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Melanoma exosomes promote mixed M1 and M2 macrophage polarization

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ABSTRACT

Macrophages are key participants in melanoma growth and survival. In general, macrophages can be classified as M1 or M2 activation phenotypes. Increasing evidence demonstrates that melanoma exosomes also facilitate tumor survival and metastasis. However, the role of melanoma exosomes in directly influencing macrophage function is poorly understood. Herein, we investigated the hypothesis that natural melanoma exosomes might directly influence macrophage polarization. To explore this hypothesis, ELISA, RT-qPCR, and macrophage functional studies were performed *in vitro* using an established source of melanoma exosomes (B16-F10). ELISA results for melanoma exosome induction of common M1 and M2 cytokines in RAW 264.7 macrophages, revealed that melanoma exosomes do not polarize macrophages exclusively in the M1 or M2 direction. Melanoma exosomes induced the M1 and M2 representative cytokines TNF-α and IL-10 respectively. Further assessment, using an RT-qPCR array with RAW 264.7 and primary macrophages, confirmed and extended the ELISA findings. Upregulation of markers common to both M1 and M2 polarization phenotypes included CCL22, IL-12B, IL-1β, IL-6, i-NOS, and TNF-α. The M2 cytokine TGF-β was upregulated in primary but not RAW 264.7 macrophages. Pro-tumor functions have been attributed to each of these markers. Macrophage functional assays demonstrated a trend toward increased i-NOS (M1) to arginase (M2) activity. Collectively, the results provide the first evidence that melanoma exosomes can induce a mixed M1 and M2 pro-tumor macrophage activation phenotype.

1. Introduction

Exosomes are cell-derived extracellular nanovesicles, approximately 100 nm in diameter, depending on their cell of origin [1]. Similar to soluble mediators, tumor exosomes can promote tumor supportive processes. For example, melanoma derived exosomes can mediate immune suppression [2]. They can directly interact with and suppress natural killer and cytotoxic CD8+ lymphocytes or induce myeloid derived suppressor cells (MDSCs). MDSCs can anergize anti-tumor CD8+ lymphocytes, promote M2 macrophage (Mφ) polarization and recruit pro-tumor regulatory T cells.

Melanoma exosomes further support melanoma growth via pro-angiogenic functions. They directly induce endothelial proliferation, endothelial spheroid growth and endothelial spheroid sprouts in a dose-dependent manner *in vitro* [3]. Some pro-angiogenic and immunomodulatory factors found in melanoma exosomes include interleukin 6 (IL-6), vascular endothelial growth factor A (VEGF-A), and matrix metalloprotease 2 (MMP2) [4]. Melanoma exosomes have also been reported to re-program bone marrow progenitor cells toward a pro-vascular phenotype [5].

Previously, we discovered that melanoma exosomes naturally home to the subcapsular sinus (SCS) of lymph nodes [6,7] and prepare them for tumor metastasis [6]. Induction of pro-angiogenic and inflammatory genes such as hypoxia inducible factor 1 alpha (HIF-1α) and tumor necrosis factor alpha (TNF-α) by melanoma exosomes in lymph nodes was observed [6]. Increased expression of these genes is suggestive of Mφ participation in melanoma exosome mediated preparation of pre-metastatic niches.

Macrophages are key participants in tumor pathogenesis. They can be divided into two general classes (M1 and M2) based on function [8]. M1 polarized Mφs possess anti-tumor functions whereas M2 tumor associated Mφs (TAMs) promote tumor growth [9]. Further subdivisions exist within the general M2 Mφ category [8]. Definitive M2 subclassification remains a work in progress given the overlap between polarization markers. Using a typical scheme, M2 Mφs can be divided into M2a, M2b, M2c, and M2d/TAM subclasses [8,10]. In general, IL-10 production is common for all M2 subclasses [8].

To date, there have been minimal investigations into the direct influence of melanoma exosomes on Mφ function. Determining whether melanoma exosomes stimulate Mφ dependent pro-tumor processes will further our basic understanding of melanoma pathogenesis. In this study, we hypothesized that natural melanoma exosomes might directly

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influence Mφ polarization.

2. Materials and methods

2.1. Cell culture

B16-F10 melanoma cells (CRL-6475) and RAW 264.7 mouse Mφs (TIB-71) were obtained directly from the American Type Culture Collection (ATCC). Primary C57BL/6 mouse bone marrow Mφs (C57-6030F) were obtained from Cell Biologics Inc. B16-F10 melanoma cells were cultured at 37 °C in 90% Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and 5% CO^2. RAW 264.7 mouse Mφs were cultured at 37 °C, and maintained in 90% DMEM with 10% heat-inactivated FBS and 5% CO^2. Primary Mφs were cultured at 37 °C, and maintained in complete Mφ medium (M3368, Cell Biologics Inc.) and 5% CO^2.

2.2. Exosome isolation

Isolation and characterization of highly purified populations of B16-F10 mouse melanoma exosomes from cell culture, by means of differential centrifugation, have previously been established [3]. Briefly, to isolate exosomes, cells were grown to 70% confluence in three 300 cm^2 flasks. Culture media was removed, and the cells were washed with DMEM. Cells were then cultured for 48 h in bovine exosome-free conditioned media. Conditioned culture media was prepared by subjecting normal culture media to overnight ultracentrifugation at 110,000*g* to remove bovine exosomes [11]. Cell culture media was harvested after 48 h, diluted 1:1 in 50 mM trehalose (cryoprotectant) PBS [12], and processed using differential centrifugation. Supernatants were collected, and the pellets were discarded, following 3400*g* for 30 min to remove residual cells and debris, and 10,000*g* for 30 min to remove microparticles. Finally, the exosome pellet was collected after 110,000*g* for 1.5 h, and then washed at 110,000*g* for 1.5 h. Exosome protein concentrations were measured using a Pierce BCA protein assay (ThermoFisher Scientific). Exosomes were stored in 50 mM trehalose PBS until use [12].

2.3. Exosome characterization

B16-F10 exosome size (hydrodynamic diameter) was determined in 1X PBS, using dynamic light scattering with a NanoBrook 90Plus (Brookhaven Instruments), according to established methods [3,6,12]. B16-F10 exosome density was determined by sucrose gradient centrifugation of fluorescently labeled exosomes as described previously [3]. Briefly, flotation of fluorescent red carbocyanine DiI (1.0 μM, ThermoFisher Scientific) labeled exosomes (400 μg of exosome protein) on a continuous sucrose gradient (2.0–0.25 M sucrose, 20 mM HEPES/NaOH, pH 7.4) was performed using a Beckman Coulter SW 41 rotor [3,12]. The gradient was produced using a Gradient Master (Biocomp Instruments, Fredericton, NB, Canada) and was spun, after loading exosomes, for > 20 h at 100,000*g*. Post centrifugation, 1 ml fractions were collected from the bottom up. The density of each fraction was calculated using a refractometer [3]. Two hundred microliters of each fraction was added to a black 96-well plate and DiI exosome fluorescence detected using a Tecan M200 infinite pro microplate reader according to established methods [3,12]. Detection of the exosomal marker CD63 on B16-F10 exosomes (10 μg of exosome protein) was determined using an ExoELISA-ULTRA CD63 Kit (EXEL-ULTRA-CD63-1, System Biosciences) according to the manufacturer's instructions. A CD63 standard, supplied with the kit, enabled quantification of CD63 + exosomes per μg of exosome protein.

2.4. Cell treatments

RAW 264.7 or primary Mφs (2500 per well of a 96 well plate) were grown for 24 h in their respective bovine exosome-free conditioned medias. Post 24 h of cell culture, media was replaced with fresh conditioned media containing either lipopolysaccharide (LPS), IL-4, B16-F10 melanoma exosomes, or a combination of LPS + exosomes, or IL-4 + exosomes for an additional 24 h. For LPS or IL-4 dosing of Mφs, 200 ng/ml LPS (*E. coli*, Sigma-Aldrich, L6529) or 40 ng/ml IL-4 (Sigma-Aldrich, SRP-3211) was used. These concentrations are based on previously established methods to induce M1 or M2 polarization in RAW 264.7 cells [13]. For exosome dosing, a concentration of 0.01 mg/ml exosome protein, measured via BCA absorbance (ThermoFisher Scientific Inc.) was used. All treatments were performed in bovine exosome-free conditioned media.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Following RAW 264.7 cell treatments with LPS, IL-4, exosomes or a combination, cell supernatants were collected and processed using affymetrix eBioscience ready-set-go ELISA kits according to manufacturers' instructions to detect RAW 264.7 cell expression of IL-10 (cat# 88-7105), TGF-β (cat# 88-8350), TNF-α (cat# 88-7324) and IL-1β (cat# 88-7013-86). Cytokine levels were normalized to cell growth using PrestoBlue® (ThermoFisher Scientific) cell viability reagent. The 2-tailed Student's *t* test was used to determine statistically significant, *P* values for $\alpha = 0.05$, differences in cytokine expression between treatment groups.

2.6. Quantitative reverse transcription PCR (RT-qPCR)

Following RAW 264.7, or primary Mφ treatments with exosomes, cell culture media was removed and cells were washed in PBS (Sigma-Aldrich, cat# D8537). RNA was isolated using Qiagen's miRNAeasy kit (cat# 217004) according to the manufacturer's instructions. RNA quantity and quality were assessed using a Tecan M200 infinite pro microplate reader. For each sample, 1 μg of cellular RNA was converted to cDNA using Qiagen's RT2 First Strand kit (cat# 33041). Following conversion to cDNA, each sample was applied to a Qiagen Mouse Cancer Inflammation and Immunity Crosstalk RT2 Profiler PCR Array (PAMM-181Z). Quantitative PCR was performed using a StepOnePlus™ Real-Time PCR system (Applied Biosystems™). Array results, normalization gene selection, and statistics were determined using Qiagen's online PCR array data analysis portal (http://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/rt2-profiler-pcr-arrays-data-analysis-center). Using Qiagen's data analysis portal, the beta actin gene was selected as the best normalization gene across RAW 264.7 Mφ arrays, and (C-X-C) motif chemokine receptor 7 (CXCR7), VEGF-A, and toll-like receptor 4 (TLR-4) were automatically selected as the best normalization genes across primary Mφ arrays.

2.7. Inducible nitric oxide synthase (i-NOS) and arginase activity assays

To determine M1 or M2 RAW 264.7, or primary Mφ function, following treatment with B16-F10 melanoma exosomes, assays to assess increased inducible nitric oxide synthase activity, indicative of M1 Mφ polarization, and increased arginase activity, indicative of M2 Mφ polarization, were performed. To assess i-NOS activity, a Nitric Oxide Synthase Detection System (Fluorometric, FCANOS1-1KT, Sigma-Aldrich) was used according to manufacturer's instructions. Briefly, 25,000 cells per well of a 96 well plate were incubated for 24 h at 37 °C in 200 μl of bovine exosome-free conditioned media containing mouse GM-CSF (0.1 mg/ml, PeproTech, 315-03) [14]. Post 24 h, cell culture supernatants were aspirated and wells were treated for an additional 24 h at 37 °C with either LPS (200 ng/ml), washed exosomes (0.01 mg/ml in 50 mM Trehalose-PBS), or in an equivalent amount of 50 mM trehalose-PBS (vehicle-control) in 200 μl of fresh bovine exosome-free conditioned media containing mouse GM-CSF (0.1 mg/ml, PeproTech, 315-03) [14]. Subsequently, i-NOS activity was determined.