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

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11 **Abbreviations used**

12 **Human cytomegalovirus (HCMV)**

13 major immediate early (MIE)

14 latency associated transcript (LAT)

15 major immediate early promoter (MIEP)

16 **Murine CMV (MCMV)**

17 polycomb repressor complex 2 (PRC2)

18 Tumour Necrosis Factor Receptor I (TNFRI)

19 Latent viral interleukin 10 (LavIL10)

20 IE1 exon 4 (IE1ex4)

Dendritic cell (DC)

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.

44 **Introduction: the opportunistic pathogen**

45 The herpesvirus human cytomegalovirus (HCMV) represents a very common infection exhibiting a
46 seroprevalence of 0-100% depending on socioeconomic status. Primary infection of healthy
47 individuals with HCMV is usually asymptomatic but results in the establishment of a lifelong infection
48 of the host [1]. HCMV is also highly immunogenic with infection and persistence leaving a large
49 indelible mark on both $CD4^+$ and $CD8^+$ T cell compartments of seropositive individuals [2]. In stark
50 contrast with the asymptomatic infection of healthy individuals, congenital infection or infection of
51 immunocompromised patients can result in significant morbidity and mortality [1, 3, 4]. As well as
52 primary infection, a profound disease burden is also associated with the reactivation of infectious
53 virus within latently infected individuals - particularly in allograft bone marrow transplant patients
54 [5]. Similarly, a 2011 meta-analysis of congenital HCMV infections in the US (between 1988-1994)
55 estimated that only 25 % of HCMV cases found at birth resulted from maternal primary infection
56 during pregnancy which highlights the importance of understanding the impact of non-primary
57 infections: i.e. re-infection and reactivation from latency in seropositive mothers [6].

58

59 HCMV latency and reactivation represents both clinical problem and challenging academic riddle.
60 Significant research efforts have been directed towards developing an understanding of the
61 mechanisms that are involved in the establishment and maintenance of viral latency as well as the
62 fundamental principles that govern the reactivation of latent virus. These strategies have involved
63 studies in primary human tissue and cell culture models, as well as using animal model systems of
64 CMV which, all told, have generated a fascinating insight into the enigmatic problem of latency.
65 From the beginnings of understanding the cellular basis of latency onto more recent studies that
66 have utilised powerful systems biology approaches to probe the molecular details the story of HCMV
67 latency and reactivation is unravelling. In this review we will focus on the recent identification of a
68 number of viral and cellular gene products that are active in latently infected cells - including the

69 detection of both viral and cellular non-coding RNAs – and how they contribute to the latent
70 phenotype. Furthermore, we will consider how the identification of these functions impacts on our
71 understanding of HCMV latency with particular emphasis on the concept that multiple latent
72 phenotypes may exist within the host. Finally, we will illustrate how these new insights resonate
73 with studies in the alpha and gamma herpes virus families through shared viral and cellular functions
74 or mechanisms that help govern the latent state.

75

76 **Keep your friends close...**

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

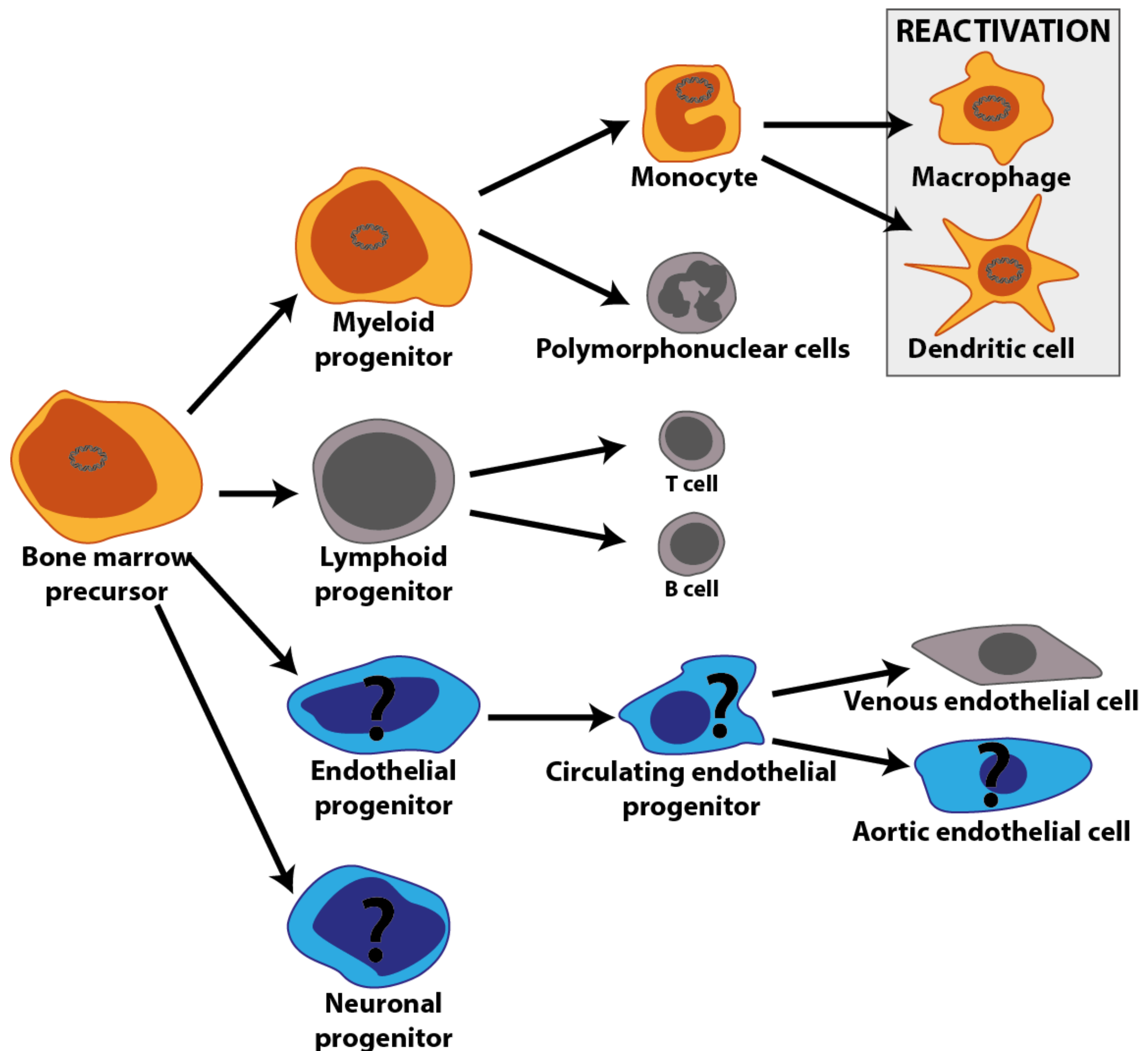
85

86 Similarly, at a molecular level, the mechanisms governing latency/persistence during murine CMV
87 (MCMV) infection appear to have resonance with studies of HCMV also – if not least when
88 considering the molecular mechanisms that dictate the regulation of major immediate early (MIE)
89 gene expression required for full lytic infection [10]. In contrast, a compelling comparative narrative
90 at the cellular level is less clear [10]. Whereas HCMV latency in the haematopoietic cell lineage well
91 established the same is not true of MCMV where studies of latency have focused on the role of
92 endothelial cells of a number of organs. Of course, it is entirely possible that the nature of studying

93 HCMV in the human host directly renders it difficult to address whether other cell types, alongside
94 cells of the haematopoietic system, are also sites of viral latency (Figure 1). For instance, attempts to
95 study human endothelial cells have proven somewhat inconclusive. Although HCMV genomes could
96 not be detected in endothelial cells isolated from saphenous vein tissue [11], in vitro studies suggest
97 that subsets of endothelial cells – for instance aortic – could support a latent or at least a persistent,
98 non-lytic, infection [12]. Thus, the vascular origin of the endothelial cells could be important and
99 suggests that an analysis of circulating endothelial cell progenitors in the peripheral blood may
100 reveal a further site of HCMV latency and represents a tractable question to address.

101

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



114
115 **Figure 1 Human cytomegalovirus natural latency in cell lineages.** Viral latency is established in the
116 haematopoietic progenitors resident in the bone marrow and the carriage of viral genomes has been defined
117 in the monocyte/myeloid lineage with reactivation occurring in the terminally differentiated myeloid
118 macrophages and DCs (Orange cells). In contrast, the viral genome is not carried in the lymphocyte population
119 nor is there any evidence for viral latency in venous endothelial cells (grey cells). Experimental infection data
120 suggest that endothelial and neuronal progenitor cells may also be sites of latency although no data from
121 natural latency currently exists (blue cells).

122

123 Finally, interesting recent experimental studies also suggest that specific neuronal progenitors may
124 be another potential site of latency. A key aspect is that the origin (fetal versus embryonic stem
125 cells) of the neural progenitors allied with the nature of the differentiation stimuli applied appears to
126 influence the outcome of infection [15-18]. Importantly, the nature of the infection of neuronal cells

127 may have implications for understanding the pathogenesis of HCMV congenital infections.
128 Furthermore, resolving the differences between the distinct types of neuronal cells and how they
129 respond to reactivation stimuli could have wider impact on our understanding of HCMV reactivation
130 in multiple cell types – particularly given that the recent study [17] identified multiple blocks to
131 reactivation of infectious virus which resonates with studies of latency in myeloid cells [19, 20].
132 However, interrogating these sites ex vivo is somewhat more challenging than the haematopoietic
133 system due to almost prohibitive access to the material required to perform the same analyses that
134 have defined the cells of the haematopoietic lineage as sites of HCMV latency.

135

136 **The silent virus?**

137 Perhaps the most significant advance in our recent understanding of HCMV latency is the
138 contribution of viral functions to this process – an area of study which, until recently, was in contrast
139 to the arguably much better defined patterns of gene expression observed with the alpha and
140 gamma herpesvirus subfamilies. The most intensively studied of all latent transcripts is the latency
141 associated transcript (LAT) of herpes simplex virus – acting as a non-coding RNA that, once subjected
142 to RNA processing, exerts an impressive number of reported functions including anti-apoptotic
143 effects, heterochromatic modification of histones as well as the generation of virally encoded miRNA
144 species with the potential to regulate viral and cellular gene expression [8, 21-23]. Similarly, for the
145 gammaherpes subfamily, untranslated RNAs have been identified during viral latency [24, 25]. It is of
146 note that a number of alternate transcriptional programmes have been described for EBV based
147 upon the analysis of transformed cell lines but, at least in long-term healthy carriers, the detection
148 of lymphocytes expressing EBV proteins is quite sporadic and is usually restricted to EBNA-1 and
149 LMP-2A positive cells [26, 27]. Evidently, the expression of non-coding RNAs during latency provides
150 a sophisticated mechanism for modulating the host cell environment without attracting an immune
151 response against the latently infected cell.

152

153 **Does HCMV express functional untranslated RNAs during latency?**

154 There are now a number of studies that have reported latent gene expression in various
155 experimental latent systems [28-33] a number of which have also been detected in natural latency
156 [29, 32, 34-37]. However, the most recent addition to this increasing repertoire of genes expressed
157 during latency was provided by a provocative study utilising an RNAseq analysis of HCMV
158 transcription during both experimental and natural latency [33]. Amongst these were non-coding
159 RNAs including beta 2.7 which was a predominant transcript in naturally latent CD14+ cells
160 (interestingly, a transcript that was not identified in the first studies that showed monocytes as a site
161 of persistence in vivo [19]) and experimentally latent CD34+ cells (exhibiting between 20-30x the
162 number of 'reads' detected for UL138 – an accepted latent gene product [35]). Similarly, beta 2.7
163 was detected in CD34+ cells isolated from the peripheral blood of healthy volunteers although at
164 relatively lower levels than observed for UL138. Indeed it is interesting to note whilst overlap was
165 observed with the experimental and natural latency transcriptional profiles they are not equivalent.
166 One possible explanation is the analysis of different cell types (cord (experimental) versus mobilised
167 (natural) CD34+ cells) which could be impacting on the expression profiles of the viral genes. That
168 aside, does HCMV infection render latently infected myeloid cells resistant to the action of rotenone
169 (and other mitochondrial complex I inhibitors) through extrapolation of the function of beta 2.7 in
170 infected neuronal cells [38]? One could speculate that if beta 2.7 is expressed during latency it may
171 be particularly important for the protection of neuronal progenitor cells from cell stress if these are
172 indeed sites of persistence in vivo and – thus displaying some functional similarities with the LAT of
173 HSV [23].

174

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

188

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

195

196 One prevailing view of the regulation of viral gene expression during latency is that higher order
197 chromatin structure and associated functions are important for maintaining MIEP quiescence and is
198 supported by a number of observations that link chromatin with the latent phenotype [33, 40, 41,

199 48]. Therefore it is intriguing that HCMV exploits additional strategies that contribute to the
200 regulation of the IE gene products during latent infection in vitro. There is the inhibitory effect of
201 mir112.1 on IE72 expression [46, 47]. Furthermore, more recent work from the Murphy laboratory
202 using the Kasumi-3 cell line model they have established to interrogate HCMV latency [49], has
203 postulated that an abundance of a cellular miRNA – mir200 – targets the UL122 transcript for
204 degradation thereby preventing IE86 protein expression also [50]. Thus, the expression of both MIE
205 products IE72 and IE86 is targeted during latency by miRNAs. Although previous work has suggested
206 that the cellular miRNAome is modulated by latent infection in vitro [51] no changes in mir200 were
207 observed suggesting that this miRNA represents a naturally abundant species in progenitor myeloid
208 cells that contributes to the latent phenotype of HCMV. Again, the presence of a cellular miRNA at
209 high levels contributing to viral latency resonates with studies in HSV where the neuronal specific
210 mir-138 species targets the ICP0 gene product to support latency [52]. It remains important that
211 when identifying functions using cell lines in experimental latency further insight into an
212 understanding of their precise contribution to latency in the host is highly dependent on the use of
213 physiologically acceptable and predictive cellular models as well as verification in studies of cells
214 from natural human infections to begin to appreciate their contribution to CMV latency in vivo.

215

216 **Expression of viral proteins during latency modulates the cellular environment**

217 The regulation of the MIEP during latency involves multi-faceted integration of viral and cellular
218 functions that act concomitantly to generate a phenotype that promotes latent infection. However,
219 it is now clear that a discrete set of protein coding transcripts is expressed during latency [29, 32, 34-
220 37]. A number of these gene products are involved in the manipulation of the cellular environment
221 to re-direct the immune response (e.g. US28 [53] and ORF94 [54]) or hijack cellular signalling
222 pathways involved in immune recognition (e.g. UL144 and Nf-kB [55]; UL138 and TNFRI [56, 57])
223 during lytic infection. In contrast, only the viral interleukin-10 homologue LAvIL10 (UL111.5A) has

224 been shown to have a defined role during latent infection (see [58] for review). Many of the
225 functions of LAvIL10 are analogous to the roles defined for its cellular counterpart and likely
226 contribute to the immune-suppressive phenotype generated in, and around the microenvironment
227 of, a latently infected cell in vitro [59-61]. Furthermore, it is hypothesised that LAvIL10 and cIL10
228 may act in unison to drive a more expansive range of outcomes from providing protection from cell
229 death stimuli, driving latent gene expression to propagating the unique cellular miRNA landscape
230 observed in latently infected CD34+ cells [51, 62, 63].

231

232 The repertoire of latent functions was expanded by, perhaps, the most provocative observation in
233 the recent RNAseq study regarding the detection of IE1 sequences in the naturally latent CD34+ cells
234 analysed – initially suggesting that the MIE region is active in the cells analysed in this study [33]. The
235 reported detection of UL123 RNA was discrepant with a number of previous studies that show major
236 IE gene expression is undetectable in the cells of naturally latent individuals [19, 29, 40, 64].
237 Importantly, more recent evidence at least addresses these concerns in part. A protein product
238 arising from exon 4 of the MIE (IE1ex4) is detectable in CD34+ cells and that the expression of the
239 coding transcript is under the control of a promoter distinct from the MIEP [65]. It was speculated
240 that this region was important for maintenance of the viral genome during latency with the IE1ex4
241 gene product having important tethering functions analogous to those observed with KSHV gene
242 product LANA [66]. Importantly, these studies also suggested evidence for latent replication (hence
243 the need for genome maintenance) in their system [65]. The posit of latent replication is at odds
244 with a number of studies in primary cells and cell lines in vitro that show a reduction in the
245 frequency of genome positive cells following long term culture and expansion of the cells [49, 67,
246 68]. Similarly, the low frequency of genome-positive cells in vivo would also suggest that replication
247 during latency is limited [69]. Possibly, there may be genome replication during latency which, whilst
248 inefficient, is sufficient to contribute to the maintenance of the latent pool and the maintenance of

249 genome positive cells as they transit into the periphery. By analogy with EBV this argument has
250 merit. EBNA1-dependent replication is clearly recognised as an important contributor to the
251 maintenance of the EBV latent pool [70] yet the frequency of EBV genome positive B cells of 1:10000
252 or lower [71] is consistent with those reported for HCMV [69]. Furthermore, the retention of a
253 chromatin tethering domain in the IE72 protein that is dispensable for lytic infection may suggest an
254 important function during latent infection [72].

255

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

263

264 **Viral reactivation – has HCMV perfected an exit strategy?**

265 It is becoming increasingly clear that the virus is directing significant activity towards the
266 maintenance of viral latency in myeloid progenitor cells. However, the other arm of the conundrum
267 is the exit from latency and reactivation of the lytic lifecycle. A key trigger of this event is the cellular
268 differentiation to a differentiated macrophage or dendritic cell [19, 40, 64, 77-80] suggesting that
269 changes in the cellular environment are promoting the reactivation event (figure 1). At the most
270 simplistic level, the first event that must likely occur is that the MIEP must transition from a
271 repressed to an active promoter state for robust lytic IE gene expression to occur. Here it is
272 important to de-lineate between the control of the MIE products IE72 and IE86 and the identified

273 IE1ex4 tethering function identified recently [65] where it is hypothesised that the regulation of the
274 IE1ex4 is via a cryptic promoter in the coding region of the MIE rather than the MIEP.

275

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

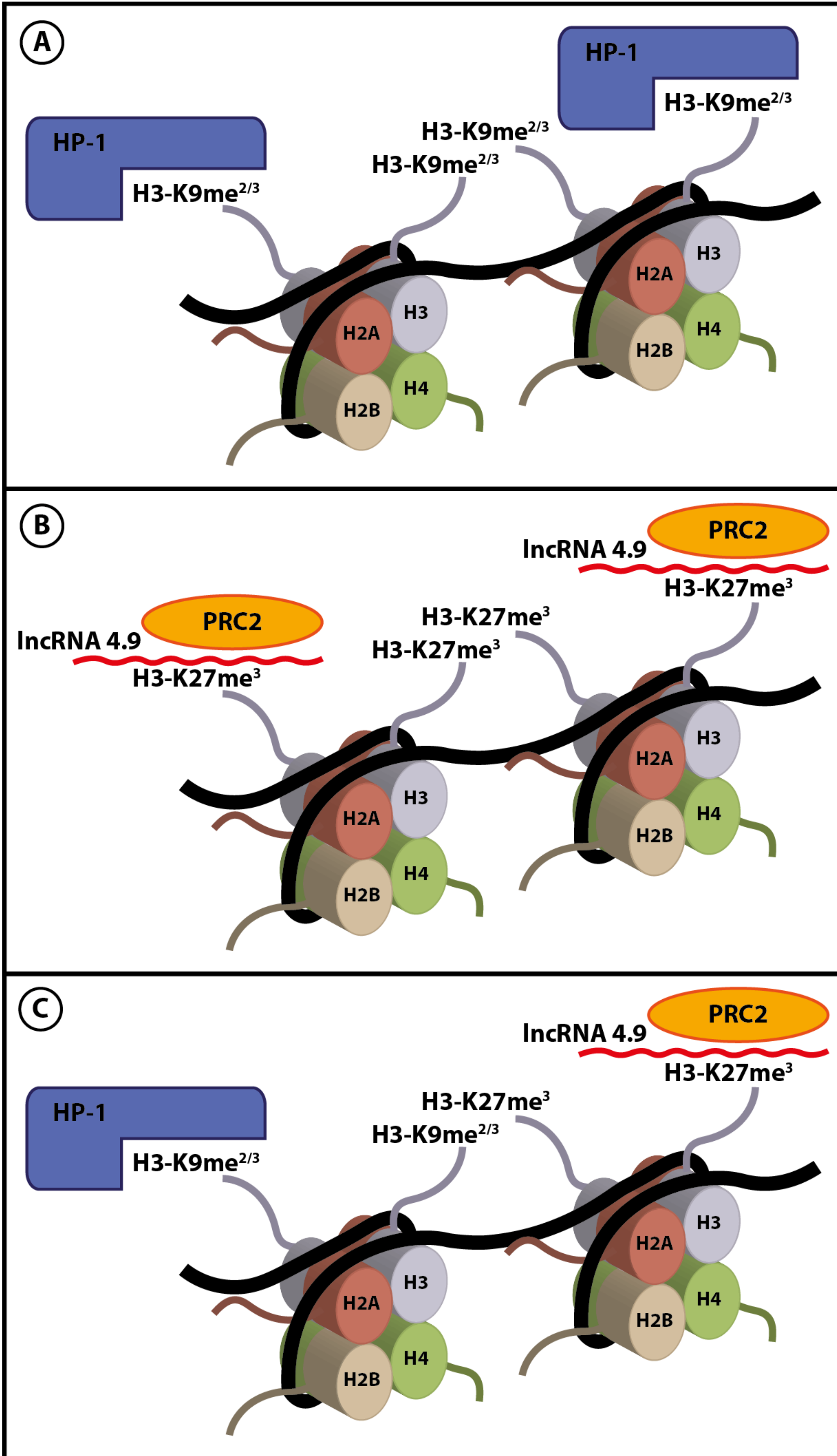
288

289 The MIEP is a complex promoter which contains multiple binding sites for both cellular
290 transcriptional repressors and activators [82]. Evidently, based on studies of the chromatin structure
291 around the MIEP, the predominant cellular (e.g. YY1/ERF and histone methyltransferases) and viral
292 (e.g. lnc4.9 and PRC2) activities during latency drive a chromatin signature that is highly repressive
293 [40, 83, 84] supporting the latent phenotype (figure 2). So the question remains: how does the MIEP
294 become activated? Although it has been demonstrated that reactivation of HCMV is concomitant
295 with extensive histone de-methylation and acetylation at the MIEP [40] it does not illuminate the
296 mechanism that drives the switch in the chromatin phenotype. However, if we reason that the MIEP

297 behaves akin to a cellular inflammatory promoter – and, in essence, consider the MIEP as another
298 genetic element influenced by the same mechanisms that control eukaryotic gene expression – we
299 can begin to unravel its regulation. Firstly, the MIEP is responsive to a number of inflammatory
300 stimuli [85-87] and, furthermore, HCMV reactivation and disease is associated with highly
301 inflammatory environments [78, 88, 89]. Secondly, the reactivation of HCMV IE gene expression is
302 seen efficiently in dendritic cell types in vitro [40, 77, 87, 90] – a cell type that is a prodigious
303 producer of inflammatory cytokines following stimulation. In itself this seems a minor link except
304 when we consider the activation of inflammation requires the de-methylation and subsequent
305 acetylation of histones bound to these cellular promoters and that this can occur in a mitogen
306 activated kinase, NF-kB and CREB dependent manner [91]. Our own recent work has illustrated that
307 the activation of ERK-MAPK signalling in DCs plays an important role in HCMV reactivation [48, 87]
308 and built on previous studies suggesting that the CREB transcription factor was an important
309 mediator of viral reactivation [92, 93]. Furthermore, studies using experimentally latent cell lines as
310 well as clinical data suggest enhanced NF-kB signalling correlates with HCMV reactivation [85, 89,
311 94]. All these data would be consistent with the hypothesis that the HCMV MIEP is mimicking the
312 promoters of cell-encoded inflammatory genes.

313 **Figure 2 Chromatin mediated regulation of viral immediate early gene expression during latency.** The MIEP is
314 bound by methylated histones and additional repressor complexes including HP1 and PRC2. The mechanism of
315 HP1 recruitment is unknown but likely occurs through a high affinity interaction with histone H3 methylated at
316 residue lysine 9. The recruitment of PRC2 is directed by the viral lnc4.9 transcript which promotes extensive
317 histone methylation of lysine residue 27 of histone H3. Multiple chromatin states could exist where individual
318 MIEPs are either bound exclusively by HP1 (a) or PRC2 (b), or the MIEP could be regulated by both marks
319 concomitantly (c). The differing functions of PRC2 in the establishment of repressive chromatin and HP1 in the
320 long term maintenance of silenced chromatin may point towards specific roles at different times during latent
321 infection.

322



323

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

325 When considering the regulation of gene expression – whether it is eukaryotic or viral – the nature
326 of the signalling response is a key determinant. There are multiple outcomes associated with any
327 specific signalling event which, in turn, are dictated by the cellular and intra-cellular phenotype. By
328 way of example, there are approximately 4,000 CREB responsive genes in the human genome yet
329 addition of a potential cAMP agonist does not trigger the uniform expression of the said 4,000 genes
330 [95]. Key to the differences are multiple signalling events acting co-operatively to generate a very
331 specific output which will be determined by the cellular receptors expressed on specific cell types as
332 well as the availability of downstream signalling molecules to elicit function [96-98]. Additionally, the
333 abundance of post-translational modifications on histone proteins in contrast to the very few
334 outputs available (i.e. gene expression versus no gene expression) hints at the chromatin structure
335 playing a key role in signal integration and defining the nature of the output in specific
336 circumstances [99]. Thus critical for the understanding of the impact of any signalling pathway on
337 gene expression requires an appreciation of the cellular and molecular context in which that
338 pathway is being activated.

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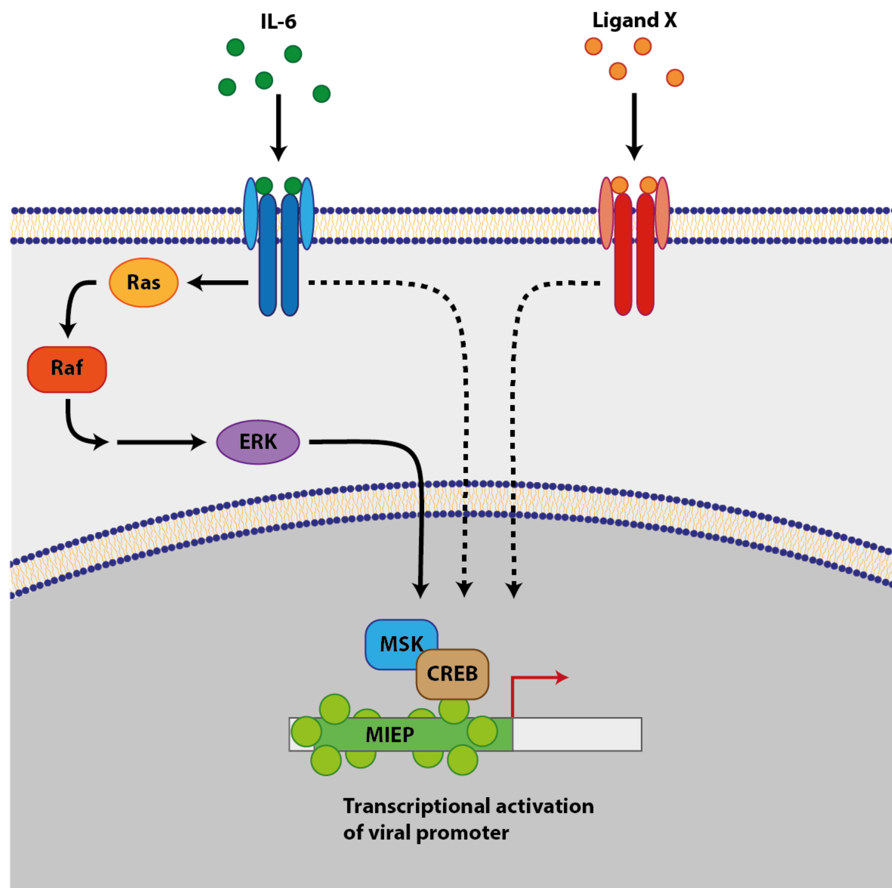

340

341 **Figure 3 Signal integration is required to trigger viral reactivation.** HCMV reactivation has been reported to be
342 ERK-MAPK dependent in DCs stimulated with IL-6. The targeting of ERK-MAPK activity to the MIEP in DCs likely
343 involves the activation of multiple pathways to generate the specific output required. Multiple mechanisms
344 could be responsible including the activation of additional IL-6 responsive pathways or the activation of
345 additional pathways via concomitant binding of additional ligands.

346

347 Our own work has focused on the role phosphorylation of two key proteins could play in the
348 reactivation of HCMV by providing a platform for signal integration at the MIEP [48]. In essence, we
349 proposed that histone phosphorylation was a key event to mediate the switch from a repressed to
350 an active chromatin form. Importantly, IL-6 stimulation of the ERK-MAPK pathway in DCs does not
351 promote global histone phosphorylation but, instead, is targeted to CREB-responsive promoters (i.e.
352 the MIEP) through the activity of mitogen and stress activated kinases [48] (figure 3). The similarities

353 with the c-FOS cellular promoter (coincidentally, itself a cellular 'immediate early' response gene)
354 suggest that this is an event associated with 'rapid response' promoters [100]. Although these
355 studies begin to hint at the mechanism involved, the full extent of signal integration required to elicit
356 this response of the MIEP in DCs remains to be understood. The high density of post-translational
357 modifications on histones heavily influences the activity of 'reader' functions associated with the
358 regulation of gene expression [99]. The specific recruitment of reader proteins, in turn, enlists the
359 modifying enzymes necessary for generating the signal and cell type specific responses we observe.
360 A prescient example is the role of histone H3 serine 10 phosphorylation. This phosphorylation event
361 de-stabilises the binding of the repressor HP-1 [101, 102] and also promotes recruitment of 14-3-3-
362 ε triggering the subsequent recruitment of transcription elongation factors [103, 104]. However, the
363 studies cited only address the reversal of HP-1 mediated silencing. As has recently been shown, the
364 PRC2 complex also may play a role in the silencing of HCMV gene expression [33, 105]. Thus are
365 similar mechanisms required to reverse PRC2 activity? This supposes that both modifications (HP-1
366 and PRC2) are active within the same MIEP (figure 2) which could not be the case based on the
367 proposed mutual exclusion of lysine 9 and lysine 27 methylation on the same histones at cellular
368 promoters [106, 107]. If so, then the proposed mechanism for alleviation of HP-1 silencing [48] may
369 only reactivate a subset of viral MIEPs, i.e. those bound predominantly by HP-1. Alternatively, PRC2
370 may be regulated by the same mechanisms since, architecturally, histone H3 serine 10 and histone
371 H3 serine 28 look highly similar [107] expressing the same ARKS domain (alanine; arginine; lysine;
372 serine). Indeed, MSKs also phosphorylate histones at serine 28 (which, like its serine 10 counterpart,
373 is adjacent to a key lysine (27) residue that is tri-methylated) and thus may trigger similar effects as
374 observed with serine 10 phosphorylation [108]. Contrastingly, recent data also suggests that PRC2
375 may be an important co-factor for the binding of HP1 to trimethylated H3-K9 arguing for these
376 marks at least acting co-operatively [109]. Indeed, this final scenario provides the most attractive
377 explanation for the recruitment of PRC2 to the MIEP by lnc4.9 [33] in light of the known recruitment
378 of HP1 to the MIEP during latency [40].

379

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

393

394 However, as it currently stands, the study of higher order chromatin structure and the control of
395 either viral or cellular gene expression are potentially approaching an impasse. Current techniques
396 rely on global analyses of cell populations that are fixed in time which, due to the low throughput
397 nature, can only analyse relatively large time frames. As such, it cannot be determined whether a
398 number of observations play a functional role or are bystander effects that are essentially passive in
399 the process. The ability to image this on a single cell scale and watch changes in real time will
400 massively impact on this. A recent study elegantly demonstrated that RNA Pol II activation is indeed
401 regulated by histone acetylation [115] suggesting that the study of chromatin dynamics at the single
402 cell scale is possible. Applying this to study the regulation of the MIEP in real time will substantially
403 illuminate our understanding of the factors directly regulating viral – and, more broadly, eukaryotic –

404 gene expression. However, the application of this approach for studying HCMV will additionally rely
405 heavily on the future development of new techniques that facilitate the isolation and enrichment of
406 HCMV genome positive cells – a technical hurdle that has not yet been overcome.

407

408 **Fighting on multiple fronts**

409 An incorrect assumption would be that there is one de facto mechanism required for HCMV
410 reactivation. This seems unlikely to be the case especially if the concept of a single latency
411 phenotype is becoming less applicable. The reactivation of HCMV in differentiated myeloid cells has
412 been reported following the stimulation of multiple progenitor cell types under a variety of
413 inflammatory conditions [31, 40, 64, 78, 79, 87]. Furthermore, the induction of IE gene expression,
414 whilst essential for initiating reactivation, does not, by itself, dictate that infectious virus will be
415 produced. It is highly likely that a number of viral gene products and cellular interactions are
416 important for driving HCMV reactivation through early and late gene expression and ultimately to
417 the production of infectious virus. Consistent with this are studies performed on both experimental
418 and natural latency. In the very first reports studying HCMV latency ex vivo from in vitro
419 differentiated monocytes both IE and early gene expression was detectable [19]. Similarly, the
420 transfection of IE proteins into latently infected THP1 cells again resulted in the induction of early
421 gene expression [20]. However, the recovery of infectious virus was not observed in either system.
422 Indeed, the first description of the reactivation of infectious virus ex vivo from CD14+ monocytes
423 was reported using a cytokine cocktail derived from allogeneically stimulated T cells [78]. This
424 suggested that signalling events associated with both differentiation and inflammation are key to
425 efficient HCMV reactivation. Arguably, cell differentiation (at least within the myeloid lineage)
426 remains the major determinant of HCMV reactivation and that inflammation increases the efficacy
427 of the reactivation phenotype. Indeed, more recent studies suggest that the key inflammatory
428 mediator IL-6 has multiple effects on HCMV reactivation ranging from increased IE gene expression,

429 less abortive reactivation events and, ultimately, more efficient reactivation of infectious progeny –
430 in part, by altering the particle to plaque forming unit ratio of the progeny virus [68] as well as
431 increasing the frequency of IE positive cells that transition into late stage gene expression [87]
432 during the reactivation process.

433

434 Studies in MCMV raise interesting questions also. The systemic addition of interferon-beta to
435 chronically infected mice had a dramatic impact on the level of reactivation in the murine model
436 [116]. Elucidating the precise mechanism of action of systemic interferon is clearly hard to dissect.
437 Interferon-beta has a direct impact on the replication of both MCMV and HCMV in vitro (for review
438 see [117, 118]) – as well as many other viruses – and thus the effects observed could be due to a
439 whole multitude of interferon-induced effects. The authors hypothesised that the well known
440 interferon induced accumulation of nuclear domain 10 bodies could be a key factor in the observed
441 phenotype [116]. However, failing a PML KO mouse there was no direct evidence for this phenotype,
442 which could potentially be a combination of anti-viral effects associated with interferon activity.
443 Furthermore, we note a recent study of the experimental infection of CD14+ monocytes which
444 suggested that latent HCMV disabled aspects of the JAK/STAT pathway which would render them
445 less sensitive to direct effects of interferons if the observations were to be extrapolated to HCMV
446 [119]. Nevertheless, what the mouse model does inform is that the complex interplay between the
447 latent cell and the extracellular environment driven by the host will be a key regulator of the latent
448 phenotype.

449

450 If we consider the transition from viral latency to reactivation analogous to pushing a rock up a hill
451 then the more factors that favour reactivation will push the rock towards the precipice of infectious
452 virus production. Countering these effects will be cellular (and viral) responses that are providing

453 resistance to progress and, ultimately, it is the dynamic changes in the activity of these processes
454 which decide the final outcome.

455

456 **Concluding remarks**

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

477 except from studies of the late stages of lytic infection which will have at least some overlap with the
478 mechanisms governing HCMV reactivation.

479

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

496

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

500

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504

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