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Schisandrin B inhibits Th1/Th17 differentiation and promotes regulatory T cell expansion in mouse lymphocytes

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ABSTRACT

Schisandrin B (Sch-B), the most abundant active ingredient of the fruit of *Schisandra chinensis*, has been proposed to have antioxidant, anti-tumor and anti-inflammatory effects. The present study was undertaken to investigate the effect of Sch-B on differentiation of T helper cells (Th). Using mouse splenic lymphocytes stimulated with concanavalin A (Con A) in vitro and ex vivo as inflammation models, we found that Sch-B significantly inhibited secretion of Th1 and Th17 related cytokines, such as IFN-γ and IL-17. In addition, we found that Sch-B suppressed the differentiation of naive CD4+ T cells into Th1 and Th17 cells, while promoted their differentiation into the regulatory T cells (Treg) in vitro. We further found that Sch-B suppressed transcription of Th1-related T-box transcription factor, T-bet, and Th17-related transcription factor, retinoid related orphan receptor gamma t (RORγt), while enhanced transcription of Treg-related transcription factor forkhead box protein 3 (Foxp3) in naive CD4+ T cells under Th cell polarization conditions. Furthermore, the effect of Sch-B on the T cell differentiation was abrogated by heme oxygenase-1 (HO-1) inhibitor zinc protoporphyrin. Taken together, we conclude that Sch-B can modulate differentiation of naïve CD4+ T cells into specific lineages of effector cells, which may have potential benefits for treatment of autoimmune diseases.

1. Introduction

CD4+ T helper cells (Th) are believed to play central roles in modulating immune responses [1]. Upon activation via T-cell receptor (TCR), Th can differentiate into different effector subsets, such as Th1, Th2, Th17 or regulatory T cells (Treg), depending on the cytokines in the microenvironment [2]. For more than two decades, the Th1/Th2 paradigm has been used to explain most of the phenomena related to adaptive immunity [3]. However, this view has been modified following the recent discoveries of Th17 and Treg cells, which are thought to be more critically involved in eliciting and regulating autoimmune responses [4]. Th1 and Th17 induce inflammation and promote immune responses that may lead to autoimmune diseases through secreting proinflammatory cytokines, such as interferon (IFN)-γ and interleukin 17 (IL-17), respectively [5,6]. By contrast, immunosuppressive Treg cells exert an important role in maintenance of immune homeostasis and immune tolerance by producing anti-inflammatory cytokines, such as IL-10 [7]. Imbalance between Th1/Th17 and Treg cells contributes to the pathogenesis of some autoimmune/inflammatory diseases, such as multiple sclerosis (MS) [8], rheumatoid arthritis (RA) [9,10], graft-versus-host disease (GVHD) [11], and type 1 and type 2 diabetes [12,13]. Thus, modulation of an appropriate balance between Th1/Th17 and Treg cells has become a new paradigm for immunomodulatory therapy [14]. Schisandrin B (Sch-B) (Fig. 1), the most abundant active ingredient in the fruit of *Schisandra chinensis*, has been shown protection against hepatitis, hepatotoxins, myocardial disorders and renal damage through inhibition of lipoperoxidative damage to the plasma membrane by removal of reactive oxygen species or reduction of inflammation [15–17]. Sch-B also has an anti-tumor role through antiproliferation and/or induction of apoptosis of tumor cells [18,19]. Recently, Checker et al. reported that Sch-B exhibited an anti-inflammatory effect by activating nuclear factor-erythroid 2-related factor 2 (Nrf2) and its target gene heme oxygenase-1 (HO-1) in lymphocytes [20]. HO-1 can stimulate the anti-inflammatory molecule IL-10 [21], a major anti-inflammatory cytokine secreted by Treg. Furthermore, HO-1 has been shown to inhibit Th17 production and promote Treg cell differentiation, thus alleviating some allergic and inflammatory diseases [22–25]. In addition, carbon monoxide (CO) is endogenously produced by HO-1, and CO releasing molecule-A1 (CRMA1) has an anti-inflammatory role through inhibition of Th1/Th17 cells in vitro [26].

Based on these reports, we hypothesized that Sch-B may have immunomodulatory effect by regulating Th1/Th17 and Treg balance. In this study, we tested this hypothesis in an in vitro system for mouse splenic naïve T cell differentiation and found that Sch-B had distinct effects on T differentiation into different effector lineages.

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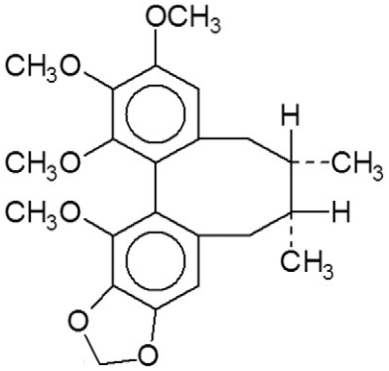

Fig. 1. Chemical structure of Sch-B.

2. Materials and methods

2.1. Chemicals

Concanavalin A (Con A), dimethyl sulfoxide (DMSO) and zinc protoporphyrin (ZnPP) were purchased from Sigma-Aldrich (USA). Schisandrin B (Sch-B) was obtained from Chengdu Must Biotechnology Co. Ltd. (Chengdu, China) with the purity over 99%. Sch-B was dissolved in DMSO at the concentration of 25 mM (stock solution) and stored at −20 °C before use. Fetal bovine serum (FBS) was obtained from Shanghai ExCell Biology Inc. (Shanghai, China). ELISA kits for the detection of cytokines IFN-γ, IL-6, TGF-β and IL-10 were purchased from Beijing 4A Biotech Co. Ltd. (Beijing, China). IL-17 was purchased from eBioscience Inc. (USA). Monoclonal antibodies against CD4 labeled with fluorescein isothiocyanate (FITC), against IL-17, and against INF-γ labeled with phycoerythrin (PE) were purchased from Biolegend Inc. (USA). Mouse Regulatory T Cell Staining Kit was purchased from eBioscience Inc. (USA), containing anti-mouse Foxp3 labeled with allophycocyanin (APC), anti-mouse CD4 labeled with FITC, anti-mouse CD25 labeled with PE and flow cytometry staining buffer. Antibodies against phosphorylated signal transducer and activator of transcription 3 (p-STAT3) (Tyr 705) and β-actin were obtained from Cell Signaling Technologies, Inc. (USA).

2.2. Animals

Pathogen-free male BALB/c mice of 6–8 weeks of age were obtained from The Center for Experimental Animals, Shanxi Medical University. License number: SCXK (Shanxi) 2009-001. The animals were kept under SPF standards with sterilized food and water in animal facility with 22 ± 2 °C and 50% humidity in a 12/12-hour light-dark cycle. They were kept for 1 week before being used for experiments. The protocol of this study was approved by Experiment Animal Ethics Committee of Shanxi Medical University.

2.3. Preparation of mouse lymphocytes

Fresh lymphocytes were prepared from the above BALB/c mice for each experiment. Mice were sacrificed by cervical dislocation. The spleens were immediately excised and gently ground through a 100-mesh screen with red blood cell lysis buffer to lyse red blood cells (RBC) for 5 min. The lymphocytes were harvested by centrifugation at 1200 rpm for 5 min and washed twice with PBS. Naïve CD4+ T cells were isolated from spleens using a CD4+CD62L+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. The purity of the cells is more than 94%.

All lymphocytes were adjusted to 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) containing 100 IU/ml penicillin and 100 μg/ml streptomycin and incubated at 37 °C with 5% CO_2.

2.4. Lymphocyte treatment

The splenic cells were pretreated with various concentrations of Sch-B and stimulated with Con A for detecting the cytokine secretion. For in vivo experiments, mice were injected intraperitoneally (IP) with Sch-B (80 mg/kg body wt.) in 100 μl DMSO. The mice in the control group were treated with an equal volume of DMSO. For cell culture, the same volume of DMSO no more than 0.1% (v/v) was added to each group. Cells of all groups were incubated with Sch-B at 37 °C in 5% CO_2 for 1 h and stimulated with Con A for 48 h for ELISA, 24 h for quantitative real-time PCR (qPCR) and 1 h for western-blot.

For differentiation experiments, Naïve CD4+ T cells were co-cultured with Sch-B in various concentrations, and activated with immobilized anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) (both from eBioscience, USA) in the presence of different cytokines and antibodies as described below for 3 days to allow for naïve CD4+ T cells to differentiate into different effector lineages. For Th1 differentiation, the cultures were supplemented with IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) (both from eBioscince, USA). For Th17 differentiation, both IL-6 (20 ng/ml) and TGF-β (5 ng/ml) (both from eBioscince, USA) were added. For Treg differentiation, the culture was supplemented with TGF-β (5 ng/ml).

2.5. Cytotoxicity and cell proliferation

Cell Counting Kit 8 (CCK-8) was used to investigate cytotoxicity and proliferation of lymphocytes. The assay reagent is a tetrazolium compound (WST-x8) that is reduced by dehydrogenases in live cells into an orange-colored formazan product measured at 450 nm [27]. The quantity of formazan product measured at 450 nm is directly proportional to the number of live cells in the culture. For cytotoxicity analysis, 100 μl of complete medium contain 2×10^5 cells per well containing Sch-B at different concentrations were cultured for 24 h. For cell proliferation experiment, 2×10^5 cells per well in 100 μl complete medium were cultured in the presence of Con A with different concentrations of Sch-B for 24 h. After incubation, 10 μl of CCK-8 solution was added to each well for additional 4 h, and absorbance at 450 nm was measured using a microplate reader (ELX 80, BioTek Instruments, Inc., USA). The well with CCK-8 in medium without cells was used as the blank control.

2.6. ELISA

The concentrations of IFN-γ, IL-10, IL-17, IL-6 and TGF-β in supernatants obtained from different groups were measured using commercially available ELISA kits according to the manufacturer's protocols.

2.7. Flow cytometry

Lymphocytes were stimulated with 50 ng/ml phorbol myristate acetate (Sigma-Aldrich, USA) and 500 ng/ml ionomycin (Sigma-Aldrich, USA) for 4 h in the presence of 1 mM brefeldin A (Sigma-Aldrich, USA). Cells were then stained with FITC-conjugated anti-mouse CD4 (eBioscience) at 4 °C in dark, followed by fixation and permeabilization, and PE-conjugated anti-mouse IFN-γ and PE-conjugated anti-mouse IL-17 were used for Th1 and Th17, respectively. Treg cells were stained according to the manufacturer's instruction. FITC-labeled rat anti-IgG1 (Biolegend) and PE-conjugated anti-rat IgG2a isotype (eBioscience) were used as negative controls. Anti-mouse CD16/32 was used to block non-specific staining. Cells were analyzed with a FACS Calibur cytometer (BD, USA) and WinMdi 2.8 software.

2.8. Quantitative real-time PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The RNA samples were reverse-transcribed into cDNA using a reverse transcription kit from