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ORIGINAL ARTICLE

EXOSOMES FROM CT26 CELLS PROMOTE TUMOR PROGRESSION BY SUBVERTING MACROPHAGE FROM M1 TO M2 MEDIATED PARTIALLY BY INTERLEUKIN-4

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ABSTRACT

Background: Macrophages are a critical component of anti-tumor immunity but may be subverted from M1 phenotype, which mediates tumor elimination, to an alternatively – activated, M2 phenotype, which promotes tumor progression.

Methodology: In this study, exosomes purified from CT26 cells culture medium were used to treat CT26 tumor – bearing mice. Then the polarization of macrophage from Balb/c mice was tested after incubation with exosomes by detection of Tumor Necrosis Factor-α (TNF-α), interleukin 6, 10 (IL6, IL10) and Chemokine (C-C motif) ligand 1 (CCL1) using ELISA assays and demonstrated that CT26 – derived exosomes effectively promote polarization of macrophage in vitro. IL4 is a strong inducer of M2 polarization, so we next measured the secretion of IL4 from macrophage after treating with exosomes and results showed that the expression of IL4 was dramatically elevated by CT26 – derived exosomes.

Results: This result was confirmed by treating macrophages from IL-4 knockout mice in vitro and in vivo.

Conclusion: Collectively, we suggest a novel mechanism by which CT26 – derived exosomes exert tumor promotion activity via subverting phenotype of macrophage.

Key words: Macrophage polarization; exosomes, IL4.

INTRODUCTION

Tumor – induced immune suppression is a significant impediment to innate immunosurveillance and immunotherapy of cancer.[1,2] Increasing evidence suggest that a variety of mechanisms are responsible for tumor related immune suppression and the polarization of tumor – associated macrophages is one of the most important mechanism.[3-5] Macrophages are the dominant population of leukocytes found in the tumor microenvironment. Accumulating evidence suggests that these tumor – associated macrophages (TAMs) actively promote all aspects of tumor vascularization, growth, and development.[6,7] TAMs exhibit an M2 – like phenotype because they express a series of markers, such as heat shock proteins, CD163 and C-type lectin domains. On the other hand, a number of chemoattractants including interleukin 4, 10 (IL4, IL10) and Transforming growth factor beta (TGF-β) in the tumor microenvironment which lead to the adoption of an M2 phenotype.[8-13]

Studies reported that TAMs existing in solid tumor tissues significantly contribute to the initiation of angiogenesis by producing numbers of substances including VEGF, PDGF, plasminogen activators and matrix metalloproteases.[8,14-16] In the absence of TAMs, the tumor cells produce the necessary stimuli to initiate tumor angiogenesis, but the initiation is delayed.[17] Furthermore, TAMs can promote lymphangiogenesis mediated by VEGF C, VEGF-D via VEGFR3.[18] In addition, TAMs also play a pivotal role in tumor growth and metastasis by secreting a series of substances. Another important mechanism is immunosuppression induced by TAMs by expressing immunosuppressive cytokines and proteases.

Exosomes range approximately from 30 – 100 nm in diameter secreted by live cells and were first observed in the early 1980s.[19] In recent years, exosomes have emerged as important molecules for inter-cellular communication that are involved both in normal and in pathophysiological conditions, such as cancers. A large number of biological functions of exosomes have been demonstrated, but to our knowledge, only few studies focused on the functions of exosomes on TAMs. In this study, we found that exosomes derived from CT26 cells culture medium could promote CT26 tumor growth by regulating polarization of macrophages.

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MATERIALS AND METHODS

Cell culture

The CT26 mouse colon carcinoma cell line purchased from the American Type Culture Collection (ATCC, MD, USA) was routinely maintained in RPMI 1640 culture medium containing 10% (v/v) fetal calf serum in a humidified CO_2 incubator at 37°C.

Mice

BALB/c mice, 6 – 8 weeks of age were obtained from Animal Biosafety Level 3 Laboratory of Wuhan University (Wuhan, China). IL4 KO BALB/c mice were purchased from The Jackson Laboratory (Marine, USA). All animal procedures were approved by the Institutional Animal Care and Use Committee of Wuhan University.

Isolation of exosomes

Exosomes were isolated from CT26 cells using the Total Exosome Isolation Kit following the manufacturers protocol (Invitrogen, Carlsbad, CA). Exosomes in serum was removed by ultracetrifugation at 100,000 g for 2 h to minimize contamination. Briefly, supernatants from CT26 cell cultures were collected and 9 ml was added to 15 ml Beckman centrifuge tubes and centrifuged at 2000*g* for 30 min to remove cells and debris. Cell – free media were transferred into 15 ml centrifuge tubes and mixed with exosome isolation reagent. Exosomes were precipitated by incubating the cell supernatant mixture at 4°C overnight and then collected by centrifugation at 10,000*g* for 1.0 h. Supernatant was carefully removed and the pellets were resuspended in PBS and stored at –80°C until use.

For isolation of control exosomes from serum of normal Balb/c mice, 100 µl of serum was transferred to a new tube and mixed with 200 µl Total Exosome Isolation reagent. After incubation at 4°C for 30 min, mixture was centrifuged at 1,000 g for 10 min at 22°C. Discard the supernatant and re-suspend the pellets with PBS.

Transmission Electron Microscopy (TEM)

Exosomes purified from CT26 and serum of normal mice were dried onto glow discharged 300 mesh formvar / carbon – coated TEM grids (Ted Pella, Redding, CA), negatively stained with 2% aqueous uranyl acetate and observed with a Hitachi H7600 TEM (Hitachi High – Technologies Corp., Tokyo, Japan) operated at 80kV. Images were captured with a side mounted 1K AMT Advantage digital camera (Advanced Microscopy Techniques, Corp. Woburn, MA)

Size distribution analysis of exosomes

CT26 exosomes or control exosomes were diluted in 1 ml ddH_2O and size distribution was analyzed at 37°C according to the manufacturer's instructions using Zetasizer Nano ZS 90 (Malvern Instruments, Orsay, France).

Bone marrow cells isolation and induction Bone marrow – derived macrophage (BMDM)

Bone marrow cells were isolated as previously reported.[20] In brief, femur and tibia bones from 6 – 8 week old mice were isolated and cut open. The marrow was flushed out into pre-cold PBS with 2% Fetal Bovine Serum (FBS). Pass marrow through 21G needle 10 times to dissociate the cells. After removing cell clumps, bone, hair and tissues by passing cells through 70 µm cell strainer, the red blood cells were removed by adding 3 volume of NH_4CL solution. Then the cells were collected by centrifugation at 1000 rpm for 5 min and re-suspended in Iscove's Modified Dulbecco's Medium with 10% FBS and 10 ng/ml M-CSF. Cells were cultured in 24 – well plates and the medium was changed every 3 days.

ELISA assays

Cytokines or chemokines were detected in supernatant of BMDM using ELISA. In brief, BMDM cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS, 100 ng/ml LPS and 50 ng/ml IFN-γ for 48 h with or without exosomes treatment. Then the concentration of TNF-α, IL6, IL10 and CCL1 in culture medium were measured by using ELISA kit from e-Bioscience (San Diego, CA, USA).

Tumor models

To test function of exosomes on CT26 tumor growth in vivo, 2 × 105 of CT26 cells were subcutaneously injected to the flank of Balb/c mice or IL4 knockout Balb/c mice. Mice were divided into three groups and treatment was initiated 3 days after tumor implantation. Mice were respectively treated with 20 µg of CT26 – derived exosomes (Exo – CT26), control exosomes (Exo-C) or PBS as negative control every 3 days for 10 times. Tumor volume was measured every five days and the survival rate of tumor – bearing mice was monitored.

Tumor size was calculated according to following formula:

Tumor size = Length (mm) × Width $(mm)^2/2$.[21]

Statistical analysis

Statistical analyses were performed using Student's t-test. For the analysis of animal experiments, two – way ANOVA with Prism 5.0 software was utilized. $P < 0.05$ was regarded as statistically significant.

RESULTS

Characterization and function of exosomes

To study function of tumor exosomes on tumor growth, CT26 tumor model was used in this study for approving concept. Exosomes were first isolated and purified from culture medium of CT26 cells (Exo-CT26) and from serum of normal mice (Exo-C). Then the morphology of exosomes was observed by TEM. As

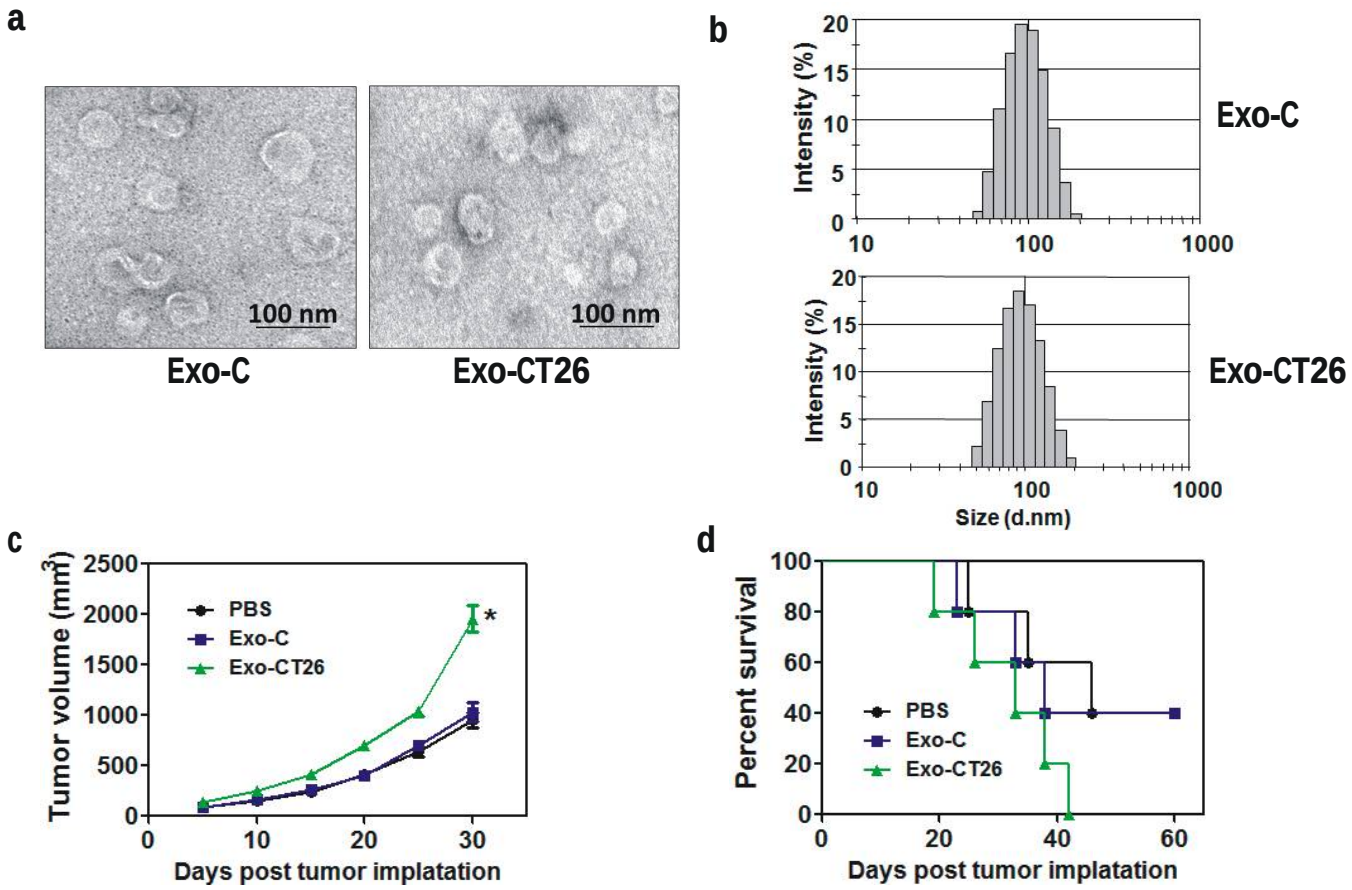

Fig. 1: *Characterization of exosomes and function test in vivo. Exosomes were purified from CT26 (Exo – CT26) and from serum of normal Balb/c mice as control (Exo C). (a) TEM was performed to observe morphology of exosomes. (b) Size distribution of exosomes. (c) CT26 tumor size was measured after treating with Exo – CT26 and Exo-C. (d) Survival rate of CT26-bearing mice. (*p < 0.05, vs PBS and Exo-C groups).*

shown in **Figure 1a**, rounded particles with approximately 100 nm in size were demonstrated. Meanwhile, dynamic light scattering was performed to measure the size distribution of exosomes, as indicated in **Figure 1b**, size of both exosomes was about 100 nm.

To test function of exosomes on tumor growth in vivo, CT26 tumor – bearing mice were treated with exosomes from CT26 cells culture medium, both tumor size (**Figure 1c**) and survival rate (**Figure 1d**) of mice demonstrated that Exo – CT26 significantly promote CT26 tumor growth (*p < 0.05) and decrease survival rate of mice (*p < 0.05).

Exo – CT26 promote polarization of macrophages from M1 to M2

To investigate whether the tumor promotion function of Exo – CT26 resulted from the regulation of phenotypes of macrophages by Exo – CT26, BMDM cells were prepared and treated with LPS / IFN-γ followed with Exo – CT26, Exo-C or PBS. Then the cytokines TNF-a, IL6 (M1 markers) and IL10, CCL1 (M2 markers) secreted by BMDM were detected using ELISA. As demonstrated in Figure 2, expression of TNF-a (**Figure 2a**, **p < 0.01) and IL6 (**Figure 2b**, *p < 0.05) were significantly inhibited by treating with Exo-CT26. In contrast, IL10 (**Figure 2c**, *p < 0.05) and CCL1 (**Figure 2d**, *p < 0.05) were increased after treatment with Exo – CT26.

Exo – CT26 evaluate the expression of IL4

IL4 is a major stimulator for M2 polarization, so we next test whether Exo – CT26 promote IL4 expression of BMDM. As indicated in **Figure 3**, Exo – CT26 further enhances expression of IL4 from LPS / IFN-γ-treated BMDM. This data implies that polarization of macrophages was regulated by Exo - CT26 possibly through IL4 pathway.

Exo – CT26 promote M2 transformation mediated by IL4

To further confirm our hypothesis, an IL4 knockout mice tumor model was used. For in vitro experiment, BMDM cells were prepared from IL4 knockout mice and cultured with LPS / IFN-γ, followed by treating

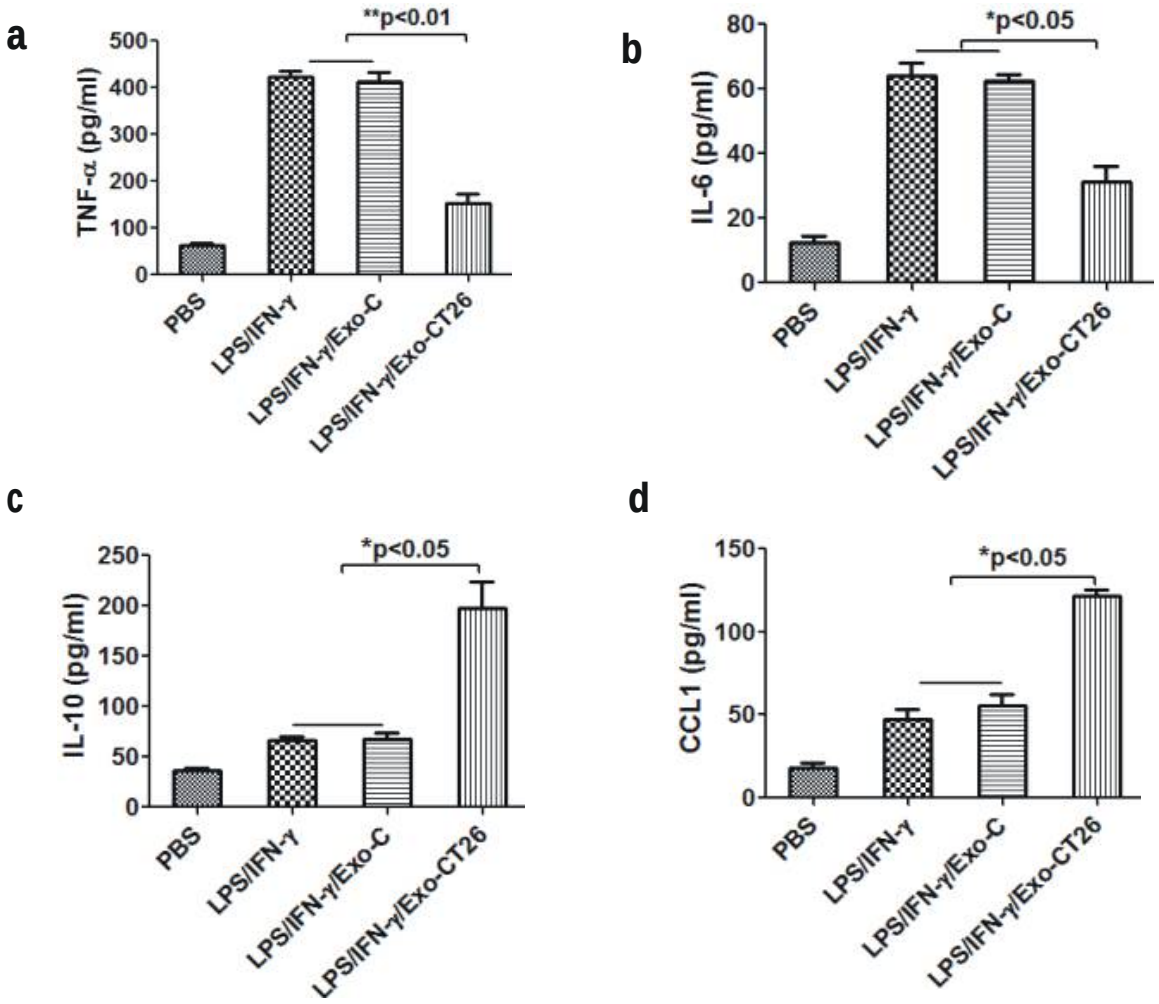

Fig. 2: *Cytokines expression in supernatants of BMDM cells. BMDM were stimulated with LPS / IFN-γ and then treated with Exo-CT26 and Exo-C, TNF-a (a), IL6 (b), IL10 (c) and CCL1 (d) were detected in supernatants by ELISA. (*p < 0.05, **p < 0.01 vs. PBS and Exo-C groups).*

with Exo – CT26, Exo-C or PBS. TNF-α (**Figure 4a**) and IL10 (**Figure 4b**) were detected and results showed that Exo – CT26 did not significantly change the expression of both cytokines.

In vivo CT26 tumor model was also performed in IL4 knockout Balb/c mice. CT26 tumor – bearing IL4 knockout mice were respectively treated with Exo – CT26, Exo-C or PBS, data from tumor growth (**Figure 4c**) and survival rate (**Figure 4d**) revealed that Exo – CT26 has no tumor promotion function in case of IL4 absence.

DISCUSSION

Cell-to-cell communication is required to keep proper coordination among different types of cells within tissues. Classically, cells communicate with each other through direct contact or via soluble factors. However, during the last few years an understanding of the function of exosomes released by cells in transferring small packages of information to target cells is rapidly

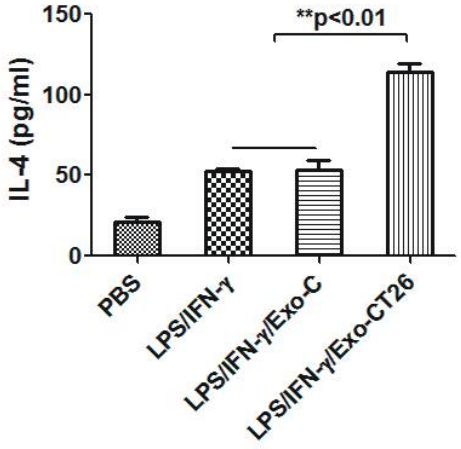

Fig. 3: *Expression of IL4 in culture medium of BMDM. BMDM were stimulated with LPS / IFN-γ and then treated with Exo – CT26 and Exo-C, IL4 was detected in supernatants by ELISA. (**p < 0.01 vs. PBS and Exo-C groups).*

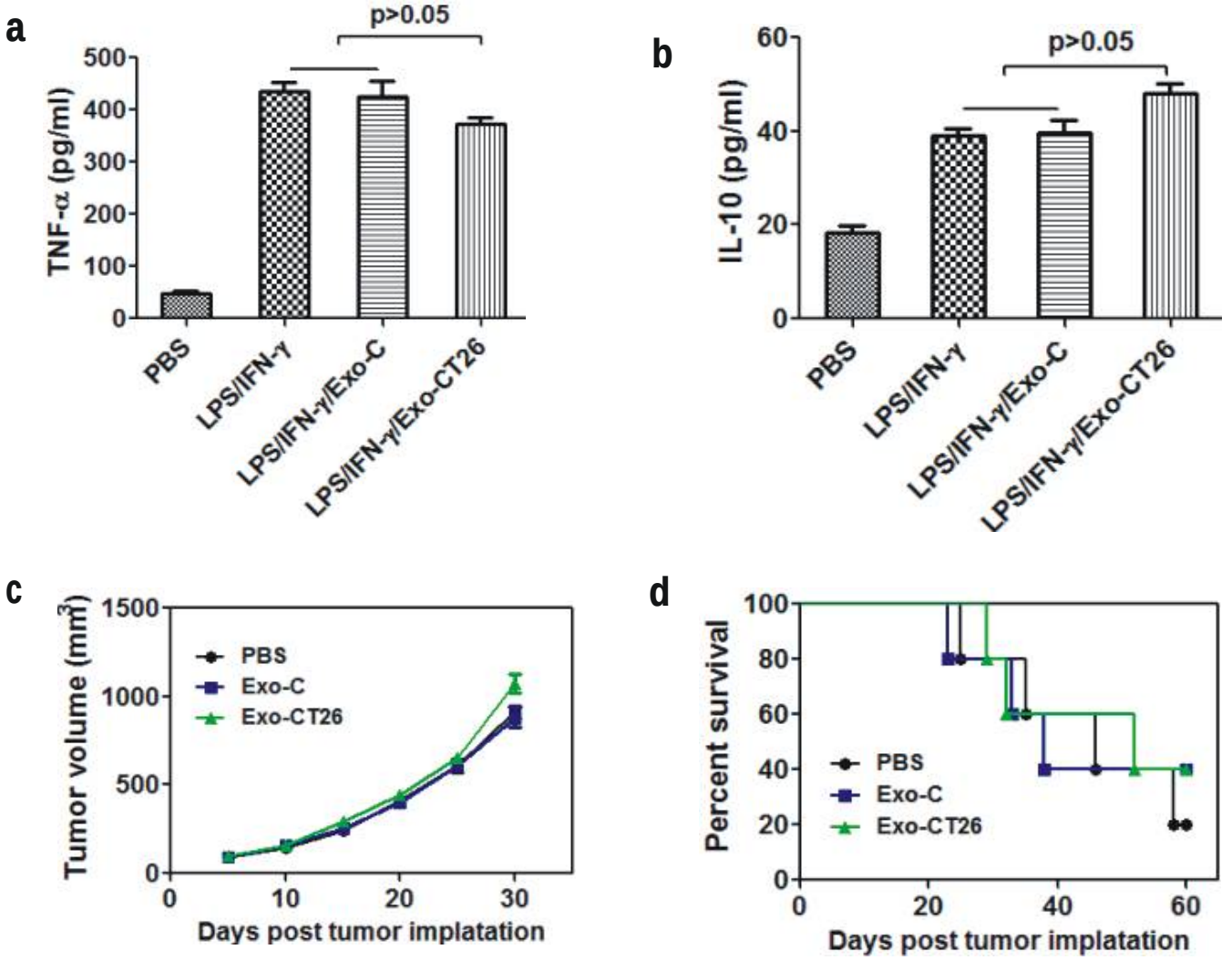

Fig. 4: *Cytokines in BMDM from IL4 knockout mice and functions of exosomes in CT26 – bearing IL4 knockout mice. BMDM cells were prepared from IL4 knockout mice and stimulated with LPS / IFN-γ and then treated with Exo – CT26 and Exo-C. TNF-a (a) and IL10 (b) in culture medium were measured using ELISA. CT26 tumor growth (c) and survival rate (d) of tumor – bearing IL4 knockout mice were analyzed.*

emerging, especially in cancer field.[22]

Tumor – derived exosomes can induce potent anti-tumor immune responses leading to tumor rejection in prophylaxis and therapy models, and inter – tumor cross – protection across tumor histology and MHC class I barriers was achieved, however, more and more studies demonstrated tumor – derived exosomes is another mechanism for tumor escaping from immune system attack[23]. Study reported that tumor exosomes can suppress the function of immune cells by inducing apoptosis of activated cytotoxic T cells or promoting differentiation, expansion of Treg cells.[24,25] Other reports suggested that tumor – derived exosomes can promote tumor growth by interfering with DC maturation,[26] favoring MDSC differentiation[27] and suppression of NK cell activities.[28] Liu et al (2013)[29] demonstrated that exosomes from murine – derived GL26 cells promote glioblastoma tumor growth by reducing number and function of CD8+ T cells Study also revealed that exosomes from bladder cancer cells inhibit tumor cell apoptosis and induce cell proliferation by up-regulating the expression of Bcl2, cyclin D1 and down – regulating levels of Bax and caspase3.30 Also, renal cancer cell – derived exosomes can promote angiogenesis by reducing the expression of hepatocyte cell adhesion molecule.[31]

Recently, studies begin to focusing on the roles of tumor exosomes in function of macrophages. Jang et al (2013)[32] suggested that exosomes derived from epigallocatechingallate treated breast cancer cells suppress tumor growth by inhibiting tumor – associated macrophage infiltration and M2 polarization. Marton et al (2012)[33] also found that melanoma cells derived exosomes may play a role in tumor progression and

metastasis formation by altering phenotype of macrophages. But effects of tumor exosomes, especially colon cancer exosomes on macrophages polarization and the mechanism are poorly investigated. As expected, exosomes from CT26 cells significantly promote tumor growth and decrease survival rate in vivo. In consist with Marton's study,[33] but in contrast with Jang's study,[32] we found that exosomes purified from CT26 cells promote polarization of macrophage from M1 to M2. We speculated that this difference resulted from two factors: (1) component difference between breast cancer and colon cancer exosomes. (2) exosomes derived breast cancer cells without epigallocatechingallate treatment have same effects on macrophage polarization as colon cancer exosomes, epigallocatechingallate changed the properties of exosomes.

Both in vitro cytokines profile and in vivo tumor model performed in IL4 knockout mice demonstrated that CT26 exosomes promote tumor progression by subverting macrophages from M1 to M2 mediated partially by IL4. This study provides us a new insight for investigating functions of tumor – derived exosomes on tumor progression for the first time. But whether this scenario is applicable to other tumors remains to be elucidated.

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