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Original Research

Sensitization of cisplatin resistant bladder tumor by combination of cisplatin treatment and co-culture of dendritic cells with apoptotic bladder cancer cells

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Abstract: Dendritic cells (DCs) are a population of professional antigen presenting cells (APCs), serving as central regulators of adaptive immunity by presenting antigens. In addition, DCs play essential roles in the connection of the innate and adaptive immune responses. Recently, therapeutic vaccines turn out to become a viable therapeutic approach for immunotherapy of cancers. Here we report a dendritic cell-based vaccine strategy which could suppress the bladder tumor growth *in vivo* and improve the efficiency of chemotherapy. In this study, we investigate the antitumor effects of mature DCs induced by antigen loading in bladder tumor *in vivo*. We demonstrate the co-culture of cisplatin induced apoptotic human bladder cancer cell line, T24 cells with immature DCs could promote the maturation of DCs. Cisplatin and these activated DCs were reintroduced into mice bearing the T24 cisplatin resistant cells-derived tumor growth to activate or boost the immune response. Mice with iDCs co-cultured with apoptotic T24 (iDCs T24 Apo) cells injection effectively initiate a cytotoxic effect against tumor growth and improve the survival rates of mice compared with controls. Moreover, we observed injection of iDCs T24 apoptosis cells combined with cisplatin into mice with T24 cisplatin resistant cancer cells-derived tumor resulted in a statistically significant suppression of tumor growth compared with mice injected with PBS alone, cisplatin alone, iDCs, iDCs T24 Apo cells, cisplatin plus iDCs. This study provides a dendritic cell-based vaccine strategy which might reduce the risk of tumor recurrence and improve the efficiency of anti-chemoresistance of bladder cancer.

Key words: Cisplatin; Bladder tumor; Dendritic cells; Immunotherapy.

Introduction

Dendritic cells (DCs) form a population of professional antigen presenting cells (APCs), which control immune responses through innate and adaptive immunity (1). DCs originate from the bone marrow hematopoietic progenitor cells (2). It is known that DCs could uptake, process and present multiple types of antigens to antigen-specific naïve T cells (3, 4). Moreover, DCs have capacities to induce or suppress the proliferation, activation, and differentiation of specific T-cell subsets (5).

DCs exhibit two wildly studied maturation states: the "immature" and "mature" (6, 7). Immature DCs express relatively low levels of surface MHC-I and MHC-II and costimulatory molecules such as CD80 and CD86 (6, 7). Immature DCs display a specialized function as antigen-capturing cells. Thus, they are unable to process and present antigens efficiently to T cells (8). Mature DCs express cell surface molecules important for T cell activation (9). Therefore, they display immunogenic functions. Maturation of DCs is associated with increased efficiency in antigen processing, which makes DCs as potent activators of T cell immunity (9). In addition, activation and maturation of DCs with different maturation stimuli is associated with the local microenvironment and can be blocked or polarized by specific factors (10).

It is known that DCs play the major role in cancer immunesurveillance as the antigen-presenting cells through the initiation of the antitumor immune responses (11, 12). Recently, DC-based vaccines have been intensively studied and vaccination strategies involving DCs have been developed (13). Clinical trials were performed to demonstrate the DC vaccines are feasible and efficient to elicit immunological responses (13). DC vaccinations have the abilities to induce tumor-specific effector T cells that can suppress tumor growth specifically and that can induce immunological memory to control tumor relapse (14). An important step is to provide DCs with tumor-specific antigens. Recent studies demonstrated this can be achieved *in vitro* through inducing DCs to take up the tumor specific antigens (15). Tumor antigen-loaded DC vaccine in combination with IL-15 and p38 mitogen-activated protein kinase inhibitor confers strong CTL activation (16). In addition, co-culture of DCs with apoptotic cancer cells has been reported as a novel approach in the tumor immunotherapy in multiple cancer types such as breast cancer (17) and bladder cancer (18). Therefore, it is important to understand the biology of DCs and how they regulate the innate and the adaptive immune responses for the improvement of the tumor immunotherapy using DC vaccination. In this study, we will investigate the maturation of the DCs by co-culturing of immature DCs with cisplatin induced-apoptotic human T24 bladder cancer cells. Moreover, the effects of the combination of cisplatin with antigen-loaded dendritic cells on the cispla-

tin resistant tumor growth *in vivo* will be assessed.

Materials and Methods

Cell culture

The human bladder cancer cell line T24 was obtained from American Type Culture Collection (ATCC). Cells were cultured in 1640 medium (Invitrogen, Carlsbad, CA) with 10 % FBS (Invitrogen) and 1 % penicillin/streptomycin (Invitrogen). All cells were cultured in a sterile incubator maintained with 5 % CO_2 at 37 °C.

Animal experiments

All the mice from this study are 4-6 weeks old, 17-20 grams C57BL/6 female mice. Mice were maintained in SPF level animal rooms.

Isolation and characterization of bone marrow derived-dendritic cells from mice

Isolation of bone marrow derived-DCs was performed according to the previous description (18). Briefly, bone marrows of C57BL/6 mice were flushed from femurs and tibiae, then filtered through a 70 μM nylon mesh. The erythrocytes were lysed by ammonium chloride. After centrifugation, the pellet was saved and washed with PBS twice with 5 min each. The pellet was re-suspended with RPMI-1640 medium with 10% fetal bovine serum. Cells were plated in a 6-well plate for 24 hours, then the medium was refreshed with addition of GM-CSF (10 μg/L) and IL-4 (5 μg/L). The characterization of bone marrow derived-DCs was performed using flow cytometry. $5x10^5$/mL cells were incubated with antibodies against CD80-PE, CDI-A-PE and CD86-PE in 5% BSA solution at 4 °C for 30 min. Cells were then washed by PBS for further analysis by flow cytometry. All antibodies were from BD Biosciences.

Generation of iDCs in vitro

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors using Ficoll-Paque Plus (DH184-1; Dingguo Biotechnology, China). PBMCs were cultured in complete RPMI 1640 medium for 2 h at 37°C after erythrocyte lysis. Monocytes were isolated by adherence to plastic culture flasks and incubated with 100 ng/mL recombinant human granulocyte/macrophage colony stimulating factor (GM-CSF) (PeproTech, USA) and 10 ng/mL recombinant human IL-4 (PeproTech) for 6 days.

Apoptosis detection

T24 parental or cisplatin resistant cells were treated with cisplatin at the indicated concentration for 72 hours, cells were cultured with RPMI-1640 medium. Apoptosis was measured by Annexin V/PI staining according to the previous description (17). T24 cells were stained with Annexin V-FITC and propidium iodide (PI) using an Annexin V: FITC Apoptosis Detection Kit I (BD Biosciences, USA) according to manufacturer instructions and then analyzed using a BD flow cytometer (FacsCalibur™) with the Flowjo software to determine the extent of apoptosis.

Western blot analysis

After treatments, proteins were extracted using RIPA buffer (Pierce, Waltham, MA, USA). Protein concentrations were measured by Bradford assay using the Quick Start™ Bradford protein assay kit including the BSA standard (#5000201, Bio-rad, Hercules, CA, USA). Equal amount of protein sample was diluted, heated for denaturation, and then subjected to dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (PVDF, Millipore). The membrane was blocked in 5 % non-fat milk buffer for 1 hour at room temperature and then probed with cleaved Caspase-3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) rabbit polyclonal primary antibody. After washing 3 times with Tris-buffered saline with Tween (TBST), the membrane was incubated in peroxidase-conjugated goat anti-rabbit IgG antibody (1:4000, Immunoway, USA). Bands were visualized by an enhanced chemiluminescence detection system. Each experiment was repeated three times.

Loading of tumor antigen

After apoptosis induction, T24 cells were cultured with RPMI-1640 medium with FBS for three days, then cells were trypsinized and co-cultured with immature DCs at 1:1 ratio for 24 hours for the following experiments in this study. The immature DCs was stimulated with LPS in control group.

T-lymphocyte proliferation and activation assay

After co-culture with cisplatin-treated or untreated T24 cells, DCs were co-cultured with autologous T-lymphocytes at a ratio of 1:8. T-lymphocytes were sorted from PBMCs by negative selection using Pan T-Cell Isolation Kit magnetic beads (Miltenyi Biotec GmbH, Germany) and were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) according to manufacturer instructions. After 5 days of co-culture, T-lymphocyte proliferation was detected by the measurement of CFSE fluorescence intensity and supernatants were collected for the measurement of secreted IFN-γ using IFN-γ ELISA kits (BD Biosciences) according to manufacturer instructions.

Tumor protection studies

Total 60 female C57BL/6 mice were randomly separated into six groups for subcutaneous pre-injection with PBS alone, cisplatin alone, iDCs, iDCs T24 Apo cells, cisplatin plus iDCs and cisplatin plus iDCs T24 Apo cells. The subcutaneous pre-injections were repeated after 7 days. All mice were then subcutaneously injected with T24 cisplatin resistant cells to develop tumors. Half mice of each group were used for the measurements of tumor sizes, others of each group were subjected to survival rate analysis.

Statistical analysis

Statistical analysis was performed using Prism 5.0 (GraphPad Software). Unpaired Student's t-tests were used to assess statistical significance. Data are reported as means ± SD. P values < 0.05 were considered significant.

Results

Characteristics of cisplatin resistant bladder cancer cells

To investigate the effects of the tumor antigen induced immunotherapy and the cisplatin-based combination chemotherapy in advanced/metastatic bladder cancer, we established cisplatin resistant human bladder cancer cell line originating from T24 cells. The cells were treated with elevated concentrations of cisplatin for 3 months to select the survival cells. Our results showed the IC50 to cisplatin of T24 parental cells was 4.68 μM, which is significantly lower than the IC50 of T24 cisplatin resistant cells (61.42 μM). In addition, compared with T24 cisplatin resistant cells, T24 parental cells displayed apoptotic morphological changes under cisplatin treatment (Fig. 1B). Taken together, the above results suggest the T24 cisplatin resistant cells could tolerate higher concentration of cisplatin treatment than parental cells.

Induction of apoptosis of T24 parental bladder cancer cells by cisplatin

It has been reported that DCs co-cultured with irradiated apoptotic tumor cells elicit stronger immune responses (17, 18). To test whether co-culture of immature DCs with apoptotic cancer cells induced by anti-cancer agent cisplatin could stimulate immune response, we first treated T24 parental and T24 cisplatin resistant bladder cancer cells with cisplatin at 5, 10 or 20 μM for 3 days to obtain the best apoptotic tumor cells. Consistently, we found the caspase-3 activity was significantly induced in T24 cells at 5, 10 and 20 μM (Fig. 2A). Moreover, the cleavage of caspase-3 was detected by Western blot, demonstrating T24 parental cells were sensitive to cisplatin treatment at 5 μM (Fig. 2B). However, T24 cisplatin resistant cells did not exhibit significantly apoptotic phenotype at 5 and 10 μM, suggesting under relative low concentration of cisplatin, the induction of apoptosis of T24 parental cells may contribute to activate immune response through co-culturing with immature DCs.

Co-culture of apoptotic T24 cells with immature DCs promotes the maturation of DCs in vitro

It was known that the bone marrow derived stem cells could be induced to become dendritic cells which initiate the immune response (1, 2). To study the tumor antigen induced immune response, it was necessary to collect adequate numbers of hematopoietic stem cell derived-mature DCs. In our previous publication, we have successfully cultured the mouse bone marrow stem cells with IL-4 and GM-CSF for obtaining mature DCs (19). In the fifth day, the IL-4 and GM-CSF induced the expression of cell surface antigen CD11c, but other markers for mature DCs, CD80, CD86 and I-A were low expression, indicating the stem cell derived-DCs retain an immature phenotype. With the stimulation of LPS,

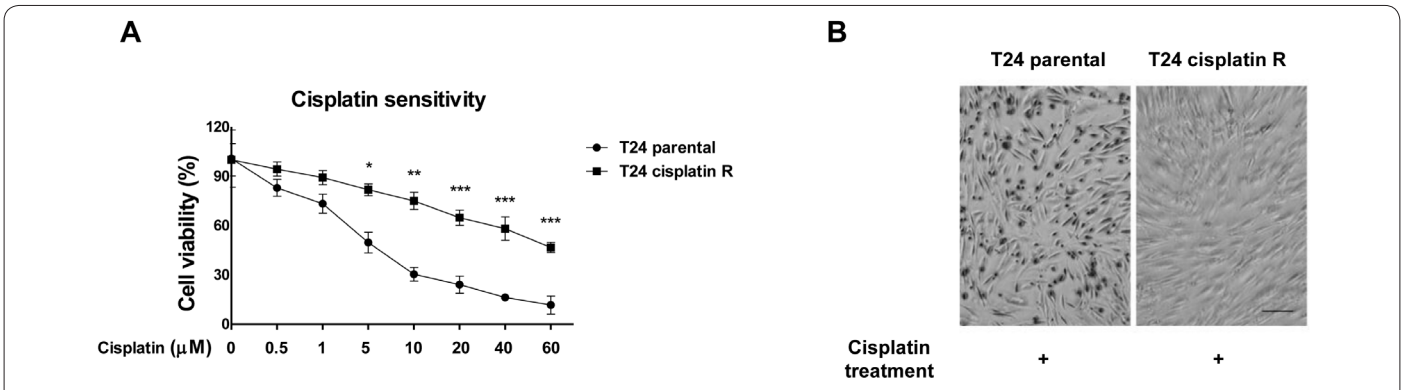

Figure 1. Establishment and characteristics of cisplatin resistant bladder cancer cells. (A) T24 parental and cisplatin resistant cells were treated with 0, 0.5, 1, 5, 10, 20, 40 or 60 μM for 72 hours. Cell survival rates were analyzed by flowcytometry. (B) T24 parental and cisplatin resistant cells were treated cisplatin at 5 μM for 72 hours, cell death and morphology changes were observed by bright field microscope. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

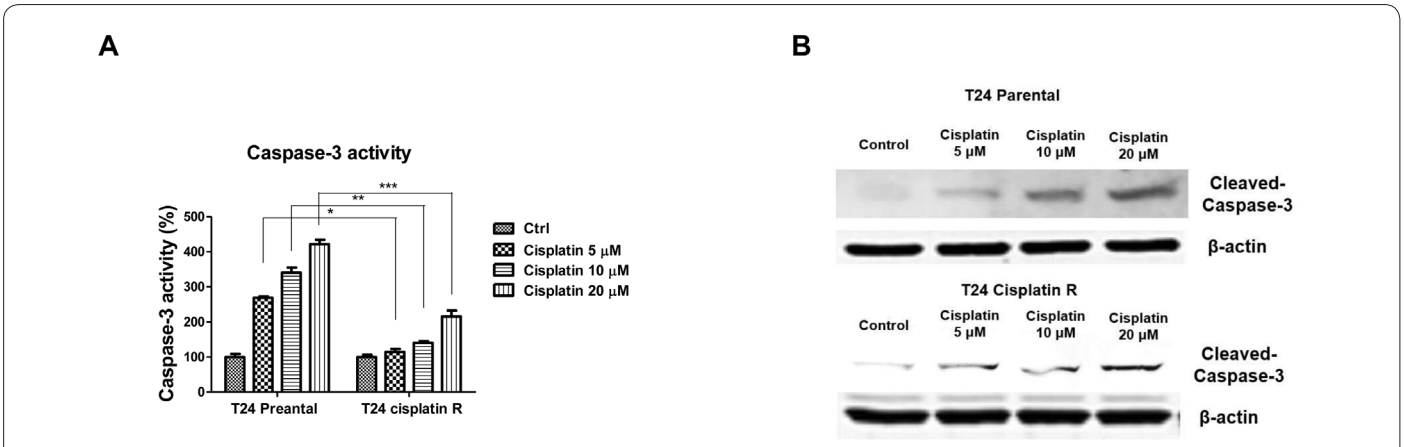

Figure 2. Induction of apoptotic T24 cells. (A) T24 parental and cisplatin resistant cells were treated with 0, 5, 10 or 20 μM for 72 hours. Caspase-3 activity was measured. (B) Cleavage of Caspase-3 was analyzed by Western blot in T24 parental and cisplatin resistant cells under 0, 5, 10 or 20 μM cisplatin treatments for 72 hours. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

the stem cell derived-DCs displayed high expressions of CD11c, CD80, CD86 and I-A (Fig. 3). These features consistently showed a mature phenotype of stem cell derived-DCs. Thus, our in vitro culture system could generate large numbers of immature DCs capable of antigen uptake and transition to mature phenotype.

To investigate whether co-culture of apoptotic human cancer cells with bone marrow derived-DCs could induce the maturation of DCs, we co-cultured the apoptotic T24 cells (T24 Apo) with DCs for 24 hours. Cells were then analyzed by flow cytometry. Our results showed in 24 hours' co-culture, the CD80, CD86 or I-A positive cells significantly increased (Fig. 3A-3C). However, iDCs co-cultured with healthy T24 cell did not induce maturation (Fig. 3A-3C), indicating co-culture of apoptotic T24 cancer cells with bone marrow derived-DCs could induce significant maturation of DCs in 24 hours (Fig 3).

Maturation of DCs by co-culture with apoptotic T24 cells stimulates the proliferation and activation of T-lymphocytes

We examined the effects of the maturation of DCs induced by apoptotic T24 cells on the T-lymphocyte proliferation and activation. The autologous T-lymphocytes were negatively selected and labeled with CFSE dye from the PBMCs of DC donors (17). T-lymphocytes were then co-cultured with the DCs induced by apoptotic T24 cells or normal T24 cells for 5 days. We measured the T-lymphocyte proliferation by flow cytometry. As shown in Figure 4A, mature DCs induced by apoptotic T24 cells could significantly promote T-lymphocyte proliferation compared with control groups (iDCs, iDCs co-culture with normal T24). In addition, T-lymphocyte activation accompanied with the secretion of IFN-γ was detected. Results in figure 4B demonstrated T-lymphocytes stimulated with DCs induced by apoptotic T24 cells exhibited significant increase in IFN-γ secretion compared with control (iDCs, iDCs co-culture with normal T24). Taken together, these results suggest DCs co-cultures with apoptotic T24 cells could effectively activate T-lymphocytes.

Overcoming cisplatin resistant bladder tumor growth by the combination of cisplatin and DC vaccine in vivo

Since the DC vaccination-based strategies have been applied clinically to tumor therapy (13, 14). To assess whether the DCs loaded with antigens from apoptotic cancer cells have significantly inhibitory effects on cisplatin resistant tumor growth, we tested the anti-tumor effects of mice which were subcutaneously pre-injected with PBS alone, cisplatin alone, iDCs, iDCs stimulated by co-cultured with apoptotic T24 cells, cisplatin plus iDCs and cisplatin plus iDCs co-cultured with apoptotic T24 cells. All mice were then subcutaneously injected with T24 cisplatin resistant cells to develop tumors. As we expected, after 20 days, mice with PBS, cisplatin alone, immature DCs and cisplatin plus iDCs began to die. Mice with iDCs stimulated by co-cultured with apoptotic T24 cells injection showed decreased mortality rates after 45 days (Fig. 5A). In contrast, injection of cisplatin plus iDCs T24 Apo cells into mice with T24 cisplatin cancer cells-derived tumor resulted in a statistically significant improvements of survival rates (Fig. 5A). Consistently, mice injected with cisplatin

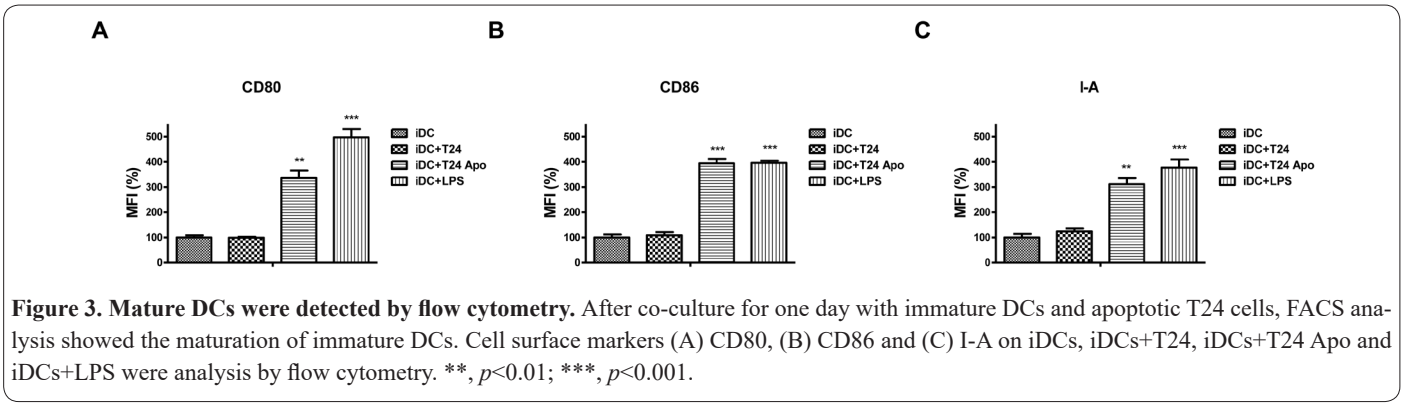

Figure 3. Mature DCs were detected by flow cytometry. After co-culture for one day with immature DCs and apoptotic T24 cells, FACS analysis showed the maturation of immature DCs. Cell surface markers (A) CD80, (B) CD86 and (C) I-A on iDCs, iDCs+T24, iDCs+T24 Apo and iDCs+LPS were analysis by flow cytometry. **, $p<0.01$; ***, $p<0.001$.

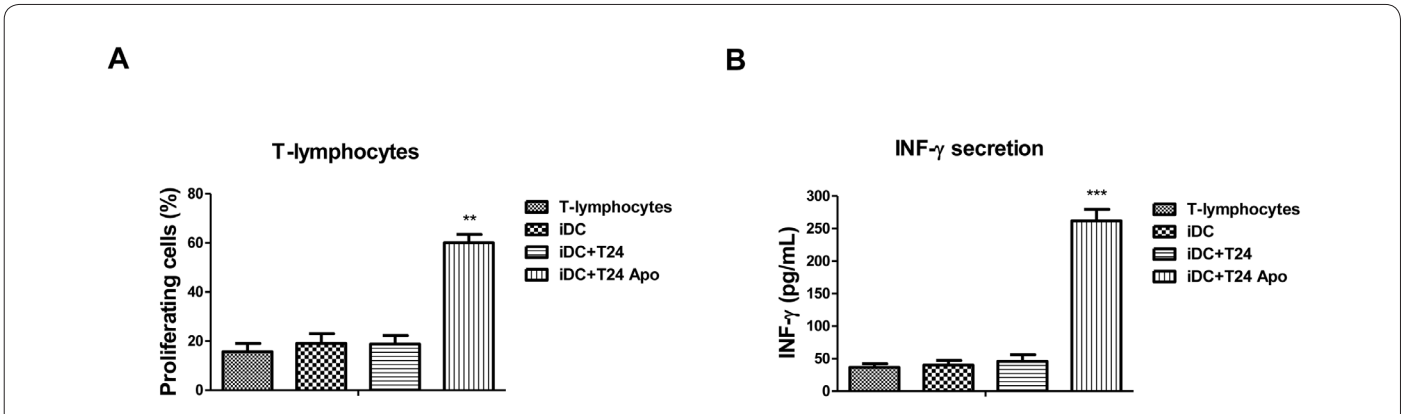

Figure 4. iDCs co-cultured with cisplatin induced apoptotic T24 cells enhance autologous T-lymphocyte proliferation and IFN-γ release. After isolation, the T-lymphocytes were co-cultured with the iDCs, iDCs+T24 or iDCs induced by apoptotic T24 (iDCs+T24 Apo) for 5 days. (A) The proliferation of T-lymphocytes was assessed by flow cytometry. (B) IFN-γ secretion was detected in the supernatants by ELISA. Experiments were performed in triplicate. **, $p<0.01$; ***, $p<0.001$.

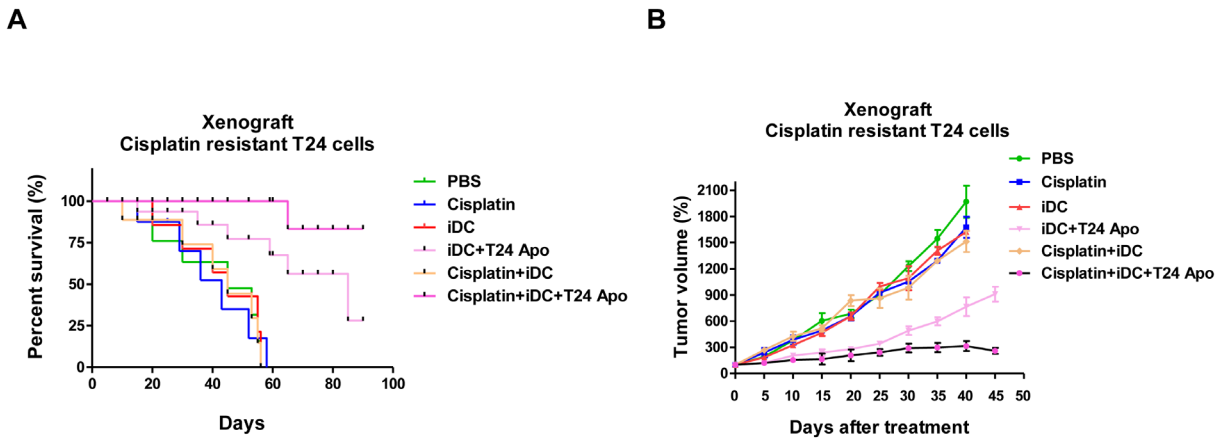

Figure 4. DCs vaccine plus cisplatin treatment displays anti-chemoresistance on tumor *in vivo*. (A) Mice were injected with T24 cisplatin resistant cells to develop tumors. Then each group of mice was injected with PBS alone, cisplatin alone, iDCs, iDCs+T24 Apo cells, cisplatin plus iDCs and cisplatin plus iDCs+T24 Apo cells. The survival rates were analyzed. (B) The tumor volumes of each mouse in each group were measured and compared.

plus iDCs T24 Apo showed remarkably decreased tumor growth compared with that with injection of PBS, cisplatin alone, immature DCs and cisplatin plus iDCs (Fig. 5B). Taken together, these *in vivo* results demonstrated the combination of cisplatin and DC loading with apoptotic T24 cells significantly overcome cisplatin resistant tumor growth.

Discussion

Dendritic cell (DC)-based immunotherapy is considered a promising approach which is under investigation in multiple cancers. As an effective antigen-presenting cells, DCs process antigen and present it to stimulate T-lymphocytes. It has been known that the tumor vaccine generated through antigen loading from apoptotic cancer cells could stimulate strong immune response since the tumor antigens originate from abnormally expressed endogenous proteins are derived from viral proteins. Recent studies demonstrated that apoptotic liver cancer cells and colorectal carcinoma cells have the abilities to stimulate the maturation of DCs (20, 21). However, whether the cisplatin-induced apoptotic cancer cells can activate iDCs maturation and the effects of the tumor antigen loaded-DCs combined with cisplatin on the sensitivity of cisplatin resistant bladder tumor growth is currently unknown.

Here we report the combination of cisplatin and dendritic cell-based vaccine strategy, which could suppress the cisplatin resistant bladder tumor growth *in vivo* and improve the survival of mice bearing cisplatin resistant xenograft bladder tumors. In this study, we investigated the antitumor effects of mature DCs induced by antigen loading in bladder tumor *in vivo*. Co-culture of apoptotic T24 cells with immature DCs could induce the maturation of DCs. Subsequently, these activated DCs were reintroduced into mice to activate or boost the immune response, resulting an effective initiation of cytotoxic effects against tumor growth.

Currently, the mechanisms for the apoptotic tumor cells induced maturation and activation of DCs are still under investigation. It has been described that irradiation of tumor cells could provide a safe source of antigens for DC uptake (22). In addition, irradiation could induce apoptotic tumor cells release endogenous signals such as HSP70, HMGB-1 to improve the immunogenicity of tumor cells (23). Moreover, loading DCs with a mixture of apoptotic tumor cells could upregulate co-stimulatory molecules on DCs and induce phagocytosis and (22, 24), resulting in an enhanced antitumor immunity. A recent study illustrated that the radiation-mediated antitumor immunity has the capacity to regulate IFN-β pathway in DCs (25). In summary, the ability of the cisplatin-induced apoptotic T24 cells to effectively induce immature bone marrow derived-DCs maturation was investigated in this study. We found co-culture of apoptotic T24 cells with immature DCs has the potential to induce the maturation of DCs, leading to the enhanced suppression of T24 cisplatin resistant bladder tumor growth in mice. We provide a dendritic cell-based vaccine strategy which might reduce the risk of tumor recurrence and improve the efficiency of anti-chemoresistance of bladder cancer.

Conflict of interests

None.

Acknowledgements

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