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Original Article

The tumor protection effect of high-frequency administration of whole tumor cell vaccine and enhanced efficacy by the protein component from *Agrocybe aegerita*

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Abstract: Whole tumor cell vaccines have been widely studied and elicits limited immune responses because of the poor immunogenicity. In the present study, we discovered that high-frequency administration of irradiated whole tumor cell vaccine triggered rejection of tumor cells (90% or 100% of the mice that were vaccinated with irradiated H22 cells or S180 respectively were protected), and provided cross-protection and long-term anti-tumor immunity in BALB/c mouse models. The antitumor activity required CD4+, CD8+ T cells and macrophage that was proved in the nude mice and cell depletion mouse models. The adoptive transfer experiment suggested that repeated whole tumor cell vaccination successfully stimulated the anti-tumor response by activation of the immune cells. A high immunization frequency within a short period of time and the presence of glycosylated molecules and nucleic acids on the surface of intact tumor cells were crucial for the successful prevention of tumor growth by whole tumor cell vaccines. Moreover, Yt, the protein component from fungus *Agrocybe aegerita*, increased whole tumor cell vaccine-mediated tumor rejection and cross-protection effect. These data indicated that the frequency of administration of whole tumor cell vaccines was of critical importance for the efficacy, which needed to be integrated into vaccine strategies for producing potential vaccines.

Keywords: High-frequency, whole tumor cell vaccine, protein component, immunodeficient mice

Introduction

In the past 50 years, many significant scientific discoveries and technological achievements have opened new avenues for studying the relationship between tumors and the immune system. As a result, tumor immunotherapies, including monoclonal antibodies, immune cell therapy and cancer vaccines, have received abundant attention as cancer treatments [1, 2]. In particular, cancer vaccines area type of active immunotherapy that specifically stimulates or activatesthe patient's immune response against tumor-specific antigens, thereby providing a targeted cancer therapy. However, many of the identified tumor-specific antigens are also expressed in normal tissues. In addition, tumor cells may undergo genetic mutations to evade immune recognition. All of these factors make itchallenging to develop effective cancer vaccines. In this context, whole tumor cell vaccines may be particularlyuseful due to their ability to target a range of tumor antigens [3, 4].

A number of cell based cancer vaccines and strategies have been tested, including autologous tumor cell vaccination [5], allogeneic tumor cell vaccination [6], tumor cell lysates [7], cytokine modified tumor cell vaccine [8], virus modified tumor cell vaccine [9]. Cell vaccines have generated promising results in early clinical trials, such as *Canvaxin* [10], *GVAX* [11], etc. However, since most tumor cells are poorly immunogenicdue to immunoediting, enhancing the immunostimulatory capacity of whole tumor cell vaccines iscritical to improving their therapeutic efficacy.

Previous studies found that the protein component Yt, which was isolated from the medicinal fungus *Agrocybe aegerita*, exhibited potent

immunomodulatory activity [12]. Therefore,Yt was combined with whole tumor cell vaccines to be determined if it could serve as an adjuvantto enhance immune stimulation. Surprisingly, the high-frequency administration of whole tumor cell vaccineswas found to greatly prevent live tumor challenge, even without Yt. The present study investigated the immune stimulation effect of the whole tumor cell vaccines, and further explored the enhanced antitumor immunity effect of Yt.

Materials and methods

Mice and cell lines

BALB/c mice (male and female; 6 weeks old) and nude mice (male and female; 5 weeks old) were purchased from the animal experimental center of the epidemic prevention sector in Hubei province, kept at standard conditions (in laminar airflow cabinets under pathogen-free conditions with 12 h light/12 h dark schedule) and fed with autoclaved Harlan Teklad sterilizable rodent diet *ad libitum*. All procedures performed in studies involving animals were approved by the Animal Welfare Committee of the Center. S180 sarcoma cells and syngeneic H22 hepatocellular carcinoma cells were purchased from ATCC (No. TIB-66) and China Center for Type Culture Collection (CCTCC, No. GDC091, Wuhan, China), and cultured under standard cell culture conditions.

Antibodies and in vivo subset depletion

Hybridomas secreting CD4-(YTS 191.1.2, YTA 3.1.2, both rat IgG2b), CD8-(YTS 169.4.2.1, YTS 156.7.7, both rat IgG2b) specific mAb were a gift from Herman Waldmann (Oxford, UK). Hybridomas were grown in culture and mAb were purified by precipitation in saturated ammonium sulfate.

For depletion of CD4+ or CD8+ T cells, mice were injected with either 0.5 mg of YTS 191.2 and YTA 3.1.2 mAb or 0.5 mg of YTS 169.4.2.1 and YTS 156.7.7 mAb every 3 days till experiment was finished. For depletion of macrophage, 1 mg/ml liposome-encapsulated Clodronate was injected intraperitoneally (0.2 mL/mouse) every 3 days till experiment was finished.

The efficacy of depletions was evaluated by fluorescence-activated cell sorting analysis with splenocytes or peritoneal macrophage that were obtained from mice after the experiments were finished.

Vaccination protocol

The mice were randomly distributed into control and vaccination groups (n =10 mice per group). Tumor cells H22 and S180 in PBS were irradiated at 10,000 rads and delivered (1×10^6 irradiated tumor cells/mL in 0.1 ml PBS) by subcutaneous injection for every other day. After 7 vaccinations, the mice were challenged by subcutaneous injection of 1×10^6 live H22 or S180 tumor cells in the right oxter. The survival of tumor challenged mice was monitored.

Whole tumor cell vaccine in nude mice, T cell-depleted mice and macrophage depleted mice

In order to study the roles of different immune cells in tumor prevention induced by the whole cell vaccine, nude mice, CD4-depleted, CD8-depleted, and macrophage depleted mice were vaccinated according to the protocol described above, and then challenged with live tumor cells. The survival of tumor challenged mice was monitored, while two perpendicular diameters of the tumor were measured by vernier caliper and the weight was determined every other day. The tumor volume was calculated using the formula $V = 1/2L_1L_2^2$, where L_1 is the long diameter, L_2 is short diameter [13].

Adoptive transfer

In the adoptive transfer model, female BALB/c donor mice were immunized for 1 time or 7 times, and boosted with irradiated tumor cells. Wild-type non-immunized mice were injected with PBS solution and used as a negative control. After 10 days, the splenocytes were harvested, suspended in PBS, and delivered (1×10^7 cells/mL in 200 μL PBS) by tail vein injection into the recipient mouse. After 3 days, the recipient mouse were challenged by live H22 cells (1×10^6 cells/mL in 100 μL PBS), and the survival was monitored.

The enzyme treatment of tumor cells

H22 tumor cells were treated by using β-galectosidase (final concentration of 10 U/10^6 cells), β-glucosidase (final concentration of 10 U/10^6 cells), RNase A (final concentration 50 μg/mL), and trypsin (final concentration 2.5

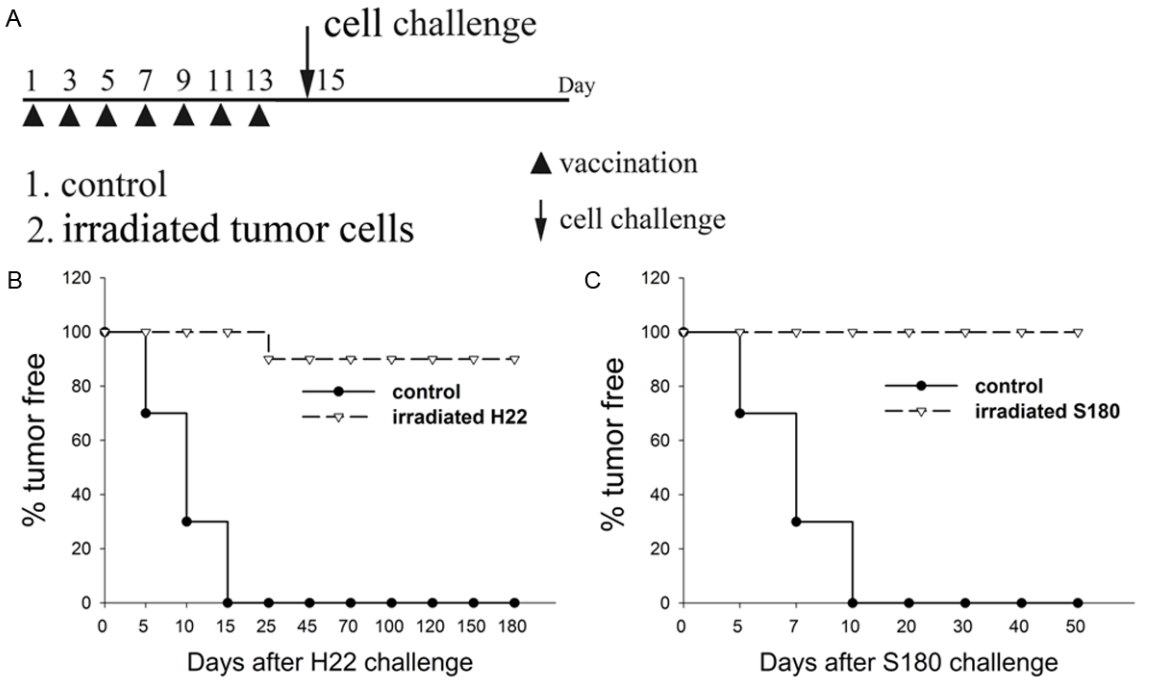

Figure 1. High-frequency administration of whole cell vaccine rejected live tumor cells in BALB/c mice. A. The schedule of tumor vaccine. The mice were vaccinated by irradiatedtumor cells H22 or S180 (1×10^6 cells/mL in 0.1 ml PBS) for every other day. After 7 vaccinations, the mice were challenged by subcutaneous injection of 1×10^6 live H22 or S180 tumor cells. B. Mice were previously vaccinated with H22 whole tumor cell vaccines, and the tumor growth was monitored until 180 days post-H22 challenge. C. Mice were previously vaccinated with S180 whole tumor cell vaccines, and the tumor growth was monitored until 50 days post-S180 challenge. n =10, and experiments repeated twice.

mg/mL) at 37°C for one hour respectively. The cell lysates were prepared by ultrasound. The vaccination procedure was according to the protocol described above. After the challenge of live H22 cell at the concentration of 1×10^6 cells/mL, the tumor growth was monitored.

Yt as adjuvant

The protein component Yt was extracted as described previously [12], and four group of BALB/c mice were given PBS, irradiated tumor cells, and combined vaccine of tumor cell/Yt (0.5 or 1 mg/mL) respectively. Mice were vaccinated according to the protocol described above, and then challenged with live tumor cells. The survival of tumor challenged mice was monitored.

Statistics

Results were expressed as mean values ± standard deviation (SD), and a Student's t test was used for evaluating statistical significance. A value less than 0.05 (P <0.05) was used for statistical significance.

Results

High-frequency administration of whole tumor cell vaccine triggers rejection of tumor cells in mice

H22 and S180 tumor cells (1×10^6 cells/mL) were irradiated prior to administration to mice-via a total of 7 consecutive vaccinations (**Figure 1A**). After a live H22/S180 tumor cell (1×10^6 cells/mL) challenge, the mice in the control group that received PBS solutionexhibited a gradual increase in the average size of H22/S180 tumors. In contrast, 90% of the mice that were previously vaccinated with H22 whole tumor cell vaccines were tumor-free until the end of the study (180 days post-H22 challenge, **Figure 1B**), and all mice (100%) that received the S180 whole tumor cell vaccine were protected against live S180 tumor development for up to 50 days (**Figure 1C**).

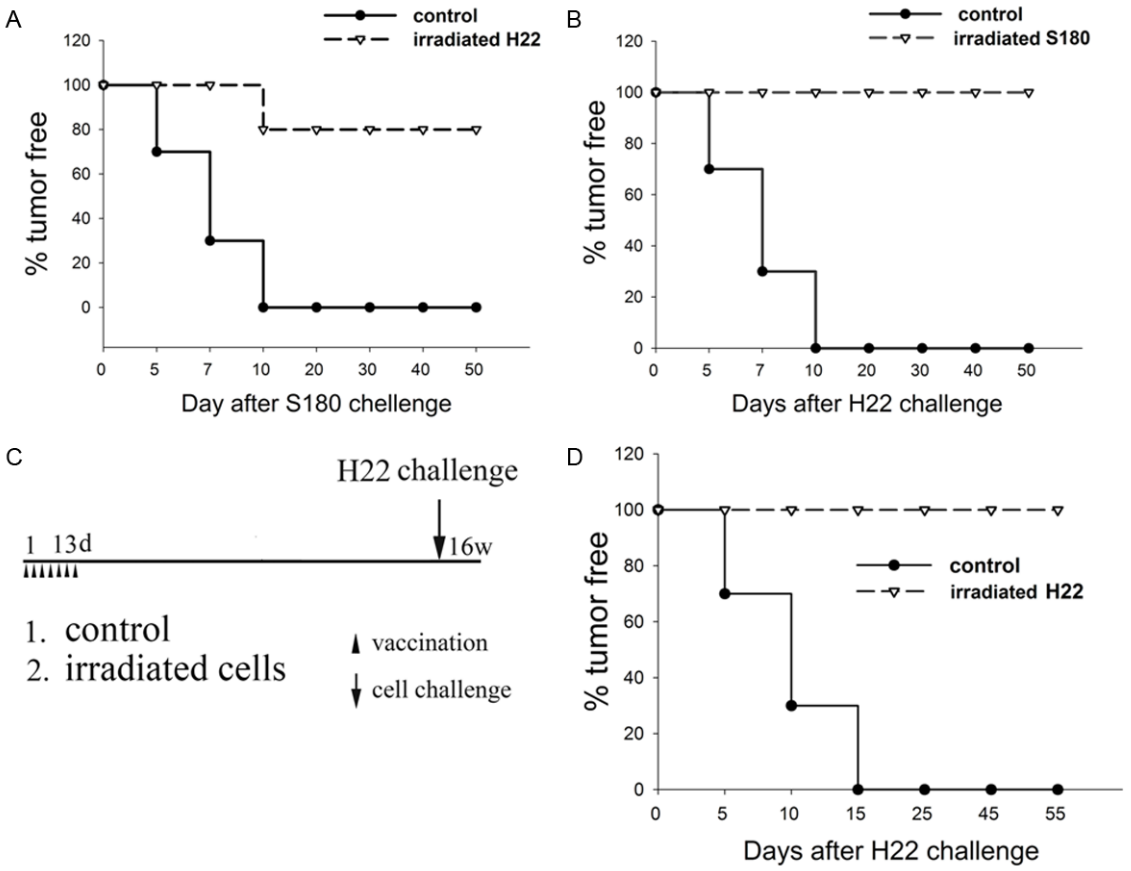

Figure 2. High-frequency administration of whole tumor cell vaccines provide cross-protection and long-term anti-tumor immunity. A. Mice were vaccinated with irradiated H22 whole tumor cell vaccines (1×10^6 cells/mL in 100 μL PBS) for 7 times, and after 2 days, the mice were challenged by subcutaneous injection of 1×10^6 live S180 cells. The tumor growth was monitored. B. Mice were vaccinated with S180 whole tumor cell vaccines, and challenged by live H22 cells. C. The schedule of tumor vaccine.Mice were vaccinated with irradiated H22 whole tumor cell vaccines (1×10^6 cells/mL in 100 μL PBS) for 7 times, and after 16 weeks, the mice were challenged by 1×10^7 live H22 cells. D.The tumor growth was monitored. n =10, and experiments repeated twice.

High-frequency administration of whole tumor cell vaccinesprovide cross-protection and long-term anti-tumor immunity

Irradiated H22 or S180 cells were injected into mice every other day for a total of 7 consecutive injections. Two days after the end of the vaccination series, the mice were challenged with either live S180 or live H22 tumor cells. The results indicated that 80% of the mice vaccinated with H22 whole tumor cellswere protectedagainst S180 tumor challenge (**Figure 2A**), and 100% of the mice vaccinated with S180 whole tumor cellswereprotected against H22 tumor growth (**Figure 2B**).

To determine whether whole tumor cell vaccines provided long-term protection against tumor development, mice that received irradiated H22 whole tumor cells every other day for 7 consecutive injectionswere subsequently housed for 16 weeks prior to challenge with live H22 tumor cells (**Figure 2C**). All micewere completely protected against tumor growth (**Figure 2D**).

Whole tumor cell vaccination is ineffective against tumor challenge in immunodeficient mice

To verify the importance of a functional immune system for this approach, we examined the anti-tumor efficacy of whole tumor cell vaccines in nude mice. As depicted in **Figure 3A**, nude mice were challenged with live H22 tumor cells after 7 consecutive immunizations with UV-irradiated low- or high-dose H22 tumor cells. All mice, regardless of the presence or absence of previous whole tumor cell vaccinations,exhibited increased tumor growth (**Figure 3B**), indicating

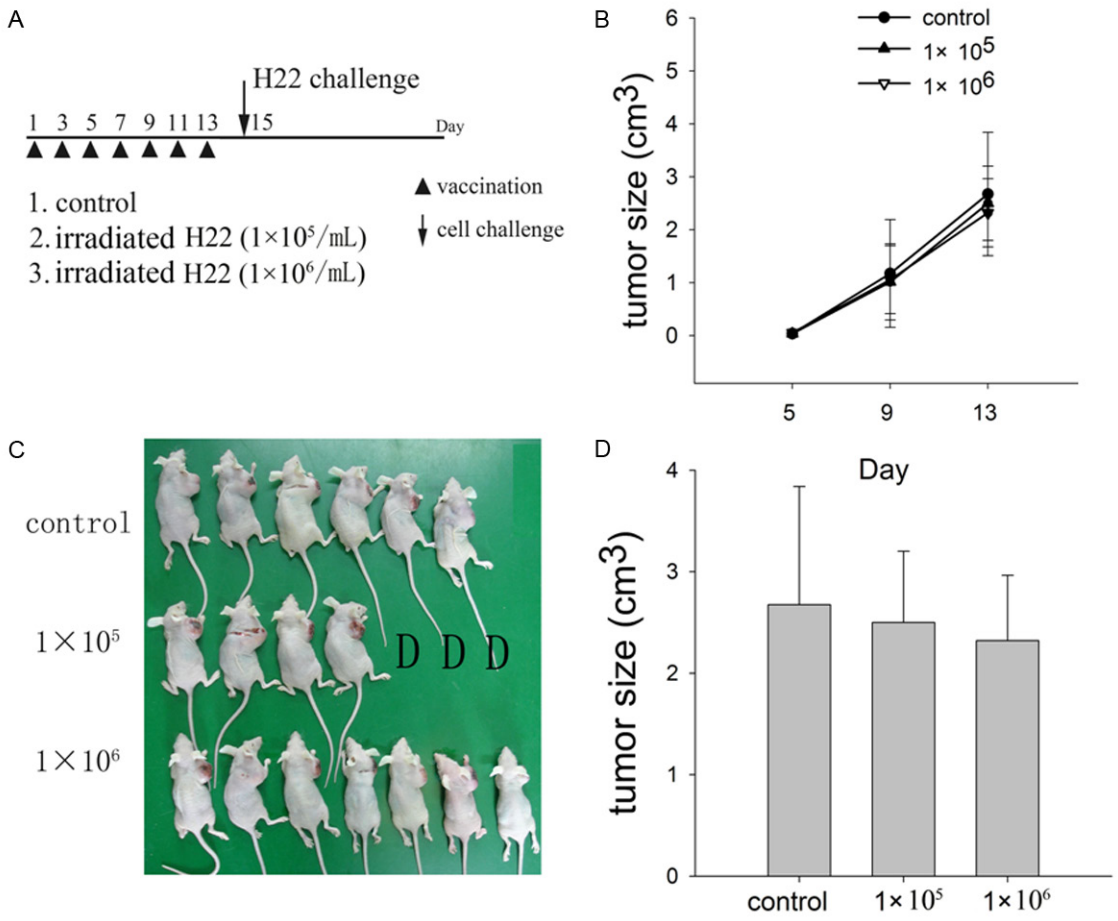

Figure 3. The effect of Whole tumor cell vaccine in nude mice. A. the schedule of vaccination. Mice were vaccinated by PBS (control), and irradiated H22 cells (1×10^5 cells/mL and 1×10^6 cells/mL) for 7 times. After 2 days, the mice were challenged by subcutaneous injection of 1×10^6 live H22 cells. B. The tumor growth was monitored, and the tumor volume was calculated using the formula $V = 1/2L_1L_2^2$. C. When the experiment was finished, the tumor size was measured. D. indicated the death of the mouse. D. The tumor volume was calculated. N =7, and $P > 0.05$.

that both immunization methods failed to protect nude mice against the H22 tumor challenge. In fact, the mice that were immunized with low-dose H22 exhibitedan even greater reduction in survival than control mice (3 mice died in the low-dose group versus 0 mice in the control group, **Figure 3C**), despite having an average tumorsizethat was similar to that of the control mice (**Figure 3D**). These results indicate that the anti-tumor efficacy of whole tumor cell vaccines is dependent upon an intact immune system.

T cells and macrophages are crucial for the induction of anti-tumor immunity by high-frequency administration of whole tumor cell vaccines

We further investigated the involvement of CD4+ T cells, CD8+ T cells and macrophages inwhole tumor cell vaccine-mediated tumor cell rejection using monoclonal antibodies and liposomes. As shown in **Figure 4A**, concomitant with whole tumor cell immunization (7 immunizations, every other day), the mice received injections of monoclonal antibodies and liposome$_{clodr}$ every 3 daysuntil the end of the study to block CD4+ T cells, CD8+ T cells and macrophages. T cell (**Figure 4B**, **4C**) and macrophage (**Figure 4D**) blockage was examined via flow cytometric analysis of splenic lymphocytes or peritoneal macrophage from the mice. The analysis revealed a 31.7%, 55.6% and 79.6% blockage of CD4+ T cells, CD8+ T cells and macrophages, respectively (**Figure 4E**). In addition, only 30% of these mice were protected against tumor growth by whole tumor cell vaccines (**Figure 4F**); this result suggests the importance of CD4+ T cells, CD8+ T cells and

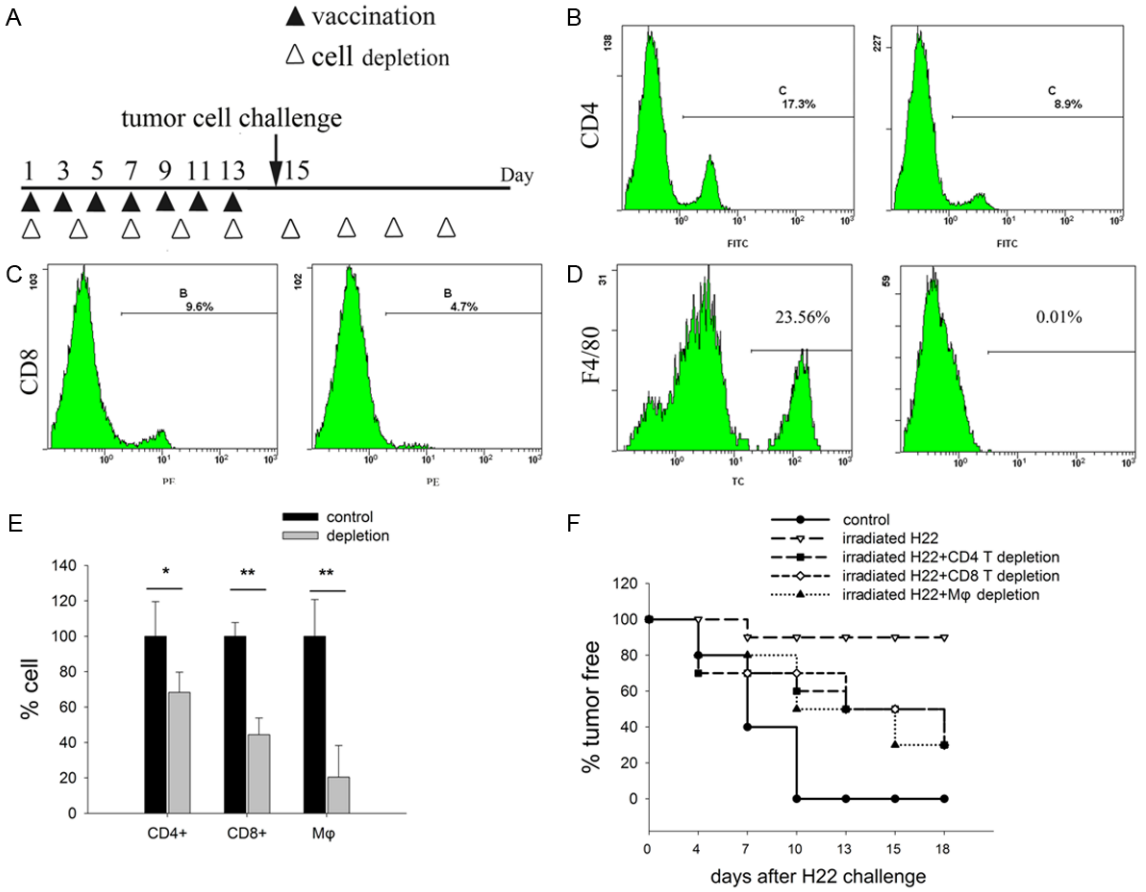

Figure 4. The tumor rejection effect of the whole tumor cell vaccine depended on T cells and macrophage. A. the schedule of the vaccination. The mice were vaccinated by irradiated H22 cell for every other day, after 7 vaccinations, the mice were challenged by live H22 cells. For depletion of CD4+, CD8+ T, or macrophage cells, the mice were injected 0.5 mg CD4 mAb or CD8 mAb or 1 mg/ml liposome-encapsulated Clodronate every 3 days till experiment was finished. The efficacy of depletion of CD4+ T cells (B), CD8+ T cells (C) and macrophage (D) was evaluated by fluorescence-activated cell sorting analysis after the experiments were finished. E. the statistical data of cell depletion efficacy. n =10, * indicated that P <0.05, and ** indicated that P <0.01. F. the tumor growth of mice were monitored.

macrophages as mediators of vaccine-induced tumor rejection.

Whole tumor cell vaccines provide adoptive immunity

To determinewhether whole tumor cell vaccine-stimulated lymphocytes confer adoptive immunity in a secondary host, we transferredthe splenic lymphocytes that wereisolated from control mice or mice immunizedwith irradiated H22 tumor cells either 1 or 7 times into recipient mice via tail vein injection. Four days after lymphocyte transfusion, the recipient mice were challenged with live H22 tumor cells and tumor growth was monitored in both groups. As shown in **Figure 5A** and **5B**, the mice infused with lymphocytes from control donorsexhibited rapid tumor growth, while the mice that received splenic lymphocytes from previously immunized donors exhibited much slower tumor development. In particular, lymphocytes isolated from mice that received 7 repeated immunizations conferred much stronger protection against tumor challenge in recipient mice than lymphocytes from non-immunized control mice (P <0.05). This result suggested that repeated whole tumor cell vaccination successfully stimulated theanti-tumor response in donor mice, whose immune cells remained active and couldprotect against tumor development even when transferred to recipient mice.

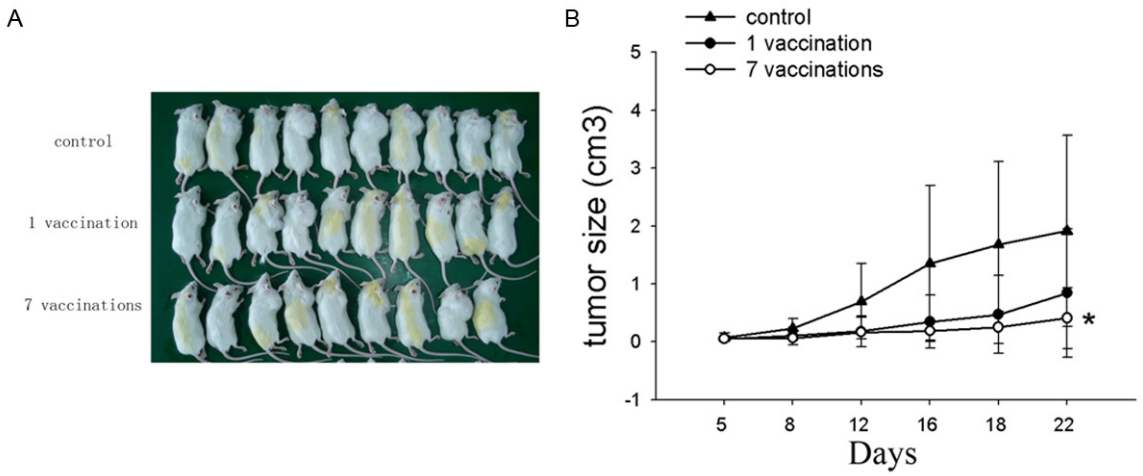

Figure 5. The adoptive transfer of splenocytes from the mice that were vaccinated. Mice were immunized for 1 time or 7 times, and boosted with irradiated tumor cells. After 10 days, the splenocytes were harvested, and delivered (1×10^7 cells/mL in 200 μL PBS) by tail vein injection into the recipient mouse. After 3 days, the recipient mouse were challenged by live H22 cells (1×10^6 cells/mL in 100 μL PBS). A. When tumors in control group reached in a volume of 2000mm^3, the mice were euthanized. B. the tumor growth was monitored. n =10, and * indicated P <0.05.

Table 1. The immunity effect of the whole cell vaccine depends on the frequency of vaccination

	Tumor free mouse/-Total mouse
control	0/10
1-time vaccination	4/10
3-time vaccination	7/10
5-time vaccination	10/10
7-time vaccination	10/10

Table 2. The immunity effect of the whole cell vaccine depends on the cell integrity

	Tumor free mouse/-Total mouse
control	0/10
UV-treated H22	10/10
β-glucosidase-treated H22	5/10
β-galactosidase-treated H22	6/10
RNase-treated H22	3/10
Trypsin-treated H22	4/10
Lysate	5/10

Adequate immunization frequency and cellular integrity were essential for successful whole tumor cell vaccination

The mice were divided into 4 groups and administered irradiated S180 whole tumor cells 1, 3, 5 or 7 times within a 13-day period. The mice were subsequently challenged with live S180 tumor cells on day 15. Tumor growth was monitored, and the results demonstrated that all mice that received 5 or 7 repeated immunizations were completely tumor-free.In contrast, only 70% and 40% of the mice that received 3 or 1 immunization (s) were protected against tumor development, respectively (**Table 1**). These results suggest that a high immunization frequency within a short period of time is crucialfor the successful prevention of tumor growth by whole tumor cell vaccines.

To determine whether intact tumor cells are necessary for effective immunization, we disrupted the membrane surface structure and cellular integrity of H22 tumor cells via enzymatic digestion, which was accomplished using two types of glycosidases, RNase A and trypsin, and ultrasonication. The mice were administered either intact H22 whole tumor cells, which received UV irradiation only as a positive control, or compromised H22 whole tumor cells for a total of 7 immunizations.The mice were then challenged with live H22 tumor cells 2 days after the vaccination. As shown in **Table 2**, both enzymatic digestion and ultrasonication reduced the number of whole tumor cell vaccine-protected mice in comparison to the positive control, as reflected by a decrease from the 100% protection rate of the control group. This

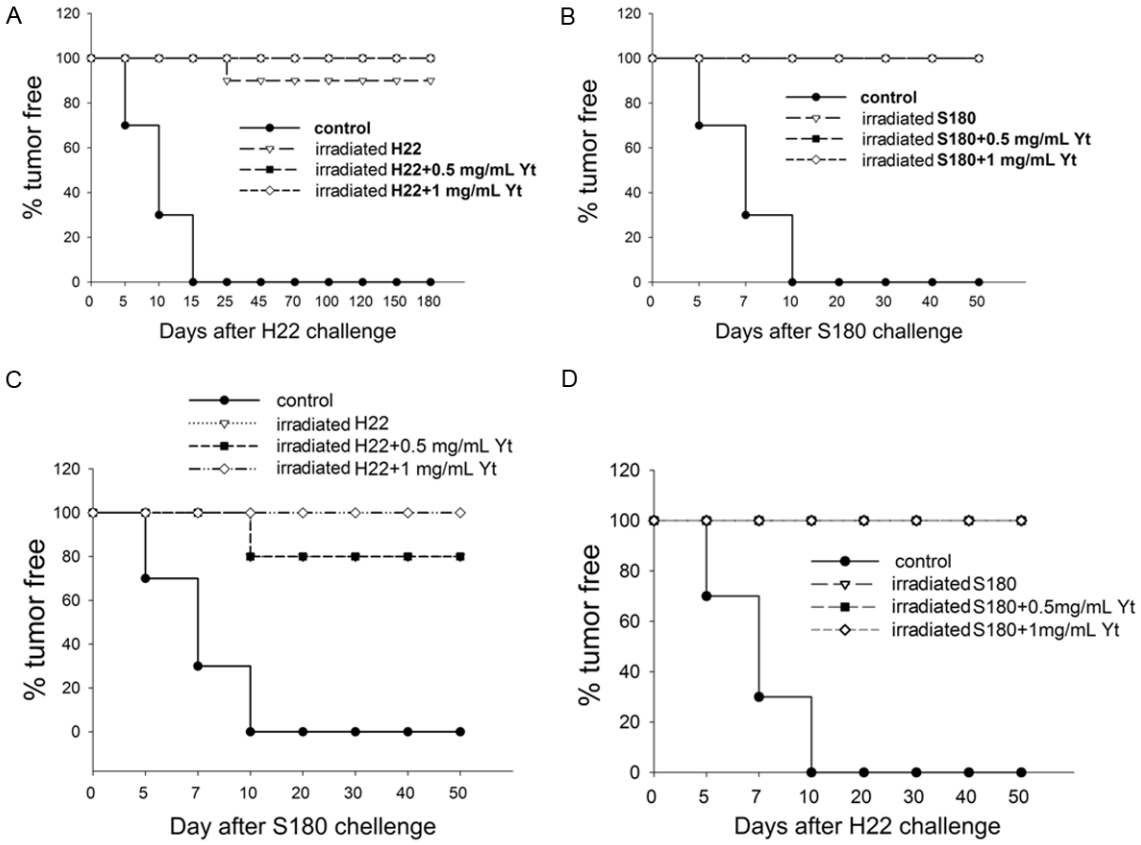

Figure 6. Yt was combined with whole tumor cell vaccines as adjuvant and improved the protection effect. The mice were vaccinated by PBS, irradiated H22 cells, and irradiated cells with Yt (0.5 mg/mLor 1 mg/mL) every other day. After 7 vaccinations, the mice were challenged by live H22 cells (A) or S180 cells (C). The mice were vaccinated by PBS, irradiated S180 cells, and irradiated cells with Yt (0.5 mg/mLor 1 mg/mL) every other day. After 7 vaccinations, the mice were challenged by live S180 cells (B) or H22 cells (D). The tumor growth was monitored. n =10.

result indicates that effective immune activation by whole tumor cell vaccines requires the presence of glycosylated molecules and nucleic acids on the surface of intact tumor cells.

Yt acts as an adjuvant and enhances the efficacy of whole tumor cell vaccines

To determine whether a combination of Yt and whole tumor cell vaccines can provide stronger protection against tumor challenge than whole tumor cell vaccines alone, we immunized the mice with either irradiated H22 cells alone or in combination with Yt prior to the live H22 tumor cell challenge. Both mono- and combinatorial vaccinations protected against tumor growth in the mice. It is worth noting that all mice that received thecombined vaccination containing Yt and H22 whole tumor cells achieved tumor-free status (100% protection, **Figure 6A**). Similarly, the injection of the S180 whole tumor cell vaccine, both alone and in combination with Yt, completely prevented tumor growth in mice challenged with live S180 tumor cells (**Figure 6B**).

Furthermore, irradiated H22 tumor cells fully protected against S180 tumor development in mice when combined with high-dose Yt. In contrast, the H22 whole tumor cell vaccine alone or in combination with low-dose Yt provided only partial cross-protection against S180 tumor development (**Figure 6C**). On the other hand, S180 whole tumor cell vaccination, alone or in combination with Yt, provided complete cross-protection against H22 tumor challenge (**Figure 6D**).

Discussion

Whole tumor cell vaccines have received increasing attention due to their expression of

multiple abnormal tumor proteins, which eliminates the need to identify specific tumor antigens. However, many tumors only weakly express or fail to expressantigenic proteins [14-17], thereby avoiding effective stimulation of the anti-tumor immune response. Therefore, whole tumor cell vaccinesare often transfected with cytokines [18], such as IL-2 [19], IL-4 [20], IL-6 [21], IFN-γ [22], IL-12 [23], GM-CSF [24], and viruses [25] or combined with other drugs [15] to enhance immune stimulation. Nevertheless, even strongly antigenic whole tumor cell vaccines have not yet to confer 100% protection against tumor development. For example, immunization with a solidified hepatocarcinoma H22 tumor vaccine 4 times at 1-week intervalsonly protected 50% of mice from developing tumors and failed to provide any protection against subsequent S180 tumor challenge [26]. When challenged with live H22 tumor cells two weeks after immunization with the same tumor cells, the mice had an overall survival time of only 30±3.23 days [27]. In the present study, by repeatedly immunizing the mice with H22 whole tumor cell vaccines within a short period of time, we achieved 90-100% and 80%protection against H22 (for up to 180 days, **Figure 1B**) and S180 tumor challenge, respectively (**Figure 2A**).

A significance difference between our whole tumor cell immunization method and previous approaches is the frequency of vaccine administration. Traditional whole tumor cell vaccinations are administered according to theviral vaccine schedule, which typically specifies a vaccination once every 1-4 weeks. In contrast, we vaccinated the mice once every other day. As demonstrated in **Table 1**, repeating the vaccination >5 times achieved the best protection against tumor challenge, whereas <5 (i.e., 1 or 3) immunizationsyielded a significantly reduced efficacy. Our high frequency immunization method not only generated 100% H22 tumor rejection but also cross-protected 80% of the S180 tumor-challenged miceagainst developing tumors. Li*et al.* discovered that UV-irradiated S180 cells exhibit strong anti-tumor activity and can be used as an adjuvant for whole tumor cell vaccines [28]. Our results are consistent with this finding and further suggest that the observed potent immunostimulatory effects of S180 cells might be closely related to the high frequency of the first 3 immunizations, which were injected daily. Thus, we conclude that frequent immunization with whole tumor cell vaccines within a short period of time can elicit strong activation of anti-tumor immunity. It is important to note that this immunization method did not appear to change the average body weight of the mice, indicating that no significant toxicity was induced. However, caution must be used, as some autoimmune conditions may be induced by intense immune stimulation. Nevertheless, further studies are needed before side effects become the primary concern [29].

In pre-clinical studies, whole tumor cell vaccines arecapable of activating the innate immune system, including natural killer (NK) cells, macrophages, eosinophilic granulocytes, and most importantly, T cells. T cell activation is the key index for evaluating the therapeutic efficacy of tumor vaccines [30]. Our studies demonstratesthat whole tumor cell vaccines stimulate CD4+ T cells, CD8+ T cells and macrophages, which may participate in the activation of the anti-tumor immune response. Although the exact mechanismsthat underlie the activation of these immune cellsremain unknown, it is possible that tumor cell immunizationdirectly or indirectly upregulates the cytokines that are upstream of these cells.

Our results also demonstratesthat whole tumor cell vaccine-mediated anti-tumor immunity requires the integrity of the cell surface structure, as biochemical or ultrasonic disruption of the membranes of vaccine cells diminish the protective effects of vaccination. Previous studieshave demonstrated that the surface glycosylation of tumor cells is crucial for inducing the expression of specific antibodies and thus for the efficacy of tumor cell vaccines [31]. Consistent with this finding, glycosidase-treated vaccine tumor cells conferred markedly reduced protection against subsequent tumor challenge. In addition, combining poly (I:C) or other nucleic acids with tumor vaccines can trigger a stronger stimulation of the immune response [32]. However, the role of surface nucleic acids in whole tumor cell vaccine-mediated immune protection remains unknown. Here, we foundthat RNase A treatment led to a larger decrease in vaccine-mediated tumor rejection than glycosidase treatment, indicating that surface nucleic acids were crucial for

immune recognition and activation by whole tumor cell vaccines. Finally, Melacine, which is a mixture of cell lysates from two types of tumors, in combination with the adjuvant DETOX induced clinical responses in 5 out of 17 patients in a phase I clinical trial [33]. In the present study, the ultrasonic disintegration of cellular membranes impairs vaccine-mediated tumor rejection, suggesting that an intact cellular structure is required for whole tumor cell vaccine-mediated immune protection.

In previous studies, Yt was shown to be an active anti-tumor agent, likely through its potent immunomodulatory effect. For example, Yt was demonstrated to enhance lymphocyte proliferation and cellular toxicity. Moreover, Yt substantially increased the mRNA levels of IL-2, IFN-γ and TNF-α in tumor-bearing mice. In the present study, Yt increased H22 whole tumor cell vaccine-mediated tumor rejection from 90% to 100% (**Figure 6A**). In cross-protection studies, high-dose Yt was able to increase the H22 tumor cell vaccine-mediated cross-protection rate to 100% (**Figure 6C**). In addition, the administration of Yt to animal models at a high frequency (every other day) caused no apparent toxicity as described before [12]. This evidence demonstrates that Yt is a promising adjuvant for tumor vaccines.

Taken together, the results of our study indicate that the administration of whole tumor cell vaccines at high frequencies has the following advantages for tumor rejection: 1. The cross-protective activity of this approach may circumvent the problemscaused by the limited number of autologous/heterologous tumor vaccine cells; and 2. The long-term protection of this approach against tumor development may be usefulfor preventing post-surgical tumor relapse in patients. Nevertheless, our future studies will focus onclarifying the mechanism that underlies the observed immune response to the whole tumor cell immunization method described in this study. Combinedwith an analysis of therapeutically significant molecular markers, our future studies willprovide new clues for developing tumor vaccines andimproving current methods of whole tumor cell vaccination.

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Disclosure of conflict of interest

None.

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