The effect of HMG-CoA reductase inhibitors on naturally occurring CD4⁺CD25⁺ T cells

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Abstract

Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are in widespread use due to their LDL reducing properties and concomitant improvement of clinical outcome in patients with and without preexisting atherosclerosis. Considerable evidence suggests that immune mediated mechanisms play a dominant role in the beneficial effects of statins. Naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs) have a key role in the prevention of various inflammatory and autoimmune disorders by suppressing immune responses. We tested the hypothesis that statins influence the circulating number and the functional properties of Tregs.

We studied the effects of in vivo and in vitro statin treatment of human and murine mononuclear cells on the number of Tregs and the expression level of their master transcription regulator, Foxp3.

Atorvastatin, but not mevastatin nor pravastatin, treatment of human peripheral blood mononuclear cells (PBMCs) increased the number of CD4⁺CD25⁺ cells, and CD4⁺CD25⁺Foxp3⁺ cells. These Tregs, induced by atorvastatin, expressed high levels of Foxp3, which correlated with an increased regulatory potential. Furthermore, co-culture studies revealed that atorvastatin induced CD4⁺CD25⁺Foxp3⁺ Tregs were derived from peripheral CD4⁺CD25⁻Foxp3⁻ cells. Simvastatin and pravastatin treatment in hyperlipidemic subjects increased the number of Tregs. In C57BL/6 mice however, no effect of statins on Tregs was evident.

In conclusion, statins appear to significantly influence the peripheral pool of Tregs in humans. This finding may shed light on the mechanisms governing the plaque stabilizing properties of statins.

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1. Introduction

Statins, the inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are effective lipid lowering agents that are widely used in medical practice [1]. The beneficial effect of lipid lowering by statins in the reduction of the atherosclerosis progression, and the risk of coronary heart disease as consequence, has been demonstrated in large clinical trials [2,3]. However, despite similar reduction in total cholesterol, it appears that the benefits of statins exceed those of other lipid-lowering agents [3,4]. This observation is one of the findings that led to the contention that statins exert additional beneficial effects, independent of lipid lowering, that have been ascribed to their modulating effect on the immune system [3–6]. As it is well established that the immune system plays an active role in the pathogenesis of atherosclerosis [7,8], attenuation of the atheroma and its stabilization by statins could be also attributed to their immunomodulatory properties [9].

Atorvastatin and pravastatin were found to attenuate T cell activation and proliferation, to inhibit the secretion of the pro-inflammatory cytokines and to enhance the secretion of anti-inflammatory cytokines [10,11]. Two principal
mechanisms for these findings were described. First, it has been demonstrated that statins inhibit interferon-gamma (IFN-gamma) induced expression of major histocompatibility complex class II (MHC-II) on antigen presenting cells (APCs), and thus repress MHC-II mediated T cell activation [1]. Secondly, it has been demonstrated that certain statins selectively bind to lymphocyte function associated antigen-1 (LFA-1), locking the receptor in an inactive conformation, thereby blocking the binding to its counter-receptor intercellular adhesion molecule 1 (ICAM-1), an adhesion mechanism which enables leukocyte adhesion to endothelium and T-cell costimulation by APCs [12].

Whereas T helper 1 (Th1) lymphocytes appear to promote atherosclerosis, there are confounding data regarding the protective or pathogenic Th2-driven responses in atherosclerosis [13,14].

Recent evidence indicate that CD4+CD25+ regulatory T cells (Tregs), play a critical role in the control of atherosclerosis by influencing Th1 and Th2 pathogenic responses [14–17]. Naturally occurring CD4+CD25+ regulatory T cells are generated spontaneously in the thymus and are crucial for the suppression of pathogenic immune responses against self or non-self antigens and the prevention of immune mediated disorders [18,19]. Although Tregs constitutively express the surface markers cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR), these molecules appear to be shared by nonregulatory activated T cells [18,20,21]. So far, there are two unambiguous markers to identify naturally occurring Tregs. Forkhead/winged helix transcription factor (Foxp3), which is expressed only by Treg cells and is thought to be the master transcriptional regulator of the development and function of this subset [8,20,21]. The second is the alpha chain of interleukin 2 (IL-2) receptor (CD25) that is highly expressed by Treg cells and downregulated by activated T cells [22].

Herein, we tested the hypothesis that the immunomodulatory properties of statins could be attributed to their effects on Tregs. We have shown that whereas statins do not exert meaningful effects on murine Tregs, several statins appear to upregulate the number of functionally active human Tregs.

2. Materials and methods

2.1. Study population

For in vitro experiments, peripheral blood mononuclear cells (PBMCs) were isolated from 5 healthy donors at the age range: 27–41 years. For ex vivo experiments PBMCs were isolated from subjects with hypercholesterolemia, starting a treatment with either 20 mg simvastatin (n = 7), or 10–40 mg pravastatin (n = 5) from Teva Pharmaceutical Industries Ltd., Israel. All experiments were approved by the institutional ethics committee and informed consent was obtained from all patients.

2.2. Animals

C57BL/6, 8–12 weeks old, were purchased from Jackson Laboratory (Bar Harbor) and grown at the local animal house. Mice were fed a normal chow diet containing 4.5% fat by weight (0.02% cholesterol).

2.3. Cell culture

PBMCs were prepared by Ficoll-Paque density gradient (Lymphoprep™, Nycomed Pharma AS, Oslo, Norway). PBMCs or C57BL/6 splenocytes were cultured (1.5 × 10⁶/ml) at 37 °C in an atmosphere of 5% CO₂ in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 1% penicillin-streptomycin and 1% glutamine (Biological Industries, Kibbutz Beil Haemek, Israel).

Mevastatin (0.5 and 1 μM), Pravastatin (20, 50, 100, 250 and 500 μM) (Sigma–Aldrich Inc., St. Louis, MO, USA) and Atorvastatin (Pfizer Inc., prescription formulation) (2, 5, 10, 25 and 50 μM) were added separately to cultured PBMCs or splenocytes, and were incubated for 96 h. These concentrations were selected since they have been previously shown to promote immunomodulatory effects of statins [12,23,24]. PBMCs or splenocytes cultured with media alone were used as controls.

2.4. The effects of in vivo treatment with statins in mice

C57BL/6 mice were intraperitoneally (i.p.) injected with pravastatin (20 mg/(kg bw day), n = 4, injection volume = 400 μl). After 3 weeks of injection, the mice were sacrificed and splenocytes and thymocytes were isolated for determination of CD4+CD25high by flow cytometry as well as Foxp3 expression by Western blotting. Mice injected with PBS served as controls (n = 4).

Similar experiments were conducted to examine the effect of atorvastatin in vivo. C57BL/6 mice were i.p. injected with atorvastatin (Lipitor™, Parke-Davis GmbH, Germany) (10 mg/(kg bw day), n = 4, injection volume = 400 μl) for 3 weeks, and Treg number was evaluated in the spleen and thymus. As a control group served mice injected with PBS + 5% ethanol (n = 4).

2.5. Flow cytometry

For CD4+CD25high detection, splenocytes were co-stained with the following monoclonal antibodies: fluorescein (FITC)-labeled anti-CD4 (7D4, Miltenyi Biotech) and phycoerythrin (PE)-labeled anti-CD25 (GK1.5, Miltenyi Biotech). FITC-labeled mouse IgG2b (KHLH2b-1-2 from SouthernBiotech) and PE-labeled mouse IgM (RKT2118 from BioLegend) were used as isotypic controls.

Human PBMCs were stained with FITC-labeled anti-CD4 (L3T4) and PE-labeled anti-CD25 (BC96). FITC-labeled Mouse IgG1 κ (MOPC-21/P3) and PE-labeled mouse IgG1 κ (P3) from eBioscience were used as isotypic controls.
CD4+CD25+Foxp3+ cells were detected by Foxp3 intracellular staining, using Human Regulatory T Cell Staining Kit (eBioscience, USA) according to manufacturer’s protocol. Briefly, cells were counterstained with FITC-labeled anti-CD25 (BC96) and APC-labeled anti-CD4 (RPA-T4). After incubation of 30 min, cells were incubated with a fixation solution, washed, and resuspended in a permeabilization solution. Fixed and permeabilized cells were stained with PE-labeled anti-Foxp3 (PCH101). FITC-labeled Mouse IgG1 κ (MOPC-21/P3), PE-labeled rat IgG2a (eBR2a) and APC labeled mouse IgG1 κ (P3) were used as isotypic controls (eBioscience, USA). Stained cells were analyzed on a FACSscape flow cytometer, using CellQuest software (Becton Dickinson).

2.6. Western blot analysis of Foxp3 protein content

Cultured or fresh PBMCs, and fresh splenocytes were lysed, and protein concentration in lysates was determined using BCA protein kit (Pierce, USA). Comparative analysis was performed by quantitative densitometry. Western blot was performed using a polyacrylamide gel (Santa-Cruz, USA) at a dilution of 1:1000 and a secondary antibody-peroxidase-conjugated AffiniPure donkey anti rat IgG (H+L) (Jackson Laboratories) for detection with chemiluminescent substrate (Santa-Cruz, USA). Cell lysates were resolved on 8% SDS-PAGE and transferred onto a nitrocellulose membrane. Western blot was performed using a clonal antibody (CD4+ T-cell isolation kit; Milteny Biotech, Bergisch Gladbach, Germany). This was followed by a step of positive selection of CD25+ cells by microbead separation (CD25 microbeads; Miltenyi Biotech), a procedure yielding a purity of 92–98% as assessed by flow cytometric counting of CD4+CD25+ cells.

2.7. Cell separation

CD4+CD25+ T cells were isolated from PBMCs by a first step of negative sorting using a cocktail of hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies and microbeads coupled to an anti-hapten monoclonal antibody (CD4+ T-cell isolation kit; Milteney Biotech, Bergisch Gladbach, Germany). This was followed by a step of positive selection of CD25+ cells by microbead separation (CD25 microbeads; Miltenyi Biotech), a procedure yielding 92–98% purity as assessed by flow cytometric counting of CD4+CD25+ cells.

2.8. Functional suppression assays

Costar 96-well plates (Corning, NY) were incubated with 5 μg/ml anti-CD3 monoclonal antibody (UCHT1 from R&D systems) overnight at 4 °C, and washed. Then, CD4+CD25− (responder T cells) and CD4+CD25+ (Tregs) were cultured (2 × 105 cells/well) in RPMI medium supplemented with 10% fetal calf serum in different responder/suppressor ratios (1:1, 1:1/2 and 1:1/4). All cells were cultured in a final volume of 200 μl in the presence of 10 μg/ml mitomycin-C treated CD4− cells/well (40 min of incubation, 50 μg/ml) (Sigma–Aldrich Inc., USA), serving as antigen presenting cells (APCs). After 72 h, 3H-thymidine (1 μCi/well) was added for 16 h before proliferation was assayed by scintillation counting (β counter). Percent inhibition of proliferation was determined as follows: 1 – (median 3H-thymidine uptake of CD4+CD25+:CD4+CD25− co-culture/median 3H-thymidine uptake of CD4+CD25− cells). The suppression was repeated in the presence and absence of pravastatin (20 and 100 μM) and atorvastatin (2 and 10 μM).

2.9. Statistical analysis

Data are presented as mean ± S.E.M. Significance between each two groups was examined by a one-way ANOVA test. p value <0.05 was considered significant.

3. Results

3.1. Atorvastatin increases the number of human Treg cells in vitro

The purpose of this study was to examine the effect of statins on the number and function of Treg cells. The effect of statins on the number of Tregs, in humans, was examined both in vitro and ex vivo. For in vitro experiments, PBMCs from healthy individuals were cultured for 96 h in the presence of mevastatin (0.5 and 1 μM), pravastatin (20, 50, 100 and 250 μM) and atorvastatin (2, 5, 10 and 25 μM). As shown in Fig. 1A and B, mevastatin did not significantly alter the expression of CD4+CD25high of total CD4+CD25+ (17.8 ± 2.1% in the presence of 1 μM mevastatin versus 17.1 ± 2.3% in control). Pravastatin increased the percentage of CD4+CD25high cells but this effect was found to be non-significant (19 ± 3.4% in the presence of 100 μM pravastatin versus 17.1 ± 2.3% in control). However, atorvastatin significantly increased in the percentage of CD4+CD25high of total CD4+CD25+ cells (27.6 ± 3.4% and 28.2 ± 5.4% in the presence of 5 and 10 μM atorvastatin, respectively). Higher concentrations of atorvastatin did not result in additional elevation.

The observed effect of atorvastatin correlated closely with the results of Foxp3 expression by FACS (Fig. 1C and D). Indeed, 10 μM atorvastatin increased the number of CD4+CD25+Foxp3+ cells of total CD4+CD25+ by 48.7 ± 22.2% relative to control, and again, this effect was not evident with the other statins. Similar results were obtained when Foxp3 expression was examined by Western blotting (Fig. 1E). 10 μM atorvastatin led to an increase of 66.8 ± 4.5% in Foxp3 expression. Kinetic analysis demonstrated that the most pronounced effects of atorvastatin on the number of CD4+CD25high, as well as on the level of Foxp3 expression were evident after 96 h of incubation, in comparison to a 48 h treatment (Fig. 1F–J).

3.2. Treatment with pravastatin and simvastatin increases the number of CD4+CD25high cells in humans

The effect of statins on the number of Tregs in humans was examined ex vivo by comparing the number of...
Fig. 1. The effect of statins on Tregs in humans in vitro. PBMCs from healthy individuals were isolated and cultured with either mevastatin, pravastatin, atorvastatin or control medium for 96 h. (A) A representative FACS analysis of CD4+CD25^{high} of total CD4+CD25+. Cultured cells were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD25. (B) FACS analysis results: %CD4+CD25^{high} of total CD4+CD25+, relative to control. (C) A representative FACS analysis of CD4+CD25^{+}Foxp3^{+} of total CD4+CD25+. Cultured PBMCs were intracellular stained for Foxp3. Cells were initially stained with APC anti-CD4 and FITC anti-CD25. After fixation and permeabilization, cells were stained with PE anti-Foxp3. (D) FACS analysis results: %CD4+CD25^{+}Foxp3^{+} of total CD4+CD25+, relative to control. (E) Western blot for determination of Foxp3 expression in cultured PBMCs. Protein quantification was performed by Tina-quant assay, and is presented as %OD-background/mm^2, relative to control. For kinetic analysis, atorvastatin was added to a 96 h culture of PBMCs at two time points: 96 and 48 h before cells were harvested. A 96 h culture of PBMCs in the absence of atorvastatin served as control. (F) A representative FACS analysis of CD4+CD25^{high} of total CD4+CD25+. (G) A representative FACS analysis of CD4+CD25^{+}Foxp3^{+} of total CD4+CD25+. (H) FACS analysis results: Delta %CD4+CD25^{+}Foxp3^{+} of total CD4+CD25+, relative to control. (I) FACS analysis results: Delta %CD4+CD25^{+}Foxp3^{+} of total CD4+CD25+, relative to control. (J) A representative Western blot for determination of Foxp3 expression, in 48 and 96 h samples. Protein quantification was accomplished as mentioned in (E) (M: mevastatin; P: pravastatin; A: atorvastatin; *: p < 0.05).
CD4+CD25^{high} and Foxp3 expression before (time 0) and after oral treatment with statins for a period of 4/8 weeks. Since there is a considerable variability in the number of Tregs between individuals [25], baseline levels in a given individual represented the referenced value. Thus, we evaluated subjects with hypercholesterolemia, initiating a treatment with either pravastatin or simvastatin. These healthy subjects appear to have mean levels of Tregs that are similar to non-hyperlipidemic subjects (data not shown). As shown in Fig. 2A and B, 8 weeks of treatment with pravastatin...
led to a median 3.7-fold increase in the percentage of CD4+CD25high of total CD4+CD25+ relative to baseline values. Similar effects were evident in patients treated with simvastatin, which led to a median 2.4-fold increase of circulating CD4+CD25high cells after 4 weeks.

Western blot analysis of Foxp3 expression revealed a similar trend: both pravastatin and simvastatin upregulated Foxp3 expression, an effect that was more pronounced 8 weeks treatment of pravastatin (Fig. 2C).

3.3. Atorvastatin and pravastatin do not alter the number of Treg cells in mice

We then sought to determine whether the effect of statins on the number of Tregs is also observed in mice. C57BL/6 murine splenocytes were cultured for 96 h in the presence of pravastatin (20–500 µM) and atorvastatin (2–50 µM). No significant increase in percentage of CD4+CD25high was observed after addition of pravastatin or atorvastatin (Fig. 3A and B). This finding was confirmed in the in vivo experiments. C57BL/6 mice were injected i.p. with either atorvastatin (10 mg/kg bw) or pravastatin (20 mg/kg bw) for a period of 3 weeks. Since naturally occurring murine Treg cells differentiate in the thymus and are found primarily in thymus and in spleen [26], the number of CD4+CD25high was evaluated in both these organs. Although atorvastatin treatment led to a slight increase of 10.3 ± 6.8% in the percentage of CD4+CD25high of total CD4+CD25+ cells relative to control, in splenocytes, and 17.9 ± 9.9% in thymocytes, both effects were found to be non-significant (Fig. 3C and D). A similar trend was observed in pravastatin treated mice with a non-significant CD4+CD25high cell increase in splenocytes and thymocytes (Fig. 3E and F). Evaluation of Foxp3 expression by Western blotting confirmed that Foxp3 was not inducible by atorvastatin or pravastatin treatment in C57BL/6 splenocytes (Fig. 3G).
Fig. 3. The effect of statins on Tregs in a murine model in vitro and in vivo. Murine splenocytes were cultured with either pravastatin or atorvastatin for 96 h. A culture of splenocytes in the absence of statins served as a control. (A) A representative FACS analysis of CD4+CD25^high of total CD4+CD25^+ in cultured splenocytes. (B) FACS analysis results: %CD4+CD25^high of total CD4+CD25^+ relative to control. Atorvastatin (10 mg/(kg bw day), n = 4) or PBS + 5% ethanol (n = 4) were i.p. injected to C57BL/6 mice for 3 weeks. After sacrifice, Treg cells number was determined in spleen and in thymus. A similar experiment was conducted in order to examine the effect of pravastatin in vivo. Mice were i.p. injected with pravastatin (20 mg/(kg bw day), n = 4) or PBS alone (n = 4) for 3 weeks, and Treg cells number was evaluated in spleen and thymus. (C) A representative FACS analysis of CD4+CD25^high in the spleen and in the thymus, in atorvastatin and control treated mice. (D) FACS analysis results: %CD4+CD25^high of total CD4+CD25^+ relative to control. (E) A representative FACS analysis of CD4+CD25^high in the spleen and thymus, in pravastatin and PBS treated mice. (F) FACS analysis results: %CD4+CD25^high of total CD4+CD25^+. Western blot for determination of Foxp3 expression in splenocytes from each treatment group. Protein quantification was estimated as shown in Fig. 1 (C: control; P: pravastatin; A: atorvastatin, none of the values reached a statistical significance).
3.4. Atorvastatin promotes the conversion of peripheral human CD4⁺ CD25⁻ Foxp3⁻ T cells to CD4⁺ CD25⁺ Foxp3⁺ Treg cells

We then examined whether the source of these “newly formed” regulatory T cells, induced in vitro by atorvastatin in humans, is the CD4⁺ CD25⁻ Foxp3⁻ T cells subset, in which Foxp3 and CD25 expression is upregulated.

Human CD4⁺CD25⁻ T cells were purified from healthy individuals by magnetic bead separation and exposed to 2 and 10 μM atorvastatin, in the presence of anti-CD3 mAb, for responders stimulation, and mitomycin-C treated CD4 depleted cells, serving as APCs. Previous studies have shown that in humans, during anti-CD3 mediated activation of CD4⁺CD25⁻ T cells, two populations of cells may arise, effector CD4⁺CD25⁺Foxp3⁻ and CD4⁺CD25⁺Foxp3⁺ with regulatory activity [27]. Indeed, stimulation of CD4⁺CD25⁻ cells with anti-CD3 alone for 96 h led to the generation of a new CD4⁺CD25⁺ T cells subset which constituted 36.2 ± 0.7% of the total number of cells. 16.1 ± 1.3% of these newly generated CD4⁺CD25⁺ cells expressed Foxp3, and the rest did not express Foxp3 signifying activated T responder cells (Fig. 4A and B).

Addition of 2 and 10 μM atorvastatin to the culture led to a significant decrease in the number of these anti-CD3 induced CD4⁺CD25⁺ cells, in a dose dependent manner (Fig. 4C). Despite the decrease in the total CD4⁺CD25⁺ cells number, the percentage of Foxp3 expressing cells of those CD4⁺CD25⁺ remaining cells, increased in the presence of atorvastatin, and 10 μM atorvastatin led to the appearance of 22.1 ± 1.3% CD4⁺CD25⁺Foxp3⁺ cells (Fig. 4B and D). These findings indicate that atorvastatin promoted the conversion of CD4⁺CD25⁻Foxp3⁻ cells to CD4⁺CD25⁺Foxp3⁺ regulatory T cells, accompanied by the inhibition of the anti-CD3 mediated T cells activation.

The possibility that the source of these induced by atorvastatin CD4⁺CD25⁺Foxp3⁺ cells were rare CD4⁺CD25⁻ Foxp3⁺ that were activated by atorvastatin and as a consequence regained CD25 expression is ruled out, since only 1.6% of the purified CD4⁺CD25⁻ cells expressed Foxp3 and this population remained stable in the presence of anti-CD3 mAb and atorvastatin (data not shown).

3.5. Atorvastatin upregulates the regulatory function of Tregs in humans in vitro

A thymidine incorporation assay was conducted in order to determine whether the newly generated statin inducible Treg population possesses improved functional suppressible properties. A quantitative analysis of the regulatory function of CD4⁺CD25⁺ Tregs was performed by co-culturing them with autologous T-responder cells (2 × 10⁴ cells/well) at different ratios (Treg/responder ratios: 1:1, 1:2 and 1:4), in the presence of APCs (10⁵ cells/well) and plate-bound anti-CD3 mAb.

As presented in Fig. 5 A, addition of 20 and 100 μM pravastatin to the co-cultured Tregs and T-responder cells at a 1:1 ratio led to a non-significant increase of 7.8 ± 2% and 11.5 ± 4.6% in the inhibition rate, correspondingly. This observation supports our previous findings that the effect of pravastatin on the Tregs pool is minor.

Atorvastatin, however, increased the extent of thymidine uptake inhibition in a significant dose-dependent manner (30.1 ± 5.4% and 49.7 ± 0.3% in the presence of 2 and 10 μM atorvastatin, respectively, at a 1:1 ratio), and this effect repeated itself in all Treg/responder ratios (Fig. 5B).
Fig. 4. CD4+CD25−Foxp3− cells transform into CD4+CD25+Foxp3+ cells in the presence of atorvastatin. CD4+CD25+, CD4+CD25− and CD4− were isolated from PBMCs of healthy individuals, using MACS, as described in Section 2. CD4+CD25− cells (responder T cells) were incubated in the presence of APCs, in pre-coated plates with 5 μg/ml anti-CD3 mAb for responder T cells stimulation. (A) A representative FACS analysis of total CD4+CD25+ before (time 0) and after a 96 h stimulation with anti-CD3 mAb (control). (B) A representative FACS analysis of CD4+CD25+Foxp3+ of total CD4+CD25+ in the presence and absence of atorvastatin. (C) %total CD4+CD25+ is increased in the presence of anti-CD3 mAb and decreased in the presence of atorvastatin in a dose-dependent manner. (D) FACS analysis results: %CD4+CD25+Foxp3+ and %CD4+CD25+Foxp3− of total CD4+CD25+ following treatment with atorvastatin (A: atorvastatin; *: p < 0.05).

Fig. 5. Atorvastatin enhances the inhibitory function of Tregs. A suppression assay was conducted as described in Section 2. CD4+CD25+ cells (Tregs) and the CD4+CD25− (responders T cells) were co-cultured in different ratios (Treg: responders, 1:1, 1:2 and 1:4), in the presence of APCs for 72 h and a thymidine incorporation assay was performed. (A) Addition of pravastatin to the culture leads to an increase in the suppression rate, in a non-significant manner (*p = 0.05, **p = 0.08). (B) Addition of atorvastatin to the culture leads to a significant increase in the suppression rate (**p < 0.05).
4. Discussion

Statins have been shown to result in a significant reduction in LDL cholesterol levels and large prospective studies have linked these metabolic effects to an impressive improvement in outcome. A large body of evidence exists to support non-metabolic effects of statins. In this study, we have shown a yet unreported potential mechanism of certain statins that relates to their influence on naturally occurring Tregs.

As immune modulating agents, statins were found to act as direct inhibitors of MHC-II expression induced by IFN-gamma and thus as repressors of T cell activation [1]. It has also been shown that statins selectively block LFA-1 that result in a decreased adhesion of lymphocytes to ICAM-1 and impaired T cell activation by APCs [14]. Inhibition of T cell activation by both mechanisms may lead to a reduction in T cell differentiation into effector cell populations (Th1 and Th2) and a reduction in other effector functions such as cytokine release.

Here we show that atorvastatin treatment of human PBMCs in vitro leads to an induction of the transcription factor Foxp3, accompanied by an increase in the number of Treg as determined by CD4+CD25+Foxp3+ staining, as well as the number of CD4+CD25high cells. The augmented number of Treg cells, induced by atorvastatin, correlated with the increase in their functional inhibitory properties. A similar trend, although not statistically significant, was observed with pravastatin treatment of human PBMCs in vitro which slightly enhanced the number and function of Treg cells, whereas no effect was evident with mevastatin. Thus, statins appear to differ with respect to their effects on Tregs. Kwak et al. [1] also noted that the potency of statins as MHC-II repressors varied between different statins, with atorvastatin being the most powerful in vitro MHC-II repressor. We believe that the differential effects of statins on the Tregs pool in the in vitro experiments result from their structure variability, leading to their characterization as lipophilic statins (e.g. atorvastatin) which are easily distributed in extrahepatic cells, or hydrophilic statins (e.g. mevastatin and pravastatin) which possess a reduced potential for uptake by peripheral cells [28].

We also provide here evidence, indicating that the source of the newly atorvastatin generated human CD4+CD25+ Foxp3+ Tregs are actually peripheral CD4+CD25−Foxp3− which acquired Foxp3 and CD25 expression in response to atorvastatin.

The ability to induce in vitro CD4+CD25+Foxp3+ conversion from the CD4+CD25−Foxp3− subset was also ascribed to other immunosuppressive compounds such as copolymer-1 [29], rapamycin [30] and anti-thymocyte globulin (ATG) [31]. The acquisition of a regulatory phenotype by CD4+CD25−Foxp3− cells was also observed in vivo upon homeostatic expansion [32], in which a physiological inducer of Foxp3 gene expression was found to be TGF-beta [33]. Analysis of subjects before and after treatment with either the hydrophilic pravastatin or the lipophilic simvastatin revealed that both agents significantly induced Foxp3 expression and enhanced the number of CD4+CD25high Tregs cells in vivo. This finding led to the assumption that in in vivo systems the hydrophilic–lipophilic properties of statins do not play a critical role in their ability to affect the Tregs pool, probably due to the penetrability of both the hydrophilic and the lipophilic statins to hepatic cells [28], leading to an indirect effect on immune cells.

Many pleiotropic effects of statins, in particular the immune modulating effects, are thought to be mediated by the reduction of the isoprenoid intermediates Farnesylpyrophosphatase (FPP) and Geranylgeranylpyrophosphatase (GGPP) synthesis, which results in a decreased activation of the small G proteins, Ras and Roo, respectively [9]. A potential mechanism for the induction of Foxp3 by statins within the CD4+CD25−Foxp3− subset might be related to this pathway.

Interestingly, neither atorvastatin, nor pravastatin, significantly increased the number of CD4+CD25high Tregs or the expression levels of Foxp3, in a C57BL/6 murine model in vitro and in vivo. How could these observations be reconciled with the immunosuppressive properties that have been ascribed to statins in experimental murine models? It has been shown that in murine experimental autoimmune encephalomyelitis (EAE), atorvastatin treatment in vitro and in vivo induced a Th2 bias and suppression of Th1 cytokine secretion [12]. The Th2 response induced by statins in murine C57BL/6 models was also observed by Hakamada-Taguchi et al. which showed that treatment of primed CD4+ T cells in vitro with cerivastatin, simvastatin, lovastatin and atorvastatin, but not pravastatin, inhibited Th1 development and promoted Th2 polarization [34]. Thus, it can be assumed that in murine models, pravastatin failed to influence either a Th2 subset bias or Treg upregulation. In contrast, the anti-inflammatory effects of atorvastatin, in murine models are probably evident by promoting a Th2 response rather than the expansion of the Treg pool.

In conclusion, our results provide evidence that several statins induce expansion of functionally active CD4+CD25+Foxp3+ Tregs in humans in vitro and in vivo. Increased number of Treg cells by statins in the atherosclerotic lesion would result in reduced pathogenic responses mediated by the effector T cells in the atheroma, and a bias to a stable plaque as a consequence [14]. Thus, this finding sheds new light on the mechanisms mediating the vasculo-protective properties of several statins that could also applicable to immune mediated disorders.

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