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, in the vast majority of cells, latency was established post-transcriptionally in the GFP+ infected cells: transcription was initially turned on, and then turned off. We also found that some of the GFP- cells were infected, suggesting that latency might be established in these cells at the outset of infection. We were not able to test this hypothesis because some GFP- cells expressed lytic genes, and thus, it was not possible to separate them from GFP- quiescent cells. In addition, we found that 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 once latency is established. We confirmed previous studies showing that TNF-α induced reactivation of infectious virus, and 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 transcriptional reactivation of IE gene expression independently of differentiation. TNF-α-mediated reactivation in Kasumi-3 cells was correlated with activation of NF-κB, KAP-1 and ATM.

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. Our observation that viral gene expression is first turned on, and then turned off to establish latency suggests that there is a host defense, which may be myeloid-specific, responsible for transcriptional silencing of viral gene expression. Our observation that

50 TNF-α induces reactivation independently of differentiation provides insight into molecular
51 mechanisms that control reactivation.
52
53 **INTRODUCTION**
54 HCMV is a ubiquitous human pathogen of the beta herpesvirus family. Infection is
55 transmitted through contact with body fluids, including saliva, urine, blood, and genital
56 secretions. Hematogenous spread of the virus leads to a systemic infection, with multiple cell
57 types infected in many organs. In immunocompetent hosts, primary infection is typically sub-
58 clinical, and is resolved by activation of innate and adaptive immunity. However, latently
59 infected cells, which carry viral DNA, but do not produce infectious virus, are able to escape
60 immune surveillance, and can persist for the life of the host.
61 Under the appropriate conditions, the virus in these cells wakes up from its dormant state
62 to reactivate the infectious cycle. In immunocompetent hosts, viral replication is controlled by
63 the immune response, but reactivation of latent virus can be a significant infectious complication
64 in immunocompromised hosts, such as recipients of solid organ or bone marrow transplants.
65 Reactivation of CMV in solid organ transplant recipients is associated with increased risk of
66 CMV disease, acute and chronic allograft rejection, infection with other opportunistic pathogens,
67 graft failure, and death (1). Risk factors for reactivation include CMV serostatus of the donor
68 (D) and recipient (R), with the highest risk in the D+/R- combination, specific
69 immunosuppression protocols, and inflammatory conditions associated with high TNF-α
70 secretion, including allograft rejection and sepsis (1).
71 The cell type harboring latent CMV DNA in solid organs has not been clearly identified.
72 However, it is known that latent virus is transmitted through blood transfusion, and monocytes in

73 the blood and hematopoietic progenitor cells (HPCs) in the bone marrow are sites of latency in
74 vivo (2-10). Experimental models have shown that these cells are less permissive to lytic
75 replication, and that they support a latent infection (11-16). Cell type-specific establishment of
76 latency is thought to be due to a combination of host and viral factors. Infection activates a host
77 intrinsic immune response, which recognizes viral DNA invading the nucleus and silences viral
78 gene expression at the outset of infection through heterochromatinization of viral genomes (13,
79 17-26). Factors present in the viral particle, including the tegument protein pp71, enter the cell
80 upon infection, and counteract this host defense response to activate viral gene expression. In
81 cells that support latency, pp71 is sequestered in the cytoplasm and is therefore unable to
82 perform this function (26-28). Differentiation of myeloid cells to dendritic cells increases
83 permissiveness of these cells to infection and also induces reactivation of both naturally and
84 experimentally latently infected cells (12, 13, 15, 20, 29-32). Thus, the generally accepted
85 paradigm is that myeloid progenitor cells are not permissive to infection due to
86 heterochromatinization and transcriptional repression of invading viral genomes. The process of
87 myeloid cell differentiation is thought to change the balance of repressive and activating cellular
88 factors that control both viral and cellular transcription, resulting in changes to the viral
89 epigenome that activate viral gene expression (17, 33, 34).
90 However, some recent observations suggest that the current paradigm may be in need of
91 revision. First, several investigators have demonstrated that, although myeloid progenitors may
92 be less permissive than other cell types, some expression of viral lytic genes is detectable at early
93 times post-infection in various models of latency, including primary CD34+ HPCs, Kasumi-3
94 cells, a CD34+ myeloblastic cell line derived from a patient with acute myeloid leukemia (AML)
95 (35), which is a tractable model for CMV latency and reactivation (27, 36, 37), and primary

96 CD14+ peripheral blood mononuclear cells (11, 12, 36-43). Expression of these genes is lost as
97 latency is established. These observations suggest that latency is not necessarily established at
98 the outset of infection, but may also be established following activation of viral transcription.
99 Second, differentiation of myeloid cells into dendritic cells *in vivo* occurs in the context
100 of infection and inflammation (44), and all of the physiologically relevant differentiation factors
101 that have been used to induce reactivation of CMV in primary hematopoietic cells, including
102 GM-CSF (45), TNF-α, IL-6, and LPS, are mediators of inflammation. Thus, it is difficult to
103 distinguish the roles of inflammation versus differentiation in these models. In contrast, Kasumi-
104 3 cells, like other transformed cell lines, are refractory to normal physiological differentiation
105 cues, and therefore can be used to dissect the contributions of these two pathways. Here, we
106 have used the Kasumi-3 model to further investigate these issues.
107
108 **RESULTS**
109 **Latency is established after activation of transcription.** The time at which latency is
110 established following infection with HCMV has not been clearly determined. Some previous
111 studies have shown that primary CD34+ myeloid progenitor cells do not express lytic viral genes
112 upon infection, but rather, that viral genomes in these cells are inactivated upon entry and remain
113 quiescent until they receive signals that induce them to differentiate (26, 46). However, other
114 studies have shown that both primary CD34+ cells and Kasumi-3 cells express an SV40
115 promoter-driven GFP reporter, as well as viral genes at early times post-infection (11, 36-41),
116 and that latency can be established in the GFP+ population following cell sorting (11, 36, 38, 40,
117 41, 47). To investigate the question of timing further, we infected Kasumi-3 cells with the
118 TB40/E*wt*-GFP strain of HCMV (48), and analyzed the cells by FACS after 24 hr (Fig. 1A).

119 GFP expression was observed in 20-50% percent of cells (Fig. 1B), as previously reported (36).
120 Following inactivation of input virus, we purified the GFP+ population by cell sorting and
121 analyzed viral RNA expression, DNA copy number, and production of infectious virus over the
122 course of infection. Latency has been operationally defined as the lack of virus production in
123 cells that carry viral DNA with the capacity to reactivate (49). Production of infectious virus
124 was detectable at 4 dpi and peaked at 8 dpi, but was no longer detectable at 11dpi (Fig. 1C).
125 A second characteristic of latency is transcriptional repression of genes involved in lytic
126 replication. We therefore analyzed expression of viral genes representative of the immediate
127 early (IE-2, UL122 and IE-1, UL123), early (viral DNA polymerase UL54), and late (UL32)
128 phases of the viral life cycle. Expression of these genes peaked at 4 dpi and then fell throughout
129 the course of infection. Viral DNA copy number also increased following infection, peaked 4-8
130 dpi, and then decreased significantly (Fig. 1D and Fig. S1). Because both viral DNA and RNA
131 copy number dropped over the course of infection, analysis of RNA expression alone does not
132 indicate repression of viral transcription. We therefore calculated the ratio of viral RNA to DNA.
133 Relative to 1 dpi, this ratio fell for all genes from 4 to 14 dpi (Fig. 1D and E). TNF-α induced
134 reactivation of infectious virus in these cells (see below). It is important to note that, because the
135 cells were sorted for GFP expression to obtain a pure population of infected cells in which viral
136 genomes were transcriptionally active, these results show that latency can be established by
137 shutting off gene expression after it has been turned on. We have introduced the term post-
138 transcriptional latency to describe this state.
139
140 **Some GFP- cells contain viral DNA and express lytic transcripts.** Although our
141 studies indicate that latency can be established post-transcriptionally, we wondered whether there
142 might be a second population of cells in which latency is established at the outset of infection.

143 These cells would carry viral DNA, but would not express GFP or viral genes. To test this
144 hypothesis, we analyzed both the GFP-positive and GFP-negative populations for the presence of
145 viral DNA at 1 dpi. To preclude cross-contamination of these fractions, the gates for sorting were
146 set far apart. qPCR analysis revealed that, although the vast majority of the DNA was present in
147 the GFP+ cells, some DNA was detectable in the GFP- population (Fig. 2C). We then analyzed
148 the RNA from these cells for expression of immediate early, early, and late genes (Fig. 2D).
149 Expression of immediate early (IE-2, UL122) RNA was detectable in the GFP- population, albeit
150 at a lower level than the GFP+ cells. When we calculated the ratio of UL122 RNA to DNA, we
151 found that there was no difference between the GFP+ and GFP- cells, indicating that
152 transcription of the IE genes was equally active in these two populations at this time (Fig. 2E).
153 However, when we analyzed the RNA/DNA ratios of early (UL54) and late (UL32) genes, we
154 found that this ratio was significantly lower in the GFP- cells (Fig. 2E), indicating that these
155 classes of genes were transcriptionally repressed in the GFP- cells. We then analyzed the GFP-
156 cells for changes in GFP expression, viral RNA expression and DNA copy number over time.
157 We found that some GFP- cells become GFP+ by 4 dpi (Fig. 2B). Expression of all classes of
158 viral RNA and DNA copy increased in these cells from 1 to 4 dpi, and then declined after 7 dpi
159 (Fig. S2).
160 In order to determine whether there was a subset of cells in the GFP- population
161 containing quiescent viral DNA, we re-sorted these cells at 4 dpi into GFP-positive and -negative
162 cells for analysis of viral DNA and RNA (Fig. 2A and B). The results show that viral DNA was
163 detectable in the GFP- population at 4 dpi (Fig. 2C, GFP-D4). Analysis of RNA expression
164 showed that some expression of lytic genes was still detectable in the GFP- population at 4 dpi
165 (Fig. 2D,GFP-D4). We conclude that *i)* the GFP reporter is regulated as an early gene in

166 TB40/E*wt*-GFP infected Kasumi-3 cells, as previously noted in fibroblasts (50); *ii)* there is
167 heterogeneity in the kinetics of viral gene expression after infection of Kasumi-3 cells, with
168 activation of early and late gene expression delayed in some cells; *iii)* it is not possible to use
169 sorting for GFP expression to determine whether a small minority of Kasumi-3 cells infected
170 with TB40/E*wt*-GFP contain viral genomes, but remain quiescent.
171
172 **US28, UL138, and 2.7 kb RNAs are not differentially expressed in latently infected**
173 **Kasumi-3 cells.** A number of studies have analyzed the viral transcriptome in latency, and some
174 of these have indicated that some lytic genes are also specifically expressed in latency, including
175 US28, UL138, and a non-coding 2.7 kb RNA (RNA2.7) (12, 14, 37, 46, 47, 49, 51-53). We
176 analyzed expression of these genes in GFP+ cells over the course of infection in Kasumi-3 cells.
177 RNA2.7 is expressed at very high levels during lytic infection of fibroblasts (54), and at early
178 times after infection of Kasumi-3 cells (Fig. 3A). UL138 and US28 RNAs are also relatively
179 abundant transcripts during lytic infection. To analyze repression of these genes relative to a
180 lytic gene, we analyzed UL32, a gene that is expressed at levels similar to UL138 and US28 at 4
181 dpi. Our results show that expression of UL138, US28, and RNA2.7 followed the same pattern
182 as that observed for UL32, with a peak of expression at 4 dpi, followed by a significant decrease
183 at 7 dpi (Fig. 3A). When we analyzed the RNA/DNA ratio, we found that expression of these
184 genes was repressed over the course of infection, with kinetics similar to that of UL32 (Fig. 3C).
185 The RNA/DNA ratio of RNA2.7 was significantly higher than other genes at 4 dpi, consistent
186 with its high level of expression during lytic infection, but there was no difference between
187 UL32 and UL138 or US28 at any time point, and there were no differences in the RNA/DNA
188 ratios between UL32 and any of the other genes analyzed after 4 dpi (Fig. 3C). Thus, there was

189 no preferential expression of UL138, US28, or RNA2.7 over the lytic gene UL32 when latency
190 was established.
191
192 **TNF-α induces reactivation of HCMV in Kasumi-3 cells independently of myeloid**
193 **cell differentiation.** Previous studies showed that TNF-α induced reactivation of HCMV in
194 latently infected Kasumi-3 cells (36). We confirmed this result using slightly different
195 conditions. Kasumi-3 cells were infected with TB40/E*wt*-GFP virus at an MOI of 1-2, flow-
196 sorted for GFP+ cells and cultured for 14 days to establish latency. The cells were then divided
197 into different pools and incubated for a further 3 days in the presence of 5 ng/ml TNF-α or left
198 untreated. Relative to untreated controls, TNF-α induced statistically significant increases in
199 viral DNA copy, RNA expression and the RNA/DNA ratio, as well as production of infectious
200 virus (Fig. 4A and additional RNA analyses of early and late genes in Fig. S3). In addition, we
201 analyzed the percentage of reactivating cells by flow cytometry after staining with an IE1/2-
202 specific antibody. Expression of IE proteins was essentially undetectable in latently infected,
203 untreated Kasumi-3 cells (Fig. 4B, upper right panel). Treatment with TNF-α reproducibly
204 induced expression of IE1/2 proteins in 2-3% of the cells (representative results shown in Fig. 4B,
205 lower left panel).
206 Differentiation of myeloid progenitor cells to a dendritic cell phenotype has long been
207 associated with transcriptional activation of HCMV IE gene expression and reactivation of
208 infectious virus (13, 20, 34, 40). Transformed cell lines are generally resistant to differentiation,
209 and therefore it seemed unlikely that differentiation would play a role in this model. However,
210 the fact that reactivation occurred in only a small percentage of latently infected Kasumi-3 cells
211 left open the possibility that reactivation occurred in a small number of cells with the capacity to
212 differentiate. To investigate this question, we used immunophenotyping to characterize the

213 differentiation state of Kasumi-3 cells carrying reactivating virus. Latently infected TNF-
214 α treated or untreated cells were stained for IE1/2 antibody in combination with the
215 hematopoietic progenitor marker CD34, and for markers of various stages of myeloid
216 differentiation, including CD64, a marker of granulo-monocytic lineage commitment (55), CD14
217 (monocytes), CD15 (granulocytes), and CD1c and CD11c, which identify major subpopulations
218 of human myeloid dendritic cells (56). Uninfected Kasumi-3 cells are homogeneous with respect
219 to CD34 expression, and do not express other markers of myeloid cell differentiation (Fig. 4B,
220 upper left and Fig. S4). Expression of CD34 was lost in a small percentage of cells after
221 establishment of latency, but latent cells did not differentiate (Fig. S4B). All the latently infected
222 cells were negative for expression of IE proteins (Fig. 4B, upper right panel). 2-3% of the cells
223 became IE+ after treatment with TNF-α, and these cells retained expression of CD34 (Fig. 4B,
224 lower left panel). We then gated on the IE+/CD34+ population to analyze expression of
225 differentiation markers in reactivating cells. ~95% of the IE+/CD34+ cells were negative for
226 CD64- and for markers of mature monocytes and dendritic cells (Fig. 4C). Thus, our data shows
227 that differentiation was not required for transcriptional reactivation of IE gene expression in this
228 model.

229 **TNF-α induces activation of NF-κB and a DNA damage response in Kasumi-3 cells.**
230 Binding of TNF-α to the TNFR1 receptor activates signaling cascades, which lead to activation
231 of transcription factors that control activation of the HCMV Major Immediate Early Promoter
232 (MIEP), including canonical NF-κB (p65/p50) (57-59). Because the IE genes are
233 transcriptionally silent in latently infected cells and they are required for activation of lytic
234 replication, activation of the MIEP is thought to be a requisite first step in reactivation of the
235 virus. Activation of NF-κB is controlled at multiple levels, including cellular localization and

236 post-translational modification at multiple sites (60). TNF/TNFR1-mediated activation of IKK
237 leads to degradation of the inhibitory IκB subunit, which permits translocation of the active
238 p65/p50 complex from the cytoplasm into the nucleus. In addition, IKK mediates
239 phosphorylation of p65 S536 (61, 62), which leads to enhanced transactivation through increased
240 CBP/p300 binding and acetylation of p65 K310 (63). We therefore analyzed total levels of p65,
241 p-p65 S536, and the ratio of p-p65 S536/total p65 in both uninfected and latently infected
242 Kasumi-3 cells treated with or without TNF-α (Fig. 5A). Our results show that all of these
243 increased following treatment with TNF-α in latently infected cells.

244 Previous studies showed that TNF-α induces activation of ATM (64), and that activation
245 of ATM is sufficient to induce reactivation of HCMV in primary hematopoietic progenitor cells
246 through phosphorylation of KAP-1 (S824) bound to latent viral genomes (40). We therefore
247 analyzed total and phospho-ATM (S1981), total and phospho-KAP-1 (S824) and the ratio of
248 phospho/total proteins. In addition, we analyzed phosphorylation of H2AX (γ-H2AX), a variant
249 histone that is recruited to sites of DNA damage (65). Our results show that all of these markers
250 of DNA damage are increased by treatment of Kasumi-3 cells with TNF-α (Fig. 5B-D).

251

252 **DISCUSSION**

253 **Mechanisms of establishment of latency**. HCMV establishes latency in myeloid
254 progenitor cells through heterochromatinization and repression of viral gene expression, but the
255 timing and molecular mechanisms that control this process are not well understood. While some
256 studies have shown that viral gene expression is silenced immediately after infection (26, 46),
257 others have shown that viral gene expression is activated in various models at early times post-
258 infection, and that latency is subsequently established in this population of cells (11, 36, 38, 40,

259 41, 47). Our studies are in agreement with this latter observation. We found that ~90% of
260 infected Kasumi-3 cells become GFP+ at 1 dpi. From 4-8 dpi, expression of all classes of viral
261 genes was activated in this population, viral DNA was amplified, and infectious virus was
262 produced. At later times, viral RNA expression and DNA copy number, and the RNA/DNA
263 ratio decreased. Some lytically infected cells die after infection, and the drop in RNA/DNA may
264 be due in part to loss of these cells. However, we observed a significant decrease in RNA/DNA
265 between live GFP+ cells isolated at 1 dpi, prior to robust viral replication, and 17 dpi, when the
266 cells maintain viral DNA, but expression of viral genes is nearly undetectable. This difference is
267 due at least in part to transcriptional repression of viral genes during the transition from a lytic to
268 latent infection.

269 Thus, we find that, although activation of the MIEP is lower in myeloid progenitor cells
270 than permissive fibroblasts (26), Kasumi-3 cells support the full cycle of lytic replication. In
271 contrast to fibroblasts, where the infection is propagated throughout the culture to kill all the
272 cells, our studies suggest that some infected Kasumi-3 cells are able to shut down viral
273 replication and survive. Our studies are in agreement with previous studies of Kasumi-3 cells,
274 which showed increased expression of lytic RNAs and amplification of viral DNA relative to
275 early times post-infection, and, in some cases, production of infectious virus, followed by
276 transcriptional repression (36, 37, 66). Although Kasumi-3 cells may be more permissive than
277 primary CD34+ HPCs, previous studies have also shown activation of viral gene expression in
278 HPCs prior to establishment of latency (11, 38, 41, 47).

279 Recent studies indicate that post-transcriptional establishment of latency may be a more
280 general feature of herpesvirus latency (67-70). Previous studies have shown that activation of
281 the immediate early ICP0 promoter was detectable in some, but not all neurons of mice latently

282 infected with HSV (68). Activation of the late gC promoter was also detectable in the acute
283 stage of infection, but not in latently infected mice. This observation suggests that, although
284 some viral genes may be expressed prior to establishment of latency, there is a point of no return,
285 and full replication of the virus is not compatible with survival. Interestingly, these authors also
286 demonstrated activation of the HCMV MIEP prior to establishment of latency in neurons
287 infected with HSV.

288 We considered the possibility that there may be two routes to establishment of latency:
289 repression prior to activation of gene expression as well as post-transcriptional repression. Our
290 results show that ~10% of the infected cells are GFP- at 1 dpi. We found that there is
291 heterogeneity in the kinetics of viral gene expression, and that some GFP- cells become GFP+,
292 activate the early and late phases of viral gene expression, and amplify viral DNA with delayed
293 kinetics. Previous studies showed that initiation of HCMV gene expression requires that the cells
294 be in the G0 or G1 phase of the cell cycle (71), and heterogeneity in cell cycle phase may
295 account for some of the observed variation in the kinetics of gene expression. The majority of
296 the cells that were GFP- at 1 dpi remain GFP- at 4 dpi. But, because some GFP- cells expressed
297 lytic viral transcripts, we were unable to determine whether there were other cells that retain viral
298 genomes, but do not express viral RNAs. However, since 90% of the infected cells are GFP+ at
299 1 dpi, these cells would constitute a very small minority of the infected cells.

300 Our findings have two important implications. First, the observation that viral gene is
301 expression is first turned on, and then turned off, suggests that there is a mechanism for shutting
302 off viral genomes that are actively engaged in transcription. This may be due to expression of
303 viral genes that facilitate establishment of latency (37, 41, 46-49, 66, 72, 73). However, these
304 genes are also expressed in models of lytic infection, and could not act directly to repress viral

305 gene expression, since they are not localized to the nucleus. An attractive hypothesis that would
306 account for the highly restricted tropism for HCMV latency is that there is a host defense
307 response that shuts off viral transcription specifically in myeloid lineage cells.

308 Second, shedding of HCMV is often observed in healthy, immunocompetent individuals,
309 suggesting that the virus reactivates with high frequency (74). The finding that HCMV
310 establishes latency in cells following activation of transcription raises the possibility that these
311 genomes may retain a memory of prior activation, and may therefore be poised to reactivate
312 readily under the appropriate conditions. Interestingly, previous studies have shown that HCMV
313 chromatin is specifically marked by H3 lysine 4 methylation following replication of viral DNA
314 in fibroblasts (75). It is tempting to speculate that there may be cell type-specific differences in
315 histone modifications bound to post-replicative viral DNA that affect the potential to establish
316 latency and to reactivate.

317 **Viral transcription in latency**. Previous studies showed that some viral genes that are
318 expressed during lytic infection, particularly UL138 and US28, facilitate establishment of
319 latency in myeloid progenitor cells (37, 41, 46-49, 66, 72, 73). However, analysis of expression
320 of these genes in latently infected cells has been contradictory and confusing. Some studies
321 have shown that some genes are selectively expressed in latency (12, 14, 37, 46, 47, 51-53). In
322 contrast, studies using unbiased transcriptome analyses to detect viral transcripts in both
323 experimental and natural latency, have identified many genes expressed under conditions of
324 latency, and none of these show specific expression of some RNAs in the absence of all other
325 lytic genes (11, 39, 42, 43, 76). This raises the possibility that detection of these transcripts is
326 due to the presence of a small population of lytically infected cells. With the exception of the
327 noncoding miR-UL148D microRNA (37), most previous studies have not included analyses of

328 the RNA/DNA ratio to demonstrate differences in transcriptional activity between lytic genes
329 and latency-associated lytic genes. With the exception of LUNA (14), there has been no analysis
330 of histones bound to latency-associated genes in latently infected cells to support the idea that
331 they are selectively active. We have investigated expression of US28, UL138, as well as the non-
332 coding RNA2.7 in the Kasumi-3 model. We find that the pattern of expression of these genes
333 parallels that of other lytic genes, including UL122, UL123, UL54, and UL32, and analysis of
334 the RNA/DNA ratio shows that transcription of all of these genes is repressed in latency. We
335 suggest that this type of analysis should be included in future studies to demonstrate latency-
336 specific expression of a gene. Collectively, our studies and others are consistent with the idea
337 that these genes facilitate establishment of latency through changes in the relative abundance of
338 viral transcripts, but that they are shut off when latency is established. This situation would be
339 analogous to that of EBV latency, where the EBNA genes are expressed in naive B cells, but are
340 shut off in memory B cells, which are the long term site of latency (77) and HSV, where ICP0 is
341 initially expressed to turn on LAT expression and facilitate heterochromatinization of viral
342 genomes (67).

343 **What is the role of myeloid cell differentiation in reactivation of HCMV?** Previous
344 studies using primary CD34-derived or monocyte-derived dendritic cells (MoDCs) showed that
345 LPS-induced reactivation of latent HCMV required differentiation to a dendritic cell phenotype
346 (9, 13, 20, 30, 32, 52, 78). Reactivation in immature dendritic cells in response to LPS occurred
347 through induction of IL-6 and activation of ERKs and mitogen and stress-activated kinases
348 (MSK) (52, 79). However, the factors that were used to differentiate dendritic cells in these
349 models are also mediators of inflammation, and it is therefore difficult to distinguish the roles of
350 a general inflammatory response, which could occur in many cell types, from a dendritic cell-

351 specific mechanism of reactivation. In contrast, Kasumi-3 cells, like many transformed cell lines,
352 are refractory to the normal cues that induce differentiation, and are therefore more suitable for
353 studies seeking to dissect the roles of these different processes. Our studies show that
354 differentiation is not required for reactivation in response to TNF-α.

355 Previous studies showed that, in latently infected primary CD34+ hematopoietic
356 progenitor cells, viral genomes are bound to KAP-1, and that KAP-1 is required to establish
357 latency through heterochromatinization and transcriptional silencing (40). Reactivation could be
358 induced by treatment of the cells with chloroquine, an agent that induces phosphorylation of
359 KAP-1 through activation of ATM, or by knock-down of KAP-1, and this effect was potentiated
360 by co-treatment with TNF-α. These cells retained expression of CD34, suggesting that
361 differentiation was not required for reactivation in this model, although expression of dendritic
362 cell markers was not analyzed (40).

363 ATM is the master regulator of the response to double-stranded DNA breaks (DSB) (80).
364 ATM is recruited to damage sites by the Mre11-Rad50-Nbs1 (MRN) complex, where it
365 phosphorylates a variety of substrates that facilitate DNA repair, including KAP-1. KAP-1 is a
366 multifunctional protein whose activity is regulated through post-translational modifications (81).
367 The sumoylated form of KAP-1 acts as a repressor of transcription through recruitment of factors
368 that mediate H3K9 de-acetylation/methylation. ATM-mediated phosphorylation of KAP-1 leads
369 to changes in its interaction partners that result in de-condensation of chromatin to allow repair
370 of damaged sites, and this is especially important in repair of breaks that occur in regions of
371 heterochromatin (81-83). The results of Rauwel, et al. (40) therefore suggest that reactivation of
372 HCMV can be induced through chromatin remodeling induced by activation of a DNA damage
373 response independently of differentiation to dendritic cells. Co-treatment with TNF-α

374 potentiated reactivation in response to chloroquine, suggesting that inflammatory mediators that
375 activate NF-κB can act synergistically with chromatin remodeling to induce reactivation of
376 HCMV. Our studies showed that TNF-α was sufficient to induce reactivation in Kasumi-3 cells
377 without inducing differentiation, and that this correlated with activation of NF-κB, as well as
378 ATM and KAP-1. Further studies will be required to fully understand the roles of differentiation,
379 inflammation, and chromatin remodeling in reactivation of HCMV. However, the results of our
380 studies, as well as those of others, suggest that the mechanisms of reactivation of HCMV are not
381 necessarily limited to a dendritic cell specific pathway, but may be induced by more general
382 pathways of inflammation and cellular damage.
383

384 **MATERIALS AND METHODS**

385 **Cells and reagents:** Kasumi-3 and MRC-5 cells were obtained from the ATCC. Human
386 recombinant TNF-α was obtained from PreproTech (#300-01A). Fluorescent conjugated
387 monoclonal antibodies used in this study are listed in Table 1. TB40/E*wt*-GFP (48) viral stocks
388 were produced and titered as described in supplementary methods.

389 **Infection of Kasumi-3 cells:** Kasumi-3 cells were infected with HCMV strain TB40/E*wt*-GFP
390 (48) (MOI 1-2) using centrifugal enhancement of infection, as described in supplementary
391 methods (84). GFP+ and GFP- cells were sorted on a FACSAria-6 laser cell sorter (BD
392 Biosciences). Infected cells were cultured for 13 days to establish latency, with media changes
393 every 2-3 days. For reactivation studies, cells were treated with 5ng/ml TNF-α at 14 dpi for 3
394 days at 37°C. A $TCID_{50}$ assay was used to determine the infectious titer of infected cell
395 supernatants.

396 **RNA and DNA extraction:** DNA was isolated with the Arcturus pico Pure DNA extraction kit
397 (Thermo Fisher Scientific) as recommended by the manufacturer. RNA was purified with the
398 Direct-zol RNA mini prep kit (Zymo Research) with a DNAse digestion step following
399 manufacturer's instructions.
400 **Real time PCR analysis**. For all analyses, mock-infected cells were analyzed in parallel with
401 infected cells. These samples were uniformly positive for cellular genes, but negative for viral
402 genes. TaqMan assays specific for viral genes were custom-designed by Life Technologies
403 (Table 2). Relative viral DNA and RNA quantity was analyzed by the 2-(ΔΔCt) method, using
404 pre-designed assays (Life Technologies) for RNaseP (catalog number 4403326) or GAPDH,
405 respectively, as the normalization controls. This method was validated by determining that the
406 efficiencies of the target and reference genes were approximately equal (85) as described in
407 supplementary methods (Fig. S5 and S6). Previous studies have shown that GAPDH is an
408 appropriate normalization control for RNA analysis of CMV-infected cells (86), and we verified
409 that GAPDH Ct values are stable following infection of Kasumi-3 cells (Fig. S7). TaqMan gene
410 expression assays purchased from Life Technologies were used for analysis of cellular RNAs
411 (Hs00174128_m1 for TNF-α and Hs99999905_m1 for GAPDH).
412 **Immunophenotyping.** Cells were stained with antibodies specific for CD34, CD64, CD14,
413 CD15, CD11c, CD1c and IE 1/2 (CH160) (Table 1) as described in supplementary methods.
414 Data on live cells was acquired on 6-laser BD LSRFortessa SORP flow cytometer and analyzed
415 using FlowJo software (version 9).
416 **Cytokine stimulation and intracellular phospho-protein analysis:** Kasumi-3 cells were
417 treated or untreated with 5 ng/ml TNF for 30' at 37°C, fixed, permeabilized and washed
418 according to previously described protocols (87, 88). Cells were stained with antibodies specific

419 for p65 (NF-κB), p-p65 (pNF-κB), ATM, p-ATM, KAP-1, p-KAP-1 or γ-H2AX (Table 1).
420 Antibodies were validated using published protocols (89) as described in supplementary methods
421 and Fig. S8. Data were acquired on BD LSRFortessa SORP flow cytometer and analyzed using
422 FlowJo (version 9). The fraction of responding cells for each population was determined as
423 described in supplementary methods as previously described (88, 89).
424

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700 **FIGURE LEGENDS**

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

715 **Fig. 2. Analysis of GFP- infected cells at 1 and 4 dpi.** (A) Schematic outlining the sorting
716 strategy to purify GFP- cells at D1 and D4 post infection. (B) Representative FACS analysis of
717 GFP sorting at D1 and D4 post infection. (C) DNA amount, (D) mRNA expression, (E)
718 RNA/DNA ratio of UL122, UL54, UL32, US28, UL138, and RNA2.7 analyzed at the indicated
719 times post infection and expressed relative to D1. RNA expression values were normalized to
720 GAPDH and the DNA amount was normalized to RNAseP. The statistical significance was
721 calculated by 2-way ANOVA with a Tukey's multiple comparison test (n=3). The error bars

722 represent the SEM and the asterisks indicate p-values (* P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001;

723 **** P ≤ 0.0001) calculated by comparison of the different genes at each time point.

724

725 **Fig. 3. Expression of latency associated RNAs.** mRNA expression (A), DNA amount (B) and

726 RNA/DNA ratio (C) of UL32, RNA2.7, UL138 and US28 analyzed at the indicated times post

727 infection and expressed relative to D1. RNA expression values were normalized to GAPDH and

728 the DNA amount was normalized to RNAseP. The statistical significance was calculated by 2-

729 way ANOVA with a Tukey's multiple comparison test (n=3). The error bars represent the SEM

730 and the asterisks indicate p-values (* P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001; **** P ≤ 0.0001)

731 calculated by comparison of the different genes at each time point.

732

733

734 **Fig 4. TNF-α induces reactivation independently of differentiation.** (A) Latently infected

735 cells (14 dpi) were treated or not with TNF-α for 3 days. On day 17, DNA and RNA from

736 infected cells was analyzed for DNA copy number and expression of viral genes as decribed in

737 Materials and Methods. The release of viral particles in the supernatant was measured by a

738 $TCID_{50}$ assay. The statistical significance was calculated by unpaired *t-test* (n=4). The error bars

739 represent the SEM and the asterisks indicate p-values (* P<0.05; ** P< 0.01). (B) Representative

740 FACS analysis of uninfected, latently infected cells and TNF-α treated cells for the expression of

741 the hematopoietic progenitor marker CD34 and the viral immediate early proteins IE1/2.

742 Reactivating cells were immunophenotyped by gating on the CD34+/IE1/2+ population and

743 analyzing expression of markers of myeloid differentiation (CD64, CD14, CD15, CD11c and

744 CD1c). (C) Percentage of undifferentiated cells within the IE1/2 expressing population in the

745 TNF-α treated cells (CD14-, CD64-, CD15-, CD11c-and CD1c-). The error bars represent the

746 SEM calculated from 3 experiments.

747 **Fig 5. TNF-α mediated reactivation is correlated with activation of NFκB and ATM**

748 **signaling.**

749 Latently infected cells (14 dpi) were treated for 3 days with or without TNF-α 5ng/ml. (A to D)

750 Flow cytometry analysis were performed to assess the activation of NFκB (p65) and DDR

751 (ATM, KAP1 and γH2AX). The activation of p65, ATM and KAP1 and are expressed as the

752 Ratio of Phosphoprotein and total protein compared to untreated cells (p-p65-S536/p65, pATM-

753 S1981/ATM, and p-Kap-S824/Kap1). γH2AX is expressed as ratio of MFI compared to

754 untreated cells. The error bars represent the SEM calculated from 3 independent experiments (*

755 P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001).

756

757 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
IE1/2	CH160	Virusys
NF-kB p65	L8F6	Cell Signaling Technology #9460
phospho-NF-kB p65 (Ser536)	93H1	Cell Signaling Technology
ATM	D2E2	Cell Signaling Technology #2873
phospho-ATM (S1981)	D25E5	Cell Signaling Technology #13050
KAP-1	EPR5216	Abcam #109287
phospho-KAP-1 (S824)	EPR5248	Abcam #133440
γ-H2AX (S139)	EP854(2)Y	Abcam #195189

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765 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

766

767 **SUPPLEMENTARY FIGURES**

768 **Fig. S1. Analysis of viral RNA and DNA over the course of infection in Kasumi-3 cells.**

769 RNAs and DNAs from representative immediate early, early and late genes were analyzed by

770 RT-qPCR and qPCR, respectively, at various times post-infection as described in Fig. 1 and

771 Materials and Methods.

772 **Fig S2. Kinetic analysis of HCMV DNA and RNA expression in GFP- cells isolated at 1 dpi**.

773 (A-C) Relative mRNA expression and DNA amount of UL122, UL54 and UL32 analyzed by

774 RT-qPCR and qPCR, respectively, at the indicated times post infection and expressed relative to

775 D1. Statistical significance was calculated by a one-way ANOVA with a Dunnett's multiple

776 comparison test (n=3). The error bars represent the SEM and the asterisks indicate p-values (* $P \leq$

777 0.05; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$*) calculated by comparison to the peak at D4.

778 **Fig. S3. TNF induces expression of HCMV early and late genes.** RNAs from the experiments

779 shown in Fig. 6 were analyzed for relative expression of the early gene UL54 and the late gene

780 UL32.

781 **Fig. S4. Phenotyping of uninfected and latently infected Kasumi-3 cells.** Representative

782 FACS analysis of uninfected (A) and latently infected (B) cells for the expression of the

783 hematopoietic progenitor marker CD34 and markers of myeloid differentiation (CD64, CD14,
784 CD15, CD11c and CD1c).

785 **Fig. S5. Analysis of the efficiencies of amplification of viral genes versus RNaseP.** Viral
786 genes and the cellular gene RNaseP were amplified in samples prepared from serial dilutions of
787 DNA isolated from lytically infected MRC-5 fibroblasts. The ΔCt values ($C_T$viral gene –
788 C_TRNaseP) for each dilution were calculated and plotted against the log ng DNA.

789 **Fig. S6. Analysis of the efficiencies of amplification of viral RNAs versus GAPDH.** Viral
790 RNAs and cellular GAPDH RNA were amplified in samples prepared from serial dilutions of
791 cDNA prepared from RNA isolated from lytically infected MRC-5 fibroblasts. The ΔCt values
792 ($C_T$viral gene – C_TGAPDH) for each dilution were calculated and plotted against the log ng
793 cDNA.

794 **Fig. S7. Validation of GAPDH as a normalization control in HCMV-infected Kasumi-3**
795 **cells.** Data shows average Ct values ± SD for GAPDH at various times after infection. N=4.

796 **Fig. S8. Antibody staining validation.** (A) Representative flow cytometric analysis of HeLa
797 cells, untreated (red) or treated with hTNF-α (20ng/ml) and Calyculin A (100nM) for 15 mins
798 (blue), using Phospho-NF-*k*B p65 (Ser563) rabbit mAb and total NF-*k*B p65.
799 (B and C) Representative flow cytometric analysis of HCT116 treated with 200nM NCS, using
800 phospho-ATM (S1981), phospho-KAP1 (S824) mAb, ATM and total KAP1 mAb (blue)
801 compared to untreated control cells (red).
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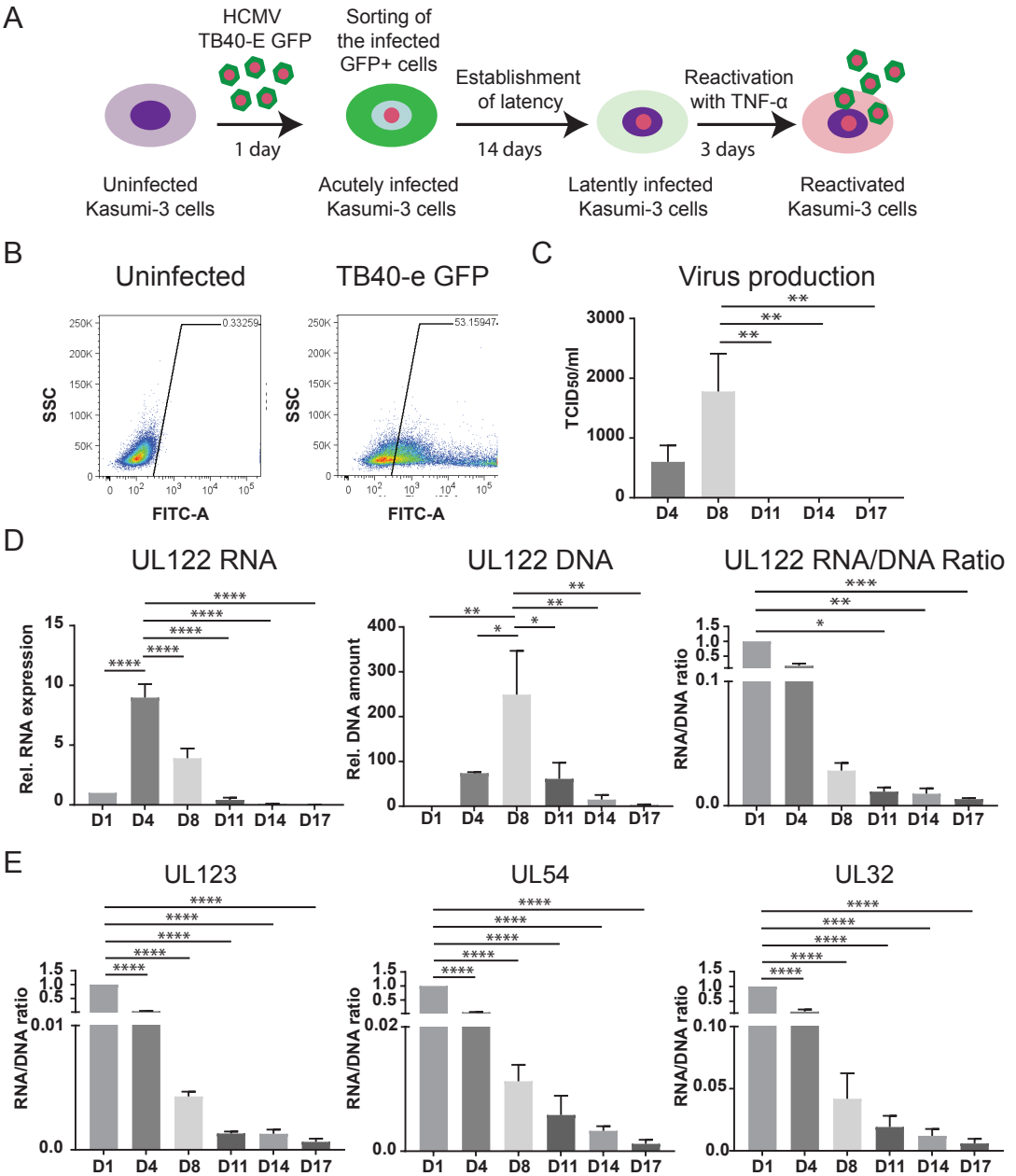

Fig. 1. HCMV latency is established after activation of transcription at 14 days post infection. (A) Schematic outlining the infection model used for studies of latency and reactivation in Kasumi-3 cells. GFP+ infected cells were purified by flow cytometry at 1 dpi. On day 14, latently infected cells 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

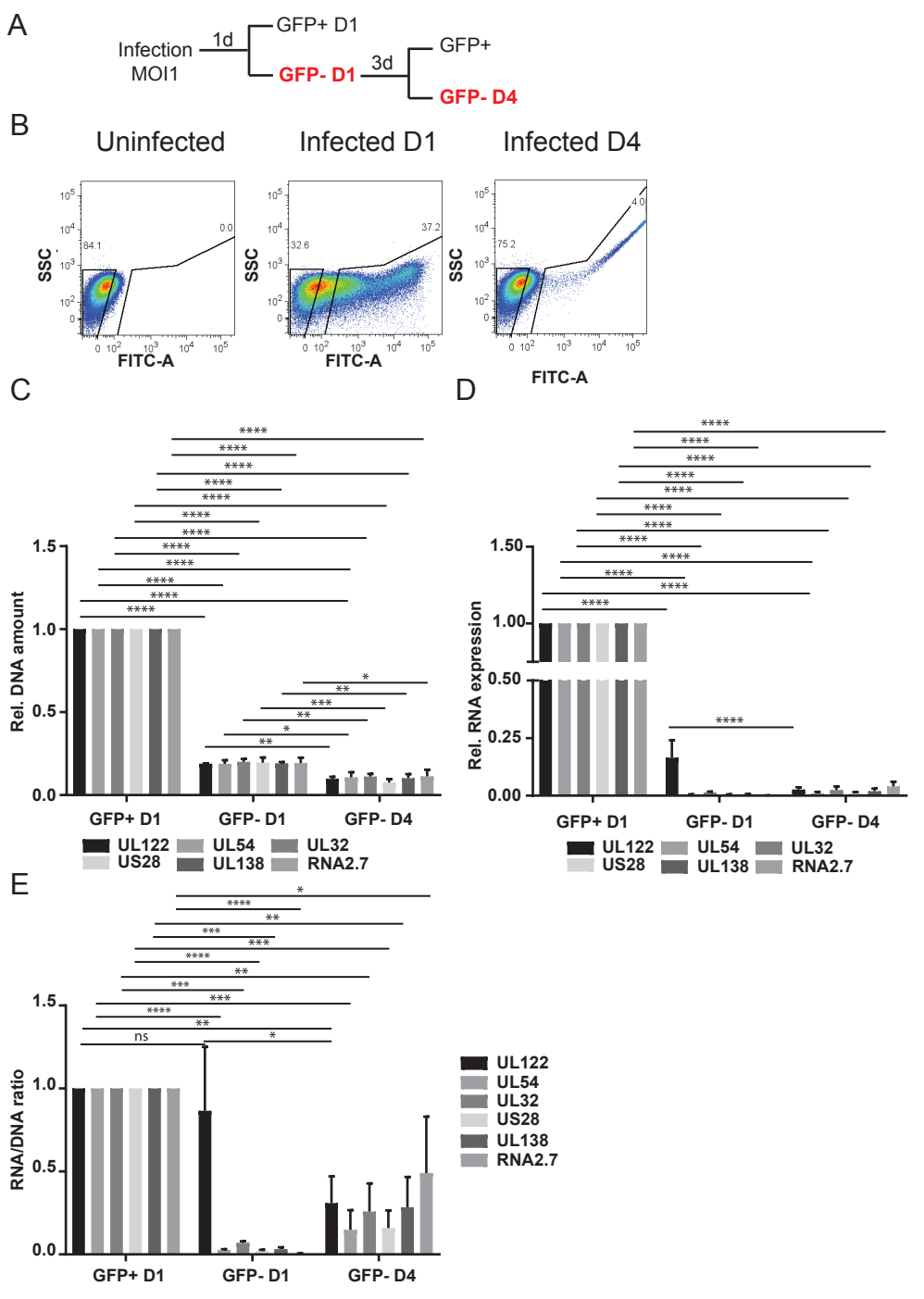

Fig. 2. Analysis of the GFP- infected cells at 1 and 4 days post infection.
(A) Schematic outlining the sorting strategy to purify GFP- cells at D1 and D4 post infection.
(B) Representative FACS analysis of GFP sorting at D1 and D4 post infection. DNA amount (C), mRNA expression (D), and RNA/DNA ratio (E) of UL122, UL54, UL32, US28, UL138, and RNA2.7 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.

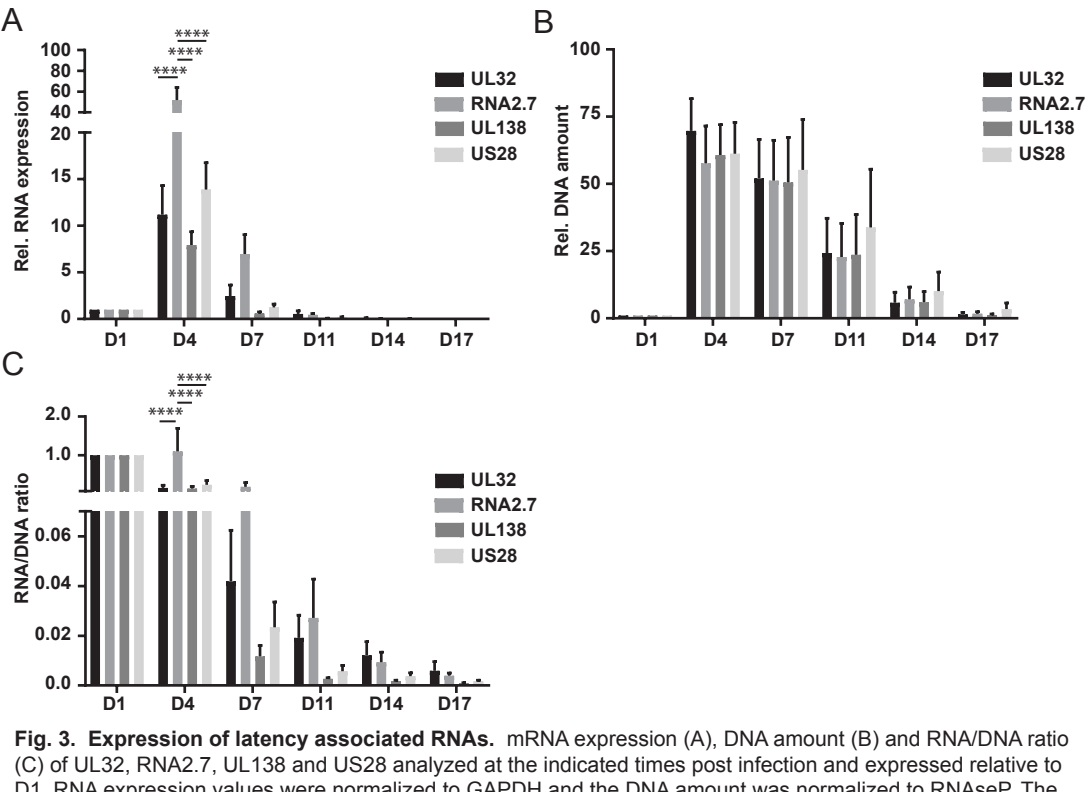

Fig. 3. Expression of latency associated RNAs. mRNA expression (A), DNA amount (B) and RNA/DNA ratio (C) of UL32, RNA2.7, UL138 and US28 analyzed at the indicated times post infection and expressed relative to D1. RNA expression values were normalized to GAPDH and the DNA amount was normalized to RNAseP. 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.

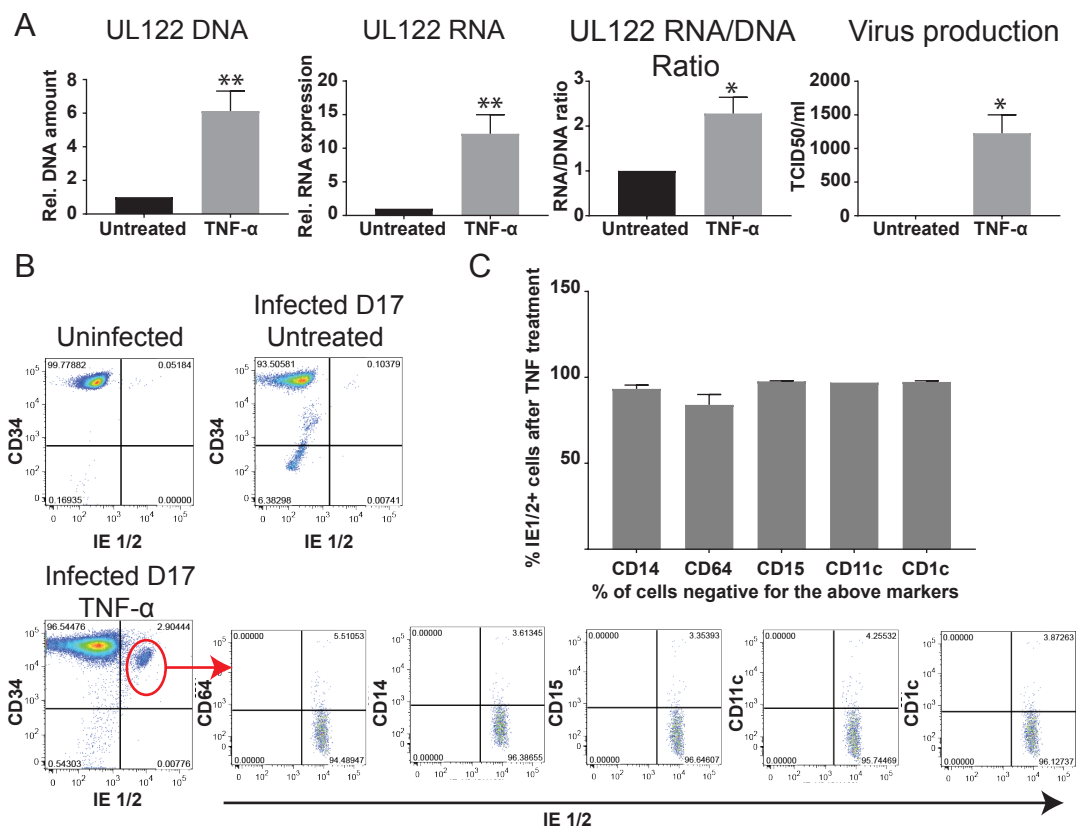

Fig 4. TNF-α induces reactivation independently of differentiation. (A) Latently infected cells (14 dpi) were treated or not with TNF-α for 3 days. On day 17, DNA and RNA from infected cells was analyzed for DNA copy number and expression of viral genes as decribed in Materials and Methods. The release 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.

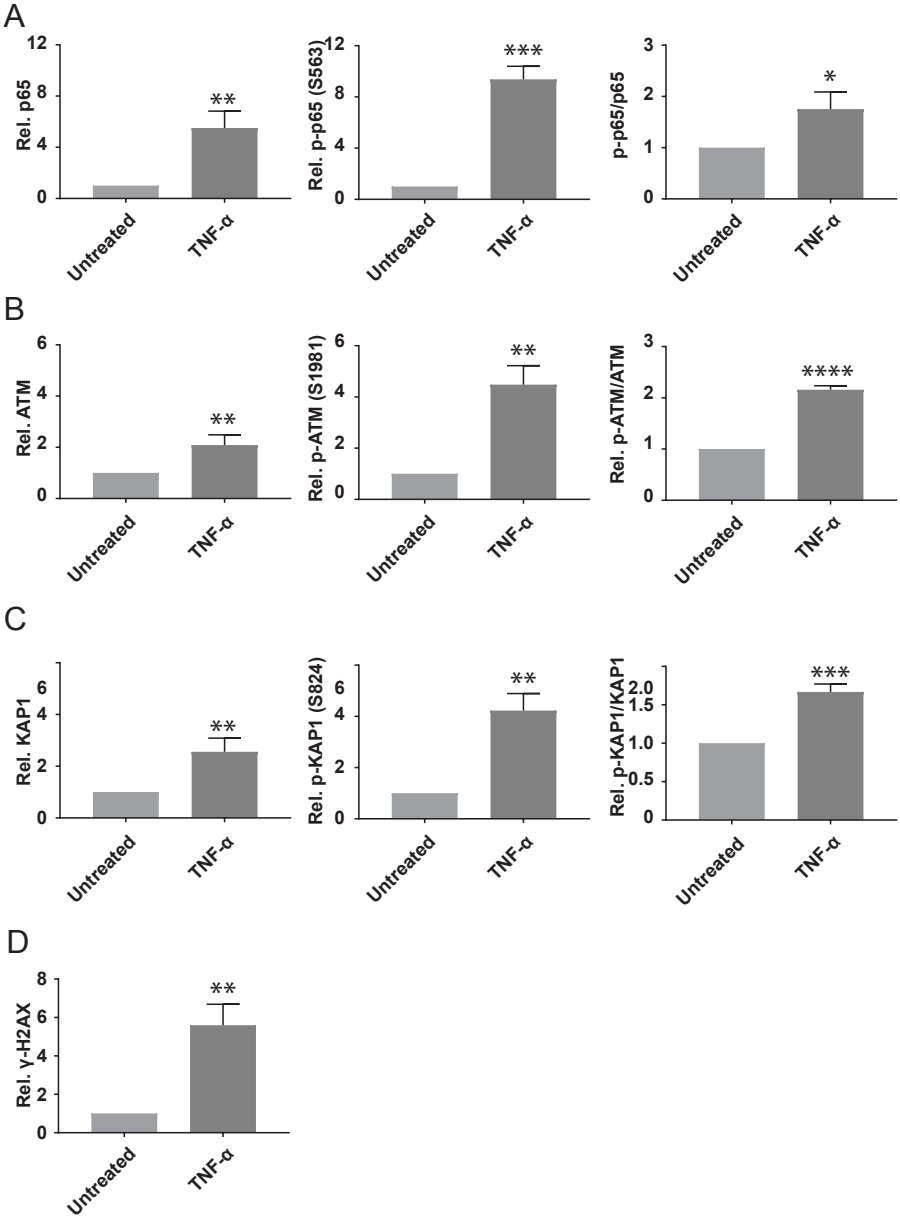

Fig 5. TNF-α mediated reactivation is correlated with activation of NFκB and ATM signaling. Latently infected cells (14 dpi) were treated for 3 days with or without TNF-α 5ng/ml. (A to D) Flow cytometry analysis were performed to assess the activation of NFκB (p65) and DDR (ATM, KAP1 and γH2AX). The activation of p65, ATM and KAP1 and are expressed as the Ratio of Phosphoprotein and total protein compared to untreated cells (p-p65-S536/p65, pATM-S1981/ATM, and p-Kap-S824/Kap1). γH2AX is expressed as ratio of MFI compared to untreated cells. The error bars represent the SEM calculated from 3 independent experiments (* P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001).