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The LPS Receptor, CD14 in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis

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Key Words

Multiple sclerosis • Experimental autoimmune encephalomyelitis • EAE • LPS receptor • CD14 • Innate immunity • Autoimmunity

Abstract

Innate immune receptors are crucial for defense against microorganisms. Recently, a cross-talk between innate and adaptive immunity has been considered. Here, we provide first evidence for a role of the key innate immune receptor, LPS receptor (CD14) in pathophysiology of experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis. Indicating a functional importance *in vivo*, we show that CD14 deficiency increased clinical symptoms in active experimental autoimmune encephalomyelitis. Consistent with these observations, CD14 deficient mice exhibited a markedly enhanced infiltration of monocytes and neutrophils in brain and spinal cord. Moreover, we observed an increased immunoreactivity of CD14 in biopsy and post mortem brain tissues of multiple sclerosis patients compared to age-matched controls. Thus, the key innate immune receptor, CD14, may be of pathophysiological relevance in experimental autoimmune encephalomyelitis and multiple sclerosis.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that is histopathologically characterised by acute demyelinating lesions [1-3]. For many years the prevailing view of MS pathogenesis was that autoreactive CD4+ T cells, formed in the periphery, play the primary role in disease initiation. According to this hypothesis, the CD4+ T cells invade the CNS, re-encounter their antigen and cause a neuroinflammatory response involving B cells, CD8+ T cells and mononuclear phagocytes, that ultimately results in myelin damage and neuronal cell death [2, 4].

Innate immune receptors recognise specific microbial components and are crucial for effective host defence against invading microorganisms. Recently, a disease-promoting role of the innate immune system in autoimmune diseases has been proposed, based e.g., on the observation that ligands of innate immune receptors modulate disease severity of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [5]. Furthermore, the innate immune receptor, Toll-like receptor 4 (TLR4) has been described to be involved in pertussis toxin-induced leukocyte recruitment into the brain tissue during EAE [6]. Apart from this, innate immune

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receptors have been considered to be required for full T cell-dependent B cell activation and antibody responses after immunisation of mice with human serum albumin and lipopolysaccharide (LPS) [7]. These findings support a central role of the innate immune system not only in acute infection but also in autoimmunity.

The LPS receptor (CD14) is a prototype innate immune receptor and plays a crucial role in inflammatory responses to bacterial pathogens i.e. by recognition of LPS, a most characteristic component of Gram negative bacteria [8-12]. Since recent studies revealed that CD14 is present on microglial cells [12, 13] and can be detected in EAE [12], we investigated, whether this key innate immune receptor could be relevant in MS pathophysiology.

Materials and Methods

Mice

Female CD14 deficient mice and C57BL/6J wt control mice (6-12 weeks) were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) via Charles River (Sulzfeld, Germany). All animal experiments were performed with approval of the local ethical committee. Mice were housed under specific pathogen-free conditions.

Induction and clinical evaluation of active EAE

Active EAE was induced as follows: each animal received 200µg of myelin oligodendrocyte glycoprotein (MOG) aa_{35-55} in incomplete Freund's adjuvant (Santa Cruz, LabForce, Nunningen, Switzerland) supplemented with 4 mg/ml non-viable, desiccated *Mycobacterium tuberculosis* (H37RA, Difco BD Bioscience Clontech, Allschwill, Switzerland) subcutaneously. 300ng pertussis toxin from *Bordetella pertussis* (Sigma, Grogg Chemie AG, Stettlen/Deisswil, Switzerland) per mouse was administered intraperitoneally at days 1 and 3 post immunisation (p.i.). Assessment of clinical disease activity was performed as described before [14], with the following scores: 0 = healthy, 0.5 = limp tail, 1 = hindleg weakness, 2 = hindleg paraparesis, 3 = hindleg paraparesis with incontinence. A total number of 47 CD14 deficient and 46 wt mice were examined in five independent experiments.

Antibodies for immunohistochemical analysis of murine CNS tissue

The following antibodies were used: rat anti-mouse CD45 (M1/9, ATCC, Rockeville, MD, USA), rat anti-human CD44 IgG2a (isotype control, 9B5, kindly provided by E. Butcher, Stanford CA, USA), rat anti-mouse CD11b (Mac-1, M1/70, ATCC), rat anti-mouse Ly-6G and Ly-6C (Gr-1, BD Pharmingen), rat anti-mouse neutrophils (7/4, Serotec Biozol, Eching, Germany), rat anti-mouse macrophages (F4/80, BD Pharmingen) and rat anti-somatostatin IgG2b (isotype control, Acris, Bad Nauheim, Germany).

Immunohistochemical analysis of murine CNS tissue

Mice were anaesthetised with isoflurane (Baxter, Arovet AG, Zollikon, Switzerland) and perfused with 1% formaldehyde (Grogg Chemie AG, Stettlen/Deisswil, Switzerland) in PBS through the left heart ventricle. Brains and spinal cords were removed, cut into three blocks, embedded in Tissue-tec (OCT compound, Haslab, Ostermundingen, Switzerland) and snap-frozen in an isopentan bath (2-Methyl-butan, Grogg Chemie AG, Stettlen/Deisswil, Switzerland) at -80°C. Cryostat sections (6µm) were air-dried overnight, acetone fixed, and stained using a three-step immunoperoxidase technique. Sections were incubated in 30-minute steps with PBS washes in between each step: primary monoclonal antibody was followed by a biotinylated secondary goat anti-rat IgG (Vectastain, Linearis, Wertheim-Bettingen, Germany), and finally horseradish peroxidase-conjugated streptavidin (Vectastain, Linearis). Sections were developed with 0.07% amino-ethylcarbazol (AEC, Sigma, Deisenhofen, Germany) and 0.009% hydrogen peroxide in 0.01 mol/l acetate buffer (pH 5.2) for 10 minutes and then counterstained with hematoxylin-eosin and coverslipped with Aquatex (Merck, Darmstadt, Germany). The whole procedure was carried out in a humidified chamber.

Immunohistochemical analysis of CD14 in human brains

Formalin-fixed and paraffin-embedded human autopsy (n=2) and biopsy (n=4) material of acute and chronic active multiple sclerosis cases as well as age-matched controls without neuropathological alteration was used. Biopsy tissue from patients with intracerebral abscess was used as positive control. Informed consent for autopsy was given by the relatives. All cases underwent detailed neuropathologic examination. Slides were pre-treated as follows: after deparaffinisation and rehydration, slides were treated in citrate buffer, pH 6.0 by incubating in a microwave 650 Watts 5×3 min. Sections were blocked with 0.2% casein for 1 hour. Staining was performed using the mouse monoclonal antibody against CD14 (1:100, NCL-L-CD14-223, Novocastra, Newcastle upon Tyne, UK) followed by anti-mouse universal immuno-alkaline polymer (Histofine®, Nichirei Biosciences, Tokyo, Japan). The substrate for the development of AP consisted of 0,01% (w/v) neofuchsin (Sigma) 0,02% sodium nitrite (Fluka, Kassel, Germany), 0,028% naphthol-ASBi-phosphate (Sigma) 0,6% N,N-dimethylformamide (Merck) in 0.05 M Tris-HCl buffer, (pH 8.7) containing 1 mM levamisole (Sigma). The slides were counter-stained with hematoxylin.

Analysis of CSF and plasma of MS patients

CSF examination included determination of CSF cell counts, calculation of albumin-quotient (Q_{Alb}) and IgG-index and assessment of oligoclonal bands by isoelectric focussing. Samples for determination of sCD14 were immediately centrifuged (5000 x g, 5 min) and stored at -80°C until used. All experiments were performed with approval of the local ethical committee.

Intrathecal release of soluble CD14

We investigated the release of the soluble isoform of CD14 (sCD14) by use of a sandwich enzyme-linked immunosorbent

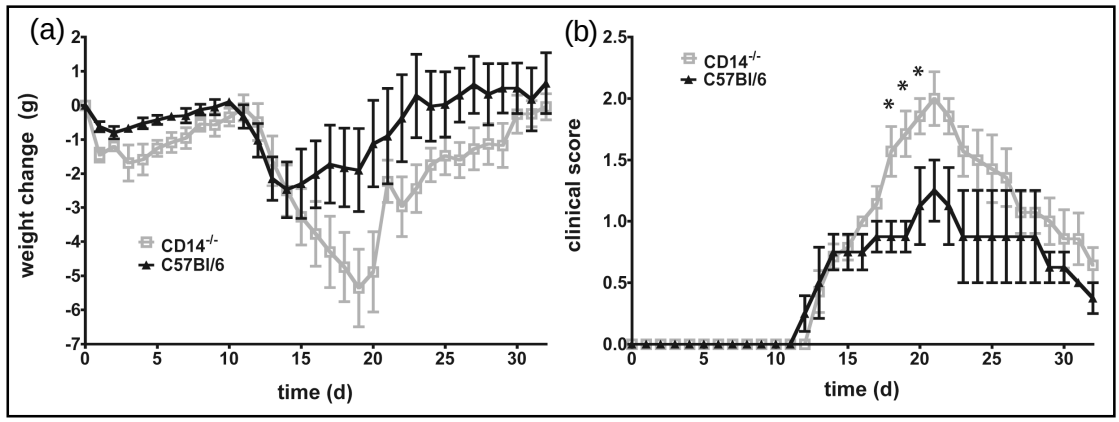

Fig. 1. CD14 deficiency enhances clinical features of active EAE. One representative experiment out of 5 is shown. Average weight loss (a) and disease scores (b) of CD14 deficient mice and wt controls were calculated daily. CD14 deficient animals showed a significant increase of disease severity at the peak of clinical symptoms between days 18-20 (* $p < 0.05$). Values represent 10 mice per group.

assay (ELISA) using two monoclonal antibodies (clones 69 and 69 peroxidase-conjugated antibodies, R&D, Wiesbaden, Germany). Serum, diluted 1:2000 and CSF samples, diluted 1:100 were incubated according to the manufactorer's instructions. Optical density readings were performed at 450nm. The lower limits of detection of sCD14 were 250 pg/ml. The intrathecal origin of CD14 was calculated in relation to a blood brain barrier leakage. Analogously to the IgG Index, a sCD14 index [(CSF sCD14: serum sCD14): (CSF albumin: serum albumin)] was calculated to correct for a possible passive transfer of sCD14 through a disrupted blood brain barrier.

We examined the relation between CSF cell count, IgG index, presence of oligoclonal bands and MRI markers of disease activity (gadolinium MRI examination).

Statistics

Data in figures and tables are shown as mean ± SD. Two-independent samples *t* test on InStat Graph Pad 3.0 was used for multiple comparisons. Statistical significance was set at $p< 0.05$.

Results

CD14 deficiency enhances clinical features of active EAE

To find out, whether CD14 is involved in the pathogenesis of EAE, we immunised CD14 deficient animals and age-matched wt control mice and compared the respective disease progression. CD14 deficiency markedly augmented neurological symptoms in active EAE. Figure 1 a,b shows one representative experiment (n=10 animals per group) of 5 independent experiments. Although both groups developed weight loss (Fig.1a) and exhibited disease at a similar interval post immunisation (p.i.) (mean onset: $CD14^{-/-}$: 13.25±0.71 p.i.; wt: 13.14±0.9 p.i.), CD14 deficient animals exhibited significantly increased peak disease severity when compared to wild type control mice ($p< 0.05$ between days 18 to 20 p.i., e.g. d19: $CD14^{-/-}$: 1.7±0.49, wt: 0.9±0.25; Fig.1b).

CD14 deficiency is associated with increased inflammatory infiltrates in the CNS

Consistent with these observations, histopathological investigation of three different regions of each, brain and spinal cord revealed that CD14 deficient mice developed a markedly increased cell response, characterised by extended, plaque-shaped infiltrates in both, grey and white matter (Fig. 2a).

Focussing on known CD14 positive cells, further differentiation allowed to identify CD11b/F4/80 positive mononuclear phagocytes (monocytes/microglia) and Gr-1/7/4 positive neutrophils as cellular constituents of these infiltrates in the brain (Fig. 2b) and spinal cord (Fig. 2c). The ratio between both cell populations did not differ between CD14 positive and deficient mice.

CD14 expression is upregulated in brain tissue, but its release is not altered in CSF of MS patients

In order to investigate CD14 also in the human disease, we analysed both CD14 expression in brains or release of its soluble variant (sCD14) in brain tissue, CSF or blood from MS patients. We observed a pronounced immunoreactivity for CD14 in parenchymal cells in biopsy and autopsy brain tissue samples of MS patients (Fig. 3). In addition, also some of the perivascular cells were CD14 positive, probably corresponding to infiltrating macrophages. By contrast, no parenchymal CD14 staining and only rare perivascular macrophage-related staining was seen in brains of age-related controls (Fig. 3).

Finally, we analysed peripheral and intrathecal release of the sCD14 in 21 patients with relapsing remitting MS and 21 healthy control subjects. For a correction of

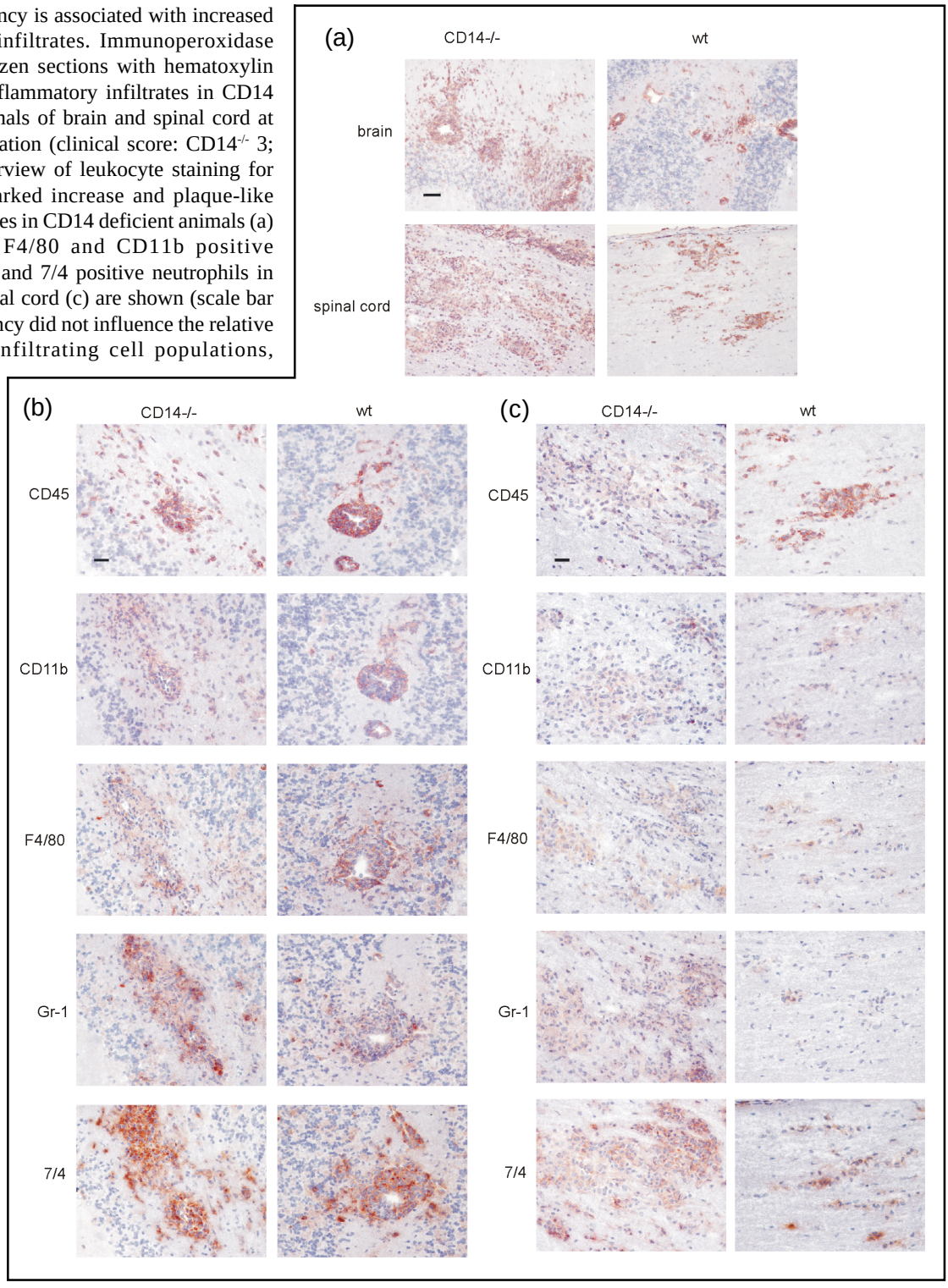

Fig. 2. CD14 deficiency is associated with increased inflammatory CNS infiltrates. Immunoperoxidase staining of serial frozen sections with hematoxylin counterstaining of inflammatory infiltrates in CD14 deficient and wt animals of brain and spinal cord at day 18 post immunisation (clinical score: $CD14^{-/-}$ 3; wt 2) is shown. Overview of leukocyte staining for CD45 revealed a marked increase and plaque-like shape of CNS infiltrates in CD14 deficient animals (a) (scale bar 50µm). F4/80 and CD11b positive monocytes and Gr-1 and 7/4 positive neutrophils in the brain (b) and spinal cord (c) are shown (scale bar 20µm). CD14 deficiency did not influence the relative proportion of the infiltrating cell populations, although the number of inflammatory cells is increased in CD14 deficient animals.

possible impairment of the blood brain barrier, a CD14 index ($CD14_{CSF}/CD14_{serum}$:$albumin_{CSF}/albumin_{serum}$) [15] was calculated. In contrast to the histopathological studies, we did not observe increased levels of sCD14 in CSF (Fig.4) or plasma (data not shown) of MS patients compared to control subjects. Moreover, patients with an acute exacerbation of MS did not differ from those with non-active disease. Thus, the sCD14 levels in CSF or blood do not reflect the pronounced parenchymal CD14 response observed in MS brains.

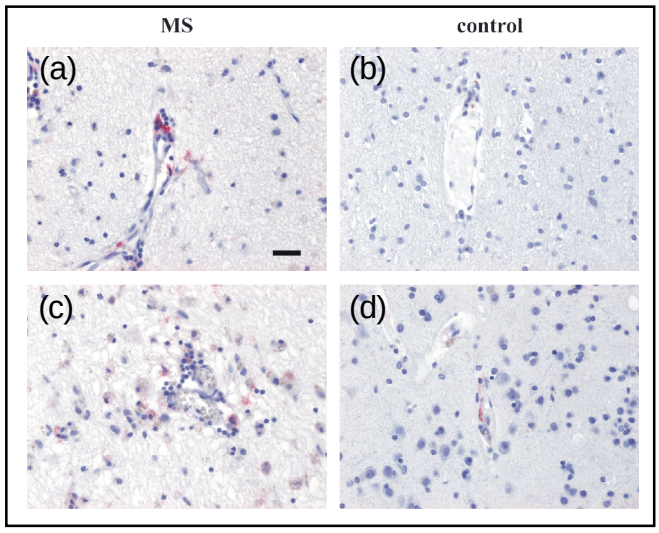

Fig. 3. CD14 is upregulated in brain tissue of MS patients. Immunohistochemical analysis of MS biopsy and autopsy tissue showed a pronounced immunoreactivity for CD14 not only in perivascular but also in parenchymal cells (a, b). Healthy control subjects only showed perivascular macrophage-related positive signals (c, d). Shown is alkaline phosphatase neofuchsin staining with hematoxylin counterstaining (scale bar 30 μm).

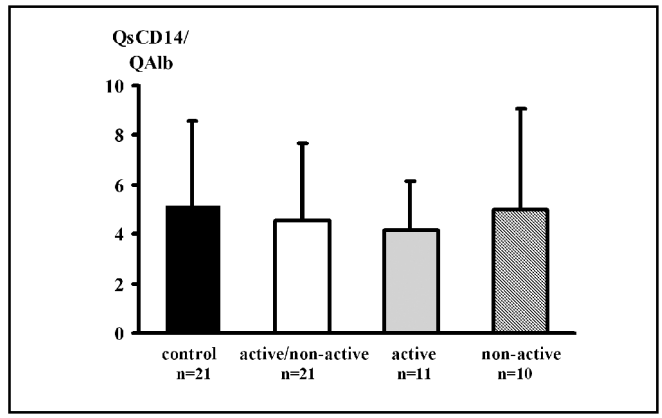

Fig. 4. Intrathecal sCD14 secretion is unchanged in MS. Intrathecal release of sCD14 receptor was analysed by ELISA in 21 patients with relapsing remitting MS and 21 healthy control subjects. SCD14 release did not significantly differ in MS, or within the MS group subclassified into active vs. non-active disease.

Discussion

The innate immune system has been originally related to the host defense against invading microorganisms, but there is increasing evidence for a role also in autoimmune diseases. In this study, we provide the first evidence for a modulating role of the key innate immune receptor, CD14 in EAE. CD14 deficiency resulted in a strong increase of clinical and histopathological inflammatory features of the disease, suggesting that CD14 possesses protective effects in this autoimmune disease. This is consistent with recent reports showing that innate immunity is involved in adaptive immune responses in diseases such as rheumatoid arthritis, asthma, atherosclerosis, diabetes [7, 18-22]. However, as an alternative explanation of our findings, we cannot rule out the possibility that experimental impairment of innate immunity (e.g. by CD14 deficiency) could cause compensatory over-regulation of the adaptive immune system.

The immunohistochemical analysis allowed identification of neutrophils as one of the contributing infiltrating cell types. Consistent with this, polymorphonuclear leukocytes have been shown to play a critical role in the EAE effector phase [23]. Interestingly, it is known that CD14 can be also expressed on neutrophils [24-26], although most research on CD14 focussed on mononuclear phagocytes. Furthermore, in a meningitis model, CD14 deficiency leads to an increased migration of polymorphonuclear leukocytes into the brain, corroborating our findings in EAE [27].

CD14 as a glycosyl-phosphatidyl-inositol-anchored molecule is known to use TLR4 as a co-receptor for full function. Interestingly, TLR4 has been described to be expressed on regulatory T cells, suggesting a modulatory role. Furthermore, TLR4 can induce maturation of dendritic cells after exposure to microorganims [28, 29]. Until now, there are no reports of CD14 being involved in these mechanisms. But, it is likely that also CD14 might play a role in regulatory T cell mediated antigen presentation, which needs further investigations.

Linking the animal experimental data to human disease, we showed a pronounced parenchymal CD14 immunoreactivity in biopsy and post mortem brain tissues of patients with MS but not in age-matched controls. As a complementary approach to analyse CD14 in patients, we quantified the secretion of sCD14 in MS patients and control subjects. However, we did not observe a significant increase in either systemic or intrathecal release of sCD14 in MS patients compared to control subjects, regardless of the presence or absence of active disease. Thus, these results stand in contrast to an earlier report of increased plasma levels of sCD14 in MS patients in peripheral blood [30] and argues against the assumption that sCD14 levels in peripheral blood or CSF can reflect cerebral CD14 responses in MS patients.

In conclusion, we show for the first time a role of CD14, the key innate immune receptor crucial in defence

against microorganism, in EAE. This, together with the detection of this receptor in brains of MS patients suggests a pathophysiological role of CD14 in MS. More generally, these observations provide further evidence for an involvement of the innate immune system in autoimmunity.

Acknowledgements

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