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The Effects of Silymarin and Cyclosporine A on the Proliferation and Cytokine Production of Regulatory T Cells

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ABSTRACT

Background: Immunosuppressive agents are necessary to enhance allograft tolerance after transplantation and the treatment of autoimmune disorders. Regulatory T cells (Tregs) play a pivotal role in improving allograft tolerance and determining the fate of transplanted organs. Therefore, the aim of this study was to investigate the immunomodulatory effects of cyclosporine A (CsA) and silymarin on the proliferation and cytokine production of Tregs.

Methods: Peripheral blood mononuclear cells (PBMCs) were obtained from healthy voluntaries and Tregs were isolated using an immunomagnetic separation method. The phenotypic characteristics of Tregs were determined by flow cytometry. Tregs were expanded and then cultured with different concentrations of CsA and silymarin. The effects of CsA and silymarin on the viability, proliferation, and transforming growth factor-beta 1 (TGF-β1) production of Tregs were determined after 3 and 5 days of culture.

Results: CsA significantly decreased Treg proliferation in a dose-dependent manner ($p < 0.01–0.05$). CsA failed to change TGF-β1 production of Tregs. On the contrary, silymarin significantly increased the proliferation of Tregs ($p < 0.01–0.05$). A statistically significant increase was also observed in the TGF-β1 production of Tregs ($p < 0.01–0.05$). Our data showed that Treg viability was not compromised by CsA and silymarin.

Conclusion: Overall, the results of this study for the first time indicate that silymarin, unlike CsA, has the ability to increase the proliferation and TGF-β1 production of Tregs and may be beneficial in the treatment of autoimmune disorders and improvement of Treg-dependent allograft tolerance after transplantation.

KEYWORDS
Silymarin; cyclosporine A; regulatory T cell; transforming growth factor-beta 1

Introduction

Regulatory T cells (Tregs) are a subset of T cells, which suppress the cells from both the innate and adaptive immune systems (Adnani et al., 2018). Tregs comprise 5 to 10% of CD4 + T cells and are characterized by the expression of some markers such as cytotoxic T lymphocyte antigen-4 (CTLA-4), CD127, CD25 markers, and Foxp3 transcription factor (Erfani et al., 2013; Singh et al., 2016). They play a crucial role in the maintenance of immunologic tolerance and prevention of autoimmune diseases through regulating

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immune responses (Chruscinski et al., 2015; Fanigliulo et al., 2015; Velásquez-Lopera et al., 2008). The inhibitory functions of Tregs are mediated by cell-cell interaction and the secretion of immunosuppressive mediators, such as interleukin-10 (IL-10), transforming growth factor-beta1(TGF-β1), interleukin-35 (IL-35), and galectin-1 (Arandi et al., 2014; Brusko et al., 2007; Lim et al., 2014; Waldmann et al., 2014; Wang and Vella, 2016). Tregs are dependent on interleukin-2 (IL-2) for their survival, generation, and suppressive function. IL-2 induces Foxp3 expression in which is the 'master switch' for development and inhibitory function of Tregs via signal transducer and activator of transcription 5 (Stat5) signaling (Ruppert et al., 2015). Absence or defect in the functions of Treg are largely associated with various autoimmune disorders and graft rejection (Chruscinski et al., 2015). It has been proposed that many immunosuppressive drugs used for reducing the risk of immune-rejection of transplanted organs exert their inhibitory functions via Tregs (Miroux et al., 2009, 2012a). Thus, a great number of studies have been carried out on the effects of immunosuppressive agents on these cells (Demirkiran et al., 2008, Miroux et al., 2012a). However, the effect of some immunosuppressive drugs on Tregs remains unclear.

Cyclosporine A (CsA) is an immunosuppressive drug that inhibits IL-2 production, leading to the inhibition of T cell proliferation (Tsuda et al., 2012). Cyclosporine A reduces the IL-2 production by binding to a cytosolic protein called cyclophilin. Cyclosporine-cyclophilin complex inhibits calcineurin phosphatase induced upon T cell activation and thereby block translocation of the nuclear factor of activated T cells (NFAT) into the nucleus, and IL-2 gene expression is subsequently down-regulated (Ruppert et al., 2015). CsA reduces Foxp3 expression and then inhibits development and suppressive functions of Treg (Miroux et al., 2009, 2012a, Wuest et al., 2008). Although CsA inhibits the induction of immune tolerance, it decreases the risk of allograft rejection (Miroux et al., 2012a). Previous reports have shown that CsA contributes to the reduction of liver transplant rejection through decreasing the risk of hepatitis C reoccurrence (Miroux et al., 2009). In spite of evidence showing CsA decreases the number of Tregs and reduces the activity of these cells by intervening in IL-2 production, there are some reports pointing CsA treatment improves Foxp3 expression in Tregs and leads to the increased number of these cells in treated patients (Fanigliulo et al., 2015; Knol et al., 2012). However, the molecular mechanisms involved in stimulatory activity of CsA on the function and development of Tregs are not yet understood.

The use of plants as a great source of products with therapeutic properties in treating the diseases has been reported since ancient times (Vargas-Mendoza and Madrigal-Santill án et al., 2014, Gharagozloo et al., 2013a). Silymarin, an active flavonolignan, is a medicinal plant derived from the seeds of milk thistle (silybum marianum). It is known as one of the top 10 most popular natural products with antioxidant activities, hepatoprotective (Vargas-Mendoza and Madrigal-Santillán et al., 2014), anti-carcinogenic (Mateen et al., 2013), immunosuppressive, and regenerative effects (Vargas-Mendoza and Madrigal-Santillán et al., 2014), consumed by Western societies (Polyak et al., 2013). Moreover, there is no report that shows the toxic effects of silymarin (Mateen et al., 2013; Schönfeld et al., 1997). Although the molecular mechanisms of immunomodulatory activity of this herbal product has remained unknown, previous studies have revealed that silymarin treatment strongly inhibits the nuclear translocation of the transcription factor-κB (NF-κB) in CD4 + T cells, which is known as a regulator of T cell activation and many

inducible genes, including interleukin-1(IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), lymphotoxin, and interferon-γ (IFN- γ) (Gharagozloo et al., 2010; Meroni et al., 1988, Gharagozloo et al., 2013a; Lovelace et al., 2015; Polyak et al., 2007; Kang et al., 2009). Other studies have provided convincing evidence that silymarin decreases T cell proliferation, which is accompanied by a reduction in IL-2 and IFN-γ production (Gharagozloo et al., 2010; McClure et al., 2012). On the other hand, others have indicated that silymarin inhibits PI3K/AKT/mTOR signaling pathway in human T lymphocytes (Gharagozloo et al., 2013a). Moreover, it has been reported that silymarin is able to reduce the activation of ERK1/2 and P38 pathways in T cells following T cell receptor (TCR) engagement (Gharagozloo et al., 2013a).

Given that frequent use of immunosuppressive drugs leads to debilitating side effects such as increased development of infectious diseases and various cancers (Kinlen et al., 1979; Poynard et al., 1997), it is required to select the drug which, with a few number of side effects, leads to better results. . Considering the fact that silymarin is a plant extract without identified toxic effects, and its effects on Tregs are not fully recognized so far, we attempted to identify the effect of silymarin on proliferation and cytokine production of the purified Tregs through the investigation and comparison of silymarin effects with CsA, as a well-known immunosuppressive agent, to introduce silymarin as the alternative drug in the treatment of autoimmune diseases and minimize the risk of immune-rejection of organ transplantations.

Materials and methods

Peripheral blood mononuclear cells (PBMCs) isolation

Heparinized blood samples (25 ml) were obtained from five healthy volunteers and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque centrifugation according to the manufacturer's guideline (Miltenyi Biotec, Germany). The study was approved by the Ethics Committee of the Isfahan University of Medical Sciences (protocol number: 394363). All participants gave written informed consent before entering the study. The isolated cells were washed twice with phosphate buffered saline (PBS, pH 7.3) and centrifuged at 200 × g for 5 min. The pellet of the cells was suspended in 3 ml of PBS and the viability of the cells was determined using trypan blue dye exclusion. Cell counts were performed with a haemocytometer.

Isolation of CD4+ CD25+ Tregs from peripheral blood

CD4+ CD25 + T cell isolation was carried out using a CD4+ CD25+ regulatory T cell isolation kit following the manufacturer's protocol (Miltenyi Biotec, Germany). A two-step procedure was used to isolate Tregs. Briefly, in the first place, the isolated PBMCs from each healthy donor were labeled with 10 µl of biotin-conjugated monoclonal antibodies cocktail (includes monoclonal antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD235a (Glycophorin A), CD123, and TCRγ/δ) and 20 µl of microbeads conjugated to monoclonal anti-biotin antibody per 10^7 cells and then incubated for 15 min at 4–8°C. Afterwards, the cell suspension was diluted with 2 ml PBS buffer (containing 0.5% bovine serum albumin, and 2 mM EDTA) and magnetic separation of

the cells was performed using a negative selection column (LS). The flow-throughs containing CD4+ cell were collected and subjected to the next step of Tregs isolation. Secondly, 10 µl of microbeads conjugated to monoclonal anti-CD25 antibody per 10^7 cells were added to the pre-enriched CD4+ cell suspension and incubated for 15 min at 4–8 °C. Subsequently, by adding 1 ml PBS buffer, the dilution of the cell suspension was performed and then passed through a positive column (MS). The columns were removed from the magnetic separator and washed with 1 ml PBS buffer, in order to magnetically labelled cells to be collected. Cell viability was determined using trypan blue dye exclusion and cell count was performed using a haemocytometer.

Assessment of the purity of Tregs by flow cytometry

To determine the purity of Tregs, the cells isolated from PBMCs were stained using a human regulatory T cell staining kit according to the manufacturer's instructions (eBioscience, USA). Accordingly, the isolated cells (3×10^5) in 100 µl PBS/1% bovine serum albumin (BSA) were stained with fluorescein isothiocyanate (FITC) anti-human CD4, Phycoerythrin (PE) anti-human CD25, and the matched-isotype control antibodies for 25 min at 4°C. The matched-isotype control antibodies were used as negative controls. Afterwards, fixation and permebilization of the cells were performed for Foxp3 staining as an interacellular marker according to the manufacturer's guidline (eBiosciences, USA). The fixed and permebilized cells were stained with Phycoerythrin/Cyanine5 (PE-cyn5) anti-human Foxp3 antibody for 25 min at 4°C. The cells were washed twice with 1 ml PBS and centrifuged at 300 × g for 5 min at room temperature. The percentage of the stained cells was measured by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (v10.1, FlowJo, Ashland, OR, USA). The CD4+, CD25 +, and Foxp3+ cells were considered as Tregs. Cell samples with the purity of >92% were used in the following experiments.

Culture and expansion of CD4+ CD25+ Foxp3+ Tregs

Tregs were cultured in 96-well round-bottom plates at a density of 1×10^5 cells per well in Roswell Park Memorial Institute (RPMI) medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin/streptomycin (Sigma-Aldrich, USA), and 500 U/ml IL-2 (Miltenyi Biotec, Germany). The cells were stimulated with CD3/CD28 MACSiBead particles (20 µl/well, Miltenyi Biotec, Germany) and incubated at 37°C with 5% CO2. After 5–8 days of culture, the expanded cells were counted using a heamocytometer and cell viability was determined by trypan blue dye exclusion.

Silymarin and CsA preparation

Silymarin (0.024 mg) and CsA (1 mg) were dissolved in 1 ml dimethyl sulphoxide (DMSO) and further diluted 1000 times with complete RPMI to yield stock concentrations of 50 mM and 1 µg, respectively. The stocks were stored at −20°C for further use. Silymarin (Cat no: S0292) and CsA (Cat no: 30024) were purchased from Sigma-Aldrich (USA).

Assessment of the effects of silymarin and CsA on Tregs

To evaluate the effects of silymarin and CsA on Treg proliferation, Tregs were labeled with carboxyfluorescein succinimidyl ester dye (CFSE) according to the manufacturer's protocol (Abcam, UK). Briefly, Tregs (2×10^6) were re-suspended in 1 ml PBS. CFSE was dissolved in DMSO to yield a stock concentration of 10 mM. Thereafter, 1 µl of a 10 mM stock was added to Treg suspension (2×10^6 cells/ml) at a final concentration of 10 µM. After 20 min of incubation at room temperature in the dark, the staining was quenched by the addition of 5 ml of ice-cold complete RPMI medium. The stained cells were washed twice with complete RPMI medium. The CFSE-labeled Tregs (1×10^5 cells/ml) were cultured in 24-well plates and treated with various concentrations of silymarin (50, 100, and 150 µM/ml) and CsA (100, 200, and 400 ng/ml). All cultures were performed in duplicate. The cells were then stimulated with CD3/CD28 MACSiBead particles (30 µl/well) and incubated at 37°C with 5% CO2, one set for 3 and another for 5 days. Tregs which were cultured in the percense or absence of DMSO and stimulated with CD3/CD28 MACSiBead particles were considered as negetive controls for silymarin and CsA concentrations. DMSO was added to negative controls with equal volumes of the highest doses of CsA and silymarin. The percentage of cell proliferation was assessed through fluorecent intensity measurement of CSFE dye using a FACSCalibur system and the data were analyzed using FlowJo software.

MTT assay

To test the possible effects of silymarin and CSA on Treg viability, MTT assay was preformed. Tregs (2×10^3 cells/well) were treated with 50, 100, and 150 µM/ml of silymarin and 100, 200, and 400 ng/ml of CsA in 96-well, flat-bottomed microtiter plates (BioFil, Canada) and stimulated with CD3/CD28 MACSiBead particles (30 µl/well). The cells which were treated with DMSO or RPMI and stimulated with CD3/CD28 MACSiBead particles (30 µl/well) served as negative controls. The final concentration of DMSO in control wells was equal to the highest doses of CsA and silymarin in test wells. All assays were performed in duplicate according to the manufacturer's guideline (Abcam, UK). After 3 and 5 days, the media of the cultured cells were removed from the wells and then 50 µl of serum-free media and 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (5 mg/ml) were added into each well. The cultures were incubated at 37°C for 3 hrs. Afterwards, 150 µl of MTT solvent was added into each well. After 15-minute incubation in the dark at room temperature, the absorbance of each well was measured by spectrophotometry at 590 nm on a microplate reader (Bio-Rad Microplate Reader 550). The cell viability index was calculated according to the following formula:

$$\text{Viability index}(\%) = \frac{OD\ of\ treated\ cells - OD\ of\ background\ control}{OD\ of\ corresponding\ control - OD\ of\ background} \times 100$$

Cytokine assay

To investigate silymarin and CsA effects on cytokine production of Tregs, the cells (1×10^5 cells/ml) were cultured in 24-well plates and stimulated with CD3/CD28 MACSiBead particles (20 µl/well). Then, the cells were treated with different doses of

silymarin (50, 100, and 150 µM/ml) and CsA (100, 200, and 400 ng/ml) and incubated at 37°C with 5% CO_2 for 3 and 5 days. Tregs which were stimulated with CD3/CD28 MACSiBead particles and cultured in the presence or absence of DMSO served as negetive controls for silymarin and CsA concentrations. The culture supernatant of Tregs was collected after 3 and 5 days and quantitative analysis of TGF-β1 was performed using an Enzyme-linked immunoasorbent assay (ELISA) kit (Mabtech, Sweden) based on the manufacturer's guideline.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) and mean ± standard deviation (SD). The results were analyzed using GraphPad Prism 6 (GraphPad software, San Diego, CA). Normal distribution of data was determined by Kolmogrov-Smirnov test. The groups with normal distribution were compared using One-way ANOVA and unpaired t-tests. Mann-Whitney and Kruskal-Wallis tests were used to compare the groups with non-normal distribution. p value <0.05 was considered statistically significant.

Results

The purity of $CD4^+CD25^+$ $Foxp3^+$ Tregs

To assess the purity of Tregs, the percentage of the cells which were positive for CD4, CD25, and Foxp3 was measured by flow cytometry. Our data revealed that more than 99% of the gated lymphocytes expressed CD4 (Figure 1(a,b)). As shown in Figure 1(c), more than 92% of the CD4+ cells were positive for CD25 and Foxp3 markers.

CsA suppresses the proliferation of $CD4^+CD25^+$ $Foxp3^+$ Tregs

Since CsA is a common drug used to suppress immune responses, this drug was considered to compare the effect of CsA and silymarin on the proliferation of Tregs

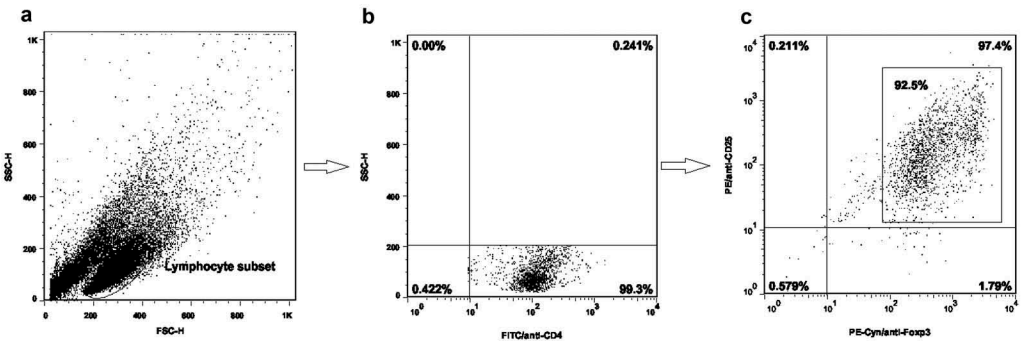

Figure 1. The percentage of CD4+ CD25+ Foxp3+ Tregs isolated from PBMCs. Tregs were isolated from PBMCs using an immunomagnetic separation method and then stained with anti-CD4, anti-CD25, and anti-Foxp3 antibodies. The percentage of the stained cells were analyzed by flow cytometry. Each plot (a, b, and c) are representative of 3 independent experiments.

in vitro. Our flow cytometry results showed that CsA, at the highest concentration (400 ng/ml), had the ability to abrogate Treg generation on day 3 of incubation ($p < 0.01$, Figure 2(a,c)). After 5 days of incubation, all concentrations of CsA exerted inhibitory activity on the proliferation of Tregs, compared to negative controls (stimulated Tregs which were stimulated with CD3/CD28 MACSiBead particles and cultured in the persence or absence of DMSO) ($p < 0.01$–0.05, Figure 2(b,d)). As shown in Figure 2(b,d), the inhibitory effect of CsA on Treg proliferation was in a dose-dependent manner ($p < 0.001$–0.01).

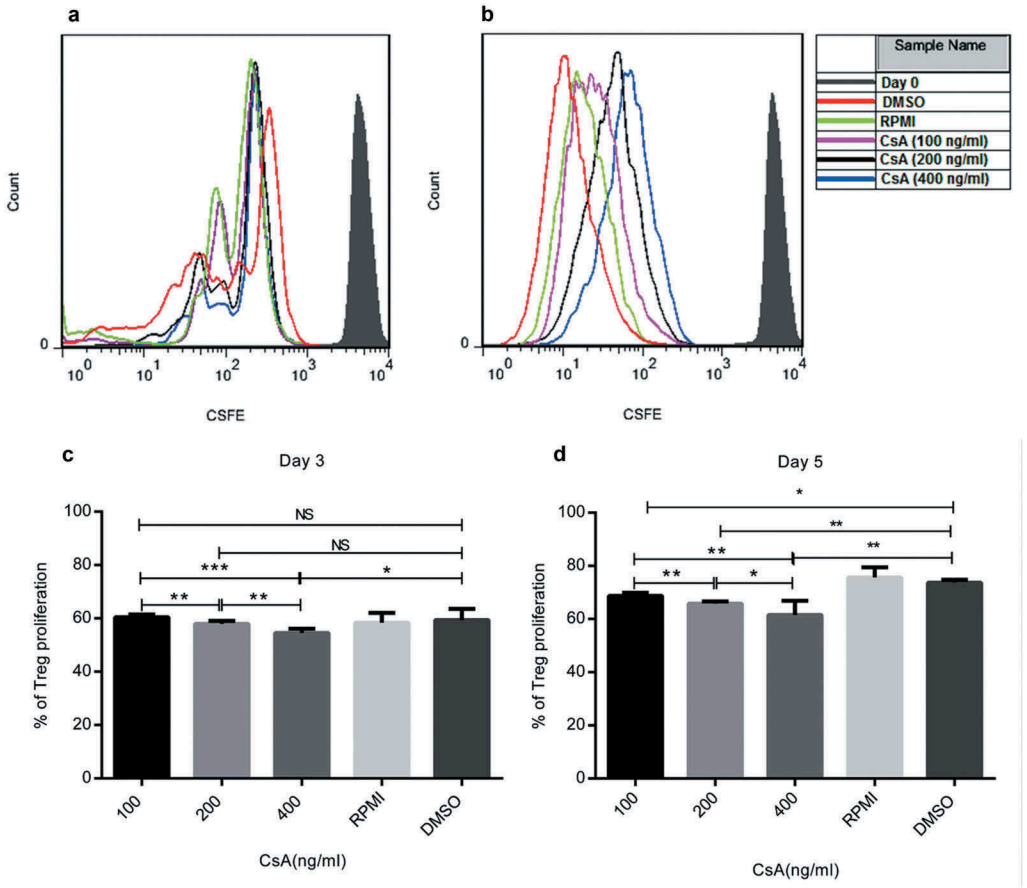

Figure 2. The effects of Cyclosporin A(CsA) on the proliferation of Tregs. Tregs were isolated from PBMCs and stianed with CSFE. The CSFE-labeled cells were cultured in 24-wall plates and treated with different concentrations (100, 200, and 400 ng/ml) of CsA. The CSFE-labeled cells which were cultured in the presence or absence of DMSO and stimulated with CD3/CD28 MACSiBead particles served as negative controls. The cells were collected from 24-wall plates and the proliferation of the CSFE-labeled Tregs was monitored by flow cytometry after 3 (a) and 5 (b) days and then analyzed (c and d). The flow cytometry plots (a and b) are representative of five independent experiments. All data show mean ± SEM. *$p < 0.05$, **$p < 0.01$.

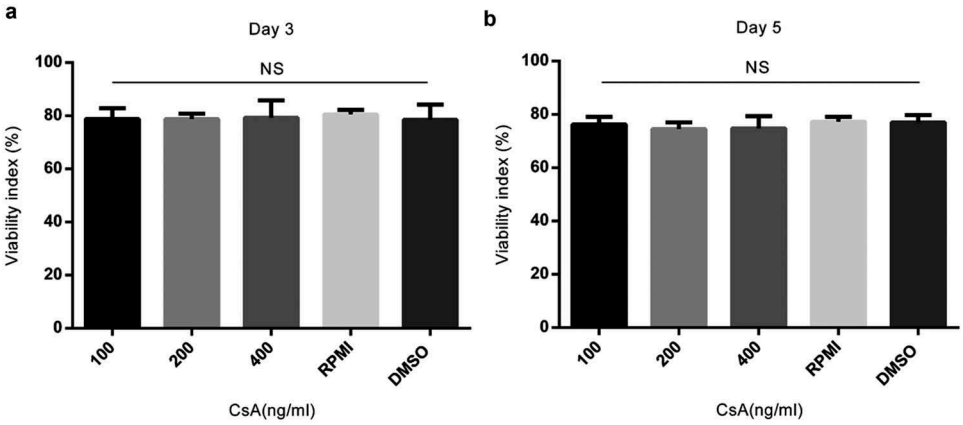

Figure 3. CsA effects on Treg viability. The isolated Tregs were cultured in the presence or absence of diferent concentrations (100, 200, and 400 ng/ml) of CsA and stimulated with CD3/CD28 MACSiBead particles. The cells were incubated under standard culture conditions (37°C, 95% humidified air and 5% CO2). Negative control cells were stimulated with CD3/CD28 MACSiBead particles and treated with DMSO and RPMI. After 3 and 5 days of incubation, the viability of the cells was measured by MTT assay. Each bar in A and B is representative of 5 independent experiments. All data show mean ± SD.

Treg viability does not affect by CsA

To investigate the viability of Tregs after treatment with different concentrations of CsA, the cell viability was assessed by MTT assay. As shown in Figure 3(a,b), Treg viability was not compromised by various doses of CsA after 3 and 5 days of culture.

CsA does not influence TGF-β1 production of Tregs

As shown in Figure 4(a,b), the results of this study indicated that there was no statistically significant change in the level of TGF-β1 in the culture supernatant of Tregs treated with different doses of CsA compared to negative controls after 3 and 5 days of culture.

Silymarin enhances the proliferation of $CD4^+CD25^+$ $Foxp3^+$ Tregs

To evaluate silymarin effect on activated Tregs, the proliferative response of Tregs stimulated with CD3/CD28 MACSiBead particles in the presence or absence of silymarin was measured by a CFSE labeling assay. After 3 days of incubation, with the exception of 150 μM/ml concentration of silymarin, the proliferation of activated Tregs was significantly induced by silymarin compared to negative controls ($p < 0.01$, Figure 5(a,c)). Interestingly, we observed that lower doses (50 and 100 μM/ml) of silymarin had more stimulatory effect on Treg proliferation than high-dose silymarin ($p < 0.001$, Figure 5(a,c)). The same trend was also observed for 5-day culture except that the proliferation of Tregs stimulated with 150 μM/ml of silymarin was also increased ($p < 0.01$–0.05, Figure 5(b,d)).

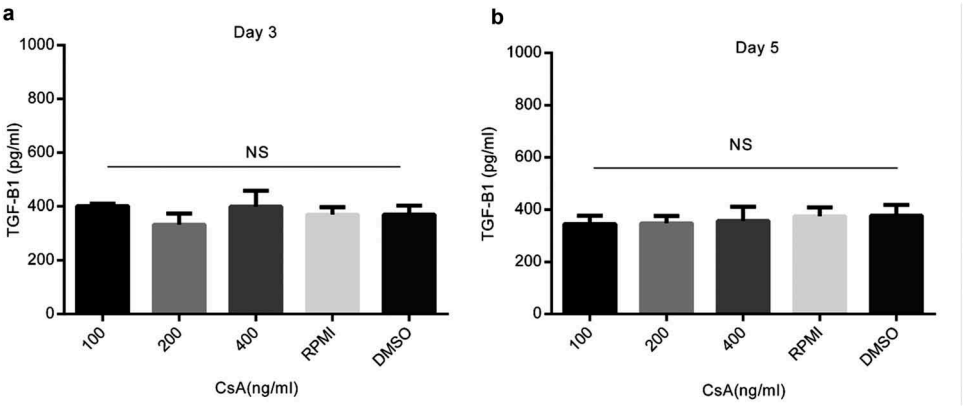

Figure 4. The effects of CsA on TGF-β1 production of Tregs. The expanded Tregs were cultured in 24-wall plates and treated with different concentrations (100, 200, and 400 ng/ml) of CsA. Tregs which were stimulated with CD3/CD28 MACSibead particles and cultured in the presence or absence of DMSO were considerd as negative controls. Culture supernatants of Tregs were collected after 3(a) and 5 days (b) and the level of TGF-β1 was measured by ELISA. The depicted results are representative of 5 individual experiments. All data show mean ± SD.

Silymarin has not the ability to change the viability of Tregs

To study the possible effect of silymarin on Treg viability, the cells were cultured in the presence or absence of this drug. Similar to the results obtained from the cells treated with CsA, our data demonstrated that different doses of silymarin had not considerable activity on the cell viability after 3 and 5 days of culture, although a numerical increase was observed in the viability of the treated-cells on day 5 (Figure 6(a,b)).

Silymarin induces TGF-β1 production of Tregs

To determine the effect of silymarin on cytokine production of Tregs, the level of TGF-β1 in the culture supernatant of Tregs treated with the different doses of silymarin was measured. As shown in Figure 7(a), a statistically significant increase in the level of TGF-β1 was observed for all concentrations of silymarin after 3 days of culture ($p < 0.01$). In agreement with the effect of silymarin on Treg proliferation, the results revealed that 50 μM/ml of silymarin had the highest stimulatory activity on TGF-β1 production of Tregs ($p < 0.05$, Figure 7(a)). Similar results were obtained from 5-day cultures except that the level of TGF-β1 in the culture supernatant of Tregs treated with 150 μM/ml of silymarin was no change compared to negative controls ($p < 0.01$–0.05, Figure 7(b)).

Discussion

Organ transplantation is an applicable treatment method for the replacement of malfunctioning organs and tissues. However, the immune response of the host to transplanted organ is a major problem commonly occurred in organ transplantations. Therefore, immunosuppressive drugs are essential to prevent graft rejection (Joffre et al., 2008).

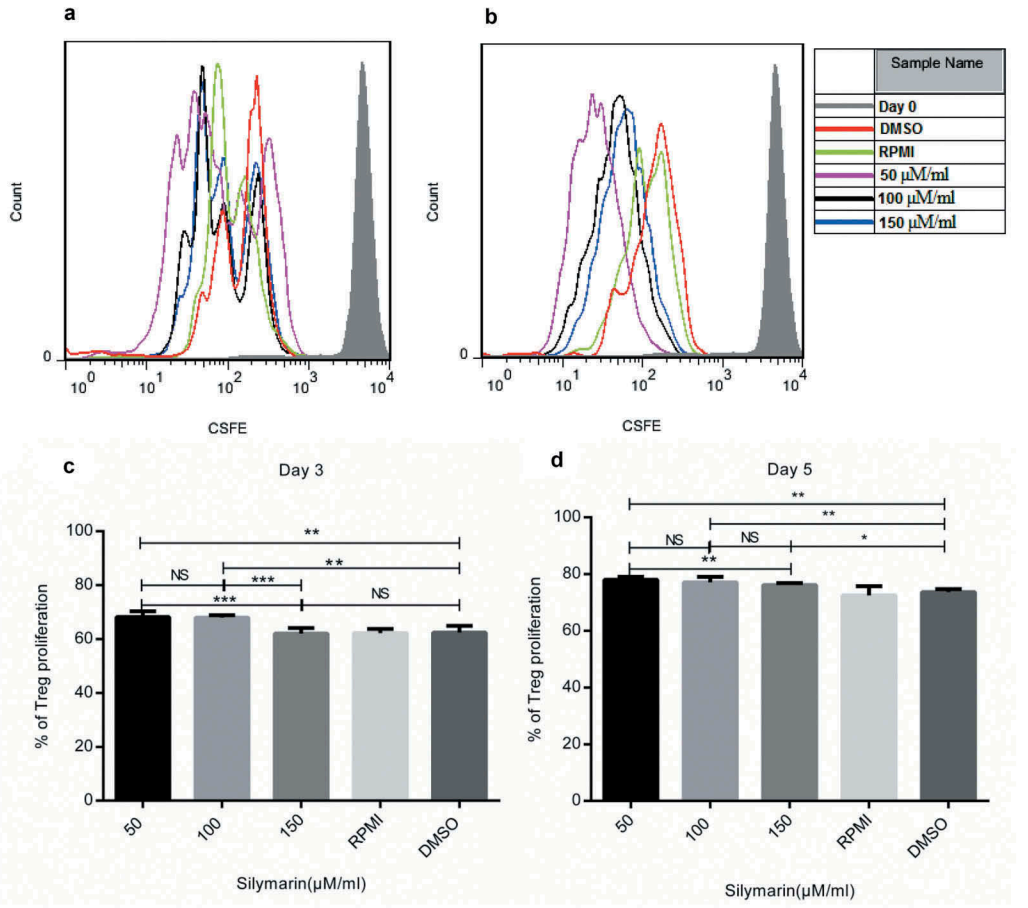

Figure 5. The effects of silymarin on the proliferation of Tregs. The expanded Tregs were labeled with CSFE and then cultured in the presence of different doses (50, 100, and 150 µM/ml) of silymarin for 3 (a) and 5 (b) days. Tregs which were cultured in the presecne or absence of DMSO and stimulated with with CD3/CD28 MACSibead particles were used as negative controls. The cells were harvested from 24-wall plates. The proliferation of Tregs was determined by flow cytometry and then analyzed (c and d). The depicted results are representative of five independent experiments. Each bar in C and D shows mean ± SEM. *$p < 0.05$, **$p < 0.01$.

Regarding that Tregs possess indispensible role in promoting immunologic tolerance to grafted organs; many studies have been carried out on the effects of immunosuppressive drugs on Tregs (Demirkiran et al., 2008; Joffre et al., 2008). In the current study, we investigated *in vitro* immunosuppressive effects of silymarin and CsA on the proliferation and cytokine production of Treg.

There is a noticeable inconsistency in literature regarding the activity of CsA on Tregs. Extensive data from the literature have shown that CsA has the ability to inhibit the development of Tregs and reduces the expression of their co-receptors and production of cytokines (Demirkiran et al., 2008; Heidt et al., 2010, Miroux et al., 2012a). It has been revealed that treatment of Tregs with different concentrations of CsA leads to a significant decrease in the number of these cells (Miroux et al., 2009, 2012a). On the contrary, other

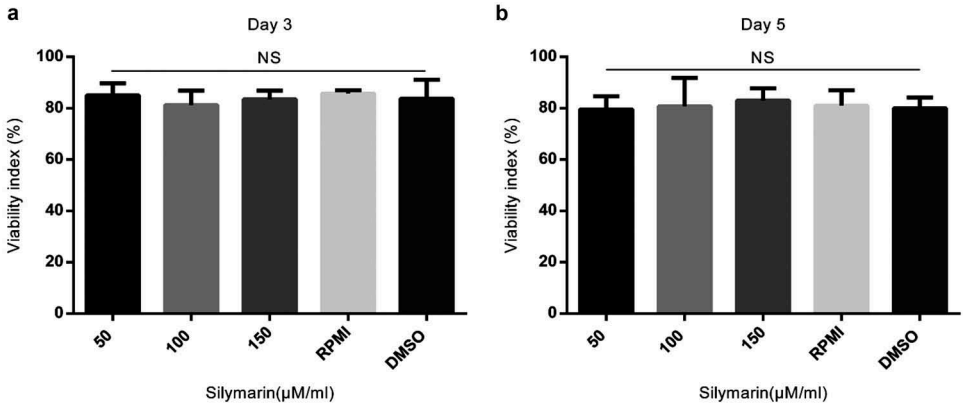

Figure 6. The effects of silymarin on Treg viability. Tregs were isolated from PBMCs and cultured in the presence or absence diferent doses (50, 100, and 150 μM/ml) of silymarin. The cells were stimulated with with CD3/CD28 MACSibead particles and incubated at 37°C with 5% CO2 for 3 and 5 days. The cells which were treated with RPMI and DMSO and stimulated with with CD3/CD28 MACSibead particles served as negative controls. After incubation, MTT assy was used to determine Treg viability. The depicted data are representative of 5 independent experiments. All data are shown as mean ± SD.

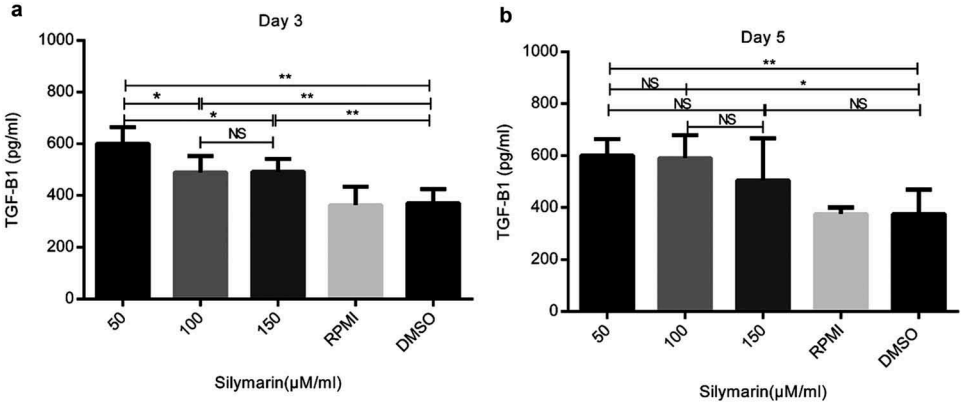

Figure 7. The effects of silymarin on TGF-β1 production of Tregs. The isolated Treg were cultured, stimulated with CD3/CD28 MACSiBead particles and incubated at 37°C with 5% CO2 for 5–8 days. The expanded Tregs were treated with different doses (50, 100, and 150 μM/ml) of silymarin. Stimulated Tregs which were cultured alone or in the presence of DMSO were used as negative controls. The culture supernatants of Treg cultured in the presence or absence of different doses of sylimarin were collected after 3 (a) and 5 (b) days. The measurement of TGF-β1 level was performed by ELISA. The depicted results are representative of 5 independent experiments. All data show mean ± SD. *$p < 0.05$, **$p < 0.01$.

reports have provided evidence regard to the stimulatory effects of CsA on Treg proliferation and function (Knol et al., 2012; Ruppert et al., 2015). Several studies have shown that CsA treatment increases the number of Tregs which is accompanied with the enhanced activity of Tregs and expression of Foxp3 (Meloni et al., 2006; Ruppert et al., 2015). Moreover, *in vitro* studies have demonstrated that CsA and rapamycin (RAPA) increase the suppressive activity of Tregs to the same extent (Fanigliulo et al., 2015). The observed

significant differences in the activities of CsA on Tregs may be explained by its concentration and/or treatment procedure. For instance, the proliferation of Tregs is inhibited by all concentrations (40 and 400 ng/ml) of CsA, while impaired suppressive activity of these cells is only mediated by low dose (40 ng/ml) of this drug (Miroux et al., 2009). Furthermore, in a study conducted by Kawai et al on rat cardiac allograft model has been revealed that administration of high-dose CsA (50 mg/kg) with pretransplant donor-specific blood transfusion (DSBT) inhibits Tregs proliferation. In contrast, low dose (10 mg/kg) of CsA enhances the generation of Tregs either in synergy with perioperative DSBT or by its own effect (Kawai et al., 2005). In line with this notion, we also observed that only 400 ng/ml dose of CsA significantly abrogated Treg proliferation after 3 days in a dose-dependent manner, but not low doses (100 and 200 ng/ml) of CsA. However, the results of 5-day cultures demonstrated that all concentrations of CsA had a significant suppressive activity on Tregs development, which may be due to longer incubation time. Another cause of discrepancy in CsA effects on Tregs may be associated with the administration of CsA with other drugs. In an *in vitro* study conducted by Miroux et al., it has been demonstrated that the combination of CsA with mycophenolic acid results in the maintenance of Treg activity. However, while used solely, it weakens the functions of Tregs (Miroux et al., 2012b).

In the next step, we investigated whether the reduced proliferation of Tregs was madiated by CsA-induced cell death in these cells. We observed that CsA failed to affect Treg viability, consistent with previous reports (Gao et al., 2007; Ruppert et al., 2015). Ruppert et al. indicated that Treg resists CsA-induced cell death via CD44-mediated signaling pathways which is an important receptor for Foxp3 expression and cell survival (Ruppert et al., 2015). These data suggest that inhibitory effect of CsA on Treg proliferation is not due to compromising cell viability.

Given that TGF-β1 is a key inhibitory cytokine of Tregs and plays a critical role in the suppressive functions of Tregs, including the inhibition of the proliferation and differentiation of the cells and induction of apoptosis (Liu et al., 2014). We assessed the level of TGF-β1 in the culture supernatant of Tregs treated with different concentrations of CsA. Similar to the activity of CsA on Treg viability, we found that CsA did not exert inhibitory effect on the TGF-β1 production of Tregs compared to control groups after 3 and 5 days. This result is consistent with other reports that indicate low doses (20 and 40 ng/ml) of CsA inhibit the functions of Tregs, while the suppressive activity of these cells are conserved when they are treated with high doses (100 and 400 ng/ml) of the drug (Miroux et al., 2009, 2012a). These observations provide evidence to reveal that CsA had not the ability to influence cytokine production of Treg, but it can restrain the generation of the cells in a dose-dependent manner. Therefore, although CsA is routinely used to prevent acute graft rejection and improve allograft survival (Hariharan et al., 2002), this immunosuppressive agent, especially at high doses, may block the potential induction of immune tolerance through the abrogation of Tregs generation.

Silymarin, a multicomponent extract from sylibum marianum, is well known for its immunosuppressive, hepatoprotective and anti-tumor properties (Zholobenko and Modriansky, 2014). A great number of *in vivo* and *in vitro* studies have indicated the effects of silymarin on CD4 + T cells (Gharagozloo et al., 2013a, Gharagozloo et al., 2013b). However, these effects on Tregs have not been reported yet. The results of this study for the first time revealed that silymarin increased the proliferation of Tregs after 3 and 5 days,

with the exception of 150 μM/ml dose on day 3. Interstingly, we observed that silymarin at low doses exreted more potently effects on Treg proliferation than higher doses. This observation may explain why 150 μM/ml dose of silymarin had not significant activity on Treg proliferation after 3 days. Although there is no evidence regard to silymarin effects on Tregs, our results were agreed with other reports showing the immunomodulatory effects of silymarin on CD4 + T cells *in vivo* (Gharagozloo et al., 2013b). However, our findings were in contrast with the mechanisms proposed for silymarin activities, including the prevention of the nuclear translocation of NF-κβ and reduction of PI3K/AKT/mTOR signaling pathway activation, which are the important roles on the activation and proliferation of T cells through inducing IL-2 production (Gharagozloo et al., 2013b). Regarding suppressive effects of silymarin on T cells (Gharagozloo et al., 2013b), the critical question regard to the results of this study is how silymarin induces the proliferation of Treg. It is thought that additional studies and more information are required to address this question and determine the mechanisms involved in silymarin effects on Tregs.

In the current study, we also evaluated the effect of silymarin on Treg viability. The results demonstrated that silymarin enhanced Treg development without affecting cell death. Our data were additional confirmation for other studies indicating silymarin did not induce apoptosis in activated T lymphocytes (Gharagozloo et al., 2013a, Gharagozloo et al., 2013b). To support this notion, Gharagozloo et al. showed that the viability of T cells was not influenced by various concentrations of silymarin (10, 50, and 100 μM) after 72 hr incubation (Gharagozloo et al., 2013a). Therefore, although there was a numerical increase in the viability of the silymarin-treated Tregs after 5 days of incubation, the results of the present study suggest that silymarin effect on Treg generation may be mediated by the mechanisms which are not involved in the cell viability.

In an attempt to discover the effect of silymarin on TGF-β1 production of Tregs, we found that the level of TGF-β1 in culture supernatant of all concentrations of silymarin was significantly higher than control groups, with the exception of 150 μM/ml of silymarin on day 5. In agreement with silymarin effect on Treg proliferation, these results revealed that silymarin at low doses had more efficient effects on cytokine production of Tregs. Thus, it is likely that disability of high-dose silymarin (150 μM/ml) in affecting the TGF-β1 level in 5-day culture supernatant of Treg was associated with different effects of silymarin in various doses and the instability and quick degradation of TGF-β1 due to longer incubation time. Other results of the current study showed a direct association between the Treg proliferation and TGF-β1 level in the cultures with silymarin, while this relationship was not observed in CsA cultures, perhaps due to the suppressive effects of CsA on Treg proliferation. This finding suggests that silymarin effect on the increased level of TGF-β1 in culture supernatants might be associated with the induction of silymarin-treated Tregs proliferation. Nevertheless, it is worthy that future studies will be designed to clarify whether silymarin effect on TGF-β1 production of Tregs is mediated by the induction of Treg proliferation or this herbal product has a direct effect on cytokine production.

In conclusion, the results of this study for the first time provide evidence to show that silymarin, unlike CsA, has useful effects on the proliferation and cytokine production of Tregs and may be a valuable drug in the treatment of various diseases, such as diseases with immune pathophysiology, and the reduction of the risk of immune-rejection of grafted organs. However, it should be noted that further studies are required to explain

the molecular mechanism involved in the immunomodulatory effects of silymarin on Tregs and how silymarin influences the functions of these cells.

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Conflict of interest

The authors have no conflicts of interest related to this study.

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