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Interaction between Herpesvirus Entry Mediator and HSV-2 Glycoproteins Mediates HIV-1 Entry of HSV-2-infected Epithelial Cells

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15 **Key Words**: HIV-1; HSV-2; Herpesvirus entry mediator; co-infection.

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17 **Abbreviations**: 7-AAD, 7-aminoactinomycin D; AChE, acetylcholinesterase; AZT,
18 azidothymidine; HIV-1, human immunodeficiency virus type 1; HSV-2, herpes simplex
19 virus type 2; HVEM, herpesvirus entry mediator; HIV-1/HVEM, HVEM-bearing HIV-1;
20 HTLV-1, human T-lymphotropic virus 1; LFA-1, integrin lymphocyte function-
21 associated antigen 1; TEM, tetraspanin enriched membrane; VCAM-1, cell adhesion

22 molecule vascular cell adhesion molecule 1; XMRV, xenotropic murine leukemia virus-

23 related virus.

ABSTRACT

HSV-2 increases HIV-1 acquisition and transmission via an unclear mechanism. HSV-2 entry receptor herpesvirus entry mediator (HVEM) is highly expressed on HIV-1 target cells $CD4^+$ T cells and may be incorporated into HIV-1 virions, while HSV-2 glycoproteins can be present on infected cell surface. Since HVEM-gD interaction together with gB/gH/gL is essential for HSV-2 entry, HVEM-bearing HIV-1 (HIV-1/HVEM) may enter HSV-2-infected cells through such interactions. To test this hypothesis, we first confirmed the presence of HVEM on HIV-1 virions and glycoproteins on HSV-2-infected cell surface. Additional studies showed that HIV-1/HVEM bound to HSV-2-infected cell surface in an HSV-2 infection-time-dependent manner via HVEM-gD interaction. HIV-1/HVEM entry of HSV-2-infected cells was dependent on HVEM-gD interaction and the presence of gB/gH/gL, and was inhibited by azidothymidine (AZT). Furthermore, peripheral blood mononuclear cells (PBMCs)-derived HIV-1 infected HSV-2-infected primary foreskin epithelial cells and the infection was inhibited by anti-HVEM/gD antibodies. Together, our results indicate that HIV-1 produced from $CD4^+$ T cells bears HSV-2 receptor HVEM and can bind to and infect HSV-2-infected epithelial cells depending on HVEM-gD interaction and the presence of gB/gH/gL. Our findings provide a potential new mechanism underlying HSV-2 infection-enhanced HIV-1 mucosal transmission and may shed light on HIV-1 prevention.

42 **INTRODUCTION**

43 Human immunodeficiency virus type 1 (HIV-1) infection is usually accompanied by co-
44 infections of other pathogens including herpes simplex virus type 2 (HSV-2) [1]. Like HIV-1,
45 HSV-2 is also sexually transmitted, but targets different cell subsets. HSV-2 predominantly
46 infects epithelial cells, causing mucosal ulceration, and can also infect immunocytes and neurons
47 [2-4]. Both epidemiological and clinical data have suggested that HSV-2 infection enhances
48 HIV-1 acquisition and transmission up to three folds, but the underlying mechanisms remain to
49 be defined [5-10]. HSV-2 is around 11.3 % (417 million) positive in the general population globe
50 wide with an annual new infection rate of 0.5 % (19.2 million) [11]. Moreover, data from World
51 Health Organization (WHO) indicate that 60-90 % of HIV-1-infected individuals are co-infected
52 with HSV-2 [12]. Consequently, understanding the mechanisms underlying HSV-2-enhanced
53 HIV-1 acquisition and transmission is crucial for HIV-1 prevention and treatment.

54

55 To date, several potential mechanisms from different perspectives have been proposed, which
56 can be generally divided into two categories. One is that HSV-2 infection would generate
57 ulceration and consequently disrupt the integrity of the mucosal barrier and allow HIV-1
58 infection of target cells underneath [10, 13-15]. The other is that inflammatory responses at the
59 site of HSV-2 infection could cause enrichment of immunocytes including HIV-1 target cells at
60 these sites [9, 16, 17]. However, all these potentials mechanisms have yet to be confirmed. In
61 addition, the above presumptions all focus on the potential changes of topical microenvironment
62 following HSV-2 infection, but little is known concerning the roles played by HIV-1 virion
63 itself.

64

65 CD4-independent HIV-1 infection of non-target cells has been described in HIV-1 co-infection
66 with other viruses. For instance, under the condition of HIV-1 and herpes simplex virus 1 (HSV-
67 1) co-infection, HIV-1 infection of $CD4^-$ keratinocytes was observed [18]. HIV-1 infection of a
68 variety of $CD4^-$ cell subsets including $CD8^+$ T cells, B cells, epithelial cells and even skeletal
69 muscle cells was reported in the context of HIV-1 and human T-lymphotropic virus 1 (HTLV-1)
70 co-infection [19]. HIV-1 infection of female genital epithelial cells was also described when co-
71 infected with xenotropic murine leukemia virus-related virus (XMRV) [20]. We hypothesized
72 that, under the circumstances of HIV-1 and HSV-2 infection, HSV-2-induced changes on
73 epithelial cells may be beneficial for HIV-1 to establish infection.
74
75 As an enveloped virus, HIV-1 obtains its membrane from host cells during viral budding, which
76 consequently contains host cell components [21]. Herpesvirus entry mediator (HVEM), an HSV-
77 2 entry receptor, is highly expressed on HIV-1 target cells $CD4^+$ T cells [22]. Theoretically,
78 HVEM is likely present on the surface of HIV-1 virion. HSV-2 is also an enveloped virus.
79 Although its viral packaging usually takes place in the Golgi apparatus, HSV-2 glycoproteins
80 may be partially present on the host cell surface owing to the overexpression of viral proteins and
81 the trafficking of cell membrane system [23]. Since HSV-2 glycoprotein D interaction with its
82 receptor HVEM together with the presence of three viral glycoproteins gB, gH and gL
83 (designated as HVEM-gB/gD/gH/gL) are essential for successful viral entry, HVEM-bearing
84 HIV-1 (designated as HIV-1/HVEM hereafter) may be able to enter HSV-2-infected cells
85 through such interactions [24, 25]. In the current study, using both cell lines and primary
86 foreskin epithelial cells as models, we tested whether HIV-1/HVEM could infect HSV-2-infected

87 epithelial cells and whether this type of infection is HVEM-gD interaction- and gB/gH/gL -

88 dependent.

89

90 **RESULTS**

91 **HVEM is present on HIV-1 virions.**

92 To test our hypothesis that HVEM-gD interaction together with gB/gH/gL may mediate HIV-1

93 entry of non-target epithelial cells, we first examined the presence of HVEM on HIV-1 virions.

94 We conducted experiments to address whether HVEM is expressed on HIV-1 target $CD4^+$ T

95 cells and whether it can be incorporated into HIV-1 virions. Freshly isolated peripheral blood

96 mononuclear cells (PBMCs) from different donors were assessed for the cell surface expression

97 of CD4 and HVEM, respectively. Our results showed that a near 100 % HVEM expression was

98 observed on PBMCs regardless of donor origins, and not surprisingly almost 100 % $CD4^+$ T cells

99 express HVEM (Fig. 1a) [22].

100

101 We next determined whether HVEM is incorporated into the viral membrane during progeny

102 viral budding. HIV-1 from various sources was concentrated by ultracentrifugation, lysed and the

103 presence of HVEM was determined by Western blot. As shown in Figure 1b, HVEM expression

104 was detected from HIV-1 pellets generated from both PBMCs and HVEM-expressing 293T

105 (293T-HVEM) cells. In contrast, no HVEM was detected from virions generated from 293T

106 cells. Pelleted HIV-1 supernatants are usually contaminated with microvesicles. Our data showed

107 that microvesicles, as indicated by acetylcholinesterase (AChE), were present in pelleted HIV-1

108 samples. To confirm that HVEM detected in the pellets was associated with HIV-1 virions rather

109 than contaminant microvesicles, we pelleted cell culture supernatants from mock-treated 293T,

110 293T-HVEM and PBMC cells, respectively, and assessed the presence of HVEM. Our data
111 showed that no HVEM was detected in the pellets of mock-treated cell culture supernatants,
112 indicating that HVEM is associated with HIV-1 but not contaminant microvesicles (Fig. 1b). To
113 further confirm these results, pelleted virus stocks were purified by 6–18% Optiprep™ density
114 gradient and HVEM presence in the purified viruses were determined. As shown in Figure 1c,
115 HVEM was successfully detected in purified HIV-1 derived from 293T-HVEM and PBMCs.
116 These data together indicate that HVEM is highly expressed on $CD4^+$ T cells and can be
117 incorporated into HIV-1 virions during virus budding.
118
119 **HIV-1/HVEM binds to HSV-2-infected cell surface via HVEM-gD interaction.**
120 Cell surface presentation of viral glycoproteins and the viability of HSV-2-infected cells are two
121 important factors determining the entry of HIV-1/HVEM in HSV-2-positive cells. Our data
122 showed that HSV-2 gB and gD on cell surfaces of infected ME-180 and HeLa cells increased in
123 a time dependent manner and peaked around 24 h (Fig.S1). Cell viability assay showed that,
124 although HSV-2 infection disrupted cell morphology of both ME-180 and HeLa cells in an
125 infection dose dependent manner, the majority of infected cells remained viable 30 h after
126 infection as evidenced by 7-aminoactinomycin D (7-AAD) staining (Fig. S2).
127
128 We subsequently investigated whether HIV-1/HVEM could bind to HSV-2 infected epithelial
129 cells. Two cervical epithelial cell lines HeLa and ME-180 were adopted in the assay. As shown
130 in Fig.2a, binding of HIV-1/HVEM to HSV-2-infected cells was significantly increased in an
131 HSV-2 infection time dependent manner, whereas HIV-1/BaL did not show such a binding
132 pattern. Similar binding profiles of HIV-1/HVEM were observed in both HeLa and ME-180 cell

133 lines following HSV-2 infection, although higher level of binding was on ME-180 than on HeLa
134 cells.
135
136 HVEM serves as a receptor for gD during HSV-2 entry. We further determined whether the
137 binding of HIV-1/HVEM to HSV-2 infected cell surface was dependent on HVEM and HSV-2
138 glycoproteins. HSV-2 glycoproteins gB, gD, gH and gL alone or in combinations was expressed
139 in HeLa and ME-180 cells, followed by the assessment of HIV-1/HVEM binding. Our results
140 showed that HIV-1/HVEM bound to cells transfected with HSV-2 gB/gD/gH/gL, and such
141 binding appeared to be gD-dependent (Fig. 2b). These data suggest that binding of HIV-
142 1/HVEM to HSV-2-infected cell surface is dependent on the interaction between HVEM and gD.
143
144 **HIV-1/HVEM enters HSV-2-infected cells in an HVEM-gB/gD/gH/gL dependent manner.**
145 We next asked whether the binding of HIV-1/HVEM to HSV-2 infected epithelial cells could
146 lead to viral infection. HeLa or ME-180 cells were infected with HSV-2 followed by co-
147 cultivation with HVEM-bearing replication-competent HIV-1_{BaL} (HIV-1_{BaL}/HVEM) in the
148 presence or absence of HIV-1 reverse transcriptase inhibitor (azidothymidine, AZT).
149 Quantification of in-cell HIV-1 p24 showed that p24 was only detected in cells pre-infected with
150 HSV-2 but not in those without HSV-2 infection. Moreover, the addition of AZT almost
151 completely inhibited p24 production, indicating that a productive HIV-1 infection took place in
152 HSV-2 infected epithelial cells (Fig.3a).
153
154 By assessing HIV-1/HVEM entry of HSV-2 gB/gD/gH/gL transfected cells, we further showed
155 that HIV-1/HVEM entry of epithelial cells required the co-expression of all four glycoproteins

156 gB/gD/gH/gL (Fig. 3b). The control virus, HIV-1/BaL without HVEM incorporation, only
157 infected CD4/CCR5- but not gB/gD/gH/gL-expressing cells. In contrast, HIV-1/HVEM, due to
158 the lack of HIV-1 Env, infected gB/gD/gH/gL-expressing cells, but not those with CD4/CCR5
159 expression (Fig. 3b).
160
161 We further addressed the influence of gB/gD/gH/gL expression level on the entry efficiency of
162 HIV-1/HVEM. Our results showed that the amount of in-cell HIV-1/HVEM increased along
163 with the gB/gD/gH/gL expression level (data not shown). Moreover, blockade of gD by an
164 antibody significantly inhibited the entry of HIV-1/HVEM into gB/gD/gH/gL-expressing
165 epithelial cells, further reinforcing the significance of gD-HVEM interaction in this process (Fig.
166 3c).
167
168 **PBMC-propagated HIV-1 infects HSV-2-infected human foreskin epithelial cells.**
169 The results above indicated that HVEM-bearing HIV-1, via an HVEM-gB/gD/gH/gL dependent
170 manner, could successfully bind to and subsequently enter HSV-2-infected epithelial cells. To
171 further confirm our findings in a physiologically relevant system, HIV-1_{BaL} was propagated in
172 PBMCs and its ability to infect HSV-2-infected primary foreskin epithelial cells was determined.
173 In accordance with the findings above, our results showed that HIV-1, albeit at a very low level,
174 could infect HSV-2-infected foreskin epithelial cells. Moreover, this infection could be
175 substantially inhibited by neutralizing antibodies against HSV-2 gD or HVEM (Fig. 4).
176

177 Taken together, our data reveal that HIV-1 can obtain HVEM from the host cell membrane and
178 such HVEM-bearing virus can infect HSV-2-infected non-HIV-1-target cells through an HVEM-
179 gB/gD/gH/gL dependent manner.
180

181 **DISCUSSION**

182 For an infection to occur, HIV-1 must cross the mucosal epithelial barrier [26]. HSV-2 infection-
183 resulted disruption of mucosal integrity has been proposed to be one of the mechanisms
184 accounted for HSV-2-enhanced HIV-1 acquisition and transmission. However, the fact that
185 enhanced HIV-1 infection can occur in HSV-2 asymptomatic stage suggests the existence of
186 other mechanisms [27]. In the asymptomatic phase, HSV-2 replication and viral shedding still
187 take place [28]. This persistent viral replication could result in phenotype changes to the infected
188 epithelial cells other than ulceration, which might be in favor of the cross of mucosal epithelial
189 barrier by HIV-1. In the current study, we revealed that HSV-2 replication results in the presence
190 of viral glycoproteins on the infected cell surface, which can be targeted by HIV-1 through viral
191 membrane-retained HVEM. HVEM interaction with HSV-2 gD in the presence of the other three
192 viral glycoproteins gB/gH/gL can subsequently lead to HIV-1 infection of epithelial cells. Our
193 findings reveal a potential new mechanism underlying HSV-2-enhanced HIV-1 mucosal
194 transmission, which may facilitate HIV-1 to cross mucosal epithelia.
195

196 HIV-1 co-infection with other viruses/pathogens is not uncommon. HIV-1 infection of CD4$^-$
197 keratinocytes has been described under the condition of co-infection with HSV-1[18], although
198 the underlying mechanism remains to be fully defined. HSV-2 has a high genetic similarity to
199 HSV-1, with about 83 % protein sequence identity. Moreover, these two viruses share almost

200 identical arrangement of open reading frames and can form various recombinant viruses in *in*
201 *vitro* culture [29-31]. Given the high resemblance of these two viruses, it is likely that the
202 incidence of HIV-1 infection of keratinocytes under HSV-1 co-infection may also take place
203 under HSV-2 co-infection. Indeed, our study here revealed that HIV-1 infection of keratinocytes
204 occurred under the condition of HSV-2 co-infection. Our results further showed that HIV-1 entry
205 of non-target cell was mediated by HVEM on HIV-1 through interaction with gB/gD/gH/gL on
206 HSV-2 infected epithelial cells. Furthermore, since HSV-1 adopts a similar entry mechanism as
207 does HSV-2, such HVEM-gB/gD/gH/gL-mediated HIV-1 infection may provide an explanation
208 for the phenomenon that HIV-1 can infect $CD4^-$ keratinocytes under the condition of HSV-1 co-
209 infection.
210
211 HIV-1 infects $CD4^+$ T cells, macrophages and dendritic cells, while $CD4^-$ keratinocytes are
212 usually non-permissive to HIV-1. As mentioned above, in the case of co-infection with viruses
213 like HSV-1, HTLV-1 and XMRV, HIV-1 infection of a range of non-target cells has been
214 described, but the efficiency of these atypical infections has not been documented [18-20]. In the
215 current study, we revealed that epithelial cells could be infected by HIV-1 providing that these
216 cells are productively infected by HSV-2. Nevertheless, the proportion of HIV-1-infected
217 epithelial cells was rather small. Of more than 90 % of HSV-2-infected primary foreskin
218 epithelial cells, approximately 2 % of them were co-infected by HIV-1. Although the reasons for
219 the low co-infection rate might be multifaceted, we believe that the cell condition at the time of
220 HIV-1 infection is critical for the success of HIV-1 infection and replication. A successful HIV-1
221 infection appeared to rely on early HSV-2 infection (4-6 h). When cells were infected for a
222 longer period of time with HSV-2 (16-20 h), the binding of HIV-1 to cell surface increased, but

223 intracellular HIV-1 dropped to a level below detection limit, suggesting that there were more
224 glycoproteins expressed on the cell surface but the cell condition might not be suitable for HIV-1
225 replication (Fig.2a and unpublished data). Twenty hours post infection is the time close to the
226 release of HSV-2 progeny viruses. At this time point, cellular resources might be exhausted by
227 HSV-2 replication and cells overloaded with HSV-2 progeny virus particles are ready for
228 extracellular transportation[32]. Therefore, the condition of the cells at this time point may not be
229 ideal for HIV-1 entry and replication. We postulate that a successful HIV-1/HVEM infection of
230 HSV-2-infected epithelial cells likely takes place only at the stage of HSV-2 early replication. At
231 the early stage of HSV-2 infection (e.g. 4-6 h after infection), although the expression levels of
232 HSV-2 glycoproteins are relatively low, gD expressed on the cell surface could mediate an
233 interaction with HIV-1/HVEM and consequently result in a low level of HIV-1 infection. It is
234 known that HIV-1 transmission often results from infection by a single transmitted/founder
235 virus, indicating that a high quantity of infection may not be necessary for HIV-1 to establish a
236 productive infection [33]. In our study, although HIV-1/HVEM-mediated infection of epithelial
237 cells was low, we did observe a productive HIV-1 infection, revealing that the in-cell HIV-1 p24
238 was inhibited by reverse transcriptase inhibitor AZT (Fig. 3a), which is in agreement with a
239 previous study that HIV-1 can replicate in epithelial cells under the coinfection condition[20].
240 Therefore, although HVEM-gB/gD/gH/gL-mediated HIV-1 infection of primary epithelial cells
241 is not efficient, such advantage that HIV-1 has acquired may significantly increase the chances
242 of successful mucosal transmission.

243

244 In addition to primary epithelial cells, we used two epithelial cell lines HeLa and ME-180 cells
245 in the current study. A similar tendency of results, albeit with difference, was observed in the

246 two cell lines. A higher level of HIV-1/HVEM binding and entry was observed in ME-180 cells
247 than in HeLa cells. In agreement, we observed that, following transfection with gD expressing
248 constructs, ME-180 had higher level of HSV-2 gD on the cell surface than HeLa. Likewise, ME-
249 180 was more sensitive to HSV-2 infection compared to HeLa (Fig. S2 and S3). Unexpectedly,
250 we found that HIV-1 infection of HSV-2-infected ME-180 was in a lower quantity than in HeLa
251 cells (Fig. 3a). Although the mechanism remains to be further addressed, this was likely caused
252 by the higher rate of cell death on ME-180 than on HeLa cells upon HSV-2 infection. In
253 addition, we observed basal level of HIV-1 binding to both HeLa and ME-180 cells and this was
254 independent of either HVEM expression or HSV-2 infection, suggesting that other interactions
255 between proteins from viral and cell membranes may be involved. Indeed, a wide range of host
256 proteins, including MHC restriction factors HLA-I and HLA-II, cell adhesion molecule vascular
257 cell adhesion molecule 1 (VCAM-1), integrin lymphocyte function-associated antigen 1 (LFA-
258 1), T cell signaling molecules CD3 and CD4, have previously been shown to be detected on
259 HIV-1 virions [34, 35]. HIV-1 infection of epithelial cells has also been described in *in vitro* and
260 clinical studies in the absence of HSV-2 co-infection, indicating the potential involvement of
261 unknown host factors during HIV-1 infection [36, 37]. Although beyond the scope of the current
262 study, it will be interesting to explore the roles of other host proteins on HIV-1 virions in future
263 studies.
264
265 It is unclear why HVEM was not identified previously in HIV-1 virions. To identify virion-
266 associated host proteins, differences in methodology and cells used for virus production likely
267 result in different outcomes. It is known that, using traditional methods like immunoblotting,
268 only very limited number of host proteins were identified [21, 34]. Although the adoption of

269 proteomic analysis has substantially increased the number of proteins identified in HIV-1 virions,
270 so far there have been only a few studies with different results. There is no guarantee to identify
271 all host proteins in virions using the technique. Of note, membrane proteins, due to their
272 heterogeneous hydrophobic nature, are in general under-represented in proteomic studies [38].
273 For instance, CD48 is highly expressed on immunocytes including T cells, but its detection in
274 HIV-1 virions requires a second purification step by affinity chromatography in addition to
275 conventional LC-MS/MS [39]. Another example is that, tetraspanin enriched membranes (TEMs)
276 are components frequently reported in HIV-1 envelope but have not been reported to be present
277 in HIV-1 virions in the previous proteomic study [35].
278
279 Taken together, our study reveal that HIV-1 can obtain HSV-2 receptor HVEM from host cells
280 during budding and that HVEM interaction with HSV-2 glycoprotein D in the presence of the
281 other three viral glycoproteins gB/gH/gL on HSV-2-infected cell surface can mediate HIV-1
282 infection of non-target epithelial cells. Findings in this study provide a potential new mechanism
283 underlying HSV-2 enhanced HIV-1 acquisition and transmission from a different perspective
284 and may shed light on new treatment strategies against HIV-1/HSV-2 co-infection.
285

286 **METHODS**

287 **Cells, plasmids and viruses**

288 Human embryo kidney cell line HEK293T, African green monkey kidney cell line Vero and
289 human cervical epithelial cell lines HeLa and ME-180 were purchased from the American Type
290 Culture Collection. U87-CD4.CCR5 was obtained from the NIH AIDS Research and Reference
291 Reagent Program, Division of AIDS, NIH. Primary PBMCs were isolated from peripheral blood
292 of healthy donors using Ficoll-Hypaque density gradient (GE Healthcare) according to the
293 manufacturer's instructions and cultured in RPMI-1640 medium supplemented with 10 % fetal
294 bovine serum (FBS) and antibiotics. For HIV-1 propagation in PBMCs, cells were pre-treated
295 with 20 U/mL interluekin-2 (IL-2; R&D Systems) and 1 μg/mL phytohaemagglutinin (PHA;
296 Sigma-Aldrich) for 7 days before HIV-1 inoculation. Foreskin samples were obtained from
297 teenagers underwent circumcision from Jiangxi Provincial Maternal and Child Health Hospital.
298 Foreskin epithelial cells were isolated using Dispase II (ThermoScientific) digestion as
299 previously described and cultured in EpiLife medium (ThermoScientific) supplemented with
300 EpiLife™ Defined Growth Supplement (ThermoScientific) and antibiotics [40].

301

302 HIV-1 *env*-deficient luciferase reporter gene-containing backbone plasmid pNL4-3.Luc.R^-E^- and
303 HIV-1 Env BaL-expressing construct were obtained from the NIH AIDS Research and
304 Reference Reagent Program, Division of AIDS, NIH. pcDNA3.1(+) was purchased from Life
305 Technology, ThermoScientific. Human HVEM, CD4 and CCR5 were cloned from human cDNA
306 library. The open reading frames (ORFs) of HSV-2 glycoproteins gB, gD, gH and gL were
307 amplified from HSV-2 genome. All these genes were inserted into pcDNA3.1(+) vector.

308

309 HSV-2 (G strain; LGC standards) was propagated in HeLa cells and titrated in Vero cells as
310 previously described [41]. For the production of pseudoviruses HIV-1/HVEM and HIV-1/BaL,
311 HIV-1 backbone plasmid pNL4-3.Luc.R^-E^- with either HVEM or BaL expression construct were
312 co-transfected at a ratio of 3:2 into 293T cells using Lipofectamine 2000 (ThermoScientific)
313 according to the manufacturer's instructions. Forty-eight hours post transfection, culture
314 supernatants were collected and filtrated through 0.45 µm filters to remove cell debris. Filtered
315 viruses were mixed with FBS to reach a final concentration of 20% and aliquoted and stored at -
316 80 °C till use. Infectious clone to generate replication-competent virus HIV-1_{BaL} was obtained
317 from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIH and
318 viruses were produced by transfection of 293T cells using Lipofectamine 2000. To generate
319 PBMC-derived HIV-1_{BaL}, viruses produced from 293T cells were used to infect PBMCs. Three
320 to four days post infection, culture supernatants were filtered, mixed with FBS, aliquoted and
321 stored at -80 °C till use. All HIV-1 viruses were titrated by p24 ELISA.
322
323 **HVEM cell surface expression**
324 The expression of HVEM on $CD4^+$ PBMCs was analyzed by flow cytometry. Freshly isolated
325 PBMCs were stained with PE-conjugated anti-HVEM antibody (Biolegend) and FITC-
326 conjugated anti-CD4 (BD Biosciences) for 30 min at 4 °C. After washes, cells were evaluated on
327 a BD LSRFortessa platform.
328
329 **Virus concentration and purification**
330 Ultracentrifugation was used for concentration of HIV-1 particles as previously described with
331 modifications [42]. In brief, virus stocks were laid onto a 10 % iodixanol cushion and

332 centrifuged at 50,000 g for 1.5 h at 4 °C using a Beckman SW32 Ti swinging-bucket rotor
333 (Beckman Coulter). The pelleted viruses were suspended with 1 mL PBS, aliquoted and stored
334 at -80 °C till use.

335

336 Virus purification was performed using 6–18% Optiprep™ density gradient as previously
337 described with modifications [43]. In brief, concentrated viruses in PBS were layered on the top
338 of a 6–18% Optiprep™ density gradient and centrifuged at 200,000 g for 1.5 h at 4 °C using a
339 Beckman SW41 Ti swinging-bucket rotor (Beckman Coulter). After centrifugation, gradients
340 were collected in 1 mL fractions by upward displacement and the purified viruses were sharply
341 in the bottom of the third of the gradients.

342

343 **Western blot**

344 Ultracentrifugation concentrated or density gradient purified viruses were lysed with 1 % Triton
345 X-100 for 1 h in a 37 °C water bath and then mixed with SDS-PAGE loading buffer and boiled
346 for 10 min. Thereafter, samples were separated by 10 % SDS-PAGE and transferred onto a
347 PVDF membrane. Membrane was blocked with 5 % non-fat milk and incubated sequentially
348 with primary antibodies and HRP-conjugated goat anti-mouse IgG (Santa Cruz) overnight at 4
349 °C and for 1 h at room temperature, respectively. Following incubations, the membrane was
350 extensively washed and immunobands were visualized with ECL substrates (Millipore) under a
351 CCD camera (Fujifilm LAS4000). The following primary antibodies were used in the current
352 study for Western blot: mouse anti-human HVEM (R&D systems), mouse anti-human AChE
353 (Millipore) and mouse anti-HIV-1 p24 Gag monoclonal (NIH AIDS Reagent Program, Division
354 of AIDS, NIAID, NIH) [44].

355

356 **Preparation of anti-gB/gD murine antibody**

357 Murine anti-gB or -gD sera were prepared as previously described [42, 45]. In brief, 6-8 week
358 old BALB/c mice were immunized intramuscularly with 20 μg of plasmids expressing HSV-2
359 gB or gD into the quadriceps of both legs, followed by *in vivo* electroporation using the ECM830
360 Square Wave Electroporation System (BTX). Immunization was repeated for three times at 3-
361 week intervals. One week after the final immunization, mice were sacrificed and sera were
362 collected and purified with protein A/G (ThermoScientific). The purified anti-gB or -gD sera
363 were designated as mouse anti-gB and anti-gD antibodies, respectively.

364

365 **gB/gD cell surface expression**

366 The expression of gB and gD on HSV-2-infected cell surface was determined by cell-based
367 ELISA (CELISA) and flow cytometry as previously described [46]. In brief, HeLa cells were
368 infected with HSV-2 at an MOI of 0.1 or 1 for up to 28 h (CELISA) or 24 h (flow cytometry)
369 before the expression of gB and gD on cell surface was determined.

370 For CELISA, cells were fixed with 4 % paraformaldehyde for 15 min, rinsed with PBS and
371 incubated sequentially with mouse anti-gB or -gD antibodies and HRP-conjugated goat anti-
372 mouse IgG both for 1 h at room temperature. After extensive washes, TMB solution was added
373 for colorimetric reaction followed by the addition of stop solution (2N H_2SO_4). OD values were
374 read by an ELISA plate reader (Tecan) at a test wavelength of 450 nm and a reference
375 wavelength of 570 nm.

376 For flow cytometry, cells were collected and washed with PBS and incubated with mouse anti-
377 gB or -gD antibodies and FITC-conjugated goat anti-mouse IgG both for 30 min at 4 °C. After

378 washes, cells were suspended in 1 % paraformaldehyde fixation solution and evaluated on a BD
379 LSRFortessa platform.
380
381 **Cell survival assay**
382 HeLa cells were infected with HSV-2 at an MOI of 0.1 or 1 for up to 36 h, and cells were
383 subsequently collected and incubated with 7-amino-actinomycin D (7-AAD, Biolegend) for 10
384 min in the dark, followed by immediate evaluation by flow cytometry on a BD LSRFortessa
385 platform.
386
387 **Virus binding assay**
388 HeLa or ME-180 cells were infected with HSV-2 (1 MOI) for 4 or 16 h, or transfected with a
389 combination of HSV-2 gB, gD, gH and gL plasmids for 24 h, followed by an incubation with
390 200 ng HIV-1/HVEM for 1 h at 4 °C. Cells were then extensively washed with PBS to remove
391 unbound viruses. To measure cell bound viruses, washed cells were lysed with 1 % Triton X-100
392 and HIV-1 p24 was quantified by ELISA as previously described [47, 48].
393
394 **Virus entry assay**
395 HeLa or ME-180 cells were either infected with HSV-2 (1 MOI) for 4-6 h or transfected with a
396 combination of HSV-2 gB, gD, gH and gL plasmids for 24 h, and then infected with 200 ng of
397 replication-competent HIV-1_{BaL}/HVEM (for HSV-2 infection) or pseudotyped HIV-1/HVEM
398 (for HSV-2 glycoprotein transfection) for 2 h. For AZT (NIH AIDS Research and Reference
399 Reagent Program, Division of AIDS, NIH) treatment, AZT at the final concentration of 10 μM
400 was added 1 h before HIV-1 infection and remained throughout the assay [20]. Thereafter, cells

401 were washed with PBS to remove unbound virus and cultured in complete medium for another
402 24 h. Cells were trypsinized to remove cell surface associated viruses followed by washes and
403 lysis [49]. The treatment with trypsin was to ensure that only in-cell p24 was measured in the
404 following experiments. HIV-1 p24 (for HSV-2 infection) or luciferase activity (for HSV-2
405 glycoprotein transfection) was measured. HIV-1/BaL infection of CD4/CCR5 transfected cells
406 was used as control. For HSV-2 glycoprotein concentration assay, cells were transfected with
407 ascendant doses of gB, gD, gH and gL. For gD blocking assay, cells transfected with gB, gD, gH
408 and gL were treated with mouse anti-gD antibody or control IgG for 1 h at 37 °C before used for
409 HIV-1/HVEM infection.
410

411 **Infection of foreskin epithelial cells**

412 Foreskin epithelial cells were first infected with HSV-2 (1 MOI) for 4-6 h, followed by infection
413 with PBMC-propagated replication-competent HIV-1_{BaL} (200 ng) for 2 h. Cells were
414 subsequently washed with PBS to remove unbound virus and cultured in complete medium for
415 another 24-30 h. For antibody blocking, anti-gD or anti-HVEM antibodies were used to treat
416 epithelial cells or HIV-1_{BaL} for 1 h at 37 °C prior to co-infection. After infection, cells were
417 trypsinized to remove cell surface viruses, washed and stained with mouse anti-gD antibody
418 followed by FITC-conjugated goat anti-mouse IgG, both for 30 min at 4 °C. After washes, cells
419 were fixed, permeabilized and stained with PE-conjugated anti-HIV-1 p24 (Beckman Coulter)
420 for another 30 min at 4 °C. Cells were then washed and evaluated by flow cytometry on a BD
421 LSRFortessa platform.
422

423 **Statistical analysis**

424 All data were expressed as mean ± standard deviation (SD) and analyzed by GraphPad Prism
425 7.02. Mann-Whitney test was used for comparisons between two groups while Kruskal-Wallis
426 test was used for comparisons among three or more groups. A p value less than 0.05 was
427 considered statistically significant.

428 **Funding information**

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431

432 **Conflicts of interest**

433 The authors have declared that no conflict of interest exist.

434

435 **Ethical statement**

436 This study involved experiments using human peripheral blood and foreskin samples as well as
437 specific-antigen-free (SPF) BALB/c mice. All protocols involving human samples and animals
438 were reviewed and approved by the institutional ethical review board of Wuhan Institute of
439 Virology, Chinese Academy of Science, and performed in accordance with the Declaration of
440 Helsinki and the guidelines of Hubei Laboratory Animal Science Association. Informed written
441 consents were obtained from human subjects participated in this study.

442

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576 **Figure Legends**

577 Figure 1. Presence of HVEM on HIV-1 virions. (a) HVEM expression on freshly isolated
578 PBMCs. Freshly isolated PBMCs were stained with PE-conjugated anti-HVEM and FITC-
579 conjugated anti-CD4 antibodies. The expression of HVEM and CD4 on PBMCs were analyzed
580 by flow cytometry. (b) 293T, 293T-HVEM and PBMC-derived HIV-1 virus stocks, and mock-
581 treated cell supernatants were pelleted by ultracentrifugation and HVEM, AChE and HIV-1 p24
582 in the pellets were determined by Western blot. One representative experiment out of three is
583 shown. (c) Concentrated 293T, 293T-HVEM and PBMC-derived HIV-1 virus stocks were
584 purified by 6–18% Optiprep™ density gradient, and HVEM, AChE and HIV-1 p24 in the
585 purified viruses were determined by Western blot. One representative experiment out of three is
586 shown.

587

588 Figure 2. Binding of HIV-1/HVEM to HSV-2-infected cell surface through HVEM-gD
589 interaction. (a) HeLa or ME-180 cells were mock infected or infected with 1 MOI of HSV-2 for
590 4 or 16 h and then incubated with 200 ng HIV-1/HVEM or HIV-1/BaL for 1 h at 4 °C.
591 Following incubation, cell-bound HIV-1 p24 was quantified. Data shown are mean ± SD of three
592 independent experiments. (b) HeLa or ME-180 cells were transfected with pcDNA3.1 or
593 plasmids expressing HSV-2 gB/gD/gH/gL for 24 h and then incubated with 200 ng HIV-
594 1/HVEM for 1 h. Following incubation, cell-bound HIV-1 p24 was quantified. Data shown are
595 mean ± SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

596

597 Figure 3. Entry of HIV-1/HVEM into HSV-2-infected cells through HVEM-gB/gD/gH/gL-
598 dependent manner. (a) HeLa or ME-180 cells were infected with 1 MOI of HSV-2 for 4-6 h and

599 then incubated with 200 ng HIV-1_{BaL}/HVEM for another 24 h in the presence or absence of
600 AZT. Following incubation, cell surface viruses were removed by trypsin treatment and in-cell
601 p24 was quantified. Data shown are mean ± SD of three independent experiments. (b-c) HeLa or
602 ME-180 cells were transfected with plasmids expressing HSV-2 gB/gD/gH/gL or CD4/CCR5 for
603 24 h, and then untreated (b) or treated with anti-gD antibody or control Ig (c), followed by
604 infection with HIV-1/HVEM or HIV-1/BaL for another 24 h. In-cell luciferase activity was
605 measured. Data shown are mean ± SD of three independent experiments. ns, not statistically
606 significant; *, $p < 0.05$; **, $p < 0.01$.
607
608 Figure 4. PBMC-propagated HIV-1_{BaL} infection of HSV-2-infected primary foreskin epithelial
609 cells. Foreskin epithelial cells were infected with HSV-2 for 4-6 h and then incubated with HIV-
610 1 for another 24-30 h in the presence or absence of inhibitory antibodies. Following infection,
611 cell surface viruses were removed by trypsin treatment. gD and in-cell p24 were stained and
612 analyzed by flow cytometry. One representative experiment out of three is shown.
613

Figure 1

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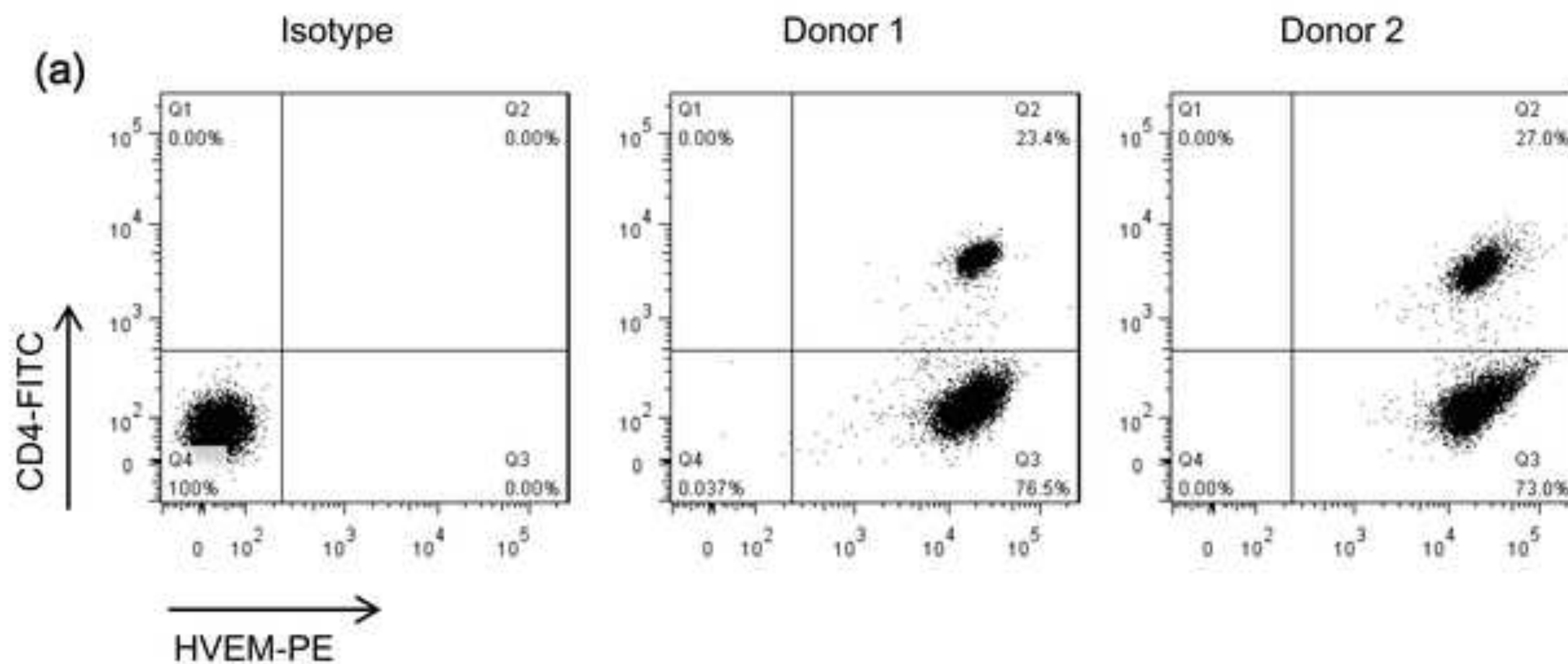

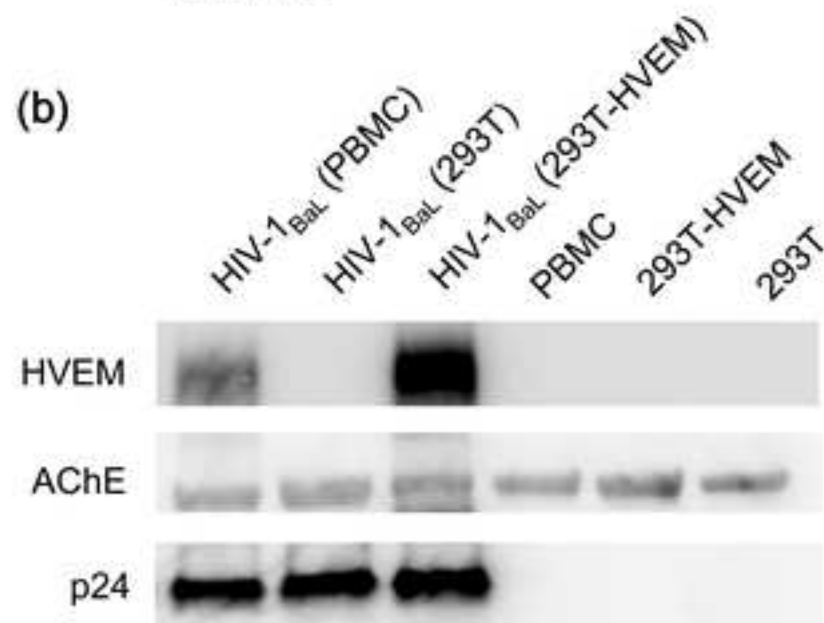

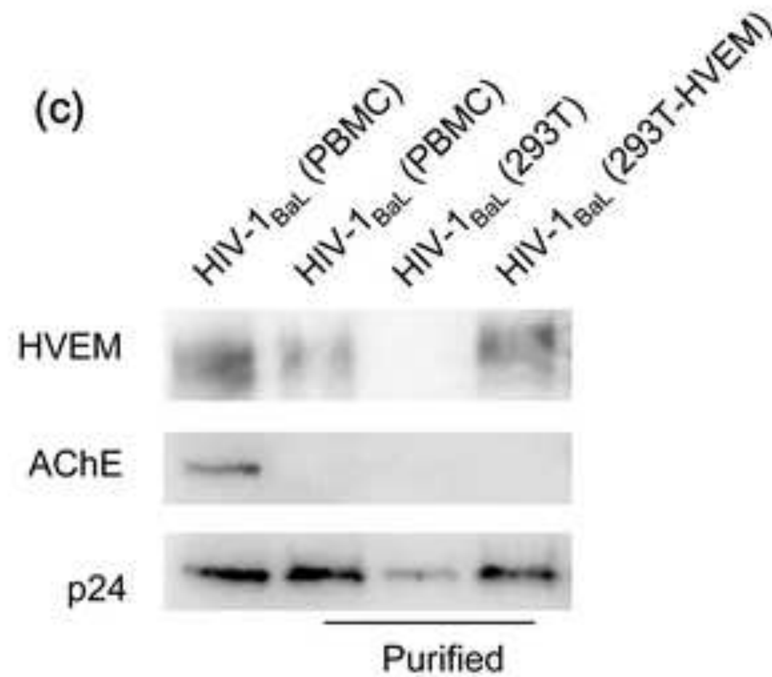

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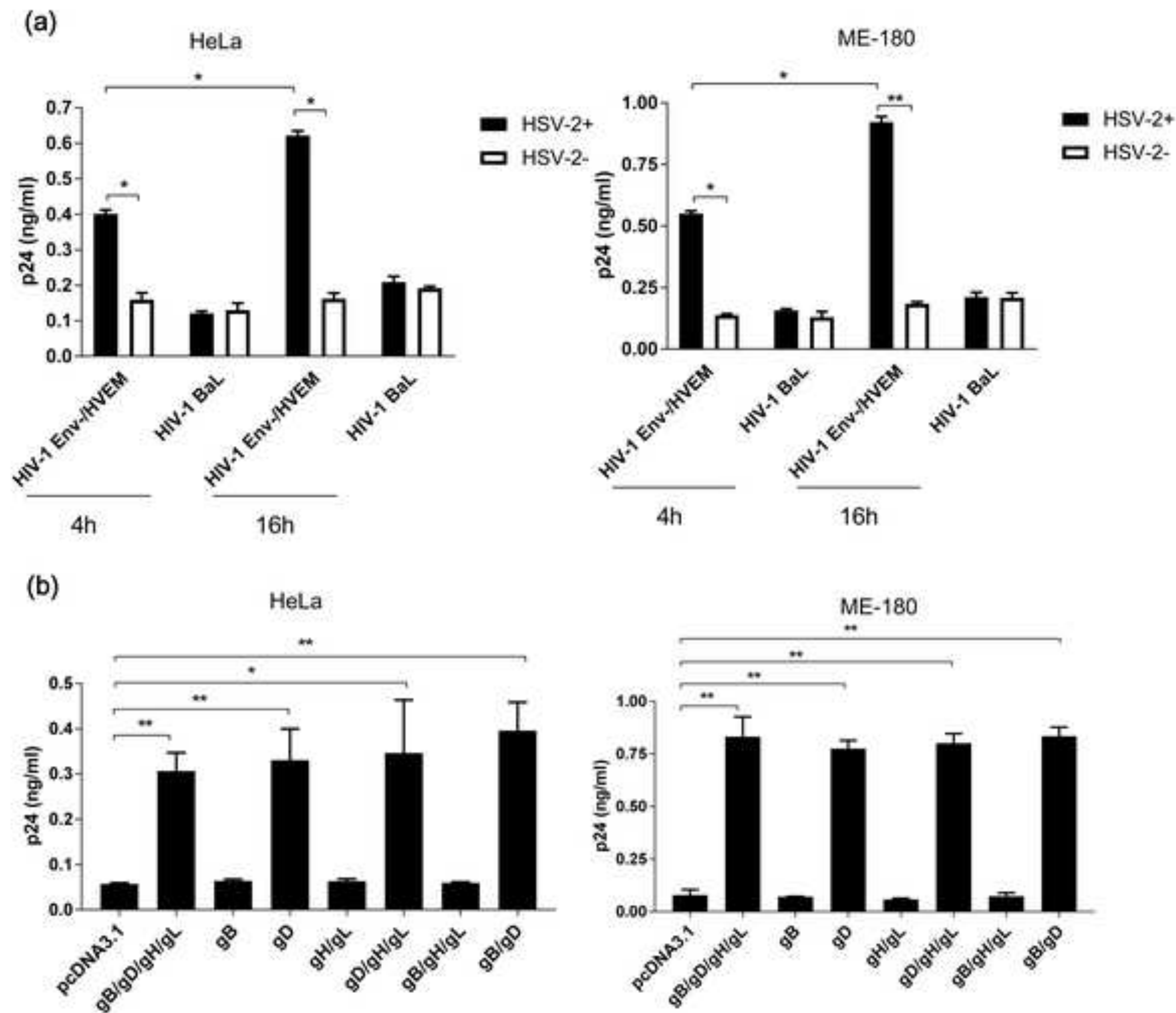

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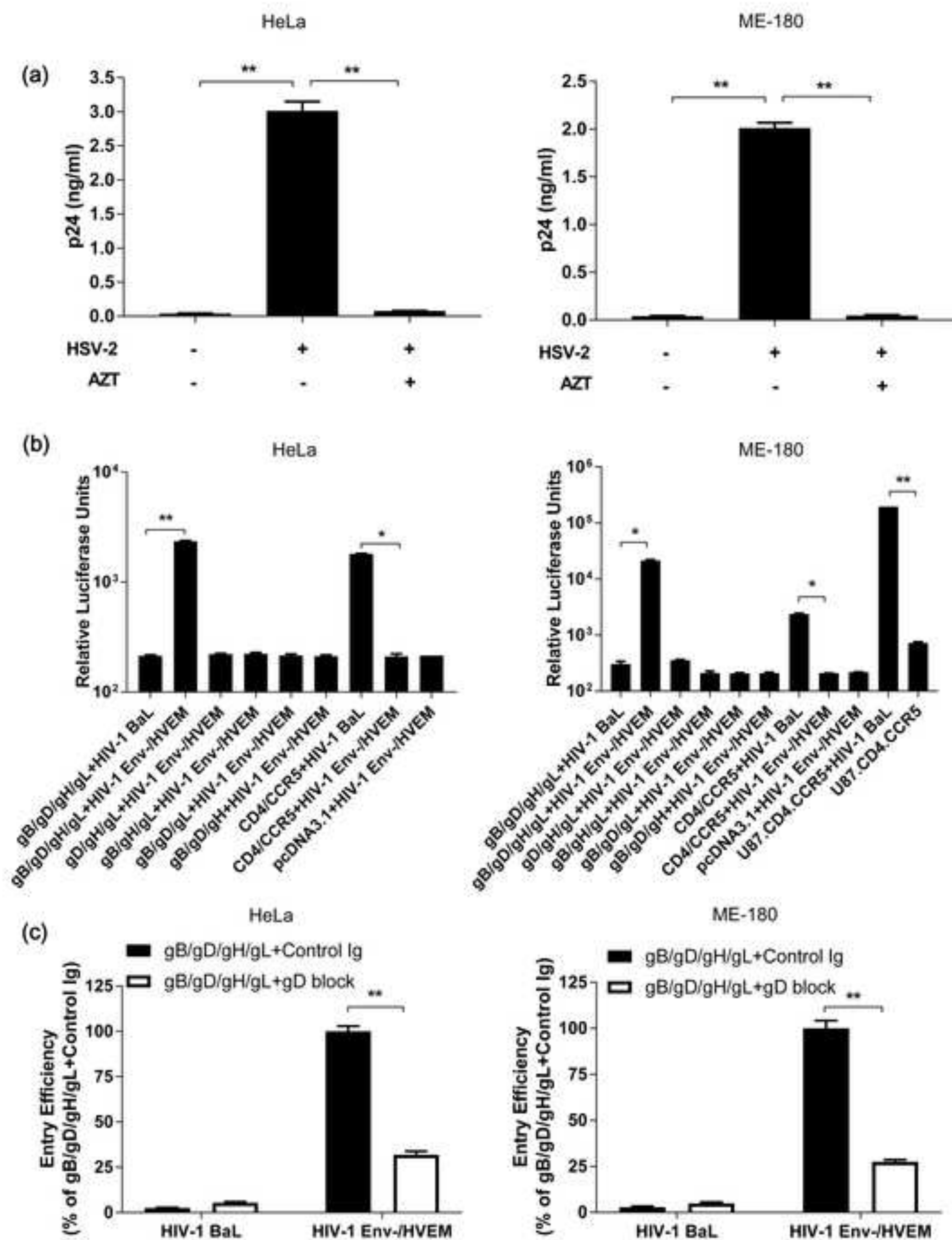

Figure 4

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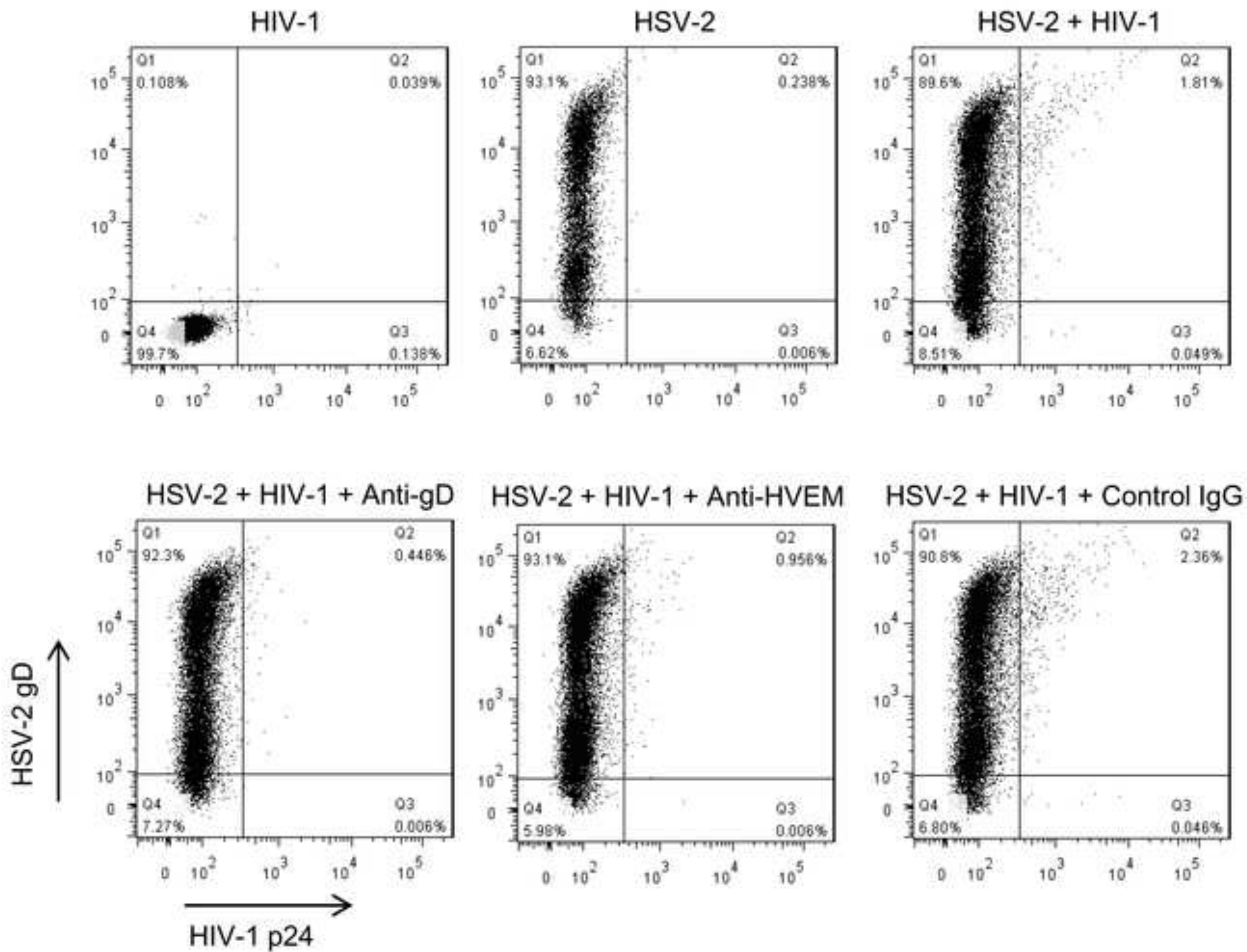

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Supplemental Materials

Figure S1: Expression of viral glycoproteins on HSV-2-infected cell surface. (a-b) HeLa cells were infected with 0.1 or 1 MOI of HSV-2 for an ascending time periods and then cell surface expression of gB (a) and gD (b) was measured by CELISA. Data shown are mean ± SD of three independent experiments. (c) HeLa cells were infected with 0.5 or 1 MOI of HSV-2 for 24 h and then cell surface expression of gB and gD was measured by flow cytometry. One representative experiment out of three is shown.

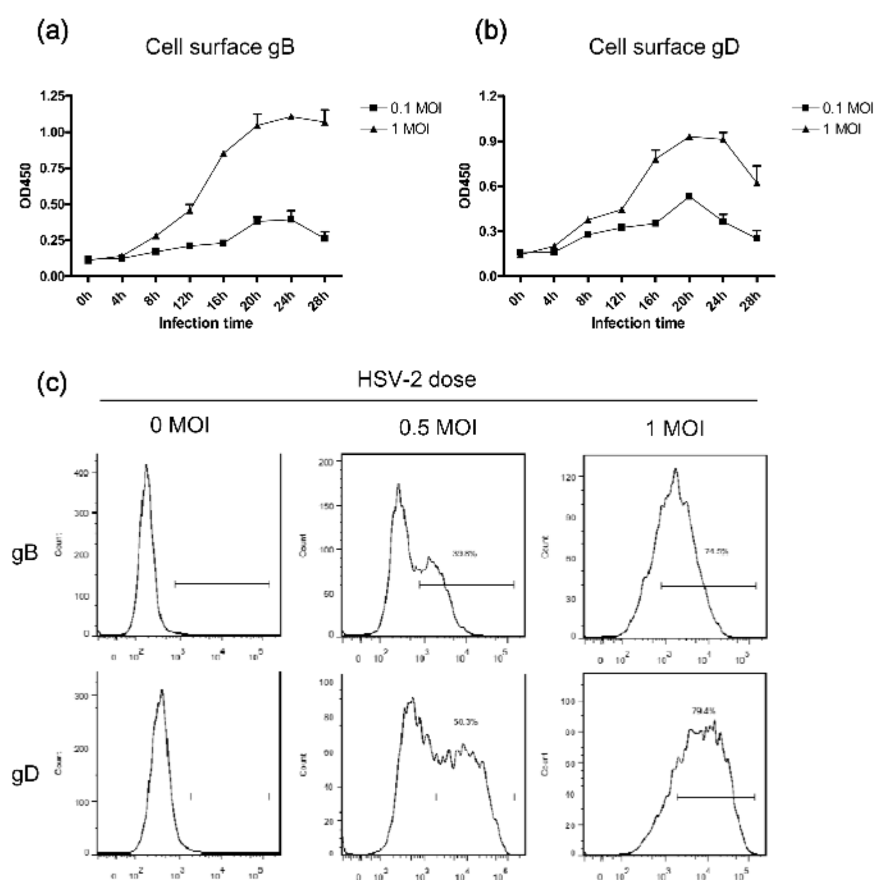

Figure S2: Impact of HSV-2 infection on cell viability. HeLa and ME-180 cells were mock-infected or infected with HSV-2 (MOI 0.1-1) for 30 h and then cell morphology was observed under microscope while cell viability was determined by 7-AAD staining. One representative experiment out of two is shown.

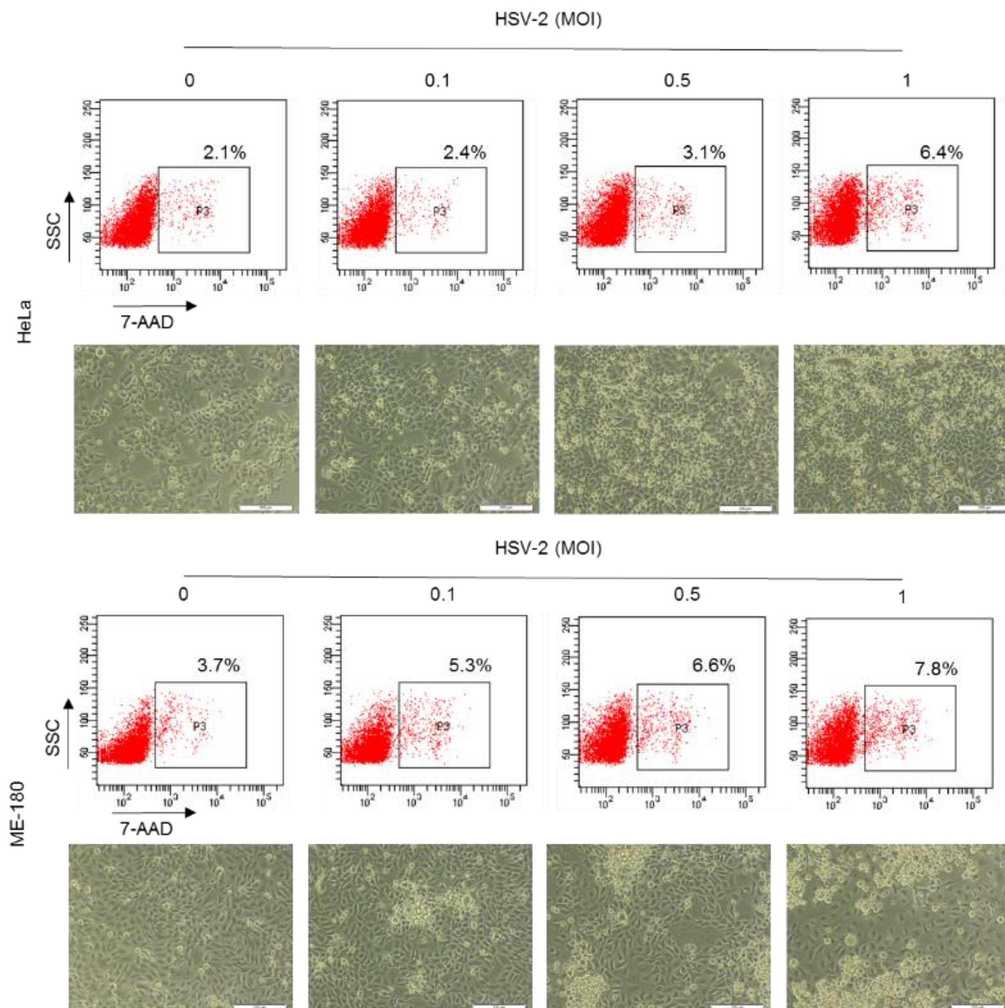

Figure S3: Viral glycoprotein expression on transiently transfected HeLa and ME-180 cells. HeLa and ME-180 cells were cotransfected with plasmids expressing HSV-2 gB, gD, gH and gL for 24 h and then cell surface gD was determined by flow cytometry. One representative experiment out of two is shown.