Irradiated whole-cell vaccine suppresses hepatocellular carcinoma growth in mice via Th9 cells

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Abstract. Liver cancer is one of the most common malignant tumors with no available satisfactory treatment. The aim of the present study was to investigate the anti-tumor effect of an irradiated hepatocellular carcinoma (HCC) whole-cell vaccine and its underlying mechanisms. Hepa1-6 and H22 HCC cell lines were irradiated in preparation for whole-cell vaccine production. Subsequently, two HCC tumor-bearing mouse models were created by injecting these Hepa1-6 and H22 cells into the abdominal skin of C57BL/6 and ICR mice, respectively. The mice were immunized with the corresponding whole-cell vaccine the next day, and then once a week until the end of the experimental period. Tumor growth, blood T helper (Th)9 cells and plasma interleukin (IL)-9 levels were monitored during the immunization period. Th9 cells were also induced by *in vitro* co-culture of the whole-cell vaccine with lymphocytes from the spleen and lymph nodes of the corresponding mice. Alterations of gene expression in transcription factor (TF) were determined by reverse transcription-quantitative PCR, and Th9 cells were detected using flow cytometry. The whole-cell vaccine effectively suppressed HCC tumor growth, as indicated by slower tumor growth and a smaller tumor size in the immunized group compared with the control. The percentage of blood Th9 cells and the concentration of plasma IL-9 were significantly increased in the immunized group. The whole-cell vaccine also induced Th9 cell differentiation and upregulated the expression of TFs PU.1, interferon regulatory factor 4 and basic leucine zipper transcriptional factor ATF-like. These results suggest that the irradiated HCC whole-cell vaccine inhibited tumor growth by increasing Th9 cell numbers in HCC mice

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Traditional and novel treatments, such as transarterial chemoembolization and stem cell therapy, are not satisfactory (1-3). Currently recognized treatment methods, such as immunotherapy, and anti-programmed cell death protein 1 or anti-cytotoxic T-lymphocyte protein 4 antibody treatment, were shown to be effective in only 10-20% of patients with HCC (4). The tumor vaccine approach has seen rapid developments. Tumor vaccine studies have primarily focusing on identifying new, or modifying existing, antigens to increase immunogenicity and tumor specificity (5). The major hepatoma vaccines include the HCC-associated antigen vaccine (6), the DNA/RNA or hepatitis B virus (HBV)-based vaccine (7,8), the peptide vaccine (9) and the autologous tumor antigen vaccine (10). Additionally, the personalized neoantigen vaccine is a current research hotspot (11,12). The aforementioned vaccines have shown varying degrees of anti-cancer effects in the liver. However, they have not been used in mainstream clinical treatment, which may be because these antigens are not expressed on all HCC tumor cells. Therefore, tumor clones without the expression of these antigens can escape anti-tumor immunity and continue to expand (13).

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Moreover, due to the prolonged preparation process and high cost, many patients are unable to access the vaccine. Only a limited number of studies using the autologous whole-cell vaccine have obtained successful cancer immunotherapy results (14), potentially because the immunogenicity of the untreated whole-cell vaccines was too poor to induce specific anti-tumor immunity. Therefore, novel strategies for further enhancement of vaccine immunogenicity are required (15,16). We hypothesize that irradiation has the potential to modify or stimulate HCC cells to express more tumor antigens and trigger a strong, specific anti-tumor immune response for suppressing tumor growth. The advantages of the irradiated whole-cell vaccine approach are: i) It includes a large number of membrane-associated tumor or mutated antigens; ii) it completely matches the major histocompatibility complexes of the donor patient; and iii) it is easy to prepare. Preparing this type of vaccine is less time-consuming and more practical than preparing other types of tumor vaccines. T helper (Th)9 cells were first described in the course of parasitic infection (17). These cells also possess pleiotropic functions and are involved in cancer, autoimmunity and other pathologies (18-20). To determine the effectiveness and possible mechanisms through which the irradiated whole-cell vaccine exerts its anti-tumor effect, the present study focused on the role of Th9 cells. Purwar *et al* (21) were the first to discover the anti-tumor effect of Th9 cells. It was found that ROR-γt-deficient mice were able to increase $CD4^+$ interleukin (IL)-9^+ cell numbers, and that the anti-tumor effect was abolished when using an IL-9 neutralizing antibody. Lu *et al* (22) have also verified the anti-tumor effects of Th9 cells, confirming that the *in vitro* transfer of Th9 cells into tumor-bearing animals suppresses tumor growth.

The differentiation mechanism of Th9 cells remains unclear. However, the transcription factors (TFs) PU.1, interferon regulatory factor 4 (IRF4) and basic leucine zipper transcriptional factor ATF-like (BATF) are indispensable in this process (23). PU.1 and IRF4 have proven critical for Th9 cell differentiation (24,25). PU.1 is induced by TGF-β (26), while IRF4 is induced by IL-4 (27) in conjunction with antigen receptor stimulation. Furthermore, the ectopic expression of PU.1 or IRF4 increases IL-9 production in the polarization of Th9 cell cultures (23). Th9 cells exert their anti-tumor effects in a variety of ways (28): i) Th9 cells promote T cell survival and secrete IL-9 and granzyme B, which directly target tumor cells (29,30); ii) IL-9 promotes the activation and proliferation of macrophages and plays a non-specific role in tumor cell destruction (31); and iii) IL-9 promotes the secretion of chemokine C-C motif chemokine ligand 2, and enhances the survival and antigen-presentation ability of C-C chemokine receptor type 6^+ dendritic cells (32)

The aim of the present study was to determine whether a single high-dose-irradiated HCC whole-cell lysate vaccine could inhibit the growth of HCC, focusing on the role of Th9 cells in this novel approach to active immunotherapy.

Materials and methods

Cell culture. Murine HCC Hepa1-6 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium, and murine HCC H22 cells, (Bio-Rad Laboratories, Inc.) in RPMI 1640 at 37˚C (5% CO_2) in a humidified incubator. The media contained 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All other reagents were purchased from Thermo Fisher Scientific, Inc., unless otherwise stated.

Vaccine preparation. Hepa1-6 or H22 cells cultured in 15-cm dishes were placed on the 1-cm tissue equivalent compensator and exposed to 8-Gy radiation using a linear accelerator (voltage, 6 MV; direction, 180˚; dose rate, 5 Gy min; irradiated volume, 10x10 cm; distance from source to skin, 100 cm). After 2 days, the cells and their conditional media were harvested and homogenized using the Ultrasonic Cell Disruptor (Scientz-IID; NingBo Scientz Biotechnology Co., Ltd.). The protein concentration of the homogenized mixtures (irradiated Hepa1-6 or H22 cell cultures) was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) and adjusted to a final concentration of 1 mg/ml. The two irradiated cell vaccine preparations were then used to induce active anti-tumor immunity in the corresponding tumor-bearing mouse models (i.e., Hepa1-6 vaccine for the Hepa1-6/C57BL/6 model, and H22 vaccine for the H22/ICR model).

Animal models. C57BL/6 or ICR mice (pathogen-free, female, 6-8 weeks old) were purchased from Slaccas Experimental Animal LLC (license no. SCXK 2012-0002). A total of $1x10^6$ Hepa1-6 or H22 cells in 0.1 ml each were subcutaneously injected into the C57BL/6 or ICR mice, respectively. The next day, the mice were randomly divided into 2 groups (10-16 mice/group, Fig. 1A); the control group was injected with media alone, and the other group was immunized with the corresponding vaccine preparation (Hepa1-6 or H22) into two-foot pads (0.1 ml/site). A vernier caliper was used to measure tumor length and width. The measurements were taken once or twice a week depending on the mouse model. Tumor volume was calculated as ½ (length x width²) (33). At the end of the study period, the mice were anesthetized with 1.5% pentobarbital sodium (40 mg/kg, Merck KGaA) and sacrificed by cervical dislocation. The experiment was performed twice. All mice had *ad libitum* access to a standard diet and water, and animal wellbeing was closely monitored. All animal experiments were approved by the Fujian Medical University Institutional Animal Ethical Committee (approval no. FJMU IACUC 2018-027).

Flow cytometry (FCM). When tumor growth in the treated mice was significantly suppressed (~2 weeks after immunization), immunological parameters were evaluated using FCM. For myeloid-derived suppressor cell (MDSC) staining, the tail blood was collected and incubated with anti-mouse CD11b-FITC (cat. no. 101206; BioLegend, Inc.) and anti-mouse Ly-6G/Ly-6C (Gr1)-PE antibody (cat. no. 108408; BioLegend, Inc.) for 30 min at 4˚C. Red cells were then lysed with ammonium-chloride-potassium (ACK) lysis buffer (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The remaining cells were assessed by FCM.

Regulatory T (Treg) cell staining was performed following the manufacturers' instructions for the one-step protocol for
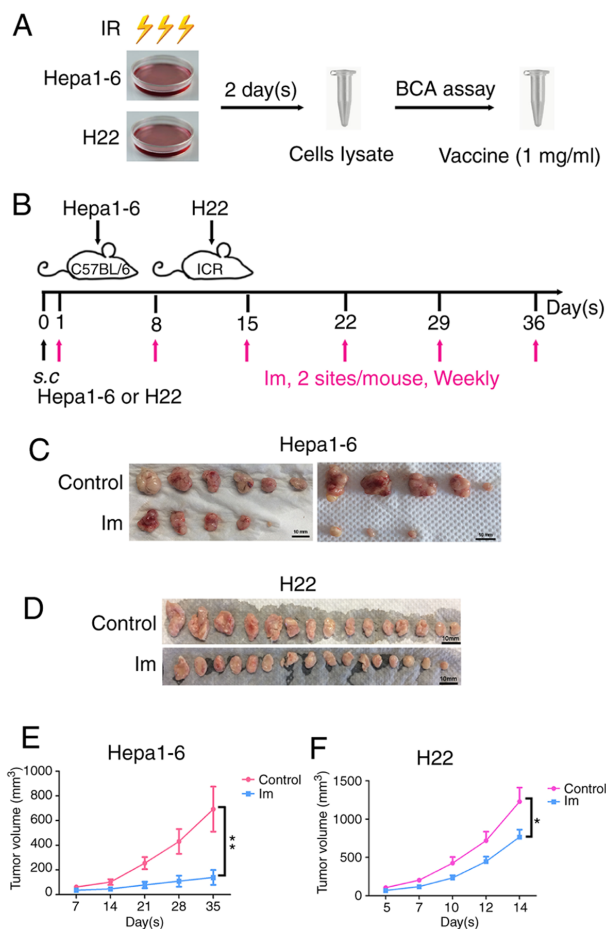
Figure 1. Single high-dose-irradiated HCC whole-cell vaccine suppresses HCC tumor growth. (A) Whole-cell vaccine preparation procedure. Hepa1-6 or H22 cells were cultured and exposed to radiation. After 2 days, the cells and the conditioned media were harvested, homogenized and adjusted to a final protein concentration of 1 mg/ml. (B) Schedule for the immunization of HCC tumor-bearing mice with the whole-cell vaccine. Hepa1-6 or H22 cells ($1x10^6$ cells in 0.1 ml /site) were subcutaneously injected into the abdominal skin of C57BL/6 or ICR mice, respectively. The next day, half of the mice were immunized with the corresponding whole-cell vaccine, while the other half were used as a control group and received culture media alone. The HCC tumor volumes were measured and calculated. (C) Final tumor sizes and (E) tumor growth curve of Hepa1-6/C57BL/6 model; 5-6 mice/group; **P<0.01. (D) Final tumor sizes and (F) tumor growth curve of H22/ICR model; 15-16 mice/group; *P<0.05. Experiments were repeated twice. HCC, hepatocellular carcinoma; BCA, bicinchoninic acid.

intracellular (nuclear) proteins. Briefly, the blood cells were incubated with anti-mouse CD4-APC (cat. no. 17-0041-81; eBioscience; Thermo Fisher Scientific, Inc.) and anti-mouse CD25-PE-Cy7 antibodies (cat. no. 25-0251-81; eBioscience; Thermo Fisher Scientific, Inc.) for 30 min at 4°C, and then lysed with ACK lysis buffer (Thermo Fisher Scientific, Inc.) to remove red blood cells. The remaining white blood cells (WBCs) were treated with fresh fixation/permeabilization working solution (Thermo Fisher Scientific, Inc.) for 30 min at room temperature, then incubated with anti-mouse Foxp3-PE antibody (cat. no. 12-5773-82; eBioscience; Thermo Fisher Scientific, Inc.) in fresh permeabilization working solution (Thermo Fisher Scientific, Inc.) for another 30 min at 4°C.

For cytokine staining, blood was diluted two-fold in RPMI 1640, stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml ionomycin (Merck KGaA) for 4 h, and

then treated with 1 µg/ml monensin (Merck KGaA) for another 2 h. Cytokine staining was subsequently performed following the manufacturers' instructions for the two-step protocol for intracellular (cytoplasmic) proteins. Briefly, the stimulated and blocked blood cells were harvested and incubated with anti-mouse CD3-FITC (cat. no. 11-0032-80; eBioscience; Thermo Fisher Scientific, Inc.), anti-mouse CD8a-FITC (cat. no. 11-0081-81; eBioscience; Thermo Fisher Scientific, Inc.), anti-mouse CD4-APC and anti-mouse CD25-PE-Cy7 antibodies separately or together for 30 min at 4°C. Red blood cells were lysed with ACK lysis buffer and the remaining WBCs were then fixed by 4% paraformaldehyde for another 15 min at 4°C. The cells were washed with permeabilization solution and then stained with anti-mouse IL-9-PE (cat. no. 514104; BioLegend, Inc.), anti-mouse IL-4-PE (cat. no. 12-7041-82; eBioscience; Thermo Fisher Scientific, Inc.), anti-mouse IL-17A-PE (cat. no. 12-7177-81; eBioscience; Thermo Fisher Scientific, Inc.), anti-mouse IFN-γ-PE (cat. no. 12-7311-82; eBioscience; Thermo Fisher Scientific, Inc.) or anti-mouse IL-10-PE antibodies (cat. no. 12-7101-82; eBioscience; Thermo Fisher Scientific, Inc.) in fresh permeabilization working solution for another 30 min at 4°C.

Following FCM (Accuri C6; BD Biosciences), the percentages of CD4 T cells (CD4+), CD8 T cells (CD8+), MDSCs (CD11b+Gr1+), Treg cells (CD4+CD25+Foxp3+), Th1 cells (CD3+CD4+IFN-γ+), Th2 cells (CD3+CD4+IL-4+), Th9 cells (CD3+CD4+IL-9+), Th17 cells (CD3+CD4+IL-17+) and T regulatory type 1 cells (Tr1 cells, CD3+CD4+CD25+IL-10+) were obtained directly from the forward scatter/side scatter-gated lymphocyte population, and the data were analyzed using FlowJo7.6 software (FlowJo LLC).

In vitro Th9 cell induction. Spleen and mesentery lymph nodes were harvested from C57BL/6 or ICR mice, and ACK lysis buffer was used to remove red cells. The remaining cells were adjusted to a final density of $2.5x10^6$/ml in culture media, either in the presence of 10 ng/ml murine IL-4 (PeproTech, Inc.) and 5 ng/ml human TGF-β (PeproTech, Inc.), alone or in combination with 5 µg/ml Hepa1-6 or 10 µg/ml H22 cell lysate vaccine. After 6 h, half of the cells were harvested and stored at -80°C for subsequent analysis, and the remainder were cultured for a further 3 days. Before harvesting, the cells were stimulated with 50 ng/ml PMA and 1 µg/ml ionomycin (Merck KGaA) for 4 h, and then treated with 1 µg/ml monensin (Merck KGaA) for another 2 h. Following stimulation and blocking, the cells were harvested and washed with PBS containing 1% BSA, and incubated with anti-mouse CD4-APC or anti-mouse CD4-FITC antibodies (cat. no. 11-0041-81; eBioscience; Thermo Fisher Scientific, Inc.) for 30 min at 4°C. The cells were then stained with anti-mouse IL-9-PE, following the manufacturers' instructions for the two-step protocol for intracellular (cytoplasmic) proteins (as aforementioned). Briefly, the cells were fixed in 4% paraformaldehyde for another 15 min at 4°C, washed with permeabilization solution, and then incubated with the anti-mouse IL-9-PE antibody (also in permeabilization solution) for another 30 min at 4°C. The percentage of Th9 cells (CD4+IL-9+) was determined by FCM (CytoFLEX; Beckman Coulter, Inc.), and the data were analyzed using FlowJo7.6 software (FlowJo LLC).

Cytokine assay. Plasma IL-9 was quantified using the mouse IL-9 ELISA Kit [cat. no. EK2092/2-96T; Multisciences (Lianke) Biotech Co., Ltd.], per the manufacturers' instructions, and measured using a SpectraMax i3x ELISA reader (Molecular Devices, LLC).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and cDNA synthesis was performed using Supermo III M-MLV Reverse Transcriptase (Bioteke Corporation) according to the manufacturer's instructions. The expression of PU.1, IRF4 and BATF mRNA was quantified in triplicate using RT-qPCR SYBR Green I dye (Promega Corporation) with the QuantStudio 5 system (Applied Biosystems, Inc.). The thermocycling conditions were as follows: Initial activation of Taq polymerase at 95°C for 5 min, followed by 40 cycles of PCR amplification at 95°C for 15 sec and annealing/elongation at 60°C for 30 sec. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Cq}$ method as previously described (34). β-actin was used as an internal control for normalizing target gene expression levels. The primer sequences used were as follows: PU.1 forward, 5'-AGGAGTCTTCTACGACCTGGA-3' and reverse, 5'-GAAGGCTTCATAGGGAGCGAT-3'; IRF4 forward, 5'-CCGACAGTGGTTGATCGACC-3' and reverse, 5'-CCTCACGATTGTAGTCCTGCTT-3'; BATF forward, 5'-CACAGAAAGCCGACACCCTT-3' and reverse, 5'-GCTGTTTGATCTCTTTGCGGA-3'; and β-actin forward, 5'-GGCTGTATTCCCCTCCATCG-3' and reverse, 5'-CCAGTTGGTAACAATGCCATGT-3'.

Statistical analysis. Quantitative data are presented as the mean ± standard deviation unless stated otherwise. Statistical significance was determined using the Student's unpaired t-test for two-group comparisons or one-way analysis of variance followed by Tukey's HSD test with ranks for multiple-group comparisons. All statistical analysis analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc.), and P<0.05 was considered to indicate a statistically significant difference.

Results

Irradiated HCC whole-cell lysate vaccines suppress HCC tumor growth. Preparation of the irradiated HCC whole-cell lysate vaccines (briefly, whole-cell vaccines), as well as the vaccine immunization schedule, is described in Fig. 1A and B. HCC tumor-bearing mouse models were used to assess the effects of the whole-cell vaccine: Hepa1-6 cells in C57BL/6 and H22 in ICR mice. HCC tumor growth was significantly suppressed in mice immunized with the whole-cell vaccine compared with that in the control mice. In the Hepa1-6/C57BL/6 model group, tumor volume was 692.4±182.3 mm³ in the control and 138.6±60.17 mm³ in the immunized group (Fig. 1C and E; P<0.01). In the H22/ICR tumor model, the tumor volume was 1228±184.6 mm³ in the control and 766.7±96.65 mm³ in the immunized group (Fig. 1D and F; P<0.05). These data demonstrate that the whole-cell vaccine effectively triggered active immunity against tumor growth in two mouse models.
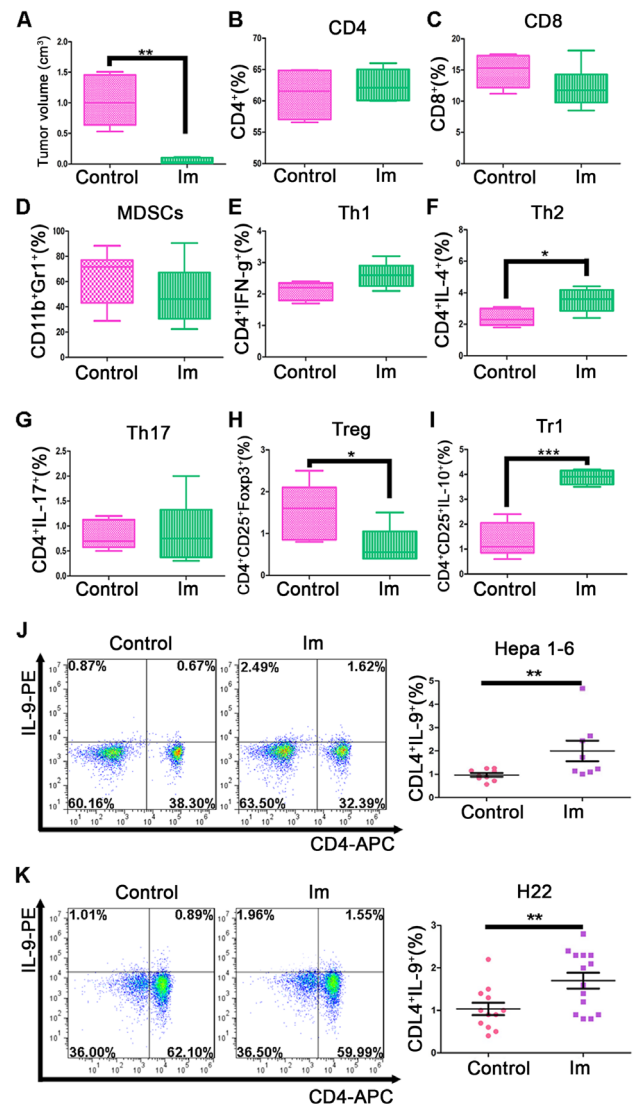
Figure 2. Increased immune cell numbers in vaccinated HCC tumor-bearing mice. (A) H22 vaccine suppressed H22 tumor growth. Systemic immunological cellular changes of the H22/ICR tumor-bearing mice immunized with the H22 whole-cell vaccine were evaluated by FCM. There were no significant differences in the percentages of (B) CD4 cells, (C) CD8 cells, (D) MDSCs, (E) Th1 cells and (G) Th17 cells between the control group and vaccine-immunized groups. However, there were significant differences in the numbers of (F) Th2, (H) Treg and (I) Tr1 cells between the two groups. Percentages of blood CD4⁺IL-9⁺ Th9 cells were increased in the vaccinated groups of the two HCC-bearing mouse models, compared with (J and K) their control groups. Significant differences between the two groups were identified by Student's t-test. N≥6. *P<0.05, **P<0.01 and ***P<0.0001. HCC, hepatocellular carcinoma; FCM, flow cytometry; MDSCs, myeloid-derived suppressor cells; Th, T helper; Treg, regulatory T cells; CD, cluster of differentiation; IL, interleukin; Im, whole-cell immunized.

Alterations in the immune cell subsets of tumor-bearing mice immunized with the whole-cell vaccine. Alterations in the immune cell subsets of whole-cell vaccine-immunized tumor-bearing mice were detected by antibody staining and FCM. In the H22/ICR mouse model, no significant differences were detected in the percentages of CD4 cells (Fig. 2B), CD8 cells (Fig. 2C), MDSCs (Fig. 2D), Th1 cells (Fig. 2E) and Th17 cells (Fig. 2G) between the control and immunized groups. However, the percentages of Th2 (Fig. 2F) and Tr1 (Fig. 2H) cells was increased, while those of Treg cells (Fig. 2I) were decreased in the immunized
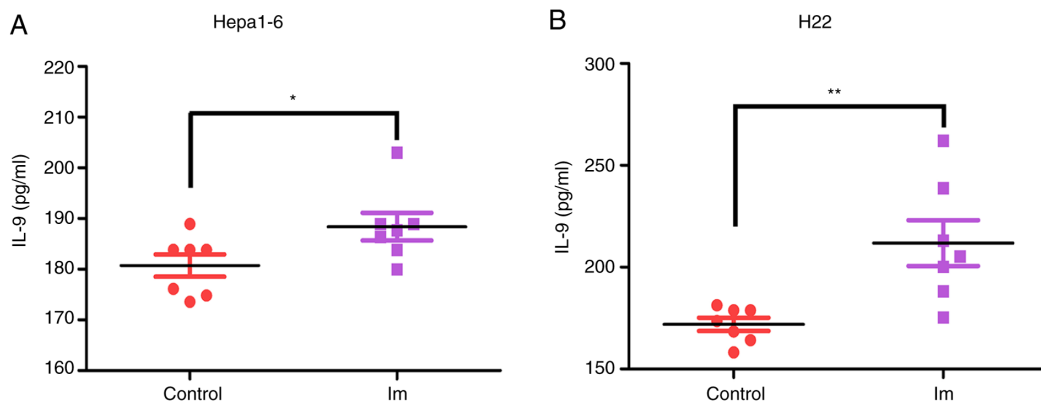
Figure 3. Plasma IL-9 is increased in vaccinated HCC tumor-bearing mice. Plasma IL-9 in two HCC tumor-bearing mouse models was measured using a mouse IL-9 ELISA kit. Concentrations of IL-9 in the immunized groups were significantly increased compared with those of the control groups. (A) Hepa1-6/C57BL/6 model and (B) H22/ICR model. Differences between the groups were analyzed by Student's t-test. N≥6. *P<0.05 and **P<0.01. IL, interleukin; HCC, hepatocellular carcinoma; Im, whole-cell vaccine immunized.
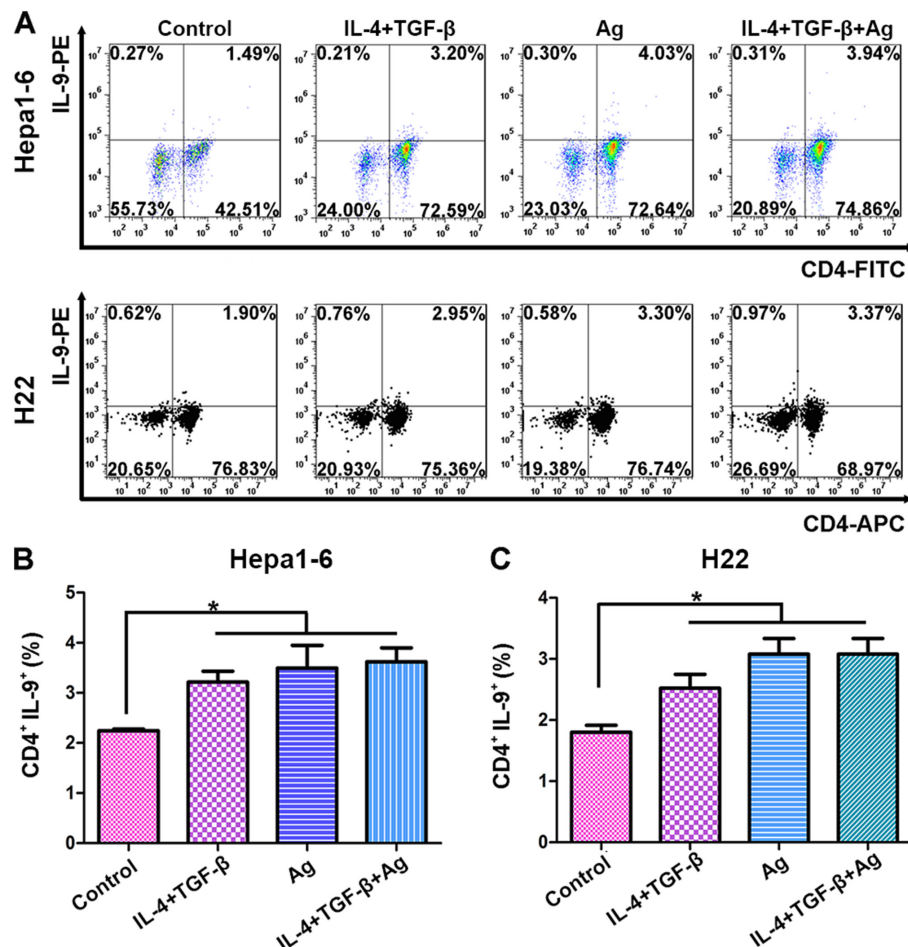

Figure 4. Vaccine-induced Th9 differentiation *in vitro*. Lymphocytes were isolated from the spleen and mesentery lymph nodes of C57/BL6 or ICR mice. Cells were then adjusted to a final density of 2.5x10⁶/ml in culture media, in the presence of 10 ng/ml IL-4 and 5 ng/ml TGF-β, alone or in combination with 5 µg/ml Hepa1-6 or 10 µg/ml H22 vaccine. After 3 days of culture, the percentage of CD4⁺IL-9⁺ Th9 cells was determined by FCM. Th0 cells were transformed into Th9 cells in the vaccine-only group or in the co-culture with TGF-β and IL-4 groups. (A) FCM for Th9 cells in two HCC tumor-bearing mouse models. Statistical analysis for the (B) Hepa1-6/C57BL/6 and (C) H22/ICR models. *P<0.05 and **P<0.01. Cellular experiments were repeated >3 times. IL, interleukin; TGF, transforming growth factor; FCM, flow cytometry; Th, helper T; HCC, hepatocellular carcinoma; Ag, whole-cell antigen.

group compared with the control group. Of note, the percentage of Th9 cells (CD3⁺CD4⁺IL-9⁺) was significantly elevated in the immunized groups compared with the control group in both HCC-bearing mouse models (Fig. 2J and K).

Plasma IL-9 level is increased in whole-cell vaccine-immunized tumor-bearing mice. As shown in Fig. 3A and B, in two HCC tumor-bearing mouse models, the concentration of plasma IL-9 was increased in the immunized compared with
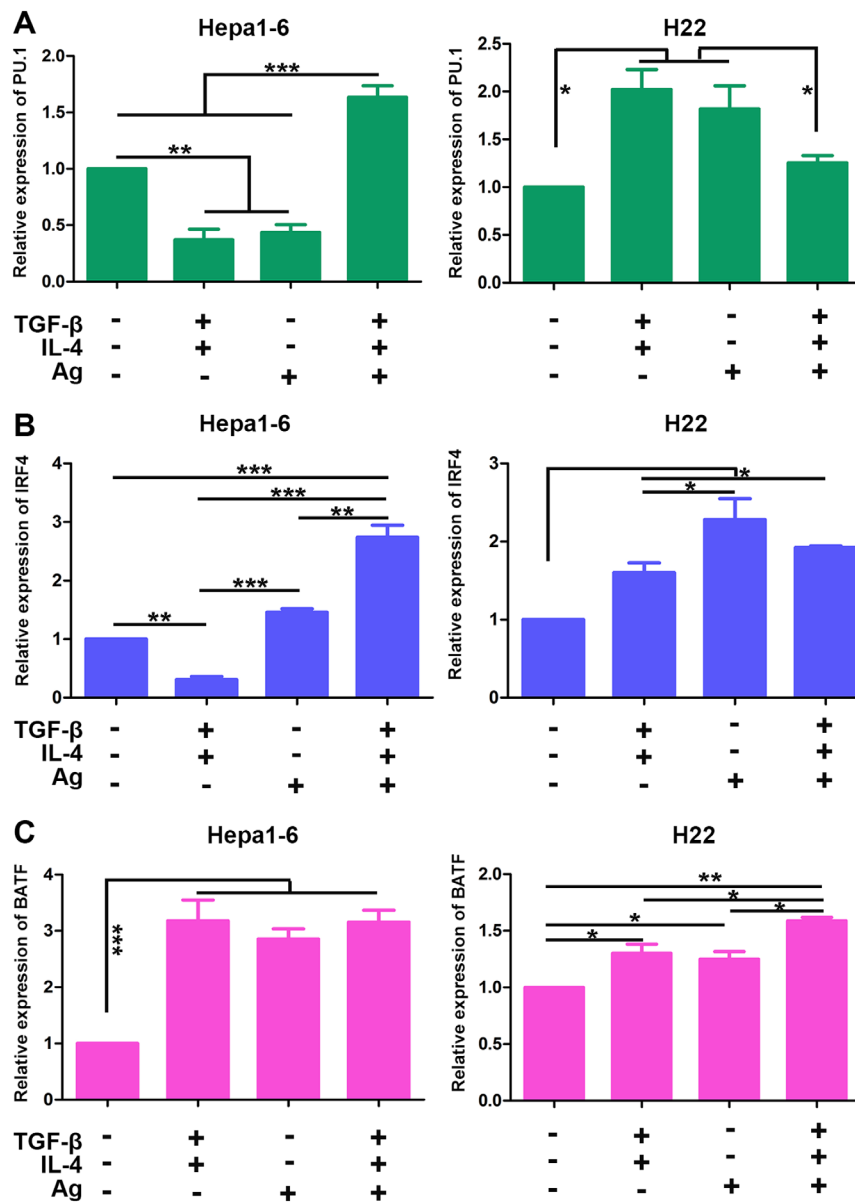
Figure 5. Vaccine-induced Th9 differentiation upregulates transcription factors PU.1, IRF4 and BATF *in vitro*. After 6 h, lymphocytes of the spleen and mesentery lymph nodes from C57/BL6 or ICR mice were cultured with 10 ng/ml IL-4 and 5 ng/ml TGF-β alone or in combination with a 5 µg/ml Hepa1-6 or 10 µg/ml H22 vaccine. Total RNA was extracted and cDNA was prepared. Reverse transcription-quantitative PCR was carried out in triplicate using SYBR Green I dye. Relative quantification was performed using the $2^{-\Delta\Delta Cq}$ method and normalized to β-actin expression. Results of (A) PU.1, (B) IRF4 and (C) BATF expression. Fold up- or downregulation is directly marked above the histogram of each group. In the control group, the reference sample value is set as 1. All other groups were compared with the reference sample. *$P<0.05$, **$P<0.01$ and ***$P<0.001$. Experiments were repeated >3 times. Th, helper T; IL, interleukin; TGF, transforming growth factor; IRF4, interferon regulatory factor 4; BATF, basic leucine zipper transcriptional factor ATF-like.

the control group, indicating that Th9 cells may promote HCC cell killing by increasing the expression of IL-9.

Vaccine-induced Th9 differentiation may be associated with the upregulation of TFs PU.1, IRF4 and BATF. Th0 cells are precursors that selectively differentiate into different Th cell subsets under divergent microenvironments. To investigate the effect of the whole-cell vaccine on Th0- to-Th9 cell differentiation, the whole-cell vaccine was added to the culture media of lymphocytes from the spleen and mesenteric lymph nodes of C57BL/6 or ICR mice. CD4⁺IL-9⁺ cells were detected by FCM after 3 days of culture. Fig. 4A shows that Th0 cells were transformed into Th9 cells in the presence of the whole-cell vaccine, TGF-β + IL-4 or a combination of the

vaccine + TGF-β + IL-4. There were no significant differences among the three treatment groups (Fig. 4B and C), indicating that the whole-cell vaccine acted in a similar manner to TGF-β and IL-4 in Th9 cell transformation.

The RT-qPCR results indicated that TFs PU.1 (Fig. 5A), IRF4 (Fig. 5B) and BATF (Fig. 5C) were upregulated in the Hepa1-6/C57BL/6 model, while only PU.1 and IRF4 were upregulated in the H22/ICR model.

Discussion

Using two HCC tumor-bearing mouse models, the present study demonstrated that a whole-cell vaccine (prepared with 8 Gy-irradiated HCC cells) exerted promising anti-HCC

tumor effects by promoting Th9 cell expansion. Th9 cell differentiation was also found to be associated with the TFs PU.1, IRF4 and BATF. Clinical trials for previous cancer vaccines designed to promote a HCC-specific immune response (including those derived from irradiated autologous whole tumor lysates) have been shown to be well tolerated and relatively safe, but to deliver unsatisfactory clinical outcomes (14,35,36). We hypothesize that if tumor cells are killed by irradiation, that the corresponding antigens will be the same as or similar to those of live tumor cells *in vivo*, which are already tolerated by, or have evaded, host immune surveillance. Herein, a novel approach was adopted using irradiation as a 'stressor' to upregulate neoantigens that could then trigger anti-tumor immunity. Both the cellular portion and conditioned media (containing the secreted fraction of the irradiated cells) were obtained. Previously, exosomes collected from cells following irradiation stress have been proven to elicit anti-tumor immunity (37). The present results suggested that the irradiated vaccine could contain more neoantigens and inhibit tumor growth more effectively than traditional tumor vaccines.

Due to its high recurrence rate and serious complications, none of the current therapeutic regimens for liver cancer produce satisfactory results. The whole-cell vaccine generated in the present study is easy to prepare and exerts a strong anti-tumor effect. Combined with surgery, radiotherapy or chemotherapy, this novel approach may indicate a novel therapeutic strategy to combat HCC recurrence.

Systemic immunity commonly destroys liver cancer cells via CD8 T or NK cells (9,38). There is now a consensus that Th9 cells comprise a subset of anti-tumor immune cells (18). Abdul-Wahid *et al* (31) found that the induction of antigen-specific Th9 cell immunity accompanied by mast cell activation blocked *in vivo* tumor cell engraftment in an IL-9-dependent manner. When IL-9 was depleted, the vaccine was found to have no effect on tumor suppression (31). The present data demonstrated that the whole-cell vaccine increased Th9 cell numbers, though the percentage of CD8 cells did not change *in vivo*. At the same time, plasma IL-9 was also increased. These results indicated that Th9 cells destroy HCC cells by secreting IL-9. In patients with HCC, Th9 cells are reportedly tumor-promoting through the C-C motif chemokine ligand 2 and signal transducer and activator of transcription 3 pathways (39). In China, the majority of patients with HCC are also believed to be chronically infected with HBV, a conclusion that was drawn based on virus-free mouse models, which admittedly differ from HCC patients with HBV. The effects of whole-cell vaccine and HBV cross-interaction on Th9 cells should be further studied to identify possible dual effects of Th9 cells in HCC (40-43).

The mechanisms underlying Th9-associated IL-9 production were further explored during treatment with the vaccine. The whole-cell vaccine comprised a number of different antigens, and as such, its composition was complex. It was therefore difficult to determine the exact antigen responsible for Th9 cell induction. The current co-culture study demonstrated that the whole-cell vaccine had the same capacity to transform Th0 cells into Th9 cells as IL-4 and TGF-β, revealing a new mechanism through which the whole-cell vaccine exerts its anti-tumor effects.

TFs are the key molecules that determine the differentiation of Th0 cells. For example, TGF-β activates Foxp3, resulting in Th0-to-Treg cell differentiation (44). TGF-β also promotes Th0-to-Th9 differentiation by inducing PU.1, which is encoded by *Sfpi1* (26), as well as IRF4, activated by IL-4 (27). BATF is another TF necessary for Th9 cell differentiation (45). When naive CD4 cells were in a BATF-deficient environment, Th9 cell differentiation was significantly inhibited (45). The TFs required for the differentiation of Th0 cells may also be strain/cell-dependent. In Hepa1-6 cells of the C57BL/6 model, antigens primarily regulated BATF, while H22 antigens mainly regulated PU.1 and IRF4, promoting Th9 cell differentiation in the ICR model of the present study. Thus, Th9 cell differentiation is a complex process involving the interaction of multiple regulatory networks.

In conclusion, in the present study, a whole-cell vaccine was found to be a new approach for promoting active immunotherapy, which works by triggering the production of Th9 cells and their related TF pathways. These Th9-associated TF pathways will be studied in greater detail, as clinical use of the whole-cell vaccine requires further investigation.

Availability of data and materials

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

Authors' contributions

JC and LZ conceived and designed the study, and are responsible for confirming the authenticity of the raw data. JC, YD, FH, RL, RC, ZW, WH, JL and BW performed the experiments. JC, YY, JH and LF analyzed the data. JC, LZ, WZ and JH interpreted the experimental results. JC and ZW prepared the figures. JC drafted the manuscript. JC, LZ, ZW, FH, JH and WZ edited and revised manuscript. All authors approved the content of the final version of the manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Fujian Medical University Institutional Animal Ethical Committee (approval no. FJMU IACUC 2018-027).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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