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Research Article

Combined Effect of Lentinan and Cisplatin on Cytokines IL-6, TNF-α, and TGF-β in Tumor Therapy

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Lentinan is a biologically active ingredient isolated from the fruiting body of Lentinula edodes (shiitake) and has antitumor properties. Cytokines, at the same time, are a key factor in regulating immune responses in physiological and pathological states. Many cytokines have shown positive effect on different malignancies in clinical trials. Studies have shown that such cytokines as IL6, TNF-α, and TGF-β play a quite important role in the formation of tumor microenvironment. In this study, we investigated the antitumor effect of combined medication of lentinan and cisplatin, another antitumor drug, and its impact on cytokines IL-6, TNF-α, and TGF-β in the treatment of tumor. The results showed that lentinan enhanced the cytotoxic effect of cisplatin on tumor cells. Furthermore, it was proved that the combined treatment of lentinan and cisplatin enhanced the inhibitory effect of cisplatin on the expression of IL-6, TNF- α, TGF-β and tumor growth in mice bearing human ovarian cancer xenograft, human prostate tumor xenograft, and human non-small cell lung cancer xenograft.

1. Introduction

Lentinan (LNT) is a structural component of the mushroom cell wall which contains two main polymers, namely, chitin and β-glucans. The single strands of both molecules are linked to a hydrogen bridge bond to form a covalent bond between the two polymers. This process leads to the formation of a strong cell wall in which chitin fibers are linked to glucans to form a network of matrix [1]. The cell wall structure of Lentinus edodes can be divided into three layers. The outer layer is heteropolysaccharide and β-(1→3)-glucans with a β-(1→6) branch, which can be extracted with water and dilute alkali solution. The middle layer is mainly β-(1→6)-glucans containing a few β-(1→3)-branched chains, which are insoluble in water and can be extracted with strong alkali solution. The inner layer is a compound of chitin, β-glucans, and a small amount of acidic polymers [2].

The extraction method of polysaccharides varies with its structure and water solubility. The basic rule is to destroy the cell wall from the outer layer to the inner layer under mild conditions. The extracted polysaccharides can be further purified by a series of combinatorial techniques. Acid solution, alkali solution, and water are the three basic extraction solvents, in which water extraction is the most commonly seen. At present, several polysaccharides have been identified through different extraction methods and purification processes. The extraction method varies depending on the chemical structure of the polysaccharides from different parts of Lentinus edodes. Lentinan is insoluble in cold water and slightly soluble in hot water [3]. Another study showed that lentinan is β-d-(1,3)-d-glucopyranose with a branched chain of β-d-(1,6)-monosaccharide, which is water soluble and has the property of $[\alpha]\, d = +20o{-}{+}22o$ (NaOH) [4]. The structures of the side chains together with their physical properties are slightly different.

Based on the fact that lentinan is harmless and does not impose additional stress on the human body, lentinan is also considered as biological response modifiers (BRMS). It has been shown that lentinan has antitumor properties and its antitumor mechanism includes prevention of tumorigenesis (cancer prevention activities, enhancement of human immunomodulatory activity, and direct antitumor activity by

inducing apoptosis of tumor cells (direct tumor suppressor activity) [5].

Lentinan also has an effect of preventing cancer, which was discovered in farmers who were engaged in the cultivation of mushrooms. The study showed that the cancer mortality rate of these farmers was remarkably 40% lower than that of the common people [6]. It was proved that cytochrome P450s (CYP) is involved in the cancer prevention of lentinan. CYP is a kind of exogenous digestive enzyme mainly expressed in the liver. The study showed that inhibition of CYP activity helps to enhance the cancer prevention of lentinan, because the CYP1A subfamily induced by exogenous substances such as polycyclic aromatic hydrocarbon can metabolize carcinogens [7]. In Hashimoto's study, the female BALB/C mice were intraperitoneally injected with lentinan and it was observed that lentinan downregulated the expression of CYP and the activity and level of CYP1A induced by 3-methylcholanthrene. In addition, it was accompanied by the inhibition of the production of tumor necrosis factor-α (TNF-α) and the DNA-binding activity of aryl hydrocarbon receptor as well as the enhancement of the DNA-binding activity of nuclear factor-κB [7, 8]. These results suggested that mushroom polysaccharides, such as lentinan, had anticancer activity, as downregulation of CYP1A was considered to prevent metabolic activation of protocancer cells [7, 8]. In addition, the cancer prevention of lentinan may also be related to the fact that lentinan can inhibit the activity of telomerase [9]. Lentinan not only has anticancer activity but also can be used as an immunopotentiator to treat tumors in combination with anticancer drugs.

Since lentinan requires a functional T cell to exert its antitumor activity, it is also considered as a T cell-directed immunopotentiator. It has also been shown that the enhancement of the host immune system by β-(1→3)-glucans is mainly manifested in three aspects—an increase in the number of T helper cells (Th), enhancement of macrophage activation, and nonimmunological activity, where proliferation of macrophage and peripheral monocyte and the activation of lymphocyte and complement system are successively affected by stimulating the host defense mechanisms and colony-stimulating factor (CSF) of acute phase proteins [10, 11].

Cytokines are key factors in regulating immune responses under physiological and pathological conditions. Many cytokines have had a positive clinical effect on different malignancies in clinical trials [12]. It was shown that the cytokines IL6, TNF-α, and TGF-β play a very important role in the formation of tumor microenvironment [13].

Interleukin-6 (IL-6) is an essential cytokine involved in innate immunity. In a series of animal cancer models, IL-6 secreted by malignant tumor cells and mesenchymal stem cells has been proved to promote tumor growth [14]. Meanwhile, IL-6 also acts as a downstream effector of the oncogene ras [15] and is considered to be an important component of certain cancer cytokine networks, such as ovarian serous and clear cell carcinoma [16], multiple myeloma [17], ricin poisoning [18], and hepatocellular carcinoma [19].

There is preclinical evidence that IL-6 can increase the survival rate of ovarian cancer cells and enhance the resistance to chemotherapy by JAK/STAT signaling in tumor cells and translocation of IL-6 receptor alpha on tumor endothelial cells. In addition, IL-6 also has angiogenic properties and regulates immune cell infiltration, matrix response, and tumorigenic effects of Th17 lymphocytes [20, 21].

Many studies have confirmed that tumor necrosis factor-α (TNF-α) is a cytokine produced by activated macrophages and has multiple biological effects. TNF-α has been shown to inhibit the growth of both tumor cells and tumor-bearing mice in some vitro experiments, but clinical trials, taking it as an antitumor agent, have not achieved positive clinical results [22]. What is more, TNF-α has also been shown to be a key cytokine that promotes tumor cell proliferation and invasion in tumor microenvironment. After treatment with anti-TNF-α antibody in mice with in situ pancreatic tumors, the tumor growth and migration ability of mice were inhibited [23]. TNF-α has been proved to be closely associated with tumor cell growth and metastasis in studies of thoracic tumors, skin tumors, and gastrointestinal cancers [24]. The level of TNF-α has a certain correlation with the extent, recurrence, metastasis, and prognosis of malignant tumors in patients with prostate cancers [25]. Meanwhile, TNF-α has also been shown to enhance the invasive ability of pancreatic cancer cell lines in vitro [26].

The specific mechanism of TNF-α promoting malignant tumor progression has not been elucidated. Certain studies have found that TNF-α promotes the inflammatory response of various cytokines and chemokines, thereby affecting the formation and development of tumor vessels and promoting tumor invasion and metastasis [27]. TNF-α may also affect the activity of tumor cells and promote tumor cell proliferation by activating transcription factors and related gene cell signaling pathways [28]. TNF-α has also been proved to directly cause genetic damages, mutations, and DNA amplification, and thereby influence tumor development [29, 30]. Besides, TNF-α can also alter the function of immune cells and promote tumor progression [30].

Transforming growth factor-β (TGF-β) is a necessary cytokine for activating tumor stroma. TGF-β plays a vital role in tumor microenvironment, promotes the recruitment of immune cells, inhibits the antitumor immune response, and affects the differentiation of epithelial cells and endothelial cells. The signaling pathway of TGF-β could be simplified as the binding of TGF-β to its TGF-β receptor II (TβRII), which promoted the activation of TβRI molecules, and phosphorylated the sites of TβRI serine and threonine, thus activated the catalytic activity of TβRI and generated the trimeric complexes of TGF-β/TβRII/TβRI proteins. The complexes could be activated by autophosphorylation and activated the TGF-β/Smad pathway through a series of signal activations, thereby inducing or inhibiting gene expression [31].

The study was to investigate the antitumor effect of the combined use of lentinan and antitumor drug cisplatin and its effect on cytokines IL-6, TNF-α and TGF-β in tumor therapy. Its specific mechanism, however, remains to be discovered in further studies. In general, the study provides a potential therapeutic method and research protocol for cancer treatment.

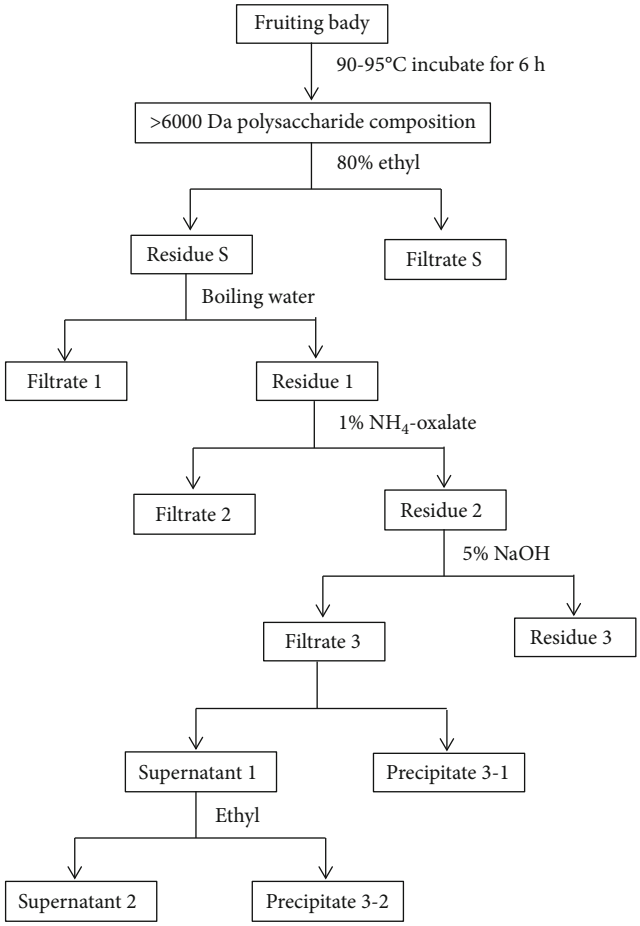

Figure 1: Extraction of lentinan from the shiitake.

2. Materials and Methods

2.1. Experimental Materials. Hey ovarian carcinoma cells, prostate cancer LNCaP cells, and non-small cell lung cancer A549 cells were all purchased from Shanghai Cell Bank of the Chinese Academy of Sciences. Fetal bovine serum (FBS), RPMI-1640 medium, EDTA trypsin, penicillin, and streptomycin were purchased from Gibco Life Technologies. The CCK-8 kit was obtained from Dojindo (Japan). IL-6 ELISA kit and TNF-α ELISA kit were purchased from Xinbosheng Biotechnology Co., Ltd. RNAiso Reagent (total RNA extraction reagent) and SYBR® Premix Ex TaqTM II Reagent and PrimeScript™ RT Master Mix (reverse transcription kit) were purchased from Takara. Ethanol, chloroform, and methanol were purchased from Sinopharm Chemical Reagent Co., Ltd. Diethyl pyrocarbonate (DEPC) was purchased from Beyotime Biotechnology. SPF male BALB/C/nu nude mice weighing 18 ± 2 g were purchased from Shanghai Slack Laboratory Animal Co., Ltd.

2.2. Preparation of Lentinan (LNT). Lentinula edodes powder dissolved in water was incubated at 90-95°C for 6 hours. The ultrafiltration membrane was used to filter components having a molecular mass greater than 6000 Da, and only Lentinula edodes extract with molecular weight less than 6000 Da was collected. The shiitake material, from which low molecular weight substances were removed using 80% ethanol, was then extracted three times with water (100°C, 3 h), 2% ammonium oxalate (100°C, 6 h), and 5% sodium hydroxide (80°C, 6 h). The polysaccharides obtained in the first time were water soluble, while those in the other two times were water insoluble. The extracted polysaccharides were further purified by different concentrations of ethanol and other technologies. Firstly, neutral and acidic polysaccharides were separated by DEAE cellulose column in ion-exchange chromatography. The neutral polysaccharides were further classified into α-glucan (adsorbed fraction) and β-glucan (nonabsorbed fraction) by gel filtration and affinity chromatography. Purified polysaccharides were obtained by the elution of acidic polysaccharides with 1 M sodium chloride [5], as shown in Figure 1.

2.3. Cell Culture. Hey ovarian carcinoma cells, prostate cancer LNCaP cells, and non-small cell lung cancer A549 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. Cells in exponential growth phase were collected for the investigation of antitumor effect.

2.4. Detection of Cytotoxic Activity of Cells by CCK8 Assay. Cell suspension (100 μL) was inoculated in a 96-well plate. The plate was then precultured at 37°C in an incubator supplied with 5% CO_2. Thereafter, 10 μL of CCK8 solution was added to each well, and the plate was incubated for 2 hours, followed by the measurement of absorbance at 450 nm.

Viability calculation : cell viability $*$ (%) = [A(dosing) − A(blank)] × 100/[A(0 dosing) − A(blank)], where A (dosing) is the absorbance of a well containing cells, CCK8 solution, and drug solution; A (blank) is the absorbance of a well having a medium and CCK8 solution but no cells; A (0 dosing) is the absorbance of a well having cells and CCK8 solution but no drug solution; and $*$ is the cell viability (cell proliferation or cytotoxic activity).

2.5. Establishment of Tumor-Bearing Mice Models

2.5.1. Mice Bearing Human Ovarian Cancer Xenograft. Hey cells in logarithmic growth phase were prepared into single cell suspension. The 0.2 ml of cell suspension (containing about 1×10^5 Hey cells) was taken and inoculated subcutaneously into the right axilla of BALB/c/nu nude mice. After the tumor grew to about 50 mm^3, the tumor-bearing mice were divided into 3 groups for drug treatment: the normal group, treated with intraperitoneal injection of the same amount of normal saline for 30 days; the cisplatin group, given intraperitoneal injection of 0.2 ml (2 mg kg^{-1}) cisplatin solution once a day for 30 days; the cisplatin-lentinan group, treated with intraperitoneal injection of 0.2 ml (cisplatin: 7 mg kg^{-1}; lentinan: 25 mg kg^{-1}) cisplatin and lentinan solution once a day for 30 days. The size and weight of tumor were measured every 3 days for generating a tumor growth curve.

2.5.2. Mice Bearing Human Prostate Tumor Xenograft. LNCaP cells in logarithmic growth phase were prepared into single cell suspension. 0.2 ml of the cell suspension (containing about

1×10^6 LNCaP cells) was taken and inoculated subcutaneously into the right axilla of BALB/C/nu nude mice. As the tumor grew to about 50 mm^3, the tumor-bearing mice were divided into 3 groups for drug treatment: the normal group, treated with intraperitoneal injection of the same amount of normal saline for 30 days; the cisplatin group, given intraperitoneal injection of 0.2 ml (7 $mg\,kg^{-1}$) cisplatin solution once a day for 30 days; and the cisplatin-lentinan group, treated with intraperitoneal injection of 0.2 ml (cisplatin: 7 $mg\,kg^{-1}$; lentinan: 25 $mg\,kg^{-1}$) cisplatin and lentinan solution once a day for 30 days. The size and weight of tumor were measured every 3 days for generating a tumor growth curve.

2.5.3. Mice Bearing Human Non-Small Cell Lung Cancer Xenograft. A549 cells in logarithmic growth phase were prepared into single cell suspension. 0.2 ml of the cell suspension (containing about 1×10^7 A549 cells) was taken and inoculated subcutaneously into the right axilla of BALB/c/nu nude mice. When the tumor size was up to 50 mm^3, the tumor-bearing mice were divided into 3 groups for drug treatment: the normal group, treated with intraperitoneal injection of the same amount of normal saline for 30 days; the cisplatin group, given intraperitoneal injection of 0.2 ml (7 $mg\,kg^{-1}$) cisplatin solution once a day for 30 days; and the cisplatin-lentinan group, treated with intraperitoneal injection of 0.2 ml (containing cisplatin: 7 $mg\,kg^{-1}$; lentinan: 25 $mg\,kg^{-1}$) cisplatin and lentinan solution once a day for 30 days. The size and weight of tumor were measured every 3 days for generating a tumor growth curve.

2.6. ELISA Test. Serum was prepared from the orbit blood sampling. 100 μl per well of serum was then added to the enzyme label plate coated with monoclonal antibody and mixed gently. After incubation for 90 min at 37°C, the supernatant liquid was discarded, and the secondary antibody was added for further incubation at 37°C for 1 h. Working solutions of the enzyme conjugate was then added, followed by incubation at 37°C for 30 min. Afterwards, the supernatant was discarded, and the substrate solution was added for further incubation in the dark for 20 min. Finally, the termination solution was added to stop the reaction. The value of optical density of each well was measured at a wavelength of 450 nm. Finally, the content of IL-6, TNF-α, and TGF-β in serum was calculated according to the standard curve.

2.7. Flow Cytometry Assessment. We investigated the expression of cluster of determinant (CD) of cell surface molecules targeting immunophenotyping of cells. Single-cell suspension was extracted from tumor-bearing mice, and the mice were cultured with monoclonal antibodies, CD3-PERCP, CD4-FITC, FOXP3-PE, and IL-17-APC. After incubation at 4°C for 3 hours, the expressing cell population was analyzed by flow cytometer (Becton Dickinson and Company).

2.8. Statistical Analysis. The experimental data were expressed as mean ± S.E.M and were analyzed by oringe 8.0 software. Significant difference was measured by SPSS 19.0 one-way analysis of variance (ANOVA). $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered significant statistically.

3. Results

3.1. Enhanced Cytotoxic Effect of Cisplatin on Tumor Cells by Lentinan. To explore the antitumor effect of lentinan, the cytotoxic effect of lentinan, cisplatin, or the combined use of both on Hey human ovarian carcinoma cells, LNCaP human prostate cancer cells and human non-small cell lung cancer A549 cells were evaluated by CCK-8 assay. The results showed that the IC50 of lentinan for killing Hey cells, LNCap cells, and A549 cells was 800 μM, and the IC50 of cisplatin for killing the cells was 30 μM. The combined use of lentinan (800 μM) and cisplatin reduced the proliferation of Hey cells, LNCap cells, and A549 cells in a dose-dependent manner (Figures 2(a) and 2(b)). In the meantime, the combined treatment of lentinan and cisplatin significantly increased the killing effect of cisplatin on the tumor cells ($P < 0.05$, as shown in Figures 2(c), 2(d), and 2(e)).

3.2. Enhanced Inhibitory Effect of Cisplatin on Expressions of IL-6, TNF-α, TGF-β, and Tumor Growth in Mice Bearing Human Ovarian Cancer Xenograft by Combined Treatment of Lentinan and Cisplatin. To explore the effect of the combined use of lentinan and cisplatin on the cytokines IL-6, TNF-α, and TGF-β in tumor therapy, the expressions of IL-6, TNF-α, and TGF-β in mice bearing human ovarian cancer xenograft after the combination therapy were detected by ELISA and qPCR. The experimental results are shown in Figure 3. The expressions of IL-6, TNF-α, and TGF-β proteins in serum of tumor-bearing mice in the normal saline group were 95.40333 ± 2.69563 pg/ml, 1536.84333 ± 41.83909 pg/ml, and 194 ± 2.5 pg/ml, respectively. The expressions of IL-6, TNF-α, and TGF-β proteins in serum of tumor-bearing mice in cisplatin group were 53.02667 ± 3.09189 pg/ml, 1270.97 ± 26.21165 pg/ml, and 153 ± 3.84 pg/ml, respectively. The expressions of IL-6, TNF-α, and TGF-β proteins in serum of tumor-bearing mice in lentinan-cisplatin group were 43.74333 ± 2.31067 pg/ml, 966.98333 ± 34.28658 pg/ml, and 137 ± 2.51 pg/ml, respectively. The expressions of IL-6, TNF-α, and TGF-β in serum of mice were significantly decreased in both the cisplatin group and lentinan-cisplatin group ($P < 0.01$) compared with the normal saline group. The expressions of IL-6, TNF-α, and TGF-β were significantly decreased in the lentinan-cisplatin group ($P < 0.05$) compared with the cisplatin group. The results indicated that the combined use of lentinan and cisplatin significantly enhanced the inhibitory effect of cisplatin on the expressions of IL-6, TNF-α, and TGF-β in the serum of mice bearing human ovarian cancer xenograft (Figures 3(a), 3(b), and 3(c)). Meanwhile, the combination enhanced the inhibitory effect of cisplatin on tumor growth of mice bearing human ovarian cancer xenograft as well (Figure 3(d)).

3.3. Enhanced Inhibitory Effect of Cisplatin on Expressions of IL-6, TNF-α, and TGF-β and Tumor Growth in Mice Bearing Human Prostate Tumor Xenograft by Combination Therapy of Lentinan and Cisplatin. To explore the effect of combined use of lentinan and cisplatin on the cytokines IL-6, TNF-α, and TGF-β in tumor therapy, the expressions of IL-6, TNF-

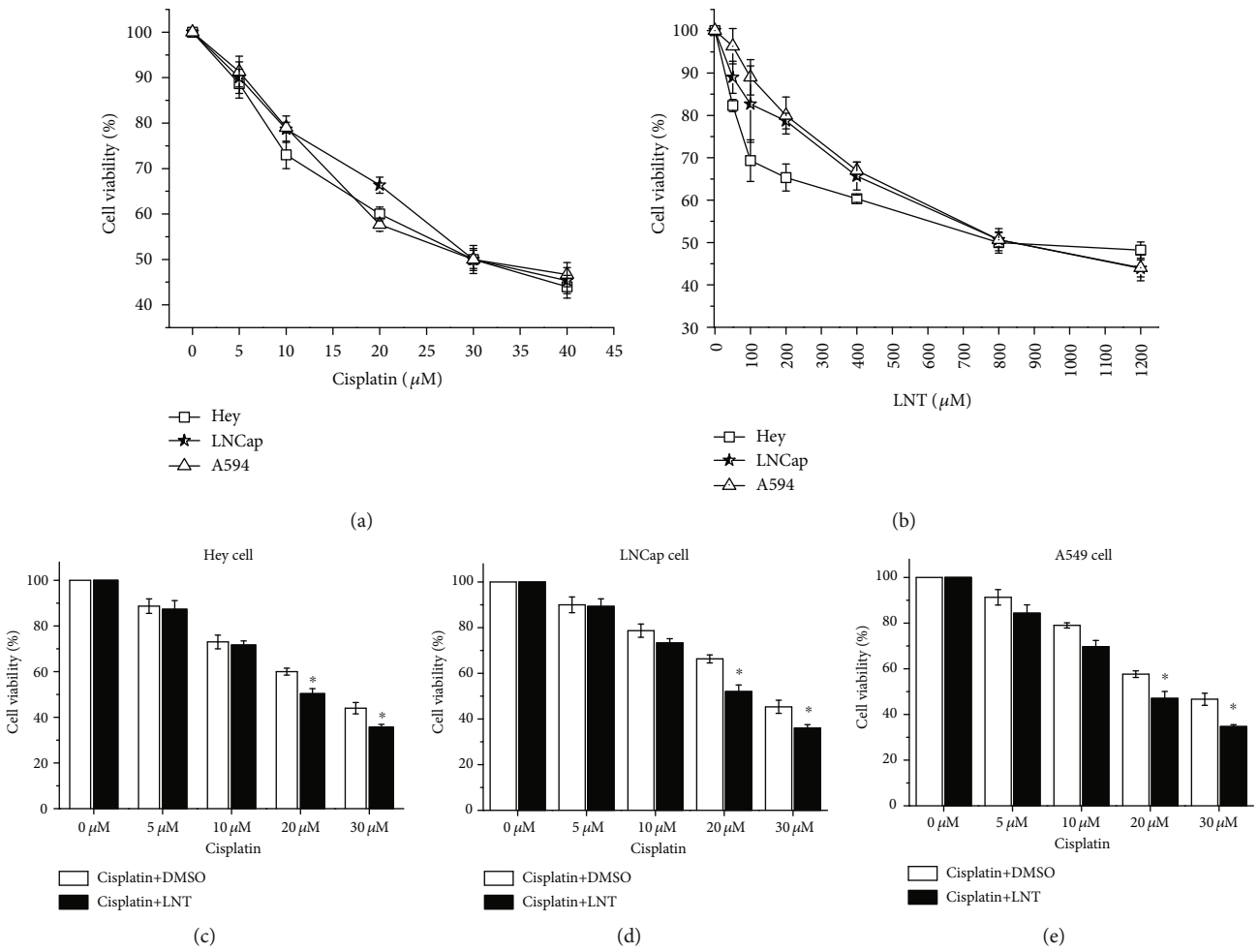

Figure 2: Cytotoxic effect of cisplatin on tumor cells enhanced by lentinan. (a) The cytolytic activity of cisplatin on different doses to Hey cells, LNCap cells, and A594 cells. (b) The cytolytic activity of LNT on different doses to Hey cells, LNCap cells, and A594 cells. (c–e) The cytolytic activity of the combination of LNT (800 μM) and cisplatin to Hey cells (c), LNCap cells (d), and A594 cells (e). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the cisplatin+DMSO group.

α, and TGF-β in mice bearing human prostate tumor xenograft after the combination therapy were detected by ELISA and qPCR. The experimental results are shown in Figure 4. The expressions of IL-6, TNF-α, and TGF-β proteins in serum of tumor-bearing mice in the normal saline group were 95.40667 ± 2.83179 pg/ml, 1542.17667 ± 18.39545 pg/ml, and 1661 ± 15.37 pg/ml, respectively. The expressions of IL-6, TNF-α, and TGF-β proteins in serum of tumor-bearing mice in the cisplatin group were 52.97 ± 2.29613 pg/ml, 1264.63667 ± 32.63896 pg/ml, and 126.331 ± 8.298 pg/ml, respectively. The expressions of IL-6, TNF-α, and TGF-β proteins in serum of tumor-bearing mice in the lentinan-cisplatin group were 44 ± 1.45039 pg/ml, 965.31667 ± 44.84394 pg/ml, and 107 ± 3.79 pg/ml, respectively. The expressions of IL-6, TNF-α, and TGF-β in serum of mice were significantly decreased in both the cisplatin group and lentinan-cisplatin group ($P < 0.01$) compared with the normal saline group. The expressions of IL-6, TNF-α, and TGF-β were significantly decreased in the lentinan-cisplatin group ($P < 0.05$) compared with the cisplatin group. The results suggested that the combined use of lentinan and cisplatin significantly enhanced the inhibitory effect of cisplatin on the expressions of IL-6, TNF-α, and TGF-β in the serum of mice bearing human prostate tumor xenograft (Figures 4(a), 4(b), and 4(c)). Meanwhile, the combination enhanced the inhibitory effect of cisplatin on tumor growth of mice bearing human prostate tumor xenograft as well (Figure 4(d)).

3.4. Enhanced Inhibitory Effect of Cisplatin on Expressions of IL-6, TNF-α, and TGF-β and Tumor Growth in Mice Bearing Human Non-Small Cell Lung Cancer Xenograft by Combination Therapy of Lentinan and Cisplatin. To explore the effect of combined use of lentinan and cisplatin on the cytokines IL-6, TNF-α, and TGF-β in tumor therapy, the expressions of IL-6, TNF-α, and TGF-β in mice bearing human non-small cell lung cancer xenograft after the combination therapy were detected by ELISA and qPCR. The experimental results are shown in Figure 5. The expressions of IL-6, TNF-α, and TGF-β proteins in serum of tumor-bearing mice in the normal saline group were 96.53 ± 2.97073 pg/ml, 1548.63 ± 10.38419 pg/ml, and 194.67 ± 3.84 pg/ml, respectively. The expressions of IL-6, TNF-α,

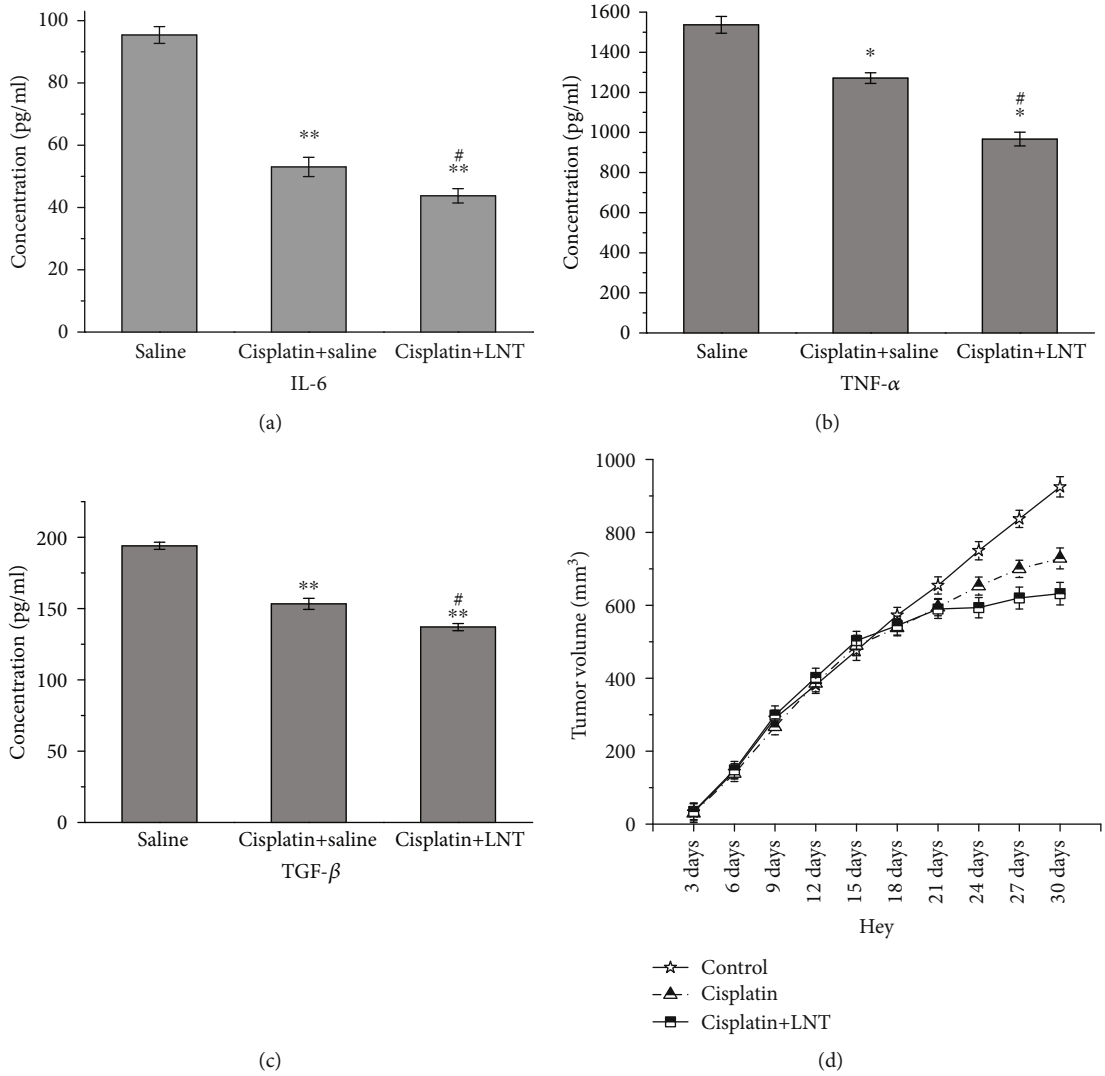

Figure 3: Enhanced inhibitory effect of cisplatin on expressions of IL-6, TNF-α, and TGF-β and tumor growth in mice bearing human ovarian cancer xenograft by combination therapy of lentinan and cisplatin. (a–c) It is enhanced by the combined treatment of lentinan and cisplatin that the expression of IL-6 (a), TNF-α (b), and TGF-β (c) on the serum of mice bearing ovarian cancer induced by cisplatin. (d) It is enhanced by the combined treatment of lentinan and cisplatin that the inhibition of tumor growth of mice bearing ovarian cancer by cisplatin. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the saline group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the cisplatin+saline group.

and TGF-β proteins in serum of tumor-bearing mice in cisplatin group were 54.03333 ± 1.92365 pg/ml, 1259.42333 ± 49.99775 pg/ml, and 140.33 ± 8.089778 pg/ml, respectively. The expressions of IL-6, TNF-α, and TGF-β proteins in serum of tumor-bearing mice in the lentinan-cisplatin group were 45.37667 ± 0.69528 pg/ml, 964.77 ± 56.22525 pg/ml, and 107.1 ± 4.37 pg/ml, respectively. The expressions of IL-6, TNF-α, TGF-β in serum of mice were significantly decreased in both the cisplatin group and lentinan-cisplatin group ($P < 0.01$) compared with the normal saline group. The expressions of IL-6, TNF-α, and TGF-β were significantly decreased in the lentinan-cisplatin group ($P < 0.05$) compared with the cisplatin group. The results indicated that the combined use of lentinan and cisplatin significantly enhanced the inhibitory effect of cisplatin on the expressions of IL-6, TNF-α, and TGF-β in the serum of mice bearing human non-small cell lung cancer xenograft (Figures 5(a), 5(b), and 5(c)). Meanwhile, the combination enhanced the inhibitory effect of cisplatin on tumor growth of mice bearing human non-small cell lung cancer xenograft as well (Figure 5(d)).

3.5. Enhanced Antitumor Effect of Cisplatin in Tumor Microenvironment in Mice Bearing Human Non-Small Cell Lung Cancer Xenograft by Combination Therapy of Lentinan and Cisplatin. To further investigate the anticancer mechanism of the combined use of lentinan and cisplatin, we detected the expressions of cytokines IL-6, TNF-α, TGF-β, regulatory T cells (Tregs), and T helper cell 17 (Th17) in tumor-infiltrating cells of non-small cell lung cancer-bearing mice. Tregs play an important role in the maintenance of immunological self-tolerance and immune homeostasis.

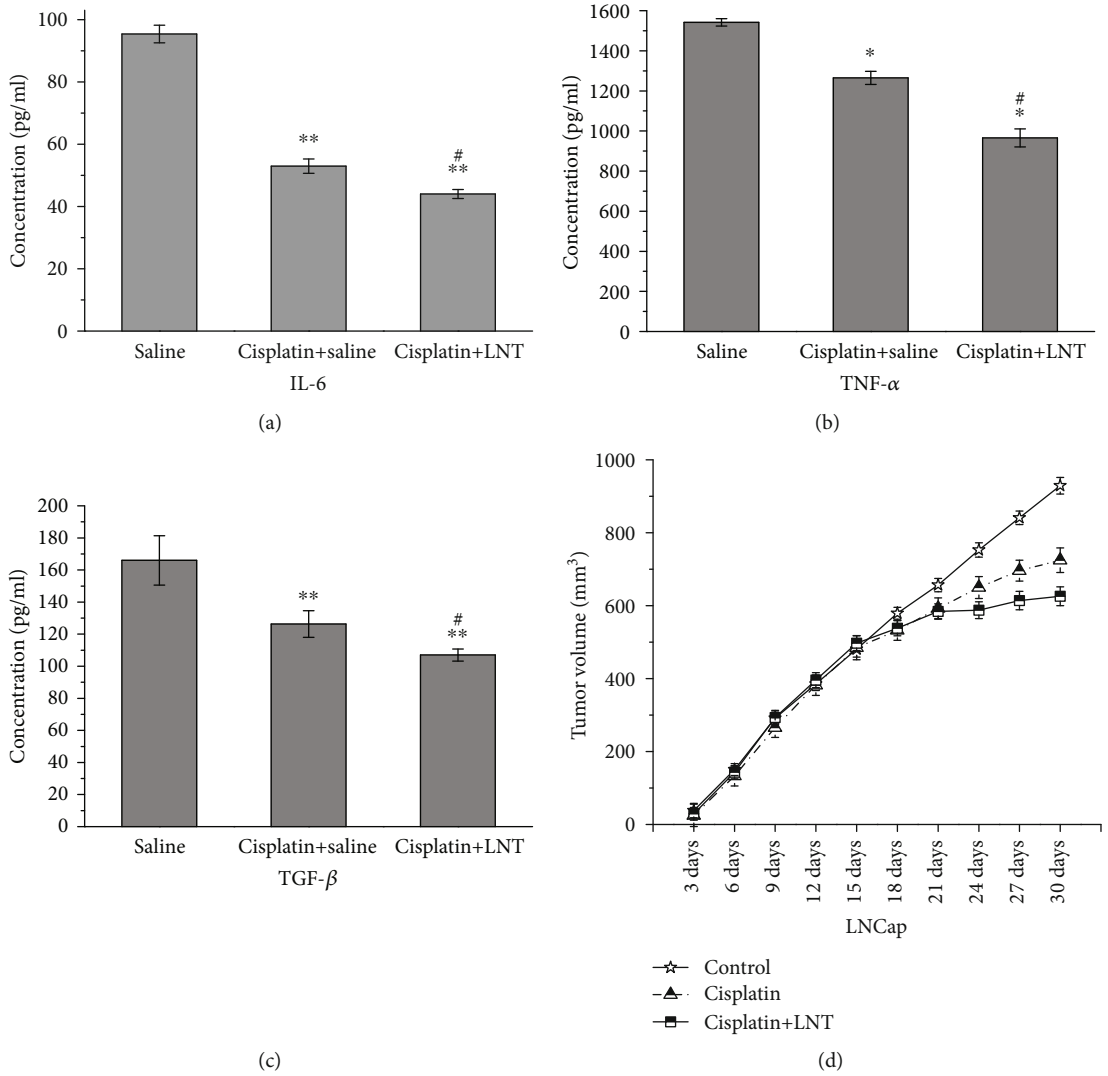

Figure 4: Enhanced inhibitory effect of cisplatin on expressions of IL-6, TNF-α, and TGF-β and tumor growth in mice bearing human prostate tumor xenograft by combination therapy of lentinan and cisplatin. (a–c) It is enhanced by the combined treatment of lentinan and cisplatin that the expression of IL-6 (a), TNF-α, and TGF-β enhanced by the combined treatment for prostate cancer induced by cisplatin. (d) It is enhanced by the combined treatment of lentinan and cisplatin that the inhibition of tumor growth of mice bearing prostate cancer by cisplatin. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the saline group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the cisplatin+saline group.

Th17 as a newly discovered T cell subpopulation capable of secreting interleukin 17(IL-17) is of great significance in the body's defense response. Transforming growth factor b (TGF-β), IL-6, IL-23, and IL-21 promote the differentiation and formation of Th17. Studies have shown that the Th17/Treg balance regulation is the focus of current studies, and Th17/Treg immune imbalance has been linked to the tumorigenesis [6].

The results of our study showed that the expressions of IL-6, TNF-α, and TGF-β in the supernatant of single-cell suspension of human non-small cell lung cancer tumor-bearing mice were significantly decreased in lentinan-cisplatin group (Figure 6) compared with the cisplatin group, which is consistent with the expressions in serum of the tumor-bearing mice.

Tregs are characterized by the expression of transcription factor FoxP3, and Th17 cells are characterized by that of IL-17. Therefore, in this study, Tregs and Th17 cells were labeled with FoxP3 antibody and IL-17 antibody, respectively. The results indicated that compared with the cisplatin group, the lentinan-cisplatin group showed significantly decreased Tregs but increased Th17 in the single-cell suspension of mice bearing human non-small cell lung cancer xenograft (Figure 7).

4. Discussion

The results of this study demonstrated that lentinan enhanced the cytotoxic effect cisplatin on tumor cells and that the combined treatment of lentinan and cisplatin

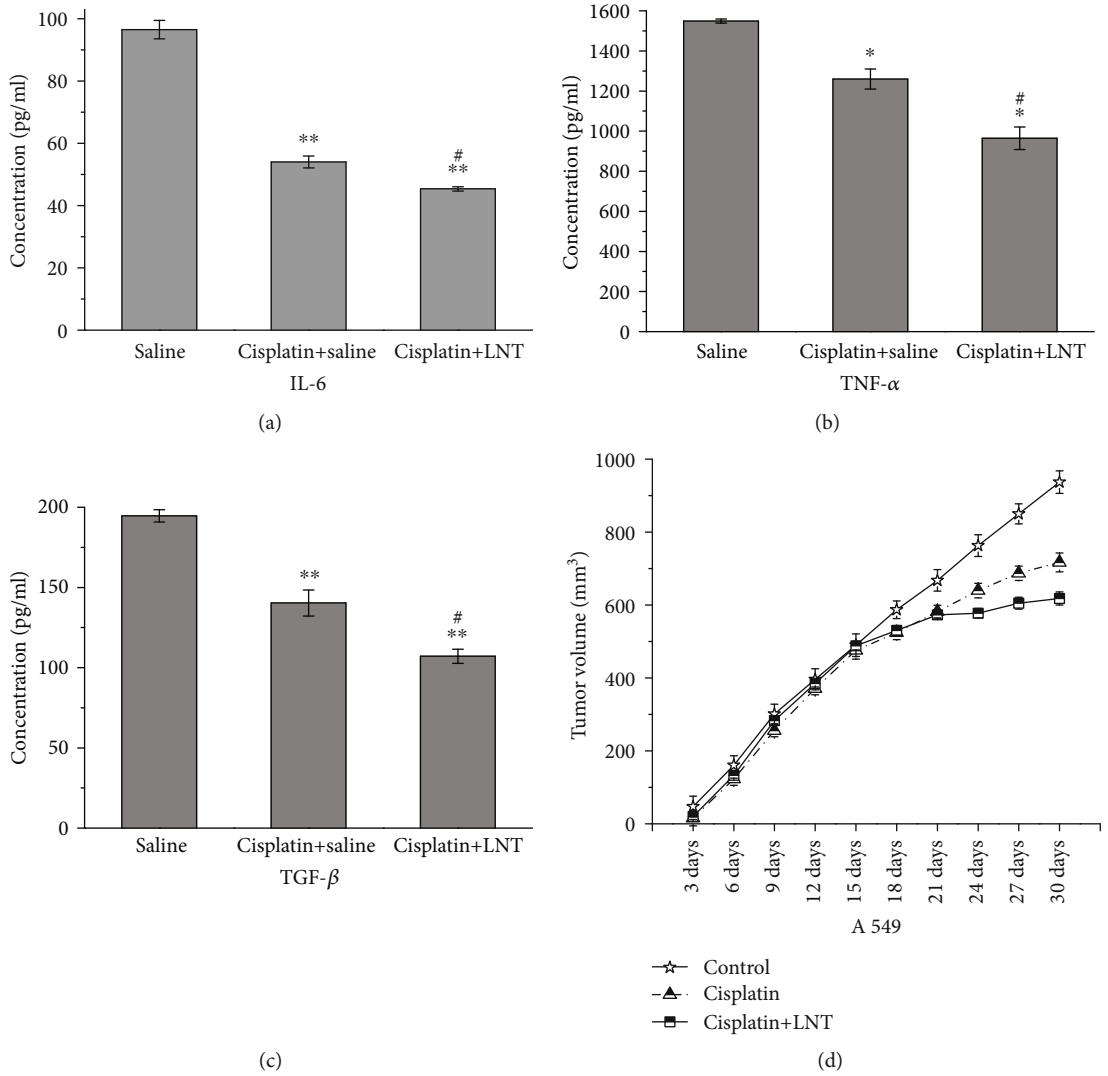

Figure 5: Enhanced inhibitory effect of cisplatin on expressions of IL-6, TNF-α, and TGF-β and tumor growth in mice bearing human non-small cell lung cancer xenograft by combination therapy of lentinan and cisplatin. (a–c) It is enhanced by the combined treatment of lentinan and cisplatin that the expression of IL-6 (a), TNF-α (b), and TGF-β (c) on the serum of mice bearing non-small cell lung cancer induced by cisplatin. (d) It is enhanced by the combined treatment of lentinan and cisplatin that the inhibition of tumor growth of mice bearing non-small cell lung cancer by cisplatin. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the saline group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the cisplatin+saline group.

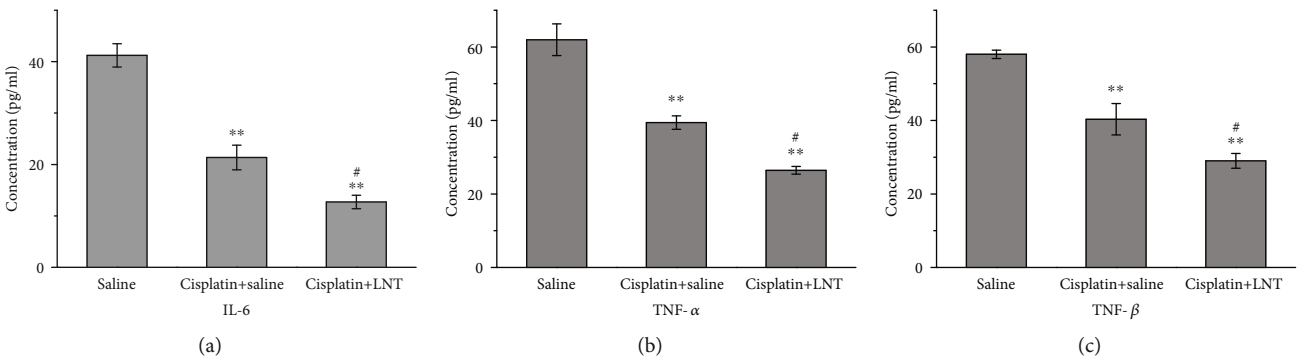

Figure 6: The expression of IL-6, TNF-α, and TGF-β on the tumor of tumor-bearing mice with non-small cell lung cancer. (a–c) The expression of IL-6 (a), TNF-α (b), and TGF-β (c) on the tumor cell suspension supernatant of tumor-bearing mice with non-small cell lung cancer after the combined treatment of lentinan and cisplatin. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the saline group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the cisplatin+saline group.

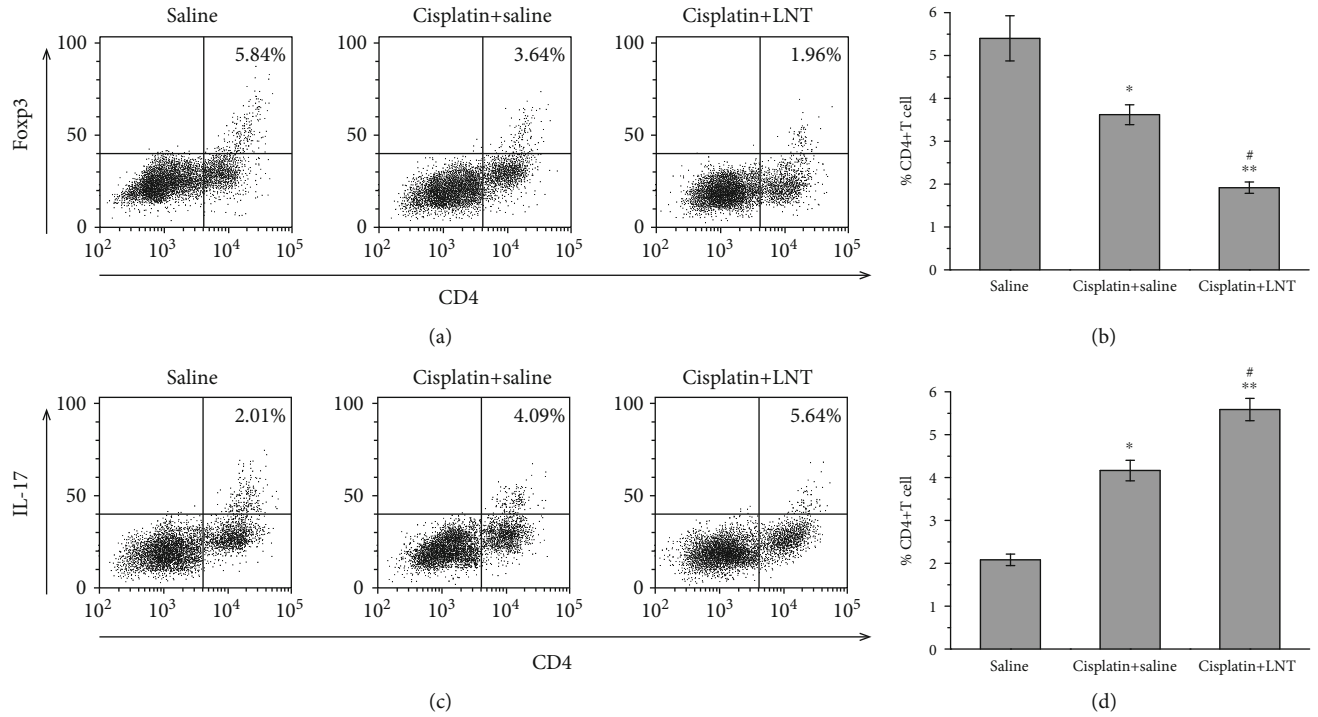

Figure 7: Change of Treg and Th17 cells in tumor tissues of human non-small cell lung cancer-bearing mice. (a, b) Tregs in mouse tumors were significantly reduced after the combined treatment of lentinan and cisplatin. (c, d) Th17 cells in mouse tumors were significantly increased after the combined treatment of lentinan and cisplatin.

enhanced the inhibitory effect of cisplatin on the expression of IL-6, TNF-α, and TGF-β and tumor growth in mice bearing human ovarian cancer xenograft, human prostate tumor xenograft, and human non-small cell lung cancer xenograft.

Cytokines are key factors in regulating immune responses under physiological and pathological conditions. Many cytokines have shown positive effect on different malignancies in clinical trials [12]. Tumor microenvironment is the result of the interaction of many factors, including natural immune cells such as macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, natural killer cells, and adaptive immune cells such as T lymphocytes and B lymphocytes, vascular and lymphatic networks, interstitial fibroblasts, and growth factors, inflammatory factors, chemokines, and the like generated by autocrine or paracrine of cells. Cytokines IL6, TNF-α, and TGF-β play a quite important role in the formation of tumor microenvironment [13].

High levels of IL-6 have been found in both plasma and malignant ascites [32] in patients with advanced ovarian cancer, which has been proved to be associated with poor prognosis of the cancer [33]. Some ovarian cancer cells constitutively secrete IL-6. When these cells are cocultured with other cells from ovarian cancer microenvironment [34, 35], the expression level of IL-6 can be found to be increased. We found that IL-6 is part of the autocrine cytokine network of malignant ovarian tumor cells [35]. The network was involved in the regulation of the cytokines TNF-α and IL-1β, CCl2, CXCL12, and VEGF and could affect angiogenesis in tumor microenvironment by a paracrine manner.

Th17 cell-mediated immunity plays a critical role in maintaining the balance of the mucosa and hematopoietic system. Meanwhile, Th17 cell response, if too intense, can induce autoimmune diseases. It was shown that Th17 cell-induced excessive inflammation and Treg-induced overimmunosuppression (i.e., imbalance of Th17/Tregs) may result in carcinogenesis [7]. In this study, we found that Tregs were significantly reduced and Th17 cells were obviously increased in single-cell suspension of mice bearing human non-small cell lung cancer xenograft in lentinan-cisplatin group as compared with the cisplatin group. And the expressions of IL-6, TNF-α, and TGF-β in the supernatant of single-cell suspension were significantly reduced. The above results suggested that lentinan may regulate the balance of Th17/Tregs by decreasing the expressions of IL-6, TNF-α, and TGF-β, thereby enhancing the antitumor effect of cisplatin.

Our study on the combination therapy of lentinan and cisplatin against tumors provides a potential solution for future cancer treatment.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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