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# Effects of $^{12}\text{C}^{6+}$ Heavy Ion Radiation on Dendritic Cells Function

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
Funds Collection G

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**Background:** Carbon ion radiotherapy has been shown to be more effective in cancer radiotherapy than photon irradiation. Influence of carbon ion radiation on cancer microenvironment is very important for the outcomes of radiotherapy. Tumor-infiltrating dendritic cells (DCs) play critical roles in cancer antigen processing and antitumor immunity. However, there is scant literature covering the effects of carbon ion radiation on DCs. In this study, we aimed to uncover the impact of carbon ion irradiation on bone marrow derived DCs.





**Material/Methods:** Bone marrow cells were co-cultured with GM-CSF and IL-4 for seven days, and the population of DCs was confirmed with flow cytometry. We used an Annexin V and PI staining method to detect cell apoptosis. Endocytosis assay of DCs was determined by using a flow cytometry method. DCs migration capacity was tested by a Transwell method. We also used ELISA assay and western blotting assay to examine the cytokines and protein expression, respectively.

**Results:** Our data showed that carbon ion radiation induced apoptosis in both immature and mature DCs. After irradiation, the endocytosis and migration capacity of DCs was also impaired. Interestingly, carbon irradiation triggered a burst of IFN- $\gamma$  and IL-12 in LPS or CpG treated DCs, which provide novel insights into the combination of immunotherapy and carbon ion radiotherapy. Finally, we found that carbon ion irradiation induced apoptosis and migration suppression was p38 dependent.

**Conclusions:** Our present study demonstrated that carbon ion irradiation induced apoptosis in DCs, and impaired DCs function mainly through the p38 signaling pathway. Carbon ion irradiation also triggered anti-tumor cytokines secretion. This work provides novel information of carbon ion radiotherapy in DCs, and also provides new insights on the combination of immune adjuvant and carbon ion radiotherapy.

**MeSH Keywords:** **Apoptosis • Dendritic Cells • Heavy Ion Radiotherapy**

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## Background

As a novel strategy for cancer therapy, carbon ion radiotherapy has drawn more and more attention. Compared to proton irradiation, carbon ion radiotherapy has potential advantages: better physical dose distribution, higher relative biological effectiveness, lower oxygen enhancement ratio, desirable features for eradication of radioresistant tumors, and unrepaired DNA damage [1]. Beside these radiobiological features, carbon ion radiation also induces systemic effects, especially strong immune responses [2,3]. Carbon ion radiation induced anti-tumor immunity, such as the release of high mobility group box 1 (HMGB1) has also been reported [3]. But the understanding of carbon ion radiation on immune microenvironments inside tumors remains unclear.

Tumor-infiltrating dendritic cells (TIDCs) play critical roles in cancer antigen processing and antitumor immunity [4,5]. TIDCs were also induced to secrete cytokines such as IL-4, IL-10, IFN- $\gamma$ , and IL-12, which regulate immune homeostasis and adaptive immunity [5]. DC-based vaccines with different modifications have been widely studied for antitumor and immune regulatory effects [6,7]. Moreover, a previous study demonstrated that the combination of  $\alpha$ -galactosylceramide-pulsed DCs and carbon ion radiotherapy inhibited tumor growth and distant metastasis [1]. However, the direct effect of carbon ion radiation on DCs was still to be uncovered.

Several studies have shown that DCs are very resistant to  $\gamma$ -irradiation, compared to lymphocytes and monocytes [8,9]. Our previous work demonstrated that  $\gamma$  ray impaired DC migration *in vitro* while promoted DC migration in whole body irradiation [10]. In the present study, we aimed to investigate the influence of  $^{12}\text{C}^{6+}$  heavy ion radiation on bone marrow derived dendritic cells (BMDCs). And we found that heavy ion radiation induced apoptosis in both immature and mature DCs, suppressed endocytosis capacity, and affected cytokines secretion by DCs.

## Material and Methods

### Mice and treatments

Six to eight weeks old female C57BL/6 mice (Chinese Academy of Science (Shanghai, China) were housed in a temperature-controlled room in separate cages as described in our previous studies. Femurs from non-irradiated mice were used for the preparation of BMDCs.

### Bone marrow derived dendritic cells culture (BMDCs)

DCs were cultured from bone marrow cells as described in our previous work [10]. Briefly, after depletion of red blood cells, bone marrow cells ( $2 \times 10^6$  cells/mL) were grown in RPMI1640

medium with 10% FCS, recombinant mouse GM-CSF (10 ng/mL) and recombinant mouse IL-4 (Peprotech Inc., USA) (1 ng/mL) in 6-well plates. Three days later, non-adherent cells were gently washed out and adherent cells were collected on day 5 as immature DCs (iDCs), which were used in the study experiments. Then immature DCs were treated with LPS (1  $\mu\text{g}/\text{mL}$ ) or CpG-ODN (10  $\mu\text{g}/\text{mL}$ ) for 24 hours and named as LPS-matured DCs or CpG-matured DCs (mDCs).

### Irradiation

Cells were irradiated with  $^{12}\text{C}^{6+}$  heavy ion radiation at the Heavy Ion Research Facility in Lanzhou (Chinese Academy of Sciences, Lanzhou, China), as described in our previous studies [11]. Briefly, the cells were irradiated in suspension in a  $10 \times 10$  cm<sup>2</sup> radiation field, dose rate 0.5 Gy/minute, 350 MeV/U. After irradiation, cells were used in the study experiments.

### Cell apoptosis assay

At 24-hours post heavy-ion irradiation, DCs with different treatments were tested for apoptosis with an apoptosis detecting kit (Invitrogen, NY, USA). Cells were washed twice with PBS and suspended in 100  $\mu\text{L}$  binding buffer. Then cells were incubated with 5  $\mu\text{L}$  Annexin V-FITC for 20 minutes, and followed with PI staining. Subsequently, cells were analyzed by flow cytometry.

### Endocytosis assay

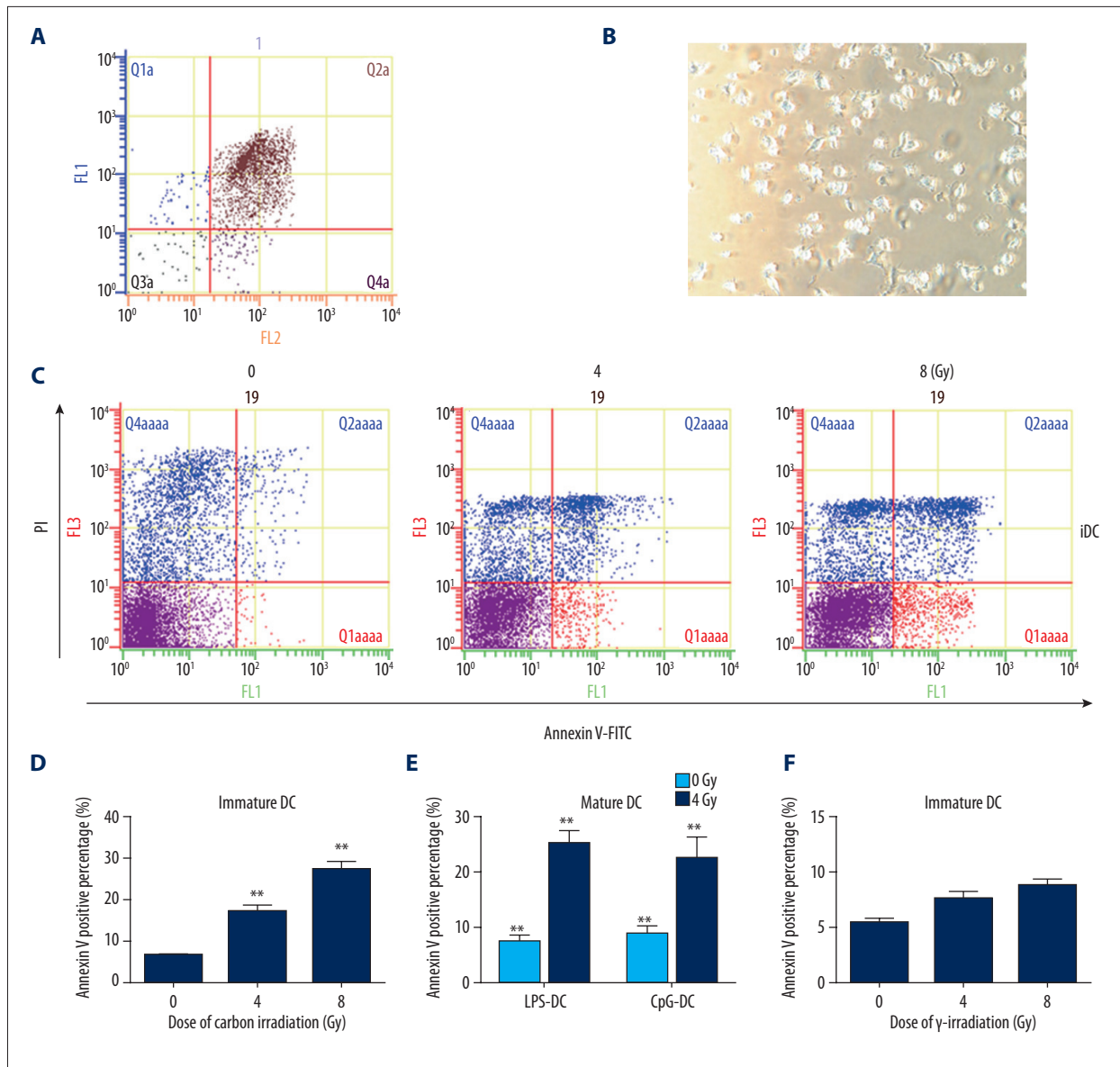
The endocytosis capacity of DCs was analyzed with a flow cytometry method as described previously. After different treatments, DCs were incubated with 5  $\mu\text{L}$  FITC-dextran (Invitrogen, US) for one hour at 37°C. The uptake of FITC-dextran was analyzed by flow cytometry (Beckman Coulter).

### In vitro cell chemo-attraction assay

A Transwell method (Corning Costar, Cambridge, MA, USA) was used for detecting the cell migration capacity of DCs, as described in our previous studies. Briefly, 100 ng/mL chemokine CCL19 in 0.6 mL RPMI1640 medium was added to the lower wells, and  $2 \times 10^5$  DCs were added to the upper wells and incubated for four hours at 37°C. Migrated cells in the lower wells were collected and analyzed with flow cytometry.

### Western blot assay

Proteins were obtained from cell lines at 0, 0.5, and 2 hours post-irradiation as previously described. The methods used in this study were described in our previous study. We detected p-p65 (Cell Signaling Tech, USA), p-Erk, p-p38, and GAPDH (Cell Signaling Technology, US; 1: 1,000). Secondary antibodies were also purchased from Cell Signaling Technology.



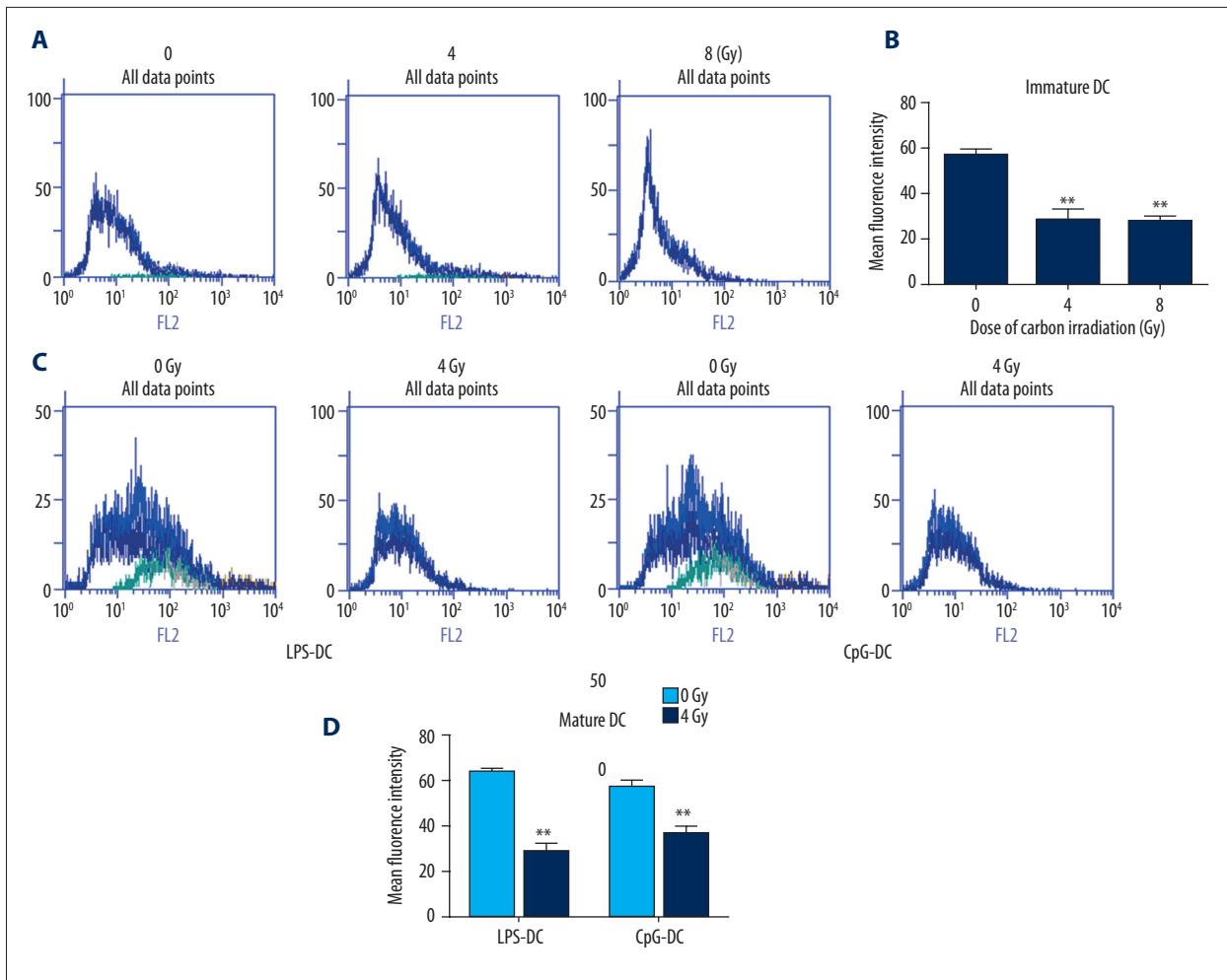
**Figure 1.**  $^{12}\text{C}^{6+}$  heavy ion radiation induced cell apoptosis in BMDCs. **(A)** Flow cytometry analysis of cultured BMDCs using CD11c-PE (FL2) and CD11b-FITC (FL1) antibodies. **(B)** Representative image of immature BMDCs. **(C)** Representative images of flow cytometry analysis of cell apoptosis in DCs. **(D)** A bar graph of apoptosis assay in immature DCs in response to carbon ion radiation. **(E)** Apoptosis assay of LPS treated and CpG treated DCs after carbon ion irradiation. **(F)** Apoptosis assay of DCs in response to  $\gamma$ -irradiation. \*\*  $p < 0.01$  versus control group ( $n=8$ ).

### ELISA assay

At 24 hours post-irradiation, supernatants of cells from different groups were collected. The cytokines concentrations in different groups were tested by ELISA (DRG International Inc., US) in accordance with the manufacturer's instructions.

### Statistical analysis

All the experiments were conducted three times, independently. Data were shown as means  $\pm$  SEM. We used analysis of variance (ANOVA) and Student-Newman-Keuls post hoc test for statistical analysis. The numbers of samples are indicated in the Figure legends.



**Figure 2.**  $^{12}\text{C}^{6+}$  heavy ion radiation suppressed the endocytosis capacity of BMDCs. (A, C) Representative images of flow cytometry of DCs incubated with FITC-dextran in iDCs and mDCs. (B, D) Bar graphs of mean FITC fluorescence in cells treated with heavy ion radiation of different doses. \*\*  $p < 0.01$  versus control group ( $n=8$ ).

## Results

### $^{12}\text{C}^{6+}$ heavy ion radiation induced apoptosis in BMDCs

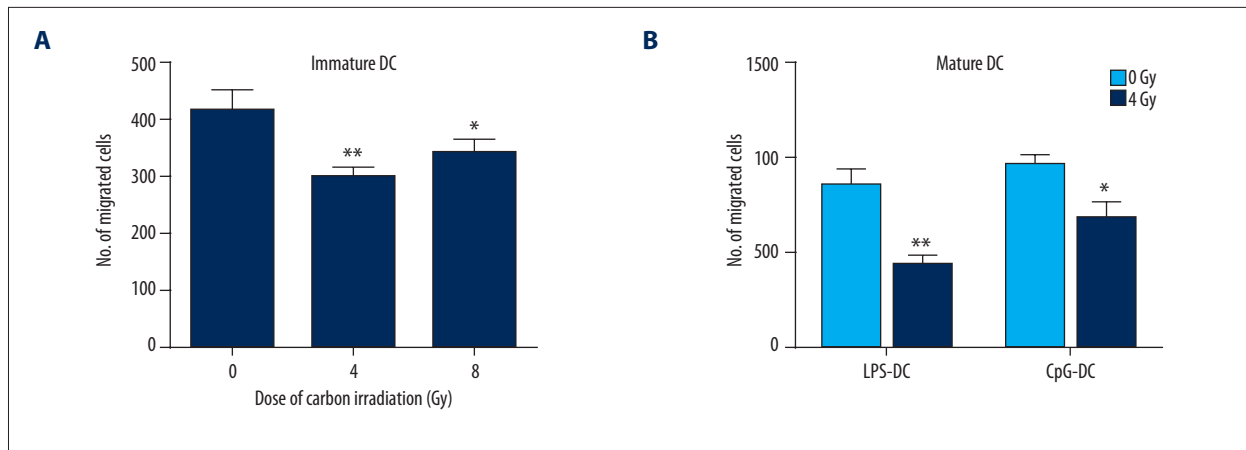
BMDCs were generated by using an *in vitro* method with GM-CSF and IL-4, and the phenotype and morphology were confirmed (Figure 1A, 1B). At 24 hours after 4 Gy and 8 Gy carbon irradiation, apoptosis of DCs was tested by using an Annexin V and PI method. We found carbon ion irradiation resulted in cell apoptosis of immature DCs in a dose-dependent manner (Figure 1C, 1D). Moreover, we used LPS or CpG treated DCs to study the effects of carbon radiation on mature DCs and found a significant increase in apoptosis in mature DCs (Figure 1E). However, no significant difference was found in DCs after  $\gamma$ -irradiation (Figure 1F).

### $^{12}\text{C}^{6+}$ heavy ion radiation suppressed the endocytosis capacity of BMDCs

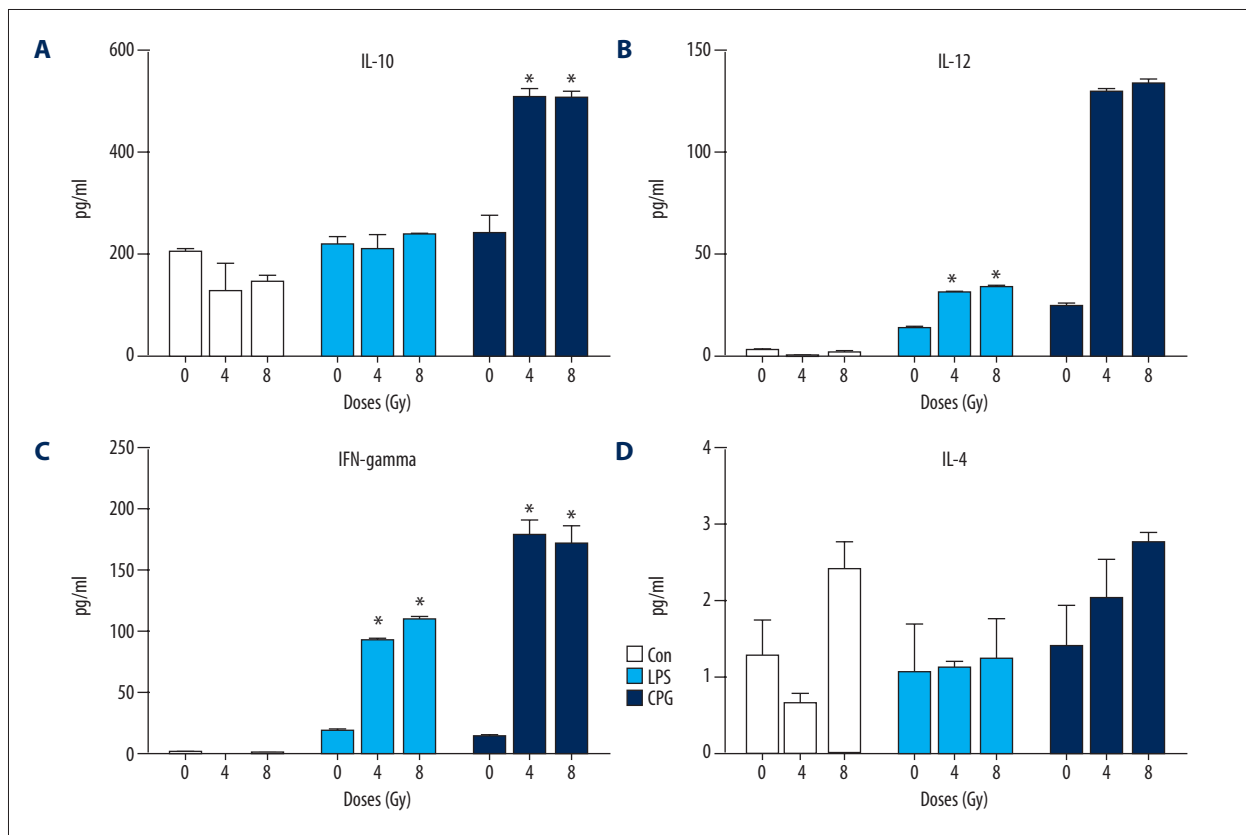
We measured the endocytosis ability of iDCs and mDCs after 4 Gy irradiation. Our data showed that carbon irradiation significantly inhibited the uptake of FITC-dextran of iDCs, but no significant inhibition was found between 4 Gy and 8 Gy irradiation (Figure 2A, 2B). Next, we found that the endocytosis capacity of mDCs was also suppressed significantly (Figure 2C, 2D).

### $^{12}\text{C}^{6+}$ heavy ion radiation inhibited cell migration in BMDCs

Using Transwell assay, we examined the migration ability of DCs in response to chemokine CCL19 as described previously [12]. Our data showed that carbon ion radiation significantly reduced the number of migrated iDCs (Figure 3A). In LPS-mDCs and CpG-mDCs, the migration ability greatly improved compared with iDCs, which was also significantly inhibited by IR (Figure 3B).



**Figure 3.**  $^{12}\text{C}^{6+}$  heavy ion radiation inhibited cell migration in BMDCs. Shown are bar graphs of numbers of migrated iDCs (A) and mDCs (B) treated with heavy ion radiation of different doses. \*  $p < 0.05$  versus control group, \*\*  $p < 0.01$  versus control group (n=8).

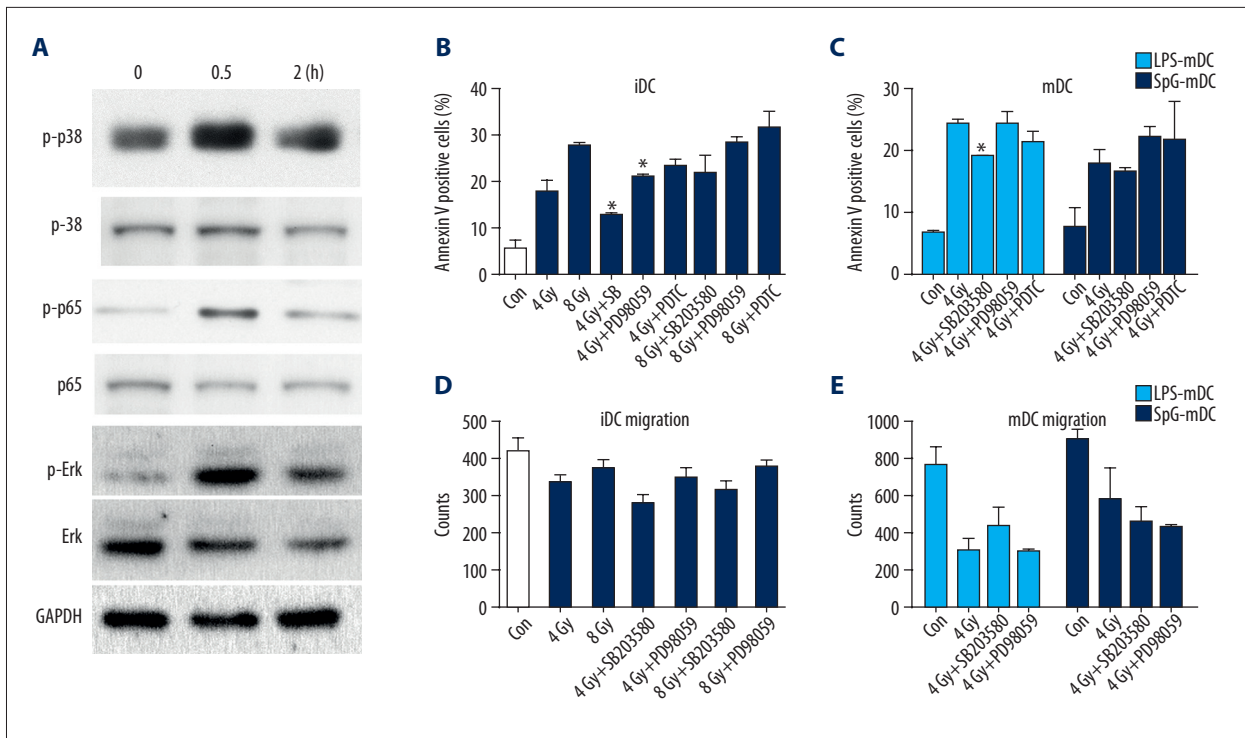


**Figure 4.**  $^{12}\text{C}^{6+}$  heavy ion radiation stimulated IFN- $\gamma$  and IL-12 in LPS or CpG treated DCs. Effects of  $^{12}\text{C}^{6+}$  heavy ion radiation on cytokines secretion of immature and mature DCs were measured by an ELISA assay. Shown are bar graphs of concentrations of IL-10 (A), IL-12 (B), IFN- $\gamma$  (C), and IL-4 (D) in DCs treated with heavy ion radiation of different doses. \*  $p < 0.05$  versus control group, \*\*  $p < 0.01$  versus control group (n=8).

### $^{12}\text{C}^{6+}$ heavy ion radiation triggered a burst in IFN- $\gamma$ and IL-12 in mature DC

In our experiments, the level of IL-4, IL-10, IL-12, and IFN- $\gamma$  in supernatants of cultured DCs was examined. We found that

carbon ion irradiation exhibited little influence on cytokines secretion of iDCs (Figure 4A–4D). Surprisingly, the level of IL-12 and IFN- $\gamma$  in LPS- and CpG-matured DCs were significantly elevated after exposure to heavy ion irradiation (Figure 4B, 4C). However, increase of IL-10 was only observed in CpG-mDCs,



**Figure 5.**  $^{12}\text{C}^{6+}$  heavy ion radiation affects DC function through p38 signaling pathway. (A) Representative images of phos-p38, phos-p65, and phos-erk examined by western blot assay. (B, C) Apoptosis assay of irradiated iDCs and mDCs treated with specific inhibitors to p38, erk and NF- $\kappa$ B p65. (D, E) DC migration assay of iDCs and mDCs after irradiation with different treatments. \*  $p < 0.05$  versus control group, \*\*  $p < 0.01$  versus control group (n=8).

not the LPS-mDCs (Figure 4A). The influence of heavy ion radiation on IL-4 showed no obvious changes (Figure 4D). These data suggested that carbon ion radiation enhanced IL-12 and IFN- $\gamma$ , while providing novel insight for the combination of heavy ion radiotherapy and immune adjuvants in cancer therapy.

### $^{12}\text{C}^6$ heavy ion radiation affects DC function through the p38 signaling pathway

It has been reported that carbon radiation activates the MAPK signaling pathway and NF- $\kappa$ B [13,14]. Our data showed that carbon irradiation induced phosphorylation of p38, Erk, and NF- $\kappa$ B p65 (Figure 5A). Then we used NF- $\kappa$ B inhibitor PDTC [15], p38 inhibitor SB203580 [16], and JNK inhibitor PD98059 [17] to check the underlying mechanisms, and we found that radiation-induced apoptosis was abolished in SB203580 treated iDCs and mDCs (Figure 5B, 5C). While the inhibitory effects of carbon radiation on LPS-mDC migration was reversed after SB203580 treatment (Figure 5D, 5E). No significant difference was found in the PDTC or PD98059 treated groups.

## Discussion

Dendritic cells (DCs) have been proven to infiltrate tumors, which affects the tumor microenvironment and initiates immunity against tumor cells [18]. On one hand, TIDCs uptake tumor antigens and present to naïve T cells, and play a role in immune surveillance [19]. DCs also regulate immune response in tumors through secreting cytokines, chemokines, and exosomes, which influences tumor environment. On the other hand, tumors could escape immune surveillance and bypass the immunity. DC maturation has also been shown to be inhibited by cytokines secreted by tumor cells, such as IL-10, PGE2, and TGF- $\beta$  [20–22]. During chemotherapy and radiotherapy, the effects of radiation and chemicals on TIDCs are quite important for outcomes.

In the present study, we found that carbon ion radiation increase apoptosis of DCs in a dose-dependent manner, whereas it was assumed to be quite resistant to photon radiation previously. We also showed that carbon ion radiation inhibited the endocytosis capacity and migration ability in both immature and mature DCs. Carbon ion radiation showed stimulating effects on cytokines production in terms of IFN- $\gamma$  and IL-12. These data will provide novel understandings for the radiotherapy of carbon ion radiation from immunological aspects.

A previous study showed that DCs were quite resistant to x-rays or  $\gamma$ -rays [23]. But there was no evidence showing changes of DCs in response to carbon ion radiation, which is more effective than photon radiation. In the present study, we found that  $^{12}\text{C}^{6+}$  heavy ion radiation significantly increased the percentage of apoptotic cells in iDCs and mDCs. DCs play critical roles in presenting antigens to T cells, thus initiating anticancer adaptive immunity. The apoptosis of TIDCs might abrogate this DC mediated anticancer function. However, DCs from the circulation could also migrated to the radiation field and exert antigen processing roles. These data provide novel information of DCs in response to heavy ion radiation.

To determine the influence of DC functions by  $^{12}\text{C}^{6+}$  heavy ion radiation, we checked the changes of endocytosis capacity, migration ability, and cytokines production. Previous studies had studied the influence of ultraviolet on endocytosis of DCs [24]. In the present study, we found that heavy ion radiation suppressed the endocytosis capacity of DCs in both iDCs and mDCs, which impairs the procession of cancer antigen. Our data also showed that DC migration capacity was inhibited by heavy ion radiation, which was consistent with our previous data using  $\gamma$ -rays. But later we proved that whole body

irradiation promoted DC migration, indicating an important role of microenvironment in DC migration regulation. By using an ELISA assay, we checked the production of cytokines in supernatants of DC culture. No significant changes were observed in iDCs treated with heavy ion radiation. But the level of IL-12 and IFN- $\gamma$  were dramatically upregulated in CpG-ODN and LPS matured DCs. Already used in clinics as an immune adjuvant, CpG-ODN might be more efficient in combination with heavy ion radiotherapy when treating cancer.

## Conclusions

Our study demonstrated heavy ion radiation could result in cell apoptosis in DCs, and suppress the endocytosis and migration function. Surprising, heavy ion radiation dramatically stimulated the secretion of cytokines in CpG-mDCs. These data provided novel understandings of the influence of heavy ion radiation on DCs, and novel information on heavy ion radiotherapy.

## Competing of interest

The authors have no conflicts of interest to disclose.

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