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ORIGINAL ARTICLE

Sinomenine Activates Gut Innate Immune Response through the Aromatic Hydrocarbon Receptor by Regulating the IL-23/IL-17 Axis

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ABSTRACT

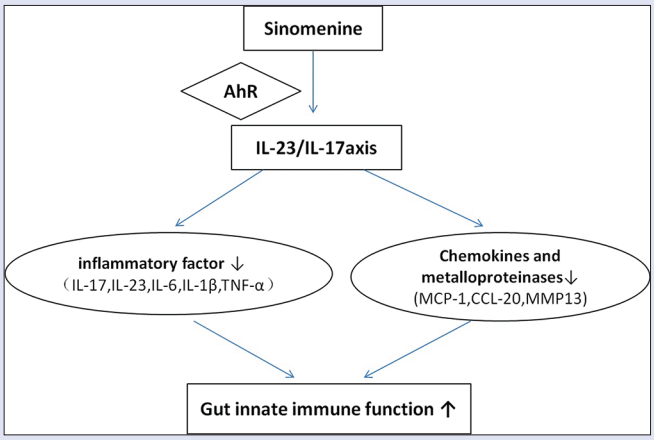
Background: Sinomenine (SIN) plays a role in regulating intestinal immune inflammation, but its effect on the intestinal immune response is unclear. **Objective:** To investigate the potential mechanism of SIN in protecting intestinal immunity. **Materials and Methods:** The mechanism by which SIN regulates intestinal immunity was detected in RAW264.7 cells using enzyme-linked immunosorbent assay, real-time quantitative polymerase chain reaction, Western blotting, and immunohistochemical staining. **Results:** Compared with the control group, the LPS group has higher cell viability and inflammatory cytokines (interleukine-1beta [IL-1β], tumor necrosis factor α[TNF-α], IL-6, IL-17A, and IL-23), chemokine, and metalloproteinase levels. SIN significantly suppressed these increases. By contrast, aromatic hydrocarbon receptor (AhR) and IL-10 levels were lower in the LPS group compared with the control group, and SIN treatment prevented increased these levels. **Conclusion:** SIN can activate the innate immune function of the intestinal tract by affecting the IL-23/IL-17 axis through the AhR.

Key words: AhR, IL-23/IL-17 axis, innate immune, intestinal immunity, sinomenine

SUMMARY

- Studies have demonstrated the role of SIN in regulating the balance of intestinal inflammatory factors and antiinflammatory factors. SIN could suppress MMP-2 and MMP-9 production by inhibiting TNBS-induced colitis in rats. In this study, we found that SIN reduces the secretion of inflammatory cytokines, such as IL-6, IL-23, and IL-17, and affects the expression of chemokines CCL-20, MCP-1, and MMP-13 in macrophages of intestinal innate immune cells. We determined that SIN affects macrophages through the AhR, thereby affecting the expression of various cytokines, chemokines, and metalloproteinases involved in intestinal innate immunity.

Abbreviations used: SIN = Sinomenine, ELISA = enzyme-linked immunosorbent assay, IF = immunofluorescence, real-time PCR = real-time polymerase chain reaction, WB = Western blotting, AhR = aromatic receptor, SD = Standard deviation, DMEM = Dulbecco's modified Eagle's medium, FBS = fetal bovine serum, CCK8 = cell counting Kit-8, TBST = TBS + Tween 20, and HRP = horseradish peroxidase.



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INTRODUCTION

Inflammatory Bowel Disease (IBD) indicates chronic bowel inflammation of unknown aetiology, including ulcerative colitis and Crohn's disease (CD). The knowledge of the immune factors involved in IBD pathogenesis is clear. Th17 cells, which mainly secrete interleukin-17 (IL-17), are a subgroup of T helper (Th) cells distinct from the Th1 and Th2 cell subsets. The discovery of Th17 cells has aided in explaining some abnormalities in the Th1/Th2 axis. IL-17 promotes inflammation by combining the adaptive immune system with the innate immune system. IL-23, a cytokine discovered in 2000, is a key factor in regulating the interaction between the intestinal innate and adaptive immune systems. Animal experiments and clinical studies have demonstrated that the IL-23/IL-17 axis plays a key role in IBD pathogenesis, thus exerting its immunological effects.

Sinomenine (SIN) is an alkaloid monomer extracted from the dried stem of *Anemone* spp. In clinical practice, SIN is used in the treatment of kidney disease, rheumatoid arthritis, lupus erythematosus, and other immune-related diseases with high efficacy. The role of SIN in regulating the balance of intestinal inflammatory

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factors and antiinflammatory factors is well supported. Although an increasing number of studies have investigated the role of SIN in intestinal immune inflammation in IBD, research on its role in antiinflammatory immunity and molecular gene levels remains in its infancy.

Macrophages play a crucial role as the first line of defence in the body's innate immune system. Due to their role in immune surveillance, macrophages can sense a broad spectrum of stimuli, including viruses, foreign compounds, parasite antigens, immune complexes, dead cells, and various regulatory factors secreted by other cells. In response to these stimuli, macrophages are activated (i.e., they enter a state of defence against a pathogen or other factor) before performing immune regulatory functions and maintaining tissue integrity. Macrophages in the lamina propria of CD patients produce large amounts of IL-23.[1] Recent studies have shown that IL-23 may play a key role in autoimmune diseases by promoting IL-17 secretion from Th17 cells. IL-17 can activate macrophages, which secrete various inflammatory factors and act on the surrounding cells to amplify the inflammatory response.[2] The aromatic hydrocarbon receptor (AhR) is a ligand-activated transcription factor of the PAS subfamily of the HLH superfamily. Hayashi *et al.*[3] first reported AhR expression in mononuclear macrophages in 1995. SIN can alleviate rheumatoid arthritis by interfering with regulatory T-cell production through aromatics receptors.[4] In this study, we evaluated the role of SIN in macrophages among innate immune cells. By interacting with aromatic receptors on macrophages and influencing their effects on other innate immune cells, SIN regulates the IL-23/IL-17 axis; inflammatory factors such as IL-6, IL-23, and IL-17; and chemokines CCL20, MCP-1, and MMP-13.

MATERIALS AND METHODS

Cell culture and treatment

The mouse macrophage line RAW264.7 (Zhongyuan Biotechnology, Beijing, China) was cultured in DMEM complete medium containing 10% (Gibco, USA) and 100 g/mL penicillin and 100 U/mL streptomycin antibiotics (Gibco) at 37°C and 5% CO_2.

Experimental groups

A control group, LPS group, and LPS+SIN group were established. When the cultured cells grew to a 70% fusion state, LPS (100 ng/mL) was added to the LPS group for immune stimulation, LPS and SIN at various concentrations were added to the LPS plus SIN group, and neither LPS nor SIN was added to the control group. The cells were cultured at 37°C in 5% CO_2 for follow-up tests.

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Japan) was used for the cell proliferation assay. Cells were inoculated at a concentration of 3×10^5/mL/well on 96-well plates. Each cell contained eight parallel holes, and different concentration of SIN (0.01, 0.05, 0.125, 0.25, 0.5, 1, and 2.5 μm) were added at multiple time points of 6, 24, 48, and 96 h. After the CCK-8 reagent was added, cells proliferated on the multifunctional enzyme marker. After 2 h, the OD value was measured at 450 nm, and the results of the control group, the LPS group, and the LPS+SIN group were compared at each time point. The appropriate concentration and stimulation time was determined for the following experiments based on the cell survival rate.

Enzyme-linked immunosorbent assay

Logarithmic-phase RAW264.7 cells were added to 12 wells at a concentration of 3×10^5/mL/well. Each cell group was grown to 60% confluency to prepare for treatment with different concentrations of SIN at specific time points. LPS was added to the LPS and LPS+SIN groups at a concentration of 1μg/mL. The supernatant was collected and cells were incubated for 24 h at −20°C, according to the manufacturer's instructions.

Real-time polymerase chain reaction analysis

After RAW264.7 cells were cultured for 24 h, the supernatant was discarded and trizol was added to cells (1 mL/group) at − 70°C after processing. Primers (Shanghai Jima Biological) were added to RAW264.7 cells to extract total RNA, which was reverse transcribed to cDNA in 20 μL; 5 μL of cDNA was used as a template to join the family of cytokines and chemokines (AhR, IL-1β, IL-6, IL-17, IL-23) upstream and downstream of the primers. Polymerase chain reaction amplification was run in a 50 μL system.

Western blot analysis

Proliferating RAW264.7 cells were added at a concentration of 3×10^5/mL to a 35-mm dish. Experimental groups were used as references, and the cell culture for each group was grown to a 60% fusion state. SIN at various concentrations was added to the LPS + SIN group at the specified time points. LPS was added to the LPS and LPS + SIN groups at a concentration of 100 ng/mL. No LPS or SIN was added to the control group. Cells continued to develop for 24 h, supernatant, after which cells were collected and Western blotting was used for detection of MCP-1, CCL20, and MMP-13 protein expression.

Immunohistochemical staining

The intestinal tissues from different groups were fixed in 4% paraformaldehyde for at least 6 h, embedded in paraffin, and then cut into 5-mm-thick slices for immunohistochemical staining. The first antidilution factor (rabbit IgG: AhR-pastes 1:50) and DAB color rendering time were obtained under the microscope. Image-Pro Plus (version 6.0), an image acquisition software program, was used to collect images. At high magnification (400×), five representative fields were randomly selected from each slice. IOD/area (density mean) within the region was selected.

Statistical analysis

SPSS (version 13.0) was used, and the results were expressed as the mean ± standard error. The difference of population means was tested by one-way ANOVA. $P < 0.05$ or $P < 0.01$ was considered statistically significant.

RESULTS

SIN altered cell proliferation

Effects of SIN on RAW264.7 cell proliferation. Growth rate of RAW264.7 cells after SIN treatment at different concentrations (0, 1, 5, 10, 50, 125, 250, and 500 μg/mL) for 24 h measured by 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide assay. As shown in Figure 1. Therefore, we selected 125μg/mL, 24 h stimulated for further experiments.

SIN altered the contents of cytokines IL-1β, TNF-α, IL-6, IL-17A, and IL-23

Effects of SIN on the expression of the proinflammatory cytokines (IL-6, IL-1β, IL-17A, TNF-α, and IL-23) in RAW264.7 cells.

Cytokines production in RAW264.7 cells before and after LPS treatment measured using ELISA. As shown in Figure 2, compared with the control group, IL-6, IL-1β, IL-17a, TNF-α, and IL-23 levels were increased in the model group and decreased in different concentrations of SIN. The results showed that SIN inhibited the production of proinflammatory cytokines and had antiinflammatory effect.

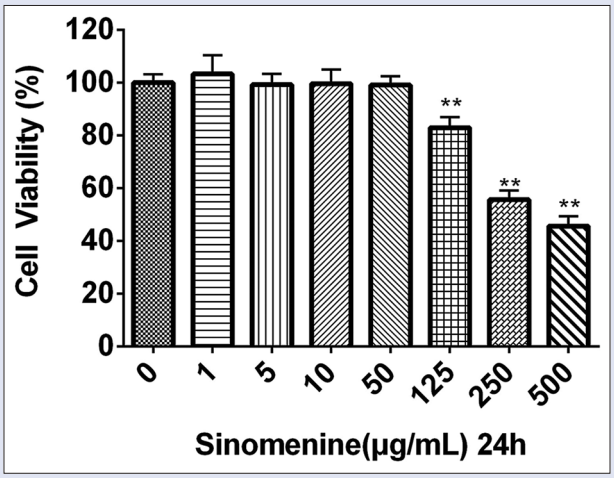

Figure 1: Effects of SIN on RAW264.7 cell proliferation. Growth rate of RAW264.7 cells after SIN treatment at different concentrations (0, 1, 5, 10, 50, 125, 250, and 500 μg/mL) for 24 h measured by 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide assay. $^{**}P < 0.01$. The statistical analysis was performed using one-way ANOVA. (131.6 mm)

Differential levels of AhR, IL-23, IL-6, IL-10, and IL-1β in each group

It has been reported that the expression of AhR in mononuclear macrophages. Therefore, in this study, we studied whether SIN affects the expression of the Th17 cell-related factor by AhR receptor and determination effect of SIN on AhR, IL-23, IL-6, TNF-α, IL-12, IL-10, IL-1β, and IL-17 levels in RAW264.7 cells through real-time polymerase chain reaction. As shown in Figure 3, in the model group, the expression of IL-10 decreased, while the expression of AhR, IL-23, IL-6 and IL-1β increased. After SIN treatment, the expression of AhR and IL-10 increased, while the expression of proinflammatory factors IL-23, IL-6, and IL-1β decreased.

Differential expression of chemokines and metalloproteinases in each group

To determine whether SIN can prevent the production of CCL-20, MCP-1, and MMP-13 by inhibiting the production of cytokines, reduce the chemotaxis and infiltration of inflammatory cells, inhibit the excessive degradation of extracellular cells, and maintain the intestinal mucosal barrier. As shown in Figure 4, compared with the control group, the protein expression levels of CCL-20, MCP-1, and MMP-13 in the model group increased, while the protein expression levels of CCL-20, MCP-1, and MMP-13 decreased after treatment with different concentrations of SIN.

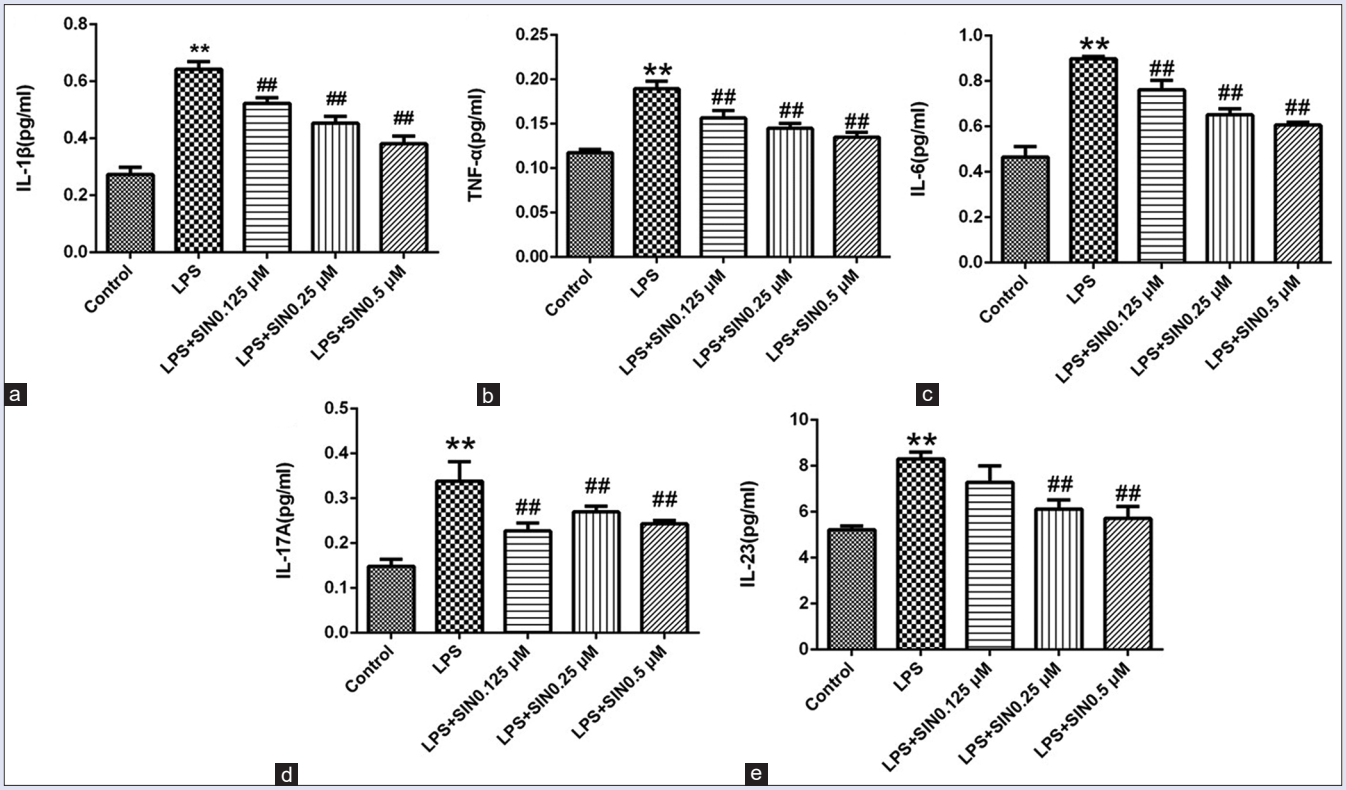

Figure 2: Effects of SIN on the expression of the proinflammatory cytokines such as (a) IL-1β content (b) TNF-α content (c) IL-6 content (d) IL-17A and (e) IL-23 in RAW264.7 cells. Cytokines production in RAW264.7 cells before and after LPS treatment measured using ELISA. Values are expressed asmean ± standard error of the mean (n = 5). $^{**}P < 0.01$ compared with normal controls; $^{\#\#}P < 0.01$ compared with the LPS control. (239.1 mm)

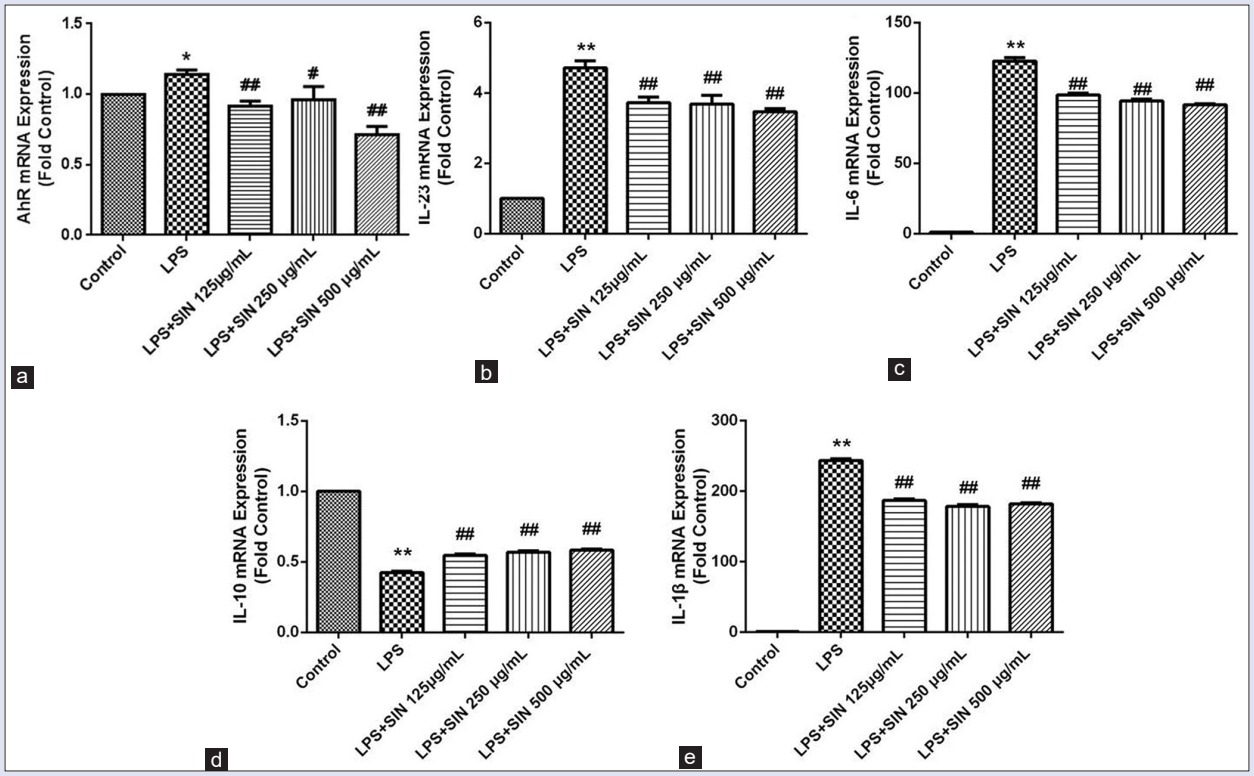

Figure 3: Determination effect of SIN on AhR, IL-23, IL-6, TNF-α, IL-12, IL-10, IL-1β, and IL-17 levels in RAW264.7 cells through real-time polymerase chain reaction (*$P < 0.05$ compared with normal controls; ** $P < 0.01$ compared with normal controls; ##$P < 0.01$ compared with LPS control). (a) SIN reduced IL-23 expression in a dose-dependent manner compared with LPS. (b) SIN treatment increased IL-10 RNA levels in a dose-dependent manner compared with LPS. (c) IL-6 RNA levels showed a stepwise decline as the concentration of SIN treatment increased compared with the LPS group. (d) IL-1β RNA in cells treated with LPS. (e) Treatment with SIN increased AhR RNA levels dose dependently compared with LPS. (236.4 mm)

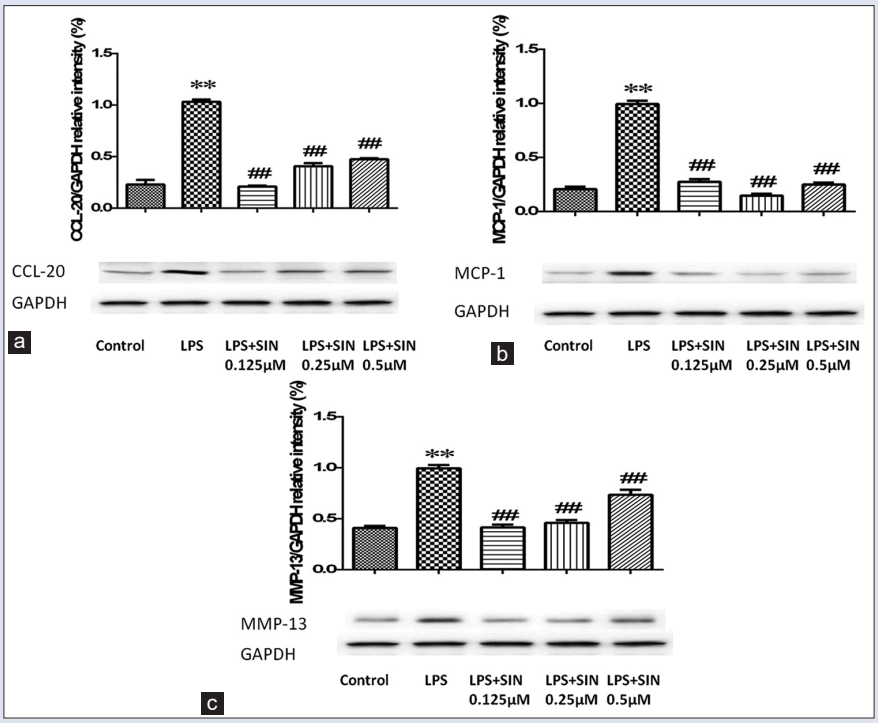

Figure 4: Determination effect of SIN on chemokines and metalloproteinases using Western blot analysis and normalized to GAPDH. (a) CCL-20, (b) MCP-1, and (c) MMP-13 in RAW264.7 cells. The levels of CCL-20, MCP-1, and MMP-13 expression were detected by Western blot analysis and normalized to β-actin. Values are expressed as mean ± standard error of the mean ($n = 3$). **$P < 0.01$ compared with normal controls; ##$P < 0.01$ compared with LPS control. (230 mm)

Differential expression of AhR translocation in each group

We further demonstrated the intestinal protective effect of SIN through AhR receptor in vivo. As shown in Figure 5, the expression level of AhR in the model group was increased compared with that in the control group, and the expression level of AhR decreased after SIN administration.

DISCUSSION

SIN, an alkaloid monomer extracted from the orientvine of *Stephania* in the Menispermaceae family, has antiinflammatory properties; moreover, it inhibits cellular and humoral immunity, scavenges free radicals, and has other pharmacological effects. Studies have shown that SIN can influence levels of miRNA-155 and related inflammatory cytokines to ameliorate colitis severity in mice.[5] In the TNBS-induced rat model of colitis, SIN blocked MMP-2 and MMP-9 production by inhibiting the synthesis of TNF-α. It also reduced the chemokines of inflammatory cells and infiltration of colon tissues, inhibited the excessive degradation of ECM, and maintained the function of the intestinal mucosal barrier.[6] The in-depth study of the intestinal immune response to SIN in the IBD model can aid in explaining its pharmacological mechanism further. This study found that LPS stimulated RAW264.7 cells to induce IL-1β, TNF-α, IL-6, IL-17A, and IL-23 production. The expression of the chemokines MCP-1 and CCL20, as well as MMP-13, were also increased. Furthermore, this study found that different doses of SIN inhibited the LPS-induced expression of MCP-1, CCL20, and MMP-13 in a dose-dependent manner. In other words, SIN inhibits the changes of various cytokines, chemokines, and metalloproteinases in macrophages.

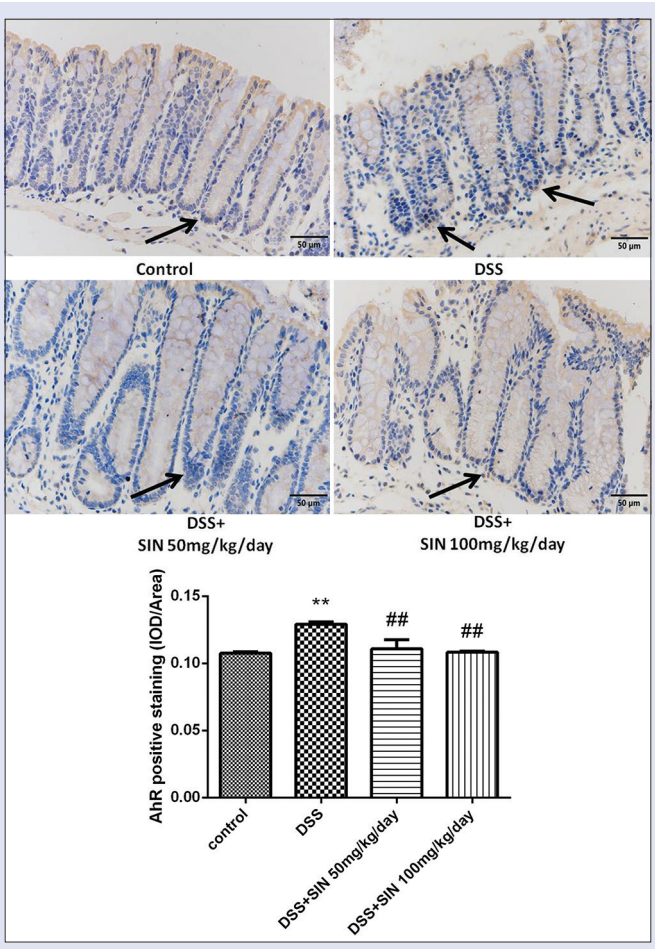

Figure 5: AhR translocation was detected by immunohistochemistry staining using AhR antibodies (original magnification, 400×). Values are expressed as means ± standard error of the mean ($n = 3$). $^{**}P < 0.01$ compared with normal controls; $^{\#\#}P < 0.01$ compared with DSS control. (177.9 mm)

AhR is the only known receptor for dioxin, a potent immunomodulating environmental contaminant. It is now known that AhR exerts an important regulatory effect on the development and function of both innate and adaptive immune cells when it responds to endogenous ligands generated from the host's cells, diet, and microbiota.[7-9] AhR connects signals from the external environment to internal cellular processes with consequences for immune cell function.[10] Although AhR seems to be a crucial cofactor in the regulation of both homeostasis and inflammation, its role in gut autoimmune pathology is poorly described. AhR is mainly expressed in barrier tissues (e.g., gut, skin, and lung tissues) by immune cells such as lymphocytes. The gut is enriched with metabolites derived from either the diet or the microflora, and some of these metabolites can function as AhR ligands, binding to AhR and consequently inducing its nuclear translocation and transcriptional activation. In the present study, we demonstrated that SIN decreased the nuclear translocation of AhR. Thus, we predict that the IL-23/IL-17 axis is activated in macrophages cultured with LPS and contributes to the enhanced nuclear translocation of AhR.

The gut also has a cytokine milieu resulting from cytokine production by immune cells such as dendritic cells, likely in response to gut microbiota.[11] We predict that SIN functions by binding the aromatics receptors of macrophages. Simultaneously, through the expression of the target gene of IL-23/IL-17, SIN also affects macrophage chemokine and metalloproteinase protein expression levels and reduces macrophages invasion.

IL-23 produced by macrophages can activate Th17 cells, a subset of CD4+ T cells that secrete IL-17.[12] These cells are currently the focus of many studies because they play a dominant, detrimental role in many autoimmune inflammatory disorders, such as psoriasis, IBD, and multiples sclerosis.[13-15] The importance of the IL-23/IL-17 signalling axis in autoimmune diseases is also evident from the many scientific studies and ongoing clinical trials that specifically target IL-23 or IL-17.[14] In this study, SIN inhibited the release of IL-23 as well as controlled the levels of IL-1, TNF-α, IL-6, and IL-17A. Interestingly, these inflammatory mediators have been shown to promote the development of Th17 cells, and IL-6 is even involved in the pathological process of Th17 cells.[16-18] IL-1 and IL-23 synergistically promote the production and proliferation of these cells.[12,14,19,20]

In this study, SIN regulated the secretion of various inflammatory cytokines and the IL-23/IL-17 axis by downregulating the aromatics receptors on macrophages, which in turn downregulated chemokines and metalloproteinases, affecting the function of the intestinal innate immune system. In addition, IL-17 is mainly secreted by Th17 cells. Additional studies of the Th17–T-cell interaction is necessary to elucidate the processes of innate and adaptive immunity and to demonstrate the efficacy of SIN.

CONCLUSION

SIN has been used in therapeutic approaches for various diseases in clinical practice. SIN affects the expression of key immune and adaptive immune factors IL-23 and IL-17 through the aromatics receptors on macrophages, thus downregulating chemokine and metalloproteinase expression. These results suggest that SIN can regulate intestinal innate immune function.

Financial support and sponsorship

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Conflict of interest

There are no conflicts of interest.

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