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Atorvastatin Induces T Cell Anergy via Phosphorylation of ERK1

Sonia Waiczies,* Timour Prozorovski,* Carmen Infante-Duarte,* Astrid Hahner,* Orhan Aktas,* Oliver Ullrich,† and Frauke Zipp2*†

Modulation of T cell response is a novel property of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors. Previously we reported the benefits of atorvastatin treatment in experimental autoimmune encephalomyelitis, the murine model of the T cell-mediated autoimmune disorder multiple sclerosis, in which a blockade of the T cell cycle by atorvastatin was attributed to an accumulation of the negative regulator p27Kip1. We show in this report that, in line with the documented role of p27Kip1 in T cell anergy, treatment with atorvastatin results in a deficient response to a second productive stimulus in human T cells. This effect of atorvastatin was dependent on HMG-CoA reduction and required IL-10 signaling. Importantly, atorvastatin induced an early and sustained phosphorylation of ERK1, but not ERK2, which was crucial for the induction of anergy. On the basis of the therapeutic impact of HMG-CoA reductase inhibitors, the present findings should pave the way for future therapeutic concepts related to tolerance induction in neuroinflammatory disorders such as multiple sclerosis. The Journal of Immunology, 2005, 174: 5630–5635.

A torvastatin is a family member of the orally administered cholesterol-lowering agents known as statins. These drugs bind to the 3-hydroxy-3-methylglutaryl (HMG-CoA)3 reductase leading to competitive displacement of the natural substrate, HMG-CoA, thereby inhibiting its catalytic conversion to t-mevalonate (1). Mevalonate is a precursor not only to cholesterol synthesis but also to intermediary lipid donors that are important for the isoprenylation of GTP-binding proteins, such as Ras, Rap1a, RhoA, and RhoB (2). These small GTPases from the Ras superfamily require lipid modifications for membrane tethering and subsequent interaction with downstream effector cascades. Only then are they capable of regulating their diverse cellular functions, which include cell survival, proliferation, differentiation, and cytoskeletal organization (3). As a result of their isoprenylation requirements, GTPases are targets of statin therapy. One function of receptor-activated Ras signaling governs the accumulation of cyclin D1-cyclin-dependent kinase (CDK)4 cell cycle complexes, via the switching of MAPK signaling cascades (4). Among these cascades, the Ras/Raf/MEK/ERK signaling cascade has been demonstrated to be crucial for both T cell development and activation (5). Although it is clear that even low levels of ERK1/2 MAPK phosphorylation are sufficient to promote T cell proliferation, higher levels have been reported to promote cell cycle arrest, via the induced expression of CDK inhibitors (6, 7). We could previously show that the expression of CDK inhibitor p27Kip1 is induced by applying atorvastatin to human T cells (8). Apart from our observations in T cells, several previous reports have documented the immunomodulatory nature of statins (9–11). These studies have in fact revealed the possible benefits of using these lipid-lowering agents for the treatment of autoimmune disorders such as multiple sclerosis (MS). According to our data and those of other groups, in vivo statin treatment could prevent as well as reverse disease progression in the murine model of MS (8, 12) and in collagen-induced arthritis (13). Based on these observations, and the fact that statins are generally well-tolerated orally administered drugs, a pilot clinical study in MS (14) and a phase II trial in rheumatoid arthritis (15) have already been reported, although further clinical trials are still in progress.

We show for the first time the induction of T cell anergy by atorvastatin treatment, as a result of a HMG-CoA reductase-dependent failure of treated cells to down-regulate the anergy factor p27Kip1. Notably, we observed a robust and rapid phosphorylation of the MAPK ERK1 following atorvastatin treatment, which was independent of T cell activation. The phosphorylation of ERK by MEK1/2 was shown to be crucial for anergy induction by atorvastatin because we were able to prevent T cell hyporesponsiveness by using U0126, a specific blocker of MEK1/2.

Materials and Methods

Reagents

Pure atorvastatin (provided by Pfizer) was dissolved in 2% DMSO/ethanol (carrier was tested as vehicle control). Mevalonate was prepared, as already described, by activating t-mevalonic acid lactone (Sigma-Aldrich) (8). MEK1/2 inhibitor U0126 (Calbiochem) was used to reverse phosphorylation of ERK1/2. Human myelin basic protein (MBP) was purified, as previously described (16), and Con A was purchased from Sigma-Aldrich.

Antibodies

Anti-IL-10 (Sigma-Aldrich) was used to neutralize human IL-10. Anti-CD3/OKT3 (Orthoclone; kindly provided by Janssen-Cilag) and anti-CD28 (R&D Systems) Abs were used together for T cell stimulation. Specific primary Abs to: p27Kip1 and CDK4 (both from Santa Cruz
Biotechnology); Ras (Oncogene Research Products); phosho-Raf, phosho-ERK1/2, ERK1/2 (all from Cell Signaling Technology); and β-actin (Sigma-Aldrich) were used for Western blot analysis.

Peripheral immune and T cells
PBMC (peripheral immune cells) were obtained from heparinized peripheral blood of healthy donors in accordance with local ethics committees and isolated by Ficoll Hypaque density gradient centrifugation. Immunophenotypic analysis of PBMC, on a FACSCalibur (BD Biosciences), showed that CD3+ T cells comprised 60.65% (range from 30 to 85%, n = 11) of PBMC. For some assays, these cells were short-term stimulated with Con A. PBMC stimulated for 4 days with TCR stimuli were termed peripheral T cells.

Human Ag-specific T cells (T cell lines)
Human CD4+ T cell lines specific for human MBP were established using a modified "split-well" protocol, as has been previously described (16). Briefly, 10^5 PBMC were seeded in culture medium (containing 5% pooled Irradiated (3000 rad) autologous APC (10^5) were added to each well of the six wells and taken from each original well and split into two wells on a new plate. After 7 days, 10^5 PBMC (in 96-well round-bottom microtiter plates. At 7–57 days later, 100 μl were from each well and split into two wells on a new plate. Irradiated (3000 rad) autologous APC (10^5) were added to each well of the master plate and to the split plate in the presence or absence of Ag. Ag specificity was tested by a standard proliferation assay. Specifically, responsive populations were selected for further expansion.

Anergy assays
The extent of T cell responsiveness to a second stimulus was performed as previously described (16). Briefly, resting T cell lines or PBMC (0.5 × 10^6/ml) were stimulated with specific Ag presented by irradiated autologous APC (1.5 × 10^6/ml) or non-specific stimuli (2 μg/ml Con A; respectively, in 24-well culture plates for 4 days in the presence or absence of atorvastatin. Surviving T cells were thoroughly washed and restimulated with the original stimulus (in the absence of atorvastatin). The extent of T cell response to a second stimulus was determined using proliferation assays.

[3H]Thymidine incorporation proliferation assay
[3H]Thymidine (0.5 μCi; Amersham Biosciences) was added to each well of a proliferation assay plate. Incorporation of radioactivity was measured after 18 h with a beta counter (Microbeta; Wallac).

Intracellular IFN-γ staining
The flow cytometric determination of intracellular IFN-γ was performed as previously described (16). Briefly, PBMC were stimulated with 0.1 μg/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin in the presence of 10 μg/ml brefeldin A. After 6 h, cells were permeabilized and stained by incubation with 2% FACS permeabilization buffer. Permeabilized and stained cells (1.5 × 10^6/ml) were added to a 96-well plate coated with 3 μg/ml aprotinin, 100 μg/ml aprotinin, 100 μg/ml PMSF with a Hamilton syringe. After 15 min incubation on ice, lysates were centrifuged for 15 min at 16,000 × g. Cellular lysates equivalent to 10 μg of protein, as determined by the bicinchoninic acid method (Pierce), were separated using 12% PAGE and blotted onto nitrocellulose membranes. Following 1 h blocking (5% milk powder, 2% BSA, 0.1% Tween 20) at room temperature, membranes were incubated overnight at 4°C with specific Abs to: p27^kip1; CD4, Ras, phosho-Raf, phosho-MEK1/2, phosho-ERK1/2, ERK1/2, and β-actin. Abs were diluted in blocking buffer. Following a series of washing steps, the membranes were incubated for 1 h with 1.25 μg/ml secondary Ab coupled to HRP (Dako). Specific bands were detected using the ECL-Plus system (Amersham Biosciences). Immunoblots were sequentially probed with β-actin Ab as a loading control. The level of protein expression was measured with BioDocII (Biometra) documentation system, adjusted in relation to the expression of actin, and expressed as arbitrary OD index.

Data analysis
The individual potencies (measured in micrometers) of atorvastatin for inhibiting proliferation (IC_{50}) in Ag-specific T cell lines were determined by plotting mean percentage proliferation response as a function of increasing concentrations of the drug. The mean T cell response was calculated as an index of stimulation (SI) optimized for Ag: cpm_{preg}, whereas the observed percentage response was calculated as follows: (SI_{ag_mixed} × 100%), where Ag is Ag without drug exposure; c, control in the absence of Ag and drug; and ag_atorva, Ag incubation in presence of drug. The concentration-response profiles were fitted to a three-parameter sigmoidal Hill model, y = (a/(b + x)) + c, using SigmaPlot 2001 for Windows. The concentration-growth effect (CGE) for the maximum effect (IC_{50}) was expressed as the geometric mean (GM) of the IC_{50} values, predicted vs observed residuals, sum of squares, and visual inspection.

Results
Atorvastatin induces T cell anergy
The dosage of atorvastatin required to produce IC_{50} on proliferation was 3 μM for both human MBP-specific T cells stimulated with specific Ag (Fig. 1a) and freshly isolated peripheral T cells stimulated with anti-CD3/anti-CD28 (data not shown). We also observed a concomitant decrease in IFN-γ production following atorvastatin incubation in freshly isolated PBMC (Fig. 1b). Similar to our previous findings in Ag-specific T cells (8), doses higher than IC_{50} interfered with the cell cycle of freshly isolated T cells following TCR stimulation in the presence of costimulation by inhibiting CDK4 induction and p27^kip1 degradation. Expression of these cell cycle regulators was semiquantitatively analyzed and OD values, normalized against β-actin expression, are shown in Fig. 1e. The lack of degradation of the negative cell cycle regulator p27^kip1 by atorvastatin (Fig. 1c) is suggestive of an induction of the cell cycle (17). In fact, apart from losing their ability to enter the cell cycle and proliferate, both Ag-specific T cell lines (Fig. 1d) and peripheral T cells (Fig. 1e) became hyporesponsive to a second stimulus following 4 days pretreatment with atorvastatin. Inhibition of IFN-γ production and induction of T cell anergy was dependent on HMG-CoA reduction because it was prevented (both

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peripheral T cells and Ag-specific T cell lines) when cells were additionally pretreated with the product of HMG-CoA reduction, mevalonate (Fig. 1, b–e).

IL-10 is necessary for anergy induction by atorvastatin
As tolerance induction and impaired degradation of p27Kip1 can be induced by inhibitory cytokines such as IL-10 and TGF-β in CD4+ T cells (18, 19), we addressed whether atorvastatin-induced T cell anergy requires IL-10 production. Indeed, using both conventional PCR (Fig. 2a) and TaqMan RT-PCR (Fig. 2b), we found that atorvastatin promotes an induction of IL10 gene expression, which mevalonate strongly suppresses, suggesting an HMG-CoA reductase-dependent regulation of IL-10. In parallel we also observed by real-time PCR a similar modulation of TGFβ gene expression, although to a lesser extent, following 4 h atorvastatin in the presence of Con A (2.3-fold increase from untreated cells) when compared with control Con A treatment alone (0.9-fold increase) and atorvastatin/mevalonate coincubation (0.6-fold increase). Furthermore, by applying a neutralizing Ab against IL-10, we also show a critical requirement for IL-10 secretion by atorvastatin-induced anergy because induction of the anergic state by atorvastatin was completely abrogated (Fig. 2c).

Rapid and strong phosphorylation of ERK1 by atorvastatin
Because atorvastatin-induced anergy in T cells was dependent on the HMG-CoA pathway, we hypothesized alterations in Ras GTP-binding protein, known to be posttranslationally modified by isoprenylation, and the protein effector pathways to be driving forces of the cell cycle machinery responsible for the observed effects of statins. We therefore analyzed the influence of atorvastatin on effector molecules of Ras, specifically the ERK group of MAPKs. Although essential for T cell survival (5), activation of ERK is also essential for specifically inducing IL-10 production (20). We preincubated T cells with 25 μM atorvastatin for 4–48 h (to differentiate short-term from long-term effects) before a brief incubation with ionomycin and PMA (for direct stimulation of the Ras signal pathway). We encountered a surprisingly strong and early phosphorylation of ERK1 but not of ERK2 in the presence of atorvastatin, in both peripheral immune cells (Fig. 3a) as well as in Ag-specific T cells (data not shown). Atorvastatin-induced ERK1 phosphorylation was clearly blocked by neutralizing Ab against IL-10 (Fig. 3b), confirming the crucial role of IL-10 in the anergic state.

FIGURE 1. Atorvastatin induces T cell anergy. a, The human CD4+ MBP-specific T cell line FN8 was stimulated with specific Ag in the presence of increasing concentrations of atorvastatin. Proliferation was measured by [3H]thymidine incorporation assays and the observed percentage proliferative response was calculated as a percentage of SI in the absence of atorvastatin. The atorvastatin concentration-response profiles were fitted to a three-parameter sigmoidal Hill model and IC50 (3 μM) calculated (see Materials and Methods). b, The histogram is representative of three experiments and depicts an inhibition of intracellular IFN-γ staining following overnight (18 h) incubation of PBMC with atorvastatin (shaded histogram, thick line) in comparison to isotype (shaded histogram), vehicle (thin line), atorvastatin/mevalonate (dotted line), and control (thick line). Cells were shortly stimulated (6 h) with PMA and ionomycin in the presence of brefeldin A before flow cytometric measurements. c, For the immunoblots PBMC from healthy human donors were stimulated for 72 h with anti-CD3/anti-CD28 in the presence or absence of atorvastatin (25 μM) or vehicle and with or without mevalonate (200 μM). Values given below p27Kip1 and CDK4 bands are OD measurements normalized against β-actin expression. d, Resting human Ag-specific T cells stimulated with specific Ag in the presence (shaded bars) or absence (□) of atorvastatin and with mevalonate (□) were restimulated with titrating doses of specific Ag (0–10 μg/ml) in the presence of APC. The extent of T cell response to the second stimulus was determined with [3H]thymidine incorporation assays as cpm (± SE). e, PBMC were stimulated with 2 μg/ml Con A under varying conditions: medium (○), 10–25 μM atorvastatin (shaded bars) and in the presence (□) or absence (□) of 200 μM mevalonate. Following 4 days atorvastatin incubation, surviving T cells were restimulated with Con A and the extent of T cell response (in the absence of both atorvastatin and mevalonate) calculated as SI (± SE) from [3H]thymidine measurements.
phosphorylation was several times stronger than after induction by PMA (Fig. 3a). The robust ERK1 phosphorylation (following 4 and 24 h atorvastatin treatment) prompted us to look at the kinetics of this phosphorylation, in the absence of T cell activation (Fig. 3b). Within 15 min, atorvastatin treatment resulted in the phosphorylation of ERK1, which persisted for at least 24 h. This result is in contrast to PMA-induced Ras-mediated activation of ERK1/2, which as already described in the literature, is only transiently up-regulated (data not shown). A semiquantitative analysis of which as already described in the literature, is only transiently up-regulated (data not shown).

Gene expression was analyzed by real-time RT-PCR and normalized against 18S gene expression. In vitro findings shed light onto the beneficial outcome of applying HMG-CoA reductase inhibitors such as atorvastatin for the treatment of the T cell-mediated autoimmune disorders MS (14) and rheumatoid arthritis (15). The negative cell cycle regulator p27^Kip1 plays a central role in blockade of clonal expansion and is a critical downstream target of anergy induced by blockade of costimulatory pathways (17). Clonal anergy may also be induced by IL-10 and TGF-β. Application of these inhibitory cytokines results in an accumulation of p27^Kip1 in alloreactive murine CD4+ T cells and renders these cells tolerant for in vivo transfer in histoincompatible recipients (18, 19). In the present study we show that atorvastatin predominantly promotes IL10 gene expression in peripheral immune cells and could prevent the atorvastatin-mediated anergic condition in T cells by blocking IL-10 signaling with neutralizing Abs. These findings, together with reports documenting an induction of in vivo tolerance following exogenous application of this inhibitory cytokine (18, 19), indicate that IL-10 plays a key role in the induction of anergy by atorvastatin and could be responsible for the accumulation of p27^Kip1 (18).

So far, most of the properties of statins, in particular those describing their immunomodulatory nature (8, 10), were shown to be dependent on the HMG-CoA pathway. The underlying mechanisms of most of these functions have been interpreted to be an inhibition of Ras GTP-binding protein isoprenylation (2). In the present study we also observed a dependency of anergy on the HMG-CoA pathway and anticipated Ras GTP-binding proteins and their effector molecules, upstream of the cell cycle machinery, to be negatively regulated by atorvastatin. Hillyard et al. (25) have

**Induction of T cell anergy by atorvastatin is mediated via early ERK1 phosphorylation**

Because a sustained activation of the Raf/MEK/ERK pathway was previously reported in the context of anergy induction (23), we wanted to know whether the T cell anergy induced by atorvastatin is dependent on the observed rapid and strong ERK1 phosphorylation. For this we used the MEK1/2 blocker U0126, a specific inhibitor of ERK phosphorylation, at the same concentration used in the phosphorylation experiments. In line with reports documenting the negative effects of blocking ERK1 activation on T cell development and survival (24), U0126 inhibited proliferation during the priming stimulus (Fig. 4a). However on restimulating surviving cells we were indeed able to prevent anergy induction by atorvastatin when additionally pretreating with the specific inhibitor of ERK phosphorylation (Fig. 4b).

**Discussion**

Induction of T cell tolerance by immunomodulatory agents is becoming an increasingly important approach for the treatment of inflammatory autoimmune disease such as MS. Anergy constitutes one means of imposing tolerance by rendering autoreactive cells functionally inactive. In the present study we report an induction of T cell anergy following in vitro application of atorvastatin, which was accompanied by an induction of IL10 as well as an accumulation of the anergy factor p27^Kip1. These in vitro findings shed light onto the beneficial outcome of applying HMG-CoA reductase inhibitors such as atorvastatin for the treatment of the T cell-mediated autoimmune disorders MS (14) and rheumatoid arthritis (15). The negative cell cycle regulator p27^Kip1 plays a central role in blockade of clonal expansion and is a critical downstream target of anergy induced by blockade of costimulatory pathways (17). Clonal anergy may also be induced by IL-10 and TGF-β. Application of these inhibitory cytokines results in an accumulation of p27^Kip1 in alloreactive murine CD4+ T cells and renders these cells tolerant for in vivo transfer in histoincompatible recipients (18, 19).

To determine whether the strong phosphorylation of ERK1 is directly mediated by the MAPK MEK1/2, we applied a selective inhibitor of MEK1/2 (U0126). Doses of U0126 specific for MEK inhibition (10 µM) (22) could delay phosphorylation of ERK1 (Fig. 3c). Atorvastatin treatment also resulted in an induction of ERK1 but not ERK2 gene expression, already within the first 4 h of treatment (Fig. 3d). Gene expression of ERK1 was normalized against GAPDH expression (Fig. 3d). Altogether these data indicate a highly specific effect of atorvastatin on ERK1 gene expression and protein phosphorylation independent of the influence on Ras.

**FIGURE 2.** Induction of T cell anergy by atorvastatin necessitates IL-10 signaling. a, PBMC were activated with Con A in the presence or absence of atorvastatin with or without mevalonate (200 µM) for various periods of time. IL10 gene expression was analyzed by RT-PCR. b, PBMC were activated with Con A for 4 h in the presence or absence of atorvastatin with or without mevalonate. IL10 gene expression was analyzed by real-time RT-PCR and normalized against 18S gene expression. c, Peripheral immune cells stimulated with Con A were cultured in medium (□) or with atorvastatin (shaded bars). Some cells pretreated for 4 days with atorvastatin were also pretreated with titrating doses (500–5000 ng/ml) of mevalonate (light gray bars) or 200 µM mevalonate (dark gray bar). Mean T cell response to a second Con A stimulus was calculated for each condition as SI (±SE) following Ag.
recently reported that a 48-h preincubation with fluvastatin inhibited FcγR-mediated activation of ERK1/2 in the monocytic cell line U937. In this study we, however, found a rapid and sustained phosphorylation of ERK1 in human T cells upon atorvastatin treatment; the level of phosphorylation was much higher than the normal threshold achieved by PMA. The anergic state of T cells treated with atorvastatin was clearly dependent on the early ERK1 phosphorylation as a consequence of Ras/Raf/MEK cascade activation, because we could prevent anergy by blocking MEK activation; the level of phosphorylation was much higher than the normal threshold achieved by PMA. In line with this, activation of ERK was previously shown to dramatically induce tyrosine phosphorylation of TCR-associated proteins and phosphorylated ERK1/2 in the cytosol (26). Although these studies are in line with the present findings, we show a highly specific effect of atorvastatin on ERK1 because we found a dramatic and sustained phosphorylation of ERK1 but not ERK2 as well as an induction of ERK1 but not ERK2 gene expression. The up-regulation of ERK1 gene expression following ERK1 phosphorylation is in line with a positive feedback loop following ERK activation proposed by Hernandez et al. (27). Also, the promoter of the ERK1 gene, unlike that of the ERK2 gene, contains no CCAAT box (28), which is important for transcription factor NF-Y/CBF binding (29). Thus it is not surprising that these two genes can be differentially regulated.

For the first time we report that atorvastatin, an immunomodulator currently under clinical investigation for the treatment of autoimmune disorders, induces anergy in human T cells as a consequence of increased IL10 expression and rapid and sustained ERK1 phosphorylation. Because survival of T cells necessitates “normal” levels of ERK1 phosphorylation, different kinetics or intensities in ERK1 signaling may determine whether autoreactive cells become tolerant or promote autoimmune processes. The cholesterol-lowering HMG-CoA reductase inhibitor atorvastatin tips over this equilibrium by inducing ERK1 phosphorylation to the extent that regulatory elements such as IL-10 and p27Kip1 may be switched on to precipitate T cell anergy.

**Acknowledgments**

We thank Andrzej Stepulak and Marco Sifringer from the Department of Neuropediatrics (University Hospital Charité) for helpful discussions and
expert technical assistance, Susan Pikol and Bibiane Seeger for technical support, and Andrew Mason for careful proofreading of this manuscript.

Disclosures
The authors have no financial conflict of interest.

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