

Atorvastatin upregulates regulatory T cells and reduces clinical disease activity in patients with rheumatoid arthritis

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Abstract In this study, we investigated the hypothesis that regulatory T cells (T_{reg}) are involved in the immunomodulatory effects of statins on rheumatoid arthritis (RA) patients. The 12-week study cohort consisted of 55 RA patients and 42 control subjects allocated to either a group treated with atorvastatin (AT) (20 mg/day) or a non-AT group. T_{reg} numbers, suppressive function, serum inflammatory markers, and disease activity were evaluated before and after the therapy. Furthermore, the effects of AT on the frequency and suppressive function of T_{reg} were determined in vitro. Our data revealed that the suppressive function of T_{reg} from RA patients significantly decreased compared with that of control subjects. AT significantly reduced erythrocytopenia, C-reactive protein, and disease activity. Concomitantly, T_{reg} numbers and suppressive functions were significantly improved by AT. Consistent with the in vivo experiments, AT promoted the generation of T_{reg} from primary T cells and enhanced preexisting T_{reg} function in vitro. Moreover, we showed that PI3K-Akt-mTOR and ERK signal pathways were involved in the induction of T_{reg} by AT. **In conclusion, AT significantly increased T_{reg} numbers and restored their suppressive function in the RA patients, and this may be relevant in the modulation of uncontrolled inflammation in this disorder.**—Tang, T-T., Y. Song, Y.J. Ding, Y-H. Liao, X. Yu, R. Du, H. Xiao, J. Yuan, Z-H. Zhou, M-Y. Liao, R. Yao, H. Jevallee, G-P. Shi, and X. Cheng. **Atorvastatin upregulates regulatory T cells and reduces clinical disease activity in patients with rheumatoid arthritis.** *J. Lipid Res.* 2011. 52: 1023–1032.

Supplementary key words HMG-CoA reductase inhibition • immunity • cell signaling • drug therapy/hypolipidemic drugs • inflammation • lymphocytes • statins

This study was supported by the National Natural Science Foundation of China (grants 30600234 and 30871067), the Program for New Century Excellent Talents in the University of China (NCET-09-0380), and the National Basic Research Program of China (973 program grants 2007CB512000 and 2007CB512005).

Manuscript received 30 August 2010 and in revised form 2 March 2011.

Published, JLR Papers in Press, March 2, 2011
DOI 10.1194/jlr.M010876

Regulatory T cells (T_{reg}), a subset of T cells that constitutively expresses CD4 and CD25, play a crucial role in preventing autoimmune disorders and actively controlling autoimmune responses (1). Foxp3, a member of the forkhead/winged-helix family of the transcriptional factor, has been identified as the best marker of T_{reg} (2). Recently, a downregulation of CD127 has been shown to be closely correlated with Foxp3 (3). Thus, it can be used as a reliable surface marker for T_{reg} . It has been well documented that T_{reg} are involved in the pathogenesis of autoimmune disorders, such as multiple sclerosis (4) and type 1 diabetes (5), and they have certain protective effects.

Rheumatoid arthritis (RA) is a chronic inflammatory arthropathy associated with systemic inflammation and often leads to clinically significant functional impairment. The etiology of RA is unclear. However, it is accepted that autoimmune responses play distinct roles in the pathogenesis of RA. Studies of experimental models of inflammatory arthritis have revealed that T_{reg} are the protective regulators of the disorder (6–8). However, to date, there is limited information about the role of T_{reg} in RA (9–14).

Statins, which inhibit 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, have been shown to possess anti-inflammatory and immunomodulatory properties and contribute to cholesterol reduction (15). A number of studies have shown that statin treatment benefits RA patients, which

Abbreviations: Con, control; DAS28, Disease Activity Score 28; DMARD, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; hs-CRP, highly sensitive C-reactive protein; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; NSAID, nonsteroidal anti-inflammatory drug; PBMC, peripheral blood mononuclear cell; RA, rheumatoid arthritis; TC, total cholesterol; TG, triglyceride; TNF- α , tumor necrosis factor- α ; T_{reg} , regulatory T cell; T_{resp} , T responder cell.

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may be partly due to its immunomodulatory properties (16, 17). In this study, we employed both in vivo and in vitro strategies to evaluate the effects of statins on T_{reg} in RA patients and tested the hypothesis that T_{reg} are involved in the immunomodulatory effects of statins on RA patients.

MATERIALS AND METHODS

Subjects

In this study, we enrolled 55 patients with active RA who fulfilled the 1987 American College of Rheumatology criteria for RA (18). A patient was determined to have active RA if he or she met at least two of the following criteria: (1) the patient had six tender joints, (2) 45 min of morning stiffness, and (3) three swollen joints. The inclusion criteria included a history of RA for at least 1 year of the ongoing active disease and the use of stable doses of disease-modifying antirheumatic drugs (DMARD) for at least 3 months before recruitment. Exclusion criteria included inability to give informed consent, pregnancy or lactation, dyslipidemia, use of any lipid-lowering medication, presence of known hepatic disease or elevated liver transaminase levels within the previous 3 months, and hydroxychloroquine treatment in the previous 3 months. We also included 42 control subjects matched by age and gender. The investigation conformed to the principles outlined in the Declaration of Helsinki. The trial was approved by the country's ethics committee, and the patients and controls provided written informed consent.

Study design

The patients continued to take stable doses of prestudy DMARD, nonsteroidal anti-inflammatory drugs (NSAID), and prednisone during the study. The subjects were randomly allocated to either a group receiving AT treatment (20 mg daily, RA-A and control [Con]-A) or a group that did not receive AT (RA-C and Con-C) for 12 weeks. RA disease activity was evaluated using the Disease Activity Score 28 (DAS28) instrument at the baseline and after the therapy. DAS28 was calculated as previously described (19). Blood samples were collected at the baseline (RA-AB, RA-CB, Con-AB, and Con-CB) and at 12 weeks after the therapy (RA-A1, RA-C1, Con-A1, and Con-C1). After centrifugation, serum was obtained for the assessment of total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), highly sensitive C-reactive protein (hs-CRP), interleukin 6 (IL-6), and intercellular adhesion molecule-1 (ICAM-1). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (Sigma-Aldrich) and were then used for serial analyses.

Flow cytometry analysis

The following antibodies were used: anti-CD4-PerCP, anti-CD25-FITC, anti-CD127-Alexa Fluor® 647, anti-CD45RO-APC, anti-CD62L-APC, anti-HLA-DR-APC, and anti-Foxp3-PE (all from eBioscience). For the surface staining, cells were incubated with antibodies for 20 min at 4°C. For the intracellular staining of Foxp3, cells were fixed and permeabilized according to the manufacturer's instructions before an antibody was added. Isotype controls were used to allow for correct compensation and to confirm antibody specificity. The samples were analyzed using flow cytometry on a FACSCalibur (BD Biosciences).

Foxp3 expression by real-time PCR

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using

random hexamer primers and RNase H-reverse transcriptase (Invitrogen). The sequences of the primers were as follows: Foxp3 forward, 5'-GAAACAGCACATTCCCAGAGTTC-3', and reverse, 5'-ATGGCCCAGCGGATGAG-3'; and GAPDH, forward, 5'-CCA-CATCGCTCAGACACCAT-3', and reverse, 5'-GGCAACAATATC-CACTTTACCAGAGT-3'. The samples were analyzed with an ABI Prism 7900 sequence detection system (Applied BioSystems) using SYBR Green Master Mix (Takara, Japan). The relative expression level of Foxp3 was normalized with GAPDH as a housekeeping gene and was calculated by the $2^{-\Delta\Delta Ct}$ method.

Cell isolation

$CD4^+CD25^-$ primary T cells and $CD4^+CD25^+CD127^{low}$ T_{reg} were magnetic-sorted using a $CD4^+CD25^+CD127^{dim/-}$ regulatory T-cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. Briefly, PBMCs were first incubated with a biotin-labeled cocktail of antibodies, $CD4^+CD127^{low}$ T cells were isolated using negative selection, and then $CD25^-$ T cells were removed using positive selection after incubation with anti-CD25 microbeads. A purity of >90% was confirmed for $CD4^+CD25^-$ T cells and $CD4^+CD25^+CD127^{low}$ T_{reg} by flow cytometry.

Cell culture

Pure AT (Honghui medicine Co., Ltd., Beijing, China) was dissolved in 2% DMSO-ethanol (the carrier was tested as a vehicle control). L-Mevalonic acid lactone (Sigma-Aldrich) was activated in 1 N NaOH and then neutralized with 1 N HCl to pH 7.2.

Magnetic-sorted $CD4^+CD25^-$ T cells from the RA patients were prepared according to the manufacturer's instructions and incubated at a density of 2×10^6 cells/ml in RPMI 1640 medium with 100 U/ml penicillin and 100 μ g/ml streptomycin, 2 mM glutamine, and 10% (v/v) heat-inactivated fetal bovine serum (Gibco BRL) in the presence of 2 μ g/ml plate-bound anti-CD3 (OKT3; eBioscience), 2 μ g/ml soluble anti-CD28 (eBioscience) antibodies, and 20 IU/ml IL-2 (Peprotech). AT (1, 5, and 10 μ M) was added, and the cells were then incubated for 5 days at 37°C in 5% CO_2 . In some experiments, 200 μ M L-mevalonate and the inhibitors of ERK (U0126, 5 μ M) and PI3K (LY294002, 5 μ M) were added to the culture by themselves or with AT.

Suppression assays

To assess the suppressive function, $CD4^+CD25^-$ T cells (T responder cells [T_{resp}]) and $CD4^+CD25^+CD127^{low}$ T_{reg} were cocultured at T_{resp}/T_{reg} ratios of 1:0, 4:1, 2:1, and 1:1 in 200 μ l of RPMI 1640 medium. Plate-bound anti-CD3 (2 μ g/ml; eBioscience) and soluble anti-CD28 (5 μ g/ml; eBioscience) (anti-CD3/28) were added at the beginning of the experiment. After 5 days of culture, 3H -labeled thymidine (1 μ Ci/well) was added 16 h before the culture was terminated. The cells were harvested and assayed by scintillation counting. Isolated $CD4^+CD25^+CD127^{low}$ T_{reg} were harvested and washed vigorously with medium after a 24-h incubation period with AT. Then, T_{reg} were cocultured with T_{resp} and tested using the suppression assay. Blood samples were obtained again on the next day, and T_{resp} were freshly isolated when T_{reg} were preincubated with AT.

Cytokines detection

Cultures of 1×10^5 magnetic-sorted $CD4^+CD25^+CD127^{low}$ T_{reg} and 1×10^5 $CD4^+CD25^-$ T cells were cocultured with anti-CD3/28 in U-shaped-bottom 96 well plates to a final volume of 200 μ l. Three days later, culture supernatants were collected, and tumor necrosis factor- α (TNF- α) and IFN- γ were quantified using ELISA kits (eBioscience) according to the manufacturer's instructions.

Western blotting

The CD4⁺CD25⁻ T cells from the RA patients were preincubated with AT (5 μM) for 2 h and then stimulated with anti-CD3/28. At different times, cell lysates were prepared using a 1% NP-40 lysis buffer, and the protein concentration in the lysates was determined using a BCA protein kit (Pierce). Protein extract (25 μg) was used for each sample and was separated by SDS-PAGE and then transferred to nitrocellulose membranes. The protein bands were subsequently probed using specific primary antibodies, phosphorylated ERK1/2, ERK1/2, phosphorylated p38, p38, phosphorylated JNK, JNK, phosphorylated Akt, Akt, phosphorylated mTOR, mTOR, phosphorylated STAT5, STAT5, phosphorylated Smad3, and Smad3 (all at 1/1,000 dilution; Cell Signaling Technology), and then by anti-rabbit-IgG-horseradish peroxidase (1/20,000 dilution). Proteins were detected using an ECL detection kit (Pierce). A comparative analysis was performed using quantitative densitometry.

Statistical analyses

Values are expressed as means ± standard deviations (SD) or percentages in text and figures. For variables with normal distribution and homogeneity of variance, an independent *t* test or one-way ANOVA was used to test differences among two or more groups. For skewed variables, a nonparametric Kruskal-Wallis (H) test or Mann-Whitney U test was used for analyses. The effect of AT in vivo was assessed using a paired-sample *t* test or Wilcoxon signed-rank test within groups, depending on normality. For the ranked data, Pearson's chi-square test or Fisher's exact test was used for the comparison among multiple groups. In all cases, a two-tailed *p* value of <0.05 was considered significant.

RESULTS

T_{reg} display compromised function in patients with RA

Table 1 summarizes the demographic and clinical characteristics of the study population. The PBMCs of RA patients (*n* = 55) and control (*n* = 42) donors were stained with fluorescent-labeled antibodies against CD4, CD25, and Foxp3. The gating strategy for the CD4⁺CD25⁺Foxp3⁺ T_{reg} analysis is shown in **Fig. 1A**. Further phenotypic analysis revealed that these CD4⁺CD25⁺Foxp3⁺ cells showed a low expression of CD127 and a high expression of CD45RO, CD62L, and HLA-DR, demonstrating their regulatory phenotype (**Fig. 1B**). CD4⁺CD25⁺Foxp3⁺ T_{reg} did not differ significantly between the RA patients and the control subjects (RA vs. Con, 5.2% ± 1.3% vs. 5.0% ± 1.5% of CD4⁺ T cells, respectively; *p* = 0.542 (**Fig. 1C**)). In line with this observation, Foxp3 mRNA expression in the PBMCs appeared to be similar between the RA patients and the control subjects (*p* = 0.432) (**Fig. 1D**).

Next, CD4⁺CD25⁺CD127^{low} T_{reg} and CD4⁺CD25⁻ T_{resp} were purified by magnetic sorting and tested in the suppression assay. CD4⁺CD25⁻ T_{resp} from the RA patients and the control subjects exhibited similar proliferation in the presence of anti-CD3/28 (*p* = 0.177) (**Fig. 2A**). Quantitative analysis of CD4⁺CD25⁺CD127^{low} T_{reg} function was performed by coculturing them with autologous CD4⁺CD25⁻ T_{resp} at different ratios (T_{reg}/T_{resp} ratios of 1:1, 1:2, and 1:4). The data indicated that the RA patients showed significantly reduced T_{reg} suppression function compared with that of the control subjects for all T_{reg}/T_{resp} ratios

TABLE 1. Demographic and clinical characteristics of the study population

Characteristics	Control group (<i>n</i> = 42)	RA group (<i>n</i> = 55)
Age (years)	50 ± 12	51 ± 13
Male/female, %	5/37, 12/88	6/49, 11/89
Rheumatoid factor-positive, no., %	—	39, 71
Disease duration (years)	—	12.03 ± 8.38
hs-CRP (mg/l)	—	14.32 ± 7.84
ESR (mm/h)	—	20.75 ± 8.05
DAS28	—	5.67 ± 0.85
IL-6 (pg/ml)	—	17.57 ± 12.37
ICAM-1 (ng/ml)	—	245.61 ± 79.67
Total cholesterol (mmol/l)	4.54 ± 0.43	4.50 ± 0.42
HDL cholesterol (mmol/l)	1.21 ± 0.32	1.25 ± 0.29
LDL cholesterol (mmol/l)	2.61 ± 0.34	2.51 ± 0.40
Triglycerides (mmol/l)	1.30 ± 0.32	1.31 ± 0.35
Mediations, no. (%)		
Methotrexate	—	36 (65)
Leflunomide	—	4 (7)
Sulfasalazine	—	6 (11)
TNF inhibitor	—	16 (29)
NSAIDs	—	42 (76)
Prednisone	—	7 (13)

Values are presented as means ± SD or percentages. RA, rheumatoid arthritis; hs-CRP, high-sensitivity C-reactive protein; ESR, erythrocyte sedimentation rate; DAS28, disease activity 28; IL-6, interleukin-6; ICAM-1, intercellular adhesion molecule-1; HDL, high density lipoprotein; LDL, low density lipoprotein; TNF, tumor necrosis factor; NSAID, nonsteroidal anti-inflammatory drugs.

tested (*p* < 0.05) (**Fig. 2A**). We also investigated whether T_{reg} from the RA patients were able to suppress TNF-α and IFN-γ production by activated CD4⁺CD25⁻ T cells. Because CD4⁺CD25⁻ T cells from the RA patients and the control subjects may differ in their respective abilities to produce TNF-α and IFN-γ upon activation, we first measured the production of cytokines by activated T cells from the two groups. The results suggested an increase in the production of TNF-α and IFN-γ in activated T cells from the RA patients compared with those of the control subjects. Therefore, T_{reg} from the two groups were cocultured with activated T cells from the RA patients to determine their effects on cytokines production. In agreement with the results of the suppression assay, T_{reg} from the RA patients were less effective at inhibiting the production of the two cytokines than those from the control group (**Fig. 2B**).

AT reduces RA clinical disease activity and enhances the frequency and suppressive function of circulating T_{reg} in vivo

The potential of statins to modulate immune responses has led to considerable interest in their use for the treatment of RA. Therefore, we set out to investigate whether the clinical response to statin therapy results in changes in the T_{reg} population. RA patients and control subjects in our study population were allocated to either a group that received AT (20 mg daily, RA-A group and Con-A group) or one that did not (RA-C group and Con-C group) for 12 weeks. **Table 2** shows that the RA patients and the control subjects with or without AT treatment were generally comparable at baseline. **Table 3** shows the effects of statins on lipids, disease activity, and inflammatory markers of RA

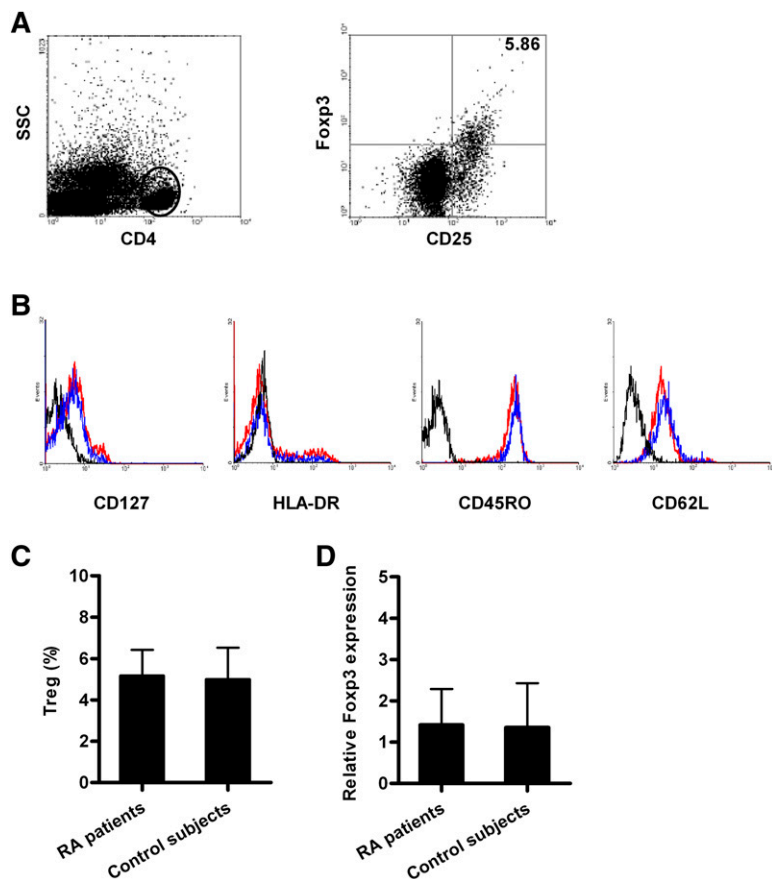


Fig. 1. Determination of T_{reg} in RA patients and control subjects is shown. (A) PBMCs from 55 RA patients and 42 control subjects were stained for CD4, CD25, and Fcpx3 and analyzed by flow cytometry. Dot plots are representative fluorescence-activated cell sorting pictures from an RA patient. (B) Histograms represent data from a RA patient when the gated T_{reg} were further phenotypically analyzed for the indicated markers (black line, isotype control; blue line, control subject; red line, RA patient). (C) Collective analyses of T_{reg} percentages in the two groups are shown. (D) Expression of Fcpx3 mRNA in PBMCs was compared between the two groups by using real-time PCR.

patients after 12 weeks. AT produced a significant reduction in both TC and LDL ($p < 0.001$). Disease activity, as assessed by DAS28, decreased significantly by AT ($p < 0.001$). hs-CRP and erythrocyte sedimentation rate (ESR) were also reduced by AT ($p < 0.05$). Neither IL-6 nor ICAM-1 was significantly affected by the drug ($p > 0.05$). A

significant reduction in TC and LDL was also observed in the control subjects receiving AT after 12 weeks ($p < 0.05$, data not shown).

Flow cytometric analyses showed a significant increase in T_{reg} in RA patients (RA-A1 vs. RA-AB or RA-C1: $6.8\% \pm 1.9\%$ vs. $5.1\% \pm 1.4\%$ or $5.2\% \pm 1.1\%$ of $CD4^+$ T cells,

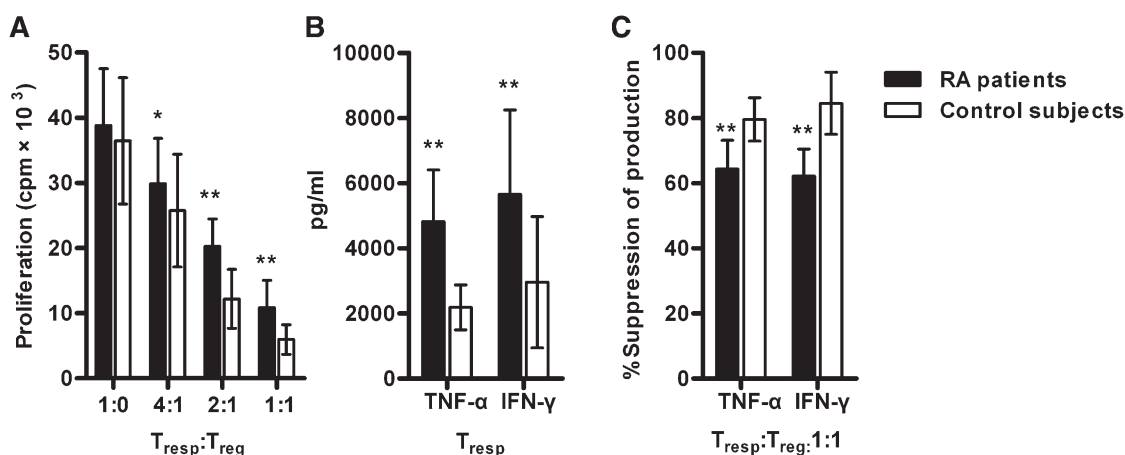


Fig. 2. Compromised T_{reg} suppressive function in RA patients is shown. (A) Magnetic-sorted $CD4^+$ $CD25^+$ $CD127^{low}$ T_{reg} and $CD4^+$ $CD25^-$ T_{resp} from RA patients and control subjects were mixed at the indicated ratios and tested in the proliferation assay. (B) Comparison of cytokines production by activated $CD4^+$ $CD25^-$ T cells from the RA patients or the control subjects is shown. (C) Comparison of cytokines production by $CD4^+$ $CD25^-$ T cells from RA patients when cells were cocultured with autologous T_{reg} or T_{reg} from control subjects is shown. *, $p < 0.05$, or **, $p < 0.01$ versus control subjects.

TABLE 2. Baseline clinical characteristics of Con-A, Con-C, RA-A and RA-C groups

Characteristics	Con-A (n = 21)	Con-C (n = 21)	p	RA-A (n = 28)	RA-C (n = 27)	p
Age (years)	50 ± 11	50 ± 13	0.920	51 ± 14	51 ± 13	0.914
Male/female, %	3/18, 14/86	2/19, 10/90	0.634	4/24, 14/86	2/24, 7/93	0.413
Rheumatoid factor-positive, no. (%)	—	—	—	18 (64)	21 (78)	0.271
Disease duration (years)	—	—	—	10.95 ± 8.52	13.15 ± 8.24	0.217
hs-CRP (mg/l)	—	—	—	14.00 ± 7.29	14.65 ± 8.50	0.822
ESR (mm/h)	—	—	—	20.86 ± 8.93	20.63 ± 7.20	0.930
DAS28	—	—	—	5.74 ± 0.86	5.60 ± 0.86	0.577
IL-6 (pg/ml)	—	—	—	16.53 ± 12.56	18.7 ± 12.3	0.348
ICAM-1 (ng/ml)	—	—	—	222.50 ± 88.45	269.6 ± 62.3	0.081
Total cholesterol (mmol/l)	4.51 ± 0.40	4.57 ± 0.46	0.687	4.51 ± 0.38	4.48 ± 0.47	0.738
HDL cholesterol (mmol/l)	1.18 ± 0.24	1.24 ± 0.38	0.970	1.23 ± 0.29	1.27 ± 0.29	0.606
LDL cholesterol (mmol/l)	2.60 ± 0.37	2.61 ± 0.33	0.970	2.50 ± 0.38	2.51 ± 0.43	0.754
Triglycerides (mmol/l)	1.24 ± 0.39	1.35 ± 0.25	0.099	1.37 ± 0.37	1.25 ± 0.31	0.105
Medication, no. (%)						
Methotrexate	—	—	—	19 (68)	17 (63)	0.703
Leflunomide	—	—	—	3 (11)	1 (4)	0.317
Sulfasalazine	—	—	—	2 (7)	4 (15)	0.362
TNF inhibitor	—	—	—	6 (21)	10 (37)	0.203
NSAIDs	—	—	—	24 (86)	18 (66)	0.096
Prednisone	—	—	—	5 (18)	2 (7)	0.245

Values are presented as means ± SD.

respectively, $p < 0.05$) and the control subjects receiving AT (Con-A1 vs. Con-AB or Con-C1: $6.5\% \pm 1.2\%$ vs. $5.0\% \pm 1.6\%$ or $5.3\% \pm 1.4\%$ of $CD4^+$ T cells, respectively, $p < 0.05$) compared with the baseline and subjects not receiving AT (Fig. 3A, B). AT showed no effect on the proliferation of T_{resp} , as a similar extent of proliferation was observed in subjects who received AT or did not receive AT both at baseline and after 12 weeks (Fig. 3C, left panel). Next, we compared the suppressive function of T_{reg} after AT therapy by using a 1:2 T_{reg}/T_{resp} ratio. As expected, T_{reg} suppressive function remained compromised in patients who did not receive AT 12 weeks later, as it was at baseline (baseline RA-CB vs. Con-CB, $p < 0.05$; 12 weeks later, RA-C1 vs. Con-C1, $p < 0.05$) (Fig. 3C, right panel). In contrast, there was a significant increase in the suppressive function of T_{reg} in subjects receiving AT (RA-A1 vs. RA-AB or RA-C1, $p < 0.05$; Con-A1 vs. Con-AB or Con-C1, respectively, $p < 0.05$) that was not seen in patients who did not receive AT.

In summary, the above-described results indicate that statins are promising drugs for RA and that they increase the frequency of T_{reg} and restore the suppressive function of these cells in RA patients.

AT induces the conversion of T_{reg} from primary T cells and enhanced the function of preexisting T_{reg} in vitro

Next, we performed experiments to investigate the origin of the newly formed T_{reg} . The $CD4^+CD25^-$ T cells of the RA patients were sorted to >90% purity by magnetic beads separation. As shown in Fig. 4A, the addition of AT to activated RA $CD4^+CD25^-$ T cells resulted in a dose-dependent increase in the percentage of $CD4^+CD25^+Foxp3^+$ cells. The increased number of $CD4^+CD25^+Foxp3^+$ cells was prevented by the product of HMG-CoA reduction, L-mevalonate, indicating that the effects of AT on T_{reg} depended on HMG-CoA reduction. However, a suboptimal increase could also be observed in the absence of AT. Although Foxp3 is the best marker of T_{reg} , previous studies (20) have reported a transient expression of Foxp3 in conventional T cells, triggered by activation in vitro. Therefore, we then tested whether $CD4^+CD25^+Foxp3^+$ cells differentiated in the presence of AT were functionally suppressive. Five days after the culture, $CD4^+CD25^+CD127^{low}$ T cells were isolated and mixed with freshly autologous T_{resp} . Our data indicated that only newly formed $CD4^+CD25^+CD127^{low}$ T cells in the presence of AT were able to suppress the proliferation of T_{resp} (Fig. 4B).

TABLE 3. Difference in serum lipids, DAS28, and inflammatory factors in RA patients after 12 weeks of therapy

Parameter	RA-A (n = 28)	RA-C (n = 27)	p
Total cholesterol (mmol/l)	-1.03 (-1.16, -0.89)	0.03 (-0.15, 0.20)	<0.001
HDL cholesterol (mmol/l)	0.05 (0, 0.09)	0.03 (-0.05, 0.12)	0.797
LDL cholesterol (mmol/l)	-0.99 (-1.13, -0.86)	0.06 (-0.11, 0.23)	<0.001
Triglycerides (mmol/l)	-0.10 (-0.21, 0.01)	-0.03 (-0.11, 0.06)	0.304
DAS28	-0.41 (-0.52, -0.30)	-0.03 (-0.15, 0.10)	<0.001
hs-CRP (mg/l)	-5.41 (-6.48, -4.34)	-0.06 (-1.77, 1.65)	<0.001
ESR (mm/h)	-5.46 (-7.31, -3.62)	0.03 (-0.15, 0.2)	0.005
IL-6 (pg/ml)	-2.12 (-5.56, 1.32)	1.04 (-0.80, 2.87)	0.104
ICAM-1 (ng/ml)	-21.32 (-43.11, 0.48)	0.76 (-23.99, 25.51)	0.174

Values are presented as means (95% confidence interval).

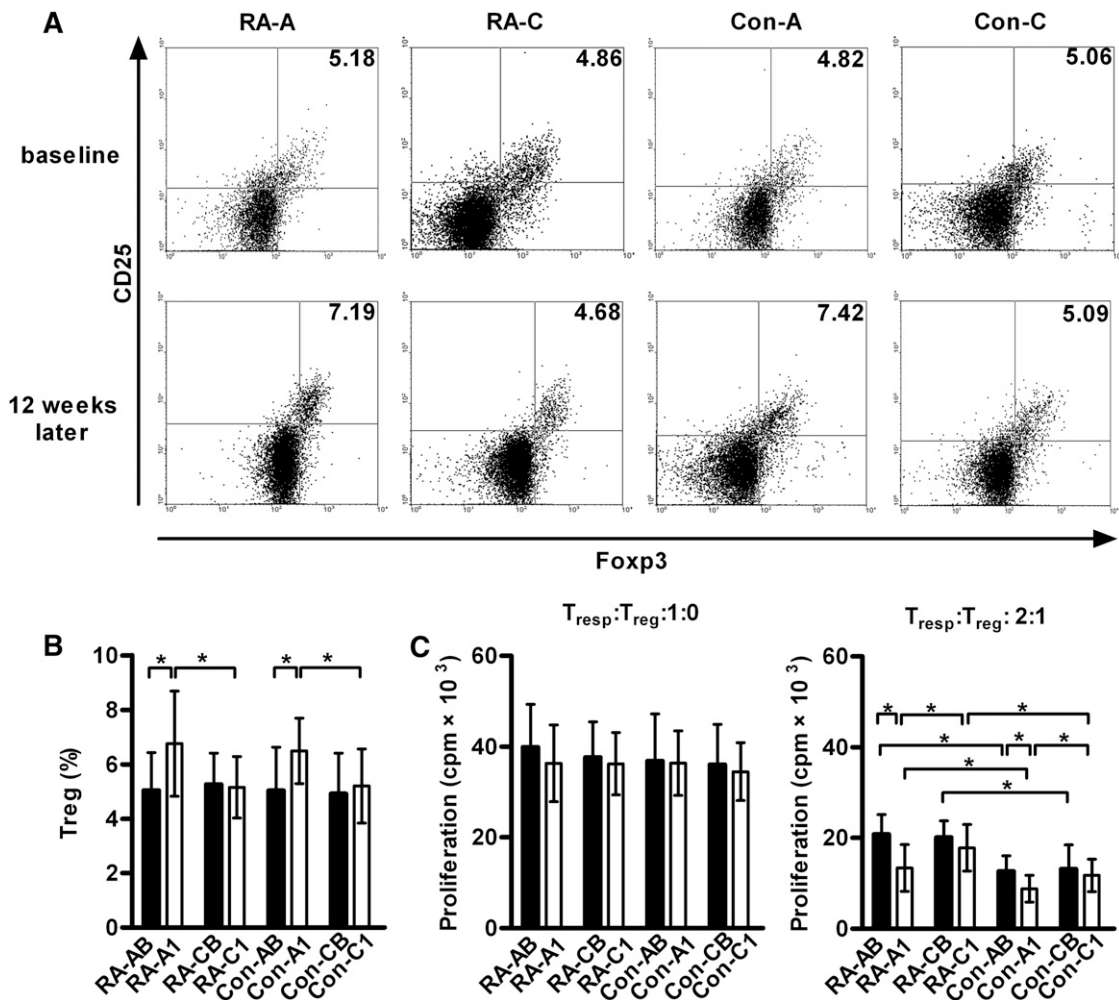


Fig. 3. Influence of AT on circulating T_{reg} in vivo is shown. (A) Dot plots show representative fluorescence-activated cell sorting pictures of CD4⁺ CD25⁺ Fop3⁺ T_{reg} from a single person in four groups. (B) The frequency of T_{reg} was compared among four groups before (RA-AB, RA-CB, Con-AB, and Con-CB) and after 12 weeks (RA-A1, RA-C1, Con-A1, and Con-C1) of treatment. (C) The proliferation of T_{resp} when they were cultured alone or together with T_{reg} at a 2:1 ratio were compared among four groups before and after 12 weeks of treatment. *, $p < 0.05$ versus the indicated group.

To determine whether AT targeted the preexisting T_{reg}, T_{reg} were isolated from the RA patients and incubated with AT for 24 h and then tested in the suppression assay. The results showed that AT increased the expression of Fop3 in the purified T_{reg} of the RA patients (Fig. 4C). Consistent with this observation, enhanced suppressive function was observed in T_{reg} that were preincubated with AT (Fig. 4D). Similar effects of AT on T_{reg} were observed in the control subjects (data not shown).

AT induces the generation of T_{reg} accompanied by the inhibition of PI3K-Akt-mTOR and ERK signal transduction pathways

To gain insight into the molecular mechanisms, we preincubated the CD4⁺CD25⁻ T cells from the RA patients with AT, induced activation in these cells using anti-CD3/28, and then probed the cell lysates for the phosphorylation of several signaling pathways. **Figure 5A** shows that AT-treated cells displayed significantly reduced phosphorylation levels of Akt, mTOR, and ERK compared with those of untreated cells. Other signaling pathways that have

been reported to be related to the conversion of T_{reg} including p38, JNK, STAT5, and smad3 were also tested; however, no alteration was observed (data not shown). To ensure that the inhibition of ERK and the PI3K-Akt-mTOR signaling pathway was downstream of the induction effect of AT on T_{reg}, we next tested whether treatment with an ERK inhibitor or a PI3K inhibitor during T-cell activation could directly induce CD4⁺CD25⁺Fop3⁺ T_{reg}. As shown in Fig. 5B, primary CD4⁺CD25⁻ T cells activated in the presence of U0126 or LY294002 showed high expression of both CD25 and Fop3, as was the case for AT.

DISCUSSION

RA is a systemic autoimmune disease characterized by a chronic relapsing–remitting joint inflammation. Accumulating evidences suggests that T_{reg} defects are involved in the suppression of immune activation in several human diseases (21, 22). Therefore, it is possible that T_{reg} are involved in the physiopathogenesis of rheumatoid arthritis.

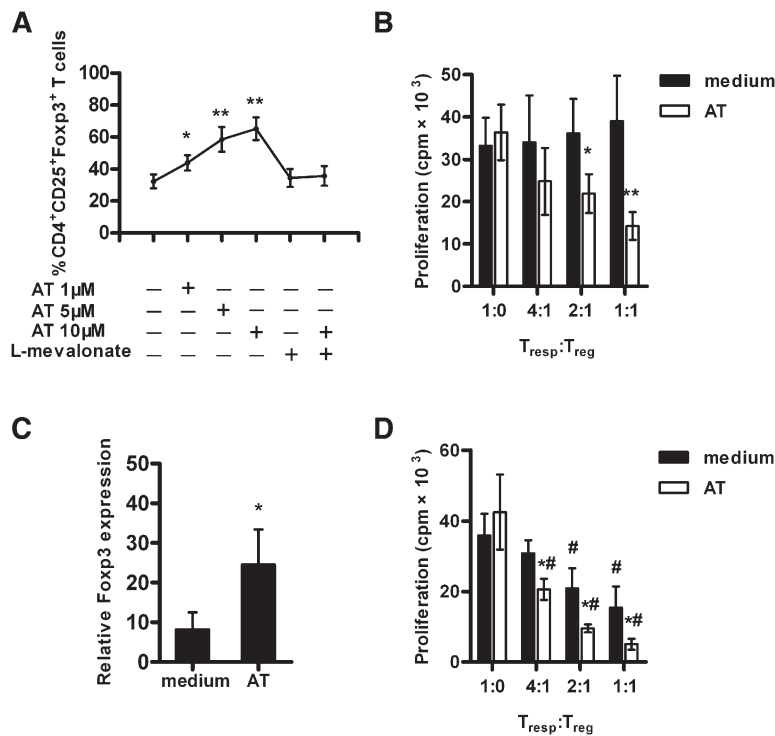


Fig. 4. Effects of AT on T_{reg} in vitro are shown. (A) AT induced CD4⁺CD25⁺Foxp3⁺ from primary CD4⁺CD25⁻ T cells in a dose-dependent manner and L-mevalonate reversed the induction of these cells by AT. (B) CD4⁺CD25⁺Foxp3⁺ induced only in the presence of AT suppressed the proliferation of T_{resp}. (C.) T_{reg} incubated with AT for 24 h showed an increased expression of Foxp3. (D.) T_{reg} preincubated with AT showed enhanced suppressive function. The data are representative of three independent experiments with different donors. *, $p < 0.05$, and **, $p < 0.01$ versus medium; #, $p < 0.05$ versus a 1:0 T_{resp}/T_{reg} ratio.

In animal models, T_{reg} depletion was shown to aggravate the progression of arthritis, whereas the adoptive transfer of T_{reg} could suppress the development of arthritis in the mouse model of collagen-induced arthritis, suggesting that there is therapeutic potential for restoring T_{reg} activity in arthritis (6–8). Until now, there has been no consensus on T_{reg} defects in RA patients. The frequency of circulating T_{reg} in RA patients was reported to be either elevated (9) or unchanged (10, 11) by different researchers. The discrepancy might be due to the use of different strategies to identify T_{reg}. In the present study, we considered T cells with the phenotypic characteristics of CD4⁺CD25⁺Foxp3⁺ to be T_{reg} and confirmed that these cells shared a phenotype identical to that in healthy subjects. Our results showed that the RA patients had numbers of circulating T_{reg} similar to that of the control donors. Consistent with a previous report (13), we observed a reduced capacity of T_{reg} to inhibit proliferation and cytokine production by Teff cells. However, another group of researchers demonstrated that T_{reg} cells from RA patients showed a similar ability to suppress Teff cell proliferation but were not effective for suppressing cytokine production by Teff cells (14). These discrepancies in findings between our study and others may be due to differences in the study populations, the medication, the methods used to identify and purify T_{reg}, or how the suppression assays were performed. In summary, our findings suggested a potential role for T_{reg} in the disease, and we hypothesized that the compromised function of T_{reg} may have contributed to the inappropriate activation of the immune system in patients with RA.

Statins act as immunomodulatory agents that block the adhesion of T cells to antigen-presenting cells, inhibiting MHC-II expression and T-cell activation, suppressing

T-cell proliferation, and downregulating Th1 cell function (23). Our previous clinical and experimental studies have demonstrated that statins regulate immune responses and can be used to treat inflammatory diseases (24–27). Although statins are not a routine treatment agent for RA patients, mounting evidence shows that statins have beneficial effects against several aspects of the disease including disease activity, inflammatory status, and endothelial function (28). The findings of the Trial of Atorvastatin in Rheumatoid Arthritis, the largest, double-blind, placebo-controlled trial of statin therapy, suggested that AT is a potentially excellent candidate agent for RA patients for reducing both RA disease activity and systemic inflammatory activity, which were assessed by DAS28 and hs-CRP, respectively (29). However, a recent cohort study showed that statins do not have any beneficial effects for reducing disease inflammation, which was evaluated by the use of oral steroids in RA patients (30). The beneficial effects of statins in RA patients were confirmed in the present study, as evidenced by reduced DAS28, ESR, and hs-CRP. Therefore, understanding the immunomodulatory effects of statins on the T_{reg} cells in vivo highlights the role of these cells in RA and might partially explain the immunomodulatory effects of AT. In the present study, we analyzed the effects of AT on the amount and function of T_{reg}. Our results demonstrated that AT significantly increased the frequency of T_{reg} in RA patients. A similar increase of T_{reg} was observed in the control subjects after AT treatment, which is consistent with the research of Karin and colleagues (31). We further confirmed the stability of “newly formed” T_{reg} because they sustained their classic phenotype and suppressive function, even after remaining in vitro for 2 weeks (data not shown). Our data also showed a restored suppressive function of T_{reg} in the RA patients undergoing

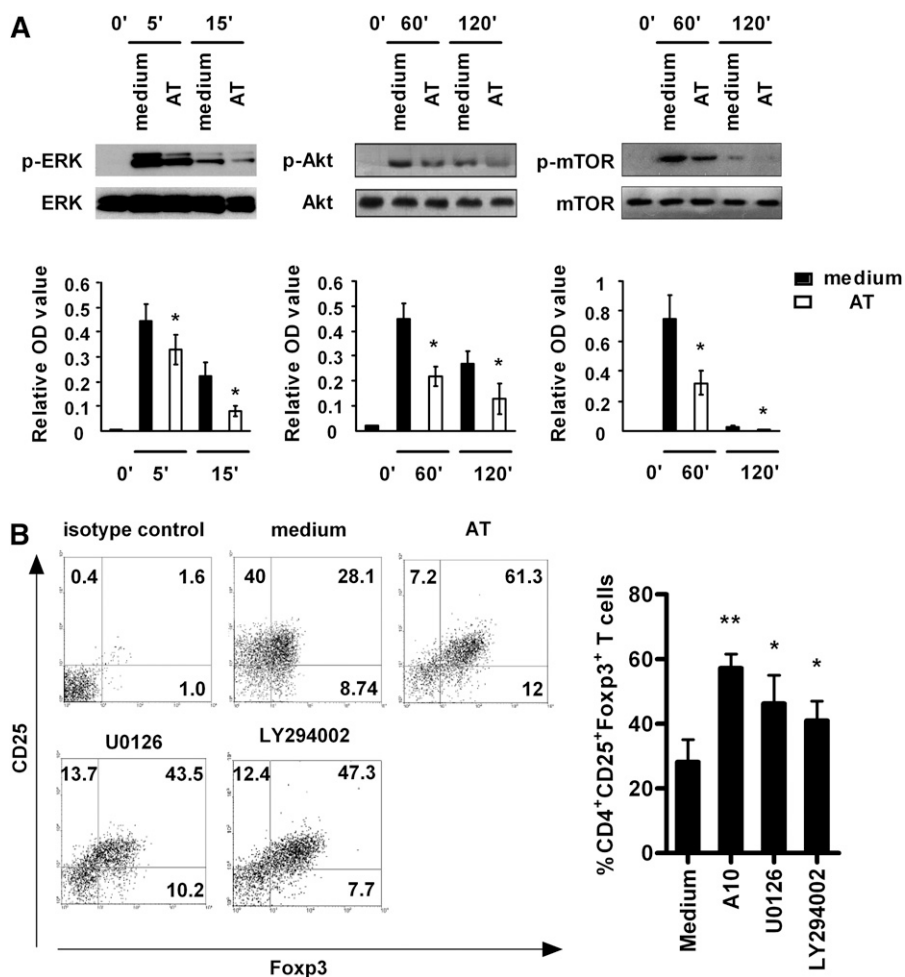


Fig. 5. AT induced the generation of T_{reg} accompanied by the inhibition of PI3K-Akt-mTOR and ERK signal transduction pathways. (A, upper panel) Representative Western blots show the indicated protein in primary $CD4^+CD25^-$ T cells treated with AT; (lower panel) mean densitometric analyses of the relative phosphorylated protein to total protein are shown. (B left) Representative fluorescence-activated cell sorting (FACS) results are shown in primary $CD4^+CD25^-$ T cells treated with AT (10 μ M), the ERK inhibitor (U0126, 5 μ M), or the PI3K-Akt inhibitor (LY294002, 5 μ M); (right.) collective analyses of T_{reg} induced by AT, U0126 or LY294002, are shown. The data are representative of three or four independent experiments with different donors. *, $p < 0.05$, and **, $p < 0.01$ versus medium.

AT therapy. Considering the protective role of T_{reg} on experimental inflammatory arthritis (32), our results suggested that the upregulation of T_{reg} is associated with the beneficial effects of AT in RA. Ehrenstein et al. (33) found that anti-TNF- α therapy could increase the number of T_{reg} by generating a newly formed population of T_{reg} that lacked CD62L expression. In our study, we did not detect the presence of a population of T_{reg} lacking CD62L in RA patients after AT therapy (data not shown). It seems that anti-TNF- α therapy and AT therapy act through different mechanisms.

In the in vitro study, $CD4^+CD25^-$ T primary cells showed a classic phenotype of T_{reg} , as evidenced by the high expression of CD25 and Foxp3 in the presence of AT following activation. However, acquisition of the T_{reg} phenotype does not exclusively mean the conversion of T_{reg} from human T cells because we also observed a population of nonsuppressive T cells with the phenotype of T_{reg} in the absence of AT, and previous research has reported a tran-

sient expression of Foxp3 in conventional T cells triggered by activation (20). Furthermore, the inhibitory function was confirmed in the presence of AT, supporting the existence of a unique ability of AT to convert T_{reg} . It has been demonstrated that AT could confer its anti-inflammatory effects both depending on (34) and independently of (35) the inhibition of HMG-CoA reductase. To clarify whether the conversion of T_{reg} is mediated by the HMG-CoA reductase pathway, we added mevalonate along with AT and found that this combination could reverse the induction of T_{reg} by AT. These data suggested that the anti-inflammatory effects of AT are mediated by the HMG-CoA reductase pathway.

Statins not only prevent the synthesis of cholesterol but also reduce the synthesis of isoprenoid intermediates, such as farnesyl pyrophosphate or geranylgeranyl pyrophosphate, thus affecting the activation of the small GTPases, including Ras and Rho-GTPases (36). Two important substrates of GTPases, PI3K-Akt-mTOR and ERK signaling

pathways, which regulate FoxP3 expression in activated naïve T cells (37, 38), have been demonstrated to be regulated by AT in some systems (39, 40). In our study, we observed that AT inhibited the phosphorylation of ERK and PI3K-Akt-mTOR and U0126 (ERK inhibitor) or LY294002 (PI3K-Akt inhibitor) treatment of activated primary T cells mimicked the effects of AT on T_{reg}. Therefore, we propose that the inhibition of ERK and PI3K-Akt-mTOR could be an intermediate step in the induction of T_{reg} by AT. Several other signal pathways, such as p38, JNK, STAT5, and smad3, have been also reported to be involved in the conversion of T_{reg} (41, 42). However, we did not observe any effects of AT on these pathways. As for the preexisting T_{reg} cells, AT enhanced their suppressive function in vitro.

In conclusion, we have demonstrated the systemic inflammatory response in RA patients assessed by decreased T_{reg} suppressive function. AT significantly upregulates the frequency and impaired function of T_{reg} and reduces clinical disease activity in patients with RA. This is the first study to prospectively analyze the effects of statin therapy on T_{reg} in RA patients. Analysis of the impact of statins on T_{reg} is essential not only to elucidate its role in the pathogenesis of the disorder but also to clarify its mechanism of action. Considering the above-described immunomodulatory effects, its oral bioavailability, and its good safety profile, statin treatment is a promising approach for treating RA and other chronic inflammatory diseases. **LR**

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