

Concentration-dependent effect of silymarin on concanavalin A-stimulated mouse spleen cells *in vitro*

Original research article/Review

Hrčková G.^{1✉}, Mačák Kubašková T.¹, Mudroňová D.², Bardelčíková A.³

¹Institute of Parasitology of the Slovak Academy of Sciences, Košice, Slovakia

²Institute of Microbiology and Immunology, The University of Veterinary Medicine and Pharmacy, Košice, Slovakia

³Annamária Bardelčíková, Department of Pharmacology, Faculty of Medicine, The University of P. J. Šafárik, Košice, Slovakia

Received 2 October, 2019, accepted 10 December, 2019

Abstract Aims: Silymarin (SIL), a mixture of phenolic compounds, has a pleiotropic mode of action on various cell types, including immune cells. In this study, we investigated the concentration-dependent effect of SIL on proliferation of concanavalin A (CoA)-stimulated mouse spleen T lymphocytes, their viability, and secretion of IFN- γ and IL-4 cytokines *ex vivo* in relation to gene expressions of transcription factors nuclear factor kappa B and Foxp3. In addition, metabolic activity of T cells was determined as changes in the mitochondrial membrane potential and apoptosis. Material/Methods: Isolated splenocytes were stimulated with lectin CoA and treated with SIL at the concentrations of 5, 10, 20, and 40 $\mu\text{g/ml}$ for 70 h and unstimulated cells served as the control. Cultures of splenocytes were evaluated for proliferation index following BrdU incorporation and viability of cells after trypan blue staining. Gene expressions of transcription factors and cytokines were assessed using real-time PCR, whereas ELISA test was applied to measure cytokine secretion. Mitochondrial membrane potential and apoptosis were determined by flow cytometry. Results: We demonstrated that CoA-activated mouse spleen T lymphocytes show different susceptibilities to low ($\leq 10 \mu\text{g/ml}$) and higher (20 and 40 $\mu\text{g/ml}$) SIL concentrations. Low concentrations resulted in increased proliferation, cytokine secretion, and mitochondrial membrane potential and reduced transition of cells to apoptosis. High concentration of SIL had the opposite effect without exerting significant cytotoxicity and upregulated genes for cytokines and transcription factors on mRNA level. It is possible that individual subpopulations of T cells induced by CoA were differentially affected by the various SIL concentrations and the dose of 40 $\mu\text{g/ml}$ had the profound suppressive effect. This correlated with the highest expression of Foxp3 factor, indicating that this dose stimulated preferential differentiation to Tregs lymphocytes. Conclusions: Treatment with suitable doses of SIL can provide potential benefits in the modulation of host immune functions in various diseases.

Keywords silymarin – mouse – splenocytes – proliferation – mitochondrial potential – apoptosis

INTRODUCTION

Numerous phytochemicals produced by plants as secondary metabolites are potent immunomodulators and their biological activity was demonstrated *in vitro* on isolated cells and *in vivo* on animal models of various diseases. One natural compound with a very long history of use in traditional medicine is silymarin (SIL), isolated from the seeds of *Silybum marianum* L. (Asteraceae). It contains a mixture of flavonolignans of which silybin A/B, silychristin A, silydianin, isosilybin A, and 2,3-dehydrosilybin form approximately 70%–

80% and other components are flavonoids, fatty acids, and some undefined molecules. SIL is subject to intensive research and has received great attention due to its wide range of pharmacological effects. SIL displays free radical scavenging and strong antioxidant abilities, which are associated with inducing superoxide dismutase activities and increasing cellular glutathione content (Kwon et al., 2013; Surai, 2015). A growing number of studies have shown that SIL and its main component, silybin A/B, exhibit hepatoprotective,

* E-mail: hrcka@saske.sk

© European Pharmaceutical Journal

anticarcinogenic, immunomodulatory, and antiangiogenic activities (Saller et al., 2001; Frascini et al., 2002; Gažák et al., 2007; Esmail et al., 2017), suggesting multiple molecular mechanisms regulated by SIL's components. Anticancer activities involve suppression of the inflammatory process that leads to neoplastic transformation, hyperproliferation, and downregulation of progression of carcinogenesis and angiogenesis (Ramasamy and Agarwal, 2008). Reports on immunomodulatory activities of SIL and silybin indicate that stimulation or suppression of inflammatory reactions in animal models of diseases is dependent on the concentration in medium as well as administered dose *in vivo*. It has been shown that SIL can modulate human T-lymphocyte proliferation and secretion of cytokines. *In vitro* treatment of peripheral blood mononuclear cells from β -thalassemia patients with low SIL concentrations up to 20 $\mu\text{g}/\text{ml}$ led to the restoration of glutathione levels and stimulated suppressed lymphocyte proliferation (Alidoost et al., 2006). On the contrary, *in vitro* treatment with higher doses (80 $\mu\text{M}/\text{ml}$) of SIL resulted in suppressed expression of T-cell activation- and exhaustion markers on CD4+ and CD8+ T cells from chronically infected HIV-positive subjects and also modulated functions of other human primary cells (Lovelace et al., 2017). SIL exerted significant inhibitory effect on proliferation of CD3-activated mouse spleen T-helper (CD4+) cells *in vitro* at a concentration of 50 $\mu\text{M}/\text{ml}$ or higher (Gharagozloo et al., 2010). Studies showed that anti-inflammatory activities of SIL are dependent on the inhibition of translocation of transcription nuclear factor kappa B (NF- κB) from the cytoplasm into the nucleus (Manna et al., 1999; Gharagozloo et al., 2010) where it regulates transcription of many genes for inflammatory mediators and cytokines.

Activation of T lymphocytes during an immune response is mediated by various T-lymphocyte receptors and triggers a series of programmed gene regulations, proliferation, and effector functions. Concanavalin A (CoA), a lectin isolated from the seeds of *Canavalia ensiformis* (the Jack bean), is a very potent mitogen for lymphocytes and can induce rapid cell proliferation, preferentially of T lymphocytes, including both helper and suppressor subsets (Dwyer and Johnson, 1981). It was shown that lymphocytes possess about 10^6 CoA receptors per cell on the cell membrane and bind to various glycosyl proteins and to α -D-mannose residues on glycolipids (Wang et al., 1971). CoA also stimulates the energy metabolism of T cells via modulation of cell respiration localized in mitochondria and the overall effects are mostly mediated by modulation of ATP turnover (Krauss et al., 1999). Research accumulated in the past decade has revealed that immune cells change their metabolic programs which are tightly regulated via mitochondrial dynamics, to support activation and differentiation into individual subsets (O'Neill et al., 2016). Mitochondria are central organelles of metabolism that provide energy during the differentiation and maintenance of immune cell phenotypes (Ozay et al., 2018). Moreover, alterations of mitochondrial membrane

potential are important in apoptosis and may precede nuclear signs of apoptosis in several different cell types. Kulkarni et al. (1998) showed that CoA-induced apoptosis in human fibroblasts is associated with breakdown of mitochondrial membrane potential and loss of calcium homeostasis in mitochondria. Immunomodulatory activities of SIL have been evaluated in various cell models *in vitro*, but it is not clear whether SIL treatment of CoA-activated mouse spleen cells involves modulation of cytokines secretion and alterations in mitochondrial homeostasis and whether it can prevent cell apoptosis.

In this study, we aimed to investigate the concentration-dependent effect of SIL on proliferation of CoA-stimulated splenocytes, viability of cells, and secretion of IFN- γ and IL-4 cytokines *ex vivo* in relation to gene expressions of transcription factors Foxp3 and NF- κB . In addition, metabolic changes in cells were evaluated by means of the mitochondrial membrane potential and apoptosis.

MATERIAL AND METHODS

Reagents

Spleen cells were incubated in RPMI medium (Biochrom-Merck, Germany) containing 2 mM of stable glutamine and supplemented with 10% heat-inactivated bovine fetal serum (Biochrom-Merck, Germany), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 10 $\mu\text{g}/\text{ml}$ gentamicin, and 2.5 $\mu\text{g}/\text{ml}$ amphotericin B (all from Sigma-Aldrich, St. Louis, USA). SIL was purchased from Sigma-Aldrich and was dissolved in 100% DMSO to obtain a concentration of 10 mg/ml. All the subsequent dilutions were made in RPMI medium and the final DMSO concentration was 0.1% or lower. Cells were treated with SIL concentrations of 2.5, 5, 10, 20, and 40 $\mu\text{g}/\text{ml}$ (final). The composition of SIL was determined by HPLC analysis (Hrčková et al., *in press*) and was as follows: taxifolin (4%), silychristin A/silychristin B and silydianin (33%), silybin A/B (52%), isosilybin A/B (11%). CoA, Rhodamine 123, and DMSO were purchased from Sigma-Aldrich.

Animals and isolation of splenocytes

Male Balb/c mice were purchased from VELAZ (Prague, Czech Republic) and used at the age of 8 weeks. Spleens were aseptically isolated from three mice in each experiment ($n = 3$). Suspensions of splenic cells were obtained by gentle squeezing spleen tissue between the glass slides in 5 ml of cold medium on ice and red blood cells were removed by incubation of suspension with lysis solution (8.02% NH_4Cl , 0.85% NaHCO_3 , and 0.37% EDTA) on ice. Splenic cells were washed with PBS, filtered through 40 μm nylon filters (BD Biosciences, Darmstadt, Germany), and resuspended in medium. Viability of the cells was more than 95% as determined by trypan blue exclusion.

T-lymphocyte proliferation

The suspensions of naive splenocytes were diluted to a concentration of 1×10^6 cells/ml and seeded into flat bottom 96-well plates in quadruplicates (Corning) for each treatment. They were used to examine the effect of SIL on the T-lymphocyte proliferation by BrdU Cell Proliferation ELISA Kit (Roche Diagnostics GmbH, Mannheim, Germany) and cytokine production *ex vivo*. Splenocytes were stimulated with CoA (3 μ g/ml) for 70 h at 37°C and 5% CO₂. BrdU was added to the cell suspensions at 5 μ M final concentration for the last 18 h of cultivation. Stimulation of cell proliferation termed as proliferation index (PI) was determined as the ratio of absorbance of stimulated versus unstimulated cells for each cell sample plated in quadruplicates and data are expressed as means $\pm$ SD from three mice/treatment.

Cytokine concentrations

Supernatants from other CoA-stimulated splenocyte cultures (not supplemented with BrdU) were collected. Samples from identical treatments in wells were pooled and stored at -80°C for determination of IFN- γ and IL-4 by ELISA kits (Mouse Ready-SET-Go ELISA; eBioscience, Germany). Then cytokine concentrations were determined in triplicates for each treatment/mouse using MaxiSorp Nunc-immuno module strips (Thermo Fisher Scientific, Roskilde, Denmark) and calculated in picogram per milliliter and expressed as mean $\pm$ SD.

Viability assessment (trypan blue exclusion test)

Viability of CoA-stimulated splenocytes was evaluated after 70 h of incubation with increasing SIL concentrations using 0.05% solution of trypan blue (Sigma-Aldrich, Merck Sigma, UK) in PBS. Splenocytes were incubated in 24-well plates at a concentration of 1×10^6 /ml in RPMI medium, stimulated with CoA, and treated with the same concentrations of SIL (in quadruplicates) as were used in the proliferation assay. After 70 h of incubation, nonadherent cells (lymphocytes) from each well were collected in tubes, and after addition of trypan blue, unstained live cells were counted using hemocytometer. Proportions (%) of live cells from total counted cells per well ($n^3 \times 100$) were calculated for control sample and each SIL concentration and were expressed as mean $\pm$ SD.

Flow cytometric analysis of mitochondrial membrane potential

Naive, unstimulated, and CoA-stimulated cells treated with SIL were incubated in 24-well plates for 70 h as described previously. Then nonadherent cells were collected into tubes and used to measure mitochondrial membrane potential (Ym) using the fluorescent dye Rhodamine 123, which is taken by mitochondria of living cells. Changes in the uptake of this dye are believed to reflect the level of Ym (Darzynkiewicz et

al., 1982). The stock solution (1 mg/ml) of Rhodamine 123 was prepared in ethanol and added to the cell suspensions. Cells were incubated with Rhodamine 123 (10 μ M final) for 20 min at 37°C and immediately used to measure Ym without washing by flow cytometry. Flow cytometry was performed with excitation of Rhodamine 123 at 505 nm and emission at 535 nm using flow cytometer FACS Canto (Becton Dickinson Biosciences, USA). Data were analyzed using FACS Diva software and were expressed as the mean fluorescence intensity of Rhodamine 123 to establish the effect of CoA and SIL on splenocytes.

Annexin V/propidium iodide apoptosis assay

Cell suspensions of nonadherent cells from cultivations in 24-well plates treated in the same way as were applied in other assays were used to study apoptotic process after 70 h of incubation. Apoptosis was detected with BD Pharmingen Annexin V-FITC Apoptosis Detection Kit (APO Alert Annexin V, ClonTech, California, USA) according to manufacturer's instructions. Briefly, treated cells were centrifuged for 10 min to remove the medium, then were washed and resuspended in 200 μ l of the binding buffer. Apoptotic cells were detected after staining with 5 μ l of Annexin V and 5 μ l of propidium iodide solutions at room temperature in the dark for 15 min. Analysis was performed by flow cytometry using BD FACS Canto (Becton Dickinson Biosciences) and data were analyzed using FACS Diva software. Data are expressed as proportions of apoptotic cells (%).

Isolation of RNA from cells and real-time PCR

Quantitative transcription profiles of genes for IFN- γ , IL-4, Foxp3, and NF- κ B and housekeeping genes β -actin as well as GAPDH in spleen cells were determined by real-time PCR (RT-PCR). Cells (1×10^6 /ml) were plated into 24-well plates in triplicate for each treatment and stimulated with CoA and/or SIL at increasing concentrations for 70 h. Then supernatants containing lymphocytes were collected into tubes, centrifuged, and pellets from three wells/treatment were pooled and used for extraction of RNA after adding Trizol reagent (Amresco, Solon, OH, USA). Then 3 μ g of total RNA was reverse transcribed with RevertAid H Minus M-MuLV Reverse Transcriptase and oligo dT primers (both from Thermo Fisher Scientific, St. Leon-Rot, Germany). Real-time quantitative analysis of the relative abundance of mRNA species was determined using the SYBR green master mix (BioRad, Hercules, CA, USA) on BioRad CFX thermocycler (BioRad). PCR was performed in 20- μ l reactions with detection primer pairs for IFN- γ (forward: 5'-TCAAGTGGCATAGATGTGGAAGAA-3'; reverse: 5'-TGGCTCTGCAGGATTTTCATG-3'), IL-4 (forward: 5'-ACAGGAGAAGGGACGCCAT-3'; reverse: 5'-GAAGCCCTACAGACGAGCTCA-3'), Foxp3 (forward: 5'-AAT AGT TCC TTC CCA GAG-3'; reverse: 5'-GAT TTC ATT GAG TGT CCT-3'), NF- κ B (forward: 5'-GGGCACTGACGCGACG-3';

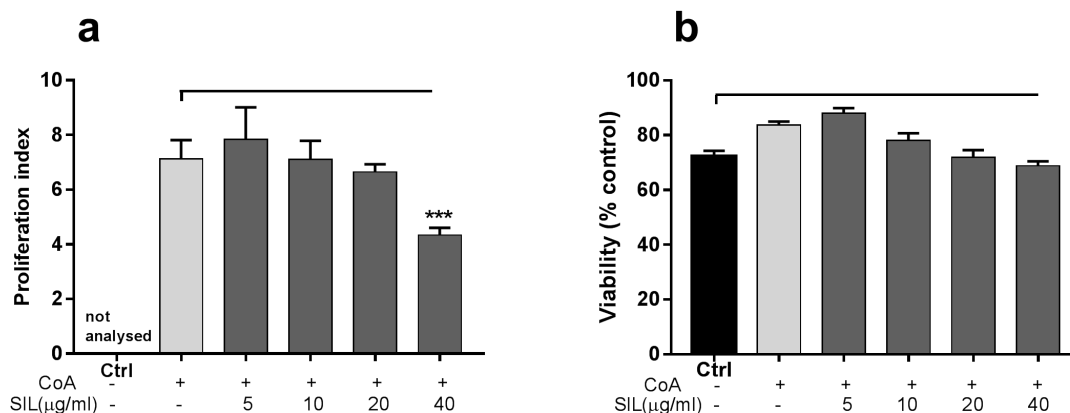


Figure 1: The effect of silymarin on CoA-activated mouse spleen cells *in vitro*: (a) proliferation index of T lymphocytes; (b) viability test of T lymphocytes using trypan blue staining. Significantly different values between CoA alone and CoA + silymarin treated groups are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

reverse: 5'-AGCGCCCCTCGCATTTATAG-3'), β -actin (forward: 5'-ACCAACTGGGACGACATGGAGAAAATC-3'; reverse: 5'-GTAGCCGCGCTCGGTGAGGATCTTCAT-3'), and GAPDH (forward: 5'-TCACCACCATGGAGAAGGC-3'; reverse: 5'-GCTAAGCAGTTGGTGGTGCA-3'). The results are expressed as fold amplification of the target gene compared with its expression in cells in naive mice by means of the comparative Ct method DDCT (Livak and Schmittgen, 2001).

Statistical analysis

The results were analyzed either by using one-way ANOVA followed by Tukey's post hoc test or with the grouped analyses utilizing two-way ANOVA and the Sidak post hoc test. Data were evaluated by GraphPad Prism (version 7) (GraphPad Software, Inc., San Diego, CA, USA), and the differences were regarded as significant at least at $p < 0.05$.

RESULTS

The effect of silymarin on proliferation of CoA-stimulated lymphocytes and their viability

CoA is a strong mitogen, activating proliferation preferentially of T-cell subsets via cell membrane receptors. Splenocytes were treated with increasing concentrations of SIL and PI was determined in BrdU incorporation assay. As shown in Fig. 1a, SIL stimulated proliferation up to a concentration of 5 μ g/ml and significant elevation was detected in CoA-alone treated cells ($p < 0.05$). On the contrary, high dose of 40 μ g/ml significantly ($p < 0.001$) decreased PI of activated T lymphocytes.

To investigate whether highly reduced proliferation is due to the cytotoxic effect of SIL on lymphocytes, we counted viable cells after staining with trypan blue and calculated the proportions of live cells from total counted cells (Fig. 1b). In

comparison with unstimulated lymphocytes, the proportions of live cells increased significantly after CoA treatment and after CoA + 5 μ g/ml of SIL exposure, which correlated with the changes of PI. However, at 40 μ g/ml of SIL no cytotoxic effect was determined as non-significantly reduced numbers of live cells were observed, indicating that reduction of proliferation by SIL was caused by other mechanisms.

The effect of silymarin on secretion and gene expression of cytokines

The effect of treatments on IFN- γ and IL-4 cytokine levels and mRNA expression of both genes were examined by ELISA test and quantitative RT-PCR, respectively. As shown in Fig. 2 a, b concentration of cytokines was barely detectable in unstimulated cells, but has markedly increased after CoA stimulation. SIL significantly enhanced production of IFN- γ at concentrations of 5 and 10 μ g/ml in comparison with CoA-alone-stimulated cells, but had no inhibitory effect at the highest concentration. In contrast, IL-4 levels were not significantly changed, except for the markedly suppressed secretion after exposure to 40 μ g/ml of SIL. However, SIL treatment had the opposite effect on mRNA transcript levels for both cytokines (Fig. 2 c, d). In unstimulated cells, the abundance of mRNA copies was several times higher than in CoA-stimulated cells. We found stimulation of genes expression with increasing concentrations of SIL in T-lymphocyte cell cultures, which was significantly higher ($p < 0.01$) after treatment with 40 μ g/ml of SIL in comparison with CoA-treated cells.

The effect of silymarin on gene expression of transcription factors

SIL can suppress nuclear transcription factor NF- κ B on the protein level but it is not clear if SIL similarly suppresses NF- κ B

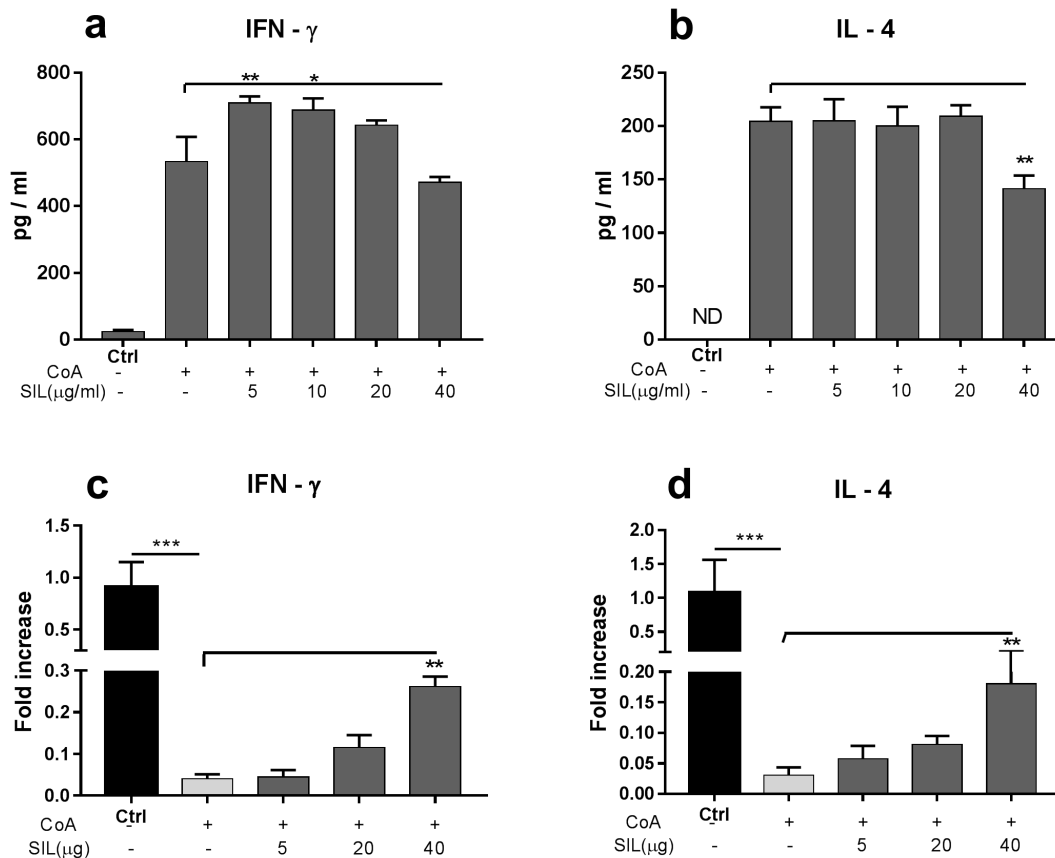


Figure 2: The effect of silymarin on cytokine secretion and mRNA levels in control unstimulated splenocytes and CoA-activated mouse spleen T lymphocytes in vitro: (a) concentration of IFN- γ and (b) IL-4 in supernatants of cells after 70 h cultivation; (c) relative gene expression for IFN- γ and (d) IL-4 in T lymphocytes after 70 h of cultivation. Significantly different values are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

on the mRNA level. To clarify concentration-dependent effect of SIL on CoA-stimulated T cells we examined the relative transcripts abundance of gene for p65 (RelA) which is one of the two subunits of NF- κ B (Fig.3a). No difference was found between unstimulated and CoA-stimulated cells. Interestingly, SIL in dose-dependent manner stimulated expression of gene for this protein, and significantly elevated mRNA copies were found at a dose of 40 μ g/ml ($p < 0.01$). Expression of transcription factors of T-helper cell subsets characterizes precisely their phenotype; therefore we evaluated the expression levels of gene encoding Foxp3, which is activated in regulatory T cells (Tregs) having suppressive effect on IFN- γ -dependent inflammatory reactions (Hori et al., 2003). As shown in Fig.3 b, transcript levels increased significantly in CoA-activated cells in comparison with untreated cells ($p < 0.001$). SIL enhanced the activity of gene for Foxp3 at transcriptional level at concentrations of 20 and 40 μ g/ml; however, low concentrations up to 5 μ g/ml slightly downregulated gene expression.

The effect of silymarin on mitochondrial membrane potential

Upon activation, T cells utilize mitochondrial energy to differentiate into distinct T-helper cells (Ozay et al., 2018) and changes in mitochondrial dynamic can be assessed by mitochondrial membrane potential (Ym). The effects of SIL on Ym in CoA-stimulated T lymphocytes are demonstrated in Fig. 4 as the mean intensity of fluorescence (MIF), and representative plots of MIF are shown in the left panel. In comparison with freshly isolated lymphocytes, incubation of naïve unstimulated cells for 70 h at standard conditions resulted in significant decrease of Ym ($p < 0.05$). In contrast, CoA activation of cells led to moderate stimulation of Ym and significant activation was found at 5 μ g/ml concentration of SIL.

The effect of silymarin on cell apoptosis

Impairment in mitochondrial biogenesis to a critical level might result in cell death in many pathological conditions. To get a deeper insight into the regulation of T-cell functions

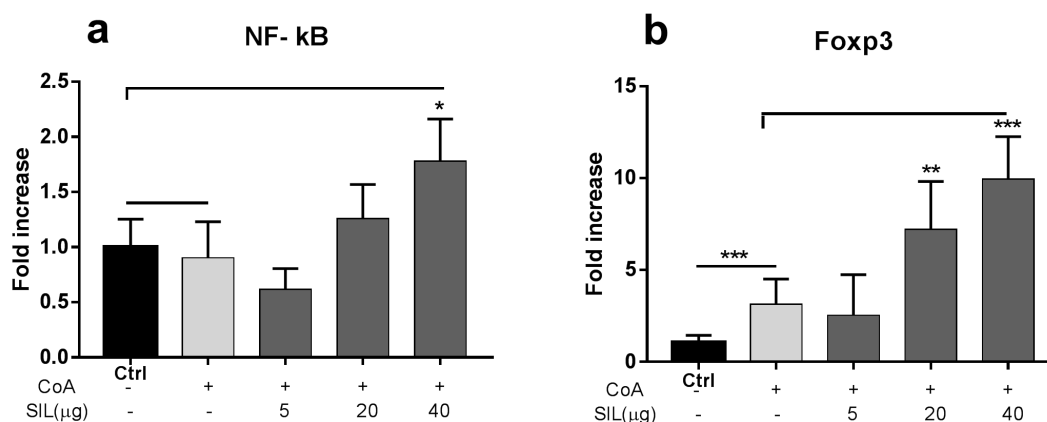


Figure 3: The effect of silymarin on mRNA levels in control unstimulated splenocytes and CoA-activated mouse spleen T lymphocytes *in vitro* for (a) transcription factor NF-κB and (b) transcription factor Foxp3. Significantly different values are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

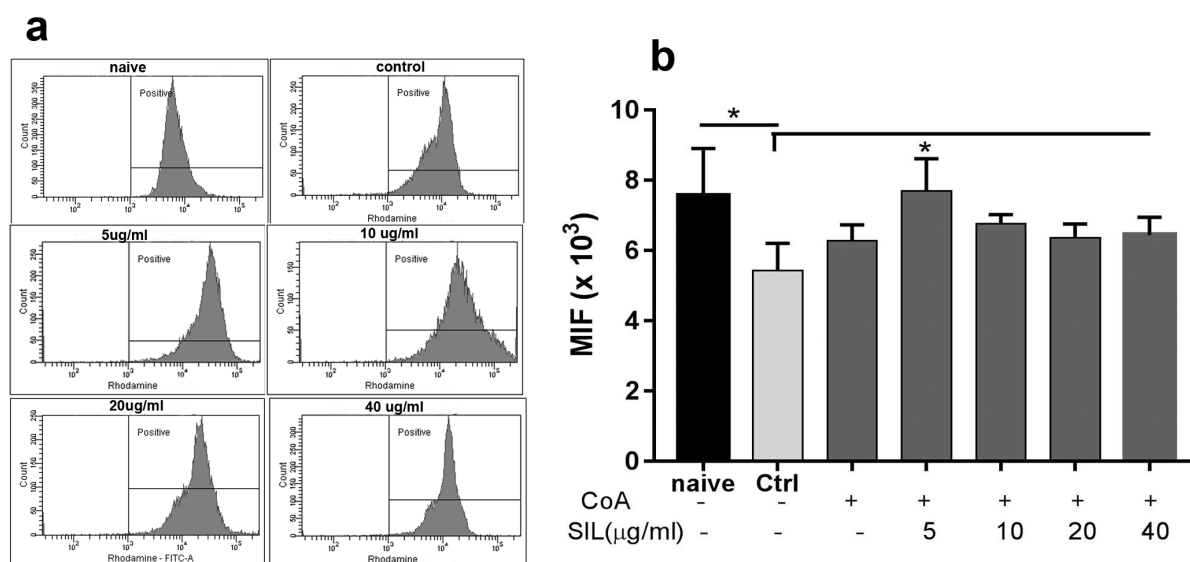


Figure 4: The effect of silymarin on mitochondrial membrane potential in naive and CoA-activated mouse spleen cells and after treatment with silymarin (b), expressed as the mean intensity of fluorescence (MIF) for Rhodamine 123: (a) representative plots of MIF for control and individual silymarin-treated groups. Significantly different values are indicated as: * $p < 0.05$.

and fate by various concentration of SIL, we determined the proportions of live cells, cells in the early stage of apoptosis, and dead cells by flow cytometry. Results of our analyses are summarized in Fig.5 and representative dot plots from analysis of freshly isolated naive spleen cells (upper left panel) and CoA-stimulated cells treated with 5 μg/ml of SIL for 70 h are on lower left panel. While in suspension of freshly isolated spleen cells, more than 93% of cells were live, cultivation for 70 h negatively influenced the physiological state of lymphocytes as manifested by their entry into the early phase of apoptosis. We found that $86.2 \pm 2.7\%$ of unstimulated cells (control group) was at the early stage of apoptosis at the end of cultivation. Following CoA stimulation the proportion of

early apoptotic cells decreased, whereas the proportions of live cells significantly increased ($p < 0.05$). In comparison with the control, further lowering of early apoptotic cells and elevation of live cells were observed after 5 μg/ml of SIL, which correlates with the elevation of mitochondrial membrane potential.

DISCUSSION

SIL is a natural product widely studied for its numerous beneficial effects on health and prevention of many diseases (Gažák et al., 2007). It is considered very safe as after oral administration the 50% lethal dose was 10 g/kg in rats

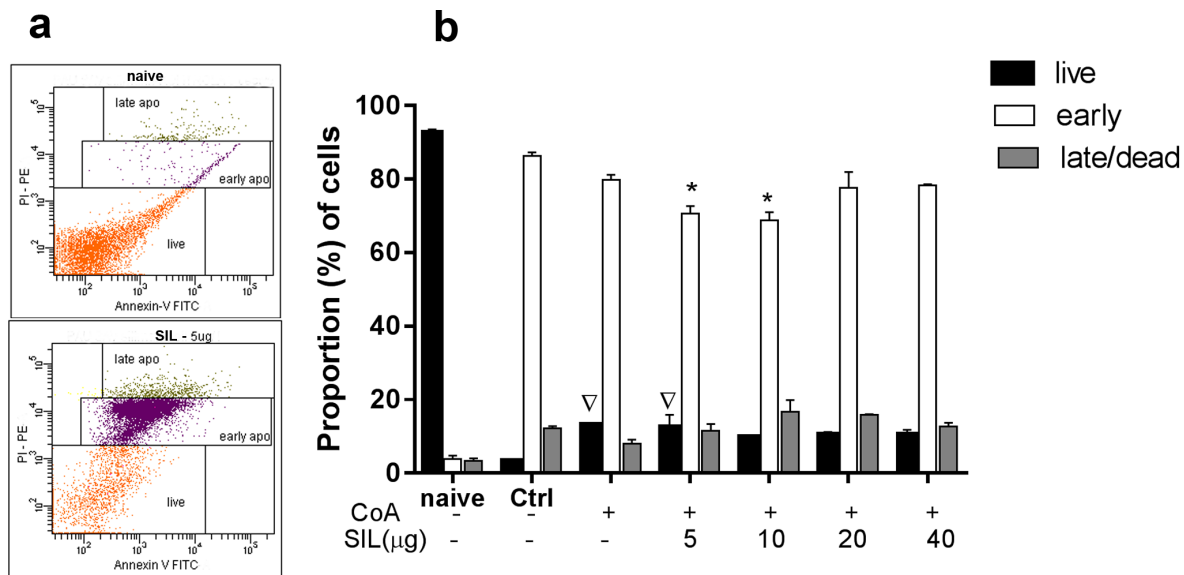


Figure 5: The proportions of live, early apoptotic, and late apoptotic/dead cells determined for the suspensions of naïve splenocytes, CoA-activated T lymphocytes, and following *in vitro* treatment with silymarin after 70 h of cultivation (b). Representative flow cytometric dot plots showing Annexin V and propidium iodide staining of naïve splenocytes and CoA-activated cells following silymarin treatment (a). Significantly different values between CoA alone and silymarin-treated groups for early apoptotic cells are indicated as * $p < 0.05$ and for live cells are indicated as $Np < 0.05$.

(Fraschini et al., 2002). Studies on immunomodulatory activities indicate concentration-dependent effect at various *in vitro* and *in vivo* experimental conditions mostly reporting on suppression of inflammatory mediators (Esmail et al., 2017). In this study, we aimed to compare the effects of lower doses (5 and 10 µg/ml) and higher doses (20 and 40 µg/ml) of SIL preparation (Sigma) in which flavonolignans silybin A/B formed about 52%. We focused on spleen T lymphocytes isolated from healthy Balb/c mice following activation by plant mitogen CoA. We first investigated the dose-dependent effect on T-cell proliferation showing that low dose of 5 µg/ml can enhance proliferation and significant suppression was observed at a concentration of 40 µg/ml. Generally, upon priming of T cells with different stimuli, heterogeneous population is generated since naïve T cells have stem-like properties and can differentiate into virtually all different types of effector, memory, or regulatory cells. Higher sensitivity to suppressive effect of SIL was found when purified mouse spleen CD4⁺ T cells were used. Namdari et al. (2018) found that concentrations of 20 µg/ml of SIL and higher significantly reduced cell proliferation. By using CD4⁺ splenocytes from C57/BL6 mice, proliferation assay revealed that SIL, at 50 µM concentration (approximately 25 µg/ml), significantly inhibited CD4⁺ cell proliferation (Gharagozloo et al., 2010). Suppressive effect of higher concentrations of SIL was not due to the direct induction of cell death, which was confirmed in our study by trypan blue test and in the study of Namdari et al. (2018) by propidium iodide staining. Moreover, we revealed that reduced proliferation was not due

to decreased cell viability as the proportions of live/dead cells remained similar as in untreated cells.

Reports on concentration-dependent effect of SIL administration to mice present various findings. Johnson et al. (2003) showed that the absolute numbers of splenic CD3⁺ T lymphocytes were reduced after intraperitoneal administration of 10 and 50 mg/kg of SIL; however, PHA-induced T-lymphocyte proliferation *ex vivo* was increased in 10 mg/kg of body weight-treated group. Wilasrusmee et al. (2002) studied the standardized milk thistle extract containing SIL and other phytochemicals in murine lymphocyte proliferation tests using CoA as the mitogen. They showed that the extract profoundly increased lymphocyte proliferation at doses of 250 µg/ml and only moderately at lower doses. This effect of milk thistle was associated with an increase in IFN-γ, IL-4, and IL-10 cytokines. Since SIL was found to form 70%–80% in the whole extract, the stimulatory activity after oral administration could be comparable with the results of another study on mice in which the purified SIL was used (Karimi et al., 2018). The authors showed that treatment of healthy mice with low dose of SIL (50 mg/kg) for 14 consecutive days stimulated both cellular and humoral immune functions. It increased the proliferation of phytohemagglutinin-A-stimulated spleen T-cell proliferation and IFN-γ secretion *ex vivo*. Pharmacokinetic studies on tissues distribution of silybin, the major active constituent of SIL, have revealed that in mice orally fed with silybin at dose of 50 mg/kg the peak levels were observed at 0.5 h after administration, and concentrations in tissues

were the following: 8.8 µg/g (liver), 4.3 µg/g (lung), 123 µg/g (stomach), and 5.8 µg/ml (pancreas) (Zhao and Agarwal, 1999). These low concentrations presented in these organs, except stomach, could support the stimulatory activities of SIL found in our *in vitro* study and *in vivo* studies mentioned earlier.

We further investigated the effect on cytokine secretion by CoA-stimulated cells and showed that only the highest concentration of SIL (40 µg/ml) leveled down the amount of IFN-γ and IL-4, probably due to diminished cell numbers as a consequence of reduced proliferation. The higher susceptibility to SIL was found for CD4+ T-helper cells activated with monoclonal anti-CD3/anti-CD28 antibodies, where SIL at concentrations of 20 and 30 µg/ml significantly decreased IFN-γ gene expression and upregulated Foxp3 transcription factor in Treg cells. Foxp3 factor plays a key role in the maintenance of lymphoid homeostasis in a number of immune circumstances, where Tregs guide immunosuppressive reactions (Feuerer et al., 2009).

In our study, SIL at the dose of 20 and 40 µg/ml stimulated expression of Foxp3 transcription factor in CoA-stimulated T lymphocytes, as well as mRNA levels for IFN-γ cytokine, suggesting that CoA probably induced a heterogeneous population of T cells with different transcriptional profiles and cytokine secretion. Stimulation of gene for NF-κB p65 subunit on mRNA level with increasing concentrations of SIL was also observed in our study. In the cytoplasm, NF-κB dimer is present in inactive form bound to inhibitory kappa B (IκB) protein and upon activation of cells the NF-κB is released from IκB and translocates to the nucleus. It was shown that SIL can block the translocation of NF-κB to nucleus via suppression of IκB degradation (Manna et al., 1999). We assume that although mRNA and probably also protein levels of NF-κB subunit were significantly elevated after 40 µg of SIL, simultaneous dose-dependent inhibition of IκB degradation occurred with increasing concentrations of SIL.

Interaction of CoA (concentration 3 µg/ml) with cell surface glycoproteins can induce apoptosis in fibroblasts and other nonimmune cells, and intrinsic pathway of apoptosis can be initiated by imbalance in the mitochondrial membrane potential Ym (Kulkarni et al., 1998). Mitochondrial control and guidance of cellular activities of T cells (Chao et al., 2017; O'Sullivan and Pearce, 2015) were recognized as a key factor in many diseases (Shinohara and Tsukimoto, 2018). In our study, cultivation of unstimulated splenocytes for 70 h decreased Ym and induced transition of naive splenocytes to early apoptosis (approximately 86%) and late apoptosis (approximately 11%). Activation with CoA stimulated Ym in T cells, which correlated with the decreased proportion of the early apoptotic cells (79%) indicating the metabolic reprogramming in activated cells. It was found that CoA bound to the cell membrane is internalized and accumulated primarily onto the mitochondria as early as

1 h posttreatment, and gradually increased mitochondrial membrane permeability. The increased mitochondrial membrane permeability would then lead to staining with Annexin V, the marker labeling the early stage, but no typical apoptosis was observed (Shinohara and Tsukimoto, 2018). We further showed that low concentrations of SIL (up to 10 µg/ml) positively modulated the mitochondrial dynamics in activated T cells which probably accounted for observed reduced proportions of the early apoptotic and late apoptotic/dead cells. Interestingly, higher concentrations (20 and 40 µg/ml) were not pro-apoptotic, in agreement with results of the study of Gharagozloo et al. (2010) on activated CD4+ T cells, supporting our hypothesis that antiproliferative effect of 40 µg/ml SIL (approximately 100 µM/ml) was not due to the cytotoxic effects on activated T cells. Detailed examination of the effect of SIL on proliferation revealed a significant G1 arrest in the cell cycle of CD3+ CD28+ activated T lymphocytes after 96 h of incubation with 100 µM SIL (approximately 50 µg/ml) without causing cell death (Gharagozloo et al., 2013). It is possible that the stimulated cells had an impaired respiratory capacity with a decreased mitochondrial membrane potential due to excess of produced reactive oxygen species. Addition of SIL to stimulated cells increased the mitochondrial membrane potential, suggesting that in CoA-stimulated cells the electron transfer, respiratory activity of the cells, and activity of the enzymes involved in oxidative phosphorylation have increased. Moreover, SIL is likely to prevent peroxidative damage of phospholipids, thereby preventing the initiation of apoptosis through cytochrome c release (Yuan, 2006; Robertson and Orrenius, 2000).

In conclusion, our study demonstrated that mouse spleen T lymphocytes activated by lectin CoA show different susceptibilities to low (10 µg/ml) and higher (20 and 40 µg/ml) SIL concentrations after 70 h of incubation *in vitro*. Treatment with low concentrations resulted in increased proliferation, cytokine secretion, and mitochondrial membrane potential and reduced transition of T cells to apoptosis. SIL at high concentration had the opposite effect without exerting significant cytotoxicity and upregulated genes for cytokines and transcription factors on mRNA level. It is possible that individual subpopulations of T cells induced by CoA were differentially affected by the various SIL concentrations and profound suppression of T lymphocyte functions was associated with the dose of 40 µg/ml. This correlated with the highest expression of Foxp3 factor indicating that this dose preferentially promoted differentiation to Tregs lymphocytes.

CONFLICTS OF INTEREST

We wish to confirm that there are no conflicts of interest associated with this publication.

ACKNOWLEDGMENTS

This study was supported by the project APVV No. 17-0410 and the project VEGA No. 02/0091/17 granted by the Ministry of Education, Science, Research and Sport of the Slovak Republic. The authors also gratefully acknowledge the bilateral mobility project SAV-AV ČR No. 18–24.

References

- [1] Alidoost F, Gharagozloo M, Bagherpour B, et al. Effects of silymarin on the proliferation and glutathione levels of peripheral blood mononuclear cells from beta-thalassemia major patients. *Int Immunopharmacol.* 2006;6:1305-1310.
- [2] Chao T, Wang H, Ho PC. Mitochondrial control and guidance of cellular activities of T cells. *Front Immunol.* 2017;8:473.
- [3] Darzynkiewicz Z, Traganos F, Staiano-Coico L, Kapuscinski J, Melamed MR. Interaction of rhodamine 123 with living cells studied by flow cytometry. *Cancer Res.* 1982;42:799-806.
- [4] Dwyer JM, Johnson C. The use of concanavalin A to study the immunoregulation of human T cells. *Clin Exp Immunol.* 1981;46:237-249.
- [5] Esmaeil N, Anaraki SB, Gharagozloo M, Moayedi B. Silymarin impacts on immune system as an immunomodulator: One key for many locks. *Int Immunopharmacol.* 2017;50:194-201.
- [6] Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol.* 2009;10:689-695.
- [7] Fraschini F, Demartini G, Esposti D. Pharmacology of Silymarin. *Clin Drug Invest.* 2002;22:51-65.
- [8] Gažák R, Walterova D, Kren V. Silybin and silymarin—new and emerging applications in medicine. *Curr Med Chem.* 2007;14:315-338.
- [9] Gharagozloo M, Javid EN, Rezaei A, Mousavizadeh K. Silymarin inhibits cell cycle progression and mTOR activity in activated human T cells: therapeutic implications for autoimmune diseases. *Basic Clin Pharmacol Toxicol.* 2013;112:251-256.
- [10] Gharagozloo M, Velardi E, Bruscoli S, et al. Silymarin suppress CD4+ T cell activation and proliferation: effects on NF-kappaB activity and IL-2 production. *Pharmacol Res.* 2010;61:405-409.
- [11] Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003;299:1057-1061.
- [12] Johnson VJ, He Q, Osuchowski MF, Sharma RP. Physiological responses of a natural antioxidant flavonoid mixture, silymarin, in BALB/c mice: III. Silymarin inhibits T-lymphocyte function at low doses but stimulates inflammatory processes at high doses. *Planta Med.* 2003;69:44-49.
- [13] Karimi G, Hassanzadeh-Josan S, Memar B, Esmaeili SA, Riahi-Zanjani B. Immunomodulatory effects of silymarin after subacute exposure to mice: A tiered approach immunotoxicity screening. *J Pharmacopuncture.* 2018;21:90-97.
- [14] Krauss S, Buttgerit F, Brand MD. Effects of the mitogen concanavalin A on pathways of thymocyte energy metabolism. *Biochim Biophys Acta.* 1999;1412:129-138.
- [15] Kulkarni GV, Lee W, Seth A, McCulloch CA. Role of mitochondrial membrane potential in concanavalin A-induced apoptosis in human fibroblasts. *Exp Cell Res.* 1998;245:170-178.
- [16] Kwon DY, Jung YS, Kim SJ, Kim YS, Choi DW, Kim YC. Alterations in sulfur amino acid metabolism in mice treated with silymarin: a novel mechanism of its action involved in enhancement of the antioxidant defense in liver. *Planta Med.* 2013;79:997-1002.
- [17] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402-408.
- [18] Lovelace ES, Maurice NJ, Miller HW, et al. Silymarin suppresses basal and stimulus-induced activation, exhaustion, differentiation, and inflammatory markers in primary human immune cells. *PloS one.* 2017;12:e0171139-e0171139.
- [19] Manna SK, Mukhopadhyay A, Van NT, Aggarwal BB. Silymarin suppresses TNF-induced activation of NF-kappa B, c-Jun N-terminal kinase, and apoptosis. *J Immunol.* 1999;163:6800-6809.
- [20] Namdari H, Izad M, Rezaei F, Amirghofran Z. Differential regulation of CD4(+) T cell subsets by Silymarin in vitro and in ovalbumin immunized mice. *Daru.* 2018;26:215-227.
- [21] O'Neill LA, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol.* 2016;16:553-565.
- [22] O'Sullivan D, Pearce EL. Targeting T cell metabolism for therapy. *Trends Immunol.* 2015;36:71-80.
- [23] Ozay EI, Sherman HL, Mello V, et al. Rotenone treatment reveals a role for electron transport complex i in the subcellular localization of key transcriptional regulators during T helper cell differentiation. *Front Immunol.* 2018;9:1284.
- [24] Ramasamy K, Agarwal R. Multitargeted therapy of cancer by silymarin. *Cancer Lett.* 2008;269:352-362.
- [25] Robertson JD, Orrenius S. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. *Crit Rev Toxicol.* 2000;30:609-627.
- [26] Saller R, Meier R, Brignoli R. The use of silymarin in the treatment of liver diseases. *Drugs.* 2001;61:2035-2063.
- [27] Shinohara Y, Tsukimoto M. Adenine nucleotides attenuate murine T cell activation induced by Concanavalin A or T cell receptor stimulation. *Front Pharmacol.* 2018;8:986.
- [28] Surai PF. Silymarin as a Natural Antioxidant: An overview of the current evidence and perspectives. *Antioxidants (Basel).* 2015;4:204-247.
- [29] Wang JL, Cunningham BA, Edelman GM. Unusual fragments in the subunit structure of concanavalin A. *Proc Natl Acad Sci U S A.* 1971;68:1130-1134.

- [30] Wilasrusmee C, Kittur S, Shah G, et al. Immunostimulatory effect of *Silybum Marianum* (milk thistle) extract. *Med Sci Monit.* 2002;8:Br439-443.
- [31] Yuan J. Divergence from a dedicated cellular suicide mechanism: exploring the evolution of cell death. *Mol Cell.* 2006;2:1-12.
- [32] Zhao J, Agarwal R. Tissue distribution of silibinin, the major active constituent of silymarin, in mice and its association with enhancement of phase II enzymes: implications in cancer chemoprevention. *Carcinogenesis.* 1999;20:2101-2108.