

Simvastatin inhibits secretion of Th17-polarizing cytokines and antigen presentation by DCs in patients with relapsing remitting multiple sclerosis

Xin Zhang¹, Yazhong Tao¹, Jinzhao Wang¹, Rafael Garcia-Mata²
and Silva Markovic-Plese^{1,3}

¹ Department of Neurology, University of North Carolina at Chapel Hill, NC, USA

² Department of Cell and Developmental Biology, University of North Carolina at Chapel Hill, NC, USA

³ Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, NC, USA

Statins, widely used cholesterol-lowering agents, have also been demonstrated to have antiinflammatory effects. Here, we characterize the capacity of simvastatin to target DCs and modulate T-cell priming and Th17-cell differentiation, in a cohort of patients with relapsing remitting multiple sclerosis (RRMS). We report that simvastatin inhibits IL-1 β , IL-23, TGF- β , IL-21, IL-12p70, and induces IL-27 secretion from DCs in RRMS patients, providing an inhibitory cytokine milieu for Th17 and Th1-cell differentiation. The effect on DCs is mediated via induction of SOCS1, SOCS3, and SOCS7 gene expression, which are associated with the inhibition of STAT1, STAT3, and ERK1/2 phosphorylation. A geranylgeranyl transferase inhibitor replicated simvastatin's effects on DC cytokine secretion, implicating that simvastatin-induced depletion of isoprenoids mediates this effect. Simvastatin inhibited antigen presentation by DCs via suppression of the MHC class I expression, costimulatory molecules CD80 and CD40, and by inducing a dramatic loss of dendritic processes. The changes in DC morphology were also mediated via inhibition of geranylgeranylation. The therapeutic use of geranylgeranylation inhibitors may provide selective inhibition of key pathogenic cytokines that drive the autoimmune response in MS; their use represents a promising therapeutic approach that requires further clinical testing.

Keywords: DCs · MS · Simvastatin · Th17 cells



Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Multiple sclerosis (MS), a chronic progressive CNS inflammatory disease, is a leading cause of disability in the young adult popula-

tion [1]. The disease is mediated by a T-cell autoimmune response against CNS myelin antigens. However, the triggering molecular events are still not fully elucidated. It is postulated that peripheral blood T cells are activated by viral or bacterial infections in the context of an innate immune response. The dysregulation of the immune response leads to chronic inflammation, whereby activated mononuclear cells migrate through the blood brain barrier and cause inflammatory perivenular CNS lesions that lead to

Correspondence: Dr. Silva Markovic-Plese
e-mail: markovic@email.unc.edu

axonal transection and myelin loss. Recent studies have indicated that T helper (Th)17-cells play a critical role in the development of the autoimmune response in MS [2]. Th17-cell differentiation in humans is orchestrated by multiple cytokines, including the stimulatory IL-1 β , IL-6 [2], IL-23 [3], and IL-21 [4], and the inhibitory cytokines IFN- γ , IL-4, IL-27, IL-12 [5,6], and IL-10, while the role of TGF- β remains controversial [7].

DCs, the most efficient APCs, play a role in T-cell priming, and are one of the key sources of the cytokines that promote T-cell differentiation. Maturation of DCs is characterized by upregulated surface expression of MHC and costimulatory molecules, which enable DCs to effectively present antigen. Serafini et al. [8] have identified myeloid (m)DCs in MS lesions, preferentially localized in perivascular cuffs in the proximity of T cells, suggestive of their role in antigen presentation within the CNS. More recently, Bailey et al. [9] have demonstrated that peripherally derived CD11b⁺ mDCs accumulate in the CNS during relapsing EAE, where they localize in the central parts of the active lesions and induce IL-17 production by CD4⁺ T cells via the production of large amounts of IL-6, TGF- β , and IL-23. The mDCs that are attracted to the inflamed CNS from the peripheral circulation acquire myelin antigens and present them to naïve autoreactive CD4⁺ T cells. mDC abundance in the CNS positively correlated with clinical disease activity [9].

It has been reported that *in vitro* differentiated monocyte-derived DCs from MS patients secrete higher levels of the proinflammatory cytokines IFN- γ , IL-6, TNF- α , and IL-23 than those from healthy controls (HCs) [10], suggesting that therapies regulating DC cytokine secretion might contribute to the control of disease activity. Indeed, our study of IFN β -1a, an effective treatment of relapsing remitting multiple sclerosis (RRMS), has reported that IFN β -1a inhibited DC secretion of IL-1 β , IL-23, and TGF- β 1 and induced IL-27 secretion, which collectively suppressed Th17-cell differentiation [11]. Glatiramer acetate, another first-line therapy for MS, modulates the cytokine secretion of human DCs, inducing a Th1 to Th2 cytokine secretion shift [12]. Statins have been reported to suppress the IFN- γ -induced MHC class II expression on APCs [13], and to inhibit DC maturation [14] and antigen presentation [15–17]. However, the effect of statins on DC cytokine secretion has not been studied in humans.

Statins are orally administered competitive inhibitors of HMG-CoA reductase, a key regulatory enzyme involved in cholesterol biosynthesis that catalyzes the HMG-CoA conversion to mevalonate [18]. The immunomodulatory effects of statins are mediated via depletion of isoprenoids, the intermediary metabolites in cholesterol synthesis [19]. Isoprenylation, a posttranslational protein modification in which lipid moieties are covalently attached to target proteins, determines protein localization to cellular membranes and enables their biological activity [20].

There are two isoprenylation pathways: geranylgeranylation and farnesylation, which are employed in the attachment of the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) to the cysteine residue in CAAX C-terminal motifs of specific GTPases [21]. Small GTPases are the major substrates for isoprenylation. Among multiple Ras-like family members, Rho, Rab, Rac, and Cdc42 are geranylgeranylated, while Ras

is preferentially farnesylated [22]. The role of small GTPases in actin polymerization and cytoskeleton formation has been extensively studied, but only a few studies have addressed the role of GTPases in signal transduction and cytokine secretion [23,24]. Following successful simvastatin treatment in patients with active RRMS [25], here we characterize the ability of simvastatin to modulate the T-cell priming and Th17-cell differentiating function of DCs. We report that selective inhibition of geranylgeranylated GTPases mediates simvastatin's immunomodulatory effects on DC cytokine secretion and antigen presentation.

Results

Simvastatin modifies DC cytokine secretion toward a Th17- and Th1-inhibitory pattern

Cytokine production by priming DCs plays a critical role in T-cell differentiation. In order to evaluate simvastatin's effect on DC cytokine secretion and to examine to what extent the effect differs between the RRMS patients and HCs, we evaluated the DCs derived from ten RRMS patients and from six HCs. In RRMS patients, simvastatin significantly inhibited IL-1 β , IL-23, TGF- β 1, and IL-12p70, and increased IL-27 secretion (Fig. 1), while the changes in IL-4 and IL-6 did not reach statistical significance (Fig. 1 and data not shown). In contrast, in HCs, simvastatin only significantly induced IL-27 secretion (Fig. 1), while changes in the other tested cytokines did not reach statistical significance.

The statistical analysis of the differences between the LPS-stimulated DC cytokine secretion revealed that DCs derived from RRMS patients secrete significantly higher levels of IL-1 β , IL-23, TGF- β , and IL-12p70 and lower levels of IL-27 in comparison with DCs derived from HCs (Supporting Information Fig. 1). In order to examine to what extent these differences reflect the baseline cytokine production of the *ex vivo* separated nonmanipulated mDCs, we isolated CD1c (BDCA-1)⁺ DCs from the peripheral blood of four RRMS patients and four HCs and measured gene expression levels of the key Th17-polarizing cytokines. Our data revealed that the gene expression of IL-23p19 and IL-21 was significantly increased in mDCs from RRMS patients in comparison with those from HCs. In contrast, mDCs derived from RRMS patients had significantly lower IL-27 gene expression (Supporting Information Fig. 2).

Dose titration experiments demonstrated that simvastatin induced a significant inhibition of IL-23 and IL-21 already at 0.1 μ M. However, since the most significant effect on all tested cytokines was detected at 10 μ M, we used this concentration in the remainder of the study.

Similar to the findings in monocytes [26], studies of the mechanisms of simvastatin-induced changes in DCs' cytokine secretion demonstrated that it upregulated *SOCS1*, *SOCS3*, and *SOCS7* gene expression (Fig. 2A). At the protein level, simvastatin inhibited STAT1, STAT3, and ERK1/2 phosphorylation (Fig. 2B), which may contribute to the inhibition of IL-12p70, IL-1 β , IL-23, IL-21, and TGF- β 1 secretion by DCs [27,28].

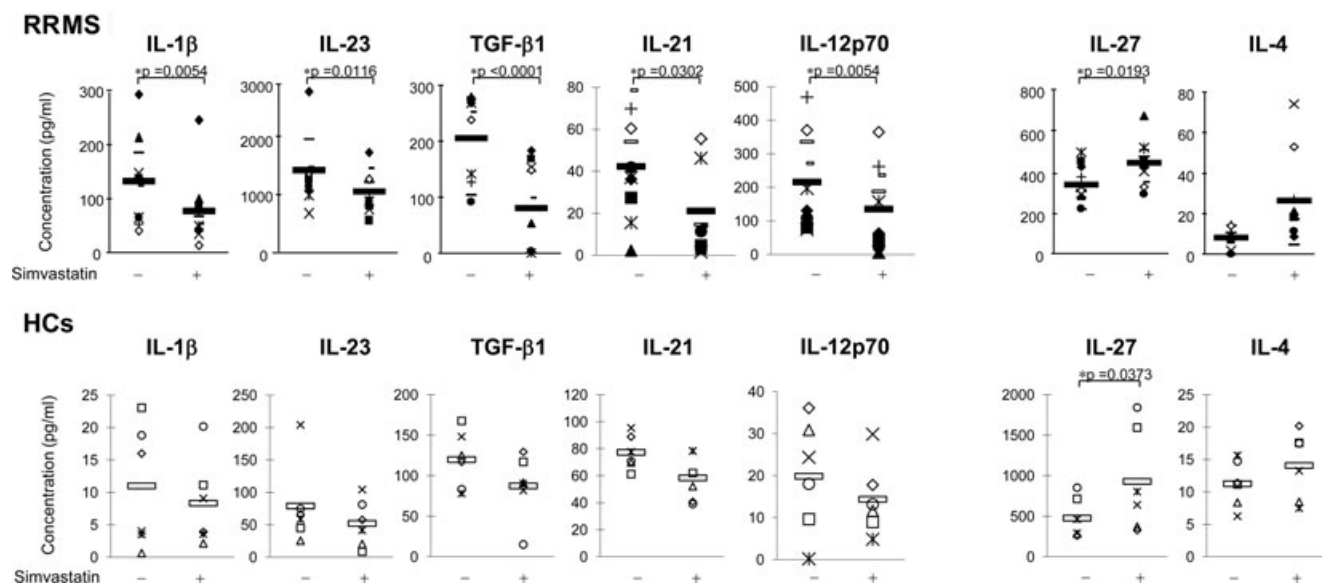


Figure 1. Simvastatin modifies DC cytokine secretion toward Th17- and Th1-inhibitory pattern. A total 3×10^6 monocyte-derived DCs per condition collected from ten RRMS patients and six HCs were cultured in the absence or presence of simvastatin (10 μ M) for 24 h in serum-free medium, and then matured with LPS (1 μ g/mL) for 48 h prior to SN collection. Cytokine secretion was measured using ELISA. The results are expressed as cytokine concentration in pg/mL; each symbol represents an individual study subject and the bar represents the mean. Statistical analysis was performed using a paired t-test

Simvastatin regulates DC cytokine secretion via inhibition of GTPase geranylgeranylation

Since GTPases play a role in the proximal activation of JAK/STAT and ERK1/2, signaling pathways that lead to cytokine transcription [29, 30], we examined to what extent was simvastatin’s effect on DCs’ cytokine secretion mediated by the selective inhibition of farnesylation or geranylgeranylation, using the farnesyltransferase inhibitor (FTI)-277 and the geranylgeranyl transferase inhibitor (GGTI)-298.

The effects of simvastatin on the inhibition of IL-1 β , IL-23, TGF- β 1, and IL-21, and the induction of IL-27 were reversed by mevalonic acid, a downstream metabolite of HMG-CoA reductase, which confirmed that simvastatin’s regulation of DC cytokine

secretion is mediated through the inhibition of HMG-CoA reductase (Supporting Information Fig. 3).

We then examined the extent to which simvastatin’s effect on DC cytokine secretion was mediated by the selective inhibition of farnesylation or geranylgeranylation, using the GGTI-298 and FTI-277. We demonstrated that GGTI replicated simvastatin’s inhibitory effect on IL-1 β and IL-23, and the induction of IL-27 and IL-4 secretion (Fig. 3A), which suggests that the geranylgeranylated GTPases Rho, Rac, and Rab may regulate the production of these cytokines. Indeed, the statin-induced secretion of IL-27 was reversed in the presence of GGPP and the inhibition of IL-23 and the induction of IL-27 and IL-4 by GGTI were reversed by GGPP (Supporting Information Fig. 3). Simvastatin’s inhibition of TGF- β 1 secretion was not replicated by GGTI or by FTI, while

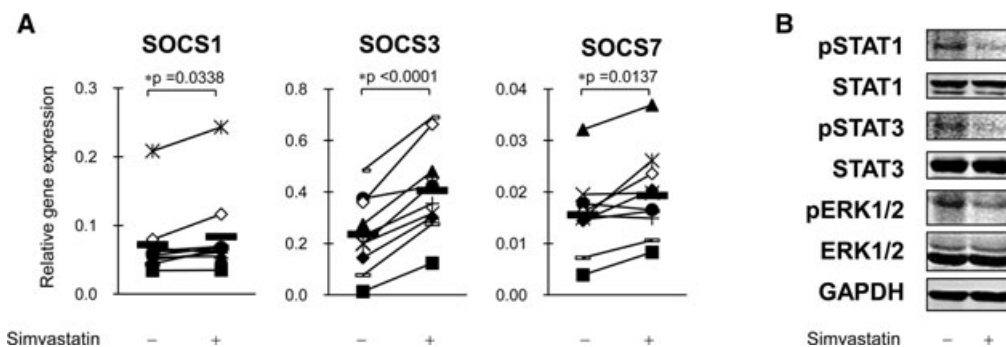


Figure 2. Simvastatin induced the expression of SOCS1, SOCS3, and SOCS7 and inhibited the phosphorylation of STAT1, STAT3, and ERK1/2. DCs derived from ten RRMS patients were treated as described in Figure 1. Cells were harvested at 2 h after maturation for RNA and protein extraction. (A) Gene expression of SOCS1, SOCS3, and SOCS7 was measured using qRT-PCR. The results are expressed as the relative gene expression normalized against the 18S mRNA. (B) Protein expression of pSTAT1, STAT1, pSTAT3, STAT3, pERK1/2, ERK1/2, and GAPDH was measured using western blotting. The results shown represent one of three similar blots. Statistical analysis was performed using a paired t-test.

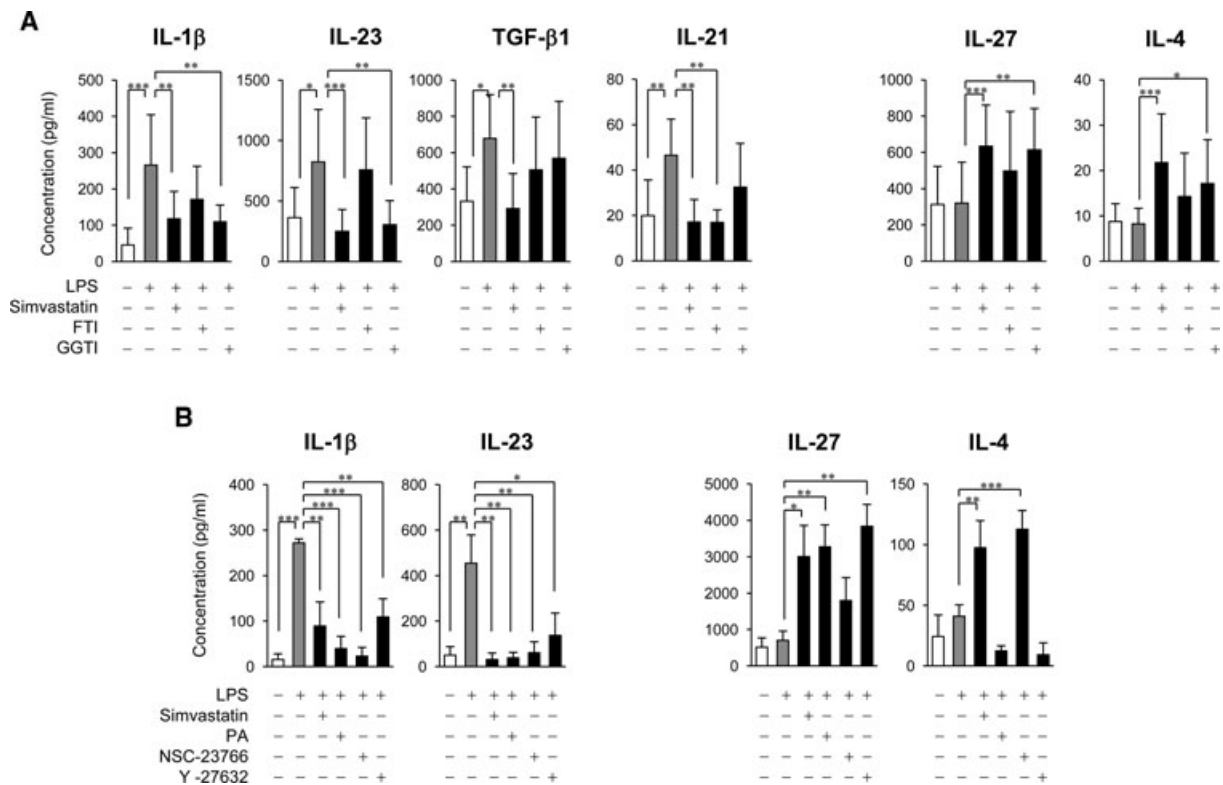


Figure 3. Simvastatin regulates DC cytokine secretion via inhibition of GTPase geranylgeranylation. (A) DCs from eight RRMS patients were cultured in the absence or presence of simvastatin, FTI-277 (FTI) and GGTI-298 (GGTI) (10 μ M) for 24 h in serum-free medium, and then matured with LPS for 48 h prior to SN collection. Secretion of the indicated cytokines was measured in SNs using ELISA. (B) DCs derived from three RRMS patients were cultured in the absence or presence of simvastatin, the Rab inhibitor perillyl alcohol (PA), the Rac inhibitor NSC-23766 (NSC) and the Rho kinase inhibitor Y-27632 (Y) at 100 μ M for 24 h in serum-free medium, and matured with LPS. The concentrations of secreted IL-1 β , IL-23, IL-27, and IL-4, which were changed by GGTI, were measured in SNs by ELISA. Data are shown as mean values \pm SD. Statistical analysis was performed using a repeated measures ANOVA; * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FTI inhibited IL-21 secretion (Fig. 3A), which was reversed in the presence of FPP (Supporting Information Fig. 3).

To further investigate simvastatin's specific targets among the geranylgeranylated GTPases, DC cytokine secretion was measured upon treatment with the Rab inhibitor perillyl alcohol (PA), the Rac inhibitor NSC-23766 (NSC), or the Rho-associated kinase (ROCK) inhibitor Y-27632. All three GTPase inhibitors reproduced simvastatin's inhibition of IL-1 β and IL-23 secretion, the Rab- and Rho/ROCK-specific inhibitors mimicked the simvastatin-increased IL-27, while the increase in IL-4 secretion was replicated only by the Rac-specific inhibitor (Fig. 3B).

Simvastatin induces loss of dendritic processes and inhibits antigen presenting capacity of DCs

Since geranylgeranylation of GTPases facilitates their proper membrane insertion and activation, we measured the extent to which simvastatin inhibits membrane binding of the geranylgeranylated GTPases Rab1, Rac1, and RhoA. Following subcellular fractionation of DCs that had been matured in the absence or presence of simvastatin, Western blotting studies on the DCs derived from three RRMS patients revealed that simvastatin inhibited the membrane binding of all three geranylgeranylated GTPases, while at the

same time it increased their inactive cytosolic fractions (Fig. 4). Simvastatin-mediated functional inhibition of all three geranylgeranylated GTPases may impair their downstream signaling, which is involved in the regulation of cytoskeletal changes and cytokine transcription.

In an attempt to characterize the simvastatin-induced changes in DC maturation, we measured the surface expression of MHC class I, class II DR, CD83, CD80, CD86, CD40, and CCR7 on DCs matured in the absence or presence of simvastatin. Our results obtained on DCs derived from ten RRMS patients revealed that simvastatin significantly decreased the percentage of MHC class I (-1.02-fold, $p = 0.0166$), CD80 (-1.16-fold, $p = 0.0404$) and CD40 (-3.14-fold, $p = 0.0228$) expressing DCs, while it did not change the expression of MHC class II DR, CD83, CD86, and CCR7 (data not shown).

In addition to the relatively small changes in DC surface marker expression, simvastatin induced dramatic changes in their morphology, characterized by a loss of dendritic processes and reduction of actin stress fibers. These effects were replicated by GGTI, but not by FTI treatment (Fig. 5A), and were reversed by mevalonic acid (data not shown). These results indicate that simvastatin-induced inhibition of small GTPase geranylgeranylation may affect DC antigen presentation. In order to determine whether the simvastatin-mediated morphological changes resulted from the

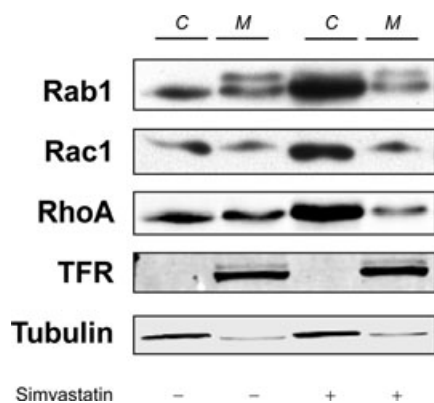


Figure 4. Simvastatin inhibits membrane binding of Rab1, Rac1, and RhoA in DCs derived from RRMS patients. A total of 4×10^6 DCs per condition derived from three RRMS patients were cultured in the absence or presence of simvastatin for 24 h in serum-free medium, and matured with LPS for 48 h prior to cell harvesting. Cells were fractionated into cytosolic (C) and membrane (M) fractions, and the protein expression of Rab1, Rac1, RhoA was measured by Western blotting. TFR and tubulin were used as loading controls for the membrane and cytosolic proteins, respectively. The results shown represent one of three similar blots.

inhibition of specific geranylgeranylated GTPases, DC cell shape was examined upon treatment with the Rab inhibitor PA, the NSC, or ROCK inhibitor Y-27632. However, none of these individual GTPase inhibitors reproduced the morphological changes in DCs mediated by simvastatin and GGTI (data not shown), which suggests that the morphological changes may be mediated by multiple geranylgeranylation-dependent GTPases.

In order to directly examine the extent to which simvastatin affects DC antigen presentation, we cultured antigen-specific T-cell clones (TCCs) with Influenza (Flu) HA_{306–316} peptide presented by autologous DCs, matured in the absence or presence of simvastatin, GGTI, or FTI. We found that simvastatin inhibited antigen presentation, since Flu peptide presented by simvastatin-pretreated DCs induced significantly lower TCC proliferation in comparison to the untreated DCs (Fig. 5B). Simvastatin inhibition of DC antigen presentation is mediated through its inhibition of geranylgeranylation, since treatment with GGTI, but not with FTI, replicated this effect.

Discussion

The current study on mature DCs, the most effective APCs and regulators of T-cell differentiation, revealed that simvastatin inhibits the secretion of IL-1 β , IL-23, TGF- β 1, IL-21, and IL-12p70, and induces the production of IL-27 in RRMS patients, providing a cytokine milieu that inhibits Th17 and Th1-cell differentiation [5, 31]. The effect in HCs showed a similar pattern, however only IL-27 induction reached statistical significance. In comparison with simvastatin's effect on monocytes [26], the present study on DCs derived from RRMS patients revealed more prominent inhibition of IL-1 β , TGF- β , and IL-23 secretion upon in vitro simvastatin treatment.

While a previous study on monocytes revealed that simvastatin-induced upregulation of *SOCS3* and *SOCS7* had a significant negative correlation with *IL-6* and *IL-23* gene expression [26], in the DCs derived from RRMS patients simvastatin's induction of *SOCS1*, *SOCS3*, and *SOCS7* gene expression was even more prominent, and was associated with the simvastatin-mediated inhibition of STAT1, STAT3, and ERK1/2 phosphorylation. Individual SOCS proteins regulate multiple cytokines and therefore represent an attractive therapeutic target in inflammatory diseases [32], as reported by Li et al. [27] who showed that *SOCS3*-transduced DCs had a decreased production of IL-12 and IL-23. *SOCS1* and *SOCS3* directly inhibit JAK tyrosine kinase activity, which inhibits the homodimerization of STAT1 and STAT3 [33]. STAT3 tyrosine phosphorylation is critical for IL-1 β [34] and IL-23 secretion [27], while ERK1/2 phosphorylation leads to upregulated TGF- β 1 production [2]. In an animal model of rheumatoid arthritis, intra-articular injection of recombinant *SOCS3* reduced joint inflammation, decreased STAT3 phosphorylation and the subsequent IL-6 production [35]. Furthermore, as recently reported from our laboratory, the supernatants (SNs) from simvastatin-pretreated DCs significantly inhibited IL-17A secretion in activated naive CD4⁺CD45RA⁺ T cells [36], suggesting that simvastatin-induced changes in DC cytokine secretion indirectly inhibit Th17-cell differentiation.

Consistent with previous studies of the antiinflammatory effects of statins [20, 22], we found that simvastatin's immunomodulatory effects on DC cytokine production are mediated by the inhibition of protein isoprenylation. In studies addressing which isoprenylation pathway (geranylgeranylation or farnesylation) is inhibited by statins, we demonstrated that the inhibitor of geranylgeranylation (GGTI) replicates simvastatin's ability to inhibit IL-1 β and IL-23 and induce IL-27 and IL-4 secretion by DCs, and that GGPP reverses simvastatin-mediated increase in IL-27 secretion.

The expression of DC surface markers is essential for antigen presentation. Yilmaz et al. [23] have reported that simvastatin inhibits DC HLA-DR expression, the costimulatory molecules CD86, CD83, and CD40, and their ability to induce T-cell proliferation in healthy individuals. In RRMS patients, we demonstrated that simvastatin significantly inhibits HLA class I, CD80, and CD40 expression, but does not inhibit HLA-DR expression, consistent with a report on simvastatin's inhibition of MHC class II antigen presentation due to the inhibition of Rab-mediated HLA DR-antigen complex endocytosis and lack of effect on the HLA-DR basal surface expression on B cells and DCs [37].

In addition to moderate inhibition of costimulatory molecule expression, simvastatin dramatically changed the morphology of DCs, which lose their long dendritic processes. It has been reported that individual GTPases mediate actin polymerization and stress fiber formation (Rho), lamellipodiae (Rac), and filopodia (Cdc42) formation, which are interrelated processes that determine cell shape, polarization, and migration [38]. The cell-shape changes induced by simvastatin were replicated by GGTI, suggesting that simvastatin-induced cytoskeletal changes may be related to the dysfunction of geranylgeranylated proteins. Our studies of the

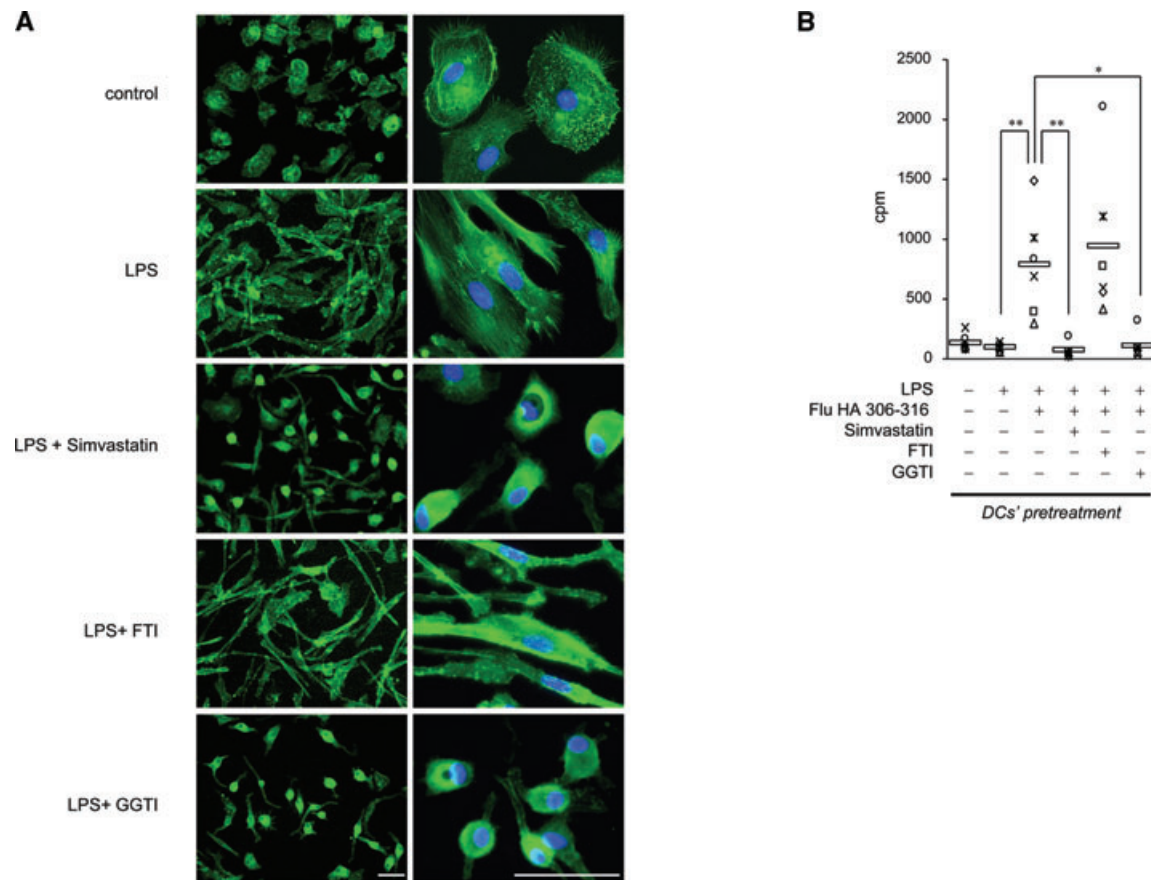


Figure 5. Simvastatin inhibits DC antigen presentation via inhibition of geranylgeranylation. (A) A total of $2 \times 10^5/\text{cm}^2$ DCs derived from four RRMS patients were cultured in the absence or presence of simvastatin, FTI, and GGTI ($10 \mu\text{M}$) for 24 h in serum-free medium, and then matured with LPS for 48 h. The cells were fixed and blocked. Actin was stained with FITC-phalloidin (green) and the nuclei were stained with Hechst dye (blue). The images shown represent the results of one of four similar experiments. Original magnification $10\times$ (left) and $40\times$ (right); scale bars = $10 \mu\text{m}$. (B) A total of 1×10^5 DCs/well were cultured in the absence or presence of simvastatin, FTI, and GGTI for 24 h in serum-free medium, and matured with LPS for 48 h. After washing, Flu peptide was presented by DCs to 10^5 /well Flu HA₃₀₆₋₃₁₆ peptide-specific autologous T-cell clones for an additional 48 h. The plates were pulsed with ^3H -thymidine and the proliferative response against the peptide was measured and shown as cpm. Each symbol represents an individual clone and bars represent average values. Statistical analysis was performed using a repeated measures ANOVA; * indicates $p < 0.05$, ** $p < 0.01$.

subcellular distribution of Rab1, Rac1, and RhoA have demonstrated that simvastatin decreased the membrane-bound fraction of all three tested GTPases, accompanied by their increased amount in the cytoplasm. Furthermore, simvastatin-mediated inhibition of geranylgeranylation significantly decreased the antigen presenting capacity of DCs, as evidenced by the inhibited proliferation of peptide-specific TCCs when cocultured with simvastatin- and GGTI-pretreated antigen-presenting DCs. While simvastatin affected multiple components of DC antigen presentation, we propose that the loss of long dendritic processes most effectively decreases the capacity of DCs to migrate and interact with T cells [16].

In conclusion, simvastatin's immunoregulatory effects on the Th17 and Th1-polarizing cytokine secretion and antigen presenting capacity of DCs represent novel biological activities of this therapy [39], which may be even more selectively achieved by using geranylgeranylation inhibitors.

Materials and methods

Study subjects

Thirty-one patients with RRMS and ten HCs were enrolled in the study upon signing an Institutional Review Board-approved informed consent form. The inclusion criteria consisted of a confirmed diagnosis of RRMS according to McDonald's diagnostic criteria [40], age 18–55, 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 weeks for IV methylprednisolone, 3 months for IFN- β and glatiramer acetate. Patients previously treated with natalizumab, fingolimod or immunosuppressive medications were not enrolled in the study.

Cell separation and culture

CD14⁺ monocytes were isolated from the peripheral blood of 31 RRMS patients and six HCs using an EasySep Negative Selection Monocyte Enrichment Kit (StemCell Technologies Inc.). The cell purity (>98%) was confirmed by flow cytometry. A total of 1×10^6 /mL monocytes were cultured with GM-CSF (1000 U/mL) and IL-4 (500 U/mL) (R&D Systems) in complete RPMI 1640 medium (RPMI 1640, 1% HEPES, 2% L-Glutamine, 1% Sodium Pyruvate, 1% Essential Amino Acids, 1% Pen/Strep, and 5% human serum) for 7 days to generate DCs. After 7 days, immature DCs phenotype was consistently CD14⁻, CD11c⁺, CD1a⁺, CD1c (BDCA-1)⁺, CD141 (BDCA-3)⁺, CD86⁺, HLA class I⁺, HLA class II⁺, and CD83⁺. After washing with PBS, 3×10^6 monocyte-derived DCs per condition were cultured as indicated, in the absence or presence of simvastatin (10 μ M), the farnesyl transferase inhibitor (FTI)-277 and the GGTI-298 (10 μ M) (EMD Biosciences), the Rab GTPase inhibitor PA (Sigma), the Rac1 GTPase inhibitor NSC-23766 (NSC) (Tocris Bioscience) and the Rho kinase inhibitor Y-27632 (100 μ M) (EMD Biosciences), mevalonic acid (100 μ M), FPP, and GGPP (20 μ M) (Sigma) for 24 h in serum-free media. They were then matured with LPS (1 μ g/mL) (Sigma) for 2 h prior to RNA and protein extraction, and for 48 h prior to flow cytometry staining and SN collection.

CD1c (BDCA-1)⁺ dendritic cell, the major subset of myeloid dendritic cells (mDCs), were isolated from the peripheral blood of four RRMS patients and four HCs using a human CD1c (BDCA-1)⁺ Dendritic Cell Isolation Kit (Miltenyi Biotec). The cell purity (>95%) was confirmed by flow cytometry.

Quantitative RT-PCR

Total RNA was extracted from monocyte derived DCs and from isolated BDCA-1⁺ mDCs using an RNeasy Kit (Qiagen), and cDNA was synthesized using a High Capacity cDNA Archive Kit (Applied Biosystems). The primers for SOCS1, SOCS3, SOCS7, IL-1 β , IL-23p19, TGF- β 1, IL-21, IL-27, IL-4, and 18S mRNA were purchased from Applied Biosystems, and the gene expression was measured by quantitative RT-PCR (qRT-PCR) using Taqman Gene Expression Assays (Applied Biosystems) in triplicate. The results are expressed for each subject as the relative gene expression normalized for 18S mRNA expression.

ELISA

SNs from the cultured DCs were collected and stored at -80°C until the cytokine measurements using ELISA. IL-1 β , TGF- β 1, IL-4, IL-6, IL-12p70 (BD Bioscience), IL-27 (R&D systems), IL-23, and IL-21 (eBiosciences) were measured in duplicate by ELISA, following the manufacturer's recommendations. The results are expressed for each subject as the cytokine concentration in pg/mL.

Flow cytometry

A total of 1×10^6 DCs/mL were stained by FITC-conjugated anti-HLA Class I and anti-HLA Class II, PE-conjugated anti-CD80, -CD86 and -CD40 and -CCR7, as well as PE-Cy5.5-conjugated anti-CD11c and allophycocyanin-conjugated anti-CD1a mAb for gating (BD Biosciences). The percentage of gated cells expressing each molecule was determined using CellQuest software (BD Biosciences).

Subcellular fractionation

A total of 4×10^6 DCs per condition derived from three RRMS patients were cultured in the absence or presence of simvastatin for 24 h in serum-free medium, and matured with LPS for 48 h prior to cell harvesting. The cells were washed and incubated for 10 min in ice-cold hypotonic lysis buffer (10 mM HEPES at pH 7.3, 1.5 mM MgCl₂, 5 mM KCl, 1 mM DTT, and protease inhibitors). The cells were scraped and homogenized with 25 strokes of a Dounce homogenizer. The homogenates were centrifuged at 700 g for 3 min at 4°C to pellet nuclei and intact cells. The SNs were spun at 40 000 g for 30 min at 4°C and the pellets were gently washed in hypotonic lysis buffer. A 15% of the cytosolic fraction and 100% of the membrane fraction were analyzed by SDS-PAGE. The expression of Rab1, RhoA (Santa Cruz), Rac1 (BD Biosciences), transferrin receptor (TFR), and tubulin (Sigma) was measured using Western blotting.

Western blotting

Proteins from the cell lysates of the DC cultures were denatured in SDS, resolved by 10% SDS-PAGE, and transferred on polyvinylidene difluoride (PVDF) membranes prewetted with 100% methanol. The membranes were incubated overnight with antibodies against STAT1, pSTAT1, STAT3, pSTAT3, ERK1/2, pERK1/2, Rab1, RhoA (Santa Cruz Biotechnology), Rac1 (BD Biosciences), TFR tubulin (Sigma), and GAPDH (Chemcon). The blots were washed with Tris-Buffered Saline Tween-20, and incubated with IRDye 680- or IRDye 800-conjugated IgG secondary antibody (LI-COR Biosciences) for 1 h. The membranes were washed and the protein bands were visualized and quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences).

Immunostaining

A total 2×10^5 DCs/cm² were fixed in 4% paraformaldehyde for 10 min at room temperature, washed and blocked with 5% goat serum in PBS and 0.1% NP40 for 1 h at room temperature. Actin was stained with FITC-Phalloidin (green) and the nuclei with Hoechst dye (blue). The fluorescent images were viewed using a LEICA DM IRB Inverted Microscope (Leica Microsystems Inc.) and were digitally captured using Q Capture software (Roper Scientific Inc.).

Proliferation assay

A total of 1×10^5 DCs were cultured in the absence or presence of simvastatin, FTI, and GGTI (10 μ M) for 24 h in serum-free medium, followed by maturation with LPS (1 μ g/mL) for 48 h. After washing and adding fresh serum-free medium, 10^5 of Flu HA_{306–318} peptide-specific autologous TCCs per condition were added to the cultures in the absence or presence of Flu HA_{306–316} peptide (10 μ g/mL) for an additional 48 h. The plates were pulsed with 1 μ Ci ³H-thymidine/well, incubated overnight, and harvested. Filters containing DNA with incorporated radioactive isotope were counted in a scintillation counter, and the proliferative response against the antigenic peptide was measured in cpm in comparison to the cells cultured without peptide.

Statistics

Statistical analyses of the paired results for the qRT-PCR, ELISA, and FACS experiments were performed using Student's paired *t*-tests with SigmaPlot 10.0 software (Systat Software Inc.). Statistical analyses of the comparisons for multiple groups (more than two groups) were performed using a repeated measure ANOVA with Graphpad InStat software (Graphpad Software Inc.). A *p* < 0.05 was considered significant.

Acknowledgments: We thank all the patients and healthy individuals for participating in the study, Luigi Troiani and Manisha Chopra for patient enrollment and blood samples collection, Neelima Choudhary for technical assistance, and Dr. Rick Meeker for critical reading of the manuscript. The study was supported by the National Multiple Sclerosis Society Research Grant (RG 4107A1/1); Y.T.'s fellowship support was provided by the National Multiple Sclerosis Society Center Award.

Conflict of interests: The authors declare no financial or commercial conflict of interest.

References

- Sospedra, M. and Martin, R., Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 2005. **23**: 683–747.
- Acosta-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A. and Sallusto, F., Interleukins 1 beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat. Immunol.* 2007. **8**: 942–949.
- Wilson, N. J., Boniface, K., Chan, J. R., McKenzie, B. S., Blumenschein, W. M., Mattson, J. D., Basham, B. et al., Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat. Immunol.* 2007. **8**: 950–957.
- Yang, L., Anderson, D. E., Baecher-Allan, C., Hastings, W. D., Bettelli, E., Oukka, M., Kuchroo, V. K. et al., IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature* 2008. **454**: 350–352.
- Amadi-Obi, A., Yu, C. R., Liu, X., Mahdi, R. M., Clarke, G. L., Nussenblatt, R. B., Gery, I. et al., T(H)17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat. Med.* 2007. **13**: 711–718.
- Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E. et al., Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 2007. **204**: 1849–1861.
- Manel, N., Unutmaz, D. and Littman, D. R., The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgamma. *Nat. Immunol.* 2008. **9**: 641–649.
- Serafini, B., Rosicarelli, B., Magliozzi, R., Stigliano, E., Capello, E., Mancardi, G. L. and Aloisi, F., Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells. *J. Neuropathol. Exp. Neurol.* 2006. **65**: 124–141.
- Bailey, S. L., Schreiner, B., McMahon, E. J. and Miller, S. D., CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ T(H)-17 cells in relapsing EAE. *Nat. Immunol.* 2007. **8**: 172–180.
- Huang, Y. M., Xiao, B. G., Ozenci, V., Kouwenhoven, M., Teleshova, N., Fredrikson, S. and Link, H., Multiple sclerosis is associated with high levels of circulating dendritic cells secreting pro-inflammatory cytokines. *J. Neuroimmunol.* 1999. **99**: 82–90.
- Zhang, X., Jin, J., Tang, Y., Speer, D., Sujkowska, D. and Markovic-Plese, S., IFN-beta1a inhibits the secretion of Th17-polarizing cytokines in human dendritic cells via TLR7 up-regulation. *J. Immunol.* 2009. **182**: 3928–3936.
- Vieira, P. L., Heystek, H. C., Wormmeester, J., Wierenga, E. A. and Kapsenberg, M. L., Glatiramer acetate (copolymer-1, copaxone) promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells. *J. Immunol.* 2003. **170**: 4483–4488.
- Kwak, B., Mulhaupt, F., Myit, S. and Mach, F., Statins as a newly recognized type of immunomodulator. *Nat. Med.* 2000. **6**: 1399–1402.
- Yilmaz, A., Reiss, C., Tantawi, O., Weng, A., Stumpf, C., Raaz, D., Ludwig, J. et al., HMG-CoA reductase inhibitors suppress maturation of human dendritic cells: new implications for atherosclerosis. *Atherosclerosis* 2004. **172**: 85–93.
- Peng, X., Jin, J., Giri, S., Montes, M., Sujkowski, D., Tang, Y., Smrtka, J. et al., Immunomodulatory effects of 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors, potential therapy for relapsing remitting multiple sclerosis. *J. Neuroimmunol.* 2006. **178**: 130–139.
- Kobayashi, M., Azuma, E., Ido, M., Hirayama, M., Jiang, Q., Iwamoto, S., Kumamoto, T. et al., A pivotal role of Rho GTPase in the regulation of morphology and function of dendritic cells. *J. Immunol.* 2001. **167**: 3585–3591.
- Shurin, G. V., Tourkova, I. L., Chatta, G. S., Schmidt, G., Wei, S., Djeu, J. Y. and Shurin, M. R., Small rho GTPases regulate antigen presentation in dendritic cells. *J. Immunol.* 2005. **174**: 3394–3400.
- Markovic-Plese, S., Singh, A. K. and Singh, I., Therapeutic potential of statins in multiple sclerosis: immune modulation, neuroprotection and neurorepair. *Future Neurol.* 2008. **3**: 153–167.
- Cantrell, D. A., GTPases and T-cell activation. *Immunol. Rev.* 2003. **192**: 122–130.
- Greenwood, J., Steinman, L. and Zamvil, S. S., Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nat. Rev. Immunol.* 2006. **6**: 358–370.

- 21 Li, X., Liu, L., Tupper, J. C., Bannerman, D. D., Winn, R. K., Sebti, S. M., Hamilton, A. D. et al., Inhibition of protein geranylgeranylation and RhoA/RhoA kinase pathway induces apoptosis in human endothelial cells. *J. Biol. Chem.* 2002. **277**: 15309–15316.
- 22 Liao, J. K., Isoprenoids as mediators of the biological effects of statins. *J. Clin. Invest.* 2002. **110**: 285–288.
- 23 Yilmaz, A., Reiss, C., Weng, A., Cicha, I., Stumpf, C., Steinkasserer, A., Daniel, W. G. et al., Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. *J. Leukoc. Biol.* 2006. **79**: 529–538.
- 24 Becker, C. E., Creagh, E. M. and O'Neill, L. A., Rab39a binds caspase-1 and is required for caspase-1-dependent interleukin-1 β secretion. *J. Biol. Chem.* 2009. **284**: 34531–34537.
- 25 Vollmer, T., Key, L., Durkalski, V., Tyor, W., Corboy, J., Markovic-Plese, S., Preiningerova, J. et al., Oral simvastatin treatment in relapsing-remitting multiple sclerosis. *Lancet* 2004. **363**: 1607–1608.
- 26 Zhang, X., Jin, J., Peng, X., Ramgolam, V. S. and Markovic-Plese, S., Simvastatin inhibits IL-17 secretion by targeting multiple IL-17-regulatory cytokines and by inhibiting the expression of IL-17 transcription factor ROR γ in CD4 $^{+}$ lymphocytes. *J. Immunol.* 2008. **180**: 6988–6996.
- 27 Li, Y., Chu, N., Rostami, A. and Zhang, G. X., Dendritic cells transduced with SOCS-3 exhibit a tolerogenic/DC2 phenotype that directs type 2 Th cell differentiation in vitro and in vivo. *J. Immunol.* 2006. **177**: 1679–1688.
- 28 Zhou, L., Ivanov, II, Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D. E. et al., IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 2007. **8**: 967–974.
- 29 Simon, A. R., Vikis, H. G., Stewart, S., Fanburg, B. L., Cochran, B. H. and Guan, K. L., Regulation of STAT3 by direct binding to the Rac1 GTPase. *Science* 2000. **290**: 144–147.
- 30 Pelletier, S., Duhamel, F., Coulombe, P., Popoff, M. R. and Meloche, S., Rho family GTPases are required for activation of Jak/STAT signaling by G protein-coupled receptors. *Mol. Cell. Biol.* 2003. **23**: 1316–1333.
- 31 Chen, Z., Tato, C. M., Muul, L., Laurence, A. and O'Shea, J. J., Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum.* 2007. **56**: 2936–2946.
- 32 Ramgolam, V. S. and Markovic-Plese, S., Regulation of suppressors of cytokine signaling as a therapeutic approach in autoimmune diseases, with an emphasis on multiple sclerosis. *J. Signal Transduct.* 2011. **2011**: 635721.
- 33 Kubo, M., Hanada, T. and Yoshimura, A., Suppressors of cytokine signaling and immunity. *Nat. Immunol.* 2003. **4**: 1169–1176.
- 34 Samavati, L., Rastogi, R., Du, W., Huttemann, M., Fite, A. and Franchi, L., STAT3 tyrosine phosphorylation is critical for interleukin 1 β and interleukin-6 production in response to lipopolysaccharide and live bacteria. *Mol. Immunol.* 2009. **46**: 1867–1877.
- 35 Wong, P. K., Egan, P. J., Croker, B. A., O'Donnell, K., Sims, N. A., Drake, S., Kiu, H. et al., SOCS-3 negatively regulates innate and adaptive immune mechanisms in acute IL-1-dependent inflammatory arthritis. *J. Clin. Invest.* 2006. **116**: 1571–1581.
- 36 Zhang, X., Tao, Y., Troiani, L. and Markovic-Plese, S., Simvastatin inhibits IFN regulatory factor 4 expression and Th17 cell differentiation in CD4 $^{+}$ T cells derived from patients with multiple sclerosis. *J. Immunol.* 2011. **187**: 3431–3437.
- 37 Ghittoni, R., Napolitani, G., Benati, D., Olivieri, C., Patrussi, L., Laghi Pasini, F., Lanzavecchia, A. et al., Simvastatin inhibits the MHC class II pathway of antigen presentation by impairing Ras superfamily GTPases. *Eur. J. Immunol.* 2006. **36**: 2885–2893.
- 38 Takai, Y., Sasaki, T. and Matozaki, T., Small GTP-binding proteins. *Physiol. Rev.* 2001. **81**: 153–208.
- 39 Zamvil, S. S. and Steinman, L., Cholesterol-lowering statins possess anti-inflammatory activity that might be useful for treatment of MS. *Neurology.* 2002. **59**: 970–971.
- 40 McDonald, W. I., Compston, A., Edan, G., Goodkin, D., Hartung, H. P., Lublin, F. D., McFarland, H. F. et al., Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* 2001. **50**: 121–127.

Abbreviations: FPP: farnesyl pyrophosphate · GGPP: geranylgeranyl pyrophosphate · GGTI: geranylgeranyl transferase inhibitor · HC: healthy control · NSC: the Rac inhibitor NSC-23766 · PA: perillyl alcohol · ROCK: the Rho-associated kinase · RRMS: relapsing remitting multiple sclerosis · TCC: T-cell clone

Full correspondence: Dr. Silva Markovic-Plese, Department of Neurology, Microbiology and Immunology, University of North Carolina at Chapel Hill, NC 27599, USA

Fax: +1-919 843 4576

e-mail: markovic@email.unc.edu

Received: 27/3/2012

Revised: 16/8/2012

Accepted: 12/10/2012

Accepted article online: 17/10/2012