TNF-α induces reactivation of human cytomegalovirus independently of myeloid cell differentiation following post-transcriptional establishment of latency

Running title: Differentiation-independent reactivation of HCMV

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ABSTRACT: We used the Kasumi-3 model to study HCMV latency and reactivation in myeloid progenitor cells. Kasumi-3 cells were infected with HCMV strain TB40/E*wt*-GFP, flow sorted for GFP+ cells, and cultured for various times to monitor establishment of latency, as judged by repression of viral gene expression (RNA/DNA ratio) and loss of virus production. We found that latency was established post-transcriptionally: transcription was initially turned on, and then turned off. We did not find evidence to support a latency model in which viral gene expression is repressed at the outset of infection. The pattern of expression of lytic genes that have been associated with latency, including UL138, US28, and RNA2.7, was the same as that of other lytic genes, indicating that there was no preferential expression of these genes in latency. By analyzing expression of the progenitor cell marker CD34 as well as myeloid cell differentiation markers in IE+ cells after treatment with TNF-α, we showed that TNF-α induced reactivation independently of differentiation. In contrast, LPS did not induce reactivation in Kasumi-3 cells, despite activation of ERK and NF-κB, and did not induce transcription of inflammatory cytokines. We speculate that reactivation occurs in two steps: 1. activation of signal-specific transcription factors that bind to the MIEP; 2. reconfiguration of viral chromatin to allow access to the MIEP. We propose that differentiation is required to render cellular chromatin competent to express inflammatory cytokines that drive reactivation in response to LPS, but it is not required for reactivation in response to TNF-α itself.

IMPORTANCE: HCMV is an important human pathogen that establishes lifelong latent infection in myeloid progenitor cells, and reactivates frequently to cause significant disease in immunocompromised people. The epigenetic mechanisms that allow the virus to establish latency specifically in myeloid cells, and to reactivate in response to inflammatory mediators are

50 not well understood. Our observation that viral gene expression is first turned on, and then
51 turned off to establish latency suggests that there is a host defense, which may be myeloid-
52 specific, responsible for transcriptional silencing of viral gene expression. Our observation that
53 TNF-α induces reactivation independently of differentiation, but LPS does not, provides insight
54 into molecular mechanisms that control reactivation.
55

56

57 **INTRODUCTION**

58 HCMV is a ubiquitous human pathogen of the beta herpesvirus family. Infection is
59 transmitted through contact with body fluids, including saliva, urine, blood, and genital
60 secretions. Hematogenous spread of the virus leads to a systemic infection, with multiple cell
61 types infected in many organs. In immunocompetent hosts, primary infection is typically sub-
62 clinical, and is resolved by activation of innate and adaptive immunity. However, latently
63 infected cells, which carry viral DNA, but do not produce infectious virus, are able to escape
64 immune surveillance, and can persist for the life of the host.

65 Under the appropriate conditions, the virus in these cells wakes up from its dormant state
66 to reactivate the infectious cycle. In immunocompetent hosts, viral replication is controlled by
67 the immune response, but reactivation of latent virus can be a significant infectious complication
68 in immunocompromised hosts, such as recipients of solid organ or bone marrow transplants.
69 Reactivation of CMV in solid organ transplant recipients is associated with increased risk of
70 CMV disease, acute and chronic allograft rejection, infection with other opportunistic pathogens,
71 graft failure, and death (1). Risk factors for reactivation include CMV serostatus of the donor
72 (D) and recipient (R), with the highest risk in the D+/R- combination, specific
73 immunosuppression protocols, and inflammatory conditions associated with high TNF-α
74 secretion, including allograft rejection and sepsis (1).

75 The cell type harboring latent CMV DNA in solid organs has not been clearly identified.
76 However, latent virus can also be transmitted through blood transfusion, and monocytes in the
77 blood and hematopoietic progenitor cells (HPCs) in the bone marrow are known to be sites of
78 latency in vivo (2-10). Experimental models have shown that these cells are less permissive to

79 lytic replication, and that they support a latent infection (11-16). Cell type-specific
80 establishment of latency is thought to be due to a combination of host and viral factors. Infection
81 activates a host intrinsic immune response, which recognizes viral DNA invading the nucleus
82 and silences viral gene expression at the outset of infection through heterochromatinization of
83 viral genomes (13, 17-25). Factors present in the viral particle, including the tegument protein
84 pp71, enter the cell upon infection, and counteract this host defense response to activate viral
85 gene expression. In cells that support latency, pp71 is sequestered in the cytoplasm and is
86 therefore unable to perform this function (26-28). Differentiation of myeloid cells to a dendritic
87 cell phenotype increases permissiveness of these cells to infection and also induces reactivation
88 of both naturally and experimentally latently infected cells (12, 13, 15, 20, 29-32). Thus, the
89 generally accepted paradigm is that myeloid progenitor cells are not permissive to infection due
90 to heterochromatinization and transcriptional repression of invading viral genomes. The process
91 of myeloid cell differentiation is thought to change the balance of repressive and activating
92 cellular factors that control both viral and cellular transcription, resulting in changes to the viral
93 epigenome that activate viral gene expression (17, 33, 34).
94 However, some observations suggest that the current paradigm may be in need of
95 revision. First, several investigators have demonstrated that, although myeloid progenitors may
96 be less permissive than other cell types, some expression of viral lytic genes is detectable at early
97 times post-infection in various models of latency, including primary CD34+ HPCs, Kasumi-3
98 cells, a CD34+ myeloblastic cell line derived from a patient with acute myeloid leukemia (AML)
99 (35), which has recently been demonstrated to be a tractable model for CMV latency and
100 reactivation (26, 36), and primary CD14+ peripheral blood mononuclear cells (11, 12, 36-40).
101 Expression of these genes is lost as latency is established. These observations suggest that

102 latency is not necessarily established at the outset of infection, but may also be established
103 following activation of viral transcription.
104 Second, differentiation of myeloid cells into dendritic cells in vivo occurs in the context
105 of infection and inflammation (41), and all of the physiologically relevant differentiation factors
106 that have been used to induce reactivation of CMV in primary hematopoietic cells, including
107 GM-CSF (42), TNF-α, IL-6, and LPS, are also mediators of inflammation. Thus, it is difficult to
108 distinguish the roles of inflammation versus differentiation in these models. In contrast, Kasumi-
109 3 cells, like other transformed cell lines, are refractory to normal physiological differentiation
110 cues, and therefore can be used to dissect the contributions of these two pathways. Here, we
111 have used the Kasumi-3 model to further investigate these issues.
112
113 **RESULTS**
114 **Latency is established after activation of transcription.** The time at which latency is
115 established following infection with HCMV has not been clearly determined. Some previous
116 studies have shown that primary CD34+ myeloid progenitor cells do not express lytic viral genes
117 upon infection, but rather, that viral genomes in these cells are inactivated upon entry and remain
118 quiescent until they receive signals that induce them to differentiate (13, 19, 20, 28, 43).
119 However, other studies have shown that both primary CD34+ cells and Kasumi-3 cells express
120 an SV40 promoter-driven GFP reporter, as well as viral genes at early times post-infection, and
121 that latency can be established in the GFP+ population following cell sorting (11, 36, 37, 39, 40,
122 44). To investigate the question of timing further, we infected Kasumi-3 cells with the
123 TB40/E*wt*-GFP strain of HCMV (36), and analyzed the cells by FACS after 24 hr (Fig. 1A).
124 Although the proportion varied, GFP expression was consistently observed in a high percentage

125 of cells (Fig. 1B), as previously reported (36). There was a broad range in the extent of GFP
126 expression among the infected cells. We then purified the GFP+ population by cell sorting and
127 analyzed viral RNA expression, DNA copy number, and production of infectious virus over the
128 course of infection. Latency has been operationally defined as the lack of virus production in
129 cells that carry viral DNA with the capacity to reactivate (45). Production of infectious virus
130 was detectable at 4 dpi and peaked at 8 dpi, but was no longer detectable at 11dpi (Fig. 1C).
131 A second characteristic of latency is transcriptional repression of genes involved in lytic
132 replication. We therefore analyzed expression of viral genes representative of the immediate
133 early (IE-2, UL122 and IE-1, UL123), early (viral DNA polymerase UL54), and late (UL32)
134 phases of the viral life cycle. Expression of these genes peaked at 4 dpi and then fell throughout
135 the course of infection (Fig. 1D and Fig. S1). Viral DNA copy number also increased following
136 infection, peaked 4-8 dpi, and then decreased significantly (Fig. 1D and Fig. S1). Because both
137 viral DNA and RNA copy number dropped over the course of infection, analysis of RNA
138 expression alone does not indicate repression of viral transcription. We therefore calculated the
139 ratio of viral RNA to DNA. Relative to 1 dpi, this ratio fell for all genes from 4 to 14 dpi (Fig.
140 1D and E). These results show that viral genomes are increasingly subject to transcriptional
141 repression throughout the course of infection. TNF-α induced reactivation of infectious virus in
142 these cells (see below). It is important to note that, because the cells were sorted for GFP
143 expression to obtain a pure population of infected cells in which viral genomes were
144 transcriptionally active, these results show that latency can be established by shutting off gene
145 expression after it has been turned on. We have introduced the term post-transcriptional latency
146 to describe this state.
147

148 **Early gene expression is delayed in some infected cells.** Although our studies indicate
149 that latency can be established post-transcriptionally, we wondered whether there might be a
150 second population of cells in which latency is established at the outset of infection. These cells
151 would carry viral DNA, but would not express GFP or viral genes. To test this hypothesis, we
152 analyzed both the GFP-positive and GFP-negative populations for the presence of viral DNA at 1
153 dpi. Fig. 2A shows a representative gating strategy used to separate the populations. qPCR
154 analysis revealed that, although the vast majority of the DNA was present in the GFP+ cells,
155 some DNA was detectable in the GFP- population (10.8%±4.8%, Fig. 2B). We then analyzed
156 the RNA from these cells for expression of immediate early, early, and late genes. Expression of
157 immediate early (IE-2, UL122) RNA was detectable in the GFP- population, albeit at a lower
158 level than the GFP+ cells. When we calculated the ratio of UL122 RNA to DNA, we found that
159 there was no difference between the GFP+ and GFP- cells, indicating that transcription of the IE
160 genes was equally active in these two populations at this time (Fig. 2C). However, when we
161 analyzed the RNA/DNA ratios of early (UL54) and late (UL32) genes, we found that this ratio
162 was significantly lower in the GFP- cells (Fig. 2D and E), indicating that these classes of genes
163 were transcriptionally repressed in the GFP- cells. We then analyzed the GFP- cells for changes
164 in GFP expression, viral RNA expression and DNA copy number over time. We found that
165 some GFP- cells become GFP+ by 4 dpi (Fig. 3A). While UL122 (IE-2) RNA expression
166 increased only modestly from 1 to 4 dpi (Fig. 3B), early and late gene expression increased 10-
167 15-fold (Fig. 3C,D). Similar to the GFP+ population, both RNA expression and DNA copy
168 number declined steadily after 7 dpi in the GFP- cells. We conclude that *i)* as previously noted
169 (46), the GFP reporter is regulated as an early gene in the TB40/E*wt*-GFP virus; *ii)* there is
170 heterogeneity in the kinetics of viral gene expression after infection of Kasumi-3 cells, with

171 activation of early gene expression delayed in some cells; *iii)* all classes of viral RNAs are
172 eventually expressed in the infected cells in the GFP- population; and *iv)* similar to the GFP+
173 cells, their expression is subsequently repressed.
174
175 **US28, UL138, and 2.7 kb RNAs are not differentially expressed in latently infected**
176 **Kasumi-3 cells.** A number of studies have analyzed the viral transcriptome in latency, and have
177 indicated that some lytic genes are also specifically expressed in latency, including US28,
178 UL138, and a non-coding 2.7 kb RNA (RNA2.7) (11, 14, 37, 43, 45, 47-56). We analyzed
179 expression of these genes in GFP+ cells over the course of infection in Kasumi-3 cells. RNA2.7
180 is expressed at very high levels during lytic infection of fibroblasts (57), and at early times after
181 infection of Kasumi-3 cells (Fig. 4A). UL138 and US28 RNAs are also relatively abundant
182 transcripts during lytic infection. To analyze repression of these genes relative to a lytic gene,
183 we analyzed UL32, a gene that is expressed at levels similar to UL138 and US28 at 4 dpi. Our
184 results show that expression of UL138, US28, and RNA2.7 followed the same pattern as that
185 observed for UL32, with a peak of expression at 4 dpi, followed by a significant decrease at 7 dpi
186 (Fig. 4A). When we analyzed the RNA/DNA ratio, we found that expression of these genes was
187 repressed over the course of infection, with kinetics similar to that of UL32 (Fig. 4C). The
188 RNA/DNA ratio of RNA2.7 was significantly higher than other genes at 4 dpi, consistent with its
189 high level of expression during lytic infection, but there was no difference between UL32 and
190 UL138 or US28 at any time point, and there were no differences in the RNA/DNA ratios
191 between UL32 and any of the other genes analyzed after 4 dpi (Fig. 4C). Thus, there was no
192 preferential expression of UL138, US28, or RNA2.7 over the lytic gene UL32 when latency was
193 established.
194

195 **TNF-α induces reactivation of HCMV in Kasumi-3 cells independently of myeloid**
196 **cell differentiation.** Previous studies showed that TNF-α induced reactivation of HCMV in
197 latently infected Kasumi-3 cells (36). We confirmed this result using slightly different
198 conditions. Kasumi-3 cells were infected with TB40/E*wt*-GFP virus at an moi of 1.5-2, flow-
199 sorted for GFP+ cells and cultured for 14 days to establish latency. The cells were then divided
200 into different pools and incubated for a further 3 days in the presence of 5 ng/ml TNF-α or left
201 untreated. Relative to untreated controls, TNF-α induced statistically significant increases in
202 viral DNA copy, RNA expression and the RNA/DNA ratio, as well as production of infectious
203 virus (Fig. 5A and additional RNA analyses of early and late genes in Fig. S2). In addition, we
204 analyzed the percentage of reactivating cells by flow cytometry after staining with an IE1/2-
205 specific antibody. Expression of IE proteins was essentially undetectable in latently infected,
206 untreated Kasumi-3 cells (Fig. 5B, upper right panel). Treatment with TNF-α reproducibly
207 induced expression of IE1/2 proteins in 2-3% of the cells (representative results shown in Fig. 5B,
208 lower left panel).
209 Differentiation of myeloid progenitor cells to a dendritic cell phenotype has long been
210 associated with transcriptional activation of HCMV IE gene expression and reactivation of
211 infectious virus (13, 15, 20, 34). Transformed cell lines are generally resistant to differentiation,
212 and therefore it seemed unlikely that differentiation would play a role in this model. However,
213 the fact that reactivation occurred in only a small percentage of latently infected Kasumi-3 cells
214 left open the possibility that reactivation occurred in a small number of cells with the capacity to
215 differentiate. To investigate this question, we used immunophenotyping to characterize the
216 differentiation state of Kasumi-3 cells carrying reactivating virus. Latently infected TNF-α-
217 treated or untreated cells were stained for IE1/2 antibody in combination with the hematopoietic

218 progenitor marker CD34, and for markers of various stages of myeloid differentiation, including
219 CD64, a marker of granulo-monocytic lineage commitment (58), CD14 (monocytes), CD15
220 (granulocytes), and CD1c and CD11c, which identify major subpopulations of human myeloid
221 dendritic cells (59). Uninfected Kasumi-3 cells are homogeneous with respect to CD34
222 expression, and do not express other markers of myeloid cell differentiation (Fig. 5B, upper left
223 and data not shown). This marker was lost in a small percentage of cells after establishment of
224 latency, but these were not the cells containing reactivating virus, since CD34- cells were also
225 negative for expression of IE1/2 (Fig. 5B, upper right and lower left panels). 5-6% of the
226 IE+/CD34+ cells acquired expression of CD64. However, 94-95% of the IE+/CD34+ cells were
227 CD64-negative, and 96% of IE+/CD34+ cells were also negative for markers of mature
228 monocytes and dendritic cells (Fig. 5C). Thus, our data shows that differentiation was not
229 required to reactivate IE gene expression in this model.

230 **LPS does not induce reactivation in Kasumi-3 cells.** The bacterial cell wall
231 component LPS is often used to drive HCMV reactivation in dendritic cell models. LPS induces
232 activation of NF-κB through a signaling pathway that is very similar to that of TNF-α, and LPS
233 induces expression of TNF-α, which is sufficient to drive reactivation of HCMV (36, 60). We
234 therefore investigated whether treatment with LPS was sufficient to induce reactivation of
235 HCMV in Kasumi-3 cells. Surprisingly, our results show that LPS did not induce reactivation in
236 this model (Fig. 6A). We then investigated whether Kasumi-3 cells are able to respond to LPS.
237 Since TNF-α is sufficient to induce reactivation of HCMV in Kasumi-3 cells, we first analyzed
238 release of TNF-α into the media in response to LPS. Monocytic THP-1 cells were analyzed as a
239 positive control (61). Our results show that Kasumi-3 cells do not produce TNF-α under
240 conditions that are sufficient to induce production in THP-1 cells (Fig. 6B). We then determined

241 whether LPS signaling pathways were intact in Kasumi-3 cells. LPS signaling is mediated by
242 TLR4 and the co-receptor MD2 (62). CD14 acts as a chaperone to transfer LPS from LBP (LPS-
243 binding protein) to its receptor. TLR4 receptor engagement activates MyD88-dependent
244 signaling, leading to activation of NF-κB and MAPKs, including ERKs, and expression of TNF-
245 α, IL-1β, and IL-6 (63, 64). Kasumi-3 cells express TLR4 and MD-2 on the cell surface, but not
246 CD14. CD14 is required for response to low concentrations of LPS, but not for response to the
247 concentrations used in this study (65), and downstream signaling components, including NF-κB
248 and ERK, were activated in response to LPS (Fig. 6D, E). However, analysis of RNA expression
249 showed that there was no increase in TNF-α RNA in Kasumi-3 in response to LPS (Fig. 6F).
250 Thus, production of TNF-α in response to LPS is blocked in Kasumi-3 cells at the level of
251 transcription. Similarly, LPS did not induce expression of IL-1β or IL-6 in Kasumi-3 cells (Fig.
252 6G,H).
253
254 **DISCUSSION**
255 **Mechanisms of establishment of latency**. HCMV establishes latency in myeloid
256 progenitor cells through heterochromatinization and repression of viral gene expression, but the
257 timing and molecular mechanisms that control this process are not well understood. While some
258 studies have shown that viral gene expression is silenced immediately after infection (13, 19, 20,
259 28, 43), others have shown that viral gene expression is activated at early times post-infection,
260 and that latency is subsequently established in this population of cells (11, 36, 38). Our studies
261 are in agreement with this latter observation, and we show that, not only is there a decrease in the
262 abundance of viral transcripts, but that the ratio of viral RNA to DNA decreases over the course
263 of infection, and therefore that viral genomes are increasingly repressed as latency is established.

264 Post-transcriptional establishment of latency has also been demonstrated in some, but not all
265 HSV-infected neurons in mice (66-68). We therefore considered the possibility that there may
266 be two routes to establishment of latency: repression prior to activation of gene expression as
267 well as post-transcriptional repression. Our results show that there is heterogeneity in the
268 kinetics of viral gene expression, and that activation of the early and late phases of viral gene
269 expression is delayed in some cells, but we do not find evidence to support establishment of
270 latency at the outset of infection. Previous studies have shown that initiation of HCMV gene
271 expression requires that the cells be in the G0 or G1 phase of the cell cycle (69), and
272 heterogeneity in cell cycle phase may account for some of the observed variation in the kinetics
273 of gene expression.

274 Our findings have two important implications. First, the observation that viral gene is
275 expression is first turned on, and then turned off, suggests that there is a cellular defense
276 mechanism for shutting off viral genomes that are actively engaged in transcription. Cell type
277 specific expression of this host defense response may account for the highly restricted tropism
278 for HCMV latency. Second, shedding of HCMV is often observed in healthy, immunocompetent
279 individuals, suggesting that the virus reactivates with high frequency (70). The finding that
280 HCMV establishes latency in cells following activation of transcription raises the possibility that
281 these genomes may retain a memory of prior activation, and may therefore be poised to
282 reactivate readily under the appropriate conditions. Interestingly, previous studies have shown
283 that HCMV chromatin is specifically marked by H3 lysine 4 methylation following replication of
284 viral DNA in fibroblasts (71). It is interesting to speculate that there may be cell type-specific
285 differences in histone modifications bound to post-replicative viral DNA that affect the potential
286 to establish latency and to reactivate.

287 **Viral transcription in latency**. Several studies have suggested that some viral genes
288 that are expressed during lytic infection also have a role in latency, including UL138 and US28,
289 and genetic analyses have shown that mutation of these genes increases viral gene expression in
290 myeloid progenitor cells (40, 43, 47-51). These studies did not include analyses of the
291 RNA/DNA ratio to demonstrate differences in transcriptional activity between lytic genes and
292 latency-associated lytic genes (12, 14, 40, 43, 48, 72, 73). We have investigated expression of
293 US28, UL138, as well as the non-coding RNA2.7 in the Kasumi-3 model. We find that the
294 pattern of expression of these genes parallels that of other lytic genes, including UL122, UL123,
295 UL54, and UL32, and analysis of the RNA/DNA ratio shows that transcription of all of these
296 genes is repressed in latency.

297 The advent of highly sensitive new technologies has allowed unbiased transcriptome
298 analyses to detect viral transcripts in both experimental and natural latency. These studies have
299 identified many genes expressed under conditions of latency, and none of these show specific
300 expression of some RNAs in the absence of all other lytic genes (11, 37, 52, 54, 74). These
301 observations raise the possibility that detection of lytic transcripts is due to the presence of a
302 small number of productively infected cells. This notion is consistent with frequent shedding of
303 virus and inflation of a CMV-specific effector memory T cell response, an indicator of repeated
304 antigen stimulation, observed in healthy individuals (70, 75).

305 Collectively, our studies and those of others suggest that the criteria for classification of a
306 gene as latency-associated should be expanded to include analysis of relative transcriptional
307 activity, as well as quantitative analysis of RNA abundance and the effect of genetic mutation on
308 viral gene expression.

309 **What is the role of myeloid cell differentiation in reactivation of HCMV?** Previous
310 studies using primary CD34-derived or monocyte-derived dendritic cells (MoDCs) showed that
311 reactivation of latent HCMV carried in myeloid progenitor cells required differentiation to a
312 dendritic cell phenotype (9, 13, 20, 30, 32, 72, 76). Reactivation in immature dendritic cells in
313 response to LPS occurred through induction of IL-6 and activation of ERKs and mitogen and
314 stress-activated kinases (MSK) (72, 77). However, the factors that were used to differentiate
315 dendritic cells in these models are also mediators of inflammation, and it is therefore difficult to
316 distinguish the roles of a general inflammatory response from dendritic cell-specific
317 differentiation in reactivation of HCMV in these cells. In contrast, Kasumi-3 cells, like many
318 transformed cell lines, are refractory to the normal cues that induce differentiation, and are
319 therefore more suitable for studies seeking to dissect the roles of these different processes. Our
320 studies show that differentiation is not required for reactivation in response to TNF-α.
321 In contrast to TNF, LPS was not able to induce reactivation in Kasumi-3 cells, despite the
322 fact that LPS induces expression of inflammatory cytokines, including TNF-α, IL-1β, and IL-6
323 in monocytic cells (63, 64). To explain this surprising result, we investigated expression of the
324 LPS signaling components and the response of Kasumi-3 cells to LPS. We found that Kasumi-3
325 cells express the LPS co-receptors TLR4 and MD2 on the cell surface. They do not express the
326 LPS chaperone protein CD14, which is required for a response to low concentrations of LPS, but
327 is not required for the concentrations used in this study (65), and downstream signaling
328 components, including NF-κB and ERK, were activated in response to LPS. Thus, the LPS
329 signaling pathways leading to activation of transcription factors that control expression of
330 inflammatory cytokines appear to be intact in these cells. However, activation of NF-κB was not
331 sufficient to induce expression of TNFα, IL-1β, or IL-6 in response to LPS in Kasumi-3 cells.

332 Previous studies have shown that treatment with LPS induces selective activation of NF-κB-
333 responsive genes in myeloid cells, and this differential response is governed in part by
334 chromatin-mediated restriction in access of NF-κB to its binding sites in the promoters of target
335 genes (78-81). These observations suggest that the promoter regions of cytokine genes are not
336 accessible in Kasumi-3 cells, and NF-κB is therefore not able to bind to its cognate sites to
337 activate transcription of the cytokines that drive reactivation in response to LPS.

338 Recent studies have begun to elucidate the molecular pathways for determination of
339 various lineages in the hematopoietic system (79, 82, 83). Cell type-specific activation of gene
340 expression is achieved through chromatin remodeling of enhancers by lineage-specific pioneer
341 transcription factors, which are able to recognize their cognate binding sites in the context of
342 nucleosome-dense heterochromatin (84). Expression of these factors in committed cells opens
343 the chromatin to create nucleosome-free promoter regions that permit binding of signal-specific
344 transcription factors, such as NF-κB, and thus, cell type-specific activation of gene expression.
345 It is known that the viral genome is heterochromatinized in latently infected cells, and that
346 reactivation is associated with reprogramming of the viral epigenome (13, 20). We hypothesize
347 that this requires both activation of signal-specific transcription factors that bind to the MIEP,
348 such as NF-κB, and remodeling of the viral chromatin to allow these transcription factors access
349 to their binding sites. We speculate that TNF-α is able to perform both of these functions, but
350 while LPS is able to activate NF-κB, it is not able to induce chromatin remodeling.
351 Differentiation of the cells may be required to perform this function.

352 Further studies will be required to fully understand the roles of differentiation,
353 inflammation, and chromatin remodeling in reactivation of HCMV. Our studies suggest that the
354 requirement for differentiation in reactivation is dependent on the context—it is not required for

355 reactivation in response to TNF-α or activation of ATM (39), but it may be required for
356 expression of myeloid lineage-specific pioneer transcription factors that mediate changes in the
357 chromatin needed to render latently infected cells competent to respond to other ligands, such as
358 LPS.

359 **MATERIALS AND METHODS**

360 **Cells and reagents:** Kasumi-3, MRC-5, and THP-1 cells were obtained from the ATCC and
361 cultured as recommended. Human recombinant TNF-α was obtained from PreproTech (#300-
362 01A) and LPS was obtained from Sigma (#L5658). Fluorescent conjugated monoclonal
363 antibodies used in this study are listed in Table 1. TB40/E*wt*-GFP (36) viral stocks were
364 produced by electroporation of BAC DNA into MRC-5 fibroblasts with pGCN-pp71 expression
365 vector (85). Culture media was collected, cleared of cellular debris by centrifugation and
366 concentrated by ultracentrifugation on a sorbitol cushion (86). Viral titers were determined using
367 the 50% tissue culture infective dose ($TCID_{50}$) limiting dilution assay after two weeks in culture
368 Titers were calculated from duplicate 96-well plates by scoring the GFP+ wells using the method
369 of Reed and Muench (87).

370

371 **Infection of Kasumi-3 cells:** Kasumi-3 cells were infected with HCMV strain TB40/E*wt*-GFP at
372 high concentration ($2x10^7$ cells/ml) using an MOI of 1.5-2 and centrifugal enhancement of
373 infection (1000x*g* for 30 minutes at 25°C). Cells were then diluted to $1.25x10^6$ cells/ml with fresh
374 media without removing the virus and incubated at 37°C for additional 23 hours. 1 day post-
375 infection (dpi), extracellular virus was inactivated by a treatment with a Citric Acid solution as
376 previously described (38) and GFP+ and GFP- cells were sorted on a FACSAria-6 laser cell
377 sorter flow cytometer (BD Biosciences). Infected cells were cultured for 13 days to establish

378 latency, with media changes every 2-3 days. For reactivation studies, cells were treated with
379 5ng/ml TNF-α or 100ng/ml LPS at 14 dpi for 3 days at 37°C. A $TCID_{50}$ assay was used to
380 determine the infectious titer of the infected cell supernatants.

381 **LPS and TNF-α treatments:** Kasumi-3 cells were seeded at $6.6x10^5$ cells/ml and treated with
382 5ng/ml TNF-α or 100ng/ml LPS for 4 hours. As positive control, THP-1 cells were treated or not
383 with LPS at the same conditions. Production of human TNF-α in the supernatants was analyzed
384 with Quantikine ELISA kit (R&D systems # DTA00C) and the cells analyzed for activation of
385 NF-κB and Erk by FACS (see below) and assayed for the expression of TNF-α, IL-6 and IL-1β
386 transcripts by qRT-PCR.

387 **RNA and DNA extraction:** DNA was isolated with the Arcturus pico Pure DNA extraction kit
388 (Thermo Fisher Scientific) as recommended by the manufacturer. RNA was purified with the
389 Direct-zol RNA mini prep kit (Zymo Research) with a DNAse digestion step following
390 manufacturer's instructions.

391 **Real time PCR analysis**. For all analyses, mock-infected cells were analyzed in parallel with
392 infected cells. These samples were uniformly positive for cellular genes, but negative for viral
393 genes. TaqMan assays specific for viral genes were custom-designed by Life Technologies
394 (Table 1). Relative viral DNA quantity was analyzed by the ΔΔCt method, using a pre-designed
395 assay for RNaseP as the normalization control (Life Technologies, catalog number 4403326).
396 This method was validated by determining that the efficiencies of the target and reference genes
397 were approximately equal, as described in the Supplementary Methods and shown in Fig. S3 (88).
398 Relative viral RNA quantity was analyzed by the ΔΔCt method using GAPDH (assay ID
399 Hs99999905_m1, Life Technologies) as the normalization control. Previous studies have shown
400 that GAPDH is an appropriate normalization control for RNA analysis of CMV-infected cells

401 (89), and we verified that GAPDH Ct values are stable following infection of Kasumi-3 cells
402 (Fig. S4) Use of GAPDH and the ΔΔCt method for analysis of viral RNAs was validated as
403 described in the Supplementary Methods (Fig. S5). TaqMan gene expression assays purchased
404 from Life Technologies were used for analysis of cellular genes (Hs00174128_m1 for TNF-α,
405 Hs00174131_m1 for IL-6 and Hs01555410_m1 for IL-1β mRNA) and GAPDH was used as the
406 normalization control.

407 **Flow Cytometry.** For the immunophenotypic and receptor analyses, cells were washed twice in
408 FACS buffer (2% BSA in phosphate-buffered saline [PBS]), stained with the appropriate
409 antibodies for 30 min at 4°C, washed twice and resuspended in PBS. For phenotyping of
410 reactivated cells and signaling analyses, cells were washed, fixed at a final concentration of 4%
411 formaldehyde and permeabilized with 80% methanol per previously defined protocols (90, 91).
412 Cells were then washed and stained for 1 hour on ice with CD34, CD64, CD14, CD15, CD11c,
413 CD1c and IE 1/2 (CH160) for phenotyping. For analysis of signaling pathways, cells were
414 stained with antibodies specific for Erk, p-Erk, p65 or p-p65, then washed twice and resuspended
415 in PBS. The specificity of the phospho-antibodies for the activated form of the protein was
416 validated according to previously published methods (92) as described in the supplementary
417 methods and shown in Fig. S6S. Data were acquired on 6-laser BD LSRFortessa SORP flow
418 cytometer and analyzed using FlowJo software (version 9). For the phospho-protein analysis, the
419 fraction of activated cells in each population was determined using the following formula as
420 previously described (91, 93)

421 $$Treated\left(\frac{MFI\ Treated\ Stained}{MFI\ Treated\ Unstained}\right) / Untreated\left(\frac{MFI\ Untreated\ Stained}{MFI\ Untreated\ Unstained}\right).$$

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701

702 **FIGURE LEGENDS**

703 **Fig. 1. HCMV latency is established after activation of transcription at 14 days post**
704 **infection**. A) Schematic outlining the infection model used for studies of latency and reactivation
705 in Kasumi-3 cells. GFP+ infected cells were purified by flow cytometry at 1 dpi. On day 14,
706 latently infected cells were treated with TNF-α for 3 days to induce reactivation. B)
707 Representative FACS analysis of GFP expression in Kasumi-3 infected cells at 1 dpi compared
708 to uninfected cells. C) Release of viral particles into the media was measured by a $TCID_{50}$ assay
709 on MRC-5 cells after 2 weeks. D) UL122 mRNA expression and DNA amount were analyzed by
710 at the indicated times post infection and expressed relative to D1 after normalization to GAPDH
711 or RNAseP. E) RNA/DNA ratios of UL123, UL54, and UL32 over the course of infection. For
712 Panels C to E, statistical significance was calculated by a one-way ANOVA with a Dunnett's
713 multiple comparison test (n=4). The error bars represent standard error of the mean (SEM) and
714 the asterisks indicate p-values (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$) calculated
715 by the comparison to the peak (D4 for RNA, D8 for DNA and virus, and D1 for the RNA/DNA
716 ratio).

717 **Fig 2. HCMV early and late gene expression is repressed in some infected cells at 1 dpi**. A)
718 Representative FACs analysis of HCMV infected cells at 1 dpi showing the gating strategy used
719 to separate GFP+ and GFP- infected cells. B) UL122 DNA amount in GFP+ and GFP- infected
720 cells was analyzed by qPCR at 1 dpi and expressed relative to GFP+ cells after normalization to

721 RNAseP. C to E) Ratio of relative RNA expression to the relative DNA amount in GFP- cells
722 compared to GFP+ cells at 1 dpi. For Panel B to E statistical significance was calculated by
723 unpaired *t-test* (n=4). The error bars represent the SEM and the asterisks indicate p-values (*ns* P>
724 0.05; * P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001).

725 **Fig 3. HCMV early and late gene expression is delayed in some infected cells**. A)
726 Representative FACS analysis of the GFP- population purified 1 dpi and cultured for an
727 additional 3 days (right panel) compared to uninfected cells (left panel). B to D) Relative mRNA
728 expression and DNA amount of UL122, UL54 and UL32 analyzed by RT-qPCR and qPCR,
729 respectively, at the indicated times post infection and expressed relative to D1. For Panel B to D
730 statistical significance was calculated by a one-way ANOVA with a Dunnett's multiple
731 comparison test (n=3). The error bars represent the SEM and the asterisks indicate p-values (* P≤
732 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001*) calculated by comparison to the peak at D4.

733
734 **Fig. 4. Expression of latency associated RNAs.** mRNA expression (A), DNA amount (B) and
735 RNA/DNA ratio (C) of UL32, RNA2.7, UL138 and US28 analyzed at the indicated times post
736 infection and expressed relative to D1. RNA expression values were normalized to GAPDH and
737 the DNA amount was normalized to RNAseP. The statistical significance was calculated by 2-
738 way ANOVA with a Tukey's multiple comparison test (n=3). The error bars represent the SEM
739 and the asterisks indicate p-values (* P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001)
740 calculated by comparison of the different genes at each time point.

741
742
743 **Fig 5. TNF-α induces reactivation independently of differentiation.** A) Latently infected
744 cells (14 dpi) were treated or not with TNF-α for 3 days. On day 17, DNA and RNA from
745 infected cells was analyzed for DNA copy number and expression of viral genes as decribed in

746 Materials and Methods. The release of viral particles in the supernatant was measured by a
747 $TCID_{50}$ assay. The statistical significance was calculated by unpaired *t-test* (n=4). The error bars
748 represent the SEM and the asterisks indicate p-values (* P< 0.05; ** P< 0.01). B) Representative
749 FACS analysis of uninfected, latently infected cells and TNF-α treated cells for the expression of
750 the hematopoietic progenitor marker CD34 and the viral immediate early proteins IE1/2.
751 Reactivating cells were immunophenotyped by gating on the CD34+/IE1/2+ population and
752 analyzing expression of markers of myeloid differentiation (CD64, CD14, CD15, CD11c and
753 CD1c). C) Percentage of undifferentiated cells within the IE1/2 expressing population in the
754 TNF-α treated cells (CD14-, CD64-, CD15-, CD11c-and CD1c-). The error bars represent the
755 SEM calculated from 3 experiments.

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757
758 **Fig. 6. LPS does not induce reactivation in Kasumi-3 cells.** A) Latently infected cells (14 dpi)
759 were treated or not with TNF-α or LPS for 3 days. On day 17, DNA and RNA from infected
760 cells were analyzed for DNA copy number and expression of UL122. Data from 4 independent
761 experiments are reported as the average ± SEM. B) Production of TNF-α was assayed by ELISA
762 in the supernatants of uninfected Kasumi-3 and THP-1 cells, treated or not with LPS, 4 hours
763 post-treatment. Data represents 3 independent experiments. C) Representative FACS analysis of
764 uninfected Kasumi-3 cells for the expression of CD34 and members of the LPS receptor complex,
765 TLR-4, CD14 and MD2. D) Uninfected Kasumi-3 cells were treated or not with TNF-α or LPS
766 for 4 hours while THP-1 cells were treated or not with LPS under the same conditions. For NF-
767 κB activity, cells were analyzed for expression of total p65 (left panel), p-p65 (S536) (middle
768 panel) and the ratio of phospho- to total protein (right panel) relative to untreated cells.
769 Activation of ERK was analyzed by measuring p44/42 MAPK (ERK) (left panel), Phospho-

770 p44/42 MAPK (p-ERK) (middle panel) and the ratio of phospho- to total protein (right panel)
771 relative to untreated cells (N=3). F to H) Uninfected Kasumi-3 cells and THP-1 cells were
772 treated or not with LPS for 4 hours and then analyzed for RNA expression of TNF-α (F), IL-6
773 (G) and IL-1β (H), relative to THP-1 by qRT-PCR (N=7). Data from B to H are reported as the
774 average ± SEM. Statistical analyses were performed using ordinary 1-way ANOVA (panel A),
775 2-way ANOVA with a Tukey's multiple comparison (panel B, D, and E), or pairwise
776 comparison based on a Wilcoxon rank sum test (panel F-H) (* $P < 0.05$; ** $P \leq 0.01$; *** $P \leq$
777 0.001; **** $P \leq 0.0001$).
778
779 Table 1. Antibodies used in this study.

Marker	Clone	Supplier
CD34	581	BD Biosciences
CD45	HI30	BD Biosciences
CD1c	F10/21A3	BD Biosciences
CD11c	B-ly6	BD Biosciences
CD14	MOP9	BD Biosciences
CD15	HI98	BD Biosciences
CD64	10.1	BD Biosciences
TLR4 (CD284)	TF901	BD Biosciences
MD2	18H10	Abcam
IE1/2	CH160	Virusys
Erk p44/42 MAPK(Erk1/2)	137F5	Cell Signaling Technology
phospho-Erk (phospho-p44/42 MAPK(Erk1/2)(Thr202/Tyr204)	D13.14.4E	Cell Signaling Technology
NF-kB p65	L8F6	Cell Signaling Technology
phospho-NF-kB p65 (Ser536)	393H1	Cell Signaling Technology

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791 Table 2. TaqMan assays for analysis of viral DNA and RNA

Assay name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Reporter sequence (5'-3')
UL122	GGCTCACCTCGTCAATCTTGA	CAACGAGAAGGTACGCAATATCATG	CCCCTTCTGCACACCC
UL123	GCACCCGACAAAACTCACTTAAGA	TGACGCTTGTATGATGACCATGT	ACGGGTCCATCTCTC
UL54	GAAACATAGCCGCCACAGAAC	ACGAATAGTGTTGCCGTGTCA	AACGCCGCTATCATCT
UL32	GGTGAAACGCGGATCTTGAC	GCTCACGGAGACCAGAGG	CTGTCGGAATCCTCG
UL138	CTTCCTCCCAACGGACCAA	CGGACAGACGATACCGTTTCT	ACGGCCCGATGAGATC
US28	CCGATCATCCTCAACGTAGAACT	CGGTAGTAGCAGTAGCTGATGAC	CATGCTCGGTGCTTTC
2.7kb RNA	TCACCGTTTTCTCTCTTCTCTCTCT	TCGAAGAATGAAAGACGACGATGATT	CCGCTCCTGCCACCCG

792

793 **Supplementary Methods.**

794 **DNA and RNA analysis**. To demonstrate that the ΔΔCt method is valid to quantify viral DNA
795 using our custom viral TaqMan assays and the cellular gene RNaseP as the normalization control,
796 the efficiency of amplification each viral gene was analyzed in parallel with RNaseP using serial
797 dilutions of DNA isolated from lytically infected MRC-5 fibroblasts as the template. The ΔCt
798 values ($C_T$viral gene – C_TRNaseP) for each dilution were calculated and plotted against the log
799 ng DNA. The slopes of these plots were <0.1 (Fig. S3). Similar analyses were performed to
800 demonstrate the validity of the ΔΔCt method to quantify viral RNAs using GAPDH as the
801 normalization control. Serial dilutions of cDNA prepared from lytically infected MRC-5 cells
802 were used as the template (Fig. S5).

803 **Validation of flow cytometric analysis of phospho-Erk and phospho-NFkB (p65).** Phospho-
804 Erk antibody staining was validated by analyzing phorbol 12-myristate 13-acetate (PMA)-
805 induced phosphorylation in Jurkat cells. Cells were either untreated or treated with 50nM PMA
806 for 15 minutes, or with the MEK inhibitor U0126 (100uM) at 37°C followed by immediate
807 fixation with 4% methanol free formaldehyde, followed by 80% methanol permeabilization.
808 U0126 is a highly selective inhibitor of both MEK1 and MEK2. Following fix-permeabilization

809 the cells were washed and stained. One set of tubes were stained with pre-titered volume of
810 Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) rabbit mAb and the second set
811 stained with matched Isotype control (Fig. S6A). In addition, dose-response and kinetic analyses
812 were used to evaluate activation of phospho-Erk in response to PMA. Similarly phospho-p65
813 (NFkB) antibody staining was validated by TNF-a-induced NFkB phosphorylation of HeLa cells.
814 HeLa cells were either untreated or treated with 100 nM Calyculin A, an inhibitor of protein
815 phosphatase 1 (PP1) and protein phosphatase 2A (PP2A-C) for 10 minutes followed by 20ng/ml
816 of human TNF-a for 5 minutes at 37°C, followed by immediate fixation and permeabilization.
817 Following these, the cells were washed, and processed for staining. One set of tubes was stained
818 with pre-titered volume of phospho-NF-kB p65 (Ser563) (93H1) rabbit mAb and the second set
819 stained with matched Isotype control (Fig. S6B).
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821 **SUPPLEMENTARY FIGURES**
822 **Fig. S1. Analysis of viral RNA and DNA over the course of infection in Kasumi-3 cells.**
823 RNAs and DNAs from representative immediate early, early and late genes were analyzed by
824 RT-qPCR and qPCR, respectively, at various times post-infection as described in Fig. 1 and
825 Materials and Methods.
826 **Fig. S2. TNF induces expression of HCMV early and late genes.** RNAs from the experiments
827 shown in Fig. 5 were analyzed for relative expression of the early gene UL54 and the late gene
828 UL32.
829 **Fig. S3. Analysis of the efficiencies of amplification of viral genes versus RNaseP.** Viral
830 genes and the cellular gene RNaseP were amplified in samples prepared from serial dilutions of

831 DNA isolated from lytically infected MRC-5 fibroblasts. The ΔCt values ($C_T$viral gene –

832 C_TRNaseP) for each dilution were calculated and plotted against the log ng DNA.

833 **Fig. S4. Validation of GAPDH as a normalization control in HCMV-infected Kasumi-3**

834 **cells.** Data shows average Ct values ± SD for GAPDH at various times after infection. N=4.

835 **Fig. S5. Analysis of the efficiencies of amplification of viral RNAs versus GAPDH.** Viral

836 RNAs and cellular GAPDH RNA were amplified in samples prepared from serial dilutions of

837 cDNA prepared from RNA isolated from lytically infected MRC-5 fibroblasts. The ΔCt values

838 ($C_T$viral gene – C_TGAPDH) for each dilution were calculated and plotted against the log ng

839 cDNA.

840 **Fig. S6. Phospho-antibody staining validation.** (A) Representative flow cytometric analysis of

841 Jurkat cells treated with 100μM UO126 (blue) or 50 nM PMA-treated (green), using Phospho-

842 p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit mAb compared to a matched Isotype control

843 (red). (B) Representative flow cytometric analysis of HeLa cells, untreated (blue) or treated with

844 hTNF-α (20ng/ml) and Calyculin A (100nM) for 15 mins (green), using Phospho-NF-*k*B p65

845 (Ser563) rabbit mAb compared to a matched Isotype control (red).

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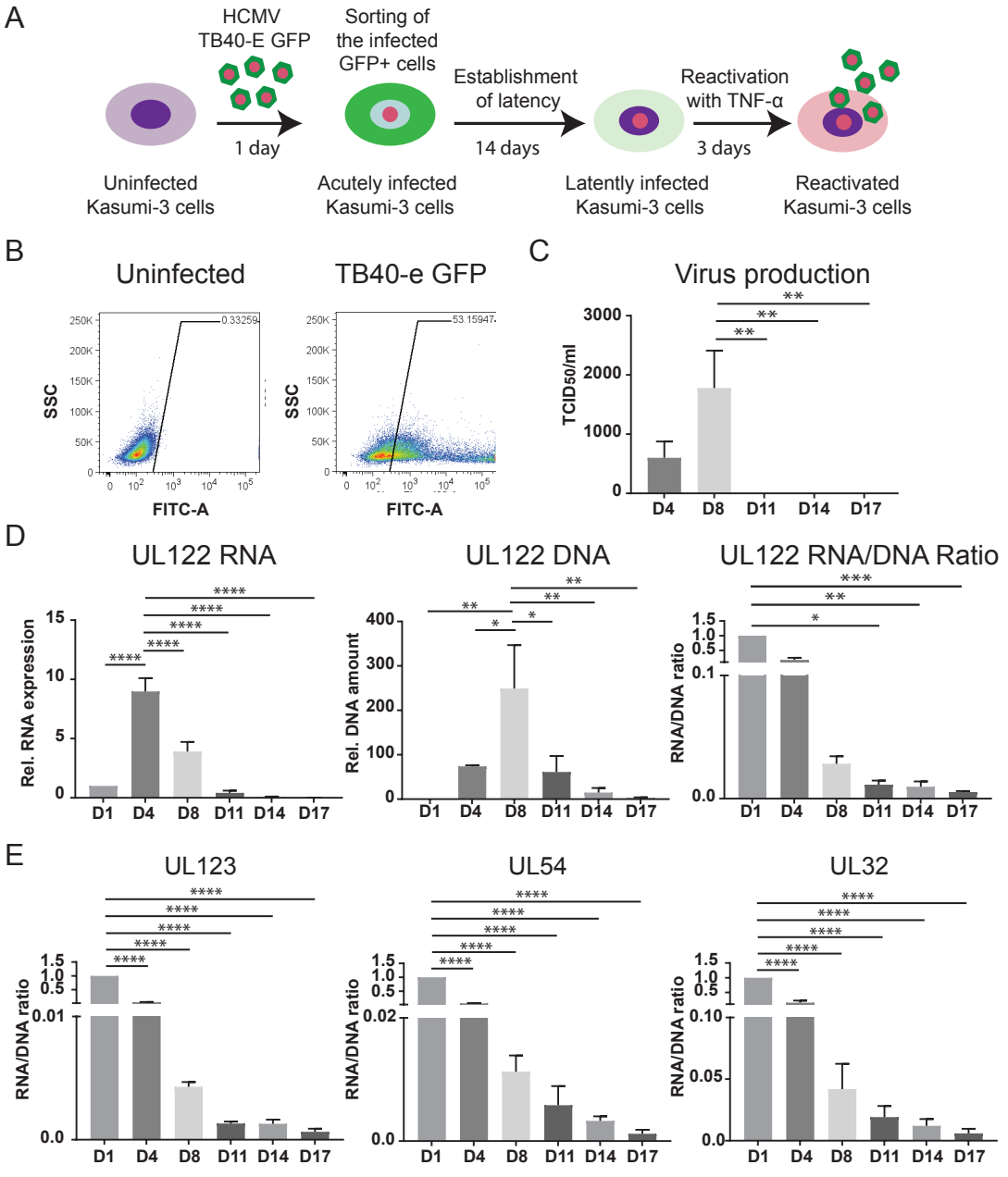

Fig. 1. HCMV latency is established after activation of transcription at 14 days post infection. A) Schematic outlining the infection model used for studies of latency and reactivation in Kasumi-3 cells. GFP+ infected cells were purified by flow cytometry at 1 dpi. On day 14, latently infected cells were treated with TNF-α for 3 days to induce reactivation. B) Representative FACS analysis of GFP expression in Kasumi-3 infected cells at 1 dpi compared to uninfected cells. C) Release of viral particles into the media was measured by a TCID50 assay on MRC-5 cells after 2 weeks. D) UL122 mRNA expression and DNA amount were analyzed by at the indicated times post infection and expressed relative to D1 after normalization to GAPDH or RNAseP. E) RNA/DNA ratios of UL123, UL54, and UL32 over the course of infection. For Panels C to E, statistical significance was calculated by a one-way ANOVA with a Dunnett's multiple comparison test (n=4). The error bars represent standard error of the mean (SEM) and the asterisks indicate p-values (* P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001) calculated by the comparison to the peak (D4 for RNA, D8 for DNA and virus, and D1 for the RNA/DNA ratio).

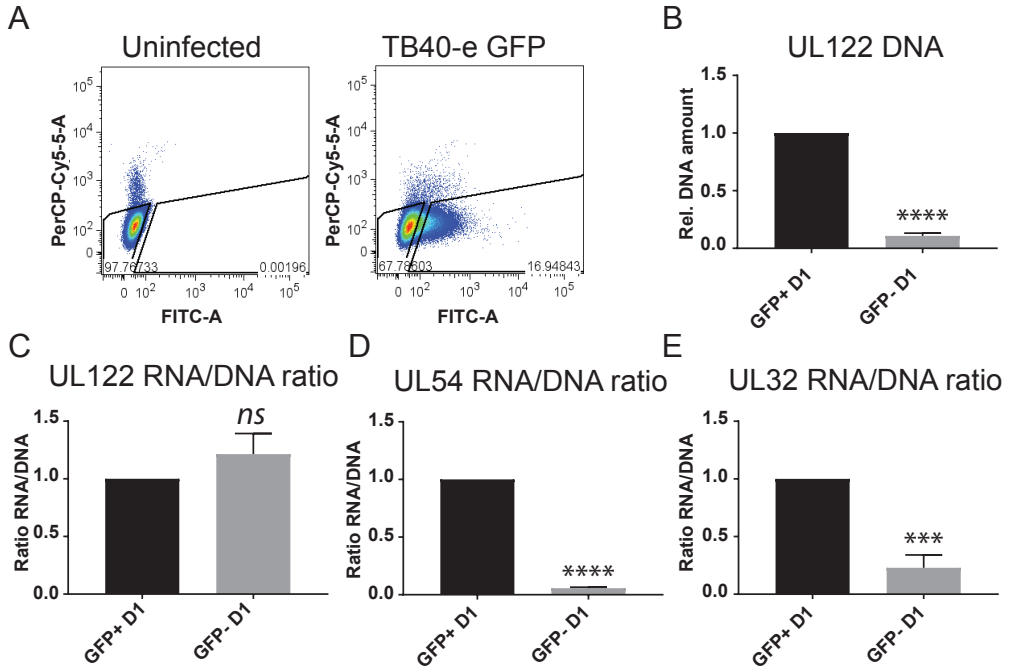

Fig 2. HCMV early and late gene expression is repressed in some infected cells at 1 dpi. A) Representative FACs analysis of HCMV infected cells at 1 dpi showing the gating strategy used to separate GFP+ and GFP- infected cells. B) UL122 DNA amount in GFP+ and GFP- infected cells was analyzed by qPCR at 1 dpi and expressed relative to GFP+ cells after normalization to RNAseP. C to E) Ratio of relative RNA expression to the relative DNA amount in GFP- cells compared to GFP+ cells at 1 dpi. For Panel B to E statistical significance was calculated by unpaired t-test (n=4). The error bars represent the SEM and the asterisks indicate p-values (ns P> 0.05; * P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001).

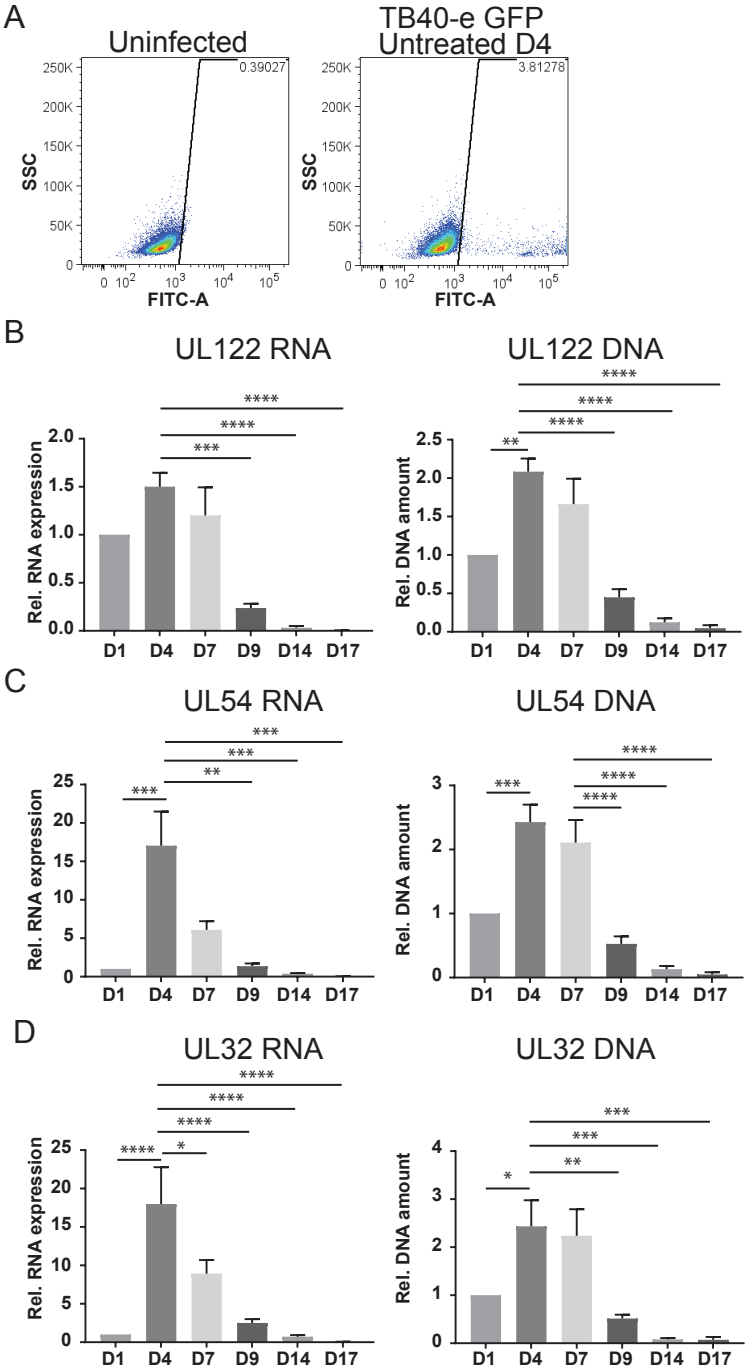

Fig 3. HCMV early and late gene expression is delayed in some infected cells. A) Representative FACS analysis of the GFP- population purified 1 dpi and cultured for an additional 3 days (right panel) compared to uninfected cells (left panel). B to D) Relative mRNA expression and DNA amount of UL122, UL54 and UL32 analyzed by RT-qPCR and qPCR, respectively, at the indicated times post infection and expressed relative to D1. For Panel B to D statistical significance was calculated by a one-way ANOVA with a Dunnett's multiple comparison test (n=3). The error bars represent the SEM and the asterisks indicate p-values (* P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001*) calculated by comparison to the peak at D4.

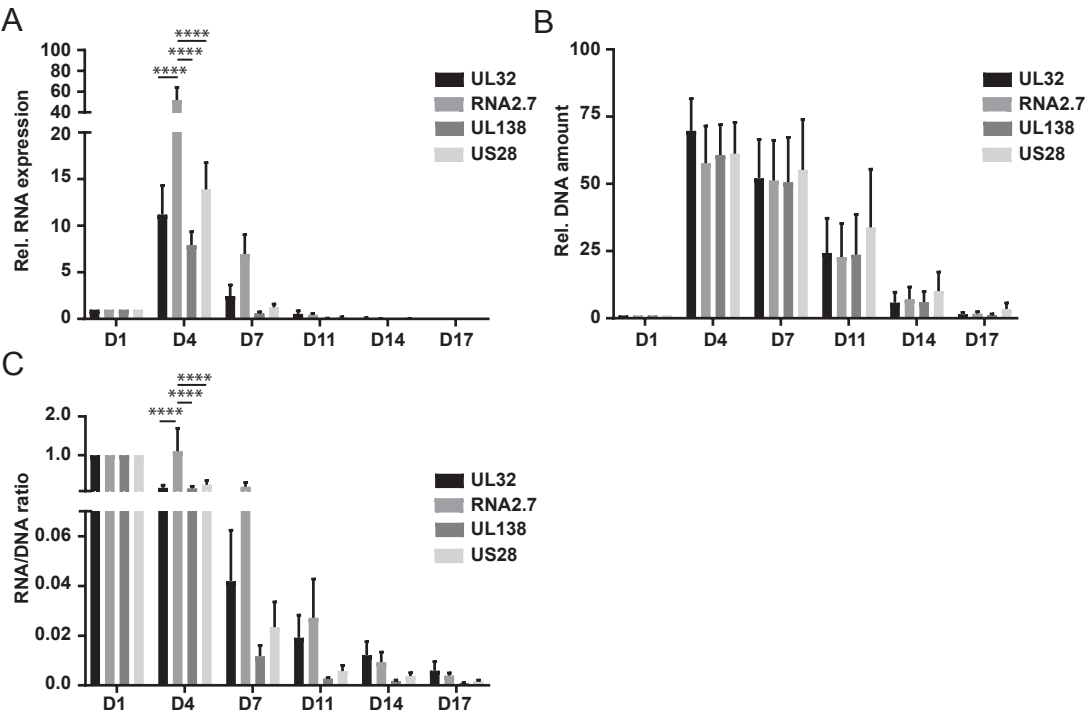

Fig. 4. Expression of latency associated RNAs. mRNA expression (A), DNA amount (B) and RNA/DNA ratio (C) of UL32, RNA2.7, UL138 and US28 analyzed at the indicated times post infection and expressed relative to D1. RNA expression values were normalized to GAPDH and the DNA amount was normalized to RNAseP. The statistical significance was calculated by 2-way ANOVA with a Tukey's multiple comparison test (n=3). The error bars represent the SEM and the asterisks indicate p-values (* P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001) calculated by comparison of the different genes at each time point.

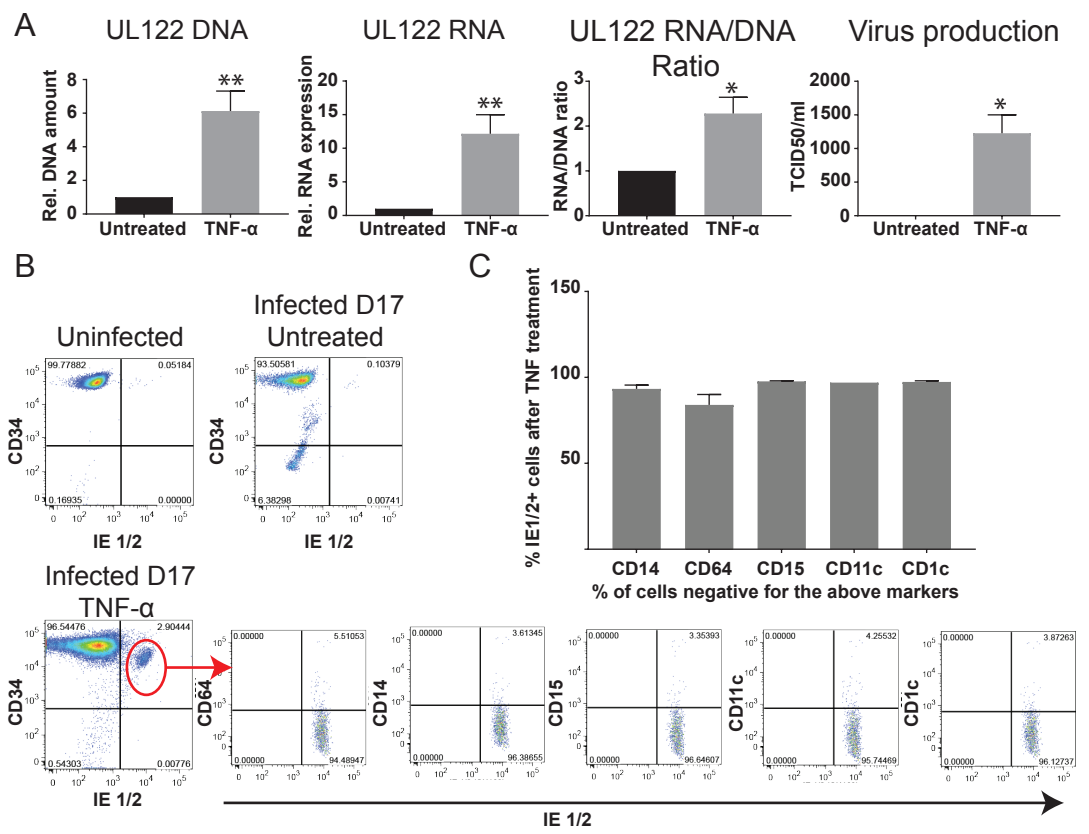

Fig 5. TNF-α induces reactivation independently of differentiation. A) Latently infected cells (14 dpi) were treated or not with TNF-α for 3 days. On day 17, DNA and RNA from infected cells was analyzed for DNA copy number and expression of viral genes as decribed in Materials and Methods. The release of viral particles in the supernatant was measured by a TCID50 assay. The statistical significance was calculated by unpaired t-test (n=4). The error bars represent the SEM and the asterisks indicate p-values (* P< 0.05; ** P< 0.01). B) Representative FACS analysis of uninfected, latently infected cells and TNF-α treated cells for the expression of the hematopoietic progenitor marker CD34 and the viral immediate early proteins IE1/2. Reactivating cells were immunophenotyped by gating on the CD34+/IE1/2+ population and analyzing expression of markers of myeloid differentiation (CD64, CD14, CD15, CD11c and CD1c). C) Percentage of undifferentiated cells within the IE1/2 expressing population in the TNF-α treated cells (CD14-, CD64-, CD15-, CD11c-and CD1c-). The error bars represent the SEM calculated from 3 experiments.

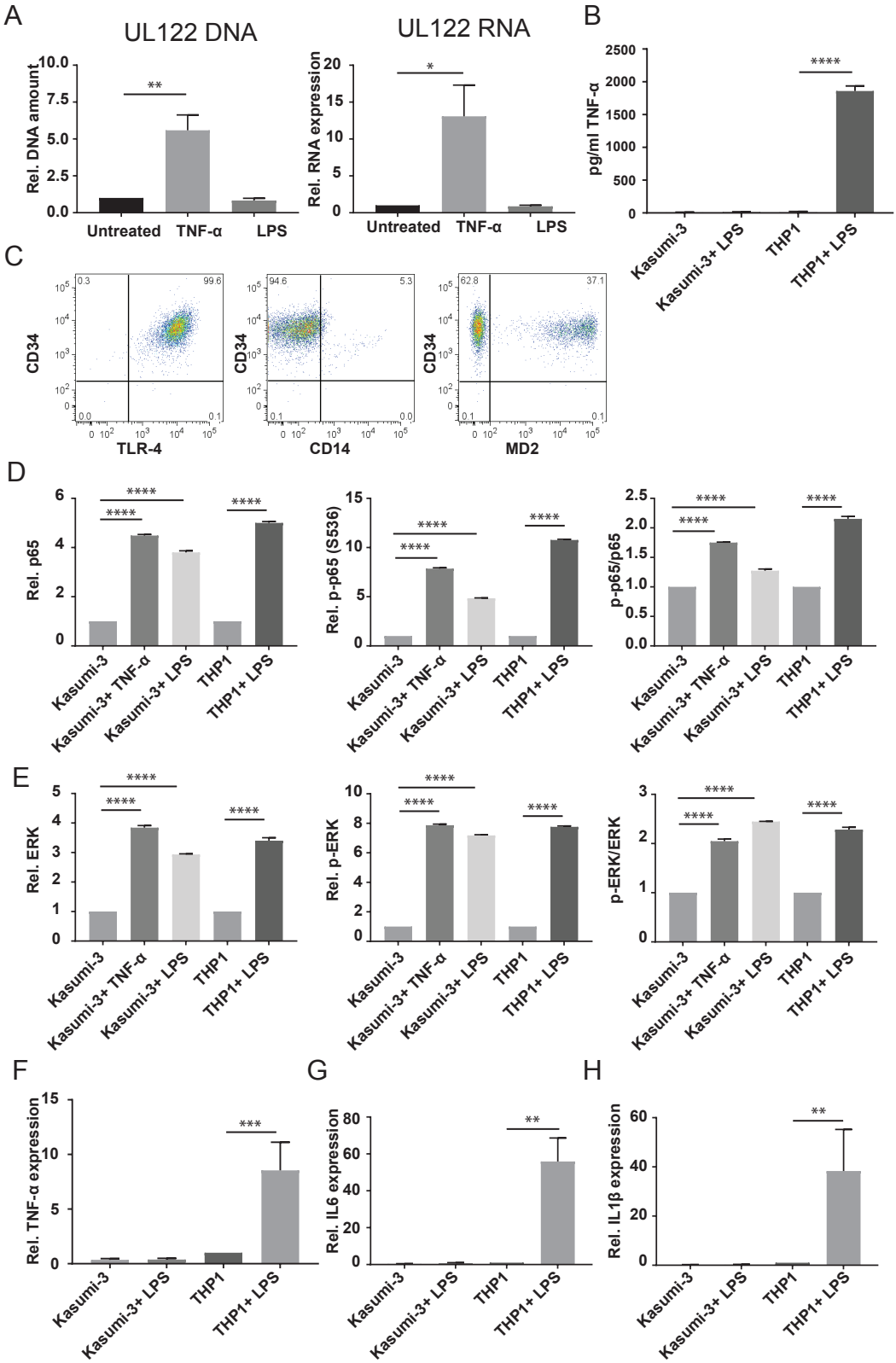

Fig. 6. LPS does not induce reactivation in Kasumi-3 cells. A) Latently infected cells (14 dpi) were treated or not with TNF-α or LPS for 3 days. On day 17, DNA and RNA from infected cells were analyzed for DNA copy number and expression of UL122. Data from 4 independent experiments are reported as the average ± SEM. B) Production of TNF-α was assayed by ELISA in the supernatants of uninfected Kasumi-3 and THP-1 cells, treated or not with LPS, 4 hours post-treatment. Data represents 3 independent experiments. C) Representative FACS analysis of uninfected Kasumi-3 cells for the expression of CD34 and members of the LPS receptor complex, TLR-4, CD14 and MD2. D) Uninfected Kasumi-3 cells were treated or not with TNF-α or LPS for 4 hours while THP-1 cells were treated or not with LPS under the same conditions. For NF- B activity, cells were analyzed for expression of total p65 (left panel), p-p65 (S536) (middle panel) and the ratio of phospho- to total protein (right panel) relative to untreated cells. Activation of ERK was analyzed by measuring p44/42 MAPK (ERK) (left panel), Phospho-p44/42 MAPK (p-ERK) (middle panel) and the ratio of phospho- to total protein (right panel) relative to untreated cells (N=3). F to H) Uninfected Kasumi-3 cells and THP-1 cells were treated or not with LPS for 4 hours and then analyzed for RNA expression of TNF-α (F), IL-6 (G) and IL-1β (H), relative to THP-1 by qRT-PCR (N=7). Data from B to H are reported as the average ± SEM. Statistical analyses were performed using ordinary 1-way ANOVA (panel A), 2-way ANOVA with a Tukey's multiple comparison (panel B, D, and E), or pairwise comparison based on a Wilcoxon rank sum test (panel F-H) (* P< 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001).