Atorvastatin Restores Lck Expression and Lipid Raft-Associated Signaling in T Cells from Patients with Systemic Lupus Erythematosus

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Atorvastatin Restores Lck Expression and Lipid Raft-Associated Signaling in T Cells from Patients with Systemic Lupus Erythematosus

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Loss of tolerance to self-Ags in patients with systemic lupus erythematosus (SLE), a prototypic autoimmune disease, is associated with dysregulation of T cell signaling, including the depletion of total levels of lymphocyte-specific protein kinase (Lck) from sphingolipid-cholesterol-enriched membrane microdomains (lipid rafts). Inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase (statins) can modify the composition of lipid rafts, resulting in alteration of T cell signaling. In this study, we show that atorvastatin targets the distribution of signaling molecules in T cells from SLE patients, by disrupting the colocalization of total Lck and CD45 within lipid rafts, leading to a reduction in the active form of Lck. Upon T cell activation using anti-CD3/anti-CD28 in vitro, the rapid recruitment of total Lck to the immunological synapse was inhibited by atorvastatin, whereas ERK phosphorylation, which is decreased in SLE T cells, was reconstituted. Furthermore, atorvastatin reduced the production of IL-10 and IL-6 by T cells, implicated in the pathogenesis of SLE. Thus, atorvastatin reversed many of the signaling defects characteristic of SLE T cells. These findings demonstrate the potential for atorvastatin to target lipid raft-associated signaling abnormalities in autoreactive T cells and provide a rationale for its use in therapy of autoimmune disease.

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Materials and Methods

Patients and controls

Seventy-one SLE patients fulfilling the American College of Rheumatology revised classification criteria for lupus (15) were assessed for disease activity using the British Isles Lupus Assessment Group index (16). Only patients with active SLE (British Isles Lupus Assessment global score ≥ 6) were included (mean age, 39 years; age range, 19–76 years; 40 women and 4 men). Sixty-one healthy individuals (mean age, 38 years; age range,
Abs and reagents
Purified Abs to human CD3 (OKT3), CD19 (RFB9), CD14 (AML), CD56 (B159), and CD16 (3G8) were obtained from Chemicon. Mouse mAb against Lck (3A5), 3A5 conjugated to Sepharose, 3A5 conjugated to PE, CD45 (55-Z6), and rabbit polyclonal Abs against Lck were obtained from Santa Cruz Biotechnology. Rabbit polyclonal Abs to the active form of Lck (phospho-src Y414) were purchased from New England Biolabs. Abs to phospho-src Y-P414 are cross-reactive with other src family kinases including Lck, and was used because specific anti-phospho-Lck pY394 was not available. Rabbit polyclonal Ab to LAT was obtained from Upstate Biomedical. Rabbit polyclonal Abs to ERK1/2 (p44/42), phospho-ERK1/2 and actin were purchased from Cell Signaling Technology. Conjugated Abs to human CD3-FITC, CD4-PE, and CD8-PE and purified mAb to CD28 (CD28.2), and flotilin-1 were obtained from BD Biosciences. HRP- and FITC-conjugated Abs, rabbit anti-mouse and goat anti-rabbit, cholera toxin B (CTB)-FITC, and filipin were purchased from Sigma-Aldrich. Atorvastatin was donated by Pfizer.

Cell culture and isolation of T lymphocytes
PBMC isolated from ~40 ml of heparinized venous blood from SLE patients or controls by centrifugation over Ficoll-Hypaque (Pharmacia Bio- tech) was cultured at 37°C in 5% CO2 for 72–96 h in RPMI 1640 medium (Invitrogen Life Technologies) with 5–10% (Invitrogen Life Technolo- gies), 100 U/ml penicillin, and 50 mg/ml streptomycin (Sigma- Aldrich), with or without 10μM atorvastatin added to the culture medium. To inhibit prenyltransferases, medium was supplemented with 10 μM GGTi-286 and 10 μM FITI-277 (Calbiochem). Cell viability was measured by trypan blue exclusion (>99%). T lymphocyte-enriched populations were obtained by negative selection using a method described previously (11). Where indicated, T cells were activated using magnetic beads coated with anti-CD3 and CD28 (Dynal), and filipin were purchased from Sigma-Aldrich. Atorvastatin was donated by Pfizer.

Flow cytometry
The purity of T lymphocytes in the enriched populations was determined by flow cytometry and was consistently >94%. To measure total Lck, T cells were surface stained with anti-CD3-FITC, fixed with 1% paraformaldehyde in PBS, and permeabilized with PBS staining buffer (PBS, 0.3% saponin, and 1% FCS) before staining with Lck-PE. GM1 levels in resting and activated T cells were analyzed using CTB-FITC. Cells were surface stained with anti-CD4-PE, fixed with 1% paraformaldehyde in PBS and stained with CTB-FITC (1/300) for 1 h on ice, washed, and analyzed. To determine the level and distribution of plasma membrane cholesterol ex vivo, resting and activated T cells were labeled with filipin. Briefly, in a method modified from Ref. 17, T cells were fixed with 4% paraformaldehyde, quenched with 50 mM glycine, and incubated with 12.5 μg/ml filipin (Sigma-Aldrich) and incubated with 12.5 μg/ml filipin for 2 h; cells were washed twice before analysis. Cyto- kine production was assessed in T cells plated in 96-well plates (5 × 10^4 cells/well) in RPMI medium with 1 μM atorvastatin where indicated) precoated with anti-CD3 Abs (5 μg/ml) and soluble anti-CD28 Ab (5 μg/ml). Following 72 h of incubation, supernatants were collected and analyzed by cytokometric bead array according to the manufacturer’s instructions (BD Biosciences). ERK phosphorylation was measured in T cells activated with anti-CD3/CD28 (5 μg/ml) for up to 60 min using a phospho-ERK1/2 (T202-Y204) Flex Set CBA kit (BD Biosciences). T cells were analyzed according to the manufacturer’s instructions and results compared with total protein were measured using the bichinchoninic acid protein assay (Biorad). All experiments were analyzed using a BD Biosciences FACScan, LSR equipped with an argon laser (filipin assay), or FACSArray (cytokine assays). ERK phosphorylation flow cytometers with CellQuest software (BD Biosciences).

Confoal microscopy
For fluorescence microscopy experiments, cells were washed in serum-free RPMI 1640. CTB-Alexa Fluor 594 (Molecular Probes) was used to label GM1. Patching was induced after CTB labeling as described previously (18). Cells were incubated with goat anti-CTB Abs (1/250 in PBS/0.1% BSA; Calbiochem) for 30 min on ice, and then 20 min at 37°C. T cells fixed in 4% paraformaldehyde were washed in PBS and applied to multi- well TESPA-coated slides (Hendly). Attached cells were stained with Abs to CD45 and Lck as described previously (13) and analyzed with a Bio-Rad MRC 1024 confocal system (Bio-Rad) equipped with an argon and helium/
SEM; by comparing the density of the bands to an actin control (AATI1006) blotting for Lck expression (AATI1006). The relative levels of Lck were estimated for the activated form of Lck. Fig. 1 (A)

Purified T cells from patients with SLE and healthy controls were isolated by negative selection following a 96-h incubation with or without 10 μM atorvastatin. Membrane GM1 labeled with CTB-FITC (A) or total Lck (B) expression were analyzed by flow cytometry (±SEM; n = 10). Whole T cell lysates were analyzed by Western blotting for Lck, the activated form of Lck (Lck pY414), and actin, as a control for protein loading (C). The semiquantitative density ratio of active Lck:total Lck is shown in D (±SEM, n = 6). Representative Western blots showing DRMs isolated from SLE and healthy T cells by floatation over dense sucrose gradients and analyzed for expression of Lck and LAT (E). Actin expression in the T cell lysates before raft isolation is used as a control for protein loading (n = 9). T cell lipid raft fractions were isolated after culture with 10 and 20 μM atorvastatin or medium alone and analyzed by Western blotting for Lck expression (F). The relative levels of Lck were estimated by comparing the density of the bands to an actin control (±SEM; n = 4).

FIGURE 1. Atorvastatin treatment restored Lck and GM1 expression to normal in lupus T cells. Purified T cells from patients with SLE and healthy controls were isolated by negative selection following a 96-h incubation with or without 10 μM atorvastatin. Membrane GM1 labeled with CTB-Alexa Fluor 594 (red) and patched with goat anti-CTB Abs. Cells were stained with Abs to CD45 (green) and analyzed by confocal microscopy. As we have previously shown, CD45 and GM1 (CTB) were colocalized in lupus but not in healthy T cells; however, stimulation with atorvastatin reversed CD45/GM1 colocalization in lupus T cells (Fig. 2A). To further confirm that the change in CD45 membrane distribution was associated with reduced CD45-Lck interaction, we immunoprecipitated Lck from T cell lysates and looked for CD45 coprecipitation. Although the expression of total Lck was increased in the atorvastatin-treated lupus T cells, there was no associated with raft domains to levels similar to those observed in T cells from healthy controls (Fig. 1E). However, another raft-associated signaling molecule, LAT, which has normal expression in lupus T cells (11), remained unaffected by atorvastatin. In contrast, atorvastatin treatment of healthy T cells reduced the levels of raft-associated proteins Lck and LAT (Fig. 1E). Using higher concentrations of atorvastatin, total Lck (and LAT) are depleted from the raft fraction in lupus T cells. Taken together these experiments not only demonstrate that atorvastatin specifically targets Lck in lupus T cells, but also illustrates that lupus T cells have an altered sensitivity to atorvastatin compared with healthy T cells, needing higher concentrations to dissociate raft-associated protein Lck from DRMs (Fig. 1F).

Atorvastatin blocked the association of CD45 with Lck leading to reconstitution of Lck in lipid rafts

In lupus T cells, reduced total Lck expression is associated with translocation of CD45 into lipid raft domains (13). CD45 dephosphorylates the inhibitory tyrosine (Tyr505) of Lck, allowing Lck to adopt an open-active conformation. Therefore, to examine whether normalization of Lck expression was coupled with reduced Lck/CD45 coassociation, we first examined the membrane distribution of CD45 by confocal microscopy. As we have previously shown, CD45 and GM1 (CTB) were colocalized in lupus but not in healthy T cells; however, stimulation with atorvastatin reversed CD45/GM1 colocalization in lupus T cells (Fig. 2A). To further confirm that the change in CD45 membrane distribution was associated with reduced CD45-Lck interaction, we immunoprecipitated Lck from T cell lysates and looked for CD45 coprecipitation. Although the expression of total Lck was increased in the atorvastatin-treated lupus T cells, there was no

FIGURE 2. Atorvastatin diminished the coassociation of Lck with CD45 in lupus T cells. Purified T cells from patients with SLE and healthy controls were isolated by negative selection following a 96-h incubation with or without 10 μM atorvastatin. Membrane GM1 was labeled with CTB-Alexa Fluor 594 (red) and patched with goat anti-CTB Abs. Cells were stained with Abs to CD45 (green) and analyzed by confocal microscopy. Representative images from three experiments are shown. B, Lck was immunoprecipitated from T cell lysates (100 μg) and analyzed for Lck and CD45 coprecipitation by Western blotting. C, The semiquantitative density ratio of Lck:CD45 in SLE T cells was compared with healthy controls (±SEM, n = 5).
corresponding increase in CD45 coassociation (Fig. 2B), thus resulting in a significant reduction in Lck/CD45 coassociation ($p = 0.04$) (Fig. 2C). These results suggest that atorvastatin can modulate the aberrant lupus T cell signaling via inhibiting Lck/CD45 association in the lipid raft.

Inhibition of protein prenylation alone did not restore Lck and GM1 expression in SLE cells

Atorvastatin could modulate lipid raft domains, possibly by inhibiting cholesterol biosynthesis and/or by inhibiting protein prenylation. Protein prenylation (geranylgeranylation or farnesylation) enables membrane targeting of the Ras and Rho family GTPases, both integral to signaling via the T cell Ag receptor (14, 20). Therefore, to test the effect of soluble inhibitors of prenylation on raft expression of Lck, healthy as well as lupus T cells were incubated with and without GGTI-286 and FTI-277 (soluble inhibitors of prenylation). No significant differences in total Lck expression were seen when Lck:flotillin-1 ratios were compared between lupus T cells cultured with or without inhibitors of prenylation (Fig. 3, A and B). In addition, specific inhibitors of prenylation did not reduce expression of membrane GM1 (Fig. 3C). Hence, these results suggest that the restoration of a normal Lck expression and distribution within the raft by atorvastatin has an independent mechanism from inhibition of protein isoprenylation.

**Atorvastatin restored the kinetics of TCR activation to normal levels in SLE T cells associated with a reduction in cholesterol**

The restoration of Lck expression within lipid rafts in SLE T cells by atorvastatin suggested that the hyperactivated T cell phenotype...
with lupus T cells was more rapid than in healthy T cells, again confirming the hyperactivated status of lupus T cells. In contrast, when SLE T cells were cultured with atorvastatin, the visible accumulation of Lck to the cell/bead contact zone was significantly delayed. Thus, a significantly reduced number of lupus T cell/bead conjugates were formed with the atorvastatin-treated cells compared with lupus T cells cultured in medium alone and healthy T cells (Fig. 4E). These results suggest that atorvastatin can modulate the immunological synapse, thereby dampening the hyperactive phenotype of SLE T cells. In summary, these results indicate that atorvastatin can reverse abnormalities in lupus T cells, specifically the proximal events involving T cell-APC interactions, which is likely due to alterations in membrane lipid/protein content and distribution.

Normalization of intracellular signaling pathways in SLE T cells treated with atorvastatin

During T cell activation, the Ras family GTPases regulate signal transduction via the mitogen-activated protein kinase pathway, leading to ERK phosphorylation (14). In lupus T cells however, TCR-driven phosphorylation of ERK1 and ERK2 is diminished in contrast to an increase in global protein tyrosine phosphorylation (24, 25). SLE T cells failed to display significant ERK1/2 phosphorylation following TCR-mediated activation (Fig. 5, A and B). However, atorvastatin recovered early ERK1/2 phosphorylation to levels similar to those observed in T cells from the healthy controls (Fig. 5, A and B), while abolishing it in the healthy controls. Thus, it appears that atorvastatin can repair abnormal TCR-stimulated signaling events in SLE T cells by recovering Lck expression, reducing global protein tyrosine phosphorylation (data not shown), and restoring signaling via the RAS-ERK pathway.

Atorvastatin reduced T cell production of IL-10 and IL-6 in SLE

Because statins have been shown to modulate cytokine production in experimental models of arthritis (2, 3) and to link the changes in lipid rafts to the pathophysiology of lupus, we explored the effect of atorvastatin on the production of cytokines relevant to the pathogenesis of SLE. T cells were purified from lupus patients and stimulated for 72 h with anti-CD3/CD28, either with or without atorvastatin. Culture supernatants were recovered and analyzed for the production of IFN-γ, IL10, and IL-6 by cytometric bead array. The results show that atorvastatin did not significantly influence the production of IFN-γ (data not shown). In contrast, the production of IL-10 and IL-6, implicated in the pathogenesis of SLE (26), were significantly reduced by atorvastatin (IL-10 and IL-6, p = 0.03; Fig. 5C).

Discussion

The key finding from this work is that the HMG-CoA reductase inhibitor, atorvastatin can reverse the raft-associated proximal signaling abnormalities present in lupus T cells. This was related to the restoration of total Lck expression and associated with reduced levels of activated Lck. Lck activity is regulated by its proximity to positive and negative regulatory molecules, CD45 and C-terminal Src kinase, respectively (27). Although CD45 is largely excluded from raft domains in healthy T cells, several investigators have reported dynamic changes in CD45 localization and raft partitioning during T cell activation (28). CD45 is abnormally localized within lipid rafts in lupus T cells, where it coassociates with, and activates, Lck (Fig. 2), resulting in increased ubiquitin-mediated Lck proteolysis and reduced levels of total Lck (11, 13). Thus, the dissociation of CD45 from Lck- and GM1-enriched membrane
microdomains by atorvastatin in unstimulated lupus T cells restored the balanced regulation of Lck activity and subsequent intracellular signal transduction during TCR activation. Despite the low levels of total Lck in lupus T cells, we demonstrate a more rapid accumulation of total Lck to the immunological synapse (a proportionately small area of the cell surface), a phenomenon that has also been described with CD3 and CD45 (12, 29). This more rapid accumulation of signaling molecules to the immune synapse in lupus T cells is associated with an increased proportion of lipid rafts in the T cell membrane (measured by GM1 and cholesterol expression) (12, 29). Hence, by disrupting the up-regulation of membrane lipids, GM1, and cholesterol, during T cell activation (Fig. 4, B and C), atorvastatin restored the rapid formation of the immune synapse measured by slower accumulation of Lck and reduced formation of bead:cell conjugates (Fig. 4E). Indeed, atorvastatin treatment of lupus T cells normalized levels of global protein tyrosine phosphorylation (data not shown) and specifically restored ERK1/2 phosphorylation following TCR activation. A novel insight into the mechanism by which atorvastatin can selectively influence ERK phosphorylation, and thereby T cell function, has been revealed recently by Zhang et al. (30). This group demonstrated that TCR-mediated phosphorylation of ERK depends on the membrane localization of CD45. Raft excluded CD45 positively regulated ERK phosphorylation, thus the normalization of ERK phosphorylation in lupus T cells may reflect the modulation of lipid and protein interactions by atorvastatin that result in the dissociation of CD45 from GM1 in the membrane.

Overall, the effects of atorvastatin on lupus T cells were more profound compared with healthy T cells and occasionally paradoxical. Lupus T cells analyzed immediately ex vivo have an anergic phenotype due to repeated autoantigenic stimulation and rounds of activation in vivo (31). This is consistent with the diminished ERK phosphorylation, despite an activated phenotype found in lupus T cells (24, 25). It is possible to reverse some abnormalities in lupus T cells by resting T cells away from sources of activation (31). We have shown that lupus T cells, after resting, still display a hyperactivated phenotype with rapid up-regulation of membrane GM1 upon stimulation with anti-CD3/CD28 (13). The experiments described here confirm that this hyperactivated state is associated with a failure to induce ERK phosphorylation. Paradoxically, atorvastatin restored ERK phosphorylation in lupus T cells upon activation, while abolishing ERK phosphorylation in healthy T cells. This variation in the effect of atorvastatin on ERK phosphorylation is reflected in previous studies where cholesterol depletion using methyl-β-cyclodextrin or statins can induce ERK phosphorylation (5, 32, 33), but has also been shown to inhibit ERK phosphorylation (34, 35) depending on the experimental conditions or immunological environment. Equally, the differential phosphorylation of ERK1/2 has been associated with either the induction or failure of T cell anergy, a process that is dysfunctional in patients with SLE (36). For instance, the inability to activate ERK has been associated with T cell anergy (37). Moreover, TCR-mediated activation of regulatory T cells, which have an anergic phenotype, also failed to induce ERK phosphorylation (38). Opposing this view, sustained activation of ERK in T cells is associated with cytokine unresponsiveness and anergy (39). Consistent with the notion that ERK phosphorylation leads to induction of anergy, sustained antigenic signaling in B cells, in the general context of lupus-type autoimmunity, leads to activation of ERK and a tolerogenic signal (40). Thus, anergy may occur through differing and sometimes apparently opposing mechanisms that may depend on the immunopathological environment. Therefore, according to some reports, the actions of statins reported here are compatible with a restoration of tolerance in lupus T cells.

A further paradoxical effect of atorvastatin was the restoration of raft-associated Lck in lupus T cells, whereas in healthy T cells we observed a depletion of Lck from raft fractions. However, at a higher dose (20 μM vs 10 μM), atorvastatin also depleted raft-associated Lck in lupus T cells. This differential sensitivity between healthy and lupus T cells has been observed previously when cellular cholesterol was depleted using methyl-β-cyclodextrin (12) and was confirmed when we examined membrane cholesterol in resting T cells. Although atorvastatin (10 μM) reduced membrane cholesterol in healthy T cells (measured by filipin binding and flow cytometry), a similar reduction was only observed at higher concentrations of atorvastatin (20 μM vs 10 μM) in the lupus T cells (data not shown), again suggesting that these cells have an altered sensitivity to the effects of atorvastatin. Increased membrane cholesterol in resting T cells has been linked to impaired T cell activation and is associated with decreased immune cell function in old age (41). It remains to be determined whether the increase in membrane cholesterol levels observed in T cells from lupus patients contributes to the many abnormalities observed in these cells.

The pleiotropic action of statins contributes to the complexity of the mechanisms underlying their immunological effects (1, 34). We, and others, have proposed that some of these effects could be attributed to a reduction in membrane cholesterol (42, 43). Moreover, a recent report demonstrates that reducing the cholesterol content of lipid rafts using simvastatin, another HMG-CoA reductase inhibitor, alters downstream signaling in prostate cancer cells (44). However, the effect of statins on membrane lipid composition remains controversial, with several reports showing no effect on membrane cholesterol levels and raft architecture (34, 45). Another important action of inhibitors of HMG-CoA reductase is the suppression of protein prenylation which has been shown to underlie the mechanism of action of statins in some animal models, particularly those associated with a marked Th1 response (46). Furthermore, no reduction in cholesterol was seen when statins were used in these Th1-driven models of autoimmunity, whereas when statins were administered to a murine model of lupus, a significant lowering of cholesterol occurred (4). In the work presented in this study, the targeted inhibition of protein prenylation was not sufficient to restore Lck or GM1 levels in lupus T cells (Fig. 3). This result is consistent with Hillyard et al. (47) who have shown that inhibitors of prenylation do not affect raft integrity or associated signaling of human monocytes. Whereas the effects of statins in a Th1-driven disease are likely because of alterations in protein prenylation, modulation of membrane lipids may play a more significant role in non-Th1 autoimmunity such as SLE.

Atorvastatin reduced the production of IL-10 and IL-6 by activated T cells, with a more marked effect on lupus T cells than healthy T cells, at least with respect to IL-6. Most studies relating to the immunomodulatory properties of statins in autoimmunity have studied their effects on animal models of disease with a predominant Th1-driven response (2, 3). However, induction of a Th2 cytokine response by statins has not been a universal feature in rodent models of autoimmune disease (48) or in human primary PBMC from patients with multiple sclerosis, where an in vitro study observed an increase in IFN-γ and reduction in IL-10 production (49). Th1 and Th2 cytokines both contribute to the pathogenesis of lupus disease, which is not so easily defined within the classical Th1/Th2 paradigm (50). Moreover, atorvastatin did not alter T cell cytokine production in lupus-prone NZB/W mice (4). Again, these results suggest that the effect of statins depends on the immunopathological environment, but that these agents have a consistent influence on the restoration of T cell tolerance.
Taken together, the results shown here suggest that a normal signaling profile and cellular function can be restored to SLE T cells by in vitro atorvastatin treatment. It is possible that atorvastatin is readjusting autoreactive lupus T cells toward a tolerant phenotype. It is tempting to speculate that part of the pleiotropic effects of statins on the immune system could result from changes in membrane lipids, at least in the context of SLE. These findings may have implications not only in the understanding and potential treatment of SLE and possibly other autoimmune diseases, but could also be applicable in the context of atherosclerosis, itself a treatment of SLE and possibly other autoimmune diseases, but effects of statins on the immune system could result from changes in phenotype. It is tempting to speculate that part of the pleiotropic signaling profile and cellular function can be restored to SLE T cells.

References


Disclosures

The authors have no financial conflict of interest.

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