

# A potent and protective human neutralizing antibody targeting a key vulnerable site of Epstein-Barr Virus

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1 **A potent and protective human neutralizing antibody targeting a key**  
2 **vulnerable site of Epstein-Barr Virus**

3

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30

31 **ABSTRACT**

32 Epstein-Barr virus (EBV) is associated with a range of epithelial and B cell  
33 malignancies as well as autoimmune disorders, for which there are still no  
34 specific treatments or effective vaccines. Here, we isolated EBV gH/gL-specific  
35 antibodies from an EBV-infected individual. One antibody, 1D8, efficiently  
36 neutralized EBV infection of two major target cell types, B cells and epithelial  
37 cells. In humanized mice, 1D8 provided strong protection against a high-dose  
38 EBV challenge by substantially reducing viral loads and associated tumor  
39 burden. Crystal structure analysis revealed that 1D8 binds to a key vulnerable  
40 interface between the D-I/D-II domains of the viral gH/gL protein, especially the  
41 D-II of the gH, thereby interfering with the gH/gL-mediated membrane fusion  
42 and binding to target cells. Overall, we identified a potent neutralizing antibody  
43 as a promising candidate for prophylactic and therapeutic interventions against  
44 EBV infection. The key vulnerable site also provides insights into the EBV  
45 vaccines design.

46

47 **KEY WORDS:** EBV, human neutralizing antibody, humanized mouse model,  
48 epitope

49 **INTRODUCTION**

50 Epstein-Barr virus (EBV) is the causative agent of a wide range of diseases  
51 in humans such as infectious mononucleosis and lymphoproliferative disorders,  
52 as well as epithelial and B cell malignancies including nasopharyngeal  
53 carcinoma and Burkitt's lymphoma(1-4). Despite decades of research, a safe  
54 and effectively vaccine against EBV still remains elusive, largely due to a lack  
55 of knowledge regarding the specificity and magnitude of immune responses  
56 required for protection(5-8). EBV-infected individuals produce broad and  
57 potent neutralizing antibodies that can inhibit infection of both epithelial cells  
58 and B cells *in vitro*(9-13). However, their specificity to viral antigens and  
59 potential mechanism of neutralization are not clear.

60 Recent studies on monoclonal antibodies (mAbs) revealed some of the  
61 intricate interactions between antibodies and viral surface antigens, providing  
62 critical insights into the potential targets for antibody neutralization and vaccine  
63 development(14-18). The reported mAbs recognize exclusively viral surface  
64 glycoproteins that work in concert in determining viral tropism and mediating  
65 viral fusion with the target cells, such as gp350, gH/gL, gB and gp42(19-22).  
66 Recently, gH/gL and gB, which together constitute the fusion machinery of EBV,  
67 have drawn increasing attention as newer generations of antibodies targeting  
68 this machinery demonstrate broad and potent inhibitory activity against EBV  
69 infection of both B cells and epithelial cells(23), as well as cross-neutralizing  
70 reactivity to related herpesviruses of non-human primate(9, 24, 25).

71 As components of the fusion machinery, gH/gL and gB demonstrate unique  
72 structural and functional features that are critical for viral entry, but they also  
73 inadvertently expose some vulnerable sites during the process, and became  
74 susceptible to antibody binding and neutralization(26-28). Structurally, gH/gL  
75 consists of four distinct domains named domain-I (D-I) to domain-IV (D-IV),  
76 forming an elongated structure(29). D-I is formed by gL and the N-terminus of  
77 gH, while D-II to D-IV are formed by the rest of gH. D-I and D-II are connected  
78 through a linker helix and form a structurally distinct groove. For viral fusion to  
79 occur, gH/gL must interact with gB, which triggers a cascade of events  
80 involving dramatic structural changes of gB from the pre- to the post-fusion  
81 conformation(27, 28). Mutations in the D-I and D-I/D-II interfaces of gH/gL  
82 were shown to affect the membrane fusion process, suggesting that these  
83 regions of gH/gL are important for the interaction and activation of gB(30, 31).

84 Apart from the fusion machinery, EBV infection requires additional surface  
85 glycoproteins to complete the entry process, but the involved accessory  
86 molecules are rather different between B cells and epithelial cells(32). For  
87 instance, EBV utilizes gp350, one of the most abundant glycoproteins on the  
88 viral envelope, to attach to the cell surface through high-affinity interaction with  
89 CD21 or CD35(33-35). Such attachment promotes the bridging effect of  
90 another surface glycoprotein, gp42, which inserts itself between gH/gL and  
91 human leukocyte antigen (HLA) class II, which triggers the downstream fusion  
92 machinery(36, 37). Interestingly, gp42 has an inhibitory effect on epithelial cell

93 infection, suggesting a different entry mechanism in B cells and epithelial  
94 cells(38). For infection of epithelial cells, gH/gL first binds to integrin and  
95 NMHC-IIA on the cell surface. The fusion machinery then interacts with  
96 neuropilin 1 (NRP1) and ephrin receptor A2 (EphA2)(39-41), which leads to a  
97 conformational transition of gB, facilitating viral fusion(27, 42).

98 Most of the current anti-gH/gL antibodies are of murine origin(36, 43). E1D1,  
99 CL59 and CL40 can block epithelial cell infection but fail to efficiently neutralize  
100 B cell infection(22). Only one human neutralizing antibody targeting gH/gL,  
101 AMMO1, was recently isolated from an EBV-infected individual(22). AMMO1  
102 can potently block infection of both B cells and epithelial cells *in vitro*. AMMO1  
103 can also protect humanized mice from EBV challenge and provide sterilizing  
104 immunity in macaques against oral challenge with rhesus lymphocryptovirus,  
105 the EBV relative that infects rhesus macaques. These findings indicate that a  
106 vaccine capable of inducing AMMO1-like neutralizing antibodies may protect  
107 human from EBV infection. Cryo-electron microscopy (cryoEM) analysis of the  
108 AMMO1-gH/gL-gp42 complex revealed that AMMO1 binds to an epitope  
109 between D-I and D-II of gH/gL(22), which serves as a more precise and clear  
110 target for future vaccine design and development.

111 Here, we sought to isolate more neutralizing antibodies from EBV-infected  
112 individuals targeting the EBV gH/gL. After screening a large number of infected  
113 individuals, we successfully isolated the anti-gH/gL antibody 1D8, which is  
114 capable of efficiently neutralizing EBV infection of epithelial cells and B cells *in*

115 *vitro*. 1D8 also provided potent protection against EBV challenge in humanized  
116 mice by significantly reducing the viral loads and associated tumor burden.  
117 Using X-ray crystallography, we determined the structure of the 1D8-gH/gL  
118 complex and showed that 1D8 recognizes a key epitope located at the top of  
119 the groove between D-I and D-II of gH/gL, especially the D-II of gH. Notably,  
120 this epitope is located on the opposite side of that recognized by AMMO1 and  
121 CL40. In addition, 1D8 also significantly inhibited viral membrane fusion and  
122 gH/gL binding to epithelial cell receptor EphA2. We believe that this new  
123 vulnerable site, together with that recognized by AMMO1 and CL40, suggests  
124 that D-I and D-II represent an attractive target for the rational design of  
125 vaccines aiming to elicit antibody responses similar to these mAbs. Finally,  
126 1D8 could also be used alone or in combination with other mAbs in  
127 prophylactic and therapeutic interventions against EBV infection either in  
128 organ transplant recipients or immunocompromised patients.

129

## 130 **RESULTS**

### 131 **Isolation of human monoclonal antibodies targeting the EBV gH/gL**

132 We first screened plasma samples from a cohort of high-risk individuals and  
133 nasopharyngeal carcinoma patients(44, 45) for those with the highest levels of  
134 binding and neutralizing activity. Of the 48 plasma samples screened, donor 27  
135 from the high-risk group had antibodies with the highest affinity for gH/gL  
136 measured by ELISA, with the half-maximal effective concentration (EC50)



137 corresponding to a 6874-fold dilution (fig. S1A). The same plasma sample also  
138 displayed the most potent neutralizing activity against EBV infection of HNE1  
139 epithelial cells and Raji B cells, with respective half-maximal inhibitory  
140 concentrations (IC<sub>50</sub>) corresponding to a 273-fold and 1250-fold dilution (fig.  
141 S1B). To isolate monoclonal antibodies, we used phycoerythrin (PE)  
142 conjugated gH/gL as baits to stain and sort the antigen-specific memory B cells  
143 from the peripheral blood mononuclear cells (PBMCs) of donor 27 using flow  
144 cytometry (Fig. 1A). Out of a total 54 sorted single B cells, we were able to  
145 clone and express 10 full-length human immunoglobulin G1 (IgG1) genes in  
146 transfected 293T cells. Two antibodies, 1D8 and 2A6, were found to have  
147 strong binding to gH/gL. As shown in Fig. 1B, 1D8 showed ~7-fold stronger  
148 binding to gH/gL than 2A6, with an EC<sub>50</sub> of 0.008µg/ml and 0.057µg/ml,  
149 respectively. 1D8 also demonstrated higher neutralizing activity than 2A6  
150 against EBV infection of HNE1 epithelial cells and Raji B cells (Fig. 1C,D and  
151 table S1). The IC<sub>50</sub> of 1D8 was about 6 times lower than that of 2A6 for both  
152 cell types. Notably, 1D8 displayed comparable binding and neutralizing  
153 activities to AMMO1, a potent gH/gL-specific neutralizing antibody previously  
154 isolated from an EBV-infected individual(22). The equilibrium dissociation  
155 constant (K<sub>D</sub>) measured by SPR was 0.59nM for 1D8 and 0.14nM for AMMO1  
156 (fig. S1C,D and table S2). When tested for neutralizing activity against EBV  
157 infection, 1D8 and AMMO1 showed IC<sub>50</sub> values of 0.238 and 0.318µg/ml in  
158 Raji B cells, as well as 0.123 and 0.127µg/ml in HNE1 epithelial cells,

159 respectively (Fig. 1C,D and table S1).

160

### 161 **1D8 protects against lethal EBV challenge in humanized mice**

162 To test the protective potential of 1D8 *in vivo*, we used a humanized mouse  
163 model reconstituted with human cord blood-derived CD34+ stem cells that  
164 became susceptible to EBV infection and disease after approximately 8 weeks  
165 of development and maturation(46-48). The entire experimental protocol and  
166 assays conducted to evaluate protection are outlined in Fig. 2A. Briefly, we  
167 administrated 400µg of 1D8, AMMO1 as positive control, or 2G4 and PBS as  
168 negative controls to groups of seven to eight humanized mice via the  
169 intraperitoneal (i.p.) route. On the following day, the animals were challenged  
170 with 1,000 50% transforming dose (TD<sub>50</sub>) Akata EBV via the intravenous (i.v.)  
171 route. In the ensuing up to 6-week period, all animals received the testing  
172 antibodies or PBS weekly via the i.p. route and were monitored for body weight,  
173 survival, as well as various virological and immunological parameters.

174 EBV DNA in the peripheral blood measured by quantitative PCR reflected  
175 the distinctions in clinical manifestation between these animals (Fig. 2B). In the  
176 animals treated with 1D8 and AMMO1, EBV DNA became detectable on week  
177 3 after challenge and slowly increased in the following two weeks, but no  
178 animals had EBV DNA copy numbers greater than 10 copies/µl blood at week  
179 5 post challenge. By contrast, in the animals treated with 2G4 and PBS, EBV  
180 DNA rapidly increased from week 3 onwards after the challenge and reached

181 about 100-fold higher copy numbers than in the groups treated with 1D8 and  
182 AMMO1 at week 5 post-challenge (Fig. 2B). All animals in the 1D8 and  
183 AMMO1 treated groups survived the challenge and demonstrated relatively  
184 stable body weight without obvious pathology (Fig. 2C,D). By contrast,  
185 negative control animals (2G4 and PBS groups) began significantly losing  
186 weight starting on day 28 (4 weeks), succumbed to disease and had to be  
187 euthanized by day 38 after the challenge.

188 By the time they were ready for the protection experiments, the reconstituted  
189 animals had about 20% human CD45+ lymphocytes in the peripheral blood,  
190 nearly 90% of which were human CD20+ B cells and <1% were human CD3+  
191 T cells (Fig. 2E-G). In addition, the dynamic change of human CD20+ B cells  
192 and CD3+ T cells in the peripheral blood was correlated with distinct pattern of  
193 disease progression between these animals. Animals in the 1D8 and AMMO1  
194 treated groups showed a relatively slower decrease in the number of CD20+ B  
195 cells compared with the 2G4 and PBS treated groups. At the same time, the  
196 increase in the number of CD3+ T cells was slower in animals treated with 1D8  
197 and AMMO1 compared to those treated by 2G4 and PBS (Fig. 2F,G).

198 Furthermore, EBV DNA copy numbers measured in the spleen, liver and  
199 kidney collected at necropsy shared a similar trend with those measured in the  
200 peripheral blood (Fig. 2H,J). The copy numbers of EBV DNA were significantly  
201 lower in the 1D8 and AMMO1 treated groups than in the 2G4 and PBS treated  
202 groups, although the copy numbers were generally higher in the spleen than in

203 the liver and kidney (Fig. 2H,J). Taken together, these results demonstrated  
204 that 1D8 as well as the positive control AMMO1 can significantly reduce viral  
205 replication and provide complete protection from a lethal EBV challenge.

206

207 **Marked reduction in viral replication and tissue damages in the protected**  
208 **animals**

209 To study the impact of protection at the tissue levels, we collected the spleen,  
210 liver and kidney of the animals at the necropsy. The most profound and visible  
211 changes were observed in the spleens. Morphologically, the spleens from the  
212 2G4 and PBS groups were clearly enlarged with a few irregular and pale  
213 tumors across the entire surface. By contrast, the spleens in the 1D8 and  
214 AMMO1 groups were normal in size and color, without visible tumors (Fig. 3).

215 We went on to perform histopathology analysis on the spleen sections using  
216 hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) for hCD3  
217 and hCD20, as well as in situ hybridization for Epstein-Barr virus-encoded  
218 RNAs (EBERs) (Fig. 3). All mice treated with 2G4 or PBS presented with  
219 typically large B-cell lymphomas in the white pulp regions, which consisted of  
220 large and atypical lymphoid cells that were positive for hCD20 and EBER.  
221 They were abundant and widely distributed across the tissue sections.  
222 Morphologically their proliferations destroyed the underlying architecture of the  
223 tissue with some infiltration by hCD3+ T cells. Additionally, areas of  
224 coagulative necrosis were often present in the spleens of mice from the 2G4

225 and PBS groups. By contrast, in the 1D8 and AMMO1 groups, the overall  
226 tissue architecture remained largely intact, even if some atypical large  
227 transformed cells could also be seen. Among the large number of hCD20+ B  
228 cells in the white pulp areas, EBER+ cells were relatively scarce. A few hCD3+  
229 T cells were also found scattered within. Similarly, in the hepatic and renal  
230 sections from 2G4 and PBS groups, a large number of hCD20+ and EBER+ B  
231 cells were identified, while they were rare in the 1D8 and AMMO1 groups ([fig.](#)  
232 [S2](#)). The infected cells were frequently found near the blood vessels in both  
233 the liver and kidney, likely the results of migration and seeding from the blood  
234 circulation. Collectively, these results show that 1D8 and AMMO1 can  
235 significantly reduce viral replication and tissue damage relative to 2G4 and  
236 PBS, offering an explanation for their impressive *in vivo* protection against a  
237 lethal EBV challenge.

238

### 239 **1D8 binds to a key epitope on gH/gL**

240 To understand the neutralizing mechanism of 1D8, we sought to determine the  
241 structure of the 1D8-gH/gL complex by X-ray crystallography. After screening  
242 nearly 100 crystals with relatively weak diffraction, we obtained a dataset with  
243 4.2 Å resolution and solved the structure by molecular replacement ([table S3](#)).  
244 The structure showed that 1D8 bound to the interface at the top of the groove  
245 formed by D-I and D-II of gH/gL, especially the D-II of gH ([Fig. 4A](#)). 1D8 bound  
246 to the unique epitope spanning of gH/gL, which comprised residues of both gH

247 and gL, burying a surface area of 969 Å<sup>2</sup>. CDRL1, CDRL3 and CDRH2 of 1D8  
248 made practically no contribution to the binding of gH/gL. CDRL2 mainly bound  
249 to 2α-2 and 2β-2. The loop between 2α-9 and 2β-11 was bound by CDRH1  
250 and CDRH3. CDRH3 also bound to the loop between 2α-1 and 2β-1. The  
251 heavy chain of 1D8 bound to the loop between Lα-1 and Lα-2 (Fig. 4B). To  
252 identify the key residue for antibody binding, we generated a series of single  
253 alanine substitutions for the contacting residues on gH/gL. Except for two  
254 (L122A and C312A), the remaining 15 mutants were successfully expressed  
255 and purified from the supernatant of transfected 293F cells. We then  
256 performed ELISA to assess the impact of these mutants on 1D8 binding. One  
257 mutant, N310A, locate in the loop between 2α-9 and 2β-11, was identified to  
258 specifically reduce the binding of 1D8 but not AMMO1 (Fig. 4B and fig. S3A,B).  
259 When measured by SPR, the binding affinity of 1D8 for this particular mutant  
260 dropped to 31.6nM, representing more than 53-fold decrease compared to the  
261 wild type gH/gL. However, no significant change of binding was found for  
262 AMMO1 compared to the wild type gH/gL (fig. S3C,D and table S2). The  
263 unique binding of 1D8 is further supported by superimposing the antibodies  
264 with known structural information onto the same gH/gL molecule. As shown in  
265 Fig. 4C,D, 1D8 bound to gH/gL at the top of the groove formed between D-I  
266 and D-II, especially the D-II of gH, while AMMO1 binds the opposite side of the  
267 molecule through a discontinuous epitope formed at the D-I/D-II interface. The  
268 mouse-derived antibody CL40 partially overlaps with the epitope of AMMO1 by

269 binding to an epitope on gH at the interface between D-II and D-III(22).  
270 Another mouse antibody E1D1, however, only recognizes gL(36, 43) (Fig.  
271 4C,D). We also used bio-layer interferometry (BLI) to confirm that 1D8 does  
272 not compete with any of these antibodies in binding to gH/gL (fig. S3E). These  
273 results indicate that 1D8 recognizes a key vulnerable site on gH/gL and  
274 provide a good rational basis for combined use with other antibodies in  
275 suppressing EBV infection.

276

### 277 **1D8 inhibits gH/gL-mediated membrane fusion and binding to B and** 278 **epithelial cells**

279 We next studied the ability of 1D8 to inhibit gH/gL mediated membrane fusion  
280 by monitoring the fusion efficiency between the effector and target cells.  
281 Specifically, effector CHO-K1 cells expressing the gH/gL and gB fusion  
282 machinery were incubated with a saturated concentration of 1D8 or relevant  
283 controls at 37°C for 1h before mixing at a 1:1 ratio with the target HEK293  
284 cells. The inhibitory activity was measured 24h afterwards via the luciferase  
285 activity in the cell lysates, which only became detectable when fusion occurred.  
286 As shown in Fig. 5A-C, the effector CHO-K1 cells expressed good levels of  
287 gH/gL as measured by flow cytometry. In the presence of 1D8 or AMMO1, the  
288 fusion activity was barely measurable and similar to the background where  
289 only the effector cells were present (Fig. 5D). By contrast, incubation with the  
290 negative controls 2G4 or PBS resulted in high levels of luciferase activity

291 beyond one million relative light unit (RLU). In addition, we tested the ability of  
292 1D8 to interfere with the binding of fluorescently labelled gH/gL to Raji B cells  
293 and HK1 epithelial cells, both of which are susceptible to EBV infection *in*  
294 *vitro*(49, 50). Gp42 was included in the assessment of the binding to B cells  
295 but not for the epithelial cells, since the gH/gL-gp42 complex is specifically  
296 required for B cell activation and fusion(36). 1D8 and relevant controls were  
297 incubated with fluorescent-labeled gH/gL at 37°C for 1h before mixing with B  
298 cells or epithelia cells and further incubation on ice for 1h. The levels of  
299 inhibition of gH/gL-mediated binding to both cell types were measured by flow  
300 cytometry. As shown in Fig. 5E, pre-incubation with 1D8 and AMMO1  
301 significantly reduced but did not completely abrogate gH/gL-gp42 binding to B  
302 cells. While no difference was found between the negative controls 2G4 and  
303 PBS, AMMO1 appeared to be more potent than 1D8 in interfering with the  
304 binding of gH/gL to B cells. Conversely, 1D8 seems to be more powerful than  
305 AMMO1 in inhibiting the binding of gH/gL to the epithelia cells, whereas the  
306 negative controls showed negligible effect (Fig. 5F). Lastly, we studied the  
307 ability of 1D8 to inhibit the interaction between gH/gL and EphA2, a recently  
308 identified receptor for EBV infection of epithelial cells that depends on an  
309 interaction with gH/gL(39, 40). Consistent with an earlier report(22), the  
310 interaction between EphA2-Fc and gH/gL was indeed rather weak as  
311 measured by BLI. Nevertheless, pre-incubation of gH/gL with 1D8 did result in  
312 a small and clear reduction in the interaction between gH/gL and EphA2 (Fig.



313 5G). Conversely, such an effect was not noticed for AMMO1 or 2G4 (Fig. 5H,I).  
314 This may explain why 1D8 was more effective than AMMO1 in inhibiting EBV  
315 infection of epithelial cells (see above), although the underlying mechanism  
316 warrants further investigation. Taken together, these findings indicate that 1D8  
317 as well as the positive control AMMO1 can significantly inhibit gH/gL-mediated  
318 membrane fusion and binding to B and epithelial cells, either through direct  
319 blocking of binding or by sterically interfering with the downstream interactions  
320 required for EBV infection.

321

## 322 **DISCUSSION**

323 Neutralizing antibodies are the major component of protective immunity  
324 against viral infection in humans(51-53). They exert their function by targeting  
325 crucial epitopes on the viral envelope glycoproteins. Identifying the neutralizing  
326 mAbs and their recognized epitopes is therefore the first crucial step for  
327 understanding the protective antibody response, which can inform the rational  
328 development of antibody-based therapy and vaccines(17, 18). In some  
329 EBV-infected individuals, high levels of serum neutralizing antibodies have  
330 been identified capable of blocking infection of both B cells and epithelia  
331 cells(9). This finding indicates that the human immune system is able to  
332 generate potent neutralizing antibodies to clear the infection and/or attenuate  
333 disease progression. However, the antigen and epitope specificity, as well as  
334 the potential mechanism of neutralization of these antibodies are not entirely

335 clear.

336 We report here the isolation and characterization of the human neutralizing  
337 antibody 1D8, which protects EBV infection in both B cells and epithelial cells.  
338 Passive delivery of 1D8 significantly reduced the viral loads and tumor burden  
339 of EBV-induced lymphoma in humanized mice. Structural analysis of the  
340 1D8-gH/gL complex identified a key epitope at the top groove of gH/gL  
341 between D-I and D-II, especially the D-II of gH, which is distinct from any of the  
342 reported antibodies. In addition, 1D8 was found to inhibit viral membrane  
343 fusion and reduce the binding of gH/gL to the epithelial cell receptor EphA2(39,  
344 40). We believe that the new vulnerable site recognized by 1D8 represents  
345 another attractive target for the rational design of vaccines capable of eliciting  
346 1D8-like neutralizing antibodies. 1D8 could also serve as a promising  
347 candidate for antibody-based therapy and prevention of EBV infection.

348 A couple of points need to be highlighted here. First, as both B cells and  
349 epithelia cells are major targets for EBV infection(54), it is highly desirable to  
350 isolate neutralizing antibodies capable of blocking the virus and protecting both  
351 cell types from infection. 1D8, together with recently reported AMMO1(22), are  
352 the only two representatives of this class of antibodies with dual tropism.  
353 However, we are uncertain how much this type of antibodies contributes to the  
354 overall neutralizing activities in the infected individuals. Given the low  
355 frequency in identifying 1D8 and AMMO1 antibodies among the isolated  
356 memory B cells(22), it is reasonable to assume they are quite rare and might

357 only be induced among a small proportion of naturally infected patients.  
358 Perhaps, this is due to the elusive nature of their epitopes, which are only  
359 transiently exposed during viral entry. It could also be due to their highly  
360 dynamic nature involving multiple conformational changes during infection.  
361 The rapid movement across the D-I and D-II groove required for binding and  
362 triggering of gB glycoprotein supports this notion(30, 31, 55, 56). Furthermore,  
363 compared to gp350, gH/gL is much less abundant and therefore has a  
364 quantitative disadvantage in immune recognition and stimulation(19). However,  
365 identification of 1D8 and AMMO1 and their epitopes around D-I and D-II offer  
366 an unprecedented opportunity to expose this vulnerable site in much more  
367 precise and persistent manner so that more focused and stronger immune  
368 response like 1D8 and AMMO1 could be generated. This could be done either  
369 by including gH/gL in the vaccine regimen(9, 23) or singling out D-I and D-II  
370 domain as epitope-focused immunogens. Both approaches would require  
371 careful design and validation to ensure proper structure and exposure of  
372 vulnerable sites recognized by 1D8 and AMMO1. In support of this notion,  
373 nanoparticles displaying gH/gL elicited a strong neutralizing antibody response  
374 against EBV infection of both target cell types(9), even if this exciting report  
375 requires further confirmation. Lastly, given the relatively conserved nature of  
376 this region among herpesviruses(24, 27), carefully designed D-I and D-II  
377 immunogens may be able to induce an even broader and stronger  
378 cross-neutralizing antibody response against a wide variety of viral strains.

379 Second, despite structural and functional insights, we are still not certain of  
380 the exact mechanism through which 1D8 neutralizes EBV infection of both  
381 target cell types. Structurally, 1D8 recognized a key epitope within the groove  
382 between D-I and D-II, especially the D-II of gH, whereas AMMO1 was found to  
383 bind a discontinuous epitope spanning D-I and D-II on the opposite ridge of the  
384 groove(22). Such convergence on D-I and D-II domains suggests a common  
385 mechanism of neutralization, either by affecting coordination within and across  
386 D-I and D-II or their interaction with other viral glycoproteins such as gB or  
387 gp42 required for downstream viral entry (Fig. 6A,B). AMMO1 was postulated  
388 to lock D-I, D-II and the linker helix, preventing proper movement required for  
389 interaction and activation of gB(56). As residues with the D-I and D-II groove  
390 also mediate membrane fusion and several of these critical residues are near  
391 the epitope of 1D8(30, 31, 55, 56), it stands to reason that 1D8 could also exert  
392 its neutralization activity by inhibiting the fusion process. Instead of acting like  
393 a molecular clamp as AMMO1, 1D8 may act more like a molecular wedge  
394 forcing into the space within the groove. Certainly, as 1D8 and AMMO1 bind  
395 distinct epitopes, there must be some differences in the exact mechanisms  
396 underlying their inhibitory effects. For example, AMMO1 appears to be more  
397 potent than 1D8 in interfering with the binding of gH/gL to B cells (Fig. 5E),  
398 perhaps due to its ability to displace the c-terminal domain of gp42 through the  
399 gp42 N173 glycan(22). Conversely, 1D8 seems to be more powerful than  
400 AMMO1 in inhibiting binding of gH/gL to epithelia cells, likely by affecting the

401 interaction between EphA2-Fc and gH/gL (Fig. 5F,G and 6A). In any case, the  
402 1D8 antibody identified in this study represents another potent human  
403 neutralizing antibody that can be used alone or in combination with other  
404 antibodies such as AMMO1 for antibody-based interventions against EBV  
405 infection. The epitope defined here will also assist the rational design of  
406 vaccines focusing more on the vulnerable sites to elicit powerful neutralizing  
407 antibodies similar to 1D8 and AMMO1.

408

## 409 **MATERIALS AND METHODS**

### 410 **Human subjects**

411 We collected plasma samples from 48 participants including 23 histologically  
412 diagnosed NPC cases and 25 non-NPC high-risk healthy controls in a  
413 screening program in Sihui County in Guangdong Province of China from 2007  
414 and 2018. Peripheral blood mononuclear cell (PBMC) sample of donors 27  
415 were collected in 2018. The screening program has been introduced in detail  
416 in other manuscript. This study was reviewed and approved by the Ethics  
417 Committee of the Sun Yat-Sen University Cancer Center (SYSUCC;  
418 Guangzhou, Guangdong, China) and was conducted in accordance with the  
419 Declaration of Helsinki.

420

### 421 **Cell lines**

422 All cell lines were cultured at 37°C in a humidified atmosphere comprising 5%

423 CO<sub>2</sub>. 293T cells (ATCC) were grown in DMEM (GIBCO) +10% FBS (GIBCO).  
424 CHO K-1 cells (ATCC) were maintained in Ham's F-12 (GIBCO) +10% FBS.  
425 Raji cells (ATCC), HNE1 cells(57) and HK1 cells(58) were maintained in  
426 RPMI1640+10% FBS. Akata B cells(59) harboring a modified EBV, in which  
427 the thymidine kinase gene has been replaced with a neomycin and green  
428 fluorescence protein (GFP) cassette (Akata-GFP), were grown in RPMI 1640  
429 (GIBCO) +5% FBS. 293F cells (ThermoFisher) were maintained in Freestyle  
430 293 medium (Union) with gentle shaking. All cells were grown with 100U/ml  
431 penicillin and 100µg/ml streptomycin.

432

### 433 **Humanized mice**

434 The construction of the humanized mice was based on  
435 NOD.*Cg-Prkdc<sup>em1IDMO</sup>Il2rg<sup>em2IDMO</sup>* mice (NOD-*Prkdc<sup>null</sup> IL2Rγ<sup>null</sup>*, NPI®)(60),  
436 which were kept in a specific pathogen free (SPF) facility and obtained from  
437 BEIJING IDMO Co., Ltd. To generate the humanized immune system, mice  
438 were i.p. injected with a single dose of Busulfan at 20mg/kg body weight. After  
439 48h post-injection, the mice received an intravenous tail injection of human  
440 CD34+ cells, which were isolated from umbilical cord blood (Beijing Novay  
441 biotech) with a purity of over 90%. Human CD45+ cells in peripheral blood of  
442 each humanized mouse were detected at 4 and 8 weeks post engraftment by  
443 flow cytometry.

444

445 **Plasmids**

446 The gH/gL [residues 19 to 679 of gH and residues 24 to 137 of gL were linked  
447 by (G4S)<sub>3</sub>] and gp42 (residue 34 to 223) fragments were amplified from the  
448 bacterial artificial chromosome (BAC) of EBV-M81 by PCR and cloned into the  
449 pcDNA3.1 plasmid with an N-terminal CD5 leader peptide and a C-terminal  
450 HIS Tag. Targeted mutations were introduced into pcDNA3.1-CD5-gH/gL using  
451 the ClonExpress MultiS One Step Cloning Kit (Vazyme) and were confirmed by  
452 Sanger sequencing. The pCAGGS expression plasmids for gH, gL, gB, and  
453 pT7EMCLuc (which carries a luciferase-containing reporter plasmid under the  
454 control of the T7 promoter) were kindly provided by Dr. R. Longnecker. The  
455 ectodomain of EphA2 was cloned in an Fc construct as described  
456 previously(61).

457

458 **Recombinant antibody cloning**

459 The VH and VK/Vλ genes of reference antibodies CL40, E1D1 and AMMO1  
460 were obtained from PDB and codon optimized genes were synthesized by  
461 Tsingke Biological Technology Company. Antibody heavy chain and light chain  
462 variable gene fragments were obtained using separate primer pairs(62) with  
463 restriction enzyme cutting sites, including VH primers with 5'Agel and 3'Sall,  
464 VK primers with 5'Agel and 3'BsiWI, and Vλ primers with 5'Agel and 3'XhoI.  
465 Then PCR products were cloned into antibody expression vectors containing  
466 the constant regions of human IgG1. The sequences of the recombinant

467 plasmids were verified by Sanger sequencing.

468

### 469 **Recombinant protein expression**

470 The 293F cells were transfected with plasmids encoding EBV glycoproteins,  
471 EphA2-Fc and recombinant antibodies at a density of  $1.5 \times 10^6$  cells/ml in  
472 Freestyle 293 medium using PEI (Polysciences) transfection reagent  
473 according to the manufacturer's instructions. After five days, the culture  
474 supernatant containing EBV glycoprotein was collected and passed through  
475 Ni-NTA resin (GE Healthcare), followed by washing (PBS with 20mM imidazole,  
476 pH7.4) and elution (PBS with 250mM imidazole, pH7.4). The proteins were  
477 further purified by size exclusion chromatography (SEC) and dialyzed into PBS.  
478 Clarified cell supernatant containing recombinant antibodies or EphA2-Fc was  
479 passed over Protein A agarose (GenScript), followed by extensive washing  
480 with PBS, and then eluted with 10 mL of 0.3M glycine, pH 2.0 into 1 mL of 1M  
481 Tris HCl, pH 8.0. Purified proteins were then dialyzed into PBS.

482

### 483 **Recombinant protein biotinylation**

484 gH/gL were biotinylated at a theoretical 1.5:1 biotin/protein ratio using the  
485 EZ-Link Sulfo-NHS-Biotin (ThermoFisher) at room temperature for 30min.  
486 Free biotin was removed by 3 successive rounds of dilution with PBS.

487

### 488 **Preparation of the antigen binding fragment**



489 1D8 Fab was obtained by digesting 1D8 IgG with Endoproteinase Lys-C  
490 (Sigma) at 37°C (1 mg IgG: 250 ng Lys C) for 12h. Fab fragments were isolated  
491 with Fc fragments using protein A agarose, then further purified by SEC.

492

### 493 **Biolayer Interferometry (BLI)**

494 Antibody competition binding assays (Octet Red 96, ForteBio, Pall LLC):  
495 250nM gH/gL was captured onto HIS1K sensors (ForteBio, Pall LLC) for 120s.  
496 The baseline interference was then read for 60 s in KB buffer (PBS, 0.1% BSA,  
497 0.02% Tween). Then the sensor was loaded with 1D8 (10µg/ml) or KB (blank)  
498 for 60s and balanced in KB for 60s, followed by association with 10µg/ml  
499 competitive antibodies (1D8, AMMO1, CL40, E1D1) for 120s and association  
500 with KB for 120s. One gH/gL-1D8 loaded sensor was immersed in buffer as a  
501 reference during the association and dissociation steps and used to subtract  
502 the background signal.

503 Antibody/EphA2 competition binding assays: 2µg/ml gH/gL-biotin was  
504 immobilized on streptavidin biosensors (ForteBio, Pall LLC), and then  
505 immersed into KB for 60s. Then the sensor was loaded with 1D8 (50nM),  
506 AMMO1 (50nM), 2G4 (50nM) or KB (blank) for 60s and balanced in KB for 60s,  
507 followed by associated with 1000nM EphA2-Fc for 100s and association with  
508 KB for 120s. One gH/gL-antibody loaded sensor was immersed in buffer as a  
509 reference during the association and dissociation steps and used to subtract  
510 the background signal.

511

## 512 **Surface Plasmon Resonance (SPR)**

513 The binding kinetics and affinity of antibodies for gH/gL or their mutants were  
514 analyzed by SPR (Biacore 8K, GE Healthcare). Anti-human IgG (Fc) antibody  
515 was covalently immobilized onto a CM5 sensor chip (GE Healthcare) via  
516 amine groups in 10 mM sodium acetate buffer (pH 5.0) for a final RU of around  
517 5000. Specifically, antibodies 1D8 or AMMO1 (2µg/ml) were captured by  
518 anti-human IgG antibody for 10s. Diluted gH/gL or their mutants were run at a  
519 flow rate of 30 µl/min in HBS-EP (aqueous buffer containing 0.01M HEPES  
520 pH7.4, 0.15M NaCl, 3mM EDTA and 0.05%(v/v) Tween 20, filtered through a  
521 0.2µm filter). The sensograms were fit to a 1:1 binding model using the Biacore  
522 Insight Evaluation Software (GE Healthcare).

523

## 524 **Enzyme-Linked Immunosorbent Assay (ELISA)**

525 For ELISA, 100ng/well of EBV glycoprotein was coated in 96-well  
526 enzyme-linked immunosorbent assay plates overnight at 4°C. Then, the plates  
527 were blocked with 5% bovine serum albumin (BSA) in PBS and 0.1%Tween-20  
528 (blocking buffer) at 37°C for 1 h. After blocking, the plates were washed three  
529 times with 0.1% Tween-20 in PBS (washing buffer). Plasma samples or  
530 recombinant antibodies were diluted serially in blocking buffer and incubated  
531 at 37°C for 1 h. Following three times of washing, a 1:4000 goat anti-human  
532 IgG-HRP (Promega) in blocking buffer was added to each well and incubated

533 at 37°C for 45 minutes. Plates were washed five times and incubated with  
534 3,3',5,5'-tetramethylbenzidine substrate (TMB) (TIANGEN) for 5 minutes at  
535 room temperature. Then 1M hydrochloric acid (HCl) was added and the OD<sub>450</sub>  
536 was read on a microplate reader (Epoch2). For the binding analysis of gH/gL  
537 mutants, 500ng/well of antibody was coated in plates overnight at 4°C. The  
538 plates were then blocked and washed. The gH/gL mutants were diluted serially  
539 in blocking buffer and incubated at 37°C for 1h. Following three times of  
540 washing, 1:3000 diluted mouse anti-his antibody (TRANSGEN BIOTECH) in  
541 blocking buffer was added to each well and incubated at 37°C for 1h. After  
542 three times of washing, a 1:5000 diluted goat anti-mouse-HRP antibody  
543 (Invitrogen) was added and incubated at 37°C for 1h. The final steps were the  
544 same as above.

545

#### 546 **B cells sorting**

547 Cryopreserved 10 million PBMC were thawed into 1 ml preheated RPMI1640,  
548 centrifuged at 300×g for 5 min, re-suspended in 500µl FACS buffer (PBS+2%  
549 FBS), and incubated with 200nM his-tagged antigen (gH/gL) for 45 min at 4°C.  
550 The PBMC were then washed two times with 1ml FACS buffer and  
551 resuspended in 100µl FACS buffer. The PBMC were stained with the following  
552 antibodies: CD3-PE-Cy5 (BD Biosciences) at a 1:25 dilution, CD14-PE-Cy5  
553 (eBioscience) at a 1:50 dilution, CD16-PE-Cy5 (BD Biosciences) at a 1:25  
554 dilution, CD235a-PE-Cy5 (BD Biosciences) at a 1:100 dilution,

555 CD19-APC-Cy7 (BD Biosciences) at a 1:100 dilution, CD20-PE-Cy7 (BD  
556 Biosciences) at a 1:200 dilution, IgG-FITC (BD Biosciences) at a 1:25 dilution,  
557 and anti-his-PE (BioLegend) at a 1:20 dilution for 30 min at 4°C. The PBMC  
558 were washed three times with 1ml FACS buffer and resuspended in 500µl  
559 FACS buffer, then subjected to FACS on a BD FACS Aria II (BD Biosciences).  
560 Antigen-positive B cells (CD3-, CD14-, CD16-, CD235a-, CD19+, CD20+,  
561 IgG+, PE+) were sorted individually into 96-well PCR vital-plates containing  
562 20µl first strand buffer (5µl first strand buffer, 0.5µl of RNase inhibitor  
563 (Invitrogen), 1.25µl of 100µM DTT, 0.06µl of IGEPAL (Sigma).

564

#### 565 **VH/VL recovery from sorted cells**

566 Wells containing sorted cells were mixed with 6µl of reverse transcription (RT)  
567 buffer containing 1.5µl mixed primers specific for human IgG, IgM, IgD, IgA1,  
568 IgA2, K and λ constant gene regions, 1.5µl of 25 mM dNTP mix (Invitrogen),  
569 and 0.25µl of superscript III reverse transcriptase (Invitrogen). The RT  
570 temperature program included 42°C for 10 min, 25°C for 10 min, 60°C for 50  
571 min, and 94°C for 5 min, followed by a hold at 4°C. The VH, VK and Vλ genes  
572 were amplified from 5µl of cDNA separately using nested PCR (HotStarTaq  
573 DNA Polymerase, QIAGEN). The PCR products were purified and subjected to  
574 Sanger sequencing. Then, the VH, VK and Vλ variable genes were assembled  
575 into functional linear Ig gene expression cassettes by overlap-extension PCR.  
576 The function of the expressed antibodies was determined using ELISA

577 screening.

578

### 579 **Virus production**

580 Akata cells carrying EBV, in which the thymidine kinase gene was interrupted  
581 with a double cassette expressing GFP and a neomycin resistance gene, were  
582 resuspended in FBS-free RPMI 1640 medium at a concentration of  $2\text{--}3\times 10^6$   
583 cells per ml, followed by induction with 0.75% (v/v) of goat anti-human  
584 immunoglobulin G serum (Shuangliu Zhenglong Biochem Lab) for 6h at 37°C.  
585 After culture in fresh RPMI1640 medium supplemented with 5% FBS for 3  
586 days, virus from the supernatant was collected under sterile conditions,  
587 passed through two Millipore filters (0.8 and 0.45µm), concentrated 100-fold by  
588 high-speed centrifugation at 50,000g, and then resuspended in fresh FBS-free  
589 RPMI1640. The virus was stored at 80°C and thawed immediately before  
590 infection. To assess the virus titer, 10-fold dilutions of EBV were used to  
591 inoculate  $2\times 10^5$  PBMC per well in 24-well plates with 2µg/ml cyclosporin A  
592 (CsA) (Sigma). The cultures were fed weekly by replacing half of the medium  
593 with fresh medium containing CsA. After 6 weeks, the  $TD_{50}$  was determined  
594 based on the number of proliferating lymphocytes in the wells (63).

595

### 596 **Neutralization assay**

597 Plasma samples from study individuals or recombinant antibodies were  
598 incubated with GFP-expressing EBV at serial dilutions for 3h at 4°C. Then the

599 mixtures were added to Raji B cells or HNE1 epithelial cells and incubated for  
600 3h at 37°C. Then the unbound virus was removed by washing with PBS twice.  
601 Infected cells were cultured in fresh medium for 48h, followed by detection and  
602 analysis of GFP-positive cells using a flow cytometer and FlowJo 10 software  
603 (FlowJo, USA). The neutralization rate of each sample was defined as:  
604 (%GFP+ cells in the positive control well containing virus alone - %GFP+ cells  
605 in the plasma or antibody containing well)/ %GFP+ cells in the positive control  
606 well×100.

607

#### 608 **EBV infection in humanized mice**

609 At 8 weeks post CD34+ stem cells transfer, 0.4 mg of experimental or control  
610 antibodies were i.p. injected per humanized mouse. After 24 h, the mice  
611 received a dose of Akata EBV equivalent to 1,000 TD<sub>50</sub> via i.v. injection. In the  
612 following period, the mice received a dose of 0.4mg antibody weekly. The  
613 blood collection and recording of body weight and health status were also  
614 done every week. The mice were euthanized 6 weeks post EBV infection or  
615 earlier if they became clinically ill (e.g. body weight loss of approximately  
616 20%).

617

#### 618 **Detection of EBV DNA in blood and tissues**

619 DNA was extracted from the peripheral blood (100µl) or tissues of the mice  
620 using commercial DNA extraction kits (Omega). The EBV genome copy

621 number was determined by real-time PCR (Roche Light Cycler 480) using the  
622 TaqMan BamHI probes (40) as described previously. The copy numbers of  
623 EBV were quantified using a standard EBV genome (BDS biotech) as control.

624

#### 625 **H&E staining, IHC, and in situ hybridization**

626 Tissues were fixed in 10% formalin and embedded in paraffin. Consecutive  
627 sections were used for staining with H&E. Immunostaining of human T cells  
628 and B cells was performed using hCD3 antibody (VENTANA) and hCD20  
629 antibody (VENTANA) at 1:200 dilution. EBERs were stained by in situ  
630 hybridization using the EBER detection kit (ZSGB-BIO), according to the  
631 manufacturer's instructions. Histological staining was evaluated by  
632 experienced pathologists.

633

#### 634 **Detection of human immune cells in the blood of humanized mice**

635 Peripheral blood of mice was treated with 1ml red blood cell lysis buffer  
636 (BioLegend) at room temperature for 10min. Then the cells were centrifuged at  
637  $300 \times g$ , washed twice with PBS, re-suspended in PBS, and stained with  
638 antibodies including anti-human CD45-PE (BD Biosciences),  
639 CD3-PerCP-Cy5.5 (BD Biosciences) and CD20-FITC (BD Biosciences) at  
640 1:100 dilution for 30 min at 4°C. After washing with PBS, the percentage of  
641 CD3+ or CD20+ cells among the CD45+ cells was quantified using a flow  
642 cytometer.

643

#### 644 **Cell surface binding assays**

645 For the cell-surface binding assay, 1 mg of gH/gL-biotin conjugated with SA-PE  
646 (gH/gL-PE) was diluted in 10 ml of PBS into individual wells of a 96 well plate.  
647 An equimolar amount of gp42 was added to select wells containing gH/gL-PE.  
648 5 mg/ml of monoclonal antibodies, including 1D8, AMMO1, or 2G4, were added  
649 to select wells containing gH/gL with or without gp42 and incubated for 1 h at  
650 37°C. At the same time, adherent HK1 cells were trypsinized (NCM Biotech),  
651 washed with RPMI 1640 and then allowed to recover at 37°C in a humidified  
652 atmosphere comprising 5% CO<sub>2</sub> for 1 h with gentle agitation twice during the  
653 period. Recovered HK1 and Raji cells were pelleted by centrifugation at 300×  
654 g for 5 min and then resuspended at a density of 2×10<sup>6</sup> cells/ml in ice-cold 0.5%  
655 bovine serum albumin (BSA) in PBS. Then, 100 μl of the HK1 or Raji cells  
656 suspension were added to wells containing SA-PE, gH/gL-PE with or without  
657 gp42 and antibodies, and incubated on ice for 1 h. The cells were pelleted by  
658 centrifugation at 300×g for 5 min, washed with 1 ml of ice cold 0.5% BSA in  
659 PBS, pelleted again and resuspended in 10% phosphate buffered formalin.  
660 The amount of PE staining was quantified using a flow cytometer.

661

#### 662 **Virus-free fusion assay**

663 Effector CHO-K1 cells were transiently transfected with expression plasmid  
664 (pCAGGS-gH, pCAGGS-gL, pCAGGS-gB and pT7EMCLuc, which carries a



665 luciferase-containing reporter plasmid under the control of the T7 promoter).  
666 Target cells (HEK-293T) were transfected with expression plasmid pCAGT7  
667 (expressing T7 DNA polymerase). After 24h, the effector cells were trypsinized  
668 and re-suspended at a density of  $1 \times 10^6$  cells/ml. Aliquots comprising  
669 250 $\mu$ l/well of effector cell suspension was pre-incubated with 2 $\mu$ g 1D8,  
670 AMMO1 or 2G4 antibody at 37 $^{\circ}$ C for 1h. Then the target cells were also  
671 trypsinized and re-suspended at a density of  $1 \times 10^6$  cells/ml. An aliquot  
672 comprising 250 $\mu$ l of the effector cell suspension was added to the effector cells  
673 with or without antibody. After 24h, the medium was aspirated and the cells  
674 were lysed in 100 $\mu$ l of luciferase agent (Dual-Glo Luciferase Assay System).  
675 Then, 75 $\mu$ l of cell lysate was transferred to a white-bottom assay plate and  
676 luciferase activity was read on a GloMax-96 Microplate Luminometer  
677 (Promega).

678

### 679 **Cell surface staining**

680 Following 24h after expression plasmid transfection, the effector CHO-K1 cells  
681 were trypsinized and re-suspended at a density of  $1 \times 10^6$  cells/ml. The  
682 expression level of gH/gL was detected using the indicated antibody. AMMO1  
683 and 1D8 were used for gH/gL staining and 2G4 as a control. Then, 10 $\mu$ g/ml of  
684 antibody was added to the cell suspension and incubated at 4 $^{\circ}$ C for 1h. The  
685 cells were washed twice with PBS and stained with human IgG-PerCP-Cy5.5  
686 (PC5.5) (BioLegend) at a 1:100 dilution. After washing with PBS, the

687 percentage of PC5.5+ cells was quantified using a flow cytometer (CytoFLEX,  
688 BECKMAN).

689

### 690 **Crystallization of the 1D8 Fab and data collection**

691 To purify the gH/gL-1D8 Fab complex, 1D8 Fab was incubated with gH/gL for 1  
692 h on ice in HBS buffer, and the mixture was then subjected to gel filtration  
693 chromatography. Fractions containing the complex were pooled and  
694 concentrated to 10 mg/ml. Crystals were successfully grown at 18°C in sitting  
695 drops, over wells containing 200 mM sodium citrate, 100 mM HEPES sodium  
696 salt, pH 7.5, 15 % w/v MPD. The drops were made by mixing 200 nl  
697 gH/gL-1D8 Fab complex in HBS buffer with 200 nl well solution. Crystals were  
698 harvested, soaked briefly in 200 mM sodium citrate, 100 mM HEPES sodium  
699 salt, pH 7.5, 15 % w/v MPD, 20% glycerol, and flash-frozen in liquid nitrogen.  
700 Diffraction data were collected at the BL17U beam line of the Shanghai  
701 Synchrotron Research Facility (SSRF). Diffraction data were processed with  
702 HKL2000 and the crystal diffracted to 4.2Å. The data processing statistics are  
703 listed in Supplementary Table 3.

704

### 705 **Structure solution and refinement**

706 The structure was determined via the molecular replacement method using  
707 PHASER in CCP4 suite. The search models were gH/gL (PDB code 5T1D)  
708 and the antibody with the highest sequence identity with 1D8. Density map

709 improvement by atoms update and refinement was performed with  
710 ARP/wARP29. Subsequent model building and refinement were performed  
711 using COOT and PHENIX, respectively. Final Ramachandran statistics  
712 indicated that 91.48% residues were in favored conformations, 7.06% allowed  
713 and 1.46% outliers for the final structure. The structural refinement statistics  
714 are listed in Supplementary Table 3. All structural figures were generated with  
715 PyMol (DeLano, 2002).

716

### 717 **Statistical analysis**

718 Unless noted otherwise, a two-tailed, unpaired *t*-test was used to assess  
719 statistical significance. Statistical calculations were performed in GraphPad  
720 Prism 8. The number of replicates and a description of the statistical method  
721 are provided in the corresponding figure legends. Differences with *P* values of  
722 less than 0.05 were considered to be statistically significant. \**P* < 0.05, \*\* *P* <  
723 0.01, \*\*\* *P* < 0.001, ns=not significant.

724

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864

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878 manuscript, and all authors contributed to editing and figure preparation.  
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880 authors declare no potential conflicts of interest. **Data availability:** The atomic  
881 model of 1D8-gH/gL (PDB ID: 7D5Z) has been deposited in the Protein Data  
882 Bank.

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889 **MAIN FIGURE LEGENDS**

890 **Fig. 1. Isolation of gH/gL-specific monoclonal antibodies using single B**  
891 **cell sorting and cloning.** (A) FACS-based sorting strategy for gH/gL-specific  
892 B cells. (B) Binding activities of 1D8 and 2A6, the positive control AMMO1, and  
893 the negative control 2G4 to EBV gH/gL measured by ELISA. The data are  
894 presented as means  $\pm$  SEM. (C) Neutralizing activities of 1D8 and 2A6, the  
895 positive control AMMO1, and the negative control 2G4 against EBV infection of  
896 Raji B cell lines and (D) HNE1 epithelia cell line. The data shown is means  $\pm$   
897 SEM. SSC-A, side-scatter area; FSC-A, forward-scatter area.

898

899 **Fig. 2. Protective efficacy of 1D8 against lethal EBV challenge in**  
900 **humanized mice.** (A) Timeline for engrafting CD34+ human hematopoietic  
901 stem cells (HSC), antibody administration, viral challenge, and monitoring for  
902 various biological and clinical outcomes. Four hundred micrograms of 1D8,  
903 positive control AMMO1, negative control 2G4, or PBS negative control were  
904 administered to the humanized mice via intraperitoneal injection either 24h  
905 prior to or weekly for 5 weeks after intravenous challenge with Akata EBV. (B)  
906 EBV DNA in the peripheral blood, (C) body weight, and (D) survival were  
907 monitored weekly. The percent changes in (E) hCD45+, (F) hCD20+, or (G)  
908 hCD3+ cells over the experiment period. On week 6 post infection, (H) virus  
909 titers in spleen, (I) liver, (J) kidney were analyzed. All data are presented as  
910 mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; ns, no significant.



911

912 **Fig. 3. 1D8 reduces viral replication and tissue damages in humanized**  
913 **mice.** Representative of macroscopic spleens and splenic sections stained for  
914 hematoxylin and eosin (H&E), EBV encoded RNA (EBER), human CD20  
915 (hCD20), and human CD3 (hCD3) at necropsy. The scale bars are indicated.

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917 **Fig. 4. 1D8 targets an unique epitope on gH/gL. (A)** Structure overview in a  
918 cartoon representation with gL in cyan, gH D-I in blue, D-II in wheat, D-III in  
919 green, D-IV in yellow, and 1D8 Fab in purple. The map is contoured at 1.2  
920 RMS to show the density. **(B)** Zoomed-in view of the interaction between 1D8  
921 and D-I and D-II. The key binding residue N310 of gH was indicated by a red  
922 star. **(C)** Cartoon representation of Fab 1D8 and other previously published  
923 Fabs AMMO1, CL40, and E1D1 bound to a single gH/gL molecule. The color  
924 scheme for gH/gL is as in **(A)** whereas 1D8 Fab in purple, AMMO1 Fab in light  
925 blue, CL40 Fab in pink, and E1D1 Fab in dark green. **(D)** Surface mapping of  
926 the four Fab epitopes on gH/gL with the same color in **(C)**. Areas in black  
927 indicate the region where structural change was found upon binding to  
928 AMMO1 or CL40.

929

930 **Fig. 5. 1D8 interferes with cell fusion and binding.** Quality control of gH/gL  
931 expression on the surface of CHO-K1 cells by staining with 1D8 **(A)**, positive  
932 control AMMO1 **(B)**, and negative control 2G4 **(C)** before proceeded to the

933 fusion experiment shown in **(D)**. **(D)** Marked reduction in cell-cell fusion in the  
934 presence of 1D8, the positive control AMMMO1, but not the negative control  
935 2G4. Marked reduction in gH/gL binding to Raji B cell **(E)** and HK1 epithelial  
936 cell **(F)** in the presence of 1D8, the positive control AMMO1, but not the  
937 negative control 2G4. Binding of EphA1-Fc to gH/gL was reduced by 1D8 **(G)**,  
938 but not by AMMO1 **(H)** or 2G4 **(I)** measured by Bio-layer interferometry (BLI).  
939 All data are presented as mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns,  
940 no significant. SSC-A, side-scatter area; PC5.5, PerCP-Cy5.5; RLU, relative  
941 light unit; SA-PE, streptavidin-phycoerythrin; MFI, mean fluorescence intensity.

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943 **Fig. 6. Possible mechanisms of 1D8-mediated neutralization.** **(A)** For  
944 epithelia cells, 1D8 could interfere the interaction between gH/gL and EphA2  
945 either by directly restricting access to the interface or by indirectly posing  
946 allosteric hindrance. It could also restrict the movement across the D-I/D-II  
947 groove of gH/gL that is required for gB interaction and triggering. **(B)** For B  
948 cells, 1D8 could also restrict the movement across the D-I/D-II groove of gH/gL  
949 that is required for downstream viral entry. 1D8 does not appear influence  
950 interaction between gp42 and its receptor HLA class II.

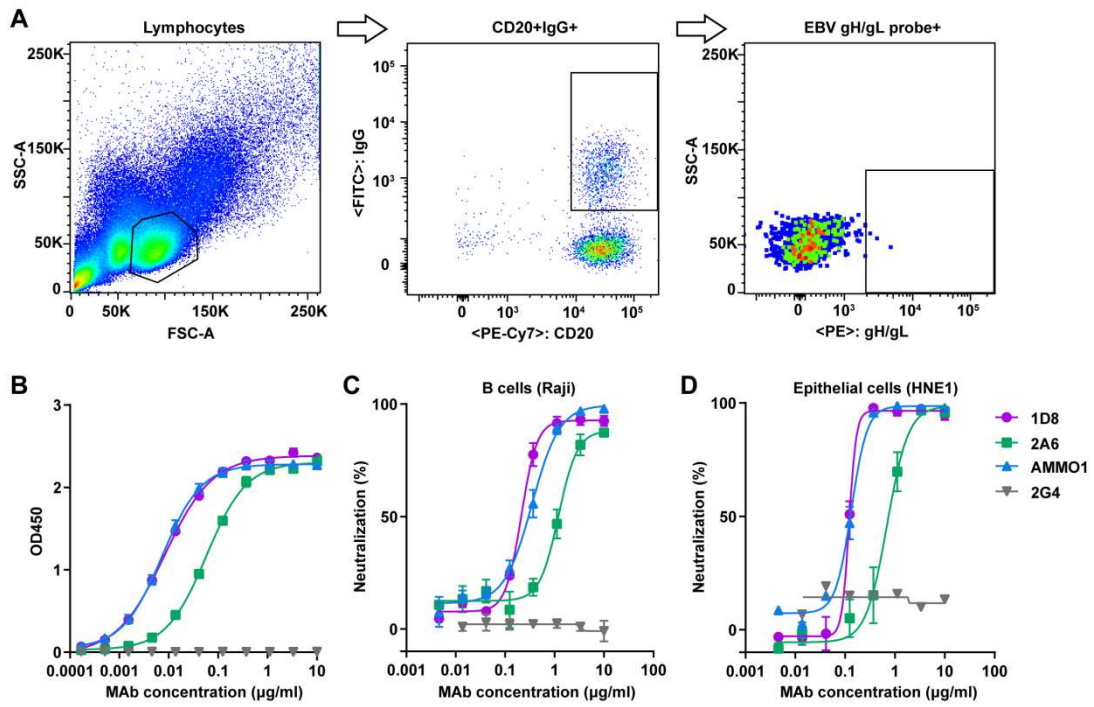
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955 **Fig. 1**



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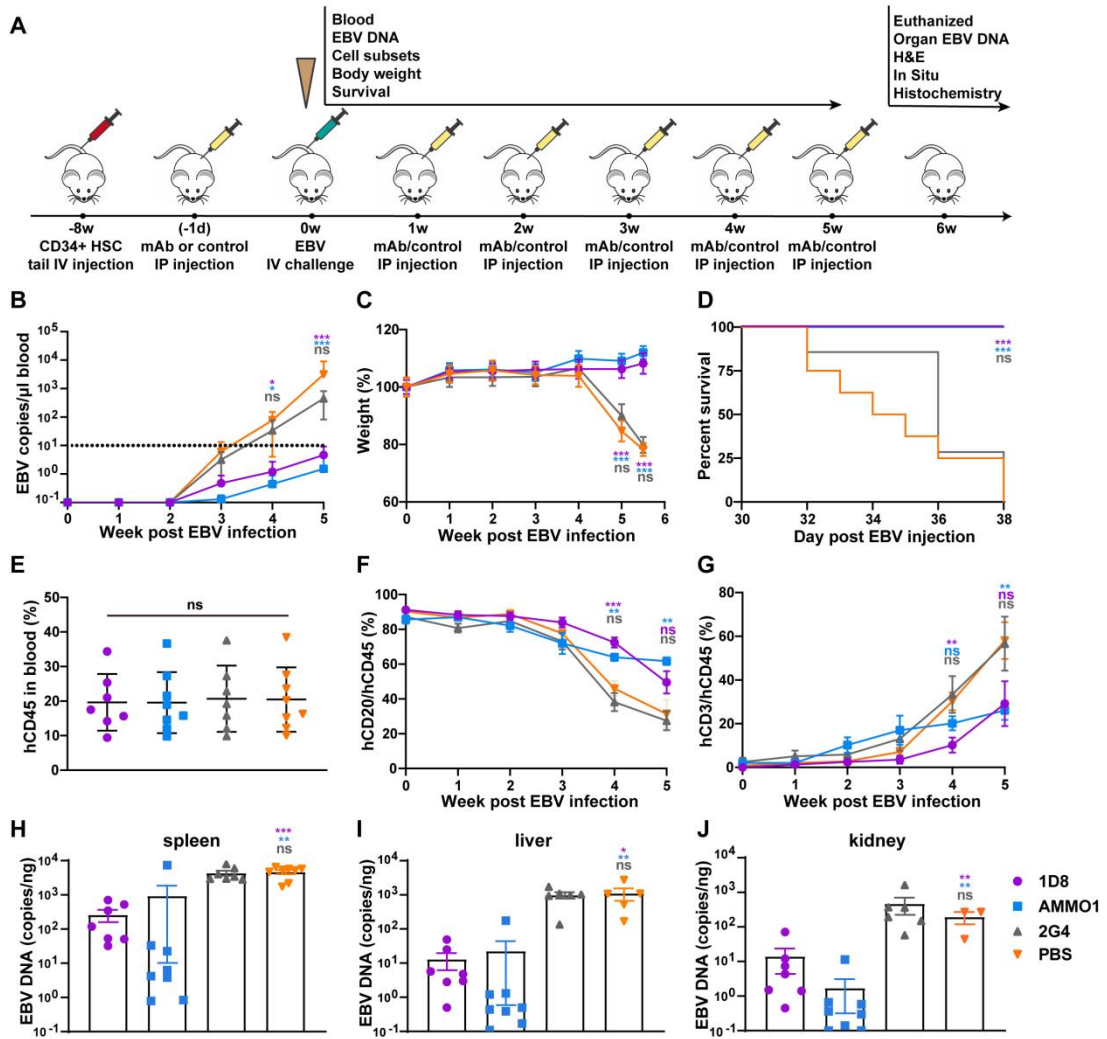
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969 **Fig. 2**



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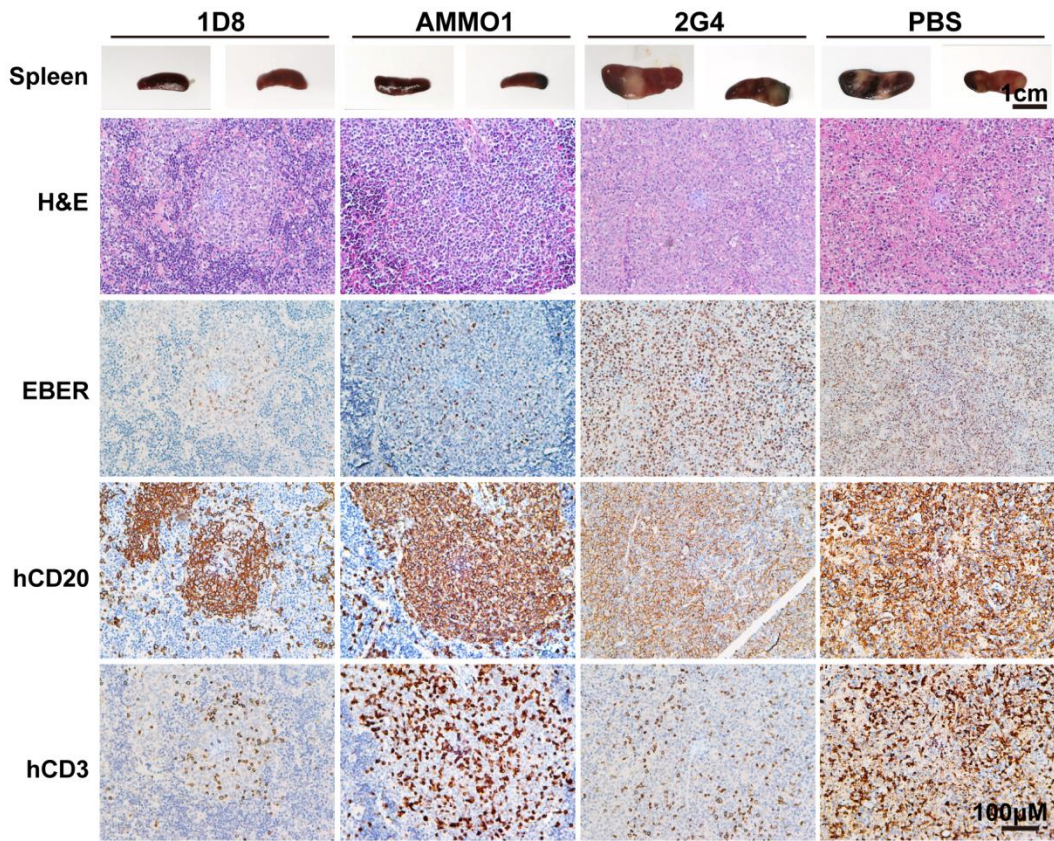
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979 **Fig. 3**



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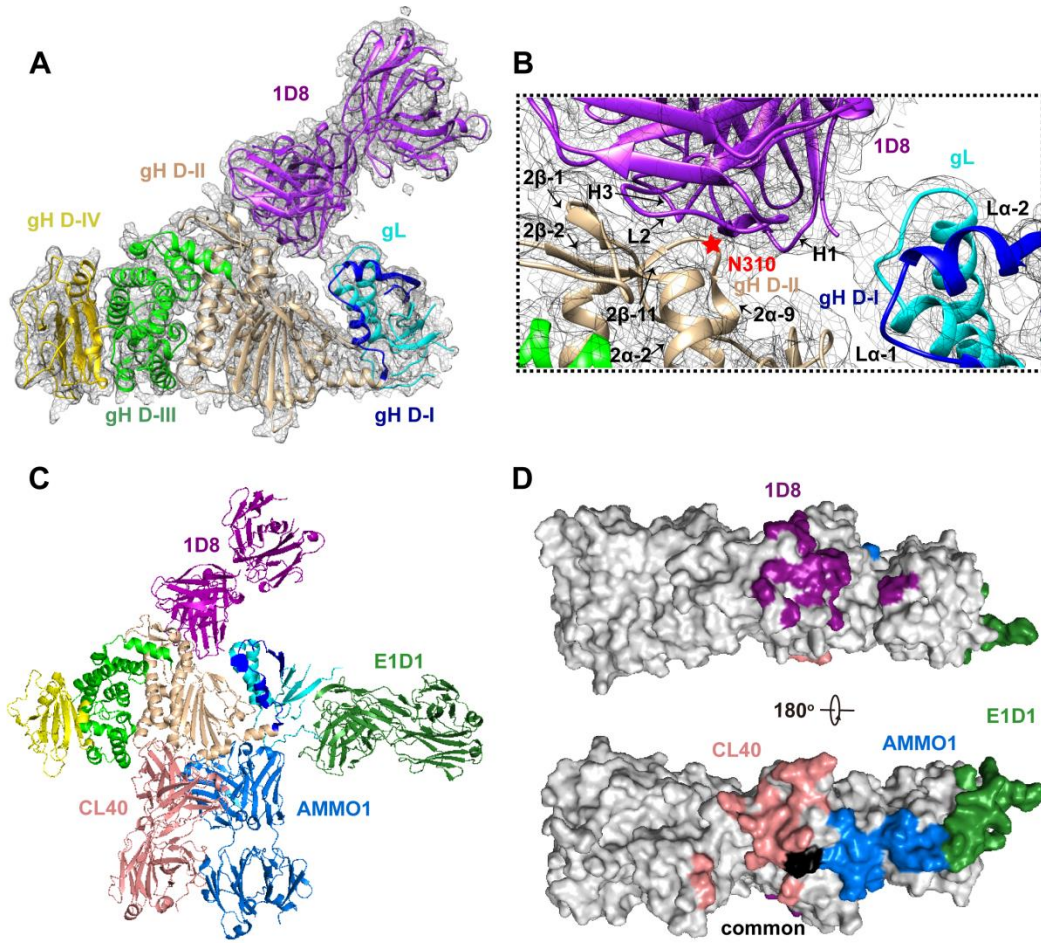
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991 **Fig. 4**



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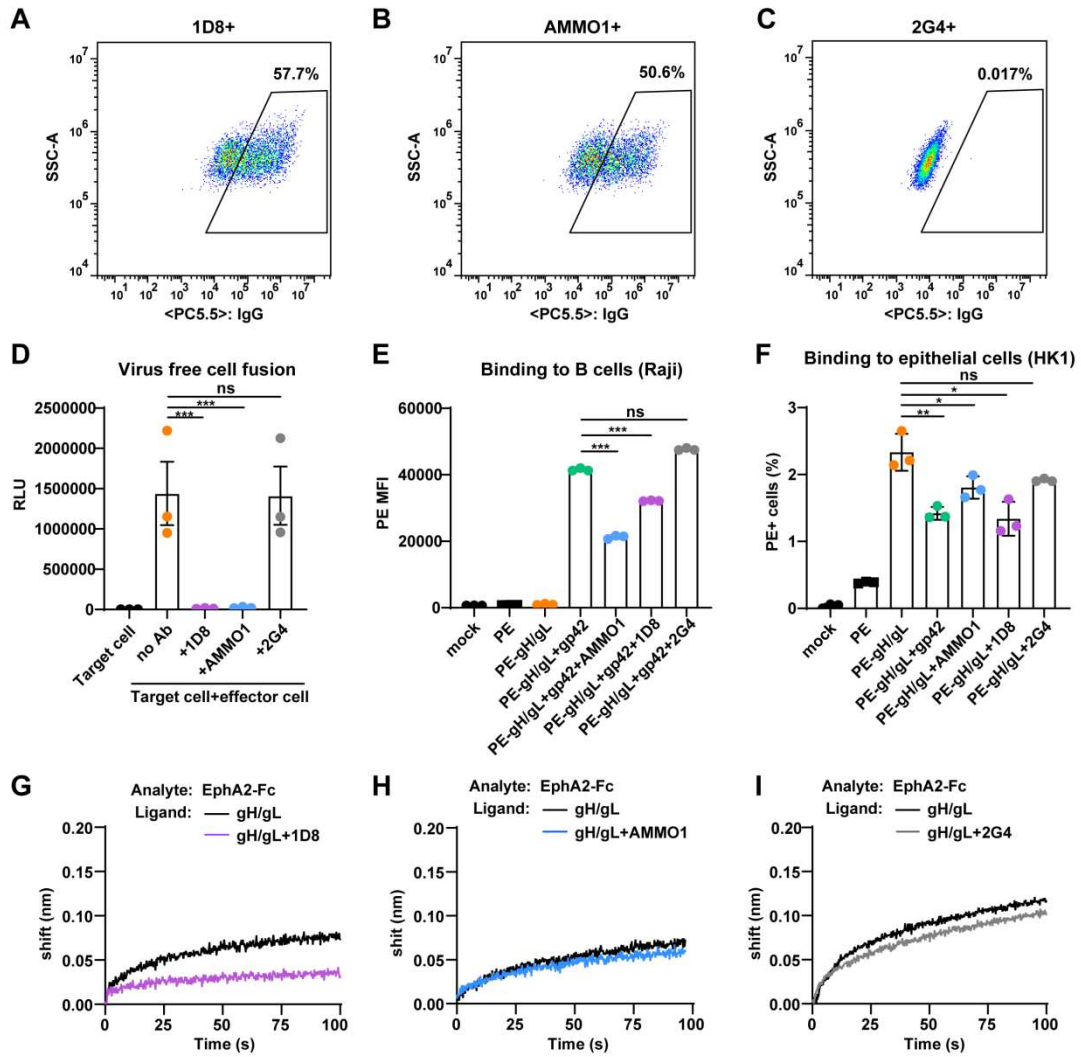
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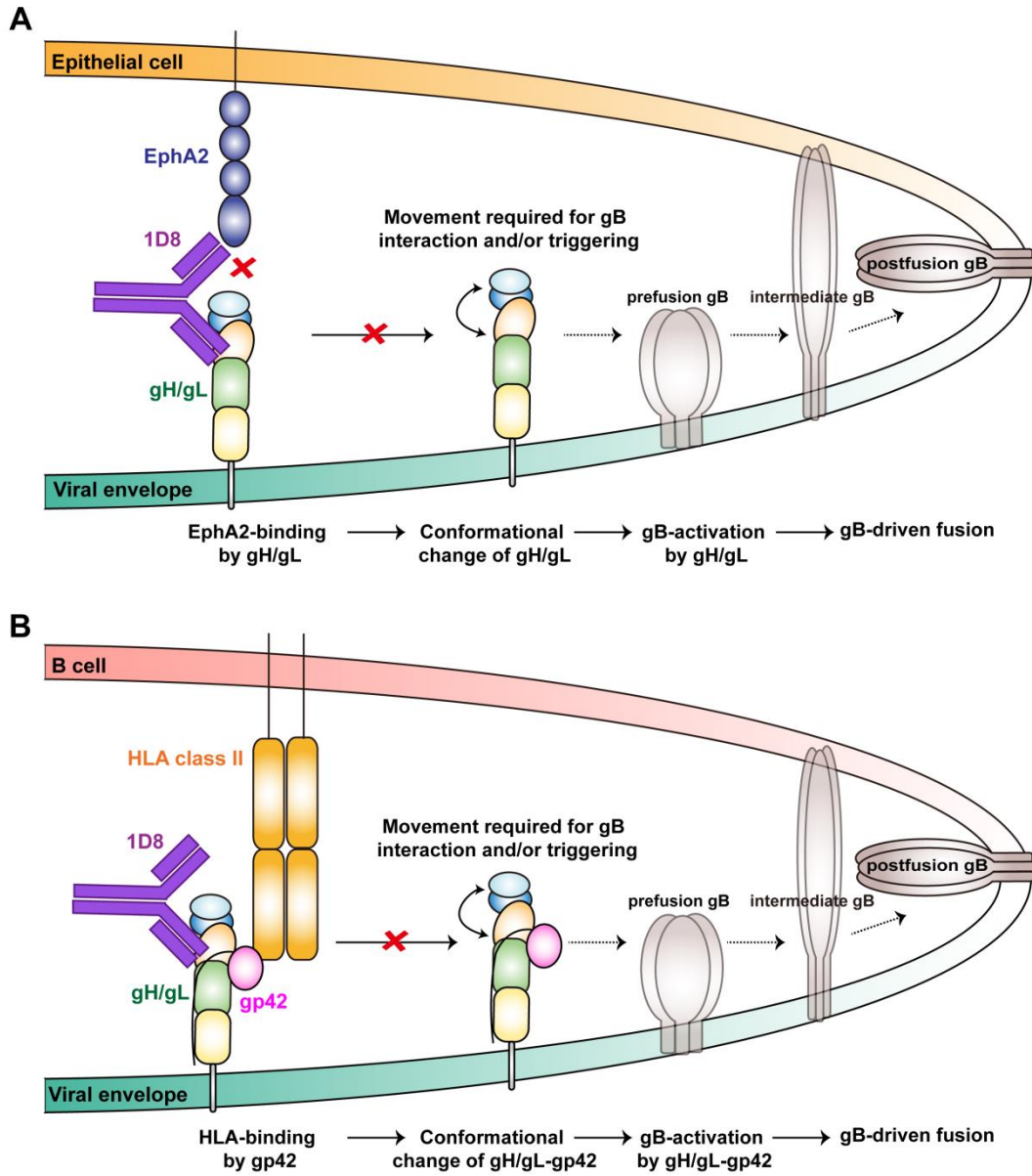
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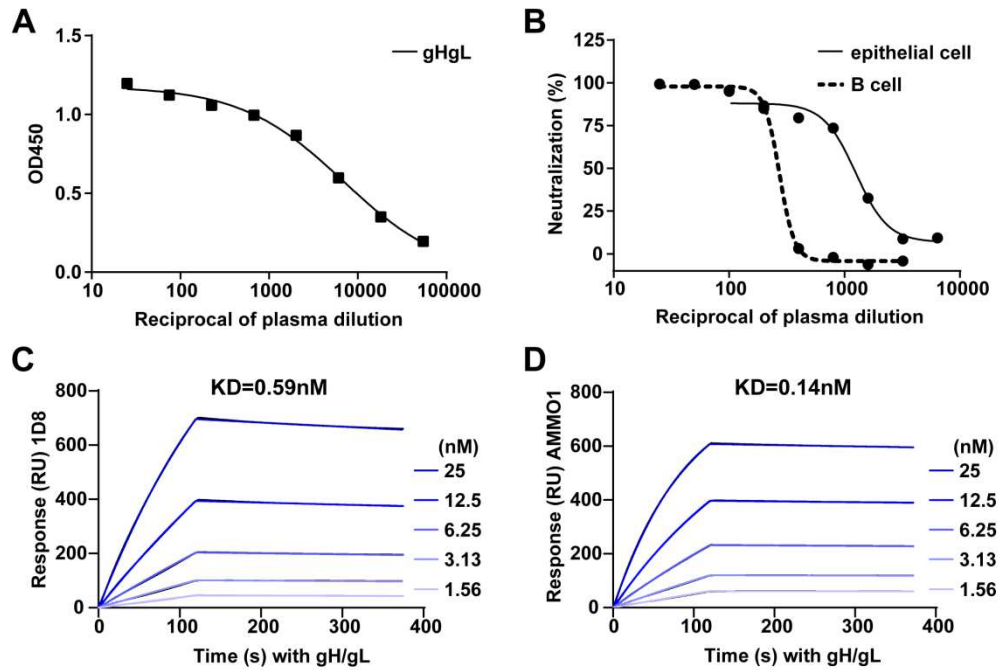
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1020 SUPPLEMENTARY MATERIALS

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1023 **Fig. S1. Plasma binding and neutralizing activities from donor 27.**

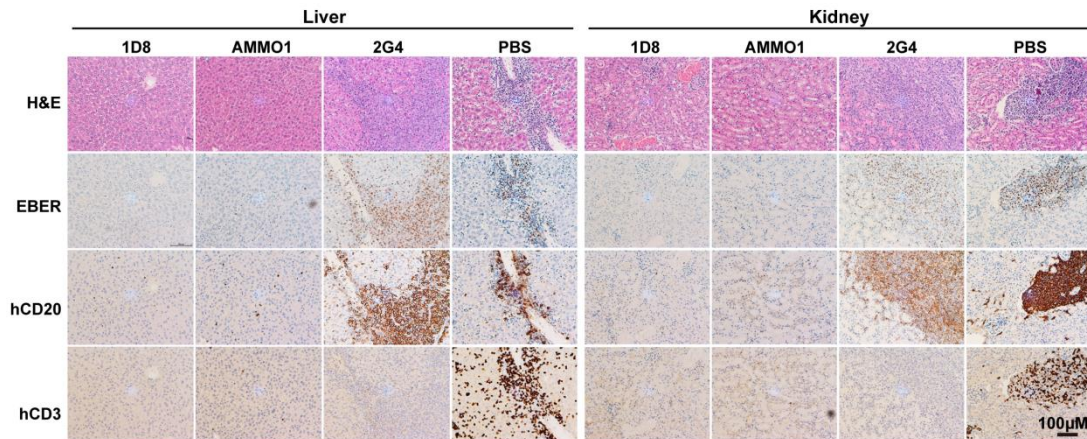
1024 (A) Plasma binding activities to gH/gL measured by ELISA. (B) Plasma  
1025 neutralizing activities against EBV infection of Raji B cells and HNE1 epithelial  
1026 cells. Binding activity of 1D8 (C) or AMMO1 (D) to gH/gL measured by surface  
1027 plasmon resonance (SPR). All experiments were performed in duplicate, and  
1028 the data shown are means with SEM.

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1034 **Fig. S2. 1D8 reduces viral replication and tissue damages in liver and**  
 1035 **kidney of mice.**

1036 Hepatic and renal sections stained for hematoxylin and eosin (H&E), human  
 1037 CD20 (hCD20), human CD3 (hCD3), and EBV encoded RNA (EBER) at  
 1038 necropsy. Scale bar of 100µm is shown.

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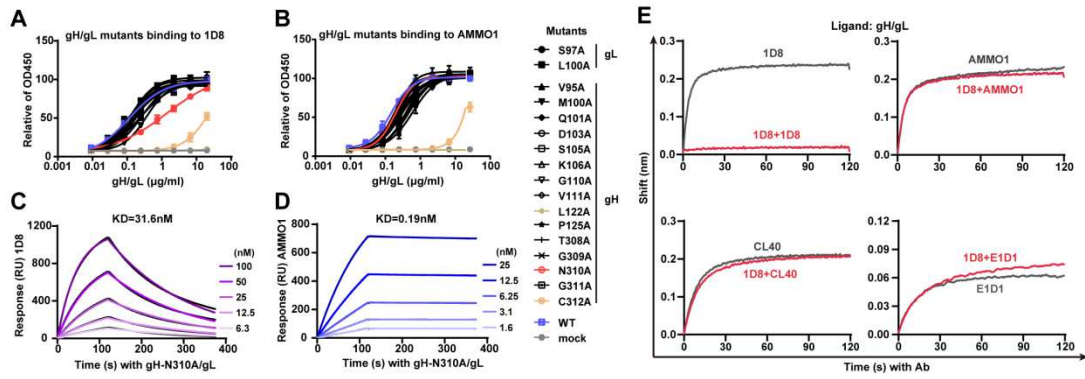
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1050 **Fig. S3. Binding of 1D8 to gH/gL mutants and its competition with**  
 1051 **gH/gL antibodies.**

1052 (A) 1D8 and (B) AMMO1 binding to various gH/gL mutants measured by  
 1053 ELISA. ELISA was performed in duplicate wells, and the data shown are  
 1054 means with SEM. Binding activity of 1D8 (C) and AMMO1 (D) to gH-N310A/gL  
 1055 mutant measured by surface plasmon resonance (SPR). (E) Competitive  
 1056 binding of 1D8 with AMMO1, CL40 or E1D1 to gH/gL measured by bio-layer  
 1057 interferometry (BLI).

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**Table S1. Neutralization potency of monoclonal antibodies.**

Mab	Epithelial cells ( $\mu\text{g/ml}$ )		B cells ( $\mu\text{g/ml}$ )	
	IC <sub>50</sub>	IC <sub>90</sub>	IC <sub>50</sub>	IC <sub>90</sub>
1D8	0.123	0.187	0.238	0.442
2A6	0.745	2.270	1.320	>50
AMMO1	0.127	0.305	0.318	1.148
2G4	NA	NA	NA	NA

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1095 **Table S2. Kinetic Analysis of Antibodies Binding to gH/gL Measured by**

1096 **SPR.**

Ligand	Anylate	kon (1/Ms) $\times 10^5$	koff (1/s) $\times 10^{-4}$	KD (nM)
1D8	gH/gL S97A	1.33	1.36	1.02
1D8	gH/gL L100A	1.07	1.55	1.44
1D8	gH V95A/gL	1.30	2.31	1.77
1D8	gH M100A/gL	1.51	1.96	1.30
1D8	gH Q101A/gL	0.95	1.95	2.03
1D8	gH D103A/gL	0.72	0.69	0.95
1D8	gH S105A/gL	0.90	0.97	1.08
1D8	gH K106A/gL	1.26	1.09	0.86
1D8	gH G110A/gL	2.66	0.26	0.10
1D8	gH V111A/gL	1.11	1.48	1.34
1D8	gH P125A/gL	0.71	2.00	2.79
1D8	gH T308A/gL	1.04	1.01	0.97
1D8	gH G309A/gL	0.95	0.95	1.00
1D8	gH N310A/gL	1.75	55.20	31.60
1D8	gH G311A/gL	0.78	2.05	2.62
1D8	gH/gL WT	2.45	1.46	0.59
AMMO1	gH N310A/gL	4.97	0.96	0.19
AMMO1	gH/gL WT	5.77	0.85	0.14

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1106 **Table S3. Data collection and refinement statistics.**

EBV gH/gL-1D8	
<b>Data collection</b>	
Space group	P4 <sub>1</sub> 2 <sub>1</sub> 2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	212.865, 212.865, 598.128
α, β, γ, (°)	90, 90, 90
Resolution (Å)	50.03-4.201(4.351-4.201) *
<i>R</i> <sub>sym</sub> or <i>R</i> <sub>merge</sub>	0.131 (1.261)
<i>I</i> / <i>sI</i>	8 (1.4)
Completeness (%)	99.34 (98.99)
Redundancy	8.1 (8.2)
 <b>Refinement</b>	
Resolution (Å)	50.03-4.201
No. reflections	100331
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub>	23.31/26.77
No. atoms	
Protein	36304
<i>B</i> -factors	
Protein	161.73
R.m.s. deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.98
Ramachandran plot (%)	
Favored	91.48%
Allowed	7.06%
outlier	1.46%

1107 One crystal was used.

1108 \*Values in parentheses are for highest-resolution shell.

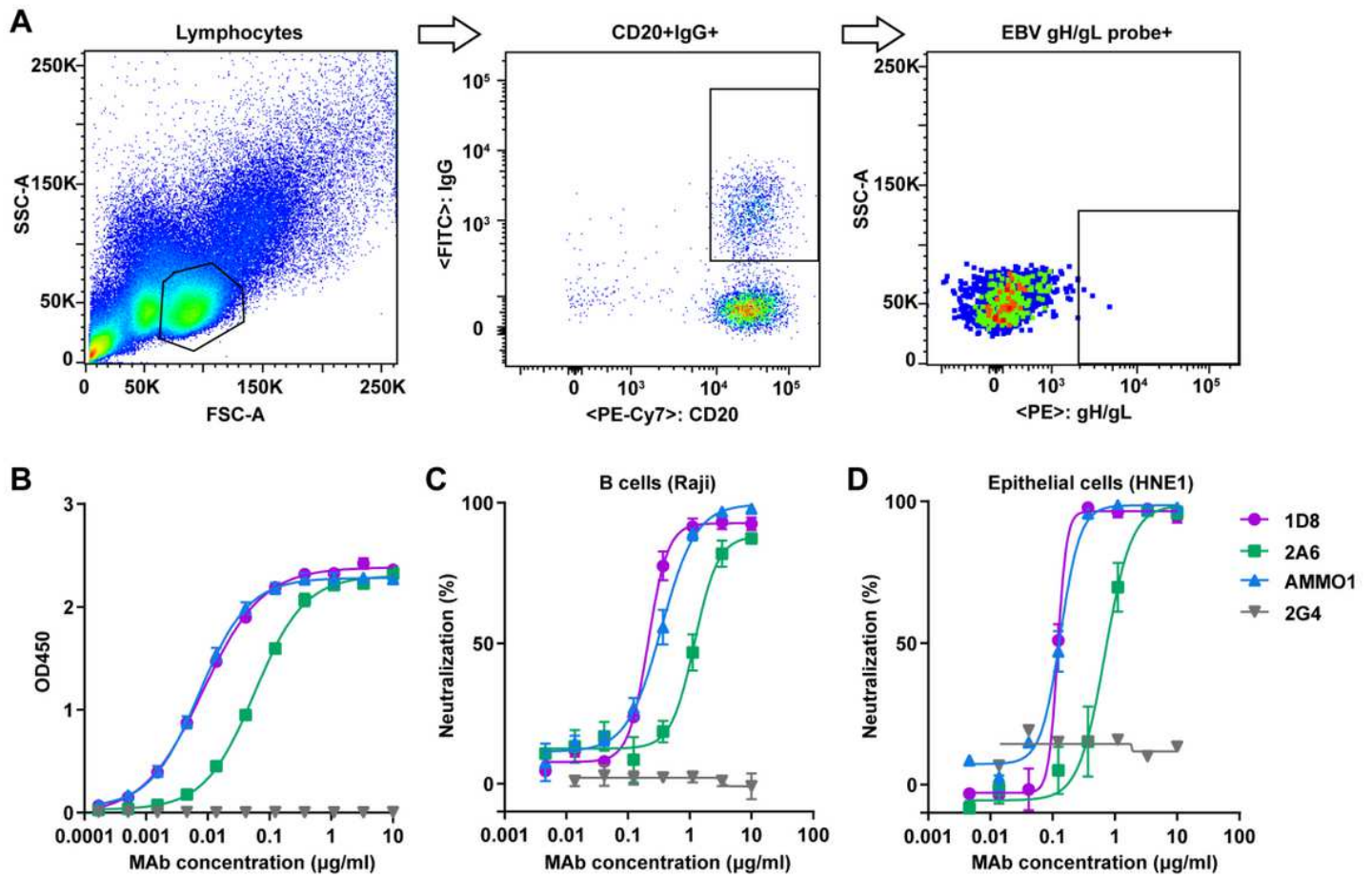
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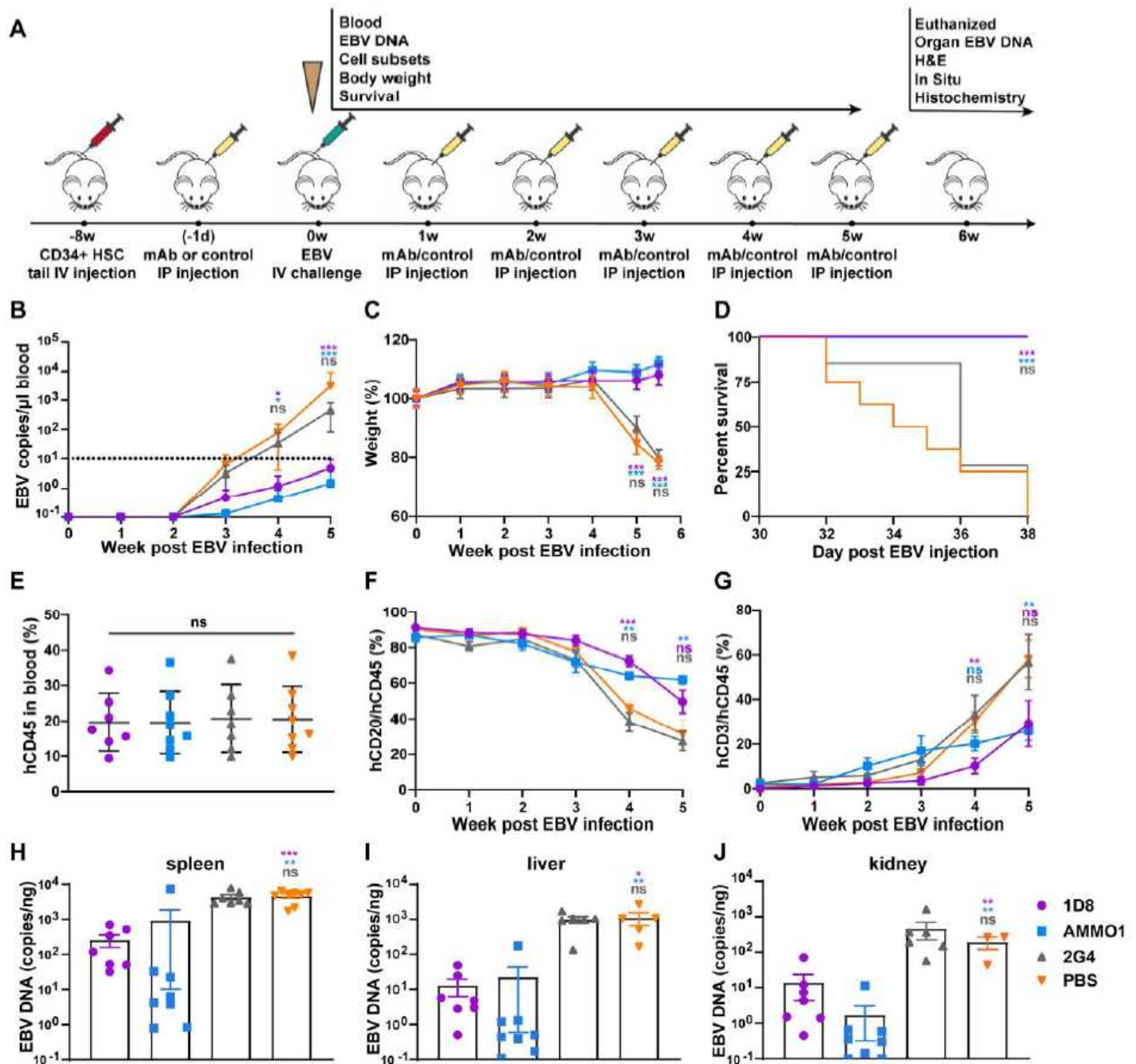
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# Figures



**Figure 1**

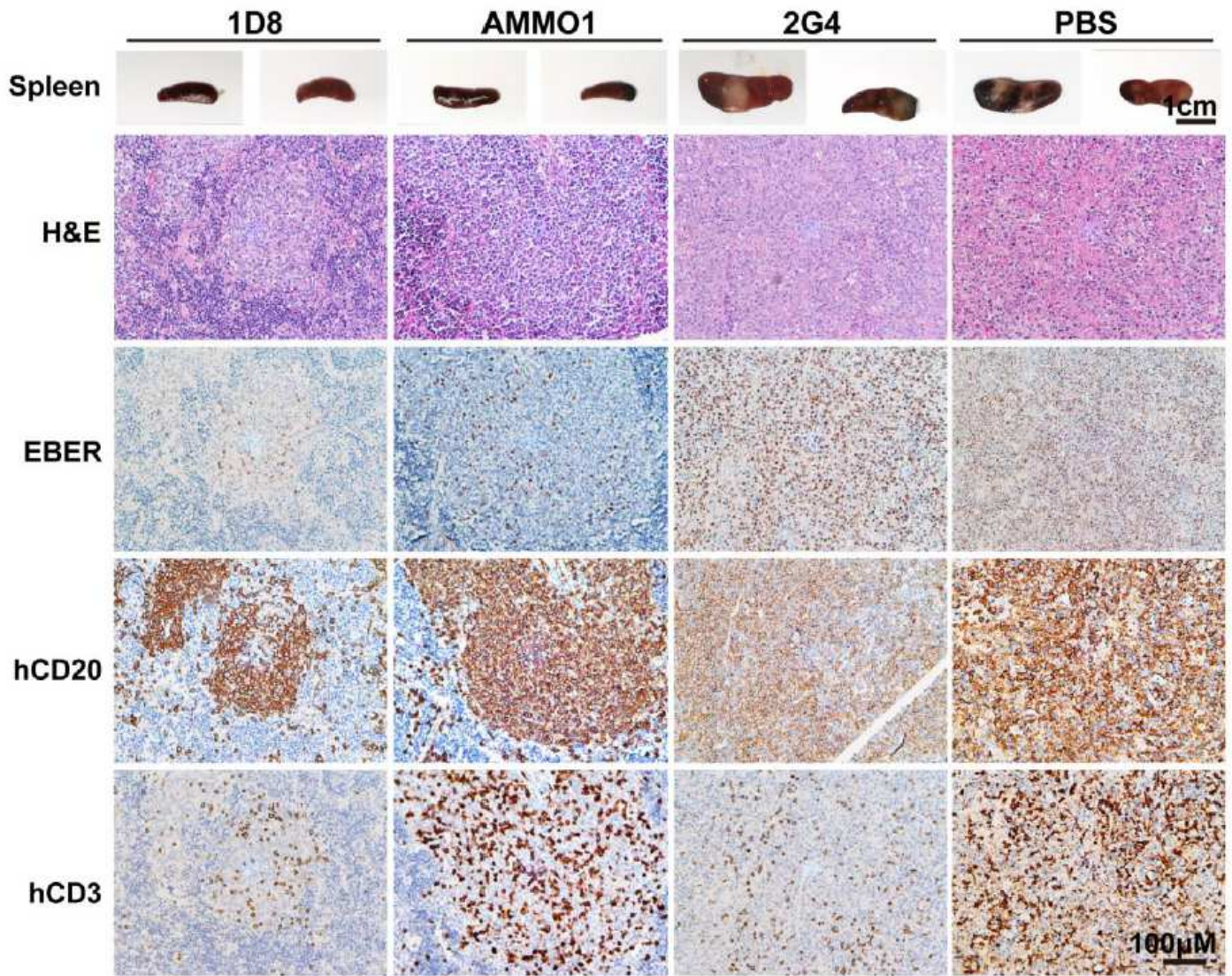
Isolation of gH/gL specific monoclonal antibodies using single B cell sorting and cloning A FACS based sorting strategy for gH/gL specific B cells. B Binding activities of 1D8 and 2A6, the positive control AMMO1, and the negative control 2G4 to EBV gH/gL measured by ELISA. The data are presented as means  $\pm$  SEM. C Neutralizing activities of 1 D8 and 2A6, the positive control AMMO1, and the negative control 2G4 against EBV infection of Raji B cell lines and D HNE1 epithelia cell line. The data shown is means  $\pm$  SEM. SSC A, side scatter area; FSC A, forward scatter area.



**Figure 2**

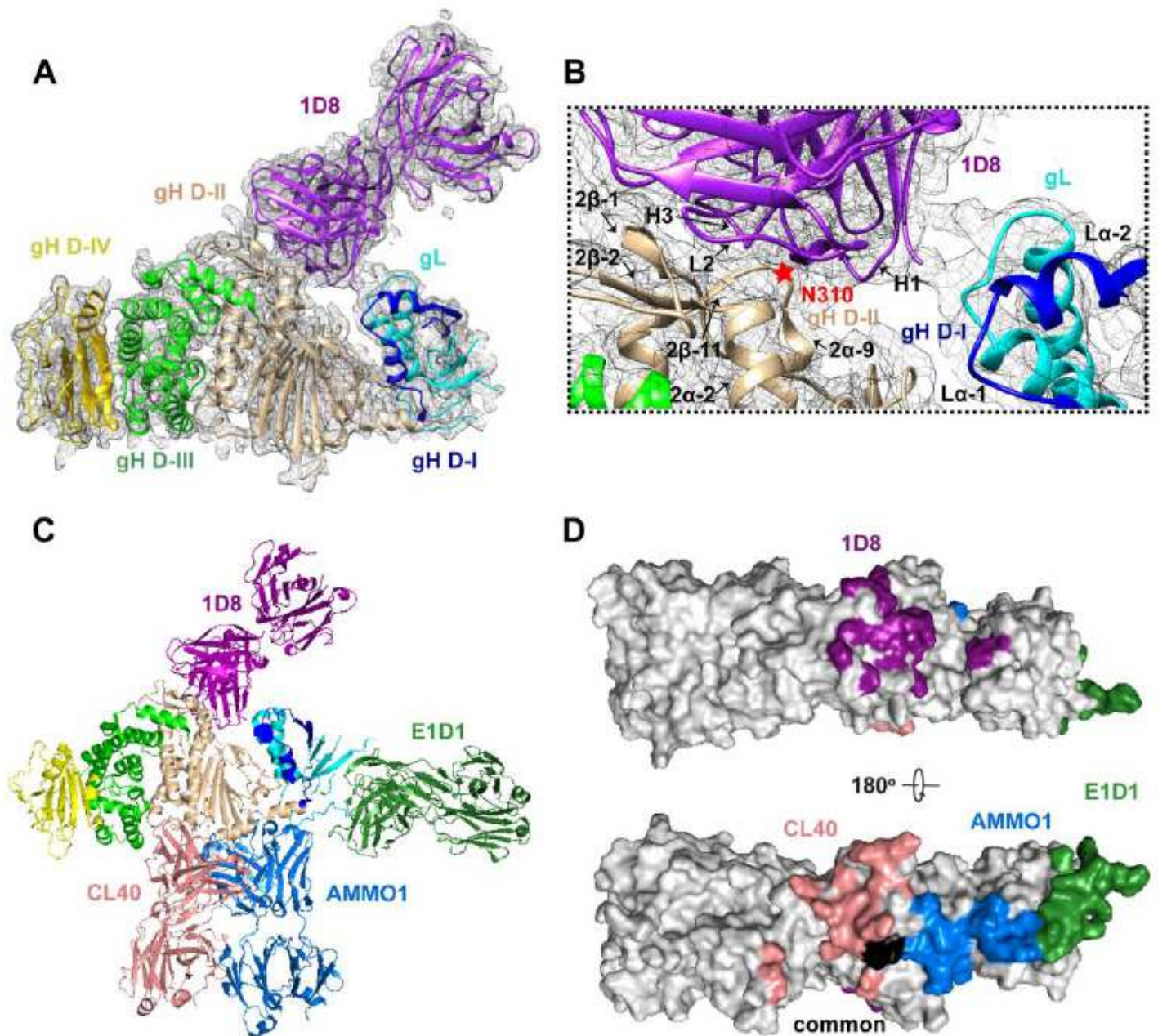
Protective efficacy of 1D8 against lethal EBV challenge in humanized mice. **A** Timeline for engrafting CD34+ human hematopoietic stem cells (HSC), antibody administration, viral challenge, and monitoring for various biological and clinical outcomes. Four hundred micrograms of 1D8, positive control AMMO1, negative control 2G4, or PBS negative control were administered to the humanized mice via intraperitoneal injection either 24h prior to or weekly for 5 weeks after intravenous challenge with Akata EBV. **(B)** EBV DNA in the peripheral blood, **(C)** body weight, and **(D)** survival were monitored weekly. The percent changes in **(E)** hCD45+, **(F)** hCD20+, or **(G)** hCD3+ cells over the experiment period. On week 6 post infection, **(H)** virus titers in spleen, **(I)** liver, **(J)** kidney were analyzed. All data are presented as mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, no significant.





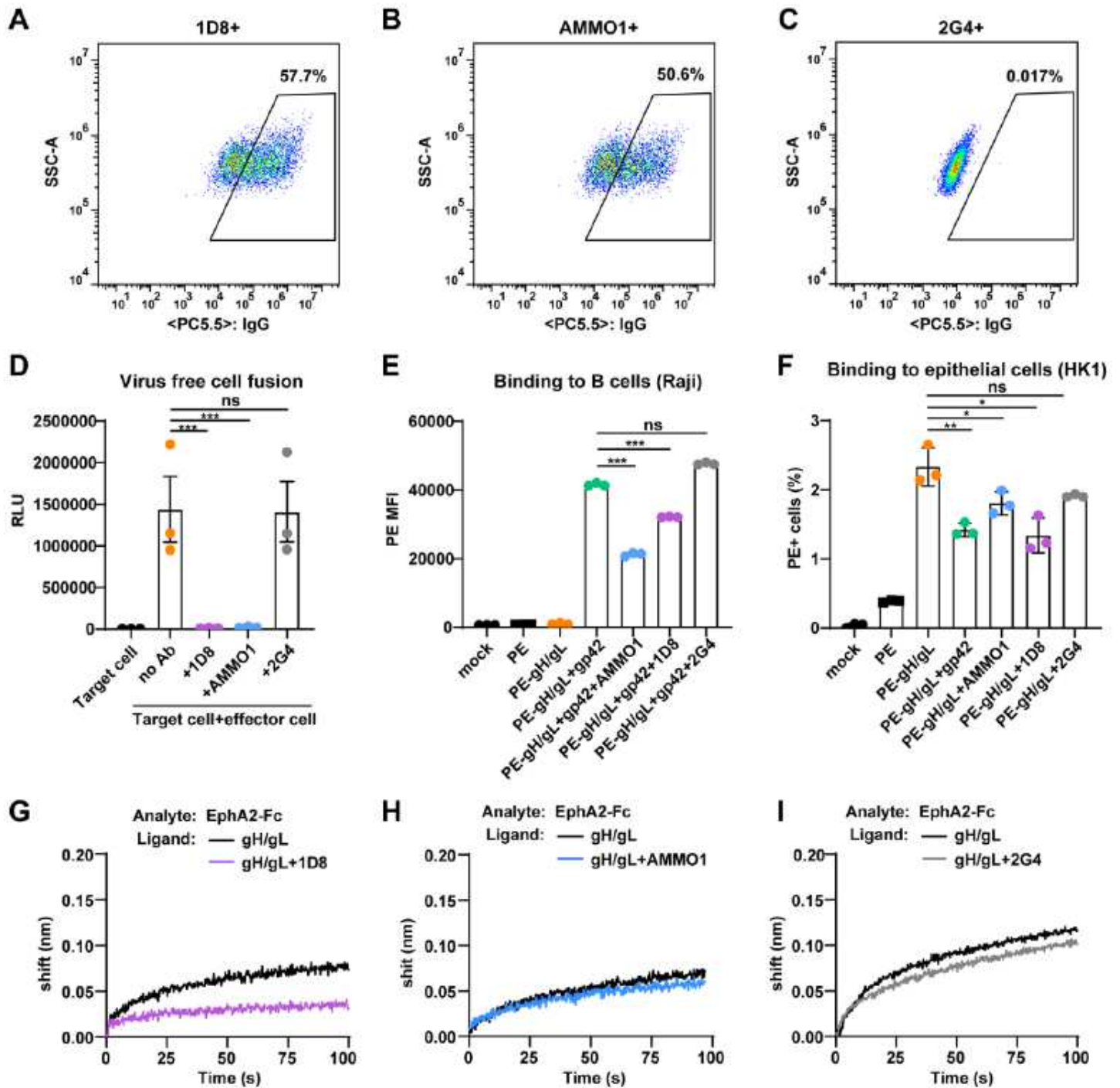
**Figure 3**

1D8 reduces viral replication and tissue damages in humanized mice Representative of macroscopic spleens and splenic sections stained for hematoxylin and eosin (H&E), EBV encoded RNA (EBER), human CD20 ( and human CD3 (hCD3) at necropsy. The scale bars are indicated.



**Figure 4**

1D8 targets an unique epitope on gH/gL A Structure overview in a cartoon representation with gL in cyan, gH D I in blue, D II in wheat, D III in green, D IV in yellow, and 1D8 Fa b in purple. The map is contoured at 1.2 RMS to show the density. B Zoomed in view of the interaction between 1D8 and D I and D II. The key binding residue N310 of gH was indicated by a red star. C Cartoon representation of Fab 1D8 and other previously published Fabs AMMO1, CL40, and E1D1 bound to a single gH/gL molecule. The color scheme for gH/gL is as in ( A ) whereas 1D8 Fab in purple, AMMO1 Fab in light blue, CL40 Fab in pink, and E1D1 Fab in dar k green. D Surface mapping of the four Fab epitopes on gH/gL with the same color in C ). Areas in black indicate the region where structural change was found upon binding to AMMO1 or CL40.



**Figure 5**

1D8 interferes with cell fusion and binding Quality control of gH/gL expression on the surface of CHO K1 cells by staining with 1D8 (A), positive control AMMO1 (B), and negative control 2G4 (C) before proceeded to the fusion experiment shown in (D). (D) Marked reduction in cell cell-cell fusion in the presence of 1D8, the positive control AMMMO1, but not the negative control 2G4. Marked reduction in gH/gL binding to Raji B cell (E) and HK1 epithelial cell (F) in the presence of 1D8, the positive control AMMO1, but not the negative control 2G4. Binding of EphA1 EphA1-Fc to gH/gL was reduced by 1D8 (G), but not by AMMO1 (H) or 2G4 (I) measured by Bio Bio-layer interferometry (BLI). All data are presented as

mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, no significant. SSC SSC-A, side side-scatter area; PC5.5, PerCP PerCP-Cy5.5; RLU, relative light unit; SA SA-PE, streptavidin streptavidin-phycoerythrin; MFI, mean fluorescence intensity.

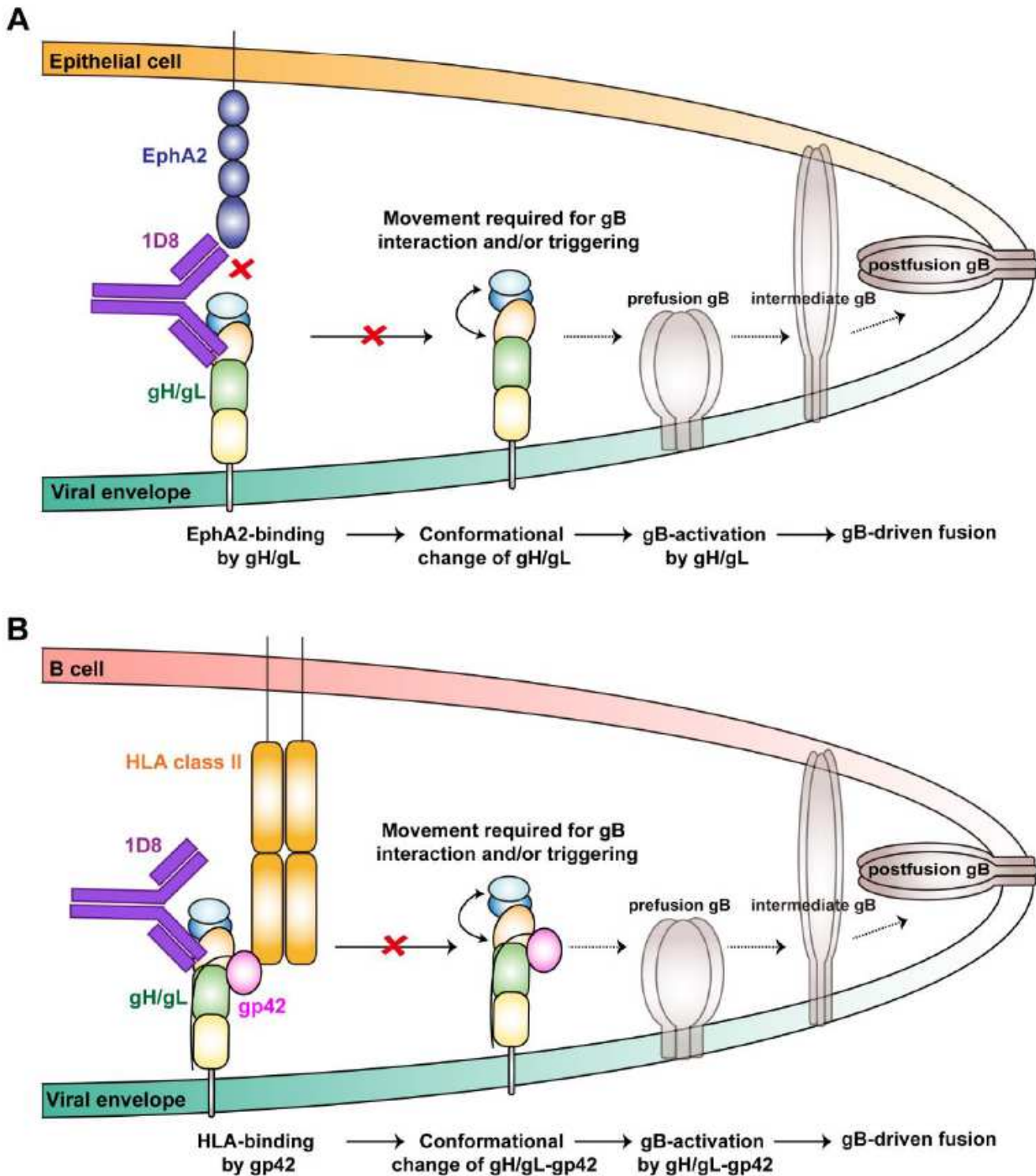


Figure 6

Possible mechanisms of 1D8 1D8-mediated neutralization neutralization. (A) For epithelia cells, 1D8 could interfere the interaction between gH/gL and EphA2 either by directly restricting access to the

interface or by indirectly posing allosteric hindrance. It could also restrict the movement across the D D-I/D -II groove of gH/gL that is required for gB interaction and triggering. (B) For B cells, 1D8 could also restrict the movement across the D D-I/D -II groove of gH/gL that is required for downstream viral entry. 1D8 does not appear influence interaction between gp42 and its receptor HLA class II.