



## Application of immunotherapy based on dendritic cells stimulated by tumor cell-derived exosomes in a syngeneic breast tumor mouse model

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### ABSTRACT

We here evaluated the therapeutic effect of tumor cell-derived exosomes (TEXs)-stimulated dendritic cells (DCs) in a syngeneic orthotopic breast tumor model. The DC line DC2.4 and breast cancer cell line E0771 originally isolated from C57BL/6 mice were used. E0771 cells stably expressing the exosomal CD63-RFP or luciferase (Luc) and DC2.4 cells stably expressing GFP were produced using lentivirus. TEXs were purified from conditioned medium of E0771/CD63-RFP cells. Breast tumor model was established by injecting E0771/Luc cells into mammary gland fat pad of mice. TEXs contained immune modulatory molecules such as HSP70, HSP90, MHC I, MHC II, TGF- $\beta$ , and PD-L1. TEXs were easily taken by DC2.4 cells, resulting in a significant increase in the *in vitro* proliferation and migration abilities of DC2.4 cells, accompanied by the upregulation of CD40. TEX-DC-treated group exhibited a decreased tumor growth compared with control group. CD8<sup>+</sup> cells were more abundant in the tumors and lymph nodes of TEX-DC-treated group than in those of control group, whereas many CD4<sup>+</sup> or FOXP3<sup>+</sup> cells were localized in those of control group. Our results suggest a potential application of TEX-DC-based cancer immunotherapy.

### 1. Introduction

Dendritic cells (DCs), which are the most potent antigen-presenting cells of the immune system, induce T cell-mediated antitumor effects [1]. DC-based immunotherapy has been used in clinical trials for patients with cancer [2–4]. *Ex vivo* methods of tumor antigen loading of DCs, with peptides, cytokines, and cell lysates (CLs) are still unfulfilled in preclinical and clinical trials for cancer immunotherapy [5].

Exosomes, which are extracellular vesicles that can carry proteins, lipids, RNAs, and DNAs originating from cells, are mediators of intracellular communication [6]. Tumor cell-derived exosomes (TEXs), which carry diverse immunomodulatory proteins such as tumor antigens, heat shock proteins (HSPs), major histocompatibility complex

class I and II (MHC I and II), and transforming growth factor beta (TGF- $\beta$ ), are involved in immune suppression and immune stimulation at the level of DCs [7–13]. TEXs can effectively taken up by DCs and can fully maturate DCs. During the development of maturity, the expression of costimulatory receptor molecules of CD40, CD80, CD86 and MHC molecules on DCs plasma membrane is increased. These activated DC molecules bind to the receptors on the T cell, which lead T cell differentiation into pro or anti-inflammatory. CD40 is a major regulator of DCs which overexpressed when DC maturate and migration into lymph nodes to initiate immune response. CD40/CD40L interaction is important for upregulation of IL-12 cytokine, which enhances proliferation of CD8<sup>+</sup>T cells [5,9,14,15].

DCs strongly boost antitumor activity by controlling T cells [16]. T

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lymphocyte antigens CD3, CD4, CD8, and FOXP3 in breast cancer tissue serve as independent prognostic factors [17,18]. DCs stimulated by TEXs, which are derived from glioma, thymoma, and hepatoma cells, elicit potent antitumor responses by activating CD8<sup>+</sup> T cells and decreasing the number of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cells [19–21]. Thus, TEX-stimulated DCs have attracted much attention as a potential source of antitumor immunotherapy.

Clinically, the treatment options for triple-negative breast cancer (TNBC) have been limited due to the lack of well-defined molecular targets. Additional immunotherapy for TNBC has led to promising regimens. In our previous study [15], 4T1-TEXs, which were purified from a mouse TNBC cell line 4T1 overexpressing the exosomal CD63-red fluorescent protein (RFP) fusion protein, increased the DC proliferation and migration capacities and the CD40, CD80, and CCR7 levels in DCs. In addition, TEXs have also been shown to promote DC migration toward lymph nodes (LNs) *in vivo* using ultrasound-guided photoacoustic imaging. Considering the findings from previous studies, we hypothesized that TEX-stimulated DCs might be relevant in strategies for antitumor treatment.

Here, we used the breast cancer cell line E0771 and DC line DC2.4 established from C57BL/6 mice for studying immunotherapy application based on TEX-stimulated DCs in a mouse syngeneic model. In this study, we investigated the *in vitro* DC activation by TEXs derived from E0771 cells and the antitumor potential of TEX-stimulated DCs in syngeneic breast tumors in mice inoculated with E0771 cells.

## 2. Materials and methods

### 2.1. Cell culture

The murine breast cancer cell line E0771 was obtained from the CH3 BioSystems (Amherst, NY, USA). DC2.4 cells were kindly provided by K. L. Rock at the Dana-Farber Cancer Institute [22]. The E0771 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (WelGene, Daegu, Korea) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL). The DC2.4 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% HEPES, 1% nonessential amino acids, 1% glutamine, 55 µM 2-mercaptoethanol, penicillin (100 units/mL), and streptomycin (100 µg/mL), in an incubator set at 5% CO<sub>2</sub> and 37 °C.

### 2.2. Stable cell line establishment

For the isolation of RFP-tagged exosomes, a stable E0771 cell line (E0771/CD63-RFP) overexpressing the exosomal CD63-RFP fusion protein was generated by lentiviral transduction using a pCT-CD63-RFP Cyto-Tracer (System Biosciences, Palo Alto, CA, USA). A stable E0771 cell line (E0771/Luc) expressing luciferase-GFP and a stable DC2.4 cell line (DC2.4/GFP) expressing GFP protein were generated by lentiviral transduction.

### 2.3. TEX isolation and nanoparticle tracking analysis

Conditioned media were obtained from E0771/CD63-RFP cells grown at subconfluence for three to four days in exosome-depleted growth media (Gibco Laboratories, Carlsbad, CA, USA). TEXs were isolated from collected conditioned media by using the Exo-spin™ Exosome Purification Kit (Cell Guidance Systems, Cambridge, UK). The size distribution and concentration of exosomes were determined using a NanoSight NS500 (Malvern, Grovewood Road, UK) equipped with a 642-nm laser and a charge-coupled device (CCD) camera, and data were analyzed using Nanoparticle Tracking Analysis software [23].

### 2.4. Confocal microscopy

For the live imaging of RFP-tagged TEX uptake by DC2.4 cells, GFP-transduced DC2.4 cells ( $1 \times 10^4$ ) were cultured in exosome-depleted media. At 24, 48, and 72 h after the administration of 30 µg/mL of TEXs into DC2.4 cells, the cellular uptake of TEXs was monitored using a laser scanning confocal microscope (Leica, Wetzlar, Germany) at 558 nm/605 nm and 488 nm/509 nm for excitation/emission, respectively.

### 2.5. Western blot

Cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). The proteins extracted from E0771 cells, DC2.4 cells, and purified TEXs were incubated with primary antibodies against CD63, Alix A, TGF-β2, HSP70 and HSP90 (Santa Cruz Biotechnology, Dallas, Texas, USA), MHC I and II, PD-L1, and MUC1 (Abcam, Cambridge, UK), Calnexin and β-actin (Sigma-Aldrich) and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Quantification of band intensities on the western blot was carried out using ImageJ software and normalized to levels of β-actin.

### 2.6. Proliferation assay

TEXs (10–50 µg/mL) were administered to DC2.4 cells for 24–72 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (250 µg/mL) was added to each well and incubated for 1 h at 37 °C. After the incubation, the Formazan crystals formed were solubilized by the addition of 150 µL of dimethyl sulfoxide to each well. Absorbance was measured at 540 nm using a microplate reader.

### 2.7. Transwell migration assay

DC2.4 cells, which were activated with TEXs (30 µg/mL) or a complex with TNF-α (20 ng/mL) and IFN-γ (20 ng/mL), were seeded in the upper chamber of the transwell plate with a 0.8-µm pore size (Corning, Lowell, MA, USA). The lower chamber was filled with CCL19 (250 ng/mL) and CCL21 (250 ng/mL). After a 24-h incubation, cells that migrated into the lower chamber were fixed in 4% paraformaldehyde solution, followed by staining with 1% crystal violet. Crystal violet from the stained membrane was extracted with 1% SDS. The optical density at 550 nm was measured using a microplate reader.

### 2.8. Flow cytometry

Cells were incubated with anti-CD40-fluorecein isothiocyanate (FITC), anti-CD80-FITC, and anti-CD86-FITC antibodies (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at 4 °C. Cell-associated fluorescence was measured immediately after the staining using a FACSCalibur flow cytometer equipped with the CellQuest™ program (BD Biosciences).

### 2.9. Ethical statement and animal research

Animal research was carried out in compliance with the ARRIVE guideline. All protocols in this study were approved by the Committee on the Ethics of Animal Experiments of Seoul National University Hospital, Seoul, Korea (IACUC No. 19-0077-S1A0(1)), in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. A total of 10 female C57BL/6 mice were used.

### 2.10. Orthotopic breast tumor mice and TEX-stimulated DC treatment

A total of  $4 \times 10^5$  E0771/Luc cells were directly injected into the mammary fat pad of five-week-old C57BL/6 female mice (Orient Bio, Sungnam, Korea). Mice were randomly divided into the PBS-injected control group (n = 5) and the TEX-DC-injected group (n = 5). In the

TEX-DC group, a total of  $2 \times 10^6$  DC2.4 cells stimulated with TEX (30  $\mu\text{g}/\text{mL}$ ) for 24 h were administered via four repeated injections into the dermis around the axillary LNs of mice at seven-day intervals, 11 days after the tumor cell injection.

### 2.11. Bioluminescence imaging

The mice were anesthetized with isoflurane and then intraperitoneally injected with 150  $\mu\text{g}/\text{g}$  of the firefly luciferase substrate D-luciferin (Promega, San Luis Obispo, CA, USA). *In vivo* bioluminescence imaging of tumors was performed using the IVIS Lumina II system (Caliper, Hopkinton, MA, USA) with the use of Living Image acquisition and analysis software.

### 2.12. Immunohistochemistry

Immunohistochemical staining was performed with primary antibodies (CD4, CD8, CD3, and FOXP3) and appropriate HRP-conjugated secondary antibodies and was subsequently stained with a counter hematoxylin solution (Millipore Ltd., Darmstadt, Germany). Histological images of stained tissues were acquired using a microscope equipped with a CCD camera (Leica).

### 2.13. Statistical analyses

All experiments were performed in three or five independent samples for each condition. The results are expressed as mean  $\pm$  standard error. GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform two-tailed unpaired *t*-test and analysis of variance. Statistical significance was set at  $P < 0.05$ .

## 3. Results

### 3.1. Analysis of TEXs isolated from E0771/CD63-RFP cells

Confocal fluorescence imaging revealed E0771/CD63-RFP cells that stably expressed a common exosomal marker of CD63 tagged with RFP (Fig. 1A). Fig. 1B shows the representative NanoSight tracking analysis video frames of TEXs isolated from E0771/CD63-RFP cells. Histograms showing the size distribution of TEXs revealed heterogeneous sizes ranging from 50 to 150 nm in diameter (Fig. 1C). The measured mean diameter was  $113.1 \pm 1.1$  nm and mode  $95.7 \pm 3.3$  nm. Western blotting revealed that purified TEX expressed specific exosomal marker proteins,

such as CD63 and Alix, but not the endoplasmic reticulum membrane marker Calnexin (Fig. 1D).

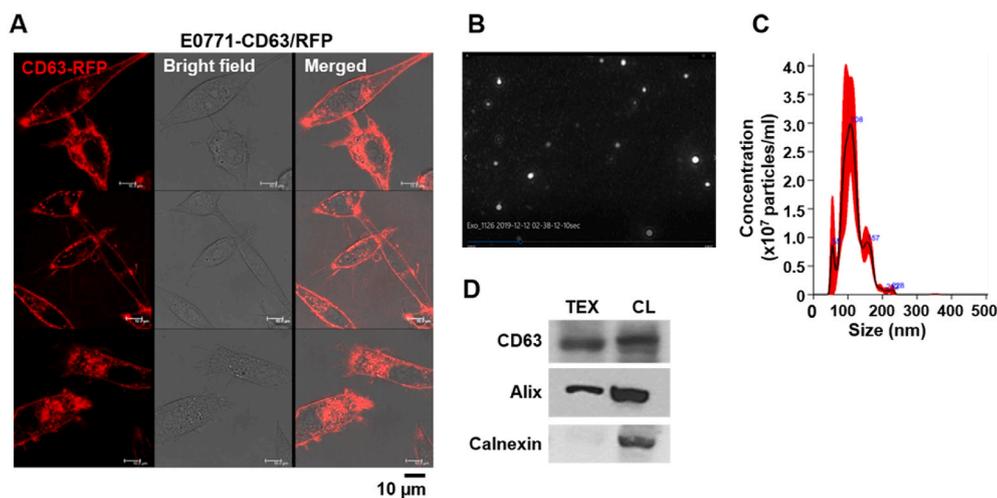
### 3.2. Properties and proliferation activity of TEX-stimulated DCs

TEXs contained molecules involved in antitumor activity and immunogenicity such as HSP70, HSP90, MHC I, MHC II, TGF- $\beta$ , and PD-L1, but not MUC1 (Fig. 2A). On live cell imaging using confocal laser scanning microscopy, DCs took up a large amount of RFP-tagged TEXs at 24 h after the administration of TEXs and displayed a spindle-shaped morphological change at 36–72 h (Fig. 2B). TEXs dose-dependently promoted the proliferation of DCs, but the administration of 50  $\mu\text{g}/\text{mL}$  TEXs for 48 h reduced DC growth (Fig. 3A). High concentration of 100–500  $\mu\text{g}/\text{mL}$  TEXs resulted in cytotoxicity of DCs (Supplementary Fig. 1). TEXs increased the migration ability of DCs toward chemokines (CCL19 and CCL21), compared with TNF- $\alpha$ /IFN- $\gamma$  and phosphate buffer solution (PBS) (Fig. 3B, TEX:  $119.5 \pm 2.3$ , TNF- $\alpha$ /IFN- $\gamma$ :  $107.2 \pm 1.4$ ,  $P < 0.0001$ ). Flow cytometry results revealed that TEXs significantly increased the levels of CD40 on DCs (Fig. 3C, TEX:  $2.7 \pm 0.02$ ,  $P < 0.0001$ ).

### 3.3. Application of immunotherapy based on TEX-stimulated DCs in a breast tumor mouse model

For TEX-DC-based immunotherapy application in a breast tumor mouse model, the study was conducted according to the experimental schedule depicted in Fig. 4A. One of five tumor-bearing mice in each group (TEX-DC and control) died during this experimental period; thus bioluminescence images of tumors were analyzed in a total of four mice per group (Fig. 4B). Total photon flux was lower in TEX-DC group than in control group 30 days post-injection (Fig. 4C, TEX-DC;  $44.17 \pm 12.83$  vs. Control;  $9.44 \pm 3.38$ ,  $P = 0.026$ ).

A large population of intratumoral CD3<sup>+</sup> T cells was observed in both groups, but a small population of CD4<sup>+</sup> T cells infiltrated the tumors (Fig. 4D). A few intratumoral CD8<sup>+</sup> T cells were present in TEX-DC group but not of control group (Fig. 4D). FOXP3<sup>+</sup> T cells were not detected in the tumors in either group. Similarly, many CD3<sup>+</sup> cells were detected in the paracortex of axillary LNs in both groups (Fig. 4E). CD4<sup>+</sup> and FOXP3<sup>+</sup> T cell populations decreased in the axillary LNs of TEX-DCs group compared with those of control group, whereas CD8<sup>+</sup> T cells were observed in the tumors of TEX-DC group but not those of control groups (Fig. 4E).



**Figure 1.** Generation of stable E0771 cells expressing the exosomal CD63-RFP fusion protein and analysis of exosomes isolated from E0771 cell culture medium. (A) Confocal images of CD63-RFP-transduced E0771 cells. Scale bar: 10  $\mu\text{m}$ . (b) Representative exosome tracking analysis video frames of TEXs. (C) Measurements of TEX sizes and concentrations. (D) Western blot of CD63, Alix, and calnexin in TEXs and E0771 cell lysates (CL).

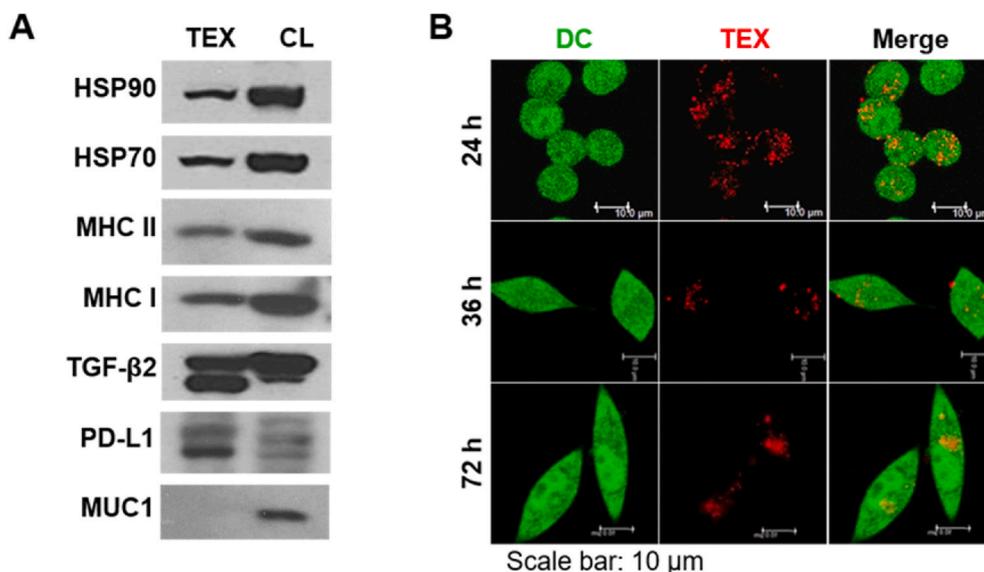


Fig. 2. Western blot for immunomodulatory proteins in TEXs and live images for TEX uptake in DC2.4 cells. (A) Western blot of HSP90, HSP70, MHC I, MHC II, TGF- $\beta$ 2, PD-L1, and MUC1 in TEXs and cell lysates (CL). (B) Confocal images of GFP-labeled DC2.4 cells after the administration of TEXs (30  $\mu$ g/mL) for 24–72 h.

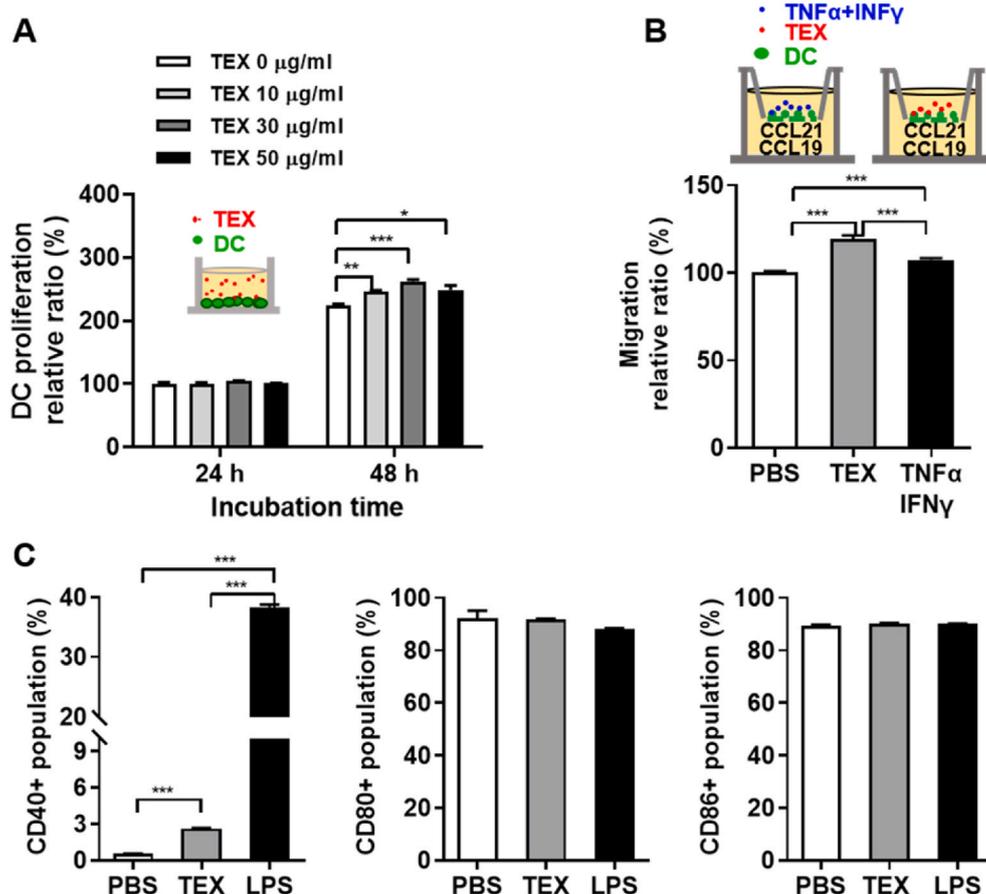
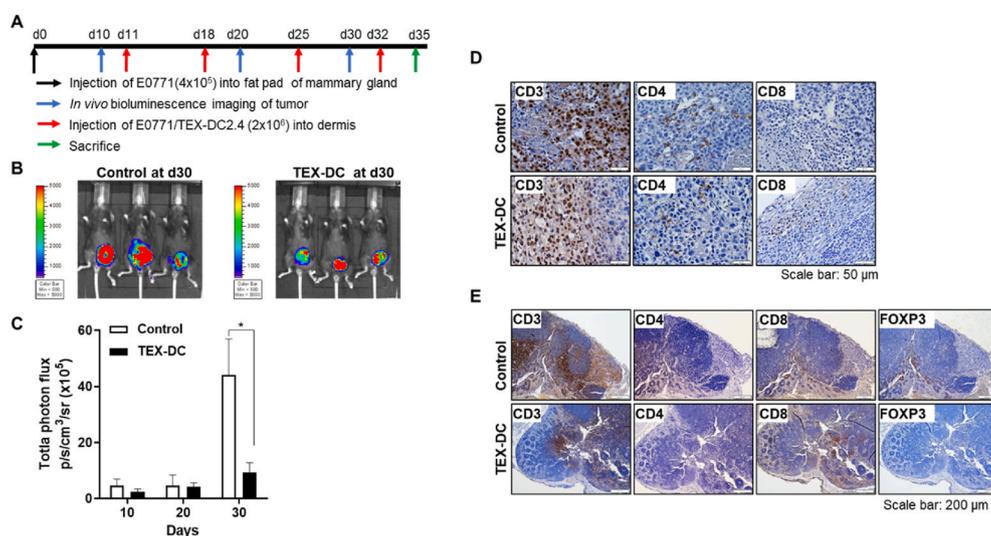


Fig. 3. Analysis of proliferation, migration and co-stimulatory molecules expression of TEX-stimulated DC2.4 cells. (A) MTT assay for evaluating the DC2.4 cell proliferation stimulated by TEXs (30  $\mu$ g/mL) for 24–48 h. (B) Transwell migration assay for evaluating the TEX (30  $\mu$ g/mL)- or TNF- $\alpha$ /IFN- $\gamma$  (20 ng/mL)-stimulated DC2.4 cell migration into CCL19/CCL21 for 24 h. (C) Flow cytometry analysis of CD40, CD80, and CD86 on DC2.4 cells treated with TEX (30  $\mu$ g/mL) or lipopolysaccharide (LPS, 100 ng/mL) for 24 h. Data are shown as mean  $\pm$  standard error.

#### 4. Discussion

TEXs carry valuable information originating from cells and play key roles in intercellular communication as biological messengers of cells [6]. TEXs have gained attention for their efficient delivery of diverse immunomodulatory proteins to DC cells [7–10,14,24–27]. E0771-TEXs

contain proteins that cause immune activation and suppression; HSP70 and HSP90 have antitumor activity, MHC I and MHC II are involved in immunogenicity. There are reports that exosomes carrying MHC complexes prime specific immune responses [7–13]. Although up to 50  $\mu$ g/mL of E0771-TEXs promoted DC2.4 cell migration and proliferation activities and increased CD40 on DC2.4 cells without



**Fig. 4.** *In vivo* bioluminescence imaging and immunohistochemical analysis. (A) Experimental schedule of treatment with DC2.4 cells ( $2 \times 10^6$ ) stimulated by TEX (20–30  $\mu\text{g}/\text{mL}$ ) and bioluminescence imaging of C57BL/6 mice implanted with E0771/Luc cells ( $4 \times 10^5$ ). (B) Bioluminescence imaging of tumors in mice 30 days after the injection of E0771/Luc cells into the fourth mammary gland fat pad. (C) Total photon flux measured from tumors of TEX-DC and control groups. Data are shown as mean  $\pm$  standard error. (D–E) Immunohistochemical analysis of CD3, CD4, CD8, and Foxp3 on tumor and lymph node microsections.

cytotoxicity, these biological changes caused by E0771-TEXs (10–50  $\mu\text{g}/\text{mL}$ ) on DC2.4 cells were weak compared with those caused by 4T1-TEXs (5–10  $\mu\text{g}/\text{mL}$ ) in our previous studies [15]. These findings suggest that the weak effect of E0771-TEXs on the proliferation and migration activities of DC2.4 cells *in vitro* may be caused by the immunosuppressive proteins within E0771-TEXs. TEXs (50–100  $\mu\text{g}/\text{mL}$ ) isolated from TNBC cells (4T1, MDA-MB-231) resulted in the upregulation of PD-L1 and suppression of CD80 and CD86 expression on DCs, causing apoptosis and blocking DC maturation and migration. However, the manipulated 4T1-TEXs of 4T1 cells induced DC maturation and improved antitumor immunity by priming cytotoxic T cells [28]. In this study, exosomal PD-L1 and TGF- $\beta$  was detected in E0771-TEXs, and the treatment with TEXs at 100–500  $\mu\text{g}/\text{mL}$  causes cytotoxicity of DCs. TGF- $\beta$  induces DC apoptosis [26,29], and an activation of PD-L1/PD-1 pathways is involved in the process of cytotoxicity of immune cells including DCs [27,30]. We figure out that a high dose of TEXs transferred a large amount of PD-L1 and TGF- $\beta$  proteins to DCs can activate the cytotoxic mechanism, and impair DC-mediated anti-tumor immunity. In order to induce the most successful anti-tumor immune response by TEX-stimulated DCs, future work on exosome engineering should be required; the deletion of immunosuppressive molecules such as PD-L1 and TGF- $\beta$  within TEXs may provide for a potent TEX-DC-based antitumor therapy in the E0771 tumor model. In addition, combination therapy with the PD-L1 and PD-1 antibodies-loaded nanoparticle might increase the efficacy of TEX-DCs.

Treatment with an immunotherapy-based combination can improve early-stage TNBC patient survival [31]. Activated DCs have been shown to act as a bridge to co-stimulate CD3<sup>+</sup> and CD8<sup>+</sup> cytotoxic T cells and CD4<sup>+</sup> helper T cells [32,33]. CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> tumor-infiltrating T cells in breast cancer tissues are related to clinicopathological variables and survival outcome [34]. CD8<sup>+</sup> T cells are the key effector cell population mediating effective antitumor immunity, resulting in better clinical outcomes, whereas CD4<sup>+</sup> T cells have negative prognostic effects on breast cancer patient outcomes [18]. FOXP3 expression, which is involved in inducing immunosuppressive functions, is reported to be higher in malignant than in normal tissue, restricted to CD4<sup>+</sup> T cells, and low or absent in CD8<sup>+</sup> T cells [35]. In the E0771 breast tumor mouse model, a large number of CD3<sup>+</sup> T cells were found in the tumors and LNs of both TEX-DC and control groups, whereas small populations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were detected. CD4<sup>+</sup> or FOXP3<sup>+</sup> T cells were more abundant in the LNs of control group than in those of TEX-DC group, whereas CD8<sup>+</sup> T cells were observed in the tumors of TEX-DC group, suggesting that TEX-DCs act as a bridge to work in concert to stimulate CD8<sup>+</sup> T cells.

A specific biomarker and drug-loaded nanoparticles have attracted the attention for targeting strategy of imaging and therapy of cancer [36, 37]. Very recently, Li Y et al. developed the platform consisting of TEXs and nanoparticles with near-infrared (NIR) fluorescence imaging and NIR light-triggered therapy [38,39]. This platform of combined TEX and nanoparticle has been growing interest for simultaneously providing an effective targeted cancer treatment. However, various types of nanoparticles can induce oxidative stress-mediated cytotoxicity by generating reactive oxygen species. Therefore, the development of a functionalized nanoparticle delivery system with antioxidant capabilities is needed. In our study, even without a delivery system such as nanoparticles, TEXs easily are uptaken by DCs, and activate DCs to inhibit tumor growth. Future perspectives of drug-loaded functionalized nanoparticle along with TEX can elicit an effective antitumor immune response by activating DCs.

Exosomes have emerged as signal messengers, mediating intercellular communications as well as inter-organ crosstalk. In many cancer cells, abnormal TEX biogenesis and secretion are linked with mitochondrial and lysosomal dysfunction to support tumor progression [40]. TEX carrying mutant mitochondrial DNA can reprogram mitochondrial function and contribute to endocrine therapy resistance in breast cancer [41]. Accelerated lysosomal exocytosis enhances the release of TEX propagating signaling molecules to neighboring cells to facilitate breast cancer growth, invasion, and metastasis [42]. The crosstalk between TEXs and mitochondria and lysosomes may represent a potential therapeutic target in cancer.

## 5. Conclusion

In the present study, we used E0771 and DC2.4 cell lines, which are derived from C57BL/6 mice [24,43,44], in order to investigate DC-induced immune responses in a syngenic breast tumor mouse model. We successfully established E0771 cells producing exosomes with a stable expression of CD63-RFP expression and a diameter of  $113.1 \pm 2.2$  nm. In TEXs isolated from E0771 cells (E0771-TEXs), the exosomal specific markers CD63 and Alix as well as immune activators and inhibitors such as HSP70 and HSP90, MHC I and II, TGF- $\beta$ 2, and PD-L1 were detected at the protein level. TEXs increased the *in vitro* proliferation and migration abilities of DCs, accompanied by the upregulation of CD40. In an orthotopic breast tumor mouse model, the administration of TEX-DCs did not elicit a potent antitumor effect, but TEX-DC group exhibited a decreased tumor growth accompanying cytotoxic CD8<sup>+</sup> T cell infiltration in tumor tissues compared with control group.

Taken together, this study has shown a potential of TEX-DC-based

cancer immunotherapy in a syngenic breast tumor mouse model models; However, to improve the effect of an antitumor immunotherapy based on TEX-DCs, immunosuppressive factors existing in TEXs must be excluded.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.101136>.

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