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Thymol as a reciprocal regulator of T cell differentiation: Promotion of regulatory T cells and suppression of Th1/Th17 cells

Haideh Namdari[a], Maryam Izad[b], Farhad Rezaei[c], Zahra Amirghofran[a,d,*]

[a] Department of Immunology, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran
[b] Department of Immunology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[c] Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[d] Autoimmune Diseases Research Center, Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

Regulatory T cells (Tregs) are critical for maintaining immune response and enhancing their differentiation has therapeutic implications for autoimmune diseases. In this study, we investigated the effects of thymol a well-known monoterpene from Thyme on differentiation and function of Tregs. *In vitro* generation of Tregs from purified naïve $CD4^+CD25^-$ T cells in the presence of thymol was carried out. Suppressor activity of generated Tregs was examined by changes in the proliferation of CFSE-labeled conventional T cells. Thymol promotes differentiation of naïve $CD4^+CD25^-$ T cells to $CD4^+CD25^+Foxp3^+$ Tregs [66.9–71.8% *vs.* control (47%)] and increased intensity of Foxp3 expression on Tregs ($p < 0.01$). In functional assay, an increased immune suppression by thymol-induced Tregs (≈2.5 times of untreated Tregs) was detected. For *in vivo* study, thymol was intraperitoneally administered to ovalbumin (Ova)-immunized mice. Flow cytometry assessment of spleens from thymol-treated Ova-immunized mice showed increased number of $CD4^+$ $Foxp3^+$ Tregs (> 8%, $p < 0.01$(and decreased levels of $CD4^+$T-bet^+ Th1 and $CD4^+ROR\gamma t^+$ Th17 cells resulted in significant decreased Th1/Treg and Th17/Treg ratios. In *ex vivo* Ova challenge of splenocytes from thymol-treated Ova-immunized mice, similarly higher levels of $CD4^+$ $Foxp3^+$ Tregs, and also elevated TGF-β expression in $CD4^+Foxp3^+$ population (48.1% *vs.* 18.9% in untreated Ova-immunized group) and reduced IFN-γ-producing $CD4^+$T-bet^+ T cells and IL-17-producing $CD4^+ROR\gamma t^+$ T cells were detected. This led to marked decreased ratios of IFNγ/TGF-β and IL-17/TGF-β expressions. In conclusion, this study revealed thymol as a compound with enhancing effects on Treg differentiation and function, which may have potential benefits in treatment of immune-mediated diseases with Th1/Th17 over-activation.

1. Introduction

Regulatory T cells (Tregs) are essential for the maintenance of self tolerance and control of autoimmunity. These cells are defined by expression of the surface markers CD4 and CD25 and the transcription factor forkhead box P3 (Foxp3) [1]. Foxp3 is necessary for Treg cell lineage commitment. Moreover, continued expression of Foxp3 is required to maintain the function of mature peripheral Tregs [2]. The importance of $Foxp3^+$ Tregs in the control of autoimmunity has been shown in the Foxp3-deficient mice, in which over-activation of CD4 + T cells lead to developing autoimmune inflammation in multiple organs due to excessive release of cytokines [3]. Impairments in Treg numbers or function have also been demonstrated in various human autoimmune diseases [4,5].

One of the main features of Tregs is the secretion of cytokines with suppressive activity. The major Treg cytokine is transforming growth factor (TGF)-β [6]. This cytokine inhibits formation of inflammatory CD4 + T helper (Th)1 and Th17 cells and represses the release of their main cytokines, interferon-(IFN)-γ and interleukin (IL)-17 [7,8]. IFNγ secreted by Th1 cells, is responsible for the cells proinflammatory effect and induction of T-bet, a lineage-defining transcription factor for Th1 cells [9]. Th1 responses against self-antigens, are predominately responsible for generation of various types of autoimmune diseases such as multiple sclerosis, type 1 diabetes and rheumatoid arthritis [10].

Evidence also shows the involvement of Th17 cells and their key effector cytokine, IL-17 in pathogenesis of a number of autoimmune disorders [11,12]. Differentiation of these cells is directed by their master transcription factor retinoic acid-related orphan receptor γt ($ROR\gamma t$) [13]. The balance between Th1 and Th17 inflammatory CD4 + T subsets and Tregs is critical for controlling the immune responses. A

* Corresponding author at: Immunology Department, Medical School, Shiraz University of Medical Sciences, 71348-45794 Shiraz, Iran.
E-mail address: amirghz@sums.ac.ir (Z. Amirghofran).

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shift in the Th17/Treg equilibrium toward the pro-inflammatory Th17 side can lead to autoimmune disorders including rheumatoid arthritis, ankylosing spondylitis and psoriasis [12,14]. Also altering the balance between Th1 and Treg responses may be responsible for repeated rheumatic flares [15].

Thymol (2-isopropyl-5-methylphenol) also known as "hydroxy cymene" is the main monoterpene phenol found in the essential oils extracted from plants belonging to the Lamiaceae family, such as those of the genera Thymus [16]. Thymol has a long history of use for medical purposes [17]. This component is known for its antioxidant, anti-inflammatory, antiseptic, antibacterial, antifungal and healing properties as well as for its beneficial effects on the cardiovascular system [17]. In various studies the immunomodulatory effect of thymol has been shown [18–20]. Thymol has been revealed to ameliorate lipopolysaccharide (LPS)–induced inflammation in murine macrophage cell lines and mouse mammary epithelial cells [17]. In our previous study, we have observed increased expression of main dendritic cells markers including CD40, CD86 and major histocompatibility complex-II (MHC-II) on these cells in the presence of thymol [21]. Thymol reduced inflammatory responses through modulation of the expression of c-Jun N-terminal kinase (JNK), stress-activated protein kinases (STAT-3), activator protein-1 (AP-1) and nuclear factors of activated T-cells (NFATs) in LPS-treated macrophages [20]. We also showed that thymol could decrease IFNγ, T-bet, IL-17A and RORγc mRNA expressions in splenocyte cultures of mice immunized with ovalbumin (Ova) [22]. By these data, the suppressive effect of thymol on Th1 and Th17 cell-related specific transcription factors at gene expression level was shown. Considering the regulatory and suppressive role of Tregs on T cell-mediated immunity, whether the observed inhibitory effects of thymol is due to its possible enhancing effect on the number or function of Tregs have not yet been studied. Therefore, in the present study, we aim to explore the role of thymol on Tregs differentiation and function, and also to find the ratio of Th1/Treg and Th17/Treg at cellular level.

2. Materials and methods

2.1. Animals

Normal Female BALB/c mice between 6 to 8 weeks-old, weighting 25–30 g, were obtained from Center for Comparative and Experimental Medicine of Shiraz University of Medical Sciences (SUMS). Mice were kept under standard conditions with *ad libitum* access to chow and drinking water. Mice were sacrificed by cervical dislocation and spleens were removed under antiseptic conditions. Spleen cells were isolated by pushing the tissue through a mesh wire and then mononuclear cells were separated by centrifugation using Lymphodex (Gibco, Germany). After washing with phosphate buffered saline (PBS), they were counted using trypan blue dye staining. The study procedure was approved by the SUMS Ethics Committee and all experiments were carried out according to the National Institute of Health guide for the care and use of laboratory animals.

2.2. Real-time PCR for mRNA expression

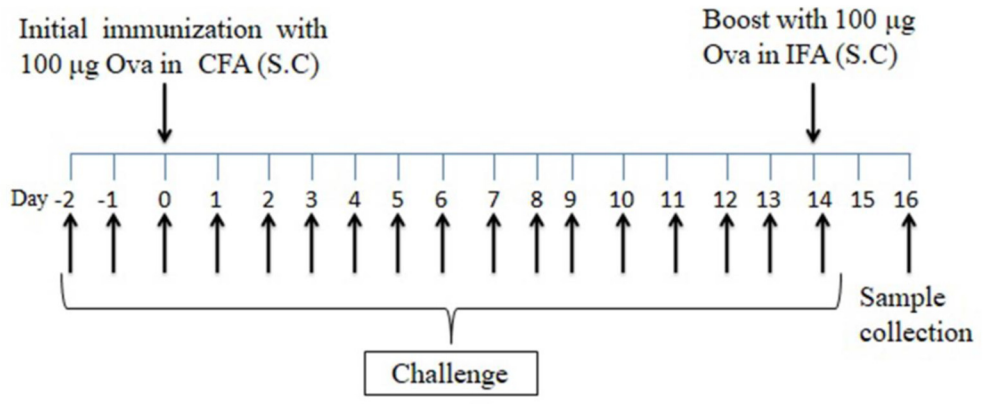
For quantifying the *in vitro* expression of Foxp3 and TGFβ mRNAs by real-time PCR, splenocytes were cultured at a density of 1×10^6 cells/well/ml in 24-well culture microplates coated with 2.5 μg/ml of anti-CD3 monoclonal antibody (mAb) (BD Biosciences, San Diego, CA). All mAbs used in this study purchased from BD Biosciences. Then, 2 μg/ml anti-CD28 mAb and thymol (Sigma-Aldrich, Germany) at 10 and 25 μg/ml concentrations were added and cells incubated in a CO_2 incubator. Wells containing antibody-stimulated cells without thymol were used as positive control and those without antibodies and thymol considered as negative control. Dimethyl sulphoxide (DMSO, Sigma-Aldrich, Canada) as the solvent (0.05%) was added to all control wells. After 72 h, cells were subjected to RNA extraction for gene expression changes using the RNX-plus buffer (Sinagen, Iran) according to the manufacturer's protocol. The integrity of total RNA was checked by agarose gel electrophoresis, as demonstrated by the presence of intact ribosomal RNA (28S and 18S bands). For determination of the purity of the extracted RNA, the relative absorbance at OD260/OD280 was measured. The concentration of the extracted RNA was measured using the NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE). Subsequently, the synthesis of complementary DNA (cDNA) was conducted using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) with the use of random primers. Primer design was carried out using Primer Express TM software (Applied Biosystems). The primer sequences used in this study were as follows: Foxp3; F 5′-AATAGTTCCTTCCCAGAG-3′, R 5′-GATTTCATTGAGTGT CCT-3′. TGF-β; F 5′-GCAACAACGCCATCTAT-3′, R 5′-AAGGTAACGCC AGGAAT-3′. GAPDH; F 5′-CGGTGTGAACGGATTTGGC-3′, R 5′-GTGAG TGGAGTCATACTGGAAC-3′. Each reaction was set in duplicate with the SYBR® Green qPCR SuperMix-UDG with ROX (Takara, Kyoto, Japan) on ABI thermocycler (Foster City, CA). Ten ng DNA and 200 nM of each primer pair were used for reaction mixes. The conditions for the RT-PCR were as follows: Initial denaturation at 96 °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. To determine the specificity of amplification, melting curve analysis was applied to all final PCR products. Real-time PCR data was analyzed using the standard ΔΔCt method. GAPDH mRNA was used as internal control for data normalization. Values for each gene after normalization were shown as the relative fold change (RFC) to positive control.

2.3. Isolation of $CD4^+$ naïve T cells and in vitro generation of $CD4^+$ $CD25^+$ Foxp3+ Tregs

Naïve $CD4^+$ $CD25^-$ T cells from mice spleens were purified using mouse Naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. For induction of naïve $CD4^+CD25^-$ T cells differentiation to Tregs, freshly isolated cells were cultured in 24-well plates coated anti-CD3 (5 μg/ml) in the presence of thymol (10 and 25 μg/ml). Then, anti-CD28 mAb (2.5 μg/ml), TGF-β (BD Biosciences) (2 ng/ml) and IL-2 (Life Technologies, MO) (100 units/ml) in X-*vivo*-15 serum free medium (Invitrogen, MO) were added. Cells stimulated at the same condition (without adding thymol) were considered as untreated control. After 96 h of incubation, a part of cells were collected for *in vitro* suppression assay and the rest were stained for CD4 and CD25 positivity using FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 mAbs. For analysis of intracellular Foxp3, cells were fixed and became permeable with Foxp3 Staining Buffer Set (BD Biosciences) and then stained with PE-labeled anti-Foxp3 mAb. Samples were run on a FACSCalibur (BD Biosciences) instrument and analyzed using Flowjo software (Treestar Inc., Ashland, OR).

2.4. In vitro suppression assay

To assess the inhibitory activities of Tregs generated in the presence or absence of thymol, $CD4^+CD25^-$ T cells were used as conventional T cells (Tconv cells). Tconv cells were labeled with 2.5 μM carboxyfluorescein-succinimidyl-ester- (CFSE, Invitrogen, Germany) and stimulated with anti-CD3 and anti-CD28 mAbs (STconv cells). Tregs (1×10^5 cells/100 μl) collected from previous experiment, were cultured with STconv cells at the same ratio in 96-well round bottom plates for 72 h. STconv cells alone and in co-cultured with Tregs in the absence of thymol (untreated) were used as controls. For analysis by flow cytometry, gating was applied on STconv cells and the intensity of CFSE staining was measured. Using the following formula the percentage of proliferation suppression (S) was determined [23]; $S = (a - b) / a \times 100$ where a and b are the percentages of proliferation in the absence and presence of Tregs, respectively.



Groups	Initial Immunization	Boost	Challenge
Ova-only	With Ova	With Ova	Olive oil
Thymol treated Ova-immunized	With Ova	With Ova	Thymol
Non-immunized	Saline	Saline	Olive oil

Fig. 1. Schematic diagram of the immunization of mice with Ova and challenge with thymol. Initial immunization with Ova was performed on day 0. Mice were boosted on day 14. Thymol was injected from two days before Ova immunization and continued to be injected every day till day 14. Two days later mice were sacrificed. The challenge periods with thymol are shown by vertical arrows. Non-immunized mice and Ova-only mice were used as controls.

2.5. Ova-immunization

We used Ova-immunized mice model to evaluate the effects of thymol on *in vivo* generation of Tregs in response to a specific antigen. Mice were randomly divided into three groups each consists of seven mice. Two groups of mice received one subcutaneous (sc) injection of 100 µg Ova (Sigma-Aldrich, Canada) emulsified 1:1 with complete Freund's adjuvant (Sigma-Aldrich). These mice were considered as Ova-immunized mice. A second immunization in these mice was performed by sc injection of 100 µg Ova in incomplete Freund's adjuvant (BioGene, Iran) two weeks later. The rest of mice were non-immunized and received only normal saline (sc). Of two groups of Ova-immunized mice, in one group thymol (80 mg/kg) was intraperitoneally (ip) administered two days before the first Ova challenge, followed by on every day for two weeks. In the other group olive oil as vehicle was injected (Ova-only mice) at the same days. Vehicle was also similarly administered in non-immunized group. On day 16, three mice from each group were randomly selected to aseptically remove their spleens for the next experiments (Fig. 1).

2.6. Determination of T cell subsets and cytokine secretion in Ova-immunized mice by flow cytometry

The spleens of above animals were directly examined for the number of Tregs and other $CD4^+$ T cell subsets. For this, staining of cells with FITC-conjugated anti-CD4 antibody was performed. After fixation and permeabilization, cells were stained with PE-conjugated anti-Foxp3, anti-T-bet and anti-RORγt mAbs.

The rest of isolated splenocytes were used for *ex vivo* analysis. Cells were cultured for 24 h in the absence (internal control) or presence of Ova (100 µg/ml) to measure the intracellular cytokine expression in Tregs and other CD4+ T cells after antigen challenge. To prevent the release of cytokines from the cells, during the first hour of culture brefeldin A was added. Cells were then transferred into tubes and stained with FITC-conjugated anti-CD4 mAb. After fixation and permeabilization of the cells, staining protocol was continued by adding PE-conjugated anti-Foxp3, anti-T-bet, anti-RORγt, and APC-labeled anti-TGFβ, anti-IFNγ and anti-IL-17 mAbs to the tubes. After washing, cells were finally re-suspended in PBS containing 2% paraformaldehyde, and then analyzed by flow cytometry as mentioned before.

2.7. Statistical analysis

Data obtained in this study were expressed as mean ± standard error (SE) of at least three independent experiments. Statistical analysis was performed using Student's *t*-test and one-way analysis of variance (ANOVA) at significance level of $p < 0.05$, with the help of SPSS (Abaus Concepts, Berkeley, CA) and GraphPad Prism 5 (San Diego, CA) softwares.

3. Results

3.1. In vitro effects of thymol on Foxp3 and TGF-β gene expressions

Real-time PCR was performed to analyze the gene expression levels of Foxp3 transcription factor in thymol treated-splenocytes. As shown in Fig. 2A, thymol significantly augmented Foxp3 mRNA levels at 10 µg/ml (2.08 ± 0.21 RFC, $p < 0.01$) and 25 µg/ml (1.73 ± 0.17 RFC, $p < 0.01$). We observed that treatment of splenocytes with both concentrations of thymol led to significant up-regulation of TGF-β mRNA expression to approximately 1.5 times greater than the positive control ($p < 0.001$) (Fig. 2B).

3.2. Effect of thymol on in vitro differentiation of naïve $CD4^+$ T cells to $CD4^+Foxp3^+$ Tregs

For *in vitro* differentiation of Tregs, we purified naïve $CD4^+CD25^-$ T cells from mice splenocytes. Fig. 3A shows the isolated cells with > 93% purity after staining with conjugated anti-CD4/anti-CD25 mAbs. Differentiation of naïve $CD4^+CD25^-$ T cells to Treg cells was conducted by simultaneous treatment of naïve cells with anti-CD3/anti-CD28 mAbs, TGF-β and IL-2 in the presence or absence of thymol. A representative flow cytometry analysis of the data from three independent experiments is shown in Fig. 3B. As illustrated in Fig. 3C, 10 µg/ml (66.95 ± 2.5%, $p < 0.01$) and 25 µg/ml (71.8 ± 5.13%, $p < 0.001$) of thymol increased the numbers of $CD4^+CD25^+Foxp3^+$ cells compared to untreated control (47%), indicating the ability of thymol to promote differentiation of naïve T cells to Foxp3+ Tregs. Quantification of Foxp3 mean fluorescence intensity (MFI) of expression disclosed that Tregs produced in the presence of 10 µg/ml ($p < 0.001$) and 25 µg/ml of thymol ($p < 0.01$) expressed greater levels of Foxp3 protein *versus* those from untreated cultures (Fig. 3D).

3.3. In vitro functional analysis of thymol–induced Treg cells

We further compared the suppressive activity of Treg cells generated in the presence of thymol (thymol-induced Tregs) with those generated in the absence of thymol (untreated). CFSE-labeled Tconv

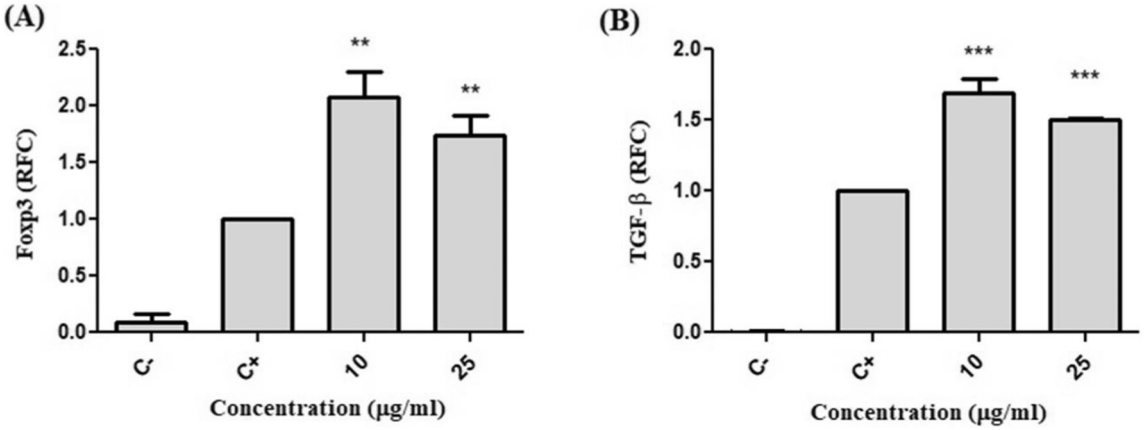


Fig. 2. Effects of thymol on Foxp3 and TGF-β gene expressions in the stimulated splenocytes of mice. Splenocytes were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies and cultured in the presence of thymol for 3 days. A) Foxp3 and B) TGF-β gene expressions were assayed by real-time PCR. Cells stimulated with antibodies cultured without thymol were used as positive control (C+) and those without antibodies and thymol considered as negative control (C−). Values represent mean ± standard error of three independent experiments. **$p < 0.01$, ***$p < 0.001$ compared to positive control. RFC, relative fold change.

cells were stimulated with anti-CD3/anti-CD28 mAbs and subsequently co-cultured with Tregs for 3 days. Fig. 4A, which is a representative flow cytometry analysis of the data, shows the results of proliferation suppression of Tconv cells in different conditions. The percentage proliferation suppression by Tregs induced in the presence of 10 µg/ml (54.3 ± 4.9%, $p < 0.01$) and 25 µg/ml (52.6 ± 8.085%, $p < 0.05$) of thymol was significantly higher than untreated Treg cells (21.7 ± 6.82%) (Fig. 4B), indicating the more functionality of thymol-induced Tregs.

3.4. *In vivo* effect of thymol on Treg differentiation and reciprocally repression of Th1 and Th17 differentiation in Ova-immunized mice

In order to find the effect of thymol on *in vivo* differentiation of Treg cells in response to specific antigen and the ratio with Th1 and Th17 subsets, we enumerated $CD4^+Foxp3^+$ Treg cells, $CD4^+T\text{-}bet^+$ Th1 cells and $CD4^+ROR\gamma t^+$ Th17 cells in spleens of non-immunized and Ova-immunized mice treated with or without thymol. As shown in Fig. 5A, Ova immunization significantly increased the number of different T cell subsets in mice. Thymol treatment of Ova-immunized mice increased the number of $CD4^+Foxp3^+$ Tregs to 8.71 ± 1.04% ($p < 0.01$(, indicated its potential to induce *in vivo* Tregs differentiation in response to specific antigen. This compound reciprocally decreased *in vivo* generation of $CD4^+T\text{-}bet^+$ Th1 (3.33 ± 1.17%, $p < 0.05$) and $CD4^+ROR\gamma t^+$ Th17 (1.96 ± 0.11, $p < 0.05$) subsets. We observed an obvious imbalance in the ratio of Th1 and Th17 to Tregs in spleens of Ova-immunized mice treated with thymol; the ratio of Th1/Treg cells was markedly decreased (0.36 ± 0.09%, $p < 0.05$) compared with untreated Ova-immunized mice (3.013 ± 0.6%) (Fig. 5A). Similarly, thymol reduced the ratio of Th17/Treg cells in spleen of treated mice (0.24 ± 0.03%) *vs.* untreated (2.09 ± 0.44) ($p < 0.05$). Fig. 5B shows a representative flow cytometry analysis of different T cell subsets in spleens of each mice group.

3.5. Effects of thymol on TGF-β secretion by Tregs and the ratio to IFN-γ and IL-17 upon ex vivo Ova challenge of splenocytes

To further elucidate the effect of thymol on Th1/Treg and Th17/Treg balance, spleen cells from Ova-immunized mice were cultured in the presence of Ova and examined for the changes in $CD4^+$ T cell subpopulations and the expression of the related specific cytokines. Representative flow cytometry data in dot plots and histograms which show the number of $CD4^+Foxp3^+$, $CD4^+T\text{-}bet^+$ and $CD4^+ROR\gamma t^+$ T cells and their positivity for TGF-β, IFNγ and IL-17 are shown in Fig. 6.

As shown, Ova challenge of splenocytes cultured from Ova-immunized mice has increased the number of $CD4^+Foxp3^+$, $CD4^+T\text{-}bet^+$ and $CD4^+ROR\gamma t^+$ T cells compared to those from non-immunized mice. Similar to the results obtained in direct assessment of $CD4^+$ T cell subsets in the mice spleen, Ova-challenge of splenocytes cultured from thymol-treated Ova-immunized mice showed increased numbers of $CD4^+Foxp3^+$Tregs (6.69 ± 1.36 *vs.* 2.33 ± 0.32 in control, $p < 0.05$) and decreased numbers of $CD4^+T\text{-}bet^+$ (0.48 ± 0.05 *vs.* 3.08 ± 1.24, $p < 0.05$) and $CD4^+ROR\gamma t^+$ cells (0.96 ± 0.13 *vs.* 6.78 ± 0.85, $p < 0.01$).

Evaluation of cytokine levels in each of these subsets demonstrated an efficient up-regulation in TGF-β protein expression, and down-regulation in IFN-γ and IL-17 expressions due to thymol treatment. We observed that the number of $CD4^+Foxp3^+TGF\text{-}\beta^+$ cells increased to 48.1 ± 5.9% relative to the level seen in cells from the untreated Ova-immunized group (18.9 ± 2.5%, $p < 0.05$) (Fig. 7A). In the thymol-treated group, the frequency of IFN-γ-producing $CD4^+T\text{-}bet^+$ T cells (4.62 ± 2.5%, $p < 0.01$) and IL-17-producing $CD4^+ROR\gamma t^+$ T cells (8.62 ± 1.22%, $p < 0.05$) was less than cells from the untreated Ova-immunized group (Fig. 7B, C). More importantly, by determination of cytokine ratios, we observed that IFNγ/TGF-β (0.10 ± 0.058%, $p < 0.01$) and IL-17/TGF-β (0.17 ± 0.024%, $p < 0.05$) ratios were markedly diminished in splenocytes from thymol-treated group after *ex vivo* Ova challenging compared with control (Fig. 7D, E). These findings provided further evidence for a shift in the Th1/Treg and Th17/Treg balance toward Treg differentiation.

4. Discussion

Thymol is a monoterpenoid phenol abundantly found in certain plants such as *Thymus vulgaris* [16]. In recent years, thymol has been considered for its significant anti-inflammatory, antioxidant and immunomodulatory effects [17]. In various studies the inhibitory effects of thymol on immune responses and on various cells and components of immune system has been shown [17]. Given the key role of Treg cells on reducing inflammation, regulation of the immune system and suppression of T cell-mediated immunity, we sought to investigate the potential effects of thymol *in vitro* and *in vivo* on Treg cells. Based on our previous study 10 µg/ml and 25 µg/ml concentrations of this compound which had shown no evidence of cytotoxicity [20], were used for the experiments. *In vitro* analysis of the effects of thymol on Tregs first was evaluated by assessing changes in Foxp3 and TGF-β gene expressions using real time-PCR. Transcription factor Foxp3 serves as a lineage specification factor of Treg cells [1] and is required for the generation and suppressive function of these cells [2]. Furthermore, TGF-β, as one of the cytokines released by Treg cells, has critical roles in suppressing

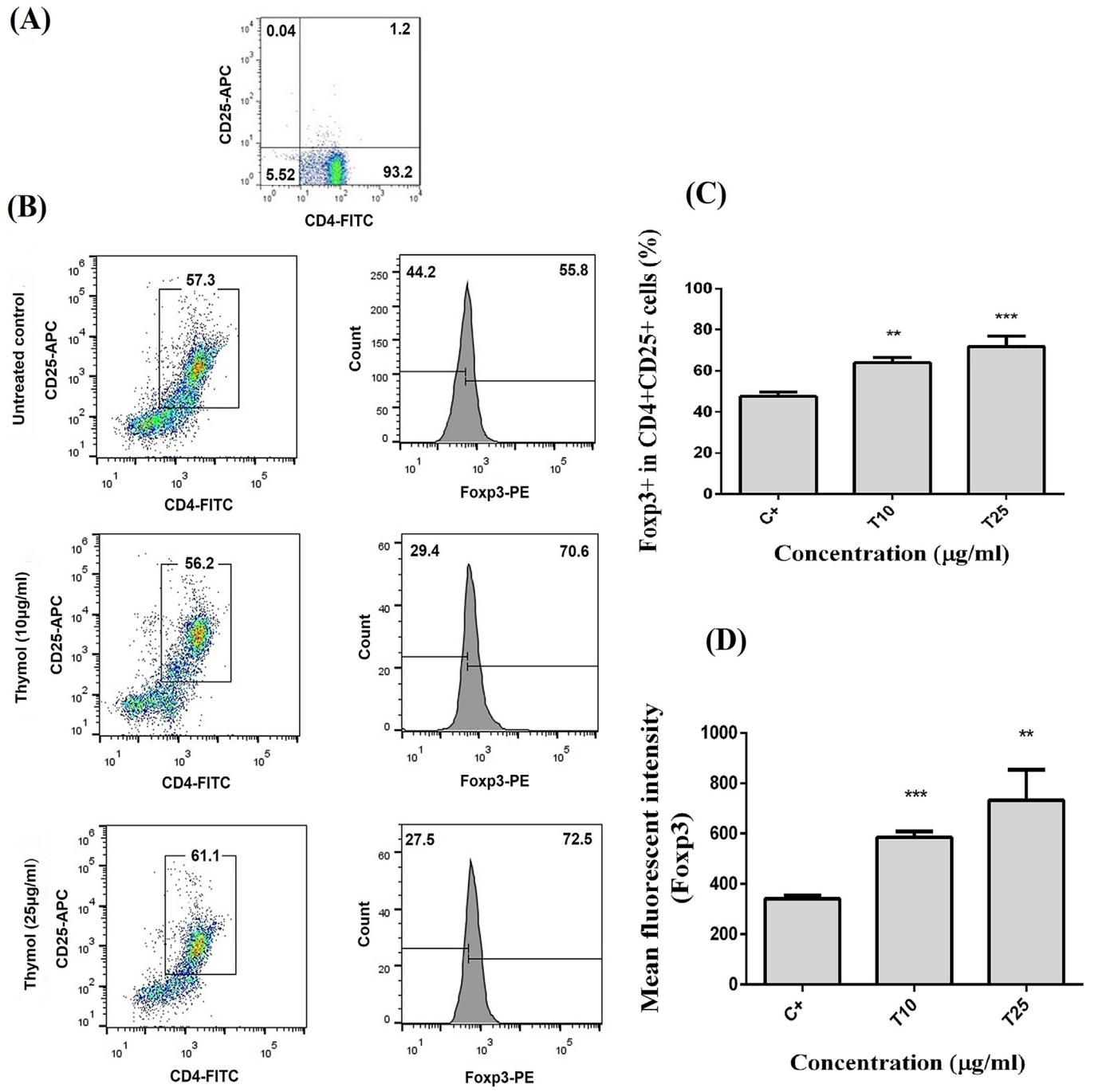


Fig. 3. Effect of thymol on *in vitro* generation of $CD4^+CD25^+Foxp3^+$ Tregs. $CD4^+CD25^-$ naïve T cells were isolated from splenocytes and activated with anti-CD3/CD28 antibodies, TGF-β and IL-2 in the presence of thymol (10 and 25 µg/ml) for 96 h. Percentage of $Foxp3^+$ cells in $CD4^+CD25^+$ population was determined using flow cytometry. Activated cells without thymol treatment were used as positive control (C^+). A) Shows the purity of isolated naïve CD4 + CD25-T cells (> 93%). B) Histograms depict Foxp3 expression in gated $CD4^+CD25^+$T cells. C). Bars indicate mean ± standard error of the percentage of $CD4^+CD25^+Foxp3^+$ Tregs. D) Foxp3 mean fluorescent intensity of expression. Flow cytometry dot plots and histograms are one representative out of three experiments. **p < 0.01, ***p < 0.001 compared to positive control.

immune responses [6]. We observed that thymol could augment the expression of Foxp3 and TGF-β expression to > 1.5 times compared to the control. In the next step we evaluated the effects of thymol on differentiation of naïve T cells to Tregs. The $CD4^+CD25^-$ from mice spleen were purified and exposed to Tregs differentiation condition in the presence of thymol. We showed that thymol had the capability to induce a higher rate of naïve T cells conversion to $CD4^+CD25^+Foxp3^+$T cells. Thymol also increased Foxp3 intensity of expression in generated Tregs compared to untreated control. These results suggested the positive impact of this compound on differentiation and development of Treg cells. As Treg cells have critical roles in induction of immune tolerance and immune homeostasis [2], using agents with the ability to promote the reduced quantities or functions of Tregs seen in certain diseases might have important therapeutic benefits [24].

We tested whether the thymol-induced Tregs were functional by evaluating their suppression function on Tconv cells. *In vitro* functional assay showed that thymol-induced Tregs suppressed Tconv

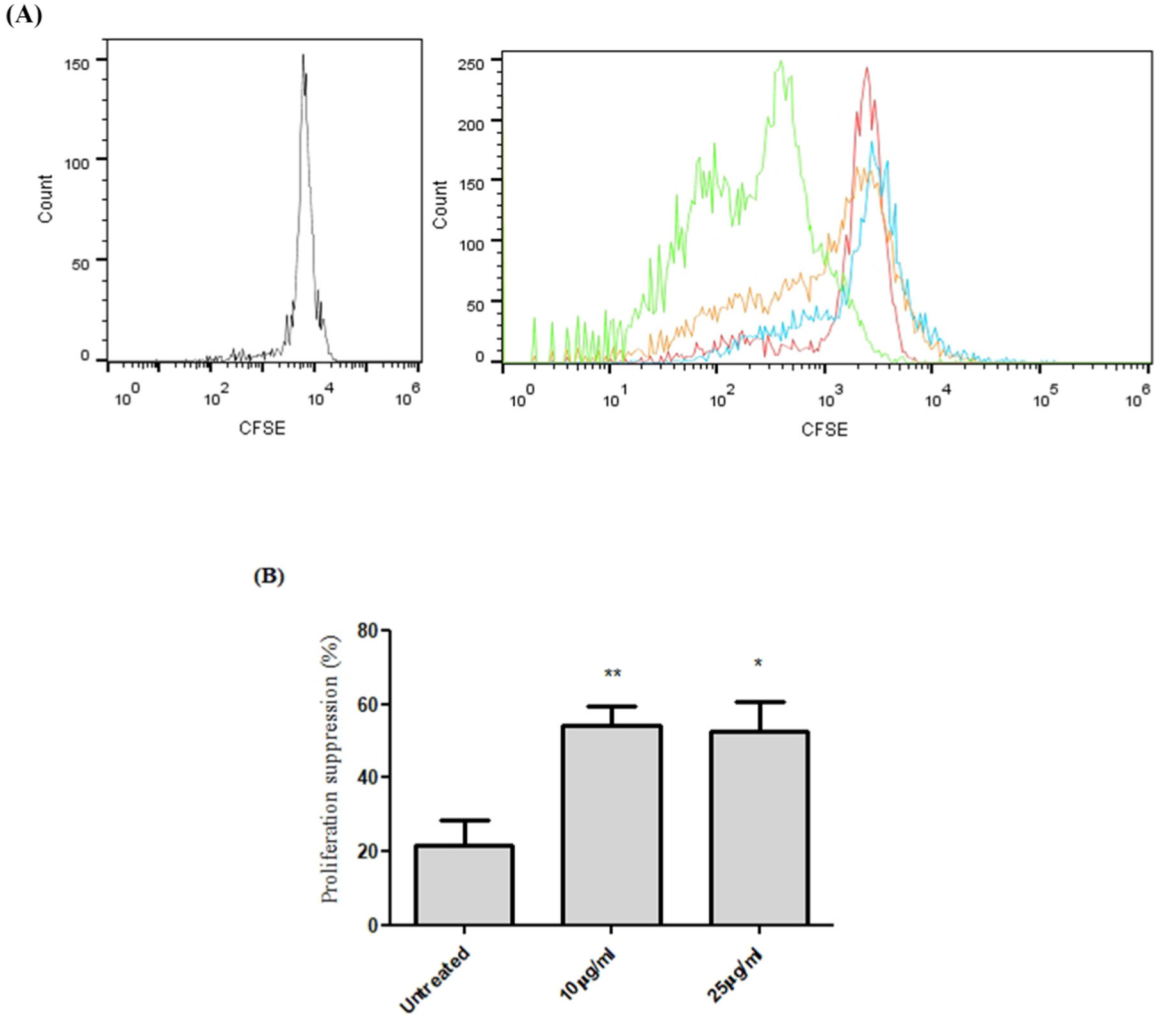


Fig. 4. Immunosuppressive potential of thymol-induced Tregs. $CD4^+CD25^+Foxp3^+$ Tregs obtained in the presence of 10 and 25 μg/ml thymol and those obtained in the absence of thymol (untreated control) were co-cultured with CFSE-labeled naïve $CD4^+CD25^-$ T cells stimulated with anti-CD3/anti CD28 antibodies (STconv cells). After 72 h, the proliferation suppression in STconv cells was determined using flow cytometry. A) Left side plot shows day 0 of CFSE labeling, right side shows the proliferation rate in STconv cells alone (green line) and in thymol-generated Tregs 10 μg/ml (red line) and 25 μg/ml (blue line) in comparison with untreated Tregs (orange line) after 3 days. Data shown is one representative out of three independent experiments at a 1:1 Treg:responder T cell ratio in all conditions. B) Bars indicate mean ± standard error of suppression of STconv cells proliferation in thymol-treated and untreated groups. *$p < 0.05$, **$p < 0.01$ compared to untreated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proliferation more efficient than untreated Tregs. The presence of Tregs with proper suppressive function against conventional T cells is a critical approach by which the immune system confines excessive T cell-mediated responses [25]. To the best of our knowledge this is the first report showing the enhancing effects of thymol on Treg cells differentiation and function.

In the *in vivo* analysis, we evaluated the modulatory effects of thymol in Ova-immunized mice model to evaluate the impact of thymol on the specific responses of Treg cells and the balance with inflammatory T cell subsets (Th1 and Th17). The splenocytes from the Ova-immunized mice treated with thymol were directly examined for the presence of Tregs, Th1 and Th17 cells. We found that, consistent with the *in vitro* results, thymol treatment of Ova-immunized mice increased the number of $CD4^+Foxp3^+$ T cells to > 8%. We also observed reduced numbers of $CD4^+T\text{-}bet^+$ and $CD4^+ROR\gamma t^+$ T cells in mice spleen showed the stimulatory effects of thymol on Treg differentiation and its suppression on Th1 and Th17 development. These results were in line with our previous study, which demonstrated down-regulation of T-bet and RORγt mRNA gene expression levels in thymol-treated splenocytes [22]. Th1 and Th17 cells have long been known to play an important role in inflammatory immune responses. Over-activation of these cells contributes to the pathogenesis of organ-specific autoimmune diseases such as type 1 diabetes and multiple sclerosis [14,15]. On the other hand, 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-β and IL-10 [26]. It is well known that imbalance of Th1 and Th17 to Tregs could derive immune system to an inflammatory state, which may contribute to the occurrence of autoimmune diseases [12,14,15].

We determined the ratio of $CD4^+T\text{-}bet^+$ cells to $CD4^+Foxp3^+$ cells and $CD4^+ROR\gamma t^+$ cells to $CD4^+Foxp3^+$ cells to find the balance of Th1/Treg and Th17/Treg cells in thymol-treated Ova-immunized mice. The results showed a marked decrease in both Th1/Treg and Th17/Treg ratios. In *ex vivo* analysis upon Ova-challenge of splenocytes from thymol-treated Ova-immunized mice, again enhancement in the number of $CD4^+Foxp3^+$ T cells and reduction in $CD4^+T\text{-}bet^+$ and $CD4^+ROR\gamma t^+$T cells compared to those from non-immunized mice was detected. As Tregs exert their main function through release of TGF-β, the intracellular expression of this cytokine in $CD4^+Foxp3^+$ T cells was examined. According to flow cytometry results, thymol up-regulated the expression level of TGF-β in $CD4^+Foxp3^+$ T cells to > 2.5 times of those from untreated Ova-immunized mice. This increase was

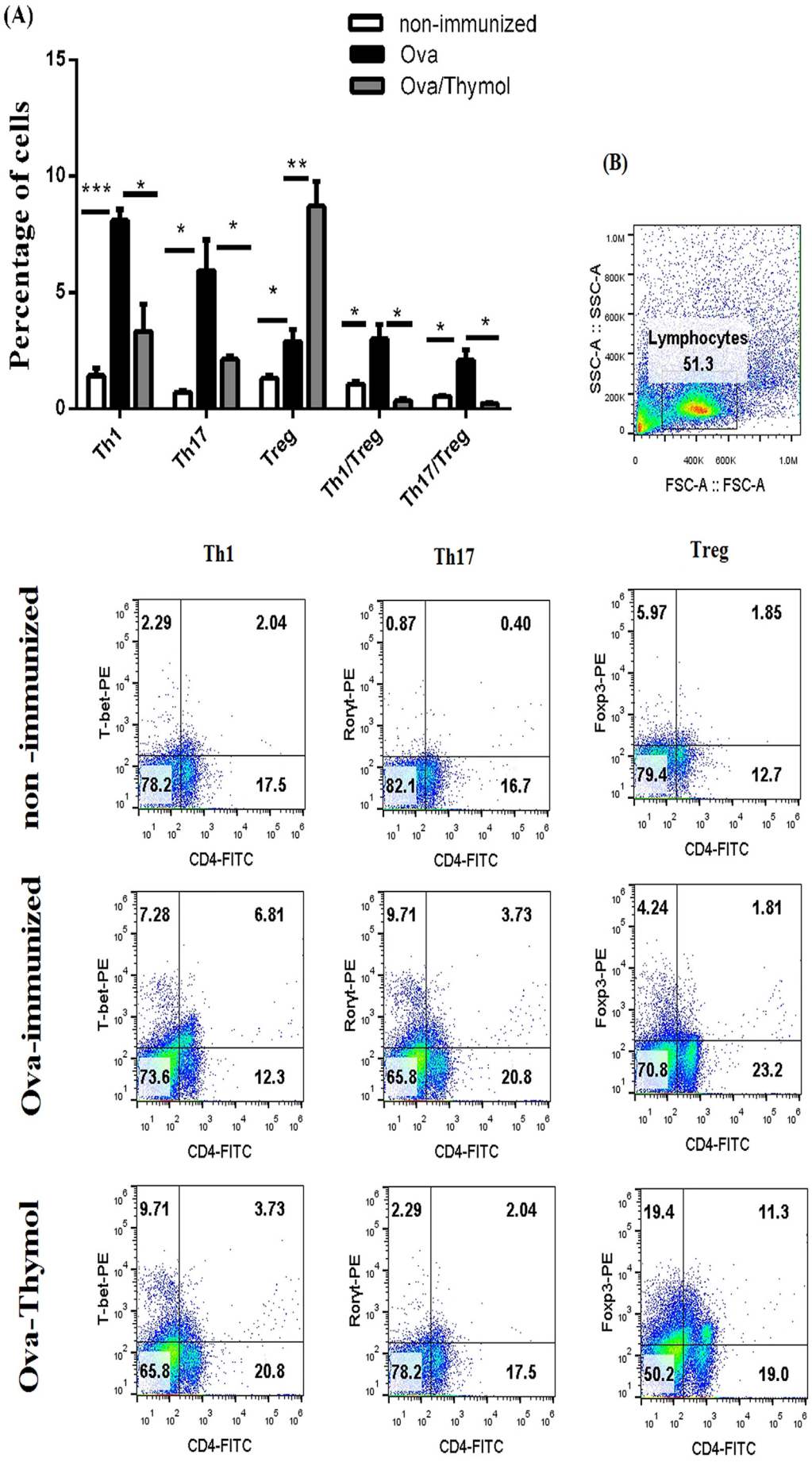


Fig. 5. Effects of thymol on the percentage of Tregs and other $CD4^+$ T cell subsets in spleen of treated Ova-immunized mice. Spleen cells from non-immunized, untreated Ova-immunized mice (Ova-only) and thymol-treated Ova-immunized mice were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-Foxp3, anti-T-bet and anti-RORγt for determining the frequencies of Treg, Th1 and Th17 cells by flow cytometry, respectively. A) Bars show mean ± standard error of the percentage of Th1, Th17 and Treg cells and the Th1/Treg and Th17/Treg ratios (n = 3/group). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to Ova-only mice. B) Representative dot plots of Th1, Th17 and Treg cells in the splenocytes of non-immunized and Ova-immunized and thymol-treated Ova-immunized mice. The values in the upper right quadrants indicate the percentages of each T cell subset.

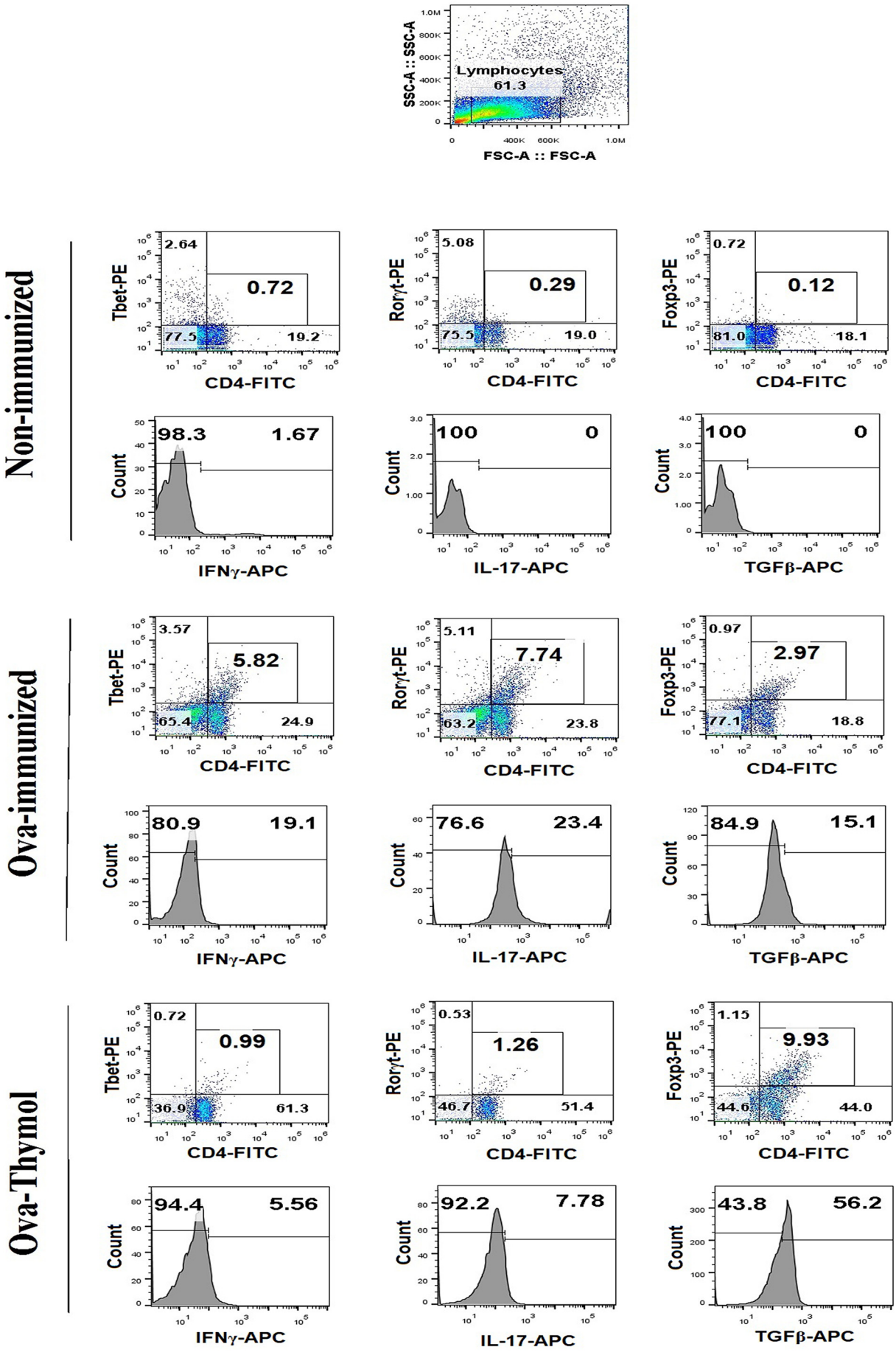


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Fig. 6. Effects of thymol on the percentage of Tregs and other $CD4^+$ T cell subsets upon *ex vivo* challenge of splenocytes with Ova. Splenocytes from non-immunized, untreated Ova-immunized mice and thymol-treated Ova-immunized mice were cultured with Ova for 24 h. Then the percentage of $CD4^+Foxp3^+$, $CD4^+T\text{-}bet^+$ and $CD4^+ROR\gamma t^+$ T cells was determined. After gating $CD4^+Foxp3^+$, $CD4^+Tbet^+$ and $CD4^+ROR\gamma t^+$ cells, intracellular expression of TGFβ, IFNγ and IL-17 in each related group was measured. One representative flow cytometry plots out of three separate experiments are shown.

accompanied by decreased IFNγ and IL-17 cytokine levels in $CD4^+T\text{-}bet^+$ and $CD4^+ROR\gamma t^+$ cells, respectively. Determination of the ratio of $IFN\gamma^+$ cells to TGF-β^+ cells and IL-17^+ cells to TGF-β^+ cells showed that thymol has markedly decreased these ratios. These findings provided further evidence for a shift in the Th1/Treg and Th17/Treg balance toward Treg differentiation.

There are a number of studies in which the modulatory effects of various plant natural products and their mechanisms of action on induction and activity of Treg cells have been shown. Baicalin, a compound with antioxidant activity isolated from the Chinese herb huangqin, induced Treg cell differentiation and promoted their *in vitro* regulatory activity. This compound restored the Foxp3 expression

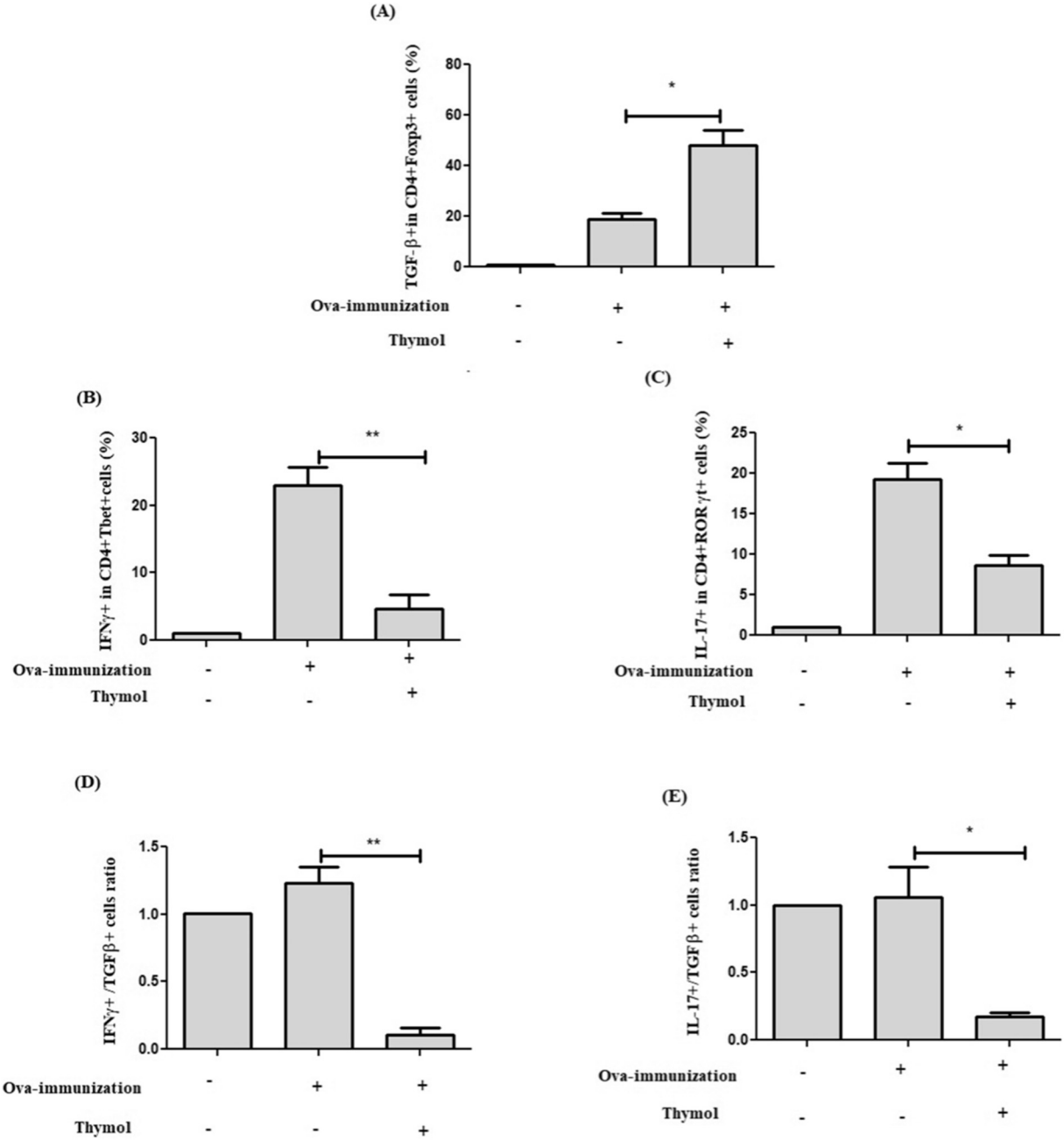

Fig. 7. Effects of thymol on the frequencies of $IFN\gamma^+$, IL-17^+ and TGF-β^+ T cells and the ratio of $IFN\gamma^+/TGF\beta^+$ and IL-$17^+/TGF\beta^+$ cells upon *ex vivo* challenge of splenocytes with Ova. Splenocytes obtained from non-immunized and Ova-immunized mice treated and untreated with thymol were cultured with Ova for 24 h in the presence of brefeldin A. The percentages of $IFN\gamma^+$, IL-17^+ and TGF-β^+ T cells were examined by intracellular staining using flow cytometry. Controls were cells from non-immunized mice and those from Ova-only mice. Data of each bar demonstrates mean ± standard error of: A–C) the percentages of $TGF\beta^+$, $IFN\gamma^+$ and IL-17^+ cells within $CD4^+Foxp3^+$, $CD4^+Tbet^+$, $CD4^+ROR\gamma t^+$ and T cells, respectively. D–E) The $IFN\gamma^+/TGF\beta^+$ and IL-$17^+/TGF\beta^+$ cells ratios. *$p < 0.05$, **$p < 0.01$ compared to Ova-only group.

following its initial IL-6-mediated inhibition [27]. In another study, grape seed proanthocyanidin extract (GSPE) acted on T cells to regulate their differentiation. Administration of this component to mice decreased the population of Th17 cells through inhibition of STAT3 activity and increased Treg cells through induction of STAT5 activity. These findings suggested the implication of this compound as a therapeutic agent in immune-mediated disorders such as autoimmune diseases [28].

Isoliquiritigenin and naringenin, two constituents from licorice promoted Treg cell induction both *in vitro* and *in vivo*. These components have also enhanced immunosuppressive function of Treg cells. Data showed that licorice could induce Treg cells by two mechanisms including inhibition of AKT and the mammalian target of rapamycin (mTOR) signaling, and activation of aryl hydrocarbon receptor (AhR) signaling [29].

At present we do not know which of the above mechanisms are involved in the thymol effects on Treg cell differentiation. Rapamycin a known inducer of Tregs has shown to act through mTOR inhibition [30]. Whether thymol works as rapamycin or as other natural products mentioned above or different pathways are involved in development of Tregs need to be evaluated in further studies.

In conclusion, to our knowledge, for the first time we revealed that thymol could improve Treg differentiation and change the Th1/Tregs and Th17/Tregs balance at cellular level. Added to its previously found anti-inflammatory functions, thymol is suggested as a safe alternative for treating autoimmune and inflammatory diseases.

Conflicts of interest

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

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