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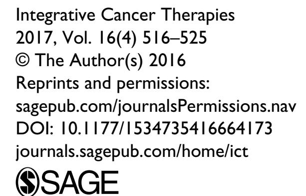

# Antitumor Activity of the Chinese Medicine JC-001 Is Mediated by Immunomodulation in a Murine Model of Hepatocellular Carcinoma

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## Abstract

JC-001 is a Chinese medicine that has been used to treat liver disease; however, its significance in cancer treatment has not been characterized. In this study, we used an immunocompetent tumor model to characterize the antitumor activity of JC-001. A total of 48 Hepa 1-6 tumor-bearing C57BL/6 mice were randomly grouped into 4 groups and treated with $H_2O$ or JC-001 via oral administration. After hepatoma cell lines, including HepG2, Hep3B, SK-Hep-1, and Hepa 1-6, underwent 96 hours of JC-001 treatment, a low cytotoxic effect was observed. In contrast, no direct cytotoxic effect of JC-001 on a normal human liver cell line, THLE-3, was observed under the same incubation conditions. Using a murine tumor model, we found that tumor growth could be inhibited by JC-001 in C57BL/6 mice but not in immunodeficient mice. Histopathological analysis of tumors from C57BL/6 mice revealed immune cell infiltration in tumors from the JC-001–treated group, as observed by hematoxylin and eosin staining; in addition, Ki67, hypoxia-inducible factor-1-α, and high mobility group box 1 expression levels were suppressed in the tumors. Both the coculture assay and murine spleen mRNA quantitative PCR analyses demonstrated that JC-001 could suppress Th17 immunity. Our data suggest that JC-001 is a Chinese medicine with low cytotoxicity that can significantly suppress tumor growth by immune regulation. This herbal remedy has great potential for future clinical application in hepatoma therapy.

## Keywords

Chinese medicine, Hepa 1-6, immunomodulation, JC-001, splenocytes, coculture, hepatocellular carcinoma

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## Introduction

Hepatocellular carcinoma (HCC), which has an extremely poor prognosis, is one of the deadliest cancers worldwide. The major factors associated with HCC include chronic hepatitis B and C viral infection, alcoholic liver disease, nonalcoholic fatty liver disease, and aflatoxin-B1-contaminated food[1,2]; all these factors can change the liver microenvironment and induce hepatocarcinogenesis.[3] The early stage of this disease responds to potentially curative treatments, such as surgical therapies, liver transplantation, and radiofrequency ablation, and certain patients have a 5-year survival rate of up to 60% to 70%. However, the efficiencies of chemotherapy, targeted molecular therapy, transarterial chemoembolization, and selective internal radiation therapy in clinical practice are low when patients are diagnosed at an advanced stage.[1,4]

$CD4^+$ T cells have multiple functions in responding to tumor stimulation and acting against tumor cells. When naïve $CD4^+$ T cells identify the major histocompatibility complex (MHC) class II molecule-peptide complexes on

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professional antigen-presenting cells (APCs), they differentiate toward different $CD4^+$ T cell subsets according to the cytokine environment. In tumors, Th1 cells are the main effectors of $CD4^+$ T cell subsets that differentiate when interleukin (IL)-12 and interferon γ (IFN-γ) are present in the surrounding environment. Th1 cells express T-bet, STAT1, and STAT4 and release IFN-γ and tumor necrosis factor (TNF)-α to enhance cellular immunity.[5] Th2 cell differentiation requires IL-4 and thymic stromal lymphopoietin (TSLP) stimulation, GATA-3, and STAT6 expression and IL-4, IL-5, IL-13, and IL-10 release to support humoral immunity.[6] A recent study showed that memory Th2 cells can enhance the antitumor activity via IL-4-mediated natural killer (NK) cells[7]; in addition, memory Th2 cells can enhance the antitumor activity of $CD8^+$ cytotoxic T lymphocytes (CTLs).[7] Th17 cells differentiate in the presence of IL-6 and transforming growth factor (TGF)-β stimulation, RORγt and STAT3 expression, and proinflammatory cytokine IL-17A, IL-17F, and IL-22 release to contribute to the inflammatory environment.[8] Regulatory T cells (Tregs) differentiate when TGF-β and IL-10 are present, when Foxp3 is expressed, and when IL-10 and TGF-β are secreted, and these cells play a modulator role in controlling the balance of the immune response. In the presence of tumors, Tregs develop and respond to inflammatory stimulation[9]; in addition, they regulate many types of T helper cells, such as Th1, Th2, and Th17 cells.

In the past, many traditional herbal prescriptions have been used to treat chronic liver disease, cancer, and cancer treatment–associated problems; these prescriptions include xiao-chai-hu-tang[10,11] and TJ-14.[12-14] JC-001 is a Chinese medicine that is usually used to treat liver diseases, such as chronic hepatitis B and C viral infections, alcoholic liver disease, and fatty liver disease. We hypothesized that JC-001 could affect the inflammatory microenvironment and halt liver cancer progression. To test this hypothesis, we used an immunocompetent tumor model and analyzed the efficacy and mechanism of this medication, which could be applied to improve the outcomes of patients with liver cancer.

## Materials and Methods

### Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI), Minimum Essential Media (MEM), LHC-9 medium, L-glutamine, trypsin-EDTA and fetal bovine serum (FBS) were purchased from Gibco, Grand Island, NY. The E.Z.N.A.® HP Total RNA Isolation Kit was purchased from OMEGA, Norcross, GA. LDH cytotoxicity assay kits were purchased from BioVision, Milpitas, CA. ELISPOT assay kits for murine IL-10, IL-17A, IL-17F, and TNF-α were purchased from eBioscience, San Diego, CA; a murine T cell activation-3 factor (TCA-3) kit was purchased from Sigma-Aldrich, Stockholm, Sweden, and a murine α-fetoprotein (α-AFP) kit was purchased from Cusabio Biotech, Wuhan, China.

### Plant Materials

JC-001 was provided by Jun Chen Biotech Co, Ltd, Tainan, Taiwan. The main components of this prescription are *Bupleurum chinense* DC, *Gentiana scabra* Bge, *Rheum palmatum* L, *Clematis montana* Buch.-Ham, *Carthamus tinctorius* L, *Prunus persica* (L) Batsch, *Angelica dahurica* (Fisch ex Hoffm) Benth et Hook f, *Siegesbeckia orientalis* L, *Glycyrrhiza uralensis* Fisch, and *Solanum incanum* L.

For cell culture, JC-001 was dissolved in autoclaved double-distilled water ($ddH_2O$) and boiled for 2 hours in a water bath. The boiled JC-001 was centrifuged at 16 000*g* for 30 minutes. Then, the supernatant was collected and centrifuged again at 16 000*g* for 30 minutes. Finally, the supernatant was filtered with a 0.22-μm filter and recovered and stored at −20°C until use.

### Animals and Cell Lines

Six to 8-week-old female C57BL/6 and male BALB/c nude mice weighing 20 to 25 g were obtained from BioLASCO Taiwan Co, Ltd. All animals in this study were treated according to the guidelines of the Instituted Animal Care and Use Committee (IACUC) of Chung Shan Medical University (CSMU) for the care and use of laboratory animals; the IACUC approval number is 1029. The mice were housed on a 12-hour light/dark cycle. All the mice were subjected to experimental procedures after adaptation for 1 week.

A human normal liver epithelial cell line, THLE-3 (ATCC number: CRL-11233), and a human liver adenocarcinoma cell line, SK-HEP-1 (ATCC number: HTB-52), were obtained from the American Type Culture Collection (ATCC). A murine hepatoma cell line, Hepa 1-6 (ATCC number: CRL-1830), and 2 human hepatocellular carcinoma cell lines, HepG2 (ATCC number: HB-8065) and Hep3B (ATCC number: HB-8064), were obtained from the Bioresource Collection and Research Center, Taiwan. All cell lines were maintained under the cell culture conditions recommended by ATCC.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

Cell viability was analyzed using the MTT assay at 96 hours after JC-001 treatment. Cells ($2 \times 10^4$) were seeded on 24-well dishes with 1 mL of culture medium. After 12 hours for adhesion, the medium was removed and replaced by fresh medium containing 0 (blank), 50, 100, 200, 400, or 800 μg/mL JC-001. Then, 500 μg/mL MTT reagent was added to each well for 4

**Table 1.** The Gene-Specific Primer Sequences for Real-Time Q-PCR Analysis.

| | Forward Primer 5′-3′ | Reverse Primer 5′-3′ |
|---|---|---|
| 18s rRNA | GGCCGTTCTTAGTTGGTGGAGCG | CTGAACGCCACTTGTCCCTC |
| T-bet | GCCT ACCAGAACGCAGAGA | GGTGTCCCCAGCCAGTAA |
| GATA3 | TCTGGAGGAGGAACGCT AAT | GAGTGGCTGAAGGGAGAGA |
| RORγt | GCCAAGACTCCTCCAGCT | AGATGCTGTCTCTGCCTTCA |
| Foxp3 | TCCCTCACCCCACCTACA | GCTCCCTGGACACCCAT |

hours. After incubation, the supernatant was removed, and 1 mL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan product. Finally, we measured the $OD_{470}$ to calculate the cell viability and compared it with the blank group. All experiments were performed in duplicate.

## C57BL/6 (Immunocompetent) Mouse Xenograft Model

The 6-week-old immunocompetent C57BL/6 mice were kept in individual ventilating cages for 1 week before being injected with cancer cells. The mice were subcutaneously inoculated with $2 \times 10^6$ Hepa 1-6 cancer cells with 100 µL of medium without FBS. Then, 7 days after inoculation, the mice were fed 200 µL of JC-001 or autoclaved dd$H_2O$. At day 30 after injection, the mice were anesthetized, and the tumors were surgically excised, imaged, weighed, and fixed in 4% paraformaldehyde.

## Histological and Immunohistochemical Examination

All tumor sample analyses were performed by Rapid Science Co, Ltd, Taichung, Taiwan. Murine tumor tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and the paraffin blocks were cut into 3-µm sections for staining with hematoxylin and eosin (H&E). The histology was examined by light microscopy. For immunohistochemistry, the slides were deparaffinized in xylene and hydrated in a graded alcohol series. After microwave antigen retrieval was performed, 3% hydrogen peroxide was used to quench the reaction of endogenous peroxidase, and the samples were blocked with 5% bovine serum in phosphate-buffered saline (PBS). For HMGB1 (ARG65636; Arigo), the slides were incubated at 4°C overnight with antimouse HMGB1 50× dilution. The antimouse HIF-1-α (GTX127309; GeneTex) primary antibodies were diluted 1:500, and the Ki67 (ab16667; Abcam) were diluted 1:200 for incubation overnight. After the slides were washed with phosphate-buffered saline with Tween 20 (PBST), they were sequentially incubated with biotinylated antirabbit IgG secondary antibody, streptavidin-biotin complex, and 3,3′-diaminobenzidine. The slides were counterstained with hematoxylin and examined by light microscopy at 200× zoom.

## Cytokine Array Analysis

For cytokine array analysis, all reagents were supplied by the RayBio Mouse Cytokine Antibody Array III Kit and used according to the manufacturer's procedures. Briefly, frozen tumors in liquid nitrogen were ground and lysed with 2 to 10 mL of lysis buffer. After the tumor samples were incubated at room temperature for 10 minutes, they were centrifuged at 16 000 rpm and 4°C for 2 hours. The tissue lysate was placed in new tubes, and the concentration was adjusted to 1 mg/mL. The 1 mL mixed lysate was added to the chip with gentle shaking for 1 hour at room temperature. The chip was washed with wash buffer I for 10 minutes in triplicate followed by treatment with wash buffer II for 10 minutes in triplicate. The chip was sequentially incubated with biotinylated primary antibodies and streptavidin-labeled secondary antibody for signal detection.

## Quantitative reverse transcription PCR of Splenocytes

The spleen mRNA quantitative reverse transcription PCR (RT-qPCR) was performed by AllBio Science Inc, Taichung, Taiwan. The spleen was kept in RNAlater after removal from the anesthetized animal. The total RNA was extracted using HP Total RNA kit; 2 µg total RNA was treated with DNase I, and complementary DNA (cDNA) was synthesized using moloney murine leukemia virus reverse transcriptase (M-MLV RT). The expression of the target genes was analyzed using an ABI PRISM® 7000 Sequence Detection System. The primer sequences are shown in Table 1. The gene expression was measured relative to the 18s rRNA using the ΔΔCt algorithm.

## Generation of Hepa 1-6–Immunized Splenocytes and Cytotoxicity Assay

For generating Hepa 1-6–immunized splenocytes, 8-week-old C57BL/6 mice were immunized with subcutaneous inoculation of $10^4$ Hepa 1-6 cells once a week for 3 cycles. After immunizing, they were killed humanely, and a routine surgical procedure was used to aseptically remove the spleen. Then, single-cell suspensions were prepared by mechanically passing spleen tissue through mesh screens.

The erythrocytes were lysed, and the cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. For the cytotoxicity assay of Hepa 1-6–immunized splenocytes, $10^4$ target cells were seeded in 96-well plates. After 12 hours, allowing for adhesion, the target cells were cocultured with splenocytes for 5 hours; the stands for effector cell to target cell (E/T) ratios were respectively 20, 40, and 80. The cytotoxic activities of splenocytes in the presence of target cells were analyzed with an lactate dehydrogenase (LDH) cytotoxicity assay kit (BioVision).

### Analysis of Cytokine Secretion in Coculture Conditioned Medium

Hepa 1-6–immunized splenocytes ($4 \times 10^6$/1 well) were cocultured with target cells ($10^5$/1 well) in 24-well plates with 2 mL of RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. After the cells were incubated for 36 hours, the culture medium was collected and cytokine secretion analyzed by ELISPOT assay. All ELISPOT assay reagents were supplied in the kit and used according to the manufacturer's procedures.

### Statistical Analyses

The 2-tailed Student's *t* test was used for statistical analyses for the in vitro experimental data, and the unpaired Student's *t*-test was used to compare the means in 2 groups in vivo. Where appropriate, a $P$ value <0.05 was considered significant.

## Results

### JC-001 Did Not Exhibit Significant In Vitro Cytotoxicity

Before the animal experiments, the potential effects of JC-001 were tested on cells. At 96 hours after JC-001 was added to cell lines, its cytotoxicity against normal and cancer cell lines was analyzed using the MTT assay. No cytotoxicity against normal human liver epithelial THLE-3 cells was observed even when the JC-001 concentration reached 800 µg/mL. In human and murine cancer cell lines, only SK-Hep-1 exhibited a dose-dependent effect on cell viability after 96 hours of treatment; most cancer cell lines were not affected, even when the JC-001 concentration reached 400 µg/mL, as shown in Figure 1. Furthermore, the survival rate of murine liver cancer cells (Hepa 1-6) in the presence of 200 µg/mL JC-001 was 76% in the control group, achieving significance ($P < .05$). However, when the JC-001 concentration was increased to 800 µg/mL, the Hepa 1-6 survival rate did not decline. Therefore, at a concentration of 400 µg/mL, JC-001 is not cytotoxic against the normal cells or most liver cancer cells tested.

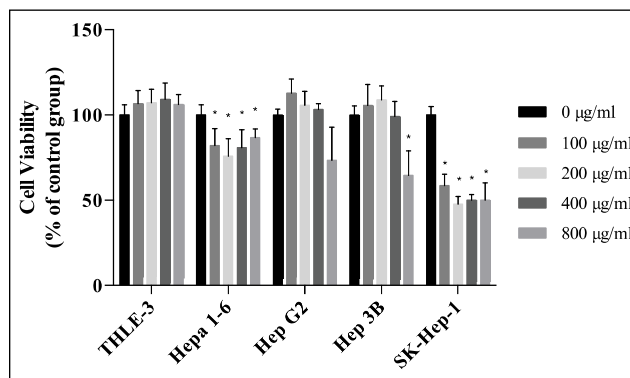


**Figure 1.** In vitro effect on viability of JC-001 treatment on normal liver epithelial cell and cancer cell strains. MTT assay was used for analysis of the cell viability of human normal liver epithelial cell (THLE-3), human hepatoma cell (HepG2, Hep3B, SK-Hep-1), and murine hepatoma cell (Hepa 1-6) for 96 hours JC-001 treatment. Each bar represents mean ± SD (n = 4). *$P < .05$ compared with the 0-µg/mL group.

### JC-001 Suppressed Hepa 1-6–Induced Subcutaneous Tumor Growth in Immunocompetent Animals

The results of the cytotoxicity test indicated that JC-001 did not exhibit significant in vitro cytotoxicity in Hepa 1-6 and other cancer cell lines. Subsequently, we used Hepa 1-6 to conduct in vivo animal experiments. Hepa 1-6 cells were subcutaneously implanted in C57BL/6 mice. After tumor formation was confirmed, the mice were randomly grouped and treated with JC-001. Three weeks after treatment, we observed successively diminished tumor growth in the mice in the JC-001 treatment group. These mice were then killed humanely to analyze tumor size. JC-001 treatment did not result in significant differences in the body weight, relative liver weight, or relative kidney weight (Table 2), and the sizes of the tumors for the 3×-dose and 10×-dose treatment groups were significantly smaller than those in the control group (25.4% and 16.5%, respectively). In addition, tumors were not observed in 2 mice from the 1×-dose treatment group (2/11), 4 mice from the 3×-dose group (4/11), and 2 mice from the 10×-dose group (2/11).

### JC-001 Induced Immune Cell Infiltration and Decreased HMGB1, Ki67, and HIF-1α Expression in Tumors From Immunocompetent Mice

The tumor tissues were evaluated using H&E staining. Observation of the tumor slices obtained from the C57BL/6 control group at 200× magnification showed a few surrounding immune cells in the tumor periphery (Figure 2). Nevertheless, the majority of the area remained structurally

**Table 2.** JC-001 Suppressed Hepa 1-6–Induced Subcutaneous Tumor Growth in Immunocompetent Animals.[a]

| Group | Body Weight (g) | Relative Liver Weight (%) | Relative Kidney Weight (%) | Tumor Weight (%) |
|---|---|---|---|---|
| Naïve | 20.50 ± 1.15 | 4.19 ± 0.17 | 1.11 ± 0.08 | — |
| Control | 20.60 ± 1.26 | 4.03 ± 0.20 | 1.05 ± 0.10 | 225.6 ± 214.7 |
| 1× JC-001 | 20.64 ± 1.29 | 3.90 ± 0.33 | 1.08 ± 0.08 | 142.1 ± 126.9 |
| 3× JC-001 | 20.55 ± 1.04 | 4.09 ± 0.36 | 1.03 ± 0.08 | 55.4 ± 94.0* |
| 10× JC-001 | 20.00 ± 1.11 | 4.25 ± 0.32 | 1.02 ± 0.06 | 37.2 ± 59.1* |

[a]Hepa 1-6 cells ($2 \times 10^6$ cells per mice) were subcutaneously injected in the left flank of C57BL/6 mice. After 8 days of tumor growth, the tumor-bearing mice were treated with 1× (740 mg/kg), 3×, 10× JC-001 or $H_2O$ once a day for 21 days.
*$P < .05$ compared with the control group.

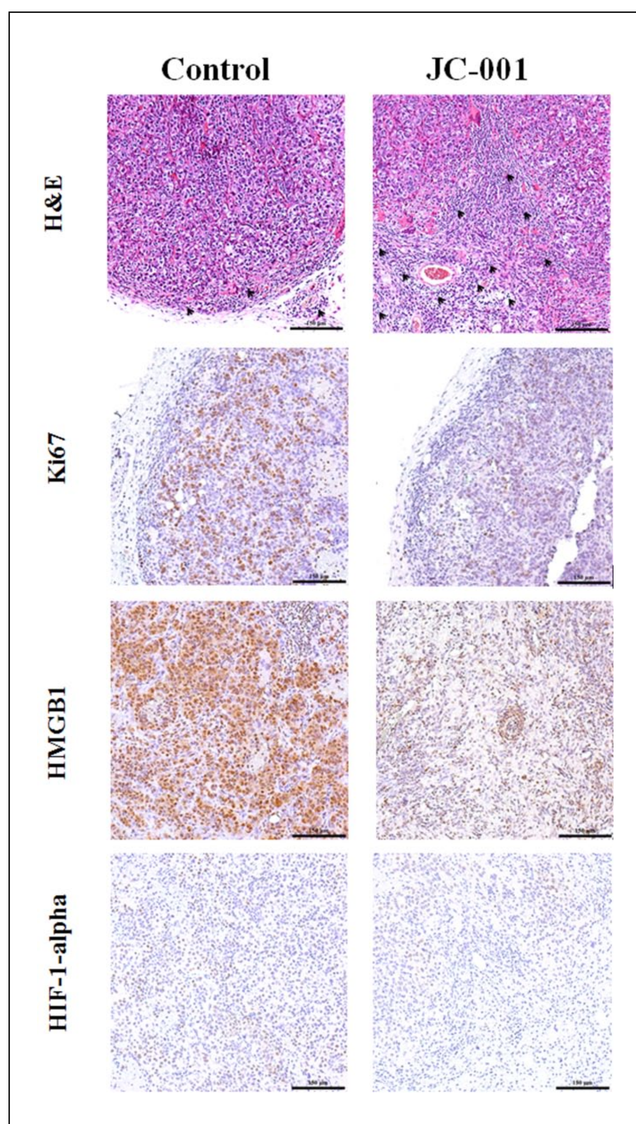


**Figure 2.** Hematoxylin and eosin and immunohistochemistry stain for Ki67, high mobility group box 1 (HMGB1), and hypoxia-inducible factor-1-α (HIF-1-α) of subcutaneous Hepa 1-6 tumors. The 3× JC-001 treatment mice tumors showed immune cell infiltration obviously and expressed low levels of Ki67, HMGB1, and HIF-1-α compared with the control group tumors.

unchanged, and the tumor cells exhibited a bundle formation that was similar to the tissue structure of clinical liver cancer. We then analyzed the tumor slices obtained from the JC-001 3×-dose treatment group, which exhibited the largest tumor weight difference. Immune cell infiltration was observed in a large portion of the tumor, and a comparatively large number of immune cells were observed in the tumor. In contrast, immune cell infiltration was not observed in the BALB/c nude mice, and no significant difference between the control and treatment groups was observed (data not shown). With regard to immunohistochemistry, Hepa 1-6 tumors expressed high levels of Ki67, high mobility group box 1 (HMGB1), and hypoxia-inducible factor-1-α (HIF-1α) in C57BL/6 mice, but the expression levels of Ki67, HMGB1, and HIF-1α were lower in biopsies from the JC-001 treatment group. In conclusion, the tumor tissue biopsy showed that JC-001 increased immune cell infiltration and decreased Ki67, HMGB1, and HIF-1α expression in the Hepa 1-6–induced tumors in immunocompetent mice.

### *The Effect of JC-001 on the mRNA Expression Levels Related to Spleen Cell Immunity*

The spleen contains the largest number of immune cells in the circulatory system. According to the above results, JC-001 treatment affected Hepa 1-6–induced tumors in immunocompetent animals but did not affect tumors in mice with thymic abnormalities (BALB/c nude). Thus, we considered that T cells might be target cells that are activated by JC-001, necessitating analysis of the immune cell pattern in the spleen. In this experiment, the naïve group exhibited stable mRNA expression levels of T-bet, GATA3, RORγt, and Foxp3 (Figure 3), suggesting that the breeding process was clean and without infection. In the Hepa 1-6 tumor-bearing mice group, the spleen cell mRNA expression levels of T-bet, GATA3, RORγt, and Foxp3 were higher than in the naïve group. However, the 3×-dose treatment of tumor-bearing mice resulted in upregulation of T-bet and GATA-3 cells in the spleen compared with untreated tumor-bearing mice, whereas the RORγt and Foxp3 expression

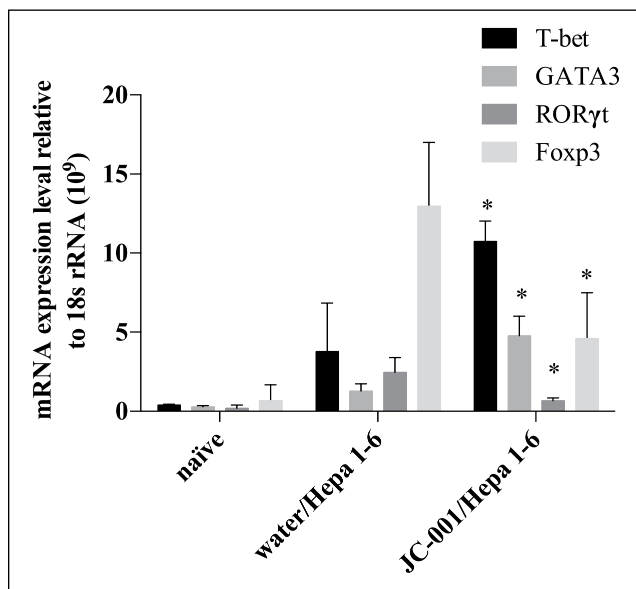


**Figure 3.** The effect of JC-001 on mRNA expression levels in spleen cell immunity. The Hepa 1-6 tumor-bearing C57BL/6 mice were treated with 2467 mg/kg JC-001 or water for 18 days, and the mRNA expression levels were analyzed by real-time Q-PCR. Each bar represents mean ± SD (n = 3). *$P < .05$ compared with Hepa 1-6 tumor-bearing group.

levels were downregulated. This result shows that the ratio of the 3×-dose JC-001 tumor treatment influenced the activation of Th1 and Th2 cells and suppressed Th17 cells, which in turn suppressed Hepa 1-6–activated Treg cells and improved the adaptive immune response.

### Analysis of the Change in the Tumor Microenvironment in Response to JC-001 Treatment of Tumor-Bearing Mice

The immunocompetent tumor models suggested that the antitumor activity of JC-001 might be achieved by immune modulation. Therefore, it is of interest to investigate whether JC-001 treatment would affect the expression of cytokines and chemokines in the tumor microenvironment. The RayBio® Mouse Cytokine Antibody Array III that contains 62 antibodies against cytokines was used for the analyses.

After JC-001 treatment, a relatively significant increase in TCA-3 (Figure 4), vascular endothelial growth factor (VEGF), TNFα, IL-12 p70, insulin-like growth factor-binding protein 3 (IGFBP3), macrophage inflammatory protein-1α (MIP-1α), IL-10, IL-6, TIMP metallopeptidase inhibitor 1, and IL-4 levels was observed in the tumor microenvironment. The relative levels of IFN-γ, thymus-expressed chemokine, IL-3, RANTES, and IL-1α were reduced after JC-001 treatment.

### JC-001 Enhanced the Cytotoxic Ability of Splenocytes in Coculture Conditions

Considering that the above results suggested that the antitumor activity of JC-001 requires a competent immune system, JC-001 might directly enhance the immune response and mediate the immune cell composition in the tumor microenvironment. Additionally, memory immune cells play the main role in tumor rejection for tumor-bearing animals. To investigate whether JC-001 enhances the memory immune response in tumors, whole splenocytes containing Hepa 1-6 memory cells from Hepa 1-6–immunized mice were cultured with Hepa 1-6 cells, and cytotoxicity against Hepa 1-6 was analyzed using the LDH release assay. After the cells were cocultured for 5 hours, cytotoxicity was significantly enhanced when the JC-001 concentration reached 200 µg/mL. This result indicated that JC-001 could promote the memory immune response in vitro (Figure 5).

### JC-001–Mediated Cytokine Secretion In Vitro

Because JC-001 could suppress Hepa 1-6 tumor growth in C57BL/6 mice in vivo and promote cytotoxicity of the Hepa 1-6–immunized splenocytes against Hepa 1-6 tumor cells in vitro, we investigated whether JC-001 could mediate cytokine and chemokine secretion via immune cells in the microenvironment. To further identify the factor that could be mediated via JC-001, we analyzed α-AFP, TCA-3, IL-10, IL-17A, IL-17F, and TNF-α levels in coculture conditions using ELISA. IL-10 and TNF-α were upregulated (Figure 6), whereas α-AFP, IL-17A, and IL-17F were downregulated after 36 hours of JC-001 treatment. TCA-3 expression was slightly enhanced when JC-001 concentration reached 200 µg/mL. This result was similar to the cytokine array data of the tumor microenvironment and the Th1/Th2/Th17/Treg paradigm of spleen cells after JC-001 treatment.

## Discussion

Because JC-001 did not exhibit cytotoxicity in a series of cancer cell strains examined in our study (data not shown), we considered the immune system when investigating the carcinostatic mechanism of this substance. The immune system is an essential factor to consider in cancer-related research, and cancer cells of various forms and organs use differing mechanisms and modes to resist the immune system and evade relevant attacks. Thus, this study examined immunocompetent animals with liver cancer to further understand the carcinostatic effects of JC-001. We found that JC-001 caused significant immune infiltration in tumors that were induced by Hepa 1-6 liver cancer cells in immunocompetent mice. Additionally, several mice experienced tumor rejection following treatment, a phenomenon that

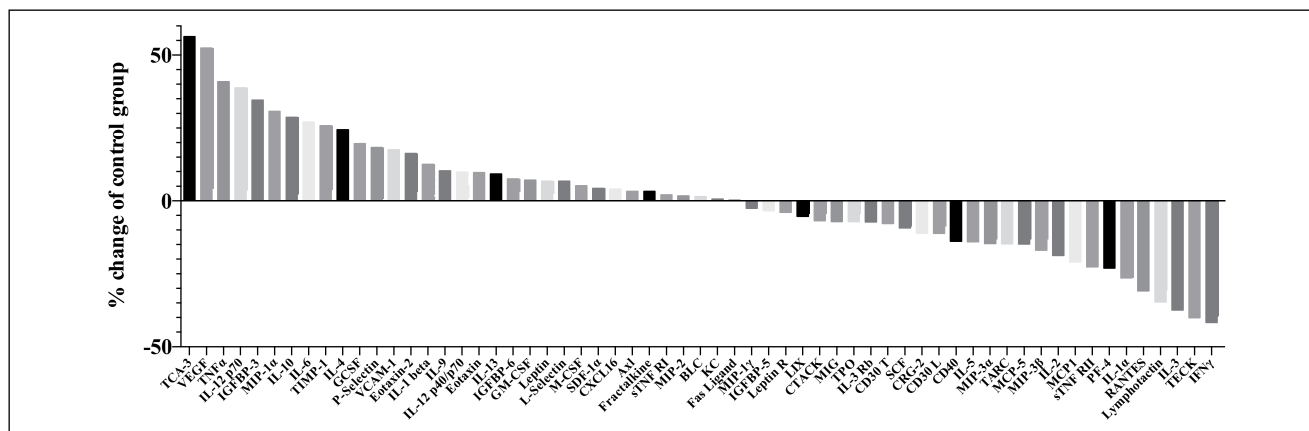


**Figure 4.** The level of cytokines in the tumor microenvironment analyzed by RayBio Mouse Cytokine Antibody Array 3.1.
Abbreviations: TCA-3, T cell activation-3 factor; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; IL, interleukin; IGFBP-3, insulin-like growth factor-binding protein 3; MIP, macrophage inflammatory protein; TIMP-1, TIMP metallopeptidase inhibitor 1; RANTES, regulated on activation, normal T cell expressed and secreted; Lymphotactin, chemokine (C motif) ligand 1; IFN, interferon.

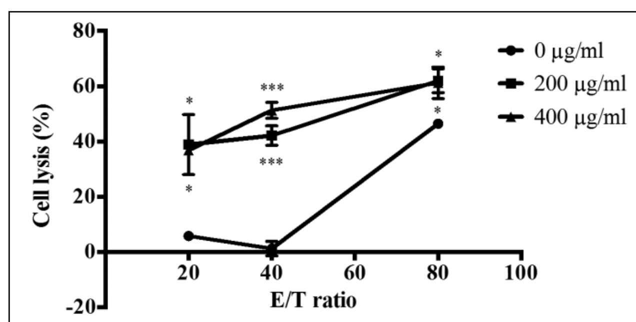


**Figure 5.** JC-001 enhanced the cell lysis activity of splenocytes from Hepa 1-6–immunized mice in vitro. Each bar represents mean ± SD (n = 3).
$^*P < .05$ and $^{***}P < .001$ compared with the 0-μg/mL group.

was absent in mice with thymic defects. Because thymic defects result in incomplete differentiation of T cells, we speculated that T cells play a key role in JC-001 induction of tumor rejection.

HMGB1 is a damage-associated molecular pattern protein that functions as a mediator in the proinflammatory effects and that affects the processes of many inflammatory diseases, such as autoimmunity and cancer.[15,16] Particularly in HCC, hepatoma cells can overexpress HMGB1 to enhance tumor cell metastasis and promote inflammation and HCC.[17] Hypoxia induces solid tumors to overexpress HMGB1[18] and negatively regulates the Th1 response.[19] In the JC-001-treatment group, the Ki67, HMGB1, and HIF-1α expression levels in the tumors were downregulated, showing that JC-001 treatment can improve hypoxia levels, reduce HMGB1 expression, and stunt growth in solid tumors. This phenomenon is likely a result of JC-001-mediated immunomodulation.

The activation of spleen T cells is a phenomenon that can be easily observed. The purpose of conducting coculture experiments was to observe whether the response of memory immune cells was enhanced by JC-001 treatment. In this study, a significant increase in mice spleen cell cytotoxicity following JC-001 treatment was observed when splenocytes and Hepa 1-6 cells were cocultured. This result showed that the response of memory immune cells was directly improved by JC-001 treatment in vitro. In addition, T cell activation was not the only cause for JC-001 suppression of Hepa 1-6–induced tumor growth. Therefore, we analyzed cytokines in the tumor microenvironment. The results of this analysis indicated that there was a significant increase in TCA-3, VEGF, TNF-α, IL-12 p70, IGFBP-3, MIP-1α, IL-10, and IL-6 levels in the tumor microenvironment.

In response to tumor stimulation, TCA-3 (CCL1), a proinflammatory glycoprotein, is secreted by the activated T cells.[20-22] Meanwhile, monocytes, NK cells, B cells, T cells, and dendritic cells travel toward the tumor microenvironment to create and promote an inflammatory response as well as suppress tumor growth.[21,23,24] VEGF is secreted by tumor and stromal cells (macrophages, endothelial cells, and fibroblasts), and it plays multiple roles in the tumor microenvironment, including primary stimulation of angiogenesis, increase in the vascular permeability, promotion of dedifferentiation and the epithelial–mesenchymal transition phenotype of tumor cells as a chemoattractant to recruit Treg cells, and enhancement of Th2 polarization.[25,26] TNF-α, which is typically secreted by M1-type macrophages, $CD4^+$ T cells, and NK cells, contributes to the systemic inflammatory response by inducing fever, apoptosis, and IL-1 and IL-6 production,[6,26] inhibiting tumor progression and viral replication. JC-001 treatment increased the TNF-α

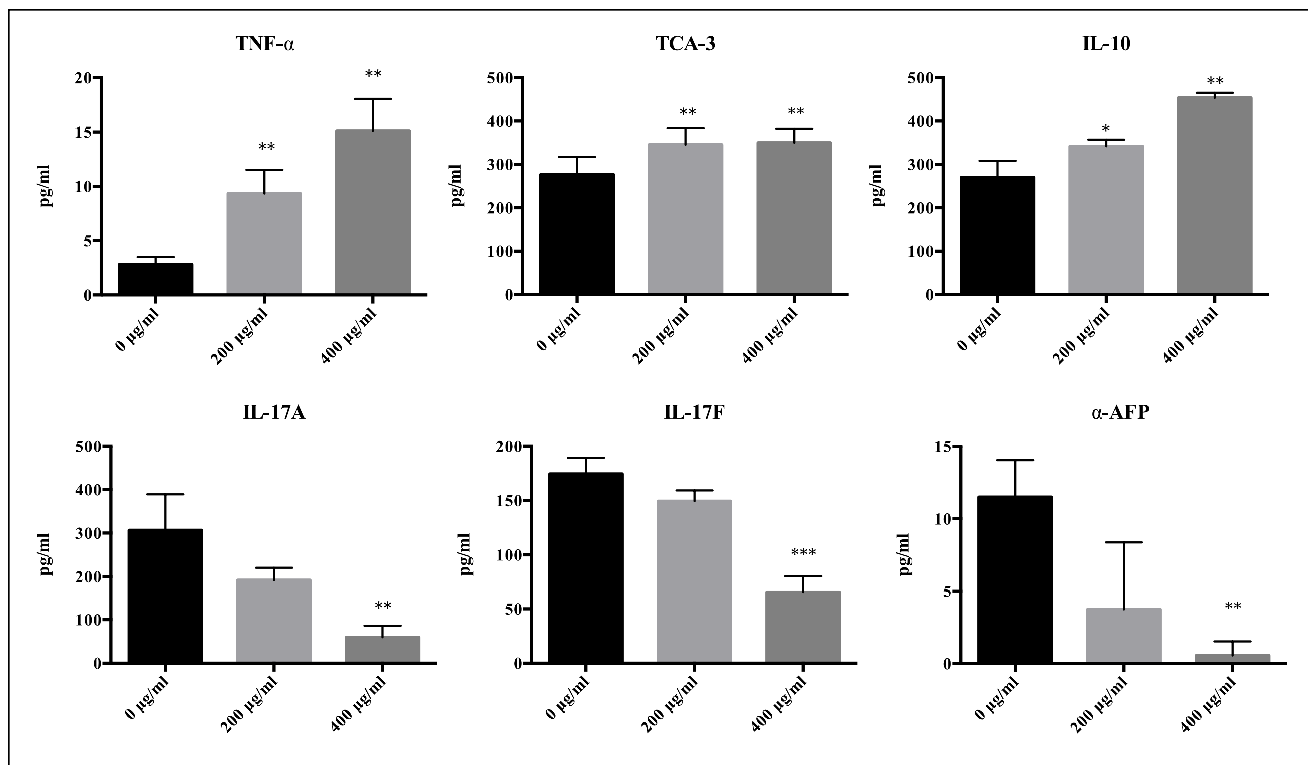


**Figure 6.** JC-001 mediated the cytokine secretion in splenocytes/Hepa 1-6 coculture condition medicine. Each bar represents mean ± SD (n = 3).
*$P < .05$, **$P < .01$, and ***$P < .001$ compared with the 0-μg/mL group.

level in the tumor microenvironment, enhancing the inflammatory response. In addition, a trend of increasing IL-1 and IL-6 levels was observed in the tumor microenvironment after JC-001 treatment, implying that TNF-α substantially affects the tumor microenvironment. IL-12 helps stimulate undifferentiated $CD4^+$ T cells to differentiate into Th1 cells and prompts T and NK cells to secrete TNF-α and IFN-γ.[27-29] In addition, IL-12 can directly augment $CD8^+$ T cell cytolysis, survival, and proliferation through CCL1 and CCL17 production.[28] An increase in IL-12 p70 in the tumor microenvironment, resulting from JC-001 treatment, can guide the development of microenvironment immunity toward Th1.

IL-10, a cytokine inhibitory factor can be expressed by Tregs, Th2 cells, APCs, macrophages, NK cells, and epithelial cells.[30,31] In the tumor microenvironment, IL-10 plays a role in immunosuppression of tumor progression.[26,30,32] However, recent studies have shown that IL-10 can elicit the IFNγ-dependent tumor immune surveillance and promote antitumor immunity by $CD8^+$ T cells.[33-35] In addition, IL-10 can limit tumor Th17 inflammation and restrain tumor progression.[36] In this study, JC-001 upregulated Th1, Th2, and Treg and downregulated Th17 in the spleens of non–tumor-bearing mice. In tumor-bearing mice, JC-001 also upregulated Th1 and Th2 while downregulating Th17. In contrast, JC-001 treatment enhanced IL-10 secretion in the tumor microenvironment and in coculture conditions. As a result, we hypothesized that JC-001 plays a modulator role in the immune system and that IL-10 is the key factor in JC-001 treatment.

To confirm the tumor microenvironment data, we cocultured Hepa 1-6–immunized splenocytes with Hepa 1-6 cells and analyzed the quantity change of cytokine secretion in culture medium. The in vitro data showed that TNF-α, TCA-3, and IL-10 secretion exhibited the same tendency as in the tumor microenvironment data but that IL-12 p70 secretion did not. This phenomenon might be a result of the composition of cells in the tumor microenvironment and differences in the coculture conditions; in addition, the target cells of JC-001 differed. IL-17A and IL-17F were produced by Th17 cells, which promoted a protumor environment.[37,38] In the tumor microenvironment, IL-17A promotes the recruitment of neutrophils and DNA damage of local tissue through the secretion of radical oxygen species.[39,40] In addition, IL-17F can regulate angiogenesis and endothelial cell cytokine production.[41] Our data showed that 400 μg/mL JC-001 significantly downregulated both IL-17A and IL-17F under coculture conditions, indicating that JC-001 could directly inhibit the activity of Th17 cells. This finding confirmed the RORγt mRNA level in the

spleen in vivo and suggested that downregulation of Th17 cells was the main function of JC-001 in this project.

α-AFP, a 70-kD glycoprotein synthesized from the fetal yolk sac, liver, and intestines, highly correlates with the prognosis of patients with HCC.[42-44] The Hepa 1-6 cells could secrete α-AFP in cell culture and in the plasma of the orthotopic tumor model. In addition, the plasma α-AFP level increased as Hepa1-6 tumors progressed in vivo.[45] In our animal model, the plasma α-AFP level was not significantly enhanced in tumor-bearing mice, and the subcutaneous tumor microenvironment likely differed from that in orthotopic tumors. However, the secretion of α-AFP in the coculture medium was downregulated when the JC-001 concentration reached 200 μg/mL. This result also confirmed the data of the CTL-mediated cytotoxicity and indicated that 200 μg/mL JC-001 could enhance the activity of Hepa 1-6–immunized splenocytes and could suppress α-AFP secretion from Hepa 1-6 cells under the coculture conditions.

In summary, this study demonstrated that JC-001 could inhibit Hepa 1-6 tumors via immune modulation in this animal model, which was not achieved through a cytotoxic effect on various tumor cells. In addition, the subsequent research focused on how JC-001 modulates the Th1/Th2/Th17/Treg paradigm in the tumor microenvironment. In conclusion, we suggest that JC-001 can be widely used in various tumor treatments, which merits further clinical testing and assessments.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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