

Inflammatory signals induce MUC16 expression in ovarian cancer cells via NF- κ B activation

JIE LIU^{1,2*}, LI LI^{2*}, NING LUO², QI LIU², LI LIU², DANDAN CHEN², ZHONGPING CHENG^{2,3} and XIAOWEI XI¹

¹Department of Obstetrics and Gynecology, Shanghai General Hospital of Nanjing Medical University, Shanghai 200080; ²Department of Obstetrics and Gynecology, Shanghai Tenth People's Hospital, Clinical College of Nanjing Medical University; ³Gynecologic Minimally Invasive Surgery Research Center, Tongji University School of Medicine, Shanghai 200072, P.R. China

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Abstract. Cancer antigen 125 (CA125), encoded by the mucin 16 cell surface associated (MUC16) gene, has been widely used as a biomarker for ovarian cancer (OC) screening. However, it has yet to be elucidated as to why its levels increase with tumor progression as well as with certain other non-malignant conditions. Based on our knowledge of the inflammatory microenvironment (IME) in OC, HEY cells were treated with several inflammation-associated factors as well as their antagonists, and it was observed that inflammation-associated factors upregulated MUC16 gene expression. Considering the role of nuclear factor (NF)- κ B in the inflammatory signaling network and our previous research on OC, chromatin immunoprecipitation was performed, and it was observed that activated NF- κ B bound to the MUC16 gene promoter and enhanced its expression, thereby elevating secreted CA125 levels. These findings demonstrated that IME and MUC16 gene expression were associated in OC, partly

elucidating the role of IME in tumor progression, explaining the elevated serum CA125 levels in some non-malignant conditions, and confirming IME as a potential target for OC therapy.

Introduction

Ovarian cancer (OC) is the most common lethal gynecological malignancy and the fifth leading cause of cancer-related mortality in women worldwide (1). The majority of patients with OC are diagnosed at an advanced stage, due to the lack of specific symptoms at the early stages of the disease (2). Cancer antigen 125 (CA125) is one of the main biomarkers of OC used widely in the clinical setting (3). Serum CA125 detection in OC is a valuable indicator of prognosis, survival time and stage (4,5). However, false-positive results upon serum CA125 detection may adversely affect women who are screened, both psychologically and in terms of unnecessary surgical intervention (6).

CA125 is a membrane-associated mucin-type glycoprotein encoded by the mucin 16 cell surface associated (MUC16) gene. Elevated MUC16 expression was reported to promote proliferation, migration and chemoresistance of OC cells (7-10), and to be associated with poor prognosis of the patients (11). These findings indicated the roles of MUC16 upregulation in OC and explained why CA125 can be used as the biomarker of OC. CA125 is generally present in normal ovarian epithelia, endometrium and decidua (12). However, its levels may increase in benign gynecological diseases and abdominal disorders, as well as in malignant diseases, including OC (13,14). Upregulation of CA125 under both benign and malignant conditions suggested that these conditions may share certain common factors, such as the inflammatory microenvironment (IME), which may be involved in MUC16 and CA125 regulation. It has been reported that malignant ascites from OC enhanced MUC16 expression and stimulated the release of CA125 in human peritoneal mesothelial cells, with stimulating factors unknown (15). IME has been found to be involved in the development and progression of tumors, including OC (16,17). Alterations of inflammatory cytokines, such as interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)- α in the IME play important roles in this process (18).

Correspondence to: Dr Zhongping Cheng, Department of Obstetrics and Gynecology, Shanghai Tenth People's Hospital, Clinical College of Nanjing Medical University, 301 Middle Yanchang Road, Shanghai 200072, P.R. China
E-mail: mdcheng18@tongji.edu.cn

Dr Xiaowei Xi, Department of Obstetrics and Gynecology, Shanghai General Hospital of Nanjing Medical University, 100 Haining Road, Shanghai 200080, P.R. China
E-mail: nmuxiaoweixi@126.com

*Contributed equally

Abbreviations: CA125, cancer antigen 125; CHIP, chromatin immunoprecipitation; HRP, horseradish peroxidase; IFN, interferon; IL, interleukin; IME, inflammatory microenvironment; LPS, lipopolysaccharides; MMP, matrix metalloproteinase; NS, negative site; OC, ovarian cancer; RT-qPCR, reverse transcription-quantitative PCR; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TNFR, TNF receptor

Key words: LPS, IL-6, IL-8, TNF- α , CA125

For example, elevated expression of IL-6 was observed in the serum, ascitic fluid and tumor tissues from patients with OC (19,20). Upregulation of the expression of IL-6 and IL-6 receptor (IL-6R) have been reported to contribute to the proliferation, migration and chemotherapy resistance of OC cells, and may be associated with poor prognosis of patients with OC (21-23). TNF- α facilitates tumor progression through promoting the expression of cytokines and matrix metalloproteinases (MMPs), as well as angiogenesis in OC (24,25). In addition, it has been reported that TNF- α and interferon (IFN)- γ stimulate the expression of MUC16 in breast and endometrial cancer, as well as OC (26). Moreover, IFN- γ and IL-8 were reported to induce MUC16 expression in human ocular surface epithelial cells (27). These findings suggested that inflammatory cytokines in the IME may contribute to the increase of MUC16 expression levels in OC; however, the underlying mechanisms remain to be elucidated.

In the present study, OC cells were treated with inflammation-associated factors, including lipopolysaccharides (LPS), IL-6, IL-8 and TNF- α , and the expression of MUC16 was investigated. The aim was to determine the effect of inflammation-associated factors on MUC16 expression in OC cells and CA125 concentration. Moreover, the effect of the activation of the canonical downstream signaling pathway of each inflammation-associated factor in the regulation of MUC16 and the role of nuclear factor (NF)- κ B in this process were investigated, in order to determine whether the IME contributes to the level of CA125 in OC, and whether it should be taken into consideration in OC diagnosis.

Materials and methods

Cell lines and culture. Four human OC cell lines (OVCAR3, HEY, A2780 and SKOV3) were purchased from the American Type Culture Collection. The OVCAR3, HEY and SKOV3 cell lines were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin (Thermo Fisher Scientific, Inc.). A2780 cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin (Thermo Fisher Scientific, Inc.). All cells were cultured in a humidified incubator with 95% air and 5% CO₂ at 37°C. Cells were routinely passaged and used when they were in the logarithmic growth phase.

Transient transfection. To overexpress or knock down NF- κ B in HEY cells, transient transfection was performed using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 1x10⁶ cells were transfected with 5 μ g pENTER-H-NF- κ B plasmid expressing human NF- κ B (Vigene Biosciences, Inc.) for overexpression. Furthermore, a total of 5x10⁵ cells were transfected with 100 pmol siRNAs targeting NF- κ B for downregulation. Three siRNAs (siRNA-1, 5'-TTGCTA GAACATGCTATAACATG-3'; siRNA-2, 5'-ACGATTGCA ACATCTCTAAGAAT-3'; siRNA-3, 5'-AAGCAATTAAC AAGTTTGTAAT-3') and non-targeting negative control

(5'-TTCTCCGAACGTGTACGTT-3') synthesized by Shanghai GenePharma Co., Ltd. were transfected. At 48 h post-transfection, the cells were used for subsequent assays.

Treatment with inflammation-associated factors. A total of 1x10⁶ HEY cells were first treated with 10, 50 and 100 ng/ml LPS (Sigma-Aldrich; Merk KGaA), IL-6 (PeproTech, Inc.) or IL-8 (PeproTech, Inc.), or 2.5, 10 and 25 ng/ml of TNF- α (PeproTech, Inc.) for 24, 48 and 72 h at 37°C, with 1X PBS used as a control. The lowest concentration and shortest stimulation time of inflammation-associated factors resulting in a statistically significant change in MUC16 mRNA expression levels were selected as the optimal concentration and duration, respectively. HEY cells were treated with LPS, IL-6, IL-8 or TNF- α at the optimal concentration and for the optimal duration. For co-treatment, HEY cells were treated with 10 ng/ml LPS combined with 500 nM Toll-like receptor 4 (TLR4) antagonist VIPER (Novus Biologicals, LLC) for 48 h, 50 ng/ml IL-6 was combined with 10 μ M membrane glycoprotein 130 (gp130) inhibitor SC144 (Selleck Chemicals) for 24 h, 50 ng/ml IL-8 was combined with 10 μ M CXCR2 antagonist SB225002 (Selleck Chemicals) for 48 h or 2.5 ng/ml TNF- α was combined with 100 nM of its inhibitor GSK2982772 (Selleck Chemicals) for 24 h.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from cell lines using TRIzol[®] reagent (cat. no. 15596; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was synthesized using a cDNA synthesis kit (cat. no. 12594; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RT-qPCR was performed with SYBR-Green Master Mix (cat. no. K0223; Thermo Fisher Scientific, Inc.) using the ABI 7300 platform (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: TLR4 forward, 5'-CCGCTTTCCTCTCTCAC-3' and reverse, 5'-CAT CCTGGCATCATCCTCAC-3'; IL-6R forward, 5'-GGT GCGAAAGGATGAAAG-3' and reverse, 5'-TAGGAT TACAGGCGTGAG-3'; gp130 forward, 5'-GAAAGGCTG CTTGGGTTTC-3' and reverse, 5'-GCTCTGGCTTCGTAT CTG-3'; CXCR2 forward, 5'-GGGCACACTTCCACTACT CTC-3' and reverse, 5'-GAACGTGGCCTCCTACTTC-3'; TNF receptor superfamily member 1A (TNFRSF1A) forward, 5'-GCCGCCTACTTGGTGCTAAC-3' and reverse, 5'-CGT CCCTCATCCTCGCAAAC-3'; TNF receptor superfamily member 1B (TNFRSF1B) forward, 5'-TGAGGCTGGGAA ATCGTTTG-3' and reverse, 5'-GCTTTGTCTGTTGGCT TGTTG-3'; JNK1 forward, 5'-GCATCTCAACTCTGTGCAT AG-3' and reverse, 5'-CAGCAGATTAGCATAGAAC-3'; p38 forward, 5'-AAGGAAGGAGGCAGACTGATG-3' and reverse, 5'-GCTGTGGATGGTGAGGATTTG-3'; extracellular signal-regulated kinase ERK2 forward, 5'-TGGGTC AGAAACAAATGG-3' and reverse, 5'-TGCTCTACACGC ATAAAC-3'; NF- κ B forward, 5'-GAATGGCTCGTCTGT AGTG-3' and reverse, 5'-TGATATCTGTGCTCCTCTC-3'; MUC16 forward, 5'-GCAGACAGCAGAGACTATC-3' and reverse, 5'-CTGGACTTCCCAACCATTTC-3'; and GAPDH forward, 5'-AATCCCATCACCATTCTTC-3' and reverse, 5'-AGGCTGTTGTCATACTTC-3'. RT-qPCR reactions were

conducted according to the following thermocycling parameters: 95°C for 10 min; 40 cycles at 95°C for 15 sec and 60°C for 45 sec. Primer specificity was assessed by melt-curve analysis. Relative mRNA quantification was calculated using the $2^{-\Delta\Delta Cq}$ method with GAPDH used as an internal control gene (28).

Western blot analysis. Cell lysates of HEY cells were prepared with RIPA lysis buffer (cat. no. 89901; Thermo Fisher Scientific, Inc.) and centrifuged at 111 x g for 15 min at 4°C. The protein concentration was measured using a BCA protein assay kit (cat. no. 23250; Thermo Fisher Scientific, Inc.) and the lysate was stored at -80°C for further experiments. Equal amounts of protein (30 μ g) were loaded in each lane, separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (EMD Millipore) by semi-dry electrophoretic transfer method for 30 min (25 V). Subsequently, the membranes were blocked using 5% non-fat milk at room temperature for 1 h and incubated for 2 h at room temperature with primary antibodies against MUC16 (dilution, 1:500; cat. no. ab110640), TLR4 (dilution, 1:500; cat. no. ab13556), TNFR-I (dilution, 1:1,000; cat. no. ab19139), TNFR-II (dilution, 1:10,000; cat. no. ab109322), IL-6R (dilution, 1:200; cat. no. ab128008), gp130 (dilution, 1:500; cat. no. ab87969), CXCR2 (dilution, 1:500; cat. no. ab14935), NF- κ B/p65 (dilution, 1:1,000; cat. no. ab16502) (all purchased from Abcam); JNK (dilution, 1:1,000; cat. no. 9252), p-JNK (dilution, 1:1,000; cat. no. 9251S), p38 (dilution, 1:1,000; cat. no. 8690), p-p38 (dilution, 1:1,000; cat. no. 9211), ERK (dilution, 1:1,000; cat. no. 4695), p-ERK (dilution, 1:1,000; cat. no. 4370) and GAPDH (dilution, 1:2,000; cat. no. 5174) (all purchased from Cell Signaling Technology, Inc.). GAPDH was used as an internal protein loading control. The membranes were washed with TBS-0.1% Tween-20 and incubated with the corresponding secondary antibodies for 1 h at 37°C. The horseradish peroxidase (HRP)-labeled donkey anti-goat IgG (dilution, 1:1,000; cat. no. A0181), HRP-labeled goat anti-rabbit IgG (dilution, 1:1,000; cat. no. A0208) and HRP-labeled goat anti-mouse IgG (dilution, 1:1,000; cat. no. A0216) secondary antibodies were purchased from Beyotime Institute of Biotechnology. The proteins were visualized by enhanced chemiluminescence (cat. no. WBKLS0100; EMD Millipore) according to the manufacturer's instructions, and the densitometric analyses of the bands were performed by ChemiDoc™ XRS + image analyzer (Bio-Rad Laboratories, Inc.).

ELISA. Cell culture supernatants were collected from cells treated as mentioned in *Treatment with inflammation-associated factors*. CA125 levels were measured using a commercial ELISA kit (cat. no. XY-E10325; Shanghai Xinyu Biological Technology Co., Ltd.) according to the manufacturer's protocols. The absorbance was measured at 450 nm on a microplate absorbance reader (MR-960; Perlong Medical Equipment Co., Ltd.). The concentration of CA125 was quantified by corresponding standard curves.

Bioinformatics analysis. To determine the potential binding sites of NF- κ B, the promoter sequence of human MUC16 (chr19:8981139-8983842) obtained from the University of California, Santa Cruz database (<https://genome.ucsc.edu/>)

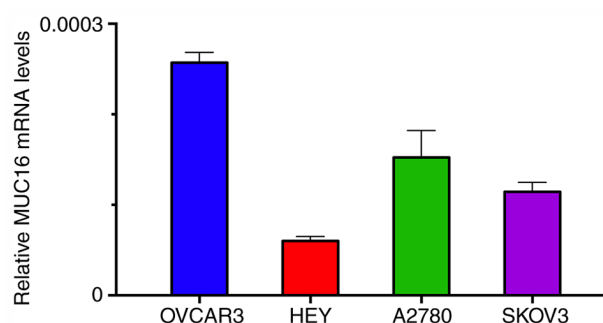


Figure 1. mRNA expression levels of MUC16 in four different OC cell lines. The basal MUC16 mRNA expression levels in four human OC cell lines were measured with reverse transcription-quantitative PCR analysis. GAPDH was used as an internal control. Data are presented as the mean \pm SEM from triplicate experiments. MUC16, mucin 16 cell surface associated; OC, ovarian cancer.

was sent to the Consit database (<http://consite.genereg.net/>) with an 85% Transcription Factor score as the cutoff value. Then, PCR primers amplifying the retrieved potential binding sites was sent for synthesis and used for chromatin immunoprecipitation (ChIP) analysis.

ChIP assay. Immunoprecipitation assays were performed with a commercial CUT&Tag kit (cat. no. S602; Vazyme Biotech Co., Ltd.) and p65 antibody (cat. no. 10745-1-APNF- κ B; ProteinTech Group, Inc.) according to the manufacturers' instructions. HEY cells were harvested using 0.25% Trypsin and washed using washing buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl and 500 mM spermidine. Cells were resuspended in antibody buffer containing 2 mM EDTA, 0.1% BSA (cat. no. E661003; Sangon Biotech Co., Ltd.) and 0.5% digitonin (Vazyme Biotech Co., Ltd.) for p65 antibody (1 μ g per immunoprecipitation) incubation at room temperature. IgG antibody (cat. no. AP162-KC; Sigma-Aldrich) was used as the negative control. After DNA collection and purification by the phenol and chloroform method, qPCR was performed with SYBR-Green Master Mix (cat. no. K0223; Thermo Fisher Scientific, Inc.) with the ABI 7300 platform (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers of two potential binding sites were used: P65-binding site primers (p65B) forward, 5'-ACCTCCACCTCCTGGGTT C-3' and reverse, 5'-GGTGGGTGGATTACTTGAAGTC-3'; NF- κ B-binding site primers forward, 5'-GTCGCCAG GCTGAAGTG-3' and reverse, 5'-CTGCTGGGCGTGGTG TCT-3'; and negative site primers (NS) forward, 5'-AGGAGA CGCAGCTTAGAACC-3' and reverse, 5'-TTCAACTTTCCA GCCTCCA-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 30 sec and 40 cycles of 95°C for 5 sec and 60°C for 10 sec. The enrichment of the binding site was calculated using the $2^{-\Delta\Delta Cq}$ method with NS used as internal control.

Statistical analysis. All data were analyzed using SPSS 20.0 software (IBM Corp.). The measurement values are presented as the mean \pm SEM from at least triplicates. One-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

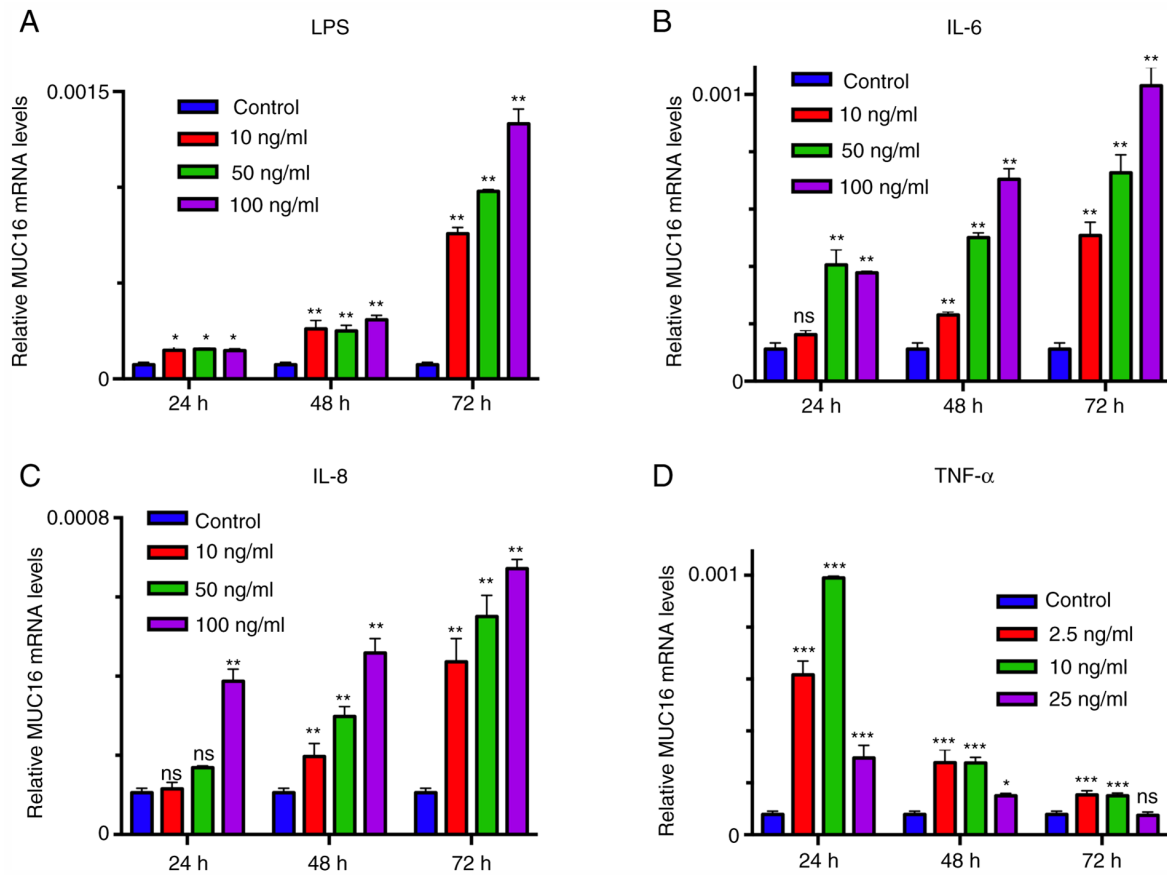


Figure 2. Screening the optimal concentration and duration of inflammation-associated factor stimulation. HEY cells were treated with different stimulation concentrations of (A) LPS, (B) IL-6, (C) IL-8 and (D) TNF- α for 24, 48 and 72 h. The mRNA expression levels of MUC16 were determined by reverse transcription-quantitative PCR analysis. GAPDH was used as an internal control. Data are presented as the mean \pm SEM from triplicate experiments. * P <0.05, ** P <0.01 and *** P <0.001 vs. the control group. LPS, lipopolysaccharides; IL, interleukin; TNF, tumor necrosis factor; MUC16, mucin 16 cell surface associated; ns, not significant.

Results

Basal MUC16 mRNA expression levels in four OC cell lines. First, the expression levels of MUC16 were assessed in four different human OC cell lines (OVCAR3, HEY, A2780 and SKOV3) by RT-qPCR to select the appropriate tumor cells for further analysis. Among these tumor cells, HEY cells exhibited the lowest and OVCAR3 the highest levels of MUC16 expression (Fig. 1). Therefore, HEY cells were selected for further experiments.

Screening the optimal concentration and duration of inflammation-associated factor stimulation. To select the optimal concentration and duration of inflammation-associated factor treatment, HEY cells were treated with different concentrations of LPS, IL-6, IL-8 or TNF- α for 24, 48 and 72 h and MUC16 mRNA expression was analyzed. The results demonstrated that the mRNA expression levels of MUC16 were increased in tumor cells treated with inflammation-associated factors in a dose and time-dependent (Fig. 2). The highest level of MUC16 expression was observed in cells treated with 100 ng/ml LPS, IL-6 and IL-8 for 72 h respectively, while lower expression levels were observed in cells treated with lower concentrations or shorter durations (Fig. 2). For TNF- α administration, 10 ng/ml incubation for 24 h induced

the highest MUC16 expression while increasing TNF- α concentration and longer duration induced lower MUC16 expression at 24 h (Fig. 2). Therefore, these concentrations of inflammation-associated factors and respective treatment durations were used in the following experiments.

Inflammation-associated factors induce the expression of MUC16 in OC cells. To investigate whether the inflammation-associated factors LPS, IL-6, IL-8 and TNF- α affect the expression of MUC16 in OC cells, the mRNA expression levels of MUC16 were assessed in OC cells treated with inflammation-associated factors. RT-qPCR analysis revealed that the mRNA expression levels of MUC16 were significantly increased in OC cells treated with LPS, IL-6, IL-8 or TNF- α compared with those in untreated cells (Fig. 3A-D). When the corresponding receptor antagonists were added, the upregulation of MUC16 was inhibited (Fig. 3A-D). In addition, the protein expression levels of MUC16 were detected in tumor cells treated with inflammation-associated factors. Western blot analysis demonstrated that the protein expression levels of MUC16 were also increased following treatment with the aforementioned inflammation-associated factors (Fig. 3E-H). In addition, the increase induced by treatment with inflammation-associated factors was inhibited by their corresponding receptor antagonists (Fig. 3E-H). These results suggested that

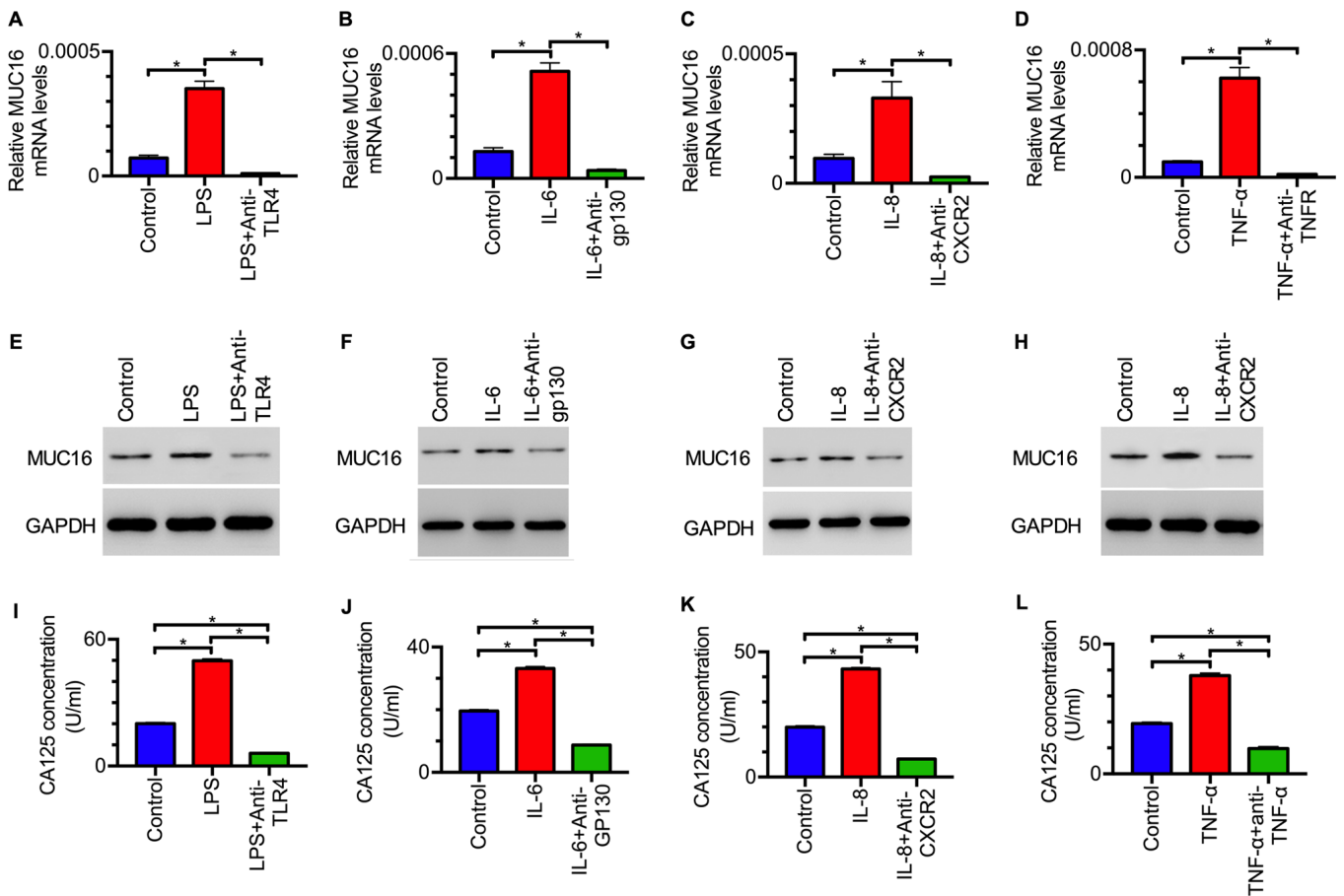


Figure 3. Inflammation-associated factors induce MUC16 expression in ovarian cancer cells. mRNA expression levels of MUC16 in HEY cells treated with (A) LPS or LPS + anti-TLR4 for 48 h, (B) IL-6 or IL-6 + anti-gp130 for 24 h, (C) 50 ng/ml IL-8 or IL-8 + anti-CXCR2 for 48 h and (D) 2.5 ng/ml TNF- α or TNF- α + anti-TNF- α for 24 h. GAPDH served as an internal control in reverse transcription-quantitative PCR analysis. Data are presented as the mean \pm SEM of three independent experiments. * P <0.05 as indicated. Western blot analysis of MUC16 protein expression levels in HEY cells treated with (E) LPS or LPS + anti-TLR4, (F) IL-6 or IL-6 + anti-gp130, (G) IL-8 or IL-8 + anti-CXCR2 and (H) TNF- α or TNF- α + anti-TNF- α . Images are representative of three independent experiments. Levels of CA125 in the supernatant of HEY cells treated with (I) LPS or LPS + anti-TLR4, (J) IL-6 or IL-6 + anti-gp130, (K) IL-8 or IL-8 + anti-CXCR2 and (L) TNF- α or TNF- α + anti-TNF- α , as determined by ELISA. Data are presented as the mean \pm SEM of three independent experiments. * P <0.05 as indicated. LPS, lipopolysaccharides; IL, interleukin; TNF, tumor necrosis factor; TLR, Toll-like receptor; MUC16, mucin 16 cell surface associated.

LPS, IL-6, IL-8 and TNF- α induced MUC16 expression in HEY cells. Moreover, the levels of CA125 in the cell culture supernatant were also investigated. ELISA demonstrated that a higher level of CA125 was observed in the supernatant from tumor cells treated with inflammation-associated factors compared with that from control cells (Fig. 3I-L). When cells were treated with both receptor antagonists and inflammation-associated factors simultaneously, CA125 levels were decreased to a lower level compared with the control group, suggesting that receptor antagonists inhibited the activation of MUC16 expression by inflammation-associated factors (Fig. 3I-L). Collectively, these data indicated that inflammation-associated factors increased MUC16 expression and the level of CA125 in OC cells.

Inflammation-associated factors activate downstream signals in OC cells. To elucidate the mechanisms underlying the regulation of MUC16 expression by inflammation-associated factors, the activation status of the downstream signaling molecules of each inflammation-associated factor was investigated. The molecules of the MAPK signaling pathway,

including JNK, p38, ERK and NF- κ B, were selected for investigation, as the MAPK signaling pathways are activated by all four factors (29-31). RT-qPCR analysis revealed that treatment with the inflammation-associated factors increased the mRNA expression levels of *JNK*, *p38*, *ERK* and *NF- κ B* in tumor cells, and the addition of the respective receptor inhibitors lowered the mRNA expression levels of these genes (Fig. 4A-D). In addition, the protein expression levels and phosphorylation status of these molecules were also investigated via western blot analysis. The results demonstrated that the expression of receptors including TLR4, IL-6R, CXCR2, TNFRI and TNFRII and the levels of p-JNK1/2, p-p38, p-ERK1/2 and NF- κ B were upregulated by inflammation-associated factors, and phosphorylation was inhibited by their receptor inhibitors, whereas the protein expression levels of JNK1/2, p-38 and ERK1/2 exhibited no marked changes in expression when compared with the control group for all four mediators (Fig. 4E-H). The upregulation of receptor (TLR4, IL-6R, CXCR2, TNFRI and TNFRII) expression and phosphorylation levels of JNK1/2, p-38 and ERK1/2 were inhibited by inflammation-associated factor receptor inhibitors, suggesting

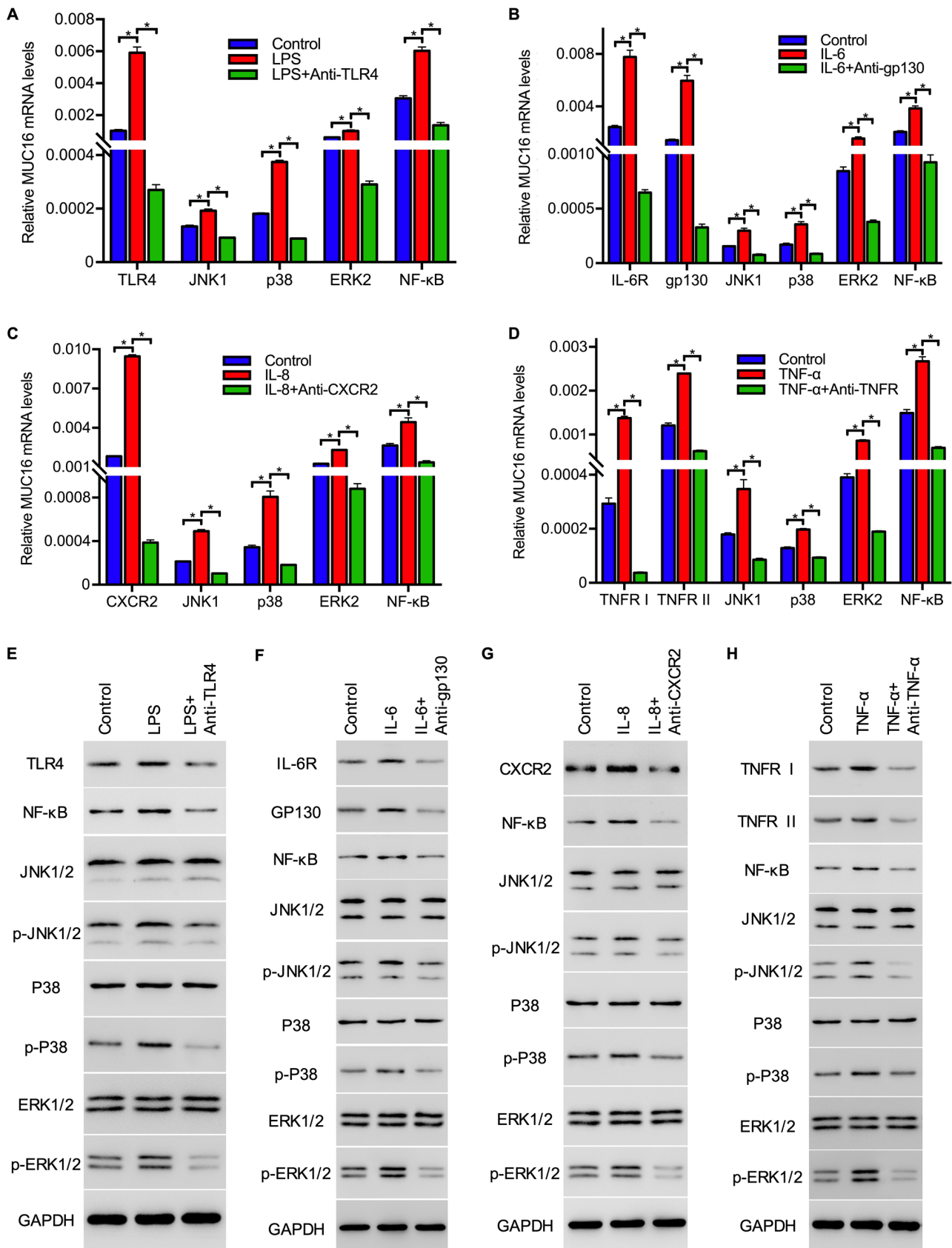


Figure 4. Inflammation-associated factors stimulate the expression and activation of cell proliferation signals in ovarian cancer cells. Expression of TLR4, JNK1, p38, ERK2 and NF-κB in HEY cells treated without or (A) with LPS or LPS + anti-TLR4, (B) IL-6 or IL-6 + anti-gp130, (C) IL-8 or IL-8 + anti-CXCR2 and (D) TNF-α or TNF-α + anti-TNF-α as detected by reverse transcription-quantitative PCR analysis. GAPDH served as an internal control. Data are presented as the mean ± SEM of three independent experiments. * $P < 0.05$ as indicated. Expression and phosphorylation levels of inflammation-associated factor receptors, molecules in the MAPK signaling pathway and NF-κB in HEY cells treated with (E) LPS or LPS + anti-TLR4, (F) IL-6 or IL-6 + anti-gp130, (G) IL-8 or IL-8 + anti-CXCR2 and (H) TNF-α or TNF-α + anti-TNF-α, as evaluated by western blotting. NF-κB, nuclear factor-κB; LPS, lipopolysaccharides; IL, interleukin; TNF, tumor necrosis factor; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; IL-6R, IL-6 receptor; TNFR, TNF receptor; gp130, membrane glycoprotein 130.

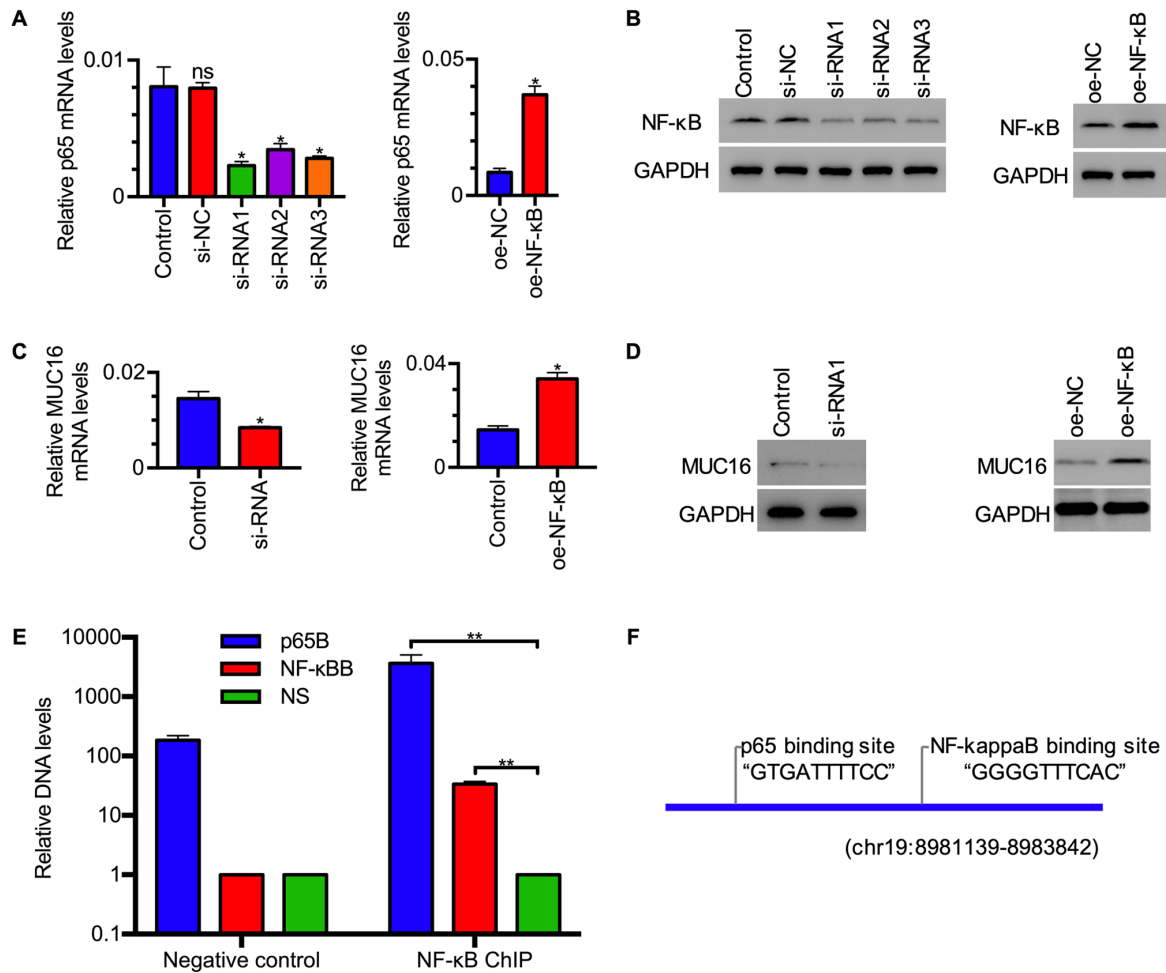


Figure 5. NF- κ B/p65 enhances MUC16 expression by binding to its gene promoter. (A) NF- κ B mRNA expression levels relative to GAPDH in HEY cells following NF- κ B knockdown and overexpression (n=3). Data are presented as the mean \pm SEM of three independent experiments. *P<0.05 vs. control or oe-NC. (B) NF- κ B protein expression levels in HEY cells following NF- κ B knockdown and overexpression. (C) MUC16 mRNA expression levels relative to GAPDH in control, NF- κ B knockdown and NF- κ B overexpression HEY cells (n=3). *P<0.05 vs. control. (D) MUC16 protein expression levels in control, NF- κ B knockdown and NF- κ B overexpression HEY cells. (E) Quantitative analysis of relative DNA levels collected after chromatin immunoprecipitation (n=3). Data are presented as the mean \pm SEM of three independent experiments. **P<0.01. (F) Potential NF- κ B/p65 binding sites on the MUC16 promoter. NF- κ B, nuclear factor- κ B; si, small interfering RNA; oe, overexpression; NC, negative control; MUC16, mucin 16 cell surface associated.

that these inflammatory mediators activated downstream signaling cascades in HEY cells (Fig. 4E-H). Taken together, these data suggested that LPS, IL-6, IL-8 and TNF- α induced the expression and activation of molecules, including JNK, p38, ERK, NF- κ B, in downstream signaling cascades.

NF- κ B/p65 enhances MUC16 expression by binding to its promoter. Having established that inflammation-associated factor treatment upregulated MUC16 and NF- κ B expression in HEY cells, the present study sought to investigate whether NF- κ B, a canonical transcription factor, mediated the upregulation of MUC16 by inflammation-associated factors. To this end, transfection of plasmid expressing NF- κ B or siRNAs targeting NF- κ B into HEY cells was performed to upregulate or downregulate NF- κ B expression, respectively (Fig. 5A and B), and then MUC16 expression was assessed. RT-qPCR analysis revealed that the mRNA expression levels of MUC16 were increased in tumor cells with NF- κ B overexpression and decreased in tumor cells with NF- κ B knockdown (Fig. 5C). Western blot analysis revealed that the protein expression levels of MUC16 exhibited a similar expression pattern as its

mRNA in tumor cells with NF- κ B overexpression or knockdown (Fig. 5D). Moreover, ChIP assay was performed using NF- κ B antibody to investigate how NF- κ B regulates MUC16 gene expression. Quantitative analysis revealed that DNA levels amplified by p65B and NF- κ B primers were higher compared with those by NS primers in samples immunoprecipitated by p65 antibody, while p65B primers amplified more DNA in p65 ChIP compared with negative control (Fig. 5E). These data revealed that two sites from the MUC16 promoter, identified as potential NF- κ B-binding sites by bioinformatics analysis (Fig. 5F), were enriched by ChIP assay, suggesting that NF- κ B binds to these sites on the MUC16 promoter. These data indicated that NF- κ B may activate MUC16 transcription by binding to its promoter.

Discussion

MUC16, one of the main biomarkers of OC, is involved in OC development and metastasis, and has been found to be associated with poor prognosis (7,8). However, little is known on the association between MUC16 expression and

inflammation-associated factors. In the present study, it was observed that the inflammation-associated factors LPS, IL-6, IL-8 and TNF- α increased the expression levels of MUC16 and enhanced CA125 release in OC. Moreover, it was demonstrated that NF- κ B mediated regulation of MUC16 via directly binding to the promoter of MUC16. The finding on the upregulation of MUC16 by TNF- α was consistent with the observations reported by Morgado *et al* (26) who indicated that TNF- α and IFN- γ stimulated MUC16 expression in OC cells via NF- κ B activation. The present study demonstrated that NF- κ B mediated not only the TNF- α regulation of MUC16, but also LPS, IL-6 and IL-8 regulation, suggesting that NF- κ B may be one of the main transcriptional factors regulating MUC16 expression in OC. These findings indicated that inflammatory factors regulated MUC16 expression in OC cells and NF- κ B may have a role in this process.

The finding that inflammatory factors regulated MUC16 expression in OC may improve the understanding of how inflammation contributes to OC. Inflammation is a hallmark of cancer that contributes to the occurrence and development of various tumors, including OC (16,32). An increasing number of studies have uncovered the role of inflammation in the initiation and progression of OC, with the proinflammatory cytokine IL-6 established as a key immunoregulatory cytokine (31,33,34). IL-6 was reported to enhance the migratory ability of tumor cells via increasing MMP9 expression (23). Furthermore, together with IL-8, IL-6 also markedly promoted the proliferation of OC cells in a time- and dose-dependent manner (35). TNF- α , another important inflammatory factor, was found to promote tumor cell migration by upregulating CXCR4 via NF- κ B activation in OC cells (36). Upregulation of MUC16 expression by these inflammation-associated factors in the present study elucidated another mechanism underlying the effects of inflammatory factors on OC.

In addition, inflammatory factor-mediated regulation of MUC16 may explain the high false-positive rate of CA125 in OC diagnosis. Inflammation is involved in diverse biological and pathological processes, including non-malignant diseases and tumors. For example, some patients with endometriosis have been found to have elevated serum and peritoneal fluid IL-6 levels (37,38), while an IL-6/TNF- α -based model has been reported as a potential predictor of chronic endometritis (39). Some inflammatory and autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and asthma, have also been associated with an increased serum IL-6 level (40). It was also reported that 8, or a combination of IFN- α with TNF- α /IL-17, increased the expression of MUC16 in human ocular surface epithelial cells (27). Moreover, anti-inflammatory agents, such as dexamethasone, were reported to upregulate MUC16 in human corneal epithelial cells (41), suggesting that some inflammatory factors exert different roles to IL-6, IL-8 and TNF- α in MUC16 regulation. These findings indicated that inflammation may increase MUC16 expression in non-malignant diseases and suggested that other factors should be taken into consideration together with CA125 in OC diagnosis.

Notably, it was observed that the expression of the receptors of inflammation-associated factors was upregulated in HEY cells when treated with their ligands. This type of positive feed-forward loop has also been reported in hepatocytes (42)

and bronchial epithelial cells (43), while IL-6 treatment decreased IL-6R expression in primary monocytes (42) and NK92 cells (an IL-2-dependent natural killer cell line) (44). These contradictory findings suggested that inflammatory signaling pathways display cell-specific regulation, which requires further investigation. In addition, unlike other inflammatory signaling molecules, JNK1/2, p38 and ERK1/2 protein expression levels in HEY cells exhibited no synchronous elevation with their mRNA expression levels when treated with inflammatory factors, which may be attributed to complex biochemical processes, such as time and space interval between mRNA transcription and protein translation, post-translational modification (45), and regulation of inflammatory signaling networks. More sophisticated experiments involving these aspects should be performed to elucidate the differences between mRNA and protein expression levels.

In conclusion, the present study demonstrated that inflammatory factors, including LPS, IL-6, IL-8 and TNF- α , upregulated the expression levels of MUC16 in OC cells via NF- κ B. These findings may improve the understanding of the molecular mechanisms underlying the regulation of MUC16 expression and uncover the association between inflammation and MUC16 expression in OC. In addition, these findings suggested that inflammatory factors may represent promising targets for OC therapy or diagnosis, along with MUC16/CA125, although further investigation is required to verify the association of inflammation with MUC16 expression and serum CA125 concentration.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JL, LLi, ZC and XX were responsible for the research conception and design, analysis and interpretation of the data, the statistical analysis and manuscript drafting. NL, QL, ZC and XX contributed to data acquisition, analysis and interpretation of the data, and critical revision of the manuscript for important intellectual content. LLi and DC performed the systematic search of the literature, and contributed to the acquisition, analysis and interpretation of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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