Salvianolic acid B: *in vitro* and *in vivo* effects on the immune system

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Abstract: Type 1 diabetes (T1D) is an autoimmune disorder with a strong inflammatory component. Autoreactive cells specifically target insulin-producing β-cells, which leads to loss of glucose homeostasis. T1D remains incurable and versatile; potentially beneficial therapeutics are being tested worldwide. Possible candidates for the treatment of autoimmune diabetes are plants and their extracts since they are rich in biophenols, substances that act as secondary metabolites, and have verified antioxidant and antiinflammatory effects. Salvianolic acid B (SalB) is a biophenol and one of the major constituents of *Origanum vulgare* ssp. *hirtum* (Greek oregano) extracts which in our previous studies was shown to exhibit an antidiabetic effect in mice. The aim of the present study was to determine whether SalB is responsible for the observed effects of Greek oregano extracts. SalB was applied *in vitro* to macrophages and lymphocytes isolated from C57BL/6 mice, as well as *in vivo* in the model of T1D induced by multiple low doses (MLD) of streptozotocin (STZ). SalB did not affect the viability of cells, but it significantly decreased secretion of nitric oxide (NO) and TNF in lipopolysaccharide (LPS)-stimulated macrophages, as well as the secretion of IFN-γ in concanavalin A (ConA)-stimulated lymphocytes. However, when applied *in vivo,* SalB at a dose of 2.5 mg/kg b.w., applied for 10 consecutive days, failed to protect mice from diabetes development. In conclusion, SalB exerts immunomodulatory effects *in vitro*, but is not effective in prevention of T1D *in vivo*. It probably requires cooperation with some other substances for the maximum efficacy exhibited by oregano extracts.

Key words: salvianolic acid B; type 1 diabetes; macrophage; lymphocyte; immune modulation

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disorder where autoreactive immune cells specifically target insulin-producing pancreatic β-cells [1]. Destruction of β-cells leads to impaired insulin secretion and ultimately to a loss of glucose homeostasis [2]. Combined genetic and environmental factors lead to the immune response against β-cells [3]. The autoimmune response directs autoreactive immune cells towards pancreatic islets and insulitis occurs [4]. Macrophages and autoreactive T lymphocytes make up most of the immune infiltrate [5], and their effector molecules, such as pro-inflammatory cytokines and nitric oxide, lead to the creation of a pro-inflammatory milieu, which is detrimental for β-beta cells [1]. While specific subsets of T helper (CD4+) cells, such as IFN-γ-producing Th1 cells and IL-17-producing Th17 subsets, lead to T1D progression, IL-4-producing Th2 cells and IL-2-producing Treg cells have an antiinflammatory role and can prevent T1D development [5-8].

T1D incidence is about 3% and is rapidly rising [9]. Currently, T1D is treated with daily insulin administration [1] and constant efforts are being made in the search for novel therapeutic strategies to treat T1D. Plants and their extracts are becoming increasingly interesting candidates for novel treatments of various ailments, including autoimmune diseases. This is mainly because plants are rich in biophenols and are considered to be less toxic and have fewer side effects than synthetic drugs. Biophenols are substances abundantly found in plants where they act as secondary metabolites [10]. A number of studies has linked intake of biophenols through diet with beneficial effects in chronic diseases [11]. In our previous studies, we showed the antidiabetic effects of methanolic and ethyl-acetate oregano extracts (*Origanum vulgare* ssp. *hirtum*) on the multiple low doses of streptozotocin (MLDS) experimental model of T1D [12,13]. Both substances are rich in salvianolic acid B (SalB), a water-soluble polyphenolic compound that

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possesses anti-oxidant and anti-inflammatory effects [14]. The aim of this study was to investigate whether SalB is the substance responsible for the antidiabetic effects of methanolic and ethyl-acetate extracts of *Origanum vulgare* ssp. *hirtum*.

MATERIALS AND METHODS

Salvianolic acid B

Salvianolic acid B was a commercial sample obtained from Fluka (Buchs, Germany).

Animals

Male C57BL/6 mice, 8-12-weeks old were housed in the animal facility of the Institute for Biological Research. Their use and all experimental procedures were approved by the Ethics Committee of the institute (App. No. 01-07/15), which is in accordance with Directive 2010/63/EU. The mice were kept under standard conditions at a 12 h light/dark cycle with free access to a standard pelleted diet and tap water.

Cell preparation and culture

In vitro analysis was performed on male C57BL/6 mice. To obtain cervical lymph node cells (CLNC), organs were mechanically disrupted by gentle teasing through a cell strainer (BD Bioscience, Bedford, USA), and the cells were collected by centrifugation. Peritoneal cells (PC) were collected by washing the peritoneal cavity of mice with cold PBS. Samples of conditioned medium, used for *in vitro* detection of cytokines and NO, were obtained by seeding the cells (5×10^6 CLNC mL^{-1} well^{-1}, or 1×10^6 PC mL^{-1} well^{-1}) for 24 h (PC) and 48 h (CLNC) in 24-well culture plates (Sarstedt, Nümbrecht, Germany) in RPMI-1640 medium (with 25 mmol/L HEPES, 2 mmol/L L-glutamine) supplemented with 10% FCS (PAA Chemicals, Pasching, Austria), penicillin/streptomycin (Sigma Aldrich, St. Louis, MO, USA), and 5 µmol/L β-mercaptoethanol at 37°C in a 5% CO$_2$ incubator. Cells were cultured in the presence of mitogen activators ConA (1 µg/mL, Sigma Aldrich; CLNC) and LPS (5 ng/mL, Sigma Aldrich; PC).

Multiple low-dose streptozotocin-induced diabetes

Immunoinflammatory T1D was induced in male 8-12-week-old C57BL/6 mice with multiple low doses of STZ (MLDS, 40 mg kg^{-1} day^{-1}, intraperitoneally (i.p.) for five consecutive days, Sigma Aldrich). Each experimental group comprised 7 animals. Salvianolic acid B was administered i.p. for 10 consecutive days (2.5 mg kg^{-1} day^{-1}), starting from the day of diabetes induction (prophylactic regimen). Mice were monitored for diabetes by weekly measurement of blood glucose levels using a glucometer (Sensimac, IMACO GmbH, Lüdersdorf, Germany). Clinical diabetes was defined by hyperglycemia in non-fasted animals (blood glucose>10 mmol/l).

Measurement of cytokine levels

Concentrations of cytokines in cell culture supernatants were determined by a sandwich ELISA using MaxiSorp plates (Nunck, Rochild, Denmark) and anti-mouse-paired antibodies according to the manufacturer's instructions. Samples were analyzed in duplicate for murine IL-17, IL-1β, TNF (BD Biosciences), IFN-γ (eBioscience) and IL-4 (R&D Systems). Absorbance was measured by a LKB 5060-006 microplate reader (LKB Instruments, Vienna, Austria) at 450 and 570 nm.

Determination of nitrite levels

Nitrite accumulation, an indicator of NO production, was measured in cell culture supernatants using the Griess reagent [15]. Absorbance was measured by a microplate reader (LKB Instruments) at 540 and 650 nm.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [16]. In brief, the cells were treated with 0.5 mg/mL MTT solution for 30 min. In viable cells, tetrazolium salts were reduced to formazan crystals in the process of cell respiration. Purple formazan was then dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured in a LKB microplate reader at 540 and 670 nm.
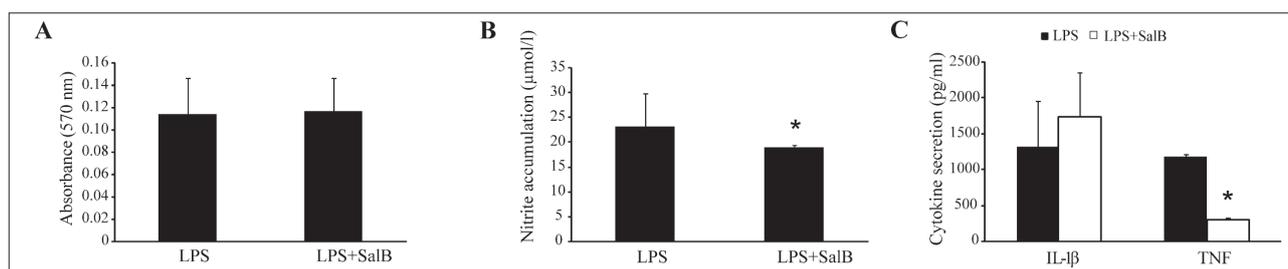
Fig. 1. *In vitro* effect of SalB on macrophage viability and function. PC isolated from C57BL/6 mice were stimulated *in vitro* by LPS (5 ng/mL) in the presence or absence of SalB (50 μg/mL). PC viability (**A**), secretion of NO (**B**) and cytokines (**C**) were measured after 24 h of incubation. Results are presented as the means±SD. *P<0.05 refers to LPS+SalB-treated vs. LPS-treated PC.

Statistical analysis

Data are presented as means±SD. Statistical analysis was performed using Statistica 6.0 (StatSoft Inc., Tulsa, Okla., U.S.A.) software. Comparisons between the groups were performed by one-way ANOVA, followed by Student-Newman-Keuls *post hoc* test and finally Student's t-test or Mann-Whitney U-test, where appropriate. Analysis of diabetes incidence was performed by the χ^2 test. A p-value less than 0.05 was considered to be statistically significant.

RESULTS

The effect of SalB on peritoneal cells *in vitro*

To evaluate the potential influence of SalB on immune cells *in vitro,* PC rich in macrophages isolated from C57BL/6 mice and stimulated with LPS were treated with SalB at a concentration of 50 μg/mL. While SalB had no effect on the viability of peritoneal cells (Fig. 1A), it significantly reduced their pro-inflammatory potential, judging by the reduced levels of NO (Fig. 1B) and reduced secretion of the pro-inflammatory cytokine TNF (Fig. 1C). The concentration of SalB for *in vitro* experiments was determined on the basis of our previous work with Greek oregano extracts [12,13].

The effect of SalB on lymphocytes *in vitro*

While macrophages are the first cells to enter pancreatic islets, lymphocytes are the key players in T1D pathogenesis [5]. We therefore assessed the effect of SalB on lymphocytes. SalB (50 μg/mL) showed no cytotoxic effect on lymphocytes stimulated with ConA, judging by the viability assay (Fig. 2A). The effector
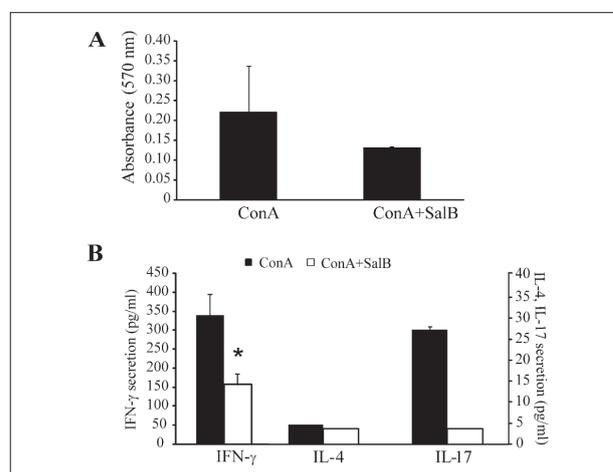
Fig. 2. *In vitro* effect of SalB on lymphocyte function and viability. Lymphocytes isolated from cervical lymph nodes of C57BL/6 mice were stimulated *in vitro* with ConA (1 μg/mL) in the presence or absence of SalB (50 μg/mL). Lymphocyte viability (**A**) and secretion of cytokines (**B**) were measured after 48 h of incubation. Results are presented as the means±SD. *P<0.05 refers to ConA+SalB-treated vs. ConA-treated lymphocytes.

function of lymphocytes was determined by measuring the secretion of pro- and anti-inflammatory cytokines (Fig. 2B). While SalB had no effect on the secretion of anti-inflammatory IL-4 and pro-inflammatory IL-17, it significantly reduced the secretion of the pro-inflammatory cytokine IFN-γ (Fig. 2B) and thereby lowered their total pro-inflammatory potential.

SalB does not prevent T1D development *in vivo*

To investigate the ability of SalB to prevent T1D development *in vivo*, MLDS-challenged C57BL/6 mice were treated with SalB (i.p.) at a dose of 2.5 mg/kg b.w. in a prophylactic regime, every day for ten days. The dose of SalB for *in vivo* experiments was determined on the basis of our previous work with Greek
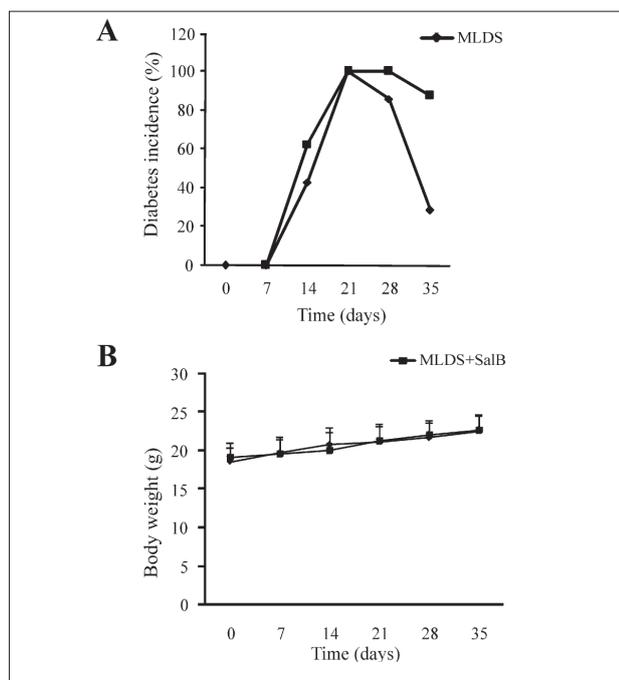
Fig. 3. *In vivo* effect on SalB on T1D. T1D was induced in male C57BL/6 mice by the administration of STZ (40 mg/kg b.w., i.p.) for five consecutive days. One group of animals was simultaneously treated with SalB (2.5 mg/kg b.w., i.p) for 10 consecutive days (prophylactic treatment). The animals were monitored weekly for changes in blood glucose levels (**A**) and body weight (**B**). The results from one representative result out of three experiments with similar results are presented as the means±SD (n=7 mice per group). Statistical comparisons were made by one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons.

oregano extracts [12,13]. Our results showed that SalB did not prevent T1D development (Fig. 3A), in fact the incidence of T1D was higher throughout the entire experiment, although not statistically significant, than in the MLDS group (Fig. 3A). Additionally, the treatment with SalB showed no visible side effects on mice in terms of body weight gain (Fig 3B), behavior and general appearance (data not shown).

DISCUSSION

In this study, we present evidence that polyphenol SalB exerts an anti-inflammatory effect on both macrophages and lymphocytes *in vitro*, but that it was unable to alter the autoimmune response *in vivo* and thus prevent T1D pathogenesis in C57BL/6 mice.

In our previous studies, we showed the antidiabetic effects of the methanolic and ethyl-acetate extracts of

Origanum vulgare ssp. *hirtum* in an MLDS model of T1D [12,13]. However, search for the active component of these extracts was less successful, since neither rosmarinic acid nor carvacrol (constituents of these extracts) prevented the development of T1D [12,13, respectively]. SalB is another compound present in oregano extracts at a high concentration. It is frequently found in potentially immunomodulatory substances that are active *in vitro*, however, its effects do not appear to be translatable to an *in vivo* system [17]. In the present research, the *in vitro* anti-inflammatory potential of SalB was mediated by a reduction of TNF from macrophages, which is in accordance with the study of Sun et al. (2016), who also showed that SalB downregulated TNF secretion [18]. Macrophages also downregulated their NO production after SalB treatment. Evidence from the literature describes the inhibitory effect of a similar compound, salvianolic acid A, on macrophage-derived NO [19]. However, SalB displayed an opposite effect *in vivo* in the model of rat hepatitis, where macrophages expressed higher levels of inducible NO synthase, the enzyme crucial for induced NO synthesis [20].

SalB also exerted its *in vitro* immunomodulatory effect through the inhibition of lymphocyte-derived IFN-γ that is produced by Th1 and cytotoxic CD8+ (and to a smaller extent by NK cells). The effect of SalB on lymphocytes has been poorly studied and there are only data from one *in vivo* study where SalB inhibited Th1 activity during experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis in rats [21].

Despite the obvious anti-inflammatory effect of SalB *in vitro*, a prophylactic treatment of MLDS-challenged mice with SalB failed to protect the mice from T1D development. In contrast, one study claims that SalB is antidiabetic *in vivo*, since it prevented the development of hyperglycemia and subsequently increased insulin levels in Wistar rats [22]. However, there are several major differences that could be responsible for the different outcomes: the authors used outbred rats in the animal model, the doses of SalB were much higher (20 and 40 mg/kg b.w.), and were applied continuously for 3 weeks. Cells that contribute the most in the creation of an inflammatory milieu in T1D are T helper cells (Th1 and Th17), cytotoxic CD8+ lymphocytes [6,23] and inflammatory macrophages [24]. Since SalB suppressed the IFN-γ-producing popula-

tion and not the IL-17-producing Th17 cells, it can be speculated that the failure of SalB to prevent T1D development *in vivo* is due to the fact that SalB showed no effect on the pathogenic Th17 population. Therefore, in order to provide the most effective treatment of autoimmune/inflammatory disorders it might be more effective to use SalB in combination with some other substances that can downregulate the Th17 immune response. Since our previous research showed the antidiabetic effect of the oregano extracts that contain SalB among other substances, possible candidates for synergistic studies could be found in the substances present in the oregano extracts.

Authors' contribution: MV, TS and IS performed the experiments and analyzed the data. IS and MV wrote and edited the text. IS conceived and designed the study.

Conflict of interest disclosure: We declare no conflict of interest.

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