DRY OLIVE LEAF EXTRACT (DOLE) DOWN-REGULATES THE PROGRESSION OF EXPERIMENTAL IMMUNE-MEDIATED DIABETES BY MODULATION OF CYTOKINE PROFILE IN THE DRAINING LYMPH NODES

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Abstract - We have recently demonstrated the beneficial effects of dry olive leaf extract (DOLE) in two preclinical models of type 1 diabetes. Here we analyze the potential mechanisms underlying diabetes amelioration at the level of lymph node drainage. Treatment of C57BL/6 mice with DOLE during induction of diabetes with multiple low doses of streptozotocin (MLD-SZ) modulated cytokine expression and production in pancreatic lymph node cells, thereby changing the balance between potentially pathogenic and down-regulating cytokines. These results support the immunoregulatory potential of DOLE which takes place at the level of lymph node drainage and preserves the target tissue from autoimmune attack.

Key words: Autoimmune diabetes, streptozotocin, olive leaf extract, cytokine, inflammation, C57BL/6 mice

INTRODUCTION

Type 1 diabetes (T1D) is a T-cell mediated disorder that results from the autoimmune destruction of pancreatic β cells. The progression of T1D in mice and possibly in humans alike, is marked by two general "checkpoints": the first is associated with islet inflammation (insulitis), but mice remain diabetes-free because of limited β cell destruction; the second corresponds with a shift from "benign" to "aggressive" insulitis when β cells are efficiently destroyed to promote overt diabetes (Pop et al., 2005; Stojanovic et al., 2008). The destruction of insulin-producing cells further results in the loss of metabolic regulation and resultant hyperglycemia and severe sequelae of the disease (Azam and Eisenbarth, 2004). There is abundant evidence in animal models and to a lesser extent in humans with T1D that islet β cell destruction results from a disorder in immunoregu-lation (Rabinovitch and Suarez-Pinzon, 2007). Pancreatic lymph nodes (PLN) are reported to be key sites for the activation and tolerance induction of β-cell-specific T cells (Hoglund et al., 1999; Tritt et al., 2008). The lymph node (LN) environment is crucial for the priming of T cells in response to autoantigen and activation of diabetogenic T cells (Hoglund et al., 1999). Importantly, regulatory T (Treg) cells present in the regional LN continuously control organ-specific autoimmune diseases and inflammatory processes (Tritt et al., 2008), underscoring the importance of the LN environment in regulating immune responses. Both macrophages and T cells are thought to exert their diabetogenic potential by releasing proinflammatory cytokines of both innate and adaptive immunity (Kolb, 1987). Thus, Th1- and Th17-type cytokines as well as monokines, correlate with development, whereas Th2 and Th3, as well as Treg-type cytokines correlate with protection from

the disease (Miljkovic et al., 2005; Rabinovitch and Suarez-Pinzon, 2007; Stojanovic et al., 2008).

Diabetes mellitus is still not completely curable with the present antidiabetic drugs. Although results obtained with islet transplantation are promising, interfering with the autoimmune response still remains a conceivable strategy for the prevention and treatment of T1D. Therefore, herbal drugs are gaining popularity in the treatment of the disease. The major merits of herbal medicine seem to be their efficacy, low toxicity, low incidence of adverse effects and low cost, which have rendered them useful ingredients in complementary alternative medical and/or nutritional supplements. With regard to this, the medical potential of the constituents and products of the olive tree, *Olea europaea*, has been used traditionally for various medicinal purposes, including several metabolic disorders (Ramirez-Tortosa et al., 1999; Esposito et al., 2007). Olive leaves present a unique opportunity to study the effects of *O. europaea*-derived polyphenols since the leaf contains polyphenols and only a small amount of oleic acid. Methanolic extracts of olive leaves contain secoiridoids (oleuropein, ligostroside, dimethyloleuropein, oleoside), flavonoids (apigenin, kaempferol, luteolin), as well as phenolic compounds (caffeic acid, tyrosol, hydroxytyrosol) (El and Karakaya, 2009). Recently, by utilizing two established preclinical models of autoimmune diabetes, cyclophosphamide-accelerated diabetes in non-obese diabetic mice and multiple low-dose streptozotocin (MLD-SZ)-induced diabetes in susceptible mouse strains, we showed that the *O. europaea*-derived components present in a polyphenol-enriched dry olive leaf extract (DOLE) with oleuropein as major component, can ameliorate disease progression (Cvjeticanin et al., 2010). In both T1D models, *in vivo* administration of DOLE significantly reduced the clinical signs of diabetes and restored insulin expression and release. In the peripheral immune system, DOLE acted as a modulator of a variety of inflammatory and immunological events, such as nitric oxide production, T lymphocyte proliferation, as well as cytokine production within the spleen. Given the role of the LN environment in regulating autoimmune disease, and based

on the findings from our previous work (Cvjeticanin et al., 2010), the present study is focused on the effect of DOLE treatment on the production of signature cytokines in the PLN as the most relevant organ in diabetes pathology.

MATERIALS AND METHODS

Reagents

The extract DOLE EFLA® 943, standardized to 18-26% of oleuropein, was from Frutarom Switzerland td (Wädenswil, Switzerland). As evaluated by phytochemical analysis (Dekanski et al., 2009), the extract contained 19.8% oleuropein, 0.52% tannins, 0.02% caffeic acid, and 0.29% total flavonoids, including 0.04% luteolin-7-*O*-glucoside, 0.07% apigenine-7-*O*-glucoside, and 0.04% quercetin. All other reagents were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated.

Type 1 diabetes induction and application of DOLE

Genetically susceptible inbred male C57BL/6 mice were maintained in our own animal facility (Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Serbia) with free access to food and potable water. The animals and feed were checked regularly by appropriate microbiological examination. They showed that the animals were not infected with common mouse pathogens and that the feed was free of microbiological contamination. Immunoinflammatory T1D was induced in 8-12 week-old animals with multiple low doses of streptozotocin (MLD-SZ, 40 mg/kg body weight, intraperitoneally for 5 consecutive days), exactly as reported previously (Cvjeticanin et al., 2009). The treatment with DOLE (40 mg/kg b.wt per day, for 10 consecutive days) started 24 h after the last MLD-SZ injection. Clinical diabetes was defined by hyperglycemia (blood glucose levels >10 mmol/l). The groups of mice (4-5 mice per group) used for *ex vivo* analyses of cytokine production were killed on day 15 of diabetes post induction, when the treatment with DOLE was completed. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the

Institute for Biological Research "Sinisa Stankovic" and run in accordance to the requirements of the European Union regarding handling of experimental animals.

Cell isolation and culture

Pancreatic lymph nodes (PLN) were isolated from the DOLE-treated or control diabetic mice organs were mechanically disrupted, passed through 40 μm nylon mesh filter and collected by centrifugation. To determine *ex vivo* cytokine production, a single cell suspension of PLN (5 x 10^6 cells) was cultured in 24-well culture plates in 1 ml of culture medium (RPMI-1640 medium supplemented with 5% FCS, 2 mM l-glutamine, 0.01% sodium pyruvate, $5x10^{-5}$ M 2-mercaptoethanol and antibiotics). After 48 h of incubation at 37°C in a humidified atmosphere with 5% CO_2, the media were harvested for cytokine assay.

Determination of cytokine secretion

Cytokines in cell culture supernatants were determined by sandwich ELISA using MaxiSorp plates (Nunck, Rochild, Denmark) and anti-mouse paired antibodies according to the manufacturer's instruc-

tions. Samples were analyzed for murine IL-17, IL-1β, TNF-α (BD Pharmingen, San Diego, CA, USA), IL-6, IL-2, IL-10 (eBioscience, San Diego, CA), IFN-γ TGF-β, and IL-4 (R&D, Minneapolis, MN, USA). The results were calculated using standard curves made on the basis of known concentrations of the appropriate recombinant cytokines.

Quantification of cytokines by real time PCR

Total RNA from the PLN cells (5×10^6) was extracted using a mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) according to the manufacturer's instructions. The RNA was reverse transcribed using Moloney leukemia virus reverse transcriptase and random hexamers (both from Fermentas, Vilnius, Lithuania). PCR amplification of cDNA (0.4 μl per 20 μl of PCR reaction) was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Warrington, UK) using SYBRGreen PCR master mix (Applied Biosystems) as follows: 10 min at 50°C for deoxyuridine triphosphate activation; 10 min at 95°C for initial denaturation of the cDNA followed by 40 cycles (15 s of denaturation at 95°C and 60 s for primer annealing and chain extension step at 60° C). The sequences of the primer pairs used for each PCR are listed in Table 1. Data

Table 1. Nucleotide sequences of primers used in the RT-PCR

Cytokine	Primer sequence (5' – 3')		GenBank acc. no.
	Forward	Reverse	
IL-1β	GCTGAAAGCTCTCCACCTCAATG	TGTCGTTGCTTGGTTCTCCTT	NM_008361.3
TNF-α	CCACGTCGTAGCAAACCAC	TGGGTGAGGAGCACGTAGT	NM_013693.2
IL-6	TTCACAAGTCCGGACAGGAG	TGGTCTTGGTCCTTAGCCAC	NM_031168.1
IL-2	CCTGAGCAGGATGGAGAATTACA	TCCAGAACATGCCGCAGAG	NM_008366.2
IFN-γ	CATCAGCAACAACATAAGCGTCA	CTCCTTTTCCGCTTCCTGA	NM 008337.2
IL-17	GGGAGAGCTTCATCTGT	GACCCTGAAAGTGAAGGG	NM 010552.3
IL-4	ATCCTGCTCTTCTTTCTCG	GATGCTCTTTAGGCTTTCC	NM 021283.1
IL-10	TGTGAAAATAAGAG-CAAGGCAGTG	CATTCATGGCCTTGTAGACAC	NM 010548.1
TGF-β	CCCTGCCCCTACATTTGGA	ACGGTGATGCGGAAGCAC	NM_011577.1
β-actin[a]	GGACCTGACAGACTACC	GGCATAGAGGTCTTTACGG	NM 007393.2

[a]Housekeeping reporter gene.
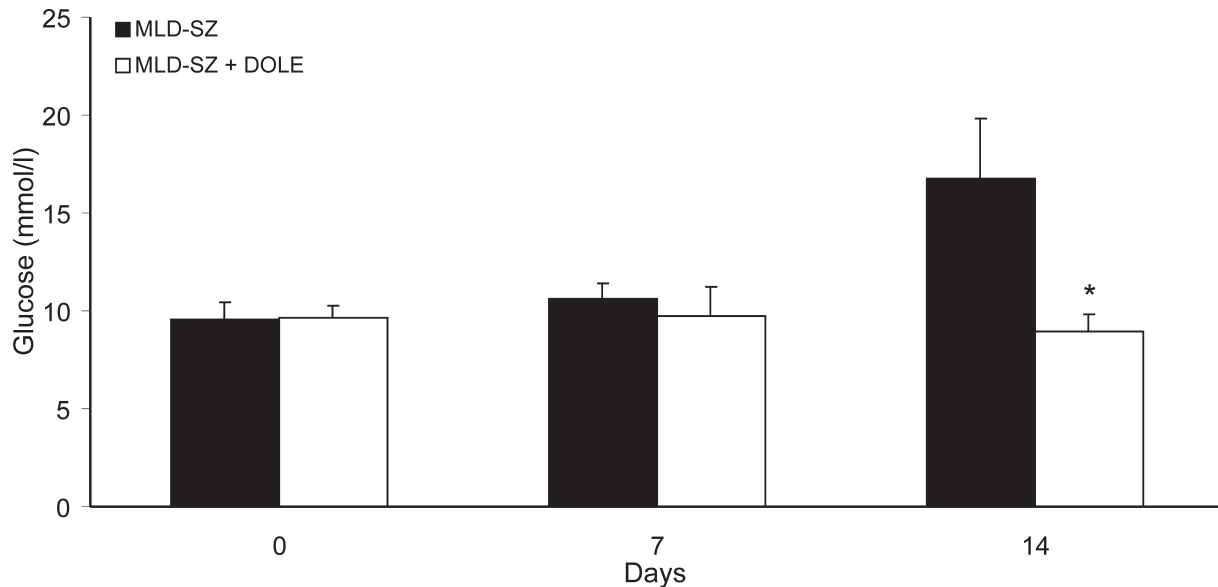
Fig. 1. Effect of DOLE treatment on the development of hyperglycemia induced by MLD-SZ. Blood glucose levels in MLD-SZ-treated control mice (n=5) and MLD-SZ + DOLE-treated mice (n=5). *P < 0.05 refers to control MLD-SZ-treated mice.

were quantitatively analyzed using SDS 2.1 software (Applied Biosystems). Gene expression was calculated according to the formula $2^{-(Cti-Cta)}$, where C_{ti} is the cycle threshold of the gene of interest and C_{ta} is the cycle threshold value of the housekeeping gene, β-actin. The obtained values from the MLD-SZ group were arbitrarily attributed a value of one (an arbitrary unit). The efficiency of real time PCR was in the optimal range of 90-110% (slope of standard curves 3.1-3.6) for all of the primer pairs used.

Statistical analysis

The results are presented as means and standard deviations. Statistical analysis of differences was made using one-way ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons, or Student's *t*-test, as appropriate. The statistical package used was Statistica 6.0 (StatSoft Inc Tulsa, OK, USA). The results were considered statistically significant with *P* values of <0.05.

RESULTS

To investigate the immunomodulatory mechanisms

that may contribute to the beneficial effect of DOLE on the autoimmune diabetogenic processes (Cvjeti-canin *et al.*, 2010), we used the established model of DOLE-induced disease modulation in MLD-SZ-induced diabetic C57BL/6 mice. As judged by blood glucose levels, the animals treated with DOLE did not develop the clinical signs of diabetes characterized by a delayed appearance of sustained hyperglycemia (Fig. 1), thus confirming the previously demonstrated beneficial effects of the extract.

Because pro-inflammatory cytokines contribute to the pathogenesis of T1D development, whereas anti-inflammatory cytokines are associated with protection from the disease, we determined the effects of DOLE treatment on diabetes-associated cytokine production by draining the pancreatic lymph nodes (PLN) obtained from animals in which the disease with MLD-SZ was induced 15 days before. The levels of both pro-inflammatory (IL-1β, TNF-α, IL-6, IL-2, IFN-γ, IL-17) and anti-inflammatory cytokines (IL-4, IL-10, TGF-β) in the supernatants of the cells cultured without any additional *in vitro* stimulation, were measured by ELISA. As can be seen in Fig. 2, DOLE treatment significantly reduced the levels of
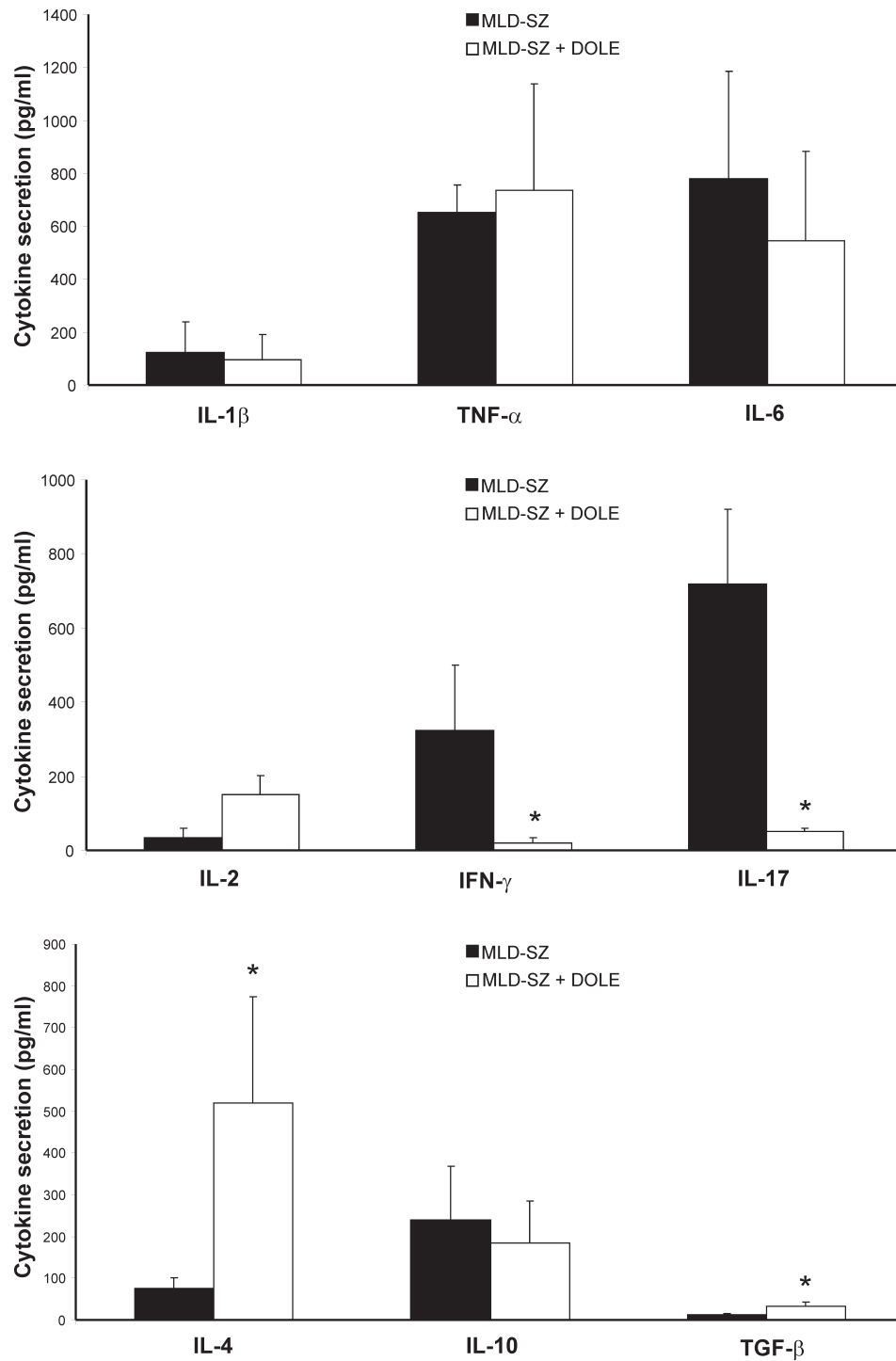
Fig. 2. Effect of DOLE treatment on PLN cytokine production. PLN were isolated from day-15 MLD-SZ-treated control mice and MLD-SZ + DOLE-treated mice. Cytokine protein levels in PLN culture supernatants were determined by ELISA. Each column represents the mean value of five mice per group ± SD. *P < 0.05 refers to control MLD-SZ-treated mice.
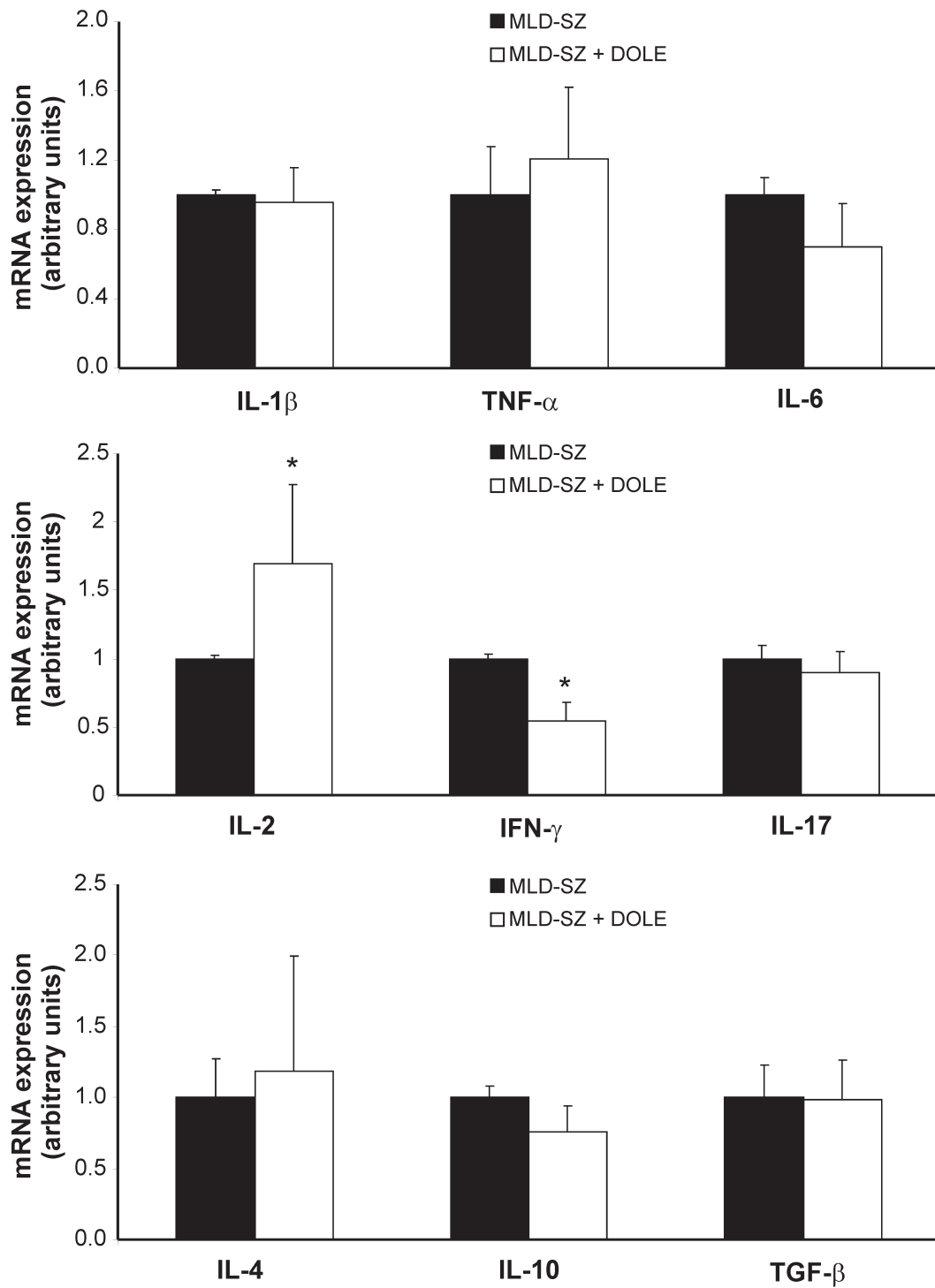
Fig. 3. Effect of DOLE treatment on PLN cytokine expression. The expression of genes in PLN obtained from day-15 MLD-SZ-treated control mice and MLD-SZ + DOLE-treated mice was determined by real-time PCR. Expression levels are shown as arbitrary units normalized to expression of the housekeeping gene β-actin. *P < 0.05 refers to control MLD-SZ-treated mice.

two pro-inflammatory cytokines, IFN-γ and IL-17, compared with the control diabetic mice. On the contrary, in the DOLE-treated mice a substantial increase of IL-4, and slight, but significantly higher TGF-β production were observed. However, there was no significant difference between the groups in the levels of the other cytokines examined, although the tendency of an IL-2 rise was observed in the DOLE-treated mice (Fig. 2).

To correlate these findings with the presence of cytokine gene expression *in vivo*, we analyzed and compared the levels of cytokine mRNA in the PLN of DOLE-treated versus untreated MLDS-induced animals. As revealed by real-time PCR (Fig. 3), IL-2 expression in the PLN of DOLE-treated mice was upregulated when compared with that in the control diabetic mice, whereas the message for IFN-γ was significantly downregulated. Regarding other cytokines examined there were no significant differences between the two groups (Fig. 3).

DISCUSSION

Effective T1D therapy requires the elimination and/or regulation of T cell clones that initiate and perpetuate the damage of the pancreatic β cells. The present study confirms our previous findings that the regulatory effects of DOLE on the immune system can be used to prevent diabetes induced with MLD-SZ (Cvjeticanin et al., 2010). Moreover, the present results demonstrate that DOLE profoundly modulates the cytokine profile which takes place in the PLN, the compartment relevant to the target tissue, which is crucial for both initiation and perpetuation of the disease (Hoglund et al., 1999; Tritt et al., 2008) and provide evidence that the observed protective effect is associated, at least in part, with a modulation of cytokine production changing the balance between potentially pathogenic and down-regulating cytokines.

The present study, to the best of our knowledge, is the first demonstration that the cytokine profiles in the PLN of DOLE-treated T1D-induced mice were clearly changed when compared to the T1D animals. Although multiple mechanisms might participate in the DOLE-induced amelioration of T1D, the initial DOLE effects at the level of PLN might have the substantial influence on the disease development. In support of this, it was reported that during the course of T1D in NOD mice, there had been changes in gene expression specific to the PLN microenvironment, and those changes may dictate the fate of the immune response even before insulitis is present (Kodama et al., 2008). In addition, the removal of PLN prevents diabetes in NOD mice, thus suggesting that PLN are required for the priming of β cell reactive T cells in NOD mice (Gagnerault et al., 2002). Moreover, it was recently suggested that genes influencing disease-specific Treg will contribute more directly to susceptibility and resistance to an organ-specific autoimmune disease when acting at the level of primary LN drainage at a given target organ than when acting systemically (Del Rio et al., 2011).

The expression of the proinflammatory cytokines IFN-γ and IL-17 is positively correlated to the induction and progression of T1D (Rabinovitch and Suarez-Pinzon, 2007; Cvjeticanin et al., 2010). In agreement with this, the present results demonstrate that DOLE profoundly suppressed the production of both these cytokines by local LN cells. Having in mind the above-mentioned role for regional LN in the diabetogenic process, the inhibition of IFN-γ and IL-17 by DOLE in this compartment seems to be a valid explanation for the beneficial effect of the extract in T1D. However, mRNA expression was significantly reduced only for IFN-γ, while IL-17 expression was only slightly impaired, without reaching statistical significance. This could be a consequence of the different kinetics of cytokine expression versus protein secretion. In addition to these Th-derived proinflammatory cytokines, the other cytokines which are mainly derived from macrophages (such as IL-1, TNF-α, and IL-6) may have prominent proinflammatory functions acting downstream of Th1/Th17 cell-mediated autoimmunity (Lukic et al., 1998; Stosic-Grujicic et al., 2008). Although we show herein the suppression of MLD-SZ-induced diabetes by DOLE in the face of ongoing inflammation, the obtained results suggest that DOLE did not significantly affect innate inflammatory cytokine production. The

observed lack of DOLE activity could be explained by a paucity of macrophages within LN tissue.

IL-2, another Th1-type cytokine long known to induce T cell growth and activate T cell immune responses, has more recently been recognized to be crucial for the induction of immune tolerance (Mallek and Bayer, 2004). Thus, IL-2 is essential in downregulating the expansion of antigen-specific autoreactive T cells by inducing apoptotic cell death and by stimulating the proliferation and function of Treg cells, thereby maintaining self tolerance (Rabinovitch and Suarez-Pinzon, 2007). In addition, immunotherapies that increase IL-2 production and/or action may correct a deficiency in IL-2 production that appears to underlie the autoimmunity of T1D, thereby restoring immune self-tolerance to islet β cells and preventing T1D (Rabinovitch and Suarez-Pinzon, 2007). Importantly, we showed that under the influence of DOLE, the expression of IL-2 was significantly up-regulated in the LN of MLD-SZ-treated mice, although the amount of released protein was not significantly influenced. Hypothetically, the lack of IL-2 protein accumulation versus high mRNA expression may have been due to the consumption of IL-2 by Treg cells. Interestingly, in a model of inflammatory bowel disease, it has been shown that deprivation of IL-2 by CD4+CD25+ regulatory T cells may induce apoptosis of effector T cells (Pandyan et al., 2007).

It is well established that Th2/3-type cytokines correlate with protection from T1D (Rabinovitch and Suarez-Pinzon, 2007; Stosic-Grujicic et al., 2008). Evidence is provided that the progression of "benign" to "aggressive" insulitis corresponds with a temporal decrease of TGF-β and FoxP3 co-expressing CD4+CD25+ Treg cells in the pancreatic lymph nodes (PLN) (Pop et al., 2005). Furthermore, TGF-β may have a direct effect on the induction of Treg function in an autocrine manner (Piccirilo et al., 2002), as well as in enhancing Th2 cell expansion and repressing the induction of Th17 (Zhu et al., 2009). From this, limiting the amounts of TGF-β that are produced during diabetes induction would be expected to reduce the efficiency of such a mechanism. Results presented herein clearly show that *in vivo* treatment

with DOLE up-regulated the production of IL-4, the key driver of Th2 responses, as well as of TGF-β. Our present findings thus revealed the dominance of Th2/3-type over Th1/Th17 signature cytokines if the cells were obtained from DOLE-treated mice, thus shifting the balance towards a beneficial cytokine milieu.

Collectively, it seems that DOLE protects mice from MLD-SZ-induced T1D by suppression of a cascade of events associated with the progression of the disease from "mini autoimmune response" into chronic immune-mediated damage of endocrine pancreas. The potent bioactivity and low toxicity of DOLE components have rendered them useful ingredients in complementary alternative medical and nutritional supplements. These data warrant additional studies for the prospective translation of DOLE to the treatment of T1D in humans.

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