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Effects of 4T1 Breast Cancer Cell-Derived Exosomes on Neutrophil Function: A Novel Axis in Tumor-Immune Cells Interactions

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Abstract

Background: Breast cancer is one of the most prevalent malignancies affecting women worldwide and remains a leading cause of mortality. Tumor-derived exosomes are critical mediators of intercellular communication within the tumor microenvironment, influencing cancer progression and immune responses. Neutrophils, the most abundant leukocytes in the bloodstream, play a pivotal role in the innate immune system and can adopt pro- or anti-tumorigenic phenotypes.

Objective: This study aims to investigate the effects of exosomes derived from the 4T1 murine mammary carcinoma cell line on neutrophil immune function, focusing on cell viability, respiratory burst activity, and antimicrobial capabilities.

Methods: Exosomes were isolated from the conditioned medium of 4T1 breast cancer cells using the Exocib kit and characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), and flow cytometry for CD63 expression. Neutrophils were isolated from human peripheral blood of healthy donors, ensuring purity and viability above 95%. The isolated neutrophils were pretreated with 100 µg/mL of 4T1-derived exosomes or fresh DMEM as a control for 2 hours. Subsequent functional assays included the MTT assay for cell viability, the nitroblue tetrazolium (NBT) reduction assay for respiratory burst activity, and a yeast killing assay to evaluate antimicrobial function.

Results: TEM and DLS confirmed the successful isolation of exosomes with spherical morphology and an average hydrodynamic diameter of approximately 172 nm. Flow cytometry validated the expression of the exosomal marker CD63. The MTT assay demonstrated that exosome treatment did not significantly affect neutrophil viability, maintaining cell vitality above 90%. The NBT reduction assay revealed a statistically significant 4% increase in respiratory burst activity in exosome-treated neutrophils compared to controls ($p < 0.05$). Additionally, the yeast killing assay indicated that neutrophils exposed to 4T1-derived exosomes exhibited enhanced antimicrobial activity against opsonized yeast cells.

Conclusion: Exosomes derived from 4T1 breast cancer cells can alter neutrophil immune functions by enhancing respiratory burst activity and antimicrobial capabilities without compromising cell viability. These findings suggest that cancer-derived exosomes may play a role in altering innate immune responses, potentially contributing to cancer progression and offering new avenues for therapeutic intervention targeting the tumor-immune interface.

Keywords: Exosomes, Breast Cancer, Neutrophils, NBT, 4T1 Cell Line.

Introduction

Cancer remains the second leading cause of mortality globally, posing a significant threat to human life and health, second only to cardiovascular diseases [1]. Among various cancers, breast cancer is one of the most prevalent malignancies affecting women worldwide [2]. While male breast cancer is relatively rare, its incidence has notably increased in recent years [2, 3]. The 4T1 murine mammary carcinoma cell line serves as a valuable model for studying breast cancer metastasis due to its ability to mimic human breast cancer by forming tumors and spontaneously spreading to other organs [4].

Exosomes are small extracellular vesicles released by all cell types, including tumor cells, and are present in various body fluids. Tumor-derived exosomes contribute to cancer growth, metastasis, and immune evasion by transporting genetic material that facilitates intercellular communication [5]. These exosomes can fuse with cells in target organs, preparing the pre-metastatic niche; specific integrins in exosomes drive metastasis, and targeting them can reduce metastatic spread [6]. Exosomes play a pivotal role in regulating the immune system in cancer through multiple mechanisms: facilitating antigen presentation to activate T cells directly or via antigen-presenting cells (APCs); activating immune cells such as macrophages, natural killer (NK) cells, and APCs to enhance T-cell responses; suppressing immune activity by inducing T-cell apoptosis, inhibiting NK and macrophage functions, and expanding immunosuppressive myeloid-derived suppressor cells; and evading immune detection by inhibiting complement activation [7].

Neutrophils are the most abundant leukocytes in the human bloodstream and serve as primary responders in the innate immune system, playing a critical role in defending against inflammation and infection [8]. Traditionally recognized for their antimicrobial functions, recent evidence indicates that neutrophils are also present within tumor tissues, where tumor-associated neutrophils (TANs) significantly influence cancer progression [9]. TANs are implicated in cancer-related inflammation, a pivotal driver of tumor development and progression. These cells modulate the tumor microenvironment through the secretion of cytokines and chemokines, thereby regulating the recruitment and activation of various inflammatory cells [10].

Moreover, neutrophils exhibit remarkable plasticity within the tumor milieu, adopting either anti-tumorigenic (N1) or pro-tumorigenic (N2) phenotypes. N1 neutrophils exert anti-tumor effects by directly destroying cancer cells and stimulating immune responses, whereas N2 neutrophils promote tumor growth and metastasis by secreting growth factors, facilitating angiogenesis, and suppressing anti-tumor immunity [11]. Despite the growing recognition of TANs' roles in cancer, the precise mechanisms that regulate neutrophil plasticity and their impact on tumor progression remain inadequately understood. Therefore, further investigation into neutrophil function within tumors is essential for developing novel therapeutic strategies aimed at modulating the tumor microenvironment.

In recent years, cell-based therapies have gained traction as effective treatment options; however, the focus is gradually shifting toward cell-derived therapies, particularly exosomes, due to their versatility and therapeutic promise. Exosomes have been extensively studied for

their ability to act as both therapeutic agents and delivery systems. Notably, these exosomes can be isolated from both normal and cancerous cells, providing diverse applications in treatment strategies. For instance, Kim et al. explored the use of cancer-derived exosomes as vehicles for clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 delivery, finding that cancer-derived exosomes displayed enhanced in vivo targeting capability by selectively accumulating in intended regions, thus highlighting their potential in cancer therapeutics [12]. Similarly, Qiao et al. examined the role of tumor cell-derived exosomes as carriers for delivering anticancer drugs, demonstrating that these exosomes could enhance drug retention and effectively eradicate tumors when loaded with Doxil compared to Doxil alone in nude mice [13]. Additionally, Lei et al. assessed the effects of periodontal ligament stem cell-derived exosomes on inflammatory bone loss in periodontitis, finding that these exosomes could enhance alveolar bone regeneration, suggesting they serve as an effective and practical cell-free alternative to mesenchymal stem cell-based therapies [14]. Furthermore, Tan et al. conducted a study on the effects of mesenchymal stem cell-derived exosomes in liver regeneration, indicating that these exosomes exert hepatoprotective effects against toxicant-induced liver injuries by activating proliferative and regenerative responses [15].

While the effects of exosomes derived from mesenchymal stem cells on neutrophils have been evaluated in various studies [16-18], similar analyses for exosomes derived from cancer cells remain unexplored. Given that exosomes contain diverse molecular contents depending on their cell of origin, it is imperative to investigate the impact of exosomes derived from 4T1 breast cancer cells on neutrophil function.

This research aims to elucidate how exosomes and conditioned medium from 4T1 breast cancer cells affect neutrophil function, a key component of the immune system. By investigating these interactions, the study seeks to uncover mechanisms by which breast cancer cells may alter immune responses, potentially leading to new biomarkers for cancer progression and the development of innovative therapies that enhance anti-tumor immunity, ultimately improving breast cancer treatment outcomes.

Materials and Methods

Cell Culture

The 4T1 murine mammary tumor cell line (obtained from the Pasteur Institute of Iran) was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Capricorn Scientific, Germany) supplemented with 10% fetal bovine serum (FBS; Anacell, Iran), 1% penicillin-streptomycin (Sigma-Aldrich, USA), and 1% L-glutamine (Sigma-Aldrich, USA). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2.

Isolation of Conditioned Medium and Exosomes

Conditioned Medium Collection

After three passages, the culture medium was replaced with a medium containing less FBS. Then 4T1 cells were seeded into T-75 culture flasks. Upon reaching 70%–80% confluence, the culture medium was removed, and cells were washed three times with sterile phosphate-buffered saline (PBS). Serum-free DMEM was then added, and cells were incubated for 48 hours at 37 °C. The conditioned medium (CM) was collected and centrifuged at 3,000 rpm for 20 minutes to remove cellular debris. The supernatant was stored at –80 °C for further use.

Exosome Isolation

Exosomes were isolated from the conditioned medium using the Exocib kit (Cib Biotech Co., Iran) following the manufacturer's instructions. Briefly, the CM was centrifuged at 3,000 rpm for 20 minutes to eliminate residual debris. Reagent A was added to the supernatant at a ratio of 1:5 (v/v), and the mixture was vortexed for 5 minutes. The solution was then incubated overnight at 4 °C with gentle shaking. Post-incubation, samples were centrifuged at 3,000 rpm for 40 minutes at 4 °C. The resulting exosome-containing pellet was resuspended in 200 µL of Reagent B and stored at –80 °C until use.

Exosome Characterization

Protein Quantification

The protein content of isolated exosomes was determined using the Nadford™ Protein Assay Kit based on the Bradford method (Navand Salamat, Iran). Exosome samples (5 µL) were added to wells of a 96-well microplate, followed by 250 µL of Nadford reagent. After incubation at room temperature for 10 minutes, absorbance was measured at 595 nm using a microplate reader. Protein concentrations were calculated using a standard curve generated with known concentrations of bovine serum albumin (BSA).

Transmission Electron Microscopy (TEM)

Exosome morphology and size were evaluated using transmission electron microscopy (TEM). Exosome suspensions were fixed in 3% glutaraldehyde solution at a 1:1 ratio (v/v). Fixed samples (2 µL) were placed on Formvar-carbon-coated copper grids and allowed to adhere for 1–2 minutes. Grids were then negatively stained with 1% uranyl acetate for contrast enhancement. After air-drying, the samples were examined using a Zeiss-Leo 906 TEM (Zeiss, Germany) at an accelerating voltage suitable for exosome imaging.

Dynamic Light Scattering (DLS)

The size distribution of exosomes was assessed by dynamic light scattering (DLS) using a Zetasizer Nano ZS instrument (Malvern Instruments, UK). Exosome suspensions were diluted with sterile PBS and filtered through a 0.22 µm syringe filter to remove aggregates. Measurements were conducted at 25 °C, and data were analyzed using the Zetasizer software to determine the average hydrodynamic diameter and polydispersity index.

Flow Cytometry Analysis of Exosomal Markers

Flow cytometry was employed to confirm the presence of exosomal surface markers. Exosome samples were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD63 antibodies (BioLegend, USA) at 4 °C for 30 minutes in the dark. Labeled exosomes were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, USA). Fluorescence intensity data were processed using FlowJo software (FlowJo LLC, USA) to assess marker expression.

Neutrophil Isolation and Pretreatment

Isolation from Human Blood

Neutrophils were isolated from fresh heparinized (10 U/mL heparin) peripheral blood samples (5 mL per donor) obtained from healthy, non-smoking adult volunteers after informed consent was received. Equal volumes of 3% dextran solution were added to the blood samples, gently mixed, and incubated at room temperature for 45 minutes to facilitate red blood cell sedimentation. The leukocyte-rich supernatant was carefully collected and subjected to centrifugation over a Ficoll-Hypaque density gradient at 400 × g for 30 minutes. Neutrophil pellets were harvested, washed with PBS, and resuspended in DMEM [19].

Purity and Viability Assessment

The purity of isolated neutrophils was confirmed by Giemsa staining, examining cell morphology under a light microscope at 1,000× magnification. Cell viability was assessed using the trypan blue exclusion assay. Only preparations with purity >95% and viability >98% were used in subsequent experiments.

Pretreatment with Exosomes

Isolated neutrophils were aliquoted into sterile tubes and pretreated with 4T1-derived exosomes at a final concentration of 100 µg/mL or fresh DMEM as a control. The cells were incubated at 37 °C for 2 hours in a shaker incubator to ensure homogeneous exposure.

Functional Assays

Cell Vitality Assessment via MTT Assay

Neutrophil Vitality post-treatment was evaluated using the methyl thiazolyl tetrazolium (MTT) assay. Following the 2-hour pretreatment, cell suspensions were adjusted to a concentration of 1×10^6 cells/mL, and 100 µL of each sample was transferred to wells of a 96-well microplate in triplicate. MTT solution (20 µL of a 5 mg/mL stock) was added to each well, and the plate was incubated at 37 °C for 2 hours. After incubation, the plate was centrifuged at 3,000 rpm for 10 minutes, and the supernatant was carefully removed. Formazan crystals formed by metabolically active cells were dissolved by adding 100 µL of dimethyl sulfoxide (DMSO) to each well. Absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to the control group.

Respiratory Burst Activity via NBT Reduction Assay

The Nitroblue tetrazolium (NBT) reduction assay was performed to assess the respiratory burst activity of neutrophils, indicative of superoxide anion production. Pretreated neutrophils (500 µL) were incubated with 500 µL of 0.1% NBT solution prepared in PBS (pH 7.4) and 500 µL of opsonized *Candida albicans* suspension. The mixture was incubated at 37 °C for 30 minutes with gentle shaking. Following incubation, tubes were centrifuged at 400 × g for 10 minutes, and supernatants were discarded. The resultant formazan deposits were dissolved by adding 1 mL of 1 M potassium hydroxide followed by 1 mL of DMSO. An aliquot of the solution (100 µL) was transferred to a 96-well microplate, and optical density was measured at 540 nm using a microplate reader. The respiratory burst activity was calculated and compared across treatment groups. This evaluation was done in triple independent.

Yeast Killing Assay

In this assay, 100 µL of blood serum was dispensed into microtubes, followed by 100 µL of pretreated neutrophils at a concentration of 5×10^6 cells/mL. The samples were incubated at 37°C for 5 minutes to activate the neutrophils. Control samples consisted of serum and DMEM only, without neutrophils. Subsequently, 100 µL of a opsonized yeasts with autologous serum—adjusted to contain the same number of yeast cells as neutrophils—was introduced to each microtube. These mixtures were then incubated at 37°C for 90 minutes to allow sufficient interaction between neutrophils and yeast. Afterward, distilled water was added to lyse the neutrophils, and the mixtures were centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was carefully transferred to microplate wells, where each well received 20 µL of MTT solution. Following a 2-hour incubation at 37°C, 100 µL of DMSO was added to dissolve the formazan crystals. Absorbance measurements were then

recorded at 490 nm to quantify the yeast viability. This evaluation was done in triple independent.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were conducted using GraphPad Prism version 10 (GraphPad Software, USA). Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

Results

Exosome Characterization

The isolated exosomes from 4T1 breast cancer cells were characterized for morphology, size, and surface marker expression.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) images revealed spherical vesicles with diameters ranging from 58 to 170nm (Figure 2).

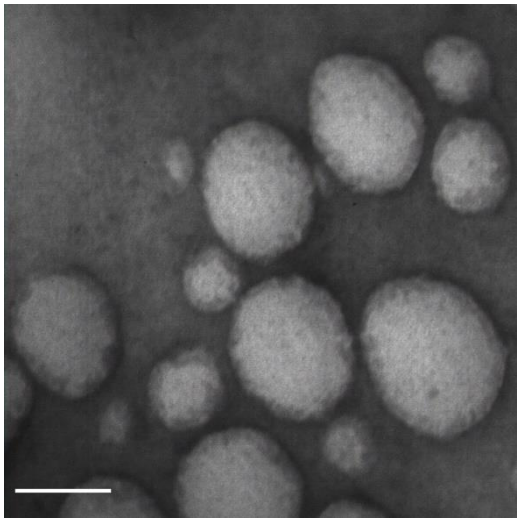


Figure 2: Transmission electron microscopy image of exosomes isolated from 4T1 cells, revealing their characteristic spherical morphology. Scale bar: 100 nm.

Dynamic light scattering (DLS)

DLS analysis confirmed an average hydrodynamic diameter of 172.3 ± 16.7nm with a polydispersity index of 2.548, indicating uniformity in size distribution (figure 3).

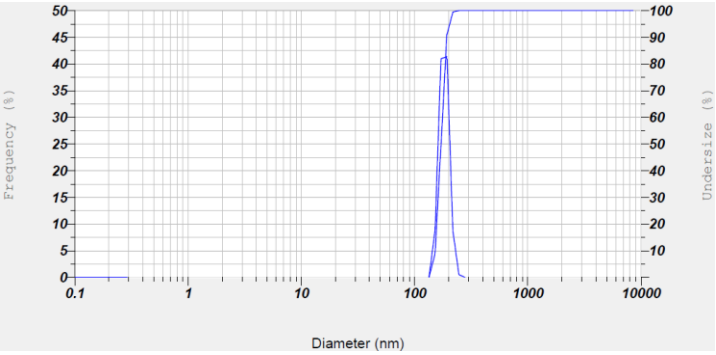
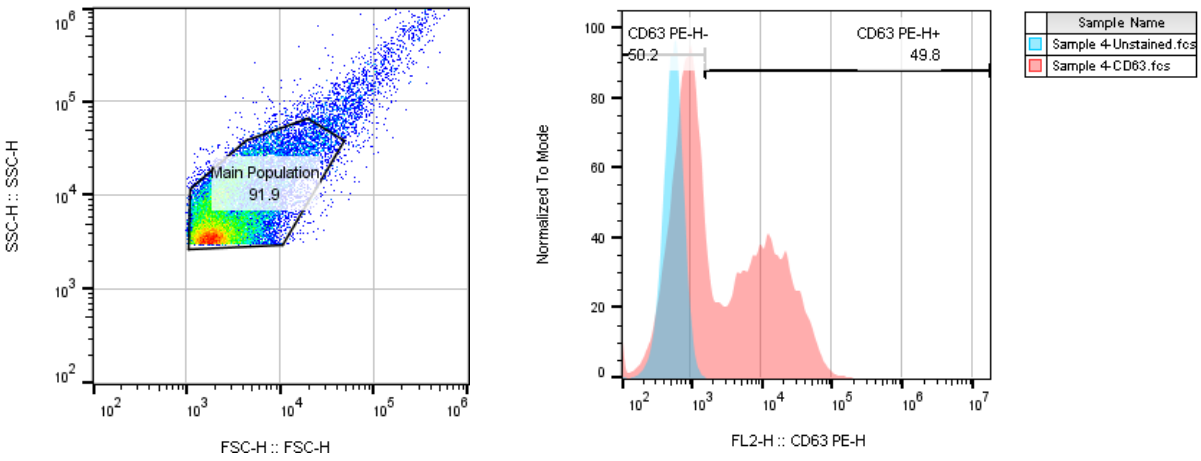


Figure 3. Representative DLS analysis of 4T1-derived exosomes.

Flowcytometry

Flow cytometric analysis (figure 4) demonstrated the expression of exosomal markers, including CD63, confirming the vesicular identity of the isolates. The protein concentration of exosomes, as determined by the Bradford assay, averaged 0.35 ± 0.05 mg/mL.



a b

Figure 4. Flow cytometric analysis of 4T1-derived exosomes. a) shows the gating strategy used to identify the main population, while b) illustrates CD63 expression via histogram.

Purity and viability of isolated neutrophils

Trypan blue exclusion and Giemsa staining (Figure 5) confirmed the purity and viability of the neutrophils to be greater than 95%.

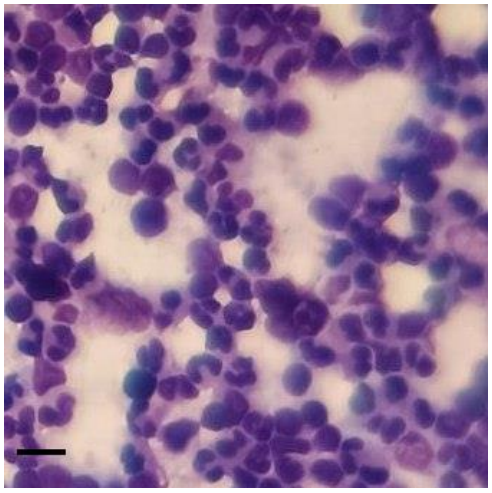


Figure 5: Light microscopy image of isolated neutrophils**.** Scale bar: 20 µm.

Neutrophil Vitality

The MTT assay revealed that pretreatment with 4T1-derived exosomes (100 µg/mL) did not significantly reduce neutrophil viability compared to the control group (DMEM alone). Cell vitality remained above 90% in all treatment groups, confirming that the experimental conditions were non-toxic for neutrophils (figure 6).

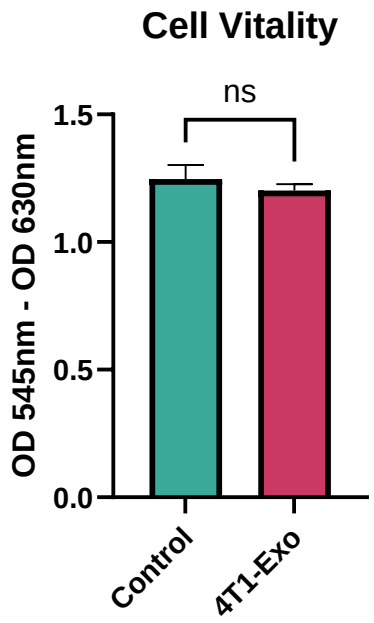


Figure 6. Cell vitality assay comparing control and 4T1-exosome–treated neutrophils, showing no significant difference in absorbance.
In the chart, «Control» denotes untreated neutrophils, while «4T1-Exo» represents neutrophils treated with 4T1-derived exosomes.

Assessment of respiratory burst capability

The NBT reduction assay showed a significant alteration in the respiratory burst activity of neutrophils following exposure to 4T1-derived exosomes. Neutrophils treated with exosomes exhibited a 4% increase in superoxide production compared to the control group ($p < 0.05$) (figure 7).

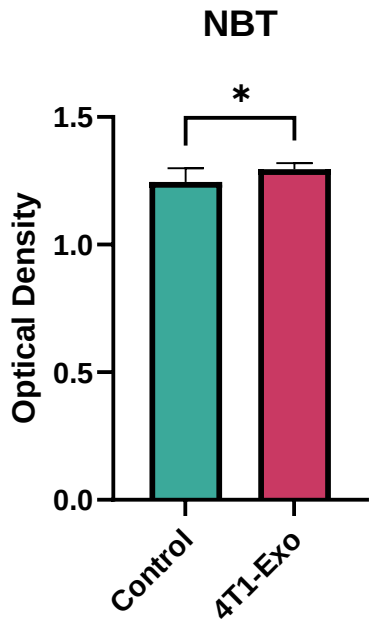


Figure 7: NBT reduction assay evaluating neutrophil respiratory burst activity. Neutrophils exposed to 4T1-derived exosomes showed a 4% increase in superoxide production compared to controls ($p < 0.05$).

Yeast Killing Assay

To evaluate how exosomes derived from 4T1 cells (4T1-Exo) influence neutrophil-mediated yeast killing, neutrophils were co-incubated with opsonized yeasts at 37°C for 90 minutes, promoting direct cell-pathogen interaction. As illustrated in Figure 8, treatment with 4T1-Exo led to a substantial increase in yeast killing compared to the control group, indicating that 4T1-Exo may enhance neutrophil capability in eliminating yeast cells.

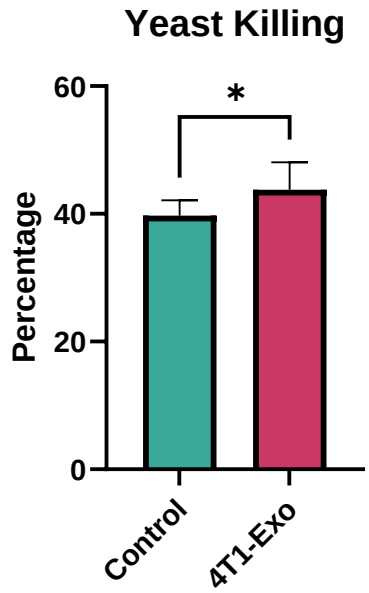


Figure 8. Yeast killing assay comparing control vs. 4T1-derived exosome-treated neutrophils, showing a significant difference ($p < 0.05$).

Discussion

Exosomes are a type of extracellular vesicles characterized by their lipid bilayer membrane and secreted by the majority of eukaryotic cells. They originate from the endocytic

pathways. Exosomes play significant roles in numerous biological processes, influencing both health and disease. The biological function of exosomes depends on their bioactive cargo, including lipids, metabolites, proteins, and nucleic acids, which can be transferred to target cells [20]. The functions of EXs are largely determined by their origin, as they are products of their parent cells and reflect their characteristics [21]. Exosomes were initially misinterpreted as cellular waste. This view shifted with the identification of dendritic cells-derived exosomes, which demonstrated their role in suppressing cancer cell growth by enhancing the proliferation of cytotoxic T lymphocytes.

Exosomes can be derived from both healthy and cancerous cells [5, 22, 23]. Tumor-derived exosomes (TEX) are involved in intercellular substance exchange and signaling between tumors and their host, playing significant roles in cancer pathogenesis, progression, invasion, and metastasis. Notably, their potential as innovative tools for precision therapy in various cancers has also been highlighted. Given the involvement of exosomes in cancer cell invasion and metastasis, TEX present a promising therapeutic avenue that could enhance the effectiveness and outcomes of cancer treatment [24].

Exosomes can regulate the immune system by promoting either immune activation or immunosuppression, thereby influencing the course of tumor progression. Tumor-derived exosomes (TEX) have been shown to influence tumor immunity through multiple mechanisms. Macrophages are among the cells that are altered by the tumor microenvironment and can be activated by TEXs. TEXs have been identified as ligands for toll-like receptor2 (TLR2). Upon stimulation of TLR2, these exosomes activate the Nuclear factor kappa B (NF-κB) pathway in macrophages, leading to the release of pro-inflammatory cytokines [25]. TEX also play a significant role in modulating the behavior of neutrophils. TEX have been found to promote a pro-tumor phenotype in neutrophils [26]. TEX have also been found to trigger the formation of neutrophil extracellular traps (NETs), which are thought to play a role in the prothrombotic phenotype associated with cancer [27].

The precise role of TEXs, particularly those derived from 4T1 cells, in their interaction with neutrophils remains poorly understood. In this study, we investigated the effects of 4T1-derived exosomes on neutrophil activity, focusing on ROS production and yeast-killing ability. The results showed that 4T1-Exo enhanced ROS production (Figure 7). ROS are produced in response to microbial invaders. These free oxygen radicals are highly toxic to pathogens and serve as a defense mechanism to prevent microbial colonization of tissues [28]. The production of ROS is elevated in tumor cells. Moderate elevations in ROS are linked to various pathological conditions, including tumor promotion and progression [29]. Similarly, a study by Dutta et al. demonstrated that exosomes derived from breast cancer cells can induce ROS production in normal mammary epithelial cells, leading to DNA damage responses, stabilization of p53, and the activation of autophagy [30]. Additionally, healthy cells-derived exosomes, including mesenchymal stem cells (MSCs), have also been shown to enhance the respiratory burst of neutrophils [31]. On the other hand, exosomes derived from Wharton's Jelly MSCs and adipose tissue MSCs demonstrated no increase in ROS production [32, 33]. These differences are due to the contents of exosomes, which vary based on the type of cell and the conditions under which they are produced.

Furthermore, the yeast killing assay demonstrated enhanced yeast killing activity in the presence of 4T1-Exo compared to the control group (Figure 8).

This finding is consistent with the ROS results, as exosomes increased ROS levels, thereby enhancing yeast-killing activity. Notably, 4T1-Exo exhibited no toxic effects on neutrophils, as their viability in the presence of exosomes was comparable to that of the control group (Figure 6). These effects are likely due to the molecular components of 4T1-Exo, which, like other TEX, carry a distinct repertoire of bioactive molecules, including proteins, lipids, DNA, microRNAs (miRNAs), messenger RNAs (mRNAs), and non-coding RNAs [24].

Beyond neutrophil modulation, TEX are known to exert diverse effects on other cell types within the tumor microenvironment, such as stromal cells, vascular endothelial cells, and fibroblasts, ultimately promoting cancer progression. For instance, exosomes enriched with microRNA-105 from highly metastatic breast cancer cells have been shown to suppress zonula occludens protein 1 (ZO-1) expression in endothelial cells, thereby disrupting vascular integrity and increasing susceptibility to cancer progression [24]. Additionally, TEX containing proteins such as Rab3D, Transforming growth factor beta-1 (TGF-β1), and Latent Membrane Protein 1 (LMP1) have been reported to facilitate epithelial-mesenchymal transition, thereby enhancing the oncogenic potential and invasive capabilities of cancer cells [24].

Therefore, 4T1-Exo could be considered a potential target for therapeutic strategies. While our findings highlight their ability to enhance ROS production and yeast-killing activity in neutrophils without inducing toxicity, further studies are necessary to elucidate the underlying molecular pathways involved in these effects. Additionally, the potential role of 4T1-Exo in promoting tumor progression warrants further investigation. Beyond their implications in tumor biology, these exosomes may also hold promise in clinical applications for individuals with compromised immune systems. Given their ability to modulate neutrophil activity and enhance microbial defense mechanisms, 4T1-Exo could be explored as a tool to strengthen immune responses in such patients, potentially reducing their susceptibility to infections. This dual role of 4T1-Exo—as both a therapeutic target in cancer and an immunomodulatory agent—underscores the need for comprehensive research to understand their biological functions and therapeutic potential fully.

Conclusion

In conclusion, this study demonstrates that 4T1-Exo can enhance ROS production and promote yeast-killing activity in neutrophils without reducing neutrophil vitality. This suggests that 4T1-Exo may modulate neutrophil function to influence the immune response within the tumor microenvironment. Given the potential of tumor-derived exosomes to impact immune cell behavior and cancer progression, targeting TEX could serve as a promising therapeutic approach to improve cancer treatment outcomes. Future studies should explore the underlying molecular mechanisms and evaluate the therapeutic potential of exosomes in enhancing immune responses, particularly in individuals with compromised immune systems.

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