



## Original Article

# Exosome-derived TXNDC5 is Required for the Inflammatory Progression of Rheumatoid Arthritis Fibroblast-like Synoviocytes

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## Abstract

**Background and objectives:** Thioredoxin domain-containing 5 (TXNDC5) is an endoplasmic reticulum (ER) residing chaperon that is associated with the inflammatory phenotype of rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs), such as high proliferation, cytokine production and invasion. However, if TXNDC5 is involved in communication between RA FLSs remains unknown.

**Methods:** Exosomes were separated and TXNDC5 expression in exosomes was detected by Western blotting, immunofluorescent staining, and flow cytometry. Cell Counting Kit-8 (CCK-8), Annexin V-APC/7-amino-actinomycin D staining, Western blotting, and enzyme-linked immuno sorbent assay (ELISA) were applied to detect the effects of exosomes on cell viability, apoptosis, activation of signaling pathways, and the production of inflammatory factors.

**Results:** TXNDC5 protein was detected in the exosomes from RA FLSs and its content in exosomes increased when RA FLSs were stimulated by ER stress inducers. Functionally, TXNDC5 overexpressing RA FLSs-derived exosomes (TXNDC5-containing Exo) increased the production of inflammatory factors and the phosphorylated levels of extracellular regulated protein kinases (ERK), protein kinase B (PKB/Akt), p65 nuclear factor kappa beta (NF- $\kappa$ B), and p38 mitogen-activated protein kinase (MAPK) signaling pathways in recipient FLSs. In addition, recipient FLSs with increased TXNDC5 expression were characterized by enhanced cell viability but a decrease in apoptosis in response to ER stress. More importantly, the introduction of TXNDC5-containing Exo protected recipient RA FLSs against the toxicity of methotrexate for viability, cytokine production, and apoptosis.

**Conclusions:** In combination, these results could provide a novel approach for TXNDC5 to communicate via exosomes between RA FLSs to exacerbate inflammation of RA and specific inhibition of exosome-mediated delivery of TXNDC5 has potential as a novel treatment strategy for RA.

**Keywords:** TXNDC5; Rheumatoid arthritis; Exosomes; ER stress; Apoptosis; Inflammation.

**Abbreviations:** TXNDC5, Thioredoxin domain-containing 5; ER, endoplasmic reticulum; RA FLSs, rheumatoid arthritis fibroblast-like synoviocytes; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MMP, matrix metalloproteinase; HSC70, heat shock cognate 70; NF- $\kappa$ B, nuclear factor kappa beta; IL, interleukin.

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## Introduction

Exosomes are extracellular vehicles (EVs) that are formed by the inward budding of the plasma membrane into the cytoplasm and are released by exocytosis, which are called multivesicular bodies.<sup>1</sup> Of interest, exosomes that encompass proteins, lipids, enzymes, DNA, and RNA are released into the extracellular space and deliver signals through exosome-cell interactions.<sup>2,3</sup> Exosomes reflect

the biological state of parent cells and are vital to homeostatic maintenance and the occurrence of disease.<sup>4</sup> Exosomes are characterized by their ability to regulate the immune response and are involved in the occurrence or progression of autoimmune diseases.<sup>5</sup> Rheumatoid arthritis (RA) is a chronic autoimmune disease where the highly proliferative fibroblast-like synoviocytes (FLSs) promote the progressive destruction of articular cartilage.<sup>6</sup> Exosomes could communicate between cells that reside in disease joints and contribute to hyper-inflammation, angiogenesis, antigen presentation, and the degradation of the extracellular matrix.<sup>7</sup> For example, RA FLSs-derived exosomes contain citrullinated proteins and a membrane form of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>8</sup> The chondrocytes-derived exosomes could increase the production of matrix metalloproteinase (MMP)-13 in FLSs.<sup>9</sup> In addition, serum exosomal proteins might act as biomarkers in patients with RA.<sup>10</sup> Therefore, exosomes are closely related with RA progression and identifying novel components in exosomes could be helpful to elucidate the pathological mechanism and to provide novel treatment strategies for RA.

Thioredoxin domain-containing protein 5 (TXNDC5) belongs to the thioredoxin family and localizes mainly in the endoplasmic reticulum.<sup>11</sup> As a chaperon to maintain homeostasis in response to endoplasmic reticulum (ER) stress, TXNDC5 could protect endothelial cells against stress-induced cell death in response to hypoxia.<sup>12</sup> A previous study identified TXNDC5 as a pathological factor in RA and its increased expression conferred RA FLSs with an inflammatory phenotype.<sup>13</sup> In addition, this mechanistic study showed that TXNDC5 synergized with heat shock cognate 70 (HSC70) to activate nuclear factor kappa beta (NF- $\kappa$ B) signaling in RA FLSs.<sup>14</sup> Of note, TXNDC5 could be detected in the fluids of RA patients, although the exact mechanism that accounts for the existence of TXNDC5 remains unclear.<sup>15</sup> Methotrexate (MTX) is the first-line therapy for RA but some patients are insensitive to MTX.<sup>16</sup> A previous study demonstrated that MTX induced ER stress and ER stress is often associated with drug resistance.<sup>17</sup> However, if TXNDC5 is related to the drug resistance of RA needs to be determined.

In this study, TXNDC5 containing exosomes (TXNDC5-containing Exo) that were derived from RA FLSs were transported into recipient FLSs. This agreed with its endogenous role in RA FLSs, TXNDC5 overexpressing RA FLSs-derived exosomes increased cell viability and production of inflammatory factors of recipient RA FLSs. Importantly, it confers RA FLSs with growth advantages but apoptotic resistance against ER stress and MTX. This study provides a novel way for TXNDC5 to exacerbate the inflammation of RA through exosome-mediated communication with the surrounding cells and targeting TXNDC5 could be promising for RA treatment.

## Materials and methods

### Culture of synovial fibroblasts

The study protocol was approved by the Institutional Review Board of Shandong First Medical University & Shandong Academy of Medical Sciences (Jinan, Shandong, China). RA FLSs were obtained by primary extraction from synovial tissue. The synovial tissues were obtained from the knee synovium of RA patients and were surgically removed during joint replacement at the Department of Orthopedics of Shandong Provincial Hospital (Jinan, Shandong, China). The patients were clinically diagnosed according to the standards of the American College of Rheumatology, and written informed consent for all patients were obtained. Synovial

tissue samples were finely minced and digested with a solution that contained 1 mg/ml collagenase (type II) (Solarbio, Beijing, China) in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific, Waltham, MA, USA) for 3 h in a 37 °C, 5% CO<sub>2</sub> incubator (Thermo Scientific, Waltham, MA, USA), followed by further digested with 0.25% trypsin (Thermo Scientific, Waltham, MA, USA) for 15 m. Then, cells were collected from supernatants by centrifuge and cultured in DMEM that contained 10% fetal bovine serum (Thermo Scientific, Waltham, MA, USA) at 37 °C in 5% CO<sub>2</sub> for 2 days, and then the medium was changed. Experiments were performed when the cell density reached 80%–90% and RA FLSs with 3–7 passages were used for the following experiments in this study.

### Western blotting analysis

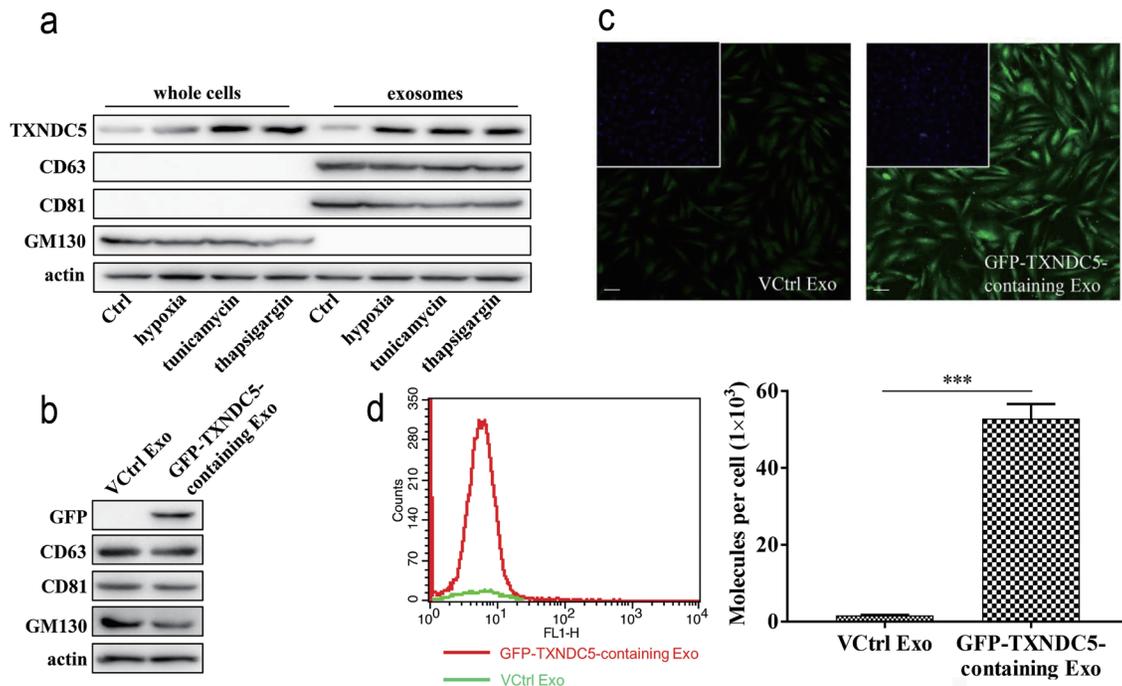
After various treatments, cells were collected for homogenization in ice-cold radio-immunoprecipitation assay buffer (Solarbio, Beijing, China) at 4 °C for 40 m and subsequently centrifuged at 16,000 × g for 30 m at 4 °C. The total protein from the supernatant was collected and the concentration was determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Then, total protein was incubated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (Beyotime, Shanghai, China) at 100 °C for 5 m, and separated by SDS-PAGE and transblotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Then the following antibodies were used to detect the indicated parameters: CD63 (ab134045), CD81 (ab109201), GM130 (ab187514), ERK (ab184699), p-ERK (ab201015), Akt (10176-2-AP), p-Akt (66444-1-Ig), p65 NF- $\kappa$ B (66535-1-Ig), p-p65 NF- $\kappa$ B (ab76302), p38 MAPK (14064-1-AP), p-p38 MAPK (ab178867), Bcl-2 (ab182858), Bax (ab32503), TXNDC5 (ab13820), GFP (ab183734), Actin (ab8226), GAPDH (ab181602), and HRP-linked secondary antibodies (ab7090, ab97040). The signals were visualized using chemiluminescence by Electro-Chemi-Luminescence (ECL) (Vazyme, Nanjing, China).

### Isolation of exosomes derived from RA FLSs

RA FLSs were cultured in an exosome-free culture medium for the indicated treatments and exosomes were isolated as described previously.<sup>18</sup> In brief, the culture medium was centrifuged at 2,000 × g for 10 m to remove the cells. Then, the supernatant was centrifuged at 12,000 × g for 30 m to eliminate debris. Then, the supernatant was filtered using 0.22  $\mu$ m polyvinylidene difluoride filter and ultracentrifuged at 100,000 × g for 70 m. Exosomes were resuspended in phosphate buffer saline ([PBS], Solarbio, Beijing, China) and stored at –80 °C for subsequent experiments.

### Immunofluorescent labeling

Primary FLSs were transfected with a lentiviral vector that expressed a GFP-TXNDC5 fusion protein. Exosomes derived from this primary RA FLSs (RA FLSs-derived exosomes) were isolated from the culture medium by sequential centrifugation as described previously. Recipient FLSs were treated with exosomes that were derived from primary RA FLSs. The recipient FLSs were fixed with 4% paraformaldehyde (Boster, Wuhan, China) at room temperature for 15 m followed by permeabilization with 0.1% Triton X-100 (Solarbio, Beijing, China) for 10 m. After washing, cells were blocked for 1 h using goat serum (Solarbio, Beijing, Chi-



**Fig. 1.** TXNDC5 shuttles between FLSs through exosome: (a) RA FLSs were exposed to thapsigargin, tunicamycin, or hypoxia at indicated dosage. The level of TXNDC5, CD63, CD81, and GM130 were analyzed by Western blotting; (b) Western blotting was performed against the protein of the recipient FLSs treated with GFP-TXNDC5 that contained exosomes (GFP-TXNDC5-containing Exo) or VCtrl exosomes (VCtrl Exo); (c) GFP-tag was detected by immunofluorescence, scale bars = 40  $\mu$ m; and (d) GFP-tag was detected by flow cytometry analysis. The results are presented from three independent experiments and each experiment was repeated three times. \*\*\* $p < 0.001$ .

na). Cells were washed three times with PBS and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Solarbio, Beijing, China) for 5 m. Images were acquired using an Olympus FV 3000 fluorescence microscope system (Olympus, Tokyo, Japan). All experiments were repeated three times.

#### Flow cytometry

Following incubation with RA FLSs-derived exosomes, recipient FLSs were fixed with 4% paraformaldehyde (Boster, Wuhan, China) at 4  $^{\circ}$ C for 15 m, and then permeabilized with 0.2% Triton X-100 (Solarbio, Beijing, China) for 10 m. After washing with PBS that contained 2% human serum albumin (blocking solution), the samples were incubated with the primary antibodies (rabbit anti-GFP [ab183734, Abcam]) with a dilution of 1:100 at 4  $^{\circ}$ C for 1 h, and subsequent the secondary antibody (goat anti-rabbit IgG H+L [ab150077]) with a dilution of 1:2,000 at 4  $^{\circ}$ C for 1 h. Then, the fluorescently labeled cells were analyzed by flow cytometry (FCM) (Becton Dickinson, USA), and the data were analyzed using FlowJo software (version 10.7.2, TreeStar, Woodburn, Oregon, USA).

#### Cell viability and ELISA

Cell viability was analyzed by CCK-8 kit (Solarbio, Beijing, China) according to the manufacturer's instruction and the signals were detected by a spectrophotometer at an absorbance of 450 nm. The supernatants were obtained from RA FLSs that were collected and subjected to measure the levels of interleukin (IL)-6, IL-8, MMP-1 and MMP-3 (Abcam, Cambridge, MA, USA).

#### Cell apoptosis analysis

Apoptosis was evaluated by Annexin V-APC/7-amino-actinomycin D staining (KeyGEN, Nanjing, China) according to the manufacturer's instruction. In total, 50,000 cells were analyzed by flow cytometry and the Annexin V-positive cells were considered apoptotic cells. The final results were analyzed using ModFit software (Verity Software House, Topsham, ME, USA).

#### Statistical analyses

Statistical analyses were carried out using SPSS V.16 software (IBM, Armonk, New York, USA). The experiments were repeated three times on different patients. A t-test was used to assess statistical differences between both groups. Multifactor analysis of variance was used for comparison between multiple groups, and an LSD-t test was used for further pairwise comparisons. Importantly,  $p$ -values  $< 0.05$  were considered significant. The data are expressed as mean  $\pm$  standard deviation (SD).

## Results

### TXNDC5 is transported into recipient FLSs through exosomes

Exosomes were isolated from the cultured media of RA FLSs. When assessed by Western blotting, the isolated pellet was positive for the exosomal markers CD63 and CD81, but negative for a Golgi membrane protein, GM130 (Fig. 1a). TXNDC5 protein was

detected in the exosomes, the levels of which increased as a result of hypoxia and ER stress stimulation (Fig. 1a).

To determine whether TXNDC5-containing Exo could be delivered into recipient FLSs, primary FLSs were transfected with a lentiviral vector that expressed a GFP-TXNDC5 fusion protein; therefore, exosomal GFP-TXNDC5 could be followed by the presence of GFP. GFP-tag was observed in the recipient FLSs as demonstrated by Western blotting (Fig. 1b), immunofluorescent analysis (Fig. 1c), and flow cytometry analysis (Fig. 1d). Therefore, it was proven that TXNDC5 could be transported between neighboring FLSs by exosomes.

#### ***Exosomes derived from TXNDC5 that overexpress RA FLSs regulate the inflammatory phenotype of recipient RA FLSs***

To investigate the role of exosomes from TXNDC5-overexpressing RA FLSs (TXNDC5-containing Exo), the production of IL-6, IL-8, MMP-1, and MMP-3 in RA FLSs was analyzed. The levels of IL-6, IL-8, MMP-1, and MMP-3 expression were strongly upregulated in RA FLSs following stimulation with IL-1 $\beta$  and TNF- $\alpha$  (Fig. 2). A further upregulation of IL-6, IL-8, MMP-1, and MMP-3 expression was detected when RA FLSs were pretreated with TXNDC5-containing Exo in contrast to the ones pretreated with Vector control (VCtrl)-derived exosomes (VCtrl Exo) (Fig. 2).

#### ***Exosomes derived from TXNDC5 that overexpressed RA FLSs increased the viability of RA FLSs and protected RA FLSs against apoptosis that results from ER stress***

The effects of TXNDC5-containing Exo were evaluated on cell viability and apoptosis. Compared with VCtrl Exo, TXNDC5-overexpressing Exo increased the viability of RA FLSs as shown by CCK-8 (Fig. 3a). Then, Annexin V staining (Fig. 3b) and Bcl-2 and Bax (Fig. 3c), were measured to detect the apoptosis of RA FLSs. The results of Annexin V staining showed that the increase in apoptosis that resulted from ER stress was diminished by pretreatment with TXNDC5-containing Exo. In addition, similar effects were evidenced by the increase in Bcl-2, but the decrease in Bax (Fig. 3c) when RA FLSs were pretreated with TXNDC5-containing Exo in the presence of ER stress.

The inflammatory signaling pathways, such as ERK and Akt are closely related to an inflammatory phenotype of RA FLSs.<sup>19,20</sup> Then, if TXNDC5-containing Exo could activate ERK, Akt, p65 NF- $\kappa$ B, and p38 MAPK signaling were examined. With the exposure of IL-1 $\beta$  and TNF- $\alpha$ , the phosphorylation of ERK, Akt, p65 NF- $\kappa$ B, and p38 MAPK signaling pathways increased in RA FLSs. Importantly, the phosphorylated levels of the previous signaling pathways increased more when RA FLSs were pretreated with TXNDC5-containing Exo in contrast to its parental controls (Fig. 3d).

#### ***Exosomes derived from TXNDC5 that overexpressed RA FLSs promoted drug resistance in RA FLSs***

In addition, if TXNDC5-containing Exo behaved as a counter-defense mechanism that leads to drug insensitivity was analyzed. To test this, RA FLSs were pretreated with TXNDC5-containing Exo or VCtrl Exo for 12 h, followed by MTX treatment for 72 h. MTX treatment reduced cell viability (Fig. 4a) but increased cell apoptosis (Fig. 4b) of RA FLSs. However, this reduction in cell vi-

ability or increase in cell apoptosis could be restored or attenuated by pretreatment with TXNDC5-containing Exo compared with the parental controls. The consistent decreases in IL-6, IL-8, MMP-1, MMP-3, and MMP-13 expression that resulted from MTX could be restored partly by treatment with TXNDC5-containing Exo (Fig. 4c). In combination, these findings established a clear role for TXNDC5 overexpressed exosomes from RA FLSs in reducing drug insensitivity in RA.

## **Discussion**

This study showed that TXNDC5 protein was observed in the exosomes from RA FLSs, and it could be transferred via exosomes into recipient FLSs to exacerbate its inflammatory phenotypes, such as cell viability and inflammatory factor production. Importantly, recipient FLSs with increased TXNDC5 expression exhibited apoptotic resistance to ER stress and MTX. Therefore, TXNDC5 could exacerbate inflammation and promote the disease progression of RA by exosome-mediated transfer into recipient cells. Exosomes could transport and transmit pathogenic signals between synovial cells, chondrocytes, and immune cells in the joints of RA patients, which destroyed the normal operation of the intra-articular environment and further aggravated the disease progression of RA.<sup>21</sup> Potentially, exosomes-derived TXNDC5 could be involved in communication between various types of cells that reside in the inflamed joint, which requires further research.

In RA, chronic ER stress is closely related to the pathogenesis and progression of RA by increased proliferation of FLSs and the production of pro-inflammatory cytokines.<sup>22</sup> Importantly, RA FLSs are characterized by apoptosis resistance against ER stress. Therefore, the ER stress-related pathway combined with its components are important targets to develop novel treatments for RA. A previous study demonstrated that TXNDC5 promoted high proliferation of RA FLSs and prostate cancer cells under inflammation or androgen-deprived conditions.<sup>23</sup> In this study, TXNDC5 facilitated cell-to-cell communication between FLSs in exosomes and treatment with exosomes from TXNDC5 that overexpressed RA FLSs could increase cell viability and cytokine production in recipient FLSs. Although MTX is regarded as a breakthrough in RA therapies, a large number of patients respond insufficiently to the therapy.<sup>24</sup> This research showed that TXNDC5-containing Exo could protect recipient FLSs from the cytotoxic effects of MTX and specific targeting toward TXNDC5-containing Exo might overcome this type of resistance and provide a new treatment strategy for RA.

Previous studies showed that EVs that were released by TNF- $\alpha$ -treated monocytes and T cells directly stimulated FLSs to secrete inflammatory mediators, such as IL-6 and IL-8.<sup>25</sup> Detailed analysis showed that exosomes from FLSs activated NF- $\kappa$ B to promote inflammation and apoptotic resistance although the exact mechanism remains unclear. A previous study showed that endogenous TXNDC5 synergized with HSC70 to increase the activity of NF- $\kappa$ B signaling, which might explain the previous effects of FLSs-derived exosomes on NF- $\kappa$ B signaling.<sup>14</sup> In addition, a recent study showed that exosomes from blood plasma activated Akt and ERK signaling pathways, which then initiate angiogenesis and promoted the expression of anti-apoptotic proteins.<sup>26</sup> In this study, TXNDC5-containing Exo activated Akt, ERK, p65 NF- $\kappa$ B, and p38 MAPK signaling; therefore, ameliorating ER stress or drug-induced apoptosis, and therefore, impairing the inflammatory phenotype of RA FLSs. Therefore, TXNDC5-containing Exo could improve the adaptation of RA FLSs to ER stress and treatment drugs, and therefore, improve inflammation in RA partly by activating the inflam-

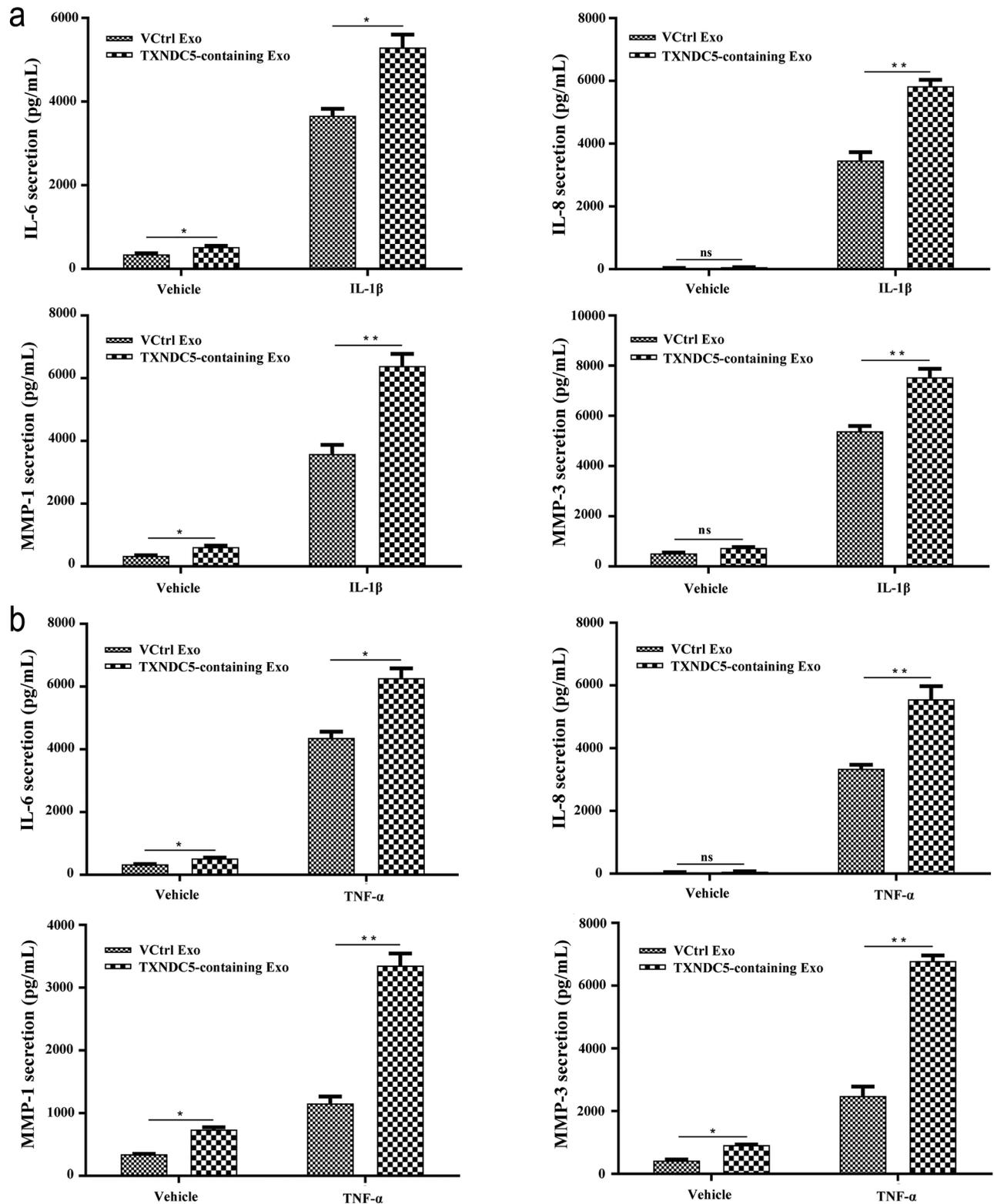


Fig. 2. TXNDC5 that overexpress RA FLSs-derived exosomes regulate the inflammatory phenotype of RA FLSs: (a and b) expression level of IL-6, IL-8, MMP-1, and MMP-3 in normal and inflammatory environments (treated with IL-1 $\beta$  or TNF- $\alpha$ ) were evaluated by ELISA following stimulation with exosomes from TXNDC5 overexpressed RA FLSs in contrast to cells treated with VCtrl Exo. The results are presented from three independent experiments and each experiment was repeated three times. ns, not significant; \* $p$ <0.05 and \*\* $p$ <0.01.

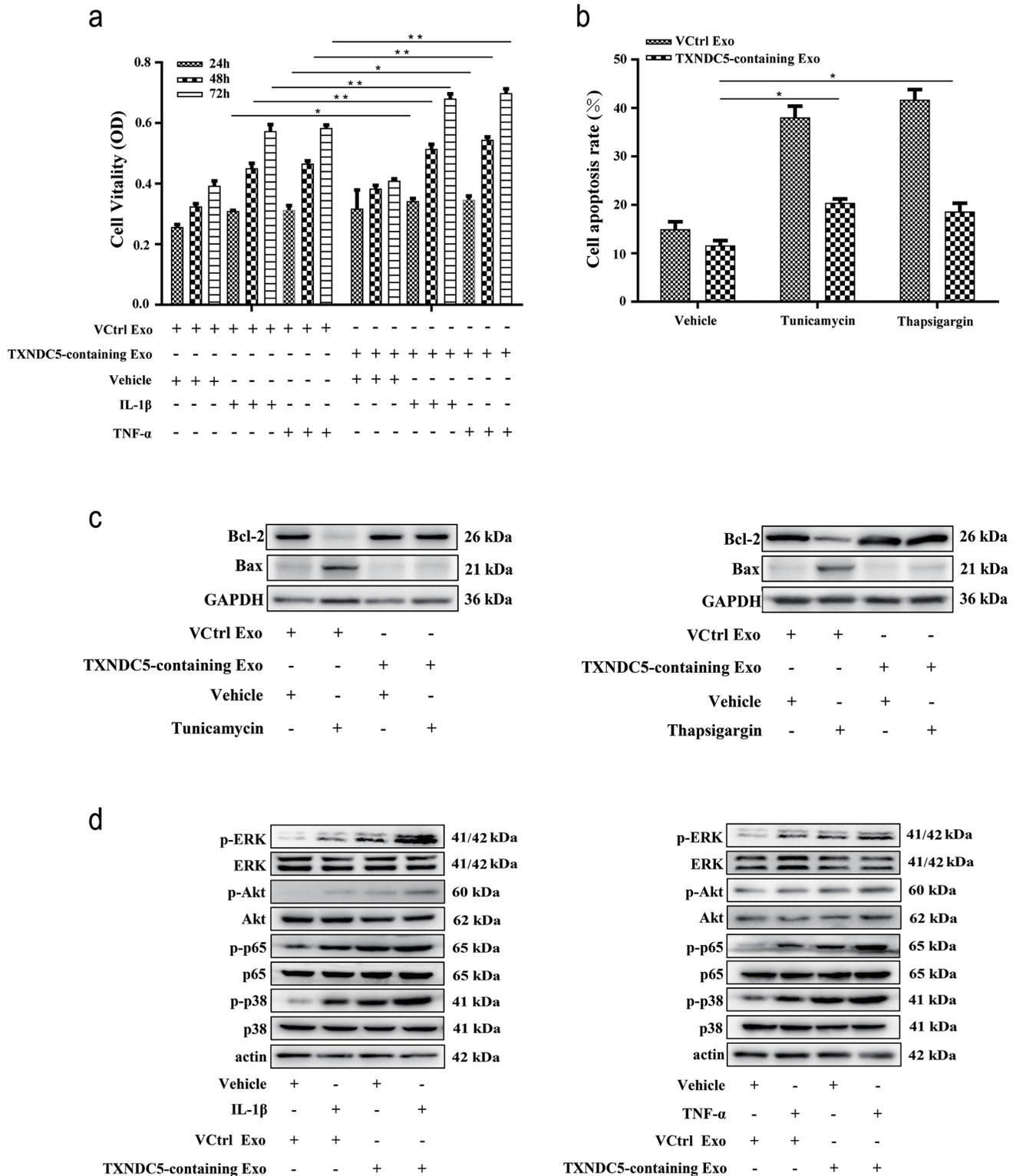


Fig. 3. TXNDC5-derived exosomes increase the viability of RA FLSs and protect RA FLSs against ER stress-induced apoptosis: (a) cell viability of RA FLSs was measured after pretreated RA FLSs with TXNDC5 or VCtlr that expressed RA FLSs-derived exosomes in normal (vehicle) or inflammation-stimulating environment (treated with IL-1β or TNF-α); (b) Annexin V staining; and (c) Western blotting were used to detect cell apoptosis after pretreated RA FLSs with TXNDC5 or VCtlr that expressed RA FLSs-derived exosomes, followed by thapsigargin or tunicamycin treatment; and (d) effect of exosomes derived from TXNDC5 overexpressed RA FLSs on the phosphorylation of ERK, Akt, p65 NF-κB, and p38 MAPK signaling pathways were analyzed by Western blotting and β-actin served as a loading control. The data are expressed as the mean ± SD of three repeated experiments. \**p*<0.05 and \*\**p*<0.01.

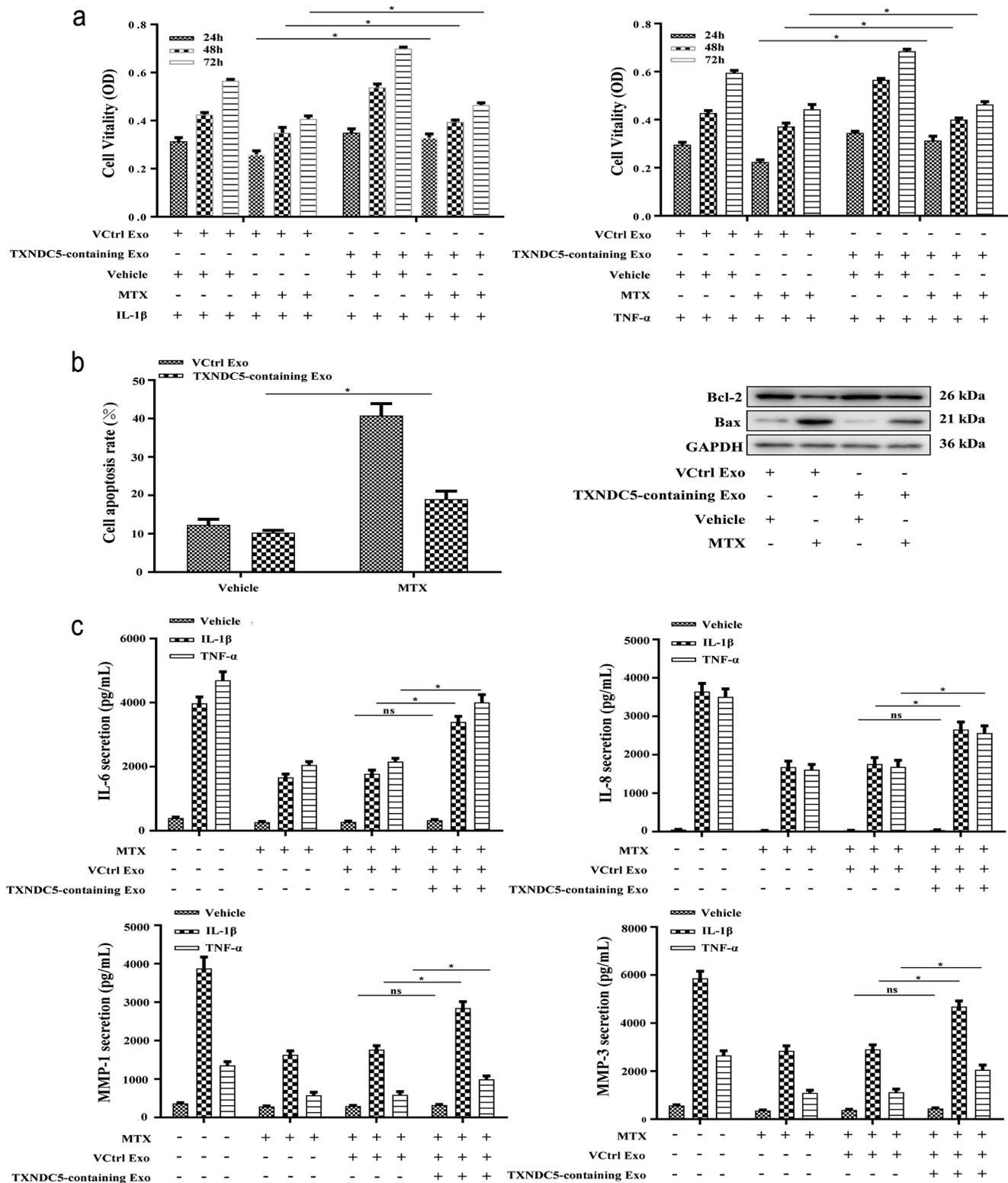


Fig. 4. TXNDC5-derived exosomes promote insensitivity of RA FLSs towards MTX: (a) cell viability of RA FLSs was measured after pretreatment with TXNDC5- or VCtrl that expressed RA FLSs-derived exosomes for 12 h, followed by MTX treatment for 72 h in the presence of IL-1β or TNF-α; (b) Annexin V staining or Western blotting was used to detect the apoptosis rates in RA FLSs; (c) RA FLSs were pretreated with exosomes from TXNDC5 that overexpressed RA FLSs followed by MTX and IL-1β or TNF-α, ELISA was performed to detect the levels of IL-6, IL-8, MMP-1, and MMP-3. The data are means ± SD of three independent experiments performed in triplicate. ns, not significant; \**p*<0.05.

mation signaling pathways.

### Future directions

More research is required to clarify if the engulfment of TXNDC5 is an active or passive process. In addition, if TXNDC5 could be transported from RA FLSs to different cell types needs to be validated. Answering these questions could provide new insights into the underlying mechanism that accounts for similar traits among different cell types that reside in an inflamed joint. Because TXNDC5 could be transferred via exosomes to impair the inflammatory phenotype of RA, the specific inhibition of exosome-mediated delivery of TXNDC5 might be a potential novel treatment strategy for RA.

### Conclusions

Exosomes that are derived from RA FLSs are carriers that transport TXNDC5 into recipient FLSs. Exosome-derived TXNDC5 could promote RA progression by removing the inflammatory phenotype and apoptotic resistance from recipient FLSs. This study identified a novel avenue in exosomes for TXNDC5 to be involved in RA and specific targeting toward exosome-derived TXNDC5 could be promising for RA treatment.

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### Conflict of interest

No potential conflicts of interest were disclosed by the authors.

### Author contributions

Study design, performance, analysis and interpretation of data and write the manuscript (LG, LW, YZ), critically revised the manuscript (LW, YZ, RZ). All authors have made a significant contribution to this study and have approved the final manuscript.

### Data sharing statement

No additional data are available.

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