

Research Article

Sparstolonin B Exerts Therapeutic Effects on Collagen-Induced Arthritis by Inhibiting the NLRP3 Inflammasome and Reducing the Activity of α 1,3-Fucosyltransferase

Yue Sun , Yun Guo , Jing Zhang , Lihua Chang , Haiyan Zhao , and Xiaofei Wang 

Department of Rheumatology and Immunology, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004, China

Correspondence should be addressed to Xiaofei Wang; wangxf@sj-hospital.org

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Objective. To explore the role of α 1,3-fucosyltransferase in the mediation of rheumatoid arthritic inflammation, the protective effect of Sparstolonin B on rheumatoid arthritis (RA), and the mechanisms that regulate the NLRP3 inflammasome. **Methods.** Forty, weighing from 260–300 g, male Sprague-Dawley rats were randomly divided into the following groups: a sham operation group (Sham group), a rheumatoid arthritis model group (RA group), an RA+Sparstolonin B treatment group (RAS group), an RA+Iguratumod group (RAI group), and an RA+SsnB+NLRP3 inflammasome activator (Nigericin) group (RASN group); ten animals were allocated to each group. We determined the arthritis index for each group of rats, and pathological changes were evaluated by hematoxylin-eosin staining. We also used ELISAs to determine the serum levels of IL-17, IL-6, TNF- α , TGF- β , IL-18, and IL-1 β . TUNEL staining was used to investigate apoptosis in synovial cells. IF was used to detect the release of ROS, ASC formation, and the expression levels of FucT-V and NLRP3. Western blotting was used to detect the protein expression levels of Bcl-2, Bax, TLR4, MYD88, NF- κ B, pro-caspase-1, NLRP3, FucT-V, E-Selectin, and P-Selectin. We also performed *in vitro* experiments with Sparstolonin B and detected changes in 1,3-fucosyltransferase activity by ELISA. The pyroptosis-related phenotype, including ASC, was identified by immunofluorescence, while levels of NLRP-3, pro-IL-1, and pro-caspase-1 were detected by western blotting. **Results.** Sparstolonin B was showed to alleviate joint swelling in RA rats, inhibited inflammatory cell infiltration and the release of ROS, reduced damage caused by oxidative stress, and suppressed the rate of apoptosis in synovial cells. The administration of Sparstolonin B inhibited the secretion of IL-17 from Th17 cells and triggered the secretion of TGF- β from Treg cells, thus leading to the reduced expression of TLR4, MyD88, and NF- κ B, and the suppression of TNF- α secretion. Moreover, Sparstolonin B downregulated the expression of NLRP3, inhibited ASC formation *in vivo* and *in vitro*, and reduced the levels of IL-18 and IL-1 β . The expression levels of FucT-V, E-Selectin, and P-Selectin were also inhibited. Interestingly, these protective effects of Sparstolonin B could be blocked in RA rats by inhibiting the activation of the NLRP3 inflammasome. **Conclusion.** Sparstolonin B improved inflammatory responses and oxidative stress by inhibiting the NLRP3 inflammasome, inhibiting the expression of FucT-V and downregulating the TLR4/MYD88/NF- κ B signaling pathway in order to rescue RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease that is characterized by synovitis [1], autoantibody production [2], cartilage and bone destruction [3], and systemic inflammatory injury. RA is the most common form of rheumatic immune disease. Research has shown that 50% of patients who have had RA for more than ten years will incur damage to the extra-articular organs, such as the car-

diovascular and nervous systems, the lungs, the kidneys, and the bones [4]. It has been hypothesized that inflammatory cytokines are the key factors that underlie RA [5].

Pyroptosis is a newly discovered form of cell death that differs from apoptosis and necrosis [6]. During apoptosis, apoptotic bodies are formed within cells. However, in pyroptosis, the cell membrane becomes perforated, resulting in the destruction of cell integrity and the release of the cell contents; this is more similar to necrosis. Pyroptosis also releases

the cell contents including IL-1 β , IL-18, and other inflammatory cytokines, causing the body to produce an inflammatory response. Pyrolysis is closely related to the occurrence and development of many diseases, such as ulcerative colitis, tumors, and cardiovascular diseases [7, 8]. Recent studies have shown that cell pyrolysis is also closely related to autoimmune diseases, especially RA [9]. Because RA can cause joint deformities and loss of function, it has become one of the main causes of disability. Therefore, finding new and effective drugs to treat or prevent the occurrence and development of RA is very important.

It has been reported that the damage-related molecular pattern of pyroptosis is initially stimulated by the activation of NOD-like receptor family pyrin domain-containing 3 (NLRP3), which is a component of the innate immune system that functions as a pattern recognition receptor (PRR) that recognizes pathogen-associated molecular patterns (PAMPs). ASC is apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD). The combination of the oligomerization of NLRP3 with ASC and cysteine protease-1 precursor (pro-caspase-1) results in the cleavage of cysteine protease-1 (CP1), thereby activating interleukin-1 β (IL-1 β) and interleukin-18 (IL-18), thus promoting the progression of inflammation and cell damage [10, 11]; this complex is referred to as the inflammasome. Previous research showed that the inflammatory response and the erosion of articular cartilage were alleviated after knocking out the caspase-1 gene in a mouse model of RA, thus indicating that caspase-1 plays an important role in the pathogenesis of RA [12]. Furthermore, the excessive activation of NLRP3 is highly correlated to the occurrence and development of various inflammatory diseases, suggesting that the downstream products of NLRP3, IL-1 β , and IL-18, may play an essential role in the inflammatory responses of RA [13].

Sparstolonin B (SsnB) is a new natural product of coumarin compounds that is derived from Jing-San-Leng, a form of traditional Chinese medicine. SsnB is an inhibitor of the toll-like receptor 2 (TLR2) and TLR4 signaling pathways and can therefore selectively block the binding of myeloid differentiation primary response 88 (MyD88) to TLR2 and TLR4, thus suppressing macrophage inflammation [14–16]. Research has showed that SsnB can significantly improve the pulmonary inflammation in mice with endotoxemia by reducing the expression levels of inflammatory factors in the serum, lung, and liver tissues, thereby improving the survival rate [14]. Furthermore, SsnB can reduce the myocardial cell inflammatory damage induced by hypoxia-reoxygenation and inhibit the lipopolysaccharide-induced inflammation of human umbilical vein endothelial cells [17, 18]. An accumulation of evidence now indicates that SsnB is a potential precursor for the development of selective TLR antagonists to treat inflammatory-related diseases [19, 20].

α 1,3-Fucosyltransferase (FucT-V) is a cell adhesion molecule that is closely related to cell inflammation and leukocyte transport [21]. It has been shown that FucT-V plays a vital role in the inflammation process of synovial tissue in RA patients, thus implying that FucT-V may represent a key mediator of the inflammatory response and participate

in the occurrence and development of RA. High expression levels of FucT-V have been detected in the synovial tissue of the knees in RA patients, thus indicating that FucT-V may promote the migration of inflammatory cells to the inflammation site by increasing the synthesis of selectin [22–24]. Igaratimod is an effective disease-modifying anti-rheumatic drug that can prevent inflammatory processes by inhibiting the production of immunoglobulins and inflammatory cytokines [25]. Igaratimod was used in this study as a positive control drug to determine the therapeutic effect of SsnB.

In this study, we successfully established a rat model of RA and then administered our rats with SsnB as an interventional therapy. We observed the protective effects of SsnB on RA rats and investigated its effects on inflammation and FucT-V. We also explored the regulatory mechanisms associated with the NLRP3 inflammasome.

2. Materials and Methods

2.1. Experimental Animals. A total of 50 specific pathogen-free grade Sprague-Dawley (SD) male rats, weighing 260–300 g, were purchased from Vital River Laboratories (Beijing, China) (Production License Number: SCXK(Beijing) 20120007). Animal experiments in this study were carried out in the Barrier System of the Department of Laboratory Animals, China Medical University (Shenyang, China) (User License Number: SYXK(Liao) 2013001). Rats were randomly divided into the following groups: a sham operation group (Sham group, $n = 10$), a RA model group (RA group, $n = 10$), a RA+SsnB group (RAS group, $n = 10$), a RA+Igaratimod group (RAI group, $n = 10$), and a RA+SsnB+NLRP3 inflammasome activator group (RASN group, $n = 10$). The animal experiments were approved by the Experimental Animal Welfare and Ethics Committee of China Medical University (Reference: IACUC NO.2019108).

2.2. Establishment of a Rat Model of RA. Bovine type II collagen was combined with Freund's complete adjuvant to form a 25 mg/mL emulsion; multiple injections of the combined emulsion were then given into the tail root of the experimental rats. In order to establish a rat model of RA, a second booster immunization was performed on the left tail root of the rats after 21 days. After 21 days of booster immunization, the arthritis index (AI) was used as a criterion for estimating whether the model was successfully established. Rats in the RAS group were given SsnB (300 mg/kg) every day for 14 days after RA modelling. Rats in the RAI group were treated with Igaratimod (10 mg/kg) every day for 14 days after RA modelling. Rats in the RASN group were treated with NLRP3 inflammasome activator Nigericin (4 mg/kg) administered intraperitoneally every two days [26] and meanwhile given SsnB (300 mg/kg) every day for 14 days after RA modelling.

The Sham group was only given the same dose of saline solution. After 14 days of continuous administration, we observed and recorded the skin color, temperature, infection status, hindfoot movement, and joint swelling status, of the hind feet and tread joints in each rat from each group.

2.3. The Scoring of Arthritis in Rats. We made regular observations of the hind feet of all rats. The arthritis score was evaluated by applying the arthritis index score method. This method is based on the degree, range, and deformation of the joint, redness and swelling, dryness and loss of lustre in the fur, and symptoms of reduced activity. The two hind feet of each rat were evaluated, and the mean arthritis score for each group was calculated. The arthritis scores were evaluated as follows: 0 point—no redness or swelling; 1 point—red spots or mild swelling; 2 points—moderate swellings at the joint site; 3 points—severe swellings; and 4 points—deformed joints and no weight bearing. The highest possible arthritis score was 16 points.

2.4. Cell Culture and Treatment. Synovial cells were obtained from CIA rats and passaged 4-5 times before being used for *in vitro* experiments. Cells (5×10^5 /well) were cultured for 48 h in 24-well plates containing DMEM with 10% fetal calf serum FCS and $5 \mu\text{g}/\text{mL}$ LPS in a humidified 5% CO_2 atmosphere at 37°C . After Sparstolonin B ($40 \mu\text{M}$) treatment for 1 h [27], we collected the supernatants for ELISA. We also collected cells from each group in order to detect apoptosis. Following cell lysis, we detected the levels of key proteins by western blotting.

2.5. Hematoxylin-Eosin (HE) Staining. CO_2 inhalation euthanasia was used to euthanize the rats. We removed the right hind limbs of all euthanized rats and removed all muscle tissues so that the bones were completely exposed. These tissues were then fixed in 10% neutral formaldehyde solution and decalcified with 10% EDTA solution. Complete decalcification was indicated by tissue softening and the lack of resistance to acupuncture. The decalcified tissues were then dehydrated with a gradient series of ethanol, cleared with xylene, embedded in paraffin, and sliced into $4 \mu\text{m}$ sections. Sections were then stained with hematoxylin for 5 min and differentiated in 1% HCl-ethanol. Then, sections were stained with eosin solution for 1 min, dehydrated, cleared, and sealed in neutral resin. A light microscope was then used to observe and evaluate histopathological changes. We compared the groups with regard to four key aspects: synovial tissue hyperplasia, inflammatory cell infiltration, pannus formation, and cartilage and bone destruction.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was used to determine the serum levels of IL-17 (SEA063Ra, USCN, USA), IL-6 (SEA079Ra, USCN, USA), tumor necrosis factor-alpha (TNF- α) (SEA133Ra, USCN, USA), transforming growth factor-beta (TGF- β) (SEA124Ra, USCN, USA), IL-18 (SEA064Ra, USCN, USA), and IL-1 β (SEA563Ra, USCN, USA). The ELISA kits were used in accordance with the manufacturer's instructions. ELISA was also used to investigate the levels of $\alpha 1,3$ -fucosyltransferase in synovial cells following treatment with Sparstolonin B. In brief, $100 \mu\text{L}$ of gradient standard solutions and diluted sample solutions was added into wells on the ELISA plates, followed by 1 h incubation at 37°C . The solutions in each well were then removed and replaced by detection reagent A. Cells were then rinsed three times with PBST and then mixed with

detection reagent B. Cells were incubated at 37°C for 1 hour after the addition of each detection reagent. Plates were then washed five times in PBST before the application of tetramethylbenzidine (TMB) reaction product for 15 min. Stop solution was then added into the plates and the plates examined for 10 min for any evidence of color change. Finally, the absorbance at 450 nm was detected using a microplate reader. The levels of each factor were calculated according to the obtained data and standard curves.

2.7. Immunofluorescence (IF). Deparaffinized tissue sections were soaked into 3% H_2O_2 solution, washed with PBS buffer, and incubated with 0.1 M sodium citrate solution for antigen retrieval. Sections were then blocked with goat serum and incubated for 30 min at 37°C . We then removed residual serum from each section and then incubated the sections at 4°C overnight with an ROS probe and antibodies against ASC (#PA5-88132, Invitrogen, USA), FucT-V, and CD3 (#PA5-102404, Invitrogen, USA). Subsequently, sections were rinsed with PBS buffer and incubated with fluorescent-labelled secondary antibody for 30 min at 37°C in the dark. Sections were then stained with DAPI for 10 min in order to stain the cell nucleus. Finally, sections were mounted with N antifade mounting reagent. The expression levels of the target antigen were then evaluated under a fluorescence microscope.

2.8. TUNEL Staining. TUNEL staining was carried out with an Apoptosis Detection kit (CI0617, Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions. Paraffin-fixed tissue sections were sliced into $5 \mu\text{m}$ sections. These sections were deparaffinized, cleared with xylene and blocked in skimmed milk solution. Sections were then incubated for 60 min with $50 \mu\text{L}$ of TUNEL working solution in a dark chamber at 37°C . Later, $50 \mu\text{L}$ of streptavidin-HRP working solution was applied to the sections and incubated for another 30 min at 37°C in the dark. Finally, cell nuclei were stained with DAPI for fluorescence staining. Sections were then analyzed by fluorescence microscopy, and apoptosis rate was calculated.

2.9. Western Blotting. Frozen tissues were lysed with RIPA lysis buffer containing 1% PMSF, followed by homogenization and centrifugation at 12,000rpm at 4°C for 15 min. Protein concentration was then determined by the BCA protein quantification assay. Protein samples were then separated by 10% SDS-PAGE, transferred to PVDF membranes, and blocked with 5% skimmed milk. Next, membranes were incubated with primary antibodies at 4°C for overnight against Bcl-2 (ab196495, Abcam, USA), Bax (ab32503, Abcam, USA), TLR4 (ab217274, Abcam, USA), MYD88 (ab2064, Abcam, USA), NF- κB (ab32360, Abcam, USA), pro-caspase-1 (ab179515, Abcam, USA), IL-1 β (ab150777, Abcam, USA), NLRP3 (ab263899, Abcam, USA), E-Selectin (ab185698, Abcam, USA), and P-Selectin (ab59738, Abcam, USA); β -actin (ab8226, Abcam, USA) was used as an internal loading control. The next morning, membranes were washed five times in TBST buffer (10 min per wash) and incubated with goat anti-rabbit secondary antibody at room

temperature for 1 h. Finally, ECL chemiluminescent substrate (ECL; Thermo Fisher Scientific, 1856136) was applied onto membranes, and the protein bands were detected using an Imaging system (Tanon, China). Grey values were quantified using ImageJ software (ImageJ 1.8.0; National Institutes of Health).

2.10. qRT-PCR. 1 mL of Trizol (15596026, Invitrogen, USA) was added to the cryopreserved joint tissues, and total RNA was extracted by the chloroform method followed by reverse transcription to obtain cDNA. Primers were designed against *TLR4*, *MYD88*, *NF- κ B*, and *β -actin* (Table 1). The reaction system was prepared using a qRT-PCR kit (Invitrogen), and reactions were performed on a PCR instrument. PCR reactions were performed as follows: predenaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. This reaction was repeated 40 times in total. Results are represented using the $2^{-\Delta\Delta Ct}$ method.

2.11. Statistical Analysis. Data were analyzed using SPSS version 21.0 statistical software (IBM SPSS Statistics, USA) and represented as mean \pm standard deviation (SD). The normal distribution of data was tested with the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was used to make comparisons between multiple groups. Fisher's least significant difference (LSD) test was used to compare between groups. The Wilcoxon rank sum test was used when groups were not normally distributed. Differences were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. SsnB Reduced the Extent of Injuries Caused by RA. Rats in the Sham group were in a good state of health; their nails were a red color and of normal appearance. In contrast, rats in the RA group were mentally inactive; these rats also had swollen and deformed joints and experienced difficulty when moving (Figure 1(a)). Consequently, RA rats had higher arthritis scores (Figure 1(b) and Table 2). Histopathological analysis of the RA rats revealed synovial cell hyperplasia and the infiltration of a large number of inflammatory cells in the interstitium (Figure 1(c)). When the RA rats were treated with SsnB or Igaratimod, we found that the histopathological changes improved and that the arthritis score was reduced, thus suggesting that SsnB and Igaratimod treatment exerted significant therapeutic effects on the progression of RA.

3.2. SsnB Inhibited Oxidative Stress Injury and Reduced Cell Apoptosis in a Rat Model of RA. RA may induce a decline in the antioxidant capacity of the body. Oxidative stress can occur if the oxidative capacity exceeds the antioxidative capacity, thus leading to the excessive release of ROS. In turn, this can result in the lipid peroxidation of biofilms, the denaturation of intracellular proteins and enzymes, and DNA damage. These consequences can lead to cell death, tissue damage, and disease. In this study, we detected the excessive release of ROS in the joints of RA rats (Figure 2(a)). This led to changes in the protein expression levels of apoptosis-

TABLE 1: Primer sequences.

Gene	Primer (5' \rightarrow 3')
TLR4	Forward: AGTAGGCCACATTACACTGCT
	Reverse: GACCCACACCTCACAAATTGA
MYD88	Forward: TCATGTGGTCAAGACGAGAT
	Reverse: GGAGGAAGGAAGGCATGA
NF- κ B	Forward: AGAATGAGGACTGGGTGAGAAAC
	Reverse: CAACGGCTCTGGATAAAAGTGTCTA
β -Actin	Forward: CCATTGAACACGGCATTG
	Reverse: TACGACCAGAGGCATACA

related proteins (Bax and Bcl-2) as the endogenous apoptosis pathway was activated by oxidative stress (Figure 2(b)). The rate of cell apoptosis decreased following the administration of SsnB (Figure 2(c)). The administration of SsnB also alleviated the changes in Bax and Bcl-2 protein expression (Figure 2). Collectively, our *in vitro* experiments suggested that SsnB can inhibit oxidative stress injury and reduce cellular apoptosis in RA, thus improving synovial function.

3.3. SsnB Inhibited the Inflammatory Response in RA Rats by Downregulating the TLR4/MYD88/NF- κ B Signaling Pathway. T-cell immune dysregulation is known to play a crucial role in the pathogenesis of RA. T-helper 17 cells (Th17) and regulatory T cells (Treg) play essential roles in mediating inflammation in RA and are known to cause erosion and destruction of the articular cartilage and bone [28–30]. And RA is characterized by the production of excessive inflammatory mediators, such as cytokines (IL-1 β , IL-6, TNF- α , and IL-17), chemokines, and autoantibodies, which cause excessive activation of immune cells [31].

We used ELISA to investigate changes in the levels of Th17 cytokines, differentiation-associated factors, and Treg cytokines. We found that the levels of IL-17 and IL-6 were increased in RA rats while the levels of TGF- β were decreased, which confirmed the successful preparation of RA rats' model. However, these changes were reversed following the administration of SsnB (Figure 3(a)). The TLR4/MYD88/NF- κ B pathway is known to be one of the most important signaling pathways in the mediation of inflammation; SsnB is a selective inhibitor of the TLR2 and TLR4 pathways. In this study, we found that SsnB can effectively reduce the expression levels of *TLR4*, *MYD88* and *NF- κ B* mRNAs (Figure 3(b)) and proteins (Figure 3(c)), thus suggesting that SsnB downregulates the TLR4/MYD88/NF- κ B pathway to inhibit the inflammatory response in a rat model of RA.

3.4. SsnB Inhibited Cellular Pyroptosis in RA by Downregulating the NLRP3 Inflammasome. Previous research has suggested that inflammasomes mediate various immune-inflammatory diseases and metabolic diseases. In this study, we observed increased levels of IL-18 and IL-1 β in our rat model of RA (Figure 4(a)), thus suggesting that molecules associated with RA-mediated damage would activate NLRP3 and be combined with the ASC to form

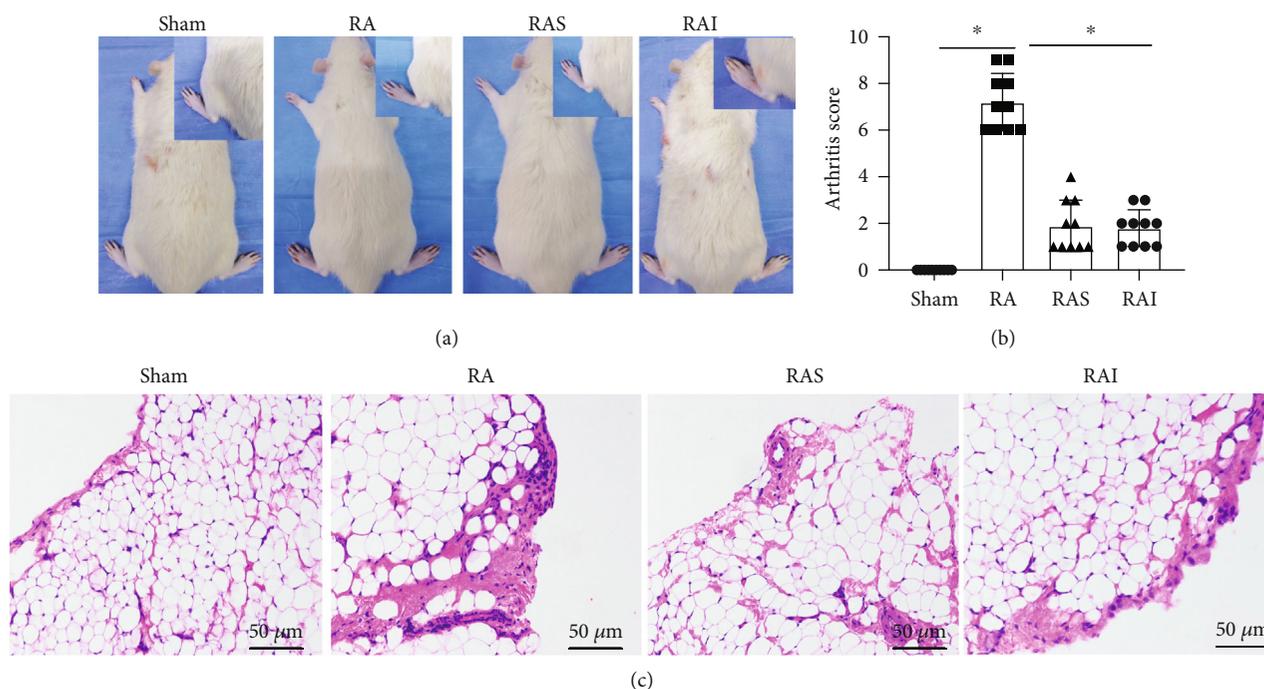


FIGURE 1: SsnB reduces RA arthritis injury. (a) Pathological changes in rats of each group; (b) Arthritis scores evaluation; (c) HE staining (scale bar = 50 μm). All the data were represented as the mean \pm SD; * $p < 0.05$.

TABLE 2: Arthritis index of each group.

Group	Arthritis index (mean \pm SD)
Sham	0
RA	7.2 \pm 1.17
RAS	1.9 \pm 1.04
RAI	1.8 \pm 0.75
RASN	6.1 \pm 0.89

inflammasomes (Figure 4(b)), thereby promoting the cleavage of pro-caspase-1 and activating IL-1 β . We also found that SsnB can downregulate the expression of NLRP3 (Figure 4(c)) and therefore inhibit the pyroptosis of RA cells. Moreover, SsnB treatment significantly inhibited the expression of pyroptosis-related protein (Figure 4(c)), thus suggesting that SsnB can inhibit pyroptosis in RA cells.

3.5. Activation of NLRP3 Inhibited the SsnB-Mediated Downregulation of FucT-V Activity in RA Rats. FucT-V has been reported to participate in the inflammatory response in the synovial tissues of patients with RA. We found that the expression levels of FucT-V and NLRP3 were both elevated in RA rats (Figure 5(a)). The overexpression of FucT-V catalyzes the formation of sialylated Lewis sugar (SLeX) on the hydroxyl group of N-acetylglucosamine [32]. SLeX, a ligand for molecules associated with cell adhesion, enhanced the expression levels of E-Selectin and P-Selectin (Figure 5(b)) and promoted leukocytes to roll on to the endothelial cells, thereby penetrating the blood vessels at sites of inflammation. SsnB also downregulated the expression levels

of FucT-V and NLRP3, thereby inhibiting the expression of E-Selectin and P-Selectin (Figure 5(b)). Interestingly, the NLRP3 inflammasome activator, Nigericin, was administered as the treatment of SsnB to activate the NLRP3 inflammasome [33]. In the present study, we demonstrated that the protective effects of SsnB were remarkably suppressed, thus resulting in the increased release of ROS (Figure 5(c)) and a higher rate of apoptosis (Figure 5(d)).

3.6. SsnB Inhibited the Apoptosis of Synovial Cells in CIA Rats, Reduced Cell Pyroptosis, and Blocked the Activity of α 1,3-Fucosyltransferase In Vitro. To further investigate the effect of SsnB on the synovial cells of CIA rats, we further analyzed cellular apoptosis, the activity of α 1,3-fucosyltransferase, and the pyroptosis-related phenotype. SsnB treatment significantly inhibited the expression of several pyroptosis-related proteins, including NLRP3, pro-IL-1 β , and pro-caspase-1 (Figure 6(a)). SsnB treatment also downregulated the expression of the ASC (Figure 6(b)). TUNEL assays also showed that SsnB treatment reduced the levels of cellular apoptosis (Figure 6(c)). In order to discover the mechanism underlying the action of SsnB, we performed ELISA to detect the levels of α 1,3-fucosyltransferase in cell supernatant; we found that the levels of α 1,3-fucosyltransferase decreased following SsnB treatment (Figure 6(d)).

4. Discussion

RA is an autoimmune disease that is primarily characterized by chronic synovial inflammation and the destruction of cartilage and bone. RA is also characterized by the swelling of synovial endothelial cells, the infiltration of inflammatory

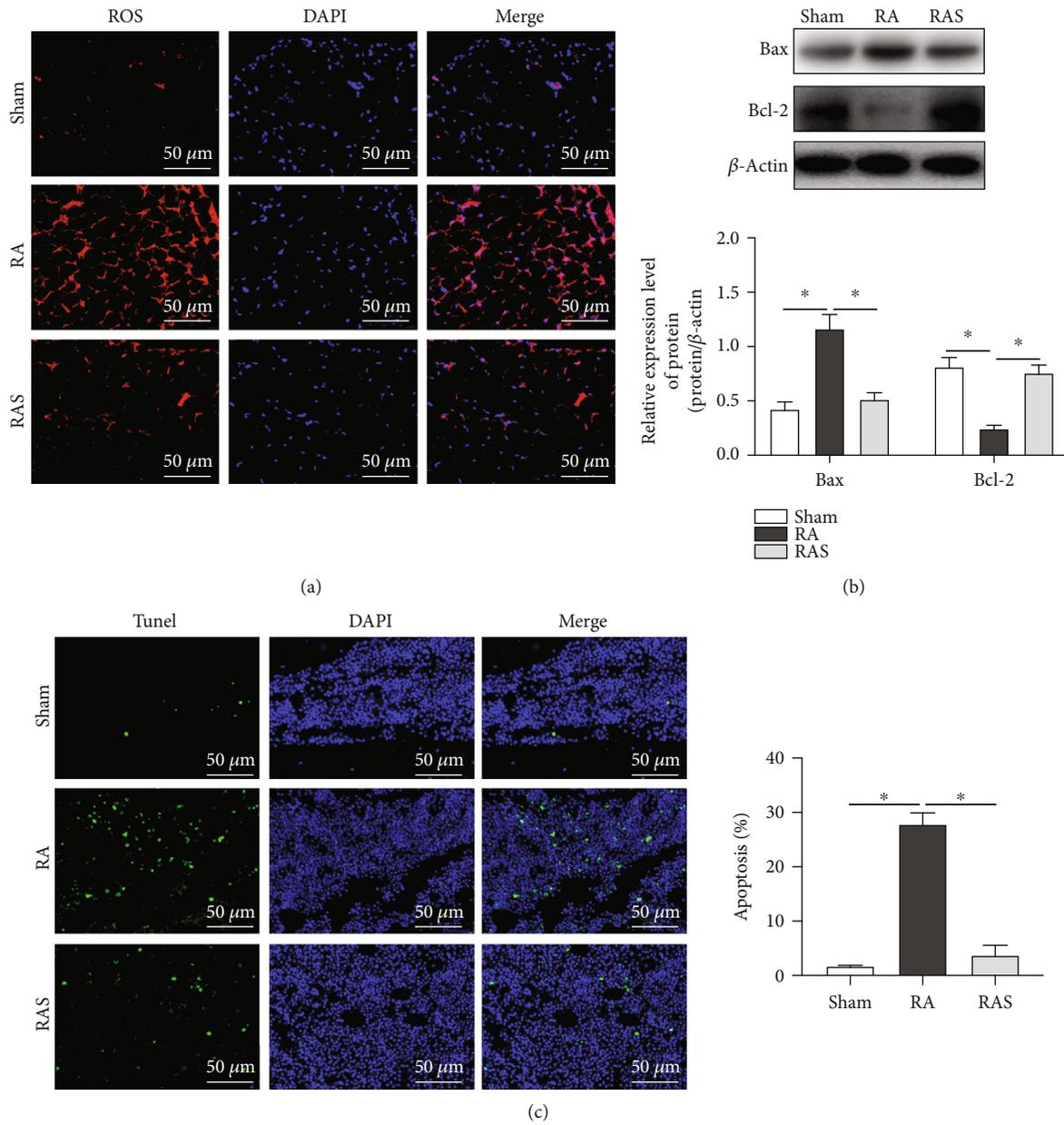


FIGURE 2: SsnB inhibits oxidative stress injury and reduces RA arthritis cell apoptosis. (a) Expression of ROS detected by IF (scale bar = 50 μm). (b) Expressions of apoptosis-associated proteins detected by western blotting. (c) Cell apoptosis rate examined by TUNEL staining (scale bar = 50 μm). All the data were represented as the mean ± SD; **p* < 0.05.

cells, the thickening of synovial tissues, and the formation of abundant villi that protrude towards the joints. These villi form in the cavity, cartilage, and subchondral bone and erode the joint cartilage and subchondral bone by releasing hydrolytic enzymes, thus resulting in the narrowing of joint spaces and fibrous rigidity. These processes cause dysfunction and destruction in the joints. Studies have shown that a large number of inflammatory factors can exacerbate the inflammation and bone destruction in RA patients by destroying the microenvironment in the synovial tissue during the pathological process of RA [34, 35]. In this study, we showed that SsnB effectively reduced arthritis injury in RA rats, inhibited synovial inflammation, and reduced oxidative stress injury.

Furthermore, FucT-V may play an important role in the process of RA synovial inflammation. The mechanisms involved in this process are highly correlated to the NLRP3 inflammasome which regulates cellular pyroptosis.

SsnB is a new natural product isolated from Jing-San-Leng and acts as a selective inhibitor of the TLR2 and TLR4 pathway. SsnB is a selective inhibitor of the TLR2 and TLR4 pathways. It has been reported that SsnB can bind to the TLR domains of TLR2 and TLR4 on cell membranes to block the recruitment of the MyD88 downstream adaptor protein by TLR2 and TLR4, thus leading to the interruption of the TLR2 and TLR4 pathway cascades, resulting to block forming a common MYD88-dependent NF-κB pathway,

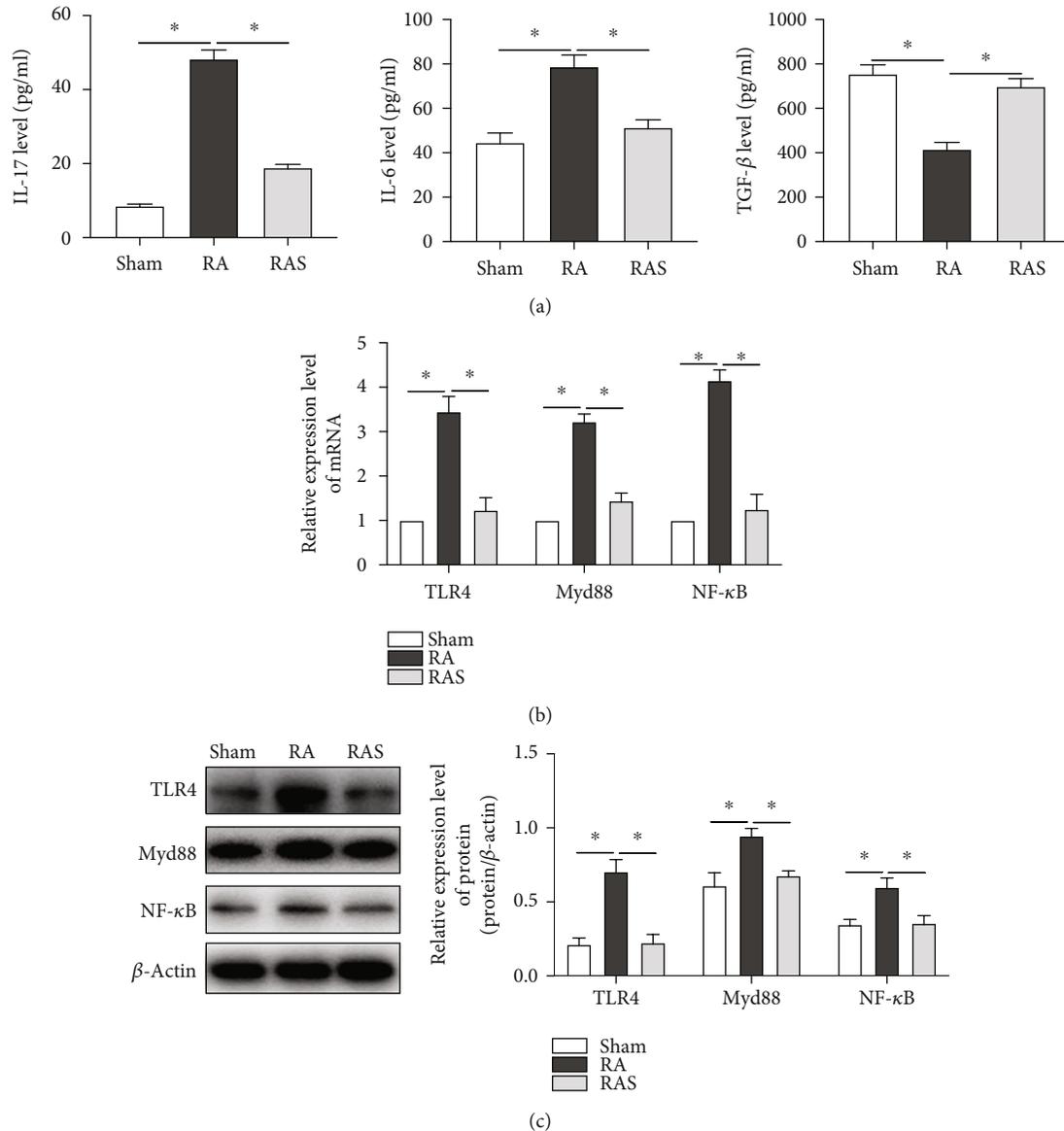


FIGURE 3: SsnB inhibits the inflammatory responses in RA rats through downregulating the TLR4/MYD88/NF- κ B pathway. (a) Contents of cytokines detected by ELISA assay; mRNA and protein expressions of TLR4, MYD88, and NF- κ B detected with qRT-PCR (b); and western blotting (c), respectively. All the data were represented as the mean \pm SD; * p < 0.05.

thereby reducing the production of cytokines and exhibiting vigorous anti-inflammatory activity [20, 36]. The TLR4/MYD88/NF- κ B pathway is known to be one of the most important signaling pathways in the mediation of inflammation [37]. In this study, we found that SsnB inhibited the release of related inflammatory factors by downregulating the expression levels of TLR4, MyD88, and NF- κ B, in RA rats.

Evidence also indicates that an imbalance of Th1/Th2 and Th17/Treg cells may be a significant underlying factor in the pathogenesis of RA. In the present study, we also discovered that SsnB can suppress the secretion of IL-17 and TGF- β from Th17 and Treg cells, respectively, thus indicating that SsnB has strong therapeutic significance with regard to rescuing inflammatory response in RA.

It is reported that inflammasomes are closely related to various immune-inflammatory diseases and metabolic diseases. The NLRP3 inflammasome is a multiprotein complex composed of NLRP3, ASC, and pro-caspase-1; this is the primary protective line in the human body [38]. The activation of caspase-1 after the complete assembly of NLRP3 inflammasome is the primary method with which to initiate cell pyroptosis. Cell pyroptosis differs from apoptosis in that it does not depend on caspase-3 and caspase-6. Instead, cell pyroptosis causes DNA breakage and attacks the cell membrane, thus increasing the difference between intra-/extracellular osmotic pressure, thus leading to inflammatory cell death. As a result, a large number of inflammatory factors in the cell, including IL-1 β and IL-18, would be released to the extracellular environment, thus leading to an expanded

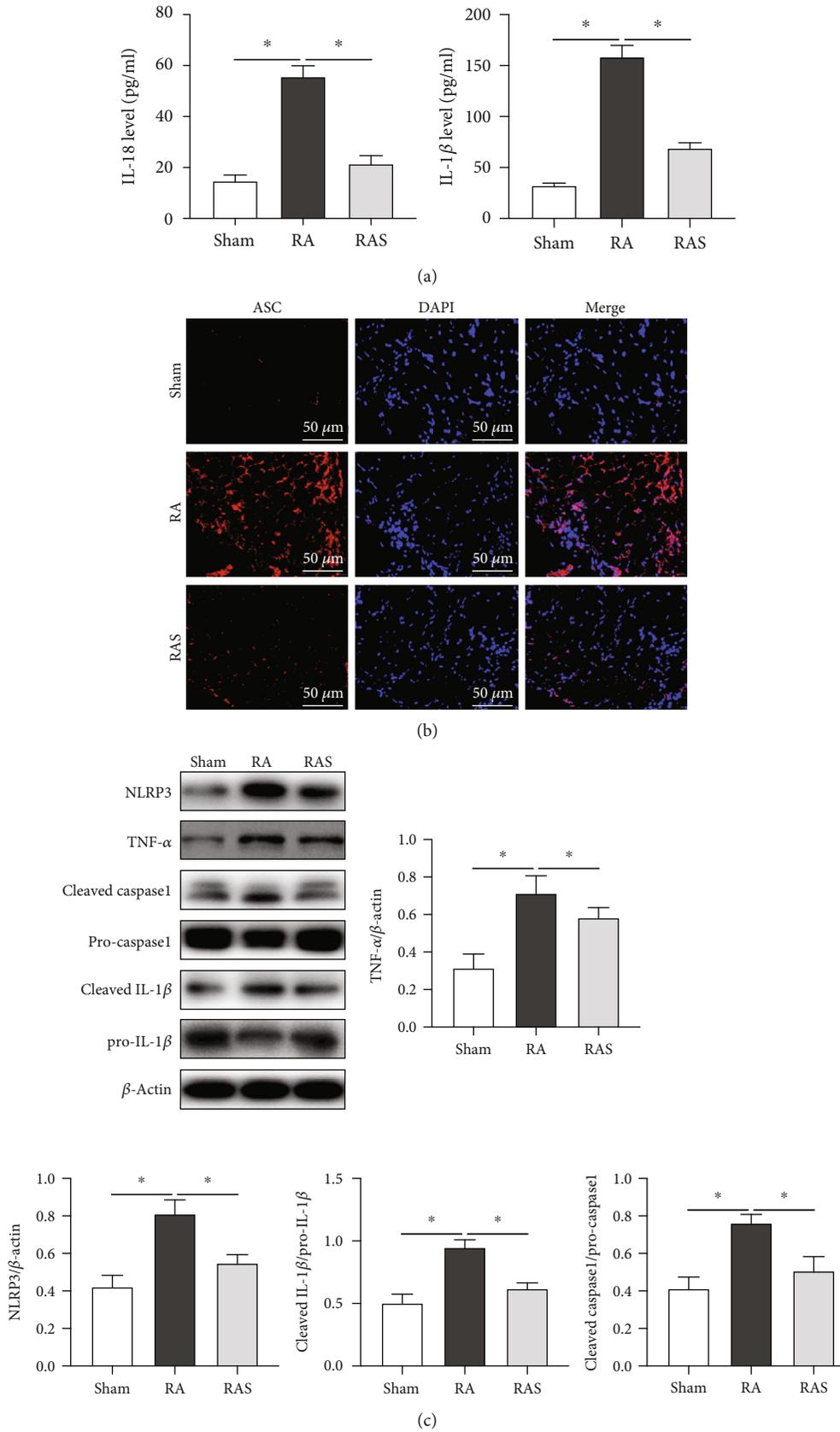
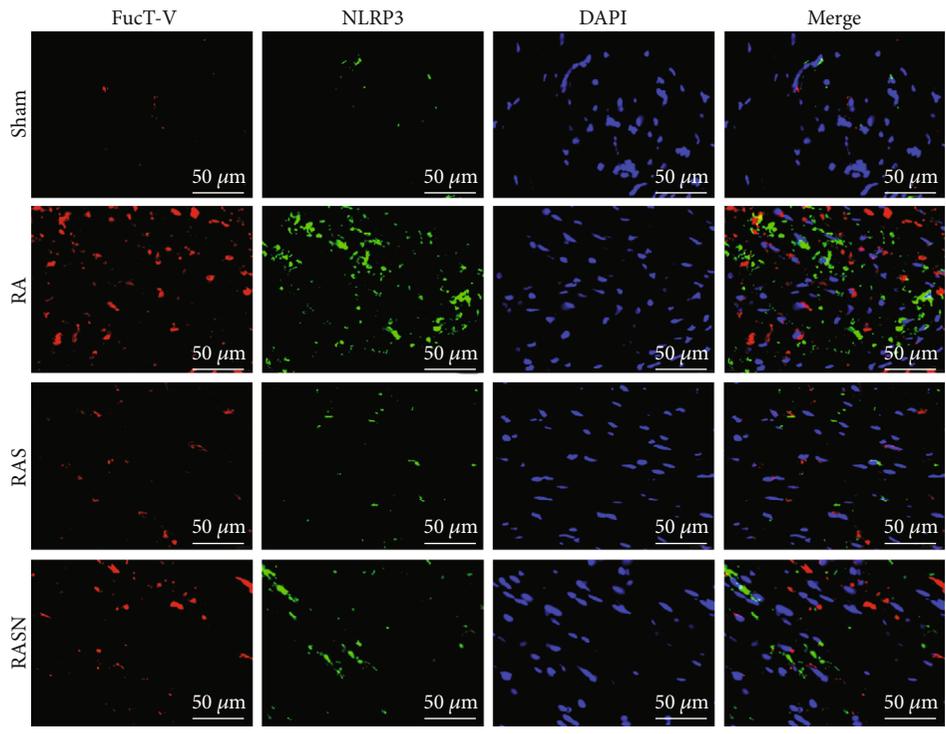
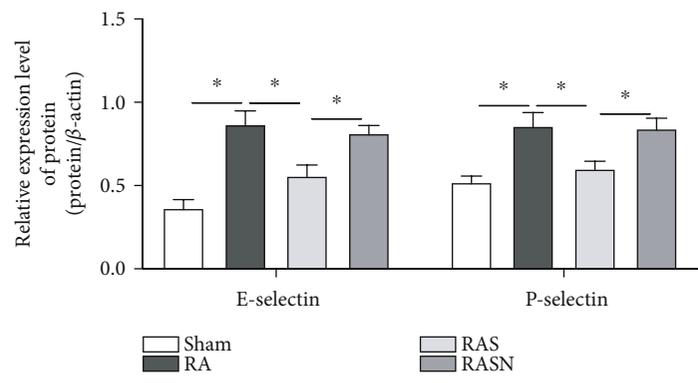
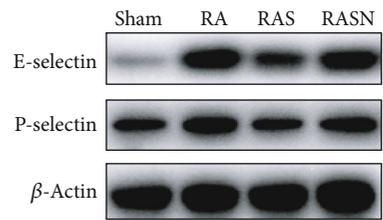


FIGURE 4: SsnB inhibits RA cells pyroptosis through downregulating NLRP3 inflammasome. (a) Levels of IL-18 and IL-1 β detected by ELISA assay. (b) Formation of ASC spots observed by IF (scale bar = 50 μ m). (c) Western blotting for detection of expressions of pyroptosis-associated proteins. All the data were represented as the mean \pm SD; * p < 0.05.



(a)



(b)

FIGURE 5: Continued.

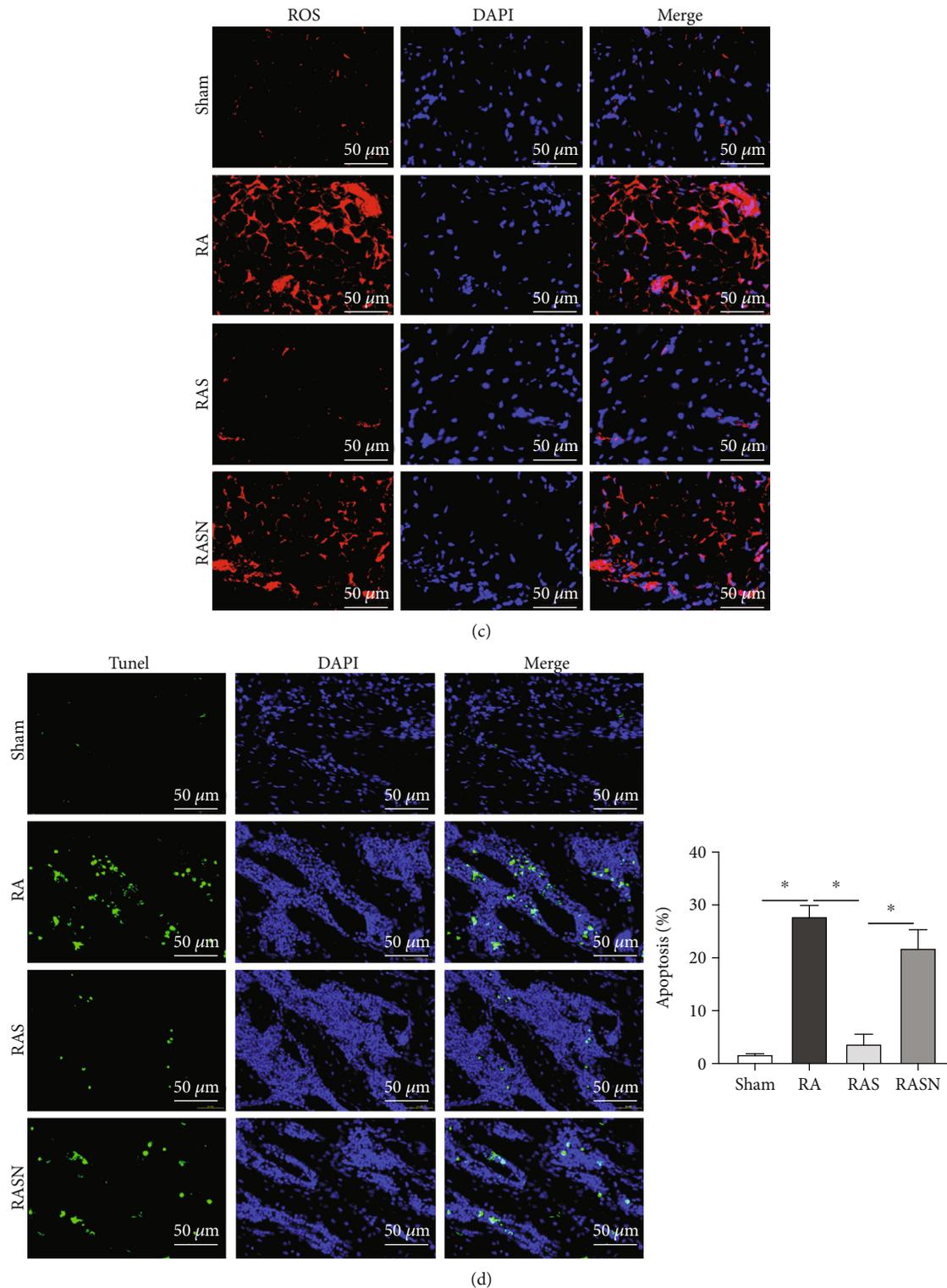


FIGURE 5: Activating NLRP3 inhibits SsnB downregulating the FucT-V activity in RA rats. (a) Expressions of FucT-V and NLRP3 detected by IF (scale bar = 50 μm). (b) Expressions of adhesion-associated proteins detected by western blotting. (c) Expression of ROS observed by IF (scale bar = 50 μm). (d) The cell apoptosis rate examined by TUNEL staining (scale bar = 50 μm). All the data were represented as the mean \pm SD; * $p < 0.05$.

cascade of inflammatory responses. In this study, we proved that RA could promote the release of ROS, one of the known intermediate products that can activate NLRP3. Spots were

formed with ASC after the activation of NLRP3, thus indicating that cell pyroptosis plays a vital role in the occurrence and progression of RA. Simultaneously, we also discovered that

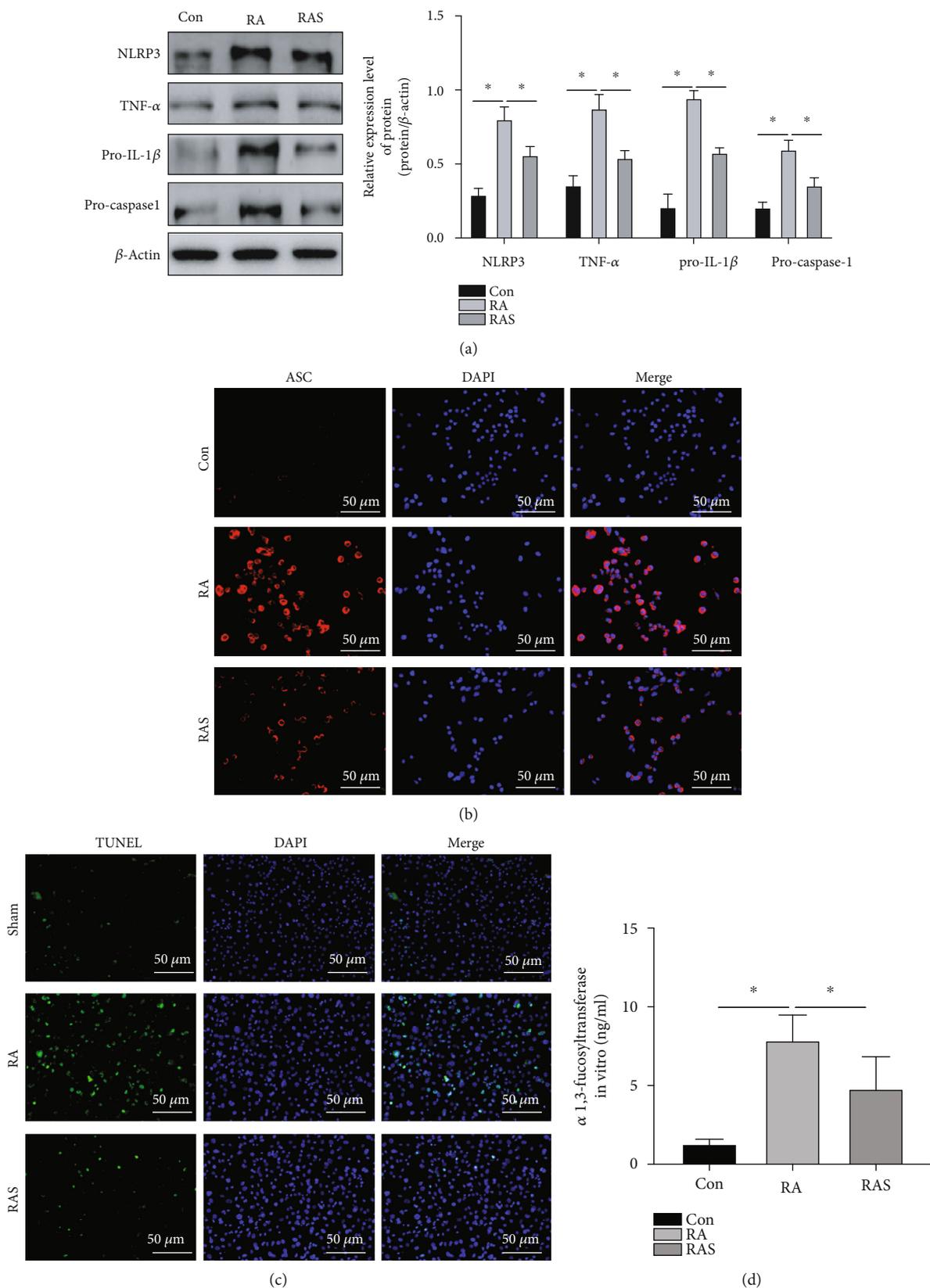


FIGURE 6: SsnB inhibits the apoptosis of synovial cells and reduces cell pyroptosis and blocks the activity of α 1,3-fucosyltransferase in vitro. (a) The expression of pyroptosis-related proteins NLRP3, pro-IL-1 β , and pro-caspase-1 detected by western blot. (b) Formation of ASC spots observed by IF (scale bar = 50 μ m). (c) Cell apoptosis rate examined by TUNEL staining (scale bar = 50 μ m). (d) The level of α 1,3-fucosyltransferase in cell supernatant examined by Elisa. All the data were represented as the mean \pm SD; * $p < 0.05$.

SsnB can downregulate the expression of NLRP3 and suppress the downstream cascade inflammatory reaction, thus suggesting that SsnB can inhibit pyroptosis in RA cells by downregulating the expression of the NLRP3 inflammasome.

FucT-V, a proinflammatory factor involved in immune responses and inflammation, has been shown to play a significant role in cell adhesion, inflammatory progression, and leukocyte transportation. FucT-V catalyzes the hydroxyl groups on L-fucosylation and sialylated N-acetylglucosamine based on GDP-fucose as a substrate to form SLeX which serves as a ligand for cell adhesion molecules, including E-Selectin and P-Selectin [32]. During inflammation and immune responses, selectin on the endothelial cell surface, along with sugar-ligands expressed on the surface of leukocytes, is combined. This causes the leukocytes to roll onto the endothelial cells, thus penetrating the blood vessels at the inflammatory sites. The successful completion of this process is highly correlated to the processing and modification of glycosyltransferase. The abnormal expression of FucT-V activity is also closely related to immune diseases. It has been reported that the expression levels of E-Selectin and P-Selectin are significantly increased in patients with RA [39]. In this study, we demonstrated that FucT-V coexpressed with NLRP3 and that this coexpression increased by upregulating the expression levels of E-Selectin and P-Selectin. In contrast, the administration with SsnB reduced the expressions of related proteins. Notably, the protective effects of SsnB were suppressed following treatment with a NLRP3 inflammasome activator, thus indicating that SsnB alleviates RA by inhibiting the NLRP3 inflammasome and downregulating the expression of FucT-V, thereby inhibiting inflammatory responses and oxidative stress injury.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no competing interests.

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