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Tumor cell-derived exosome-targeted dendritic cells stimulate stronger $CD8^+$ CTL responses and antitumor immunities

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ABSTRACT

Tumor cell-derived exosomes (TEX) have been widely used to induce antitumor immune responses in animal models and clinical trials. However, the efficiency of the antitumor immunity that is induced by TEX is still relatively weak. In this study, we compared the antitumor immunities between EG7 tumor cell-derived exosomes (EXO_{EG7}) and EXO_{EG7}-targeted dendritic cells (DC_{EXO}). We found that EXO_{EG7} harbored OVA and peptide major histocompatibility complex I (pMHC-I), which were expressed on its parental EG7 tumor cells, and they could transfer OVA and pMHC-I to dendritic cells (DCs) *in vitro*. DC_{EXO} could more efficiently induce antitumor immunity than EXO_{EG7}. In addition, we showed that the immune stimulatory effects of EXO_{EG7} were dependent on the host DCs and, whereas those of DC_{EXO} were not, indicating the important role of the host DCs in TEX vaccines. Taken together, TEX-targeted DCs may be more effective for EXO-based vaccines for the induction of antitumor immunity.

1. Introduction

Tumor cells express a series of antigens that are recognized by cytotoxic T lymphocytes (CTL) [3,23]. However, most of the special tumor antigens have not been identified, and the nature of the tumor antigens that mediate efficient immune responses that lead to tumor rejection remains unclear [13].

One general characteristic of tumor cells is the releasing or shedding of membrane vesicles, which are called exosomes (EXO). EXOs are small membrane-bound vesicles that are released by a variety of cell types [10,28]. Many types of cells, such as dendritic cells (DCs) and B lymphocytes, as well as various tumor cells, that excrete EXOs are of increased interest in tumor immunotherapy [29,33]. Functional analyses have shown that EXOs that are derived from antigen-presenting cells contain a number of antigen-presenting molecules, such as major histocompatibility complex class I and II (MHC-I and MHC-II), costimulatory molecules (CD80 and CD86), and heat shock proteins 70 and 90 (HSP70/HSP90) [11,27]. Some reports have shown that DC-derived EXOs (EXO_{DC}) have antigen-presenting capabilities, which make them a potentially attractive vehicle for immunotherapy [14,18]. Recently, reports from Altieri, Andre and Wolfers have demonstrated that tumor cell-derived exosomes (TEXs) and those isolated from malignant effusions can induce antigen-specific CTL responses and antitumor immunities [1,2,29]. Thus, TEXs have attracted much attention as a potential source of tumor antigens [5,20].

Zitvogel and our previous studies have shown that peptide-pulsed DCs and EXO_{DC} elicit potent antitumor immune responses [15,33].However, the efficiency of the antitumor responses induced by EXO_{DC} is higher than that of EG7 tumor cell-derived EXO (EXO_{EG7}), and this is perhaps due to the lower levels of expression of MHC-II and costimulatory molecules on EXO_{EG7} compared to the expression on EXO_{DC}. In another study, we have demonstrated that EXO_{DC} target other DCs *in vitro*, and EXO_{DC}-targeted DCs can more strongly stimulate antigen-specific $CD8^+$ T-cell proliferation *in vitro* and *in vivo* and more efficiently induce antigen-specific CTL responses and antitumor immunities than EXO_{DC} [14]. Therefore, it is hypothesized that TEXs may transfer tumor antigens to DCs and that TEX-targeted DCs may more efficiently induce antitumor immunity.

In this study, we first investigated the phenotype characteristics of EXO_{EG7} with flow cytometry and compared the efficiency of EXO_{EG7} and EXO_{EG7}-targeted DCs in the stimulation of antigen-specific CTL responses and antitumor immunity.

2. Materials and methods

2.1. Reagents, cell lines, and animals

OVA was obtained from Sigma–Aldrich China, Inc. (Shanghai, China). Biotin or fluorescein isothiocyanate (FITC)-labeled antibodies (Abs) specific for H-$2K^b$, Ia^b, CD40, CD54, or CD80 were all

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obtained from Becton Dickinson Medical Devices Co., Ltd. (Shanghai, China). Anti-Mouse OVA257-264 (SIINFEKL) peptide that was bound to H-2K^b PE-Cy7 was purchased from eBioscience (Shanghai, China). The anti-H-2K^b/OVA-I (pMHC-I) complex Ab was produced by the Chinese Peptide Company (Hangzhou, China). Mouse recombinant IL-4 and GM-CSF were purchased from PeproTech (Shanghai, China). The carboxyfluorescein succinimidyl ester (CFSE) was obtained from Invitrogen China Limited (Shanghai, China). A CytoTox96 Non-Radioactive Cytotoxicity Assay Kit was purchased from Shanghai Promega Biological Products, Ltd. (Shanghai, China). The OVA-transfected tumor cell line EG7 (H-2b), BL6-10$_{OVA}$ melanoma cell lines, and wild-type mouse thymoma tumor cell line EL-4 (H-2b) were generated in the Shanghai Laboratory Animal Center (Shanghai, China). Female C57BL/6 and the diphtheria toxin receptor (DTR)-transgenic mice were obtained from the Shanghai Laboratory Animal Center and used at 6–14 weeks. They were allowed to adapt to their environment for 1 week before the initiation of the experiments, and all animals were maintained under standard environmental conditions with free access to food and water. Mice were treated according to the animal care committee guidelines of the School of Medicine of Shanghai Jiaotong University.

2.2. Generation of bone marrow-derived DCs

The generation of bone marrow-derived DCs has been described previously [16]. Briefly, bone marrow cells prepared from the femora and tibiae of naive C57BL/6 mice were depleted of red blood cells with 0.84% ammonium chloride and plated in DC culture medium containing 10% FCS, GM-CSF (10 ng/mL), and IL-4 (10 ng/mL). On day 3, the nonadherent granulocytes and T and B lymphocytes were gently removed and fresh media was added, and, 2 days later, the DCs aggregates were dislodged and replated. On day 7, DCs were harvested. To generate OVA-pulsed DCs (DC$_{OVA}$), DCs were pulsed with OVA protein (0.3 mg/mL) in AIM-V medium (GIBCO) for overnight culture.

2.3. Generation and purification of exosomes

EXOs were isolated as described previously [6,25]. Briefly, the supernatants of EG7 cells were subjected to 4 successive centrifugations at 300*g* for 5 min and 1200*g* for 20 min and 10,000*g* for 30 min to remove cells and cellular debris, and 100,000*g* for 1 h to pellet EXOs. The EXO pellets were washed twice in a large volume of PBS and recovered by centrifugation at 100,000*g* for 1 h. The amount of exosomal proteins recovered was measured using Bradford assay. EG7 cell-derived exosomes were defined as EXO$_{EG7}$.

2.4. Phenotypic characterization of EG7 cells and EXO$_{EG7}$

For the phenotypic analysis of EG7 cells and EXO$_{EG7}$, both EG7 cells and EXO$_{EG7}$ were stained with a panel of biotin-labeled and FITC-labeled Abs and analyzed by flow cytometry with FACScan (Coulter EPICS XL, Beckman Coulter, Inc., Brea, CA) as previously described [8,14].

2.5. Exosomes taken up by DCs

To test whether EXO$_{EG7}$ could be taken up by DCs *in vitro*, DCs were co-cultured with CFSE-labeled EXO$_{EG7}$ (EXO$_{CFSE}$) for 4 h, and CFSE-positive cells were then detected by flow cytometry [14]. To further confirm that EXO transferred its molecules to DCs, DCs were analyzed for the expression of OVA and the pMHC-I molecule after incubating with EXO$_{EG7}$. DCs pulsed (targeted) with EXO$_{EG7}$ were defined as DC$_{EXO}$.

2.6. Tetramer staining

C57BL/6 mice were intravenously immunized with EXO$_{EG7}$ (10 μg/mouse) or DC$_{EXO}$ (1×10^6/mouse). Six days later, tail blood was harvested and incubated with 10 μL of the PE-H-2K^b/OVA$_{257-264}$ tetramer (eBioscience) and FITC-CD8 for 30 min at room temperature, and the cells were then analyzed by flow cytometry. To investigate the involvement of the host DCs in the TEX vaccines, we also used DTR-transgenic mice, in which CD11c$^+$ DCs were sensitive to diphtheria toxin (DT) [19]. The DTR-transgenic and wild-type C57BL/6 mice were intraperitoneally injected with 2 doses of DT (1.5 ng/g weight) every 3 days. After 2 days, almost no CD11c$^+$ DCs were detectable in the spleen (data not shown) [19]. The treated mice were then intravenously injected with EXO$_{EG7}$ or DC$_{EXO}$, and CD8$^+$ T cells expressing the H-2K^b/OVA$_{257-264}$ tetramer were detected.

2.7. Cytotoxicity assay

In vitro cytotoxicity was analyzed with the lactate dehydrogenase (LDH) releasing method with the CytoTox96 Cytotoxicity Assay Kit according to the manufacturer's instructions. Spleen T lymphocytes from the above immunized mice were harvested with nylon wool columns after 7 days of immunization. Spleen T lymphocytes (5 × 106) were co-cultured with γ-irradiated (6000 rad) EG7 cells (1 × 105) in Dulbecco's Modified Eagle's Medium plus 10% FCS containing IL-2 (20 U/mL) in a 24-well plate (Costar, Shanghai, China). Five days later, T cells were harvested and used as effectors. This enzymatic assay colorimetrically measured the amount of LDH that was released from lysed target cells, including EG7 or control EL-4 cells that were mixed with different ratios of effectors for 4 h at 37 °C. The spontaneous/maximal release ratio was <20% in all experiments. Specific lyses (%) was calculated with the following formula: (experimental LDH release − effectors spontaneous LDH release − target spontaneous LDH release)/(target maximum LDH release) × 100.

2.8. Animal studies

To examine whether EXO$_{EG7}$ or DC$_{EXO}$ induced antitumor protective immunity, wild-type C57BL/6 and DC-knockout mice ($n = 8$) were intravenously injected with EXO$_{EG7}$ (10 μg/mouse) and DC$_{EXO}$ (1×10^6/mouse), respectively. After 6 days, the immunized mice were intravenously challenged with BL6-10$_{OVA}$ (0.5×10^6/mouse). To investigate the involvement of the host DC in EXO vaccines, we also used diphtheria toxin receptor (DTR) transgenic mice, in which, CD11c$^+$ DC sensitive to diphtheria toxin (DT) [19]. The DTR transgenic and wild-type C57BL/6 mice were injected single dose of DT (1.5 ng/g weight), after 2 days, almost no CD11c$^+$ DC were detectable in spleen of DTR transgenic mice [19]. The treated mice ($n = 8$) were then injected i.v. with EXO$_{EG7}$ (10 μg/mouse) and DC$_{EXO}$ (1×10^6/mouse), respectively, and then i.v. challenged with BL6-10$_{OVA}$ (0.5×10^6/mouse) 6 days later. The mice were sacrificed 4 weeks after the tumor cell injection, and the lung metastatic tumor colonies were counted in a blind fashion. Metastases on freshly isolated lungs appeared as discrete black pigmented foci that were easily distinguishable from normal lung tissues, and they were confirmed with histological examination. Metastatic foci that were too numerous to count were assigned an arbitrary value of >100 [30].

3. Results

3.1. Phenotypic characterization of EG7 tumor cells and EXO$_{EG7}$

EG7 tumor cells expressed MHC-class I, CD54, and OVA, but MHC-class II, CD40, and CD80 were almost undetectable. In