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Suppression of Th1 and Th2 Immune Responses in Mice by Sinomenine, an Alkaloid Extracted from the Chinese Medicinal Plant *Sinomenium acutum*

Abstract

The present study was designed to investigate the effect of sinomenine (SIN), an alkaloid extracted from *Sinomenium acutum*, on Th1 and Th2 immune responses in mice. For this investigation, mice were s.c. immunized with ovalbumin (OVA) emulsified with complete Freund's adjuvant (day 0). Varying doses of SIN were orally administered daily over a period of 21 days, commencing on day 0. On day 21, anti-OVA IgG and proliferative responses of spleen cells to the antigen were measured. Anti-OVA IgG2a and IFN- γ were measured as indicators of Th1 immune responses and anti-OVA IgG1, IgE, and IL-5 as those of Th2 responses. TGF- β was measured as an indicator of Th3 immune responses. The results showed that treatment with SIN was followed by decreases in anti-OVA IgG and the antigen-specific splenocyte

proliferation. Production of all isotypes of antibodies including anti-OVA IgG2a, IgG1 and IgE as well as secretion of cytokines such as IFN- γ and IL-5 was suppressed by SIN, although the suppression of anti-OVA IgG2a and IFN- γ by the alkaloid appeared to be greater than that of anti-OVA IgG1, IgE, and IL-5. In addition, SIN enhanced the secretion of TGF- β . These results suggest that SIN appears to have suppressive effects on both Th1 and Th2 immune responses. The results also suggest that Th1 responses may be more preferentially suppressed by the *Sinomenium acutum*-derived alkaloid compared to Th2 responses. TGF- β may at least in part contribute to the suppression of Th1 as well as Th2 immune responses.

Key words

Sinomenine · Th1 · Th2 · cytokines

Introduction

CD4⁺ T cells are mainly classified into two subsets, types I (Th1) and II (Th2), on the bases of their distinct pattern of cytokine profiles [1]. Th1 cells predominately secrete cytokines such as IL-2 and IFN- γ involved in cellular immunity, whereas Th2 cells produce cytokines including IL-4, IL-5, and IL-6 implicated in humoral immunity [2]. There have been a number of studies showing that Th1 and Th2 cytokines play an important role in diseases. For instance, IFN- γ is seen in the joints of patients with rheumatoid arthritis (RA), and attenuating the production of IFN- γ ameliorates the severity of the disease [3]. Increased ex-

pression of IL-4 is observed in patients with asthma [4], and neutralization of IL-4 blocks the development of acute airway hypersensitivity in a murine asthma model [5]. Th1 cytokines suppress secretion of Th2 cytokines while Th2 cytokines inhibit production of Th1 cytokines [6], [7]. Therefore, it is anticipated that modulation of the secretion of Th1 and Th2 cytokines influences Th2- and Th1-dominant diseases, respectively.

Sinomenine (7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinan-6-one) (SIN, Fig. 1) is an alkaloid extracted from the Chinese medicinal plant *Sinomenium acutum*. SIN has long been used for the treatment of patients with rheumatic diseases

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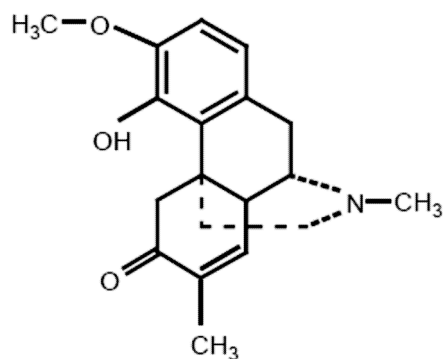


Fig. 1 The chemical structure of sinomenine.

including RA for over 2000 years [8]. Previous studies demonstrated that SIN might have suppressive or therapeutic effects on inflammation, arthritis, immune responses, and arrhythmia [9], [10], [11]. For example, the alkaloid reduces the mRNA expression of proinflammatory cytokines including $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ in rats with adjuvant arthritis [12]. However, little is known about its effect on Th1 and Th2 immune responses in detail.

In the present study, we show that oral treatment of mice with SIN was followed by suppression of both Th1 and Th2 immune responses, although the suppression of Th1 responses by the alkaloid appeared to be greater than that of Th2 responses.

Materials and Methods

Materials

Female DBA/1J mice, 9 to 10 weeks of age, were used in all experiments. The mice were maintained in a temperature-controlled environment with free access to standard rodent chow and water. Experiments were performed in accordance with the ethical guideline of Kobe Pharmaceutical University.

Immunization with ovalbumin

One hundred micrograms of ovalbumin (OVA; Sigma-Aldrich Fine Chemicals; St Louis, MO, USA) were dissolved in 50 μL of phosphate-buffered saline (PBS, $\text{pH} = 7.0$) and emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco Laboratories; Detroit, MI, USA). One hundred microliters of the emulsion were s.c. injected into the base of the tail (day 0).

Administration of sinomenine

Varying doses of sinomenine (SIN, > 99.2% HPLC; Tiancheng Drug and Bio-engineering Co.; Xian, China) including 30, 100 and 300 mg/kg were dissolved in 200 μL of PBS and orally administered daily from days 0 to 20. PBS and the steroidal anti-inflammatory drug dexamethasone (2 mg/kg) (DEX; Sigma) were given as negative and positive controls, respectively. There were no significant differences in body-weight gains between the PBS, SIN, and DEX treatment groups (Table 1).

Proliferation assay

A total of 5×10^5 spleen cells in 0.1 mL of RPMI 1640 (Sigma) containing 1 mM L-glutamine, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 5×10^{-5} M 2-mercaptoethanol and 5% heat-inactivated fetal bovine serum were cultured with 500 $\mu\text{g}/\text{mL}$ of OVA in each well of 96-well tissue culture plates. Thirty-six hours later, each

Table 1 Effect of sinomenine (SIN) on body weight gains

Dose (mg/ kg)	Body weight (g)	
	Day 0	Day 21
0 (PBS)	18.8 $\pm$ 0.6	20.1 $\pm$ 0.7
30	19.7 $\pm$ 0.6	20.6 $\pm$ 0.8
100	18.9 $\pm$ 0.6	20.1 $\pm$ 0.6
300	19.0 $\pm$ 0.9	19.1 $\pm$ 0.8
2 (DEX)	19.7 $\pm$ 0.6	20.4 $\pm$ 0.8

Mice were orally treated with the indicated doses of SIN, daily over a period of 21 days, commencing on the day of immunization with OVA (day 0). PBS and dexamethasone (DEX) were given as negative and positive controls, respectively. Values are the mean $\pm$ s.e.m. of 8 mice.

well was pulsed with 0.5 μCi of tritiated thymidine, and the cells were cultured for another 24 h. The cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques. The values, indicated in cpm, were the average of triplicate cultures of cells pooled from 8 mice.

Measurement of antibodies to OVA

Sera were obtained on day 21 and heat-inactivated at 56 $^{\circ}\text{C}$ for 30 min. Anti-OVA IgG, IgG2a, IgG1 and IgE were measured using an ELISA as described previously [1]. In brief, for the measurement of IgG, IgG2a, IgG1, 96-well flat-bottom microtiter plates were incubated with 100 $\mu\text{L}/\text{well}$ of OVA (100 $\mu\text{g}/\text{mL}$) at 37 $^{\circ}\text{C}$ for 60 min and washed 4 times with PBS containing 0.05% Tween-20. The wells were then blocked by incubation with 150 μL of PBS containing 1% casein (Sigma) at 37 $^{\circ}\text{C}$ for 60 min. After washing, the plates were incubated with 100 μL of a 1 : 125 000 (for IgG and IgG1) or 1 : 1000 (for IgG2a) dilution of each serum sample at 37 $^{\circ}\text{C}$ for 60 min. The plates were washed, and 100 $\mu\text{L}/\text{well}$ of a 1 : 1000 dilution of alkaline phosphatase-labeled rat anti-mouse IgG (Sigma) or 1 : 1000 dilution of alkaline phosphatase-labeled anti-mouse IgG1 and IgG2a (PharMingen; San Diego, CA, USA) were added and incubated at 37 $^{\circ}\text{C}$ for 60 min. After washing, 100 μL of 3 mM *p*-nitrophenyl phosphate (Bio-Rad, Richmond, CA, USA) were added per well, and the plates were incubated in the dark at room temperature for 20 min. The absorbance was then measured at 405 nm using an IMMUNO-MINI NJ-2300 (Nalge Nunc International K.K.; Tokyo, Japan). The values are shown as absorbance units at $\text{OD}_{405} \pm \text{S.E.M.}$

For the measurement of IgE, 96-well flat-bottom microtiter plates were incubated with 100 $\mu\text{L}/\text{mL}$ well $^{-1}$ of rat anti-mouse IgE (Monosan; Uden, Netherlands) at 37 $^{\circ}\text{C}$ for 60 min and washed 4 times with PBS containing 0.05% Tween-20. The wells were then blocked by incubation with 150 μL of PBS containing 1% casein (Sigma) at 37 $^{\circ}\text{C}$ for 60 min. After washing, the plates were incubated with 100 μL of a 1 : 5 dilution of each serum sample at 37 $^{\circ}\text{C}$ for 60 min. The plates were washed, and 100 $\mu\text{L}/\text{well}$ of biotinylated OVA (1 $\mu\text{g}/\text{mL}$) were added and incubated at 37 $^{\circ}\text{C}$ for 60 min. After washing, 100 μL of streptavidin-HRP (horseradish peroxidase streptavidin; Vector Laboratories, Inc.; Burlingame, CA, USA) were added and incubated at 37 $^{\circ}\text{C}$ for 60 min. The plates were washed, and 100 μL of TMB (Pierce Biotechnology, Inc.; Rockford, IL, USA) substrate solution were added to each

well. The plates were incubated in the dark at room temperature for 30 min and stopped by adding 100 μ L of sulfuric acid (1 mol/L) (Wako Pure Chemical Industries, Ltd.; Osaka, Japan). The absorbance was then measured at 450 nm using an IMMUNO-MINI NJ-2300. The values are expressed as absorbance units at OD450 $\pm$ S.E.M.

Measurement of cytokines

Spleens were removed on day 21 and cell suspensions prepared [2]. Spleen cells were depleted of erythrocytes by lysis with Tris-NH₄Cl. A total of 5×10^5 spleen cells in 0.1 mL of RPMI 1640 (Sigma) containing 1 mM of L-glutamine, 100 U/mL of penicillin, 100 μ g/mL of streptomycin, 5×10^{-5} M of 2-mercaptoethanol and 5% of heat-inactivated fetal calf serum were cultured in 24-well tissue culture plates with or without 500 μ g/mL of OVA. Four days later, supernatants were harvested and stored at -40°C until assayed. IFN- γ , IL-5 (Pierce Biotechnology, Inc.; Rockford, MD, USA) and TGF- β (Bioscience International Inc.; Camarillo, CA, USA) were measured by ELISA kits available commercially. We failed to determine levels of IL-4 in the collected samples because the amount of cytokine produced was too small to be measured by ELISA.

Statistical analysis

The data obtained were statistically evaluated using a one-way analysis of variance followed by Bonferroni's test; P values < 0.05 were considered to be significant.

Results

As shown in Table 2, anti-OVA IgG in sera decreased following treatment with SIN in a dose-related fashion. The level of anti-OVA IgG in mice treated with SIN was 51 % lower than that in animals given PBS. Dose-related decreases in proliferation of spleen cells to the antigen were also observed in the SIN treatment group. The highest dose of SIN suppressed the cellular proliferative responses up to 48%. DEX (2 mg/kg) suppressed both anti-OVA production and splenocyte proliferation to the extent achieved by treatment with 300 mg/kg of SIN.

Table 2 Effects of sinomenine(SIN) on production of anti-OVA IgG and proliferative responses of spleen cells to the antigen

SIN (mg/kg)	Anti-OVA IgG (A405) ^a	Proliferation (cpm) ^b
0 (PBS)	0.77 $\pm$ 0.11	42 925 $\pm$ 1 772
30	0.58 $\pm$ 0.08 **	38 187 $\pm$ 1 690
100	0.51 $\pm$ 0.11 **	30 703 $\pm$ 1 119 **
300	0.38 $\pm$ 0.06 **	22 424 $\pm$ 2 486 **
2 (DEX)	0.35 $\pm$ 0.08 **	22 046 $\pm$ 849 **

Mice were orally treated with the indicated doses of SIN daily over a period of 21 days, commencing on the day of immunization with OVA (day 0). PBS and dexamethasone (DEX) were given as negative and positive controls, respectively. On day 21, anti-OVA IgG in sera and proliferative responses of spleen cells to OVA were measured as described in Materials and Methods.

^a Mean $\pm$ s.e.m. of 8 mice.

^b Mean $\pm$ s.e.m. of triplicated cultures of cells pooled from 8 mice.

** P < 0.01 vs PBS treatment group.

The effects of SIN on the production of anti-OVA IgG2a as a Th1 cell-dependent antibody [13] and the antigen-specific IgG1 and IgE as Th2 cell-dependent antibodies [14] were examined. Fig. 2 shows the results. All the isotypes of anti-OVA antibodies measured were suppressed by SIN dose-dependently although the extent of the suppression of IgG2a production appeared to be greater than those of IgG1 and IgE. The highest dose (300 mg/kg) of SIN showed 71 % of inhibition of IgG2a production whereas identical treatment resulted in 40 and 44 % inhibition of IgG1 and IgE production, respectively. The similar extent of suppression of these isotypes of antibodies was seen in mice given DEX.

The effect of SIN on secretion of the Th1 cytokine IFN- γ and the Th2 cytokine IL-5 was examined. The effect of SIN on regulatory CD4⁺ T cell was also examined by measuring TGF- β . Treatment with SIN resulted in significant suppression of secretion of IFN- γ and IL-5, as shown in Fig. 3, although the suppression of IFN- γ secretion by SIN appeared to be greater than that of IL-5. DEX also suppressed the secretion of IFN- γ and IL-5 and the extent of the suppression of these cytokines by the drug was similar to that by 300 mg/kg of SIN. Treatment with SIN resulted in enhancement of the production of TGF- β .

Discussion

The present study indicates that SIN, an alkaloid extracted from the Chinese medicinal plant *Sinomenium acutum* used for the treatment of rheumatic diseases for more than 2000 years, may regulate both Th1 and Th2 immune responses since SIN suppressed the production of anti-OVA IgG2a and IFN- γ (Th1) as well as the antigen-specific IgG1, IgE and IL-5 (Th2). Previous studies demonstrated that treatment of patients with SIN was followed by decreases in IgG, IgM, and IgA [15], supporting the result that SIN suppressed anti-OVA IgG production as shown in our studies. *In vitro* studies revealed that SIN down-regulated the proliferation of spleen cells to mitogens including concanavalin A, lipopolysaccharide, and anti-CD3 [16]. Our studies also showed that OVA-specific splenocyte proliferation was inhibited by SIN. However, few studies previously defined the effects of SIN on Th1 and Th2 responses. To our knowledge, this is the first report demonstrating that SIN was able to regulate Th1 as well as Th2 immune responses.

It is worthy of note that the extent of the suppression of Th1 by SIN appeared to be greater than that of Th2. The precise mechanism by which SIN suppressed Th1 more markedly than Th2 is unknown. However, the following mechanisms in terms of the preferential suppression of Th1 by the alkaloid might be involved. Firstly, SIN suppresses the NF- κ B activity of macrophages and synoviocytes from rats with adjuvant arthritis [12]. Studies have proven that NF- κ B plays an important role in Th1 but not in Th2 *in vivo* [17], [18]. Therefore, suppression of NF- κ B by SIN might lead to a stronger suppressive effect on Th1 than on Th2. Secondly, et al. [19] implicated that extracellular signal-regulated protein kinase (ERK) had a preferentially suppressive effect on Th1, because ERK1^{-/-} mice showed higher expression of the Th1 cytokine IFN- γ and lower expression of the Th2 cytokine IL-5. SIN has been shown to activate ERK in the murine macrophage cell line RAW264.7 [20]. Therefore, SIN might more strongly sup-

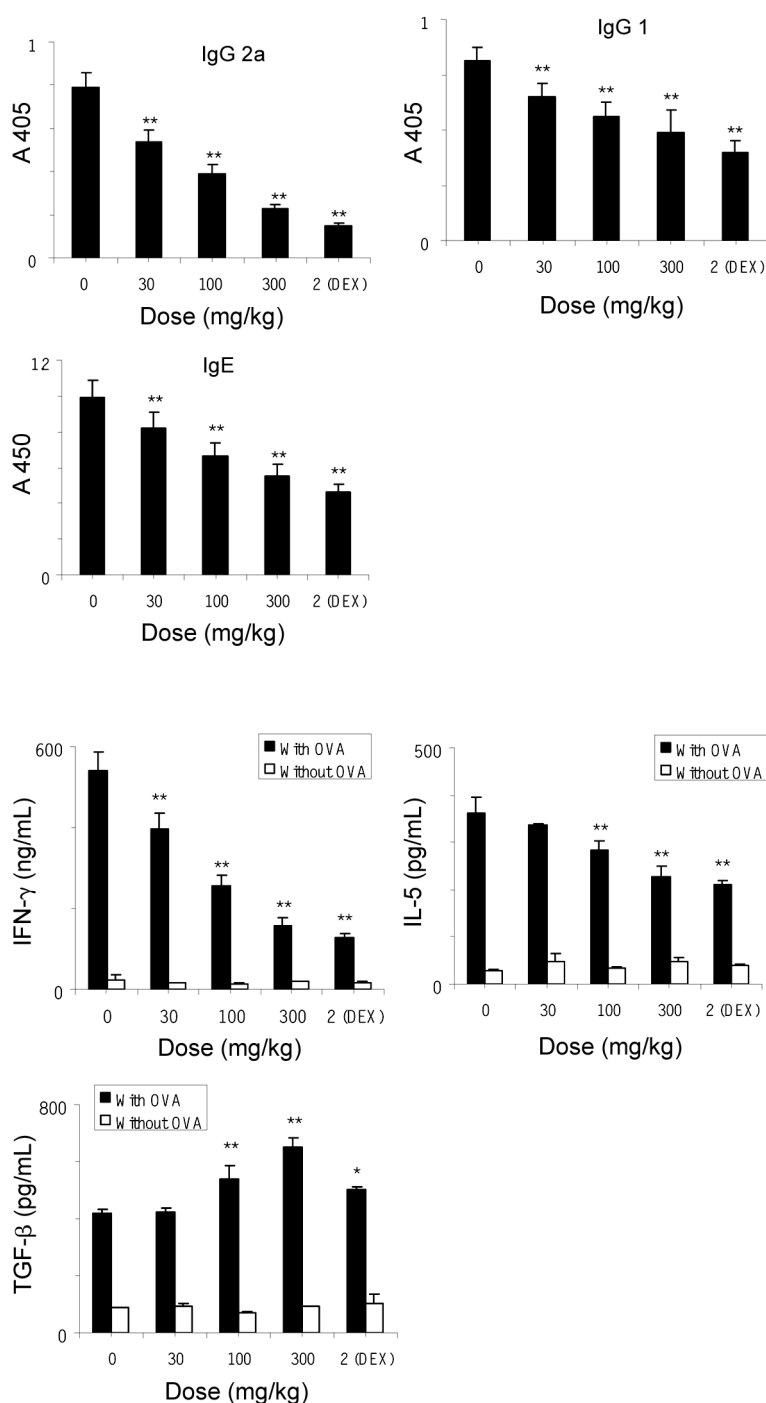


Fig. 2 Effect of sinomenine (SIN) on the production of anti-OVA IgG2a, IgG1 and IgE in sera. Mice were immunized with OVA emulsified with complete Freund's adjuvant on day 0. The indicated doses of SIN were orally given commencing on day 0 daily over a period of 21 days. PBS and the indicated dose of DEX were given as negative and positive controls, respectively. On day 21, anti-OVA IgG2a, IgG1 and IgE in sera were measured as described in Materials and Methods. Bars show the mean $\pm$ s.e.m. of 8 mice. ** $P < 0.01$ vs. PBS treatment group, Bonferroni's test.

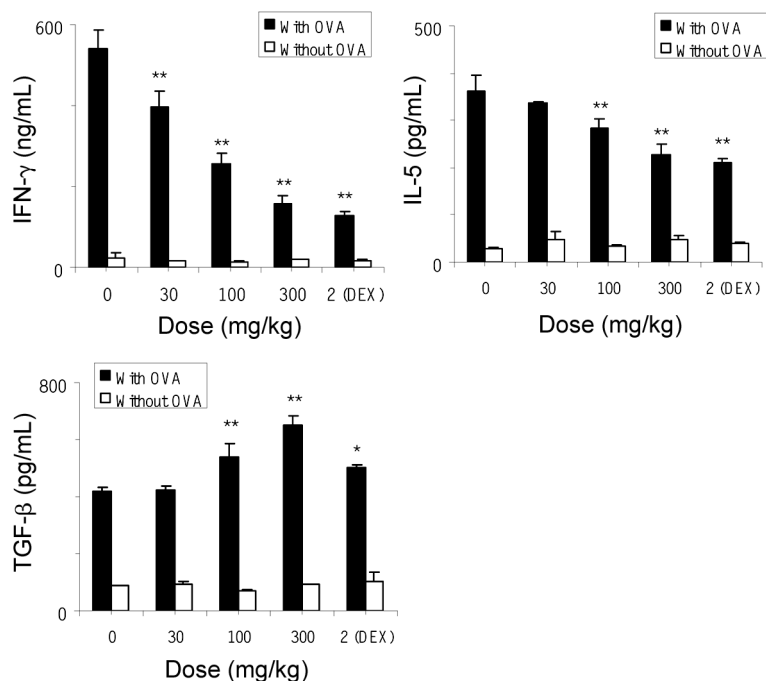


Fig. 3 Effect of sinomenine (SIN) on the production of IFN γ , IL-5 and TGF- β . Mice were immunized with OVA emulsified with complete Freund's adjuvant on day 0. The indicated doses of SIN were orally given commencing on day 0 daily over a period of 21 days. PBS and the indicated dose of DEX were given as negative and positive controls, respectively. On day 21, mice were sacrificed and spleen cells were cultured for 4 days. IFN γ , IL-5 and TGF- β were measured as described in Materials and Methods. Bars show the mean $\pm$ s.e.m. of triplicate cultures of cells pooled from 8 mice. ** $P < 0.01$ vs. PBS treatment of group, * $P < 0.05$ vs. PBS treatment of group, Bonferroni's test.

press Th1 than Th2 by the activation of ERK. Thirdly, Roy et al. [21] found that chronic morphine treatment *in vivo* and *in vitro* decreases the production of Th1 cytokines. Furthermore, an opioid antagonist increases Th1 cytokines production [22]. SIN also has been used for the treatment of opioid abstinence symptoms [23]. Therefore, SIN may more preferentially suppress Th1 than Th2 via the opioid receptor since its chemical structure is similar to that of morphine [23], [24]. Fourthly, the more marked suppression of Th1 by SIN might be in part explained by the suppression of expression of B7-DC and B7-H1 belonging to the B7 family by the alkaloid [25]. B7-DC has been shown to play a role in Th1 but not Th2 cell activation [26]. Furthermore, Matsumoto et al. [27] found that treatment of mice with anti-B7-DC monoclonal antibody was followed by a decrease in IFN- γ but an in-

crease in IL-5 and IL-13. B7-H1 has been shown to play a role in both Th1 and Th2 responses [28], [29]. Therefore, the suppression of both B7-DC and B7-H1 by SIN might have resulted in more marked decrease in Th1 than Th2.

Liu et al. [9], [30] showed that SIN in plasma lasted longer when it was given orally instead of *i.v.* or *i.p.* and that SIN was quickly absorbed from the gastrointestinal tract. Therefore, we selected the route of oral administration of SIN to test its effect on Th1 and Th2. SIN is also given orally to the patients with RA [15].

TGF- β has been shown to play an important role in the function of regulatory T cells. Antigen-specific Th1 and Th2 responses are

down-regulated by Tr1 cells through secretion of TGF- β [31]. In our studies, treatment with SIN was followed by an enhancement of TGF- β secretion. Therefore, the down-regulation of Th1 as well as Th2 responses by SIN may be related to the role of this T regulatory cytokine in these immune responses.

Interestingly, the extent of the suppression by 300 mg/kg of SIN on anti-OVA IgG production, the proliferative responses to the antigen as well as Th1 and Th2 responses were comparable to that by 2 mg/kg of the steroidal anti-inflammatory drug DEX used as a positive control that was expected to suppress all these immune responses [32].

SIN has long been used for the treatment of rheumatic diseases including RA. The mechanisms of the beneficial effect of SIN on RA include the suppression of secretion of proinflammatory cytokines such as IL-1 β , IL-8, and TNF- α by the alkaloid [33]. SIN was also previously shown to down-regulate the expression of NO and cyclooxygenase-2 [11]. Our studies appear to add a new mechanism of action of SIN on RA, that is, this drug may suppress Th1 immune responses that play a role in the joint inflammatory disease [34]. This may be further supported by the finding that the experimental model of RA adjuvant arthritis in rat in which Th1 cells specific for arthritogenic antigens are critically involved can be ameliorated by SIN [9].

In summary, SIN suppressed both Th1 and Th2 responses when anti-OVA IgG2a and IFN- γ (Th1) and the antigen-specific IgG1, IgE and IL-5 (Th2) were measured. SIN appeared to show more marked suppressive effects on Th1 than Th2 responses. The Th3 cytokine TGF- β may play a role in the regulations of Th1 and Th2 by SIN. The beneficial effect of SIN on RA may be at least in part explained by the suppression of pathogenic antigen-specific Th1 by the Chinese medicinal plant-derived alkaloid.

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