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Differential regulation of CD4$^+$ T cell subsets by Silymarin in vitro and in ovalbumin immunized mice

Haideh Namdari[1] · Maryam Izad[2] · Farhad Rezaei[3] · Zahra Amirghofran[1,4] ID

Abstract

CD4$^+$ T cell subsets including regulatory T cells (Tregs), Th1 and Th17 are critical for control and development of inflammation and autoimmunity. We investigated the in vitro and in vivo effects of silymarin, a well-known herbal medicine on differentiation and function of Tregs and Th1 and Th17 responses. For in vitro study, mice splenocytes treated with 20–30 μg/ml silymarin were evaluated for gene expressions of specific transcription factors and cytokines of CD4$^+$ T cell subsets using real-time PCR. Induction of Treg cell development in the presence of silymarin was performed on isolated naïve CD4$^+$ T cells. Effect of silymarin-induced Tregs on T cell suppression was determined by CFSE labeling method. Results of this part showed that silymarin significantly decreased IFNγ, RORγt and IL-17 gene expressions and upregulated Foxp3, TGF-β and IL-10 mRNA. More silymarin-enhanced naïve CD4$^+$ T cells differentiated to Tregs (67%) than the control (47%). Silymarin-induced Tregs reduced proliferation of naïve activated T cells (<50%). For in vivo study, mice were immunized with ovalbumin (Ova) on days 1 and 14. Silymarin (100 mg/Kg) was intraperitoneally administered two days before the first Ova challenge followed by on every day for two weeks. Splenocytes were then isolated for assessment of CD4$^+$ T cell subsets and ex vivo analysis using flow cytometry. Treatment of Ova-immunized mice with silymarin increased Tregs (11.24 ± 1.2%, $p < 0.01$(but decreased Th1 (1.72 ± 0.4%, $p < 0.001$) and Th17 (1.07 ± 0.04%, $p < 0.001$) cells. Ex vivo Ova challenge of splenocytes from Ova-immunized mice treated with silymarin decreased proliferation of splenocytes, IFNγ (2.76% of control) and IL-17 (<8%) along with increased TGF-β (59.7) expressions in CD4$^+$T-bet$^+$, CD4$^+$RORγt$^+$ and CD4$^+$Foxp3$^+$ T cells, respectively. In conclusion, silymarin promoted Treg differentiation and function and decreased Th1 and Th17 cells. Silymarin may differentially regulate CD4$^+$ T cell responses which can provide potential benefits for its use as treatment of immune-related diseases.

Keywords Silymarin · Regulatory T cells · Th1 · Th17 · Ovalbumin

Background

T helper cells (Th) as the most important cells in cellular immunity are essential for nearly all aquired immune

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responses. Th1 and Th17 are two main subsets of Th cells that have critical roles in inflammatory and cellular immune responses [1] .Th1 cells express T-bet transcription factor and predominantly secrete interferon (IFN)γ. They are important for host protection against intracellular pathogens and induction of delayed type hypersensitivity responses [2]. Overactivation of Th1 responses against self-antigens, can result in the development of autoimmunity. Multiple lines of evidence implicate Th1 cells as the main effector T cells accountable for induction of experimental autoimmune encephalomyelitis and multiple sclerosis [3]. Th17 cells produce interleukin (IL)-17 and express the transcription factor retinoic acid receptor related orphan receptor (ROR)γt. Th17 responses are essential for host protection against fungi and extracellular pathogens [4]. However, immunopathology can result from prolonged and excessive responses [5]. These cells, due to the essential role of IL-17 in tissue inflammation, are particularly suited for the promotion of autoimmunity [6].

Regulatory T cells (Tregs) are a subpopulation of CD4^{+}T cells with a distinct function and development compared to conventional Th cells. Tregs have a critical role in maintenance of immune self-tolerance and inflammation induced by microbial and environmental agents [7]. The balance between Th17 and Th1 responses with Tregs is essential for immune homeostasis. Numerous inflammatory conditions, such as autoimmunity, allograft rejection and tumorigenesis are the result of imbalances between Th17 and Th1 responses with Tregs [8]. There is a reduction in the number and activity of Tregs during various inflammatory and autoimmune diseases [9]. Due to the pivotal role of Treg cells in control of immune responses, differentiation modulation of these cells has been suggested as an approach for treatment and limitation of autoimmune and chronic inflammatory diseases [10]. Tregs can be induced from naive T cells in the presence of transforming growth factor (TGF)-β and IL-2 [11]. Forkhead box P3 (Foxp3) is the master regulator of Treg differentiation and function. A deficiency in Foxp3 can lead to systemic autoimmunity [12].

Silymarin is an active ingredient derived from the seeds of the milk thistle plant *Silybum marianum* L. Gaertn. (Asteraceae). It consists of one flavonoid (taxifolin) and a family of flavolignans including silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin and also contains a small amount of fatty acids and polyphenolic compounds [13]. Silymarin has anti-inflammatory, anti-fibrotic and antioxidant properties, therefore, this extract is used to reduce inflammatory reactions and fibrogenesis in chronic liver diseases [14–17]. In several clinical trials the protective effects of silymarin in patients with cirrhosis as well as cancer, hepatitis C, diabetes and hypercholesterolemia have been shown [18].

Previous studies investigated the immunomodulatory function of silymarin. The results indicated that this extract prevented the production of proinflammatory cytokines from CD4^{+} T cells and attenuated the proliferation of these cells in response to specific antigens such as those of hepatitis C virus, candida and tetanus, mitogenic stimulation and anti-CD3 antibody [19, 20]. Silibinin, the main compound of silymarin, inhibited the expression levels of the cytokines TNFα, IFNγ, IL-4, IL-2 and inducible nitric oxide synthase (iNOS) in the liver [21]. Mice received intraperitoneal injections of silymarin had evidence of suppressed T cell function [13]. Almasi et al. in their study showed that silymarin inhibited the proliferation of mitogen-stimulated T cells. The authors suggested that silymarin had significantly stronger suppressive activity on T cell proliferation compared to FK506 and rapamycin [22]. Gharagozloo et al. reported that silymarin repressed in vitro cell proliferation by inducing arrest in the G1 phase of the cell cycle and inhibited mTOR signaling pathway in human stimulated T cells. Their research showed the capability of silymarin to reduce T lymphocyte

activation and proliferation in mice by inhibition of nuclear factor (NF)-κB activation and preventing its translocation to the nucleus [23].

These data have indicated that silymarin has various significant anti-inflammatory and immunomodulatory effects which are especially noted by inhibition of CD4^{+} T cell proliferation and function. However, to the best of our knowledge there is a lack of adequate data on the influence of silymarin on T cell subsets particularly Treg cells as the central cells of immunoregulation and Th17 cells. Th17 cells similar to Th1 cells are the major cells that contribute to T cell-mediated inflammation and autoimmune disease. Therefore, in the present study we aim to investigate the in vivo and in vitro effects of silymarin on induction and differentiation of Treg cells. Inhibition of immune responses due to suppressive activities attributed *to* Treg cells is closely related to the presence of the inflammatory T cell subsets, Th1 and Th17 and their balance with Treg cells. Therefore, we have also assessed the effects of silymarin on Th1 and Th17 responses.

Materials and methods

Reagents

Dimethyl sulfoxide (DMSO), trypan blue, propidium iodide, Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium and silymarin were obtained from Sigma St. Louis, MO. The silymarin was free of endotoxin as described before [24]. Fetal bovine serum (FBS) was obtained from Roche (Germany), 5-bromo-20-deoxy-uridine (BrdU) kit from Gibco (Ashland, KY) and Lymphodex from Inno-Train Diagnostic (Kornberg, Germany). Phosphate-buffered saline (PBS) was purchased from Lonza (Switzerland) and Concanavalin A (Con-A) from Fluka (Germany). Anti-CD3 and anti-CD28 monoclonal antibodies (mAb)s were purchased from Becton Dickinson (BD) Biosciences (Pharmingen, San Diego, CA). RNXTM-plus solution kit for RNA extraction was obtained from Sinagen (Tehran, Iran). High-capacity cDNA reverse transcription kit was purchased from Applied Biosystems (ABI, Foster City, CA) and SYBR® Green qPCR SuperMix-UDG with ROX from TaKaRa (Shuzo, Kyoto, Japan). Reagents used for flow cytometry including PE-conjugated anti-Foxp3 antibody, FITC-conjugated anti-CD4 antibody, APC-conjugated anti-CD25 antibody, APC-labeled anti-TGFβ, anti-IFNγ, anti-IL-17 antibodies and fixation/permeabilization kit were purchased from BD. Mouse naïve CD4^{+} T cell isolation kit was obtained from Miltenyi Biotec, Germany. Serum free culture medium X-vivo-15 from Invitrogen, MO. TGFβ, IL-2 and CellTrace CFSE cell proliferation kit from Life Technologies, MO. Freund's complete adjuvant (FCA) and ovalbumin (Ova) were purchased from Sigma. Freund's incomplete adjuvant (FIA)

was obtained from BioGene (Mashhad, Iran). Other chemicals and solvents were of reagent grade and available.

Animals

BALB/c female mice at 6–8 weeks of age (25–30 g body weight) were obtained from Center for Comparative and Experimental Medicine of Shiraz University of Medical Sciences. Mice were kept under standard conditions and were provided standard laboratory chow and drinking water ad libitum. A group of mice were used for studying the in vitro effects of silymarin on CD4+ T cell subsets and induction of Treg cells. Other groups of mice were used for studying the in vivo and ex vivo effects of silymarin on CD4+ T cell subsets following immunization with an antigen (Ova). The study protocol was approved by the Ethics Committee, Shiraz University of Medical Sciences and all experiments were performed in accordance with the National Institute of Health guide for the care and use of laboratory animals.

In vitro splenocyte proliferation assay

After the mice sacrificed, spleens were removed and completely dissociated. Mononuclear cells were then separated by centrifugation over Lymphodex cell separation medium and after washing twice with PBS they were counted using 0.4% trypan blue stain. Cells were seeded into 96-well culture microplates (10^5 cells/well/100 μl) to determine the effects of silymarin on splenocytes proliferation using BrdU incorporation assay as previously described [17] . Briefly, cells were treated with various concentrations of the silymarin (1–50 μg/ml) in the presence of Con-A (2 μg/ml) for 72 h. The positive control was cells treated only with Con-A and the negative cells were those with no treatment. After labeling with BrdU, DNA was denatured and the cells were incubated with peroxidase-conjugated anti-BrdU mAb for detecting incorporated BrdU. The substrate solution was added and then the optical density (OD) of the samples measured in a microplate reader at 450 nm and a reference wavelength of 690 nm. The OD of cells treated with silymarin to the OD of positive control was determined and considered as the proliferation index. Experiments were done in triplicate and each experiment was performed at least three times.

Cell viability assay

To determine the non-cytotoxic concentrations of silymarin, the viability of silymarin-treated splenocytes was evaluated by propidium iodide staining using flow cytometry. As mentioned above, stimulated cells were seeded into 96-well culture microplates and treated with various concentrations of silymarin. Cisplatin, a cytotoxic drug, (50 μg/ml) was added in a triplicate wells as positive control. Negative control was cells treated with Con-A containing DMSO (0.05%). Cells were incubated at 37 °C in 5% CO_2 incubator with humidified atmosphere for 72 h. After incubation time, propidium iodide assay was performed according to the method previously described with some modifications [25]. Briefly, cells were collected and after centrifugation to remove media, they were resuspended in PBS containing 5 μg/ml propidium iodide. During 5 min, the fluorescence intensity was analyzed using a FACSCalibur flow cytometer (BD Biosciences). Cell death was expressed as the percentage of high red fluorescence for each treatment versus the negative control. Data analysis was performed using the FlowJo software version 7.6.5 (TreeStar Inc., Ashland, OR).

In vitro treatment of cells with silymarin for studying gene expressions using real-time PCR

The effects of silymarin on mRNA expression of T cell specific transcription factors and cytokines was examined by real time-PCR. Splenocytes were cultured at a density of 1×10^6 cells/well/ml in 24-well culture microplates coated with anti-CD3 mAb (2.5 μg/ml). After addition of anti-CD28 mAb (2 μg/ml) and non-cytotoxic concentrations of silymarin (20 and 30 μg/ml), cells were incubated at 37 °C in a humidified incubator with 5% CO_2. Cells treated only with antibodies without silymarin were considered as positive control and cells without any treatment, neither antibodies nor silymarin, were considered as negative control. DMSO as the solvent control was added to all control wells. After 72 h, cells were washed with cold PBS and then RNA was extracted using an RNA extraction kit according to the manufacturer's instructions. For determination of the concentration and quality of the extracted RNA, the relative absorbance of OD260/OD280 was measured and the banding pattern of RNA in agarose gel electrophoresis was visualized under UV illumination. Then, cDNA synthesis was carried out using a High-capacity cDNA reverse transcription kit with the use of random primers. Primer design was carried out using Primer Express ™ software (Applied Biosystems, Foster City, CA). The sequences of primers are shown in Table 1.

The real time-PCR was performed in duplicate with the SYBR® Green qPCR SuperMix-UDG with ROX on ABI thermocycler (Foster City, CA). Reaction mixes were prepared with 200 nM of each primer pair and then cDNA (10 ng) was added. Each reaction consisted of 1 cycle of 95 °C for 30 s, followed by 40 cycles of 95 C for 5 s, annealing temperature (specific for each primer) for 18 s and 72 °C for 30 s. Results of target mRNA levels were normalized against GAPDH mRNA in each sample and shown as the relative fold change (RFC) to positive control.

Table 1 Primer sequences for real-time PCR

Gene	Accession Number	Primer sequences	Annealing temperature (°C)
GAPDH	NM_001289726	Forward 5′-CGGTGTGAACGGATTTGGC-3′ Reverse 5′-GTGAGTGGAGTCATACTGGAAC-3′	55.6
IFN-γ	NM_008337	Forward 5′- GAGGAACTGGCAAAAGGATGGT −3′ Reverse 5′- CGCTTATGTTGTTGCTGATGGC -3′	59.2
IL-10	NM_010548	Forward 5′- GCAGGACTTTAAGGGTTACTTGGG-3′ Reverse 5′- GATTTCTGGGCCATGCTTCTC -3′	59.8
IL-17A	NM_010552	Forward 5′- AACACTGAGGCCAAGGACTTC -3′ Reverse 5′- GTCTTCATTGCGGTGGAGAGT −3′	62.4
TGF-β	NM_011577	Forward 5′- GCAACAACGCCATCTAT-3′ Reverse 5′- AAGGTAACGCCAGGAAT −3′	58.6
Tbet	NM_019507	Forward 5′- TAAGCAAGGACGGCGAAT −3′ Reverse 5′- GGTGGACATATAAGCGGTTC -3′	63.3
RORγt	NM_001293734	Forward 5′- AAGAGAAGAGGAGAGTGGAA −3′ Reverse 5′- GTGGAGGTGCTGGAAGAT −3′	60.7
Foxp3	NM_001199347	Forward 5′- AATAGTTCCTTCCCAGAG −3′ Reverse 5′- GATTTCATTGAGTGTCCT −3′	58.3

CD4+ naïve T cells isolation and in vitro induction of Treg cells differentiation

Splenocytes were washed three times with PBS and naïve CD4+ T cells were separated using mouse naïve CD4+ T cell isolation kit according to the manufacturer's instruction. Briefly, CD44+ activated/memory T cells and non-CD4+ T cells were depleted by indirect magnetic labeling using a cocktail of biotin-conjugated antibodies against various markers including CD8a, CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b (DX5), CD105, anti-MHC class II, Ter-119 and TCRγ/δ followed by addition of anti-biotin microbeads. Isolated cells (1×10^6 cells/well in x-vivo15 culture medium) were activated with 5 μg/ml plate-bound anti-CD3, 2 μg/ml anti-CD28, 2 ng/ml TGFβ and 100 Unit/ml IL-2 in the presence of 20 and 30 μg/ml of silymarin in 24-well culture plates. Cells cultured at the same condition without adding silymarin considered as untreated control. After 4 days of incubation, a part of cells were collected for in vitro suppression assay and the rest were stained with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 antibodies. After washing, the cells were fixed and made permeable using permeabilization buffer and then stained with PE-labeled anti-Foxp3 antibody. Samples were run in a FACSCalibur flow cytometer and analyzed using FlowJo software.

In vitro suppression assay

Naïve CD4+CD25− T cells (T conventional/Tconv cells) were isolated, labeled with 2.5 μM CFSE and stimulated with anti-CD3/anti-CD28 antibodies (STconv cells). These cells were co-cultured with Tregs obtained in the presence of silymarin collected from previous experiment at equal ratio (1×10^5 cells/100 μl) in 96-well round bottom plates. Controls were STconv cells co-cultured with Tregs obtained in the absence of silymarin (untreated) and STconv cells alone. After 72 h, the proliferation suppression was assessed using flow cytometry based on CFSE dilution in gated STconv cells. Percentage of suppression (S) was calculated as follows [26]: $S = (a\text{-}b)/a \times 100$ where a is the percentage of proliferation in the absence of Tregs and b is the percentage of proliferation in the presence of Tregs.

Ova-immunization

To study the influence of silymarin on in vivo CD4+ T cell subsets following a specific immune response, we used Ova-immunized mice model. A total of 21 mice were divided into three groups, each consisting of seven animals. Ova (2 mg/ml in normal saline) was emulsified in an equal volume of FCA and then 0.1 ml of emulsion was injected subcutaneously (sc) into the shaved backs of mice in two of the groups (Ova-immunized mice). The third group was injected (sc) only with normal saline and considered as non-immunized control. A group of Ova-immunized mice were treated with intraperitoneal (ip) injections of silymarin (100 mg/kg) Ova and the other group received olive oil as vehicle (Ova-only mice) on each of two days before the first Ova challenge, followed by on every day for two weeks. The non-immunized control group received vehicle injections in parallel with the test groups. A boosting sc injection of Ova in FIA (at the same concentration as in the first challenge) was given on day 14. Two days later, mice were euthanized by cervical dislocation and their spleen removed.

Flow cytometry for T cell subsets, cytokine secretion and cell proliferation in Ova-immunized mice and in ex vivo splenocyte culture

Splenocytes from all mice were isolated. A part of splenocytes were directly examined for the percentage of CD4$^+$ T cell subsets in vivo. Cells were first stained with FITC-conjugated anti-CD4 antibody and then fixed and made permeable using permeabilization buffer and stained with PE-labelled anti-Foxp3, anti-T-bet and anti-RORγt antibodies.

Another part of the isolated splenocytes were cultured in the presence of Ova (100 μg/ml) to study intracellular cytokine content in different CD4$^+$ T cell subsets after antigen challenge ex vivo. During the first hour of culture, brefeldin A was added and then after 24 h, cells were collected and stained with FITC-conjugated anti-CD4. The cells were fixed and made permeable using permeabilization buffer and then stained with PE- labelled anti-Foxp3, anti-T-bet, anti-RORγt and APC-labeled anti-TGFβ, anti-IFNγ and anti-IL-17 antibodies.

The rest of isolated splenocytes (5×10^6 cells) were labeled with CFSE and cultured with or without Ova (100 μg/ml) in 96-well plates for 72 h. The proliferation of cells was determined using flow cytometry analysis for dilution of CFSE as mentioned before. Cells cultured in the absence of Ova were used as internal control.

Statistical analysis

All data were expressed as mean ± standard error (SE) of at least three independent experiments. The differences between treatments for different tests were analyzed using Student's t test and one-way analysis of variance (ANOVA) at significance level of $p < 0.05$, using SPSS (Abaus Concepts, Berkeley, CA) and GraphPad Prism 5 (San Diego, CA) softwares.

Results

Effects of silymarin on in vitro splenocytes proliferation and their viability

We used the BrdU incorporation assay to analyze the in vitro effects of silymarin on proliferation of stimulated mice splenocytes. The results showed that various concentrations (10–50 μg/ml) of silymarin reduced splenocyte proliferation (Fig. S1). Propidium iodide staining with flow cytometry analysis showed that silymarin at concentrations less than 50 μg/ml had no cytotoxic effects (Fig. S2). Therefore, we selected the 20 and 30 μg/ml concentrations of silymarin for the following experiments.

Effects of silymarin on in vitro gene expressions of T cell transcription factors and cytokines

We performed real-time PCR analysis of the mRNA levels of T-bet, RORγt and Foxp3 transcription factors in silymarin treated-splenocytes in vitro. As shown in Fig. 1a-c, the mRNA level of the T-bet transcription factor at the higher concentration of silymarin showed a non-significant decrease to approximately half of the control (0.54 ± 0.28 RFC). Treatment of cells with 20 μg/ml of silymarin significantly decreased RORγt gene expression (0.60 ± 0.1 RFC, $p < 0.01$). In contrast, silymarin significantly increased Foxp3 mRNA levels at 20 μg/ml (1.20 ± 0.02 RFC, $p < 0.01$) and 30 μg/ml (1.45 ± 0.11 RFC, $p < 0.05$). The influence of silymarin on gene expressions of T cell cytokines showed that 30 μg/ml of silymarin decreased IFNγ (0.47 ± 0.03 RFC, $p < 0.01$) and IL-17 (0.37 ± 0.15 RFC, $p < 0.01$) gene expressions (Fig. 1d, e). We observed significant upregulation in IL-10 gene expression at 30 μg/ml (1.30 ± 0.3 RFC, $p < 0.001$) of silymarin (Fig. 1f). Treatment of splenocytes with 20 μg/ml (1.1 ± 0.009 RFC, p < 0.001) and 30 μg/ml (1.35 ± 0.03 RFC, p < 0.001) of silymarin increased TGF-β expression (Fig. 1g).

### Effects of silymarin on in vitro differentiation of Tregs

We isolated naïve CD4$^+$ T cells from mice splenocytes for in vitro differentiation of Tregs. Figure 2a shows the purity of these cells had greater than 93% purity after they were stained by labeled anti-CD4/anti-CD25 antibodies. The isolated cells were cultured in the presence of anti-CD3/anti-CD28 antibodies, TGF-β and IL-2 in order to allow for differentiation into Treg cells in the presence of silymarin. Figure 2b shows a representative flow cytometry analysis from the three independent experiments. We found that 20 μg/ml (66.3 ± 2.45%, $p < 0.01$) and 30 μg/ml (67.9 ± 5.28%, p < 0.01) of silymarin increased the numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells compared to the untreated control (47.4 ± 2.3%) as shown in Fig. 2ca. These data showed the ability of silymarin to potentiate the differentiation of naive CD4$^+$CD25$^-$ T cells to CD4$^+$CD25$^+$Foxp3$^+$ Tregs. Analysis of the mean fluorescence intensity (MFI) of Foxp3 expression revealed that Tregs generated in the presence of silymarin expressed higher amounts of Foxp3 protein on a per-cell basis compared to those from untreated cultures ($p < 0.001$; Fig. 2cb).

Effects of silymarin-induced Tregs on in vitro T cell suppression

We sought to determine the possible suppressive function of the Tregs obtained in the presence of silymarin. CFSE-labeled Tconv cells were stimulated with anti-CD3/anti-CD28 antibodies and subsequently co-cultured with silymarin-induced

Fig. 1 In vitro effects of silymarin on the gene expressions of T cell specific transcription factors (**a-c**) and cytokines (**d-g**) in activated splenocytes by real-time PCR. Mouse splenocytes were stimulated with anti-CD3/anti-CD28 antibodies and treated with 20 and 30 μg/ml of silymarin for 72 h. Antibody-only treated cells considered as positive control (C+) and cells with no treatments as negative control (C-). Control wells had DMSO as the solvent (0.05%). Total RNA was extracted from the cells and reverse-transcribed to cDNA, after which real time-PCR was performed. Values represent mean ± standard error of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to positive control. RFC, relative fold change
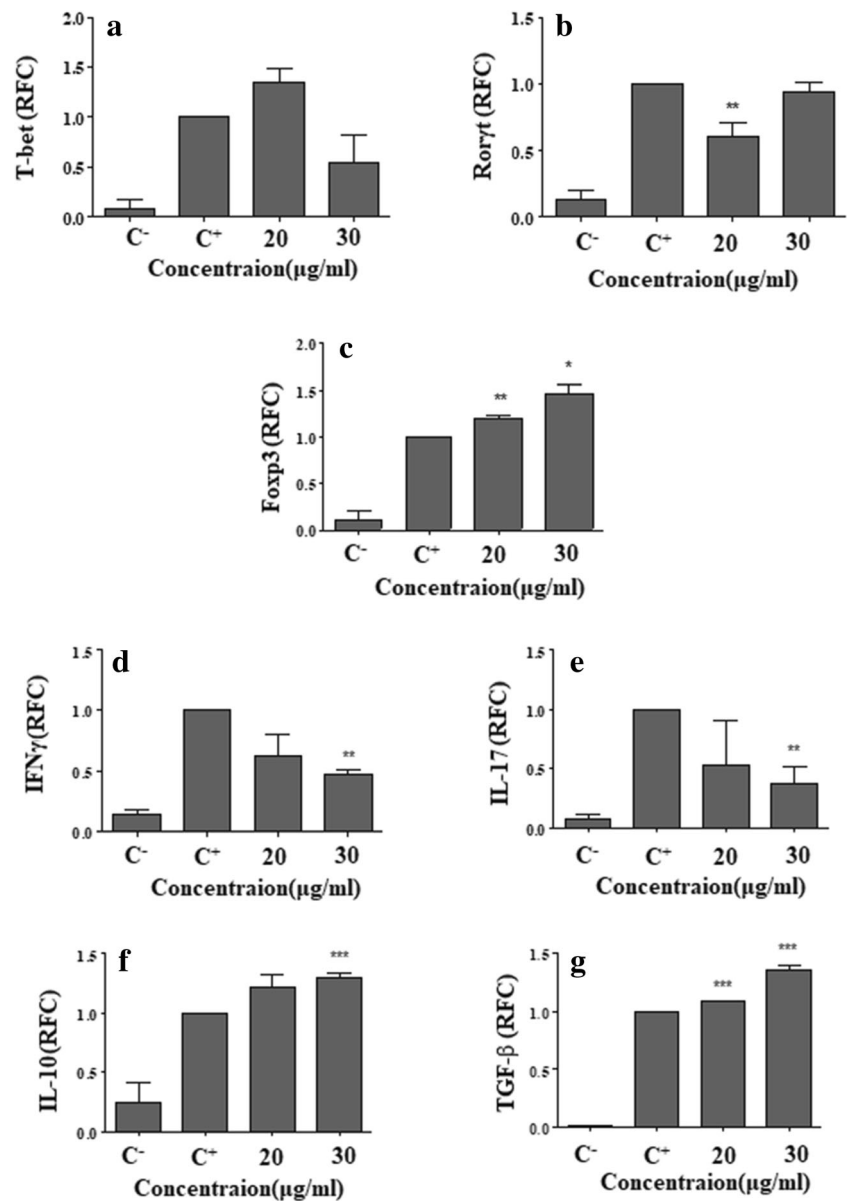
Treg cells. Figure 3(a-c) show a representative flow cytometry analysis of the data from three independent experiments. As shown in Fig. 3d, the difference between the percentage of proliferation suppression in untreated ($22.09 \pm 6.9\%$) and silymarin 20 μg/ml ($46.12 \pm 2.79\%$) and 30 μg/ml ($46.19 \pm 4.61\%$) treated–Tregs was statistically significant ($p < 0.05$).

## Effects of silymarin on CD4⁺ T cell subsets in Ova-immunized mice

Splenocytes from non-immunized and ovalbumin (Ova)-immunized mice were directly stained for CD4, T-bet, RORγt and Foxp3 positive cells. The Fig. 4(a-c) indicate that Ova immunization in mice resulted in significant increases in the number of different T cell subsets compared to the non-immunized mice. In Ova-immunized mice, silymarin

treatment increased the percentage of CD4⁺Foxp3⁺ Tregs to $11.24 \pm 1.2\%$ ($p < 0.01$), whereas there was a decrease in the CD4⁺T-bet⁺ Th1 ($1.72 \pm 0.4\%$, $p < 0.001$) and CD4⁺RORγt⁺ Th17 (1.07 ± 0.04, $p < 0.001$) subpopulations.

Effects of silymarin on cytokine secretion by different CD4⁺ T cell subsets in ex vivo Ova challenge of splenocytes

Spleen cells from Ova-immunized mice cultured in the presence of Ova were examined for the percentages of Th1, Th17 and Treg subpopulations and the expressions of related cytokines. Figure 5(a-c) show that splenocytes from Ova-immunized mice cultured in the presence of Ova increased the numbers of CD4⁺T-bet⁺, CD4⁺RORγt⁺ and CD4⁺Foxp3⁺ T cells compared to non-immunized mice.

Fig. 2 In vitro effects of silymarin on Tregs differentiation by Foxp3 staining of CD4$^+$CD25$^+$ cells using flow cytometry. Naïve CD4$^+$ T cells were isolated from mouse splenocytes and stimulated with anti-CD3/anti-CD28 antibodies, TGFβ and IL-2 cytokines in the presence of 20 and 30 μg/ml of the silymarin for 4 days. Control was stimulated cells without silymarin containing DMSO (0.05%) (untreated). **a** Isolation of highly pure naive CD4$^+$CD25$^-$ T cells is shown after immune staining with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 antibodies. **b** Percentage of Foxp3$^+$ Treg cells determined by gating of Foxp3$^+$ cells in CD4$^+$CD25$^+$ population. Flow cytometry dot plots and histograms are representative of three experiments. **c** Values shown are mean ± standard error of the number of CD4$^+$CD25$^+$Foxp3$^+$ Tregs (a) and the mean fluorescent intensity (MFI) of Foxp3 expression (b) in cultures treated with silymarin compared to untreated control (**c**). **$P < 0.01$, ***$P < 0.001$
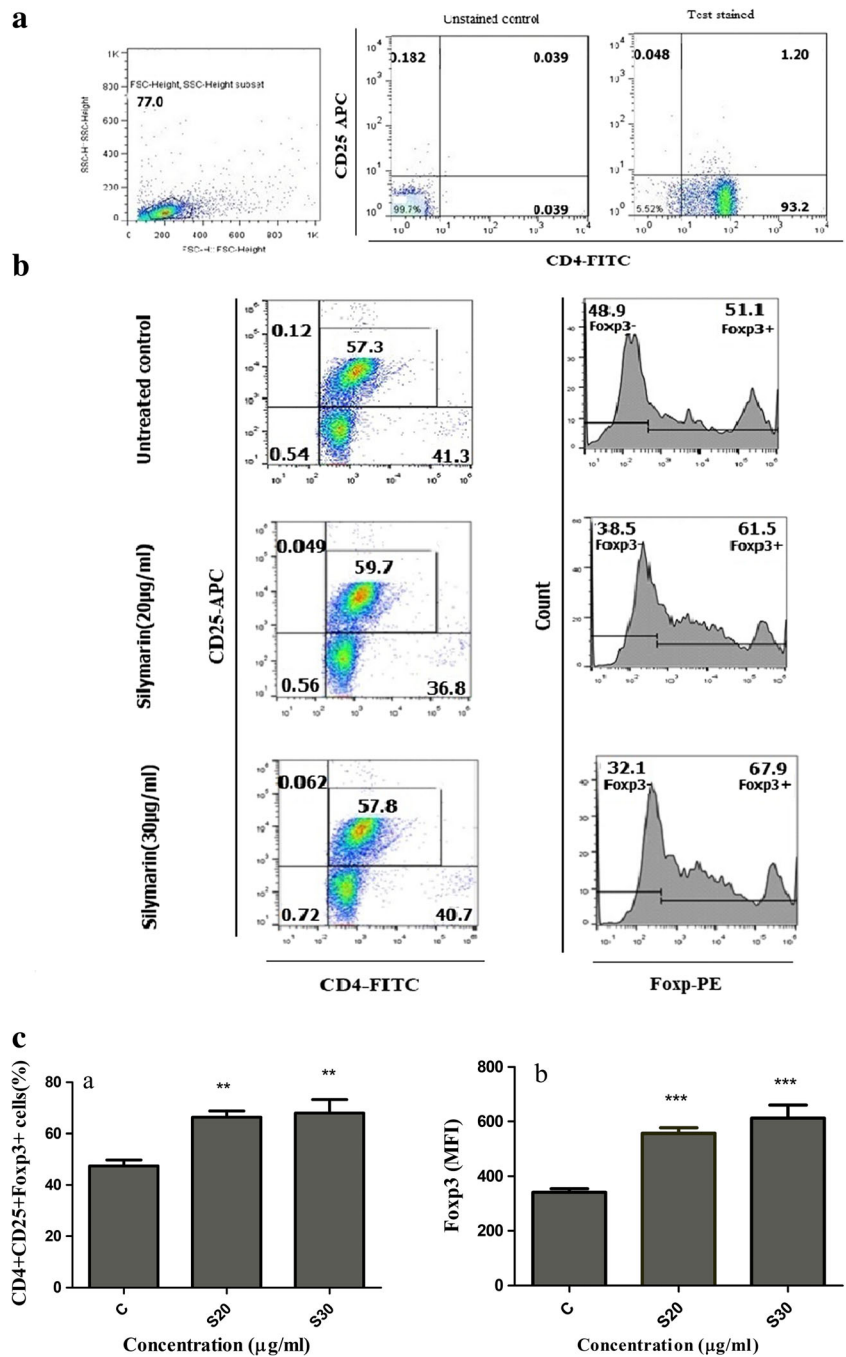
Similar to the results for direct analysis of the splenocytes for the CD4$^+$ T cell subsets, we found that Ova challenge of the splenocytes isolated from silymarin-treated mice reduced Th1 and Th17 cells and increased Treg cells [Fig. 5(a-c)]. Evaluation of cytokine levels in each of these subsets showed that in the silymarin-treated group, there was less IFNγ expression in CD4$^+$T-bet$^+$ T cells (2.76 ± 0.36%, $p < 0.01$) and IL-17 expression in CD4$^+$RORγt$^+$ T cells (9.8 ± 1.24%,

$p < 0.05$) compared to cells from the Ova-only group (Fig. 5d-e). We observed less intense IFNγ and IL-17 expressions in parallel with reduced percentages [Fig. 5(g-h)]. Silymarin increased TGF-β protein expression in the CD4$^+$Foxp3$^+$ subpopulation to 59.7 ± 6.12% (p < 0.01) relative to the level seen in cells from the Ova-only group (18.9 ± 2.5%; Fig. 5f). MFI of TGF-β expression also increased in the presence of silymarin (p < 0.05; Fig. 5i). Figure 6 shows representative
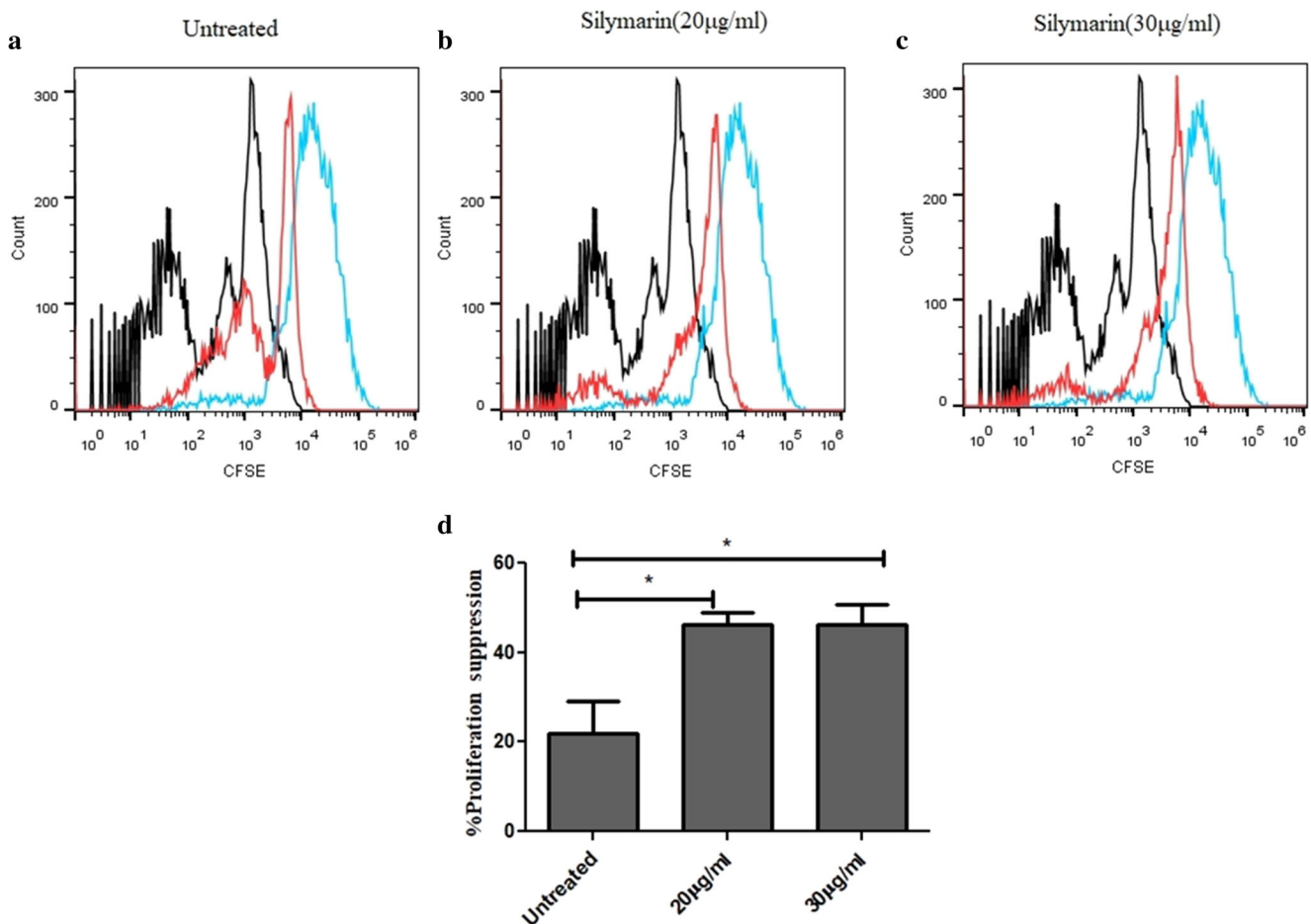
Fig. 3 In vitro effect of silymarin-induced Tregs on T cell proliferation. CFSE-labeled naïve CD4$^+$CD25$^-$ T cells stimulated with anti-CD3/anti CD28 antibodies (STconv cells) were co-cultured with Tregs obtained in the presence of silymarin (20 and 30 μg/ml) and those obtained in the absence of silymarin (untreated) for 72 h. The suppression of cell proliferation was assessed using flow cytometry by measuring CFSE dilution in gated STconv cells. a–c overlay of day 0 of CFSE labeling (blue line) with STconv cells (black line) and silymarin-treated and untreated Tregs (red line) at day 3. d bar graph shows mean ± standard error of suppression of STconv cells proliferation in silymarin-treated and untreated groups. *$p < 0.05$ compared to STconv cells

flow cytometry data in dot plots and histograms which show the percentages of T cell subsets (CD4$^+$T-bet$^+$, CD4$^+$RORγt$^+$ and CD4$^+$Foxp3$^+$ T cells) and expression of IFNγ, IL-17 and TGF-β cytokines in these cells, respectively.

Effects of silymarin on cell proliferation in ex vivo Ova challenge of splenocytes

Proliferation of CFSE-labeled spleen cells from the three groups of mice cultured in the presence of Ova was measured after 72 h. As seen in Fig. 7, splenocytes isolated from Ova-immunized mice ($67.9 \pm 5.4\%$, $p < 0.01$) had greater proliferation compared to those from non-immunized animals ($14.03 \pm 3.13\%$). Treatment of Ova-immunized mice with silymarin resulted in decreased proliferation of splenocytes ($32.2 \pm 2.3\%$, $p < 0.01$), which showed the inhibitory effect of silymarin on antigen specific immune responses in mice.

Discussion

Silymarin, the seed extract of milk thistle (*Silybum marianum*) is a mixture of seven flavonolignans and one flavonoid that has antioxidant, hepatoprotective and anti-inflammatory effects on various diseases. Several studies have reported the immunomodulatory activity of this component of *Silybum marianum*. Inhibitory effects of silymarin on maturation of dendritic cells, decreased proliferation of T cells and reduced levels of various proinflammatory cytokines have been reported [19–21]. In the present study, we aimed to evaluate the in vitro and in vivo effects of silymarin on Tregs and inflammatory Th subsets (Th1 and Th17) to elucidate the possibility of Treg involvement in the inhibitory effects of silymarin. We first examined the effect of various concentrations of this extract on mice splenocyte proliferation and found that the 20 μg/ml and higher concentrations of silymarin significantly reduced cell proliferation. In an attempt to
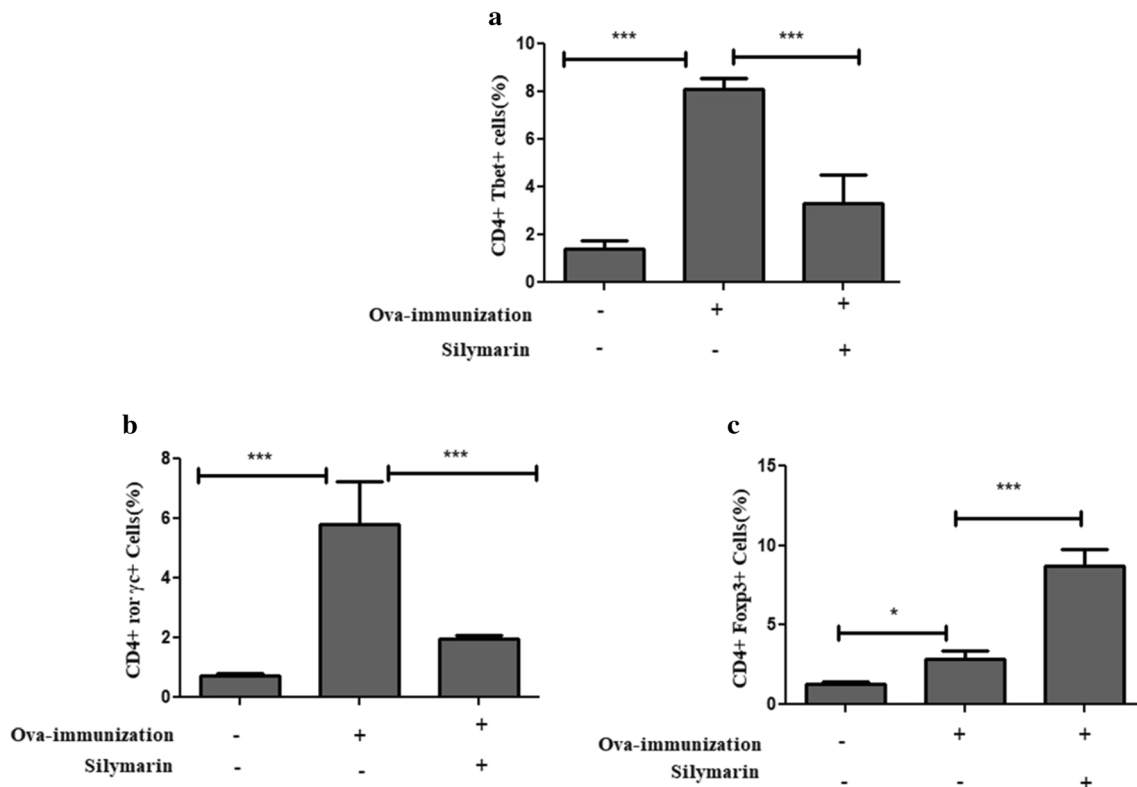
Fig. 4 Effect of silymarin on CD4$^+$ T cell subsets in Ova-immunized mice. Splenocytes from non-immunized and Ova-immunized mice treated and untreated with silymarin were isolated and stained for CD4, T-bet, RORγt and Foxp3 markers using flow cytometry to determine the percentages of Th1, Th17 and Treg cells. Controls were cells from non- immunized mice and those from untreated Ova-immunized mice (Ova-only). **a**–**c** Bars shown are mean ± standard error of the number of Th1 (CD4$^+$T-bet$^+$), Th17 (CD4$^+$RORγt$^+$) and Treg (CD4$^+$Foxp3$^+$) cells in gated spenocytes ($n = 7$/group). *$p < 0.05$, ***$p < 0.001$ compared to Ova-only mice

exclude any probable cytotoxic effects, we use propidium iodide staining to assess cell viability. The results showed that 50 μg/ml of silymarin decreased cell viability. Therefore, in the next step we used the two non-cytotoxic inhibitory concentrations of silymarin (20 and 30 μg/ml) to investigate their effects on Tregs and the Th cell subsets by measuring the expressions of lineage-specific transcription factors and cytokines. Analysis of the in vitro activity of silymarin on T cells showed that 30 μg/ml of silymarin non-significantly reduced T-bet expression and decreased IFNγ cytokine levels. These results suggested an inhibitory effect on the Th1 pathway and were in line with a previous study where silymarin inhibited the release of IFNγ, IL-2 and TNFα proinflammatory cytokines by peripheral blood mononuclear cells of hepatitis C virus-infected patients [27].

Similarly, silymarin significantly decreased RORγt and IL-17 gene expressions in treated splenocytes which suggested its inhibitory effects on Th17 responses. To the best of our knowledge, this effect has not been previously reported. Th17 cells play important roles in inflammation by secretion of IL-17 and recruitment of immune cells to the site of tissue injury. They are involved in the pathophysiology of various autoimmune and inflammatory diseases [19, 28].

We have conducted in vitro analysis of the effects of silymarin on Tregs by examining the expression of Foxp3, as a major transcription factor of Treg cells. We observed increased expression of Foxp3 mRNA in silymarin-treated cells accompanied by increased levels of IL-10 and TGF-β cytokine expressions. We isolated naive CD4$^+$CD25$^-$ T cells and cultured them in the presence of TGF-β and IL-2 to differentiate toward Tregs. Silymarin effectively increased conversion of naive T cells into CD4$^+$CD25$^+$Foxp3$^+$ Tregs (≈1.4% of untreated cells). In the T cell suppression assay, we assessed the inhibitory function of silymarin-induced Treg cells on in vitro proliferative responses of *Tconv cells. We observed that* silymarin-induced Treg cells suppressed proliferation of stimulated Tconv cells by approximately 50%. Altogether, the results from our in vitro analysis suggested that silymarin had the ability to increase functional Tregs and reduce Th1 and Th17 responses.

In the in vivo analysis we evaluated the modulatory effects of silymarin in an Ova-immunized mice model to investigate the effect of silymarin on the specific
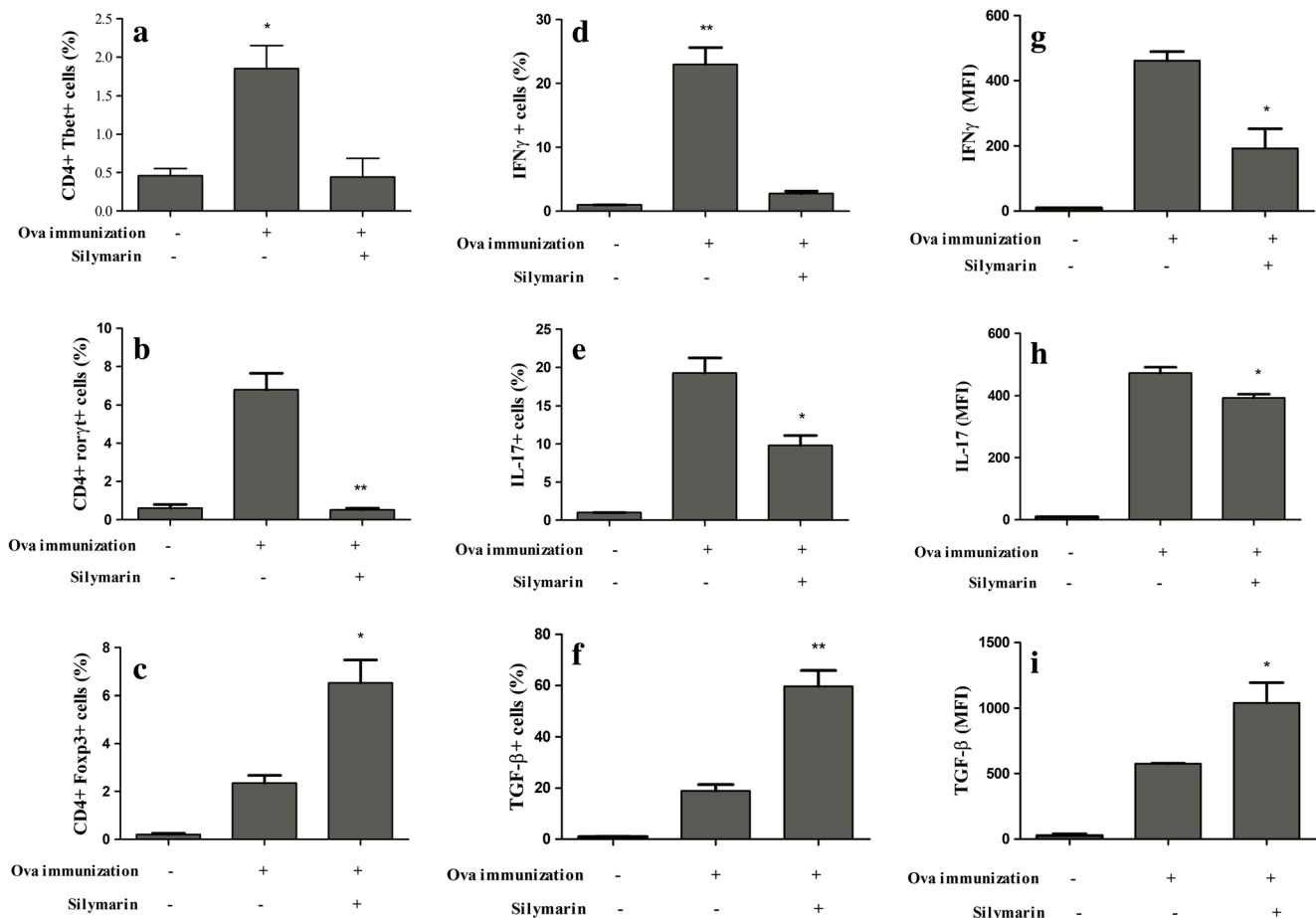
Fig. 5 Effects of silymarin on cytokine expressions in different CD4$^+$ T cell subsets after ex vivo challenge of spenocytes with antigen. Splenocytes from non-immunized and Ova-immunized mice treated and untreated with silymarin were isolated and cultured in the presence of Ova for 24 h. Then cells were harvested and CD4$^+$Tbet$^+$, CD4$^+$RORγt$^+$ and CD4$^+$Foxp3$^+$ T cell subpopulations were assessed for IFNγ, IL-17 and TGFβ expressions, respectively using flow cytometry. Controls were cells from non-immunized mice and those from Ova-only mice. **a–f** Bars shown are mean ± standard error of the number of CD4$^+$Tbet$^+$/IFNγ^+, CD4$^+$RORγt$^+$/IL-17$^+$ and CD4$^+$Foxp3$^+$/TGFβ^+ T cells (n = 3/group). *p < 0.05, **p < 0.01 compared to Ova-only group. **g–i** Mean fluorescence intensity (MFI) of IFNγ expression in CD4$^+$Tbet$^+$ cells, IL-17 in CD4$^+$RORγt$^+$ cells and TGFβ in CD4$^+$Foxp3$^+$ cells

responses of T cell subsets. The splenocytes from the Ova-immunized mice treated with silymarin were directly evaluated for the presence of Th1, Th17 and Treg cells. We observed that in accordance with the in vitro results, silymarin treatment of mice reduced the numbers of CD4$^+$T-bet$^+$ and CD4$^+$RORγt$^+$ T cells and caused a greater than 11% increase in the number of CD4$^+$Foxp3$^+$ T cells. These results showed the positive effects of silymarin on in vivo Treg development.

In a series of ex vivo experiments, we cultured the isolated splenocytes from silymarin-treated mice in the presence of Ova. We examined T cell proliferation and evaluated the number of different subsets of CD4$^+$ T cells and cytokine secretion pattern after antigen challenge. Treatment of Ova-immunized mice by silymarin led to an approximately 32% decrease in

splenocyte proliferation which showed the inhibitory effects of silymarin on specific immune responses in mice. This finding was possibly due to induction of functional Tregs and agreed with our in vitro data and previous report [19]. Analysis of the changes in the levels of different T cell subsets showed decreases in CD4$^+$T-bet$^+$ and CD4$^+$ RORγt$^+$ T cell subsets in splenocytes from silymarin-treated Ova-immunized mice compared to those from untreated Ova-immunized mice.

Fig. 6 Flow cytometry representative dot plots and histograms showing ▶ IFNγ IL-17 and TGFβ positive cells in CD4$^+$T-bet$^+$, CD4$^+$RORγt$^+$ and CD4$^+$Foxp3$^+$ T cell subpopulations, respectively in ex vivo Ova challenge of splenocytes from non-immunized, untreated Ova-immunized and silymarin-treated Ova-immunized groups as described in Fig. 5
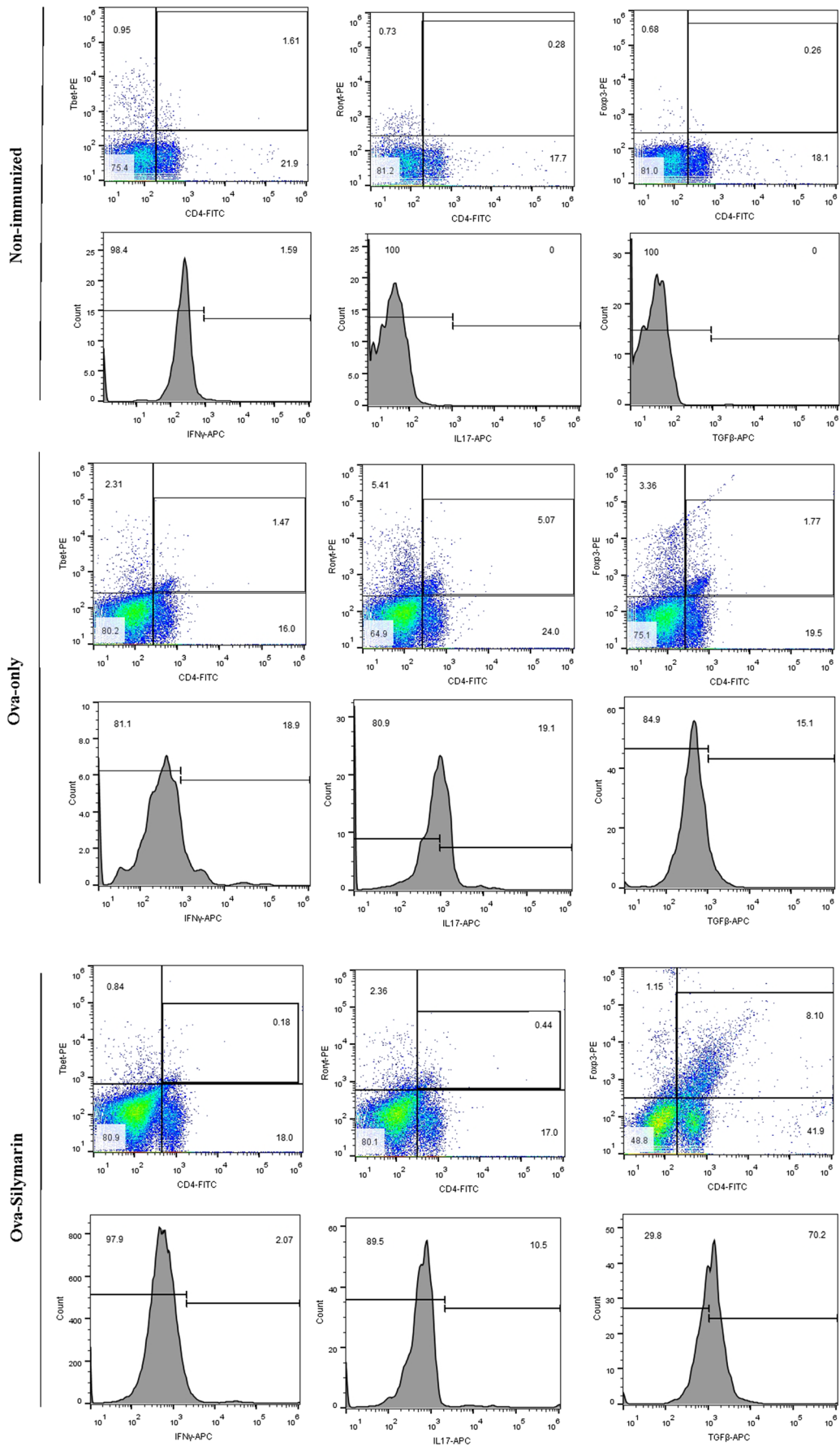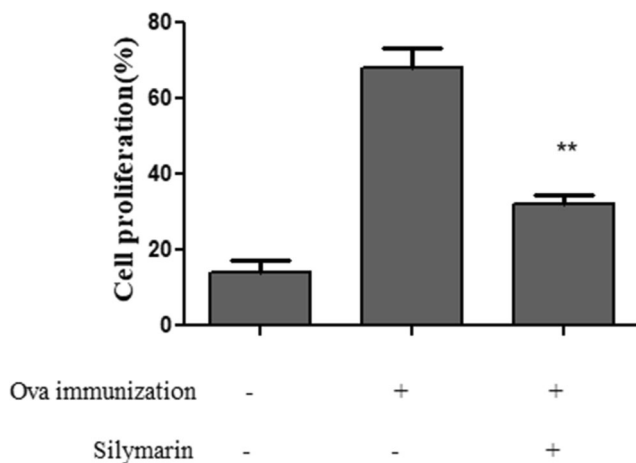
Fig. 7 Effect of silymarin on cell proliferation after ex vivo challenge of spenocytes with antigen. Splenocytes from non-immunized and Ova-immunized mice treated and untreated with silymarin were isolated and labeled by CFSE. Then challenged with ovalbumin (Ova) and cultured for 72 h. Cell proliferation was assessed using CFSE dilution method. Controls were cells from non-immunized mice and those from untreated Ova-immunized mice. Values represent mean ± standard error of cell proliferation (n = 3/group). **p < 0.01 compared to untreated Ova-immunized group

These decreases were accompanied by downregulation of IFNγ and IL-17 cytokines in the Th1 and Th17 subpopulations, respectively. We observed a greater than 6% increase in CD4$^+$Foxp3$^+$ T cells in the splenocyte culture of silymarin-treated mice compared to the untreated mice. Staining for intracellular TGF-β cytokine in this group showed approximately a 3-fold upregulation of TGF-β expression compared to the control. Taken together, these data supported the in vitro data and showed that silymarin administration significantly promoted functional Tregs and decreased Th1 and Th17 cells.

There are few studies of the effects of silymarin on Tregs. A group of patients with chronic hepatitis C have shown no changes in the frequency of CD4$^+$Foxp3$^+$ or CD4$^+$CD25^{high} Tregs compared to the non-treated group [29]. Another study showed that silymarin did not alter Treg markers (CD4$^+$CD25$^+$/CD127^{low}) on peripheral blood mononuclear cells from human immunodeficiency virus (HIV)-infected individuals [30]. It might not be logical to compare these results with the results obtained in this study because of the assessments in humans and mice along with differences in the type of antigen. Usually viruses *can influence the* immune system by creating immunosuppression. Therefore, the use of silymarin with Treg induction may be more effective in autoimmune and inflammatory diseases rather than in infections.

Th1 and Th17 cells have major roles in inflammatory immune responses. Their overactivation has long been recognized to contribute to the pathogenicity of organ-specific autoimmune diseases such as type 1 diabetes

and multiple sclerosis [31]. IFN-γ and IL-17 production by these cells have been associated with disease activity in several autoimmune conditions [1, 32]. In contrast, Treg cells play a crucial role in maintaining the immune balance and prevention of autoimmunity by regulating cell function through the release of suppressive cytokines, such as TGF-β1 and IL-10. Decreased numbers of Tregs have been reported in patients with psoriatic arthritis, autoimmune liver diseases, systemic lupus erythematosus, and Kawasaki disease [33]. Thereby, strategies to either induce Tregs in vivo or their ex vivo expansion are being considered as suitable approaches for the treatment of these diseases. The results of this study have shown the ability of silymarin to differentially regulate CD4$^+$ T cell subsets and change the Tregs/Th1 and Tregs/Th17 balance. This finding implies a possible benefit of this extract in the treatment of autoimmune and inflammatory diseases. Further studies are needed to determine the active components of silymarin that elicited Treg enhancing and antiproliferative effects.

Conclusion

In this study, we showed the ability of silymarin to enhance Treg differentiation and function, and decrease Th1 and Th17 levels. These findings have provided new evidence for the underlying anti-inflammatory and immunomodulatory mechanisms of action by silymarin and on the other hand, support the potential usefulness of this natural product in treatment of immune-related diseases and conditions associated with immune over-activation.

Authors' contributions HN and ZA wrote this manuscript, designed this study and analyzed the data; HN performed the experiments. MI and FR help in flow cytometry analysis. All authors read and approved the final manuscript.

Data availability Datasets analyzed during the current study available from the corresponding author on reasonable request.

Compliance with ethical standards

Consent for publication All authors agree to publish our manuscript.

Competing interests The authors declare that they have no conflict of interest in this work.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. All protocols for animal care and treatment were approved by the local ethics committee.

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