Suppressive Effect of *Solanum lyratum* Aqueous Extract Via Down-regulation of TNF-α and IFN-γ Production on Collagen-induced Arthritis in Mice

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Solanum lyratum Thunb (Solanaceae) has multiple applications in korean traditional medicine because of its cytotoxic, immunological and anti-inflammatory activities. However, no study on the anti-arthritic activity of *Solanum lyratum* Thunb has been reported in vivo. Rheumatoid arthritis (RA) is a systemic autoimmune disease with chronic inflammation characterized by hyperplasia of synovial cells in affected joints, which ultimately leads to the destruction of cartilage and bone. Cytokine production were assessed during CIA(collagen-induced arthritis) model mice in lymph node (LN), in knee joint and spleen, using ELISA. DBA/1j mice were immunized with bovine type II collagen. After a second collagen immunization, mice were treated with *Solanum lyratum* Thunb (SLT) orally at 400, 200 mg/kg once a day for 4 weeks. The severity of arthritis within the knee joints was evaluated by histological assessment of cartilage destruction and pannus formation. SLT significantly suppressed the progression of CIA and inhibited the production of TNF-alpha and IFN-gamma in serum and spleen cell culture supernatant. The erosion of cartilage was dramatically reduced in mouse knees after treatment with SLT. In conclusion, our results demonstrates that SLT significantly suppressed the progression of CIA. This action was characterized by suppression of arthritis index, cartilage erosion and synovial cell infiltration and the decreased production of TNF-α, IFN-γ, CD4+, CD19+, CD3+/CD69+ cells (in lymph node), CD11b+/Gr-1+ (in knee joint).

Key words : *Solanum lyratum* Thunb (SLT), arthritis, TNF-a, IFN-γ, CD4+, CD19+

Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease characterized by hyperplasia of synovial cells and angiogenesis in affected joints, which ultimately leads to the destruction of cartilage and bone[1]. RA is characterized by the inflammation of synovial joints infiltrated by CD4+ T cells, macrophages, and plasma cells that play major roles in the pathogenesis of the disease[2]. T cells have a direct impact on TNF-α, IFN-γ induction in RA joints. TNF-α is known to play a critical role in the pathogenic mechanisms of a number of chronic inflammatory diseases, including RA. Also, it is the major dominant regulator of inflammatory cytokines (interleukine-1, interleukine-6 and interleukine-8)[3]. B cells may play an important role in the pathogenesis of RA through cellcell interaction with T cells, dendritic cells, synovial nurse-like cells and fibroblasts[4]. CD4+ CD25+ regulatory T cells are potent suppressors of T cell responses and suppress the IFN-γ and TNF-α production of effector T cells. CD4+ CD25+ regulatory T cells were able to diminish the clinical severity of arthritis despite a lack of reduction in systemic CII-specific T and B cell responses[5].

Solanum lyratum Thunb, well known as 'Back-Mo-Deung' in Korea, has been used for regulating body immune function and ability, and it still occupies an important place in traditional Korean medicine[6]. This herb contains solalyrantines A and B, respectively, together with several furostanol, spirostanol and spirosolane glycosides[7]. The major contents of SLT are steroid and alkaloids, such as tomatidenol, solasodine, solanidine S, soladulcinine, a-solamarine, b-solamarine etc.

Kim et al.[6] showed that SLT acts as a modulator of

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mouse peritoneal macrophages activation by rIFN-γ via a process involving L-arginine dependent NO production. Some work has been done on specific active properties of various extracts from SLT[8], but data on water-soluble polysaccharides of SLT are very scarce, which have not been investigated in detail.

Moreover, no study on anti-arthritic and anti-inflammatory activity of *Solanum lyratum* Thunb has been reported. In order to verify its anti-arthritic effects, we have investigated the immunomodulatory and anti-inflammatory activities of the plant. The aim of this study was to evaluate the control activity of *Solanum lyratum* Thunb aqueous extract on TNF-alpha, IFN-γ cytokines production, inhibition of total cells influx in lymph node, knee joint and spleen. The effects of *Solanum lyratum* Thunb aqueous extract on total cell number, T, B, regulatory T cell number in lymph node, knee joint and spleen by flow cytometric analysis and cytokines production in serum by ELISA were determined. Moreover, to determine whether SLT prevented articular destruction, we histologically analyzed the knee joints of mice.

In this study, the various immunomodulatory effects of the SLT were investigated in order to determine the potential bioactivity of SLT on RA using collagen-induced arthritis (CIA) mice.

Materials and Methods

1. Plant material and preparation of extracts

The sample of *Solanum lyratum* Thunb were purchased from local market(Busan, Korea) in September, 2005. The plant was identified by Professor Young-Cheol Lee, College of Oriental Medicine, Sangji University in Wonju, Korea, and a voucher specimens (SLT) are deposited in our laboratory (Department of Herbology, College of Oriental Medicine, Sangji University Wonju 220-702, Republic of Korea). Plant material (200 g) was extracted three times with distilled water. Then, the extract was filtered and evaporated on a rotatory evaporator (Rotary evaporator, BUCHI B-480, Switzerland) and finally dried by a freeze drier (Freeze dryer, EYELA FDU-540, Japan) to yield the extract SLT (15 g). The yield (w/w) of the extract was about 7.5%.

2. Animals

Seven to eight-week-old male DBA/1J mice were obtained at SLC (Hamamatsu, Japan). All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

3. Preparations of murine CIA model

All of the animal procedures were approved by the Experimental Animal Commission of the Institute of Traditional Medicine and Bioscience at Daejeon University. Male DBA/1J mice (79 weeks old; SLC, Hamamatsu, Japan) received 200 ug of bovine type II collagen (Sigma) in Freund's complete adjuvant (Sigma) by intradermal injection at the base of the tail on day 0 and a booster injection on day 21 (n = 6 mice per group). Mice were monitored daily for signs of arthritis, and each paw was scored individually as follows: 0 = normal, 1 = slight erythema and edema, 2 = increased edema with loss of landmarks, 3 = marked edema, and 4 = marked edema with ankylosis on flexion. Each mouse was assigned an arthritis score (articular index) that equaled the sum of the scores for each paw, so that the possible maximum score per mouse was 20. Mice were orally administered with SLT (400 or 200 mg/kg dissolved in distilled water) daily from day 1 to day 28 after arthritis incidence, or intraperitoneally injected methotrexate (MTX; 0.3 mg/kg) and monitored for disease incidence and the severity of arthritis up to day 28. CIA control mice received an intraperitoneal injection of PBS alone.

On the final day of above experiment, all of the mice were anesthetized with ethyl ether and then blood was collected from each by cardiac puncture; the mice were then killed by cervical dislocation. The mice spleen, knee joint and lymph node were taken out and used for ELISA analysis and total cell counts.

4. Cell proliferation assay

Isolated splenocytes (2×10^4 cells/well) from CIA model mice were plated on 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) coated collagen II(1 μg/ml) in 100 μl of RPMI 1640 supplemented with 20% FBS in the absence or the presence of anti-CD3 (0.5 μg/ml) or anti-CD28 mAb (1 μg /ml). For the [3H] thymidine uptake assay, cells were incubated for 72hrs in a humidified 5% CO_2 incubator at 37℃. The cells were pulsed with 0.5 μCi of [3H] thymidine (specific activity, 84.8 Ci/mmol; NewEngland Nuclear, Boston, MA) for the last 6-16 h of incubation, and were then harvested onto a glass-fiber filter using an automated cell harvester (Inotech, Zurich, Switzerland).The amount of radioactivity incorporated into the DNA was determined using a liquid scintillation counter (LS 6000A; Beckman, Palo Alto, CA).

5. Antibodies and flow cytometric analysis.

All antibodies(CD3, CD4, CD8, CD19, CD25, CD11b, Gr-1) for flow cytometric analysis were purchased from Becton Dickinson (BD) PharMingen (San Diego, CA). Cells from lymph node, knee joint and spleen were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS

and 0.01% NaN_3) for 10 min on ice, and analyzed by flow cytometry on a FACScan using CellQuest software (BD Biosciences, Mountain View, CA). Absolute cell numbers were counted manually in a hemocytometer chamber(Fisher). 2~4×10^3 cells were spun onto glass slides (Cytospin centrifuge ,Cellspin, Hanil,, Korea) (400 g for 4 minutes). Differential count was made according to standard morphologic criteria.

6. Enzyme-Linked Immunosorbent Assay (ELISA)

TNF-α in serum and IFN-γ production in the presence of anti-CD3 (0.5 ㎍/ml) or anti-CD28 mAb (1 ㎍/ml) in supernatant of spleen cells isolated from the indicated mice (n=6) were measured by ELISA according to the manufacturer's instruction on a monoclonal antibody-based mouse cytokineELISA kit (R&D system). Moreover, IgG antibody responses to type II collagen in CIA mice treated with MTX and SLT were measured in the serum obtained on day 28. All data represent the standard deviation of at least three different determinants and were compared using Student's paired t-test.

7. H&E (Hematoxylin and eosin) and M-T (Masson trichrome) staining of knee joint in CIA induced murine model

For histologic analysis of the knee joints, the hind limbs of the mice were removed postmortem and fixed in 10% neutral-buffered formalin, decalcified in 5% formic acid, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin or Masson's trichrome.

8. Statistical Analysis.

For statistical analysis of data, P-values were analyzed using a paired Student's t-test software program (Startview 5.1; Abacus Concepts, Berkeley, CA). Results were considered statistically significant when P values were *p<0.05, **p<0.01, ***p<0.001.

Results

1. Effects of SLT on the clinical characteristics of CIA

To determine whether SLT suppresses the immune-mediated pathologic process in arthritic mice, we investigated the effect of SLT on the arthritis incidence and articular index of CIA in DBA/1J mice. SLT had no significant effect on the mean body weight of the mice nor did it elicit any behavioral changes in the mice in either treatment group, which suggests that SLTis not toxic in vivo at the concentrations that were tested (data not shown). In our results, 3 weeks of treatment with SLT (400, 200 mg/kg) beginning 1 day after the booster injection of collagen, suppressed the incidence and articular index and severity of CIA significantly($P < 0.1$)(Fig. 1).

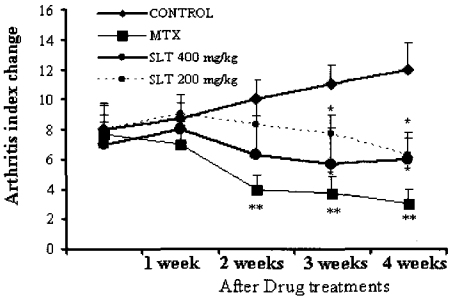

Fig. 1. Effect of SLT clinical evident collagen-induced arthritis (CIA). Mice with CIA were treated with SLT therapeutic dosing models. DBA/1J mice (n=6 mice per treatment group) were immunized with bovine II collagen in Freund's complete adjuvant on days 0 and 21 and were monitered daily for signs of arthritis. In the therapeutic dosing model, treatment was initiated at 1 day after the onset of clinically evident articular disease, and mice were treated for a total of 4 weeks with SLT (400, 200 mg/kg). The severity of the disease was assessed by calculating the mean articular index. Bars show the mean and SEM (*p<0.05, **p<0.01 versus CIA control group).

2 Inhibitory effect of SLT on spleen cells proliferation in CIA model mice

To study the effects of SLT on spleen cells proliferation, isolated spleen cells from each group, in the absence or the presence of anti-CD3 (0.5 ㎍/ml) or anti-CD28 mAb (1 ㎍/ml) were tested for their ability to proliferate in response to different stimuli. As shown in Fig. 2 ,the tritiated thymidine uptake in the SLT (400 mg/kg 6177.25±1012.0 cpm, 200 mg/kg; 8569.5±1176.2 cpm) or methotrexate (MTX) stimulated states (7429.5±1382.3 cpm) were decreased significantly.

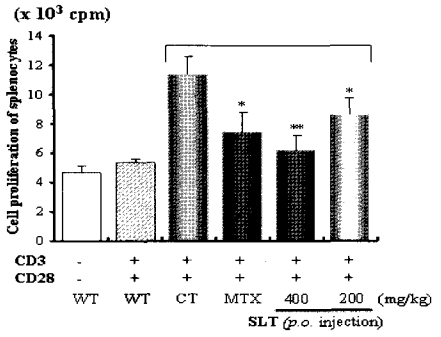

Fig. 2. Effect of the SLT on spleen cells proliferation. Cell proliferation was measured by [3H] thymidine uptake as described above in Materials and Methods. Briefly, Isolated splenocytes (2 x 10^4 cells/well) from CIA model mice were plated on 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) coated collagen II (1 ㎍/ml) in the absence or the presence of anti-CD3 (0.5 ㎍/ml) or anti-CD28 mAb (1 ㎍/ml). For the [3H] thymidine uptake assay, cells were incubated for 72hrs and pulsed with 0.5 ㎍i of [3H] thymidine for the last 6-16 h of incubation, and harvested onto a glass-fiber filter using an automated cell harvester (Inotech, Zurich, Switzerland). Radioactivity was determined by a scintillation counter. Each point represents the mean of three independent experiments. Results are representative of three independent experiments and are expressed as the mean ± SE of triplicate cultures (*p<0.05, **p<0.01 vs cpm of control group).

3. Inhibitory effects of SLT on total cell accumulation in lymph node, knee joint and spleen

To evaluate the effect of SLT on CIA model mice, we investigated the recruitment of total cells to lymph node, knee joint and spleen. In SLT treated groups, absolute cell number were reduced compared with CIA model control group(Fig. 3).

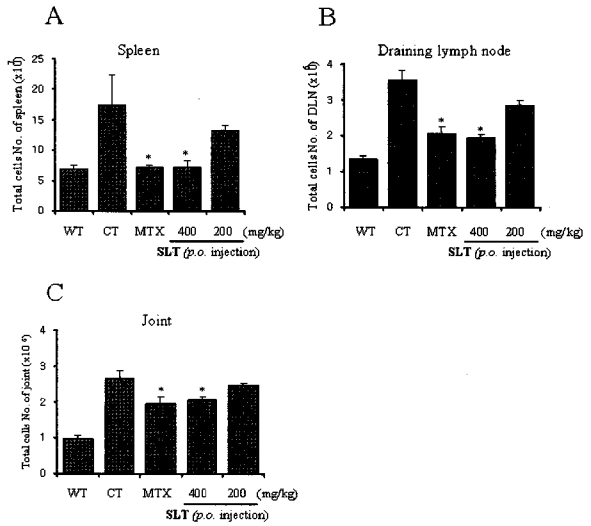

Fig. 3. Effect of SLT on absolute number of total cells in lymph node, knee joint and spleen. On the final day of above experiment(previously described in materials and methods), all of the mice were anesthetized with ethyl ether and then blood was collected from each by cardiac puncture; the mice were then killed by cervical dislocation. The mice lymph node, joint and spleen were taken out and used for total cell counts. $2\sim4\times10^3$ cells were spun onto glass slides (Cytospin centrifuge ,Cellspin, Hanil,, Korea) (400 g for 4 minutes). Differential count was made according to standard morphologic criteria. WT: wild type (normal DBA/1J mice), CT: CIA control type

4. Determination of FACS analysis

1) Inhibitory effect of SLT on CD3+, CD4+, CD8+ T cells population in CIA model mice

The cell surface expression of CD4+ T helper was analyzed by flow cytometry. To detect CD4+expression on spleen and lymph node, immunofluorescence staining was performed using FITC-conjugated anti-CD8 with PE-conjugated anti-CD4 mAb as described in Materials and Methods. To evaluate the efficacy of SLT treatment on CD4+ T cell, CD8+ T cell population, we compared the effects of SLT on CD4+ T cell, CD8+ T cell expression in CIA model mice by using flow cytometry. The absolute number of CD4+ cells in SLT treated group were decreased when compared with control group in lymph node(400 mg/kg)(Fig. 4).

2) The effect of SLT on, CD19+ B cells, CD3+CD69+ early activated T cells, CD4+CD25+ regulatory T cells surface marker Effects of SLT on CD4+CD25+ regulatory T cells, CD19+ B cells, CD3+CD69+, there were marked change of CD19+ B cells, CD3+CD69+ early activated T cells in SLT treated group compared to control group. The deficits in CD4+ T cells(Th cells) were accompanied by concurrent decreases absolute number of CD19+ B cells(Fig. 4). However, the absolute number of CD8+ cytotoxic T cells, CD4+CD25+ regulatory T cells in lymph node were not changed significantly(Fig. 4, 5).

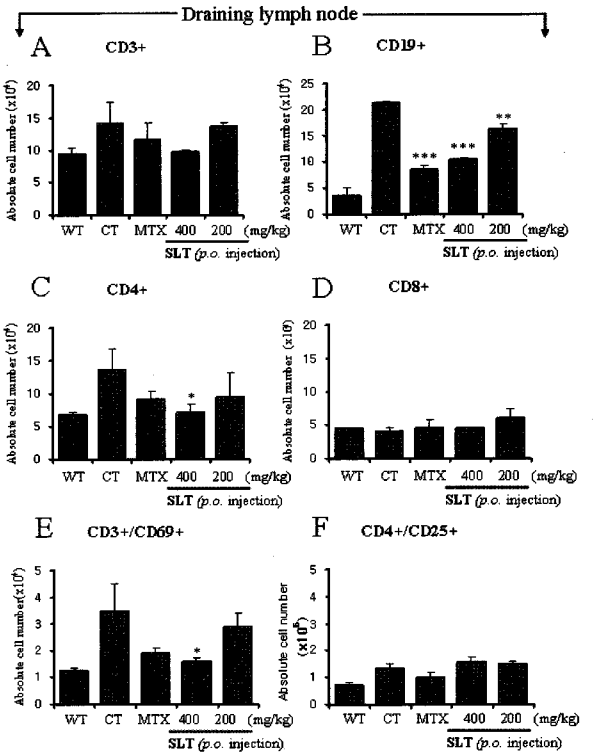

Fig. 4. Effect of SLT on absolute numbers of CD3+, CD19+, CD4+, CD8+, CD3+/CD69+, CD4+/CD25+ cells in lymph node. Cells from lymph node were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01% NaN_3) for 10 min on ice, and analyzed by flow cytometry on a FACScan using CellQuest software (BD Biosciences, Mountain View, CA). Absolute cellnumbers were counted manually in a hemocytometer chamber(Fisher). $2\sim4\times10^3$cells were spun onto glass slides (Cytospin centrifuge ,Cellspin, Hanil,, Korea) (400 g for 4 minutes). Differential count was made according to standard morphologic criteria. LN: Lymph node

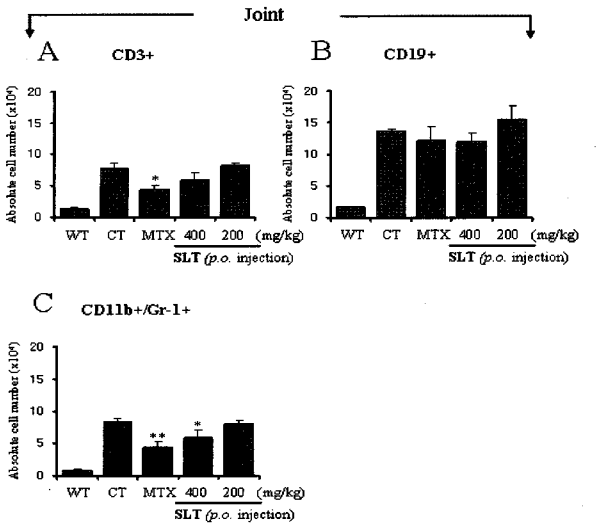

Fig. 5. Effect of SLT on absolute numbers of CD3+, CD19+, CD11b+/G-1+ cells in knee joint.

5. Inhibition of cytokines production in serum

TNF-α, IFN-γ have central roles in the maintenance of chronic inflammation and tissue damage during the progression of RA. Especially, TNF-α is known to play a critical role in the pathogenic mechanisms of a number of chronic inflammatory diseases, including RA. To study whether SLT was related to inflammatory cytokines production, the productions of TNF-α, IFN-γ in serum were analyzed by ELISA, respectively. As shown in Fig. 6, TNF-α, IFN-γ productions in serum were suppressed by SLT. The levels of totalIgG anti-collagen antibodies were lower in murine CIA treated with MTX and SLT than in control group. However, there were no significant differences in the levels of IgG anti-collagen antibodies between murine CIA treated with control and SLT. These results support the conclusion that SLT suppressed the generation of pro-inflammatory cytokines including TNF-α and IFN-γ.

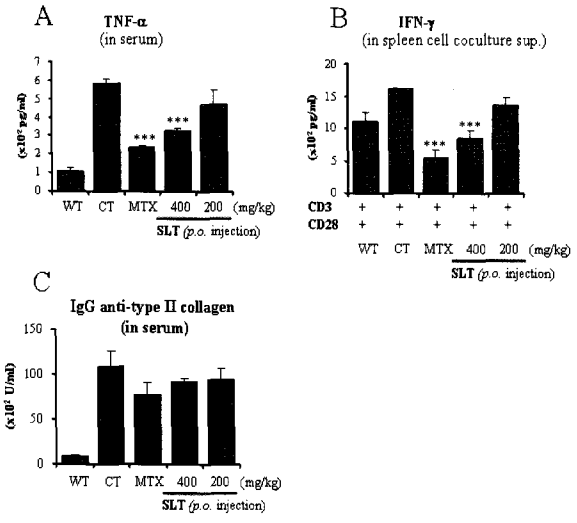

Fig. 6. Effect of SLT on TNF-α, IFN-γ cytokines production in serum and coculuture supernatant in spleen. On the final day of above experiment (previously described in materials and methods), all of the mice were anesthetized with ethyl ether and then blood was collected from each by cardiac puncture; the mice were then killed by cervical dislocation. The mice spleen and serum were taken out and used for ELISA assay. The results are expressed the mean±S.E (N=6). Statistically significant value compared with control group data by Student's t-test (***p<0.001).

6. Histological analysis of knee joint from CIA model mice

To determine whether SLTprevented articular destruction, we analyzed the knee joints of mice histologically. Cartilage erosion and synovial cell infiltration were severe in mice with CIA(Fig. 7B), but SLT group administered at concentration of 400 mg/kg prevented these signs of disease severity condiderably(Fig. 7D). A small increase in synovial cell infiltration was detected in the joints of animals receiving 200 mg/kg of SLT(Fig. 7E), but no discernable cartilage erosion was observed in the knee joints of these animals. These histopathlogic results suggest that SLTsuppresses the immune-mediated pathologic process in CIA model mice.

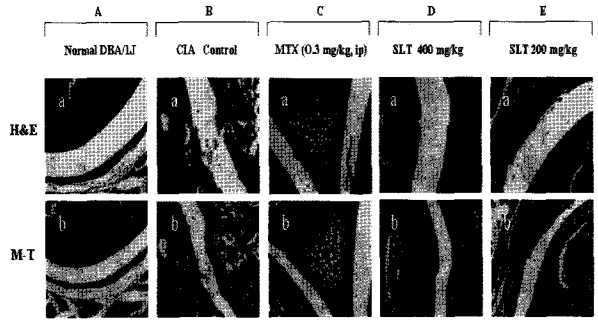

Fig. 7. Pathologic morphology of the knee joints following administration of SLT to mice after the onset of CIA. Mice were treated with PBS only (A), CIA control (B) and MTX (C) or 400, 200 mg/kg SLT (D and E) following the detection of arthritis, as described in Materials and Methods. After 4 weeks of SLT administration, arthritic knee joints were removed and stained with hematoxylin and eosin or Masson's trichrome. Profound cartilage erosion and synovial infiltration (B) were observed in the CIA controls, whereas SLTtreated mice exhibited significantly reduced histologic evidence of destruction and inflammation (D and E). Representative knee joints of mice from each group are shown.

Discussion

SLT has been used in Korean traditional medicine for treatment anti-inflammatory and many allergic diseases. The major contents of SLT are steroid and alkaloids, such as tomatidenol, solasodine, solanidine S, soladulcinine, a-solamarine, b-solamarine etc. The most effective sterol components of the plant (pregnane, steroidal saponin etc.) have cytotoxic, antitumor activities[9,10].

CIA is a well-established in vivo model that has been used in numerous studies to investigate the pathogenesis of RA and for identification of potential therapeutic targets[11]. Collagen-induced arthritis and human RA have been shown to exhibit common immunological and pathological features[12,13], including the involvement of inflammatory mediators in the arthritic etiology.

T cells play a fundamentalrole in the initiation and perpetuation of RA immunopathology, leading to downstream inflammation and, ultimately, soft tissue and joint destruction. CD4+ T-helper cells and macrophages infiltrate the synovial membrane (SM) in chronic, destructive rheumatoid arthritis (RA) and probably play a central role in promoting and maintaining the disease process[14]. In several systems, CD4+ T-cells differentiate into a Th1-like subpopulation, characterized by predominant production of IFN-γ. The Th1-like and Th2-like subpopulations appear to be differentially involved in autoimmune disorders, probably exerting proinflammatory and regulatory functions, respectively[15]. A predominance of proinflammatory Th1-type cells has indeed been postulated in RA[16].

In this study, we report the effect of SLT on CD4+ cells in lymph node and spleen with collagen induced arthritis model mice. CD4+ cells in periphery take an important role in

the induction and development of CIA. CD4+ T cells are required for the induction of CIA, and CD8+ T cells might have a suppressive role in the etiology of CIA[17]. CD4+ and CD8+ T cells are involved in resistance to arthritis though the relative importance of each subset changes during the course of the process leading to the development of resistance to CIA[18]. In our study, SLT could lower the absolute number of CD4+ T cells in spleen and lymph node, and suggest that SLTmight induce immunosuppressive response by lowering the CD4+ T cells and enhancing CD8+ T cells.

In recent years, the role of B cells as part of the immune system and in the pathogenesis of RA has increased dramatically. B cells are known to act as antigen-presenting cells, to secrete pro-inflammatory cytokines, to produce rheumatoid factor (RF) autoantibody and to activate T cells[19]. Such recent developments spurred an interest in the potential role of B cells as a therapeutic target in RA and, specifically, their depletion by use of monoclonal antibody technology.

CD11b and Gr-1 are the most common markers found in suppressor cells and the CD11b+Gr-1+ population is heterogeneous, enclosing mature granulocytes, monocytes etc[20]. The contact between T cells and monocytes leading to the production of TNF and IL-1[21].

In our study, population (absolute number) of CD19+ B cells, CD11b+Gr-1+ population were reduced, but further investigations such as functional studies, signal transduction need to be performed to demonstrate above results.

SLT inhibited various aspects of CIA model mice: arthritis index, spleen cell proliferation, the secretion of pro-inflammatory cytokines, local inflammatory cells influx etc. At present, which compound is responsible for each of these anti-inflammatory effects is not clear. However, it is noteworthy that the secretion of TNF-α, IFN-γ were accompanied by concurrent decreases in absolute number of CD4+ T cells, CD19+ B cells, CD11b+Gr-1+.

In summary, SLT have profound effects on collagen induced arthritis model through suppression of TNF-α, IFN-γ, CD4+ T cells, CD19+ B cells in lymph node. The therapeutic activity of SLT on RA in oriental medicine may be partly related to CD4+ T cells, CD19+ B cells, pro-inflammatory cytokines including TNF-α, IFN-γ. The novel therapeutic roles of SLT that may be applicable to RA. We currently study to clearly understand the mechanism of therapeutic role for SLT in CIA model mice.

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