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ORIGINAL ARTICLE

Immunomodulatory Effects of IFN-β and Lovastatin on Immunophenotype of Monocyte-Derived Dendritic Cells in Multiple Sclerosis

Halina Bartosik-Psujek · Jacek Tabarkiewicz · Krystyna Pocinska · Sebastian Radej · Zbigniew Stelmasiak · Jacek Rolinski

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Abstract Multiple sclerosis (MS) is an autoimmune disease of the central nervous system and current MS treatment is only partially effective. Recent data suggest that statins may be potent immunomodulatory agents. In order to evaluate their role in MS, we analyzed the in vitro effects of interferon (IFN)-β and lovastatin on the differentiation and maturation of monocyte-derived dendritic cells (DCs) of MS patients. Twenty-seven patients with relapsing–remitting MS were recruited for the study. DC differentiation and maturation were evaluated based on surface phenotypic changes and the expressions of CD14, CD83, CD1a, CD80, CD86, CD206, and C209 were analyzed by flow cytometry. The results showed that IFN-β and lovastatin affect DC phenotype. Both agents decrease the expression of CD1a, which indicates a weakened presentation of glycolipid antigens. IFN-β causes up-regulated and lovastatin down-regulated expression of CD86, which results in a biased Th-cell responses in MS. Furthermore, high doses of lovastatin cause a decrease in CD209 expression on the surface of DCs and can limit their migration to various tissues. One of the mechanisms of the beneficial action of IFN-β and statins may be associated with their influence on DCs.

Keywords Dendritic cells · Interferon beta · Lovastatin · Multiple sclerosis

H. Bartosik-Psujek (✉) · K. Pocinska · Z. Stelmasiak
Department of Neurology, Medical University of Lublin, Jaczewskiego 8, 20-090 Lublin, Poland
e-mail: bartosikpsujek@op.pl

J. Tabarkiewicz · S. Radej · J. Rolinski
Department of Clinical Immunology, Medical University of Lublin, Lublin, Poland

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play a major role in the immune system. The ability of DCs to regulate immunity is dependent on their maturation. Several factors trigger their maturation: whole bacteria or bacteria-derived antigens, viruses, and inflammatory cytokines. DC maturation involves the down-regulation of antigen internalization, redistribution of major histocompatibility complex (MHC) molecules from the intracellular space to the DC surface, an increase in the surface expression of co-stimulatory molecules, and the secretion of cytokines along with chemokines and chemokine receptors. Mature DCs lose the ability to take up antigens, but they upregulate surface MHC II and co-stimulatory molecules, becoming the most potent APCs in the body. Terminally differentiated, mature DCs can efficiently induce T-cell activation as well as proliferation and are able to initiate an antigen-specific immune response, whereas immature DCs may be involved in the maintenance of peripheral tolerance (Cella et al. 1997).

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by an inflammatory reaction, demyelination, and axonal loss, which lead to CNS damage. MS is thought to be mediated by autoreactive T helper (Th1) cells and DCs, which are required for primary T-cell activation as well as T cell-dependent immunity and are known to play an important role in the initiation and maintenance of CNS inflammation in MS (Hohlfeld et al. 1995; Serafini et al. 2006). Different classes of immunomodulatory agents (e.g. interferon (IFN)-β, glatiramer acetate) are approved for MS treatment. Although the mechanisms of action of these agents are not completely understood, they have pleiotropic effects on various immune system components, which

contributes to treatment efficacy. Immunomodulatory therapy also influences DCs, as shown both in vitro and in vivo (Huang et al. 2005; Then Bergh et al. 2004; Vieira et al. 2003; Zang et al. 2004). Current MS treatment is only partially effective, however, and it is important to identify existing or novel classes of drugs that may benefit MS therapy. Recent studies have demonstrated that oral cholesterol-lowering HMG-CoA reductase inhibitors (known as statins) have immunomodulatory properties (Ghittoni et al. 2006; Markovic-Plese et al. 2008). In experimental autoimmune encephalomyelitis (EAE), a model of MS, and in vitro, statins have been shown to promote the differentiation and expansion of myelin protein-reactive regulatory Th2 cells and to suppress the upregulation of MHC class II and co-stimulatory molecules on APCs (Neuhaus et al. 2002; Youssef et al. 2002).

Numerous studies have assessed various aspects of MS therapy, but only a few have evaluated the effects of immunomodulators on DCs. Moreover, there are no reports regarding the influence of statins on DCs in MS patients. Therefore, in order to explore the potential immunomodulatory activity of IFN-β and HMG-CoA reductase inhibitors on DCs, we analyzed their effects on the differentiation and maturation of monocyte-derived DCs from MS patients. For this purpose we incubated DCs with IFN-β and lovastatin and subsequently analyzed the expressions of maturation markers as well as antigen-presenting molecules. Lovastatin was chosen because it readily crosses the blood–brain barrier (Johnson-Anuna et al. 2005) and its activity was confirmed in EAE (Nath et al. 2004).

Materials and methods

Patients

Twenty-seven patients (19 female and 8 male) with relapsing–remitting MS according to the McDonald criteria (McDonald et al. 2001) were recruited for the study. The mean age of the patients was 29.9 ± 6.6 (range 19–46) years, with a mean expanded disability status scale score of 2.7 ± 0.8 (range 1–4.0) and a mean duration of MS of 3.5 ± 1.45 (range 1–7) years. All patients were in the stable stage of the disease; they had no relapses or steroid therapy in the 3 months preceding the study. None of the MS patients had ever received immunosuppressive or immunomodulatory therapy and no coincidental infections were reported at the time of sample collection or within the month preceding the study. Moreover, we excluded patients with other inflammatory or infectious diseases, any severe general medical conditions, or abnormal results of routine laboratory tests (e.g. blood chemistry, complete blood count, CRP, thyroid function tests, urinalysis). Thirteen age- and sex-matched healthy individuals served as controls. The study was approved by the Local Ethics Committee and all subjects gave informed consent.

In Vitro Generation of Monocyte-Derived DCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood samples by density gradient centrifugation (Gradisol-L, Poland). After isolation, the cells were washed twice in PBS without Ca^{2+} or Mg^{2+} (PAA, Austria). To enrich the monocyte population, the resulting cells were resuspended in RPMI 1640 culture medium (PAA, Pasching, Austria) supplemented with 2% human albumin (ZLB Bioplasma AG, Switzerland), 100 IU/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin (Sigma–Aldrich, Germany) and allowed to adhere in culture dishes (BD Falcon, USA) at 37°C in humidified 5% CO_2 for 90 min. Nonadherent cells were washed out with PBS w/o Ca^{2+} or Mg^{2+}. The adherent cells were cultured in the appropriate culture medium. For the induction of DCs, rhIL-4 (500 U/ml, Miltenyi Biotec GmbH, Germany) and rhGM-CSF (1,000 U/ml, Leukine Bayer HealthCare Pharmaceuticals, USA) were added at day 0 and refreshed on day 2, and recombinant human tumor necrosis factor (TNF)-α (50 ng/ml, Miltenyi Biotec GmbH, Germany) was added to each culture well on day 5 to induce DC maturation. To evaluate immunomodulatory effects, the following mediators were added simultaneously with TNF-α to the experimental wells: 1,000 U/ml of IFN-β1a (Avonex, Biogen Idec) and two different doses of lovastatin at a concentration of 1 or 10 μM (Calbiochem, USA). On day 7, DCs were harvested using Trypsin/EDTA solution (Biochrome, Germany) and washed twice in PBS w/o Ca^{2+} or Mg^{2+}. Viability was checked by Trypan Blue (Sigma–Aldrich, Germany) staining with light microscopy.

Flow Cytometry Analysis

The generated DCs were washed twice with PBS w/o Ca^{2+} or Mg^{2+} and stained with mouse anti-human monoclonal antibodies. We used FITC-conjugated antibodies against CD83, CD80, and CD209, PE-conjugated antibodies against CD1a, CD86, and CD206 (all from BD Pharmingen, USA), and the BD Simultest CD45-FITC/CD14-PE (Becton–Dickinson, USA). We checked the co-expression of the DCs markers in the following combinations: CD45/CD14, CD83/CD1A/HLA-DR, CD80/CD86/HLA-DR, and CD209/CD206/HLA-DR. Cells were incubated with mo-Abs at 4°C for 30 min. After immunofluorescent staining, the cells were analyzed by a FACSCalibur using CellQuest software (Becton–Dickinson, USA). Appropriate isotype-matched immunoglobulins (BD Pharmingen, USA) were used as negative controls. Dead cells were gated out

according to propidium iodide staining. Monocyte-derived DCs were gated according to morphology on the FSC/SSC dot-plot and the expression of CD45 antigen. Mean fluorescence intensity (MFI) was calculated for the cells of monocytoid morphology in the experimental gate.

Statistical Analysis

Statistical analyses were performed using the nonparametric Mann–Whitney *U* test and the Wilcoxon test. The *p* value was calculated with the Benjamini and Hocheberg correction and was considered statistically significant when it was <0.05.

Results

DCs Exposed to TNF-α

Under all culture conditions we found a lack of completely immature $CD83^{-}/CD1a^{+}$ DCs; all cells positive for CD1a co-expressed the maturation marker CD83 and a high expression of costimulatory molecules and HLA-DR (data not shown), so we considered them mature DCs. On day 7, most monocytes differentiated into DCs in both MS and healthy control cultures (97 vs. 96%, respectively), and similar percentages (Table 1) and MFIs (8.4 ± 13.9 vs. 7.9 ± 7.0) of CD14 were found in the cultures from MS patients and healthy controls. DCs from MS patients had significantly higher CD1a expression compared with the healthy subjects, expressed as the percentage of positive cells (Table 1) and as the MFI (307.6 vs. 85.1, $p < 0.05$). In similar manner, the MFIs of CD86 and CD209 were found to be significantly different in cultures from MS patients and controls. On the other hand, the expressions of CD83, CD80, and CD206 were similar and did not differ between the groups. These findings demonstrate differences in the expression of DC surface molecules between MS patients and healthy individuals. After TNF stimulation, DCs from MS patients displayed a mature phenotype like the DCs from healthy subjects (similar expressions of CD83 and more than 70% of cells were $CD1a^{+}CD83^{+}$). However, DCs of MS patients displayed higher potency to present glycolipid antigens as well as cell migration. The expressions of surface antigens on the DCs from the MS patients and healthy volunteers are presented in Fig. 1 and Table 1.

DCs Exposed to IFN-β

The addition of IFN-β changed the DC phenotype. Compared with the culture with TNF-α alone, the expression of CD1a was found to be significantly lower (Table 1; Fig. 1)

Table 1 Analysis of the immunophenotype of monocyte-derived DCs shown as the percentage of positive cells

	TNF		TNF + IFN		TNF + LOV1		TNF + LOV10	
	Control	MS	Control	MS	Control	MS	Control	MS
CD14	1.7 ± 1.6 (1.2)	2.1 ± 2.4 (1.4)	2.0 ± 2.6 (1.0)	2.1 ± 2.9 (1.3)	4.0 ± 2.9 (4.4)	3.32 ± 2.3 (2.5)	3.0 ± 2.9 (2.4)	3.72 ± 4.3 (2.5)
CD83	92.7 ± 9.4 (99.4)	99.0 ± 1.2 (99.5)	99.4 ± 4.0 (99.7)	97.0 ± 5.0 (99.4)	98.2 ± 3.8 (96.90	97.7 ± 3.8 (98.0)	98.2 ± 11.8 (95.9)	96.7 ± 5.8 (99.0)
CD1a	17.0 ± 13.7 (13.1)	79.6 ± 17.2# (82.8)	15.1 ± 12.9 (12.4)	54.0 ± 22.3*# (60.2)	28.2 ± 14.8 (24.7)	60.6 ± 15.2 (69.9)	19.9 ± 14.8 (17.7)	70.6 ± 19.2# (69.9)
CD206	30.4 ± 8.3 (28.8)	39.2 ± 19.7 (37.8)	35.0 ± 13.1 (37.0)	34.9 ± 21.9 (33.3)	42.8 ± 17.7 (39.9)	35.5 ± 15.8 (35.2)	41.8 ± 19.7 (39.2)	34.5 ± 19.8 (35.2)
CD80	96.2 ± 3.9 (97.3)	98.6 ± 1.7 (99.1)	96.3 ± 5.5 (99.4)	97.4 ± 5.7 (99.3)	93.5 ± 5.7 (96.5)	90.4 ± 8.0 (96.0)	94.6 ± 4.7 (95.9)	97.4 ± 4.0 (99.0)
CD86	80.9 ± 15.3 (85.4)	85.5 ± 13.2 (87.9)	96.3 ± 4.4 (99.8)	98.3 ± 3.6* (98.0)	84.3 ± 15.9 (85.2)	70.2 ± 10.6*# (71.7)	81.3 ± 17.9 (88.2)	82.3 ± 7.5 (85.5)
CD209	95.5 ± 6.6 (98.8)	98.3 ± 2.6 (99.2)	93.3 ± 9.5 (98.8)	98.9 ± 1.9 (99.3)	93.7 ± 6.1 (93.2)	94.7 ± 4.9 (95.7)	93.8 ± 4.5 (93.2)	94.5 ± 2.9 (93.7)

Results are expressed as the mean ± SD, with the median in parenthesis

* Indicates a significant difference versus TNF-treated culture and $*p < 0.05$

# Indicates a significant difference versus the control and $\#p < 0.05$

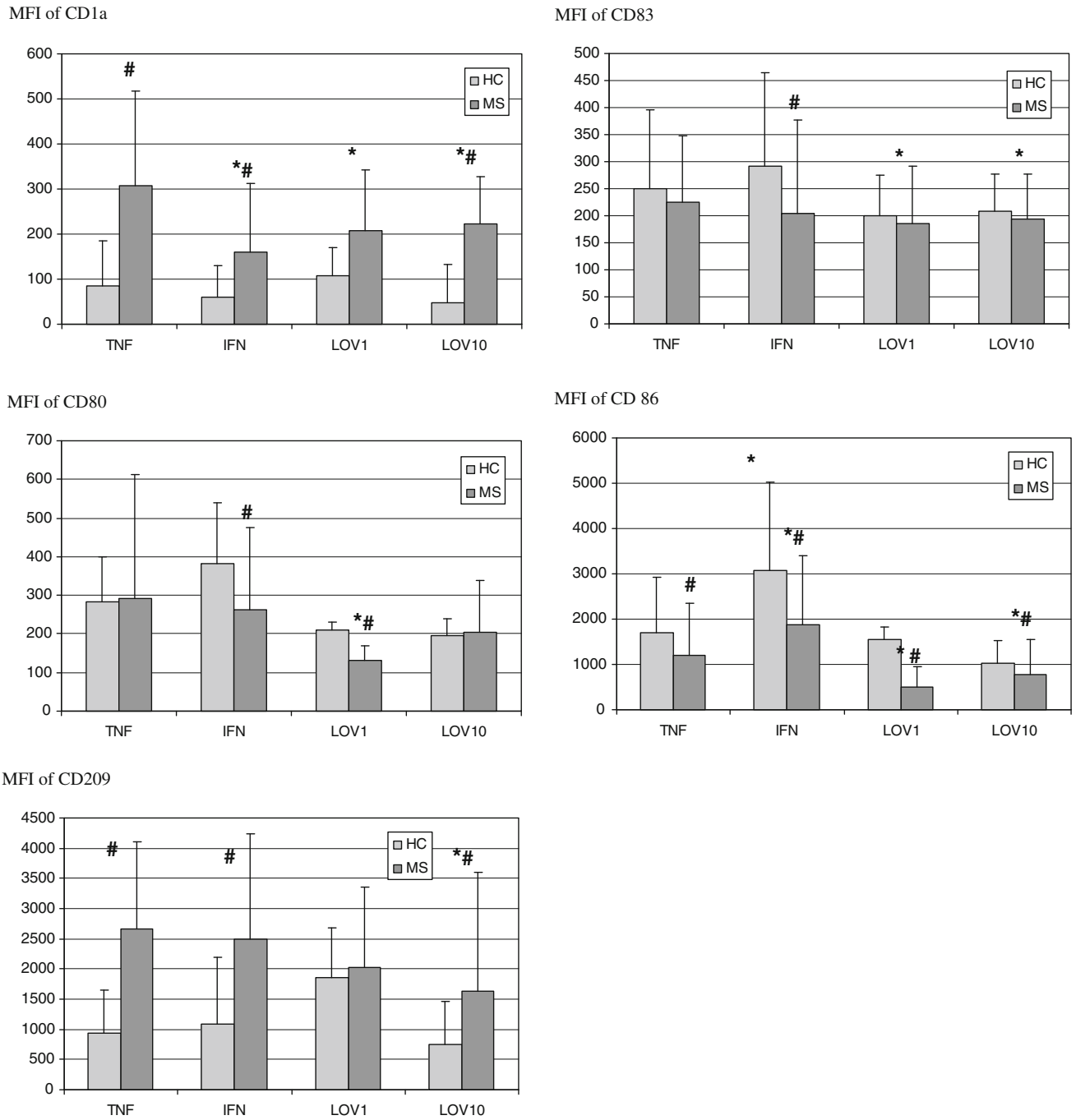

Fig. 1 Analysis of the immunophenotype of monocyte-derived dendritic cells of MS patients (MS) and healthy volunteers (HC) displayed as the mean fluorescence intensity (MFI) indicating the density of expression of the molecules per cell. Results are expressed as the mean ± SD. *Indicates a significant difference versus TNF-α alone-treated culture (Wilcoxon test, $p < 0.05$). #Indicates a significant difference versus the healthy controls (Mann–Whitney test, $p < 0.05$)

in the cultures from MS patients. Moreover, compared with cultures from healthy volunteers, DCs from MS patients exposed to IFN-β demonstrated a significantly higher expression of CD1a, expressed both as MFI and as the percentage of positive cells, and a lower expression of CD83 expressed as the MFI. The expression of CD86 on DCs from MS patients was found to be significantly higher than in the culture with TNF-α alone. Moreover, the MFI of CD86 was higher than in the lovastatin cultures. No statistically significant differences in the expression of CD206 were found following the administration of IFN compared with TNF-α alone using the MFI (MS 216.8 ± 155.1 vs. 266.9 ± 237.5, control 165.4 ± 162.6 vs. 200.8 ± 188.9) and the percentage of positive cells.

The expression of surface antigens on DCs from the MS patients and healthy volunteers are presented in Fig. 1 and Table 1. Our findings reveal that IFN-β has diverse effects on DCs. It down-regulates the expression of CD1a in both groups; however, this is more pronounced in MS patients than in healthy individuals. Furthermore, the effects of IFN-β on co-stimulatory molecules varies. It inhibits up-regulation of CD80 and stimulates CD86 in MS patients, which induces a Th2 response.

DCs Exposed to Statins

To investigate the effects of statins on the maturation of DCs, two different concentrations of lovastatin (1 and 10 μM) were added to DC cultures from both MS patients and healthy subjects. No statistically significant differences in CD14 expression using MFI were found following the administration of the low and high concentrations of lovastatin compared with the culture with TNF-α alone (8.8 ± 9.1 vs. 8.4 ± 13.9, and 8.5 ± 10.8 vs. 8.4 ± 13.9, respectively, $p > 0.05$). Compared with the culture with TNF-α alone, however, the expressions of CD1a and CD83 decreased after the low and high doses of lovastatin in the MS patients. Moreover, the expression of CD1a in cultures from MS patients and healthy controls were significantly different following the high dose of lovastatin according to both MFI and the percentage of positive cells. Incubation of DCs from MS patients with lovastatin resulted in a significant decrease in the MFIs of CD80 and CD86 compared with the culture with TNF-α alone and the expression of CD86 in the cultures from MS patients was significantly lower than in those from the healthy controls. A dose-dependent decrease in the MFI of CD209 was also seen following the higher dose of lovastatin, which was statistically significant in the cultures from the MS patients (1,612.7 vs. 2,659.7, $p < 0.05$) and healthy controls (707.6 vs. 942.7, $p < 0.05$) compared with the culture with TNF-α alone. The expression of CD206 was not significantly different from the control cultures or between low and high concentrations of lovastatin (MFI of CD206: 231.7 ± 308.1 and 254.3 ± 339.9, respectively). Our observations indicate that lovastatin inhibits DC maturation independent of dose, significantly decreases the expression of CD1a, and inhibits the up-regulation of CD86 in cultures from MS patients. Additionally, high doses of statins are likely to limit the migration properties of DCs.

Discussion

Human peripheral blood monocytes can be differentiated into immature DCs by culturing with GM-CSF and interleukin (IL)-4. During this process, they down-regulate the monocyte marker CD14 and express the DC marker CD1a. Immature DCs can be induced to mature by incubation with TNF-α. DC maturation is accompanied by a slight down-regulation of CD1a, decreased expression of the mannose receptor CD206, induction of the maturation marker CD83, and upregulation of HLA-DR, CD40, CD80, and CD86 (Cella et al. 1997). The addition of substances with immunomodulatory properties to the culture is likely to substantially change both the differentiation of monocytes and the maturation of monocyte-derived DCs. In our study, IFN-β and various doses of lovastatin were added and we did not observe any significant influence on DC differentiation. A similar effect of IFN-β on the differentiation of monocytes in MS patients was shown previously (Hussien et al. 2001; Zang et al. 2004). The authors found that IFN-β only delayed differentiation and after 10 days the percentage of CD14 was not significantly different from that of a control group (Zang et al. 2004). Some authors reported earlier that IFN enhances the terminal differentiation of DCs. However, in that study, different methods were used: both IFN-α and IFN-β were assessed, IFNs were added daily between days 14 and 17, and FACS analysis was performed on day 17 (Luft et al. 1998).

Among the immunomodulatory agents that we evaluated, IFN-β was found to significantly decrease the expression of CD1a and to markedly up-regulate CD86 in DCs of MS patients; however, the phenotypic DC changes in the cultures from healthy subjects and MS patients caused by IFN-β were similar. Earlier studies demonstrated that IFN-β caused a dose-dependent down-regulation of CD1a and increased expressions of CD83, CD80, and particularly CD86 in DCs generated in vitro from MS patients (Huang et al. 2005; Zang et al. 2004). Unfortunately, there are no studies evaluating the effects of lovastatin on the maturation of DCs in MS patients. Such studies carried out in healthy individuals showed that various doses of atorvastatin and simvastatin suppressed the upregulation of CD40, CD86, and CD83 on LPS-stimulated DCs and may inhibit DC maturation (Yilmaz et al. 2004, 2006). In our study, lovastatin also decreased the expression of CD1a and inhibited the upregulation of CD83 and CD86 in cultures from both MS patients and healthy subjects. However, statistically significant differences were seen only in the MS patient cultures and the influence of lovastatin on CD80 was ambiguous. We found that incubation with a low dose of lovastatin significantly inhibits the up-regulation of MFI of CD80, but this was not observed in the percentage of positive cells and for the high dose of lovastatin. Similar results were presented recently in DCs derived from PBMCs of patients with monosymptomatic optic neuritis. In that study the authors reported that simvastatin causes a marked decrease in the expressions of CD1a and CD83 (Tsakiri et al. 2010). Furthermore, in our study a decrease in CD209 expression following high doses

of lovastatin were observed. The expression of CD209 is related to the migration of DCs to various tissues and organs, including the CNS (Geijtenbeek et al. 2000). Therefore, it might be assumed that the migration of DCs is impaired by lovastatin, that could be potentially effective in MS therapy, but in vivo studies are needed to confirm this hypothesis. Other authors have demonstrated a significant reduction in the expression of CD209 on DCs from MS patients in in vitro cultures treated with IFN-β (Zang et al. 2004). In our study, a reduction in the expression of CD209 after IFN-β treatment was also observed; however, it was not statistically significant.

Experimental autoimmune encephalomyelitis and in vitro studies have shown that statins suppress several key functions of the immune system that influence the development of autoimmune disease. The authors showed that statins exert immunomodulatory effects on both APCs (macrophages) and T cells. On APCs, statins inhibited the expression of MHC-II, CD80, and CD86. Moreover, they inhibited the secretion of proinflammatory cytokines (IL-2, IL-12, IFN-γ and TNF-α), induced the secretion of Th2 cytokines (IL-4, IL-5, IL-10), and decreased antigen-specific T-cell activation (Kwak et al. 2000; Nath et al. 2004; Neuhaus et al. 2002; Youssef et al. 2002). Recently, Zhang et al. reported that simvastatin inhibits the production of IL-6 and IL-23 and induced IFN-γ, IL-4, and IL-27, which together suppressed IL-17 secretion in the $CD4^+$ cells derived from MS patients. In addition, simvastatin directly suppressed IL-17 transcription factor RORC (retinoic acid-related orphan nuclear hormone receptor C) and IL-17 gene expression as well as IL-17 secretion from the $CD4^+$ T cells (Zhang et al. 2008).

Our findings also indicate that IFN-β and lovastatin affect the phenotype of DCs and are thus likely to modify their function in different ways. All of them decrease the expression of CD1a, which is responsible for the presentation of lipid and glycolipid antigens to T lymphocytes (Porcelli and Modlin 1999). Hence both immunomodulatory agents we examined weaken, at least partially, the autoaggressive reactions against self antigens in MS patients by blocking the activity of CD1a. On the other hand, the effects of individual immunomodulatory agents on co-stimulatory molecules vary. IFN-β caused essential up-regulation of CD86, whereas lovastatin inhibits up-regulation of both CD80 and CD86, resulting in biased Th-cell responses in MS patients. Since the co-stimulatory molecules CD80 and CD86 lead to the activation of Th1 and Th2 pathways, respectively (Kuchroo et al. 1995), our findings from the IFN-β-treated culture may reflect a dominance of Th2 versus Th1 responses. Moreover, lovastatin may inhibit DC maturation. This is extremely relevant since the degree of DC maturation determines the generation and regulation of interactions between DCs and T cells. Mature DCs induce naïve T cells and most commonly initiate Th1-cell antigen-specific activation, while immature DCs have tolerogenic properties (Adorini et al. 2004). As statins have different mechanisms of action from IFN-β, they might be useful in combined therapy. However, results of recent studies with combined therapy with IFN-β and statins are controversial. Both beneficial and harmful results of combined therapy were reported (Birnbaum et al. 2008; Paul et al. 2008; Rudick et al. 2009).

In summary, one of the mechanisms of the beneficial action of IFN-β and statins may be associated with their influence on DCs. Delayed maturation of DCs resulting in their tolerogenic properties, inhibition of Th1 responses, induction of Th2 responses, and impact on migration of DCs are likely to be relevant to MS, with its dominating T-cell pathology. The present study is the first to demonstrate that lovastatin affects the in vitro on maturation of monocyte-derived DCs from MS patients. Further studies are needed to determine the functional properties of DCs after statin treatment and whether combined therapy with agents of various mechanisms of action on DCs is likely to lead to synergistic action and more beneficial effects than monotherapy.

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