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Chemokine-Containing Exosomes Are Released from Heat-Stressed Tumor Cells via Lipid Raft-Dependent Pathway and Act as Efficient Tumor Vaccine

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Exosomes derived from dendritic cells or tumor cells are a population of nanometer-sized membrane vesicles that can induce specific antitumor immunity. During investigation of the effects of hyperthermia on antitumor immune response, we found that exosomes derived from heat-stressed tumor cells (HS-TEX) could chemoattract and activate dendritic cells (DC) and T cells more potently than that by conventional tumor-derived exosomes. We show that HS-TEX contain chemokines, such as CCL2, CCL3, CCL4, CCL5, and CCL20, and the chemokine-containing HS-TEX are functionally competent in chemoattracting CD11c+ DC and CD4+CD8+ T cells both in vitro and in vivo. Moreover, the production of chemokine-containing HS-TEX could be inhibited by ATP inhibitor, calcium chelator, and cholesterol scavenger, indicating that the mobilization of chemokines into exosomes was ATP- and calcium-dependent and via a lipid raft-dependent pathway. We consistently found that the intracellular chemokines could be enriched in lipid rafts after heat stress. Accordingly, intratumoral injection of HS-TEX could induce specific antitumor immune response more efficiently than that by tumor-derived exosomes, thus inhibiting tumor growth and prolonging survival of tumor-bearing mice more significantly. Therefore, our results demonstrate that exosomes derived from HS-TEX represent a kind of efficient tumor vaccine and can chemoattract and activate DC and T cells, inducing more potent antitumor immune response. Release of chemokines through exosomes via lipid raft-dependent pathway may be a new method of chemokine exocytosis. *The Journal of Immunology, 2011, 186: 2219–2228.

Exosomes are membrane microvesicles (50–100 nm in diameter) released by various cells and originating from the endosomal compartment with characteristic protein content (1). Although the sources of exosomes are diverse, they have common characteristics, such as structure (bilipidic layer), size, density, and overall protein composition (1, 2). Abundant proteins have been detected in exosomes, including cytoplasmic proteins, such as tubulin, actin, actin-binding proteins, annexins, molecules responsible for signal transduction (protein kinases, heterotrimeric G proteins) and heat-shock proteins (e.g., HSP70 and HSP90), and membrane-associated molecules, such as MHC molecules, adhesion molecules, membrane receptors, and tetraspanins (e.g., CD9, CD63, CD81, and CD82) (1–3). To characterize the purified exosomes, morphological examination by electron microscopy and protein composition examination by Western blot or flow cytometry have been widely used. To date, exosomes have been implicated in the regulation of immune response, development, ectodomain shedding of membrane proteins, and retrovirus particle release (1–4).

Exosomes derived from dendritic cells (DC) or tumor cells, designated as DEX and TEX, respectively, have drawn the most intense attention because of the immunogenic activities of these exosomes in induction of antitumor immune response (5, 6). DEX pulsed with peptide acid eluted from a variety of different tumors mediated tumor growth retardation in an MHC- and CD8+ T cell-dependent manner (5). DEX can directly or indirectly present the pulsed tumor Ags to CD4+ and CD8+ T cells and activate T cells both in vitro and in vivo (7–9). Additionally, DEX secreted from bone marrow-derived or monocyte-derived DC harbor functional membrane-bound NKGD2 ligands and IL-15Rα, which may trigger NK cell activation (10). Because of the efficient induction of antitumor immunity elicited by DEX, clinical trials using modified DEX in treatment of melanoma and non-small cell lung cancer have been undertaken (10–12). In many aspects, cancer-derived exosomes resemble those of DC origin in their biophysical and biochemical properties (2, 4). However, the effects of TEX in tumor immunity have been controversial. It has been reported that TEX can induce apoptosis of T cells, impair DC differentiation, inhibit NK activity, and propagate immunosuppressive myeloid suppressor cells and regulatory T cells (13–24). In contrast, other studies using TEX derived from ascites of melanoma patients and colon cancer patients (25, 26), TEX pulsed with superantigens or engineered with heat shock protein (HSP)70 and cytokines (26–30), or TEX derived from heat-stressed tumor cells (31, 32) suggest that TEX can also activate T cells and be applied in...
immunotherapy of cancer. Therefore, further investigations of biological characteristics and functions of TEX may be helpful in the development of more efficient tumor cell-based vaccines and in better understanding of mechanisms of tumor escape from immunological surveillance.

During investigations of local hyperthermia-elicited (42–43°C) antitumor immunity, we found that heat stress can induce the production of chemokines by tumor cells via an autocrine activity of secreted HSP70 (33). Further studies show that exosomes derived from heat-stressed tumor cells (HS-TEX) contain chemokines that can chemoattract and activate DC and T cells. Previously, several reports have suggested that cytokines and chemokines, such as TNF-α, TGF-β, IL-1β, IL-6, IL-10, and CCL5, can be released in exosomes (19–23, 32, 34, 35). However, the mechanisms involved in exosome-mediated cytokine/chemokine exocytosis as well as the roles of chemokines contained in exosomes have not been elucidated. In this study, we report that heat stress can induce the release of chemokine-containing HS-TEX that can chemoattract and activate DC and T cells in vitro and induce tumor-specific antitumor immune response in vivo. HS-TEX thus may serve as an efficient tumor vaccine. We show that the release of chemokine-containing exosomes is dependent on ATP-induced calcium influx and on enrichment of chemokines in lipid rafts after heat stress. Our study suggests that exosomes may represent a novel machinery for chemokine exocytosis.

Materials and Methods

Mice, cells, Abs, and reagents

Male C57BL/6 (H-2K b) mice, 6–8 wk of age, were purchased from Joint Ventures Sipper BK Experimental Animal Company (Shanghai, China). Mice were housed under specific pathogen-free conditions for at least 1 wk before any experiments. Lewis lung carcinoma 3LL cell line (H-2K b) and melanoma cell line (H-2K b) were obtained from American Type Culture Collection (Manassas, VA), and were cultured as recommended. Mouse bone-marrow derived dendritic cells (BMDC) were prepared by culturing with 20 ng/ml recombinant GM-CSF and 10 ng/ml IL-4 (Genzyme, Cambridge, MA) for 7 d as described previously (36). CD11c+ DC and T cells, including CD3+ , CD4+, and CD8+ populations, were isolated by immunomagnetic separation (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) and as described previously (33). Purity for each MACS-isolated cell type was more than 95% as confirmed by FACS assays. The monoclonal Abs against HSP70 (inducible HSP70), HSC70 (constitutive HSP70), HSP90, HSP90, CD54, CD80, CD86, and MHC class I (Ia) and the polyclonal Abs against annexin II and CCL20 were obtained from Abcam (Cambridge, MA). Monoclonal Abs against CD71, CD107a, CCL2, CCL3, CCL4, CCL5, CD18, and MHC class II were obtained from eBioscience (San Diego, CA). Fluorescent Abs against CD80, CD86, Ia α, CD11c, CD4, CD8, and isotype control Abs were obtained from BD Pharmingen (San Diego, CA). ELISA kits for measuring cytokines were from R&D Systems (Minneapolis, MN). Chemical inhibitors, including monensin, suramin, verapamil, 1,2-bis(o-amino-phenoxo)ethane-N,N,N’,N’-tetraacetic acid (BAPTA), methyl-β-cyclodextrin (MβCD), and other nonspecified reagents were from Sigma.

Isolation and characterization of exosomes from tumor cells with or without heat stress

For purification of TEX, fresh culture medium containing no serum was added into the tumor cell culture system 4 h before the isolation procedure. For the purification of HS-TEX, fresh culture medium containing no serum was added before the heat treatment at 42°C for 1 h in a thermal incubator with subsequent recovery at 37°C for 4 h as described previously (28, 33). TEX and HS-TEX were purified as described previously by three successive centrifugations at 300 × g (5 min), 1200 × g (20 min), and 10,000 × g (30 min) to eliminate cells and debris, followed by ultracentrifugation for 1 h at 110,000 × g (28). The pellet was washed once in a large volume of PBS, centrifuged at 110,000 × g for 1 h, and resuspended in 50–200 µl PBS. The amount of exosomal proteins recovered was measured by BCA assays. Results are presented as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
was measured by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The morphology of the isolated exosomes was determined by electron microscopy as described (28). Flotation of exosomes on a continuous sucrose gradient was performed as described (26). Fractions of the gradient (1 ml each) were diluted in 2 ml PBS, centrifuged for 1 h at 100,000 \* g. 

**Western blot analysis**

Protein components contained in TEX and HS-TEX were examined by Western blotting as described previously (36). Finally, Supersignal West Femto Maximum Sensitivity substrate (Pierce) was used for demonstration of chemiluminescence.

**In vitro chemoattraction assay**

Chemotaxis of mouse DC and splenocytes (CD4+ or CD8+) was assayed as described previously (36) by diluting equal amounts of TEX and HS-TEX to the upper chamber in a volume of 0.1 ml. Migration of DC and T cells was assayed by counting the migrated cells with the assistance of FACS.

**DC adhesion assay**

Forty-eight hours after transduction of 3LL cells with GFP-encoding adenovirus (Ad-GFP), TEX and HS-TEX, which were GFP+, were isolated. CD11c+ DC were co-incubated with 10 \* 10^5 TEX and HS-TEX for 1 h at 4°C, extensively washed in cold PBS three times, and finally analyzed by FACS as described (36).

**In vitro T cell activation assays and CTL induction**

To evaluate the effects of TEX and HS-TEX in activating T cells, CD4+ and CD8+ T cells were isolated from wild-type mice 14 d after the last immunization with 3LL cell lysates (twice at an interval of 7 d). Then, CD4+ and CD8+ T cells were cocultured with 100 ng/ml LPS for 24 h were used as control. Results are presented as mean \pm SD of triplicate samples. **p < 0.01, ***p < 0.001.
To analyze the efficiency of TEX and HS-TEX in acting as artificial Ag-presenting vesicles, we cocultured CD8+ T cells (2 × 10^6/well) derived from wild-type mice with 5 μg or 10 μg TEX or HS-TEX as indicated in the presence or absence of day 7 BMDC in medium containing 30 U/ml IL-2 (Peprotech, Rocky Hill, NJ) for 7 d in 24-well plates in a final volume of 2 ml/well (one DC per five CD8+ T cells). Seven days later, CD8+ T cells were restimulated twice. On day 21, CD8+ T cells were collected, and functions of stimulated CD8+ T cells were evaluated by IFN-γ levels and cytotoxicity assay. For IFN-γ release assay, stimulator cells were prepared by pulsing the day 7 BMDC with 10 μg/ml MUT1 (FEYNY AQL, H-2Kb) peptide (a specific MHC class I (MHC-I) epitope expressed by 3LL cells) for 8 h, and the BMDC pulsed with 10 μg/ml OVA (257-264, SIINFEKL, H-2Kb) were used as control. After stimulating the stimulator cells with 100 ng/ml LPS for 48 h, they were cocultured with the stimulated CD8+ T cells at a 1:5 ratio. Forty-eight hours later, the culture supernatants were tested for the level of IFN-γ by ELISA assay. Cytotoxicity assays were performed using standard 4-h 31Cr-release assay against 3LL cells as described (37).

**Tumor-bearing mice preparation, therapeutic protocols, and functional assessment of tumor-infiltrating DC and T cells**

The 3LL tumor models were established and monitored as described previously (37). Seven days after 3LL inoculation (1 × 10^6 cells per mouse). TEX and HS-TEX were injected intratumorally (5 μg per mouse) at multiple sites. The injection was repeated three times at an interval of 3 d. Tumor volume was monitored every 3 d with a caliper and was presented as mean ± SEM of 10 mice in each group. CTL activity was evaluated by a standard 31Cr-release assay 14 d after the last intratumoral injection as described (33). For protection assay, C57BL/6 mice were s.c. immunized with 10 μg TEX and HS-TEX every 7 d for three times total. Seven days (day 0) after the last immunization, C57BL/6 mice were challenged with 5 × 10^6 3LL cells contralaterally as described (37).

To evaluate the functional status of DC and T cells isolated from tumor-infiltrating mononuclear cells, the mononuclear cell suspensions were incubated with magnetic beads specifically for CD11c+ and CD3+ cells, respectively, and then isolated by immunomagnetic separation (Miltenyi Biotec) on RS1 columns as described previously (33). DC and T cells were subjected to quantitative PCR assays as described previously (38).

**In vivo chemoattraction assay**

Five micrograms TEX and HS-TEX were injected into the preestablished tumor mass. At the indicated time points, tumors were excised and frozen-sectioned. DC were stained with FITC–anti-CD11c Ab, and T cells were examined by staining with fluorescent anti-CD4+ or CD8+ Abs (Pharmingen) as described (37). Otherwise, immune cells infiltrated in the tumor mass were evaluated by FACS assays of isolated tumor-infiltrating mononuclear...
cells after staining with fluorescent anti-CD11c, anti-CD4, or anti-CD8 Abs as described (33).

Measurement of extracellular ATP and intracellular calcium

An ATP bioluminescence assay kit containing luciferin/luciferase reagent (The ENLITEN ATP Assay System; Promega, Madison, WI) was used to measure ATP concentration in the culture supernatant according to the manufacturer’s instructions. To determine the time course of ATP release, media samples were taken at each time point and immediately frozen at −80°C for further analysis.

Ca²⁺ imaging was performed as described previously with minor modification (38). 3LL cells were plated on coverslips and loaded for 1 h at 37°C with 3 M fluo 3-AM (Invitrogen, San Diego, CA) in the presence of 0.02% pluronic acid in medium. Cells were washed with PBS, and preheated (42°C) medium was added to the chamber. Then the chamber was held on the LEICA Microscope Heating Stage set at 42°C. Fluo 3-AM fluorescence imaging was recorded at baseline and scanned at 15-s intervals using a Leica TCS SP2 confocal laser microscope (Leica, Wetzlar, Germany) under a 100X.40 CS objective lens. Images were analyzed using Leica Confocal software. Data were collected as the average fluorescence detected from 10 cells per microscope field at each time point.

Lipid raft isolation

Lipid rafts were isolated by sucrose density gradient centrifugation essentially as described (39). Cells were harvested in ice-cold PBS and lysed in 1 ml MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl) containing 1% Triton X-100, 10 μg/ml benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM NaVO₄, 10 mM NaF, and 1 mM PMSF, incubated on ice for 30 min, and vortexed extensively. One milliliter of an 80% sucrose solution in MES-buffered saline was mixed with the lysate, and this was overlaid with 2 ml of a 30% sucrose solution in MES-buffered saline. The samples were centrifuged in a Beckman SW55Ti rotor at 200,000 × g overnight at 4°C as described. Fractions of 0.5 ml were taken from the top of the gradient to which 250 μl 2 X SDS gel-loading buffer was added. Thirty microfilters of each fraction was subjected to SDS-PAGE and Western blotting. A similar procedure was used for isolation of the Triton-X 100 insoluble fractions in TEX or HS-TEX.

Statistical analysis

All experiments were independently performed three times. Results are given as means ± SE or means ± SD. Comparisons between two groups were done using Student t test, and comparisons between multiple groups were done using Kruskal–Wallis tests. Survival estimates and median surivals were done using an Kruskal–Wallis tests. Survival estimates and median survival were done using Student test, and comparisons between multiple groups were done using Kruskal–Wallis tests. Survival estimates and median survival were determined using the method of Kaplan and Meier. Statistical significance was determined as p values <0.05.

Results

Characterization of exosomes derived from tumor cells with or without heat stress

After isolation of TEX and HS-TEX from 3LL lung cancer cells treated or not treated with heat stress, we characterized their morphology and components. We found that TEX and HS-TEX were membrane vesicles ranging in size from 50–100 nm by electron microscopy (Fig. 1A). These vesicles were positive for several protein markers characteristic of TEX, such as CD18, CD54, CD63, CD71, CD80, CD86, CD107a, MHC-I, and HSP (Fig. 1B). Therefore, exosomes were contained in the pellets isolated after ultracentrifugation from tumor cell culture supernatants before and after heat stress.

As HS-TEX were isolated from heat-stressed (42°C for 60 min) tumor cells, the HS-TEX may contain apoptotic bodies (heterogeneous in size and most of them larger than 100 nm) or released intracellular vesicles. We stained the 3LL cells with hypotonic propidium iodide and annexin V after heat stress and 4-h recovery and found that the heat treatment could not induce significant apoptosis and necrosis of 3LL cells (data not shown). It has been previously reported that the stepwise centrifugation method used in this study can yield exosomes without apoptotic bodies (40). Therefore, the heat stress would not affect the general characteristics of HS-TEX. However, as further evidence for the quality of the isolated exosomes, we floated them on a sucrose gradient, and we found that MHC-I and HSC70 molecules were enriched in the typical exosome fractions (fractions 6–9, corresponding with sucrose density of 1.12–1.17 g/ml; Fig. 1C), indicating that the isolated HS-TEX were exosomes.

Then we quantified the isolated TEX and HS-TEX by BCA assays, and we found that heat stress could increase the release of exosomes from 3LL tumor cells (Fig. 1D). As early as 10 min after heat stress plus 1-h recovery, when protein expression of HSP70 was not induced (data not shown), the production of HS-TEX was increased. After 30-min heat-stress treatment plus 4-h recovery, the production of HS-TEX was elevated to ~2.5- to ~3.5-fold compared with that of TEX derived from 4-h culture supernatant of 3LL cells without heat stress. These data suggest that heat stress...
may promote the release of exosomes and that the general features of HS-TEX are similar to normal TEX.

**HS-TEX chemoattract, adhere with, and activate DC more significantly**

To compare the immunological effects of HS-TEX on DC with that of TEX, we examined the migration capacity, cytokine production, phenotype, and allostimulatory capacity of DC in the absence of TEX or HS-TEX. Surprisingly, we found that HS-TEX could potentiate the migration (Fig. 2A), increase the production of IL-12p70 (Fig. 2B), promote the maturation (Fig. 2C, 2D) and elevate the allostimulatory capacity of DC (Fig. 2E).

Because molecules responsible for DC adhesion were detected in HS-TEX (Fig. 1B), we determined whether HS-TEX were more effective in adhering with DC. It has been reported that GFP was found in exosomes secreted by tumor cells transfected with gene encoding this protein (6). Thus, we isolated both TEX and HS-TEX from 3LL cells transfected with Ad-GFP and confirmed GFP expression by Western blot (data not shown). We found that DC could more efficiently adhere with HS-TEX than with TEX (Fig. 2F), which may be due to more adhesion molecules contained in HS-TEX than in TEX (Fig. 1B). After blocking the adhesion molecules that were potentially involved in intercellular adhesion, we found that CD54 (and other unknown molecules) may be the major molecule involved in HS-TEX adhesion with DC (Fig. 2G).

**HS-TEX chemoattract, activate, and present tumor Ags to CD4+ and CD8+ T cells more potently**

Similar to the effects of HS-TEX on DC, we found that HS-TEX could chemoattract CD4+ and CD8+ splenic T cells (Fig. 3).
Moreover, when CD4+ and CD8+ T cells isolated from mice immunized twice with 3LL lysates were cocultured with TEX or HS-TEX, the proliferation (Fig. 3B) and the production of IL-2 (Fig. 3C) and IFN-γ (Fig. 3D) were potentiated by HS-TEX. In the presence of DC, the proliferating capacity and the production of IL-2 and IFN-γ by T cells were further potentiated (Fig. 3B–D). These data suggest that HS-TEX can directly activate T cells by functioning as Ag-presenting vesicles and possibly by presenting Ags through either MHC-I or MHC class II (MHC-II) pathways.

To examine the potential of HS-TEX in induction of antitumor immunity, we performed in vitro CTL induction experiments. We found that coculture of HS-TEX with splenic CD8+ T cells could induce higher levels of IFN-γ and more strong cytotoxic activity against 3LL cells (Fig. 3E, 3F), indicating that HS-TEX were competent in inducing Ag-specific antitumor immune response in vitro.

**HS-TEX more efficiently chemoattract and activate DC and T cells in vivo**

To examine the effects of HS-TEX on DC and T cells in vivo, we intratumorally injected HS-TEX into 3LL tumors. At different time points, DC and T cells infiltrated into tumor mass were evaluated by immunofluorescence microscopy or FACS assays. We found that HS-TEX could rapidly chemoattract more DC (Fig. 4A, 4B) and T cells (Fig. 4A, 4B) to the tumor tissue than TEX did.

To evaluate the functional status of infiltrated DC and T cells after HS-TEX injection, we isolated DC (CD11c+) and T (CD3+) cells from the tumor mass 4, 8, or 24 h after HS-TEX injection. We found that higher levels of MHC-II and CCR7 mRNA were detected in DC derived from HS-TEX–treated tumor compared with that detected in DC derived from TEX-treated tumor (Fig. 4C). Higher levels of IL-2 and IFN-γ mRNA were detected in T cells derived from HS-TEX–treated tumor compared with that detected in T cells derived from TEX-treated tumor (Fig. 4D). These data suggest that DC and T cells infiltrated into tumors after HS-TEX injection are significantly activated locally in tumor.

To investigate the effects of HS-TEX on the T cell activation by DC in vivo, we isolated splenic CD3+ T cells from HS-TEX–treated mice 24 h or 48 h after HS-TEX intratumoral injection. We found that the IL-2 and IFN-γ mRNA levels were significantly elevated by HS-TEX treatments (Fig. 4E), indicating that HS-TEX could promote the DC–T cell interaction and the activation of T cells in spleen.

**HS-TEX are more efficient in inhibiting tumor growth, activating CTL, and inducing tumor-specific protection**

TEX have been used to treat established murine tumors and can be used as a kind of vaccine that can protect mice from tumor challenge (6). And more importantly, exosomes derived from DC and tumor cells can induce Ag-specific antitumor response (5, 6). We performed the following experiments using 3LL tumor cells as a model to evaluate the effects of HS-TEX as therapeutic vaccines. First, we assessed the capacity of HS-TEX and TEX administration to induce antitumor immunity in a preestablished 3LL tumor model. We intratumorally injected HS-TEX or TEX 7 d after 3LL tumor inoculation (day 0). We found that administration of HS-TEX could more significantly inhibit 3LL tumor growth (p < 0.01; Fig. 5A) and could more significantly increase the survival rate of mice bearing 3LL tumor (p < 0.01; Fig. 5B) compared with those of the TEX-treated group. In the group treated with TEX, 20% of mice could completely eradicate the preestablished tumors; whereas in mice treated with HS-TEX, 50% of mice could completely eradicate the preestablished tumors (Fig. 5C). In pre-established B16 tumors (in C57/B6 mice) and CT26 tumors (in BALB/c mice), the intratumoral injection of HS-TEX derived from corresponding tumor cells could also significantly inhibit tumor growth and prolong the survival (data not shown) compared with those of the TEX-treated group.

CTL induction is an important indicator of tumor-specific antitumor immune response during tumor treatment. We found that CD8+ T cells derived from spleens of mice treated with HS-TEX could more significantly lyse 3LL tumor cells than that by the CD8+ T cells derived from spleens of mice treated with TEX (p < 0.01; Fig. 5D).

Next we investigated whether TEX or HS-TEX immunization could protect mice from tumor challenge. We found that HS-TEX immunization could protect 90% of the mice from subsequent 3LL tumor cell challenge, whereas TEX could only protect 60% of the mice from tumor challenge (Fig. 5E), supporting the idea that HS-TEX as well as TEX could serve as efficient tumor vaccines.
Chemokines are packaged in HS-TEX, and a fusion with target cells is required for the chemotactic activity

The above results demonstrated that HS-TEX were different to TEX in that they could more significantly chemotact and activate DC and T cells both in vitro and in vivo. The chemotactic activity of HS-TEX suggests that HS-TEX may contain chemokines. To test this proposal, we performed Western blot assays and found that chemokines, including CCL2, CCL3, CCL4, CCL5, and CCL20, were contained in HS-TEX (Fig. 6A). To confirm further this observation, we performed sucrose gradient ultracentrifugation assays of HS-TEX, and we found that chemokines were detected in gradients with the density between 1.12 and ∼1.19 g/ml (Fig. 6B). The data suggest that these chemokines were associated with exosomes.

Most of the components of exosomes, such as MHC molecules, adhesion molecules, and even HSP, were presented on the surfaces of TEX (1, 2). However, it may not be true for chemokines because chemokines were always included within vesicular structures (41). To examine the location of chemokines in HS-TEX, we treated the isolated HS-TEX with proteinase K for 30 min and reisolated the HS-TEX by ultracentrifugation. Whereas the MHC-I and HSC70 molecules were diminished after proteinase K treatment, we found that the association of chemokines (CCL2 and CCL5) with HS-TEX as well as the chemotactic activity toward DC were unaffected (Fig. 6C, 6D), indicating that chemokines were possibly packaged within HS-TEX.

Then we asked how HS-TEX could elicit the chemotactic activity to target cells. Considering that exosomes were of bilipidic layer structures that were capable of fusing with cells to intercommunicate between cells (1, 2), we speculated that HS-TEX may function as chemokines via membrane fusion. To test this hypothesis, we immobilized HS-TEX on the lower surface of the Transwell chamber by using anti-CD63 Ab and then performed in vitro chemotactraction assays. We found that chemotactic activity of HS-TEX to DC was significantly inhibited (Fig. 6D). In the presence of monensin and latrunculin A, inhibitors for liposome membrane fusion and endocytosis, respectively, the chemotactic activity of HS-TEX was also inhibited (Fig. 6D).

These data suggest that chemokines contained in HS-TEX may require a fusion and a subsequent endocytosis process for eliciting their chemotactic activity.

Release of HS-TEX is dependent on ATP release and calcium influx

Extracellular ATP is an important mediator for induction of vesicular release, and heat stress can induce ATP release (42). Therefore, we speculated that heat stress may induce ATP release, and then chemokine-containing HS-TEX is released. To test this possibility, we measured extracellular ATP after heat stress and found that heat stress could induce a rapid release of ATP as early as 2 min after heat treatment (Fig. 7A).

As one of the effects of extracellular ATP, mobilization of calcium influx is an important event in regulating vesicular transport (34, 41). We found that intracellular calcium concentration is increased by heat stress (Fig. 7B), which could be inhibited by apryase (for hydrolysis of ATP), suramin (ATP antagonists), EDTA (chelator of extracellular calcium), verapamil (calcium channel blocker) and BAPTA (selective calcium chelator) (Fig. 7C). Correspondingly, we found that the release of HS-TEX by heat stress (Fig. 7D) and the release of chemokines by exosomes (Fig. 7E) were inhibited by the above inhibitors. These data suggest that heat stress may induce extracellular ATP release, which, in turn, mobilizes calcium influx and induces the release of chemokine-containing HS-TEX.

FIGURE 8. Chemokines contained in HS-TEX are derived from lipid raft. A, HS-TEX obtained from 3LL cells pretreated or not pretreated with MβCD (10 mM) for 30 min and then treated with heat stress as indicated ("0" indicates TEX isolated from 3LL cells cultured for 1 h) were examined by Western blot for the indicated molecules. B, HS-TEX (5 μg) in A were examined for chemotactic activity to day 7 BMDC. Results are presented as mean ± SD of triplicate samples. *p < 0.05, ***p < 0.001. C and D, 3LL cells pretreated or not pretreated with MβCD (10 mM) for 30 min were treated with heat stress for 30 min and were isolated for lipid raft (C). TEX and HS-TEX obtained from the treated cells were also lysed in 1% Triton-X 100 and subjected to gradient ultracentrifugation (D). Then, an equal volume of each fraction was examined for indicated molecules by Western blot.
with lipid raft, we treated 3LL tumor cells with MβCD and examined the chemokines contained in HS-TEX. We found that MβCD treatment could significantly decrease the chemokine contained in HS-TEX (Fig. 8A). Correspondingly, we found that HS-TEX isolated from MβCD-treated cells failed to chemoattract DC (Fig. 8B).

To examine further the association of chemokines with lipid raft, we isolated lipid raft from the cells 30 min immediately after heat stress and examined the chemokines in lipid raft. We found that heat stress could induce the enrichment of CCL2 and CCL5 in lipid raft fractions (fractions 3–5), similar to the distribution of HSC70 in lipid raft (Fig. 8C). In HS-TEX isolated after heat stress, we found that the levels of chemokines, together with HSC70, but not CD71, were increased in lipid raft-insoluble fractions after heat stress (Fig. 8D). These results suggest that heat stress can induce the sorting of chemokines into lipid raft and then promote the release of chemokines in exosomes.

Discussion
Exosomes derived from DC and tumor cells, DEX and TEX, respectively, have been regarded as a tumor vaccine (1–4). For DEX, it is agreed by many researchers that they can induce antitumor immunity, which has been verified by clinical trials (3). However, controversies exist for TEX. Generally, TEX, also called tumor-derived microvesicles, lethal vesicles, and tolerosomes, have been suggested to be a mechanism for tumor escape and demonstrated to inhibit immune response (4). Several studies have shown that TEX can promote tumor progression and metastasis, in contrast to its known ability to inhibit immune response (4). Several studies have shown that TEX can promote tumor progression and metastasis, in contrast to its known ability to inhibit immune response (4).

Heat stress can induce the production and release of HSP, which, in turn, may serve as a source of tumor Ags and as a stimulator of immune cells (36). We have shown previously that HS-TEX derived from colon cancer cells and B lymphoma can efficiently induce tumor-specific antitumor immunity in mouse models (31, 32). In the current study, we demonstrated that HS-TEX from 3LL tumor cells could activate DC and T cells both in vitro and in vivo, leading to inhibition of tumor progression and even induction of tumor eradication, which suggests that HS-TEX may be a more efficient tumor vaccine than TEX. More HSP (e.g., HSP60, HSP70, HSC70, and HSP90) and membrane molecules (e.g., CD54, CD86) were detected in HS-TEX, which may explain the activating but not inhibiting effects of HS-TEX in eliciting antitumor immunity. Although we have not examined the effects of HS-TEX on NK cells, it may be inferred that HS-TEX may also increase NK activity via increased HSP expression, based on the study showing that TEX derived from melanoma cells over-expressing membrane-bound HSP70 can activate CD8+ T cells and NK cells (30). After heat stress, inhibitory molecules (such as FasL and TGF-β) were not increased (data not shown), whereas positive regulators (HSP, adhesion molecules, and, especially, chemokines) of immune response were significantly induced in HS-TEX, which together may revert the functions of TEX and lead to antitumor immunity. Therefore, our study suggests that HS-TEX may be an efficient tumor vaccine, and heat stress may change the functional features of TEX.

One unique finding in the current study is that chemokines are enriched in TEX after heat stress. Previously, it has also been found by several studies that cytokines, such as IL-1β, IL-10, and membrane TGF-β and TNF-α, can be present in exosomes (19–23, 32, 34, 35), indicating that TEX may be a method for release of cytokines. It has been well established that chemokines and cytokines with signal peptide are secreted via classical secretory routes (endoplasmic reticulum–Golgi–secretory vesicles), whereas molecules without signal sequence such as fibroblast growth factor and IL-1β are secreted via a nonclassical route (41). Notably, TEX derived from tumor cells genetically modified with cytokines may contain functional cytokines (26, 28, 29). In our study, we have detected several chemokines in HS-TEX, which are functionally competent in chemoattracting DC and T cells in vitro and in vivo. Our study suggests that these chemokines are packaged in exosomes and may elicit chemotactic activity through membrane fusion and endocytic pathway. Until now, it has not been elucidated how exosomes are used by cells as a method for release of cytokines. There are studies suggesting that molecules involved in membrane transport, such as Rab4, Rab5, Rab7, and Rab9, can be detected in exosomes (1, 2), suggesting that exosomes may be a physical machinery for cytokine release. Heat stress may dramatically affect the intracellular transport of cytokine/chemokine-containing vesicles. As a kind of physical stress, heat may directly affect lipid metabolism. We have shown that chemokines can be enriched in lipid rafts after heat stress, indicating that heat stress may alter the lipid composition of intracellular chemokine-containing vesicles. Considering that heat stress can also increase the yield of exosomes, it may be proposed that heat stress can alter not only the intracellular localization of chemokines but also subsequent exocytosis of chemokine-containing vesicles. One important finding to explain the alteration of the chemokine exocytosis pathway is that heat stress can induce ATP release and ATP-dependent calcium influx. Thus, calcium may regulate the translocation of chemokine-containing vesicles, as has been described by previous studies (41, 45). It has also been reported that SNARE molecules (e.g., SNAP-23, SNAP-25, syntaxins, and VAMPS), basic machinery to regulate membrane transport, can be detected in lipid rafts and may be involved in cytokine secretion (46–50). Therefore, release of chemokine-containing exosomes may be a physical mechanism for cytokine secretion, and heat stress may promote the presence of chemokines in cholesterol-rich lipid rafts and the subsequent vesicular release via exosomes. Further investigations are required to determine the structures (e.g., recycling endosomes) in which chemokines are packaged.

In sum, our study has extensively characterized the HS-TEX and suggests that HS-TEX may be an efficient tumor vaccine in induction of antitumor immune response potentially by chemotaxing and activating DC and T cells. It may be tempting to test the possibility of applying HS-TEX in clinical trials (or in combination with autologous DC) in the future. Our study also highlights that heat stress may induce the translocation of chemokines to cholesterol-rich lipid rafts and regulate the subsequent exocytosis of exosomes via ATP-induced calcium-regulated pathway, thus expanding the view of regulatory mechanisms involved in chemokine exocytosis.

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Disclosures
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