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Cytomegalovirus latency and reactivation: Recent insights into an age old problem

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Summary

Human cytomegalovirus (HCMV) infection remains a major cause of morbidity in patient populations. In certain clinical settings it is the reactivation of the pre-existing latent infection in the host that poses the health risk. The prevailing view of HCMV latency was that the virus was essentially quiescent in myeloid progenitor cells and that terminal differentiation resulted in the initiation of the lytic lifecycle and reactivation of infectious virus. However, our understanding of HCMV latency and reactivation at the molecular level has been greatly enhanced through recent advancements in systems biology approaches to perform global analyses of both experimental and natural latency. These approaches, in concert with more classical reductionist experimentation, are furnishing researchers with new concepts in cytomegalovirus latency and suggest that latent infection is far more active than first thought. In this review we will focus on new studies that suggest that distinct sites of cellular latency could exist in the human host which, when coupled with recent observations that report different transcriptional programmes within cells of the myeloid lineage, argues for multiple latent phenotypes that could impact differently on the biology of this virus in vivo. Finally, we will also consider how the biology of the host cell where the latent infection persists further contributes to the concept of a spectrum of latent phenotypes in multiple cell types which can be exploited by the virus.

Introduction: the opportunistic pathogen

The herpesvirus human cytomegalovirus (HCMV) represents a very common infection exhibiting a seroprevalence of 0-100% depending on socioeconomic status. Primary infection of healthy individuals with HCMV is usually asymptomatic but results in the establishment of a lifelong infection of the host [1]. HCMV is also highly immunogenic with infection and persistence leaving a large indelible mark on both $CD4^+$ and $CD8^+$ T cell compartments of seropositive individuals [2]. In stark contrast with the asymptomatic infection of healthy individuals, congenital infection or infection of immunocompromised patients can result in significant morbidity and mortality [1, 3, 4]. As well as primary infection, a profound disease burden is also associated with the reactivation of infectious virus within latently infected individuals - particularly in allograft bone marrow transplant patients [5]. Similarly, a 2011 meta-analysis of congenital HCMV infections in the US (between 1988-1994) estimated that only 25 % of HCMV cases found at birth resulted from

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maternal primary infection during pregnancy which highlights the importance of understanding the impact of non-primary infections: i.e. re-infection and reactivation from latency in seropositive mothers [6].

HCMV latency and reactivation represents both clinical problem and challenging academic riddle. Significant research efforts have been directed towards developing an understanding of the mechanisms that are involved in the establishment and maintenance of viral latency as well as the fundamental principles that govern the reactivation of latent virus. These strategies have involved studies in primary human tissue and cell culture models, as well as using animal model systems of CMV which, all told, have generated a fascinating insight into the enigmatic problem of latency. From the beginnings of understanding the cellular basis of latency onto more recent studies that have utilised powerful systems biology approaches to probe the molecular details the story of HCMV latency and reactivation is unravelling. In this review we will focus on the recent identification of a number of viral and cellular gene products that are active in latently infected cells - including the detection of both viral and cellular non-coding RNAs – and how they contribute to the latent phenotype. Furthermore, we will consider how the identification of these functions impacts on our understanding of HCMV latency with particular emphasis on the concept that multiple latent phenotypes may exist within the host. Finally, we will illustrate how these new insights resonate with studies in the alpha and gamma herpes virus families through shared viral and cellular functions or mechanisms that help govern the latent state.

Keep your friends close…

A key characteristic of human herpesvirus infection is the ability to establish a lifelong latent infection in the host. The establishment of herpes virus latency can occur in multiple cell types with the alpha herpesviruses exhibiting a neuronal tropism whereas the cells of the haematopoietic system represent important reservoirs for the beta and gamma herpesviruses. Although the cellular identity of the latently infected cell can vary, latency at a molecular level is characterised by overall suppression of viral lytic gene expression attributable to epigenetic regulation via histone modification machinery, a very limited but specific transcriptional profile during latency, and a responsiveness to host derived cues to exit latency and re-enter the lytic lifecycle [7–9].

Similarly, at a molecular level, the mechanisms governing latency/persistence during murine CMV (MCMV) infection appear to have resonance with studies of HCMV also – if not least when considering the molecular mechanisms that dictate the regulation of major immediate early (MIE) gene expression required for full lytic infection [10]. In contrast, a compelling comparative narrative at the cellular level is less clear [10]. Whereas HCMV latency in the haematopoietic cell lineage well established the same is not true of MCMV where studies of latency have focused on the role of endothelial cells of a number of organs. Of course, it is entirely possible that the nature of studying HCMV in the human host directly renders it difficult to address whether other cell types, alongside cells of the haematopoietic system, are also sites of viral latency (Figure 1). For instance, attempts to study human endothelial cells have proven somewhat inconclusive. Although HCMV genomes could not be detected in endothelial cells isolated from saphenous vein tissue [11], in vitro studies suggest that

subsets of endothelial cells – for instance aortic – could support a latent or at least a persistent, non-lytic, infection [12]. Thus, the vascular origin of the endothelial cells could be important and suggests that an analysis of circulating endothelial cell progenitors in the peripheral blood may reveal a further site of HCMV latency and represents a tractable question to address.

Finally, it is important to recognise that multiple sites of viral latency could exist in the host. Although many studies of MCMV latency focus on endothelial cells this does not preclude myeloid cells as also being important. The detection of MCMV genomes in bone marrow and macrophages has been observed and, importantly, these genomes can be reactivated [13]. Recent work analysing MCMV infection suggested that MCMV hijacked the function of a subset of monocytic cells (‘patrolling monocytes’) to promote viral dissemination [14]. The inference from this study is that the immuno-privileged phenotype of the patrolling monocytes exploited for dissemination could also be important for the establishment of long term persistence in the host. Although a preceding study reported that patrolling monocytes were not important sites of MCMV reactivation, this study focused on liver tissue and thus it is plausible that specific cell types contribute to reactivation in discrete organs within the host and, secondly, may point towards a specific role for monocytes in the seeding of MCMV latency in endothelial cells rather than as a site of long term latency themselves.

Finally, interesting recent experimental studies also suggest that specific neuronal progenitors may be another potential site of latency. A key aspect is that the origin (fetal versus embryonic stem cells) of the neural progenitors allied with the nature of the differentiation stimuli applied appears to influence the outcome of infection [15–18]. Importantly, the nature of the infection of neuronal cells may have implications for understanding the pathogenesis of HCMV congenital infections. Furthermore, resolving the differences between the distinct types of neuronal cells and how they respond to reactivation stimuli could have wider impact on our understanding of HCMV reactivation in multiple cell types – particularly given that the recent study [17] identified multiple blocks to reactivation of infectious virus which resonates with studies of latency in myeloid cells [19, 20]. However, interrogating these sites ex vivo is somewhat more challenging than the haematopoietic system due to almost prohibitive access to the material required to perform the same analyses that have defined the cells of the haematopoietic lineage as sites of HCMV latency.

The silent virus?

Perhaps the most significant advance in our recent understanding of HCMV latency is the contribution of viral functions to this process – an area of study which, until recently, was in contrast to the arguably much better defined patterns of gene expression observed with the alpha and gamma herpesvirus subfamilies. The most intensively studied of all latent transcripts is the latency associated transcript (LAT) of herpes simplex virus – acting as a non-coding RNA that, once subjected to RNA processing, exerts an impressive number of reported functions including anti-apoptotic effects, heterochromatic modification of histones as well as the generation of virally encoded miRNA species with the potential to regulate viral and cellular gene expression [8, 21–23]. Similarly, for the gammaherpes subfamily,

untranslated RNAs have been identified during viral latency [24, 25]. It is of note that a number of alternate transcriptional programmes have been described for EBV based upon the analysis of transformed cell lines but, at least in long-term healthy carriers, the detection of lymphocytes expressing EBV proteins is quite sporadic and is usually restricted to EBNA-1 and LMP-2A positive cells [26, 27]. Evidently, the expression of non-coding RNAs during latency provides a sophisticated mechanism for modulating the host cell environment without attracting an immune response against the latently infected cell.

Does HCMV express functional untranslated RNAs during latency?

There are now a number of studies that have reported latent gene expression in various experimental latent systems [28–33] a number of which have also been detected in natural latency [29, 32, 34–37]. However, the most recent addition to this increasing repertoire of genes expressed during latency was provided by a provocative study utilising an RNAseq analysis of HCMV transcription during both experimental and natural latency [33]. Amongst these were non-coding RNAs including beta 2.7 which was a predominant transcript in naturally latent CD14+ cells (interestingly, a transcript that was not identified in the first studies that showed monocytes as a site of persistence in vivo [19]) and experimentally latent CD34+ cells (exhibiting between 20-30x the number of ‘reads’ detected for UL138 – an accepted latent gene product [35]). Similarly, beta 2.7 was detected in CD34+ cells isolated from the peripheral blood of healthy volunteers although at relatively lower levels than observed for UL138. Indeed it is interesting to note whilst overlap was observed with the experimental and natural latency transcriptional profiles they are not equivalent. One possible explanation is the analysis of different cell types (cord (experimental) versus mobilised (natural) CD34+ cells) which could be impacting on the expression profiles of the viral genes. That aside, does HCMV infection render latently infected myeloid cells resistant to the action of rotenone (and other mitochondrial complex I inhibitors) through extrapolation of the function of beta 2.7 in infected neuronal cells [38]? One could speculate that if beta 2.7 is expressed during latency it may be particularly important for the protection of neuronal progenitor cells from cell stress if these are indeed sites of persistence in vivo and – thus displaying some functional similarities with the LAT of HSV [23].

A second non-coding transcript identified was lnc4.9 [33]. Intriguingly, the authors hypothesise that (in concert with the novel latent expression of UL84) lnc4.9 interacts with members of the polycomb repressor complex 2 (PRC2). PRC2 is one of two complexes (the other being PRC1) that control gene expression. The PRC2 comprises 4 subunits with histone binding and histone methyltransferase activity [39]. Thus the interaction of lnc4.9 with PRC2 is hypothesised to promote extensive histone methylation (H3-K27) at the major IE promoter (MIEP) [33] which would contribute to the known epigenetic silencing of IE gene expression during the establishment of latency [40, 41]. Again, there are similarities with the role of LAT and the establishment of facultative chromatin on HSV promoters during latency [42] although the HSV studies show that the initial recruitment and silencing by PRC2 was independent of a physical interaction with the LAT RNA in HSV latency [42]. Additionally, the Pan RNA encoded by KSHV promotes the formation of facultative chromatin on the Rta promoter to induce silencing [43] thus highly similar mechanisms appear to be active across the herpesvirus family.

As well as the expression of long non-coding RNAs during latency there is emerging evidence that HCMV also expresses a repertoire of miRNAs during all phases of infection [44, 45]. These have both viral and cellular targets and thus provide the ability to 'fine tune' the cellular environment to optimise viral replication or persistence. Pertinently, the virally encoded mir112.1 targets the UL123 mRNA preventing translation of the IE72 protein [46] with major implications for the control of MIE gene expression (i.e. UL122 and UL123) during latency [47].

One prevailing view of the regulation of viral gene expression during latency is that higher order chromatin structure and associated functions are important for maintaining MIEP quiescence and is supported by a number of observations that link chromatin with the latent phenotype [33, 40, 41, 48]. Therefore it is intriguing that HCMV exploits additional strategies that contribute to the regulation of the IE gene products during latent infection in vitro. There is the inhibitory effect of mir112.1 on IE72 expression [46, 47]. Furthermore, more recent work from the Murphy laboratory using the Kasumi-3 cell line model they have established to interrogate HCMV latency [49], has postulated that an abundance of a cellular miRNA – mir200 – targets the UL122 transcript for degradation thereby preventing IE86 protein expression also [50]. Thus, the expression of both MIE products IE72 and IE86 is targeted during latency by miRNAs. Although previous work has suggested that the cellular miRNAome is modulated by latent infection in vitro [51] no changes in mir200 were observed suggesting that this miRNA represents a naturally abundant species in progenitor myeloid cells that contributes to the latent phenotype of HCMV. Again, the presence of a cellular miRNA at high levels contributing to viral latency resonates with studies in HSV where the neuronal specific mir-138 species targets the ICP0 gene product to support latency [52]. It remains important that when identifying functions using cell lines in experimental latency further insight into an understanding of their precise contribution to latency in the host is highly dependent on the use of physiologically acceptable and predictive cellular models as well as verification in studies of cells from natural human infections to begin to appreciate their contribution to CMV latency in vivo.

Expression of viral proteins during latency modulates the cellular environment

The regulation of the MIEP during latency involves multi-faceted integration of viral and cellular functions that act concomitantly to generate a phenotype that promotes latent infection. However, it is now clear that a discrete set of protein coding transcripts is expressed during latency [29, 32, 34–37]. A number of these gene products are involved in the manipulation of the cellular environment to re-direct the immune response (e.g. US28 [53] and ORF94 [54]) or hijack cellular signalling pathways involved in immune recognition (e.g. UL144 and Nf-kB [55]; UL138 and TNFRI [56, 57]) during lytic infection. In contrast, only the viral interleukin-10 homologue LAvIL10 (UL111.5A) has been shown to have a defined role during latent infection (see [58] for review). Many of the functions of LAvIL10 are analogous to the roles defined for its cellular counterpart and likely contribute to the immune-suppressive phenotype generated in, and around the microenvironment of, a latently infected cell in vitro [59–61]. Furthermore, it is hypothesised that LAvIL10 and cIL10 may

act in unison to drive a more expansive range of outcomes from providing protection from cell death stimuli, driving latent gene expression to propagating the unique cellular miRNA landscape observed in latently infected CD34+ cells [51, 62, 63].

The repertoire of latent functions was expanded by, perhaps, the most provocative observation in the recent RNAseq study regarding the detection of IE1 sequences in the naturally latent CD34+ cells analysed – initially suggesting that the MIE region is active in the cells analysed in this study [33]. The reported detection of UL123 RNA was discrepant with a number of previous studies that show major IE gene expression is undetectable in the cells of naturally latent individuals [19, 29, 40, 64]. Importantly, more recent evidence at least addresses these concerns in part. A protein product arising from exon 4 of the MIE (IE1ex4) is detectable in CD34+ cells and that the expression of the coding transcript is under the control of a promoter distinct from the MIEP [65]. It was speculated that this region was important for maintenance of the viral genome during latency with the IE1ex4 gene product having important tethering functions analogous to those observed with KSHV gene product LANA [66]. Importantly, these studies also suggested evidence for latent replication (hence the need for genome maintenance) in their system [65]. The posit of latent replication is at odds with a number of studies in primary cells and cell lines in vitro that show a reduction in the frequency of genome positive cells following long term culture and expansion of the cells [49, 67, 68]. Similarly, the low frequency of genome-positive cells in vivo would also suggest that replication during latency is limited [69]. Possibly, there may be genome replication during latency which, whilst inefficient, is sufficient to contribute to the maintenance of the latent pool and the maintenance of genome positive cells as they transit into the periphery. By analogy with EBV this argument has merit. EBNA1-dependent replication is clearly recognised as an important contributor to the maintenance of the EBV latent pool [70] yet the frequency of EBV genome positive B cells of 1:10000 or lower [71] is consistent with those reported for HCMV [69]. Furthermore, the retention of a chromatin tethering domain in the IE72 protein that is dispensable for lytic infection may suggest an important function during latent infection [72].

What is not clear how the amino acid sequence of the latent IE1 sequence compares with classical IE72 although its detection with an exon-4 specific antibody suggests high similarity [65]. This becomes important when considering the prodigious immune response directed against IE72 [73–75] which, if also present in the latent IE1, would presumably flag the latent cell to the immune system. Possibly, the multi-faceted antagonism of the immune response in the micro-environment of the latent cell [59, 76] would afford some protection from recognition if the VLE epitope [73] was generated from latent IE1.

Viral reactivation – has HCMV perfected an exit strategy?

It is becoming increasingly clear that the virus is directing significant activity towards the maintenance of viral latency in myeloid progenitor cells. However, the other arm of the conundrum is the exit from latency and reactivation of the lytic lifecycle. A key trigger of this event is the cellular differentiation to a differentiated macrophage or dendritic cell [19, 40, 64, 77–80] suggesting that changes in the cellular environment are promoting the reactivation event (figure 1). At the most simplistic level, the first event that must likely

occur is that the MIEP must transition from a repressed to an active promoter state for robust lytic IE gene expression to occur. Here it is important to de-lineate between the control of the MIE products IE72 and IE86 and the identified IE1ex4 tethering function identified recently [65] where it is hypothesised that the regulation of the IE1ex4 is via a cryptic promoter in the coding region of the MIE rather than the MIEP.

However, we must also consider whether the MIEP is completely silent in latently infected cells – or rather that transcription is substantially reduced to undetectable levels in our assay systems. This concept of RNA Polymerase II activity occurring from a 'repressed' promoter has been postulated for cellular promoters repressed in ES cells [81] and RNA Polymerase II binding to the MIE region has been reported in latently infected cells [65]. In the analysis of ES cells, evidence of transcription did not result in functional outputs and, similarly, there is no evidence of IE72 and IE86 protein expression during latency although several transcripts have been identified to arise from this region in latently infected cells [28, 33, 36] – the expression of which is thought to be independent of regulation by the MIEP. Whether the MIEP is completely repressed or is exhibiting low level activity during latency is not definitively known however it is clear that the activity of the MIEP substantially higher in terminally differentiated myeloid cells but not their progenitors and this change in activity is considered a pivotal event for viral reactivation.

The MIEP is a complex promoter which contains multiple binding sites for both cellular transcriptional repressors and activators [82]. Evidently, based on studies of the chromatin structure around the MIEP, the predominant cellular (e.g. YY1/ERF and histone methyltransferases) and viral (e.g. lnc4.9 and PRC2) activities during latency drive a chromatin signature that is highly repressive [40, 83, 84] supporting the latent phenotype (figure 2). So the question remains: how does the MIEP become activated? Although it has been demonstrated that reactivation of HCMV is concomitant with extensive histone de-methylation and acetylation at the MIEP [40] it does not illuminate the mechanism that drives the switch in the chromatin phenotype. However, if we reason that the MIEP behaves akin to a cellular inflammatory promoter – and, in essence, consider the MIEP as another genetic element influenced by the same mechanisms that control eukaryotic gene expression – we can begin to unravel its regulation. Firstly, the MIEP is responsive to a number of inflammatory stimuli [85–87] and, furthermore, HCMV reactivation and disease is associated with highly inflammatory environments [78, 88, 89]. Secondly, the reactivation of HCMV IE gene expression is seen efficiently in dendritic cell types in vitro [40, 77, 87, 90] – a cell type that is a prodigious producer of inflammatory cytokines following stimulation. In itself this seems a minor link except when we consider the activation of inflammation requires the de-methylation and subsequent acetylation of histones bound to these cellular promoters and that this can occur in a mitogen activated kinase, NF-kB and CREB dependent manner [91]. Our own recent work has illustrated that the activation of ERK-MAPK signalling in DCs plays an important role in HCMV reactivation [48, 87] and built on previous studies suggesting that the CREB transcription factor was an important mediator of viral reactivation [92, 93]. Furthermore, studies using experimentally latent cell lines as well as clinical data suggest enhanced NF-kB signalling correlates with HCMV reactivation [85, 89, 94]. All these data would be consistent with the hypothesis that the HCMV MIEP is mimicking the promoters of cell-encoded inflammatory genes.

An epigenetic platform for signal integration – and induction of viral gene expression

When considering the regulation of gene expression – whether it is eukaryotic or viral – the nature of the signalling response is a key determinant. There are multiple outcomes associated with any specific signalling event which, in turn, are dictated by the cellular and intra-cellular phenotype. By way of example, there are approximately 4,000 CREB responsive genes in the human genome yet addition of a potential cAMP agonist does not trigger the uniform expression of the said 4,000 genes [95]. Key to the differences are multiple signalling events acting co-operatively to generate a very specific output which will be determined by the cellular receptors expressed on specific cell types as well as the availability of downstream signalling molecules to elicit function [96–98]. Additionally, the abundance of post-translational modifications on histone proteins in contrast to the very few outputs available (i.e. gene expression versus no gene expression) hints at the chromatin structure playing a key role in signal integration and defining the nature of the output in specific circumstances [99]. Thus critical for the understanding of the impact of any signalling pathway on gene expression requires an appreciation of the cellular and molecular context in which that pathway is being activated.

Our own work has focused on the role phosphorylation of two key proteins could play in the reactivation of HCMV by providing a platform for signal integration at the MIEP [48]. In essence, we proposed that histone phosphorylation was a key event to mediate the switch from a repressed to an active chromatin form. Importantly, IL-6 stimulation of the ERK-MAPK pathway in DCs does not promote global histone phosphorylation but, instead, is targeted to CREB-responsive promoters (i.e. the MIEP) through the activity of mitogen and stress activated kinases [48] (figure 3). The similarities with the c-FOS cellular promoter (coincidentally, itself a cellular ‘immediate early’ response gene) suggest that this is an event associated with ‘rapid response’ promoters [100]. Although these studies begin to hint at the mechanism involved, the full extent of signal integration required to elicit this response of the MIEP in DCs remains to be understood. The high density of post-translational modifications on histones heavily influences the activity of ‘reader’ functions associated with the regulation of gene expression [99]. The specific recruitment of reader proteins, in turn, enlists the modifying enzymes necessary for generating the signal and cell type specific responses we observe. A prescient example is the role of histone H3 serine 10 phosphorylation. This phosphorylation event de-stabilises the binding of the repressor HP-1 [101, 102] and also promotes recruitment of 14-3-3-ε triggering the subsequent recruitment of transcription elongation factors [103, 104]. However, the studies cited only address the reversal of HP-1 mediated silencing. As has recently been shown, the PRC2 complex also may play a role in the silencing of HCMV gene expression [33, 105]. Thus are similar mechanisms required to reverse PRC2 activity? This supposes that both modifications (HP-1 and PRC2) are active within the same MIEP (figure 2) which could not be the case based on the proposed mutual exclusion of lysine 9 and lysine 27 methylation on the same histones at cellular promoters [106, 107]. If so, then the proposed mechanism for alleviation of HP-1 silencing [48] may only reactivate a subset of viral MIEPs, i.e. those bound predominantly by HP-1. Alternatively, PRC2 may be regulated by the same mechanisms since,

architecturally, histone H3 serine 10 and histone H3 serine 28 look highly similar [107] expressing the same ARKS domain (alanine; arginine; lysine; serine). Indeed, MSKs also phosphorylate histones at serine 28 (which, like its serine 10 counterpart, is adjacent to a key lysine (27) residue that is tri-methylated) and thus may trigger similar effects as observed with serine 10 phosphorylation [108]. Contrastingly, recent data also suggests that PRC2 may be an important co-factor for the binding of HP1 to trimethylated H3-K9 arguing for these marks at least acting co-operatively [109]. Indeed, this final scenario provides the most attractive explanation for the recruitment of PRC2 to the MIEP by lnc4.9 [33] in light of the known recruitment of HP1 to the MIEP during latency [40].

An interesting aspect of all such signalling mechanisms is the feedback loops encoded within them. For instance, following mitogenic stimulation, histone phosphorylation on target promoters is a transient event declining one hour post stimulation through the concomitant activation of phosphatases [110]. We note that in our studies of the MIEP during reactivation histone phosphorylation was more prolonged in comparison [48]. It would be interesting to determine whether this is just a result of asynchronous induction in a total analysed population or whether HCMV actively manages the cellular environment to favour sustained activation by a concomitant down-regulation of the negative feedback loop. During lytic infection, HCMV actively manages the chromatin landscape on the viral genome [84, 111–114] – largely via the activity of IE72 and IE86 – and thus it is possible latent functions contribute to a phenotype more conducive for pro-reactivation stimuli. This in itself would be consistent with the common theme that pathogens hijack signalling pathways by isolating and re-directing the facets that are beneficial away from the non-beneficial aspects.

However, as it currently stands, the study of higher order chromatin structure and the control of either viral or cellular gene expression are potentially approaching an impasse. Current techniques rely on global analyses of cell populations that are fixed in time which, due to the low throughput nature, can only analyse relatively large time frames. As such, it cannot be determined whether a number of observations play a functional role or are bystander effects that are essentially passive in the process. The ability to image this on a single cell scale and watch changes in real time will massively impact on this. A recent study elegantly demonstrated that RNA Pol II activation is indeed regulated by histone acetylation [115] suggesting that the study of chromatin dynamics at the single cell scale is possible. Applying this to study the regulation of the MIEP in real time will substantially illuminate our understanding of the factors directly regulating viral – and, more broadly, eukaryotic – gene expression. However, the application of this approach for studying HCMV will additionally rely heavily on the future development of new techniques that facilitate the isolation and enrichment of HCMV genome positive cells – a technical hurdle that has not yet been overcome.

Fighting on multiple fronts

An incorrect assumption would be that there is one de facto mechanism required for HCMV reactivation. This seems unlikely to be the case especially if the concept of a single latency phenotype is becoming less applicable. The reactivation of HCMV in differentiated myeloid

cells has been reported following the stimulation of multiple progenitor cell types under a variety of inflammatory conditions [31, 40, 64, 78, 79, 87]. Furthermore, the induction of IE gene expression, whilst essential for initiating reactivation, does not, by itself, dictate that infectious virus will be produced. It is highly likely that a number of viral gene products and cellular interactions are important for driving HCMV reactivation through early and late gene expression and ultimately to the production of infectious virus. Consistent with this are studies performed on both experimental and natural latency. In the very first reports studying HCMV latency ex vivo from in vitro differentiated monocytes both IE and early gene expression was detectable [19]. Similarly, the transfection of IE proteins into latently infected THP1 cells again resulted in the induction of early gene expression [20]. However, the recovery of infectious virus was not observed in either system. Indeed, the first description of the reactivation of infectious virus ex vivo from CD14+ monocytes was reported using a cytokine cocktail derived from allogeneically stimulated T cells [78]. This suggested that signalling events associated with both differentiation and inflammation are key to efficient HCMV reactivation. Arguably, cell differentiation (at least within the myeloid lineage) remains the major determinant of HCMV reactivation and that inflammation increases the efficacy of the reactivation phenotype. Indeed, more recent studies suggest that the key inflammatory mediator IL-6 has multiple effects on HCMV reactivation ranging from increased IE gene expression, less abortive reactivation events and, ultimately, more efficient reactivation of infectious progeny – in part, by altering the particle to plaque forming unit ratio of the progeny virus [68] as well as increasing the frequency of IE positive cells that transition into late stage gene expression [87] during the reactivation process.

Studies in MCMV raise interesting questions also. The systemic addition of interferon-beta to chronically infected mice had a dramatic impact on the level of reactivation in the murine model [116]. Elucidating the precise mechanism of action of systemic interferon is clearly hard to dissect. Interferon-beta has a direct impact on the replication of both MCMV and HCMV in vitro (for review see [117, 118]) – as well as many other viruses – and thus the effects observed could be due to a whole multitude of interferon-induced effects. The authors hypothesised that the well known interferon induced accumulation of nuclear domain 10 bodies could be a key factor in the observed phenotype [116]. However, failing a PML KO mouse there was no direct evidence for this phenotype, which could potentially be a combination of anti-viral effects associated with interferon activity. Furthermore, we note a recent study of the experimental infection of CD14+ monocytes which suggested that latent HCMV disabled aspects of the JAK/STAT pathway which would render them less sensitive to direct effects of interferons if the observations were to be extrapolated to HCMV [119]. Nevertheless, what the mouse model does inform is that the complex interplay between the latent cell and the extracellular environment driven by the host will be a key regulator of the latent phenotype.

If we consider the transition from viral latency to reactivation analogous to pushing a rock up a hill then the more factors that favour reactivation will push the rock towards the precipice of infectious virus production. Countering these effects will be cellular (and viral) responses that are providing resistance to progress and, ultimately, it is the dynamic changes in the activity of these processes which decide the final outcome.

Concluding remarks

The control of HCMV latency and reactivation remains a complex problem. At a molecular level the virus establishes a non-lytic infection that provides a cellular reservoir for HCMV. Key to the establishment of latency is the inhibition of the lytic lifecycle – requiring the repression of viral lytic gene expression, some form of maintenance of the viral genome – or at least maintenance of the viral reservoir through constant re-seeding. Although the themes regarding the molecular control are becoming increasingly understood: e.g. cellular factors are required to repress the MIEP; an important virion transactivator is sequestered in the cytoplasm; viral functions target the repression of the MIEP; the virus responds to signalling cues triggered by inflammation and differentiation; the integration of these themes is not so well understood For instance, what is the cellular factor that sequesters pp71 in the cytoplasm of CD34+ cells [41]? Furthermore studies of HCMV latency are often centred on the mechanisms that regulate the MIEP and less emphasis is placed onan understanding of the precise contribution of latent functions towards the maintenance of the latent state. For instance, the activity of the UL133-138 locus has been shown to contribute to the reactivation/dissemination phenotype in the humanised mouse model although the precise mechanism has yet to be elucidated [120] but is likely to contribute to the lifelong persistence of HCMV. Also, why is reactivation more efficient in differentiating cell types? – after all the pathways identified to be important for driving reactivation are also active in undifferentiated cells so what is it about DCs and macrophages specifically that makes them sites of HCMV reactivation? Finally, we are not really any closer to understanding the role of viral or cellular factors important for the transition from induction of IE gene expression in latency to the reactivation of infectious virus except from studies of the late stages of lytic infection which will have at least some overlap with the mechanisms governing HCMV reactivation.

It is becoming increasingly important to consider whether a spectrum of latent infections exists. For instance, different profiles of latent gene expression have been reported depending on the experimental system employed to identify them [28, 29, 31, 33]. More importantly, the expression of viral transcripts in natural latency also displays a level of heterogeneity which, again, appears to be dependent on the haematopoietic cell type analysed [19, 33, 36, 40]. Furthermore, these analyses always represent population analyses yet it is possible that, as described for the gamma herpes viruses, that different patterns of latent viral gene expression occur even within these populations. We noted that in a recent study defining a strategy to remove latently infected cells through the targeting of a UL138 associated function the data suggest that the elimination of the HCMV infected cells was never complete [121]. This could of course be due to the efficacy of vincristine but alternatively, could suggest a latent population with a different transcriptional profile. Additonally, latent gene expression is predicted to be dictated by the cellular transcriptional milieu thus if multiple sites of cellular latency exist (i.e. neuronal versus endothelial versus haematopoietic) then the latent transcriptional profile could be markedly different. This seems highly plausible given that transcriptional differences are observed within the different cell populations of the haematopoietic lineage alone.

As we begin to unravel the complexity of HCMV latency and reactivation, the use of the phrase ‘quiescent infection’ interchangeably with ‘latency’ is increasingly becoming a misnomer that fails to do justice to the increasingly complex and active regulation of the latent lifecycle of, and by, HCMV.

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Abbreviations used

HCMV	Human cytomegalovirus
MIE	major immediate early
LAT	latency associated transcript
MIEP	major immediate early promoter
MCMV	Murine CMV
PRC2	polycomb repressor complex 2
TNFRI	Tumour Necrosis Factor Receptor I
LavIL10	Latent viral interleukin 10
IE1ex4	IE1 exon 4
DC	Dendritic cell

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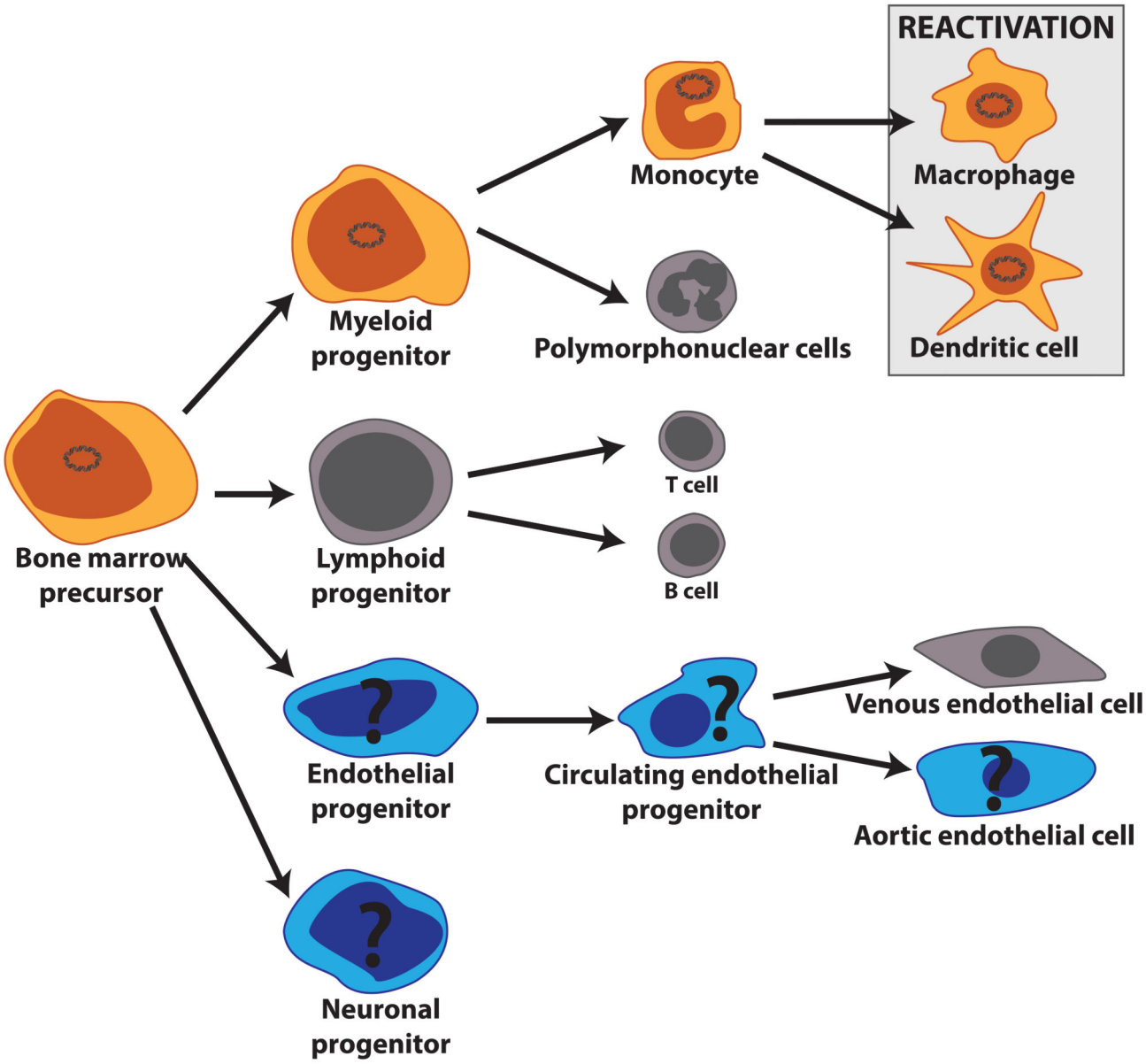

Figure 1. Human cytomegalovirus natural latency in cell lineages.

Viral latency is established in the haematopoietic progenitors resident in the bone marrow and the carriage of viral genomes has been defined in the monocyte/myeloid lineage with reactivation occurring in the terminally differentiated myeloid macrophages and DCs (Orange cells). In contrast, the viral genome is not carried in the lymphocyte population nor is there any evidence for viral latency in venous endothelial cells (grey cells). Experimental infection data suggest that endothelial and neuronal progenitor cells may also be sites of latency although no data from natural latency currently exists (blue cells).

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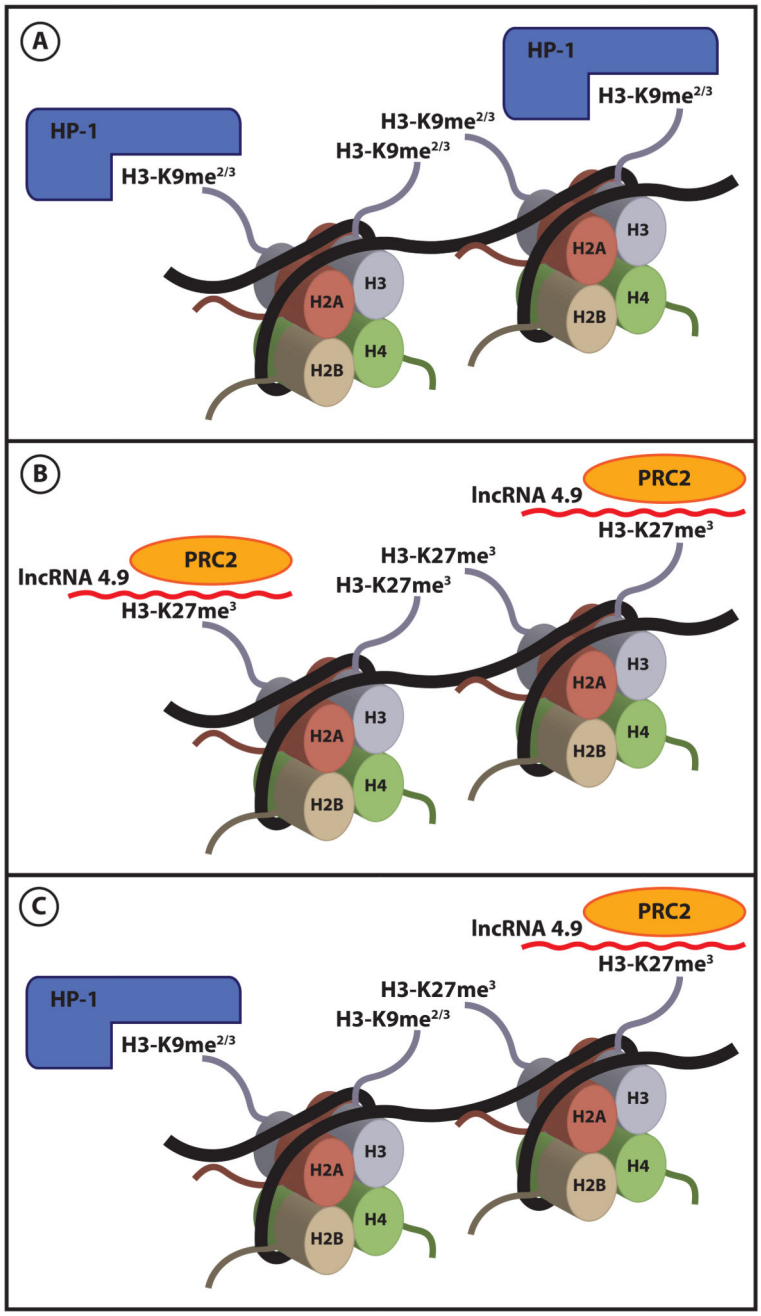

Figure 2. Chromatin mediated regulation of viral immediate early gene expression during latency.

The MIEP is bound by methylated histones and additional repressor complexes including HP1 and PRC2. The mechanism of HP1 recruitment is unknown but likely occurs through a high affinity interaction with histone H3 methylated at residue lysine 9. The recruitment of PRC2 is directed by the viral lnc4.9 transcript which promotes extensive histone methylation of lysine residue 27 of histone H3. Multiple chromatin states could exist where individual MIEPs are either bound exclusively by HP1 (a) or PRC2 (b), or the MIEP could be regulated by both marks concomitantly (c). The differing functions of PRC2 in the establishment of

repressive chromatin and HP1 in the long term maintenance of silenced chromatin may point towards specific roles at different times during latent infection.

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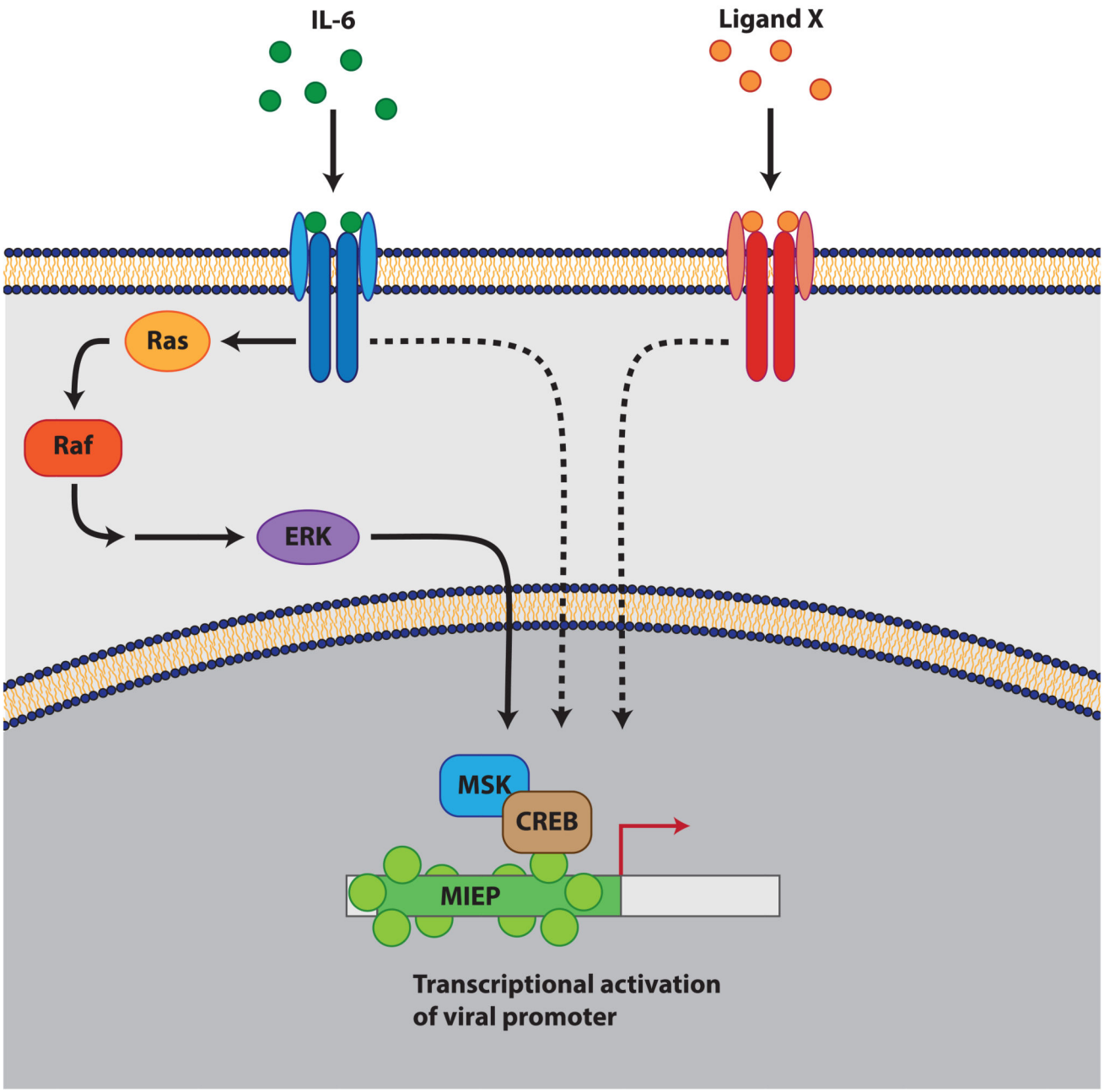

Figure 3. Signal integration is required to trigger viral reactivation.

HCMV reactivation has been reported to be ERK-MAPK dependent in DCs stimulated with IL-6. The targeting of ERK-MAPK activity to the MIEP in DCs likely involves the activation of multiple pathways to generate the specific output required. Multiple mechanisms could be responsible including the activation of additional IL-6 responsive pathways or the activation of additional pathways via concomitant binding of additional ligands.