Abrogation of Local Cancer Recurrence After Radiofrequency Ablation by Dendritic Cell-based Hyperthermic Tumor Vaccine

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Local recurrence is a therapeutic challenge for radiofrequency ablation (RFA) in treatment of small solid focal malignancies. Here we show that RFA induced heat shock proteins (HSPs) expression and high mobility group box-1 (HMGB1) translocation in xenografted melanoma, which might create a proinflammatory microenvironment that favors tumor antigen presentation and activation of the effector T cells. On this basis, we investigate whether a prime-boost strategy combining a prime with heat-shocked tumor cell lysate-pulsed dendritic cell (HT-DC) followed by an *in situ* boost with radiofrequency thermal ablation can prevent local tumor recurrence. The combination treatment with HT-DC and RFA showed potent antitumor effects, with ≥90% of tumor recurrence abrogated following RFA treatment. By contrast, prevaccination with unheated tumor lysate-pulsed DC had little effect on tumor relapse. Analysis of the underlying mechanism revealed that splenocytes from mice treated with HT-DC plus RFA contained significantly more tumor-specific, IFN-γ-secreting T cells compared with control groups. Moreover, adoptive transfer of splenocytes from successfully treated tumor-free mice protected naive animals from tumor recurrence following RFA, and this was mediated mainly by $CD8^+$ T cells. Therefore, the optimal priming for the DC vaccination before RFA is important for boosting antigen-specific T cell responses and prevention of cancer recurrence.

Received 19 February 2009; accepted 24 August 2009; published online 22 September 2009. doi:10.1038/mt.2009.221

INTRODUCTION

Hyperthermia has become a vital adjunct in regional control of unresectable focal malignancies. The most commonly used heating method in clinical settings is capacitive heating using a radiofrequency ablation (RFA) electric field. RFA has been proven to provide favorable survival with excellent local control and achieve survival time comparable to surgery in selected patients.[1] Much of the allure of RFA is its ability to achieve local tumor destruction with little morbidity and mortality compared with surgical resection. However, the overall therapeutic efficacy of this approach has been limited. Many of these patients will die of intrahepatic recurrence and multiple metastases that remain untreated.[2,3] Therefore, the addition of a relevant systemic therapy would be highly desirable to enhance its potency.

RFA induces hyperthermia within the tumor lesion which may cause immunologic and biologic effects.[4–6] In conjunction with the generation of thermally altered tumor antigens, the unspecific inflammatory stimulus induced by RFA might help to overcome immune-tolerance and induce a systemic immune response including tumor-specific T cell activation.[4,7] The proinflammatory effects of thermally necrotic cells appear to be caused by the release of endogenous adjuvants, such as the nuclear protein high mobility group box-1 (HMGB1) and heat shock proteins (HSPs) such as HSP70 or gp96,[8–11] which can stimulate a primary antitumor immune response both locally and systemically via activation of dendritic cells (DCs). DCs are regarded as the most potent antigen-presenting cells for naive T cell activation.[12] RFA thus appears to create an *in situ* environment resembling T cell vaccination *ex vivo*.[4] However, in patients with cancer, the effectiveness of such *in situ* vaccination might be compromised because the hyperthermic tumor microenvironment is always not conducive to the activation and emigration of dysfunctional DCs.[11,13,14]

Immunization with *ex vivo* antigen-loaded DC could circumvent possible defects in the DCs of patients with cancer and might significantly boost antitumor immune responses.[15] Hyperthermic tumor lysate is superior to other ways of DC pulsing as hyperthermia-induced HSPs have the promiscuous ability to chaperone and present a broad repertoire of tumor antigens to DCs,[16] thus circumventing the need of prior identification of tumor-associated antigens from individual cancers. Furthermore, HSPs

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deliver maturation signals to DCs by upregulating the expression of costimulatory and antigen-presenting molecules, including CD80, CD86, and MHC (major histocompatibility complex) class II molecules.[17–19] More importantly, it is conceivable that heat-shocked tumor cell lysate-pulsed DCs (HT-DCs) might be able to prime a set of tumor-specific T cells that could more efficiently recognize and eradicate the surviving heat-shocked tumors cells till remain unkilled by RFA treatment. To test this possibility, we evaluated the effect of combining RFA with HT-DC vaccination on the poorly immunogenic B16F10-luc melanoma. The data show that this combined treatment induces strong and durable T cell-mediated tumor-specific immunity that results in the efficient destruction of remnant tumor cells and prevents tumor recurrence following RFA.

RESULTS

Hyperthermia induces HSPs expression and HMGB1 translocation in animal tumor models and cultured cells

We first investigated the hyperthermic effects on expression and localization of several immune-stimulatory molecules such as HSPs and HMGB1 following RFA treatment. Immunohistologically or immunofluorescently, tumor cells showed typical signs of cytoplasmic and nuclear-thermic alterations of RFA treatment (data not shown). The untreated xenografted B16F10-luc melanoma of the control group showed only sparse HSP70- or gp96-positive tumor cells. After RFA treatment, a highly elevated amount of HSP70- or gp96-positive tumor cells could be observed in tumor lesions (**Figure 1a**). Furthermore, RFA treatment induced a dramatic translocation of nuclear HMGB1 into the cytoplasma and intercellular space, indicative of active release of this protein (**Figure 1b**). Because B16F10-luc melanoma has a much higher recurrence rate after RFA than other sygeneic tumor cells tested (data not shown), we used this poorly immunogenic murine tumor as a model to mimic cancer relapse after thermal ablation.

To assess the *in vitro* effects of heat shock, B16F10-luc melanoma cells were exposed to temperatures of 37, 42, or 43 °C for 0.5 hours, followed by a recovery period at 37 °C for 5 hours. After heat shock treatment, an evident enhancement of HSP70 and gp96 expression by the tumor cells was observed using western blot analysis. Quantification of western blot signals showed that the expressions of HSP70 and gp96 were increased in 43 °C-cultured tumor cells by about twofold and threefold, respectively, when compared with 37 °C-cultured tumor cells (**Figure 1c**). Consistently, immunofluorescent staining showed increased cytoplasmic distribution of HMGB1 in heated tumor cells as compared to unheated cells (**Figure 1d**). The effects of heat shock were temperature dependent as observed for HSP70 and gp96. For subsequent experiments, we used a 43 °C heating because exposure to temperatures above 43 °C led to decreased cell viability. These data suggest that hyperthermia may potentiate immune activation by upregulating immunochapones such as HSPs and HMGB1 both *in vivo* and *in vitro*.

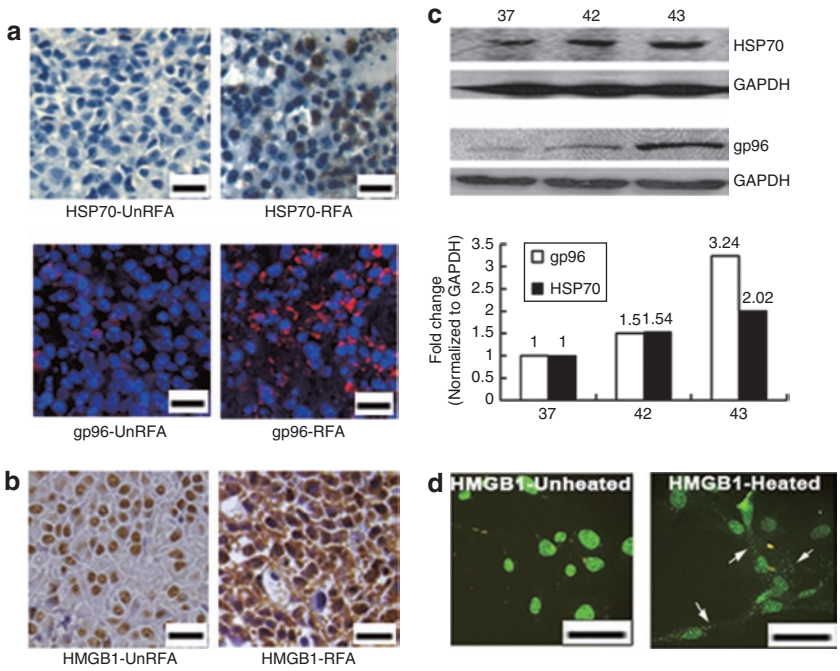

Figure 1 Hyperthermia induces HSPs expression and HMGB1 translocation in xenografted tumor and cultured cells. (**a**) Immunohistochemical staining for HSP70 expression and immunofluorescence assay for gp96 in the RFA-treated and untreated B16F10-luc melanoma tissue. (**b**) Immunohistochemical staining of HMGB1 in the RFA-treated and untreated B16F10-luc melanoma tissue. (**c**) Western blot analysis of HSP70, gp96, and GAPDH in B16F10-luc cells exposed to temperatures of 37 °C,. For quantification, band intensities were first normalized to the respective GAPDH signal and then calculated as fold change of 42 or 43 °C-treated tumor lysates relative to 37 °C-treated tumor lysates. (**d**) Immunofluorescence staining of HMGB1 in B16F10-luc cells exposed to 37 or 43 °C. Arrow, cytoplasmic HMGB1. Data are representative of three independent experiments (bar = 50 μm). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMGB1, high mobility group box- 1; HSP, heat shock proteins; RFA, Radiofrequency ablation.

Heat shock of cultured tumor cells promotes DC maturation

Because both HSPs and HMGB1 are known to activate DCs, we then assessed whether heated tumor cells influence the maturation status of DCs. Considering that RFA causes *in situ* tumor necrosis, we used tumor cell lysate as an antigen source to pulse DCs *in vitro*, which mimicked the *in vivo* situation. As shown in **Figure 2a**, DCs pulsed with heated tumor cell lysate manifested higher MHC class II and CD80/CD86 expression than those pulsed with unheated tumor lysate or phosphate-buffered saline (PBS) control. Furthermore, heat-shock of tumor cells also induced a higher expression of chemokine receptor CCR7 in DCs, which is necessary to direct DCs to secondary lymphoid nodes and to elicit an adaptive immune response. In contrast, the chemokine receptor CCR5, which is thought to be involved in recruitment of immature DC to tissues, was slightly reduced in DCs pulsed with heated tumor cell lysate as compared with DCs pulsed with unheated cell lysate or PBS (**Figure 2b**). In addition, lipopolysaccharide and heat-shock costimulation led to a synergistic increase in DCs maturation (**Figure 2a**). Taken together, these results indicate that heat-shocked tumor cells induce DC maturation *in vitro*.

Vaccination with HT-DC is necessary to protective immunity against tumor recurrence after RFA

Our preliminary experiments have shown that nearly 80% of RFA-treated mice bearing B16F10-luc melanoma will experience a rapid recurrence and progression of the disease (**Supplementary Figure S1**, C and D, blank group), which suggest that the induction of antitumor immunity by *in situ* tumor destruction was either insufficient or unreasonable possibly owing to the lack of effective T-cell priming. We postulated that a prime-boost vaccination with heated tumor lysate-pulsed DC mimicking RFA effects *in vivo* might improve anticancer immune responses. To test this hypothesis, bone marrow-derived immature DC from syngeneic mice were pulsed with heat-shocked (43 °C) tumor cell lysate (HT-DC), unheated (37 °C) tumor cell lysate (UT-DC) or PBS, matured by lipopolysaccharide treatment, and injected through foot pads 4 days before subcutaneous inoculation of B16F10-luc melanoma cells. A detailed time schedule of the different treatments is given in **Figure 3a**. None of the above vaccination protocols could protect mice from orthotopic challenge with B16F10-luc tumor as shown in **Figure 3b**. On day 0, the tumors treated with DC vaccines in the different groups of

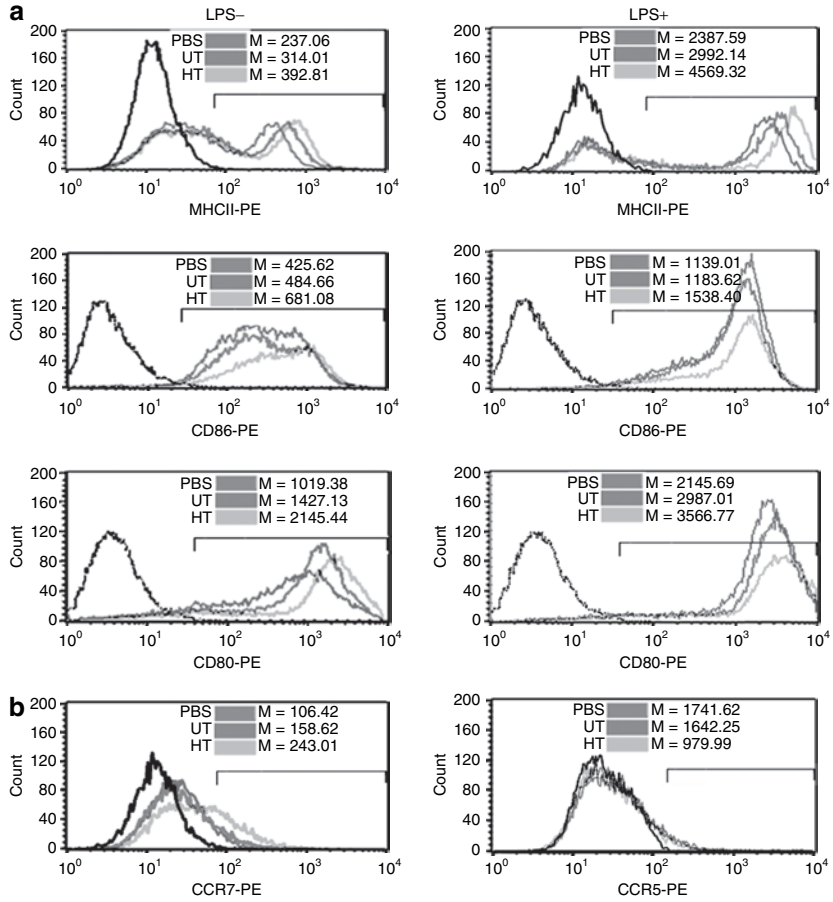

Figure 2 DC phenotype after coculture with heated tumor cell lysate. Bone marrow-derived immature DCs were loaded with heated or unheated B16F10-luc cell lysates. Their phenotypes were then assessed by FACS with or without overnight maturation by lipopolysaccharide (100 ng/ml). (**a**) FACS analysis using PE-conjugated mAbs against MHC class II, CD80, CD86 shows that heat-shocked DCs express higher levels of MHC and costimulatory molecules. The figure shows the mean fluorescence intensity value (M). Data are representative of three independent experiments with similar results. (**b**) FACS analysis for cell surface expression of CCR5 or CCR7 also shows the increase in DCs maturation. Experiments were repeated three times with similar results. DC, dendritic cell; FACS, fluorescence-activated cell sorting.

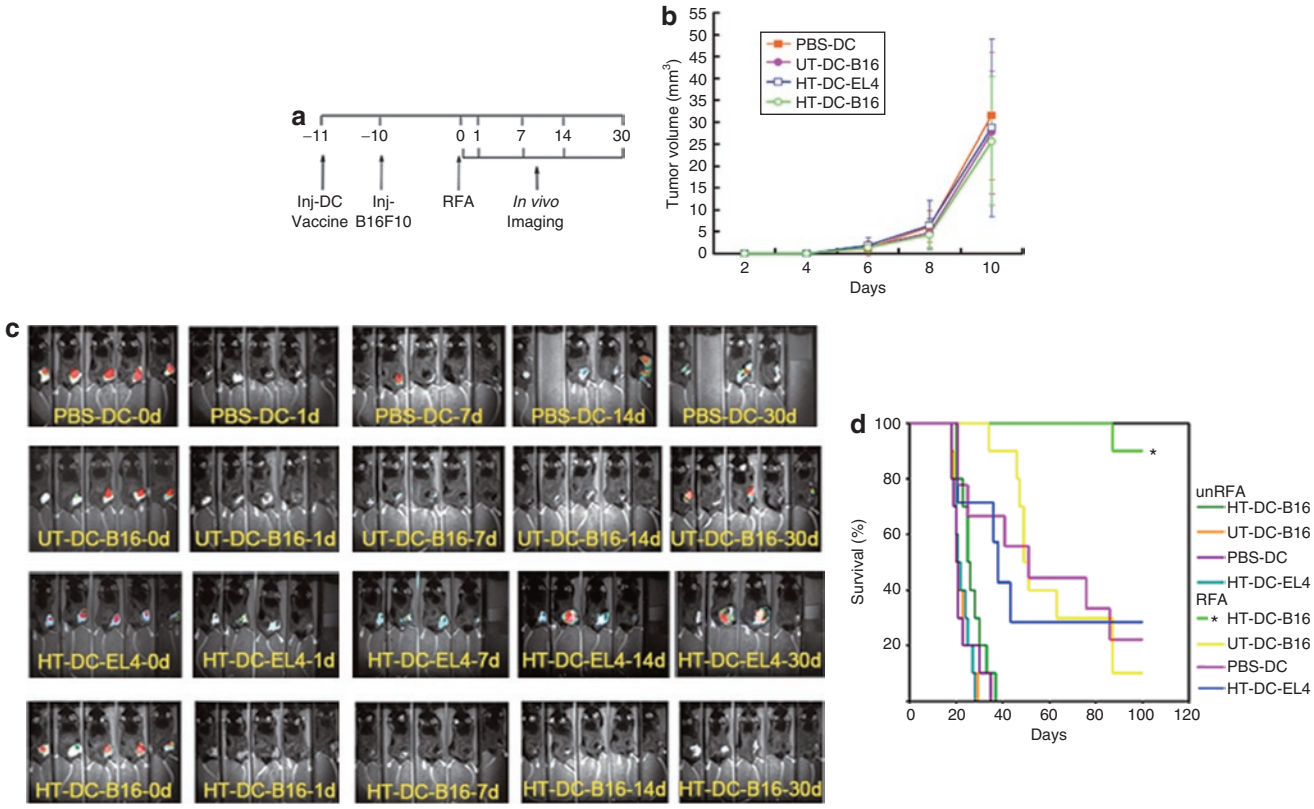

Figure 3 Effect of combined HT-DC + RFA on the recurrence of B16F10-luc tumor. (**a**) Time schedule outlining the different treatments as used in the experiments. (**b**) HT-DC or UT-DC was given 4 days before B16F10-luc inoculation (1×10^5). Mean tumor volumes of each group before RFA treatment are shown in the graph ($n = 15$ per group). There is no evident effect of vaccination on the outgrowth of the tumors prior to RFA. (**c**) Bioluminescence imaging of different groups of mice bearing B16F10-luc tumor on day 0,1,7,14, and 30 after RFA treatment. Experiments were repeated three times with similar results. (**d**) The overall survival of the vaccinated mice with or without subsequent RFA treatment, were formulated using Kaplan–Meier method ($n = 10$, $P < 0.01$ versus other groups). HT-DC-B16, heated B16F10-luc tumor lysate-pulsed DCs; UT-DC-B16, unheated B16F10-luc tumor lysate-pulsed DCs; HT-DC-EL-4, heated EL4 tumor lysate-pulsed DCs; PBS-DC, mock-pulsed DC. DC, dendritic cell; HT-DC, heat-shocked tumor cell lysate-pulsed dendritic cell; UT-DC, unheated tumor cell lysate-pulsed dendritic cell; RFA, radiofrequency ablation.

equal size (4–5 mm) were subjected to RFA using two consecutive treatment cycles of 60 seconds with the power limited to 5 W and temperature limited to 85 °C, together covering the whole tumor area (6 mm in diameter). The tumor ablation and recurrence were monitored by whole-body bioluminescence imaging (**Figure 3c**). Remarkably, the local cancer recurrence after RFA was abrogated in ≥90% of the mice, which received HT-DC priming and remained tumor free until the end of the experiment, whereas the tumors regrew rapidly following treatment with the combination of UT-DC vaccination or PBS with RFA (**Figure 3c,d**). Of note, neither HT-DC nor UT-DC vaccination alone could prolong mice survival (**Figure 3d**). As it is not known whether the protective effects of HT-DC require the presentation of specific antigens from certain tumor cells, a group of mice that received DCs pulsed with irrespective heat-shocked EL4 tumor cells was included. However, no protection was observed against relapse of B16F10-luc melanoma in this group, thus showing that the activation was tumor antigens specific and not solely due to a nonspecific activation of DCs by hyperthermic tumor lysates (**Figure 3c,d**). Collectively, our data clearly indicate that prevaccination with HT-DC vaccine elicits highly efficient tumor-specific immunity in the setting of regional hyperthermia cancer therapy. To draw our approach near the clinical situation, we modified the treatment protocol and performed DC vaccination 3 days after tumor implant followed by RFA treatment 7 days later. Interestingly, similar preventive effects of HT-DC on tumor recurrence were observed (**Supplementary Figure S1**), suggesting that DC vaccination after tumor inoculation was still able to protect mice from tumor relapse.

Combination treatment with HT-DC and RFA elicits tumor-specific T cell responses

To determine whether treatment of B16F10-luc-bearing mice with HT-DC and RFA could elicit tumor-specific T cell responses, 3.5×10^7 splenocytes from combinatorially treated mice were transferred to naive mice 1 day before tumor cell inoculation and the survival time of tumor-bearing mice was monitored for 30 days. Interestingly, transfer of splenocytes of HT-DC + RFA-treated mice resulted in delayed tumor outgrowth and improved survival after a lethal B16F10-luc challenge. However, no tumor growth delay was observed after transfer of splenocytes from either UT-DC + RFA or PBS + RFA control groups (**Figure 4a**). Subsequently, we evaluated the tumor-specific cytolytic activity of lymphocytes from each group. As shown in **Figure 4b**, $CD8^+$ T lymphocytes from the spleens of mice that received HT-DC + RFA treatment displayed significantly stronger cytolytic activity against B16F10-luc cells than that in control groups (**Figure 4b**).

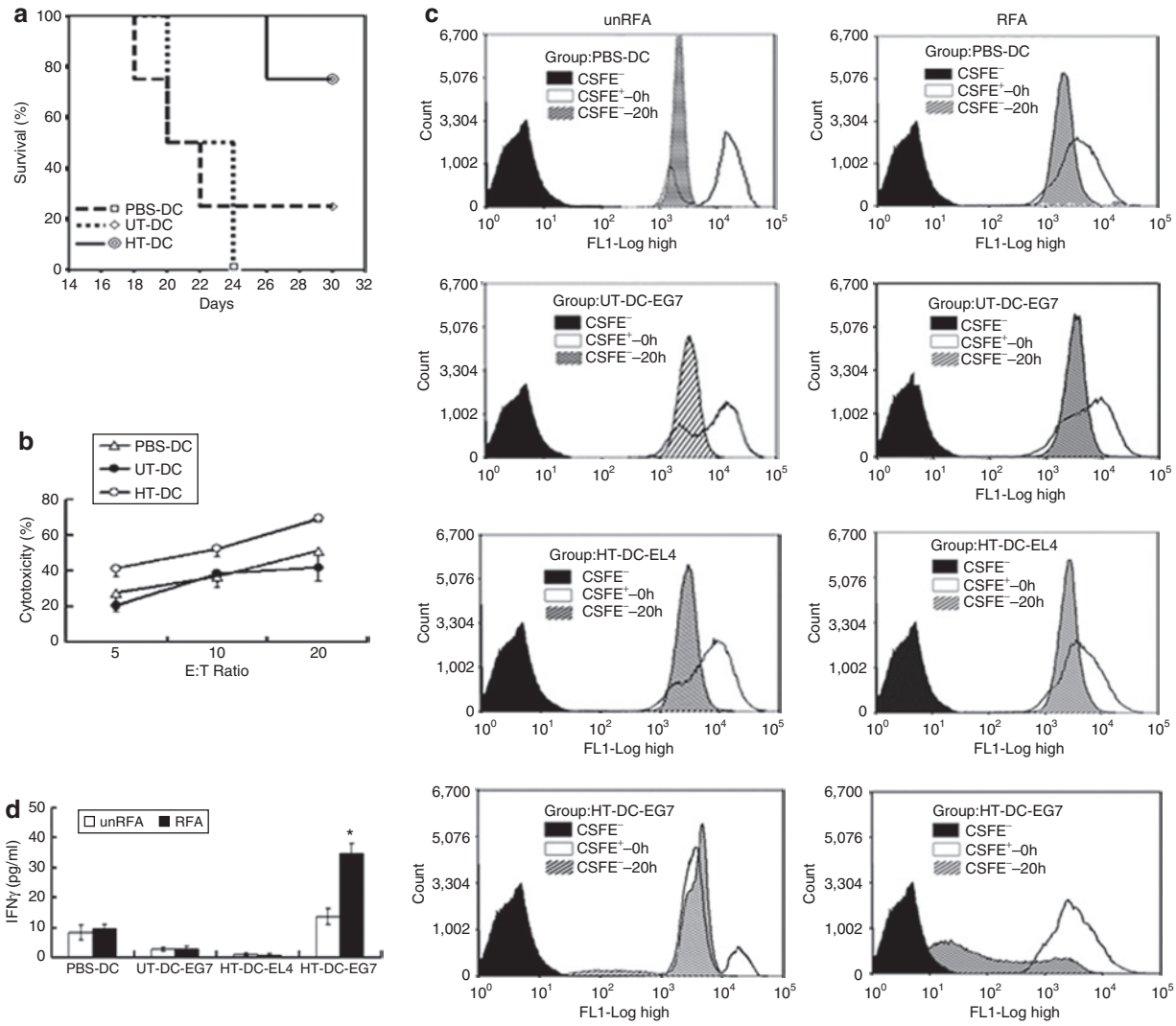

Figure 4 Tumor-specific T cell responses induced by the combination treatment. (**a**) Adoptive transfer of immune reactivity. Fourteen days after RFA, 3.5×10^7 splenocytes from the combinatorially treated mice were transferred to naive mice 1 day before B16F10-luc tumor cell inoculation (1×10^5) and the survival time of tumor-bearing mice were monitored for 30 days. Shown are the pooled results of two independent experiments ($n = 6$). (**b**) Cytotoxic activity of $CD8^+$ T cells after the combination therapy. $CD8^+$ T cells derived from combinatorially treated mice were used as effector cells. B16F10-luc was used as target cells. Effector cell:target cell (E:T) ratios ranged from 20:1 to 5:1. Data represent means ± SE ($n = 3$) from one of three independent experiment. **c** and **d**, mice were injected subcutaneously with 0.5×10^6 EG7 cells and received DC vaccines alone (heat-shocked tumor cell lysate-pulsed dendritic cell-EG7 or unheated tumor cell lysate-pulsed dendritic cell-EG7) or followed by RFA treatment. (**c**) Purified $CD8^+$ T cells from the draining lymph nodes were labeled with carboxyfluorescein diacetate succinimidyl ester *ex vivo*, plated at 10^6 cells/well in 24-well plates in the presence of OVA-pulsed DCs and analyzed by flow cytometry. Experiments were repeated two times with similar results. (**d**) Interferon-γ levels were measured in duplicate using enzyme-linked immunosorbent assay in supernatants collected from cultures of purified $CD8^+$ T cells in C. *$P < 0.01$. RFA, radiofrequency ablation.

These findings indicate that the observed immune response is mainly T cell mediated and depends on the DC vaccine type applied before RFA.

We then asked whether the observed antitumor immunity was due to enhanced priming of antigen-specific cytotoxic T lymphocytes (CTLs) by HT-DC vaccine. To test this postulation, OVA-expressing EL-4 (EG-7) tumor cells were used to replace B16F10-luc in the combination regimens. Two weeks later, $CD8^+$ T cells of RFA-treated or untreated mice were isolated from the draining lymph nodes of each experimental group, labeled with carboxyfluorescein diacetate succinimidyl ester and analyzed for OVA-specific T cell proliferation *ex vivo*. We found that after being restimulated with OVA peptide *in vitro*, $CD8^+$ T cells from mice that prevaccinated by HT-DC proliferated much faster and produced much higher IFN-γ than those derived from UT-DC vaccinated or PBS control mice (**Figure 4c,d**). Interestingly, prevaccination with HT-DC alone was able to elicit a weak but specific $CD8^+$ T cell response as evidenced by the increase of IFN-γ production and carboxyfluorescein diacetate succinimidyl ester dye dilution (**Figure 4c,d**), but this effect was markedly enhanced by *in situ* tumor ablation. These results clearly indicate that the prime-boost regimen utilizing HT-DC priming and RFA boost indeed evoked highly efficient tumor-specific CTL responses.

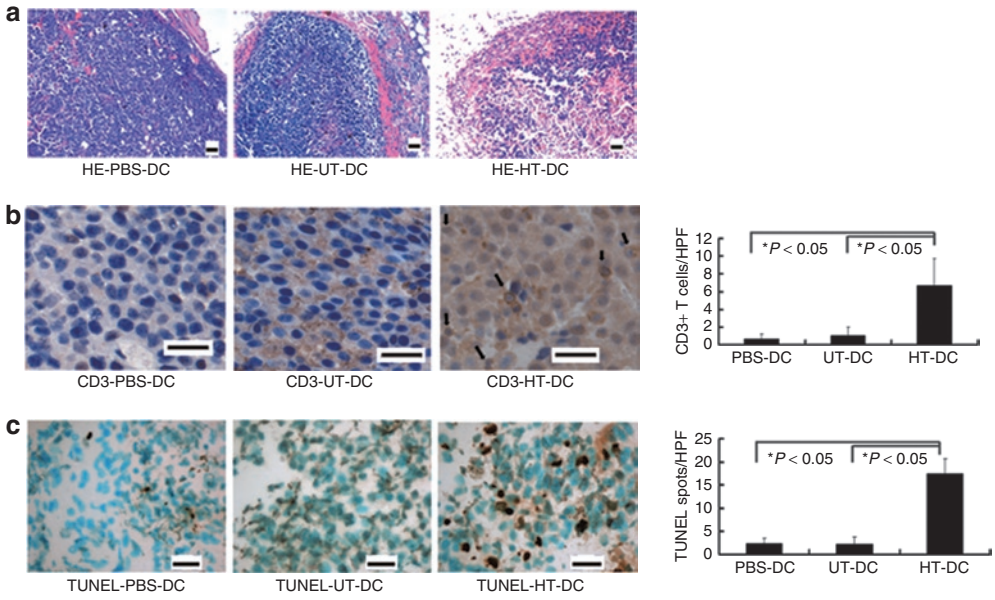

Figure 5 HT-DC vaccination stimulates lymphocytes infiltration and tumor killing after RFA. (**a**) Tumors were excised 3 day after the combination treatment, hematoxylin and eosin staining shows marked increase of necrosis and inflammatory infiltration in B16F10-luc tumor treated with HT-DC + RFA. (**b**) Immunostaining for CD3 in tumor implants with combination treatment of different groups. (**c**) TdT-mediated dUTP nick end labeling staining shows extensive cell apoptosis or necrosis in HT-DC + RFA-treated tumor. Bar = 50 μm. Histological quantification of tumor-infiltrating T cells and apoptotic cells are shown in the right panel. *$P < 0.05$. HT-DC, heat-shocked tumor cell lysate-pulsed dendritic cell; RFA, radiofrequency ablation.

HT-DC vaccination stimulates lymphocytes infiltration and tumor killing *in vivo*

To determine the cytotoxicity of DC vaccine primed lymphocytes *in vivo*, the C57BL/6 mice were prevaccinated with HT-DC, UT-DC, or PBS, inoculated subcutaneously with B16F10-luc and treated by RFA with lower output and shorter ablation time to preserve enough tumor tissue for examination. Hematoxylin and eosin staining showed much more pronounced tumor necrosis and inflammatory infiltration in HT-DC + RFA group (**Figure 5a**). Furthermore, many CD3 positive T-lymphocytes were observed in the center of the tumor of HT-DC primed mice. By contrast, within the treated tumors of the control groups, nearly no CD3-positive lymphocytes could be found (**Figure 5b**). Concordantly, the increased infiltrating lymphocytes in HT-DC vaccinated mice were accompanied by a marked tumor apoptosis as observed by TdT-mediated dUTP nick end labeling staining (**Figure 5c**). Histological quantification of tumor-infiltrating T cells and apoptotic cells confirmed the remarkable differences between HT-DC + RFA group and control groups (**Figure 5b,c**, $P < 0.05$). These results suggest that preimmunization with HT-DC stimulated T cell motility and enhanced its cytotoxicity against remnant tumor cells after RFA.

Identification of the T-cell subset responsible for tumor-specific T-cell responses

To further evaluate the role of CTLs in HT-DC + RFA-induced tumor protection, naive mice were inoculated with B16F10-luc melanoma cells and allowed to grow for 10 days when tumors reached an average diameter of 5 mm. Splenocytes from HT-DC + RFA-treated mice or from control groups were then transferred to these mice 1 day before RFA. Splenocytes from naive mice were used as negative controls (blank group). As expected, most of mice receiving splenocytes from the HT-DC + RFA-treated mice, but not from other mice, were efficiently protected from tumor recurrence (**Figure 6a**). To determine the role of $CD4^+$ and $CD8^+$ T cells in tumor protection, we repeated this experiment using splenocytes depleted of $CD4^+$ or $CD8^+$ T cells, or purified $CD4^+$ or $CD8^+$ T cells from HT-DC + RFA–treated mice. One day later, these tumor-bearing mice were treated with RFA and tumor recurrence was monitored. As shown in **Figure 6b**, whereas $CD4^+$ T exerted little effect on tumor protection, $CD8^+$ T cells mediated most of the effect. Interestingly, mice transferred with $CD4^+$-depleted T cells exhibited lower recurrence rate than those with CD4 enriched T cells. These results are consistent with the concept that the net impact of open repertoire $CD4^+$ T cells was negative in the anti-tumor setting, which was possibly due to the presence of $CD4^+$ regulatory T cells (Treg).

DISCUSSION

The pathological interactions between cancer cells and host immune cells in the tumor microenvironment create an immunosuppressive network that prevent enough presentation of tumor-associated antigens, dampen stimulatory capacity of antigen-presenting cells and inhibit cytotoxic activity of effector T cells or effector cytokines.[20] RFA is a minimally invasive therapy for local tumor destruction that generates large amounts of tumor debris,[21] which is a potential antigen source for the induction of antitumor immunity. On the other hand, RFA induces hyperthermia within the tumor lesion, which leads to the transcriptional activation of a series of molecular chaperones such as HSP70 and gp96 (**Figure 1**) that may form complexes with antigenic peptides, enhance their antigenicity and direct them to MHC class I and II pathways for presentation to T cells.[22–24] In addition, RFA treatment may result in the generation of thermally altered tumor

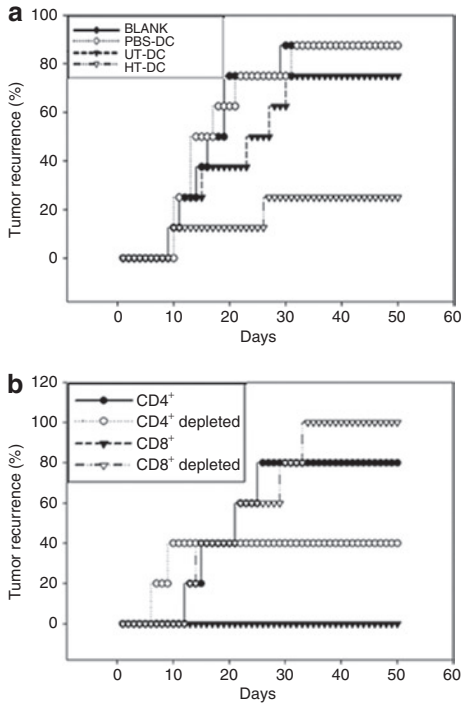

Figure 6 ***In vivo*** **characterization of the antitumor immune responses induced by HT-DC + RFA.** (**a**) Naive mice were inoculated with 1 × 10^5 B16F10-luc melanoma cells for 10 days. Splenocytes (1 × 10^7) from HT-DC + RFA-treated mice or from control groups were then transferred to these mice 1 day before RFA and tumor recurrence were monitored for 50 day. Shown are the pooled results of two independent experiments (n = 8). Splenocytes from naive mice served as a control group (blank). (**b**) Role of CD4 and CD8 T-cell subsets in protection against tumor recurrence after HT-DC + RFA treatment. Splenocytes were harvested from tumor-free mice treated with HT-DC + RFA. After magnetic selection, 1 × 10^7 $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+$ T-cell-depleted, $CD8^+$ T-cell-depleted splenocytes were infused intravenously into tumor-bearing mice. One day later, these mice were subjected to RFA treatment and then monitored for tumor recurrence for 50 day (n = 5). HT-DC, heat-shocked tumor cell lysate-pulsed dendritic cell; RFA, radiofrequency ablation.

antigens that are displayed in the vicinity of the residual tumor cells and a small number of normal cells but not in the most of the peripheral normal cells. The appearance of these immunologically hidden tumor antigens that are complexed with HSPs can therefore help lower the threshold for tumor antigenicity, disrupt the tolerance of the immune system and eventually eliminate the residual unkilled tumor cells after RFA treatment.

However, it is apparent that the host's antitumor immune responses, even when tumors are necrotic, are usually very weak.[20] RFA-induced HSPs and heat-dependent tumor antigen expressions are transient and will soon weaken due to rapid protein turnover, thus reducing the antigenicity of cancer cells. Therefore, a prime-boost vaccination with heated tumor lysates mimicking RFA effects *in vivo* is expected to improve anticancer immune responses. Here we show that mice immunized with HT-DC either before or after tumor inoculation were efficiently protected against local cancer recurrence after RFA, whereas preimmunization with mature DCs pulsed with unheated tumor lysates failed to augment efficacious adaptive immunity against cancer and enjoyed no protection from tumor recurrence following RFA. These effects are likely attributed to following reasons. First, heat-induced HSPs-peptide complexes provide both danger signals and antigenic peptides to DCs. Damage associated molecular patterns released from dying cells, including HMGB1 and HSPs, can act on multiple pattern recognition receptors and synergize with lipopolysaccharide in the activation and maturation of DCs *ex vivo*[20] (**Figure 2**). Second, HT-DC immunization may yield a T-cell repertoire that is highly skewed toward tumor recognition in the circumstance of RFA treatment because most of remnant tumor cells will be under heat shock stress and display tumor specific antigenic peptides in complex with HSPs. Thus, necrotic antigen exposure in the context of "danger" signals may make the residual heated tumor cells more vulnerable to CTL-mediated killing. Third, RFA-induced *in situ* "danger" environment at the tumor site might promote the homing of effector lymphocytes to the tumor and induce sufficient arousal of fully functional effector T cells.[20] Although regional antigen overload may favor tolerance induction, the inflammatory "danger" environment induced by hyperthermic treatment may help break the tolerance and tilt the balance towards immune activation. Indeed, incorporation of danger signals as a strategy to boost tumor immunity has been suggested.[25,26] Recent studies proposed HSPs and HMGB1 as dominant danger signals for the host's cellular immune system even in the absence of immunogenic peptides.[27,28] We hypothesize that HT-DC vaccine-primed memory T cells and RFA-boosted effector T cells might play central roles in the control of tumor recurrence. T lymphocytes can be found infiltrating human tumors and have been associated with both an improved or poorer prognosis. For example, it was shown that a high density of $CD8^+$ effector memory T cells in colorectal cancers was associated with increased survival. Similarly, an inverse correlation between the expression of IFN-γ and tumor recurrence was observed, suggesting that an upregulation of genes related to Th1 adaptive immune response was associated with a decreased risk of tumor relapse.[29] On the contrary, the presence of $CD4^+$ Treg cells, which is thought to inhibit the activities of tumor specific $CD8^+$ T lymphocytes, was reported to be correlated with a poor prognosis in human breast cancer and hepatocellular carcinoma.[30,31] Here we found that the bulk population of unseparated $CD4^+$ T cells was detrimental to tumor treatment whereas $CD4^+$-depleted T cells confer protection against tumor recurrence, indicating that a distinct subset of $CD4^+$ T cells, *e.g.*, Treg, may carry out specialized immunoregulatory functions to inhibit immune responses. However, as $CD4^+$ T-cell responses are an important element of effective vaccine for cancer, concurrent depletion of conventional $CD4^+$ Th cells may result in the decreased protection. It is conceivable that selective elimination of Treg cells might enhance the effect of this experimental regimen.

RFA has become the standard of care for liver cancer. To improve the survival in patients treated with RFA, the combination with several other approaches aimed at boosting antitumor immunization have been explored. For example, a phase I clinical trial combining intratumoral DC immunotherapy with RFA is currently underway.[6] Another attempt to improve antitumor immunization is blocking T cell-autonomous inhibitory circuit or regulatory T cells by monoclonal antibodies against CTLA-4 or CD25 in mice undergoing RFA.[13,21] Together with our results,

these data suggest that RFA might be viewed as immunosupportive therapy that have the potential to unmask tumor antigens and turn the tumor itself into a form of polyvalent *in vivo* cellular vaccine, and that tumor-specific T cell priming with or without additional immune checkpoint blockade might provide the immunological adjuvant necessary to realize a true therapeutic impact.

In addition to RFA, conventional chemotherapy- and radiotherapy-based cancer treatments can also elicit specific cellular responses that render tumor-cell death immunogenic. Interestingly, vaccination against cancer-specific antigens can sensitize the tumor to subsequent chemotherapeutic treatment.[32] However, such combinations, whereas potentially effective, might also be fraught with significant toxicity due to immunosuppressive side effects of massive chemotherapy.[33] Combining *in situ* tumor destruction using RFA with HT-DC preimmunization may represent a relatively safer way of *in situ* immune response induction and may prove beneficial in the treatment of patients with a high risk of relapse.

MATERIALS AND METHODS

Cell lines and animal models. The luciferase-expressing B16F10 (B16F10-luc) melanoma cells, T-lymphoid EL4 cells and OVA-expressing EL4 (EG7) cells were grown in Roswell Park Memorial Institute 1640 medium (Gibco BRL, Gaithersburg, MD), containing 10% fetal bovine serum. Male C57BL/6 mice (age, 6–8 weeks) were purchased from Shanghai Experimental Center (Chinese Science Academy) and maintained in the barrier facility under pathogen-free conditions. The mice were injected subcutaneously B16F10-luc cells on the right flank and then subjected to RFA treatment 10 days later when tumor attained a size of 4–5 mm in diameter. For bioluminescence imaging, B16F10-luc tumor-bearing mice were injected with luciferin substrate at a dose of 0.15 mg/g mouse body weight by intraperitoneal injection. Ten minutes after substrate injection, mice were anesthetized and imaged using the Roper Imaging System luminescence-sensitive CCD camera (Roper Scientific, Tucson, AZ). Data were collected and analyzed using Slidebook software (version 4.1, Denver, CO). All of the experiments were performed according to "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23 revised 1985).

Preparation of tumor antigens and DC vaccines. B16F10-luc melanoma cells in the logarithmic growth phase were heated at 37, 42, or 43 °C for 30 minutes, washed in PBS and subjected to five freeze (liquid nitrogen) and thaw (37 °C water bath) cycles to obtain crude lysates. DCs were generated from bone marrow as described previously.[34] After 5 days of culture in GM-CSF, immature DC were collected and replaced in serum-free Roswell Park Memorial Institute 1640. Prepared tumor antigens were added at 100 μg/10^6 DCs in 1.5 ml and incubated overnight at 37 °C in a 5% CO_2 atmosphere.

RFA. Animals were anesthetized by isoflurane inhalation and properly shaved at the tumor (white patches shown in **Figure 3b**) and on the ventral area. After placement and proper attachment of the ventral side onto an electricity-conducting pad (grounding pad), the tumor area was disinfected with alcohol. An RFA needle with active tip of 8 mm (MSI SA Electrodes, MedSphere International, Shanghai, China) was inserted subcutaneously and placed in the middle of the tumor. After placement of the RFA needle, impedance could be evaluated on the radiofrequency lesion generator system (Model S-500L RF Generator, MedSphere International, Shanghai, CN). Treatment then was started by delivering RFA energy. During two treatment cycles of 60 seconds, temperature could be monitored using a thermistor and thermocouple in the tip of the probe. Treatment was considered successful if a tip temperature of 80–85 °C could be reached. To evaluate histologically the immunological reactions after RFA treatment, paraffin-embedded samples were immunohistochemically examined for the expression and localization of HSP70, gp96 (Neomarker, Cheshire, UK) or HMGB1 (Abcam, Cambridge, MA), and for specific infiltrations of T-lymphocytes with mouse CD3 mAb (Abcam, Cambridge, MA).

Assay for CTL activity. Cytotoxic activity was determined by lactate dehydrogenase release assay (Promega, Madison, WI). $CD8^+$ lymphocytes derived from the spleens of mice treated by combination therapy at 14 days were isolated and used as effector cells. Target cells (B16F10-luc, 2×10^4 cells/ml) in a volume of 50 μl were placed in wells of a 96 well V bottom plate, then 50 μl of effector cells, at various concentrations, were added to each assay wells. Plates were centrifuged for 5 minutes at 250 g, incubated in a 37 °C 5% CO_2 atmosphere for 4 hours, and supernatants harvested and tested according to manufacturer's instructions. Percent cytotoxicity was calculated as follows: % cytotoxicity = [(experimental − culture medium background)/(maximum lactate dehydrogenase release − culture medium background)] × 100%.

Assay for $CD8^+$ T cells proliferation and IFN-γ production. Mice were injected subcutaneously with 0.5×10^6 EG7 cells, and then received combination treatment. Fourteen days later, the draining lymph nodes were removed and single-cell suspensions were obtained by crushing and passing through nylon mesh. $CD8^+$ T cells were collected by positive selection using the $CD8^+$ T cell isolation kit (Stemcell Technologies, Vancouver, Canada). A total of purified $CD8^+$ T cells were labeled with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR; 1 μmol/l) *ex vivo*. Carboxyfluorescein diacetate succinimidyl ester-labeled $CD8^+$ T cells were then plated at 10^6 cells/well in 24-well plates in Roswell Park Memorial Institute 1640 medium, containing 10% fetal bovine serum and OVA peptide-pulsed DCs. Before culture and after cultured for 24 hours, cells were collected and analyzed by flow cytometry and the supernatants were also collected for IFN-γ detection by enzyme-linked immunosorbent assay (eBioscience, San Diego, CA). Data were analyzed for statistical significance by Student's *t* test.

Adoptive cell transfer. For adoptive transfer experiments, combinatorially treated mice were used as donors, from which splenocytes were isolated. Lymphocytes were concentrated by density gradient centrifugation. Recipient naive mice then received 3.5×10^7 lymphocytes intravenously These mice were challenged with 2×10^4 B16F10-luc cells 1 day later. Tumor sizes were measured every 2 days. For identification of the T-cell subset responsible for tumor-specific T-cell responses, naive mice were inoculated with B16F10-luc melanoma cells for 10 days before transfer. When transfer, $CD4^+$ and $CD8^+$ T cells from donors were isolated or depleted using magnetic bead cell sorting kit (Stemcell Technologies, Vancouver, Canada), 1×10^7 specifically purified or depleted T cells were then transferred intravenously into naive tumor-bearing C57BL/6 mice. One day after transfers, recipient naive mice accepted treatment of RFA. Tumor sizes were measured every 2 days.

Western blotting and flow cytometry analysis. Samples of heated or unheated tumor lysates were run on 10% SDS-PAGE gels. HSP was detected using the anti-HSP70 and anti-gp96 mAb (Neomarker, Fremont, CA), followed by anti-mouse HRP (Santa Cruz, CA). Specific bands were developed using ECL (Amersham Biosciences, Piscataway, NJ). For flow cytometric analysis, DCs (2×10^5 cells) were suspended in PBS containing 1% BSA and were stained with various fluorochrome-conjugated monoclonal antibodies (mAbs) for 20 min on ice. The following anti-mouse mAbs and isotype-matched controls were used: anti-MHC class II (IAIE), anti-CD80, anti-CD86, anti-CCR7 and anti-CCR5 (eBioscience, San Diego, CA). Flow cytometric analysis was performed using Moflo-XDP (Beckman Coulter, Fullerton, CA) equipped with Summit 5.1 Software.

Statistical analysis. Data are presented as mean ± SE. Statistical analysis was done using Student's *t*-test. Statistical significance was determined at

the < 0.05 level. Survival estimates were determined using the method of Kaplan and Meier.

SUPPLEMENTARY MATERIAL

Figure S1. HT-DC vaccination after tumor inoculation protected mice from tumor relapse after RFA.

ACKNOWLEDGMENTS

We thank Chuan-Gui Wang (Ph.D., East China Normal University Institute of Biomedical Science), Lin-Na Guo, Dan Cao, Dong-Ping Hu and Wei-Wei Zhang for their helpful technical assistances. We also thank Wei-Zhong Tang for kindly providing apparatus for RFA. This work was supported by National Natural Science Foundation of China (30871293, 30921006, and 30620130434), Chinese National Programs for High Technology Research and Development (2007AA02Z166, 2006AA02A310), Chinese National Key Project (2008ZX10002-020), Shanghai Venus and Dawn Scheme for Young Scholars grant 07QA14070, 07SG45, and 06DZ22025. There is no conflict of interest to disclose.

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