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Evaluation of TAK-242 (Resatorvid) Effects on Inflammatory Status of Fibroblast-like Synoviocytes in Rheumatoid Arthritis and Trauma Patients

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ABSTRACT

Fibroblast-like synoviocytes (FLSs) produce lots of inflammatory molecules that trigger immune responses and intensification the inflammation and thereby play important roles in Rheumatoid Arthritis (RA) pathogenesis. Due to the important roles of toll-like receptor 4 (TLR4) in cytokine production and inflammation, we aimed to evaluate the effects of TAK-242 (Resatorvid) on *interleukin (IL)1-β, IL-6, TNF-a,* and *TLR4* expression and two important proteins of nuclear factor-κB (NF-κB) signaling pathway (Ikβα and pIkβα) in RA and trauma FLSs.

FLSs were isolated from synovial tissues of trauma (n=10) and RA (n=10) patients and cultured in Dulbecco's Modified Eagle Medium (DMEM). 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was performed to evaluate the cytotoxicity effects of TAK-242 on the RA FLSs. Real-time PCR was performed to measure the expression level of *IL1-β, IL-6, TNF-a*, and *TLR4* genes in Lipopolysaccharide (LPS) and TAK-242 treated FLSs. Furthermore, the treated FLSs were evaluated for protein levels of Ikβα and pIkβα by western blot.

The baseline expression of *IL1-β, IL-6, TNF-a,* and *TLR4* showed no significant differences between healthy and RA FLSs. LPS stimulated FLSs significantly increased mRNA levels of *IL-1β, IL-6, TNF-a,* and *TLR4* genes in both the healthy and RA FLSs compared with that of their

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control groups, and pretreatment with TAK-242 reversed the effect. Furthermore, LPS-stimulated FLSs significantly increased the level of pIkβα in both the healthy and RA FLSs compared with that of their control groups, and pretreatment with TAK-242 reversed the effect.

We provide the data that TAK-242 through inhibiting the NF-κB signaling pathway may modulate TLR4-mediated inflammatory responses and could be considered as a potential therapeutic agent for RA patients.

Keywords: Rheumatoid arthritis; Trauma and stressor related disorders; Toll-like receptor 4

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by autoantibody production, synovial hyperplasia (pannus formation), and joint damage. Systemic complications and progressive disability are burdens that are leading to socioeconomic costs.[1] Fibroblast-like synoviocyte (FLSs), type B synoviocytes or known as synovial fibroblasts, are mainly located in the intimal layer of the synovium and form a 2-3 layer of cells in healthy synovium of human[2-4] FLSs through interactions with extracellular matrix (ECM) and also with other cells in the synovium constitute a normal intimal layer.[5] These cells play critical roles in the organization and formation of the synovial lining. Indeed, FLSs can develop a three-dimensional complex structure in the synovial lining and also secrete various lubricant compositions.[6] In an inflammatory situation like RA disease, the typical 2-3 layers of the cells are proliferated and transformed into hyperplastic cell layers of synoviocytes in the synovial lining. These cells secrete lots of inflammatory cytokines and chemokines that lead to recruitment, differentiation, proliferation, and activation of immune and resident none-immune cells.[7,8] Furthermore, FLSs secrete different types of matrix metalloproteases (MMPs) that are implicated in the degradation of ECM and finally led to joint damage in RA patients.[9] FLSs in normal conditions are neutral, while inflammation could be activated through various extrinsic and intrinsic factors. Factors with the potential ability to activate FLSs exist in different stages of RA.[10-12] These factors usually use specific receptors or channels outside or inside of the FLSs and trigger signaling pathways and activate the cells.[13-16]

Various studies have suggested that toll-like receptor 4 (TLR4)-mediated inflammation may play important roles in RA development.[17,18] TLR4 is one of the important TLRs that are highly expressed in granulocytes, macrophages, and monocytes. Moreover, *TLR4* expression in synoviocytes, osteoblasts, and chondrocytes indicates the TLR4 involvement in the pathophysiology of the musculoskeletal system.[19] Thus, it seems that TLR4 could be more implicated in RA pathogenesis than other TLRs.[20] Moreover, *TLR4* upregulation has been noticed in synovial fibroblasts and macrophages of RA patients in comparison to osteoarthritis patients or healthy controls.[21,22] Investigation on *TLR4-/-* mice model of arthritis showed a significant decrease in Th17 number and IL17 production, and also alleviated the severity of the disease.[23] Altogether, these results emphasize TLR4 importance in RA pathogenesis and also provide us knowledge that TLR4 might be a potential therapeutic target for RA patients. Thus, inhibition of *TLR4* signaling during disease progression might alleviate disease manifestations and improve the disease outcome.

TLR4 blockers such as TLR4 blocking antibodies, TLR4 antagonists, small molecule inhibitors, and decoy peptides have been widely developed to block the TLR4 signaling pathway.[24,25] Many TLR4 blocking agents have been introduced to inhibit the TLR4 signaling pathway in different diseases, including RA[25] TAK-242 (Resatorvid), an important TLR4 signaling blocker, selectively binds to the intracellular domain of TLR4 and disrupts TLR4 interactions with its adaptor proteins; TRAM (toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β-related adaptor molecule) and/or TIRAP (toll/interleukin-1 receptor domain-containing adaptor protein). Disruption between TLR4 and TIRAP by TAK-242 could impair NF-κβ activation, which is associated with low levels of inflammatory mediators. Moreover, TAK-242 could also disrupt the interaction between TLR4 and TRAM leads to inhibition of both the interferon-sensitive response element (ISRE) and

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NF-κβ activation, which leads to downregulation of interferons and cytokines.[26] The nuclear factor-κB (NF-κB) signaling pathway could modulate the expression of chemokines, cytokines, and adhesion molecules and thereby be involved in inflammatory processes. Thus, deregulated NF-κB activation contributes to the pathogenesis of inflammatory diseases such as RA. NF-κB activation is implicated in the proliferation and apoptosis resistance of RA FLSs that results in hyperplasia in RA synovium.

The decade of researches documented that only 60% of patients show an appropriate response to current therapies.[27] Many attempts have been done to develop therapeutic agents to control or treat RA patients. For instance, biologic therapies such as antibodies against interleukin (IL)1-β, IL-6, or TNF-α showed promising results in alleviating symptoms of inflammatory diseases, while 30–50% of RA patients showed no efficacious response to the antibodies.[28] Thus, new therapeutic agents are necessary for the treatment of RA disease.

Due to the suppressive roles of TAK-242 in the expression of inflammatory cytokines, it might show promising results in the treatment of inflammatory and autoimmune diseases such as RA.[29] The effect of TAK-242 on Lipopolysaccharide (LPS)/TLR4 mediated inflammation in RA might be NF-κB dependent.[17] Therefore, we aimed to evaluate the effects of TAK-242 on IL1-β, IL-6, TNF-α, and TLR4 expression levels and two important proteins of the NF-κB signaling pathway (Ikβα and pIkβα) in RA and trauma FLSs.

MATERIALS AND METHODS

Subjects

Synovial tissues were collected from RA and trauma patients who were undergoing joint replacement or synovectomy. We had two groups of patients, 10 RA patients during knee replacement surgery (4 men and 6 women) and 10 trauma individuals mostly due to sports trauma and during arthroscopy for ligament reconstructions or meniscus surgeries (4 men and 6 women) all with Iranian ethnicity. The mean age of RA and trauma patients were 57.44±11.45 and 51.7±12.73, respectively. Tissue samples are collected from Shariati and Laleh hospitals, Tehran, Iran. RA patients were age-, gender-, and ethnicity-matched with trauma patients. RA patients were end-stage and diagnosed according to the 2010 revised criteria of the American College of Rheumatology.[30] All the participants signed the informed consent to participate in our survey. The study was approved by the ethics committee of Iran University of Medical Sciences (IR.IUMS.FMD.REC.1398.123) and Tehran University of Medical Sciences (IR.TUMS.VCR.REC.1397.037). Smoker RA subjects were excluded from the study, and the control individuals had no history of autoimmune, rheumatologic, and cancer diseases, or glucocorticoid medications.

FLS Isolation and Cell Culture

The synovial tissues of RA and trauma patients were obtained, transferred to our lab, and washed with phosphate-buffered saline (PBS, Gibco Invitrogen, USA), 70% ethanol, and PBS containing 1% penicillin-streptomycin. The tissues were sectioned into pieces in a microplate containing Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Biosera, France) and 1% penicillin-streptomycin (Biosera, France). Then, for tissue digestion, the collagenase VIII (1 mg/mL, Sigma–Aldrich, USA) was added into a 50 mL conical centrifuge tube (SPL, Life Sciences, Korea) containing the dissected synovial tissues and incubated in a water bath at 37°C for 80 min. During the incubation time, the tissues were vortexed and resuspended 2-3 times. After incubation time, the digested tissues were centrifuged, the supernatant was discarded, and the cell pellet was resuspended with 1 mL of complete DMEM and transferred into two T25 flasks (SPL, Life Sciences, Korea) contained 4 mL of complete DMEM media. The T25 flasks were transferred in a 37°C, 5% CO2 incubator. Culture media were replaced with fresh media every other day. When the confluence reached 80%, the cells were sub-cultured into two T75 flasks (SPL, Life Sciences, Korea). After three passages, a homogenous population of FLSs was obtained. Thus, the third to the sixth passage of FLS cells were used for subsequent evaluation and intervention.

Immunocytochemistry (ICC) and Flow Cytometry

ICC was conducted to confirm that the isolated cells have a fibroblastic origin. Firstly, 5×10^4 cells of FLS cells were seeded into a 24-well plate containing DMEM medium supplemented with 20% FBS and incubated for 24 h. The media was discarded, and the cells were washed with PBS. Cultured FLSs were

incubated with cold methanol for 5 min to fix the cells. Then, the cells were washed with PBS and incubated with a blocking agent, phosphate-buffered saline with Triton-X100 contain 1% BSA, for 1 h on the shaker. Then, the cells were incubated with the primary antibody, anti-fibroblast surface protein antibody (ab11333, Abcam, UK) overnight at 4°C. Next, the cells were incubated with secondary antibody, sheep anti-mouse Ig (human Ig absorb)-FITC conjugated (Ibn Sina, ARI2011F, Iran), for 1 h at room temperature in a dark place. To counterstaining nuclei, the 4′, 6-diamidino-2-phenylindole (DAPI) was used. Finally, an inverted fluorescence microscope was used for the assessment of the stained FLSs.

For further confirmation of fibroblast as FLSs, some of the important surface CD markers of the cells were evaluated by the flow cytometry technique. For this purpose, FLSs were collected and washed with PBS three times. Then, FLSs were incubated with the fluorescein isothiocyanate (FITC)-conjugated antibodies against some of the surface CD markers at 37°C for 1 h. The surface CD markers were CD13, CD44, CD68, and CD90.[31,32] The antibodies used included anti-CD13 antibody (ab227663), anti-CD44 antibody (ab6124), anti-CD68 antibody (ab31630), and anti-CD90 antibody (ab225), which were all purchased from Abcam Inc. (Cambridge, UK). Unlabeled cells were used in other FACS assays as negative controls.

MTT Assay

RA FLS cells (7.5×10^3 cells per well) were seeded into a 48-well plate in 500 µL of complete DMEM medium and incubated overnight. Next, the medium was discarded, and the fresh medium was replaced. Cells were treated with various concentrations of TAK-242 (Resatorvid, Cayman, Michigan, USA) (16, 32, and 48 µM) for 24 h. Then, 50 µL of MTT [3-(4, 5-Dimethylthiazol-2-yl) -2, 5-Diphenyltetrazolium Bromide] assay solution (5 mg/mL) was added into each well and incubated in a 37°C, 5% CO2 incubator for 4 h. After the incubation period, the medium containing MTT was discarded then, 500 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) was added to each well and shaken for 15 min to solubilize formazan crystals. A microplate reader (Bio-Tek, USA) was used to measure optical density (OD) at a wavelength of 570 nm. MTT was obtained from Sigma-Aldrich (St. Louis, MO, USA). All experiments were triplicate.

FLS Cell Grouping and Intervention

Firstly, FLS cells with 80% confluency were trypsinized and harvested, then seeded into a T25 flask (1.5×10^5 cells). Each FLS sample was divided into 3 experimental groups: 1) control (no treatment), 2) LPS (100 ng/mL), 3) TAK-242 (32 µM) + LPS (100 ng/mL). The last group was pretreated with TAK-242 for 1 h. Then, groups' numbers 2 and 3 were treated with 100 ng/mL of LPS and incubated in a 37°C, 5% CO2 incubator for 6 h. Finally, cells were harvested by trypsinization and lysed for molecular assessments such as gene expression and western blot analysis.

RNA Extraction and qPCR

Total RNA was extracted from all three groups of harvested cells using the RNA extraction kit (SinaColon Co., Tehran, Iran) according to the manufacturer's instructions. Next, the purity and yield of total RNA were evaluated by NanoDrop spectrophotometer (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA). The extracted RNA samples were stored at -80ºC for the next molecular evaluation. The RNA reverse transcription kit (RT-ROSET, ROJE, Iran) was used for cDNA synthesis. The PCR reaction for cDNA synthesis consisted of 300 ng template RNA, 10 µL RT mix (2x), 2 µL MixZyme, and RT-PCR Grade water up to 20 µL.

The PCR conditions for cDNA synthesis were as follows: 10 min at 25°C, 60 min at 47°C, and 5 min at 85°C. Next, 50 ng of cDNA was used for real-time RT-PCR. The qPCR reaction (17 µL) consisted of 7.5 µL Real Q Plus 2x Master Mix Green (AMPLIQON, Denmark), 4.5 µL distilled water, 3 µL cDNA template, and 1 µL each of reverse and forward primers. The qPCR reaction conditions were as follows; stage one; 95ºC for 15 min, stage two; 40 cycles of 95ºC for 15s, 60ºC for 1 min. The Applied Biosystems StepOnePlus Real-Time PCR System (Foster City, CA, USA) was used for real-time PCR. The primers and their sequences will be provided upon request.

The $2^{-\Delta\Delta CT}$ formula was used to measure the relative mRNA level of the genes.[33] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was used to normalize the RNA levels of the genes.

Protein Extraction and Western Blot

Western blot analysis was carried out to evaluate the Ikβα and pIkβα protein levels. After 6 h of treatment

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with TAK-242 and LPS, the cells were washed with ice-cold PBS, and the radioimmunoprecipitation assay (RIPA) lysis buffer containing complete EDTA-free protease inhibitor cocktail tablets (Roche, Germany) was used to extract total proteins according to the manufacturer's instructions. The Lowry method was used to measure the protein concentrations. Protein lysates from various experimental groups of the cells were prepared. Then, an equal amount of protein extracts (50 µg) was used to separate them by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 90 V for 2.5 h. Then, the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Scientific, USA) at 100 V for 90 min. To block free spaces, the membrane was incubated with 5% skim milk (Sigma-Aldrich, USA) in 1 X Tris-buffered saline with Tween (TBST) for 1h at room temperature. Next, the membrane was incubated with primary antibodies (anti-Ikβα (ab97783, 1:1000), anti pIkβα (#2859, 1:1000), and anti-βactin (ab8226, 1:1000), Abcam, UK) at 4˚C for overnight. The unbounded antibodies were deleted after washes with 1X TBST. Next, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (PZ5610, 1:3000) for 2 h at room temperature. The blots were washed again, and the protein bands were visualized with enhanced chemiluminescence detection reagent (ECL, GE Healthcare, USA). The results were semi quantified by Image J software (NIH, USA) and normalized with β-actin as an internal control. The experiment for every protein detection was repeated two times.

Statistical Analysis

SPSS (version 26.0, SPSS Inc.) was used to analyze our results, and for design our graphs, the GraphPad Prism software (Version 8.0 for Windows) was used. Firstly, our data were evaluated for normality through the normality test. Our data were not normally distributed (Kolmogorov-Smirnov<0.05). The Friedman was applied for comparison among multiple paired groups. The Mann Whitney and Wilcoxon tests were used for comparison between two unpaired and paired groups, respectively. Data are represented as mean±standard error of the mean (SEM) and *p* values lower than 0.05 ($p<0.05$) were considered statistically significant.

RESULTS

Identification of FLSs

Firstly, we showed that the isolated cells are FLS. For this purpose, the microscopic evaluation showed a spindle-shaped morphology, which is following the normal morphological features of FLSs (Data not shown). After the third passage, ICC was performed to confirm that the fibroblastic origin of isolated cells, and the results illustrated a homogeneous population of fibroblasts (Figure 1). For absolute confirmation, the cells were evaluated through flow cytometry by some important surface CD markers. Flow cytometry data represent high expression of CD13 (97.14%±2.06%), CD44 (99.12%±2.21%), and CD90 (94.67%±3.7%) but a rare expression of surface marker CD68 (0.23%±4.01%) (Figure 2). Flow cytometric data indicated that CD13, CD44, and CD90 were stained

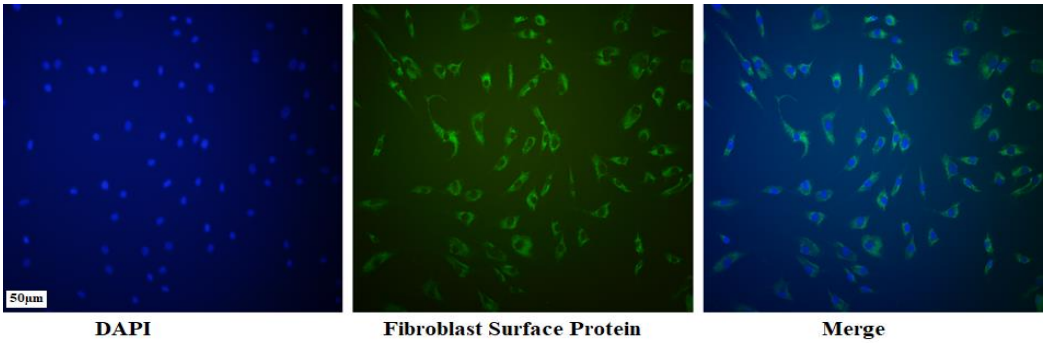

Figure 1. Immunocytochemistry (ICC) identification of fibroblast-like synoviocytes (FLS); using the fibroblast surface marker expression. To counterstain nuclei, the 4′, 6-diamidino-2-phenylindole (DAPI, blue color) was used. The primary antibody was the anti-fibroblast surface protein antibody (ab11333). The secondary antibody was sheep anti-mouse Ig (human Ig absorb)-FITC conjugated (Ibn Sina, ARI2011F) (green color).

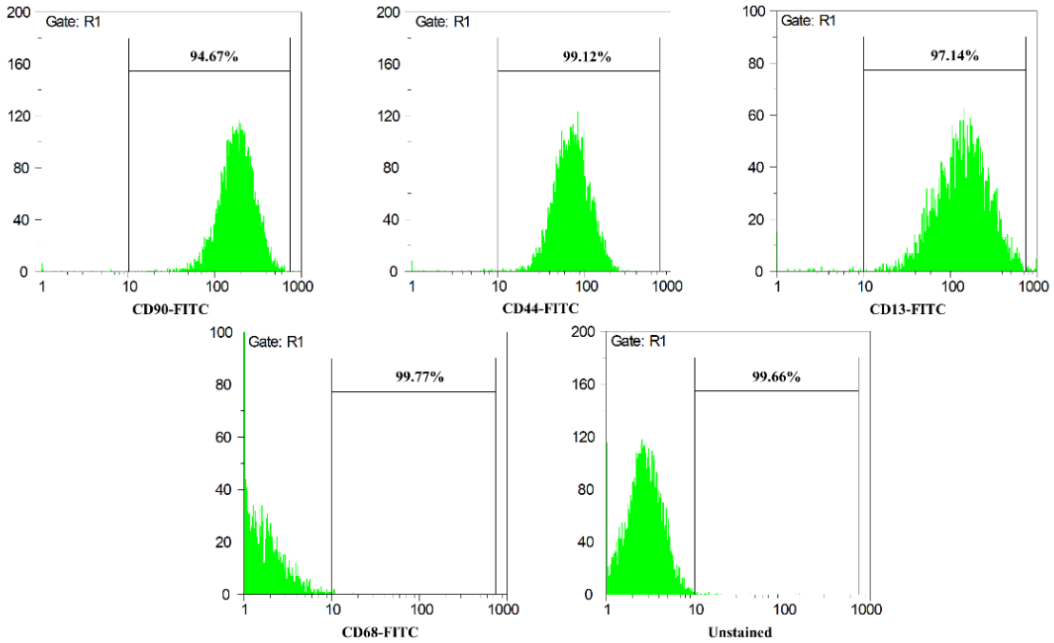

Figure 2. Flow cytometry for detection of fibroblast-like synoviocytes (FLS) surface markers (CD13, CD44, CD68, and CD90). Flow cytometric data indicated that CD13, CD44, and CD90 were stained positive while CD68 was stained negative, suggesting the successful separation of FLSs from synovial tissues.

positive while CD68 was stained negative, suggesting the successful separation of FLSs from synovial tissues. Altogether, our experiments documented that the isolated cells are FLS, and the third to the sixth passages were used for subsequent experiments.

Effect of TAK-242 on Viability of the Cells

To test the TAK-242 effects on the viability of RA FLSs, different concentrations of TAK-242 (16, 32, and 48 µM) were added to the cells, and an MTT assay was carried out to measure the cytotoxic effects of TAK-242 for the cells at 24 h (Figure 3). As TAK-242 concentration increased, the survival rate of the cells decreased at 24 hours. The high concentration of TAK-242 (48 µM) for 24 hours, reduced the FLS number to about half of the control group. Our data shows that TAK-242 has no cytotoxic effects for FLS at 32 µM until 24 h.

Effect of TAK-242 on Expression of Inflammatory-related Genes in LPS-stimulated FLSs

We aimed to determine the therapeutic effects of TAK-242 on FLSs. To achieve this goal, RA and healthy FLSs were pretreated with TAK-242 (32 µM) in culture media for 1 h, then stimulated by LPS (100 ng/mL) for 6 h. First, the baseline expression of the *IL1-β, IL-6, TNF-α,* and *TLR4* genes was measured and compared between healthy and RA FLSs (Figure 4). Baseline expression of *IL1-β*, *IL-6*, *TNF*, and *TLR4* showed no significant differences between RA and healthy FLS cells (p=0.43, p=0.82, p=0.32, and p=0.39 respectively). Moreover, the effect of TAK-242 on the expression of the proinflammatory cytokines and TLR4 in LPS-stimulated FLSs was evaluated (Figure 5). FLS treatment with LPS revealed a significant increase in the mRNA levels of *IL1-β, IL-6*, *TNF-α,* and *TLR4* in both the healthy FLSs (p=0.004, p=0.008, p=0.02, and p=0.004 respectively) and RA FLSs (p=0.001, p=0.002, p=0.005, and p=0.004 respectively) compared with that of their control groups. On the other hand, pretreatment with TAK-242 could significantly decrease expression of *IL1-β, IL-6*, *TNF-α*, and *TLR4* in both the healthy FLSs (p=0.004, p=0.002, p=0.008, and p=0.004, respectively) and RA FLSs (p=0.002, p=0.002, p=0.002, and p=0.004 respectively) compared with that of their LPS groups. Altogether, our data indicate that TAK-242 can suppress inflammatory responses by downregulating the expression of the proinflammatory cytokines in LPS-stimulated FLS cells.

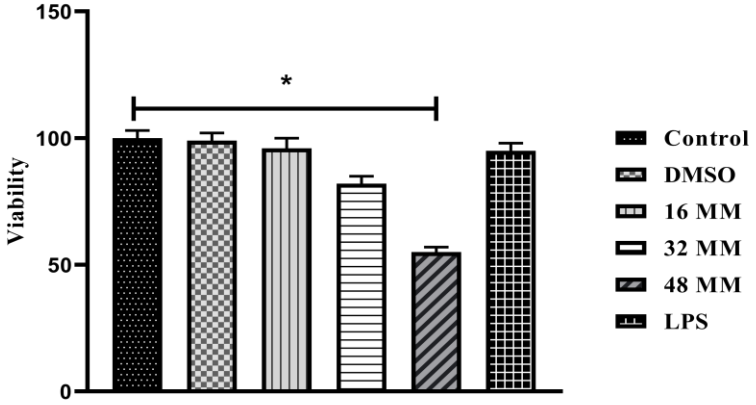

Figure 3. The effects of TAK-242 on the viability of rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs). Cytotoxic effects were evaluated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) assay after treatment with different concentrations of TAK-242 (16, 32, and 48 µM) for 24 h. TAK-242 was dissolved in Dimethyl sulfoxide (DMSO) and the DMSO was used as a control group to exclude the DMSO effects on the cell viability. We treated our cells with Lipopolysaccharide (LPS) to evaluate the TAK-242 effects. Therefore, the LPS group was used as a group to investigate the LPS effect on the viability of the cells. Data are shown as mean±SEM of three independent experiments (* p<0.05).

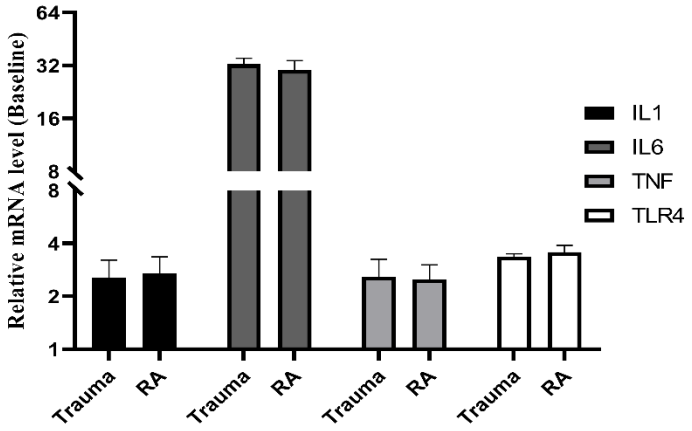

Figure 4. Baseline expression of the interleukin (*IL)1-β, IL-6, TNF-α,* and *toll-like receptor 4 (TLR4)* in rheumatoid arthritis (RA) and healthy fibroblast-like synoviocytes (FLSs). Baseline expression of *IL1-β, IL-6, TNF-α,* and *TLR4* showed no significant differences between RA and healthy FLS cells (p=0.43, p=0.82, p=0.32, and p=0.39 respectively).

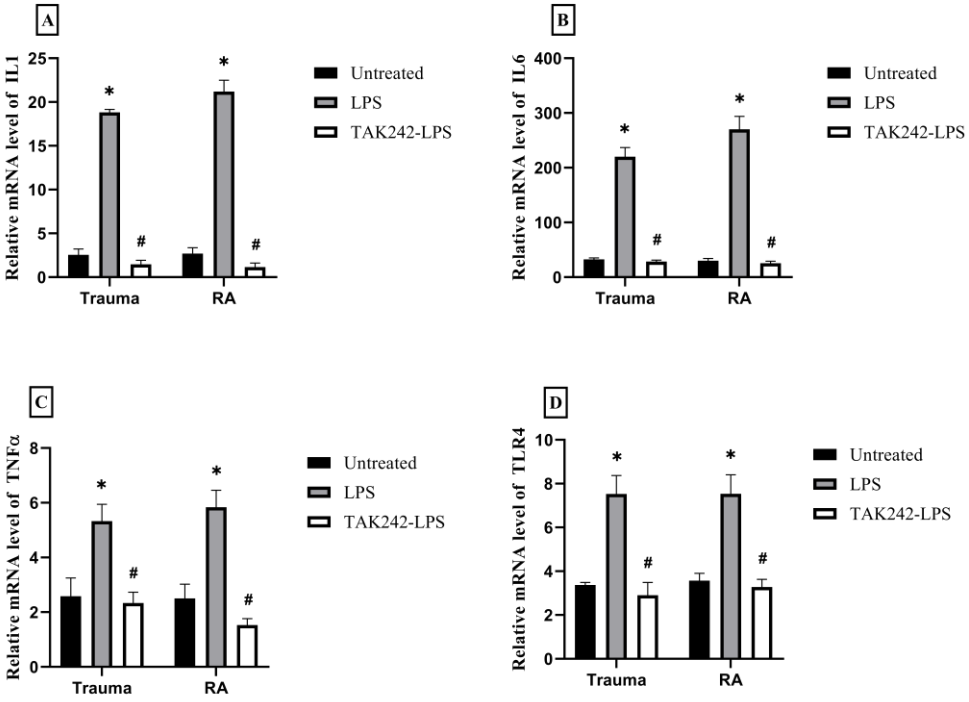

Figure 5. Effect of TAK-242 on the expression of the proinflammatory cytokines and *toll-like receptor 4 (TLR4)* in Lipopolysaccharide (LPS)-stimulated fibroblast-like synoviocytes (FLSs): A. FLS treatment with LPS revealed a significant increase in mRNA levels of interleukin (*IL)-1β* in both the healthy FLSs (*p*=0.004, *FC*=7.36) and rheumatoid arthritis (RA) FLSs (*p*=0.001, *FC*=7.84) compared with that of their control groups. Pretreatment with TAK-242 could significantly decrease expression of the *IL-1β* in both the healthy FLSs (*p*=0.004, *FC*=0.08) and RA FLSs (*p*=0.002, *FC*=0.05) compared with that of their LPS groups. B. FLS treatment with LPS revealed a significant increase in mRNA levels of *IL-6* in both the healthy FLSs (*p*=0.008, *FC*=6.76) and RA FLSs (*p*=0.002, *FC*=8.97) compared with that of their control groups. Pretreatment with TAK-242 could significantly decrease expression of the *IL-6* in both the healthy FLSs (*p*=0.002, *FC*=0.13) and RA FLSs (*p*=0.002, *FC*=0.09) compared with that of their LPS groups. C. FLS treatment with LPS revealed a significant increase in mRNA levels of *TNF-α* in both the healthy FLSs (*p*=0.02, *FC*=2.07) and RA FLSs (*p*=0.005, *FC*=2.34) compared with that of their control groups. Pretreatment with TAK-242 could significantly decrease expression of the *TNF-α* in both the healthy FLSs (*p*=0.008, *FC*=0.44) and RA FLSs (*p*=0.002, *FC*=0.26) compared with that of their LPS groups. D. FLS treatment with LPS revealed a significant increase in mRNA levels of *TLR4* in both the healthy FLSs (*p*=0.004, *FC*=2.23) and RA FLSs (*p*=0.004, *FC*=2.11) compared with that of their control groups. Pretreatment with TAK-242 could significantly decrease expression of the *TLR4* in both the healthy FLSs (*p*=0.004, *FC*=0.39) and RA FLSs (*p*=0.004, *FC*=0.44) compared with that of their LPS groups. *$p < 0.05$ vs. untreated group, #$p<0.05$ vs. FLSs treated with LPS. FC; fold change

Effect of TAK-242 on TLR4/NF-κB Signaling Pathway in LPS-stimulated FLSs

To understand the molecular mechanisms involved in TAK-242 anti-inflammatory effects, we evaluated some of the important proteins with critical roles in TLR4/NF-κB signaling pathway. Thus, FLSs of both groups were pretreated with TAK-242, and then stimulated with LPS. Western blot was carried out to evaluate protein levels of IκBα and pIκBα (Figure 6).

Although protein levels of IκBα and pIκBα were higher in RA FLSs, compared with that of the healthy FLSs, our analysis showed no significant differences between RA and healthy FLSs (p=0.44 and p=0.86 respectively). LPS-stimulated FLSs showed a significant increase in pIkβα in both the healthy FLSs (p=0.03) and RA FLSs (p=0.02) compared with that of their control group. On the other hand, pretreatment with TAK-242 revealed a significant decrease in pIkβα

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in both the healthy FLSs (*p*=0.01) and RA FLSs (*p*=0.01) compared with that of their LPS groups. Indeed, TAK-242 was able to inhibit the LPS-induced phosphorylation of IκBα. These data are suggesting that TAK-242 could downregulate the expression level of the proinflammatory mediators in LPS-stimulated FLS cells by inhibiting TLR4/NF-κB pathway in FLSs.

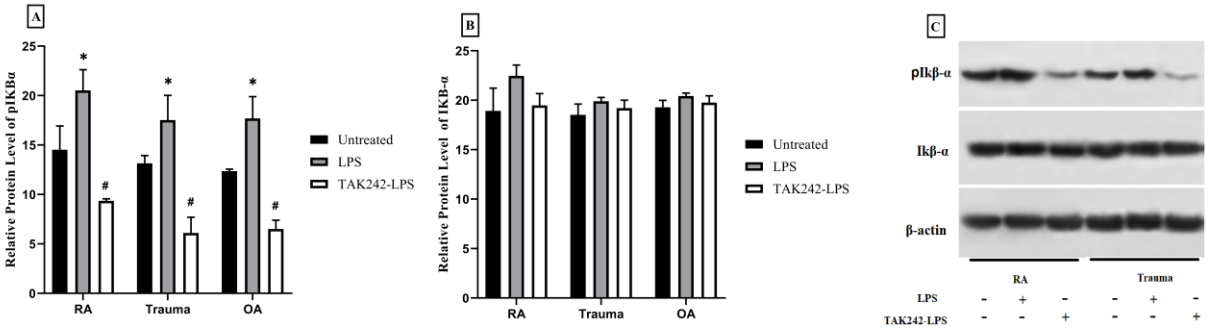

Figure 6. Effect of TAK-242 on protein levels of IκBα and pIκBα in Lipopolysaccharide (LPS)-stimulated fibroblast-like synoviocytes (FLSs): 6A. Although the protein level of pIκBα was higher in rheumatoid arthritis (RA) FLSs, compared with that of the healthy FLS, our analysis showed no significant differences between RA and healthy FLSs (*p*=0.86). FLS treatment with LPS showed a significant increase in protein levels of pIκBα in both the healthy FLSs (*p*=0.03, *FC*=1.3) and RA FLSs (*p*=0.02, *FC*=1.4) compared with that of their control groups. On the other hand, pretreatment with TAK-242 could significantly decrease protein levels of pIκBα in both the healthy FLSs (*p*=0.01, *FC*=0.34) and RA FLSs (*p*=0.01, *FC*=0.45) compared with that of their control groups. 6B. Although the protein level of IκBα was higher in RA FLSs, compared with that of the healthy FLS, our analysis showed no significant differences between RA and healthy FLSs (*p*=0.44). 6C. IκBα and pIκBα protein levels were detected by specific antibodies in LPS, LPS+TAK-242, and untreated groups and β-actin was used as an internal control by western blot. **p*<0.05 vs. untreated group, #*p*<0.05 vs. FLSs treated with LPS.FC; fold change.

DISCUSSION

The therapeutic effect of TAK-242 was evaluated on a mouse model of sepsis and showed promising results in decreasing production of cytokine levels and also improved their survival, which makes the TAK-242 an efficient therapeutic agent for sepsis.[34] Moreover, the therapeutic effect of TAK-242 was evaluated in the RA FLS line MH7A, primary FLS, and adjuvant-induced arthritis (AIA) rat model. According to their results, they concluded that the TAK-242 shows potential for cost-effective development as a remedy for rheumatoid arthritis or to control the progression of RA[29] Nonetheless, it could not pass the human clinical trial in phase III due to its failure in alleviating the sepsis symptoms especially cytokine storm.[35] Indeed, due to TLR4 importance, many TLR4 inhibitors are introduced and showed successful results in preclinical trials, while their clinical successes were not satisfactory.[36] Therefore, to modulate the immune responses through TLR4 signaling in inflammatory diseases such as RA the TLR4-mediated immune responses need to be more elucidated.

Mounting evidence exists for the despicable role of the aberrant immune response in the pathogenesis of rheumatoid arthritis (RA), where TLR4 can activate synovial fibroblasts that lead to chronic inflammation and bone and cartilage destruction, thus making TLR4 a potent drug target in RA.[37,38] TLR4-antagonizing peptides were able to inhibit in vitro and in vivo inflammatory responses. Furthermore, it was documented that TLR4-antagonizing peptides were able to alleviate inflammatory symptoms and synovial tissue destruction in the RA rat model. Cumulatively, they suggest that the TLR4-antagonizing peptides hold strong promise and could be of great value in curbing TLR-related immune complications including RA.[39] The body of evidence indicating that TLR signaling pathways are involved in RA progression, especially in the late stages of the disease. Investigation in this era documented that the TLR4 signaling pathway is more important than other TLR signaling pathways in RA

pathogenesis and progression.[40] Thus, in our study, inhibitory effects of TAK-242, TLR4 inhibitor, were evaluated to find a novel potential therapeutic agent for RA patients. In our study, we could not find any significant differences in the expression of *IL1-β, IL-6, TNF-α,* and *TLR4* between trauma and RA patients. Our RA patients were at the end-stage, and they received immunosuppressive drugs, and this could be the reason that we did not find any significant differences at the baseline level. We revealed that TAK-242 could downregulate the expression levels of *IL1-β, IL-6, TNF-α,* and *TLR4* genes in the LPS stimulated FLSs in both the RA and healthy groups. Indeed, we illustrated the inhibitory effects of TAK-242 on the expression levels of the proinflammatory mediators and *TLR4* in the LPS stimulated FLSs. Samar pita and their colleagues.[29] reported that TAK-242 dose dependently could inhibit the increased expression of IL-6, IL-8, MMP-1, and VEGF in LPS stimulated MH7A cells, which is consistent with our study. Possible molecular mechanism TAK-242 in suppressing the expression of the genes is inhibiting of NF-κB translocation from the cytosol into the nucleus.[(41)] Indeed, NF-κB is an important downstream signal transducer of TLR4 and could be activated by TLR4 ligands. NF-κB is trapped by its binding to IκB molecules in the cytoplasm and once the IκB is phosphorylated and degraded the NF-κB subunits, p50 and p65, are allowed to enter the nucleus and activate target genes.[42] To find out the participation of the TLR4/NF-κB signaling pathway in the gene expression modulations by TAK-242, we measured the protein levels of the TLR4/NF-κB signaling pathway, IκBα, and pIκBα, in the absence or presence of TAK-242. We illustrated that LPS increases phosphorylation levels of IκBα (pIκBα) and activate the NF-κB signaling pathway and thereby increases the gene expression in the LPS stimulated FLSs. On the contrary, TAK-242 inhibits phosphorylation levels of IκBα and prohibits the translocation of NF-κB subunits into the nucleus and there by downregulates the gene expression in the TAK-242 pretreatment FLSs. In addition, it is evident that theTLR4/NF-κB pathway is implicated in chronic inflammation and bone and cartilage destruction in RA disease[43] These data provide knowledge that TAK-242 could effectively inhibit the NF-κB pathway, the important pathway in the induction and progression of arthritis. Indeed, TAK-242 inhibited the mobilization of NF-κB into nuclei, which is similar to the Samar pita and their colleagues' study.[29]

Collectively, we provide the data that TAK-242 through inhibiting the NF-κB signaling pathway may modulate TLR4-mediated inflammatory responses in the RA FLSs and could be considered as a potential therapeutic agent for RA patients. Due to the unresponsiveness of some RA patients to conventional and biological drugs, TLR4 inhibitors, especially TAK-242, could be an option to control inflammatory settings and related complications in RA disease. More studies on the effect of TAK-242 and other TLR4 inhibitors in RA FLSs are required to develop a cost-effective medication for RA patients. We had limitations such as lack of access to synovial tissues of patients and also lack of access to synovial tissues of RA patients with different stages to compare gene expression between stages.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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