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MECHANISMS OF HUMAN CYTOMEGALOVIRUS PERSISTENCE AND LATENCY

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1. ABSTRACT

Human cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus that causes severe disease primarily in immunosuppressed individuals. A major characteristic of HCMV with obvious clinical importance is the ability of the virus to establish lifelong infection within the host following the initial acute infection. One strategy used by HCMV to maintain itself within the host is the establishment of cellular sites of persistent infection and viral latency. Recent studies have identified endothelial cells and monocyte-derived macrophages (MDM) as sites of HCMV persistence and latency. These studies show that endothelial cell origin and MDM differentiation pathway are critical factors that influence the characteristics of HCMV replication in these cell types. The specific HCMV genes involved in endothelial cell and MDM tropism are unknown. However, studies in the closely related murine cytomegalovirus (MCMV) model have provided considerable insight into viral genes that enable replication in these cell types. This review will focus on mechanisms of HCMV replication in endothelial cells and MDM, and on the viral genes involved in regulation of viral replication in these important cell types.

2. INTRODUCTION

HCMV infection is extremely common in the human population with an incidence of 40-100% depending on age and socioeconomic status. HCMV infection is generally acquired during childhood and results in an asymptomatic life-long infection in normal immunocompetent individuals. However, in immunocompromised individuals HCMV causes severe and life-threatening disease (1). HCMV is the most common congenital viral infection and is the leading

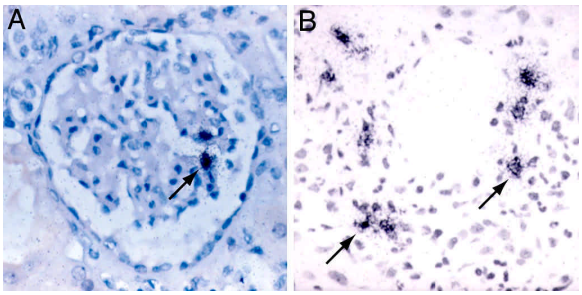

Figure 1. HCMV-infected cells in kidney (A) and liver (B) from SOT patients. Viral DNA (arrows) was visualized by *in situ* hybridization with a HCMV-specific probe, and cells were counterstained with hematoxylin and eosin.

infectious cause of central nervous system maldevelopment in neonates (2-4). In a recent Swedish study, 0.5% of newborns were infected with HCMV and of these 18% had long-term neurological sequelae (primarily sensorineural deafness and microcephaly) (5). Currently, HCMV is considered the major cause of sensorineural deafness in neonates (2). In AIDS patients, HCMV is the most common life-threatening opportunistic viral infection. However, the use of highly active antiretroviral therapy (HAART) for the treatment of patients is dramatically decreasing the incidence of HCMV disease in this population (6, 7). In solid organ transplant (SOT) and allogeneic bone marrow transplantation (BMT) patients, HCMV disease remains a serious complication. In the absence of prophylaxis, 8-39% of SOT and 20-35% of BMT patients develop symptomatic HCMV disease (7). Furthermore, the increased use of bone marrow transplantation for the treatment of diseases such as sickle cell anemia and multiple sclerosis, combined with the emergence of drug resistant HCMV strains, is increasing the population at risk for HCMV disease (7-9).

The clinical pathology associated with HCMV disease varies according to the etiology of immune suppression. In SOT and BMT patients, HCMV infection frequently results in disease of the gastrointestinal (GI) system and the lung. HCMV disease of the GI tract occurs in approximately 5% of transplant patients and can involve any region of the GI tract (9). HCMV infection of the lung is most severe in BMT patients where it causes pneumonia in approximately 15% of recipients and is associated with 20% mortality (1). In AIDS patients, HCMV infection of the lung is frequently observed, but the infection progresses more slowly and rarely results in pneumonia. HCMV infection of the retina is common in AIDS patients, but is rarely observed in transplantation patients. In one Australian study, only 1% of solid organ recipients developed HCMV retinitis (10). This is compared to HCMV retinitis occurring in 85% of AIDS patients with AIDS-related HCMV disease (7). The reason for the differences in clinical pathology of HCMV disease associated with different etiology of immunosuppression is unclear.

Histological analyses of autopsy tissues obtained from patients with HCMV disease have identified infected cells in virtually every organ (Figure 1). Cell types including MDM, T lymphocytes, granulocytes, endothelial cells, epithelial cells, fibroblasts, stromal cells, neuronal cells, smooth muscle cells and hepatocytes are infected in HCMV-infected patients (11-19). The most frequently infected cell types during acute disease are epithelial cells, endothelial cells, and macrophages. In the peripheral blood, leukocytes are a major source of HCMV during acute disease (11, 14, 20), and HCMV has been shown to be transmitted to patients by transfusion of the leukocyte fraction (21, 22). Examination of separated cell populations from the peripheral blood of asymptomatic HCMV seropositive individuals has identified monocytes as the predominant infected cell type in the circulation. This observation is consistent with the identification of macrophages as a major HCMV-infected cell type in tissues (20, 23-25). Although HCMV can be isolated from both the mononuclear and polymorphonuclear cell fractions, only a low percentage of HCMV-infected blood cells are detected in these individuals (11, 12, 14, 18, 20, 26-31). In contrast, a high frequency of HCMV positive leukocytes are observed in biopsies of transplanted kidneys and liver tissues during HCMV disease (13). Together, these results suggest a model wherein monocytes in the peripheral blood serve as a reservoir of latent virus. Following stimulation of these cells in response to inflammatory and/or allogeneic events, reactivation of the latent virus results in active HCMV replication and disease. In studies from our laboratory, we have demonstrated that $CD14^+$ monocytes harbor latent HCMV and that allogeneic stimulation of these cells results in the generation of a unique population of macrophages in which virus is reactivated (see below) (32).

3. ENDOTHELIAL CELLS AND MACROPHAGES AS SITES OF HCMV PERSISTENCE AND LATENCY

Endothelial cells and macrophages have been implicated as sites of HCMV persistence and latency, respectively. Consequently, the ability of HCMV to replicate in these two cell types is believed to play an important role in enabling the virus to maintain a life-long infection within the host. A number of studies also indicate that HCMV may modulate interactions between these two cell types to facilitate the spread of infection throughout the host (33, 34). In these studies, HCMV infection of endothelial cells was shown to increase the expression of adhesion molecule ICAM-1, which corresponded to an increased interaction between endothelial cells and monocytes resulting in monocyte infection (34). ICAM-1 was upregulated at the transcriptional level by the interaction of two HCMV transcriptional activators (IE1 and IE2) with the ICAM-1 promoter (35). Interestingly, monocytes infected in this manner were capable of transmitting virus to uninfected endothelial cells suggesting a possible mechanism for HCMV dissemination *in vivo* (33).

3.1. Growth of HCMV in endothelial cells

Endothelial cells form the inner lining of blood vessels and are involved in a variety of processes regulating tissue homeostasis and inflammation. Examination of

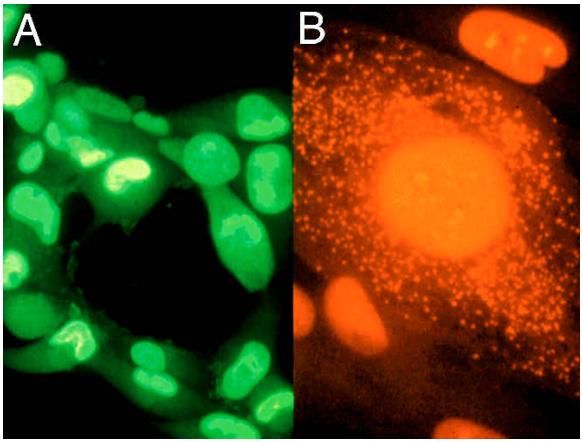

Figure 2. HCMV infects aortic endothelial cells (AEC). Immunofluorescence microscopy of HCMV-infected AEC at day 3 post-infection showing the presence of a viral antigens IE (A) and pp65 (B).

individuals with acute disease has found that endothelial cells constitute one of several cell types that are fully permissive for virus replication (17). Interestingly, aggregated endothelial cells containing HCMV were observed in the circulation of patients with active HCMV infection (36). This observation led to the speculation that HCMV-induced damage to vessel endothelium may result in shedding of infected endothelial cells into the peripheral blood facilitating virus dissemination. Examination of arterial specimens from HCMV seropositive individuals without active infection has revealed the presence of viral DNA in vessel walls (37-40). These findings have generated interest in the association of HCMV with atherosclerosis (41, 42). Although the role of HCMV in the development of atherosclerosis is controversial, the presence of virus in the vessel walls suggests that arteries may serve as sites for viral persistence.

Results from studies investigating the growth of HCMV in endothelial cells support a role of endothelial cells as an important reservoir of HCMV in the host. These studies showed that endothelial cell origin has a major influence on the characteristics of HCMV replication in this cell type. Endothelial cells exhibit phenotypic differences that are dependent on the source (adult versus fetal), vessel size (micro- versus macrovascular) and anatomical location of the endothelial cells (43, 44). For example, human brain microvascular endothelial cells (BMVEC), which together with astrocytes compose the blood brain barrier, possess specific transporter systems to allow the transfer of specific metabolites from the blood to the brain parenchyma. Due to these specialized functional requirements, BMVEC are functionally and biochemically distinct from endothelial cells derived from other anatomical locations such as aortic macrovascular endothelial cells (AEC) (43-46). Studies in our laboratory comparing HCMV infection in BMVEC and AEC have shown that HCMV replication differs dramatically between these two cell types (47). Although both BMVEC and AEC express viral proteins and support HCMV replication (Figure 2), virus fails to accumulate intracellularly in AEC resulting in a reduced level of cell-associated compared to supernatant virus. In contrast, virus accumulates intracellularly in BMVEC resulting in comparable levels of cell-associated and supernatant virus. This difference in the distribution of virus corresponds to HCMV infection resulting in a lytic infection in BMVEC, but not AEC, suggesting that efficient removal of mature intracellular virions (by either export or degradation) may enable prolonged cell survival. Importantly, this ability of HCMV to produce a persistent long-term productive infection in AEC suggests that AEC may represent a site of persistence within the host.

3.2. Determinants of HCMV endothelial cell tropism

HCMV replication in endothelial cells is also influenced by viral strain suggesting that specific viral gene(s) are required for efficient replication in this cell type (48-50). A number of early studies observed a loss of endothelial tropism of clinical HCMV isolates following passage in human fibroblasts - a cell type commonly used for production of high titer virus stocks. Recent studies investigating the phenotypic changes associated with HCMV passage in fibroblasts compared to endothelial cells has given some insight into the determinants of endothelial tropism (49, 50). Essentially all clinical isolates were initially able to replicate in both endothelial and fibroblast cells. However, passage in fibroblasts consistently resulted in a loss of viral ability to replicate in endothelial cells. Plaque-purification of endothelial cell tropic strains (resulting in a single clone) resulted in the stable maintenance of endothelial cell tropism even after long-term passage in fibroblasts (49). Consequently, the loss of endothelial tropism appeared to result from the selection of viral variants from the unpurified patient isolates as opposed to selection of mutant viruses following *de novo* mutation or non-genetic alteration of virion composition. Additionally, these studies showed that recent clinical isolates exhibited a gradation in their ability to replicate in endothelial cells suggesting that endothelial tropism is mediated by more than one viral gene (49). The existence of multiple genes directing endothelial cell tropism is supported further by the observation that co-infection of endothelial cells with two distinct non-endothelial tropic viruses resulted in production of an endothelial-tropic recombinant virus (49). This result could be explained by two mutations present in the same gene having been repaired by the recombination event. However, the more likely scenario is one of recombination relocating two distinct genetic loci required for endothelial tropism within the same recombinant genome.

As an initial step to identifying the genetic determinants of endothelial cell tropism, a number of studies have compared the growth characteristics of endothelial and non-endothelial tropic HCMV strains (51-53). Although both virus types were shown to be comparable in their ability to enter endothelial cells, the non-endothelial strains were impaired in their ability to translocate the viral genome to the nucleus. The specific viral factors responsible for this difference in nuclear transport, as well as other genetic determinant(s) required for HCMV endothelial cell tropism, have not been defined. However, some insight can be gained from studies

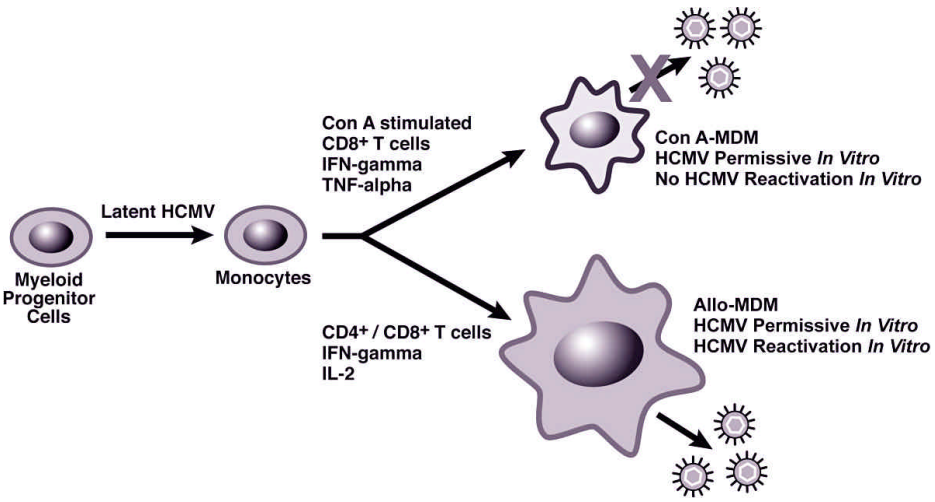

Figure 3. Schematic showing cellular and cytokine factors necessary for generation of Con A- and Allo-MDM.

performed in the related mouse cytomegalovirus (MCMV) model. In this system, a novel random transposon mutagenesis screen of the MCMV genome in a bacterial artificial chromosome (BAC) identified a MCMV gene (M45) with homology to ribonucleotide reductase that was necessary for MCMV growth in endothelial cells *in vitro* (54). Endothelial cells infected with M45 deletion mutants exhibited rapid cellular apoptosis indicating that this gene enabled MCMV replication in endothelial cells by preventing apoptosis of the infected cell. Interestingly, M45 was also required for normal MCMV *in vitro* replication in macrophages, but not fibroblasts, bone marrow stromal cells or hepatocytes. This observation suggests that viral determinants of endothelial and macrophage tropism may be closely linked, which may reflect the common derivation of these two cell types. Recently, the MCMV homologue of HCMV UL36 (M36) has also been shown to be required for efficient replication in endothelial cells and macrophages (Dr. Markus Wagner, University of Munich, personal communication). In these studies, M36 was also shown to have an anti-apoptotic function suggesting that the ability to inhibit apoptosis is a common requirement for growth in endothelial cells and macrophages. The role of the M45 and M36 HCMV homologues (UL45 and UL36, respectively) in cellular tropism has not been investigated. However, four HCMV proteins (IE1, IE2, pUL36/vICA and pUL37/vMIA), which include the product of the UL36 gene, have been shown to inhibit apoptosis following over-expression of the recombinant protein (55-57). The role of these proteins in HCMV cellular tropism has not been investigated.

3.3. Growth of HCMV in macrophages

3.3.1. CON A-MDM

A major focus of our laboratory has been the generation and characterization of macrophage culture systems that can support HCMV replication *in vitro*. The first primary MDM system that was able to support HCMV replication *in vitro* used co-cultivation with Con A-stimulated autologous non-adherent peripheral blood mononuclear cells (PBMC) to induce monocyte differentiation (58). HCMV infection of these MDM (designated Con A-MDM) was productive (although at a low frequency; <5%), nonlytic and virus remained exclusively cell-associated (58, 59). Generation of the HCMV-permissive Con A-MDM phenotype required $CD8^+$ T lymphocytes and the cytokines IFN-gamma and TNF-alpha (but not IL-1, IL-2, TGF-beta or GM-CSF) (59). Furthermore, addition of either recombinant IFN-gamma or TNF-alpha to monocyte cultures produced MDM that were comparable to Con A-MDM in their level of virus production (59). These observations suggest a model wherein production of IFN-gamma and TNF-alpha by Con A-activated $CD8^+$ T lymphocytes induces monocyte differentiation into HCMV permissive Con A-MDM, and indicate a critical role of immune stimulation for the production of HCMV-permissive macrophages (Figure 3). However, a concern with the Con A-MDM system was the inability to reactivate HCMV from latently infected Con A-MDM of sero-positive individuals. This inability to reactivate virus from latently infected monocytes combined with the relatively low level of infection *in vitro* suggested that the Con A-MDM culture system induced a state of monocyte differentiation that was suboptimal for production of HCMV.

3.3.2. ALLO-MDM

More recently, we have developed a MDM system that is permissive to HCMV replication and enables reactivation of HCMV from latently infected $CD14^+$ monocytes (32). In this system, PBMC are allogeneically stimulated by coculture of two HLA-mismatched donor PBMC populations followed by isolation of the resultant MDM (Allo-MDM). Allo-MDM express both macrophage (CD14/CD64) and dendritic (CD1a/CD83) cell markers. In contrast to Con A-MDM, *in vitro* infection of Allo-MDM is characterized by vigorous virus replication in a large number of cells (>50%) (Figure 4). This infection is lytic and results in the release of extracellular virus (60). Importantly, latent HCMV can be reactivated from Allo-MDM, which is not possible using other MDM culture systems (Figure 3) (32, 59, 60). These studies emphasize the importance of differentiation pathway on characteristics of HCMV infection and reactivation in MDM.

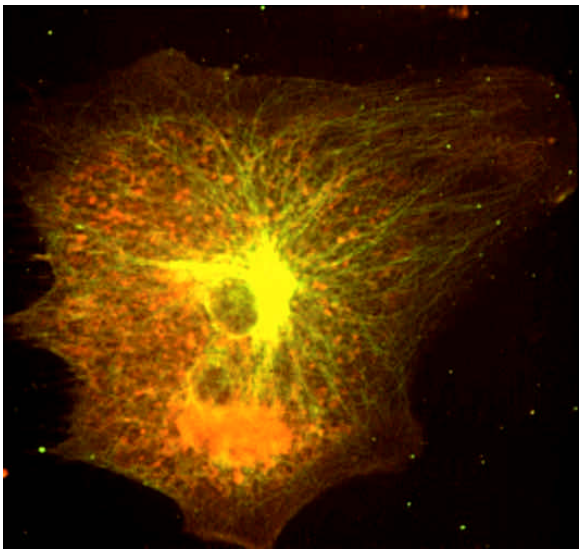

Figure 4. HCMV infection in $CD14^+$ Allo-MDM. Immunofluorescence microscopy showing infection of Allo-MDM observed by presence of HCMV antigen (gB; red) in a macrophage counterstained for alpha-tubulin (green).

Cellular depletion and cytokine neutralization experiments showed that both $CD4^+$ and $CD8^+$ T lymphocytes and the cytokines IFN-gamma and IL-2 (but not IL-1, TNF-alpha or TGF-beta) were required for generation of the HCMV-permissive Allo-MDM phenotype (60). While these cytokines were shown to be required for induction of an Allo-MDM phenotype critical for the support *in vitro* HCMV infection, reactivation of latent virus from these cells appeared to require additional factors. IFN-gamma (but not IL-1, IL-2, TNF-alpha, TGF-beta or GM-CSF) was shown to be necessary within the first 48 hours of allogeneic stimulation for efficient reactivation of latent HCMV. However, IFN-gamma alone was not sufficient for the induction of a MDM phenotype capable of reactivating virus. Allo-MDM-conditioned media was able to induce HCMV reactivation in the absence of allogeneic stimulation. This result indicates that soluble factors released during allogeneic stimulation are sufficient for production of a MDM phenotype capable of HCMV reactivation. Analysis of the cytokine profiles released during Allo- compared to Con A-MDM differentiation have revealed considerable differences in the kinetics as well as in the type of cytokines released by the two differentiation pathways. Interestingly, IL-13 was observed only in Allo-MDM cultures. Since IL-13 has previously been shown to increase HCMV protein expression in alveolar macrophages *in vitro*, this cytokine may be important in the reactivation of HCMV from latency (61). Together, these studies of Allo-MDM differentiation suggest a model (Figure 5), wherein latently infected monocytes in the peripheral blood are activated by an immune response (ie., during blood transfusion). During this response, $CD4^+$ and $CD8^+$ lymphocytes play a critical role with IL-2 released from $CD4^+$ lymphocytes inducing the release of IFN-gamma. The IFN-gamma, in addition to other soluble factors, then mediates induction of the Allo-MDM phenotype required for reactivation of latent virus.

Since monocytes are unable to replicate and are short-lived, the presence of latent virus in $CD14^+$ monocytes suggests that HCMV is maintained in a renewable or long-lived precursor population of the myeloid cell lineage. HCMV has been reported to infect $CD34^+$ pluripotent stem cells *in vivo* (62, 63). However, as $CD34^+$ stem cells are a common precursor of all peripheral blood cell types, the absence of virus from many peripheral cell lineages suggests that $CD34^+$ stem cells represent a minor site of HCMV persistence or latency (16, 18, 63, 64). Another potential site of HCMV latency are $CD33^+$ myelomonocytic lineage granulocyte-macrophage progenitor cells (GM-Ps). HCMV has been shown to infect GM-Ps *in vitro*, and these cells produce infectious HCMV following differentiation into $CD14^+$ macrophages (65, 66). CMV latency-associated transcripts (CLTs) that map to a specific region of the viral genome were detected in the *in vitro* infected GM-Ps. However, CLTs are not observed in Allo-MDM, and inactivation of the major conserved gene expressed from CLTs (ORF94; UL126a) did not effect HCMV reactivation in an *in vitro* GM-P latency model. These results indicate that CLTs play a minimal role in regulation of HCMV latency and reactivation (67).

3.4. Determinants of HCMV macrophage tropism

Currently, viral genes associated with growth in MDM have been identified only in the MCMV system. In addition to M45 and M36 (see above), M140 and M141 gene products (pM140 and pM141) have been shown to be required for normal *in vitro* replication in macrophages (54, 68-70). In animal studies, M140 and M141 were required for normal replication in the macrophage-rich spleen, but were not required for replication in the liver suggesting their role in mediating macrophage tropism *in vivo*. (68). Importantly, M140 and M141 were required for normal virus pathogenicity suggesting an important role of virus macrophage tropism in the disease process. The pM140 (56 kDa) and pM141 (52 kDa) are homologues of HCMV genes in the US22 family. Although the mechanisms by which these proteins mediate macrophage tropism are unknown, pM140 appears to have a dual role, which includes stabilization of pM141. The function of the HCMV US22 genes in viral replication in macrophages is unknown.

4. SUMMARY AND FUTURE DIRECTIONS

Endothelial cells and macrophages appear to play an important role in enabling HCMV to maintain a lifelong infection of the host by serving as sites of persistent infection and viral latency. The ability of HCMV to produce a long-term productive infection in AEC suggests that endothelial cells may represent a site of persistence within the host. The capacity to reactivate HCMV from latently infected $CD14^+$ monocytes identifies the macrophage as an important source of latent virus that appears to be critical for spread of infection through the host. Results from both of these culture systems emphasize the importance of the specific cellular environment in

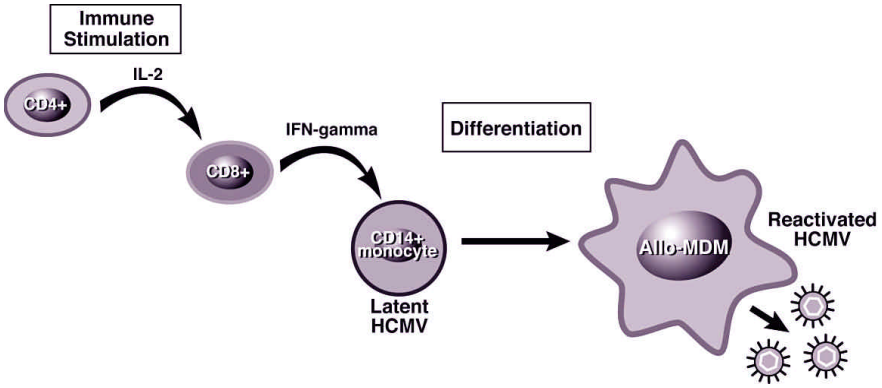

Figure 5. Model for reactivation of latent HCMV *in vivo*. Immune response is activated by allogeneic reaction or response against pathogens. This activation event induces production of IL-2 by $CD4^+$ lymphocytes stimulating IFN-gamma secretion by $CD8^+$ lymphocytes. In combination with other factors, IFN-gamma induces $CD14^+$ monocytes to differentiate into Allo-MDM reactivating latent virus resulting in the production of infectious virus.

determining the characteristics of HCMV infection. Cell origin and pathway of differentiation are critical factors for HCMV persistence in endothelial cells, and for viral latency and reactivation in MDM. Viral genetic determinants are also important in enabling HCMV replication in these cell types. Although the specific HCMV genes required for replication in endothelial cells and MDM have not yet been determined, a number of novel tropism genes have been identified in the closely related MCMV model. Identification of the viral mechanisms that enable growth in endothelial cells and MDM, and the role of endothelial and MDM tropism in viral pathogenesis represent an exciting area of ongoing research.

5. ACKNOWLEDGEMENTS

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