

## Original Article

# Microwave ablation combined with dendritic cell vaccine: a potential synergistic therapy for hepatocellular carcinoma

Yan Zhou<sup>1,3\*</sup>, Jiapeng Jiang<sup>2\*</sup>, Jianmin Ding<sup>1,3</sup>, Peng Wang<sup>3</sup>, Hui Liu<sup>3</sup>, Ying Luo<sup>3</sup>, Zhengyan Zhu<sup>3</sup>, Yingtang Gao<sup>3</sup>, Xiang Jing<sup>1,3</sup>

<sup>1</sup>Department of Ultrasound, Tianjin Third Central Hospital, Tianjin 300170, China; <sup>2</sup>The Third Affiliated Hospital of Tianjin Medical University, Tianjin 300170, China; <sup>3</sup>Tianjin Institute of Hepatobiliary Disease, Tianjin Key Laboratory of Artificial Cell, Artificial Cell Engineering Technology Research Center of Public Health Ministry, Tianjin third Central Hospital, Tianjin 300170, China. \*Equal contributors.

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**Abstract:** Hepatocellular carcinoma (HCC) is one of the most common malignant cancers with a high recurrence rate. An effective therapeutic strategy for HCC is, therefore, in urgent demand. This work aims to investigate the *in vivo* anti-HCC effect of microwave ablation (MWA) followed by tumor lysate activated dendritic cell (DC) vaccine. C57BL/J mice were rechallenged with Hepa1-6 tumor cells with different treatments (single MWA, single DC injection and MWA combined with DC injection). Then tumor growth was monitored and compared. The populations of Treg and Th17 cells were detected by flow cytometry and immunohistochemistry before and after different treatments. The migration of DCs was monitored using optimal imaging. A general volume decrease in the rechallenged tumors was observed both in the MWA and MWA + DC groups. The volume of the rechallenged tumors in the MWA + DC group showed a more significant downward trend compared with that in the MWA group. The migration of DCs from the injected site to the inguinal lymph node region was detected 24 h after injection by optimal imaging. A significant decrease of Treg and Th17 cells in the peripheral blood and lymph nodes was observed in the mice of the MWA + DC group. In summary, MWA combined with DC vaccination showed an effective synergistic therapy for HCC, with few tumor recurrences.

**Keywords:** Microwave ablation, hepatocellular carcinoma, dendritic cell, vaccine, synergistic therapy

## Introduction

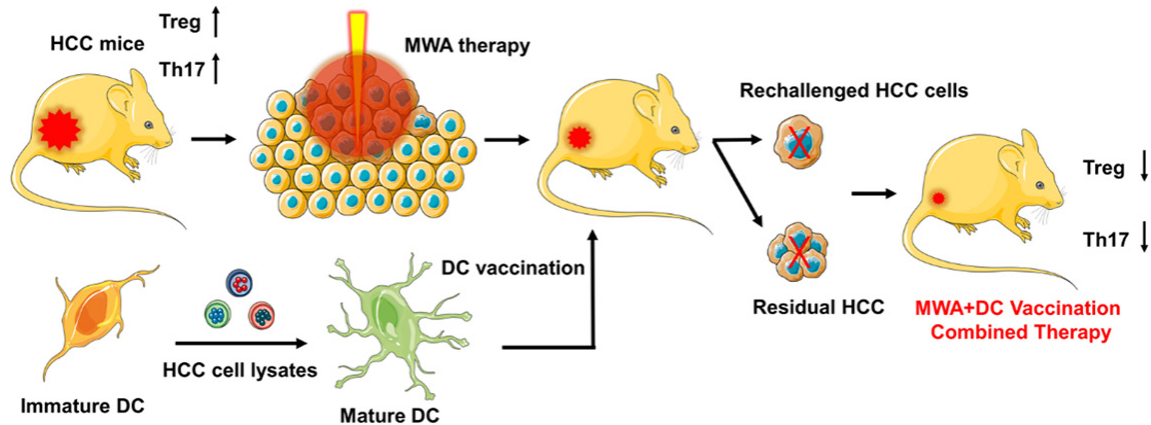
Hepatocellular carcinoma (HCC) is one of the most common malignant cancers with high mortality [1]. Currently, several methods, such as liver resection, transplantation, local thermal ablation and chemotherapy, were developed to treat HCC [2, 3]. Among these treatments, microwave ablation (MWA)-based thermal therapy has obtained increasing attention due to its minimal invasiveness and fewer side effects compared with other methods. However, MWA was also found to have non-negligible tumor recurrence on account of the incomplete ablation [4]. The surviving HCC cells in the residual tumor mass or satellite lesions often result in tumor recurrence. Therefore, developing a new treatment strategy that can ablate the primary HCC mass and

eliminate residual and metastatic HCC cells is in of great importance.

Recently, vaccine-based immunotherapy, especially the tumor lysate activated dendritic cell (DC) vaccination, has received great attention due to the antigen-specific immunities and the long-term immune-memory effect [5-8]. However, with immunotherapy it is hard to eradicate primary tumors. Integration of DC vaccination-based immunotherapy and other complementary therapies makes it possible to eliminate both primary HCC masses and residual or metastatic HCC cells.

MWA has been reported to evoke immune response and improve immunologic defense in patients with HCC due to the antigen exposure from the necrotic tumor cells after ablation [9, 10]. However, immune response evoked by

## Anti-tumor effect of MWA combined with DC vaccine



**Figure 1.** Schematic illustration of the Microwave Ablation Combined with Dendritic Cell Vaccine in treating hepatocellular carcinoma

thermal ablation is insufficient for tumor obliteration. Therefore, in this study, tumor lysate activated DC vaccination was administrated to the HCC mice after MWA. The synergetic anti-HCC effect of MWA combined with DC vaccination was investigated (**Figure 1**).

### Methods

#### *Cell lines and animal models*

All the animal experiments were carried out in the animal unit, Tianjin Third Central Hospital (Tianjin, China) according to procedures authorized by the institutional ethical committee. Murine HCC cell line hepa1-6 and C57BL/J mice were purchased from Military Medical Sciences. Cells were maintained at 37°C and 5% CO<sub>2</sub> in DMEM supplemented with 10% fetal bovine serum. Mice ages 6-8 weeks were used.

Hepa1-6 cells ( $3 \times 10^6$ ) were injected subcutaneously into the right side of the back of C57BL/6 mice. In the tumor challenging test, cells were injected into the left side of the back. The tumors were measured every 2 days. Their volumes were calculated according to the sphere formula:  $(4/3 \times 3.14 \times L \times W \times (L + W)/2)$ , where *L* and *W* are the tumor length and width, respectively. The mice with tumor diameter of 5-7 mm were selected for the next experiment.

#### *Experimental design protocol*

Mice were randomly divided into 7 groups: the control group (without tumor), tumor-bearing group; MWA group (tumor-bearing mice with

MWA treatment alone), MWA + DC group (tumor-bearing mice with combined MWA and DC therapy), DC group (tumor-bearing mice with DC therapy), MWA-Re group (tumor rechallenging was performed 10 days after MWA) and MWA + DC-Re group (tumor rechallenging followed by DC treatment 10 days after MWA). Each group had six mice, which were monitored every day. Tumor volume was measured every 2 days. All mice were killed to obtain lymph node drainage and peripheral blood as experimental specimens according to the study protocol. The proportions of Treg cells and Th17 cells in the peripheral blood, spleen and lymph were detected in the control, tumor-bearing (10 days after tumor onset), MWA (2 days and 10 days), DC (2 days and 10 days) and MWA + DC group (2 days and 10 days).

#### *Preparation of mature tumor lysate-derived DC vaccine*

Hepa1-6 cells ( $3 \times 10^7$ ) were resuspended in 10 mL PBS, heated to 43°C for 30 minutes, subjected to freezing of five cycles (-80°C refrigerator) and thawed (37°C water bath) to obtain crude lysates. The lysates were passed through 0.22 µm filters and stored at -80°C. The total protein concentration was measured by BCA assay according to the manufacturer's protocol (BCA Protein Assay Kit, Thermo).

Bone Marrow cells were obtained from the tibias and femurs. These cells were then cultured for 5 days in RPMI 1640 medium containing 20 ng/mL GM-CSF and 10 ng/mL IL-4 (PetroTech). Immature DCs were resuspended in RPMI

1640 medium on the fifth day at a concentration of  $10^6$  cells/mL. One Hundred  $\mu\text{g/mL}$  tumor lysate was added into the medium. Twenty ng/ml TNF- $\alpha$  (PetroTech) was added after 2 days.

### *Microwave ablation and DC vaccination*

MWA procedures were carried out using a MTC-3 microwave instrument (Forsea Microwave & Electronic Research Institute, Nanjing, China) with a frequency of 2,450 MHz and an output power of 0-100 W. The microwave antenna was a 16 G unipolar cooled-shaft needle with a length of 15 cm and a 5 mm active tip, which is specifically designed to meet the requirements of the animal experiment. Aloka 5000 (Aloka, Tokyo, Japan) with a 10 MHz linear array probe was used to guide and monitor the MWA procedures. Microwave output power was 15 W. Ablation time was determined according to the tumor size and the ultrasound observation of the changes of the tumors, which was usually about 1 minute. For MWA + DC treatment, the ablated tumors were cooled for 1 hour before DC injection.  $3 \times 10^6$  DCs resuspended in 150  $\mu\text{L}$  PBS were then injected into peripheral area of the ablated region.

### *Flow cytometry*

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Treg cells: Peripheral blood cells were stained with CD4-FITC and CD25-APC (eBioscience) for 30 min at 4°C. Then erythrocytes were lysed and washed. Permeabilization and fixation of cells were performed using fix/perm (eBioscience) according to the manufacturer's instruction. After permeabilization, cells were incubated with FoxP3-PE (eBioscience) for 30 min at 4°C. Flow-cytometric analysis was performed using Canto II flow cytometer (BD Biosciences) equipped with CellQuest Software (BD Biosciences).

CD4<sup>+</sup>IL17<sup>+</sup>Th17 cells: cells were stimulated for 5 h with Leukocyte Activation Cocktail (BD), stained with CD4-FITC (BD Biosciences), fixed and permeated. Cells were then incubated with IL17A-PE (BD Biosciences). Flow analysis was carried out.

### *Immunohistochemical staining*

Spleen and draining lymph nodes were embedded in paraffin and cut into sections of 3  $\mu\text{m}$ . The sections were deparaffinized in xylene and rehydrated in ethanol-water mixture. Sections

were heated in EDTA buffer for 3 min at 100°C and treated with 3%  $\text{H}_2\text{O}_2$  for 15 min. Then they were blocked by 5% BSA at room temperature for 15 min. The sections were incubated with mouse anti-mouse Foxp3 monoclonal antibody (Abcam) or rabbit anti-mouse IL17 polyclonal antibody (Abcam) at 4°C overnight. After being washed by PBS three times, the sections were incubated with the secondary antibody for 30 min at 37°C. Subsequently, the sections were colored through DAB chromogen kits and recolored of the nuclei or cytoplasm with hematoxylin.

The distribution of Treg or Th17 T cells in tissue sections was observed under the Olympus BX51 optical microscope ( $\times 400$ ). Five pictures were taken for each section. IPP software was used to count positive cells. The number of Treg and Th17 T cells was obtained by averaging the 5 pictures.

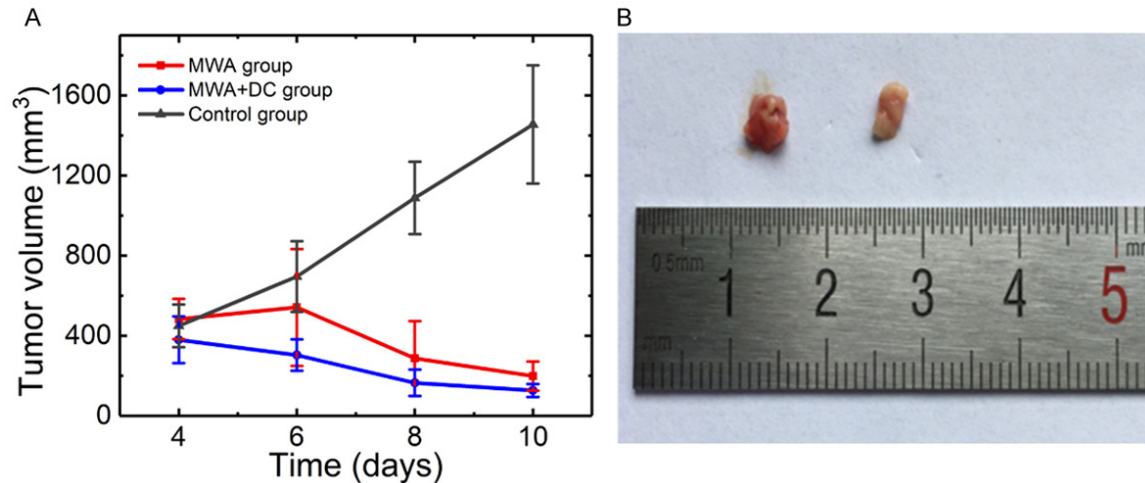
### *Optical imaging of dendritic cell*

DC cells were labeled by DiR (Invitrogen), a near-infrared (NIR) lipophilic carbocyanine dye, with a concentration of 20  $\mu\text{g/mL}$ . After that, half of the cells were fixed by 1% paraformaldehyde (as the control group). Stained cells were washed three times with PBS and then resuspended in PBS to a concentration of  $2 \times 10^7/\text{mL}$ . One hundred fifty  $\mu\text{L}$  DC or 150  $\mu\text{L}$  paraformaldehyde fixed DCs were injected into the peripheral area around the ablated region of the anesthetized mice.

The *in vivo* imaging system with an excitation wavelength of 748 nm and an absorption wavelength of 800 nm was used to monitor the migration of DCs. Fluorescence signals in the dorsal and vertical area were collected 0 h, 4 h, 24 h and 48 h after DC injection.

### *Statistical analysis*

SPSS 22.0 was used for data analysis. Continuous variables were expressed as mean  $\pm$  standard deviations (SD). Categorical variables were presented by frequencies and percentages. Student's t-test was used to compare two samples. The repeated measures analysis of variance (ANOVA) was used to compare the proportions of Treg and Th17 cells at different times. Wilcoxon and Kruskal-Wallis tests were used to compare data that are not normally distributed. A *p* value < 0.05 indicates statistical significance.



**Figure 2.** A: The growth of rechallenged tumors in the three groups. A significant difference was observed between the MWA and tumor-bearing group at 8 and 10 days ( $p < 0.05$ ) and the MWA + DC and tumor-bearing groups at 6, 8, 10 days ( $p < 0.05$ ). B: A gross tumor in the MWA group (left) was larger than that in the MWA + DC group (right) at the 10th day.

## Results

### *Rechallenged tumor growth curves of the MWA and MWA + DC group*

Tumor volume was monitored by ultrasonography 4, 6, 8 and 10 days after tumor rechallenging in both in the MWA and MWA + DC group. The tumor-bearing mice are regarded as the control group. Tumor volume in the control group gradually increased over time, whereas those in the MWA and MWA + DC group generally decrease. Although no statistical significance of the rechallenged tumor volume is observed between the MWA and MWA + DC group, the volume of the rechallenged tumors in MWA + DC group showed an earlier tendency of decrease compared with those in the MWA group (Figure 2).

### *Optical imaging of DC migration in vivo*

DIR labeled DCs (DIR DC) and DIR labeled DCs fixed with paraformaldehyde (PF-DIR DC) were injected into the peripheral area of the ablated region in tumor-bearing mice after MWA. Diffused fluorescence signal was detected during the first 48 hours both in the DIR DC and PF-DIR DC group. The weak fluorescence signal at the right inguinal lymph node area was observed in the DIR DC group, while strong signal was detected 48 hours after DC injection. In contrast to the DIR DC group, no fluorescence signal was observed in the ventral side

after the injection in the PF-DIR DC group during 0 to 48 h, as shown in Figure 3.

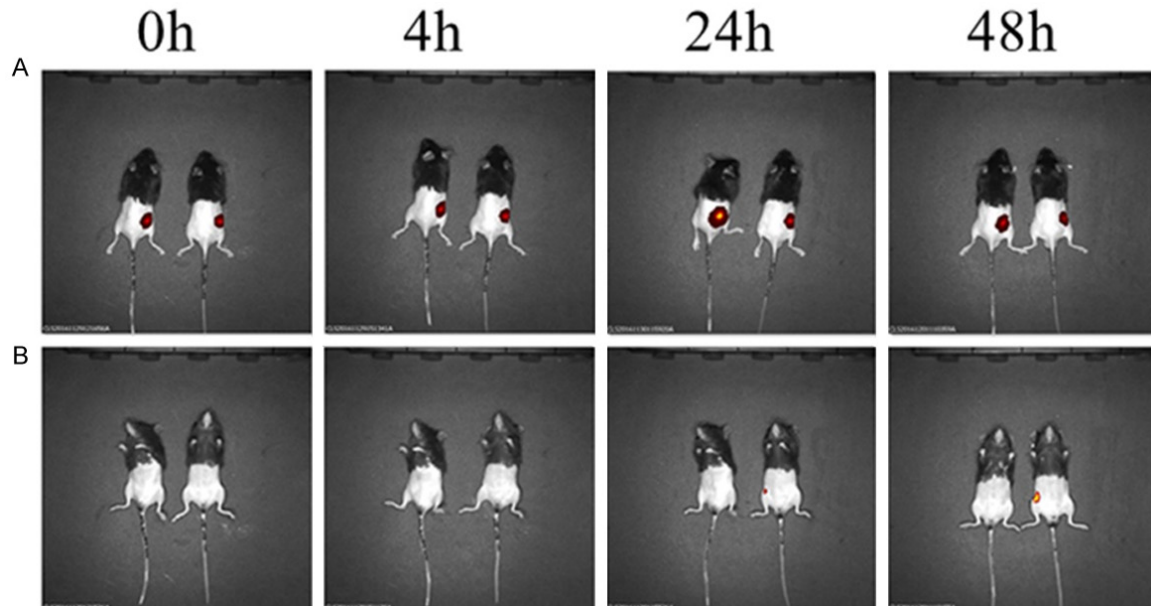
### *Alteration of Treg and Th17 cells in tumor-bearing mice*

The frequency of Treg and Th17 cells in the peripheral blood is significantly higher in the tumor-bearing group compared with that in the control group (Treg cells:  $(9.93 \pm 0.53)\%$  vs  $(8.48 \pm 0.81)\%$ ,  $p = 0.004$ ; Th17 cells:  $(4.58 \pm 1.04)\%$  vs  $(2.82 \pm 0.51)\%$ ,  $p = 0.007$ ). For the tumor-bearing group, the average numbers of per HP of Treg cells in the spleen and lymph node are  $40.0 \pm 13.38$  and  $18.81 \pm 8.04$ , respectively; while the average numbers of Th17 cells in spleen and lymph node are  $22.00 \pm 19.87$  and  $41.50 \pm 9.27$ , respectively. The expression of both of the T cells in the spleen and lymph node increase significantly in the tumor-bearing group. For the control group, in the spleen and lymph node, the average numbers of Treg cells are  $5.33 \pm 4.32$  and  $6.50 \pm 2.81$ , and of Th17 cells,  $2.00 \pm 1.41$  and  $9.67 \pm 6.71$ , respectively (compared with the tumor-bearing group: Treg cells,  $p < 0.05$ ; Th17 cells,  $p < 0.05$ ).

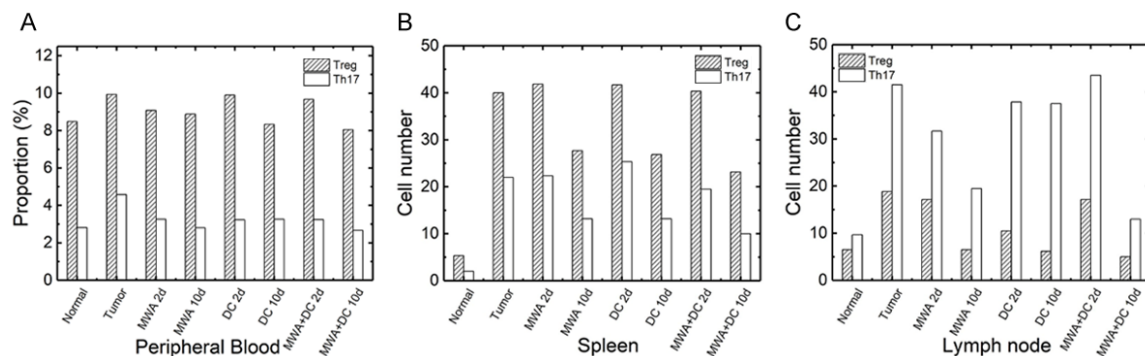
### *Alteration of Tregs and Th17 cells in the peripheral blood after different treatments*

Compared with the tumor-bearing group, the proportions of Treg cells in the peripheral blood in the three experimental groups (MWA group, DC group and MWA + DC group) did not signifi-





**Figure 3.** A: Optical imaging of DCs cell in the dorsal side at 0, 4, 24 and 48 hours after DC injection (left: PF-DIR DC; right: IR DC). The fluorescence signal was detected in the region of injection from 0 to 48 hours and with the maximum intensity at the 24 hours. B: Optical imaging of DCs cell in the ventral side at 0, 4, 24 and 48 hours after DC injection (left: PF-DIR DC; right: IR DC). The fluorescence signal was observed in the right inguinal lymph node region in the DIR DC group but not PF-DIR DC group.



**Figure 4.** Dynamic change of proportions of Treg and Th17 cells in (A) peripheral blood, (B) spleen and (C) lymph node in different groups.

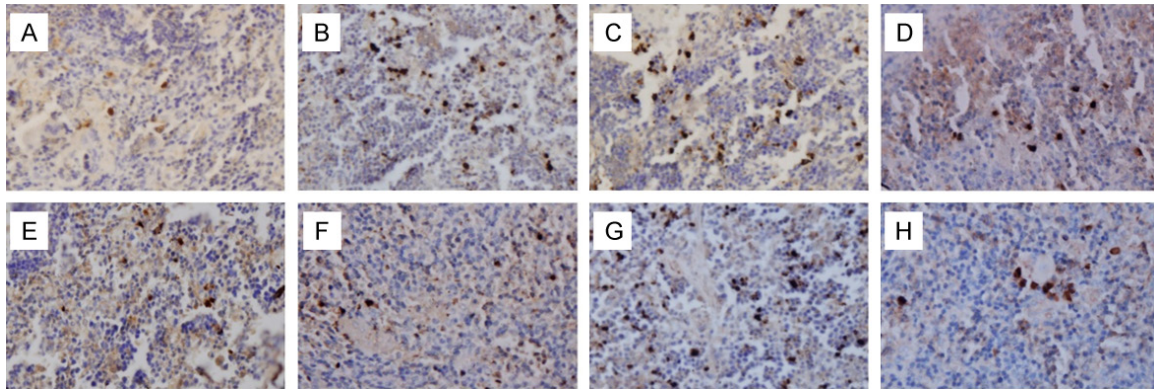
cantly change 2 days after treatment. They, however, significantly decreased 10 days after treatment in all of the three groups. For Th17 cells, the proportions decreased 2 days after the treatment by MWA, DC or MWA + DC, and then continuously decreased during the 10-day period in the three groups (**Figure 4**).

#### *Alteration of Tregs and Th17 cells in the spleen and lymph node after different treatments*

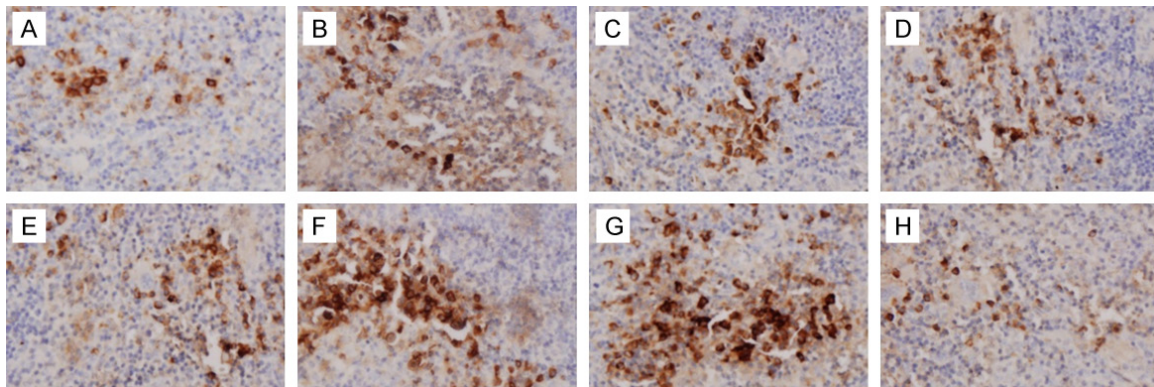
Two days after treatment, the frequencies of Treg cells in the lymph node in the MWA group, DC group and MWA + DC group were similar to

that in the tumor-bearing group. Ten days after treatment, however, the frequencies of Treg cells in the three groups were significantly decreased. Different from the changes of Treg cells in lymph node, the decrease of Th17 cells is statistically significant only in the MWA and MWA + DC group compared with the tumor-bearing group 10 days after treatment.

The frequencies of Treg and Th17 cells in the spleen 2 days after MWA, DC or MWA + DC treatment were comparable with those in the tumor-bearing group. A decrease in frequencies of Treg and Th17 cells appeared 10 days after



**Figure 5.** Immunohistochemical typical images of lymph node Treg cells (400×) in the (A) normal-control group; (B) tumor-bearing group; (C) MWA 2d group; (D) MWA 10d group; (E) DC 2d group; (F) DC 10d group; (G) MWA + DC 2d group; (H) MWA + DC 10d group.



**Figure 6.** Immunohistochemical typical images of lymph node Th17 cells (400×) in the (A) normal-control group; (B) tumor-bearing group; (C) MWA 2d group; (D) MWA 10d group; (E) DC 2d group; (F) DC 10d group; (G) MWA + DC 2d group; (H) MWA + DC 10d group.

treatment. This decrease is not statistically significant in the MWA and DC group, while it is statistically significant in the MWA + DC group, as shown in **Figures 4-6**.

### Discussion

Thermal ablation, including RFA and MWA, is minimally invasive for local tumor destruction and is recommended as a radical treatment method for HCC by several guidelines [2, 3]. Previous studies [11-13] demonstrated that RFA can evoke an immune response by changing the levels of T cells and inducing infiltration and amplification of antigen-presenting cells. Gao et al., [14] however, reported that thermal ablation stabilizes the level of Treg in the peripheral blood rather than increase it. In this study, dynamic changes of Treg and Th17 cells after MWA, which play an important role in the hepatocarcinogenesis [15-17], have been investi-

gated. We have found that the Treg and Th17 cells in the peripheral blood of tumor-bearing mice significantly increased compared with those of non-tumor-bearing mice, consistent with previous studies [18, 19]. Moreover, the changes of Treg and Th17 cells have been found in the spleen and lymph node of tumor-bearing mice. This change is similar to that in peripheral blood. We have also found that the Treg and Th17 cells in the peripheral blood and lymph node but not in the spleen decreased after MWA. This result indicates that Treg and Th17 cells play an essential role in the development of HCC and that MWA can evoke a minor immune response.

According to the results of previous studies, although the immune response evoked by thermal ablation can be used for immunotherapy for HCC, the weak immune response induced by thermal ablation may not be sufficient to

avoid tumor recurrence [20]. Therefore, DC vaccination activated by tumor lysate has been presented as a novel method to address the insufficient immune response induced by thermal ablation [6-8]. In this study, tumor lysate activated DC vaccination has been used to enhance the anti-tumor effect induced by MWA. We have found that the injection of activated DC vaccination after MWA treatment can induce the decrease in Treg and Th17 cells in the peripheral blood, lymph node and spleen of tumor-bearing mice. However, using MWA or DC injection alone leads to a decrease in Treg and Th17 cells only in the peripheral blood and lymph node but not spleen. This difference implies a stronger anti-tumor effect induced by MWA combined with DC injection compared with MWA or DC injection alone. We have further compared the growth curves of tumors in the MWA group and the MWA + DC group. The results show that the rechallenged mice in both of the MWA and the MWA + DC groups have significantly smaller tumor volumes compared with the original tumor-bearing mice. The original tumor-bearing mice show a gradual increase in tumor volume over time, whereas those in the MWA and MWA + DC group show a general decrease in tumor volume. The tumor regression observed in the MWA and MWA + DC group corresponds to the decrease of Treg and Th17 cells. This phenomenon indicates that the reduction of Treg and Th17 cells induced by MWA and (or) DC injection may result in tumor regression. Although no statistical significance in tumor volume has been observed between the MWA and MWA + DC group, the volumes of rechallenged tumors show a more obvious downward trend in the MWA + DC group compared with that of the MWA group, which implies a stronger anti-tumor effect induced by MWA combined with DC injection. Thus, MWA combined with immunotherapy may be an effective treatment strategy to prevent tumors from recurring after thermal ablation [7, 8, 21].

To confirm the anti-tumor effect of DC vaccination and clarify the migration of DCs, optical imaging has been performed. Optical imaging clearly shows the migration of DCs from periablated to the inguinal lymph node region 24 h after injection. An aggregation of DCs in the inguinal lymph node area has also been observed 48 h after DC injection. The migration of DCs revealed by optical imaging may imply

how DCs play a role in tumor regression *in vivo*. However, there may be more complex mechanisms of the anti-tumor effects of DCs.

Although the present study shows a strong anti-tumor function of MWA combined with DC vaccination. Some limitations still exist in this study. First, we focused only on two subsets of CD4<sup>+</sup> T cells, while the effects of other subsets of T cells or cytokines on tumorigenesis have not been studied. Second, the underlying mechanism of the anti-tumor effect of DCs has not been investigated in this study.

In summary, MWA can evoke immune response by decreasing the proportion of Treg and Th17 cells in the peripheral blood and lymph node. Compared with MWA or DC vaccination alone, MWA combined with DC vaccination has a greater influence on Treg and Th17 cells. The rechallenged HCC tumors can be effectively suppressed by tumor lysate-activated DC vaccination after MWA. MWA combined with DC vaccination, therefore, may be an effective therapy to prevent HCC from recurrence.

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## Disclosure of conflict of interest

None

**Address correspondence to:** Xiang Jing, Department of Ultrasound, Tianjin Third Central Hospital, No. 83 Jintang Road, Hedong, Tianjin 300170, China. Tel: 86-022-84112323; Fax: 86-022-84118095; E-mail: dr.jingxiang@aliyun.com

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