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EBV down-regulates COX-2 expression via TRAF2 and ERK signal pathway in EBV-associated gastric cancer

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ABSTRACT

Epstein-Barr virus-associated gastric cancer (EBVaGC) accounts for nearly 10% of gastric cancer. Cyclooxygenase-2 (COX-2) plays a crucial role in cancer progression. However, there is no experimental study on the regulation mechanism of EBV on COX-2 in EBVaGC. To understand more about the tumorigenic mechanism of EBVaGC, the study investigated the role of EBV encode latent membrane protein LMP1 and LMP2A in the regulation of COX-2. The expression of COX-2 was examined in EBVaGC and EBV negative gastric cancer (EBVnGC) cell lines. The plasmids were transfected in SGC7901 to overexpress LMP1/2A. Small interfering RNA (si-RNA) targeting LMP1/2A in GT38 and targeting TRAF2 in SGC7901 were used to detect the expression of COX-2. Furthermore, si-ERK1/2 and the MEK inhibitor PD0325901 were used to investigate whether p-ERK participate in the regulation of COX-2 in SGC7901. The overexpression of LMP1 or LMP2A in SGC7901 down-regulates both COX-2 and TRAF2 expression, and knockdown of LMP1 or LMP2A in GT38 resulted in a certain recovery of COX-2 and TRAF2 expression. Moreover, si-TRAF2 indicated that a sharp down-regulation of COX-2. And the decrease of p-ERK also mediates the inhibitory effect of LMP1 on COX-2. In summary, overexpression of LMP1 and LMP2A inhibits COX-2, which is mediated by a decrease of TRAF2, and p-ERK is involved in the inhibition of COX-2 by LMP1 in gastric cancer.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous gamma-type oncogenic herpes virus that infects more than 90% of the population (Kim et al., 2015), and is increasingly reported in various malignancies such as certain T-cell lymphomas, Hodgkin's lymphoma (HL), Nasopharyngeal carcinoma (NPC) and gastric cancer (Zhang et al.). EBV-associated gastric cancer (EBVaGC) accounts for about 10% of global gastric cancer and ranges from 1.3% to 20.1% in different countries (Chen et al., 2012). EBVaGC has characteristic clinicopathological features, including advantages in males, proximal sites in the stomach, lymphoid epithelioid histology (Camargo et al., 2014; van Beek et al., 2004; Yau et al., 2014).

Latent membrane protein 1 (LMP1) and LMP2A are integrin expressed in EBV cell membranes and notable for playing a major role in the development of cancer (Lee et al., 2004; Vrzalikova et al., 2018). LMP1 can regulate epithelial cell migration and invasion pathways by inducing expression of IL-6 and IL-8 (Eliopoulos et al., 1999), epidermal growth factor receptor (EGFR) (Miller et al., 1995), matrix metalloproteinase (MMP) (Liu et al., 2003), and down-regulating the expression of E-cadherin (Fahraeus et al., 1992). However, LMP1 expression is rarely described in EBVaGC, and it was mentioned in a review by Ribeiro J et al. in 2017 that LMP1 was found to be present in only 10% (21/199) of EBVaGC cases (Ribeiro et al., 2017). Shin et al. (1996) and Ham et al.(Harn et al., 1995) reported 3/6 and 2/12 cases of LMP1 positive expression in EBV positive gastric cancer, respectively. Further investigation about the role of LMP1 in EBVaGC should be studied, because some authors believe that the low expression of LMP1 in EBVaGC is due to technical limitations (Imai et al., 1994; Shin et al., 1996), and other have shown that LMP1 expression can indeed be inhibited in gastric cancer to help tumor cells escape the immune surveillance of the host immune system (Sheu et al., 1998). It has been found that about 40%˜50% of EBVaGC expresses LMP2A, and its expression is closely related to poor survival results (Iizasa et al., 2012). LMP2A not only activates signaling pathways in epithelial cells including phosphoinositide 3-kinase (PI3K)/Akt, nuclear factor (NF)-κB, and signal transduction activator (STAT) (Lu et al., 2006; Pan et al., 2008), it also induces cell growth, enhances cell adhesion and

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Table 1
Sequences of primers for real-time PCR and small interfering RNA.

	Forward Primers Sequences (5'-3')	Reverse Primers Sequences (5'-3')
GAPDH	CAAATTCCATGGCACCGTCA	ATCGCCCCACTTGATTTTGG
LMP1	AATTTGCACGGACAGGCATT	GTGCGCCTAGGTTTTGAGAG
LMP2A	TGTCGCTGGCATACTCTTCA	GCGTGTTAGTCATCACCGTC
	Sense (5'-3')	**Antisense (5'-3')**
Negative control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
LMP1	AAGAGCCUUCUCUGUCCACUTT	AGUGGACAGAGAAGGCUCUUTT
LMP2A	GAACGAUGAGGAACGUGAAT	UUCACGUUCCUCAUCGUUCT
MAPK3(ERK1)	GAAACUACCUACAGUCUCUTT	AGAGACUGUAGGUAGUUUCTT
MAPK1(ERK2)	GUGCUCUGCUUAUGAUAAUTT	AUUAUCAUAAGCAGAGCACTTT
TRAF2#1	GCUCAUGCUGACCGAAUGUTT	ACAUUCGGUCAGCAUGAGCTT
TRAF2#2	UCUGGAAGAUCUCAGACUUTT	AAGUCUGAGAUCUUCCAGATT

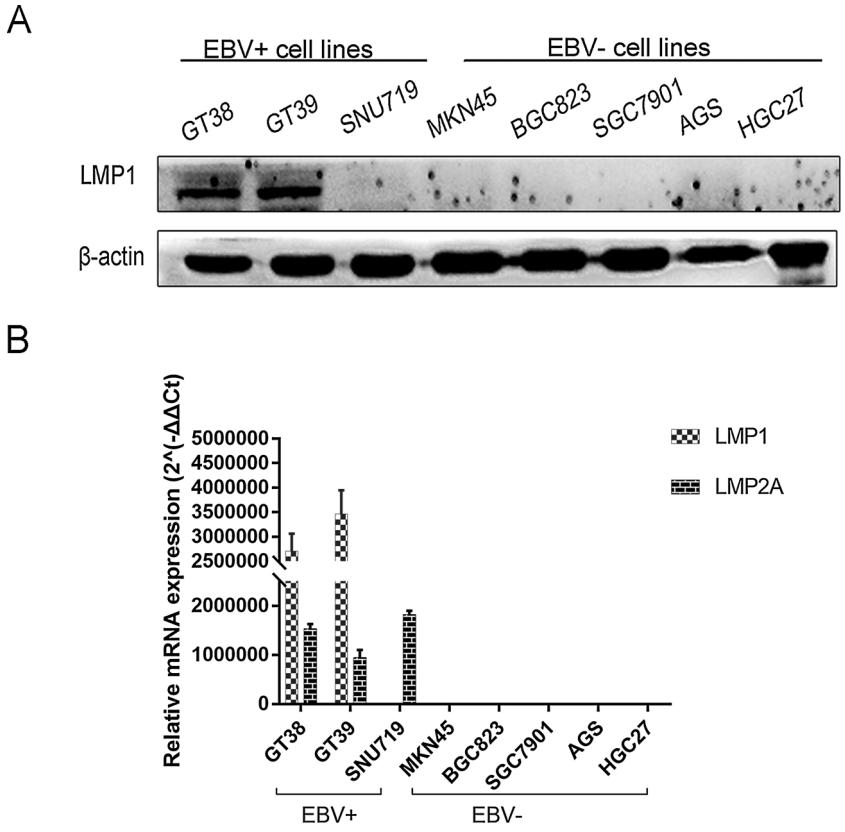

Fig. 1. The expression of LMP1 andLMP2A in EBV-associated/-negative gastric carcinoma (EBVa/nGC) cells.
A. Western-blot shows the expression level of LMP1 in gastric cancer cell lines. **B.** qRT-PCR shows the expression of LMP1 and LMP2A mRNA in gastric cancer cell lines

movement, and inhibits epithelial cell differentiation (Allen et al., 2005; Fotheringham et al., 2012), which indicated that it may be a potential target for gene therapy for EBVaGC therapy.

Cyclooxygenase-2 (COX-2) is an inducible isoenzyme of cyclooxygenase that catalyzes the formation of prostaglandin E2 (PGE2) in response to various inflammatory stimuli or growth factors (Nakao et al., 2002). Abnormalities of COX-2 expression are found in epithelial tumors such as rectal cancer, lung cancer, and breast cancer (Kim et al., 2017). Binding of an inducible transcription factor to a cis-acting element in the promoter mediates transcriptional activation of COX-2, including NF-κB, cyclic AMP response element (CRE) etc. (Hashemi Goradel et al., 2019; Hashemi Goradel et al., 2019). Studies have shown that COX-2 is frequently overexpressed in EBV positive tumors like NPC and HL (Al-Salam et al., 2013; Gandhi et al., 2015). CTAR1 and CTAR2, the two functional domains of LMP1, can induce COX-2 expression to promote NPC progression by activating the NF-κB signaling pathway (Murono et al., 2001). At the same time, one study revealed that EBV inhibits PGE2 biosynthesis in monocytes, which involves inhibition of inducible COX-2 isoform expression at the transcriptional and translational levels, possibly representing another mechanism of EBV pathogenicity (Savard et al., 2000). However, no studies have been found between LMP2A and COX-2 in any of the EBV-associated tumors. And whether LMP1 plays a role in COX-2 in EBVaGC is also requires further research.

The expression of tumor necrosis factor receptor-associated factor 2 (TRAF2) is significantly increased in gastric cancer tissues, which is associated with tumor invasion and metastasis, and is also an independent prognosis for patients with gastric cancer (Zhao et al., 2018). It binds to the carboxy-terminal cytoplasmic domain (CT) of LMP1 in vitro and is capable of co-precipitation with LMP1 in B lymphoblastoid cells (Kaye et al., 1996). As a key effector of NF-κB activation, LMP1 and LMP2A affect the expression of COX-2 by affecting TRAF2 was suspected. Numerous studies have shown that MAPK signaling pathway plays an important role in regulating the expression of COX-2 in a

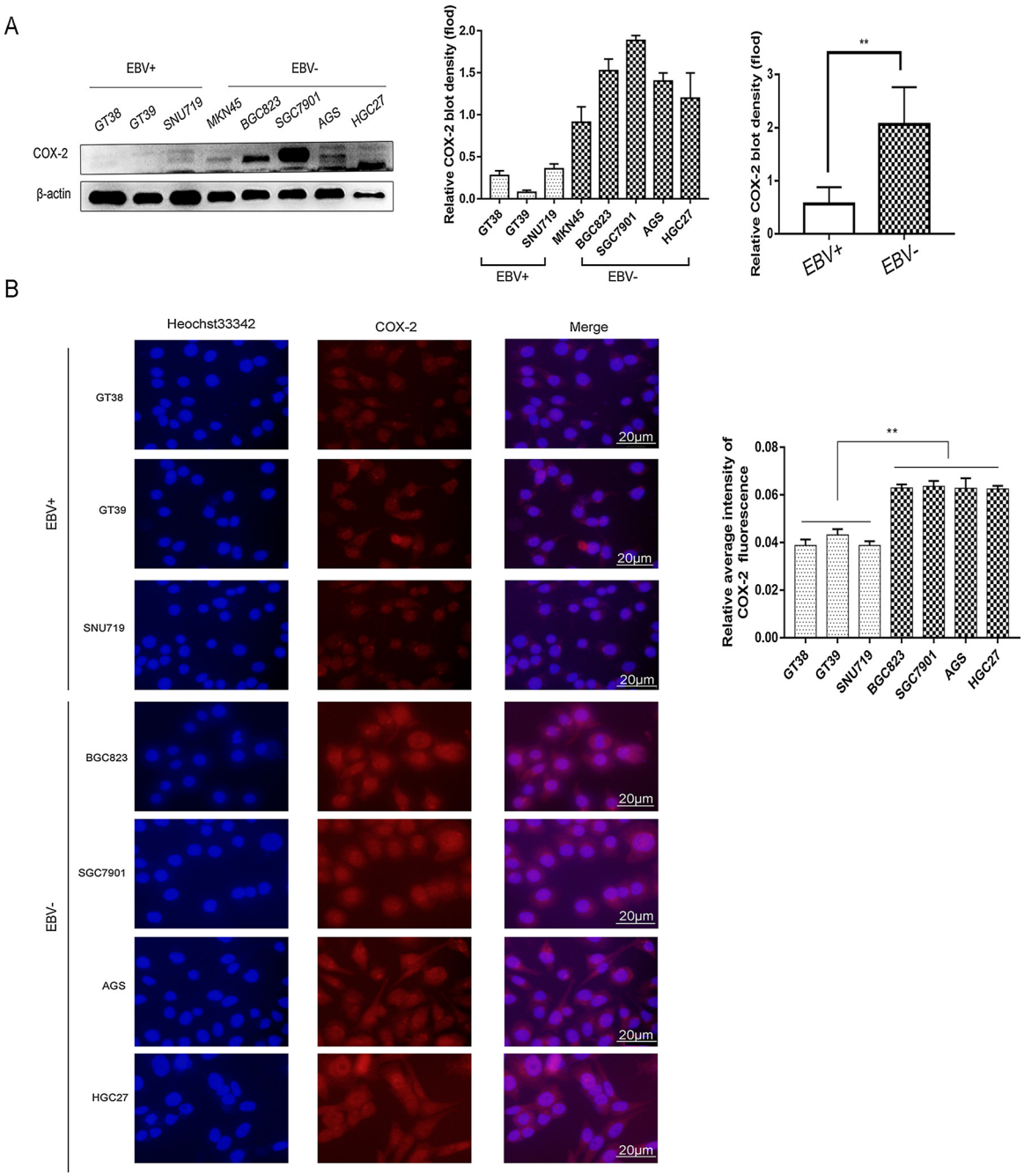

Fig. 2. The expression of COX-2 in EBVaGC and EBVnGC cells.
A. Western detection of protein expression of COX-2 in EBVaGC and EBVnGC cells, $^{**}p < 0.01$. **B.** COX-2 localization in GC cells by immunofluorescent staining (400×, 20 μm). Exponential optical density curve of COX-2 labeling measured by Image *J. The average fluorescence intensity of the EBV^{+} cell lines was significantly weaker than that of the EBV^{-}* cell lines, $^{**}p < 0.01$.

variety of tumors (Chi et al., 2016; Kitanaka et al., 2017; Patel et al., 2012) but in a test for early infection of Kaposi's sarcoma-associated herpesvirus (KSHV), ERK alone did not significantly inhibit COX-2 promoter activity, gene expression or protein levels or PGE2 secretion (Sharma-Walia et al., 2010). Will the ERK pathway play a role in the regulation of COX-2 expression by EBV in gastric cancer? Therefore, this study sought to explore the presence of TRAF2 regulation between EBV and COX-2, and whether p-ERK is involved in.

2. Materials and methods

2.1. Cell lines and culture conditions

3 EBVaGC cell lines GT38, GT39 and SNU719; 5 EBV-negative GC (EBVnGC) cell lines AGS, HGC27, BGC823, MKN45, and SGC7901 were used in this study. In GT38 and GT39, both LMP1 and LMP2A exist, and SNU719 expresses only LMP2A. SNU719 was a genuine gift provided by Qian Tao (Chinese University of Hong Kong). GT38 and GT39 were a grateful gift from Sarienji T (Tottori University). BGC823, this EBVnGC cell line was obtained from Chunkui Shao (Sun Yat-sen University). Mycoplasma contamination in cell lines is routinely tested. When

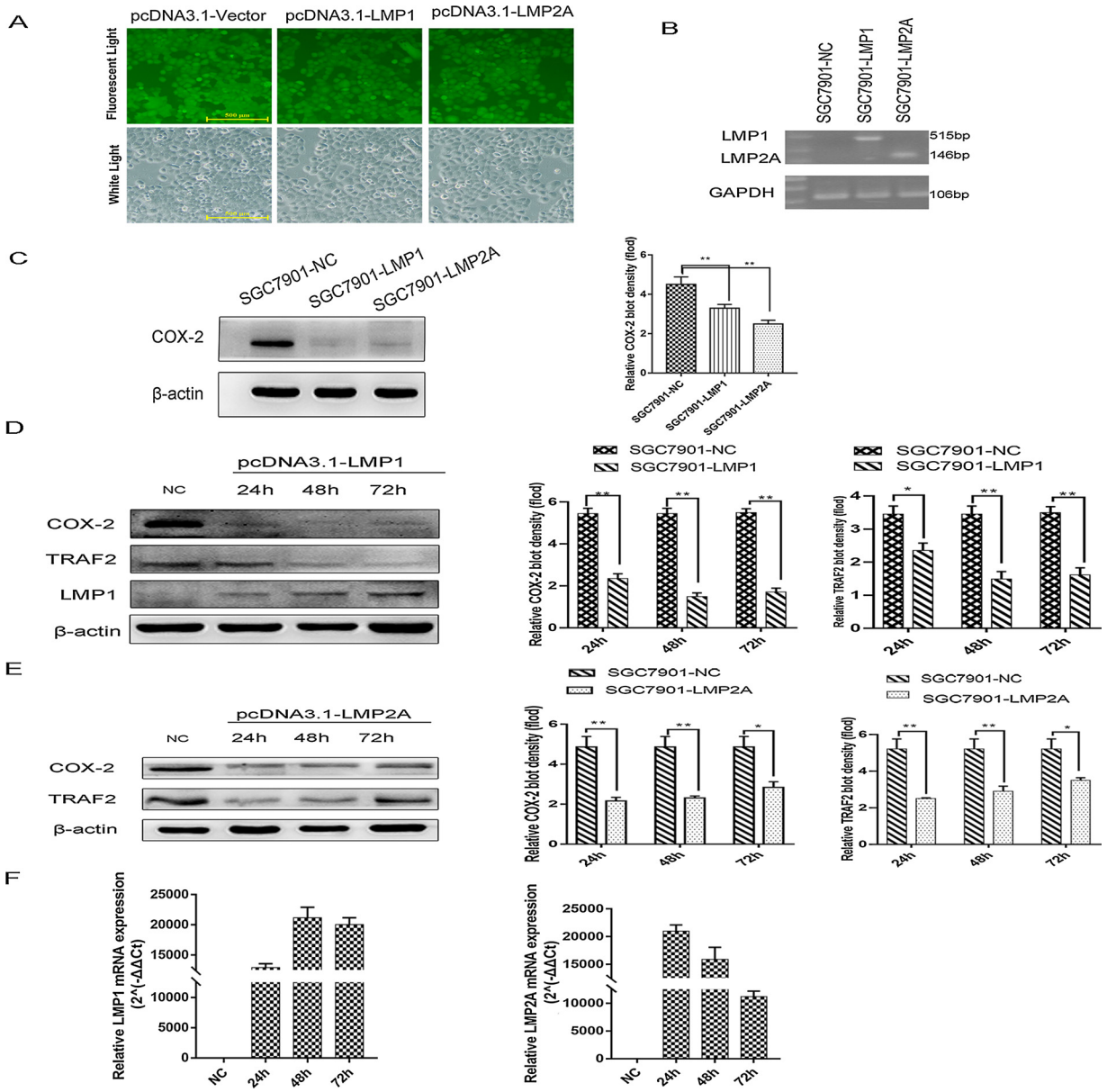

Fig. 3. EBV oncogenes latent membrane protein 1 (LMP1) and LMP2A decreased the expression of COX-2 and TRAF2.
A. SGC7901-NC, SGC7901-LMP1 and SGC7901-LMP2A, under a fluorescence microscope, respectively. **B.** The mRNA expression of LMP1 and LMP2A in transfected SGC7901 cells. **C.** The expression of COX-2 in SGC7901-LMP1 and SGC7901-LMP2A was down-regulated compared to SGC7901-NC, $^{**}p < 0.01$. **D.** Transiently transferred LMP1 in SGC7901 to investigate the time-dependent effect of LMP1 on COX-2 and TRAF2 inhibition. **E.** Transiently transferred LMP2A in SGC7901 to investigate the time-dependent effect of LMP2A on COX-2 and TRAF2 inhibition. F. The relative mRNA expression level was determined after transfection of the LMP1 or LMP2A plasmid in SGC7901.

needed, cells were treated with PD0325901 (a potent inhibitor of ERK1/2 phosphorylation) in a six-well plate. The cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Thermo Fisher Scientific, Germany) supplemented with 10% fetal bovine serum (Biological Industries, Israel), 1% penicillin-streptomycin at 37℃ in a humidified incubator with 5% CO_2.

This study was approved by the Medical Ethics Committee at the Medical College of Qingdao University. All the methods mentioned in this manuscript were performed according to the approved guidelines.

2.2. RNA isolation and RT-PCR

Total RNA was isolated from the cultured cells using the TRIzol reagent (Ambion, USA) and then 1 μg of total RNA was reverse-transcribed into cDNA for detecting messenger RNA (mRNA) according to the manufacturer's protocol. cDNA was used as template and qRT-PCR was run in a Faststart Essential DNA Green Master kit (Roche) using a LightCycler96 SN10700 Sequence Detection System (Roche). Samples were performed biologically in triplicate and their relative expression was determined by normalizing expression of each target to GAPDH. The products were detected by 1.2% agarose gel electrophoresis. Primers used in these experiments were shown in Table 1.

2.3. Western-blot analysis

Whole-cell extracts were prepared from the treated cells with RIPA buffer containing 1% protease inhibitor phenylmethanesulfonylfluoride (PMSF) and 1% phosphatase inhibitors mixture. Cell lysates were centrifuged at 12,000 rpm for 15 min and the supernatant was collected. A bicinchoninic acid assay kit (CWBIO, Beijing, China) was

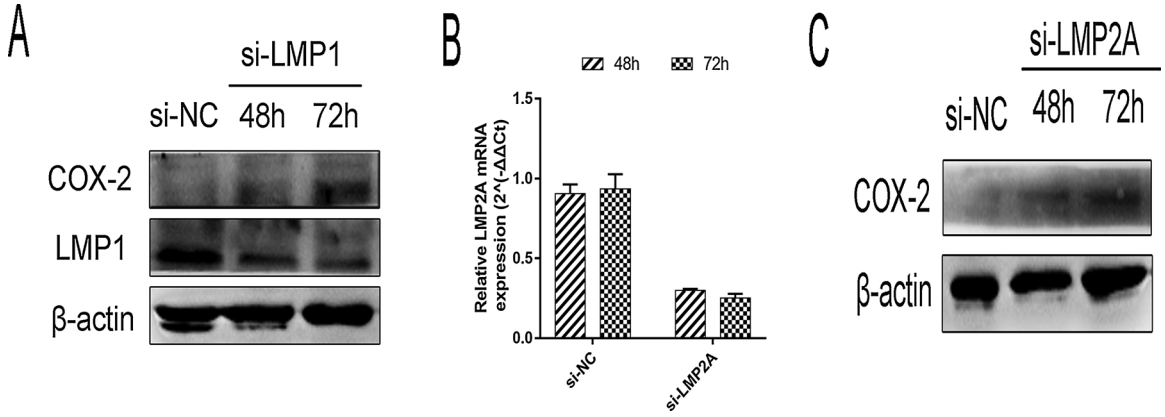

Fig. 4. Knockdown of LMP1 or LMP2A upregulated the expression of COX-2 in GT38.
A. Western-blot of COX-2 was performed after knockdown 48 h and 72 h of LMP1. **B.** The amount of mRNA expressed after 48 h and 72 h after knockdown of LMP2A. **C.** Western-blot of COX-2 was performed after knockdown 48 h and 72 h of LMP2A.

used to measure total protein concentration. Proteins were mixed with loading buffer (5x) and boiled for 5 min for denaturation, then were electrophoretically separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membranes. Then the membranes were blocked with 5% fat-free milk for 2 h at room temperature before being incubated overnight with primary antibodies against COX-2 (Abcam, Cambridge, UK, ab15901, 1:1000), anti-p-ERK1/2 antibody (CST, Chicago, USA, #43,701:1000), anti-TRAF2 antibody (CST, Chicago, USA, #4724 T, 1:1000), anti-LMP1 antibody (Abcam, Cambridge, UK, ab178113, 1:1000). After being washed in TBST three times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) (1:5000) for 2 h at room temperature. The protein bands were then detected using the enhanced chemiluminescence system. Anti-beta-actin antibody (Abcam, Cambridge, UK, ab8226, 1:1000) was used as a control for equal loading of samples.

2.4. Immunofluorescence assay

The plates were placed in an incubator overnight in which sterile coverslips and a density of 1, 2×10^5 cells per well were placed. The cells grown on the coverslips were manipulated in the following order, briefly washed with PBS, fixed with 4% paraformaldehyde for 10 min, and then blocked and permeabilized with buffer (1% BSA, 0.15% Triton-X100, 22.52 mg / ml glycine in PBS) at room temperature for 1 h. Incubate with COX-2 primary antibody (1:200) overnight at 4 °C. Fluorescent secondary antibodies conjugated to Alexa Fluor 555 (CST, USA) were incubated for 1 h at room temperature, and then the nuclei were counterstained for 5 min with Hoechst 33342 (Solaibio, China). Finally, coverslips were covered on a fluorescent anti-quenching agent (Beyotime, Shanghai, China), and the localization of COX-2 in the cells was observed with a fluorescence microscope (Nikon, Japan).

2.5. Transfection with small interfering RNAs (siRNAs) and Overexpression of LMP1 and LMP2A

The cells were seeded in a 6-well plate at 1×10^6 cells per well, and transfected with siRNA directed against LMP1 (EB-Virus), LMP2A (EB-Virus), ERK1 (MAPK3), ERK2 (MAPK1), TRAF2#1 and TRAF2#2, see Table 1 for the sequence. LMP1 and LMP2A were cloned into pcDNA3.1-EF1a-mcs-3flag-CMV-EGFP vector. 3 μg LMP1 (pcDNA3.1-LMP1), LMP2A (pcDNA3.1-LMP2A) or a negative control vector (pcDNA3.1-vector) plasmid was transfected into SGC7901 using the lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Germany) according to the manufacturer's protocol. Cells were harvested for assay at the indicated times. Stable transfection of LMP1 and LMP2A was obtained by geneticin screening and observed under fluorescence microscope. At the same time, GT38 expressed strong LMP1 and LMP2A, so we knocked down LMP1/2A in this cell line to detect if it could reverse the low expression of COX-2. Since the expression of TRAF2 and p-ERK are too low in EBVaGC, the small interfering RNA can not play a role in the cell or the effect is meaningless. Therefore, we used the EBVnGC cell line SGC7901 for interference inhibition test.

2.6. Statistical analysis

Statistical analyses were conducted using SPSS 19.0 statistical software (SPSS, Chicago, IL). Association between COX-2 expression levels were determined using the Chi-square ($\chi 2$) test and Fisher's exact test, as appropriate. The statistical analysis of EBV-negative versus EBV-positive levels of COX-2 determined by combining the levels of COX-2 from all of the cell lines. All data were expressed as means ± Standard Error of Mean (SEM). The significance of the treatment group compared to the control was determined by Student's t-test. ($p < 0.05$ was considered significant; ns, no significant; $*p < 0.05$, $**p < 0.01$).

3. Results

3.1. Expression of LMP1 and LMP2A in cell lines

Both Western-blot (Fig. 1A) and qRT-PCR (Fig. 1B) results showed strong expression of LMP1 in GT38 and GT39. LMP2A transcripts were also clearly detected in 3 EBV^+GC cell lines (Fig. 1B). None of the negative cell lines expressed EBV-encoded membrane proteins.

3.2. Lower expression of COX-2 in EBVaGC cell lines than EBVnGC cell lines

As shown in Fig. 2A, Western-blot was used to detect the expression level of COX-2 protein. The results displayed that COX-2 expression was obviously higher in negative cell lines than in EBV positive GC cell lines and was stronger in SGC7901 and BGC823. Immunofluorescence was performed to investigate the cellular localization of COX-2. At the same exposure time intensity, the three EBV^+ cell lines exhibited weaker fluorescence intensities compared to the three EBV^- cell lines (Fig. 2B). COX-2 is clearly localized at the nucleus and cytoplasm in EBVnGC cell lines, and show more COX-2 localization in the nucleus rather than cytosol. Fluorescence intensity analysis showed that the average fluorescence intensity in EBV^+ group was significantly weaker than that in the EBV^- group under the same exposure time.

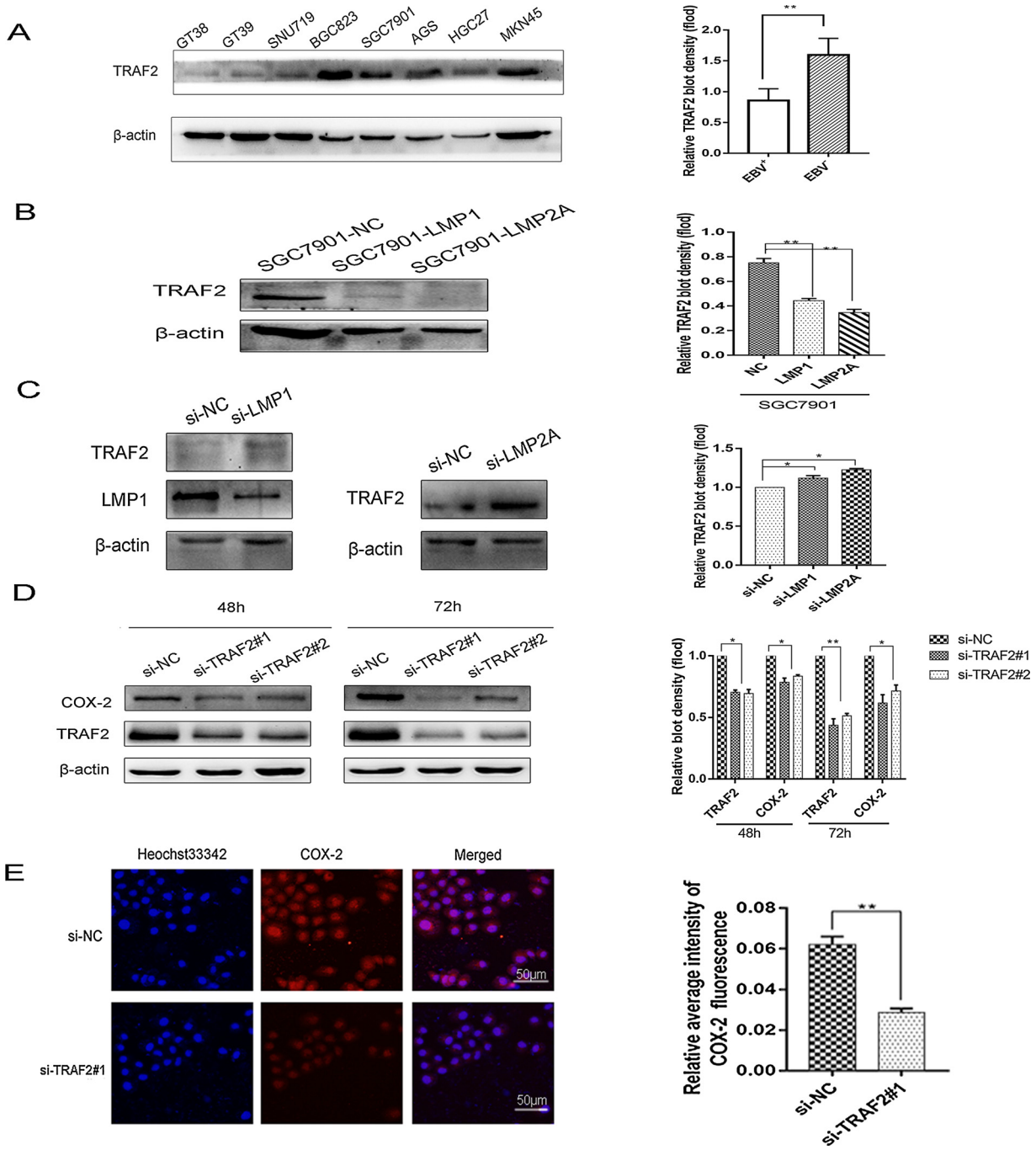

Fig. 5. TRAF2 is involved in the inhibition of COX-2 by LMP1 and LMP2A.
A. The expression of TRAF2 protein was higher in EBVnGC cells than in EBVaGC cells (**p < 0.01). **B.** Both SGC7901-LMP1 and SGC7901-LMP2A inhibited TRAF2 protein. **C.** Western-blot of TRAF2 was performed after knockdown LMP1 or LMP2A 72 h in GT38. **D.** si-TRAF2 were transferred into SGC7901. **E.** Immunofluorescence was used to detect the localization of COX-2 after 48 h of si-TRAF2#1 treatment (200×, 50 μm).

3.3. Overexpression of LMP1 and LMP2A inhibit the expression of COX-2 in SGC7901

To observe the effect of EBV-encoded latent membrane proteins on COX-2, the study selected SGC7901 cells with the strongest COX-2 expression to transfect LMP1 (SGC7901-LMP1), LMP2A (SGC7901-LMP2A) by geneticin screening. Microscopic fluorescence and RNA detection showed that the plasmids were successfully integrated into the cells and expressed the corresponding proteins (Fig. 3A/B). Western-blot showed that COX-2 expression in SGC7901-LMP1 and SGC7901-LMP2A were significantly inhibited compared to the control plasmid SGC7901-NC ($p < 0.05$, Fig. 3C). Transiently transfected LMP1 and LMP2A in SGC7901 were used to further investigate the time-dependent effect of the two on COX-2 inhibition (Fig. 3D/E). Fig. 3F showed the mRNA expression levels of LMP1 and LMP2A after transfection. The results showed that both LMP1 and LMP2A showed significant COX-2 inhibition at 24 h after transfection ($p < 0.01$), and sustained down-regulation of COX-2 expression at 48h and 72h.

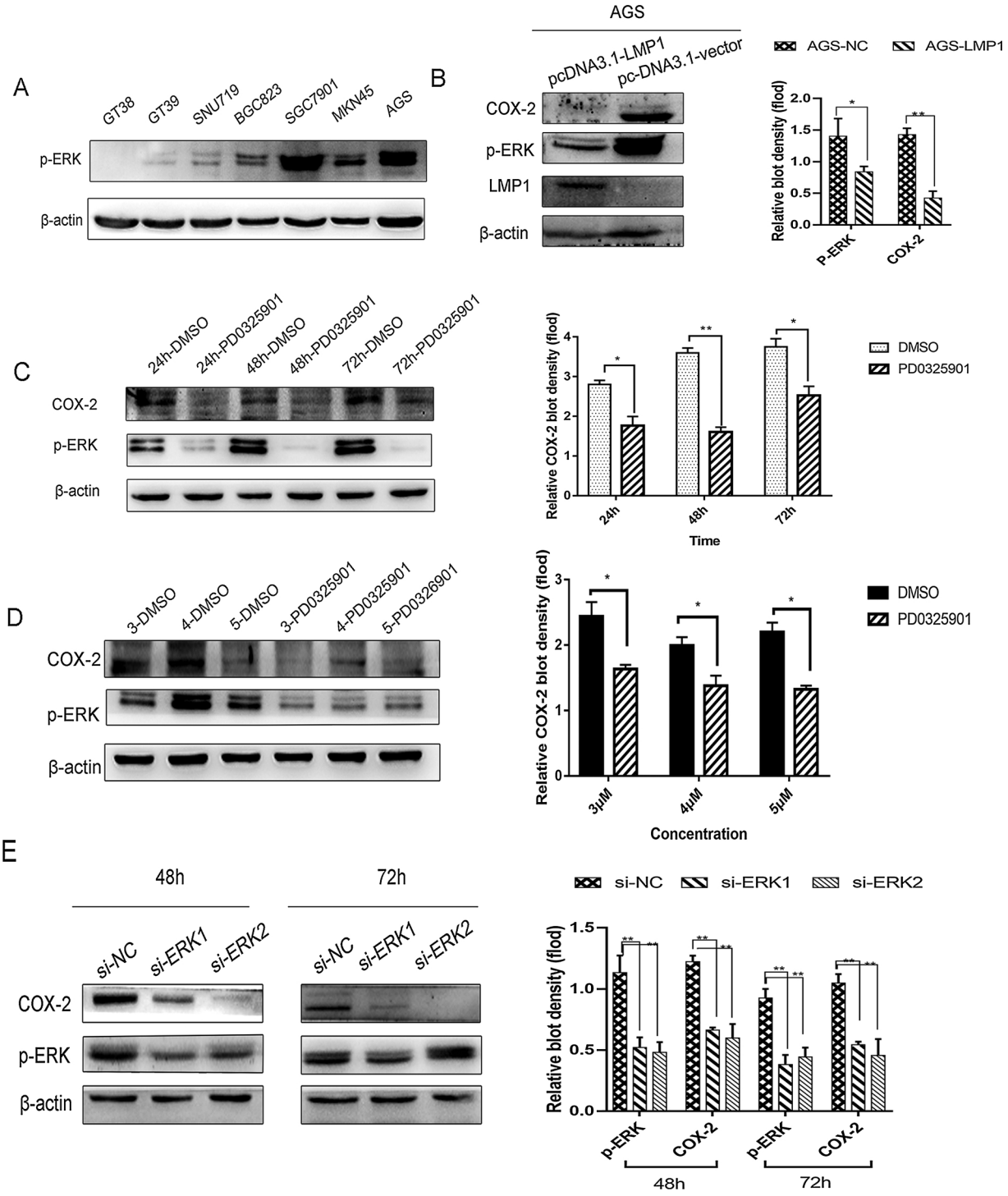

Fig. 6. LMP1 down-regulated the expression of COX-2 by inhibiting phosphorylation of ERK.
A. The protein level of p-ERK1/2 was significantly higher in EBVnGC cells than in EBVaGC cells but not ERK. **B.** LMP1 was transferred into AGS cells for 48 h, the sharp drop in p-ERK (*$p < 0.05$) as well as COX-2 (**$p < 0.01$). **C.** The MEK1/2 inhibitor PD0325901 at a concentration of 3 μM was applied to SGC7901 cells for 24 h/48 h/72 h. **D.** SGC7901 cells were treated with different inhibitor concentrations 3 μM, 4 μM, 5 μM for 48 h. **E.** Small interference targeting for ERK1/ERK2 (si-ERK1/2) were transferred into SGC7901.

3.4. Knockdown of LMP1 or LMP2A upregulated the expression of COX-2 in GT38

To further validate the negative regulation of COX-2 by LMP1 and LMP2A, we used siRNA to interfere with the expression of LMP1 and LMP2A in GT38. The results showed that a gradual increase in COX-2 protein after knockdown LMP1 (Fig. 4A). qRT-PCR showed that the transcript was decreased at 48 h and 72 h after the action of si-LMP2A (Fig. 4B). At the same time, Western blot showed that the expression of COX-2 were higher than that of si-NC (Fig. 4C).

3.5. LMP1 and LMP2A reduce COX-2 via TRAF2

For the purpose of figuring out whether TRAF2 is involved in the

inhibition of COX-2 by LMP1 and LMP2A, its expression was detected in the EBVaGC and EBVnGC cell lines at first (Fig. 5A). The results demonstrated that the expression of TRAF2 is much lower in the EBVaGC compared with EBVnGC cell lines ($p < 0.01$). In this case, the effects of LMP1 and LMP2A on TRAF2 were examined. Both stably transfected LMP1 and LMP2A in SGC7901 showed strong down-regulation of TRAF2 (Fig. 5B). Then the study detected the effect of transient transfection of LMP1 and LMP2A on TRAF2 (Fig. 3D/E). After 48 h of LMP1 transfection, a violently reduced effect on TRAF2 was observed and this inhibition was sustained until 72 h. As for LMP2A, the transfection of the plasmid into the cell also produced a strong inhibitory effect on TRAF2, and significant expression downregulation was observed in all three periods. At the same time, we examined the protein expression of TRAF2 after interfering with LMP1/2A for 72 h in GT38. Fig. 5C indicated that the expression of TRAF2 is elevated.

So subsequently two small interfering RNAs were used to interfere the expression of TRAF2 to explore whether it affects the expression of COX-2 (Fig. 5D). According to the results obtained, dramatically down-regulation of COX-2 with the decrease of TRAF2 protein was observed after 48 h and 72 h. At the same exposure time, the fluorescence intensity was significantly weakened compared with the control group, and the localization results showed that the COX-2 in the nucleus of si-TRAF2 was greatly reduced (Fig. 5E).

3.6. LMP1 down-regulated the expression of COX-2 by inhibiting phosphorylation of ERK

Previous data in laboratory showed that the Ras/MEK/ERK pathway was more activated in EBVnGC than the EBVaGC (Qianqian Zhang, unpublished data). The current results showed that the protein level of p-ERK1/2 was significantly higher in EBVnGC cells than in EBVaGC cells also confirmed this ($p < 0.01$, Fig. 6A). In order to make the experimental results more comprehensive and accurate, in this experiment, LMP1 was transferred into AGS cells, the same sharp drop in p-ERK as well as COX-2 were obtained after 48 h (Fig. 6B).

The MEK1/2 inhibitor PD0325901 at a concentration of 3 μM was applied for 24/48/72 h in six-well plates in SGC7901 cells, respectively. Western-blot showed that the COX-2 protein level in the inhibitor-treated group was significantly decreased. And the inhibitory effect of COX-2 reached the strongest after 48 h of inhibitor treatment compared to the control group ($p < 0.01$, Fig. 6C). Therefore different inhibitor concentrations (3 μM, 4 μM, 5 μM) were used for 48 h, and a similar decrease of COX-2 protein was observed in the inhibition group (Fig. 6D). In order to more directly verify that the inactivation of p-ERK is a key factor in the reduction of COX-2 expression, small interferences targeting for ERK1/ERK2 (si-ERK1/2) were used in this experiment (Fig. 6E). Immunoblot results showed that with the inactivation of ERK, the expression of COX-2 protein was drastically reduced both after 48 h and 72 h, which was consistent with the results of the treatment group treated with MEK pathway inhibitor.

4. Discussion

The data indicated that COX-2 was overexpressed in NPC biopsies, which was associated with lymph node involvement induced by LMP1(Murono et al., 2001) and metastasis via NF-κB site induced by EBNA3C (Kaul et al., 2006). However, PGE2 secretion and COX-2 mRNA expression were reduced in EBV-infected monocytes (Savard et al., 2000), which means that the regulation mechanism of EBV for COX-2 may be diverse. Moreover, there is no research on LMP2A and COX-2 in EBV-associated cancers. In the case of EBV-associated gastric cancer, the study of COX-2 is still not comprehensive.

Experimental data showed that significant differences in COX-2 expression were observed in the EBV^+GC and the EBV^-GC cell lines, and the negative group was conspicuous higher than the positive group. Consistent with Western-blot results, semi-quantitative immunofluorescence analysis also showed a low abundance of COX-2 in positive cells. Lower fluorescence in the cytoplasm may be due to low total expression. Stable overexpression of EBV-encoded LMP1 and LMP2A produced significant inhibition of COX-2, which was confirmed again by transient transfection. Fig. 3F shows that LMP2A mRNA expression is reduced at 72 h compared to 48 h resulted in partial recovery of COX-2, but they were still lower than that of standardized NC, respectively. At the same time, transient overexpression of LMP1 and LMP2A activates many signaling pathways, whether the activation of these pathways is weakened over time and affects the expression of COX-2 is worthy of further study. Although there was reported that LMP1 recruits TRAF1 and TRAF2, which is important for the downstream MAP kinase and classical NF-κB activation in human renal epithelial cell line 293 cells (Greenfeld et al., 2015), the current experimental results indicate that overexpression of LMP1 and LMP2A by stable transfection or transient transfection both have significant inhibitory effects on TRAF2 and may lead to a stable lower expression state of TRAF2 in EBVaGC. The inhibitory effect of LMP2A is consistent with the previous report (Vrazo et al., 2012). Studies have demonstrated that overexpression of LMP1 and LMP2A in SGC7901 can induce NF-κB activation by inhibiting IκBα and up-regulating TRAF1 (Zhang et al., 2019a,b), TRAF1/2/3 shares a common LMP1 binding site, whereas TRAF1 binds more strongly than TRAF2/3 (Devergne et al., 1996). Therefore, down-regulation of TRAF2 may be an optimization mechanism chosen by the virus for the continuous activation of NF-κB.

Hepatitis B virus (HBV)-infected large hepatitis delta antigen (LHDAg) promotes TRAF2-induced NF-κB activation and expression of COX-2 in Huh7 cells (Fotheringham et al., 2012). Western-blot results showed that COX-2 protein was decreased sharply after effective interference with TRAF2 in this study. The optimal duration of action of the two small interfering RNAs are inconsistent and we provide a control group for each time period. Moreover, compared with the control group, siTRAF2#1 immunofluorescence showed a significant decrease in the fluorescence intensity of COX-2, and the nuclear reduction was particularly strong, which further confirmed the effect of TRAF2 on COX-2. Based on all above experimental results, the inference of LMP1 and LMP2A inhibit the expression of COX-2 by inhibiting TRAF2 is reasonable. The might pathways involved in can be MAPK, JAK-STAT (Nagashima et al., 2018), Wnt (Araki et al., 2003; Masuda et al., 2015), etc. which requires further research.

Laboratory studies have shown that SGC7901-LMP1 has a significant inhibitory effect on p-ERK compared to SGC7901-NC when it is in the same cell cycle and was confirmed in AGS cells in this study, it was found that both p-ERK and COX-2 were decreased sharply with overexpression of LMP1. The current results indicated that the protein level of p-ERK1/2 was significantly higher in EBVnGC cells than in EBVaGC cells. To investigate whether highly activated p-ERK is responsible for the high expression of COX-2, SGC7901 cells were treatment with ERK upstream factor MEK inhibitor PD0325901 and intracellular si-ERK1/2. COX-2 expression was significantly inhibited with decreased ERK phosphorylation as the results displayed. And the down-regulation of COX-2 was most significant after 48 h of inhibitor inhibition. COX-2 promotes cell proliferation and inhibits apoptosis. Therefore, we suspect that the reason for the 72 h increase over 48 h may be caused by prolonged cell growth slowing or increased cell death, which in turn causes compensatory up-regulation of COX-2, and our data was shown that the COX-2 level in the control group also increased with the prolonged treatment time, which further confirmed that the COX-2 expression level of the treatment group was not only affected by the inhibitor, but also some regulatory mechanisms by up-regulating COX-2 to antagonize the cell damage caused by its solvent DMSO. The outcome of the study certificated that inhibition of COX-2 by LMP1 is at least a result of impeding p-ERK function.

A series of studies have shown that COX-2 protein expression is associated with intestinal histology subtype, proximal location, tumor size, clinical stage of lymph node metastasis (Cheng and Fan, 2013). In

MKN-45 cells, inhibition of COX-2 by NS-398 inhibitors reduced proliferation and induced apoptosis, which may be related to down-regulation of Bcl-2 (anti-apoptotic gene) and up-regulation of Bax (apoptotic gene) (Sun et al., 2008). COX-2 induction can up-regulate the expression of Akt to promot angiogenesis and can reduce the activity of E-cadherin to enhance the invasiveness of cancer cells. The metastatic effect is attenuated after inhibition of COX-2 (Cheng and Fan, 2013). Chan MW et al. demonstrated that tumor-infilable Treg cells with increased Foxp3 expression can mediate the mechanism of immunosuppression through COX-2/PGE(2) production in the gastric microenvironment (Yuan et al., 2010). Transfection of COX-2 siRNA with MKN45 also enhanced the chemosensitivity of gastric cancer cells (Chan et al., 2007). Clinically, the incidence of lymph node metastasis (LNM) in EBVaGC is significantly reduced compared to EBVnGC and shows a longer disease-free period leading to a better prognosis (Nishikawa et al., 2018). Experimental data indicate that COX-2 is significantly inhibited in EBVaGC cells, suggesting that lower levels of COX-2 expression in EBVaGC may be associated with better prognosis. Low expression of LMP1 in EBVaGC contributes to immune escape, and high expression exposes cells to the immune environment (Leen et al., 2001). Studies have shown that COX-2 promotes gastric cancer progression in part through immunosuppression (Yuan et al., 2010). Therefore, when LMP1 is overexpressed, the immunosuppressive effect of COX-2 will be weakened, which also supports our experimental results. Although COX-2 acts as an important inflammatory factor and plays a significant role in promoting cancer, the association between EBV and inflammation is still uncertain in gastric cancer. The involvement of EBV in progressive inflammatory lesions is primarily a negative result (Morales-Sanchez and Fuentes-Panana, 2017). Chronic gastritis tissue biopsy to detected latent EBV-infected cells found that EBV-positive gastritis samples are < 3% and differed from the previously reported that higher prevalence of cells carrying viruses in inflammatory conditions elsewhere in the gastrointestinal tract. These findings refute the direct involvement of EBV in the pathogenesis of chronic gastritis (Hungermann et al., 2001). The mechanism of EBVaGC is not fully understood until now. The inhibition of COX-2 can be an important complement to the relationship between EBVaGC and inflammation.

The transcription factor C/EBPβ is required for the induction of expression of the COX-2 gene in macrophages, but not in fibroblasts (Gorgoni et al., 2001). Acute parenteral LPS provides gastric protection against ethanol damage through increased blood flow in the gastric microcirculation and overexpression of COX-2 and enhanced endogenous PG release (Katori and Majima, 2000). The regulation and function of COX-2 expression is complex and appears to involve diverse mechanisms of different cell types and conditions. Therefore, it is critical to clarify the expression of COX-2 in gastric cancer and to study its possible regulatory mechanisms. For the first time, this study explored the negative regulatory relationship between LMP1/2A and COX-2 in EBV-associated gastric cancer. This down-regulation of COX-2 by LMP1/2A was found to be achieved by inhibiting the expression of TRAF2. Simultaneously, LMP1 acts on COX-2 via reducing the activation of p-ERK. The study of the relationship between LMP2A and COX-2 was first revealed and has certain significance, but more mechanisms need to be explored. Researchers wish this study will give people a more comprehensive understanding of the role of EBV in tumors and play a role in the broader research of future scholars.

Author contributions

Yi-fan Qi, Wen Liu, Bing Luo designed research.
Yi-fan Qi, Mengyang Liu, Hua Xiao analyzed data.
Yi-fan Qi performed research.
Yi-fan Qi, Yan Zhang wrote the paper.

Declaration of Competing Interest

None.

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