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Ligustilide inhibits the activation of cancer-associated fibroblasts

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ABSTRACT

The purpose of this work was to study the effects and underlying molecular mechanisms of ligustilide on cancer-associated fibroblasts (CAFs). The effects of ligustilide on the growth of CAFs and splenocytes were detected by MTT assay, and flow cytometry was used to detect effects on T-cell proliferation. Western blotting was used to detect the expression levels of CAF-related proteins after ligustilide treatment. This study found that ligustilide had no effect on the growth of splenocytes but that it could change the immunosuppressive function of CAFs through the TLR4-NF-κB pathway and restore T-cell proliferation previously inhibited by the CAF supernatant. Thus, ligustilide is expected to be a candidate for new antitumor drugs.

1. Introduction

Due to increasing morbidity and mortality, cancer is becoming the leading cause of death in China and an important public health issue [1–3]. However, surgery, radiotherapy and chemotherapy as conventional cancer treatments have obvious side effects and high rates of disease recurrence [4–6]. Therefore, the current treatment for malignant tumors has shifted from traditional surgical treatment to multidisciplinary comprehensive treatments in which tumor immunotherapy plays a critical role in clinical practice [7,8].

Cancer-associated fibroblasts (CAFs) are the major stromal cell types in the tumor microenvironment. Many studies have shown that CAFs play a significant functional role in cancer progression and metastasis by producing a favorable inflammatory microenvironment, including CXCL1, CXCL2, IL-1β and IL-6. CXCL1 and CXCL2 are the chemoattractants for neutrophils and macrophages, and IL-1β and IL-6 are proinflammatory cytokines [9–13]. Compared with normal fibroblasts in healthy tissues, activated fibroblasts in the tumor microenvironment have a significant change in phenotype and function [14]. Increasing levels of transforming growth factor in the tumor microenvironment can induce normal fibroblasts to obtain a CAF phenotype and express CAF-specific marker proteins such as α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) [15–18]. In addition, the chemokines TGF-β and IL-10 secreted by CAFs inhibit the recruitment of T cells and attract inhibitory T cells into the tumor while also causing suppression of any effector T-cell activity. This process interferes with the interaction between T cells and tumor cells, ultimately preventing tumor destruction [19,20]. CAFs can promote tumor to grow by secreting osteopontin. However, manipulation of the immunosuppressive functions of cancer-associated fibroblasts can inhibit the growth of tumors, allowing CAF-targeted immunotherapy to be achieved [21].

Ligustilide (3-butenyl-4,5-dihydro-1(3H)-isobenzofuranone) is one of the main components of traditional Chinese medicines such as *Angelica* and Chuanxiong. Ligustilide has a strong pharmacological effect on the cerebrovascular circulatory system and immune system, acting on TLR4 and TLR2 receptors to regulate immune function through the NF-κB pathway [22–24]. It was found that ligustilide has antitumor effects and has obvious positive outcomes on the treatment of breast cancer, glioblastoma, melanoma and prostate cancer [25–27]. Ligustilide may sensitize the breast cancer cells, which is resistant to tamoxifen, by inhibiting the autophage and promoting the caspase-independent cell death. However, whether ligustilide affects the tumor microenvironment, especially CAFs and their mechanisms, has not been reported.

In this study, we investigated the effect of ligustilide on CAFs. We

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found that ligustilide has no direct effect on CAF and splenocyte growth but can restore T-cell proliferation previously inhibited by CAF supernatant, indicating that ligustilide inhibits the immunosuppressive function of CAFs. This study revealed an important mechanism of ligustilide in the treatment of tumor immunotherapy, potentially providing new strategies and means for antitumor treatments by stimulating the conversion of CAFs to normal fibroblasts.

2. Materials and methods

2.1. Materials

Ligustilide has a molecular formula of $C_{12}H_{14}O_2$ and molecular weight of 190.24. The compound was obtained from Sichuan Weikeqi Biotechnology Co., Ltd. at a purity of 98.64%. Carboxyfluoresceinsuccinimidyl ester (CFSE) was purchased from Sigma-Aldrich (Darmstadt, Germany). The TLR4 inhibitor (CIL-095) and RIPA lysis buffer were purchased from Beyotime Biotechnology (Suzhou, China). DMEM and RPMI-1640 medium were purchased from HyClone (Logan, Utah, USA). Fetal bovine serum (FBS) was purchased from PAN (Aidenbach, Germany). Penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). The primary antibodies phospho(P)-p65, p-IKb, IKb, p-TAK and TAK, TRAF6, MyD88, α-SMA were purchased from Cell Signaling Technology (Massachusetts, USA). GAPDH was purchased from Sanjian (Tianjin, China). Secondary anti-mouse and anti-rabbit antibodies were obtained from ZSGBbio (Beijing, China). Antibodies including APC-anti-CD4, PE-anti-CD8a were purchased from Biolegend (Cambridge, UK). All other chemicals and reagents used in this study were of analytical grade.

2.1.1. Preparation of CAF supernatant

Prostate CAF cells were seeded in 24-well plates in 1 ml of DMEM medium per well at a density of 5×10^4 cells/well. The cells were then incubated with differing concentrations of ligustilide (0, 15, 30 and 45 μM). After 24 h of incubation, the culture supernatant was collected and filtered through a 0.22 μm filter.

2.2. Cell culture

CAFs were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified incubator at 5% CO_2.

2.3. MTT assay

The effect of ligustilide on the growth of immune cells can be determined by an MTT cell proliferation assay. Splenocytes were obtained from wild-type mice and cultured in 96-well U-bottom plates at a cell density of 3×10^5 cells/well, using Concanavalin A (ConA) as the positive control and DMEM + / + medium without ligustilide as the negative control. For the experimental group, ligustilide at concentrations of 0, 15, 30 and 45 μM was added into the culture medium. The plates were placed in a humidified cell incubator at 37 °C and 5% CO_2 for 48 h. Next, 10 μL of MTT reagent was added to each well and incubated for 4 h in the dark. Subsequently, triple solution (10% SDS, 5% isobutanol, 0.012 mol/L HCl, dissolved in distilled water) was added, and after incubation for 4–6 h, the absorbance at 570 nm was measured with a microplate reader (BIO-RAD Laboratories, Philadelphia, PA, USA).

2.4. T-cell proliferation assay

T-cell proliferation was measured using the intracellular dye carboxyfluoresceinsuccinimidyl ester (CFSE). Splenocytes were isolated from wild-type mice and incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified incubator at 5% CO_2 for 4 h. Cell suspensions were then collected and labeled with 1 mM CFSE at 37 °C for 5 min, followed by quenching in 10% FBS-RPMI 1640 medium. CFSE-labeled splenocytes were incubated with 0.6 mg/ml ConA and 30% CAF supernatant derived from CAFs treated with different concentrations of ligustilide. After 3 days, the cells were stained with APC-anti-CD4 and PE-anti-CD8a, and the CFSE signal of gated splenocytes was analyzed.

2.5. Flow cytometry

Cells were labeled for immunofluorescence and analyzed by flow cytometry for cell surface molecules. Antibodies including APC-anti-CD4 and PE-anti-CD8a were diluted at 1:200 in PBS with 2% FBS.

2.6. Western blot analysis of cell proteins

CAFs treated with different concentrations of ligustilide (0, 15, 30 and 45 μM) for various durations were harvested from a 6-well plate and lysed in RIPA buffer to isolate whole cell proteins. The cell extract containing proteins (30 μg) was separated on 12% SDS-polyacrylamide gels and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked in 3% BSA in PBS-T (0.1% Tween-20) at 4 °C overnight and probed with the primary antibodies as follows: Phospho(P)-p65, p-IKb, IKb, p-TAK and TAK, TRAF6, MyD88, and α-SMA, which were diluted at 1:1000, and GAPDH, which was diluted at 1:3000. After washing three times with PBS-T, HRP-conjugated goat anti-mouse or goat anti-rabbit IgG was used as the secondary antibody. Specific bands were visualized using a Chemiluminescence Imaging System (Clinx Science Instruments Co. Ltd., Shanghai, China).

2.7. Cell immunofluorescence

CAFs were seeded in 35 mm culture dish with a glass bottom designed for confocal microscopic applications at a density of 5 × 104/ml. After CAF stays overnight, ligustilide at various concentrations were added. After incubation for an additional 24 h, the cells were washed twice with PBS and were fixed by 4% PFA for 20 min. Then, cells were fully washed and treated with 0.1% Triton for 5 min. After fully wash, cells were blocked with 3% BSA and stained with 1:200 diluted primary antibody (Cell Signaling, USA) for overnight at 4 °C. Then, samples were incubated with secondary antibody (Invitrogen, USA,) for 60 min at 37 °C. After 3 times washes and stained with DAPI (Invitrogen, USA) for 20 min, the samples were fully rinsed with PBS and examined under a digital microscope (OLYMPUS, Tokyo, Japan).

2.8. Statistical analysis

Quantitative data are presented as the mean ± standard deviation (SD) from three or more independent experiments, and evaluated by one-way ANOVA. $p < 0.05$ was considered statistically significant. All experimental procedures were independently repeated at least three times.

3. Results

3.1. Ligustilide has no effect on the growth of CAFs and splenocytes

An MTT assay was used to analyze the effect of ligustilide on CAFs and splenocytes. Prostate CAFs and wild-type mouse splenocytes were treated with different concentrations of ligustilide for 24 h. Ligustilide had no effect on prostate-CAF (Fig. 1A) or spleen cell (Fig. 1B) growth.

3.2. Ligustilide reverses the immunosuppressive function of CAFs on T cells

To investigate the effect of ligustilide on the immunosuppressive function of CAFs on T cells, flow cytometry analysis (Fig. 2A) was performed to assess T-cell proliferation. The results indicate that

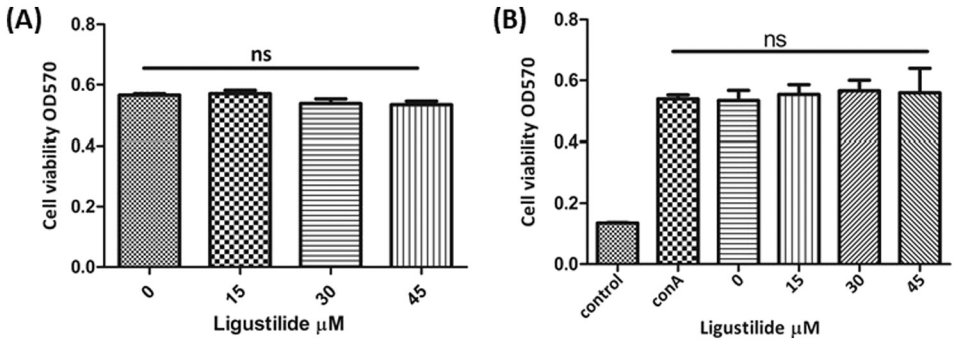

Fig. 1. The effect of ligustilide on prostate-CAFs (A) and splenocytes (B). Splenocytes (3×10^5 cells/well in 96-well culture plates) and CAFs (5×10^3 cells/well in 96-well culture plates) were treated with various concentrations of ligustilide for 24 h. Each concentration group contains four replicates; DMEM was used as the control. ns represents statistically nonsignificant differences.

proliferation of CD4+ (Fig. 2B) and CD8+ (Fig. 2C) T cells at 0.6 mg/ml ConA was inhibited by the supernatant of CAF cultures (at 30% CAF supernatant). However, when CAF cells were incubated with different concentrations of ligustilide for 24 h and this supernatant was used in the assays, T-cell proliferation could be restored; furthermore, this effect was dose-dependent. These results show that ligustilide can eliminate prostate-CAF-mediated T-cell suppression.

Ligustilide reverses the immunosuppressive function of prostate-CAF in the co-culture system. The experiment in supernatant culture system showed that could inhibit the proliferation of T cells by changing the function of CAFs and influence the levels of immunosuppressive factors in the supernatant. To further validate the interaction of CAFs directly with T cells, we designed a co-culture system. The results showed that untreated CAFs of ligustilide significantly reduced the proliferation of T cells, but its inhibition on T cell proliferation was significantly impaired after stimulation with ligustilide (Fig. 2E). These results suggest that prostate-CAFs may directly interact with T cells, thereby affecting its proliferation, but ligustilide may reverse the inhibitory effect of CAFs on T cells.

3.3. Ligustilide acts on the TLR4 receptor and activates the NF-κB pathway

The results of flow cytometry indicated that ligustilide could reverse the immunosuppressive function of CAFs and that the degree of reversal was dose-dependent with the concentration of ligustilide. We next studied the mechanism of this reversal by performing a Western blot test or cellular immunofluorescence. The expression levels of p-p65, p65, pIκB, IκB, pTAK, TAK, MyD88, TRAF6, and TLR4 were found to vary with the concentration of ligustilide (Fig. 3A). To further explore the main receptor of the NF-κB pathway activated by ligustilide, TLR4 receptor blockers were added to the experimental system for 6 h. Western blot analysis showed that the levels of MyD88, TRAF6, TAK1, p-IκB, p-p65 and other proteins did not change in CAFs after treatment with ligustilide (Fig. 3B). At the same time, we found the same results using cellular immunofluorescence (Fig. 3C).

3.4. Ligustilide reverses the immunosuppressive function of CAF through the TLR4 receptor

We added TLR4 receptor blockers when preparing the CAF supernatant and found that even the CAF supernatant incubated with ligustilide did not restore T-cell proliferation (Fig. 4). We can conclude that ligustilide acts mainly on the TLR4 receptor, which in turn activates the NF-κB pathway and reverses the immunosuppressive function of CAFs.

3.5. Ligustilide decreases the expression level of α-SMA CAF

Based on Western blot analysis and cellular immunofluorescence, the expression of α-SMA was found to be reduced in CAFs 24 h after ligustilide incubation. This result shows that ligustilide can induce CAF transformation to normal fibroblasts (NFs). At the same time, we also found that the addition of TLR4 receptor blockers in the CAF medium did not alter the expression of α-SMA (Fig. 5), further supporting that the action of ligustilide on CAFs alters the properties of TLR4 receptors.

4. Discussion

Angelica is a traditional Chinese medicine containing the compound ligustilide, and one study found that ligustilide from *Angelica* extract can relax blood vessels and has a protective effect against brain ischemia-reperfusion injury [28]. It was also found that ligustilide has a direct antitumor effect itself or in conjunction with chemotherapy drugs.

Though it has been demonstrated that ligustilide can inhibit tumor growth, there has been no report on the use of ligustilide in tumor immunotherapy. Therefore, we studied the role of ligustilide in the tumor microenvironment, especially its effects on CAFs and the associated mechanisms. We found that low concentrations of ligustilide had no effect on CAF and splenocyte growth (Fig. 1). CAFs are known to significantly inhibit proliferation of T cells after ConA stimulation and have been shown to have obvious immunosuppressive effects including inhibition of T-cell proliferation, reduction in cytotoxic T-cell effects on tumor cells, and formation of a tumor microenvironment that is conducive to tumor growth [29]. However, we found that when ligustilide is added to the system, T-cell proliferation previously inhibited by untreated CAF supernatant can be restored, indicating that ligustilide can abolish CAF-mediated T-cell inhibition (Fig. 2). Ligustilide can alter certain properties of the original CAF cells, reversing their immunosuppressive effect, which may in turn change the tumor microenvironment and enhance the body's immune regulation so that immune cells can better carry out their function.

In the NF-κB family of transcription factors, RelA has long been recognized as an oncogene for many solid cancer and hematological malignancies [30]. However, some reports have also shown that RelA can act as a biphasic regulator with antitumor effects [31,32]. Studies have shown that activation of the NF-κB pathway can reverse the immunosuppressive function of myeloid derived suppressor cells (MDSCs) [33]. However, we found that the expression of key proteins (p-p65, p65, pIκB, IκB, p-TAK, TAK, MyD88, and TRAF6) in the NF-κB pathway in CAFs was changed after being subjected to the action of ligustilide (Fig. 3A). It has been found that TLR4 plays a role in antitumor drugs. Moreover, TLR4, has been shown to have an important role in innate immune response and can be expressed in many important immune cells and tumor cells [32,34,35]. TLR4 recruits Mal and MyD88, then MyD88 mediates the binding of TRAF6 to preformed membrane-bound complexes of TAB1, TAK1, and TAB2; and activated TAK1 then phosphorylates IKK complexes. IKK can then activate the p65/p50 NF-κB dimer, which is freely transferred into the nucleus, binds its target DNA sequences and promotes the expression of pro-inflammatory cytokines [36]. After treatment of CAFs with the TLR4 blocker CIL-095, we found no change in the proteins associated with the NF-κB pathway (Fig. 3B). At the same time, after pretreatment of CAFs with TLR4 receptor blocker, ligustilide could not reverse the immunosuppressive effect of CAFs (Fig. 4). Our study confirms that ligustilide acts on TLR4 to

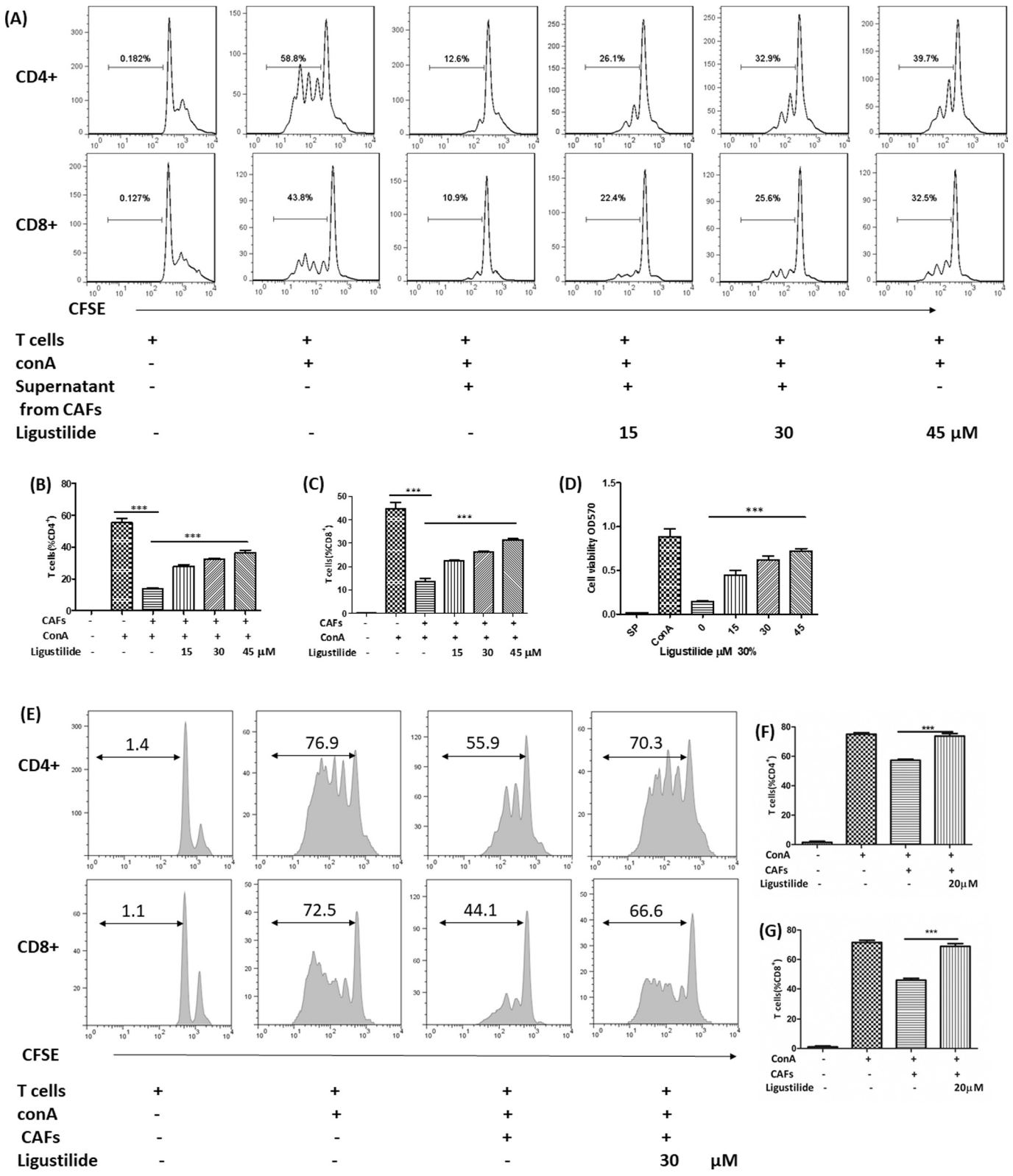

Fig. 2. Ligustilide reverses the immunosuppressive function of CAF cells. T cells from splenocytes of wild-type mice (3×10^5 cells/well in a 96-well culture plate) were treated with various concentrations of supernatant from CAFs for 3 days, stained with fluorescent antibodies (APC-anti-CD4, PE-anti-CD8a) and subjected to a fluorescence-activated cell sorting analysis with a flow cytometer. (A) Data were analyzed using FlowJo7.6, a bar chart of (B) CD4+ or (C) CD8+ T-cell proliferation was analyzed by GraphPad. (D)Splenocytes (3×10^5cells/well in 96-well culture plates) were treated with various concentrations of supernatant from CAFs for 3 days. Cell viability was analyzed by MTT assay. CAFs pretreated with or without ligustilide (20 μM) for 4 days were cocultured with CFSE-labeled splenocytes at the ratio of 1:6000 in a 96-well round bottom plate, and 0.6 μg/ml ConA was added at the same time. The proliferation ratio of CD4+ and CD8+ T cells in splenocytes was measured with a flow cytometer after cultured at 37 °C for 3 days. (E) Data were analyzed using FlowJo7.6, a bar chart of (F) CD4+ or (G) CD8+ T-cell proliferation was analyzed by GraphPad. DMEM was used as the control. ***$p < 0.001$, and ns represents non-statistically significant differences.

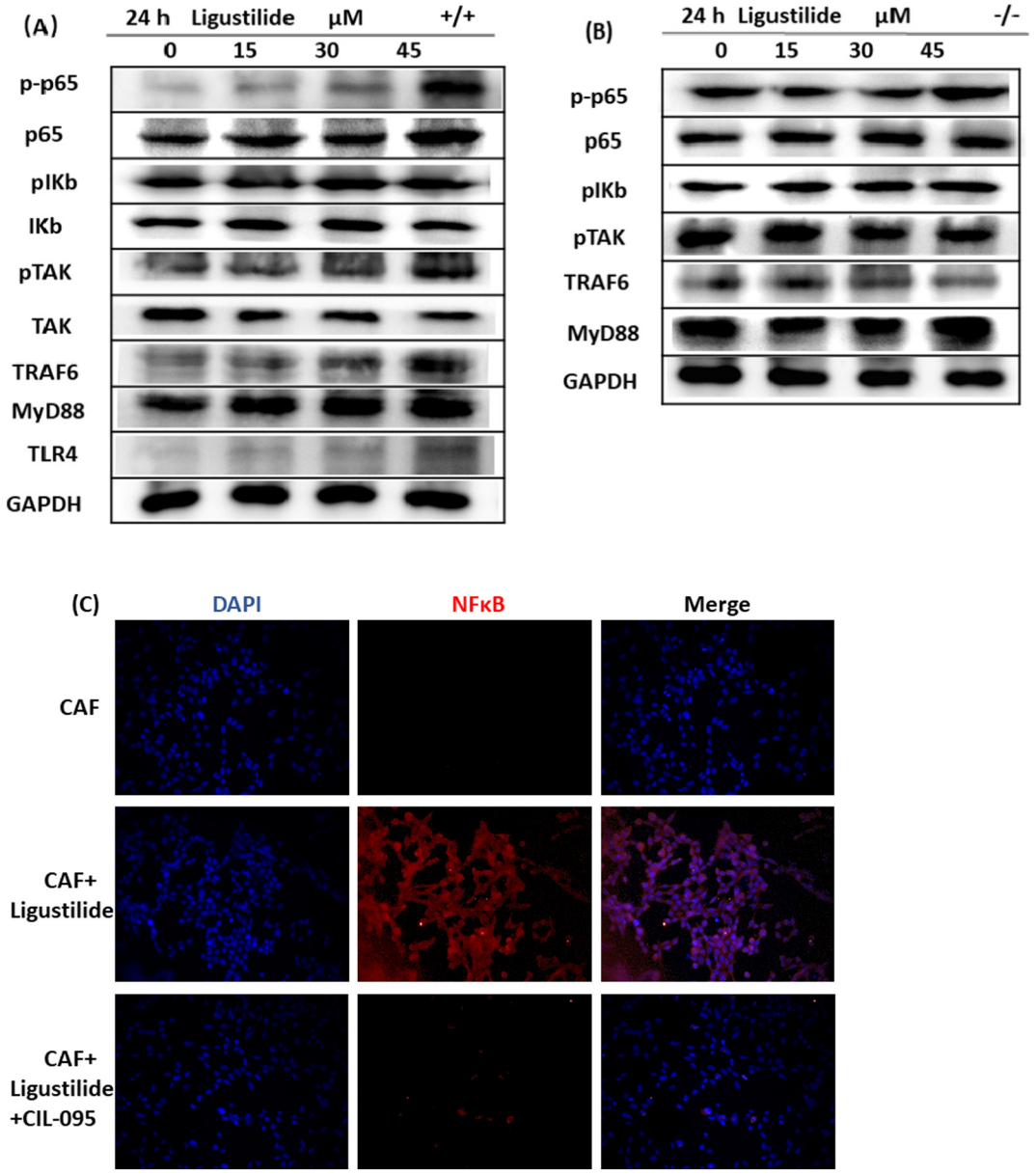

Fig. 3. Ligustilide activated the NF-κB pathway in CAFs. (A) CAFs were treated with 0,15,30 and 45 μM ligustilide for 24 h, and the protein expression levels of p-p65, p65, pIκB, IκB, p-TAK, TAK, MyD88, TRAF6, TLR4 and GAPDH were determined by Western blot. (B) TLR4 receptor blockers were added to the experimental system for 6 h, and the protein expression levels of p-p65, p-IκB, p-TAk, MyD88, TRAF6 and GAPDH were determined again by Western blot. (C) The expression of NF-κB in CAFs treated under different conditions was detected by cellular immunofluorescence.

activate the NF-κB pathway. Activation of NF-κB may cause CAFs to promote the immune response of secretory factors, thereby reversing immunosuppression, but the exact mechanism requires further study.

We suspect that the effect of ligustilide on prostate CAFs may change some CAF properties. α-SMA is expressed in cancer-associated fibroblasts. We found that ligustilide can downregulate the expression of α-SMA in CAFs, and we also found that the expression of α-SMA was not changed after TLR4 receptor blockers were added to the system (Fig. 5). This result suggests that the effect of ligustilide on TLR4 may change certain properties of CAF, and ligustilide may cause CAFs transformation to NFs. However, this is only the current conjecture. The effect of ligustilide on CAFs remains to be further studied.

In summary, our study shows that ligustilide can reverse the immunosuppressive function of CAF cells and is beneficial to the body's immune function against tumors, suggesting that ligustilide can be used as a candidate for the design and development of novel antitumor drugs. It is possible to reverse the tumor-promoting effects of CAFs by selective activation of NF-κB signaling pathways in CAF cells. (See Fig. 6.)

5. Conclusions

In conclusion, we found that ligustilide activates the NF-κB pathway and promotes the conversion of CAFs to NFs by acting on the TLR4 receptor. Altogether, our results revealed that ligustilide may serve as a potential anticancer agent by reversing T-cell inhibition by CAFs.

Conflicts of interest

The authors declare no conflict of interest.

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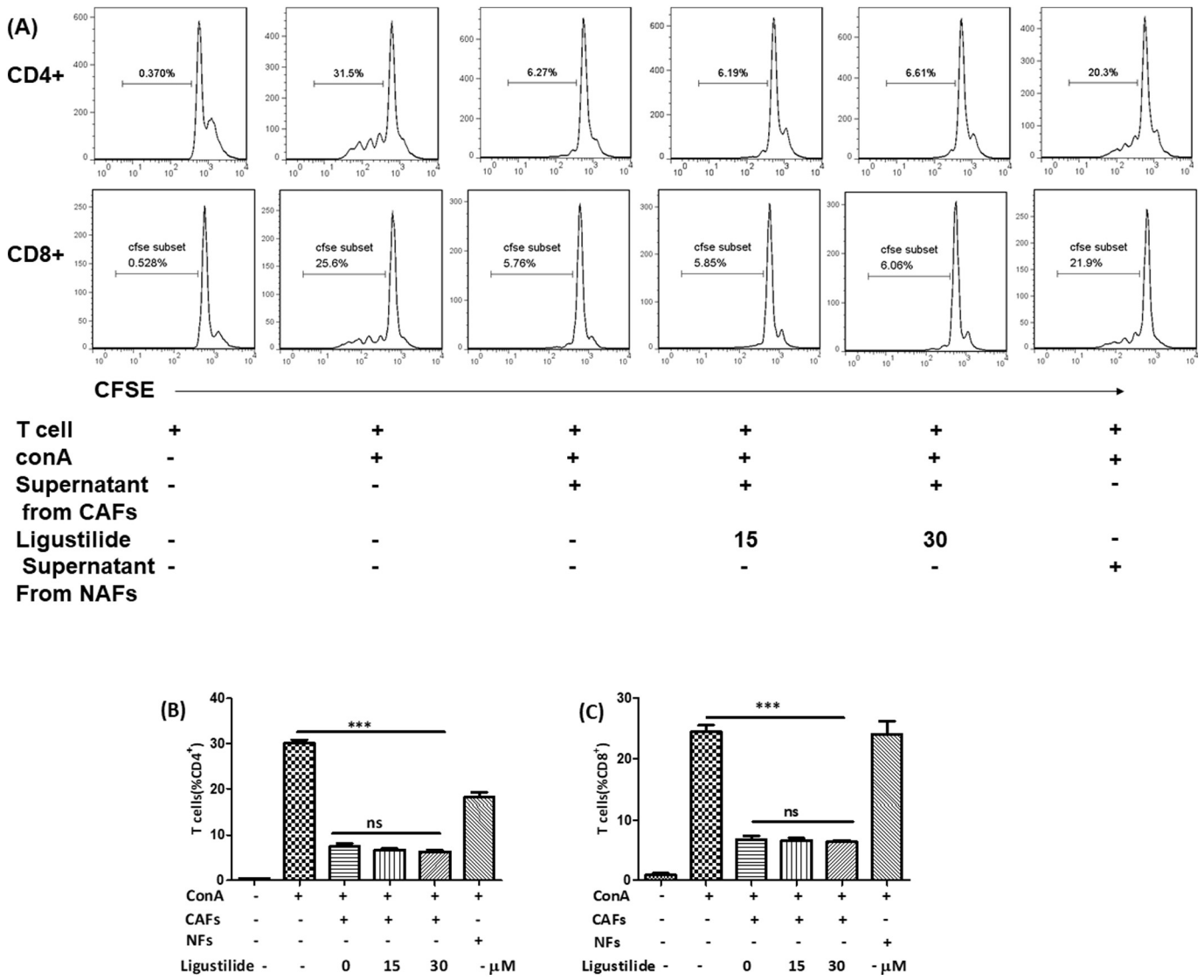

Fig. 4. Ligustilide reverses the immunosuppressive function of CAF cells through an effect on Toll-like receptor 4. (A) TLR4 receptor blockers were added to the experimental system for 6 h. T cells from splenocytes of wild-type mice (3×10^5 cells/well in a 96-well culture plate) were treated with various concentrations of supernatant from CAFs for 3 days, stained with fluorescent antibodies (APC-anti-CD4, PE-anti-CD8a) and subjected to a fluorescence-activated cell sorting analysis with a flow cytometer. Quantification of (B) CD4+ or (C) CD8+ cell proliferation was expressed as the mean ± SD and analyzed by one-way ANOVA. ***$p < 0.001$, and ns represents non-statistically significant differences.

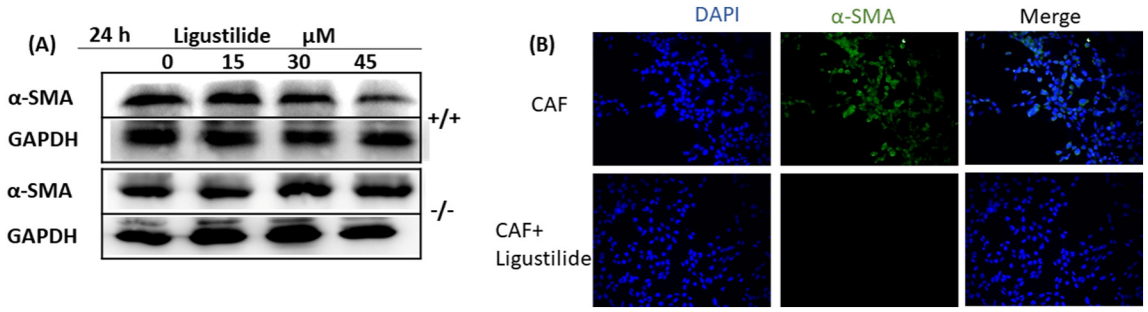

Fig. 5. Reduced activity of prostate-CAFs. After treatment with ligustilide for 24 h, the expression of α-SMA was analyzed by Western blot as a marker of CAF activity (A). TLR4 receptor blockers were added to the experimental system for 6 h, and the expression of α-SMA was determined by Western blot. GAPDH served as the control. After treatment with ligustilide for 4 days, the expression of α-SMA was analyzed by cellular immunofluorescence (B).

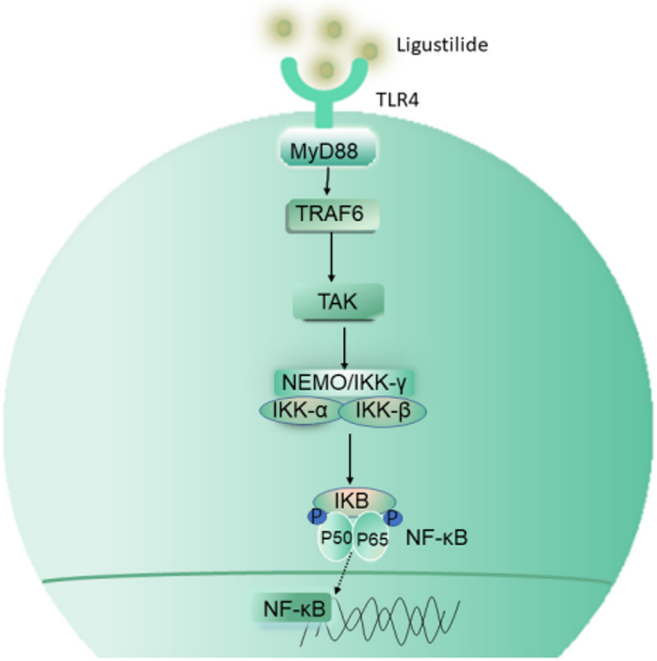

Fig. 6. The signaling pathway of CAFs by ligustilide.

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