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Original Article

Prophylactic administration of PEPITEM in experimental autoimmune encephalomyelitis delays disease onset, inhibits leukocyte infiltration, and alleviates severity

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Abstract: Background: Multiple sclerosis (MS) is a chronic, immune-mediated neurological disorder in which the immune system mistakenly attacks the myelin sheath, affecting the communication between the brain and the rest of the body. Objective: This study investigated the prophylactic use of peptide inhibitor of trans-endothelial migration (PEPITEM), a novel peptide, in alleviating experimental autoimmune encephalomyelitis (EAE), a mouse model for Multiple Sclerosis (MS). Methods: Female C57BL/6 female mice were assigned to the control, untreated EAE, or PEPITEM group. EAE was induced in mice in the untreated EAE and PEPITEM groups through immunization by injecting an emulsion containing myelin oligodendrocyte glycoprotein 35-55 in complete Freund's adjuvant. Mice in these groups subsequently received PEPITEM or scramble peptide injections daily for 21 days. Then, all mice were euthanized to obtain samples for histologic and immunohistochemical analyses of central nervous system lymphocytic infiltrate. Levels of biomarkers, including myelin basic protein, microtubule-associated protein 2 (MAP-2), interleukin-17 (IL-17), and forkhead box P3 (Foxp3), were evaluated in both serum and spinal cord lysates using western blotting and enzyme-linked immunosorbent assay. Results: In the PEPITEM group, EAE onset was significantly delayed and disease severity was reduced compared to the untreated EAE group. Analysis of spinal cord tissues revealed a marked reduction in inflammatory cell infiltration following PEPITEM administration. Furthermore, PEPITEM treatment led to significantly reduced IL-17 and Foxp3 levels, highlighting its potential in mitigating inflammatory responses. Conclusion: PEPITEM has potent prophylactic potential against MS, providing a robust foundation for further exploration.

Keywords: Multiple sclerosis, prophylactic, experimental autoimmune encephalomyelitis, PEPITEM, demyelination, leukocyte trafficking

Introduction

Multiple sclerosis (MS) is a chronic, immune-mediated neurological disorder affecting the central nervous system (CNS). In MS, the immune system mistakenly attacks the myelin sheath, the protective covering of nerve fibers, leading to communication problems between the brain and the rest of the body [1]. This attack results in axonal damage and demyelin-

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ation, leading to a range of neurological symptoms [2, 3]. Hence, MS can cause deterioration or permanent damage to nerves. The specific cause of MS remains unknown, but its etiology is believed to involve a combination of genetic, environmental, and possibly infectious factors. The risk factors for MS include genetic predisposition (having a first-degree relative with MS increases risk), Epstein-Barr virus (EBV) exposure, smoking, and low vitamin D levels [4-6]. Epidemiologic studies examining environmental risk factors for MS, including vitamin D, smoking, and EBV infection, are the most prevalent in MS prevention research [4, 7, 8]. MS can present with various symptoms depending on the location of the CNS lesions. Common symptoms include fatigue, visual disturbances (e.g., optic neuritis), muscle weakness or spasticity, and coordination and balance issues [9, 10]. MS has four major types. Relapsing-remitting MS (RRMS), the most common type, is characterized by distinct episodes of neurologic symptoms followed by periods of partial or complete recovery. Secondary progressive MS features an initial RRMS phase followed by a progressive phase with or without relapses. Meanwhile, primary progressive MS is characterized by the steady deterioration of neurologic function from the onset without distinct relapses. Progressive-relapsing MS is a rare form characterized by steady disease progression with occasional relapses [3, 11]. MS affects approximately 2.8 million people globally [12, 13]. The prevalence of MS is estimated at 35.9 per 100,000, although with significant variation between countries and regions [14]. Particularly, the prevalence of MS increases with distance from the equator, being higher in countries in North America, Europe, and parts of Australia than in Asian and African countries [12, 15]. Regionally, there is increasing awareness of MS in the Gulf Cooperation Council region, and its prevalence appears to be increasing in this region [15, 16]. However, because of challenges in accurately diagnosing MS and the absence of mandatory reporting, its prevalence is likely underestimated. Moreover, MS has a female predominance (2:1 to 3:1), and the disease is most commonly diagnosed in people aged 20-40 years, although it can occur at any age [17-19].

The primary goals of current therapeutic approaches for MS are to modify the disease course, treat relapses, manage symptoms, and improve patients' quality of life. Disease-modifying therapies are used to reduce relapse rates and delay disease progression, corticosteroids are applied for acute relapses, and symptomatic treatments are employed to treat issues such as spasticity, fatigue, and bladder problems. Additionally, current treatments for managing harmful immune reactions in autoimmune diseases typically involve drugs that suppress the general activity of various immune cells throughout the system. However, widespread immunosuppression can lead to severe, and occasionally fatal, side effects [20]. Consequently, although available drugs effectively reduce joint damage, they neither cure the disease nor eliminate the ongoing necessity for treatment. There has been limited focus on disease prevention [21] even though prevention is inherently more beneficial than treatment after symptoms appear, which can strain healthcare resources. To date, immune-related diseases such as MS and rheumatoid arthritis (RA) have not received intensive focus in prevention strategies in terms of public health and precision prevention [22]. Scientists continue to debate the exact causes of MS, suspecting a combination of genetic and environmental factors. Identifying the elements responsible for MS could lead to the development of more effective treatment and prevention. In an effort to promote the development of MS prevention strategies, Tremlett et al. highlighted the potential of investigating the MS prodrome, a presymptomatic phase detectable years before a clinical diagnosis of MS is made [23], specifically considering the possibility of reversing the cause during the prodromal period. Deeper comprehension of the risk factors and elements influencing disease progression in this phase could offer insight into prevention and early intervention strategies before the appearance of neurological symptoms [23, 24]. Hence, exploring prophylactic treatments for MS is crucial, as early intervention may alter the disease course. This approach focuses on preventing or minimizing the onset of neurological deficits by targeting the early stages of the disease process.

Given the complex nature of the pathophysiology of MS, the development of novel preventive strategies has relied on the utilization of robust and relevant experimental models. The experi-

mental autoimmune encephalomyelitis (EAE) model has emerged as the basis of these models, primarily because of its close resemblance to MS in terms of immunopathogenesis and clinical manifestations. This model has been pivotal in unraveling the complex interactions of immune mechanisms underlying MS and in the preclinical evaluation of therapeutic agents. Historically, the EAE model has facilitated significant advancement in MS studies, including the elucidation of various aspects of the disease's pathogenesis and potential intervention points [8, 25]. These studies collectively highlight the EAE model's capacity to simulate the autoimmune attack on the CNS, thereby emulating key aspects of MS, such as demyelination, neuroinflammation, and axonal damage.

Peptide inhibitor of trans-endothelial migration (PEPITEM) is a novel peptide that modulates immune responses, particularly T cell migration across the endothelial barrier, a key event in the pathogenesis of MS [26]. Additionally, it plays a role in the adiponectin-PEPITEM pathway, which regulates immune reactions and inflammation in the body [26]. Previously, we demonstrated that treatment with PEPITEM alleviated symptom severity in the EAE model [27]. In addition to MS, PEPITEM is promising in controlling inflammation in other autoimmune diseases. For instance, a recent study suggested that it is beneficial for conditions related to obesity and type 2 diabetes owing to its ability to suppress inflammation and enhance glucose metabolism [28]. Furthermore, PEPITEM reduces the recruitment of T cells in inflamed areas in various conditions, including peritonitis, hepatic ischemia-reperfusion damage, *Salmonella* infection, uveitis, and Sjögren's syndrome [26]. Additionally, Kemble et al. investigated the therapeutic abilities of PEPITEM in a mouse model of arthritis; synthetic PEPITEM administration before disease onset prevented the progression of collagen-induced arthritis in mice [29]. This promising finding aligns with recent results demonstrating the ability of synthetic PEPITEM to either prevent or postpone RA onset in animal models and restore the proper movement of white blood cells in human tissues [30].

The integrated analysis of clinical and histopathologic variables, cytokine profiles, and protein expression can provide a comprehensive understanding of PEPITEM's ability to modulate the EAE phenotype. Therefore, this study explored the prophylactic potential of PEPITEM in mitigating EAE symptoms in mice. Our investigation specifically assessed how prophylactic PEPITEM influences disease progression, with a focus on its capacity to reduce severity and modify disease outcome.

Materials and methods

Animals

Female C57BL/6 mice (9-13 weeks old; weight, 21 ± 3 g) were obtained from the animal facility of King Saud University's (KSU) Experimental Surgery Center. The experiment involved three groups of mice, with each group comprising 10 female mice aged between 9-13 weeks and weighing approximately 21 ± 3 g. These mice were group-housed in standard cages with proper bedding and nesting materials. The environment was maintained at 22°C ± 2°C and 50% ± 10% relative humidity under a 12-h/12-h light/dark cycle (lights on from 7:00 am to 7:00 pm). The mice had uninterrupted access to water, a water gel on the cage floor, and food. Their weight and health were monitored daily by veterinarians. The experimental design was approved by the Institutional Animal Care and Use Committee at King Abdullah International Medical Research Center (reference number: RC-19/084/R). Additionally, the study received approval from the Research Ethics Committee of KSU (KSU-SE-19-10).

Experimental design

Mice were randomly assigned to three experimental groups (n = 10 per group). Group 1 (G1) served as the normal control, group 2 (G2) underwent EAE induction and treatment with a scrambled peptide for 21 days, and group 3 (G3) underwent EAE induction and treatment with PEPITEM for 21 days.

Reagents

EAE induction kits were obtained from Hooke Laboratories (Lawrence, MA, USA), and they included pre-filled syringes containing a myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) emulsion combined with complete Freund's adjuvant (CFA), as well as vials containing pertussis toxin (PTX) in glycerol buffer. PEPITEM

and the scrambled peptide were synthesized by Thermo Fisher Scientific (Waltham, MA, USA). Additionally, we procured recombinant anti-myelin basic protein (MBP, Cat. No. ab216668), microtubule-associated protein-2 (MAP-2, Cat. No. ab32454), anti-CD3 (Cat. No. ab11089), anti-CD163 (Cat. No. ab182422), recombinant anti-N-cadherin (Cat. No. ab76011), recombinant anti-GAPDH cadherin antibodies, and the Quant-TI protein assay kit from Abcam (Cambridge, UK). Furthermore, acrylamide/bis solution and thick blot filter paper were acquired from Bio-Rad (Hercules, CA, USA). Finally, mouse interleukin 17 (IL-17) and mouse forkhead box protein P3 (Foxp3) enzyme-linked immunosorbent assay (ELISA) kits were purchased from MyBioSource, Inc. (Cat. Nos. MBS455642 and MBS452652, respectively, San Diego, CA, USA).

Induction of EAE in mice

EAE kits were prepared freshly, ensuring that they were used before their expiration date, and stored in a refrigerator at 2°C-8°C. In accordance with the manufacturer's instructions, mice were immunized by injecting an emulsion containing MOG35-55 in CFA. All mice received two injections of PTX in phosphate-buffer saline (PBS) on days 0 and 1. Specifically, using a mouse restraint cage, the antigen emulsion was subcutaneously administered at the midline of the upper and lower back in a volume of 0.1 mL per site (total of 0.2 mL per mouse). Two hours later, PTX was freshly prepared, diluted per the manufacturer's instructions, and administered intraperitoneally in a volume of 0.1 mL on days 0 and 1. Mice in G1 were injected with normal saline.

PEPITEM administration

Given the preventive nature of this study, daily intraperitoneal (IP) injections of PEPITEM (100 mg/mL in a total volume of 200 µL per injection) commenced on day 0, coinciding with the start of disease induction, and continued until the end of the experiment on day 20 and animal sacrificing by day 21 as described previously [26, 27].

Animal observations

Mice were monitored daily for changes in weight, behavior, clinical signs, and mortality. Additionally, the EAE scoring system was utilized according to the kit's instruction manual. Both animal caretakers and the research team documented EAE scores each day. The scoring system was as follows: 0, no symptoms; 0.5, slight tail limpness; 1, complete tail flaccidity; 1.5, tail flaccidity with walking imbalance; 2, tail flaccidity with hindlimb inhibition; 2.5, tail flaccidity and hindlimb weakness; 3, complete tail and hindlimb paralysis; 3.5, inability to return to a normal position when placed on its side, in addition to tail and hindlimb paralysis; 4, complete tail and hindlimb paralysis with partial forelimb inhibition; and 5, severe complete paralysis or mortality, at which point euthanasia was recommended.

Tissue collection

As the study focused on the acute phase of the disease, EAE-induced mice were humanely euthanized on day 21 by an overdose of isoflurane. The isoflurane concentration was adjusted to ≥ 5% or higher, and exposure was maintained until one minute after breathing ceased. Following euthanasia, half of the mice were perfused with cold PBS and paraformaldehyde through the left ventricles. Organs were then collected, fixed, and embedded in paraffin for hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) analysis. For the remaining mice, their organs were promptly harvested, frozen in liquid nitrogen, and preserved at -80°C for western blotting.

Histopathological analysis

This analysis focused on the lumbar region of the spinal cord for two main reasons. First, this region is rapidly and frequently affected in this model. Second, the motor neurons responsible for innervating the hindlimb muscles are located in the lumbar region. To conduct this analysis, 4-µm-thick sections were obtained from paraffin blocks of the spinal cord using a microtome. The sections were dewaxed in xylene, dehydrated in a gradient of alcohol for 5 min, and stained with Harris hematoxylin for 1 min followed by eosin for 2 min. Subsequently, the sections were dehydrated with alcohol and mounted for examination by light microscopy using an Axio Imager 2 microscope (Zeiss, Oberkochen, Germany) and Aperio ScanScope AT slide scanner (Leica, Nussloch, Germany).

Immunohistochemistry

IHC analysis was conducted automatically utilizing the Ventana BenchMark system (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, tissue slides were fixed in 10% neutral buffered formalin. The slides were first incubated with primary antibodies: anti-CD3 (1:200 dilution, Cat. No. ab11089, Abcam) and anti-CD163 (1:400 dilution, Cat. No. ab182422, Abcam). This was followed by incubation with a secondary antibody coupled to a chromogenic substrate.

Spinal cord homogenate preparation

Spinal cord samples were weighed and homogenized in ice-cold M-PER™ mammalian protein extraction reagent (Cat. No. 78501, Thermo Fisher Scientific (Waltham, Massachusetts, United States)) at a concentration of 10 μL/μg tissue using a plastic grinding rod. The homogenates were then centrifuged at 14,000 rpm and 4°C for 15 min. The protein concentration in the supernatant of each sample was determined utilizing Qubit™ protein assay kits (Cat. No. Q33211, Thermo Fisher Scientific (Waltham, Massachusetts, United States)) per the manufacturer's instructions.

Western blotting

One microgram of the mouse spinal cord homogenate was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride blotting membranes (Cat. No. 10600021, GE Healthcare, Chicago, IL, USA). The membranes were blocked with 5% dried skim milk for 60 min and subsequently incubated overnight at 4°C with primary antibodies including anti-β-actin (1:2000, Cat. No. ab-6276, Abcam), anti-MAP-2 (1:1000), anti-MBP (1:1000) (Catalog No. ab216668), and anti-N-cadherin (1:1000) (Catalog No. ab76011), all sourced from Abcam (Cambridge, United Kingdom), in blocking solution. After three washes (5 min each), the membranes were incubated with either goat anti-rabbit (Catalog No. ab205718, Abcam) or goat anti-mouse (Catalog No. ab205719), secondary antibody (1:10,000, Cat. No. ab205719, Abcam (Cambridge, United Kingdom)). Following four additional washes (5 min each), the membranes were exposed to an ECL western blotting substrate kit (Cat. No. ab65623, Abcam (Cambridge, United Kingdom)) for 2 min. Each band on the membranes was detected using a ChemiDoc Touch imaging system (Bio-Rad (Hercules, California, United States)). The data were semi-quantitatively analyzed using Image Lab software (Bio-Rad).

Quantification of the serum and spinal cord levels of Foxp3 and IL-17

Foxp3 and IL-17 levels in serum and spinal cord samples were quantified using ELISA kits from MyBioSource, Inc. (USA), following the manufacturer's instructions. Briefly, the microtiter plate was pre-coated with IL-17 or Foxp3, and standards or samples (serum or spinal cord homogenate) were added to the designated wells. These wells were then treated with a biotin-conjugated antibody specific for IL-17 or Foxp3. Subsequently, avidin-conjugated horseradish peroxidase was added to each well, followed by incubation. Changes in color occurred in the wells containing IL-17 or Foxp3, which were measured spectrophotometrically at 450 nm. Finally, the concentrations of IL-17 or Foxp3 were calculated using the optical density of the samples in relation to the standard curve.

Statistical analysis

Quantitative experimental results are presented as the mean ± standard error of the mean (SEM). Data were analyzed using GraphPad Prism 8 (GraphPad Prism 8 software Inc., La Jolla, CA; Software, Boston, MA, USA). To compare the means of ≥ 3 groups, one-way analysis of variance was employed, followed by the Tukey-Kramer multiple comparisons test as a post-hoc analysis. $P < 0.05$ indicated statistical significance.

Results

Prophylactic administration of PEPITEM delayed the onset of EAE symptoms and alleviated disease severity

To investigate the therapeutic potential of prophylactic PEPITEM in mitigating the symptoms of EAE, IP injections of PEPITEM or scrambled peptide were administered to mice on days 0-20. Then, mice were weighed and regularly observed using the EAE scoring system.

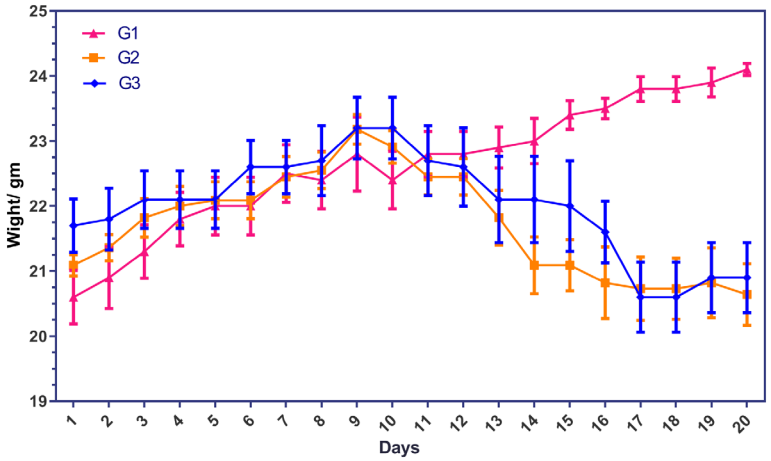


Figure 1. Daily weight changes in EAE-induced and control mice. Weight changes were assessed in mice over 21 days. G1 (pink triangles), normal control mice; G2 (orange squares), EAE-induced mice treated with daily IP scramble peptide injections for 21 days; G3 (blue diamonds), EAE-induced mice treated with daily IP PEPITEM injections for 21 days.

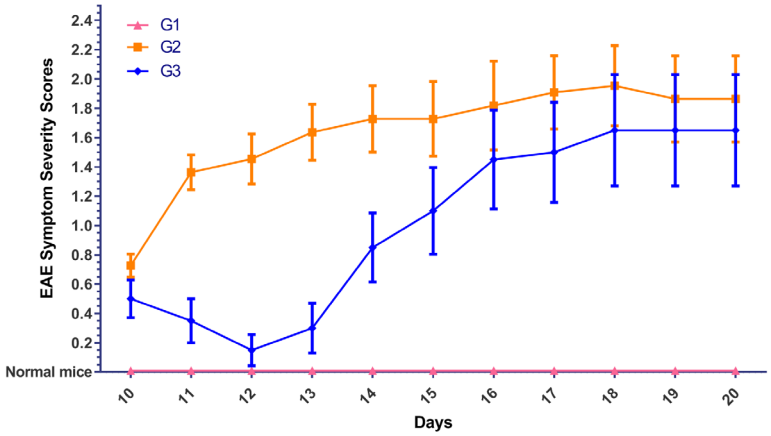


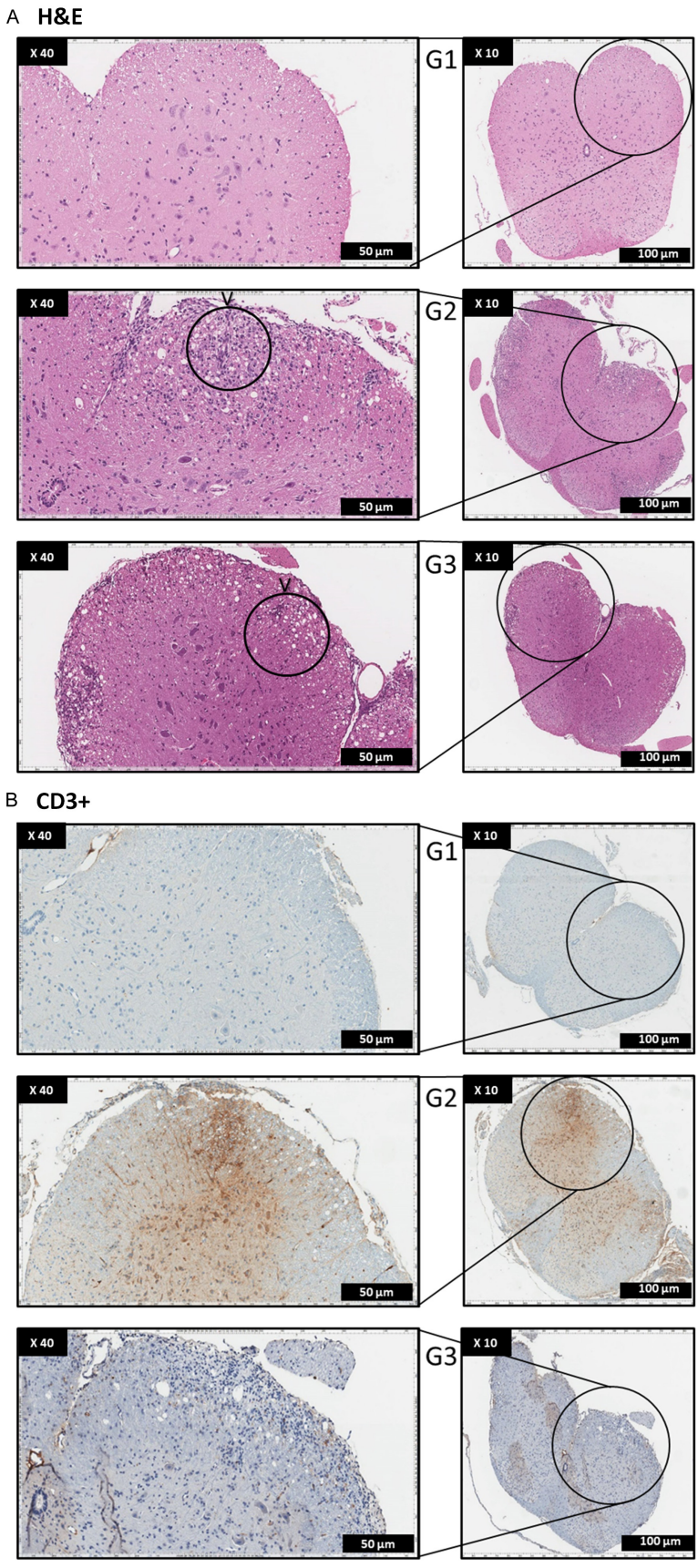
Figure 2. EAE symptom severity scores across the three mouse groups over time. EAE symptom scores were recorded from day 10 to day 20 for G1 (pink triangles, normal control mice), G2 (orange squares, EAE-induced mice treated with daily IP scrambled peptide injections), and G3 (blue diamonds, EAE-induced mice treated with daily IP PEPITEM injections). Scores range from 0 (no symptoms) to 5 (severe complete paralysis or mortality).

Body weight

The initial weight of mice was similar in the three groups, although slightly lower in G1 than in G2 and G3. Body weight steadily increased over the 20-day treatment period in G1, as expected in healthy mice. The weight of G2 mice initially increased similarly as observed in G1 mice until approximately day 9, after which a significant decline was recorded until day 15, followed by a slight recovery. This could be indicative of the disease's impact on the health of the mice, leading to weight loss. The weight trend in G3 was initially similar to that in G2, but a less pronounced decline in weight was observed. This suggests that PEPITEM can mitigate the effects of the disease to some extent. By day 20, a consistent increase in weight was noted in G1, the initial weight had not been recovered in G2, and body weight was higher in G3 than in G2, although lower than the starting value (**Figure 1**).

EAE scoring

Using the EAE scoring system, we confirmed the development of EAE in G2 and G3 mice. Conversely, the EAE score was consistently 0 throughout the experimental period in G1. Typical symptoms developed in the experimental groups (G2 and G3) 10 ± 1 days after immunization, such as limpness of the tip of the tail and hindlimb weakness. Symptoms such as complete hindlimb paralysis, partial forelimb paralysis, and death peaked on day 17 (**Figure 2**). No symptoms were initially observed in G2 mice, but a rapid increase in the EAE score from 0 to 2 was recorded from day 10 to day 14. The score then plateaued, suggesting that symptoms remained steady without further treatment. Similarly, no symptoms were initially observed in G3 mice. However, the increase in the EAE score was more gradual in this group, reaching a lower peak of approximately 1.5 by day 16. Additionally, after reaching this peak, the symptoms of G3 mice appeared to stabilize, indicating the maximum response to EAE induction or a natural plateau in disease progression. The lower peak EAE score and stabilization of symptoms in G2 highlight the potential efficacy of PEPITEM (**Figure 2**).



Histopathologic analysis

To evaluate the prophylactic impact of PEPITEM on the central nervous system (CNS), we conducted histopathologic examinations of the spinal cord in mice with EAE. A certified neuropathologist analyzed the histopathologic alterations in the spinal cord of these mice by H&E staining. This analysis primarily focused on distinguishing between white and gray matter, detecting the presence or absence of demyelination, evaluating the condition of the leptomeninges, and identifying any signs of inflammation, such as lymphocytic infiltration, in the leptomeninges or CNS parenchyma. Our findings revealed the infiltration of inflammatory cells accompanied by patchy vacuolization predominantly in G2 and G3 mice, suggesting disease progression and an inflammatory response (**Figure 3A**). Furthermore, we performed IHC analysis to examine protein levels in the spinal cord in mice with EAE, specifically targeting CD3+ T cells and assessing microglial activation by CD163 staining (**Figure 3B**, **3C**). CD3+ T cells were dispersed through the white matter in G2 mice, indicating an active inflammatory response. Conversely, G3 mice displayed moderate CD3+ T cell infiltration, although weaker than that in G2 mice but greater than that in G1 mice, indicating efficacy of PEPITEM. Moreover, CD163+ staining in G2 mice revealed pronounced microglial activation throughout the spinal cord, including the notable presence of macro-

C **CD163+**

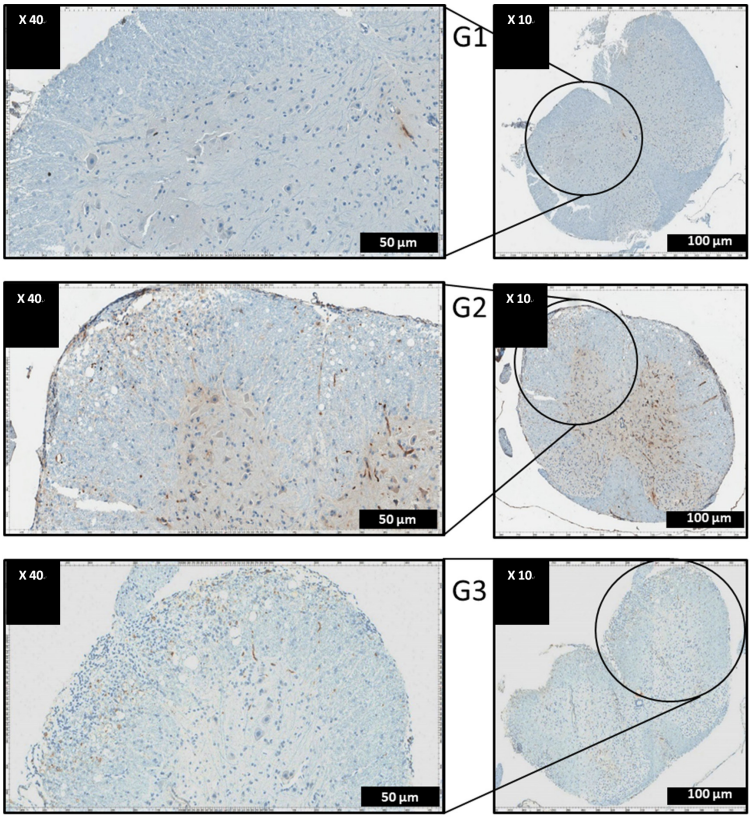


Figure 3. Histopathologic analysis of spinal cord sections in EAE-induced C57BL/6 mice. A. Tissues were collected, fixed, and stained with H&E to illustrate the characteristics of the spinal cord in the three groups. G1 mice exhibited a normal histologic architecture with clear differentiation between white and gray matter and no evidence of inflammatory cell infiltration. G2 mice displayed marked infiltration of inflammatory cells, disrupting the normal architecture of both white and gray matter, with vacuolization (V) indicating a moderate-to-severe inflammatory response. G3 mice exhibited reduced inflammatory cell infiltration and less vacuolization (V) than G2 mice. G1, normal control mice; G2, EAE-induced mice treated with daily IP scramble peptide injections for 21 days; G3, EAE-induced mice treated with daily IP PEPITEM injections for 21 days (n = 3 per group). B, C. Spinal cord sections were stained with anti-CD3 and anti-CD163 antibodies to detect CD3+ T cells and CD163+ macrophages, respectively. G1 mice exhibited minimal staining for both CD3 and CD163, indicating no significant inflammation. G2 mice exhibited pronounced staining for both markers, reflecting the intense infiltration of CD3+ T cells and CD163+ macrophages and indicating severe inflammation. G3 mice displayed moderate staining and vacuolation, reduced levels of T cell and macrophage infiltration compared to G2 mice, although with ongoing inflammation. G1, normal control mice; G2, EAE-induced mice treated with daily IP scramble peptide injections for 21 days; G3, EAE-induced mice treated with daily IP PEPITEM injections for 21 days (n = 3 per group).

phages in the affected white matter areas. In comparison, G3 mice exhibited foamy macrophages within the damaged white matter regions; however, there was no marked activation of microglia across the spinal cord.

Prophylactic PEPITEM administration controlled IL-17 and Foxp3 levels in the spinal cord lysate

Figure 4 presents the protein concentrations of IL-17 and Foxp3 in the serum and spinal cord lysate among the three groups. The serum levels of IL-17 and Foxp3 remained within a similar range across all three groups, displaying no significant statistical variation. Conversely, PEPITEM administration resulted in marked reductions in both IL-17 and Foxp3 levels in the spinal cord lysates of mice with EAE, indicating a significant effect of the treatment at this site.

Western blotting for MBP, MAP-2, and N-cadherin expression

To validate our findings, the protein expression of MBP, MAP-2, and N-cadherin in the mouse spinal cord was assessed on day 21 through western blotting. The results revealed significant reductions in MBP, MAP-2, and N-cadherin expression in G3 mice compared with G2 mice, indicating fewer inflammatory changes in PEPITEM-treated mice (**Figure 5**).

Discussion

Early treatment with PEPITEM may be beneficial in controlling neuroinflammatory disorders. Previous studies have revealed that immunization with a MOG-vitD mixture exerts preventive effects on EAE development [31, 32]. The present study results provide a comprehensive evaluation of the potential prophylactic effects of PEPITEM in an EAE mouse model, with an emphasis on clinical outcomes

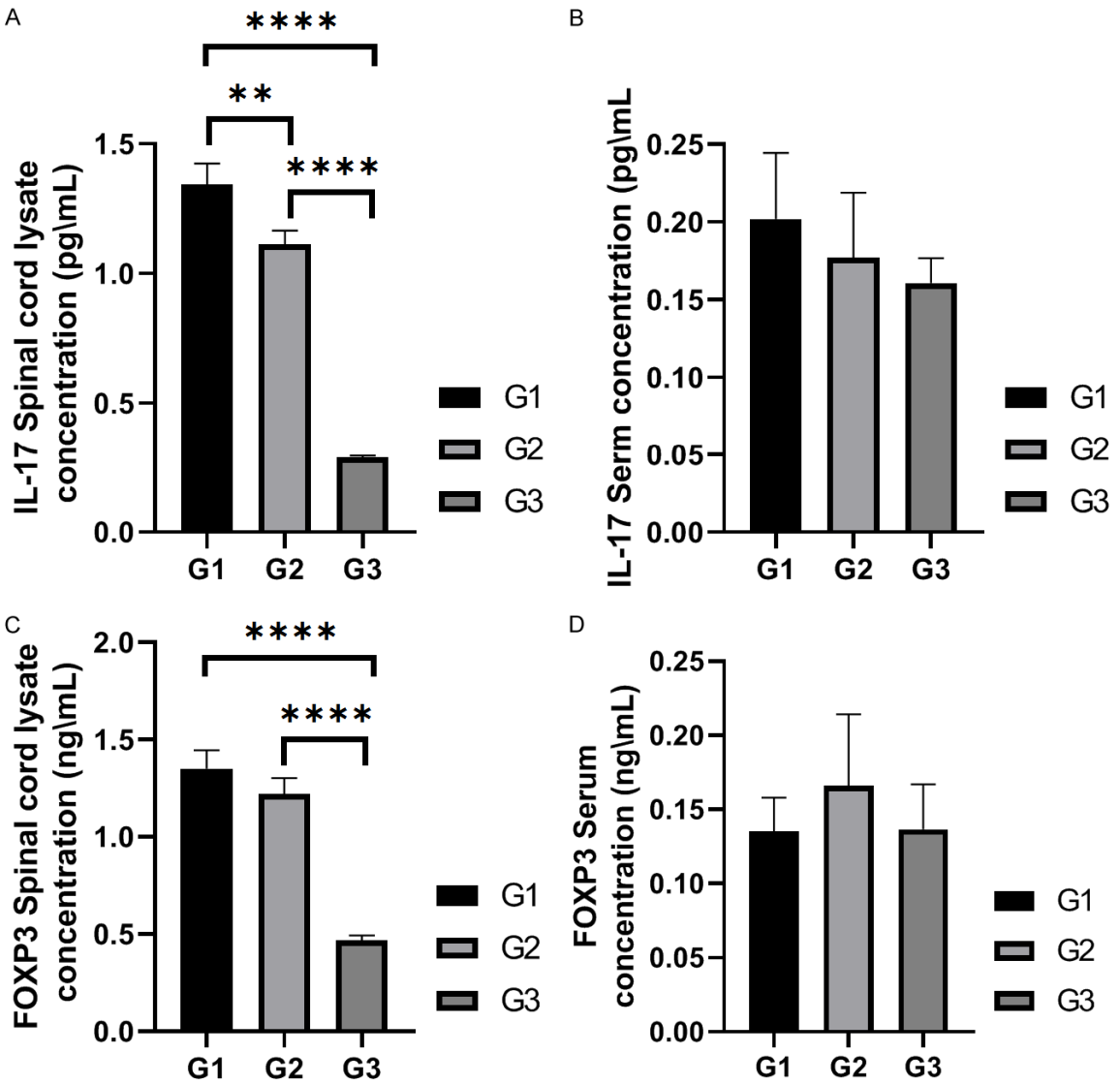


Figure 4. IL-17 and Foxp3 protein concentrations in spinal cord lysate and serum from mice with EAE. Bar graphs present IL-17 (A, B) or Foxp3 (C, D) concentrations in the spinal cord lysates or serum of control mice (G1), EAE-induced mice receiving a scrambled peptide (G2), or EAE-induced mice receiving PEPITEM treatment (G3). Mice in G2 and G3 received daily IP injections for 21 days. Data are expressed as the mean ± SEM (n = 3 per group in duplicate). **P < 0.005, ****P < 0.0001.

such as disease severity, body weight, histologic changes, cytokine levels, and the expression of proteins related to neural integrity.

One approach to evaluating disease severity in EAE is the EAE scoring system, which focuses on clinical indicators of paralysis. However, this method can be subject to evaluator bias. To minimize this bias, the evaluators were blinded to the specific treatment groups of the animals they were assessing. We observed distinct disease progression patterns across the three groups over 11 days. No EAE symptoms were observed in G1, as expected. The EAE score rapidly increased in G2 from day 10 to day 14 and then stabilized, indicating swift disease onset and rapid progression to peak disease severity. Conversely, the score gradually increased in G3, with a delayed and lower peak than that observed in G2, followed by a subsequent plateau. These data suggest that G3 mice experienced slower disease progression or a possible mitigating effect compared to the more severe and rapid progression observed in G2 mice. The appearance of symptoms provides evidence that PEPITEM did not prevent

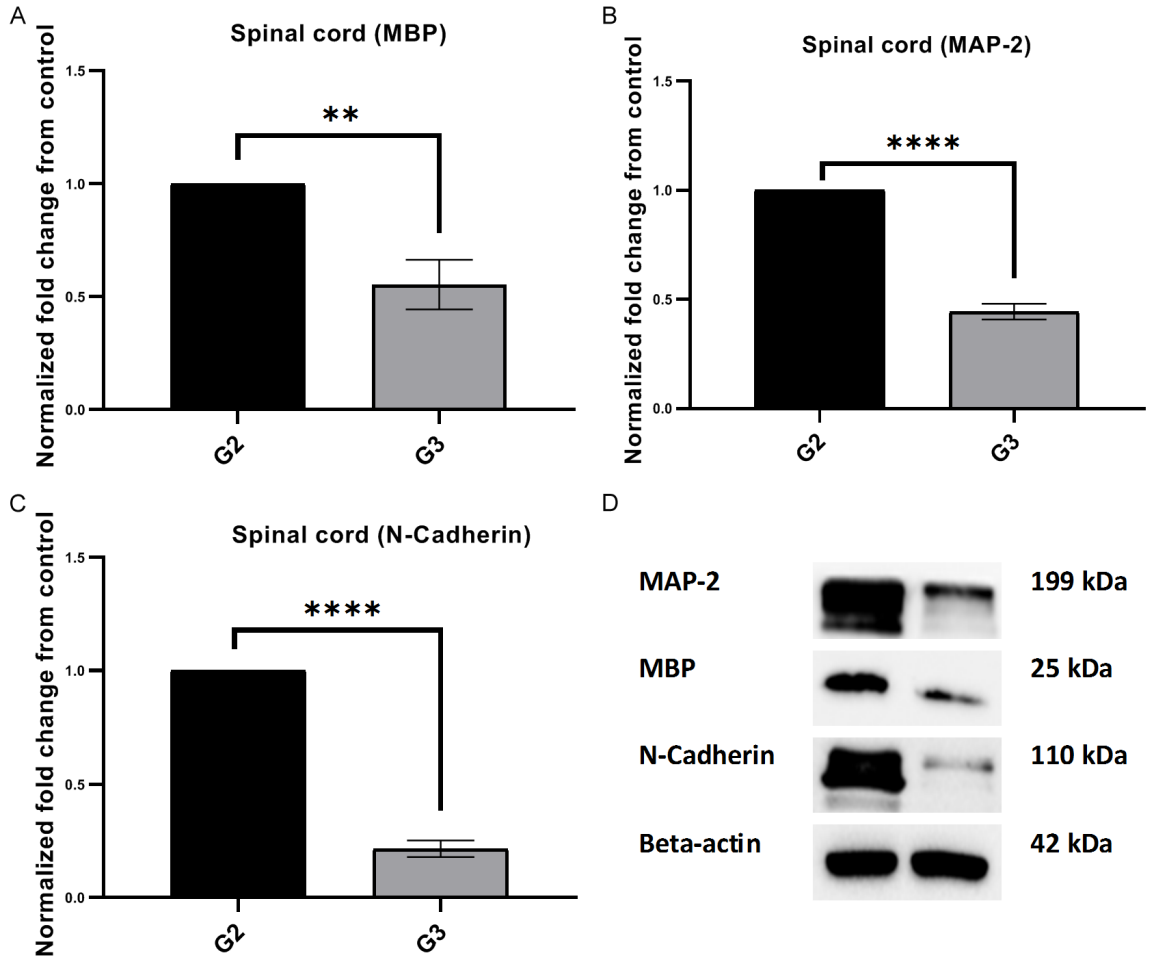


Figure 5. Quantitative analysis of MBP, MAP-2, and N-cadherin protein levels in EAE-induced mice. Bar graphs present the normalized protein levels in spinal cord lysates from EAE-induced mice receiving a scrambled peptide (G2), or EAE-induced mice receiving PEPITEM treatment (G3). A. MBP. B. MAP-2. C. N-cadherin. D. Representative blot. Data are presented as the mean ± SEM (n = 4 per group in triplicate). **P < 0.0018, ****P < 0.0001.

the induction of EAE, contradicting previously reported findings that PEPITEM halted the initiation of collagen-induced arthritis [29]. Nevertheless, the anti-inflammatory effect of PEPITEM was confirmed, supporting the findings of our previous study wherein PEPITEM was administered after the appearance of symptoms [27].

Body weight tended to increase over the experimental period in G1, suggesting minimal effect of the disease or experimental conditions. Meanwhile, G2 mice exhibited significant weight gain until day 9, followed by a distinct, steady decline up to day 17 and a subsequent plateau, which could reflect the onset of disease-related symptoms or adverse treatment effects. Similarly, body weight in G3 mice increased until day 9, followed by a gradual but persistent decrease, although less severe than that in G2 mice, indicating milder disease progression or a response to treatment. These patterns support the impact of PEPITEM on the health and well-being of mice with EAE. The potential stress caused by daily injections cannot be overlooked, as this could contribute to or exacerbate the observed weight loss in G2 and G3 mice given that stress associated with handling and injection can significantly affect the well-being and weight of subjects [33].

The histopathologic findings showed the effect of PEPITEM on the spinal cord at the cellular level. The observed inflammatory changes in both G2 and G3 confirmed the development of an autoimmune response characteristic of EAE. However, comparative analysis revealed that inflammatory marker levels were substan-

tially lower in G3 mice, including less demyelination and a reduced number of leukocyte infiltrates in the leptomeninges, than in G2 mice. Moreover, the reduced CD3+ T cell infiltration in G3 mice, as evidenced by CD3+ IHC analysis, indicates that PEPITEM can modulate the adaptive immune response, specifically influencing T cell dynamics, which play a pivotal role in EAE pathogenesis. These intriguing results align with existing research indicating that PEPITEM selectively inhibits T cell migration without affecting the total adhesion leukocyte pool [26]. Additionally, PEPITEM can counter the systemic, low-grade inflammation associated with obesity and mitigate obesity's effects on pancreatic stability by regulating leukocyte trafficking in the context of a high-fat obesogenic diet [28]. These findings highlight the fact that although PEPITEM does not entirely inhibit disease induction, it does appear to ameliorate the intensity of the inflammatory response. The detection of CD163, a marker of microglial activation, in G2 mice confirmed an active state of neuroinflammation characterized by the presence of a significant number of macrophages within the areas of white matter damage. This finding is consistent with the aggressive immune response and substantial disease pathology in EAE, since microglial activation is a hallmark of neuroinflammatory processes and it often correlates with disease severity [34, 35]. In G3 mice, the presence of foamy macrophages within the areas of white matter damage suggested that microglia were not significantly activated despite evidence of ongoing disease pathology and immune cell activity. This may indicate a more controlled inflammatory environment, possibly because of the prophylactic administration of PEPITEM, which might modulate the immune response and limit the activation of microglia.

To evaluate PEPITEM's efficacy in the EAE mouse model, specific biomarkers were chosen to assess its impact on neural integrity and inflammation. MBP, a crucial component of the myelin sheath enveloping nerves, served as a primary indicator of myelin integrity. Its relevance is particularly marked in demyelinating diseases such as EAE, in which myelin degradation is a hallmark of disease progression. Concurrently, MAP-2, integral to neuron structure stabilization, was used as a neuronal marker. The presence and concentration of MAP-2 offer insights into the structural integrity and health of neurons, which are aspects potentially affected by the pathophysiologic processes of EAE [36]. Additionally, N-cadherin, a protein pivotal in cell-cell adhesion and in maintaining the architecture of neural tissue, was also used. As a marker of neural integrity, N-cadherin also reflects the inflammatory state of neural tissues, making it a valuable indicator for our study [37]. By quantifying the concentrations of these markers, the prophylactic effect of PEPITEM on various aspects of neural health and integrity was comprehensively investigated in the EAE model. Western blotting for neuronal and myelin proteins such as MAP-2, MBP, and N-cadherin provided further insight into the neuroprotective potential of PEPITEM. The preservation of MAP-2 and MBP levels in G3 versus G2 suggests that PEPITEM reduced neuronal damage and myelin degradation, which are hallmarks of EAE pathology [38]. The lower EAE scores in PEPITEM-treated mice, indicating less severe clinical manifestations of the disease, support these findings. Additionally, the significant decrease in N-cadherin expression in G3 further reinforces the potential of PEPITEM to maintain cellular integrity and support neural network stability, which could contribute to the maintenance of neurological function in mice with EAE [27].

To assess whether the systemic response (serum levels) was correlated with local inflammation within the CNS (spinal cord lysate), ELISA was performed to measure Foxp3 and IL-17 levels. IL-17 has been established as a key proinflammatory cytokine in the pathogenesis of EAE, often correlating with disease severity. Notably, PEPITEM treatment resulted in reduced IL-17 levels in both compartments, although the reduction was more pronounced in the spinal cord. This suggests that PEPITEM exerts a more localized anti-inflammatory effect within the CNS, possibly explaining the mitigation of clinical symptoms as indicated by reduced EAE scores [39]. Although IL-17 levels were reduced by PEPITEM treatment, the difference between G2 and G3 did not reach significance. Moreover, the levels of Foxp3, a transcription factor associated with regulatory T cell development and function, typically provide insight into the regulatory aspects of immune responses. The Foxp3 protein concentration in the spinal cord lysate significantly varied among the three groups. Particularly, its levels were markedly increased in G2 mice, presumably

indicating the stimulation of regulatory mechanisms in response to the induced disease. Conversely, Foxp3 levels were significantly lower in G3 mice than in G2 mice, suggesting that PEPITEM has a modulatory effect on the immune response in EAE. Our current understanding typically links a decrease in Foxp3 expression with proinflammatory responses. However, this view has been challenged by recent research. For instance, Zhou et al. found that a reduction in Foxp3+ T regulatory cell numbers can actually inhibit the proliferation of cancer cells and restrict tumor growth [40]. Furthermore, another study posited that Foxp3 expression does not invariably correlate with immunosuppressive activity [41]. In light of these findings, the observed reduction in Foxp3 levels in the spinal cord in our EAE model might underpin the mechanism by which PEPITEM exerts its anti-inflammatory effects. This suggests that a decrease in Foxp3 levels, contrary to traditional views, does not always signify a proinflammatory state, and this reduction could be instrumental in the therapeutic action of PEPITEM.

Overall, these results suggest that PEPITEM attenuates the inflammatory response. This attenuation could manifest as a reduction in T cell infiltration and more regulated microglial activation, which could translate into less severe clinical manifestations of the disease. The differential impact of PEPITEM on various immune cells, including diminished T cell trafficking and altered macrophage activity, without complete blockade of microglial activation provides insight into the complex role of PEPITEM in neuroinflammatory conditions and offers a promising avenue for therapeutic intervention in EAE and possibly other inflammatory diseases of the CNS.

Conclusions

PEPITEM can reduce the severity of the autoimmune response in EAE. Our findings indicate that PEPITEM may be beneficial in managing EAE and enhance patients' quality of life by mitigating severe inflammatory responses. However, further studies are needed to elucidate the precise mechanisms by which PEPITEM exerts its effects, evaluate its long-term efficacy, and explore its applicability to human autoimmune conditions such as MS.

Future directions and limitations

Increasing the sample size in future studies can minimize the impact of variation between animals, enhancing statistical power. The use of different PEPITEM doses and administration routes is recommended to optimize the treatment effect. Additionally, confirming the anti-inflammatory effects of PEPITEM by assessing additional markers in treated animals could provide valuable insight. This study's findings might not apply to other doses or injection methods, necessitating further research. The EAE scoring system might not capture subtle CNS changes, highlighting its limitations. The subjectivity of mouse scoring is another possible limitation.

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Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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