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Simvastatin Inhibits IFN Regulatory Factor 4 Expression and Th17 Cell Differentiation in CD4⁺ T Cells Derived from Patients with Multiple Sclerosis

Xin Zhang,^{*,1} Yazhong Tao,^{*,1} Luigi Troiani,^{*} and Silva Markovic-Plese^{*,†}

Subsequent to the clinical trial of simvastatin in patients with relapsing remitting multiple sclerosis (RR MS), which demonstrated the ability of simvastatin to inhibit new inflammatory CNS lesion formation, the current in vitro study has characterized the mechanisms through which simvastatin inhibits Th17 cell differentiation. The anti-inflammatory effects of statins are mediated by the inhibition of isoprenylation, which ensures proper membrane insertion and function of proteins. Small GTPases, involved in multiple signal transduction pathways, are the key targets for isoprenylation. We report that simvastatin, one of the most hydrophobic statins with good CNS penetration, inhibited Th17 cell differentiation and IL-17A, IL-17F, IL-21, and IL-22 secretion in in vitro-differentiated naive CD4⁺ T cells from RR MS patients. Simvastatin exerted a less prominent effect on the cells from healthy controls, as it inhibited only IL-17F secretion. The inhibition of Th17 cell differentiation was mediated via inhibition of IFN regulatory factor 4 (IRF4) expression, which was identified as a key transcription factor for human Th17 cell differentiation using both IRF4 gene knockdown and overexpression experiments. In studies addressing which isoprenylation pathway—geranylgeranylation or farnesylation—is inhibited by simvastatin, we demonstrated that the geranylgeranyl transferase inhibitor replicated the effect of simvastatin. Selective inhibition of geranylgeranylated RhoA-associated kinase replicated the effect of simvastatin on the inhibition of IRF4 expression and IL-17A, IL-17F, IL-21, and IL-22 secretion, presenting a promising new therapeutic approach for this disabling disease. *The Journal of Immunology*, 2011, 187: 3431–3437.

Multiple sclerosis (MS) is the most prevalent inflammatory, presumably autoimmune, CNS disease that leads to chronic progressive disability in the young adult population (1). According to current understanding of the pathogenesis of the disease, the autoimmune response is initiated in genetically susceptible individuals by environmental triggers: ubiquitous viral or bacterial infections. The innate immune response against microbial pathogens leads to the activation of autoreactive T cells, which penetrate the blood–brain barrier and initiate within the CNS a chronic inflammatory response against myelin Ags. While the available first-line therapies (IFN- β and glatiramer acetate) are only moderately effective, therapies that provide more effective suppression of the disease activity (mitoxantrone, natalizumab) are associated with a significant toxicity.

All but the recently approved fingolimod therapy are parenterally administered, which contributes to the side effect profile and relatively poor compliance. Therefore, new therapies that may target some of the key pathways in the development of the autoimmune response and that have oral bioavailability and better tolerability are actively sought.

Th17 cells play a critical role in the development of the autoimmune response. Their role in relapsing remitting MS (RR MS) is supported by a recent report on the increased frequency of IL-17A-producing cells, which is positively associated with clinical disease activity (2). Th17 cell differentiation is orchestrated by multiple cytokines, including IL-6, IL-1 β , TGF- β , IL-21, and IL-23, which stimulate Th17 cell differentiation, and IFN- γ , IL-4, IL-12, IL-10, and IL-27, which inhibit Th17 cell differentiation (3). Mouse studies of the transcriptional regulation of Th17 cell differentiation have established a central role of retinoic acid-related orphan nuclear hormone receptor (ROR) γ . However, our understanding of the human Th17 cell differentiation is still elusive (3). Recently, IFN regulatory factor 4 (IRF4) was identified as a transcription factor for Th17 cell differentiation, as *Irf4*-deficient mice lack Th17 cells and were completely resistant to experimental autoimmune encephalomyelitis (EAE) (4). Consistent with the EAE susceptibility, IRF4 is required for the induction of ROR γ _t and ROR α upon exposure to Th17-polarizing cytokines. RhoA-associated kinase (ROCK)2 is activated under Th17-polarizing conditions and phosphorylates IRF4, which directly binds to the IL-17A and IL-21 promoters and induces their transcriptional activation (5, 6). In contrast to several recent animal studies on the regulation and function of IRF4 in T cell differentiation (7), the role of IRF4 in human Th17 differentiation has not been extensively studied.

Statins are orally administered competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonic

^{*}Department of Neurology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; and [†]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

¹X.Z. and Y.T. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Silva Markovic-Plese, 6109 Neuroscience Research Building, 103 Mason Farm Road, Chapel Hill, NC 27599. E-mail address: markovic@email.unc.edu

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Abbreviations used in this article: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; FTI, farnesyl transferase inhibitor; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; GGTI, geranylgeranyl transferase inhibitor; HC, healthy control; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A; IRF4, IFN regulatory factor 4; L-DC, LPS-treated DC; MA, mevalonic acid; MS, multiple sclerosis; ROCK, RhoA-associated kinase; ROR, retinoic acid-related orphan nuclear hormone receptor; RR MS, relapsing remitting multiple sclerosis; shRNA, short hairpin RNA; SL-DC, simvastatin plus LPS-treated DC; SN, supernatant.

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acid (MA) (8). As effective cholesterol-lowering agents, statins have been extensively used for prevention of cardiovascular disease. Recent studies have reported on their immunomodulatory effects, including induction of a Th1 to Th2 cytokine shift (9, 10), inhibition of inflammatory cell migration to the CNS mediated by suppression of matrix metalloproteinase-9 activity (11), and allosteric LFA-1 blockade (12). The best-characterized mechanism of the immunomodulatory effects of statins is the inhibition of isoprenylation mediated via depletion of farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), isoprenoids generated as intermediary metabolites in cholesterol synthesis (13). Isoprenylation, a posttranslational protein modification in which lipid moieties are covalently attached to target proteins, determines protein localization to cellular membranes and their biological activity (14). Small GTPases, which play a role in multiple signal transduction cascades (15), are the major substrates for such posttranslational modifications.

Two isoprenylation pathways—geranylgeranylation and farnesylation—are used in the attachment of GGPP and FPP to the cysteine residue in the specific GTPase CAAX C-terminal motifs (16). Among multiple Ras-like family members, Rho, Rab, Rac, and Cdc42 are geranylgeranylated, whereas Ras is preferentially farnesylated (17). Although the role of small GTPases in actin polymerization and cytoskeleton formation has been extensively studied, their role in signal transduction and cytokine secretion has not been characterized in detail (18, 19). Subsequent to the simvastatin treatment clinical trial in RR MS patients, which demonstrated the ability of simvastatin to inhibit new inflammatory CNS lesion formation (20), our study has characterized the immunomodulatory mechanisms through which simvastatin targets the differentiation of Th17 cells, which play a critical role in the development of the autoimmune response in MS.

Materials and Methods

Study subjects

Forty RR MS patients were enrolled in the study upon signing an institutional review board-approved informed consent form. The inclusion criteria consisted of a confirmed diagnosis of RR MS according to McDonald's diagnostic criteria (21), age 18–55 y, and an extended disability status score of 1.5–5.5. The exclusion criteria were a history of hyperlipidemia treated with statins and immunomodulatory or immunosuppressive therapy at the time of blood sample collection. The treatment-free period was at least 4 wk for i.v. methylprednisolone and 3 mo for IFN- β and glatiramer acetate. Patients previously treated with immunosuppressive medications were not enrolled in the study.

Cell separation and culture

Naive CD45RA⁺CD4⁺ T cells were isolated from the peripheral blood of 40 RR MS patients and 3 healthy controls (HCs) using a Naive CD4⁺ T-Cell Isolation Kit (Miltenyi Biotec). The cell purity (>98%) was confirmed by flow cytometry. Naive CD4⁺ T cells (2×10^6 per condition) were cultured in serum-free medium and stimulated with plate-immobilized anti-CD3 (1 μ g/ml) and anti-CD28 (5 μ g/ml) mAbs (BD Biosciences) to induce cell differentiation in the absence or presence of Th17-polarizing cytokines IL-1 β , IL-6, IL-23 (50 ng/ml; R&D Systems), and anti-IL-4 (BD Biosciences) and anti-IL-27 (GeneTex) mAbs (10 μ g/ml). When indicated, 2×10^6 naive CD4⁺ T cells per condition were cultured in the absence or presence of simvastatin (10 μ M; EMD Biosciences), MA (100 μ M; Sigma), farnesyl transferase inhibitor (FTI)-227 or geranylgeranyl transferase inhibitor (GGTI)-298 (10 μ M; EMD Biosciences), Rab GTPase inhibitor perillyl alcohol (Sigma), Rac1 GTPase inhibitor NSC-23766 (Tocris Bioscience), or Rho kinase inhibitor Y-27632 (EMD Biosciences) (100 μ M) for 24 h in serum-free medium, and then stimulated with Th17-polarizing cytokines to induce T cell differentiation. Th1 cell differentiation was induced in the presence of IL-12 (10 ng/ml), IFN- γ (3 ng/ml), and anti-IL-4 mAb (10 μ g/ml), and Th2 cell differentiation was induced in the presence of IL-4 (10 ng/ml) and anti-IL-12 mAb (10 μ g/ml). Media containing the same concentration of IFN- γ and IL-4 that was used in Th1- and Th2-polarizing cytokines were incubated for the same time (12 d) and used as the control medium whose cytokine con-

centrations were subtracted from the IFN- γ and IL-4 produced by T cells upon Th1 and Th2 differentiation, respectively. After 2 h, 48 h, or 12 d, the cells were harvested for RNA and protein extraction, and the supernatants (SNs) were collected for cytokine measurements using ELISA.

Coculture experiments

Dendritic cells (DCs; 2×10^6 per condition) were cultured in the absence or presence of simvastatin for 24 h in serum-free medium and matured with LPS. After 48 h of DC maturation, the SNs from the simvastatin plus LPS-treated DCs (SL-DCs) or the LPS-treated DCs (L-DCs) were transferred into the empty wells, and the DCs were washed in serum-free medium. Allogenic naive CD4⁺ T cells (2×10^6) derived from four RR MS patients were added to the DCs in the fresh media, the DC SNs, or to serum-free medium in the absence or presence of simvastatin. After 24 h, the cells were stimulated with plate-immobilized anti-CD3 and anti-CD28 mAbs for 12 d, the cells were harvested for RNA extraction, and the SNs were collected for the cytokine measurements. Media containing the same concentration of LPS and simvastatin as in DC cultures were incubated for the same time to be used as the LPS control medium (L-medium) or as the simvastatin plus LPS control medium (SL-medium), whose effects were subtracted from the DC SNs transferred to the T cells to eliminate any direct effect of LPS and simvastatin on the cytokine production of the T cells.

ELISA

SNs from the above cultures were collected and stored at -80°C until the cytokine measurements. IL-17A, IL-17F, IL-21 (eBioscience), IL-22 (Antigenix America), IL-4, and IFN- γ (BD Biosciences) were measured in duplicate using ELISA following the manufacturer's recommendations. The results are expressed for each subject as the cytokine concentration in pg/ml.

Western blotting

Proteins from cell lysates of the naive CD4⁺ T cell cultures were denatured in SDS, resolved by 10% SDS-PAGE, and transferred on polyvinylidene fluoride membranes prewetted with 100% methanol. Membranes were incubated with Abs against RORc, IRF4 (Santa Cruz Biotechnology), STAT3, p-STAT3 (Cell Signaling), Def6 (a kind gift from Dr. B. Pernis, Columbia University, New York, NY), and GAPDH (Chemcon) overnight. The blots were washed with TBST and incubated with IRDye 680 or IRDye 800 conjugated IgG secondary Ab (LI-COR Biosciences) for 1 h. The membranes were washed and protein bands visualized and quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences).

Short hairpin RNA gene knockdown

Naive CD4⁺ T cells (2×10^6 per condition), derived from three RR MS patients, were plated in 24-well plates at a concentration of 10^6 cells/ml in the absence or presence of *IRF4* or copepod (copGFP) control short hairpin RNA (shRNA) lentiviral particles with 10 μ g/ml polybrene (Santa Cruz Biotechnology) following the manufacturer's instructions. Cells were placed in the selection medium containing 2.5 μ g/ml puromycin for 4 d. After washing, the cells were cultured in serum-free medium in the absence or presence of simvastatin for 24 h and then stimulated with plate-immobilized anti-CD3 and anti-CD28 mAbs in the absence or presence of Th17-polarizing cytokines. The cells were harvested for Western blotting after 2 h, and the SNs were collected after 12 d for the cytokine measurements.

Overexpression of *IRF4*

The pCMV empty vector and *IRF4* construct (OriGene) were transformed into *Escherichia coli* DH5 α competent cells. Plasmid extraction was performed using an Endofree Plasmid Maxi Kit (Qiagen) following the manufacturer's instructions. *IRF4* and a control plasmid were nucleofected at 5 μ g into freshly isolated 6×10^6 naive CD4⁺ T cells following the manufacturer's instructions (Lonza). After washing and 24-h incubation in fresh serum-free medium, the cells were cultured in the absence or presence of simvastatin for 24 h and then stimulated with plate-immobilized anti-CD3 and anti-CD28 mAbs in the absence or presence of Th17-polarizing cytokines. The cells were harvested after 2 h for Western blotting, and the SNs were collected after 12 d for the cytokine measurements.

Quantitative RT-PCR

Total RNA was extracted using an RNeasy kit (Qiagen), and cDNA was synthesized using a High Capacity cDNA Archive Kit (Applied Bio-

systems). The primers for RORc, IRF4, and 18S were purchased from Applied Biosystems, and the gene expression was measured by quantitative RT-PCR using TaqMan Gene Expression Assays (Applied Biosystems). The results are expressed for each subject as the relative gene expression normalized for the 18S mRNA expression.

Statistics

Statistical analyses of the comparisons for multiple groups (more than two groups) were performed using a repeated-measures ANOVA with GraphPad InStat software (GraphPad Software). A p value <0.05 was considered significant.

Results

Simvastatin-induced inhibition of Th17-cytokine secretion is predominantly mediated by its direct effect on T cells

To compare the effects of simvastatin on Th17 cell differentiation via its effect on DC surface marker expression (18, 22), on DC cytokine secretion (23), and its direct effect on T cells, we cultured naive CD45RA⁺ CD4⁺ cells with LPS-matured DCs in the absence (L-DC) or presence of simvastatin (SL-DC), with the SNs of DCs matured in the absence (SL-SN) or presence of simvastatin (SL-SN) or on T cells alone in the absence or presence of simvastatin, and then measured their Th17 cytokine secretion. The direct effect of simvastatin on the T cells provided the strongest inhibition of IL-17A (-22 -fold, $p < 0.001$, Fig. 1), IL-17F (-3.26 -fold, $p < 0.01$), IL-21 (-9.05 -fold, $p < 0.05$), and IL-22 (-3.47 -fold, $p < 0.05$) secretion (data not shown). The effect of cytokine secretion by DCs also inhibited to a lesser degree IL-17A secretion (-4.6 -fold, $p < 0.01$), whereas the inhibition of the DC surface molecule expression involved in cell-to-cell contact did not significantly inhibit the IL-17A secretion by T cells (Fig. 1).

Simvastatin inhibits Th17 cell differentiation through inhibition of IRF4 expression

To identify the optimal polarizing cytokines in human Th17 cell differentiation, we treated naive CD4⁺ T cells derived from nine RR MS patients with IL-1 β , IL-6, IL-23, or TGF- β 1. Similar to a previous report in healthy individuals (24), IL-1 β and IL-6 in-

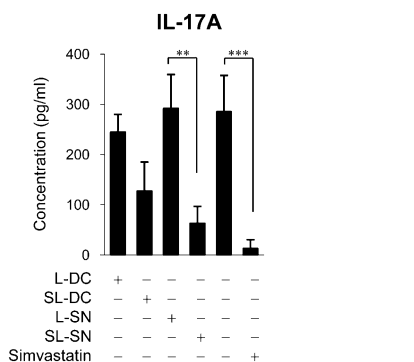


FIGURE 1. Simvastatin predominantly inhibits IL-17A secretion by its direct effect on T cells. DCs (2×10^6 per condition) derived from four RR MS patients were matured with LPS and cultured in the absence (L-DC) or presence of simvastatin (SL-DC) for 24 h. After 48 h, the supernatants from LPS-matured DCs (L-SN) and from simvastatin plus LPS-treated DCs (SL-SN) were collected, and the DCs were washed in serum-free medium. Allogenic naive CD4⁺ T cells (2×10^6) derived from four RR MS patients were cocultured with 1) DCs, 2) the DC SNs, or 3) alone in the absence or presence of simvastatin. After 24 h, the cells in all conditions were stimulated with plate-immobilized anti-CD3 and anti-CD28 mAbs for 12 d, when the SNs were collected for the cytokine measurements. The results are expressed as cytokine concentration in pg/ml. Statistical analysis was performed using repeated-measures ANOVA. ** $p < 0.01$, *** $p < 0.001$.

duced a significant increase in IL-17A secretion (2.0-fold and 1.8-fold, respectively) (Supplemental Fig. 1A) but failed to induce RORc gene expression (Supplemental Fig. 1B). TGF- β 1 induced RORc gene expression (3.2-fold) (Supplemental Fig. 1B) but not IL-17A production (Supplemental Fig. 1A), indicating that human Th17 differentiation may not be directly regulated by RORc, as has been reported in mice (3). In contrast, the gene expression of IRF4 was significantly induced by IL-1 β (5.4-fold) and by IL-6 (5.5-fold) (Supplemental Fig. 1B). A linear regression analysis revealed a positive correlation between the IRF4 and IL-17A gene expression ($p = 0.0002$, $r = 0.5484$), indicating that IRF4 may play an important role in human Th17 cell differentiation.

We next examined the effect of simvastatin on the expression of RORc, p-STAT3, and IRF4 during Th17 differentiation. Th17-polarizing cytokines (IL-1 β , IL-6, IL-23, anti-IL-4 and anti-IL-27 mAbs) (25) induced IRF4, RORc, and p-STAT3 expression at 2 h (Fig. 2A). Simvastatin at 2 h inhibited IRF4 and minimally decreased RORc expression, and at 48 h it inhibited IRF4, RORc, and p-STAT3 expression (Fig. 2A), indicating that the simvastatin-mediated inhibition of IRF4 expression may play a key role in the early inhibition of Th17 differentiation, as revealed by decreased secretion of IL-17A and IL-17 F (Fig. 2B). Although Chen et al. (6) have reported that the expression of the IRF4 binding factor Def6 regulates IRF4 function by inhibiting its binding to the IL-17A and IL-21 cytokine promoters, and Canonigo-Balancio et al. (26) have reported that it induced Th17 cell differentiation, in our study Def6 was not induced by Th17 cell differentiation, and its expression was not changed by simvastatin treatment (data not shown).

To demonstrate that the effect of simvastatin on Th17 cell differentiation is mediated via HMG-CoA reductase inhibition, we used its downstream metabolite mevalonic acid and demonstrated that the inhibition of IRF4 was reversed by the addition of MA (Fig. 3A). Consistent with the IRF4 inhibition, simvastatin significantly inhibited Th17 cytokine secretion, including IL-17A, IL-17F, IL-21, and IL-22, which was reversed by MA (Fig. 3B) in the differentiated naive CD4⁺ T cells derived from RR MS patients. In contrast, in HCs simvastatin inhibited only IL-17F secretion, and the other Th17 cytokines showed only a trend similar to the changes observed in RR MS patients (Fig. 3C).

IRF4 is a transcription factor required for human Th17 cell differentiation

To confirm further the role of IRF4—which was inhibited by simvastatin—in human Th17 cell differentiation, we knocked down the IRF4 gene expression in naive CD4⁺ T cells using shRNA IRF4 lentiviral particles (gene silencing efficiency 75–85%, Fig. 4A). Upon IRF4 knockdown, Th17-polarizing cytokines failed to induce Th17 cell differentiation and the secretion of IL-17A, IL-17F, IL-21, and IL-22 (Fig. 4B). IRF4 knockdown also inhibited RORc expression (Fig. 4A), indicating that IRF4 signals upstream of RORc.

To investigate if IRF4 is sufficient to induce Th17 cell differentiation, we overexpressed IRF4 in the naive CD4⁺ T cells derived from RR MS patients (Fig. 4C). IRF4 overexpression significantly increased IL-17A, IL-17F, IL-21, and IL-22 secretion (Fig. 4D), even in the absence of Th17-polarizing cytokines. In the presence of Th17-polarizing cytokines, IRF4 overexpression induced significantly higher secretion of all tested Th17 cytokines, whose inhibition by simvastatin was significantly decreased in comparison with the control plasmid-transfected Th17 differentiated cells (Fig. 4D). These results directly confirm that the IRF4 transcription factor is sufficient for the induction of human Th17 cell differentiation independent of RORc.

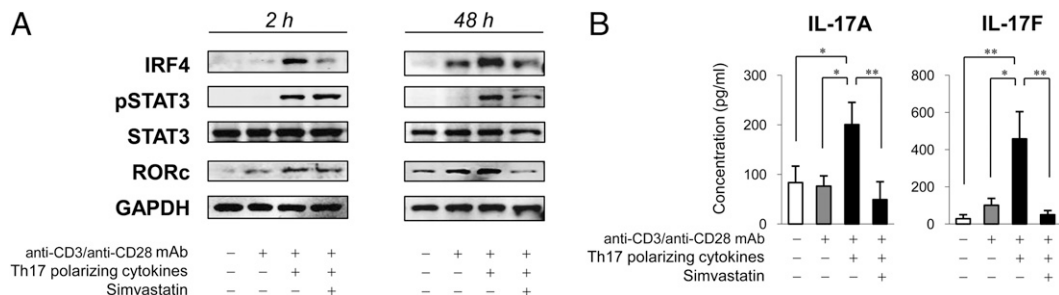


FIGURE 2. Simvastatin inhibits Th17 differentiation by suppressing IRF4 expression. *A*, Naive CD4⁺ T cells were derived from six RR MS patients and cultured in the absence or presence of simvastatin for 24 h and then stimulated with plate-immobilized anti-CD3 plus anti-CD28 mAbs for 2 h and 48 h in the absence or presence of Th17-polarizing cytokines. The protein expression was measured by Western blotting; one of six similar experiments is presented. *B*, The SNs were collected from the same cultures, and IL-17A and IL-17F concentrations were measured in duplicate using ELISA. Statistical analysis was performed using repeated-measures ANOVA. * $p < 0.05$, ** $p < 0.01$.

Simvastatin inhibits Th17 differentiation through the inhibition of small GTPase geranylgeranylation

To identify which isoprenylation pathway is selectively inhibited when simvastatin inhibits Th17 cell differentiation, we compared its effects with those of selective farnesylation and geranylgeranylation inhibitors. Signaling experiments revealed that GGTI inhibited IRF4 expression at 2 h (Fig. 5A) and mimicked the simvastatin-induced inhibition of IRF4 expression and the subsequent IL-17A, IL-17F, and IL-22 secretion (Fig. 5B), whereas FTI failed to do so.

We next compared the magnitude of statin-mediated inhibition of Th17 cell differentiation with the effect on Th1 and Th2 T cell differentiation. The results revealed that simvastatin significantly inhibited IFN- γ secretion, which was replicated with GGTI ($p < 0.05$). The secretion of the Th2 cytokine IL-4 was significantly induced in the presence of simvastatin, whose effect was mimicked by FTI ($p < 0.05$; Supplemental Fig. 2). Comparison of the magnitude of simvastatin-inhibited Th17 cell differentiation with its effects on the differentiation of other T cell lineages revealed that simvastatin more significantly inhibited the production of the Th17 cytokines IL-17A (-4.7-fold) and IL-17F (-14-fold) than it inhibited the secretion of Th1 IFN- γ (-1.7-fold) and induced the secretion of the Th2 prototypical cytokine IL-4 (2.6-fold).

Simvastatin inhibits IRF4 expression and Th17 cell differentiation via Rho kinase/ROCK inhibition

Subsequent studies have identified the extent to which individual Rab, Rac, and Rho GTPases play a role in the induction of Th17 signature cytokine secretion by using selective inhibitors of each GGPP-modified GTPase. Western blotting experiments revealed that Rho kinase/ROCK inhibitor Y-27632 decreased IRF4 expression at 2 h (Fig. 6A). Consistent with previous reports on the mutual induction of GTPases (27, 28), we demonstrated that all three tested GTPase inhibitors significantly suppressed IL-22 secretion in differentiated Th17 cells. Rac and Rho kinase inhibitors suppressed IL-21, simvastatin-inhibited IL-17F was replicated by Rab and Rho-kinase inhibitor, whereas simvastatin-inhibited IL-17A secretion was only replicated by the Rho kinase/ROCK-specific inhibitor Y-27632 (Fig. 6B).

Discussion

Our current study compared the relative contribution of the simvastatin-mediated effect on DC cell-to-cell contact, cytokine secretion, and its direct effect on T cell differentiation. We found that its direct effect on T cells most significantly inhibited IL-17A, IL-17F, IL-21, and IL-22 secretion, whereas the culture of naive CD4⁺ T cells with the SNs from simvastatin-pretreated DCs provided less prominent inhibition of IL-17A secretion.

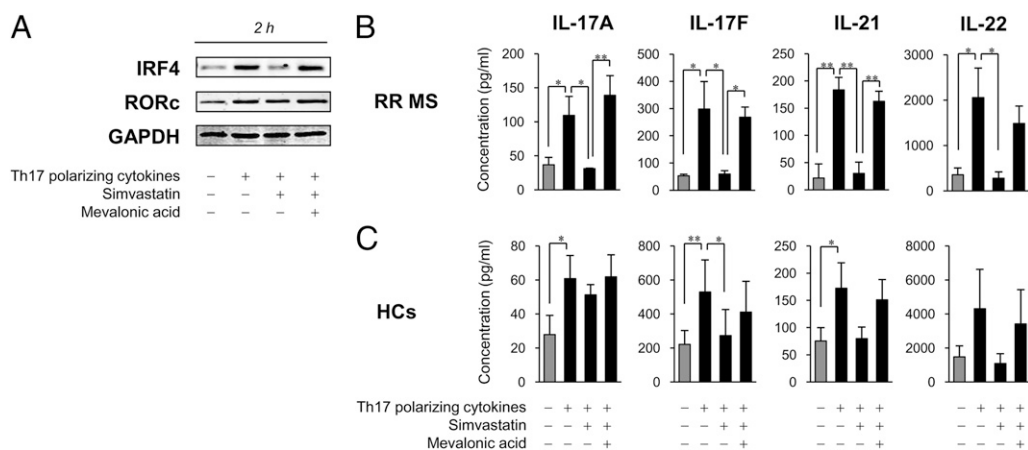


FIGURE 3. Simvastatin-mediated HMG-CoA reductase inhibition induces more prominent suppression of Th17 cell differentiation in patients with RR MS than in HCs. Naive CD4⁺ T cells were derived from three RR MS patients and HCs, cultured in the absence or presence of simvastatin and MA (100 μ M) for 24 h, and stimulated with plate-immobilized anti-CD3 plus anti-CD28 mAbs for 2 h in the absence or presence of Th17-polarizing cytokines. *A*, The protein expression was measured by Western blotting. GAPDH was used as a loading control. One of three similar blots is shown. *B*, The SNs from the same cultures were collected after 12 d, and the concentrations of IL-17A, IL-17F, IL-21, and IL-22 were measured by ELISA. The figure presents an average of three experiments. *C*, SNs from the cultures derived from HCs were used for the ELISA cytokine measurements. Statistical analysis was performed using a repeated-measures ANOVA. * $p < 0.05$, ** $p < 0.01$.

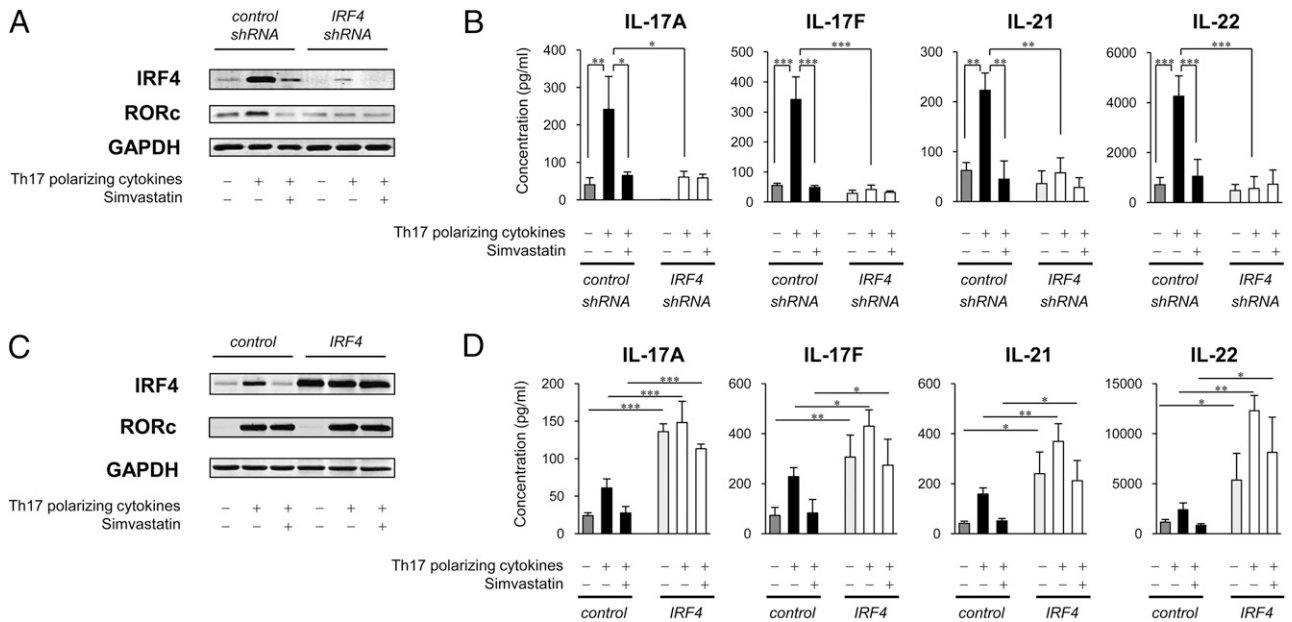


FIGURE 4. IRF4 is a transcription factor required for human Th17 cell differentiation. *A*, Naive CD4⁺ T cells derived from three RR MS patients were seeded at 10⁶ cells/ml in the presence of copepod (copGFP) control or *IRF4* shRNA lentiviral particles following the manufacturer’s instructions. Cells were placed in selection media for 4 d, cultured in serum-free medium in the absence or presence of simvastatin for 24 h, followed by stimulation with plate-immobilized anti-CD3 and anti-CD28 mAbs in the absence or presence of Th17-polarizing cytokines. Cells were harvested after 2 h and the SNs collected after 12 d. The protein expression of IRF4, RORc, and GAPDH was measured by Western blotting. The figure presents one of three similar blots. *B*, The cytokine production of IL-17A, IL-17F, IL-21, and IL-22 was measured by ELISA, and an average of three experiments is presented. *C*, IRF4 signaling is sufficient to induce Th17 cell differentiation. IRF4 and control plasmids were nucleofected into naive CD4⁺ T cells, which were cultured in the absence or presence of simvastatin (10 μM) for 24 h and then stimulated with plate-immobilized anti-CD3 and anti-CD28 mAbs in the absence or presence of Th17-polarizing cytokines. Cells were harvested for Western blotting after 2 h, and the SNs were collected for the cytokine measurements after 12 d of differentiation. The protein expression of IRF4, RORc, and GAPDH was measured by Western blotting. The figure presents one of three similar blots. *D*, The cytokine production was measured by ELISA. Statistical analysis was performed using a repeated-measures ANOVA. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Several reports on the direct effect of statins on T cell cytokine secretion in EAE have identified a shift from Th1 to Th2 cytokine secretion (9, 10). Our study examines previously unexplored effect of statins on Th17 cell differentiation in humans. Similar to the findings of Acosta-Rodriguez et al. (24), we found that IL-1β and IL-6 induce IL-17A secretion. These cytokines also induced the expression of IRF4, a transcription factor recently proposed to play a critical role in Th17 differentiation and EAE induction in mice (4). In contrast, TGF-β induced *RORc* expression but not IL-17A secretion (3). A positive correlation between *IRF4* and *IL-17A* further suggests the critical role of IRF4 as a transcription factor in human Th17 cell differentiation.

Simvastatin inhibited early (2 h) IRF4 expression and to some extent RORc expression in the naive cells differentiated in the

presence of Th17-polarizing cytokines, leading to a later (48 h) inhibition of IRF4, RORc, and p-STAT3 expression. The specificity of effect, mediated via HMG-CoA reductase inhibition, was confirmed by the reversal of the inhibition of IRF4 protein expression and IL-17A, IL-17F, IL-21, and IL-22 secretion by MA, a product of HMG-CoA reductase. Study of the effect of simvastatin on Th17 cell differentiation in HCs showed a similar pattern but less prominent Th17 cytokine inhibition; simvastatin inhibited only IL-17F secretion but did not significantly change the production of other Th17 cytokines. Studies of larger numbers of HCs may be needed to definitively establish their differential response to simvastatin in comparison with RR MS patients. Whereas recent studies have demonstrated that IRF4-deficient mice failed to differentiate Th17 cells and are completely resistant to EAE (4),

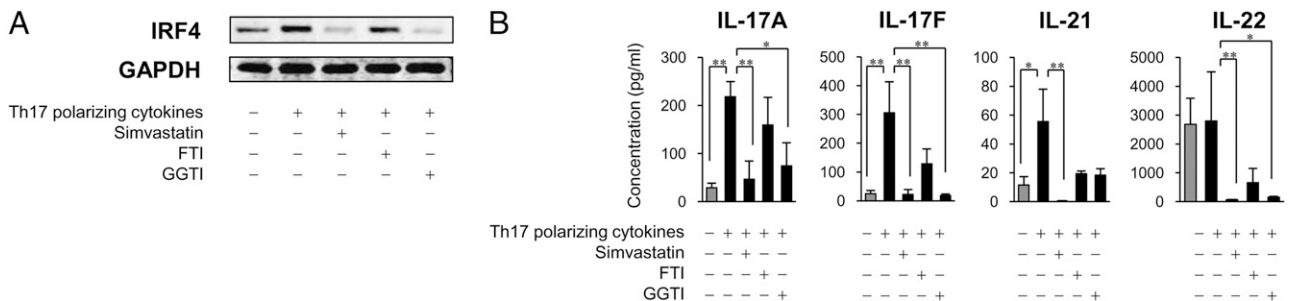


FIGURE 5. Simvastatin inhibits IRF4 expression and Th17 cell differentiation via inhibition of geranylgeranylation. Naive CD4⁺ T cells (2 × 10⁶ per condition) derived from three RR MS patients were cultured in the absence or presence of simvastatin, FTI, or GGTI for 24 h and then stimulated with plate-immobilized anti-CD3 and anti-CD28 mAbs for 12 d in the presence of Th17-polarizing cytokines. *A*, The protein expression of IRF4 and GAPDH were measured by Western blotting. The results present one of three similar experiments. *B*, The cytokine production of IL-17A, IL-17F, IL-21, and IL-22 was measured by ELISA. Statistical analysis was performed using a repeated-measures ANOVA. **p* < 0.05, ***p* < 0.01.

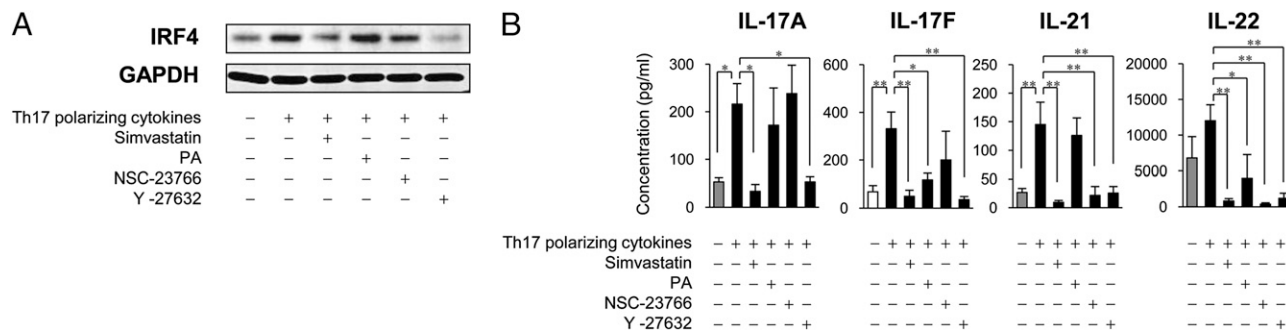


FIGURE 6. Simvastatin inhibits Th17 cell differentiation via selective inhibition of Rho kinase ROCK. **A**, Naive CD4⁺ T cells derived from three RR MS patients were cultured in the absence or presence of simvastatin, Rab GTPase inhibitor perillyl alcohol (PA), Rac1 GTPase inhibitor NSC-23766, and Rho kinase inhibitor Y-27632 for 24 h and then stimulated with plate-immobilized anti-CD3 and anti-CD28 mAbs for 12 d in the presence of Th17-polarizing cytokines to induce T cell differentiation. The protein expression was measured by Western blotting. The results present one of three similar experiments. **B**, The cytokine production was measured using ELISA; the results present an average of three experiments. Statistical analysis was performed using a repeated-measures ANOVA. * $p < 0.05$, ** $p < 0.01$.

human studies only indirectly pointed to the role of IRF4 in Th17 cell differentiation by demonstrating elevated IRF4 expression in IL-17⁺ cells, which are committed to Th17 differentiation (29). Our study using shRNA *IRF4* knockdown has directly demonstrated the IRF4 requirement for human Th17 cell differentiation. We found that loss of IRF4 expression abolished the Th17 cell differentiation. Furthermore, IRF4-deficient naive CD4⁺ cells had a decreased ROR γ c expression, whereas STAT3 phosphorylation was unchanged. These findings are consistent with the results from *IRF4*-deficient mice, which exhibited decreased ROR α and ROR γ t expression and intact STAT3 phosphorylation upon IL-21 stimulation (30). IRF4 overexpression experiments further confirmed the dominant role of IRF4 in Th17 cell differentiation. IRF4 transfection, even in the absence of Th17-polarizing cytokines, induced a Th17 cell phenotype that included IL-17A, IL-17F, IL-21, and IL-22 secretion independent of RORc. However, simvastatin failed to inhibit exogenous IRF4 expression, suggesting that it does not directly regulate IRF4, but most likely inhibits it through an inhibition of Rho/ROCK signaling.

The effects of simvastatin on T cell differentiation are mediated by the inhibition of geranylgeranylation, as GGTI inhibited IRF4 protein expression and IL-17A, IL-17F, and IL-22 cytokine secretion. We further extended our studies on this medication's effects on Th1 and Th2 cell differentiation and demonstrated that it significantly inhibited IFN- γ secretion upon Th1 differentiation, whereas it induced IL-4 production in the presence of Th2-polarizing cytokines. However, the inhibition of IL-17A and IL-17F secretion was more prominent than the changes in Th1 and Th2 differentiation, demonstrating that simvastatin preferentially inhibits Th17 cell differentiation in humans. The anti-inflammatory effect of statins in patients with rheumatoid arthritis and cardiac disease was characterized by a change from the Th1 to the Th2 cytokine secretion profile, but the effect on the differentiation of recently identified Th17 cells has not been examined (31, 32). Although animal studies have reported that GGTI inhibited T cell proliferation and that FTI induced Th1 and Th2 immune responses (33), our results have demonstrated that the simvastatin-mediated inhibitory effect on human Th1 and Th17 differentiation is mediated via inhibition of geranylgeranylation, and the inhibition of farnesylation induces Th2 differentiation. Because several studies have reported that statins may also induce increased IFN- γ production (34) and matrix metalloproteinase secretion (35) in PBMCs derived from MS patients, it is important to monitor their potential proinflammatory effects. However, the majority of the available reports indicate that statins predomi-

nantly exhibit anti-inflammatory effects, targeting multiple molecular mechanisms.

Biswas et al. (36) have recently reported that Rho kinase ROCK2 is activated under Th17-skewing conditions and phosphorylates IRF4, suggesting that therapeutic targeting of the Rho-mediated signaling pathway may selectively inhibit Th17 cell differentiation. We demonstrated that the ROCK inhibitor Y-27632 replicated the effect of simvastatin, indicating that simvastatin inhibits IRF4 expression and Th17 cell differentiation via inhibition of the Rho kinase ROCK. Although the statin-mediated inhibition of geranylgeranylation was implicated in the inhibition of mouse Th17 cell differentiation (37), our study has identified that the Rho/ROCK inhibitor selectively suppresses IRF4 expression and subsequent IL-17A, IL-17F, IL-21, and IL-22 secretion. However in *Def6*-deficient mice characterized by spontaneous autoimmune disease, the use of the same inhibitor did not affect IRF4 expression or its cellular localization (36). The more prominent effect of Rho/ROCK inhibitor in humans suggests that species-specific differences may exist in IRF4 regulation and in its role in Th17 cell differentiation; however, the release of IRF4 in *Def6*-deficient mice should also be considered. It is important to note that the inhibitory role of *Def6* on the Th17 cell differentiation reported in this TCR-transgenic mouse model was not confirmed in the nonrestricted TCR repertoire of BALB/c mice (26). However, in our study, Th17 cell differentiation was not associated with increased *Def6* expression, and simvastatin did not change it.

In contrast to statin-mediated inhibition of cholesterol synthesis, which raises some concerns about the inhibition of remyelination in the CNS (38), Rho kinase inhibitors may provide a desired selective inhibition of the key pathogenic cytokines, which drive the autoimmune response in MS. Because Rho kinase expression is induced by inflammatory stimuli and Rho expression is increased in MS lesions (39), the selective Rho kinase inhibitors represent a promising therapeutic approach (40) that requires further clinical testing.

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Disclosures

The authors have no financial conflicts of interest.

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