Foxp3 expression in A549 cells is regulated by Toll-like receptor 4 through nuclear factor-κB

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Abstract. Foxp3 is a master regulator of the development and function of $CD4^+CD25^+$ regulatory T cells (Tregs). Previous studies have reported that Foxp3 is also expressed in tumour cells and promotes tumour immune evasion. However, the regulation of the expression of Foxp3 in tumour cells remains unclear. Toll-like receptor 4 (TLR4), a member of the pattern recognition receptor family, is also expressed in tumour cells. Previous studies have found that the TLR4 signaling pathway is involved in tumour immune evasion in lung cancer cells, and that the transcription factor, nuclear factor-κB (NF-κB), plays a key role in the TLR4 signaling pathway. Moreover, recent studies found that NF-κB promotes the transcription of Foxp3. We hypothesised that TLR4 may also be involved in the regulation of Foxp3 in A549 cells through NF-κB. Therefore, we examined the effect of TLR4 and NF-κB on the expression of Foxp3 in the A549 lung cancer cell line. The results showed that Foxp3 and TLR4 are expressed in A549 cells; the expression of Foxp3 increased after lipopolysaccharide (LPS) stimulation and decreased after blocking the TLR4 signaling pathway. In addition, the expression of NF-κB (p65) increased after LPS stimulation and the expression of Foxp3 decreased after blocking NF-κB. These results suggest that TLR4 is involved in the regulation of Foxp3 in A549 cells through NF-κB.

Introduction

Lung cancer has the highest morbidity and mortality of all malignant tumours. Lung cancer is divided into two categories based on tumour histology: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for approximately 80% of lung cancer cases. The chemotherapy sensitivity of NSCLC is very poor, and the mortality rate is extremely high. Recurrence and metastasis are both critical factors in determining the prognosis of patients with NSCLC. Therefore, it is critical to study the molecular mechanisms of invasion and metastasis, and identify a target to reverse or prevent the invasion and metastatic processes, to improve the survival of lung cancer patients.

Foxp3 is a member of the forkhead/winged-helix transcription factor family. Foxp3 has been identified as a master regulator of the development and function of regulatory T cells (Tregs) (1,2). Tregs are a subset of T cells that are involved in immune suppression and play an important role in the process of maintaining tolerance (3,4). Previous studies have reported that Foxp3 is not only expressed in Tregs, but also in tumour cells. Since Hinz *et al* first reported the expression of Foxp3 in human pancreatic carcinoma cells (5), other studies have shown that Foxp3 is expressed in other tumour cells, including human breast cancer, prostate cancer, lung and melanoma cells (6-10). In addition, other studies have shown that the expression of Foxp3 within tumour cells is associated with tumour progression and metastasis, and thus it is a poor prognostic factor (5,6). However, it is unclear how the expression of Foxp3 is regulated in tumour cells.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are mainly expressed in immune cells and are considered to be an important link between innate and adaptive immunity (11,12). To date, 13 types of TLRs have been identified (13). Recent studies have found that TLR4, while mainly expressed in immune cells, is also expressed in many tumour cell lines and tumour tissues, including adrenocortical, breast, lung and bladder cancer (14-17). Notably, lung cancer cells expressing TLR4 are functionally associated with higher tumour invasiveness and metastasising potential as well as anti-apoptotic activity. The expression levels of TLR4 also positively correlate with lung cancer malignancy (16,18). Nuclear factor-κB (NF-κB) plays a key role in the TLR4 signaling pathway (19). NF-κB represents a family of five Rel proteins: c-Rel, RelA/p65, RelB, NF-κB1 (p50 and its precursor, p105) and NF-κB2 (p52 and its precursor, p100) (6,20). In many types of cancer, NF-κB is persistently activated, which protects developing tumour cells from death and thereby contributes to tumourigenesis and cancer therapy resistance (20). Moreover, two earlier studies using a murine breast cancer model as well as *in vitro* colorectal cancer cells demonstrated that lipopolysaccharide (LPS), a TLR4 ligand, promoted tumour invasion through the NF-κB pathway (21,22). Recent studies have also reported that NF-κB promotes the transcription of Foxp3 (23,24).

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We hypothesised that the TLR4 signaling pathway may be involved in the regulation of Foxp3 expression in lung tumour cells through NF-κB. Therefore, we examined the effect of TLR4 and NF-κB on the expression of Foxp3 in the A549 lung cancer cell line.

Materials and methods

Cell lines and reagents. The Type II pneumocyte-derived lung cancer cell line A549 was obtained from the Department of Immunology at the Norman Bethune College of Medicine at Jilin University, Changchun, China, and maintained at 37˚C under 5% CO_2 in complete RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA). Polymyxin B sulfate (PMB) and a monoclonal antibody for NF-κB p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The NF-κB specific inhibitor, ammonium pyrrolidine dithiocarbamate (PDTC), was purchased from Sigma (St. Louis, MO, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR) and RT-qPCR. Total RNA was extracted with RNAiso™ Plus (Takara, Shiga, Japan) as described by the manufacturer. For reverse transcription, cDNA was generated using 1 μg of total RNA, oligo(dT) primers (Promega) and reverse transcriptase (M-MLV; Takara) in a total volume of 20 μl.

For PCR, the primers sequences were as follows: Foxp3 sense, 5'-TCCCAGAGTTCCTCCACAAC-3' (29) and antisense, 5'-TCTGGCTCCGTTTCTTGC-3'; TLR4 sense, 5'-ACCTGT CCCTGAACCCTATGAA-3' (30) and antisense, 5'-CTTCT AAACCAGCCAGACCTTG-3'; GAPDH sense, 5'-ATGGGG AAGGTGAAGGTCG-3' and antisense, 5'-GGGTCATTGAT GGCAACAATATC-3' (31). For Foxp3, PCR was performed at 94˚C for 30 sec, 53˚C for 30 sec and 72˚C for 60 sec for 35 cycles. For TLR4, PCR was performed at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 60 sec for 25 cycles. For GAPDH, PCR was performed at 94˚C for 30 sec, 52˚C for 30 sec and 72˚C for 60 sec for 25 cycles, followed by a 10-min elongation at 72˚C. The RT-PCR products were analysed by 2% agarose gel electrophoresis and visualised by ethidium bromide staining. The band intensity was analysed using quantitative scanning densitometry. The ratio of TLR4 or Foxp3 to GAPDH was used as the level of mRNA expression.

For real-time PCR, the primers were as follows: Foxp3 sense, 5'-GAGAAGCTGAGTGCCATGCA-3' and antisense, 5'-AGGAGCCCTTGTCGGATGAT-3' (32). The TLR4 and GAPDH primer sequences were the same as those used for RT-PCR. Real-time PCR amplification of a cDNA template corresponding to 20 ng total RNA was conducted with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) using SYBR-Green PCR Master Mix II (Applied Biosystems) following the manufacturer's instructions. PCR conditions were 50˚C for 2 min, 95˚C for 10 min, then 40 cycles consisting of 15 sec at 92˚C and 1 min at 60˚C, followed by a dissociation plot. For data analysis, the comparative threshold cycle (CT) value for GAPDH was used to normalise loading variations in the real-time PCR data. The ΔΔCT value was then obtained by subtracting the control ΔCT values from the corresponding experimental ΔCT values. The ΔΔCT values were converted to fold difference over the control using the equation $2^{-\Delta\Delta CT}$.

Flow cytometry (FCM) for the expression of TLR4 and Foxp3. A549 cells ($1x10^6$) were harvested and fixed in 2% paraformaldehyde (1 ml/tube) for 60 min at 4˚C. Cells were then centrifuged at 1,000 rpm for 5 min, the cell pellet was resuspended in 100 μl 0.1% saponin (eBioscience) and 1 μg of purified anti-Foxp3 antibody (eBioscience) was added for 50 min at 4˚C. For the detection of TLR4 expression, $1x10^6$ A549 cells were resuspended in 100 μl 0.1 M PBS and 2 μg of purified anti-TLR4 (eBioscience) was added for 50 min at 4˚C. The cells were then washed and resuspended in 100 μl 0.1% saponin or 0.1 M PBS; 0.5 μg of FITC-conjugated goat anti-mouse IgG was added for 50 min at 4˚C. The cells were washed with 0.1 M PBS and fixed in 2% paraformaldehyde (350 μl/tube) for detection using FCM.

Preparation of nuclear extracts for western blot analysis. Nuclear proteins from A549 cells were extracted as previously described (33). Western blot analysis was performed as follows. Protein lysates (30-50 μg) were separated by 10% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were washed in 5% non-fat dry milk in phosphate-buffered saline plus 0.03% Tween-20 (PBST) for 1 h. Immunoblotting was performed using NF-κB and Lamin A monoclonal antibodies at a dilution of 1:100 in PBST and 1:200 in non-fat dry milk Tris-buffered saline, respectively. The membrane was probed with a secondary antibody (donkey anti-goat IgG/HRP and goat anti-mouse IgG/HRP) at a dilution of 1:4,000. Protein expression was detected with a GeneGnome Imaging System (Syngene, Cambridge, UK) using BeyoECL Plus (Beyotime, Haimen, China).

Results

TLR4 and Foxp3 are expressed in A549 cells. The expression of TLR4 and Foxp3 in A549 cells was determined by RT-PCR, RT-qPCR and FCM. The results showed that both TLR4 and Foxp3 were expressed in A549 cells (Fig. 1).

Activation of TLR4 promotes the expression of Foxp3. In order to investigate whether the TLR4 signaling pathway is involved in the regulation of the expression of Foxp3, the expression of Foxp3 in A549 cells was detected after LPS stimulation and blocking of the TLR4 signaling pathway.

Expression of Foxp3 mRNA in A549 cells after LPS stimulation at various concentrations. The mRNA expression of Foxp3 in A549 cells after LPS treatment at various concentrations in A549 cells was detected by RT-PCR and RT-qPCR. The results showed that the expression of Foxp3 mRNA in the LPS-stimulated groups was significantly increased compared to the control group, particularly at the concentration of 10 μg/ml of LPS stimulation (Fig. 2).

Expression of Foxp3 mRNA in A549 cells after LPS stimulation for different periods of time. To determine the effect of LPS stimulation on the expression of Foxp3 mRNA, A549 cells were stimulated for 12, 24, 36 or 48 h with LPS (10 μg/ml) and Foxp3 mRNA was detected by RT-PCR and RT-qPCR. The

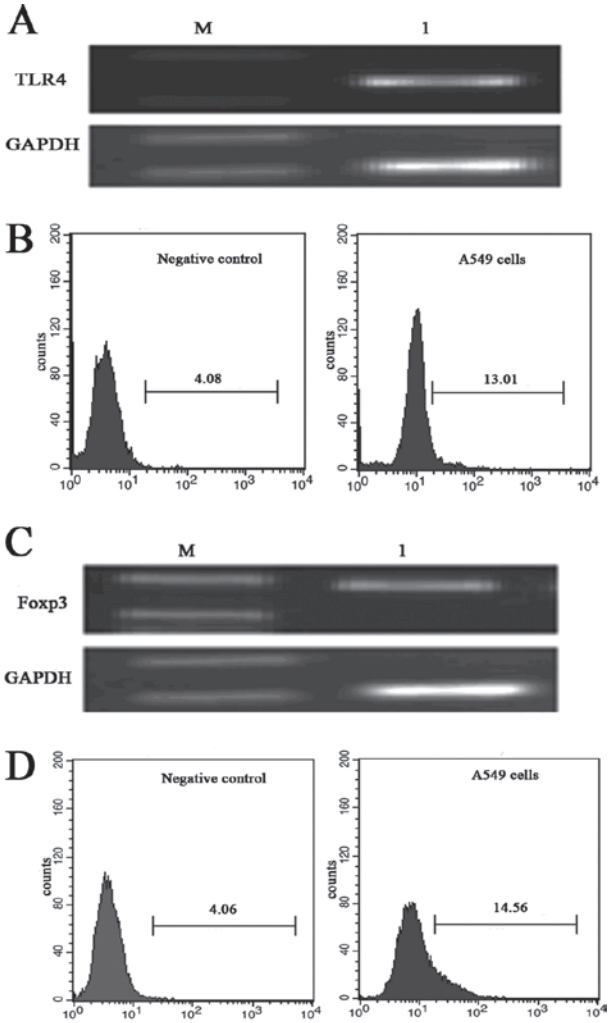

Figure 1. Expression of Toll-like receptor 4 (TLR4) and Foxp3 in A549 cells. (A) Gel electrophoresis of TLR4 PCR products. M, marker; 1, A549 cells. (B) A549 cells were harvested, stained with purified anti-human TLR4 and then with FITC-conjugated goat anti-mouse IgG. (C) Gel electrophoresis of Foxp3 PCR products. M, marker; 1, A549 cells. (D) A549 cells were harvested, stained with purified anti-human Foxp3 and then with FITC-conjugated goat anti-mouse IgG.

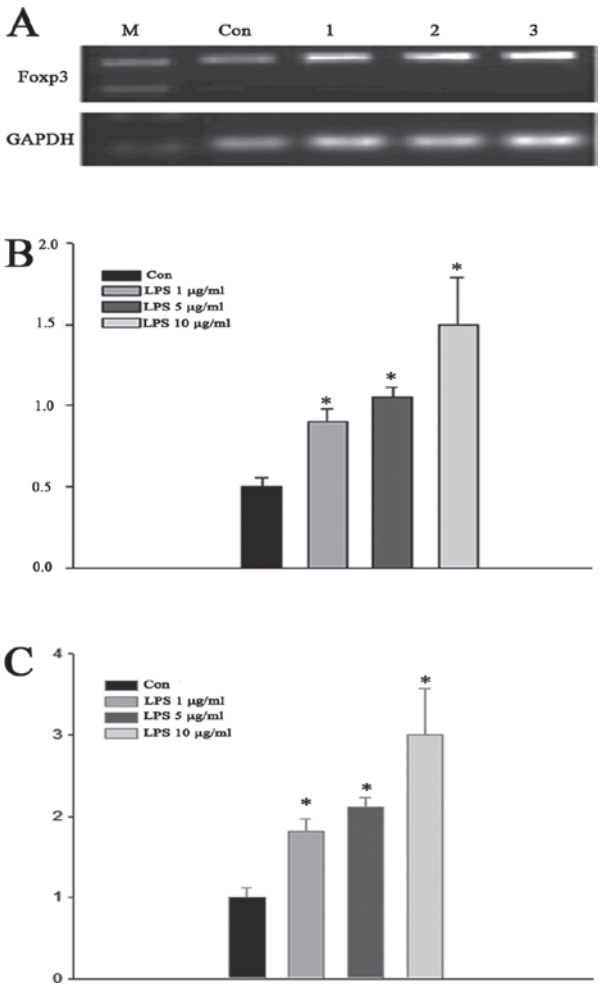

Figure 2. Expression of Foxp3 mRNA after treatment with various concentrations of lipopolysaccharide (LPS) in A549 cells by RT-PCR and RT-qPCR. (A) Gel electrophoresis of Foxp3 PCR products. M, marker; Con, control group; 1, LPS 1 μg/ml; 2, LPS 5 μg/ml; 3, LPS 10 μg/ml. (B) Graph of Foxp3 mRNA expression in A549 cells. (C) A549 cells were treated with LPS at different concentrations for 24 h, and the mRNA expression of Foxp3 was determined by RT-qPCR. *P<0.05 vs. control (0 μg/ml LPS).

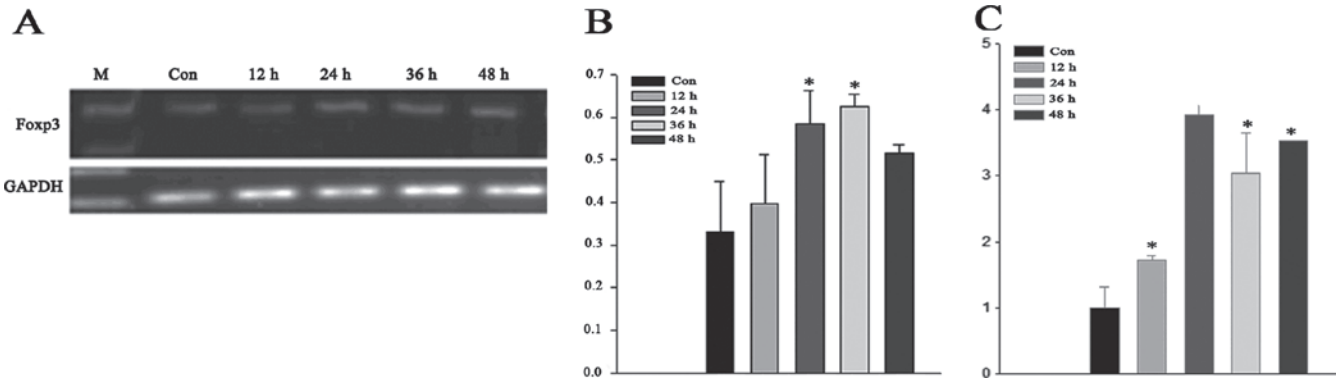

Figure 3. Expression of Foxp3 mRNA after lipopolysaccharide (LPS) treatment for different periods of time in A549 cells by RT-PCR and RT-qPCR. (A) Gel electrophoresis of Foxp3 PCR products. M, marker; Con, control group. (B) Graph of Foxp3 mRNA in A549 cells. (C) The A549 cells were treated with LPS (10 μg/ml) for different periods of time, and the mRNA expression of Foxp3 was determined by RT-qPCR. *P<0.05 vs. control (0 μg/ml LPS).

results showed that the expression of Foxp3 mRNA increased upon LPS stimulation, peaking at 24 h (Fig. 3).

Expression of Foxp3 protein increases after LPS stimulation. Foxp3 protein expression was examined after LPS stimulation (10 μg/ml) for 24 h and analysed by FCM. The results showed that the mean fluorescence intensity (MFI) of Foxp3 in the LPS-stimulated group was significantly increased (Fig. 4).

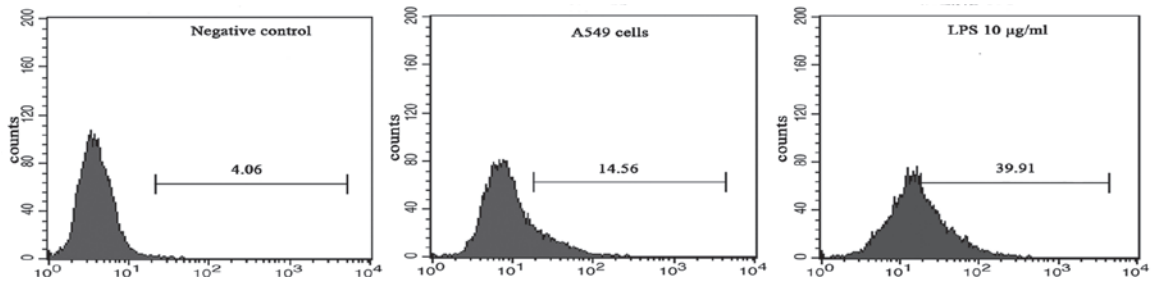

Figure 4. Expression of Foxp3 protein after LPS (10 μg/ml) treatment for 24 h in A549 cells determined by flow cytometry (FCM). A549 cells were treated with LPS (10 μg/ml) for 24 h and the cells were fixed, permeabilised and stained with purified anti-human Foxp3 and FITC-conjugated goat anti-mouse IgG. The protein expression of Foxp3 was determined by FCM.

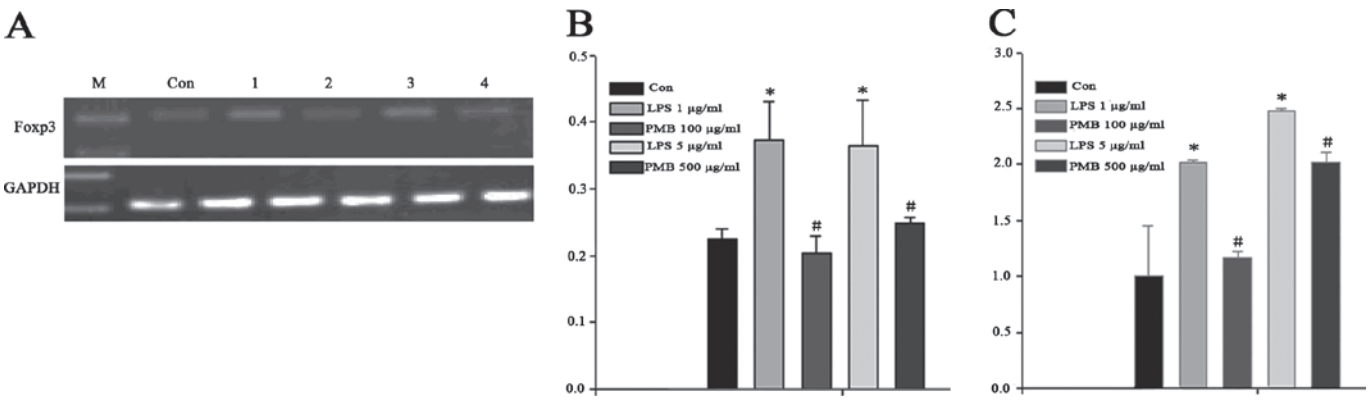

Figure 5. Expression of Foxp3 mRNA in A549 cells after inhibition of the Toll-like receptor 4 (TLR4) signaling pathway. A549 cells were incubated with polymyxin B sulfate (PMB) (100 and 500 μg/ml) for 1 h before the addition of lipopolysaccharide (LPS) (1 and 5 μg/ml) for 24 h, and the expression of Foxp3 mRNA was determined by RT-PCR and RT-qPCR. (A) Gel electrophoresis of Foxp3 PCR products. M, marker; Con, control group; 1, LPS 1 μg/ml; 2, LPS 1 μg/ml + PMB 100 μg/ml; 3, LPS 5 μg/ml; 4, LPS 5 μg/ml + PMB 500 μg/ml. (B) Graph of Foxp3 mRNA in A549 cells. (C) A549 cells were treated with LPS or LPS + PMB, and the mRNA expression of Foxp3 was determined by RT-qPCR. *P<0.05 vs. LPS 0 μg/ml; $^{\#}$P<0.05 vs. LPS-stimulated group.

Expression of Foxp3 decreases after inhibiting the TLR4 signaling pathway. To further verify that the TLR4 signaling pathway is involved in the regulation of the expression of Foxp3, we examined the expression of Foxp3 mRNA following treatment with PMB to block the TLR4 signaling pathway. The results showed that the expression of Foxp3 mRNA was significantly decreased with PMB treatment compared to the control LPS-stimulated group (Fig. 5). We then examined the expression of Foxp3 protein by FCM. The results showed that the MFI of Foxp3 was significantly decreased with PMB treatment compared to the control LPS-stimulated group (Fig. 6). These data indicate that the expression of Foxp3 is regulated by the TLR4 signaling pathway in A549 cells.

NF-κB is involved in the expression of Foxp3 induced by the TLR4 signaling pathway

Expression of p65 protein increases after LPS stimulation. NF-κB plays a key role in the TLR4 signaling pathway, translocating to the nucleus after the activation of the TLR4 signaling pathway. To explore whether NF-κB is involved in the regulation of Foxp3 by the TLR4 signaling pathway, the expression of the p65 protein after LPS (1 μg/ml) stimulation was detected by western blot analysis. The results showed that nuclear p65 expression in the LPS-stimulated group was significantly higher compared to the control group (Fig. 7). Therefore, this suggests that NF-κB is associated with the TLR4 signaling pathway.

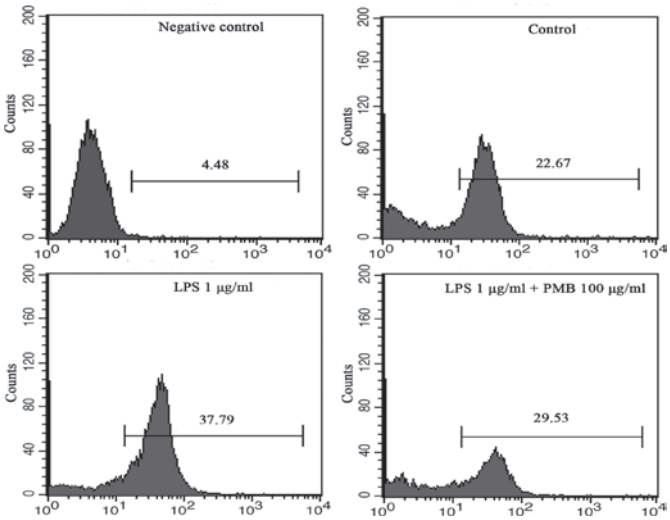

Figure 6. Expression of Foxp3 protein after treatment with lipopolysaccharide (LPS) or LPS + polymyxin B sulfate (PMB) at 24 h in A549 cells determined by flow cytometry (FCM). A549 cells were treated with PMB (100 μg/ml) for 1 h and then LPS (1 μg/ml) was added for 24 h. The cells were then fixed, permeabilised and stained with purified anti-human Foxp3 and FITC-conjugated goat anti-mouse IgG. The protein expression of Foxp3 was determined by FCM.

Expression of Foxp3 in A549 cells after NF-κB inhibition. To investigate whether the expression of Foxp3 is regulated by the TLR4 signaling pathway through NF-κB, PDTC was used to inhibit NF-κB. The expression of Foxp3 mRNA was then

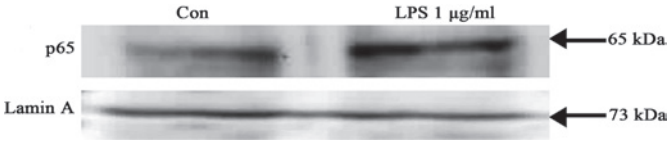

Figure 7. Expression of p65 after lipopolysaccharide (LPS) (1 μg/ml) treatment at 24 h in A549 cells determined by western blot analysis.

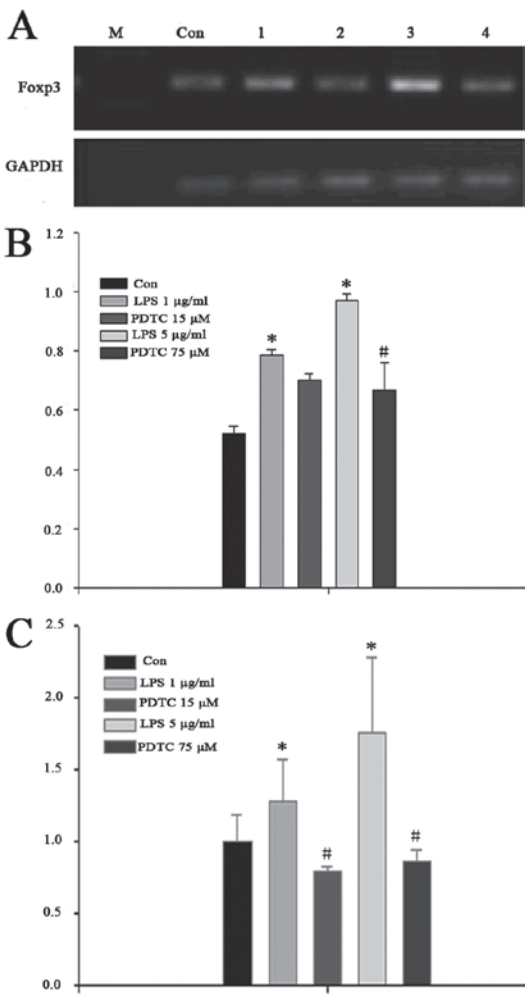

Figure 8. Expression of Foxp3 mRNA in A549 cells after nuclear factor-κB (NF-κB) inhibition. A549 cells were incubated with the NF-κB inhibitor, ammonium pyrrolidine dithiocarbamate (PDTC), (15 and 75 μM) for 30 min, followed by stimulation with lipopolysaccharide (LPS) (1 and 5 μg/ml) for 24 h. (A) Gel electrophoresis of Foxp3 PCR products. M, marker; Con, control group; 1, LPS 1 μg/ml; 2, LPS 1 μg/ml + PDTC 15 μM; 3, LPS 5 μg/ml; 4, LPS 5 μg/ml + PDTC 75 μM. (B) Graph of Foxp3 mRNA in A549 cells. (C) A549 cells were treated with LPS or LPS + PDTC, and the mRNA expression of Foxp3 was determined by RT-qPCR. *P<0.05 vs. LPS 0 μg/ml; #P<0.05 vs. LPS-stimulated group.

examined by RT-PCR and RT-qPCR. The results showed that the expression of Foxp3 mRNA was significantly decreased upon PDTC treatment compared to the control LPS-stimulated group, particularly at 75 μM PDTC and 5 μg/ml LPS (Fig. 8). We then examined the expression of the Foxp3 protein by FCM. The results showed that the expression of the Foxp3 protein significantly decreased upon PDTC treatment compared to the control LPS-stimulated group (Fig. 9). These results suggest that NF-κB is involved in the regulation of Foxp3 expression.

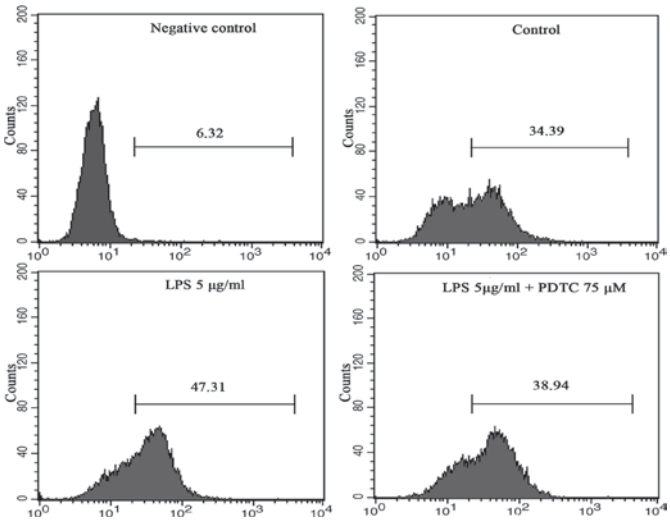

Figure 9. Expression of Foxp3 protein after treatment with lipopolysaccharide (LPS) or LPS + ammonium pyrrolidine dithiocarbamate (PDTC) for 24 h in A549 cells determined by flow cytometry (FCM). A549 cells were incubated with the nuclear factor-κB (NF-κB) inhibitor, PDTC, (75 μM) for 30 min, followed by stimulation with LPS (5 μg/ml) for 24 h. The cells were then fixed, permeabilised and stained with purified anti-human Foxp3 and FITC-conjugated goat anti-mouse IgG. The protein expression of Foxp3 was determined by FCM.

Discussion

The forkhead transcription factor, Foxp3, is highly expressed in Tregs and has been identified as a key player in mediating their inhibitory function. It has been reported that the expression of Foxp3 within tumour cells is associated with tumour progression and metastasis, and thus it is a poor prognostic factor. Hinz *et al* indicated that Foxp3 is expressed in pancreatic carcinoma cells and that these cells share similar growth-suppressive effects with Tregs, mimicking Treg function. This may represent a new mechanism of immune evasion in pancreatic cancer (5). Merlo *et al* indicated that Foxp3 expression in primary breast carcinoma specimens is associated with worse overall survival probability, and this risk increases with the intensity of Foxp3 immunostaining (6). However, Zuo *et al* showed that Foxp3 is a novel transcriptional repressor for the oncogene, SKP2 (7). Um *et al* showed that Foxp3 is not only expressed in patients with NSCLC, but also in the NCI-H460 and A549 lung cancer cell lines (8). The mechanisms that control Foxp3 expression in lung cancer cell lines remain unclear. In this study, we determined that A549 cells express Foxp3, and that its expression is partially regulated by TLR4 signaling.

TLR4 is extensively expressed in APC cells and epithelial cells, and recent studies showed that TLR4 is expressed in many tumour cells, such as adrenocortical, lung, breast and bladder cancer cells (25). The expression of TLR4 in tumour cells is associated with tumour invasion and metastasis. He *et al* determined that TLR4 expressed in human lung cancer cells is functionally active, and may play an important role in promoting the immune evasion of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance (18). Zhang *et al* showed that TLR4 is strongly expressed in lung cancer tissue. They also reported for the first time a positive correlation between the expression levels of TLR4

and lung cancer malignancy (16). NF-κB plays a key role in the TLR4 signaling pathway. Previous reports have shown that LPS, a TLR4 ligand, stimulates the activation of NF-κB and induces the resistance of human lung cancer to TNF-α or TRAIL-induced apoptosis (18,26). Xu *et al* showed that nickel promotes the invasive potential of human lung cancer cells via TLR4/MyD88 signaling (27). Wang *et al* suggested that the high expression of TLR4 and MyD88 is associated with liver metastasis and is an independent predictor of poor prognosis in patients with colorectal cancer (28). In this study, the expression of Foxp3 increased after LPS stimulation in a concentration-dependent manner, and the expression of Foxp3 decreased after inhibition of the TLR4 signaling pathway.

A number of studies have shown that Foxp3 is closely connected with NF-κB. Milkova *et al* found that the NF-κB signaling pathway is involved in the LPS/IL-2-induced upregulation of Foxp3 expression in human $CD4^+CD25^{high}$ Tregs (29). NF-κB also promotes the transcription of Foxp3 (24). In this study, the expression of p65 increased after LPS stimulation, and the expression of Foxp3 decreased after NF-κB inhibition. These results indicate that the TLR4 signaling pathway is involved in the regulation of Foxp3 expression through NF-κB.

In the present study, we determine that Foxp3 expression is regulated by the TLR4 signaling pathway through NF-κB in A549 cells, which may provide some clues for understanding the mechanism of Foxp3 induction in tumour cells, and may also provide some insight on immunotherapy for lung cancers.

Acknowledgements

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