Allergologia et immunopathologia

Sociedad Española de Inmunología Clínica, Alergología y Asma Pediátrica

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ORIGINAL ARTICLE

OPEN ACCESS

DUOX1 inhibits the progression of rheumatoid arthritis by regulating the NF-κB pathway in vitro

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Received 19 December 2024; Accepted 6 February 2025
Available online 1 March 2025

KEYWORDS
rheumatoid arthritis;
DUOX1;
NF-κB;
inflammation;
oxidative stress;
fibroblast-like synoviocytes

Abstract

Background: This study investigates the role of dual oxidase 2 (DUOX1) in fibroblast-like synoviocytes associated with rheumatoid arthritis (RA) and to elucidate its potential mechanism of action.

Method: The anti-inflammatory effects of DUOX1 were assessed using IL-1β (interleukin-1 beta)-stimulated synovial fibroblasts (MH7A). Cell viability and migration were evaluated using the Cell Counting Kit-8 and Transwell assays, respectively. Enzyme-linked immunosorbent assay (ELISA) was performed to measure cellular inflammatory factor levels, and immunofluorescence and specific kits were used to assess reactive oxygen species (ROS) production and redox indicators. Western blotting was performed to confirm the antiarthritic mechanism of DUOX1.

Result: The findings revealed that the stimulation if IL-1β downregulates DUOX1 expression in MH7A cells, leading to increased proliferation, migration, inflammatory responses, and oxidative stress. Conversely, DUOX1 overexpression increased the production of IL-1β inducing excessive proliferation, migration, inflammation, and oxidative stress in MH7A cells, and inhibited the activation of the nuclear factor kappa B (NF-κB) inflammatory pathway.

Conclusion: DUOX1 significantly suppresses the proliferation, migration, inflammation, and oxidative stress of RA synovial cells through the inhibition of the NF-κB signaling pathway.

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https://doi.org/10.15586/aei.v53i2.1293

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovitis, prolonged inflammation, and synovial membrane hyperplasia, ultimately leading to cartilage degradation and joint bone erosion.[1] The key pathological features of RA include angiogenesis and irreversible joint destruction, both driven by the excessive proliferation of synovial cells, whilr also contributing to the global prevalence of RA with rising incidence and disability rates reported in many regions.[2] The progression of RA is characterized by the activation and invasive behavior of synovial cells, resulting in bone erosion and cartilage damage. Clinically, patients frequently experience chronic joint pain, swelling, and stiffness. In advanced cases, these localized symptoms can extend to systemic complications, affecting the cardiovascular and pulmonary systems and other organs.[3,4] It is reported that inflammatory and oxidative factors, along with activated fibroblast-like synoviocytes, play a pivotal role in the pathogenesis of RA, particularly during bone degradation and synovitis. Oxidative stress, characterized by an overproduction of free radicals, depletes antioxidant defenses, thereby accelerating bone destruction.[5] Despite advances made in understanding RA, its precise pathophysiology remains unclear, necessitating further research to uncover mechanisms that could inform clinical management and therapeutic development.

Dual oxidase 2 (DUOX1), a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family, is widely expressed across various tissues, playing a role in diverse cellular functions, including host defense, acid generation, fluid homeostasis, mucin regulation, and cell death.[6] In macrophages, DUOX1 contributes to type 2 inflammation and mucus metaplasia observed in allergic respiratory conditions.[7] Similarly, in virus-infected respiratory epithelial cells, DUOX1 regulates processes that promote neutrophil recruitment and activation.[8] Diet-induced obesity increases DUOX1 activity, thereby exacerbating allergen-induced type 2/17 respiratory inflammation.[9] Additionally, DUOX1 knockdown has been found to enhance wound healing by modulating reactive oxygen species (ROS) levels and inhibiting NF-κB (nuclear factor kappa B) signaling.[10] In another context, vitamin D alleviates hypoxia-induced oxidative stress in primary rat neurons by downregulating DUOX1 expression.[11] DUOX1-induced oxidative stress is also implicated in the pathogenesis of heart failure through the upregulation of caspase-1-dependent pyroptosis.[12] In RA, a bioinformatics analysis identified lower expression of DUOX1 as a differentially expressed gene, suggesting that DUOX1 may play a role in slowing disease progression.[13] Despite these findings, detailed investigations into the specific mechanisms of DUOX1 in RA remain limited.

The aim of this study was to investigate the role and underlying mechanisms of DUOX1 in modulating proliferation, migration, inflammation, and oxidative stress in IL-1β (interleukin-1 beta)-stimulated fibroblast-like synoviocytes to enhance understanding of DUOX1's functions and highlight its potential as a therapeutic target for RA treatment.

Methods

Cell culture

The human RA fibroblast-like synoviocyte cell line MH7A was obtained from the American Type Culture Collection (ATCC). Briefly, the cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 (carbon dioxide), using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To simulate RA conditions, MH7A cells were treated with IL-1β 10 mg/L (Sigma-Aldrich Corp., St. Louis, MO, USA) for 24 hours.[14]

Adenovirus infection

In vitro, the human DUOX1 gene was amplified by PCR (polymerase chain reaction) using the pcDNA3.1 vector as a template and subsequently cloned into a linearized adenoviral vector using the Adeno-X Adenoviral System 3 kit. The recombinant plasmid was verified through gene sequencing and DNA (deoxyribonucleic acid) electrophoresis before being prepared for packaging. MH7A cells were then transfected with the recombinant plasmid, following which AdenoX-DUOX1 (ad-DUOX1)-infected MH7A cells were used to evaluate the infection efficiency. AdenoX-LacZ (ad-NC) provided by the kit was used as a negative control. Western blot analysis was performed to determine DUOX1 expression in each cell line.

Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's instructions. The CCK-8 solution was added to the cultured MH7A cells, which were then incubated at 37°C for 2 hours. Absorbance was measured at 450 nm using a microplate reader to determine cell viability.

Transwell assay

The migratory capacity of transfected MH7A cells was evaluated using transwell chambers coated with Matrigel and equipped with 8 μm porous membranes (Biotechnology, Shanghai, China). According to the manufacturer's instructions, transfected MH7A cells (4×10^5 cells/mL) were seeded into the upper chamber of the transwell device, whereas the lower chamber was filled with a medium containing 10% FBS as a chemoattractant. After 48 hours of incubation, nonmigrated cells on the upper surface of the membrane were removed using a cotton swab and repeatedly rinsed with phosphate-buffered saline (PBS). Migrated cells on the lower surface were fixed with 4% paraformaldehyde for 20 minutes and stained with 0.2% crystal violet for 15 minutes (Biotechnology, Shanghai, China). Images were captured, and cell migration was quantified using an inverted microscope (Nikon, Tokyo, Japan).

Enzyme-linked immunosorbent assay

The secretion levels of interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor alpha (TNF-α) were assessed using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Zhuocai Biotechnology Co. Ltd., Shanghai, China) following the manufacturer's instructions.

Reactive oxygen species detection

Intracellular ROS levels were measured using DCFH-DA (dichlorodihydrofluorescein diacetate) (Sigma-Aldrich, MO, USA). MH7A cells were incubated with 5 μM DCFH-DA in DMEM for 30 minutes. After incubation, the cells were washed twice withPBS and collected. Fluorescence intensity was quantified using a fluorescence microscope (1X71) (Olympus Corp., Tokyo, Japan).

Oxidative markers analysis

The levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) in MH7A cells were determined using commercially available detection kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's protocols.

Real-time quantitative PCR

Total RNA (ribonucleic acid) was extracted from MH7A cells using TRIzol® reagent (Thermo Fisher Scientific, Inc., MA, USA). Complementary DNA (cDNA) synthesis and quantitative PCR (qPCR) were performed using the SYBR Premix Ex Taq™ II Kit (Takara Bio Inc., Shiga, Japan) following the manufacturer's guidelines. The qPCR reactions were conducted using the following thermal cycling parameters: an initial denaturation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 5 seconds, 60°C for 30 seconds, and 70°C for 60 seconds. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method[15] on an ABI 7500 PCR machine (Applied Biosystems; Thermo Fisher Scientific Inc., MA, USA). The primer sequences used were:

DUOX1, F: 5′-AACCCTACCTGCCTAACCC-3′,
R: 5'-CTGTCCAGTGCTGCGGTC-3';
GAPDH, F: 5′-GGAGTCTACTGGCGTCTTCAC-3′,
R: 5'-ATGAGCCCTTCCACGATGC-3'.

Western blotting

MH7A cell lysates were prepared using the RIPA (radioimmunoprecipitation assay) buffer and protein concentrations were quantified with a BCA (bicinchoninic acid assay) kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The total number of proteins were separated using 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto PVDF (polyvinylidene fluoride) membranes, which were then blocked with 5% skimmed milk at room temperature for 1 hour and incubated with the following primary antibodies: anti-DUOX1 (1:1000; cat. no. ab230209; Abcam), anti-IκB-α (1:1000; cat. no. ab230341; Abcam), anti-p-IκB-α (1:1000; cat. no. ab154090; Abcam), anti-p65 (1:500; cat. no. ab16502; Abcam), anti-p-p65 (1:1000; cat. no. ab86299; Abcam), and anti-GAPDH (1:1000; cat. no. ab8245; Abcam). Next, the membranes were treated with an HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG (immunoglobulin G) secondary antibody (1:5000; cat. no. BA1054) (Boster Biological Technology, Pleasanton, CA, USA). The protein bands were visualized using ECL (Enhanced Chemiluminescence) reagents (Affinity Biosciences, Cincinnati, OH, USA) on a Bio-Rad gel imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA) and band intensities were analyzed using Image Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

Statistical analysis

The data are presented as the mean ± standard deviation (SD), with all the experiments performed in triplicate. Statistical analyses were conducted using GraphPad Prism 7.0 software (San Diego, CA, USA). Differences between two groups were analyzed using the t-test and one-way analysis of variance (ANOVA) was employed for comparisons among multiple groups. A *P*-value of < 0.05 was considered statistically significant.

Results

DUOX1 is lowly expressed in rheumatoid arthritis fibroblast-like synoviocytes

To determine whether DUOX1 directly influences RA inflammation in vitro, MH7A cells were stimulated with 10 ng/mL IL-1β for 24 hours. Western blot and qRT (quantitative reverse transcription)-PCR analyses revealed that IL-1β treatment significantly reduced DUOX1 expression in inflamed synoviocytes (Figure 1A and B). These findings suggest that DUOX1 may play a protective role in preventing the progression of RA.

Overexpression of DUOX1 inhibits MH7A cell viability and invasion

To further validate the role of DUOX1, we overexpressed DUOX1 in MH7A cells via the adenovirus infection. Western blot results confirmed the successful overexpression of DUOX1 (Figure 2A). The cell viability of MH7A cells was evaluated using the CCK-8 assay, which showed that IL-1β treatment significantly enhanced cell viability; however, DUOX1 overexpression markedly reduced the IL-1β-induced increase in cell viability compared to the empty vector group (Figure 2B).

Given the aggressive behavior of RA fibroblast-like synoviocytes, we assessed the invasive potential of MH7A cells. The number of invasive cells was significantly higher in IL-1β-treated MH7A cells compared to the control group. In addition, overexpression of DUOX1 reversed the IL-1β-induced increase in cell invasion (Figure 2C and D).

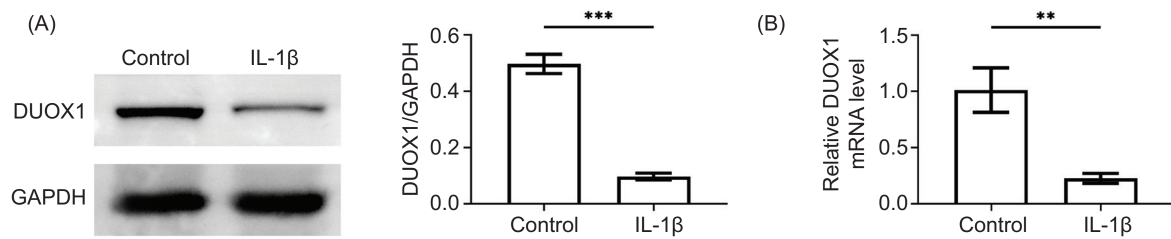

Figure 1 DUOX1 expression is reduced in rheumatoid arthritis fibroblast-like synoviocytes. (A) Protein expression of DUOX1 in MH7A cells. (B) mRNA expression of DUOX1 in MH7A cells. Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group (n = 3).

Figure 2 Overexpression of DUOX1 inhibits MH7A cell viability and migration. (A) Protein expression of DUOX1 in MH7A cells. (B) Cell viability assessed using the CCK-8 assay. (C) Quantification and representative images of cell migration. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (n = 3).

These results indicated that DUOX1 inhibits both cell viability and invasion in MH7A cells.

Overexpression of DUOX1 inhibits the inflammatory response of MH7A cells

The effects of DUOX1 on the inflammatory response in MH7A cells were evaluated by measuring the secretion levels of IL-6, TNF-α, and IL-8 using ELISA. IL-1β treatment significantly increased the levels of these inflammatory cytokines compared to the control group (Figure 3). DUOX1 overexpression, however, mitigated the elevated levels of IL-6, TNF-α, and IL-8 induced by IL-1β treatment. These findings suggest that DUOX1 reduces the inflammatory response in MH7A cells.

Overexpression of DUOX1 inhibits oxidative stress in MH7A cells

We further investigated the role of DUOX1 in oxidative stress. The results showed that IL-1β treatment reduced SOD and GSH-Px levels, increasing MDA and ROS levels in MH7A cells; however, DUOX1 overexpression reversed these effects, restoring SOD and GSH-Px levels and reducing MDA and ROS levels (Figure 4A and B). These results indicate that DUOX1 prevents oxidative damage and alleviates oxidative stress in MH7A cells.

DUOX1 inhibits the activation of the NF-κB signaling pathway

The NF-κB signaling pathway plays a pivotal role in RA-associated inflammation. To investigate whether DUOX1 affects this pathway, we measured the levels of proteins associated with NF-κB signaling in MH7A cells treated with IL-1β. IL-1β stimulation increased the levels of p-p65 and p-IκB-α, indicating NF-κB activation. However, overexpression of DUOX1 significantly reversed these increases, suggesting that DUOX1 inhibits the activation of the NF-κB signaling pathway (Figure 5).

Discussion

RA is a common synovial disease characterized by the invasion, activation, and inflammation of synovial cells, ultimately destroying bone and cartilage.[16] Results from numerous studies have proven that oxidative stress and inflammation are interconnected processes that significantly contribute to the pathophysiology of RA. For example, oxidative stress can result from the excessive accumulation of ROS, which is known to promote the proliferation of synovial cells, joint damage, and cartilage degradation. Additionally, inflammatory mediators can produce harmful free radicals, further exacerbating joint injury and cartilage erosion. This bidirectional relationship highlights oxidative stress as a critical driver of RA progression.[17,18]

Altered gene expression has also been identified as a key factor in RA pathogenesis.[19] Despite significant efforts to identify specific genes and pathways involved in RA, the functional roles and mechanisms of many candidate genes remain poorly understood. Among these, DUOX1 has recently emerged as a potential anti-inflammatory mediator in various diseases, including Alzheimer's disease, intestinal inflammation, and respiratory inflammation.[20-22] However, the precise role and mechanism of DUOX1 in RA have not been fully elucidated. In this study, we established an RA cell model by treating MH7A cells with IL-1β, which mimics the inflammatory microenvironment of RA.

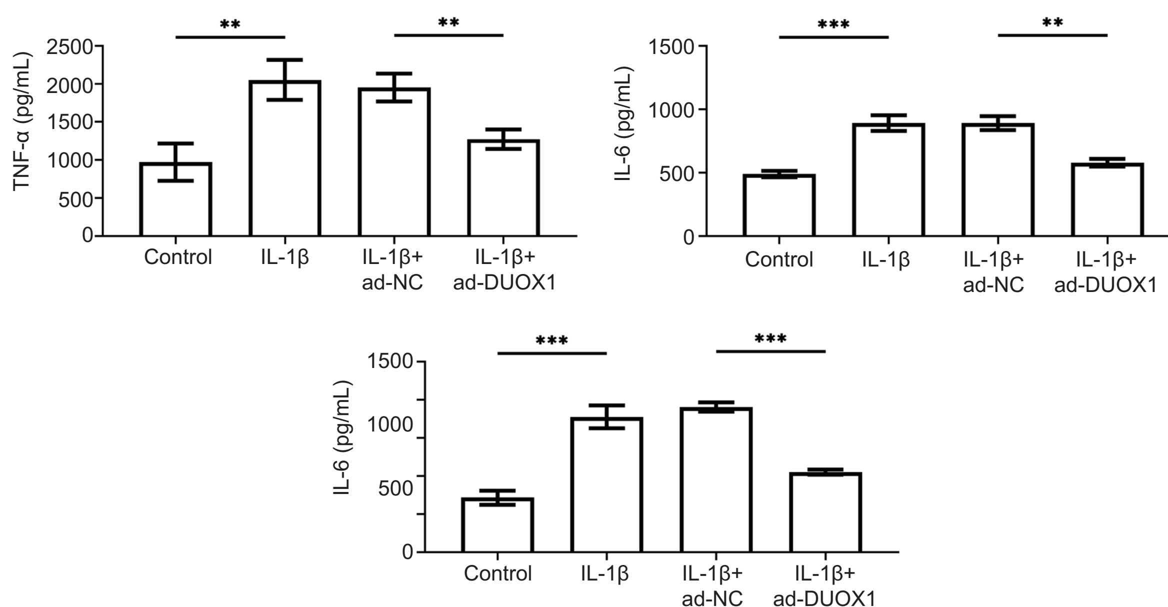

Figure 3 Overexpression of DUOX1 inhibits the inflammatory response in MH7A cells. Levels of inflammatory factors (IL-6, IL-1β, and TNF-α) in the culture medium were measured using ELISA. Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ (n = 3).

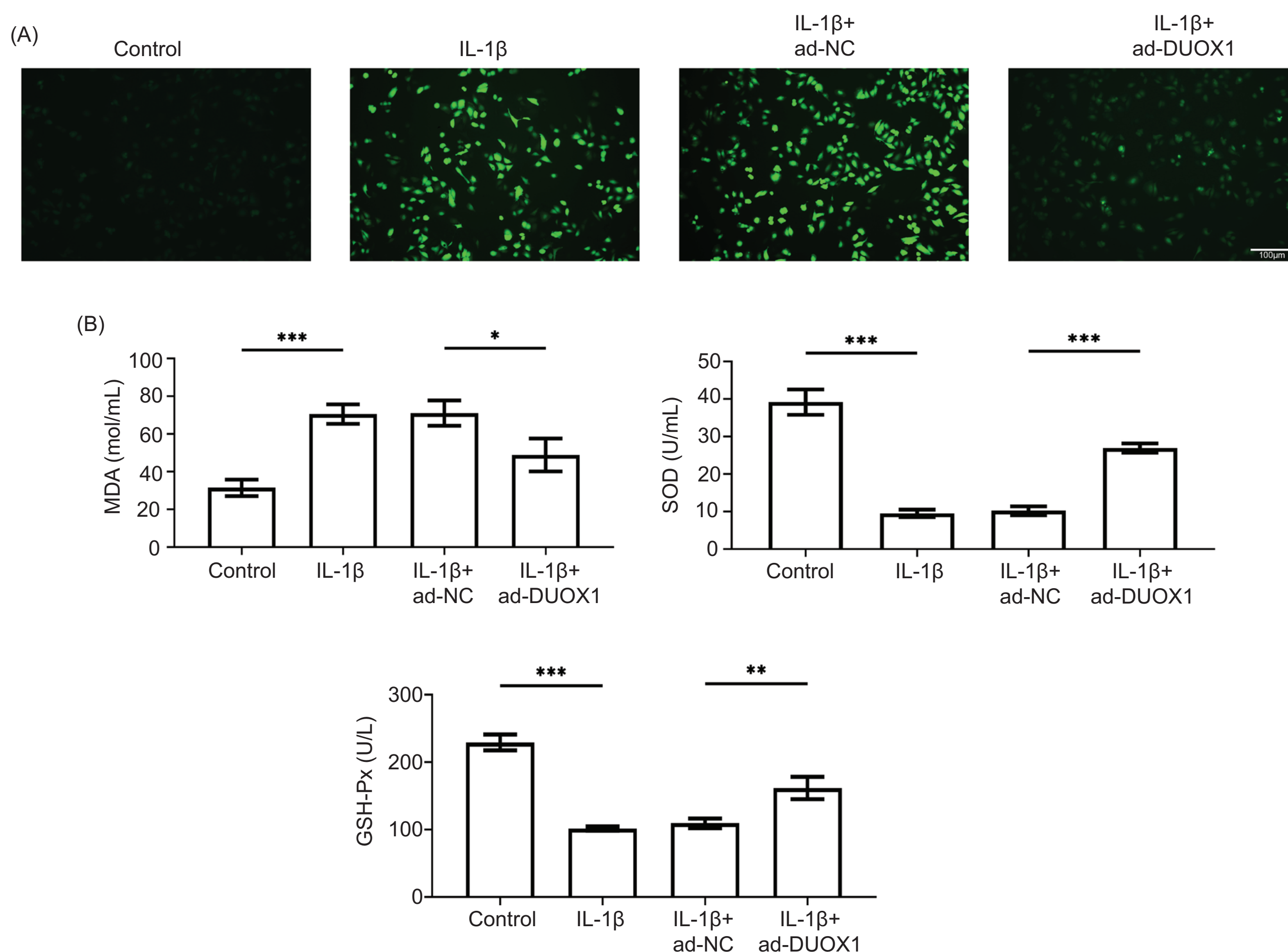

Figure 4 Overexpression of DUOX1 inhibits oxidative stress in MH7A cells. (A) ROS fluorescence intensity was measured using DCFHDA. (B) Levels of SOD, GSH-Px, and MDA in MH7A cells were determined using detection kits. Data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 (n = 3).

The biological behavior of IL-1β-stimulated synovial cells closely resembles that of tumor cells, making this model suitable for investigating disease mechanisms.[23] The expression of DUOX1 was significantly downregulated in IL-1β-treated MH7A cells. Given that DUOX1 overexpression has been shown to inhibit synovial cell migration, survival, inflammation, and oxidative stress (key processes involved in the progression of RA), it may represent a promising therapeutic target for patients with this condition.

In RA, synovial cells perform dual roles: they respond to the inflammatory microenvironment and exhibit aggressive behavior within the rheumatoid synovium.[24] When activated, these synovial cells secrete several inflammatory molecules, including IL-6, IL-8, and TNF-α, contributing to the pathogenesis of RA. The aggressive phenotype of synovial cells and the associated bone loss observed in RA may be driven by these pro-inflammatory factors.[25] Joint swelling and synovial hyperplasia are distinctive features of RA, driven by the inflammatory proliferation of synovial cells. The inflammatory microenvironment promotes the invasion and migration of fibroblast-like synoviocytes (FLS) into intra-articular tissues, resulting in damage to subchondral bone and cartilage.[26,27] In this study, overexpression of DUOX1 significantly inhibited the proliferation, migration, and inflammatory response of IL-1β-stimulated MH7A cells (demonstrated by CCK-8, ELISA, and Transwell assays). These findings further support the anti-inflammatory role of DUOX1 in RA and underscore its potential therapeutic value in mitigating synovial cell-driven joint damage.

Inflammation and oxidative stress are closely interconnected, both being significantly implicated in the pathophysiology of RA.[28] Antioxidants play a vital role in mitigating inflammation, whereas free radicals such as ROS act as potent mediators that can initiate and exacerbate inflammatory responses.[17] The production of ROS induces lipid peroxidation, leading to an increase in MDA and a decrease in SOD, which scavenges superoxide free radicals.[29] DUOX1 downregulation has been shown to significantly reduce ROS production.[10] According to our findings, DUOX1 overexpression was found to counteract the effects of IL-1β, reducing SOD and GSH-Px levels and increasing MDA and ROS levels in MH7A cells.

In RA, NF-κB is constantly active, contributing to the pro-inflammatory, destructive, and proliferative behavior

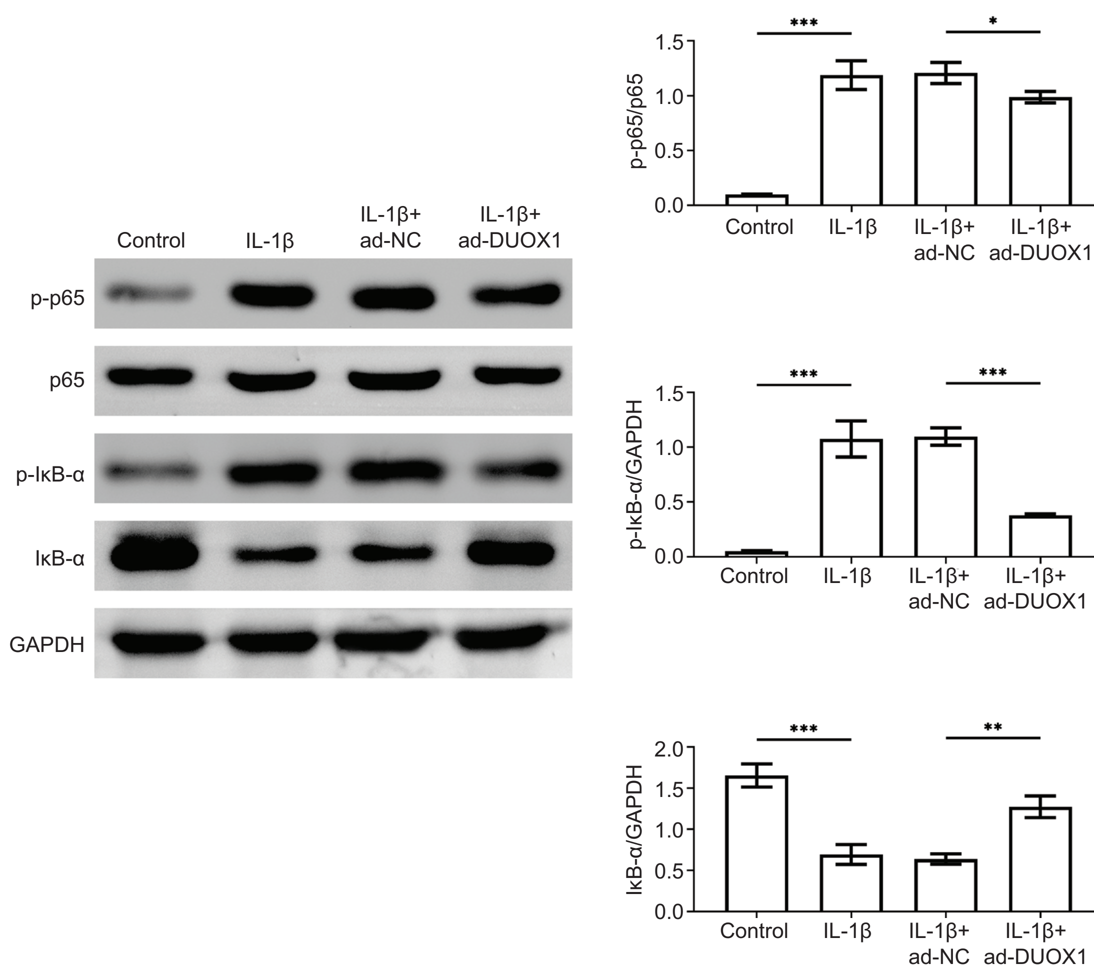

Figure 5 DUOX1 inhibits the activation of the NF-κB signaling pathway. Protein expression levels of p65, p-p65, IκB-α, and p-IκB-α in MH7A cells were analyzed by Western blotting. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (n = 3).

of synoviocytes.[30] Under normal conditions, NF-κB forms an inactive complex in the cytoplasm with IκB-α. IκB-α undergoes proteasomal degradation when it is phosphorylated, allowing NF-κB (p65) to translocate to the nucleus and initiate gene transcription.[31,32] Our results indicate that DUOX1 overexpression significantly reduces the phosphorylation of p65 and IκB-α, thereby inhibiting the activation of the NF-κB signaling pathway.

This study has several limitations. First, the findings have not been validated through in vivo experiments. Second, the direct interaction between DUOX1 and NF-κB has not been confirmed in this investigation; further research is needed to clarify the role of DUOX1 in the progression of RA. Third, the pathogenesis of RA involves multiple cell types, and focusing on a single cell type provides only a partial understanding. Fourth, RA is driven by numerous mechanisms, many of which were not explored in this study.

Conclusion

In summary, this study demonstrates that DUOX1 reduces IL-1β-induced synoviocyte proliferation, migration, inflammation, and oxidative stress while also inhibiting the activation of the NF-κB signaling pathway. These findings suggest that enhancing DUOX1's protective effects could represent a promising strategy for the targeted treatment of RA.

Acknowledgments

Not applicable.

Ethics Approval

Not applicable.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article. Additional datasets used or analyzed are available from the corresponding author upon reasonable request.

Authors Contribution

Dan Xuan and Dandan Feng designed the study, conducted the experiments, and supervised data collection. Fuyong Qiang analyzed and interpreted the data. Yonghui Xia prepared the manuscript for publication and reviewed the manuscript draft. All authors have read and approved the final manuscript.

Conflicts of Interests

The authors declare no conflicts of interest.

Funding

None.

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