



Melanoma cell-derived exosomes alter macrophage and dendritic cell functions *in vitro*

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ABSTRACT

To clarify controversies in the literature of the field, we have purified and characterized B16F1 melanoma cell derived exosomes (mcd-exosomes) then we attempted to dissect their immunological activities. We tested how mcd-exosomes influence CD4⁺ T cell proliferation induced by bone marrow derived dendritic cells; we quantified NF- κ B activation in mature macrophages stimulated with mcd-exosomes, and we compared the cytokine profile of LPS-stimulated, IL-4 induced, and mcd-exosome treated macrophages. We observed that mcd-exosomes helped the maturation of dendritic cells, enhancing T cell proliferation induced by the treated dendritic cells. The exosomes also activated macrophages, as measured by NF- κ B activation. The cytokine and chemokine profile of macrophages treated with tumor cell derived exosomes showed marked differences from those induced by either LPS or IL-4, and it suggested that exosomes may play a role in the tumor progression and metastasis formation through supporting tumor immune escape mechanisms.

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1. Introduction

Previous studies showed that cultured human tumor cells release exosomes, *i.e.*, microvesicles of 20–100 nm diameters. Exosomes bear the molecular markers of the originating tumor cells' plasma membrane. Among others, exosomes contain structural proteins, MHC molecules and cell surface molecules typically associated with apoptosis; therefore they are possible inducers of anti-tumor immune responses [1]. The immunomodulatory activities of tumor exosomes are poorly understood, with reported activating [2] and inhibitory effects [3]. The wide range of observed biological effects seems to depend on tumor type and staging.

The size of exosomes may facilitate their *in vivo* penetration and interaction with different host cell types, even distant from the tumor mass. Exosomes have been shown to participate in

cell-to-cell communication by various mechanisms. The most obvious mechanism involves membrane proteins, partially identical with those of the originating tumor cells. Upon endocytosis, exosomes may deliver their active components, proteins and RNA, directly into the cytoplasm of bystander cells. These transport processes can influence invasion of tumor cells, stimulate antigen specific T-cell responses, modulate cell polarity and have a role in the developmental patterning of tissues [4]. Exosomes may also have an important role in tumor immune evasion by direct suppression of immune cell activation.

By dissecting the immunomodulatory effects of melanoma cell derived exosomes we wished to clarify some of the controversies in the literature of the field. We tested how mcd-exosomes influence the CD4⁺ T cell proliferation induced by bone marrow derived dendritic cells (DCs). We quantified the NF- κ B activation in mature macrophages stimulated with mcd-exosomes. We qualified the production pattern of inflammatory cytokines and chemokines that have previously been identified as mediators or regulators of anti-cancer responses. Our findings suggest that, in spite of their immune activating effects detectable in our model, the concomitant

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immune suppressive activity of tumor-derived exosomes could contribute to tumor immune escape.

2. Materials and methods

2.1. Isolation of exosomes

mcd-exosomes were prepared according to an earlier protocol [5]. B16 supernatants were centrifuged at $3900 \times g$ for 10 min at 4°C to remove cells, then filtered through a $0.2 \mu\text{m}$ membrane (Millipore, Billerica, MA) to remove cell debris. Exosomes were pelleted by ultracentrifugation at 150,000 RCF (Ti 70 rotor 38,200 rpm for 1 h, Optima LE-80K ultracentrifuge, Beckman/T1270 rotor at 40,500 rpm for 1 h, Sorvall Discovery 90SE ultracentrifuge, Hitachi) at 4°C . Pellet was washed twice and resuspended in DPBS and stored at -80°C .

2.2. Exosome characterization

Exosomes were let to adsorb to the freshly cleaved muscovite mica (SPI-Chem™ Mica Sheets, Structure Probe, Inc., West Chester, PA, USA) surface directly from buffer solution (20 mM Tris, 130 mM NaCl, pH = 7). All AFM measurements were carried out with an Asylum MFP-3D head and Molecular Force Probe Controller (Asylum Research, Santa Barbara, CA), using MFP-3D Xop driver program written in Igor Pro software (version 6.2.2, Wavemetrics, Lake Oswego, OR). Image procession and data calculation were made in the same software. Silicon nitride rectangular cantilevers with “V” shaped tips (Bio-Lever BL-RC150VB-C1, Olympus Optical Co., Ltd., Tokyo, Japan with the typical spring constant of 0.03 N/m in liquid) were used for the experiments. Typically 512×512 points was taken at 1 line/s scan rate in AC mode under buffer solution. The measurements presented here are $1 \mu\text{m} \times 1 \mu\text{m}$ flattened height and amplitude images, and height profile.

For transmission electron microscopy, the pelleted exosomes were washed in 0.1 M PBS then fixed overnight in a fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB). Samples were then washed in 0.1 M PB with two rinses, 30 min in each, and postfixed in 1% aqueous OsO_4 for 1 h. After $3 \times 30'$ wash in distilled water, pellets were dehydrated in graded series of ethanol, processed through propylene oxide and embedded in plastic (Durcupan ACM). Blocks were polymerized at 56°C for 2 days. Plastic blocks were trimmed then sectioned with a Leica Ultracut S ultramicrotome at a nominal thickness of 60 nm. Ultrathin sections were mounted on formvar coated 150 mesh copper grids, stained with uranyl acetate and lead citrate and examined under the electron microscope (Zeiss CEM 902). Digital images were taken at $85,000\times$ and $140,000\times$ instrumental magnification using a 4 M pixel monochrome CCD camera (Diagnostic Instruments, model #14).

2.3. Cell cultures and cell lines

Murine immature DCs were generated from bone marrow (BM). C57BL/6 mice were provided by the Animal Production Area of the NCI (Frederick, MD). NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council, 1996; National Academy Press; Washington, D.C.). BM was obtained by aspirating cells from the femurs and tibias of 8–10-week-old female C57BL/6 mice followed by culture in RPMI-1640 medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum (HyClone, Logan, Utah), 2 mM glutamine, 100 U/ml penicillin and streptomycin (Quality Biological, Gaithersburg, MD) and murine recombinant GM-CSF

at 20 ng/ml. 6 days old cell cultures were induced by exosomes ($5 \mu\text{g/ml}$ total protein), recombinant RANTES ($1 \mu\text{g/ml}$) (Peprotech) and anti-CD3+ anti-CD28 ($2.5 \mu\text{g/ml}$ each) (BD Biosciences, San Jose, CA).

2.3.1. B16F1 melanoma cell line

Murine melanoma cell line, obtained from DTP, DCTP Tumor Repository, were cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech) containing 10% fetal bovine serum (HyClone), 1% sodium pyruvate, 1% MEM non-essential amino acids, 1% MEM vitamin solution (Invitrogen) 2 mM glutamine, 100 U/ml each penicillin and streptomycin (Quality Biological).

2.3.2. CD4+ cells

Spleens from 8- to 10-week-old female C57BL/6 mice were dissected and CD4+ cells were purified using R&D Systems (Minneapolis, MN), Mouse T Cell CD4 Subset Column Kit following the manufacturer's instruction.

2.3.3. RAW 264.7 macrophage cell line

The RAW 264.7 murine macrophages were cultured in MIX MEM [1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 plus 10% (v/v) FCS (all from Sigma)].

2.3.4. Transfection of cell lines

RAW 264.7 macrophages were transfected with pNF- κ B-luc4 plasmid as described previously [6].

2.4. Luciferase assay

One-day-old cultures RAW 264.7 cells, grown on luminoplates (Corning-Costar; Zenon Biotechnology Ltd., Szeged, Hungary) were used. Cells (5×10^5 cells/well in MIX MEM, 10% FCS) were induced by mcd-exosomes and 100 ng/ml LPS (*E. coli* 055:B5; Sigma). After 6 h of incubation with inducers, the medium was removed, and the cells were washed and lysed for 10 min at room temperature in Cell Culture Lysis Reagent ($20 \mu\text{l/well}$; Promega Bio-Science Hungary, Budapest). Substrate was added ($20 \mu\text{l/well}$; Promega, Bio-Science Hungary, Budapest), and luciferase activity was measured in a Luminoskan Ascent (Thermo Electron Corporation, Waltham, MA) scanning luminometer. Cell viability was routinely determined using trypan blue exclusion test during the assays to make sure that assays were always carried out on viable cells.

2.5. Proteoma profiling of mcd-exosomes induced macrophages

10^6 /sample mature macrophages were induced by 100 ng/ml LPS (Sigma), 20 ng/ml IL-4 (R&D Systems, Minneapolis, MN) and mcd-Exosomes (total protein content adjusted for $600 \mu\text{g/ml}$) for 24 h. To define basal cytokine and chemokine production of the mature macrophages, they were cultured in absence of the stimulators. After induction, supernatants were collected and analyzed with R&D Systems Proteomes Profiler Assay following the manufacturer's instruction.

3. Results

3.1. B16 murine melanoma cells produce exosomes

At the first step, we established a standardized protocol of melanoma cell culture for exosome production. For an optimal exosome recovery, 2×10^7 melanoma cells were seeded at T75 flasks and cultured for 2–4 (typically 3) days. Melanin content of the supernatants was used as a surrogate marker of copious exosome production. Size and form distribution of the exosomes was determined by atomic force microscopy (Fig. 1a, b, and d). Fig. 1 shows

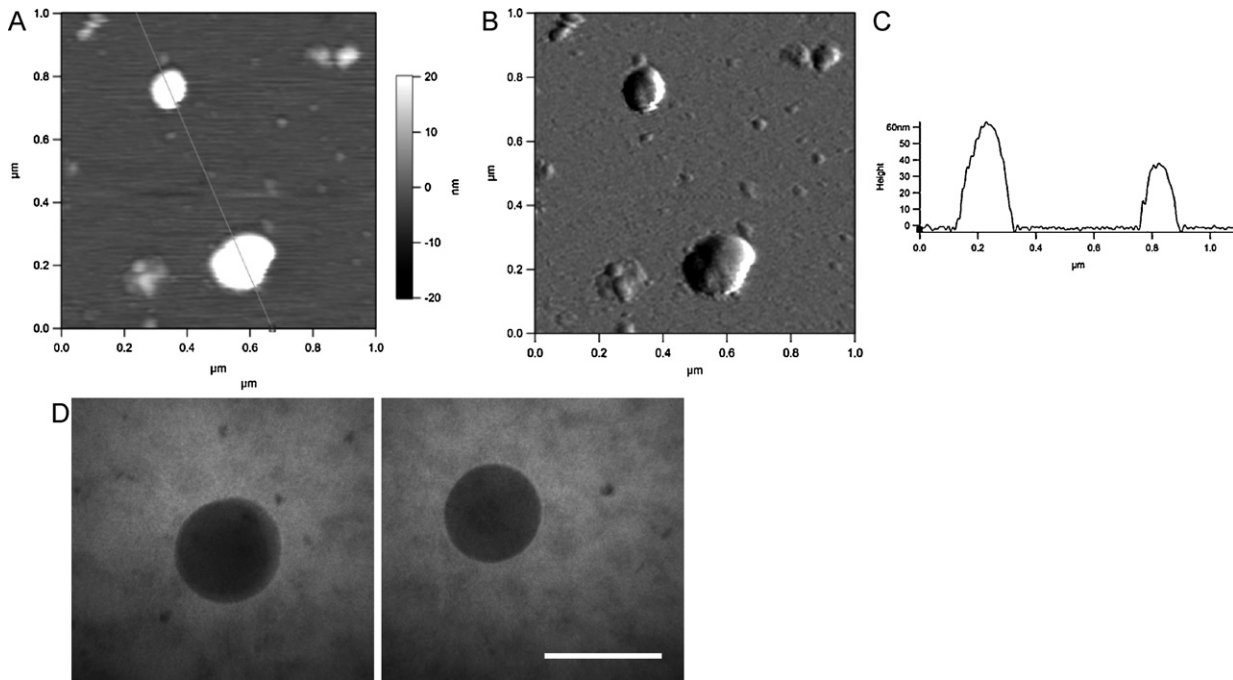


Fig. 1. B16F1 melanoma cells release exosomes into the culture medium. Exosomes prepared from the supernatants of cultured melanoma cells were visualized by two different methods. (A–C) Height and amplitude image, and height profile of exosomes measured in buffer by AFM. The size of the exosomes was found to be well defined and characteristic. (D) Transmission electron microscopic images of B16 exosomes. While the diameter of the particles is comparable to those seen by atomic force microscopy, no internal structure is noticeable. The scale bar represents 100 nm.

that the diameter of the exosomes (indicated in the AFM height image section) is in the range of 30–70 nm, respectively. This data corresponds to the typical size range of exosomes found in literature [1]. Transmission electron microscopy images showed no internal structure, thus ruling out contamination of our samples with viral particles (Fig. 1c and d).

3.2. Melanoma cell derived exosomes enhance T cell proliferation induced by DCs

Immature murine DCs were generated from bone marrow of 8–10-week-old female C57BL/6 mice. In the next step, the DCs cultures were stimulated with B16 exosomes. LPS served as a positive control for DC activation.

CD4⁺ T cells (5×10^5 cells/well) were seeded in U-bottom 96-well plates together with 10^4 cells/well of induced or control DCs as described below. Anti-CD3 and anti-CD28 antibodies (2.5 μg/ml) were added as positive controls for inducing T cell proliferation and RANTES (1 μg/ml) as a negative control. The proliferation was determined after 72 h of incubation. Cells were pulsed with 1 μCi [³H] thymidine per well for the last 6 h of culture period. We found that the mcd-exosome pretreatment significantly increased the T cell proliferation inducing capacity of DCs. As shown in Fig. 2, CD4⁺ T cells from B6 mice were highly responsive to DC induced by purified exosomes, LPS or CD3/CD28 antibody stimulation, indicating that mcd-exosome stimulated DCs were indeed activated. In contrast, DCs pretreated by RANTES were hyporesponsive.

To assess the eventual differences between the different stimuli, effect of mcd-exosomes on T cell proliferation, bone marrow derived DCs were incubated with exosomes, LPS (100 ng/ml) or IL-4 (20 ng/ml IL-4) for 24 h. Splenic T cells separated with CD4⁺ T cell selection columns were co-cultured with the induced DCs. After incubation, T cell surface markers were analyzed by flow cytometry. We have found that the stimuli induced significant T cell

proliferation, but no differences were detected in the ratio of the activated T cells (CD4⁺CD44⁺CD25⁺), neither resting T cells between the different treatment groups. We concluded that mcd-exosomes, just like LPS or IL-4, could induce T cell proliferation, but no signs of different types of polarization were detected in this model (data not shown). These results, however, again underlined that melanoma derived exosomes are not immunologically inert particles.

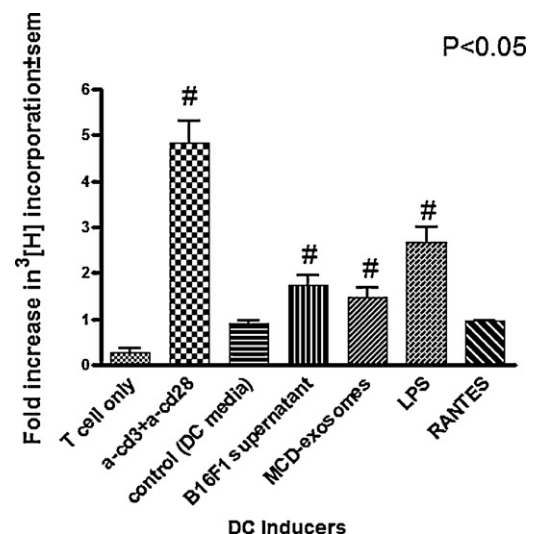


Fig. 2. B16F1 melanoma exosomes enhance T cell proliferation induced by dendritic cells. Purified T cells were stimulated with bone marrow derived C57BL/6 CD4⁺ dendritic cells in the presence of exosomes, positive control (LPS 100 ng/ml) or negative control (RANTES). The background T cell proliferation was determined by using T cells only, while the maximal T cell proliferation was measured after stimulation with anti-CD3 and anti-CD28 antibodies.

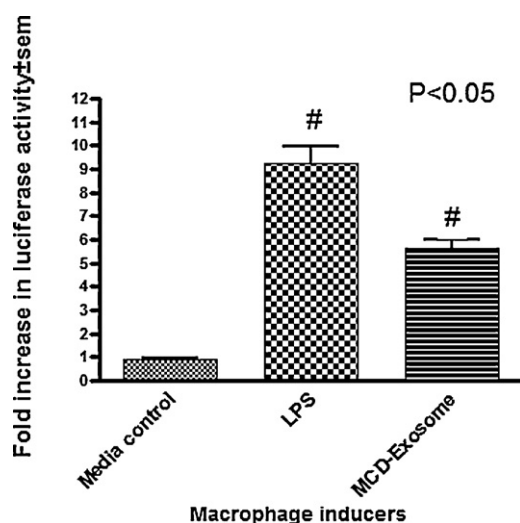


Fig. 3. B16F1 melanoma exosomes activate macrophages. B16F1 exosomes induced the activation of pNF- κ B-luc4 reporter gene in RAW 264.7 mouse macrophages treated for 6 h. LPS (100 ng/ml) was applied as positive control.

3.3. Melanoma cell derived exosomes activate NF- κ B in macrophages

Mature RAW 264.7 reporter macrophages stably transfected by the pNF- κ B-luc4 reporter plasmid were induced with purified melanoma cell derived exosomes; the total protein concentration of the exosome suspension was adjusted to 600 μ g/ml. *E. coli* LPS was used as a positive control (Fig. 3). We have found that the mcd-exosomes increased the luciferase activity ($P < 0.05$). NF- κ B activation in macrophages is involved in immune activation leading to inflammatory cytokine gene transcription. The activation of this central pathway supports our hypothesis that tumor derived exosomes are able to stimulate elements of immune system. Though the increased T cell proliferation and macrophage activation verified the immunological activity of mcd-exosomes, the type of activation, i.e., the contribution of M1 type, inflammatory activation that leads to tumor rejection, or M2 type activation leading to tumor progression, was not clear at this point.

3.4. Melanoma cell derived exosomes alter the cytokine and chemokine profile of macrophages

To investigate the possible type 1/type 2 bias after exosome induction, we screened the cytokine profile of mature murine macrophages. We induced 10^6 RAW 264.7 murine macrophages with B16F1 exosomes (600 μ g/ml total protein), 100 ng/ml LPS (Sigma) and 20 ng/ml IL-4 (R&D Systems, Minneapolis, MN) then the supernatants were harvested and tested by a cytokine and chemokine profiling assay. The proteoma analysis of the supernatants was performed by a multi-dot blot assay (Proteome Profiler Array, R&D Systems); the areas of maximal density were quantified by the ImageJ 1.45s, NIH, USA software. Fig. 4 shows a representative result of four experiments that gave similar results. From the cytokines quantified only those that showed the most characteristic changes are plotted at the figure. Since the used RAW 264.7 murine macrophage cell line is of a mature phenotype, we detected a basal production of numerous cytokines and chemokines in absence of stimuli. After different types of induction, we found that mcd-exosomes reduced the level of TIMP1, IFN- γ and IL-16, as compared with non-stimulated controls. In contrast, we detected a marked increase in the levels of IL-8, CCL2, MIP2 and IL-1Ra. The mcd-exosome induced protein pattern was distinctly different both from the LPS- and the IL-4-induced profile. IL-4 induction decreased the

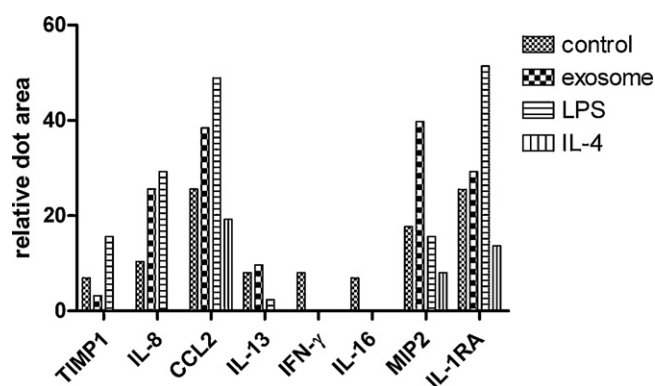


Fig. 4. B16F1 exosomes alter the cytokine and chemokine profile of mature macrophages. The basal levels of MIP2, IL-8, CCL2, IL-13 and IL-1Ra proteins were increased by the treatment with melanoma cell derived exosomes, while the levels of TIMP1, IFN- γ and IL-16 decreased. The mcd-exosome induced protein pattern was distinctly different both from the LPS- and the IL-4 induced profile.

level of CCL2, IL-1Ra and MIP2, while mcd-exosomes increased it. In contrast to LPS induction, mcd exosomes induced IL-13 and MIP2 production of the macrophages.

Beside the cytokines mentioned above, we analyzed the expression of further cytokines and chemokines, taken together, 40 proteins, including CXCL13, IL-3, CXCL17, C5a, IL-4, CXCL11, TIMP1, G-CSF, IL-5, IL-8, TNF α , GM-CSF, IL-6, M-CSF, TREM1, CCL-1, IL-7, CCL2, CCL11, IL-10, CCL12, CD54, IL-13, CXCL9, IFN γ , IL-12p70, CCL3, IL-1 α , IL-16, CCL4, IL-1 β , IL-17, MIP2, IL-1Ra, IL-23, CCL5, IL-2, IL-27 and CXCL12. The levels of IL-3, CXCL17, CXCL11, IL-5, IL-6, CCL11, IL10, IL-12p70, IL-2 and CXCL12 were below the detection limit of our assay. In repeated experiments we did not find consequent, markedly differences between effect of different types of inductions in case of CXCL13, CXCL10, C5a, IL-4, G-CSF, TNF α , GM-CSF, M-CSF, TREM1, CCL-1, IL-7, CCL12, CD54CXCL9, CCL3, CCL4, IL-23, CCL5 and IL-27.

4. Discussion

In the first step of our studies we have set up a standardized method of reproducible *in vitro* production of exosomes, then their purification by differential filtration and ultracentrifugation steps. The surface composition of exosomes is generally identical to the cell membrane of the originating cells, thus no specific surface markers are available for their identification. Because of that, we turned to ultrastructural and AFM methods for quality control of the exosome suspensions. As it was expected based on literature data, the obtained exosomes had a diameter of 20–100 nm, and the lack of their internal structure showed that they are not contaminated with viral particles; a fact that could have resulted in misinterpretation of the obtained data.

Then we showed that melanoma cell derived exosomes promoted the maturation of dendritic cells, after which they induced an enhanced T cell proliferation. These results are in contrast to several earlier papers suggesting that exosomes suppress, rather than promote DC maturation, therefore they might not enhance anti cancer immunity [7,8]. Other publications suggested that tumor exosomes do enhance tumor immunity [1]. Multhoff et al. found that exosomes originating from Hsp70/Bag-4 membrane-positive pancreas and colon tumor cells stimulate migration and reactivity in NK cells [9]. These NK cells activated with exosomes effectively lysed autologous colon tumor cells. Since tumor cell derived exosomes are formed from the cell membrane of the originating cells, these discrepancies might reflect the inherent genetic instability of tumors as reflected in different molecular patterns of plasma membranes of different tumors. Based on these data, B16F1 melanomas

seem to activate, rather than suppress DC maturation and by that induction of T cell proliferation, however the phenotype of the activated T cell is not clear yet.

Although slightly less effectively than the positive control LPS, exosomes induced NF- κ B activation in RAW macrophages, demonstrating that they have activatory effect in this model also. The transcription factor NF- κ B is known to regulate the expression of a number of crucial genes in macrophages. Activation of these cells in response to M1 stimuli, such as TLR ligands, TNF- α and IL-1 β , is regulated primarily by NF- κ B [10,11]. In addition, transcription of several tumor-promoting genes, such as VEGF, IL-6, TNF- α , and COX2, is also regulated, in part, by NF- κ B, pointing to its key role in the activation of tumor infiltrating macrophages [12–14].

On the other hand, Feng-Hua Wu et al described that the activated NF- κ B not only promoted the proliferation of B16 cells, but also enhanced apoptosis resistance of B16 cells by up-regulating Bcl-2 and Bcl-xL and down-regulating Bax [15].

All these data suggest that the development of malignant processes is supported by exosome-induced activation of NF- κ B.

The chemokine/cytokine expression changes induced by the exosome treatment are complex and more difficult to interpret.

The exosome induction resulted in a “mixed” macrophage cytokine and chemokine profile, different both from Th1 and Th2 type responses. The cytokine profile detected might be in connection with the malignant phenotype of the tumors. The reduced TIMP1 matrix metalloproteinase inhibitor level could support metastasis formation. The decreased level of IFN γ and IL-16 might reflect a reduced anti-tumoral Th1 type response, together with the IL-1Ra and IL-13, both well known suppressors of the Th1 type immune response.

CCL2 and MIP-2, expressed in higher quantities in our model, have been shown to be involved in the process of inflammation, angiogenesis, tumorigenesis, and wound healing [16,17].

TIMP-1 has also multi-faceted role in tumor growth and rejection. Although it blocks the matrix metalloproteinases, essential for tumor invasion and metastasis, it also possesses an array of tissue specific metalloproteinase-independent activities, including mitogenic and anti-apoptotic effects [18,19]. To summarize our findings, we can state that exosomes produced by B16F1 are immunologically active. They are capable of transforming dendritic cell and macrophage functions, inducing T cell proliferation and NF- κ B activation *in vitro*. mcd-exosomes can trigger the secretion of cytokines and chemokines by macrophages, some of which may have anti-tumor and tumor-supporting properties, depending on the circumstances, while others may facilitate tumor progression.

In conclusion, our observations suggest that nanometer-sized exosomes produced by tumor cells may play a role in the tumor progression and metastasis formation of malignant diseases through supporting tumor escape mechanisms.

Taken together, our observations suggest that melanoma derived exosomes interact with macrophages as well as dendritic cells, although the mixed biological effects observed in our experiments may contribute not only to anti-tumor immune response but also to tumor immune escape. The fact that, in the absence of therapeutical interventions, the tumor microenvironment harbors mostly type 2 tumor associated macrophages and suppressive

dendritic cells indicates that melanoma derived exosomes might typically skew the balance in the direction of immune suppressive mechanisms.

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