Tumor-derived exosomes elicit tumor suppression in murine hepatocellular carcinoma models and human in vitro

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/hep.28549
Key words: Exosome, hepatocellular carcinoma, immunotherapy, shared antigens, cross-protection

Financial Support: This research was supported by Chinese National Basic Research Program (973) (no. 2012CBA01305, 2012CB932503), National Natural Science Foundation of China (grant no. 81273420, 81361128013, 81501531 and 81301526) and Research Fund for the Doctoral Program of Higher Education of China (no. 20111202110002).
Abstract

Hepatocellular carcinoma (HCC) remains a global challenge due to high morbidity and mortality rates and poor response to treatment. Immunotherapy, based on introduction of dendritic cells (DCs) activated by tumor cell lysates as antigens ex vivo, shows limited response rate in HCC patients. Here, we demonstrate that tumor cell-derived exosomes (TEXs), displaying an array of HCC antigens, can elicit a stronger immune response than cell lysates in vitro and in vivo. Significant tumor growth inhibition was achieved in ectopic and orthotopic HCC mice treated with TEX-pulsed DCs. Importantly, the tumor immune microenvironment was significantly improved in orthotopic HCC mice treated by TEX-pulsed DCs, demonstrated by increased numbers of T lymphocytes, elevated levels of γ-interferon (IFN-γ) and decreased levels of interleukin-10 (IL-10) and tumor growth factor-β (TGF-β) in tumor sites. As expected, T cells played an essential role in TEX-pulsed DC-mediated immune response. Notably, exosomes from HCC cells not only promoted HCC-specific cytolysis but also provided cross-protective effects against pancreatic cancer cells. Moreover, HCC-specific cytolysis, elicited by DCs pulsed with human HepG2 cell-derived exosomes, was observed across different human HCC cells irrespective of human leukocyte antigen (HLA) types. Conclusions: Our findings provide evidence for the first time that HCC TEXs can potently carry HCC antigens, trigger strong DC-mediated immune response and improve HCC tumor micro-environment.
Introduction

Ranked as the sixth most lethal malignancy, hepatocellular carcinoma (HCC) attracts significant attention due to its aggressive nature, high mortality rate and low response rates to treatments in the clinic (1). Although surgical resection can extend HCC patients’ lifespan, it is only amenable to early-stage HCC patients and the high recurrent rate is far from ideal. Chemotherapeutic or radiotherapeutic interventions such as sorafenib, transcatheter arterial chemoembolization and radiofrequency ablation are intensively used in the clinic, however the survival benefit is limited (2).

Therefore, other interventional approaches have been rigorously pursued. Among these, dendritic cell (DC)-based immunotherapy is a promising strategy with clinical studies in man (3-5). The first-in-man clinical trial in HCC patients demonstrated proof-of-concept evidence for DC-based immunotherapy with HCC cell lysates as antigens, however the response rate was marginal and the efficacy was low (5), necessitating further investigation into other immunotherapy options.

Recently, the discovery of physiological roles of exosome, a nanovesicle secreted by cells, as an intercellular courier of protein, mRNA and miRNA have garnered considerable interest (6). Exosomes have been extensively studied for diagnostic purposes and drug delivery vehicles (7, 8). Particularly, exosomes from tumor cells (TEXs) can efficiently deliver a variety of tumor antigens to DCs and thus have been employed as antigen carriers for cancer immunotherapy (9). Wolfers et al demonstrated that a broad spectrum of TEXs could be employed as a source of shared
antigens for priming cytotoxic T lymphocytes (CTLs) and eliciting an immune response stronger than cell lysates in mice (10). This concept has also been adopted in other tumor models including leukemia and renal cell carcinoma (11-14). However, the potency of HCC TEXs remains to be determined in HCC models. The unique liver immune microenvironment, reflected by the inherent tolerogenic character of normal liver, renders HCC poorly immunogenic. It likely contributes to the limited efficacy observed in DC immunotherapy with cell lysates as antigens in clinical trials (5). The immune tolerogenicity of liver, resulting in immune escape in HCC, represents a critical challenge to immunotherapeutics in HCC. It will profoundly impact on DC-based immunotherapies for HCC if HCC TEXs could overcome this limitation by targeted presentation of a smaller subset of antigens.

Here we examine the feasibility and functionality of HCC TEXs to stimulate immune response in HCC models. Mouse HCC TEXs were investigated for their ability to transfer antigens to DCs and elicit antitumor immunity in vitro and in vivo. Our study demonstrates that HCC TEX-pulsed DCs (DC_{TEX}) could induce significantly stronger immune response and suppress tumor growth in HCC mice, compared with DCs pulsed with cell lysates. Notably, significant tumor inhibition was achieved in DC_{TEX}-treated HCC mice prophylactically and therapeutically. Tumor immune microenvironment was reshaped in DC_{TEX}-treated orthotopic HCC mice. Importantly, DC_{TEX} provided cross protection against allogeneic HCC cells and other major histocompatibility complex (MHC)-matched tumor cells, for example pancreatic...
cancer cells. HCC-specific cytolysis effect, elicited by $\text{DC}_{\text{TEX}}$, was also observed in human HCC cells, independent of human leukocyte antigen (HLA) types. Our findings provide evidence for the first time that TEXs can trigger DCs to mount an effective immune response in HCC mice and human HCC cells. This suggests that TEXs from different tumor cells could be used as a source of antigens to stimulate patient-derived DCs $\text{ex vivo}$ for DC-based immunotherapy in HCC and thus make it easier for timely intervention in HCC patients. More importantly, TEXs show superiority to clinically tested cell lysates as antigen carriers for DC-mediated immunotherapy in HCC, indicating that TEXs can be a competent antigen source.

**Methods and Materials**

**Mice**

$\text{C57BL/6}$ wild-type (H-2$^b$) and $\text{BALB/C}$ nude mice (H-2$^d$) (6-8 weeks old) were used in all experiments (5 or 10 mice were used in each group for ectopic or orthotopic studies, respectively, and the experiments were repeated for 3 times unless otherwise specified). All the animal experiments were carried out in the animal unit, Tianjin Medical University (Tianjin, China) according to procedures authorized and specifically approved by the institutional ethical committee (Permit Number: SYXXK 2009–0001). Mice were sacrificed by CO$_2$ inhalation or cervical dislocation at desired time-points, and tissues were either snap-frozen in liquid nitrogen-cooled isopentane or fixed with Bouin’s solution (Sigma, US) and embedded with paraffin for histological study.
Cell lines

Murine dentritic cell line DC2.4 (referred to DC) (H-2<sup>b</sup>) (kindly provided by Dr De Yang, Center for Cancer Research, NIH, US) was used for murine studies. DC cells were cultured as described previously (15). Briefly, DC cells were cultured in DMEM medium with 1% antibiotics, 1% glutamine (Gln), 1% β-mercaptoethanol and 10% depleted fetal bovine serum (FBS, Hyclone, US), obtained by centrifugation at 100,000g for 1 h to remove possible FBS-containing exosomes. Murine HCC cell line hepa1-6 (derived from C57L mice, H-2<sup>b</sup>) was purchased from Boster Biological Technology Ltd (Wuhan, China) and cultured in DMEM medium with 2 mM Glutamine and 10% FBS as per manufacturer’s instructions. Murine pancreatic cancer cell line (Panc02, H-2<sup>b</sup>) and non-small cell lung cancer cell line (LLC1, H-2<sup>b</sup>) were cultured as previously described (16, 17). Murine HCC cell line H22 (H-2<sup>d</sup>) (kindly provided by Professor Bo Huang, Huazhong University of Science and Technology, Wuhan, China) was cultured as described previously (18). Human HCC cell lines including HepG2 and Hep3B were purchased from ATCC biobank and cultured as per manufacturer’s instructions (19). Human HCC LM3 was cultured as described previously (20).

Establishment of orthotopic HCC mouse models by tissue implantation

Subcutaneous HCC mouse models were established by subcutaneous injection of hepa1-6 cells (3x10<sup>6</sup>) into left axilla of C57BL/6 mice. Then subcutaneous tumors with
a longitudinal diameter of 1 cm were peeled from subcutaneous mouse models after schedule 1 killing. Tumor tissues were washed in D-hanks buffer. Necrotic tissues were removed from tumors and tumor tissues were cut into about 1 mm$^3$ pieces. 2-3 tumor pieces were implanted in the left lobe of liver in the recipient mice under anesthesia.

**Isolation of human DCs and lymphocytes**

For human *in vitro* studies, human peripheral blood was obtained from healthy volunteers (provided by Tianjin Blood Center, Tianjin, China). Peripheral blood mononuclear cells (PBMCs) were isolated with human Lymphoprep solution (Axis-shield PoC AS, Oslo, Norway) as per manufacturer’s instruction, then cells were seeded in 10 cm Petri-dish with 10 ml RPMI 1640 medium plus 10% FBS and incubated for 2 h to allow cells to adhere to the surface. Adherent cells were harvested and induced to form immature DCs by culturing in RPMI 1640 medium containing 20% FBS, 120 ng/ml recombinant human GM-CSF (PeproTech, US) and 60 ng/ml recombinant human IL-4 (PeproTech, US) for 5 days. Non-adherent cells were recovered and cultured in DF12 medium containing 10% FBS for 7 days and were used as human lymphocytes.

**Preparation of exosomes and cell lysates**

Cell culture medium was sequentially centrifuged at 1000g for 10 min, followed by 10000g for 30 min. The supernatant was collected and filtered with a 0.22 µm filter
(Millex, Germany), followed by ultracentrifugation at 100,000g for 1 h to pellet exosomes. Exosome pellets were washed in a large volume of PBS and recovered by centrifugation at 100,000g for 1 h. The total protein concentration of exosomes was quantified by Bradford assay (Sangon Biotech, US). Hepa1-6 cell lysates were obtained by five successive cycles of freeze and thawing. Lysates were then fractionated by centrifugation (3000g x 30 min), and the supernatant was collected and filtered with a 0.22 µm filter. The concentration of cell lysates was quantified by Bradford assay.

**T cell proliferation assay**

Totally 1x10^7 T lymphocytes derived from lymph nodes of MHC-matched C57BL/6 mice were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technology, US) according to the manufacturer’s instruction. CFSE-labelled T cells (3x10^5) were co-incubated with DC or DC_{TEX} at different ratios (20:1; 10:1 or 5:1) for 4 days as described previously (22). DCs were pretreated with 10 µg/ml mitomycin C (Sigma, UK) for 2 h to inhibit cell division. Subsequently, PE-labelled anti-mouse CD3 monoclonal antibody (ebioscience, US) was used to counter-stain specific T cells at 4 ºC for 1 h, followed by flow cytometry.

**Cytolysis assay**

The cytotoxicity detection kit (R&D systems, US) was used to measure the cytolysis rate elicited by effector T cells against different tumor cells. The amount of lactate...
dehydrogenase (LDH) released from lysed target cells was used as an indicator for cytolysis. Cytolysis rate (%) was calculated based on the equation: cytotoxicity (%) = (effector/target cell mix - effector cell control – low control)/ (high control-low control) × 100.

**Measurement of T lymphocytes in mouse serum and tumor tissues**

Blood from HCC mice treated with DC$_{TEX}$, DC$_{lys}$, DC or PBS was collected with 1% heparin, followed by lysis with the ammonium chloride-potassium (ACK) lysis buffer for 5 min at room temperature to generate lymphocyte suspensions. Mixture of lymphocytes was stained with rat anti-mouse monoclonal antibodies including FITC-labelled CD3e, APC-labelled CD8a, PE-labelled CD4 and PE-cy7-labelled CD25, FITC-labelled Fox3P antibodies (ebioscience, US) at 4°C for 45 min, followed by flow cytometry. Isolation of T lymphocytes from mouse tumor tissues was adopted from previous report (23). Briefly, tumor tissues were minced into small pieces with surgical scissors, gently forced through a 200 µm-gauge stainless steel mesh with a sterile syringe plunger and digested in collagenase type IV suspension (0.05 mg/ml, Worthington Biochem. Corp., NJ, US) for 40 min at 37°C. The resulting suspension was filtered through the 70 µm cell strainer. The extract was centrifuged at 528g for 10 min and the supernatant was removed. The mixture was re-suspended with 10 ml 40% percoll (Pharmacia, Sweden) followed by centrifugation at 850g for 30 min at 22°C to remove the supernatant. Cell pellets were re-suspended in ammonium chloride-potassium (ACK) lysis buffer to remove red blood cells and the rest cells
were stained with different fluorescence-labelled antibodies as described above.

**Statistical analysis**

All data are reported as mean values ± SEM. Statistical differences between treatment and control groups were evaluated by SigmaStat (Systat Software, London, UK). Both parametric and non-parametric analyses were applied, in which the Mann-Whitney Rank Sum Test (Mann-Whitney U test) was used for samples on a non-normal distribution whereas a two-tailed t test was performed for samples with a normal distribution, respectively.

**Results**

**Enrichment of tumor-associated antigens and antigenic chaperones in TEXs**

To test the feasibility of hepa1-6 TEXs as a source of antigens for DC-mediated antitumor immunity in HCC, we first characterized TEXs with exosomal biomarker proteins including transmembrane protein CD63 and Alix. Consistent with previous reports (8), the TEX sample contained exosome protein markers but not cellular protein (Fig. 1A) and exhibited a characteristic saucer-cup shape structure (Fig.1B). The yield of hepa1-6 TEXs was about 0.24-0.4 µg of protein per million tumor cells in 24 h, which is similar to other tumor cells (10). It is reported that exosomes bear various molecular constituents of their cells of origin, and thus we examined the expression of two well-characterized HCC antigens - alpha-fetoprotein (AFP) and glypican 3 (GPC3) (24, 25). Strikingly, enriched AFP expression was detected in
TEXs compared with their parental cells, whereas similar levels of GPC3 expression were found between TEXs and their parental cells (Fig.1C). Further investigation on other immuno-modulators revealed the presence of antigenic chaperone - heat shock protein 70 (HSP70) in TEXs (26, 27) (Fig. 1C).

To test whether hepa1-6 TEXs can be taken up by DCs, PKH67-labelled TEXs were co-incubated with DCs and active cellular uptake was readily detectable at 2 h and peaked at 24 h after co-incubation (Supplementary Figs. S1A, B). Internalized PKH67-labelled TEXs were perinuclear and punctuate in appearance (Supplementary Fig. S1C). Notably, TEX uptake promoted DC maturation and activation as demonstrated by elevated levels of CD11c, a myeloid DC marker (28), major histocompatibility complex class I and II (MHC I and MHC II), co-stimulatory factors CD80, CD86 and intercellular adhesion molecule (ICAM) expression on DC TEX surface compared with untreated DCs (Fig.1D), suggesting TEXs is capable of activating DCs.

TEXs show superiority to cell lysates in eliciting DC-mediated antitumor immunity in vitro and in vivo

To examine if DC TEX could trigger T cell-mediated cytolysis against hepa1-6 cells, we optimized TEX concentrations required for effective antitumor cytolysis in vitro. Three different concentrations (20, 40 and 80 µg/ml) were tested with the same number of DCs (1x10^5), respectively. An evident dose-dependent trend was
established with TEXs and up to 50.4% cytolysis against hepa1-6 cells was yielded with effector T cells, primed by DC_{TEX} at the final concentration of 80 µg/ml (Fig. 2A). In contrast, 34.8% tumor lysis efficiency was obtained with the same number of effector T cells, primed by DCs pulsed with a saturated concentration of hepa1-6 cell lysates (100 µg/ml) (DC_{lys}) (Fig. 2A). To confirm the antigen-specific effect of hepa1-6 TEXs, control exosomes from a muscle cell (H_{2}K, H-2^{b}) were tested side-by-side and the results showed significantly lower cytolysis efficiency elicited by DCs pulsed with H_{2}K exosomes than DC_{TEX} (Supplementary Fig. S2). To exclude the possibility of technical artifacts, such as protein aggregates or membrane particles from fetal bovine serum (FBS), accounting for the observed antitumor effect elicited by TEXs, “mock” exosomes prepared from cell-free culture medium were loaded into DCs and used for cytolysis study in vitro. “Mock” exosome-pulsed DCs elicited a significantly low cytolysis rate compared with DC_{TEX}, similar to that of DCs alone (Supplementary Fig. S2). Also no endogenous murine retroviruses or mycoplasma contamination were found in parental cells (data not shown). These data demonstrated that hepa1-6 TEXs could induce antigen-specific cytolysis.

To investigate the capability of TEXs in triggering immune rejection in vivo, DCs (2x10^{6}) were pulsed with a suboptimal dose of TEXs (40 µg/ml) and compared with a saturated concentration of cell lysates (100 µg/ml). Activated DCs were injected intravenously into MHC-matched C57BL6 mice (H-2^{b}) bearing ectopic tumors (4.2±0.2 mm longitudinal diameter) 3 times at weekly interval. DC_{TEX}-treated mice
showed slower tumor growth with significantly reduced tumor volume compared with DC_{lys}-treated mice (Fig. 2B). Significantly stronger tumor suppression was achieved in DC_{TEX}-treated mice compared with DC_{lys}, DC and PBS treatment groups on day 11 (P=0.034), 13 (P=0.0003), 15, 17, 19, 21, 23 and 24 (P<1.0E-05, n=15) after inoculation under identical dosing conditions (Fig. 2C). Examination on T lymphocytes in serum from DC_{TEX}-treated mice indicated a significant increase in the number of CD8^{+} T lymphocytes, compared with DC_{lys}, DC and PBS treatment groups, though no difference was observed in the number of CD4^{+} T lymphocytes (Fig. 2D). Consistently, a significant increase in the number of CD8^{+} T lymphocytes was achieved in tumor tissues from DC_{TEX}-treated mice, compared with DC_{lys}, DC and PBS treatment groups (Fig. 2E), indicating that DC_{TEX} functions primarily through CTL priming. Notably, significantly reduced number of CD25^{+}FoxP3^{+}CD4^{+} regulatory T cells (Treg) was detected in tumor tissues from DC_{TEX}-treated mice, compared with DC_{lys}, DC and PBS treatment groups (Fig. 2F), suggesting DC_{TEX} treatment improved the tumor microenvironment in ectopic HCC mice. Overall, in vitro and in vivo studies demonstrated the superiority of TEXs to cell lysates in eliciting DC-mediated antitumor immunity.

**TEXs induce effective tumor suppression prophylactically**

Considering individuals with increased risk of HCC e.g. HBV carriers might benefit from immunization, we investigated the immunomodulatory effect of TEXs on HCC prophylactically. To boost the effect, C57BL6 mice were immunized intravenously
with $1 \times 10^6$ \( \text{DC}_{\text{TEX}} \) for 3 times at weekly interval, followed by challenge with $7 \times 10^5$ live hepa1-6 cells subcutaneously (Fig. 3A). Significant retardation of tumor growth was observed in \( \text{DC}_{\text{TEX}} \)-treated mice compared with DC or PBS treatment groups under identical dosing conditions, respectively (Fig. 3B). The difference was more evident between \( \text{DC}_{\text{TEX}} \) and DC or PBS groups at later time-points (Fig. 3C). An average tumor size of $14.38 \pm 1.39$ mm longitudinally was observed in \( \text{DC}_{\text{TEX}} \)-treated mice at 35 days after tumor challenge, whereas $21.2 \pm 1.8$ or $21.1 \pm 1.1$ mm detected in DC or PBS treatment groups under identical dosing conditions, respectively (Fig. 3C). Importantly, a long-lasting immune response with prolonged survival rate was achieved in 100% \( \text{DC}_{\text{TEX}} \)-treated mice at 58 days after tumor challenge, whereas no mice survived in DC or PBS treatment groups (Fig. 3D), indicating that TEXs are potent in conferring DC tumor-specific, long-lasting antitumor immunity in a prophylaxis setting.

**TEXs improve immune and tumor microenvironment in orthotopic HCC mice**

It is known that tumor microenvironment plays a critical role in tumor progression and treatment, particularly for HCC, thus HCC is notoriously difficult to treat due to its unique immunotolerogenic nature (29). To mimic the tumor microenvironment closely, we tested \( \text{DC}_{\text{TEX}} \) in \( C57BL6 \) mice bearing day-five-established orthotopic HCC. \( \text{DC}_{\text{TEX}} \) ($4 \times 10^6$) were injected intravenously into orthotopic HCC mice four times every four days apart with last injection 7 days after (Fig. 4A). Significant reduction in tumor growth was observed in \( \text{DC}_{\text{TEX}} \)-treated mice compared with DC or PBS treatment groups at 23 days after inoculation, with average tumor sizes of
2.4±0.3, 6.5±1.2 or 6.4±1.8 mm for DC_{TEX}, DC or PBS groups, respectively (Fig. 4B). DC_{TEX} treatment significantly prolonged the survival time and rate in 100% mice compared with DC or PBS treatment groups (Fig. 4C). Notably, DC_{lys} also resulted in significantly reduced tumor volume (an average tumor size of 3.9±0.26 mm) compared with DC and PBS groups, but to a much less extent than DC_{TEX} treatment under identical dosing conditions (Supplementary Fig. S3A). Importantly, repeated administration of DC_{TEX} resulted in significant improvement in immune microenvironment of orthotopic tumor-bearing mice, with significantly increased numbers of CD8^{+} T lymphocytes in serum from DC_{TEX}-treated mice compared with DC or PBS groups (Fig. 4D), to a less extent with DC_{lys} under identical dosing conditions (Supplementary Fig. 3B). Analysis of serum cytokines from DC_{TEX}-treated mice indicated a significant increase in γ-interferon (IFN-γ), a functional parameter of T-cell immune response (30) (Fig. 4E) and dramatic reductions in immuno-inhibitory interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (Fig. 4F). These results demonstrated that DC_{TEX} treatment altered the immune milieu from immuno-inhibitory to immuno-stimulatory and it is critical for the prognosis of HCC patients (31).

To investigate whether there is active recruitment of effector T lymphocytes to primary tumor sites in orthotopic HCC mice, we examined levels of T cell infiltration in tumor tissues. Quantitative analysis of T cells in tumor tissues demonstrated significantly increased numbers of CD8^{+} T cells and reduced numbers of
CD4+CD25+FoxP3+ Treg cells in tumor tissues from DC_{TEX}-treated mice compared with DC and PBS treatment groups (Fig. 5A, B), suggesting that DC_{TEX} treatment reshapes the tumor microenvironment in orthotopic HCC mice. Consistently, further immunohistochemical staining of CD3+ and FoxP3+ Treg cells in tumor tissues revealed a substantial number of CD3+ T lymphocytes and fewer FoxP3+ Treg cells in tumors from DC_{TEX}-treated mice compared with DC or PBS groups (Fig. 5C). Analysis of cytokines in tumor tissues also proved the improvement in tumor microenvironment demonstrated by significantly elevated levels of IFN-γ (Fig. 5D) and decreased levels of IL-10 and TGF-β (Fig. 5E). DC_{lys} also improved the tumor microenvironment in orthotopic HCC mice, but to a much less extent than DC_{TEX} under identical dosing conditions (Supplementary Fig. S3C, D). These findings supported the conclusion that DC_{TEX} can trigger a strong antitumor immune response and reshape the tumor microenvironment in orthotopic HCC mice.

TEXs activate T-cell dependent antitumor immunity

Given the striking antitumor effect of DC_{TEX} on immunocompetent mice, we next examined whether DC_{TEX} can mount antitumor immunity in immuno-deficient BALB/c nude mice (H-2b), a model for T cell deficiency due to the lack of thymus (32). The same dosing regimen used in immunocompetent mice was applied in immuno-deficient nude mice (BALB/C) with day-five established ectopic tumors. No difference in tumor volume was detected between DC_{TEX}-treated immuno-deficient nude mice compared with DC or PBS treatment groups, respectively (Fig. 6A),
suggesting that T cell is important for the functionality of DC_{TEX}. Further *in vitro* CFSE assay demonstrated a higher stimulatory capacity of DC_{TEX} (stimulator) at activating lymph node-derived naïve T cell (responder) clonal expansion compared with DC at different ratios of stimulator: responder (22). The ratio of 1:10 (stimulator: responder) showed the most evident difference between DC_{TEX} and DC (Supplementary Fig. S4). To determine the functionality of activated T cells, we measured levels of IFN-γ and IL-2 in supernatants from T cells activated by DC_{TEX} or DC for 3 days with a ratio of 1:10 (stimulator/responder). The results showed significantly increased levels of IFN-γ and IL-2 in T cells activated by DC_{TEX} compared with DC and T control groups (Fig. 6B), indicating T cells are responsive to DC_{TEX}. Effective cytolysis against hep1-6 cells was elicited with effector T cells activated by DC_{TEX} at different E:T (effector : target) ratios (Fig. 6C). Up to 72% cytolysis efficiency was observed in DC_{TEX}, whereas only 17.2% and 11.6% in DC or naïve T cell control groups at the ratio of 20:1 (E:T), respectively (Fig. 6C), supporting the conclusion that DC_{TEX} provokes T cell-dependent antitumor immunity. Similar levels of IFN-γ, IL2 and cytolysis were obtained in T cells activated by DC_{TEX} at a ratio of 1:5 (stimulator: responder) (Supplementary Fig. S5). These findings indicate that TEXs are competent antigen carriers for triggering DC-mediated and T cell-dependent cytolysis.

**TEXs confer DCs reciprocal cross-protection in mouse and human cells**

It is reported previously that tumor cell lysates demonstrated the capability of eliciting
cross-protection in a broad spectrum of HCC patients independent of HLA types (5), therefore we investigated whether TEXs, harboring various signatures of parental cells, could be a source of shared antigens for T cell cross-priming in other HCC cells with different genetic backgrounds. DC\textsubscript{TEX}-activated syngeneic effectors T cells (H-2\textsuperscript{b}), derived from spleen naïve T cells, were applied to lyse against allogeneic H22 HCC cells (H-2\textsuperscript{d}). Notably, a comparable tumor-specific cytolysis rate was achieved between MHC-matched hepa1-6 (41.6±0.61%) and allogeneic H22 cells (44.6±2%) (Fig. 6A). Furthermore, cross-protective effect was also detected for MHC-matched pancreatic cancer cells - Panc02 (C57BL6, H-2\textsuperscript{d}) (41.3±1.17%) but not for MHC-matched non-small cell lung cancer cells – LLC1 (C57BL6, H-2\textsuperscript{d}) (15.9±2%) with effector T cells activated by hepa1-6 TEX-pulsed DCs (Fig. 7A). This result suggests that hepa1-6 TEXs are a source of shared antigens not only for allogeneic HCC but also for MHC-matched Panc02. Further examination on the immune capacity of syngeneic DCs, pulsed with Panc02 or LLC1 TEXs, demonstrated that Panc02 or LLC1 TEX-pulsed syngeneic DCs effectively activated syngeneic spleen-derived naïve T cells and resulted in increased levels of IL-2 and IFN-γ release, compared with DC or T cell control groups, respectively (Supplementary Fig. S6A). Effective tumor-specific cytolysis was detected for both Panc02 and LLC1 cells with T cells activated by Panc02 or LLC1 TEX-pulsed DCs, respectively (Supplementary Fig. S6B, C). Importantly, effective cytolysis against hepa1-6 and H22 cells was observed with T cells activated by Panc02 TEX-pulsed DCs but not by LLC1 TEX-pulsed DCs (Supplementary Figs. S6B, C). Interestingly, no cross-protection
was detected in LLC1 cells with Panc02 TEXs (Supplementary Fig. S6) and the same for hepa1-6, H22 and Panc02 cells with LLC1 TEXs (Supplementary Fig. S6C), suggesting that potentially shared antigens exist between HCC and pancreatic cancer but not for non-small cell lung cancer.

To determine whether TEXs could induce tumor-specific cytolysis in human settings, we evaluated exosomes derived from HepG2, a human HCC cell line showing high levels of AFP expression (19). DCs were derived from healthy volunteers’ peripheral blood mononuclear cells (PBMCs) and pulsed with HepG2 TEXs. HepG2 TEX-pulsed DCs activated syngeneic human lymphocytes demonstrated by elevated levels of IL-2 and IFN-γ compared with DC or lymphocyte control groups (Fig. 7B). Co-incubation of activated effector lymphocytes with HepG2 cells resulted in a significantly higher tumor-specific cytolysis rate compared with DC or unprimed lymphocyte control groups (Fig. 7C). Notably, this tumor-specific cytolysis was also observed in other AFP-expressing human HCC cells such as Hep3B and LM3 (Fig. 7C) (19, 20), indicating human HepG2 TEXs are a source of competent antigens for providing cross-protection on human HCC cells, independent of HLA types.

Discussion

Therapeutic interventions in HCC represent a significant challenge due to the unique immune tolerogenicity of liver. DC-based immunotherapy showed promise in earlier human trials, but the effect was inadequate (5). Effective treatments are urgently
needed; particularly treatments that can counterbalance the immunosuppressive nature of liver tissue and augment tumor-specific immunity. Here we demonstrate that exosomes from HCC cells could serve as a carrier of a broad spectrum of antigens and elicit DC-mediated tumor rejection in both ectopic and orthotopic HCC mouse models. Of importance, DCTEX treatments improved the immune and tumor microenvironment of orthotopic HCC mice, demonstrated by increased levels of immuno-stimulatory cytokines and infiltrating CD8+ T cells, reduced levels of immuno-inhibitory Treg cells and cytokines. These results indicate the potential of TEXs as a source of potent antigens for amplifying DC-based antitumor immunity. This is the first study, to our knowledge, to investigate the potency of HCC TEXs in promoting DC-mediated antitumor effect in orthotopic HCC mice. Importantly, HCC TEXs not only promoted tumor-specific antitumor effect on MHC-matched cells but also on allogeneic HCC cells and MHC-matched pancreatic cancer cells, consistent with previous reports on other tumor-derived exosomes (10). Our data indicate that TEXs likely provide shared antigens for a variety of tumor types. Moreover, the tumor-specific cytolysis was also observed in human HCC cells with human HCC TEXs, independent of HLA types.

Although significant tumor growth inhibition was achieved with DCTEX in vivo, no complete tumor eradication was detected in our study, underlining the tenacity of HCC at overcoming current therapeutics. The dosing regimens and delivery routes are also likely to impact the immunotherapeutic effect of DCTEX (33), thus there is more room for optimization. When we compared the in vitro cytolysis effect of DC cells
(DC2.4 cell line) used in this study with the more clinically acceptable bone
marrow-derived primary DCs pulsed by hepa1-6 TEXs under an identical protocol,
we obtained similar results (data not shown). However the low yield of bone
marrow-derived DCs limits its scale-up, particularly for \textit{in vivo} studies; therefore we
used DC cell line (DC2.4) throughout murine studies.

Compared to ectopic mice, lower survival rate was obtained for the treatment groups
in orthotopic HCC mice, reflecting the immunotolerogenic nature and complexity of
orthotopic HCC and further underlining the importance of orthotopic models in
developing therapeutic approaches for HCC. Although orthotopic HCC
transplantation models used in the current study may not accurately recapitulate the
immunosuppressive nature of local tumor immune microenvironment manifested in
HCC patients, an elevated level of IFN-\(\gamma\), decreased levels of immunosuppressive
IL-10 and TGF-\(\beta\) and increased T lymphocyte infiltration in tumor tissues in
DC\textsubscript{TEX} treated orthotopic HCC mice does suggest that DC\textsubscript{TEX} treatment elicits strong
immunomodulatory effects. Our data demonstrated that DC\textsubscript{TEX} can activate functional
T cells and mount an adequate antitumor immune response to slow down the
progression of HCC. Further long-term investigation with repeated administration of
DC\textsubscript{TEX} in spontaneous HCC models is warranted.

The selection of the source of antigens is critical for DC-mediated immunotherapy as
no single antigen is ubiquitously expressed by HCC cells. Previous studies with
HLA-restricted peptides failed to trigger immune response and also there is a risk of immune escape with a single antigen (34, 35). Here we used HCC TEXs as a source of multiple antigens for DC-mediated immunotherapy. This approach took advantage of the unique characteristic of TEXs carrying a broad spectrum of antigens and could elicit antigen-specific responses from both CD4+ helper and CD8+ cytotoxic T cells, thus resulting in amplified immunotherapeutic effect of DCs, demonstrated by cross-protective reactivity against different HCC cells from mouse and human, independent of MHC or HLA types. The cross-protective effect between HCC and pancreatic cancer cells may represent a novel avenue for treating both HCC and pancreatic cancer but not limited to. We speculated that the cross-protective effect observed can be attributed to the presence of relevant shared candidate antigens in TEXs and further studies are warranted. Interestingly, the ability to target multiple tumors with the same TEX suggests that a small subset of TEXs derived from cultured tumor cells, when combined with patient-derived DCs, can form the basis for a larger set of tumors, thus streamlining the production of TEXs and allowing faster treatment than if one had to specifically harvest patient-derived TEXs.

Taken together, our study demonstrate for the first time that HCC TEXs can provide a source of multiple antigens at amplifying DC-mediated immunotherapeutic effect in HCC mouse models and human HCC cells. Importantly, HCC TEXs confer DC not only tumor-specific cytolysis but also provide cross protection for other types of HCC cells and pancreatic cancer cells, demonstrating its potential as an alternative antigen.
carrier to cell lysates for eliciting and augmenting DC-mediated immunotherapeutic
effect for HCC.

Acknowledgements
The authors thank Drs Yiqi Seow (Biomedical Sciences Institutes, A*STAR,
Singapore) and Mary McMenamin (Department of Physiology, Anatomy and Genetics,
Oxford University) for critical review of the manuscript.

Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Figure Legends

Figure 1. Characterization of hepa1-6 cell-derived exosomes (TEXs) and surface protein in TEX-pulsed DCs (DCTEX). (A) Western blot analysis for detecting the expression of exosomal biomarkers and cellular protein in hepa1-6 TEXs. Total protein (20 µg) was loaded for hepa1-6 cell lysates and TEXs, respectively. (B) Transmission electron microscopy (TEM) image for hepa1-6 TEXs (scale bar = 100 nm). (C) Western blots for examining levels of HCC-specific antigens and immune-related chaperone in hepa1-6 TEXs. Total protein (20 µg) was loaded for hepa1-6 cell lysates and TEXs to measure the expression of GPC3 and HSP70. Various amounts of hepa1-6 cell lysate and TEX loading used were noted in the image. (D) Flow cytometry for analyzing levels of surface proteins in DC or DCTEX. The experiments were repeated 3 times with triplicates each time (n=9).

Figure 2. Comparative studies of DCTEX and DCs pulsed with cell lysates (DClys) in vitro and in vivo. (A) In vitro comparison of cytolysis rates against hepa1-6 cells between DCTEX and DClys at different concentrations. A LDH-releasing cytotoxic assay was performed to measure the cytolysis efficiency of effector T cells activated by DCTEX or DClys, respectively. Significant difference was detected between 40 µg/ml DCTEX and saturated concentration of DClys (n=9, two-tailed t test, **P<0.01). The data represents mean±sem. (B) In vivo comparison between DCTEX and DClys in the capability of inhibiting tumor growth in day-five established ectopic HCC mice at final concentrations of 40 or 100 µg/ml, respectively. The experiments were repeated 3 times with 5 biological replicates each time (n=15). (C) Cross-comparison between
mice treated with DC_{TEX}, DC_{lys}, DC or PBS, respectively. Significant difference was achieved between DC_{TEX} and other three treatment groups on day 11 (two-tailed t test, P=0.034), 13 (two-tailed t test, P=0.0003), 15, 18, 19, 21, 23 and 24 (two-tailed t test, P<1.0E-05, n=15) after inoculation, respectively. (D) Analysis of CD4^{+} and CD8^{+} T lymphocytes in serum from ectopic tumor-bearing mice treated with DC_{TEX}, DC_{lys}, DC or PBS, respectively. Significant increase in the number of CD8^{+} T lymphocytes was detected in DC_{TEX}-treated mice compared with DC_{lys} treatment (two-tailed t test, P=0.042, n=15). (E) Analysis of CD4^{+} and CD8^{+} T lymphocytes in tumor tissues from ectopic tumor-bearing mice treated with DC_{TEX}, DC_{lys}, DC or PBS, respectively. Significant increase in the number of CD8^{+} T lymphocytes was detected in DC_{TEX}-treated mice compared with DC_{lys} treatment (two-tailed t test, P=0.012, n=15). (F) Analysis of CD25^{+}FoxP3^{+}CD4^{+} Treg cells in tumor tissues from ectopic tumor-bearing mice treated with DC_{TEX}, DC_{lys}, DC or PBS, respectively. Significant reduction in the number of CD25^{+}FoxP3^{+}CD4^{+} Treg cells was detected in DC_{TEX}-treated mice compared with DC_{lys} treatment (two-tailed t test, P=0.028, n=15).

**Figure 3. Prophylaxis studies on the antitumor effect of DC_{TEX} in MHC-matched C57BL/6 mice (H-2^{b}).** C57BL6 mice were immunized with PBS(●), DC (■) or DC_{TEX} (▲) for three times intravenously at weekly interval before challenged with 7\times10^{5} hepa1-6 cell suspensions at one week after the last immunization. (A) Schematic diagram for the dosing regimen of DC_{TEX} in C57BL6 mice prophylactically. I.v denotes intravenous injection. (B) Measurement of subcutaneous tumor weight at
35 days after inoculation in prophylactic settings. Significant difference was obtained between DC\textsubscript{TEX} and DC (two-tailed t test, \(P=0.036\)) or PBS (two-tailed t test, \(P=0.0013\), \(n=15\)). (C) Analysis of tumor volume from subcutaneous tumor-bearing mice immunized with PBS, DC or DC\textsubscript{TEX} at 35 days after inoculation, respectively (two-tailed t test, \(*P<0.05\), \(**P<0.01\), \(n=15\)). (D) Survival rate of subcutaneous tumor-bearing mice immunized with PBS, DC or DC\textsubscript{TEX}, respectively (two-tailed t test, \(**P<0.01\), \(n=15\)).

Figure 4. Systemic studies of DC\textsubscript{TEX} in MHC-matched HCC mice orthotopically.

MHC-matched orthotopic HCC mouse models were established by implanting 1 mm\(^3\) tumor tissues on the left lobe of liver of \textit{C57BL6} mice. Four consecutive intravenous treatments were applied to orthotopic HCC mice with PBS (○), \(4 \times 10^6\) DC (■) or DC\textsubscript{TEX} (▲), respectively, 4 days after implantation. (A) Schematic diagram for the dosing regimen of DC\textsubscript{TEX} in MHC-matched \textit{C57BL6} mice orthotopically. I.v denotes intravenous injection. (B) Measurement of tumor volume in orthotopic tumor-bearing mice treated with PBS, DC or DC\textsubscript{TEX}, respectively. The tumor volume was calculated on day 23 after implantation. Significant tumor suppression was yielded between DC\textsubscript{TEX} and DC or PBS (two-tailed t test, \(**P<0.001\), \(n=30\)). (C) Survival rate of tumor-bearing mice treated with PBS, DC or DC\textsubscript{TEX}, respectively (two-tailed t test, \(**P<0.01\), \(n=30\)). (D) Flow cytometric analysis of CD4\(^+\) and CD8\(^+\) T lymphocytes in serum from orthotopic tumor-bearing mice treated with PBS, DC or DC\textsubscript{TEX}, respectively, on day 23 after implantation. Significant increase in the number of CD8\(^+\)
T lymphocytes was detected between DC_{TEX} and DC or PBS (two-tailed t test, **P<0.001, n=30). (E) Measurement of IFN-γ in serum from tumor-bearing mice treated with DC_{TEX}, DC or PBS on day 23 after implantation. Significant difference was found between DC_{TEX} and DC or PBS (two-tailed t test, **P<0.001, n=30). (F) Measurement of immunosuppressive cytokines including TGF-β and IL-10 in serum from treated mice with ELISA. The comparison was conducted between DC_{TEX} and DC or PBS groups (two-tailed t test, *P<0.05, n=30).

Figure 5. Investigation of the tumor microenvironment in orthotopic HCC mice treated by repeated intravenous administration of DC_{TEX}. (A) Analysis of CD4^+ and CD8^+ T lymphocytes in tumor tissues from orthotopic tumor-bearing mice treated with DC_{TEX}, DC or PBS, respectively. Significant increase in the number of CD8^+ T lymphocytes was detected in DC_{TEX}-treated mice compared with DC or PBS groups (two-tailed t test, *P<0.05, n=30). (B) Analysis of CD25^+FoxP3^+CD4^+ Treg cells in tumor tissues from orthotopic tumor-bearing mice treated with DC_{TEX}, DC or PBS, respectively. Significant reduction in the number of CD25^+FoxP3^+CD4^+ Treg cells was detected in DC_{TEX}-treated mice compared with DC and PBS treatments (two-tailed t test, *P<0.05, n=30). (C) Immunohistochemistry of CD3^+ and FoxP3^+ Treg cells in tumor sections from mice treated with PBS, DC or DC_{TEX}, respectively (scale bar = 100 µm). Arrowhead points to CD3^+ or FoxP3^+ T cells. (D) Measurement of IFN-γ in tumor tissues from treated mice with ELISA. The comparison was conducted between DC_{TEX} and DC or PBS groups (two-tailed t test, **P<0.01, n=30).
(E) Measurement of TGF-β and IL-10 in tumor tissues from treated mice with ELISA. The comparison was conducted between DC_{TEX} and DC or PBS groups (two-tailed t test, **P<0.01, n=30).

**Figure 6. DC_{TEX} mediated T cell-dependent antitumor effect.** (A) Assessment of DC_{TEX} mediated antitumor effect in immunodeficient BALB/C nude mice. Nude mice were immunized intravenously with PBS (●), DC (■) or DC_{TEX} (▲), respectively, for three times at weekly interval followed by challenge with 7x10^5 hepa1-6 cell suspension a week after the last immunization (n=5). (B) Measurement of IFN-γ and IL-2 in supernatants from the co-culture of spleen naïve T cells with DC or DC_{TEX} at the ratio of 10:1 (T: DC) (two-tailed t test, **P<0.01, n=15). (C) Cytolysis assay for effector T cells activated by DC_{TEX} or DC against hepa1-6 target cells at different E: T (effector: target) ratios (two-tailed t test, **P<0.01, n=15).

**Figure 7. Studies of tumor-specific cytolysis in mouse and human HCC cells and cross-protective effect against pancreatic cancer cells with TEX-pulsed DCs.** (A) Tumor-specific cytolysis for allogeneic HCC cell and cross-protection for MHC-matched pancreatic cancer cells with hepa1-6 TEX-pulsed DCs. The comparison was conducted between DC_{TEX} and PBS or DC groups (two-tailed t test, **P<0.01, n=15). (B) Measurement of IL-2 and IFN-γ in the culture medium after the co-incubation of human lymphocytes with human DCs or HepG2 TEX-pulsed DCs (two-tailed t test, *P<0.05, n=15). (C) Tumor-specific cytolysis of different human HCC cells with human lymphocytes activated by human DCs or HepG2 TEX-pulsed
DCs, respectively. The comparison was conducted between DC\textsubscript{TEC} and DC or PBS groups (two-tailed t test, *P<0.05; **P<0.01, n=15).
Figure 1. Characterization of hepa1-6 cell-derived exosomes (TEXs) and surface protein in TEX-pulsed DCs (DCTEX). (A) Western blot analysis for detecting the expression of exosomal biomarkers and cellular protein in hepa1-6 TEXs. Total protein (20 µg) was loaded for hepa1-6 cell lysates and TEXs, respectively.
(B) Transmission electron microscopy (TEM) image for hepa1-6 TEXs (scale bar = 100 nm).

50x26mm (300 x 300 DPI)
(C) Western blots for examining levels of HCC-specific antigens and immune-related chaperone in hepa1-6 TEXs. Total protein (20 µg) was loaded for hepa1-6 cell lysates and TEXs to measure the expression of GPC3 and HSP70. Various amounts of hepa1-6 cell lysate and TEX loading used were noted in the image.
(D) Flow cytometry for analyzing levels of surface proteins in DC or DCTEX. The experiments were repeated 3 times with triplicates each time (n=9).

178x126mm (300 x 300 DPI)
Figure 2. Comparative studies of DCTEX and DCs pulsed with cell lysates (DClys) in vitro and in vivo. (A) In vitro comparison of cytolysis rates against hepa1-6 cells between DCTEX and DClys at different concentrations. A LDH-releasing cytotoxic assay was performed to measure the cytolysis efficiency of effector T cells activated by DCTEX or DClys, respectively. Significant difference was detected between 40 µg/ml DCTEX and saturated concentration of DClys (n=9, two-tailed t test, **P<0.01). The data represents mean±sem.
(B) In vivo comparison between DCTEX and DClys in the capability of inhibiting tumor growth in day-five established ectopic HCC mice at final concentrations of 40 or 100 µg/ml, respectively. The experiments were repeated 3 times with 5 biological replicates each time (n=15).

148x111mm (300 x 300 DPI)
(C) Cross-comparison between mice treated with DCTE, DClys, DC or PBS, respectively. Significant difference was achieved between DCTE and other three treatment groups on day 11 (two-tailed t test, $P=0.034$), 13 (two-tailed t test, $P=0.0003$), 15, 18, 19, 21, 23 and 24 (two-tailed t test, $P<1.0E-05$, $n=15$) after inoculation, respectively.
(D) Analysis of CD4+ and CD8+ T lymphocytes in serum from ectopic tumor-bearing mice treated with DCTEX, DClys, DC or PBS, respectively. Significant increase in the number of CD8+ T lymphocytes was detected in DCTEX-treated mice compared with DClys treatment (two-tailed t test, P=0.042, n=15).
(E) Analysis of CD4+ and CD8+ T lymphocytes in tumor tissues from ectopic tumor-bearing mice treated with DCTEX, DClys, DC or PBS, respectively. Significant increase in the number of CD8+ T lymphocytes was detected in DCTEX-treated mice compared with DClys treatment (two-tailed t test, \( P=0.012, n=15 \)).
(F) Analysis of CD25+FoxP3+CD4+ Treg cells in tumor tissues from ectopic tumor-bearing mice treated with DCTEX, DClys, DC or PBS, respectively. Significant reduction in the number of CD25+FoxP3+CD4+ Treg cells was detected in DCTEX-treated mice compared with DClys treatment (two-tailed t test, $P=0.028$, $n=15$).

125x76mm (300 x 300 DPI)
Figure 3. Prophylaxis studies on the antitumor effect of DCTEX in MHC-matched C57BL/6 mice (H-2b). C57BL6 mice were immunized with PBS(λ), DC (ν) or DCTEX (▲) for three times intravenously at weekly interval before challenged with 7x10^5 hepa1-6 cell suspensions at one week after the last immunization. (A) Schematic diagram for the dosing regimen of DCTEX in C57BL6 mice prophylactically. I.v denotes intravenous injection.
56x14mm (300 x 300 DPI)
(B) Measurement of subcutaneous tumor weight at 35 days after inoculation in prophylactic settings. Significant difference was obtained between DCTEX and DC (two-tailed t test, \( P=0.036 \)) or PBS (two-tailed t test, \( P=0.0013, \) \( n=15 \)).

120x200mm (300 x 300 DPI)
(C) Analysis of tumor volume from subcutaneous tumor-bearing mice immunized with PBS, DC or DCTEX at 35 days after inoculation, respectively (two-tailed t test, *P<0.05, **P<0.01, n=15).

93x65mm (300 x 300 DPI)
(D) Survival rate of subcutaneous tumor-bearing mice immunized with PBS, DC or DCTEX, respectively (two-tailed t test, **P<0.01, n=15).
Figure 4. Systemic studies of DCTEX in MHC-matched HCC mice orthotopically. MHC-matched orthotopic HCC mouse models were established by implanting 1 mm3 tumor tissues on the left lobe of liver of C57BL6 mice. Four consecutive intravenous treatments were applied to orthotopic HCC mice with PBS (λ), 4 x10^6 DC (ν) or DCTEX (▲), respectively, 4 days after implantation. (A) Schematic diagram for the dosing regimen of DCTEX in MHC-matched C57BL6 mice orthotopically. I.v denotes intravenous injection.
(B) Measurement of tumor volume in orthotopic tumor-bearing mice treated with PBS, DC or DCTEX, respectively. The tumor volume was calculated on day 23 after implantation. Significant tumor suppression was yielded between DCTEX and DC or PBS (two-tailed t test, **P<0.001, n=30).
(C) Survival rate of tumor-bearing mice treated with PBS, DC or DCTEX, respectively (two-tailed t test, **P<0.01, n=30).

62x47mm (300 x 300 DPI)
(D) Flow cytometric analysis of CD4+ and CD8+ T lymphocytes in serum from orthotopic tumor-bearing mice treated with PBS, DC or DCTEX, respectively, on day 23 after implantation. Significant increase in the number of CD8+ T lymphocytes was detected between DCTEX and DC or PBS (two-tailed t test, **P<0.001, n=30).
(E) Measurement of IFN-γ in serum from tumor-bearing mice treated with DCTEX, DC or PBS on day 23 after implantation. Significant difference was found between DCTEX and DC or PBS (two-tailed t test, **P<0.001, n=30).

61x42mm (300 x 300 DPI)
(F) Measurement of immunosuppressive cytokines including TGF-β and IL-10 in serum from treated mice with ELISA. The comparison was conducted between DCTEX and DC or PBS groups (two-tailed t test, *P<0.05, n=30).

62x21mm (300 x 300 DPI)
Figure 5. Investigation of the tumor microenvironment in orthotopic HCC mice treated by repeated intravenous administration of DCTEX. (A) Analysis of CD4+ and CD8+ T lymphocytes in tumor tissues from orthotopic tumor-bearing mice treated with DCTEX, DC or PBS, respectively. Significant increase in the number of CD8+ T lymphocytes was detected in DCTEX-treated mice compared with DC or PBS groups (two-tailed t test, *P<0.05, n=30).
(B) Analysis of CD25+FoxP3+CD4+ Treg cells in tumor tissues from orthotopic tumor-bearing mice treated with DCTEX, DC or PBS, respectively. Significant reduction in the number of CD25+FoxP3+CD4+ Treg cells was detected in DCTEX-treated mice compared with DC and PBS treatments (two-tailed t test, *P<0.05, n=30).
(C) Immunohistochemistry of CD3+ and FoxP3+ Treg cells in tumor sections from mice treated with PBS, DC or DCTEX, respectively (scale bar = 100 µm). Arrowhead points to CD3+ or FoxP3+ T cells.
(D) Measurement of IFN-γ in tumor tissues from treated mice with ELISA. The comparison was conducted between DCTEX and DC or PBS groups (two-tailed t test, **P<0.01, n=30).

68x46mm (300 x 300 DPI)
(E) Measurement of TGF-β and IL-10 in tumor tissues from treated mice with ELISA. The comparison was conducted between DCTEX and DC or PBS groups (two-tailed t test, **P<0.01, n=30).

69x23mm (300 x 300 DPI)
Figure 6. DCTEX mediated T cell-dependent antitumor effect. (A) Assessment of DCTEX mediated antitumor effect in immunodeficient BALB/C nude mice. Nude mice were immunized intravenously with PBS (○), DC (■) or DCTEX (▲), respectively, for three times at weekly interval followed by challenge with 7x10^5 hepa1-6 cell suspension a week after the last immunization (n=5).
(B) Measurement of IFN-γ and IL-2 in supernatants from the co-culture of spleen naïve T cells with DC or DCTEX at the ratio of 10:1 (T: DC) (two-tailed t test, **P<0.01, n=15).

57x19mm (300 x 300 DPI)
(C) Cytolysis assay for effector T cells activated by DCTEX or DC against hepa1-6 target cells at different E:T (effector: target) ratios (two-tailed t test, **P<0.01, n=15).

101x83mm (300 x 300 DPI)
Figure 7. Studies of tumor-specific cytolysis in mouse and human HCC cells and cross-protective effect against pancreatic cancer cells with TEX-pulsed DCs. (A) Tumor-specific cytolysis for allogeneic HCC cell and cross-protection for MHC-matched pancreatic cancer cells with hepa1-6 TEX-pulsed DCs. The comparison was conducted between DCTEX and PBS or DC groups (two-tailed t test, **P<0.01, n=15)
(B) Measurement of IL-2 and IFN-γ in the culture medium after the co-incubation of human lymphocytes with human DCs or HepG2 TEX-pulsed DCs (two-tailed t test, *P<0.05, n=15)
68x23mm (300 x 300 DPI)
(C) Tumor-specific cytolysis of different human HCC cells with human lymphocytes activated by human DCs or HepG2 TEX-pulsed DCs, respectively. The comparison was conducted between DC TEX and DC or PBS groups (two-tailed t test, *P<0.05; **P<0.01, n=15).
Figure S1. Cellular uptake of PKH67-labelled hepa1-6 TEXs in DCs. (A) Fluorescence microscopic analysis of cellular uptake of PKH67-labelled hepa1-6 TEXs in DCs at different time-points. (B) Quantitative analysis of hepa1-6 TEX cell uptake in DCs with flow cytometry at 24 h or 48 h, respectively. (C) Confocal fluorescence microscopic image of cellular localization of hepa1-6 TEXs in DCs. Green represents PKH67-labelled exosomes (TEX-PKH67); blue is for nuclei counterstained with Hoechst 33342 (scale bar = 10 μm).
Figure S2. Evaluation of “mock” and control exosomes in promoting DC-mediated antitumor cytolysis in vitro. “Mock” or control exosomes were derived either from cell-free culture medium or MHC-matched immortalized mouse myoblasts (H2K, H-2b). Significantly greater cytolysis efficiency was achieved with DCTEX compared to DCmock and DCH2K (two-tailed t test, P=1.07E-06, n=15). DCH2K referred to DCs pulsed with exosomes from H2K cells.
Figure S3. Investigation of cell lysate-pulsed DCs (DC_{lys}) in MHC-matched orthotopic HCC mice. DCs (4x10^6) pulsed with a saturated concentration of cell lysates (100 µg/ml) (DC_{lys}) were injected intravenously into orthotopic C57BL/6 HCC mice for 4 times every 4 days with the last injection 7 days after. (A) Measurement of tumor volume in orthotopic tumor-bearing mice treated with PBS, DC or DC_{lys} respectively. The tumor volume was calculated on day 23 after implantation. Significant tumor suppression was yielded between DC_{lys} and DC or PBS, respectively (two-tailed t test, **P<0.001, n=30). (B) Analysis of CD4^+ and CD8^+ T lymphocytes in serum from orthotopic tumor-bearing mice treated with DC_{lys}, DC or PBS, respectively. Significant increase in the number of CD8^+ T lymphocytes was detected in DC_{lys}-treated mice compared with DC and PBS groups (two-tailed t
test, \( P<0.05, \) \( n=30 \). (C) Analysis of CD4\(^+\) and CD8\(^+\) T lymphocytes in tumor tissues from orthotopic tumor-bearing mice treated with DC\(_{\text{lys}}\), DC or PBS, respectively. Significant increase in the number of CD8\(^+\) T lymphocytes was detected in DC\(_{\text{lys}}\)-treated mice compared with DC and PBS groups (two-tailed t test, \( P<0.05, \) \( n=30 \)). (D) Analysis of CD25\(^+\)FoxP3\(^-\)CD4\(^+\) Treg cells in tumor tissues from orthotopic tumor-bearing mice treated with DC\(_{\text{lys}}\), DC or PBS, respectively. Significant reduction in the number of CD25\(^+\)FoxP3\(^+\)CD4\(^+\) Treg cells was detected in DC\(_{\text{lys}}\)-treated mice compared with DC and PBS groups (two-tailed t test, \( P<0.05, \) \( n=30 \)).
Figure S4. Carboxyfluorescein diacetate succinimidyl ester (CFSE) assay for T cell proliferation in vitro. Naïve T lymphocytes were collected from lymph nodes of MHC-matched C57BL/6 mice and stained with CFSE followed by priming with DC or DC_{TEX} at different ratios for 4 days. Activated T lymphocytes were recovered and stained with FITC-labelled CD3 mouse monoclonal antibody and analyzed by flow cytometry.
Figure S5. Tumor-specific cytokine release and cytolysis elicited by activated T cells primed by DC_{TEX} in vitro. (A) Measurement of cytokines released by splenic lymphocytes activated by DC or DC_{TEX} at a ratio of 5:1 (T cell: DC). (B) Quantitative analysis of tumor-specific cytolysis with different ratios of effector T cells and hepa1-6 target cells. The comparison was performed between DC_{TEX} and DC or PBS groups, respectively (two-tailed t test, **P<0.01, n=15).
Figure S6. Reciprocal cross-protection between pancreatic cancer and HCC TEXs in vitro. (A) Cytokine release assay from activated splenic T lymphocytes primed by either Panc02 TEX-pulsed DCs (DC_{P-TEX}) or LLC1 TEX-pulsed DCs (DC_{L-TEX}). IL-2 and IFN-γ secreted by activated T cells were measured with ELISA. (B) Tumor-specific cytolysis and cross-protective effect of activated T cells primed by pancreatic cancer TEX-pulsed DCs on autologous parental cells or HCC cells. (C) Tumor-specific cytolysis of activated T cells primed by non-small cell lung cancer (LLC1) TEX-pulsed DCs on autologous parental cells. The comparison was performed between DC_{TEX} and DC or PBS groups, respectively (two-tailed t test, *P<0.05; **P<0.01, n=15).
Supplementary Methods and Materials

Cellular uptake assay and electron microscopy

Hepa1-6 TEXs were labelled with a membrane dye PKH67 (Sigma, US) and co-incubated with murine DCs at the final concentration of 10 μg/ml. Cellular uptake was monitored with conventional fluorescence microscopy (Olympus, Japan) at different time-points from 0 to 48 h, followed by quantification with flow cytometry (BD, US). Confocal fluorescence microscopy (Olympus, Japan) was used to examine the localization of PKH67-labelled exosomes in DCs and nuclei were counter-stained with Hoechst 33342 (Sigma, US). For electron microscopic analysis, hepa1-6 TEXs were loaded onto a copper grid and stained with 1% phosphotungstic acid for 2 min at room temperature and observed with transmission electron microscopy (Hitachi HT7700, Japan).

Cell lines

Murine immortalized muscle cell line H2K (H-2b) was kindly provided by Professor Terry Partridge (Children’s National Medical Center, Washington D.C., US) and cultured in DMEM medium with 20% FBS at 33°C under 10% CO₂.

Western blot

Protein extraction and western blot were carried out as previously described (21). Various amounts of protein prepared from hepa1-6 cell lysates and TEXs were loaded onto SDS–polyacrylamide gel (10%). The membrane was then washed and blocked
with 5% skimmed milk and probed for 1 h with different primary antibodies including mouse monoclonal antibodies: Alix and Cytochrome C (Cell signaling technology, US); and rabbit polyclonal antibodies: CD63 (Santa Cruz, US), Hsp70 (Cell signaling technology, US), AFP (abcam, UK) and GPC3 (ImmunoWay, US) with a dilution of 1 in 1000, respectively. The bound primary antibodies were detected by peroxidase-conjugated rabbit anti-mouse or goat-anti-rabbit IgGs (Sigma, US), respectively, and the ECL Western blot analysis system (Millipore, Billerica, MA) was used.

**Flow cytometry**

Murine DCs were co-incubated with hep1-6 TEXs for 24 h and stained with FITC-labelled anti-mouse isotype control IgG (Biolegend, UK), CD80, CD86, ICAM1 (abcam, UK), MHCI (ebioscience, US), MHCII (Biolegend, UK) and Percp-cy5.5-labelled anti-mouse CD11c antibodies (ebioscience, US) at the final concentration of 1 μg/ml at 4 °C for 45 min, respectively, followed by flow cytometry (BD, US).

**Cytokine release assay**

Murine or human DCs were pulsed with hep1-6 or HepG2 TEXs for 24 h at the final concentration of 40 μg/ml, respectively. T cell proliferation assay was carried out as described above. After T lymphocytes were activated with DC or DCTEX for 3 days, levels of IFN-γ and IL-2 in supernatant were detected. For mouse in vitro study, IFN-γ and IL-2 ELISA kits were purchased from R&D systems and ebioscience (US). For in
vivo cytokine assay, mouse serum was harvested from orthotopic tumor-bearing mice 2 days after the last immunization and centrifuged at 3000 g for 30 min at room temperature, followed by analysis of IFN-γ (R&D systems, US), IL-10 (MultiSciences Biotech Co., Ltd., China) and TGF-β (MultiSciences Biotech Co., Ltd., China), respectively. For human in vitro study, ELISA kits for IFN-γ (DAKEWEI, China) and IL-2 (DAKEWEI, China) were used, respectively.

**Immunohistochemistry**

To examine the presence of T lymphocytes and regulatory T cells in tumor sections from orthotopic HCC mice treated with DC_{TEX} or control groups, mouse tumor tissues were fixed in Bouin’s solution (Sigma, US) and embedded with paraffin. CD3⁺ or Foxp3⁺ T lymphocytes were stained with rabbit-anti-mouse polyclonal antibodies CD3 (Novus, US) or Foxp3 (abcam, UK) at the dilution of 1 in 250, respectively, and detected by goat-anti-rabbit secondary antibody.