Journal of General Virology

Human cytomegalovirus miR-UL112-1 promotes the down-regulation of viral immediate early gene expression during latency to prevent T cell recognition of latently infected cells

--Manuscript Draft--

Manuscript Number:	JGV-D-16-00275R1
Full Title:	Human cytomegalovirus miR-UL112-1 promotes the down-regulation of viral immediate early gene expression during latency to prevent T cell recognition of latently infected cells
Short Title:	HCMV miR-UL112-1 prevents CTL detection of latency
Article Type:	Standard
Section/Category:	Animal - Large DNA Viruses
Corresponding Author:	John H. Sinclair Cambridge University Cambridge, UNITED KINGDOM
First Author:	Betty Lau, PhD
Order of Authors:	Betty Lau, PhD
	Emma L. Poole, PhD
	Ellen Van Damme, PhD
	Lieve Bunkens
	Madeleine Sowash Sowash, BSc
	Harry King, BSc.
	Eain Murphy, PhD
	Mark R. Wills, PhD
	Marnix Van Loock, PhD
	John H. Sinclair
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3 Running title: HCMV miR-UL112-1 prevents CTL detection of latency

4 Betty Lau[1a†], Emma Poole[1†], Ellen Van Damme [2], Lieve Bunkens [2], Madeleine Sowash [1b], Harry King [1],
5 Eain Murphy[3], Mark Wills [1], Marnix Van Loock [2] and John Sinclair [1]*

6 [†]These authors contributed equally to the work.

7 [1] Department of Medicine, University of Cambridge, Level 5, Addenbrooke's Hospital, Hills Road,
8 Cambridge CB2 2QQ, United Kingdom.

9 [2] Janssen Pharmaceutica NV, Turnhoutseweg 30, 2340 Beerse, Belgium.

10 [3]Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio
11 44195, United States of America

12 [a]Present address: Medical Research Council-University of Glasgow Centre for Virus Research,
13 Garscube campus, Glasgow G61 1QH, United Kingdom

14 [b]Present address: Department of Medicine, Columbia University, 630 W 168th Street, New York, NY
15 10032, United States of America.

16 *Corresponding author: John Sinclair, email: js152@hermes@ac.uk, phone: 01223 336850

17 Keywords: latency; human cytomegalovirus (HCMV); viral miRNAs; immune survelliance

18 Subject category: Standard research article (Animal – DNA Viruses)

19 Abstract word count: 244

20 Article word count (excluding title page, figure legends and references): 5883

21 Number of main figures and tables: 6

Abstract

Human cytomegalovirus (HCMV), a member of the herpesvirus family, can cause significant morbidity and mortality in immune compromised patients resulting from either primary lytic infection or reactivation from latency. Latent infection is associated with a restricted viral transcription programme compared to lytic infection which consists of defined protein coding RNAs but also includes a number of virally encoded microRNAs (miRNAs). One of these, miR-UL112-1, is known to target the major lytic IE72 transcript but, to date, a functional role for miR-UL112-1 during latent infection has not been shown. To address this, we have analysed latent infection in myeloid cells using a virus in which the target site for miR-UL112-1 in the 3' untranslated region of IE72 was removed such that any IE72 RNA present during latent infection would no longer be subject to regulation by miR-UL112-1 through the RNAi pathway. Our data show that removal of the miR-UL112-1 target site in IE72 results in increased levels of IE72 RNA in experimentally latent primary monocytes. Furthermore, this resulted in induction of IE expression detectable by IE-specific cytotoxic T cells (CTLs); no such CTL recognition of monocytes latently infected with wild-type virus was observed. We also recapitulated these findings in the more tractable THP-1 cell line model of latency. These observations argue that an important role for miR-UL112-1 during latency is to ensure tight control of lytic viral IE gene expression thereby preventing recognition of latently infected cells by the host's potent pre-existing anti-viral CTL response.

Introduction

Human cytomegalovirus (HCMV) is a species specific herpesvirus carried by 60-80% of human populations, depending upon demographics (Ross & Boppana, 2005). In the immune competent, primary infection rarely results in clinical problems. However, in the immune compromised and the neonate, primary HCMV infection can cause significant disease sequelae (Ho, 1990; Zaia, 1990).

45 Like all herpesviruses, HCMV is never cleared after primary infection but maintains a life-long
46 persistence which is underpinned by the ability of the virus to undergo latent infection (Sinclair &
47 Sissons, 2006). As with primary infection, reactivation of virus from latency in the immune
48 compromised or during pregnancy, can also result in severe disease to the immunocompromised
49 individual or the foetus in utero (Adler, 1983; Griffiths & Walter, 2005; Rubin, 1990; Sissons &
50 Carmichael, 2002).

51 One established site of latent carriage, in vivo, is in cells of the myeloid lineage, including $CD14^+$
52 monocytes and their $CD34^+$ progenitors (Hahn *et al.*, 1998; Khaiboullina *et al.*, 2004; Mendelson *et*
53 *al.*, 1996; Taylor-Wiedeman *et al.*, 1991). However, differentiation of these cells to dendritic cells
54 (DCs) or macrophages results in full virus reactivation (Reeves *et al.*, 2005c; Soderberg-Naucler *et al.*,
55 2001; Taylor-Wiedeman *et al.*, 1994).

56 During latent infection of monocytes or $CD34^+$ cells, carriage of latent viral genomes is associated
57 with the expression of a restricted pattern of viral genes compared to lytically infected cells
58 (Slobedman *et al.*, 2010); the absence of infectious virion production during this life cycle mitigates
59 the need for expression of much of the virus genome and likely, in itself, reduces the ability of the
60 host's immune response to detect and clear virus infected cells. Instead, viral gene expression
61 during latency is likely reduced to those that play an important role in establishment and
62 maintenance of the latent life cycle, and functions for proteins encoded by many of these latency-
63 associated transcripts are now beginning to be elucidated (Keyes *et al.*, 2012b; O'Connor & Murphy,
64 2012; Poole *et al.*, 2014; Poole *et al.*, 2015; Tarrant-Elorza *et al.*, 2014; Weekes *et al.*, 2013). In
65 addition to these, it is also becoming clear that known viral non-coding RNAs are also expressed
66 during latency (Rossetto *et al.*, 2013) and these include viral miRNAs (Fu *et al.*, 2014; Meshesha *et*
67 *al.*, 2016; Shen *et al.*, 2014). Although the functional targets of a number of viral miRNAs expressed
68 during lytic infection have been described (for reviews see: Dhuruvasan *et al.*, 2011; Goldberger &

69 Mandelboim, 2014; Hook *et al.*, 2014a) , little is known about the role of HCMV encoded miRNAs
70 during latency.

71 One viral miRNA expressed during latency in primary myeloid cells is miR-UL112-1 (Meshesha *et al.*,
72 2016) (Betty Lau et al unpublished observations). A number of cellular and viral targets of this
73 miRNA have been validated during lytic infection (Grey, 2015; Huang *et al.*, 2013; Lee *et al.*, 2012);
74 these include multiple components of the host secretory pathway (Hook *et al.*, 2014b), the NK cell
75 activatory ligand MICB (Stern-Ginossar *et al.*, 2008), cellular transcription factor BclAF1 (Lee *et al.*,
76 2012) as well as the viral IE72 immediate early gene and viral UL114 (Grey *et al.*, 2007; Murphy *et al.*,
77 2008; Stern-Ginossar *et al.*, 2009). Furthermore, the targeting of MICB and BclAF1 by miR-UL112-1
78 was shown to aid immune evasion and virus spreading, respectively, during lytic infection (Lee *et al.*,
79 2012; Stern-Ginossar *et al.*, 2008). Despite a relative wealth of information about the role of this
80 viral miRNA during lytic infection, nothing is known about the role of miR-UL112-1 during latency.
81 On the basis that miR-UL112-1 targets IE72 expression during lytic infection, we reasoned that an
82 even more crucial role for this viral miRNA could be its ability to target IE72 during latent infection;
83 thereby helping to ensure tight negative regulation of lytic gene expression, and hence virus
84 production, during latent carriage - a known hallmark of latency.

85 Here, we have analysed whether the ability of miR-UL112-1 to target IE72, specifically, is important
86 for latent carriage. Since miR-UL112-1 is known to have other cellular targets (Hook *et al.*, 2014b;
87 Huang *et al.*, 2013; Lee *et al.*, 2012) besides viral IE72, we approached this by generating
88 recombinant virus in which the miR-UL112-1 target site in the 3' untranslated region (UTR) of IE72
89 was removed (Δ112-1 target virus) rather than by deleting the miR-UL112-1 miRNA coding sequence
90 itself - so ensuring that any phenotype we observed during latency was a result of lack of targeting
91 of IE72 by miR-UL112-1 rather than its other known or unknown targets.

92 Our results show that, as expected, latent infection with wild-type virus result in very low levels of
93 IE72 RNA expression. In contrast, latent infection with Δ112-1 target virus results in latently infected

94 cells expressing substantial levels of IE72 RNA. Importantly, as opposed to cells latently infected with
95 wild-type virus, cells latently infected with Δ112-1 target virus now become overt targets for IE72
96 specific $CD8^+$ cytotoxic T cells.

97 Finally, the difficulty in using primary cells from healthy donors led us to interrogate whether cell
98 line models of HCMV latency could be used for similar immunological analyses of HCMV latent
99 infection. Using the THP-1 cell line model of latency, we have recapitulated our observations on the
100 effects of latent infection in the absence of miR-UL112-1; as with primary cells, THP-1 cells latently
101 infected with Δ112-1 target virus also become overt targets for IE72 specific $CD8^+$ cytotoxic T cells.

102 Taken together, our data define an important role for miR-UL112-1 in the maintenance of latency as
103 well as demonstrating the applicability of the THP-1 model system for intricate analysis of HCMV
104 latency at the molecular and immunological level. We believe that expression of miR-UL112-1 during
105 latency is important to "mop up" leaky IE RNA expression to prevent recognition of latently infected
106 cells by the robust IE72 specific CTL response known to be present in normal HCMV carriers
107 (Gillespie *et al.*, 2000; Kern *et al.*, 1998).

108 **Results**

109 *Deletion of the miR-UL112-1 target site in the IE72 3' UTR results in low level IE expression in primary*
110 *monocytes but not full reactivation of virus production*

111 The viral miR-UL112-1 miRNA is expressed during latency in a number of experimental latent models
112 including experimentally latent primary monocytes and $CD34^+$ haemopoietic progenitor cells
113 (Meshesha *et al.*, 2016) (Betty Lau et al unpublished observations). Besides the documented ability
114 of miR-UL112-1 to target IE72, this viral miRNA is also known to target cellular MICB (Stern-Ginossar
115 *et al.*, 2008), multiple components of the host secretory pathway (Hook *et al.*, 2014b), BclAF1 (Lee
116 *et al.*, 2012), IL-32 (Huang *et al.*, 2013) and is likely to have other unidentified viral and cellular target
117 RNAs as many biochemical analyses suggest that miRNAs may typically target dozens of targets. As

118 we wanted to specifically address the effect of lack of IE72 RNA regulation by miR-UL112-1, without
119 potential confounding effects of miR-UL112-1 on other targets, we decided to construct a
120 recombinant virus (Δ112-1 target) in which the miR-UL112-1 target binding site was removed (Fig.
121 1a); so allowing an analysis of the effect of miR-UL112-1 on IE72 expression, in isolation.

122 We initially analysed the establishment of latency after 4 days infection in $CD14^+$ monocytes as we
123 have done previously (Krishna *et al.*, 2016; Weekes *et al.*, 2013) with both wild- type (WT) or Δ112-1
124 target virus which resulted in similar numbers of GFP+ latently infected cells (approximately 10-20%,
125 data not shown) as well as similar levels of expression of the latency associated gene UL138 (Fig. 1b).
126 However, the suppression of IE gene expression in comparison to levels of UL138 expression (which
127 is a hallmark of latent infection), was only observed with the WT virus; perhaps not unexpectedly,
128 cells latently infected with Δ112-1 target virus resulted in a discernible increase in levels of IE72 RNA
129 (Fig. 1b). Importantly, although cells latently infected with Δ112-1 target virus did show less
130 suppressed IE72 expression, there was no evidence of virus production from these cells or, as
131 expected, from cells latently infected with WT virus (Fig. 1c). Consistent with this, no late viral gene
132 expression (pp28) was observed in cells latently infected with either virus (Fig. 1b). In contrast,
133 reactivation of cells latently infected with WT or Δ112-1 target virus showed similar levels of viral
134 gene IE and late gene expression (Fig. 1b) and both produced similar levels of infectious virions (Fig.
135 1c).

136

137 *The expression of IE by cells otherwise latently infected with the miR-UL112-1 target mutant virus*
138 *can be detected by IE specific T cells*

139 Given that cells latently infected with Δ112-1 target virus expressed detectable levels of IE72 RNA,
140 we reasoned that, if translated, this would make these cells vulnerable to surveillance by the well
141 characterized IE72-specific $CD8^+$ cytotoxic T cell (CTL) response known to be present in the

142 peripheral blood of many healthy HCMV carriers; up to 10% of all circulating CTLs in healthy virus
143 carriers are IE72 specific (Gillespie *et al.*, 2000; Kern *et al.*, 1998). Consequently, we next tested
144 whether cells latently infected with Δ112-1 target virus were differentially recognised by IE72-
145 specific CTLs compared to cells latently infected with WT virus.

146 To do this, we generated a T cell line which recognised the previously mapped IE72 peptide VLE
147 (Khan *et al.*, 2002), presented by MHC Class I HLA-A2. Using Fluorospot analysis, we first checked
148 that the T cell line produced IFNγ when cultured with both peptide pulsed monocytes and DCs.
149 These T cells were then used against latently infected monocyte targets as well as reactivating DC
150 targets. As expected, the T cell line recognised monocytes latently infected with WT or Δ112-1
151 target virus which had been induced to reactivate by their differentiation to DCs (Fig. 2). In contrast,
152 monocytes latently infected with WT virus showed little IE72 specific CTL recognition, whereas
153 monocytes latently infected with Δ112-1 target virus were clearly recognised by IE-specific CTLs (Fig.
154 2). Taken together, these data suggest that miR-UL112-1 is important for reinforcing the suppression
155 of IE gene expression during latency to help prevent T cell recognition of latently infected cells by
156 IE72-specific CTLs.

157 *Validation of the THP-1 cell model system*

158 All the analyses, so far, were carried out using latently infected primary human monocytes obtained
159 from healthy blood donors; this requires local ethical approval and access to clinical services which
160 are not always readily available. On this basis, we wanted to determine if the effects of a lack of the
161 miR-UL112-1 target site on T cell surveillance could be recapitulated in other, more tractable,
162 models of HCMV latency.

163 A number of models of HCMV latency which use established myeloid cell lines have been described
164 and these include myeloid cells expressing the $CD34^+$ marker, such as Kasumi-3 (O'Connor &
165 Murphy, 2012), or $CD14^+$ monocytic cells, such as THP-1s (Keyes *et al.*, 2012a), which can

166 recapitulate many aspects of HCMV latency and reactivation observed in primary cells (Albright &
167 Kalejta, 2013). The ability to use such established cell line model systems in the analysis of HCMV
168 latency has a number of advantages which include their ease of use compared with primary cells,
169 the lack of a requirement to recruit donors and, importantly, there would be no need to tissue type
170 match each individual donor for e.g. immunological assays. Such cell lines are also homogenous and
171 more easily to manipulate by e.g. transfection.

172 As observed by others (Keyes *et al.*, 2012a), THP-1 cells acted as a tractable model of latent
173 infection. However, we found that growing cells in 2% FCS, rather than 10% FCS (as is standard),
174 after infection reduced large scale expansion of the cell population and the associated loss of viral
175 genomes (see Supplementary Fig. S1). Fig. 3a shows that latent infection of THP-1 cells resulted in
176 cells which express good levels of viral UL138 RNA in a background of much reduced expression viral
177 IE72 RNA, compared to levels of IE72 RNA expressed upon reactivation (e.g. see Fig. 3a, tracks
178 labelled +PMA). Consistent with this low level of IE72 RNA expression, we observed extremely low
179 levels of IE72 protein expression using indirect immunofluorescence (Fig. 3b, left panel). In contrast,
180 differentiation of these latently infected cells resulted in induction of IE72 expression at the level of
181 RNA (Fig. 3a) and detection of numerous IE72 positive cells (Fig. 3b, right panel). Perhaps most
182 importantly, on the basis that lack of infectious virus is a hallmark of latency, co-culture of the
183 undifferentiated latently infected THP-1 cells (shown in the left panel of Fig. 3b) with fully permissive
184 indicator fibroblasts showed no infectious virus production (Fig. 3c, left panel). In contrast,
185 reactivated virus was clearly detectable (Fig. 3c, right panel) after co-culture of these differentiated
186 latently infected THP-1 cells (shown in right panel, Fig. 3b) with indicator fibroblasts. Taken together,
187 these data show that infection of THP-1 cells results in latent HCMV infection which can be
188 reactivated by differentiation.

189

190

191 *THP-1 cells latently infected with Δ112-1 target virus also show de-repressed IE expression and*
192 *become targets for IE-specific CTLs*

193 Fig. 2 clearly showed that removal of the miR-UL112-1 target site in IE72 prevented the repression of
194 IE gene expression during HCMV latency in primary monocytes. Consequently, we tested whether
195 latently infected THP-1 cells also showed the same effect. Fig. 4a shows that infection of THP-1 cells
196 with both wild-type virus and Δ112-1 target virus resulted in equivalent levels of UL138 expression;
197 this was not the case for expression of IE72. Although infection with WT virus did result in some
198 detectable levels of IE72 expression, consistent with the less robust control of expression of IE72
199 known to occur upon latent infection of THP-1 cells (Keyes *et al.*, 2012a) compared to monocytes,
200 infection with Δ112-1 target virus resulted in substantially increased levels of IE72 RNA. This argues
201 that, during latent infection of THP-1 cells, the Δ112-1 target virus is less able to suppress IE gene
202 expression during latency in the absence of the miR-UL112-1 target site; exactly as observed for
203 primary monocytes.

204 In order for us to determine if THP-1 cells latently infected with Δ112-1 target virus were also more
205 susceptible to surveillance by IE72-specific CTLs, we needed to determine whether THP-1 cells could
206 act as targets for IE-specific T cells in an MHC Class I restricted manner. THP-1 cells are known to
207 express high levels of HLA-A2 and, consequently, when loaded with the HLA-A2 restricted VLE
208 polypeptide of IE72 should present and be recognised by VLE-specific CTLs (Khan *et al.*, 2002). Fig. 5a
209 shows that, as expected, THP-1 cells express high levels of HLA-A2 and, when pulsed with a VLE
210 polypeptide, are recognised in a dose dependent manner by a VLE-specific CTL line using
211 IFNϒELISpot analysis (Fig. 5b). Given that THP-1 cells can act as IE-specific CTL targets in this setting,
212 we next tested whether these IE-specific T cells recognised latently infected THP-1 cells, lytically
213 infected differentiated THP-1 cells or latently infected THP-1 induced to reactivate by differentiation.
214 Fig. 5c shows that differentiated THP-1 cells infected with HCMV, which undergo lytic infection, are
215 recognised by IE-specific CTLs, as expected. In contrast, THP-1 cells latently infected with HCMV are

216 poorly recognised by VLE-specific CTLs but do become targets when these latently infected cells are
217 induced to reactivate. This is consistent with the targeting of infected THP-1 by IE72-specific T cells
218 only occurring when IE is fully expressed i.e. during lytic infection and reactivation, but not latency.

219 Finally, we analysed whether THP-1 cells latently infected with Δ112-1 target virus were also targets
220 for IE-specific CTLs, as we had observed using primary monocytes. To do this, we analysed the ability
221 of IE-specific CTLs to reduce the number of GFP-tagged latently infected cells after latent infection
222 with WT or Δ112-1 target virus. Fig. 5d shows that, as expected, irrelevant EBV-specific T cells had no
223 effect on the number of GFP-tagged latently infected THP-1 cells regardless of whether latency was
224 established with WT or Δ112-1 target virus. In contrast, if IE-specific CTLs were used in the assay, the
225 number of GFP-tagged latently infected THP-1 cells was reduced almost three fold when latency was
226 established using Δ112-1 target virus compared to cells latently infected with WT virus.

227 Formally, whilst it is possible that this removal of these otherwise latently infected THP-1 cells
228 expressing IE antigen results from CTL-mediated repression of IE gene expression and that these
229 THP-1 cells still carry reactivatable virus, we think this unlikely as cells which are induced to
230 transiently express IE72 by treatment of latently infected cells with histone deacetylase inhibitors
231 (HDACi) are killed by IE-specific CTLs (Krishna *et al.*, 2016).

232 Collectively, these data show THP-1 cells is a tractable experimental model of latency in which we
233 were able to reproduce the observations with regards to the downregulation of IE72 by miR-UL112-1
234 during latency.

235

236 **Discussion**

237 The inability of HCMV latently infected cells to be cleared by the host after primary infection, even
238 though host T cell responses to HCMV are profound and long-term, argues that latent reservoirs are
239 unable to be surveilled and targeted by the extremely large HCMV-specific $CD8^+$ and $CD4^+$ T cell

240 response known to be present in normal healthy HCMV carriers (Wills *et al.*, 2015). Indeed, up to
241 10% of the $CD8^+$ T cell compartment in peripheral blood of some healthy HCMV seropositive carriers
242 recognise IE72 antigen (Gillespie *et al.*, 2000; Kern *et al.*, 1998). Consequently, robust repression of
243 IE72 expression during latent carriage is likely to be of critical importance in order to prevent latently
244 infected cells from being recognised and cleared by the well established IE72-specific anti-viral T cell
245 response.

246 We and others have shown that latent infection of both experimentally and naturally latent myeloid
247 lineage cells results in a latency-associated transcription programme in which IE gene expression is
248 profoundly suppressed; this results from repression of the viral major immediate early promoter
249 (MIEP) mediated by concerted actions of cellular transcriptional suppressor proteins (Murphy *et al.*,
250 2002; Rauwel *et al.*, 2015; Reeves *et al.*, 2005c) and viral regulators of IE expression (Lee *et al.*, 2015)
251 reflected in repressive histone post-translational modifications around the MIEP (Murphy *et al.*,
252 2002; Reeves *et al.*, 2005b; Reeves *et al.*, 2005c). Our view is that, as with transcription in general,
253 such repression is likely to be leaky, resulting in incomplete silencing of IE gene expression and,
254 hence, requiring additional levels of IE suppression. The viral miR-UL112-1 miRNA has been shown to
255 attenuate IE72 expression during lytic infection at low multiplicities of infection (Grey *et al.*, 2007;
256 Murphy *et al.*, 2008). This led us to analyse the role of miR-UL112-1 during latent infection when, we
257 predict, such fine tuning of IE72 expression may be particularly important. Consistent with this,
258 experimental latent infection of $CD14^+$ monocytes with the Δ112-1 target virus consistently resulted
259 in a significant increase in IE72 RNA compared to latency established with WT virus. Despite this, the
260 increase in IE72 RNA in cells latently infected with Δ112-1 target did not result in virus reactivation
261 as determined by virus co-culture analysis. It did, however, result in the ability of these cells latently
262 infected with Δ112-1 target virus to be detected by IE72 specific CTLs, resulting in their killing; again,
263 no such killing of cells latently infected with WT virus was observed.

264 We also addressed whether we could recapitulate these findings in an established cell line model of
265 HCMV latency, as there are a number of advantages associated with using such cell lines over
266 primary cells for HCMV latency studies; their ease of use, the lack of requirement for ethical
267 approval, the ability to manipulate them by e.g. transfection as well as their homogeneity as a cell
268 population. Using these cells, we are able to recapitulate our key observations using primary
269 monocytes and, therefore, believe that the THP-1 latency model represents a tractable model for
270 also addressing immune control of HCMV latency and reactivation.

271 Taken together, we posit that miR-UL112-1 has a key role during latency which is to ensure robust
272 control of expression of viral IE72 by minimising translation of any low levels of IE72 transcription
273 which escapes latency-associated repression of the MIEP; if left uncontrolled, expression of this lytic
274 antigen would otherwise result in latently infected cells becoming targets for the robust IE72
275 antiviral response routinely observed in healthy HCMV carriers.

276

277 **Materials and Methods**

278 *Cells and viruses*

279 For latent infections, primary monocytes were isolated from venous blood of an HLA-A2 HCMV
280 seronegative donor by $CD14^+$ MACS microbeads positive selection (Miltenyi Biotec) as instructed by
281 the manufacturer. Briefly, peripheral blood mononuclear cells were extracted from venous blood by
282 Lymphoprep density gradient centrifugation (Axis-Shield), as previously described (Mason *et al.*,
283 2013). PBMCs were then incubated with CD14 direct microbeads before application of a magnetized
284 LS column, and subsequently eluting the bound cells after washing the column. The purity of isolated
285 cells were analysed by flow cytometry which routinely showed 98.1% cells expressed CD14 (range
286 97.4–98.9%). Primary monocytes were cultured in X-Vivo-15 (Lonza) supplemented with 2.5mM L-
287 glutamine (GE healthcare).

288 THP-1 cells (TIB-202, ATCC) were propagated in RPMI (Lonza) supplemented with 10% heat-
289 inactivated fetal calf serum (FCS, F7524, Sigma-Aldrich) and 0.04% gentamicin (Gibco – Life
290 Technologies). During latency and reactivation experiments, THP-1 cells were cultured in RPMI
291 supplemented with 2% FCS.

292 ARPE-19 cells (CRL-2302, ATCC) and neonatal NHDF fibroblasts (CC-2509, Lonza) were grown
293 respectively in DMEM:F12 with L-glutamine (733-1713, Biowhittaker) and MEM (11095-098, Gibco –
294 Life Technologies) containing 10% FCS and 0.04% gentamicine. Stocks of HCMV TB40/E (Chou &
295 Scott, 1988) were generated on ARPE-19 to obtain highly endothelial tropic virus. Virus yield was
296 determined by an immune-fluorescence assay adapted from Chou et al. (Chou & Scott, 1988). After
297 scoring, the virus titre (TCID50/ml) was calculated based on the Spearman-Kärber method.

298 TB40E-GFP Δ112-1 target virus was derived from TB40E-eGFP (O'Connor & Murphy, 2012), based on
299 the HCMV bacterial artificial chromosome (BAC)-derived strain TB40/E (clone 4) and which was
300 previously engineered to express enhanced green fluorescent protein (eGFP). TB40-eGFP was used
301 as the wild-type virus where indicated and was used to generate the TB40/E*gfp* Δ112-1 mutant using
302 *galK* BAC recombineering protocols. The TB40/E*gfp* Δ112-1 mutant contains 12 single base pair
303 substitutions that abrogate expression of miR-112-1, while maintaining the coding potential of the
304 UL114 ORF located on the opposite strand. Mutagenesis was performed on the BAC clone of
305 TB40/E*gfp*, using the *galK* selection and counter selection recombineering protocols as previously
306 described (Warming *et al.*, 2005). Briefly, the TB40/E*gfp*-BAC was transformed into *E. coli* SW102,
307 which has the heat inducible recombination genes required for BAC-mediated recombineering. PCR
308 products were generated with primers that contain 50 bases pairs of HCMV flanking sequence
309 targeting recombination of the *galK* expression cassette to the location of interest. The forward and
310 reverse primers to insert *galK* into the miR-UL112-1 sequence were 5'-
311 GGCGCTCTGACAGCCTCCGGATCACATGGTTACTCAGCGTCTGCCAGCCcctgttgacaattaatcatcggcga-3' and
312 5'-GCGACGCGGCGTGCTGCTGCTCAACACCGTGTTCACCGTGGTGCACGGACtcagcactgtcctgctcctt-3',

313 respectively (capitalized nucleotides correspond to the HCMV sequence). TB40-eGFP-BAC-containing
314 SW102 cells were made competent and transformed with the HCMV flanking *galK* PCR product.
315 Transformed cells were plated on M63 minimal salt agar plates supplemented with chloramphenicol
316 (12.5 µg/ml) and 0.2% galactose. TB40- eGFP-BAC clones that underwent homologous
317 recombination were additionally screened for growth on McConkey's medium supplemented with
318 galactose. Positive colonies (red) were picked and further screened by PCR to confirm the proper
319 location of the *galK* insert. Positive colonies were made electrocompetent and transformed with 80-
320 base pair double-stranded DNA with HCMV sequences that flank the *galK* integrated sequence
321 surrounding the sequence to be substituted into the viral genome. The double-stranded DNA used
322 to mutate the mature miRNA sequence of miR-UL112-1 was generated by annealing the following
323 oligonucleotides: 5'-
324 GTGCTGCTGCTCAACACCGTGTTCACCGTGGTGCACGGACAACCCGGGAGCCATAGGCATCTGGGCTGGCAG
325 ACGCTGAGTAACCATGTGATCCGGAGGCTG-3' and 5'-
326 CAGCCTCCGGATCACATGGTTACTCAGCGTCTGCCAGCCCAGATGCCTATGGCTCCCGGGTTGTCCGTGCACC
327 ACGGTGAACACGGTGTTGAGCAGCAGCAC -3'.Positive recombinants are selected by growth on m63
328 minimal salt agar plates containing 0.2% 2-deoxy-galactose (2-DOG) and 0.2% glucose. All mutations
329 were validated by sequencing the recombinant viruses. Virus was generated by electroporation of
330 primary fibroblasts with BAC DNA (20 µg) plus an HCMV pp71-expressing plasmid (pCGNpp71). After
331 a 100% cytopathic effect was achieved, virus was harvested by ultracentrifugation through a 20%
332 sorbitol cushion. Viral pellets were resuspended in X-VIVO 15 medium (Lonza) supplemented with
333 1.5% bovine serum albumin. Aliquots were flash frozen in liquid nitrogen and stored at −80°C until
334 further use. Stock titers were assessed by 50% tissue culture infective dose assays on primary
335 fibroblasts.

336 *THP-1 latency and reactivation model*

337 Twenty four hours prior to infection, THP-1 cells were pre-incubated in RPMI with 2% heat-
338 inactivated FCS (RPMI/2% FCS). This reduced large scale expansion of the cell population and the
339 associated loss of viral genomes (see Supplementary Fig. S1). To obtain latently infected cells,
340 undifferentiated THP-1 cells were infected at MOI 5 with HCMV for 3-4 hours at 37°C in 0.5ml
341 RPMI/2% FCS per $6x10^5$ cells with occasional agitation to avoid settling. After infection, the cells
342 were washed once with 2ml RPMI/2% FCS and spun down at 300*g* (5 minutes). The cells were
343 resuspended in RPMI/2% FCS, plated in a 24-well plate and incubated for 3 days at 37°C/5% CO2. To
344 induce reactivation, the cells were differentiated to THP-1 macrophages using 100nM phorbol 12-
345 myristate 13-acetate (PMA, Sigma-Aldrich).

346 A similar protocol was used for immunological assays with small adaptations. After the
347 establishment of latency (72 hours post infection), the medium was replaced by fresh medium and
348 24 hours later cells were used as stimulator cells in IFNγ ELISPOT assays and in T cell co-culture
349 experiments (see below). To obtain reactivated cells for IFNγ ELISPOT assays, latently infected cells
350 were treated with PMA as described above and incubated for 96 hours. As controls, lytically infected
351 cells were obtained by pre-treatment with PMA (100nM) for 48 hours. Subsequently, the THP-1
352 macrophages were infected with TB40/E virus at MOI 5, incubated for a further 96 hours and then
353 used as stimulator cells in IFNγ ELISPOT assays.

354 *Immediate-early antigen immunofluorescence assay*

355 On the day of read-out, undifferentiated THP-1 cells in suspension were collected by centrifugation
356 at 300*g* for 5 minutes. The cells were washed once with PBS, resuspended in 200µl PBS and adhered
357 to a glass adhesion slide (0901000, Mariënfeld Laboratory Glassware) as described by the
358 manufacturer. Differentiated adherent THP-1 cells were stained directly in the 24-well plate.

359 Fixation of the cells was carried out with 100% ice-cold methanol (10 minutes at -20°C). The slides or
360 dishes were washed twice with PBS and incubated for 20 minutes at RT with anti-immediate-early

361 antigen antibody (11-003, Argene) diluted 1/100 in PBST (PBS containing 0.05% tween-20). Before
362 adding the secondary antibody (1/100, Alexa Fluor 488 goat anti-mouse IgG, A-11001, Life
363 Technologies), the slides or dishes were washed twice with PBS. After 20 minutes of incubation with
364 the secondary antibody, the cells were washed once with PBS. Positive cells were manually counted
365 under an Axiovision fluorescence microscope.

366 Fluorescent images were taken using an Axiovision fluorescence microscope (Bright field: 5-9
367 milliseconds exposure; pseudo colour green fluorescence at laser intensity 25%: 500 milliseconds
368 exposure). Overlays were made automatically using the Axiovision software.

369 *Analysis of viral RNA and DNA*

370 DNA and RNA samples were prepared from monocytes obtained from one well or three wells,
371 respectively, of a 24-well plate. Non-adherent cells were collected by centrifugation (5 minutes,
372 300*g*) to remove the medium and were subsequently washed once with PBS. The cells were
373 collected again by centrifugation (300*g*, 5 minutes) and lysed in 500µl lysis buffer (Invisorb® Spin Cell
374 Mini Kit) or 600µl RLT Plus buffer (Qiagen RNeasy Plus kit) to obtain DNA and RNA samples,
375 respectively. After removing the medium, adherent cells were washed and lysed in the well. DNA
376 was prepared according to the manufacturer's instructions. RNA samples were prepared using a
377 Qiagen RNeasy Plus kit with an additional on-column DNase digestion step according to the
378 manufacturer's instructions.

379 Reverse transcription was carried out with oligo-dTs using the Superscript III kit (both from Life
380 Technologies) according to the manufacturer's instructions with an RNA input of 250-500ng. Gene
381 specific primers were used to amplify GAPDH, IE1/UL123 and UL138 by PCR using the Expand High
382 Fidelity PCR system (Roche) with the following cycling conditions: 95°C (5 minutes), 30 cycles of 94°C
383 (40 seconds), 55°C (40 seconds), and 72°C (60 seconds), and a final extension at 72°C for 5 minutes

384 (for primers see Table 1). PCR products were analyzed using agarose gel electrophoresis (2% E-gel,
385 Life Technologies). Images were taken using an ImageQuant LAS 4000.

386 RT-qPCR analyses were performed using the QuantiTect virus kit (Qiagen) as the manufactuer
387 describes, using primers and probes listed in Table 1. The reaction mixtures consisted of 1x
388 QuantiTect Virus NR Master Mix, 1x ROX Dye solution, 0.4µM of each forward and reverse primer,
389 0.2µM probe, 1x QuantiTect Virus RT Mix and 20-50ng sample RNA. The RT-qPCRs were performed
390 on 7500 Real-Time PCR System (Applied Biosystems) with cycling conditions as follows; 20 minutes
391 at 50°C, 5 minutes at 95°C, then 50 cycles of 15 seconds at 95°C, 45 seconds at 60°C. All qPCR
392 reactions were performed in duplicate with minus template controls and minus reverse
393 transcriptase controls. Viral transcript levels were normalised to the housekeeping transcript
394 GAPDH. The sensitivities of the qPCRs for UL138 and IE1/UL123 were approximately equivalent with
395 both having a detection limit of about 10 copies of HCMV genome (Betty Lau, unpublished
396 observations). In contrast, the conventional RT-PCRs with products detected by agarose gel
397 electrophoresis were not quantitative and detect UL138 more efficiently than IE72 (Emma Poole,
398 data not shown).

399 *Preparation of peripheral blood mononuclear cells (PBMC)*

400 Venous blood was obtained from a HLA-A2 HCMV seropositive individual and collected in sterile
401 50ml tubes containing 4ml heparin sodium (100IU/ml) in PBS. The blood was diluted 1:2 with RPMI-
402 1640 containing no serum (PAA laboratories, Austria) supplemented with 105IU/ml penicillin,
403 100mg/ml streptomycin, and 2mM L-glutamine. PBMC were isolated by Ficoll-Hypaque density
404 gradient centrifugation, 25ml blood was layered onto 12.5ml Lymphoprep (Axis-Shield, Norway) and
405 centrifuged at 800*g* for 15 minutes without the brake. PBMC were washed twice in PBS and
406 resuspended in RPMI supplemented with 10% FCS (RPMI-10). Autologous serum was also collected
407 and incubated 56°C for 30 minutes in a water bath to inactivate complement. Post incubation the
408 serum was UV-irradiated for 30 minutes and aliquoted for freezing at -20°C.

409 *Expansion of HCMV specific CD8$^+$ T-cells*

410 CD8$^+$ T-cells were isolated from the PBMCs of an HLA-A2 HCMV seropositive individual using a
411 magnetic CD8$^+$ T-cell enrichment kit (StemCell technologies) and then resuspended in RPMI
412 containing 10% FCS (Invitrogen) and 10% heat inactivated autologous donor serum. Cells were
413 stimulated with irradiated autologous PBMC which had been pulsed with HCMV IE72 peptide VLE
414 (Khan *et al.*, 2002) or EBV specific GLC peptide (Steven *et al.*, 1996). T cell cultures were carried out
415 in the presence of 2.5IU/ml human recombinant IL-2 (National Institute for Biological Standards and
416 Control, Potters Bar, United Kingdom) in 96-well plates at 37°C and a humidified CO_2 atmosphere for
417 10 – 14 days with feeding of the culture with the same media every five days. The specificity of
418 expanded CD8$^+$ cultures for mapped peptides was assessed by VLE HLA A2 specific pentamer
419 staining. Briefly, cells were harvested and washed and then stained with the specific unlabelled
420 pentamer (ProImmune), washed and then further stained with pentamer specific PE fluorophore
421 (ProImmune) and CD8 and CD3 antibodies conjugated to PerCP-Cy5.5 and FITC, respectively. After
422 this, cells were fixed and acquired on a FACS Sort using CellQuest software (BD Biosciences). Data
423 was analyzed using FlowJo software. All expanded cell lines were tested for specificity using IFNγ
424 ELISpot or Flourospot assays against THP-1 cells, monocytes and DCs. Antigen specific CD8$^+$ T cells
425 were frozen in Liquid Nitrogen at 2 X 10^6 cells/ml.

426 *Detection of cytokine production by ELISPOT and Fluorospot*

427 Human IFNγ ELISPOT (Ready-SET-Go!, eBioscience, San Diego, USA) assays were preformed
428 according to the manufacturer's instructions using 96-well PVDF membrane plates (Millipore,
429 Billerica, USA). VLE specific CD8$^+$ T-cells were rapidly defrosted at 37°C and diluted into 25mls of
430 warm RPMI-10 washed once and incubated at 37oC for 2hrs before being counted and then added
431 to wells at 12.5K, 25K or 50K T-cells per well in triplicate. The T-cells were stimulated with 50K THP-1
432 cells that had been latently or lytically infected with HCMV or latently infected cells that had been

433 reactivated. Positive controls were THP-1 cells pulsed with cognate peptide and negative controls
434 were unpulsed and uninfected THP-1 cells.

435 Plates were incubated for 48 hours at 37°C and then developed according to manufacturer's
436 instruction. Images of each well were taken using an ELISPOT plate scanner (ELISPOT Reader System,
437 AID) and spots enumerated using ImageJ (National Institutes of Health).

438 Human IFNγ Fluorospot plate (Mabtech AB, Nacka Strand, Sweden) assays were preformed
439 according to the manufacturer's instructions. Monocytes from an HLA-A2 HCMV seronegative donor
440 were latently infected for 6 days with WT or Δ112-1 target virus and then left untreated or treated
441 with IL-4/GM-CSF for an additional 6 days and then matured with LPS for 1 days to reactivate latent
442 virus. These were then cocultured with and HLA-A2 restricted VLE specific $CD8^+$ T-cells at 37ºC in a
443 humidified CO_2 atmosphere for 48 hours. The cells and medium were decanted from the plate and
444 the assay developed following the manufacturer's instructions. Developed plates were read using
445 an AID iSpot reader (AID) and counted using AID EliSpot v7 software.

446

447 *THP-1 co-culture*

448 THP-1 cells were plated at 5 x 10^4 cells per well in 48 well plates. At 3 days post infection, VLE or EBV
449 (GLC) specific $CD8^+$ T-cells were rapidly defrosted at 37°C and diluted into 25mls of warm RPMI-10
450 washed once and incubated at 37**°C** for 2hrs before being counted and then added at the
451 effector:target ratio of 3:4. The numbers of GFP expressing cells across entire wells were
452 enumerated 24 hours later.

453 *Ethical approval*

454 All human samples were obtained under ethical approval and after approval of protocols from the
455 Cambridgeshire 2 Research Ethics Committee (REC reference 97/092) and these protocols were

456 conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained
457 from all of the volunteers included in this study prior to providing blood samples and all experiments
458 were carried out in accordance with the approved guidelines.

459

460 **Acknowledgements**

461 We gratefully acknowledge funding from the UK Medical Research Council (J.H.S. and M. R. W.
462 G:0701279) which supports the current research in our laboratory and also the support of NIHR UK
463 Biomedical Research Centre (J.H.S. and M. R. W.).

464

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610

611 **Table legends**

612 **Table 1.** Primer sets for PCR and qPCR used in this study.

613 **Figure legends**

614 **Fig. 1.** Removal of the miR-UL112-1 target site leads to induction of IE gene expression in the
615 absence of virion production. (a) The target site of miR-UL112-1 in the 3' UTR of IE72 was deleted to
616 generate a recombinant virus in which IE72 is no longer targeted by miR-UL112-1. (b) Monocytes
617 (mono) were either uninfected, infected with wild type TB40E (WT) or the miR-UL112-1 target site
618 mutant virus (Δ112-1) and latency was established for 4 days before harvesting and analysing mRNA
619 levels for viral products UL138, IE and pp28 relative to cellular gene GAPDH. Alternatively, these
620 gene products were analysed following reactivation from latency by differentiation to dendritic cells
621 (DC) (reactivation). (c) Both latent monocytes and reactivating DCs, described in (a), were also co-
622 cultured with indicator fibroblasts and supernatants harvested and seeded onto fresh fibroblasts for
623 24h before fixing and immunofluorescence staining for IE protein, which was used to determined the
624 IE foci forming units.

625 **Fig. 2.** Monocytes latently infected with HCMV Δ112-1 target site mutant are recognized specifically
626 by IE-specific $CD8^+$ T cells. Monocytes (Mono) were latently infected with either wild-type HCMV
627 (WT) or miR-UL112-1 target site mutant virus (Δ112-1) and a proportion of cells from each
628 population were also differentiated to dendritic cells (DC) by IL-4 and GM-CSF treatment to induce
629 reactivation. Both the undifferentiated and differentiated cells were then co-cultured with IE (VLE)
630 specific CD8 T cells and then assayed for IFNY secretion using Fluorospot assays. Mock infected
631 monocytes (Mono mock) and dendritic cells (DC mock), were also co-cultured with IE (VLE) specific
632 CD8 T cells. The levels of T cell recognition is shown relative to the level of T cell recognition of
633 reactivating THP-1 cells (induced to reactivate by PMA). Error bars denote standard deviation of 3
634 biological replicates. T cells alone showed no background spots and reactivating THP-1 routinely
635 showed in the region of 4-50 spot forming units.

636 **Fig. 3.** Characterization of the THP-1 latency and reactivation model in low serum. (a) THP-1 cells
637 were infected with TB40/E at MOI 5 and left for 3 days to obtain a latently infected population. PMA
638 was added on day 3 and left on the cells for 24, 48 or 72 hours; cDNA was produced and amplified

639 using IE, UL138 and GAPDH primers. RT minus control samples (no RT) were run in parallel to
640 exclude genomic DNA contamination. A representative experiment of three independent replicates
641 is shown. Lane 1: 100bp DNA ladder (Life Technologies); Lane 2: mock infected, untreated; Lane 3:
642 mock infected, 100nM PMA; Lane 4: TB40/E infected, untreated; Lane 5: TB40/E infected, 100nM
643 PMA; Lane 6: TB40/E infected, untreated; Lane 7: TB40/E infected, 100nM PMA; Lane 8: TB40/E
644 infected, untreated; Lane 9: TB40/E infected, 100nM PMA. (b) Microscopic images of TB40/E
645 infected, undifferentiated THP-1 cells (latency) (left panel) and TB40/E latently infected THP-1 cells,
646 differentiated on day 3 post infection with 100nM PMA (reactivation) (right panel). (c) Cells from fig.
647 b, left panel or right panel were co-cultured with fibroblasts for 5 days (left panel or right panel,
648 respectively). For all panels in (b) and (c), IE expression was visualised using immunofluorescence
649 staining with anti-IE72/86 antibody. Arrows indicate IE expressing cells.

650 **Fig. 4.** Latent infection with Δ112-1 target site mutant virus results in increased levels of IE72 RNA
651 expression compared to wild-type virus. THP-1 cells were infected at MOI 5 with the parental WT
652 virus or the Δ112-1 target site mutant. Latently infected cells were sorted for GFP expression at 2
653 days post infection, before mRNA expression was analysed by RT-qPCR at 3 dpi. The level of UL138
654 and IE72 normalised to the mRNA level of housekeeping gene GAPDH is shown in (a) and (b),
655 respectively.

656 **Fig. 5.** IE (VLE) specific $CD8^+$ T cells recognises lytically but not latently infected THP-1 cells. (a) THP-1
657 cells were stained with monoclonal antibody specific for MHC Class I HLA-A2 (filled plot) or an
658 isotype matched control antibody (open plot). (b) THP-1 cells were pulsed with IE (VLE) peptide
659 (pulsed) and assayed with control THP-1 cells (unpulsed) for their ability to be recognised by
660 increasing numbers of IE (VLE) specific $CD8^+$ T cells; as measured by IFNγ production in ELISPOT
661 assays. (c) Latently infected THP-1 cells (latent), lytically infected differentiated THP-1 cells (lytic) or
662 latently infected THP-1 cells differentiated with PMA to induce HCMV reactivation (reactivated)
663 were co-cultured with increasing numbers of IE (VLE) specific $CD8^+$ T cells (as detailed in the figure)

664 and assayed for IFNϒ using ELISPOT assay. (d) Δ112-1 target mutant allows recognition and removal
665 of latently infected cells. THP-1 cells were infected at MOI 5 with the parental WT virus (WT) or the
666 Δ112-1 target virus before latently infected cells were sorted for GFP expression at 2 days post
667 infection. At 3 days post infection, the cells were co-cultured with $CD8^+$ T cells as indicated for a
668 further 24 hours before the numbers of GFP expressing cells were enumerated. Changes in GFP+ cell
669 numbers are shown as fold change over the number of GFP+ cells present in wild type virus infected
670 THP-1 cells cultured in the absence of T cells. Statistical analysis was performed using t-test, with *
671 indicating significant difference ($p<0.05$). Error bars denote standard deviation of technical
672 replicates.

673

674 **Table 1.**

Conventional PCR		
Gene	Sequence	Reference
IE Fwd	CAT CCA CAT CTC CCG CTT AT	(Reeves *et al.*)
IE Rev	CAC GAC GTT CCT GCA GAC TAT G	
GAPDH Fwd	GAG TCA ACG GAT TTG GTC GT	
GAPDH Rev	TTG ATT TTG GAG GGA TCT CG	
UL138 Fwd	TGC GCA TGT TTC TGA GCT AC	(Goodrum *et al.*, 2007)
UL138 Rev	ACG GGT TTC AAC AGA TCG AC	
Probe-based qPCR		
Gene	Sequence	Reference
IE72 Fwd	CAA GAA CTC AGC CTT CCC TAA GAC	(Visconti *et al.*, 2004)
IE72 Rev	TGA GGC AAG TTC TCG AAT GC	
IE72 probe	[6FAM] CCA ATG GCT GCA GTC AGG CCA TG [TAM]	
GAPDH Fwd	GGA AGC TTG TCA TCA ATG	(Poole *et al.*, 2014)
GAPDH Rev	CCC CAC TTG ATT TTG GAG	
GAPDH probe	[JOE] ATC ACC ATC TTC CAG GAG CGA G [BHQ1]	
UL138 Fwd	CGC TGT TTC TCT GGT TAG	(Krishna *et al.*, 2016)
UL138 Rev	CAG ACG ATA CCG TTT CTC	
UL138 probe	[Cy5] CCG ACG ACG AAG ACG ATG AAC [BHQ2]	
pp28 Fwd	CGA ACT CTG CAA ACG AAT A	(Krishna *et al.*, 2016)
pp28 Rev	GAG GGA TGT TGT CGT AGG	
pp28 probe	[Cy3] CGT AGA GAC ACC TGG CGA CC [BHQ2]	

675

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680

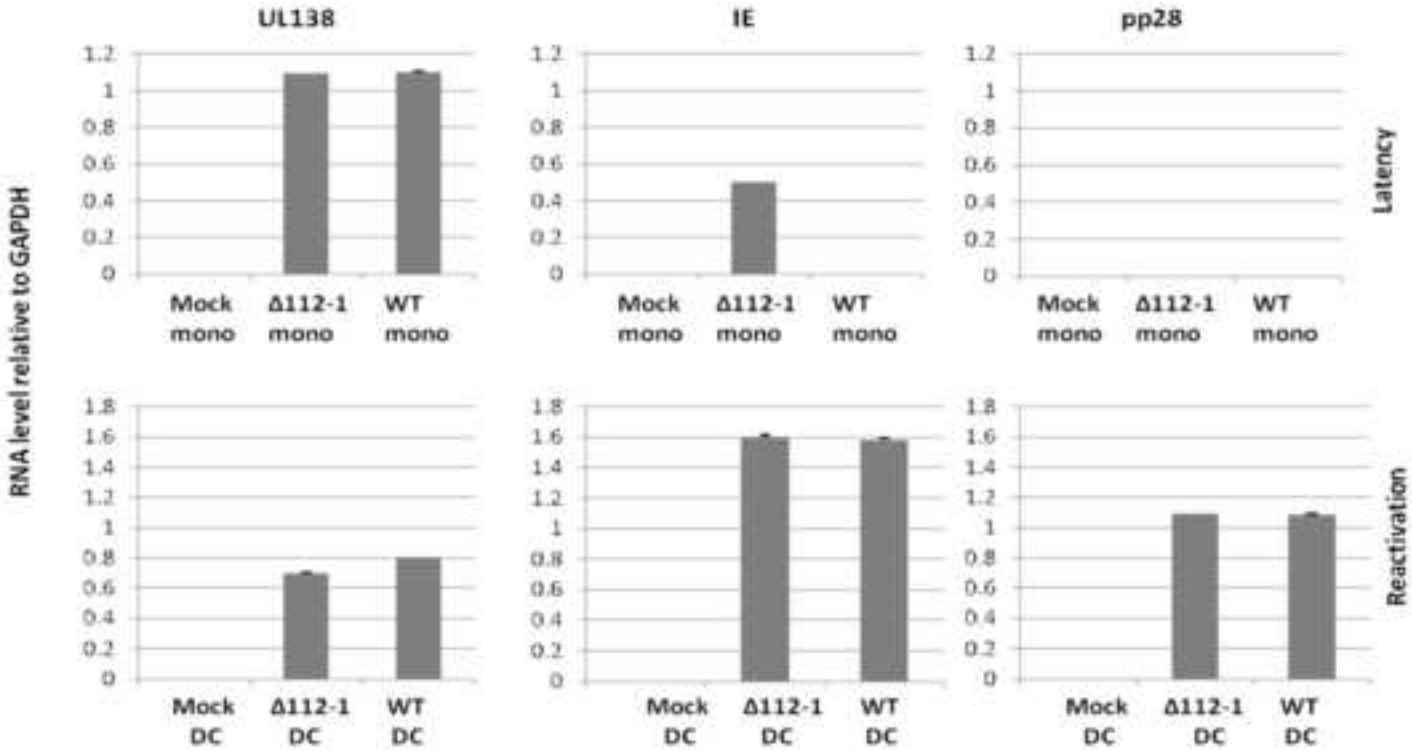
681

Figure 1

A

IE72
3' UTR
UL112-1 target site

B


UL138
IE
pp28
RNA level relative to GAPDH
Latency
Reactivation
Mock mono
Δ112-1 mono
WT mono
Mock DC
Δ112-1 DC
WT DC

C

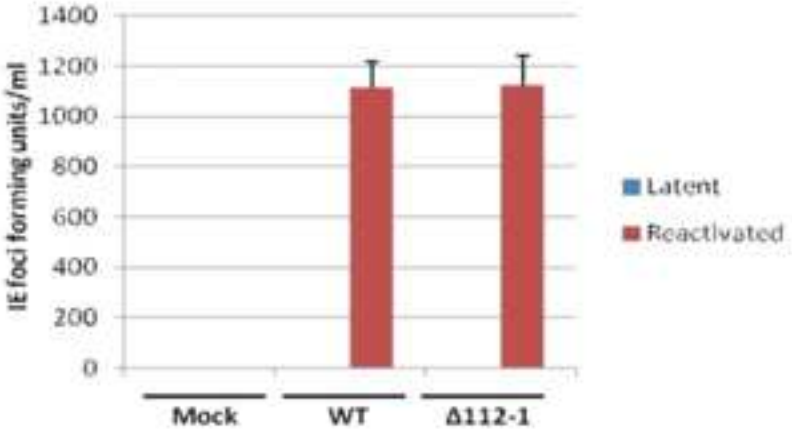

IE foci forming units/ml
Latent
Reactivated
Mock
WT
Δ112-1

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Figure 2

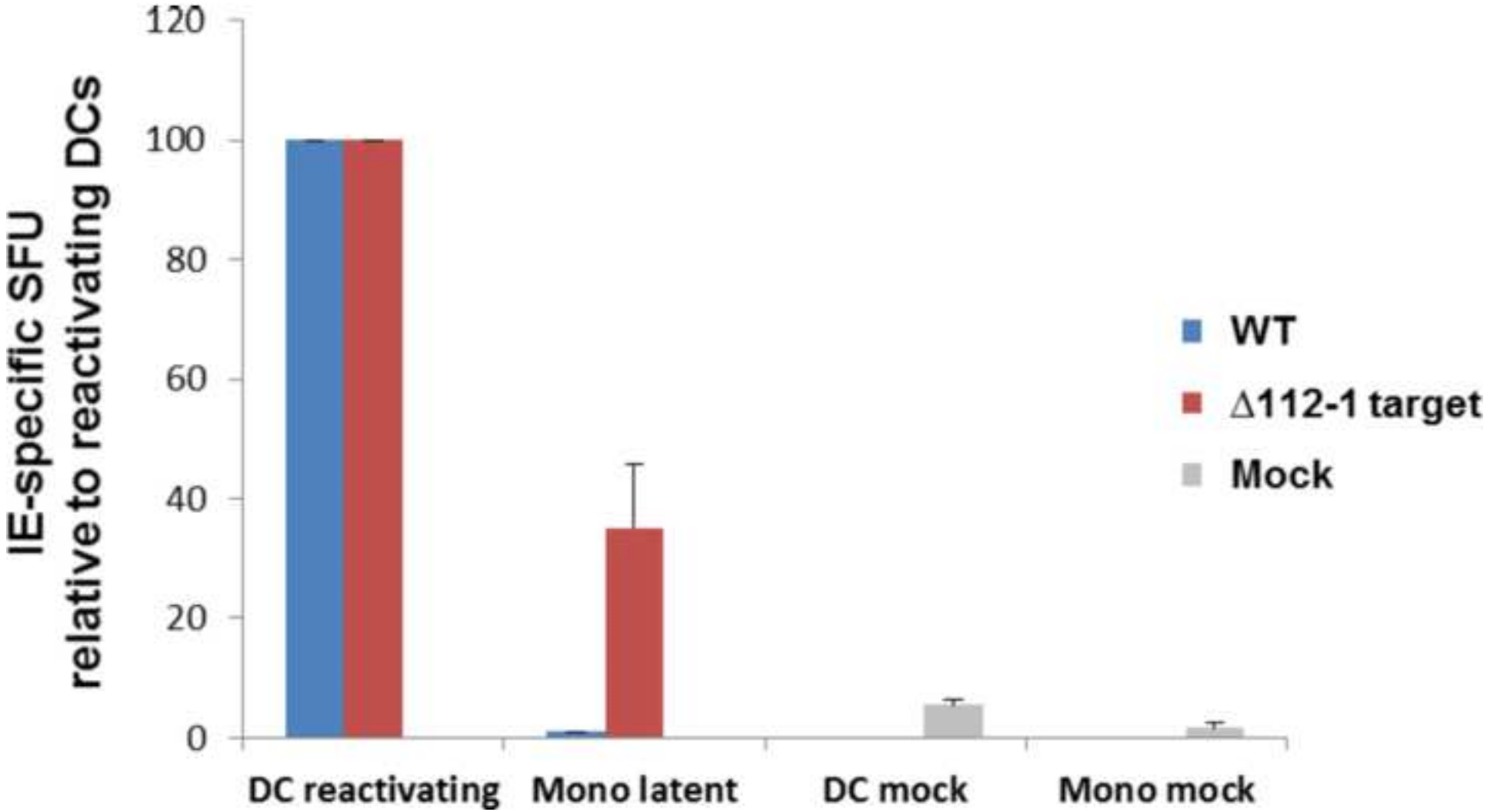

Figure 3

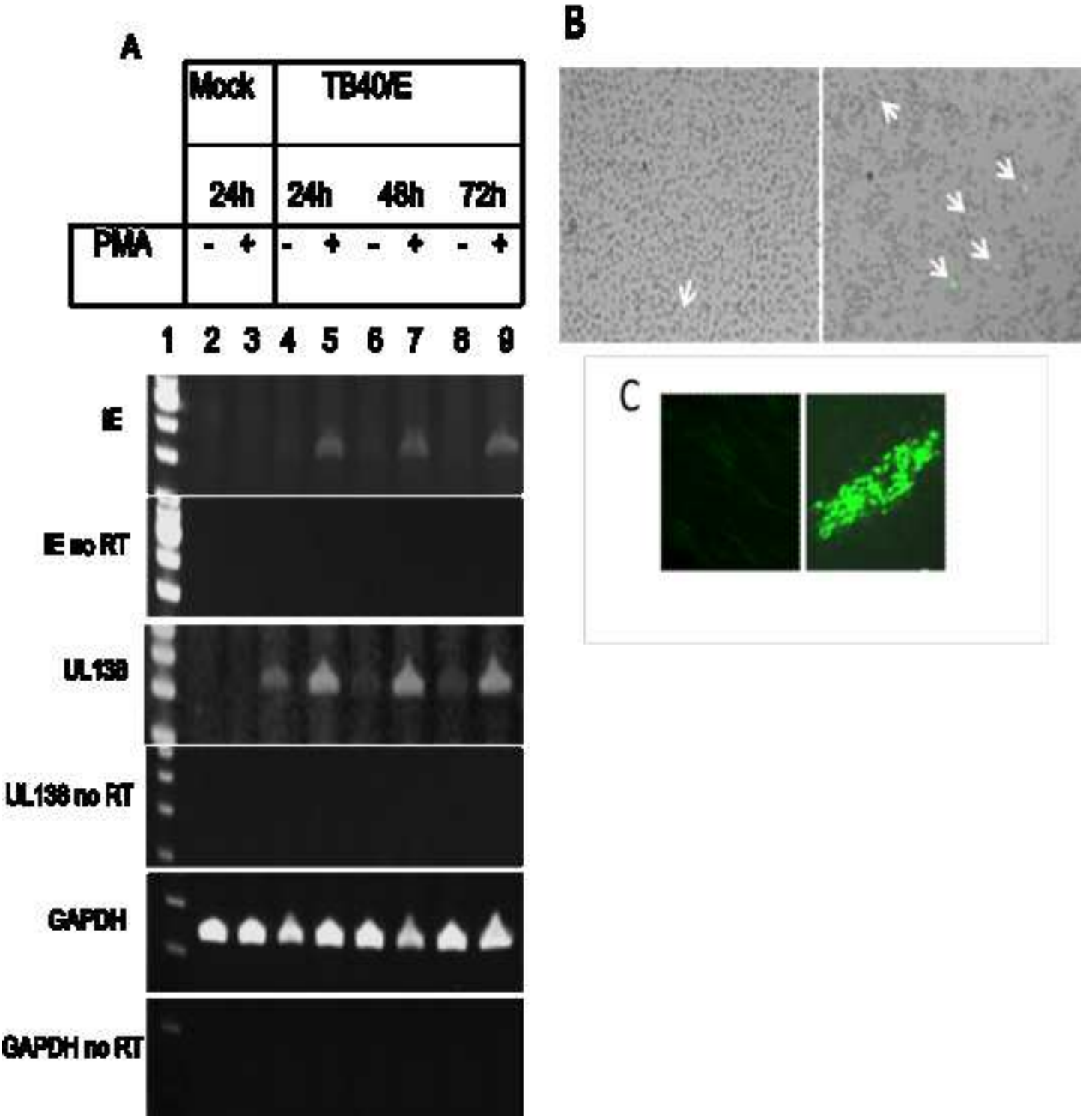
A
Mock
TB40/E
24h
24h
48h
72h
PMA
- + - + - + - +
1 2 3 4 5 6 7 8 9
IE
IE no RT
UL138
UL138 no RT
GAPDH
GAPDH no RT
B
C

figure 4

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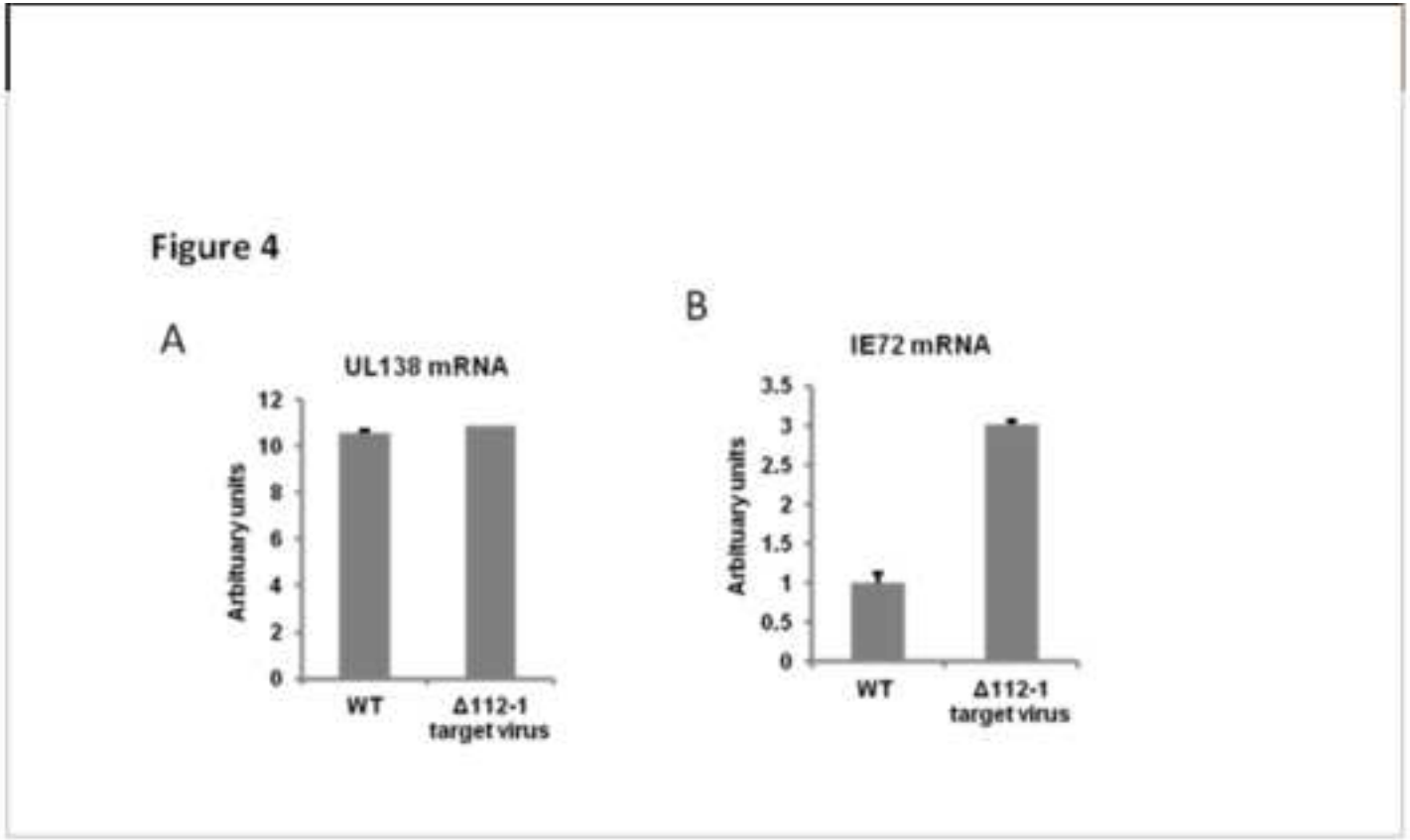

figure 5

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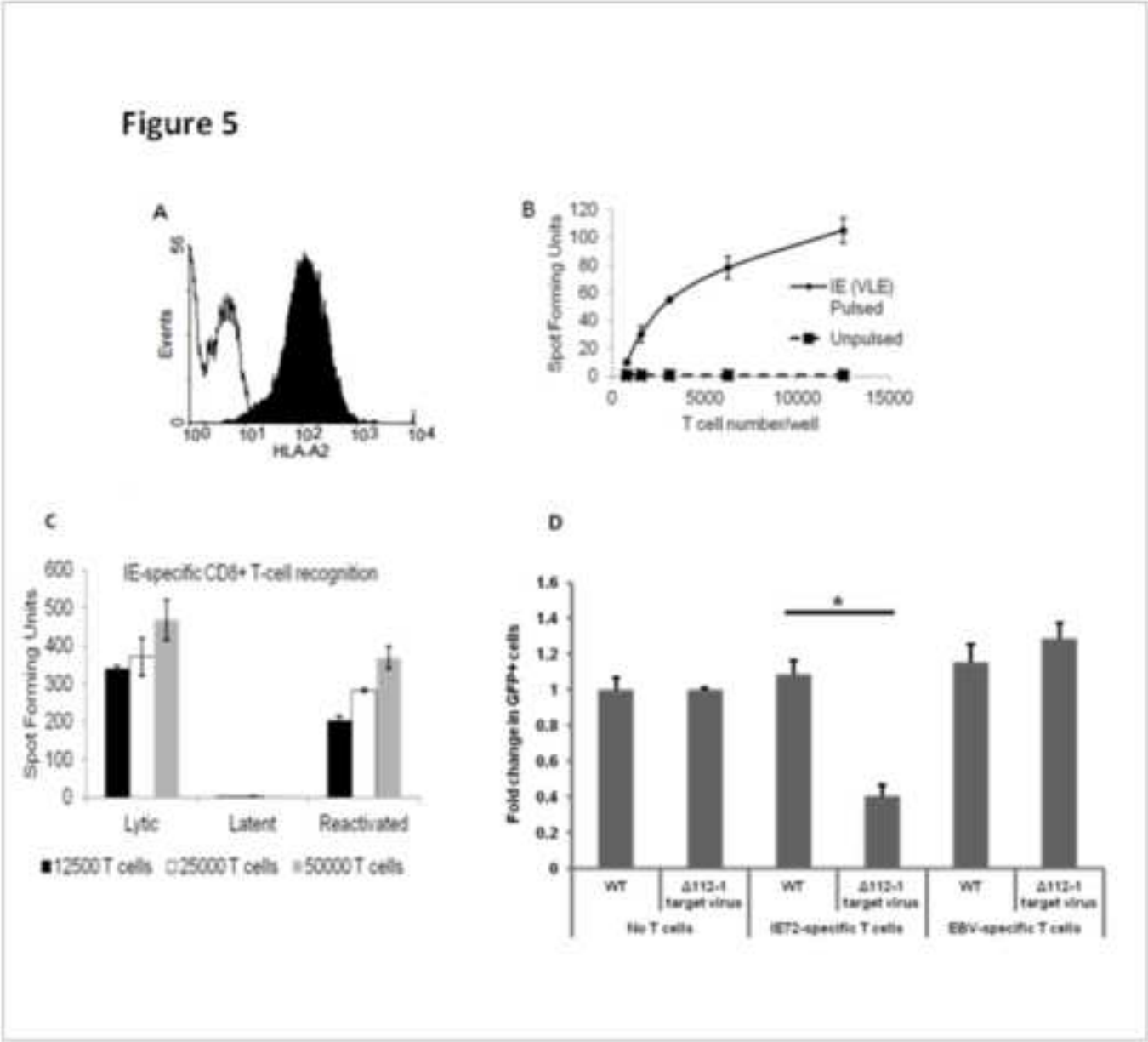

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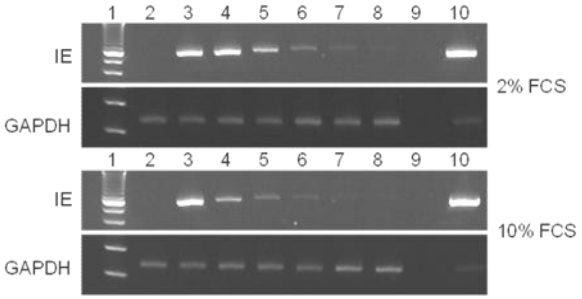
Supplementary Figures



Supplementary Fig. S1

A representative image for the genome maintenance over time in reduced and 10% serum conditions as determined by end-point PCR. THP-1 cells grown in serum-reduced conditions or in 10% serum were mock infected (lane 2) or infected at MOI 5 (lanes 3-10). DNA samples were prepared 1 day (lane 2 and lane 3), 3 days (lane 4), 6 days (lane 5), 9 days (lane 6), 13 days (lane 7) and 20 days post-infection (lane 8). End-point PCR for the viral IE region and the cellular control GAPDH was performed on input normalized total DNA. A 100bp ladder (Life Technologies) was used to verify the amplicon size (lane 1), as a positive control DNA of infected HFF (lane 10, MOI 5, 3 days p.i.) was amplified. A no template control is shown in lane 9.

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