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RESEARCH

# 4-1BB Protects Dendritic Cells from Prostate Cancer-Induced Apoptosis

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**Abstract** It has been shown that human prostate cancer (PCa) cells induced apoptotic death of the most potent antigen-presenting cells, dendritic cells (DCs), which are responsible for the induction of specific antitumor immune responses. Here, we investigated the function of 4-1BB on protecting DCs from prostate cancer-induced apoptosis with an agonistic mAb to 4-1BB. RM-1 cells and DCs were co-incubated for 48 h and DC apoptosis was assessed by Annexin Vassay. TNF-α and IL-12 production were assessed by enzyme-linked immunosorbent assay (ELISA) and Bcl-2 and Bcl-xL on DCs were analyzed by Western blot. We have shown that co-incubation of RM-1 cells with DCs is accompanied by an increased level of DCs apoptosis. Triggering 4-1BB on DCs resulted in increased resistance of DCs to RM-1 cells-induced apoptosis, which was owing to the up-regulated expression of Bcl-2 and Bcl-xL, and increased secretion of TNF-αand IL-12. These results demonstrate that triggering 4-1BB on DCs could increased resistance of DCs to PCa-induced apoptosis.

**Keywords** Dendritic cells · Co-stimulatory molecules · 4-1BB · Prostate cancer

## Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in old men and also the second leading cause of male cancer death in the Western countries [1]. In addition, the incidence and mortality of carcinoma of prostate are increasing in China. Treatment of advanced prostate cancer is still far from satisfactory, especially when the tumor reaches the hormone-resistant state. In the past decade, a wide array of immunotherapeutic strategies has been developed for prostate cancer and successfully tested in animals and humans. Among these, the dendritic cells (DCs)-based vaccine has been one of the treatment modalities most extensively studied in a variety of preclinical models [2], and clinical trials [3, 4]. DCs, which are the most potent APCs, are known to be deficient in number and functional activity in patients with cancer [5]. Elimination of DCs from the tumor environment significantly diminishes the initiation of specific immunologic responses. However, recent research demonstrated that human prostate cancer cell not only cause apoptotic death of DCs but also markedly inhibit the generation of DCs in cultures [6, 7]. Therefore, effective protection of DCs from PCa-induced apoptosis may significantly improve the efficacy of DCs-based therapies in cancer clinical trials.

There are several things that can increase the survival of DCs. Activation of DCs with cytokines, including CD154, IL-12, IL-15 and TNF-α has been shown to increase the resistance of DCs to tumor-induced apoptosis [7, 8]. However, although 4-1BB signal plays a important role in

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T cell proliferation, long-term survival, anti-apoptosis of activation-induced $CD8^+$ T cells [9], its role in protection of DCs from tumor-mediated cell death has not yet been evaluated. 4-1BB is a TNFR superfamily member expressed by activated T lymphocytes [10]. The systemic treatment of mAbs against 4-1BB or gene transfer of 4-1BB ligand into tumor cells induces strong cell-mediated immune responses against tumors [11, 12]. Administration of anti-4-1BB mAb in tumor-bearing mice lead to regression of established tumors in many mouse models [13]. We and others have demonstrated that activated DCs expressed higher 4-1BB [14]. So we hypothesized that activation of 4-1BB signal on DCs could protect DCs from PCa-induced apoptosis.

To demonstrate the hypothesis, we used an agonistic mAb against 4-1BB to trigger 4-1BB signal and detected its effection on protection of DCs from PCa-induced apoptosis.

## Materials and Methods

### Animals, Cell Lines and Antibodies

Female C57BL/6 (H-2 $K^b$) mice, 6–8 weeks old, were obtained from SHANGHAI SLAC LABORATORY ANIMAL CO. LTD (Shanghai, China). Animals were maintained at the Central Animal Facility of Wuhan University according to standard guidelines and experiments were conducted according to the guidelines of the China Council for Animal Care. RM-1, a murine prostate cancer cell line, was obtained from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium with 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5 % $CO_2$.

Anti-4-1BB mAb (clone 158,321) was purchased from R&D systems. Rabbit anti-Bcl-2 mAb, rabbit anti-Bcl-xL mAb and hamster IgG isotypecontrol mAb were purchased from Cell Signaling Technology.

### DCs Generation and Coculture with RM-1 Cells

Mouse DCs were generated from bone marrow suspensions harvested from 6 to 8 weeks old C57BL/6 mice according to the publication [15] with slight modifications. Briefly, bone-marrow cells were harvested from femurs and tibias, depleted of red blood cells, and washed twice in PBS. Cells were resuspended in a DCs medium consisting of RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS) (Gibico, America), 10 ng/mL GM-CSF (R&D Systems, Minneapolis, MN, USA), 10 ng/mL IL-4 (R&D Systems, Minneapolis, MN, USA), and 50 mM 2-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin and cultured (37 °C, 5 % $CO_2$) in 6-well plates at $1\times10^6$ cells/3 ml/well. On day 3 and 5 of culture, floating cells were gently removed, and fresh mGM-CSF/mIL-4-containing medium was added. On day 6, non-adherent cells and loosely adherent proliferating DCs aggregates were collected. Mature DCs (mDCs) were generated by the inclusion of 10 ng/ml LPS (Sigma) for another 24 h of culture.

mDCs were collected and co-cultured with the murine prostate cancer cell line RM-1 in 6-well plates. Cells were separated using membrane inserts with 0.4 μm pore size, which prevented direct cell-to-cell contact, but allowed a free exchange of soluble substances. $6\times10^5$ mDCs were placed in 6-well plates in 3 ml of medium with 100ug anti-4-1BB Ab, hamster IgG isotypecontrol Ab or none added. $3\times10^6$ RM-1 cells incubated in 2 ml of medium were placed into the inserts. DCs were cultured alone without RM-1 cells as negative control. After additional of 48 h co-incubation, mDCs were collected for the following experiments.

### Apoptosis Analysis by Flow Cytometry

For apoptosis analysis, 4-1BB Ab-triggered DCs ($4\times10^5$) were collected and apoptosis was analyzed by flow cytometry, staining with FITC-conjugated annexin V and/or propidium iodide (PI) according to manufacturer instructions. All Annexin V positive cells were considered apoptotic and their percentage was calculated among the total number of cells. Cells taking the vital dye PI were considered dead. Samples were analyzed by flow cytometry analysis (Apoptosis Kit, BD Pharmingen, Germany).

### Cytokine Production by DCs

For cytokine assays, co-culture supernatants were harvested and used for enzyme-linked immunosorbent assay (ELISA). A mouse IL-12 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) and a mouse TNF-α Quantikine ELISA Kit (R&D Systems) were used to detect IL-12 and TNF-α, respectively, following the manufacturer's instructions.

### Western Blot Analysis

DCs were collected and lysed. The lysates were separated on 10 % SDS-PAGE. After electrophoresis, the protein blots were transferred to a nitrocellulose membrane (Amersham, Waukesha, Wisconsin, USA). The membrane was blocked with 5 % non-fat milk in TBST for 1 h and incubated overnight with rabbit anti-Bcl-2 or rabbit anti-Bcl-xL mAb at 4 °C. After three washes with TBST, the membrane was incubated at 37 °C for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody diluted with TBST. The detected protein signals were visualized by an enhanced chemilluminescence reaction system. Western blot for β-actin was used as an internal sample.

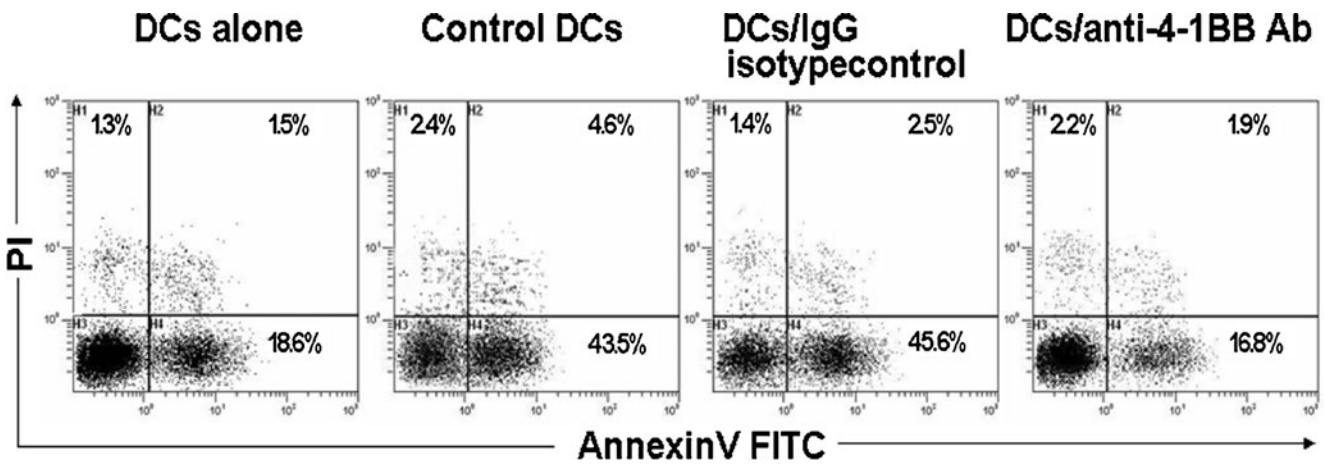


**Fig. 1** Murine PCa cells increase the number of Annexin V positive PI negative apoptotic cells in DCs cultures during co-incubation. Mature DCs treated with anti-4-1BB Ab, hamster IgG isotypecontrol Ab or none were co-incubated with RM-1 cells or cultured alone. mDC were collected after 48 h for detecting apoptosis by staining with FITC-conjugated annexin V and PI. The results are representative of three independent experiments

## Statistical Analysis

SPSS13.0 was used for data variation analysis. Data are reported as mean ± SD and were analyzed by the Student *t*-test, *P* values less than 0.05 were considered statistically significant.

## Results

### Prostate Cancer Cells Induced the Apoptosis of Murine DCs upon Co-Incubation

To detect whether triggering 4-1BB on mDCs could pretect DCs apoptosis from prostate cancer cells, mature DCs treated with anti-4-1BB Ab, hamster IgG isotypecontrol Ab or none were co-incubated with RM-1 cells or cultured alone. mDC were collected after 48 h for detecting apoptosis by staining with FITC-conjugated annexin V and/or PI. The rate of apoptosis of anti-4-1BB Ab-treated DCs was lower than that of IgG isotypecontrol Ab-treated DCs and none-treated DCs (Fig. 1). Results of the Annexin V binding assay revealed that apoptotic rate, determined as percentage of Annexin V+PI- cells, among mDCs treated with anti-4-1BB Ab was 18.12 %, lower than that in hamster IgG isotypecontrol Ab-treated mDCs (45.6 %), none-treated mDCs (48.1 %). The differences were statistically significant ($P<0.05$) (Fig. 2).

### Cytokine Production by DCs

To determine the mechanism of affection of mDCs induced by 4-1BB-mediated signal, we analyzed cytokine

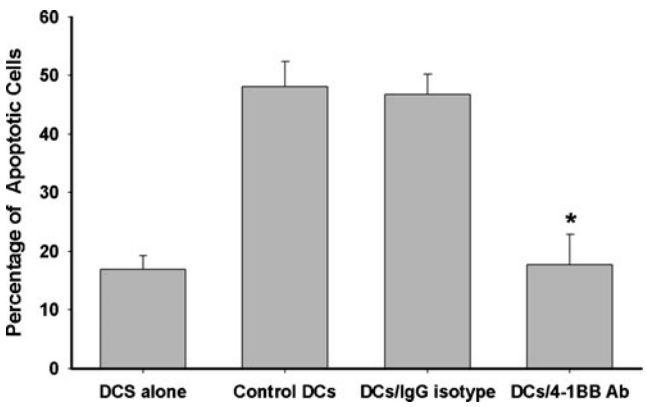


**Fig. 2** Treatment of DC with anti-4-1BB Ab resulted in the increased resistance of DCs to prostate cancer-induced apoptosis. Mature DCs treated with anti-4-1BB Ab, hamster IgG isotypecontrol Ab or none were co-incubated with RM-1 cells or cultured alone. mDC were collected after 48 h for detecting apoptosis by an Annexin V binding assay. *$P<0.05$

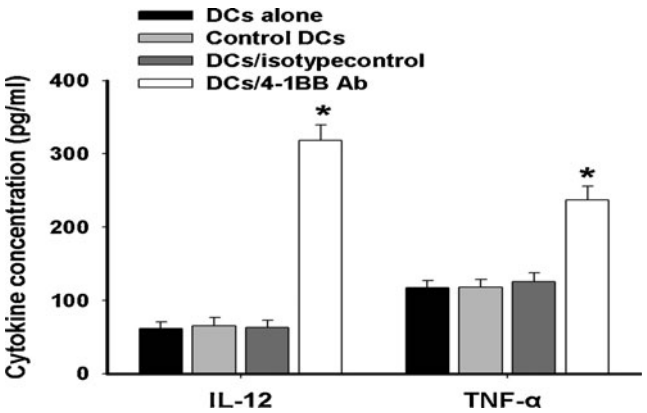


**Fig. 3** Cytokine (IL-12/TNF-α) production by DCs. Mature DCs treated with anti-4-1BB Ab, hamster IgG isotypecontrol Ab or none were co-incubated with RM-1 cells or cultured alone for 48 h. Then culture supernatants were collected for analyzing production of IL-12 and TNF-α by ELISA. Data are expressed as mean ± SD. Similar results were obtained from three independent experiments. (*$P<0.05$)

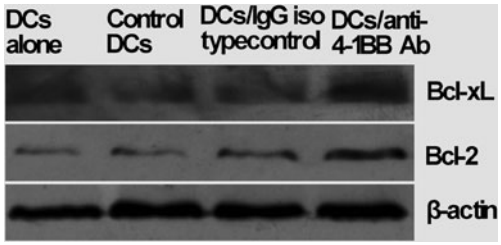


**Fig. 4** Analysis of antiapoptotic molecules of DCs. Mature DCs treated with anti-4-1BB Ab, hamster IgG isotypecontrol Ab or none were co-incubated with RM-1 cells or cultured alone for 48 h. Then DCs were collected, and equal amounts of cell lysates were applied to Western blotting of Bcl-2 and Bcl-xL

production by mDCs. Mature DCs treated with 100 μg anti-4-1BB Ab, hamster IgG isotypecontrol Ab or none were co-incubated with RM-1 cells or cultured alone for 48 h. Then the culture supernatants were harvested and analyzed for the production of IL-12 and TNF-α by ELISA . The level of IL-12/TNF-α in 4-1BB Ab-treated supernatant was more than that in hamster IgG isotypecontrol Ab-treated mDCs and none-treated mDCs (Fig. 3).

Western Blot Analysis

To determine the effect of triggering 4-1BB involved in the anti-apoptotic molecules of mature DCs from RM-1 cells. mDCs were collected, and equal amounts of cell lysates were applied to Western blot of Bcl-2 and Bcl-xL. Both anti-apoptotic proteins were detected in 4-1BB-mediated DCs, and their levels were slightly increased by treatment with anti-4-1BB mAb (Fig. 4).

## Discussion

Dendritic cells (DCs) are professional antigen-presenting cells (APCs), which display an extraordinary capacity to induce, sustain, and regulate T-cell responses providing the opportunity of DC-based cancer vaccination strategies. Due to their various antitumor effects, DCs emerged as promising candidates for the treatment of PCa patients. Consequently, several clinical trials enrolling PCa patients were conducted, which were based on the administration of DCs pulsed with tumor-associated antigens [3, 4]. However, a number of treated PCa patients were resistant to DC-based immunotherapies, suggesting that this approach should be further improved [16]. It has been known that DCs numbers reduce in PCa tissues compared to benign prostatic hyperplasia (BPH) tissues and the levels of infiltrating DCs further lessen with the advancement of anaplastic grade [17, 18]. Pirtskhalaishvili G et al. [7] demonstrated that prostate cancer cells induced apoptosis of human DCs during the in vitro co-incubation. Due to their crucial role in the mounting of host antitumor immune responses, it is imaginable that DCs elimination might effectively inhibit the immune system. Therefore, it is likely that protection of DCs from PCa-induced apoptosis could improve the host immunity and strengthen the antitumor responses.

4-1BB, a number of TNFR superfamily, has emerged as an important mediator of survival signaling, and studied as a stimulatory receptor on T cells responding for a number of years. Extensive evidence has shown that signals through 4-1BB delivered by agonistic antibodies or by over-expressed ligand can augment T-cell activation or survival [9]. Recently, it has been shown that DCs express 4-1BB constitutively and signaling via 4-1BB using 4-1BB ligand (4-1BBL)-transfected cells up-regulates B7-1 and B7-2, and increases IL-6 and IL-12 production by DCs [19]. In addition, administration of in vivo agonistic anti-4-1BB monoclonal antibody (mAb) to naive mice could enhance the ability of DCs to stimulate in vitro T cell-proliferative responses to both alloantigens and nominal antigens [14]. More interestingly, 4-1BB$^{-/-}$ mice have increased frequencies of DCs [20], but these DCs have decreased survival rates [21], highlighting the importance of 4-1BB in DC regulation. Here we have demonstrated that 4-1BB signal protects DCs from prostate cancer-induced apoptotic death in vitro. In our system, agonistic anti-4-1BB mAb performed protective functions probably by stabilizing the expression of anti-apoptotic protein Bcl-2 and Bcl-xL. In addition, activation of 4-1BB induced up-regulation of IL-12/TNF-α secretion by DCs (Fig. 3). IL-12 and TNF-α could also protect DCs from tumor-induced apoptosis [7, 8].

In summary, activation of 4-1BB on DCs could enhance their anti-apoptotic features and protect DCs from PCa induced apoptosis. Therefore, use of agonistic anti-4-1BB mAb-stimulated DCs in the treatment of PCa might enhance the efficacy of DCs-based therapy.

**Disclosure Statement** All authors have no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. All authors have read and approved the manuscript.

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