Amelioration of CIA by Asarinin is associated to a downregulation of TLR9/NF-κB and regulation of Th1/Th2/Treg expression

Qiaomei Dai\textsuperscript{a}, Meiqiao Wang\textsuperscript{a}, Yaozhang Li\textsuperscript{a}, Ji Li\textsuperscript{* b}

\textsuperscript{a} Department of pathology, Heilongjiang University of Chinese Medicine, Harbin, China

\textsuperscript{b} Department of Chinese formulae, Heilongjiang University of Chinese Medicine, Harbin, China

*Correspondence to: Ji Li, Department of Chinese formulae, Heilongjiang University of Chinese Medicine, 24 He ping Road, Xiangfang Dist., Harbin 150040, China. TEL: 86-451-82195940. E-mail: 3078763598@qq.com.
Abstract

To study the role of asarinin on collagen-induced arthritis (CIA) and its treatment mechanism on dendritic cells (DCs) and T cells. Before the onset of arthritis, asarinin were given orally to CIA mouse. Macroscopic scoring and micrometer caliper measurement were used to assess arthritis. The occurrence of cartilage destruction and bone erosion were assessed by histology of knee. Sandwich ELISA and PCR were used to assess the level of cytokines in hindpaw and arthritic joint. The CD11c MicroBeads were employed to isolate CD11c+ cells from the spleen. Quantitative PCR was used to determine DCs surface molecules of spleen. Macroscopic score and the frequency of arthritis were inhibited by asarinin. Swelling of hindpaws, inflammatory cell infiltration in the synovium, cartilage destruction, and bone erosion were delayed with asarinin. Asarinin treatment suppressed the expression of Th1 cytokines and increased the levels of Th2 cytokines (IL-10), TGF-β and Foxp3 in the synovium and hindpaw, however T-bet mRNA levels in synovium decreased. Lower expression of TLR9 and NF-kB were found in DCs after asarinin treatment. There was no difference in the expression of ICAM-1, OX40-L, and 4-1BBL in spleen DCs between the asarinin group and model control group. Asarinin can treat CIA. TLR9/NF-κB pathway may be involved in the asarinin treatment of CIA by skewing the balance of Th1/Th2/Treg to a Th2 type.

Key words: asarinin, collagen induced arthritis, dendritic cells, regulatory T cells, T helper cells
Introduction

Rheumatoid arthritis (RA) is a complex autoimmune disease that mainly affects the synovial tissue of the joints of patients, and leads to chronic immune inflammatory reaction in long-term development, and eventually leads to cartilage and bone erosion and joint destruction (1). The cytokines involved in the chronic synovial inflammation included inflammatory cytokines and anti-inflammatory cytokines, and T cells play a critical role on cytokine production. Although the roles of T cells in the pathogenesis of RA are not fully understood, Th1 cytokines (interferon-γ [IFN-γ], tumor necrosis factor-α [TNF-α], interleukin-1 [IL-1]), Th2 cytokines (IL-4 and IL-10), Th17 cytokines (IL-17 and IL-22), and CD4+CD25+FoxP3+ regulatory T cells (Treg cells) have been shown to be the contributing factors that interact with cell surface molecules to activate synovial inflammatory cells, synovial cells, and osteoclasts, leading to the inflammation and destruction of joint (1,2). TGF-β alone induces the Treg transcription factor Foxp3 and is essential for the development of Treg cells in the periphery (3). The differentiation of T cells is mainly related to professional antigen presenting cells (DCs). When DCs detect pathogen-associated molecular patterns or damage-associated molecular patterns and antigen activation signals, they will enter a mature state and reach the secondary lymphoid organs and present the antigens to naive T cells, and drive naive T cells to differentiate into Th cells (4).

Type II collagen is the first collagen fiber identified to induce polyarticular lesions in the experiment, and causes erosive arthritis in DBA/1 mice and primates. This polyarticular lesion is called collagen-induced arthritis. Unlike the onset of RA, CIA is an arthritis that is induced by a strong heterologous antigen, but the erosion of articular cartilage and bone tissue was found in the CIA model, similar to the pathological changes in human RA, and the immune recognition of CII was similar to human RA (5-8).

XiXin, the dried roots and rhizomes of Asarum heterotropoides Fr. Schmidt var. mandshuricum (Maxim.) Kitag., A. sieboldii Miq. var. seoulense Nakai, and A. sieboldii Miq. (Aristolochiaceae), is a common herb used in
China and other countries. Asarinin is one of major active chemical components isolated from Xixin with a molecular weight of 354.35. Similar to its progenitor plant, asarinin has a variety of pharmacological properties, including antipyretic effects, anti-inflammatory effects and immunosuppressive properties (9-12).

The pharmacological characteristics of asarinin prompted us to study the potential effect of asarinin on RA (13). T cells and DCs are involved in the inflammatory response of RA, so we focus our research on the immunoregulatory effects of asarinin on CIA, and the possible roles of T cells and DCs in the pathological process of arthritis.

**Materials and Methods**

*Animals*  DBA/1 male mice were obtained from Shenyang Slac Laboratories (Shenyang, China). The age of 8-10 weeks of DBA/1 mice were immunized to make the CIA model. The mice used in this experiment were treated in accordance with the guidelines of the Animal Ethics Committee of Heilongjiang University of Traditional Chinese Medicine.

*Materials* Freund’s complete adjuvant (FCA) of Sigma and bovine type II collagen of Chondrex were purchased from Beijing Boleide Development of Science and Technology CO., LTD (Beijing, China). TaKaRa RNA PCR Kit (AMV) Ver.3.0 and EX TAQ R-PCR Version 2.1 were purchased from Takara Biomedical Technology (Beijing, China). TRIzol reagent of Invitrogen was obtained from Thermo Fisher Scientific (Shanghai, China). Asarinin was obtained from Chengdu Must Bio-Technology Co., Ltd (Chengdu, China). Primers and probes were purchased from Pharmacia Biotech (Roosendaal, Netherlands); Q-plex array was obtained from Beijing Taize Ruida Technology (Beijing, China). CD11c MicroBeads was obtained from Beijing Beads Biotechnologies Co., Ltd (Beijing, China).

*Induction of CIA* On day 1, The mice were immunized with 100μl of emulsion (100μg of bovine type II collagen) at the base of the tail after the bovine type II collagen was emulsified with FCA(1:1 Vol), and the
intraperitoneal injection was administered to the animals with 100μg of bovine type II collagen on day 21(5).

**Treatment protocol** To study the curative effects of asarinin on RA, CIA mice without symptoms of arthritis on the 21st day following the administration of control, asarinin and MTX groups randomly. Asarinin was administered orally to mouse once every 2 days for 60 days at dosage of 4mg/kg, MTX were injected intraperitoneally daily for 60 days at dosage of 0.2mg/kg, and PBS without collagen II was administered orally to model group and blank group. Two independent observers performed erythema and swelling scores on the mice every other day.

**Assessment of CIA** Arthritis responses was assessed from day 1 of the second immunization, and arthritis was judged visually by redness or swelling of the toes, ankles and knees. Arthritis scoring criteria were from the paper described previously. Arthritis scores were 0, 0.25 or 0.5 respectively, depending on the onset of arthritis, the number of joints involved in arthritis, and the severity of the arthritis. Two independent observers performed erythema and swelling scores on the mice every other day. Two independent observers performed erythema and swelling scores on the mice every other day, and the foot depth was measured every six day (14-16).

**Histology** The mice were anesthetized and killed by ether. Knee joints were removed and fixed in 4% formalin for 4 days. After 5% decalcification of formate, the sample was treated for paraffin embedding. Hematoxylin and eosin (H&E) or Van Gieson stain were used to stain tissue sections (7 pans). The occurrence of cartilage damage and bone erosion was detected by histopathology (15, 16).

**Cytokines measurement of hindpaw** Mice were sacrificed after anesthesia. The hind paws were removed and the protein extracts of the hind paws were homogenized by mixing the joints of DTT and protease inhibitors. According to the manufacturer's instructions, the level of cytokines in the hindpaw protein extract was detected using a Q-plex array by a sandwich ELISA.

**CD11c+DCs acquisition** After the CIA mice were anesthetized and killed, the spleen of mice was taken and
grounded with 200 mesh stainless steel to obtain spleen cell suspension. CD11c + DCs cells were isolated from
the suspension of spleen cells using CD11c MicroBeads according to the manufacturer's instructions (17).

RT-PCR and quantitative PCR  CIA mice were sacrificed after anesthesia, RNA was obtained from synovium
and CD11c+ cell suspension. Reverse transcription-PCR (RT-PCR) and quantitative PCR were used to amplify
related cytokines in synovium of CIA (1, 14-16), and the expression of TLR-9, NF-κB and adhesion molecules
and costimulatory molecules on the surface of DCs was determined by Quantitative RT-PCR(18). Primers for
IL-12, TGF-β, IL-18, Foxp3, TNF-α, TLR9, IL-10, NF-κB, T-bet, ICAM-1, GATA-3, OX-40L, and 4-1BBL
genes sequences for PCR were from the paper described previously or synthesized by Pharmacia Biotech
(1,14,19). Primers sequences were shown in Table I. RT-PCR and quantitative PCR were performed according
to the manufacturer's instructions.

Statistical analysis  Data are presented as the means ± SDs. The statistical significance of differences was
analyzed by Student’s t test, Wilcoxon rank test, and one-way analysis of variance (ANOVA), and a p value
below 0.05 was considered significant.

Results

Therapeutic role of asarinin in collagen-induced arthritis

CIA mice were treated with asarinin before the onset of arthritis, and CIA mice without arthritic symptoms
were chose for experiment on the 21st day of immunization. Asarinin inhibited the onset of CIA and arthritis
symptoms of CIA, and the mean arthritis score and frequency of arthritis significantly decreased than those in
control (Fig. 1a and Fig.1b). The animals in the model control group showed more extreme symptoms than
those in the treatment group (Fig.1c). Joint thickness (paw swelling) significantly decreased than that in control
after asarinin treatment (Fig. 1d).Infiltration of inflammatory cells in the knee and destruction of bone and
cartilage were significantly improved in the asarinin treatment group (Fig. 1e) (Table 2).
**Cytokine protein expression in the hindpaw**

Cytokine protein extracts of the hind paws from CIA mice are presented in Fig. 2. A significant decrease was observed in levels of TNF-α, IL-12 and IL-18 in the asarinin-treated mice compared with that of vehicle-treated animals. After asarinin treatment, levels of TGF-β, IL-10 and Foxp3 increased even more impressively (Fig. 2).

**Asarinin inhibits CIA by skewing the balance of Th1/Th2/Treg to a Th2 type**

In order to further clarify the Th1/Th2/Treg cells in collagen induced arthritis after the treatment of asarinin, mRNA of Th1/Th2/Treg cells related cytokines was extracted from synovial tissue and patellar cartilage on day 81. Cytokine and transcription factor mRNA expression were determined by RT-PCR (Fig.3.a) and quantitative RT-PCR (Fig. 3b) as shown in Fig.3. TNF-α, IL-12 and IL-18 were significantly reduced in asarinin-treated mice compared to the control group. Increased IL-10 and TGF-β mRNA levels were even more impressive (Fig. 3a and Fig. 3b). The expression of Foxp3 was up-regulated after asarinin treatment. A series of intracellular transcription factors is activated, resulting in helper T-cell activation and the differentiation of Th1/Th2, so GATA-3 and t-bet in the synovium were tested. GATA-3 mRNA expression was not different between the two groups (data not shown). However, T-bet mRNA expression was significantly lower in the asarinin-treated mice than in the control group (Fig. 3a and Fig. 3b).

**DCs surface molecules expression**

To illustrate the mechanism of DCs in treatment of CIA with asarinin, we investigated DCs surface molecules expression (Fig. 4). The lower expression of TLR9 and NF-κB was found in asarinin group when compared with model control group, and the expression of ICAM-1, OX40L, and 4-1BBL was not significantly different (Fig. 4).
Discussion

There were significant differences in the asarinin-treated group compared with the control group with respect to mean macroscopic score, frequency of arthritis, swelling of hind paws and the degree of histopathological progress. No significant difference was observed between the asarinin-treated group and MTX-treated group. MTX, a cytotoxic immunosuppressant, was first used to treat tumors in the 1820s, and was used to treat RA in the 1850s. MTX is an anti-rheumatoid drug with an obvious curative effect that plays an important role in the treatment of RA. MTX relieves arthritis by preventing or slowing the destruction of articular cartilage and bone. Asarinin had a definite therapeutic effect on collagen-induced arthritis.

The incidence of RA is related to T cell immune abnormalities. T cells can be classified into helper T cells, such as Th1 cells and Th2 cells, cytotoxic T cells, and regulatory T cells, according to immune effector functions. Th1, Th2, or Treg cells are activated by the initial CD4+ T cell differentiation, and they become functionally and phenotypically different effector T cells (20-22). There are local responses in RA patients with inflamed joints that are Th1-dominant and Th2 deficient. RA is thought to be a disease mediated by the Th1 immune response (23). On the other hand, Treg cells infiltrate the synovial tissue of RA patients, and this infiltration is associated with RA diseases activity. Treg cells in patients with RA are fewer in number and function abnormally (24, 25). Inflammatory cytokines in RA are selectively recruited into the joint cavity, so this study examined various cytokines in the hindpaw and synovium (23, 26). Asarinin, like MTX, significantly reduced the expression levels of IL-18, IL-12 and TNF-α in the hindpaw and synovium. However, the levels of Th2 cytokines (IL-10) were up-regulated, and Foxp3 expression and TGF-β expression in synovial membrane tissue and hindpaw increased. The levels of Th2 cytokines(IL-4 and IL-5) were not amplified by RT-PCR and quantitative PCR (data not shown). A series of intracellular transcription factors is activated, resulting in helper T-cell activation and the differentiation of Th1/Th2, so GATA-3 (Th2-specific transcription factor) and t-bet.
(Th1-specific transcription factor) in the synovium were tested. The results showed that while the levels of GATA-3 were not significantly different among the groups (data not shown), the levels of t-bet were significantly lower in the asarinin group than in the control group. The evidence suggested that in the asarinin group, the activation of the Th1 immune response was inhibited through inhibition of the transcription factor t-bet.

Foxp3 is essential for T-cell homeostasis and is specifically expressed by regulatory T cells in the body (27, 28). The immunosuppression of Treg cells relies on a large quantity of cytokines such as IL-10 and TGF-β (29). The PCR results showed that asarinin significantly increased the expression levels of TGF-β and FOXP3 in the synovium. Regulatory Th2 gene expression was inhibited when GATA-3 combined with FoxP3. The level of GATA-3 decreased as the level of FOXP3 increased due to the interaction of GATA-3 with FOXP3 (30-32). IL-10 and TGF-β expression levels increased after asarinin treatment, which promoted Treg cells by activating the transcription factor FOXP3. The treatment with asarinin increased the number of Treg cells and inhibited the development of inflammation in collagen-induced arthritis.

Intercellular cell adhesion molecule-1 (ICAM-1) is both a cell surface glycoprotein and a costimulatory molecule of the immunoglobulin superfamily, providing signals for cytotoxic T lymphocytes and NK cells. ICAM-1 and (vascular cell adhesion molecule-1) VCAM-1 contribute to angiogenesis and leukocyte migration in RA synovial tissue (33). The TNF/TNFR family members include 4-1BB/4-1BBL, CD27/CD70, ICOS/ICOSL, and OX40/OX40L, which have been reported to play an important role in T cell activation (34). OX-40 is originally thought to be a surface marker activation of CD4+ T cells in rats (35), which is mainly induced at the effector stage of T cells and is mainly expressed on Th2 cells (36). Dendritic cells express TLR9 in cells and specifically identify non-methylated CpG motifs, which has been shown to promote Th1 immune responses in L. major-infected susceptible mice. TLR9 promotes the expression of inflammatory cytokines by
activating NF-κB (37). Previous studies have confirmed that the production of CD4 + CD25 + regulatory T cells was induced by human plasmacytoid DCs activated by CpG oligonucleotide (38). Lupus is prevented by TLR9 signaling by regulating the activity of regulatory T cells (39, 40). Pyrrolidine dithiocarbamate (PDTC) is inhibitor of NF-κB. Experimental autoimmune uveoretinitis (EAU) is a T helper type 1 cell-mediated autoimmune disease. EAU can be inhibited by PDTC (41). E6446 is inhibitor of TLR9. E6446 can alleviate the disease of spontaneous mouse lupus model (42). Lower expression of TLR9 and NF-kB were found in asarinin group in our study. Although the mechanism of action of methotrexate in the treatment of RA has not yet been fully understood, it is recognized as one of the most effective DMARD drugs because of its quick effect, convenient administration, mild side effects and no long-term carcinogenic effects, and related studies have confirmed that MTX can regulate the balance of Th1/Th2/Treg to treat experimental RA(43). Asarinin maybe inhibit CIA by skewing the balance of Th1/Th2/Treg to a Th2 type through inhibiting the activation of TLR9/ NF-κB pathway.

Acknowledgments

This work was supported by the Fund of Heilongjiang Science and Technical Office (QC2011C059), Harbin Science and Technology Bureau of Heilongjiang Province (2012RFQXS020), Postdoctoral Foundation of China and Heilongjiang Province (2013M531081, LBH-Z11004, LBH-Q15138). Excellent Innovative Talents Support Project of Heilongjiang University of Traditional Chinese Medicine (Excellent Young Academic Leader).

Conflict of Interest

The authors declare no conflict of interest.
References


Fig. 1 Therapeutic effect of asarinin. Fig. 1a Frequency of arthritis. Fig. 1b Clinical arthritis score. The mean macroscopic score and frequency of arthritis were monitored throughout the study, with data representing arthritis score and the average incidence of arthritis in each group.*indicates $P<0.05$ between dose group (asarinin or MTX) and model control groups by Wilcoxon rank test. Fig. 1c Paw swelling of control group. Fig. 1d The thickness of
The hindpaw depth was measured by micrometer caliper every six days to observe the effect of asarinin on arthritis responses. Fig. 1e Representative histological findings in the knee joint of CIA mice after asarinin treatment. H&E or Van Gieson stain was used to stain knee joint of CIA collected on day 81. Left: Severe cartilage surface disruption, infiltrate of inflammatory cells, and bone erosion were found in model control group (arrows). Right: CIA mice of asarinin group, the intact cartilage surface is shown to be significantly improved (arrows). Note: C: cartilage, js: joint space, s: Synovium. Left (×10 by H&E), right (×40 by Van Gieson stain).

(Color figure can be accessed in the online version.)
Fig. 2 Regulation of cytokine secretion in the hindpaw in asarinin-treated mice. Levels of IL-12, IL-18, TNF-α, IL-10, TGF-β, and Fox-P3 were determined by using ELISA kits. *indicates \( P<0.05 \) or **indicates \( P<0.01 \) between treated (asarinin or MTX) and model control groups by one-way analysis of variance.
Figure 3

Fig. 3a Detection of expression of cytokines and transcription factors in knee joints by RT-PCR. On day 81, total RNA was extracted from synovial tissue of five CIA mice per experimental group, RT-PCR was used to determine the expression of IL-18, IL-10, Fox-P3, TNF-α, IL-12, TGF-β, and t-bet, and with β-actin as loading control. Fig. 3b Detection of expression of cytokines and transcription factors in knee joints by quantitative RT-PCR. Quantitative RT-PCR was employed to determine the expression of IL-12, IL-18, TNF-α, IL-10, TGF-β, Fox-P3, and t-bet, and with β-actin as loading control. The relative expressions of IL-12, IL-18, TNF-α, IL-10, TGF-β, Fox-P3, and t-bet were obtained from three-separated quantitative RT-PCR analysis (mean±SE), expressed by density histogram data. *indicates $P<0.05$ or **indicates $P<0.01$. 
$P<0.01$ between treated (asarinin or MTX) and model control groups by Student’s t test.
Fig. 4 Effect of asarinin on expression of surface signaling pathways molecules of DCs. The density histogram data showing changes in the expression of ICAM-1, OX-40L, 4-1BBL, TLR9 and NF-κB in DCs of spleen, determined by quantitative PCR analysis at 81 days after immunization. *, *p < 0.05 vs. control by Student’s t test, **p < 0.01 vs. control by Student’s t test.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primers</th>
<th>Antisense Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Sense 5’tgg aat cct gtg gca tcc atct aac c 3’</td>
<td>Antisense 5’taa aac gca gct taa cag tcc g 3’</td>
</tr>
<tr>
<td>IL-12</td>
<td>Sense 5’gac cct gcc cat tga act ggc 3’</td>
<td>Antisense 5’caa cgt tgc ata gta gga teg 3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>Sense 5’gaa gac cct cag gat gge 3’</td>
<td>Antisense 5’cca agg agt tgc cta gta 3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Sense 5’ctg tga agg gaa tgg ggt tt 3’</td>
<td>Antisense 5’cag gga gga gta ctt gaa ggt 3’</td>
</tr>
<tr>
<td>IL-18</td>
<td>Sense 5’acc gaa ttc act gta cca cgg cag taa tac gga 3’</td>
<td>Antisense 5’gcc tct cag gtt aac att cac gat tta tcc cca 3’</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Sense 5’aaa cgg aag cgc atc gaa 3’</td>
<td>Antisense 5’ggg act ggc gag cct tag tt 3’</td>
</tr>
<tr>
<td>t-bet</td>
<td>Sense 5’tca cta agc aag gac ggc gaa t 3’</td>
<td>Antisense 5’ggc tct ggc tct cca tca tca a 3’</td>
</tr>
<tr>
<td>GATA-3</td>
<td>Sense 5’cct tat cca gcc cca ggc aa 3’</td>
<td>Antisense 5’gge ctc gct cta acc ca 3’</td>
</tr>
<tr>
<td>FOX-P3</td>
<td>Sense 5’tca agt acc aca ata tgc gac cc 3’</td>
<td>Antisense 5’gtg ggc gat ggc att ctt c 3’</td>
</tr>
<tr>
<td>TLR-9</td>
<td>Sense 5’ atg gtt ctc cgt cga agg act 3’</td>
<td>Antisense 5’gag gct tca gct cac agg g 3’</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Sense 5’ gga ggc atg ttc ggt agt gg 3’</td>
<td>Antisense 5’ccc tgc gtt gga ttt cgt g 3’</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Sense 5’ gtg atg ctc agg tat cca ccc a 3’</td>
<td>Antisense 5’cac agt tct cca agc aca cgg 3’</td>
</tr>
<tr>
<td>OX40L</td>
<td>Sense 5’aat ctt gaa aac gga gca tca agg c 3’</td>
<td>Antisense 5’cag gca gac ata gat gaa gca c 3’</td>
</tr>
<tr>
<td>4-1BBL</td>
<td>Sense 5’cgg cgc tcc tca gag ata c 3’</td>
<td>Antisense 5’atc cgg aag att aac cgc agg 3’</td>
</tr>
<tr>
<td>Group</td>
<td>Infiltrate</td>
<td>Cartilage destruction</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Control</td>
<td>1.2±0.5</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>MTX</td>
<td>0.7±0.5*</td>
<td>0.21±0.3*</td>
</tr>
<tr>
<td>Asarinin</td>
<td>0.9±0.4</td>
<td>0.19±0.28*</td>
</tr>
<tr>
<td>Sham</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Note: *P <0.05 versus controls. (x±s, n=5)