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Immunosuppressive, Anti-Inflammatory, and Antioxidant Effects of Simvastatin on Pristane Induced Arthritis

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ABSTRACT

The current therapeutic approaches to the autoimmune disease rheumatoid arthritis depend mainly on synthetic anti-arthritis compounds that usually cause many adverse effects. The anti-arthritic effect of the hypocholesterolemic drug simvastatin (Sim) has been confirmed, however, its actual mechanism of action has not been investigated yet. Therefore, this study aimed to unveil the biochemical and molecular changes that accompany the application of Sim as anti-arthritic in a mouse model of pristane-induced arthritis. Female Swiss albino mice (20-30g) were randomly divided into 5 groups (n= 10/group): control, Sim control, pristane-induced arthritis, Sim co-treated, and Sim post-treated group. Sim treatment significantly 1) downregulated the expression of immunomodulatory genes [interferon gamma (IFNγ) and lactoferrin (LF)], 2) decreased the expression of inflammation-related genes [tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL1β)], 3) declined the expression of matrix metalloproteinase-3 (MMP3), transforming growth factor beta (TGFβ), and oxidized-LDL receptor (OLR1), 4) upregulated the expression of the anti-inflammatory gene IL10, 5) reduced the levels of the oxidative markers [lipid peroxide marker malondialdehyde (MDA) and nitric oxide (NO)], 6) increased the levels of antioxidant markers [reduced glutathione (GSH) and superoxide dismutase (SOD)]. These findings conclude that administration of Sim relieved pristane-induced arthritis, with best improvement in the Sim co-treated (prevention) group. Thus, Sim could be used as a protective drug against rheumatoid arthritis based on its immunomodulatory, anti-inflammatory, and antioxidant effects.

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AE and TA presented basic concept and idea, did formal analysis of data and supervised the project. TA revised the manuscript. RM conducted the experiment and wrote the first draft. ME analysed data and interpreted it, wrote and revised the manuscript.

Key words
Simvastatin, Pristane, Arthritis, Anti-inflammatory, Antioxidant

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune, chronic inflammatory disease affecting mainly the synovial membrane (Adamopoulos, 2015; Yang *et al*., 2021) with higher susceptibility in females than in males especially in the elderly with 45-50 years of age (Kustiarini *et al*., 2019). The synovial membrane (synovium) cells, synoviocytes , showed higher activities of CD 4^+ T-lymphocytes, and higher levels of NF-kβ which induced the production of inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL1β), adhesion molecules such as transforming growth factor β (TGFβ), and matrix-degrading molecules like matrix metalloproteinase-3 (MMP3) in RA patients (Edupuganti *et al*., 2015; Zaiss *et al*., 2021). However, the anti-inflammatory cytokine interleukin in 10 (IL10) was significantly reduced in the synoviocytes and sera of RA patients (Barbosa *et al*., 2017). Moreover, these cytokines attract a very large number of inflammatory cells that release reactive oxygen species (ROS) causing overproduction of the lipid peroxidation biomarker MDA and reduction of antioxidant markers such as reduced glutathione (GSH) and superoxide dismutase (SOD) in the blood of RA patients and animals (Ahmed *et al*., 2015; Yu *et al*., 2015).

Among the different methods used to induce RA in animals, pristane induced arthritis is preferable due to earlier onset of (Hou *et al*., 2011). It also specifically targets large joints that show severe inflammation with little involvement of other organs (Faisal *et al*., 2015; Sangaletti *et al*., 2021). Accordingly, pristane-induced arthritis has been extensively used in rodents as a model of RA (De Franco *et al*., 2014). Pristane can induce RA by activating macrophage and overproducing ROS, causing oxidative stress damage for the synovium (Mohamed *et*

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al., 2014; Sangaletti *et al*., 2021).

The currently used anti-arthritic drugs such as glucocorticoids and non-steroidal anti-inflammatory drugs cause several complications and side effects on many organs such as stomach, liver, and kidney, especially when used for long-duration with overdoses (Cai *et al*., 2021). Therefore, it is an urgent demand to find new, safe compounds to treat RA. The hypocholesterolemic drugs, statins, are recommended for the treatment of chronic inflammatory diseases (Mohamed *et al*., 2021). These drugs decrease cholesterol levels through inhibition of 3-hydroxy-3-methylglutary-CoA reductase (McCarey *et al*., 2004). In addition to their hypocholesterolemic effects, statins such as simvastatin (Sim) can also induce immunosuppressive effects through blocking major histocompatibility complex class II (MHCII) and interferon γ (IFNγ) (Barbosa *et al*., 2017). Moreover, statins can inhibit the release of chemokines and other factors from the vascular endothelium leading to inhibition of macrophages and lymphocytes recruitment with a subsequent reduction in proinflammatory cytokines (Greenwood and Mason, 2007). Sim can also inhibit the activation of macrophages and lymphocytes in different rodent models of arthritis (Ahmed *et al*., 2015; Gottschalk *et al*., 2014).

The oxidized low-density lipoprotein (oxLDL) plays a crucial role in the pathogenesis of atherosclerosis (Shaw, 2004). High levels of oxLDL were found in joints of RA patients (Dai *et al*., 2000; McMahon *et al*., 2006). On the other hand, lactoferrin (LF), an essential first-line defense molecule against infection that induces innate immune cells proliferation, is the main target for the majority of autoimmune responses (Hu *et al*., 2017). A large number of LF-specific autoantibodies were detected in the blood of RA patients (Kida *et al*., 2011; Wong *et al*., 2009). However, little is known regarding the effect of statins, as powerful hypolipidemic and anti-inflammatory agents, on the levels of oxLDL or LF.

With the aforementioned observations, the immunosuppressive effects of statins along with the fact that statins are mostly well-tolerated drugs (Maron *et al*., 2000) offer a rationale for investigating the underlying mechanisms of Sim action during the treatment of a mouse model of pristane-induced arthritis. Therefore, this study was conducted to investigate these mechanisms using molecular and biochemical approaches.

MATERIALS AND METHODS

Mice

This study was carried out in accordance with US National Institutes of Health Guidelines, agreed with the principle of Helsinki's animal ethics, and was approved by the Research Ethics Committee of Faculty of Veterinary Medicine, Kafrelsheikh University. The present study was carried out on 50 female Swiss albino mice of 20-30g body weight. They were housed in wire cages with soft-wood chips for bedding. They were given a commercial basal diet and water *ad libitum* with a light/dark system of 12 h/12h.

Experimental design

The mice were randomly divided into 5 groups (n = 10/ group). In the control group **(**Cnt), mice were intraperitoneally injected with 0.5 ml/mouse saline (as a vehicle) twice with 7 weeks interval. In the Sim control group (Sim), mice were orally (by gavage) received 10 mg/kg/day simvastatin (ADWIC Chemical Co. Cairo, Egypt) for 65 days (Palmer *et al*., 2004). In the pristane-induced arthritis group (RA), each mouse was intraperitoneally injected with 0.5 ml pristane (2, 6, 10, 14 tetramethylpentadecane, Sigma-Aldrich Chemical Co. St. Louis, USA) twice with 7 weeks interval (Patten *et al*., 2004). The severity of arthritis was rated visually by evaluating the degree of inflammation in joints as previously described (De Franco *et al*., 2014; Leung *et al*., 2003). In the Sim co-treated group (RA+Sim-co), mice were intraperitoneally injected with 0.5 ml/mouse pristane twice at 7 weeks interval and orally given 10 mg/kg/day simvastatin daily for 65 days starting from the first pristane injection. In the Sim post-treated group (RA+Sim-post), mice were intraperitoneally injected with 0.5 ml/mouse pristane twice with 7 weeks interval and given 10 mg/kg/day simvastatin orally daily for 65 days starting from the appearance of arthritis clinical manifestations at the 60th day of the experiment.

Sampling

At the end of the treatment period (Day 125), mice were euthanized by exsanguination, knee and ankle joints were immediately excised and the synovium was carefully dissected from each joint. The tissue specimens were split into two parts; the first part was homogenized (for biochemical assay) and the second one was kept at -70°C (for RNA extraction).

Real-time PCR (qPCR)

Real-time PCR (qPCR) was used to assess the altered expression of *IFNγ*, *LF*, *TNFα*, *IL1β*, *IL10*, *MMP3*, *TGFβ*, and lectin type oxidized LDL receptor 1 (*OLR1*) in the synovium following treatment with Sim. Total RNA was extracted using a commercially available kit (Gene JET RNA Purification Kit, Thermo Scientific, # K0731, USA) following the manufacturer protocol and as previously detailed (Abou-Easa *et al*., 2014). RNA integrity was

determined by electrophoresis on 1.5% agarose gels, and concentration and purity were evaluated by Quawell nanodrop Q5000 (USA). The obtained total RNA was reverse transcribed to cDNA following the manufacturer's procedure (Thermo Scientific, #EP0451, USA). A PCR mixture (20µl) included cDNA, 2XMaster Mix (Quanti Tect SYBR Green), and mice-specific primers (Table I) was then prepared and placed in the thermal cycler (Step One Plus, Applied Biosystem, USA). We used the following thermal cycles for all genes: Initial denaturation (94°C/ 4 min/1 cycle), denaturation (94°C/40 s/40 cycles), annealing (60°C/30 s//40 cycles), and extension (72°C/30 s/40 cycles). The melting curve condition and fold change calculation based on cycle threshold (Ct) of target genes and the housekeeping (*β actin*) gene using the Livak method ($2^{-\Delta\Delta Ct}$) were performed as previously detailed (Elgazar *et al*., 2018; Saleh *et al*., 2014; Selim *et al*., 2019). The samples were analyzed in triplicates along with non-template control (NTC) and negative reverse transcription controls in each plate.

Table I. Primers used for real-time PCR.

Gene	Forward primer 5`→3`
INFγ	F ACTGGCAAAAGGATGGTGAC R TGAGCTCATTGAATGCTTGG
LF	F AAACAAGCATCGGGATTCCAG R ACAATGCAGTCTTCCGTGGTG
IL1β	F AAATCTCGCAGCAGCACATCAA R CCACGGGAAAGACACAGGTAGC
IL10	F CGGGAAGACAATAACTGCACCC R CGGTTAGCAGTATGTTGTCCAGC
TNFα	F GACAAGGCTGCCCCGACTACG R CTTGGGGCAGGGGCTCTTGAC
TGFβ	F GCAACATGTGGAACTCTACCAGA R GACGTCAAAAGACAGCCACTCA
MMP3	F CTCTGGAACCTGAGACATCACC R AGGAGTCCTGAGAGATTTGCGC
OLR1	F GTCATCCTCTGCCTGGTGTTGT R TGCCTTCTGCTGGGCTAACATC
β actin	F ACTATTGGCAACGAGCGGTT R CAGGATTCCATACCCAAGAAGGA

Biochemical assays

Synovial membrane samples were homogenized with phosphate buffer saline (PBS), centrifuged at 12000 xg for 15 min at 4 °C, and then the supernatant was collected for measurment of oxidative and antioxidant parameters. The levels of oxidative stress markers [lipid peroxide marker malondialdehyde (MDA) and nitric oxide (NO)] and the levels of the antioxidant marker [reduced glutathione (GSH)] and the activities of the antioxidant enzyme superoxide dismutase (SOD) were measured in homogenized synovial membrane specimens using commercially available kits (Biodiagnostics, Egypt) and as previously described (El-Bayomi *et al*., 2018; Saleh and El-Magd, 2018).

Statistical analysis

The difference between the groups was determined by one-way ANOVA using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as mean ± standard error of the mean (SEM). Duncan's multiple range test (DMRT) was applied as a post hoc test and significance was set at $p< 0.05$.

RESULTS

Sim downregulated the expression of immunomodulatory genes

Changes in the relative expression of the two immunomodulatory genes interferon γ (*INF*γ) and lactoferrin (*LF*) genes in the synovium of pristane-induced arthritic mice following treatment with Sim were determined using real-time PCR (qPCR). The results of qPCR are shown in Figure 1. We found a significant upregulation in the expression of INFγ and LF genes in the untreated pristane-induced arthritic group (G3, RA) as compared to the control (G1, Cnt) and Sim control (G2, Sim) group. However, pristane-induced arthritic mice (G3, RA) treated with Sim [G4 (RA+Sim-co) and G5 (RA+Sim-post)] showed a significantly downregulated expression relative to the RA group. Among the two treated groups, the co-treated (prophylactic) group (G4, RA+Sim-co) showed significantly lower INFγ and LF expression than the post-treated (G5, RA+Sim-post) group. On the other hand, no significant difference in gene expression was noticed between the two controls groups (G1 (Cnt) and G2 (Sim)). These findings infer a potent immunosuppressive potential for Sim against pristane-induced arthritis, particularly when applied as a prophylactic treatment.

Sim inhibited the expression of inflammatory genes

Mice injected with pristane (G3, RA) exhibited a significant elevation in the expression of the two inflammatory genes IL1β, TNFα in the synovium compared to the two control groups [G1 (Cnt) and G2 (Sim)] (Fig. 2). Administration of Sim significantly downregulated this elevated expression with lowest expression in the co-treated group (G4, RA+Sim-co). On the other hand, the anti-inflammatory IL10 gene was significantly decreased in the arthritic group (G3) compared to the two controls groups (G1 and G2). Treatment with Sim significantly

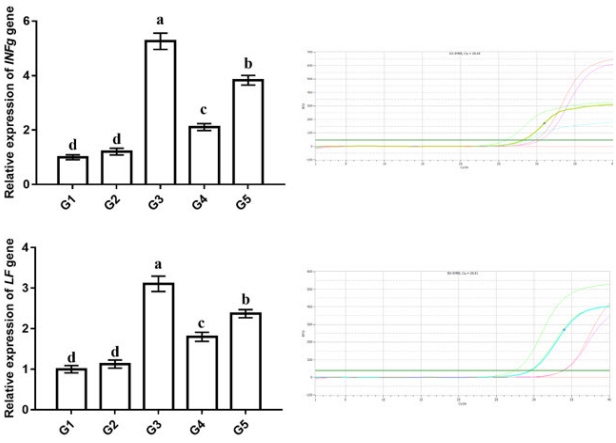

Fig. 1. Effect of Sim on the expression of immunomodulatory *INFγ* and *LF* genes in the synovium of pristane-induced arthritic mice as detected by qPCR. Data were expressed as mean fold change ± SEM (n = 5) from the Cnt group (G1). Amplification curves showing the cycle threshold (Ct) values were displayed next to each gene. Columns with different letters (a-d) are significantly different at $P<0.05$. G1, Cnt; G2, Sim; G3, RA; G4, RA+Sim-co; G5, RA+Sim-post group.

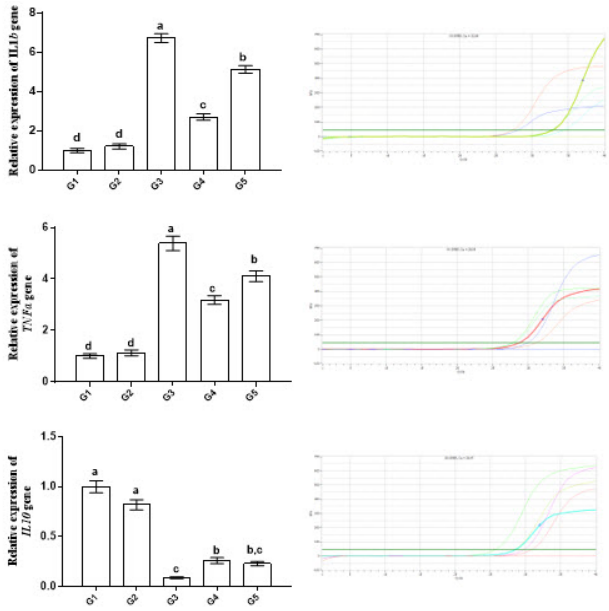

Fig. 2. Effect of Sim on the expression of inflammation-related genes (*IL1β*, *TNFα*, *IL10*) in the synovium of pristane-induced arthritic mice as detected by qPCR. Data were expressed as mean fold change ± SEM (n = 5) from the Cnt group (G1). Amplification curves showing the cycle threshold (Ct) values were displayed next to each gene. Columns with different letters (a-d) are significantly different at $P<0.05$. G1, Cnt; G2, Sim; G3, RA; G4, RA+Sim-co; G5, RA+Sim-post group.

upregulated IL10 expression, with higher expression in the co-treated group (G4), relative to the arthritic group (G3). These results imply that Sim had an anti-inflammatory effect on arthritis induced by pristane with best effect when given as a prevention therapy.

Sim reduced expression of arthritis-related genes

The expression of TGFβ1, MMP3, and OLR1 in the synovium was significantly higher in the arthritic group (G3) than in the control groups (G1 and G2) (Fig. 3). Arthritic mice treated with Sim (G4 and G5) exhibited significant downregulated expression of TGFβ1, MMP3, and OLR1 genes, with lowest expression in the co-treated group (G4), relative to the untreated arthritic mice (G3). However, the expression of these genes in the treated groups (G4 and G5) was still significantly higher than the control groups (G1 and G2). Again, no significant difference in TGFβ1, MMP3, and OLR1 expression was observed between the two control groups (Fig. 3). These results suggest that Sim had a notable anti-arthritic effect with better influence for the prevention approach.

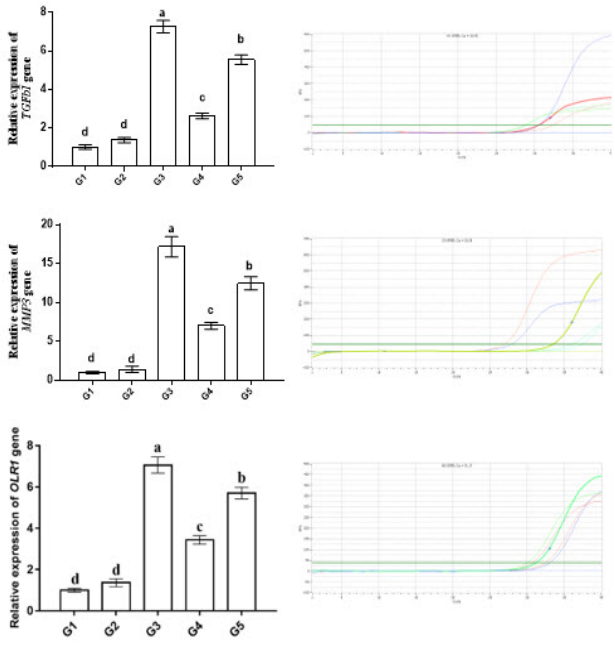

Fig. 3. Effect of Sim on the expression of *TGFβ*, *MMP3*, and *OLR1* genes in the synovium of pristane-induced arthritic mice as detected by qPCR. Data were expressed as mean fold change ± SEM (n = 5) from the Cnt group (G1). Amplification curves showing the cycle threshold (Ct) values were displayed next to each gene. Columns with different letters (a-d) are significantly different at $P<0.05$. G1, Cnt; G2, Sim; G3, RA; G4, RA+Sim-co; G5, RA+Sim-post group.

Sim decreased oxidative stress and enhanced antioxidant status

Figure 4 shows the effect of Sim treatment on the lipid peroxide MDA and the oxidative stress marker NO as well as the activities of antioxidant enzymes GSH and SOD in the synovium of the pristane-induced arthritic mice. Arthritic mice (G3) showed significant elevation in MDA and NO levels and significant reduction in GSH and SOD levels relative to the control animals (G1 and G2). Administration of Sim restored these markers to levels close to the control. Again, the co-treated group (G4) exhibited lower levels of oxidants marker (MDA and NO) and higher levels of antioxidant markers (GSH and SOD). These data indicate that Sim could diminish oxidative stress damage in the synovium generated by pristane with a better effect in the co-treated group.

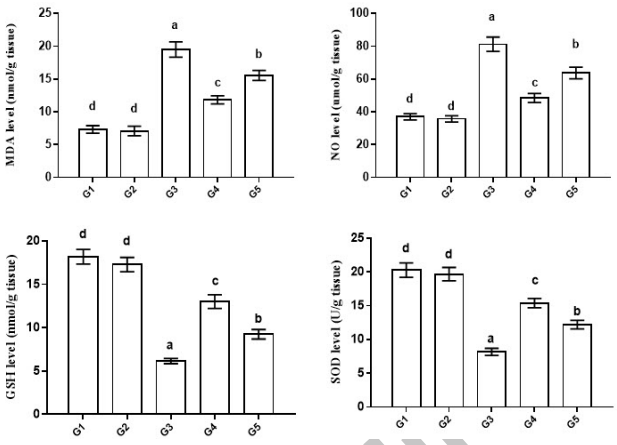

Fig. 4. Effect of Sim on oxidative stress (MDA and NO) and antioxidant (GSH, and SOD) markers in the synovium of pristane-induced arthritic mice. Data were expressed as mean ± SEM (n = 5) from the Cnt group (G1). Columns with different letters (a-d) are significantly different at $P<0.05$. G1, Cnt; G2, Sim; G3, RA; G4, RA+Sim-co; G5, RA+Sim-post group.

DISCUSSION

Several previous studies reported a notable antiarthritic effect of the hypocholesterolemic drugs statins including simvastatin (Sim). However, little is known regarding the biochemical and molecular alterations associated with this effect. To the best of our knowledge, this is the first study to report that treatment with Sim ameliorated arthritis triggered by pristane with best improvement when animals pre-treated with Sim (prophylactic group). This anti-arthritic effect could be mediated through, at least in part, potent immunomodulatory, anti-inflammatory, and antioxidant effects of Sim.

The immunomodulatory potential is one of the main mechanisms by which Sim can induce its anti-arthritic effect. Several evidence obtained from experimental trials showed that statins possess powerful immunomodulatory potential independent of their hypocholesterolemic effects (Cai *et al*., 2021; Sparrow *et al*., 2001; Yang *et al*., 2021). Supporting this notion, we also found a potent immunosuppressive effect for Sim as revealed by downregulated expression of the immunoregulatory INFγ and LF genes in the synovium of pristane-induced arthritic mice treated with Sim compared to the untreated arthritic mice. Consistent with our findings, a large body of previous *in vivo* and *in vitro* studies revealed that treatment with statins including Sim could significantly inhibit the expression of IFNg and MHCII in synoviocytes, endothelial cells, synovial fluid neutrophils, and macrophages (Cross *et al*., 2003; Mostafa *et al*., 2020; Mulhaupt *et al*., 2003). Additionally, Sim inhibits T lymphocytes and macrophages proliferation and activation in vitro (Leung *et al*., 2003) and in patients with hypertriglyceridemia (Krysiak and Okopień, 2013). LF regulates the functions of several immune cells and ultimately induces both innate and adaptive immune responses (Ibrahim *et al*., 2019; Legrand, 2011). To the best of our knowledge, this is the first study to report an inhibitory effect of Sim on LF expression levels in the synovium of arthritic animals.

The anti-arthritic potential of Sim can also be attributed to its anti-inflammatory effect. Indeed, we reported a significant downregulation in gene expression of the proinflammatory cytokines IL1β and TNFα and a significant upregulation of the anti-inflammatory IL10 in synovium of arthritic mice following treatment with Sim. Sim does not only reduce the production of pro-inflammatory cytokines (TNFα, IL8, and I1β) in animals and patients (Marino *et al*., 2014; Shevchuk *et al*., 2020; Wang *et al*., 2015) but it also inhibits the release of inflammatory cytokines in RA patients and animals (Ahmed *et al*., 2015; Barbosa *et al*., 2017; Pereira *et al*., 2014). Sim and other statins could also exert anti-inflammatory effects through inhibition of iNOS and COX1 expression (Colucci *et al*., 2013; Myasoedova *et al*., 2020; Tan *et al*., 2016). The anti-inflammatory cytokine IL10 found in the synovial fluid and synovium of RA patients (Katsikis *et al*., 1994) derived mainly from T and B lymphocytes and macrophages (Brennan and Foey, 2002). As an anti-inflammatory molecule, we found an increase in the expression of the IL10 gene after treatment of arthritic group with Sim. Similarly, Sim induced the production of IL10 in the synovium of a rat model of complete Freund's adjuvant-induced arthritis (Barbosa *et al*., 2017) and serum of patients with chronic obstructive pulmonary disease (Maneechotesuwan *et al*., 2015). Moreover, the in vitro

inhibition of IL10 significantly elevates TNFα and IL1β (Katsikis *et al*., 1994). IL10 decreases MHC class II that subsequently inactivates T cells and prevents cytokines formation (Fillatreau *et al*., 2008; O'Garra and Vieira, 2007). It also triggers activation of CD8 T cells, enhancing their cytotoxic potential (Moore *et al*., 2001).

The lipoprotein-associated phospholipase A2 (Lp-PLA2), is a platelet-activating factor that can also catalyze the cleavage of the oxLDL into its highly immunogenic metabolites (Tselepis and Chapman, 2002). A large variety of epitopes on oxLDL metabolites were recognized by particular autoantibodies in humans with a higher activity of Lp- PLA2 (Lourida *et al*., 2006; Nezos *et al*., 2021; Papathanasiou *et al*., 2008; Tsouli *et al*., 2006). A higher level of oxLDL was detected in joints of RA patients (Dai *et al*., 2000; McMahon *et al*., 2006). To the best of our knowledge, this is the first study to report that administration of Sim resulted in a significant downregulation of the *OLR1* gene in the synovium of arthritic mice.

MMP3 is mainly formed by immune cells activated by inflammatory cytokines and can destroy different members of collagens that are the main tissue constituents of the joint (Guerrero *et al*., 2021; Yamaguchi *et al*., 2008). MMP3 is another arthritis-related marker that could be targeted by Sim. Our results showed a significant decrease in *MMP3* expression following treatment with Sim. In support, Aktas *et al*. (2011) reported that the antiarthritic potential of Sim is mediated through inhibition of MMP3 expression in the joint of osteoarthritic rats. MMP3 levels were also elevated in the blood and synovial fluid of RA patients (Cunnane *et al*., 2001). Statins reduced the expression of MMP3 in rabbit macrophages and human chondrocytes (Abeles and Pillinger, 2006).

In the present study, treatment with pristane significantly upregulated *TGFβ* expression in arthritic mice. These findings agreed with Bira *et al*. (2005) who found elevated expression of *TGFβ* and its receptor in synovial fibroblasts of patients with RA. To the best of our knowledge, this is the first study to report downregulated expression of *TGFβ* in the synovium of the arthritic mice following treatment with Sim.

In addition to inflammation, oxidative stress participated in the damage of joint tissue during the pathogenesis of RA (Mahajan and Tandon, 2004). The anti-arthritic influence of Sim could also be mediated by its antioxidant properties. In support, we found that Sim decreased the oxidant (MDA, NO) and increased the antioxidant (GSH, SOD) markers in the synovium of arthritic mice. In agreement, previous studies reported the similar antioxidant potential for Sim in rat models of arthritis (Ahmed *et al*., 2015) and ischemic heart failure (Cho *et al*., 2014). Statins can inhibit oxidative stress by lowering the release of lipid peroxide MDA and NO (Fenster *et al*., 2003; van Boheemen *et al*., 2021). Elevation of MDA levels in the arthritic mice could trigger cell membrane damage of synovial membrane cells and increase free radicals production leading to oxidative stress damage particularly when endogenous antioxidant systems fail to compensate for the ROS.

CONCLUSIONS

Sim has a potent anti-arthritic effect against pristane-induced arthritis and this effect could be mediated through, at least in part, its immunomodulatory, anti-inflammatory, and antioxidant effects. In general, mice co-treated with Sim and pristane conferred better effects against arthritis than post-treated animals. This highlights the impact of using Sim as a prophylaxis (prevention) better than treatment. Therefore, Sim could be used as a protective drug against rheumatoid arthritis.

Statement of conflict of interest

The authors have declared no conflict of interest.

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