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ORIGINAL ARTICLE

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Parthenolide enhances the metronomic chemotherapy effect of cyclophosphamide in lung cancer by inhibiting the NF-kB signaling pathway

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Abstract

BACKGROUND

Parthenolide (PTL), a sesquiterpene lactone derived from the medicinal herb Chrysanthemum parthenium, exhibits various biological effects by targeting NF-kB, STAT3, and other pathways. It has emerged as a promising adjunct therapy for multiple malignancies.

AIM

To evaluate the *in vitro* and *in vivo* effect of PTL on cyclophosphamide (CTX) metronomic chemotherapy.

METHODS

The cytotoxicity of PTL and CTX on Lewis lung cancer cells (LLC cells) was assessed by measuring cell activity and apoptosis. The anti-tumor efficiency was evaluated using a tumor xenograft mice model, and the survival of mice and tumor volume were monitored. Additionally, the collected tumor tissues were analyzed for tumor microenvironment indicators and inflammatory factors.

RESULTS

In vitro, PTL demonstrated a synergistic effect with CTX in inhibiting the growth of LLC cells and promoting apoptosis. *In vivo*, metronomic chemotherapy combined with PTL and CTX improved the survival rate of tumor-bearing mice and reduced tumor growth rate. Furthermore, metronomic chemotherapy combined with PTL and CTX reduced NF-κB activation and improved the tumor immune microenvironment by decreasing tumor angiogenesis, reducing Transforming

growth factor β, and α-SMA positive cells.

CONCLUSION
PTL is an efficient compound that enhances the metronomic chemotherapy effects of CTX both *in vitro* and *in vivo*, suggesting its potential as a supplementary therapeutic strategy in metronomic chemotherapy to improve the chemotherapy effects.

Key Words: Lung cancer; Parthenolide; Cyclophosphamide; Rhythmic chemotherapy; NF-κB pathway

Core Tip: Our present study found that as a specific inhibitor of NF-kB, Parthenolide can promote the efficiency of cyclophosphamide in lung cancer *via* inhibiting NF-kB signaling *in vitro* and *in vivo*. Our present study will help scientists and clinicians to draw up novel rhythmic chemotherapy strategies.

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INTRODUCTION

Toxic effects and chemotherapy resistance are the main obstacles to traditional chemotherapy clinically. To avoid these problems caused by traditional chemotherapy regimens, a new type of administration method called "metronomic chemotherapy" has emerged[1]. This approach involves frequent administration of conventional chemotherapeutics at very low doses. The term “metronomic chemotherapy” was mentioned preclinically as early as 2000[2,3]. Apart from its advantages including minimizing side effects and reducing opportunities for acquired drug resistance, it is proposed that metronomic chemotherapy targets activated endothelial cells in tumors, modulates hosts’ immune system, and affects tumor progenitor cells and neighboring stromal cells[4]. The most studied drugs for metronomic chemotherapy include cyclophosphamide (CTX), methotrexate, capecitabine, and vinorelbine[5]. CTX is a prodrug that undergoes conversion *in vivo* by hepatic cytochrome P450 enzymes to active forms of metabolites, such as phosphoramide mustard. These active forms undergo covalent cross-linking with DNA, leading to DNA strand breaks and cross-links, which impede the process of DNA replication and transcription[6-8]. However, CTX involves a wide range of cytotoxicity and can cause several serious side effects such as myelosuppression, immunosuppression, alopecia, and urinary toxicity. Because metronomic chemotherapy has a good weakening effect on toxic effects and chemotherapy resistance, CTX is the most widely used metronomic chemotherapy agent preclinically and clinically[9,10]. CTX has been used in combination with other chemotherapy agent, such as vincristine, lomustine, and etoposide, possibly by regulating the host immune system to enhance the efficacy of lung cancer therapy[11,12]. However, uncertainty about treatment efficacy, challenges with compliance, and patient applicability limit the use of CTX-related metronomic chemotherapy. Therefore, there is an urgent need to find more effective drugs for lung cancer treatment in combination with CTX.

Natural products have long been used as the primary arsenal for anti-cancer drug development[13]. Novel natural products act on tumors solely or in combination with traditional chemotherapy drugs. Feverfew (*Tanacetum parthenium L.*) is a traditional medicinal plant that has been used for centuries to treat fevers, migraine headaches, rheumatoid arthritis, and various other inflammation-related diseases[14]. In addition, feverfew extracts have demonstrated the ability to inhibit the proliferation of tumor cells in laboratory studies[15]. This plant contains a large number of natural products, its active compounds include sesquiterpene lactones, like parthenolide (PTL)[14]. PTL was isolated from feverfew extract half a century ago and has been reported to exhibit tumor cell growth inhibition effects *in vitro*[16,17]. In 2001, it was discovered that PTL directly targets Ikappa B kinase, providing a clear explanation for its anti-inflammatory mechanism of action[18]. NF-kB signaling is also involved in tumor progression[19], its activation suppresses the apoptotic potential of chemotherapeutic agents and contributes to drug resistance. It also reported that NF-kB inhibitors could sensitize metronomic chemotherapy[20].

Several chemicals have been reported to sensitize the efficacy of metronomic chemotherapy. However, whether PTL can sensitize CTX metronomic chemotherapy has never been investigated. Considering the reported characteristics of PTL, especially its strong effect on NF-kB inhibition, we hypothesized that PTL can enhance the efficacy of CTX. In the present study, we tested the biological effect of PTL on CTX both *in vitro* and *in vivo* and observed that PTL strongly enhanced the efficacy of CTX, partially by NF-kB inhibition.

MATERIALS AND METHODS

Cell line and mice

Mouse Lewis lung carcinoma (LLC) cell line was purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium containing 10% FBS at 37 °C in a humidified 5% CO_2 incubator as previously described[21]. For *in vitro* cell assays, LLC Cells were transfected with CYP2B6 constant express plasmid, and the expression of CYP2B6 was quantified by Q-PCR and Western blot. The cell medium was changed every day and the cells were used in their logarithmic growth phase for all experiments.

Male wild-type C57BL/6J mice were used for all experiments. Mice weighed 20-22 g were purchased from Kunming Medical University [Grade SPF II, SCXK(Yunnan)K2020-0004] and fed a standard laboratory diet with water ad libitum and were kept under constant environmental conditions in the Biological Center Lab of Kunming Medical University. All the experimental procedures were approved by the ethics committee of Kunming Medical University and performed following the institutional animal care guidelines and the ARRIVE guidelines.

Cell proliferation assay

LLC cells transfected with CYP2B6 were exposed to a culture medium with different concentrations of CTX and/or PTL. Cell viability was assessed by an Enhanced CCK8 Cell Proliferation Assay kit (Elabscience, Wuhan, China) according to the manufacturer's instructions. About 2 × 10^3 cells in 200 μL of cell culture medium were seeded into 96-well plates. After 24 hours of attachment, different concentrations of CTX and/or PTL were added to the culture medium. The cell viability was assayed every 24 hours.

PI/annexin double staining

Apoptosis was assayed by PI/annexin V double staining using a cell apoptosis assay kit (Elabscience, Wuhan, China) according to the manufacturer's instructions. A total of 5 × 10^4 cells were collected and resuspended in 195 μL Annexin V-FITC binding buffer and then stained with 5 μL Annexin V-FITC and 10 μL PI, then incubated at room temperature in the dark for 20 minutes. The apoptotic cells were monitored by flow cytometry assay using a flow cytometer (BD Bioscience).

Western blot assay

Protein expression levels were quantified by Western blot. In brief, total proteins were extracted using RIPA Lysis (Beyotime Biotechnology, Shanghai, China) according to the protocols provided by the manufacturer. Then, proteins were isolated by 10% SDS-PAGE and electro-transferred on PVDF membranes (Millipore, United States). Subsequently, the membranes containing proteins were seriatim incubated with the primary and secondary antibodies for the indicated time. Antibodies used in the experiment including CYP2B6 antibody (A1463, ABclonal, Wuhan, China) anti-NF-kB P65 (ab16502, Abcam, Cambridge, MA, United States), Anti-NF-kB p65 (phospho S536) (ab76302, Abcam), anti-β-Actin (E-AB-20034, Elabscience) and Goat Anti-Rabbit IgG (H + L) (peroxidase/HRP conjugated) (E-AB-1003, Elabscience). The protein bolts were developed by the Immobilon Western HRP Substrate (Millipore) and pictured with a chemiluminescence imager (Tanon, China), then quantified with Image J software (NIH, Bethesda, MA, United States).

NF-κB p65 transcription factor activity assay

Transcription factor activity for NF-kB was carried out with a commercial NF-κB p65 transcription factor activity assay kit (RayBiotech Inc., United States) which coded plate with NF-κB binding DNA sequence that specifically captured the active NF-κB p65 contained in whole cell lysate or nuclear extracts. The active NF-κB p65 was further quantified by NF-κB p65 primary antibody and HRP-conjugated secondary antibody. After a short incubation with CTX and PTL, the nuclear protein was extracted from 1 × 10^7 LLC cells using a Nuclear/Cytoplasmic Protein Extraction Kit (SINP001, Viagene Biotech Inc., United States). The Nuclear protein lysis was loaded onto the NF-κB p65 transcription factor activity detecting plate provided by the manufacturer, and the signal was detected according to the manufacturer's instructions.

Subcutaneous tumor inoculation

LLC cells (1 × 10^6 cells/mouse) re-suspended in serum-free DMEM were injected under the skin of C57BL/6 mice in the back or neck area. Fourteen days after tumor cell injection, the tumor-bearing mice were euthanatized by cervical dislocation, the tumor was harvested, segmented, and digested with collagenase IV (Sigma), about 1 × 10^8 cells were harvested after digestion, and 2 × 10^6 cells/mouse were subcutaneously injected in the back of 40 mice. Randomly distributed these mice into four groups, *i.e.*, control, CTX, PTL, and CTX + PTL group, each including 10 mice. After one week of the tumor cell implantation, the daily CTX and/or PTL administrated was performed by intragastric infusion and lasted for about 3 weeks. The survival of mice and the tumor size (measured with a vernier caliper) were recorded. When the tumor's long diameter reached 2 cm, the mouse was killed, and the tumor was isolated from the skin and weighed after exsanguination. The tumor mass was kept in a formalin solution and embedded in paraffin for Immunohistochemistry (IHC). Both the natural death and euthanasia of mice were recorded as death events in the Kaplan-Meier assay.

Immunohistochemistry assay

Immunohistochemistry assay was carried out as previously described[22]. Briefly, the paraffin section of tumor tissues from mice was dewaxed and rehydrated. Antigen retrieval was first carried out. Then tissue sections were quenched in 0.3% hydrogen peroxide and blocked using 5% goat serum. The slides were incubated with the primary antibody at 4 °C

overnight and then probed with HRP-conjugated secondary antibody at room temperature for 1 hour. Afterward, the slides were stained using diaminobenzidine (DAB). The presentation of dark brown was considered positive. Antibodies used in this section were as followed: Ant-CD31 (ab124432, Abcam), anti-F4/80 (A1256, ABclonal), Anti-alpha smooth muscle Actin [1A4] (ab7817, Abcam), Anti-Mannose Receptor/CD206 (ab64693, Abcam), Anti-NF-kB p65 (phospho S536) (ab76302; Abcam), Anti-transforming growth factor β (TGF β1) antibody (ab215715, Abcam), tumor necrosis factor α (TNF-α) antibody (A11534, ABclonal), IL-6 antibody (A0286, ABclonal), Goat Anti-Rabbit IgG (H + L) (peroxidase/HRP conjugated) (E-AB-1003, Elabscience) and Goat Anti-mouse IgG (H + L) (peroxidase/HRP conjugated) (E-AB-1001, Elabscience).

Statistical analysis

Survival data were analyzed using the Kaplan-Meier method, and survival curves were compared using the log-rank test in univariate analysis. A one-way ANOVA test was performed to evaluate the difference between groups. Two-group comparisons were performed using the Student *t*-test.

RESULTS

Overexpression of CTX metabolic enzyme P450 in LLCs

CTX is a prodrug that requires metabolic activation by cytochrome P450 enzymes in the liver to convert it into its active forms. There are currently 57 known human cytochrome P450 genes, which exhibit significant inter-individual genetic variation. CTX hydroxylation can be catalyzed by various P450 (CYP) enzymes, including CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, and CYP2J2[23-30]. Among them, CYP2B6 and CYP2C19 are the enzymes with the highest bioactivation activity for CTX[31]. Several studies have shown that overexpression of P450, especially CYP2B6, in tumor cells can enhance the sensitivity of tumor cells to CTX[32-35].

To evaluate the synergistic effect of PTL in combination with CTX *in vitro*, we developed a lung cancer cell line with constitutive expression of cytochrome P450 enzymes to enable efficient metabolism of CTX. We confirmed the overexpression of CYP2B6 in the P450-overexpressing (P540-OE) LLC cells at both the mRNA and protein levels (Figure 1A and B). While the wild-type LLC cells, human lung cancer cells (A549, H838, Hcc827, H1299, H1975, H1734, and XWCL-05), and normal bronchial epithelial cells (Beas-2B), expressed a very low level of P450 protein (Figure 1B and Supplementary Figure 1). It is worth noting that the expression of exogenous genes may impact cell growth. Therefore, to evaluate the inhibitory effect of CTX on P540-OE LLC cells, it is essential to account for any potential influence exerted by the exogenous expression of CYP2B6 on cell growth. The results of the CCK8 cell activity assay showed that P450 overexpression or vector transfection had no significant effect on cell growth during the 4-day growth process (Figure 1C). Therefore, in subsequent experiments, P540-OE LLC cells were used to evaluate the antitumor activity of PTL and CTX treatment regimens.

PTL enhanced cytotoxicity of CTX on P450 overexpressed LLC cells

To assess whether overexpression of cytochrome P450 sensitizes LLC cells to CTX, we used the CCK8 assay to evaluate cell proliferation activity. P450 overexpression significantly increased the sensitivity of LLC cells to CTX (Figure 2A and B). As the treatment time and concentration increased, there was a remarkable decrease in the proliferation activity of LLC P450-OE cells compared to the control group (Figure 2A and B). These findings indicate that P450 overexpression enhances the cytotoxic effect of CTX. By comparing the percentage of inhibition, we observed that the highest inhibitory effect was achieved at 48 hours of treatment (Figure 2C). Therefore, for subsequent experiments, we selected the 48-hour treatment as the optimal time for collecting cell samples.

To assess the cytotoxic effects of CTX and PTL on LLC cells, this study established a range of concentration gradients for individual treatments of CTX and PTL on P450-OE LLC cells. PTL inhibited the proliferation of P450-OE LLC cells in a dose-dependent manner within the concentration range of 2.5 uM to 20 uM (Figure 2D and E). Similarly, CTX also exhibited a dose-dependent inhibition of P450-OE LLC cell proliferation within a concentration range of 2.5 ug/mL to 20 ug/mL (Figure 2D and E). To determine the synergistic potential of combining these two drugs, we calculated the combination index (CI). Inhibition of cell proliferation was more pronounced when CTX and PTL were used in combination (Figure 2F). Specifically, when the inhibitory effect ranged from 0.2 to 1.0, the CI remained less than 1, and it gradually decreased as the inhibitory effect increased (Figure 2F). These findings collectively demonstrate that the combination of CTX and PTL produces a synergistic effect on cell toxicity.

To further elucidate the cytotoxic effects of the combined treatment of CTX and PTL, we examined the changes in apoptosis levels in P450-OE LLC cells when exposed to CTX and PTL individually as well as in combination. When administered alone, CTX at a concentration of 5 ug/mL or PTL at 5 uM significantly promoted apoptosis in P450-OE LLC cells. Specifically, the apoptosis ratio was 22.88% ± 1.67% for CTX at 5 ug/mL and 16.1% ± 1.62% for PTL at 5 uM (Figure 2G). Strikingly, the proportion of induced apoptosis dramatically increased to 48.57% ± 1.86% when the two drugs were combined (Figure 2G). Importantly, the overall level of apoptosis observed in the combined treatment group surpassed that observed with either drug alone. These findings underscore the enhanced efficacy of the combination therapy, suggesting a promising approach for targeted tumor cytotoxicity.

Further, this study evaluated the effects of 5 μg/mL CTX and 5 μM PTL individually as well as in combination on the viability of hepatic (LO2), alveolar (RLE-6TN), and renal (HK2) cells. As indicated in Figure 2H, 5 μg/mL CTX was significantly cytotoxic to LO2 and HK2, and 5 μM PTL enhanced the cell activity of LO2 and HK2 after 48 hours of treatment. Moreover, the combination of CTX and PTL alleviated the cytotoxicity of CTX on LO2 and HK2. Notably, CTX

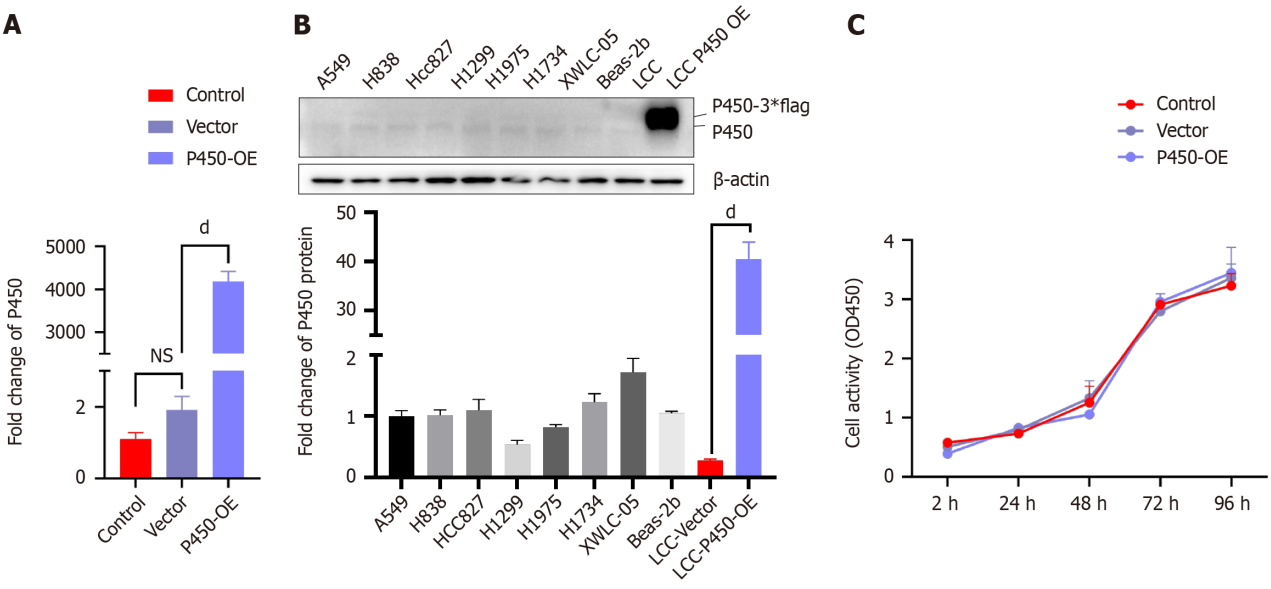

Figure 1 P450 expression detection in different cell lines and the effect of its overexpression on Lewis lung cancer cells cell activity. A: Q-PCR detection of P450 mRNA expression in Lewis lung cancer cells (LLC) cells transfected with P450 overexpression plasmid (P450-OE); B: Western blot detection of P450 protein in different cell lines. Full undamaged Gels and Blots images are shown in Supplementary Figure 1; C: CCK8 assay of LLC cells transfected with P450-OE plasmid or the vector plasmid. $n = 3$, $^{d}P < 0.0001$; NS: Not significant; LCC: Lewis lung cancer cells.

and PTL had no significant effect on the cell activity of RLE-6TN. This suggests that CTX is toxic to normal hepatic and renal cells and that its toxicity can be mitigated by combination therapy with PTL.

PTL improved the survival of tumor-bearing mice in combination with low-dose CTX

In a study conducted by Dynes *et al*[36], it was demonstrated that metronomic chemotherapy using a dosage of 20 mg/kg/day CTX in mice was effective. Therefore, for this study, metronomic chemotherapy was administered to mice with a CTX dose of 20 mg/kg/day. The reported anti-tumor dose of PTL ranged from 4 to 40 mg/kg[37-39]. Generally, doses of 4-12 mg/kg were administered intravenously or peritoneally, while a dose of 40 mg/kg/day was given *via* intragastric administration as per the former report[38]. For this study, the reference dosage of 40 mg/kg/day was employed for intragastric administration of PTL.

The animal experiment procedure is displayed in Figure 3A. A significant difference in survival among the four groups of mice is indicated in Figure 3B. Survival curve analysis revealed significant differences between the PTL and Control group, as well as between the CTX and Control group (Figure 3C and D). Notably, the PTL + CTX combination group exhibited the longest survival time, with 40% of tumor-bearing mice living extended to the end of the experiment (Figure 3B and E). Additionally, a statistical difference in survival was observed between the CTX treatment group and the CTX + PTL combined administration group (Figure 3E), indicating an enhanced tumor inhibition effect of the combination of PTL and CTX.

The tumor growth in the control group exhibited rapid progression, with a mean tumor diameter of 2 cm reached as early as day 19. While there were no significant differences in the tumor growth curves between the CTX-only, PTL-only, and CTX + PTL combined chemotherapy groups (Figure 3F), a noteworthy observation was the significant difference in tumor growth rates when normalized by growth time between the control group and the CTX + PTL combined groups (Figure 3G). Although the tumor growth rate in the PTL and CTX treatment group was lower than that in the control group, statistical analysis showed no significant difference, suggesting that the effect of PTL and CTX combined chemotherapy had a more favorable impact compared to their individual use.

Effects of PTL combined with CTX on tissue morphology, angiogenesis, and immune microenvironment of transplanted tumors

To further evaluate the impact of PTL combined with CTX on tumor tissue pathology, we conducted Hematoxylin and eosin staining on the collected tumor tissues. Compared to the control group, the CTX group displayed a significant amount of cell detachments and shrinkage within the tumor, accompanied by the presence of necrotic areas. In the PTL group, there were loose connections between tumor cells, along with a considerable amount of cell detachments and fragmented cell nuclei, indicating apoptotic tumor cell death. Notably, the PTL combined with the CTX group exhibited extensive necrotic regions within the tumor, along with disrupted tumor morphology and visible congestion, suggesting a more severe collapse of the tumor tissue structure (Figure 4A). A combination of PTL and CTX significantly enhanced CTX anti-angiogenesis effects, reduced the vascular density to nearly 50% percent (Figure 4B), and demonstrated a superior effect on tumor toxicity and angiogenesis inhibition compared to single CTX.

To assess the inhibitory effects of PTL combined with CTX on tumor graft growth and changes in the tumor immune microenvironment, we conducted IHC staining for macrophage marker genes CD206 and F4/80 in the tumor tissues.

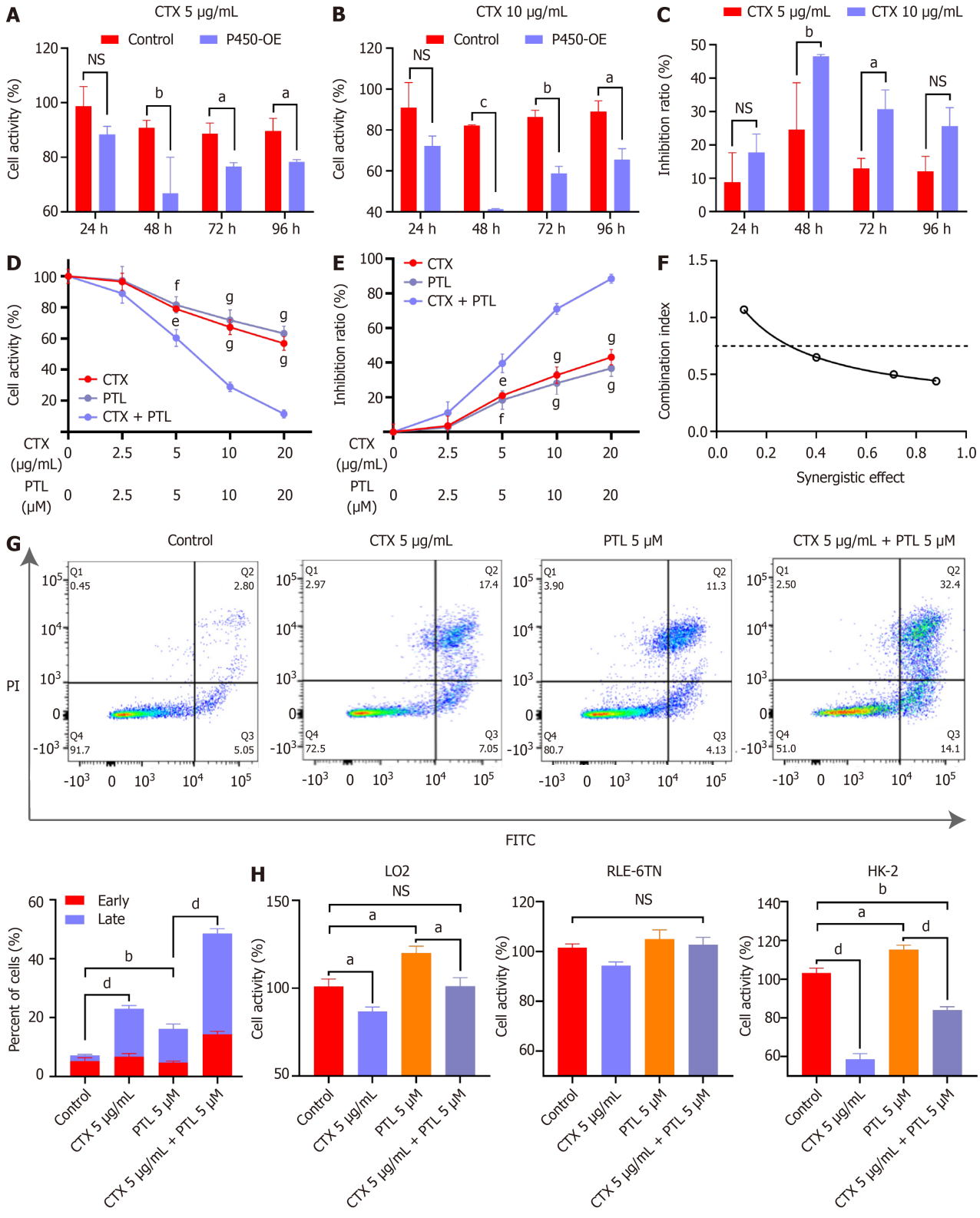

Figure 2 Parthenolide enhanced cytotoxicity of cyclophosphamide on P450 overexpressed Lewis lung cancer cells. A and B: P450 overexpression plasmid (P450-OE) and Control groups were treated with 5 ug/mL (A) or 10 ug/mL (B) cyclophosphamide (CTX), and its inhibitory effect on cell proliferation was evaluated using CCK8 assay; C: Comparison of proliferation inhibition percentage of P450-OE Lewis lung cancer cells (LLCs) treated with 5 and 10 ug/mL CTX; D: Cells were treated with different concentrations of CTX, Parthenolide (PTL), or a combination of the two drugs for 48 hours, and cell viability was detected by CCK-8 assay; E: Inhibition curve of different groups; F: The Combination Index was calculated using CompuSyn software; G: The apoptosis percentage of P450-OE LLC cells was evaluated after 48 hours of exposure to 5 μg/mL CTX and 5 μM PTL individually as well as in combination; H: CCK-8 evaluated the viability of LO2, RLE-6TN and HK-2 cells after treatment with 5 μg/mL CTX and 5 μM PTL individually as well as in combination for 48 hours. $n = 3$, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. Compared with CTX + PTL group, $^{e}P < 0.01$; $^{f}P < 0.001$; $^{g}P < 0.0001$; NS: Not significant. PTL: Parthenolide; CTX: Cyclophosphamide.

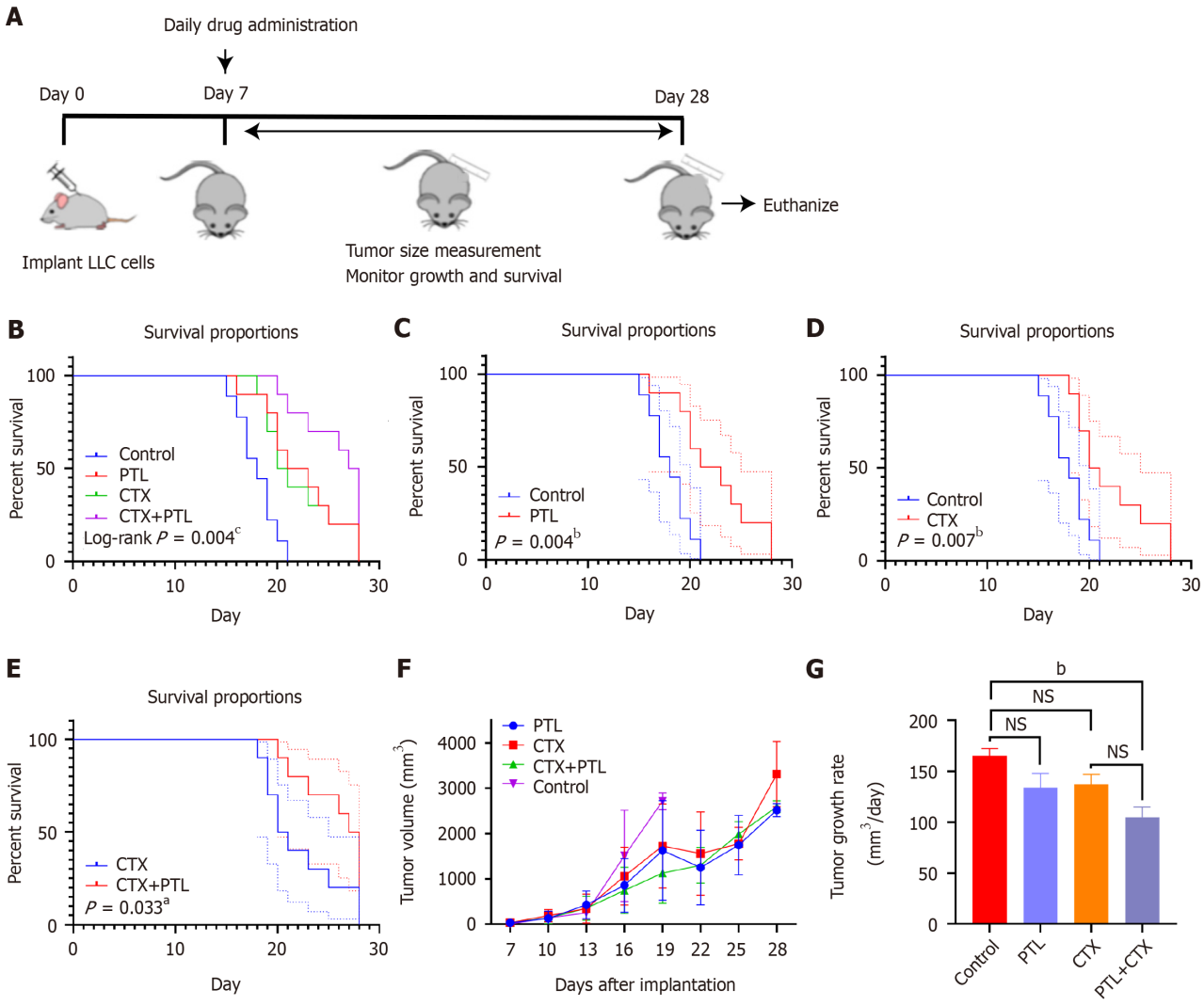

Figure 3 Survival analysis of tumor-bearing mice. A: Animal experiment process; B: Survival curve and log-rank analysis of 4 groups of tumor-bearing mice; C: Survival comparison between control and parthenolide (PTL) groups; D: Survival comparison between control and cyclophosphamide (CTX) chemotherapy groups; E: Comparison between CTX and PTL + CTX combined groups; F: Tumor growth curve of different groups; G: Comparison of tumor growth rate. n = 10, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. NS: Not significant; PTL: Parthenolide; CTX: Cyclophosphamide.

Additionally, we analyzed immune regulatory factors TGF-β, pro-inflammatory factors IL-6 and TNF-a, and invasive marker gene α-SMA expression in tumor tissues. The proportion of F4/80-positive cells significantly increased after PTL or CTX treatment. However, when the two drugs were combined and administrated, the proportion of the F4/80-positive cell population did not further increase (Figure 4C). The proportion of CD206-positive cells showed no significant differences among tumors of the four groups (Figure 4C). Hence, the increase of F4/80 positive macrophages might be due to the import of proinflammatory M1 cells, whereas PTL combined CTX administration did not enhance or decrease macrophage recruitment. Similar expression of the inflammatory factors (IL-6 and TNF-α) among the control group, the CTX treatment group, and the PTL treatment group. However, in the PTL combined with the CTX group, there was a slight decrease in the expression levels of IL-6 and TNF-α; however, this decrease was not statistically significant (Figure 5A). There was no significant change in the expression level of TGF-β in the tumor tissues of the CTX group when compared to the control group. In contrast, PTL treatment led to a significant down-regulation in the expression level of TGF-β (Figure 5A). Furthermore, the combination of PTL and CTX exerted a substantial inhibitory effect on the expression level of TGF-β in the tumor microenvironment (Figure 5A). CTX alone had no significant effect on the expression level of α-SMA (Figure 5B). PTL treatment alone significantly downregulated the expression level of α-SMA, and PTL combined with CTX also significantly inhibited the expression level of α-SMA (Figure 5B). Thus, the expression level of α-SMA is primarily influenced by PTL treatment.

In conclusion, these results indicated that PTL promotes CTX efficiency not only by acting on tumor cells but also by influencing the tumor microenvironment. PTL could enhance the angiogenesis inhibition effect of CTX. It also inhibited TGF-β and a-SMA expression in tumor tissue. Since the involvement of TGF-β and a-SMA in tissue reparation and tumor metastasis, the inhibition of them might contribute to the tumor sensitivity to CTX.

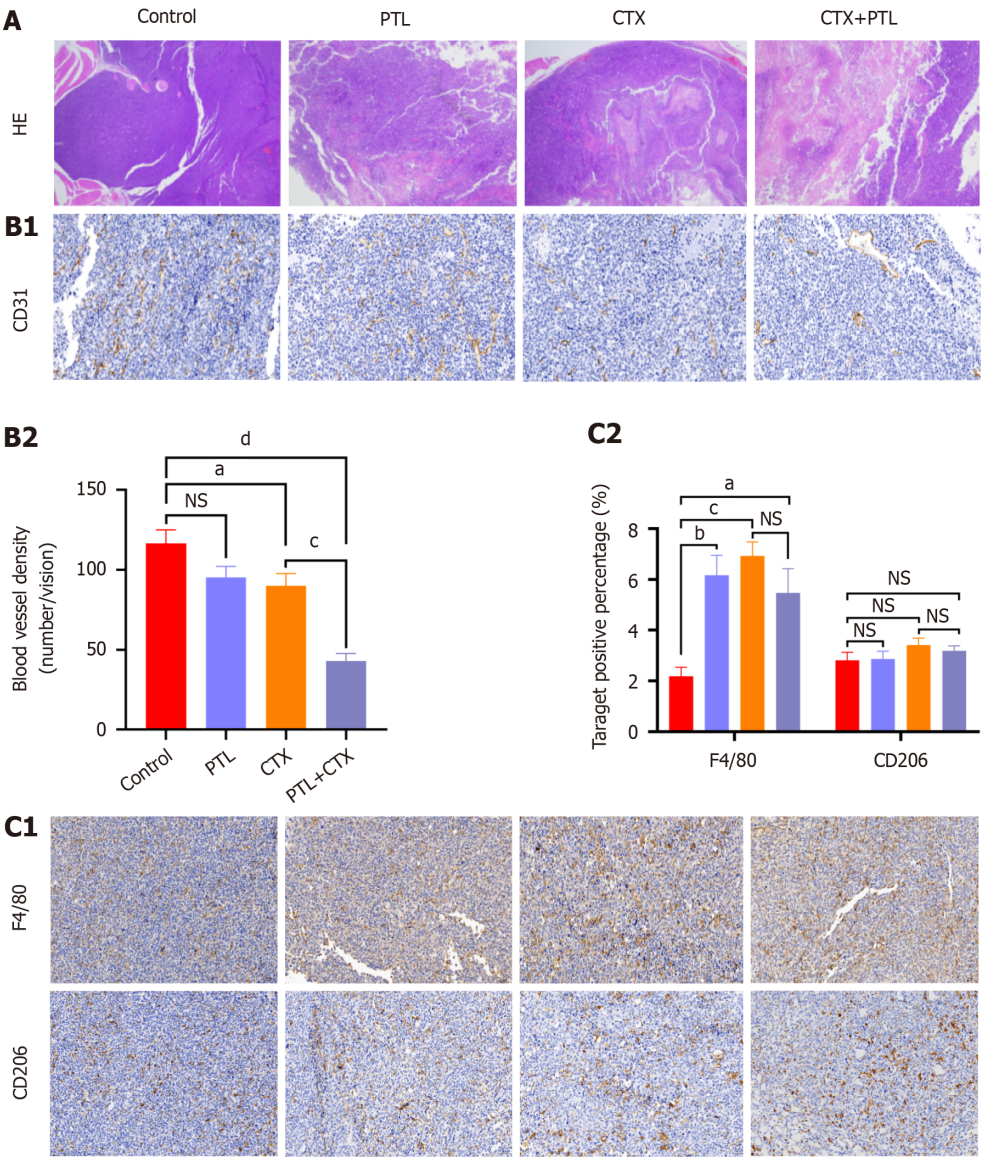

Figure 4 Effects of parthenolide combined with cyclophosphamide on tissue morphology and macrophage infiltration of transplanted tumors. A: Hematoxylin and eosin staining; B: CD31 staining and statistical results of tumor vascular density; C: F4/80 and CD206 staining in tumor tissues as well as comparison of F4/80 and CD206 positive cell proportion. Magnification: 200 ×. n = 10; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$; NS: Not significant. Con: Control; PTL: Parthenolide; CTX: Cyclophosphamide; HE: Hematoxylin and eosin staining.

PTL alleviated CTX-induced NF-kB activation

Many literatures have reported that the inhibition of PTL on NF-κB is the basis of its anti-tumor activity, and the activation of NF-κB leads to its transfer into the nucleus. CTX alone had no significant effect on the overall expression level of NF-κB in transplanted tumors, but could slightly increase (without significance) the proportion of NF-κB staining in the nucleus (Figure 6A). Compared to the CTX group, PTL combined with CTX significantly inhibited the expression level of NF-κB in transplanted tumor tissues and the transfer of NF-κB to the nucleus (Figure 6A), suggesting that PTL combined with CTX could inhibit the activation of NF-κB induced by CTX chemotherapy. To further verify the inhibitory effect of PTL on NF-κB in CTX, P450 OE LLC cells treated with CTX or PTL + CTX were collected and the expression of NF-κB was detected by Western blot. CTX did not induce the up-regulation of NF-κB p65 expression but could induce the phosphorylation of NF-κB p65 (Figure 6B). The up-regulation of p-NF-κB p65 induced by CTX could be down-regulated when PTL is combined with CTX (Figure 6B and Supplementary Figure 2). The results of the transcription factor activity assay further confirmed that PTL combined with CTX can inhibit CTX-induced NF-κB p65 transcription activity (Figure 6C). Therefore, PTL combined with CTX may have a synergistic anticancer effect by inhibiting CTX-induced NF-κB activation. Overall, these results demonstrated that parthenolide, as an NF-kB inhibitor, alleviated CTX-induced NF-kB activation, and thus may improve the sensitivity of LLC cells to CTX.

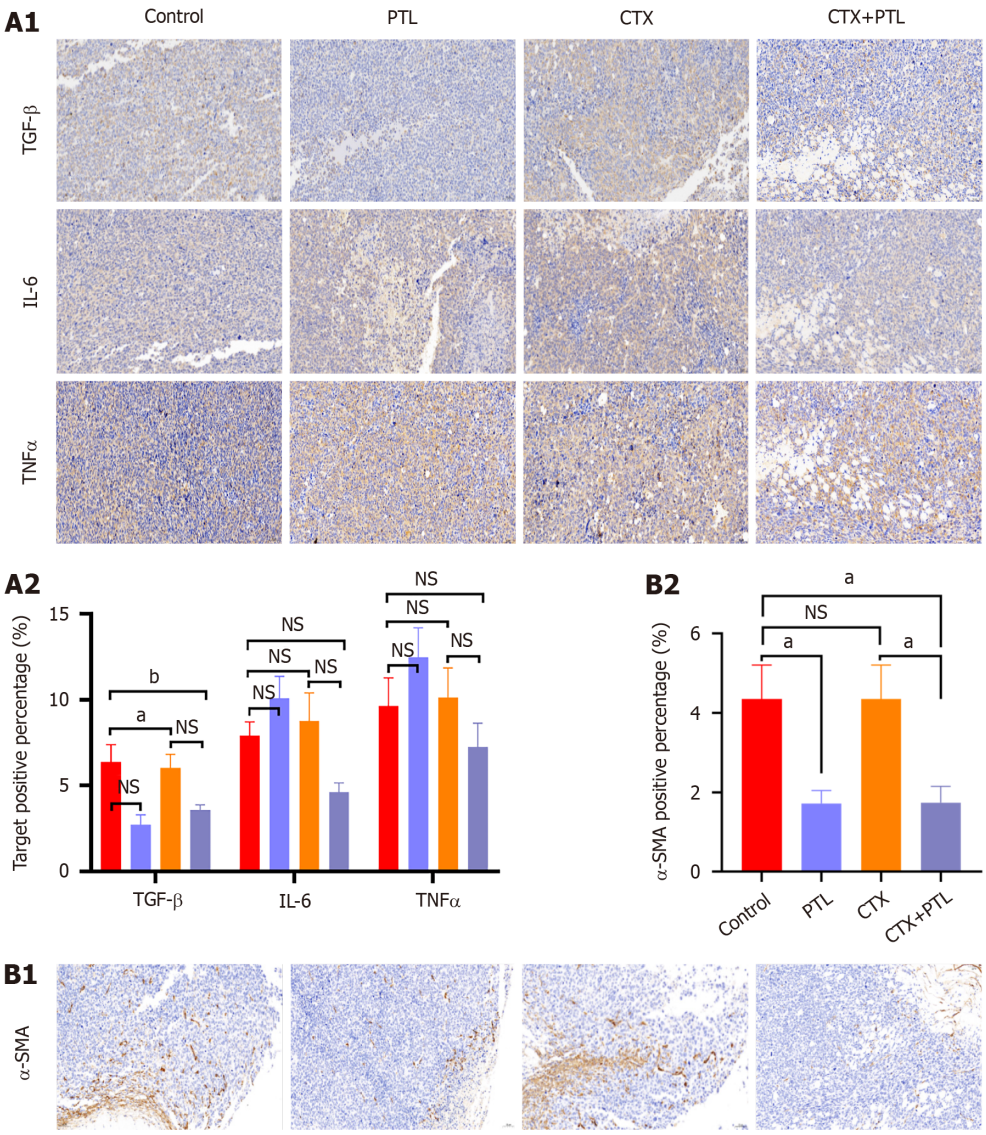

Figure 5 Influence of parthenolide combined with cyclophosphamide on the immune microenvironment in the transplanted tumor. A: Immunohistochemistry (IHC) staining of transforming growth factor β (TGF-β), IL-6, and tumor necrosis factor α (TNF-a), and the percentage of TGF-β, IL-6, and TNF-a positive cells in different treatment groups were compared; B: A-SMA IHC staining in tumor tissues. Magnification: 200 ×. n = 10; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$; NS: Not significant. PTL: Parthenolide; CTX: Cyclophosphamide.

DISCUSSION

There are a variety of ways for cancer therapy, including surgery, radiation therapy, chemotherapy, immunotherapy, and targeted drug therapy. And most of them relied on novel chemicals or combinations of chemicals. Conventional therapies including surgery, radiation therapy, and chemotherapy for cancer treatment have posed many challenges, including toxicity, and multidrug resistance. Newly developed therapies, such as immunotherapy and targeted drug therapy have other challenges such as high economic expenses[40]. Complementary alternative medicine by employing phyto-chemicals received increased attention because of their capability to modulate a myriad of molecular mechanisms with a less toxic effect. Phytochemicals can favorably inhibit several signaling pathways involved in cancer development and progression. Some combinations of phytochemicals promote cell death, inhibit cell proliferation and invasion, sensitize tumor cells, or boost the immune system, thus making them striking alternatives in cancer therapy. Some chemicals have also been proven to promote the efficiency of CTX, such as dexamethasone and celecoxib[41]. However, the role of natural products including phytochemicals in metronomic chemotherapy has not been widely investigated. Our present study proposed a novel combination of PTL and low-dose CTX to enhance the efficiency of metronomic chemotherapy. The present study demonstrated that the combination of CTX (5 μg/mL) and PLT (5 μM) enhanced the toxicity of CTX on LLC cells overexpressing P450 *in vitro*. In addition, CTX exhibited toxicity in normal hepatic and renal cells. This is consistent with previous reports[42,43]. Surprisingly, PLT alleviated CTX-induced activity inhibition of normal hepatic and renal cells. This suggests that PLT can effectively control the toxic and side effects of CTX on normal cells.

Though metronomic chemotherapy showed many advantages including minimizing side effects and reducing opportunities for acquired drug resistance, many signal pathways have still been activated to promote tumor cell survival, such as NF-kB. NF-kB has long been considered a double sword in cell death and cell survival[44]. Targeting

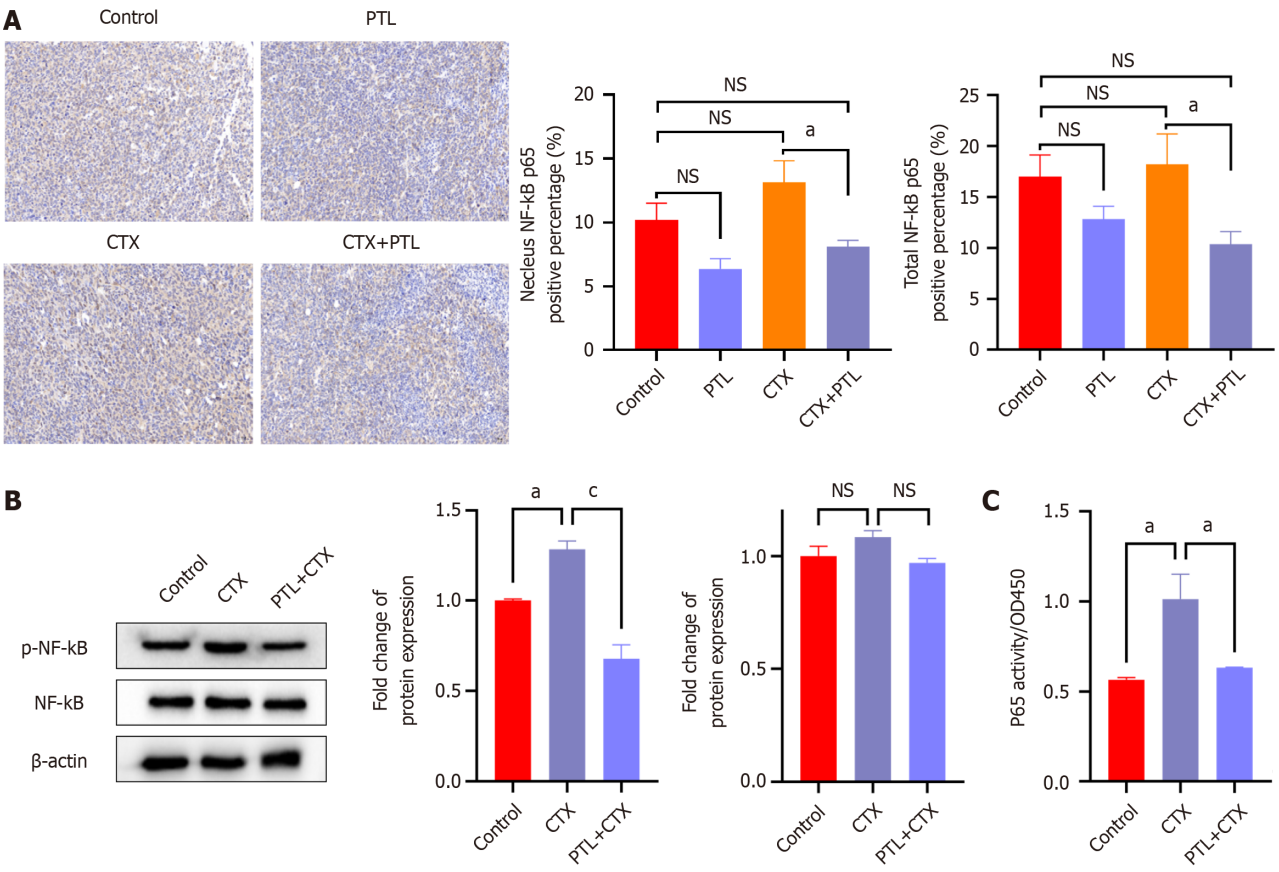

Figure 6 Parthenolide alleviated cyclophosphamide-induced NF-kB activation. A: NF-κB immunohistochemistry staining as well as comparison of the percentage of total and nuclei NF-κB positive cells in transplanted tumors of mice in different treatment groups. Magnification: 200 ×; B: P-NF-κB and NF-κB protein immunoblotting as well as gray analysis. Full undamaged Gels and Blots images are shown in Supplementary Figure 2; C: Analysis of NF-κB P65 transcriptional activity. n = 10, $^{a}P < 0.05$; $^{c}P < 0.001$; NS: Not significant. PTL: Parthenolide; CTX: Cyclophosphamide.

nuclear factor-kappa B can be used to overcome resistance to chemotherapy[45]. However, whether NF-kB inhibition boosts CTX efficiency has never been investigated. Our present study showed that parthenolide, which is a well-accepted NF-kB inhibitor, significantly increased the efficiency of CTX to kill tumor cells either *in vitro* or *in vivo*. The results showed in our present study will help scientists and clinicians draw up novel metronomic chemotherapy strategies.

The schedule of standard therapy with CTX was a 21-day cycle of either 100 or 150 mg/kg CTX, administered intraperitoneally once every other day over 6 days. And 10-40 mg/kg daily are considered low dose CTX[46]. Our present study showed that even at low doses, CTX can activate NF-kB (shown in Figure 6). While PTL significantly attenuated NF-kB activity induced by CTX. These results reminded us that NF-kB inhibition in CTX may be useful clinically to promote the therapy efficiency. This is also consistent with previous studies that CTX can activate NF-kB in other cells and tissues[47], and inhibition of NF-kB activation by CTX was involved in the reduction of side effects.

Angiogenesis is critical for solid tumor formation[48], and vascular formation is even considered the hallmark of solid tumors[49]. NF-kB activation has been reported to be critical in tumor angiogenesis[50]. Though the efficacy of CTX can be significantly increased when administered in combination with anti-angiogenic drugs[51], whether NF-kB inhibition can inhibit angiogenesis in CTX has never been investigated. Our present study showed that NF-kB inhibition in CTX significantly reduced the CD31 positive staining in the tumors, indicating reduced angiogenesis.

In many solid tumor types, tumor-associated macrophages are important components of the tumor microenvironment. And macrophage infiltration is strongly associated with poor survival in solid tumor patients[52]. Here we demonstrated that CD206 positive M2-like tumor-associated macrophages were not reduced by either CTX or PTL administration, but both CTX and PTL increased the total F4/80 positive cells, this indicated the infiltration of CD206 negative macrophages, possibly M1 macrophages. Thus, CTX or PTL administration might boost the immune system. However, we did not observe the synergistic effect of the two drugs in macrophage recruitment. Proinflammatory factors IL-6 and TNF-a also have no significant difference among groups. Overall, these results indicate that PTL combined with CTX did not synergistically enhance the inflammatory reaction.

TGF-β expression in tumors enables cancer cells to undergo epithelial-to-mesenchymal transition and correlates with chemoresistance[53]. TGF-β expression in tumors hinders the release of cancer cell antigens, subverts dendritic cell function, reduces T cell trafficking and infiltration to tumors, antagonizes recognition of cancer cells by T cells, and hinders the killing of cancer cells[54]. TGF-β expression was directly or indirectly related to the treatment activity. High levels of TGF-β are associated with therapeutic resistance[55]. Elevated levels of TGF-β are associated with desmoplasia, which results from abnormal signaling of the TGF-β pathway and the remodeling of the microenvironment[54].

Desmoplasia refers to the dense extracellular matrix (ECM) composed of fibrous collagen, hyaluronic acid, fibrin, proteoglycan, and tendinous protein C[56], which can compress the blood vessels within the tumor, resulting in hypoperfusion, thus obstructing the delivery of therapeutic drugs, and renders the tumor hypoxic and drug resistant[54]. Here, our results indicated that NF-κB inhibition with PTL reduces TGF-β expression, which might render cancer cells sensitive to CTX.

α-SMA is another ECM and tissue-remolding-associated gene in cancer progression. Tumor cells that express α-SMA are predicted to be the cells that have an invasive nature and tend to metastasize[17]. Here we demonstrated that NF-kB inhibition with PTL reduced the proportion of α-SMA positive cells. It might be another mechanism of PTL's synergistic effect on CTX.

CONCLUSION

Thus, our present study found that NF-kB inhibition in CTX is not only involved in the balance of cell survival and cell death of tumor cells but also involved in the tumor microenvironment by reducing angiogenesis, macrophage infiltration, and inhibiting metastasis. In conclusion, our present study for the first time finds that as a specific inhibitor of NF-kB, PTL can promote the efficiency of CTX in lung cancer *via* inhibiting NF-kB signaling *in vitro* and *in vivo*. By directly promoting the cytotoxicity of CTX or improving the tumor microenvironment. Our present study will help scientists and clinicians to draw up novel metronomic chemotherapy strategies.

FOOTNOTES

Author contributions: Cai Z and Wang QM conceived and designed the experiments; Cai Z, Gao L, Hu K, and Wang QM performed the experiments; Cai Z and Gao L analyzed and interpreted the data; Hu K, and Wang QM contributed reagents/materials/analysis tools; Cai Z wrote original draft; Gao L, Hu K, and Wang QM reviewed and edited draft; All authors have read and agreed to the published version of the manuscript.

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