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The cerebrospinal fluid cytokine signature of multiple sclerosis: A homogenous response that does not conform to the Th1/Th2/Th17 convention

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ABSTRACT

In this cross-sectional study, we wanted to identify key cytokines characteristic of different stages of multiple sclerosis (MS). To this end, cerebrospinal fluid from patients with MS was investigated with a multiplexed fluorescent bead-based immunoassay. In total 43 cytokines were assessed and related to clinical and imaging data. Increased levels of CCL22, CXCL10 and sCD40L characterized relapsing–remitting MS patients with the presence of gadolinium-enhancing lesions; decreased CCL2 and increased CXCL1 and CCL5 were typical of relapsing–remitting MS patients irrespectively of the presence of gadolinium-enhancing lesions. These homogenous patterns of cytokine activation do not conform to conventional Th1/Th2/Th17 responses.

1. Introduction

Multiple sclerosis (MS) is a complex disease with inflammation and degeneration in the central nervous system (CNS). Inflammation is considered to be most important in the early stages of this disease, when transient loss of neurological function due to inflammatory damage to the CNS is followed by periods of recovery, in which inflammation is low-grade or absent (relapsing–remitting MS, RRMS).

Eventually, most patients will develop a secondary progressive course (SPMS), characterized by, fewer and milder relapses (Lublin and Reingold, 1996) but incessant deterioration of function (Confavreux et al., 2003). It is widely believed that a degenerative process is predominant in this stage of the disease. However, it has been hypothesized that inflammation carries on in a compartmentalized state (Meinl et al., 2008) which is supported by some evidence from histopathological studies (Hochmeister et al., 2006; Frischer et al., 2009).

Cytokines play a pivotal role in the regulation of inflammatory responses and tissue repair. They orchestrate all phases of immune responses and act in highly complex, dynamic networks in a paracrine and/or autocrine fashion. Immune responses have been characterized by the pattern of cytokines produced; the distinction between Th1 and Th2 type responses is the classical example (Mosmann et al., 1986). MS has been associated with the Th1 response and the more recently discovered Th17 type response (Sospedra and Martin, 2005; Segal, 2010). Of late, the stereotyped cytokine responses have come into question and it has been theorized that the tissue shapes the immune response locally and Th1 and Th2 responses are only crude simplifications (Matzinger and Kamala, 2011).

The multiplex bead assay technology provides an opportunity to measure a multitude of analytes simultaneously in a small sample volume. The analytes are captured by spectrally distinct microbeads and tagged with fluorescent-labeled markers, which are excited and detected by flow cytometry (Nolan and Mandy, 2001). This technology enables a systematic and unbiased study of cytokine signatures in different stages of MS.

The purpose of this cross sectional exploratory study was to investigate whether and how different stages of MS are characterized by different patterns of cytokines. To this end, cerebrospinal fluid (CSF) from MS patients and controls were analyzed and the levels of 43 different cytokines were determined.

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2. Material and methods

2.1. Ethics statement

The study was approved by the ethics committee of Uppsala University (DNr 2008/182). All subjects provided written informed consent.

2.2. Subjects

An outpatient clinic cohort comprised of 37 RRMS patients and 19 SPMS patients was used. All patients met the revised McDonald's criteria for MS diagnosis (Polman et al., 2011). SPMS patients were clinically deteriorating in the absence of clinical relapses. No transitional cases of SPMS were included. Ten controls were used. They were patients with other, non-inflammatory, neurological disease (*e.g.* thunderclap headache or idiopathic intracranial hypertension) or patients investigated for symptoms mimicking neurologic disorder where no signs of disease could be demonstrated with clinical examination, MRI, and routine CSF analysis.

All patients underwent a clinical evaluation and lumbar puncture at inclusion. Clinical relapses were assessed by an experienced neurologist (JB). A clinical relapse was defined as new or recurrent neurologic symptoms lasting ≥24 h, not associated with fever or infection. To identify inflammatory disease activity, MRI of the brain was performed within one week of the lumbar puncture. All but five patients underwent MRI of the entire spinal cord as well. Three patients repeated MRI and lumbar puncture after three weeks and three months.

Seventeen RRMS patients were on treatment (glatiramer acetate, n = 5; beta interferon, n = 5; IVIG, n = 3; natalizumab, n = 3, mitoxantrone, n = 1) as was one SPMS patient (natalizumab, n = 1). No subject was treated with corticosteroids within one month before sampling. The characteristics of subjects are summarized in Table 1.

2.3. CSF collection

Following lumbar puncture, CSF was collected in a polypropylene tube and centrifuged at 250 ×*g* for 5 min. The supernatant was pipetted off, gently mixed, and aliquoted in polypropylene tubes stored at −80 °C without being thawed and re-frozen.

2.4. MR examinations

MRI was performed on all patients at 1.5 T using the same imager and imaging protocol in all examinations. Gadopentetate dimeglumine (Magnevist®, Bayer AG, Leverkusen, Germany; 0.4 mL/kg body weight, *i.e.* double dose) was used as a contrast agent. The number of gadolinium enhancing (Gd+) lesions was counted on T1-weighted images. At least one Gd+ lesion was found in 21/37 RRMS patients; 12 of them had a clinical relapse with onset ≤1 month before inclusion. In 2/19 SPMS patients, one Gd+ lesion could be detected.

2.5. Multiplexed fluorescent bead-based immunoassays

Two different kits for cytokine detection and measurement were used: a 25-plex HTH17MAG-14K-25 kit and an 18-plex HCYTOMAG-60K-18 kit (both Merck Millipore, Darmstadt, Germany). The HTH17MAG-14K-25 kit has been designed to detect cytokines associated with Th17 type immunity and contained the following analytes: IL-1b, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-31, IL-33, GM-CSF, IFN-γ, MIP-3a (CCL20), TNF-α and TNF-β. The HCYTOMAG-60K-18 kit was designed to detect mainly chemokines and contained the following analytes: CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CCL11 (eotaxin), MDC (CCL22), CX3CL1 (fractalkine), CXCL1 (GRO), CXCL10 (IP-10), EGF, FGF-2, Flt-3 ligand, PDGF-AA, PDGF-AB/BB, sCD40L, TGF-α and VEGF.

Samples were prepared according to the manufacturer's instructions, with the addition of one extra standard point. The samples were run in duplicates on a Magpix machine (Luminex, Austin, TX, U.S.A.) on two separate plates. Care was taken to balance the number of controls and patients in different stages of MS on each plate to minimize the potential effects of inter-plate variance.

2.5.1. Quality of analysis

The manufacturer provides two standardized quality controls for each analyte (high and low), which were used on each plate. The values of these fell within the specified range for all quality controls with exception for one quality control of MIP-3a, TNF-α and FGF-2 and two quality controls of IL-6, IL-10 and sCD40L. Notably, both aberrant quality controls of sCD40L were just below the lower limits on one plate. The pooled CVs of intra-assay CVs were 3.75 (IL-15); 2.79 (IL-27); 2.32 (CCL2); 1.48 (CCL5); 2.90 (CCL22); 1.68 (CXCL1); 2.06 (CXCL10); and 4.89 (sCD40L). A CV of 0 was replaced with the lowest recorded value.

2.5.2. Quality control

As a further quality control, samples were sent to another laboratory to be analyzed for CCL2 with a well-established sandwich immunochemical method using electrochemiluminescence detection (Meso Scale Discovery, MSD Human MCP-1, Meso Scale Discovery, Gaithersburg, MD). Although absolute values were about 1.7 times higher with the fluorescent beads, the relative differences were similar, and the correlation between values analyzed with the two methods was very strong (Spearman $r = 0.95$, $p < 0.0001$, Appendix A, Fig. A1).

2.6. Statistical analysis

Statistical analyses were done with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). For establishing a statistically significant difference between two groups, the Mann–Whitney test was used; for statistical significance between three or more groups, the Kruskal–Wallis test was used. Dunn's multiple comparison test was used for *post hoc* analysis. Correlations were described with Spearman's rank correlation coefficient. A two-tailed p value of <0.05 was considered significant.

Table 1
Clinical data.

	Controls	Gd− RRMS	Gd+ RRMS	SPMS
n	10	16	21	19
Female/male	7/3	12/4	15/6	10/9
Age, years (range)	33.5 (19–65)	39.5 (28–70)	37 (18–51)	62 (35–68)
EDSS (range)	n/a	2 (0–3.5)	2 (0–7.5)	6 (3–7.5)
Disease duration, months (range)	n/a	123 (6–346)	57 (1–342)	280 (113–538)
Duration of progressive disease, years (range)	n/a	n/a	n/a	11 (6–26)
Clinical relapse < 1 month	n/a	0/16	11/21	0/19
On treatment	n/a	7/16	10/21	1/19

Gd+, gadolinium enhancing; RRMS, relapsing–remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; EDSS, expanded disability status scale. Age, EDSS and disease durations are expressed as medians.