

The TLR4/ERK/PD-L1 axis may contribute to NSCLC initiation

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Abstract. Infection and inflammation serve an important role in tumor development. Toll-like receptor 4 (TLR4) is a pivotal component of the innate and adaptive immune response during infection and inflammation. Programmed-death ligand 1 (PD-L1) is hypothesized as an important factor for non-small cell lung cancer (NSCLC) immune escape. In the present study, the relationship between TLR4 and PD-L1, in addition to the associated molecular mechanism, were investigated. TLR4 and PD-L1 expression in lung cancer tissues were detected using immunohistochemistry, whilst overall patient survival was measured using the Kaplan-Meier method. The A549 cell line stimulated using lipopolysaccharide (LPS) was applied as the *in vitro* inflammatory NSCLC model. Associated factors were investigated using reverse transcription-quantitative PCR and western blotting. Lung cancer tissues exhibited increased PD-L1 and TLR4 levels compared with those of adjacent para-cancerous tissues, where there was a positive correlation between TLR4 and PD-L1 expression. In addition, increased expression of these two proteins was found to be linked with poorer prognoses. Following the stimulation of A549 cells with LPS, TLR4 and PD-L1 expression levels were revealed to be upregulated in a dose-dependent manner, where the ERK and PI3K/AKT signaling pathways were found to be activated. Interestingly, in the presence of inhibitors of these two pathways aforementioned, upregulation of PD-L1 expression was only inhibited by the MEK inhibitor PD98059, which can inhibit ERK activity. These data suggested that the ERK signaling pathway is necessary for the TLR4/PD-L1 axis. In conclusion, data from the present study suggest that TLR4

and PD-L1 expression can serve as important prognostic factors for NSCLC, where TLR4 activation may induce PD-L1 expression through the ERK signaling pathway.

Introduction

According to data from the global cancer statistics in 2018, the most common and malignant form of cancer is that of lung cancer, which accounts for 11.6% of all cancers and 18.4% of all cancer-associated mortality globally (1). In China, lung cancer is the most prominent cause of mortality associated with cancer in both males and females, with mortality rates of 52.47 and 26.29 per 100,000, respectively (2). Non-small cell lung cancer (NSCLC) cases make up ~85% of all lung cancer cases, with ≤40% of cases not being identified until advanced stages (III-IV), where treatment options and the probability of survival become limited (3).

In total, ~15% cancer cases have recently been suggested to be associated with an infectious origin, accounting for 1.2 million cases annually (4). Previous clinical and experimental findings suggest that chronic infection and inflammation are closely linked with lung cancer (5-7). Toll-like receptors (TLRs) are key receptors that can detect and then respond to infections through the innate immune and inflammatory response mechanisms. TLR4 was the first identified human toll homolog family of proteins that can be activated by lipopolysaccharides (LPS) and induces the secretion of proinflammatory cytokines among others to combat pathogenic infections (8). Although recent studies found TLR4 to be expressed in lung cancer cells and tissues (9,10), the role of TLR4 in lung cancer remains controversial. The programmed-death 1 receptor/PD-ligand 1 (PD-L1) pathway has been reported to be a key inhibitory mechanism in lung cancer cells, the activation of which leads to effector T cell exhaustion and immune escape (11,12). Previous studies have demonstrated that PD-L1 expression could be induced by TLR4 in macrophages and colonic stromal cells (13,14), though the functional relationship between PD-L1 and TLR4 in lung cancer remains elusive.

In the present study, PD-L1 and TLR4 expression were measured in lung cancer tissues, where TLR4 and PD-L1 expression were found to be upregulated in lung cancer tissues, with a positive correlation being observed between the expression levels of these two proteins. In addition, the mechanism in which TLR4 influenced the expression of PD-L1 and

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associated signaling pathways in the A549 lung cancer cell line was also investigated.

Materials and methods

Patients. The Ethics Committee of The First Affiliated Hospital of Nanchang University approved the present study (Nanchang, China). Informed consent was obtained from all patients. All patients were pathologically diagnosed as NSCLC and had not undergone any radio- or chemotherapy and should have a complete set of clinicopathological and follow-up data. Patients with other malignant tumors and diseases that may affect survival, including diabetes and heart failure were excluded. In total, 60 patients that underwent pulmonary resection from thoracic surgery department of The First Affiliated Hospital of Nanchang University were enrolled between January and December 2010. Overall survival (OS) was defined as the time from diagnosis to mortality or the final follow-up. Table I highlights the clinicopathological parameters from all enrolled patients.

Immunohistochemistry (IHC). A total of 60 NSCLC samples and 20 matched adjacent para-cancerous tissues (>2 cm from the edge of tumor tissue) were collected in this study. Once resected, tissues were fixed in 10% formalin overnight at room temperature (RT), embedded in paraffin and cut into 4- μ m thick tissue sections. Polyclonal rabbit anti-TLR4 (1:100, cat. no. ab13556; Abcam), and polyclonal rabbit anti-PD-L1 (1:100, cat. no. 13684; Cell signaling Technologies, Inc.) primary antibodies were used in the present study for IHC. Matched adjacent para-carcinoma tissues served as controls. Briefly, the samples were first heated at 70°C for 20 min prior to de-paraffinization in xylene, followed by rehydration with a descending ethanol series. Antigen retrieval was performed at 100°C and in citrate buffer (10 mM, pH 6.0) for 2 min and permeabilized in 0.5% Triton X-100 at RT for 20 min, following which 0.3% hydrogen peroxide was added to block the activity endogenous peroxidase for 10 min at RT. The sections were then blocked with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at RT before the tissue sections were incubated with the primary antibodies at 4°C overnight. Samples were then washed with PBS before the addition of horseradish peroxidase (HRP)-conjugated secondary antibodies (1:200; cat. no. G1213; Wuhan Servicebio Technology Co., Ltd.) at 37°C for 30 min. After washing, the tissues were then treated with 3,3'-diaminobenzidine (MXB Biotechnologies) for chromogenic development. Nuclei were next stained using hematoxylin (30 mg/ml; Sigma-Aldrich; Merck KGaA) for 1 min at RT before the sections were then added to coverslips and assessed by light microscopy (magnification, x100; Olympus Corporation), with all sections assessed by two pathologists independently in a blinded manner. Sections were deemed to be PD-L1 or TLR4 positive based on the presence of cell membrane or cytoplasmic staining, with a semi-quantitative system applied for IHC scoring. Five random areas with typical staining were identified, with 100 cells were assessed per area to determine the strength and frequency of staining. Staining strength was determined based on the following color scale: i) Negative, 0; ii) light brown, 1; iii) brown, 2; and iv) dark brown, 3. The frequency of cells staining positive for

the indicated antigen was determined based on the following scale: i) $\leq 5\%$, 0; ii) 6-25%, 1; iii) 26-50%, 2; iv) 51-75%, 3; and v) 76-100%, 4. These two scores were then multiplied together to yield a final staining score, with scores ≤ 1 being deemed negative (-), whereas those ≥ 1 were considered positive (+).

Cell culture. The A549 cell line was a gift from Dr. Jianbin Wang, Translation Medical Department of Nanchang University (Nanchang, China). RPMI-1640 (Biological Industries) supplemented with 10% newborn bovine serum (Biological Industries) and penicillin/streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) was used to culture cells at 37°C under a humidified atmosphere containing 5% CO₂. Confluent cells were collected using trypsin/EDTA for 3 min, after which cells (3×10^5 cells/ml) were plated into six-well plates (1 ml/well) for LPS treatment. LPS (Sigma-Aldrich; Merck KGaA) was used to treat the cells (0.5, 1 and 2 μ g/ml) for a range of time points (15 and 30 min, or 1, 2, 4 or 24 h) at 37°C. PD98059 (cat. no. M1822; Abmole Bioscience Inc.) and LY294002 (cat. no. 9901; Cell Signaling Technology, Inc.) were utilized as inhibitors for MEK and AKT, respectively. A549 cells were pretreated with PD98059 or LY294002 at different concentrations for 1 h at 37°C, following which LPS (1 μ g/ml) was added into the medium.

Western blotting. RIPA buffer (Applygen Technologies, Inc.) was used to lyse the A549 cells for protein extraction, following which a bicinchoninic acid protein assay kit (Vazyme Biotech Co., Ltd.) was used to quantify protein concentration. A total of 25 μ g protein of each sample was separated by 10% SDS-PAGE and transferred onto PVDF membranes, which were then blocked for 1 h with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) at RT before being probed overnight at 4°C with the following primary antibodies: Anti-TLR4 (1:500; cat. no. ab13556; Abcam), anti-PD-L1 (cat. no. 13684; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-p44/42MAPK (1:1,000; cat. no. 9101; Cell Signaling Technology, Inc.), anti-p44/42MAPK (1:1,000; cat. no. 4695; Cell Signaling Technology, Inc.), anti-Akt (1:1,000; cat. no. 4691; Cell Signaling Technology, Inc.), anti-p-Akt (Ser473; 1:2,000; cat. no. 4060; Cell Signaling Technology, Inc.) and anti- β -tubulin (1:2,000; cat. no. TA506805; OriGene Technologies, Inc.). The membranes were then washed three times and probed again with HRP-conjugated goat anti-mouse IgG (1:5,000; cat. no. SE131; Beijing Solarbio Science & Technology Co., Ltd.) or goat anti-rabbit IgG (1:5,000; cat. no. SE134; Beijing Solarbio Science & Technology Co., Ltd.) at RT for 1 h. Electro-chemiluminescence Plus supersensitive luminescent solution (Beijing Solarbio Science & Technology Co., Ltd.) was used for protein detection, following which the Image J software (v1.43j; National Institutes of Health) was used to perform densitometric analysis using β -tubulin as a loading control for normalization.

Reverse transcription-quantitative-PCR (RT-qPCR). RNAsimple Total RNA Kit (Tiangen Biotech Co., Ltd.) was used for RNA isolation according to manufacturer's protocols. Subsequently, reverse transcription was performed using FastQuant RT kit (Tiangen Biotech Co., Ltd.) according to manufacturer's protocols. The temperature protocol was 42°C

Table I. Association between PD-L1 and TLR-4 expression levels and the clinicopathological parameters of patients with non-small cell lung cancer.

Parameters	Cases	PD-L1			P-value ^a	TLR-4			P-value ^b
		+	-	%		+	-	%	
Gender					0.791				0.071
Male	48	30	18	62.5		22	26	45.8	
Female	12	7	5	58.3		9	3	75	
Age (years)					0.559				0.123
>60	21	14	7	66.7		8	13	38.1	
≤60	39	23	16	59		23	16	59.0	
Histology type					0.729				0.010
SCC	27	16	11	59.3		9	18	33.3	
ADC	33	21	12	63.6		22	10	69.7	
Grade					0.602				0.866
High-middle	42	25	17	35.7		22	20	52.4	
Low	18	12	6	66.7		9	9	50.0	
Lymphatic invasion					0.791				0.796
Negative	30	18	12	60.0		15	15	50	
Positive	30	19	11	63.3		16	14	53.3	
Tumor size (cm)					0.453				0.715
≤3	20	11	9	55.0		11	9	55.0	
>3	40	26	14	65.0		20	20	50.0	
TNM stage					0.090				0.025
I + II	39	21	18	53.8		16	23	41	
III	21	16	5	76.2		15	6	71.4	

^aPD-L1⁺ vs. PD-L1⁻; ^bTLR4⁺ vs. TLR4⁻. Pearson's χ^2 test was used to analyze the data above. PD-L1, programmed cell death ligand 1; TLR4, toll-like receptor 4; SCC, squamous cell carcinoma; ADC, adenocarcinoma.

for 15 min followed by 95°C for 3 min for cDNA synthesis. SuperReal PreMix Plus (SYBR Green; Tiangen Biotech Co., Ltd.) was then used for qPCR in an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following thermocycling conditions: Initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Sequences of the primers were as follows: PD-L1 forward, 5'-GCCGACTACAAGCGAATTAC-3' and reverse, 5'-TCTCAGTGTGCTGGTCACAT-3' and β -actin forward, 5'-CGGGAAATCGTGCGTGAC-3' and reverse, 5'-TAGAAGCATTTGCGGTGG-3'. The $2^{-\Delta\Delta C_q}$ method was utilized when assessing relative expression (15).

Statistical analysis. SPSS statistical software (version 19.0; IBM Corp.) and GraphPad Prism 5.0 (GraphPad Software, Inc.) were utilized when performing statistical assessments. All experiments were repeated 3 times and data were presented as the mean \pm SD or SEM. Spearman rank correlation coefficient was used for correlation analyzes. χ^2 -test was used for comparisons between categorical variables and the Kaplan-Meier method was used when assessing overall survival based on the IHC scores with log-rank tests used for comparisons of significance. A Cox proportional hazard model was applied for multivariate analyses of the independent factors associated

with survival. Statistical comparisons between >2 groups were performed using one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TLR4 and PD-L1 expression are both increased in NSCLC tissues, which exhibit positive association with each other. To assess TLR4 and PD-L1 expression in NSCLC tissues, IHC was performed in 60 cases of NSCLC tissues and 20 matched adjacent para-cancerous tissues. Positive TLR4 and PD-L1 staining was mainly observed at the membrane and cytoplasm of the cancerous tissues (Figs. 1 and S1). The rate of TLR4-positive expression was observed in 31/60 (51.7%) NSCLC tissues, compared with 1/20 (5%) observed in adjacent para-cancerous tissues, which was found to be significant ($P < 0.001$; Table SI). The rate of PD-L1-positive expression was observed in 37/60 (61.7%) NSCLC tissues and 2/20 (10%) adjacent para-cancerous tissues, with the difference found to be significant ($P < 0.001$; Table SI). Both TLR4 and PD-L1 positive rates were found to be significantly higher in NSCLC tissues compared with those in para-cancerous tissues (Fig. 1). A total of 24 tissues exhibited positive staining for both TLR4

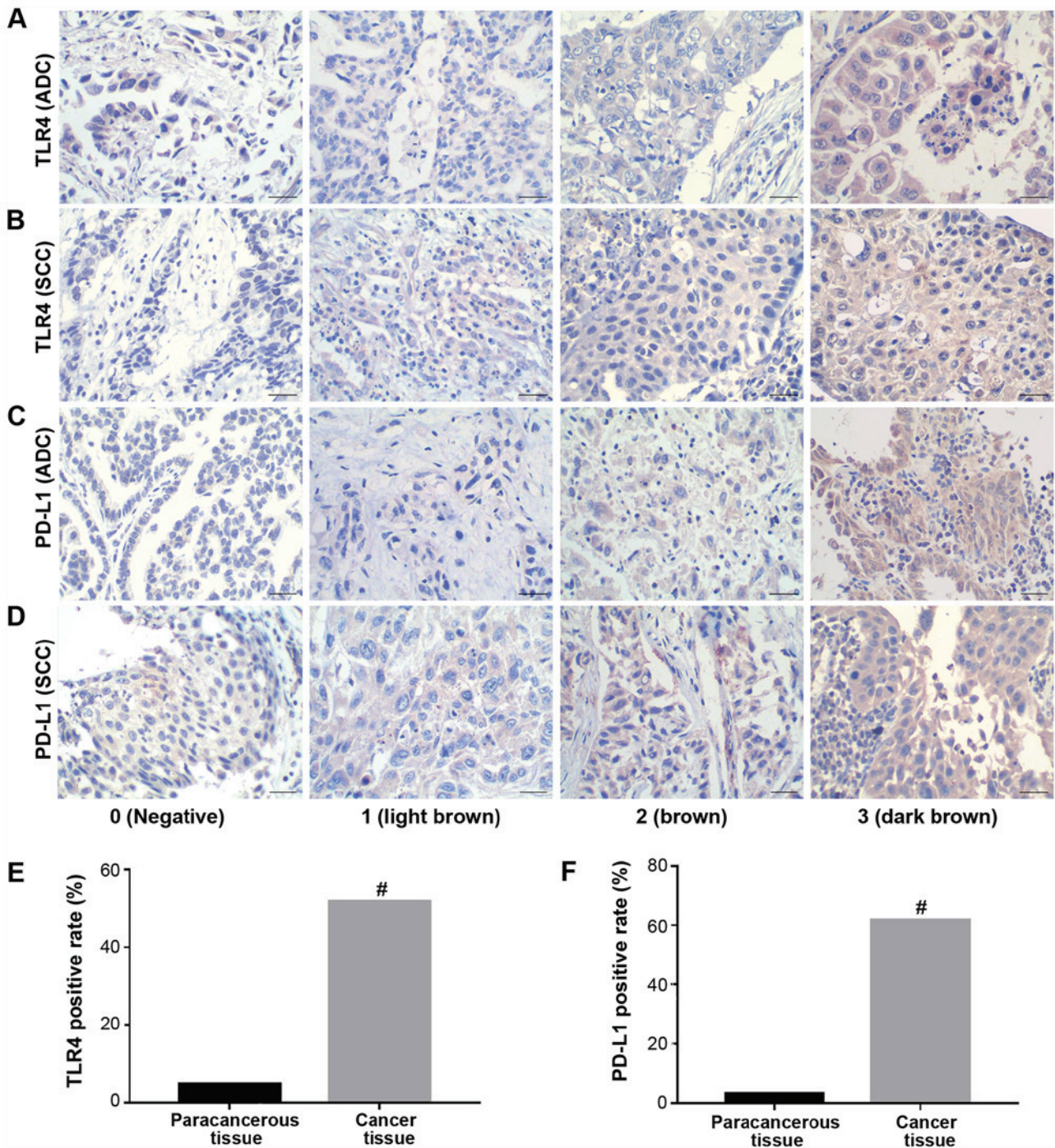


Figure 1. Positive TLR4 and PD-L1 expression are more readily observed in lung cancer tissues. (A-D) Representative images of (A) lung adenocarcinoma and (B) lung squamous cell carcinoma tissues exhibiting TLR4 staining. Representative images of (C) lung adenocarcinoma and (D) lung squamous cell carcinoma tissues exhibiting PD-L1 staining. The number of tissues staining positive for (E) TLR4 and (F) PD-L1 expression in cancer and para-cancerous tissues. Scale bar, 50 μ m. [#] $P < 0.01$ vs. para-cancerous tissue. TLR4, toll-like receptor; PD-L1, programmed-death ligand 1; ADC, adenocarcinoma; SCC, squamous cell carcinoma.

and PD-L1, whilst 16 tissues were tested negative for both TLR4 and PD-L1 staining among the 60 lung cancer tissues (Table SII). χ^2 -test revealed a positive association between the incidence of positive TLR4 and that of positive PD-L1 expression based on the IHC scores ($\chi^2 = 6.733$, $P = 0.0095$).

To assess the clinical relevance of TLR4 and PD-L1, the association between the clinicopathological characteristics, including age, gender, histological type, stages of pathological differentiation, lymphatic invasion, tumor size and TNM stage,

and the expression of TLR4/PD-L1 was analyzed in Table I. TLR4 expression was found to associate with the histological type ($P = 0.01$) and TNM stages ($P = 0.025$) but PD-L1 expression did not associate with any of the clinicopathological parameters tested.

Elevated TLR4 and PD-L1 correspond to poorer prognoses in patients with NSCLC. The average follow-up time for the patients was 38.33 ± 2.814 months (range, 5-68 months),

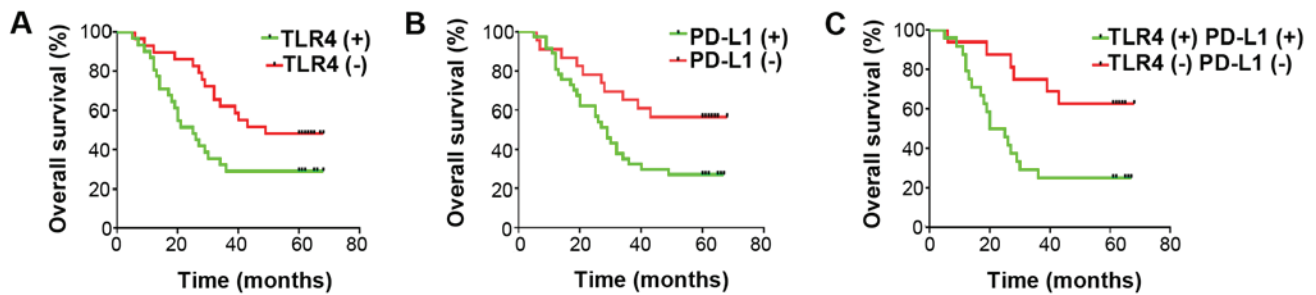


Figure 2. OS rates of non-small cell lung cancer according to TLR4 and PD-L1 expression levels. (A) Positive TLR4 staining is associated with decreased OS ($P=0.0143$). (B) Positive PD-L1 staining is associated with decreased OS ($P=0.0353$). (C) Positive combined PD-L1 and TLR4 staining is associated poorer OS ($P=0.0091$). OS, overall survival; TLR4, toll-like receptor; PD-L1, programmed-death ligand 1.

where 37 died and 23 surviving to the final follow-up on 31st December, 2015. The 1-, 3- and 5-year OS rates for these individuals were found to be 88, 45 and 38.3%, respectively. Patients with positive TLR4 and/or PD-L1 staining were found to associate significantly with lower OS compared with those with negative staining at each time point (Fig. 2). Although it was found by univariate analysis that the OS rate was significantly associated with lymphatic invasion ($P=0.01$), tumor stage ($P=0.01$), TLR4 ($P=0.02$) and PD-L1 expression ($P=0.01$), subsequent multivariate analysis did not demonstrate these to be independent prognostic factors (Table II).

LPS induces TLR4 and PD-L1 expression in a dose-dependent manner. LPS is a potent agonist for TLR4 (9). In the present study, A549 cells were treated with different concentrations (0.5, 1 and 2 $\mu\text{g/ml}$) of LPS for 24 h. TLR4 expression was demonstrated to be significantly increased by LPS treatment in a dose-dependent manner compared with that in the control group, which peaked at 1.0 $\mu\text{g/ml}$ (Fig. 3). PD-L1 expression was also increased after LPS treatment compared with that in control group with the optimal concentration found to be 1.0 $\mu\text{g/ml}$ (Fig. 3). A higher concentration of LPS (2.0 $\mu\text{g/ml}$) was not able to upregulate the TLR4 and PD-L1 expression further. Therefore, 1.0 $\mu\text{g/ml}$ was used as the concentration for subsequent LPS stimulation experiments.

ERK and PI3K/AKT signaling pathway are activated by LPS stimulation. To explore the mechanism underlying the TLR4 and PD-L1 upregulation by LPS treatment, expression of proteins associated with the ERK and PI3K/AKT signaling pathway were measured using western blotting. ERK and AKT phosphorylation were found to be significantly increased following treatment with LPS compared with those in control cells (Fig. 4). The levels of phosphorylation peaked at 30 min after LPS treatment, which then decreased thereafter (Fig. 4). These results suggest that the ERK and PI3K/AKT signaling pathway was activated by LPS stimulation.

LPS-induced PD-L1 expression is mediated via ERK signaling but not the PI3K/AKT pathway. Since both ERK and PI3K/AKT signaling pathway were activated by LPS treatment, pharmacological inhibitors of the ERK and PI3K/AKT signaling pathway applied in the present study to investigate

which pathway is necessary for the induction of PD-L1 expression. PD98059 is a selective inhibitor of MAPK/ERK kinase (MEK), which binds to the inactive form of MEK to prevent the activation of MEK1 and MEK2 by upstream kinases (16). PD98059 was therefore used to inhibit ERK activity. By contrast, LY294002 is a broad-spectrum PI3K inhibitor that has been demonstrated to block PI3K-dependent AKT phosphorylation and kinase activity (17). As shown in Fig. 5, PD98059 significantly inhibited ERK phosphorylation, whilst LY294002 significantly inhibited AKT phosphorylation compared with cells treated with LPS alone.

PD-L1 expression was subsequently measured. Western blotting and RT-qPCR analysis revealed that the LPS-induced PD-L1 upregulation was significantly reversed by the MEK inhibitor PD98059 but not by the PI3K inhibitor PD294002 on both protein and mRNA levels (Fig. 6). These data indicated that LPS induced PD-L1 upregulation via ERK signaling pathway.

Discussion

Lung cancer ranks number one in the number of mortalities associated with cancer in men and second in women, with 1.8 million newly diagnosed cases and 1.6 million deaths resulting from this disease globally each year (18). In total, ~80-85% lung cancer cases are of the NSCLC type. Since clinical manifestations and symptoms are nonspecific, the majority of patients with NSCLC are diagnosed after the occurrence of metastasis (19), greatly diminishing the efficacy of surgery. Although the introduction of targeted therapies such as immunotherapy have improved survival to a certain degree, the overall survival rate remains unsatisfactory (19-21). Therefore, early diagnosis and treatment are crucial in preventing tumor progression and reducing the mortality of patients.

There is accumulating evidence demonstrating that cancer is associated with infectious agents and inflammation (4,22-26). It is estimated that $\leq 20\%$ of all cancers are preceded by inflammation as a result of pathogenic infection, with hepatocellular carcinoma and hepatitis B virus-induced hepatitis, gastric cancer and *H. pylori*-induced gastritis, cervical cancer and human papillomavirus infection among the well documented examples (27-29). Denholm *et al* (30) previously found the incidence of lung cancer to be significantly associated with chronic bronchitis and emphysema,

Table II. Univariate and multivariate analysis of the clinicopathologic factors in patients with NSCLC with respect to overall survival.

Parameters	Cases	Univariate analysis		Multivariate analysis	
		Survive time	P-value	HR (95% CI)	P-value
Gender			0.609		
Male	48	36.06±3.29			
Female	12	35.42±5.16			
Age (years)			0.845		
>60	21	39.1±4.75			
≤60	39	37.92±3.54			
Histology type			0.293		
SCC	27	41.63±4.48			
ADC	33	35.64±3.57			
Grade			0.177		
High-middle	42	40.83±3.36			
Low	18	32.5±5.03			
Lymphatic invasion			0.013		
Negative	30	31.47±3.73		0.557 (0.246-1.258)	0.159
Positive	30	45.2±3.88			
Tumor size (cm)			0.355		
≤3	20	42.05±4.79			
>3	40	36.48±3.48			
TNM stage			0.009		
I +II	39	43.67±3.33		1.24 (0.538-2.86)	0.614
III	21	28.43±4.47			
PD-L1			0.023		
Positive	37	33.32±3.36		0.663 (0.31-1.422)	0.291
Negative	23	46.39±4.57			
TLR4			0.014		
Positive	31	31.71±3.85		0.591 (0.298-1.17)	0.131
Negative	29	45.41±3.75			

Overall survival time was presented as mean ± SEM and was determined by the Kaplan-Meier method with log-rank test. Multivariable analysis of the independent factors was performed using the Cox proportional hazard model. PD-L1, programmed cell death ligand 1; TLR4, toll-like receptor 4; SCC, squamous cell carcinoma; ADC, adenocarcinoma; HR, hazards ratio; CI, confidence interval.

with the presence of both conditions associating more strongly with lung cancer compared with chronic bronchitis alone. Interestingly, a growing body of evidence are supporting an association between *H. pylori* infection with lung cancer (31). However, the mechanisms through which inflammation promotes cancer are not fully understood.

TLR4 is a key mediator of innate immunity, which specifically recognizes conserved motifs expressed by pathogens to mediate immune responses (32). Huang *et al* (33) previously demonstrated that TLR4 is expressed by many types of cancer cells, including colon, breast, prostate and lung cancer cells. Following TLR4 activation, tumor cells can synthesize a number of factors, including interleukin-6, interleukin-12 and PD-L1, which is a co-stimulator of T cell function. Interaction between PD-L1 and PD-1 expressed on cytolytic T cells leads to the negative co-stimulation of TCR signaling, resulting in

effector T cell exhaustion (34). PD1/PD-L1-induced immune evasion by tumor cells is an important mechanism for NSCLC, the blockade of which has improved the survival of a small percentage of patients with NSCLC (35,36). However, the majority of patients showed little to no response or acquire resistance during treatment (37).

A number of studies have previously reported that TLR4 and PD-L1 were aberrantly expressed in cancer tissues or cell lines (13,14,38-40). PD-L1 expression can be induced by extracellular vesicles from melanoma cells via TLR4 signaling (41). Both TLR4 and PD-L1 were upregulated in ~50% of peripheral T-cell lymphomas, which were found to be associated with poor prognoses (42). Therefore, in the present study it was hypothesized that TLR4-induced PD-L1 expression could be the mechanism underlying lung cancer progression. TLR4 and PD-L1 expression levels were

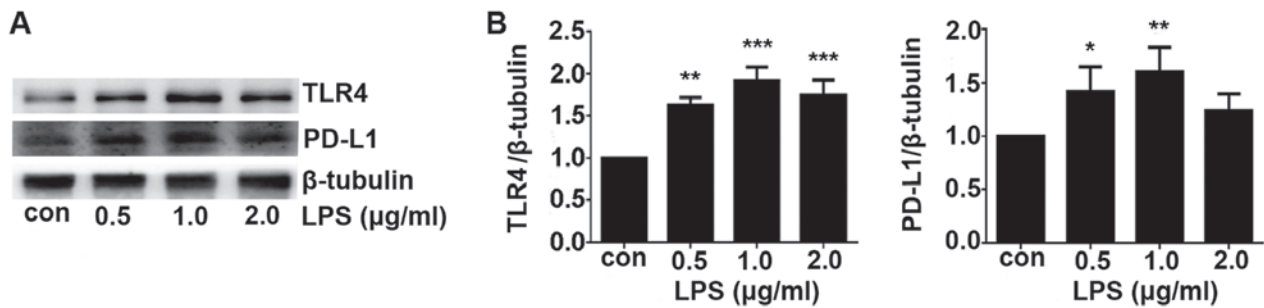


Figure 3. LPS treatment increases TLR-4 and PD-L1 expression in a dose-dependent manner. A549 cells were subjected to 0.5, 1, 2 μg/ml LPS for 24 h prior to western blotting. (A) Representative images of the TLR-4 and PD-L1 blots. (B) Quantification of the TLR4 and PD-L1 expression levels shown in (A). Data are shown as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 vs. con. LPS, lipopolysaccharide; Con, control; TLR4, toll-like receptor; PD-L1, programmed-death ligand 1.

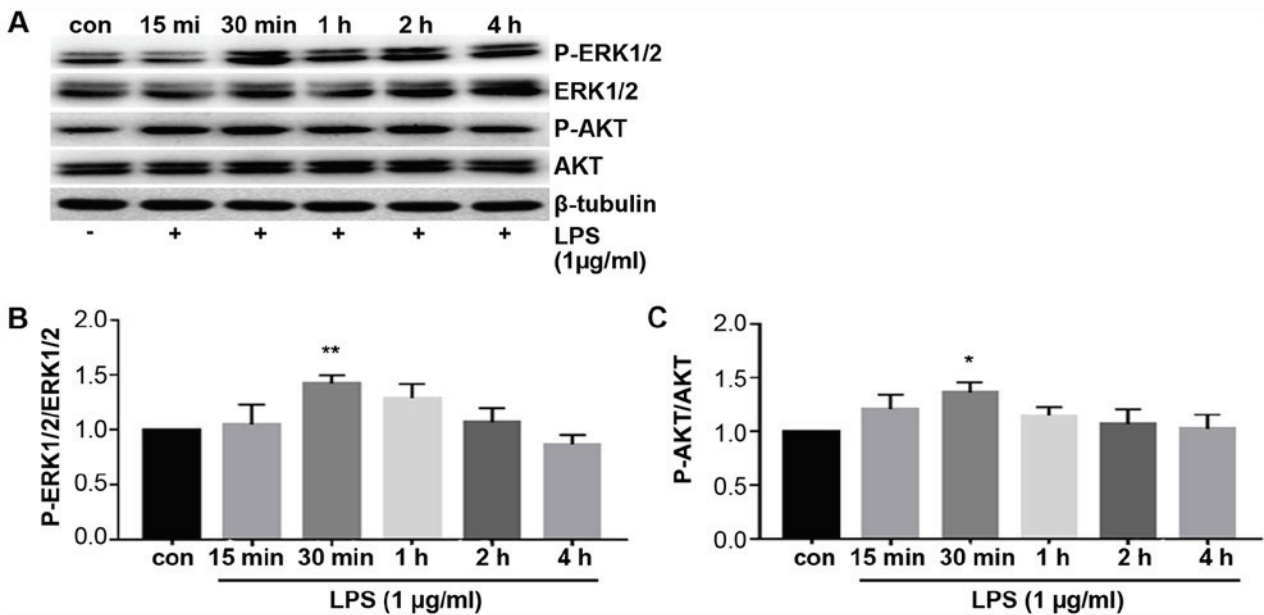


Figure 4. ERK and PI3K/AKT signaling pathway are activated by LPS stimulation. A549 cells were treated with LPS (1 μg/ml) for 15 and 30 min or 1, 2 and 4 h prior to western blotting. (A) Representative images of the blots showing ERK1/2 and AKT phosphorylation. Semi-quantitative densitometric analysis measuring relative (B) p-ERK1/2 and (C) p-AKT levels following treatment with/without LPS. *P<0.05 and **P<0.01 vs. con. LPS, lipopolysaccharides; Con, control.

first measured in NSCLC tissues, which demonstrated that both TLR4 and PD-L1 were significantly more prominent in NSCLC tissues compared with those in para-cancerous tissues. In addition, a statistically significant positive correlation was observed between TLR4 and PD-L1 expression, whilst overall survival was also revealed to associate significantly with TLR4 and PD-L1 expression. However, none were demonstrated to be independent prognostic factors for NSCLC. Wang *et al* (40) reported different findings, who determined that higher expression of TLR4 in lung cancer tissues was significantly associated with poorer OS and disease-free survival, where TLR4 was found to be a independent prognostic factor for NSCLC through multivariate analysis. There are several studies that revealed contradictory results. Wei *et al* (43) assessed the relevance of serum levels of soluble TLR4 (sTLR4) in NSCLC, who found lower sTLR4 levels to be indicative of reduced survival among patients with early-stage NSCLC that had recently undergone tumor resection surgery. Another previous study by Bauer *et al* (10)

also supported the notion that increasing TLR4 expression may improve outcomes, but no significance was found. A possible explanation for this inconsistency may be due to different sample sizes, whilst another explanation could be that the complex formed by sTLR4 and the adaptor protein myeloid differentiation factor-2 (MD-2) may attenuate TLR4-mediated signaling, since TLR4 requires MD-2 to respond efficiently to LPS (44,45).

Although the present study didn't uncover a prognostic value of TLR4 and PD-L1. It is believed that inflammation can lead to carcinogenesis (46,47). TLR4 is a component of the innate and adaptive immune response to infection and inflammation, whilst PD-L1 also has a pivotal role in immune escape by lung cancer. Therefore, the relationship between TLR4 and PD-L1 was explored further *in vitro* in the present study, using LPS as the inflammatory stimulator. TLR4 activation by LPS was found to induce PD-L1 expression in A549 cells. LPS stimulation can induce TLR4 pathway activation, in turn activating the NF-κB, MAPKs, p38, ERK and PI3K/AKT

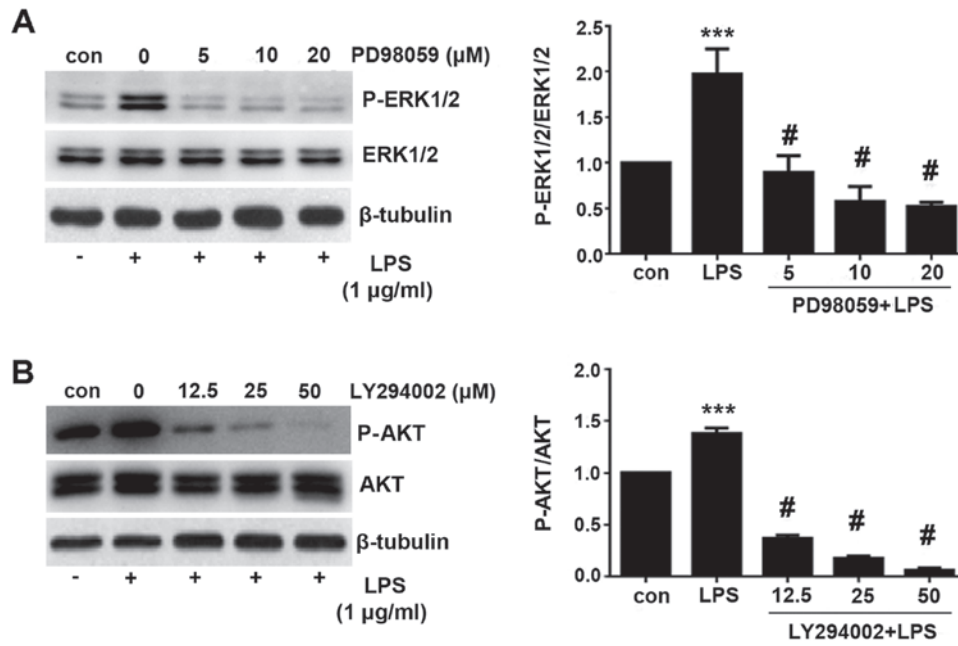


Figure 5. LPS-induced ERK and PI3K/AKT signaling activation can be blocked by their corresponding pharmacological inhibitors. A549 cells were first treated with 1 μg/ml LPS and the MEK inhibitor PD98059 or PI3K inhibitor LY294002 at the indicated concentrations prior to western blotting analysis. (A) Representative images of the blots showing ERK1/2 phosphorylation and corresponding semi-quantitative densitometric analysis showing relative p-ERK1/2 levels following respective treatments. (B) Representative images of the blots showing AKT phosphorylation and corresponding semi-quantitative densitometric analysis showing relative p-AKT levels following respective treatments. Data are presented as the mean ± SD. ****P*<0.001 vs. con and #*P*<0.05 vs. LPS. LPS, lipopolysaccharide; con, control.

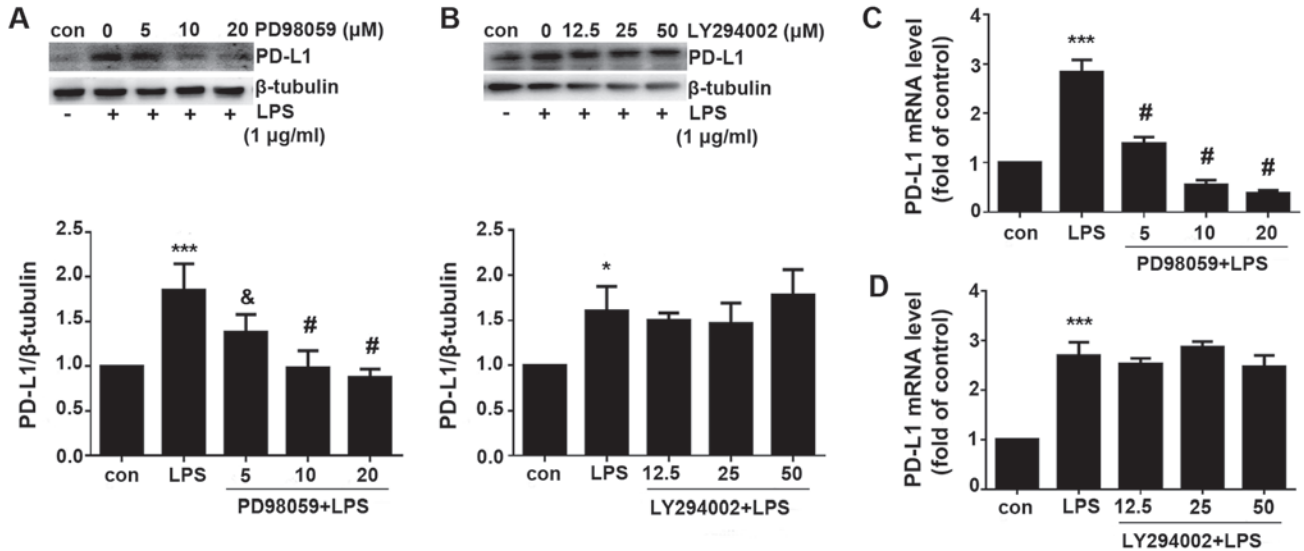


Figure 6. LPS-induced PD-L1 upregulation is inhibited by the MEK inhibitor but not PI3K inhibitor. A549 cells were treated with LPS and the MEK inhibitor PD98059 or the PI3K inhibitor LY294002 at their indicated concentration prior to western blotting. (A) Representative image of the blots of PD-L1 and corresponding semi-quantitative densitometric analysis following treatment with LPS and/or PD98059. (B) Representative image of the blots of PD-L1 and corresponding semi-quantitative densitometric analysis following treatment with LPS and/or LY294002. (C) Measurement of PD-L1 mRNA expression following treatment with LPS and/or PD98059. (D) Measurement of PD-L1 mRNA expression following treatment with LPS and/or LY294002. Data are presented as the means ± SD. **P*<0.05 and ****P*<0.001 vs. con. &*P*<0.05 and #*P*<0.001 vs. LPS. LPS, lipopolysaccharides; Con, control; PD-L1, programmed-death ligand 1.

signaling pathways (48,49). Data from the present study confirmed that the ERK and PI3K/AKT signaling pathways were activated by LPS treatment. By using the MEK inhibitor to inhibit ERK activity and PI3K inhibitor to inhibit AKT activity respectively, it was revealed that LPS-induced PD-L1 upregulation was dependent on the TLR4/ERK but not the TLR4/PI3K/AKT signaling pathway. These results are consis-

tent with those previously reported by Qian *et al* (38) and Wang *et al* (39) on bladder cancer tissues and cells.

A number of limitations remain associated with the present study. The cancer tissue sample size obtained for IHC is relatively small, whilst the *in vitro* part of the present study is restricted to A549 cell line. Although the underlying mechanism between TLR4 and PD-L1 was mainly focused on ERK and PI3K/AKT

signaling pathway in the present study, other signaling pathways downstream of TLR4 may also be involved in the process, such as the NF- κ B and interferon regulatory factor 5 (IRF5) pathway. The NF- κ B pathway activation has been demonstrated to contribute to PD-L1 upregulation in LPS-treated gastric cancer cells (50), but whether the same phenomenon exists in lung cancer cells remain poorly understood and require further investigations.

Despite its limitations, the present study contributed to the understanding of the functional relationship between TLR4 and PD-L1 in NSCLC. TLR4 and PD-L1 expression are found to be significantly associated with OS, whilst TLR4 can induce PD-L1 expression through the ERK signaling pathway following stimulation by LPS. Taken together, during conditions of chronic inflammation, TLR4 induced PD-L1 expression may contribute to NSCLC initiation.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GW contributed to the design of the study and editing of the manuscript. XK, PL, CZ processed the experiments. XK also responsible for manuscript writing. YZ was responsible for collecting and organizing the clinical data. HH was responsible for following up the patients and revising the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethical Committee of The First Affiliated Hospital of Nanchang University (Nanchang, China) approved the present study. All patients gave informed consents to participate.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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