Koyuncu et al. 2013; Purdy et al. 2015). These VLCFAs are used to build the viral envelope. Targeting various metabolic enzymes in these pathways blocks infection, including targeting fatty acid synthase (FAS), fatty acid elongase 7 (ELOVL7), and acetyl-CoA synthetase short-chain family member 2 (ACSS2). For example, depleting cells of ELOVL7 activity decreases saturated VLCFA production resulting in infected cells releasing viral particles that are non-infectious due to a loss of the lipids required to build a functional envelope.

Considerable progress has been made on the identification of enzymes and pathways of core metabolism that are required for HCMV infection; however, much less is known about how the virus hijacks these pathways. Kinases that act as metabolic regulators in uninfected cells are co-opted by HCMV to remodeling metabolism. Amp-activated protein kinase (AMPK) and calmodulin-dependent kinase kinase (CAMKK) are important for changes in central carbon metabolism (McArdle et al. 2012; McArdle et al. 2011; Terry et al. 2012). Viral changes to fatty acid metabolism involve mTOR signaling (Spencer et al. 2011; Purdy et al. 2015). Protein kinase RNA-like endoplasmic reticulum kinase (PERK), an ER stress-responsive factor, contributes to HCMV-induced lipid synthesis (Yu et al. 2013). Although these cellular kinases provide critical insight into the mechanisms HCMV is using to alter host metabolism, they only provide a partial picture. Indeed, there are likely many more host factors involved that have yet to be identified. Similarly, the viral factors required for hijacking metabolism are unknown. The

viral activator of mTOR, UL38, is important for fatty acid metabolism, but the other viral factors have yet to be elucidated.

Our current knowledge is limited to the metabolic contributions to lytic infection. Metabolites or lipids may also be important for establishing a latent infection and/or reactivation. If HCMV reactivation and release of virions in hematopoietic cells involve metabolic contributions similar to those observed in fibroblasts, then targeting host metabolism may prevent reactivation. It is important to note that metabolism is not fixed. Like infection, metabolism also contributes to aging. How does aging-associated metabolic changes contribute to HCMV infection or its immune control? Further metabolism investigations into aging, HCMV latency, and reactivation are required.

Regulation of latency

Following a primary infection, HCMV establishes a lifelong latent infection in the host. The molecular basis of the latent infection is not well understood. The virus will sporadically reactivate from latency in response to ill-defined stimuli in the host, and the healthy host will asymptomatically shed low levels of virus to ensure spread. During primary infection, children will asymptomatically shed virus for months to years in their saliva and urine (Britt 2008). By contrast, it is not known how viral latency and persistence changes as the host ages. Does HCMV reactivate more frequently or have heightened level of viral gene expression in the aged host, which may contribute to T cell expansion? Is the latent state truly static or in a state of flux that changes with age? Approaching these questions and other regarding latency required understanding the molecular basis of latency.

The Goodrum lab is focused on identifying viral determinants of latency—virus genes that promote viral latency or reactivation from latency—as a means to understand the basic molecular mechanisms contributing to latency (Goodrum et al. 2007; Umashankar et al. 2011; Umashankar et al. 2014). HCMV coordinates the expression of two viral genes, UL135 and UL138, which have opposing roles in regulating viral replication (Umashankar et al. 2014). UL135 promotes reactivation from latency and virus replication, in part, by overcoming replication-suppressive effects of UL138. To define the mechanism by which UL135 and UL138 oppose one another, we identified viral and host proteins interacting with UL138 and UL135 proteins to begin to define the mechanisms by which these proteins function. We show that UL135 and UL138 both target the same receptor tyrosine kinase (RTK), epidermal growth factor receptor (EGFR) (Buehler et al. 2016). EGFR is a major homeostatic regulator involved in cellular proliferation, differentiation, and survival, making it an ideal target for viral manipulation during infection. UL135 promotes the turnover of EGFR, whereas pUL138 preserves cell surface expression and activation of EGFR. Activated EGFR is sequestered within the infection-induced, juxtanuclear viral assembly compartment and is unresponsive to stress. Further, EGFR is localized in both Rab 5 and Rab 11 vesicles, suggesting that EGFR trafficking has been stalled and diverted to the endocytic recycling compartment. Intriguingly, EGFR sequestered within these vesicles remains active, as indicated by phosphorylation at Tyrosine 1068, even under stress conditions that should deactivate EGFR. Taken together, these findings suggest that CMV insulates active EGFR in the cell and that UL135 and UL138 function to fine-tune EGFR levels at the cell surface to allow the infected cell to respond to extracellular cues. Consistent with the role of UL135 in promoting replication, inhibition of EGFR or downstream phosphoinositide 3-kinase (PI3K) favors reactivation from latency and replication. We propose a model whereby UL135 and UL138 together with EGFR comprise a molecular switch that regulates states of latency and replication in HCMV infection by regulating EGFR trafficking to fine tune EGFR signaling (Buehler et al. 2016). Understanding the mechanisms by which HCMV modulates its latent cycle is critical to ultimately control latency and reactivation (Goodrum 2016). For example, if HCMV is a driver of aging, then suppressing or limiting viral reactivation may assuage age-related risks or pathology associated with HCMV seropositivity. Further, HCMV-mediated modulation of RTKs, such as EGFR, may broadly impact the host environment and homeostasis beyond the infected cell and contribute to age-related pathologies of CMV persistence. Further, this work defines viral determinants that, based on their interactions in the host cell, can positively and negatively regulate replication of the virus and facilitate transitions into and out of latency—suggesting a role for viral mechanisms regulating latent latency and replication. Both viral and host determinants of latency are important candidate targets for antiviral intervention to

ameliorate age-related pathologies or prevent reactivation and disease in the setting of transplantation.

T cell "memory inflation" as a result of latency-associated gene expression

After termination of productive primary CMV infection, when viral latency is established in multiple host tissues, CMV-specific $CD8^+$ T cells persist at extra-lymphoid sites such as the lungs. Although the overall absolute number of tissue-infiltrating $CD8^+$ T cells declines, CD8 T cells specific for certain viral epitopes accumulate over time also in absolute numbers and assume the phenotype $CD44^+CD62L^-CD127^-KLRG1^+$ characteristic of terminally differentiated T cells known as short-lived effector cells (SLECs) (Holtappels et al. 2000; Snyder et al. 2008; Seckert et al. 2012). This phenomenon has been coined with the now popular term "memory inflation" (MI) (Karrer et al. 2003; Klenerman and Oxenius 2016). Although we have in recent years learned a lot about the immunology of MI, the link to the specific biology of CMVs as well as the "molecular drivers" of MI and the epitope selectivity are still incompletely understood.

To understand the molecular mechanisms of MI, it is important to note that latency does not mean total transcriptional quiescence of the viral genome at all its genetic loci and at all times. Instead, in a murine model of mCMV infection after experimental hematopoietic cell transplantation, latency has been shown to be a highly dynamic state in which, at any given time, most of the viral gene loci are silenced while some hotspots are transiently in an "on" state (Kurz et al. 1999; Grzimek et al. 2001; Simon et al. 2006). This fact was recently coined with the term "sleepless latency" (Poole and Sinclair 2015). Such a transient gene desilencing results in a stochastic expression of so-called transcripts expressed in latency (TELs) (Seckert et al. 2011), but without completion of the productive viral replication cycle. The presence of low numbers of TELs is a hallmark of latency, but TELs are not necessary to keep a cell in a latent status.

A well-described hotspot of transcriptional activity during mCMV latency is the major immediate-early (MIE) gene locus that codes for at least two different TELs, the *immediate-early* transcripts *ie1* and *ie2* (Grzimek et al. 2001). Both TELs are expressed independently from a bidirectional gene pair flanking the MIE promoter-enhancer, and show a random expression pattern in the lungs of latent mice that follows the Poisson distribution. The Poisson distribution function allows us to calculate the frequency of transcriptional activity as one *ie1* TEL-expressing cell out of 2500 latently infected cells at any moment (Seckert et al. 2012). To date, the *ie1* TEL of mCMV is the only known TEL that corresponds to an epitope-specific MI, although the amount of IE1 protein was below the limit of molecular detection. Furthermore, it remained open to question if the epitope is actually presented during latency, resulting in CD8 T cell expansion, or if MI is driven by a latency-associated MI-promoting cytokine microenvironment.

It has been proposed that the "inflationary" $CD8^+$ T cells are involved in the immune surveillance of viral latency by sensing and terminating episodes of viral transcriptional reactivation (Reddehase et al. 2008) and that latent gene expression results in the presentation of antigenic peptides that stimulate virus-specific CD8 T cells (Seckert et al. 2012). This implies transcription of epitope-encoding viral genes (Aiello et al. 2008), translation of these transcripts to the respective antigenic proteins (Borza and Hutt-Fletcher 2002), and efficient antigen processing and epitope presentation (Britt 2008), all of which represent bottlenecks for MI to occur. We tried to address these points in our group over the past years and provided evidence strongly supporting the hypothesis that MI is driven by viral epitopes presented by latently infected host-tissue cells of non-hematopoietic origin (Seckert et al. 2011; Torti et al. 2011). We are currently investigating if mCMV gene *m164*, a second driver of MI in the $H2^d$ haplotype (Holtappels et al. 2002; Fink et al. 2014), is also expressed in latency as a TEL.

Chronic CMV infection in older adults: diagnostic evaluation and translational research

Chronic CMV infection is currently diagnosed by a positive anti-HCMV IgG serology, and is believed to contribute significantly to immunosenescence and adverse health outcomes in older adults (Pawelec et al. 2005; Koch et al. 2007; Schmaltz et al. 2005; Wang et al. 2010; Aiello et al. 2008; Roberts et al. 2010; Strandberg et al. 2009). However, critical evaluation of the literature reveals the heterogeneity of HCMV-seropositive older adult population and inconsistent

findings. For example, while studies reported that HCMV-seropositive older persons had significantly higher mean value of HCMV-specific T cell frequencies than seronegative controls, many seropositive individuals had minimal or no such increase (Czesnikiewicz-Guzik et al. 2008; Vescovini et al. 2010). Recent studies showed no association between HCMV seropositivity and frailty or functional disability (Goldeck et al. 2016; Mathei et al. 2015; Leng 2011; Mathei et al. 2011). This inconsistency is likely due to inadequacy of the anti-HCMV IgG serology-based diagnostic paradigm because a positive IgG titer is a crude measure that merely indicates prior exposure to the virus and does not distinguish between past (resolved) or chronic (persistent or latent) infections. The Leng lab has set out to address this challenge. Our approach is based on the biology that HCMV is known to persist with its viral DNA genome harbored in myeloid lineage cells including the peripheral blood monocytes, establishing latent (persistent) infection in some immunocompetent persons. We have developed and optimized a nested PCR-based assay for HCMV DNA detection in the peripheral blood monocytes from older adults. Our data from cross-sectional and longitudinal studies indicate that (a) HCMV DNA in monocytes is present in only about 55–60% of HCMV-seropositive older persons, (b) this subset has high HCMV pp65-specific $CD8^+$ T cell frequencies and elevated serum levels of IL-6 (chronic inflammation) and neopterin (immune activation) relative to CMV-seropositive persons with no HCMV DNA in the peripheral blood monocytes, and (c) gain or loss of HCMV DNA in monocytes is closely associated with increases or decreases, respectively, in HCMV-specific $CD8^+$ T cells and IL-6 levels, even when anti-HCMV IgG titers do not change appreciably (Leng et al. 2011b; Leng et al. 2011a; Li et al. 2014b). Consistent with these results, one study employing digital droplet PCR (ddPCR) also reported the utility of HCMV DNA detection in the peripheral blood monocytes for the evaluation of latent/chronic HCMV infection in older adults (Parry et al. 2016).

There is little doubt that chronic CMV infection has significant impact on T cell immunity. Frequently reported readouts for this effect include T cell frequencies specific to or T cell responses to two most commonly studied HCMV gene products, pp65 (encoded by UL83) and IE1 (encoded by UL123), through either tetramer (pentamer or other multimer) analysis or ex vivo stimulation assay to HCMV peptide pools, respectively. However, this is a grossly inadequate sampling because the genome of HCMV has at least 213 open reading frames (ORFs) that are expressed differentially over the course of infection. Among HIV-infected and uninfected MSM participants in the MACS, we conducted an extensive study of T cell responses to peptide pools encoded by 19 HCMV ORFs that elicited the largest responses (correlation of 0.9 with total responses) in healthy donors. Our results demonstrate that T cell responses to pp65 and IE1 combined only represented <12% of the total $CD4^+$ and <40% of the total $CD8^+$ T cell responses (Li et al. 2014a), suggesting the need for more comprehensive evaluation of the impact of chronic HCMV infection on T cell immunity beyond the commonly tested HCMV pp65 and IE1 epitopes.

Current FDA-approved therapeutic agents for CMV infection are based on DNA polymerase inhibitors. They have significant toxicities with emerging drug resistance. The Leng laboratory has developed a fibroblast culture of HCMV infection at very low viral titer, i.e., multiplicity of infection (MOI) of 0.01. This culture system mimics low-level HCMV infection in vivo likely from reactivation of latent infection. For example, while previous studies have shown anti-HCMV effect of resveratrol, a sirtuin activator, Mao and colleagues have recently demonstrated surprising, but potent suppressive effects of sirtinol, a sirtuin antagonist, on HCMV infection and HCMV-induced activation of molecular mechanisms of senescence and production of reactive oxygen species in human fibroblasts (Mao et al. 2016). Leng's laboratory is actively pursuing this line of translational research exploring other agents for their anti-HCMV effects with the long-term goal of developing more effective and better tolerated pharmacological therapy for chronic HCMV infection.

Concluding remarks and future directions

Taken together, research findings presented at the workshop represent the current state of knowledge in these important fields. Investigators at the workshop have also concluded that a number of areas deserve further and more in-depth investigation. For example, pertinent topics related to latency and reactivation were discussed (Goodrum 2016; Dupont and Reeves 2016). How do we define latency in an infected human? Does a latent state exist or are some cells latently infected while others are

replicating the virus? If the former is true, how often and at what circumstances of reactivations occur? An in-depth longitudinal cohort study with frequent sampling will be required to answer this question. However, latency may be far more dynamic than it is appreciated, termed "restless sleep." As such, the current "immediate early (IE) → early (E) → late (L)" linear model of viral gene expression should no longer be the only predominant model to account for the biology of latent versus lytic infections and reactivation. For example, infected cells can express IE genes, which does not necessarily lead to reactivation, but would potentially lead to stimulation of T and B cells. Factors that impact CMV driving immune remodeling, i.e., immunosenescence, are another important topic. Is the maintenance of CMV genome copies or viral load, or the number of latently infected cells important to pathologies associated with CMV infection and reactivation? On the reverse side, is the viral load or sources required for adequate immune surveillance, particularly that in the T cell compartment, or suppression of the infection? It is also important to further investigate cell type-specific contributions to viral tropism, acute/lytic and latent infection, and reactivation, as well as cell-cell dynamics and interaction. Last but not least, more efforts should be devoted to build connections between mCMV and HCMV research.

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