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RESEARCH

Macrophage-mediated tumorigenic effects of breast cancer cell exosomes

Meme kanseri hücre ekzozomlarının makrofaj aracılı tümörijenik etkileri

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Abstract

Purpose: As a heterogeneous disease, breast cancer is one of the most commonly diagnosed cancers due to hereditary/genetic factors and non-hereditary factors and is a leading cause of disease-associated death among women all over the world. Macrophages are one of the cell types that have essential roles in physiological and pathological cellular processes containing hemostasis, inflammation, and carcinogenesis. Exosomes secreted by cancer cells regulate cellular processes such as the polarization of macrophages, different organ metastasis, and drug resistance via internalized macrophages. In this study, we aimed to reveal the exosome-mediated regulation of the tumor microenvironment in specific to macrophage cells.

Materials and Methods: THP-1 monocyte cells are cultured with RPMI-1640 medium supplemented with 20 ng/ul phorbol-12-myristate-13-acetate (PMA) for 48 hours to induce macrophage differentiation. Exosomes of MCF-10A and MDA-MB-231 cells were isolated using a commercial kit. Fold changes in proliferation capacities of MCF-10A treated with exosomes of MDA-MB-231 cells were calculated.

Results: A statistically significant increase in the expression of genes involved in the inflammation-related JAK/STAT pathway in THP-1 cells treated with MDA-MB-231 exosomes was observed. Besides, we detected morphological and proliferative changes in MCF-10A cells that were co-cultured with THP-1/MDA-MB-231exo cells.

Conclusion: This study may contribute to the literature by showing that phenotypic differences in immune system cells can occur through exosomes in the tumor microenvironment.

Keywords:. Macrophage differentiation, exosomes, breast cancer, inflammation

Öz

Amaç: Kalıtsal ve genetik faktörler ile kalıtsal olmayan faktörler nedeniyle heterojen bir hastalık olan meme kanseri, tüm dünyada kadınlar arasında en sık görülen kanserlerden biridir ve hastalığa bağlı ölümlerin önde gelen nedenlerinden biridir. Makrofajlar hemostaz, inflamasyon ve karsinogenezi içeren fizyolojik ve patolojik hücresel süreçlerde önemli rollere sahip hücre tiplerinden biridir. Kanser hücreleri tarafından salgılanan eksozomlar, makrofajların polarizasyonu, farklı organ metastazı ve ilaç direnci gibi hücresel süreçleri düzenleyebilirler. Bu çalışmada makrofaj hücrelerine özgü tümör mikro çevresinin eksozom aracılı regülasyonunu ortaya koymayı amaçladık.

Gereç ve Yöntem: THP-1 monosit hücreleri, makrofaj farklılaşmasını indüklemek için 48 saat boyunca 20 ng/ul forbol-12-miristat-13-asetat (PMA) içeren RPMI-1640 besiyeri ile kültüre edildi. MCF-10A ve MDA-MB-231 hücrelerinden ticari bir kit kullanılarak eksozom izolasyonu gerçekleştirildi. MDA-MB-231 hücrelerinin eksozomları ile muamele edilen MCF-10A hücrelerinin proliferasyon kapasitelerindeki kat değişimleri değerlendirildi.

Bulgular: MDA-MB-231 eksozomları ile muamele edilen THP-1 hücrelerinde inflamasyon ile ilişkili JAK/STAT yolağında görev alan genlerin ifadesinde istatistiksel olarak anlamlı bir artış saptandı. Ayrıca THP-1/MDA-MB-231exo hücreleri ile birlikte kültüre edilen MCF-10A hücrelerinde morfolojik ve proliferatif değişiklikler saptandı.

Sonuç: Bu çalışmada tümör mikro çevresindeki eksozomlar aracılığıyla immün sistem hücrelerinde fenotipik farklılıkların oluşabileceğini göstererek literatüre katkı sağlayabilecektir.

Anahtar kelimeler: Makrofaj farklılaşması, eksozomlar, meme kanseri, inflamasyon

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INTRODUCTION

Breast cancer is one of the most commonly diagnosed cancers and is a leading cause of disease-associated death among women all over the world[1]. The important challenge associated with breast cancer is that it is a heterogeneous disease with hereditary/genetic factors and non-hereditary factors[2]. Recently, the type of extracellular vesicles (EVs) named exosomes have been found to be involved in breast cancer pathogenesis[3]. Exosomes that are secreted by cancer cells into their local environment lead to biological modifications of recipient cells as endothelial, fibroblastic, and immune cells in the tumor environment[4]. Therefore, exosomes have been identified as important mediators of tumor cells and other cell types in the tumor microenvironment[5]. Recent evidence indicated that exosomes may transfer biologically functional molecules to the recipient cells including other cancer cells, immune cells, fibroblastic, endothelial, and epithelial cells that enhance cancer progression, angiogenesis, metastasis, drug resistance, and immunosuppression activity of cancer cells[6]. For example, Yang et.al. demonstrated that exosomes derived from breast cancer cells activate cancer-associated fibroblasts and exosomes via mir-146a to promote cancer progression[7].

The tumor environment (TME) is organized by tumor cells, including a variety of micro- and macro-molecules. In the TME, macrophages are one of the cell types, and they have been found to have essential plays in physiological and pathological cellular processes containing hemostasis, inflammation, and carcinogenesis[8]. A previous study demonstrated that macrophage-secreted interleukin-35 induces cancer cell plasticity[9]. Exosomes secreted by cancer cells regulate cellular processes such as the polarization of macrophages, different organ metastasis, and drug resistance via internalized macrophages[10]. Breast cancer-derived exosomes trigger reactive oxygen species, inflammation, and autophagy. Increasing evidence demonstrated that tumor-derived exosomes were transferred into monocytic cells, and these induce the production of various genes containing interleukin (IL)-6, IL-8, IL-1β, STAT3, NF-ϰB, tumor necrosis factor (TNF)-α that play roles in pro-inflammatory pathways in the immune cells[11]. On the other hand, tumor-associated macrophages (TAM) are commonly associated with a poor prognosis in solid tumors. Mostly for patients, TAMs are known to resist responses to standard-of-care therapeutics, including chemotherapy, irradiation, and different inhibitors[12]. Exosomes transport different molecular components including coding and non-coding RNAs, proteins, lipids, etc., affecting a variety of aspects of cancer progression. In gastric cancer, tumor-derived exosomes promoted the expression of proinflammatory factors in THP-1 cells[13]. Metastatic melanoma cells-secreted exosomal PD-L1 inhibits the function of CD8 T cells[14]. Furthermore, previous studies showed that exosomal miRNAs regulate Treg/Th17 Cell Imbalance in ovarian cancer[15].

In current kinds of literature, the effects of the tumor on the microenvironment are partially known, but the mechanisms involved in this process have not been clarified. Therefore, we hypothesized to investigate the exosome-mediated regulation of the tumor microenvironment specifically of macrophage cells. In this study, we aimed to reveal the effects of tumor-associated macrophage-derived exosomes on phenotype and pathways associated with cellular differentiation in breast cancer.

MATERIALS AND METHODS

All stages of the study were carried out in Pamukkale University Faculty of Medicine, Department of Medical Genetics. This study is conducted with cell lines and does not need ethical approval.

Cell culture

Normal breast epithelial cell line MCF-10A is cultured in DMEM / F12 (Sigma, Germany) medium containing 1% Penicillin-Streptomycin, L-glutamine, 5% heat-inactivated Horse FBS (GIBCO, USA), 10ug / ml insulin (GIBCO, America), 20ng / ml EGF (Miltenyi Biotec, Germany), 0.5 µg / ml hydrocortisone (Sigma, Germany); metastatic breast cancer cell line MDA-MB-231 and monocyte cell line THP-1 are cultured in RPMI1640 Medium (GIBCO, USA) containing heat-inactivated 10% FBS (GIBCO, USA), 1% Penicillin-Streptomycin (GIBCO, USA) and 1% L-glutamine (GIBCO, USA). Cells are incubated at 37 °C, 5% CO_2 and 95% humidity in air.

Monocyte differentiation into macrophage

THP-1 monocyte cells are cultured with RPMI-1640 medium supplemented with 20 ng/ul phorbol-12-myristate-13-acetate (PMA) for 48 hours to induce macrophage differentiation.

Exosome isolation

Norgen Cell Culture Media Exosome Purification kit protocol (Norgen Biotek Corp., Canada) for isolating EVs from the conditioned culture medium of MCF-10A and MDA-MB-231 cells was applied according to the manufacturer's protocol. Briefly, cell-free media of MCF-10A and MDA-MB-231 cells were treated with 25 µL of ExoC Buffer and 200 µL of Slurry E added. Then, centrifuged for 2 minutes at 2,000 rpm. The pellets were resuspended in 200 µL of ExoR Buffer followed by centrifugation at 2 minutes at 500 rpm. The supernatant was filtered into a Mini Filter Spin column assembled with a 2 mL tube and centrifuge for 1 minute at 6,000 rpm to obtain exosomes.

Exosome Uptake Assay

MDA-MB-231 cell lines transfected with vector a vector expressing GFP-fused tetraspanin CD63 under the control of the CMV promoter (pCT-CD63-GFP) (CYTO120-PA-1, System Biosciences). Following to generation of cells that expressed CD63-GFP, exosomes were harvested. MCF-10A cells were incubated with MDA-MB-231 cells-secreted exosomes containing the CD63-GFP protein and the incorporation of exosomes into the targeted MCF-10A cells was analyzed by fluorescence microscopy (Olympus CKX53).

Proliferation assay

To investigate the proliferation of MCF-10A and MDA-MB-231 cells incubated with exosomes derived from MCF-10A, MDA-MB-231, THP1/MDA-MB-231exo treated, and THP1/MCF-10A exo treated cells were plated onto 6-well plates (1×10^5 per well). After 24h, fresh medium containing exosomes (equivalent to 40 µg protein) was added to MCF-10A cells. Fold changes of proliferation capacities of supernatant-treated and exosome-treated cells were performed as described earlier[16].

Statistical analysis

The graphs, calculations, and statistical analyses were performed using GraphPad Prism software version 8.0.1 (GraphPad Software, San Diego, CA, USA). Two-way ANOVA and Paired t-tests were used for comparisons of differential expressions of genes. Statistical results with $*p<0.05$, $**p<0.01$, $***p<0.001$, or $****p<0.0001$ were considered statistically significant.

RESULTS

Phenotypic macrophage differentiation as a result of exosomal activity in breast cancer cells

In order to investigate the effect of exosomal activity originating from breast cancer cell lines on tumor-associated macrophage polarization, THP-1 monocyte cells were first induced to transform into macrophages by PMA treatment. 48 hours after, THP-1 macrophage cells were treated with both the supernatant obtained from MDA-MB-231 and MCF-10A cell cultures as well as their isolated exosomes (indicated as MDA-MB-231 exo and MCF-10A exo). Data have shown that in THP-1 cells treated with MDA-MB-231 exosomes, there was a statistically significant increase in the expression of genes involved in the inflammation-related JAK/STAT pathway (Figure 1).

In addition, to investigate whether the resulting effect is an exosome-specific effect, THP-1 cells were treated with both supernatant and exosomes of MDA-MB-231 cells. We observed a statistically significant increase in the expressions of the relevant genes of THP1 cells treated with MDA-MB-231 exosomes (Figure 2).

Biological changes (morphological and proliferative) in MCF-10A cells

After observing the change in THP-1 cells, MCF-10A cells which are breast epithelial cells, another cell group in the tumor microenvironment, were treated with tumor-derived exosomes of THP-1 cells (indicated as THP-1/MDA-MB-231exo) in order to evaluate their impact. Exosome uptake of MCF-10A cells was evaluated under a fluorescent microscope (Figure 3). When MCF-10A cells were co-cultured with THP-1/MDA-MB-231exo cells, their morphology started to change from epithelial to fibroblast in 48 hours (Figure 4). Besides, the proliferative index has also increased (Figure 5a). At the same time, when metastatic breast cancer cell line MDA-231 cells were treated with exosomes originating from MCF-10A cells, it was found to have a reducing effect on proliferative activity, in contrast to the tumor-derived exosomal effect, which was consistent with our initial data (Figure 5b).

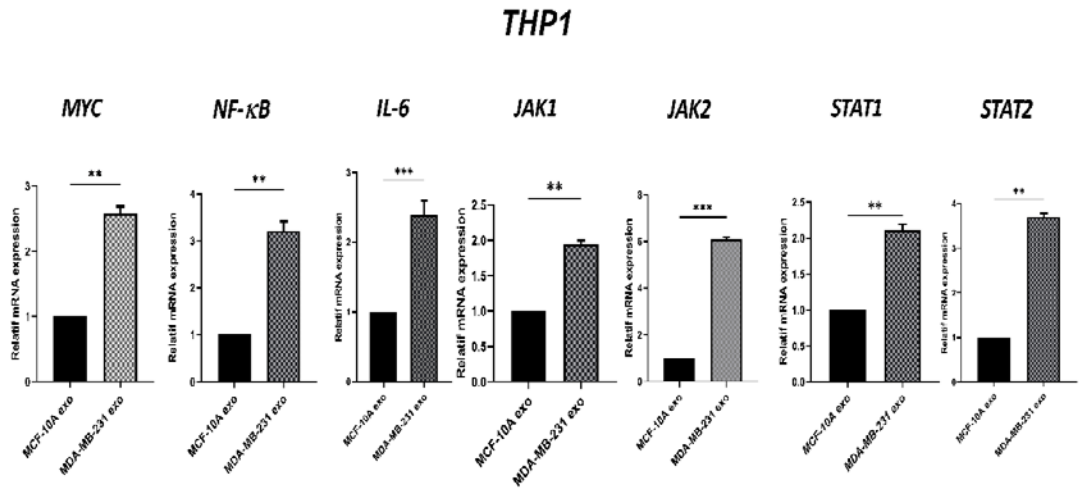

Figure 1. Relative expressions of genes related to the JAK-STAT pathway in THP-1 cells treated with the exosomes of MCF-10A and MDA-MB-231 cells ($p<0.01$, ***$p<0.001$).**

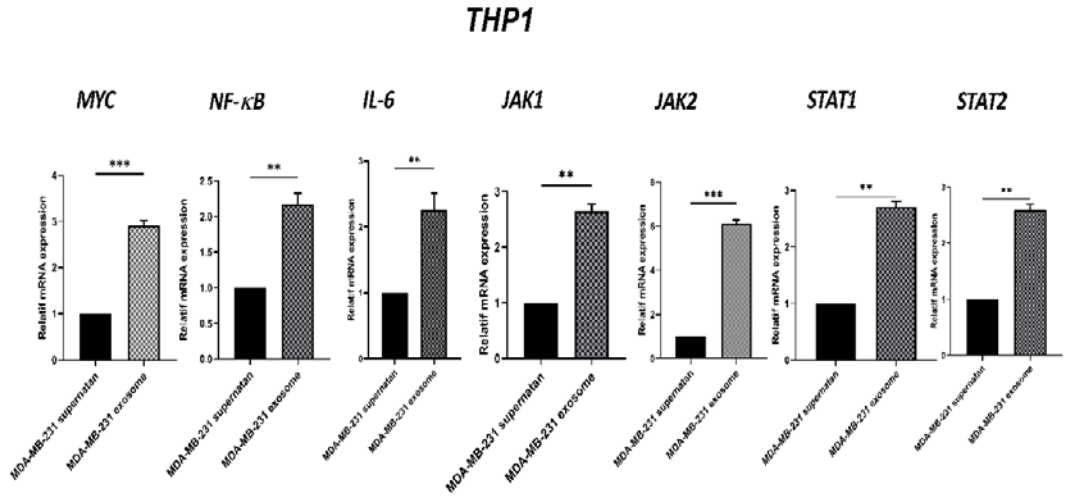

Figure 2. Comparison of the expressions of the genes involved in JAK-STAT pathway in THP-1 cells treated with both supernatant and exosomes of MDA-MB-231 cells ($p<0.01$, ***$p<0.001$).**

In addition, in contrast to the effect of tumor-derived exosomes on THP1 cells, THP-1 cells were found to have a tumor-suppressive effect when THP1 cells were treated with normal epithelial cell line MCF-10A exosomes. In line with the data obtained, we think that tumor-derived exosomes have an enhancing effect on tumorigenesis in THP1 cells compared to exosomes originating from normal cells.

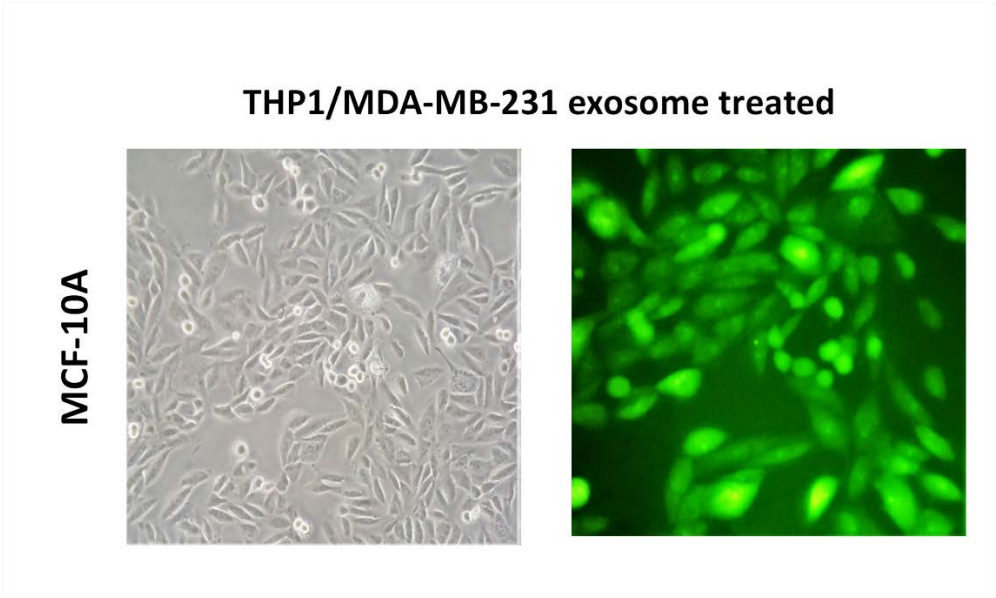

Figure 3. MCF-10A cells were incorporated with the exosomes of MDA-MB-231 cells.

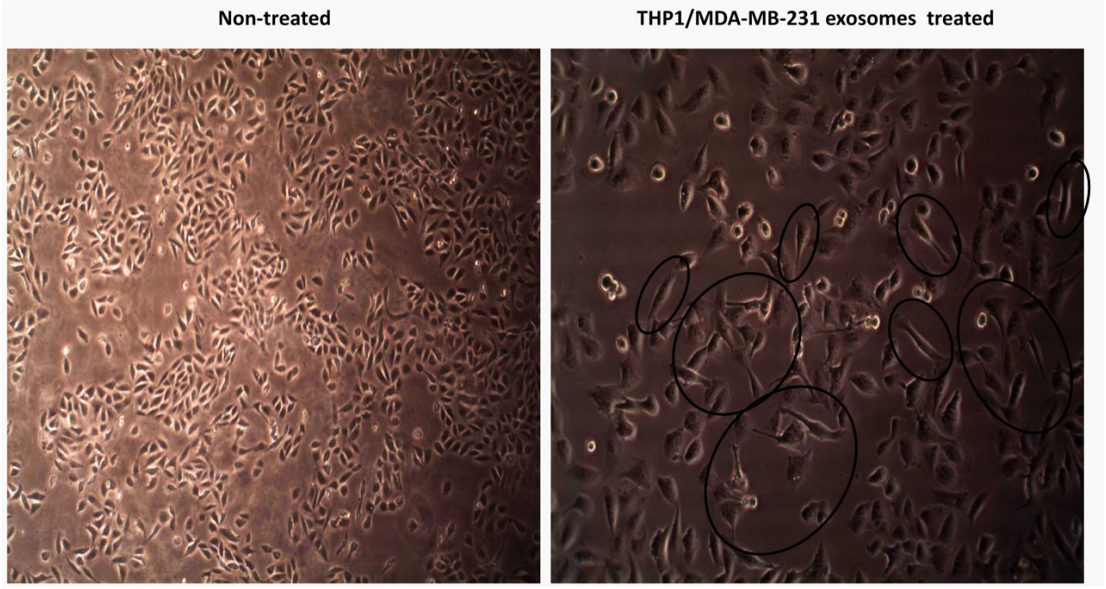

Figure 4. Morphological changes of MCF-10A cells from epithelial to fibroblast.

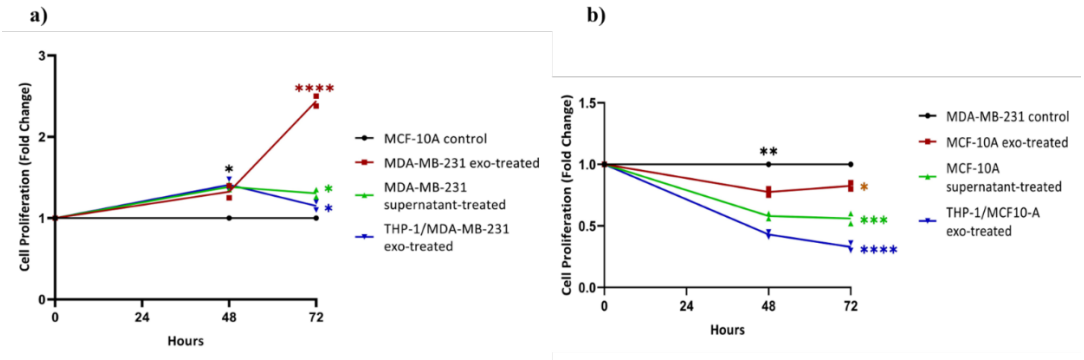

Figure 5. The proliferative index of MCF-10A cells a) MCF-10A cells b) MDA-MB-231 cells (*$p<0.05$, $p<0.01$, ***$p<0.001$, ****$p<0.0001$).**

DISCUSSION

Monocytes which play important roles in the induction of inflammatory response are heterogeneous and can be differentiated into inflammatory macrophages that play essential roles in antigen presentation, phagocytosis, and immunomodulation in inflammation[17,18]. Stimulations that trigger monocyte/macrophage differentiation occur in response to IL-34, macrophage colony-stimulating factor (M-CSF), and granulocyte-macrophage colony-stimulating factors (GM-CSF)[19-21]. Besides, CXCL12 has been shown to regulate monocyte/macrophage differentiation by downregulating RUNX3[22]. In this study, we treated THP-1 monocytes with 20 ng/µl phorbol 12-myristate 13-acetate (PMA) for differentiation into macrophages. The human leukemia monocyte cell line, THP-1, is the most widely used cell as a representative of primary macrophages in vitro[23,24].

There are two types of macrophages: classically activated macrophages (M1) and alternatively activated macrophages (M2). When monocytes are recruited into the tumor microenvironment they are differentiated into tumor-associated macrophages (TAMs)[25]. TAMs have been shown to be detected in several tumor tissues and thus, found to be associated with tumor development[26]. Extracellular vesicles (EVs) released from macrophages transport miRNAs and lncRNAs to recipient cells, stimulating the pro-inflammatory response. Monocyte/macrophage-derived EVs can modulate cell proliferation and migration, inflammation, apoptosis, and angiogenesis[27-29]. When compared to M1 macrophages, exosomes of M2 macrophages are shown to promote the invasion and proliferation of breast cancer cells[30]. In order to differentiate monocytes into TAMs we co-cultured THP-1 cells with the supernatant of metastatic breast cancer cells (MDA-MB-231). The exosomes are collected and normal breast cells were treated with exosomes of THP-1/MDA-MB231 cells. When exosomes of breast cancer-derived macrophages are co-cultured with MCF-10A cells, we observed both morphological and proliferative changes. The cell morphology has started to change from epithelial to fibroblast form and proliferation was found to be increased.

Exosomes are known to regulate the biological functions, metastasis and immunity of cancer cells. Therefore, exosomes of cancer cells have emerging roles in the diagnosis and treatment. It has been shown in some studies that the HER2 family was associated with exosomes in some cancers including breast cancer and serum exosomal-annexin A2 levels were higher in triple-negative breast cancer patients[31,32]. In another study, M2 macrophages are found to be responsible for cell migration and invasion of colon cancer resulting in the downregulation of BRG1 in response to macrophage-derived exosomes[33]. Exosomes also deliver lipids, proteins, and non-coding RNAs such as miRNAs, lncRNAs, and circRNAs from cell to cell[34]. It has been reported that exosomes regulate TAMs via miRNAs, lncRNAs, and circRNAs in several cancers[35-37]. New shreds of evidence are highlighting the importance of exosomes in macrophage polarisation in cancers. The lack of knowledge is the underlying mechanism involved in these processes.

In many cancers, inflammatory NF-κB, MAPK, JAK-STAT, and PI3K/AKT pathways are most commonly dysregulated. Besides, IL-6 is reported to be expressed by polarized macrophages in TME[38]. It has been reported that breast cancer-derived exosomes containing IL-6 receptor gp130 have the capacity to change macrophage phenotype and help to generate a pro-tumorigenic cancer microenvironment[39]. In our study, THP-1-induced proliferation and morphological differences in MCF-10A cells suggest that macrophage cells may have a tumor-promoting effect. When macrophage cells were treated with MDA-MB-231 supernatant and exosomes alone, the effects were found to be different compared to control cells. Based on this observation, this effect may be exosome-specific and tumor-derived exosomes in the tumor microenvironment may also affect the immune system cells, altering the expression of inflammation-related genes, and tending to create a more favorable microenvironment.

In terms of our study, the limitation is that it is an in vitro study on breast cancer to investigate the exosome-mediated effects. It will be useful to support the hypothesis with studies that can be performed on different cancer types and also in vivo modeling.

In conclusion, the necessity of developing different strategies for halting the progression and treatment of breast cancer is striking. Revealing a treatment strategy targeting the tumor microenvironment will enable new treatment options to be created. We believe that this study may contribute to the literature by showing that phenotypic differences in immune system cells can occur through exosomes in the tumor microenvironment. New studies are required for the determination of exosomal cargo context in order to understand the mechanism that results in phenotypical differences.

Yazar Katkıları: Çalışma konsepti/Tasarımı: ST, PET, OT, KI, BÇ; Veri toplama: ST, PET, OT, Kİ, BÇ; Veri analizi ve yorumlama: ST, PET, OT, KI; Yazı taslağı: ST, PET, OT, KI; İçeriğin eleştirel incelenmesi: ST, PET, OT, KI, BÇ; Son onay ve sorumluluk: ST, PET, OT, KI, BÇ; Teknik ve malzeme desteği: ST, PET, OT; Süpervizyon: ST, PET, OT, KI, BÇ; Fon sağlama (mevcut ise): yok.
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