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ARTICLE

Overexpression of *Helicobacter pylori* VacA N-terminal fragment induces proinflammatory cytokine expression and apoptosis in human monocytic cell line through activation of NF-κB

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Abstract: Vacuolating cytotoxin (VacA) is an important virulence factor in the pathogenesis of *Helicobacter pylori*-related diseases. The aim of this study was to investigate the function of the amino-terminal 476 residue fragment (p52) of VacA and the possible molecular mechanisms responsible for its induction of proinflammatory cytokines secretion and apoptosis. Human acute monocytic leukemia cell line THP-1 was used as an in vitro model to study proinflammatory cytokines secretion and apoptosis induced by transfection of a recombinant plasmid encoding the amino-terminal 476 residue fragment (p52) of VacA. The results showed that VacA p52 overexpression induced the production of tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), nitric oxide, and reactive oxygen species in THP-1 cells in a time-dependent manner. VacA p52 overexpression also promoted THP-1 cells apoptosis. In addition, VacA p52 triggered the activation of nuclear factor kappa B (NF-κB), indicating a possible mechanism for its induction of proinflammatory cytokines secretion and cell apoptosis. Our study demonstrated that the induction of cytokines secretion and apoptosis by VacA p52 in THP-1 cells could be mediated through activation of nuclear factor kappa B.

Key words: Helicobacter pylori, VacA, THP-1 cells, proinflammatory cytokines, apoptosis, nuclear factor kappa B (NF-κB).

Résumé : La cytotoxine vacuolante (VacA) est un important facteur de virulence participant à la pathogenèse de maladies engendrées par *Helicobacter pylori*. Le but de la présente étude était de faire la lumière sur la fonction du fragment de 476 résidus de la partie amino-terminale (p52) de VacA et sur les mécanismes moléculaires qui seraient responsables de sa capacité à stimuler la production de cytokines proinflammatoires et l'apoptose. On a eu recours à la lignée cellulaire humaine monocytique leucémique THP-1 en tant que modèle pour l'étude de la sécrétion de cytokines proinflammatoires et de l'apoptose provoquées par la transfection d'un plasmide recombinant codant le fragment de 476 résidus de la partie amino-terminale (p52) de VacA. Les résultats illustrent que la surexpression de la p52 de VacA déclenche la production du facteur de la nécrose tumorale alpha, de l'interleukine-1 bêta, d'oxyde nitrique et de réactifs oxygénés chez les cellules THP-1, qui varie en fonction du temps. La surexpression de la p52 de VacA a également entraîné l'apoptose des cellules THP-1. En outre, la p52 de VacA a déclenché l'activation du facteur nucléaire kappa B, un mécanisme qui expliquerait son induction de la sécrétion de cytokines proinflammatoires et de l'apoptose. Notre étude démontre que l'induction de la sécrétion de cytokines et de l'apoptose chez les cellules THP-1 par la p52 de VacA pourrait passer par l'activation du facteur nucléaire kappa B. [Traduit par la Rédaction]

Mots-clés : Helicobacter pylori, VacA, cellules THP-1, cytokines proinflammatoires, apoptose, facteur nucléaire kappa B.

Introduction

Helicobacter pylori is a Gram-negative bacterium infecting the human gastric mucosa, causing gastritis and peptic ulcer and, in some cases, gastric cancer (Ding et al. 2010). VacA, an 88 kDa vacuolating cytotoxin, is a major protein produced by *H. pylori*. VacA is an important virulence factor in the pathogenesis of peptic ulceration and gastric cancer (Cover and Blanke 2005). VacA can cause a wide range of alterations in eukaryotic cells in vitro. One of the most prominent activities of VacA is its capacity to induce the formation of large cytoplasmic vacuoles (Leunk et al. 1988). Other specific functions of VacA are also reported, such as depolarization of the membrane potential (McClain et al. 2003), induction of apoptosis (Manente et al. 2008), detachment of epithelial cells from the basement membrane (Fujikawa et al. 2003), interference with the process of antigen presentation (Molinari et al. 1998), activation of mitogen-activated protein kinases (Nakayama et al. 2004), and inhibition of activation-induced proliferation of T lymphocytes (Torres et al. 2007). Many of these roles are dependent on the capacity of VacA to form anion-selective membrane channels (Cover and Blanke 2005).

VacA is expressed in *H. pylori* as a 140 kDa protoxin that undergoes amino- and carboxyl-terminal processing, yielding the secreted, mature 88 kDa VacA toxin (Cover et al. 1994). The 88 kDa VacA monomers can assemble into large water-soluble flower-shaped structures comprising 6–14 monomers (Cover et al. 1997). The assembly of VacA into oligomeric structures may be required for membrane channel formation, as well as for many roles of VacA in mammalian cells (Torres et al. 2004).

The mature 88 kDa VacA passenger domain can undergo proteolytic cleavage to yield a 33 kDa amino-terminal fragment (p33) and a 55 kDa carboxy-terminal fragment (p55), which remain physically associated. The p33 and p55 fragments are considered to represent 2 domains or subunits of VacA (Torres et al. 2005). Several lines of evidence indicate that the p33 domain is required for the formation of anion-selective membrane channels (McClain et al. 2001, 2003) and that the p55 domain is responsible for VacA binding to mammalian cells (Wang and Wang 2000) (Fig. 1A).

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By analyzing truncated VacA fragments for intracellular vacuolating activity, Ye et al. (1999) demonstrated that an amino-terminal fragment of approximately 422 residues comprises a minimal VacA domain that can mediate vacuole formation from the host cell cytosol. More than 50% of the 953 amino acids can be deleted from the VacA carboxyl terminus without affecting intracellular vacuolating activity. These investigations revealed that a discrete VacA fragment can function from the host cell cytosol to induce vacuoles with properties similar to those caused by full-length toxin added externally to cells.

It is believed that proinflammatory cytokines released by host cells in response to *H. pylori* infection might increase the risk of severe pathological outcomes such as cancer. Proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) are 2 of the potent proinflammatory cytokines elicited by *H. pylori* infection. Recent studies have demonstrated that mucosal levels of IL-1β and TNF-α are significantly higher in *H. pylori*-positive patients than in *H. pylori*-negative patients (Senthilkumar et al. 2011). Reactive oxygen species (ROS) and reactive nitrogen species may play important roles in cellular injury and carcinogenesis of gastric epithelial cells infected with *H. pylori*. *Helicobacter pylori* induces oxidative stress, and increased levels of ROS were detected in gastric epithelial cells, which may lead to altered epithelial proliferation and oxidative DNA damage (Ding et al. 2007). Nitric oxide (NO) is produced by the iNOS (inducible nitric oxide synthase) enzyme expressed by macrophages and neutrophils in gastric mucosa upon *H. pylori* infection. It has been speculated that increased levels of NO can damage host cell DNA, cause tissue injuries (Cooper and Magwere 2008), and increase cell proliferation (Pepine 2009). Enhanced production of NO has been reported to be associated with gastric carcinogenesis (Jun et al. 1999).

Macrophages are essential components of innate immunity, and apoptosis of these cells impairs mucosal defense to microbes. Induction of macrophage apoptosis is a method employed by various pathogens to escape host immune responses (Navarre and Zychlinsky 2000). Furthermore, macrophages play a central role in the inflammatory response to infection with *H. pylori* (Gobert et al. 2002*a*). A recent study demonstrated that *H. pylori* induces apoptosis of macrophages in association with alterations in the mitochondrial pathway (Menaker et al. 2004). Elimination of these immune-modulatory cells may represent a mechanism employed by the bacterium to evade host immune responses (Menaker et al. 2004). THP-1, a human acute monocytic leukemia cell line, is one of the most widely used cell lines to investigate the function and regulation of monocytes and macrophages in the study of bacterial pathogenicity. This monocyte cell line can be differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (PMA) (Park et al. 2007). Nuclear factor kappa B (NF-κB) is a critical transcriptional regulator of genes involved in inflammation, cell proliferation, and apoptosis (Barnes and Karin 1997; Baeuerle and Baltimore 1996). A number of genes encoding the cytokines, chemokines, and pro-inflammatory mediators are known to be activated by NF-κB and many of these are part of the pathogenesis of *H. pylori*-associated diseases (Sharma et al. 1998; Isomoto et al. 2000). Pyrrolidine dithiocarbamate (PDTC), a stable pyrrolidine derivative of dithiocarbamates, has been widely used as an inhibitor of the NF-κB activation (Nakashima et al. 2011). To determine whether vacuoles formed by the cytosol-expressed fragment of VacA are similar to those produced by full-length VacA, we transfected THP-1 cells with a plasmid expressing VacA p52. The aim of this study was to investigate the effects and the underlying molecular mechanisms of *H. pylori* VacA N-terminal p52 fragment on cytokine secretion, ROS production, and apoptosis of PMA-differentiated THP-1 cells.

Materials and methods

Bacterial strains and plasmid construction

Wild-type *H. pylori* strain 26695 (expressing type s1–m1 form of VacA, GenBank accession No. NC_000915) was grown on Columbia agar plates containing 5% defibrinated sheep blood (Oxoid Basingstoke, England) at 37 °C in a humidified atmosphere with 5% CO_2. The bacteria were collected from the agar plates into phosphate-buffered saline, and the genomic DNA was extracted by conventional phenol–chloroform method (Sambrook and Russel 2001). For cloning the sequence encoding the 52 kDa N-terminal domain (1428 bp) of VacA, which corresponds to 100–1531 bp of *vacA* gene, polymerase chain reaction (PCR) was performed with the following primers, which include *Xho*I and *Bam*HI restriction sequences (indicated by the underlined sequences), at their 5′ terminals: *vacA*-F (5′-TT*CCTCGAG*GCCTTTTTCACAACCGTGATC-3′), *vacA*-R (5′-TGCC*GGATCC*ACCTTTAAAATTAGCTGTATGAG-3′). The resulting PCR product was digested with *Xho*I and *Bam*HI and ligated to *Xho*I- and *Bam*HI-digested pDsRed-Monomer-C1 (Clontech, Palo Alto, California) to create the recombinant plasmid pDsRed-Monomer-C1/VacA p52. The recombinant plasmid was confirmed by PCR and restriction enzyme digestion followed by sequencing (Sangon Company, China).

Cell cultures and transfection

The human acute monocytic leukemia cell line THP-1 (China Center for Type Culture Collection, Wuhan University) was cultured in RPMI 1640 medium (Hyclone, USA) containing 10% fetal bovine serum (Hyclone), 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. For differentiation, THP-1 cells were plated at 4×10^5 cells/well in 24-well plates (Costor, New York, USA), and stimulated for 48 h with 50 nmol/L PMA (Sigma-Aldrich) (Park et al. 2007). Before transfection, PMA-differentiated THP-1 cells were cultivated in 1% fetal bovine serum overnight in 24-well plates at a concentration of 10^5 cells/mL. Recombinant plasmid (pDsRed-Monomer-C1/VacA p52) or the pDsRed-Monomer-C1 empty vector was transfected into THP-1 cells by Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. After 5 h the cells were placed in normal growth medium. Twenty-four hours after transfection, cells were harvested by trypsinisation and centrifuged at 1000 r/min (106*g*) for 5 min. The supernatant was discarded and the cell pellet was collected for protein extraction.

Western blotting

Protein was prepared from cells with protein extraction reagent (Pierce, USA). After shaking for 5 min, the lysates were collected and centrifuged at 12 000 r/min (15 294*g*) for 5 min at 4 °C. Protein concentration was determined using the Bradford method (Bradford 1976; Stoscheck 1990), and the lysates were separated by electrophoresis in a 12% polyacrylamide gel, followed by electrotransfer onto nitrocellulose membranes (Hybond ECL). The nitrocellulose membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in the Tris-buffered saline Tween-20 (TBST) (137 mmol/L NaCl, 20 mmol/L Tris, pH 7.6, 0.1% Tween-20), and blotted with polyclonal goat anti-VacA antibody (1:1000 dilution in TBST with 5% non-fat milk) overnight at 4 °C. After incubation, the membranes were washed 3 times with TBST for 10 min, followed by incubation with secondary horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, USA) antibody at a 1:5000 dilution in blocking buffer for 1 h at room temperature. The membranes were then washed 3 times with TBST. The membrane was also blotted with goat polyclonal antibody to β-actin (Abcam, UK) at a dilution of 1:1000 as internal control. The bands were visualized by enhanced chemiluminescence detection system (Amersham Biosciences) according to the manufacturer's instructions.

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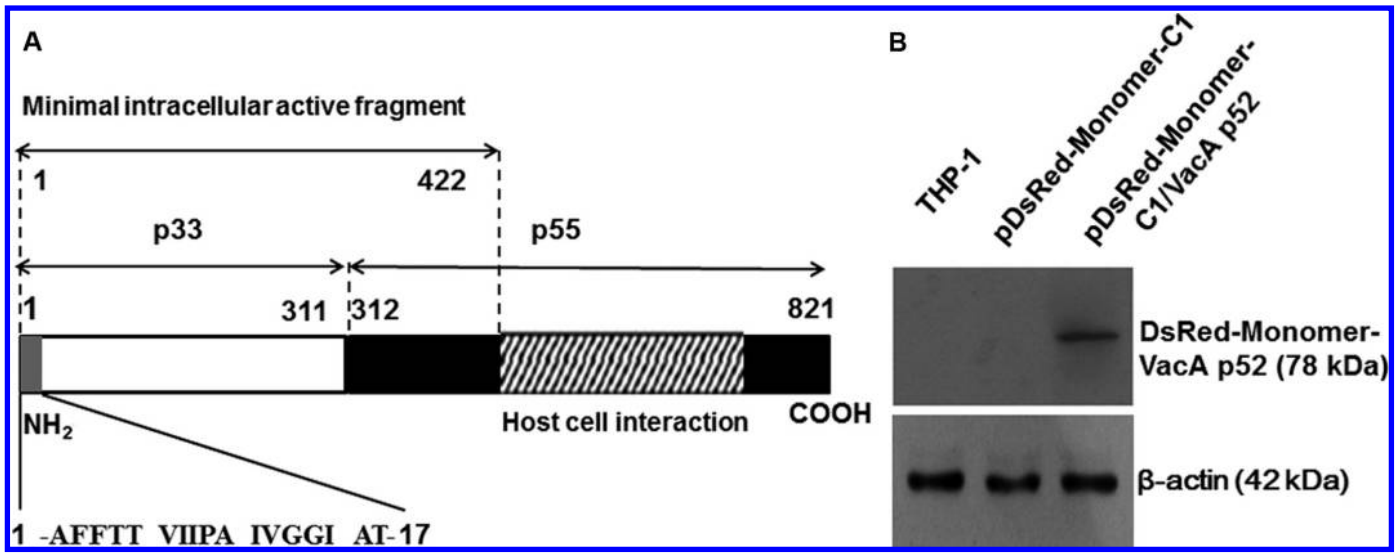
Fig. 1. Expression of DsRed-Monomer-VacA p52 in THP-1 cells. (A) Structure of mature VacA. VacA is composed of a discrete amino-terminal domain (p33) (white bar) and a carboxyl-terminal domain (p55) (black bar). The minimal VacA fragment that induces cellular vacuolation when directly expressed within mammalian cells comprises residues 1 to 422. The putative VacA receptor-binding domain has been localized to the central region of p55 (hatched region). The amino acid sequence of the VacA amino-terminal region (residues 1 to 17) for its importance to intracellular VacA mediated vacuolation is highlighted (gray bar). (B) Western-blot analysis of p52 VacA in THP-1 cells. THP-1 cells were untreated or transfected with pDsRed-Monomer-C1 empty vector or pDsRed-Monomer-C1/VacA p52, and the cell lysate were analyzed by Western blot. The resulting band of DsRed-Monomer-VacA p52 is about 78 kDa. β-actin (42 kDa) was used as internal control.



Vacuolating assay

For transmission electron microscopy, cells were grown to confluence in tissue culture flasks and transfected with *H. pylori* VacA p52 plasmid as described above. The cells were then fixed with 2% glutaraldehyde (*v*/*v*) in 0.1 mol/L phosphate buffer, post-fixed in 2% osmium tetroxide, and dehydrated through a series of graded acetone washes. Samples were embedded in Epon, and ultrathin sections were placed onto 300-mesh copper grids (Dytoc et al. 1994). The grids were then stained with uranyl acetate and lead salts as described previously (Jones et al. 1999). Grids were examined for the presence of vacuolated cells with a Hitachi H-7500 transmission electron microscope (Hitachi Corp., Tokyo, Japan) at an accelerating voltage of 60 kV.

For neutral red uptake assay, the transfected cells were assayed for vacuole formation by quantitative measurement of neutral red uptake as described previously (Cover et al. 1991). The experiments were performed in 96-well plates, and the optical density at 540 nm of wells was determined by iMark microplate absorbance reader (Bio-Rad). All assays were performed in triplicate.

NO measurement

The production of NO was determined using Griess reagent solution (Promega). THP-1 cells were preincubated with 25 μmol/L PDTC (Sigma-Aldrich) for 30 min before transfection to evaluate the effect of PDTC. Cells seeded in 24-well plates transfected with pDsRed-Monomer-C1 empty vector and the untreated cells were used as negative controls, and cells stimulated with 100 ng/mL lipopolysaccharide (*Escherichia coli* O55:B5, Sigma) were used as positive control. At 6, 12, 24, 36, and 48 h after transfection, 50 μL of cell culture supernatant was mixed with 100 μL of Griess reagent solution and incubated for 10 min at room temperature. Absorbance of the mixture was measured at 550 nm by the microplate reader (Bio-Rad) and NO concentration was determined from a standard curve of $NaNO_2$ (1 to 125 μmol/L final concentrations).

Assessment of intracellular oxygen species (ROS)

The generation of intracellular ROS was measured using 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA, Sigma) as probe. For the assay, the cells were seeded in 96-well plates and transfected as described above. At different time points (6, 12, 24, 36, 48 h) after transfection, the cell culture medium was substituted by 1 mL of freshly prepared DCFH-DA (10 μmol/L) and incubated for 30 min at 37 °C. Cells were harvested and the fluorescence intensity was measured immediately using a microplate fluorimeter (F-4500, Hitachi, Germany) with the excitation filter of 488 nm and the emission filter of 525 nm at 37 °C.

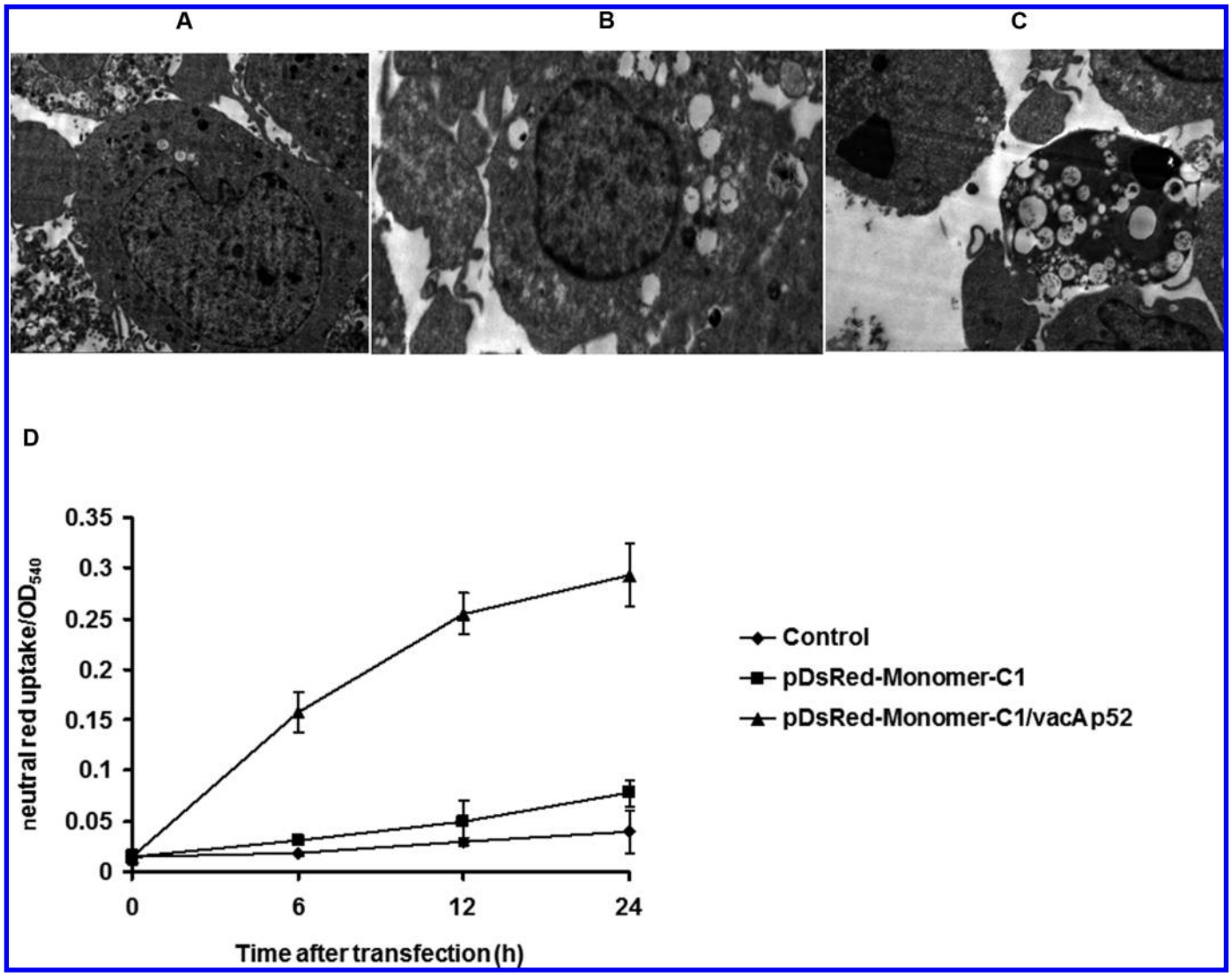
Enzyme-linked immunosorbent assay (ELISA)

The culture supernatants were collected from the 24-well plates at different time points (6, 12, 24, 36 h) after transfection. TNF-α and IL-1β in the supernatants were measured using sandwich ELISA kits (Jingmei Biotech, China) according to the manufacturer's instruction. The absorbances of the microwell were read by an ELISA microplate reader (Bio-Rad) at 450 nm. The cytokine concentration was calculated by a standard curve of recombinant TNF-α and IL-1β generated by known concentrations.

Detection of apoptosis

Cell apoptosis was analyzed using FITC-conjugated Annexin V (BD Pharmingen, San Jose, California) and 7-aminoactinomycin D (7-AAD). During early apoptosis, phosphatidylserine (PS) was translocated from the cytoplasmic face of the plasma membrane to the cell surface, thereby exposing PS to the extracellular environment with the plasma membrane left intact. Annexin V had a strong, Ca^{2+}-dependent affinity for PS and, therefore, served as a probe for detecting apoptosis. Annexin V staining preceded the loss of membrane integrity associated with necrosis or late stage of apoptosis. Therefore, staining with Annexin V was typically used in conjunction with a vital dye such as 7-AAD to identify early apoptotic cells (Annexin V positive and 7-AAD negative). Cells were harvested at 16 h post-transfection, washed twice in cold PBS and resuspended in Annexin V binding buffer (10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) (BD Pharmingen) at a concentration of 3×10^6 cells/mL. A 100 μL volume of suspension was stained with 5 μL of Annexin V-FITC and 5 μL of 7-AAD. The cells were gently vortexed and incubated for 15 min at room temperature in dark. After addition of 400 μL of binding buffer to each tube, cells were analyzed by flow cytometry

Fig. 2. Expression of p52 VacA increased vacuolating activity in THP-1 cells. (A–C) Representative transmission electron micrographs showing vacuolated cells (magnification, ×10 000). (A) Untreated THP-1 macrophage. (B–C) THP-1 cells transfected with VacA p52 plasmid for 24 h, showing the cytoplasm of THP-1 macrophage filled with vacuoles with normal form of the cell nucleus (B), and the cytoplasm of THP-1 macrophage filled with vacuoles with cell nucleus pushed aside (C). (D) Vacuolating cytotoxic activity of p52 VacA in THP-1 cells. THP-1 cells were transfected with pDsRed-Monomer-C1/VacA p52, and the vacuolating activity was measured by neutral red uptake assay. Result was represented as A_{540} values (mean ± standard deviation) from 3 independent experiments, each with triplicate samples.



(FACSCalibur, BD Biosciences). Cells treated with hexadecadrol (1 μmol/L) were used as a positive control.

Electrophoretic mobility shift assay (EMSA)

After transfection, the cells were harvested at the indicated time points for preparation of nuclear protein. The cells were lysed by incubation at 4 °C for 10 min in 400 μL of buffer A (10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.2 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 0.2% Nonidet P-40, 0.5 mmol/L DTT (dithiothreitol, reducing agent), 0.2 mmol/L PMSF (phenylmethylsulfonyl fluoride)). After centrifugation at 13 000 r/min (17 949*g*) for 10 min, the nuclear pellet was resuspended in 100 μL of ice-cold buffer B (20 mmol/L HEPES (pH 7.9), 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 20% (*v*/*v*) glycerol, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L PMSF), followed by incubation at 4 °C for 20 min. After centrifugation, the supernatant was collected, aliquoted, and stored at –70 °C. The nuclear protein concentration was determined using the BCA protein assay kit (Pierce). The BCA protein assay combined the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The purple-colored reaction product of this assay was formed by the chelation of 2 molecules of BCA with 1 cuprous ion. This water-soluble complex exhibited a strong absorbance at 562 nm that was nearly linear with increasing protein concentrations.

Activated NF-κB in the nuclei of untransfected and transfected cells was detected by the biotin-labeled EMSA kit (Viagene, Ningbo, China), according to the manufacturer's instruction. The EMSA is a rapid and sensitive method to detect protein – nucleic acid interactions. It is based on the observation that the electrophoretic mobility of a protein – nucleic acid complex is typically less than that of the free nucleic acid. From the experiments we can detect the activated transcription factors that can bind DNA or RNA in nucleus. The consensus NF-κB oligonucleotides included in the kit were 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′. The specificity of binding was examined by competition with an unlabeled oligonucleotide.

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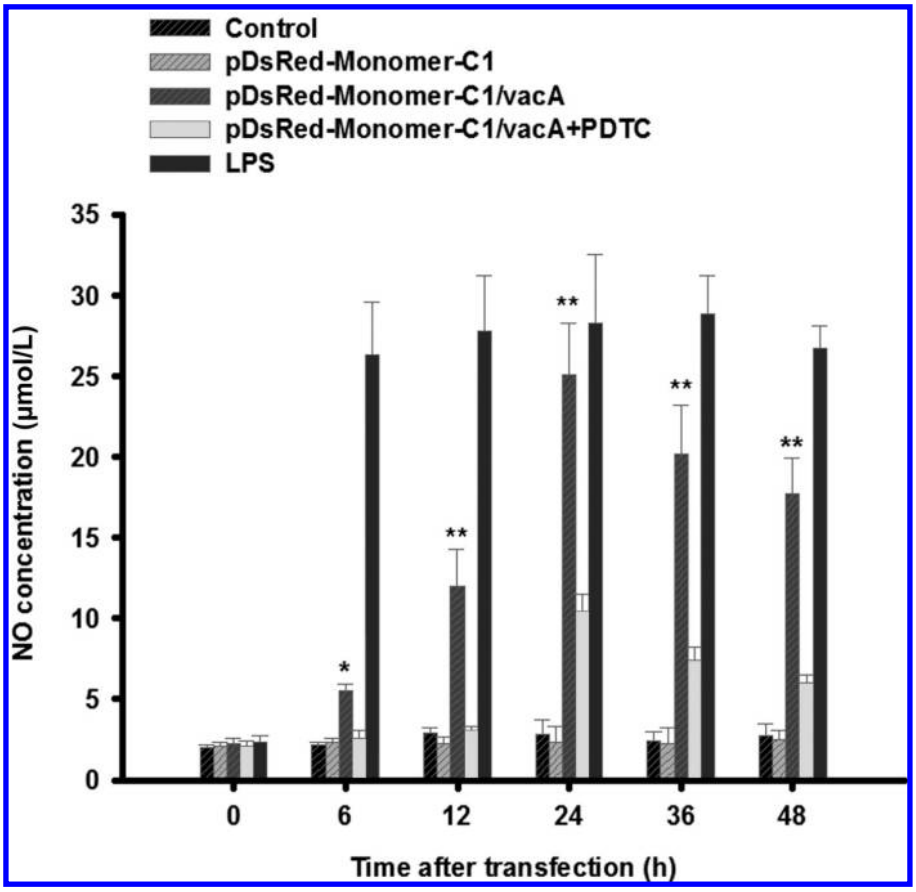
Fig. 3. Expression of nitric oxide (NO) in VacA p52 transfected THP-1 cells. THP-1 cells were transfected with pDsRed-Monomer-C1/VacA p52 or pDsRed-Monomer-C1 empty vector for different time (6, 12, 24, 36, or 48 h) and the level of NO in the cell culture medium was determined by Griess reagent solution. To analyze the effect of pyrrolidine dithiocarbamate (PDTC), cells were preincubated with 25 μmol/L PDTC for 30 min before transfection. Untreated THP-1 cells and THP-1 cells stimulated with 100 ng/mL lipopolysaccharide (LPS) were used as negative and positive controls, respectively. Results are shown as mean ± standard deviation and are representative of 3 independent experiments, each with triplicate samples. *, significantly different from the control group at $P < 0.05$ by repeated measures ANOVA. **, significantly different from the control group at $P < 0.01$ by repeated measures ANOVA.



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Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analysis was performed with SPSS software version 18.0 (SPSS, Chicago, Illinois, USA). Differences between the groups were analyzed with repeated measures ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

Expression of recombined plasmid pDsRed-Monomer-C1/VacA p52 in THP-1 cells

The recombined plasmid pDsRed-Monomer-C1/VacA p52 was first confirmed by enzyme digestion and sequencing. After excising the plasmid with *Xho*I and *Bam*HI, the 1428 bp desired product was obtained. Moreover, sequencing result of the amplified fragment was consistent with that of *H. pylori vacA* deposited in GenBank (RefSeq No. NP_207680). Next the recombined plasmid was transfected into THP-1 cells. Red fluorescent cells were detected by fluorescence microscopy 24 h after transfection, and an immunoreactive band of 78 kDa corresponding to DsRed-Monomer-VacA p52 fusion protein was detected by Western blotting (Fig. 1B). The results suggested that pDsRed-Monomer-C1/VacA p52 was expressed in THP-1 cells, which could be used in the following experiments.

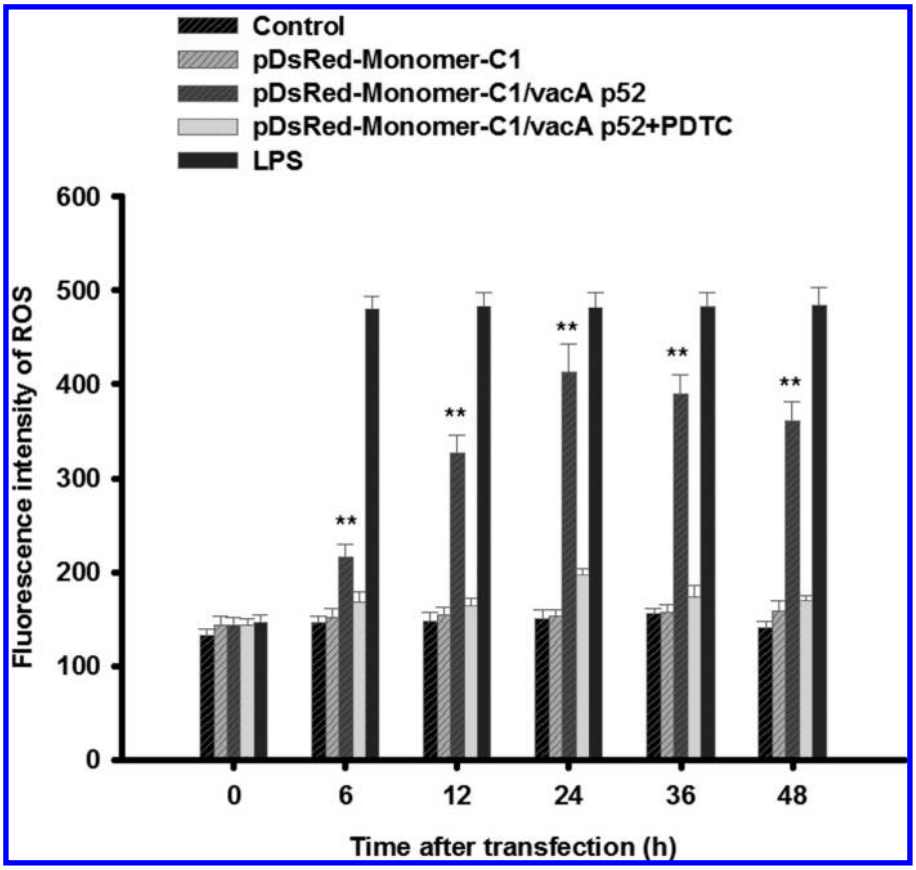
Overexpression of VacA p52 in THP-1 cells increased vacuolating activity

To investigate whether expression of VacA p52 could exert vacuolating activity in THP-1 cells, cells were fixed for transmission electron microscopy observation or for neutral red uptake assay on live cells. Before transfection, no vacuole could be observed in THP-1 cells and the intact cell structure can be seen (Fig. 2A). When THP-1-derived macrophages were transiently transfected with eukaryotic expression vectors pDsRed-Monomer-C1/VacA p52, a clear vacuolated phenotype was observed, resulting in either the cytoplasm filled with vacuoles and a normal form of the cell nucleus (Fig. 2B), or the cytoplasm filled with vacuoles with the cell nucleus pushed aside (Fig. 2C). Results of the neutral red uptake assay also showed that transfection with pDsRed-Monomer-C1/VacA p52 could increase vacuolating activity dramatically compared with cells untransfected or transfected with the empty plasmid (Fig. 2D).

VacA p52 induced inflammatory mediators in THP-1 cells

Next, the levels of the inflammatory mediators NO and intracellular ROS were determined after VacA p52 recombinant plasmid transfection. THP-1 cells were transfected transiently with pDsRed-Monomer-C1 empty plasmid or pDsRed-Monomer-C1/VacA p52. THP-1 cells induced by 100 ng/mL lipopolysaccharide were used as positive control. Moreover, the antioxidant PDTC was added before plasmid transfection to inhibit the NF-κB pathway. Compared with untreated cells, cells transfected with the empty vector did not upregulate the level of NO during the 48 h period after transfection ($P > 0.05$, Fig. 3), whereas VacA p52 transfection increased NO secretion from 6 h to 48 h after

Fig. 4. Induction of reactive oxygen species (ROS) in THP-1 cells after VacA p52 transfection. The formation of intracellular ROS was assayed by measuring the fluorescence of 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA). THP-1 cells were transfected with pDsRed-Monomer-C1/VacA p52 or pDsRed-Monomer-C1 empty vector for different times (6, 12, 24, 36, or 48 h) and the DCF fluorescence intensity was determined. The effect of pyrrolidine dithiocarbamate (PDTC) was analyzed by preincubation of cells with 25 μmol/L PDTC for 30 min before transfection. Untreated THP-1 cells and THP-1 cells stimulated with 100 ng/mL lipopolysaccharide (LPS) were used as negative and positive controls, respectively. Results are shown as the mean ± standard deviation and are representative of 3 independent experiments, each with triplicate samples. **, significantly different from the control group at $P < 0.01$ by repeated measures ANOVA.



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transfection compared with the control group ($P < 0.01$, Fig. 3). The expression of NO increased from 6 h and achieved peak at 24 h after transfection. Moreover, PDTC administration decreased the upregulation of the NO level induced by VacA p52 plasmid transfection (Fig. 3).

To measure intracellular ROS concentration, THP-1 cells after transfection were incubated with fluorophore DCFH-DA, and the level of 2′,7′-dichlorofluorescein (DCF) fluorescence was used as a sensitive, albeit indirect, measure of intracellular ROS (Korystov et al. 2007). Quantitative measurements demonstrated that VacA p52 expression led to a significant increase of DCF fluorescence compared with untransfected cells or cells transfected with empty vector, which was attenuated by PDTC treatment (Fig. 4). The intensity of DCF fluorescence began to increase at 6 h and peaked at 24 h after the transient transfection of pDsRed-Monomer-C1/VacA p52. These data indicated that at 6–48 h after transfection, the levels of NO and ROS secretion in macrophages transfected with the recombinant plasmids were significantly higher than that of the control group.

VacA p52 induced the production of proinflammatory cytokines in THP-1 cells

To determine the effect of VacA p52 transfection on the levels of proinflammatory cytokines, ELISA was performed to measure TNF-α and IL-1β secretion. Compared with control cells, the levels of TNF-α and IL-1β in THP-1 cells were markedly increased in a time-dependent manner in response to VacA p52 transfection. However, empty vector transfection did not significantly change their levels versus control cells (Figs. 5A and 5B). Pretreatment with PDTC significantly inhibited the expression of TNF-α and IL-1β induced by VacA p52 transfection.

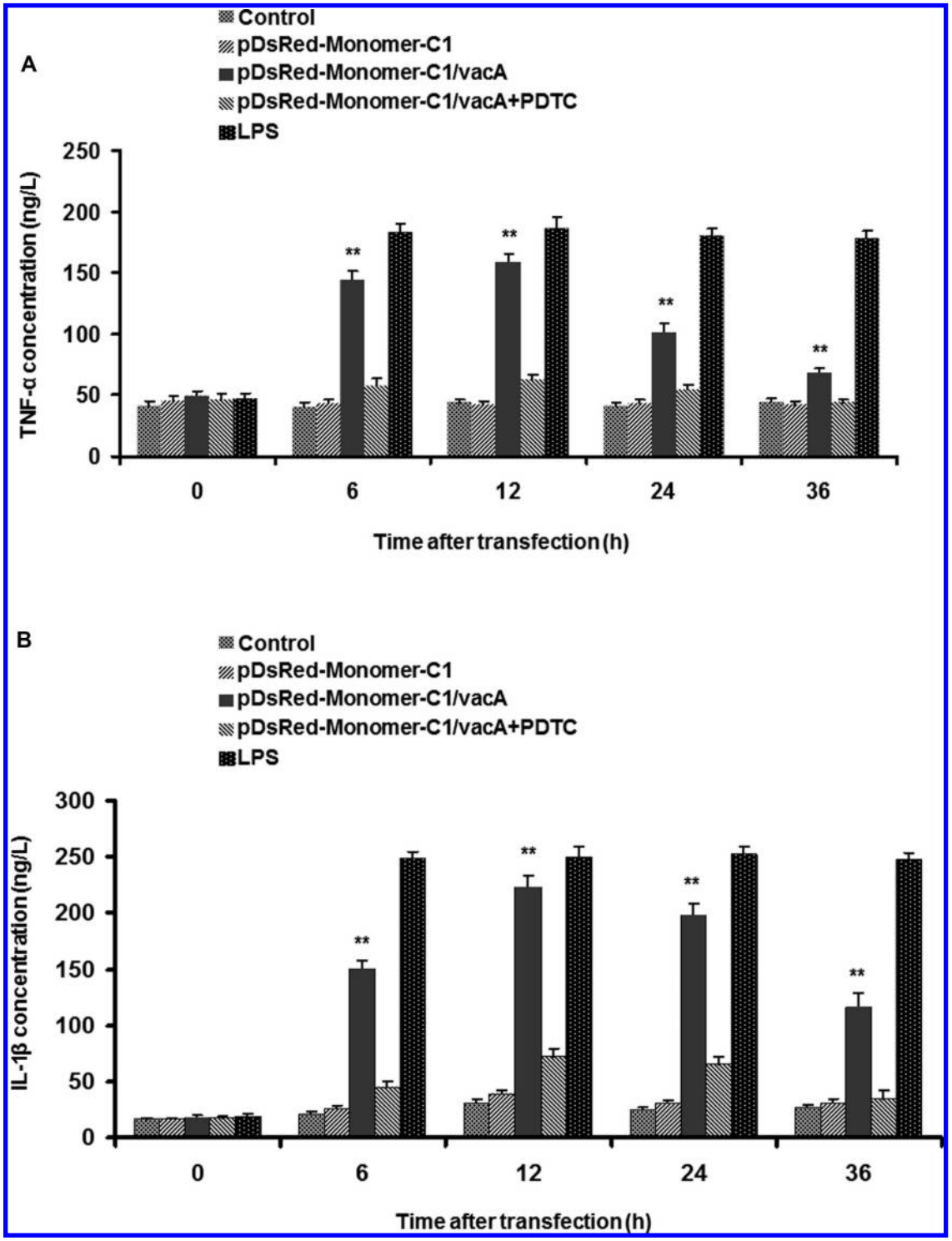
VacA p52 promoted apoptosis in THP-1 cells

Next the apoptosis of THP-1 cells after transfection were analyzed by Annexin V – 7-AAD staining, and the results are shown in Table 1 and Fig. 6. At 16 h post-transfection, the apoptosis rate of cells transfected with the empty vector was not significantly different from that of the control group ($P > 0.05$, Figs. 6B and 6C), while the apoptosis rates of THP-1 cells transfected with the recombinant vector increased significantly compared with the control cells or cells transfected with the empty vector ($P < 0.05$, Fig. 6D). The addition of PDTC significantly attenuated VacA p52-induced apoptosis (Fig. 6E). Cells treated with hexadecadrol (5 mg/mL) were analyzed as positive controls (Fig. 6F).

VacA p52 triggered NF-κB activation

To investigate whether VacA p52 overexpression is capable of triggering NF-κB activation, EMSA was performed in transfected cells. THP-1 cells were transfected with pDsRed-Monomer-C1/VacA p52 and harvested at different time intervals after transfection. NF-κB DNA binding activities in the nuclear extracts were determined by biotin-labeled EMSA. As shown in Fig. 7, after stimulation with lipopolysaccharide, a significant NF-κB activation was

Fig. 5. Proinflammatory cytokine levels determined by enzyme-linked immunosorbent assay (ELISA). THP-1 cells were transfected with pDsRed-Monomer-C1/VacA p52 or pDsRed-Monomer-C1 empty vector for different time (6, 12, 24, or 36 h) and the production of TNF-α (A) and IL-1β (B) was analyzed. A group of cells were preincubated with 25 μmol/L PDTC for 30 min before transfection. THP-1 cells with no treatment and stimulated with 100 ng/mL lipopolysaccharide (LPS) were used as negative and positive control, respectively. Results are shown as mean ± standard deviation and are representative of 3 independent experiments, each with triplicate samples. **, significantly different from the control group at $P < 0.01$ by repeated measures ANOVA.



detected in THP-1 cells. The DNA binding activity of NF-κB could be detected as early as 3 h after VacA p52 recombinant plasmid transfection, peaked at 12 h and then declined. NF-κB activity was detectable in the blank control or the empty vector transfection THP-1 cells but at much lower levels than in the cells transfected with the recombinant plasmid. The specificity of NF-κB DNA binding was verified by competition analysis with an excess of unlabeled specific (NF-κB) or unspecific oligonucleotides (AP-1). These data indicated that VacA p52 could activate NF-κB pathway in THP-1 cells.

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Fig. 6. Apoptosis of the THP-1 cells induced by VacA p52 transfection. (A) Dot plot depicting FSC versus SSC profile of THP-1 cells. (B) Untreated THP-1 cells. (C–F) THP-1 cells were transfected with pDsRed-Monomer-C1 empty vector (C), pDsRed-Monomer-C1/VacA p52 (D), pDsRed-Monomer-C1/VacA p52 after 25 μmol/L PDTC treatment (E), or treated with 5 mg/mL hexadecadrol (F) for 16 h. The cells were then stained with Annexin V and 7-aminoactinomycin D (7-AAD) and analyzed by fluorescence-activated cell sorting (FACS).

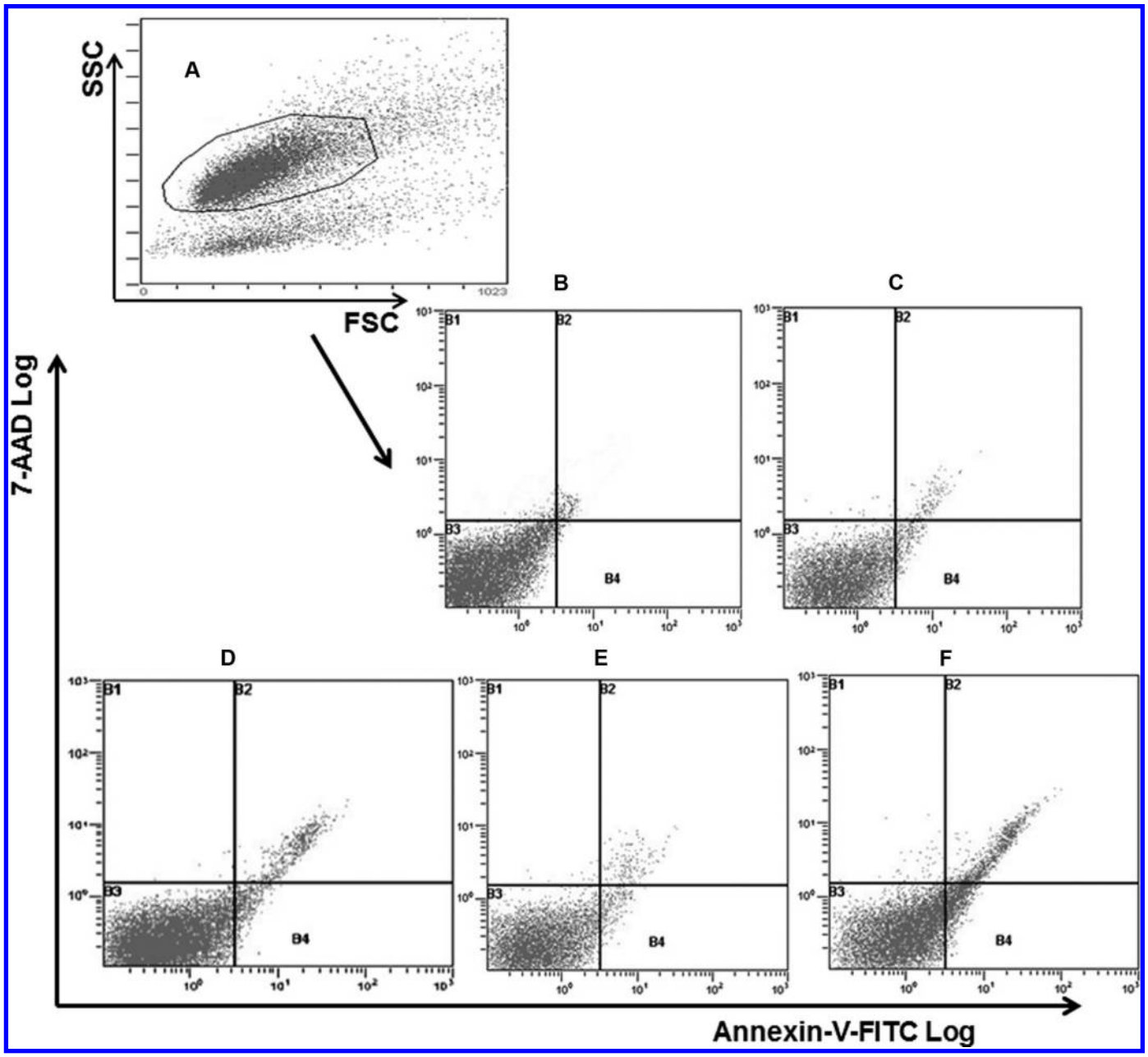

Table 1. Apoptosis rate of the THP-1 cells induced by VacA p52 with Annexin V–7-AAD staining method 16 h after transfection.

Group	Apoptosis rate (%) (mean ± SD, n = 3)
Control	2.78±0.79
pDsRed-Monomer-C1	6.43±1.36
pDsRed-Monomer-C1/VacA	16.42±5.76*
pDsRed-Monomer-C1/VacA + PDTC	6.82±0.79
Hexadecadrol	29.89±4.08*

Note: 7-AAD, 7-aminoactinomycin D; PDTC, pyrrolidine dithiocarbamate. *, significantly different from the control group at $P < 0.05$.

Discussion

Previous study demonstrated that when VacA is transiently transfected into HeLa cells (thus eliminating the requirement for toxin binding to the cell surface and internalization), the minimum portion of VacA required for cell vacuolation comprises a 422 amino acid protein, corresponding to the entire p33 domain and about 111 amino acids from the amino-terminal portion of p55 (Ye et al. 1999). Our data indicate that VacA p52 exhibits detectable vacuolating cytotoxic activity.

While *H. pylori* is typically considered to be noninvasive because most of the bacteria reside in the mucus layer of the stomach in contact with the epithelium, studies have demonstrated that the bacterium and its products can contact with lamina propria immune cells directly. Consequently, infection of *H. pylori* results in

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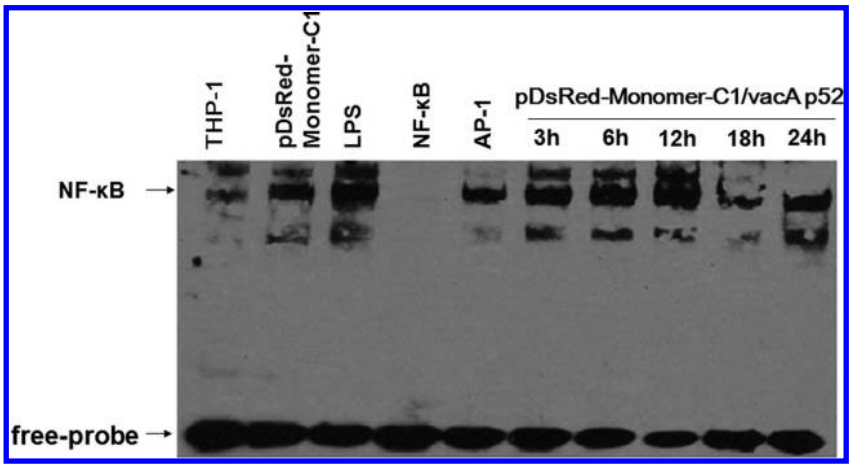
Fig. 7. Electrophoretic mobility shift assay assay of NF-κB activity in different groups of THP-1 cells. NF-κB activation was measured using a biotin-labeled oligonucleotide encompassing the NF-κB consensus motif. THP-1 cells were transfected with pDsRed-Monomer-C1/VacA p52 for different time periods (3, 6, 12, 18, and 24 h). THP-1 cells untreated or treated with 100 ng/mL lipopolysaccharide (LPS) were used as negative and positive controls, respectively. The specificity of DNA binding was assessed by preincubating extracts with unlabeled specific (NF-κB) or unspecific (AP-1) competitor oligonucleotide. The upper arrow indicates specific NF-κB band.



a large influx of immune cells, including neutrophils, macrophages, dendritic cells, and lymphocytes, and an associated innate and adaptive immune response (Algood et al. 2007).

Macrophages play a central role in the inflammatory response to *H. pylori* infection (Gobert et al. 2002*a*). For example, an increased number of macrophages are observed in the mucosa of gastric biopsy specimens obtained from infected children and correlate with the severity of gastritis (Dzierzanowska-Fangrat et al. 2008). It is well known that macrophages play important roles in innate immune responses against bacteria by releasing cytokines. In the present study, we have demonstrated that *H. pylori* VacA p52 is capable of inducing THP-1 cells to produce proinflammatory cytokines and to induce THP-1 cells apoptosis by activating NF-κB.

In an attempt to clarify the potential pathogenicity of *H. pylori* VacA, we have demonstrated in this study that VacA p52 could trigger THP-1 cells to produce NO, ROS, TNF-α, and IL-1β in a time-dependent manner. We have also found that PDTC, an inhibitor of NF-κB, could attenuate the effects of VacA p52. One primordial mechanism of antimicrobial host defense is the generation of high levels of NO by the enzyme iNOS. Previous studies have demonstrated that *H. pylori* could induce the expression and activity of iNOS in macrophages both in vivo and in vitro (Gobert et al. 2002*b*). Further, other studies have shown that co-culture of macrophages with *H. pylori* can kill the bacterium by an NO-dependent mechanism (Lewis et al. 2010). However, it has been speculated that increased NO may induce DNA damage and angiogenesis. The production of ROS resulting from oxidative burst is an important mediator that eliminates internalized pathogens in macrophages (Lowrie and Andrew 1988). For pathogens, it is important to avoid the rapid onslaught of innate response by effector cells, particularly the engulfment by phagocytes and being destroyed by the reactive oxygen. Extensive recent studies have revealed that *H. pylori*-induced ROS production in gastric epithelial cells might affect gastric epithelial cell signal transduction, resulting in gastric carcinogenesis (Handa et al. 2010). TNF-α and IL-1β are important inflammatory cytokines in that they can stimulate rapid influx of neutrophils, which are effective cells of host defense until antigen-specific mechanisms are induced to eliminate the pathogens. However, the expression and production of various cytokines could also inevitably influence the development of inflammatory reactions, which may directly or indirectly contribute to pathogenesis and tissue damage (Senthilkumar et al. 2011).

NF-κB is known as a widespread rapid-response transcription factor that is normally expressed in the cytoplasm of a variety of cells. Recent studies have demonstrated that there are NF-κB binding sites in the 5′ transcriptional regulation regions of TNF-α and IL-1β gene. Since the induction of gene-specific recognition elements always locates in the upstream promoter region, we investigated whether the production of TNF-α and IL-1β in THP-1 cells transfected with VacA p52 vector were associated with the activation of NF-κB. By using electrophoretic mobility shift and transactivation assays, we have clearly demonstrated that VacA p52 can induce the transcriptional activation of NF-κB, and the activation peaked at 12 h post-transfection. The above results indicate that VacA p52 are potent activators of NF-κB, and NF-κB activation may be of great importance to induce the production of TNF-α and IL-1β.

Three distinct bands representing the VacA p52-induced NF-κB activity were detected in THP-1 cells by EMSA, suggesting that multiple protein–DNA complexes were detectable. The multiple protein–DNA complexes were possible, since it is well known that besides the p50–p65 association yielding the classical NF-κB heterodimer, p50 and p65 subunits may interact with other members of the Rel protein family in the formation of heterodimers. In addition, NF-κB subunits may be associated with transcription factors of other families (Guijarro et al. 1996) to form protein–protein complexes.

Increasing evidence indicated that apoptosis of immune cells plays an important role in modulating the pathogenesis of a number of bacterially induced diseases (Ashida et al. 2011). Since infection causes the accumulation of immune cells in the stomach, understanding the interaction of *H. pylori* with the immune cells is also important in delineating the pathogenesis of infection. Indeed, *H. pylori* induces apoptosis of macrophages (Gobert et al. 2002*a*) and T lymphocytes (Ganten et al. 2007). The question that remains to be answered is whether the VacA p52 could induce apoptosis of macrophages in addition to inducing inflammatory cytokine production. In this study, cell apoptosis was detected in THP-1 cells transfected with VacA p52 vector by Annexin V – 7-AAD staining, and the apoptosis-inducing activity of VacA p52 was inhibited by PDTC, which demonstrated that VacA p52-induced THP-1 cell apoptosis was partially associated with the activation of NF-κB. Figure 6C showed that the empty vector transfection cause low levels of apoptosis. We speculated that this might be caused by the cytotoxicity of lipofectamine. Lipofectamine is widely used

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as nonviral gene delivery vector, but the agents have been shown to induce apoptosis by its cytotoxicity (Kongkaneramit et al. 2008).

DCFH-DA is one of the most commonly used probes for the detection of intracellular ROS (Wang and Joseph 1999). This probe is cell-permeable and is hydrolyzed intracellularly to 2′,7′-dichloro-dihydro-fluorescein (DCFH), which is then oxidized by ROS into DCF. DCF is a highly fluorescent compound that can be monitored by several fluorescence-based techniques (e.g., confocal microscopy and flow cytometry). Despite its advantages, the DCFH-DA technique is often criticized. The intracellular redox chemistry of DCFH is complex and there are several limitations and artifacts associated with the DCF assay for intracellular ROS measurement. One should be careful to interpret results obtained using this probe, and the use of alternate probes is encouraged (Halliwell and Whiteman 2004; Kalyanaraman et al. 2012).

Helicobacter pylori VacA is reported to produce multiple effects on mammalian cells. Previous study indicated that the use of different host cell lines has produced inconsistent vacuolating activity results (Yahiro et al. 1997), indicating that VacA may affect cell function in a host-cell-dependent manner. Accordingly, the effect of the VacA p52 on gastric epithelial cells requires further investigation.

In summary, this study showed that *H. pylori* VacA p52 increases the production of proinflammatory cytokines and induces apoptosis in macrophages through a NF-κB-dependent pathway. Thus, in addition to other strategies to evade host immune responses, such as disruption of phagosome maturation (Zheng and Jones 2003) and disruption of cytokine signaling (Mitchell et al. 2004), induction of macrophage apoptosis may represent a mechanism by which *H. pylori* impairs the host immune response to establish chronic infection in humans.

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