International Immunopharmacology 71 (2019) 93–99

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

TGR5 agonist INT-777 mitigates inflammatory response in human endometriotic stromal cells: A therapeutic implication for endometriosis

Dan Lyu[a], Ning Tang[b], Jianye Wang[b], Yan Zhang[b], Jianfang Chang[b], Zhitao Liu[b], Haiping Liu[b,*]

[a] Department of Pain Management, Tianjin First Center Hospital, Tianjin, China
[b] Reproductive Medicine Center, The 960th Hospital of the PLA Joint Logistics Support Force, Jinan, China

ARTICLE INFO

Keywords:
Endometriotic stromal cells (ESCs)
TNF-α
TGR5
INT-777
JNK
AP-1
NF-κB

ABSTRACT

Endometriosis is a condition characterized by the presence of endometrial tissues outside the uterus. Endometriotic stromal cells (ESCs) are known to undergo regeneration and are linked to the causation of endometriosis. Activation of stromal cells by local inflammatory cytokines is proposed to be one of the mechanisms of endometriosis development. Takeda-G-protein-receptor-5 (TGR5) is a G protein-coupled bile acid receptor that plays multiple roles in various cells and tissues. In this study, we show that activation of TGR5 by its specific agonist, INT-777, protects ESCs from inflammation and oxidative stress induced by tumor necrosis factor-α (TNF-α). TGR5 is fairly expressed in cultured ESCs, and TNF-α treatment suppresses TGR5 expression. Activation of TGR5 by its synthetic agonist, INT-777, dramatically reduces the production of pro-inflammatory cytokines and adhesion molecules by TNF-α, including interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Moreover, INT-777 suppresses TNF-α-induced NADPH oxidase 4 (NOX4) expression and ameliorates cellular oxidative stress. Mechanistically, our findings demonstrate that INT-777 suppresses TNF-α-induced c-Jun N-terminal kinase (JNK) activation via suppression of p-JNK. INT-777 inhibits TNF-α-induced activation of the activator protein-1 (AP-1) pathway owing to its suppression of c-Jun and c-fos as well as transfected AP-1 promoter. INT-777 also inhibits nuclear factor-κB (NF-κB) activation as revealed by its suppression of TNF-α-induced nuclear p65 accumulation and NF-κB promoter. Collectively, our data indicate that activation of TGR5 by its agonist has protective effects against inflammation and reactive oxygen species (ROS) in cytokine-induced activation of ESCs. Therefore, INT-777 may have an implication in the clinical treatment of endometriosis.

1. Introduction

Endometriosis is a common condition in which endometrial tissue grows outside the uterus. Endometriosis affects about 10% of women of reproductive age, which equates to approximately 176 million women around the world [1]. Endometriosis is marked by ectopically growing endometrial cells and local inflammation and is often associated with pain and infertility in women [2]. Diagnosis of endometriosis remains a challenge due to the wide variety of symptoms and clinical manifestations. Endometriosis often remains undiagnosed or at latency due to the dependence on surgical diagnosis, laparoscopy or other imaging techniques [3]. There is no cure for endometriosis and current treatments are limited to mere symptomatic relief rather than targeting the mechanism of disease as this remains largely unknown [4]. The most common treatments aim to reduce inflammation and remove ectopic lesions, but recurrence or therapy resistance is very common [5].

In recent years, endometriotic stem or stromal cells (ESCs) have been identified as playing a key role in the regeneration of human endometrium [6]. There are data showing that ESCs derived from endometriosis patients show altered ESC function [7]. Cell fate tracing experiments have shown that the presence of endometriosis may deplete ESCs in blood circulation and even cause stromal cells to migrate from endometriotic lesions into the uterus [8]. These evidences indicate that mesenchymal stromal cells could contribute to the development of endometriosis. Therefore, by targeting the functions of ESCs, we can further elucidate the pathological mechanisms of endometriosis and develop better treatment options for this disease. Various biomolecular changes including increased cytokine production have been associated with the development of lesions in endometriosis. Excessive production of the pro-inflammatory cytokine TNF-α plays a key role in increased

* Corresponding author at: Reproductive Medicine Center, The 960th Hospital of the PLA Joint Logistics Support Force, 25 Shifan Road, Tianqiao District, Jinan, Shandong 250031, China.
E-mail address: jeniss512@163.com (H. Liu).

https://doi.org/10.1016/j.intimp.2019.02.044
Received 25 December 2018; Received in revised form 16 February 2019; Accepted 25 February 2019
Available online 14 March 2019
1567-5769/ © 2019 Published by Elsevier B.V.

inflammatory activity in ESCs by stimulating prostaglandin production and triggering the release of vascular endothelial growth factor [9]. TNF-α can also activate the transcriptional factor nuclear factor-κB (NF-κB), which acts as a central regulator of inflammatory signaling in ESCs [10]. Activated NF-κB is responsible for upregulating the gene expression of several chemokines including monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) [11]. Additionally, anti-inflammatory drugs including TNF-α inhibitors and antibody against matrix metalloproteinases (anti-MMP) exert therapeutic effects on endometriosis [12].

TGR5 was first identified as a bile acid-specific G-coupled protein receptor [13]. Since then, a variety of studies have found that TGR5 is widely expressed in different tissues and can activate various intracellular signaling pathways upon interaction with bile acids [14]. More recently, TGR5 was found to be the receptor for multiple selective synthetic agonists including INT-777 and agonism of TGR5 has been shown to regulate cell function and metabolism. Thus, TGR5 has emerged as a potential target for the treatment of metabolic disorders [15]. TGR5 has also been reported to promote human endometrial cancer cell proliferation, thereby suggesting that it plays a regulatory role in the function of endometrial cells [16]. We hypothesize that TGR5 may be present in ESCs and may regulate endometrial function. In this study, we investigated the involvement of ESCs in endometrial stem cells.

2. Materials and methods

2.1. Isolation of human ESCs, cell culture, and treatment

Human subject experiments were designed in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects [17]. The study was approved by the ethics committee of our institute. All patients signed informed consent before taking part in the study. Primary ESCs cells were isolated from the ovarian endometrioma of donors during a laparoscopic procedure. The tissues were minced and treated for 1 h with 2.5 mg/mL of type I collagenase (Sigma Aldrich, Saint Louis, MO, USA) in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Logan, UT, USA) at 37 °C in 5% CO_2. Dissociated cells were then centrifuged at 1000 ×*g*, suspended in complete DMEM with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin and streptomycin, and plated in culture dishes. After culturing overnight, non-adherent cells were removed and adherent cells had begun proliferating. The culture medium was replaced every 3 days. The purity of the cells was tested by flow-cytometry analysis using fluorescein isothiocyanate (FITC)-conjugated anti-CD45 antibody (Abcam, USA). At passage 3, the cells were morphologically homogeneous and exhibited the appearance of stromal cells. ESCs from passages 3–8 were used in each experiment. Recombinant human TNF-α was from R&D Systems (#210-TA); INT-777 was from MedChemExpress (#HY-15677). For cytokine TNF-α treatment experiments, primary ESCs were plated in 6-well plates and grown at confluence. The cells were then treated with 10 ng/mL of TNF-α for 24 h. For INT-777 agonist treatment experiments, INT-777 at the concentrations of 5 and 10 μM was added to cell growth media for 24 h and 48 h. Human endoC-βH1 β cells were purchased from the American Type Culture Collection (ATCC) (USA) and maintained in DMEM media with 10% fetal bovine serum (FBS). All cells were grown in an incubator at 37 °C in a 5% CO_2 atmosphere.

2.2. Semi-quantitative and quantitative polymerase chain reaction (PCR) analysis

The total RNA from human ESCs was extracted using a micro RNeasy Micro Kit from Qiagen (Cat.74004) in accordance with the manufacturer's instructions. RNA concentrations were quantified by Nanodrop. RNA (1 μg) was used to synthesize cDNA using iScript™ Reverse Transcription Supermix (BioRad, #1708840) for RT-qPCR from Invitrogen. For semi-quantitative PCR of TGR5, the PCR mixture was run using a standard 3-step PCR procedure for 35 cycles on 1.5% agarose gel and the results were quantified using Image J software. For quantitative PCR of TGR5, SYBR-based real time PCR experiments were performed to detect the total mRNA transcripts of human TGR5 on a ABI 7500 platform. For each sample, differences in threshold cycle (ΔCt) values were calculated by correcting the Ct of the gene of interest to the Ct of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative gene expression was expressed as $2^{-\Delta\Delta Ct}$ with respect to basal condition cells. The following primers were used in this study:

IL-6: forward, 5′-TTGGGAAGGTTACATCAGATC-3′; reverse, 5′-GGGTTGGTCCATGTCAATTT-3′;

IL-8: forward, 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′; reverse, 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′;

MCP-1: forward, 5′-AGCCAGATGCAGTTAATGCCC-3′; reverse, 5′-ACACCTGCTGCTGGTGATTCTC-3′;

VCAM-1: forward, 5′-CTTAAAATGCCTGGGAAGATGGT-3′;
reverse, 5′-GTCAATGAGACGGAGTCACCAAT-3′;

ICAM-1: forward, 5′-GGCTGGAGCTGTTTGAGAAC-3′; reverse, 5′-CTGACAAGTTGTGGGGGAGT-3′;

GAPDH: forward, 5′-ACT GGC GTC TTC ACC ACC AT-3′; reverse, 5′-AAG GCC ATG CCA GTG AGC TT-3′.

2.3. Nuclear extract

Nuclear extracts of ESCs were extracted using a kit from Thermo Fisher Scientific in accordance with the manufacturer's instructions. The nuclear protein lamin B1 was used as a positive control, and the nuclear fraction of p65 protein level was examined to determine NF-κB activation.

2.4. Western blot analysis

Cells from the different conditions were lysed by radio-immunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitor. Protein concentration was determined using the DC Bio-Rad Laboratories protein reagent (Bio-Rad, 500-0116) Cell lysates or nuclear extracts (20 μg) were immobilized by polyacrylamide gel electrophoresis (PAGE) gel. The separated protein mixes were then transferred to polyvinylidene fluoride (PVDF) members and blotted against their specific antibodies. Blots were developed with enhanced chemiluminescent (ECL) substrate (Thermo-Fisher Scientific, USA). The results were scanned, and the sum of the optical densities of target immuno-bands was quantitatively analyzed using Image J software. First, the background was subtracted. Then, target bands were selected and signal intensities were quantified. Data were exported for statistical analysis or graphical comparisons. Expression of target proteins was normalized to β-actin [18].

2.5. Enzyme-linked immunosorbent assay (ELISA) analysis

To measure the secreted levels of IL-6, IL-8, and MCP-1 by ELISA, ESC culture media was collected for analysis. The following ELISA kits were from R&D Systems: Human IL-6 Quantikine ELISA Kit (#D6050, R &D Systems), Human IL-8 Quantikine ELISA Kit (#D8000C, R&D Systems), Human MCP-1 Quantikine ELISA Kit (#DCP00, R&D Systems). The procedures were performed in accordance with the manufacturer's instructions. Data were collected via 96-plate reader spectrometry. The absolute values were obtained from a standardized 4-PL curve. Relative levels of these cytokines are presented as normalized to total protein amounts in each condition.

2.6. Measurement of reactive oxygen species (ROS)

Cellular reactive oxygen species (ROS) production was measured by quick staining the cells from the different conditions with 2′, 7′–dichlorofluorescin diacetate dye (DCFH-DA, Invitrogen). ESCs were treated with TNF-α (10 ng/mL) in the presence or absence of INT-777 (5, 10 μM) for 24 h. Upon completion of the treatment, cells were washed 3 times with phosphate buffer saline (PBS), followed by being loaded with 5 μM DCFH-DA in phenol red free medium and incubated for 30 min at 37 °C in darkness. Cells were then washed 3 times and fluorescence signals were recorded using an IBE2000 inverted fluorescence microscope (Zeiss, Germany) with excitation at 488 nm and emission at 525 nm. The fluorescent density of images was quantified using Image J software to index intracellular ROS [19].

2.7. Promoter assay

AP-1 promoter luciferase vector was from Addgene (#11783), and NF-κB luciferase vector was purchased from Thermo Fisher Scientific, USA. Cells were co-transfected with AP-1 or NF-κB promoter with a firefly luciferase promoter as a reference by Lipofectamine 2000 reagent from Invitrogen (cat 11,668,027). At 24 h post transfection, the cells were treated with 10 ng/mL TNF-α in presence or absence of 5 and 10 μM INT-777 for additional 24 h. The total cell lysates were collected to measure the dual luciferase activity of renilla and firefly luciferase. The relative luciferase value was calculated by normalizing the activity of firefly luciferase to renilla luciferase.

2.8. Statistical analysis

Data are shown as means ± standard derivation (S.D.). Experiments were repeated at least 3 times. Comparisons among different groups were performed using the one-way analysis of variance (ANOVA) test, followed by the Bonferroni post-hoc test. A P value < 0.05 was determined to be statistically significant.

3. Results

3.1. TGR5 is fairly expressed in human endometriotic stromal cells (ESCs)

Firstly, we examined the expression level of TGR5 in human endometriotic stromal cells (ESCs). We compared relative amounts of TGR5 mRNA and protein in ESCs and EndoC-βH1 β cells, which are derived from pancreatic islets and known to express TGR5. Our semi-qualitative PCR experiment showed that the TGR5 mRNA level of ESCs is comparable to that of EndoC-βH1 β cells (Fig. 1A). Furthermore, our western blot experiment confirmed that the expression of TGR5 protein in ESCs is fairly comparable to that of EndoC-βH1 β cells (Fig. 1B).

3.2. TGR5 expression is suppressed by TNF-α in endometriotic stromal cells

To explore the possible involvement of TGR5 in the development of endometriosis, we applied the cytokine TNF-α to cultured ESCs to mimic the high-inflammation environment of the disease, and then examined the expression level of TGR5. Compared with non-treated cells, 5, 10 and 20 ng/mL TNF-α treatment caused roughly 30%, 50% and 65% reductions in TGR5 expression, respectively, at the mRNA level (Fig. 2A). We were also able to confirm this reduction at the protein level. Using non-treated cells as a control, 5, 10 and 20 ng/mL TNF-α caused roughly 25%, 45% and 55% reductions in TGR5 at the protein level, respectively (Fig. 2B). These data suggest that cytokines such as TNF-α exert a dose-dependent inhibitory effect on TGR5 expression in ESCs. As an important G protein receptor for multiple bile acids, reduced TGR5 could have an abnormal influence on intracellular downstream signaling. Next, we attempted to manipulate the activity of TGR5 using a selective agonist and conducted a series of experiments to explore its function in ESCs. Agonists generally activated the receptor by increasing receptor interactions and expression. We employed the compound INT-777, which is known to be a potent TGR5 agonist. The results of real time PCR and western blot analysis in Supplementary Fig. 1A and B revealed that treatment with INT-777 could restore the expression of TGR5 against TNF-α in ESCs.

3.3. Activation of TGR5 by its agonist INT-777 inhibits TNF-α-induced pro-inflammatory cytokine production

We carefully examined the influence of TGR5 activation on pro-inflammatory gene panels upon inflammatory stimulation by applying TNF-α with and without INT-777. We chose three key pro-inflammatory factors (IL-6, IL-8 and MCP-1) and examined changes in their mRNA and protein expression upon these treatments. At the mRNA level and referenced to non-treated cells, 10 ng/mL of TNF-α resulted in about 4- to 4.5-fold higher IL-6, IL-8 and MCP-1 transcripts. However, the presence of 5 and 10 μM INT-777 significantly attenuated these inductions unanimously. All three of these factors had 2.5- to 3-fold higher expression in the presence of 5 μM INT-777, and 1.8- to 2-fold higher expression in the presence of 10 μM INT-777 (Fig. 3A). At the protein level and referenced to non-treated cells, TNF-α treatment gave rise to about 3- to 3.5-fold higher expression of IL-6, IL-8 and MCP-1. However, the presence of 5 and 10 μM INT-777 significantly suppressed these inductions. The three factors were 1.8- to 2-fold higher in the presence of 5 μM INT-777 and were all returned to near control levels in the presence of 10 μM INT-777 (Fig. 3B). These data indicate that inflammatory stimulation by TNF-α normally reduces TGR5 and induces pro-inflammatory factors, but the regain of TGR5 activation by its agonist reverses the effect of TNF-α. In other words, TGR5 agonist INT-777 prevents TNF-α-induced cytokine induction. As TGR5 is constitutively expressed in human endometriotic stromal cells, we also investigated the effects of INT-777 alone on inflammatory cytokine expression in ESCs. Interestingly, real time PCR results in

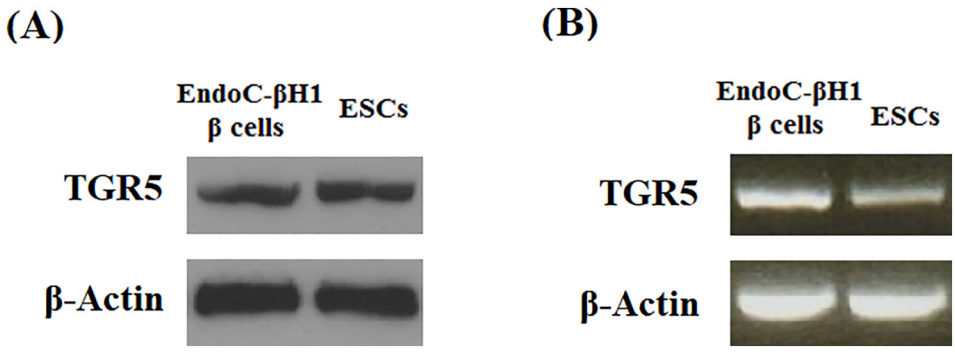

Fig. 1. The G-protein-coupled bile acid receptor Gpbar1 (TGR5) is fairly expressed in human endometriotic stromal cells (ESCs). EndoC-βH1 β cells were used as a positive control. (A). Reverse transcription PCR analysis reveals that TGR5 is expressed in ESCs; (B). Western blot analysis reveals that TGR5 is expressed in ESCs. Experiments were repeated in triplicate.

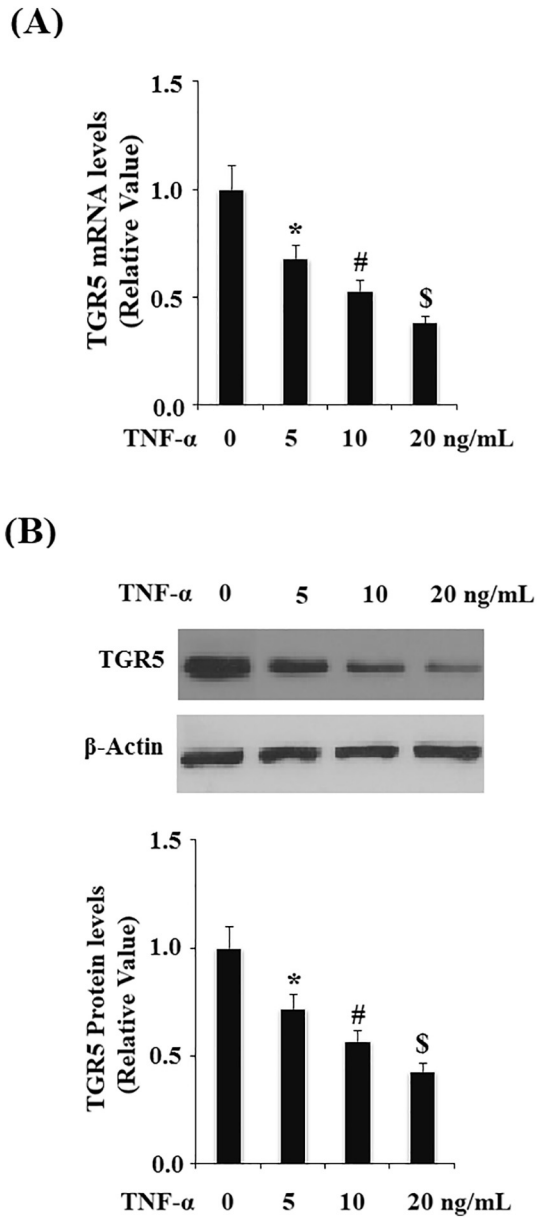

Fig. 2. TNF-α reduces TGR5 expression in a dose-dependent manner in human endometriotic stromal cells (ESCs). ESCs were treated with TNF-α at the concentrations of 5, 10, and 20 ng/mL for 24 h. (A). Expression of TGR5 at the mRNA level as determined by real time PCR analysis in ESCs; (B). Expression of TGR5 at the protein level as determined by western blot analysis in ESCs (*, $P < 0.01$ vs. control group; #, $P < 0.01$ vs. 5 ng/mL TNF-α group; $, $P < 0.01$ vs. 10 ng/mL TNF-α group; $n = 5$).

Supplementary Fig. 2 revealed that treatment with INT-777 alone significantly reduced the expression of IL-6, IL-8, and MCP-1 at the mRNA level in a dose-dependent manner in ESCs.

3.4. INT-777 suppresses TNF-α-induced production of adhesion molecules

Using a similar approach, we were able to examine the influence of TGR5 activation on two adhesion molecules in ESCs cells: ICAM and VCAM-1. At the mRNA level and referenced to non-treated cells, 10 ng/mL of TNF-α resulted in about 5- to7-fold higher ICAM-1 and VCAM-1 transcripts. However, the presence of the two doses of INT-777 both significantly attenuated these inductions. Upon treatment with 5 μM INT-777, TNF-α only caused 3.5- to 4-fold higher ICAM-1 and VCAM-1 transcripts. Upon treatment with 10 μM INT-777, TNF-α only induced approximately 2- to 2.5-fold higher ICAM-1 and VCAM-1 transcripts (Fig. 4A). At the protein level, ICAM-1 and VCAM-1 were barely detectable in non-treated cells, but these two molecules were greatly induced upon TNF-α treatment. However, TNF-α only induced ICAM-1 and VCAM-1 by roughly 60% in the presence of 5 μM INT-777 as compared to its absence. TNF-α only induced ICAM-1 and VCAM-1 production by roughly 40% when 10 μM INT-777 was present as compared to its absence (Fig. 4B). These experiments support that INT-777 has an inhibitory effect on cytokine-induced production of adhesion molecules. We can conclude that INT-777-mediated TGR5 activation has an anti-inflammatory effect in ESCs.

3.5. INT-777 inhibits TNF-α-induced oxidative stress

To further explore the possible protective effect of INT-777 in the setting of inflammatory stimulation, we tested the oxidative stress response of ESCs in the presence or absence of INT-777 and TNF-α. We examined NOX-4, a key component of cellular NAPDH oxidase, and cellular ROS signals via DCFH-DA florescence staining. For NOX-4 expression in ESCs and referenced to non-treated cells, TNF-α induced roughly 3.5-fold higher NOX-4. However, the presence of the two doses of INT-777 reduced TNF-α-induced NOX-4 production to roughly 2- and 1.5-fold, respectively (Fig. 5A). Again, referenced to non-treated cells, TNF-α treatment generated roughly 3.5-fold higher production of ROS, but TNF-α only yielded roughly 2.5- and 1.5-fold production of ROS in the presence of the two doses of INT-777 (Fig. 5B). These data suggest that INT-777-mediated TGR5 activation exerts an anti-ROS effect in ESCs.

3.6. INT-777 inhibits TNF-α-induced activation of JNK kinase

Next, we sought to define the possible molecular pathways involved in the effects of INT-777 in ESCs. The inflammatory response is a classic stress inducer in various immune cells, which often involves the stress-induced JNK pathway. We examined the influence of the addition of INT-777 on TNF-α-induced JNK kinase activation by determining the phosphorylation of JNK kinase. Based on blot intensity, we quantitated the levels of p-JNK and total JNK. Compared to non-treated cells, total JNK levels were stable and remained unchanged, while TNF-α induced roughly 3.5-fold p-JNK activation, but only caused roughly 2.2- and 1.5-fold p-JNK in the presence of 5 and 10 μM INT-777 (Fig. 6). This experiment implies that INT-777 ameliorates cytokine-induced JNK activation.

3.7. INT-777 inhibits TNF-α-induced activation of the AP-1 pathway

We then looked into other molecular pathways involved in the effects of INT-777 in ESCs. Since activation of the AP-1 complex is well-known to be involved in inflammation modulation, we were prompted to look into the influence of INT-777 on TNF-α-induced AP-1 activation by investigating two subunits of the AP-1 complex: c-Jun and c-fos. Intensity quantitation and comparisons of the protein levels of these two subunits to non-treated cells were performed as previously. TNF-α treatment increased expression of both c-Jun and c-fos by roughly 3.5-fold, but only caused approximately 2-fold expression of both proteins in the presence of 5 μM INT-777. Importantly, TNF-α nearly lost its effect in the presence of 10 μM INT-777 (Fig. 7A). To further confirm the inhibitory effect of INT-777 on the AP-1 pathway, we transfected AP-1 promoter fused with luciferase into ESCs and measured the change in promoter activity. Normalized to transfected cells without any treatment, TNF-α caused roughly 28-fold higher AP-1 promoter activity, but only caused roughly 15- and 5-fold higher promoter activity in the presence of the two doses of INT-777, respectively (Fig. 7B). These experiments verified that INT-777 inhibits cytokine-induced AP-1 activation.

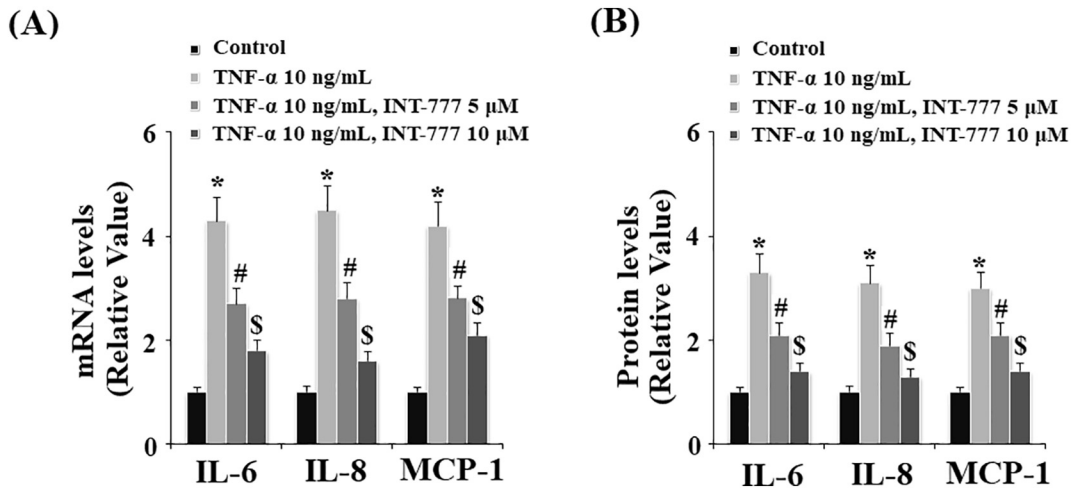

Fig. 3. Activation of TGR5 by its agonist INT-777 inhibits TNF-α-induced expression of pro-inflammatory cytokines including IL-6, IL-8, and MCP-1 in ESCs. ESCs were treated with TNF-α (10 ng/mL) in the presence or absence of INT-777 (5, 10 μM) for 24 h. (A). Expressions of IL-6, IL-8, and MCP-1 at the mRNA level as determined by real time PCR analysis; (B). Expressions of IL-6, IL-8, and MCP-1 at the protein level as determined by ELISA (*, $P < 0.01$ vs. control group; #, $P < 0.01$ vs. TNF-α group; $, $P < 0.01$ vs. TNF-α + 5 μM INT-777 group; n = 4–5).

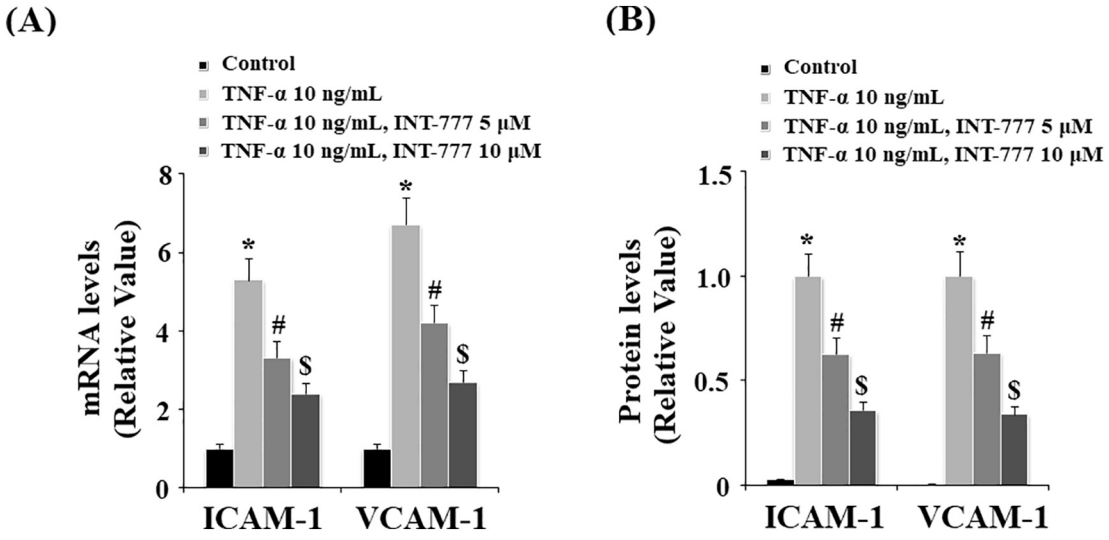

Fig. 4. INT-777 inhibits TNF-α-induced expression of ICAM-1 and VCAM-1 in human ESCs. ESCs were treated with TNF-α (10 ng/mL) in the presence or absence of INT-777 (5, 10 μM) for 24 h. (A). Expressions of ICAM-1 and VCAM-1 at the mRNA level as determined by real time PCR analysis; (B). Expressions of ICAM-1 and VCAM-1 at the protein level as determined by ELISA (*, $P < 0.01$ vs. control group; #, $P < 0.01$ vs. TNF-α group; $, $P < 0.01$ vs. TNF-α + 5 μM INT-777 group; n = 4–5).

3.8. INT-777 inhibits TNF-α-induced activation of the NF-κB pathway

Finally, we examined the influence of INT-777 on the NF-κB pathway. Since the NF-κB pathway is the most studied inflammatory pathway, we investigated nuclear NF-κB signaling in the presence or absence of INT-777 with a similar approach. In the nucleus, accumulation of p65 ultimately determines NF-κB transcriptional activity. TNF-α treatment increased p65 accumulation by roughly 3.5-fold, but only caused roughly 2.2-fold p65 accumulation in the presence of 5 μM INT-777. Interestingly, TNF-α had no effect in the presence of 10 μM INT-777 (Fig. 8A). To further confirm the inhibitory effect of INT-777 on the NF-κB pathway, we transfected AP-1 NF-κB promoter plasmid with luciferase into ESCs and measured the change in promoter activity. Normalized to transfected cells without any treatment, TNF-α caused roughly 22-fold higher activity of NF-κB promoter, but only caused roughly 10- and 3-fold higher promoter activity in the presence of the two doses of INT-777, respectively (Fig. 8B). These experiments confirmed that INT-777 has an inhibitory effect on cytokine-induced NF-κB activation.

4. Discussion

The diagnosis of endometriosis remains a challenge due to a lack of understanding of the disease mechanism and its complex appearance. Early studies endorse endometriosis as an estrogen-dependent inflammatory disease [13]. Therefore, current medical treatments are based on anti-inflammation and hormonal therapy. Endometriosis exhibits increased endometrial cell proliferation, survival, inflammation, and deficient differentiation. All these abnormalities have been linked to a stromal cell producing excessive estrogen and prostaglandin as well as progesterone resistance [14]. Various immunological aspects have been shown to contribute to the pathogenesis of endometriosis, and the disease shows similarities with autoimmune diseases. The transcription factors AP-1 and NF-κB mediate the production of major pro-inflammatory cytokines in ESCs and have been shown to be involved in the pathophysiology of endometriosis [15]. ESCs have the potential to

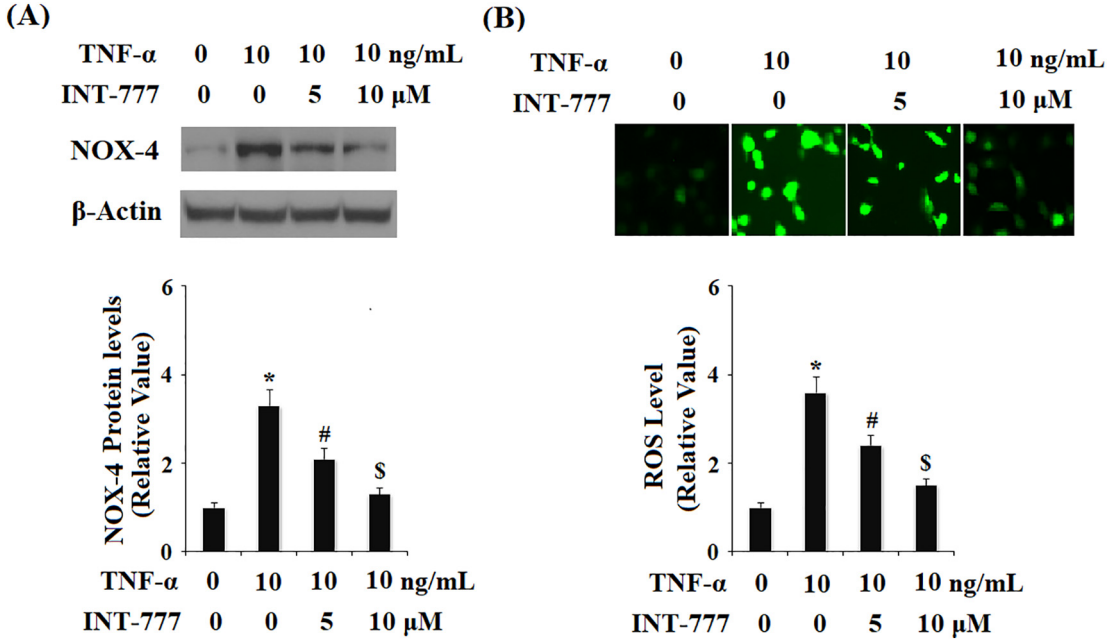

Fig. 5. INT-777 inhibits TNF-α-induced oxidative stress in human ESCs. ESCs were treated with TNF-α (10 ng/mL) in the presence or absence of INT-777 (5, 10 μM) for 24 h. (A). Expression of NOX-4 as determined by western blot analysis; (B). Production of reactive oxygen species (ROS) as determined by DCFH-DA assay (*, $P < 0.01$ vs. control group; #, $P < 0.01$ vs. TNF-α group; $, $P < 0.01$ vs. TNF-α + 5 μM INT-777 group; n = 5).

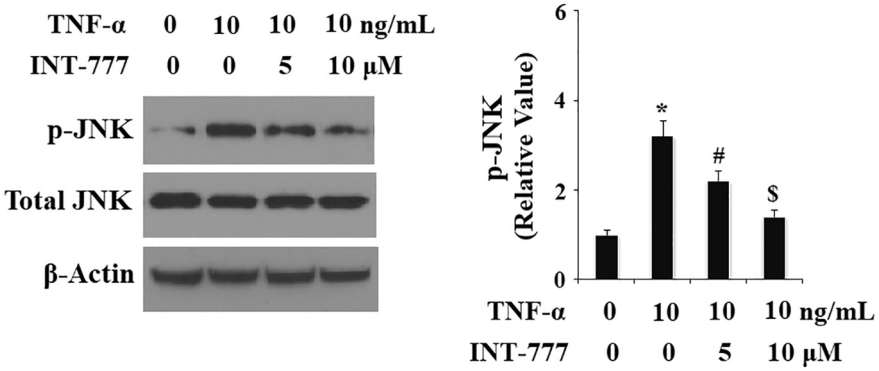

Fig. 6. INT-777 inhibits TNF-α-induced activation of JNK in human ESCs. ESCs were treated with TNF-α (10 ng/mL) in the presence or absence of INT-777 (5, 10 μM) for 2 h. Phosphorylated and total levels of JNK were determined by western blot analysis (*, $P < 0.01$ vs. control group; #, $P < 0.01$ vs. TNF-α group; $, $P < 0.01$ vs. TNF-α + 5 μM INT-777 group; n = 5).

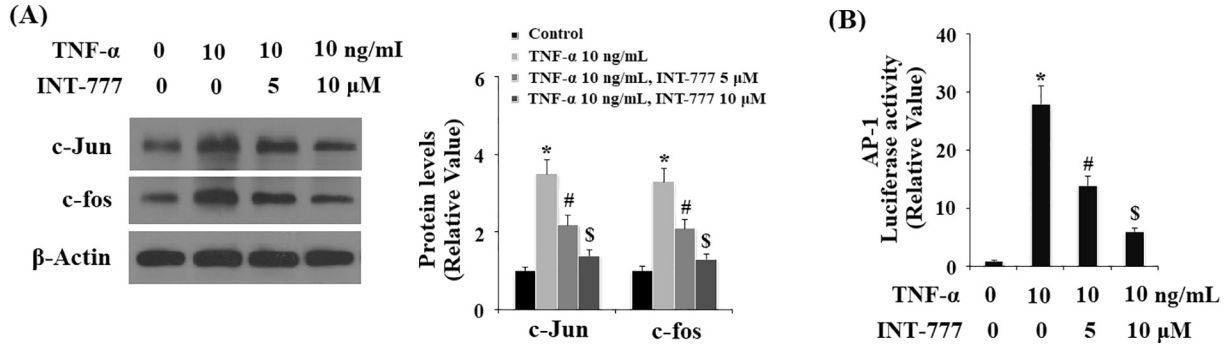

Fig. 7. INT-777 inhibits TNF-α-induced activation of the transcriptional factor AP-1. ESCs were treated with TNF-α (10 ng/mL) in the presence or absence of INT-777 (5, 10 μM) for 24 h. (A). Expressions of c-Jun and c-fos as determined by western blot analysis; (B). Luciferase activity of AP-1 (*, $P < 0.01$ vs. control group; #, $P < 0.01$ vs. TNF-α group; $, $P < 0.01$ vs. TNF-α + 5 μM INT-777 group; n = 4–5).

differentiate into multiple cell lineages and are an ideal candidate for regenerative medicine and cell-based therapies.

Activation of TGR5 in immune cells exhibits a potent anti-inflammatory response, suggesting that activation of TGR5 could have therapeutic potential in inflammatory diseases [16,17]. Our study investigated the effect of TGR5 activation in ESCs. Our findings provide several lines of evidence to support the notion that activation of TGR5 in ESCs could relieve cytokine-induced stressed cellular function. Firstly, our data demonstrate that TGR5 is fairly expressed in isolated human ESCs and provide the fundamental basis for its involvement in ESC function. Secondly, we show that TNF-α treatment reduces stromal TGR5 expression, implying that TGR5 is responsive to inflammation

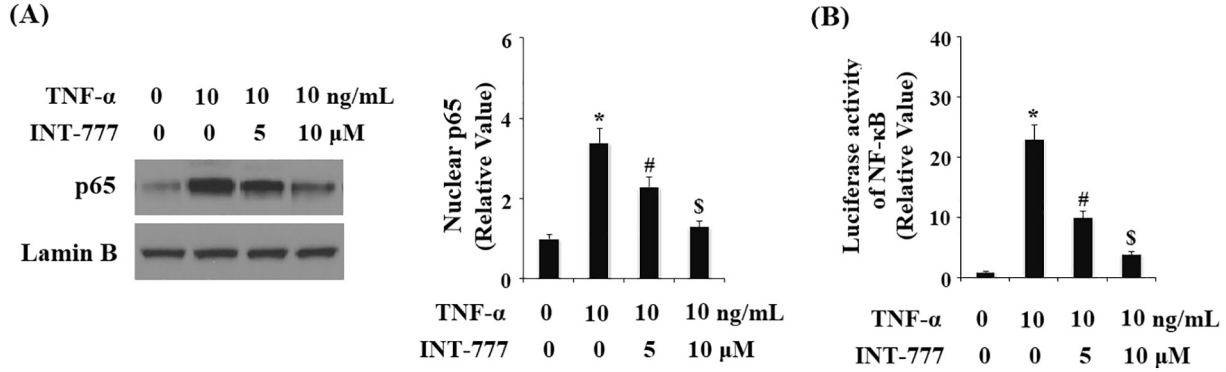

Fig. 8. INT-777 inhibits TNF-α-induced activation of NF-κB. ESCs were treated with TNF-α (10 ng/mL) in the presence or absence of INT-777 (5, 10 μM) for 24 h. (A). Nuclear translocation of p65 as determined by western blot analysis; (B). Luciferase activity of NF-κB promoter as measured by a luminator (*, $P < 0.01$ vs. control group; #, $P < 0.01$ vs. TNF-α group; $, $P < 0.01$ vs. TNF-α + 5 μM INT-777 group; n = 4–5).

triggered by cytokines such as TNF-α which reduces its expression. We could infer that TGR5 has fairly normal expression in a healthy state, but is reduced by the high-inflammation state of endometriosis. Therefore, we hypothesized that ESCs of endometriosis patients have reduced expression of TGR5 due to the increased inflammatory environment. We then conducted experiments on two aspects of TGR5 expression to explore the effect of reduced TGR5 activation. Firstly, our findings show that activation of TGR5 by its specific antagonist INT-777 indeed weakened cytokine-induced expression of the pro-inflammatory cytokines IL-6, IL-8, MCP-1, ICAM-1 and VCAM-1. Secondly, we show that INT-777 treatment reduced cytokine-induced cellular ROS production and decreased expression of the oxidative stress-associated NAPDH oxidase subunit NOX-4. The suppression of these major pro-inflammatory factors and production of ROS by INT-777 implies that this compound could have a potential therapeutic implication to target ESCs and ameliorate inflammatory endometriosis. Mechanistically, our study shows that the protective effect on ESCs exerted by INT-777 involves suppression of JNK pathway activation as revealed by its direct inactivation of p-JNK. Importantly, INT-777 reduces the transcriptional activity of the two master inflammation regulators AP-1 and NF-κB as revealed by its suppression of both major pathway components and promoters. The AP-1 and NF-κB signaling pathways have been shown to be activated in the endometriosis [15]. Activation of TGR5 by INT-777 counters these pathways, which indicates that INT-777 is a potent compound and has an explicit mechanism to support this effect. Owing to their specificity to the endometrium, ESCs constitute an endometrium-specific cell population and have been considered as promising candidates for targeting endometrial disorders including endometriosis [18]. A very recent report showed that human endometrium is regenerated on a monthly basis by endometrial stem cells, and ESCs likely mediate angiogenesis and stromal regeneration [19]. Although we have no evidence to show that TGR5 signaling could contribute to the regeneration of ESCs, a genetic knockout mice study has shown that TGR5 activation is essential for bile acid-mediated hepatic stem cell formation [20]. The role of TGR5 in the regeneration of ESCs would be our next study goal.

In conclusion, our understanding of TGR5 and its role in endometrial stromal cells provides mechanistic insights into the beneficial effect of TGR5 activation by its agonist INT-777. Targeted activation of TGR5 by INT-777 has promising potential for use in clinical trials in order to develop a new therapeutic strategy for the treatment of this dreadful disease.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.02.044.

References

[1] P.A. Rogers, T.M. D'Hooghe, A. Fazleabas, C.E. Gargett, L.C. Giudice, G.W. Montgomery, L. Rombauts, L.A. Salamonsen, K.T. Zondervan, Priorities for endometriosis research: recommendations from an international consensus workshop, Reprod. Sci. 16 (4) (2009) 335–346.
[2] C. Mehedintu, M.N. Plotogea, S. Ionescu, M. Antonovici, Endometriosis still a challenge, Journal of Medicine and Life 7 (3) (2014) 349–357.
[3] T. Bloski, R. Pierson, Endometriosis and chronic pelvic pain: unraveling the mystery behind this complex condition, Nurs Womens Health 12 (5) (2008) 382–395.
[4] Greene AD, Lang SA, Kendziorski JA, Sroga-Rios JM, Herzog TJ, Burns KA. Endometriosis: where are we and where are we going? Reproduction. 2016; 152(3): R63–78.
[5] P. Vercellini, P. Vigano, E. Somigliana, L. Fedele, Endometriosis: pathogenesis and treatment, Nat. Rev. Endocrinol. 10 (5) (2014) 261–275.
[6] D. Hufnagel, F. Li, E. Cosar, G. Krikun, H.S. Taylor, The role of stem cells in the etiology and pathophysiology of endometriosis, Semin. Reprod. Med. 33 (5) (2015) 333–340.
[7] C.E. Gargett, S. Gurung, Endometrial mesenchymal stem/stromal cells, their fibroblast progeny in endometriosis, and more, Biol. Reprod. 94 (2016) 1291–1295.
[8] N. Pluchino, H.S. Taylor, Endometriosis and stem cell trafficking, Reprod. Sci. 23 (12) (2016) 1616–1619.
[9] A. Suardika, T.G. Astawa Pemayun, New insights on the pathogenesis of endometriosis and novel non-surgical therapies, J Turk Ger Gynecol Assoc 19 (3) (2018) 158–164.
[10] G. Grandi, M. Mueller, N. Bersinger, A. Papadia, K. Nirgianakis, A. Cagnacci, B. McKinnon, Progestin suppressed inflammation and cell viability of tumor necrosis factor-α-stimulated endometriotic stromal cells, Am. J. Reprod. Immunol. 76 (4) (2016) 292–298.
[11] R. González-Ramos, S. Defrère, L. Devoto, Nuclear factor-kappaB: a main regulator of inflammation and cell survival in endometriosis pathophysiology, Fertil. Steril. 98 (3) (2012) 520–528.
[12] H. Falconer, J.M. Mwenda, D.C. Chai, C. Wagner, X.Y. Song, A. Mihalyi, P. Simsa, C. Kyama, F.J. Cornillie, A. Bergqvist, G. Fried, T.M. D'Hooghe, Treatment with anti-TNF monoclonal antibody (c5N) reduces the extent of induced endometriosis in the baboon, Hum. Reprod. 21 (7) (2006) 1856–1862.
[13] Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, et al. Identification of membrane-type receptor for bile acids (M-BAR). Biochem. Biophys. Res. Commun. 2002; 298: 714–719.
[14] H. Duboc, Y. Taché, A.F. Hofmann, The bile acid TGR5 membrane receptor: from basic research to clinical application, Dig. Liver Dis. 46 (4) (2014) 302–312.
[15] D. Philipot, D. Guérit, D. Platano, P. Chuchana, E. Olivotto, F. Espinoza, A. Dorandeu, Y.M. Pers, J. Piette, R.M. Borzi, p16INK4a and its regulator miR-24 link senescence and chondrocyte terminal differentiation-associated matrix remodeling in osteoarthritis, Arthritis Res Ther 16 (1) (2014) R58.
[16] I. Casaburi, P. Avena, M. Lanzino, D. Sisci, F. Giordano, P. Maris, S. Catalano, C. Morelli, S. Ando, Chenodeoxycholic acid through a TGR5-dependent CREB signaling activation enhances cyclin D1 expression and promotes human endometrial cancer cell proliferation, Cell Cycle 11 (2012) 2699–2710.
[17] World Medical Association, World medical association declaration of Helsinki: ethical principles for medical research involving human subjects, JAMA 310 (20) (2013) 2191–2194.
[18] R.J. Castellani, Y. Gupta, B. Sheng, S.L. Siedlak, P.L. Harris, J.M. Coller, G. Perry, H.G. Lee, M. Tabaton, M.A. Smith, X. Wang, X. Zhu, A novel origin for granulovacuolar degeneration in aging and Alzheimer's disease: parallels to stress granules, Lab. Investig. 91 (12) (2011) 1777–1786.
[19] B. Sheng, K. Gong, Y. Niu, L. Liu, Y. Yan, G. Lu, L. Zhang, M. Hu, N. Zhao, X. Zhang, P. Tang, Y. Gong, Inhibition of gamma-secretase activity reduces Abeta production, reduces oxidative stress, increases mitochondrial activity and leads to reduced vulnerability to apoptosis: implications for the treatment of Alzheimer's disease, Free Radic. Biol. Med. 46 (10) (2009) 1362–1375.
[20] L.C. Giudice, L.C. Kao, Endometriosis, Lancet 364 (2004) 1789–1799.