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ORIGINAL ARTICLE

Immunoregulatory Effects of Silymarin on Proliferation and Activation of Th1 Cells Isolated from Newly Diagnosed and IFN-ß$_{1b}$-Treated MS Patients

Fereshteh Navabi,[1] Vahid Shaygannejad,[2,3] Faezeh Abbasirad,[1] Elaheh Vaez,[1] Fahimeh Hosseininasab,[1] Mohammad Kazemi,[2] Omid Mirmosayyeb,[2,4] Fereshteh Alsahebfosoul,[1] and Nafiseh Esmaeil[1,5]

Abstract—Multiple sclerosis (MS) is a central nervous system autoimmune disease characterized by demyelination. Autoreactive T cells mainly interferon gamma (IFN-γ) producing T helper cells (Th1) have an important role in MS pathogenesis. Silymarin is a unique blend produced from milk thistle (*Silybum marianum*) plant which its imunomodulatory role has been indicated in studies. In the present study, the effects of silymarin on isolated Th1 cells were investigated in newly diagnosed MS patients and those who received betaferon. PBMCs were separated from newly diagnosed and IFN-β-treated MS patients. The Th1 cell isolation from PBMCs was performed using a human Th1 cell isolation kit. Th1 cells were cultured in the presence of silymarin (50, 100, and 150 μM for 48, 72, and 120 h). Th1 cell proliferation and CD69 expression were assessed by flow cytometry. Also, IFN-γ production and T-bet gene expression were measured by ELISA and real-time PCR respectively. *In vitro* cultured Th1 cells showed that silymarin suppresses Th1 cell proliferation dose and time dependently in newly diagnosed and IFN-β-treated MS patients in comparison to DMSO control. Also, CD69 expression as an early activation marker was changed after Th1 cell treatment with different doses of silymarin at different times. T-bet gene expression was significantly decreased in Th1 cells isolated from newly diagnosed and IFN-β-treated RRMS patients after treatment with silymarin compared to DMSO control. Additionally, IFN-γ production by Th1 cells was decreased after treatment silymarin in newly diagnosed patients; however, in IFN-β treated after 48-h treatment with silymarin, IFN-γ concentration was decreased at concentrations of 100 and 150 μM, and after 120 h, a significant increase was observed in the

[1] Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, 81744-176, Iran
[2] Isfahan Neurosciences Research Center, Alzahra Hospital, Department of Neurology, Isfahan University of Medical Sciences, Isfahan, Iran
[3] Department of Neurology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[4] Student Research Committee, Isfahan University of Medical Sciences, Isfahan, Iran
[5] To whom correspondence should be addressed at Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, 81744-176, Iran. E-mail: nafesm5@gmail.com

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IFN-γ level at a concentration of 100 μM in comparison with DMSO. Our findings here clearly show that silymarin is an effective regulator for Th1 response *in vitro* condition. It not only suppresses Th1 proliferating activity but also inhibits T-bet gene expression and IFN-γ production by these cells.

KEY WORDS: multiple sclerosis; silymarin; Th1 cells; interferon-β therapy; immunoregulatory.

INTRODUCTION

Multiple sclerosis (MS) is a central nervous system autoimmune disease characterized by demyelination and brain damage [1]. The disease generally occurs at the age of 20–40 years, and women are twice as likely to suffer [2]. A type of disease that is seen in about 85% of patients with MS is relapsing-remitting MS (RRMS), which is associated with recurrence of defects and acute neurological attacks resulting in disability, while some improvements are experienced in the intervals between recurrences [3]. MS is an inflammatory disease in which the breakdown of the blood-brain barrier (BBB) indicates the onset of disease progression through the migration of pathogenic lymphocytes to the central nervous system (CNS). In this regard, the association between endothelial cells, immune cells, and CNS cells allows permeation of self-reactive lymphocytes and then their performance and stability in the parenchymal CNS [4–7].

Based on the evidence, it is believed that self-reactive T cells are mainly interferon gamma (IFN-γ) producing T helper cells (Th1) [8, 9]. Controversial results have been reported the role of IFN-γ in MS disease [10, 11]. Factors such as dosage, specificity, and activity time as well as interactions with other cytokines and cells can determine the pure effects of IFN-γ. Earlier studies have shown that IFN-γ can activate CNS-residing cells, induce expression of MHC molecules, and subsequently strengthen the myelin-uptake process. IFN-γ can also have different effects on the BBB. Also, the role of other immune cells such as Th17 cells and Treg cells in the pathogenesis of MS has indicated in many studies [12].

To date, there is no treatment that improves disability in MS patients; however, early treatment with existing drugs may alter the long-term course of the disease. Interferon beta is a choice for the treatment of MS patients, because it reduces the incidence of relapse, creation of new lesions, and accumulation of disabilities over time [13].

Additionally, plants are an extraordinary resource to discover valuable pharmaceutical products and develop this industry. Flavonoids are a group of natural compounds that are commonly found in most plants and affect a significant range of biochemical activities including primary cell function such as proliferation, differentiation, and apoptosis [14]. Among these flavonoids, silymarin is a unique blend produced from milk thistle (*Silybum marianum*) plant. Silymarin has anti-inflammatory, hepatoprotective, and anticoagulant effects [15]. Today, silymarin has been shown to have immunomodulatory effects, and several studies have focused on it [16–19]; however, its effect mechanisms have not yet been fully elucidated, while it is clear that silymarin inhibits the activity and proliferation of T cells [17–19]. Studies have shown silymarin exerts its anti-inflammatory effects through suppression of the transcription factor nuclear factor-kB (NF-kB) signaling pathway [20]. Also, the immunosuppressive effect of silymarin on MAPK signaling pathway and its inhibitory effects on T cell proliferation and cytokine production have been indicated [21].

Consequently, since the effect of silymarin as a regulator of immune response on the function of Th1 cells as the main cell in the pathogenesis of MS has not yet been determined, in the present study, the effects of silymarin on isolated Th1 cells were investigated in newly diagnosed MS patients and those who received betaferon.

MATERIALS AND METHODS

Silymarin Preparation

To obtain at 100 mM stock solution of *Silybum marianum*, powdered extract (Sigma, St. Louis, MO, USA, Lot No. 286061) was dissolved in dimethyl sulphoxide (DMSO). The aliquots were stored at −20 °C for no longer than 30 days. In order to prepare silymarin with the concentrations of 50, 100, and 150 μM, all subsequent dilutions were made in Roswell Park Memorial Institute (RPMI) medium.

Study Patients

Two groups of patients with MS in 2017 from the clinic of MS center Kashani Hospital in Iran were selected for this study: one group had clinically definite RRMS according to the McDonald criteria [22, 23] and were treated with IFN-β and the other group was newly diagnosed patients. These patients have not received any immunosuppressive drugs, as well as those with RRMS, for at least 6 months of betaferon treatment.

Blood samples were collected from eight newly diagnosed (one man and seven women) and eight (two men and six women) IFN-$ß_{1b}$-treated patients injection and the mean age of the patients was 33.91 + 8.4. Blood collection from IFN-$ß_{1b}$-treated patients was performed 24–48 h after IFN-$ß_{1b}$ injection. The research protocols were approved by the Ethics Committee of Isfahan University of Medical Sciences, and all the participants signed an informed consent.

Blood Samples and Isolation of Peripheral Blood Lymphocytes

Peripheral blood mononuclear cells (PBMCs) from all donors were obtained after Ficoll density gradient centrifugation and processed within 3 h (freshly isolated PBMC) at room temperature from EDTA blood samples.

Magnetic Cell Separation

The separation of Th1 cells from PBMCs was performed using a human Th1 cell isolation kit according to the manufacturer's instructions (EasySep™ Human Th1 cell isolation kit).

In brief, unwanted cells were extracted by negative selection using EasySep™ Human CD4+CXCR3+ T Cell Pre-Enrichment Cocktail. Tetrameric antibody complexes (TAC) recognizing CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A, CD45RAhigh, CXCR3, and dextran-coated magnetic particles were added to PBMC and the unwanted cells separated out with a magnet. After this step, highly pure CD4+ T cells were obtained and then CXCR3+ cells were enriched by positive selection. Finally, the isolated Th1 cells were incubated at 37 °C for 12 h to ensure that the dextran-coated magnetic particles were completely separated from the Th1 cells. Purification of isolated Th1 cells was determined by flow cytometry after stimulation of Th1 cells with PMA (5 ng/mL; Sigma-Aldrich) and ionomycin (50 ng/mL; Sigma-Aldrich) in RPMI 1640 in the presence of the protein transport inhibition brefeldin A (Golgiplug) for 6 h, as per the manufacturer's instructions (BD Biosciences) and using antibodies against the CD4 and IFN-γ (eBioscience, USA). Sorted Th1 cell subsets were on average > 95% pure as determined by triple staining with CD4+, CXCR3+, and CCR4− markers and also typically ≥ 28% of cells were CD4+/IFN-γ+ (data not shown).

Cell Culture

Isolated Th1 cells were cultured (10^4/well) in complete RPMI 1640 medium (BIO-IDEA, USA), containing 10% FBS (Gibco, USA) and 1% penicillin/streptomycin. Cells were stimulated with anti-CD3 monoclonal antibody (mAb) OKT-3 coated on plates at 4 µg/ml (eBioscience, San Diego, CA, USA) and soluble anti-CD28 mAb (eBioscience) at 2 µg/ml. After 24 h, soluble IL-2 at 200 unit/ml (10 ng) was added to cells and the cells were treated with silymarin at 50, 100, and 150 µM for 48, 72, and 120 h. Negative control cells were treated with DMSO. The final concentration of DMSO in control wells was equal to test wells and was less than 0.1%. All experiments were repeated at least three times independently.

Flow Cytometry and CFSE Labeling

For CFSE labeling, 1–2 10^4 cells/ml in PBS were incubated with 1 µM carboxyfluorescein succinimidyl ester (CellTrace™ CFSE Cell Proliferation Kit; Invitrogen) before culture initiation. After stopping the labeling reaction by addition of 1 vol FBS, the cells were washed three times and cultured under conditions, as indicated in the text, for 48, 72, and 120 h.

CFSE-labeled or -unlabeled Th1 cells were stained for CD69 surface marker. PE-labeled anti-CD69 was used to stain Th1 cells according to eBioscinec's (eBioscience, USA) recommended method. Cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA).

Detection of IFN-γ Cytokine

Cell culture supernatants were collected and stored at − 20 °C. The concentration of the IFN-γ cytokine was measured using ELISA kit (PeproTech® EC Ltd., UK) according to the manufacturer's instructions.

Real-Time PCR for T-bet Gene Detection

RNA of all culture groups were extracted using a total RNA extraction kit (Yekta Tajhiz, Tehran, Iran) according to the manufacturer's protocol and the concentration and purity of RNA were assessed by spectrophotometry. Reverse transcription of RNA was performed using Thermo Fisher Scientific kit (Thermo Fisher Scientific, Lithuania). For real-time

PCR, the following primer pairs were used: *T-bet* (50-ACCAGAATGCCGAGATTACT_30 forward, 50-GGTAGGAGAGGAGAGTAGTGA_30 reverse); *β-ACTIN* (50-AGCACAGAGCCTCGCCTTT_30 forward, 50-GTTGTCGACGACGAGCG_30 reverse). Quantitative real-time polymerase chain reaction (qPCR) was performed in duplicate with 1 ng of complementary DNA (cDNA), using the 7900HT Fast Real-Time PCR System (Applied Biosystems). For RT-PCR, the reaction mixture (20 mL) contained 1 mL of diluted cDNA and 1 ml of each pair of oligonucleotide primers 7 ml water and 10 ml SYBR Green Master Mix. The real-time PCR conditions included an initial denaturation at 95 °C for 10 min, followed by a 40-cycle amplification consisting of denaturation at 95 °C for 15 s, and extension at 60 °C for 60 s followed by melting curves to verify qPCR product identity. All reactions were assayed in duplicate for each sample. All primer pairs were checked for primer-dimer formation using the three-step protocol described above without the addition of the RNA template. The geometric mean of housekeeping gene *β-ACTIN* was used as an internal control to correct the raw values for the gene of interest. Mean cycle threshold (Ct) values were standardized by calculating ΔCt using the housekeeping gene *β-ACTIN* and calculating using the 2-ΔΔCT method.

Statistical Analysis

Data are represented as mean ± SEM for replicate values. Data were analyzed by one-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) with post-Dunnett's multiple comparison test. All statistical calculations were performed using the GraphPad Prism 6.0 software. The null hypothesis was discarded when $P < 0.05$.

RESULTS

Silymarin Suppresses Th1 Cell Proliferation Dose and Time Dependently in Newly Diagnosed and IFN-β-Treated RRMS Patients

To determine the effect of silymarin on Th1 cell proliferation, we labeled Th1 cells with CFSE and CFSE profiles were assessed by flow cytometric analysis (Fig. 1a). After 48 h of Th1 cell cultured isolated from newly diagnosed MS patients, we found that the Th1 cell proliferation in the presence of 100 μM silymarin concentration was significantly lower than that in DMSO control ($P = 0.016$). However, after 72 h of treatment with Th1 cells at concentrations of 50, 100, and 150 μM of

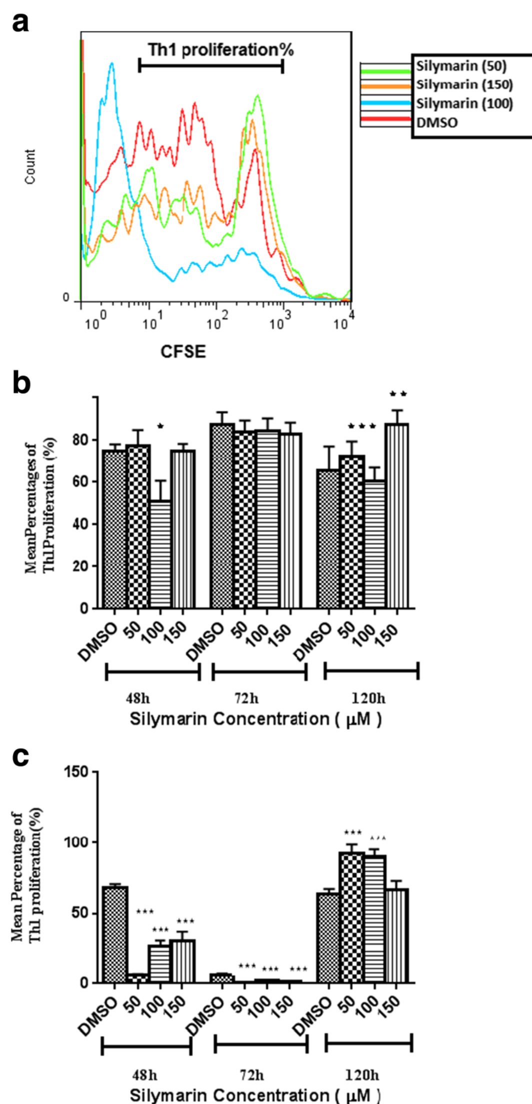

Fig. 1. Silymarin effects on Th1 cell proliferation isolated from MS patients. **a** CFSE histogram plots of Th1 cells following of the co-culture of these cell and silymarin (50, 100, 150 μM). Following 48, 72, and 120 h of culture, cells were harvested and analyzed by flow cytometry to identify proliferating cell populations. CFSE histograms depict the number of events (y-axis) and the fluorescence intensity (x-axis), with proliferating cells displaying a progressive 2-fold loss in fluorescence intensity following cell division, indicative of proliferating cells. Graphical representation of the proliferating Th1 cells isolated from newly diagnosed (**b**) and IFN-β-treated RRMS patients (**c**). Th1 cells following 48, 72, and 120 h of culture in the presence of DMSO (control) or in the presence of three concentrations of silymarin (50, 100, 150 μM). Data are pooled from three independent experiments. *$P < 0.05$, ***$P < 0.001$ indicate statistically significant differences between DMSO and silymarin co-culture conditions, using a one-way analysis of variance (ANOVA) ($P < 0.05$).

silymarin, no significant difference was observed between different concentrations of silymarin and DMSO ($P \geq 0.05$). After 120 h of treatment with Th1 cells at concentrations of 50, 100, and 150 μM of silymarin, a significant decrease was observed at 100 μM concentration ($P = 0.001$) in comparison with DMSO; however, at 150 μM silymarin concentration, significant increase in proliferation was found compared to DMSO ($P = 0.005$) (Fig. 1b).

In patients treated with betaferon, after 48 and 72 h of treatment with Th1 cells at concentrations of 50, 100, and 150 μM of silymarin, a significant decrease in proliferation was observed at different concentrations of silymarin compared to DMSO ($P < 0.0010$). However, after 120 h of treatment at 50, 100, and 150 μM of silymarin, increased Th1 cell proliferation at 50 μM silymarin ($P < 0.001$) and 100 μM silymarin ($P < 0.001$) was found compared to DMSO (Fig. 1c).

Effect of Silymarin on CD69 Expression on Th1 Cells Isolated from Newly Diagnosed and IFN-β-Treated RRMS Patients

To investigate whether the silymarin affected the activity of Th1 cells, the expression of CD69 as an early activation marker was assessed by flow cytometry after Th1 cells co-cultured with 50, 100, and 150 μM for 48,72, and 120 h (Fig. 2a).

After 48 h of Th1 cell treatment with silymarin at concentrations of 50, 100, and 150 μM, the activity of Th1 cells at a concentration of 100 μM of silymarin was decreased compared to DMSO ($P = 0.048$). Treatment of Th1 cells with different concentrations of silymarin (50, 100, and 150 μM) after 72 h had no significant effect on CD69 expression. However, after 120 h of Th1 cell treatment in the presence of 50, 100, and 150 μM silymarin, a significant increase was observed in the CD69 expression at a concentration of 150 μM of silymarin compared to DMSO ($P < 0.001$) (Fig. 2b).

In patients treated with betaferon, after 48 h of treatment with Th1 cells in adjacent 50, 100, and 150 μM silymarin concentrations, a significant increase in CD69 expression was observed at 50 μM of silymarin compared with DMSO ($P = 0.000$). However, after 72 h of culture, there was no significant difference in CD69 expression of Th1 cells in the presence of different concentrations of silymarin ($P \geq 0.05$). Also, after 120 h of Th1 cell treatment with 50, 100, and 150 μM silymarin, there was a significant decrease in the CD69 expression at 50 μM ($P < 0.01$) and 100 μM ($P < 0.01$) silymarin concentrations compared to DMSO (Fig. 2c).

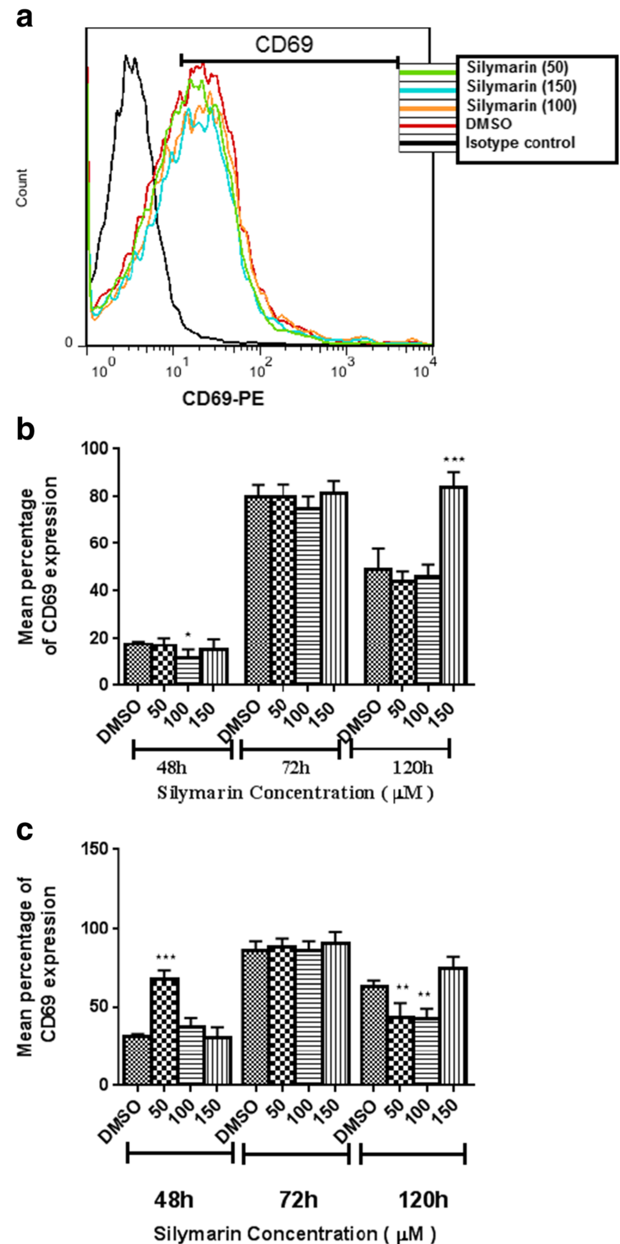

Fig. 2. Silymarin effects on CD69 expression on Th1 cells isolated from MS patients. **a** Histogram plots of Th1 cells following of the co-culture of these cell and silymarin (50, 100, 150 μM). Following 48, 72, and 120 h of culture, cells were harvested and stained with CD69 mAbs and analyzed by flow cytometry. Graphical representation of the percentage of $CD69^+$ Th1 cells isolated from newly diagnosed (**b**) and IFN-β-treated RRMS patients (**c**).Th1 cells following 48, 72, and 120 h of culture in the presence of DMSO (control) or in the presence of three concentrations of silymarin (50, 100, 150 μM). Data are pooled from three independent experiments. Data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as determined by one-way ANOVA.

T-bet Gene Expression Was Significantly Decreased in Th1 Cells Isolated from Newly Diagnosed and IFN-β-Treated RRMS Patients after Treatment with Silymarin

The expression of *T-bet* in the Th1 cells after 48, 72, and 120 h was evaluated and, as indicated in Fig. 3a, b, the expression of this gene at concentrations of 50, 100, and 150 μM of silymarin significantly decreased compared to DMSO ($P \leq 0.001$).

Effect of Silymarin on IFN-γ Production by Th1 Cells Isolated from Newly Diagnosed and IFN-β-Treated RRMS Patients

After 48 and 120 h, Th1 cell treatment with 50, 100, and 150 μM silymarin, the level of IFN-γ cytokine was not significantly different from that of the DMSO control ($P \geq 0.05$). However, after 72 h of Th1 cell treatment with silymarin concentrations of 50, 100, and 150 μM of silymarin, IFN-γ concentration was significantly decreased at 100 ($P < 0.01$) and 150 μM ($P < 0.001$) compared to DMSO (Fig. 4a).

In betaferon-treated subjects, after 48 h of Th1 cell treatment with silymarin, IFN-γ concentration was decreased at concentrations of 100 ($P < 0.001$) and 150 μM ($P < 0.05$) of silymarin in comparison with DMSO. After 72 h of culture, there was no significant difference in the IFN-γ concentration between groups ($P \geq 0.05$). In the last stage of cytokine production after 120 h, a significant increase was observed in the IFN-γ level at a concentration of 100 μM of silymarin compared to DMSO ($P < 0.05$) (Fig. 4b).

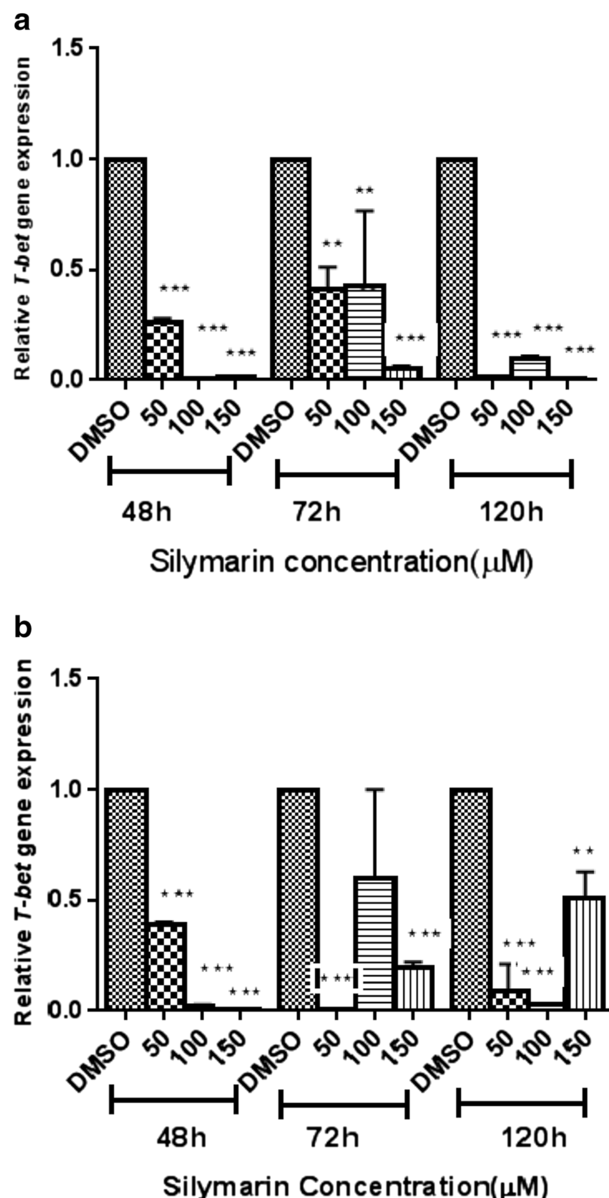

Fig. 3. Silymarin effects on *T-bet* gene expression in Th1 cells isolated from MS patients. **a** Relative gene expression of *T-bet* gene as measured by RT-qPCR in Th1 cells isolated from newly diagnosed (**a**) and IFN-β-treated RRMS patients (**b**) at different doses of silymarin (50, 100, 150 μM) after 48, 72, and 120 h. Data are pooled from three independent experiments. Data are expressed as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as determined by one-way ANOVA.

DISCUSSION

Numerous studies have highlighted effects of silymarin as anti-inflammatory and immunomodulatory phytochemical. Mechanistically, silymarin exerts its anti-inflammatory effects through the inhibition of NF-kB, STAT3, and MEK/ERK signaling pathways [20, 24–27].

Here, we showed that silymarin suppressed proliferation of Th1 cells isolated from newly diagnosed patients after 48 and 120 h at 100 μM concentration and Th1 proliferation was increased at 150 μM after 120 h. Many of the molecular and cellular interactions observed induce in low concentrations of silymarin and some of these interactions are associated with higher doses. Our findings are in agreement with Garagozloo et al. study which has been reported silymarin inhibits peripheral blood mononuclear cells (PBMCs) proliferation after 120 h at 100 and 200 μM concentrations. They found that silymarin targets the mammalian target of rapamycin (mTOR) and thus intervenes in the cell cycle. They also have shown that a significant decrease in mTOR activity in T cells in the presence of 100 μM of silymarin and in a similar manner to rapamycin compared to DMSO [18].

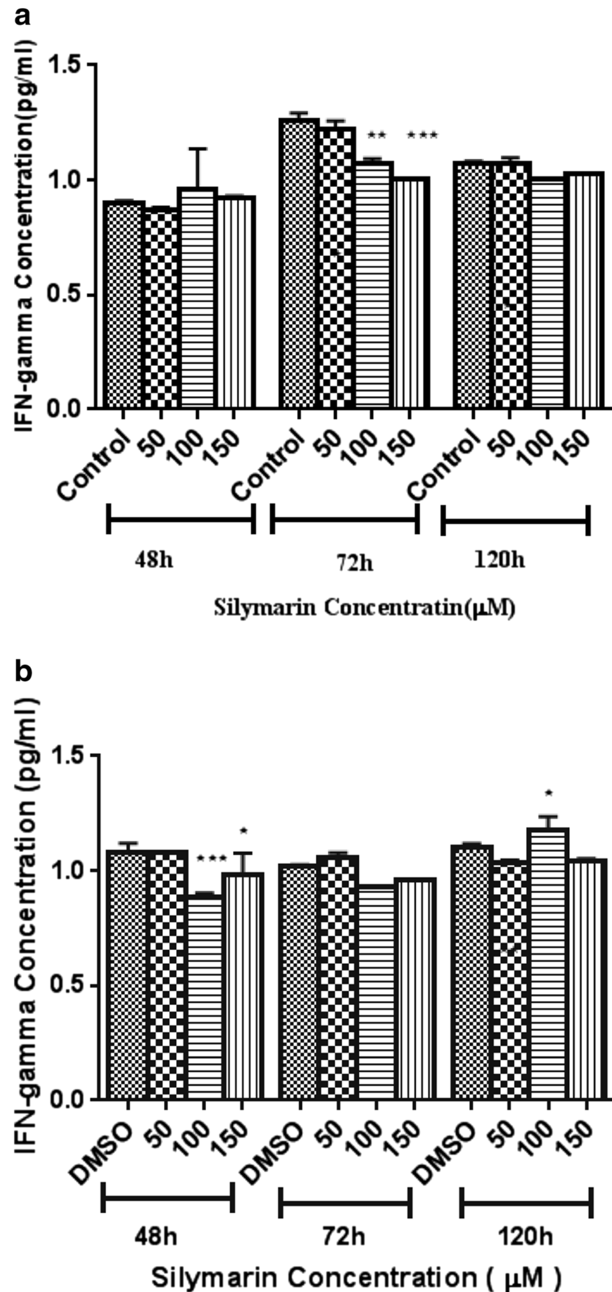

Fig. 4. **a**, **b** Silymarin effects on IFN-γ production by Th1 cells isolated from MS patients. ELISA measurements of IFN-γ concentrations in conditioned supernatants collected from co-cultures of Th1 cells with different doses of silymarin (50, 100, 150 μM) after 48, 72, and 120 h. Data are pooled from three independent experiments and are calculated as the average cytokine concentration from duplicate wells. Data are expressed as means ± SEM. $*P<0.05$, $**P<0.01$ as determined by one-way ANOVA.

mTOR is a member of phosphatidylinositol 3-kinase (PI3K)-related Ser/Thr kinase family that integrates signals from energy sufficiency nutrients, and growth factors to regulate cell growth in a variety of organisms [28, 29].

The experimental data suggest that silymarin exerts its immunosuppressive effect, at least in part, through the PI3K/Akt/mTOR pathway by inhibiting the phosphorylation of p70S6K and p-S6 proteins [30]. In T cells, mTOR has also been identified as one downstream target of interleukin (IL)-2-dependent signaling that is linked to cycle progression due to its role in Cdk2 and Cdc2 kinase activation, p27Kip1 downregulation, and G1/S checkpoint transition [31].

Therefore, the decrease of Th1 cell proliferation in the presence of silymarin in our study may be related to the suppression of PI3K/Akt/mTOR pathway by silymarin. In betaferon-treated RRMS patients, Th1 proliferation was significantly decreased after 48 and 72 h in the presence of different concentration (50, 100, and 150 μM) of silymarin.

Studies have demonstrated in the sustained treatment of multiple cell lines with IFN, type I IFNs, induce autophagy, and mTOR inhibition [32–34]. Also, Zhao et al. have shown IFN-β inhibits PI3K/AKT/NF-kB axis and p38, JNK-MAPK, as well as regulates mTOR complexes. So, we can conclude from our results that silymarin and IFN-β synergistically suppress Th1 cell proliferation.

In order to assess the effect of silymarin on the initial activation of T cells, expression of CD69 as a primary marker for activation of T cells was investigated. We found that CD69 expression on Th1 cells was significantly decreased at 100 μM of silymarin after 48 h.

Treatment of Th1 cells with different concentrations of silymarin (50, 100, and 150 μM) after 72 h had no significant effect on CD69 expression. However, after 120 h of Th1 cell treatment in the presence of 50, 100, and 150 μM silymarin, a significant increase was observed in the CD69 expression at a concentration of 150 μM of silymarin compared to DMSO ($P<0.001$). Also, in betaferon-treated patients, a significant increase in CD69 expression was observed at 50 μM of silymarin compared with DMSO. After 72 h of culture, there was no significant difference in CD69 expression of Th1 cells in the presence of different concentrations of silymarin. However, after 120 h of Th1 cell treatment with 50, 100, and 150 μM silymarin, there was a significant decrease in the CD69 expression at 50 μM and 100 μM silymarin concentrations compared to DMSO.

The decrease of CD69 expression in the presence of 100 μM silymarin concentration in newly diagnosed MS patients was consistent with decreasing cell proliferation. Accordingly, Maino et al. have shown CD69 expression is correlated with PBMC proliferative response [35]. On the

other side, studies have indicated CD69 is the earliest marker which expresses on both antigen or mitogen-activated PBMC prior to lymphocyte proliferation but its expression is not comparable to PBMC proliferative response [36–38].

However, the nature of antigenic and mitogenic stimulation probably affects not only cell proliferation but also its activation. Numerous studies have shown silymarin can target and suppress the products of NF-kB signaling pathway, including cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and inflammatory cytokines [17, 24, 39].

Therefore, the reduction of D69 expression onTh1 cells may be due to inhibitory effects of silymarin on the NF-kB transcription factor. Also, our results suggest that treatment with betaferon influences the inhibitory effect of silymarin on CD69 expression. However, more detailed studies are needed to assess the regulatory effects of silymarin on the expression of activatory molecules on T cells.

In order to investigate the immunoregulatory effect of silymarin on Th1 cells, we decided to study the effect of this compound on the expression of T-bet as a specificTh1 cell transcription factor and IFN-γ production. We found that silymarin significantly decreased T-bet expression in newly diagnosed and betaferon-treated MS patients. Also, IFN-γ levels were significantly decreased dose and time dependently in both groups. The *T-bet* transcription factor promotes the production of Th1 specific cytokines, especially IFN-γ, thereby enhances the differentiation of Th1 cells through the creation of a positive feedback loop [40, 41].

On the other hand, Zhu et al. have demonstrated IFN-γ alone is enough for T-bet expression in cells responding to TCR and CD28 co-stimulation [42]. Th1 cell differentiation from naive T cells is dependent on IFN-γ and IL-12, which activate signal transducers such as STAT1 and STAT4 respectively [43, 44]. Signal transducer and activator of transcriptions (STATs) are widely recognized as an oncogene that involves a variety of biological processes, including cell proliferation, conversion, apoptosis, differentiation, angiogenesis, inflammation, and immune response [45–47].

In a study, silibinin pre-treatment (50–200 μM) strongly reduced the cytokine mixture-induced phosphorylation of STAT1 (Tyr-701) in the total cell lysates. Silibinin treatment also decreased the total STAT1 level compare to cytokine mixture treatment alone [48].

Therefore, the inhibition of STAT1 molecule by silymarin probably leads to decrease of IFN-γ production by Th1 cells which followed by reduced *T-bet* gene expression. In addition, STAT4 is responsible for T-bet-independent IFN-γ production [42].

Nguyen et al. have indicated type 1 interferons (IFNs), including IFN alpha/beta activate STAT4 directly and induce IFN-γ production [49]. So, no significant difference in IFN-γ levels in the presence of silymarin in betaferon-treated MS patients may be due to induction of STST4 and T-bet-independent IFN-γ production. However, further investigation is needed to confirm such regulation.

At the same time, we also assessed silymarin impacts on Th17 and Treg cells and we found silymarin also suppressed Th17 cells, while its effect on Treg cells was different. Our findings have presented in a manuscript that is now under review by a journal.

Overall, our report here clearly shows that silymarin is an effective regulator for Th1 response *in vitro* condition. It not only suppresses Th1 proliferating activity, but it also inhibits T-bet expression and IFN-γ production by these cells.

Most importantly, silymarin suppresses an immune cell response that in addition to MS has a pathogenic role in many autoimmune diseases and is favorable for regulation of Th1 immune response in MS and other autoimmune disorders.

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COMPLIANCE WITH ETHICAL STANDARDS

The research protocols were approved by the Ethics Committee of Isfahan University of Medical Sciences, and all the participants signed an informed consent.

Conflict of Interest. The authors declare that they have no conflict of interest.

REFERENCES

1. Compston, A., A. Coles, et al. 2008. *Lancet (London, England)* 372 (9648): 1502–1517.
2. Bar-Or, A., E.M. Oliveira, D.E. Anderson, and D.A. Hafler. 1999. Molecular pathogenesis of multiple sclerosis. *Journal of Neuroimmunology* 100 (1–2): 252–259.

3. Ellwardt, E., and F. Zipp. 2014. Molecular mechanisms linking neuroinflammation and neurodegeneration in MS. *Experimental Neurology* 262 Pt A: 8–17.
4. Kebir, H., K. Kreymborg, I. Ifergan, A. Dodelet-Devillers, R. Cayrol, M. Bernard, F. Giuliani, N. Arbour, B. Becher, and A. Prat. 2007. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nature Medicine* 13 (10): 1173–1175.
5. Tsuge, M., K. Yasui, T. Ichiyawa, Y. Saito, Y. Nagaoka, M. Yashiro, N. Yamashita, and T. Morishima. 2010 Jul. Increase of tumor necrosis factor-alpha in the blood induces early activation of matrix metalloproteinase-9 in the brain. *Microbiology and Immunology.* 54 (7): 417–424.
6. Minagar, A., A. Long, T. Ma, T.H. Jackson, R.E. Kelley, D.V. Ostanin, M. Sasaki, A.C. Warren, A. Jawahar, B. Cappell, and J.S. Alexander. 2003. Interferon (IFN)-beta 1a and IFN-beta 1b block IFN-gamma-induced disintegration of endothelial junction integrity and barrier. *Endothelium: Journal of Endothelial Cell Research* 10 (6): 299–307.
7. Yang, F., K. Zhao, X. Zhang, J. Zhang, and B. Xu. 2016. ATP induces disruption of tight junction proteins via IL-1 beta-dependent MMP-9 activation of human blood-brain barrier *In Vitro*. *Neural Plasticity* 2016: 8928530.
8. Szabo, S.J., B.M. Sullivan, S.L. Peng, and L.H. Glimcher. 2003. Molecular mechanisms regulating Th1 immune responses. *Annual Review of Immunology* 21: 713–758.
9. Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annual Review of Immunology* 23: 683–747.
10. Fletcher, J.M., S.J. Lalor, C.M. Sweeney, N. Tubridy, and K.H. Mills. 2010. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clinical and Experimental Immunology* 162 (1): 1–11.
11. Lovett-Racke, A.E., Y. Yang, and M.K. Racke. 2011. Th1 *versus* Th17: are T cell cytokines relevant in multiple sclerosis? *Biochimica et Biophysica Acta* 1812 (2): 246–251.
12. McFarland, H.F., and R. Martin. 2007. Multiple sclerosis: a complicated picture of autoimmunity. *Nature Immunology* 8 (9): 913–919.
13. Markowitz, C.E. 2007. Interferon-beta: mechanism of action and dosing issues. *Neurology* 68 (24 Suppl 4): S8–S11.
14. Middleton, E., Jr., C. Kandaswami, and T.C. Theoharides. 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews* 52 (4): 673–751.
15. Gazak, R., D. Walterova, and V. Kren. 2007. Silybin and silymarin–new and emerging applications in medicine. *Current Medicinal Chemistry* 14 (3): 315–338.
16. Wilasrusmee, C., S. Kittur, G. Shah, J. Siddiqui, D. Bruch, S. Wilasrusmee, et al. 2002. Immunostimulatory effect of *Silybum marianum* (milk thistle) extract. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research* 8 (11): Br439–Br443.
17. Gharagozloo, M., E. Velardi, S. Bruscoli, M. Agostini, M. Di Sante, V. Donato, et al. 2010. Silymarin suppress CD4+ T cell activation and proliferation: effects on NF-kappaB activity and IL-2 production. *Pharmacological Research* 61 (5): 405–409.
18. Gharagozloo, M., E.N. Javid, A. Rezaei, and K. Mousavizadeh. 2013. Silymarin inhibits cell cycle progression and mTOR activity in activated human T cells: therapeutic implications for autoimmune diseases. *Basic & Clinical Pharmacology & Toxicology.* 112 (4): 251–256.
19. Morishima, C., M.C. Shuhart, C.C. Wang, D.M. Paschal, M.C. Apodaca, Y. Liu, et al. 2010. Silymarin inhibits *in vitro* T-cell proliferation and cytokine production in hepatitis C virus infection. *Gastroenterology* 138 (2): 671–681 81.e1–2.
20. Kang, J.S., Y.J. Jeon, S.K. Park, K.H. Yang, and H.M. Kim. 2004. Protection against lipopolysaccharide-induced sepsis and inhibition of interleukin-1beta and prostaglandin E2 synthesis by silymarin. *Biochemical Pharmacology* 67 (1): 175–181.
21. Gharagozloo, M., S. Jafari, N. Esmaeil, E.N. Javid, B. Bagherpour, and A. Rezaei. 2013. Immunosuppressive effect of silymarin on mitogen-activated protein kinase signalling pathway: the impact on T cell proliferation and cytokine production. *Basic & Clinical Pharmacology & Toxicology.* 113 (3): 209–214.
22. Miller, D.H., B.G. Weinshenker, M. Filippi, B.L. Banwell, J.A. Cohen, M.S. Freedman, et al. 2008. Differential diagnosis of suspected multiple sclerosis: a consensus approach. *Multiple Sclerosis (Houndmills, Basingstoke, England)* 14 (9): 1157–1174.
23. Charil, A., T.A. Yousry, M. Rovaris, F. Barkhof, N. De Stefano, F. Fazekas, et al. 2006. MRI and the diagnosis of multiple sclerosis: expanding the concept of "no better explanation". *The Lancet Neurology* 5 (10): 841–852.
24. Kim, E.J., J. Kim, M.Y. Lee, M.S. Sudhanva, S. Devakumar, and Y.J. Jeon. 2014. Silymarin inhibits cytokine-stimulated pancreatic beta cells by blocking the ERK1/2 pathway. *Biomolecules & Therapeutics* 22 (4): 282–287.
25. Wang, M.J., W.W. Lin, H.L. Chen, Y.H. Chang, H.C. Ou, J.S. Kuo, J.S. Hong, and K.C.G. Jeng. 2002. Silymarin protects dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting microglia activation. *The European Journal of Neuroscience.* 16 (11): 2103–2112.
26. Agarwal, C., A. Tyagi, M. Kaur, and R. Agarwal. 2007 Jul. Silibinin inhibits constitutive activation of Stat3, and causes caspase activation and apoptotic death of human prostate carcinoma DU145 cells. *Carcinogenesis* 28 (7): 1463–1470.
27. Grandis, J.R., S.D. Drenning, Q. Zeng, S.C. Watkins, M.F. Melhem, S. Endo, et al. 2000. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America* 97 (8): 4227–4232.
28. Thomson, A.W., H.R. Turnquist, and G. Raimondi. 2009. Immunoregulatory functions of mTOR inhibition. *Nature Reviews Immunology.* 9 (5): 324–337.
29. Weichhart, T., and M.D. Saemann. 2009. The multiple facets of mTOR in immunity. *Trends in Immunology.* 30 (5): 218–226.
30. Garcia-Maceira, P., and J. Mateo. 2009. Silibinin inhibits hypoxia-inducible factor-1alpha and mTOR/p70S6K/4E-BP1 signalling pathway in human cervical and hepatoma cancer cells: implications for anticancer therapy. *Oncogene* 28 (3): 313–324.
31. Reif, K., B.M. Burgering, and D.A. Cantrell. 1997. Phosphatidylinositol 3-kinase links the interleukin-2 receptor to protein kinase B and p70 S6 kinase. *The Journal of Biological Chemistry* 272 (22): 14426–14433.
32. Kim, S., M.G. Choi, H.S. Lee, S.K. Lee, S.H. Kim, W.W. Kim, et al. 2009. Silibinin suppresses TNF-alpha-induced MMP-9 expression in gastric cancer cells through inhibition of the MAPK pathway. *Molecules (Basel, Switzerland)* 14 (11): 4300–4311.
33. Larsen, L., J. Storling, M. Darville, D.L. Eizirik, C. Bonny, N. Billestrup, et al. 2005. Extracellular signal-regulated kinase is essential for interleukin-1-induced and nuclear factor kappaB-mediated gene expression in insulin-producing INS-1E cells. *Diabetologia* 48 (12): 2582–2590.
34. Burke, J.D., L.C. Platanias, and E.N. Fish. 2014. Beta interferon regulation of glucose metabolism is PI3K/Akt dependent and important for antiviral activity against coxsackievirus B3. *Journal of Virology* 88 (6): 3485–3495.
35. Maino, V.C., M.A. Suni, and J.J. Ruitenberg. 1995. Rapid flow cytometric method for measuring lymphocyte subset activation. *Cytometry* 20(2):127–133

36. Caruso, A., S. Licenziati, M. Corulli, A.D. Canaris, M.A. De Francesco, S. Fiorentini, et al. 1997. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry* 27 (1): 71–76.
37. Lopez-Cabrera, M., A.G. Santis, E. Fernandez-Ruiz, R. Blacher, F. Esch, P. Sanchez-Mateos, et al. 1993. Molecular cloning, expression, and chromosomal localization of the human earliest lymphocyte activation antigen AIM/CD69, a new member of the C-type animal lectin superfamily of signal-transmitting receptors. *The Journal of Experimental Medicine.* 178 (2): 537–547.
38. Hara, T., L.K. Jung, J.M. Bjorndahl, and S.M. Fu. 1986. Human T cell activation. III. Rapid induction of a phosphorylated 28 kD/32 kD disulfide-linked early activation antigen (EA 1) by 12-o-tetradecanoyl phorbol-13-acetate, mitogens, and antigens. *The Journal of Experimental Medicine.* 164 (6): 1988–2005.
39. Bannwart, C.F., E. Nakaira-Takahagi, M.A. Golim, L.T. de Medeiros, M. Romao, I.C. Weel, et al. 2010. Downregulation of nuclear factor-kappa B (NF-kappaB) pathway by silibinin in human monocytes challenged with Paracoccidioides brasiliensis. *Life Sciences* 86 (23–24): 880–886.
40. Afkarian, M., J.R. Sedy, J. Yang, N.G. Jacobson, N. Cereb, S.Y. Yang, T.L. Murphy, and K.M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nature Immunology* 3 (6): 549–557.
41. Lighvani, A.A., D.M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B.D. Hissong, B.V. Nguyen, M. Gadina, A. Sher, W.E. Paul, and J.J. O'Shea. 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proceedings of the National Academy of Sciences of the United States of America* 98 (26): 15137–15142.
42. Zhu, J., D. Jankovic, A.J. Oler, G. Wei, S. Sharma, G. Hu, L. Guo, R. Yagi, H. Yamane, G. Punkosdy, L. Feigenbaum, K. Zhao, and W.E. Paul. 2012. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity* 37 (4): 660–673.
43. Wei, L., G. Vahedi, H.W. Sun, W.T. Watford, H. Takatori, H.L. Ramos, H. Takahashi, J. Liang, G. Gutierrez-Cruz, C. Zang, W. Peng, J.J. O'Shea, and Y. Kanno. 2010. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* 32 (6): 840–851.
44. Thieu, V.T., Q. Yu, H.C. Chang, N. Yeh, E.T. Nguyen, S. Sehra, and M.H. Kaplan. 2008. Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. *Immunity* 29 (5): 679–690.
45. Karamouzis, M.V., P.A. Konstantinopoulos, and A.G. Papavassiliou. 2007. The role of STATs in lung carcinogenesis: an emerging target for novel therapeutics. *Journal of Molecular Medicine (Berlin, Germany)* 85 (5): 427–436.
46. Li, Y., H. Du, Y. Qin, J. Roberts, O.W. Cummings, and C. Yan. 2007. Activation of the signal transducers and activators of the transcription 3 pathway in alveolar epithelial cells induces inflammation and adenocarcinomas in mouse lung. *Cancer Research* 67 (18): 8494–8503.
47. Meyer, T., and U. Vinkemeier. 2007. STAT nuclear translocation: potential for pharmacological intervention. *Expert Opinion on Therapeutic Targets* 11 (10): 1355–1365.
48. Chittezhath, M., G. Deep, R.P. Singh, C. Agarwal, and R. Agarwal. 2008. Silibinin inhibits cytokine-induced signaling cascades and down-regulates inducible nitric oxide synthase in human lung carcinoma A549 cells. *Molecular Cancer Therapeutics.* 7 (7): 1817–1826.
49. Nguyen, K.B., W.T. Watford, R. Salomon, S.R. Hofmann, G.C. Pien, A. Morinobu, et al. 2002. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science (New York, NY)* 297 (5589): 2063–2066.