Remifentanil modulates the TLR4-mediated MMP-9/TIMP1 balance and NF-κB/STAT3 signaling in LPS-induced A549 cells

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Abstract. Remifentanil is a widely used in general anesthetic that has been found to suppress the inflammatory response in aortic endothelial cells. Therefore, it was hypothesized that remifentanil can inhibit inflammatory dysfunction in lung epithelial cells to alleviate acute lung injury (ALI). The present study aimed to examine the effects of remifentanil on inflammatory injury, MMP-9/tissue inhibitor of metalloproteinase 1 (TIMP1) balance and the potential associated regulatory pathways in A549 cells. Lipopolysaccharide (LPS) was used to treat A549 cells to establish ALI models. The possible roles of different concentrations of remifentanil in cell viability was then determined by CCK-8 and Lactate dehydrogenase release assay. Apoptosis was assessed by flow cytometry analysis and western blotting. Inflammation and oxidative stress were measured by ELISA and corresponding kits respectively. Subsequently, the effects of remifentanil on Toll-like receptor 4 (TLR4) expression and the MMP-9/TIMP1 balance were assessed by western blotting and ELISA. In addition, the effects of remifentanil on NF-κB/STAT3 signaling were evaluated by measuring the protein expression levels of associated pathway components and the degree of NF-κB nuclear translocation using western blotting and immunofluorescence respectively. Remifentanil was found to increase cell viability whilst reducing apoptosis, inflammation and oxidative stress in the LPS-treated cells. In addition, TLR4 inhibitor CLI-095 suppressed MMP-9 expression and secretion while potentiating TIMP1 expression and secretion in LPS-challenged cells. Remifentanil treatment was able to modulate TLR4 to mediate LPS-induced MMP-9/TIMP1 imbalance and suppress the phosphorylation of NF-κB/STAT3 signaling components, in addition to inhibiting NF-κB nuclear translocation. Taken together, remifentanil downregulated TLR4 to reduce MMP-9/TIMP1 imbalance to inhibit inflammatory dysfunction in LPS-treated A549 cells, by regulating NF-κB/STAT3 signaling.

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Introduction

The pathology of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) is characterized by elevations in alveolar-capillary permeability caused by inflammation, trauma and burns (1). This in turn triggers atelectasis and excessive inflammatory responses, resulting in progressively worsening respiratory failure (2). In particular, sepsis is one of the main causes of ALI, which is frequently mediated indirectly by lipopolysaccharides (LPSs) released from the outer layer of the cell wall of gram-negative bacteria (3). LPS can activate inflammation by interacting with cell surface receptors including LBP, CD14, MD2 and TLR4 to promote structural damage and dysfunction in the physiological epithelial and endothelial barriers (4,5). This is facilitated following the activation of second messengers including cAMP and cGMP and related signaling pathways including NFκB signaling and the PI3K/Akt pathway (6). Although numerous studies have previously revealed that inflammatory factors including TNF-α and IL-6 can become activated during ALI (7-9), the use of anti-inflammatory drugs (such as sivelestat and simvastatin) in clinical practice was not able to effectively reduce the mortality rate which is estimated as 30-40% globally (10). To date, no effective prevention or treatment measures exist for septic ALI (11). Clinically, mechanical ventilation is the main supportive treatment strategy (12); however, the mechanical tension generated by the ventilation process itself can also cause lung injury (13). Therefore, novel treatment methods for ALI remain in demand.

Remifentanil is a synthetic piperidine derivative and a selective opioid receptor agonist (14). Remifentanil has high affinity for μ-opioid receptors but weaker affinity for δ and κ receptors (15). Its analgesic effect has been reported to be 250 times that of morphine (16). The pharmacokinetics of remifentanil are characterized by a rapid onset of action followed by rapid clearance (17). Since its clearance is mainly dependent on non-specific esterase degradation in the plasma or tissue, it is not affected by liver and kidney function, sex,

age and body weight; Remifentanil has a short half-life and high plasma clearance rate, and is the first choice for special populations (over 65 years old, liver and kidney insufficiency, children or obesity) (18). In addition, there were no alterations in its metabolic rate or accumulation in the body after long-term infusion or repeated injections (19). Owing to these aforementioned advantages, remifentanil is used extensively as a general anesthesia (20). Previous studies have reported that remifentanil pre-treatment can alleviate myocardial ischemia/reperfusion injury, the mechanism of which is associated with the inhibition of oxidative stress and apoptosis (21,22). Remifentanil can also inhibit the LPS-induced inflammatory response in human aortic endothelial cells through the poly (ADP-ribose) polymerase 1 (PARP-1)/NF-κB signaling pathway (23). Furthermore, remifentanil has been found to inhibit the expression and release of high mobility group box protein 1 in the liver, lungs, and kidney tissues of septic rats (24). TLR4 is a member of TLRs family, regulating inflammatory response (25). Increased expression of MMP9 and increased ratio of MMP-9/tissue inhibitors of metalloproteinase 1 (TIMP1) expression are considered to be primary indicators of chronic airway injury and emphysema (26). Notably, TLR4 has been reported to regulate hippocampal MMP/TIMP imbalance in perioperative neurocognitive disorder in diabetes (27). Therefore, remifentanil treatment may exert regulatory effects on TLR4-mediated MMP-9/TIMP1 balance and inhibit inflammatory injury in lung epithelial cells.

A549 lung adenocarcinoma cells have been extensively used as a cell model for type II pulmonary epithelial cells (28,29). Therefore, in the present study, A459 cells were selected and LPS was used as the treatment to establish inflammatory damage models. The aim of the present study was to investigate the effects of remifentanil on inflammatory injury in the cells, the MMP-9/TIMP1 balance and possible regulatory pathways.

Material and methods

Cell culture. A549 lung adenocarcinoma cells (Procell Life Science & Technology Co., Ltd.) were cultured in the DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a humidified chamber at 37˚C with 5% CO_2. Remifentanil was obtained from Jiangsu Nhwa Pharmaceutical Co., Ltd. (Xuzhou, China), with the concentrations (0.625, 1.25 and 2.5 μM) set at 37˚C according to previous studies (23,30). LPS (10 μg/ml; Beyotime Institute of Biotechnology) was used to treat the cells at 37˚C for 24 h (31). The TLR4 inhibitor CLI-095 (also known as resatorvid; 3 μM; Selleck Chemicals) was added at 37˚C for 6 h to suppress TLR4 signaling (32). Cells in the treatment group were treated with remifentanil for 30 min prior to LPS stimulation. Untreated cells were regarded to be the control group.

Cell Counting Kit-8 (CCK-8) assay. A549 cells were seeded into 96-well plates at a density of $5x10^3$ cells/well (100 μl) and treated with remifentanil or LPS, as aforementioned. Following 24 h of incubation, 10 μl CCK-8 reagent (AmyJet Scientific, Inc.) was added to each well before the cells were incubated at 37˚C for a 1 h. The optical density in each well was measured using a microplate reader (450 nm; Nanjing Detie Laboratory Equipment Co., Ltd.).

Lactate dehydrogenase (LDH) release assay. A549 cells ($5x10^3$ cells/well; 150 μl) were treated with remifentanil and LPS at 37˚C, as aforementioned. At 23 h incubation, the LDH release reagent (15 μl) provided by the LDH Cytotoxicity Assay Kit (cat. no. C0016; Beyotime Institute of Biotechnology) was added to the wells, and the cells were incubated for 1 h (24 h total incubation). The cells were then centrifuged at 400 x g for 5 min at room temperature before 120 μl supernatant was taken from each well for measurement. LDH release was measured by plotting a standard curve after OD values were obtained using a microplate reader (490 nm).

Flow cytometry. Apoptosis was analyzed by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (cat. no. C1062; Beyotime Institute of Biotechnology). Briefly, treated or untreated A549 cells ($1x10^5$), aforementioned, were washed twice with pre-cooled PBS and suspended in 195 μl binding buffer. The cell suspension ($5x10^5$ cells/ml) was then transferred into a tube and incubated with Annexin V-FITC (5 μl) and propidium iodide (10 μl) at room temperature in the dark for 15 min. Results were obtained using a BD FACSCanto II flow cytometer (BD Biosciences) and FlowJo version 10 software (FlowJo LLC).

Western blotting. Protein extracts of A549 cells were obtained using the RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), cytoplasmic and nuclear protein were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol and protein concentrations were determined using the BCA assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein samples (30 μg/lane) were subjected to SDS-PAGE (10%) and transferred onto PVDF membranes (MilliporeSigma). Following blocking in 5% non-fat milk for 1 h at room temperature, the membranes were incubated with the indicated primary antibodies at 4˚C overnight and then with the respective HRP-conjugated secondary antibodies at room temperature for 2 h; all antibodies used in the present study are listed in Table I. Protein bands were visualized using an DBI TOP-ECL chemiluminescence reagent (Shanghai Xinghan Biotechnology Co., Ltd.) and quantified using ImageJ software (version 1.52v; National Institutes of Health).

ELISA. ELISA kits were used to measure the levels of inflammatory factors, including IL-6 (cat. no. ZN2272), IL-1β (cat. no. ZN2236) and TNF-α (cat. no. ZN2460; Beijing Biolab Technology Co., Ltd.), as well as the secretion levels MMP9 (cat. no. BMS2016-2; Invitrogen; Thermo Fisher Scientific, Inc.) and TIMP1 (cat. no. PT888; Beyotime Institute of Biotechnology). The operating steps were performed according to the respective protocols. Briefly, cell culture media (500 μl) from A549 cells treated with remifentanil and LPS following 18 h of incubation at room temperature was centrifuged at 2,000 x g for 10 min at room temperature to remove debris before the supernatant was collected for assaying. The samples (100 μl) were then incubated at room temperature with (in order): Biotin-labeled antibodies (100 μl)

Table I. Antibodies used for western blot analysis.

Antibody	Catalog number	Host	Dilution ratio	Company
Bcl-2	AB112	Rabbit	1:1,000	Beyotime Institute of Biotechnology
Bax	AF1270	Rabbit	1:2,000	Beyotime Institute of Biotechnology
Cleaved-PARP	ab32064	Rabbit	1:5,000	Abcam
PARP	orb88930	Mouse	1:1,000	Biorbyt, Ltd.
Cleaved-caspase3	ab32042	Rabbit	1:500	Abcam
Caspase3	GTX110543	Rabbit	1:5,000	GeneTex, Inc.
IL-6	ab233706	Rabbit	1:1,000	Abcam
IL-1β	ab283818	Rabbit	1:1,000	Abcam
TNF-α	ab183218	Rabbit	1:1,000	Abcam
Toll-like receptor 4	ab13556	Rabbit	1:500	Abcam
MMP9	ab283575	Rabbit	1:1,000	Abcam
Tissue inhibitor of metalloproteinase 1	MA1-773	Mouse	1:500	Invitrogen; Thermo Fisher Scientific, Inc.
p-NF-κB p65	MA5-15160	Rabbit	1:1,000	Invitrogen; Thermo Fisher Scientific, Inc.
NF-κB p65	14-6731-81	Rabbit	1:1,000	Invitrogen; Thermo Fisher Scientific, Inc.
p-STAT3	44-384G	Rabbit	1:1,000	Invitrogen; Thermo Fisher Scientific, Inc.
STAT3	MA1-13042	Mouse	1:5,000	Invitrogen; Thermo Fisher Scientific, Inc.
H3	ab1791	Rabbit	1:1,000	Abcam
β-actin	orb181785	Rabbit	1:500	Biorbyt, Ltd.
HRP-conjugated anti-rabbit IgG	A0208	Goat	1:1,000	Beyotime Institute of Biotechnology
HRP-conjugated anti-mouse IgG	A0216	Goat	1:1,000	Beyotime Institute of Biotechnology

P-, phosphorylated; PARP, poly (ADP-ribose) polymerase.

for 1 h, avidin-peroxidase complex (100 μl) for 25 min, TMB chromogenic solution (100 μl) for 20 min and stop solution (50 μl). Protein levels were measured by plotting a standard curve after the OD values were obtained using a microplate reader (450 nm).

Oxidative stress assessment. Superoxide dismutase (SOD; cat. no. A001-1), glutathione peroxidase (GSH; cat. no. A005-1) and malondialdehyde (MDA; cat. no. A003-4-1; Nanjing Jiancheng Bioengineering Institute) assay kits were used to assess their levels. Briefly, following indicated treatment, supernatant of A549 cells ($1x10^6$) were collected using centrifugation at 1,000 x g for 5 min at 4˚C and 10-50 μl of supernatant was used to analyze the levels of oxidative stress according to the manufacturer's protocol of corresponding kits. The OD values at 450 nm were measured using a microplate reader.

Immunofluorescence (IF). IF was performed to verify the localization of NF-κB p65. Briefly, treated or untreated A549 cells ($5x10^4$ cells/well) plated in a 24-well plate for 24 h were fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized with 0.5% Triton X-100 at room temperature for 15 min. The cells were then blocked with 10% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature, incubated with the NF-κB p65 primary antibody (1:100) overnight at 4˚C and FITC-conjugated goat anti-rabbit secondary antibodies (1:1,000; cat. no. orb688925; Biorbyt, Ltd.) for 1 h at room temperature in the dark. The nuclei were stained with DAPI (5 μg/ml) for 5 min at room temperature, and the immunostaining was examined under a fluorescence microscope (Leica Microsystems GmbH) at x200 magnification.

Statistical analysis. All experiments were performed at least three times, and normally distributed data were expressed as the mean ± standard deviation. The data were analyzed by one-way ANOVA followed by Tukey's post hoc test using the SPSS version 13 software (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Remifentanil improves the viability in LPS-treated cells. The chemical structure of remifentanil is displayed in Fig. 1A. The effects of different concentrations of remifentanil on A549 cell viability was determined using CCK-8 assay (Fig. 1B); the highest remifentanil concentration tested (2.5 μM) did not affect cell viability, suggesting it is a safe cell treatment concentration. The viability of cells treated with LPS was decreased significantly compared with untreated cells (Fig. 1C), whereas the viability of cells co-treated with remifentanil at a concentration of 2.5 μM was markedly increased compared with

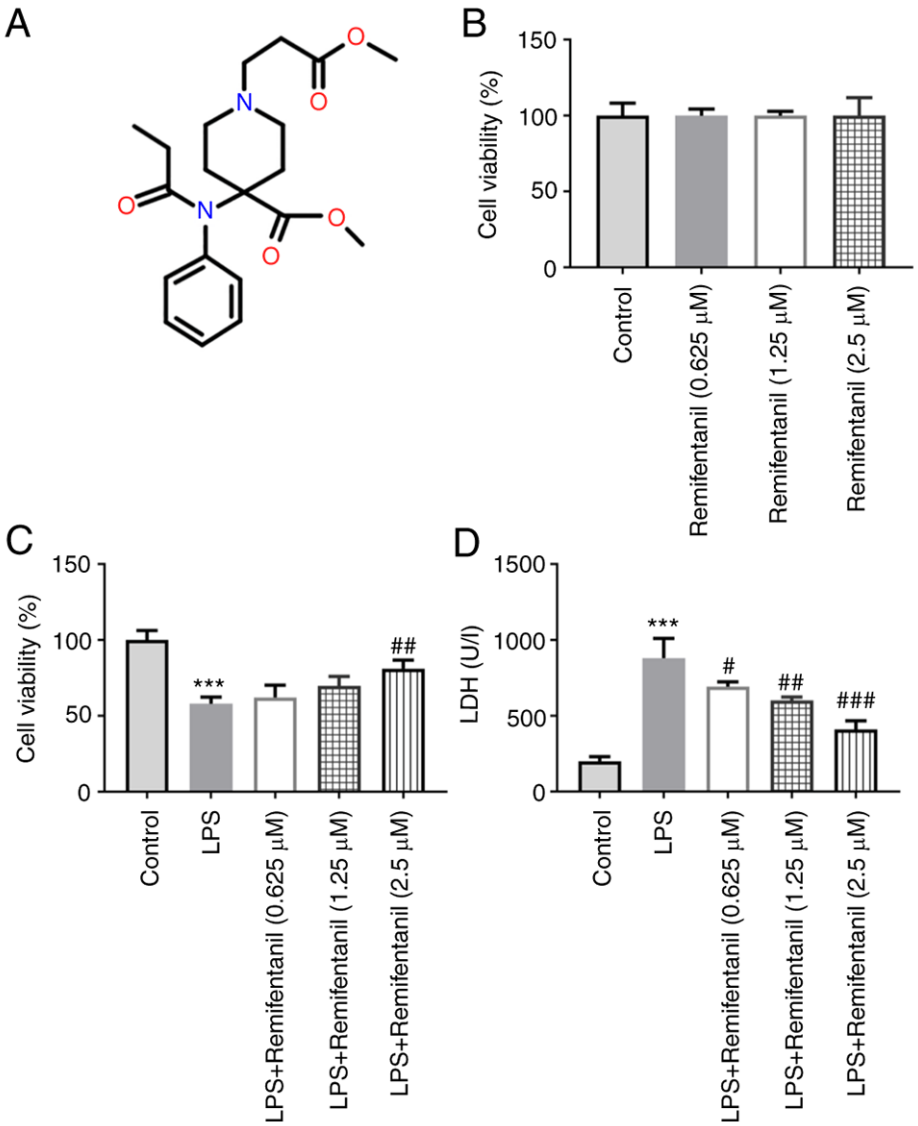

Figure 1. Remifentanil improves viability in LPS-treated cells. (A) Chemical structure of remifentanil. (B) The effect of different concentrations of remifentanil on A549 cell viability was determined using a CCK-8 assay. (C) The effect of LPS and LPS + remifentanil co-treatment on cell viability was determined using a CCK-8 assay. (D) LDH assay kit was used to determine the release level of LDH in each group of cells. ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS. CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase; LPS, lipopolysaccharide.

that in the LPS-only group. In addition, the level of LDH release in each treatment group of cells was measured. The results revealed that the degree of LDH release by cells in the LPS-only group was significantly higher compared with that in the control group (Fig. 1D). The LDH levels in cells co-treated with remifentanil were significantly reduced compared with that in the LPS group.

Remifentanil suppresses LPS-induced apoptosis. To evaluate the effect of remifentanil on apoptosis, the apoptotic rates of cells in each treatment group were detected by flow cytometry. The proportion of early-(Q3) and late-stage (Q2) apoptotic cells in the LPS group was significantly increased compared with that in the control group (Fig. 2A and B). Compared with that in the LPS-only group, the proportion of apoptotic cells in the remifentanil co-treated groups was all significantly decreased. In addition, the expression levels of apoptosis-related proteins were measured using western blotting (Fig. 2C). Compared with the control group, the protein expression levels of Bax, cleaved PARP and cleaved caspase 3 in the LPS group were significantly increased, whereas those of the Bcl-2 protein were significantly decreased. By contrast, remifentanil co-treatment reversed the alterations in the protein expression levels, with the differences reaching significance at higher concentrations tested. These data suggested that remifentanil may alleviate LPS-induced apoptosis.

Remifentanil reduces inflammation and oxidative stress caused by LPS. Inflammation-related cytokine levels were determined using western blotting and ELISA (Fig. 3A and B, respectively). The levels of IL-6, IL-1β and TNF-α expression and secretion were significantly elevated in the LPS-only group compared with the untreated control group, and these were reversed by remifentanil co-treatment in a concentration-dependent manner.

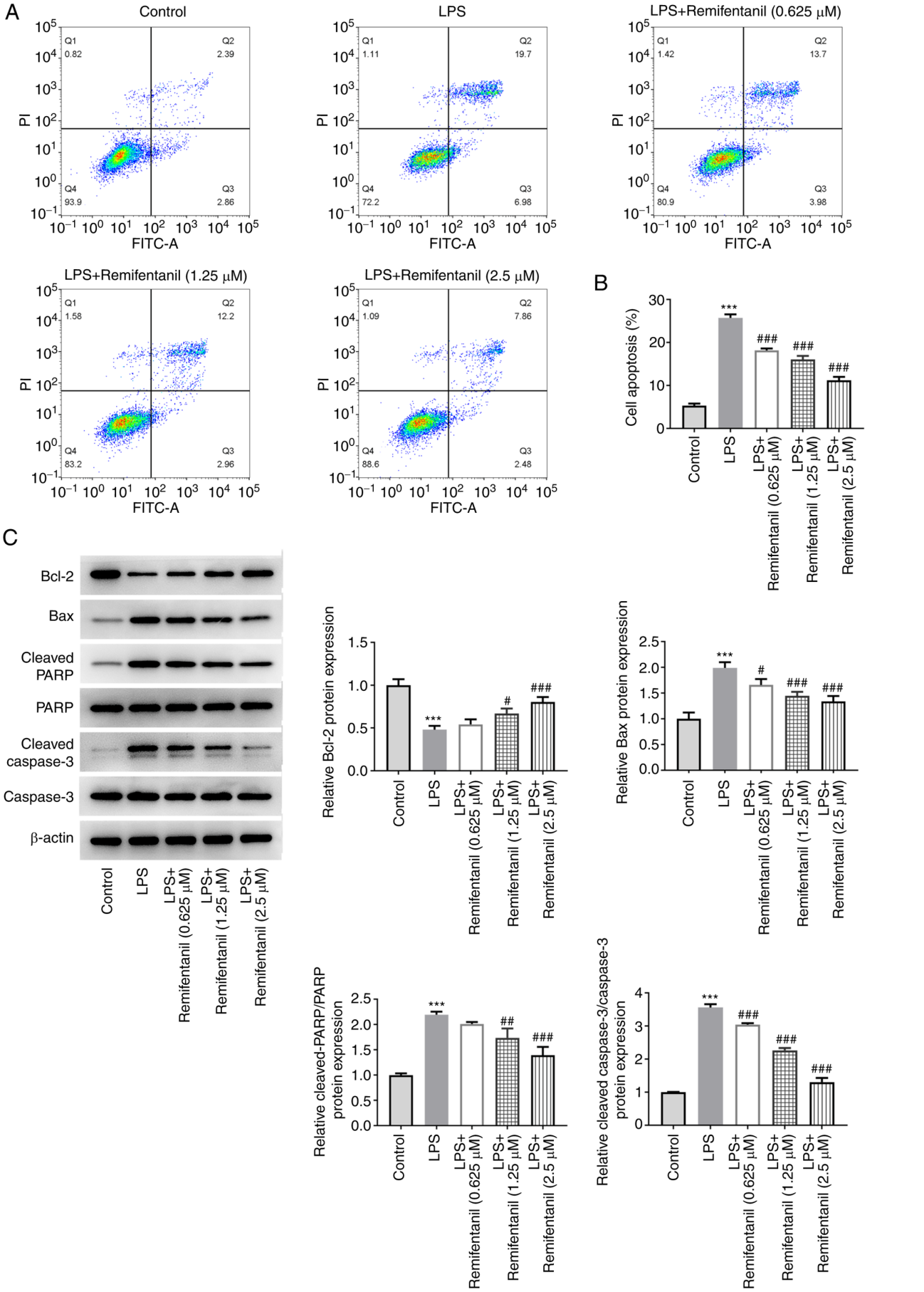

Figure 2. Remifentanil suppresses apoptosis in LPS-treated cells. (A) Apoptotic rates of cells in each group were detected by flow cytometry and (B) quantified. (C) The expression levels of apoptosis marker proteins were determined with western blotting. ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS. LPS, lipopolysaccharide; PARP, poly (ADP-ribose) polymerase.

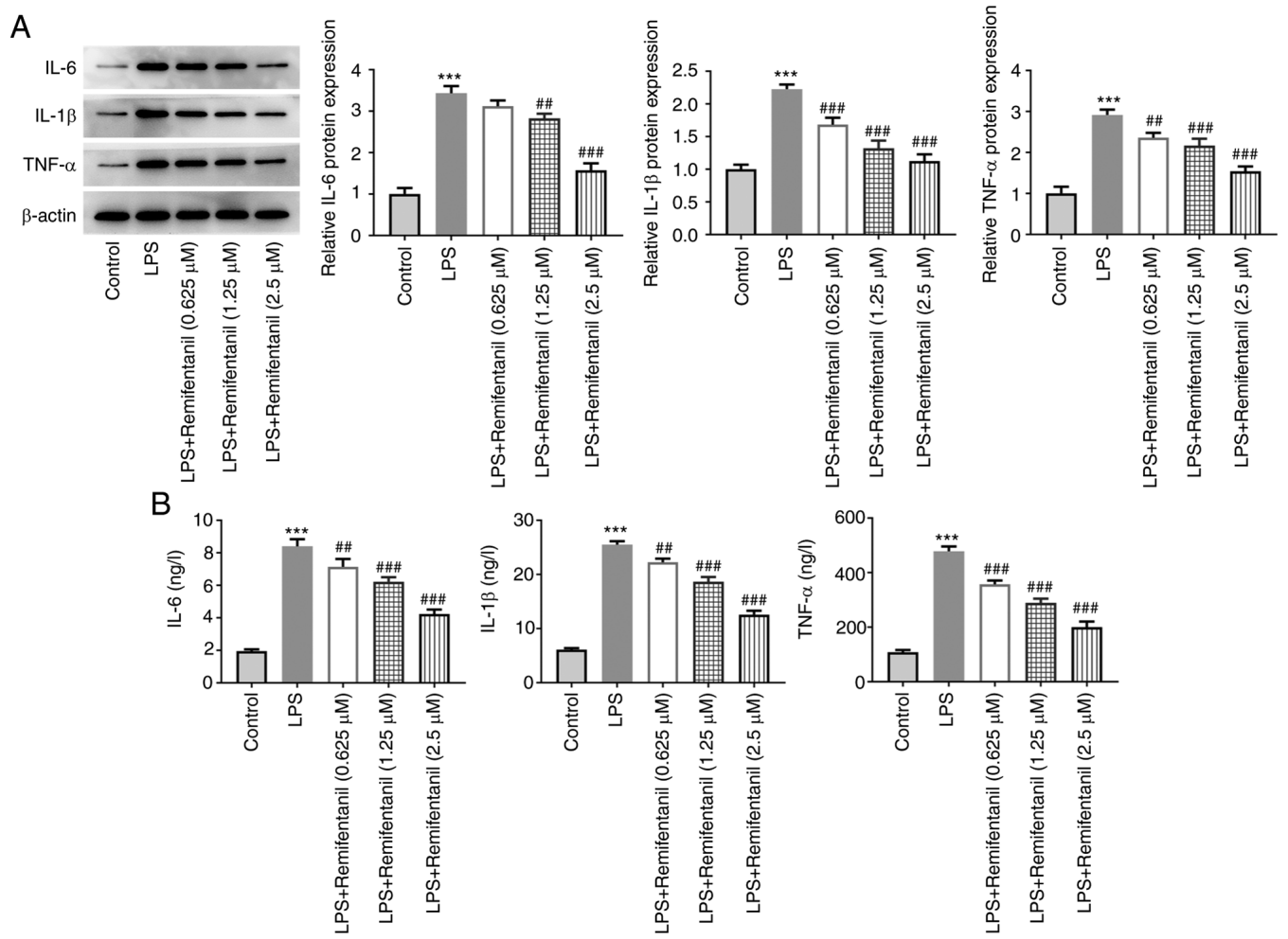

Figure 3. Remifentanil reduces inflammation-related cytokine levels in LPS-treated cells. The expression levels of inflammatory factors were determined using (A) western blotting and (B) ELISA. ***P<0.001 vs. control; ##P<0.01 and ###P<0.001 vs. LPS. LPS, liposaccharide.

The levels of oxidative stress markers were also measured using their corresponding assay kits. The levels of SOD and GSH-Px were significantly reduced in the LPS group compared with the control group whereas those of MDA were increased (Fig. 4). Co-treatment with remifentanil significantly reversed the aforementioned LPS-induced effects (Fig. 4). These observations suggested that remifentanil treatment may reduce oxidative stress in the cells caused by LPS.

Remifentanil modulates TLR4 to mediate MMP-9/TIMP1 imbalance in LPS-treated cells. The expression levels of TLR4, MMP-9 and TIMP1 were evaluated using western blotting (Fig. 5A and B). The expression levels of TLR4 and MMP-9 were significantly elevated in the LPS-treated group, whilst those of TIMP1 were decreased. MMP-9/TIMP1 ratio significantly elevated upon LPS treatment. By contrast, remifentanil co-treatment significantly reversed the LPS-induced increase in TLR4 and MMP-9 expressions and the decreased TIMP1 expression. To further assess the role of TLR4 in the MMP-9/TIMP1 expression balance, the cells were treated with the TLR4 inhibitor, CLI-095. Compared with those in the LPS group, CLI-095 co-treatment significantly reduced the expression of TLR4. Moreover, relative to LPS group, additional CLI-095 treatment weakened MMP-9 expression and content, whilst increasing those of TIMP1, resulting in a decrease in MMP-9/TIMP1 ratio. The effect of CLI-095 on the secretion of MMP-9 and TIMP1 was similar to that induced by LPS + 2.5 μM remifentanil group (Fig. 5C and D). These data suggested that remifentanil may restore MMP-9/TIMP1 imbalance to the normal level in LPS-treated cells via mediatingTLR4 signaling.

Remifentanil regulates the NF-κB/STAT3 signaling pathway. The expression levels of proteins in the NF-κB/STAT3 signaling pathway were assessed using western blotting. The phosphorylation levels of NF-κB and STAT3 proteins were found to be significantly elevated in the LPS group compared with the control, and these were significantly reversed by remifentanil co-treatment (Fig. 6A). To verify whether NF-κB signaling was activated or blocked, nuclear translocation of NF-κB p65 was examined using IF assay (Fig. 6B). The fluorescence of NF-κB p65 in the nucleus appeared to be enhanced in the LPS-treated group compared with the control group, and remifentanil co-treatment appeared to be lower compared with the LPS-only group, suggesting that remifentanil suppressed the nuclear translocation of NF-κB p65. However, the difference in the IF images was not obvious, so western blotting was performed. According to the western blotting data, the protein

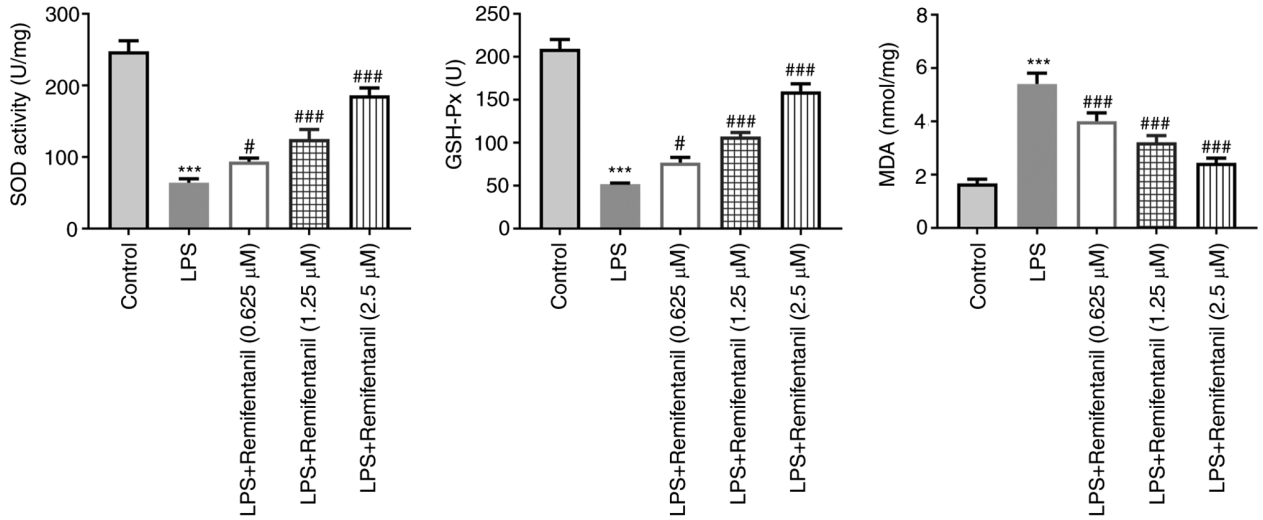

Figure 4. Remifentanil reduces oxidative stress in LPS-treated cells. The levels of oxidative stress markers SOD, GSH-Px and MDA were assessed using their corresponding assay kits. ***P<0.001 vs. control. #P<0.05 and ###P<0.001 vs. LPS. GSH-Px, glutathione peroxidase; LPS, lipopolysaccharide; MDA, malondialdehyde; SOD, superoxide dismutase.

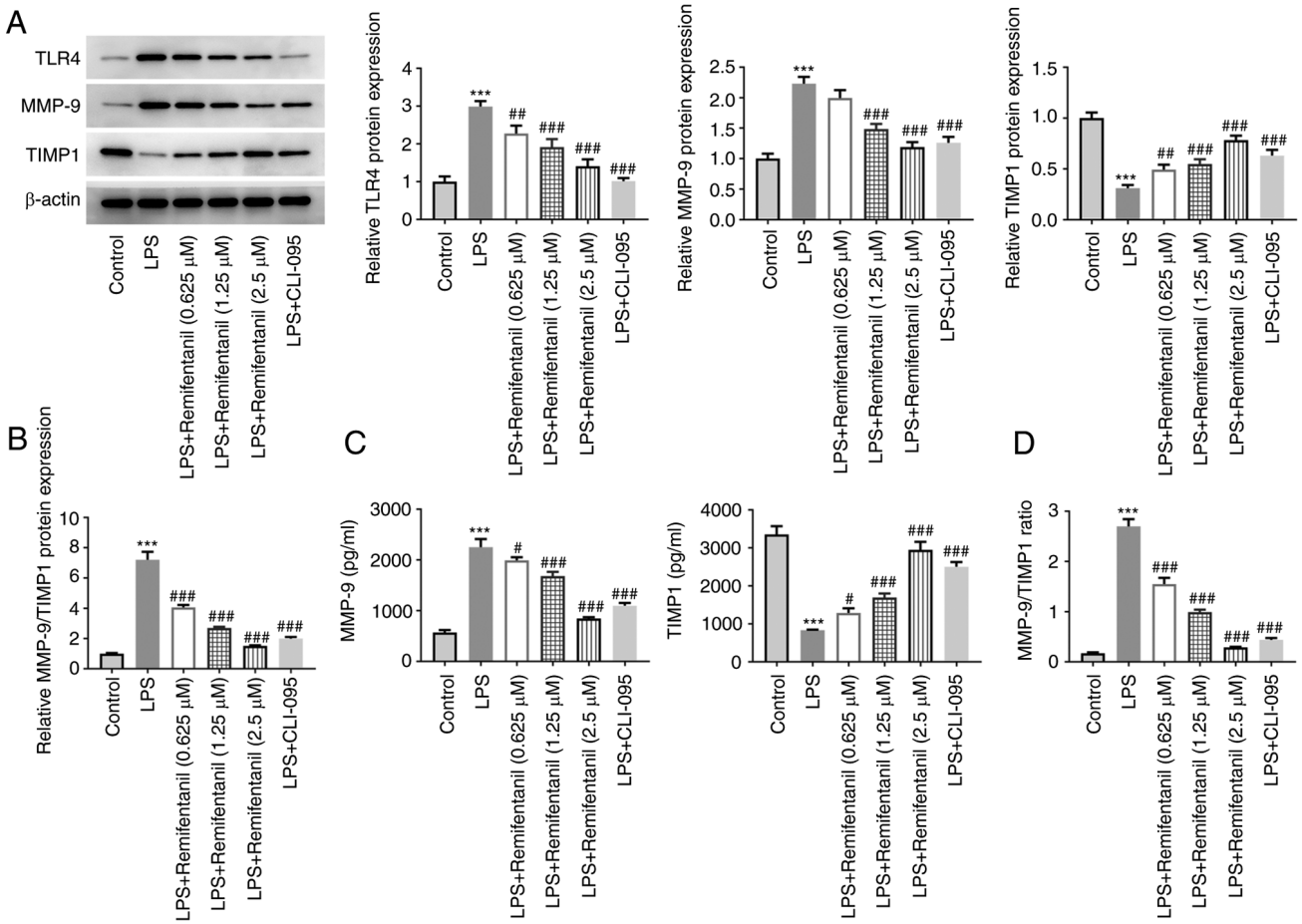

Figure 5. Remifentanil restores TLR4-mediated MMP-9/TIMP1 balance in LPS-treated cells. (A) The expression levels of TLR4, MMP-9 and TIMP1 were measured using western blotting. (B) The ratio of the expression levels of MMP-9 and TIMP1. (C) TIMP1 and MMP-9 secretion levels in the cell supernatant were determined using ELISA. (D) The ratio of the secretion levels of MMP-9 and TIMP1. ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. LPS. LPS, lipopolysaccharide; TIMP1, tissue inhibitor of metalloproteinase 1; TLR4, Toll-like receptor 4.

expression level of NF-κB p65 in the nucleus was significantly increased following LPS stimulation, which was accompanied by its reduction in the cytoplasm (Fig. 6C). After remifentanil co-treatment, the protein expression level of NF-κB p65 decreased in the nucleus and increased in the cytoplasm compared with that in the LPS-only group (Fig. 6C).

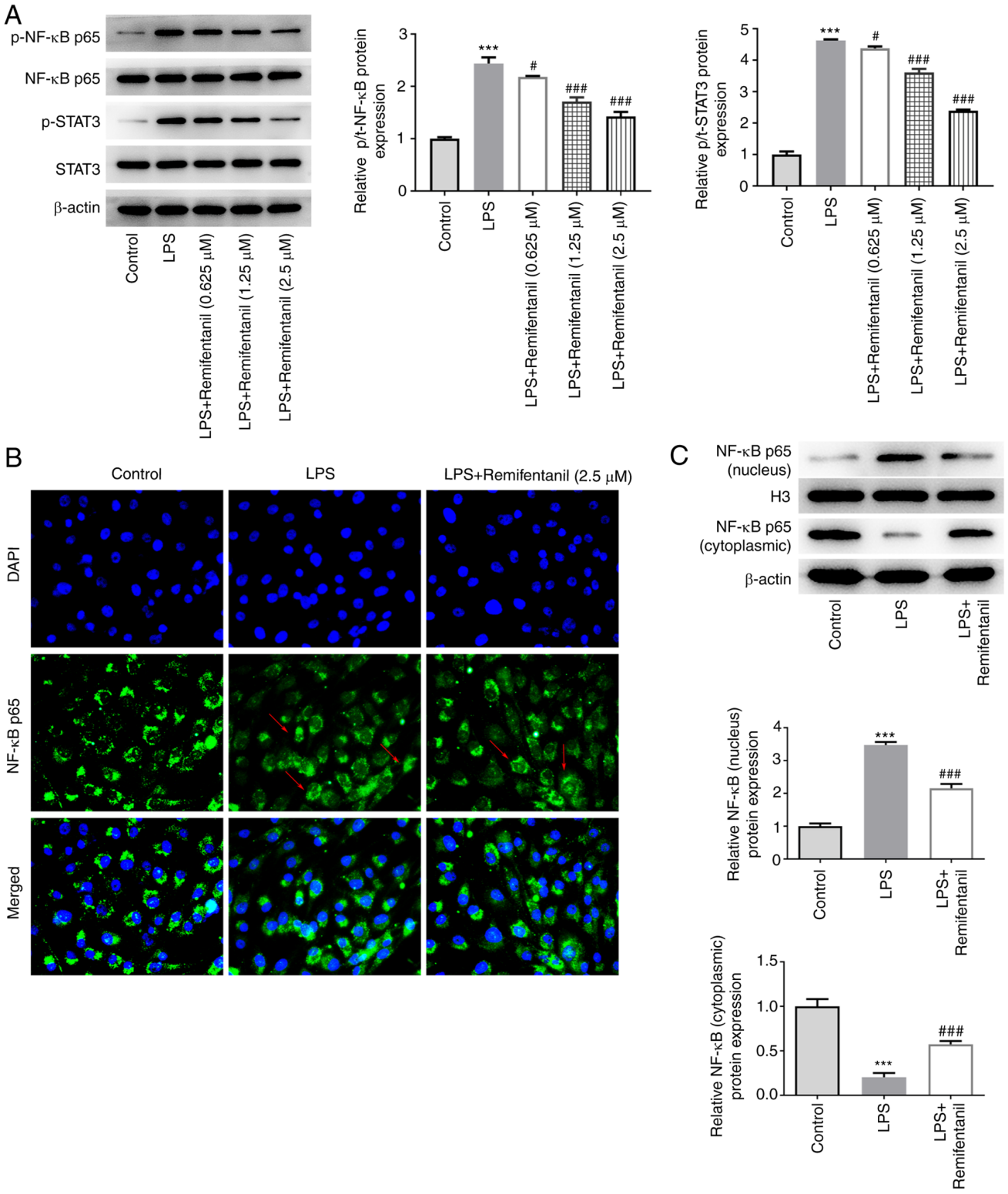

Figure 6. Remifentanil regulates the NF-κB/STAT3 signaling pathway. (A) The expression levels of proteins in the NF-κB/STAT3 signaling pathway were examined using western blotting. (B) Nuclear translocation of NF-κB p65 was examined using immunofluorescence and (C) western blotting. ***P<0.001 vs. Control. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. LPS. LPS, lipopolysaccharide; p-, phosphorylated.

Discussion

Previous studies reported that aberrant coagulation, fibrinolysis and extravascular fibrosis form the key features of ALI (33-35). Among them, coagulation and fibrinolysis are closely associated with the inflammatory response and together promote ALI progression (36). When ALI occurs, endogenous endotoxemia is induced through a multitude of mechanisms, such as stress and the immune response, which stimulates the release of inflammatory mediators IL-1β and IL-6 to enhance the inflammatory response and aggravate lung dysfunction (37,38). By contrast, fibrous deposits activate endothelial cells to also produce

proinflammatory cytokines including TNF-α, IL-1 and IL-6 to induce activated neutrophil aggregation (39). Persistent protein deposition can also cause vascular wall thickening, pulmonary artery thrombosis and increased risk of mortality (34). These previous findings suggest that the inflammatory response is an important cause of ALI, where the imbalance between the systemic inflammatory response and compensatory anti-inflammatory response serves a key role in its pathogenesis, during which LDH will be released into the bloodstream (40). Therefore, from the perspective of inflammation, seeking a novel therapeutic strategy for ALI prevention and treatment would be of significance for reducing the risk of morbidity and mortality. The results of the present study suggest that remifentanil may reduce apoptosis whilst alleviating inflammation and oxidative stress in LPS-treated A549 cells. Therefore, remifentanil may serve an anti-inflammatory role in ALI.

In the present study, it was found that remifentanil reduced the expression level of TLR4 and ratio of MMP-9/TIMP1 in LPS-treated cells. TLRs belong to a class of pattern recognition receptors that can mediate the inflammatory response to pathogens by inducing proinflammatory effects (25). It has been previously revealed that TLR4 is involved in the regulation of immune defense responses and inflammatory mediators in the respiratory tract during ALI progression in an animal model (4). In addition, another previous study demonstrated that remifentanil can ameliorate hepatic ischemia-reperfusion injury by upregulating the expression of β-arrestin 2, a well-known μ-opioid receptor desensitizer (41). Since β-arrestin 2 is also a negative regulator of the TLR4-mediated inflammatory response, β-arrestin 2 may serve as a key molecule connecting the μ-opioid receptor and TLR4 pathways (41). These findings imply that remifentanil might elevate β-arrestin 2 expression to suppress TLR4-mediated inflammatory response, which was consistent with the results in the present study. Reactive oxidants and free radicals can directly obstruct cell function, causing proteases, such as MMP-9, to be released. MMP-9 serves a role in various physiological processes, including extracellular matrix degradation and lung tissue remodeling (42-44), both of which have been reported to be associated with the occurrence of various lung diseases including chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis (42-44). MMP9 activity is regulated by the specific endogenous inhibitor TIMP1 (45). Under the action of external stimuli, the proportion of their expression and the interaction between the two can be regulated, the dysregulation of which can result in lung pathology (45). Moreover, increased expression of MMP9 and increased ratio of MMP-9/TIMP1 expression are considered to be primary indicators of chronic airway injury and emphysema (26). Notably, TLR4 has been reported to regulate hippocampal MMP/TIMP imbalance in perioperative neurocognitive disorder in diabetes (27). Therefore, identifying the upstream pathway(s) that may alleviate the imbalance and, in turn, designing putative intervention methods is currently garnering research interest. In the present study, remifentanil was found to restore their imbalance to the normal level, indicating it has the potential to treat ALI.

As a proinflammatory factor, LPS induces the inflammatory response, mainly by activating the NF-κB pathway (6). Previous studies have shown that remifentanil can alleviate endothelial cell inflammation (23) and myocardial injury (46) through inhibiting NF-κB signaling, where activating TLR4 can lead to the activation of NF-κB signaling (47). NF-κB is one of the most important nuclear transcription factors in cells and serves a central role in the transcriptional regulation of cellular messages mediated by stimuli including inflammation and infection (48). NF-κB activation can promote the expression and release of ILs and TNF-α, which can be applied for diagnosis and severity assessment of ALI (49). Furthermore, activation of the STAT3 signaling pathway can also promote the production of inflammatory cytokines and chemokines to aggravate the process of ALI (50). Following the action of Janus kinase, STAT3 in the cytoplasm dimerizes due to following Y705 and S727 phosphorylation in its protein structure, where STAT3 can also be activated by reversible acetylation (51). Activated STAT3 will then translocate into the nucleus and bind to genomic DNA to regulate transcription (52). Previous studies have revealed that in innate immunity, adaptive immunity, acute and chronic inflammation, and tumor occurrence, STAT3 activity is aberrantly activated with high frequencies (43,53-55). STAT3 cooperates with NF-κB to drive inflammatory response (56). Therefore, the apparent suppressive effects of remifentanil on NF-κB/STAT3 signaling may be one of the mechanism by which it alleviates ALI. However, the present study is limited to *in vivo* experiments using A549 cells. Other cell types, such as primary cells, in addition to *in vivo* studies, are required.

In conclusion, the present study found that remifentanil restored MMP-9/TIMP1 imbalance to the normal level via mediating TLR4 and inhibited inflammatory injury in LPS-treated cells by regulating NF-κB/STAT3 signaling. It is hoped that these findings may facilitate the development of novel ALI treatment methods.

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Availability of data and materials

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

Authors' contributions

JC and WZ contributed to the experimental design, conducting experiments and data analysis. JC contributed to the writing of the manuscript. JC and WZ approved the final version of the manuscript and confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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