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Cathepsin E in neutrophils contributes to the generation of neuropathic pain in experimental autoimmune encephalomyelitis

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Abstract

Pain is a frequent and disabling symptom in patients with multiple sclerosis (MS); however, the underlying mechanisms of MS-related pain are not fully understood. Here, we demonstrated that cathepsin E (CatE) in neutrophils contributes to the generation of mechanical allodynia in experimental autoimmune encephalomyelitis, an animal model of MS. We showed that CatE-deficient (*CatE*$^{-/-}$) mice were highly resistant to myelin oligodendrocyte glycoprotein (MOG_{35-55})-induced mechanical allodynia. After MOG_{35-55} immunization, neutrophils immediately accumulated in the dorsal root ganglion (DRG). Adoptive transfer of MOG_{35-55}-stimulated wild-type neutrophils into the dorsal root ganglion induced mechanical allodynia in the recipient C57BL/6 mice. However, the pain threshold did not change when MOG_{35-55}-stimulated *CatE*$^{-/-}$ neutrophils were transferred into the recipient C57BL/6 mice. MOG_{35-55} stimulation caused CatE-dependent secretion of elastase in neutrophils. Behavioral analyses revealed that sivelestat, a selective neutrophil elastase inhibitor, suppressed mechanical allodynia induced by adoptively transferred MOG_{35-55}-stimulated neutrophils. MOG_{35-55} directly bound to toll-like receptor 4, which led to increased production of CatE in neutrophils. Our findings suggest that inhibition of CatE-dependent elastase production in neutrophil might be a potential therapeutic target for pain in patients with MS.

Keywords: Mechanical allodynia, Cathepsin E, Neutrophil elastase, Experimental autoimmune encephalomyelitis

1. Introduction

Multiple sclerosis (MS) is a progressive inflammatory demyelinating disease of the central nervous system (CNS). Multiple sclerosis manifests as neurological deficits, including motor, cognitive, and neuropsychiatric symptoms. It is now well established that pain is a major concern for patients with MS. In fact, the prevalence of pain in patients with MS has been estimated to be 29% to 86%, depending on the patient population and the survey method used.[24,37] However, currently available analgesics provide insufficient relief from pain due to the complex pathobiology of the disease. The majority of studies on experimental autoimmune encephalomyelitis (EAE), an animal model for MS, indicate that mechanical allodynia often precedes motor dysfunction, which is observed on day 9 to 10 after immunization with immunogenic peptides.[20,38] Notably, neuropathic pain in patients with MS can appear before or exactly at the onset of neurological symptoms.[39] However, the induction mechanisms underlying mechanical allodynia in the preclinical phase of EAE model animals and patients with MS remain unclear.

Immune and glial cells are known crucial factors in neuropathic pain in EAE.[38] However, their activation in the spinal dorsal horn (SDH) was not observed in the preclinical phase of EAE (days 3-5 after immunization).[16,38] In addition, the expression levels of pain-related peptides in laminae I-II of the superficial dorsal horn of mice remained unchanged even in the clinical phase of EAE (on days 10-12 after immunization).[38] Hence, it is possible that other factors contributed to the induction of mechanical allodynia during the preclinical phase of EAE. Neutrophils, the most abundant white blood cells, contribute to the first line of defense in inflammatory response. A recent study uncovered an emerging role for neutrophils in motor dysfunction in EAE mice.[42] Notably, a neutrophil expansion had already occurred in the bone marrow, blood, and spleen during the preclinical phase of EAE (days 3-5 after immunization), and the number of neutrophils increased persistently even after the clinical onset of EAE.[42] However, whether neutrophils are involved in mechanical allodynia during the preclinical phase of EAE remains unclear.

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Cathepsin E (CatE) is an intracellular aspartic protease of the pepsin superfamily localized in the endosomal structures, plasma membrane, endoplasmic reticulum, and Golgi complex.[44,45,51,57,59] Although little is known about its function, we found that *CatE*-deficient mice are resistant to mechanical allodynia in EAE. CatE is exclusively expressed in microglia of the CNS,[34,35] and microglia activation is a critical factor to induce mechanical allodynia.[50] However, activation of microglia was not observed in the SDH during the preclinical phase of EAE.[16,38] We thus hypothesized that CatE has an important role in neutrophils but not in microglia during the preclinical phase of EAE. The goal of this study was to evaluate the role of CatE in neutrophils in the development of mechanical allodynia during the preclinical phase of EAE.

2. Materials and methods

2.1. Animals

Female mice were used for all the experiments because MS is most frequently diagnosed in women.[10] Cathepsin E-deficient ($CatE^{-/-}$) mice[52] were kindly provided by Prof Kenji Yamamoto (Kyushu University). $CatE^{-/-}$ mice and wild-type (WT) littermates (8-12 weeks old) were used for the experiments. C57BL/6 mice (8-12 weeks old) were purchased from CLEA Japan (Tokyo, Japan). All animals were housed at a temperature of 22 ± 1°C with a 12-hour light–dark cycle (light on 8:00-20:00) under specific pathogen-free conditions and fed food and water ad libitum. All animal experiments and care were performed in accordance with the protocols approved by the Institutional Animal Care and Use committee regulations at Kyushu University.

2.2. Induction of experimental autoimmune encephalomyelitis and behavioral testing

Mice were immunized with subcutaneous injection of 50-μL emulsion containing myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}, MEVGWYRSPFSRVVHLYRNGK, 300 μg; GenScript, Tokyo, Japan) and complete Freund's adjuvant (CFA, 300 μg; Difco Laboratories, Detroit, MI) with heat-inactivated *Mycobacterium tuberculosis* H37Ra (300 μg; Becton Dickinson, Sparks Glencoe, MD) in the bilateral inguinal region. Pertussis toxin (PTX, 500 ng; Sigma, St Louis, MO) was injected intraperitoneally at the time of immunization and 2 days after MOG_{35-55} immunization. For the negative control experiments, mice were immunized with CFA/PTX or ovalbumin ($OVA_{323-339}$, non-CNS peptide, 200 μg; Peptide Institute, Inc, Osaka, Japan)/CFA/PTX. All procedures were performed under isoflurane anesthesia (1%-3%) in oxygen. The von Frey test was performed for 12 days after MOG_{35-55} immunization. Details were described below. For neutrophil depletion experiments, anti-Ly6G mAb (clone 1A8, 500 μg, BP0075-1; BioXCell, West Lebanon, NH) or isotype control rat IgG2a (clone 2A3, 500 μg, BP0089; BioXCell) were injected intraperitoneally into C57BL/6 mice[11,55] on days 0 and 3 after MOG_{35-55} immunization. Behavioral testing was performed on days 0 and 5 after MOG_{35-55} immunization. For adoptive transfer experiments, MOG_{35-55}-stimulated neutrophils were injected intravenously or intrathecally into naive C57BL/6 mice. Neutrophils (8.0×10^6 cells) that were stimulated with MOG_{35-55} (25 μg/mL) or $OVA_{323-339}$ (25 μg/mL) for 6 hours were injected into naive C57BL/6 mice through the external jugular vein at a volume of 100 μL in saline. MOG_{35-55}-stimulated monocytes (4.0×10^5 cells) were injected into naive C57BL/6 mice. Needle (30-gauge) was inserted into the vein penetrating through the pectoral muscle. Isolation of neutrophils and monocytes was described below. The intrathecal injection of cells or reagents in naive C57BL/6 mice using 30-gauge needle was performed according to the method described previously.[18,19] MOG_{35-55}-stimulated neutrophils (2.0×10^5 cells/5 μL in saline) were injected into intrathecal space of naive C57BL/6 mice. For the treatment of sivelestat, sivelestat sodium tetrahydrate (Abcam, Cambridge, MA) was injected intraperitoneally (100 mg/kg) or intrathecally (100 ng per mice) into naive C57BL/6 mice 30 minutes before the intravenous transfer of MOG_{35-55}-stimulated neutrophils. Some neutrophils were incubated with VIPER (a specific inhibitor for toll-like receptor 4 [TLR4] that directly interacted with the TLR4 adaptor proteins MyD88 adaptor-like and TRIF-related adaptor molecule, 4 μM; IMGENEX, San Diego, CA)[28] or CP7 (a negative control peptide of VIPER, 4 μM; IMGENEX)[28] 1 hour before MOG_{35-55} stimulation. Recombinant mouse neutrophil elastase protein (rmNE, 1 μg, 5 μL in saline; R&D systems, Minneapolis, MN) was injected intrathecally into naive WT and $CatE^{-/-}$ mice. Intrathecal injection of pepstatin A conjugated with penetratin (PepA-p, 3, 30, 300, or 3000 ng/5 μL in saline; Merck Millipore, Darmstadt, Germany) was performed on days 5 to 7 after MOG_{35-55} immunization. Behavioral testing was performed 3 and 24 hours after PepA-p injection. All mice were habituated to the testing environment for 3 days and were tested for mechanical allodynia. The room temperature remained stable at 22 ± 1°C. The mice were placed in an acrylic cylinder (6 cm diameter) with wire mesh floors and allowed to habituate to the environment for 1 hour. Calibrated von Frey filaments (0.02-2.0 g; North Coast Medical, Inc, Morgan Hill, CA) were applied to the midplantar surface of the hind paw.[18,19] The 50% paw withdrawal thresholds (PWTs) were calculated using the up–down method.[9] For measuring clinical scores, mice were monitored daily according to the severity graded as follows: 0 = normal; 1 = paralyzed tail; 2 = loss in coordinated movement; paralysis of hind limb; 3 = paralysis of both hind limbs; 4 = paralysis of forelimbs; and 5 = moribund. Investigators were blinded to the genotype of mice or treatment.

2.3. Isolation of neutrophils and monocytes

Neutrophils were isolated from the bone marrow of mice with Percoll density gradient centrifugation. Both legs were cut and the muscles were carefully removed after deep anesthesia (somnopentyl, 200 mg/kg, intraperitoneally [i.p.]; Kyoritsu Seiyaku, Tokyo, Japan). Bone marrow cells were then flushed from femurs and tibias with RPMI 1640 (Thermo Fisher Scientific, Waltham, MA) using a 25-gauge needle. Cells were treated with RBC lysis solution (Miltenyi Biotec, Surrey, United Kingdom). Neutrophils were purified over a 62.5% Percoll gradient in Hanks' Balanced Salt Solution with centrifugation for 30 minutes at 1200*g*.[32] Collecting cells were stained with the anti-mouse Ly6G-PE (1: 160; clone: 1A8; BioLegend, San Diego, CA) and anti-mouse CD11b-APC (1: 2500; clone: M1/70; BioLegend) and verified as neutrophil using a FACSVerse (BD Biosciences, San Jose, CA). Gating was based on isotype control using PE Rat IgG2a, κ isotype Ctrl (CatNo: 400507; BioLegend) and APC Rat IgG2b, κ Isotype Ctrl (CatNo: 400611; BioLegend). Neutrophil purity was greater than 95%. For the immunoblot, neutrophils (5×10^5 cells) were stimulated with MOG_{35-55} (25 μg/mL) for 6 hours. In some experiments, neutrophils were treated with LPS-*Rhodobacter sphaeroides* Ultrapure (LPS-RS, 10 μg/mL; InvivoGen, San Diego, CA), an inhibitor for TLR4, before MOG_{35-55} stimulation. Some neutrophils were stimulated with LPS-*Escherichia coli* (100 ng/mL; Sigma) for 6 hours. For qPCR, 1×10^5 cells of neutrophils

were stimulated with MOG_{35-55}. The ratio of neutrophils in the bone marrow was analyzed using FACSVerse and BD FACSuite software. Monocytes were isolated from the bone marrow using EasySep Mouse Monocyte Isolation kit (Stemcell Technologies, Vancouver, Canada), and then stimulated with MOG_{35-55} (25 μg/mL) for 6 hours in Dulbecco's Modified Eagle's medium (DMEM).

2.4. Endotoxin test

Limulus amebocyte lysate endotoxin assay was performed by using ToxinSensor (GenScript) according to the manufacturer instructions. The activity of endotoxin in synthesized MOG_{35-55} and its diluent medium was 0.00751 and 0.00000 endotoxin units per milliliter (EU/mL), respectively. A negligible amount of endotoxin activity does not influence cellular activity and was extremely lower than maximum permissible endotoxin levels for drugs determined by the U.S. Food and Drug Administration.

2.5. Immunohistochemistry

Mice were deeply anesthetized with somnopentyl (200 mg/kg, i.p.) 5 days after MOG_{35-55} immunization and perfused transcardially with 0.1 M phosphate buffer, pH 7.4, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The lumber 5 (L5) spinal cord, L5 dorsal root ganglion (DRG), trigeminal ganglion (TG), and brainstem segments were dissected and further fixed by immersion in 4% PFA overnight at 4°C. Segments were further incubated with 30% sucrose overnight to protect cryolesion. Sections were made at a thickness of 40 μm for spinal cord and brainstem and 10 μm for DRG and TG. Blocking was performed using 1% normal donkey serum (Jackson ImmunoResearch, West Grove, PA), 1% BSA (Sigma), and 0.1% Triton-X (Sigma) in PBS for 1 hour. The specimens were incubated with a primary anti-Iba1 antibody (1:1000; 019-19741; FUJIFILM Wako Pure Chemical Corp, Tokyo, Japan), anti-CD3 antibody (1:500; 555273, BD Biosciences), anti-cathepsin E antibody (1:200; AF1130, R&D), anti-myeloperoxidase antibody (1:200; AF3667; R&D systems), or anti-ELA2 antibody (a marker for neutrophil elastase [NE], 1:200; AF4517; R&D systems) for overnight at 4°C. The specimens were washed 3 times in PBS, and then stained with the secondary antibodies conjugated with Alexa 488 or Cy3 (1:400; Jackson ImmunoResearch), NeuroTrace 435/455 Blue Fluorescent Nissl Stain (1:100; N21479; Thermo Fisher Scientific), or 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; D1306; Thermo Fisher Scientific) for 2 hours at 4°C. The specimens were mounted in the antifading medium Vectashield (Vector Laboratories, Burlingame, CA). Images were captured using a C2si Confocal Laser Microscope (Nikon, Tokyo, Japan).

2.6. Immunoblot

Neutrophils, L4–5 DRG, and HEK293T were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and protease inhibitor cocktail) and mixed with sample buffer. Proteins (5-20 μg) were loaded into each lane and separated by 10% to 12% SDS-PAGE gel. After transfer, the membranes were blocked with TBS-T (0.2% Tween-20 in TBS) containing 5% Blocking One (Nakarai Tesque, Kyoto, Japan) for 1 hour at room temperature, and then incubated with the primary antibodies diluted in TBS-T containing 5% Blocking One for overnight at 4°C. Primary antibodies were used as follows: an anti-cathepsin E antibody (1:2000), anti-myeloperoxidase antibody (1:400), anti-ELA2 antibody (1:2000), anti-DDDDK (flag)-HRP antibody (1:2000; M185-7; Medical & Biological Laboratories, Nagoya, Japan), anti-MD-2 antibody (1: 1000; NB100-56655, Novus Biologicals, Littleton, CO), anti-IκB-α antibody (1:2,000, sc-847; Santa Cruz, Santa Cruz, CA), or anti-β-actin antibody (1:10,000; ab8226, Abcam). After being washed with TBS-T, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody (1:1000; GE Healthcare, Princeton, NJ) for 1 hour at room temperature. The membrane-bound horseradish peroxidase–labelled antibodies were detected using Immobilon ECL Ultra Western HRP Substrate (Merck Millipore) with an image analyzer (LAS-4000; Fuji Photo Film Co, Tokyo, Japan). The bands that were evaluated by apparent molecular size were quantified using the ImageJ software program (http://rsbweb.nih.gov/ij/). The band intensity was normalized to β-actin.

2.7. PCR

The total RNA was extracted from neutrophils, L4–5 DRG, and HEK293T with RNAiso Plus (Takara, Shiga, Japan) according to the manufacturer instructions. The amount of total RNA was determined by measuring the OD260/280 with a NanoPhotometer spectrophotometer (Implen, München, Germany). Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) with 500 ng of extracted RNA. Quantitative RT-PCR of CatE, CatD, and Elane mRNA were performed for 35 to 50 cycles (95°C for 5 seconds, 60°C for 10 seconds) and processed in triplicate with a Corbett Rotor-Gene RG-3000A Real-Time PCR System (Qiagen) using a Rotor-Gene SYBR Green RT-PCR Kit (Qiagen). The data were analyzed using the RG-3000A software program (version Rotor-Gene 6.1.93, Corbett). To quantify the expression levels of target mRNAs, the relative standard curve method was used. All values were normalized to β-actin expression. Semiquantitative PCR of TLR4 and MD-2 mRNA was performed with Phusion polymerase (New England Biolabs, Ipswich, MA) for 35 cycles (98°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds). The PCR products were run on 1% agarose gel to analyze forced expression of mouse TLR4 and MD-2 in HEK293T. The primer sequences were used as follows: *CatE*-forward; 5′-CAGTCCGACACATACACG-3′, *CatE*-reverse; 5′-TGGCCTGGCTCCTTGAC-3′, *CatD*-forward; 5′-CTGAGTGGCTTCATGGGAAT-3′, *CatD*-reverse; 5′-CCTGACAGTGGAGAAGGAGC-3′, *Elane*-forward; 5′-AGCACTGGCCTCAGAGAT TG-3′, *Elane*-reverse; 5′-AGTTCCTGGCAATGAGGGTG-3′, *β-actin*-forward; 5′-AGAGGGAAATCGTGCGTGAC-3′, *β-actin*-reverse; 5′-CAATAGTGATGACCTGGCCGT-3′, *Tlr4*-forward; 5′-GCCTGACACCAGGAAGCTTGA-3′, *Tlr4*-reverse; 5′-TGGACGTGTAAACCAGCCAGG-3′, *Md-2*-forward; 5′-ACCTATTCATCAGTGTCAACTCCA-3′, *Md-2*-reverse; 5′-ACGGCGGTGAATGATGGTGA-3′, *Gapdh*-forward; 5′-AGGTCGGTGTGAACGGATTTG-3′, and *Gapdh*-reverse; 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

2.8. Measurement of CatE enzymatic activity

Whole-cell lysate of neutrophils was made 6 hours after MOG_{35-55} (25 μg/mL) stimulation. Enzymatic activity of CatE in them was measured using a fluorescence-quenching substrate (KYS-1, MOCAc-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys(Dnp)-D-Arg-NH_2; Peptide Institute, Inc). The increase in fluorescence intensity produced by substrate cleavage was measured at an emission wavelength of 393 nm with excitation at 328 nm using a microplate reader Infinite M200 Spectrophotometer (Tekan, Männedorf, Switzerland). The data represent relative value of CatE enzymatic activity that normalized to WT neutrophil (MOG_{35-55} negative).

2.9. Measurement of elastase activity

Elastase activity in the supernatant of neutrophil culture after MOG_{35-55} (25 μg/mL) stimulation for 6 hours and the whole-cell lysate of DRG from MOG_{35-55}-immunized mice were examined using the NE-specific synthetic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (600610; Cayman Chemical, Ann Arbor, MI). PepA-p injection (intrathecally [i.t.]) was conducted on days 5 to 7 after MOG_{35-55} immunization. The L4–5 DRG were collected 3 hours after i.t. injection of PepA-p on day 7 of MOG_{35-55}-immunized mice. Then, we made cell lysate of DRG for measuring elastase activity. Total concentration of protein in each specimen was determined using bicinchoninic acid assay. Ten μg of specimen was incubated in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and 1 mM substrate for 1 hour at 37°C. In some experiments, whole-cell lysate of DRG was made from *CatE*$^{-/-}$ mice 5 days after MOG_{35-55} immunization. Ten μg of specimen was mixed with recombinant mouse cathepsin E protein (rmCatE, 1.25 μg; R&D systems). Pepstatin A (10 μM; sigma), a specific inhibitor of aspartic protease, was treated 1 hour before rmCatE treatment. Reaction was conducted under pH 4.0 for 6 hours at 37°C, and then the samples were incubated with elastase-specific synthetic substrate. Finally, p-nitroaniline was measured using a microplate reader Infinite M200 Spectrophotometer at 405 nm. Relative NE activity was normalized to WT neutrophils (MOG_{35-55} negative) or cell lysate of DRG from saline-treated MOG_{35-55}-immunized mice.

2.10. MOG_{35-55} binding assay

For generating the mMD-2 plasmid, mouse MD-2 coding sequence was amplified from a RIKEN FANTOM clone (2810443A20, accession number AK019283, DNAFORM, Tokyo, Japan) using PrimeStar (Takara), which was replaced with the *Kpn*I-*Apa*I sites of mTLR4 plasmid, provided from Ruslan Medzhitov (#13085; Addgene, Cambridge, MA). Human Embryonic Kidney cells 293T (HEK293T) was harvested on a 13ϕ glass in a 24-well plate and transfected with mTLR4-flag (0.4 μg, Addgene #13087 from Ruslan Medzhitov), mMD-2 (0.4 μg), and Polyethylenimine Max (4 μg, PolySciences, Niles, IL) in Opti-MEM (Thermo Fisher Scientific). The next day, the medium was replaced with DMEM. Cells were treated with FITC (fluorescein isothiocyanate)–conjugated MOG_{35-55} (FITC-MOG_{35-55}, 25 μg/mL; GenScript) for 24 hours. Mouse TLR4 and MD-2 in HEK293T were confirmed using Western blot and semiquantitative PCR analyses described above. FITC fluorescence was captured using C2si Confocal Laser Microscope and FACSVerse. Six independent experiments were performed.

2.11. Statistical analyses

Genotype selection, reagent treatment, behavioral test, and statistical analyses were separately and blindly performed. All data are shown as the mean ± SEM. Data distribution was assumed to be normal but this was not formally tested. We used one-way analysis of variance (ANOVA) and two-way ANOVA to analyze 3 or more levels of 1 factor and 2 factors each with multiple levels, respectively. To compare every mean with every other mean, we used the post hoc Tukey test. The *t* test was used to compare one variable between 2 groups. Two-way ANOVA for repeated measures followed by the Tukey test was used to determine statistical significances in all behavioral experiments. The number of neutrophils in the DRG, CatE enzymatic activity in the neutrophils, relative NE activity, and Elane mRNA were analyzed using two-way ANOVA (genotype and treatment as between factors of variation). Western blot for CatE, the number of CatE-positive cells, and CatE mRNA were analyzed using an unpaired *t* test. Relative NE activity and protein levels of CatE and IκB-α were analyzed using the one-way ANOVA Tukey test. Unless otherwise indicated, the data met the assumptions of equal variances. Differences were considered to be significant for values at $P < 0.05$.

3. Results

3.1. MOG_{35-55} immunization in mice induces accumulation of neutrophils in the dorsal root ganglion

For the preparation of EAE model, we injected MOG_{35-55} (CNS-derived peptide, 300 μg) with CFA (300 μg) and pertussis toxin (PTX, 500 ng) in C57BL/6 mice. Mechanical allodynia was measured by the stimulation with von Frey filaments. The mice exhibited reduction in PWT to mechanical stimulation 4 days after MOG_{35-55} immunization. The decreased PWT lasted for 12 days after MOG_{35-55} immunization (**Fig. 1A**). First significant change in motor dysfunction appeared at day 12 after MOG_{35-55} immunization (two-way ANOVA Tukey test, vs day 0 [WT]: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; vs day 0 [*CatE*$^{-/-}$]: †$P < 0.05$, †††$P < 0.001$; **Fig. 1B**). Mechanical allodynia preceded motor dysfunction in EAE model mice, in concordance with past evidence.[20,38] We found that *CatE*$^{-/-}$ mice were highly resistant to mechanical allodynia caused by MOG_{35-55} immunization compared with WT mice (two-way ANOVA, $F_{(1, 234)}$ genotype = 82.15, $P < 0.0001$; **Fig. 1A**), which indicates the involvement of CatE in mechanical allodynia. During the preclinical phase of EAE, *CatE*$^{-/-}$ mice also did not show any motor dysfunction (**Fig. 1B**). Further experiments were performed at day 5 after EAE induction because we observed a significant difference in mechanical allodynia between WT and *CatE*$^{-/-}$ mice at the preclinical stage of EAE (day 5). The PWT remained unchanged in the CFA/PTX- or ovalbumin ($OVA_{323-339}$, 200 μg; non-CNS peptide)/CFA/PTX-immunized mice, which are negative controls for MOG_{35-55} immunization, during the study period (**Fig. 1C**). T cells and microglia in the SDH play an important role in neuropathic pain in EAE.[38] However, we could not detect T cells in the SDH as well as DRG, TG, and brainstem during the preclinical phase of EAE (day 5 after immunization) (Supplement Fig. 1A, available at http://links.lww.com/PAIN/A791). Immunoreactivity for T cells was observed in the several regions during the clinical phase of EAE (clinical score 3; day 18) (Supplement Fig. 1B, available at http://links.lww.com/PAIN/A791), consistent with the findings reported by Frezel et al.[16] We also could not detect any obvious morphological changes in microglia of the SDH during the preclinical phase of EAE (Supplement Fig. 2, available at http://links.lww.com/PAIN/A791), which was distinct from the characteristic feature of activated microglia during the clinical phase of EAE.[38] We therefore speculated that other factors contributed to the induction of mechanical allodynia during the preclinical phase of EAE. According to the fact that neutrophil expansion occurs during the preclinical phase of EAE (days 3-5 after immunization),[42] neutrophils might be involved in the mechanical allodynia. As we expected, we detected ELA2, a marker for neutrophils, in the DRG after 5 days of MOG_{35-55} immunization, which was merged with CatE (arrowheads in **Fig. 1D**). By contrast, DRG neurons did not express CatE (a broken line in **Fig. 1D**). To further confirm whether $ELA2^{+}$ cells were neutrophils, we conducted myeloperoxidase (MPO; another marker for neutrophils) staining. The $ELA2^{+}$ cells in the DRG were found to be positive for MPO

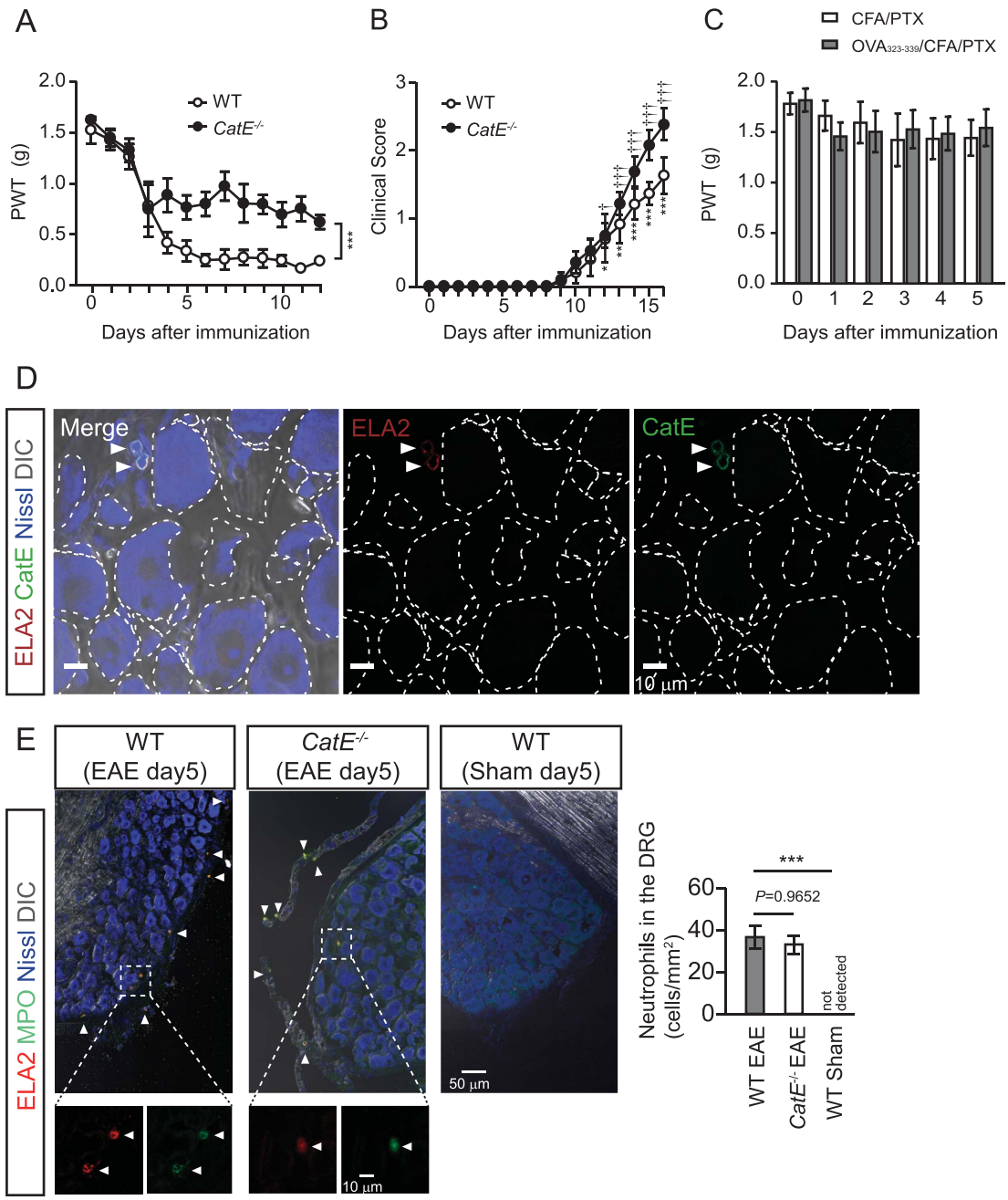

Figure 1. Lack of cathepsin E attenuates mechanical allodynia in EAE. (A and B) The time course of PWT and clinical score in WT and $CatE^{-/-}$ mice after MOG_{35-55}/CFA/PTX immunization. n = 10 mice per group, two-way repeated-measures ANOVA, $F_{(1, 234)}$ genotype = 82.15, $P < 0.0001$ in (A), two-way ANOVA Tukey test, vs day 0 (WT): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; vs day 0 ($CatE^{-/-}$): †$P < 0.05$, †††$P < 0.001$ in (B). (C) The time course of PWT in WT mice after CFA/PTX or $OVA_{323-339}$/CFA/PTX immunization. n = 6 mice per group, two-way ANOVA Tukey's test, vs day 0 (CFA/PTX): day 1: $P > 0.9999$; day 2: $P = 0.9998$; day 3: $P = 0.9534$; day 4: $P = 0.9610$; day 5: $P = 0.9691$; vs day 0 ($OVA_{323-339}$/CFA/PTX): day 1: $P = 0.9517$; day 2: $P = 0.9827$; day 3: $P = 0.9904$; day 4: $P = 0.9719$; day 5: $P = 0.9940$. (D) Images show ELA2 (a marker of neutrophil, red), CatE (green), and Nissl (blue) in the L5 DRG of WT mice on day 5 after MOG_{35-55} immunization. Scale bar = 10 μm. Arrowheads indicate cells positive to ELA2 and CatE. Broken lines indicate DRG neurons.)(E) Images show ELA2 (red), MPO (green), and Nissl (blue) in the DRG of WT or $CatE^{-/-}$ mice on day 5 after MOG_{35-55} immunization. Scale bars = 50 μm and 10 m in upper and lower panels, respectively. Lower images are the enlarged images of inset. The columns represent the density of $ELA2^{+}/MPO^{+}$ cells in the DRG of MOG_{35-55}-immunized mice. n = 6 mice per group, two-way ANOVA Tukey test, $^{***}P < 0.001$. Data represent the mean ± SEM. ANOVA, analysis of variance; CFA, complete Freund's adjuvant; DIC, differential interference contrast; DRG, dorsal root ganglion; EAE, experimental autoimmune encephalomyelitis; PWT, paw withdrawal threshold; WT, wild-type.

(arrowheads in **Fig. 1E**). Accumulation of neutrophils in the DRG 5 days after MOG_{35-55} immunization is consistent with neutrophil expansion during the preclinical phase of EAE.[42] However, $ELA2^{+}$ cells were absent in the spinal cord, trigeminal nucleus caudalis, area postrema, facial motor nucleus of brainstem, pyramidal tract, and TG during the preclinical phase of EAE (Supplement Fig. 3A, available at http://links.lww.com/PAIN/A791). During the clinical phase of EAE (clinical score 3, day 18), a large number of neutrophils was accumulated in these regions, except for TG (Supplement Fig. 3B, available at http://links.lww.com/PAIN/A791). These results suggest that neutrophils are immediately accumulated in the DRG after MOG_{35-55} immunization, and CatE in neutrophils contributes to the induction mechanical allodynia.

3.2. Neutrophils are required for the mechanical allodynia in experimental autoimmune encephalomyelitis

To address the involvement of neutrophils in mechanical allodynia during the preclinical phase of EAE, we conducted antibody-mediated neutrophil depletion in C57BL/6 mice with Ly6G-specific mAb (clone 1A8), which did not influence the number of monocytes and lymphocytes.[11,55] MOG_{35-55} immunization–induced mechanical allodynia was almost completely abolished in neutrophil-depleted mice (two-way ANOVA Tukey test, $^{***}P < 0.001$; **Fig. 2A**). The protein levels of both ELA2 and MPO in the DRG of MOG_{35-55}-immunized mice were also abolished by neutrophil depletion on treatment with Ly6G-specific mAb (unpaired *t* test, $^{***}P < 0.001$; **Fig. 2B**). Moreover, both the CatE protein levels and CatE-positive cells in the DRG of MOG_{35-55}-immunized mice were abolished by Ly6G-specific mAb (unpaired *t* test, $^{***}P < 0.001$; **Figs. 2C and D**). Considering the role of neutrophils in EAE-induced mechanical allodynia, we speculated that the attenuation of mechanical allodynia in MOG_{35-55}-immunized $CatE^{-/-}$ mice may be attributed to the decreased neutrophil count. However, the ratio of $Ly6G^{+}/CD11b^{high}$ cells (neutrophils) in the mice bone marrow remained unchanged by the loss of CatE, as per FACS analysis (unpaired *t* test, $P = 0.4757$; WT mice [n = 5]: 15.64 ± 0.08%, $CatE^{-/-}$ mice [n = 6]: 15.76 ± 0.15%). Based on the above-mentioned observations, we presumed that CatE contributes to the generation of mechanical allodynia through modulation of neutrophil functions.

3.3. CatE in neutrophils is directly induced after MOG_{35-55} stimulation

Considering the short lifespan of neutrophils,[30] it is possible that MOG_{35-55} directly activates them without engaging cells of the adaptive immune response through direct cell–cell contact or soluble mediators. To explore this possibility, we isolated neutrophils from the bone marrow of mice with a >95% purity (**Fig. 3A**), and then stimulated them with MOG_{35-55} (25 μg/mL) for 6 hours. MOG_{35-55}-stimulated neutrophils significantly upregulated CatE, as determined by its protein levels, mRNA expression, and enzymatic activity (unpaired *t* test, $^{**}P < 0.01$, $^{***}P < 0.001$; **Figs. 3B and C**, two-way ANOVA Tukey test, $^{**}P < 0.01$; **Fig. 3D**). In addition, CatE immunoreactivity was observed in MOG_{35-55}-stimulated neutrophils, which clearly merged with ELA2 (**Fig. 3E**), consistent with the data of the DRG in MOG_{35-55}-immunized mice (**Figs. 1D and E**). These results indicate that MOG_{35-55} directly induces CatE in neutrophils.

3.4. CatE is required for the enzymatic activity of elastase in neutrophils after MOG_{35-55} stimulation

To analyze the pathogenicity of MOG_{35-55}-stimulated neutrophils, we intravenously transferred them into naive C57BL/6 mice. A single intravenous injection of MOG_{35-55}-stimulated WT neutrophils (8.0×10^6 cells) induced mechanical allodynia in naive C57BL/6 mice (two-way ANOVA Tukey test, vs 0 hours: $^{***}P < 0.001$; **Fig. 4A**). Monocytes that are also expanded in the bone marrow and blood during the preclinical phase of EAE[42] were possibly involved in mechanical allodynia. However, MOG_{35-55}-stimulated monocytes (4.0×10^5 cells) did not elicit mechanical allodynia (**Fig. 4A**), suggesting that monocytes are not involved in the generation of mechanical allodynia. We further analyzed whether neutrophils were specifically activated by MOG_{35-55}. Distinct from MOG_{35-55} (CNS-derived peptide) stimulation, $OVA_{323-339}$ (non-CNS peptide)-stimulated neutrophils did not induce mechanical allodynia (**Fig. 4A**). These data indicate that MOG_{35-55}-specific neutrophil activation has occurred in EAE model mice. To analyze the role of CatE in neutrophils in mechanical allodynia, we isolated neutrophils from the bone marrow of $CatE^{-/-}$ mice. We found that MOG_{35-55}-stimulated $CatE^{-/-}$ neutrophils did not elicit mechanical allodynia in naive C57BL/6 mice (two-way ANOVA Tukey test, MOG_{35-55}-stimulated WT neutrophils vs MOG_{35-55}-stimulated $CatE^{-/-}$ neutrophils: $\dagger\dagger\dagger P < 0.001$; **Fig. 4A**). MOG_{35-55}-stimulated neutrophils were further transferred into the intrathecal space of naive C57BL/6 mice to assess region-specific roles of neutrophils in mechanical allodynia. As seen in the intravenous-transfer experiments, MOG_{35-55}-stimulated WT (2.0×10^5 cells/5 μL saline), and not the $CatE^{-/-}$ neutrophils, rapidly triggered mechanical allodynia, and lasted for 12 hours (two-way ANOVA Tukey test, vs 0 hours: $^{***}P < 0.001$; WT neutrophils [MOG_{35-55}] vs $CatE^{-/-}$ neutrophils [MOG_{35-55}]: $\dagger\dagger\dagger P < 0.001$; **Fig. 4B**). These data support the accumulation of neutrophils in DRG when mice exhibited mechanical allodynia after MOG_{35-55} immunization (**Figs. 1D and E**).

Increasing evidence supports the claim that elastase induces pain.[1,53] We predicted that MOG_{35-55}-stimulated neutrophils might release NE because neutrophils are one of the main sources of elastase. Therefore, we used sivelestat, a specific NE inhibitor. Systemic (10 mg/kg) or intrathecal (100 ng/5 μL saline) injections of sivelestat in naive C57BL/6 mice were administered 30 minutes before adoptive transfer of MOG_{35-55}-stimulated neutrophils, based on previous reports.[1,53] Sivelestat significantly abolished mechanical allodynia induced by adoptive transfer of MOG_{35-55}-stimulated neutrophils (two-way ANOVA Tukey test, Saline [i.p.] vs Sivelestat [10 mg/kg, i.p.]: $\dagger\dagger\dagger P < 0.001$; Saline [i.t.] vs Sivelestat [100 ng, i.t.]: $\S P < 0.05$, $\S\S P < 0.01$, $\S\S\S P < 0.001$; **Fig. 4C**). The dose of sivelestat (i.p.) used in our study is half the effective concentration reported by Vicuna et al.[53] Sivelestat-treated mice did not show a significant reduction in PWT after the adoptive transfer of neutrophils during the study period (two-way ANOVA Tukey test, vs 0 hours [i.p.], 2 hours [i.p.]: $P = 0.6131$; 4 hours [i.p.]: $P > 0.9999$; 6 hours [i.p.]: $P = 0.9827$, vs 0 hours [i.t.], 2 hours [i.t.]: $P = 0.999$; 4 hours [i.t.]: $P = 0.9766$; 6 hours [i.t.]: $P = 0.943$; **Fig. 4C**). These observations suggest that NE released from neutrophil sensitizes DRG neurons.

Next, to test whether neutrophils release NE in response to MOG_{35-55}, NE activity was measured using the supernatant of MOG_{35-55}-stimulated neutrophils. MOG_{35-55} dramatically increased NE activity in WT neutrophils, which was dependent on CatE (two-way ANOVA Tukey test, $^{***}P < 0.001$; **Fig. 4D**). The functional link between CatE and NE activities was further analyzed. Considering above data, NE remains unprocessed as an immature form in $CatE^{-/-}$ neutrophils after MOG_{35-55} stimulation. We therefore collected the DRG from MOG_{35-55}-immunized $CatE^{-/-}$ mice that showed neutrophil accumulation (**Fig. 1E**). Whole-cell lysate from the DRG of MOG_{35-55}-immunized $CatE^{-/-}$ mice were treated with recombinant mouse CatE (rmCatE), and then the enzymatic activity of NE was measured. The enzymatic activity of NE in the DRG of MOG_{35-55}-immunized $CatE^{-/-}$ mice was facilitated by the treatment of rmCatE (1.25 μg) for 1 hour at 37°C, which was abrogated in the presence of pepstatin A (PepA, 10 μM), an inhibitor for aspartic protease (one-way ANOVA Tukey test, $^{*}P < 0.05$, $^{**}P < 0.01$; **Fig. 4E**). We further analyzed the involvement of CatE in the synthesis of NE. The increased expression levels of Elane (neutrophil elastase) mRNA in neutrophils after MOG_{35-55} stimulation were not altered by *CatE* deficiency (two-way ANOVA Tukey test, vs MOG_{35-55} negative: $^{*}P < 0.05$; WT MOG_{35-55} vs

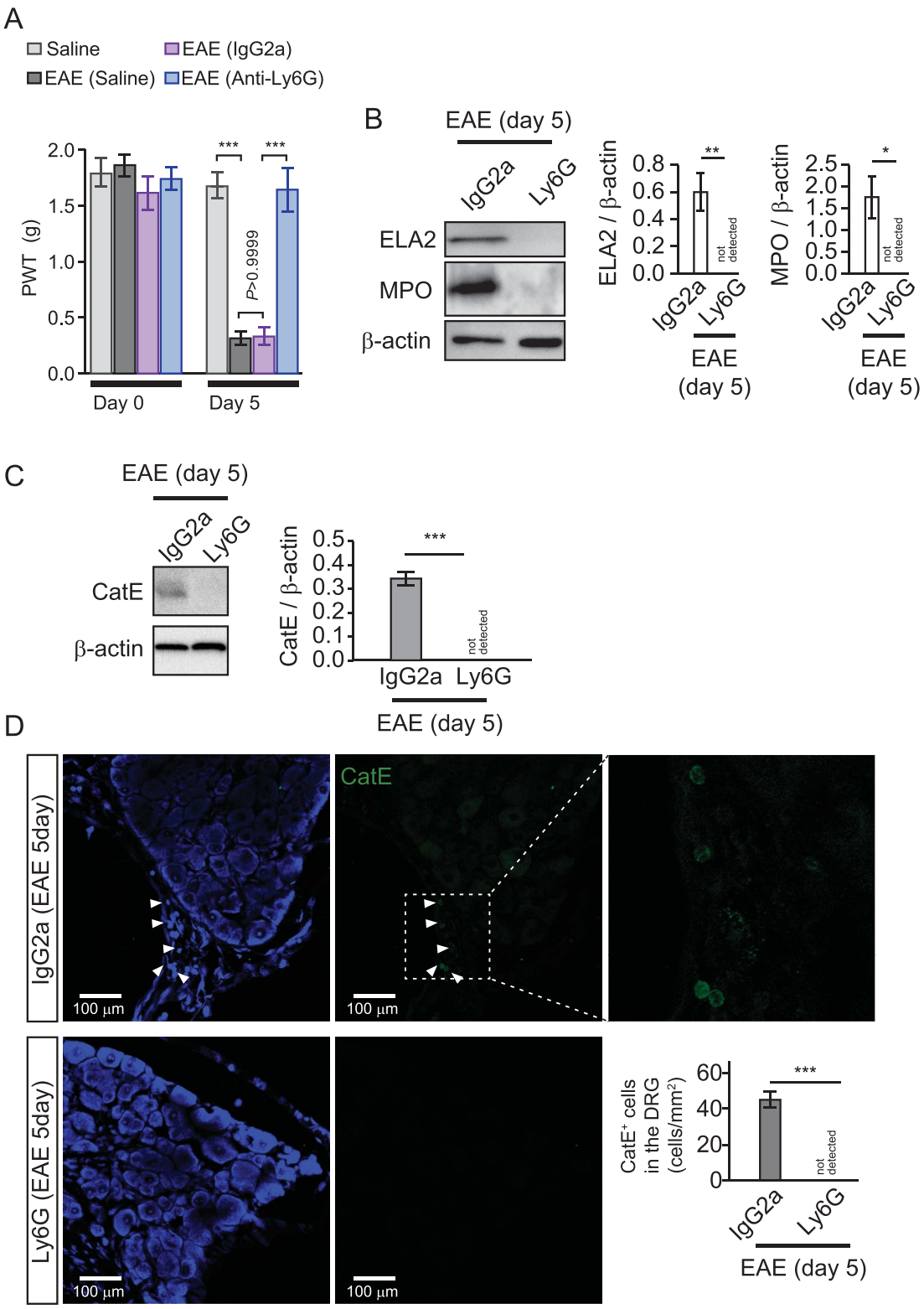

Figure 2. Neutrophil depletion attenuates mechanical allodynia and accumulation of CatE-positive cells in the DRG during the preclinical phase of EAE. (A) The PWT in MOG_{35-55}-immunized WT mice that were treated with Ly6G-specific antibody (500 μg, i.p.) or isotype control IgG2a (500 μg, i.p.). n = 6 mice per group, two-way ANOVA Tukey test, $^{***}P < 0.001$. (B) Immunoblot for ELA2 and MPO in the L5 DRG of neutrophil-depleted mice on day 5 after MOG_{35-55} immunization. The columns represent the relative values of ELA2 or MPO proteins normalized to β-actin. n = 3 mice per group, unpaired *t* test, $^{***}P < 0.001$. (C) Immunoblot for CatE in the L5 DRG of neutrophil-depleted mice on day 5 after MOG_{35-55} immunization. The columns represent the relative values of CatE protein normalized to β-actin. n = 3 mice per group, unpaired *t* test, $^{***}P < 0.001$. (D) Images show CatE (green) and Nissl (blue) in the L5 DRG of neutrophil-depleted mice on day 5 after MOG_{35-55} immunization. Arrowheads indicate CatE-positive cells. Bottom image indicates the enlarged image of inset. Scale bars = 100 μm and 10 μm. The columns represent the density of CatE-positive cells in the L5 DRG. n = 5 mice per group, unpaired *t* test, $^{***}P < 0.001$. Data represent the mean ± SEM. ANOVA, analysis of variance; DRG, dorsal root ganglion; EAE, experimental autoimmune encephalomyelitis; PWT, paw withdrawal threshold; WT, wild-type.

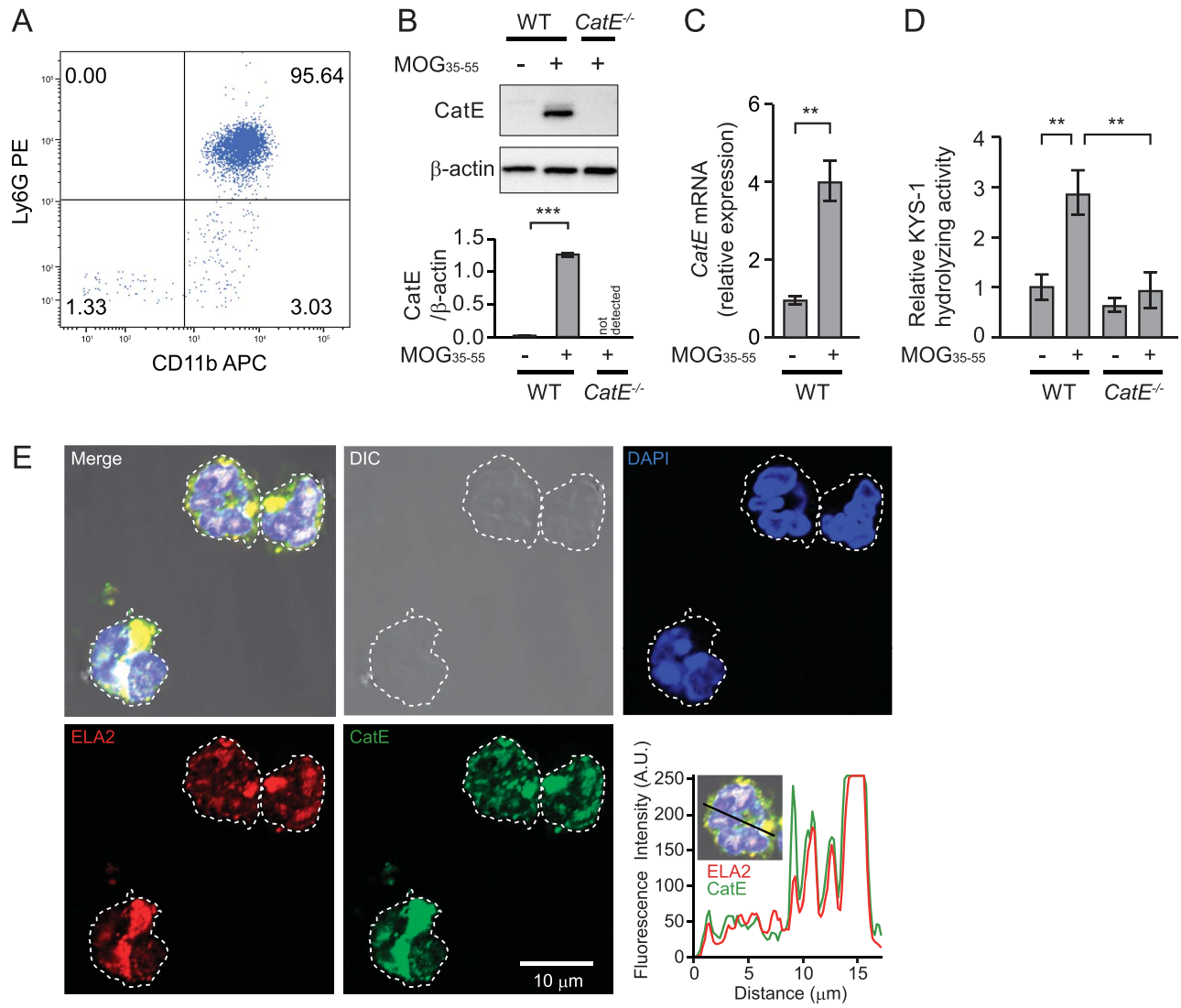

Figure 3. MOG_{35-55} induces CatE in neutrophils. (A) Representative dot plot for flow cytometry of $Ly6G^+/CD11b^+$ cells in isolated neutrophils from the bone marrow of WT mice. Gating is based on isotype control for Ly6G and CD11b. (B) Immunoblot for CatE in neutrophils after stimulation with MOG_{35-55} (25 μg/mL) for 6 hours. The band for CatE is absent in $CatE^{-/-}$ neutrophils. The columns represent the relative values of CatE protein normalized to β-actin. n = 3 mice per group, unpaired *t* test, $^{***}P < 0.001$. (C) Quantitative PCR analyses of CatE mRNA in MOG_{35-55}-stimulated neutrophils. The columns represent the relative values of CatE mRNA normalized to β-actin. n = 3 mice per group, unpaired *t* test, $^{***}P < 0.001$. (D) Enzymatic activity for CatE in cell lysate of MOG_{35-55}-stimulated neutrophils. n = 3 mice per group, two-way ANOVA Tukey test, $^{**}P < 0.01$. (E) Images show CatE (green), ELA2 (red), and Nissl (blue) in MOG_{35-55}-stimulated neutrophils. Broken lines indicate outline of neutrophils. Scale bars = 10 μm. The profile plots fluorescence intensity (values from 0 to 255) of CatE and ELA2 fluorescence at the position along the black line in the image that was cropped from merged image. Data represent the mean ± SEM. ANOVA, analysis of variance; WT, wild-type.

$CatE^{-/-}$ MOG_{35-55}: $P = 0.6437$; **Fig. 4F**). These results indicate that CatE is involved in NE processing and secretion but not its synthesis. Note that intrathecal injection of NE elicited mechanical allodynia in both naive WT and $CatE^{-/-}$ mice (two-way ANOVA Tukey test, vs 0 hours: $^{***}P < 0.001$, $\dagger\dagger\dagger P < 0.001$; **Fig. 4G**), which suggests that mechanical allodynia response is unchanged by *CatE* deficiency.

It is still unclear whether accumulated neutrophils in the DRG are sufficient to induce mechanical allodynia because neutrophils are also reported to be circulating in the blood during EAE.[1] To address this, we tried to inhibit the CatE-dependent NE activity of accumulated neutrophils in the DRG of MOG_{35-55}-immunized mice by intrathecal injection of PepA-conjugated penetratin (PepA-p), a cell-penetrating peptide. It is well known that PepA can inhibit enzymatic activity of both CatE and cathepsin D (CatD). We therefore analyzed the involvement of CatD in NE release from neutrophils. The expression levels of CatD mRNA in neutrophils were not changed by MOG_{35-55} stimulation (Supplement Fig. 4, available at http://links.lww.com/PAIN/A791). Based on these data, we ruled out the possible involvement of CatD on NE release from neutrophils. We administered 4 dose of PepA-p in MOG_{35-55}-immunized mice. Low concentrations of PepA-p (3 and 30 ng, i.t.) had minimal or no recovery from EAE-induced mechanical allodynia 3 hours after intrathecal injection (two-way ANOVA Tukey test, vs day 5 pre: $^{*}P < 0.05$; **Fig. 4H**). However, a higher concentration of PepA-p (300 and 3000 ng, i.t.) showed significant recovery from EAE-induced mechanical allodynia (two-way ANOVA Tukey's test, vs day 5 pre, $\dagger P < 0.05$, $\dagger\dagger\dagger P < 0.001$, $\#\#\#P < 0.001$; **Fig. 4H**). We also found that the effects of PepA-p (300 and 3000 ng) persisted 24 hours after intrathecal injection (day 7 in **Fig. 4H**). High dose of PepA-p (3000 ng) exhibited more strong analgesic efficacy than 300 ng of PepA-p (two-way ANOVA Tukey test, $\S P < 0.05$). We further examined whether enzymatic activity of NE in the DRG of

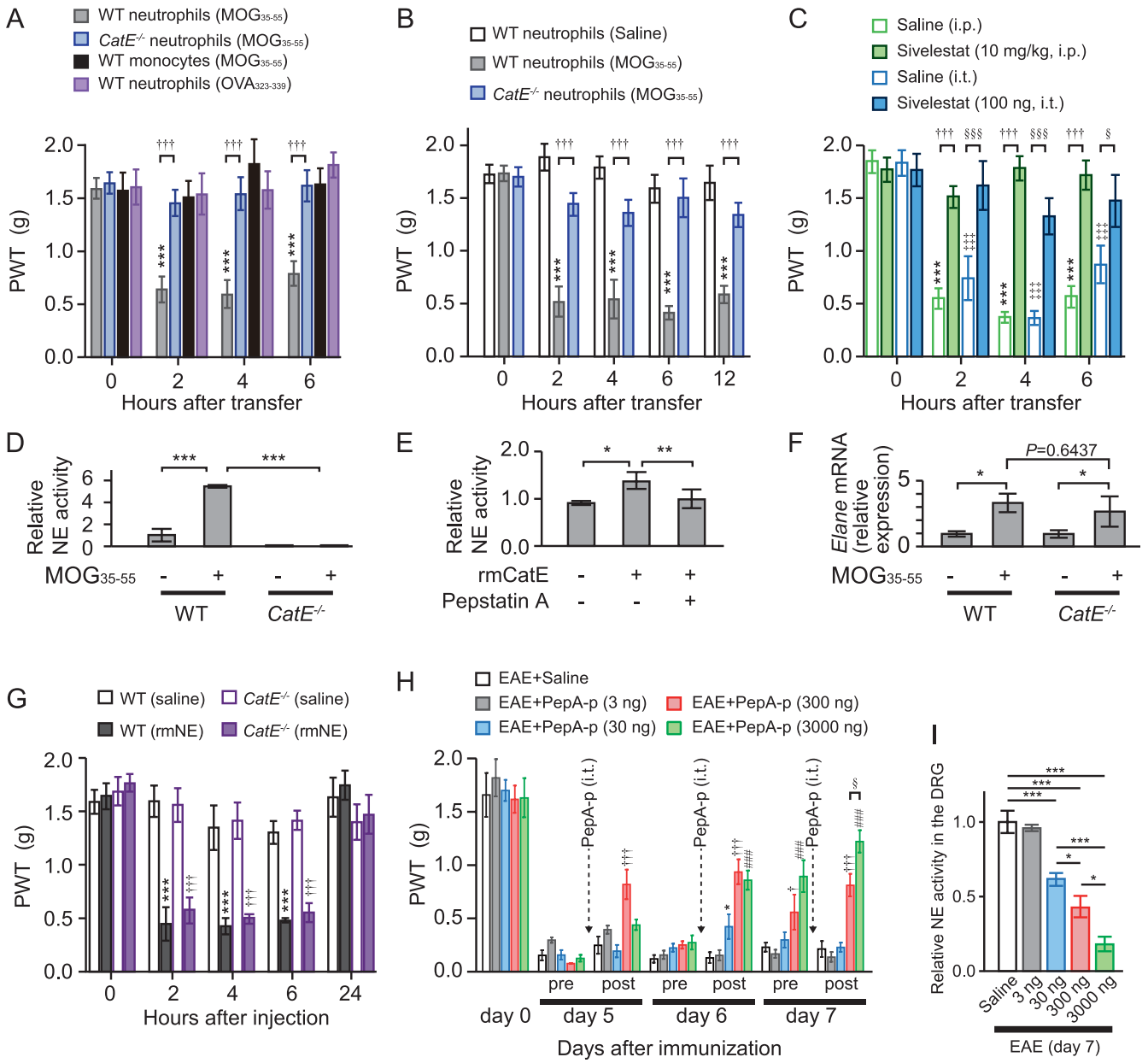

Figure 4. CatE-dependent production of elastase in neutrophils contributes to the induction of mechanical allodynia during the preclinical phase of EAE. (A) The PWT of naive C57BL/6 mice after intravenous injection of MOG_{35-55} (25 μg/mL)-stimulated WT neutrophils (n = 10), MOG_{35-55}-stimulated *CatE*$^{-/-}$ neutrophils (n = 10), MOG_{35-55}-stimulated WT monocytes (n = 6), or $OVA_{323-339}$ (25 μg/mL)-stimulated WT neutrophils (n = 6). Two-way ANOVA Tukey test, vs 0 hours: ***$P < 0.001$, †$P < 0.05$; WT neutrophils (MOG_{35-55}) vs *CatE*$^{-/-}$ neutrophils (MOG_{35-55}): ††$P < 0.01$, †††$P < 0.001$; two-way ANOVA Tukey test, vs 0 hours (MOG_{35-55}-stimulated WT monocytes): 2 hours: $P > 0.9999$; 4 hours: $P = 0.9986$; 6 hours: $P > 0.9999$; vs 0 hours ($OVA_{323-339}$-stimulated WT neutrophils): 2 hours and 4 hours: $P > 0.999$; 6 hours: $P = 0.9998$. (B) The PWT of naive C57BL/6 mice after intrathecal injection of saline- or MOG_{35-55}-stimulated WT neutrophils and MOG_{35-55}-*CatE*$^{-/-}$ neutrophils. n = 10 mice per group, two-way ANOVA Tukey test, vs 0 hours: ***$P < 0.001$, WT neutrophils (MOG_{35-55}) vs *CatE*$^{-/-}$ neutrophils (MOG_{35-55}): †††$P < 0.001$. (C) Systemic (100 mg/kg, n = 10) or intrathecal injection (100 ng, n = 6) of sivelestat attenuated mechanical allodynia caused by intravenous injection of MOG_{35-55}-stimulated neutrophils. Two-way ANOVA Tukey test, vs saline (i.p.), 0 hours: ***$P < 0.001$, saline (i.t.), 0 hours: ‡‡‡$P < 0.001$; saline (i.p.) vs sivelestat (i.p.): †††$P < 0.001$; saline (i.t.) vs sivelestat (i.t.): §$P < 0.05$, §§$P < 0.01$, §§§$P < 0.001$. (D) Neutrophil elastase (NE) activity in the supernatant of WT and *CatE*$^{-/-}$ neutrophils 6 hours after MOG_{35-55} (25 μg/mL) stimulation. n = 3 mice per group, two-way ANOVA Tukey test, ***$P < 0.001$. (E) Whole-cell lysate of DRG from MOG_{35-55}-immunized *CatE*$^{-/-}$ mice was incubated with recombinant mouse CatE (rmCatE, 1.25 μg) for 6 hours with or without pepstatin A (10 μM), a specific inhibitor of aspartic protease. n = 4 mice per group, one-way ANOVA Tukey test, *$P < 0.05$, **$P < 0.01$. (F) Quantitative PCR analyses of Elane mRNA in MOG_{35-55}-stimulated neutrophils. The columns represent the relative values of Elane mRNA normalized to β-actin. n = 3 mice per group, two-way ANOVA Tukey test, *$P < 0.05$. (G) The PWT of naive WT or *CatE*$^{-/-}$ mice after intrathecal injection of recombinant mouse neutrophil elastase protein (rmNE, 1 μg). n = 10 mice per group, two-way ANOVA Tukey test, vs 0 hours (WT, rmNE): ***$P < 0.001$; vs 0 hours (*CatE*$^{-/-}$, rmNE): †††$P < 0.001$. (H) The PWT of MOG_{35-55}-immunized mice 3 and 24 hours after intrathecal injection of pepstatin A-penetratin (PepA-p). n = 6 mice per group, two-way ANOVA Tukey test, vs day 5 pre: *$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, ### $P < 0.001$; PepA-p (300 ng) vs PepA-p (3000 ng): §$P < 0.05$. (I) Neutrophil elastase activity in the DRG of MOG_{35-55}-immunized mice. The DRG was collected from PepA-p-treated mice after behavioral analyses on day 7. n = 6 mice per group, one-way ANOVA Tukey test, *$P < 0.05$, ***$P < 0.001$. Data represent the mean ± SEM. ANOVA, analysis of variance; DRG, dorsal root ganglion; EAE, experimental autoimmune encephalomyelitis; PWT, paw withdrawal threshold; WT, wild-type.

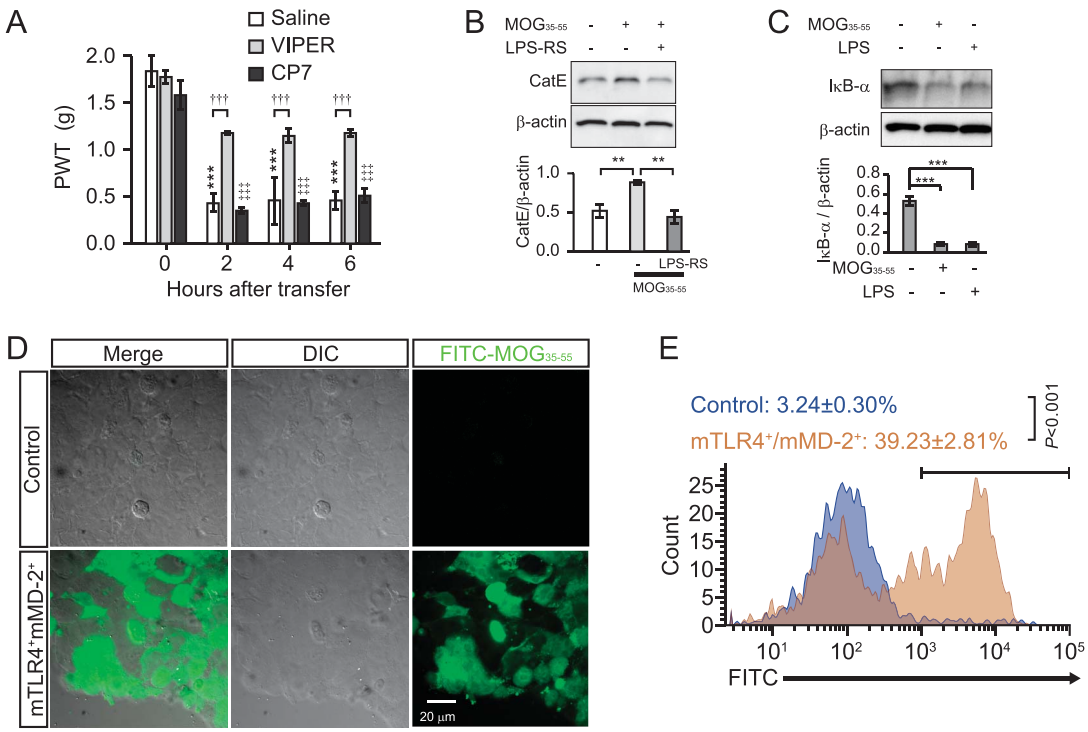

Figure 5. MOG_{35-55} binds and activates TLR4 on neutrophils. (A) The time course of PWT in recipient mice that were intravenously transferred neutrophils stimulated with MOG_{35-55} in the presence of VIPER (an inhibitor for TLR4, 4 μM) or CP7 (a negative control of VIPER, 4 μM). n = 10 mice per group, two-way ANOVA Tukey test, vs 0 hours: $^{***}P < 0.001$, ‡‡‡$P < 0.001$; Saline vs VIPER: †††$P < 0.001$. (B) The immunoblot for CatE in MOG_{35-55}-stimulated WT neutrophils with or without LPS-RS (10 μg/mL). The columns represent the relative values of CatE protein normalized to β-actin. n = 3 mice per group, one-way ANOVA Tukey test. $^{*}P < 0.05$, $^{**}P < 0.01$. (C) Degradation of IκB-α in neutrophils was observed 6 hours after stimulation with MOG_{35-55} (25 μg/mL). LPS *Escherichia coli* (100 ng/mL) was used as the positive control for TLR4 ligand. The columns represent the relative expression of IκB-α normalized to β-actin. n = 3 mice per group, one-way ANOVA Tukey test, $^{***}P < 0.001$. (D) Mouse TLR4 and MD-2 expressing HEK293T was treated with FITC-conjugated MOG_{35-55} (25 μg/mL) for 6 hours. Images show DIC and FITC fluorescence (green). DIC: differential interference contrast; FITC: fluorescein isothiocyanate. Scale bar = 20 μm. (E) Quantitative analyses of FITC-positive HEK293T by flow cytometry analyses. Gating is based on control HEK293T. n = 6 independent experiments, unpaired *t* test, vs control: $^{***}P < 0.001$. Data represent the mean ± SEM. ANOVA, analysis of variance; PWT, paw withdrawal threshold; WT, wild-type.

MOG_{35-55}-immunized mice is really inhibited by intrathecal injection of PepA-p. The DRG was collected 3 hours after PepA-p injection in MOG_{35-55}-immunized mice on day 7. PepA-p showed dose-dependent inhibition of NE activity in the DRG of MOG_{35-55}-immunized mice (**Fig. 4I**). These results indicate that CatE is required for NE enzymatic activity, and neutrophil accumulation in the DRG is sufficient to induce mechanical allodynia.

3.5. MOG_{35-55}-induced CatE upregulation in neutrophils is mediated through toll-like receptor 4

Finally, we addressed how neutrophils were directly activated by MOG_{35-55}. Sivelestat attenuates lipopolysaccharide (LPS)-induced inflammation,[56] which implies that TLR4 pathway may get activated by MOG_{35-55} in neutrophils. To investigate whether MOG_{35-55}-induced events in neutrophils were mediated through TLR4, we treated neutrophils with VIPER (4 μM, a viral inhibitory peptide of TLR4) 1 hour before MOG_{35-55} stimulation. Subsequently, we transferred them into naive C57BL/6 mice. MOG_{35-55}-stimulated neutrophils whose TLR4 were pharmacologically blocked by VIPER did not elicit mechanical allodynia in naive C57BL/6 mice, whereas CP7 (4 μM, a negative control peptide of VIPER) showed no effect on neutrophil-induced mechanical allodynia (two-way ANOVA Tukey test, vs 0 hours: $^{***}P < 0.001$, ‡‡‡$P < 0.001$; control vs VIPER: †††$P < 0.001$; **Fig. 5A**). Further analyses revealed that MOG_{35-55}-driven CatE production in neutrophils was significantly inhibited by LPS-*Rhodobacter sphaeroides* Ultrapure (10 μg/mL), an antagonist for TLR4 by competitive binding to the TLR4/myeloid differentiation factor 2 (MD-2) complex (one-way ANOVA Tukey test, $^{**}P < 0.01$; **Fig. 5B**). These data implicated a direct binding of MOG_{35-55} on TLR4. To confirm whether functional TLR4 signaling in neutrophils occurred after MOG_{35-55} stimulation, we analyzed the degradation of inhibitor kappa B-α (IκB-α). As seen with the stimulation by LPS-*Escherichia coli* (100 ng/mL), a TLR4 ligand, MOG_{35-55} (25 μg/mL) stimulation for 6 hours caused the reduction of protein levels of IκB-α in neutrophils (one-way ANOVA Tukey test, $^{***}P < 0.001$; **Fig. 5C**). The agonistic activity of TLR4 by MOG_{35-55} may be attributed to the endotoxin contamination in peptide; however, a negligible amount of endotoxin activity was detected in the synthesized MOG_{35-55} (0.00751 EU/mL) and its diluent medium (0 EU/mL), by a limulus amebocyte lysate endotoxin assay. To visualize direct binding between MOG_{35-55} and TLR4/MD-2, HEK293T expressing both mouse TLR4 and MD-2 were treated with FITC-conjugated MOG_{35-55}. (Supplement Fig. 5, available at http://links.lww.com/PAIN/A791). FITC fluorescence was detected in $mTLR4^+$/$mMD\text{-}2^+$ HEK293T but not in the nontransfected HEK293T (**Fig. 5D**). We further quantified $FITC^+$ HEK293T using flow cytometry analyses. The average fraction of $FITC^+$ HEK293T in total cells significantly increased after the cotransfection of mTLR4 and mMD-2 plasmids (control: 3.24 ± 0.30%; $mTLR4^+$/$mMD\text{-}2^+$: 39.23 ± 2.81%; unpaired *t* test, $^{***}P < 0.001$; **Fig. 5E**). Not all HEK293T were positive for FITC fluorescence due to transfection efficacy of plasmids. These results suggest that MOG_{35-55} acts as a ligand for TLR4/MD-2.

4. Discussion

We initially hypothesized that CatE in microglia might be involved in induction of mechanical allodynia during the preclinical phase of EAE, based on the facts that CatE is exclusively expressed in microglia of the CNS,[34,35] and microglia activation is a critical factor to induce mechanical allodynia.[50] Contrary to our expectations, we observed that *CatE*$^{-/-}$ mice were resistant to mechanical allodynia after MOG_{35-55} immunization without affecting microglial activation (Supplement Fig. 2, http://links.lww.com/PAIN/A791). In this study, we observed that mechanical allodynia during the preclinical phase of EAE in mice is induced by elastase, which is released from accumulated neutrophils in the DRG. Furthermore, elastase processing in neutrophils is dependent on enzymatic activity of CatE. Adoptively transferred neutrophil-induced mechanical allodynia is significantly attenuated by sivelestat. We thus suggest that a neuroimmune communication sustained by neutrophil activity is dependent on CatE. Moreover, we found that MOG_{35-55}, a classically known CNS peptide, acts as a ligand for the TLR4/MD-2 complex. To date, known endogenous ligands for TLR4 include heme,[4] fetuin-A,[40] galectin-3,[6] and high-mobility group box 1 (see review Ref. 58). Thus, the MOG_{35-55}-mediated TLR4 pathway is a novel finding.

Neuropathic pain in MS can appear before or exactly at the onset of neurological deficits.[39] This pain symptom is similar to the observation that allodynia precedes neurological dysfunction in EAE animals. Thus, the preclinical phase of EAE is a good time frame to identify the mechanisms of mechanical allodynia. In the current study, we identified the pathogenic role of neutrophils in mechanical allodynia. However, severe motor impairment impedes pain behaviors during the chronic phase of EAE, confounding the involvement of neutrophils in chronic pain. Because persistent and incremental expansion of neutrophils occurs in the bone marrow, blood, and spleen after EAE induction,[42] neutrophils may contribute to the chronic phase of pain. Although granulocytes are relatively rare in mature MS lesions, expansion of neutrophils and increased amount of NE were detected in the serum of patients with MS.[33,42] We have shown that mechanical allodynia is mitigated by the inhibition of NE from accumulated neutrophils in the DRG, and completely attenuated by sivelestat. Thus, not only accumulated neutrophils in the DRG but also circulating neutrophils release NE, which elicits mechanical allodynia through activation of protease-activated receptor 2 in DRG neurons.[61] We propose that sivelestat may be an effective drug for management of MS-related pain. Patients with MS experience a wide range of neuropathic pain symptoms, including ongoing dysesthetic pain and paroxysmal pain.[24,37] In future, we need to assess the type of pain in which neutrophils were specifically involved.

Loss of CNS inhibitory controls is believed to be responsible for neuropathic pain in MS. Infiltration of T cells is observed in several brain regions, which is considered as one of the causes for loss of CNS inhibitory controls. We observed infiltration of neutrophils in the brain and spinal cord during the clinical phase of EAE. However, neutrophils and T cells were never detected in the brain and spinal cord during the preclinical phase of EAE. This implied that the loss of CNS inhibitory control is not involved in mechanical allodynia during the preclinical phase of EAE. Surprisingly, segmental differences in neutrophil accumulation between the DRG and TG were observed. We expected to observe neutrophil accumulation in the TG because it lacks the blood–brain barrier.[13] However, we could not observe neutrophil accumulation in the TG even in the clinical phase of EAE (Supplement Fig. 3, http://links.lww.com/PAIN/A791). RNA sequencing studies have revealed differential molecular profiles between the DRG and TG in both mice and humans.[15,27] Distinct release probability of peptides from soma might contribute to the different accumulation patterns of neutrophils between the DRG and TG. It has been reported that P/Q-, N-, and L-type Ca^{2+} channels are involved in the release of calcitonin gene-related peptide from the soma of TG neurons,[54] but not DRG neurons.[14] Sensory neuron–derived calcitonin gene-related peptide is capable of suppressing neutrophil migration.[2,41] Hence, we inferred that neutrophil accumulation was not observed in the TG of MOG_{35-55}-immunized mice.

Trigeminal neuralgia is one of the most common neuropathic pains in patients with MS.[24] However, the DRG was chosen in the current study for the ease of reagent treatment and cell injection. There is disagreement on the mechanical allodynia initiation time window in facial region during the preclinical phase of EAE.[12,47] Hence, appropriate methods are needed for analyzing trigeminal pain. Given the distinct accumulation profile of neutrophils between the DRG and TG, the neutrophil/CatE/elastase pathway may be restricted in the DRG. However, we can infer the possible involvement of NE from circulating neutrophils in trigeminal pain because of the increased serum levels of neutrophils and NE in the patients with MS.[33,42] As previously mentioned, circulating NE also has an important role in the induction of mechanical allodynia, possibly leading to activation of the TG neurons in patients with MS.

The effectiveness of sivelestat is demonstrated using animal models, including spared nerve injury model of neuropathic pain, cancer-induced bone pain, and diabetic neuropathic pain, by inhibiting both T-cell- and neutrophil-derived elastase.[1,53] Although distinct from the neuropathic pain models, sivelestat completely attenuated mechanical allodynia in our model, at doses similar (100 ng, i.t. injection) or lower (10 mg/kg, i.p. injection) to that from the previous reports.[1,53] Considering the involvement of microglia, macrophages, T cells, and neutrophils in neuropathic pain caused by nerve injury, cancer, and diabetes, sivelestat may not fully attenuate mechanical allodynia in these models.

Although we investigated the role of neutrophils in EAE-induced mechanical allodynia, further studies are necessary to determine the mechanisms underlying neutrophil migration into the DRG after MOG_{35-55} immunization. For instance, Rumble et al.[42] reported that CXC motif chemokine ligand 1 (CXCL1) was elevated immediately after MOG_{35-55} immunization in various tissues, including the spleen, liver, lungs, and spinal cord, which might evoke neutrophil mobilization. Considering that MOG_{35-55} is a ligand for TLR4, a TLR4-CXCL1 pathway[17,48] may contribute to the upregulation of CXCL1 in numerous tissues. In fact, accumulating evidence indicates the existence of TLR4 in both rodent and human DRG neurons.[26,31,49] Therefore, induction of CXCL1 in DRG neurons through TLR4 after MOG_{35-55} immunization may trigger neutrophil recruitment to the DRG. Similar neuroimmune crosstalk may occur in chronic pain in sepsis[3] and sickle cell disease[60] because LPS is a ligand of TLR4 and heme is a derivative of hemoglobin after hemolysis,[4] which respectively take part in these conditions. We believe that our results will help to comprehend the induction mechanism of neuropathic pain. Another inducer of CXCL1 is interleukin-17A from T helper 17 cells.[21] Notably, interleukin-17A in the DRG and SDH plays an important role in nociception in the neuropathic pain model[25,46] and EAE model,[20] respectively. Based on these observations, cumulatively, we cannot eliminate the possible involvement of circulating effector cells in EAE-induced pain.

CatE is reported to be expressed in activated microglia,[34–36] dendritic cells,[8] macrophages,[43] and lymphocytes,[43] and is localized in distinct cellular compartments including the endosomal structures,[45,59] plasma membrane,[57] endoplasmic reticulum, and Golgi complex.[44,45,51] It is suggested that CatE is involved in major histocompatibility complex class II–mediated antigen presentation.[5,23,36] As shown in our data, CatE immunofluorescence is detected in neutrophils, and exhibits a granule-like pattern, which clearly merged with NE. These data suggest that CatE is presumably located in the azurophilic granules, involved in the processing of proelastase. According to the enzymatic property that CatE recognizes precursor sequence (proline-X (X is any amino acid)-X'-hydrophobic amino acid at the P4–P1 position) of proteins,[22] which is included in the signal peptide sequence of proelastase, it is reasonable to assume that CatE cleaves peptide bond in the carboxyl-terminal sequences of a NE signal peptide and generates enzymatically active form of NE. Enzymatic activity and immunoreactivity, but not the secretion of NE, were still observed in the DRG of MOG_{35-55}-immunized *CatE*$^{-/-}$ mice (**Figs. 1E and 4E**). From these observations, we suggest that CatE may contribute to boosting of neutrophil functions in response to MOG_{35-55} without affecting the basal cellular functions, such as migration.

We could not confirm the involvement of neutrophil/CatE/elastase pathway in MS-associated neuropathic pain because EAE can be induced by several immunogenic peptides, including myelin basic protein and proteolipid protein. However, involvement of neutrophils in EAE has been previously reported in the myelin basic protein- and proteolipid protein-model.[7,29] Thus, we believe that neutrophil/CatE/elastase pathway may be a general pathway during the preclinical phase of EAE, although further analyses are needed.

In summary, our results demonstrate that neutrophils were accumulated in the DRG of EAE mice. Neutrophils produced elastase in a CatE-dependent manner, which in turn activated the DRG neurons, during the preclinical phase of EAE. Our findings offer new insights into the pain mechanisms in EAE, and may contribute to the development of therapeutics targeting CatE to alleviate MS-associated neuropathic pain.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Author contributions: Y. Harada and Y. Hayashi designed research; Y. Hayashi supervised research; Y. Harada, J. Zhang, K. Imari, R. Yamasaki, K. Yamamoto, J.-i. Kira, and Y. Hayashi performed research; J. Ni and Z. Wu assisted in selection of genotype and reagents; Y. Harada, J. Zhang, K. Imari, H. Nakanishi, and Y. Hayashi analyzed data; and Y. Harada and Y. Hayashi wrote the manuscript.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A791.

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