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Increased immunopotency of monocyte derived dendritic cells from patients with optic neuritis is inhibited *in vitro* by simvastatin

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ABSTRACT

Multiple sclerosis (MS) is an autoimmune disease where myelin-reactive lymphocytes and their activation depend on interactions with antigen presenting cells (APCs).
Dendritic cells (DC) are professional APCs dependent on maturation to attain full T-cell priming capacity. The immunomodulatory properties of simvastatin influence the function of both T cells and APCs and could thus be a potential therapy for MS.
The phenotype of myeloid DC in untreated patients with monosymptomatic optic neuritis (ON) was determined by flow cytometry and the impact of simvastatin on the function of myeloid DC derived from peripheral blood mononuclear cells (PBMC) was analysed in vitro. DC from ON patients had more mature phenotype compared with healthy controls (HC). Particularly the fraction of DC expressing CD1a and CD80 was significantly higher in ON than in HC ($P<0.05$). Addition of 10 μM simvastatin significantly inhibited the maturation of DC in the ON group. Furthermore, ON derived DC induced stronger T-cell proliferation in the mixed leukocyte reaction (MLR), and simvastatin solely inhibited this proliferation of T-cells in the ON group and not in HC.
In conclusion DC from ON patients have a more mature phenotype and an increased stimulatory capacity. Simvastatin has an inhibitory effect on the differentiation and maturation of DC, and selectively reduce the T-cell proliferation induced by DC from patients with ON.
The results from these *in vitro* assays suggest potential beneficial inhibitory effects of Simvastatin in the inflammation in ON and early MS, but we need more clinical trials to confirm it.

Introduction

Optic neuritis (ON) is an inflammatory demyelinating disease which affects the optic nerve and has a similar pathophysiology as multiple sclerosis (MS) (Germann et al., 2007; Klistorner et al., 2008). About 2/3 of patients with ON will develop MS later in Sorensen et al. (1999). MS is a chronic disease of the CNS that is characterized by inflammation, demyelination and axonal injury. The immune system has a central role in the pathogenesis of the disease, where myelin-reactive lymphocytes and their generation depend in large part on interactions with Antigen Presenting Cells (APC) (Hemmer et al., 2006; Peng et al., 2006).

APC process and present antigen on major histocompatibility complex (MHC) molecules for recognition by T cells through the T-Cell Receptor (TCR) (Wu and Laufer, 2007).

Dendritic cells (DC) are professional APCs with the unique ability to initiate a primary immune response by expression of co-stimulatory molecules and the production of pro-inflammatory cytokines necessary for priming of T cells. However, DC are dependent on maturation to attain full T-cell priming capacity. The establishment of MS requires not only initial priming of naïve auto-reactive T cells but also their reactivation after presentation of CNS-associated antigens (Ags) by local APC upon entry into the CNS (Wu and Laufer, 2007; Svane et al., 2006; Yilmaz et al., 2004). It has not yet been determined whether DC present in the CNS are generated from resident cells or result from migration of peripheral DC (Wu and Laufer, 2007; El et al., 2005; Aktas et al., 2003).

In MS studies, DC generated from adherent peripheral blood mononuclear cells (PBMC) from patients with MS do not differ from those obtained from HC in terms of yield and morphology. However,

Abbreviations: Ag(s), Antigen(s); APC(s), Antigen Presenting Cell(s); BBB, Blood Brain Barrier; CIS, Clinically Isolated Syndrome; CM, Culture Medium; CSF, Cerebrospinal Fluid; DC, Dendritic Cells; FACS, Fluorescence Activated Cell Sorting; FCS, Fetal Calf Serum; HC, Healthy Control; LFA-1, Lymphocyte function-associated antigen 1; MFI, Mean Fluorescence Intensity; MHC, Major Histocompatibility Complex; MLR, Mixed Leukocyte Reaction; MRI, Magnetic Resonance Imaging; MS, Multiple Sclerosis; OB, Oligoclonal Bands; ON, Optic Neuritis; PBL, Peripheral Blood Lymphocytes; PBMC, Peripheral Blood Mononuclear Cells; VEP, Visual Evoked Potential.

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DC generated from MS patients' blood showed high CD1a expression and higher secretion of pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-6) than HC (Huang et al., 1999).

It has become evident that the 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors or statins possess both antiproliferative and immunomodulatory properties that alter the function of both T cells and APC (Kofler et al., 2008; Weber et al., 2006). *In vitro* studies have shown that statins suppress several key functions of the immune system that influence the development of autoimmune disease. Therefore, there is a considerable interest in their therapeutic potential for the treatment of autoimmune diseases including MS (Weber et al., 2006; Greenwood et al., 2006).

The mechanism by which statins may play a role in autoimmunity involves the inhibition of HMG-CoA reductase. Besides the biosynthesis of cholesterol, this enzyme regulates the biosynthesis of isoprenoids that mediate the membrane association of certain GTPases. Decrease in the production of isoprenoids results in a decrease of T helper cell differentiation and, consequently, decrease in autoimmune responses (Paraskevas et al., 2007).

Cell culture and animal experiments have shown that statins suppress Th1 and boost Th2 development by suppression of IL-12 and by reducing NF-κB activation (Ortego et al., 1999). In accordance, statins are able to bind and block lymphocyte function-associated antigen-1 L-site (LFA-1) resulting in stimulation of Th2 development (Weitz-Schmidt et al., 2001).

The aim of this study is to determine the phenotype of peripheral blood monocyte derived DC in patients with mono-symptomatic ON (as part in early multiple sclerosis) and to examine the effect of simvastatin on maturation and function of DC from ON patients measured by the ability to induce T-cells proliferation.

Materials and methods

Patients

Five (3 females, 2 males) untreated consecutive patients with acute monosymptomatic optic neuritis with ON onset within 24 days was included in the study. All the patients had been examined by an ophthalmologist to ensure a correct diagnosis before the entry to the study. Median age was 29.5 years (range 24–37 years) and median delay for study blood samples and the lumbar puncture from ON onset was 15 days (range 5–24 days). None of the patients had experienced symptoms that might be suspicious of neurological disorder prior to ON symptoms and no patients had received glucocorticoid or other immunomodulating treatment prior to study entry. Visual acuity was assessed by Snellen chart, and contrast sensitivity by Arden plates (Arden and Gucukoglu, 1978). The colour vision was assessed by the Lanthony desaturated 15 hue test (Lanthony, 1978). Visual Evoked Potential (VEP) latency was recorded using the pattern reversal technique (Frederiksen et al., 1996). All patients had extended VEP latency (results are not shown). MRI of the cerebrum was performed by a 3.0 T Phillips Intera Achieva scanner. Gadolinium injection of 0.2 mmol/kg was used. T1 and T2 weighted images as well as the Gd.-enhancing lesions were counted by a radiologist and the patients were classified according the Mc'Donald criteria (Polman et al., 2005).

For the comparison, peripheral blood from an age and gender matched HC group $n=5$ (3 females, 2 males) was analysed. Age median was 30 years (range 28–40 years).

A panel of blood analyses (included red and white blood cell counts, liver enzymes, cholesterol levels, thyroid-stimulating hormone (TSH), sedimentation rate, vitamin B12 levels , autoantibody and syphilis screening) was performed in both groups to further exclude other potential causes of visual loss in ON group and ensure that HC had normal blood tests.

All the above-mentioned tests were performed within 1 week prior to study blood sample and lumbar puncture. The CSF was analysed for cell count, glucose, and proteins, as for oligoclonal bands and IgG index. (Table 1).

Table 1
Pt ID: patient Identification, T1: T1-weighted images, T2: T2-weighted images, Gd: gadolinium-enhanced lesions, McDonald: number of the McDonald criteria met (out of four), L: leucocytes, E: erythrocytes, mio/L: million/Liter, OB: oligoclonal bands.

MRI of the cerebrum and cerebrospinal fluid examination IN ON								
MRI of the cerebrum					CSF analysis			
Pt ID	T1	T2	Gd	McDonald	L (mio/L)	E (mio/L)	OB	IgG index
1	0	>9	0	3	78	5	+	1.98
2	0	9	1	3	20	1	+	0.65
3	3	>9	1	4	2	2	+	0.51
4	0	>9	2	4	10	0	+	0.87
5	3	>9	3	4	3	0	+	1.16

The study was approved by the local Ethical Committee and informed consent of all participating subjects was obtained.

Generation of DC

Freshly obtained PBMC were isolated by lymphoprep (Greiner bioOne Germany). PBMC were washed and resuspended in culture medium (CM) X-Vivo 15 with 2% glutamax and 1% heat inactivated human AB serum at 7×10^6 cells/ml and monocytes were separated by their adherence to plastic in 6-well plates (Nunc Biotech Line Slangerup DK). Non-adherent cells were removed, and adherent cells were subsequently cultured for 7 days in CM supplemented with 250 U/ml of IL-4 (CellGenix, Freiburg Germany) and 1000 U/ml of GM-CSF (Leukine from Berlex, Richmond, CA, USA) (Sallusto and Lanzavecchia, 1994).

The immature DC were preincubated with different concentrations (0, 1, 5 and 10 μM) of simvastatin (activated compound) for 24 h. (Yilmaz et al., 2004) Simvastatin was kindly provided by MERCK and CO INC. Rahway, New Jersey. DC maturation was then induced by the addition of a cytokine cocktail to DC cultures for 48 h. The cytokine cocktail consisted of 1000 U/ml IL-1β (CellGenix), 1000 U/ml TNF-α (CellGenix), 1000 U/ml IL-6 (CellGenix), and 1 μg/ml PGE2 (ProstinE2 Pfizer Brussels). This is a standard maturation cocktail which has been used for preparation of clinical grade DC for several years (Jonuleit et al., 1997; Krause et al., 2007). The viability of the propagated DC was 85–95% determined by Trypan blue test.

Flow cytometric analysis (FACS)

DC ($\sim5\times10^5$ cells) were harvested and washed once with wash buffer (FACS-PBS with 0.5% bovine albumin (SIGMA A-4503) /100 ml FACS-PBS) and resuspended in 100 μl wash buffer. In order to avoid non-specific binding blocking was performed by adding 25 μl polyclonal human IgG 0.2 mg / ml to 100 μl DC. DC were incubated for 10 min at room temperature before antibodies were added.

Monoclonal antibodies against the following antigens were used in recommended concentrations: 2.5 μl CCR7 (FITC) and 1,25 μl CD54 (FITC) from R and D systems Minneapolis MN USA), 2.5 μl CD80 (FITC), 1,25 μl IgG1 (PerCP-Cy5), 0,6 μl CD14 (PerCP-Cy5) and 0.6 μl CD86 (APC) (BD-Pharmingen San Diego CA), 1.25 μl IgG1 (FITC), 1.25 μl IgG1 (PE), 1.25 μl IgG1 (APC), 1.25 μl CD3 (PE), 1.25 μl CD19 (PE), 1.25 μl CD14 (PE), 1.25 μl CD38 (PE), 1.25 μl HLADR (PerCP-Cy5) from BD-Bioscience (San Jose, CA, USA) and 1.25 μl CD83 (APC) from Caltag Laboratories (Burlingame CA, USA). Aliquots of cells were stained with antibodies for 30 min at 4 °C and washed once. 200 μl Wash Buffer was added, before flow cytometric analysis. PE-Lineage cocktail was prepared for simultaneous labelling of T-cells and B-cells by antibodies against CD3 and C D19 (BD-Bioscience).

Four-colour analysis of DC was performed on a FACS Calibur flow cytometer (BD-Bioscience). A minimum of 10,000 DC was acquired