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*J Immunol* 2007; 179:3613-3621; doi: 10.4049/jimmunol.179.6.3613
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Atorvastatin Inhibits T Cell Activation through 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase without Decreasing Cholesterol Synthesis

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The localization of the TCR and other signaling molecules in membrane rafts (MR) is essential for the activation of T lymphocytes. MR are stabilized by sphingolipids and cholesterol. Activation of T lymphocytes leads to the confluence of small MR and the formation of an immunological synapse that is essential for sustained activation and proliferation. In this study, we investigated the effect of statins on MR and T cell activation in superantigen-stimulated human PBMC. Atorvastatin significantly inhibited cellular activation and proliferation. The binding of cholera toxin B subunit to isolated MR and to whole cells was inhibited by low doses of statins. Statins reduce the association of critical signaling proteins such as Lck and linker of activation in T cells with MR in stimulated T cells. The expression of activation markers CD69 and CD25 was inhibited. Several statin-mediated mechanisms, such as a lower stimulation with MHC-II, an inhibition of costimulation by direct binding of statins to LFA-1, a reduced secretion of cytokines, or a depletion of cellular cholesterol pools, were excluded. Inhibition of protein prenylation had a similar effect on T cell proliferation, suggesting that a reduced protein prenylation might contribute to the statin-mediated inhibition of T cell activation. Statins induce both lower levels of low-density lipoprotein cholesterol and inhibition of T cell activation, which might contribute to an inhibition of atherosclerosis. The Journal of Immunology, 2007, 179: 3613–3621.

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Life Technologies), and 10% heat-inactivated FCS (Invitrogen Life Technologies)). PBMC (4 × 10^6/ml), FACS-sorted CD4^+CD25^+ , or Jurkat E6-1 T cells were preincubated with atorvastatin for 24 h to decrease the cellular cholesterol pool. PBMC were stimulated by addition of 10 ng/ml staphylococcus enterotoxin B (SEB) (Sigma-Aldrich), and CD4^+CD25^+ T cells were stimulated with anti-CD3 (OKT-3) and anti-CD28 (clone 9.3). The incubation was continued for up to 4 days, as indicated. The viability of cultured cells was determined by 0.1% trypan blue staining (80%).

Reagents
Atorvastatin was initially purchased from Calbiochem-Merck Biosciences and was later on provided as a gift from Pfizer (Karlsruhe, Germany). Atorvastatin was dissolved in DMSO to obtain a 260 μM stock solution. Mevalonic acid (Sigma-Aldrich) was dissolved in PBS as 1 M stock solution. Farnesyltransferase inhibitors (FTT-277, L744,832; Calbiochem-Merck) and a geranylgeranyltransferase inhibitor (GGTI-298; Calbiochem-Merck) were dissolved in DMSO as 10 mM stock solution. PS 341 (Bertzozmib; Janssen-Cilag Pharma) was dissolved in DMSO to obtain a 200 μM stock and diluted to a final concentration of 260 nM.

Proliferation assays
A total of 10^5 PBMC or Jurkat T cells was preincubated with or without atorvastatin for 24 h in a 96-well plate (Cellstar; Greiner BioOne). Control experiments were incubated with atorvastatin and 1 mM mevalonic acid, as indicated. Cells were activated with or without 10 ng/ml SEB and cultured for additional 3 days. Proliferating cells were labeled with 0.5 μCi of [3H]thymidine/well for 8 h at 37°C. Labeled cells were transferred to a glass fibre filtermat, and [3H]activity was counted using a 1205 Betaplate counter (Wallac Oy). Quadruplicates of each sample were analyzed from five independent healthy donors. Hexaplicates were used for Jurkat proliferation experiments. Data show mean ± SD.

The IFN-γ-mediated proliferation was inhibited by incubation of SEB-stimulated PBMC with 20 μg/ml neutralizing anti-IFN-γ Ab (clone NIB42; eBioscence, NatuTec).

Viability
Viable PBMC were characterized by the exclusion of apoptotic and necrotic cells. Briefly, 10^6 cells from four healthy donors were cultured, as described above. Cells were incubated with or without 0.5 ml of a staining solution (25 μl FITC-labeled AxFV (FLUOS; Roche) and 25 μl of propidium iodide (PI) (10 mg/ml PI; Sigma-Aldrich) in 50 μl of Ringer solution) for 30 min at 4°C in the dark, and subsequently analyzed by cytometry (EPICS XL; Coulter Pharmaceutical). Viable PBMC were negative for AxFV-FITC and PI staining. A minimum of 10^6 cells was measured. Data analysis was performed with Coulter XL software, version 3.

Marker analysis
Cells were cultured, as indicated, stained, and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences). HLA-DR expression was determined on PBMC and on CD14-positive cells using FITC-conjugated anti-HLA-DR and PE-conjugated anti-CD14 Abs (BD Biosciences).

Quantification of cytokines
A total of 10^5 PBMC/ml from three healthy donors was preincubated with or without inhibitors in a 48-well plate (Cellstar; Greiner BioOne), stimulated with SEB and cultured for the indicated time. Each sample was cultured in duplicates. Cytokines in cell culture supernatant were measured using an IFN-γ ELISA kit from R&D Systems, according to the manufacturer’s recommendations. IFN-γ was determined after 48 h stimulation with SEB.

Cholera toxin staining and isolation of MR
A total of 10^5 PBMC was preincubated for 24 h with or without inhibitors and was subsequently stimulated with SEB for up to 72 h, as indicated. The MR isolation and quantification were performed, as previously described in detail (24), with some modifications. Briefly, SEB-stimulated cells were centrifuged at 450 × g for 5 min, and culture medium was discarded. Cells were resuspended in 100 μl of PBS and incubated with 2 μl of the cholera toxin B subunit (CTB)-HRP stock solution for 30 min on ice. The CTB-HRP stock solution contained 40 μg of CTB and 100 μg of HRP (Calbiochem) dissolved in 100 μl of double-distilled water with a final CTB-HRP activity of 191 mU/ml. Cells were washed in 3 ml of ice-cold PBS to remove unbound CTB-HRP. Thereafter, cells were lysed at 37°C for 10 min in 2 ml of TNEV lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 5 mM Na2VO4 (Merck) (pH 7.4)), supplemented with one tablet Complete mini protease inhibitor (Roche)/10 ml and 1% Brij-98 or 1% Triton X-100 (w/v) (Sigma-Aldrich), as indicated. The protein content was determined, and the A405 of each sample was calculated into CTB-HRP activity of 191 mU/ml. Was transferred into an ultra-centrifugation tube (Beckman Coulter), and overlayed with 2 ml of double-distilled water with a final CTB-HRP activity of 191 mU/ml. Was transferred into an ultra-centrifugation tube (Beckman Coulter), and overlayed with 2 ml of double-distilled water with a final CTB-HRP activity of 191 mU/ml. Was transferred into an ultra-centrifugation tube (Beckman Coulter), and overlayed with 2 ml of double-distilled water with a final CTB-HRP activity of 191 mU/ml.
Confocal images were obtained with CD4+CD25+ FACS-sorted T cells. Cells were pretreated with atorvastatin, stimulated with SEB for 72 h, fixed with paraformaldehyde (PFA), stained with CTB-FITC, and fixed again. Images were acquired using a Leica DMRBE microscope with a ×40 objective and TCS NT software.

**Immunoblot detection**

MR were isolated and quantified, as described above. Proteins from sucrose fractions (pooled 2–7 for MR and 9–12 for non-MR) were precipitated (25) and were separated using SDS-PAGE (12.5% (w/v) polyacrylamide gels). Immunoblots were performed by semidyed transfer of the proteins onto nitrocellulose membranes. The membranes were sequentially incubated with specific Abs against Lck (BD Transduction Laboratories) and LAT (Upstate Biotechnology). Specific bands were visualized using HRP-conjugated goat anti-mouse IgG (or anti-rabbit; both from Jackson ImmunoResearch Laboratories), followed by ECL. Further detections were performed after stripping the membranes by incubation in 62.5 mM NaCl, 100 mM 2-ME, and 2% SDS for 20 min at 55°C.

**Cholesterol quantification by high performance thin layer chromatography (HPTLC) and filipin staining**

A total of 5 × 10⁶ PBMC from two healthy donors was used immediately or treated with statin in cell culture flasks (Greiner BioOne) and stimulated with SEB for 2, 4, or 7 days. Whole cell pellets were thoroughly washed in PBS to remove serum lipids. Alternatively, MR were isolated from 10⁶ cultured SEB-stimulated T cells or from 10⁶ Jurkat T cells by detergent-resistant lysis and sucrose gradient centrifugation.

Cellular lipids were extracted according to the Folch method (26) with 400 μl of chloroform:methanol (2:1 v/v). Addition of 200 μl of CaCl₂ 0.02% resulted in a two-phase separation, and the upper aqueous phase was discarded. The lower phase contained cholesterol and lipids and was dried under vacuum. Lipids were redissolved in chloroform and spotted on a HPTLC plate (silica gel 60, No. 1.05547; Merck). HPTLC was performed using two runs over 3 cm in chloroform:methanol:H₂O (40:20:2.5 v/v) and a final run over 8 cm in hexane:ether:acetic acid (65:5:0.15 v/v). Lipids were visualized after short incubation in 5% (w/v) molybdatophosphoric acid and heating at 100°C for 10 min. Background staining was bleached in a 25% (v/v) NH₃ atmosphere for 1 min. Lipids were identified by lipid standards, documented with a charge-coupled device camera, and quantified.

Flow cytometry analysis was performed with PBMC that were preincubated with atorvastatin, stimulated with SEB for 72 h, fixed with PFA, stained with 100 μg/ml filipin, fixed again with PFA, and stained with CD3-PE. Only CD3-gated T cells were analyzed. Control experiments were performed with FACS-sorted CD4+CD25+ resting T cells with or without 3 mM methyl-β-cyclodextrin (MβCD) to extract membrane cholesterol.

PBMC were pretreated with or without 10 μM atorvastatin and stimulated with SEB for 72 h. Jurkat cells were incubated with or without 10 μM atorvastatin. MR were extracted by sucrose gradient centrifugation. MR lipids were extracted by the Folch method and separated by HPTLC, as described above. Control experiments were performed with 3 or 10 mM MβCD, as indicated.

**ERK phosphorylation and Ca²⁺ fluxing**

A total of 10⁶ PBMC or 10⁶ magnetic head-sorted CD4⁺ T cells was preincubated with or without 2 μM atorvastatin and stimulated with 1 μg/ml PHA and 10 ng/ml PMA (Sigma-Aldrich) for 2 min or 2 h. Lysates containing 20 μg of protein were subjected for PAGE and blotted on a nitrocellulose membrane. Western blot detection was performed with Abs against phospho-ERK (clone E4; Santa Cruz Biotechnology). Membranes were stripped and reprobed against total ERK1 (clone K23; Santa Cruz Biotechnology).

A total of 3 × 10⁶ PBMC was loaded with 2 μM Fluo-3-AM (Fluka-Sigma-Aldrich) for 20 min at 37°C and 20 min at room temperature, and stained with CD4-CD5-Cy5. Ca²⁺ fluxes were analyzed by flow cytometry. Only CD4⁺ cells were analyzed. After 1 min, baseline cells were stimulated with 5 μg/ml anti-CD3 and subsequently with 12 μg/ml goat anti-mouse F(ab')₂ (STAR105; Serotec).

**Statistical analysis**

Data represent mean values ± SD. Statistical analysis was performed using paired two-tailed Student’s t test.

**Results**

**Atorvastatin reduces T cell proliferation**

The so-called pleiotropic effects of statins implicate that different effects might require different concentrations of statins. Therefore, we tested a wide range of statin concentrations for the inhibition of T cell proliferation. Atorvastatin treatment inhibited the SEB-stimulated proliferation of PBMC, which was significant for concentrations higher than 0.2 μM atorvastatin (p = 0.0005, Fig. 1A). The IC₅₀ for the inhibition of proliferation was ~1.5 μM. Incubation with 10 μM atorvastatin inhibited the proliferation to the background level (without SEB) of nonstimulated PBMC (Fig. 1A). Statin-mediated specific inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was demonstrated by rescue of proliferation with 1 mM mevalonic acid (Fig. 1A). Cytototoxic statin effects were excluded by annexin V FITC and PI staining, which was not altered by any statin concentration (data...
not shown). These results were confirmed with MACS-sorted CD4+ cells that were stimulated with anti-CD3 and anti-CD28 (Fig. 1B). Proliferation is dependent on costimulation with anti-CD28 in these cells, and the IC50 for the inhibition of proliferation was a little lower (0.5 mM). To differentiate whether the inhibition of T cell proliferation is mediated by inhibition of T cell activation or by inhibition of the proliferation itself, in vitro generated lymphoblasts (Fig. 1C) and Jurkat T cells (Fig. 1D) were incubated with atorvastatin. The proliferation of T cell lymphoblasts was also inhibited by statin treatment (Fig. 1C). Mevalonic acid restored most of the proliferative capacity of lymphoblasts, but failed to maintain full proliferation (Fig. 1C). High concentrations of statins are required to inhibit proliferation in Jurkat T cells (Fig. 1D). These data indicate that higher concentrations of >1 μM atorvastatin might exert additional antiproliferative effects.

**Atorvastatin inhibits the expression of GM1 in stimulated T cells**

Because MR are composed in part by cholesterol, we investigated whether statins influence MR formation, which might explain the inhibition of T cell activation and proliferation. CTB is a specific ligand for GM1, and therefore a good marker for MR. PBMC were stimulated with superantigen (SEB) for up to 72 h with or without atorvastatin. GM1-containing MR were labeled with CTB-HRP and were isolated and quantified, as described in Material and Methods. Binding of CTB to MR increased with the duration of stimulation with SEB (Fig. 2A, left). Atorvastatin treatment inhibited the binding of CTB to MR, indicating a reduced expression of GM1 (Fig. 2A, right). MR-resident GM1 was quantified as cumulative HRP activity from all MR-containing fractions (Fig. 2B). In medium-cultured cells, the MR-bound CTB-HRP was 86.8 ± 44.7 arbitrary units (a.u.). Treatment with 1 μM atorvastatin inhibited raft-bound CTB-HRP to 62.3 ± 32.2 a.u. and with 5 μM atorvastatin to CTB-HRP 37.3 ± 37.4 a.u. The atorvastatin-mediated inhibition of the expression of GM1 in stimulated T cells was significant for 5 μM atorvastatin (p = 0.02). These results were confirmed by confocal images of FACS-sorted CD4+ CD25− T cells that were pretreated with or without atorvastatin, stimulated with anti-CD3 and anti-CD28 for 72 h, and stained with CTB-FITC (Fig. 2C). Fig. 2C shows that the up-regulation of CTB-FITC binding is almost completely blocked by preincubation with 2 μM atorvastatin.

**The membrane distribution of signaling proteins is sensitive to statin treatment**

The localization of signaling proteins such as the Src kinase Lck and the linker of activation in T cells (LAT) in MR is essential for the transduction of TCR signals. MR were isolated with Brij-98 at 37°C (Fig. 3A) or with Triton X-100 at 4°C (Fig. 3B). LAT and Lck were isolated from MR, soluble membranes (= sol), and insoluble compartments (= insol), and were quantified by immunoblot detection. Isolated MR of atorvastatin-treated cells exhibited markedly lower quantities of LAT and Lck in comparison with untreated cells (Fig. 3A). Similar amounts of proteins were loaded, as indicated by an actin control (Fig. 3A). Comparable results were obtained with MR isolated with Triton X-100 (Fig. 3B). In MR isolated from unstimulated T cells, only small amounts of LAT and a larger proportion of Lck can be detected (Fig. 3B). However, the association of LAT and Lck with MR is increased upon T cell stimulation, and this association is inhibited by statin treatment (Fig. 3B). The statin-mediated decrease of the absolute amount of Lck in MR is minimal, but the relative distribution of Lck between MR and soluble membrane compartments is shifted toward the soluble fraction in statin-treated cells (Fig. 3B). A loss of LAT and Lck to the insoluble sediment can be excluded. A densitometric quantification of Lck and LAT bands confirms these results (Fig. 3, C and D). These experiments show that statin treatment can inhibit the association of signaling molecules with MR in stimulated T cells.

To exclude the possibility of an increased degradation of signaling proteins, we investigated whole cell lysates from superantigen-stimulated and unstimulated T cells (Fig. 3E). Incubation with neither statins nor proteasome inhibitor PS 341 or a combination of both significantly changed the whole cellular content of signaling proteins under these conditions (Fig. 3E). Degradation of signaling proteins was not observed. These data exclude protein
7.5% of unstimulated CD4 T cells expressed CD69, which was significant after 6 h (A). Expression of CD25 was positive on approximately 1.1% of unstimulated CD4 T cells at various time points. Approximately 1.1% of unstimulated CD4 T cells expressed CD69, which increased to 24.9% after 24 h of stimulation with SEB (Fig. 4B). Incubation with 2 μM atorvastatin resulted in an inhibition of sCD25 of 70.5% at both time points (p = 0.03). These data indicate that statin prevents an up-regulation of CD25 on Ag-stimulated T cells and exclude a statin-mediated cleavage of CD25.

**Atorvastatin does not reduce MHC class II on APCs**

Superantigens cross-link MHC molecules and the TCR complex and stimulate T cells. Therefore, stimulation of T cells with SEB depends on the expression of MHC molecules. The expression of MHC class II was investigated during short-term culture of stimulated PBMC. Cells were preincubated without or with 1 or 5 μM atorvastatin for 24 h and were stimulated with SEB for additional 24 or 48 h (Fig. 4C). Negative controls were conducted to dissect SEB-mediated and atorvastatin-mediated effects on APCs. The expression of HLA-DR on PBMC was determined and was normalized to the HLA-DR density after 24-h culture without any treatment (□ in Fig. 4C). The HLA-DR density (mean fluorescence intensity) on PBMC increased from 100 to 145 arbitrary units without SEB stimulation (Fig. 4C). Stimulation with SEB for 24 and 48 h significantly decreased the expression of HLA-DR (p = 0.02, Fig. 4C). Atorvastatin treatment itself did not further reduce the expression of HLA-DR on PBMC (Fig. 4C). These data show that the inhibition of proliferation cannot be explained by a down-regulation of HLA-DR on APCs.

**IFN-γ-mediated proliferation is independent of atorvastatin**

Next, we tested whether statins modulate secretion of IFN-γ. Unstimulated PBMC (without SEB) did not secrete IFN-γ, but SEB stimulation of PBMC increased IFN-γ concentration in the culture supernatant (A). Negative control cells were incubated without SEB. Proliferation of atorvastatin-treated cells was determined, as described above. IFN-γ-mediated effects were dissected by culturing cells with a neutralizing Ab or control IgG1 (B). Data represent mean ± SD of three independent experiments.

*FIGURE 4. Atorvastatin inhibits expression of T cell activation markers, but does not reduce HLA-DR expression on APCs. PBMC were preincubated with 5 μM atorvastatin for 24 h and stimulated with SEB for 6, 24, 48, and 72 h. Expression of activation markers (CD69, A; CD25, B) was determined on CD4+ cells at each time point. Data represent mean ± SD of four independent experiments. Insets. Show a representative staining after 24 h. Expression of HLA-DR was determined in cells cultured for 24 h without superantigen and statins, and was set to 100% (C, □). Cells were preincubated with or without atorvastatin (0, 1 μM, or 5 μM) for 24 h, and were subsequently stimulated with or without superantigen for 24 and 48 h, as indicated. Data show mean ± SD of three independent experiments.*

*FIGURE 5. IFN-γ-mediated proliferation is independent of atorvastatin treatment. PBMC were preincubated with up to 5 μM atorvastatin for 24 h, and were stimulated with SEB for additional 48 h. IFN-γ was quantified in the cell culture supernatant (A). Negative control cells were incubated without SEB. Proliferation of atorvastatin-treated cells was determined, as described above. IFN-γ-mediated effects were dissected by culturing cells with a neutralizing Ab or control IgG1 (B). Data represent mean ± SD of three independent experiments.*
superantigen to 360 pg/ml (Fig. 5A). Secretion of IFN-γ significantly increased upon incubation with 2 μM (666 pg/ml) and 5 μM (849 pg/ml) atorvastatin (Fig. 5A), respectively. This increment was confirmed with superantigens from three different normal donors. To dissect IFN-γ-mediated and statin-mediated effects on proliferation, SEB-stimulated PBMC were cultured with an IFN-γ-neutralizing Ab. The proliferation decreased from 20,000 to 1,000 upon withdrawal of IFN-γ (Fig. 5B). Incubation with up to 5 μM atorvastatin decreased proliferation in both medium-cultured and IFN-γ-depleted PBMC to the same extent. These data indicate that IFN-γ is essential for the proliferation of superantigen-stimulated PBMC. However, the statin-induced inhibition of T cell activation and proliferation is not mediated by an impaired synthesis of IFN-γ because statins and IFN-γ blockade additively decrease T cell activation after stimulation with SEB.

Atorvastatin does not reduce cellular cholesterol

To test whether statins inhibit cholesterol synthesis in T cells, we extracted total cellular lipids from atorvastatin-treated and untreated PBMC. The total cellular cholesterol of cells treated with 10 μM atorvastatin was only moderately reduced after 4 days of incubation (Fig. 6A). Cellular cholesterol was reduced to 83% at day 4 when compared with freshly isolated PBMC. A prolonged incubation of SEB-stimulated PBMC over 7 days lowered cellular cholesterol in untreated cells and slightly increased cholesterol in statin-treated cells (p = NS). These data might reflect an activation-induced cell death of activated cells that were not treated with statins. These data were confirmed by measuring the cellular cholesterol with filipin staining. PBMC were stimulated with superantigen for 72 h. After gating CD3+ T cells, resting and proliferating T cells were identified according to their light-scattering properties (FSC/SSC, Fig. 6B). Proliferating cells showed a higher level of filipin fluorescence and were analyzed separately from nonproliferating T cells (Fig. 6B). Total cell cholesterol was determined by HPTLC and was 0.65 ± 0.14 μg/10⁷ in MACS-isolated resting CD4+ cells and 2.42 ± 0.65 μg/10⁴ in Jurkat cells, respectively. Membrane cholesterol was extracted by incubation with up to 10 mM MbCD. The efficacy of cholesterol extraction was 34 ± 8% in CD4+ cells and 54 ± 22% in Jurkat cells, as determined by HPTLC. The inset in Fig. 6C shows a representative HPTLC experiment. The sensitivity of the filipin staining was determined with 3 mM MbCD because 10 mM MbCD was toxic to CD4+ cells. Filipin staining showed a small reduction of membrane cholesterol with 3 mM MbCD, as determined by filipin staining (Fig. 6B). Incubation with up to 5 μM atorvastatin did not decrease the cellular cholesterol content (Fig. 6B). However, the filipin-staining method seems to have a limited sensitivity to detect small changes of membrane cholesterol. Because the total cellular cholesterol might not reflect smaller changes of the cholesterol content in MR, we isolated MR from superantigen-stimulated PBMC or from Jurkat cells and quantified...
Phosphorylation of ERK and Ca

Before, we investigated early events of T cell activation such as phosphorylation of ERK. Total ERK was detected as loading control. Cells were treated with 2 μM atorvastatin for 2 min and 2 h, as indicated (A and B). Ca

flux was analyzed in PBMC loaded with Fluo3AM. Cells were incubated with or without 5 μM atorvastatin and were stimulated with anti-CD3 (5 μg/ml OKT3) and secondary Abs (12 μg/ml goat anti-mouse) (C).

Discussion

In the present study, we investigate the inhibitory effects of statin treatment on the activation and proliferation of human T lymphocytes in vitro. Our data demonstrate an almost complete inhibition of superantigen-stimulated proliferation by atorvastatin treatment, which is consistent with recently published data (11, 12). The IC

for the inhibition of proliferation is ~1.5 μM and confirms previous data with simvastatin (21, 29). Our data show that statins inhibit the expression of cellular activation markers such as CD69 and CD25. Furthermore, statins reduce the up-regulation of GM1 during T cell activation and prevent the association of Lck and LAT with MR in stimulated T cells. Earlier reports suggested several molecular mechanisms such as an inhibition of HMG CoA reductase (29), prenylation (30), coactivation by integrins like LFA-1 (23), Ag presentation (9), and modulation of lipid microdomains (20, 21). The variety of mechanisms might also depend on the used statin concentration. Most of these mechanisms were demonstrated in experiments using 10 μM atorvastatin, which is a rather high concentration that cannot be detected in human serum (31, 32). Our experiments show that superantigen-induced proliferation is restored when cells are cultured in presence of atorvastatin and mevalonic acid, indicating that inhibition of hydroxymethylglutaryl coenzyme A reductase is a critical step. Therefore, we can exclude statin binding to LFA-1 as the main mechanism for inhibition of T cell proliferation in our experiments. The earliest steps in T cell activation are tyrosine phosphorylation and Ca

flux. However, our experiments exclude statin-mediated effects on ERK phosphorylation (Fig. 8, A and B) or Ca

mobilization (Fig. 8C). The earliest detectable effects were an inhibition of CD69 expression after 6 h, which was more pronounced after 24 h. Therefore, we conclude that statins interfere with T cell activation within 24 h, but not with the initiation of T cell activation. The time frame for statin-mediated effects includes a possible inhibition of pathways that depend on prenylation (Fig. 7) or transcription and protein synthesis. The inhibitory effect of inhibitors of protein prenylation suggests that members of the Ras protein family might be involved in the action of statins. An early report showed that p21

is involved in the expression of CD69 (33). A statin-mediated inhibition of protein prenylation was also suggested by other groups (27, 28). In addition, statins also inhibited proliferation of lymphoblasts, which is independent of TCR signaling (Fig. 1C). The inhibition of proliferation can be partially rescued by mevalonic acid, which indicates that inhibition of HMG-CoA is relevant. However, the rescue is not complete. This experiment indicates that either toxic effects occur in lymphoblasts or statins might interfere with the activation of transcription factors such as NF-AT, AP-1, or NF-κB, and thereby keep cells in a hypoproliferative state. This hypothesis deserves further investigations.

An alternative hypothesis for the statin-mediated inhibition of T cell activation might be a down-regulation of Lck and LAT by degradation. However, our data exclude a general decrease of signaling molecules in membrane fractions (Fig. 3, A–D) or total cellular proteins (Fig. 3E). Neither incubation with statins nor inhibition of the proteasome or a combination of both changed cellular Lck and LAT levels.

Previous reports showed that immunomodulatory effects of statins might be explained by the inhibition of an IFN-γ-inducible

their cholesterol and lipid contents by HPTLC (Fig. 6C). We were unable to detect any difference between MR isolated from PBMC or Jurkat cells treated with or without 10 μM atorvastatin. We conclude that the inhibition of T cell activation and proliferation cannot be mediated by a depletion of cellular cholesterol or MR cholesterol.

Inhibition of prenylation

Because statins were shown to prevent prenylation of Ras family proteins (27, 28), we considered this effect for an explanation of the inhibitory effects of statins. The isoprenyl compounds geranylgeranol and farnesol are intermediate products in the cholesterol synthesis pathway. We investigated whether inhibition of prenylation can also inhibit proliferation. PBMC were treated with an inhibitor of farnesyl transferase (FTI-277) or geranylgeranyl transferase (GGTI-298). Only the highest FTI concentration (33 μM) showed a moderate inhibition of proliferation, which probably indicates toxicity (Fig. 7A). In contrast, 10 μM GGTI showed a moderate inhibition of proliferation, and proliferation was completely blocked with 33 μM GGTI, which might indicate toxicity (Fig. 7B). These results indicate that an inhibition of geranylation, but not farnesylation, might contribute to the inhibition of proliferation with high statin concentrations. Whether an inhibition of geranylation can also occur with low statin concentrations remains to be shown.

Atorvastatin does not interfere with ERK phosphorylation or Ca

flux

Statins seem to exert their function within the first 6–24 h. Therefore, we investigated early events of T cell activation such as phosphorylation of ERK and Ca

fluxing. PBMC or MACS-sorted CD4

CD25

cells were stimulated with PHA and PMA to induce phosphorylation of ERK. However, we did not detect any interference of statin treatment with phosphorylation of ERK within 2 min or 2 h of stimulation of PBMC (Fig. 8A) or sorted CD4

CD25

cells (Fig. 8B). Mobilization of Ca

was induced by stimulation of PBMC with anti-CD3 (OKT3) and a cross-linking secondary Ab (goat anti-mouse) and was analyzed in CD4

gated T cells. We did not detect any effect of statin treatment on the Ca

fluxes. These data show that ERK phosphorylation and Ca

fluxes can be excluded as targets of statin action.
promoter (type IV) of CIITA (pIV-CIITA) (9). However, these effects are restricted to the inducible, but not to the constitutive expression of pIV-CIITA and MHC-II on endothelial cells and monocytes-macroagglutinins (9). In the report of Kwak et al. (9), the inhibition of inducible MHC-II on endothelial cells was inhibited by <2 μM atorvastatin, and the allogenic T cell proliferation after 5 days was inhibited to ~40% with 10 μM atorvastatin. The statin-mediated inhibition of pIV-CIITA was confirmed by others (34). However, it was shown recently that simvastatin neither inhibited CIITA or HLA-DR transcription, nor inhibited their promoter activity in cell lines and in activated human T cells (35).

Our experiments, due to endogenous IL-2, sCD25 can be detected in the cell culture might be an alternative explanation of the reduced expression of GM1-negative MR. This hypothesis was not investigated in our experiments. Statins also inhibit proliferation in CD3^+CD28-stimulated cells that do not require MHC-II (Fig. 1B).

Additionally, our data show that statins increase the secretion of IFN-γ into the culture supernatant (Fig. 5A) and blocking of IFN-γ does not eliminate the statin-mediated inhibition of proliferation (Fig. 5B). Higher concentrations of IFN-γ might promote the inducible expression of MHC-II at later time points, but this was not observed in our experiments. However, we cannot exclude an inhibition of inducible MHC-II during long-term culture and with higher concentrations of atorvastatin. Kwak et al. (9) showed that 10 μM atorvastatin significantly inhibited IL-2 release in IFN-γ-stimulated macrophages and allogenic T cells, which could explain the inhibition of T cell proliferation with 10 μM atorvastatin. In our experiments, 5 μM atorvastatin did not inhibit IL-2 release (data not shown), but significantly stimulated the secretion of IFN-γ. At the time being, we cannot postulate an up-regulation of IFN-γ de novo synthesis. An increased secretion of IFN-γ from an intracellular compartment could be an alternative explanation. Our data show that the antiproliferative effects of statins are not mediated through decreased IFN-γ secretion. Moreover, we show that statins and IFN-γ blockade exert additive effects on lymphocyte proliferation.

Mevalonic acid counteracted the antiproliferative effects of statins in our experimental model. This suggests that the anti-inflammatory action of atorvastatin is mediated by inhibition of HMG-CoA reductase. However, in our experiments, statins reduced neither total cellular cholesterol nor MR cholesterol. The possibility remains that in the presence of serum, T cells maintain their cellular cholesterol pools by cholesterol uptake using the low-density lipoprotein receptor (36, 37).

An alternative explanation of the reduced amount of GM1 in statin-treated cells might be an even distribution of GM1 between both the MR and detergent-soluble membrane fractions. However, previous data from the literature show that GM1 is up-regulated during cellular activation (38–40) and that GM1 is highly enhanced in MR fractions with only little GM1 in soluble fractions (41–43). However, we did not detect GM1 in Western blot lysates, and therefore cannot exclude this hypothesis. An alternative explanation could be that MR are probably heterogeneous and that GM1-positive and GM1-negative MR might exist. The current standard isolation procedure cannot separate GM1-positive from GM1-negative MR. Therefore, the possibility remains that statins might selectively affect GM1-positive MR without any perturbation of the GM1-negative MR. This hypothesis was not investigated in this study.

An increased cleavage of CD25 or other stimulatory molecules might be an alternative explanation of the reduced expression of CD25 on statin-treated cells and for an inhibition of proliferation due to endogenous IL-2. sCD25 can be detected in the cell culture supernatant of stimulated T cells, but it is actually decreased in statin-treated cells. We can exclude this hypothesis for CD25, but we did not investigate cleavage products of other cell surface molecules. Therefore, we cannot exclude a hypothetical down-regulation of other costimulatory molecules by statin treatment.

In conclusion, our data show that statins can prevent T cell activation and proliferation. To our knowledge, this is the first report that quantifies cellular cholesterol and excludes a statin-mediated alteration of cellular cholesterol pools. We show that the association of signaling proteins with MR is strongly altered by statin treatment, which is a consequence, but not the cause of an inhibited T cell activation. Thus, our data provide further insight into the molecular mechanisms responsible for the anti-inflammatory properties of statins.

Disclosures

The authors have no financial conflict of interest.

References


