# Stemucronatoside L, a Pregnane Glycoside from the Roots of *Stephanotis mucronata*, Inhibits Th1/Th2 Immune Responses *in vitro*

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Stemucronatoside L (SML), isolated from *Stephanotis mucronata*, could suppress the activation of T cells *in vitro*. However, the mechanisms responsible for its immunosuppressive activity remain poorly understood. The purpose of this study was to investigate whether SML could suppress Th1/Th2 immune responses and to characterize the cellular mechanisms involved. Effects of SML on T-lymphocyte subsets and the production of Th1 cytokines IL-2 and IFN-$\gamma$, and Th2 cytokines IL-4 and IL-10 from ConA-stimulated mice splenocytes were detected by flow-cytometric analysis and ELISA method, respectively. Furthermore, effects of SML on mRNA expression level of Th1/Th2 cytokines and transcription factors T-bet and GATA-3 were evaluated by RT-PCR analysis. SML not only significantly decreased the percentage of $CD4^+$ T cells and the $CD4^+/CD8^+$ ratio, but reduced the production of Th1/Th2 cytokines in a concentration-dependent manner. The mRNA expression levels of Th1/Th2 cytokines and transcription factors (T-bet and GATA-3) were also suppressed by SML. These results suggested that SML could simultaneously inhibit Th1/Th2 immune responses by suppressing gene expression of Th1/Th2 cytokines and transcription factors.

**Introduction.** – *Stephanotis mucronata* (BLANCO) MERR. (Asclepiadaceae) is used for the treatment of rheumatoid arthritis and to relieve rheumatic aches in folk medicine in southern China [1]. We have previously isolated a series of pregnane glycosides from this plant and reported their immunomodulatory activity *in vitro* [2–6]. The compound reported in a previous paper is named as stemucronatoside L (SML; *Fig. 1*) here, which is a new pregnane glycoside and proved to inhibit the concanavalin A (ConA)- and lipopolysaccharide-stimulated mice splenocyte proliferations in a concentration-dependent manner *in vitro* [6]. However, the mechanisms responsible for its immunomosuppressive activity remain poorly understood. To investigate whether SML could inhibit Th1/Th2 immune responses and to characterize the cellular mechanisms involved, in this study, we investigated the immunosuppressive effects of SML on T-lymphocyte $CD4^+/CD8^+$ subsets, Th1- and Th2-type cytokine (IFN-$\gamma$, IL-2, IL-10, and IL-4) productions, and mRNA expression of cytokines and specific transcription factors (T-bet, GATA-3) in ConA-induced mice splenocytes *in vitro*. The results indicated that SML could significantly inhibit Th1/Th2 immune responses *in vitro* by suppressing gene expression of cytokines and specific transcription factors, which provided some pharmacological evidence to support the therapeutic effects of this herb for treatment of rheumatoid arthritis.

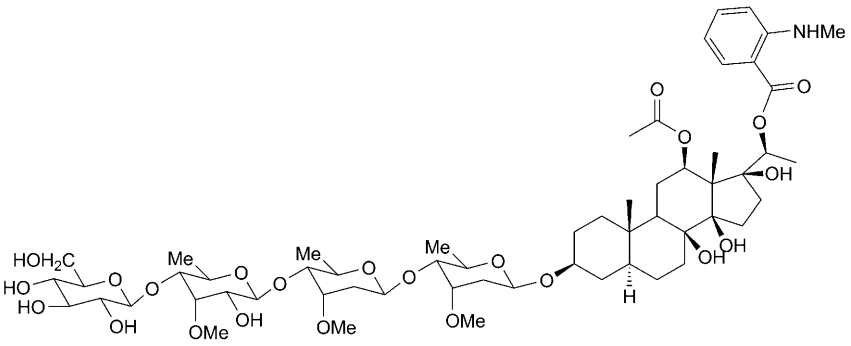


Fig. 1. *Chemical structure of stemucronatoside L* (SML)

**Results.** – 1. *Effect of SML on Splenocytes CD4⁺/CD8⁺ Subsets.* The effect of SML on splenocytes $CD4^+/CD8^+$ subsets was shown in *Table 1*. The percentage of $CD4^+$ T cells and the $CD4^+/CD8^+$ ratio was significantly increased after the mice splenocytes were stimulated by ConA. However, SML markedly decreased the percentage of $CD4^+$ T cells and the ratios of $CD4^+/CD8^+$ in ConA-induced mice splenocytes at the concentration of 2 and 10 μg/ml ($P<0.01$).

Table 1. *Effects of Stemucronatoside L* (SML) *on Splenocytes CD4⁺/CD8⁺ Subsets.* Mouse splenocytes were incubated with SML at the concentrations of 0–10 μg/ml and ConA (final concentration 3 μg/ml) for 48 h. The cells were harvested, and cellular populations were measured by flow cytometer as described in the text. The values are presented as means ± SE ($n=3$).

| Groups | $CD4^+$ [%] | $CD8^+$ [%] | $CD4^+/CD8^+$ |
|---|---|---|---|
| Control | 8.92 ± 1.73 | 4.58 ± 1.39 | 1.98 ± 0.23 |
| ConA | 17.59 ± 1.82 | 5.12 ± 1.16 | 3.48 ± 0.43 |
| ConA + SML (0.08 μg/ml) | 16.10 ± 1.27 | 5.06 ± 1.47 | 3.28 ± 0.70 |
| ConA + SML (0.4 μg/ml) | 13.97 ± 1.79 | 4.52 ± 1.27 | 3.16 ± 0.49 |
| ConA + SML (2.0 μg/ml) | 8.83 ± 1.60[a]) | 4.50 ± 1.27 | 1.99 ± 0.21[a]) |
| ConA + SML (10 μg/ml) | 8.55 ± 1.34[a]) | 4.31 ± 1.00 | 2.00 ± 0.15[a]) |

[a]) Significant differences with ConA groups were designated as $P<0.01$.

2. *Effect of SML on Cytokine Productions from ConA-Stimulated Splenocytes.* The calibration curves of IL-2, IL-4, IL-10, and IFN-$\gamma$ were constructed with mouse cytokine standards, and all their correlation coefficients were greater than 0.9980. As shown in *Table 2*, the contents of cytokines IL-2, IL-4, IL-10, and IFN-$\gamma$ in the culture supernatants from the mice splenocytes treated with SML were significantly lower than those in ConA control ($P<0.05$, $P<0.01$, or $P<0.001$), suggesting that SML significantly inhibited the production of the Th1 and Th2 cytokines from ConA-stimulated mice splenocytes in concentration-dependent manner.

Table 2. *Effects of Stemucronatoside L* (SML) *on Cytokine Production from Con A-Stimulated Mice Splenocytes.* Mouse splenocytes were incubated with SML at the concentrations of 0–10 μg/ml and ConA (final concentration 3 μg/ml) for 24 h. The culture supernatants were collected, and the contents of cytokines IL-2, IL-4, IL-10, and IFN-$\gamma$ were determined by ELISA. The values are presented as means ± SE ($n=3$).

| Groups | Cytokines [pg/ml] | | | |
|---|---|---|---|---|
| | IL-2 | IL-4 | IL-10 | IFN-$\gamma$ |
| Control | 20 ± 10 | 2.33 ± 0.33 | 20 ± 1 | 936 ± 197 |
| ConA | 636 ± 15 | 9.13 ± 0.11 | 204 ± 18 | 3366 ± 265 |
| ConA ± SML (0.08 μg/ml) | 495 ± 58[a]) | 6.35 ± 0.57[b]) | 165 ± 5[a]) | 1931 ± 216[b]) |
| ConA ± SML (0.4 μg/ml) | 488 ± 51[b]) | 5.92 ± 0.66[b]) | 146 ± 15[a]) | 1266 ± 127[c]) |
| ConA ± SML (2.0 μg/ml) | 452 ± 41[b]) | 4.83 ± 0.88[b]) | 135 ± 8[b]) | 1241 ± 91[c]) |
| ConA ± SML (10 μg/ml) | 301 ± 56[c]) | 2.60 ± 0.28[c]) | 76 ± 15[c]) | 1011 ± 63[c]) |

Significant differences with ConA groups were designated as [a]) $P<0.05$, [b]) $P<0.01$, and [c]) $P<0.001$.

3. *Effect of SML on Expression of Cytokines and Transcription Factor mRNAs in ConA-Stimulated Splenocytes.* The effect of SML on the mRNA expression of Th1/Th2 cytokines and transcription factors in ConA-stimulated mice splenocytes was evaluated, and the results were shown in *Fig. 2* and *Table 3*. SML not only significantly decreased the Th1 cytokines IL-2, IFN-$\gamma$, and transcription factor T-bet mRNA expression ($P<0.05$, $P<0.01$, or $P<0.001$), but reduced the Th2 cytokines IL-4 and IL-10, and transcription factor GATA-3 mRNA expression ($P<0.05$, $P<0.01$, or $P<0.001$) in Con A-stimulated splenocytes in concentration-dependent manner. These results indicate that SML suppressed gene expression of Th1/Th2 cytokines and transcription factors in splenocytes upon stimulation by ConA.

**Discussion.** – Pregnane glycosides isolated from the Asclepiadaceae family have attracted much attention mainly for their antitumor, antiepilepsy, antifertility, and anti-

Table 3. *The mRNA Expression Level of Cytokines and Transcription Factors in Mice Splenocytes Treated with Stemucronatoside L* (SML) *and Con A.* Mice splenocytes were incubated with stemucronatoside L (SML) at the concentrations of 0–10 μg/ml and Con A (final concentration 3 μg /ml) for 16 h. The expression of IL-2, IFN-$\gamma$, IL-4, IL-10, T-bet, and GATA-3 mRNA was analyzed by RT-PCR as described in the text. The values were normalized by dividing the amount of GAPDH gene expression. Data are shown as mean ± SE ($n=3$).

| Gene | Concentration of SML [μg/ml] | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.016 | 0.08 | 0.4 | 2 | 10 |
| IL-2 | 0.49 ± 0.01 | 0.42 ± 0.02[a]) | 0.41 ± 0.02[b]) | 0.30 ± 0.03[c]) | 0.28 ± 0.04[c]) | 0.09 ± 0.01[c]) |
| INF-$\gamma$ | 0.45 ± 0.03 | 0.21 ± 0.03[c]) | 0.19 ± 0.03[c]) | 0.18 ± 0.03[c]) | 0.15 ± 0.03[c]) | 0.09 ± 0.02[c]) |
| T-bet | 0.39 ± 0.03 | 0.33 ± 0.01[a]) | 0.33 ± 0.01[a]) | 0.33 ± 0.01[a]) | 0.27 ± 0.02[b]) | 0.22 ± 0.02[c]) |
| IL-4 | 0.52 ± 0.04 | 0.42 ± 0.04[a]) | 0.41 ± 0.01[a]) | 0.30 ± 0.01[c]) | 0.28 ± 0.01[c]) | 0.05 ± 0.01[c]) |
| IL-10 | 0.39 ± 0.02 | 0.34 ± 0.02[a]) | 0.26 ± 0.01[c]) | 0.22 ± 0.01[c]) | 0.21 ± 0.01[c]) | 0.13 ± 0.01[c]) |
| GATA-3 | 0.51 ± 0.07 | 0.38 ± 0.03[a]) | 0.37 ± 0.01[a]) | 0.37 ± 0.05[a]) | 0.37 ± 0.02[a]) | 0.27 ± 0.01[b]) |

Significant differences with 0 μg/ml were designated as [a]) $P<0.05$, [b]) $P<0.01$, and [c]) $P<0.001$.

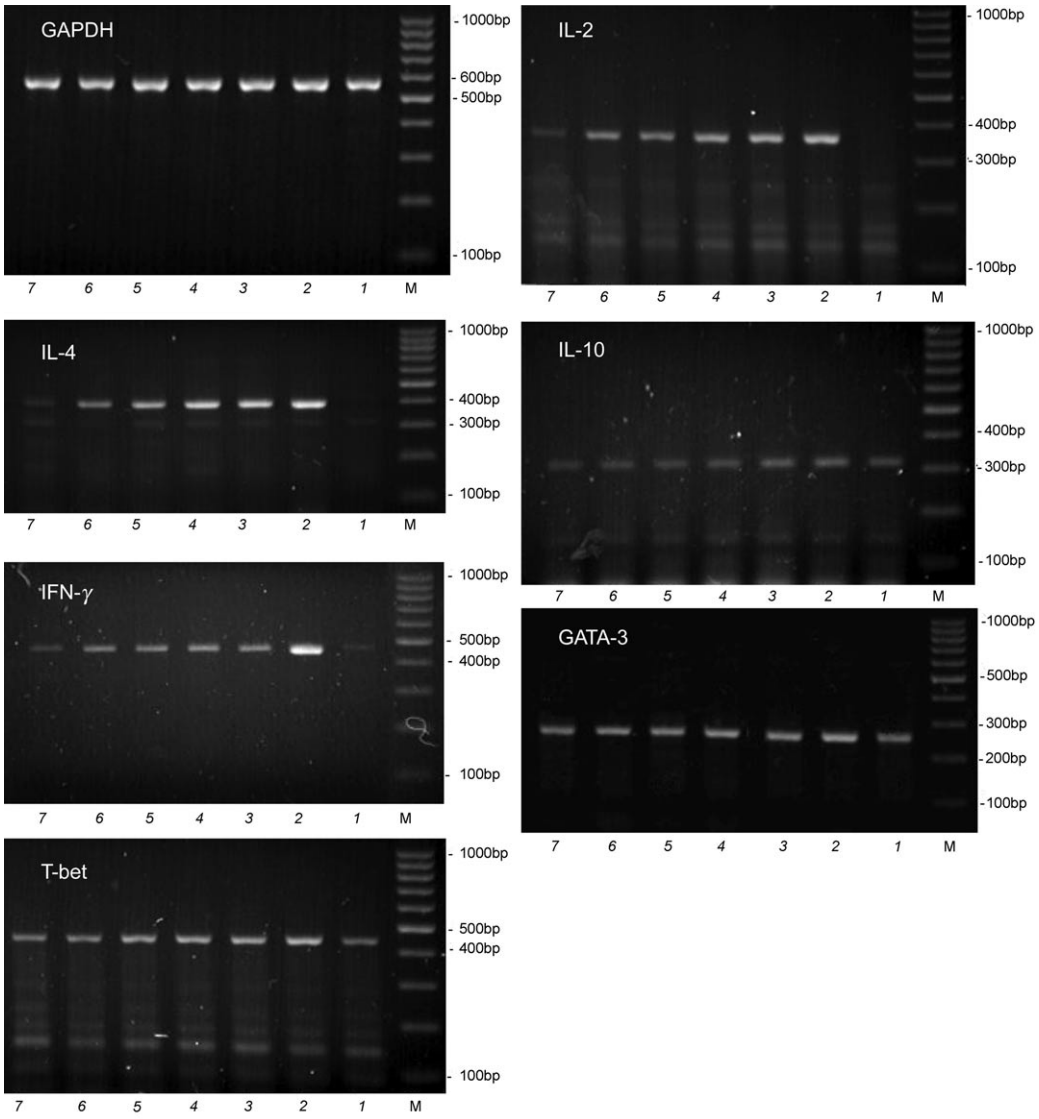


Fig. 2. *The mRNA expression level of GAPDH, cytokines and transcription factors in mice splenocytes treated with stemucronatoside L* (SML) *and ConA*. Splenocytes were incubated with SML at the concentrations of 0–10 μg/ml and ConA (3 μg/ml) for 16 h. The mRNA level of GAPDH, IL-2, IFN-γ, IL-10, IL-4, T-bet, and GATA-3 were detected by RT-PCR using specific primers. The amplified cDNA were resolved on 1.5% (*w*/*v*) agarose gel and visualized by goldview. *Lane M:* DNA marker, *Lane 1:* untreated cell control, *Lane 2:* ConA, *Lane 3:* ConA + SML (0.016 μg/ml), *Lane 4:* ConA + SML (0.08 μg/ml), *Lane 5:* ConA + SML (0.4 μg/ml), *Lane 6:* ConA + SML (2 μg/ml), *Lane 7:* ConA + SML (10 μg/ml).

acetylcholinesterase (AChE) effects [7–12], and have recently been reported to possess immunosuppressive activities [13].

The dried root of *S. mucronata* has been used for the treatment of rheumatoid arthritis and to relieve rheumatic aches in folk medicine. In an effort to elucidate its biological active constituents, we have isolated 18 pregnane glycosides from this plant and reported their *in vitro* immunological activities [2–6]. Here, we further report the inhibitory effect of SML on the Th1/Th2 immune responses *in vitro*.

The immune system encompasses various cell populations such as antigen presenting cells, natural killer cells, and B and T lymphocytes. Among them, T cells, in particular $CD4^+$ T cells, play an important role in the development and progression of human autoimmune diseases, such as rheumatoid arthritis, allergic asthma, multiple sclerosis, and systemic lupus erythematosus [14]. According to the cytokine profiles after activation, $CD4^+$ T cells are subdivided into different subclasses termed T helper lymphocyte type 1 (Th1), Th2, and others, which originate from a common precursor known as Th0 [15][16]. In general, inappropriate Th1 responses have been implicated in inflammatory and autoimmune diseases, while enhanced Th2 responses may cause atopy and allergic inflammation [17]. Th1 cytokines, including IFN-$\gamma$, IL-2, and IL-12, generally promote Th1 cell-mediated immune responses. In contrast, Th2 cytokines, including IL-4, IL-10, IL-5, and IL-13, which are required for IgE production and activation of mast cells and eosinophils, are involved in Th2 cell-mediated humoral immune responses [18]. IL-2 is considered as a T cell mitogen and required for T cell proliferation. IFN-$\gamma$ promotes Th1 cell differentiation and activates macrophages to secrete various cytokines [19]; IL-10, as a suppressive cytokine, can down-regulate Th1 responses and control Th2-mediated inflammatory processes, however, it also displays immunostimulatory properties especially on B cells and activated $CD8^+$ T cells [20]. IL-4 promotes Th2 cell differentiation and can regulate autoimmunity by antagonizing the development of type 1 responses in the development of many autoimmune diseases [21].

The fate of activated T helper cell differentiation is determined by several factors, including antigen form, dose, type of antigen presenting cells, co-stimulatory molecules, and chromatin structure, and the strongest determinant of T helper cell differentiation is the complex mixture of cytokines in the local microenvironment. It is now clear that cytokines IFN-$\gamma$ and IL-4 exert the effects on Th1 and Th2 differentiation by controlling the expression of transcription factors T-bet and GATA-3 [22]. T-bet, a member of the T-box family of transcription factors, is a Th1-specific transcription factor that plays a central role in Th1 development. Its expression is up-regulated by IFN-$\gamma$, through a STAT-1-dependent mechanism. In turn, T-bet is an IFN-$\gamma$ activator, thus creating an indirect positive feedback. Furthermore, it has been shown that ectopic T-bet is able to induce the transcription of its own gene. This effect occurs in the absence of a functional IFN-$\gamma$/IFN-$\gamma$R signaling pathway, since it is observed even in Th2 cells, and hence suggests an IFN-$\gamma$-independent positive feedback of T-bet. T-bet expression is also able to re-differentiate Th2 cells into Th1 cells in an IFN-$\gamma$R-independent manner [17]. GATA-3, a member of the GATA family of Zn finger proteins, is a Th2-specific transcription factor and up-regulated during Th2 differentiation. Its expression is up-regulated mainly by IL-4. IL-4 binding to its receptor on Th precursor (Thp) cells activates the signalling factor STAT6, which then translocates

to the nucleus and rapidly induces the expression of GATA-3. GATA-3 may also augment expression of IL-4 by interaction at sites distant from the proximal promoter [22]. These two transcription factors interact with each other; GATA-3 may inhibit T-bet expression, and T-bet is capable of inhibiting GATA-3 and the cytokines that induce them through feedback loops [17]. Since T-bet and GATA-3 inhibit each other and are involved in direct and indirect positive feedback loops, these two molecules constitute the central feature of models of T helper differentiation.

As shown in *Tables 1* and *2*, ConA significantly elevated the percentage of $CD4^+$ T cells, the $CD4^+/CD8^+$ ratio, and promoted the production of cytokines IL-2, IFN-$\gamma$, IL-10, and IL-4 in mice splenocytes. SML not only significantly decreased the percentage of $CD4^+$ T cells and the $CD4^+/CD8^+$ ratio, but reduced the production of IL-2, IFN-$\gamma$, IL-4, and IL-10 from ConA-stimulated mice splenocytes in a concentration-dependent manner. These results suggest that SML simultaneously inhibited Th1/Th2 cell activation.

To further elucidate the mechanism responsible for the changes in the amounts of type-1 and type-2 cytokines, we utilized RT-PCR to analyze the mRNA expression of IL-2, IFN-$\gamma$, IL-10, and IL-4 in ConA-stimulated mice splenocytes. Furthermore, we measured Th1- and Th2-specific transcription factor T-bet and GATA-3 gene expression in splenocytes cultured with SML and ConA. SML was shown to significantly and concentration-dependently suppress the expression of all Th1/Th2 cytokines and transcription factor mRNAs (*Fig. 2* and *Table 3*).

These results indicate that SML, a new pregnane glycoside isolated from *Stephanotis mucronata*, is a potent inhibitor of T-cell activation, which could simultaneously inhibit Th1/Th2 immune responses by suppressing gene expression of Th1/Th2 cytokines and transcription factors. This study would be beneficial in understanding the therapeutic effects of *S. mucronata* on rheumatoid arthritis and relieving rheumatic aches in folk medicine.

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## Experimental Part

*General.* Concanavalin A (ConA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from *Sigma Chemical Co.*, St. Louis, MO, USA; *RPMI 1640* medium was from *Gibco BRL*, Grand Island, NY, USA; fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (L3T4, clone H129.19) and phycoerythrin (PE)-conjugated rat anti-mouse CD8 (LY-2, clone 53-6.7) monoclonal antibody were from *BD Biosciences Pharmingen*, CA, USA; mouse cytokine (IL-2, IFN-r, IL-4, and IL-10) detecting ELISA kits were from *Wuhan Boster Biological Technology., Ltd.*, Wuhan, Hubei, P. R. China. *Trizol* was purchased from *Invitrogen*, Carlsbad, CA, USA; PCR primers and other PCR reagents were from *Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.*, Shanghai, P. R. China. Fetal bovine serum (FBS) was provided by *Hangzhou Sijiqing Corp.*, Hangzhou, Zhejiang, P. R. China.

*Extraction, Isolation, and Identification of SML.* Stemucronatoside L (SML; $C_{58}H_{91}NO_{23}$, $M_r$: 1192.5914) was isolated from the roots of *Stephanotis mucronata* according to the method described in [6], and identified on basis of chemical and spectroscopic evidence (IR, MS, and NMR experiments including $^1H$- and $^{13}C$-NMR, $^1H,^1H$-COSY, HMQC, and HMBC). The purity of SML was determined to be $>99\%$ by HPLC analysis.

The stock soln. of SML in DMSO was prepared, and then diluted as desired with *RPMI-1640* medium (without fetal calf serum). The final concentration of DMSO in the assays was less than 0.1% in all experiments and did not show any detectable effect on cell growth.

*Experimental Animals.* Female ICR mice (grade II, five-weeks old) weighing 18–22 g were purchased from *Zhejiang Experimental Animal Center* (Certificate No. SCXK 2003-0001, Hangzhou, China) and acclimatized for 7 d before use. Rodent laboratory chow and tap water were provided *ad libitum*, and maintained under controlled conditions: temp. $24\pm1°$, humidity $50\pm10\%$, 12-h light/12-h dark cycle. All the procedures were in strict accordance with the P. R. China legislation on the use and care of laboratory animals, and with the guidelines established by the *Institute for Experimental Animals*, and were approved by the *Committee for Animal Experiments*.

*Preparation of Splenocytes.* Single splenocytes were prepared as described in [23]. Spleen collected under aseptic conditions, in *Hank*'s Balanced Salt Solution (HBSS; *Sigma*), was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension, and the erythrocytes were lysed with ammonium chloride (0.8% (*w*/*v*)). After centrifugation ($1500\times g$ at 4° for 10 min), the pelleted cells were washed three times with PBS and resuspended in complete medium (*RPMI 1640* supplemented with 12 mM *HEPES* (pH 7.1), 0.05 mM 2-sulfanylethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS). Cell numbers were counted with a haemocytometer by the Trypan Blue dye exclusion technique. Cell viability exceeded 95%.

*Flow Cytometry.* Splenocytes were seeded into a 24-well flat-bottom culture plate (*Costar*, Cat. No. 3524) at $5\times10^6$ cell/ml in 1 ml of complete medium, thereafter ConA (final concentration 3 μg/ml) or *RPMI 1640* medium with SML (final concentrations 0.016, 0.08, 0.4, 2, 10 μg/ml) was added to give a final volume of 2 ml (triplicate wells). After 48 h of incubation in a humidified incubator with 5% $CO_2$ at 37°, the cells were harvested and washed with PBS three times, then stained with FITC-conjugated rat anti-mouse CD4 and PE-conjugated rat anti-mouse CD8 monoclonal antibodies. A FACScan flow cytometer with *CellQuest 3.0f* software (*BD Biosciences Pharmingen*, CA, USA) was used for data acquisition and analysis.

*Measurement of Cytokines.* Splenocytes were incubated with ConA and SML in a 24-well flat-bottom culture plate in a humidified incubator with 5% $CO_2$ at 37°. After 24 h, the plate was centrifuged at $1400\times g$ for 5 min, and culture supernatants were collected. IL-2, IL-4, Il-10, and IFN-$\gamma$ levels were assayed using commercial ELISA kits. Briefly, culture supernatants or cytokine standards were added to 96-well flat-bottom microtiter plates coated with coating antibody, and plates were then incubated at 37° for 1.5 h (IFN-$\gamma$ for 2 h). Plates were washed, and a detecting antibody was added to each well. Plates were incubated at 37° for 1 h before addition of avidin-biotin-peroxidase complex (ABC). After incubation for 30 min, plates were washed and developed with TMB (tetramethyl benzidine) at 37° for 15 min. The reaction was quenched by addition of 100 μl of stop soln. The absorbance was measured with an ELISA reader at 450 nm.

*RT-PCR for Cytokine and Transcription-Factor Gene Expression.* Splenocytes were seeded into a culture flask at $5\times10^6$ cell/ml in 4 ml of complete medium, then ConA (final concentration 3 μg/ml) or *RPMI 1640* medium with SML (final concentrations 0.016, 0.08, 0.4, 2, and 10 μg/ml) was added to give a final volume of 8 ml. After 16 h of incubation in a humidified incubator with 5% $CO_2$ at 37°, the cells were harvested and washed with PBS three times. Splenocytes were lysed in 0.5 ml of *Trizol* reagent, and the total RNA was isolated according to the manufacture's protocol. The concentration of total RNA was quantified by determining the optical density at 260 nm. cDNA Synthesis was performed according to the instruction of *M-MuLV Reverse Transcriptase* (*Fermentas*, Cat. No.EP0441).

Amplification was performed with 1 μl of synthesized cDNA, 1 U Taq DNA polymerase, 10 mM dNTP mixture, 25 mM $MgCl_2$, 0.5 μl of primer each in 20 μl of PCR buffer (50 mM KCl, 20 mM *Tris*·HCl, pH 8.4). PCR was performed for 27 (GAPDH and IFN-$\gamma$), 29 (GATA-3), 31 (IL-2 and T-bet) , or 33 (IL-4 and IL-10) cycles using a *PTC-200 Thermal Cycler* (*MJ Research*, Watertown, MA) with the following program of denaturation at 94° for 2 min, annealing at 55° (IL-2, GATA-3, and T-bet), 57° (GAPDH), or 58° (IL-4, IL-10, and IFN-$\gamma$) for 50 s, and elongation at 72° for 0.5 min. At last, the reaction was terminated at 72° for 10 min. The following primers were used for gene amplification: 5′-CCCACAGTAAATTCAACGGCAC-3′ and 5′-CATTGGGGTTAGGAACACGGA-3′ for GAPDH (product size 570 bp) [24], 5′-CTCTACAGCGGAAGCACAGC-3′ and 5′-CATCTCCTCAGAAAGTC-

CACCA-3′ for IL-2 (381 bp), 5′-TGAACGCTACACACTGCATCTTGG-3′ and 5′-CGACTCCTTTTCCGCTTCCTG AG-3′ for IFN-$\gamma$ (459 bp) [25], 5′-ATGGGTCTCAACCCCCAGC-TAGT-3′ and 5′-GCTCTTTAGGCTTTCCAGGAAGTC-3′ for IL-4 (399 bp) [26], 5′-CCAGTT-TTACCTGGTAGAAGTGATG-3′ and 5′-TGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3′ for IL-10 (324 bp) [27], 5′-AACCA GTATCCTGTTCCCAGC-3′ and 5′-TGTCGCCACTGGAAGGA-TAG-3′ for T-bet (436 bp) [28], 5′-GAAGGCATCCAGA CCCGAAAC-3′ and 5′-ACCCATGGCGGT-GACCATGC-3′ for GATA-3 (255 bp) [11]. Semi-quant. RT-PCR was performed using GAPDH as an internal control to normalize gene expression for the PCR templates. The PCR products were studied on a 1.5% agarose gel, and the amplified bands were visualized using *ImmageMaster VDS* Software (*Phamacia Biotech*, USA) after staining with *GoldView*. The size of the amplified fragments was determined by comparison with a standard DNA marker (*Tiangen*, China).

*Statistical Analysis.* The data were expressed as mean ± standard errors (SE) and examined for their statistical significance of difference with ANOVA and the post-hoc test. *P* Values of less than 0.05 were considered to be statistically significant.

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