

ORIGINAL ARTICLE

Norcantharidin enhances antitumor immunity of GM-CSF prostate cancer cells vaccine by inducing apoptosis of regulatory T cells

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Norcantharidin (NCTD) is a promising antitumor drug with low toxicity. It was reported to be able to regulate immunity, but the mechanism is not yet clear. Here we explored whether NCTD could enhance the antitumor immunity induced by prostate cancer cell vaccine. The results of the in vitro study showed that NCTD induced apoptosis and inhibited proliferation of regulatory T cells (Tregs). Mechanistic research showed that NCTD inhibited Akt activation and activated FOXO1 transcription, resulting in a pro-apoptotic effect. The results of the in vivo study showed that more tumor-infiltrating Tregs existed within peripheral blood and tumor tissue after treatment with the vaccine. Adding NCTD to vaccine treatment could decrease the number of tumor-infiltrating Tregs and increase the number of CD4⁺ and CD8⁺ T cells. Combination therapy with NCTD and vaccine was more effective in inhibiting tumor growth than the vaccine alone. In general, this is the first report that NCTD could induce apoptosis of Tregs and enhance the vaccine-induced immunity.

KEYWORDS

cancer immunotherapy, mGM-CSF vaccine, norcantharidin, prostate cancer, Tregs

1 | INTRODUCTION

Prostate cancer is a common type of cancer in men in Europe and the USA.¹ Immunotherapy with tumor cell vaccine has been proven to be effective in treating PCa.² However, suppressive immunity could negatively affect the outcome of tumor cell

vaccine treatment. Regulatory T cells are a subpopulation of CD4 T cells expressing CD25 and FoxP3, which mainly confer immunity suppression.³ Several studies had reported that Tregs widely infiltrate into tumor tissues and exist in peripheral blood of patients with malignant cancer.⁴⁻⁶ Increasing numbers of Tregs negatively correlate with patients' survival and prognosis.⁴⁻⁸ As reported by several studies, depleting Tregs by CD25-specific antibody⁹ and cytotoxic T-lymphocyte-associated protein 4 antibody¹⁰ could improve the antitumor immune response. Therefore, eliminating Tregs or blocking their inhibitory functions is essential in cancer immunotherapy.

Abbreviations: CTD, cantharidin; DC, dendritic cell; FasL, Fas ligand; FOX, Forkhead box; GM-CSF, granulocyte macrophage colony-stimulating factor; mGM-CSF, mouse GM-CSF; NCTD, norcantharidin; p-, phosphorylated; PCa, prostate cancer; PE, phycoerythrin; PI, propidium iodide; SA, streptavidin; Tcon, conventional T cell; Treg, regulatory T cell.

Lijun Mo and Xinji Zhang contributed equally to this work.

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Norcantharidin, the demethylated analogue of CTD,¹¹ has strong anti-oxidant, antitumor, and antimetastatic activity,¹² but much less toxicity than CTD in the clinical application.¹³ Published studies have reported that NCTD could significantly induce apoptosis and inhibit proliferation of tumor cells through different signaling pathways.^{14,15} Mechanistic studies showed that NCTD-induced apoptosis is mediated by regulating some transcription factors, including nuclear factor- κ B, activator protein-1, Forkhead box O, and signal transducer and activator of transcription, which can independently or collaboratively regulate the expression of apoptotic or anti-apoptotic genes.^{15,16} Norcantharidin was reported to have an effect on immune regulation,¹⁷ but its effect on Tregs is not yet clear.

In our previous research, a cancer cell vaccine was developed by anchoring streptavidin-tagged GM-CSF on the surface of prostate cancer cells.¹⁸ We found that the vaccine could enhance antitumor immune responses, but the number of Tregs also increased in the vaccine-treated mice at the same time. The present study was undertaken to evaluate the effect of NCTD on apoptosis and proliferation of Tregs *in vitro* and explore the underlying mechanism. A mouse prostate cancer model was used to evaluate the efficacy of a combination therapy with NCTD and cancer cell vaccine.

2 | MATERIALS AND METHODS

2.1 | Materials

Tumor tissues were obtained from 20 PCa patients and normal tissues were selected from 10 patients who received pathological biopsy of benign disease in Nanfang Hospital (Guangzhou, China). None of these patients had received immune-regulating drugs or radiation therapy before surgery. The study was approved by the ethics committee and informed consent was obtained from all patients. Norcantharidin, CCK-8, and DAPI reagents were purchased from Sigma (St. Louis, MO, USA). Streptavidin-tagged mouse GM-CSF (SA-mGM-CSF) fusion protein was prepared by our laboratory. Western blotting antibodies against p-ERK1/2, p-Akt, ERK1/2, Akt, Bcl-2, FOXO1, FasL, and GAPDH were purchased from Abcam (Cambridge, UK). Phycoerythrin-labeled anti-mGM-CSF, FITC-labeled anti-mCD11c, PE-labeled anti-mCD80, FITC-labeled anti-mCD4, FITC-labeled anti-mCD8 and PerCP-Cyanine 5.5-labeled anti-mFoxP3 antibodies were purchased from eBioscience (San Diego, CA, USA). C57BL/6 male mice (6-8 weeks old) were purchased from the experimental animal center of Southern Medical University (Guangzhou, China). All animal studies were undertaken in accordance with the Southern Medical University guidelines for experimental animals.

2.2 | Cell sorting

To obtain Tcons, the spleens were collected from C57/BL6 mice, then ground and isolated by lymphocyte separation liquid (Solarbio). Regulatory T cells were isolated from Tcons according to the procedure of the CD4⁺ CD25⁺ Regulatory T Cells Isolation Kit (Miltenyi Biotec, Germany), and the rest of the cells were considered as non-

Tregs. Their purity and the ratio of viability were assessed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

2.3 | Cell culture and stimulation

The mouse prostate cancer cell line (RM-1) was purchased from ATCC (Manassas, VA, USA). The cells were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/L streptomycin at 37°C in a humidified atmosphere of 5% CO₂. The Tcons, Tregs, and non-Tregs were cultured in the presence of 1 μ g/mL soluble anti-mouse CD3 and anti-mouse CD28 (1:1) antibody.

2.4 | Cell proliferation assay

The Tcons, Tregs, and non-Tregs were inoculated into 96-well plates (1×10^4 cells/well) and then incubated with different concentrations of NCTD (0-80 μ mol/L) for 24 hour or with 20 μ mol/L NCTD for 0, 24, 48, or 72 hour. The effect of NCTD on cell proliferation was determined by the CCK-8 assay. Cell viability was assessed by microplate reader (Bio-Rad, Hercules, CA, USA).

2.5 | Apoptosis assay

The Tcons, Tregs, and non-Tregs were cultured in 24-well plates as previously described and treated with various concentrations of NCTD (0-80 μ mol/L) for 24 hours or with 20 μ mol/L NCTD for 0, 24, 48, or 72 hours. The cell apoptosis was measured by flow cytometry using an annexin V/FITC and PI apoptosis detection kit (eBioscience). Annexin V(+)/PI(-) and annexin V(+)/PI(+) were defined as early apoptosis and late apoptosis, respectively.

2.6 | Western blot analysis

Regulatory T cells were cultured and treated with NCTD as previously described. Cells were collected and dissolved in RIPA buffer. Equal amounts of protein were separated on 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA), then the membrane was blocked with 5% milk and incubated with primary antibodies in TBST overnight at 4°C. Primary antibodies against the following proteins were used: ERK1/2, p-ERK1/2, Akt, p-Akt, FOXO1, Bcl2, FasL, and GAPDH. The membranes were washed four times for 7 minutes with TBST and then incubated with HRP-conjugated IgG secondary antibody for 1 hour at room temperature. Protein binding was visualized using an Immobilon Western-HRP substrate (Millipore, Billerica, MA, USA).

2.7 | Real-time RT-PCR

Total RNA of Tregs was extracted using RNAiso Plus reagent (Takara) and reversed transcribed using a PrimeScript RT reagent Kit with gDNA Eraser (Takara). For each gene, the mRNA level was

normalized against GAPDH expression. The mRNA levels of Fas, FasL, FOXO1, FOXO3a, and FOXO4 were determined using SYBR Premix Ex Taq II (Takara). Primers for PCR were designed as follows: GAPDH, AGGTCGGTGTGAACGGATTTG (forward) and GGGGT CGTTGATGGCAACA (reverse); FOXO1, ATGCTCAATCCAGAGG-GAGG (forward) and ACTCGCAGGCCACTTAGAAAA (reverse); FOXO3a, GGGGAACCTGTCCTATGCC (forward) and TCATTCT-GAACGCGCATGAAG (reverse); FOXO4, CTTCCTCGACCAGACC TCG (forward) and ACAGATCGGTTCCGGAGTGT (reverse); Fas, TATCAAGGAGGCCATTTTGC (forward) and TGTTTCCACTTC-TAAACCATGCT (reverse); and FasL, CAGCCATGAATTACCCATGT (forward) and ATTTGTGTGTGGTCCTTCT (reverse).

2.8 | Vaccine preparation

The RM-1 cells were fixed in 30% ethanol (v/v) for 30 minutes at room temperature. Ethanol-fixed RM-1 cells (1×10^7 cells/mL) were incubated with 10 mmol/L fresh EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology) for 3 minutes at room temperature, washed with PBS, then incubated with SA-mGM-CSF at 200 ng/ 10^6 cells for 1 hour and washed with PBS again. The presence of SA-GM-CSF on the surface of tumor cells was detected by flow cytometry (Becton Dickinson) using PE-anti-mGM-CSF mAb.

2.9 | Efficacy of combination therapy with vaccine and NCTD

To establish the s.c. tumor model, each mouse was injected with 2×10^6 RM-1 cells in the hind leg of C57BL/6/male mice (6-8 weeks old). The mice were randomly divided into four groups (PBS, NCTD, Vaccine, and Vaccine + NCTD [combination group]). For the Vaccine group, the mice were injected s.c. into the right hind leg with 1×10^6 the RM-1 tumor cells vaccine on day 0 (10 days after inoculation of tumor cells). The process was repeated on days 4, 8, and 12. For the NCTD or combination group, the mice were i.p. injected with NCTD beginning at day 10 after inoculation of tumor cells at a dose of 10 mg/kg/day for 14 days.

2.10 | Analysis of leukocytes in the blood by flow cytometry

Blood samples (100 μ L) were collected from each group on day 30 after inoculation of tumor cells, and the red blood cells were then lysed by ACK lysing buffer (Solarbio). To detect DCs, cells were stained with FITC-labeled anti-mCD11c and PE-labeled anti-mCD80 antibody for 30 minutes. To qualify the CD4⁺/CD8⁺ T populations, cells were stained with FITC-labeled anti-mCD4 or FITC-labeled anti-mCD8

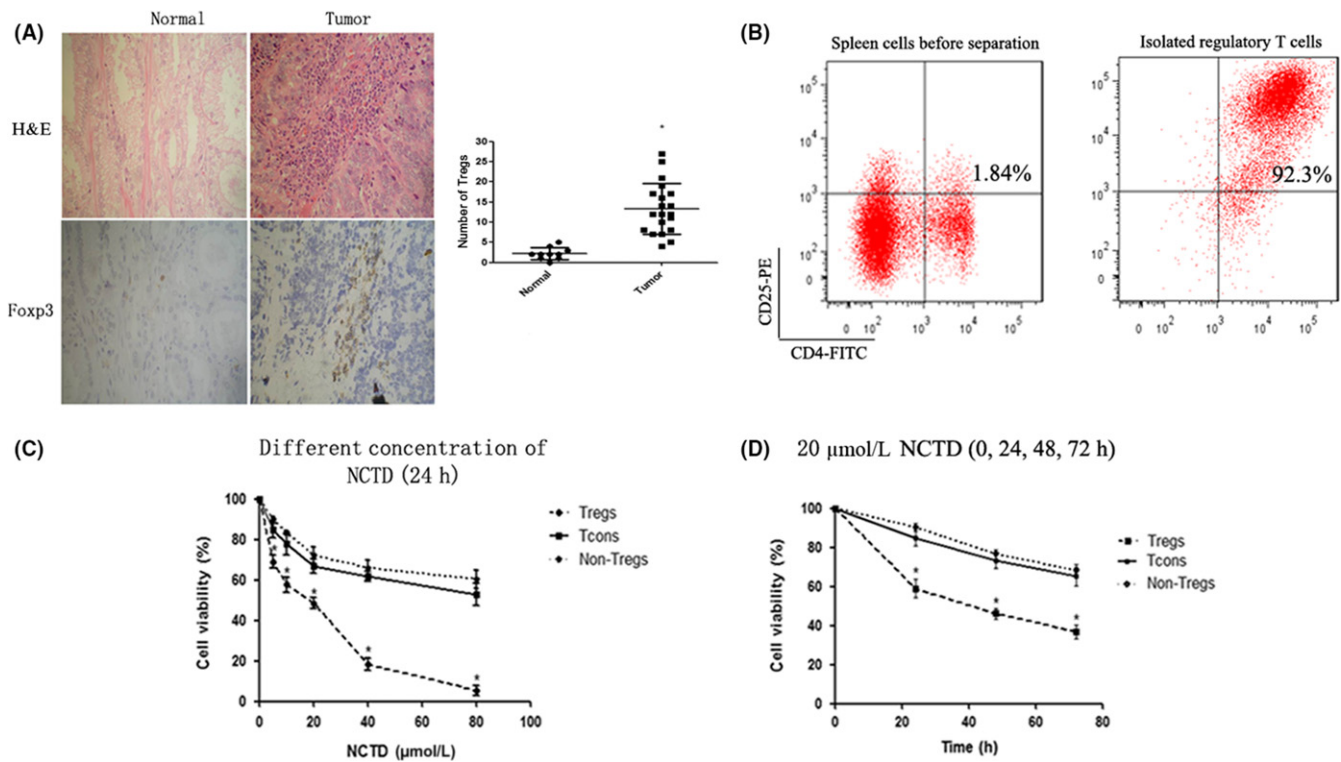


FIGURE 1 Tumor-infiltrating regulatory T cells (Tregs) in human prostate cancer and the effect of norcantharidin (NCTD) on the proliferation of Tregs. A, Twenty tumor tissues and 10 normal tissues were collected from patients and analyzed by immunohistochemistry. Sections are representative of the total vital tumor tissue (magnification, $\times 200$). B, Tregs were isolated from spleens by magnetic selection and the purity of Tregs was measured by flow cytometry. Conventional T cells (Tcons), non-Tregs, and Tregs were treated with different concentrations of NCTD (0-80 μ mol/L) for 24 h or with 20 μ mol/L NCTD for 0, 24, 48, or 72 h. Cell viability was determined by the CCK-8 assay. C, NCTD (0-80 μ mol/L) for 24 h. D, 20 μ mol/L NCTD for 0, 24, 48, and 72 h. All experiments were repeated at least three times. * $P < .05$. PE, phycoerythrin

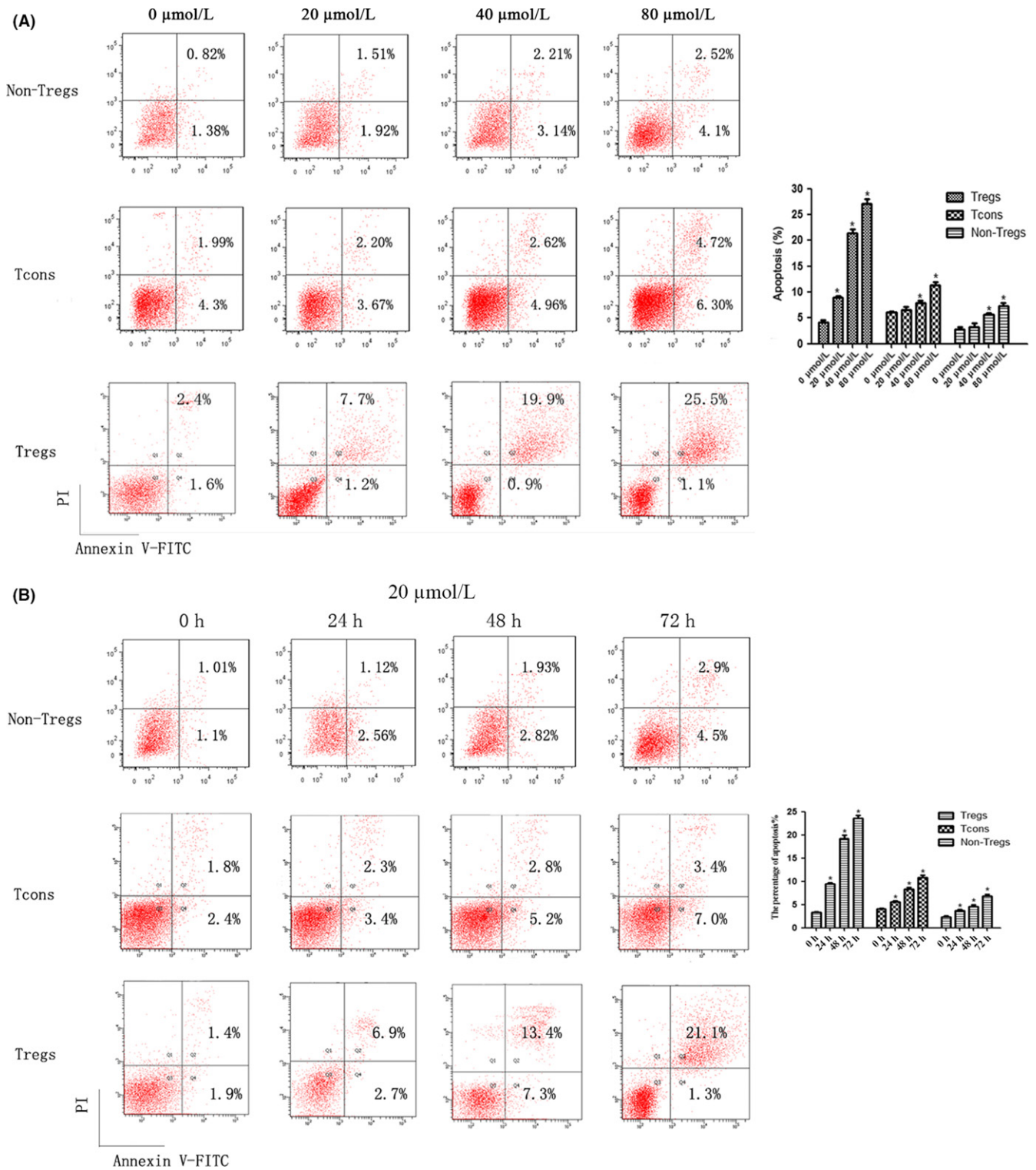


FIGURE 2 Effect of norcantharidin (NCTD) on the apoptosis of regulatory T cells (Tregs), non-Tregs, and conventional T cells (Tcons). Tcons, non-Tregs, and Tregs were treated with different concentrations of NCTD (0–80 μmol/L) for 24 h or with 20 μmol/L NCTD for 0, 24, 48, or 72 h. A, NCTD (0–80 μmol/L) for 24 h. B, 20 μmol/L NCTD for 0, 24, 48, and 72 h. All experiments were repeated at least three times. **P* < .05. PI, propidium iodide

antibody for 30 minutes. To detect the Treg populations in the blood of each experimental group, the cells were incubated with FITC-labeled anti-mCD4 for 30 minutes, then the cells were stained with PerCP-

Cyanine 5.5-labeled anti-mFoxP3 antibodies for 30 minutes after fixed and permeabilized using the Foxp3 staining kit (eBioscience). T-cell subsets were measured by flow cytometry (Becton Dickinson).

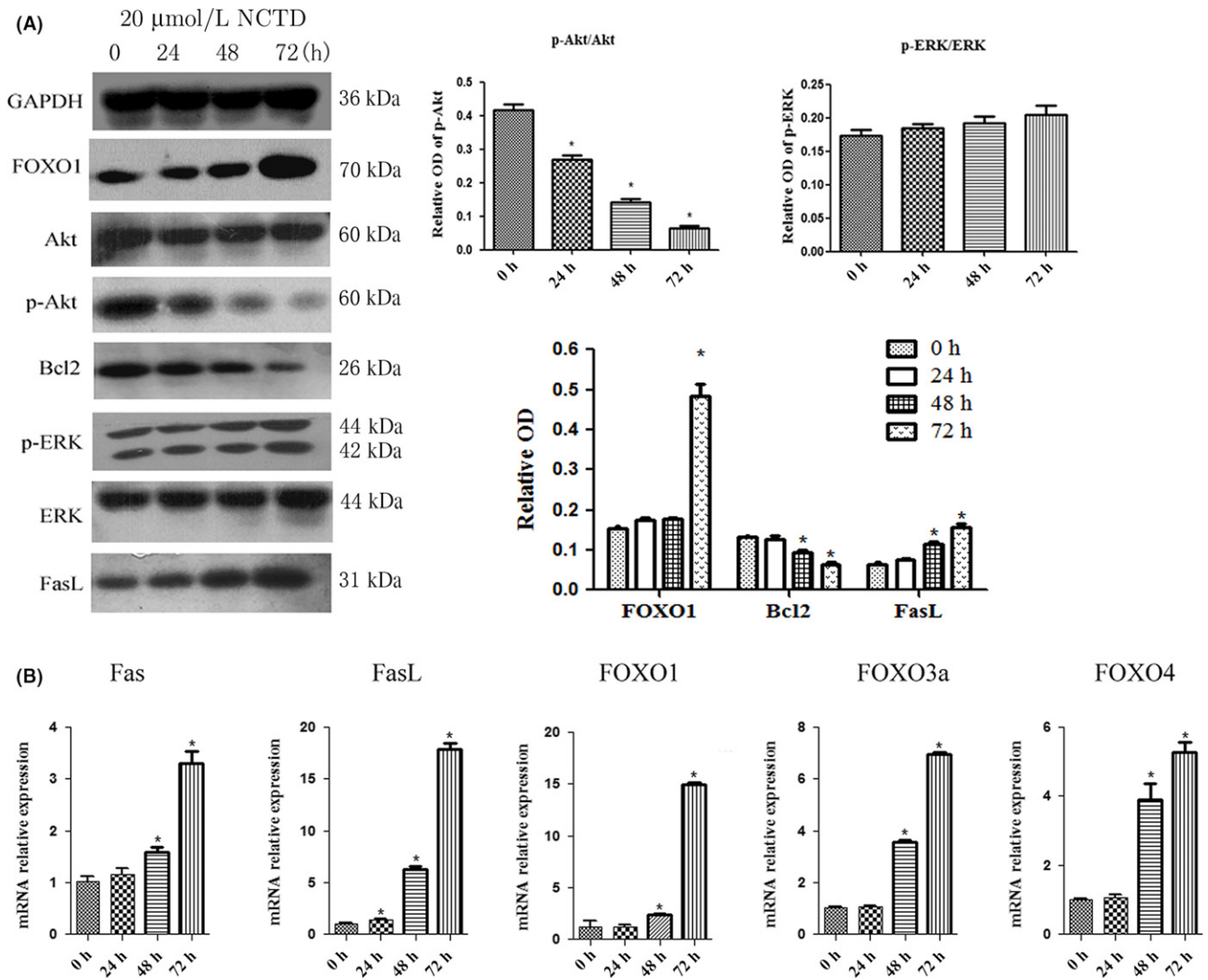


FIGURE 3 Norcantharidin (NCTD) induces apoptosis of regulatory T cells (Tregs) by regulating phosphorylated (p-)Akt, Forkhead box (FOX) O1, and Fas ligand (FasL) expression. Tregs were treated with 20 $\mu\text{mol/L}$ NCTD for 0, 24, 48, or 72 h. A, ERK1/2, p-ERK1/2, Akt, p-Akt, FOXO1, Bcl2, and FasL levels were measured by Western blot analysis, with GAPDH as control. B, FOXO1, FOXO3a, Fas, FasL, and FOXO4 mRNA levels were determined by RT-PCR. * $P < .05$. OD, optical density

2.11 | Immunohistochemical analysis

A total of 20 tumor tissues and 10 normal tissues were obtained from patients in Nanfang Hospital. Subcutaneous tumor tissues of mice were collected from each group on day 30 after inoculation of tumor cells. Tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin sections (5 μm) were deparaffinized and rehydrated and treated with hydrogen peroxide and then carried out with antigen retrieval. The sections of patients were blocked for 10 minutes and then were incubated with anti-FoxP3 antibody (1:3000; Abcam) for 1 hour, the sections of mouse tissues were incubated with anti-mCD4 (1:700; Abcam), and anti-mCD8 (1:1000; eBioscience) antibody according to the operation manual of rabbit-specific HRP/DAB (ABC) Detection IHC Kit (Abcam). All tissues were counterstained with H&E.

2.12 | Immunofluorescent analysis

Tissue samples were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin sections (4–5 μm) were deparaffinized, rehydrated, and antigen retrieval was carried out. The sections were then incubated with rat anti-mouse FoxP3 (eBioscience) antibodies after blocking. The Alexa Fluor 647-labeled goat anti-rat (Cell Signaling Technology) for FoxP3 as secondary antibodies and then were detected by the fluorescence microscope (Nikon Eclipse Ti-SR) under 200 \times magnification.

2.13 | Statistics

The results show the most typical of three independent experiments. Data are shown as mean \pm SD and were analyzed with IBM SPSS version 20.0. Differences between groups were analyzed using the

repeated measures or one-way ANOVA, or *t* test, with significant difference defined as $P < .05$.

3 | RESULTS

3.1 | Tumor-infiltrating Tregs in human prostate cancer

Immunohistochemistry was carried out to detect the infiltration of Tregs in human prostate cancer tissue. The results indicated that the quantity of Tregs in tumor tissues was significantly higher than that in normal tissues ($P < .05$; Figure 1A).

3.2 | Effect of NCTD on proliferation and apoptosis of Tregs

Magnetic cell sorting collected a high purity of Tregs from the spleens of mice (>90%; Figure 1B). To investigate the effect of NCTD on the proliferation and apoptosis of Tregs, non-Tregs, and Tcons, cells were incubated with different concentrations of NCTD (0–80 $\mu\text{mol/L}$) for 24 hours, or with 20 $\mu\text{mol/L}$ NCTD for 0, 24, 48, or 72 hours. The results of the CCK-8 analysis showed that NCTD could dramatically inhibit the proliferation of Tregs compared with Tcons ($P < .05$; Figure 1C,D), but there was no difference between Tcons and non-Tregs. In addition, NCTD dramatically induced the apoptosis of Tregs ($P < .05$; Figure 2). All effects were in a dose- and time-dependent manner.

3.3 | Norcantharidin downregulated p-AKT and activated FOXO1

Western blot analysis was used to explore the associated mechanism regarding apoptosis of Tregs. The results showed that NCTD downregulated p-Akt, whereas the total Akt protein level unchanged. Treatment with NCTD increased the levels of FOXO1 and FasL protein and decreased the level of Bcl2 protein ($P < .05$; Figure 3A), but there is no obvious change in the level of ERK or p-ERK. Real-time PCR analysis showed that mRNA levels of Fas, FasL, FOXO3a, FOXO4, and FOXO1 were significantly upregulated in NCTD-treated Tregs ($P < .05$; Figure 3B). It is suggested that NCTD might induce apoptosis through inhibiting AKT and hence activating the FOXO1–FasL axis.

3.4 | Antitumor efficacy of vaccine therapy combined with NCTD

Flow cytometry revealed that SA-mGM-CSF could be efficiently anchored on the surface of biotinylated RM-1 cells (Figure 4A). The RM-1 s.c. mouse model was used to evaluate the antitumor efficacy of the vaccine combined with NCTD. Our results showed that the RM-1 cell vaccine modestly inhibited tumor growth. The addition of NCTD to the vaccine treatment was more effective in restraining tumor growth than vaccine alone ($P < .05$; Figure 4B). These results suggest that NCTD could promote the antitumor immunity of tumor cell vaccines.

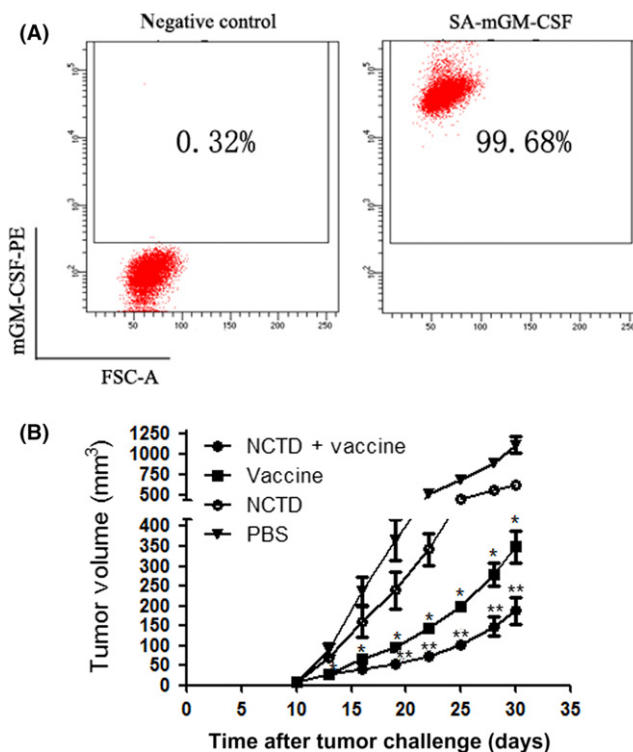


FIGURE 4 Efficacy of combination therapy with the vaccine and norcantharidin (NCTD) in prostate cancer. A, Presentation of streptavidin-tagged mouse granulocyte macrophage colony-stimulating factor (SA-mGM-CSF) on the surface of RM-1 cells was measured by flow cytometry, with unbiotinylated RM-1 cells as a negative control. B, All mice were injected s.c. with 2×10^6 RM-1 cells (right hind leg), and the tumor volume was recorded. The tumor size in the vaccine group was much smaller than that in the control group; the tumor size in the combination group was smallest among the four groups. * $P < .05$ vs PBS; # $P < .05$ vs Vaccine. FSC-A, forward scatter area; PE, phycoerythrin

3.5 | Flow cytometry analysis of DCs in blood

To evaluate the effect of the GM-CSF surface-modified tumor cell vaccine on mature DC cells, cells were collected from the blood and analyzed by flow cytometry. The results showed that mature DCs were distinctly increased after treatment with GM-CSF surface-modified vaccine. Treatment with NCTD alone had no significant effect on DC maturation ($P < .05$; Figure 5A).

3.6 | Norcantharidin therapy decreased Treg populations in vivo

Flow cytometry was used to investigate the quantity of Tregs in blood. As anticipated, the frequency of Tregs was increased in the Vaccine group and decreased in the NCTD group compared to the PBS group. Importantly, the frequency was much lower in the Vaccine + NCTD group compared to the Vaccine group ($P < .05$; Figure 5B).

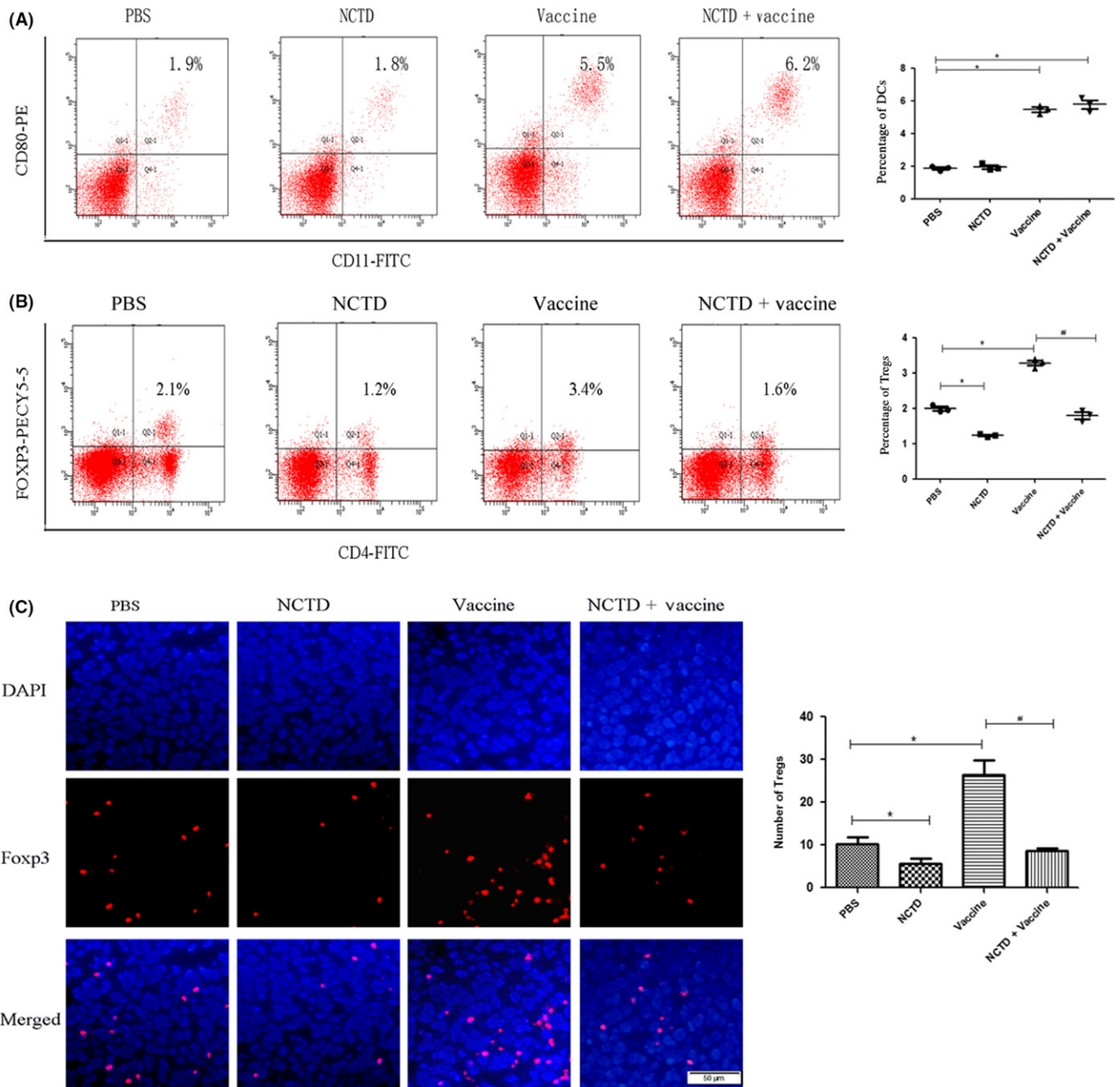


FIGURE 5 Proportion of mature dendritic cells (DCs) and regulatory T cells (Tregs) in mice after combination therapy with norcantharidin (NCTD) and vaccine. A, To detect the mature DCs, blood samples were collected from each treatment group on day 30 after tumor cell injection. Cells were stained with FITC-labeled anti-mCD11c and phycoerythrin (PE)-labeled anti-mCD80 antibodies, and measured by flow cytometry. B, Blood samples were collected from mice on day 30 after tumor cell injection and stained with FITC-labeled anti-mCD4 and Pcy5.5-labeled anti-mFoxp3 antibodies. C, Tumor tissues were collected from each group. Tregs were detected by immunofluorescence (red) (magnification, $\times 200$). All experiments were repeated at least three times. * $P < .05$ vs PBS; # $P < .05$ vs Vaccine

The proportions of Tregs in tumor tissues were detected using immunofluorescence. The results showed that more Tregs were infiltrated into the tumor tissues after the vaccine therapy. In the combination group, the number of Tregs was less than that in the Vaccine group ($P < .05$; Figure 5C). These results indicated that NCTD could decrease the number of infiltrating Tregs in vaccine treatment.

3.7 | Flow cytometry analysis of CD4⁺ and CD8⁺ T lymphocytes in blood after vaccine treatment

The rates of CD4⁺ and CD8⁺ T lymphocytes in peripheral blood of mice were analyzed by flow cytometry. Results showed that the proportions of CD4⁺ and CD8⁺ T cells were increased after treatment with vaccine or NCTD ($P < .05$; Figure 6A). The proportions of CD4⁺

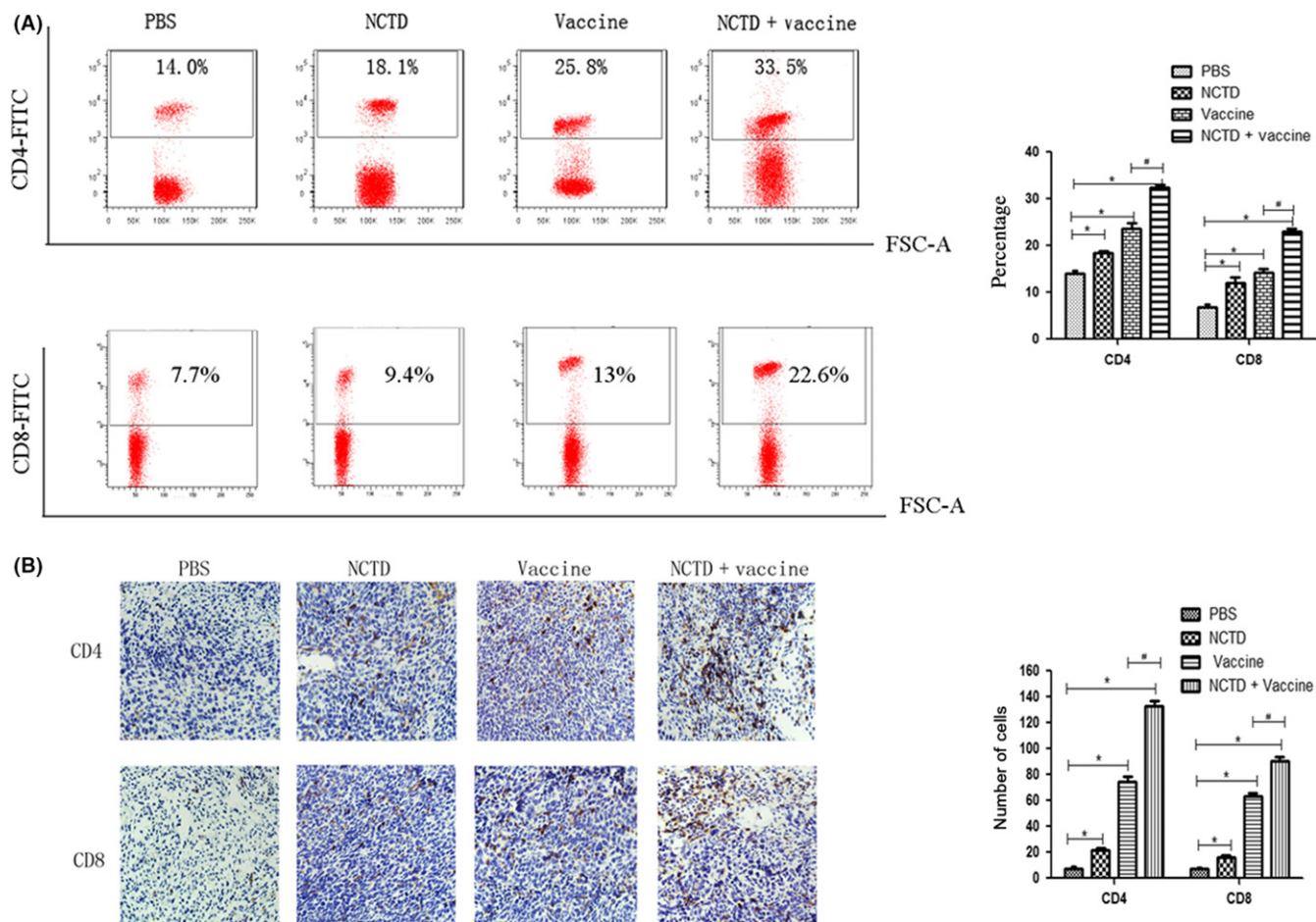


FIGURE 6 Analysis of T lymphocytes after combination therapy with vaccine and norcantharidin (NCTD). A, Blood samples were collected from each group on day 30 after tumor cell injection. Cells were stained with FITC-labeled anti-mCD4 and FITC-labeled anti-mCD8 antibodies and analyzed by flow cytometry. B, Tumor tissues were collected from each group on day 30 after tumor cell injection. Tumor-infiltrating CD4⁺ and CD8⁺ T lymphocytes were detected by immunohistochemistry. Sections are representative of the total vital tumor tissue (magnification, $\times 200$). All experiments were repeated at least three times. * $P < .05$ vs PBS; # $P < .05$ vs Vaccine

and CD8⁺ T cells in the combination group were the highest among the four groups ($P < .05$; Figure 6A).

The numbers of CD4⁺ and CD8⁺ T cells in the tumor tissues were detected by immunohistochemistry assay. The results revealed that the trend in the tumor tissue was the same as that in blood. Tumor-infiltrating T lymphocytes were increased after vaccine or NCTD treatment, and combination therapy induced more T-lymphocyte infiltration compared with vaccine or NCTD alone ($P < .05$; Figure 6B). These results indicated that NCTD could enhance the vaccine-induced immune responses.

4 | DISCUSSION

In this study, we showed that Tregs widely existed in human prostate cancer tissue. Norcantharidin could induce apoptosis and inhibit proliferation of Tregs and its mechanism is probably associated with the Akt-FOXO1-FasL pathway. Moreover, our results indicated that the SA-mGM-CSF vaccine could effectively induce antitumor

immune response. However, more Tregs existed in the blood and infiltrated into the tumor tissues after vaccine therapy. The addition of NCTD could decrease the number of Tregs and significantly enhance the vaccine-induced immunity. This is the first report that NCTD could induce apoptosis of Tregs. Norcantharidin therapy might overcome the vaccine-induced immunosuppression to enhance the vaccine-induced antitumor immune response in mouse prostate cancer.

Vaccine therapy is an important and effective approach in treating many malignancies.^{19,20} Several published studies have shown that GM-CSF-modified cell vaccines could induce effective antitumor immunity.^{19,21} Granulocyte macrophage colony-stimulating factor plays a major role in maturation of DCs and activation of CD4⁺ and CD8⁺ T cells. In this research, the numbers of mature DCs and activated T cells were increased after vaccine treatment. However, more immune-suppressive Tregs infiltrated into the tumor tissue and blood after vaccine therapy, and NCTD therapy could deplete the infiltrated Tregs. Therefore, it seems that NCTD could decrease Tregs and enhanced the vaccine-induced immunity in vivo.

FoxP3-expressing Tregs are an important subset of CD4 T cells, which suppress the effective immune response.²² Many studies have reported that the infiltration of a large number of Tregs into tumor tissues is closely related to poor prognosis.^{7,23} Consistent with previous research, our results also showed that there were abundant tumor-infiltrated Tregs in human prostate cancer tissue.⁸ Previous studies have shown that depletion of tumor-infiltrating Tregs is able to enhance the antitumor immune response.^{24,25} Therefore, it is essential to reduce Tregs to enhance antitumor immunity.

Norcantharidin, a demethylated form of CTD, has less toxicity and higher antitumor ability than CTD,²⁶ and could increase the number of white blood cells by stimulating the bone marrow.²⁷ Our results also indicated that NCTD could increase the number of leukocytes. A previous study indicated that NCTD inhibited the proliferation and promoted apoptosis of cancer cells,¹⁴ but its effect on Tregs is not yet clear. This study revealed that NCTD could inhibit proliferation and enhance apoptosis of Tregs in a dose- and time-dependent manner. These findings suggest that NCTD might be an effective drug to decrease Tregs in vitro.

It has been shown that NCTD could regulate the activity of FOXO transcription factors,²⁸ which play an important role in regulating diverse immune functions such as proliferation, differentiation, apoptosis, DNA repair, longevity, and stress resistance.^{29,30} FOXO families, especially FOXO1 and FOXO3a, promote cell apoptosis through upregulating FasL transcription.³¹ However, PI3K/AKT could negatively regulate FOXO1 and lead to apoptosis.³² Previous observations suggested that FOXO1 is a pivotal regulator of Treg cell function,³³ and negatively regulates Treg differentiation.³⁴ In this study, we analyzed the Akt-FOXO1-FasL pathway in NCTD-induced apoptosis. The results showed that the level of p-Akt was decreased, which was closely associated with increased FOXO1 transcription in NCTD-treated Tregs. Furthermore, the expressions of apoptosis-related genes, including *Fas* and *FasL*, were increased in the experimental group compared with the control group. Therefore, the mechanism of NCTD-induced apoptosis of Tregs might be related to the inhibition of p-Akt and activation of FOXO1 transcription protein.

This is the first study to report that NCTD could induce apoptosis of Tregs and enhance the antitumor immunity of tumor cell vaccines. The molecular mechanism of NCTD-induced apoptosis of Tregs might be through the inhibition of AKT and activation of FOXO1-FasL. We speculate that NCTD could represent a new method of eliminating Tregs. This study provides valuable information in cancer immunotherapy.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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