Haptoglobin deficiency facilitates the development of autoimmune inflammation

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Haptoglobin (HP) is an acute phase protein synthesized by liver cells in response to IL-6. HP has been demonstrated to modulate the immune response and to have anti-inflammatory activities. To analyze HP's effect on autoimmune inflammation, we here studied the course of EAE induced by immunization of Hp knockout (Hp-/−) and syngeneic WT mice with myelin oligodendrocyte glycoprotein peptide (MOG35–55). Hp-/− mice suffered from a more severe disease that was associated with increased expression of IL-17A, IL-6, and IFN-γ mRNA in the CNS and with a denser cellular infiltrate in the spinal cord. During the recovery phase, a significantly higher number of myeloid DC, CD8+ cells, IL-17+ CD4+ and IFN-γ+ CD4+ cells persisted in the CNS of Hp-/− mice. Absence of HP affected the priming and differentiation of T cells after MOG35–55 immunization, as levels of Th2 cytokines produced in response to MOG stimulation by Hp-/− T cells were reduced. These results suggest that HP plays a modulatory and protective role on autoimmune inflammation of the CNS.

Key words: Autoimmunity · Haptoglobin · EAE · MOG · Th17 cells

Introduction

Along with inflammation caused by injury, trauma, autoimmunity or infection, acute phase proteins (APP) are massively synthesized and released systemically [1]. This process is called the "acute phase response", and it is supposedly induced in order to minimize and repair tissue damage. APP expression is regulated by pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 [1–3]. Haptoglobin (HP) is an APP synthesized primarily by hepatocytes, and to a lesser extent in other tissues including lung, skin, spleen, and kidney under inflammatory conditions [3, 4]. Systemic and local concentrations of HP increase several-fold in response to the pro-inflammatory cytokine IL-6 [5]. One of the main functions of HP is to bind free hemoglobin, in order to facilitate its clearance by macrophages, to prevent damage caused by ROS generated by free hemoglobin, and to avoid the loss of iron through the kidney [6]. HP modulates both innate and adaptive immune responses. HP has been demonstrated to bind activated neutrophils and to inhibit several of their functions [7]. HP suppresses secretion of TNF-α, IL-10, and IL-12p70 by macrophages upon LPS triggering [8]. CD11b has been identified as a macrophage receptor for HP [9]. The HP-hemoglobin complex also binds the CD163 molecule on macrophages, leading to anti-inflammatory cytokine secretion [10]. HP acts on Langerhans cells of the skin, preventing their differentiation and function during in vitro culture [11]. HP affects proliferation and cytokine production by stimulated T cells and B cells [12]. Since HP rises during inflammatory processes in response to systemically released cytokines, and since it exerts various anti-inflammatory activities, the general picture is that HP has a feedback anti-inflammatory function. Such effects might also be relevant for the development and course of inflammatory diseases.

To determine whether HP might indeed have a protective role against autoimmunity, we induced EAE in Hp-deficient mice (Hp-/−). EAE is an extensively studied animal model of MS [13, 14]. EAE can be induced by immunization of mice with encephalitogenic myelin antigens in the presence of adjuvants. The disease process is initiated by activated myelin-specific T cells that migrate along with macrophages and DC into the brain and spinal cord, and cause inflammation and demyelination of the CNS,
leading to progressive ascending paralysis [14]. Pro-inflammatory cytokines such as IFN-γ, IL-23, IL-12, and IL-17 are thought to contribute to the pathogenesis of EAE, via inflammatory cell attraction and activation or by directly damaging the oligodendrocytes and myelin sheath [14–17]. The anti-inflammatory cytokines IL-4, IL-13, TGF-β, and IL-10 on the other hand ameliorate the inflammatory process, and are important for disease resolution and prevention of EAE [18–21]. The aim of the present study was to investigate the potential protective function of HP on the development, course and/or severity of autoimmune inflammation of the CNS.

Results

EAE is more severe in Hp-deficient mice

We induced EAE in WT and Hp−/− mice using myelin oligodendrocyte glycoprotein (MOG35–55) peptide emulsified in CFA. As shown in Fig. 1, Hp−/− mice developed more severe clinical symptoms compared with WT mice. A representative experiment is shown in panel A and the results of five pooled experiments are shown in panel B. The mean clinical score at day 21 was significantly higher in Hp−/− mice compared with WT mice (score in Hp−/− mice 2.36±1.07 versus WT mice 1.52±1.15, p = 0.009) (Fig. 1C). The onset of the disease (day on which mice reached clinical score = 1) for Hp−/− mice was at day 16.0±0.3 and for WT mice at day 17.4±0.4 (p = 0.01) (Fig. 1B). Spontaneous recovery occurred in WT and Hp−/− mice, but was delayed in Hp−/− mice (Fig. 1A). These results suggest that Hp deficiency promotes EAE.

Inflammatory cell infiltration into the CNS is more dense in Hp−/− mice

We performed histopathological analysis on spinal cords of WT and Hp−/− mice with EAE and compared the extent of the cellular infiltrate in these two groups (Fig. 2). This revealed a higher mean pathological score in Hp−/− mice compared with WT mice. For a detailed analysis of the cellular infiltrate in the CNS (Fig. 3), we isolated mononuclear cells from brain and spinal cord at different time points: at day 0 (no disease), at the onset (day 14 after immunization), at the peak (day 21 after immunization), and in the recovery phase (28 days after immunization) of the disease. We found similar kinetics of CNS infiltration in WT and Hp−/− mice, with the highest cell number for all the subpopulations at the peak of the clinical disease, and a decline thereafter. At the peak of the disease, the total number of CD8+ cells in the CNS was significantly higher in Hp−/− mice compared with WT mice. Also the number of myeloid DC was increased (but not significantly) in Hp−/− compared with WT mice. During the recovery phase, both CD8+ cells and myeloid dendritic cells persisted in significantly higher numbers in Hp−/− mice. There was no difference in the number of CD4+CD25+ Foxp3+ T cells in the CNS of Hp−/− mice compared with WT mice. Thus, the inflammatory cell infiltration is more intense and persists longer during EAE in Hp-deficient mice.

Infiltration into the CNS of IL-17- and IFN-γ-producing T cells

Both Th1 and Th17 cells have been shown to have a pathogenic role in EAE [16, 17]. Therefore, we performed a kinetic study to assess the presence of CD4+ Th17 (IL-17A-producing T cells) and Th1 (IFN-γ-producing T cells) cells in the CNS (Fig. 4). At the onset of the disease, CD4+ IFN-γ+ and CD4+ IL-17+ T cells were present in the CNS of Hp−/− and WT mice in equal numbers. At the peak of the disease, day 21, the number of CD4+ IFN-γ+ and of CD4+ IL-17+ T cells was slightly (but not significantly) higher in Hp−/− mice compared with WT mice. Most strikingly, in the recovery phase, both cell subsets remained significantly higher in Hp−/− mice than in WT mice. We conclude that the lack of HP is associated with a more intense infiltration into the CNS of pathogenic CD4+ Th17 and Th1 cells, and especially to a longer persistence of these cells.

Figure 1. Clinical scores of EAE in Hp−/− and WT mice. WT and Hp−/− mice were immunized with MOG35–55 peptide in CFA. Disease severity was scored daily according to a 0–5 severity scale. (A) Representative experiment with WT (n = 5) and Hp−/− mice (n = 6) and follow-up for 24 days. (B). Pooled results of five independent experiments (each with n = 5 per group). Data show mean ± SEM. (C) Clinical scores of individual mice at day 21 after sensitization. Horizontal bar represents the mean. *p<0.05, Mann–Whitney U test.
Hp deficiency affects MOG-induced cytokine production by splenocytes and lymph node cells

We then sought to determine whether the higher intensity of EAE in Hp−/− mice could be the result of enhanced T-cell sensitization. On day 14 and 21 after immunization, splenocytes and footpad draining lymph node (DLN) cells were isolated and cultured in the presence of 10 μg/mL of MOG35–55 peptide. The proliferative response of splenocytes and DLN cells was equivalent in WT and Hp−/− mice (Fig. 6A). Hp−/− lymph node cells showed a trend toward elevated production of IFN-γ and IL-17A (at day 14 and 21 after immunization). Hp−/− spleen T cells produced significantly less IL-4 and IL-5 compared with WT cells (Fig. 6B). Thus, in Hp−/− mice there was an elevated production in peripheral lymphoid tissues of inflammatory cytokines implicated in the progression of EAE and a reduced expression of Th2 (anti-inflammatory) cytokines. This influence of Hp deficiency on Th2 cytokine production is in accordance with previous data [12]. The results suggest an altered sensitization pattern to MOG-peptide in Hp−/− mice, which can contribute to the severity of the disease in these mice.

MOG-specific antibody production is increased in Hp−/− mice

MOG antibody levels represent another approach to evaluate the sensitization process. We measured the production of MOG35–55-specific IgG1 and IgG2a antibodies in serum from Hp−/− and WT mice by ELISA at different time points after immunization. The amount of anti-MOG35–55 antibodies was increased in Hp−/− mice at day 21 (Fig. 7). Lack of HP thus promotes B cell sensitization and MOG-specific antibody production.

Hp production in brain

Besides its production by liver cells with systemic release, HP is produced locally during inflammation [3, 22, 23]. We detected Hp mRNA at low levels in the spinal cord of naive mice, but at higher levels in the CNS of EAE mice (Fig. 8).

Discussion

In this study, we evaluated whether the APP HP could modulate the development of an organ-specific autoimmune disease. EAE was induced by immunization with MOG35–55 peptide in Hp−/− and WT C57BL/6 mice. We showed that the lack of HP is accompanied by a clinically more severe and pathologically exacerbated expression of EAE. Increased production of the pro-inflammatory cytokines IFN-γ, IL-17A, and IL-6 was found in the CNS of Hp−/− mice. EAE in Hp−/− mice was characterized by a denser CNS infiltration and by longer persistence of inflammatory cells and of IL-17A and IFN-γ producing cells in the inflamed tissue. Among the inflammatory cells, CD8+ T cells and DC were

Hp deficiency enhances the mRNA expression of inflammatory cytokines in spinal cord

To further corroborate the above findings on increased infiltration of the CNS with Th1 and Th17 cells, expression of mRNA for cytokines was measured in spinal cord tissue. At the peak of the disease (21 days after sensitization), spinal cord mRNA expression of IFN-γ, IL-17A, and IL-6 was significantly higher in Hp−/− mice compared with WT mice (Fig. 5). mRNA expression of IL-10 and TGF-β showed a trend toward augmented expression in Hp−/− mice (but this was not statistically significant). mRNA expression of IL-12 and IL-23 was similar in both groups. These results indicate that the lack of HP is associated with enhanced production in the CNS of several inflammatory cytokines implicated in the development and severity of EAE.

Figure 2. Spinal cord histology. WT and Hp−/− mice were immunized with MOG35–55 in CFA and sacrificed at day 21 or 24 after immunization. Mice were perfused and spinal cords harvested and fixed. Sections of 7 μm were stained with H&E. (A) Pathological scores for inflammation in the CNS of four pooled experiments (n = 19 per group). (B) Representative sections of spinal cord (lumbar portion) from WT and Hp−/− mice stained with H&E. Arrows point to the sites of cellular infiltration and inflammation. *p < 0.05, Mann–Whitney U test.
significantly increased at the peak of the disease and in the recovery phase. We further demonstrate that Hp deficiency did not enhance T-cell priming, but modulated the MOG-induced cytokine balance, as reflected by decreased IL-4 and IL-5 production of MOG-restimulated spleen cells.

Since EAE development depends on the balance between pro- and anti-inflammatory cytokines [15–21], we explored whether alteration of this balance in Hp−/− mice could account for more severe disease. First, we observed higher expression of IL-17A mRNA and higher numbers of Th17 cells in the CNS of MOG-immunized Hp−/− mice compared with WT mice. IL-17A is a crucial cytokine in autoimmunity. IL-17A-deficient mice develop a delayed and attenuated disease. Administration of IL-17A-blocking antibody to mice with EAE prevents development of EAE, and reduces the expression of chemokines involved in the pathogenesis of EAE [15, 16, 17, 24]. Th17 cells require IL-6 and TGF-β for their differentiation [25]. In the CNS of Hp−/− mice, the production of IL-6 and TGF-β was higher compared with WT mice, and this might contribute to the higher production of IL-17A in the CNS of Hp−/− mice. The high expression of IL-6 in the CNS of Hp−/− mice might also account for more severe disease by another mechanism, as IL-6 contributes to the resistance of myelin-specific effector T cells to be suppressed by regulatory T cells [26], and inhibits the conversion of...
CD4+ T cells into Foxp3+ regulatory T cells that potentially can control inflammation of the CNS [27]. In the second place, we found elevated expression of IFN-γ and increased infiltration of IFN-γ-producing T cells in the CNS of Hp−/− mice. IFN-γ is produced by neuro-antigen specific T cells in the CNS. Myelin-specific effector T cells expanded under Th1 conditions are able to induce EAE in naive mice [16]. Thus, the higher levels of IFN-γ might also contribute to the more severe inflammation in the CNS of Hp−/− mice. Conversely, we found that Hp−/− MOG-stimulated T cells in spleen produced lower amounts of IL-4, IL-5, and IL-10.

Figure 5. Cytokine mRNA expression in spinal cord from Hp−/− and WT mice with EAE. MOG-immunized Hp−/− and WT mice were sacrificed on day 21 after immunization and spinal cords were harvested. mRNA expression of IL-12 (p35), IFN-γ, IL-23 (p19), IL-17A, IL-10, IL-6, and TGF-β was quantified by RT-PCR. Data represent relative gene expression in individual spinal cords. The expression of each gene was calculated relative to the expression of the housekeeping gene β-actin. Results pooled from two independent experiments are shown (7–10 mice per group). *p<0.05, Mann–Whitney U-test.

Figure 6. Proliferation and cytokine production of splenocytes and DLN cells of WT and Hp−/− mice in response to MOG35–55. Spleen and footpad DLN were harvested at day 14 and 21 post immunization. Splenocytes and footpad DLN cells (5 × 10^6/mL) were cultured with MOG35–55 at 10 μg/mL. (A) Cell proliferation of DLN and spleen cells of WT and Hp−/− mice. (B) Levels of inflammatory cytokines (IFN-γ and IL-17A) and of Th2 and anti-inflammatory cytokines (IL-4, IL-5, and IL-10) in culture supernatants. Data represent mean values from 5–10 mice per group and are pooled from two experiments. IL-4, IL-5, IL-6, and IL-10 were not detectable in cultures of DLN cells. *p<0.05, Mann–Whitney U-test. Data show mean ± SEM.
important in mediating several types of adhesive interactions. Mac-1 is expressed on monocytes, macrophages, DC, and on cytotoxic cells, and is CD18 (Mac-1) integrin molecule [9]. Mac-1 is expressed on the spinal cord of mice with autoimmune inflammation of the CNS and also by production of IFN-γ, which in turn cause more severe inflammation of the CNS.

Besides Th1 and Th17 cells, we found an increased number of CD8+ T cells infiltrating the CNS of Hp−/− mice. In EAE, CD8+ T cells have previously been shown to have a pathogenic role [29, 30]. Moreover, CD8+ T cells dominate the T-cell infiltrate in active MS lesions [31]. Their detrimental function is mediated by their ability to kill oligodendrocytes and neuronal cells that upregulate MHC class I expression during autoimmune inflammation, and also by production of IFN-γ and TNF.

HP has no known receptor on T cells, but binds to the CD11b/CD18 (Mac-1) integrin molecule [9]. Mac-1 is expressed on monocytes, macrophages, DC, and on cytotoxic cells, and is important in mediating several types of adhesive interactions. Therefore, we postulate that HP may act on the T helper subset balance indirectly through effects on APC (such as DC) or macrophages. HP has already been demonstrated to inhibit the activation of skin DC (Langerhans cells) [11]. Mac-1 regulates the stimulatory capacity of DC expressing this integrin [32], and Mac-1 has been demonstrated to play an important role in the development and progression of demyelinating disease, as Mac-1-deficient mice developed a delayed and mild EAE [33]. Attenuated EAE in Mac-1-deficient mice seems to be related with a deficiency in the phagocytic activity of macrophages and microglia in the CNS and with an altered T-cell cytokine profile, but not with a deficiency in the migration of mononuclear cell into the CNS [33, 34]. In the absence of HP we could expect more free Mac-1 molecules on DC and macrophages resulting in altered activity of these cells.

Another issue is whether the protective effect of HP results either from a systemic effect, or from a local effect in the brain. HP is mainly synthesized in the liver and released in the blood during acute or chronic inflammation [1, 2], but HP is also produced locally at the site of inflammation by neutrophils [22], and it is synthesized locally in the CNS cells in reactive astrocytes after ischemia reperfusion [23]. We found Hp in mRNA in the spinal cord of EAE mice, pointing to the possibility that both systemically released and locally produced HP can have an effect on the brain inflammatory response. Our efforts to detect HP by immunohistochemistry in the brain parenchyma were, however, not successful (our unpublished observation).

In conclusion, exacerbated EAE in Hp−/− mice is accompanied by an increased expression of IFN-γ, IL-6 and IL-17A in the CNS of these animals. Furthermore, the number of IL-17+ and IFN-γ+ cells in the CNS is increased, especially in the recovery phase. It is therefore likely that HP has a physiological role in reducing the severity of an autoimmune inflammatory process. The exact mechanism should still be further explored.

**Materials and methods**

**Mice**

Hp−/− mice on the C57BL/6J background were provided by Dr. F. Berger (University of South Carolina, Columbia, SC, USA). To generate Hp−/− mice, Hp exons 2, 3, and 4 were deleted in a constructed targeting vector, which was electroporated into ES cells that were later injected into C57BL/6J blastocysts. The chimeras were mated with C57BL/6J females to produce heterozygous mice that were then intercrossed to produce mice homozygous for the mutation [6]. The congenic strain of Hp−/− mice used has been backcrossed for eight generations into C57BL/6J mice. Hp−/− mice were housed in the animal facility at Gasthuisberg University Hospital (Leuven, Belgium). Sex- and age-matched WT C57BL/6J (Horst, the Netherlands) mice were used as controls. For all experiments, 6- to 8-week-old female mice were used. All mice, also paralyzed animals, were afforded access to food and water. Experimental protocols were approved by the ethical committee for animal experimentation of the
Peptide

MOG<sub>35-55</sub> peptide (MEVGWYRSPFSRVVHLRNYK) was synthesized by EUROGENTEC (Liege, Belgium) and purified by HPLC. Peptide purity was more than 99%.

Induction and clinical evaluation of EAE

Mice were immunized with 300 μg of MOG<sub>35-55</sub> peptide and 400 μg killed Mycobacterium tuberculosis (H37 RA) (Difco Laboratories, Detroit, MI, USA) emulsified in CFA (Pierce qb Perbio, Rockford, IL, USA). Fifty microliters of the emulsion was injected in each footpad. At days 0 and 2 post immunization, 500 ng of pertussis toxin (from Bordetella pertussis Sigma, St Louis, MO, USA) were administered i.p. Clinical score was assessed daily on a scale of 0–5: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and forelimb paralysis; 5, moribund/dead state.

MOG<sub>35-55</sub>-specific T-cell proliferation and cytokine release

DLN (inguinal and popliteal) and spleen cells were harvested and single-cell suspensions were prepared by passage through a cell strainer (BD Biosciences, Erembodegem, Belgium). DLN cells and splenocytes (5 x 10<sup>5</sup>) were cultured in the absence or presence of MOG<sub>35-55</sub> peptide (10 μg/mL) in 96-well plates for 96 h. [3H]-Thymidine incorporation was measured by liquid scintillation counting. DLN cells and splenocytes were also cultured in 24-well plates (Greiner Bio-one, Germany) with or without 10 μg/mL of MOG<sub>35-55</sub>. Supernatants were recovered after 72 h of culture. IL-4, IFN-γ, IL-17A was measured using an ELISA kit (OPTEIA Mouse IL-17 ELISA (Mouse cytossets kit, Biosource Europe, Nivelles, Belgium). IL-17A was measured using an ELISA kit (OPTEIA Mouse IL-17 set, Pharmingen). Detection limit for IFN-γ was 10 pg/mL; IL-10: 50 pg/mL; IL-4: 10 pg/mL; IL-17A: 20 pg/mL; IL-5:50 pg/mL; IL-13: 20 pg/mL.

CNS inflammatory cell isolation

The animals were given a lethal dose (6 mg/mouse) of sodium pentobarbital (Nembutal) (CEVA sante animale, Brussels, Belgium). Mice were then perfused with 35 mL of ice-cold PBS. Spinal cord and brain tissues were dissected out, cut into small pieces, and digested with 2.5 mg/mL collagenase A (Roche, Mannheim, Germany) for 30 min at 37°C. Samples were then passed through a cell strainer to obtain a single-cell suspension, which was resuspended in 40% Percoll, and loaded onto 70% Percoll (Sigma). After centrifugation at 800 x g for 25 min, cells were retrieved from the 40–70% Percoll interface and washed in PBS.

RT-PCR analysis

Total cellular RNA was extracted from the spinal cord using RNeasy mini kit (Qiagen, Gaithersburg, MD, USA). cDNA was prepared from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, CA, USA). The cDNA samples were then subjected to real-time quantitative PCR, performed in the ABI prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences for Hp, IFN-γ, TGF-β, IL-6, IL-10, IL-23p19, IL-12p35, IL-17A, and β-actin were designed with Primer Express (Applied Biosystems) as shown in Table 1. cDNA plasmid standards, consisting of purified plasmid DNA specific for each individual target, were used to quantify the target gene in the unknown samples. All results were normalized to β-actin mRNA.

Cell surface marker and intracellular staining

Isolated cells were labeled with anti-CD45-PerCP (30-F11), anti-CD4 either PE (L3T4) or PerCP (RM4-5), anti-CD11c-FITC (HL3) (BD Biosciences), anti-CD11b either PE or PE-Cy5.5 (M1/70), anti-CD8-FITC (53.6.7) (eBioscience), anti-neutrophils-FITC (BD Biosciences), anti-CD11b either PE or PE-Cy5.5 (M1/70), and anti-Ly6G-PE (1A8) (Miltenyi Biotec) antibodies for 30 min at 4°C. Proper isotype controls were also included (rat-IgG2a-FITC or PE (CR19-15), rat-IgG1-PE, hamster IgG1-FITC, rat IgG2b-PE-Cy5.5).

For intracellular staining, cells obtained from the CNS were restimulated with 50 ng/mL of phorbol myristate acetate plus 1 μg/mL of ionomycin (Sigma) for 18 h. A total of 0.7 μg/mL monensin (Sigma) was added for the final 4 h of culture. Cells were first stained with anti-CD4-PerCP (RM4-5) and anti-CD25-PE (PC61), then fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen), and finally stained intracellularly with anti-IL-17E and anti-IFN-γ-FITC (BD Biosciences) or Foxp3-Alexa Fluor® 488 (FJK-16s) (eBioscience). Corresponding isotype controls were used (rat IgG2a Alexa Fluor® 488, rat IgG1-PE, rat IgG1-FITC). The stained cells were resuspended in 1% paraformaldehyde in PBS and analyzed on FACS Calibur (BD Biosciences) using CellQuest software.

Histology

Hp<sup>−/−</sup> and WT mice were sacrificed at day 21 after induction of EAE and perfusion with ice-cold PBS through the left ventricle was performed. Spinal cords were isolated and fixed in 4% formalin (Merck, Germany), embedded in paraffin (VWR prolabo, France), and sectioned transversally (7 μm). Sections were stained with H&E. Histopathology was assessed according to Dubois et al. [35]. Several sections of the whole spinal cord were evaluated. The inflammatory score was given as follows: 0 = normal; 1 = presence of a...
Table 1. Primer and probe sequences

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<tr>
<td>Hp-RV</td>
<td>CCG TAG TCT GTA GAA GGT TGG GC</td>
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<td>Hp-TP</td>
<td>AAC GGC TAT GTG GAG CAC TCT TGG</td>
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perivascular inflammatory cuff; 2 = circumferential presence of inflammatory cell groups around the spinal cord; and 3 = circumferential inflammation with parenchymal invasion.

ELISA assay for analysis of MOG35–55-specific antibodies

96-well plates (Nunc™, Denmark) were coated overnight at 4 °C with 1 μg of MOG35–55 peptide in PBS. A 1:50 dilution of each serum sample was added at 100 μL/well in duplicate and incubated for 2 h at room temperature. Then, 1 μg/mL of anti-IgG1-biotin or anti-IgG2a-biotin (100 μL/well) (BD Pharamingen) was added and incubated 1.5 h at room temperature. After washing the plates four times with PBS 0.05% Tween-20, horseradish peroxidase-conjugated streptavidin was added (R&D systems, Minneapolis, MN, USA). Plates were incubated 30 min and bound MOG-specific antibodies were detected with 3,3′,5,5′-Tetramethylbenzidine (TMB). Optical density readings at 450 nm were performed on Microplate Reader (Thermo Labsystems Multiskan RC).

Statistical analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA) by using the non-parametric two-tailed Mann–Whitney U test. A difference was considered to be significant when p<0.05.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

References


Abbreviations: APP: acute phase proteins  ·  DLN: draining lymph nodes  ·  HP: haptoglobin  ·  mDC: myeloid dendritic cell  ·  MOG: myelin oligodendrocyte glycoprotein

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