Original Article

Protective antitumor immunity induced by tumor cell lysates conjugated with diphtheria toxin and adjuvant epitope in mouse breast tumor models

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Abstract

Cancer cell vaccine-based immunotherapy has received increasing interest in many clinical trials involving patients with breast cancer. Combining with appropriate adjuvants can enhance the weak immunogenic properties of tumor cell lysates (TCL). In this study, diphtheria toxin (DT) and two tandem repeats of mycobacterial heat shock protein 70 (mHSP70) fragment 407-426 (M_2) were conjugated to TCL with glutaraldehyde, and the constructed cancer cell vaccine was named DT-TCL-M_2. Subcutaneous injection of DT-TCL-M_2 in mice effectively elicited tumor-specific polyclonal immune responses, including humoral and cellular immune responses. High levels of antibodies against TCL were detected in the serum of immunized mice with ELISA and verified with Western blot analyses. The splenocytes from immunized mice showed potent cytotoxicity on Ehrlich ascites carcinoma cells. Moreover, the protective antitumor immunity induced by DT-TCL-M_2 inhibited tumor growth in a mouse breast tumor model. DT-TCL-M_2 also attenuated tumor-induced angiogenesis and slowed tumor growth in a mouse intradermal tumor model. These findings demonstrate that TCL conjugated with appropriate adjuvants induced effective antitumor immunity *in vivo*. Improvements in potency could further make cancer cell vaccines a useful and safe method for preventing cancer recurrence after resection.

Key words Cancer immunotherapy, tumor cell lysates, cancer cell vaccine, breast cancer, adjuvant epitope

Breast cancer accounts for an estimated 23% (1.38 million) of all new female cancer cases and 14% (4.58 million) of total female cancer deaths, making it the most commonly diagnosed malignancy and leading cause of cancer death in women in the world[1]. Surgical resection, chemotherapy, radiotherapy, and hormone therapy are considered effective treatments for breast cancer. However, nonsurgical approaches are generally not selective and kill both normal and cancer cells, which causes side effects that limit treatment. In addition, women with advanced breast cancer often develop metastatic disease and are refractory to conventional treatments. At present, metastatic breast cancer is incurable, and the main goals of treatment are to prolong life and reduce symptoms to maintain quality of life. Therefore, it is critical to develop novel strategies to prevent breast tumor recurrence and improve the survival and quality of life for patients with advanced or metastatic breast cancer.

Immunotherapy for breast cancer is a highly attractive alternative approach because it induces a highly specific immune response. Cancer vaccines can activate the immune system and induce antitumor immune responses in patients with primary tumors and metastases [2,3]. More importantly, the strong immune response induced by cancer vaccines is expected to

elicit long-lasting immune memory, thereby protecting against minimal residual disease and preventing tumor recurrence.

Breast cancer antigen-specific vaccines use proteins and peptides that are expressed on normal tissues but overexpressed or mutated on tumor cells as immunogens, including mucin (MUC-1), human epidermal growth factor receptor (HER-2), carcinoembryonic antigen (CEA), human telomerase reverse transcriptase (hTERT), p53, and carbohydrate antigens [4,5]. Results from clinical trials demonstrated that vaccines targeting these immunogens are safe, showing low toxicity. However, in phase I/II trials, these vaccines used alone have not induced significant objective responses in patients with breast cancer[6].

To improve this limitation, a promising approach is the use of whole tumor cell vaccines in breast cancer. There are many reports of various preparations of whole tumor cell vaccines, including irradiated tumor cells [7], formalin-fixed cells [8], ethanol-fixed cells [9], glutaraldehyde-fixed cells[10], and tumor cell lysates (TCL) [11,12]. Unfortunately, tumor cells that lack significant levels of major histocompatibility complex proteins and other co-stimmulatory factors are poorly immunogenic and often induce tolerance. Interestingly, combination with immunomodulating adjuvants, such as bacillus Calmette-Guerin (BCG) [13], OK432 [11], and CpG oligodeoxynucleotides (ODN)[7], can enhance the immunogenicity of tumor cells. Tumor cell vaccines supplemented with cytokines, including granulocyte macrophage colony-stimulating factor (GM-CSF) [14], interleukin-12 (IL-12) [15], interleukin-2 (IL-2) [16], and tumor necrosis factor-α (TNF-α) [9], can augment the immune recognition and responsiveness of the host. Genetic modification of tumor cells to express co-stimulatory molecules, including OX40 ligand[17] and B7-1 (CD80)[18], can trigger an immune response to develop antitumor immunity. Dendritic cells (DCs) pulsed with tumor cells elicited T-cell-mediated immune responses against breast cancer *in vitro*[19,20]. However, there have been no clinical reports of cases in which cancer cell vaccines combined with adjuvants have induced impressive protection against cancer. Therefore, further studies are warranted to identify novel and potent adjuvants for cancer vaccines.

TCL, with multiple known and unknown antigens, was used as an immunogen to mount tumor-specific polyclonal immune responses [21]. To break the tolerance against TCL, carrier protein diphtheria toxin (DT) was conjugated to TCL, serving as vector for delivering B-cell and T-cell epitopes[22]. Furthermore, to boost immunogenicity and elicit strong immune responses, mycobacterial heat shock protein 70 (mHSP70) fragment 407-426 (M_2) was introduced as an immunoadjuvant[23,24].

In the present study, a breast cancer vaccine was designed to generate antitumor effects by inducing tumor-specific immune responses. We sought to determine if the vaccine could enhance the immunogenicity of TCL and induce tumor-specific polyclonal immune responses. Furthermore, we investigated the ability of the vaccine to inhibit the growth of breast tumors and tumor-induced angiogenesis in mouse models.

Materials and Methods

Tumor cell line and mice

The Ehrlich ascites carcinoma (EAC) cell line was kindly provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The cells were maintained *in vivo* in female Swiss albino mice by weekly intraperitoneal injection of 0.3 mL freshly drawn ascites fluid (diluted at 1:3 in sterile saline). After three passages *in vivo*, EAC cells were used for the study.

Female Swiss albino mice (strain ICR, SCXK-Jiangsu2007-0001), 4–5 weeks of age, were purchased from Jiangsu Experimental Animal Center (Yangzhou, Jiangsu Province, China). They were provided with standard pellet diet and tap water *ad libitum* under hygienic conditions. All experiments involving mice were performed in strict compliance with guidelines from the China Pharmaceutical University Ethics Committee.

Peptide synthesis

The two tandem repeats of fragment M_2 were synthesized by GL Biochemical cooperation (Shanghai, China). The sequence of M_2 is QPSVQIQVYQGEREIAA HNKGSSQPSVQIQVYQGEREIAAHNK.

Preparation of the conjugated tumor cell lysate vaccine

EAC cells were collected from the peritoneal cavity of tumor-bearing mice, washed in sterile saline three times and then suspended in normal saline to a concentration of 1×10^8 cells/mL. Cells were lysed by five cycles of 30-minute freezing in liquid nitrogen and 10-minute thawing at 37℃.

DT was obtained from Shanghai Institute of Biological Products. The conjugation method with glutaraldehyde (GA) was similar to one previously described[25]. Briefly, DT (1.2 mg in 2.4 mL), M_2 (1.2 mg in 1.2 mL), and normal saline (388 µL) were mixed with EAC TCLs (2×10^8 cells in 2 mL), followed by addition of 12 µL of 25% GA. The final concentration of GA was adjusted to 0.2% (*v/v*). Conjugation between EAC TCL, DT, and M_2 was

slowly performed in small rotating columns. After stirring for 2 h at room temperature, the reaction was stopped using glycine (1 mol/L) and dialyzed against phosphate buffer saline (PBS, pH 7.4) overnight. The resultant vaccine was named DT-TCL-M_2. Similarly, DT (1.2 mg in 2.4 mL), M_2 (1.2 mg in 1.2 mL), or normal saline were conjugated to EAC TCLs separately using the same protocol, and the resultant vaccines were named TCL-DT, TCL-M_2, and TCL-NS, respectively. Mice were immunized with 100 μL DT-TCL-M_2 vaccine, which contained 3.3×10^6 TCLs, 20 μg DT, and 20 μg M_2.

Immunization procedure and tumor model

Swiss albino mice were randomly divided into 5 groups of 9 mice each: TCL (control group), TCL-NS, TCL-DT, TCL-M_2, and DT-TCL-M_2. Mice were immunized subcutaneously at the right inguinal lymph node area once weekly for 3 continuous weeks with each vaccine. Two weeks after the last immunization, 1×10^6 EAC cells were subcutaneously injected into the left flank of all mice[26]. One week later, tumor growth was evaluated using calipers to measure in two dimensions every two days. Tumor volume was calculated using the formula $V = 0.52ab^2$, with "a" as the longest diameter and "b" as the shortest diameter. In each group, 3 mice were killed for cytotoxic T lymphocyte (CTL) killing assay 1 week after the last immunization, and 6 were killed for tumor weight analysis 4 weeks after the last immunization.

To establish an intradermal tumor model, 5 groups of mice (3 in each group) were vaccinated with TCL, TCL-NS, TCL-DT, TCL-M_2, or DT-TCL-M_2 three times at weekly intervals. Mice were then inoculated intradermally with 5×10^5 EAC cells in 50 μL of normal saline at two sites bilaterally on the lower ventral side (after shaving this area). One week later, all mice were killed for angiogenesis analysis.

Detection of antibody response to EAC cells by ELISA

Humoral immune responses to EAC cells were measured by an indirect enzyme-linked immunosorbent assay (ELISA) as described previously[27], with slight modification. Briefly, 96-well flat-bottomed ELISA plates were coated with 5 μg/well of EAC TCLs in 0.1 mmol/L carbonate bicarbonate buffer and kept overnight at 4℃. Plates were washed and blocked, and serum from immunized and control mice were added to the wells at a 1:100 dilution. This was followed by incubation in HRP-conjugated goat anti-mouse IgG. The reaction was completed with 3,3',5,5'-tetramethylbenzidine and stopped with H_2SO_4 (2 mol/L). Then, the absorbance value at 450 nm (A_{450}) was measured with an ELISA reader.

To determine the specific isotypes, serum from mice was diluted at 1:100 and tested in duplicate. Anti-mouse Ig subclass-specific, HRP-conjugated secondary antibodies (Abs) were used to detect the mouse IgG1 and IgG2a isotypes.

Western blot analysis

To determine the specificity of Abs elicited in immunized mice and to identify which protein bands in the TCLs react with serum from mice in each group, the TCLs were used as antigens for Western blotting. Western blotting was performed as described previously[28]. The EAC TCLs were electrophoresed on a 12% SDS-PAGE gel under denaturing conditions (the same sample was fractionated in 5 wells on one gel) and then transferred to nitrocellulose membrane (Millipore, USA). The membrane was blocked with 5% non-fat dry milk and then was washed, cut, and probed with serum from each group at a dilution of 1:50. Blots were washed and incubated with HRP-conjugated goat anti-mouse IgG. The reaction was completed by using 0.05% 3,3'-diaminobenzidine and 0.012% H_2O_2 for 15 min at 37℃.

Mouse CTL killing assay

Splenocytes were isolated from the immunized mice in each group and then were incubated with TCLs for 72 h in DMEM supplemented with 10% fetal bovine serum *in vitro*. The ratio of splenocytes to EAC TCLs was 1:1. Then, the stimulated splenocytes were recollected and co-cultured with EAC cells at ratios of 1:100, 1:50, and 1:25 in 96-well U-bottomed plates (1×10^4 cells in 0.1 mL) for 8 h at 37℃ in a 5% CO_2 humidified atmosphere. CTL activity was measured by MTT assay, and the absorbance at 570 nm (A_{570}) was read. Percent cytotoxicity (C, %) was calculated: $C = [1 - (A_{MIX} - A_{EFF}) / (A_{TAR} - A_{MED})] \times 100\%$. Here, A_{MIX} is the A_{570} of the wells containing a mixture of effector cells, A_{EFF} is the A_{570} of the wells with effector cells, A_{TAR} is the A_{570} of the wells with target cells, and A_{MED} is the A_{570} of culture medium without cells[21, 29].

Quantification of angiogenesis *in vivo*

An intradermal tumor model was used to investigate the induction of angiogenesis by tumors *in vivo*. In the model, neovasculature, observed predominantly at the periphery of the tumor, was quantified by the vessel counting method as described elsewhere[27,28]. When the tumors of the TCL control group reached 5 mm in diameter, the flap of the abdominal wall skin containing the injected cells was removed. Tumor sections were examined using light microscopy at low magnification (× 10), and the total number of blood vessels (major

vessels and branch points) was determined within a 1-cm^2 area around each implant site. The volume was then calculated using the formula $V = 0.52ab^2$, with "a" as the longest diameter and "b" as the shortest diameter.

Histopathologic observation of normal tissues

Normal tissue samples were fixed with 10% buffered formaldehyde, routinely processed, and embedded in paraffin. Paraffin sections (4 μm) were cut and stained with hematoxylin and eosin (HE). All sections were evaluated by an experienced pathologist for histopathologic changes that could be related to treatment.

Statistical analysis

Statistical comparison was made by using the two-tailed Student's t test. A value of $P < 0.05$ was considered significant.

Results

Tumor cell vaccine elicited production of specific antibodies against EAC TCLs

DT and M$_2$ as adjuvants can enhance the immunogenicity of polysaccharide antigens, therefore, DT and M$_2$ were used as cell vaccine adjuvants. To analyze whether

the vaccine could enhance the immunogenicity of EAC TCLs, an ELISA assay was performed to determine the levels of Abs against EAC TCLs in serum collected from mice immunized with TCL (control group), TCL-NS, TCL-DT, TCL-M$_2$, or DT-TCL-M$_2$ (Figure 1). Among the tumor cell vaccines, antibody levels in DT-TCL-M$_2$, TCL-DT, and TCL-M$_2$ groups were high, but in TCL and TCL-NS groups were low. The levels of specific IgG Abs were significantly higher in TCL-DT, TCL-M$_2$, and DT-TCL-M$_2$ groups than in TCL-NS and TCL groups ($P < 0.01$), but were similar between TCL-DT and TCL-M$_2$ groups or between TCL-NS and TCL groups ($P > 0.05$). Most importantly, the levels of IgG Abs were significantly higher in DT-TCL-M$_2$ group than in TCL-DT or TCL-M$_2$ group ($P < 0.05$), indicating that the highest level of IgG Abs was stimulated in DT-TCL-M$_2$ group among all groups.

To indirectly demonstrate the relative contributions of Th2 versus Th1 responses, we measured the production of IgG1 (Th2 response) and IgG2a (Th1 response) Abs against EAC TCLs in our experiments. The levels of IgG1 Abs were significantly higher in TCL-DT and DT-TCL-M$_2$ groups than in other groups ($P < 0.01$) and were significantly higher in TCL-DT group than in DT-TCL-M$_2$ group ($P < 0.05$), but were similar among TCL-M$_2$, TCL-NS, and TCL groups ($P > 0.05$). On the other hand, DT-TCL-M$_2$ induced the highest levels of IgG2a Abs. In addition, the levels of IgG2a Abs were significantly higher in TCL-M$_2$ group than in other 3 groups ($P < 0.05$) (Figure 1). The Abs induced by TCL-DT were almost exclusive of the IgG2a subclass,
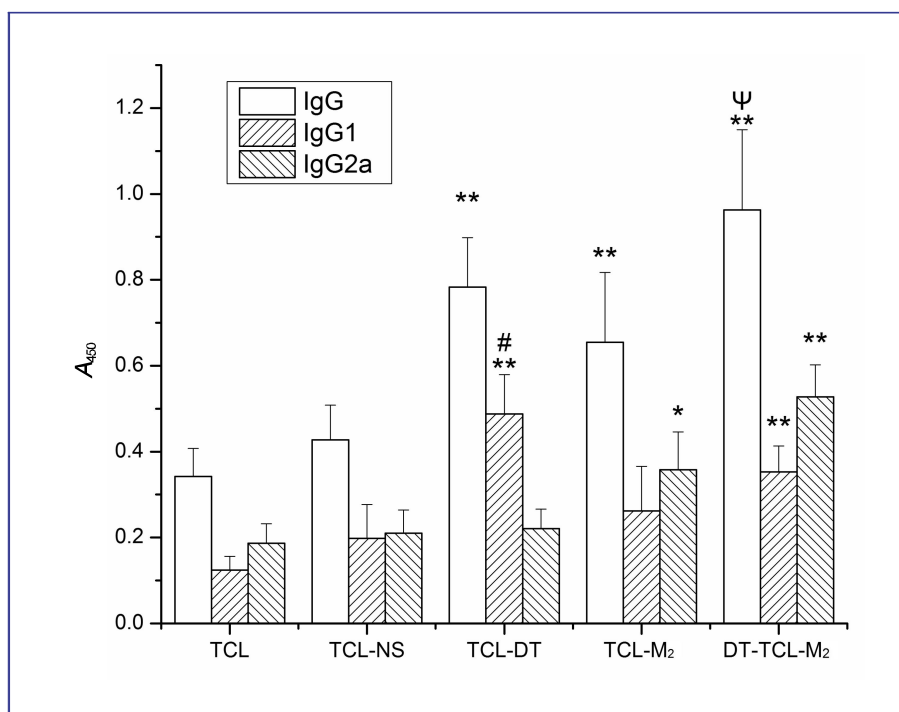
Figure 1. Detection of antibody responses to Ehrlich ascites carcinoma (EAC) tumor cell lysates (TCLs). Each group of mice (9 per group) underwent three weekly subcutaneous injections of TCL, TCL-NS, TCL-DT, TCL-M$_2$, or DT-TCL-M$_2$. Serum samples were collected from each mouse 2 weeks after the last immunization and were tested for the presence of antibodies IgG, IgG1, and IgG2a against EAC lysates. *$P < 0.05$, **$P < 0.01$, vs. TCL and TCL-NS; #$P < 0.05$, vs. DT-TCL-M$_2$; Ψ$P < 0.05$, vs. TCL-DT and TCL-M$_2$.

which is indicative of Th2 response. However, DT-TCL-M$_2$ and TCL-M$_2$ induced the IgG2a subclass more than the IgG1 subclass, which is indicative of Th1 response.

Anti-EAC antibodies analysis by Western blotting

The specificity of antibodies elicited in immunized mice was established in immunoblots using EAC TCLs as antigens. TCLs showed multiple positive bands when probed with serum from mice immunized with DT-TCL-M$_2$ and TCL-DT but negative staining when probed with serum from mice immunized with TCL-M$_2$, TCL-NS, and TCL (Figure 2). These findings suggest that the Abs in the serum specifically from mice immunized with DT-

TCL-M$_2$ and TCL-DT recognized many antigens in EAC TCLs at a dilution of 1:50.

CTLs generated by DT-TCL-M$_2$ can kill target cells

To investigate the functional effector properties of CTLs generated by different vaccines, we performed *in vitro* cytotoxicity assays of splenocytes isolated from mice treated with TCL, TCL-NS, TCL-DT, TCL-M$_2$, or DT-TCL-M$_2$. The tumor-specific cytotoxic activities were significantly stronger in DT-TCL-M$_2$ and TCL-M$_2$ groups than in TCL group ($P < 0.01$), and significantly stronger in DT-TCL-M$_2$ group than in TCL-M$_2$ group ($P < 0.05$), but were similar among the TCL-DT, TCL-NS, and TCL groups (Figure 3).
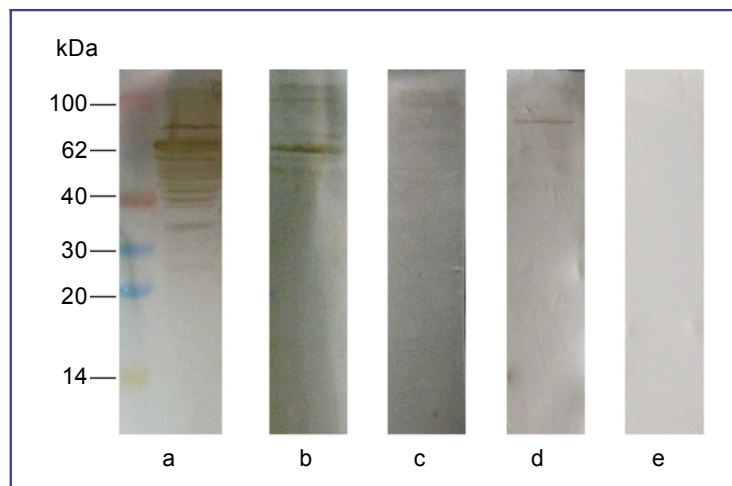
Figure 2. Western blot analysis of the specificity of antibodies against EAC TCLs. The same TCL sample was electrophoresed in 5 wells on one gel and then transferred onto nitrocellulose membranes that were blocked, washed, cut, and incubated with serum from each group. TCLs in lanes a, b, c, d, and e were stained with serum isolated from mice immunized with DT-TCL-M$_2$, TCL-DT, TCL-M$_2$, TCL-NS, and TCL, respectively.
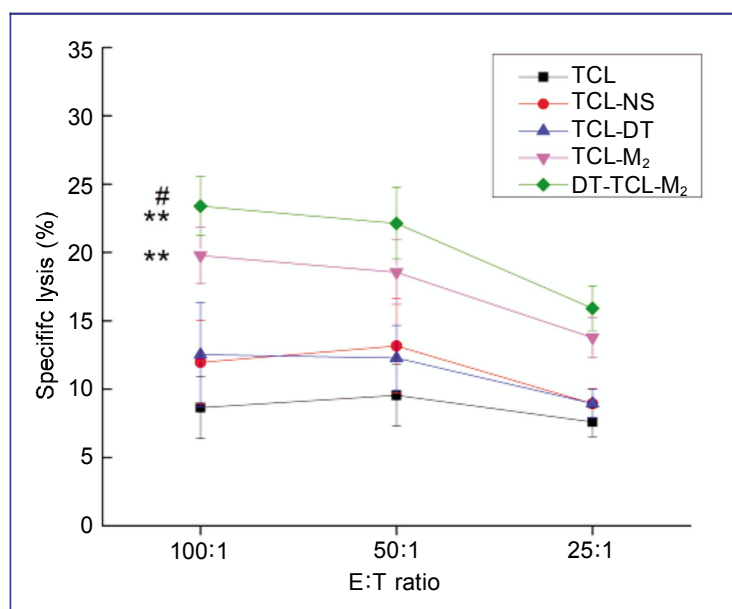
Figure 3. EAC-specific cytotoxicity of splenocytes from different groups. Splenocytes were isolated from the spleen of each mouse on day 7 after last immunization. Effector cells were stimulated by EAC lysates. A cytotoxic splenocyte killing assay was performed for 8 h using EAC TCLs as target. Percentages of cytotoxicity are shown at different effector:target (E:T) ratios. **$P < 0.01$, vs. TCL; #$P < 0.05$, vs. TCL-M$_2$.

DT-TCL-M$_2$ vaccination suppressed the growth of subcutaneous EAC solid tumor

To determine if the vaccines could inhibit the growth of EAC tumors *in vivo*, we generated an EAC solid tumor mouse model and immunized the mice with DT-TCL-M$_2$, TCL-M$_2$, TCL-DT, or TCL-NS. Compared to TCL group, vaccination with DT-TCL-M$_2$ significantly retarded EAC solid tumor growth ($P < 0.05$, from day 10 to 12; $P < 0.01$, from day 12 to 14), whereas no significant change was noted among TCL-DT, TCL-M$_2$, TCL-NS, and TCL groups. On day 14 after tumor inoculation, tumor volumes for DT-TCL-M$_2$ group were only 24% of that for TCL group (Figure 4A-C), and the mean weight of excised tumors was significantly lower in DT-TCL-M$_2$ group than in TCL group [(0.80 ± 0.68) g vs. (2.22 ± 0.84) g, $P < 0.01$], but showed no significant differences between other groups and TCL group (Figure 4D).

DT-TCL-M$_2$ inhibited angiogenesis associated with intradermal EAC tumor

Tumor cells implanted intradermally were found to induce significant angiogenesis when the intradermal tumor grew up to 5 mm in diameter. The total number of blood vessels around each implant site from mice immunized with DT-TCL-M$_2$ was significantly lower than that from mice immunized with TCL (49.5 ± 12.4 vs. 96.7 ± 16.0, $P < 0.01$), but there was no significant difference between other groups and TCL group (Figure 5A and 5B). In addition, the volumes of intradermal tumors were similar to those of the solid tumor model described above (Figure 5C). These observations suggest that vaccination with DT-TCL-M$_2$ can retard intradermal EAC tumor growth and angiogenesis.
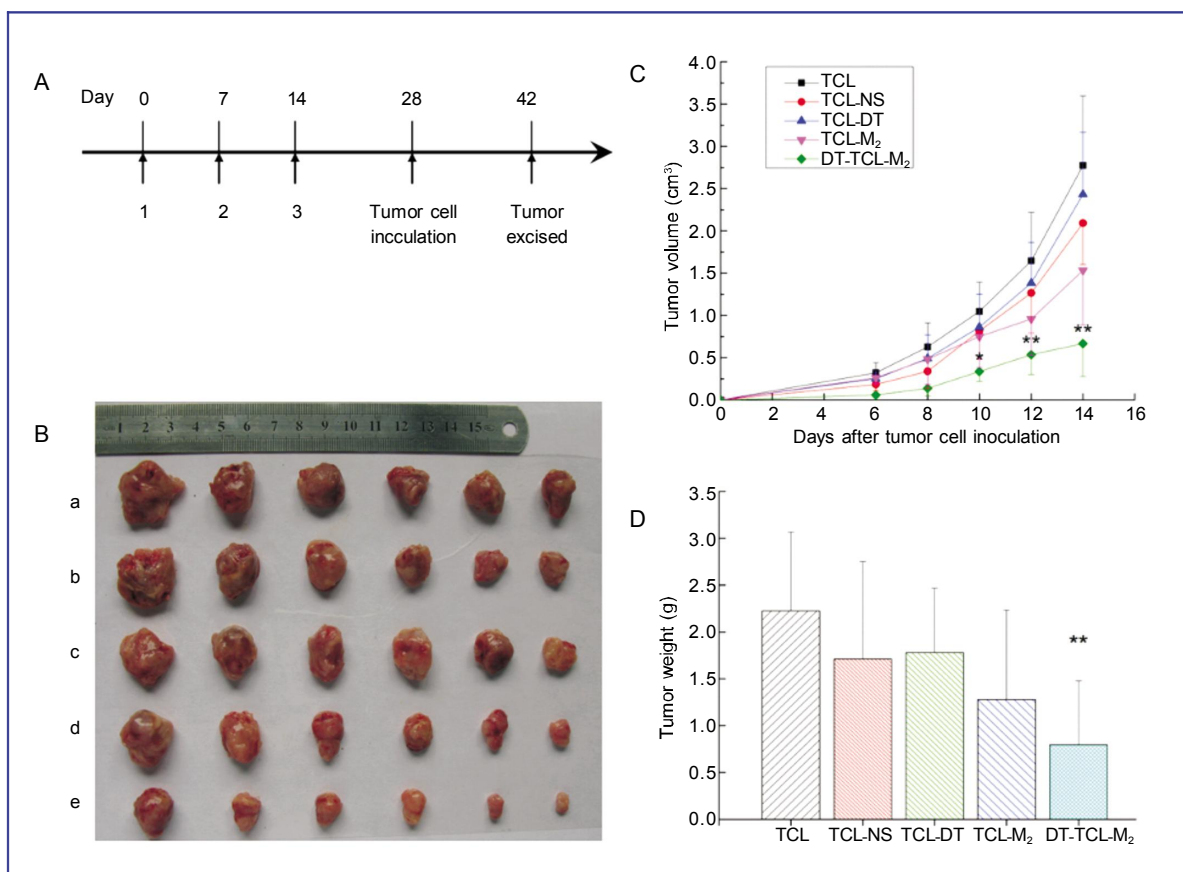

Figure 4. **Prophylactic immunization of mice with vaccines.** A, the picture shows the immunization scheme by arrows. The mice were vaccinated subcutaneously three times at weekly intervals with the vaccines at days 0, 7, and 14. At day 28, 1×10^6 EAC cells were injected subcutaneously for tumor cell inoculation. All mice were killed and tumors were excised 2 weeks later. B, solid tumors from mice immunized with TCL (a), TCL-NS (b), TCL-DT (c), TCL-M$_2$ (d), or DT-TCL-M$_2$ (e). C, *in vivo* measurement of tumor growth. The tumor volume was measured with calipers every two days beginning at day 6 after tumor cell inoculation. D, weight of tumors from mice immunized with TCL, TCL-NS, TCL-DT, TCL-M$_2$, or DT-TCL-M$_2$. **$P < 0.01$, vs. TCL.

No toxicity of tumor cell vaccine for tissues

To assess the safety of the vaccines, pathologic images with HE staining were observed (Figure 6). No significant pathologic changes were found between DT-TCL-M_2 and TCL groups in the liver, lung, heart, kidney, and spleen, indicating that these organs were all in a normal state. Vaccinated mice had no noticeable changes in fur, body weight, behavior, feeding, and life span.
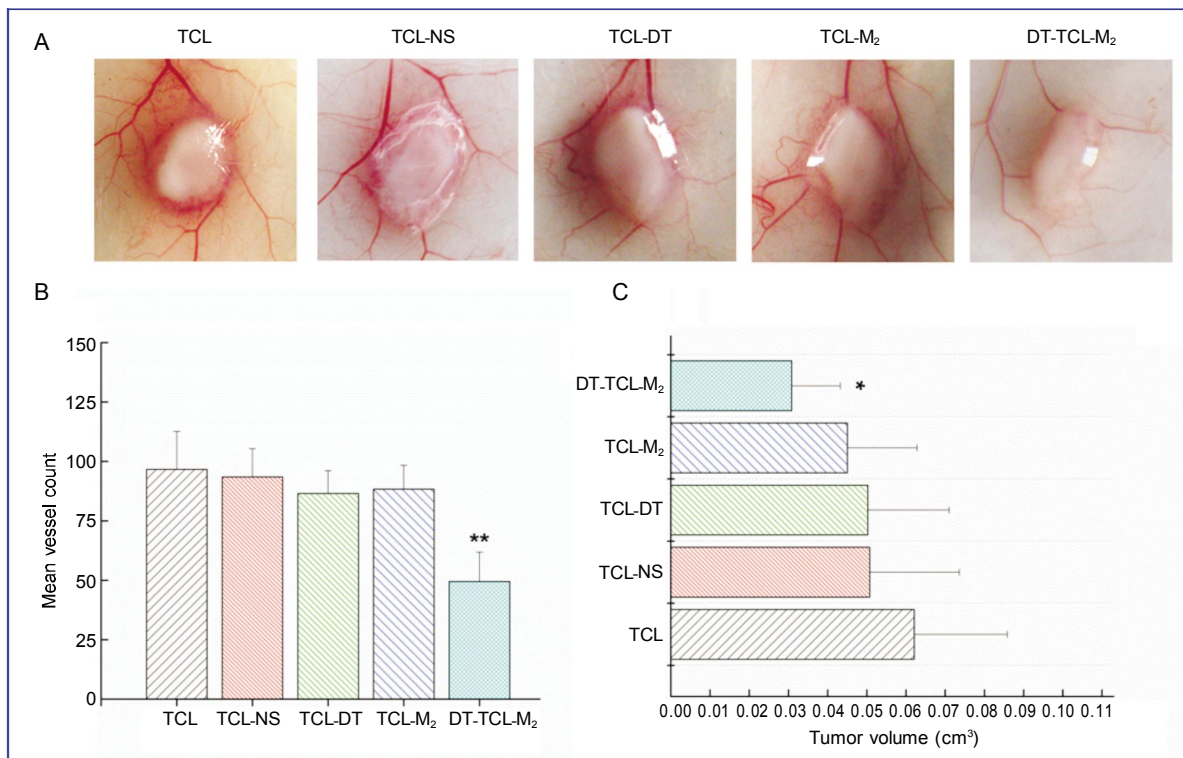
Figure 5. **Effect of the immune response on tumor-induced angiogenesis.** A, to visualize the induction of angiogenesis by a tumor *in vivo*, EAC tumor cells were implanted intradermally and new blood vessel formation was assessed 7 days later by light microscopy. The effects of immunization with TCL, TCL-NS, TCL-DT, TCL-M_2, and DT-TCL-M_2 on angiogenesis are shown in representative images (×1). B, the total number of blood vessels (major vessels and branch points) was determined within a precise 1-cm area among each implant site. C, the results of tumor volume are shown. $*P < 0.05$, $**P < 0.01$, vs. TCL.
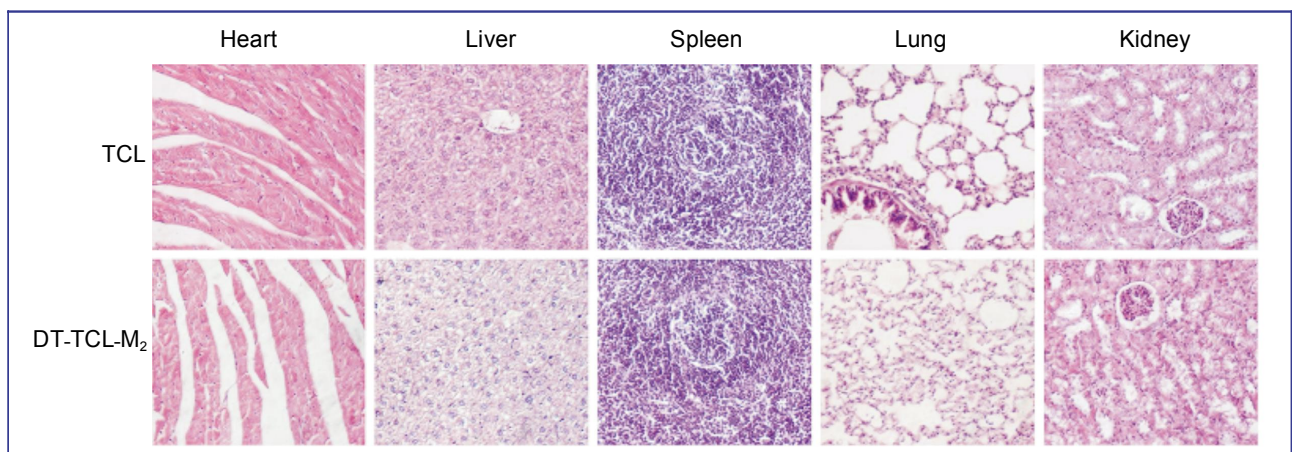
Figure 6. **Pathologic examination of the heart, liver, spleen, lung, and kidney in recipient mice (HE ×200).** No significant pathologic changes were found between TCL and DT-TCL-M_2 groups.

Discussion

This study was to test the hypothesis that DT and M_2 conjugated to TCLs will improve the weak immunogenicity of TCLs and develop tumor-specific polyclonal immune responses that can suppress tumor growth. Our data support this hypothesis. Immunization with DT-TCL-M_2 effectively evoked a preventive antitumor immunity against highly aggressive and weakly immunogenic EAC breast cancer cells. In addition, DT-TCL-M_2 vaccination effectively inhibited the growth of intradermal tumors and angiogenesis. More importantly, the administration of DT-TCL-M_2 induced tumor-specific Abs and CTL immune responses that are essential for antitumor immunity.

The challenging goal of effective tumor vaccination is to induce tumor-specific polyclonal immune responses to hinder tumor progression or recurrence. The immune system is composed of effector cells that can remove target cells. B and T cells can recognize antigens expressed only by tumors and generate tumor-specific responses because they have a vast array of clonally distributed antigen receptors. Establishing immunity against a single antigen might be ineffective in tumors with heterogeneous cell populations and could possibly induce tumor antigen escape variants[30]. In addition, a large tumor is capable of evading the immune system, and immunity against a single antigen may not break immune tolerance[4,31,32]. TCLs contain a crude mixture of all kinds of cellular components, including fragments of the destroyed cellular membrane, intracellular organelles, and cellular RNA and DNA. Therefore, TCLs contain multiple known and unknown antigens that can be presented to B and T cells and generate tumor-specific polyclonal immune responses, more likely to circumvent tumor escape and heterogeneity[21]. Therefore, in the current study, TCLs were used as the antigen source for the development of a breast cancer vaccine.

Antigens in TCLs are often poorly immunogenic and induce immune tolerance. The widely used DT as a carrier protein greatly enhances the immunogenicity of polysaccharide antigens, enabling host defense against diseases caused by encapsulated pathogens. Extensive information about the immune potential and safety profile of this carrier in clinical use is available[22,33]. Recently, mHSP70, an intracellular molecular chaperone in most microorganisms, has been widely investigated for its antitumor use because it can conjugate peptides and inactive tumor cells[21,34]. There are several properties of mHSP70 that might contribute to the generation of an antitumor immune response. For example, mHSP70 has been shown to facilitate cross-presentation of bound antigenic peptides. Furthermore, mHSP70 is capable of activating DCs. mHSP70$_{407-426}$ was identified as a major epitope within mHSP70 that is responsible for its stimulatory functions, including cytokine and chemokine production and DC maturation[23]. Combination with DT and M_2 increased the immunogenicity of weak TCLs, potentiating the host's tumor-specific polyclonal immune responses and providing a key advance in antitumor immunity.

The choice of two copies of mHSP70$_{407-426}$ as a T-helper epitope is based on our previous investigation that showed the introduction of two copies of mHSP70$_{407-426}$, but not one or three copies, into an anti-gastrin releasing peptide (GRP) DNA vaccine resulted in stronger immune stimulatory potential and impressive antitumor activity[35]. M_2 might act as an agonist for mHSP70$_{407-426}$, which might substitute for CD40 ligand in the stimulation of CD40. Thus, this may enhance antitumor immune responses by means of DC maturation[23]. When activated by an agonist of CD40, DCs exhibit increased survival and cytokine production. Additionally, CD40 activation induces up-regulation of co-stimulatory molecules, such as MHC-II, CD80 and CD86, and promotes antigen presentation, priming and cross-priming of Th and CTL[36,37]. Our data have shown that the induction of significant IgG2a is indicative of Th1 immune responses when M_2 was introduced into the vaccine. Furthermore, this is further supported by our observation of tumor-specific CTL response (Figure 3).

In the present study, TCL-DT induced the production of specific IgG1 Abs. TCL-M_2 elicited tumor-specific CTL immune response and the production of specific IgG2a Abs. However, the two vaccines led to weak results without significantly enhancing antitumor activity. The strongest humoral immune response and tumor-specific CTL response were elicited by DT-TCL-M_2. Thus, DT-TCL-M_2 significantly inhibited tumor growth in mouse breast tumor models. Our results show that the DT-TCL-M_2 vaccine is superior to the TCL-DT and TCL-M_2 vaccines alone.

Although TCLs have been reported to elicit cellular immune response for antitumor activity[21], there is little information about the humoral immune response to TCLs. Using an ELISA assay, we detected high levels of tumor-specific Abs in the serum of mice immunized with DT-TCL-M_2 (Figure 1). Cytokine B-lymphocyte stimulator protein (BLyS, also known as BAFF, TALL-1, THANK, and zTNF4) produced by DCs via CD40 signaling can

up-regulate B-cell antibody response [37]. In addition, M_2 might activate CD40 on the surface of B cells and promote germinal center formation, Ig isotype switching, somatic hypermutation of Ig to enhance affinity for antigens, and, finally, formation of long-lived plasma cells and memory B cells [36]. Furthermore, DT might serve as the vector for delivering B-cell and T-cell epitopes to the immune system, resulting in the production of Abs (Figure 2). The generation of a wider IgG subclass suggests that polyclonal expansion of antigen-reactive B cells might be possible in this model. This is supported by our results showing that many reactive bands could be clearly observed in DT-TCL-M_2 by Western blotting. To better understand the role of antibodies in inhibiting tumor growth, further studies are required. There are several potential antitumor mechanisms of Abs in this study. First, Abs may mediate antitumor effects by antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity [5,38]. Second, binding surface receptors by Abs might interfere with ligand binding or receptor clustering. For instance, Abs can act directly by blocking signal transduction pathways when targeted to growth factor receptors[27,28,39].

The tumor-associated neovasculature, which is generated by the process of angiogenesis, is important for tumor growth and progression. Recent data indicate that angiogenesis is not only essential for rapidly growing macroscopic tumors, but also contributes to the microscopic premalignant phase of neoplastic progression, further cementing its status as an integral hallmark of cancer [40]. A novel DNA vaccine targeting GRP significantly reduced tumor-associated angiogenesis and vascularization of intradermal tumors of C57BL/6 mouse-derived B16 melanoma cells (B16- F10)[41]. GRP has been reported to stimulate GPR receptor-induced pro-angiogenic gene expression as well as the expression of various angiogenic markers, including platelet-endothelial cell adhesion molecule (PECAM-1) and vascular endothelial growth factor (VEGF)[35,41]. High titers of GRP-specific Abs could neutralize elevated levels of the GRP self-peptide made by tumor cells and block the GRP/GRPR autocrine loop of GRP-dependent tumors. In this study, we investigated the anti-angiogenic activity of a tumor cell vaccine in an intradermal tumor model. Our results show that vaccination with DT-TCL-M_2 retarded intradermal EAC tumor growth and angiogenesis. The anti-angiogenic effects of DT-TCL-M_2 may further hamper tumor progression, contributing to the inhibition of tumor growth *in vivo*. Our results of

intradermal tumor volumes support this hypothesis. Notably, the results in the intradermal tumor model were similar to those observed in the solid tumor model. Tumor cell vaccines contain multiple known and unknown antigens that may generate tumor-specific polyclonal immune responses, including an immune response targeting tumor angiogenesis. The exact mechanism of this phenomenon is now under detailed investigation in our lab.

A challenge of cancer cell vaccines is the possibility of inducing substantial autoimmune reactions to antigen epitopes that are shared by normal tissues [42]. However, in animal studies and clinical trials using whole tumor cells as the source of antigen, no clinically relevant autoimmune responses were detected [19,43]. Our animal studies have suggested that the TCL immunization is apparently harmless to normal mice.

In summary, we developed a novel method of generating the cancer cell vaccine DT-TCL-M_2 with high immunogenicity by introducing a carrier protein DT and an efficient molecular adjuvant M_2 for immunotherapy of breast cancer. DT-TCL-M_2 can activate tumor-specific polyclonal immune responses against EAC breast cancer, therefore inhibiting tumor growth and enhancing antitumor activity. Clearly, the in-depth mechanism of DT-TCL-M_2 against breast cancer warrants further study.

We hope that such a novel approach might be a simple and feasible way to produce efficient autologous and allogeneic cancer cell vaccines. This potent cancer cell vaccine might provide a supplement to standard therapies including surgery, chemotherapy, and radiation or in combination with other therapeutic vaccines. These approaches will be promising for suppressing recurrence and metastasis and for producing a therapeutic effect on residual small lesions of breast cancer and other cancers after resection.

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