

***Helicobacter pylori* cytotoxin-associated gene A activates tumor necrosis factor- α and interleukin-6 in gastric epithelial cells through P300/CBP-associated factor-mediated nuclear factor- κ B p65 acetylation**

QIONG LIN, HUI XU, XINTAO CHEN, GUORONG TANG, LAN GU and YEHONG WANG

Department of Gastroenterology, Wuxi Children's Hospital Affiliated to Nanjing Medical University, Wuxi, Jiangsu 214023, P.R. China

Received October 19, 2014; Accepted July 2, 2015

DOI: 10.3892/mmr.2015.4143

Abstract. *Helicobacter pylori*-initiated chronic gastritis is characterized by the cytotoxin-associated gene (Cag) pathogenicity island-dependent upregulation of pro-inflammatory cytokines in gastric epithelial cells, which is largely mediated by the activation of nuclear factor (NF)- κ B as a transcription factor. However, the precise regulation of NF- κ B activation, particularly post-translational modifications in the CagA-induced inflammatory response, has remained elusive. The present study showed that *Helicobacter pylori* CagA, an important virulence factor, induced the expression of P300/CBP-associated factor (PCAF) in gastric epithelial cells. Further study revealed that PCAF was able to physically associate with the NF- κ B p65 sub-unit and enhance its acetylation. More importantly, PCAF-induced p65 acetylation was shown to contribute to p65 phosphorylation and further upregulation of tumor necrosis factor (TNF)- α and interleukin (IL)-6 in gastric adenocarcinoma cells. In conclusion, the results of the present study indicated that *Helicobacter pylori* CagA enhanced TNF- α and IL-6 in gastric adenocarcinoma cells through PCAF-mediated NF- κ B p65 sub-unit acetylation.

Introduction

Infection with *Helicobacter pylori* is one of the most common types of bacterial infection. *Helicobacter pylori* infection mainly occurs in economically underdeveloped regions, with the infection rates of pediatric patients in China, Japan and

Korea being higher than those in developed countries (1-5). *Helicobacter pylori* colonizes the stomach and duodenum areas, causing chronic inflammation of the gastric mucosa, and the development of stomach ulcers and neoplasms (gastric cancer and mucosa-associated lymphoid tissue) (6,7). However, the mechanism by which *Helicobacter pylori* infection causes pathological changes of the gastric mucosa remains to be fully elucidated.

Inflammatory responses have been known to have a key role in the pathogenesis of *Helicobacter pylori* infection and contribute to chronic gastritis as well as gastric and duodenal ulcers in the patients (8-10). A growing body of evidence has demonstrated that elevated levels of inflammatory factors, including tumor necrosis factor (TNF)- α , interferon- γ , interleukin (IL)-6, IL-8 and IL-32 were associated with more serious pathogenesis of patients infected with *Helicobacter pylori* (10-15). However, the underlying regulatory mechanisms of the production of inflammatory factors have largely remained elusive.

Nuclear factor (NF)- κ B has an important role in the regulation of inflammatory responses in mammals (16,17). It has been demonstrated that *Helicobacter pylori* infection activates NF- κ B and its target genes, particularly inflammatory factors in epithelial cells, which is thought to be critical for *Helicobacter pylori*-initiated chronic inflammation (9,18-21). The virulence factor cytotoxin-associated gene A (CagA) encoded by the *Helicobacter pylori* Cag pathogenicity island, has an important role in the pathogenicity of *Helicobacter pylori*, including *Helicobacter pylori*-induced activation of NF- κ B and expression of NF- κ B target genes (22,23). However, the exact function of CagA in the activation of NF- κ B and the NF- κ B-dependent inflammatory response has not been well characterized.

Emerging evidence indicated that post-translational modification, such as acetylation, has important roles in various biological events, including inflammation (24-26). It has been reported that the acetylation of the NF- κ B p65 sub-unit has an important role in its activation (27-29). In addition, recent studies showed that P300/CBP-associated factor (PCAF), also frequently referred to as lysine (K) acetyltransferase 2B, is a transcription co-activator that contains several nuclear

Correspondence to: Dr Yehong Wang, Department of Gastroenterology, Wuxi Children's Hospital Affiliated to Nanjing Medical University, 299 Qingyang Road, Wuxi, Jiangsu 214023, P.R. China
E-mail: wangyhwuxi@163.com

Key words: *Helicobacter pylori*, cytotoxin-associated gene A, inflammation, nuclear factor- κ B p65, P300/CBP-associated factor, acetylation

receptor-interacting domains and can function as an acetyl transferase (30–33). PCAF has also been reported to be associated with infection and inflammation (34–37). Therefore, there is a requirement to explore the roles of PCAF in mediating p65 activation and the production of TNF- α and IL-6 in gastric adenocarcinoma cells.

The present study aimed to investigate the implication of *Helicobacter pylori* CagA in the inflammatory response of human gastric adenocarcinoma AGS cells *in vitro*, as well as PCAF-mediated p65 acetylation and its role in regulating the production of TNF- α and IL-6 as two important inflammatory factors in AGS cells.

Materials and methods

Reagents. Monoclonal rabbit antibodies against human total p65 (cat. no. 8242), phospho (p-)p65 (cat. no. 3031), as well as monoclonal mouse antibodies against acetylated-Lysine (cat. no. 9681) and human β -actin (cat. no. 3700) were from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibodies against human PCAF (cat. no. sc-13124) and Hemagglutinin (HA)-tag (cat. no. sc-7392) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). For western blot analysis, horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody (cat. no. 7076) and anti-rabbit IgG antibody (cat. no. 7074) were purchased from Cell Signaling Technology. Enhanced chemiluminescence (ECL) Western Blotting Substrate, co-immunoprecipitation (co-IP) assay buffer, radioimmunoprecipitation assay (RIPA) lysis buffer, a bicinchoninic acid (BCA) protein assay kit and protein G-Sepharose beads were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Polyvinylidene difluoride (PVDF) membranes were obtained from Roche (Basel, Switzerland). TRIzol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Moloney Murine Leukemia Virus reverse transcriptase and oligo (dT) 15 primer were purchased from Promega (Madison, WI, USA). TaqMan® Fast Advanced Master Mix was from Applied Biosystems (Thermo Fisher Scientific). The plasmids pGCsi-U6/neo/green fluorescence protein (GFP) were obtained from Shanghai Genkan Biotechnology Co., Ltd (Shanghai, China). The pcDNA3.1 vector was purchased from Invitrogen Life Technologies. The incision enzymes *Hind*III and *Xho*I as well as T4 DNA ligase were purchased from Takara Bio Inc. (Tokyo, Japan). The *Escherichia coli* strain DH5 α was purchased from Molecular Cloning Laboratories (San Francisco, CA, USA). QIAprep spin miniprep kit was obtained from Qiagen (Hilden, Germany). PDTC was purchased from Abcam (Cambridge, UK).

Cell culture and treatment. The human AGS cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in the medium of RPMI-1640 (Gibco-BRL; Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and antibiotics (50 U/ml penicillin and 100 μ g/ml streptomycin) at 37°C in a 5%-CO₂ incubator. AGS cells were incubated with *Helicobacter pylori* CagA protein at 37°C for different time points. For certain studies, the cells were incubated with 10 μ M PDTC at 30 min before treatment with *Helicobacter pylori* CagA protein.

Construction of overexpression plasmids. The open reading frame of human PCAF mRNA (National Center of Biotechnology Information reference sequence, NM_003884.4) containing a HA tag was amplified by polymerase chain reaction (PCR) from cDNA of human AGS cells. The PCR products and pcDNA3.1 vector were further digested with the two restriction enzymes of *Hind*III and *Xho*I, and then ligated with each other by using T4 DNA ligase. The recombinant plasmids were amplified in the *Escherichia coli* strain DH5 α at 37°C for 16 h. Subsequently, the plasmids were extracted by QIAprep spin miniprep kit according to the manufacturer's instructions. Finally, the constructed plasmids (pcDNA3.1/PCAF-HA) were sequenced to confirm the nucleotide sequence by GenScript (Nanjing, China).

Construction of small hairpin (sh)RNA expression plasmids. Various shRNA sequences targeting human PCAF mRNA (NM_003884.4) were designed to silence the PCAF gene in human AGS cells. The various DNA segments for the expression of PCAF shRNA were synthesized and inserted into the shRNA pGCsi-U6/neo/GFP plasmids by Shanghai Genkan Biotechnology Co., Ltd. The most effective shRNA expression plasmid to silence human PCAF gene was selected to be used in subsequent experiments. The PCAF shRNA sequence was as follows: AAGATGGCCGTGTTATTGGTG and the Scrambled shRNA sequence was as follows: AATGACGGGCTTGTTATGGGT.

Cellular transfection. Plasmids were transfected into AGS cells by using Lipofectamine 2000 according to the manufacturer's instructions. For transfection, 4 μ g plasmid was mixed with 250 μ l serum-free RPMI-1640. At the same time, 10 μ l Lipofectamine 2000 was mixed with 250 μ l serum-free RPMI-1640. The plasmids and Lipofectamine 2000 were further incubated with each other for 20 min at room temperature. Finally, the 500- μ l mixture was added into each well in a 6-well plate containing 4 \times 10⁵ AGS cells. The medium was replaced with serum containing RPMI-1640 at 5 h after transfection, and the cells were incubated sequentially.

Production of the CagA protein. *Helicobacter pylori* CagA-His-tag was constructed in the pET21a plasmid from Novagen (Madison, WI, USA). The plasmid was then transformed into BL21(DE3) Singles™ Competent Cells (Novagen) for protein expression and purification according to the manufacturer's instructions. The protein was extracted by B-PER® bacterial protein extraction reagent (Thermo Fisher Scientific) from 50 ml bacteria according to the manufacturer's instructions. Subsequently, HisPur™ Cobalt Spin Columns (Thermo Fisher Scientific) were used for His-tag purification of the *Helicobacter pylori* CagA protein according to the manufacturer's instructions.

co-IP experiment. AGS cells were lysed in co-IP assay buffer at 4°C for 30 min. Cell lysates were then centrifuged at 15,000 \times g for 20 min at 4°C to remove any insoluble material. 300 μ g cell lysate was incubated with 40 μ l protein G-Sepharose beads in co-IP assay buffer at 4°C for 2.5 h under constant agitation and then centrifuged at 1,000 \times g for 2 min at 4°C. The recovered supernatant was then incubated with the antibody

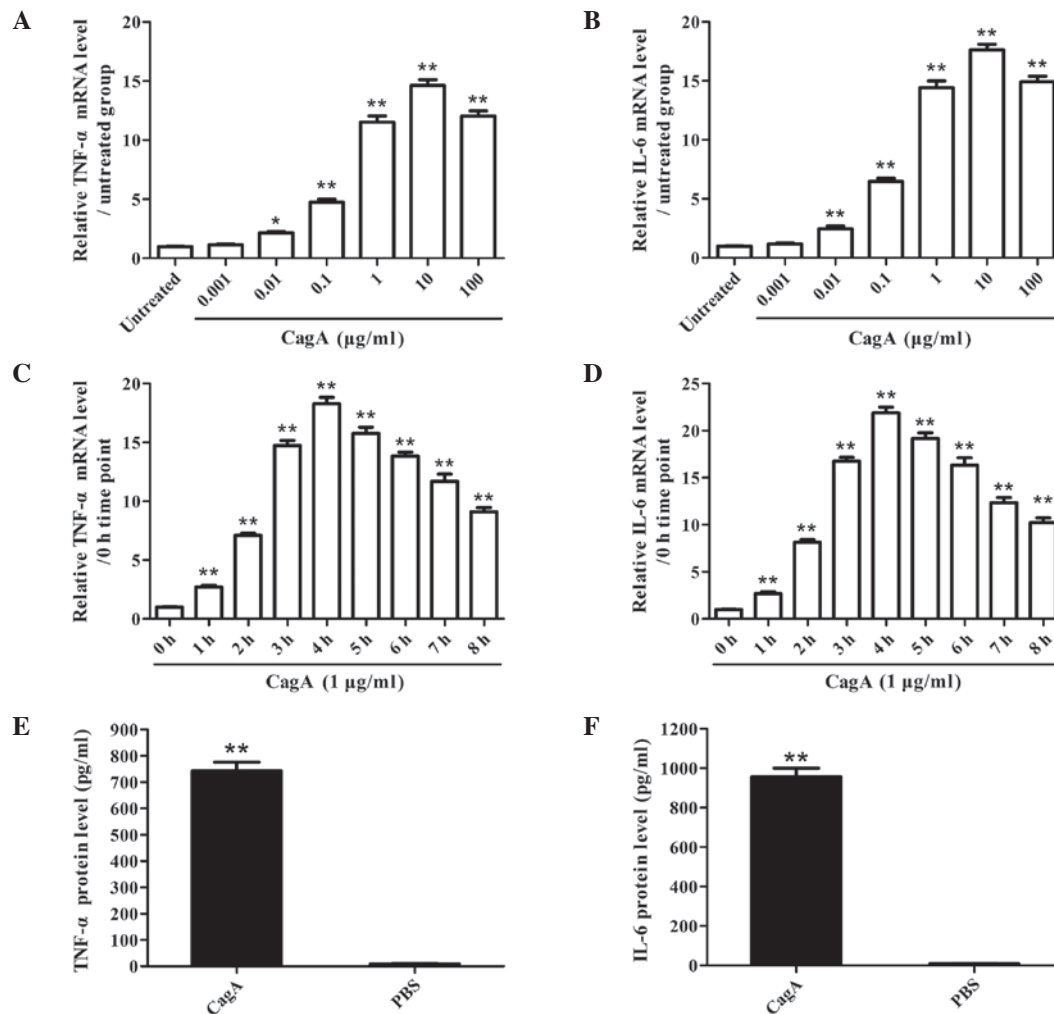


Figure 1. CagA induces the production of TNF- α and IL-6 in human AGS cells. (A and B) AGS cells were cultured with various concentrations of CagA for 6 h to induce the production of TNF- α and IL-6. RT-qPCR showed that CagA enhanced the mRNA levels of TNF- α and IL-6 in human AGS cells in a dose-dependant manner, with a maximum at the dose of 10 μ g/ml. n=4 for each group. *P<0.05, **P<0.01 compared to untreated group. (C and D) CagA (10 μ g/ml) was used to induce the production of TNF- α and IL-6 in AGS cells over various time periods. RT-qPCR showed that CagA enhanced the mRNA levels of TNF- α and IL-6 in human AGS cells in a time-dependant manner, with a maximum at 4 h. n=4 for each group. **P<0.01 compared to 0 h group. (E and F) ELISA showed that CagA (10 μ g/ml) enhanced the secretion of TNF- α and IL-6 in human AGS cells at 4 h. n=3 for each group. **P<0.01 compared to PBS group. Results shown are from one experiment, representative of three independent experiments. Values are expressed as the mean \pm standard deviation. TNF, tumor necrosis factor; IL, interleukin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; PBS, phosphate-buffered saline; CagA, cytotoxin-associated gene A.

against human total p65 (1.5 μ g for each sample) at 4°C overnight under constant agitation. The sample was then incubated with 40 μ l protein G-Sepharose beads at 4°C for 2.5 h under constant agitation. Protein G-protein complex was precipitated by centrifugation and finally re-suspended in 40 μ l SDS lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The samples were then analyzed by SDS-PAGE followed by staining with specific antibodies against total p65 (1:1,000), acetylated-Lysine (1:1,000), PCAF (1:200) and HA (1:500), respectively. At the same time, 40- μ g aliquots of whole-cell extract from the AGS cells were used to detect the protein expression as an input control.

RNA isolation and reverse transcription quantitative (RT-q)PCR. Cells were collected at the indicated time-points and RNA was extracted using TRIzol reagent. A total of 1 μ g RNA was used for the first-strand cDNA synthesis using MMLV and oligo (dT) 15 primer according to the

manufacturer's instructions. The cDNA was amplified by using TaqMan® Fast Advanced Master Mix to detect the expression of human PCAF gene using primers purchased from GenScript (forward 5'-TACCTCGGTACGAAA CCACA-3' and reverse 5'-TCCTGTCTTGCTTGTTCAG-3'; Fam/Tamra-labeled probe, 5'-CGAGCGAAGCAATGTTCT CCCA-3'). The human β -actin gene was used as an internal control using primers purchased from GenScript (forward 5'-TGGACTTCGAGCAAGAGATG-3' and reverse 5'-GAA GGAAGGCTGGAAGAGTG-3'; Fam/Tamra-labeled probe, 5'-CGGCTGCTTCCAGCTCCTCC-3'). The 7500 Real-time PCR system (Applied Biosystems) was used to perform qPCR. The reaction program included an initial step for denaturation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 60 sec. Each sample was assayed in triplicate. The relative levels of the gene expression were obtained using the $2^{-\Delta\Delta Ct}$ method as described previously (38).

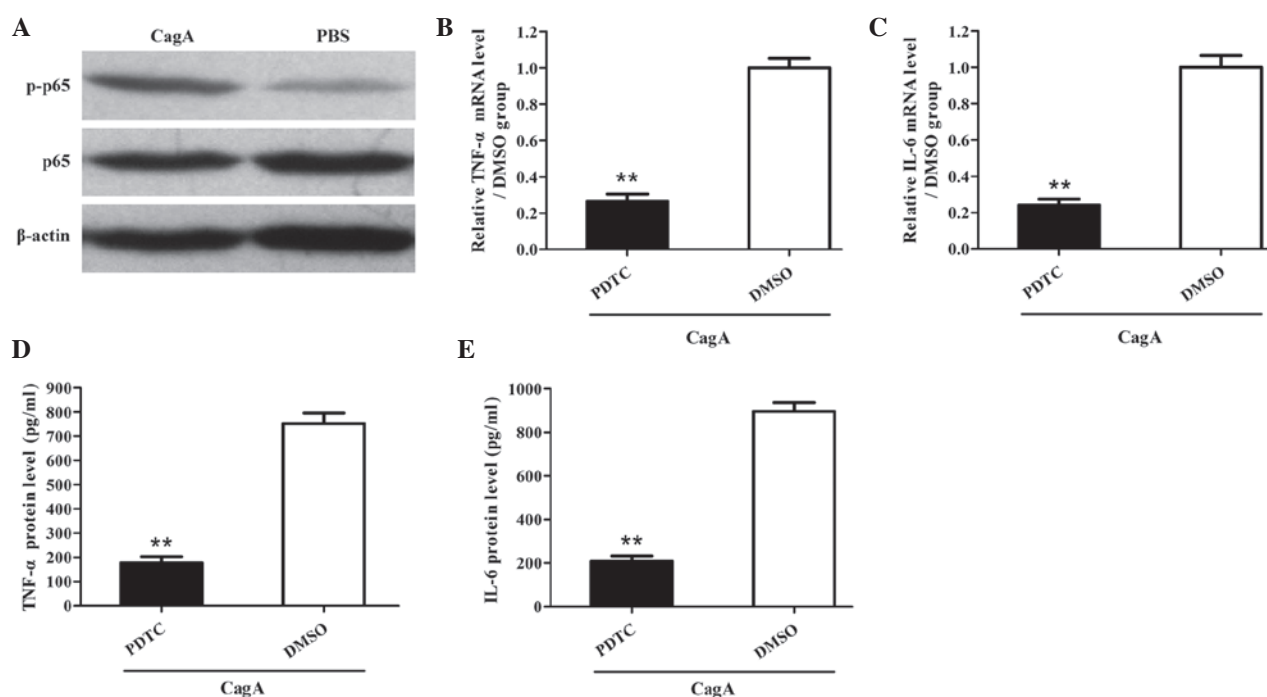


Figure 2. Activation of NF- κ B promotes the production of TNF- α and IL-6 in human AGS cells exposed to CagA. (A) Western blot analysis showed that CagA (10 μ g/ml) was able to induce the phosphorylation of NF- κ B p65 subunit in human AGS cells at 1.5 h after stimulation. (B and C) Reverse transcription quantitative polymerase chain reaction and (D and E) ELISA showed that inhibition of NF- κ B with PDTC significantly reduced the production of TNF- α and IL-6 in AGS cells induced by CagA (10 μ g/ml for 4 h). Results shown are from one experiment, representative of three independent experiments. Values are expressed as the mean \pm standard deviation ($n=3$ for each group). ** $P<0.01$ compared to DMSO group. NF, nuclear factor; TNF, tumor necrosis factor; IL, interleukin; DMSO, dimethyl sulfoxide; PDTC, pyrrolidine dithiocarbamate; p, phosphorylated; CagA, cytotoxin-associated gene A.

Western blot analysis. Cells were lysed in RIPA lysis buffer at 4°C for 30 min. The cellular lysates were then centrifuged at 15,000 \times g for 20 min at 4°C to remove any insoluble material. The concentration of the protein was determined by a BCA protein assay kit according to the manufacturer's instructions. The protein (40 μ g/well) was subjected to 10-12% SDS-PAGE and transferred onto PVDF membranes using the Mini-Protein System (Bio-Rad Laboratories, Inc., Hercules, CA USA). The PVDF membranes were incubated in blocking buffer [5% skimmed milk in Tris-buffered saline containing Tween 20 (TBS-T)] at room temperature for 1 h and then incubated with the specific antibodies (anti-total p65, 1:1,000; anti-p-p65, 1:1,000; anti-acetylated-Lysine, 1:1,000; anti-PCAF, 1:200; anti-HA, 1:500) at 4°C overnight. β -actin expression in each sample was used as an internal standard (anti- β -actin, 1:1,000). After five washes with TBS-T, the PVDF membranes were further incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2,000) or anti-rabbit IgG (1:2,000) at 37°C for 1 h. The bands were visualized using X-ray film (Life Technologies, Carlsbad, CA, USA) and the ECL detection system after washing the PVDF membranes five times. Finally, the density of the radiographic bands on the PVDF membranes was analyzed using the Quantity One software v44 (Bio-Rad Laboratories, Inc.).

ELISA. The levels of TNF- α and IL-6 in the cell media were determined using commercial human TNF- α and IL-6 ELISA kits (cat. no. 555212 and 550799) from BD Biosciences (Franklin Lakes, NJ, USA). ELISA was performed according to the manufacturer's instructions.

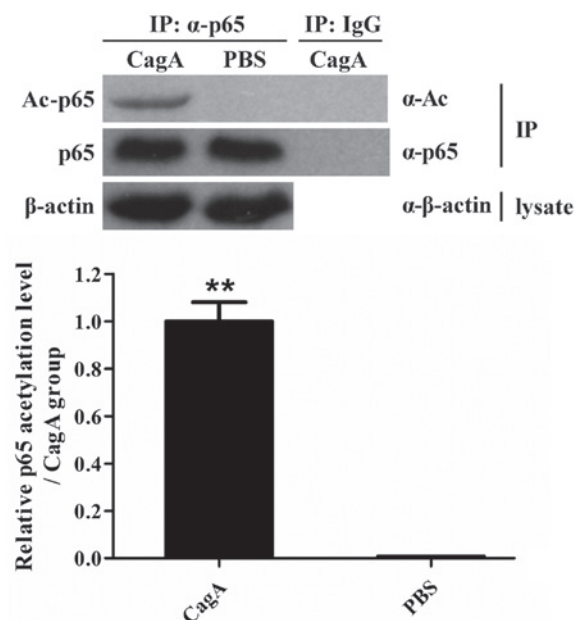


Figure 3. Acetylation of p65 is increased in human AGS cells stimulated by CagA. Human AGS cells were treated with 10 μ g/ml CagA for 1.5 h and cell lysates were subjected to immunoprecipitation using anti-p65 antibodies. The levels of Ac-p65 and total p65 in immunoprecipitated complexes were detected by western blot analysis with specific anti-Ac-p65 and anti-p65 antibodies. β -actin without immunoprecipitation was used as a loading control. The results showed that the level of p65 acetylation was significantly enhanced in human AGS cells exposed to CagA. Results shown are from one experiment, representative of three independent experiments. Values are expressed as the mean \pm standard deviation ($n=3$ for each group). ** $P<0.01$ compared to PBS group. CagA, cytotoxin-associated gene A; PBS, phosphate-buffered saline; Ac, acetylated; IP, immunoprecipitation; IgG, immunoglobulin G.

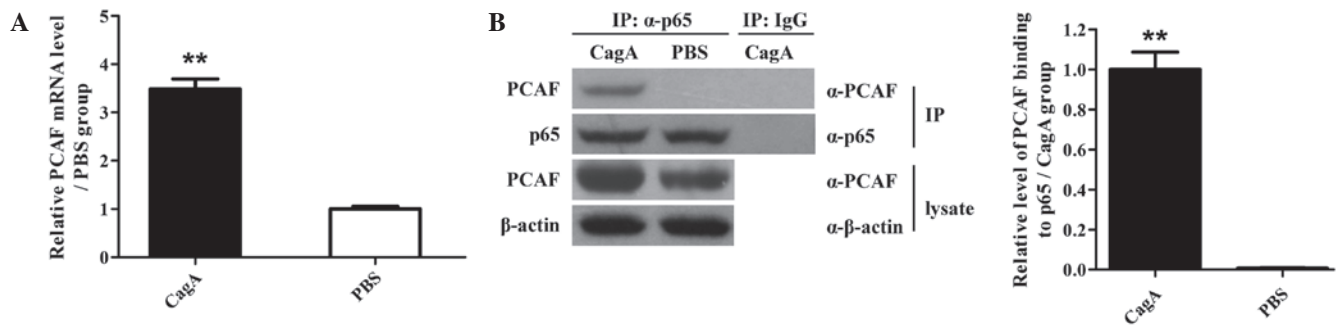


Figure 4. Expression of PCAF and its association with p53 are enhanced in human AGS cells induced by CagA. (A) The expression of PCAF at the mRNA level was detected in human AGS cells induced with CagA (10 μ g/ml for 1.5 h). Reverse transcription quantitative polymerase chain reaction analysis showed that CagA stimulation markedly increased the expression of PCAF mRNA in human AGS cells. (B) Anti-p53 antibodies were used for immunoprecipitation from lysates of human AGS cells stimulated with CagA (10 μ g/ml for 1.5 h). The levels of PCAF and p53 in the immunoprecipitated complex were detected by western blot analysis with anti-PCAF and anti-p53 antibodies. Western blot analysis was also used to detect PCAF and β -actin expression in cell lysates without immunoprecipitation. The results showed that the molecular interaction of PCAF with p53 at the protein level was markedly enhanced in human AGS cells upon CagA stimulation. Results shown are from one experiment, representative of three independent experiments. Values are expressed as the mean \pm standard deviation (n=3 for each group). **P<0.01 compared to PBS group. PCAF, P300/CBP-associated factor; CagA, cytotoxin-associated gene A; PBS, phosphate-buffered saline; IP, immunoprecipitation; IgG, immunoglobulin G.

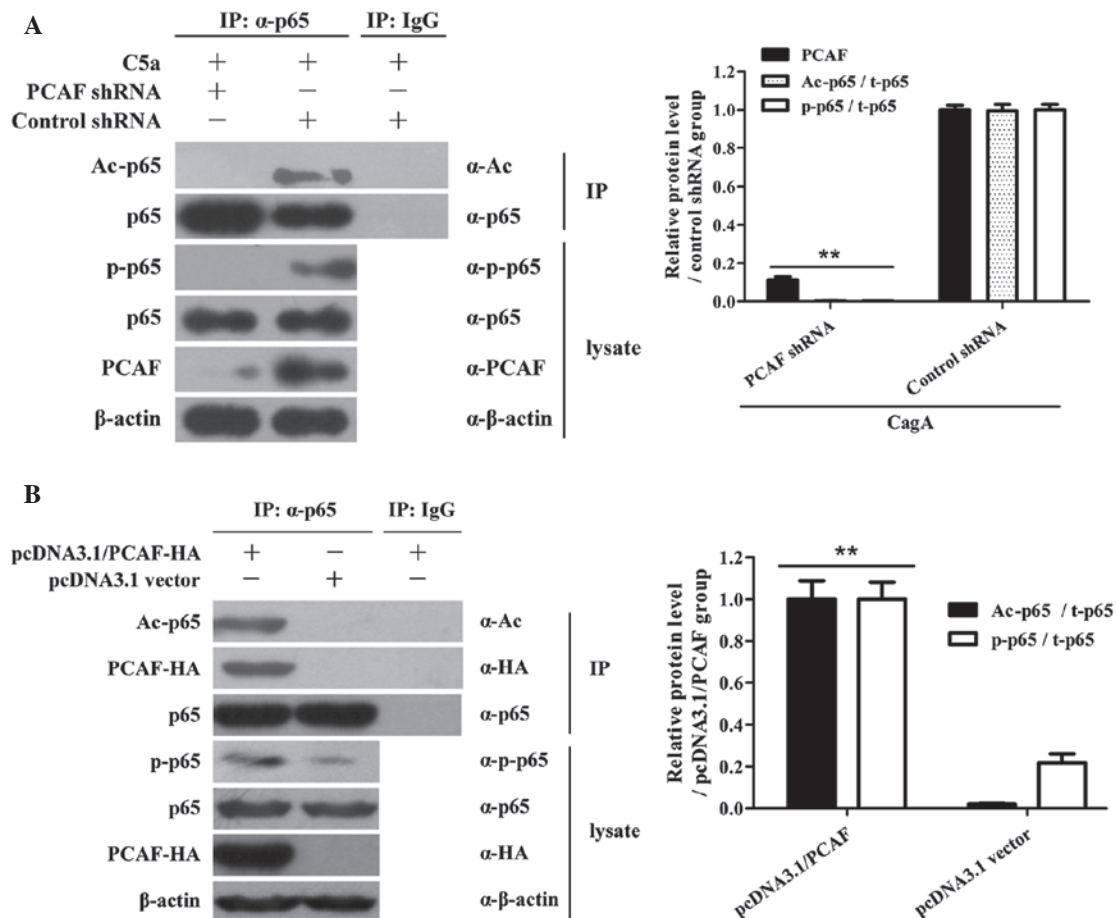


Figure 5. PCAF induction promotes p53 acetylation in AGS cells stimulated by CagA. (A) AGS cells were transfected with PCAF shRNA for 36 h and then stimulated with 10 μ g/ml CagA for 1.5 h. Cell lysates were then subjected to immunoprecipitation with anti-p53 antibodies. Western blot analysis was then used to detect the levels of Ac-p53 and total p53 in the immunoprecipitated complex with anti-A-p53c and anti-p53 antibodies. In addition, western blot analysis was used to detect p-p53, p53, PCAF and β -actin levels in cell lysates without immunoprecipitation. The results showed that PCAF shRNA decreased CagA-induced p53 acetylation and phosphorylation in AGS cells. **P<0.01 compared to control shRNA + CagA group. (B) AGS cells were treated with pcDNA3.1 PCAF-HA overexpression vector for 48 h. Cell lysates were then subjected to immunoprecipitation with anti-p53 antibodies. Subsequently, the levels of PCAF-HA, Ac-p53 and total p53 in the immunoprecipitated complexes were detected by western blot analysis with anti-HA, anti-Ac-p53 and anti-p53 antibodies. In addition, the levels of p-p53, p53, PCAF-HA and β -actin were detected by western blot in cell lysates without immunoprecipitation. The results showed that overexpression of PCAF triggered p53 acetylation and phosphorylation in AGS cells. **P<0.01 compared to pcDNA3.1 vector group. Results shown are from one experiment, representative of three independent experiments. Values are expressed as the mean \pm standard deviation (n=3 for each group). CagA, cytotoxin-associated gene A; PCAF, P300/CBP-associated factor; PBS, phosphate-buffered saline; IP, immunoprecipitation; IgG, immunoglobulin G; shRNA, small hairpin RNA; Ac, acetylated; p, phosphorylated; HA, hemagglutinin tag.

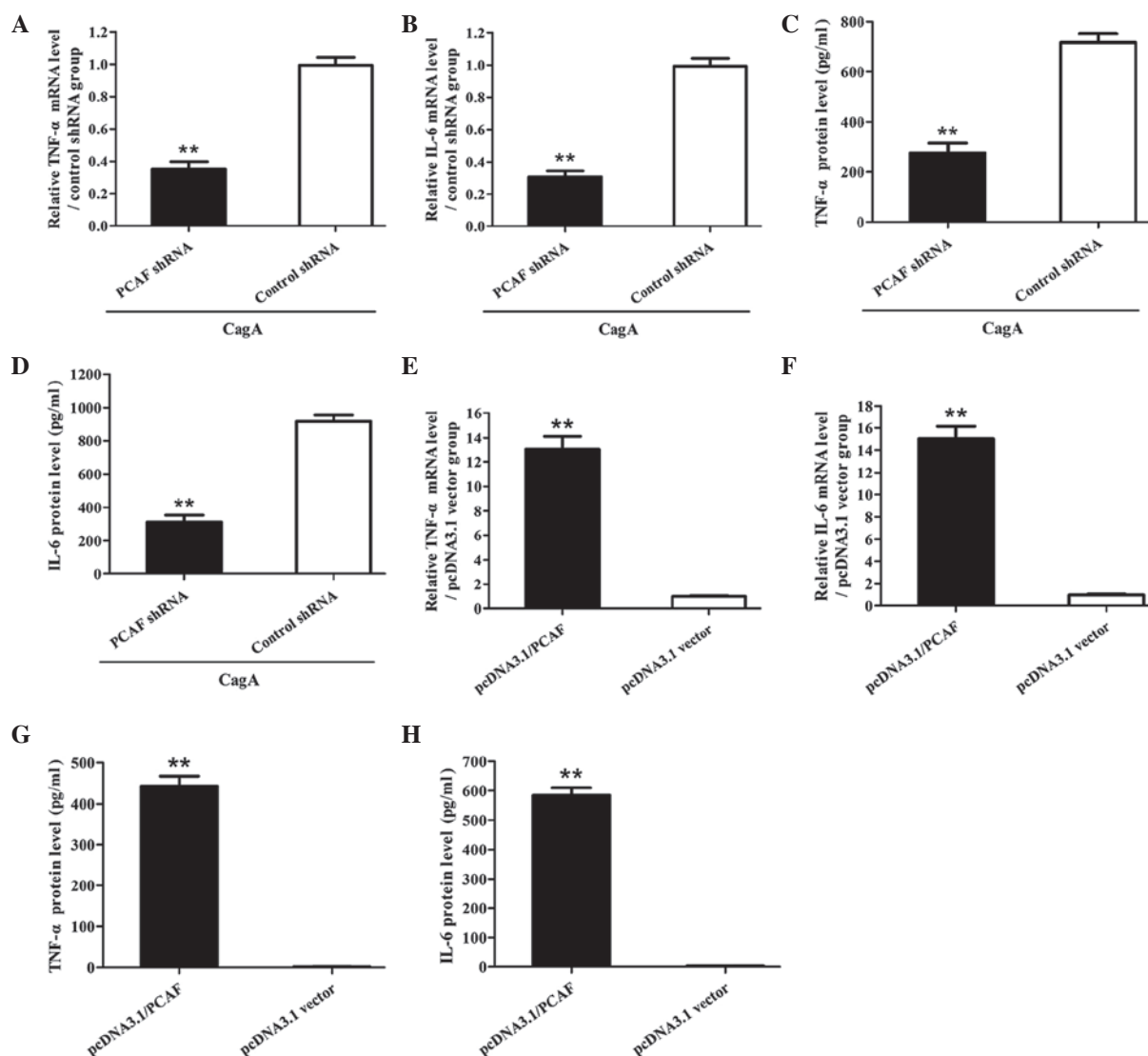


Figure 6. PCAF-mediated p65 acetylation contributes to the CagA-induced secretion of TNF- α and IL-6 by AGS cells. (A-D) AGS cells were transfected with PCAF shRNA for 36 h and then stimulated with 10 μ g/ml CagA for 4 h. Reverse transcription quantitative polymerase chain reaction and ELISA analyses showed that suppression of PCAF reduced the mRNA and protein levels of (A and C) TNF- α and (B and D) IL-6 in AGS cells stimulated with CagA (10 μ g/ml). ** P <0.01 compared to PBS group. (E-H) AGS cells transfected with PCAF overexpression vector for 48 h exhibited enhanced expression of (E and G) TNF- α and (F and H) IL-6 at the mRNA and protein level. ** P <0.01 compared to pcDNA3.1 vector group. Results shown are from one experiment, representative of three independent experiments. Values are expressed as the mean \pm standard deviation (n =3 for each group). TNF, tumor necrosis factor; IL, interleukin; CagA, cytotoxin-associated gene A; PCAF, P300/CBP-associated factor; PBS, phosphate-buffered saline; shRNA, small hairpin RNA.

Statistical analysis. All statistical analyses were performed using SPSS 12 software (SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean \pm standard deviation. The statistical significance of differences between groups was evaluated by one-way analysis of variance with simultaneous multiple comparisons between groups by the Bonferroni method. P <0.05 was considered to indicate statistically significant differences.

Results

CagA promotes the activation of NF- κ B and the production of TNF- α and IL-6 in human AGS cells. Human AGS cells were cultured with purified *Helicobacter pylori* CagA and the production of TNF- α and IL-6 was subsequently observed. The results showed that CagA enhanced the production of

TNF- α and IL-6 in human AGS cells *in vitro* in a dose- and time-dependent manner (Fig. 1). Furthermore, CagA was able to induce the activation of NF- κ B in human AGS cells *in vitro* (Fig. 2A). Of note, inhibition of NF- κ B significantly reduced the production of TNF- α and IL-6 in AGS cells induced by CagA (Fig. 2B-E). These findings indicated that CagA promoted the production of TNF- α and IL-6 by human AGS cells *in vitro* via activation of NF- κ B.

CagA enhances p65 acetylation in human AGS cells. Since *Helicobacter pylori* CagA was found to have the ability to stimulate the activation of NF- κ B and the production of TNF- α and IL-6 in human AGS cells (Figs. 1 and 2), the effects of CagA stimulation on the acetylation of p65 were further assessed in AGS cells. The level of p65 acetylation was found to be significantly enhanced in human AGS cells exposed to

CagA *in vitro* (Fig. 3), suggesting that p65 acetylation may have an important role in promoting p65 activation in human AGS cells induced by CagA.

PCAF and p65 association is increased in human AGS cells exposed to CagA. The expression of PCAF at the mRNA and protein level was detected in human AGS cells induced by CagA. It was found that incubation with CagA significantly increased the mRNA and protein expression levels of PCAF in human AGS cells (Fig. 4A and B). Furthermore, the interaction of PCAF with p65 was assessed at the protein level by IP. The results showed that the molecular interaction of PCAF with p65 at the protein level was markedly enhanced in human AGS cells upon stimulation with CagA (Fig. 4B). These findings indicated that stimulation with CagA induced the expression of PCAF and further enhanced the molecular interaction of PCAF with p65 at the protein level in human AGS cells, suggesting a potential ability of PCAF to acetylate p65.

PCAF induction contributes to p65 acetylation in AGS cells stimulated by CagA. To further determine the role of PCAF expression in p65 acetylation and phosphorylation, AGS cells were treated with PCAF shRNA expression vector for 36 h followed by CagA stimulation for 1.5 h. A Co-IP assay showed that PCAF knockdown decreased CagA-induced p65 acetylation and phosphorylation in AGS cells (Fig. 5A). Conversely, overexpression of PCAF triggered p65 acetylation and phosphorylation in AGS cells (Fig. 5B). These results suggested that PCAF is necessary for p65 acetylation and phosphorylation in CagA-induced AGS cells.

PCAF-mediated p65 acetylation is required for CagA-induced production of TNF- α and IL-6 in AGS cells. Since PCAF was shown to be required for p65 acetylation in AGS cells stimulated by CagA (Fig. 5), the present study further explored the role of PCAF-mediated p65 acetylation in the production of TNF- α and IL-6 in AGS cells induced by CagA. The results showed that suppression of PCAF markedly reduced the production of TNF- α and IL-6 in AGS cells stimulated by CagA (Fig. 6A-D). Conversely, AGS cells overexpressing PCAF exhibited enhanced production of TNF- α and IL-6 (Fig. 6E-H). These results indicated that PCAF-mediated p65 acetylation is involved in the production of TNF- α and IL-6 in AGS cells stimulated with CagA.

Discussion

Helicobacter pylori is a Gram-negative microaerophilic bacterium that selectively colonizes in human gastric and duodenal mucosa. It is generally known that persistent *Helicobacter pylori* infection leads to chronic gastritis and severe gastric pathologies, including peptic ulcers and even gastric cancer (39-41). It has been reported that infection induces not only innate immune responses but also adaptive immune responses against microorganisms; however in most cases, this fails to eradicate the microorganisms. By contrast, permanent infection ultimately triggers chronic inflammation and further leads to the damage of the body (42,43).

It is well accepted that *Helicobacter pylori* infection triggers production of various cytokines, including IL-1 β , IL-6,

IL-8, IL-18, IL-32, TNF- α and IL-6, which are known to have important roles in chronic inflammation and ultimate disease outcome (10,14,44-46). Among these inflammatory cytokines, TNF- α and IL-6 are considered to be two important inflammatory factors that can also be produced in other chronic inflammatory diseases, including Crohn's disease, rheumatoid arthritis and atherosclerosis (47-49). These studies indicated that overproduction of TNF- α and IL-6 may have a key role in mediating the inflammatory response in the process of *Helicobacter pylori* infection. In addition, CagA is considered to be an important virulence factor for *Helicobacter pylori* (46,50). Therefore, the present study aimed to investigate the implication of *Helicobacter pylori* CagA in the inflammatory response of human AGS cells *in vitro*. The results revealed that CagA markedly stimulated the production of TNF- α and IL-6 in human AGS cells *in vitro*, which is in accordance with the findings of previous studies (50,51).

NF- κ B is a major transcriptional factor that participates in the regulation of numerous cellular functions including inflammation (52-54). It has been demonstrated that *Helicobacter pylori* infection activates NF- κ B and its target genes in gastric epithelial cells, which is thought to be critical for *Helicobacter pylori*-initiated chronic inflammation (9,21). The virulence factor CagA, encoded by the *Helicobacter pylori* Cag pathogenicity island, has an important role in the pathogenicity of *Helicobacter pylori*, including *Helicobacter pylori*-induced activation of NF- κ B and expression of NF- κ B target genes (22,23). However, the exact function of CagA in the activation of NF- κ B and the NF- κ B-dependent inflammatory response have not been well characterized. Emerging evidence indicated that post-translational modification, including ubiquitination and acetylation, has important roles in various biological events, including inflammation (55-60). Among these, the acetylation of the NF- κ B p65 sub-unit has been shown to have an important role in its activation (27-29). The present study showed that the acetylation level of the NF- κ B p65 sub-unit was significantly enhanced in human AGS cells incubated with CagA *in vitro*. Therefore, the present study aimed to investigate the regulatory mechanism of p65 acetylation in CagA-induced gastric adenocarcinoma cells.

Recent studies showed that PCAF functions as an acetyl transferase (30-33); furthermore, PCAF has been reported to be linked to infection and inflammation (34,35,61). However, the roles of PCAF in mediating p65 activation and the production of TNF- α and IL-6 in CagA-induced AGS cells has remained largely elusive. The present study therefore investigated the expression of PCAF in human AGS cells induced by CagA, and showed that CagA increased the mRNA and protein expression levels of PCAF in human AGS cells. Furthermore, the results of the present study demonstrated that PCAF was required for p65 acetylation and phosphorylation in human AGS cells stimulated with CagA, suggesting that p65 acetylation contributed to p65 phosphorylation. Of note, PCAF was demonstrated to have an important role in p65 acetylation, although PCAF expression was not strongly increased. This result indicated that, in addition to the expression of PCAF, the activation of PCAF may have had a predominant role in the regulation of p65 acetylation in human AGS cells stimulated with CagA.

In the present study, it was also demonstrated that PCAF-mediated p65 acetylation functionally contributed to the production of TNF- α and IL-6 in human AGS cells stimulated by CagA, indicating that PCAF may have an important role in promoting the production of inflammatory factors. Kiernan *et al* (62) revealed that acetylation of p65 may have a key role in inhibitor of NF- κ B-mediated attenuation of NF- κ B transcription. The present study showed that p65 acetylation promoted its activation as a transcription factor to activate TNF- α and IL-6 gene expression. This difference may be due to different acetylated sites of p65. Therefore, further studies are required to identify the differential sites of PCAF-induced acetylation in AGS cells stimulated by CagA.

In conclusion, the results of the present study suggested that *Helicobacter pylori* CagA promoted the production of TNF- α and IL-6 in AGS cells through PCAF-mediated p65 acetylation. The present study therefore provided novel insight into the pathomechanism of *Helicobacter pylori* infection.

References

- Ueda J, Goshio M, Inui Y, Matsuda T, Sakakibara M, Mabe K, Nakajima S, Shimoyama T, Yasuda M, Kawai T, *et al*: Prevalence of *Helicobacter pylori* infection by birth year and geographic area in Japan. *Helicobacter* 19: 105-110, 2014.
- Shiota S, Murakami K, Okimoto T, Kodama M and Yamaoka Y: Serum *Helicobacter pylori* CagA antibody titer as a useful marker for advanced inflammation in the stomach in Japan. *J Gastroenterol Hepatol* 29: 67-73, 2014.
- Bhuiyan TR, Islam MM, Uddin T, Chowdhury MI, Janzon A, Adamsson J, Lundin SB, Qadri F and Lundgren A: Th1 and Th17 responses to *Helicobacter pylori* in Bangladeshi infants, children and adults. *PLoS One* 9: e93943, 2014.
- Guo S, He L, Zhang J, Zhou L, Ding Z, Zhang J, Yu F, Wang G, Zhou J, Guan D, *et al*: Antibiotic resistance of *Helicobacter pylori* in children and macrolide-resistant genotypes in *Helicobacter pylori*. *Zhonghua Yi Xue Za Zhi* 94: 563-566, 2014 (In Chinese).
- Yu Y, Su L, Wang X, Wang X and Xu C: Association between *Helicobacter pylori* infection and pathological changes in the gastric mucosa in Chinese children. *Intern Med* 53: 83-88, 2014.
- Basiri Z, Safaralizadeh R, Bonyadi MJ, Somi MH, Mahdavi M and Latifi-Navid S: *Helicobacter pylori* vacA d1 Genotype predicts risk of gastric adenocarcinoma and peptic ulcers in Northwestern Iran. *Asian Pac J Cancer Prev* 15: 1575-1579, 2014.
- Zhang L, Sung JJ, Yu J, Ng SC, Wong SH, Cho CH, Ng SS, Chan FK and Wu WK: Xenophagy in *Helicobacter pylori*- and Epstein-Barr virus-induced gastric cancer. *J Pathol* 233: 103-112, 2014.
- Sokolova O, Maubach G and Naumann M: MEKK3 and TAK1 synergize to activate IKK complex in *Helicobacter pylori* infection. *Biochim Biophys Acta* 1843: 715-724, 2014.
- Devi S, Ansari SA, Vadivelu J, Mégraud F, Tenguria S and Ahmed N: *Helicobacter pylori* antigen HP0986 (TieA) interacts with cultured gastric epithelial cells and induces IL8 secretion via NF- κ B mediated pathway. *Helicobacter* 19: 26-36, 2014.
- Peng LS, Zhuang Y, Li WH, Zhou YY, Wang TT, Chen N, Cheng P, Li BS, Guo H, Yang SM, *et al*: Elevated interleukin-32 expression is associated with *Helicobacter pylori*-related gastritis. *PLoS One* 9: e88270, 2014.
- Zhao Y, Wang JW, Tanaka T, Hosono A, Ando R, Tokudome S, Soeripto, Triningsih FX, Triono T, Sumoharjo S, *et al*: Association between TNF- α and IL-1 β genotypes vs. *Helicobacter pylori* infection in Indonesia. *World J Gastroenterol* 19: 8758-8763, 2013.
- Allison CC, Ferrand J, McLeod L, Hassan M, Kaparakis-Liaskos M, Grubman A, Bhathal PS, Dev A, Sievert W, Jenkins BJ and Ferrero RL: Nucleotide oligomerization domain 1 enhances IFN- γ signaling in gastric epithelial cells during *Helicobacter pylori* infection and exacerbates disease severity. *J Immunol* 190: 3706-3715, 2013.
- Abdollahi H, Shams S, Zahedi MJ, Darvish Moghadam S, Hayatbakhsh MM and Jafarzadeh A: IL-10, TNF- α and IFN- γ levels in serum and stomach mucosa of *Helicobacter pylori*-infected patients. *Iran J Allergy Asthma Immunol* 10: 267-271, 2011.
- Nakagawa H, Tamura T, Mitsuda Y, Goto Y, Kamiya Y, Kondo T, Wakai K and Hamajima N: Significant association between serum interleukin-6 and *Helicobacter pylori* antibody levels among H. pylori-positive Japanese adults. *Mediators Inflamm* 2013: 142358, 2013.
- Zhang X, Yang Y, Zhu R, Bai J, Tian Y, Li X, Peng Z, He Y, Chen L, Fang D, *et al*: H. pylori induces the expression of Hsp1 in gastric epithelial cells via interleukin-8/STAT3 phosphorylation while suppressing Hsp1. *J Cell Biochem* 113: 3740-3751, 2012.
- Zhang MS, Zhang KJ, Zhang J, Jiao XL, Chen D and Zhang DL: Phospholipases A-II (PLA2-II) induces acute pancreatitis through activation of the transcription factor NF-kappaB. *Eur Rev Med Pharmacol Sci* 18: 1163-1169, 2014.
- Cuadrado A, Martin-Moldes Z, Ye J and Lastres-Becker I: Transcription factors NRF2 and NF- κ B are coordinated effectors of the Rho family, GTP-binding protein RAC1 during inflammation. *J Biol Chem* 289: 15244-15258, 2014.
- Saravanan S, Islam VI, Babu NP, Pandikumar P, Thirugnanasambantham K, Chellappandian M, Raj CS, Paulraj MG and Ignacimuthu S: Swertiamarin attenuates inflammation mediators via modulating NF- κ B/I κ B and JAK2/STAT3 transcription factors in adjuvant induced arthritis. *Eur J Pharm Sci* 56: 70-86, 2014.
- Kim GD, Oh J, Park HJ, Bae K and Lee SK: Magnolol inhibits angiogenesis by regulating ROS-mediated apoptosis and the PI3K/AKT/mTOR signaling pathway in mES/EB-derived endothelial-like cells. *Int J Oncol* 43: 600-610, 2013.
- Tan GK and Tabata Y: Chondroitin-6-sulfate attenuates inflammatory responses in murine macrophages via suppression of NF- κ B nuclear translocation. *Acta Biomater* 10: 2684-2692, 2014.
- Chen JP, Wu MS, Kuo SH and Liao F: IL-22 negatively regulates *Helicobacter pylori*-induced CCL20 expression in gastric epithelial cells. *PLoS One* 9: e97350, 2014.
- Kang DW, Hwang WC, Park MH, Ko GH, Ha WS, Kim KS, Lee YC, Choi KY and Min DS: Rebamipide abolishes *Helicobacter pylori* CagA-induced phospholipase D1 expression via inhibition of NF- κ B and suppresses invasion of gastric cancer cells. *Oncogene* 32: 3531-3542, 2013.
- Lamb A, Yang XD, Tsang YH, Li JD, Higashi H, Hatakeyama M, Peek RM, Blanke SR and Chen LF: *Helicobacter pylori* CagA activates NF-kappaB by targeting TAK1 for TRAF6-mediated Lys 63 ubiquitination. *EMBO Rep* 10: 1242-1249, 2009.
- Chen H, Li J, Jiao L, Petersen RB, Li J, Peng A, Zheng L and Huang K: Apelin inhibits the development of diabetic nephropathy by regulating histone acetylation in Akita mouse. *J Physiol* 592: 505-521, 2014.
- Yang H, Lee SM, Gao B, Zhang J and Fang D: Histone deacetylase sirtuin 1 deacetylates IRF1 protein and programs dendritic cells to control Th17 protein differentiation during autoimmune inflammation. *J Biol Chem* 288: 37256-37266, 2013.
- Qiu W, Zhou J, Zhu G, Zhao D, He F, Zhang J, Lu Y, Yu T, Liu L and Wang Y: Sublytic C5b-9 triggers glomerular mesangial cell apoptosis via XAF1 gene activation mediated by p300-dependent IRF-1 acetylation. *Cell Death Dis* 5: e1176, 2014.
- Hah YS, Cheon YH, Lim HS, Cho HY, Park BH, Ka SO, Lee YR, Jeong DW, Kim HO, Han MK and Lee SI: Myeloid deletion of SIRT1 aggravates serum transfer arthritis in mice via nuclear factor- κ B activation. *PLoS One* 9: e87733, 2014.
- Hwang YJ, Lee EW, Song J, Kim HR, Jun YC and Hwang KA: MafK positively regulates NF- κ B activity by enhancing CBP-mediated p65 acetylation. *Sci Rep* 3: 3242, 2013.
- Cuccurrazzu B, Bortolotto V, Valente MM, Ubezio F, Koverech A, Canonico PL and Grilli M: Upregulation of mGlu2 receptors via NF- κ B p65 acetylation is involved in the Proneurogenic and antidepressant effects of acetyl-L-carnitine. *Neuropsychopharmacology* 38: 2220-2230, 2013.
- Puttugunta R, Tedeschi A, Sória MG, Hervera A, Lindner R, Rathore KI, Gaub P, Joshi Y, Nguyen T, Sehmandke A, *et al*: PCAF-dependent epigenetic changes promote axonal regeneration in the central nervous system. *Nat Commun* 5: 3527, 2014.
- Hu P, Wang X, Zhang B, Zhang S, Wang Q and Wang Z: Fluorescence polarization for the evaluation of small-molecule inhibitors of PCAF BRD/Tat-AcK50 association. *Chem Med Chem* 9: 928-931, 2014.
- Zhu LH, Sun LH, Hu YL, Jiang Y, Liu HY, Shen XY, Jin XY, Zhen X, Sun HX and Yan GJ: PCAF impairs endometrial receptivity and embryo implantation by down-regulating β 3-integrin expression via HOXA10 acetylation. *J Clin Endocrinol Metab* 98: 4417-4428, 2013.

33. Zheng X, Gai X, Ding F, Lu Z, Tu K, Yao Y and Liu Q: Histone acetyltransferase PCAF up-regulated cell apoptosis in hepatocellular carcinoma via acetylating histone H4 and inactivating AKT signaling. *Mol Cancer* 12: 96, 2013.
34. Zhao J, Gong AY, Zhou R, Liu J, Eischeid AN and Chen XM: Downregulation of PCAF by miR-181a/b provides feedback regulation to TNF- α -induced transcription of proinflammatory genes in liver epithelial cells. *J Immunol* 188: 1266-1274, 2012.
35. Bastiaansen AJ, Ewing MM, de Boer HC, van der Pouw Kraan TC, de Vries MR, Peters EA, Welten SM, Arens R, Moore SM, Faber JE, *et al*: Lysine acetyltransferase PCAF is a key regulator of arteriogenesis. *Arterioscler Thromb Vasc Biol* 33: 1902-1910, 2013.
36. Ong SP, Lee LM, Leong YF, Ng ML and Chu JJ: Dengue virus infection mediates HMGB1 release from monocytes involving PCAF acetylase complex and induces vascular leakage in endothelial cells. *PLoS One* 7: e41932, 2012.
37. Li W, Cun W, Liu L, Hong M, Wang L, Wang L, Dong C and Li Q: The transactivating effect of HSV-1 ICP0 is enhanced by its interaction with the PCAF component of histone acetyltransferase. *Arch Virol* 154: 1755-1764, 2009.
38. Hou F, Wang L, Wang H, Gu J, Li M, Zhang J, Ling X, Gao X and Luo C: Elevated gene expression of S100A12 is correlated with the predominant clinical inflammatory factors in patients with bacterial pneumonia. *Mol Med Rep* 11: 4345-4352, 2015.
39. Talaiezhadeh A, Hajiani E and Tarshizi MA: The relative frequency of the *Helicobacter pylori* Infection in proximal gastric cancers. *Pol Przegl Chir* 85: 657-662, 2013.
40. Zhu Y, Zhou X, Wu J, Su J and Zhang G: Risk factors and prevalence of *Helicobacter pylori* infection in persistent high incidence area of gastric carcinoma in Yangzhong City. *Gastroenterol Res Pract* 2014: 481365, 2014.
41. Repetto O, Zanussi S, Casarotto M, Canzonieri V, De Paoli P, Cannizzaro R and De Re V: Differential proteomics of *Helicobacter pylori* associated with autoimmune atrophic gastritis. *Mol Med* 20: 57-71, 2014.
42. Satkunanathan S, Kumar N, Bajorek M, Purbhoo MA and Culley FJ: Respiratory syncytial virus infection, TLR3 ligands and proinflammatory cytokines induce CD161 ligand LLT1 expression on the respiratory epithelium. *J Virol* 88: 2366-2373, 2014.
43. Mota A, Areias J and Cardoso MF: Chronic liver disease and cirrhosis among patients with hepatitis B virus infection in northern Portugal with reference to the viral genotypes. *J Med Virol* 83: 71-77, 2011.
44. Srivastava R, Kashyap A, Kumar M, Nath G and Jain AK: Mucosal IgA & IL-1 β in *Helicobacter pylori* infection. *Indian J Clin Biochem* 28: 19-23, 2013.
45. Kim DJ, Park JH, Franchi L, Backert S and Núñez G: The Cag pathogenicity island and interaction between TLR2/NOD2 and NLRP3 regulate IL-1 β production in *Helicobacter pylori* infected dendritic cells. *Eur J Immunol* 43: 2650-2658, 2013.
46. Bartchewsky W Jr, Martini MR, Masiero M, Squassoni AC, Alvarez MC, Ladeira MS, Salvatore D, Trevisan M, Pedrazzoli J Jr and Ribeiro ML: Effect of *Helicobacter pylori* infection on IL-8, IL-1 β and COX-2 expression in patients with chronic gastritis and gastric cancer. *Scand J Gastroenterol* 44: 153-161, 2009.
47. Augustine MV, Leonard MB, Thayu M, Baldassano RN, de Boer IH, Shults J, Denson LA, DeBoer MD, Herskovitz R and Denburg MR: Changes in vitamin D-related mineral metabolism after induction with anti-tumor necrosis factor- α therapy in crohn's disease. *J Clin Endocrinol Metab* 99: E991-E998, 2014.
48. Lv Q, Yin Y, Li X, Shan G2, Wu X, Liang D, Li Y and Zhang X: The status of rheumatoid factor and anti-cyclic citrullinated peptide antibody are not associated with the effect of anti-TNF α agent treatment in patients with rheumatoid arthritis: A meta-analysis. *PLoS One* 9: e89442, 2014.
49. Schuett H, Oestreich R, Waetzig GH, Annema W, Luchtefeld M, Hillmer A, Bavendiek U, von Felden J, Divchev D, Kempf T, *et al*: Transsignaling of interleukin-6 crucially contributes to atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 32: 281-290, 2012.
50. Abbas Z, Yakoob J, Usman MW, Shakir T, Hamid S and Jafri W: Effect of *Helicobacter pylori* and its virulence factors on portal hypertensive gastropathy and interleukin (IL)-8, IL-10 and tumor necrosis factor- α levels. *Saudi J Gastroenterol* 20: 120-127, 2014.
51. Figura N, Palazzuoli A, Vaira D, Campagna M, Moretti E, Iacoponi F, Giordano N, Clemente S, Nuti R and Ponzetto A: Cross-sectional study: CagA-positive *Helicobacter pylori* infection, acute coronary artery disease and systemic levels of B-type natriuretic peptide. *J Clin Pathol* 67: 251-257, 2014.
52. Peng Q, Liu H, Shi S and Li M: Lycium ruthenicum polysaccharide attenuates inflammation through inhibiting TLR4/NF- κ B signaling pathway. *Int J Biol Macromol* 67: 330-335, 2014.
53. Luo JG, Zhao XL, Xu WC, Zhao XJ, Wang JN, Lin XW, Sun T and Fu ZJ: Activation of spinal NF- κ B/p65 contributes to peripheral inflammation and hyperalgesia in rat adjuvant-induced arthritis. *Arthritis Rheumatol* 66: 896-906, 2014.
54. Heymann MC, Winkler S, Luksch H, Flecks S, Franke M, Ruß S, Ozen S, Yilmaz E, Klein C, Kallinich T, *et al*: Human procaspase-1 variants with decreased enzymatic activity are associated with febrile episodes and may contribute to inflammation via RIP2 and NF- κ B signaling. *J Immunol* 192: 4379-4385, 2014.
55. Corn JE and Vucic D: Ubiquitin in inflammation: The right linkage makes all the difference. *Nat Struct Mol Biol* 21: 297-300, 2014.
56. Liu Z, Qin T, Zhou J, Taylor A, Sparrow JR and Shang F: Impairment of the ubiquitin-proteasome pathway in RPE alters the expression of inflammation related genes. *Adv Exp Med Biol* 801: 237-250, 2014.
57. Khan YM, Kirkham P, Barnes PJ and Adcock IM: Brd4 is essential for IL-1 β -induced inflammation in human airway epithelial cells. *PLoS One* 9: e95051, 2014.
58. Herbert C, Shadie AM, Bunting MM, Tedla N, Garthwaite L, Freeman A, Yoo H, Park SH and Kumar RK: Anti-inflammatory and anti-remodelling effects of ISU201, a modified form of the extracellular domain of human BST2, in experimental models of asthma: Association with inhibition of histone acetylation. *PLoS One* 9: e90436, 2014.
59. Qiao G, Ying H, Zhao Y, Liang Y, Guo H, Shen H, Li Z, Solway J, Tao E, Chiang YJ, *et al*: E3 ubiquitin ligase Cbl-b suppresses proallergic T cell development and allergic airway inflammation. *Cell Rep* 6: 709-723, 2014.
60. Soe NN, Sowden M, Baskaran P, Kim Y, Nigro P, Smollock EM and Berk BC: Acetylation of cyclophilin A is required for its secretion and vascular cell activation. *Cardiovasc Res* 101: 444-453, 2014.
61. Chan C, Wang Y, Chow PK, Chung AY, Ooi LL and Lee CG: Altered binding site selection of p53 transcription cassettes by hepatitis B virus X protein. *Mol Cell Biol* 33: 485-497, 2013.
62. Kiernan R, Brès V, Ng RW, Coudart MP, El Messaoudi S, Sardet C, Jin DY, Emiliani S and Benkirane M: Post-activation turn-off of NF- κ B-dependent transcription is regulated by acetylation of p65. *J Biol Chem* 278: 2758-2766, 2003.