Synergistic effects of atorvastatin and all-trans retinoic acid in ameliorating animal model of multiple sclerosis

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One suitable approach to enhancing multiple sclerosis (MS) treatment is combination of available medications to provide more desirable outcomes. Immunomodulatory effects of atorvastatin and/or all-trans retinoic acid (ATRA) were determined in previous studies. The present study was set out to investigate the synergistic effects of combination therapy by suboptimal doses of atorvastatin and ATRA in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. EAE was induced by MOG\textsubscript{35–55} in female C57BL/6 mice. Therapies were initiated at day 12 post immunization when the mice developed a disability score and continued throughout the study until the day 33 when animals were sacrificed. Therapeutic treatment with half doses of atorvastatin and ATRA in combination has synergistic benefits causing the regression of clinical and neuropathological features of EAE more favorable than treatment with full doses of either drug alone. Without any advantage in anti-proliferative effect, combination treatment significantly reduced the secretion of pro-inflammatory cytokine interleukin-17 and conversely, increased the production of anti-inflammatory cytokine interleukin-10 more prominent than either drug alone. Furthermore, FoxP3\textsuperscript{+} Treg cells were significantly increased only in combination treatment. In conclusion, combined atorvastatin and ATRA have immunomodulatory synergistic benefits and this pharmacological approach may be as a useful strategy to control MS.

Keywords Multiple sclerosis, experimental autoimmune encephalomyelitis, Atorvastatin, all-trans retinoic acid, combination therapy

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS with underlying immunological abnormalities that lead to various neurological and autoimmune manifestations. MS is the major cause of non-traumatic disability in young adults (Mao & Reddy, 2010). Diverse classes of immunomodulator and immunosuppressive agents are accepted for MS treatment such as various interferon-\(\beta\)s, glatiramer acetate and mitoxantrone.
Nevertheless, many patients have a less than perfect response. On the other hand, MS is a very complex and heterogeneous disease. Therefore, it is impossible for a monotherapy to be beneficial in all patients during all periods of the disease (Conway & Cohen, 2010). One proper approach to control MS is to identify a rational combination of new drugs or existing medications (Conway & Cohen, 2010; Milo & Panitch, 2011; Mosayebi et al., 2011).

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for studying MS. Some therapies (mitoxantrone, copaxone and natalizumab) currently approved for MS were developed based on EAE studies (Conway & Cohen, 2010; Steinman & Zamvil, 2006).

Statins like atorvastatin, in addition to their lipid lowering effects, have anti-inflammatory, immunomodulatory and neuroprotective properties (van der Most et al., 2009). In vitro studies indicated that immunomodulatory properties of statins are comparable to interferin-β (Neuhaus et al., 2005). Early studies indicated possible beneficial effects of statins in EAE (Youssef et al., 2002) and MS (Völlmer et al., 2004). Along with these findings, appropriate safety margin, low cost compared with other MS therapies and oral once-daily prescription, place statins at the forefront of ideal candidates for combination with other MS medications (Milo & Panitch, 2011).

However, previous studies have determined anti-neoplastic and immunomodulatory properties of retinoids, a class of natural and synthetic derivatives of vitamin A. Clinically, they have been administrated for the treatment of cancers such as acute promyelocytic leukemia and Kaposi sarcoma and inflammatory diseases including acne and psoriasis (Klemann et al., 2009). Former studies demonstrated that all-trans retinoic acid, a member of retinoid family, suppressed inflammatory responses and tissue destruction and alleviated a variety of autoimmune diseases in murine models, such as rheumatoid arthritis (Nozaki et al., 2006), type I diabetes (Zunino et al., 2007), inflammatory bowel disease (Osanai et al., 2007) and EAE (Racke et al., 1995).

However, there is no or limited information about the role of combined atorvastatin and ATRA on MS and EAE. Also, to investigate combined treatment in the EAE mice by drugs that are individually effective, it is essential to test these medications in combination by suboptimal doses (Soos et al., 2002). According to the preceding descriptions, we tested whether combining the half doses of these agents could provide synergistic benefits in EAE.

MATERIALS AND METHODS

Materials

Complete Freund’s adjuvant (CFA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), pertuisis toxin, atorvastatin, all-trans retinoic acid (ATRA), dimethyl sulfoxide (DMSO), hematoxylin and eosin were procured from Sigma-Aldrich (St. Louis, MO, USA). Myelin oligodendrocyte glycoprotein 35–55 amino acid peptide (MOG35–55) was purchased from the AnaSpec Inc. (Fremont, CA). Mycobacterium tuberculosis H37RA was obtained from Difco (Detroit, MI, USA). Fetal calf serum and RPMI 1640 were purchased from GIBCO/Life Technologies Inc. (Gaithersburg, MD, USA). The cytokine assay...
by enzyme-linked immunosorbent assay (ELISA) kits for interferon gamma (IFN-γ), interleukin (IL)-10 and IL-17 were from Bender MedSystems (Vienna, Austria). Mouse Regulatory T Cell Staining Kit was bought from eBioscience (San Diego, CA, USA).

**Animals**

Female C57Bl/6 mice, 6–8 weeks of age, were purchased from Pasteur Institute of Iran. The mice were maintained under constant condition of temperature (22–24°C) and 12-h light/dark cycle and received food and water ad libitum. Animal welfare was observed in compliance with the regulations of the Ministry of Health, I.R. Iran approved by the Medical Ethics Committee of the University for Animal Studies.

**EAE induction and clinical evaluation**

EAE induction was performed as previously described (Mosayebi et al., 2007). In brief, mice were immunized subcutaneously in the flanks with 200 μg of MOG35–55 per animal emulsified in complete Freund’s adjuvant (CFA) containing 4 mg/ml of Mycobacterium tuberculosis H37RA. On the day of immunization and 48 h later, mice also received 400 ng of pertussis toxin by intraperitoneal (i.p.) injection. Individual animals were monitored daily, and clinical scores were assessed by two researchers blinded to the treatment group according to the following criteria: 0, no disease; 1, loss of tail tonicity; 2, tail paralysis; 3, weakness of hind limb; 4, hindlimb paralysis more marked at one side; 5, paralysis of both hindlimbs; 6, hind and forelimb paralysis; 7, moribund or death. Moreover, mice were weighed daily.

Mean clinical score was calculated by adding the daily clinical score for all mice in a group from day 12 post immunization (p.i.), when the mice developed a disability score, and then dividing by the total number of mice. Maximum mean clinical score was the mean clinical score at the peak of disease. Average mean clinical score was calculated by adding the mean clinical score from day 12 p.i. to day 33 p.i. and then dividing by 21.

**Treatment of mice**

Pharmacotherapy was initiated when all animals had developed signs of EAE. Mice were randomly divided into the following groups: vehicle-treated EAE, atorvastatin-treated EAE, ATRA-treated EAE, combination-treated EAE and normal control mice. Each group had seven animals and all medications were administrated by intraperitoneal (i.p.) injection. Atorvastatin (1 mg/ml) was dissolved in 2% DMSO and was administrated to the atorvastatin-treated EAE group at a daily dose of 200 μg per mouse (corresponding to 10 mg/kg body weight).

ATRA (2.5 mg/ml) was suspended in 2% DMSO and stored at −80°C as aliquots before use. ATRA was injected to ATRA-treated group at 500 μg per mouse (corresponding to 25 mg/kg body weight), every other day. These dosages were chosen in accordance with previous work on murine autoimmune models (Eller et al., 2010; Kinoshita et al., 2003; Nozaki et al., 2006; Van et al., 2009; Youssef et al., 2002). Each mouse of combination-treated EAE group received 100 μg of Atorvastatin, daily and 250 μg of ATRA, every other day. Vehicle-treated EAE mice received an equal volume of phosphate-buffered
saline (PBS) containing 2% DMSO. Normal control mice were immunized similar to EAE mice without MOG<sub>35–55</sub>. Also, these mice received an equal volume of PBS containing 2% DMSO with the same schedule of EAE mice. Treatment was continued at day 33 post induction when the mice were sacrificed.

**Splenocytes proliferation and cytokines production**
To determine the ex vivo cytokines production, spleen cells were aseptically isolated from mice on day 33 following induction of EAE and single-cell suspensions of splenocytes were made in RPMI 1640 medium supplemented with 10% fetal calf serum. Red blood cells (RBCs) were removed by RBC lysis buffer. Cell suspensions (2 × 10<sup>6</sup> cells/ml) were cultured in 24-well plates and stimulated with MOG<sub>35–55</sub> (50 μg/ml). The culture supernatants were collected after 72 h. IFN-γ, IL-17 and IL-10 production were determined by ELISA according to the manufacturer’s instructions. Proliferation was checked by MTT assay.

The splenocytes were plated in 96-well flat-bottomed plates in RPMI 1640 medium supplemented with 10% fetal calf serum (1 × 10<sup>5</sup> cells/100 μl/well) and stimulated with MOG<sub>35–55</sub> (50 μg/ml) or medium alone. After 72 h incubation, cultures were pulsed with 20 μl of the MTT solution (final concentration 5 mg/ml) for 4 h at 37°C. Then 150 μl DMSO was added and shaken vigorously to dissolve formazan crystal. The optical density (OD) at 550 nm was measured using a microplate reader (Dynatech, Denkendorf, Germany). The experiments were performed in triplicate sets. The results are expressed as the proliferation index according to the ratio of OD<sub>550</sub> of stimulated cells with MOG<sub>35–55</sub> to OD<sub>550</sub> of non-stimulated cells.

**Treg analysis by flow cytometry**
FoxP3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (FoxP3<sup>+</sup>Treg) cells were stained using Mouse Regulatory T Cell Staining Kit according to manufacturer’s instructions. In brief, 100 μl of freshly prepared splenocytes (1 × 10<sup>6</sup> cells) were stained with anti-mouse CD4 and anti-mouse CD25 for 30 min at 4°C. After staining surface markers, cells were incubated with fixation/permeabilization working solution for 30 min at 4°C. Next, the cells were washed with permeabilization buffer and incubated with anti-mouse Foxp3 antibody or isotype control at 4°C for at least 30 min in the dark. The cells were then washed with permeabilization buffer and suspended in flow cytometry staining buffer. All samples were analysed on DAKO flow cytometer (Partec, Germany), using Cyflogic software (version: 1.2.1).

**Histopathological examination**
On day 33 after disease induction, mice were euthanized under deep anesthesia. Brains were rapidly removed and fixed in 10% buffered formalin and embedded in paraffin. Sections (5 μm thick) were stained by hematoxylin and eosin. All sections were evaluated for inflammation and demyelination by two researchers blinded to the treatment group. Inflammation was scored as previously described (Chen et al., 2010): 0, no inflammation; 1, cellular infiltrate only in the perivascular areas and meninges; 2, mild cellular infiltrates in parenchyma (1–10/section); 3, moderate cellular infiltrates in.
parenchyma (11–100/section); 4, marked cellular infiltrate in parenchyma (>100/section). Demyelination was evaluated by scoring 0 to 3 for no, mild, moderate and severe lesions, respectively (Tafreshi et al., 2008).

**Statistical analysis**

Data are presented as means ± SEM and analysed by SPSS 18.0 software. Parametric data (cytokines production, splenocytes proliferation and FoxP3+ Treg cells analysis) were analysed using the one-way ANOVA plus Tukey’s post-hoc test. When data were nonparametric (clinical and histopathological scores), ranks were compared by Mann-Whitney U-test. *p* values less than 0.05 were considered statistically significant.

**RESULTS**

**Therapeutic treatment with combination of half doses of atorvastatin and ATRA alleviated EAE severity more favorable than either drug alone**

Following EAE induction, mice were monitored every day for clinical signs of disease. In order to evaluate the therapeutic effect of these drugs on established EAE, drug treatment was started at day 12 p.i. when individual mice developed a clinical score of ≥1. As shown in Table 1, maximum mean clinical score and average mean clinical score were markedly reduced in all drug-treated EAE mice, compared to vehicle-treated EAE group. However, reduction in average mean clinical score in EAE mice received half doses of atorvastatin and ATRA in combination was significantly more pronounced than treatment with each individual drug at its full doses (Table 1). Also, combination therapy decreased maximum mean clinical score further than treatment with either drug alone, although there was no statistical difference.

Statistical analysis of clinical scores showed that treatment with atorvastatin and ATRA in combination led to significant reduction of disease disability from day 17, compared to vehicle-treated EAE group. This period was started from

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>MMCS (mean ± S.E.M.)</th>
<th>AMCS (mean ± S.E.M.)</th>
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<tbody>
<tr>
<td>Combination</td>
<td>3.85 ± 0.24**</td>
<td>2.28 ± 0.12***</td>
</tr>
<tr>
<td>ATRA</td>
<td>4.28 ± 0.42*</td>
<td>3.09 ± 0.14***,#</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>4.85 ± 0.26*</td>
<td>3.39 ± 0.16**,#</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5.80 ± 0.18</td>
<td>4.16 ± 0.12</td>
</tr>
</tbody>
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Following EAE induction, the clinical signs of mice scored everyday from day 0 to 33. Drug treatment was initiated at day 12 p.i. when the mice developed a disability score and continued thought the study. The data from 7 mice per group were achieved and the mean maximum clinical score (MMCS) and average mean clinical score (AMCS) were calculated from day 12 post-induction as detailed under materials and methods. MMCS was significantly attenuated in all drug-treated mice without any advantage between groups, compared to vehicle-treated group EAE group. However, AMCS was significantly reduced in combination-treated mice at suboptimal doses, compared to mice received each of drugs alone at optimal doses. (*p* < 0.05, **p < 0.001, ***p < 0.0001 versus PBS-treated EAE group; #p < 0.001 versus combined- treated EAE group).
day 23 and 26 p.i. in ATRA- and atorvastatin-treated animals, respectively (Figure 1). Moreover, combination-treated mice showed maximum mean clinical score on day 17 p.i., whereas other groups showed maximum mean clinical score on day 18 p.i. (Figure 1). Together; these data implied that combination therapy can synergistically reverse the progression of EAE.

Although the ATRA-treated group lessened clinical disability more rapidly than atorvastatin-treated mice (Figure 1), clinical outcomes (maximum mean clinical score and average mean clinical score) of the two treatment groups weren’t significantly different (Table 1).

**Combination therapy reversed the weight loss of EAE mice further than each drug alone**

As shown in Table 2, Weight gaining in EAE mice was impeded and the mean body weight of these mice significantly reduced, compared to normal control mice. The extent of weight loss significantly inhibited in all drug-treated group, compared to vehicle-treated EAE animals. However, this beneficial effect was significantly more profound in EAE mice treated with atorvastatin plus ATRA compared to mice received each of the drugs alone.

Without any advantage in anti-proliferative effect, combined treatment attenuated pathogenic IL-17 and IFN-γ cytokines and conversely, increased
anti-inflammatory IL-10 greater than either drug alone. To determine the mechanism of disease amelioration, we measured the MOG\textsubscript{35–55}-induced secretion of cytokines from spleen cells in culture. A significant decrease in IL-17 and IFN-\(\gamma\) production in cells from drug-treated groups and a significant increase in IL-10 secretion except for cells from ATRA-treated group were found, compared to cells from the vehicle-treated group. Of note, combination treatment markedly attenuated IL-17 and conversely, increased IL-10 production more prominent than the similar findings brought about by each drug alone; compared with vehicle-treated EAE mice (Figures 2A–C).

In addition, a significant reduction in MOG-specific proliferation was observed in all drug-treated mice compared to the vehicle-treated EAE group. However, combined treatment didn’t have any synergistic effect in anti-proliferation effect compared with those treated individually with these drugs (Figure 2D).

Table 2. Weight evaluation of normal and EAE mice.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mean body weight (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control mice</td>
<td>20.14 ± 0.14</td>
</tr>
<tr>
<td>Vehicle-treated EAE mice</td>
<td>16 ± 0.12*</td>
</tr>
<tr>
<td>ATRA-treated EAE mice</td>
<td>17.78 ± 0.4*,$,##</td>
</tr>
<tr>
<td>Atorvastatin-treated EAE mice</td>
<td>17.4 ± 0.31*,$</td>
</tr>
<tr>
<td>Combined-treated EAE mice</td>
<td>18.38 ± 0.13*,$</td>
</tr>
</tbody>
</table>

The mice were weighed daily from day 0 to 33 post EAE induction. Pharmacotherapy was started when the mice developed a disability score (day 12) and continued throughout the study. The mean body weight of the EAE mice significantly decreased compared to normal control mice. The extent of weight loss significantly inhibited in all drug-treated group, compared to vehicle-treated EAE animals. Nevertheless, this advantage was significantly more favorable in combined-treated EAE group. (*\(p<0.0001\) versus Normal control mice; #\(p<0.001\) versus vehicle-treated EAE group; $\(p<0.05\), **\(p<0.001\) versus combined-treated EAE group).

Combination therapy promoted the expansion of Foxp3\textsuperscript{Treg}

To further explore the mechanism of disease attenuation, we next examined the level of Foxp3\textsuperscript{Treg} cell population in the splenocytes of mice at time of sacrifices. Evaluation method of Foxp3\textsuperscript{Treg} cells is shown in Figure (3A–C). Combination treatment with atorvastatin and ATRA significantly increased Foxp3\textsuperscript{Treg} cells compared to the vehicle-treated EAE group (Figure 3D). However, no significant change in the expression of these cells was observed upon treatment with either drug alone compared to the vehicle-treated EAE group (Figure 3D).

Combination therapy decreased the neuropathology of EAE more than each drug alone

To assess neuropathological effects of treatments, we studied two main characters of EAE: Inflammation and demyelination. In vehicle-treated mice, results showed that mononuclear cells tended to be concentrated in the meninges and then progressed into the parenchyma of the brain (Figure 4A).
Most of the lesions in this group predominated in meninges, cerebellum and subcortex with extensive perivascular cuffing into adjacent parenchyma (Figure 4).

Blinded analyses indicated that the severity of inflammation and demyelination in the brain sections was significantly regressed in EAE mice treated with atorvastatin plus ATRA, compared to vehicle-treated group (Figure 5). However, the severity of these pathological changes was markedly alleviated in the brain of EAE mice treated with these drugs individually at full doses, compared to the vehicle-treated EAE group, but these reduction weren't as profound as that observed from their combination at half doses (Figure 5). Although, we only report the results of neuropathology in the brain sections, the data obtained from spinal cords were parallel with brain pathologies (Data not shown).

**DISCUSSION**

Pharmacotherapy in MS patients is generally started after individuals have showed clinical symptoms of disease. Thus, we administrated all treatments when mice had developed signs of neurological dysfunction.
The goal of combination therapy is that the combination causes suppression of the disease and improves clinical outcomes better than treatment with either drug alone. In this regard, combination of two drugs with different mechanisms of action is a logical decision. Previous studies revealed that immunomodulatory effects of atorvastatin take place primarily through inhibition of synthesis of isoprenoid compounds in the mevalonate pathway, which are required for isoprenylation of several important cell-signaling proteins (Chow, 2009; van der Most et al., 2009; Zhang & Markovic-Plese, 2008). In contrast, retinoic acid has multiple effects on cell proliferation, differentiation and survival through specific family of nuclear receptors, the retinoic acid receptors (RARs) (Klemann et al., 2009). More importantly, our results showed that combination therapy with atorvastatin and ATRA at half doses synergistically ameliorated established EAE more impressive than full doses of either drug alone. This result was consistent with immunological and neuropathological assessments.

It is worthwhile that each medication considered for combination therapy has a good safety margin and doesn’t create additional toxicities when used in combination. In this regard, combination of two drugs with different mechanisms of action is a logical decision. Previous studies revealed that immunomodulatory effects of atorvastatin take place primarily through inhibition of synthesis of isoprenoid compounds in the mevalonate pathway, which are required for isoprenylation of several important cell-signaling proteins (Chow, 2009; van der Most et al., 2009; Zhang & Markovic-Plese, 2008). In contrast, retinoic acid has multiple effects on cell proliferation, differentiation and survival through specific family of nuclear receptors, the retinoic acid receptors (RARs) (Klemann et al., 2009). More importantly, our results showed that combination therapy with atorvastatin and ATRA at half doses synergistically ameliorated established EAE more impressive than full doses of either drug alone. This result was consistent with immunological and neuropathological assessments.

It is worthwhile that each medication considered for combination therapy has a good safety margin and doesn’t create additional toxicities when used in combination. So in the present study, we used two drugs that frequently prescribed in human diseases (Chow, 2009; Klemann et al., 2009; van der Most et al., 2009). In our study, no remarkable adverse event was observed in treatment groups. Moreover, mice in combined treatment group showed a significant weight gaining compared to other groups with EAE. Notably, the

![Figure 3](image-url)
combined treatment did not have any synergistic advantage in anti-proliferation effect and therefore immunosuppression effect compared with using each of drugs alone. Nevertheless, further studies need to be designed for evaluation of the exact mechanism(s) of the lack of synergistic effects on the anti-proliferation effect.

Previous data proposed that the cytokinic milieu produced by infiltrating CD4⁺ T lymphocytes has a substantial role in determining the extent of EAE and MS lesions (Jager et al., 2009; Lees et al., 2008). EAE and MS have long been believed to be mediated by Th1 cells. However, this view was doubted because mice deficient in components of the IL-12/Th1 axis such as IL-12α (IL-12p35), IFN-γ or IFN-γ receptor were more susceptible to EAE. Based on current findings, it seems that IL-17-producing CD4⁺ T cells (Th17) are essential to the pathology of EAE because mice lacking IL-17 are resistant to EAE (Fletcher et al., 2010; Korn et al., 2007a), treatment of mice with an IL-17-blocking antibody suppresses development of EAE (Hofstetter et al., 2009) and transfer of Th17 cells result in severe disease (El-Behi et al., 2010; Fletcher et al., 2010).

**Figure 4.** Histological outcomes in the brain sections of EAE mice treated with vehicle (A–E) and combination therapy (F). Representative sections of vehicle-treated EAE groups depict mononuclear cell infiltrates in the meninges (A, X400), subcortex along with deep brain parenchyma (B, X100) and cerebellum (C, X400); and also depict extensive perivascular cuffing in the brain parenchyma (D, X100) and extensive demyelination in the vicinity of a blood vessel with perivascular cuff in the brain white matter (E, X400). Conversely, Representative section of combination treated EAE mice depicts few infiltrates only around perivascular area and mild demyelination (F, X400). All sections were stained with hematoxylin and eosin.
IL-17 (also called IL-17A) has a potent pro-inflammatory property (Korn et al., 2007a) and is a crucial factor for Th17-mediated encephalopathogenesis (Jadidi-Niaragh & Mirshafiey, 2011). The main function of IL-17 in immunopathogenesis of EAE and MS is the breakdown of blood–brain barrier, which leads to large infiltration of various inflammatory cells like Th1 into the CNS and finally neuroinflammation and demyelination (Jadidi-Niaragh & Mirshafiey, 2011). Overall, it seems that Th17 cells are more pathogenic than Th1 cells (Korn et al., 2007a). Although, previous findings have determined the immunomodulatory potential of atorvastatin and ATRA in EAE model, the immunomodulation was mostly justified to reduction in IFN-\(\gamma\) production (Chow, 2009; Massacesi et al., 1987; Racke et al., 1995; van der Most et al., 2009).

Our results showed that treatment with ATRA and/or atorvastatin reversed EAE, at least partly by suppressing of IL-17 production. These findings offer new insight into the potential mechanisms underlying the beneficial effects of ATRA and/or atorvastatin in ameliorating EAE. Moreover, combination therapy synergistically suppressed IL-17 level greater than either drug alone upon antigen-specific restimulation. However, the level of IFN-\(\gamma\) did not show any significant differences between drug-treated groups.

IL-10, a cytokine with anti-inflammatory properties, plays an important function in limiting and terminating inflammatory reactions and subsequent preventing of tissue destruction (Asadullah et al., 2003; Saraiva & O’Garra, 2010). While IL-10 was initially described as a Th2 cytokine, further evidence revealed that IL-10 was produced by a wide range of immune cells, including Th1, Th2 and Th17 cell subsets and Treg cells, verifying its indispensable
function as a feedback regulator of various immune responses (Saraiva & O'Garra, 2010). Our data showed that combination therapy synergistically increased anti-inflammatory IL-10 more than Individual therapy upon antigen-specific restimulation.

FoxP3⁺ Treg cells possess a crucial role in maintaining self-tolerance via their inhibitory properties on effector T lymphocytes especially during Th17 immune responses (Oukka, 2007). Some evidence suggests that differentiation of Th17 and FoxP3⁺ Treg is reciprocally regulated by transforming growth factor β (TGF-β) and IL-6. TGF-β is an essential factor for development of FoxP3⁺ Treg, while TGF-β plus IL-6, an inflammatory cytokine, promote differentiation of Th17 cells (Dong, 2009). In vitro studies have demonstrated that ATRA and/or atorvastatin can suppress Th17 differentiation and promote generation of FoxP3⁺ Treg cells (Elias et al., 2008; Kagami et al., 2009; Klemann et al., 2009). Unlike the in vitro studies, individual treatment with ATRA or atorvastatin in our study had no significant effect on FoxP3⁺ Treg cell population.

A former study showed a significant reduction of Th17 cells development without any change in Treg cell population in mice orally infected with Listeria monocytogenes and treated with Retoinc acid. Authors suggested that TGF-β might be a limiting factor in the decline of Treg cell expression induced by exogenous Retoinc acid in vivo (Mucida et al., 2007). Also, it has been reported that the high level of IL-6 could inhibit de novo generation of antigen specific Treg cells (Korn et al., 2007b). While ATRA administration increased FoxP3 expression and decreased IL-17 expression in colon tissues and mesenteric lymph node cells of BALB/C mice with autoimmune colitis (Bai et al., 2009), similar treatment with ATRA in diabetic NOD mice up-regulated only differentiation of Treg cells in splenocytes without any change in Th17 population (Van et al., 2009). Moreover, a recent study indicated that atorvastatin down-regulated the Th1 and Th17 response in C57Bl/6 mice with anti-glomerular basement membrane glomerulonephritis without affecting Treg cell population (Eller et al., 2010).

Importantly, in the present report, FoxP3⁺ Treg cells were significantly increased only in combination treatment. We propose that the minor expansion of Treg cells by individual therapy in vivo may be due to the profound production of inflammatory cytokines (such as IL-6) or conversely low level production of TGF-β in cytokinic milieu induced by MOG35–55 and CFA immunization. But combination treatment has synergistically more anti-inflammatory effect, therefore promote expansion of Treg cells. This hypothesis can also be considered that differences can be seen in the mentioned reports may be due to difference in cytokinic profile induced following specific autoimmune diseases induction or inbred strain variations.

The capability of these drugs to down-regulate MOG35–55-specific lymphocyte proliferation limits the number of potentially encephalitogenic T lymphocytes, which has been known to be as an essential factor in EAE pathogenesis (Lovett-Racke & Racke, 2002). Recently, it was demonstrated that attenuation property of standard administrated medications for MS individuals is somewhat due to the inhibition of Th17 cells (Jadidi-Niaragh & Mirshafiey, 2011). Based on our findings in mouse model of MS, combined treatment caused a profound reduction in IL-17 level. Therefore, adding this combination in the treatment regimens of MS may have beneficial effects.
CONCLUSION

Our study demonstrated for the first time that therapeutic treatment with combination of half doses of atorvastatin and ATRA provides synergistic effects and leads to better outcomes than their individually administered full doses in EAE mice. Moreover, in this report we demonstrated that the beneficial effects of this combination in treatment of EAE may be partly due to immune deviation from pro-inflammatory cytokine IL-17 to anti-inflammatory cytokine IL-10 and Foxp3⁺Treg induction more pounced than that observed from monotherapy. However, this survey is a preliminary study. Probably, other mechanisms may also be involved in the synergistic benefits of this combination, and these remain to be clarified. Altogether, this pharmacological approach may be as a promising strategy for the therapy of MS.

DECLARATION OF INTEREST

This study was fully sponsored by Urmia University, Urmia, Iran. The authors report no conflicts of interest. All authors read and approved the final manuscript.

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