Identification of soluble TREM-2 in the cerebrospinal fluid and its association with multiple sclerosis and CNS inflammation

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Triggering receptor expressed on myeloid cells 2 (TREM-2) is a membrane-bound receptor expressed by microglia and macrophages. Engagement of TREM-2 on these cells has been reported to reduce inflammatory responses and, in microglial cells, to promote phagocytosis. TREM-2 function is critical within the CNS, as its genetic deficiency in humans causes neurodegeneration with myelin and axonal loss. Blockade of TREM-2 worsened the mouse model for multiple sclerosis. In the present study, a soluble form of TREM-2 protein has been identified by immunoprecipitation and by ELISA. Soluble TREM-2 protein (sTREM-2) was detected in human CSF, and was compared among subjects with relapsing-remitting multiple sclerosis (RR-MS; n = 52), primary progressive multiple sclerosis (PP-MS; n = 21), other inflammatory neurologic diseases (OIND; n = 19), and non-inflammatory neurologic diseases (NIND; n = 41). Compared to NIND subjects, CSF sTREM-2 levels were significantly higher in RR-MS (P = 0.004 by ANOVA) and PP-MS (P < 0.001) subjects, as well as in OIND (P < 0.001) subjects. In contrast, levels of sTREM-2 in blood did not differ among the groups. Furthermore, TREM-2 was detected on a subset of CSF monocytes by flow cytometry, and was also highly expressed on myelin-laden macrophages in eight active demyelinating lesions from four autopsied multiple sclerosis subjects. The elevated levels of sTREM-2 in CSF of multiple sclerosis patients may inhibit the anti-inflammatory function of the membrane-bound receptor suggesting sTREM-2 to be a possible target for future therapies.

Keywords: multiple sclerosis; neuroinflammation; microglia; macrophages; immune regulation

Abbreviations: DC = dendritic cells; EAE = experimental autoimmune encephalomyelitis; EDSS = Expanded Disability Status Score; MSSS = multiple sclerosis severity score; NHD = Nasu–Hakola Disease; NIND = non-inflammatory neurological disease; OIND = other inflammatory neurological disease; PP-MS = primary progressive multiple sclerosis; RR-MS = relapsing remitting multiple sclerosis; sTREM-2 = soluble TREM-2; TREM-2 = triggering receptor expressed on myeloid cells-2


Introduction

Triggering receptor expressed on myeloid cells 2 (TREM-2) is an innate immune receptor expressed on the surface of some myeloid cells, including microglia, macrophages, monocyte-derived dendritic cells (DC) in vitro, and osteoclasts. The natural ligand of TREM-2 has not yet been identified. Until now, TREM-2 has been described as a membrane-bound receptor associated with the adaptor protein DAP12, which delivers its intracellular signal through an immunoreceptor tyrosine-based activation motif (ITAM). Mutations in TREM-2 or DAP12 genes cause Nasu–Hakola disease (NHD), a genetic disorder that...
is characterized by fatal presenile dementia and bone cysts. Neuropathologic findings include loss of myelin and axons in the brain, with reactive astrocysis and microglial activation (Klunemann et al., 2005).

A diverse range of TREM-2 functions in myeloid cells is becoming elucidated. In microglial cells, TREM-2 promotes the phagocytosis of apoptotic neurons and resolution of inflammation (Takahashi et al., 2005). TREM-2 expression has been reported on alternatively activated (also called M2 or type II) macrophages in vitro (Turnbull et al., 2006). In this regard, two recent studies have demonstrated that TREM-2 activation delivers an inhibitory signal that attenuates the inflammatory response in macrophages stimulated through toll-like receptors (Hamerman et al., 2006; Turnbull et al., 2006). These data unveil an inhibitory effect of the TREM-2/DAP12 complex on macrophage activation, despite the presence of the cytoplasmic DAP12 activating ITAM motif (Klesney-Tait et al., 2006).

Our group and another have recently demonstrated that TREM-2 expression on microglia and macrophages in the CNS exerts a protective effect during experimental autoimmune encephalomyelitis (EAE), the leading animal model of multiple sclerosis. Blockade of TREM-2 during the EAE effector phase worsened EAE (Piccio et al., 2007), while the injection at disease peak of myeloid cells genetically modified to over-express TREM-2 limited tissue destruction (Takahashi et al., 2007).

The present study describes for the first time a human soluble TREM-2 variant protein. A soluble protein has been characterized for TREM-1 (Gibot et al., 2004a), the first TREM receptor identified, and putative transcripts for soluble forms have been described for other receptors in this family, including TREM-2 (Schmid et al., 2002; Gattis et al., 2006). Herein, soluble TREM-2 (sTREM-2) protein was detectable in the cerebrospinal fluid (CSF) and serum, and was elevated in the CSF of subjects with multiple sclerosis and other CNS inflammatory diseases compared to subjects with non-inflammatory neurological diseases. The release of soluble forms may represent a mechanism for counter-regulation of the TREM receptors (Klesney-Tait et al., 2006). In the present studies, TREM-2 receptor was also detected on CSF monocytes, and this is the first demonstration of TREM-2 on human cells in vivo. Furthermore, TREM-2 receptor was also demonstrated on myelin-laden ‘foamy’ macrophages in actively demyelinating multiple sclerosis lesions in tissues of autopsied patients. Foamy macrophages are believed to be important for resolution of CNS inflammation. The findings reported herein support a possible role of TREM-2 and its soluble form in CNS inflammatory diseases, including multiple sclerosis.

Materials and Methods
Subjects and tissue sampling
This study was performed at Washington University in St Louis, Missouri under appropriate Institutional Review Board approval. CSF and blood samples were obtained from patients of the Washington University Department of Neurology except for ten CSF and serum specimens included in the study that were collected at the Department of Neurological Sciences, University of Milan, Italy. Written consent was obtained from all participants.

CSF samples were collected during diagnostic procedures from 52 subjects with relapsing-remitting multiple sclerosis (RR-MS) and 21 subjects with primary progressive multiple sclerosis (PP-MS). All subjects with multiple sclerosis fulfilled the McDonald criteria for the diagnosis (McDonald et al., 2001). Twenty-six RR-MS subjects were on disease modifying therapy for multiple sclerosis (interferon-β) or glatiramer acetate, one was taking corticosteroids, and one was taking azathioprine. PP-MS subjects were not taking any immunomodulatory or immunosuppressive medications for multiple sclerosis. The Expanded Disability Status Score (EDSS) (Kurtzke, 1983) and the Multiple Sclerosis Severity Score (MSSS) (Roxburgh et al., 2005) were calculated for multiple sclerosis subjects from clinical records by an experienced clinician (AHC) without knowledge of the TREM-2 results (Table 1). CSF samples were also obtained from 19 subjects with other inflammatory neurological diseases (OIND), including viral meningitis, encephalitis, optic neuritis, neuromyelitis optica and acute disseminated encephalomyelitis, and from 41 subjects with non-inflammatory neurological diseases (NIND), including headache, degenerative disk disease, normal pressure hydrocephalus, seizure disorder and stroke or small vessel disease. All NIND subjects had either a normal brain MRI or evidence of small vessel disease or stroke, and a CSF analysis without evidence of CNS inflammation or autoimmune process such as intrathecal immunoglobulin (Ig) production or presence of oligoclonal Ig. All OIND subjects showed evidence of CSF inflammation with increased leukocyte count and protein with exception of one subject with recurrent optic neuritis included in this group who had a normal CSF analysis (Table 1).

Routine laboratory tests on CSF (cell count, protein, glucose) and immune tests (IgG, IgG index, oligoclonal Ig, albumin ratio) were performed through the Barnes-Jewish Hospital laboratory except for 10 specimens that were analysed at the Ospedale Maggiore Policlinico, Milan, Italy. Paired serum samples were also collected from 44 RR-MS subjects, 19 PP-MS, 30 NIND and 10 OIND. CSF and serum samples were centrifuged at 10 000g to remove cells before storage. CSF samples were also filtered through a 0.22 μm filter. All specimens were then frozen in aliquots at −80°C until analysis.

Brain tissues
Studies were performed on fresh-frozen brain and spinal cord tissues taken at autopsy from four female subjects with multiple sclerosis—two with PP-MS and two with secondary progressive multiple sclerosis. Post-mortem interval was within 5 h in all four cases. CNS areas studied included brainstem, periventricular white matter, cerebellum and spinal cord. Eight active demyelinating multiple sclerosis lesions were identified by haematoxylin-eosin/Luxol fast blue-periodic acid-Schiff + (LFB-PAS+) histochemistry and by the presence of macrophages containing myelin debris, and were confirmed as active by a neuropathologist (RES). Oil red O staining was performed to identify macrophages filled with neutral lipids as a product of myelin degradation.
**Table I** Patient characteristics and CSF findings

<table>
<thead>
<tr>
<th></th>
<th>RR-MS</th>
<th>PP-MS</th>
<th>NIND</th>
<th>OIND</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td>52</td>
<td>21</td>
<td>41</td>
<td>19</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>31/21</td>
<td>10/11</td>
<td>31/10</td>
<td>8/11</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>45/7</td>
<td>19/2</td>
<td>34/7</td>
<td>15/5</td>
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<tr>
<td>Age at LP, years</td>
<td>38 ± 10</td>
<td>54 ± 9</td>
<td>44 ± 15</td>
<td>41 ± 17</td>
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<tr>
<td>EDSS at LP</td>
<td>3.5 (1–6.5)</td>
<td>6 (2–9)</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>MSSS Therapy</td>
<td>7.6 (4.8–9.7)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>None</td>
<td>24</td>
<td>21</td>
<td>NA</td>
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<td>Steroids</td>
<td>1</td>
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<td>NA</td>
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<td>1</td>
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<td>DMT</td>
<td>26</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CSF soluble TREM-2</td>
<td>0.90 ± 0.55</td>
<td>1.08 ± 0.81</td>
<td>0.43 ± 0.23</td>
<td>1.5 ± 1.04</td>
</tr>
<tr>
<td>Median (range)</td>
<td>0.7 (0.1–2.5)</td>
<td>0.9 (0.2–3)</td>
<td>0.3 (0.1–1)</td>
<td>1.4 (0.1–3.8)</td>
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<tr>
<td>P-value compared with NIND</td>
<td>P = 0.004</td>
<td>P = 0.0005</td>
<td>P = 0.0005</td>
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<tr>
<td>Serum soluble TREM-2</td>
<td>0.72 ± 0.14 (n = 44)</td>
<td>1.05 ± 0.78 (n = 19)</td>
<td>1.09 ± 1.63 (n = 30)</td>
<td>1.13 ± 1.03 (n = 10)</td>
</tr>
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<td>Median (range)</td>
<td>0.5 (0–6.8)</td>
<td>0.7 (0.1–2.8)</td>
<td>0.5 (0.8–8)</td>
<td>0.6 (0.2–3.1)</td>
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<tr>
<td>P-value compared with NIND</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
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<td>TREM-2 index</td>
<td>0.43 ± 0.46</td>
<td>0.34 ± 0.48</td>
<td>0.21 ± 0.16</td>
<td>NA</td>
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<tr>
<td>CSF cell count per l ml</td>
<td>6 ± 1.2</td>
<td>3.1 ± 2.1</td>
<td>1.1 ± 1.2</td>
<td>1.9 ± 2.25</td>
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<tr>
<td>CSF/plasma albumin ratio</td>
<td>6.8 ± 4.1</td>
<td>4.9 ± 1.4</td>
<td>4.3 ± 1.9</td>
<td>10 ± 6.3</td>
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<tr>
<td>CSF IgG</td>
<td>6.8 ± 5.2</td>
<td>5 ± 3.3</td>
<td>2.1 ± 1</td>
<td>5.3 ± 3.6</td>
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<tr>
<td>CSF IgG index</td>
<td>1 ± 0.5</td>
<td>0.9 ± 0.5</td>
<td>0.5 ± 0.07</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Oligoclonal bands in CSF (positive/total)</td>
<td>4/1/52</td>
<td>19/21</td>
<td>0/41</td>
<td>1/0^*</td>
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<tr>
<td>CSF protein (mg/dl)</td>
<td>48.5 ± 26.4</td>
<td>38.5 ± 9.7</td>
<td>34.3 ± 16.5</td>
<td>100.5 ± 60</td>
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</tbody>
</table>

^aThe number of tested subjects is indicated in parenthesis; ^bFor nine subjects oligoclonal band test was not available; TREM-2 index was calculated as (CSF/TREM-2/SerumTREM-2)/(CSFalbumin/Serumalbumin). Numerical data are given as mean ± SD except EDSS and MSSS which are median (range) as well as where indicated. LP = lumbar puncture. DMT = disease modifying therapy (beta-interferon or glatiramer acetate); NA = not applicable/available.

**ELISA for soluble human TREM-2**

To quantify the levels of sTREM-2, an enzyme-linked immunosorbent assay (ELISA) was developed using two different monoclonal anti-TREM-2 antibodies directed against the extracellular portion of human TREM-2 protein. Soluble TREM-2 levels were determined by ELISA on all CSF and serum specimens collected during this study, and on cultured DC supernatants. Briefly, anti-human TREM-2 monoclonal antibody (mAb) (clone 20G2) (Bouchon et al., 2001) was used as capture antibody and coated overnight at 4°C on Maxisorp 96-well plates (Nalge Nunc International, Rochester, NY) at a final concentration of 5 μg/ml in sodium bicarbonate coating buffer (0.015 M Na$_2$CO$_3$ + 0.035 M NaHCO$_3$, pH 9.6). After washing, wells were blocked for 3 h at 37°C with PBS 10% fetal bovine serum (FBS). Freshly thawed CSF, serum, DC supernatants and standards were incubated in duplicate overnight at 4°C. Soluble human TREM-2-Fc was used as standard in all assays. For detection, biotinylated mouse anti-human TREM-2 mAb (clone 29E3) (Bouchon et al., 2001b) was diluted in assay buffer (PBS 5% FBS) at the concentration of 0.5 μg/ml and incubated for 1 h at room temperature (RT). After washing, wells were incubated with hors eradish peroxidase labelled streptavidin (BD Biosciences, San Jose, CA) for 1 h at RT. HRP visualization was performed with 3, 3’, 5, 5’-tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO) added to each well for 10 min at RT in the dark. Colour development was stopped by adding equal volume of 2.5 N H$_2$SO$_4$. Optical density of each well was determined at 450 nm. Washes between the different steps were done with PBS 0.1% Tween 20 (Sigma-Aldrich). An internal standard (IS), consisting of a single batch of human serum positive for sTREM-2 aliquotted and frozen, was run in all the assays. Soluble TREM-2 values for each CSF, serum and DC supernatant sample tested by ELISA were calculated relative to this IS in each assay (Fig. 2 and Table 1). The average value of the IS calculated over all assays was 2.6 ± 0.5 ng/ml (mean ± SD).

**Human DC cultures**

Peripheral blood mononuclear cells (PBMCs) were purified from human blood on Ficoll-Paque PLUS density gradient (Amersham Biosciences, Piscataway, NJ). Monocytes were isolated from PBMCs via positive selection using human CD14 + Microbeads (Miltenyi Biotec, Auburn, CA). To generate DCs, monocytes were cultured with 1,000 U/ml IL-4 and 50 ng/ml GM-CSF and 50 ng/ml IL-10 for 2 days. Supernatants from DC cultures were collected at different time points, centrifuged at 21,000 g for 15 min and filtered through a 0.22 μm filter to remove all cells and membrane debris. DC supernatants were then frozen in aliquots at −80°C until use.

**Flow cytometry**

In a subset of subjects with sufficient CSF available (29 NIND, 10 stable multiple sclerosis, three multiple sclerosis during relapse and six OIND), TREM-2 receptor expression was examined by flow cytometry on cells in freshly isolated paired CSF (10–20 ml) and blood (3 ml) samples. Total 10–20 ml of CSF were collected and kept on ice. Staining for flow cytometry was performed within 30 min after the lumbar puncture. CSF was first centrifuged at 300g for 15 min and supernatants collected, and then the pellet cells were gently re-suspended in PBS 2% FBS and stained.
A blood sample from the same subject was analysed in parallel. CSF and peripheral blood cells were stained with the anti-human TREM-2 mAb (clone 10B11) followed by the PE-conjugated goat anti-mouse IgG, (Southern Biotechnology Associates, Birmingham, AL). The APC-conjugated mouse anti-human CD14 and the FITC-conjugated mouse anti-human CCR5 were from BD Biosciences; FITC-conjugated mouse anti-human CD16 was from DiACLone (Besancon, France). Cells were analysed on a FACSCalibur cytometer using CELLQuest-pro software (Becton Dickinson). Dead cells were excluded by gating on propidium iodide-negative cells.

**Immunohistochemistry**

Eight active lesions from four multiple sclerosis autopsies were analysed. Frozen tissue was embedded in optimal cutting temperature (OCT). Eight micron thick sections were collected on slides, air-dried, fixed in acetone for 10 min and then incubated with 3% hydrogen peroxide for 10 min. Next, tissue sections were permeabilized with 0.1% saponin (Sigma-Aldrich) and non-specific antibody binding was blocked with 5% goat serum for 1 h at RT. Mouse anti-human TREM-2 mAb (clone 21E10) (Cella et al., 2003) was applied overnight at 4°C followed by a biotin conjugate anti-mouse IgG antibody (Southern Biotechnology Assoc.) for 1 h at RT. TREM-2 immunostaining was detected using a tyramide signal amplification kit (Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer’s instructions. Control sections were stained with a mouse IgG isotype control mAb or no antibody. Sections were also stained with the goat anti-human CD11b antibody (R&D Systems, Minneapolis, MN), specific for microglia and macrophages, for 1 h at RT. FITC-conjugated donkey anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used as a secondary antibody. Tissue sections were then mounted in Vectashield hard-set mounting medium with DAPI (Vector laboratories, Burlingame, CA) to stain the nuclei before microscopic analyses.

**Immunoprecipitations and western blots**

Freshly thawed DC supernatants or CSF aliquots were precleared for 1 h at 4°C with protein G-sepharose 4B (Sigma-Aldrich), and then soluble TREM-2 protein in these fluids was immunoprecipitated overnight at 4°C with 3 μg of purified anti-TREM-2 mAbs (clone 20G2 and 29E3) or mouse IgG1 isotype control antibody (BD Biosciences) previously conjugated to protein G-sepharose 4B. Precipitates were washed four times in cold phosphate buffer saline (PBS) with 1% Triton and 2 μg/ml aprotinin (Sigma-Aldrich), separated by SDS–PAGE, transferred to nitrocellulose membranes and probed with the mouse anti-TREM-2 mAbs supernatants (clones 10B11 and 21E10). In some experiments the immunoblot was performed with the polyclonal goat anti-TREM-2 antibody (R&D systems). In deglycosylation experiments the precipitates were treated for 3 h at 37°C with the enzymatic protein deglycosylation kit (Sigma-Aldrich), according to manufacturer’s protocol, to remove N-linked and O-linked carbohydrates.

**Statistical analyses**

Data sets were analysed to determine whether they were parametric. To compare two groups, a two-tailed t-test was used when data were parametric, applying the Welch’s correction factor for unequal variances when appropriate, and using the Mann–Whitney test when the data were nonparametric. Soluble TREM-2 CSF data were log-transformed to approximate a Gaussian distribution prior to analyses by ANOVA, with adjustments for multiple comparisons using the Hochberg method. Comparisons of the proportion of TREM-2 positive monocytes in CSF and serum sTREM-2 were done using the Kruskal–Wallis test for non-parametric data with adjustment for multiple comparisons using Dunn’s test. For comparisons of proportions of subjects in each group with positive TREM-2 CSF monocytes, a Fisher’s exact test was used. For analyses of correlations, either Pearson’s test (for parametric data) or Spearman’s test (for non-parametric data) were used. P<0.05 was considered significant.

**Results**

**Identification of soluble TREM-2 protein in human CSF and serum**

TREM-2 has previously been described only as a membrane-bound receptor on cultured myeloid cells, including human monocyte derived DCs, but the possibility of a soluble form has been suggested (Begum et al., 2004; Melchior et al., 2006). To investigate the existence of a soluble TREM-2 (sTREM-2) form at the protein level, human DCs were generated *in vitro* from blood monocytes by culturing with IL-4 and GM-CSF. TREM-2 was immunoprecipitated from the filtered supernatant using a monoclonal anti-human TREM-2 antibody directed against the extra-cellular TREM-2 portion (Fig. 1A and B). This novel sTREM-2 protein had molecular weights ranging between about 24 and 40 kDa. After O- and N-deglycosylation the sTREM-2 was reduced to a single 20 kDa protein, suggesting that the varied size was due to different levels of glycosylation (Fig. 1A). In the supernatants of cultured DCs from two different time points, Day 6 and Day 10 in culture, it was observed that the amount of sTREM-2 protein increased with time (Fig. 1B).

The presence of the sTREM-2 protein was also assayed in the CSF. A soluble form of the protein was detectable, as demonstrated by immunoprecipitation of TREM-2 from the CSF of a subject with NIND (chronic headache) (Fig. 1B). Supernatants from human macrophages generated *in vitro* from macrophage colony-stimulating factor (M-CSF)—treated blood monocytes were also positive for sTREM-2 (data not shown).

The same specimens (CSF and supernatants from DCs) previously tested by immunoprecipitation were then measured by ELISA. ELISA results were consistent with the qualitative levels observed in the immunoprecipitations (Fig. 1C). Soluble TREM-2 was also detected in human serum by ELISA (data not shown), but was undetectable in the serum of a patient with NHD (Fig. 1C), which was characterized by a TREM-2 mutation that leads to a truncated protein (Paloneva et al., 2002; Salmaggi et al., 2003).
Soluble TREM-2 protein is elevated in the CSF of subjects with multiple sclerosis and with OIND

Levels of sTREM-2 protein were measured by ELISA in the CSF and serum of subjects with RR-MS, PP-MS, non-inflammatory neurologic diseases (NIND) and other inflammatory neurologic disease (OIND). CSF from 52 subjects with RR-MS, 21 with PP-MS, 41 with NIND and 19 with OIND were studied. Serum samples matched with the CSF specimens were available for 44 of the RR-MS subjects, 19 PP-MS subjects, 30 subjects with NIND and 10 with OIND. Among the RR-MS subjects, 18 CSF samples were obtained during a relapse. NIND subjects served as a non-inflammatory comparison group. Clinical and CSF characteristics of the subjects are summarized in Table 1. Soluble TREM-2 levels in the CSF, given as a ratio to a positive IS, were significantly higher in RR-MS (mean ± SD: 0.9 ± 0.55; P = 0.004) and PP-MS subjects (1.08 ± 0.81; P = 0.0005) compared with subjects with NIND (0.43 ± 0.23) (Fig. 2A and Table 1). OIND subjects also had significantly higher CSF sTREM-2 levels (1.5 ± 1.04) than NIND subjects (P = 0.0005). sTREM-2 levels were not significantly different between the RR-MS and PP-MS subjects (P = 0.49; Fig. 2A). Relapsing RR-MS subjects or those subjects on immunomodulatory/immunosuppressant therapy for multiple sclerosis did not differ in sTREM-2 levels compared with the other multiple sclerosis subjects. Serum samples were available to measure sTREM-2 in blood from most of the subjects from whom there were CSF samples. No significant difference in blood sTREM-2 was found among the four groups (mean ± SD: RR-MS 0.72 ± 1.04; PP-MS 1.05 ± 0.78; NIND 1.09 ± 1.63; OIND 1.13 ± 1.03) (Fig. 2B and Table 1).

The sTREM-2 levels in the CSF were not affected by age at lumbar puncture and, in multiple sclerosis subjects, were not correlated with disability (EDSS) (Kurtzke, 1983) or MS severity (MSSS) (Roxburgh et al., 2005). No correlations were found with the total CSF cell numbers and, in the multiple sclerosis groups, with the IgG index. However, there were significant positive correlations of CSF sTREM-2 with absolute concentration of IgG and with protein in CSF detectable as a single 20 kDa band. (B) sTREM-2 glycoprotein was identified in human CSF (from a NIND subject with chronic headache) following immunoprecipitation with anti-TREM-2 mAb (first lane from left). In the supernatants of human monocyte-derived DCs after 6 or 10 days in culture, the amount of sTREM-2 protein increased with time (second and third lanes). In A and B the bands at 25 and 50 kDa represent the light (Ig L) and heavy (Ig H) chains of Ig, respectively. Molecular weight markers and specific protein bands are indicated. (C) Levels of sTREM-2 in the same specimens tested in B were quantitated by ELISA with results that were consistent with the qualitative levels observed in the immunoprecipitations. STREM-2 was undetectable in the serum of a patient with a TREM-2 gene mutation (TREM-2^−/−) leading to a truncated protein. sTREM-2 values are reported as the ratio between the samples and a positive IS.
within each multiple sclerosis group and in the OIND group. These correlations were not seen in the non-inflammatory control group.

To assess for the possibility that CSF sTREM-2 was due to the abnormal transfer of sTREM-2 from blood to CSF, the group comparisons for sTREM-2 levels were re-done excluding those subjects with an albumin level in CSF relative to blood (albumin ratio) that was above the normal range. Elevated albumin ratios indicate a disturbance of the blood brain barrier (BBB) (Tibbling et al., 1977). The differences continued to be significant ($P_{RR-MS, NIND} = 0.001; P_{PP-MS, NIND} < 0.0001$).

Comparisons with the OIND group could not be done because albumin indices were not obtained for most of the OIND subjects. Also, a linear relationship between the quantity of sTREM-2 in the CSF and in matched serum samples from the same subject was not found ($r = 0.09$), and in 46 cases the CSF TREM-2 levels were higher than the corresponding serum levels. These results suggested production of sTREM-2 within the CNS.

To further investigate the possibility that sTREM derived from blood versus the CNS, the TREM-2 ratio (CSF sTREM-2/Serum sTREM-2) and the TREM-2 index, calculated as:

$$\frac{\text{CSF sTREM-2}}{\text{Serum sTREM-2}} / \frac{\text{CSF albumin}}{\text{Serum albumin}}$$

were determined for those subjects that did not show any signs of blood brain barrier disruption (albumin ratio in the normal range) (Table 1). TREM-2 ratio and TREM-2 index were compared between NIND subjects and multiple sclerosis subjects (combined RR and PP-MS). Statistically significant elevations of the TREM-2 ratio and the TREM-2 index in multiple sclerosis subjects compared to NIND subjects were observed ($P = 0.007$, Mann–Whitney test, and $P = 0.02$, $t$-test, respectively). Together, all of these findings strongly indicate that sTREM-2 was produced within the CNS rather than gaining access to the CSF via a damaged BBB.

**TREM-2 is expressed on cerebrospinal fluid monocytes, but not on blood monocytes**

TREM-2 receptor expression was evaluated on CSF cells immediately ex vivo from subjects with or without multiple sclerosis using flow cytometry. Normal CSF contains few leukocytes (0–5/mm$^3$) that are primarily lymphocytes, with a smaller proportion of monocytes. These cells are believed to derive from the circulating blood (Fishman, 1980). CSF monocytes were identified by flow cytometry using the marker CD14. TREM-2 was expressed on a subpopulation of CSF monocytes in some subjects, but was never expressed on CD14 negative CSF cells (Fig. 3A). In most cases TREM-2 expression on monocytes was analysed by flow cytometry on parallel CSF and blood samples obtained from the same subject. Consistent with prior reports (Bouchon et al., 2001b) TREM-2 was never detected on circulating blood monocytes (Fig. 3A). CD14 positive CSF monocytes also expressed CD16 and CCR5 (Fig. 3B), suggesting that these cells resemble mature tissue macrophages as previously shown (Ziegler-Heitbrock et al., 1993; Gordon and Taylor, 2005).

The proportions of CSF monocytes that were TREM-2 positive were compared among multiple sclerosis ($n = 13$), NIND ($n = 29$) and OIND ($n = 6$) subject groups. Despite a wide variability, the percentage of CSF monocytes expressing TREM-2 was significantly higher in subjects with NIND [median (range): 6% (0–61)] compared with multiple sclerosis subjects [0% (0–46), $P < 0.01$]. As some...
of the subjects had no detectable expression of TREM-2 on CSF monocytes, additional analyses were done comparing the proportion of subjects in each group with positive TREM-2 expression. In this regard, TREM-2 could be detected on CSF monocytes in 31% of subjects with multiple sclerosis (4 out of 13), and in 33% of subjects with OIND (two out of six), while 83% of NIND subjects had detectable TREM-2+ CSF monocytes (24 out of 29) (Fig. 4).

The proportion of subjects with TREM-2+ CSF monocytes was significantly different for the multiple sclerosis group vs. NIND (\(P = 0.003\), Fisher’s exact test) and for OIND vs. NIND (\(P < 0.03\)), but not for multiple sclerosis vs. OIND (\(P = 1.0\)). TREM-2 was not detected on CSF monocytes in any of the three multiple sclerosis subjects experiencing a relapse at the time of the lumbar puncture.

Factors within the CSF milieu could regulate TREM-2 on CSF monocytes thus explaining the observed broad variability of its expression. The presence of a CSF soluble factor(s) responsible for TREM-2 receptor down-regulation on the cell membrane was investigated. Human monocyte-derived DCs that expressed TREM-2 on the cell surface in vitro (Bouchon et al., 2001b) were cultured in media containing none, 50 or 100% of CSF obtained from subjects whose CSF monocytes did not express TREM-2. In three different experiments, after 24 h in culture, TREM-2 expression on DCs was not altered from baseline with
either 50 or 100% CSF (data not shown). The possibility of a CSF soluble factor(s) responsible for TREM-2 upregulation on CSF monocytes was also tested. In subjects whose CSF monocytes expressed TREM-2, TREM-2 negative monocytes isolated from the peripheral blood were cultured with varying percentages of CSF obtained from that same subject. In two different experiments, after 24 or 48 h in vitro, TREM-2 expression was not induced on the cell surface of blood monocytes (data not shown). It was concluded that TREM-2 expression on monocytes is unlikely to be modulated by soluble factors present in the CSF.

In 23 NIND, eight multiple sclerosis and five OIND subjects, TREM-2 receptor expression on monocytes was assayed by flow cytometry and sTREM-2 was measured by ELISA in the same CSF specimen. In these subjects, sTREM-2 levels did not correlate with either the number of CD14+ monocytes/ml of CSF ($r = -0.1, P = 0.49$) or with the number of TREM-2+ monocytes/ml in the CSF ($r = 0.1, P = 0.4$). This suggests that CSF monocytes are not likely to be the main source of the sTREM-2.

TREM-2 is highly expressed on foamy macrophages in actively demyelinating multiple sclerosis lesions

Given the high levels of sTREM-2 found in the CSF of multiple sclerosis subjects, TREM-2 expression was evaluated by immunohistochemistry in CNS sections displaying typical multiple sclerosis pathology and derived from autopsy tissues of four multiple sclerosis subjects. Active multiple sclerosis lesions, characterized by the presence of perivascular inflammatory infiltrates and demyelination (Fig. 5A–C), displayed numerous myelin-laden macrophages identified by their characteristic ‘foamy’ appearance and the presence of neutral lipids detected by Oil red O histochemistry (Fig. 5D–F). Dual-labelled immunohistochemistry with monoclonal anti-human TREM-2 and anti-CD11b to identify microglia and macrophages showed that TREM-2 was highly expressed on CD11b positive lipid-laden macrophages (Fig. 5G). Less intense TREM-2 expression, as determined by dim staining intensity, was detected on CD11b positive cells with the morphology of...
resident microglial cells in the normal appearing white matter (data not shown).

Discussion

The present study identified a novel soluble form of TREM-2 which was quantitated by ELISA in the CSF and serum of multiple sclerosis and non-multiple sclerosis subjects. Soluble TREM-2 was found to be significantly elevated in CSF of multiple sclerosis subjects, as well as in subjects with other inflammatory neurologic diseases, compared with subjects with non-inflammatory neurologic diseases. TREM-2 receptor expression was also demonstrated for the first time directly ex vivo on a subset of human monocytes isolated from the CSF, whereas blood monocytes were always TREM-2 negative. Moreover, TREM-2 receptor was found to be highly expressed in the human CNS on myelin-laden macrophages in actively demyelinating multiple sclerosis lesions.

TREM-2 expression in the human CNS has an important role as demonstrated by the fact that loss-of-function mutations of the TREM-2 gene result in neurodegeneration, with prominent myelin and axonal loss in CNS white matter. Although clinical and pathological features of TREM-2 deficiency have been described, studies to define TREM-2 distribution and function within the human CNS are still lacking. The present study was undertaken to characterize TREM-2 expression in the human CNS and its potential role in the most common human demyelinating disease, multiple sclerosis. Prior to this study, only one published paper had described TREM-2 expression in human microglia using immunohistochemistry (Sessa et al., 2004). Recently, an additional paper reported TREM-2 mRNA to be upregulated in chronic active multiple sclerosis lesions (Koning et al., 2007). However, to date most of what is known about TREM-2 expression and function in myeloid cells derives from mouse studies. Murine microglia and macrophages have been shown to express TREM-2 as a cell surface receptor (Takahashi et al., 2005; Turnbull et al., 2006). In microglia, TREM-2 has been shown to promote phagocytosis of apoptotic neurons and cell debris (Takahashi et al., 2005). In both microglia and macrophages, TREM-2 has been demonstrated to downregulate expression of inflammatory cytokines (Takahashi et al., 2005; Turnbull et al., 2006). Furthermore, two different studies have reported a protective role of TREM-2 in the animal model for multiple sclerosis (Piccio et al., 2007; Takahashi et al., 2007).

In the present study, TREM-2 was shown to be highly expressed by lipid-laden macrophages within active demyelinating multiple sclerosis lesions. These macrophages, also called 'foamy macrophages', originate from both resident microglia and infiltrating monocytes (Li et al., 1996). Foamy macrophages clear myelin and cell debris, and have anti-inflammatory characteristics, resembling alternatively activated type II macrophages (Boven et al., 2006). They are believed to promote resolution of CNS inflammation, which is critical for limiting damage in multiple sclerosis lesions (Boven et al., 2006). Moreover, studies have shown that TREM-2 deficient murine macrophages activated via toll-like receptors display enhanced inflammatory responses (Turnbull et al., 2006). The present findings further support the notion that TREM-2 is expressed by a subset of human macrophages and microglia with important protective functions for CNS homeostasis and during pathology. In this regard, several recent publications have begun to address a role for regulatory macrophages in human autoimmunity, and enhancement of type II monocytes may be a mechanism of action of one approved multiple sclerosis therapy (Weber et al., 2007).

Human studies have been limited by the failure to detect TREM-2 protein on human cells isolated directly ex vivo. DCs and osteoclasts differentiated in vitro from human monocytes have been shown to express TREM-2 on the cell surface (Bouchon et al., 2001b; Cella et al., 2003). The only demonstration of TREM-2 expression directly ex vivo has been on mouse alveolar macrophages and newly recruited peritoneal macrophages (Turnbull et al., 2006). The present study demonstrates TREM-2 expression on human monocytes that have been recruited to the CSF compartment, but not on blood monocytes obtained from the same subject at the same time. Human monocytes are heterogeneous, and differential expression of antigenic markers can distinguish monocyte subsets. On CD14+ human monocytes the expression of the cell surface marker CD16 (FcγRIII) and the chemokine receptor CCR5 defines a subset resembling mature tissue macrophages (Gordon and Taylor, 2005). In the present study, all CSF monocytes were found to be CD16+ CCR5+, in contrast to blood monocytes which are known to be predominantly negative for these two markers (Weber et al., 2000). Together, the above findings suggest that TREM-2 is expressed on a subtype of differentiated myeloid cells that have entered tissues and acquired features of tissue macrophages. The present data support the hypothesis that TREM-2 is expressed on a monocyte subset with a specific effector function.

TREM-2 expression on CSF monocytes was observed less often in subjects with multiple sclerosis or OIND compared with those with NIND. In vitro, TREM-2 expression on macrophages and DCs is rapidly abrogated after treatment with LPS or IFN-γ and can be induced with IL-4 (Turnbull et al., 2006). Although it might be postulated that CSF soluble factors modulate TREM-2 expression on CSF monocytes, our results did not support this hypothesis. Another possibility for the diminished level of TREM-2 expression by CSF monocytes in multiple sclerosis and OIND is that TREM-2 has been shed from their cell surface. However, lack of correlation of sTREM-2 levels with number of CSF monocytes does not support this either. Perhaps CSF sTREM-2 derives from TREM-2+ cells within CNS tissue.
In the present study sTREM-2 levels were increased in the CSF of multiple sclerosis and OIND subjects compared with subjects with NIND, with evidence of intrathecal production of the protein. Soluble TREM-2 might originate in two non-mutually exclusive ways: it might be shed by proteolytic cleavage of membrane-anchored TREM-2 molecules, or it may originate by alternative splicing of TREM-2 leading to secreted TREM-2. The finding of high concentrations of sTREM-2 in the supernatant fluid of cultured human monocyte-derived DC and macrophages indicates that it can be shed or secreted. Indeed, TREM-2 transcripts originated via differential splicing lacking the transmembrane domain and corresponding to a putative sTREM-2 variant have been described in human and mouse myeloid cells (Schmid et al., 2002; Begum et al., 2004; Melchior et al., 2006). The predicted molecular weight of this putative secreted sTREM-2 protein would be ~27 kDa. However, the molecular mass of the deglycosylated soluble protein in the present data was only ~20 kDa and this suggests that alternative splicing may not be the source of sTREM-2. Moreover, the soluble form of the cognate receptor TREM-1 has been recently demonstrated to result from proteolytic cleavage and shedding of the TREM-1 ectodomain by metalloproteinases (Gomez-Pina et al., 2007). Preliminary observations made by our group suggest that a similar mechanism may exist for TREM-2.

The release of soluble forms of TREM receptors could represent a mechanism to counter-regulate their activity. According to this, sTREM-2 may act as a soluble ‘decoy’ receptor for the endogenous ligand, effectively inhibiting engagement of the ligand for the TREM-2 receptor. Supporting this possibility, a chimeric TREM-2-Fc protein was able to interfere with the function of TREM-2 on cultured osteoclasts during osteoclastogenesis (Kim et al., 2005). In addition, the soluble form of the TREM-1 receptor has been shown to attenuate the inflammatory response and improve survival in animal models of septic shock by inhibiting the TREM-1 receptor-mediated amplification of the inflammatory response in neutrophils and monocytes (Bouchon et al., 2001a; Gibot et al., 2004b). Therefore the hypothesis that sTREM-2 functions to counteract cellular TREM-2 receptor activity in vivo seems to be the most compelling. TREM-2, as well as TREM-1, may be one of several membrane-bound proteins that also exist as soluble forms originating via proteolytic cleavage, which serves to provide additional post-translational regulation of their activity (Garton et al., 2006). In some cases, soluble ectodomains are biological active as mediators of functions ascribed to their counterparts expressed by cells (Garton et al., 2006). However, since the nature of TREM-2 ligand(s) is still elusive, it remains premature to speculate extensively on sTREM-2 function.

In conclusion, in this study TREM-2 receptor expression has been demonstrated on myelin-laden macrophages within active multiple sclerosis lesions and, for the first time, on human CSF monocytes directly ex vivo. TREM-2 was expressed by a macrophage subset with known functions in clearing myelin debris and down-regulating inflammation. Most importantly, a novel soluble TREM-2 form has been identified at the protein level, and it was increased in the CSF in multiple sclerosis and other CNS inflammatory diseases in comparison to non-inflammatory conditions. Although sTREM-2 is not likely to be a diagnostic marker for multiple sclerosis, it might have a role in disease pathogenesis. Soluble TREM-2 may be involved in multiple sclerosis by inhibiting the presumed regulatory function of TREM-2 ligation on myeloid cells. The findings herein shed additional light on the biology of TREM-2 and its relation to human disease and suggest that TREM-2 and sTREM-2 may be targets for future therapeutics.

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