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ORIGINAL ARTICLE

The comparison of selected cerebrospinal fluid and serum cytokine levels in patients with multiple sclerosis and normal pressure hydrocephalus

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Abstract

OBJECTIVES: Cytokine production and immune activation are associated with various pathological conditions including neurodegenerative disorders. One of them is multiple sclerosis (MS), known autoimmune disease. Inflammatory changes were also reported in normal pressure hydrocephalus (NPH), neurodegenerative disorder, which pathophysiology remains still unclear. The aim of this research was to compare the group of MS subjects with NPH patients and controls and to evaluate the potential inflammatory substance of NPH in comparison with autoimmune inflamed MS.

METHODS: The levels of IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, INF-γ, sCD40L and TNF-α were measured in cerebrospinal fluid (CSF) and plasma in subjects with MS (n=15), NPH (n=18) and controls (n=11) by multiplex assay.

RESULTS: The increased levels of IL-1β, IL-6, IL-10, IL-21 and TNF-α in cerebrospinal fluid of NPH subjects in comparison with MS patients and controls were found. Regarding the MS patients, we have confirmed increased IL-33 levels in cerebrospinal fluid and periphery as well as the increase of IL-1β and IL-10 in cerebrospinal fluid and IL-4 and sCD40L in plasma.

CONCLUSION: The enlarged brain ventricles in NPH may repress and activate brain structures to the production of IL-1β, IL-6, IL-10, IL-21 and TNF-α, reflecting the inflammatory basis in NPH affected brain. The elevation of the above mentioned cytokines in MS was confirmed.

Abbreviations:

CSF	- cerebrospinal fluid
MS	- Multiple sclerosis
NPH	- Normal pressure hydrocephalus
CNS	- central nervous system
IL	- interleukine
LLoQ	- lowest limit of quantification
IND	- inflammatory neurological diseases
NIND	- non-inflammatory neurological diseases

INTRODUCTION

Cytokines are a diverse group of polypeptides comprised of interleukines (IL), interferons (INF), tumor necrosis factors (TNF), chemokines and growth factors. They are well-known as mediators of inflammation; regulation of cell growth and differentiation acting in very low concentrations (pico- or nanomolar range). Cytokines are rapidly upregulated in response to some diseases, injuries and infections and have an important role in tissue repair in various pathological conditions. In the brain, they possess diverse actions, some of which may facilitate either neurodegeneration or neuroprotection (Rothwell & Strijbos 1995; Smith *et al.* 2012; Allan & Rothwell 2001). Recently, the cytokine involvement in the neurodegenerative processes has received considerable attention. Systemic and brain inflammation were reported for a lot of neurodegenerative diseases, i.e. Alzheimer's and Parkinson's diseases, multiple sclerosis (MS) as well as normal pressure hydrocephalus (NPH) (Blum-Degena *et al.* 1995; Li *et al.* 2007; Mogi *et al.* 1996; Seppi *et al.* 2014; Tarkowski *et al.* 2003b; Sosvorova *et al.* 2014).

Cytokines have typically been classified as either pro-inflammatory or anti-inflammatory ones based on their actions in peripheral tissues. In relation to the neurodegeneration, the major pro-inflammatory cytokines are known to be IL-1β, IL-6 and TNF-α, while the anti-inflammatory cytokines are IL-4 and IL-10 (Sosvorova *et al.* 2015; Smith *et al.* 2012). Efforts have been made to understand which cytokines are detrimental and which beneficial for the nervous system. However, recent research still shows controversial data (Smith *et al.* 2012; Probert 2015). In general, it is assumed that the pro-inflammatory cytokines exacerbate and sustain neurodegenerative processes, while the anti-inflammatory ones promote cell regeneration, protection and survival. In addition, of importance are the functional state and the type of cells stimulated by the cytokines, the concentration and the type of cytokines expressed and the exposure duration (Viviani *et al.* 2004).

The immune activation in the nervous system is associated with various pathological conditions including the neurodegenerative disorders, one of which is multiple sclerosis (MS). MS is a degenerative autoimmune-mediated inflammatory disease, characterized by demyelination and axonal loss in central nervous system (CNS). The etiology of the disease remains still unclear and it is likely that both environmental and genetic factors are also involved in the disease development (Lassmann *et al.* 2007; Nylander & Hafler 2012). Regarding the MS immunological essential, cytokines were reported to play a prominent role in modulating the autoimmune inflammatory cascade (Pasquali *et al.* 2015; Burman *et al.* 2014; Kallaur *et al.* 2013).

Another neurodegenerative disease is normal pressure hydrocephalus (NPH), which came in the focus of interest and research in the past decades (Pyykko *et al.* 2014; Tarkowski *et al.* 2003b). NPH is known to be one of the treatable neurodegenerative disorders, characterized by excessive accumulation of cerebrospinal fluid (CSF) in the cerebral ventricles. NPH is manifested as balance impairment, urinary incontinence and dementia development. However, if diagnosed early enough, it is treatable by shunt insertion leading to complete or partial amelioration of patient's state (Rigamonti 2014). Altered cytokine levels in NPH were reported predominately in CSF, reflecting the inflammatory changes in NPH affected brain (Sosvorova *et al.* 2014; Tarkowski *et al.* 2003b). In a previously published study, the increased cerebrospinal fluid levels of IL-1β, IL-6 and IL-10 in patients suffering from NPH were detected (Sosvorova *et al.* 2014). In addition, increased CSF IL-33 and decreased IL-4 levels were also described in NPH before the shunt treatment (Sosvorova *et al.* 2015).

The aim of this study was to compare the levels of some cytokines in the CSF and periphery in patients with MS, NPH patients and a control group and to evaluate the potential inflammatory substance of NPH in comparison with the autoimmune inflamed MS.

MATERIALS AND METHODS

Subjects

The MS patient group included 15 subjects aged 23–62 years (4 men, 11 woman). The diagnosis of MS was confirmed by spinal fluid analysis and magnetic resonance imaging. Before plasma and CSF drown, the patients did not received any medication against MS. All patients were treated with adequate medication and none of the used drugs can be suspected of influence of cytokine levels. CSF and plasma were collected in the morning, CSF was collected by lumbar punction. All the sample collections were performed at the 1st Faculty of medicine in Prague under the same conditions.

The NPH patient group included 18 subjects (11 men, 7 women), 65–80 years old. Non-obstructive idiopathic normal pressure hydrocephalus was diagnosed on the basis of a combination of clinical presentation, magnetic resonance imaging and a lumbar drainage test (Walchenbach *et al.* 2002). All patients were treated with adequate medication and none of the used drugs can be suspected of elevation of cytokine levels. CSF and plasma were collected in the morning on the beginning of lumbar drainage test. The ventriculoperitoneal shunt was further introduced to all of the patients diagnosed with NPH after finishing the lumbar drainage test. The shunt implementation led to an improvement of the disease symptoms in all patients.

The control group (11 subjects; 3 men, 8 women) consisted of age-matched subjects, without diagnosed MS and apparent symptoms of hydrocephalus. To ensure that control group consists of neurologically healthy patients, samples were obtained from patients with incidental (unrupted) aneurysm for which lumbar drainage is used during the exercise to relax brain and

facilitate the surgical approach to the aneurysm. All the surgeries were performed at the Department of Neurosurgery of the University Military Hospital in Prague under the same conditions.

Samples (3–10 mL) were collected in plastic tubes, subsequently frozen and stored at –79 °C until the analysis (up to 1 year). For the cytokine measurement, the samples were aliquoted and each aliquot was thawed and used only once. The protocol was approved by the Ethics Committee of the Institute of Endocrinology. Informed consent was obtained from all participants in the project prior to any procedure.

Sample analysis

All samples were analyzed using Multiplex immunoanalytic xMAP (Luminex Corporation) technology employing Bio-Plex® 200 system (Bio-Rad Laboratories, Inc.). The measurements were performed using multiplex assay (Bio-Plex ProTM Human Th17 Cytokine Panel, catalog #171AA001M (Bio-Rad, Hercules, CA, USA) designed for the determination of IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, INF-γ, sCD40L and TNF-α (Geng *et al.* 2012). The method details were described elsewhere (Sosvorova *et al.* 2014).

Data statistical analysis

The relationships between dependent variables and effects of status (MS, NPH, controls) of the experiment were evaluated using repeated measures of the ANOVA model. The ANOVA model was followed by least significant difference (LSD) multiple comparisons. To eliminate skewed data distribution and heteroscedasticity, the original data were transformed by a power transformation to attain Gaussian distribution and constant variance before further processing. Statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA) was used for data processing.

RESULTS

The CSF as well as plasma levels of IL-17A, IL-17F, IL-22, IL-23, IL-25, IL-31 and INF-γ were under the lowest limit of quantification (LLoQ) in the vast majority of the determined samples. The levels of cytokines in the CSF are shown in Figure 1 and in plasma in Figure 2.

The elevated CSF levels of IL-1β, IL-6, IL-10, IL-21 and TNF-α were found in NPH patients compared to MS and the control group (p=0.0001; 0.0001; 0.0001; 0.018 and 0.0001, respectively). The CSF levels of IL-1β and IL-10 were higher in MS than in the controls.

The CSF and plasma levels of IL-33 were increased in MS patients compared to the NPH and the controls (p=0.0009; 0.0001).

CSF sCD40L levels were significantly lower in MS patients in comparison with the NPH and control group (p=0.0001).

In plasma, among IL-33, significantly increased levels of IL-4 and sCD40L were detected in the MS patients (all p=0.0001).

Plasma TNF-α levels were decreased in MS group of subjects (p=0.0036).

DISCUSSION

The major pro-inflammatory cytokines are known to be IL-1β, IL-6 and TNF-α. The interdependence and mutual upregulation of these cytokines is well documented (Chakraborty *et al.* 2010; Tobinick *et al.* 2006). They are known to mediate cellular responses during immune activation and inflammation. In MS, these cytokines might be responsible for T-cell activation (IL-1β), for demyelination (TNF-α), and for immunoglobulin synthesis (IL-6) within the CNS (Maimone *et al.* 1991). Maimone *et al.* (Maimone *et al.* 1991) determined these major pro-inflammatory cytokines in subjects with MS in comparison with patients with inflammatory (IND) and non-inflammatory (NIND) neurological diseases. The authors reported that the CSF IL-6 and TNFα levels were significantly higher in MS and IND than in NIND. In serum samples, low levels of IL-6 were detected in MS and NIND patients and TNFα was detected only in the minority of MS sera. Concerning IL-1β, it was detected only rarely across the studied groups and biological materials. Maimone *et al.* concluded, that increased CSF levels of the cytokines IL-6 and TNF-α occur frequently in MS and IND. Their results confirmed the role of pro-inflammatory cytokines in inflammatory diseases; however no comparison with healthy subjects was performed.

The comparison of cytokine levels in MS patients and healthy controls was performed by Kallaur *et al.* (Kallaur *et al.* 2013). The authors published increased serum IL-6 and IL-4 levels in MS patients. Their results indicated that MS patients exhibit a complex system of pro- and anti-inflammatory cytokines that may interact to modulate the progression and activity of the disease. The increased plasma IL-4 levels in the MS patients in our study are in accordance with the above mentioned results, however, we cannot confirm the finding of increased IL-6 in the circulation. Our data also indicated decreased plasma levels of TNF-α in the MS subjects, which is in accordance with Maimone *et al.* (Maimone *et al.* 1991).

Concerning the most widely known pro-inflammatory cytokine – IL-1β and its levels in MS patients, controversial results were reported. Several works published unaffected CSF and serum levels in MS (Maimone *et al.* 1991; Tsukada *et al.* 1991), however other authors reported increased CSF IL-1β levels (Hauser *et al.* 1990; Olsson 1992), which is in accordance with our results. CSF IL-1β in the MS group was significantly increased compared to the controls and significantly decreased compared to the NPH patients. The same was also observed for the neuroprotective anti-inflam-

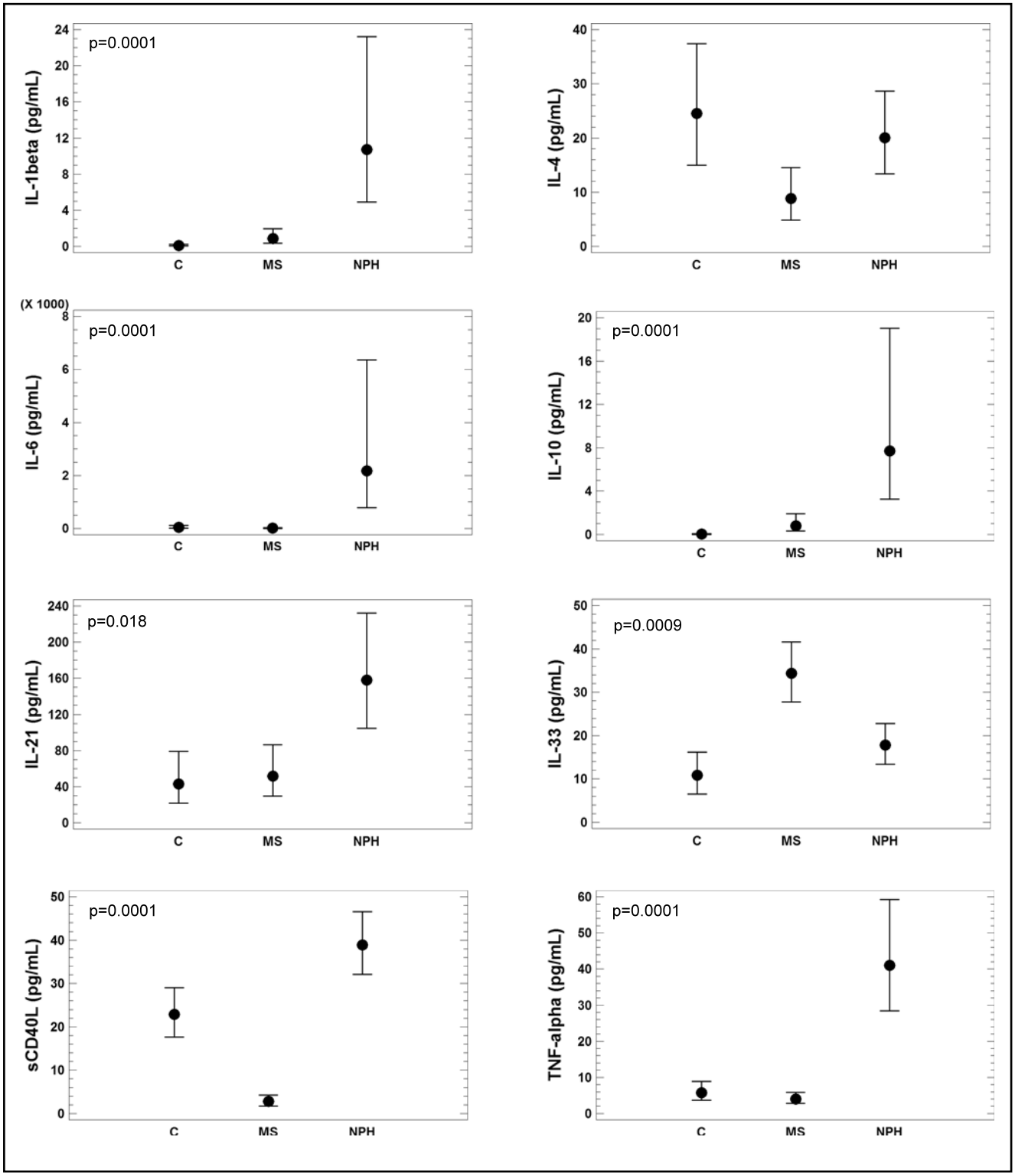

Fig. 1. The cerebrospinal fluid levels (pg/mL) of cytokines in patients with multiple sclerosis (MS) and normal pressure hydrocephalus (NPH) in comparison with the control group. Means and 95.0 percent confidence intervals are presented. The statistical important significances are indicated above graphs.

matory CSF IL-10, having potential to downregulate the production of IL-1β and TNF-α (Park *et al.* 2007). The protective properties of IL-10 in MS were reported by Gayo *et al.* (Gayo *et al.* 1998). IL-10 is considered to be an important anti-inflammatory modulator of glial activation, operating to maintain a balance between the pro- and anti-inflammatory cytokines in the CNS (Qian *et al.* 2006; Park *et al.* 2007; Sawada *et al.* 1999; Mizuno *et al.* 1994). Our results showing increased CSF IL-10 are in accordance with the already published

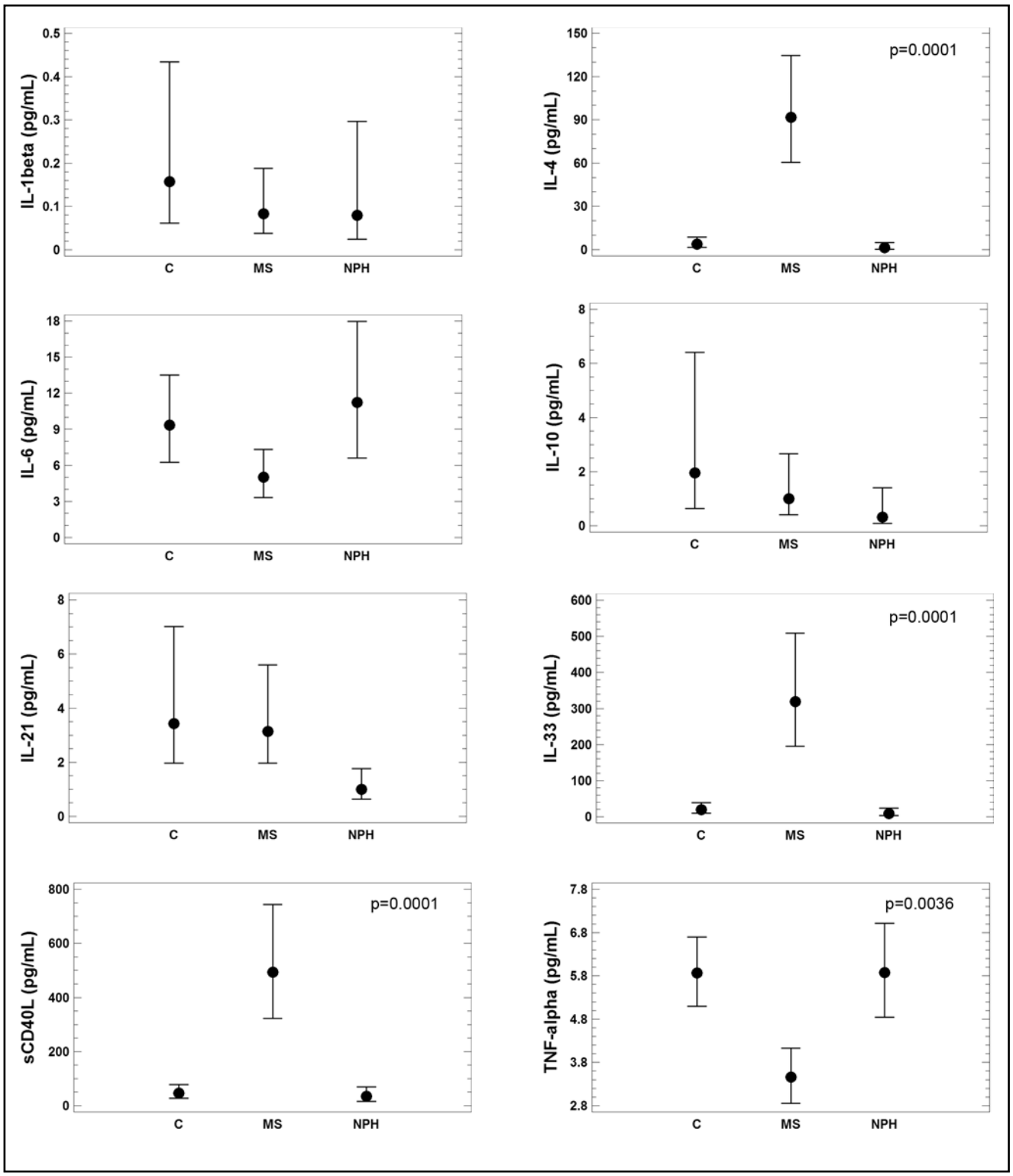

Fig. 2. The plasma levels (pg/mL) of cytokines in patients with multiple sclerosis (MS) and normal pressure hydrocephalus (NPH) in comparison with the control group. Means and 95.0 percent confidence intervals are presented. The statistical important significances are indicated above graphs.

articles. The increased CSF IL-10 levels were previously described also in NPH and this cytokine was reported to be important in the disease diagnosis as well as progression (Sosvorova *et al.* 2014; Sosvorova *et al.* 2015).

Together with the increased levels of CSF IL-10, also increased CSF IL-1β and IL-6 were estimated in NPH compared to the MS patients as well as the controls. Increased levels of these cytokines were also reported to be elevated in our previously published study com-

paring NPH subjects and subjects, where the NPH diagnosis was excluded. (Sosvorova *et al.* 2014). Taking into account the immune background of MS, the significantly increased CFS levels of these pro-inflammatory cytokines in NPH are surprising. The results reflected the inflammatory basis of NPH and one may consider that the enlarging brain ventricles may activate the production of IL-10 in NPH affected brain.

The brain production of IL-1β and IL-6 is upregulated by TNF-α, important pro-inflammatory cytokine. TNF-α is a key initiator of the immune-mediated inflammation in multiple organ and systems, including brain. This cytokine is responsible for neurotoxicity, neuronal death and neuronal dysfunction (Tobinick *et al.* 2006). It can be synthesized in the CNS and its expression increases in number of chronic neurodegenerative disorders (Viviani *et al.* 2004). CSF TNF-α levels were reported to be elevated in NPH (Tarkowski *et al.* 2003b) and MS (Sharief & Hentges 1991; Maimone *et al.* 1991) as well as in other neurodegenerative disorders, i.e. Parkinson's disease (Mogi *et al.* 1994), Alzheimer's disease and vascular dementia (Tarkowski *et al.* 2003a).

Together with the major pro- and anti-inflammatory cytokines found to be elevated in NPH, also the CSF IL-21 levels were increased. IL-21 is produced by activated T-cells and has regulatory effects on all classes of lymphocytes (B, T and NK cells) (Parrish-Novak *et al.* 2000; Wang *et al.* 2003). IL-21 has been associated with various inflammatory and autoimmune diseases, including the neurological ones. The levels of IL-21 were significantly increased in MS, neuromyelitis optica and models of experimental inflammation (Wu *et al.* 2012; Nohra *et al.* 2010). According to the authors' best knowledge the levels of IL-21 in NPH patients have been first reported to be elevated in this study. The increased CSF levels in NPH patients support the hypothesis of inflammatory changes in NPH.

IL-33 is a newly described member of the IL-1 family. It is expressed by many cell types following pro-inflammatory stimulation and is thought to be released during cell lysis (Liew *et al.* 2010). It has been proven, that IL-33 plays an important role in inflammation, infection and autoimmune diseases. There are evidence, that IL-33 may change some of the symptoms of various autoimmune disorders including MS. The highest expression of IL-33 was observed in brain and spinal cord (Schmitz *et al.* 2005). This cytokine can induce proliferation of brain microglia and also improve the expression of pro-inflammatory IL-1β and TNF-α, while increasing the expression of anti-inflammatory IL-10 at the same time. Christophi *et al.* documented for the first time the elevated Il-33 levels in both periphery and CNS in MS patients compared to healthy subjects, implicating IL-33 in the pathogenesis of MS (Christophi *et al.* 2012). Ours, as well as the other author's results, supported the finding of elevated IL-33 in the whole body system in MS (Zhang *et al.* 2014). The results of the current study are in accordance with the previously published research and support the important role of IL-33 in the pathogenesis of MS (Zhao & Chen 2014; Zhang *et al.* 2014; Christophi *et al.* 2012). In NPH, the IL-33 levels were not altered in comparison with these ones in MS patients and controls. However, decreased CSF IL-33 levels were reported after the shunt insertion, reflecting the attenuation of the inflammation processes after the surgical intervention leading to amelioration of NPH (Sosvorova *et al.* 2015).

The soluble CD40 ligand (sCD40L) levels in MS subjects were also studied. This cytokine is a platelet-derived mediator with pro-inflammatory, procoagulant and immunomodulatory functions. It serves as a link between inflammation, hemostasis and vascular dysfunction. Soluble CD40 ligand belongs together with its receptor CD40 to TNF family. Legation of CD40 leads to a wide range of immune responses including cytokine secretion (IL-10), proliferation and differentiation of B cells (Peters *et al.* 2009; Schlom *et al.* 2013; Wykes 2003). It was observed, that circulating sCD40L levels are associated with a poor outcome in diseases with combined inflammatory and vascular pathology (Johansson *et al.* 2012). Burman *et al.* published increased CSF sCD40L levels in patients suffering from other non-inflammatory neurological diseases (Burman *et al.* 2014). Our results showed decreased CSF sCD40L levels in MS compared to the other investigated groups. However, the plasma sCD40L levels were significantly higher in MS compared to other examined groups in our study. The results are in accordance with the research performed by Johansson *et al.* (Johansson *et al.* 2012).

CONCLUSION

We determined selected cytokines in the CSF and plasma of patients with MS, NPH and controls. Our results showed increased CSF levels of IL-1β, IL-6, IL-10, IL-21 and TNF-α in NPH subjects when compared with MS patients and controls. These results suggest that the enlarged brain ventricles in NPH subjects may repress and activate some brain structures to produce the mentioned cytokines. These findings indicate the inflammatory basis in NPH affected brain. Regarding the patients with multiple sclerosis, we have confirmed increased IL-33 levels in the CNS and periphery of these patients.

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