Blockade of proteinase-activated receptor 2 (PAR 2) attenuates neuroinflammation in experimental autoimmune encephalomyelitis

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Abbreviations

Amino acids are abbreviated by their one-letter code (A = alanine; K = Lysine etc.); BMDM = bone marrow-derived macrophages; CFA = complete Freund’s adjuvant; CFSE = carboxy-fluorescein diacetate succinimidyl ester; CNS = central nervous system; DMSO = Dimethyl sulfoxide; EAE = experimental autoimmune encephalomyelitis model of multiple sclerosis; FBS = fetal bovine serum; GM-CSF = granulocyte-macrophage colony-stimulating factor; LPS = Lipopolysaccharide from E-coli; MOG = myelin oligodendrocyte glycoprotein antigen; MS = multiple sclerosis; PAR = proteinase-activated receptor; PE = Phycoerythrin; PerCP = Peridinin-Chlorophyll-Protein; P2pal-18S = PAR2 antagonist, palmitoyl-RSSAMDENSEKKRKSAIK-amide; WT = wild type

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Abstract

Proteinase-activated receptor-2 (PAR2), which modulates inflammatory responses, is elevated in the central nervous system (CNS) in multiple sclerosis (MS) and in its murine model, experimental autoimmune encephalomyelitis (EAE). In PAR2-null mice, disease severity of EAE is markedly diminished. We therefore tested whether inhibiting PAR2 activation in vivo might be a viable strategy for the treatment of MS. Using the EAE model, we show that a PAR2 antagonist, the pepducin P2pal-18S, attenuates EAE progression by affecting immune cell function. P2pal-18S treatment markedly diminishes disease severity and reduces demyelination, as well as the infiltration of T-cells and macrophages into the CNS. Moreover, P2pal-18S decreases GM-CSF production and T cell activation in cultured splenocytes and prevents macrophage polarization in vitro. We conclude that PAR2 plays a key role in regulating neuroinflammation in EAE and that PAR2 antagonists represent promising therapeutic agents for treating MS and other neuroinflammatory diseases.
Proteinase-activated receptor-2 (PAR2) modulates inflammatory responses and is increased in multiple sclerosis (MS) lesions. We show that the PAR2 antagonist P2pal-18S reduces disease in the murine EAE model of MS by inhibiting T cell and macrophage activation and infiltration into the CNS, making it a potential treatment for MS.
Introduction

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disease of the central nervous system (CNS) (Giralt et al., 2018; Lassmann, 2018; Reich et al., 2018). Demyelination and axon loss, closely associated with leukocyte infiltration and activation of glia, are hallmarks of the MS-affected CNS. Both the innate and adaptive immune system play roles in the neuropathogenesis of disease (Ristori et al., 2000; Hemmer et al., 2015; Van Kaer et al., 2019). Murine experimental autoimmune encephalomyelitis (EAE) is a well-characterized neuroinflammatory animal model of MS (Stromnes and Goverman, 2006; Rangachari and Kuchroo, 2013). It involves an autoimmune response triggered by a myelin antigen, such as myelin oligodendrocyte glycoprotein (MOG), that results in immune cell infiltration into the CNS, accompanied by demyelination and motor dysfunction in the affected animals (Giralt et al., 2018). Indeed, activation and infiltration of myelin-specific T cells (Sospedra and Martin, 2005; Stromnes and Goverman, 2006), and monocytes and macrophages (Henderson et al., 2009) into the CNS are hallmarks of both the murine EAE model and human disease.

Proteinase-activated receptors (PARs) are G-protein coupled receptors (GPCRs; seven-transmembrane receptors that respond to a variety of stimuli) that are activated in pathological settings by microenvironment serine proteinases. These receptors play a broad role in a number of pathophysiologies in a variety of tissues (Peach et al., 2023) and are widely distributed in the CNS (Striggow et al., 2001; Noorbakhsh et al., 2003). Proteinase-activated receptor-2 (PAR2) is expressed by a variety of cell types, including epithelial cells (Heuberger and Schuepbach, 2019), macrophages (Rayees et al., 2020), T cells (Mari et al., 1996; Eftekharinejad et al., 2018), neurons and astrocytes (Bushell, 2007; Bushell et al., 2016), and plays a key role in
inflammation and neuronal function (Noorbakhsh et al., 2003; Bushell et al., 2006; Yoon et al., 2020). In previous work, we have shown that PAR2 expression is elevated in the CNS of MS patients and mice with EAE. Moreover, we found that PAR2-null mice exhibited a marked reduction in EAE neuropathology and had preserved motor function, implying a key role for PAR2 in EAE pathogenesis (Noorbakhsh et al., 2006). However, it is yet unclear which cell type is affected by PAR2 activation in neuroinflammation. Moreover, it is unknown whether targeting PAR2 signaling in wild-type mice affects EAE pathogenesis. Therefore, to facilitate the potential translation of these findings to a therapy, we tested the potential of blocking PAR2 activation to treat neuroinflammatory demyelination in EAE.

Since 2000, a number of PAR1 antagonists have been synthesized (Peach et al., 2023), leading to the successful development and clinical use of the PAR1 antagonist, Vorapaxar® (Andrade-Gordon et al., 1999; Gupta et al., 2023). The development of PAR2 antagonists has been more challenging (McIntosh et al., 2020). However, the peptidomimetic “pepducin” class of PAR2 antagonists (Sevigny et al., 2011) and, more recently, the nonpeptide small molecule PAR2 antagonists I-343 and I-191 (Jiang et al., 2018; Jimenez-Vargas et al., 2018), have shown great potential for specifically blocking PAR2 signaling. Of the PAR2 antagonists available, the pepducin P2pal-18S, has proved of particular value to attenuate inflammation in vivo in mice (Sevigny et al., 2011; Michael et al., 2013). Pepducins are a family of synthetic peptides that can bind to the intracellular loops of GPCRs and inhibit their function (O'Callaghan et al., 2012). P2pal-18S (palmitoyl-RSSAMDENSEKEKRKSAIK-amide) represents a well-studied PAR2 antagonist that efficiently blocks PAR2 signaling in vivo (Sevigny et al., 2011; Michael et al., 2013). P2pal-18S is thought to act intracellularly by interdicting the interaction of the intracellular domains of PAR2 with its downstream effectors (O'Callaghan et al., 2012).
inhibition has proved to be remarkably successful in mitigating peripheral inflammation in a number of disease models (Sevigny et al., 2011; Michael et al., 2013). Therefore, we chose this PAR2 antagonist to test its ability to inhibit neuroinflammation, such as is present in EAE, which has not yet been evaluated to date.

In this study, we tested the hypothesis that the selective PAR2 antagonist P2pal-18S attenuates disease in the murine EAE model by affecting the function of immune cells, which are known to be regulated by PAR2 (Shpacovitch et al., 2008). We therefore treated MOG-immunized mice with P2pal-18S and assessed the animals for both motor dysfunction and the development of spinal cord demyelination. Furthermore, we studied the infiltration of inflammatory cells into the CNS and changes in serum cytokine levels, including granulocyte-macrophage-colony-stimulating factor (GM-CSF), a cytokine shown to play a role in MS pathology (Imitola et al., 2018; Monaghan and Wan, 2020). In addition, we evaluated the impact of P2pal-18S treatment on the activation and cytokine secretion of splenocytes and on macrophage polarization in vitro.

**Materials and Methods**

**Peptides.** The PAR2 antagonist P2pal-18S (palmitoyl-RSSAMDENSEKKRKSAIK-amide) was prepared by solid phase synthesis in the University of Calgary Faculty of Medicine peptide synthesis facility, with an on-column palmitoylation step as the last procedure. Peptide was isolated by HPLC to >95% purity as verified by mass spectrometry. Stock solutions of P2pal-18S peptide were routinely prepared in dimethyl sulfoxide (DMSO). For experiments, the stock solutions were diluted using cell culture medium or 1 mM HEPES buffer pH 7.4 to obtain the final concentrations of 3, 8 and 17 µM in the cell culture media. Myelin oligodendrocyte
glycoprotein-derived peptide 35-55 (MOG$_{35-55}$; > 95% purity) used for animal immunization was generated by solid phase synthesis and HPLC purification by the University of Calgary Faculty of Medicine peptide synthesis facility.

**Animals, EAE model and treatment with P2pal-18S.** Female C57BL/6 wild-type (WT) mice (7-9 weeks of age) were purchased from the Jackson Laboratory and housed under pathogen-free conditions at the animal facility of University of Calgary, Canada. PAR2-null mice, for the isolation of bone marrow-derived macrophages (BMDM), were from the Jackson Laboratory. All procedures were done in keeping with the Canadian Council on animal care, as approved by a University of Calgary Animal Care Committee. All mice used for EAE induction in this study were sex and age-matched: 7–9-week-old female wild-type mice. After anesthesia by intraperitoneal (i.p.) injection of ketamine-xylazine (100 mg/kg and 10 mg/kg, respectively), mice were subcutaneously (s.c.) injected with 50 mg MOG$_{35-55}$ peptide emulsified in 200 μL of complete Freund’s adjuvant (CFA) containing 0.5 mg/mL *Mycobacterium butyricum* (Becton Dickinson, Mississauga ON, Canada). The dose of MOG peptide was selected to optimize our ability to see an effect of the PAR2 antagonist on degree of motor disability. EAE mice also received an i.p. injection of 100 μl pertussis toxin in saline containing a final dose of 300 ng, at days 0 and 2 post-immunization. For treatment, P2pal-18S or vehicle was added to the MOG/CFA emulsion at a final dose of 10mg/kg and administered on day 0 and again, emulsified in 200 μL olive oil (10mg/kg, subcutaneously), on day 10 post-induction. Disease severity was recorded daily by using a 0-5 scoring system as outlined previously (Stromnes and Goverman, 2006). The rating scale used was as follows: 0 = asymptomatic; 0.5 = partial tail weakness; 1 = limp tail; 1.5 = partial limb limping; 2 = uncoordinated movement, hindlimb weakness; 2.5 = partial hindlimb paralysis (single limb); 3 = complete paralysis of both limbs; 3.5 = hindlimb
paralysis with forelimb weakness; 4 = forelimb paralysis; and 4.5–5 = morbidity/death. Mice were randomly assigned treatment and littermates were treated with vehicle (olive oil) as controls. One observer scored disease severity throughout the experiment and was blinded for the treatment. Disease was monitored for a maximum of 30 days, after which mice were euthanized with CO2. For analysis of serum and spinal cord tissue, mice were euthanized with CO2 at day 15 post-induction. Blood was obtained by cardiac puncture and serum was collected and quick-frozen for multiplex cytokine analysis, as outlined below (Discovery assay, Eve Technologies, Calgary AB, Canada). In addition, spinal cord tissues were removed and fixed in 10% neutral buffered formalin before paraffin embedding for histological evaluation of demyelination and immune cell infiltration.

**Splenocyte isolation and proliferation assay.** Splenocytes were obtained from 7- to 8-week-old female C57BL/6 mice. Spleens were gently crushed using a pestle and the suspension was transferred through a 70 μm cell strainer (352350, BD) into a 50 mL tube containing 5 mL phosphate-buffered isotonic saline, pH 7.4 (PBS). Next, the solution was transferred into a 15 mL tube containing 5 mL Lympholyte cell separation medium (polysucrose 400 and sodium diatrizoate, density 1.0875 ± 0.0010 g/cm3, pH 6.9 ± 0.3; Cedarlane, Burlington, Canada). The tube was centrifuged for 30 minutes at 500 × g with no brake to isolate splenocytes based on density gradient. The “buffy layer”, the interphase containing a mixed splenocyte preparation, was collected and transferred to a 15 mL tube. The collected cells were washed by adding 10 ml PBS and centrifuged for 10 min at 300 × g. To study the effect of PAR2 antagonist on the proliferation of splenocytes, cells were labeled with carboxy fluorescein diacetate succinimidyl ester (CFSE, a dye that is diluted with every cell division, resulting in halving of the fluorescence intensity in each daughter cell; Thermo Fisher Scientific, Waltham, MA) as described previously.
In brief, cells were incubated with CFSE in a reaction volume of 2 mL PBS, at a final concentration of 2.5 mM and a cell concentration of $1 \times 10^6$ cells/mL, for 12 min at 37°C. To stop the reaction, 10 ml T cell medium (RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA), 1 mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA), 50 μM beta-mercaptoethanol (Sigma Aldrich) and 1X antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA) was added. The cells were then centrifuged for 10 min at 300 × g. The pellet was resuspended in T cell medium and then incubated with P2pal-18S at three final concentrations (3, 8, 17 μM) or equivalent volume of DMSO (0.8%), at 37°C for 30 minutes, followed by plating ($10 \times 10^4$ cells in 100 μl/well) into a 96-well round bottom plate (353077, Corning) that was pre-coated with 10 μg/mL of anti-CD3 (555273, BD Biosciences, Mississauga ON, Canada) and 10 μg/mL anti-CD28 antibodies (553294, BD Biosciences) for 2 h at 37°C. After 48 hours of incubation, the cells were collected and centrifuged at 300 × g for 5 minutes, and 100 mL supernatant aliquots were collected, quick-frozen, and saved for measurement of cytokine production (Discovery assay, Eve Technologies, Calgary AB Canada). Flow cytometry (Attune® Acoustic Focusing Flow Cytometer, Applied Biosystems) was used to assess cell proliferation and viability. To assess lymphocyte proliferation by CFSE dilution, we included an undivided cell control (i.e. cells without treatment with anti-CD3 and anti-CD28 antibodies providing the maximum, undiluted, CFSE staining) and a non-labeled cell control (i.e. cells without CFSE labeling). The impact of the PAR2 antagonist, P2pal-18S, on splenocyte viability was determined using the LIVE/DEAD™ Fixable Green Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham MA, USA) according to manufacturer instructions. Data was analyzed using FlowJo software (version 10.7.1).
Isolation and culture of bone marrow-derived macrophages (BMDM). For BMDM isolation, 7- to 8-week-old female C57BL/6 WT or PAR2-null mice were used. Femoral bones were isolated, and total bone marrow cell populations were collected by flushing the femur bones with PBS using a 10 mL syringe and a 25 G needle. The cell suspension was centrifuged at 300 × g for 10 minutes and the pellet was thoroughly resuspended with macrophage differentiation medium comprising Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 10% (v/v) L929-cell–conditioned media as a source of M-CSF, and 2 mM L-glutamine (Thermo Fisher) and 1X antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA). Cells were plated at a density of 10 ×10^6 cells in 10 mL (1 million cells/mL) in 10 cm diameter Petri dishes (25384-302, VWR) and cultured at 37°C under an atmosphere of 5% CO₂ for 10 days. At day 5, half the medium and at day 7, the entire medium was replaced with the fresh macrophage differentiation medium. After 10 days, macrophages were collected using a cell scraper (Corning), washed with PBS and centrifuged at 300 × g for 10 minutes. Aliquots of 1x10^6 cells in 1 mL macrophage medium were treated with P2pal-18S (17 μM), or equivalent volume of DMSO (vehicle) at 37°C for 30 minutes and subsequently plated for either polarization experiments or activation with LPS.

Pro- and anti-inflammatory macrophage polarization. To determine the effect of PAR2 antagonist on macrophage polarization, day 10 BMDM were pretreated with P2pal-18S (17 μM), or vehicle (DMSO) at 37°C for 30 minutes, after which they were plated at 5 × 10^6 cells/well in 2 mL macrophage medium in 6-well plates (10861-556, VWR) and stimulated with 100 ng/mL recombinant mouse IFN-g (produced in Escherichia coli (E. coli); CL9209R, Cedarlane) to induce an M1 (pro-inflammatory) or 20 ng/mL recombinant mouse IL-4 (produced in E. coli; CL121-04, Cedarlane) and 10 ng/mL recombinant mouse IL-13 (CL9313R, Cedarlane) to induce
an M2 (anti-inflammatory) phenotype, in the presence or absence of P2pal-18S. Cells were then incubated at 37°C overnight, followed by RNA extraction for assessment of polarization-induced changes in RNA expression of M1/M2 markers to typify the macrophage phenotype. To assess the effect of PAR2 inhibition on cytokine production, 50 × 10^3 cells/well were plated in a flat-bottom 96-well plate (353072, Corning) for 30 minutes with P2pal-18S or vehicle (DMSO) treatment. After that, either IFN-γ (100 ng/mL) or a combination of IL-4 (20 ng/mL) and IL-13 (10 ng/mL) were added to the wells. Plates were incubated at 37°C overnight, and 100 mL supernatants were collected and quickly frozen. Non-polarized macrophages were harvested from untreated cells and considered an M0 phenotype.

**Activation of macrophages.** BMDMs were seeded in a flat-bottom 96-well plate (Corning) at 50 × 10^3 cells/well in a total volume of 100 μL culture medium, in the presence or absence of 100 ng/mL LPS (L4005, E. Coli 055:B5, Sigma) and incubated overnight at 37°C. To evaluate the effects of PAR2 antagonist on cytokine production, 100μL conditioned medium was collected and quick-frozen. Cytokine content was determined by a Mouse Cytokine Array Proinflammatory Focused 10-plex assay (MDF10 Discovery assay, Eve Technologies, Calgary AB, Canada).

**Measurement of calcium signalling.** Measurements of PAR2-induced increases in intracellular calcium were done as outlined previously, with minor modifications (Mihara et al., 2013), using a Fluo4-No-Wash calcium indicator (36206, Life technologies). Measurements were done at 24°C as per the published protocol to optimize observing the kinetics of the calcium signal. The mobilization of intracellular calcium was measured in isolated murine splenocytes and BMDM after PAR2 stimulation by the PAR2-selective agonist peptide 2-furoyl-LIGRLO-NH₂ (2fL1).
Measurements of calcium transients were done at 24 °C (Kawabata et al., 1999), using a Perkin-Elmer fluorescence spectrophotometer with an excitation wavelength of 480 nm and a fluorescence emission recorded at 530 nm. To prepare cells for calcium transient measurements, 8-10 × 10^6 cells were collected by centrifugation at 300 x g for 10 minutes and washed with EDTA-free isotonic phosphate-buffered saline pH 7.4 (PBS) in 15 mL Falcon polystyrene tubes (BD Biosciences). The final cell suspension was harvested by centrifugation (at 300 x g for 5 minutes) and the cell pellet was resuspended in 1 ml of Fluo4-NW calcium indicator at a final concentration of 25mg/ml dissolved in Hanks’ buffered salt solution (HBSS, containing 1.5 mM CaCl₂, 1.5 mM MgCl₂) pH 7.4 supplemented with 10 mM HEPES (HBSS⁺). Indicator uptake was allowed to proceed by incubation for 45 minutes at room temperature. Aliquots (100 mL) of the Fluo4-loaded cell suspension (approximately 5-10 × 10^6 cells/ml) were then added into the magnetically stirred fluorometer cuvette at room temperature, containing 2 mL of HBSS⁺. PAR2 agonist, 2-furoyl-LIGRLO-NH₂ (2fLI), was then added at a final concentration of 20 mM. Fluorescence emission at 530 nm (excitation wavelength, 480 nm) was monitored over10 minutes at room temperature as an index of the increases in intracellular calcium concentrations. The changes in fluorescence were normalized (% of Ionophore signal, E530) relative to the peak fluorescence increase caused by the addition of 2.5 μM of the calcium ionophore (CI), A23187 (7522, Sigma), to equal aliquots of the same cell suspension.

**Quantitative reverse-transcriptase PCR.** RNA was isolated from splenocytes and BMDMs using the RNAeasy plus mini kit (Qiagen, Toronto ON, Canada). 1 mg RNA was used to synthesize cDNA using SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA according to manufacturers’ instructions in a 20 μl reaction volume). Quantitative reverse-transcription PCR was performed using a Quanti Nova Sybr Green PCR kit (Qiagen,
Toronto ON, Canada). Primers were designed using the database of http://primerdepot.nci.nih.gov/, http://mouseprimerdepot.nci.nih.gov/ or were purchased from Real-Time Primers (Melrose Park, PA USA) and yielded a single melt curve and. The following murine primers were used: GAPDH (forward: 5¢-CTGGAGAAACCTGCCAAGTA-3¢, reverse: 5¢-TGTTGCTGTAGCCGTATTCA-3¢), iNOS (forward: 5¢-CTTTGTGCGAAGTGTCAGTG-3¢, reverse: 5¢-CACCTGGAACAGCAGTCTCTCT-3), Arginase1 (forward: 5¢-GTGAGAAACCGTGCTGTCTGT-3¢, reverse: 5¢-CTGGTTGTCAGGGGAGTGTT-3”). mRNA expression levels were normalized to the housekeeping gene GAPDH. CT values for each marker were determined to calculate $2^{-\Delta\Delta{CT}}$ referenced to undifferentiated macrophage (M0).

**Multiplex cytokine analysis.** The concentration of a number of cytokines and chemokines in mouse serum were analyzed using a Mouse Cytokine Array / Chemokine Array 31-Plex assay (MD31 Discovery assay, Eve Technologies, Calgary AB, Canada). After collecting supernatants from splenocytes which were cultured as described above and treated with the PAR2 antagonist or vehicle, cytokine and chemokine levels were determined by a Mouse High Sensitivity T-Cell Discovery Array 18-plex assay (MDHSTC18 Discovery assay, Eve Technologies, Calgary AB, Canada). Conditioned media were harvested from cytokine activated and non-activated macrophages and analyzed for cytokine content by a Mouse Cytokine Array Proinflammatory Focused 10-plex assay (MDF10 Discovery assay, Eve Technologies, Calgary AB, Canada).

**Histology.** For histology, animals were euthanized 15 days after EAE induction. The spinal cords and cerebella were removed and fixed in 10% buffered formalin. The isolated thoracic and lumbar tissues were paraffin-embedded and longitudinal sections were cut (6 mm thickness) using a Leica RM2135 Microtome. After deparaffinization sections were stained with Erichrome
Cyanine R, (E2502-256; Sigma-Aldrich, Oakville ON, Canada) for visualizing the demyelinated areas. Images were taken using a bright field microscope (BX51; Olympus). For immunofluorescence staining, after antigen retrieval with sodium citrate buffer (pH 6), sections were incubated for 1 h with blocking solution (PBS containing 10% horse serum, 1% BSA, 0.1% goldfish gelatin, 0.1% Triton X-100 and 0.05% Tween 20) to prevent non-specific antigen binding. Next, sections were incubated overnight at 4 °C with primary antibodies against Myelin Basic Protein (ab40390 and ab7349; Abcam, Cambridge MA, USA, 1:100), SMI312 (837902-4; Biolegend, San Diego CA, USA, 1:2000), CD3 (ab11089; Abcam, Cambridge MA, USA, 1:200) and Iba1 (019-19741; Wako, Chemicals U.S.A. Richmond VA USA, 1:1000), diluted in antibody dilution buffer (PBS including: 1% BSA, 0.1% goldfish gelatin and 0.5% Triton X-100). The sections were then washed with PBS supplemented with 0.05% Tween 20, followed by incubating at room temperature for 1 h with secondary antibodies conjugated with Alexa 488, 546, and 594 (Jackson ImmunoResearch, West Grove PA, USA, 1:500) and nuclear yellow (1:1000) for staining of cell nuclei. Each section was washed as described above and was kept in the dark at 4 °C before imaging. The fluorescence images were taken on a confocal microscope (Fluoview FV10i; Olympus). Images were captured using a 60x objective. All samples processed for imaging were observer-blinded.

**Flow cytometry.** Mice were anesthetized at day 15 post-EAE induction, transcardially perfused with 20 mL of ice-cold phosphate-buffered saline (PBS) and then spinal cords were removed. Leukocytes were dissociated from homogenized spinal cord tissue and harvested using isotonic percoll via discontinuous density gradient centrifugation (Agrawal et al., 2011; Ewanchuk et al., 2018). Cell suspensions were then stained with the following antibodies (all BD Biosciences, San Jose CA, USA): FITC-conjugated anti-mouse CD3 (BD555274), PerCP-conjugated anti-
mouse CD4 (BD553052), PE-conjugated anti-mouse CD8 (BD562283), PE-conjugated anti-mouse CD11b (BD557397) and PerCP-conjugated anti-mouse CD45 (BD557235). After immunostaining, cells were analysed by flow cytometry using an Attune Acoustic Focusing Flow Cytometer, at the Cumming School of Medicine flow cytometry core facility. Cell populations were identified as follows: CD4⁺ T cells (CD3⁺/CD4⁺), CD8⁺ T cells (CD3⁺/CD8⁺), macrophages (CD11b⁺/CD45high) and microglia (CD11b⁺/CD45low).

**Splenocyte viability assay.** The impact of the PAR2 antagonist, P2pal-18S, on splenocyte viability was determined using the LIVE/DEAD™ Fixable Green Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham MA, USA) according to manufacturer instructions. Isolated splenocytes were treated with different concentrations of P2pal-18S (3, 8, 17 μM) and the percentage of live/dead cells (APC-Cy7) was determined using flow cytometry (Attune® Acoustic Focusing Flow Cytometer, Applied Biosystems). Cells were gated, and data were analyzed using FlowJo software (version 10.7.1).

**Statistical analysis.** All statistical analyses were performed with Prism (GraphPad Software, Version 9.4.1, USA). Non-parametric tests were used for categorical data (EAE scores) and data that did not pass a Shapiro-Wilk test for normality. Parametric test were used for all data that passed normality testing. For comparing the clinical score of EAE mice between groups over time, a Wilcoxon-matched pairs signed rank test was used. The statistical differences of incidence of EAE were tested by Chi-Square. To compare the score at day 15 of the EAE protocol for antagonist-treated vs untreated mice, we performed a Mann-Whitney test. For parametric data with 2 groups, a 2-tailed Student’s t-test was used; one-way ANOVA was performed to analyze multiple groups with Tukey’s multiple comparison post hoc test.
statistical tests and sample size were reported in the individual figure legends. The p values <0.05 were considered significant. All experiments were reproduced at least twice.
Results

PAR2 inhibition attenuates EAE

To assess the effect of PAR2 inhibition on the development of neuroinflammation in vivo, we treated EAE mice with the PAR2 antagonist P2pal-18S, given as a subcutaneous bolus injection on days 0 and 10 after induction of EAE (Figure 1A), at a dose of 10 mg/kg, which has previously been shown to effectively reduce inflammation (Sevigny et al., 2011; Michael et al., 2013). P2pal-18S treatment caused a marked reduction in EAE severity (Table 1, Figure 1B, Supplemental Figure 1), resulting in a reduction in the clinical scores at peak of disease (day 15, Figure 1C) and throughout the disease course (Table 1 and Figure 1B). Consistent with this result, myelin staining of thoracolumbar longitudinal sections revealed that P2pal-18S treatment significantly reduced demyelination in the spinal cord at the peak of disease (day 15 post-immunization, Figure 1D, E). To visualize the myelin sheath around axons, myelin and axons were stained using antibodies for myelin basic protein (MBP) and neurofilament (SMI312), respectively. Immunofluorescent labelling revealed that the P2pal-18S treatment attenuated myelin degeneration surrounding axons, resulting in preservation of axon/myelin structures (Figure 1F). Together, these results show that inhibition of PAR2 activation results in reduced disease severity in EAE, in accord with reduced demyelination.

PAR2 antagonist blocks immune cell infiltration into the CNS

We next assessed immune cell infiltration into the spinal cords of P2pal-18S- and vehicle-treated animals at peak of disease (day 15 post-immunization) by immunofluorescence. This revealed
that P2pal-18S treatment significantly reduced the infiltration of CD3+ T-cells (Figure 2A, B) and the abundance of Iba1+ macrophages and microglia (Figure 2C, D) in the spinal cord. It can be noted that demyelination (loss of immunofluorescence for myelin basic protein; MBP) occurred at the sites of cell accumulation (Figure 2A, C). Moreover, flow cytometric analysis of cells present in spinal cord tissue (gating strategy for different cell populations shown in Supplemental Figure 2) showed that P2pal-18S treatment markedly reduced the total number of infiltrating CD4+ T cells (Figure 2E, upper left quadrant, and G) and macrophages (CD11b+/CD45high, Figure 2F and G) in spinal cord tissue. In contrast, the reduction in the numbers of CD8+ T cells was less pronounced, and the difference in numbers of resident microglia (CD11b+/CD45low) did not reach statistical significance (Figure 2G). Together, these results show that inhibition of PAR2 activation reduces the infiltration of CD4+ T cells and macrophages into the CNS.

**P2pal-18S treatment decreases GM-CSF levels in the serum of EAE mice**

Since P2pal-18S treatment of EAE mice reduced neuroinflammation in the CNS, and specifically the infiltration of immune cells, we measured the level of cytokines in the serum of these mice to determine if circulating inflammatory mediators were changed by antagonist treatment. In our study, we collected blood samples from EAE mice with and without P2pal-18S treatment. The blood was isolated at peak disease (day 15 post EAE induction), and a panel of 31 serum-borne cytokines and chemokines was evaluated in the serum of each group (Figure 3A).
We observed decreased levels of GM-CSF, a cytokine shown to play a role in MS pathology (Imitola et al., 2018; Monaghan and Wan, 2020), in the serum of EAE mice treated with P2pal-18S (Figure 3B). In addition, levels of Eotaxin were also modestly, but significantly decreased. However, there was no change in the levels of key proinflammatory T helper (Th) 1 and Th17 cell cytokines IFN-g and IL-17 (Figure 3C). Additionally, levels of anti-inflammatory cytokines, such as IL-4 and IL-10, produced by Th2 cells and regulatory T cells, respectively, did not change in the antagonist-treated mice (Figure 3D).

All other cytokines and chemokines that were analyzed remained unchanged in the serum of animals treated with P2pal-18S, compared to untreated animals (Supplemental Figure 3). Together, these results demonstrate that inhibition of PAR2 with P2pal-18S treatment in vivo leads to a reduction of circulating GM-CSF and Eotaxin, supporting the reduction of neuroinflammation.

**PAR2 inhibition reduces murine CD4⁺ T cell proliferation and cytokine secretion**

CD4⁺ T-cells play an important role in the pathogenesis of EAE (Goverman, 2009). Our observation that infiltration of CD4⁺ T-cells into the CNS was reduced after treatment with P2pal-18S (Figure 2G) prompted us to evaluate the effect of PAR2 inhibition on the activation of these cells. To assess first whether T cells functionally respond to PAR2 activation, we isolated splenocytes from C57BL/6 mice and evaluated their response to PAR2 agonists. To this end, we stimulated these cells with the PAR2 activating enzyme trypsin and the highly selective PAR2 agonist 2-furoyl-LIGRLO-NH₂ (2fL1) and measured intracellular calcium
levels to evaluate the pharmacological functionality of PAR2 in these cells. We found that splenocytes responded to PAR2 activation by both trypsin and 2fLI with an increase in intracellular calcium, indicating that they express functional PAR2 (Supplemental Figure 4A and B). We then assessed the effect of PAR2 inhibition on the activation and proliferation of T cells, by stimulating splenocytes with anti-CD3 and anti-CD28 antibodies in the presence of increasing concentrations of P2pal-18S (3, 8, 17 µM) or vehicle. Treatment with the PAR2 antagonist caused a modest, but concentration-dependent decrease in CD4+ T-cell proliferation (Figure 4A). In contrast, the proliferation of CD8+ T cells was not significantly affected by P2pal-18S treatment (Figure 4B). P2pal-18S treatment had no impact on T-cell viability (Supplemental Figure 5).

To determine whether PAR2 inhibition affects T-cell function, we collected supernatants from these anti-CD3/CD28-activated splenocytes to analyze their cytokine and chemokine profile. We found that, in vitro, P2pal-18S treatment caused a concentration-dependent reduction in the secretion of several cytokines and chemokines. These included GM-CSF, but also the general proinflammatory cytokines IL-1β and IL-6, which could be produced by myeloid cells present in the splenocyte preparations, as well as the Th17 specific cytokine IL-17 and the Th2 cytokines IL-4, IL-5, and IL-13 (Figure 4C). In addition, secretion of several chemokines was reduced by P2pal-18S treatment (Supplemental Figure 6). Thus, the inhibitory action of P2pal-18S on immune cell function in vivo is paralleled by a general ability of the antagonist to reduce CD4+ T-cell activation and cytokine secretion by splenocytes in vitro.

**PAR2 inhibition reduces macrophage polarization but does not affect their cytokine production**
Because macrophages play an important role in the pathogenesis of MS and EAE (Ajami et al., 2011; Liu et al., 2013; Wang et al., 2019), we evaluated the impact of PAR2 expression and inhibition on macrophage differentiation in vitro. To this end, we again first established that macrophages express functional PAR2. We evaluated bone marrow-derived macrophages (BMDM) obtained from 7-9 week-old female mice for their response to activation by trypsin and 2fLI. We found that the cells respond to activation with both PAR2 agonists with a pronounced increase in intracellular calcium (Supplementary Figure 4C and D). Having established the presence of functional PAR2 on macrophages, we then treated BMDM from wild-type and PAR2-null mice with either IFN-γ, or IL-4 and IL-13 to stimulate polarization into pro-inflammatory (M1) and anti-inflammatory (M2) macrophages, respectively, either in the absence or presence of the PAR2 antagonist P2pal-18S. We then assessed mRNA expression of iNOS as a marker for M1 macrophages and Arginase1 as a marker for M2 macrophages. Our results show that P2pal-18S reduced the extent of differentiation of wild-type BMDM into both M1 and M2 phenotypes (Figure 5A and B). In parallel, BMDM from PAR2-null mice showed a diminished polarization to either an M1 or M2 phenotype (Figures 5A and B). As expected, P2pal-18S did not have an effect on PAR2-null cells.

To evaluate the influence of PAR2 inhibition on macrophage-mediated cytokine secretion, we pre-treated wild-type BMDM for 30 minutes with P2pal-18S (17 μM) or DMSO (0.8%), then polarized the cells to M1 and M2 macrophages overnight before collecting supernatant. Of the classical M1 cytokines, we only observed a slight increase in TNF-α secretion after polarization in the presence of P2pal-18S, but no changes in IL-1β or IL-12 (Figure 5C). Secretion of the M2 cytokine IL-10 was not affected by P2pal-18S treatment either (Figure 5D). Likewise, there were no significant differences in the remaining cytokines that were analyzed.
In addition, we activated wild-type BMDM with LPS for 12h with or without P2pal-18S (17 µM) and measured cytokine levels in the supernatants. No significant differences were found in LPS-stimulated macrophages in the absence or presence of the PAR2 inhibitor (Supplementary Figure 7).

Taken together, these data indicate that inhibition of PAR2 attenuates the polarization of macrophages into either a pro-inflammatory or an anti-inflammatory phenotype but does not significantly affect cytokine production from M1/M2 polarized or LPS-stimulated macrophages.
Discussion

We show here that inhibition of PAR2 in vivo markedly reduces neuroinflammation and demyelination in the EAE model of MS. The administration of only two subcutaneous doses of P2pal-18S significantly reduced the incidence and severity of EAE. Moreover, our results show that inhibiting PAR2 with P2pal-18S reduces T cell activation and macrophage polarization in vitro and reduces infiltration of these cells into the CNS in vivo. To our knowledge, this is the first study demonstrating a direct role in vivo for PAR2 in the neuroinflammatory response in the EAE model as envisioned some time ago by Bushell (Bushell, 2007; Bushell et al., 2016) and showing a potential therapeutic efficacy of P2pal-18S in reducing neuroinflammation.

PAR2 is implicated in the pathogenesis of murine EAE and human MS (Noorbakhsh et al., 2006) and our findings here corroborate these findings. We show that the protective effect of PAR2 inhibition on demyelination in EAE model may be due to its ability to reduce the influx of inflammatory cells into the central nervous system (CNS). P2pal-18S reduces CD4+ T-cell infiltration into the CNS, and proliferation in vitro. This impact on T cells is of importance, given their presumed role as effector cells in the pathophysiology of EAE and MS (Kaskow and Baecher-Allan, 2018). Our data also show that PAR2 inhibition reduces the production of a wide range of cytokines by in vitro (anti-CD3/D28) activated splenocytes, including IL1-β, IL-17 and GM-CSF, which play key roles in the pathogenesis of EAE and MS (Sutton et al., 2006; Lin and Edelson, 2017; Aram et al., 2019; Di Filippo et al., 2021; Janoschka et al., 2023). PAR2 activation in patient-derived cardiomyocytes has been linked to IL1-β expression and blocking PAR2 has been shown to reduce the production of IL1-β (Sasaki et al., 2022). Furthermore, lymph node cells isolated from PAR2-null mice produce less IL-17 (Crilly et al., 2012) and
PAR2 activation has been shown to increase GM-CSF production in epithelial cells (Carey et al., 2020), supporting the \textit{in vitro} findings we describe here.

In addition to reducing \textit{in vitro} secretion of these cytokines by T cells, \textit{in vivo} treatment with the PAR2 antagonist reduces the abundance of circulating GM-CSF in EAE. GM-CSF levels are elevated in the cerebrospinal fluid (CSF) and CNS lesions of MS patients (Aram et al., 2019) (Manivasagam et al., 2022), and T cells have been found to be a major source of GM-CSF production in patients with MS and in the EAE model (Croxford et al., 2015; Galli et al., 2019; Wheeler et al., 2020). An important role for GM-CSF in MS (Rasouli et al., 2015) is also supported by the finding that GM-CSF deficient mice are resistant to EAE induction (McQualter et al., 2001; Ghosh et al., 2016). GM-CSF promotes immune cell infiltration into the CNS (Duncker et al., 2018). PAR2 activation induces GM-CSF production (Vesey et al., 2013) and one of the main mechanisms underlying the ability of PAR2 inhibition to block disease in EAE could therefore be its inhibition of GM-CSF production. We did not detect a significant reduction in circulating cytokines reflective of a specific peripheral CD4$^+$ T cell response, however, such as IFN-g and IL-17. This result could be because at the time of sampling (peak disease) the immune response is localized within the CNS, rather than in lymphoid organs or circulation.

In addition to affecting T-cell proliferation and cytokine production, P2pal-18S diminishes macrophage polarization to both M1 and M2 subsets, known to play distinct roles in inflammatory responses. Macrophage polarization is also markedly attenuated in cells from PAR2-null mice, paralleling the effect of the PAR2 antagonist on this cell type. In addition, infiltration of macrophages into the CNS is significantly reduced \textit{in vivo} by P2pal-18S treatment.
in the EAE MS model. Macrophages play a crucial role in the loss of myelin and inflammation in MS lesions, due to their capacity to phagocytose myelin, produce inflammatory mediators and activate T cells (Henderson et al., 2009) (Montilla et al., 2023) (Spiteri et al., 2022). Likewise, infiltration of macrophages is crucial for the progression of EAE to severe disease (Ajami et al., 2011). While PAR2 inhibition does not seem to affect either polarized phenotype of macrophage subsets in particular, the ability of P2pal-18S to diminish inflammatory demyelination could be due in part to its suppression of macrophage activation and infiltration in general.

The role we suggest for PAR2 in MS and the EAE model parallels the involvement of PAR2 in inflammatory disease models like osteoarthritis (Ferrell et al., 2010) and antigen-induced arthritis (Busso et al., 2007). PAR2-null animals show reduced disease in the arthritis model (Busso et al., 2007). Similar to white matter tissue of patients with MS (Noorbakhsh et al., 2006), PAR2 is also upregulated in the synovium of rheumatoid arthritis patients (Busso et al., 2007). In addition, blocking PAR2 has been shown to reduce inflammation in an allergen model of lung inflammation (Asaduzzaman et al., 2015). Moreover, in a mouse adjuvant-induced carrageenan/kaolin model of paw inflammation, P2pal-18S markedly reduced the inflammatory response, supporting the therapeutic potential of PAR2 inhibition (Sevigny et al., 2011). Of note, our data show that P2pal-18S attenuates the progression and severity of motor disability when administered during immunization. It remains to be seen if PAR2 inhibition also reduces established neuroinflammation. Future studies will be directed at this issue, as well as the evaluation of orally available PAR2 inhibitors that may be of better use in humans. Nevertheless, our current data show that pharmacological inhibition of PAR2 can mitigate a number of key processes in EAE pathogenesis and therefore can be considered as a potential therapeutic target in autoimmune demyelinating diseases such as MS.
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Availability of data and materials

All data collected, generated, and analyzed during this study are included in this article and its additional information.
Authorship contributions

Participated in research design: Hollenberg, Noorbakhsh, Kuipers, Yates, Eftekhar

Conducted Experiments: Eftekhar, Ewanchuk, Rawji

Performed data analysis: Hollenberg, Noorbakhsh, Kuipers, Yates, Eftekhar, Ewanchuk, Rawji

Wrote or contributed to the writing of the manuscript: Hollenberg, Noorbakhsh, Kuipers, Yates, Eftekhar, Ewanchuk, Rawji
References


Footnotes

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The authors declare no competing interest.

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Figure Legends

Figure 1. Treatment of EAE with the PAR2 antagonist P2pal-18S attenuates disease severity. A) Overview of the P2pal-18S treatment protocol used. EAE mice received P2pal-18S (10mg/kg subcutaneously) or vehicle on days 0 and 10 post-immunization. B) EAE disease severity (average ± SEM) in animals receiving P2pal-18S treatment (open squares, N=22) compared to animals treated with vehicle only (black circles, N=24). Shown are pooled data for 3 independent experiments. Individual experiments are shown in Supplemental Figure 1. **** P < 0.0001, Wilcoxon matched pairs signed rank test. C) Average clinical score at peak disease (day 15) in vehicle (black bar) and P2pal-18S treated (white bar) mice. Data shown are mean ± SEM, ** P<0.001, Mann-Whitney test. B, C) Data shown are an aggregate of 3 independent EAE experiments. D-F) Immunohistochemical and immunofluorescent analysis of longitudinal sections of spinal cord tissue from vehicle (Control) and P2pal-18S treated mice (Treatment) at day 15 post EAE induction. D) 10X Erichrome cyanine R staining for myelin (blue). Tissue was counterstained for nuclei with eosin (red). E) Quantification of demyelinated area of panel D, * P<0.05, N=3, pooled triplicate data from 3 independent experiments. F) Immunofluorescent staining for myelin basic protein (MBP, green) and neurofilament (SMI312, red). Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI; blue). Shown are representative images from n = 2-3 mice. Scale bars: black, 100 mm, Panel D; White, 20 mm Panel F.

Figure 2. Immune cell infiltration into the CNS is reduced by P2pal-18S treatment.

Analysis of spinal cord tissue harvested from P2pal-18S-treated and control mice at day 15 post EAE induction. A and B) Immunofluorescence labelling of CD3+ (T cells, red) and C and D) Iba-1+ (macrophage/microglia, magenta) cells, and myelin basic protein (MBP, green). Nuclei
were counterstained by DAPI (blue). White scale bar in panels A and C: 20 mm. **E and F**

Representative flow cytometry plots of immune cell infiltration into the spinal cord of untreated (Control) or P2pal-18S -treated (Treatment) mice. **E** Identification of T cell subsets, based on CD4 and CD8 expression (fluorescence intensity of PerCP-conjugated anti-CD4 antibody staining shown on y-axis, PE-conjugated anti-CD8 staining on x-axis). Total T cells were gated on CD3 expression (Supplemental Fig 2) and the percentage of CD4^{+} T and CD8^{+} T cells within this population was quantified. **F** Identification of microglia and infiltrating macrophages, based on CD11b and CD45 expression (fluorescence intensity of PerCP-conjugated anti-CD45 antibody staining shown on y-axis, PE-conjugated anti-CD11b an x-axis). Cells were gated on CD45 expression first (Supplemental Fig 2) and the percentage of macrophages (CD11b^{+}/CD45^{high}) and microglia (CD11b^{+}/CD45^{low}) cells was quantified. **G** Quantification of the effect of PAR2 antagonist (white bars) or vehicle (black bars) treatment on the number of infiltrating CD4^{+} and CD8^{+} T cells, and macrophages, and CNS resident microglia. Total number of cell populations was quantified using FlowJo software. Data are represented as mean ± SEM (Error bars). N=8-10, pooled data from two independent experiments. (Student’s t-test; *, P ≤ 0.05).

**Figure 3.** P2pal-18S treatment decreases GM-CSF and Eotaxin levels in the serum of EAE mice. Serum was collected at day 15 post-immunization from PAR2 antagonist-treated (treatment) and vehicle-treated (control) EAE mice. Multiplex analysis of **A** Heatmap visualizing relative levels of cytokines (blue, low; yellow high), comparing untreated (EAE) and P2pal-18S-treated (EAE+ P2pal-18S) EAE mice, as quantified by Luminex assay. **B** Serum level of inflammatory mediators involved in the pathogenesis of multiple sclerosis, **C** pro- and **D** anti- inflammatory cytokines. Mean ± SEM (error bars) as well as individual sample values.
(circles) are shown. Values below the detection limit were substituted by lower detection limit/Ö2. N= 8-10, pooled data from 2 independent experiments. *, P ≤ 0.05), Student’s t-test.

**Figure 4. P2pal-18S reduces proliferation and cytokine production of CD4⁺ T cell.**

Splenocytes (SPL) were isolated from C57BL/6 mice and labelled with the proliferation dye CFSE as outlined in Methods. Isolated splenocytes were then treated with increasing concentrations of P2pal-18S (3, 8 and 17 µM) or vehicle (DMSO; back bars) and stimulated with anti-mouse CD3/CD28 antibodies (activated) or left unstimulated (non-activated). **A and B)** Representative flow cytometry plots were used to quantify cell proliferation in **A)** CD4⁺ T cells and **B)** CD8⁺ T cells. Dilution of CFSE dye (shown in representative histograms) is indicative of cell proliferation. Bar graphs on the right summarize cell proliferation rates as percentage of cells that have proliferated, quantified using FlowJo. **C)** Supernatants from non-activated or activated splenocytes were harvested for multiplex cytokine analysis. Changes in the cytokine levels caused by increasing concentrations of P2pal-18S upon antibody activation are shown by the shaded bars, compared with antagonist-untreated cells (black bars). Values below detection limit were substituted by lower detection limit/Ö2. SPL: splenocytes. Data are represented as mean + SEM (Error bars), for N=4. *, P ≤ 0.05, ***, P ≤ 0.001, One-way ANOVA, Tukey’s multiple comparison test.

**Figure 5. P2pal-18S blocks both pro- (M1) and anti- (M2) inflammatory macrophage polarization but not cytokine production.** **A and B)** Bone marrow-derived macrophages from wild-type (WT) or PAR2-null mice (KO) were treated overnight with either 100 ng/mL IFN-g to induce pro-inflammatory (M1) polarization, or a combination of 20 ng/mL IL-4 and 10 ng/mL IL-13 to induce anti-inflammatory (M2) polarization, in the absence or presence of the PAR2
inhibitor P2pal-18S (17 μM). mRNA expression of **A** iNOS and **B** Arginase1 was then determined by qRT-PCR as markers of pro-inflammatory and anti-inflammatory macrophages, respectively. mRNA values for each gene were normalized to values for GAPDH and the expression of iNOS mRNA (panel A) and Arginase1 mRNA (Panel B), relative to non-polarized macrophages (relative expression) was then calculated. Data shown are means +SEM; ns, not significant, **, P ≤ 0.01, ***, P ≤ 0.001 One-way ANOVA, Tukey’s multiple comparison test. N=8-12, pooled data from three experiments performed independently. **C and D** P2pal-18S (17 μM) or vehicle (an equivalent concentration of diluted DMSO) were used to treat bone marrow-derived macrophages, which were then polarized to M1, M2 macrophages as outlined above in the continued presence of P2pal-18S. After 12 h, the supernatants were harvested for multiplex cytokine analysis. Data are represented as mean ± SEM (Error bars). Values below detection limit were substituted by lower detection limit/Ö2. Data were analyzed by One-way ANOVA, Tukey’s multiple comparison test, for N=4-5 pooled data from two experiments.
Table 1. Clinical scores of P2pal-18S- and vehicle- treated EAE mice.

Day of onset: first day of symptoms. Maximum score: highest score throughout the disease course. Cumulative disease score: sum of each daily score, calculated from day 0 to day 30 after immunization. Data are represented as the mean ± SEM. * P<0.05, ** P<0.001, Chi-Square was used to evaluate the statistical differences.

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### Table

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### Graph A

- **A.** Treatment History and Flow Cytometry

### Graph B

- **B.** Clinical Score
  - Control (N=24)
  - Treatment, P2pal-18S 10mg/kg (N=22)

### Graph C

- **C.** Day 15
  - Control
  - Treatment

### Graph D

- **D.** Control vs. Treatment

### Graph E

- **E.** % Demyelinated Area
  - Control
  - Treatment

### Graph F

- **F.** Immunostaining
  - DAPI
  - MBP
  - SMI312

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Blockade of proteinase-activated receptor 2 (PAR 2) attenuates neuroinflammation in experimental autoimmune encephalomyelitis

Supplemental figure 1. P2pal-18S treatment attenuates EAE disease severity.

EAE mice received P2pal-18S (10mg/kg subcutaneously) or vehicle on days 0 and 10 post-immunization. **A** EAE disease severity (average clinical score ± SEM) in animals receiving P2pal-18S treatment (open squares, N=6) compared to those treated with vehicle only (black circles, N=6) up to day 15. Data represents the first individual experiment. **B** EAE disease severity (average clinical score ± SEM) in mice receiving P2pal-18S treatment (open squares, N=7) compared to mice treated with vehicle only (black circles, N=7) up to day 15. Data represents the second experiment. **C** EAE disease severity (average clinical score ± SEM) in mice receiving P2pal-18S treatment (open squares, N=11) compared to mice treated with vehicle only (black circles, N=9) up to day 30. Data represents the third experiment. Statistical significance is denoted as follows: * P < 0.05, ** P < 0.01, **** P < 0.0001, determined using the Wilcoxon matched pairs signed rank test.
Supplemental figure 2. Flow cytometry gating strategy. Cells were isolated at day 15 from spinal cord of EAE mice treated or not with PAR2 antagonist. **A)** Total cells were first gated on a side scatter (SSC) vs forward scatter (FSC) to discriminate leukocytes, and then gated on **B)** CD3, and subsequently CD4 or CD8, for T cell populations, or **C)** CD45 and CD11b for macrophages/microglia.
Supplemental figure 3. Serum levels of multiple cytokines and chemokines in EAE mice.

Serum concentrations of the indicated cytokines in EAE mice untreated or treated with the PAR2 inhibitor P2pal-18S were measured at day 15 post EAE induction. Multiple inflammatory and anti-inflammatory mediators were analyzed as shown by the data for each cytokine. Mean ± SEM (error bars) as well as individual sample values (circles) are shown. *, P ≤ 0.05, Student’s t-test.
Supplemental figure 4. Splenocytes and macrophages express functional PAR2. Splenocytes and bone marrow-derived macrophages (BMDM) were obtained from healthy mice as outlined in the methods to investigate the functionality of the PAR2 receptor by measuring Calcium signaling in response to PAR2 agonists. Increases in intracellular calcium levels (E_{530}) were measured in resting and non-polarized cells after activation of PAR2 with the PAR2-activating enzyme trypsin (Trp, 50 U/mL) and the receptor-selective PAR2 activating peptide 2fLI (20 μM) (Panels B and D). A and C) Calcium responses were quantified relative to a positive control calcium response (%I) generated in the same cell suspension by 2.5 μM of the ionophore A23187 (A23187 response being 100%). Bar graphs show the quantification of Calcium responses induced by trypsin and 2fLI (mean ± SEM). B and D) Representative tracings of Calcium signals (fluorescence measured at 530 nm, E_{530}) in B) splenocytes and D) bone marrow-derived macrophages (BMDM). The time scale (minutes) is indicated by the red arrow.
Supplemental figure 5. P2pal-18S is not cytotoxic for T cells. Splenocytes were activated with anti-mouse CD3/CD28 antibodies as described in methods in the presence of P2pal-18S at 3 different concentrations (3, 8 and 17µM), or vehicle control and stained with a live-dead dye after 48 hrs. Non-activated T cells (NAT) and unstained cells were used to calculate the percentage of dead cells. Representative dot plots and histograms show gating strategy to determine live and dead populations. Bar graphs show the average % of dead cells + SEM (Error bars) for N=4. Data were analyzed by One-way ANOVA, Tukey’s multiple comparison test. P<0.001 for non-activated vs. activated cells; no significant difference (ns) between activated cells treated or not with P2pal-18S.
Supplemental figure 6. P2pal-18S reduces T cell production of cytokines and chemokines \textit{in vitro}. Isolated splenocytes (SPL) were cultured with increasing concentrations of P2pal-18S (3, 8 and 17µM: grey shaded bars) or vehicle (DMSO, black and white bars). The cells were either stimulated with 10 µg/mL anti-CD3/CD28 antibodies (black and grey bars) or kept inactivated (white bars). Supernatants were collected and a number of inflammatory and anti-inflammatory factors were measured as shown above each panel. Data are represented as means ± SEM (Error bars) for N=4. *, P ≤ 0.05, ***, P ≤ 0.001, ****, P ≤ 0.0001, one-way ANOVA, Tukey’s multiple comparison test.
Supplemental figure 7: P2pal-18S has no effect on the production of cytokines by LPS-stimulated macrophages. Bone marrow-derived macrophages from wild-type (WT) were pre-treated with P2pal-18S (17µM) or vehicle (DMSO) for 30 minutes prior to activation by LPS (100 ng/mL). Supernatants were harvested after 12h for multiplex cytokines analysis. Data are represented as mean ± SEM (Error bars). Values below detection limit were substituted by lower detection limit/√2. IL-2 and IFN-γ were analyzed but were not detectable. Data were analyzed by One-way ANOVA, Tukey’s multiple comparison test, for N=4-5 pooled data from two experiments performed independently.