

RESEARCH ARTICLE

Kanglaite upregulates CD4+ T cells by inhibiting the expression of IL-1 β in A549 cells

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Abstract

Background: Superior clinical efficacy and safety have been demonstrated in Kanglaite combined with cisplatin compared with cisplatin- or Kanglaite-alone treatment on advanced non-small cell lung cancer (NSCLC). However, the mechanism of how Kanglaite enhances antitumor effect of cisplatin is not fully addressed.

Methods: An A549 tumor-bearing mouse model was applied in this study. The tumor-bearing mice were treated with 0.9% saline, Kanglaite-alone, cisplatin-alone, or Kanglaite and cisplatin combination. Immune cell population was determined by flow cytometry assay. The production of cytokine was measured by ELISA assay.

Results: The results demonstrated that Kanglaite and cisplatin combination treatment exerted the most potent effects on reducing tumor growth and prolonging mouse survival. Further studies revealed that the upregulation of CD4+ T cells and downregulation of IL-1 β might be responsible for the Kanglaite-promoted antitumor effect of cisplatin on A549 tumor.

Conclusion: Kanglaite serves as an excellent supplement for cisplatin in treating patients with advanced NSCLC.

Keywords: *Kanglaite; antitumor; cisplatin; NSCLC*

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Because of lacking specific symptoms and aggressive tumor growth, 85% of all diagnosed lung cancer cases are advanced or late-stage non-small cell lung cancer (NSCLC) (1). Lung tumors already metastasis to the extrathoracic organs, including liver, bone, brain, and Kidney, in the late-stage NSCLC cancer patients. Thus, the prognosis of NSCLC patients is dismal, with a 5-year overall survival rate of less than 5% (2, 3).

Platinum-based chemotherapies, such as cisplatin or carboplatin, have been used as a first-line drug for the treatment of most patients with advanced NSCLC (4). Because platinum compounds can target nuclear DNA causing cytotoxic effects to all rapid growth cells, they can effectively kill cancer cells while also attack normal cells, leading to severe side effects (5, 6). Besides, platinum-based drug

resistant is commonly observed in patients with lung cancer who received several cycles of platinum-based chemotherapy treatments. Therefore, platinum-based combination treatment therapies yield better responses and enhanced overall survival than single drug treatment therapies.

Recently, traditional Chinese medicine is being used to combine with conventional chemotherapy drugs to mitigate side effects, improve curative effects, and enhance quality of life. As a Chinese medicine extracted from Semen Coicis Yokuinin, Kanglaite injection (Kanglaite) employed modern advanced pharmaceutical technology (7). Kanglaite was approved by China Food and drug administration (CFDA) for lung cancer treatment in 2010. Numerous studies have proved that Kanglaite has a synergistic effect with cisplatin chemotherapy and has noticeable anti-malignant pathogenic

and analgesic effects on patients with advanced lung cancer (8, 9). However, the underlying mechanism of Kanglaite-promoted antitumor effect of cisplatin is not fully addressed.

Materials and methods

Cell culture

The A549 cell line was obtained from ATCC (Manassas, VA) and was cultured in Dulbecco's Modified Eagle Medium supplied with fetal bovine serum (10%, Gibco, Grand Island, NY). Cells were maintained in an incubator with 5% CO₂ at 37°C.

Animal model

A549 cells (5×10^6) were subcutaneously injected into the left flank of the BALB/c nude mice. Then, tumor-bearing mice were randomly split into four groups ($n = 20$ mice per group). Group 1 mice were treated with 0.9% saline; group 2 mice were given Kanglaite (10 mg/kg) by intraperitoneal injection every 3 days; group 3 mice were intraperitoneally injected with cisplatin (5 mg/mL) every 3 days; and group 4 mice were received intraperitoneal injection of Kanglaite (10 mg/kg) and cisplatin (5 mg/mL) every 3 days. Mouse survival was recorded. Tumor tissue was collected from each mouse, tumor size was measured, and tumor weight was recorded. This study was approved by the LONGHUA Hospital Shanghai University of Traditional Chinese Medicine.

Flow cytometry analysis

Total peripheral blood mononuclear cell (PBMC) isolated from each mouse was blocked with Fc antibody in cold phosphate-buffered saline (PBS) buffer for 15 min. Then, cells were stained with mixed antibodies (anti-NK 1.1, anti-CD3, anti-CD19, anti-CD8, and anti-CD4) in cold staining buffer for 30 min. Cells were washed three times with cold PBS and were subjected to flow cytometry analysis using the FACSCalibur™ platform.

ELISA

Serum levels of interleukin (IL)-10, tumor necrosis factor (TNF)- α , IL-1 β , and IL-6 were determined by corresponding ELISA kits purchased from RayBiotech (GA, USA).

Quantitative reverse transcription PCR (RT-qPCR)

Total RNA from decidua was isolated using TRIzol reagent (ThermoFisher Scientific, Waltham, MA). 1 μ g RNA was synthesized into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). RT-qPCR amplification was performed on a CFX Opus Real-Time PCR Systems (BioRad, Hercules, CA) using the SYBR™ Green PCR Master Mix (ThermoFisher Scientific) with cDNA and primers.

Statistical analysis

Data were shown as mean \pm standard error of mean (SEM). One-way ANOVA analysis with a Tukey's post hoc test was applied to assess the difference between two groups. A linear regression approach was used to determine the correlation of serum level of IL-1 β and CD4+/CD8+ T cells ratio.

Results

Kanglaite enhanced the antitumor effects of cisplatin on A549 lung cancer mouse model

To confirm that Kanglaite can enhance the antitumor effect of cisplatin on lung cancer, we established a lung tumor mouse model by subcutaneous injection of A549 cells into nude mice. Five days after tumor implantation, tumor-bearing mice were treated daily with 0.9% saline, Kanglaite-alone, cisplatin-alone, or a combination of Kanglaite and cisplatin (K+C). As depicted in Fig. 1A–C, cisplatin treatment significantly reduced A549 tumor size and weight as well as prolonged the survival probability of mice, while Kanglaite-alone had negligible effects on tumor growth and mouse survival. Notably, the combination of Kanglaite and cisplatin exhibited markedly stronger effects on tumor growth inhibition and survival probability extension than cisplatin-alone, suggesting that supplementation of Kanglaite can promote cisplatin-induced antitumor effect on A549 tumor-bearing mice.

Upregulation of CD4+T cells might be responsible for Kanglaite-promoted antitumor effect of cisplatin

To explore which immune cell population is involved in Kanglaite-promoted antitumor effect of cisplatin. Peripheral blood samples were collected from each mouse and were, respectively, stained with three sets of antibodies to define NK cells (NK1.1 positive and CD3 negative), B cells (CD19 positive and CD3 negative), and CD4+/CD8+ T cells (CD4 and CD3 double-positive or CD8 and CD3 double-positive). The results in Fig. 2A–B presented that both NK cells and B cells were only slightly increased in the combination treatment (K+C) group compared to the cisplatin treatment group. Importantly, significant upregulation of CD4 T cells and downregulation of CD8 T cells were observed in the combination treatment (K+C) group compared to the cisplatin treatment group, leading to substantially enhanced CD4+/CD8+ T cell ratio. These data suggested that CD4+ T cells might play the predominant role in the regulation of Kanglaite-promoted antitumor effect of cisplatin.

IL-1 β downregulation was identified in Kanglaite and cisplatin combination-treated group

To find out which cytokine is responsible for Kanglaite-augmented antitumor effect of cisplatin. We

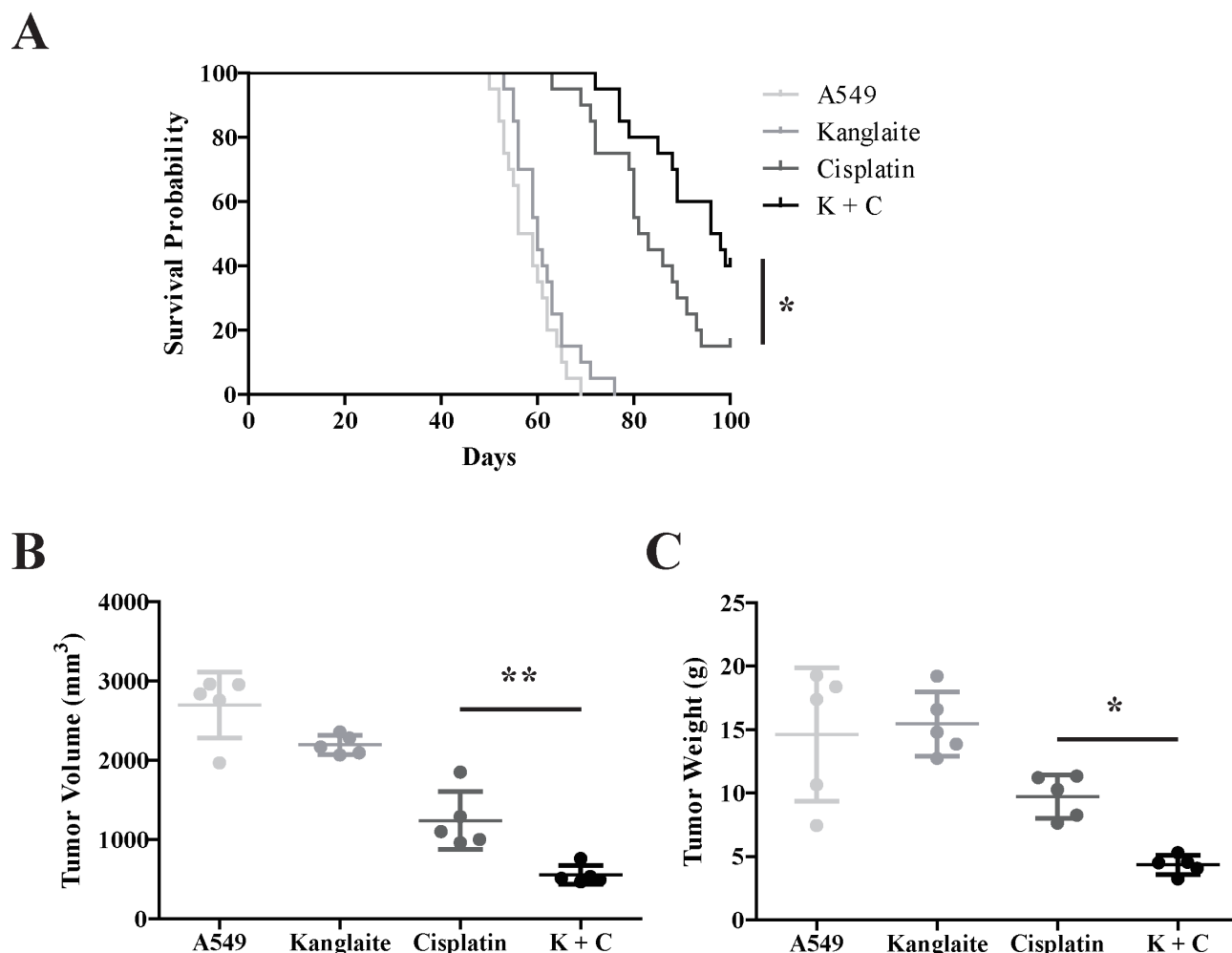


Fig. 1. Effects of Kanglaite on the growth of A549 cell-transplanted tumor in nude mice. Mice were subcutaneously injected with 5×10^6 A549 cells. (A) Survival probability was shown in the indicated group ($n = 20$ for each group). (B) Tumor size was measured every 5 days and the volume at day 50 was shown in the indicated group. (C) Tumor weight at day 50 was shown in the indicated group. Data shown as mean with SEM ($n = 5$) and $*P < 0.05$ and $**P < 0.01$.

measured the expression levels of TNF- α , IL-10, IL-6, and IL-1 β in the serum samples from different groups of mice. As illustrated in Fig. 3A–D, the mean expression levels of TNF- α , IL-10, and IL-6 were comparable between the cisplatin treatment group and the combination treatment (K+C) group. Interestingly, the IL-1 β expression levels were considerably lower in the combination treatment (K+C) group than that in the cisplatin treatment group. These results implied that Kanglaite promoted the antitumor effect of cisplatin via regulating IL-1 β levels.

Serum IL-1 β levels were negatively correlated with CD4+/CD8+ T cell ratio

Based on the data from Figs. 3 and 4, we hypothesized that Kanglaite might regulate CD4 and CD8 T cells ratio by affecting IL-1 β levels in the combination-treated (K+C) group. To address this hypothesis, we performed

a linear regression analysis of serum IL-1 β levels and CD4+/CD8+ T cell ratio. The results showed a negative correlation between serum IL-1 β levels and CD4+/CD8+ T cell ratio, implying that IL-1 β could negatively regulate CD4+/CD8+ T cell ratio.

Downregulation of IL-1 β was observed in tumor tissue

To further identify which cells/tissues expressed the downregulated IL-1 β , which was mediated by Kanglaite, we collected total mRNA from blood lymphocytes, spleen lymphocytes, and tumor tissues. The relative mRNA levels of IL-1 β in these cells/tissues were determined by RT-qPCR. The results in Fig. 5A–C exhibited that the IL-1 β mRNA levels were substantially reduced in tumor tissues, but not in blood lymphocytes or spleen lymphocytes, from combination-treated (K+C) group compared to cisplatin-treated group. These results

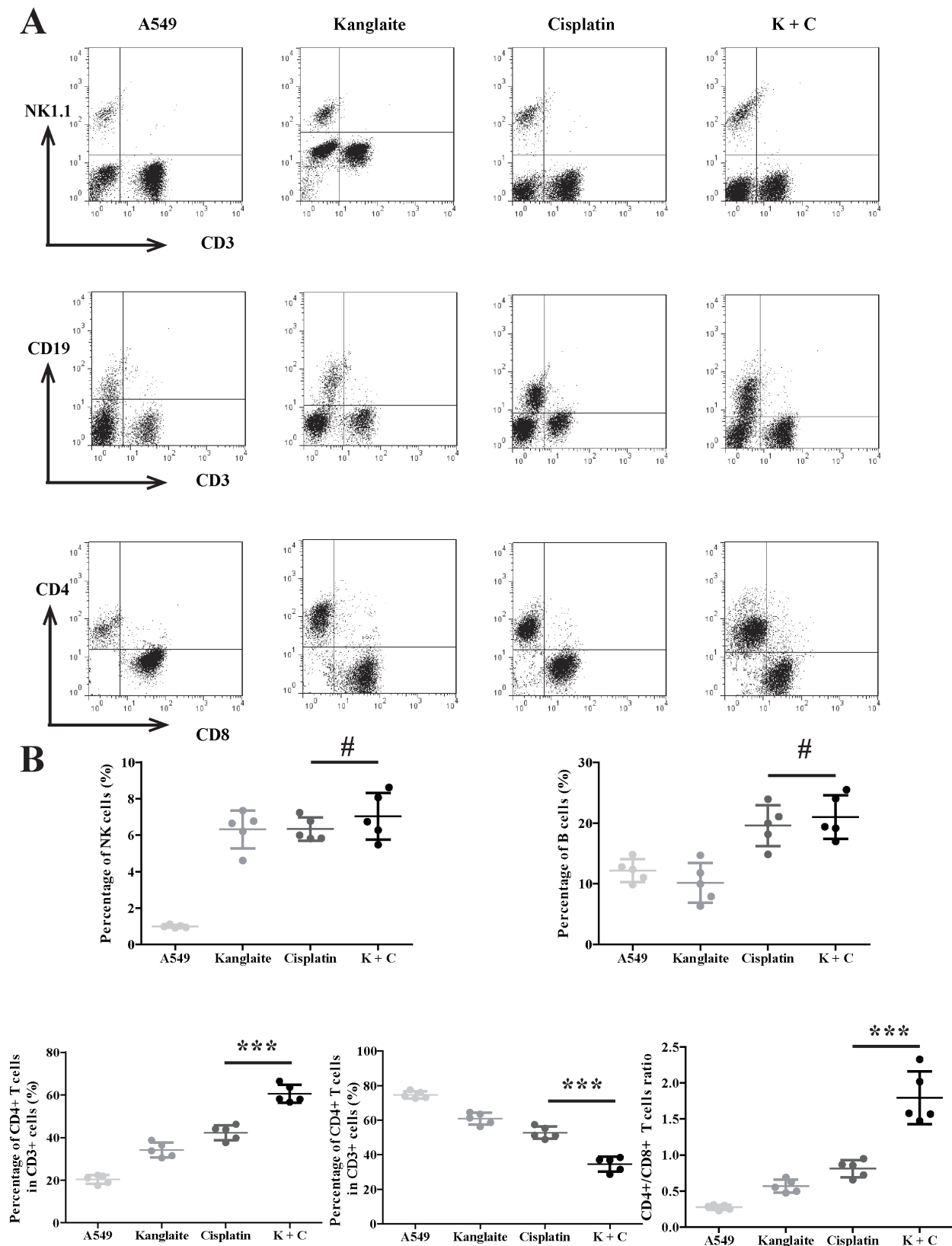


Fig. 2. Effects of Kanglaite on the relative percentages of host lymphocyte subgroups in the blood. (A) The percentages of natural killer cells (CD3-NK1.1+), B cells (CD3-CD19+), CD4+ T (CD3+ group and CD4-CD8-), and CD8+ T cells (CD3+ group and CD4-CD8+) in the peripheral blood of mice in each group were determined by flow cytometry. (B) Statistic analysis of natural killer cells, B cells, CD4+ T cells, CD8+ T cells, and CD4+/CD8+ ratio was shown. Data shown as mean with SEM ($n = 5$) and $\#P > 0.05$ and $***P < 0.001$.

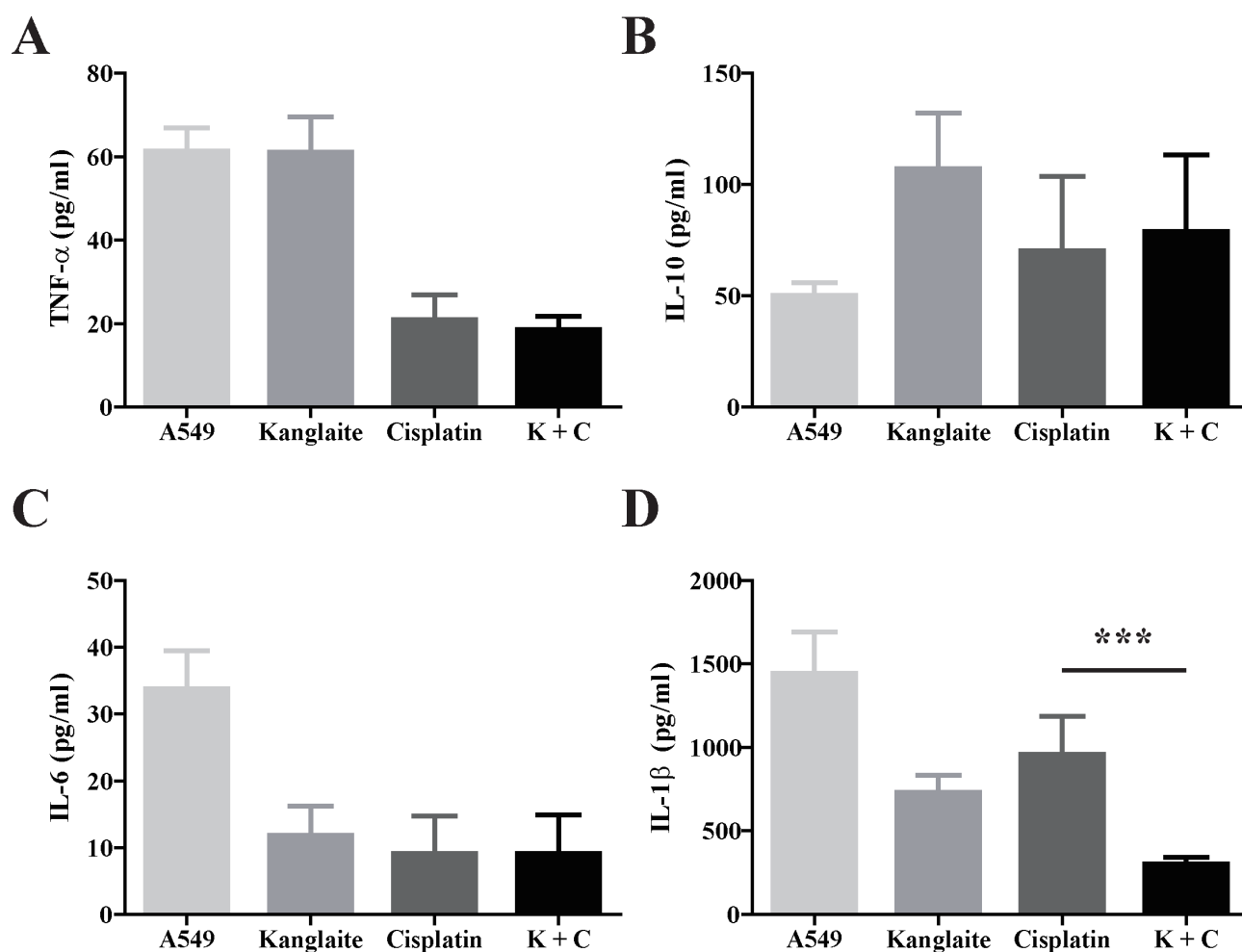


Fig. 3. Effects of Kanglaite on the serum concentration of the cytokine in the mice model. Serum level of TNF- α (A), IL-10 (B), IL-6 (C), and IL-1 β (D) was determined by ELISA. Data shown as mean with SEM ($n = 5$) and *** $P < 0.001$.

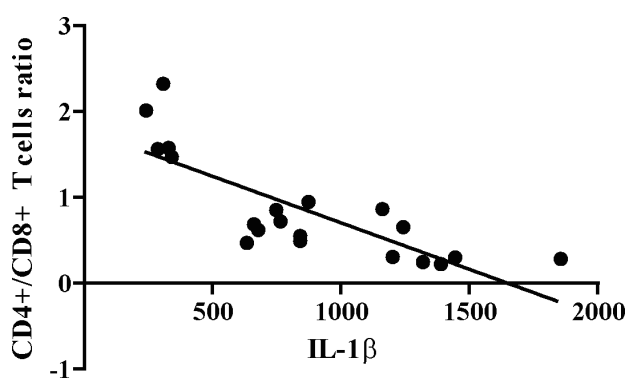


Fig. 4. The serum level of IL-1 β and CD4+/CD8+ T cells ratio was negatively correlated on the treatment of Kanglaite. The serum level of IL-1 β in Fig. 3 and the CD4+/CD8+ T cells ratio in Fig. 2 were analyzed using linear regression, and they were negatively correlated. $R^2 = 0.6410$ and $P < 0.0001$.

suggested that Kanglaite specifically downregulated IL-1 β expression in tumor tissues without affecting circulating lymphocytes.

Discussion

Traditional chemotherapy drugs can target both cancer cells and normal cells, leading to various unfavorable side effects to cancer patients. Reduced immunity of the cancer patients is one of the major adverse effects of chemotherapy, which might cause cancer patients vulnerable to exogenous bacterial and viral infections (10, 11). Thus, identifying a supplement that not only processes antitumor effect but also serves as an immune system booster is highly desirable. Chinese herbals have been used as traditional medicine mainly in Asian countries for treatment of various cancers for thousand years (12). Recently, more and more traditional Chinese medicine has been used as a supplementary to modern chemotherapy drugs. Accumulating evidence has shown encouraging clinical outcomes, such as enhanced antitumor effects, reduced side-effects, decreased drug resistance, and strengthened immunity of patients (13).

Numerous studies demonstrated superior clinical efficacy and/or safety in patients with advanced NSCLC

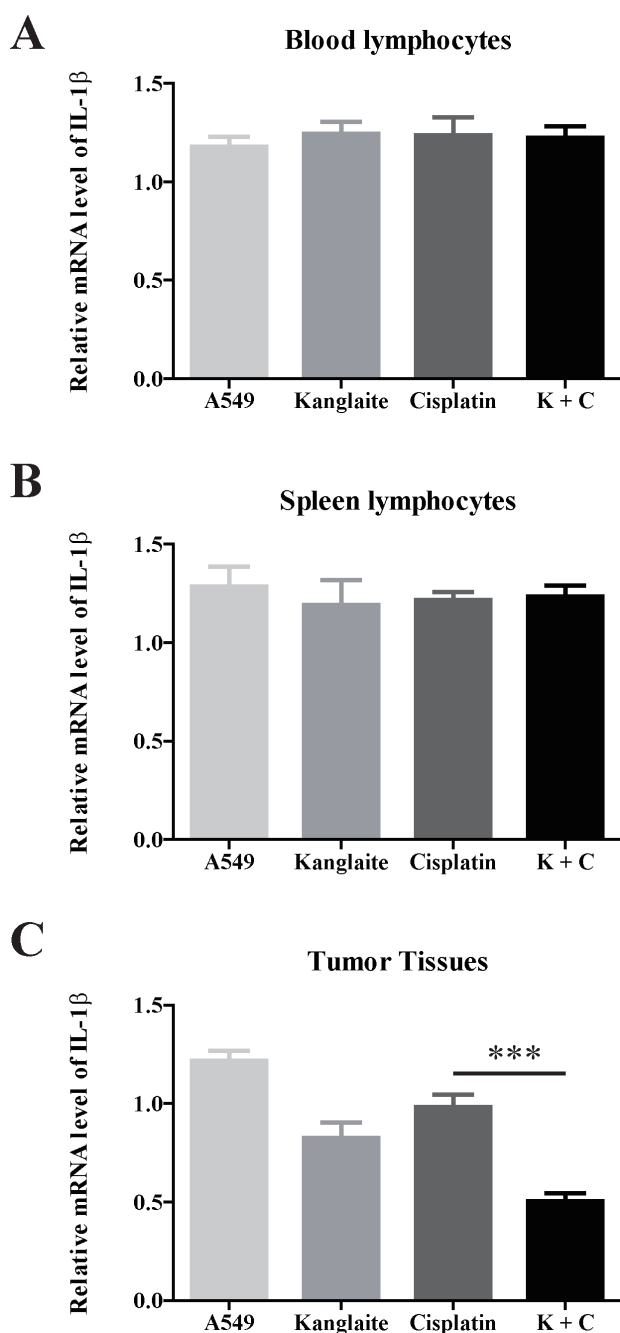


Fig. 5. Kanglaite reduced the IL-1 β expression in A549 cells in vivo. The relative expression level of IL-1 β in blood lymphocytes (A), spleen lymphocytes (B), and tumor tissue (C) was detected by real-time PCR. Data shown as mean with SEM ($n = 5$) and *** $P < 0.001$.

when Kanglaite was combined with a chemotherapy drug, including cisplatin, vinorelbine, gefitinib, and gemcitabine, compared to single chemotherapy drug treatment (14). The enhanced clinical efficacy for NSCLC patients treated with a combination regimen was demonstrated by reduced tumor size, prolonged overall survival rate, and reduced adverse events, such as vomiting, nausea, thrombocytopenia, and leukopenia (15, 16). The

enhanced antitumor effect in Kanglaite combined with chemotherapy drug may attribute to enhanced immune system in NSCLC patients. Because significant upregulation of peripheral blood T lymphocytes subsets (CD3+ T cells, CD4+ T cells, and CD8+ T cells) and Natural killer cells, as well as peripheral blood immunoglobulins (IgA, IgG, and IgM), are identified in Kanglaite and chemotherapy drug combination treatment arm compared to single treatment arm (17–19). In our study, by using a A549 tumor-bearing mouse model, we proved that Kanglaite and cisplatin combination treatment exhibited more potent antitumor effects than Kanglaite or cisplatin single treatment. More importantly, we revealed that CD4+ T cell population, but not CD8+ T, NK cells, or B cells, was markedly upregulated in the Kanglaite and cisplatin combination treatment group compared to the Kanglaite or cisplatin single treatment group, implying that increased CD4+ T population plays a critical role in killing A549 cells in nude mice. However, we cannot exclude the possibility that other immune cells (e.g. CD8+ T cells, NK cells, or B cells) may play a major role against tumor cells when applied to different animal models treated with Kanglaite cisplatin combination therapy.

A previous study reported that Kanglaite exerted immunostimulatory and antitumor effects on partially through promoting IL-2 expression (20). Similarly, another study showed that Kanglaite enhanced antitumor effect of cisplatin in HepG2-tumor-bearing nude mice. They found Kanglaite and cisplatin combination treated mice had higher IFN- γ and IL-2 as well as CD4+ T cell population than Kanglaite- or cisplatin-treated mice (21). Interestingly, we identified IL-1 β was significantly downregulated in the combination-treated group when compared to the single-treated group. However, the detailed mechanisms of how IL-1 β modulates CD4+/CD8+ T cell population and how IL-1 β downregulation enhances antitumor effect require further investigation.

Conclusion

In this study, we confirmed that Kanglaite combined with cisplatin exhibited preferable effects on inhibiting A549 tumor growth in mice, leading to prolonged mice survival. Upregulated CD4+ T cell and downregulated IL-1 β were observed in Kanglaite and cisplatin combination-treated group, implying that Kanglaite promoted antitumor effect of cisplatin through modulating IL-1 β production and CD4+ T cell population.

Conflict of interest and funding

The authors declare that they have no conflicts of interest. This work was supported by the National Natural Science Foundation of China (82174199).

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