Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi: 10.1111/cei.13487

# Peroxiredoxins are involved in the pathogenesis of multiple sclerosis and neuromyelitis optica spectrum disorder

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Accepted for publication 2 July 2020

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## Summary

**Peroxiredoxins (PRXs) are intracellular anti-oxidative enzymes but work as inflammatory amplifiers under the extracellular condition. To date, the function of PRXs in the pathogenesis of multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD) is not fully understood. The aim of this study was to investigate whether PRXs play a role in the pathogenesis of MS and NMOSD. We analyzed levels of PRXs (PRX1, PRX5 and PRX6) in the cerebrospinal fluid (CSF) and serum of 16 patients with MS, 16 patients with NMOSD and 15 patients with other neurological disorders (ONDs). We identified potential correlations between significantly elevated PRXs levels and the clinical variables in patients with MS and NMOSD. Additionally, pathological analyses of PRXs (PRX1-6) in the central nervous system (CNS) were performed using the experimental autoimmune encephalomyelitis (EAE), animal model of MS. We found that serum levels of PRX5 and PRX6 in patients with MS and NMOSD were higher compared with those in patients with ONDs ($P < 0{\cdot}05$). Furthermore, high levels of PRX5 and PRX6 were partly associated with blood–brain barrier dysfunction and disease duration in NMOSD patients. No significant elevation was found in CSF PRXs levels of MS and NMOSD. Spinal cords from EAE mice showed remarkable PRX5 staining, especially in $CD45^+$ infiltrating cells. In conclusion, PRX5 and PRX6 may play a role in the pathogeneses of MS and NMOSD.**

**Keywords:** blood–brain barrier, CD45, experimental autoimmune encephalomyelitis, multiple sclerosis, neuromyelitis optica spectrum disorder, peroxiredoxin

## Introduction

Multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD) are demyelinating inflammatory diseases of the central nervous system (CNS) [1,2]. Many previous studies have revealed the important roles of inflammatory mediators, including cytokines, in the pathogenesis of MS and NMOSD [3–8]. Substances derived from damaged autologous tissues (damage-associated molecular patterns: DAMPs), are attracting attention in the field of autoimmune diseases because they function as inflammatory mediators when released from cells [9,10]. We previously reported increased levels of high mobility group box 1 (HMGB1), a DAMP, in the serum of MS patients and the cerebrospinal fluid (CSF) from MS and NMOSD [4]. We also reported a significant positive correlation between CSF HMGB1 levels and CSF cells in patients with MS and NMOSD [4]. These findings indicate that HMGB1 may be involved with inflammation in MS and NMOSD. Also, the administration of anti-HMGB1 monoclonal antibodies ameliorated clinical symptoms, CNS inflammation, demyelination and serum interleukin (IL)-17 up-regulation in experimental autoimmune encephalomyelitis (EAE) [11]. These findings suggest that HMGB1 may control autoimmune responses by stimulating the release of inflammatory cytokines in MS. Recently, peroxiredoxins (PRXs), which are intracellular anti-oxidant

enzymes, attract attention as novel DAMPs. Extracellular PRXs also induce the production of inflammatory cytokines and trigger inflammation; the proinflammatory effects of PRXs are reported to be stronger than that of HMGB1 [12]. Besides increasing inflammation, PRXs were reported to affect the blood–brain barrier function [13], which is another important factor in the pathogenesis of MS and NMOSD [14]. We speculate that PRXs could be key players in the inflammatory response of demyelinating CNS disorders, including MS and NMOSD, and could be a future therapeutic target. However, the exact function of PRXs in the pathogenesis of MS and NMOSD is not yet fully understood. The main aim of this study was to determine whether PRXs are involved in promoting inflammatory processes and affecting the blood–brain barrier function in patients with MS and NMOSD.

## Materials and methods

### Patients

Patients with relapsing–remitting MS ($n$ = 16) [1] and anti-aquaporin 4 (AQP4) antibody-positive NMOSD ($n$ = 16) [2] were included in this study. Fifteen patients (nine men, six women; mean age = 61·4 years) with other neurological disorders (ONDs), including nine patients with amyotrophic lateral sclerosis and six with spinocerebellar degeneration, were recruited as controls. The following patient variables were reviewed: gender, age, disease duration, Kurtzke's expanded disability status scale (EDSS) scores, the presence of serum anti-AQP4 antibody [15], positivity for oligoclonal bands, CSF cell counts, CSF protein, CSF/serum albumin ratio (albumin quotient, Qalb) and immunomodulatory treatment at the time of sampling. The ethics committee of the Chiba University School of Medicine in Chiba, Japan, approved the study (approved no. 842). Written informed consent was obtained from all study subjects.

### PRX1, PRX5 and PRX6 measurements in patients with MS and NMOSD

Commercial enzyme-linked immunosorbent assay (ELISA) kits were only available for the detection of PRX1, PRX5 and PRX6 (MyBioSource, Inc., San Diego, CA, USA). CSF samples were obtained during the active disease phase (within 1 month of clinical attack and before treatment for the attack) from patients with MS ($n$ = 16) and NMOSD ($n$ = 16) and patients with ONDs ($n$ = 15). Serum samples were simultaneously obtained in 10 patients with MS, 10 with NMOSD and 10 with ONDs at the time of CSF sampling. All samples were stored at −80°C until use. The CSF and serum levels of PRX1, PRX5 and PRX6 were measured using the ELISA kits according to the manufacturer's instructions.

### Correlations between the PRXs levels and clinical parameters in patients

The possible associations between the significantly elevated levels of PRXs and clinical variables, such as the duration of disease, EDSS, CSF cell counts, CSF protein levels and Qalb, were examined in patients with MS and NMOSD.

### EAE induction and pathological analyses

To identify associations between PRXs and CNS inflammation, we isolated the spinal cords from EAE mice for pathological analysis. EAE was induced in mice using the same method described in our previous study [16]. Simply, a total of 200 μg myelin oligodendrocyte glycoprotein peptide 35–55 in complete Freund's adjuvant containing killed *Mycobacterium tuberculosis* H37Ra was subcutaneously administered to wild-type C57BL/6 mice (10 weeks old, female) (day 1). Then, the mice received intraperitoneal injections of 400 ng pertussis toxin (days 1 and 2). EAE mice were scored using the following scale: 0·0 = no clinical signs, 1·0 = partial paralysis of the tail, 2·0 = limp tail and mild bilateral hind leg paralysis, 3·0 = limp tail and complete paralysis of the hind legs, 4·0 = limp tail and complete hind leg and partial front leg paralysis and 5·0 = complete hind and front leg paralysis. In this study, we used spinal cords from EAE mice ($n$ = 2) whose score was 4·0 on day 18 to investigate the role of PRXs in the established inflammatory CNS lesions. Untreated normal (naive) mice ($n$ = 2) were used as controls. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of Chiba University (approved no. 1–9).

Histopathological examinations were performed using paraffin-embedded sections of spinal cords, and the sections were stained with hematoxylin and eosin (H&E), Luxol fast blue (LFB), Klüver–Barrera (KB), rabbit anti-CD11b (ab133357; Abcam, Cambridge, UK), mouse anti-glial fibrillary acidic protein (GFAP) (NCL-GFAP-GA5; Novocastra, Newcastle upon Tyne, UK), rabbit anti-PRX1 (15816-1-AP; ProteinTech Group, Manchester, UK), rabbit anti-PRX2 (10545-2-AP; ProteinTech Group,), rabbit anti-PRX3 (10664-1-AP; ProteinTech Group), rabbit anti-PRX4 (10703-1-AP; ProteinTech Group), rabbit anti-PRX5 (17724-1-AP; ProteinTech Group), rabbit anti-PRX6 (13585-1-AP; ProteinTech Group), rat anti-CD3 (ab56313; abcam) and rat anti-CD45 (SC-53665; Santa Cruz Biotechnology, Dallas, TX, USA). Alexa Fluor594 chicken anti-rabbit IgG (A21442; Invitrogen, Carlsbad, CA, USA) and goat anti-rat secondary antibody Alexa Fluor488

(A-11006; ThermoFisher, Fremont, CA, USA) were used as secondary antibodies.

### Statistical analyses

Statistical analyses were performed using the JMP Pro version 12.0.1 software (SAS Institute Inc., Cary, NC, USA). Groups were compared using the Mann–Whitney *U*-test for unpaired continuous variables. Spearman's rank correlation coefficient was used to test correlations between variables. *P*-values of $< 0{\cdot}05$ were considered statistically significant.

## Results

### Clinical profiles of patients

The clinical characteristics of the patients with MS, NMOSD and ONDs are summarized in Table 1. The age, duration of disease, positivity for serum anti-AQP4 antibodies, negativity for oligoclonal bands, CSF cells, CSF protein and Qalb were higher in patients with NMOSD than those in patients with MS. The ages of patients with ONDs were similar to those of the NMOSD patients, but higher than those of MS patients. CSF cells, CSF protein and Qalb were higher in MS and NMOSD patients compared with patients with ONDs.

### CSF and serum PRXs levels in patients

CSF PRX1 levels were $2{\cdot}10 \pm 2{\cdot}72$ [ng/ml, mean ± standard deviation (s.d.)], $1{\cdot}27 \pm 1{\cdot}54$ and $1{\cdot}84 \pm 1{\cdot}68$; CSF PRX5 levels (ng/ml) were $1{\cdot}17 \pm 0{\cdot}28$, $2{\cdot}20 \pm 4{\cdot}74$ and $1{\cdot}26 \pm 0{\cdot}41$; and CSF PRX6 levels (ng/ml) were $3{\cdot}94 \pm 5{\cdot}49$, $2{\cdot}15 \pm 1{\cdot}91$ and $2{\cdot}42 \pm 3{\cdot}70$ in patients with NMOSD, MS and ONDs, respectively. There were no significant differences in the levels of PRXs in the CSF among the groups of patients (Fig. 1).

Serum PRX1 levels (ng/ml) were $13{\cdot}88 \pm 20{\cdot}74$, $7{\cdot}24 \pm 10{\cdot}50$ and $2{\cdot}28 \pm 3{\cdot}98$; serum PRX5 levels (ng/ml) were $5{\cdot}47 \pm 6{\cdot}54$, $1{\cdot}77 \pm 1{\cdot}28$ and $0{\cdot}98 \pm 0{\cdot}00$; and serum PRX6 levels (ng/mL) were $43{\cdot}81 \pm 41{\cdot}21$, $22{\cdot}72 \pm 11{\cdot}47$ and $6{\cdot}25 \pm 4{\cdot}75$ in patients with NMOSD, MS and ONDs, respectively. Serum PRX5 and PRX6 levels in patients with NMOSD and MS were significantly higher than those in patients with ONDs (PRX5: NMOSD *versus* ONDs, $P = 0{\cdot}013$; MS *versus* ONDs, $P = 0{\cdot}031$, PRX6: NMOSD *versus* ONDs, $P = 0{\cdot}0003$; MS *versus* ONDs, $P = 0{\cdot}0009$) (Fig. 1).

### Correlations between elevated serum levels of PRX5 and PRX6 and clinical variables in patients

Table 2 shows possible associations between significantly elevated serum levels of PRX5 and PRX6 and clinical parameters in patients with MS and NMOSD. In MS patients, no significant correlation was observed between serum PRX5 and PRX6 levels and clinical variables (CSF cell counts, CSF protein levels, Qalb, EDSS scores, duration of disease or oligoclonal band positivity). Among NMOSD patients, serum PRX5 levels correlated positively with disease duration (Spearman's $\rho = 0{\cdot}7198$, $P = 0{\cdot}019$); and serum PRX6 levels correlated positively with CSF protein levels (Spearman's $\rho = 0{\cdot}6727$, $P = 0{\cdot}033$), Qalb (Spearman's $\rho = 0{\cdot}6727$, $P = 0{\cdot}033$) and the duration of disease (Spearman's $\rho = 0{\cdot}6748$, $P = 0{\cdot}032$). Significant correlations between serum PRX5 and PRX6 levels were confirmed in patients with NMOSD (Spearman's $\rho = 0{\cdot}7047$, $P = 0{\cdot}023$) but not in patients with MS (Spearman's $\rho = 0{\cdot}5924$, $P = 0{\cdot}071$).

### Immunohistochemical staining of PRXs in EAE spinal cords

H&E and KB staining revealed cellular infiltration in the meninges and parenchyma and LFB staining showed

**Table 1.** Clinical manifestations of patients with MS, NMOSD and ONDs

| | MS ($n = 16$) | NMOSD ($n = 16$) | ONDs ($n = 15$) |
|---|---|---|---|
| Men : women | 4 : 12 | 2 : 14 | 9 : 6 |
| Age, years | 36·5 (18–51) | 61·0 (27–86) | 61·4 (42–75) |
| Disease duration, years | 4·9 (0–20) | 5·8 (0–40) | – |
| EDSS | 6·0 (1·0–9·0) | 6·3 (1·5–9·0) | – |
| Positive AQP4 antibody | 0/16 (0%) | 16/16 (100%) | – |
| Positive OCB | 10/14 (71%) | 2/14 (14%) | – |
| CSF cells, /mm$^3$ | 3·5 (0–43) | 4·7 (1–64) | 0·7 (0–3) |
| CSF TP, mg/dl | 32 (19–50) | 51 (17–160) | 37 (18–71) |
| Qalb, $\times 10^{-2}$ | 4·9 (2·7–11·7) | 6·0 (2·8–37·0) | 4·0 (2·7–6·2) |
| Immunomodulating therapy | 4/16 (25%) | 5/16 (31%) | 0/15 (0%) |

AQP4 = aquaporin 4; CSF = cerebrospinal fluid; EDSS = expanded disability status scale; MS = multiple sclerosis; NMOSD = neuromyelitis optica spectrum disorder; OCB = oligoclonal bands; ONDs = other neurological disorders; Qalb = albumin quotient.

Values show median (range) unless indicated.

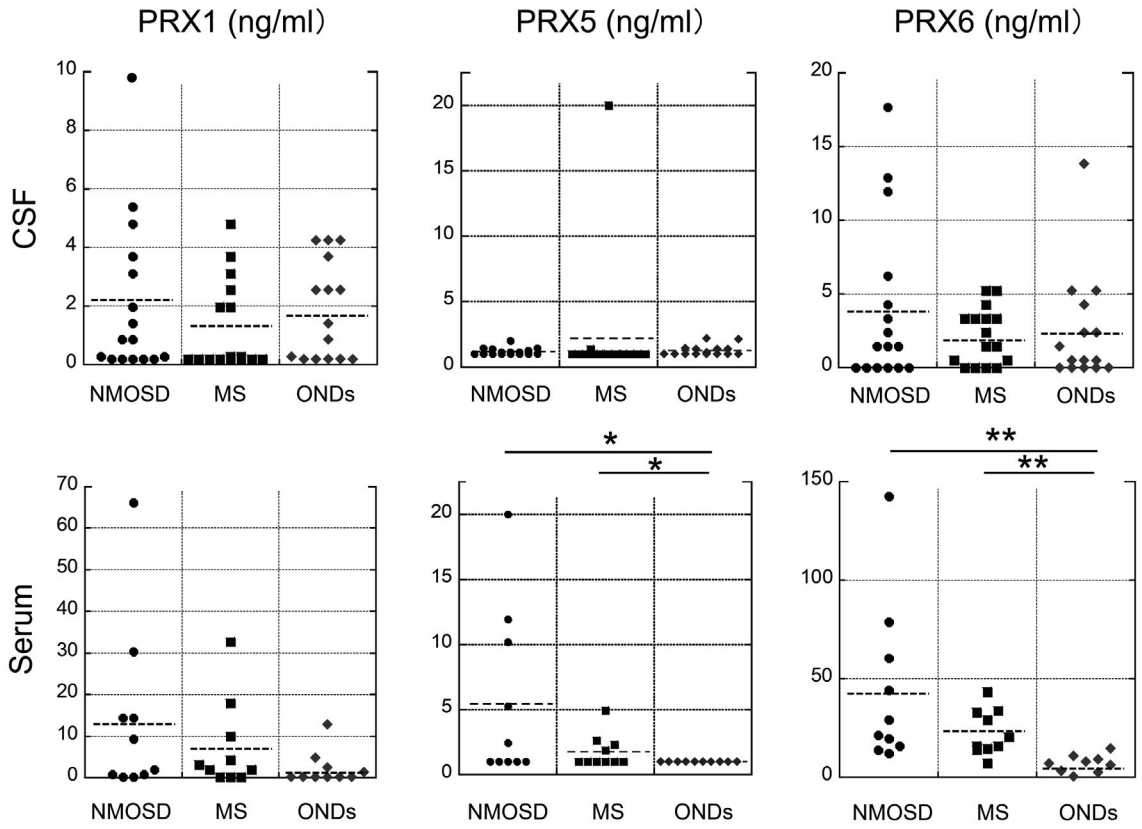


**Fig. 1.** Cerbrospinal fluid (CSF) and serum levels of peroxiredoxins (PRXs) in patients. No significant differences in the CSF levels of PRXs were identified among patients with multiple sclerosis (MS) ($n$ = 16), neuromyelitis optica spectrum disorder (NMOSD) ($n$ = 16) and other neurological disorders (ONDs) ($n$ = 15). Serum PRX5 and PRX6 levels were significantly elevated in patients with MS ($n$ = 10) and NMOSD ($n$ = 10) compared with patients with ONDs ($n$ = 10). Dashed lines indicate mean values. *$P$ < 0·05; **$P$ < 0·01.

**Table 2.** The relationships between serum PRX5 and PRX6 levels and clinical variables in patients with MS and NMOSD

| | MS ($n$ = 10) | | NMOSD ($n$ = 10) | |
|---|---|---|---|---|
| | Serum PRX5 | Serum PRX6 | Serum PRX5 | Serum PRX6 |
| CSF cells | ρ = 0.127 ($P$ = 0.727) | ρ = −0.006 ($P$ = 0.987) | ρ = 0.614 ($P$ = 0.059) | ρ = 0.212 ($P$ = 0.556) |
| CSF protein | ρ = −0·278 ($P$ = 0·436) | ρ = −0·064 ($P$ = 0·860) | ρ = 0·356 ($P$ = 0·313) | ρ = 0·673 ($P$ = 0·033*) |
| Qalb | ρ = −0·416 ($P$ = 0·266) | ρ = −0·142 ($P$ = 0·715) | ρ = 0·407 ($P$ = 0·243) | ρ = 0·673 ($P$ = 0·033*) |
| EDSS | ρ = 0·062 ($P$ = 0·865) | ρ = 0·543 ($P$ = 0·105) | ρ = −0·111 ($P$ = 0·760) | ρ = 0·275 ($P$ = 0·442) |
| Disease duration | ρ = 0·198 ($P$ = 0·583) | ρ = −0·055 ($P$ = 0·881) | ρ = 0·720 ($P$ = 0·019*) | ρ = 0·675 ($P$ = 0·032*) |
| Serum PRX5 | – | ρ = 0·592 ($P$ = 0·071) | – | ρ = 0·705 ($P$ = 0·023*) |

CSF = cerebrospinal fluid; EDSS = expanded disability status scale; MS = multiple sclerosis; NMOSD = neuromyelitis optica spectrum disorder; Qalb = albumin quotient; PRX = peroxiredoxin.

*Statistically significant.

demyelinated lesion of EAE spinal cords (Fig. 2). Majority of invasive cells to EAE lesions were CD11b$^+$ cells. Among the PRXs families (PRX 1–6), only PRX5 staining was confirmed in EAE spinal cords (Fig. 3). PRX5 staining was observed in CD45$^+$ infiltrating cells in the inflammatory lesions (Fig. 4).

## Discussion

In this study, we identified the elevations of serum PRX5 and PRX6 levels in patients with MS and NMOSD and their associations with blood–brain barrier dysfunction and disease duration in NMOSD patients. Pathological analyses

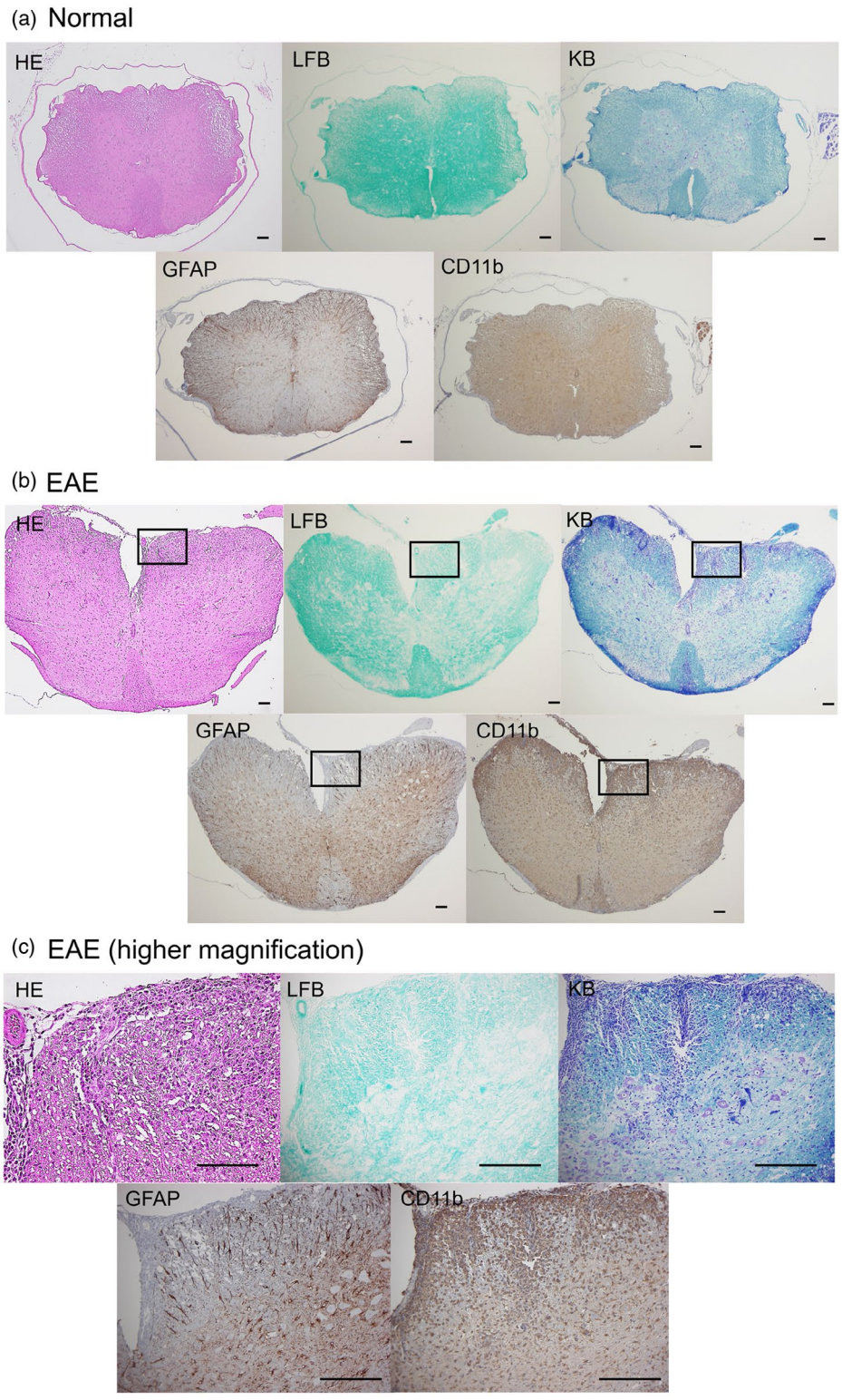


**Fig. 2.** Pathological findings of the experimental autoimmune encephalomyelitis (EAE) spinal cords. Hematoxylin and eosin (H&E), Luxol fast blue (LFB), Klüver–Barrera (KB), glial fibrillary acidic protein (GFAP) and CD11b immunoreactivity in the spinal cords of normal (a) and EAE mice (at day 18) (b: lower magnification; c: higher magnification) were performed ($n$ = 2 in each group; representative images are shown). Inflammation, demyelination, and CD11b positivity were remarkable in EAE mice but not in normal mice. Black blank squares indicate the area of high magnification. Bars indicate 100 μm.

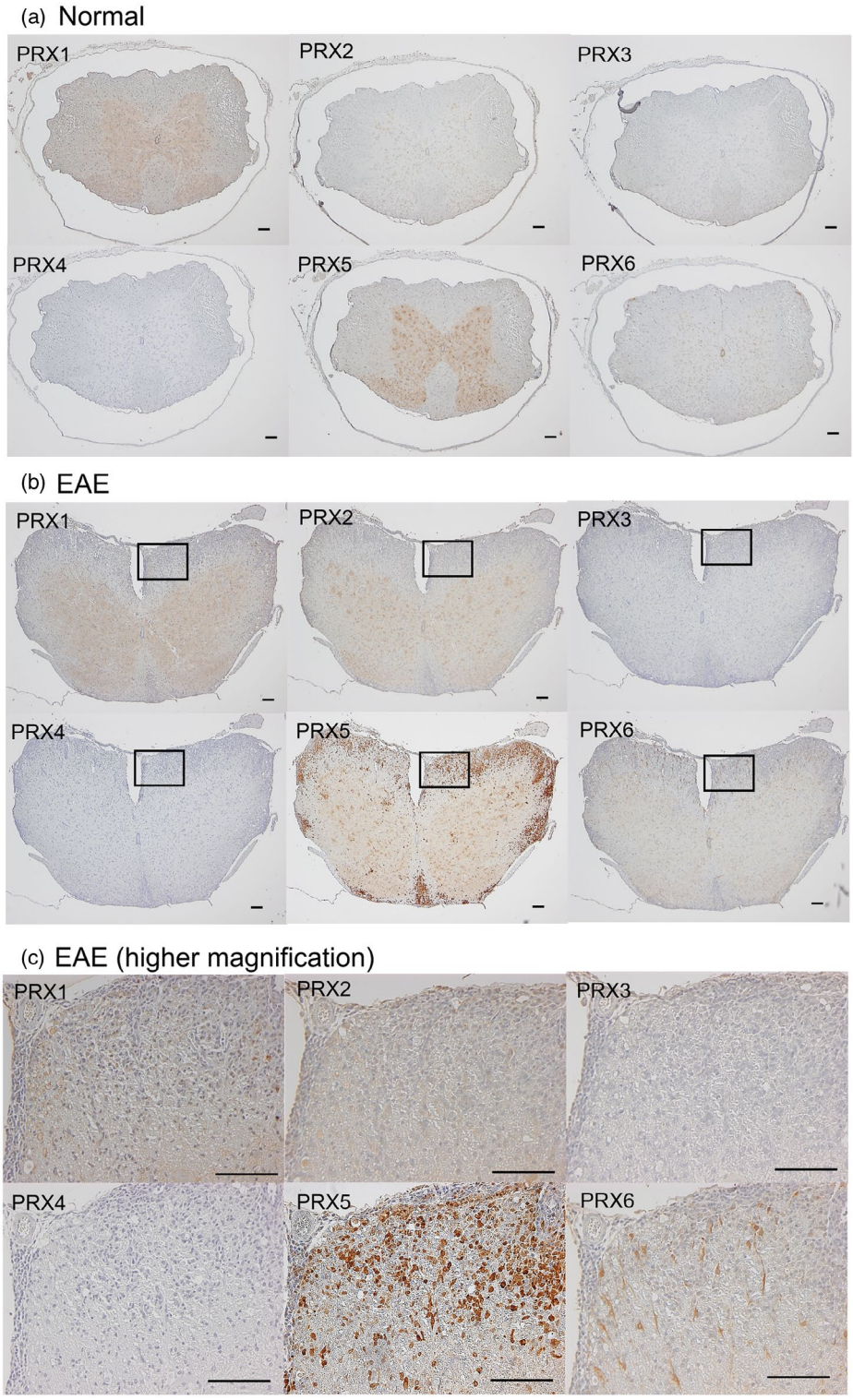


**Fig. 3.** Immunohistochemical staining of peroxiredoxins (PRXs) in the EAE spinal cords. PRX immunostaining in the spinal cords of normal (a) and experimental autoimmune encephalomyelitis (EAE) mice (at day 18) (b: lower magnification; c: higher magnification) were performed ($n = 2$ in each group; representative images are shown). PRX5 staining was remarkable in EAE mice but not in normal mice. Black blank squares indicate the area of high magnification. Bars indicate 100 μm.

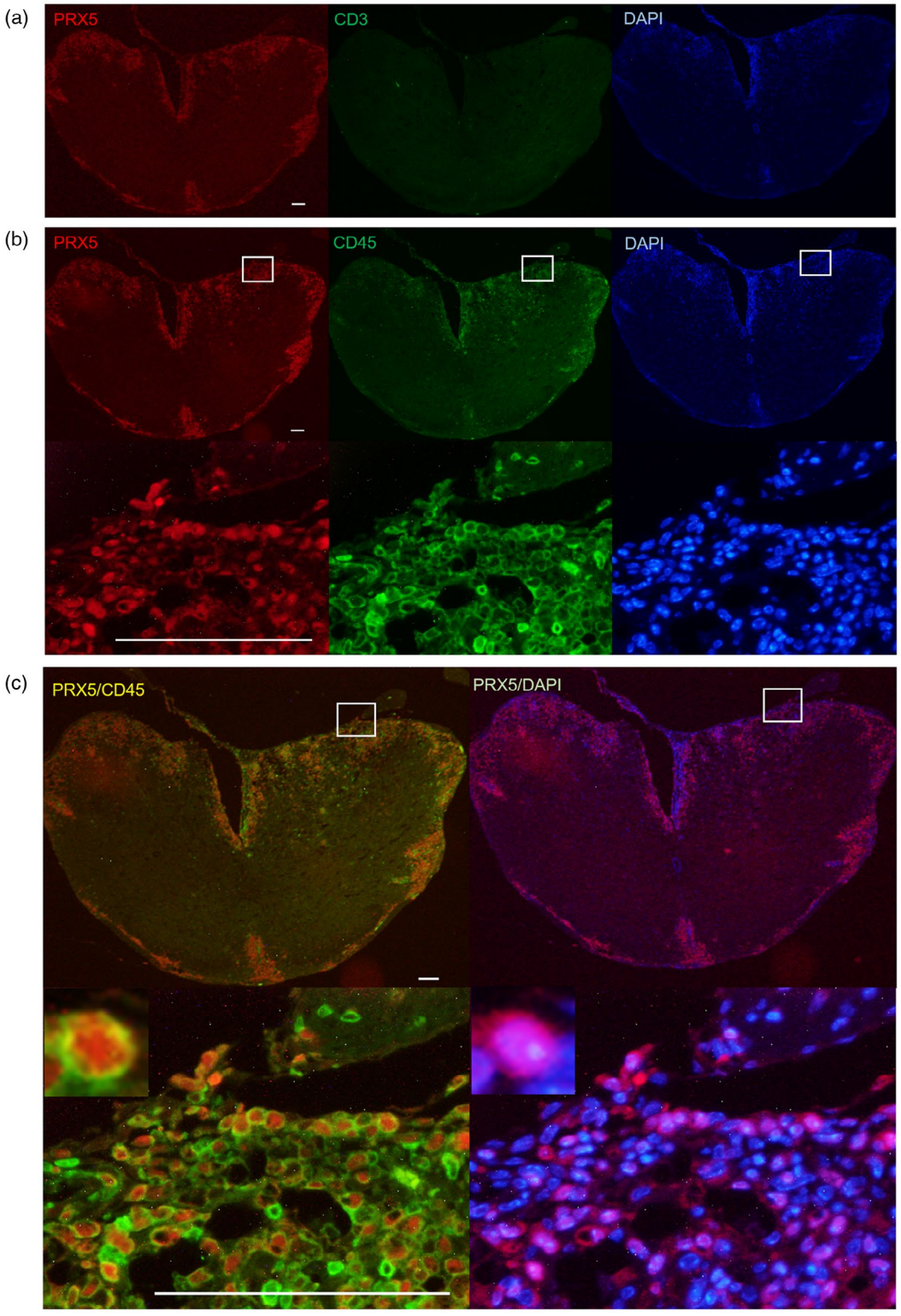


**Fig. 4.** Immunofluorescent staining of the experimental autoimmune encephalomyelitis (EAE) spinal cords. (a,b) Peroxiredoxin 5 (PRX5) (red), CD3/CD45 (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) staining of the spinal cords of EAE (representative images are shown). PRX5 and $CD45^+$ cells were confirmed in EAE spinal cords but CD3-positive cells were not identified. (c) Merged images of PRX5 and CD45 (yellow) and PRX5 and DAPI (purple). PRX5 was expressed in $CD45^+$ cells. White blank squares indicate the area of high magnification. Bars indicate 100 μm.

of the spinal cords from EAE mice showed remarkable PRX5 staining in $CD45^+$ infiltrating cells. These results indicate that PRXs may play some role in CNS inflammation, probably at a later stage both in MS and NMOSD. Indeed, it is nearly conclusive that the early underlying cellular and molecular mechanisms of the causation of MS and NMOSD are very different [17–20].

PRXs have recently received attention as novel DAMPs. Among the PRX subtypes (PRX1-6), PRX1, PRX2, PRX5 and PRX6 are expressed in the brain and can trigger the release of several cytokines. Furthermore, PRX5 has the strongest effect on the activation of T helper type 17 (Th17) activation via the secretion of IL-23 [12]. Th17 cells play a dominant role in the development of EAE [21], and are also thought to be involved in the pathogenesis of MS and NMOSD [5]. Therefore, we must consider that PRXs may play a role in triggering autoimmunity during MS and NMOSD.

Thus far, some papers about the role of PRXs in patients with MS have been published but not in patients with NMOSD. Holley *et al*. reported that $PRX5^+$ hypertrophic reactive astrocytes were observed in the acute and chronic brain lesions of MS patients. They also speculated that ongoing oxidative stress occurred during the acute and chronic phases of MS, and PRX5 was up-regulated in astrocytes to neutralize oxidative stress [22]. Voigt *et al*. reported that PRX2 was up-regulated mainly in astrocytes of white matter lesions. Furthermore, its expression level was positively correlated with the degree of inflammation and oxidative stress in patients with MS, which suggests that PRX2 contributes to the resistance of astrocytes against oxidative damage [23]. Yun *et al*. reported that PRX6 was strongly expressed by cells with astrocyte-like morphology in the MS lesions of human patients [13]. The increased PRX6 expression in astrocytes of MS patients reduced matrix metallopeptidase 9 (MMP9) expression, fibrinogen leakage, chemokines and free radical stress, leading to decreased blood–brain barrier disruption [13]. These findings suggest that PRX6 expression may represent a therapeutic way to restrict inflammation in the CNS and potentiate oligodendrocyte survival. Therefore, PRX6 may have potential as a new neuroprotective therapy for MS [13]. Taken together, up-regulated PRXs may help to protect astrocytes and maintain blood–brain barrier function. However, no paper, to our knowledge, has described the protein levels of PRXs in patients with MS and NMOSD thus far. In our study, serum PRX5 and PRX6 levels were significantly elevated in MS and NMOSD patients and partly associated with Qalb (as a marker of blood–brain barrier function), CSF protein and disease duration in NMOSD patients. In general, longer disease duration correlates with more severe blood–brain barrier dysfunction in MS and NMOSD patients. Additionally, serum PRX6 levels were significantly associated with PRX5 levels in NMOSD patients. Our findings indicate that serum PRX6 and PRX5 may be associated with blood–brain barrier dysfunction in NMOSD, like pathological analyses in patients with MS [13]. However, there was no significant elevation in PRX levels within the CSF of MS and NMOSD patients. Further studies are needed to confirm the definite mechanism of PRXs in patients with MS and NMOSD.

Conversely, there is a limited number of papers regarding the role of PRXs in EAE. It has been reported that mRNA levels of PRX1, PRX3 and PRX6 were increased in the spinal cords of EAE mice compared with that of control mice. Also, PRX6 was strongly expressed on cells with astrocyte-like morphology in EAE lesions [13]. PRX6-transgenic EAE mice exhibited less severe pathology, which indicates that the up-regulation of astrocytic PRX6 has an important role in inhibiting the destruction of myelin via microglial activation, blood–brain barrier breakdown and immune cell infiltration [13]. Similar to pathological analyses in patients with MS [13], up-regulated PRXs inside astrocytes may protect blood–brain barrier function and inhibit CNS inflammation. Conversely, PRXs have an opposite appearance, having the ability to induce inflammatory cytokine production as inflammatory mediators [12]. In our study, only PRX5 was up-regulated in $CD45^+$ cells (probably monocytes), which had infiltrated into the spinal cord lesions of EAE mice. We speculate that $CD45^+$ cells may accumulate in the lesion and amplify the inflammatory response via PRX5. Another possible explanation is that $CD45^+$ cells were dealing with ongoing oxidative stress. Although previous papers described the up-regulation of astrocytic PRX6 in EAE [13], we did not observe the expression of PRXs in astrocytes.

Some limitations of our study need to be addressed. First, increased PRXs levels were not confirmed in the CSF, but only in the serum from patients with MS and NMOSD. The small sample size in our study may explain this discrepancy between serum and CSF levels of PRXs. We could not examine the pathological analyses of CNS tissue from patients. Also, it is still unclear as to which cells secreted PRXs into the serum. Additional basic research studies are required to determine the source of these PRXs. In this study, we showed that serum PRX6 levels correlated with Qalb, indicating that high levels of PRXs were partly associated with blood–brain barrier dysfunction. However, the increased protein or albumin levels in CSF may result from the increased production of proteins from CNS cells. Finally, our pathological findings of EAE were different from previous reports; EAE spinal cords showed PRX5 expression in $CD45^+$ infiltrating cells but no PRX expression in

astrocytes. One possible explanation for this discrepancy concerns species-specific differences between humans and mice. It is also possible that pathological characteristics may differ according to disease status. Further studies are needed to confirm the definite mechanism between PRXs and EAE pathogenesis.

From our results, we suggest that the elevations of serum PRX5 and PRX6 levels are associated with the pathogenesis of MS and NMOSD and partially responsible for blood–brain barrier dysfunction in patients with NMOSD. We also showed that up-regulated PRX5 in $CD45^+$ infiltrating cells amplify inflammation in EAE. In summary, the expression of PRXs was significantly altered in CNS inflammatory demyelinating diseases, which suggests that they may regulate blood–brain barrier function or CNS inflammation. Consequently, the development of new treatment targeting PRXs may reduce symptoms of MS and NMOSD.

## Acknowledgements

This work was supported by JSPSKAKENHI Grant no. JP16K09691.

## Disclosures

The authors declare no conflicts of interest.

## Author contributions

All authors were involved in collecting clinical and experimental data, drafting the article or revising it critically for important intellectual content and have read and approved the final version of the manuscript.

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