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Constitutive activation of the canonical NF-κB signaling pathway in EBV-associated gastric carcinoma

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ABSTRACT

EBV-associated gastric carcinoma (EBVaGC) is a specific subgroup of gastric carcinoma, and the multifunctional transcriptional factor NF-κB may contribute to its tumorigenesis. In this study, we comprehensively characterized NF-κB signaling in EBVaGC using qRT-PCR, western blot, immunofluorescence assays, ELISA, and immunohistochemistry staining. NF-κB-signaling inhibitors may inhibit the growth of EBVaGC cells and induce significant apoptosis. IκBα is a key regulatory molecule, and repression of IκBα can contribute to aberrant NF-κB activation. Overexpression of LMP1 and LMP2A in the EBV-negative GC cell line SGC7901 could inhibit the expression of IκBα and induce NF-κB activation. These findings indicate that the canonical NF-κB signal is constitutively activated and plays an important role in EBVaGC tumorigenesis.

1. Introduction

Epstein–Barr virus (EBV), also known as human herpesvirus 4, was the first virus to be isolated from human tumor cells, in 1964 (Epstein et al., 1964). EBV infects more than 90% of adults worldwide via salivary transmission, and its latent infection has been closely associated with many human lymphoid and epithelial tumors, such as Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma (Fukayama et al., 1994; Kutok and Wang, 2006; Young and Rickinson, 2004; zur Hausen et al., 1970). During its latent cycle, EBV expresses six EBV-encoded nuclear antigen proteins (EBNA1, EBNA2, EBNA-3A, -3B, and -3C, and EBNA-LP) and three latent membrane proteins (LMP1, LMP2A, and LMP2B), and establishes persistent, long-term latency (Amon and Farrell, 2005; Tsurumi et al., 2005). EBV-associated gastric carcinoma (EBVaGC) is considered to be a subtype of gastric carcinoma, and EBV has been accepted as a significant infective agent implicated in its oncogenesis (Fukayama et al., 2008). EBV infection can be classified into three patterns, and EBVaGC belongs to latency type I/II, based on its latent genes EBNA1, LMP1, and LMP2 (Borozan et al., 2018; Niller and Minarovits, 2016). EBVaGC can be differentiated from EBV-negative gastric carcinoma (EBVnGC) because it shows characteristic clinicopathological features and unique oncogenic mechanisms, such as a relatively favorable prognosis, promoter hypermethylation, and viral gene expression (Fukayama and Ushiku, 2011; Network CGAR, 2014; Kaneda et al., 2012). Although there have been many investigations into EBVaGC, the exact pathogenesis of this disease remains poorly understood and requires further study. For example, the dysregulation of particular signaling pathways may contribute to EBVaGC carcinogenesis (Fukuda and Longnecker, 2007; Scholle et al., 2000). In order to determine the role of EBV infection in the tumorigenesis of EBVaGC, we focused on the nuclear factor-κB (NF-κB) signaling pathway.

The NF-κB transcriptional factor family is composed of homo- or hetero-dimers consisting of five members: RelA/p65, RelB, p50, p52, and c-Rel (Hayden and Ghosh. 2012). The DNA binding domain of these five subunits can bind to the κB sites at the promoter region of target genes to induce transcription. In most quiescent cells, the prototypical heterodimer complex NF-κB is heavily suppressed in the cytoplasm by inhibitory proteins (IκBs). Once stimulated by extracellular signals, IκBs are phosphorylated by activated IκB-kinase (IKK), which leads to ubiquitination by E3 ubiquitin ligases followed by rapid degradation (Shih et al., 2011). The dissociated NF-κB exposes nuclear localization and DNA binding sites and is then transported into the nucleus, binding the target DNA and modulating its transcription.

NF-κB, as a multifunctional transcriptional factor, is well-known to regulate various biological progresses, including cell proliferation, apoptosis, transformation, and immune escape, as well as playing a critical role in the initiation and development of various human cancers (Cahir-McFarland et al., 2004; Guttridge et al., 1999; Huber et al., 2004). It has been reported that EBV could directly activate NF-κB

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signaling in some EBV-associated diseases (Chang et al., 2013; Guasparri et al., 2008; Stewart et al., 2004; Takada et al., 2017). In the present research, we found that during EBV latency, the canonical NF-κB signaling pathway in EBVaGC is persistently activated and plays an important role in EBVaGC tumorigenesis. We tried to evaluate the exact mechanisms that could decrease the expression of IκBα, enabling the continuous activation of the NF-κB pathway in EBVaGC to be comprehensively sustained. Our results contribute to a new understanding of the molecular mechanisms of EBVaGC pathogenesis.

2. Materials and methods

2.1. Cell lines and primary tumors

In this study, the EBVaGC cell lines GT38 and GT39, expressing EBNA1, EBNA2, and LMP1, were isolated from human EBV-positive gastric carcinoma tissues, kindly provided by Sairenji T (Tottori University, Japan) (Tajima et al., 1998). SNU719 was identified as an EBV-positive gastric carcinoma cell line expressing EBNA1 and LMP2A (Oh et al., 2004) and was a kind gift from Qian Tao (the Chinese University of Hong Kong). The EBVnGC cell line BGC823 was a kind gift from Chunkui Shao (Sun Yat-sen University). The other EBVnGC cell lines used, SGC7901 and HGC27, were purchased from the Cell Bank of the Chinese Academy of Sciences. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Biological Industries Company, Israel), at 37 °C with 5% CO_2.

All GC paraffin-embedded and fresh tumor tissues were collected from the Affiliated Hospital of Qingdao University. EBV-positive specimens were determined by *in situ* hybridization of EBV-encoded small-RNA1 (EBER1), as previously described (Khan et al., 1992). This study received permission from the Medical Ethical Committee of the Medical College of Qingdao University.

2.2. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, USA) and cDNA was synthesized using a cDNA reverse transcription kit (Roche, Switzerland), according to the manufacturer's instructions. qRT-PCR was performed using a Faststart Essential DNA Green Master kit (Roche) using a LightCycler96 SN10700 Sequence Detection System (Roche). The sequences of the specific forward and reverse primers were as follows: for *NFKBIA* (IκBα), 5′- GGGCTATTCTCCCTACCAGC-3′ and 5′-TCATCATAGGGCAGCTCGTC-3′, for LMP1, 5′- CCTTGGTCTAC TCCTACTGATGATCA -3′, and 5′- CAGCACAATTCCAAGGTACAATG-3′, for LMP2A, 5′-TGTCGCTGGCATACTCTTCA-3′, and 5′-GCGTGTTAGTC ATCACCGTC-3′, for GAPDH, 5′-CAAATTCCATGGCACCGTCA-3′ and 5′-ATCGCCCCACTTGATTTTGG-3’.

The reaction condition was 95 °C for 5 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 10 s, and then 72 °C for 15 s. After expansion, the reaction mixtures were incubated at 95 °C for 10 s and heated from 65 °C to 97 °C for the dissociation-curve analysis, to determine the specificity of the amplification products. Relative gene expression was calculated using the comparative cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$), using *GAPDH* as the internal standard.

2.3. Western blot analysis, antibodies, and other reagents

The cells were washed twice with cold phosphate buffer solution (PBS) and then lysed in radioimmunoprecipitation (RIPA) (GBCBIO Technologies INC, China) buffer containing protease and phosphatase inhibitors (100:1:1). The cells were mixed with loading buffer (4 ×) and heated at 95 °C for 5 min for protein denaturation. The protein was separated on 10% SDS-PAGE gels and then blotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon, USA). The primary antibodies used were as follows: anti-IκBα, anti-phospho-IκBα (Ser32), anti-NF-κB p65, anti-Bax, anti-Bcl-2, anti-Cleaved Caspase-3, anti-TRAF1, anti-TRAF2, anti-TRAF3, and anti-TRAF6. All above antibodies were purchased from CST (Mass, USA), at a dilution of 1:1000. Anti-LMP1 and anti-FLAG antibodies were purchased from Abcam (USA) with a dilution of 1:1000. After incubation overnight at 4 °C, the blots were incubated with HRP-linked anti-mouse/rabbit secondary antibodies at a dilution of 1:1000 (CST) for 1 h at room temperature. After washing, the protein bands were visualized using an enhanced chemiluminescence detection system, Quantum-ST5 (Vilber Lourmat, France) with chemiluminescence substrates (Millipore WBKLS0100 ECL). Tubulin (Abcam, USA; 1:5000) or β-actin antibodies (CST, USA; 1:1000) were used as controls. MG-132, a proteasome and calpain inhibitor was obtained from CST. Another NF-κB inhibitor, BAY 11-7082, was purchased from Beyotime (China).

2.4. Immunofluorescence assay

Cells grown on coverslips were washed with PBS, fixed with 4% paraformaldehyde for 10 min, and then blocked with blocking buffer (1% BSA, 0.15% Triton-X100, 22.52 mg/ml glycine, PBS) for 1 h at room temperature. Cells were incubated with the primary antibody p65 (CST, USA, 1:400) overnight at 4 °C, then with the secondary antibody conjugated with Alexa Fluor 555 (CST, USA) for 1 h at room temperature. Cells were then counterstained with Hoechst 33258 (Beyotime, China) for 5 min. Images were obtained using a laser confocal fluorescence microscope (Leica TCS SP8, Germany) and a fluorescence microscope (Nikon, Japan).

2.5. CCK-8 cell proliferation assay

Cell proliferation was detected using the Cell Counting Kit 8 (CCK-8) (Bosterbio, USA). The cells were seeded at 5×10^3 cells/100 μl per well into 96-well plates and transfected with different concentrations of BAY 11-7082. After incubation for 24 h, 10 μl CCK-8 solution was added to each well. After 1 h, absorbance was tested using Soft-Max apparatus (Bio-Tek ELx808, USA) at a wavelength of 450 nm.

2.6. Annexin V apoptosis detection

Cells were washed with cold PBS and resuspended in 500 μl Annexin V binding buffer (eBioscience™ Annexin V Apoptosis Detection kit FITC; Invitrogen, USA), containing fluorochrome-conjugated Annexin V and propidium iodide (PI). The assay was performed according to the manufacturer's instructions. Annexin V was used to label cells undergoing apoptosis by detecting phosphatidylserine (PS) on the outer plasma membrane, while PI was used to detect dead cells. After incubating for 10–15 min at room temperature in the dark, the specimens were analyzed by fluorescence-activated cell sorting using FACSCalibur apparatus (BD Biosciences, USA), acquiring 30,000 events.

2.7. Nuclear protein extraction and detection of NF-κB p65 transcription activity

The nuclear protein was collected by a Nuclear and Cytoplasmic Protein Extraction Kit (P0027, Beyotime, China), according to the manufacturer's instructions. To assess the NF-κB p65-DNA binding activity, we used an NF-κB p65 Transcription Factor Assay Kit (ab133112, Abcam, USA), according to the manufacturer's instructions. Intracellular p65-DNA binding activity was determined using a spectrophotometer with absorbance at OD 450 nm. All measured values were detected using Soft-Max apparatus (Bio-Tek ELx808).

2.8. Immunohistochemical staining

Surgically resected tumor tissue samples were fixed in 10% formalin and embedded in paraffin. Sections 4 μm thick were mounted on poly-L-

lysine-coated slides. After dewaxing and rehydration, the sections were sequentially covered with 3% H_2O_2 in methanol for 10 min, to eliminate endogenous peroxidase activity. After washing with PBS, the sections were first covered by blocking solution A (SP-0023 kit, ZYMED, USA) normal goat serum at 37 °C for 30 min, then incubated with monoclonal rabbit anti-human p65 (CST, 1:800) or monoclonal mouse anti-human IκBα (CST, 1:100) at 4 °C overnight. The sections were thoroughly washed then treated with biotinylated anti-rabbit/mouse immunoglobulin G for 30 min. Sections were washed again, then treated with streptavidine/HRP complex for 30 min. Diaminobenzidine-H_2O_2 was used as a coloring substrate. Sections were counterstained with hematoxylin.

2.9. Overexpression of LMP1 and LMP2A

LMP1 and LMP2A were cloned into pcDNA3.1-EF1a-mcs-3flag-CMV-EGFP vectors and transfected into SGC7901 using lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol. SGC7901 cells were seeded at 1×10^6 cells per well in 6-well plates and transfected with 2.5 μg LMP1, LMP2A, or negative control vector plasmids. The cells were collected for assay at the designated time. Stable transfections of LMP1 and LMP2A were selected by G418.

2.10. Transfection with small interfering RNAs (siRNAs)

Cells were seeded at 1×10^6 cells per well in 6-well plates and transfected with siRNAs to TRAF1 (CCCUGCAGCACUUCAUGAATT UUCAUGAAGUGCUGCAGGGTT), LMP1 (Guasparri et al., 2008), and negative control (antisense 5′-ACGUGACACGUUCGGAGAATT-3′) using lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The cells were incubated with 50 nM siRNA for 48 or 72 h to achieve knockdown of expression of the target genes.

2.11. Determination of EBV-DNA copy numbers

An EBV-PCR fluorescence quantitative diagnostic kit (DaAn gene, China) was used to assess EBV-DNA copy numbers by real-time, fluorescence-based quantitative polymerase chain reaction (Agilent Mx3000P, USA). The DNA extraction and qRT-PCR procedures for detection were performed according to the manufacturer's instructions.

2.12. Statistical analysis

Statistical analyses were performed using a two-tailed unpaired Student's *t*-test, the Chi-square test, and Fisher's exact test. The EBV-DNA copy number was converted to a common logarithm value for the analysis. Differences were considered statistically significant if $P < 0.05$. Data were reported as means ± SD (standard deviation). All experiments were repeated at least three times.

3. Results

3.1. Activation of specific NF-κB signaling in EBVaGC cell lines and tissues

To explore the activation of NF-κB signaling in EBVaGC and the influence of EBV infection, we examined the expression of key molecules in the NF-κB signaling pathway in EBV-positive and -negative cell lines and tissues. We found that IκBα mRNA expression was significantly decreased in EBVaGC compared with EBVnGC cell lines ($P < 0.001$) by qRT-PCR (Fig. 1a). We subsequently evaluated IκBα protein levels and found they were noticeably down-regulated in EBVaGC cell lines (Fig. 1b). We analyzed NF-κB p65-DNA binding activity using an ELISA kit and found it was higher in EBVaGC cell lines compared with EBVnGC cell lines (Fig. 1c). In addition, we analyzed p65 nuclear translocation using an immunofluorescence assay. As shown in Fig. 1d, there was more than 50% nuclear translocation of p65 in EBV-positive cell lines, while little or no nuclear translocation was observed in EBV-negative cell lines.

We then analyzed IκBα mRNA expression in tissue samples from 15, 18, and 15 EBVaGC, EBVnGC, and pericarcinomatous cases, respectively, and found that the expression of IκBα was lower in EBVaGC compared with both EBVnGC and pericarcinomatous tissues (Fig. 2a). Immunohistochemistry staining showed more p65 nuclear translocation and less IκBα staining in EBVaGC tissues ($P < 0.05$), which was consistent with the immunofluorescence assay and western blot of cell lines (Fig. 2b and Table 1). These results indicate that there is constitutive activation of NF-κB signaling in both EBVaGC cell lines and tissues.

3.2. Inhibition of the NF-κB pathway could suppress the growth of EBVaGC cell lines

In order to investigate the role of NF-κB in relation to the growth of EBV-positive cells, we used two different NF-κB inhibitors, the proteasome and calpain inhibitor MG-132, to prevent the degradation of IκBα, and BAY 11-7082, to prevent the phosphorylation of IκBα. Immunofluorescence staining and ELISA were used to detect the effect of these NF-κB inhibitors. We found that both NF-κB inhibitors prevented the nuclear translocation of p65, particularly MG-132 (50 μM, 12 h) and BAY 11-7082 (10 μM, 12 h) treatment of SNU719 (Fig. 3a and b). Cell survival rates were analyzed using a CCK-8 assay following 24 h treatment with BAY 11-7082 at concentrations of 5, 10, 15, 20, 25, and 30 μM. The dose-response curve indicated that SNU719 was more sensitive to BAY 11-7082 than the EBVnGC cell line SGC7901, and that BAY 11-7082 concentrations of more than 15 μM almost completely killed EBV-positive cells after treatment for 24 h (Fig. 3c).

3.3. Inhibition of the NF-κB pathway could induce cell apoptosis

To investigate the potential anti-apoptosis effects of NF-κB activation in EBVaGC, we evaluated cell apoptosis in SNU719 cells after treatment with NF-κB inhibitors. The Annexin V staining assay indicated that the inhibition of NF-κB activation by BAY 11-7082 induced a significant increase in the percentage of early and late apoptotic cells compared with the DMSO control, in a dose-dependent manner (Fig. 4a). A low concentration of BAY 11-7082 (2.5 μM) did not significantly affected apoptosis ($P > 0.05$), whereas higher concentrations (5 and 10 μM) significantly induced apoptosis ($P < 0.05$). Similar results were obtained in two other independent experiments, and the means and SDs of these three independent experiments are also shown in Fig. 4a.

To further analyze the effects of reduced NF-κB activation on apoptosis, the expression of apoptosis-associated genes was investigated using western blotting. As shown in Fig. 4b, IκBα expression increased following MG-132 treatment for 2 h at the designated concentration. The results also showed that the expression of Cleaved Caspase-3 gradually increased with increasing MG-132 concentration. We then analyzed another pro-apoptosis protein, Bax, and found that the expression of Bax had increased slightly. However, the pro-survival protein Bcl-2 increased following treatment for a short time. Similar results were observed with BAY 11-7082 treatment for 1 h at designated concentrations.

We also investigated the expression of apoptosis genes with the concentration of 50 μM (MG-132) and 10 μM (BAY 11-7082) for several designated times. Following increased treatment time, Cleaved Caspase-3 was gradually up-regulated; conversely, Bcl-2 was down-regulated. The expression of Bax was up-regulated after MG-132 treatment, while no obvious elevated with the BAY11-7082. These results indicated that the activation of NF-κB could regulate mitochondrial apoptosis by influencing the expression of related genes. We speculated that the temporary increase in Bcl-2 observed following treatment for a short time was due to elevated stress. These results reveal that constitutive activation of NF-κB is indispensable for EBVaGC

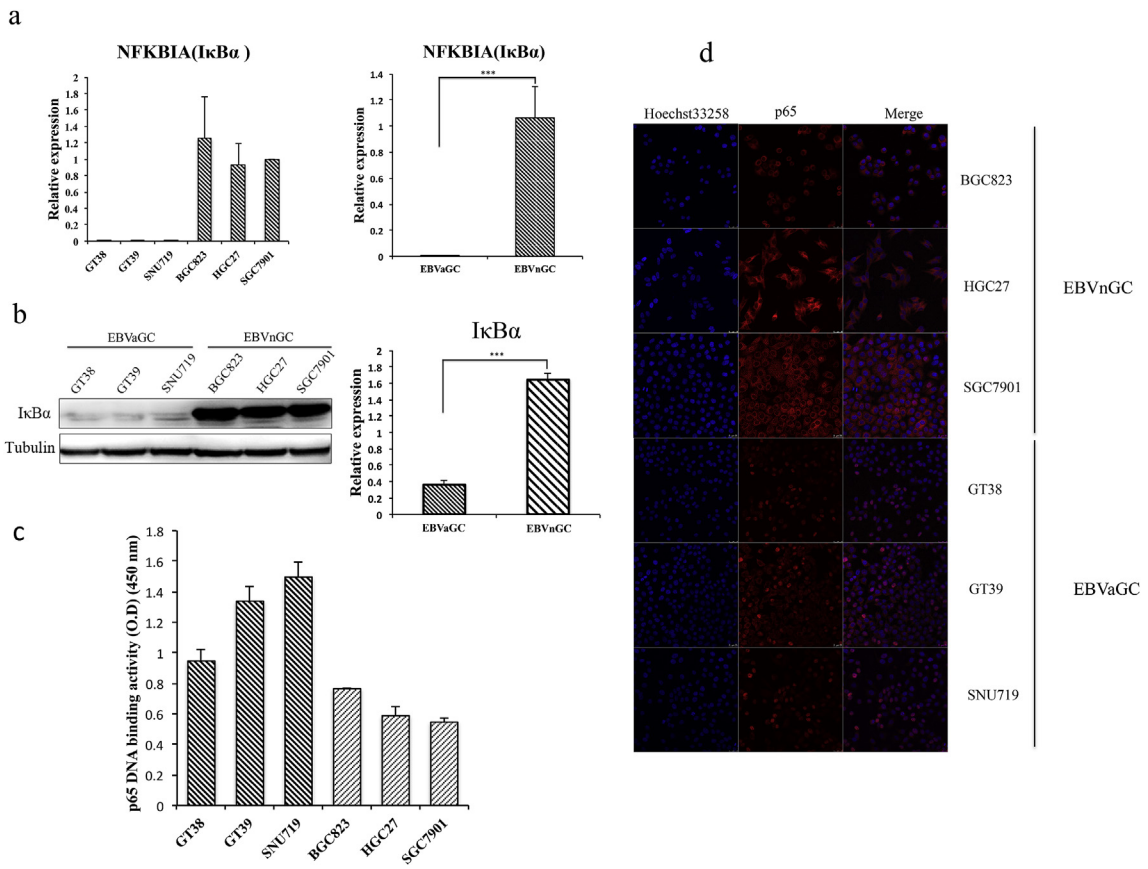

Fig. 1. Activation of NF-κB in EBV-associated gastric carcinoma (EBVaGC) cells. (a) RNA was extracted from EBVaGC and EBV-negative gastric carcinoma (EBVnGC) cells, and quantitative real-time PCR was performed in triplicate for the *NFKBIA* (IκBα) gene. Three independent experiments were performed for each cell line and relative gene expression was calculated using the comparative cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$) and GAPDH as the internal standard. The relative expression of the *NFKBIA* (IκBα) gene in EBVaGC and EBVnGC groups was significantly different (***$P < 0.001$). Data represent means ± standard deviation (SD). (b) Western blotting showed that the expression of IκBα in EBVaGC was much lower than in EBVnGC cell lines. The means ± SD of three independent experiments are shown (***$P < 0.001$). (c) p65-DNA binding activity was analyzed using an ELISA kit. Data represent means ± SD. (d) Immunofluorescent staining for NF-κB p65 location in EBVaGC and EBVnGC cells. Hoechst 33258 was used for nuclear staining. The stained cells were analyzed using confocal microscopy.

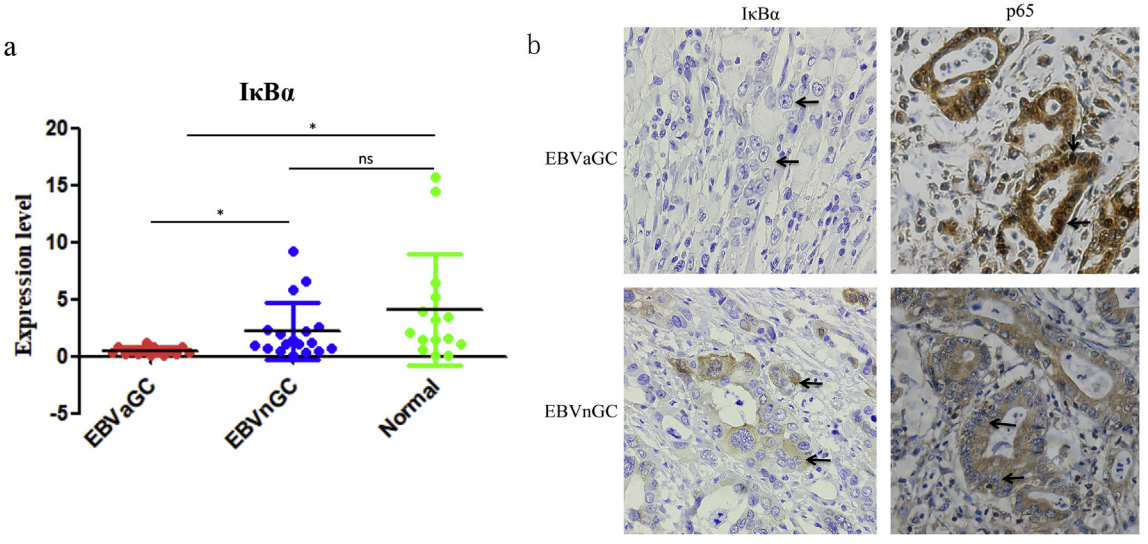

Fig. 2. Activation of NF-κB in EBVaGC tissues. (a) The relative expression of the IκBα in tissue samples from 15, 18, and 15 cases of EBVaGC, EBVnGC, and pericarcinomatous tissue, respectively (*$P < 0.05$, ns: not significant). (b) Representative microscopic images of gastric tissues. Examples of IκBα and p65 were demonstrated in primary tumors (200 ×).

Table 1
Summary of nuclear accumulation of p65 and IκBα in GC tissues.

	EBVaGC (n = 22)	EBVnGC (n = 21)	*P* value
NF-κB RelA (p65)			
Nuclear translocation	12(54.55)	5(23.81)	
No nuclear translocation	10(45.45)	16(76.19)	0.039
IκBα			
positive	5(22.73)	11(52.38)	
negative	17(77.27)	10(47.62)	0.044

EBVaGC: EBV-associated gastric carcinoma; EBVnGC: EBV-negative gastric carcinoma.

cell growth.

3.4. NF-κB signaling could modulate the expression of LMP1 and LMP2A in EBVaGC cells

Latent membrane proteins (LMPs) are recognized to be major EBV oncoproteins that make a critical contribution to pathogenesis and disease phenotypes. Therefore, we investigated the influence of the NF-κB signaling pathway on the expression of LMP1 and LMP2A. EBV copy numbers were consistent with or without inhibitor treatment (Fig. 5a and d). When the NF-κB signaling pathway was inhibited by BAY 11-7082, the expression of LMP1 of GT38 notably decreased, both at the levels of mRNA and protein (Fig. 5b and c). When SNU719 was treated with an inhibitor, there was a marked decrease in the expression of LMP2A. These results indicate that the activation of NF-κB plays an important role in latent infection established by EBV.

3.5. LMP1 and LMP2A inhibit the expression of IκBα and induce activation of the NF-κB signaling pathway

LMP1 and LMP2A are transmembrane proteins, with cytoplasmic fragments that may activate several signaling pathways. To evaluate the effect of LMP1 and LMP2A on the activation of the NF-κB signaling pathway, we transfected the EBV-negative cell line SGC7901 with pcDNA3.1-LMP1/LMP2A plasmids and pcDNA3.1 as a control (Hanbio, China). Using an ELISA assay, it was determined that EBV-negative SGC7901 cells exhibited higher p65-DNA binding activity following transfection with LMP1 and LMP2A for 48 h, compared with the vector control (Fig. 6a). Weak green fluorescence EGFP transferred by vectors was detected 6 h post-infection, and showed stable expression 24 h post-infection by western blotting (Fig. 6b and c). Phosphorylated IκBα increased following LMP1/LMP2A treatment, while the total expression of IκBα decreased considerably. We also found that phosphorylated p65 was elevated while total p65 was slightly down-regulated. These results indicated that NF-κB signaling was activated following the expression of LMP1 or LMP2A. As shown in Fig. 6d, IκBα decreased following the stable transfection of LMP1 and LMP2A. We silenced the expression of LMP1 using siRNA on GT39 and found that this elevated the expression of IκBα by 1.34-fold (Fig. 6e).

Tumor necrosis factor receptor-associated factors (TRAFs) function as signal transducers in co-operation with LMPs to activate the NF-κB pathway (Siegler et al., 2003). To clarify which TRAFs might induce the

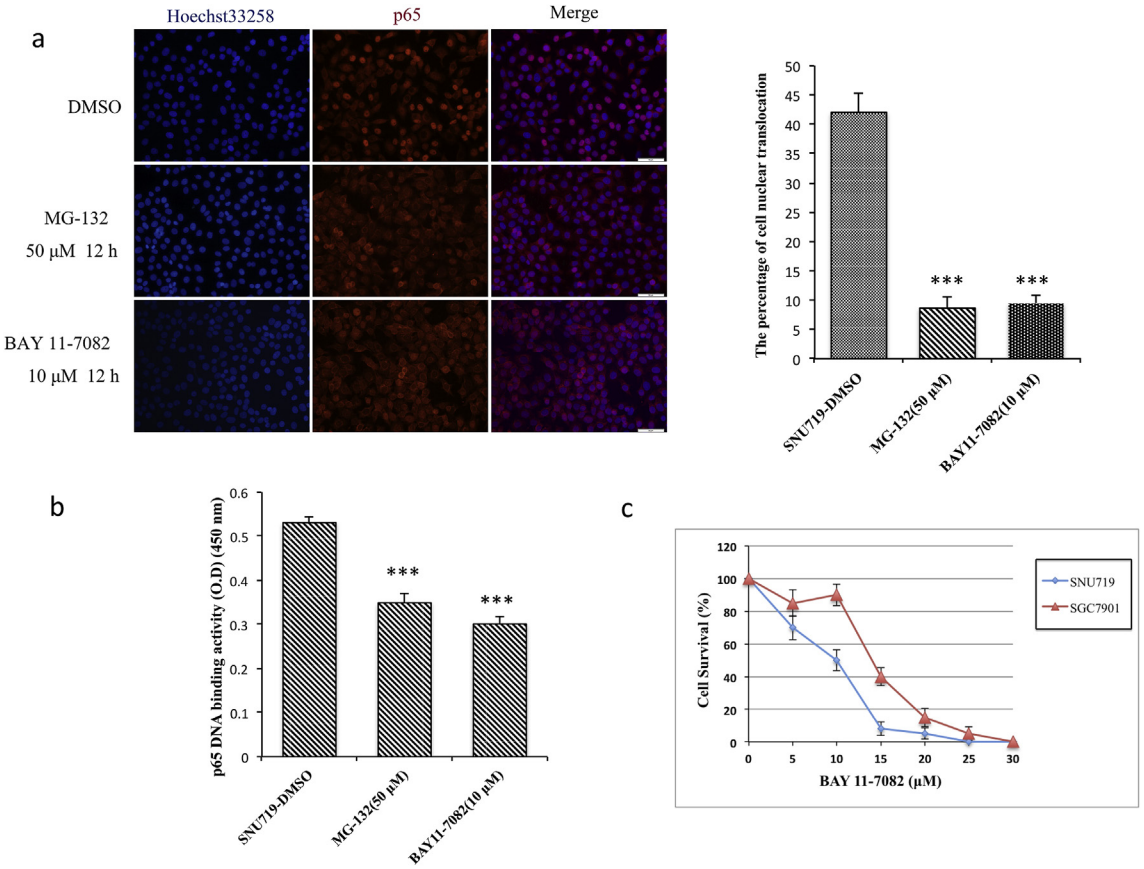

Fig. 3. Effect of NF-κB inhibition on EBV-positive GC SNU719 cells. (a) Immunofluorescent staining for NF-κB p65 location in SNU719 cells treated with MG-132 and BAY 11-7082. The cells were transfected with 50 μM MG-132 or 10 μM BAY 11-7082 for 12 h, and DMSO was used as a control. A significant reduction in nuclear location was found in treated cells, indicating that NF-κB activation was inhibited effectively. (b) p65 DNA binding activity was analyzed using an ELISA kit. The activity was reduced following treatment with MG-132 and BAY 11-7082. Data represent means ± SD. (c) EBVaGC cell line SNU719 and EBVnGC cell line SGC7901 cells were treated with BAY 11-7082 at designated concentrations, and cell viability was analyzed using a CCK-8 assay over 24 h. The dose-dependent curve of GC cell lines indicated that the EBVaGC cell line SNU719 was more sensitive to BAY 11-7082 compared with the EBVnGC cell line SGC7901.

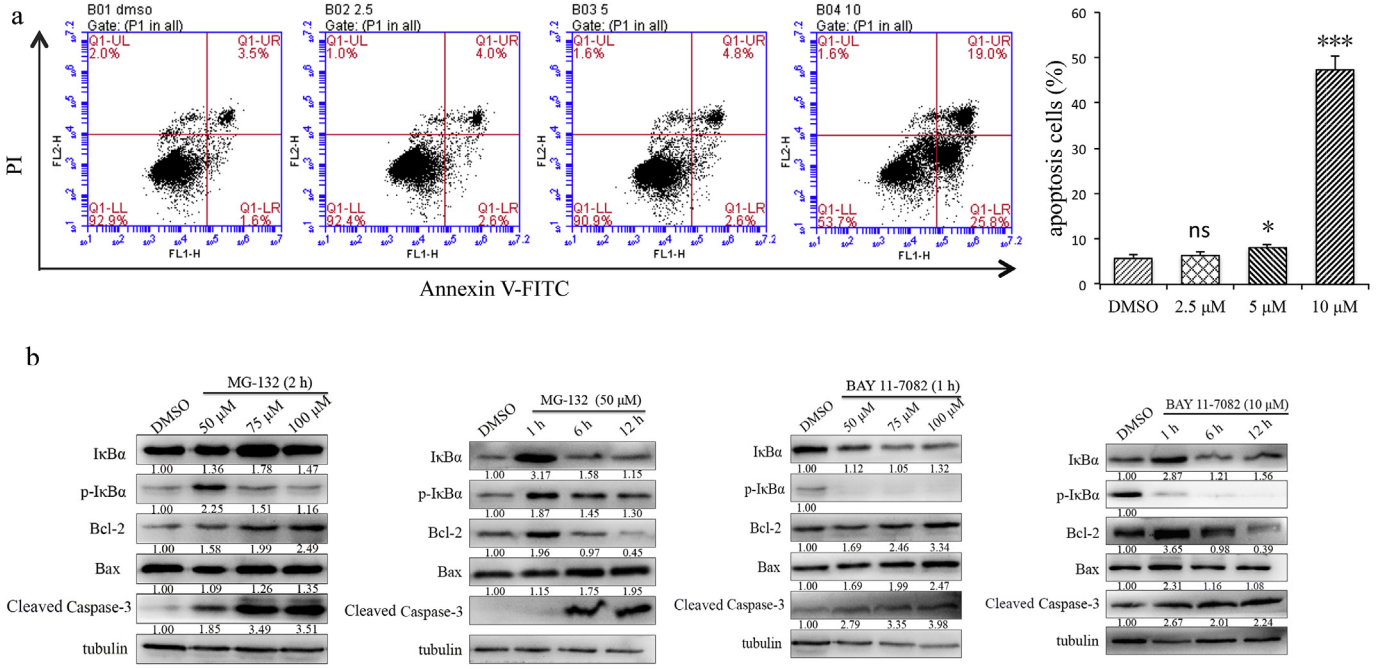

Fig. 4. NF-κB inhibitors induce apoptosis in a time- and dose-dependent manner. (a) The apoptosis fractions of SNU719 cells following different treatments with BAY 11-7082 were analyzed using Annexin V-FITC and PI double-staining. Quantitative descriptions of apoptosis were shown compared with the control (treated with DMSO). ns: not significant, $*P < 0.05$, $***P < 0.001$. Data represent three independent experiments shown as means ± SD. (b) Protein expression of total and phosphorylated IκBα, and the apoptosis-related genes Bcl-2, Bax, Cleaved Caspase 3 in SNU719 after the treatments indicated were analyzed by western blotting. Tubulin served as a control of loading. Densitometry values of the bands were expressed as fold-increases above the DMSO control (lane 1).

NF-κB pathway, we analyzed the protein expression of TRAF1, TRAF2, TRAF3, and TRAF6 following treatment with LMP1/LMP2A. We found that only TRAF1 expression was increased, while TRAF2, 3, and 6 were noticeably repressed or showed negligible change (Fig. 6f and g). These results were similar with the stable transfection of LMP1 and LMP2A (Fig. 6d). These results, of overexpression with LMP1 and LMP2A, were consistent with the original expression of TRAFs in GC cell lines (Fig. 6h). We wanted to know whether TRAF1 is the primary adaptor molecular that contributes to the activation of NF-κB. We silenced the expression of TRAF1 using siRNA and found no obvious change in the expression of p65, p-p65, or IκBα (Fig. 6i). We hypothesize that increased TRAF1 expression is a phenomenon related to the activation of NF-κB, and we plan to investigate the exact role of TRAF1 in future work.

4. Discussion

EBVaGC is a specific subgroup of GC and accounts for about 8.7% of all GC cases worldwide, according to a large-scale meta-analysis (Murphy et al., 2009). Although EBVaGC incidence seems low, annual worldwide deaths from EBVaGC are the highest among EBV-associated diseases (Khan and Hashim, 2014). EBV has two forms of infection lifecycle: lytic and latent infections. The latent infection, in which EBV genomic DNA exists as an episome in the nucleus of host cells, has been reported to be involved in many types of cancers (Tsurumi et al., 2005). This “silent” form of infection benefits the virus by enabling it to persist and establish long-term latency because only a few viral products are expressed. EBVaGC has previously been reported to belong to the latency I program, and expresses the viral proteins EBNA1 and LMP2A to enable its latent infection to persist, without LMP1 expression. However, more recent research suggests that almost all EBVaGC tissues express LMP1 mRNA, and that this contributes to cancer progression (Borozan et al., 2018; Niller and Minarovits, 2016; Strong et al., 2013). The differences in reported LMP1 expression may be due to differences in method sensitivities and the level of LMP1 in different gastric tissues. Research has shown that the EBVaGC cell lines GT38 and GT39 express LMP1, while the SNU719 cell line expresses LMP2A. The epidemiological and clinicopathological features of EBVaGC have been generally well-studied (Akiba et al., 2008), while the exact mechanisms of tumorigenesis in EBVaGC remain unclear.

The NF-κB signal is very important in cancer development and is highly activated in many malignant diseases. The constitutive activation of the NF-κB signaling pathway could induce the proliferation and prevent the apoptosis of cancer cells via the up-regulation of its downstream genes. The importance of NF-κB signaling in cancer development is well recognized for some EBV-associated cancers, however little research has been conducted into the precise activation mechanism of NF-κB in EBVaGC. Chung et al. (2013) revealed that constitutive activation of distinct NF-κB signaling was found in EBV-associated nasopharyngeal carcinoma and modulated the expression of multiple oncogenes. Yu et al. (2017) revealed the important role of NF-κB signaling and its potential for therapeutic opportunities in major types of EBV-associated lymphoid tumors. In the current study, the precise nature of NF-κB activation in gastric carcinomas was elucidated. The NF-κB pathway was found to be activated in three EBV-positive gastric carcinoma cell lines, but not in EBV-negative gastric carcinoma cell lines. Consistent with the cell line results, nuclear accumulation of NF-κB was more common in EBV-positive tissues than EBV-negative ones, which is similar to previous studies (Stewart et al., 2004; Yu et al., 2017). NF-κB is regarded as the first line of defense against extracellular stimuli or other stressful conditions. In our study, when LMP1 or LMP2A was transfected into EBV-negative cells, phosphorylated IκBα and p65 were elevated, and this was accompanied with a decrease in total IκBα expression. These findings support the hypothesis that during the initiation of EBV infection, host cells provide a rapid response to the viral stimuli by activating NF-κB signaling.

In order to unveil the precise role of activated NF-κB signaling in EBVaGC, we blocked NF-κB signaling using chemical inhibitors. IκBα is a key molecule in the NF-κB signaling pathway, and the dysregulation of IκBα contributes to the constitutive activation of NF-κB. Considering

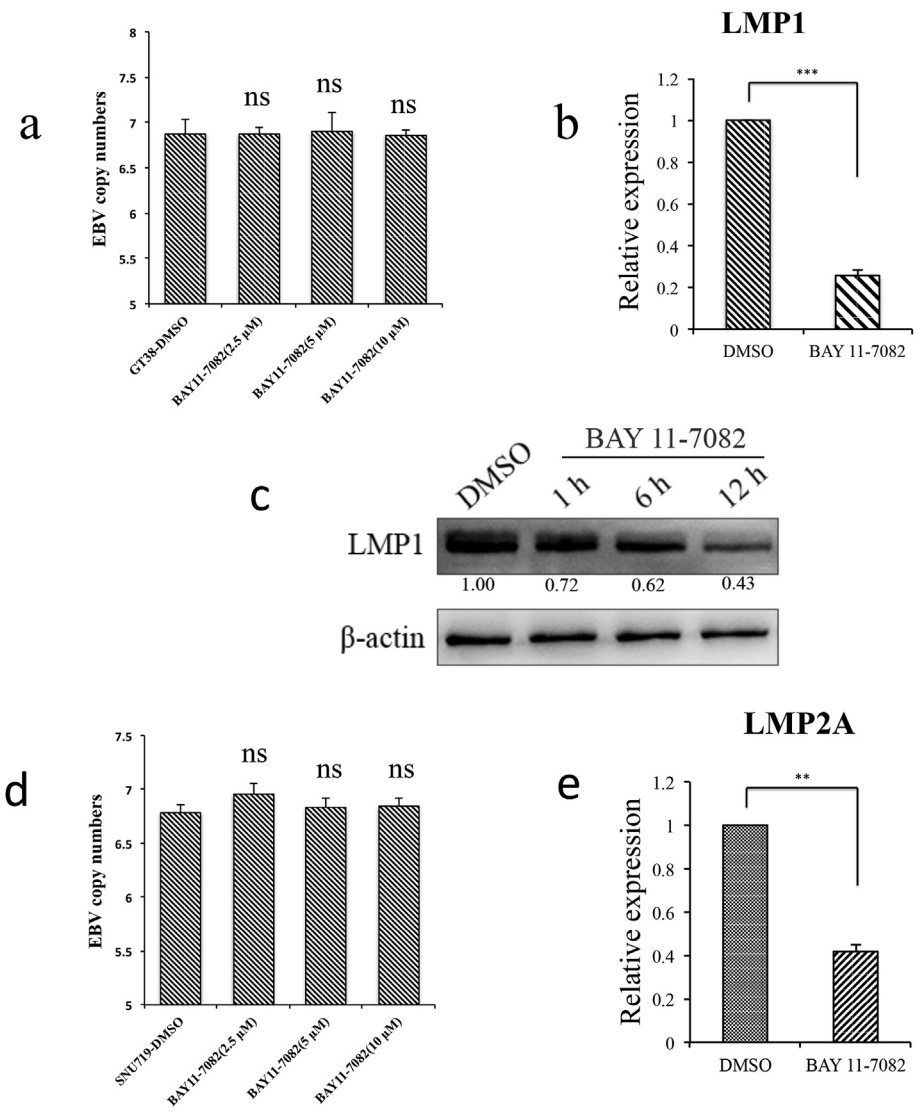

Fig. 5. The effects of BAY 11-7082 on EBV latent membrane proteins (LMP1 and LMP2A). (a) GT38 cells were treated with 2.5, 5, 10 μM BAY 11-7082 for 12 h then harvested for DNA and RNA extraction. EBV copy numbers were detected by an EBV-PCR fluorescence quantitative diagnostic kit. Relative gene expression of LMP1 was calculated using the comparative cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$) and GAPDH as the internal standard. Quantitative RT-PCR showed down-regulation of LMP1 compared with the DMSO group (***$P < 0.001$). Western blotting showed reduced expression of LMP1 following increased treatment with BAY 11-7082, while β-actin served as a control of loading. (b) The SNU719 cells were treated with 2.5, 5, 10 μM BAY 11-7082 for 12 h then harvested for DNA and RNA extraction. EBV copy numbers were detected by an EBV-PCR fluorescence quantitative diagnostic kit. Relative gene expression of LMP2A was calculated using the comparative cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$) and GAPDH as the internal standard. Quantitative RT-PCR showed down-regulation of LMP2A compared with the DMSO group (**$P < 0.01$).

the critical significance of IκBα in the NF-κB pathway, the NF-κB inhibitors we chose were focused on IκBα; BAY 11-7082 prevents the phosphorylation of IκBα, while MG-132 directly restrains the degradation of IκBα. Treatment with these two inhibitors decreased NF-κB nuclear translocation. It has been reported that NF-κB might regulate many genes implicated in cell proliferation and apoptosis (Dong et al., 2001). We found that inhibition of the NF-κB pathway using BAY 11-7082 significantly suppressed the growth of EBVaGC cells compared with EBVnGC cells. In addition, the inhibition of NF-κB induced apoptosis in EBVaGC cells in a dose- and time-dependent manner, via the regulation of various apoptosis-associated genes (e.g., BCL-2, Bax, and Cleaved Caspase-3). More importantly, in some EBV-associated cancers the activation of NF-κB might inhibit viral replication and reactivation, promoting persistent infection by inducing the expression of latent genes (Brown et al., 2003; Liu et al., 2008; Kis et al., 2010). Activated NF-κB could bind to the LMP1 promoter and up-regulate its expression (Johansson et al., 2009). Our data were consistent with these notions, and the expression of LMP1 and LMP2A was down-regulated following treatment with NF-κB inhibitors. These results indicate that the activation of NF-κB is essential to EBVaGC proliferation and the maintenance of latent viral infection. Therefore, the blockage of NF-κB using a therapeutic chemical inhibitor might be a promising strategy for the treatment of EBVaGC.

As NF-κB activation is essential to EBVaGC proliferation, we focused on the precise mechanism by which signaling activation can be induced and sustained. IκBα was the most important inhibitor of NF-κB and prevented its nuclear translocation (Baldwin, 1996). The IκBα DNA promoter has an NF-κB binding site, so elevated NF-κB activity could induce the resynthesis of IκBα. Newly synthesized IκBα could be transported to the nucleus to prevent basal NF-κB activity by stripping NF-κB from the DNA, then activating NF-κB shut-down (Hoffmann et al., 2002). The low level of IκBα expression in EBVaGC contributes to the constitutive activation of NF-κB. In our research, we discussed the regulation of IκBα at the levels of transcriptional, mRNA degradation, and translational control.

First, we analyzed the level of methylation of the IκBα promoter region as the transcriptional control. The global CpG island methylation at the tumor suppressor gene promoter region is considered to be the primary molecular abnormality in EBVaGC, and may therefore lead to the development of EBVaGC (Fukayama, 2010). We found that there was no obvious difference in methylation status in EBV-positive cell lines by BGS analysis (data not shown). This indicated that the low expression of IκBα was unrelated to promoter region methylation. It has been reported that various alterations in genes involved in the NF-κB pathway could contribute to the activation of this pathway in tumors (Chung et al., 2013; Liu et al., 2010). The low expression of IκBα mRNA needs further study.

Many studies have reported that the products encoded by EBV could

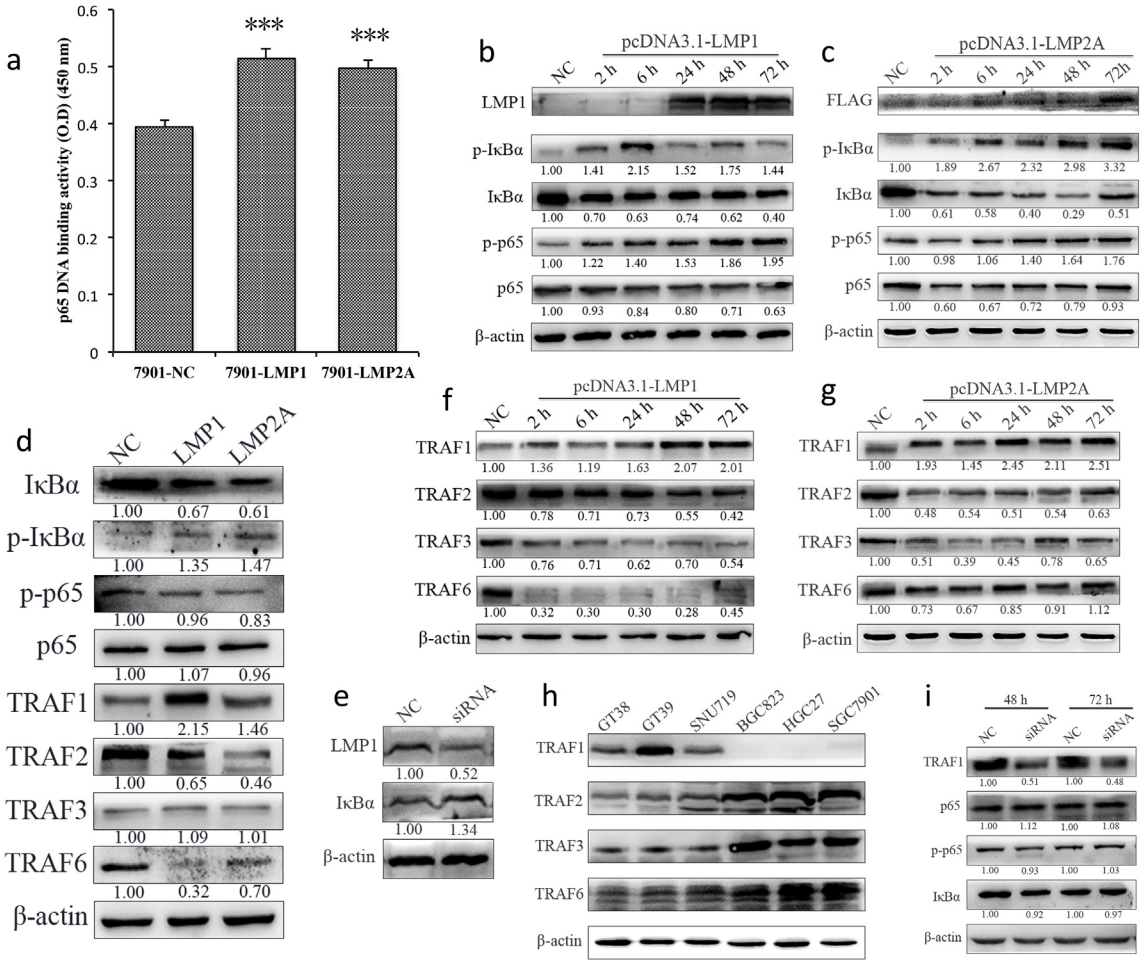

Fig. 6. LMP1 and LMP2A enhance NF-κB activity by association with IκBα and TRAFs. EBVnGC SGC7901 cells were transfected with pcDNA3.1-LMP1, pcDNA3.1-LMP2A or pcDNA3.1 empty vectors and harvested at the indicated times for further analysis. (a) NF-κB p65-DNA binding activity was detected in SGC7901 cells after 48 h transfection with vectors (***$P < 0.001$). Data represent three independent experiments shown as means ± SD. (b and c) The protein expressions were detected after 2, 6, 24, 48, and 72 h of transfection of vectors. Densitometry values of the bands were expressed as fold-increase above the NC control (lane 1). (d) LMP1 and LMP2A means stable transfection of LMP1 and LMP2A in SGC7901. (e) siRNA-LMP1 was used to silence the expression of LMP1 and the cells were collected after 48 h transfection. (f and g) The protein expressions of TRAF1, 2, 3, and 6 in SGC7901 with LMP1/LMP2A transfection. (h) The protein expression of TRAF1, 2, 3, and 6 in EBVaGC and EBVnGC cell lines. (i) siRNA-TRAF1 was used to silence the expression of TRAF1. The cells were collected after 48 h and 72 h transfection for western blotting.

induce both the canonical and non-canonical NF-κB pathways. EBV encodes two kinds of LMPs that mimic signals in EBV-associated cancers. LMP1 directly interacts with TRAFs to mediate the NF-κB signaling pathway via its two effect regions in its carboxy-terminal cytoplasmic domain (Huen et al., 1995), and LMP1 is considered to be a consistently activated CD40 receptor mimic that mediates B-cell proliferation (Kilger et al., 1998). LMP2A can modify signals by mimicking the B-cell antigen receptor using its N- terminal cytoplasmic region (Longnecker and Miller, 1996). The NF-κB signaling pathway is activated by LMP2A through the up-regulation of survivin expression, and NF-κB inhibitors could decrease survivin levels (Hino et al., 2008). EBV-encoded BARF1 could facilitate the proliferation of EBVaGC via up-regulation of the NF-κB/cyclin D1 pathway (Chang et al., 2013). In EBV-positive lymphocytes, fascin is induced by LMP1 through the activation NF-κB signaling to increase invasive migration (Mohr et al., 2014). In nasopharyngeal carcinoma (NPC) with EBV infection, LMP1 and genetic mutations contribute to aberrant NF-κB activation (Chung et al., 2013). In the present study, the EBVnGC cell line SGC7901 was transfected with LMP1 and LMP2A, and results from ELISA and western blotting indicated that both LMP1 and LMP2A could induce the activation of the NF-κB signaling pathway. This is different from some previous reports, which suggested that LMP2A could augment LMP1 signals but did not activate NF-κB signaling on its own (Dawson et al., 2001). LMP1 directly interacts and is constitutively associated with TRAF1, 2, 3, and 6 to induce the NF-κB signaling pathway (Arcipowski and Bisshop, 2012; Sandberg et al., 1997), but in distinct EBV-associated tumors, TRAF molecules are used in a different manner in a wide variety of cell types to interact with LMP1 (Arcipowski et al., 2011). In the present study, we analyzed these four TRAF members to clarify those associated with LMP1 and LMP2A in GC cells. When SGC7901 cells were infected with LMP1 and LMP2A, TRAF1 expression increased while TRAF2 and TRAF3 expression decreased, which was consistent with some earlier studies (Siegler et al., 2003). In other research, the presence of TRAF1 and TRAF2 was associated with a high level of influence over LMP1, while TRAF3 was suggested to be a negative modulator by displacing TRAF1 and TRAF2 from the LMP1 binding site (Devergne et al., 1996). The expressions of TRAF2 and TRAF3 decreased, while TRAF1 expression increased following the activation of NF-κB. A possible explanation is that while TRAF1, TRAF2, and TRAF3 can occupy a single site on LMP1, TRAF1 has a higher affinity for binding with LMP1 than TRAF2 or TRAF3 (Devergne et al., 1998). Different from the three TRAFs discussed above, TRAF6 has a unique binding site at CD40 that is associated with the shared binding site of LMP1. In our research, TRAF6 expression sharply decreased with LMP1 treatment, which is consistent

with previous research (Devergne et al., 1998), and while had no distinct change with the overexpression of LMP2A. This raises the possibility that LMP1 and LMP2A may induce the activation of NF-κB through regulating the expression of TRAF1 specifically, rather than other TRAFs. The present results have indicated that the ways LMPs mediate the NF-κB pathway are more various and complex than have previously been appreciated. The decreased expression of TRAF1 led to no obvious change in p65 and phosphorylated p65 levels, and whether LMP1–TRAF1- or LMP2A–TRAF1-binding is critical in EBVaGC is unknown and needs further research. During long-term latency, EBV-encoded LMP1 and LMP2A maintain the persistent activation of the NF-κB signaling pathway, and this activated NF-κB signal induces the expression of LMP1 and LMP2A. The existence of this positive feedback loop between NF-κB and LMP1 and LMP2A is essential for the development of EBVaGC.

In summary, the present study highlights the important role of constitutive NF-κB-activation in the proliferation of EBVaGC. The constitutive activation of NF-κB contributes to the malignant progression of EBVaGC via the up-regulation of genes involved in proliferation, anti-apoptosis, and maintaining latent infection. The key oncogenes LMP1 and LMP2A could induce the activation of NF-κB by regulating the expression of IκBα and TRAF1. Further investigation into the precise mechanisms of EBV infection and the role of NF-κB should be the focus of future research.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Medical Ethics Committee at the Medical College of Qingdao University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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