

Critical role for prokineticin 2 in CNS autoimmunity

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ABSTRACT

Objective: To investigate the potential role of prokineticin 2 (PK2), a bioactive peptide involved in multiple biological functions including immune modulation, in CNS autoimmune demyelinating disease.

Methods: We investigated the expression of PK2 in mice with experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS), and in patients with relapsing-remitting MS. We evaluated the biological effects of PK2 on expression of EAE and on development of T-cell response against myelin by blocking PK2 in vivo with PK2 receptor antagonists. We treated with PK2 immune cells activated against myelin antigen to explore the immune-modulating effects of this peptide in vitro.

Results: *Pk2* messenger RNA was upregulated in spinal cord and lymph node cells (LNCs) of mice with EAE. PK2 protein was expressed in EAE inflammatory infiltrates and was increased in sera during EAE. In patients with relapsing-remitting MS, transcripts for *PK2* were significantly increased in peripheral blood mononuclear cells compared with healthy controls, and PK2 serum concentrations were significantly higher. A PK2 receptor antagonist prevented or attenuated established EAE in chronic and relapsing-remitting models, reduced CNS inflammation and demyelination, and decreased the production of interferon (IFN)- γ and interleukin (IL)-17A cytokines in LNCs while increasing IL-10. PK2 in vitro increased IFN- γ and IL-17A and reduced IL-10 in splenocytes activated against myelin antigen.

Conclusion: These data suggest that PK2 is a critical immune regulator in CNS autoimmune demyelination and may represent a new target for therapy. *Neurol Neuroimmunol Neuroinflamm* 2015;2:e95; doi: 10.1212/NXI.0000000000000095

GLOSSARY

DMSO = dimethyl sulfoxide; **EAE** = experimental autoimmune encephalomyelitis; **G-CSF** = granulocyte colony-stimulating factor; **IFN** = interferon; **IL** = interleukin; **LNC** = lymph node cell; **MOG** = myelin oligodendrocyte glycoprotein; **mRNA** = messenger RNA; **MS** = multiple sclerosis; **PBMC** = peripheral blood mononuclear cells; **PBS** = phosphate-buffered saline; **PK2** = prokineticin 2; **PKR** = PK receptor; **PKR1** = PK receptor 1; **PKR2** = PK receptor 2; **PLP** = proteolipid protein; **qRT-PCR** = quantitative real-time PCR; **Th** = T helper cell; **TNF** = tumor necrosis factor.

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the CNS characterized by demyelination and neurodegeneration. CD4⁺ T lymphocytes activated against myelin autoantigens secreting T helper cell (Th) 1 cytokines and interleukin (IL)-17 are regarded as critical for initiation and perpetuation of inflammation in MS and its animal model, experimental autoimmune encephalomyelitis (EAE).^{1,2} Although immune-modulating therapies can reduce relapse rate and time to disease progression, there are currently no definitive cures for MS.³ A better understanding of the pathobiology of this complex disease is crucial in order to develop better therapies.

Supplemental data
at Neurology.org/nn

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Prokineticin 2 (PK2) is a bioactive peptide member of the prokineticin family, which comprises 2 small secreted proteins (8–12 kDa) highly conserved across species, namely prokineticin 1 (also known as endocrine gland vascular endothelial factor) and PK2 (also known as Bv8).^{4,5} PK2 regulates multiple biological functions including circadian rhythm,⁶ angiogenesis,^{7,8} neurogenesis of olfactory bulb,⁹ neuronal survival,¹⁰ reproduction,¹¹ and inflammation.^{12,13} It activates 2 similar G protein-coupled receptors, PK receptor 1 (PKR1) and PK receptor 2 (PKR2).¹⁴ Many cells and tissues, including the CNS and the immune system, express PK2.^{10,15,16} PK2 and PKRs are expressed by bone marrow cells and circulating leukocytes.^{4,8,17,18} PK2 was shown to induce hematopoietic cell mobilization and differentiation.^{8,18} PK2 increases in inflamed tissues^{5,15,19} and promotes inflammation.^{5,12,13} Moreover, PK2 promotes a Th1 phenotype by increasing the secretion of IL-1 β and IL-12 and reducing the secretion of IL-10 in mouse macrophages,¹³ and decreasing the production of IL-10 and IL-4 in mouse splenocytes.¹²

In this study, we investigated the potential role of PK2 in CNS autoimmunity.

METHODS EAE induction and assessment. Chronic EAE was induced in C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG)_{35–55} peptide, as described.²⁰ Relapsing-remitting EAE was induced in SJL/J mice, as described.²¹ All mice were female and 8–12 weeks old (Charles River Laboratories, Calco, Italy). Animals were assessed daily for clinical signs of EAE.²¹ During pharmacologic studies, experimenters were blinded to the treatment regimen.

Human samples. Blood samples were obtained from 24 European adults who were diagnosed with relapsing-remitting MS according to McDonald criteria²² (11 women and 13 men; mean age 34.7 \pm 1.7 years; Expanded Disability Status Scale score 1.7 \pm 1.4; disease duration 7.9 \pm 6.9 years, range 19–51 years). Patients were clinically stable, had not started any immune-modulating therapy before blood collection, and did not have other acute or chronic inflammatory disorders. Sampling was performed at least 4 weeks after the last clinical attack or steroid treatment. Twenty-four individuals (12 women and 12 men; age 33.7 \pm 2.1 years, range 23–57 years) who had no acute or chronic inflammatory diseases or autoimmune disorders were included as controls.

Study approval. All procedures involving animals were approved by the Ethical Committee of the Neurological Institute Foundation Carlo Besta and by the Italian General Direction for Animal Health at the Ministry for Health. The study on human samples was approved by the Ethical Committee of the San Raffaele Scientific Institute. Patients and controls gave their written informed consent.

Treatments. The triazinic derivatives PKR1-preferential antagonists PC7²³ and PC1²⁴ and the amphibian ortholog of PK2, Bv8,^{5,12,13} were used in the study. For in vivo use, PC7 and PC1 were diluted in phosphate-buffered saline (PBS) 1% dimethyl sulfoxide (DMSO) in a final volume of 100 μ L. As a control, mice were treated with an equal volume of PBS 1% DMSO (vehicle). Bv8 was isolated from skin secretions of the frog *Bombina variegata* as previously described⁵ and purified to 98% as assessed by high-performance liquid chromatography.

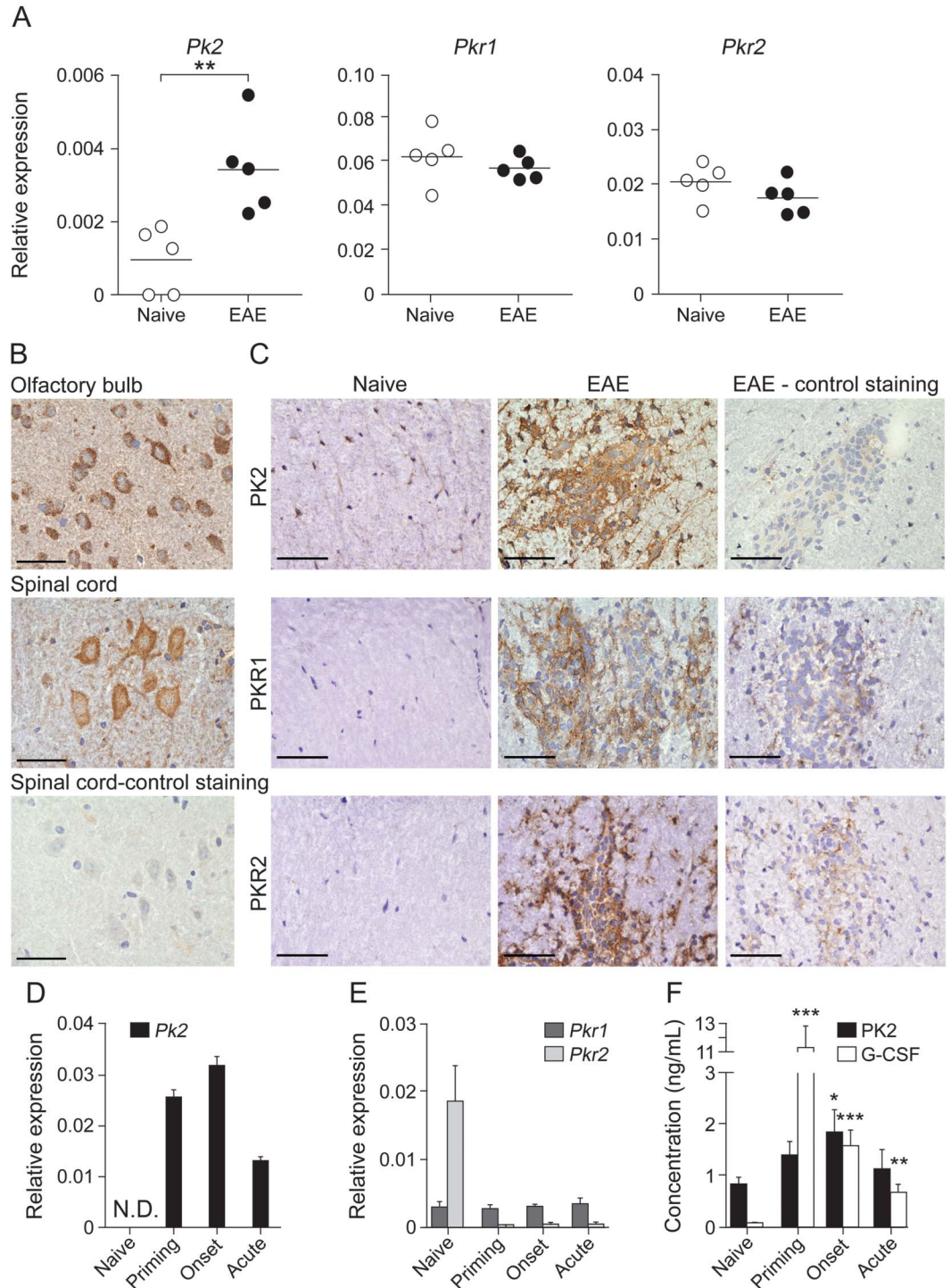
Immunohistochemistry studies. For PK2 and PKR tissue detection, 5- μ m spinal cord sections from snap frozen fresh tissues embedded in optimal cutting temperature compound were stained with rabbit anti-PK2 (1:200 dilution, Covalab, Villeurbanne, France) and rabbit anti-PKR1 and anti-PKR2 (1:200 dilution, Alomone, Jerusalem, Israel).²⁵ As negative controls, we used preadsorption of primary antibodies with PK2 (Covalab, 1:2), PKR1, and PKR2 (Alomone, 1:10) peptides, following manufacturer's instructions. All antibodies were revealed with EnVision+ rabbit horseradish peroxidase labeled polymer system and 3,3'-diaminobenzidine as a chromogen according to the manufacturer's protocol (Dako, Glostrup, Denmark). Sections were counterstained with hematoxylin.

Statistical analysis. Unless otherwise specified, results are expressed as mean \pm SEM. Statistical analyses were performed using GraphPad Prism software (La Jolla, CA). For EAE scores, differences between groups were examined for statistical significance by the nonparametric analysis Mann-Whitney *U* test. In all other cases, 2-tailed unpaired Student *t* test was used to detect differences between independent groups. *p* Values of <0.05 were considered statistically significant.

Information on RNA extraction and real-time PCR for PK2, PKR1, and PKR2; measurement of PK2 and granulocyte colony-stimulating factor (G-CSF) on serum samples; pathologic studies, proliferation assay, and cytokine analysis by ELISA; in vitro treatment with PK2/Bv8; and additional details on PC7 and PC1 compounds can be found in the supplemental material at Neurology.org/nn.

RESULTS PK2 expression increases in the CNS during chronic EAE. We first evaluated whether the expression of PK2 was increased in the target organ, the CNS, during EAE. We used quantitative real-time PCR (qRT-PCR) to investigate the messenger RNA (mRNA) expression of *Pk2* and its receptors in spinal cords of C57BL/6 mice with MOG_{35–55}-induced chronic EAE and naive mice. *Pk2* expression was significantly increased in spinal cords obtained from mice with EAE compared with naive control mice (figure 1A). Both *Pkr1* and *Pkr2* mRNA were expressed in spinal cords of naive mice, and their expression did not change significantly in mice with EAE compared with naive mice. We next wanted to verify whether PK2 expression was increased in the CNS of EAE mice at the protein level. We then performed immunohistochemistry studies using anti-PK2 antibody. As expected,^{10,25} in naive mice, PK2 was expressed in neurons of the olfactory bulb and spinal cord gray matter (figure 1B). In spinal cord white matter of naive mice, only a weak PK2

Figure 1 PK2 increases during EAE



(A) Quantitative real-time RT-PCR (qRT-PCR) analysis to determine relative messenger RNA (mRNA) expression of *Pk2*, *Pkr1*, and *Pkr2* in spinal cords of C57BL/6 mice with chronic experimental autoimmune encephalomyelitis (EAE) 14 days after immunization with myelin oligodendrocyte glycoprotein₃₅₋₅₅ or naive mice ($n = 5$ mice per group). Data are expressed as percentage of the housekeeping gene *Gapdh*. Each dot represents an individual mouse and horizontal lines indicate means. $**p < 0.01$ by Student *t* test. (B) Prokineticin 2 (PK2) staining in olfactory bulb (upper panel) and spinal cord gray matter (middle panel) of naive mice. Preabsorption of anti-PK2 antibody with PK2 peptide inhibited PK2 staining in spinal cord gray matter neurons (control staining, lower panel). Scale bars = 50 μm . (C) Representative immunohistochemical staining of PK2, PK receptor 1 (PKR1), and PK receptor 2 (PKR2) as well as control stainings (preabsorption with PK2, PKR1, or PKR2 peptides, respectively) in spinal cord white matter of naive mice and in inflammatory infiltrates of EAE mice. Scale bars = 50 μm . (D, E) qRT-PCR analysis of mRNA expression levels of *Pk2*, *Pkr1*, and *Pkr2* in lymph node cells pooled from naive mice ($n = 14$) or mice with chronic EAE at different time points ($n = 4-5$ for each time point) (priming, 7 days after immunization; onset, 9-11 days after immunization; acute, 16 days after immunization). Data are representative of 1 of 2

staining was detected on glial cells (figure 1C, upper left panel). In contrast, in spinal cord white matter of mice with EAE, staining revealed that PK2 was highly expressed within inflammatory infiltrates (figure 1C, upper middle panel). Staining for PKR1 and PKR2, which was absent in spinal cord white matter of naive mice, was detected in inflammatory infiltrates in mice with EAE (figure 1C, middle and lower panels). These results show that inflammatory EAE infiltrates in the CNS express both PK2 and the receptors for PK2.

PK2 increases in lymph node cells and sera of mice with EAE. Lymph nodes are known to play a pivotal role in the development of EAE by acting as a site for priming of T cells targeting CNS self-antigens. We evaluated mRNA expression of *Pk2* in lymph node cells (LNCs) of naive mice and mice with EAE. Transcripts for *Pk2* were undetectable in LNCs of naive mice. However, *Pk2* expression increased in LNCs during EAE (figure 1D). The receptors for PK2 were both expressed at the mRNA level in naive LNCs. While the expression of *Pkr1* did not change in LNCs during EAE, *Pkr2* expression was dramatically reduced (figure 1E).

PK2 is a secreted protein. Because *Pk2* mRNA was increased in LNCs of mice with EAE, we measured PK2 protein in serum by ELISA. We observed a significant increase of PK2 in sera of mice with EAE compared with naive mice (about a 2-fold increase at EAE onset) (figure 1F). G-CSF is a major inducer of PK2 in vitro and in vivo in both mice and humans, and treatment with G-CSF increases PK2 in serum.^{8,18} We therefore measured G-CSF in sera of mice with EAE and found a significant increase of G-CSF during the disease compared with naive mice (figure 1F). In particular, G-CSF concentrations peaked during the priming phase of EAE (7 days after immunization) and remained elevated during disease. Thus, the increase of G-CSF preceded that of PK2.

PK2 is increased in patients with relapsing-remitting MS. To determine whether these findings could be potentially relevant to the human disease, we also analyzed *PK2* mRNA expression in peripheral blood mononuclear cells (PBMC) from patients with relapsing-remitting MS and healthy controls by qRT-PCR. To limit variations in the gene expression, all patients were clinically stable and none of them had started any immunomodulatory therapy before sampling. *PK2* transcripts were significantly increased in PBMC of

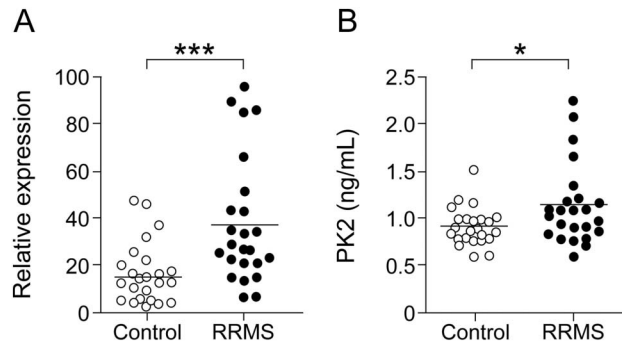
patients with relapsing-remitting MS compared with healthy controls (figure 2A). We next evaluated the serum concentrations of PK2 by ELISA and found that PK2 was significantly increased in sera of patients with relapsing-remitting MS compared with healthy controls (figure 2B).

PKR antagonist reduces disease severity and CNS pathology in chronic and relapsing-remitting EAE. We next set out to investigate the potential role of PK2 in CNS demyelinating disease by pharmacologic interference with PK2 receptors. Among the 2 known receptors for PK2, it was suggested that PKR1 is more important with regard to the proinflammatory effects induced by PK2 on immune cells in mice.^{5,12,13} In EAE studies, we used the nonpeptidic triazinic compound PC7 (figure 3A), which is a small molecule that competes for the binding of PK2 to its receptors, preferentially PKR1, and inhibits the ability of PK2 to activate downstream pathways.^{23,24} PC7 has a half maximal inhibitory concentration of 56 ± 12 nM for PKR1 and $5,230 \pm 700$ nM for PKR2 (figure 3B). We treated mice with MOG₃₅₋₅₅-induced chronic EAE with daily intraperitoneal injections of PC7 (0.5 mg/kg/day) or vehicle. Treatment with PC7 started on the day of immunization (preventive treatment) significantly delayed the onset of EAE clinical signs and reduced the severity of the disease (figure 3C and table e-1). In line with the observed disease attenuation, histopathology of CNS tissue revealed reduced inflammation and demyelination in spinal cord of mice treated with PC7 compared with vehicle-treated control mice (figure 3D). Next, we evaluated whether PC7 could reduce neurologic signs in mice with established EAE (therapeutic treatment). Treatment with PC7 started at the onset of EAE inhibited disease progression and reduced the severity within 4 days of treatment start and continued to confer protection over the next 2 weeks of treatment (figure 3E and table e-1).

We next examined whether antagonizing PKRs with PC7 was effective in reducing disease severity in a different EAE model. In contrast to MOG₃₅₋₅₅ peptide, which causes chronic EAE in C57BL/6 mice, proteolipid protein (PLP)₁₃₉₋₁₅₁ peptide induces relapsing-remitting EAE in SJL/J mice.²¹ Treatment with PC7 started at the time of EAE induction significantly reduced disease severity and CNS inflammation and demyelination (figure 4, A and B and table e-1). Moreover, treatment with PC7

independent experiments that gave similar results, each including an equal number of mice, and are expressed as percentage of the housekeeping gene *Gapdh* (mean \pm SD). (F) PK2 and granulocyte colony-stimulating factor (G-CSF) concentration measured by ELISA in sera collected from naive or chronic EAE mice at different time points, as described in D and E. Data are pooled from 2 independent experiments that gave similar results, each including 4–5 mice per group, and are shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ by Student t test vs naive mice. N.D. = not detectable.

Figure 2 PK2 is increased in patients with RRMS



(A) Quantitative real-time RT-PCR (qRT-PCR) analysis to determine relative *PK2* messenger RNA expression in peripheral blood mononuclear cells from patients with relapsing-remitting multiple sclerosis (RRMS) and healthy controls ($n = 24$ per group). Data are expressed as percentage of the housekeeping gene *ALG8*. (B) PK2 concentrations measured by ELISA in sera collected from patients with RRMS and healthy controls ($n = 24$ per group). In both A and B, each dot represents an individual and horizontal lines indicate the means. * $p < 0.05$, *** $p = 0.001$ by Student *t* test.

inhibited disease progression when started at the onset of neurologic signs (figure 4C and table e-1). To confirm the results obtained with PC7, we used another triazinic PKR1-preferential antagonist, PC1, previously used *in vivo* in rats to block PK2 and reduce hyperalgesia in a model of inflammatory pain.^{5,24} Treatment with PC1 started at the time of immunization with PLP_{139–151} resulted in reduced disease severity in the relapsing-remitting EAE model (figure 4D and table e-1).

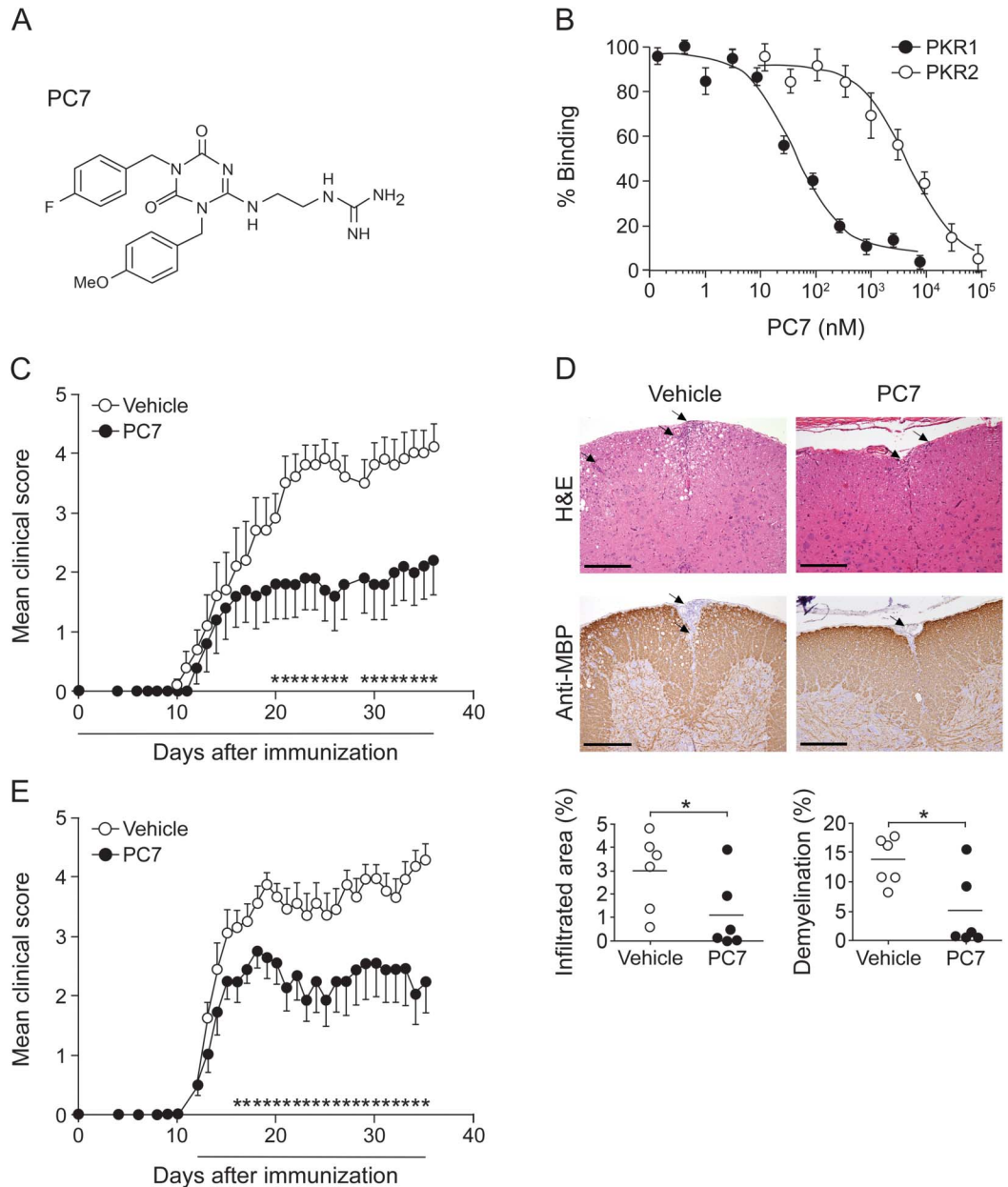
PKR antagonist modulates Th1 and Th17 responses in EAE. We next evaluated whether the protective phenotype observed in EAE mice treated with the PKR antagonist PC7 could be explained by an alteration of T-cell activation and Th regulation. We isolated LNCs from vehicle-treated and PC7-treated EAE mice during the priming phase of EAE and examined the recall response to peptide stimulation *in vitro*. In mice with MOG_{35–55}-induced EAE, LNCs from PC7-treated mice displayed significantly reduced proliferation in response to peptide stimulation compared with vehicle-treated mice (figure 5A). Moreover, in LNCs of PC7-treated mice, the secretion of proinflammatory cytokines interferon (IFN)- γ , IL-17A, and, to a lesser extent, tumor necrosis factor (TNF) was significantly reduced, whereas secretion of the suppressor cytokine IL-10 was significantly increased compared with vehicle-treated mice (figure 5A). Of interest, IL-6, known to promote Th17 polarization, was significantly increased in LNCs from PC7-treated mice compared with vehicle-treated controls. Similarly, in PLP_{139–151}-induced relapsing-remitting EAE, we observed a reduction of immune cell proliferation and of IFN- γ and TNF production in antigen-stimulated LNCs of EAE mice treated with

PC7 compared with those of vehicle-treated mice (figure 5B). However, in this model, PC7 treatment did not affect IL-17A production. As in the chronic model, IL-6 and IL-10 were increased in LNCs from PC7-treated SJL mice. These results demonstrate that antagonizing PKRs induced an important modulation of proinflammatory responses against CNS myelin in 2 murine models of EAE. This impairment could represent an important mechanism by which PC7 treatment reduced EAE severity.

PK2/Bv8 promotes Th1 and Th17 responses *in vitro*. We next examined the effects of PK2 *in vitro* on splenocytes from mice immunized with MOG_{35–55}. We used Bv8, the amphibian ortholog of PK2 (hereafter referred to as PK2/Bv8), a potent agonist of PKRs and widely used to study the effect of PKR activation in mammals.^{5,12,13} We isolated splenocytes from MOG_{35–55}-induced EAE mice during the priming phase and examined the recall response *in vitro*. Treatment with PK2/Bv8 increased immune cell proliferation and secretion of IFN- γ and IL-17A and reduced the production of IL-10 (figure 5C). These effects were reversed by pretreatment of cells with PC7 antagonist.

DISCUSSION The bioactive peptide PK2 has recently been shown to have immune-modulating properties and to promote inflammation. The potential role of this peptide in MS and autoimmune disorders has never been investigated. We present evidence that supports an important immune regulatory role for PK2 in CNS autoimmunity. EAE induction resulted in increased expression of PK2 in mouse spinal cord at both the mRNA level and the protein level in white matter inflammatory infiltrates. This finding is in agreement with different studies reporting an overexpression of PK2 in human and rodent inflamed tissues with different pathologic conditions.^{5,15,19} This EAE-associated increase of PK2 was not limited to the inflamed CNS but was also observed in peripheral immune cells. Indeed, in lymph nodes, *Pk2* mRNA that was undetectable in naive mice was highly expressed during EAE. Moreover, PK2 increased in sera of mice with EAE. In parallel with these results, we showed that in patients with relapsing-remitting MS, *PK2* mRNA expression was increased in PBMC and PK2 protein concentration was higher in sera than in healthy controls. Neutrophils and macrophages are the major sources of PK2 in the immune system.^{8,16} In MS and EAE, myeloid cells such as macrophages are a prominent constituent of CNS inflammatory infiltrates, and monocytes increase in the bloodstream right before EAE relapses.²⁶ Thus, macrophages might be important sources of PK2 in

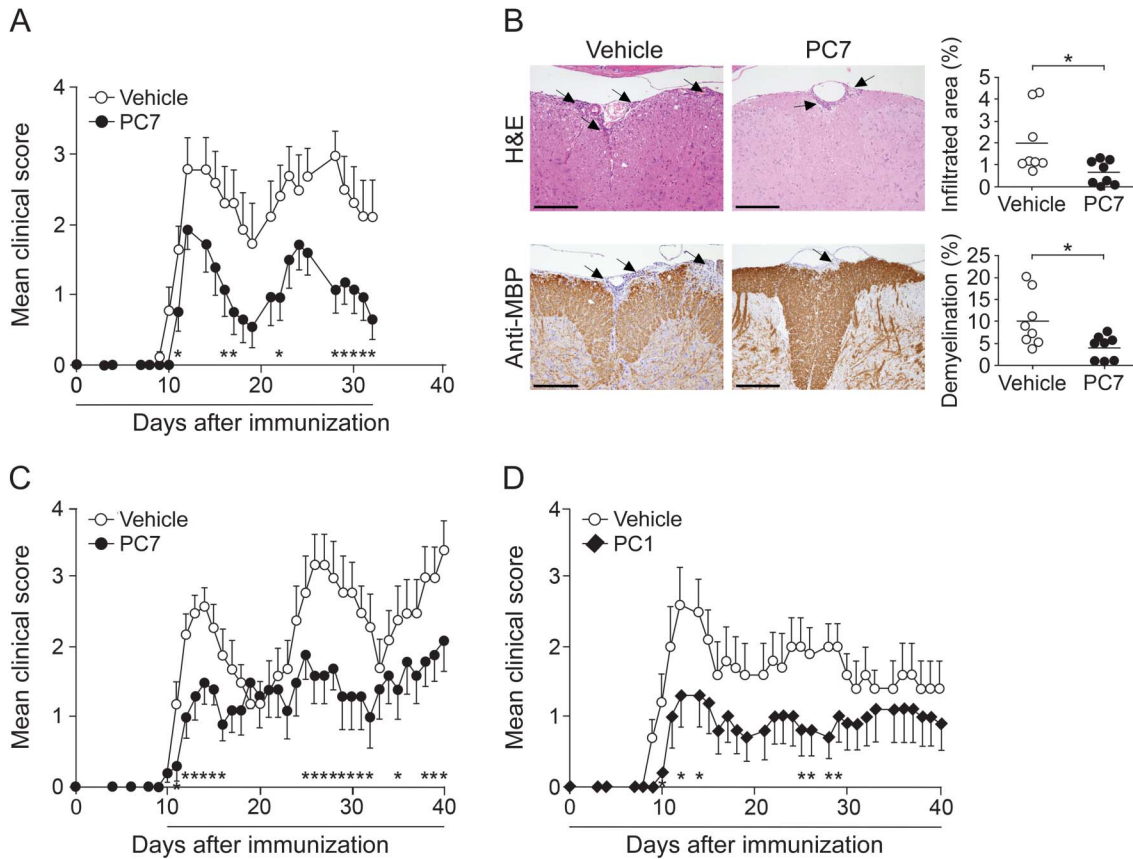
Figure 3 PKR antagonist PC7 reduces the severity of chronic EAE



(A) Chemical structure of the prokineticin receptor (PKR) antagonist PC7. (B) Affinity for PKR1 and PKR2 assayed on membrane preparations from Chinese hamster ovary cells stably transfected with PKR1 or PKR2 (see e-Methods). (C) Treatment with PC7 given daily intraperitoneally ameliorated myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅-induced experimental autoimmune encephalomyelitis (EAE) when started at the time of disease induction (n = 10 mice per group). Data are representative of 1 of 3 independent experiments that gave similar results. (D) Hematoxylin & eosin (H&E) and anti-myelin basic protein (MBP) staining of spinal cord sections from MOG₃₅₋₅₅-immunized mice treated with PC7 or vehicle from the day of immunization visualizing immune cell infiltration and demyelination, respectively (arrows). For the analysis, mice were sacrificed 36 days after immunization. Scale bars = 200 μ m. The graphs below represent comparative analysis of infiltrated and demyelinated area (% of total area). Each dot represents an individual mouse and horizontal lines indicate the means. **p* < 0.05 by Student *t* test. (E) Daily treatment with PC7 ameliorates EAE when started at the onset of clinical signs (n = 10 mice per group). Mice were scored and randomized at the onset of neurologic signs immediately before first treatment. In C and E, solid lines beneath each panel indicate PC7 treatment. Filled circles, PC7 (0.5 mg/kg); open circles, vehicle only. Data are shown as mean \pm SEM. The clinical EAE traits for these experiments are reported in table e-1. **p* < 0.05 by Mann-Whitney *U* test.

EAE and MS. G-CSF is the main inducer of PK2 in myeloid cells.^{8,27} We showed that PK2 increases early in LNCs and in serum during EAE and that G-CSF serum levels peak rapidly during the priming phase.

These results suggest that G-CSF could play a key role in promoting PK2 transcription and secretion during EAE. Of interest, in studies published more than a decade ago, G-CSF expression in MS lesions



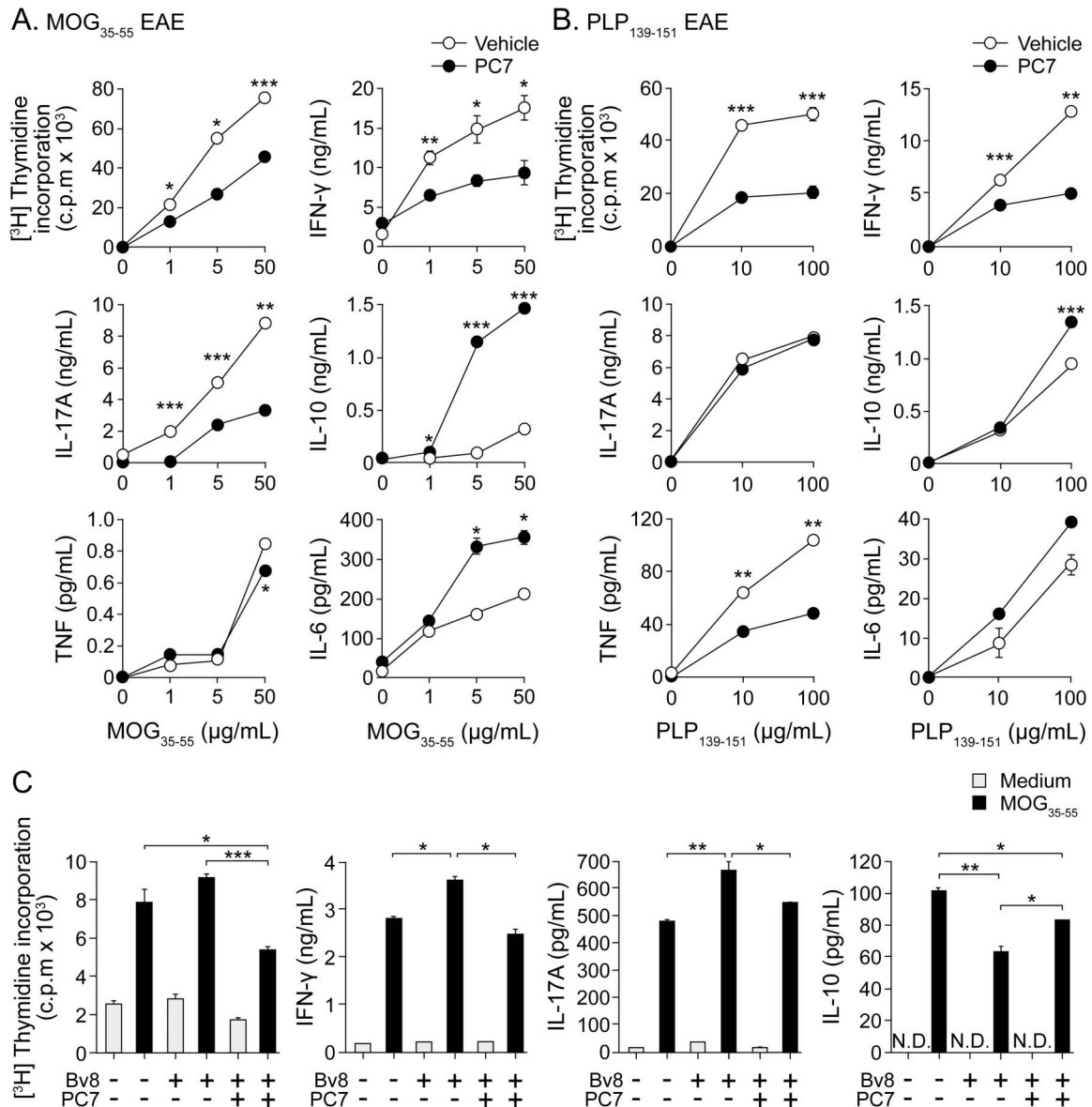
(A) Treatment with PC7 given daily intraperitoneally ameliorated proteolipid protein (PLP)₁₃₉₋₁₅₁-induced experimental autoimmune encephalomyelitis (EAE) when started at the time of disease induction (n = 9 in PC7 group, n = 10 in vehicle group). (B) Hematoxylin & eosin (H&E) and anti-myelin basic protein (MBP) staining of spinal cord sections from PLP₁₃₉₋₁₅₁-immunized mice treated with PC7 or vehicle from the day of immunization visualizing immune cell infiltration and demyelination, respectively (arrows). For the analysis, mice were sacrificed 32 days after immunization. Scale bars = 200 μm. The graphs on the right represent comparative analysis of infiltrated and demyelinated area (% of total area). Each dot represents an individual mouse and horizontal lines indicate the means. *p < 0.05 by Student t test. (C) PC7 ameliorates EAE when given at the onset of clinical signs (n = 10 mice per group). Mice were scored and randomized at the onset of neurologic signs immediately before first treatment. (D) Treatment with PC1 given daily intraperitoneally ameliorated PLP₁₃₉₋₁₅₁-induced EAE when started at the time of disease induction (n = 10 mice per group). In A, C, and D, solid lines beneath each panel indicate treatment with PC7 (A, C) or PC1 (D). Filled circles, PC7 (0.5 mg/kg); filled diamonds, PC1 (0.5 mg/kg); open circles, vehicle only. Data are shown as mean ± SEM and are representative of 1 of 2-4 independent experiments that gave similar results. *p < 0.05 by Mann-Whitney U test. The EAE clinical traits for these experiments are reported in table e-1.

was found to be upregulated in acute lesions by gene microarray analysis (about 13-fold relative to chronic silent plaques).²⁸ Furthermore, treatment with G-CSF induced flares in patients with MS²⁹ and patients with neuromyelitis optica,³⁰ another demyelinating disease of the CNS in which inflammatory lesions are rich in granulocytes. Of note, in EAE, unlike MS, treatment with G-CSF has been shown to reduce disease severity.^{28,31} However, similar paradoxical results between MS and EAE are also seen with anti-TNF, for example.³

The increased expression of PK2 that we observed in EAE and MS suggests that this molecule could play a role in neuroinflammation. In support of this hypothesis, we showed that blocking PK2 receptors with the PKR1-preferential antagonist PC7 inhibited proinflammatory T-cell responses and reduced neurologic signs and CNS damage in chronic and relapsing

EAE. A second PKR1-preferential antagonist, PC1, also ameliorated EAE. PK2 has been shown to promote the production of proinflammatory cytokines from different immune cells and to modulate T-cell function by reducing anti-inflammatory cytokine production while promoting Th1 responses.^{4,5,12,13,16} The amelioration of EAE induced by PC7 and PC1 could be explained at least in part by an overall impairment of proinflammatory responses against CNS myelin in EAE mice treated with PC7. Indeed, treatment reduced the production of IFN-γ in LNCs and, in chronic EAE, the production of IL-17A, known to play a crucial role in EAE development and progression, while increasing production of the suppressor cytokine IL-10, which is protective in EAE. In line with these data, we showed that PK2/Bv8 in vitro increased Th1 and Th17 responses in splenocytes activated against myelin antigen and

Figure 5 Effects of prokineticin receptor antagonist and PK2/Bv8 on T-cell response against myelin antigens



Lymph node cell (LNC) recall responses from (A) C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ or (B) SJL/J mice immunized with proteolipid protein (PLP)₁₃₉₋₁₅₁ treated daily intraperitoneally with PC7 (0.5 mg/kg) or vehicle. Treatments were started at the time of disease induction and LNCs were isolated from draining lymph nodes 7–10 days after immunization (5 mice/group). Filled circles, PC7 (0.5 mg/kg); open circles, vehicle only. (C) Splenocytes isolated from MOG₃₅₋₅₅-immunized C57BL/6 mice 7 days after immunization were treated *in vitro* with prokineticin 2 (PK2)/Bv8 (10⁻⁹ M) or with PC7 (10⁻⁷ M) followed by PK2/Bv8 (10⁻⁹ M) before restimulation with antigen (50 μg/mL) or medium alone. T-cell proliferation was assessed by [³H] thymidine incorporation in triplicated cultures. Cytokine production was tested by ELISA in supernatants of parallel cultures. Data are shown as mean ± SEM and are representative of 1 of 2 consecutive independent experiments that gave similar results. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by Student *t* test. EAE = experimental autoimmune encephalomyelitis; IFN = interferon; IL = interleukin; N.D. = not detectable; TNF = tumor necrosis factor.

reduced IL-10, and these effects were blocked by pre-treatment with PC7. It is interesting that PK2 has recently been shown to activate STAT3,³² which is required for Th17 generation and plays critical roles in the development of EAE and MS.³³ In our study, PC7 treatment *in vivo* also resulted in an increase of IL-6 in LNCs of mice with EAE. This cytokine is usually considered detrimental in EAE, as it promotes Th17 responses.³⁴ However, IL-6 can also limit inflammation³⁵ and exert neuroprotective effects.^{36,37}

Our experiments demonstrated that PC7 antagonist importantly modulates autoimmune response against myelin antigen in peripheral immune cells. PC7 might also have an effect on CNS immune cells, such as microglial cells, which express PKRs.¹⁵ However, whether or not PC7 can cross the blood-brain barrier and enter the CNS parenchyma during EAE still needs to be ascertained. Moreover, other mechanisms might account for the beneficial role of PC7 in EAE. In fact, other cells expressing prokineticin and

PKRs aside from immune cells might be important targets of PC7 in EAE. For example, within the CNS, neurons and astrocytes also express PKRs.¹⁵ These cells also express PK2.¹⁵ Hypoxia, reactive oxygen species, and excitotoxic glutamate, known to play a crucial role in ischemic brain injury and to contribute to myelin and axonal damage in MS,^{38,39} increase *Pk2* mRNA in primary cortical cultures.¹⁵ Intracerebral administration of exogenous PK2 increases injury and immune cell recruitment into the CNS in a rat model of ischemic injury, and PKR antagonist reduced ischemic damage.¹⁵ Moreover, recent work demonstrates that peripheral nerve damage induces a significant increase of PK2 immunoreactivity in sensitive neurons and activated astrocytes in mouse spinal cord.²⁵ Although our results suggest that immune cells infiltrating the CNS, likely macrophages, are an important source of PK2 during EAE, we cannot rule out the possibility that neuronal cells might also be important sources of PK2 during the disease.

The efficacy of PC7 and PC1 in reducing EAE severity is consistent with an important role for PKR1 in EAE but does not exclude a possible role for PKR2 in the disease, as both antagonists are also able to block PKR2. Thus, the contribution of each PKR to the pathogenesis of EAE and MS requires further investigation. Moreover, identification of PK2-producing cells and of their targets expressing PKRs during EAE and MS seems to be very important in order to understand the contribution of each molecule to disease pathogenesis. Of note, given the involvement of PK2 and its receptors in many important physiologic processes, extreme caution should be exercised when proposing PKR antagonists as a possible treatment for EAE and MS, and potential collateral effects associated with the use of PKR antagonists should be carefully determined.

In conclusion, our results identify PK2 as an important mediator of CNS autoimmunity and provide the basis for further investigation of the role of PK2 in MS and, potentially, for therapeutic application of PKR inhibitors in this disease.

AUTHOR CONTRIBUTIONS

M.A.-H. performed the main experimental work, designed the experiments, analyzed the data, and wrote the manuscript. M.C. designed, conducted, and analyzed gene expression studies in mice and performed some EAE experiments. E.F. and P.L.P. performed and evaluated pathologic and immunohistochemical studies. M.D.D. performed RT-PCR on human samples and helped with the PK2 ELISA study in human sera. S.M. conducted some EAE experiments. C.C. and V.O. synthesized PC1 and PC7. R.L. performed PC7 inhibition assays on transfected CHO cells. M.R. and V.M. provided human samples. S.S. conceived and contributed to the synthesis of PKR antagonist. L.N. provided PK2/Bv8, gave advice for Bv8 and PC7 use, and revised the manuscript. C.F. coordinated the experiments with human samples and revised and commented on the manuscript. G.B. made substantial contributions to project conception, synthesized PKR antagonists, and revised and commented on the manuscript. L.S. discussed results, gave feedback, and

helped write the manuscript. R.P. conceived and supervised the work and wrote the paper.

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DISCLOSURE

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