RESEARCH PAPER

CSF high-mobility group box 1 is associated with intrathecal inflammation and astrocytic damage in neuromyelitis optica

Akiyuki Uzawa, Masahiro Mori, Saeko Masuda, Mayumi Muto, Satoshi Kuwabara

Department of Neurology, Graduate School of Medicine, Chiba University, Chiba, Japan

Correspondence to
Dr Akiyuki Uzawa, Department of Neurology, Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8670, Japan; auzawa@chiba-u.jp

Received 27 August 2012
Revised 19 November 2012
Accepted 21 November 2012
Published Online First 19 December 2012

ABSTRACT

Objective High-mobility group box 1 (HMGB1) acts as a proinflammatory mediator when released by cells. Recent studies implicate extracellular HMGB1 in the pathogenesis of various autoimmune diseases. Our main aim of this study is to determine whether HMGB1 is involved in the neuromyelitis optica (NMO) inflammatory process.

Methods Cerebrospinal fluid (CSF) and serum HMGB1 levels in 42 NMO patients were compared with those in 30 multiple sclerosis (MS) patients, and 30 patients with other noninflammatory neurological disorders (ONNDs). We also tested the possible correlation between CSF HMGB1 levels and the clinical and laboratory variables in NMO patients.

Results CSF HMGB1 levels in NMO patients were higher than those in MS and ONNDs patients ($p<0.001$), and these levels in MS patients were higher than those in ONNDs patients ($p<0.001$). After treatment, the CSF HMGB1 levels in NMO patients decreased to normal. In addition, CSF HMGB1 levels correlated with CSF cell counts, CSF protein levels, CSF interleukin-6 levels, CSF glial fibrillary acidic protein levels, and CSF/serum albumin ratio ($p\leq0.001$). Serum HMGB1 levels in MS patients were significantly higher than those in ONNDs patients ($p=0.002$).

Conclusions HMGB1 could play a key role in central nervous system inflammation in NMO patients.

INTRODUCTION

High-mobility group box 1 (HMGB1) was recently identified as a damage-associated or pathogen-associated molecular pattern molecule.[1 2] HMGB1 has a dual function: intracellularly, it binds to DNA in the nucleus and is important for gene regulation and transcription,[3] and extracellularly, HMGB1 can act as inflammatory trigger and is involved in inflammatory processes.[4 5] Purified recombinant HMGB1 added to cultures of human monocytes stimulates the release of inflammatory cytokines which amplify inflammation.[6] HMGB1 is released by activated immunocytes or damaged, dying cells during necrosis and the late phase of cellular apoptosis.[7 8] The proinflammatory cytokine activity of HMGB1 is attributed mostly to its ligation with the receptor for advanced glycation end products (RAGE), toll-like receptor (TLR) 2, TLR4 and TLR9.[9–13] HMGB1 can activate autoreactive B cells,[14] and upregulate adhesion molecules, leading to the recruitment of inflammatory cells, thereby promoting cell migration.[4 15]

Neuromyelitis optica (NMO) and multiple sclerosis (MS) are known to differ in terms of various aspects of their pathology,[16] neuroimaging,[17] immunology[18] and response to certain immunotherapies[19 20] since the discovery of anti aquaporin-4 (AQP4) antibody in NMO,[21] but these diseases are similar in terms of autoimmune-mediated inflammatory disorders of the central nervous system (CNS).[22 23]

Recent studies have detected associations between HMGB1 and autoimmune diseases. High HMGB1 levels are present in rheumatoid arthritis (RA), Sjögren syndrome (SS), Churg–Strauss syndrome, and systemic lupus erythematosus (SLE),[24–27] and MS patients,[28] but the correlation between HMGB1 levels and NMO pathogenesis has not been studied. The objective of this study was to determine whether HMGB1 is involved in the NMO inflammatory process.

MATERIALS AND METHODS

Study participants

Forty-two NMO patients, including 33 patients with definite NMO, who met the 2006 Wingerchuk's criteria[23] and nine with anti-AQP4-positive optic neuritis and/or myelitis (NMO spectrum disorders: NMOSD), and 30 with relapsing-remitting MS, who met the 2005 revised McDonald criteria,[29] were involved in this study. We included patients with obtainable CSF samples who visited the Chiba University Hospital between 2000 and 2012. Thirty patients (11 women and 19 men; mean age=59.1 years) with other non-inflammatory neurological disorders (ONNDs) were recruited as controls, including 11 with amyotrophic lateral sclerosis, 10 with spinocerebellar degeneration, 5 with Parkinson's disease, and 4 with progressive supranuclear palsy.

We recorded the sex, age, disease duration, expanded disability status scale (EDSS) scores, presence of longitudinally extensive transverse myelitis (LETM), defined as acute myelitis with spinal cord lesions extending over ≥3 vertebral segments as observed on MRI, positivity for oligoclonal bands, positivity for serum anti-AQP4 antibody using ELISA, as described previously (with minor modifications),[30] cerebrospinal fluid (CSF) variables, including CSF interleukin (IL)-6 levels, CSF glial fibrillary acidic protein (GFAP) levels, CSF/serum albumin ratio (QAlb), and IgG index, and whether the patient was receiving immunomodulatory or immunosuppressive therapy at the time of sample collection.

To cite: Uzawa A, Mori M, Masuda S, *et al. J Neurol Neurosurg Psychiatry* 2013;**84**:517–522.

The ethics committee of the Chiba University School of Medicine, Chiba, Japan approved the study, and written informed consent was obtained from all study subjects.

CSF and serum collection

CSF samples were obtained by lumbar puncture from NMO and MS patients during the active disease phase (within 1 month of clinical relapse) and before acute relapse treatment that included high-dose intravenous methylprednisolone and plasmapheresis therapy. The serum samples were collected concurrently with lumbar puncture. QAlb as a marker of blood-brain barrier integrity and IgG index as a marker of intrathecal IgG synthesis were available in 35 NMO patients, 25 MS patients and 15 ONNDs patients. CSF samples after relapse treatment were obtained from only four NMO patients because lumbar puncture was rarely performed during remission and after relapse treatment. All samples were stored at −80°C until analysis.

CSF and serum HMGB1 measurements

The CSF and serum HMGB1 levels were determined in NMO, MS and ONNDs patients using an HMGB1 ELISA kit II (Shino-Test Corporation, Tokyo, Japan), according to the manufacturer's instructions. In brief, an ELISA plate was coated with anti-HMGB1 antibody and incubated with 110 μl of diluted samples (40 μl of CSF and 70 μl of diluent) for 24 h at 37°C. After five washes, 100 μl of detection antibody was added to each well and the plate was incubated for 2 h. The plate was washed five times and 100 μl of colour reagent (3,3′,5,5′-tetramethylbenzidine and H_2O_2 buffer solution) was added to each well, followed by another 30 min incubation. Finally, 100 μl of stop solution was added. The optical density (OD) was measured at 450 nm. HMGB1 levels were calculated with reference to a standard curve. The HMGB1 detection range was 0.25–20 ng/ml.

CSF IL-6 and GFAP measurements

CSF IL-6 levels were measured using a chemiluminescent enzyme immunoassay (CLEIA) at our clinical laboratory, employing a two-step sandwich method using a cartridge for IL-6 measurement (Human IL-6 CLEIA Fujirebio, Fujirebio, Tokyo). The intensity of chemiluminescence was measured using a luminometer to determine IL-6 levels.

The CSF GFAP levels were measured using a human GFAP ELISA kit (BioVendor, Minneapolis, Minnesota, USA). OD was measured at 450 nm; however, a second reading at 405 nm was performed when the microplate reader was incapable of reading absorbance greater than the highest standard. These procedures were performed according to the manufacturer's instructions.

Correlation between the CSF HMGB1 levels and the clinical and laboratory variables in NMO and MS patients

We examined the possible correlation between the CSF HMGB1 levels and the clinical and laboratory variables, including EDSS, CSF cell counts and CSF protein levels in 42 NMO patients and 30 MS patients. We also analysed the association between the CSF HMGB1 levels and CSF IL-6 levels, CSF GFAP levels, QAlb, IgG index, length of the spinal lesion and OD at 450 nm of serum anti-AQP4 antibody in 42 NMO patients.

Statistical analyses

All data were analysed according to the intention-to-treat principle. For the baseline variables, groups were compared using the Mann–Whitney U test for unpaired continuous measures. Spearman's rank correlation coefficient was used to evaluate the statistical dependency of two variables. All comparisons were planned and statistical tests were two-sided. $p<0.05$ was considered statistically significant. In addition, for multiple comparisons, we applied the Bonferroni correction method to the computed p values to reduce type I errors.

RESULTS

Clinical and biochemical profiles of NMO and MS patients

The clinical characteristics of NMO and MS patients are summarised in table 1. The patients' proportion of female, presence of LETM, negativity for oligoclonal bands, positivity for serum anti-AQP4 antibody, and receipt of immunomodulatory or immunosuppressive therapy, as well as age, disease duration and EDSS at sampling were higher in NMO patients than in MS patients. The CSF findings of NMO, MS and ONNDs patients are summarised in table 2. The CSF cell counts, protein levels, IL-6 levels, GFAP levels and QAlb in NMO patients were significantly higher than in MS and/or ONNDs patients.

Table 1 Clinical characteristics of NMO and MS patients

Clinical characteristics	NMO (n=42)	MS (n=30)
Men:women	3 : 39	9 : 21
Age, year; mean (range)	51.5 (27.9–86.0)	33.2 (18.1–51.4)
Disease duration, month; mean (range)	129.5 (0.0–486.8)	71.2 (0.5–269.1)
Site of relapse at sampling		
Spinal cord (%)	30/42 (71)	23/30 (77)
Brain (%)	6/42 (14)	7/30 (23)
Optic nerve (%)	6/42 (14)	4/30 (13)
EDSS; median (range)	6.8 (1.5–9.0)	3.5 (1.0–7.5)
≥3 vertebral segments spinal cord lesions (%)	38/42 (90)	3/30 (10)
Positive antiaquaporin-4 antibody (%)	36/42 (86)	0/30 (0)
Positivity for oligoclonal bands (%)	5/31 (16)	18/27 (67)
Immunomodulatory or immunosuppressivetherapy (%)	17/42 (40)	9/30 (30)
Interferon-β (%)	3/42 (7)	7/30 (23)
Azathioprine (%)	5/42 (12)	0/30 (0)
Oral prednisolone (%)	14/42 (33)	2/30 (7)

EDSS, Expanded Disability Status Scale; MS, multiple sclerosis; NMO, neuromyelitis optica.

Table 2 CSF findings of NMO, MS and ONNDs patients

CSF findings	NMO (n=42)	MS (n=30)	ONNDs (n=30)	p Value NMO versus MS	NMO versus ONNDs	MS versus ONNDs
Cell count (cells/mm^3)	21.6±33.7	6.9±8.5	0.9±0.9	0.040	<0.001*	<0.001*
Protein level (mg/dl)	98.8±150.5	32.0±8.4	34.4±12.1	<0.001*	<0.001*	0.371
IL-6 (pg/ml)	838.9±2715.4	3.0±1.1	2.3±1.1	<0.001*	<0.001*	0.018
GFAP (ng/ml)	3888.5±6621.1	0.8±0.5	0.8±0.6	<0.001*	<0.001*	0.859
QAlb×10^3	13.4±11.2	5.05±1.78	4.29±1.15	<0.001*	<0.001*	0.158
IgG index	0.68±0.21	0.70±0.27	0.50±0.1	0.799	<0.001*	0.001*

Each value represents mean±SD. Mann–Whitney U test was used for statistical analysis.
*Statistically different even after Bonferroni correction.
CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; MS, multiple sclerosis; NMO, neuromyelitis optica; ONNDs, other non-inflammatory neurological disorders; QAlb, cerebrospinal/serum albumin ratio.

Increased serum HMGB1 levels in MS patients

The mean (SD) serum HMGB1 levels were 2.65 (3.91), 2.94 (2.78) and 1.55 ng/ml (2.07) in NMO, MS and ONNDs patients, respectively. The values in MS patients were significantly higher than those in ONNDs patients (p=0.002), but there were no significant differences after applying Bonferroni correction between NMO and ONNDs (p=0.041), as well as NMO and MS (p=0.060) (figure 1).

Increased CSF HMGB1 levels in NMO and MS patients

The mean CSF HMGB1 levels were 3.56±3.76 ng/ml (mean±SD), 1.43±0.82 ng/ml, and 0.85±0.21 ng/ml in NMO, MS and ONNDs patients, respectively. The CSF HMGB1 levels in NMO patients were significantly higher than those in MS patients (p<0.001) and ONNDs patients (p<0.001), and these levels in MS patients were higher than those in ONNDs patients (p<0.001). We obtained the CSF samples from four NMO patients after treatment, of which three patients received high-dose intravenous methylprednisolone treatment, and one received plasmapheresis therapy after the high-dose intravenous methylprednisolone treatment as relapse treatment. The mean interval between clinical relapse and sampling after relapse treatment was 24.8 days. The CSF HMGB1 levels after relapse treatments in NMO patients (n=4) decreased to near the levels found in ONNDs patients (5.64±3.76 ng/ml at relapse, which decreased to 0.63±0.16 ng/ml after treatment) (figure 2).

CSF HMGB1 levels were correlated with the CSF cell counts, protein levels, IL-6 levels, GFAP levels and QAlb in NMO patients

There were significant positive correlations among the CSF HMGB1 levels and CSF cell counts (r=0.832; p<0.001), CSF protein levels (r=0.640; p<0.001), CSF IL-6 levels (r=0.506; p=0.001), CSF GFAP levels (r=0.511; p=0.001), and QAlb

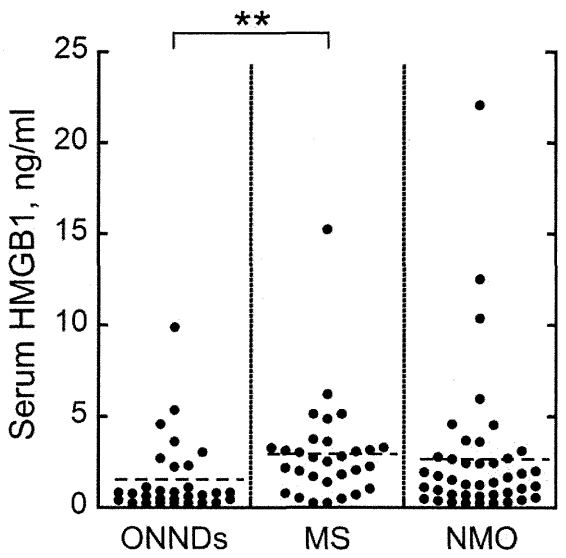


Figure 1 Serum high-mobility group box 1 (HMGB1) levels in neuromyelitis optica (NMO), multiple sclerosis (MS), and other non-inflammatory neurological disorders (ONNDs) patients. HMGB1 levels in sera of 42 NMO patients, 30 MS patients, and 30 ONNDs patients. Levels in MS patients were significantly higher than those in ONNDs (p = 0.002). There were no significant differences after applying Bonferroni correction between NMO and ONNDs (p = 0.041) as well as NMO and MS (p = 0.060). Dashed lines indicate the mean concentration in each group.

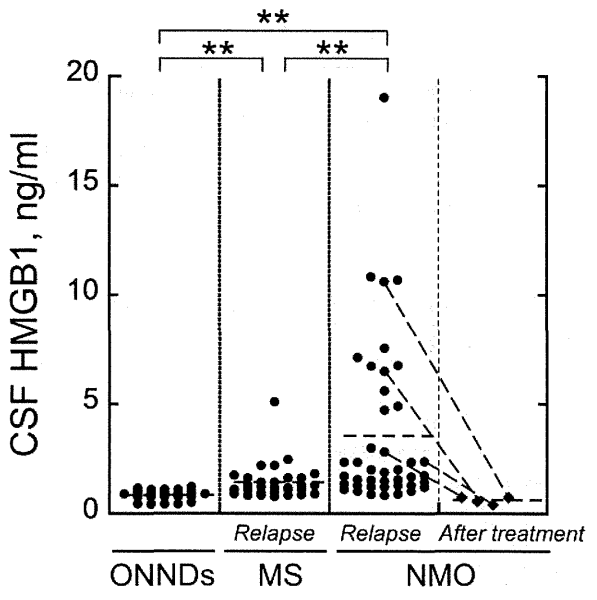


Figure 2 Cerebrospinal fluid (CSF) high-mobility group box 1 (HMGB1) levels in neuromyelitis optica (NMO), multiple sclerosis (MS), and other non-inflammatory neurological disorders (ONNDs) patients. HMGB1 levels in CSF of 42 NMO patients, 30 MS patients and 30 ONNDs patients. Levels in NMO patients were significantly higher than those in MS (p < 0.001) and ONNDs patients (p < 0.001), while the levels in MS patients were more elevated than those in ONNDs patients (p < 0.001). The CSF HMGB1 levels after relapse treatment in NMO patients (n = 4) sharply dropped to near the levels in the ONNDs patients. Dashed lines indicate the mean concentration in each group.

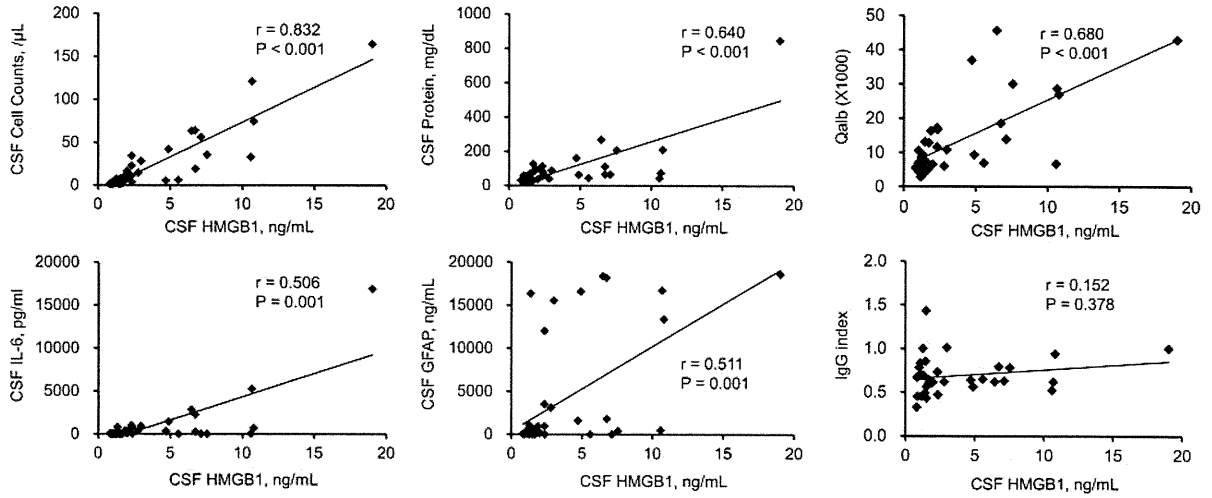


Figure 3 Correlations between cerebrospinal fluid (CSF) high-mobility group box 1 (HMGB1) levels and CSF variables in neuromyelitis optica (NMO) patients. Significant positive correlations were detected between the CSF HMGB1 levels and CSF cell counts, CSF protein levels, CSF IL-6 levels, CSF glial fibrillary acidic protein (GFAP) levels and cerebrospinal/serum albumin ratio (Qalb) values in 42 NMO patients.

(r=0.680; p<0.001) in NMO patients (figure 3). There were no significant correlations among the CSF HMGB1 levels and EDSS scores (r=0.206; p=0.203), length of spinal lesion (r=0.183; p=0.811), IgG index (r=0.152; p=0.378), or anti-AQP4 antibody titres (r=0.021; p=0.895) in NMO patients. In MS patients, there was a positive correlation between the CSF HMGB1 levels and CSF cell counts (r=0.800; p<0.001) but no correlation between the CSF HMGB1 levels and CSF protein levels (r=0.348; p=0.061) or EDSS scores (r=−0.332; p=0.074).

DISCUSSION

In this study, we found significant increases in the CSF HMGB1 levels in NMO patients compared with those in MS and ONNDs patients, while the CSF HMGB1 levels in NMO patients were also positively correlated with the CSF cell counts, CSF protein levels, CSF IL-6 levels, CSF GFAP levels and QAlb. The increased CSF protein content and QAlb may have been due to blood-brain barrier disruption, as increased permeability of the blood-brain barrier may have facilitated the access of anti-AQP4 antibody to astrocytes and further infiltration of the immune components into the CNS. Indeed, severe blood-brain barrier disruption occurred during the acute phase in NMO patients.[31] CSF IL-6 has important roles in CNS inflammation, and it may be a useful biomarker of NMO activity,[18 32] while elevated CSF GFAP levels indicate astrocytic damage, which is a primary pathological process during NMO.[33] Moreover, the CSF HMGB1 levels decreased to normal levels after relapse treatment in NMO patients. These findings suggest that CSF HMGB1 is involved in and may trigger CNS inflammation and astrocytic damage observed in NMO. We also conducted subanalysis, and found the mean CSF HMGB1 levels were 3.88 and 1.64 ng/ml in the anti-AQP4-positive NMO/NMOSD (n=36) and anti-AQP4 antibody-negative NMO groups (n=6), respectively. Their levels were greater in AQP4-positive patients compared with AQP4-negative patients, but the difference was not statistically significant. In this regard, there seemed to be similar pathogenesis between them. On the other hand, serum HMGB1 levels in NMO were higher than those in ONNDs patients, but the difference was not significant after applying Bonferroni correction (p=0.041). Although there may be some activation of HMGB1 outside the CNS in NMO, we believe the activation mainly occurs inside the CNS of NMO.

Several studies have detected systemically upregulated HMGB1 levels, and shown that HMGB1 has important roles in inflammatory autoimmune-mediated diseases. However, the role of HMGB1 in NMO has not been elucidated. In SLE patients, increased serum HMGB1 levels correlated with SLE disease activity index scores, proteinuria and anti-HMGB1 monoclonal antibody levels, indicating that HMGB1 and HMGB1 anti-HMGB1 immune complexes play a role in SLE pathogenesis.[27 34] HMGB1 was also strongly expressed in the synovial fluid of RA patients, while synovial fluid macrophages released proinflammatory cytokines after HMGB1 stimulation, suggesting that HMGB1 plays important roles in RA pathogenesis.[26] In addition, increased amounts of extracellular HMGB1 in the salivary glands of SS patients indicated that HMGB1 was involved in the inflammatory processes associated with SS.[24] NMO often co-occurs with collagen diseases, such as SLE and SS;[35] therefore, similar mechanisms may be present in these disorders. To date, there has been only one previous report of a possible correlation between HMGB1 and MS. HMGB1 was secreted by macrophages and microglia in the CNS, and its receptors RAGE, TLR2 and TLR4 were highly expressed in the active lesions of MS patients.[28] Cytoplasmic HMGB1 immunoreactivity was detected in active MS lesions, whereas nuclear HMGB1 immunoreactivity was detected in inactive lesions.[28] The distribution of HMGB1 immunoreactivity suggests that HMGB1 is released from the cell nucleus during CNS inflammation. HMGB1 also stimulated the release of inflammatory cytokines.[6] Thus, there are potential interactions among these molecules during the inflammatory processes involved in MS pathogenesis, and the presence of HMGB1 in the CSF may be a useful biomarker for CNS inflammation. Our study revealed an increase in the CSF and serum HMGB1 levels in MS patients similar to that found previously,[28] and there was a marked CSF HMGB1 elevation in NMO patients compared with that in MS and ONNDs patients, indicating that CSF HMGB1 is implicated more in the pathogenesis of NMO than MS. The increased CSF HMGB1 levels in NMO patients might be because of their more severe CNS inflammation compared with MS patients. Another possible mechanism of HMGB1 release may occur during cell death by necrosis or apoptosis. The

significant positive correlations between CSF HMGB1 and CSF GFAP levels suggest that the damage (necrosis or apoptosis) to astrocytes could be the source of CSF HMGB1. Therefore, increased CSF HMGB1 levels may be a consequence of initial cell destruction by anti-AQP4 antibody and an epiphenomenon. Meanwhile, in MS patients, HMGB1 levels were elevated not only in the CSF but also in the serum. These findings would suggest that HMGB1 and inflammatory activations in the peripheral circulation in MS are more active than those in NMO.

Recently, a therapeutic anti-HMGB1 monoclonal antibody intervention has been described in a few reports. Specific therapeutic inhibition of endogenous HMGB1 reversed the lethality of established sepsis, presumably by abrogating HMGB1-induced IL-6 and tumour necrosis factor-α release from macrophage-like cells.[6] Anti-HMGB1 monoclonal antibody therapy could also be effective in the treatment of brain ischaemia,[36 37] models of arthritis[38] and experimental autoimmune myocarditis.[39] Similarly, prevention of CNS inflammation by inhibiting HMGB1 may be a novel treatment for NMO and MS. Recently, we have reported that anti-HMGB1 monoclonal antibody ameliorates experimental autoimmune encephalomyelitis.[40] Further testing of HMGB1-blocking therapies will be required in animal models of NMO and MS.

CONCLUSION

Our study showed that CSF HMGB1 levels were high during the relapse phase of NMO and MS, especially NMO. HMGB1 may play a key role in controlling the autoimmune responses by stimulating the release of inflammatory cytokines. The control of HMGB1 is considered to be a critical factor in the pathogenesis of autoimmune diseases, including NMO and MS. CSF HMGB1 could be involved in NMO inflammation and be a biomarker for CNS inflammation in NMO. Anti-HMGB1 interventions could have the potential for treating NMO and MS in the future.

Acknowledgements We would like to thank Dr Fumio Nomura, Setsu Sawai and Michie Arai, Division of Clinical Laboratory Medicine, Chiba University Hospital for technical support.

Contributors Study concept and design: AU, MM and SK. Acquisition of data: AU, MM, SM and MM. Analysis and interpretation of data: AU and MM. Drafting of the manuscript: AU. Critical revision of the manuscript for important intellectual content: MM, SM and SK. Study supervision: MM and SK. All authors have read and approved the final version of the manuscript.

Funding This research was partly supported by the Ministry of Education, Science and Technology, Japan (AU) (grant number 24790873).

Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was supported by the ethics committee of the Chiba University School of Medicine, Chiba, Japan.

Provenance and peer review Commissioned; externally peer reviewed.

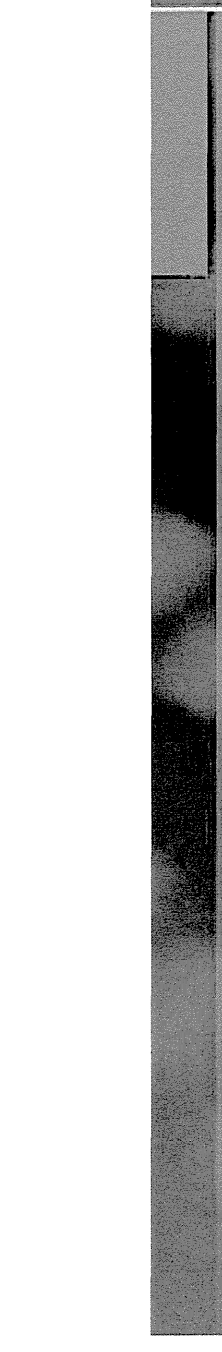
REFERENCES

1 Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 2007;81:1–5.
2 Harris HE, Raucci A. Alarmin(g) news about danger: workshop on innate danger signals and HMGB1. *EMBO Rep* 2006;7:774–8.
3 Bustin M, Reeves R. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. *Prog Nucleic Acid Res Mol Biol* 1996;54:35–100.
4 Fiuza C, Bustin M, Talwar S, *et al*. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* 2003;101:2652–60.
5 Hreggvidsdottir HS, Ostberg T, Wähämaa H, *et al*. The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation. *J Leukoc Biol* 2009;86:655–62.
6 Andersson U, Wang H, Palmblad K, *et al*. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med* 2000;192:565–70.
7 Bell CW, Jiang W, Reich CF III, *et al*. The extracellular release of HMGB1 during apoptotic cell death. *Am J Physiol Cell Physiol* 2006;291:C1318–25.
8 Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002;418:191–5.
9 Kokkola R, Andersson A, Mullins G, *et al*. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scand J Immunol* 2005;61:1–9.
10 Yang H, Hreggvidsdottir HS, Palmblad K, *et al*. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci USA* 2010;107:11942–7.
11 Park JS, Svetkauskatie D, He Q, *et al*. Involvement of Toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem* 2004;279:7370–7.
12 Tian J, Avalos AM, Mao SY, *et al*. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol* 2007;8:487–96.
13 Park JS, Gamboni-Robertson F, He Q, *et al*. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol* 2006;290: C917–24.
14 Avalos AM, Kiefer K, Tian J, *et al*. RAGE-independent autoreactive B cell activation in response to chromatin and HMGB1/DNA immune complexes. *Autoimmunity* 2010;43:103–10.
15 Rouhiainen A, Kuja-Panula J, Wilkman E, *et al*. Regulation of monocyte migration by amphoterin (HMGB1). *Blood* 2004;104:1174–82.
16 Misu T, Fujihara K, Kakita A, *et al*. Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. *Brain* 2007;130:1224–34.
17 Ito S, Mori M, Makino T, *et al*. "Cloud-like enhancement" is a magnetic resonance imaging abnormality specific to neuromyelitis optica. *Ann Neurol* 2009;66:425–8.
18 Uzawa A, Mori M, Arai K, *et al*. Cytokine and chemokine profiles in neuromyelitis optica: significance of interleukin-6. *Mult Scler* 2010;16:1443–52.
19 Watanabe S, Misu T, Miyazawa I, *et al*. Low-dose corticosteroids reduce relapses in neuromyelitis optica: a retrospective analysis. *Mult Scler* 2007;13:968–74.
20 Uzawa A, Mori M, Hayakawa S, *et al*. Different responses to interferon beta-1b treatment in patients with neuromyelitis optica and multiple sclerosis. *Eur J Neurol* 2010;17:672–6.
21 Lennon VA, Kryzer TJ, Pittock SJ, *et al*. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med* 2005;202:473–7.
22 Compston A, Coles A. Multiple sclerosis. *Lancet* 2008;372:1502–17.
23 Wingerchuk DM, Lennon VA, Pittock SJ, *et al*. Revised diagnostic criteria for neuromyelitis optica. *Neurology* 2006;66:1485–9.
24 Ek M, Popovic K, Harris HE, *et al*. Increased extracellular levels of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in minor salivary glands of patients with Sjögren's syndrome. *Arthritis Rheum* 2006;54:2289–94.
25 Taira T, Matsuyama W, Mitsuyama H, *et al*. Increased serum high mobility group box-1 level in Churg-Strauss syndrome. *Clin Exp Immunol* 2007;148:241–7.
26 Taniguchi N, Kawahara K, Yone K, *et al*. High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum* 2003;48:971–81.
27 Urbonaviciute V, Furnrohr BG, Meister S, *et al*. Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. *J Exp Med* 2008;205:3007–18.
28 Andersson A, Covacu R, Sunnemark D, *et al*. Pivotal advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis. *J Leukoc Biol* 2008;84:1248–55.
29 Polman CH, Reingold SC, Edan G, *et al*. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 2005;58:840–6.
30 Hayakawa S, Mori M, Okuta A, *et al*. Neuromyelitis optica and anti-aquaporin-4 antibodies measured by an enzyme-linked immunosorbent assay. *J Neuroimmunol* 2008;196:181–7.
31 Uzawa A, Mori M, Masuda S, *et al*. Markedly elevated soluble intercellular adhesion molecule 1, soluble vascular cell adhesion molecule 1 levels, and blood-brain barrier breakdown in neuromyelitis optica. *Arch Neurol* 2011;68:913–17.
32 Uzawa A, Mori M, Sato Y, *et al*. CSF interleukin-6 level predicts recovery from neuromyelitis optica relapse. *J Neurol Neurosurg Psychiatry* 2012;83:339–40.
33 Takano R, Misu T, Takahashi T, *et al*. Astrocytic damage is far more severe than demyelination in NMO: a clinical CSF biomarker study. *Neurology* 2010;75:208–16.
34 Abdulahad DA, Westra J, Bijzet J, *et al*. High mobility group box 1 (HMGB1) and anti-HMGB1 antibodies and their relation to disease characteristics in systemic lupus erythematosus. *Arthritis Res Ther* 2011;13:R71.
35 Pittock SJ, Lennon VA, de Seze J, *et al*. Neuromyelitis optica and non organ–specific autoimmunity. *Arch Neurol* 2008;65:78–83.
36 Liu K, Mori S, Takahashi HK, *et al*. Anti–high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J* 2007;21:3904–16.

37 Zhang J, Takahashi HK, Liu K, *et al*. Anti-high mobility group box-1 monoclonal antibody protects the blood-brain barrier from ischemia-induced disruption in rats. *Stroke* 2011;42:1420–8.
38 Schierbeck H, Lundbäck P, Palmblad K, *et al*. Monoclonal anti-HMGB1 (high mobility group box chromosomal protein 1) antibody protection in two experimental arthritis models. *Mol Med* 2011;17:1039–44.
39 Su Z, Sun C, Zhou C, *et al*. HMGB1 blockade attenuates experimental autoimmune myocarditis and suppresses Th17-cell expansion. *Eur J Immunol* 2011; 41:3586–95.
40 Uzawa A, Mori M, Taniguchi J, *et al*. Anti-high mobility group box 1 monoclonal antibody ameliorates experimental autoimmune encephalomyelitis. *Clin Exp Immunol* 2013;172:37–43.

Marie-Ève Tremblay · Amanda Sierra
Editors

Microglia in Health and Disease



Springer

Contents

Springer

http://www.springer.com/978-1-4939-1428-9

Microglia in Health and Disease
Tremblay, M.-È.; Sierra, A. (Eds.)
2014, XIV, 486 p. 32 illus., 20 illus. in color., Hardcover
ISBN: 978-1-4939-1428-9

PLOS | ONE

RESEARCH ARTICLE

Interleukin-34 Restores Blood–Brain Barrier Integrity by Upregulating Tight Junction Proteins in Endothelial Cells

Shijie Jin, Yoshifumi Sonobe, Jun Kawanokuchi, Hiroshi Horiuchi, Yi Cheng, Yue Wang, Tetsuya Mizuno, Hideyuki Takeuchi*, Akio Suzumura

Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan

*htake@riem.nagoya-u.ac.jp

OPEN ACCESS

Citation: Jin S, Sonobe Y, Kawanokuchi J, Horiuchi H, Cheng Y, et al. (2014) Interleukin-34 Restores Blood–Brain Barrier Integrity by Upregulating Tight Junction Proteins in Endothelial Cells. PLoS ONE 9(12): e115981. doi:10.1371/journal.pone.0115981

Editor: Jun-ichi Kira, Kyushu University, Japan

Received: July 11, 2014

Accepted: December 1, 2014

Published: December 23, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: This work was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a grant from the Advanced Research for Medical Products Mining Program of the National Institute of Biomedical Innovation (NIBIO) of Japan; and grants from the Ministry of Health, Labour and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Akio Suzumura is a PLOS ONE Editorial Board member. This does not alter the authors' adherence to PLOS ONE Editorial policies and criteria.

Abstract

Interleukin-34 (IL-34) is a newly discovered cytokine as an additional ligand for colony stimulating factor-1 receptor (CSF1R), and its functions are expected to overlap with colony stimulating factor-1/macrophage-colony stimulating factor. We have previously shown that the IL-34 is primarily produced by neurons in the central nervous system (CNS) and induces proliferation and neuroprotective properties of microglia which express CSF1R. However, the functions of IL-34 in the CNS are still elucidative. Here we show that CNS capillary endothelial cells also express CSF1R. IL-34 protected blood–brain barrier integrity by restored expression levels of tight junction proteins, which were downregulated by pro-inflammatory cytokines. The novel function of IL-34 on the blood–brain barrier may give us a clue for new therapeutic strategies in neuroinflammatory and neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease.

Introduction

Interleukin-34 (IL-34) has been identified as an additional ligand for colony stimulating factor-1 receptor (CSF1R), and it is broadly expressed in various organs including heart, brain, lung, liver, kidney, spleen, and colon [1]. IL-34 and colony stimulating factor-1/macrophage-colony stimulating factor (CSF-1/M-CSF) bind to the different regions of CSF1R and share no overt sequence homology [2]. Recent studies showed that IL-34 produced by epithelial lineage cells (e.g. keratinocytes, splenic vascular endothelial cells, and neurons) is necessary for the development of tissue macrophage-like cells (e.g. Langerhans cells, osteoclasts, and microglia) [3–5]. We also have shown that IL-34 is

exclusively produced by neurons in CNS and induces proliferation of microglia [6]. We also showed that IL-34 attenuated the neurotoxic effect of oligomeric amyloid beta (Aβ) *in vitro* and intracerebroventricular administration of IL-34 ameliorates the impairment of associative learning in an AD mouse model [6]. Another study also demonstrated that IL-34 rescued neuronal damage in mouse models of AD and kinate-induced neurotoxicity [7]. These findings suggest distinct functions of IL-34 in the development of various CNS disorders. However, the precise functions of IL-34 in the CNS still remain to be elucidated.

The blood-brain barrier (BBB) is a tight seal composed of capillary endothelial cells, pericytes, and astrocytes [8]. The BBB contributes to maintenance of CNS homeostasis by limiting the entry of plasma components, erythrocytes, and immune cells from the circulating blood [9–11]. Tight junction (TJ) plays an important role in the barrier function of the BBB, which is composed by TJ proteins including claudins, occludin, and zonula occludens-1 (ZO-1) [12]. BBB disruption is frequently associated with synaptic and neuronal dysfunction in various neurological disorders such as multiple sclerosis (MS), AD, Parkinson's disease, and amyotrophic lateral sclerosis [13, 14]. Pro-inflammatory cytokines such as IL-1β, tumor necrosis factor-α (TNF-α), interferon-γ, and IL-17, are thought to downregulate the expression of tight junction proteins and contribute to the transmigration of inflammatory immune cells into the CNS, which exacerbates neuroinflammation in these diseases [14–19].

In this study, we found that the CNS capillary endothelial cells as well as microglia express CSF1R. We also showed that IL-34 restored pro-inflammatory cytokine–induced BBB disruption by upregulating the expression levels of tight junction proteins such as claudin-5 and occludin. These findings suggest the presence of neuronal regulation of BBB functions via IL-34, and upregulation of IL-34 in the CNS may be a novel therapeutic strategy against neuroinflammatory and neurodegenerative disorders.

Materials and Methods

Reagents

Recombinant mouse IL-1β, TNF-α, and IL-34 were purchased from R&D Systems (Minneapolis, MN, USA). The c-fms/CSF1R tyrosine kinase inhibitor GW2580 was used as a blocker of CSF1R signaling (Millipore, Bedford, MA, USA). Dylight 594–labeled tomato lectin was used as a capillary endothelial cell marker (Vector Laboratories, Burlingame, CA, USA).

Animals

All protocols were approved by the Animal Experiment Committee of Nagoya University (approved number: 14018). C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan).

Cells

Primary neuronal cultures were prepared from the cortices of C57BL/6 mouse embryos at embryonic Day 17 as described previously [20]. Briefly, cortical fragments were dissociated into single cells in dissociation solution (Sumitomo Bakelite, Akita, Japan), and resuspended in neuron culture medium (Sumitomo Bakelite). Neurons were seeded onto 12-mm polyethylenimine-coated glass coverslips (Asahi Techno Glass Corp., Chiba, Japan) at a density of 5.0×10^4 cells/well in 24-well culture plates and were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. The purity of the cultures was >95% as determined by NeuN-specific immunostaining. Mouse brain capillary endothelial cell line MBEC4 [21] was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Confluent monolayer of MBEC4 cells was used as an established BBB model as described previously [22].

BBB Permeability assay

The permeability of MBEC4 cell monolayers was measuring transendothelial electrical resistance (TER) as described previously [22]. Confluent monolayer of MBEC4 cells on the 24-well transwell inserts (3-μm pore size) were incubated with or without 20 ng/ml TNF-α, 20 ng/ml IL-1β, 0–100 ng/ml IL-34, or 1 μmol/L GW2580 for 24 h. TER was measured using a Millicell-ERS (Millipore). Resistances of blank filters were subtracted from those of filters with cells before final resistances ($\Omega \cdot cm^2$) were calculated. Assays were carried out in five independent trials.

Immunocytochemistry

Primary neurons and MBEC4 cells were fixed with 4% paraformaldehyde for 10 min, permeabilized using 0.1% Triton X-100 for 5 min, and blocked using 5% normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature. Neurons were incubated with rabbit anti-mouse IL-34 polyclonal antibodies (ProSci, Poway, CA, USA), mouse anti-mouse microtubule-associated protein–2 (MAP-2) monoclonal antibody (Chemicon, Temecula, CA, USA) overnight at 4℃ followed by a 1-h incubation with Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). MBEC4 cells were stained using rabbit anti-mouse CSF1R polyclonal antibodies (Abcam, Cambridge, UK) overnight at 4℃ followed by a 1-h incubation with Alexa-conjugated secondary antibodies (Invitrogen). Nuclei were counterstained with Hoechst 33342 (Invitrogen). Images were analyzed using a deconvolution fluorescent microscope system (BZ-8000, Keyence, Osaka, Japan).

Immunohistochemistry

Brains and lumbar spinal cords from C57BL/6J mice were fixed with 4% paraformaldehyde overnight, equilibrated in 20% sucrose with PBS for 48 hours,

embedded in Tissue Tek O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and frozen at −80℃ overnight. Coronal brain sections and transverse spinal cord sections (20 μm-thick) were prepared using a cryostat. Sections were permeabilized using 0.3% Triton X-100 after blocking with 5% normal goat serum in PBS for 1 h. Sections were incubated with rabbit anti-mouse IL-34 polyclonal antibodies (ProSci), mouse anti-mouse MAP-2 monoclonal antibody (Chemicon), rabbit anti-mouse CSF1R polyclonal antibodies (Abcam), and Dylight 594–labeled tomato lectin (Vector Laboratories) overnight at 4℃ followed by a 1-h incubation with Alexa-conjugated secondary antibodies (Invitrogen). Images were analyzed using a deconvolution fluorescent microscope system (BZ-8000, Keyence).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

MBEC4 cells were cultured at a concentration of 4×10^5 cells/well in 24-well culture plates and stimulated with 100 ng/ml IL-34 for 24 h. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs encoding mouse IL-34 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were generated by RT-PCR using SuperScript II (Invitrogen), Blend Taq DNA polymerase (Toyobo, Osaka, Japan), and the following specific primer sets:

CSF1R forward primer: 5′-AAGCAGAAGCCGAAGTACCA-3′
CSF1R reverse primer: 5′-GTCCCTGCGCACATATTTCAT-3′
GAPDH forward primer: 5′-TGTGTCCGTCGTGGATCTGA-3′
GAPDH reverse primer: 5′-CCTGCTTCACCACCTTCTTGA-3′

Western Blotting

MBEC4 Cells were lysed in TNES buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, and 0.1% SDS) with protease inhibitor mixture (Complete Mini EDTA-free; Roche Diagnostics, Basel, Switzerland). Cell lysate proteins dissolved in Laemmli sample buffer (20 μg/well) were separated on 4–20% SDS-polyacrylamide gels (Mini-Protean TGX; Bio-Rad, Hercules, CA, USA) and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA) as described previously [23]. The membranes were blocked for 1 h at room temperature with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20, and then incubated overnight at 4℃ with rabbit anti-mouse Zonula Occludens–1 (ZO-1) polyclonal antibodies, rabbit anti-mouse occludin polyclonal antibodies, rabbit anti-mouse claudin-5 polyclonal antibodies (Invitrogen), and mouse anti-β-actin monoclonal antibody (Sigma). After an overnight incubation with primary antibodies at 4℃, each blot was probed with horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare). Blots were then visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and quantitated using

a CS Analyzer 3.0 system (Atto, Tokyo, Japan). Assays were carried out in five independent trials.

Statistical analysis

Statistical significance was analyzed with one-way analysis of variance followed by post-hoc Tukey's test, using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

IL-34 is exclusively expressed in CNS neurons

In the previous study, we have reported that IL-34 protein is primarily expressed in neurons whereas IL-34 mRNA expression was detected in neurons and astrocytes [6]. First, we confirmed the expression pattern of IL-34 in the CNS using immunostaining in mouse primary cortical neurons, brains, and spinal cords. As shown in Fig. 1, IL-34 protein was exclusively expressed in neurons in the CNS.

CNS capillary endothelial cells expressed IL-34 receptor CSF1R

Next, we examined the expression pattern of IL-34 receptor CSF1R protein in the CNS using immunostaining. In addition to microglia, CNS microvessels were also immunopositive for CSF1R (Fig. 2A, green). Its staining pattern in the microvessels was identical to that of tomato lectin (Fig. 2A, red and arrows in the overlap images) which selectively binds to the surface of capillary endothelial cells [24], suggesting that CNS capillary endothelial cells express CSF1R. Meninges and large vessel adventitia were also stained with CSF1R and tomato lectin. Although CSF1R has been detected on fibroblasts and smooth muscle cells which are the main components of meninges and adventitia [1, 7], the meninges and adventitia showed strong non-specific binding of antibodies and lectin. Therefore, the positive staining in meninges and adventitia may be artifact.

Furthermore, mouse brain capillary endothelial cell line MBEC4 cells strongly express CSF1R protein (Fig. 2B, green). MBEC4 cells constitutively express CSF1R mRNA, and stimulation with IL-34 did not alter CSF1R expression level (Fig. 2C). These data indicate that CNS capillary endothelial cells constitutively express CSF1R and are potential target of IL-34 in the CNS, as well as microglia.

IL-34 restored BBB disruption via CSF1R signaling in endothelial cells

BBB disruption is a common pathological feature of various neurological diseases, and inflammatory cytokines such as IL-1β and TNF-α have been considered as causative factors that damage BBB integrity by downregulating TJ proteins in BBB endothelial cells [14–16, 25, 26]. To investigate whether IL-34 affects the BBB

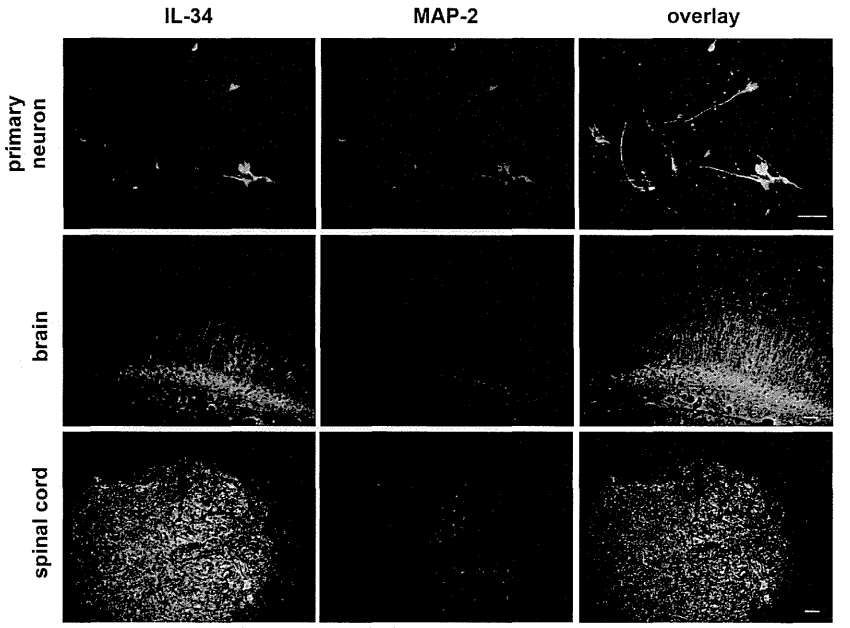


Fig. 1. IL-34 is produced by neurons in the CNS. Immunofluorescence images of primary cortical neurons, brain sections, and lumbar spinal cord sections. Green, IL-34; red, MAP-2; blue, Hoechst nuclear counterstain. Scale bar, 50 μm.

doi:10.1371/journal.pone.0115981.g001

integrity, we evaluated BBB permeability by measuring TER in MBEC4 cell monolayer as an *in vitro* BBB model [22]. IL-34 significantly ameliorated a decrease in TER induced by IL-1β and TNF-α in a dose dependent manner (Fig. 3), whereas treatment with IL-34 alone did not alter untreated BBB integrity (data not shown). Moreover, addition of CSF1R signal inhibitor GW2580 ablated the effect of IL-34 on BBB (Fig. 3). These results indicate that IL-34 restored pro-inflammatory cytokine–mediated BBB disintegrity via CSF1R signaling in endothelial cells.

IL-34 upregulated TJ proteins in BBB endothelial cells

Next, we assessed whether IL-34 alters the expression levels of TJ proteins that are sensitive to pro-inflammatory cytokines [14–16]. Western blotting analysis detected that major TJ proteins such as claudin-5 and occludin were significantly downregulated by treatment with IL-1β and TNF-α (Fig. 4). Addition of IL-34 reversed the expression levels of these TJ proteins (Fig. 4), whereas treatment with IL-34 alone did not alter the expression levels of TJ proteins in untreated MBEC4 cells (data not shown). Addition of GW2580 canceled the effect of IL-34 on the expression of claudin-5 and occludin (Fig. 4). These data suggest that IL-34 rescues pro-inflammatory cytokine–induced BBB disruption via upregulating TJ proteins such as claudin-5 and occludin in BBB endothelial cells.

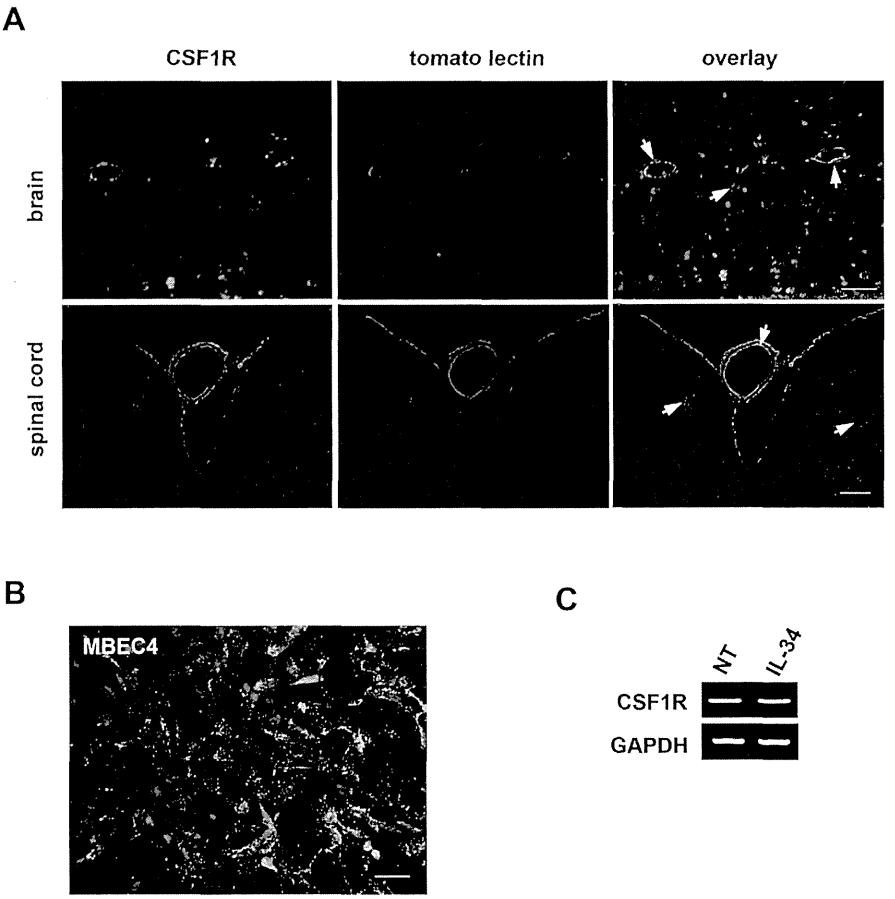


Fig. 2. CNS endothelial cells express CSF1R. (A) Immunofluorescence images of brain sections, and lumbar spinal cord sections. Green, CSF1R; red, tomato lectin; blue, Hoechst nuclear counterstain. Arrows indicate CSF1R-immunopositivity in the capillary endothelial cells. Scale bar, 50 μm. (B) Immunofluorescence image of mouse brain capillary endothelial cell line MBEC4. Green, CSF1R; blue, Hoechst nuclear counterstain. Scale bar, 50 μm. (C) RT-PCR data for CSF1R. Stimulation with IL-34 did not alter the expression of CSF1R in MBEC4 cells.

doi:10.1371/journal.pone.0115981.g002

Discussion

IL-34 is widely expressed in a variety of tissues including brain. Because IL-34 shares the same receptor with CSF-1/M-CSF, it has similar functions on monocyte lineage cells such as induction of proliferation and differentiation of macrophages, Langerhans cells, osteoclasts, and microglia [3–5, 27, 28]. In the CNS, IL-34 is mainly released by neurons, especially when they are damaged [6]. Like CSF-1/M-CSF, IL-34 induces microglial proliferation. In addition, IL-34 enhances microglial neuroprotective functions by inducing anti-oxidant enzyme heme oxigenase-1 (HO-1), and amyloid degrading enzyme insulin degrading enzyme (IDE). Moreover, we also found that IL-34 induces microglial production of TGF-β which negatively regulates microglial activation [29]. TGF-β dose-dependently suppressed microglial proliferation by IL-34 but attenuated oligomeric amyloid

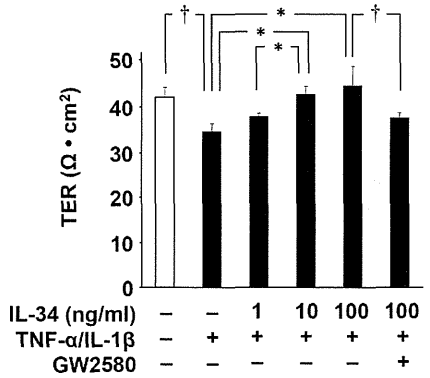


Fig. 3. IL-34 restores damaged BBB integrity. MBEC4 cells were treated with TNF-α (20 ng/ml) and IL-1β (20 ng/ml) in the presence of IL-34 (0–100 ng/ml) and GW2580 (1 μmol/l). TER of MBEC4 cell monolayer was measured after a 24-h incubation. Values are means ± SEM (n=5). *, $p<0.05$; †, $p<0.01$.

doi:10.1371/journal.pone.0115981.g003

β–mediated neurotoxicity [29]. The neuroprotective functions of IL-34 was partially suppressed by blockade of TGF-β receptor signaling, suggesting that neuroprotective effect of IL-34 was in part mediated by microglial TGF-β production in response to IL-34. Thus, IL-34 released from damaged neurons acts as a "Help-me" signal which induces microglial neuroprotective effects with

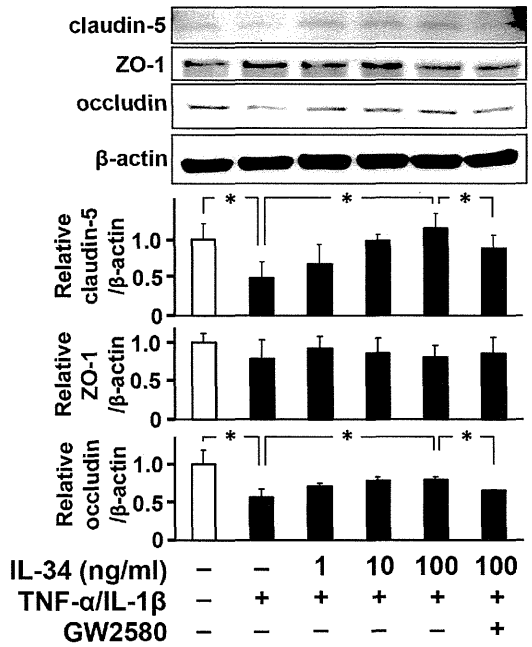


Fig. 4. IL-34 upregulates tight junction proteins in MBEC4 cells. MBEC4 cells were incubated with TNF-α (20 ng/ml) and IL-1β (20 ng/ml) in the presence of IL-34 (0–100 ng/ml) and GW2580 (1 μmol/L) for 24 h. Upper, representative images of Western blots for tight junction proteins. Bottom, quantified expression levels of tight junction proteins relative to those in untreated cells. Values are means ± SEM (n=5). *, $p<0.05$.

doi:10.1371/journal.pone.0115981.g004

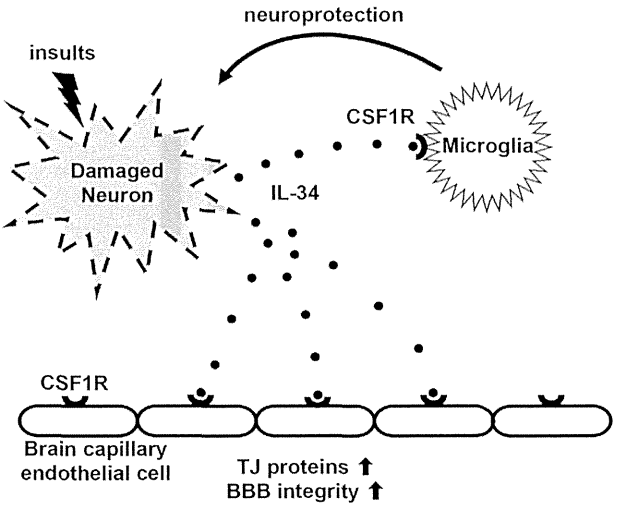


Fig. 5. Model of the roles of IL-34 in the CNS. Damaged neurons secrete IL-34 as a "Help-me" signal. IL-34 binds its receptor CSF1R which is mainly expressed in microglia and BBB endothelial cells. CSF1R signaling enhances neuroprotection in microglia and restores BBB disruption by upregulating TJ proteins in capillary endothelial cells.

doi:10.1371/journal.pone.0115981.g005

subsiding microglial activation (Fig. 5). The expression of CSF1R is reportedly high during early postnatal development, and is very low in adult brain [30]. IL-34 exhibited a broader regional expression than CSF-1/M-CSF, mostly without overlap, suggesting important role of IL-34–CSF1R signaling in regional neurogenesis. A previous study reported that CSF1R expression is increased in microglia of AD brains and microglia overexpressing CSF1R are neuroprotective [31]. Therefore, IL-34 produced by neurons [6] as well as CSF-1/M-CSF produced by astrocytes [32] may be involved in the development of neurodegenerative disesases such as AD via microglial CSF1R signaling.

In this study, we have shown that BBB endothelial cell expresses CSF1R and is a novel target of IL-34. BBB disruption has been implicated as a pathogenesis of various neurological disorders including MS and AD. A recent study showed that amyloid β suppressed expression of TJ protein ZO-1 in BBB endothelial cells via receptor for advanced glycation end products (RAGE) and claimed that amyloid β-RAGE interaction may be a potential molecular pathway in breakage of BBB integrity [33]. In addition, pro-inflammatory cytokines such as IL-1β, TNF-α, IFN-γ, and IL-17 have been considered as the candidates to increase BBB leakage [14–19]. Our findings revealed a novel function of IL-34–CSF1R signaling on the maintenance of BBB integrity via upregulating major TJ proteins claudin-5 and occludin in capillary endothelial cells (Fig. 5). A major downstream target of CSF1R signaling is cAMP responsive element-binding protein (CREB), which modulates the transcription of TJ proteins [34, 35]. Taken together, IL-34 released from damaged neurons may functions as a "Help-me" signal toward restoration of CNS homeostasis via microglia and BBB endothelial cells (Fig. 5). Our study clarified the presence of neuronal regulation of BBB functions via IL-34–CSF1R

signaling. IL-34–CSF1R pathway may be novel therapeutic target for neuroinflammatory and neurodegenerative disorders such as MS and AD.

Author Contributions

Conceived and designed the experiments: SJ HT AS. Performed the experiments: SJ. Analyzed the data: SJ HT TM AS. Contributed reagents/materials/analysis tools: YS JK TM HH YC YW. Wrote the paper: SJ HT AS.

References

1. **Lin H, Lee E, Hestir K, Leo C, Huang M, et al.** (2008) Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. Science 320: 807–811
2. **Chihara T, Suzu S, Hassan R, Chutiwitoonchai N, Hiyoshi M, et al.** (2010) IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. Cell Death Differ 17: 1917–1927
3. **Wang Y, Szretter KJ, Vermi W, Gilfillan S, Rossini C, et al.** (2012) IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. Nat Immunol 13: 753–760
4. **Greter M, Lelios I, Pelczar P, Hoeffel G, Price J, et al.** (2012) Stroma-derived interleukin-34 controls the development and maintenance of langerhans cells and the maintenance of microglia. Immunity 37: 1050–1060.
5. **Nakamichi Y, Mizoguchi T, Arai A, Kobayashi Y, Sato M, et al.** (2012) Spleen serves as a reservoir of osteoclast precursors through vitamin D-induced IL-34 expression in osteopetrotic op/op mice. Proc Natl Acad Sci U S A 109: 10006–10011.
6. **Mizuno T, Doi Y, Mizoguchi H, Jin S, Noda M, et al.** (2011) Interleukin-34 selectively enhances the neuroprotective effects of microglia to attenuate oligomeric amyloid-β neurotoxicity. Am J Pathol 179: 2016–2027.
7. **Luo J, Elwood F, Britschgi M, Villeda S, Zhang H, et al.** (2013) Colony-stimulating factor 1 receptor (CSF1R) signaling in injured neurons facilitates protection and survival. J Exp Med 210: 157–172.
8. **Risau W, Engelhardt B, Wekerle H** (1990) Immune function of the blood-brain barrier: incomplete presentation of protein (auto-)antigens by rat brain microvascular endothelium in vitro. J Cell Biol 110: 1757–1766.
9. **Abbott NJ, Romero IA** (1996) Transporting therapeutics across the blood-brain barrier. Mol Med Today 2: 106–113.
10. **Abbott NJ** (2002) Astrocyte-endothelial interactions and blood-brain barrier permeability. J Anat 200: 629–638.
11. **Begley DJ** (2003) Understanding and circumventing the blood-brain barrier. Acta Paediatr Suppl 92: 83–91.
12. **Hawkins BT, Davis TP** (2005) The blood-brain barrier/neurovascular unit in health and disease. Pharmacol Rev 57: 173–185.
13. **Zhong Z, Deane R, Ali Z, Parisi M, Shapovalov Y, et al.** (2008) ALS-causing SOD1 mutants generate vascular changes prior to motor neuron degeneration. Nat Neurosci 11: 420–422.
14. **Afonso PV, Ozden S, Prevost MC, Schmitt C, Seilhean D, et al.** (2007) Human blood-brain barrier disruption by retroviral-infected lymphocytes: role of myosin light chain kinase in endothelial tight-junction disorganization. J Immunol 179: 2576–2583.
15. **Fabry Z, Topham DJ, Fee D, Herlein J, Carlino JA, et al.** (1995) TGF-beta 2 decreases migration of lymphocytes in vitro and homing of cells into the central nervous system in vivo. J Immunol 155: 325–332.