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Tumor-derived exosomes: the next generation of promising cell-free vaccines in cancer immunotherapy

Marzieh Naseri[a,b], Mahmood Bozorgmehr[b], Margot Zöller[c], Ehsan Ranaei Pirmardan[d], and Zahra Madjd[a,b]

[a]Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran; [b]Oncopathology Research Center, Iran University of Medical Sciences, Tehran, Iran; [c]Section Pancreas Research, University Hospital of Surgery, Heidelberg, Germany; [d]Molecular Biomarkers Nano-imaging Laboratory, Brigham & Women's Hospital, Department of Radiology, Harvard Medical School, Boston, MA, USA

ABSTRACT

Identification of immunogenic tumor antigens that are efficiently processed and delivered by dendritic cells to prime the immune system and to induce an appropriate immune response is a research hotspot in the field of cancer vaccine development. High biosafety is an additional demand. Tumor-derived exosomes (TEXs) are nanosized lipid bilayer encapsulated vesicles that shuttle bioactive information to the tumor microenvironment facilitating tumor progression. However, accumulating evidence points toward the capacity of TEXs to efficiently stimulate immune responses against tumors provided they are appropriately administered. After briefly describing the function of exosomes in cancer biology and their communication with immune cells, we summarize in this review *in vitro* and preclinical studies eliciting the potency of TEXs in inducing effective anti-tumor responses and recently modified strategies further improving TEX-vaccination efficacy. We interpret the available data as TEXs becoming a lead in cancer vaccination based on tumor antigen-selective high immunogenicity.

Introduction

Chemotherapy and radiotherapy are the most frequent types of adjuvant therapies in progressed cancer, which are curative for some tumor types.[1,2] Although they have been shown to have pivotal benefits in the elimination of primary tumors, the challenges imposed by tumor recurrence and metastasis remain largely unsolved. Therefore, alternative therapeutic modalities are urgently warranted. Immunotherapy is a promising pillar in cancer therapy with the advantage of having no or minor side effects. Unfortunately, despite exciting progress, therapeutic efficacy has been limited and rather sporadic; this has been ascribed to the poor or absent immunogenicity of most tumor antigens as well as immunosuppressive features of many tumor cells.[3,4]

Dendritic cells (DCs) are known as the most potent professional antigen-presenting cells, which activate helper T cells (Th) via presenting antigenic peptides in MHCII molecules. This finding has created new hopes for boosting cancer immunotherapy, these as activated Th cells can support maturation and activation of CD8^{+} cytotoxic T cells.[5] DCs also could strengthen NK activity through by facilitating antibody secretion via Th or directly via B cells.[6,7] Indeed, the elaboration of *in vitro* expansion and activation of DCs, and loading DCs with tumor antigens provided considerable advantages in tumor immunotherapy.[8]

However, in spite of clinical benefits of DC vaccination in cancer treatment, not all patients respond and missing responses could not consistently be coordinated with the patients' data. Low tumor antigen immunogenicity, immune escape, and tumor-induced immunosuppression were frequently identified as responsible factors.[9,10] Nonetheless, progress in chemotherapy and checkpoint inhibitors together with the increasing knowledge on the power of exosomes in intercellular communication and their mode of action are promising that these drawbacks can be overcome such that immunotherapy may become a reliable and efficient adjuvant therapy during cancer progression.

Tumor-derived exosomes (TEXs) have discrete sets of proteins such as major histocompatibility complex class I and II (MHC-I and MHC-II), phosphatidylserine, milk fat globulin-E8 (MFGE8), rab7, liposome-associated membrane protein 1 (LAMP1), CD9, CD81, Annexin II, CD54, and CD63 that facilitate exosome-binding and uptake by relative ligands on DCs.[11-14] In addition, TEXs express and transfer a wide spectrum of tumor-associated antigens to DCs that can prime tumor-specific cytotoxic T lymphocytes (CTL) and induce potent antitumor immunity.[15-18] Further, immunostimulatory components are enriched in exosomes as compared to cells and previous studies in mice showed that TEXs improved vaccine efficacy compared to tumor lysates.[14,19,20] This relies on uptaken TEXs being preferably transferred to the MHC-II-loading compartment, which is accompanied by a minor loss due to lysosomal degradation. Finally, the peptide-loaded DCs promote CD4^{+} T helper cell activation.[14] DCs recruit TEXs via exosomal LFA-1 and CD54 that are major ligands for exosomes.[21] The majority of previous studies used DC-

derived exosomes as vaccines ignoring the potential of TEXs as an independent vaccine to stimulate DCs.[22-27] However, TEXs being a rich reservoir of the whole panel of tumor antigens, TEXs can stimulate a broad array of T cell clones to respond toward the multiple antigenic epitopes.[28] Moreover, TEXs can easily be isolated and purified from patients' sera and malignant effusions. Thus, TEXs are an attractive alternative source of tumor antigens for cell-free cancer vaccines in personalized tumor immunotherapy,[16,29] and it is becoming increasingly appreciated that TEXs can serve as a new promising cell-free therapeutic tool in cancer immunotherapy.[13,14,30-33]

To our knowledge, there has been no comprehensive review on the stimulatory efficacy and the antitumor immune responses induced by TEXs. Here, we will first introduce exosomes with a particular focus on the composition and targets of TEXs and their crosstalk with the tumor and the immune system. Following that, we will focus on the TEX application to induce immune responses.

Exosomes: biogenesis, structure, composition and function

Exosomes are cell-derived nanoscale (30–140 nm in diameter) vesicles possessing a lipid bilayer. They are found in almost all biological fluids including blood, serum, urine, breast milk, amniotic fluid, nasal secretions, saliva, cerebrospinal fluid (CSF), and bile as well as cell culture supernatants.[34-41] Exosomes were first identified as small vesicles involved in the maturation of sheep reticulocytes. Subsequently, these functional vesicles were named as exosomes by Johnstone in 1989.[42,43]

The most significant factor in exosomes discrimination from other extracellular vesicles is their mode of biogenesis, target binding, and uptake. Exosomes are formed by endocytosis of several plasma membrane microdomains and creation of early and late endosomes, which receive their selective cargo and become integrated into multivesicular bodies (MVBs). When MVBs fuse with the plasma membrane, exosomes are released into the extracellular environment through exocytosis.[44-49] Exosomes are the body's most efficient system in mediating biological data exchange.

In addition to constitutive exosome membrane and cytosolic molecules, exosomes contain a large variety of membrane proteins and soluble factors related to cell-type specific functions (e.g. integrins, selectins, Rab proteins, SNAREs, tetraspanins such as CD9, CD81, CD63, growth receptors), lipids (e.g. steroids, sphingolipids, glycerophospholipids), nucleic acids (mRNAs, miRNAs, sRNAs, DNAs), and others.[40,45,50-54] According to the current version of Exocarta (http://www.exocarta.org), the largest exosome content database, 41,860 proteins, more than 7,540 RNA and 1,116 lipid molecules have been identified from more than 286 exosomal studies.[55] These exosomal-shuttle molecules play key roles in exosome function. Exosomes can interact (by deliver or uptake) with their recipient cells via different mechanisms such as specific receptor binding, direct fusion with the plasma membrane, and phagocytosis.[51] By their distribution throughout the body, these vesicles transfer information from host cells to target cells over long distances. Furthermore, due to the presence of exosomes in biofluids and origin-dependent content, which closely reflects various physiological and pathological conditions, they may also serve as an ideal noninvasive or minimally invasive tool for diagnosis and monitoring the efficacy of treatment regimes.

Depending on the cell or tissue origin, exosomes have diverse biological functions in both normal and pathophysiological conditions which include beside others elimination of unnecessary proteins and molecules, blood coagulation, propagation of pathogens (prions and viruses), programmed cell death, angiogenesis, inflammation, modulation and regulation of immune response, and antigen presentation, where cell-cell communication promotes signaling and transcription.[56-61]

Several exosome isolation methods can be applied based on the sample volume, experimental design, research main questions, and type or origin of exosomes from cell culture supernatants or biological fluids. Most commonly employed isolation methods are differential ultracentrifugation, density gradients, and commercial exosome isolation kits making use of precipitation, bead-based, or immunoaffinity-based methods.[61-64] Exosomes are visualized and characterized based on size distribution, specific exosomal markers, enriched proteins and RNAs and other selective contents. Most common techniques are transmission electron microscopy (TEM), cryo-EM, flow cytometry, ELISA, western blot, RNA profile using chip-based capillary electrophoresis, RNA sequencing, RNA microarrays, polymerase chain reaction (PCR), nanoparticle tracking analysis (NTA), and Dynamic Light Scattering (DLS).[62,65] Each of the aforementioned isolation and characterization methods has some potential advantages as well as limitations, which warrants careful attention to the research purposes before choosing the isolation and characterization protocols.

TEXs in tumor cell and stroma modulation

Malignant cells secrete larger amounts of exosomes than non-transformed cells. There is evidence that TEXs can be involved in all steps of cancer development including oncogenesis and tumor growth via apoptosis inhibition and promotion of drug resistance, tumor cell spreading and metastatic settlement as well as angiogenesis, tumor immune escape, and immunosuppression.[64] In the following, we briefly summarize and give some examples on these multitude of activities sparing the communication with the immune system that will be discussed in detail in the following section.

The crosstalk of TEXs with non-transformed cells might suffice to promote oncogenesis.[66] Thus, uptake of malignant breast cancer cell-derived exosomes has been shown to educate non-tumorigenic epithelial cells to generate tumors.[67] Similarly, TEXs have been demonstrated to induce malignant transformation in prostate cancer.[68]

Furthermore, the heterogeneous tumor cell mass contains a small subpopulation so-called cancer stem cells (CSCs) with self-renewal and differentiation potential, considered to be the main cause of tumor recurrence.[69-71] One of the prominent features of CSCs is their plasticity and dynamic equilibrium, where CSC-TEXs play a crucial role. Although their mechanisms of action are rarely investigated, CSC-TEXs can induce

a stemness phenotype by stemness-related molecule transfer and targeting upstream or downstream genes, by recruiting and altering the phenotype and function of stromal cells, as well as by enhancing tumor aggression and metastatic features.[72-74] Thus, CSC-TEXs can remodel the tumor niche through influencing resident tumor cells as well as the tumor microenvironment including fibroblasts and immune cells, which leads to local tumor progression.[68] In prostate cancer, differential microRNA patterns were observed in bulk cell-TEXs and CSC-TEXs. The CSC-TEXs miRNA profile suggested an involvement in angiogenesis, proliferation, and pre-metastatic niche formation.[75] Similar results were found for CD90+ liver cancer cell-derived exosomes and CD105+ CSC-TEXs have a set of pro-angiogenic mRNAs, microRNAs, and lncRNA known to contribute to the stimulation of angiogenesis via endothelial cell phenotype modulation and lung pre-metastatic niche formation.[76,77] Other studies indicated TEXs being involved in oncogenic cell signaling pathways especially mediated by molecules such as p53, MAPK, and Wnt.[78] For example, CD82 and CD9 tetraspanins, which are enriched in exosomes, can suppress Wnt signaling.[79]

Further, TEXs molecular constituents can be associated with enhanced tumor growth. For instance, in colorectal cancer, TEXs enhance the proliferation of endothelial cells and tumor growth by their enriched content of cell cycle-involved mRNAs.[80] Moreover, activation of PI3K/Akt and MAPK/ERK pathways by TEXs could promote cell proliferation in gastric cancer.[81] TEXs have also been shown to inhibit the differentiation of bone marrow cells and alter macrophage physiology and function in favor of tumor growth.[82-85] There are similar reports on the induction of tumor growth by TEXs from melanoma, hepatoma, glioblastom, and many other cancers.[86-88]

TEX exchange can instigate migratory behavior and metastatic potential in recipient cells through transfer of their cytoplasmic contents such as miRNA and pre-miRNA transcripts into either tumor cells or cells in the tumor microenvironment.[89,90] CD44 transfer to surrounding peritoneal mesothelial cells through ovarian TEXs can promote invasive potential of cancer cells.[91] Findings also suggested that deregulation of the extracellular matrix (ECM) through proteinases, glycoproteins, and matrix metalloproteinases released from TEXs could help invadopodia maturation and fibroblast remodeling of the tumor ECM leading to tumor cell invasiveness and migration.[92-95] Furthermore, circulating TEXs have been proposed to prepare a pre-metastatic niche for incoming tumor cells.[96] Exosomal release of cytokines, growth factors, and microRNAs can support recruitment of bone marrow-derived cells (BMDCs) to potential metastatic sites modulating pre-metastatic organ cells.[87,97] Moreover, exosomal integrin expression patterns were demonstrated dictating organ sites of forthcoming metastasis.[98]

TEXs may also contribute to epithelial-to-mesenchymal transition (EMT), one of the hallmarks of tumor progression and invasion, through oncogenic transmission and TGFβ upregulation.[99,100] Additionally, TEXs can inhibit apoptosis in many cancer cell lines.[101] For instance, bladder TEXs inhibit apoptosis through Bcl-2 and Cyclin D1 proteins upregulation

and Bax and caspase-3 proteins reduction.[102] Exosomal survivin released from cancer cells also mediates inhibition of apoptosis *in vitro*.[103]

Angiogenesis is one of the crucial steps in tumor growth. In various types of cancers, uptake of TEXs by endothelial cells accelerates angiogenesis.[95,104-109] These pro-angiogenic activities of exosomes occur through reprogramming of endothelial cells by different molecules including exosomal miR-135b in multiple myeloma, miR-130 in gastric cancer, and miRNA-210 in leukemia and breast cancer.[110-113] Hence, TEXs can affect tumor progression and metastasis through vascular remodeling.

Of special interest for immunotherapy is the capacity of TEXs to promote cancer resistance to conventional therapies. Thus, TEXs horizontally transfer exosomal miRNAs involved in target cell drug resistance.[114,115] Similarly, the transfer of long non-coding RNAs via TEXs led to increased tamoxifen resistance in breast cancer cells.[116] Also, high survivin level in TEXs decreased radiation efficacy.[117] Moreover, exosome-mediated miR-32-5p delivery could increase drug resistance through activation of the PI3K/AKT pathway and inhibition of PTEN.[118]

TEXs in tumor immunology

As mentioned, TEXs are enriched in tumor antigens, but also promote immune escape.[119,120] The literature abounds with evidence suggesting TEXs as mediators of immune cell-tumor cell communication, and regulators of immune responses through both immunosuppressive and immunostimulatory functions, promoting either tumor progression or regression. In the following section, the double-edged role of TEXs in suppression and activation of immune responses against cancer is summarized based on immune cell types (Figure 1).

Natural killer (NK) cells

TEXs have been shown to inhibit tumor immune surveillance by exerting immunosuppressive impacts on NK cells as the main anti-tumor weapons of the innate immune system. Indeed, TEXs hamper NK at multiple levels by interfering with activation and, particularly, effector functions. Thus, TEXs account for significant down-regulation of NK-specific triggering surface molecules (natural killer group 2D (NKG2D) and CD69) by inhibition of Stat5, Jak3, CyclinD3 expression, increasing the number of myeloid-derived suppressor cells (MDSCs), decreasing of perforin secretion, all resulting in impaired migration and cytotoxic potential of peripheral NK cells.[121-124] In addition, TEXs promote a decrease in the number of NK cells in the spleen and lungs through induction of tumor-infiltrating natural killer (TINK) through activation of the TGF-ß/SMAD pathway mediated by exosomal TGF-ß1, which also suppress IL-2 stimulated NK cell cytotoxicity.[121,125-127] In contrast, some TEX constitutive markers facilitate NK activation; For instance, co-culture of NK cells with heat shock protein 70 (HSP-70) surface-positive TEXs, as an attractant and target structure, stimulates NK recruitment and cytolytic activity through granzyme B release of CD94+ NK cells.[128]
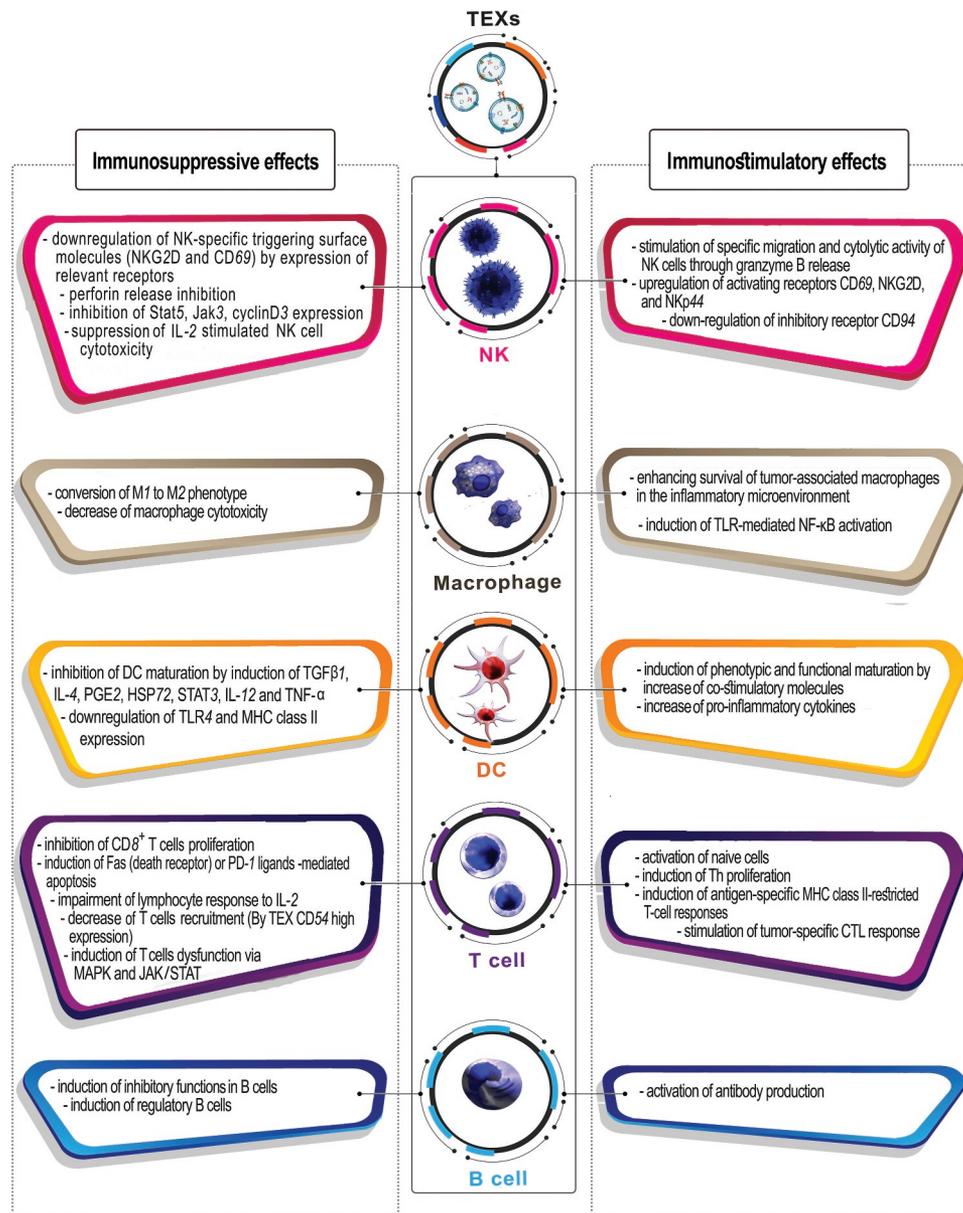
Figure 1. Schematic representation of dual functions of TEXs in suppression and activation of immune responses against cancer.

Moreover, the HSP-70-stimulated NK up-regulate the expression of CD69, NKG2D, and NKp44 stimulating receptors, while the CD94 inhibitory receptor becomes down-regulated.[129]

Macrophages

TEXs exert an important impact on macrophages by forcing them toward the immunosuppressive M2 phenotype.[130] Previous studies reported that TEXs stimulate pro-inflammatory activity and TLR-mediated NF-κB activation in macrophages, resulting in increased secretion of pro-inflammatory cytokines/chemokines, such as IL-6, TNFα, GM-CSF, and CCL2; this leads to prolonged survival of tumor-associated macrophages in the inflammatory microenvironment.[83,131] Indeed, TEXs mediate upregulation of Wnt 5α in macrophages; subsequently macrophage-

derived exosomes transfer Wnt 5α into tumor cells which leads to enhanced tumor invasion through the activation of β-catenin-independent Wnt signaling.[84]

Dendritic cells (DCs)

Differentiation of monocytes toward DCs, the professional antigen-presenting cells (APCs) and the bridge between the innate and adaptive immunity, can be modulated by TEXs via expression of PGE2, HSP72, and TGF-β, that have been reported to induce the production of inhibitory cytokines, decrease the expression of co-stimulatory molecules, increase the expression of STAT3, and inhibit the maturation and T cell stimulatory capacity of DCs through induction of IL-6 phosphorylation.[82,132,133] Further, under TEXs impact, DC maturation and antigen-specific responses can become impaired via downregulation of TLR4 and MHC-II

expression as well as induction of IL-4, TNF-α, IL-12, and TGFβ1.[132,134] Moreover, TEXs uptake by immature DCs has been demonstrated to block DC maturation by exosome-mediated mechanisms and also to induce tumor suppression via redirecting toward myeloid-derived suppressor cell (MDSCs) differentiation and proliferation.[133,135]

Despite these immunosuppressive effects, TEXs express tumor antigens as well as markers, which facilitate TEXs uptake by DCs and direct tumor antigens toward multivesicular bodies. After processing, peptides are loaded into newly generated MHC-II molecule grooves and are presented to Th cells promoting their activation. Several studies documented that *in vitro* uptake of TEXs by DC enhance the expression levels of co-stimulatory receptors (CD80, CD86), CD11c and MHC, and induce phenotypic and functional DC maturation.[130,136,137]

T cells

TEXs block CD8^{+} T cells proliferation, activation and cytotoxic activity and increase their immunosuppressive functions. They are also responsible for induction of Fas or programmed cell death-1 (PD-1) ligands-mediated apoptosis of antitumor CD8^{+} effector T cells in several cancers.[130,138-140] The expression of CD73, CD39 immunoregulatory proteins, TGF-β1 cytokine, and galectin-1 (Gal-1) in TEXs can induce a suppressive phenotype in CD8^{+} and CD4^{+} T cells.[130,141,142] In addition, TEXs impair the lymphocyte response to IL-2[143] and have been shown to induce IL-8 production in epithelial cells suppressing T cell responses.[144] Finally, TEXs can contribute to tumor escape via induction of CD4^{+} CD25^{+} FOXP3^{+} T regulatory cell (Treg) expansion, accompanied by increasing expression levels of TGF-β, IL-10, and CTLA4. They also indirectly facilitate Treg generation through inducing tolerogenic DCs.[133,145]

On the other hand, through expression of MHC class I and class II complexes on their surface, TEXs can directly present antigen, activate T cells, and induce antigen-specific MHC class II-restricted T-cell responses. Also, many studies unraveled TEXs expressing some markers that could facilitate CD8^{+} T cell activation and stimulate tumor-specific CTL responses *in vivo* and anti-tumor immune responses in mice.[13,140,146] However, the TEXs armament may preferentially suffice for memory T cell activation, whereas activation of naïve T cells requires TEXs modulation.

B cells

The effect of TEXs on B cell activation and function is not well delineated. Few studies have shown that TEXs induce differentiation of naïve B cell to a regulatory phenotype with production of inhibitory cytokines that leads to antitumor immune response inhibition.[147,148] In addition, TEXs could reduce antibody-dependent cell cytotoxicity (ADCC) through interfering with tumor-reactive antibodies binding to tumor cells.[149] In contrast, TEXs were also described to drive antibody production, the underlying mechanism remaining to be explored.[139]

TEX-based cancer vaccination

Many studies proved the feasibility and functionality of TEXs to stimulate immune responses against cancer in mouse models. For instance, a study by Bu et al., in syngeneic mice showed that vaccination with L1210 leukemia-released exosomes prevented tumor formation and elicited protection to tumor challenge.[150] Lee and colleagues demonstrated that vaccination with TEXs not only elicited significant protection against tumor growth and primed Th1 immune responses to an established melanoma but also could inhibit pulmonary metastasis in metastatic melanoma mouse models.[151] This capability of TEXs to trigger T cell-mediated antitumor immune responses and suppress tumor growth has been reported in other studies, as well.[14,15,33,137] Furthermore, TEXs harbor tumor antigens from their donor cells. Following uptake by DCs, these tumor antigens are presented as peptides in the MHC grove and can prime naïve T cells to generate anti-tumor responses.[136] However, because of TEXs-induced immunosuppression and limited TEX immunogenicity, TEXs alone application frequently resulted in unsatisfying anti-tumor immune effects *in vivo*. Thus, several strategies were developed to improve the efficacy of TEXs vaccination. Some of which are listed in Table 1 and will be discussed in the following sections.

TEXs modifications to advance tumor immunotherapeutic efficacy

Tumor cells and TEXs can be modified (either genetically or non-genetically) to enrich tumor antigens, microRNAs, and immunostimulatory molecules in TEXs with the aim of enhancing tumor cell elimination directly or in concert with killing by immune cells. Heat shock proteins (HSPs), highly enriched in cancer cells and TEXs, have potent adjuvant capability. They served as one of the approaches in strengthening cancer immunotherapy. Indeed, HSP can increase immunogenicity of TEXs and improve cancer vaccine efficacy.[158,159] Chen and colleagues demonstrated that heat shock treatment increases TEX proteins relevant to potentiate immune response induction and reported on efficient immunostimulatory functions of A20 lymphoma/leukemia cell line-derived heat-shock exosomes (HS-TEX) via the up-regulation of MHC, co-stimulatory molecules and cytokines, including IL-1β, IL-12p40, TNF-α, RANTES, and also MIP-1α in DC. The stronger protective antitumor immunity of HS-TEXs compared to untreated TEXs in immunized mice confirmed these results.[30] Similar findings also were obtained with Hsp70-enriched TEXs isolated from heat-treated MUC1-expressing CT26 cells. These HS-TEXs showed increased expression of MHC-II and enhanced immune-stimulating activity. HS-TEXs, as an MHC-independent vaccine, stimulated strong Th1 type immune responses via the increased production of IgG2a antibody and IFN-γ, resulting in elimination of cancer cells in autologous and allogeneic tumor-bearing mice.[167] This approach has also been employed by Xie and colleagues via transfection of heat-shocked J558 tumor cells with vectors expressing membrane-bound inducible HSP70 to generate TEX_HSP expressing membrane-bound HSP70. They demonstrated that TEXs bearing membrane-bound HSP stimulated IL-12 secretion by

Table 1. A summary of vaccine studies utilizing tumor-derived exosomes in *in vitro* and *in vivo* preclinical animal models.

Source of tumor exosomes	Purification method	Aim of study	Treatment regimen	Key results	Ref.
-HeLa cell line	Ultracentrifugation/ sucrose gradient	*In vitro* investigation of HeLa-derived exosomes immunogenicity.	Capacity of HeLa-TEX for inducing immune responses was examined.	• Loading with HeLa-TEX-induced DC maturation, *in vitro* T lymphocyte proliferation and specific CTL activity.	135
-EG7 expressing ovalbumin (OVA- transfected EL4 mouse thymoma) cell line	Ultracentrifugation	Comparison between TEX and dendritic cell-derived exosomes (DEXs) based vaccination.	Melanoma-bearing mice were immunized intravenously with OVA pulsed DC-derived exosomes (EXO_{DC}) or tumor-derived exosomes (TEX_{EG7}) (10 μg/mouse).	• EXO_{DC} were more immunogenic compared to TEX_{EG7}. • EXO_{DC} more strongly stimulated CD8$^+$ T cell proliferation and differentiation in compared to TEX_{EG7}. • EXO_{DC} induced more efficient CTL activity and stronger antitumor immunity both *in vitro* and *in vivo* compared to TEX_{EG7}.	11
-EG7 expressing ovalbumin (OVA- transfected EL4 mouse thymoma) cell line	Ultracentrifugation	Comparison of immunogenicity between OVA protein pulsed DC-derived exosomes (EXO_{DC}) and DC pulsed with TEX_{OVA} (DC_{EXO}).	Melanoma-bearing mice were treated intravenously with EXO_{DC} or DC_{EXO} vaccines.	• TEX uptake by DC was mediated by LFA-1, CD54, and CLR. • DC_{EXO} stimulated OVA-specific T cell proliferation *in vitro* and *in vivo*. • DC_{EXO} more strongly eradicated established tumors and induced protection against lung metastasis.	144
-A20 (H-2d) mouse B lymphoma/leukemia cell line (Exo) -Heat-shocked A20 (H-2d) mouse B lymphoma/ leukemia cell line (HS-Exo) - murine colon cancer CT-26 cell line	Ultracentrifugation	Evaluation of heat shock induction in improvement of exosome-based tumor vaccines.	Lymphoma/leukemia-bearing BALB/c mice were immunized subcutaneously with TEX and HS-TEX (10 μg/mouse)	• Heat-shock treatment did not affect the amount of A20 exosomal protein secretory rate, in contrast increased the exosomal proteins relevant to efficient immune responses. • HS-TEX contained more MHCI, HSP60, HSP90, RANTES, and IL-1b compared to TEX and CD40 was only in HS-TEX. • Efficacy of both therapeutic and prophylactic vaccines was investigated. • 80% of the vaccinated mice with HS-TEX remained tumor-free 90 days after tumor challenge and showed higher levels of IFN-γ and IL-2 compared with TEX-immunized mice ($p < .05$).	28
- B16F1 murine melanoma cell line - CIITA gene (MHC class II transcriptional activator) transduced B16F1 cell line	Ultracentrifugation/ sucrose gradient	Investigation of CIITA transduction effect in enhancing immune stimulation as compared to exosomes from parental tumor cells (TEX).	-For preventive model, C57BL/6 mice were intradermally vaccinated with PBS, TEX or CIITA-TEX (5 or 20 μg/mouse), and then challenged subcutaneously with melanoma B16F1 cells. -For therapeutic model, B16F1 melanome-bearing mice were immunized intradermally with PBS, TEX and CIITA-TEX (20 μg/mouse).	• MHC class II molecules were highly enriched in CIITA-TEX as compared to parental TEX. • It was found that *in vitro* loading of DCs with CIITA-TEX significantly induced higher level expression of immunostimulatory molecules such as MHC class II molecules and CD86 compared with DCs loaded with TEX. • Increased proliferation and IL-2 production of naïve splenocytes were observed in mice treated with CIITA-TEX vaccine when compared to mice treated with parental TEX. • Mice treated with CIITA-TEX had improved preventive and therapeutic anti-tumor immune response by both cellular and humoral mechanisms in a dose-dependent manner with increased survival rate and the best control of tumor growth.	29
-Murine colon cancer CT-26 cell line -CIITA gene transduced CT-26 cell line	Ultracentrifugation/ sucrose gradient	Transduction of CIITA gene into CT26 cell line and evaluation of immune response induction by CT-26-CIITA TEX compared to parental CT-26 TEX.	Balb/c mice were intradermally immunized with PBS, CT-26 TEX and CT-26 -CIITA TEX (10 or 20 μg/mouse), and challenged subcutaneously with CT-26 cells.	• CT-26 -CIITA TEX expressed high level of MHC class II molecules and promoted DC maturation. • Preventive CT-26-CIITA TEX vaccinated mice models showed high expression of TNF-α, IFN-γ, IL-12 cytokines, and decrease in IL-10 expression and also effectively inhibited tumor growth in compared to CT-26 TEX.	30
-K562 leukemia cell line -L1210 acute lymphoblastic leukemia cell line	Ultracentrifugation/ sucrose gradient	Examination of L1210-TEX and DC- L1210 TEX ability in induction of protective antitumor immunity in leukemia mice model.	- For therapeutic model, DBA/2 mice were immunized subcutaneously with PBS, L1210-TEX (30 μg/mouse), non-pulsed DC and DC-L1210 TEX ($1.0{-}4.0 \times 10^6$ cells/ mouse), then challenged with L1210 leukemia cells. - For preventive model, after challenging with L1210 leukemia cells, DBA/2 mice were immunized.	• L1210-TEX harbored ABL specific protein from their parental cells as well as HSP70 that facilitates antigen presentation. • Both L1210-TEX and TEX-pulsed DCs induced antigen-specific anti-leukemic CTL immune response *ex vivo* and *in vivo*. • L1210 TEX-pulsed DCs were more potent in protective and therapeutic anti-leukemic immunity induction compared to L1210-TEX alone.	31

(*Continued*)

Table 1. (Continued).

Source of tumor exosomes	Purification method	Aim of study	Treatment regimen	Key results	Ref.
-Myeloid leukemia WEHI3B cell line -Renal cell carcinoma (RENCA) cell line	Ultracentrifugation/ sucrose gradient	Efficacy evaluation of TEX- vs. tumor lysate-loaded DC as vaccines in WEHI3 myeloid leukemia and RENCA bearing mice.	Balb/c mice were challenged intravenously or subcutaneously with WEHI3B and RENCA cells, and then were vaccinated with DC-lysate, DC-TEX and TEX alone. (200 μg/ mouse)	• WEHI3B-TEX showed the marker profile of WEHI3B tumor cells with higher level of exosome markers, chemokines, and receptors. • Delayed tumor growth and prolonged survival rate were observed in all vaccinated mice. • The therapeutic efficacy was significantly stronger with more Th and CTL activation in DC-TEX compared to DC-lysate. • General applicability of DC-TEX was confirmed by the RENCA tumor-bearing mice.	12
- UNKC6141 pancreatic adenocarcinoma cell line (UNKC)	Ultracentrifugation/ sucrose gradient	Strengthen of the therapeutic efficacy of DC-TEX vaccination by blocking of MDSC maturation and activation in pancreatic cancer (PaCa).	UNKC-bearing mice (subcutaneously or orthotopic) after AGS (ATRA, Gemcitabine and Sunitinib) application were immunized intravenously with TEX-loaded DC (DC-TEX).	• UNKC-derived TEXs expressed PaCa markers and several common tumor markers. • DC-TEX vaccination prolonged the survival rate and delayed the onset of tumor growth as well as reduced UNKC dissemination. • Drug combination strengthened DC-TEX efficacy through reduction of MDSCs in vaccinated mice.	152
-Murine breast cancer 4T1 cell line	Gradient ultracentrifugation	Modification of miRNA content of TEXs for enhancement of DC maturation and effector cells activation.	TEX loaded with mimics of miRNAs (miR-155, miR-142, and let-7i) to induce potent dendritic cells (DC) and improve immune stimulation.	• Manipulated TEXs enhanced expression of associated markers to DC maturation (MHCII, CD80, and CD40) in both protein and mRNA level and induced CTL activity.	153
-Murine melanoma B16BL6 cell line	Ultracentrifugation	Co-delivery of tumor antigens and adjuvant by genetic modification of exosomes.	B16BL6 cells were transfected with immunostimulatory CpG DNA and then their derived TEXs were utilized as a vaccine in melanoma bearing mice.	• CpG DNA-modified TEX enhanced tumor antigen presentation capacity and cytokine release of DCs. • More potent cellular and humoral immune response was observed in vaccinated mice model with modified TEXs as compared to unmanipulated TEXs.	154
-HEK293 cell line	Ultracentrifugation	Direct vaccination of murine models with antitumor let7a microRNA and GE11 binding peptide-modified TEX.	Let-7a – GE11 containing TEX were administrated in human tumor xenograft models.	• Genetically engineered TEXs conferred greater immunogenicity and inhibition of tumor development in vivo.	155
-AB1 mouse mesothelioma cell line	Ultracentrifugation	Direct comparison of TEX-DC and lysate-DC vaccination in mesothelioma cancer model.	Mesothelioma bearing mice were treated with TEX-loaded DC and lysate-loaded DC vaccines.	• Significant reduction in tumor growth and increased survival rate was observed in vaccinated mice with TEX-loaded DC when compared to mice treated with tumor lysate-loaded DC vaccine.	156
-Autologous glioma tumor cells (from patients with glioblastoma multiform)	Ultrafiltration/ centrifugation/ sucrose Gradient/ ultracentrifugation	Assessment of the capacity of autologous glioma tumor cells-derived exosomes in CTLs activity induction compare to tumor lysate.	DCs were pulsed with glioma cells and their derived TEXs, and in vitro CTL activity was then evaluated.	• TEXs expressed high level of MHC-I, HSP70, ICAM-1, and MAGE-1 molecules compared with tumor lysate. • TEX-loaded DC was more efficient than tumor lysate-loaded DC in induction of glioma specific CTLs.	157
-Myeloma JS58 cell line expressing P1A tumor antigen	Ultracentrifugation/ sucrose gradient	The comparison of immune responses induced by EXO$_{HSP}$ expressing membrane-bound HSP70 (TEX$_{HSP}$) or EXO expressing cytoplasmic HSP70 (TEX$_{HS}$).	Myeloma JS58 cell line were transfected by membrane-bound inducible HSP70 expressing vectors and then myeloma bearing mice were treated with TEX$_{HSP}$ or TEX$_{HS}$ pulsed DCs.	• TEX$_{HSP}$-loaded DC efficiently stimulated DC maturation and tumor-specific CD4$^+$Th1, CD8$^+$ CTL, and NK cells responses in tumor-bearing mice. • TEX$_{HSP}$-loaded DC more strongly inhibited tumor growth and increased survival in vaccinated mice compared with TEX$_{HS}$-loaded DC.	158
-MUC1-expressing CT26 cell line	Ultracentrifugation/ sucrose gradient	Enhancement of the immune-stimulating activity of TEXs by heat treatment of tumor cells.	MUC1-CT26 cells were exposed to heat shock, then tumor bearing mice were vaccinated by HS-TEX or untreated TEX.	• HS-TEX elicited Th1 cells activation through increased IgG2a and IFN-γ production. • HS-TEX strongly suppressed tumor growth in autologous and allogeneic mouse models compared with untreated TEX.	159
-CT26 murine colon carcinoma cell line -TA3HA murine mammary carcinoma cell line	Ultracentrifugation/ sucrose gradient	Genetic manipulation of TEXs for tumor antigens delivery.	Immunogenic potential of MUC-containing exosomes was tested in autogenic and allogeneic tumors.	• MUC-containing TEXs were capable to induce DC maturation and IFN-γ secretion as well as greater inhibition of tumor growth in both allogeneic and autologous tumor-bearing mice compared to non-MUC1-containing TEXs.	24
-EG7 expressing ovalbumin cell line (OVA- transfected EL4 mouse thymoma cell line)	Ultracentrifugation/ sucrose gradient	Protein transfer of SEA on TEXs in order to increase their immunogenicity.	C57BL/6 mice were immunized subcutaneously with TEX, SEA-TEX or mixture of TEX and SEA, and were challenged with EG7-OVA cells.	• SEA-TEX more efficiently induced tumor-specific CD 8$^+$T, NK, and CD4$^+$ T cells, and increased IL-2 and IFN-γ production compared to TEX or mixture of TEX and SEA. • SEA-TEX elicited greater immune response in tumor-bearing mice.	160

(Continued)

Table 1. (Continued).

Source of tumor exosomes	Purification method	Aim of study	Treatment regimen	Key results	Ref.
-L1210 murine B lymphocytic leukemia cell line	Ultrafiltration Centrifugation/ sucrose gradient/ ultracentrifugation	Assessment of L1210-derived TEXs impact as a prophylactic vaccine in syngeneic mice models.	DBA/2 mice were subcutaneously immunized with L1210-TEXs (2.5 or 5 μg) and then challenged with L1210 leukemia cells.	• Mice treated with L1210-TEXs showed tumor growth suppression. • L1210-TEXs stimulated *in vitro* tumor-specific CTL responses.	149
-Fon (HLA-A2⁺) and Mel-888 (HLA-A2⁻) human melanoma tumor cell lines	Ultracentrifugation/ sucrose gradient	The comparison of immune responses induced by TEXs and tumor cells lysate in prophylactic and therapeutic mice models.	Mice models were immunized by different doses of TEXs and tumor cell-derived lysate as alone or loaded on DC.	• More protective antitumor activity was observed significantly in syngeneic and allogeneic mice models which treated with TEXs when compared to mice treated with tumor lysate. • Lower doses of TEXs were more potent in suppressing of tumor growth compared to tumor cell lysate.	13
-AB1 mouse mesothelioma cell line	Ultracentrifugation	Study of the impact of antigen source in cancer immunotherapy outcome.	Mesothelioma bearing mice were treated with DCs loaded by different antigen sources (tumor cell line lysate, *ex vivo* tumor lysate and tumor cell line-derived TEX).	• Although DC-EXO was effective in prolonging overall survival, but tumor cell line lysate-pulsed DCs were more effective.	161
- B16-OVA and B16 melanoma cell lines	sucrose gradient/ ultracentrifugation	Evaluation of the effect of VSV-G incorporation in TEXs on their immunogenicity.	Melanoma cell lines B16-OVA and B16 were transduced by VSV-G-expressing vectors and then melanoma bearing mice were treated with VSV-G containing TEXs loaded on DC.	• VSV-G improved TEXs antigenic transfer to DCs and more potently stimulated phenotypic and functional maturation of DCs. • VSV-G bearing TEXs elicited strong *in vivo* antitumor CTL responses.	162
-E.G7-OVA tumor cells expressing Ovalbumin (OVA)	Serial centrifugation/ sucrose gradients/ ultracentrifugation	Further improvement of TEX-based vaccination through expression of IL-2 in them.	E.G7-OVA tumor cells were transfected with IL-2 gene and then tumor bearing mice were immunized with TEX/IL-2, TEX+IL-2 and TEX alone.	• TEX/IL-2 conferred more potent CTL, NK, and CD4⁺ T cell activation, also significant suppression of tumor growth compared to TEX+IL-2 or TEX. • TEX/IL-2 increased IL-2 and IFN-γ production in vaccinated mice.	163
-Human RC-2 renal cancer cell line	Ultracentrifugation	Improving TEX immunogenicity by IL-12 gene modification in tumor cells.	Human renal tumor cells were genetically modified with IL-12 gene and then *in vitro* immune responses of TEX/IL-12 derived from these cells were examined.	• TEX/IL-12 activated T-cell proliferation and increased release of IFN-γ. • TEX/IL-12 induced *in vitro* antigen-specific cytotoxic effect.	164
-B16F1 melanoma cell line	Ultracentrifugation	*In vitro* assessment of immunological activity of melanoma cell-derived TEXs (mcd-TEXs).	Immunogenic potential of mcd-TEXs was tested *in vitro*.	• Mcd-TEXs could stimulate *in vitro* CD4⁺ T cell proliferation and NF-kB activation in macrophages.	83
- Hepa1-6 murine hepatocellular carcinoma (HCC) cell line -Human HepG2 and Hep3B HCC cell lines	Ultracentrifugation	Human *in vitro* and murine *in vivo* study of immune efficacy of HCC cell line-derived TEXs compare to cell lysate.	-For murine *in vivo* study, C57BL6 mice were immunized subcutaneously with PBS, TEX-pulsed DC (30 μg/mouse), lysate-pulsed DC in therapeutic and preventive as well as ectopic and orthotopic models. -For human *in vitro* study, DCs were pulsed with TEXs and then *in vitro* CTL activity was evaluated.	• Immunization with TEX-pulsed DC exhibited stronger *in vivo* antitumor effects and decreased TNF-β and IL-10 levels in tumor sites compared to lysate-pulsed DC. • TEX-induced efficient *in vitro* human dendritic cells maturation and showed HCC-specific cytolysis as well as cross-protective effects against pancreatic cancer cells.	165
-B16BL6 melanoma cell line -Murine melanoma tumor	Homogenization/ sonication/ ultracentrifugation	Assessment of the therapeutic effect of autologous TEXs in melanoma tumor and metastatic mice models.	Autologous murine melanoma tumors were dissected and homogenized, and then immunogenicity of their isolated TEXs was evaluated *in vitro* and *in vivo*.	• TEXs plus poly I:C (as adjuvant) increased secretion of IL-12p70 and Th1-related antibody, IgG2a. • TEXs vaccination exhibited strong antitumor effects in primary and metastatic *in vivo* cancer models.	139
- Hepa1-6 cell line	Ultracentrifugation	*In vivo* evaluation of the effect of DC-TEX and sorafenib combination on treatment outcome.	Orthotopic HCC mice were vaccinated with different combinations of DC-TEX, sorafenib and PD-1 Ab, and then *in vivo* immune responses were evaluated.	• DC-TEX activated CD8⁺T cells and decreased Treg cells. • DC-TEX conferred more PD-1⁺CD8⁺T cells in orthotopic HCC mice. • Combination of sorafenib, PD-1 Ab and DC-TEX promoted the treatment outcomes in vaccinated mice.	166

TEX$_{HSP}$-activated DCs and induced stronger antitumor immunity mediated by CD4$^+$ Th1, CD8$^+$ CTL, and NK cells than TEXs expressing cytoplasmic HSP70.[160] Thus, HSP70 up-regulation in TEX membranes may be an effective strategy to enhance immune response induction.

Another modification strategy to improve TEXs immunogenic properties relies on the manipulation of cancer cells to increase the expression of tumor-specific antigens that are transferred into TEXs. Thus far, this was approached with a variety of highly immunogenic tumor antigens. For example, high expression of the MUC1 tumor antigen is related to cancer progression and poor prognosis in many types of cancer;[168] In a study by Cho et al., MUC1-containing TEXs from MUC1 transduced CT26 and TA3HA cell lines induced DC maturation and IFN-γ secretion by Th1, and more efficiently inhibited autologous and allogeneic tumor growth than non-MUC1-containing control TEXs.[26]

TEXs-promoted immune reactions can also be strengthened by the superantigen staphylococcal enterotoxin A (SEA), which forms a complex with MHC-II on APCs. Xiu and colleagues generated TEXs containing SEA or SEA tailed with a highly hydrophobic transmembrane domain (SEA-TM) by protein transfer. Immunization of mice with SEA-membrane-anchored TEXs led to increased IL-2 and IFN-γ secretion, strong in vivo CTL responses as well as stimulation of anti-tumor effects of both CD4$^+$ T cells and NK cells. Additionally, SEA-TEXs inhibited tumor growth and prolonged the survival of tumor-bearing mice more strongly than un-manipulated TEXs or a mixture of TEXs and SEA. These effects could possibly be ascribed to SEA facilitating TEXs binding to DC and a concomitant decrease of the immunosuppressive activity of Tregs.[162] Thus, protein transfer is an appropriate method to modify TEXs toward expression of tumor antigens or other immune-enhancing proteins on their surface.

Since the recognition of MHC class II-peptide complexes by CD4$^+$ helper T cells is required for optimal and efficient induction of anti-tumor immunity, another candidate molecule for increasing the efficacy of TEXs in cancer immunotherapy is MHC class II. Master regulatory gene MHC class II transcriptional activator (CIITA) controls expression of MHC class II molecules and introduction of CIITA gene in tumor cells can stimulate tumor-specific CD4$^+$ and CD8$^+$ T cells.[155] To investigate the effect of CIITA expression on the immune-stimulating capability of TEXs, Lee et al., transduced melanoma B16F1 cells with the CIITA gene and assessed the impact of CIITA-enrichment in TEXs (CIITA-TEXs) on tumor regression. Compared to parental TEXs, CIITA-TEXs provoked enhanced immune responses reflected by the higher surface expression of MHC class II and CD86 and higher mRNA levels of inflammatory cytokine, TNF-α, and maturation marker CCR-7 on pulsed DCs. Notably, induction of IL-2 cytokine production by naïve splenocytes in vaccinated mice suggested the capability of CIITA-TEXs to induce CD4$^+$ helper T cells activity. Moreover, CIITA-TEXs vaccination delayed tumor growth and improved the survival rate in both therapeutic and prophylactic immunized melanoma-bearing mice.[31] Similar results were reported by Wen Fan et al., applying exosomes from the CIITA-transduced murine colon cancer CT-26 cell line. CT26-CIITA-derived TEXs increased Th1

immune responses reflected by significantly increased TNF-α, IFN-γ, and IL-12, and decreased IL-10 expression.[32] Thus, MHC-II expressing TEXs are powerful in strengthening anti-tumor immune responses.

Incorporation of some viral fusion proteins in TEXs was shown to enhance their uptake by DCs and to improve immunogenicity. One of these viral fusion proteins is the G protein of vesicular stomatitis virus (VSV-G), which facilitates binding of the viral particles to the cell surface. Temchura et al. elaborated that co-expression of VSV-G and tumor antigens on TEX membranes not only accelerated internalization and presentation of TEX antigens by DC but also enhanced phenotypic and functional DC maturation. Up-regulation of CD86, CD80, CD40 co-stimulatory molecules and increased IL-12 release in DC resulted in effective and specific in vivo CTL immune responses.[163] Results obtained from vaccination of mice with EGFR-expressing breast cancer xenografts, where TEXs were manipulated with GE11 peptide, which specifically binds to the EGFR, confirmed the efficacy of this approach.[164]

Expression of certain cytokines in tumor cells and subsequently in their TEXs might be another promising strategy to enhance TEXs immunogenicity. Interleukin 2 (IL-2), an important growth factor and vaccine adjuvant, mediates regulation and activation of immune cells including CTL, NK, B cells, and macrophages, and can generate clinically significant anti-tumor activity in cancer patients. Yang and colleagues represented a new method for improving TEXs application as a vaccine, where E.G7-OVA tumor cells were genetically engineered to express IL-2 to deliver IL-2-containing TEXs (TEX/IL-2). Immunization with TEX/IL-2 strongly enhanced T cell proliferation and affected NK and CD4$^+$ T cells accompanied by elevated secretion of IL-2 and IFN-γ cytokines, and resulting in efficient inhibition of tumor growth.[153] Similar results were obtained with IL-12-containing TEXs (TEX/IL-12), where human renal cancer cells were genetically modified with the IL-12 gene to produce TEX/IL-12, IL-12 being an essential co-stimulatory signal for cellular immune response activation. TEX/IL-12 was capable of increase in vitro CTL activity and strengthened IFN-γ release compared to non-manipulated TEXs.[154]

TEXs transfer diverse types of cargo including microRNAs into cancer cells. Hence, modification of TEXs to overexpress miRNAs that repress immunosuppressive targets might elevate TEX promoted immune responses. Ohno and colleagues introduced let-7a miRNA into HEK293 cells and observed that let-7a containing TEXs potently inhibited breast tumor development.[164] Also, in a recent study by Taghikhani et al., TEXs modified with miR-155, miR-142, and let-7i efficiently delivered antitumor miRNAs to DCs inducing DC maturation and enhancing in vitro CTL activity.[169] In another study, efficient in vitro tumor antigen presentation and higher cytokine release (TNF-α, IL-6, and IL-12p40) were observed in DCs after uptake of TEXs modified with CpG DNA, a well-known immune response modifier. Moreover, immunization with these TEXs resulted in potent cellular and humoral immunity along with upregulation of Th-1 related IgG2a as well as protective and therapeutic antitumor immunity in immunized mice.[165]

Taken together, all these reports indicate that appropriately modified TEXs can be a valuable tool for cancer immunotherapy.

Improvement of TEX-based vaccination by DC loading

DCs are the professional antigen-presenting cells (APCs) of the immune system with the capability to stimulate key adaptive immune cells (naïve CD8☐ T cells and CD4☐ helper T-cells, B-cells). DCs loaded with different tumor-specific antigens to enhance initiation of primary and secondary immune responses, have been used as an efficacious vaccine in numerous murine cancer models as well as in clinical trials.[10]

In vitro activation and loading of DCs, as a potent source for initiating immune responses has several advantages compared to TEXs as vaccine. This strategy would help to overcome some limitations of using TEXs alone like the risk of eliciting immunosuppressive features by free TEXs and also inadequate induction of immune responses. It would guarantee the presentation of exclusively MHC-I or MHC-II grove fitting peptides from digested tumor antigens as well as abundant availability of co-stimulatory molecules. Indeed, several studies showed that TEXs are efficiently taken up and presented by DCs and may even support DC maturation. *In vitro* studies uncovered that besides stimulating peptide-specific clonal T cell expansion, TEX-loaded DCs also are able to stimulate naïve CD8$^+$ T cells maturation toward antigen-specific CTL and induce NF-κB activation in macrophages that are involved in tumor cytotoxicity via tumor necrosis factor (TNF) release.[15,85,157,160] Thus, one important advantage of TEX-loaded DC compared to free TEXs relies on tumor antigen processing and peptide loading of MHC-I molecules. Ren et al. showed that HeLa-TEXs alone were not capable of *in vitro* T cell activation and proliferation, whereas HeLa-TEXs loaded-DCs successfully induced T lymphocyte activation.[137] In a similar study, Wolfer and colleagues observed that when TEXs were derived from the Fon melanoma cell line and loaded onto DCs, they promoted activation and IFN-γ production in CTL clones *in vitro*; whereas they could not raise CTL clones using free TEXs.[15] In line with these studies, Yao and colleagues examined the ability of leukemia-derived exosomes (LEXs) and LEX-pulsed DCs to induce antileukemic immunity in both prophylactic and therapeutic leukemia mouse models. They demonstrated that TEX-pulsed DCs significantly enhanced the survival rate of tumor-challenged mice and more effectively induced CTL immune responses in a dose-depended manner.[33] This dose-dependency was also observed in cytolysis induced by effector T cells primed with TEX-pulsed DCs in hepatocellular carcinoma (HCC) models.[156] Similar results were also obtained by Gu et al., who studied the impacts of TEX-loaded DC (DC-TEX) as a vaccine in WEHI3 myeloid leukemia-bearing mice.[14] Beside tumor antigens, the availability of TEX markers facilitating uptake by DC contributes to pronounced CTL activation. Bu and colleagues purified autologous TEXs from glioma cell culture supernatants from patients with glioblastoma multiform and noted that these TEXs were enriched in MHC-I, HSP70, ICAM-1, and MAGE-1 molecules, which are involved in TEX uptake and antigen presentation. The authors describe higher cytotoxic capacity of TEX-DC- than tumor lysate-DC-stimulated T cells in autologous glioma cell cultures.[170] These results support the hypothesis that TEXs can be used as a promising and robust platform to improve personalized cancer immunotherapy.

Taken together, one great advantage of loading DC with TEX relies on the processing of the TEX tumor antigens and the presentation of tumor antigen peptides in the MHC groove, which strongly facilitates the capture of tumor-peptide specific CD8$^+$ T cells driving their expansion and activation. The enrichment of tumor antigens in TEXs and the equipment of TEX with markers that facilitate the uptake by DC add to the superiority of TEXs-pulsed DC to CTL activation. Furthermore, the preferable processing of TEXs in the MHC-II-loading compartment leads to CD4$^+$ Th activation resulting in more efficient activation of CTLs.[14]

Both dendritic-derived exosomes (DEXs) and TEXs have been used in tumor vaccination. Only few studies compared the efficacy of the two exosome sources as vaccine. Nevertheless, the functionality examination of DC-OVA-derived EXO (EXO$_{DC}$) and EG7 tumor cell line-derived EXO (TEX$_{EG7}$) indicated that EXO$_{DC}$ can more efficiently stimulate T cell proliferation and differentiation, and also promote stronger killing activities against tumor cells compared to EXO$_{EG7}$ immunized mice. Similar results were observed regarding antitumor immunity and protection against lung tumor metastases. The higher immunogenicity of EXO$_{DC}$ could be ascribed to expression of co-stimulatory molecules such as CD40, CD80 on EXO$_{DC}$. TEX$_{EG7}$ not expressing these co-stimulatory molecules could well explain their weaker efficacy compared to EXO$_{DC}$.[13] However, reverse results were obtained when using of TEXs for DC loading. In an early study, Hao et al. evaluated immune response induction of loading mature DC with TEX, where ovalbumin (OVA) served as tumor antigen. They observed that TEX uptake by DC is mediated by LFA-1, CD54, and CLR. Interestingly, TEX-loaded DCs expressed higher level of the co-stimulatory molecules CD40, CD80, CD54, and of MHC-II than OVA-loaded DCs. Moreover, vaccination with TEX-loaded DCs induced excessive *in vitro* and *in vivo* T cell proliferation and exerted higher protective immunity against the primary tumor and lung metastasis in tumor-bearing mice than OVA-loaded DC, exosomes derived thereof or TEX.[146]

We interpret these findings that the second advantage of loading DC with TEX can be ascribed to the pronounced uptake of TEX and guidance into the MHC-II processing compartment, where TEX tumor antigens are processed for loading into newly arranged MHC-II, which are transported to the DC membrane and together with the expanded repertoire of costimulatory molecules of activated DC suffice for tumor antigen-specific Th activation. This will have bearing not only on CTL activation but also on B cell activation, antibody secretion, and NK cell stimulation. These latter activities still await detailed exploration.

Before recognizing the abundant recovery of tumor antigens in TEXs, DC frequently was loaded with tumor lysates. However, a direct comparison of DC-TEXs versus DC-lysate indicated stronger anti-tumor immunity and superior therapeutic efficacy, when loading with TEXs than tumor lysate. In

a comparative study, Wolfer et al. demonstrated that melanoma TEX-loaded human DCs induced *in vitro* IFN-γ production in CTL clones; the efficacy was comparable to that by loading with synthetic peptides and far higher than that of tumor lysate-loaded DC. Furthermore, TEX elicited more efficient protective antitumor immune responses than tumor lysate in syngeneic and allogeneic settings, even when a higher amount of lysate was applied. Interestingly, boosting with a low dose of TEXs (20 μL) protected vaccinated mice from a lethal challenge, whereas the equivalent (lysate of 2×10^4 tumor cells) was inefficient.[15] The authors suggest the efficient uptake of TEXs by DCs as underlying mechanism, where the presence of CD54, CD9, and CD63 DC-ligands on TEXs facilitates uptake. Similar results were obtained by other investigators in different tumor models using, e.g. leukemia and pancreatic cancer cell-derived exosomes, where TEX-DC more efficiently than lysate-DC increased survival and suppressed tumor growth in pancreatic, renal cell carcinoma and leukemia tumor-bearing mice.[14,152] The superiority of DC-TEX was due to highly efficient TEX uptake and long-lasting TEX processing in the MHC-II-loading compartment, which led to pronounced IL-12 up-regulation in DC and tumor-specific CD4$^+$ Th and CTL activation.[14] Besides activation of a wider range of T cell clones, the presence of classical DC costimulatory molecules may also contribute to TIL, MΦ, and NK cells recruitment.

Notably, due to the immunotolerogenic nature of some organs and tumors deriving thereof, tumor cell lysates can be hardly immunogenic.[166] To overcome this challenge, Rao et al. investigated the efficacy of HCC TEXs to stimulate immune responses *in vitro* and *in vivo*. TEX-pulsed DCs showed superiority with respect to the induction of immune response compared to cell lysates-pulsed DC in both prophylactic and therapeutic HCC mouse models. Stimulation with HCC TEXs efficiently induced the expansion of antigen-specific CTLs, provoked elevated IFN-γ levels, and decreased the release of immunosuppressive IL-10 and TGF-β, which together resulted in stronger tumor growth inhibition in both ectopic and orthotopic HCC bearing mice. Remarkably, HCC TEXs-promoted cytotoxicity exerted MHC-independent cross-protection against different HCC and pancreatic cancer cells.[156] This may be due to the presence of tumor antigens in TEXs, which are shared by multiple tumors. The finding also is in line with TEX-derived tumor peptides being presented in newly generated MHC molecules of the host DC.

In brief, DC loading with TEXs rather than tumor lysate has several advantages. Although autologous TEXs and tumor lysates share avoiding allogeneic immune response, compared to tumor lysates, tumor antigens are enriched in TEXs. Furthermore and importantly, TEXs are particularly equipped for binding and uptake, and uptaken TEX are guided in DC toward the antigen processing compartment.

Finally, we want to point out that the efficacy of vaccination with TEX-pulsed DC can profit from a concomitant treatment with drugs that particularly affect immunosuppressive cells or factors. Xiao et al. focused on improving the efficacy of DC-TEX vaccination in pancreatic cancer through combination with cytotoxic drugs that attack MDSC. Akin to previous studies, they confirmed that TEX-loaded DC could activate T lymphocytes in DC-TEX vaccinated UNKC6141 PaCa-bearing mice, prolong survival, and significantly decrease the metastatic capacity of UNKC tumor cells. Interestingly, combining DC-TEX vaccination with the application of most frequent adjuvant drugs in PaCa treatment, such as Gemcitabine (GEM), ATRA, and Sunitinib (SUN), which interfere with MDSC maturation and/or persistence, resulted in higher numbers of activated T cells in the tumor tissue and significantly improved survival rates compared to only DC-TEX vaccinated mice.[161] Combining DC-TEX vaccination with sorafenib, a chemotherapeutic drug for advanced HCC, as well as PD-1 antibody, promoted immune responses in orthotopic HCC vaccinated mice. The combination of DC-TEX and sorafenib significantly reduced Treg cells and increased CD8$^+$ T cells, although in this model the combination therapy did not significantly improve the survival rate of vaccinated mice.[171] Thus, drug combinations, as a coping strategy for preventing activation and recruitment of immunosuppressive cells and factors should be kept in mind as a possibly strong support in DC-TEX vaccination.

Taken together, DC vaccination keeps the lead role in cancer immunotherapy. Unexpectedly, the mostly immunosuppressive TEXs turn into the currently most efficient "immunogenic" tumor antigen source, provided they are used for DCs loading.

Conclusions, challenges and future direction

Throughout the evolution, the immune system has become a highly efficient organ that keeps the human body's integrity without external support. With the discovery of the memory of the adaptive immune system of higher developed organisms and the notion of forcing the immunological memory by vaccination, the power of the immune system was expanded toward disease prevention. Yet, despite some progress, the success rates by forcing the immune system to cope with tumor growth and progression remained below expectations. One of the so far most promising anti-tumor vaccination strategies makes use of the professional arm of the innate immune system, DCs. Recent research in this regard center on TEXs, which are tumor cell-derived small vesicles that are well defined for not only promoting tumor progression but also for their immunosuppressive activities. Fortunately, clarifying the underlying mechanisms has not only paved the way for tumor growth prevention but also opened new doors toward using TEXs as immunotherapeutic drugs. The most important features of TEX are their preferential collection of tumor antigens (which is not fully explored yet), their distribution throughout the body, their membrane composition, which facilitates TEX binding and uptake for the good or the bad, and lastly, the efficient delivery of the function-competent TEX content. Furthermore, owing to their membrane organization, uptaken TEXs are preferentially guided toward MVB. TEXs have additional advantages for a wide range of clinical applications. They can be collected by non- or minimally invasive methods from all biological fluids as well as from tumor cell culture supernatants. In addition, long-term storage does not strongly impair TEXs functional competence. Thus far, the therapeutic application of TEXs has mostly been explored in
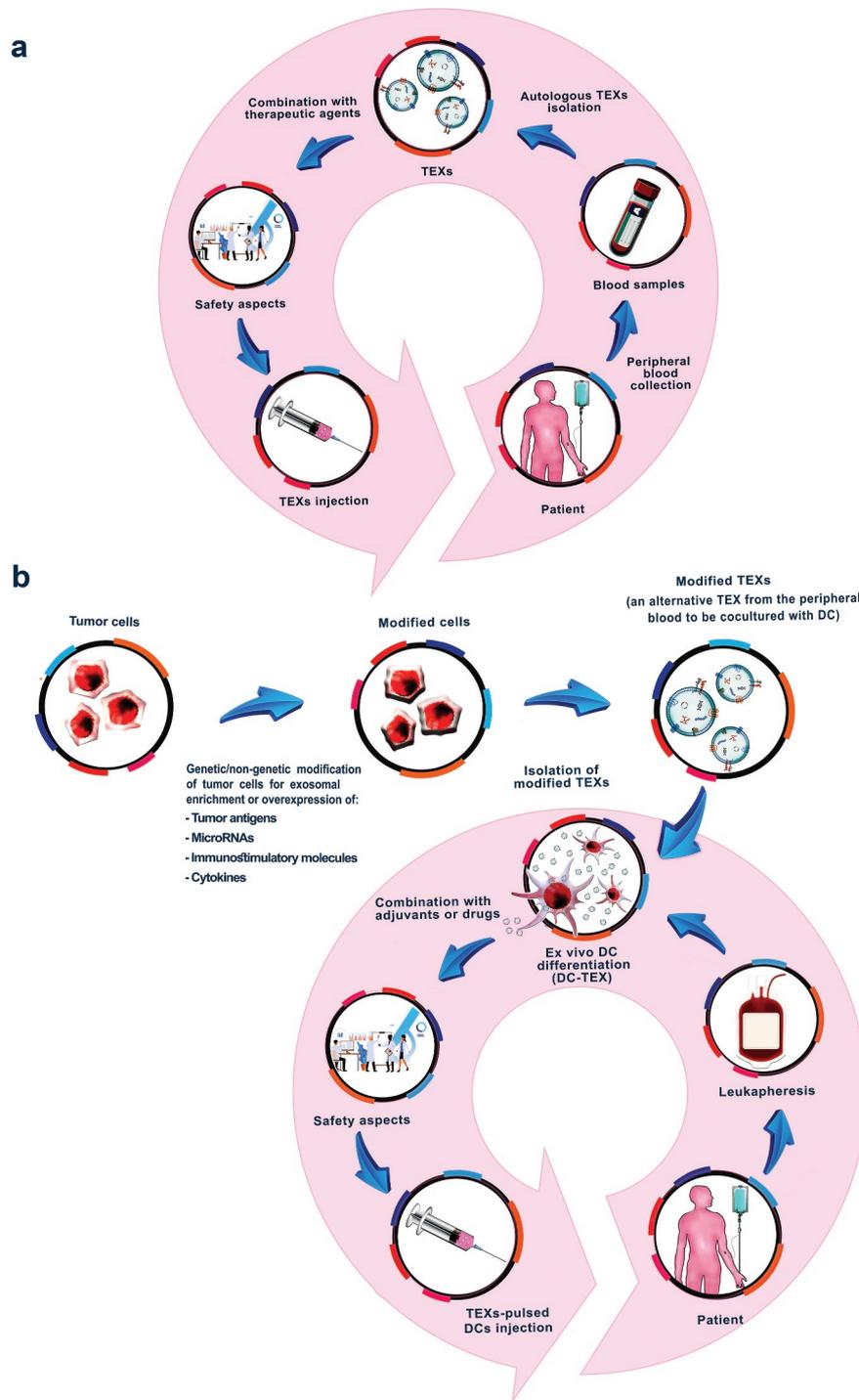
Figure 2. Tumor-derived exosome modulations aiming for increased efficacy of future TEX-based vaccines. (a). Autologous TEXs can be collected from the patients' peripheral blood. They can be modified by transfection with immune response promoting agents to strengthen the efficacy as vaccine, which treatment may be combined with additional therapeutic agents, either loaded into TEX or independently applied. (b) TEXs can be directly or indirectly modified through overexpression of some tumor antigens, miRNAs, immunostimulatory molecules and cytokines that increase their immunogenic potential. Native or modified TEXs can be used for DC pulsing, the transfer of which provoking a strong immune response, at present, considered as the most effective cancer vaccines. The efficacy of DC-TEX can be supported by cytotoxic drugs, preferentially hampering immunosuppressive cells and agents.

animal studies, which can be divided into two categories: the application of modified TEXs and the use of TEXs as antigen providers for DC in vaccination.

Manipulated TEXs have either been derived from genetically modified tumor cells or have been directly transfected. The former strategy is advantageous for membrane integrated proteins that facilitate binding to ligands on selected target cells. It has the additional advantage of a persisting donor for preparation. Direct loading of TEXs will be the method of choice for transferring high quantities of densely packed therapeutic agents, which could be non-coding RNA (e.g. miRNA), cytokines, and chemokines, as well as immune response checkpoint proteins, antibodies, and cytotoxic drugs. Obviously, combining the genetic

modification of the TEX owner cells with direct TEX loading will be beneficial, as TEX content delivery can be targeted toward specified cells. This option has rarely been taken into account and requires a scrutinized analysis of TEX ligands on the aimed for target cell. As far as DC-TEX vaccination is concerned, TEXs manipulation may be required for tumors that display tumor-associated antigens at a level too low for promoting sufficient MHC loading of DC. In many studies, TEXs were used as a vaccine for CD8^{+} T cell maturation and activation. However, one should be aware that this requires profound engineering to equip TEX with targeting units, cytokines, and sufficient amounts of tumor antigens as well as immunosuppressive molecule blockers. Using TEX after a proceeding vaccination with DC for stimulation of memory CD8^{+} T cells avoids these drawbacks, will be far less demanding, and may be considered as a clinically most relevant option (Figure 2a).

Many studies have demonstrated the efficacy of TEX-loaded DC to induce *in vitro* and *in vivo* CTL and T helper cells, to stimulate B cells, NK cells, and macrophages. An important advantage of loading DCs with autologous TEXs is provided by the individual patient's complete tumor-antigen repertoire being presented. An additional benefit relies on a single peripheral blood collection sufficing for DC and concomitant TEXs preparation. The loading that being performed *in vitro* at an appropriate stage of DC maturation, does not require support for targeting or processing, and only for TEX from very low level antigen expressing tumors it might be required to additional loading of TEX with an excess of the relevant antigens. Nonetheless, the preparation of DCs under the required safety conditions is cost and time intensive. It remains to be hoped that the pharmacy succeeds in reducing these factors, which so far hamper a desirable wide range of clinical application (Figure 2b).

The wide range clinical application of TEXs as a therapeutic drug or an antigen provider for DC vaccination remains a hopefully soon realized desire. Particularly, the broad field of potentially therapeutic TEXs requires further experimental studies to guarantee efficacy and negligible side effects. Nonetheless, TEXs as a major contributor to tumor progression obviously have a second face, which suggests them as a possibly most efficient tumor defense item. Taken together, there is some hope that TEXs may become the breakthrough in tumor immunotherapy.

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