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Evaluation of the immunosuppressive activity of artesunate *in vitro* and *in vivo*

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ABSTRACT

Artemisinin and its derivatives have been reported to have immunosuppressive activity in some laboratory studies. However, the detail of mechanism remains to be demonstrated. The objective of this study is to clarify the immunosuppressive activity of artesunate (AST), one kind of artemisinin derivatives, and to find its unexplored mode of action. *In vitro*, the proliferation of T lymphocytes and its cytotoxicity were measured by WST-1 and MTT assay. *In vivo*, the immunomodulatory effect of AST was evaluated in a mouse model of delayed type hypersensitivity reaction (DTH), which was based on a T cell-mediated immune response. The data displayed that AST had a relatively high immunosuppressive activity with low toxicity, and could inhibit T lymphocyte proliferation induced by mitogen and alloantigen. Meanwhile, topical administration of AST could suppress DTH response significantly. Moreover, AST could also increase the secretion of TFG-β, coupling with the striking enhance of NF-κB/p65 and Smad2/3 signaling. The promotion of $CD4^+CD25^+$ regulatory T cells (Tregs) was shown to be a possible mechanism involved in AST-mediated regulation. Taken together, these observations exhibit the potential of developing AST as a novel safe remedy for the treatment of T cell-mediated immune disorders.

1. Introduction

People often treat patients who suffered from autoimmune diseases, allergies and organ transplantation with immunosuppressive drugs. The clinical application of cyclosporin A and tacrolimus makes the treatment of these diseases more successful. However, the use of immunosuppressive drugs could bring with the arising risks of systemic immunosuppression [1]. Thus, it's important to find some new agents with superiority. Many drugs derived from traditional Chinese medicine have been proved to possess some special advantages as with high activity and low toxicity [2]. For instance, artemisinin (formerly referred to as arteannuin or as qinghaosu in Chinese), the active component of *Artemisia annua* L, has a long history as an antimalarial remedy [3]. Until very recently, artemisinin and its derivatives have been demonstrated to work on anti-inflammation and immune regulation [4]. Artesunate (AST), one of semi-synthetic derivatives of artemisinin, has also been reported to have a good effect on systematic lupus erythematosus, rheumatoid arthritis and allergic contact dermatitis with little toxicity, which makes it to be tolerated [5–8]. Despite recent progresses in understanding its immunosuppressive activity, the exact mechanism and signaling pathway of AST remain unclear.

It's well known that regulatory T cells (Tregs), expressing the IL-2 receptor α chain (CD25) and the forkhead family transcription factor Foxp3, are very important in the maintenance of immune homeostasis [9]. Enhancement of Tregs may regulate immune response negatively and protect individual from autoimmune and rejection [10]. In the present study, AST could inhibit the proliferation of T lymphocytes and T cell-mediated immune response *in vivo* and *in vitro*. The promotion of Tregs might be a possible mechanism involved in the immunoregulation of AST.

Abbreviations: ConA, concanavalin A; DNBS, 2, 4-dinitrobenzene sulfonic acid; DNFB, dinitrofluorobenzene; DTH, delayed type hypersensitivity reaction; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; Foxp3, forkhead/winged helix transcription factor3; LNs, lymph nodes; MLR, mixed lymphocyte culture reaction; MMC, mitomycin C; NF-κB, nuclear factor-κB; RT-PCR, reverse-transcriptase polymerase chain reaction; TGF-β, transforming growth factor-β; Tregs, regulatory T cells.

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2. Material and methods

2.1. Chemicals and reagents

AST (98% purit), ConA, DNFB, DNBS, MTT and MMC were purchased from Sigma Aldrich (St. Louis, MO, USA). WST-1 cell proliferation assay kit was from Beyotime (Haimen, Jiangsu, China). Anti-mouse NF-κB/p65 antibody was acquired from Bioworld (Nanjing, Jiangsu, China). Antibodies to TBP and Smad2/3 were purchased from Abcam (Cambridge, MA, USA) or Cell Signaling (Danvers, MA, USA). RPMI 1640, penicillin, streptomycin, and FCS were purchased from GIBCO (Grand Island, NY, USA).

2.2. Experimental animals

BALB/c and C57BL/6 mice (6 to 8 weeks old) were purchased from the Experimental Animal Centre of the Academy of Military Medical Science. All experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Logistics University of the Chinese People's Armed Police Force.

2.3. Cytotoxicity assay

Cytotoxicity was measured by MTT assay as described by Mosmann with some modifications [11]. Briefly, splenocytes (5×10^5 cells/well) were cultured in RPMI1640 complete medium (supplemented with 2 mmol/L L-glutamine, 10% (v/v) heat-inactivated FCS and 100 U/mL penicillin–streptomycin) for 48 h at 37 °C in a humidified atmosphere of 95% air/5% CO_2. 5 mg/mL MTT was added 4 h before the end of culture. After removal of MTT, the formazan precipitate was solubilized in DMSO. $OD_{570\ nm}$ was measured on a microplate reader (Model 680, Bio-Rad). The percentage of cell viability was calculated according to the following formula: viability rate (%) = $OD_{treated}$ / $OD_{control}$ × 100% [12].

2.4. Determination of proliferation response

The proliferation of splenocytes in response to ConA was determined by WST-1 assay, which was a colorimetric assay for the quantification of proliferation and viability in viable cells [13]. Briefly, splenocytes (5×10^5/well) were cultured with 5 μg/mL of ConA in a 96-well flat-bottomed plate for 48 h. Then, WST-1 reagent (1:10) was added and the absorption was measured at 450 nm. The inhibitory rate was calculated as follows: inhibitory rate (%) = [($OD_{control}$ − $OD_{treated}$) / $OD_{control}$] × 100.

2.5. Mixed lymphocyte culture reaction (MLR)

The MLR assay was conducted as described by Zhou [14]. Responder cells (5×10^5 cells/well) were obtained from BALB/c strain, whereas stimulator cells (5×10^5 cells/well) from C57BL/6 strain were pretreated with 50 μg/ml of MMC for 30 min. These cells were co-cultured for 48 h, and determined by WST-1 assay.

2.6. Antigen-specific T lymphocyte proliferation assay

Antigen-specific T lymphocyte proliferation induced by DNBS (the water-soluble analog of DNFB) *in vitro* [15] was further carried out to explore the immunomodulatory effect of AST. Firstly, dinitrophenyl-modified spleen cells (DNP-SC) were prepared as described before [16]. Spleen cells (SC) obtained from normal BALB/c mice were mixed with equal volume of 10 mM DNBS, and incubated for 30 min at 37 °C. These cells were washed three times and cultured with MMC (50 μg/mL) for 40 min, which DNP-SC were used as stimulator cells. Meanwhile, BALB/c mice were sensitized with 0.5% (w/v) DNFB dissolved in 4:1 acetone/olive oil on the shaved abdomen (day 0 and day 1). LN cells were collected from the sensitized mice on day 7, which were used as responder cells. Then, the 5×10^5 cells/mL stimulator cells and 5×10^5 cells/mL responder cells were co-cultured for 48 h. WST-1 assay was used to measure the antigen-specific T lymphocyte proliferation.

2.7. DNFB-induced DTH

The experiment for investigating DTH reaction was performed as Wang [17]. BALB/c mice were sensitized as described previously, and were challenged with 0.3% (v/v) DNFB on the right ears on day 7. Meanwhile, 25 mg AST (2%, 4%, 8%) ointment and an equivalent amount of vehicle were administered topically to the mice for 10 consecutive days (day −1 to day 8). The mice were divided into five groups, 10 animals each: normal control group, vehicle control group and three AST groups. The increase of ear patch weight and ear thickness between left and right ear was measured before and at 48 h after challenge. Meanwhile, the auricular and mandibular lymph nodes (LNs) and ear pinnae were collected for further analysis.

2.8. Histopathologic examination

All ears of mice were collected and subjected to histological examination. These ears were fixed in 10% neutral buffered formalin for 24 h, subsequently embedded in paraffin, and sectioned (4 μm) successively through the midsagittal plane, stained with hematoxylin and eosin (H&E), and examined by Motic digital microscope (Olympus). A certified pathologist analyzed and scored the samples in a blinded manner. A minimum of 3 sections per experimental animal was examined for the presence and degree of thickening of the epidermis and inflammation of the epidermis and dermis.

2.9. Flow cytometric analysis

Flow cytometry was used to characterize the effect of AST on Tregs as described previously [18]. $CD4^+CD25^+Foxp3^+$ cells were isolated from the LNs using a regulatory T cell staining kit (eBioscience) according to the manufacturer's protocol.

2.10. RT-PCR

Total RNA was extracted from T cells of the LNs using Trizol reagent (Invitrogen). RNA was quantitated by optical density measurement at 260 and 280 nm, and integrity was confirmed by running RNA on a 1% agarose gel. Equal amounts of RNA (1 μg) were reverse transcribed into cDNA using oligo(dT) primers. Amplification was performed using the following primers: Foxp3 (324 bp): 5′-TCCTTCCCAGAG TTCTTCCA-3′ and 5′-GGCTAGGTTGGAACTAGGGG-3′ GAPDH (225 bp): 5′-TGGAGAAACCTGCCAAGTATG-3′ and 5′-CCCTGTTGCTGTAGCCGTAT-3′. The PCR conditions were optimized to keep the amplification in the linear range to avoid the plateau effect. After amplification, PCR products were analyzed on a 1% agarose gel, visualized under UV light and quantified by Imager (GelDoc 2000, BIO RAD).

2.11. ELISA

The experiment to study cytokine was performed as described previously [18]. T cells were purified from the LN cells using immunomagnetic negative selection, and the purity of the resulting T-cell population was more than 95% as assessed by flow cytometric antibody (BD Biosciences). The splenocytes of normal mice were incubated for 30 min at 37 °C with 10 mmol/L DNBS and treated with 50 μg/mL MMC for 30 min, which were used as antigen presenting cells (APCs). Purified T cells (5×10^6 cells/mL) from the