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Research Article

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Study on the mechanism of LMP2A maintaining Epstein-Barr virus latency infection through interaction with CXCR4

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Abstract

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

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

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

EBV can participate in the regulation of multiple signaling pathways through its encoded latent genes, such as NF-\( \kappa \)B, JNK, PI3K/AKT, etc., and then regulate the expression of downstream target genes to promote the occurrence and development of tumors. Longnecker et al. found that LMP2A could simulate B cell receptor (BCR) signaling and regulate the activation of related signaling pathways through its cytoplasmic n-terminal region [16]. In EBVaGC, LMP2A activates NF-\( \kappa \)B 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-ΔΔCt), 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′-TCCTGTGGCATACTTTCA-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 5 min for protein denaturation. The proteins obtained were separated by
10% SDS–PAGE, and were transferred onto PVDF membranes (Millipore, USA) using a transfer device. The blotted PVDF membranes were blocked with 5% non-fat milk for 2h at room temperature and incubated with the primary antibody at 4°C overnight, followed by a second antibody for incubating 2h. Finally, proteins of interest were visualized using the enhanced chemiluminescence detection system. The specific antibodies used were as follows: Anti-β-actin, anti-ERK1/2, anti-phospho -ERK1/2, anti-NF-κB p65, anti-NF-κB p-p65, anti-IκBα, anti-LMP2A, anti-MMP-2, anti-Vimentin, HRP-linked anti-mouse/rabbit secondary antibody .All above antibodies were purchased from CST (Mass, USA), at a dilution of 1∶1000. Anti-CXCR4, anti-E-cadherin, anti-N-cadherin antibodies were purchased from Abcam (USA) with a dilution of 1∶1000. anti-phospho-IκBα, anti-BZLF1, anti-EBNA1 antibodies were purchased from SANTA (USA) with a dilution of 1∶200.

2.4 Transfection with siRNAs of LMP2A, CXCR4 and ERK

The siRNA target sequences for LMP2A, CXCR4 and ERK mRNA were designed and synthesized by GenePharma (China). The sequences are as follows: hs-CXCR4-si-1: 5′ - AGAUUAUCACUUCAAGUAAdTd - 3′, 5′ - UUAUCUGAAGUGUAUA
UCUdTd - 3′, hs-CXCR4-si-2: 5′ - GAAGCAUGACGGACAAGUAdTd - 3′, 5′ - UACUUGUCGUCAUGCUUCdTd - 3′, hs-MAPK3/ERK1: 5′ - GAAACUACCUACAGUCUTT - 3′, 5′ - GAACGUACUGAGGUAGUUUCTT - 3′, hs-MAPK1/ERK2: 5′ - GUGCUCUGCUUAAGUAATT - 3′, 5′ - AUUAUCAUGACAGACATTTT - 3′, hs-EBV-LMP2A-1: 5′ - GAACGAUGGAGGAACGUGAAAdTd - 3′, 5′ - UUCACGUUCCUCAUCGUUCdTd - 3′, hs-EBV-LMP2A-2: 5′ - GACUUAUCAACCACUAGGAAAdTd - 3′, 5′ - UUCCUAGUGGUAUAGUCdTd - 3′. 50μM siRNA was transfected using Lipofectamine 2000 Reagent (Invitrogen, Thermo Fisher Scientific, Germany). Then, the cells were harvested to assay relative gene expression. All experiments were performed in triplicate.

2.5 CCK-8 cell proliferation assay

Cell proliferation was detected using the Cell Counting Kit 8 (CCK8, Boster Biological
Technology, China). The cells were seeded at $5 \times 10^3$ cells/100 μl per well into 96-well plates. After the cells were attached to the wall, added 10μl CCK-8 solution to each well, after incubation for 24, 48, 72 and 96h, respectively. And then the cells were incubated for an additional 1h at 37°C, the absorbance was measured using Soft-Max apparatus (Bio-Tek ELx808, USA) at a wavelength of 450nm.

2.6 Migration assay

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

2.7 Overexpression of LMP2A and CXCR4

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

2.8 Statistical analysis

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

3 Results

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 CXCR4 regulates LMP2A and EBNA1 expression through the ERK pathway
In SNU719-CXCR4 cells that stably expressed CXCR4, the protein expressions of LMP2A and EBNA1 were increased. It was further found that CXCR4 overexpression induced the expression of ERK and phosphorylated ERK (p-ERK) (Fig. 3a). Meanwhile, after CXCR4 siRNA treatment, the expressions of ERK and p-ERK in GT38 and SNU719 cells were significantly decreased (Fig. 3b, Fig. 3c). In order to further understand the mechanism of CXCR4 overexpression leading to the up-regulation of LMP2 and EBNA1, the ERK1/2 inhibitor PD0325901 at 2 μM and 4 μM concentrations was cultured in six-well plates inoculated with GT38 cells for 48h, respectively. Western-blot results showed that p-ERK was significantly reduced in the inhibitor treatment group, and LMP2A and EBNA1 were also reduced to different degrees in a dose-dependent trend (Fig. 4a).

Similarly, 2μM and 4 μM ERK1/2 inhibitor PD0325901 were added to SNU719 cells, respectively, with the same results as GT38 cell line (Fig. 4b). In addition, we found that knockdown ERK1/2 had a strong inhibitory effect on the expression of LMP2A and EBNA1 (Fig. 4c, Fig. 4d). In conclusion, these results suggest that LMP2A and EBNA1 expression in EBVaGC is partially dependent on the CXCR4-ERK pathway.

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

CXCR4 was overexpressed in SNU719 to explore the effect of CXCR4 on cell phenotypic function. The effect of CXCR4 on the migration ability of SNU719 cells was detected by Transwell migration experiment. Fig. 5a showed that the overexpression of CXCR4 significantly promoted the migration of SNU719-CXCR4 cells compared with SNU719-NC cells. CCK-8 detection showed that CXCR4 overexpression significantly increased the OD value of SNU719 cells (Fig. 5b). In addition, the Western blot results showed that compared with the control group, the protein expressions of EMT-related proteins N-Cadherin, MMP-2, and Vimentin in SN719-CXCR4 were increased, while the expression of epithelial Cadherin E-Cadherin was decreased. (Fig. 5c). In addition, we further investigated the effect of CXCR4 expression on the latency of EBV based on the discovery that CXCR4 promotes LMP2A and EBNA1, the latent genes encoded by EBV. 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-κB/NRF-1/CXCR4 circuit-mediated TMP inhibition of neovascularization [27]. At the same time, many studies have shown that CXCR4
can affect the occurrence and development of tumors through the NF-κB pathway, and the NFκB-CXCR4 axis can also mediate the biological phenotype of tumors. By adding inhibitors of NF-κB pathway into GT38 cells, we found that the expression of CXCR4 decreased with the increase of inhibitor concentration, and the result was the same in SNU719. It was demonstrated that the NF-κB pathway activates CXCR4 in EBVaGC. In addition, CXCR4 binding to its ligand induces conformational changes in itself that activate MAPK/ERK, PI3K/AKT, and JAK/STAT signaling pathways [5, 8, 12, 19, 24, 26, 29]. Chang et al. found that CXCR4 activates phosphorylation of Ras/Raf/MEK/ERK pathway conjunctin Shc, thereby regulating cell cycle progression [3]. In this study, we found that overexpression or knockdown of CXCR4 in EBVaGC cells increased or decreased total ERK protein and phosphorylated ERK protein, confirming that the activation of ERK signaling pathway is related to CXCR4 expression.

Our previous studies have demonstrated that LMP2A, the EBV coding product, induces NF-κB activation by regulating the expression of IκBα and TRAF1. Structural activation of NF-κB is involved in the malignant progression of EBVaGC by upregulation of genes involved in proliferation, anti-apoptosis, and the maintenance of latent infection [35]. In this study, LMP2A plasmid was transfected into EBV negative gastric cancer cells and LMP2A-siRNA was transfected into EBV positive gastric cancer cells. We demonstrated that LMP2A could induce the activation of NF-κB. It was verified that the EBV coding product LMP2A activated CXCR4 through the NF-κB pathway. In order to further study the interaction between CXCR4 and EBV coding products in EBVaGC, based on the activation of ERK signaling pathway by CXCR4, we added inhibitors of ERK pathway in EBV positive cells, and found that the expression of LMP2A and EBNA1 decreased with the increase of inhibitor concentration, and the results were the same in SNU719. At the same time, we also transfected ERK1/2-siRNAs into EBV positive gastric cancer cells, and the result was the same as that of adding inhibitors. The results of Liu et al. showed that the double-target knockout of miR-21 and CXCR4 could more effectively inhibit the proliferation, migration, invasion and growth of glioma, and promote cell apoptosis, which may be related to PI3K/AKT and Raf/MEK/ERK signaling pathways [14]. CXCR4-ERK signaling pathway mediates proliferation,
migration, and invasion of cancer cells in multiple types of tumors [2, 15, 18]. In this study, EBV
positive cell SNU719-CXCR4 was transfected with CXCR4 plasmid and screened by G418, and
SNU719-CXCR4 cell line was successfully established. We found that after CXCR4 overexpression,
ERK pathway signal increased, EBV coding products LMP2A and EBNA1 were significantly and
stably increased, and the cell migration and proliferation ability of SNU719-CXCR4 group was
significantly higher than that of the control group, and the expression level of EMT-related proteins
was also significantly increased. In conclusion, EBV-encoded LMP2A activates CXCR4 through
the NF-κB pathway, and CXCR4 increases the expression of LMP2A and EBNA1 through the ERK
pathway, and affects related cell biological functions. LMP2A-NF-κB-CXCR4 and CXCR4-ERK-
LMP2A/EBNA1 may be potential therapeutic targets.

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

In conclusion, there is a positive feedback between EBV latency coding products and CXCR4,
and LMP2A can activate CXCR4 through the NF-κB pathway. In addition, CXCR4 can be fed back
to LMP2A and EBNA1 through the ERK signaling pathway. Meanwhile, CXCR4 can reduce the
expression of the immediate early protein BZLF1, which plays an important role in maintaining the
latency infection of EBV. Therefore, the interaction of EBV coding products with CXCR4 is
important in the development of EBVaGC and provides a therapeutic option for successful
treatment. However, the specific mechanisms need to be further studied to provide further reliable basis for targeted therapy.

**Statements and Declarations**

The authors declare that they have no competing interests.

**Author contributions**

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

**Ethical approval and consent to participate**

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

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Figure legends:

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

(a) Fluorescence efficiency of SNU719 and SGC7901 cells transfected with plasmids. Original magnification×200 for all panels. (b) Relative expression of LMP2A and CXCR4 were calculated using the comparative Cycle threshold (Ct) value (2^−ΔΔCt) and actin as the internal standard. Data was represented as mean ± 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.

**Fig2: 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 lane 3) (*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 lane 3) (**p < 0.01, *p < 0.05, ns: no significant). Data are representative of three independent experiments.

**Fig3: 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.

**Fig4: 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 lane 3) (**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 lane 3) (**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.

**Fig5:** 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 independent replicates are shown.

**Fig6:** Graphic summary.

EBV encoded latent membrane protein 2A activates seven transmembrane proteins CXCR4 through the NF-κB signaling pathway, and CXCR4 upregulates EBV encoded latent membrane protein 2A and EBV nuclear antigen 1 through the ERK signaling pathway.
Interaction between LMP2A and CXCR4 at the expression level. (a) Fluorescence efficiency of SNU719 and SGC7901 cells transfected with plasmids. Original magnification ×200 for all panels. (b) Relative expression of LMP2A and CXCR4 were calculated using the comparative Cycle threshold (Ct) value (2−ΔΔCt) and actin as the internal standard. Data was represented as mean ± 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
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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.
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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

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