Study On the Mechanism of LMP2A Maintaining Epstein-barr Virus Latency Infection Through Interaction With CXCR4

Ni Qin

Qingdao University Medical College

Yan Zhang

Zibo Central Hospital

Lin Xu

Qingdao Municipal Hospital Group

Wen Liu

Qingdao University Medical College

Bing Luo (✉ qdluobing@163.com)

Qingdao University Medical College <https://orcid.org/0000-0002-6541-5958>

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28 **Abstract**

29 Epstein-Barr virus (EBV) belongs to the γ -herpesvirus subfamily and is the first human tumor
30 virus to be discovered. The global adult infection rate exceeds 90%. EBV can participate in the
31 regulation of multiple genes and multiple signal pathways through its latent genes. Many studies
32 have reported that CXCR4 is involved in the development of gastric cancer, but there are few studies
33 on the specific mechanism of its role in EBV-associated gastric cancer (EBVaGC). In this study, we
34 explored the mechanism by which EBV-encoded products maintain EBV latent infection through
35 interaction with CXCR4, and the role of CXCR4 in EBV positive cells. The results show that there
36 is a positive feedback between the EBV-encoded products and CXCR4, and LMP2A can activate
37 CXCR4 through the NF- κ B pathway. In addition, CXCR4 can be fed back to LMP2A and EBNA1
38 through the ERK signaling pathway. At the same time, CXCR4 can promote the proliferation and
39 migration of EBV-positive cells, reduce the expression of the immediate early protein BZLF1, and
40 play an important role in maintaining the incubation period of EBV infection. These findings are
41 conducive to the further targeted therapy of EBVaGC.

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55 **1 Introduction**

56 Epstein-barr virus (EBV) belongs to the γ -herpesvirus subfamily, and the infection rate of
57 adults in the world is more than 90%. EBV is a widely recognized tumor-associated virus [34],
58 which is closely related to the occurrence and development of various tumors. Most EBV-related
59 tumors are caused by the virus establishing a long-term latent infection in host cells after the initial
60 lytic infection. EBV has two modes of infection: latent infection and lytic infection. The latent
61 infection status of EBV is an important carcinogenic basis. According to the expression of EBNA
62 and LMP [28], EBV shows three different types of latent infection, while EBV-associated gastric
63 cancer (EBVaGC) belongs to type I latent cancer, mainly expressing LMP2A and EBNA1 [21]. In
64 addition, BZLF1 is the earliest expression protein of EBV genome after activation from latent state,
65 and the transcriptional activation function of this gene plays a key role in viral gene from latent
66 activation to proliferation [25].

67 C-X-C motif chemokine receptor 4 (CXCR4), a 352 amino acid rhodopsin like GPCR, was
68 first studied as a chemokine receptor associated with breast cancer metastasis to lung tissue [30].
69 CXCR4 is the most widely expressed chemokine receptor in more than 23 human cancers, including
70 breast cancer, ovarian cancer, melanoma, etc., and is involved in tumor growth, angiogenesis,
71 metastasis, etc. [4]. Several studies have reported that CXCR4 is involved in the proliferation and
72 metastasis of gastric cancer and is closely related to the clinical prognosis of gastric cancer [31, 32].
73 EBVaGC is a unique molecular subtype of gastric cancer that accounts for 8.7% of the total number
74 of gastric cancers according to big data [20]. Based on the high incidence and mortality of gastric
75 cancer worldwide, the pathogenesis of EBVaGC is important.

76 EBV can participate in the regulation of multiple signaling pathways through its encoded latent
77 genes, such as NF- κ B, JNK, PI3K/AKT, etc., and then regulate the expression of downstream target
78 genes to promote the occurrence and development of tumors. Longnecker *et al.* found that LMP2A
79 could simulate B cell receptor (BCR) signaling and regulate the activation of related signaling
80 pathways through its cytoplasmic n-terminal region [16]. In EBVaGC, LMP2A activates NF- κ B
81 signaling by upregulating Survivin expression [7]. CXCR4-ERK signaling pathway mediates

proliferation, migration, and invasion of cancer cells in multiple types of tumors [2, 15, 18]. However, the specific mechanisms by which CXCR4 plays a role in EBVaGC are poorly studied. The purpose of this study was to investigate the mechanism of interaction between CXCR4 and EBV coding products and the involvement of CXCR4 in maintaining EBV latent infection.

2 Materials and Methods

2.1 Cell lines and culture conditions

In this study, SNU719 and GT38 cell lines were EBV-positive gastric epithelial cell lines. SNU719 cells were provided by Professor Qian Tao (The Chinese University of Hong Kong), expressing LMP2A and EBNA1. GT38 cells were kind gifts by Sairenji T (Tottori University, Japan) and expressed LMP1, EBNA1 and EBNA2. SGC7901, an EBV-negative gastric epithelial cell line, was purchased from the Cell Bank of the Chinese Academy of Sciences. All cell lines were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Biological Industries, Israel) and 2% penicillin-streptomycin, at 37 °C with 5% CO₂.

2.2 RNA isolation and Quantitative Real-time PCR(qRT-PCR)

Total RNA was extracted from cell lines with TRIzol reagent (Invitrogen, USA) and reverse transcribed using First Strand cDNA synthesis kit (Takara, Japan), according to the manufacturer's instructions. These products were using a FastStart DNA Master SYBR Green Kit (Roche, Germany) in the Light Cycler 96 sequence detection system. All reactions were done in triplicate and relative gene expression was calculated using the comparative cycle threshold (Ct) value ($2^{-\Delta\Delta C_t}$), using β -actin as the internal standard. The sequences of the specific forward and reverse primers were as follows: for LMP2A, 5'-TGTCGCTGGCATACTCTTCA-3', and 5'-GCGTGTTAGTCATCACCGTC-3', for β -actin 5'-TCCTGTGGCATCCACGAAACT-3', and 5'-GAAGCATTTGCGGTGGACGAT-3'.

2.3 Western blotting analysis

All cells were washed twice with cold phosphate buffer solution (PBS) and lysed in RIPA buffer mixture (RIPA : PMSF : phosphatase inhibitors, 100 : 1 : 1), and then mixed with loading buffer (4 ×) and heated at 95°C for 5min for protein denaturation. The proteins obtained were separated by

109 10% SDS-PAGE, and were transferred onto PVDF membranes (Millipore, USA) using a transfer
 110 device. The blotted PVDF membranes were blocked with 5% non-fat milk for 2h at room
 111 temperature and incubated with the primary antibody at 4°C overnight, followed by a second
 112 antibody for incubating 2h. Finally, proteins of interest were visualized using the enhanced
 113 chemiluminescence detection system. The specific antibodies used were as follows: Anti-β-actin,
 114 anti-ERK1/2, anti-phospho -ERK1/2, anti-NF-κB p65, anti-NF-κB p-p65, anti-IκBα, anti-LMP2A,
 115 anti-MMP-2, anti-Vimentin, HRP-linked anti-mouse/rabbit secondary antibody. All above
 116 antibodies were purchased from CST (Mass, USA), at a dilution of 1 : 1000. Anti-CXCR4, anti-E-
 117 cadherin, anti-N-cadherin antibodies were purchased from Abcam (USA) with a dilution of 1 : 1000.
 118 anti-phospho-IκBα, anti-BZLF1, anti-EBNA1 antibodies were purchased from SANTA (USA) with
 119 a dilution of 1 : 200.

120 **2.4 Transfection with siRNAs of LMP2A、CXCR4 and ERK**

121 The siRNA target sequences for LMP2A, CXCR4 and ERK mRNA were designed and
 122 synthesized by GenePharma (China). The sequences are as follows: hs-CXCR4-si-1: 5' -
 123 AGAUAUACACUUCAGAUAAAdTd - 3', 5' - UUAUCUGAAGUGUAUA
 124 UCUDTd - 3', hs-CXCR4-si-2: 5' - GAAGCAUGACGGACAAGUAdTd - 3', 5' -
 125 UACUUGUCCGUCAUGCUUCdTd - 3', hs-MAPK3/ERK1: 5' -
 126 GAAACUACCUACAGUCUCUTT - 3', 5' - AGAGACUGUAGGUAGUUUCTT - 3', hs-
 127 MAPK1/ERK2: 5' - GUGCUCUGCUUAUGAUAAUTT - 3', 5' -
 128 AUUAUCAUAAGCAGAGCACTTT - 3', hs-EBV-LMP2A-1: 5' -
 129 GAACGAUGAGGAACGUGAAAdTd - 3', 5' - UUCACGUUCCUCAUCGUUCdTd - 3', hs-EBV-
 130 LMP2A-2: 5' - GACUAUCAACCACUAGGAAdTd - 3', 5' -
 131 UUCCUAGUGGUUGAUAGUCdTd - 3'. 50μM siRNA was transfected using Lipofectamine
 132 2000 Reagent (Invitrogen, Thermo Fisher Scientific, Germany). Then, the cells were harvested to
 133 assay relative gene expression. All experiments were performed in triplicate.

134 **2.5 CCK-8 cell proliferation assay**

135 Cell proliferation was detected using the Cell Counting Kit 8 (CCK8, Boster Biological

136 Technology, China). The cells were seeded at 5×10^3 cells/100 μ l per well into 96-well plates. After
137 the cells were attached to the wall, added 10 μ l CCK-8 solution to each well, after incubation for 24,
138 48, 72 and 96h, respectively. And then the cells were incubated for an additional 1h at 37°C, the
139 absorbance was measured using Soft-Max apparatus (Bio-Tek ELx808, USA) at a wavelength of
140 450nm.

141 **2.6 Migration assay**

142 In the transwell migration assay, the transwell chamber was topped with a non-coated
143 membrane with 8 μ m pores (Corning, USA), to which a serum-free culture medium containing
144 1×10^5 cells were added, and to which 20% fetal bovine serum was added as a chemical attractant in
145 the lower chamber. After incubation for 24h, the cells were fixed with methanol, stained with
146 hematoxylin, and the stained cells were counted under 200 $\times$ magnification under a microscope. All
147 experiments had three independent repetitions.

148 **2.7 Overexpression of LMP2A and CXCR4**

149 The plasmids of LMP2A (pcDNA3.1-LMP2A) and CXCR4 (pcDNA3.1-CXCR4) were
150 purchased from Invitrogen Inc. (Hanbio, China) and were transfected using Lipofectamine 2000
151 Reagent (Invitrogen, USA). Fluorescent activity was measured under a fluorescence microscope.
152 SGC7901 cells and SNU719 cells were seeded at 1×10^6 cells per well in 6-well plates and
153 transfected with 2.5 μ g LMP2A, CXCR4 or negative control vector plasmids. Stable transfections of
154 LMP2A and CXCR4 were selected by G418. After successful transfection and screening, cells were
155 collected for experiments.

156 **2.8 Statistical analysis**

157 Data were analyzed with using Student's t-test. Analyses were performed with using GraphPad
158 Prism software (GraphPad Software, USA). Differences were considered statistically significant if
159 $P < 0.05$. All Data was expressed as means $\pm$ standard error of the mean (SEM) and all experiments
160 were repeated at least three times.

161 **3 Results**

162 **3.1 Interaction between LMP2A and CXCR4 at the expression level**

EBV-positive gastric cancer cell line SNU719 was stably transfected with CXCR4 gene and EBV-negative gastric cancer cell line SGC7901 was stably transfected with LMP2A gene. Fig.1a showed the transfection efficiency of the plasmid in cells. The corresponding mRNA and protein expression was detected by qRT-PCR and Western blot, Fig.1b showed that the expression of LMP2A increased in SGC7901 cells after transfection with LMP2A (SGC7901-LMP2A) compared with SGC7901-NC cells. Fig.1c showed that the expression of CXCR4 in SGC7901-LMP2A cells was higher than that in SGC7901-NC cells. Meanwhile, after treatment with LMP2A siRNA, the expression of LMP2A was significantly decreased in GT38 cells, and the expression of CXCR4 was down-regulated at the protein level (Fig.1d). Fig.1e showed the up-regulated expression of LMP2A and EBNA1 in SNU719-CXCR4 cell line that stably expressed CXCR4. In addition, Western blot was used to detect the expression of LMP2A and EBNA1 related proteins after CXCR4 siRNA treatment (Fig.1f). The results showed that CXCR4 siRNA significantly reduced the expression of LMP2A and EBNA1 in SNU719 cells compared with the control group.

3.2 LMP2A regulates CXCR4 expression through the NFκB pathway

Data showed that CXCR4 protein expression increased in SGC7901-LMP2A cells with stable LMP2A expression. We intend to further explore the specific ways in which LMP2A regulates CXCR4. In a follow-up validation experiment, we found higher expression levels of NF-κB pathway related protein p65 and phosphorylated p65(p-p65) in SGC7901 compared with the control group (Fig.2a). Meanwhile, the expression of p65 and p-p65 in GT38 cells was significantly decreased after LMP2A siRNAs treatment (Fig.2b). In order to make the experimental results more comprehensive and accurate, the concentrations of 2 μM and 4 μM of NF-κB inhibitor BAY-7085 were cultured in six-well plates inoculated with GT38 cells for 24h, respectively. Western blot results showed that NF-κB pathway related proteins were significantly reduced in the inhibitor group and showed a dose- dependent manner (Fig.2c). Similarly, when 2 μM and 4 μM NF-κB inhibitor BAY-7085 were added to SNU719 cells, NF-κB pathway related proteins were significantly reduced in the inhibitor group, also showing a dose-dependent manner (Fig.2d).

3.3 CXCR4regulates LMP2A and EBNA1 expression through the ERK pathway

190 In SNU719-CXCR4 cells that stably expressed CXCR4, the protein expressions of LMP2A
191 and EBNA1 were increased. It was further found that CXCR4 overexpression induced the
192 expression of ERK and phosphorylated ERK(p-ERK) (Fig.3a). Meanwhile, after CXCR4 siRNA
193 treatment, the expressions of ERK and p-ERK in GT38 and SNU719 cells were significantly
194 decreased (Fig.3b, Fig.3c). In order to further understand the mechanism of CXCR4 overexpression
195 leading to the up-regulation of LMP2 and EBNA1, the ERK1/2 inhibitor PD0325901 at 2 μ M and
196 4 μ M concentrations was cultured in six-well plates inoculated with GT38 cells for 48h, respectively.
197 Western-blot results showed that p-ERK was significantly reduced in the inhibitor treatment group,
198 and LMP2A and EBNA1 were also reduced to different degrees in a dose-dependent trend (Fig.4a).
199 Similarly, 2 μ M and 4 μ M ERK1/2 inhibitor PD0325901 were added to SNU719 cells, respectively,
200 with the same results as GT38 cell line (Fig.4b). In addition, we found that knockdown ERK 1/2 had
201 a strong inhibitory effect on the expression of LMP2A and EBNA1 (Fig.4c, Fig.4d). In conclusion,
202 these results suggest that LMP2A and EBNA1 expression in EBVaGC is partially dependent on the
203 CXCR4-ERK pathway.

204 **3.4 Effects of CXCR4 on cell migration, proliferation and maintenance of EBV latent** 205 **infection**

206 CXCR4 was overexpressed in SNU719 to explore the effect of CXCR4 on cell phenotypic
207 function. The effect of CXCR4 on the migration ability of SNU719 cells was detected by Transwell
208 migration experiment. Fig.5a showed that the overexpression of CXCR4 significantly promoted the
209 migration of SNU719-CXCR4 cells compared with SNU719-NC cells. CCK-8 detection showed
210 that CXCR4 overexpression significantly increased the OD value of SNU719 cells (Fig.5b). In
211 addition, the Western blot results showed that compared with the control group, the protein
212 expressions of EMT-related proteins N-Cadherin, MMP-2, and Vimentin in SN719-CXCR4 were
213 increased, while the expression of epithelial Cadherin E-Cadherin was decreased. (Fig.5c). In
214 addition, we further investigated the effect of CXCR4 expression on the latency of EBV based on
215 the discovery that CXCR4 promotes LMP2A and EBNA1, the latent genes encoded by EBV.
216 SNU719 cells were treated with DMSO, DMSO+ transfected CXCR4, TPA and TPA+ transfected

CXCR4 respectively. The expression of BZLF1 in TPA treated cells was significantly higher than that in the other three groups. Decreased BZLF1 expression was observed in TPA+ transfected CXCR4 group. In addition, the expression of BZLF1 in DMSO+ transfected CXCR4 group was lower than that in the other three groups (Fig.5d). These data suggest that CXCR4 can inhibit the expression of BZLF1 and promote the expression of LMP2A and EBNA1, the latent genes encoding EBV. In conclusion, CXCR4 promotes EBVaGC cell migration and proliferation, and may play an important role in maintaining EBV latent infection during EBVaGC development.

4 Discussion

CXCR4 is a seven-transmembrane G-protein-coupled receptor, with chemokine 12 (CXCL12) as its typical ligand [11]. CXCR4 is highly expressed in various types of tumor cells and tissues, and can be involved in tumor growth, invasion, angiogenesis and metastasis, such as breast cancer, colorectal cancer, pancreatic cancer, etc. [13, 17, 23]. Studies have shown that CXCR4 is regulated by a variety of factors, including its activation by binding to the chemokine CXCL12, and activation by macrophage migration inhibitory factor (MIF) and extracellular ubiquitin [1, 22]. In addition, EBV-encoded products can also regulate the expression of CXCR4. Huo *et al.* found that EBNA1, as a tumor promoter, enhanced the chemoinductance of Treg cells by targeting CXCL12-CXCR4 to promote NPC immune escape [9]. In Xu's study, the results showed that in NPC, LMP1 affects cell motility and invasion by inducing CXCR4 [33]. According to the different expression patterns of EBV protein, EBVaGC was classified as latent type I or II, mainly expressing EBER, EBNA1, LMP2A and other latent products [6]. In this study, CXCR4 expression increased or decreased in EBVaGC cells as LMP2A was overexpressed or knocked down. In addition, CXCR4 overexpression can up-regulate the expression of LMP2A and EBNA1, and knockdown CXCR4 can also reduce the expression of LMP2A and EBNA1. In conclusion, there is a positive feedback between CXCR4 and EBV encoding products to influence the occurrence and development of EBVaGC, which is regulated by some mechanism.

The results of Tang *et al* showed a new mechanism of NF- κ B/NRF-1/CXCR4 circuit-mediated TMP inhibition of neovascularization [27]. At the same time, many studies have shown that CXCR4

244 can affect the occurrence and development of tumors through the NF- κ B pathway, and the NF κ B-
245 CXCR4 axis can also mediate the biological phenotype of tumors. By adding inhibitors of NF- κ B
246 pathway into GT38 cells, we found that the expression of CXCR4 decreased with the increase of
247 inhibitor concentration, and the result was the same in SNU719. It was demonstrated that the NF-
248 κ B pathway activates CXCR4 in EBVaGC. In addition, CXCR4 binding to its ligand induces
249 conformational changes in itself that activate MAPK/ERK, PI3K/AKT, and JAK/STAT signaling
250 pathways [5, 8, 12, 19, 24, 26, 29]. Chang *et al.* found that CXCR4 activates phosphorylation of
251 Ras/Raf/MEK/ERK pathway conjunctin Shc, thereby regulating cell cycle progression [3]. In this
252 study, we found that overexpression or knockdown of CXCR4 in EBVaGC cells increased or
253 decreased total ERK protein and phosphorylated ERK protein, confirming that the activation of ERK
254 signaling pathway is related to CXCR4 expression.

255 Our previous studies have demonstrated that LMP2A, the EBV coding product, induces NF-
256 κ B activation by regulating the expression of I κ B α and TRAF1. Structural activation of NF- κ B is
257 involved in the malignant progression of EBVaGC by upregulation of genes involved in
258 proliferation, anti-apoptosis, and the maintenance of latent infection [35]. In this study, LMP2A
259 plasmid was transfected into EBV negative gastric cancer cells and LMP2A-siRNA was transfected
260 into EBV positive gastric cancer cells. We demonstrated that LMP2A could induce the activation of
261 NF- κ B. It was verified that the EBV coding product LMP2A activated CXCR4 through the NF- κ B
262 pathway. In order to further study the interaction between CXCR4 and EBV coding products in
263 EBVaGC, based on the activation of ERK signaling pathway by CXCR4, we added inhibitors of
264 ERK pathway in EBV positive cells, and found that the expression of LMP2A and EBNA1
265 decreased with the increase of inhibitor concentration, and the results were the same in SNU719. At
266 the same time, we also transfected ERK1/2-siRNAs into EBV positive gastric cancer cells, and the
267 result was the same as that of adding inhibitors. The results of Liu *et al.* showed that the double-
268 target knockout of miR-21 and CXCR4 could more effectively inhibit the proliferation, migration,
269 invasion and growth of glioma, and promote cell apoptosis, which may be related to PI3K/AKT and
270 Raf/MEK/ERK signaling pathways [14]. CXCR4-ERK signaling pathway mediates proliferation,

271 migration, and invasion of cancer cells in multiple types of tumors [2, 15, 18]. In this study, EBV
272 positive cell SNU719-CXCR4 was transfected with CXCR4 plasmid and screened by G418, and
273 SNU719-CXCR4 cell line was successfully established. We found that after CXCR4 overexpression,
274 ERK pathway signal increased, EBV coding products LMP2A and EBNA1 were significantly and
275 stably increased, and the cell migration and proliferation ability of SNU719-CXCR4 group was
276 significantly higher than that of the control group, and the expression level of EMT-related proteins
277 was also significantly increased. In conclusion, EBV-encoded LMP2A activates CXCR4 through
278 the NF- κ B pathway, and CXCR4 increases the expression of LMP2A and EBNA1 through the ERK
279 pathway, and affects related cell biological functions. LMP2A-NF- κ B-CXCR4 and CXCR4-ERK-
280 LMP2A/EBNA1 may be potential therapeutic targets.

281 EBV has two life cycles: latent infection and lytic infection. During the latent infection, EBV
282 DNA exists in the nucleus of infected cells and only expresses a small amount of latent proteins
283 such as EBNA1 and LMP2A. After infection, EBV remains latent in the host cell, but to produce
284 the virus, it must induce the lytic phase of the virus cycle. [10]. BZLF1, an immediate early protein
285 of EBV, regulates the transition from latent infection to lytic infection. We found that BZLF1 was
286 significantly up-regulated in SNU719-NC cells with PKC activator (PMA/TPA) added. The
287 expression of BZLF1 was down-regulated in SNU719-CXCR4 cells. These results suggested that
288 CXCR4 was a negative regulator of BZLF1 expression. Meanwhile, the expression levels of LMP2A
289 and EBNA1 in SNU719-CXCR4 cells were significantly higher than those in SNU719-NC cells.
290 CXCR4 was confirmed to be involved in maintaining latent EBV infection by inhibiting BZLF1 and
291 subsequently inducing potential EBV products LMP2A and EBNA1.

292 In conclusion, there is a positive feedback between EBV latency coding products and CXCR4,
293 and LMP2A can activate CXCR4 through the NF- κ B pathway. In addition, CXCR4 can be fed back
294 to LMP2A and EBNA1 through the ERK signaling pathway. Meanwhile, CXCR4 can reduce the
295 expression of the immediate early protein BZLF1, which plays an important role in maintaining the
296 latency infection of EBV. Therefore, the interaction of EBV coding products with CXCR4 is
297 important in the development of EBVaGC and provides a therapeutic option for successful

298 treatment. However, the specific mechanisms need to be further studied to provide further reliable
299 basis for targeted therapy.

300 **Statements and Declarations**

301 The authors declare that they have no competing interests.

302 **Author contributions**

303 Bing Luo and Ni Qin conceived and designed the experiments; Ni Qin, Yan Zhang, Lin Xu,
304 and Wen Liu performed the experiments; Ni Qin, Yan Zhang and Lin Xu analyzed the experimental
305 data; Ni Qin drafted the manuscript; Ni Qin and Yan Zhang revised the manuscript. All authors
306 reviewed and approved the final manuscript.

307 **Ethical approval and consent to participate**

308 We declare that this manuscript does not involve animal or human experiments, and does not
309 involve informed consent.

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443 **Figure legends:**

444 **Fig1: Interaction between LMP2A and CXCR4 at the expression level.**

445 (a) Fluorescence efficiency of SNU719 and SGC7901 cells transfected with plasmids. Original
446 magnification $\times$ 200 for all panels. (b) Relative expression of LMP2A and CXCR4 were calculated
447 using the comparative Cycle threshold (Ct) value ($2^{-\Delta\Delta C_t}$) and actin as the internal standard. Data
448 was represented as mean $\pm$ standard error of mean (SEM) and came from at least three replicates.

449 (c) The protein level of CXCR4 in SGC7901-LMP2A cell was detected by Western blot ($*p < 0.05$).
 450 (d) The effects on LMP2A and CXCR4 were analyzed by Western blot after interference of LMP2A
 451 in GT38 cell line ($**p < 0.01$, $*p < 0.05$, ns: no significant). (e) The protein level of EBNA1, LMP2A
 452 and CXCR4 in SNU719-CXCR4 cell was detected by Western blot ($***p < 0.001$, $*p < 0.05$). (f) The
 453 effects on EBNA1, LMP2A and CXCR4 were analyzed by Western blot after interference of CXCR4
 454 in SNU719 cell line ($**p < 0.01$, $*p < 0.05$). Data are representative of three independent
 455 experiments.

456 **Fig2: LMP2A regulates CXCR4 expression through the NF- κ B pathway.**

457 (a) The protein level of p65 and p-p65 in SGC7901-LMP2A cell was detected by Western blot ($**p$
 458 < 0.01). (b) The effects on p65, p-p65 and LMP2A were analyzed by Western blot after interference
 459 of LMP2A in GT38 cell line ($**p < 0.01$, $*p < 0.05$). (c) Protein expression of I κ B α , p-I κ B α , p65,
 460 p-p65 and CXCR4 in GT38 after treated with 2 μ M and 4 μ M BAY -7085 for 24 h were analyzed by
 461 Western blot. β -actin served as a control of loading. Densitometry values of the bands were
 462 expressed as fold-increases above the DMSO control (lane 1 and lane3) ($*p < 0.05$, ns: no
 463 significant). (d) Protein expression of I κ B α , p-I κ B α , p65, p-p65 and CXCR4 in SNU719 after treated
 464 with 2 μ M and 4 μ M BAY -7085 for 24 h were analyzed by Western blotting. β -actin served as a
 465 control of loading. Densitometry values of the bands were expressed as fold-increases above the
 466 DMSO control (lane 1 and lane3) ($**p < 0.01$, $*p < 0.05$, ns: no significant). Data are representative
 467 of three independent experiments.

468 **Fig3: The effect of CXCR4 on ERK signal pathway.**

469 (a) The protein level of ERK, p-ERK and CXCR4 in SNU719-CXCR4 cell was detected by Western
 470 blot ($*p < 0.05$). (b) The effects on ERK, p-ERK and CXCR4 were analyzed by Western blot after
 471 interference of CXCR4 in GT38 cell line ($**p < 0.01$, $*p < 0.05$). (c) The effects on ERK, p-ERK
 472 and CXCR4 were analyzed by Western blot after interference of CXCR4 in SNU719 cell line ($**p$
 473 < 0.01 , $*p < 0.05$). Data are representative of three independent experiments.

474 **Fig4: The effect of ERK signaling on LMP2A and EBNA1 expression.**

475 (a) Protein expression of ERK, p-ERK, LMP2A and EBNA1 in GT38 after treated with 2 μ M and 4

476 μ M PD0325901 for 48 h were analyzed by western blotting. β -actin served as a control of loading.
 477 Densitometry values of the bands were expressed as fold-increases above the DMSO control (lane
 478 1 and lane3) ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$, ns: no significant). (b) Protein expression of ERK,
 479 p-ERK, LMP2A and EBNA1 in SNU719 after treated with 2 μ M and 4 μ M PD0325901 for 48 h
 480 were analyzed by Western blot. β -actin served as a control of loading. Densitometry values of the
 481 bands were expressed as fold-increases above the DMSO control (lane 1 and lane3)
 482 ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$). (c) The effects on ERK, p-ERK, LMP2A and EBNA1 were
 483 analyzed by Western blot after interference of ERK in GT38 cell line ($**p < 0.01$, $*p < 0.05$, ns: no
 484 significant). (d) The effects on ERK, p-ERK, LMP2A and EBNA1 were analyzed by Western blot
 485 after interference of ERK in SNU719 cell line ($**p < 0.01$, $*p < 0.05$, ns: no significant). Data are
 486 representative of three independent experiments.

487 **Fig5: Effect of CXCR4 on cell migration and proliferation and maintenance of EBV latent**
 488 **infection.**

489 (a) The role of CXCR4 on cell migration were assessed by transwell migration assay at 24 h under
 490 the microscope (200x). (b) The proliferative capacity of CXCR4 was evaluated by CCK-8 assay in
 491 SNU719-CXCR4 cell ($**p < 0.01$, $*p < 0.05$). (c) The protein level of N-cadherin, E-cadherin,
 492 MMP-2, Vimentin and CXCR4 in SNU719-CXCR4 cell was detected by Western blot ($**p < 0.01$,
 493 $*p < 0.05$). Data are representative of three independent experiments. (d) Western blotting was used
 494 to compare BZLF1, CXCR4, LMP2A and EBNA1 protein expression in the DMSO,
 495 DMSO+transfected CXCR4,TPA and TPA+transfected CXCR4 groups. The averages of three
 496 independent replicates are shown.

497 **Fig6: Graphic summary.**

498 EBV encoded latent membrane protein 2A activates seven transmembrane proteins CXCR4 through
 499 the NF- κ B signaling pathway, and CXCR4 upregulates EBV encoded latent membrane protein 2A
 500 and EBV nuclear antigen 1 through the ERK signaling pathway.

501

Figures

Figure 1

Interaction between LMP2A and CXCR4 at the expression level. (a) Fluorescence efficiency of SNU719 and SGC7901 cells transfected with plasmids. Original magnification $\times 200$ for all panels. (b) Relative expression of LMP2A and CXCR4 were calculated using the comparative Cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$) and actin as the internal standard. Data was represented as mean $\pm$ standard error of mean (SEM) and came from at least three replicates. (c) The protein level of CXCR4 in SGC7901-LMP2A cell was detected by Western blot ($*p < 0.05$). (d) The effects on LMP2A and CXCR4 were analyzed by Western blot after interference of LMP2A in GT38 cell line ($**p < 0.01$, $*p < 0.05$, ns: no significant). (e) The protein level of EBNA1, LMP2A and CXCR4 in SNU719-CXCR4 cell was detected by Western blot ($***p < 0.001$, $*p < 0.05$). (f) The effects on EBNA1, LMP2A and CXCR4 were analyzed by Western blot after

interference of CXCR4 in SNU719 cell line (**p < 0.01, *p < 0.05). Data are representative of three independent experiments.

Figure 2

LMP2A regulates CXCR4 expression through the NF-κB pathway. (a) The protein level of p65 and p-p65 in SGC7901-LMP2A cell was detected by Western blot (**p < 0.01). (b) The effects on p65, p-p65 and LMP2A were analyzed by Western blot after interference of LMP2A in GT38 cell line (**p < 0.01, *p < 0.05). (c) Protein expression of IκBα, p-IκBα, p65, p-p65 and CXCR4 in GT38 after treated with 2μM and 4 μM BAY -7085 for 24 h were analyzed by Western blot. β-actin served as a control of loading. Densitometry values of the bands were expressed as fold-increases above the DMSO control (lane 1 and lane3) (*p < 0.05, ns: no significant). (d) Protein expression of IκBα, p-IκBα, p65, p-p65 and CXCR4 in SNU719 after treated with 2μM and 4 μM BAY -7085 for 24 h were analyzed by Western blotting. β-actin served as a control of loading. Densitometry values of the bands were expressed as fold-increases above the DMSO control (lane 1 and lane3) (**p < 0.01, *p < 0.05, ns: no significant). Data are representative of three independent experiments.

Figure 3

The effect of CXCR4 on ERK signal pathway. (a) The protein level of ERK, p-ERK and CXCR4 in SNU719-CXCR4 cell was detected by Western blot (* $p < 0.05$). (b) The effects on ERK, p-ERK and CXCR4 were analyzed by Western blot after interference of CXCR4 in GT38 cell line (** $p < 0.01$, * $p < 0.05$). (c) The effects on ERK, p-ERK and CXCR4 were analyzed by Western blot after interference of CXCR4 in SNU719 cell line (** $p < 0.01$, * $p < 0.05$). Data are representative of three independent experiments.

Figure 4

The effect of ERK signaling on LMP2A and EBNA1 expression. (a) Protein expression of ERK, p-ERK, LMP2A and EBNA1 in GT38 after treated with 2μM and 4 μM PD0325901 for 48 h were analyzed by western blotting. β-actin served as a control of loading. Densitometry values of the bands were expressed as fold-increases above the DMSO control (lane 1 and lane3) (**p<0.001, **p < 0.01, *p < 0.05, ns: no significant). (b) Protein expression of ERK, p-ERK, LMP2A and EBNA1 in SNU719 after treated with 2μM and 4 μM PD0325901 for 48 h were analyzed by Western blot. β-actin served as a control of loading. Densitometry values of the bands were expressed as fold-increases above the DMSO control (lane 1 and lane3) (**p<0.001, **p < 0.01, *p < 0.05). (c) The effects on ERK, p-ERK, LMP2A and EBNA1 were analyzed by Western blot after interference of ERK in GT38 cell line (**p < 0.01, *p < 0.05, ns: no significant). (d) The effects on ERK, p-ERK, LMP2A and EBNA1 were analyzed by Western blot after interference of ERK in SNU719 cell line (**p < 0.01, *p < 0.05, ns: no significant). Data are representative of three independent experiments.

Figure 5

Effect of CXCR4 on cell migration and proliferation and maintenance of EBV latent infection. (a) The role of CXCR4 on cell migration were assessed by transwell migration assay at 24 h under the microscope (200x). (b) The proliferative capacity of CXCR4 was evaluated by CCK-8 assay in SNU719-CXCR4 cell (** $p < 0.01$, * $p < 0.05$). (c) The protein level of N-cadherin, E-cadherin, MMP-2, Vimentin and CXCR4 in SNU719-CXCR4 cell was detected by Western blot (** $p < 0.01$, * $p < 0.05$). Data are representative of three independent experiments. (d) Western blotting was used to compare BZLF1, CXCR4, LMP2A and EBNA1 protein expression in the DMSO, DMSO+transfected CXCR4,TPA and TPA+transfected CXCR4 groups. The averages of three 495 independent replicates are shown.

Figure 6

Graphic summary. EBV encoded latent membrane protein 2A activates seven transmembrane proteins CXCR4 through the NF- κ B signaling pathway, and CXCR4 upregulates EBV encoded latent membrane protein 2A and EBV nuclear antigen 1 through the ERK signaling pathway.