



3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor Atorvastatin mediated effects depend on the activation status of target cells in PLP-EAE

Eilhard Mix^a, Saleh M. Ibrahim^b, Jens Pahnke^a, Anne Glass^c, Ignacio Mazón-Peláez^b,
 Susanne Lemcke^b, Dirk Koczan^b, Ulrike Gimsa^a, Sven Bansemer^c, Thomas Scheel^c,
 Thomas Karopka^c, Tobias Böttcher^a, Jana Müller^{a,d}, Eike Dazert^e, Veronica Antipova^{a,d},
 Raimund Hoffrogge^a, Andreas Wree^d, Marlies Zschiesche^a, Ulf Strauß^a,
 Günther Kundt^c, Rolf Warzok^e, Lothar Gierl^c, Arndt Rolfs^{a,*}

^a Department of Neurology, University of Rostock, Gehlsheimer Str. 20, D-18147 Rostock, Germany

^b Department of Immunology, University of Rostock, Schillingallee 70, D-18055 Rostock, Germany

^c Department of Medical Informatics and Biometry, University of Rostock, Rembrandtstr. 16/17, D-18057 Rostock, Germany

^d Department of Anatomy, University of Rostock, Gertrudenstr. 9, D-18057 Rostock, Germany

^e Department of Pathology, University of Greifswald, F.-Löffler Str. 23e, D-17487 Greifswald, Germany

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Abstract

The effect of Atorvastatin on transcriptional activity in murine experimental autoimmune encephalomyelitis (EAE) induced by PLP peptide 139–151 was analyzed by DNA microarray technique in lymph nodes and spinal cord at onset (10 days), height (20 days) and first remission (30 days) of disease. Fourteen genes were selectively influenced by Atorvastatin in EAE mice. They are mainly related to immune cell functions and regulation of cell-to-cell interaction. Interestingly, seven genes were also differentially regulated in CFA-injected control mice. But qualitative and quantitative differences to EAE mice argue for a dependency of statin effects on the activation status of target cells. Differential regulation of the newly detected candidate genes of statin effects COX-1 and HSP-105 and the previously known statin-responsive genes ICAM-1 and CD86 was confirmed by Western blot and immunohistochemistry. Flow cytometric analysis of lymph node cells revealed that the effect of Atorvastatin treatment in non-immunized healthy animals resembled the effect of immunization with PLP peptide regarding changes of T helper cells, activated B cells and macrophages. In EAE mice, these effects were partially reversed by Atorvastatin treatment. Monitoring of expression of the newly identified candidate genes and patterns of lymphocyte subpopulations might predict the responsiveness of multiple sclerosis patients to statin treatment.

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Keywords: Experimental autoimmune encephalomyelitis (EAE); Statin; Oligonucleotide microarray; Lymph nodes; Spinal cord; Leukocyte subpopulations; Western blot; Immunohistochemistry

Abbreviations: EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; MS, multiple sclerosis; CFA, complete Freund's adjuvant; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PCR, polymerase chain reaction; COX-1, cyclooxygenase-1; HSP-105, heat shock protein-105; ICAM-1, intercellular adhesion protein-1; PTX-3, pent(r)axin; p.i., post-immunization; CNS, central nervous system.

* Corresponding author. Tel.: +49 381 494 9540; fax +49 381 494 9542.

E-mail address: arndt.rolfs@med.uni-rostock.de (A. Rolfs).

1. Introduction

Statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which is the rate limiting enzyme for the catalysis of the conversion of HMG-CoA to L-mevalonate. Consequently, there also occurs a reduction of the downstream metabolites of L-mevalonate including isoprenoid

intermediates like geranylgeranylpyrophosphates and farnesylpyrophosphates. Isoprenoids such as the GTP-binding proteins Ras and Ras-like proteins Rac, Ral, Rap and Rho, are essential for the assembly of plasma and organelle membranes [1] and have pleiotropic effects on leukocytes, endothelial cells and osteocytes [2].

In the last years, a broad spectrum of pleiotropic effects of statins in various cell types and tissues has been described, including the central nervous system (CNS) and the immune system. These pleiotropic effects include the inhibition of expression and secretion of pro-inflammatory cytokines [3, 4], the inhibition of lymphocyte migration [5], the inhibition of T-cell activation and proliferation [6, 7] and the inhibition of adhesion molecules such as LFA-1, VCAM-1 and E-selectin [8, 9]. Statins have the advantage of oral application, relatively few adverse side-effects and permission for clinical use in certain diseases such as hypertension.

Promising results mostly from animal experiments have raised broad interest in the potential for treating multiple sclerosis (MS) with statins [10]. Findings in pilot studies point to anti-inflammatory effects of statins in autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE) [6,11], as well as to anti-apoptotic and neuroprotective properties [12]. Studies of Refs. [13] and [14] suggested a benefit of oral statin therapy in patients with relapsing-remitting MS. Consequently, there are several ongoing clinical trials investigating the clinical statin efficacy in MS [10].

Recently, new paradigms have changed our understanding of the pathogenesis and the therapeutic options of MS and its animal model EAE. PLP 139–151 peptide-induced EAE in SJL mice mimics clinical, histological and electrophysiological features of the most common MS type, i.e. the relapsing-remitting form [15]. EAE is mainly driven by a proinflammatory T-cell response that involves type 1 T helper cells (Th1 cells). Atorvastatin, a prototype reagent of type 2 statins containing an open ring structure and a strong hydrophobic group linked to a HMG-like moiety [16] has been proven to be a potent inhibitor of EAE [5,6,11]. These effects have also been seen in some studies even when the treatment was started after the onset of disease [6, 11]—a constellation which would be necessary in the clinical setting. Also Lovastatin, another type 2 statin, could attenuate active and adoptive EAE, if given prophylactically or therapeutically [3]. It induced a shift from Th1 to Th2 cytokines via the expression of the transcription factor GATA3 and phosphorylation of STAT6 [17]. A similar Th2 cytokine shift was induced by Atorvastatin via reduction of ERK and p38 phosphorylation [18].

The analysis of global transcriptional activity by DNA microarray technique (RNA profiling) has been a useful tool to identify genes, which are involved in the initiation and progression of EAE [19,20] including those responsible for EAE resistance in C57BL/B10 mice [21,22], and for evaluation of the effect of therapeutic drugs such as interferon- β [23,24], 17 β -estradiol [25], histamine receptor type 1 antagonists [26] and Lovastatin [27].

It was the aim of the present study to identify new targets which are important for the effect of Atorvastatin in EAE

mice compared to healthy mice. The global gene expression approach by microarray technique was chosen in order to achieve a most comprehensive analysis of statin-sensitive genes during the course of the disease. To avoid any analytical bias we did not intend to set any premise, neither regarding presumably relevant metabolic or signal pathways, nor known intercellular mediators or contact molecules. The animal model of PLP 139–151 peptide-induced EAE was chosen because it best resembles the most wide-spread relapsing-remitting type of MS. In addition, the effect of Atorvastatin on lymph node cell subpopulations in both healthy and EAE mice has been compared in order to disclose statin-specific changes of the cells responsible for the induction and maintenance of this most prevalent autoimmune disease of the nervous system.

2. Materials and methods

2.1. Immunization, statin treatment and assessment of disease

SJL mice were obtained from Charles River Wiga (Sulzfeld, Germany) and kept under standard conditions at the animal facility of the University of Rostock. Seven-weeks-old male mice were immunized subcutaneously with 150 μ g of the encephalitogenic peptide corresponding to amino acids 139–151 of mouse PLP [28] purchased from American Peptide Company (Sunnyvale, CA) in complete Freund's adjuvant (CFA, Sigma, St. Louis, MO). Male mice were preferred to female mice in order to achieve a potentially milder disease imitating the typically stealthy beginning of human MS and to avoid influences of the female hormone cycle on the distribution of lymphocyte subpopulations and on gene expression to be investigated. Mice received an intraperitoneal injection of 500 ng pertussis toxin (Sigma) simultaneously with the PLP peptide and at day 3 post-immunization (p.i.). For statin treatment, animals received 200 μ g Atorvastatin (Sortis, Pfizer, Karlsruhe, Germany) dissolved in 100 μ l of phosphate-buffered saline (PBS) per mouse and day applied orally using a microliter pipette. The dosage is equal to 10 mg/kg body weight. Control EAE mice received 100 μ l of vehicle substance PBS only. To mimic a clinical situation, Atorvastatin treatment was started only after onset of first signs of disease, i.e. on day 10 p.i. (Fig. 1). The following groups of experimental animals consisting of four mice each were compared (see Fig. 1): (A) normal healthy control mice corresponding to day 0 p.i., (B–D) EAE mice of days 10, 20 and 30 p.i., respectively, (E, F) control mice receiving no immunization but Atorvastatin for 10 and 20 days, respectively, (G, H) EAE mice receiving Atorvastatin for 10 and 20 days, respectively, starting on day 10 p.i. The clinical scores of animals were assessed by two blinded investigators before immunization (day 0) and thereafter daily until day 30 p.i. Severity of paresis was graded as follows: 0, normal; 1, flaccid tail; 2, moderate paraparesis; 3, severe paraparesis; 4, tetraparesis. Mice were sacrificed at days 10, 20 and 30 p.i., respectively, corresponding to onset of first signs of disease, height of disease and recovery from disease, respectively (for experimental design see Fig. 1). Control mice were injected with CFA only

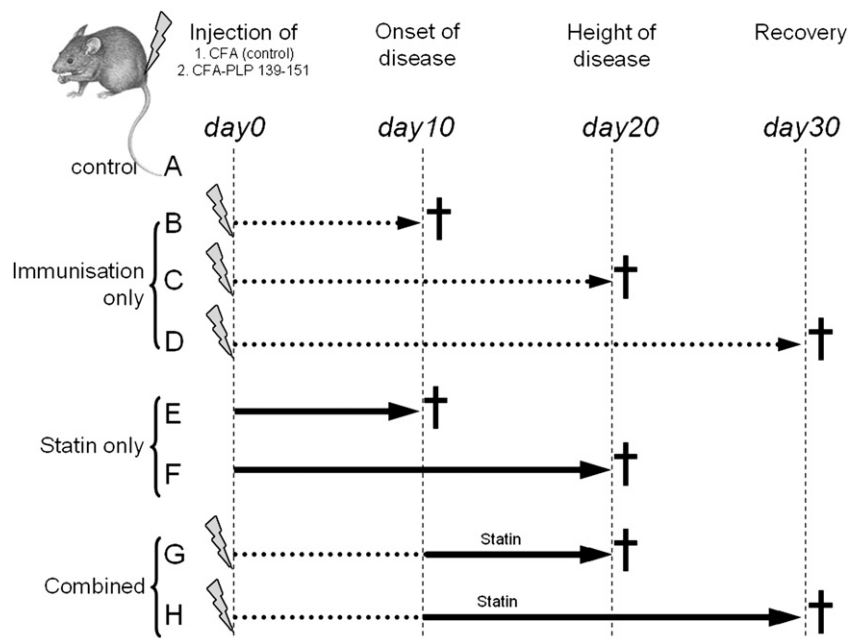


Fig. 1. Experimental design of the study to investigate immune response and gene expression in lymph nodes and spinal cord of EAE mice with and without Atorvastatin treatment. Mice of groups B–D were immunized with PLP peptide 139–151 and CFA at day 0 and sacrificed at days 10 (group B), 20 (group C) and 30 (group D) post-immunization (p.i.), respectively. Mice of groups G and H additionally received Atorvastatin orally from day 10 to day 20 p.i. and day 10 to day 30 p.i., respectively. Control mice of groups E and F were injected with CFA and received Atorvastatin for 10 and 20 days post-injection, respectively. Control mice of group A were neither immunized nor Atorvastatin treated.

and four of these mice (group A) were sacrificed for comparative analyses, too. Lymph nodes of the popliteal, preperitoneal, inguinal, mesenteric and axillary regions of each individual mouse of the experimental animal groups (A–H) were aseptically removed and used for cell cultures, flow cytometry, and RNA and protein extraction as described below. The spinal cords of each individual mouse of the experimental animal groups (A–H) were dissected into cervical, thoracic and lumbo-sacral parts to be used for histochemical staining, and RNA and protein extraction, respectively, as described below. Experiments were approved by the authorities of the county of Mecklenburg-Western Pomerania, Germany.

2.2. Isolation of lymph nodes MNC and cell culture

For proliferation assay, lymph nodes of each individual mouse of the above mentioned experimental groups (A–H) were removed and single cell suspensions of mononuclear cells (MNC) of pooled lymph nodes from individual mice were prepared and cultured as described [21]. The specific antigen PLP 139–151 peptide was added to cultures to a final concentration of 10 and 25 $\mu\text{g}/\text{ml}$ and the mitogen concanavalin A (ConA) (Difco, Detroit, MI) to 4 $\mu\text{g}/\text{ml}$.

2.3. Proliferation Assay

After 60 h of incubation, cells were pulsed with 10 μl ^3H -methylthymidine (1 $\mu\text{Ci}/\text{well}$, Amersham Pharmacia Biotech, Freiburg, Germany) and cultured for additional 12 h. Cells were harvested onto glass fiber filters (Titertek, Skatron,

Lierbyen, Norway). ^3H -thymidine incorporation was analyzed in a liquid β -scintillation counter (Wallac 1214, LKB, Bromma, Sweden). The results were measured as counts per minute (cpm).

2.4. Flow cytometric characterization of leukocyte populations

Single cell suspensions were prepared from pooled lymph nodes from each individual animal as described for lymph node cell cultures [21] of the four mice belonging to one of the experimental groups (see Fig. 1). Cell suspensions were divided into four aliquots, and double- or triple-stained for CD3-FITC/CD25-PE/CD4-Cy5, CD25-PE/CD8-FITC, CD19-FITC/CD25-PE, and CD11c-FITC/CD4-Cy5/CD11b-PE, respectively at 2 $\mu\text{g}/\text{ml}$ in PBS/0.5%BSA for 10 min at room temperature. The staining allowed for quantification of the following lymph node cell subpopulations: (1) CD3⁺ total T cells, (2) CD4⁺CD3⁺ T helper cells, (3) CD4⁺CD3⁺/CD3⁺ proportion of T helper cells among total T cells, (4) CD25⁺CD4⁺ activated or regulatory T helper cells, (5) CD25⁺CD4⁺/CD4⁺CD3⁺ proportion of activated or regulatory T helper cells among total T helper cells, (6) CD8⁺ cytotoxic T cells, (7) CD25⁺CD8⁺ activated cytotoxic T cells, (8) CD25⁺CD8⁺/CD8⁺CD3⁺ proportion of activated among total cytotoxic T cells, (9) CD19⁺ total B cells, (10) CD25⁺CD19⁺ activated B cells, (11) CD25⁺CD19⁺/CD19⁺ proportion of activated among total B cells, (12) CD11b⁺ macrophages, (13) CD11c⁺ dendritic cells. Isotype control staining was performed to exclude non-specific binding. CD4-Cy5 was a gift of the German Rheumatology Research Center, Berlin. All other antibodies were purchased

from BD Biosciences Pharmingen (Heidelberg, Germany). Cells were subsequently fixed in 2% paraformaldehyde for 10 min. After washing, cellular composition was analyzed by flow cytometry on a FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA) with respect to the different leukocyte subpopulations.

2.5. Oligonucleotide array hybridization

For gene expression analysis, individual mRNA of the mice of the experimental groups (A–H) was investigated as described [21]. RNA from thoracic spinal cord and pooled lymph nodes of individual animals was used. Analysis of gene expression was carried out with the MOE430 gene chip (Affymetrix, Santa Clara, CA) with a capacity of more than 22 000 probe sets. Hybridization and washing of gene chips were done as previously described [29,30].

2.6. Analysis of microarray data

We used the Affymetrix Microarray Suite (MAS) 5.1 (Affymetrix) to process raw microarray probe set data (for details see [21] and www.affymetrix.com, Statistical Algorithms Reference Guide, Part No. 701110 Rev2). The MAS expression data were analyzed with the software suite gEnOM [31,32]. We analyzed four individual mRNA samples from spinal cord and lymph nodes of each experimental group (A–H, Fig. 1). The individual gene “signals” of replicate samples were averaged by the median, if at least three out of the four corresponding “detection call” values were “present”. The medians were used for group comparisons. The differential expression of genes is presented as fold changes of expression levels comparing different experimental animal groups (see Fig. 1). Genes with at least two-fold changes in gene expression are to be considered relevant. Our former microarray experiments and published work by others [19,21,22,24,29,30,33–37] showed that a change of two-fold in the expression level is a cut off to consider expression changes to be significant.

2.7. Western blot

The lumbo-sacral parts of spinal cord and pooled lymph nodes, respectively, of each individual mouse of the experimental animal groups (A–H, Fig. 1) were dissected and lysed, the lysates were mixed with Laemmli-buffer, denaturated and fractionated by SDS-polyacrylamide gel electrophoresis and the proteins were blotted according to standard procedure [21]. The membranes were blocked with Odyssey® Blocking Buffer (LI-COR Bioscience, Bad Homburg, Germany) and incubated with the following primary antibodies: rat anti-mouse CD86 (B7-2) monoclonal antibody (BD Biosciences Pharmingen), rabbit anti-cyclooxygenase-1 (COX-1) polyclonal antibody (Chemicon, Hofheim, Germany), goat anti-mouse intercellular adhesion molecule-1 (ICAM-1) polyclonal antibody (R&D Systems, Wiesbaden, Germany), goat anti-heat shock protein-105 (HSP-105) polyclonal antibody (Santa Cruz, Heidelberg, Germany), goat anti-suppressor of cell signaling-3 (SOCS-3)

polyclonal antibody (Santa Cruz), mouse anti- β -actin monoclonal antibody (Sigma). For detection the following secondary antibodies were used: Alexa Fluor 680 goat anti-rabbit IgG, Alexa Fluor 680 goat anti-rat IgG, Alexa Fluor 680 donkey anti-goat IgG (all from Molecular Probes, purchased from Invitrogen, Karlsruhe, Germany), and IRDye 800CW conjugated goat-anti-mouse IgG (Rockland, purchased from BIOMOL, Hamburg, Germany). Quantitative assessment of proteins was enabled by Odyssey® Infrared Imaging System (LI-COR Bioscience). The intensity of the signals was quantified by Odyssey software version 1.2 using normalization to β -actin values thereby compensating for different protein concentrations in the samples.

2.8. Immunohistochemistry

Cervical parts of spinal cord of each individual mouse of the experimental animal groups (A–H, Fig. 1) were fixed in 4% paraformaldehyde, snap frozen and cut into 12- μ m-thick sections. Sections were immunostained according to standard procedure [38–40] using the primary antibodies monoclonal rat anti-mouse CD86 (BD Biosciences Pharmingen) and polyclonal rabbit anti-mouse COX-1 (Chemicon) and the corresponding biotinylated secondary antibodies donkey anti-rabbit IgG and donkey anti-rat IgG from Jackson ImmunoResearch (West Grove, PA). Morphometric examination was done by analyzing four digital pictures from each section of the grey matter of the spinal cord (200-fold magnification, 3CCD color camera, Hitachi HV-C20M Hitachi-Denshi, Rodgau, Germany) using KSRun software (imaging system KS400, release 3.0; Zeiss, Vision GmbH, Munich, Germany) (for illustration see Fig. 8D). The optical density of the white background was standardized to 250 and the mean optical density and the quantity of pixels that showed a positive immune reaction for CD86 and COX-1 were assessed.

2.9. Statistics

Data of clinical score have been tested for significant differences between statin-treated ($n = 9–18$ depending on the day p.i.) and untreated ($n = 8–22$ depending on the day p.i.) animals by Mann–Whitney’s U test. Data of proliferation assay and flow cytometric analysis of the four individual mice belonging to one group were averaged and groups were compared using Student’s t -test statistics. Statistical analysis of microarray data was done as described in the respective section.

3. Results

3.1. Clinical course of the disease

First clinical signs of EAE usually appear around day 10 p.i., first remission around day 20 p.i., and complete recovery around day 30 p.i. Therefore, these time points were chosen to sacrifice animals and take out lymph nodes and spinal cord for further investigations (cell culture, flow cytometry, microarray analysis, Western blot and immunohistochemistry).

The PLP-EAE imitates best the most common type of MS, i.e. the relapsing-remitting type [15]. It also induces mild symptoms of the disease, which resemble the stealthy beginning of MS. Atorvastatin was applied at the dosage of 10 mg/kg body weight, which exceeds the recommended dose for treatment of hypercholesterolaemia in humans, but reduces maximally the clinical signs of EAE in PLP-EAE according to previous reports [6,11], although the disease was more severe in those reports. In our study, Atorvastatin-treated animals revealed a significantly lower ($p < 0.05$) clinical score than control mice receiving CFA and vehicle (PBS) without statin only at day 12 p.i. A tendency towards a lower clinical score ($p < 0.1$) in statin-treated animals was also seen at days 13–15. The sequential usage of animals for lymph node and spinal cord extirpation precluded a scoring of clinical symptoms of all animals over the whole study period.

3.2. Immune response in lymph nodes

3.2.1. Proliferative response as analysed by ^3H -thymidine incorporation

In order to compare the strength of the immune response to the mitogen ConA and to the specific immunizing antigen PLP 139–151 peptide lymph node cell reactivity was assessed by proliferation assay in EAE mice on days 10, 20 and 30 p.i. with and without Atorvastatin treatment and in control mice with and without Atorvastatin treatment (Fig. 2). The basal ^3H -thymidine incorporation corresponding to the spontaneous DNA synthesis was 10–40 times higher in the control mice (about 6000 cpm in group A) compared to all other animal groups (between 150 and 400 cpm in groups B–H). Exposure of cells to mitogenic ConA resulted in higher levels of ^3H -thymidine incorporation being for each group between 40 000 and 60 000 cpm. Hence, any treatment either by immunization or by Atorvastatin application led to a decrease of the basal lymphocyte proliferation but did not change the maximum proliferation induced by mitogen. The specific immune response to the immunizing peptide PLP 139–151 was pronounced with an about 10-fold stimulation above background at 10 days p.i. However, prolonged disease was accompanied by a reduction of DNA synthesis to basal background level independent on Atorvastatin treatment, although there was a trend to a small increase of proliferation at the higher PLP 139–151 concentration (25 $\mu\text{g}/\text{ml}$). This phenomenon was seen in all immunized animals with (groups G and H) and without Atorvastatin treatment (groups C and D), but also in Atorvastatin-treated control animals (groups E and F).

3.3. Leukocyte subpopulations as analyzed by flow cytometry

Changes of leukocyte subpopulations were found in lymph nodes with respect to both (1) response to the immunization of the mice with PLP 139–151 peptide, i.e. during the course of EAE, and (2) response to Atorvastatin treatment in healthy, but CFA-injected as well as in EAE mice. The following significant changes were registered (Fig. 3).

Effect of immunization with PLP 139–151 for EAE induction (compare white columns of Fig. 3B and D to white columns of Fig. 3A and C, respectively): (i) T helper cells increased among total lymph node cells and among total T cells at both time points of analysis (10 and 20 days p.i.), (ii) activated or regulatory T helper cells decreased among total T helper cells at both time points of analysis (10 and 20 days p.i.), (iii) activated B cells increased among total lymph node cells and among total B cells after prolonged immunization (20 days p.i.), and (iv) macrophages increased at both time points of analysis (10 and 20 days p.i.). The observed changes of leukocyte subpopulations are statistically significant ($p < 0.05$; not indicated in Fig. 3 to avoid confusion with the effect of Atorvastatin).

Effect of Atorvastatin treatment in non-immunized control animals (Fig. 3A and C): (i) T helper cells increased among total lymph node cells and among total T cells at both time points of analysis, (ii) activated or regulatory T helper cells increased among total lymph node cells at both time points of analysis, (iii) activated or regulatory T helper cells among total T helper cells decreased at the later time point of analysis (Fig. 3C only), (iv) total B cells and activated B cells, but also the proportion of activated among total B cells increased at both time points of analysis, and (v) macrophages increased at both time points of analysis. The observed changes of leukocyte subpopulations are statistically significant as indicated in Fig. 3A and C.

Effect of Atorvastatin treatment in PLP-immunized EAE animals (Fig. 3B and D): (i) T helper cells increased among total T cells initially (Fig. 3B only), (ii) cytotoxic T cells decreased initially (Fig. 3B) and increased after prolonged treatment (Fig. 3D), (iii) B cells decreased after prolonged treatment (Fig. 3D only). The observed changes of leukocyte subpopulations are statistically significant as indicated in Fig. 3B and D).

CD11c⁺ dendritic cells comprised less than 1% of total lymph node cells in all samples tested and did not change significantly in response to PLP-immunization or Atorvastatin treatment (data not shown).

Comparison of PLP-immunization (EAE induction) with Atorvastatin treatment (Fig. 4): Analysis of flow cytometric data revealed the interesting finding that changes in the proportions of T helper cells, activated B cells and macrophages during PLP-induced EAE were similar to those observed after Atorvastatin treatment of healthy, only CFA-injected control mice. This is illustrated in Fig. 4 by direct comparison of data of Atorvastatin-treated CFA-injected animals (black columns) with data of corresponding non-Atorvastatin-treated PLP-immunized animals (white columns), which were not significantly different.

3.4. Gene expression in lymph nodes and spinal cord

In order to identify candidate genes that are differentially regulated in response to (i) the immunization of SJL mice with PLP 139–151 peptide and (ii) more importantly in response to Atorvastatin treatment, we compared gene expression levels between different groups of animals as indicated in Fig. 5.

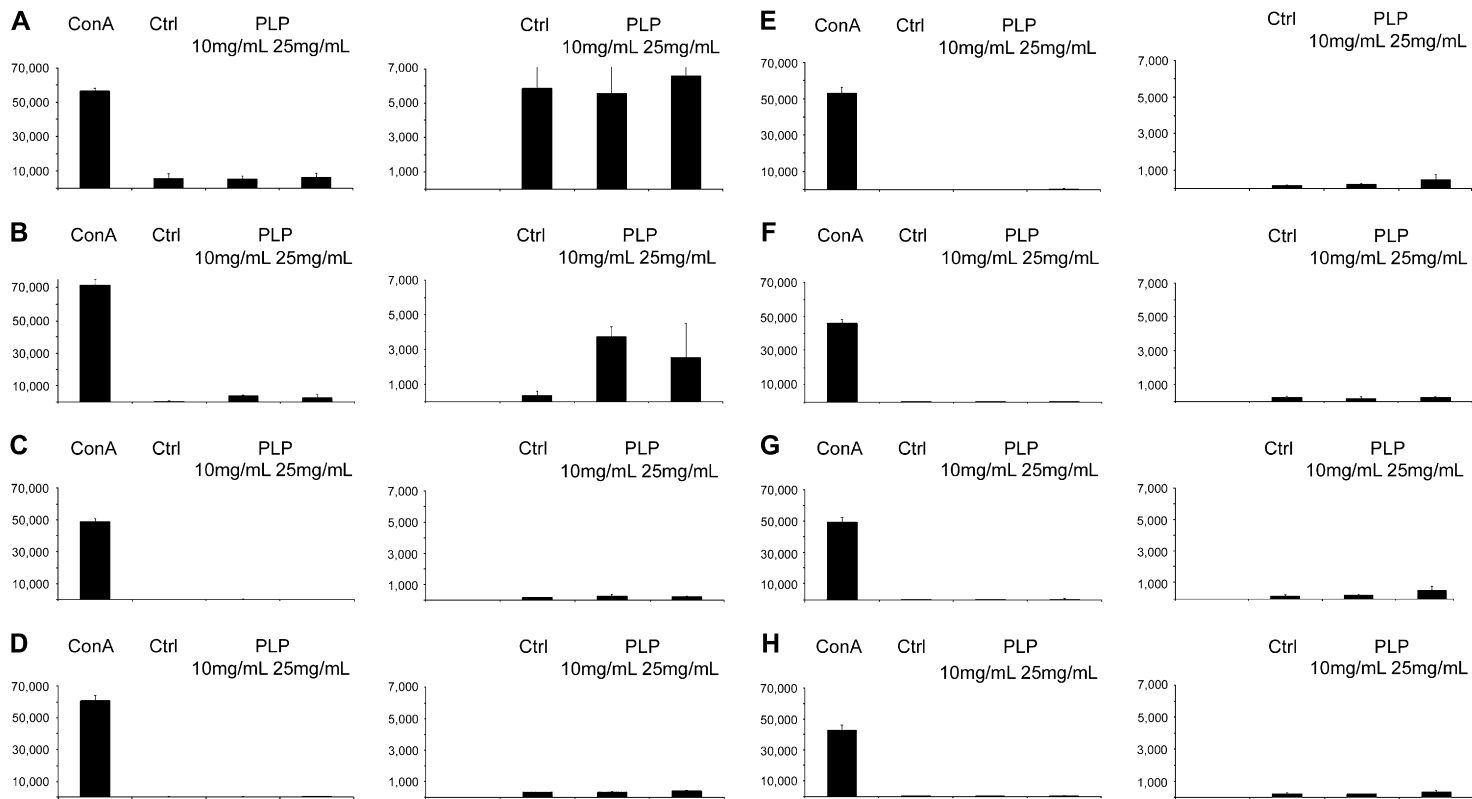


Fig. 2. Proliferative response of lymph node cells of EAE mice to the mitogen ConA and to two concentrations of the immunizing peptide PLP 139–151 as measured by ³H-thymidine incorporation in vitro. The y-axis indicates the radionuclide uptake in counts per minute (cpm). For better illustration of the different levels of ³H-thymidine incorporation in mitogen and antigen stimulated cells, left hand graphics represent results including mitogen stimulation and right hand graphics represent the corresponding results excluding mitogen stimulation. The designation of the experimental animal groups A–H corresponds to Fig. 1. “Ctrl” indicated control without in vitro stimulation.

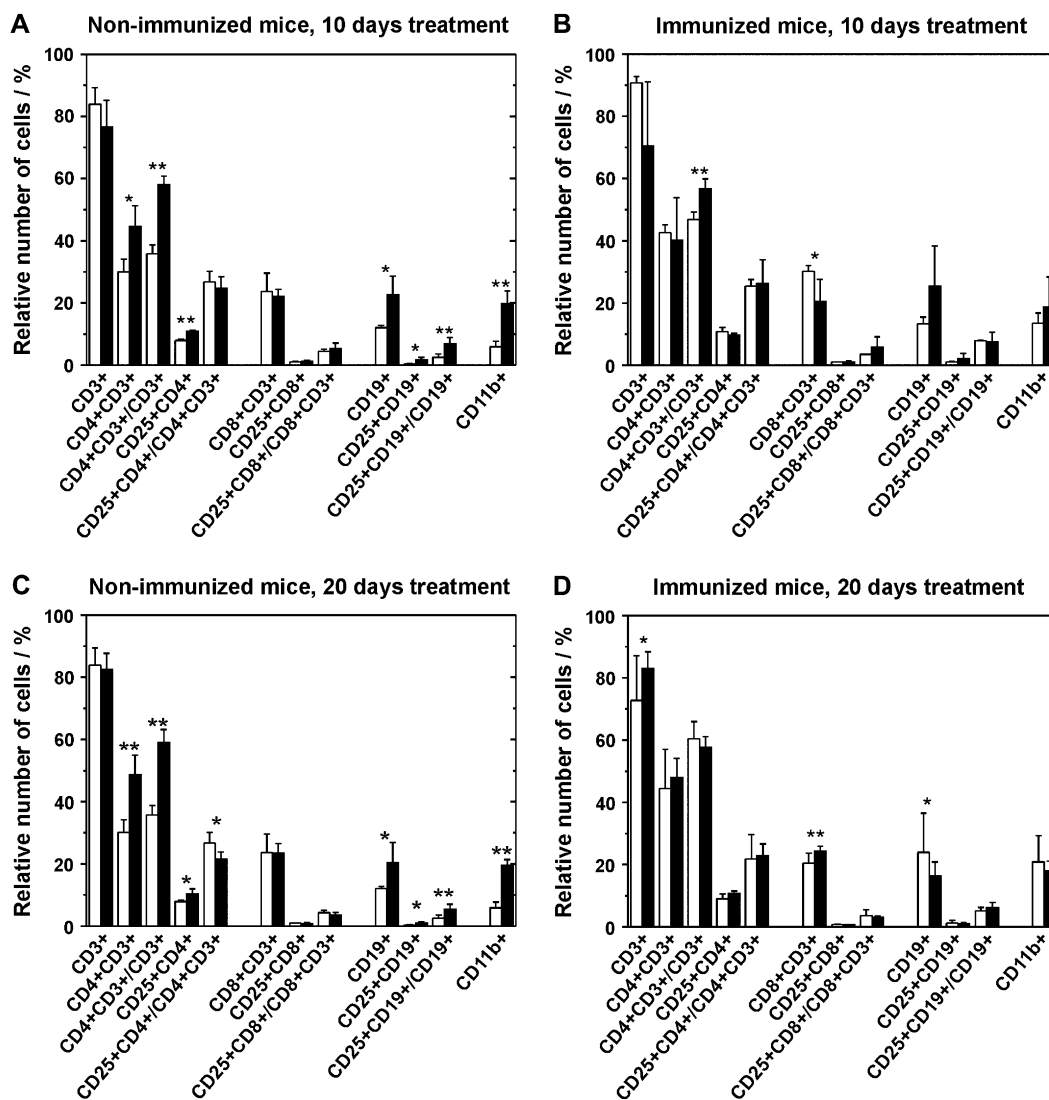


Fig. 3. Changes of leukocyte subpopulations in lymph nodes of EAE mice (B and D) and CFA-injected healthy control mice (A and C) with and without Atorvastatin treatment as detected by flow cytometry. Mice correspond to the experimental animal groups A–H described in Fig. 1 as follows: (A) black columns (group E, CFA-injected and 10 days Atorvastatin treated), white columns (group A, no immunization and no Atorvastatin treatment); (B) black columns (group G, 20 days PLP 139–151 immunized and 10 days Atorvastatin treated), white columns (group C, 20 days PLP 139–151 immunized only); (C) black columns (group F, CFA-injected and 20 days Atorvastatin treated), white columns (group A, no immunization and no Atorvastatin treatment); (D) black columns (group H, PLP 139–151 immunized and 20 days Atorvastatin treated), white columns (group D, 30 days PLP 139–151 immunized only). Columns and bars represent mean values \pm SD of groups of four animals per experiment. The Atorvastatin effect was tested for significance by Student's *t*-test of corresponding values of leukocyte subpopulations of animals with and without Atorvastatin treatment. *p*-Values are indicated by asterisks as follows: **p* < 0.05 and ***p* < 0.01.

The analysis of replicated samples of gene expression values showed a high similarity of arrays within all experimental groups (A and C–H) and both tissues (lymph nodes and spinal cord) with $r^2 \geq 0.85$. Thus, we confirmed homogeneous experimental material within groups (replicate samples) as a basis for the analysis of differential gene expression between groups.

The comparison of gene expression differences allows for detection of changes of gene expression in lymph nodes and spinal cord of EAE mice during the course of the disease with and without Atorvastatin treatment and in Atorvastatin-treated control mice. Fig. 5 demonstrates the total numbers of differentially regulated genes. The Atorvastatin-responsive genes have been attributed to functional groups with emphasis

on immune-related and CNS-related genes. The results of this detailed analysis as well as a complete list of all statin-dependently differentially expressed genes are provided by Tables S1–S6 as supplementary information (made available on-line at www.neurobiology.med.uni-rostock.de or at doi: 10.1016/j.jaut.2006.09.006). In Tables S5 and S6 additionally the fold changes of the respective comparisons between experimental animal groups (E and F versus A, G versus C and H versus D) are given. Concisely, one can summarize the major findings of microarray analysis as follows: (1) the highest numbers of differentially regulated genes were found in the spinal cord of mice in response to EAE induction (Fig. 5). However, Atorvastatin treatment influenced more genes in lymph nodes than in spinal cord. (2) The majority of regulated genes could

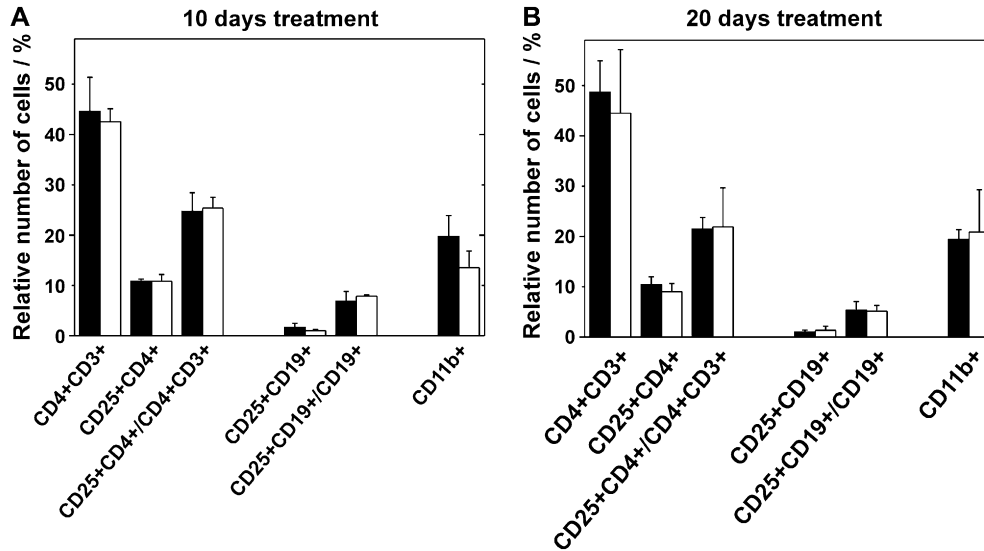


Fig. 4. EAE induction (without Atorvastatin treatment) has similar effects on proportions of total $CD4^+CD3^+$ and activated $CD25^+CD4^+$ T helper cells, $CD25^+CD19^+$ activated B cells and $CD11b^+$ macrophages as Atorvastatin treatment of healthy (only CFA-injected) control mice. Mice correspond to the experimental animal groups A–H described in Fig. 1 as follows: (A) black columns (group E, CFA-injected and 10 days Atorvastatin treated), white columns (group C, 20 days PLP 139–151 immunized only); (B) black columns (group F, CFA-injected and 20 days Atorvastatin treated), white columns (group D, 30 days PLP 139–151 immunized only). Columns and bars represent mean values \pm SD of groups of four animals per experiment as detected by flow cytometry.

be attributed to general cell functions such as regulation of cell cycle, gene transcription, protein processing and turnover, signal transduction, assembly of cytoskeleton, oxidative stress response and DNA repair (Tables S1–S4). (3) If more specifically immune-related genes and CNS-related genes are regarded, the most surprising finding was that CNS-related genes were expressed in lymph nodes and that many of them were up-regulated by Atorvastatin in both EAE and control mice (Tables S1–S4). (4) Fourteen genes have been found to be

selectively influenced by Atorvastatin, i.e. they were not affected by induction of EAE alone (Table 1). Five of them were only influenced in EAE mice and nine in both EAE and control mice. None of them was only affected in control mice.

With regard to their function, the 14 solely Atorvastatin-responsive genes belong to rather heterogeneous groups of molecules, which prevents their attribution to common pathways or mediators of the disease (see Table 1). Most of them

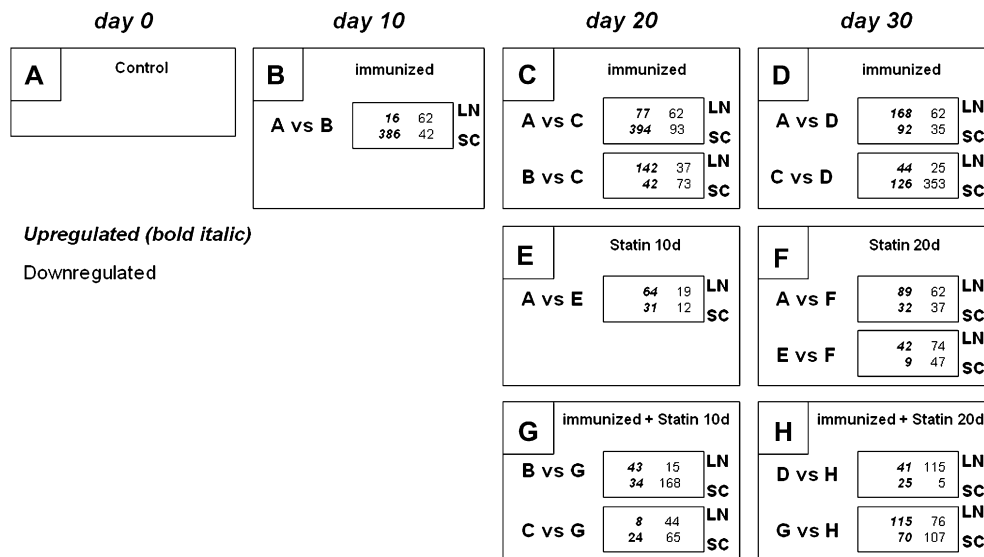


Fig. 5. Changes of gene expression detected by Affymetrix microarray assay in lymph nodes (LN, upper line values) and spinal cord (SC, lower line values) by comparing the experimental animal groups that are indicated according to the designation in Fig. 1. Numbers refer to genes that are more than twofold upregulated (bold italic) or downregulated (normal typing), when comparing the group at position two with the group at position one. For example, in (B) the comparison of group B (10 days after PLP 139–151 immunization) with group A (no immunization) revealed 16 upregulated and 62 downregulated genes in lymph nodes and 386 upregulated and 42 downregulated genes in spinal cord. A complete list of statin-dependently differentially regulated genes is available on-line as supplementary information (Table S5). The list comprises comparisons between groups E and F (Atorvastatin-treated control mice) versus group A (untreated control mice) and between groups G and H (Atorvastatin-treated EAE mice) and groups C and D (corresponding untreated EAE mice).

Table 1
Atorvastatin-responsive genes in EAE mice and control mice

Gene	Accession no.	Direction of change in EAE mice	Time of Atorvastatin treatment (days)	Tissue	Direction of change in control mice	Time of Atorvastatin treatment (days)	Tissue	Functional group
Thrombospondin-1 (TBS-1)	AI385532	Up Down	20 10	ln ^a ln	Up	10	ln	Adhesion molecules/ adhesion regulators
Connexin-43	NM_010288	Down	10	ln	Up	10	ln	
Galectin-3	X16834	Up Down	20 10	sc ^b sc	Down	20	sc	
Dectin-1	NM_020008	Up	20	sc				
Pentraxin-3 (PTX-3)	NM_008987	Up Down	20 10	ln, sc ln, sc	Up	10 20	ln ln	Cytokines/chemokines/ cytokine regulators
Suppressor of cell signaling-3 (SOCS-3)	NM_007707	Up Down	20 10	sc sc	Up	10 20	ln, sc ln	
Chemokine (CC motif) ligand-11 (CCL-11)	NM_011330	Up	20	ln	Up	10	ln	
B cell marker CD20	BE686578	Up	10	sc				B cell molecules
B cell receptor component CD79B	NM_008339	Up	10	sc				
Cyclooxygenase-1 (COX-1)	NM_008969	Down	10	ln				Metabolic enzymes
Carbonic anhydrase-3 (CAR-3)	AK003671	Up	10 20	sc sc	Up	20	sc	
Ca ⁺⁺ -binding protein S100-A4	D00208	Up	20	sc				Signaling molecules
Heat shock protein-105 (HSP-105)	BI499717	Up	20	ln	Up	20	ln	Stress molecules
Inhibin-B1	BB353211	Up Down	20 10 10	ln ln sc	Up	10	ln	Hormones

^a ln = lymph nodes.

^b sc = spinal cord.

belong to adhesion molecules or regulators of cell adhesion (4) and cytokines, chemokines or regulators of cytokine expression (3), while the remaining genes were scattered among B cell molecules (2), metabolic enzymes (2), signal molecules (1), stress molecules (1), and hormones (1). The majority of these genes (12) was upregulated in EAE mice, either in lymph nodes (thrombospondin-1 = TBS-1, chemokine ligand-11 = CCL-11, HSP-105, inhibin-B1, all upregulated after 20 days of Atorvastatin treatment) or in spinal cord (CD20, CD79B, carbonic anhydrase-3 = CAR-3, all upregulated after 10 days of Atorvastatin treatment; galectin-3, dectin-1, SOCS-3, S100-A4, CAR-3, all upregulated after 20 days of Atorvastatin treatment) or in both tissues (pentraxin-3 = PTX-3, not PiTX-3 biocid!, after 20 days of Atorvastatin treatment). Only connexin-43 and COX-1 were downregulated, i.e. in lymph nodes after 10 days of Atorvastatin treatment. In control mice, almost all (8/9) Atorvastatin-responsive genes were upregulated and mainly in lymph nodes (7/9).

3.5. Protein expression in lymph nodes and spinal cord

To compare the changes on transcription level observed by microarray technique with changes on protein level, the expression of four genes was investigated by quantitative Western blot (Odyssey system, LiCor technology) and

immunohistochemistry. Two of these genes (COX-1, HSP-105) have been found to be differentially regulated in microarray analysis and two genes (ICAM-1 and CD86) reported to be statin-responsive in the literature [41–46]. The gene expression of both molecules was not found to be differentially regulated in our study. However, Western blot and immunohistochemistry revealed changes of the expression of these molecules on the protein level. The genes have been selected on the basis of optimum technical results of preliminary experiments for protein detection in tissues of normal control animals (group A).

For illustration, Western blot detection of ICAM-1 is shown in lymph nodes of the EAE animals (Fig. 6). For quantitative Western blot analysis, the relative protein expression levels in lymph nodes and spinal cord of the animal groups with different types of treatment (groups B–H) were calculated with reference to the animal group of healthy control mice (group A = 100%) (Fig. 7). Generally, we found higher protein expression levels in lymph nodes than in spinal cord, whereby the differences were most pronounced for ICAM-1, smaller for CD86 and HSP-105 and not striking for COX-1 (data not shown). The highest relative changes of protein expression during the natural course of EAE as well as in response to Atorvastatin treatment were seen for HSP-105, which was strikingly upregulated by Atorvastatin treatment in lymph nodes and spinal cord of both

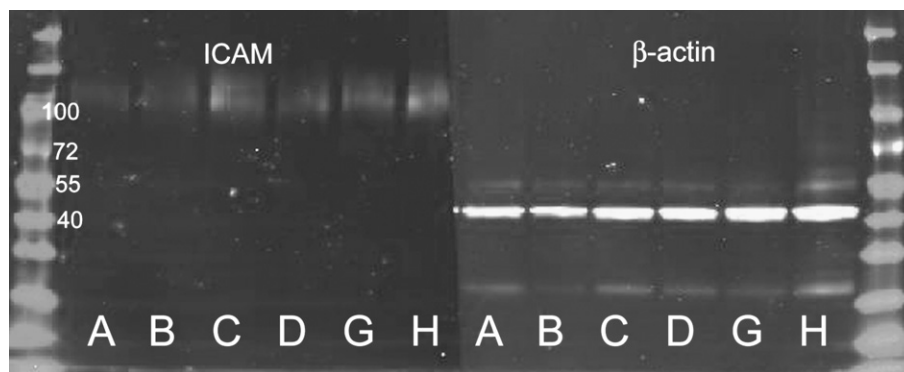


Fig. 6. Protein expression of the ICAM-1 gene in spinal cord of EAE mice during the course of disease with and without Atorvastatin treatment as detected by quantitative Western blot technique. Western blot was performed on spinal cord extracts of mice of the different experimental animal groups as designated in Fig. 1, i.e. group A (no immunization and no Atorvastatin treatment), group B (10 days after PLP 139–151 immunization, no Atorvastatin treatment), group C (20 days after PLP 139–151 immunization, no Atorvastatin treatment), group D (30 days after PLP 139–151 immunization, no Atorvastatin treatment), group G (20 days after PLP 139–151 immunization, 10 days of Atorvastatin treatment) and group H (30 days after PLP 139–151 immunization, 20 days of Atorvastatin treatment). Primary antibodies were directed against ICAM-1 and against the reference household protein β -actin. Near infrared emitting fluorescence-labeled antibodies served as secondary antibodies. Scanning of fluorescent bands and quantitative evaluation of fluorescence signals was performed by LiCor technology (Odyssey software). Results of fluorescence data of ICAM-1-labeled bands normalized to β -actin-labeled bands are the basis for the calculation of relative changes of ICAM-1 expression in groups B–H compared to group A given in Fig. 7. The molecular weight of the markers is given in kDa.

EAE and control mice (Fig. 7). This finding correlates to the finding on the transcriptional level only in lymph nodes (see Table 1). COX-1 expression was downregulated by Atorvastatin treatment in spinal cord of both EAE and control mice and in lymph nodes of EAE mice only. Only the latter finding correlates with the microarray results (see Table 1). Unfortunately, there was not enough lymph node material available from all animal groups to allow for Western blot analysis of all proteins. Therefore, the Western blot results of COX-1 from groups B, C and E are missing. Prominent findings of ICAM-1 and CD86 expression were (1) an Atorvastatin-related upregulation of both proteins in lymph nodes of control mice, i.e. after 20 days of Atorvastatin treatment (group F), and (2) an Atorvastatin-related downregulation of ICAM-1 in spinal cord of both EAE and control mice, i.e. after 10 days of Atorvastatin treatment (groups E and G).

At the cellular level, protein expression was assessed for the candidate genes of statin effects COX-1 and CD86 by immunohistochemistry. For this purpose, spinal cord sections were used. For illustration, an example of immunostaining is given for CD86 in Fig. 8. Relative expression levels of COX-1 and CD86 in the spinal cord of the eight animal groups were done by semi-quantitative analysis. The main results were (see Fig. 9): (1) an increase of the COX-1 expression by Atorvastatin treatment of control mice (group E) and (2) a downregulation of CD86 expression by Atorvastatin treatment of EAE mice (groups G and H) and by 20 days of Atorvastatin treatment of control mice (group F). The latter finding is in accordance with the Western blot results.

4. Discussion

In the present study, statin effects on PLP-EAE of SJL mice resembling the relapsing-remitting form of MS have been investigated with two major aims, (1) to define statin-dependent changes of leukocyte subpopulations in the peripheral immune system and (2) to disclose statin-induced changes of gene

expression in the immune and nervous system. Thereby, it was anticipated to eventually identify new predictors of statin-responsiveness in EAE and probably also MS.

Investigations were performed at onset of clinical symptoms (day 10 p.i.), at height of the disease (day 20 p.i.) and at first remission (day 30 p.i.). Atorvastatin was applied between days 10 and 30 p.i. to simulate the clinical situation. As control, healthy CFA-treated animals received Atorvastatin for 10 and 20 days, respectively. The peripheral immune response as measured by lymphocyte proliferation after mitogenic (ConA-induced) and antigen-specific (PLP peptide 139–151-induced) stimulation was not significantly different between Atorvastatin-treated and untreated EAE mice. However, the basal lymphocyte proliferation was decreasing over time both in EAE mice and in Atorvastatin-treated control mice. This finding raises the question whether the statin influences signaling pathways in lymphocytes that are concomitantly activated during EAE, but which may nevertheless counteract the disease process. The missing Atorvastatin effect on mitogenic and antigenic immune response at later stages of the disease is not contradictory to this hypothesis, since there was no pronounced specific immune response seen in lymph nodes of EAE mice at height of the disease and during the recovery phase. Deviating findings of Refs. [6] and [11] may be due to the following differences to our study: (1) the clinical signs of the disease were milder in our study, (2) we used male animals only, (3) we studied draining lymph node cells, but not splenocytes and T cell lines, (4) the basic proliferation of control lymphocytes from healthy animals was higher in our study than in the studies of [6] and [11]. We intended to approach the human situation of MS as close as possible by applying an initially mild disease and by analysing draining lymph nodes that are supposed to represent the major site of the primary autoimmune reaction to degenerated myelin cross-reacting antigens [47] and to release myelin-specific T cells to the CNS [48,49].

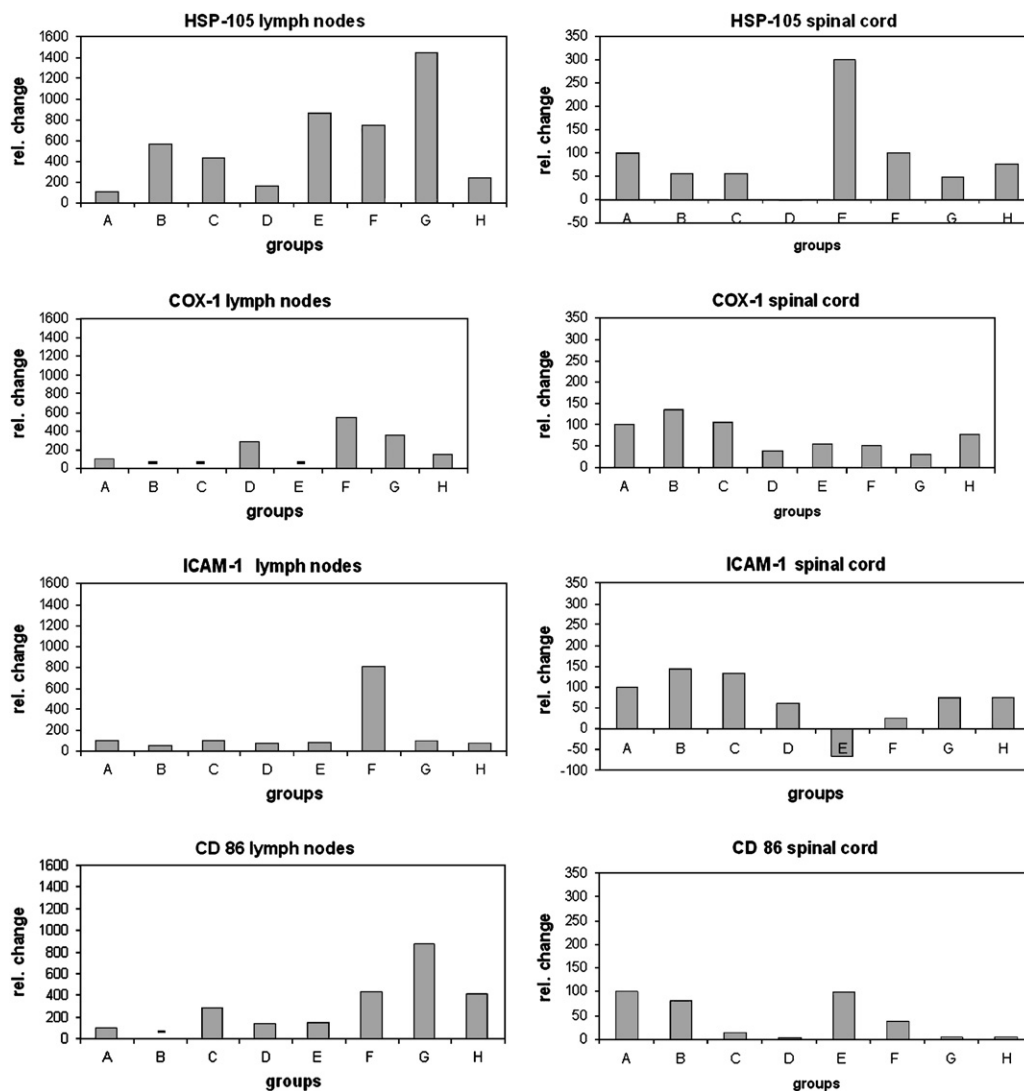


Fig. 7. Quantitative evaluation of protein expression of the genes for CD86, HSP-105, ICAM-1 and COX-1 in lymph nodes and spinal cord of EAE and control mice with and without Atorvastatin treatment by Western blot technique. Western blot was performed with fluorescence-labeled secondary antibodies and assessed by fluorescence scanner (Odyssey system, Licor) as illustrated for ICAM-1 in Fig. 6. The fluorescence signals of bands of the indicated proteins are normalized to fluorescence signals of parallel assessed β -actin bands by division of fluorescence signals of the candidate proteins of Atorvastatin effect through the fluorescence signals of the house-keeping protein β -actin. Relative changes in percent (rel. change) of normalized protein expressions refer to the comparison of groups B–H with group A, i.e. the reference group of healthy control animals, which has been set to 100%. The designation of the experimental animal groups corresponds to that described in Fig. 1. “-” indicates not done.

The anti-inflammatory statin effect may involve changes of the distribution of lymphocytes and macrophages within the lymphatic system and between the lymphatic and the nervous system. This is indicated by flow cytometric analysis of leukocyte subpopulations in the peripheral lymph nodes in our study, which argue for a strong effect of Atorvastatin on the distribution of functional subpopulations of T cells, B cells and macrophages. Interestingly, the effect of Atorvastatin treatment in non-immunized healthy animals resembled the effect of disease-inducing immunization with PLP peptide 139–151. Particularly, the proportion of T helper cells among total T cells, the proportion of activated among total B cells and the proportion of macrophages among all lymph node cells increased in both situations and the proportion of activated or regulatory T helper cells among total T helper cells decreased after

prolonged disease and after Atorvastatin treatment of healthy animals, respectively. These effects were partially reversed by Atorvastatin treatment in EAE mice. The mechanisms of action behind the changes of leukocyte subpopulations are presently not known, but influences on signaling pathways controlling the cytoskeleton, cell-to-cell interaction via adhesion molecules, cell migration and homing between the peripheral immune system and the CNS are feasible. Therefore, Atorvastatin-specific alterations of gene and protein expression in the EAE mice in comparison to healthy animals are of special interest.

A respective COX analysis was the second focus of our study. To approach this aim and to avoid a hypothesis-driven premise regarding metabolic and signal pathways as well as intercellular mediators and contact molecules that may be involved in statin

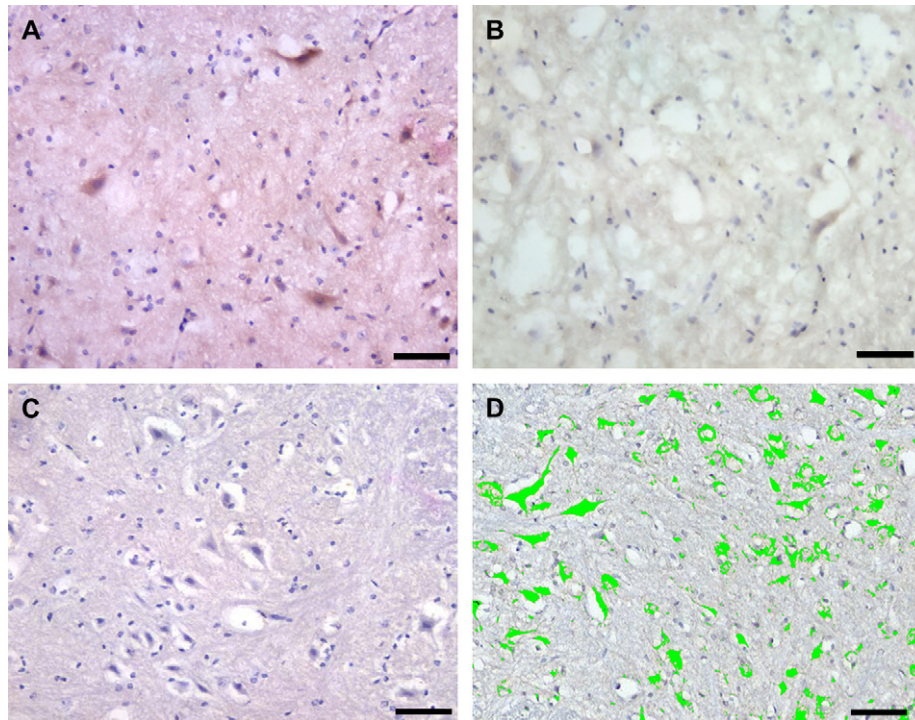


Fig. 8. Protein expression of the gene CD86 in spinal cord of EAE SJL mice without (A, B and D) and with 10 day Atorvastatin treatment (B) corresponding to the experimental animal groups C and G described in Fig. 1 as detected by immunohistochemistry. Primary antibodies were rat anti-mouse CD86 monoclonal antibodies. The antigen CD86 was visualized by biotinylated donkey anti-rat polyclonal antibodies and avidin–peroxidase complex. For negative control, the primary antibody was omitted (shown in C). The micrograph (D) illustrates the segmentation for quantitative evaluation of the histochemical staining of using KSRun software (Imaging System KS400, Zeiss). The demonstrated areas are localized in the anterior horn of the spinal cord. They illustrate the prevailing CD86 expression in immunized mice (shown in A and D), which is downregulated by Atorvastatin treatment (shown in B). Scale bar: 50 μ m.

effects, we applied the microarray technique for gene expression analysis. This method has been proven to be a useful tool to elucidate gene expression changes that differentiate between autoimmune diseases [50], tissue compartments [51], disease stages [20] and therapeutic interventions

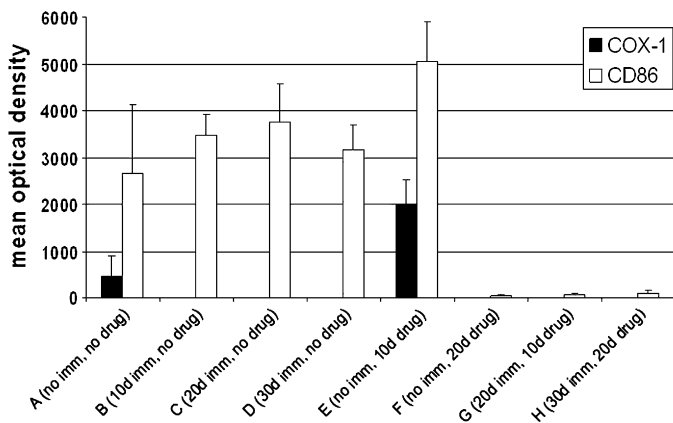


Fig. 9. Results of the quantitative evaluation of histochemical staining of spinal cord for COX-1 and CD86 using KSRun software (Imaging System KS400, Zeiss) as illustrated for CD86 in Fig. 8. The designation of the experimental animal groups corresponds to Fig. 1. Note that COX-1 is upregulated by 10 day Atorvastatin treatment in control mice (group E), whereas CD86 is downregulated by 10 and 20 days of Atorvastatin treatment in EAE mice (groups G and H), but only by 20 days of Atorvastatin treatment in control mice (group F).

[23,24,27,52]. As a major result Atorvastatin treatment influenced general cell functions such as regulation of cell cycle, cell–cell interaction, gene transcription, protein processing and turnover, signal transduction, assembly of cytoskeleton, oxidative stress response and DNA repair, and immune cell functions such as T cell, B cell and cytokine responses, rather than specific nerve cell functions (see Tables S1–S4). A somewhat surprising finding was the expression of CNS-related genes in lymph nodes. However, this might be explained by the fact that these genes belong to the neurotrophic factors and their receptors, which are actually pleiotropic in nature. The most striking differences in the expression of statin-responsive genes were found between EAE mice and healthy control mice rather than between different phases of the disease. However, only 14 genes have been found to be solely influenced by the Atorvastatin treatment (see Table 1). They belong to rather heterogeneous functional groups, whereby the majority of genes could be attributed to adhesion molecules and their receptors (TBS-1, connexin-43, galectin-3, dectin-1) and cytokines/chemokines and their regulators and receptors (PTX-3, SOCS-3, CCL-11). The remaining Atorvastatin-responsive genes represented B cell markers (CD20, CD79), metabolic enzymes (COX-1, CAR-3), signal molecules (S100), stress molecules (HSP-105) and hormones (inhibin-B1). Interestingly, 12 genes were upregulated by Atorvastatin in lymph nodes and/or spinal cord of EAE mice and only connexin-43 and COX-1 were downregulated. Also in control

mice, the majority of Atorvastatin-responsive genes (8/9) was upregulated. This result points to a general activating role of statins in EAE, which might affect cells and pathways that are involved in attenuation of the disease process and eventually even in restorative processes. In this context it is of interest that galectin-3, a promoter of neural cell adhesion and neurite growth, was downregulated by Atorvastatin in spinal cord of control mice and of EAE mice at height of the disease. However, it was upregulated in the recovery phase of the disease. Generally, it appears that Atorvastatin affects multiple pathways and mediators of EAE and that the outcome of its action depends on the activation state of target tissues as exemplified by the changes of galectin-3 expression in the spinal cord, which is not infiltrated by inflammatory cells in healthy control mice, but heavily infiltrated in EAE mice [22].

In addition to the microarray investigation, we liked to compare in selected cases the gene expression results with the expression of the gene products on protein level. Thereby, it turned out that the protein expression of the statin-responsive genes HSP-105 and COX-1 as assessed by quantitative Western blot and immunohistochemistry was only partially concordant with the microarray results. This was also true for ICAM-1 and CD86, which were additionally investigated because of their pathogenic role in autoimmune diseases [41–43] and their statin-induced downregulation reported in the literature [44–46], although they were not found to be downregulated by our microarray method. Reasons for these discordant results could be (1) changes of gene expression are not detected by our threshold of two-fold changes for the definition of significance (e.g. some authors [53,54] recently preferred a threshold value of 1.5 for the definition of significant changes of gene expression detected by microarray technique), (2) gene expression and protein expression follow different kinetics (e.g. the suppression of ICAM-1 and CD86 proteins in spinal cord of control mice is a result of earlier downregulation of their respective genes, which is not seen as late as 10 days p.i. in microarray analysis), (3) suppression of protein expression results from inhibition of posttranslational lipid modification (protein prenylation) of signaling proteins controlling the level of the respective proteins.

Our data on gene expression, protein expression, leukocyte subpopulation distribution and T-cell proliferation in PLP-EAE leads to the general conclusion that the statin effects strongly depend on the immunological status of the animals. Most interestingly, the statin effects on healthy animals seem to mimic at least partially the effect of disease-inducing immunization, especially with regard to the pattern of leukocyte subpopulations in the lymph nodes. This issue raises the question, whether at least during early phases of EAE (and perhaps of MS) signal pathways are activated, which counteract the promotion of the disease, but which do not overcome the proinflammatory, demyelinating and degenerative mechanisms during the natural course of the disease. Statins may serve to amplify anti-inflammatory and regenerative mechanisms by inhibiting RhoA-dependent signal pathways such as the RhoA/ROCK pathway responsible for cytoskeleton arrangement and

cell motility and the RhoA/FAK/AKT pathway responsible for cell proliferation [37]. This hypothesis will be tested by loss-of-function (knockdown) and gain-of-function (overexpression) experiments in the same system.

In summary, there are two most surprising results of our study: (1) similarity of Atorvastatin effects on leukocyte subpopulations with changes seen during the natural course of PLP-EAE, and (2) detection by microarray technique of new statin-sensitive genes, which do not belong to the genes published before in reports of Atorvastatin-treated PLP-EAE [6,11]. Since in our study the disease was milder and the investigations have been done on draining lymph nodes of the injection site and CNS, the results may reflect the conditions in human MS more closely and, therefore, the findings imperatively warrant respective investigations in MS. The clinical relevance of our results, especially the position of candidate genes in the chain of pathological events leading to a clinical endpoint, i.e. the correlation with disease activity, will be tested within the scope of clinical studies, i.e. phase I/II proof-of-principle trials [55].

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This work is dedicated to our good friend Lothar Gierl who left this world much too early.

Appendix A. Supplementary material

Supplementary material for this manuscript can be downloaded at doi:10.1016/j.jaut.2006.09.006.

References

- [1] Vancura A, Sessler A, Leichus B, Kuret J. A prenylation motif is required for plasma membrane localization and biochemical function of casein kinase I in budding yeast. *J Biol Chem* 1994;269:19271–8.
- [2] Laufs U, Endres M, Stagliano N, Amin-Hanjani S, Chui DS, Yang SX, et al. Neuroprotection mediated by changes in the endothelial actin cytoskeleton. *J Clin Invest* 2000;106:15–24.
- [3] Stanislaus R, Pahan K, Singh AK, Singh I. Amelioration of experimental allergic encephalomyelitis in Lewis rats by lovastatin. *Neurosci Lett* 1999;269:71–4.
- [4] Pahan K, Sheikh FG, Nambodiri AM, Singh I. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J Clin Invest* 1997;100:2671–9.
- [5] Neuhaus O, Strasser-Fuchs S, Fazekas F, Kieseier BC, Niederwieser G, Hartung HP, et al. Statins as immunomodulators: comparison with interferon-beta 1b in MS. *Neurology* 2002;59:990–7.

- [6] Aktas O, Waiczies S, Smorodchenko A, Dorr J, Seeger B, Prozorovski T, et al. Treatment of relapsing paralysis in experimental encephalomyelitis by targeting Th1 cells through Atorvastatin. *J Exp Med* 2003;197:725–33.
- [7] Waiczies S, Prozorovski T, Infante-Duarte C, Hahner A, Aktas O, Ullrich O, et al. Atorvastatin induces T cell anergy via phosphorylation of ERK1. *J Immunol* 2005;174:5630–5.
- [8] Weitz-Schmidt G, Welzenbach K, Dawson J, Kallen J. Improved lymphocyte function-associated antigen-1 (LFA-1) inhibition by statin derivatives: molecular basis determined by x-ray analysis and monitoring of LFA-1 conformational changes in vitro and ex vivo. *J Biol Chem* 2004;279:46764–71.
- [9] Prasad R, Giri S, Nath N, Singh I, Singh AK. 5-Aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside attenuates experimental autoimmune encephalomyelitis via modulation of endothelial–monocyte interaction. *J Neurosci Res* 2006;84:614–25.
- [10] Menge T, Hartung HP, Stuve O. Statins—a cure-all for the brain? *Nat Rev Neurosci* 2005;6:325–31.
- [11] Youssef S, Stuve O, Patarroyo JC, Ruiz PJ, Radosevich JL, Hur EM, et al. The HMG-CoA reductase inhibitor, Atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* 2002;420:78–84.
- [12] Simon B, Fava E, Pinon L, Nicotera P. Neuroprotective effects of statins in central neurons. *Restor Neurol Neurosci* 2002;20:292.
- [13] Sena A, Pedrosa R, Graca Morais M. Therapeutic potential of lovastatin in multiple sclerosis. *J Neurol* 2003;250:754–5.
- [14] Vollmer T, Key L, Durkalski V, Tyor W, Corboy J, Markovic-Plese S, et al. Oral simvastatin treatment in relapsing-remitting multiple sclerosis. *Lancet* 2004;363:1607–8.
- [15] Whitham RH, Bourdette DN, Hashim GA, Herndon RM, Ilg RC, Vandenbark AA, et al. Lymphocytes from SJL/J mice immunized with spinal cord respond selectively to a peptide of proteolipid protein and transfer relapsing demyelinating experimental autoimmune encephalomyelitis. *J Immunol* 1991;146:101–7.
- [16] Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* 2001;292:1160–4.
- [17] Nath N, Giri S, Prasad R, Singh AK, Singh I. Potential targets of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor for multiple sclerosis therapy. *J Immunol* 2004;172:1273–86.
- [18] Dunn SE, Youssef S, Goldstein MJ, Prod'homme T, Weber MS, Zamvil SS, et al. Isoprenoids determine Th1/Th2 fate in pathogenic T cells, providing a mechanism of modulation of autoimmunity by Atorvastatin. *J Exp Med* 2006;203:401–12.
- [19] Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* 2002;8:500–8.
- [20] Baranzini SE, Bernard CC, Oksenberg JR. Modular transcriptional activity characterizes the initiation and progression of autoimmune encephalomyelitis. *J Immunol* 2005;174:7412–22.
- [21] Mix E, Ibrahim S, Pahnke J, Koczan D, Sina C, Bottcher T, et al. Gene-expression profiling of the early stages of MOG-induced EAE proves EAE-resistance as an active process. *J Neuroimmunol* 2004;151:158–70.
- [22] Mix E, Pahnke J, Ibrahim SM. Gene-expression profiling of experimental autoimmune encephalomyelitis. *Neurochem Res* 2002;27:1157–63.
- [23] Wandinger KP, Sturzebecher CS, Bielekova B, Detore G, Rosenwald A, Staudt LM, et al. Complex immunomodulatory effects of interferon-beta in multiple sclerosis include the upregulation of T helper 1-associated marker genes. *Ann Neurol* 2001;50:349–57.
- [24] Sturzebecher S, Wandinger KP, Rosenwald A, Sathyamoorthy M, Tzou A, Mattar P, et al. Expression profiling identifies responder and non-responder phenotypes to interferon-beta in multiple sclerosis. *Brain* 2003;126:1419–29.
- [25] Matejuk A, Dwyer J, Zamora A, Vandenbark AA, Offner H. Evaluation of the effects of 17beta-estradiol (17beta-e2) on gene expression in experimental autoimmune encephalomyelitis using DNA microarray. *Endocrinology* 2002;143:313–9.
- [26] Pedotti R, DeVoss JJ, Youssef S, Mitchell D, Wedemeyer J, Madanat R, et al. Multiple elements of the allergic arm of the immune response modulate autoimmune demyelination. *Proc Natl Acad Sci U S A* 2003;100:1867–72.
- [27] Paintlia AS, Paintlia MK, Singh AK, Stanislaus R, Gilg AG, Barbosa E, et al. Regulation of gene expression associated with acute experimental autoimmune encephalomyelitis by Lovastatin. *J Neurosci Res* 2004;77:63–81.
- [28] Tuohy VK, Lu Z, Sobel RA, Laursen RA, Lees MB. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J Immunol* 1989;142:1523–7.
- [29] Bottcher T, Mix E, Koczan D, Bauer P, Pahnke J, Peters S, et al. Gene expression profiling of ciliary neurotrophic factor-overexpressing rat striatal progenitor cells (ST14A) indicates improved stress response during the early stage of differentiation. *J Neurosci Res* 2003;73:42–53.
- [30] Pahnke J, Mix E, Knoblich R, Muller J, Zschiesche M, Schubert B, et al. Overexpression of glial cell line-derived neurotrophic factor induces genes regulating migration and differentiation of neuronal progenitor cells. *Exp Cell Res* 2004;297:484–94.
- [31] Glass A, Gierl L. A system architecture for genomic data analysis. *In Silico Biol* 2002;2:207–11.
- [32] Bansemer S, Scheel T, Glass A, Koaropka T. gEnOM—software. *Proc Eur Conf Comput Biol* 2003;2:397–8.
- [33] Ibrahim SM, Mix E, Bottcher T, Koczan D, Gold R, Rolfs A, et al. Gene expression profiling of the nervous system in murine experimental autoimmune encephalomyelitis. *Brain* 2001;124:1927–38.
- [34] Whitney LW, Ludwin SK, McFarland HF, Biddison WE. Microarray analysis of gene expression in multiple sclerosis and EAE identifies 5-lipoxygenase as a component of inflammatory lesions. *J Neuroimmunol* 2001;121:40–8.
- [35] Mycko MP, Papoian R, Boschert U, Raine CS, Selmaj KW. cDNA microarray analysis in multiple sclerosis lesions: detection of genes associated with disease activity. *Brain* 2003;126:1048–57.
- [36] Nicot A, Ratnakar PV, Ron Y, Chen CC, Elkabes S. Regulation of gene expression in experimental autoimmune encephalomyelitis indicates early neuronal dysfunction. *Brain* 2003;126:398–412.
- [37] Denoyelle C, Albanese P, Uzan G, Hong L, Vannier JP, Soria J, et al. Molecular mechanism of the anti-cancer activity of cerivastatin, an inhibitor of HMG-CoA reductase, on aggressive human breast cancer cells. *Cell Signal* 2003;15:327–38.
- [38] Spaeth N, Wyss MT, Pahnke J, Biollaz G, Trachsel E, Drandarov K, et al. Radioimmunotherapy targeting the extra domain B of fibronectin in C6 rat gliomas: a preliminary study about the therapeutic efficacy of iodine-131-labeled SIP(L19). *Nucl Med Biol* 2006;33:661–6.
- [39] Vogler S, Pahnke J, Rousset S, Ricquier D, Moch H, Miroux B, et al. Uncoupling protein 2 has protective function during experimental autoimmune encephalomyelitis. *Am J Pathol* 2006;168:1570–5.
- [40] Vogelgesang S, Warzok RW, Cascorbi I, Kunert-Keil C, Schroeder E, Kroemer HK, et al. The role of P-glycoprotein in cerebral amyloid angiopathy; implications for the early pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* 2004;1:121–5.
- [41] Stuart RW, Racke MK. Targeting T cell costimulation in autoimmune disease. *Expert Opin Ther Targets* 2002;6:275–89.
- [42] Dedrick RL, Bodary S, Garovoy MR. Adhesion molecules as therapeutic targets for autoimmune diseases and transplant rejection. *Expert Opin Biol Ther* 2003;3:85–95.
- [43] Ledebuer A, Wierinckx A, Bol JG, Floris S, Renardel de Lavalette C, De Vries HE, et al. Regional and temporal expression patterns of interleukin-10, interleukin-10 receptor and adhesion molecules in the rat spinal cord during chronic relapsing EAE. *J Neuroimmunol* 2003;136:94–103.
- [44] Okouchi M, Okayama N, Omi H, Imaeda K, Shimizu M, Fukutomi T, et al. Cerivastatin ameliorates high insulin-enhanced neutrophil–endothelial cell adhesion and endothelial intercellular adhesion molecule-1 expression by inhibiting mitogen-activated protein kinase activation. *J Diabetes Complications* 2003;17:380–6.
- [45] Giguere JF, Tremblay MJ. Statin compounds reduce human immunodeficiency virus type 1 replication by preventing the interaction between virion-associated host intercellular adhesion molecule 1 and its natural cell surface ligand LFA-1. *J Virol* 2004;78:12062–5.

- [46] Yilmaz A, Reiss C, Tantawi O, Weng A, Stumpf C, Raaz D, et al. HMG-CoA reductase inhibitors suppress maturation of human dendritic cells: new implications for atherosclerosis. *Atherosclerosis* 2004;172:85–93.
- [47] Gran B, Hemmer B, Vergelli M, McFarland HF, Martin R. Molecular mimicry and multiple sclerosis: degenerate T-cell recognition and the induction of autoimmunity. *Ann Neurol* 1999;45:559–67.
- [48] Knopf PM, Cserr HF, Nolan SC, Wu TY, Harling-Berg CJ. Physiology and immunology of lymphatic drainage of interstitial and cerebrospinal fluid from the brain. *Neuropathol Appl Neurobiol* 1995;21:175–80.
- [49] Mohindru M, Kang B, Kim BS. Functional maturation of proteolipid protein(139–151)-specific Th1 cells in the central nervous system in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2004;155:127–35.
- [50] Mandel M, Gurevich M, Pauzner R, Kaminski N, Achiron A. Autoimmunity gene expression portrait: specific signature that intersects or differentiates between multiple sclerosis and systemic lupus erythematosus. *Clin Exp Immunol* 2004;138:164–70.
- [51] Lindberg RL, De Groot CJ, Certa U, Ravid R, Hoffmann F, Kappos L, et al. Multiple sclerosis as a generalized CNS disease—comparative microarray analysis of normal appearing white matter and lesions in secondary progressive MS. *J Neuroimmunol* 2004;152:154–67.
- [52] Neuhaus O, Farina C, Wekerle H, Hohlfeld R. Mechanisms of action of glatiramer acetate in multiple sclerosis. *Neurology* 2001;56:702–8.
- [53] Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, et al. Gene regulation and DNA damage in the ageing human brain. *Nature* 2004;429:883–91.
- [54] Hong J, Zang YC, Hutton G, Rivera VM, Zhang JZ. Gene expression profiling of relevant biomarkers for treatment evaluation in multiple sclerosis. *J Neuroimmunol* 2004;152:126–39.
- [55] Bielekova B, Martin R. Development of biomarkers in multiple sclerosis. *Brain* 2004;127:1463–78.