Synergistic effects of low-dose chemotherapy and T cells in renal cell carcinoma

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Abstract. Renal cell carcinoma (RCC) is not sensitive to conventional radiotherapy and chemotherapy, and the effectiveness rate of molecular targeted therapy is low. Therefore, it is urgent to identify new treatment methods. Recently, adoptive T-cell therapy has provided a new option for cancer treatment. Furthermore, low-dose chemotherapy not only has no evident side effects and inhibitory effects on the human immune system, but can also enhance the immune activity of some effector cells. Therefore, it is surmised that the combination of different mechanisms of chemotherapy and immunotherapy could be a new treatment concept. In the present study, the effects of low-dose chemotherapy combined with T cells in the treatment of renal cell carcinoma were explored using cytotoxicity assays, enzyme-linked immunosorbent assay (ELISA), western blot analysis and flow cytometric analysis. The results revealed that low-dose chemotherapy and T cells had synergistic effects on tumor cell elimination *in vitro*. The transforming growth factor (TGF)-β signaling pathway may be involved in the inhibition of T-cell functions. The targeted inhibition of TGF-β signals may be a promising therapeutic strategy for the treatment of renal cancer. The present results provided a novel strategy for the combination of low-dose chemotherapy and T cells to enhance the therapeutic efficacy of RCC treatment.

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Introduction

Renal cell carcinoma (RCC) is a common malignant tumor of the urinary system in adults, accounting for 3% of all cancer cases (1). In recent years, the incidence of RCC has been increasing annually; its degree of malignancy is extremely high, and it is difficult to detect in the early stage (2). By the time it is first diagnosed, 30% of RCC patients present with tumor metastases (3), and the survival rate of patients with metastatic RCC can be as low as 9.5% (4). Renal cancer is not sensitive to conventional chemo-radiotherapy and the effectiveness of molecular targeted therapy is low. Therefore, exploring effective treatment methods for RCC is of great significance for improving the survival rate of patients with RCC.

Cytotoxic T lymphocytes (CTLs) are specific immune defense effector cells of the immune system. Several factors affect their killing effects on tumors, such as the secretion of interferon (IFN)-γ and tumor necrosis factor (TNF) and the distance between CTLs and tumors (5). Clinical results have revealed that T-cell persistence can improve therapeutic responses, and immune memory can enhance T-cell persistence (6,7). As a result, immune memory is essential for therapeutic responses. Preclinical animal models have also confirmed that memory T cells are key to antitumor effects, including central memory T (T_{CM}) and effector memory T (T_{EM}) cells (8). $CD4^+$T cells are indispensable for functional $CD8^+$T cell memory formation (9). In addition, $CD4^+$T cells play several roles in cancer treatment, including enhancing the activation, recruitment, proliferation and cytotoxic function of $CD8^+$T cells and directly inhibiting tumor growth (10,11). Given the functions of $CD4^+$T cells, these T cells were used in the present experiments. CTLL-2 cells isolated from C57/BL/6 inbred mice are mouse-derived cytotoxic T cells, and the signaling pathway in CTLL-2 cells can be used as a model in CTL cells (12).

As an important tumor treatment method, chemotherapy can kill cancer cells in the rapid proliferation stage. However, the dose and duration of the effects of chemotherapeutic drugs are limited by acute and cumulative toxicity in normal tissues (13,14). Hence, it is urgent to identify anticancer treatments with natural properties, low toxicity, and high efficacy. Low-dose chemotherapy not only has no significant side effects or inhibitory effects on the immune system, but can also improve the immune activity of some effector cells (15). In the present study, diaminedichloroplatinum (DDP) and mitomycin C (MMC), two traditional broad-spectrum chemotherapeutic drugs used for various types of tumors (16), were selected to study the toxicity of low-dose drugs in tumor cells. DDP is a nonspecific chemotherapeutic drug that can inhibit proliferation and promote apoptosis in tumor cells (17), and it has been used for several years in clinical tumor treatment (18). MMC is also widely used for the treatment of various types of cancer, such as bladder, stomach and cervical cancer (19). These two drugs were selected with the purpose of extending this approach to more tumors than just renal cancer.

The rapid development of immunotherapy has generated a fourth therapy for cancers, which can be used in parallel with surgery, chemotherapy and radiotherapy (20). Cellular immunotherapy can recognize a small number of tumor cells and has powerful lethal and targeting effects (21). Therefore, low-dose chemotherapy combined with cellular immunotherapy is of great significance for clearing small lesions and reducing recurrence rate in patients. In the present study, the synergistic killing effect of low dose-chemotherapy combined with T cells on RCC cells *in vitro* was observed and the possible underlying synergistic mechanisms were explored. The present results provided a new concept and an experimental basis for the comprehensive treatment of RCC.

Materials and methods

Cell culture and chemotherapy treatment. The RCC cell lines RENCA and ACHN and the mouse-derived cytotoxic T cells CTLL-2 were obtained from the Jiangsu Cancer Biotherapy Institute, Xuzhou Medical University (Xuzhou, China). RENCA and CTLL-2 cells were cultured in RPMI-1640 medium and ACHN cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Life Technologies; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (FBS; Gibco; Life Technologies; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Sangon Biotech Co., Ltd.). RCC cells were detached using 0.25% trypsin (Beyotime Institute of Biotechnology). All cells were maintained in incubators (Thermo, Fisher Scientific, Inc.) with 95% air and 5% CO_2 at 37˚C.

DDP and MMC were purchased from Jiangsu Hansoh Pharmaceutical Group Co., Ltd. and Vicmed, respectively. The concentration of the DDP storage solution was 5 mg/ml. Two milligrams of MMC powder was dissolved in phosphate-buffered saline (PBS) to prepare a 2 mg/ml stock solution. For chemotherapy treatment and antitumor analysis, renal cancer cells were treated with serially diluted doses of DDP or MMC for 24 h. In order to avoid the cumulative toxicity of drugs, tumor cells treated with drugs for 24 h were used to co-incubate with T cells (Fig. 1A).

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 detection kit (Nanjing KeyGen Biotech Co., Ltd.) was used to select a low-dose drug concentration with an inhibition rate of <30% that kills tumor cells and avoids toxic effects on normal tissues (22) and to evaluate the sensitivity of tumor cells to chemotherapy drugs. Briefly, the two cell lines were inoculated into 96-well plates at $4.0x10^3$ cells/well for 24 h, and then the cells were treated with drugs for another 24 h. RENCA cells were exposed to various concentrations of DDP diluent (0, 0.25, 0.5, 1, 2.5, 5 and 10 μg/ml) or MMC diluent (0, 0.05, 0.1, 0.5, 1, 5 and 10 μg/ml), ACHN cells were exposed to various concentrations of DDP diluent (0, 0.5, 1, 2.5, 5, 10 and 20 μg/ml) or MMC diluent (0, 0.005, 0.01, 0.05, 0.1, 0.5 and 2 μg/ml). Subsequently, the cells were incubated in 100 μl serum-free medium with 10 μl CCK-8 solution at 37˚C for 2 h. The relative cell viability was determined by measuring the absorbance of the converted dye at 450 nm. RENCA and ACHN cells were exposed to 1 μg/ml DDP and 0.1 μg/ml MMC diluent separately for 0, 24, 48 and 72 h to detect tumor cell proliferation.

Preparation of T cells. Ten 4-week-old female BALB/c mice weighing 16 g (Huafukang Bioscience) were raised with the feed and sterile water for 1 week at our well-ventilated animal facility with 25˚C, humidity of 60% and a 12-h light/dark cycle before testing was initiated. The Animal Experimental Ethics Committee of Xuzhou Medical University approved all the animal studies. The mice were sacrificed by cervical dislocation and the disappearance of a blink reflex indicated the death of the mice. T cells were isolated from the lymph nodes and spleen of mice with an EasySep™ Mouse T Cell Isolation Kit (STEMCELL Technologies, Inc.). T cells were cultured in RPMI-1640 complete medium at a cell density of $1x10^6$/ml and activated by CD3 antibody (5 μg/ml) and CD28 antibody (2 μg/ml; both from Sigma-Aldrich; Merck KGaA). Peripheral blood mononuclear cells (PBMCs) from a 25-year-old female healthy blood donor who agreed to use her samples in scientific research were collected and isolated by density gradient centrifugation at our institute. The inclusion of human subjects in our study was approved by the Ethics Committee of the First People's Hospital of Lianyungang and the informed consent was obtained from the blood donor. T cells were isolated with an EasySep™ Human T Cell Isolation Kit (STEMCELL Technologies, Inc.) and amplified using a Dynabeads® Human T-Expander CD3/CD28 Kit (Thermo, Fisher Scientific, Inc.). T cells were activated in RPMI-1640 complete medium with 300 IU/ml interleukin (IL)-2 (SL PHARM) at a cell density of $1x10^6$/ml. After 48 h of activation, the T cells were used for subsequent experiments.

Flow cytometric analysis. Flow cytometry was used to detect the surface expression of CD4 and CD8 in T cells. The antibodies used for staining were purchased from BD Biosciences and included anti-mouse CD4-fluorescein isothiocyanate (FITC; cat. no. 557667), anti-mouse CD8-peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5; cat. no. 551162), anti-human CD4-FITC (cat. no. 557695) and anti-human CD8-phycoerythrin (PE; cat. no. 557086). A total of $1x10^6$ T cells were incubated in 100 μl PBS with 0.5 μl fluorescence-labeled antibody for 30 min at room temperature in the

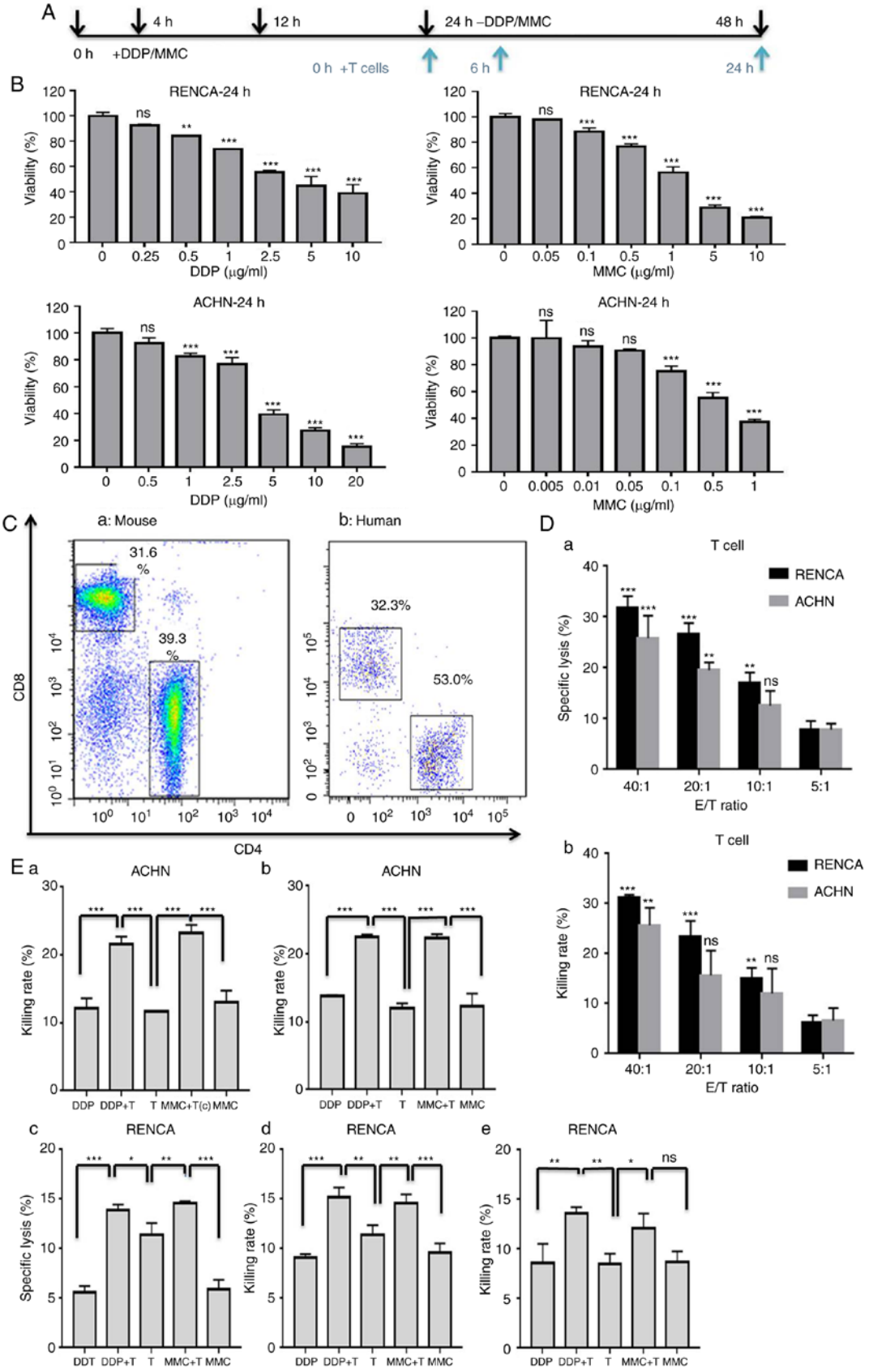

Figure 1. Killing effect of T cells combined with low-dose chemotherapy on renal cancer cells. (A) Time axis of low-dose chemotherapy combined with T cells. (B) Determination of low-dose drug concentrations. A CCK-8 assay was performed to determine the concentrations of DDP and MMC that resulted in an inhibition rate of <30% after 24-h treatment in RCC cells. (C) Expression of CD4 and CD8 in purified T cells. CD4 and CD8 expression levels in T cells isolated and purified from (a) mice and (b) humans were verified by FACS analysis. (D) Specific cytotoxicity exhibited by T cells toward RCC. The cytotoxic activity of T cells toward RCC cells was determined by (a) LDH and (b) CCK-8 assays. (E) Synergistic effects of low-dose chemotherapy and T cells on RCC cells. RCC cells treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h were coincubated with T cells for 6 h. The cytotoxic activity of T cells was determined by (a and d) CCK-8 assay, (b and e) luciferase assay and (c) LDH release assay. *P<0.05; **P<0.01; ***P<0.001; ns, not significant. RCC, renal cell carcinoma; DDP, diaminedichloroplatinum; MMC, mitomycin C; LDH, released lactate dehydrogenase; CCK-8, Cell Counting Kit-8.

dark, washed, and then analyzed using a FACS instrument (FACSCanto II; BD Biosciences).

An FITC-Annexin V Apoptosis Detection Kit (Nanjing KeyGen Biotech Co., Ltd.) was used to detect the effects of chemotherapeutic drugs on apoptosis in RCC cells. RENCA and ACHN cells, which had been exposed to 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h, were stained with 5 μl Annexin V-FITC and 5 μl propidium iodide in 500 μl binding buffer for 15 min at room temperature in the dark, washed, and then analyzed with a FACS instrument. T cells were mixed with RCC cells that had been treated with DDP and MMC in a 10:1 ratio and cultured in a 24-well plate for 24 h. After removing the T cells, $1x10^5$ RCC cells were stained for analysis.

The effects of chemotherapeutic drugs on T cell phenotypes were detected using flow cytometry. RENCA cells were exposed to 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h, and T cells were then mixed with the RENCA cells at a 10:1 ratio and cultured in a 24-well plate for 24 h. The antibodies used for staining were purchased from BD Biosciences and included anti-CD8-PerCP-Cy5.5 (cat. no. 551162), anti-CD62L-allophycocyanin (APC; cat. no. 561919), anti-CD44-PE (cat. no. 561860), and anti-programmed death factor 1 receptor/ligand (PD-L1)-APC (cat. no. 564715). A total of $1x10^6$ T cells were stained with 0.5 μl fluorescence-labeled antibody in 100 μl PBS for 30 min at room temperature in the dark, and then the stained T cells were detected using a flow cytometer.

Cytotoxicity assay. T cells were cultured with target cells (RENCA/ACHN) in 96-well plates at effector-to-target (E/T) ratios of 40:1, 20:1, 10:1 and 5:1 for 6 h (6,23). The released lactate dehydrogenase (LDH) in the supernatants was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Test Kit (Promega Corporation) according to the manufacturer's recommendations. The specific cytotoxicity was calculated according to the formula: % Cytotoxicity=100x [(experimental release-effector spontaneous release-target spontaneous release)/(target maximal release-target spontaneous release)]. The CCK-8 method was also utilized to evaluate the killing effect of T cells on RCC cells. Before testing, the T cells were removed, and RCC cells were then incubated in 100 μl serum-free medium with 10 μl CCK-8 solution at 37˚C for 2 h. The viability was determined by measuring the absorbance of the converted dye at 450 nm.

RCC cells (RENCA/ACHN) were exposed to 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h. T cells were mixed with RCC cells at a 10:1 ratio and cultured in 96-well plates for 6 h. LDH and CCK-8 assays were used to explore the cell toxicity effects induced by T cells combined with chemotherapy. Additionally, luciferase transfected RCC cell lines preserved in our laboratory were used to determine the cell killing rate by measuring the absorbance at 135 nm within 15 min after adding fluorescein (TCI) to the 96-well plates in the dark as previously described (24).

Enzyme-linked immunosorbent assay (ELISA). T cells were mixed with RCC cells that had been treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h at an E/T ratio of 10:1 in a 96-well plate for 6 or 24 h. The IFN-γ level in the supernatant was analyzed by ELISA kits [MultiSciences (Lianke) Biotech, Co., Ltd.] according to the manufacturer's recommendations.

RCC cells (RENCA/ACHN) were exposed to 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h, and then the TGF-β in the supernatant was analyzed. RCC cells that had been exposed to 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h were cultured in medium for another 24 h. The TGF-β level in the supernatant was assayed by an ELISA kit [MultiSciences (Lianke) Biotech, Co., Ltd.] according to the manufacturer's recommendations.

Cell chemotaxis analysis. The effects of drugs (DDP/MMC) on the chemotaxis of T cells were assessed using Transwell chambers (5-μm polycarbonate membrane; Costar; Corning, Inc). Briefly, the top chamber was loaded with 0.3 ml $2x10^6$ T cells in media, and the bottom chamber was loaded with 0.5 ml $2x10^5$ RCC cells in medium. After 6 and 24 h of treatment, the penetrating T cells were collected, stained with a 0.4% trypan blue solution (Sigma-Aldrich; Merck KGaA), and counted under a light microscope.

Western blot analysis. After 24 h of treatment with DDP or MMC, the drugs were removed and the RCC cells were cultured in medium for another 24 h. RCC cells that had been treated with drugs for 4, 12, and 48 h were collected, and T cells were collected after a 24-h incubation with RCC cells treated with DDP or MMC at a 10:1 ratio. Protein from these cells was extracted in lysis buffer (Beyotime Institute of Biotechnology). Equal amounts of protein (20 μg) after protein concentration determination by BCA on a microplate reader (ELx800™; BioTek USA) were loaded, separated on 10% sodium dodecyl sulfate (SDS) gels and transferred onto nitrocellulose membranes (Amersham; GE Healthcare). Then, the membranes were blocked with 5% nonfat milk at room temperature for 2 h and immunoblotted with anti-TGF-βR1 (3712), Smad2 antibody (5339; 1:1,000; Cell Signaling Technology, Inc.) or β-actin antibody (4967; 1:1,000; Cell Signaling Technology, Inc.) overnight at 4˚C. A horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse antibody (BA1056; 1:10,000; Wuhan Boster Biological Technology, Ltd.) was reacted with the membrane at room temperature for 2 h. The immobilized antibodies were then detected by an ECL chemiluminescent reagent (EMD Millipore) and visualized with the ChemiDoc XRS system (Bio-Rad laboratories, Inc.).

Statistical analysis. The data are presented as the mean ± standard deviation (SD) of three independent experiments. The data were analyzed using SPSS statistical software version 18.0 (SPSS, Inc.). Comparisons between two groups were performed using a two-tailed Student's t-test. One-way ANOVA and Tukey's post hoc test were used when more than two groups were compared. Differences with $P<0.05$ were considered statistically significant.

Results

Killing effects of T cells combined with low dose chemotherapy on renal cancer cells in vitro. CCK-8 assays were performed to determine the concentrations of DDP and MMC with an inhibitory rate of <30% in the early stage (24 h). The inhibitory effects of DDP or MMC on RCC cells were revealed to be dose-dependent. As drug concentrations increased, the survival rate was obviously decreased in RCC

Table I. Effects of DDP and MMC on the viability of RCC cells (mean ± SD).

RENCA cells		ACHN cells	
Drug concentration (μg/ml)	Viability at 24 h (%)	Drug concentration (μg/ml)	Viability at 24 h (%)
DDP			
0	100.00±2.95	0	100.00±3.35
0.25	92.52±1.07[a]	0.5	92.49±3.94[a]
0.5	84.28±0.01[b]	1	82.89±2.07[b]
1	73.85±0.35[b]	2.5	77.07±4.72[b]
2.5	55.42±1.58[b]	5	39.38±3.44[b]
5	44.85±7.32[b]	10	27.25±2.29[b]
10	38.86±6.87[b]	20	15.57±2.07[b]
MMC			
0	100.00±2.69	0	100.00±1.49
0.05	97.99±0.41[c]	0.005	99.84±13.33[c]
0.1	88.58±2.68[d]	0.01	93.84±4.25[c]
0.5	76.82±2.00[d]	0.05	90.56±1.32[c]
1	56.28±4.47[d]	0.1	75.35±3.75[d]
5	28.78±2.05[d]	0.5	55.46±3.86[d]
10	20.97±1.06[d]	1	37.55±1.69[d]

[a]P>0.05 (ns) or [b]P<0.05, compared with the untreated DDP (0 μg/ml) group; [c]P>0.05 (ns) or [d]P<0.05, compared with the untreated MMC (0 μg/ml) group. RCC, renal cell carcinoma; DDP, diamminedichloroplatinum; MMC, mitomycin C; ns, not significant.

cells when compared with untreated cells (Fig. 1B). After 24 h that of treatment with drugs, the viabilities of RENCA and ACHN cells were 73.85±0.35 and 82.89±2.07%, respectively, in the DDP (1 μg/ml) group and 88.58±2.68 and 75.35±3.75%, respectively, in the MMC (0.1 μg/ml) group (Table I). Follow-up experiments were carried out with 1 μg/ml DDP and 0.1 μg/ml MMC.

FACS was used to assess the surface expression of CD4 and CD8 in purified mouse or human T cells. The percentages of CD4 and CD8 cells were 38.1±0.14 and 31.7±0.42%, respectively, in mouse T cells and 52.1±1.27 and 32.65±0.49%, respectively, in human T cells (Fig. 1C). To evaluate the cytotoxic effects of T cells against renal cancer cells, lactate dehydrogenase (LDH) release assays and CCK-8 assays were performed. As revealed in Fig. 1D, with the increase in the E/T ratio, the killing activity of T cells against RENCA and ACHN cells was enhanced compared to that in the 5:1 E/T ratio group. Therefore, an E/T ratio of 10:1 was used for all subsequent *in vitro* studies.

To explore whether DDP or MMC treatment could increase the sensitivity of RCC cells to T cell-mediated lysis, RENCA and ACHN cells were treated with drugs for 24 h and then used as target cells in LDH release assays, CCK-8 assays, and Luc assays. The results revealed that DDP and MMC significantly increased the sensitivity of RENCA and ACHN cells to the effects of T cells (Fig. 1E). Collectively, the results revealed that DDP and MMC was capable of altering renal cancer cells to render them more amenable to T cell-mediated attack, and low-dose chemotherapy and the T cells exhibited a synergistic killing effect on renal cancer cells *in vitro*.

Effect of low-dose chemotherapy on renal cancer cells in vitro. First, the CCK-8 method was utilized to assay the effect of DDP and MMC on the proliferation of RENCA and ACHN cells. RENCA and ACHN cells were exposed to 1 μg/ml DDP or 0.1 μg/ml MMC for 0, 24, 48, or 72 h, and the viability of RENCA and ACHN cells was significantly decreased as the reaction time increased (Fig. 2A; Table II). Then, FACS was used to assess the apoptosis rates of RENCA and ACHN cells treated with chemotherapy drugs (24 h) or chemotherapy combined with T cells (48 h). Compared with those of untreated cells, the apoptosis rates of RCC cells treated with DDP or MMC were significantly increased (Fig. 2B; Table III). These data also revealed that low-dose chemotherapy and T cells had a synergistic apoptotic effect on renal cancer cells *in vitro*. Finally, western blot analysis was performed to detect the expression of transforming growth factor-β receptor 1 (TGF-βR1) in renal cancer cells treated with DDP or MMC for 4 h and 12 h. The results revealed that DDP and MMC significantly decreased the expression of TGF-βR1 compared with untreated cells, indicating that chemotherapy drugs could inhibit the expression of immune-related proteins in the early stage of treatment to enhance the anti-tumor effect of T cells (Fig. 2C).

Effect of low-dose chemotherapy on T cells in vitro. To investigate whether chemotherapy drugs could enhance the specificity and activation of T cells, cytokine release assays were performed. T cells were co-cultured with RCC cells that had been treated with DDP or MMC for 24 h at an E/T ratio of 10:1. After 6 h of incubation, the IFN-γ cytokine expression of CTLL-2 cells in the drug groups was significantly higher than that in the CTLL-2 untreated group (Fig. 3A). These results

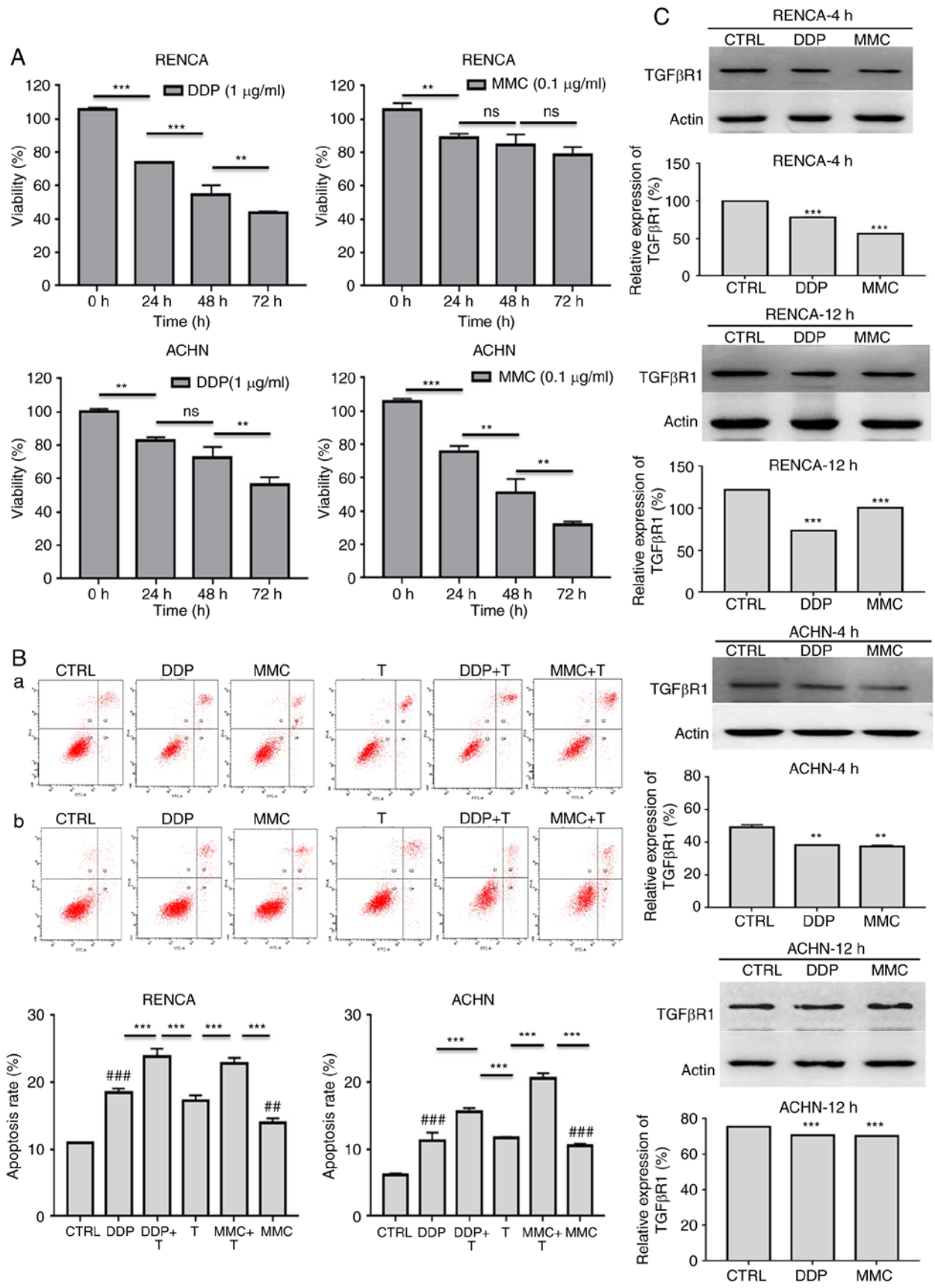

Figure 2. Effects of low-dose chemotherapy on renal cancer cells. (A) Effects of DDP and MMC on the viability of RCC cells. RENCA and ACHN cells treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 0, 24, or 72 h were assayed for viability by a CCK-8 assay. (B) Effects of the drugs (DDP or MMC) or drugs combined with T cells on the apoptosis of RCC cells. FACS was used to assess the apoptotic rates of (a) RENCA and (b) ACHN cells treated with 1 μg/ml DDP, 0.1 μg/ml MMC for 24 h or drugs combined with T cells sequentially for another 24 h. (C) Effects of DDP and MMC on TGF-βR1 expression in RCC cells. The TGF-βR1 expression in RENCA and ACHN cells treated with DDP or MMC for 4 and 12 h was assessed by western blot analysis. Actin was used to normalize the protein content. **P<0.01; ***P<0.001; $^{\#\#}$P<0.01 vs. the CTRL; $^{\#\#\#}$P<0.001 vs. the CTRL; ns: not significant. RCC, renal cell carcinoma; DDP, diaminedichloroplatinum; MMC, mitomycin C; CCK-8, Cell Counting Kit-8; TGF-βR1, transforming growth factor-β receptor 1; CTRL, control.

Table II. Effects of DDP and MMC on the proliferation of RCC cells.

Group	Drug concentration (μg/ml)	Viability (%, mean ± SD)			
		0 h	24 h	48 h	72 h
RENCA	DDP (1)	105.70±1.16	73.85±0.35[a]	54.71±5.63[b]	43.51±1.11[d]
	MMC (0.1)	105.65±4.03	88.58±2.68[a]	84.44±6.39[c]	78.58±4.64[e]
ACHN	DDP (1)	100.20±1.71	82.89±2.07[a]	72.69±6.39[c]	56.31±4.63[d]
	MMC (0.1)	105.60±1.85	75.35±3.75[a]	51.10±8.21[b]	31.65±1.95[d]

[a]P<0.05, compared with the drug-treated 0-h group; [b]P<0.05 or [c]P>0.05 (ns), compared with the drug-treated 24-h group; [d]P<0.05 or [e]P> 0.05 (ns), compared with the drug-treated 48-h group. RCC, renal cell carcinoma; DDP, diamminedichloroplatinum; MMC, mitomycin C; ns, not significant.

indicated that the DDP and MMC could specifically promote IFN-γ cytokine expression to enhance the killing effect of T cells in early stages. However, the level of the cytokine IFN-γ in the drug-treated group was significantly reduced after 24 h of incubation (Fig. 3A).

Cell migration assays were performed using a Transwell system. As revealed in Fig. 3B, a significantly higher number of CTLL-2 or T cells passed through the membrane into the lower chamber in the drug group than in the untreated group after 6 h of incubation with RCC cells. These results indicated that DDP and MMC could promote the migration of T cells to tumor sites to enhance the killing effect of T cells in the early stages. However, the migration of T cells in the drug group was significantly inhibited, and even the number of T cells was decreased compared to that in the untreated CTLL-2 or T cell group after 24 h of incubation (Fig. 3B).

It has been previously reported that chemotherapy can alter the phenotype of T cells to render them more sensitive to tumor cells (25). To determine whether DDP or MMC could regulate the cell surface marker expression, T cells were co-cultured with RENCA cells that had been treated with DDP or MMC for 24 h, and then the T cells were stained and analyzed by flow cytometry. DDP increased the percentage of $CD8^+CD44^+CD62L^+$T cells and decreased the percentage of $CD8^+CD44^+CD62L^-$T cells compared with the untreated cell group (Fig. 3C; Table IV). These data indicated that low-dose DDP could promote the transformation of effector memory T cells into central memory T cells to enhance the cytotoxicity of T cells toward tumor cells. Compared with the untreated cell group, DDP and MMC also significantly decreased PD1 expression in T cells (Fig. 3C; Table IV). The altered expression of these markers may aid in T cell cancer-killing effects.

Possible causes of the functional inhibition of T cells in vitro. To explore the causes of the decreased secretion of T-cell immune factors and the inhibition of T-cell migration toward tumor sites at 24 h, cytokine release assays and western blot analyses were performed to detect the expression of the cytokine TGF-β and the TGF-βR1 and Smad2 proteins in renal cancer cells. Compared with those in the control (CTRL) group, RCC cells in the DDP and MMC groups had stronger expression of TGF-β cytokine and TGF-βR1 and Smad2 proteins, although the increase in TGF-βR1 expression in RENCA cells in the DDP group was not statistically significant (Fig. 4A and B; Table V). TGF-βR1 and Smad2 protein expression in CTLL-2 and T cells was also detected. In the DDP and MMC groups, TGF-βR1 and Smad2 protein expression was significantly higher than that in the control group (Fig. 4C). These results indicated that the TGF-β signaling pathway may be involved in inhibiting T-cell function.

Table III. Apoptosis of RCC cells treated with drugs or combined with T cells (%, mean ± SD).

Group	RENCA	ACHN
CTRL	10.90±0.14	6.15±0.21
DDP + T	23.90±1.13	15.65±0.49
MMC + T	22.80±0.85	20.55±0.78
DDP	18.50±0.57[a,b]	11.20±1.27[a,b]
MMC	13.90±0.17[a,c]	10.50±0.28[a,c]
T	17.25±0.78[b,c]	11.70±0.14[b,c]

[a]P<0.05, compared with the CTRL group; [b]P<0.05, compared with the combined group (DDP + T); [c]P<0.05, compared with the combined group (MMC + T). RCC, renal cell carcinoma; CTRL, control, DDP, diamminedichloroplatinum; MMC, mitomycin C.

Discussion

RCC is a malignant tumor that seriously affects human health. Thirty percent of RCC patients have metastatic lesions at the time of diagnosis. At present, there are no effective drugs for patients with metastasis (4,26). Thus, the treatment of RCC represents an unmet clinical need and the discovery of innovative approaches for this purpose is urgently required. In the present study, low-dose chemotherapy combined with T cell immunotherapy, and the combination of the two methods had synergistic effects on renal cancer cell treatment *in vitro*. However, the tumor microenvironment (TME) in the body is a complex system composed of numerous cells that regulate different immune responses (27). The development of standardized, individualized chemotherapy combined with

Table IV. Effects of DDP and MMC on T cell subtypes (%, mean ± SD).

Groups	$CD8^+$ ($CD44^+CD62L^+$)	$CD8^+$ ($CD44^+CD62L^-$)	PD1
T	81.55±0.21	15.23±0.59	19.93±0.01
T+DDP	83.25±0.07[a]	13.17±0.61[a]	13.07±0.88[a]
T+MMC	82.05±1.20[b]	15.17±1.05[b]	14.93±0.32[a]

[a]P<0.05 or [b]P>0.05 (ns), compared with each T-cell group. DDP, diamminedichloroplatinum; MMC, mitomycin C; PD1, programmed death factor 1; ns, not significant.

Table V. Effects of DDP and MMC on TGF-β secretion of RCC cells (%, mean ± SD).

	RENCA		ACHN	
Drug concentration (μg/ml)	24 h	48 h	24 h	48 h
CTRL (0)	734.29±7.56	922.14±9.20	242.31±18.20	1,566.4±20.50
DDP (1)	851.97±26.22[a]	1,172.8±6.70[a]	530.13±16.16[a]	657.25±36.20[a]
MM (0.1)	880.53±36.8[a]	1,363.90±16.0[a]	459.38±0.00[a]	606.27±21.87[a]

The 48-h groups were another 24-h culture without drugs after 24-h treatment with drugs. [a]P<0.05, compared with each CTRL group. RCC, renal cell carcinoma; DDP, diamminedichloroplatinum; MMC, mitomycin C; TGF-β, transforming growth factor-β; CTRL, control.

immunotherapy with few side effects is the main direction of future research.

DDP and MMC are two of the earliest discovered chemotherapeutic agents and are the most widely used drugs (28). To avoid long-term and delayed side effects, low-dose chemotherapy drugs combined with T cell therapy was used to treat RCC. One of the main methods of cancer treatment is to induce apoptosis in cancer cells (29). Most chemotherapy drugs rely on the induction of apoptosis for efficacy (28). It has been reported that MMC and DDP can effectively inhibit tumor growth or significantly increase tumor apoptosis in rapidly proliferating cell populations (15,28,30). The present study revealed that low-dose chemotherapy could also inhibit proliferation and induce apoptosis in renal cancer cells. When combined with T cells, low-dose chemotherapy had a synergistic effect in inducing the apoptosis of renal cancer cells. Several studies have revealed that some chemotherapeutic drugs, such as DDP or MMC, in addition to their direct cytotoxic effects on tumor cells, can also regulate the antitumor immune response (25,31,32). In the present study it was revealed that DDP and MMC inhibited the expression of the immune-related protein TGF-βR1 in renal cancer cells in the early stage (4 or 12 h). TGF-βR1 is an important element of the TGF-β/SMAD signaling pathway, which has emerged as a central mediator of cancer progression due to its capacity to regulate cell growth, differentiation, and migration (33,34).

Adoptive T cell therapy provides a new treatment option for advanced cancer patients. Chemotherapeutic drugs can effectively induce immune responses by increasing the cross-presentation of tumor antigens, influencing the expression of cytokines and the migration of effector T cells, affecting production of immune memory, and reducing the number of immunosuppressive cells (35,36). The present study revealed that the combination of T cells with DDP or MMC increased the killing effect on renal cancer cells. Cytokines are important in maintaining or enhancing the function of T cells, and IFN-γ secretion was detected in T cells. The results revealed that low doses of the chemotherapeutic drugs DDP and MMC promoted IFN-γ secretion in the early stage (6 h) to enhance the antitumor effect. T cells must penetrate tumor sites to play their antitumor role effectively. Therefore, the effect of chemotherapeutic drugs on T-cell chemotaxis was examined. The results revealed that DDP and MMC could promote T cell infiltration into tumor cell sites at the early stage (6 h) to enhance the antitumor effect of T cells. $CD8^+$ ($CD44^+$ $CD62L^+$) T_{CMs}, with greater proliferation and survival potential than with $CD8^+$ ($CD44^+$ $CD62L^-$) T_{EM}, are pivotal for antitumor efficacy (6,8). The present results revealed that DDP could induce the transformation of T cells from effector memory T cells into central memory T cells, which is of great significance for T cells in exerting sustainable killing effects on tumor cells. Programmed death factor 1 receptor/ligand (PD1/PD-L1) plays an important role in regulating T cell immunity and has become a research hotspot in immunotherapy in recent years. PD1/PD-L1 can inhibit T-cell activation and proliferation, weaken the antitumor immune response, and negatively regulate immune responses (37). PD1 is expressed at various levels mainly on the surface of activated T cells (38). The present results revealed that chemotherapeutic drugs (DDP/MMC) could reduce PD1 expression in T cells to improve the persistence of T cells. A limitation of the present study was that FACS did not contain an isotype control. However, the antitumor

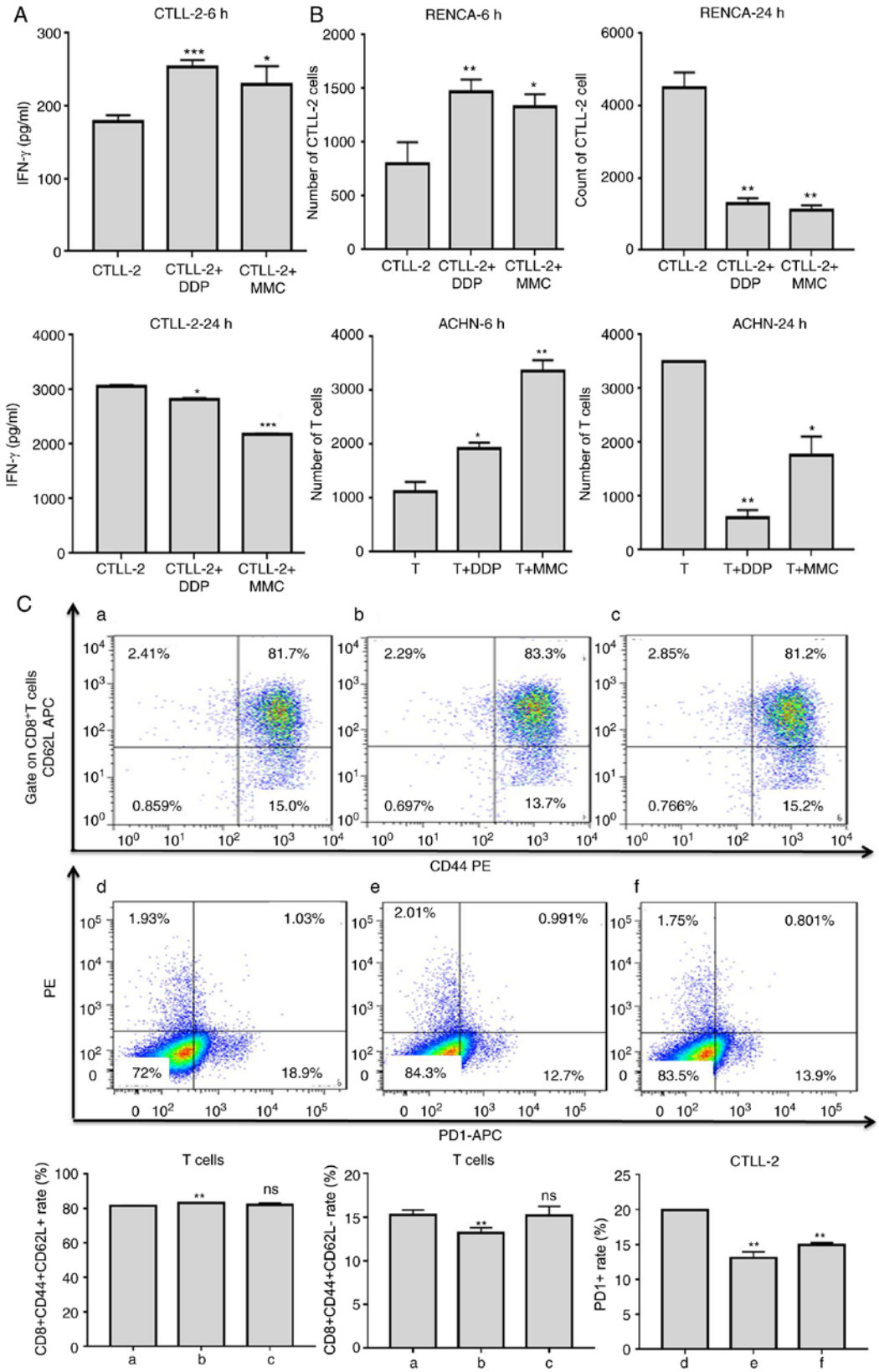

Figure 3. Effects of low-dose chemotherapy on T cells. (A) Effects of DDP and MMC on the cytokine IFN-γ release of T cells. RENCA cells treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h were coincubated with T cells for 6 and 24 h. The levels of the cytokine IFN-γ released by T cells were measured by ELISA after incubation with RENCA cells at an E/T ratio of 10:1. (B) Effects of DDP or MMC on the migration of T cells. RENCA and ACHN cells treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h were coincubated with T cells for 6 and 24 h. The migration of T cells was measured by a Trypan blue counting assay after incubation with RENCA and ACHN cells at an E/T ratio of 10:1. (C) Effects of DDP or MMC on T cell phenotypes. RENCA cells treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h were coincubated with T cells for another 24 h. Phenotypes of T cells and CTLL-2 cells were analyzed by flow cytometry for $CD8^+CD44^+CD62L^+$, $CD8^+CD44^+CD62L^-$ and PD1 surface expression. (a) T-cell group; (b) T cells combined with the DDP group; (c) T cells combined with the MMC group; (d) CTLL-2-cell group; (e) CTLL-2 cells combined with the DDP group; (f) CTLL-2 cells combined with the MMC group. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns, not significant. DDP, diaminedichloroplatinum; MMC, mitomycin C; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon γ; E/F, effector-to-target; PD1, programmed death factor 1 receptor.

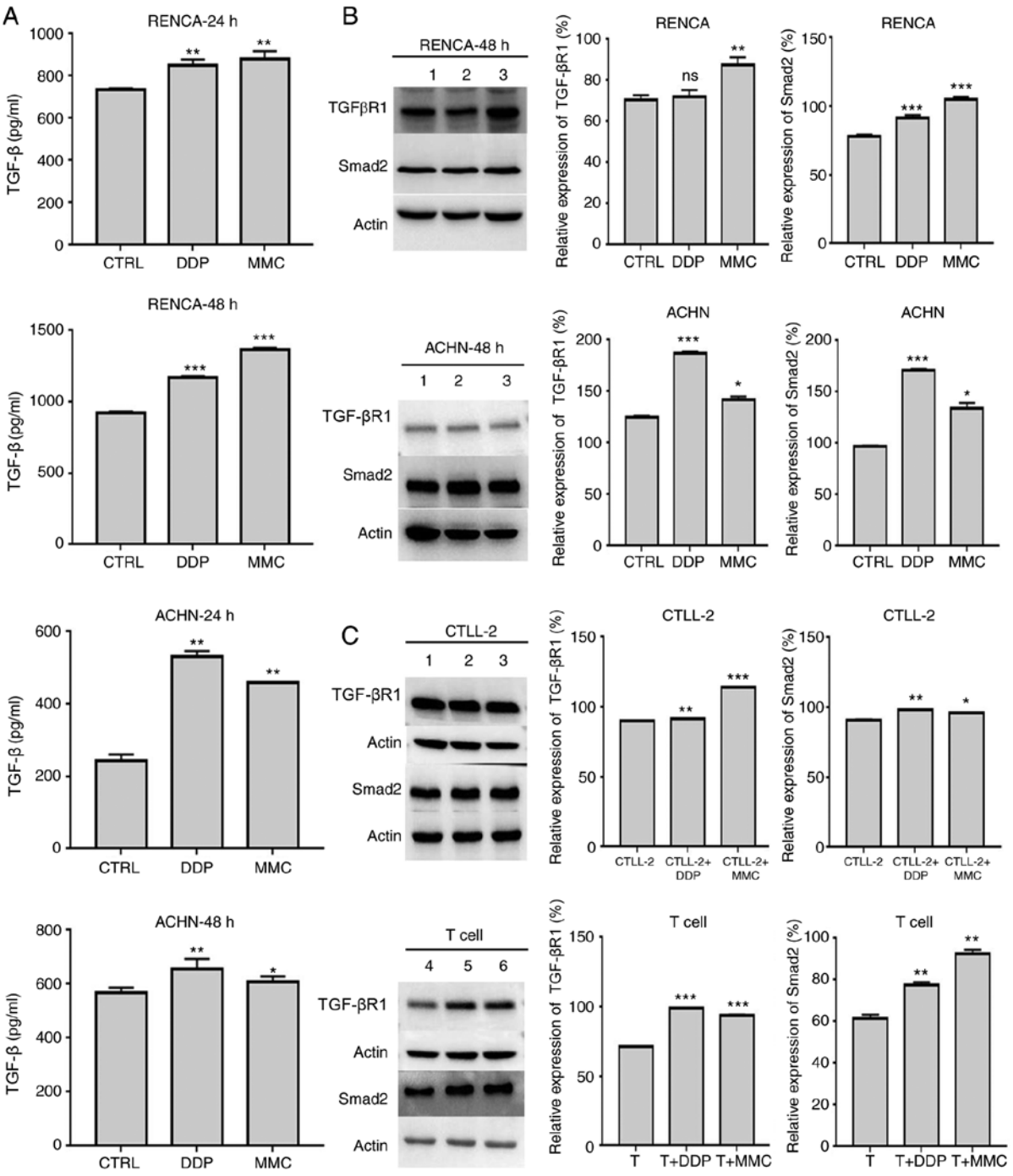

Figure 4. Possible causes of the functional inhibition of T cells *in vitro*. (A) TGF-β release by RCC cells treated with DDP and MMC. RENCA and ACHN cells were treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h and then incubated with medium for another 24 h. The levels of the cytokine TGF-β released by RENCA and ACHN cells were measured by ELISA. (B) TGF-βR1 and Smad2 expression in RCC cells treated with DDP and MMC. RENCA and ACHN cells were treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h and then incubated with medium for another 24 h. TGF-βR1 and Smad2 expression in RENCA and ACHN cells was assessed by western blot analysis. Actin was used to normalize the protein content. 1, CTRL group; 2, DDP group; 3, MMC group. (C) TGF-βR1 and Smad2 expression in T cells combined with DDP or MMC in RCC. RENCA and ACHN cells treated with 1 μg/ml DDP or 0.1 μg/ml MMC for 24 h were coincubated with CTLL-2 and T cells for another 24 h. TGF-βR1 and Smad2 expression in CTLL-2 and T cells was assessed by western blot analysis. Actin was used to normalize the protein content. 1, CTLL-2-cell group; 2, CTLL-2 cells combined with the DDP group; 3, CTLL-2 cells combined with the MMC group; 4, T-cell group; 5, T cells combined with the DDP group; 6, T cells combined with the MMC group. *P<0.05; **P<0.01; ***P<0.001; ns, not significant. RCC, renal cell carcinoma; DDP, diaminedichloroplatinum; MMC, mitomycin C; ELISA, enzyme-linked immunosorbent assay; TGF-β, transforming growth factor β; TGF-βR1, transforming growth factor β receptor 1.

effects of T cells depend on their activation, which is limited by a dual-signal system (39). Recently, chimeric antigen receptor-modified T (CAR-T) cells, which can transcend the dual signaling pathway and are not restricted by the major histocompatibility complex (MHC), have attracted wide attention (40,41). The present study provided a theoretical basis for the use of CAR-T cells combined with low-dose chemotherapy in the future.

In the later stage (24 h), it was revealed that chemotherapeutic drugs (DDP/MMC) inhibited IFN-γ secretion

and T-cell chemotaxis, which may be related to the TGF-β signaling pathway. TGF-β/Smad2 signaling is a strong immunosuppressive factor. TGF-β can effectively inhibit the proliferation, activation, and infiltration of T cells and induce T-cell apoptosis to attenuate the killing activity of T cells in tumors (42-44). To study the possible causes, the expression of TGF-β, TGF-βR1, and Smad2 was examined in renal cancer cells and the expression of TGF-βR1 and Smad2 in T cells. It was determined that the expression of TGF-β, TGF-βR1, and Smad2 in renal cancer cells was significantly increased following DDP and MMC exposure in the later stage (48 h), and the expression of TGF-βR1 and Smad2 in T cells was also significantly increased. Therefore, it was theorized that the decreased secretion of immune factors and the inhibitory infiltration of T cells may be related to the TGF-β signaling pathway. It has been reported that the inhibitory effects of TGF-β on T-cell function could be reversed by using a small molecule inhibitor of TGF-βR1 signaling, and this molecule may function as an antitumor drug in the future (44). However, whether TGF-βR1 silencing could reverse the effects of DDP and MMC on renal cells was not explored, which is a limitation of the present study.

In conclusion, the present results revealed that low-dose chemotherapy drugs (DDP and MMC) have the potential to kill RCC cells, and treatment with DDP or MMC can inhibit proliferation and promote apoptosis in RCC. Low-dose chemotherapy drugs (DDP and MMC) can synergistically enhance the immunotherapeutic effects of T cells on RCC *in vitro* by inhibiting TGF-βR1 expression in tumor cells, promoting IFN-γ secretion, chemotaxis and phenotype changes of T cells and inhibiting PD1 expression. It was also revealed that the TGF-β signaling pathway may be involved in inhibiting the function of T cells by decreasing immune factor secretion, preventing chemotaxis toward tumor cells and even promoting the apoptosis of T cells. The targeted inhibition of TGF-β signals may be a promising therapeutic strategy for the treatment of renal cancer. The present study was conducted in RCC cell lines *in vitro*. Future studies of this therapeutic strategy need to be performed *in vivo*. The present study provided a novel therapeutic option for sequential immune chemotherapy to enhance the therapeutic efficacy of T cells.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

NL and XJF conceived and designed the project. DDX, PT, YYC, WYG and YL performed the experiments. DDX and MD drafted and revised the manuscript. DDX, PT, YYC and MD analyzed and interpreted the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All applicable international, national, and institutional guidelines for the care and use of animals were followed. The inclusions of mouse and human subjects in the present study were approved by the Animal Experimental Ethics Committee of Xuzhou Medical University and the Ethics Committee of the First People's Hospital of Lianyungangand, respectively. Written informed consent was obtained from the blood donor.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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