The Immunotherapeutic Effects of *Astragalus* Polysaccharide in Type 1 Diabetic Mice

Ru-Jiang Li, Shu-Dong Qiu, Hong-Xia Chen, Hong Tian, and Hai-Xu Wang

*Department of Histology and Embryology, Key Laboratory of Environment and Genes Related to Disease (Ministry of Education), Medical School of Xi’an Jiaotong University; Xi’an 710061, China; and Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences; Beijing 100850, China.

Received August 31, 2006; accepted December 1, 2006

The present study investigated whether *Astragalus* polysaccharide (APS) possessed immunotherapeutic effects on type 1 diabetes mellitus. Diabetic mice induced by multiple low dose streptozotocin (MLD-STZ) were administered either APS (100, 200, 400 mg/kg body weight) or saline intraperitoneally daily, and sacrificed after 15 or 30 d of treatment. Meanwhile normal mice not treated with STZ nor with APS were offered into non-diabetic group. Blood glucose and serum insulin levels were measured, histologic and morphometric analyses of the pancreas were performed to determine the effect of APS on pancreatic islets. Further investigations on immune changes in spleens were tested by ELISA, semi-quantitative RT-PCR and Western blot. Downregulated blood glucose level, upregulated serum insulin concentration, increased β cell mass, decreased apoptotic β cell percentage, downregulation of Th1/Th2 cytokine ratio and upregulation of peroxisome proliferator-activated receptor gamma (PPARγ) gene expression in spleens were significantly time- and dose-dependent on APS treatment, when compared to saline controls. These results show that APS seems to be helpful to attenuate insulitis and preserve β cells from apoptosis, but it can’t entirely rescue type 1 diabetes mellitus. APS ameliorates both the clinical and histological parameters of the MLD-STZ induced diabetic mice in a long-lasting fashion, most likely through immunoregulatory actions on Th1/Th2 cytokine ratio, strongly associated with PPARγ gene expression in spleens.

**Key words** *Astragalus* polysaccharide; type 1 diabetes mellitus; Th1; Th2; PPARγ

Type 1 diabetes mellitus (T1DM) is one of the most frequently occurring chronic diseases in puberty. It afflicts millions of individuals worldwide, and its prevalence and incidence continue to rise annually. This disease results from selective destruction of the insulin-producing β cells in the pancreatic islets, and is primarily a T cell-mediated autoimmune disease directed against one or more β cell autoantigens. T cells specific for islet β cell autoantigens may exist normally but are restrained by immunoregulatory mechanisms (the self-tolerant state). However, T1DM develops when one or another immunoregulatory mechanism fails, allowing β cell autoreactive T cells to become activated, expand clonally and entrain a cascade of immune and inflammatory processes in the islets. This autoimmune inflammatory process is caused by a switch of the immune balance between T helper 1 and 2 (Th1 and Th2) lymphocytes towards Th1 response, initiated and propagated by the effect of Th1 cells and their secreted cytokines (e.g. interferon γ), and suppressed by Th2-secreted anti-inflammatory cytokines (e.g. IL-4, IL-10).

Usual clinical treatment for T1DM, such as insulin or insulin replacement, may ameliorate the symptoms of the endocrine disease, but it does not affect the autoimmune process. To successfully recover from T1DM, concomitant immunosuppressive therapy is indispensable to protect β cells from immune-mediated destruction. However, the broad spectrum immunosuppressive diabetic therapy is not completely free of islet toxic side effects and is associated with deleterious systemic complications. Although new immunomodulatory therapeutic regimens focused on GAD and heat shock protein 60 (Diapep277) which is designed to inhibit β cell destruction specifically, are in the early clinical trial phase, they are used only in new-onset of T1DM or in individuals with latent autoimmune diabetes of the adult, and appear to have marginal efficacy in the inhibition of insulitis and in decreasing insulin requirements. Therefore, today, there is an urgent need to search for alternative therapies that may have a similar degree of efficacy on the protection of exogenously or endogenously expanded β cell mass from the prevailing anti-β cell leukocyte population without undesirable side effects. The identification of compounds with immunomodulatory activity from medicinal plants may provide an opportunity to develop a new class of antidiabetic agents. According to previous reports, *Astragalus* polysaccharide (APS) is one of the most important and valuable compounds with immunomodulatory properties.

Polysaccharides from natural sources are a class of macromolecules that can profoundly affect the immune system and therefore have the potential as immunomodulators with wide clinical applications. *Astragalus* polysaccharide (APS) is extracted from *Astragalus membranaceus* (Huangqi). Scientific investigation in the last two decades has revealed much insight into the pharmacological functions of different components of Huangqi, especially its polysaccharide fractions.

*Astragalus* polysaccharide (APS) is reported to have antioxidant, antidiabetic, and immunomodulatory activities. In vivo studies, administering APS or Huangqi extract markedly decreases normal murine Th1/Th2 cytokine ratio. This immunopharmacological profile of APS may be related to its beneficial effects in the diseases with imbalance of Th1/Th2 cytokine ratio, such as T1DM. Although APS may improve the metabolism of streptozotocin (STZ) induced diabetic rats, enable insulin-sensitizing and antihyperglycemic activity in fat-fed STZ-treated rats, and before onset of T1DM in NOD mice pretreated with APS, diabetes mellitus can be partly prevented, there is no data on immunotherapeutic effects of APS on T1DM after the disease.
happens, and no reports on immunopharmacological profile of APS on T1DM. The purpose of our study was to investigate the therapeutic effects of APS on hyperglycemia, and elucidate the immunotherapeutic molecular mechanisms of how APS attenuated the development of T1DM induced by multiple low dose streptozotocin (MLD-STZ).

MATERIALS AND METHODS

Type 1 Diabetic Mice Model and Treatment Protocol
C57BL/6J male mice at the age of 12 weeks were obtained from Experimental Animal Center of Xi’an Jiaotong University Medical College (SPF, No. SCXK11-00-0004). The mice were housed in a controlled temperature environment with a 12 h light/dark cycle, and allowed free access to food and water, except when fasted before experiments. All experiments were carried out in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals.

The C57BL/6J male mice were rendered diabetic by intraperitoneal injection of STZ (40 mg/kg body weight on 5 consecutive days; Sigma, U.S.A.) in citrate buffer (pH 4.2). After confirmation that all mice were diabetic (blood glucose greater than 11.1 mmol/l) for at least 3 d, they were randomly allocated into four groups: group V (T1DM mice >11.1 mmol/l for at least 3 d), group APS200 (T1DM + APS 200 mg/kg BW/d), group APS100 (T1DM + APS 100 mg/kg BW/d), and group saline (T1DM + saline vehicle). Each diabetic group of mice received daily intraperitoneal injections of saline or APS (100, 200 or 400 mg/kg BW; Pharmagenesis Inc., U.S.A.) respectively. To evaluate the therapeutic effects of APS on diabetic mellitus, normal C57BL/6J male mice not treated with STZ nor with APS were offered into non-diabetic group (group N).

The mice were sacrificed by exsanguination after 15 or 30 d of treatment. The spleens were removed aseptically for preparation of murine splenocytes, semi-quantity RT-PCR and Western-blot, and the pancreata were excised for morphologic analyses. During all experiments, glycemia was assessed on blood collected from the tail vein using an One-Touch Ultra blood glucose meter (LifeScan, U.S.A.), and serum insulin levels were determined by radio-immunoassay (RIA) with a kit from Beifang Biotech Research Center (Beijing, China).

Microscopy and Morphometric Analysis of Pancreatic Islets
On pancreatic excision, each organ was weighed and then fixed in 4% paraformaldehyde. Samples were embedded in paraffin, 3 sections 4- to 5 µm thick were taken from each pancreas in multiple 100-µm increments, and sections were stained as follows: (1) for insulin (peroxidase staining) and haematoxylin for light microscopy; (2) insulin and cleaved caspase-3 double stained as follows: (1) for insulin (peroxidase staining) and Cy3-conjugated goat anti-rabbit IgG and Cy2-conjugated goat anti-porcine insulin (Dako, Denmark; 1:400) or a mixture of fluorescein-5-isothiocyanate (FITC)-tagged goat anti-guinea pig IgG and Cy3-conjugated goat anti-rabbit IgG (both from West Grove, U.S.A.; 1:100), 40 min at 37 °C, then the latter were washed extensively and mounted in 80% glycerol in PBS, and the former were washed and incubated with avidin–biotin complexed with horseradish peroxidase (Dako) at 37 °C for 40 min, developed using 3,3′-diaminobenzidine (DAB) tetrahydrochloride (Sigma), then counterstained with Harris’ hematoxylin (Sigma).

To determine β-cell mass (mg/pancreas), each gland was sectioned along its longitudinal axis to avoid any sampling bias resulting from regional variation in islet distribution and cell composition. Islet cross-sectional areas were traced manually with the aid of an Olympus BX60 microscope connected by video camera to a computer equipped with Image-Pro Plus software version 5.1. Briefly, islets from insulin-stained sections were traced and thresholded using the Image-Pro Plus software system to determine stained tissue area. The total pancreatic tissue was also measured and a percent β cell area was calculated. This percent was then multiplied by the organ weight to determine the β cell mass.

Apoptotic β cells were identified in deparaffinized pancreatic sections using dual staining with Caspase-3/Insulin. Caspase-3/Insulin double positive cells per section were counted in 100 low-power microscopic fields, and an average of 40 fields in three immunostained slides was surveyed for each animal. Ten animals per group were used. Results were expressed as percentage of double positive cells per total β cells.

Splenocyte Culture and Cytokine Determination
After aseptic removal, spleens were placed in cold Hanks solution and teased apart with a pair of forceps and a needle. A single cell suspension from the teased tissue was obtained by passing it through a 200-mesh net and hemolysed by the buffer solution containing 1 mM Tris·HCl and 1% NH4Cl (pH 7.2). Subsequently, the macrophage cell content was depleted by incubation of the cell suspension in tissue culture dishes at 37 °C (air + 5% CO2) to allow these cells to adhere to the bottom of the culture dishes. Remaining free floating cells were seeded on culture dishes at a density of 5×106 cells/ml in RPMI 1640 (Sigma) with 10% foetal calf serum (Sigma), 2 mM l-glutamine (Sigma). Cell viability was determined by Trypan blue dye exclusion.

The splenocytes (5×106 cells/ml) were treated with 2 µg/ml concanavalin A (Con A; Sigma) for 48 h or 72 h, and cell supernatants were collected, then levels of IL-4 (48 h) and IFN-γ (72 h) were measured by ELISA kits (R&D Systems, U.S.A.) according to the manufacturer’s instructions.

Semi-quantitative RT-PCR for PPARY Gene Expression
Total RNA of spleens were extracted by TRIZOL Reagent kit (GIBICOL), the RNA concentrations were adjusted to 1 µg/µl by measuring the OD value, and stored at −80 °C. Using BcaBEST RNA PCR Kit Ver.1.1 (Takara, Japan), reverse transcriptions were performed with 1 µg of total RNA. The cDNA was amplified by polymerase chain reaction (PCR) in a 25 µl reaction volume containing 2.5 µl of 10×PCR buffer, 1 µl of each 2.5 mMol/l dNTP mixture, 1 unit of Ex Taq DNA polymerase (Takara, Japan), 1 µl of
cDNA, 1 μl of each 10 pmol/l primers (PPARγ, sense 5'-GAG ATG CCA TTC TGG CCC ACC ACC AAC TTC GGA-3' and antisense 5'-TAT CAT AAA TAA GCA TCA ATC GGA TGG TTC-3'; β-actin, sense 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' and antisense 5'-TAAAAC GCA GCT CAG TAA CAG TCC G-3'). The condition of PCR amplification was: one cycle at 97 °C for 5 min, 30 (26) cycles at 95 °C for 30 s, 55 °C (50 °C) for 30 s and 72 °C for 1 min, and a final extension cycle at 72 °C for 8 min. PCR reactions for PPARγ gene were restricted to the linear range of amplification by limiting the cycle number to 30 (annealing at 55 °C), and amplification of the β-actin control fragment, owing to its higher level of expression, was subjected to only 26 cycles (annealing at 50 °C). PCR-amplified fragments were run beside molecular weight markers on 2% agarose gels stained with ethidium bromide. Gels were photographed using the electrophoresis gel imaging system (UVP). The semi-quantitative measure of gene expression was using the ratios of PPARγ/β-actin absorption density of bands on a gel.

**Western Blot Analysis** Spleens were homogenized in PBS containing 2 mmol/l EDTA, 5 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride, 1% TritonX-100 (pH 7.4). Homogenized samples were centrifuged at 13000×g for 15 min at 4 °C and the resulting supernatants were saved. The protein content in the supernatant was determined by bichoninic acid assay using bovine serum albumin as the standard. Ten milligrams of protein was subjected to 12% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes, and the membranes were blocked in Tris-buffered saline containing 15% powder milk and 0.1% Tween-20. Immunoblotting was carried out with a rabbit polyclonal antibody against PPARγ (at 4 °C overnight; Santa Cruz). β-Actin antibody was used for internal controls (Sigma). After incubation with the primary antibody, membranes were washed with 1×PBS containing 0.1% Tween-20 4 times. Secondary antibodies, IgG-horse-radish peroxidase conjugate (Santa Cruz) were used, respectively, for PPARγ and β-actin antibodies (at room temperature for 2 h). After washing, equal amounts of enhanced chemiluminescent reagent (ECL, Amersham Pharmacia Biotech) were applied to membranes for 1 min. Membranes were then wrapped in plastic, placed in X-ray film cassettes containing Kodak Medical X-ray film (Kodak Medical Systems), and exposed in a dark room for 45—120 s. The exposed film was scanned using a densitometer (Furi, Shanghai) to determine the optical densities of each band, and the density ratio of PPARγ to β-actin bands was calculated. All experiments were repeated for triplicate.

**Statistical Analysis** All values are expressed as the means±S.E.M. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test. A value of p<0.05 was considered statistically significant.

**RESULTS**

**Characteristics of Experimental Animals** Administration of APS for 15 d did not affect blood glucose and insulin levels. All STZ-induced diabetic mice with APS treatment remained hyperglycemic on day 15, no significant difference in blood glucose levels was demonstrated between APS (APS100, APS200 or APS400) and saline vehicle group. Moreover, plasma insulin levels of the APS-treated animals at the end of the 15-d period were not significantly different from saline controls. However, the levels of glucose in diabetic mice were significantly reduced (p<0.01) after treatment with APS (200, 400, except for 100 mg/kg BW/d) for 30 d, when compared to the values of saline control mice, whilst their insulin concentrations were significantly increased (p<0.05, p<0.01). Unfortunately, the levels of glucose and insulin in TIDM mice with APS treatment were still significantly different from normal mice (p<0.01) (Fig. 1).

**Microscopy and Morphometric Analysis of Pancreatic Islets** To investigate the reasons of antihyperglycemia of APS, histological analysis of pancreas in MLD-STZ induced diabetic mice was performed on day 15 and day 30 with APS treatment. In contrast to normal mice, in which islets were spherical, islets from both APS-treated or saline-treated diabetic mice showed altered shape and lymphocytic infiltration. Although no difference of insulitis was observed between APS- and saline-treated diabetic mice on day 15 or between group APS100 of mice and saline controls on day 30, lymphocytic infiltration in islets was attenuated greatly after treatment with APS (200, 400 mg/kg BW/d) for 30 d (Fig. 2). The mice of group APS200 or APS400 on day 30 had a high percentage of noninfiltrated islets and islets with less than 25% infiltration, whereas most of saline controls of islets had a high level of infiltration. These suggest that APS attenuates insulitis development.

Furthermore, to study whether APS protected β cells from apoptosis in TIDM in vivo, we performed double immunofluorescence analysis of insulin and Caspase 3 to identify apo-
totic β cells and β cell mass. On day 15 with APS treatment, the percentage of apoptotic β cells and β cell mass in diabetic mice were not significantly different from the values of saline controls (p>0.05). After 30 d of treatment, although a similar value of apoptotic β cell percentage or β cell mass was observed between group APS100 and V group of mice (p/H11022 0.05), a significant decline of apoptotic β cell percentage (about 40%, 50% respectively; p/H11021 0.05) and increase of β cell mass (about 2-fold, 2.4-fold respectively; p/H11021 0.01) were noted in APS200 or APS400 group of diabetic mice compared to saline controls (Figs. 3, 4). Moreover, the most striking feature of pancreata in APS200 and APS400 group of animals was small scattered clusters of insulin-producing cells, budding from intralobular and terminal ductules (data not shown). However, apoptotic β cell percentage and β cell mass in groups APS200 or APS400 of mice on day 30 with APS treatment were still significantly different from the values of normal mice (Figs. 3, 4). All of these results suggest that APS may protect, but not entirely prevent β cells from apoptosis resulted from insulitis after 30 d of APS treatment.

Effects of APS on Cytokine Production in Type 1 Diabetic Murine Splenocytes

To investigate the immunotherapeutic effects of APS on T1DM, the productions of IL-4 and IFN-γ, important cytokines of Th1/Th2 cell differentiation process, were evaluated by ELISA method. Culture of splenocytes without Con A did not yield any detectable levels of cytokines (data not shown). In order to maintain cytokine production in vitro from activated spleen cells, Con A was added. Ex vivo determination of IL-4 or IFN-γ secretion from splenocytes demonstrated no significant difference between group APS (APS100, APS200 or APS400) and saline vehicle after 15 d of treatment (p>0.05). On day 30 with

Fig. 2. Pancreata Immunolabeled for Insulin and Counterstained for Lymphocytic Infiltration in Pancreas Islets after 30 d of Treatment with APS

N, group N of mice (normal mice); V, group V of mice (diabetic control mice); APS100, group APS100 of mice (diabetic mice administered APS at 100 mg/kg BW/d); APS200, group APS200 of mice (diabetic mice administered APS at 200 mg/kg BW/d); APS400, group APS400 of mice (diabetic mice administered APS at 400 mg/kg BW/d). No differences of insulitis were observed between group APS100 and V of mice. However, lymphocytic infiltration in islets was significantly attenuated in group APS200 or APS400 of mice compared with group V. Magnification: 200×.

Fig. 3. Double Immunofluorescence Staining for Apoptotic β Cells by Insulin (Green), Cleaved Caspase 3 (Red) in Pancreatic Islets of MLD-STZ Induced Diabetic Mice after 30 d of APS Treatment

N, group N of mice (normal mice); V, group V of mice (diabetic control mice); APS100, group APS100 of mice (diabetic mice administered APS at 100 mg/kg BW/d); APS200, group APS200 of mice (diabetic mice administered APS at 200 mg/kg BW/d); APS400, group APS400 of mice (diabetic mice administered APS at 400 mg/kg BW/d). Arrow indicates a small scattered cluster of insulin-producing cells. Arrowheads show insulin and cleaved caspase 3 overlap (yellow). Magnification: 200×.

Fig. 4. Effect of APS on Frequency of Apoptosis in β Cells (a) and β Cell Mass (b) in MLD-STZ Induced Diabetic Mice after 10 or 30 d of Treatment

N, group N of mice (normal mice); V, group V of mice (diabetic control mice); APS100, group APS100 of mice (diabetic mice administered APS at 100 mg/kg BW/d); APS200, group APS200 of mice (diabetic mice administered APS at 200 mg/kg BW/d); APS400, group APS400 of mice (diabetic mice administered APS at 400 mg/kg BW/d). All data were means±S.E.M. calculated from 10 diabetic mice. p<0.05, p<0.01 compared with normal mice; *p<0.05, **p<0.01 compared with saline vehicle group.
STZ induced C57BL/6J mice were chosen to evaluate the diabetic mice. All data were not significantly different from the values of non-immune insulitis and specially study the immune pathways and modulations of autoimmune insulitis and β cell death. As such, MLD-STZ induced C57BL/6J mice were chosen to evaluate the an-
treatment, however, the splenocytes obtained from group APS200 or APS400 of diabetic mice, had lower IFN-γ, higher IL-4 release and lower IFN-γ/IL-4 ratio in comparison to saline vehicle group (p<0.05, p<0.01). In contrast, there was no difference between the group V and APS100 at the same time point (p>0.05). Meanwhile, it should be noted that the production of IL-4, IFN-γ and IFN-γ/IL-4 ratio in group APS200 or APS400 of mice on day 30 with APS treatment were not significantly different from the values of normal mice (p>0.05) (Table 1). These data show that APS may rescue successfully imbalance of Th1 and Th2 in type 1 diabetic mice.

Effects of APS on Expressions of PPARγ in Spleens of Type 1 Diabetic Mice To characterize the PPARγ gene expression in spleens of diabetic mice, semi-quantitative RTPCR and Western blot analysis were carried out after 15 or 30 d of treatment. Our results showed PPARγ gene expressed time- and dose-dependently in spleens with APS treatment (Fig. 5). The gene mRNA and protein expressions were markedly abundant in APS400 group of spleens after treatment for 15 d, but not markedly different in APS100 or APS200 group from in saline vehicle group or non-diabetic group (Fig. 5c, the data of protein expressions not shown). After 30 d of treatment, PPARγ both mRNA and protein levels in spleens were significantly increased in group APS200 or APS400, and no markedly different in group APS100, compared to group V or group N (Fig. 5).

DISCUSSION

In susceptible rodents (e.g. C57BL/6J), MLD-STZ (40 mg/kg on five consecutive days) is able to induce an insulopenic diabetes in which immune destruction plays a role, as a T-cell-dependent autoimmune disease resulting in selective destruction of the β cells, exhibiting histoinnunological and clinical similarities to type 1 diabetes in humans. This model of diabetes offers some advantages, such as simultaneous appearance of diabetes mellitus in all animals and not having immune abnormalities that may complicate studies in spontaneous diabetes in NOD mice or BB rats. Therefore, this model as a good model has been applied extensively to investigate the type 1 diabetes mellitus, especially study the immune pathways and modulations of autoimmune insulitis and β cell death. As such, MLD-STZ induced C57BL/6J mice were chosen to evaluate the an-

Table 1. Effect of APS on Productions of IL-4 and IFN-γ of Diabetic Murine Splenocytes Induced by Con A ex Vivo

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>V</th>
<th>APS100</th>
<th>APS200</th>
<th>APS400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>245±7</td>
<td>194±17A</td>
<td>201±12A</td>
<td>199±18A</td>
<td>193±14A</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>230±6</td>
<td>290±21A</td>
<td>293±14A</td>
<td>296±11A</td>
<td>291±13A</td>
</tr>
<tr>
<td>IFN-γ/IL-4</td>
<td>0.97±0.09</td>
<td>1.62±0.1A</td>
<td>1.57±0.69A</td>
<td>1.50±0.69A</td>
<td>1.69±0.12A</td>
</tr>
<tr>
<td>Day 15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>241±6</td>
<td>181±16A</td>
<td>189±10A</td>
<td>191±13A</td>
<td>192±10A</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>272±18</td>
<td>303±14A</td>
<td>281±13A</td>
<td>284±12A</td>
<td>285±16A</td>
</tr>
<tr>
<td>IFN-γ/IL-4</td>
<td>0.94±0.06</td>
<td>1.71±0.1A</td>
<td>1.90±0.60A</td>
<td>1.52±0.66A</td>
<td>1.49±0.47A</td>
</tr>
<tr>
<td>Day 30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>238±21</td>
<td>156±12A</td>
<td>167±10A</td>
<td>205±16*</td>
<td>222±14**</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>243±29</td>
<td>308±12A</td>
<td>277±13</td>
<td>265±12*</td>
<td>255±9**</td>
</tr>
<tr>
<td>IFN-γ/IL-4</td>
<td>1.02±0.06</td>
<td>2.05±0.12A</td>
<td>1.81±0.10A</td>
<td>1.34±0.09**</td>
<td>1.17±0.07**</td>
</tr>
</tbody>
</table>

N, group N of mice (normal mice); V, group V of mice (diabetic control mice); APS100, group APS100 of mice (diabetic mice administered APS at 100 mg/kg BW/d); APS200, group APS200 of mice (diabetic mice administered APS at 200 mg/kg BW/d); APS400, group APS400 of mice (diabetic mice administered APS at 400 mg/kg BW/d). All data were means±S.E.M. calculated from 10 diabetic mice. *p<0.05, **p<0.01 compared with normal mice; ∗p<0.05, ∗∗p<0.01 compared with diabetic control mice.

Fig. 5. Expression of PPARγ in Spleens of MLD-STZ Induced Diabetic Mice with APS Treatment. RT-PCR Analysis of PPARγmRNA on Day 30 (a); Western Blot Analysis of PPARγ Protein on Day 30 (b); Expression of PPARγmRNA (c). Used the Ratios of PPARγ/β-Actin Absorption Density of Bands on a Gel.

Lane M, DL 2000 marker; Lane 1, group N of mice; Lane 2, group V of mice; Lane 3, group APS100 of mice; Lane 4, group APS200 of mice; Lane 5, group APS400 of mice. All data were expressed as mean±S.E.M.β-Actin was used as an internal control. ∗p<0.05, ∗∗p<0.01 compared with group N of mice (normal mice); ∗p<0.05, ∗∗p<0.01 compared with group V of mice (diabetic control mice), n=5 per group.

thyperglycemic activity of APS and investigate the immunotherapeutic molecular mechanisms of how APS attenuated the development of T1DM.

Previous studies have shown APS lowers blood glucose levels in diabetic animals, but in normal mice, it can’t. The mechanisms for improved hyperglycemia associated with APS may be multifaceted and remain unclear so far. According to Wu report, by decreasing the elevated expression and activity of PTP1B (protein tyrosine phosphatase 1B, a negative regulator of insulin-receptor signal transduction) in the skeletal muscles, APS may prevent insulin resistance, increase insulin action and decrease blood glucose levels in
type 2 diabetic rats. As previous reports, the results from the present studies also indicated that APS possessed an antihyperglycemic property, furthermore, hypoglycemia of APS was time- and dose-dependent, though normal blood glucose levels couldn't be recovered successfully from hyperglycemia in diabetic mice with APS treatment. Daily administration of 200 or 400 mg/kg of APS on day 30 appeared to significantly decrease blood glucose levels compared with saline controls, whereas at a dose of 100 mg/kg/d or before day 15 APS didn't possess such an antihyperglycemic function. More importantly, the present data were the first to demonstrate APS could increase serum insulin concentrations in type 1 diabetic mice, strongly associating with antihyperglycemia. This finding was supported by histologic and morphometric evidences, showing β cell mass or apoptotic β cells was significantly higher or lower accordingly at the same time point.

While we indicated serum insulin levels were increased with APS treatment, there are reports in the published literature that APS treatment can't alter serum insulin levels. Wu et al. indicate that administration of APS to fat-fed STZ-treated rats, animal models of type 2 diabetes mellitus (T2DM), results in no alteration of serum insulin levels.15) Obviously, the effect of APS on the serum insulin levels is dependent on the animal model used. The fat-fed STZ-treated rats show typical characteristics similar to T2DM, where impaired glucose tolerance and a compensatory excess insulin release are caused by decreased peripheral insulin sensitivity, which is partly corrected by APS treatment.15) However, the MLD-STZ models of autoimmune diabetes we used are characterized as T1DM by T-cell-dependent autoimmune destruction of the β cells and decreased insulin levels. APS (200, 400 mg/kg/d i.p. for 30 d) treatment in this model caused milder, even no lymphocytic infiltration in pancreas islets, markedly lowered percentage of apoptotic β cells, and significantly hi-ghered β cell mass. These results strongly suggest that APS is able to ameliorate insulinitis, at least partially protect β cells from apoptosis resulted from insulinitis. Therefore, it is reasonable to postulate that with APS treatment, attenuated lymphocytic infiltration in the pancreas islets contributes to improve insulin levels and decrease hyperglycemia in MLD-STZ induced diabetic mice.

To further elucidate the immunotherapeutic molecular mechanisms of how APS attenuated mellitus, we examined cytokine IL-4 and IFN-γ release from the splenocytes by ELISA assay. Splenocytes were investigated for two main reasons. Firstly, we wanted to get an assessment of cytokine productions by peripheral immune cells. Secondly, it was very difficult to isolate islets with infiltrating immune cells from MLDSTZ-treated mice, since the islet mass was reduced and islet structure was disrupted. Based on our study, APS altered Th1 and Th2 cytokine secretion profiles time- and dose-dependently, even recovered them from T1DM. APS dramatically reduced the levels of IL-4 along with augmenting the level of IL-4 until the dose of APS was up to 200 mg/kg/d and treatment lasted 30 d. In normal rodents, similar conclusions are also previously reported by Weng and Kang,12,13) who analyze effects of APS or Huangqi extract on Th1 and Th2 cytokine secretion patterns. In NOD mice, it is postulated that APS is able to rescue imbalance of Th1 and Th2 because of partially preventing onset of T1DM with APS treatment16,17) However, in vitro fluorescence-labeled APS is able to selectively stain murine B cells and macrophages but T cells, activates murine B cells and macrophages, not T cells.22) These data together with our studies suggest in vivo APS alters Th1 and Th2 cytokine secretion profiles through indirect actions, not direct.

In our studies, in accordance with the notion that towards the Th1 cytokine IFN-γ promotes inflammatory insulinitis and diabetes,23–25) whereas preponderance of the Th2 cytokine IL-4 prevents β cell destruction,2,20,24,25) the histologic and morphometric results including increase of β cell mass, decrease of apoptotic β cell percentage and appearance of small scattered neogenetic insulin-producing cells, were strongly associated with effects of APS on improved imbalance of Th1/Th2 cytokine ratio. Therefore, the immunopharmacological profiles of APS along with the data shown here rather indicate that modulation of endogenous cytokines by the drug may have been centrally implicated in its antidiabetogenic action.

PPARγ is a member of a class of nuclear hormone receptors intimately involved in the regulation of expression of myriad genes that regulate energy metabolism, cell differentiation and apoptosis. Recently, emerging evidence indicates that PPARγ and its ligands are indeed important for the modulation of immune and inflammatory reactions.29) In the present studies, PPARγ gene expressions including mRNA and protein in spleens of the diabetic mice were upregulated time- and dose-dependently with APS treatment. Their changing trends were same as IL-4 release from splenocytes, and opposite to IFN-γ level. Our present data are the first to demonstrate effects of APS on PPARγ gene expressions of spleens in diabetic mice.

Previous data show PPARγ may function as modulators to influence inflammatory cytokine expressions. PPARγ negatively regulates the IFN-γ target genes in macrophages,27) and PPARγ ligands reduce production of Th1 cytokines resulting in encephalomyelitis,28,29) whilst PPARγ has an impact in the maturation of dendritic cells and in orientation of immune responses by favoring Th2 responses,30) and PPARγ ligands increase IL-4 levels in colon tissue,31) heart tissue and splenocyte.32) Recently, two transcription factors T-bet and GATA-3 have been reported to play important roles in Th1/Th2 cell differentiation.33) T-bet induces naïve T cells into Th1 cells, meanwhile GATA3 induces naïve T cells into Th2 cells. PPARγ has been reported to activate GATA3 and show the beneficial effects on colitis.31) As previous studies, the present results suggest that similar mechanisms may be involved in the immunotherapeutic effect on imbalance of Th1/Th2 cytokine ratio through PPARγ.

Prior to IL-4 upregulation and IFN-γ downregulation on day 30 with APS treatment, PPARγ gene expressions including mRNA and protein in group APS400 of diabetic murine spleens were already significantly upregulated on day 15. This result, combined with previous reports,27–33) suggest that PPARγ may play a pivotal role in the onset of decreased Th1/Th2 cytokine ratio in T1DM with APS treatment.

In T1DM, islet infiltration is not synchronized at all. Islets are under various stages of infiltration, as well as healthy and completely destroyed islets at any given time. Over time, this process will lead to the destruction of more and more islets, to discrete, stochastic yet, sequential elimination of individual islets within the entire pancreas. In this way, new islets
from self-duplication of \( \beta \) cells or stem cell differentiation, which is driven by insulin insufficiency in type 1 diabetic mice, can become affected continuously, until their \( \beta \) cells are completely destroyed.\(^{33}\) In the present studies, the immunopharmacological profiles of APS suggest that with APS treatment, healthy islets, new islets and functional \( \beta \) cells in inflammatory islets may be protected, even be prevented from insulitis. Under these circumstances, such islets, and \( \beta \) cells, especial new islets, can play a significant part in influencing the course of the disease. However, new islets only come every week or so in type 1 diabetic mice.\(^{34}\) In addition, our present data together with previous results suggest that in vivo APS alters Th1 and Th2 cytokine secretion profiles through indirect actions, such as upregulation of PPAR\( \gamma \) gene expressions in splenocytes. It is reasonable to believe that with APS treatment, pathological autoimmune response in the type 1 diabetic mice is induced to become a well regulated autoimmune step by step at a slow rate. Therefore, the clinical, histological, and immune parameters of the MLD-STZ induced diabetic mice with APS treatment aren’t able to be improved immediately. As the present studies, they were not improved greatly within 15 d of APS treatment until after 30 d.

In conclusion, our data suggest that APS may possess immunotherapeutic effects on T1DM, since it leads to decrease Th1/Th2 cytokine ratio, strongly associated with increased PPAR\( \gamma \) gene expressions in spleens. The altered Th cytokine profiles are helpful to protect \( \beta \) cells from apoptosis resulted from insulitis, increase \( \beta \) cell mass, improve serum insulin levels and lower hyperglycemia. The results of this study support this hypothesis, at least in this animal model.

**Acknowledgements** We thank Hai-lin Teng for valuable discussion and critical review of the manuscript. This work was supported by a grant from Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences.

**REFERENCES**