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Tumor exosomes block dendritic cells maturation to decrease the T cell immune response

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ABSTRACT

Tumors can induce the generation and accumulation of immunosuppression in a tumor microenvironment, contributing to the tumor's escape from immunological surveillance. Although tumor antigen-pulsed dendritic cell can improve anti-tumor immune responses, tumor associated regulatory dendritic cells are involved in the induction of immune tolerance. The current study sought to investigate whether exosomes produced by tumor cells had any effect on DCs in immune suppression. In this study, we examined the effect of tumor exosomes on DCs and found that exosomes from LLC Lewis lung carcinoma or 4T1 breast cancer cell blocked the differentiation of myeloid precursor cells into $CD11c^+$ DCs and induced cell apoptosis. Tumor exosome treatment inhibited the maturation and migration of DCs and promoted the immune suppression of DCs. The treatment of tumor exosomes drastically decreased $CD4^+IFN\text{-}\gamma^+$ Th1 differentiation but increased the rates of regulatory T (Treg) cells. The immunosuppressive ability of tumor exosome-treated DCs were partially restored with PD-L1 blockage. These data suggested that PD-L1 played a role in tumor exosome-induced DC-associated immune suppression.

1. Introduction

Dendritic cells (DCs) play important roles in initiating the innate and adaptive immune responses. DCs are derived from the hemopoietic progenitor cells within the bone marrow (BM). Normally, DCs capture antigens and undergo maturation, then migrate to lymph nodes, where DCs active T cells to stimulate the host antitumor immune response [1]. However, specific signaling in microenvironment might inhibit DCs maturation, resulting in the formation of DCs with immunosuppressive or tolerogenic potential [2,3]. It is well known that the presence of tumor-educated DCs is a double-edged sword, as they are able to initiate potent antitumor responses but may also support angiogenesis, suppress anti-tumor immunity and promote cancer proliferation and progression [2,4–6]. It has been demonstrated that DCs are not activated in tumor microenvironment and their immune responses are inhibited [7,8]. The suppression of DCs maturation in cancer is thought to contribute to the disappointment of anti-tumor immunotherapy. Our previous data also showed that tumor-educated DCs induced antigen-specific T cells unresponsiveness and regulatory T cells differentiation, then inhibited anti-tumor immune responses in vitro and in vivo [9]. Changes in tumor immunity due to manipulations in the tumor microenvironment and DCs function are mediated by various tumor-derived elements, many of which remain to be elucidated.

Exosomes are nanometric membrane vesicles and can be secreted by almost all kinds of cells, including cancer cells [10]. When released from a donor cell, they can transfer a lot of biological contents to target cells. Wrapped in bilayered membranes, these elements are stable even after being transferred to a distant site. Therefore, exosomes are an effective mode to affect gene expression in distant cells. The role of tumor-derived exosomes (TEXs) in tumor progression is disputed [11]. On one hand, TEXs can mediate immunosuppression by impairing DC differentiation and maturation via the induction of cytokines [12]. Mice pretreated with tumor exosomes showed an accumulation of myeloid-derived suppressor cells (MDSCs) in the spleen and peripheral blood [13]. On the other hand, tumor antigen-containing exosomes carry tumor antigens and can also promote the competent antigen presentation of APCs [14,15]. Andre et al. reported that antigens in tumor exosomes could be transferred to DCs and induce specific CTL activation [16].

In this study, we demonstrated that tumor exosomes suppressed the

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maturation and migration of mouse bone marrow derived-DCs and the secretion of pro-inflammatory factors. Exosomes from LLC Lewis lung carcinoma or 4T1 breast cancer cell blocked DCs differentiation and induced cell apoptosis. The interaction of the exosomes with DCs in vitro induced the expression of PD-L1. The blockage of PD-L1 partially restored the $CD4^+$ T cell proliferation and differentiation inhibited by exosome-treated DCs.

2. Material and methods

2.1. Mice and cell lines

C57BL/6J mice were purchased from Cavens Lab Animal (Changzhou, Jiangsu, China). OT-II mice were provided generously by Prof. Hai Qi, Tsinghua University. OT-II mice express the mouse T cell receptor specific for chicken ovalbumin (OVA) in the context of $I\text{-}A^b$. Immune response can be investigated by direct administration of OVA. All mice were housed under specific pathogen-free animal laboratory and used at 6–8 weeks old. Animal care and all experimental protocols involving animals were performed using institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

The LLC Lewis lung cancer cells (CRL-1642) and 4T1 breast cancer cells (CRL-2539) were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI 1640 complete medium supplemented with 10% FBS (Thermo Fisher Scientific Inc.). LLC is a cell line established from the lung of a C57BL mouse bearing a tumor resulting from an implantation of primary Lewis lung carcinoma. 4T1 is a 6-thioguanine resistant cell line selected from the 410.4 tumor without mutagen treatment. When injected into BALB/c mice, 4T1 spontaneously produces highly metastatic tumors.

2.2. Reagents

Recombinant mouse cytokines, FMS-related tyrosine kinase 3 ligand (Flt-3L), Chemokine (C-C motif) ligand 19 (CCL19) and CCL21, and ELISA kits for murine IFN-γ, IL-12 p70, IL-4, TNF-α and IL-10 were purchased from Biolegend (San Diego, CA). The antibodies of CD11c-PE, CD11b-FITC, MHC-II-APC, CD80-PerCP/cy5.5 and CD86-APC; isotype control mAbs; CFSE and GolgiPlug were purchased from BD Bioscience. CD11c microbeads, CD4 microbeads and MACS column were obtained from Miltenyi Biotec (Shanghai, China), and IFN-γ-FITC, Foxp3-APC, PD-L1-PE and CD40-APC were from purchased eBioscience (Shanghai, China). PD-L1 monoclonal antibody (MIH5) was purchased from Thermo Fisher Scientific Inc. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were obtained from Sigma-Aldrich.

2.3. Isolation of tumor exosomes

Exosomes derived from LLC or 4T1 cells were isolated from culture medium containing $> 10^7$ tumor cells. Cell culture medium was sequentially centrifuged at 300*g* for 5 min, followed by 2000 g for 10 min and 12000 g for 35 min at 4 °C. The supernatant was collected and filtered with a 0.22-μm filter, followed by ultracentrifugation at 120,000*g* for 3 h to pellet exosomes. Exosome pellets were washed with iced PBS and recovered by ultracentrifugation at 120,000*g* for 2 h. Exosome pellets were re-suspended in PBS at a ratio of 10 μL/1 mL medium. The total protein concentration of exosomes was quantified by an Epoch microplate spectrophotometer (BioTek, Shanghai) according to the manufacturer's instructions.

2.4. In vitro differentiation of DCs from BM precursors

BM-derived DCs were generated from primary cultures of femoral marrow from C57BL/6 mice as described previously [17,18]. In brief, BM cells were flushed from the femurs of mice using a medium-filled syringe to obtain a cell suspension. After erythrocytes were lysed, the precursor cells were washed with medium and then re-suspended in RPMI 1640 complete medium with 10% fetal bovine serum, non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 nM 2-mercaptoethanol, and 100 ng/ml Flt-3L, 2×10^6 cells per microliter were plated in 6-well plates and cultured at 37 °C in a humidified 5% CO_2 atmosphere incubator (Thermo Fisher Scientific Inc.). Various concentrations of LLC or 4T1 tumor exosomes were added to the precursor cell culture medium on days 0 and 3. The exosomes from primary isolated normal tissues (lung or mammary gland) are as control, respectively. The half medium was exchanged with fresh medium every 3 days. After in vitro culture, the resulting DCs were collected, washed and used for co-stimulatory/inhibitory molecular expression (flow cytometry), functional characterization (co-culture assays) and gene expression (quantitative RT-PCR) as described below, whereas supernatants were frozen for cytokines evaluated by ELISA.

2.5. Flow cytometry

For blocking of Fc receptors, Cells (4×10^5) in 100 μL PBS were pre-incubated with anti-CD16/CD32 monoclonal antibody for 15 min on ice prior to staining. Cells were stained with fluorochrome-tagged mAbs against CD11c, CD11b, CD40, CD86, PD-L1, MHC-II, CD80 or isotype controls on ice for 30 min, washed with ice-cold staining buffer and the phenotype was analyzed by flow cytometry (FACSCanton II, BD Biosciences).

2.6. Enzyme-linked immunosorbent assay

After treatment, cell culture medium was collected and centrifuged at 10,000 rpm for 5 min. The concentrations of IL-12p70, TNF-α, IL-10 and IL-6 were measured by the respective ELISA kits and determined by the Epoch microplate spectrophotometer according to the manufacturer's instructions.

2.7. qRT-PCR

Cells were removed medium and lysed with TRIzol reagent (ThermoFisher Scientific). The high quality total RNA from cells was isolated and the reverse transcription of RNA to cDNA was performed with TaqMan Reverse Transcription kit (ThermoFisher Scientific). The mRNA levels of indicated molecules were measured by quantitative RT-PCR (qRT-PCR) amplification using the ABI ViiA 7 real-time PCR system. Briefly, cDNA was amplified in a 25-μL reaction system, containing 12.5 μL of SYBR Green mix (Invitrogen), 100 ng of cDNA templates and indicated primers (200 nM) using thirty-eight cycles. The primer sequences were designed with NCBI/primer-BLAST and summarized in Supplemental Table 1.

2.8. DC trafficking

Chemotaxis of DCs, in vitro, was measured using 8 μm-pore transwell chambers (Corning Costar). Briefly, in lower chamber, there were 200 ng/ml recombinant CCL19 or CCL21 in the medium without FBS, and tumor exosomes pre-treated DCs were added to the upper chamber. After twenty-four hours, the cells in lower chambers were collected and counted by FACS.

To analyze the migration of tumor exosomes treated DCs in vivo, 2×10^6 pre-treated DCs were labeled with 2.5 μM CFSE in vitro. Then CFSE labeled-DCs were injected into the hind footpads of syngeneic C57BL/6 mice. After 4 h, the mice were sacrificed and the draining popliteal lymph nodes were extracted. The lymph nodes were digested by collagenase IV and DNase. The CSFE labeled-single cells were analyzed by flow cytometric.