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Research article

Anti-allodynic effect of intrathecal antibodies against macrophage-inducible C-type lectin in spinal nerve ligation model in rat

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A B S T R A C T

Introduction: Macrophage-inducible C-type lectin (Mincle) has emerged as a potential contributor to neuropathic pain induction and neuro-inflammatory responses within the spinal cord. Moreover, evidence suggests a close association between toll-like receptor (TLR) and Mincle expression in myeloid cells. This study evaluated the effectiveness of Mincle antibodies in neuropathic pain and identified the epitope of these antibodies. In addition, the mode of interaction between Mincle and TLR inhibition was explored using isobolographic analysis.
Methods: Three different Mincle antibodies and a specific TLR4 inhibitor (TAK-242) were intrathecally administered, and mechanical allodynia was evaluated using the von Frey test in a rat model of spinal nerve ligation (SNL). Isobolographic analysis was conducted on the effect of combination of TAK-242 and Mincle Ab. Microarray analysis examined the specific region of Mincle targeted by the antibodies.
Results: All Mincle antibodies and TAK-242 significantly alleviated mechanical allodynia in a dose-dependent manner. However, the maximal possible effects (MPE) produced by the antibodies ranged widely from 37.1 % to 91.8 %, comparable to that of TAK-242 (88.7 %). The combination of TAK-242 and the antibody with the highest MPE resulted in an additive interaction for their anti-allodynic effects. Epitope mapping revealed that each antibody targeted the extracellular domain, with epitope lengths ranging from 5 to 15 amino acids.
Conclusions: The current study demonstrates the anti-allodynic effect of Mincle antibodies and additive interaction with TLR4 inhibition in spinal nerve ligation model, suggesting the potential of blocking of Mincle signaling with its antibodies as a novel treatment strategy for neuropathic pain.

1. Introduction

Neuroinflammation driven by the activation of immune system has emerged as a crucial factor contributing to the onset and persistence of neuropathic pain [1,2]. This inflammatory response involves the release of various pro-inflammatory molecules including cytokines, sensitizing pain-sensing neurons, amplifying pain signals, and reducing pain thresholds [1,3]. While toll-like receptors (TLRs) are well-established for their critical role in neuroinflammatory responses within the central nervous system contributing to chronic and neuropathic pain [4], the involvement of C-type lectin receptors (CLRs) in neuropathic pain remains poorly understood [5,6].

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Mincle (Clec4e), macrophage-inducible C-type lectin, belongs to CLR family of pattern recognition receptor, serving an important component of the innate immune system [7]. Mincle expression is found in neurons, as well as immune cells such as microglia and macrophages in the nervous system, and its role in neuroinflammation has been noted in several CNS disease models [8–10]. Activation of Mincle by intrathecal administration of its ligand (trehalose-6,6-dibehenate) has shown to induce allodynic responses and microglial activation in the spinal cord of naïve rat [11]. Additionally, the peripheral nerve injury increased the expression of Mincle mRNA in the spinal nerve and dorsal root ganglion with knockout of Mincle resulting in the attenuation of mechanical allodynia in a murine model of peripheral nerve injury [5]. However, the effect of direct inhibition of Mincle following the development of neuropathic pain has yet to be studied. In addition, the close relationship between Mincle and TLR has been observed, particularly in terms of controlling responsiveness, receptor expression and immune modulation [12–15]. However, the precise interaction between Mincle and TLR in the central nervous system remains unknown.

The current study investigated the effect of Mincle antibodies on mechanical allodynia and explored the interaction of the Mincle antibodies and TLR antagonist in the anti-allodynic effect using a rat model of spinal nerve ligation. Furthermore, the epitopes of the antibodies were compared using epitope mapping with microarray analysis.

2. Results

Epitope analysis for Mincle antibodies revealed different binding sites among them, and all were found in the extracellular domain of Mincle as expected (Fig. 1). The antibody response measured by fluorescence intensity was strongest in the order of Santa Cruz Bio Ab > Novus Bio Ab > InvivoGen Ab. The size of epitope for Santa Cruz Bio and Novus Bio Ab was 5 amino acid peptides, '58 QLPEN 62′ and '165 WDVGE 169′ of human Clec4e, respectively. In contrast, the binding site of InvivoGen Ab was found at '121 QEEQEFLFRTKPKRK 135′ of mouse Clec4e, with a size of 15 amino acids, which is relatively long and could be considered atypical for an epitope.

All Mincle antibodies significantly attenuated the mechanical allodynia compared to vehicle treatment in animals with spinal nerve ligation. The anti-allodynic effect was produced in a dose-dependent manner, peaking at 2–3 h after intrathecal treatment. However, the maximal possible effects of each antibody varied widely, ranging from 37.1 ± 2.9 % (Novus Bio Ab) and 81.7 ± 23.8 % (InvivoGen Ab) to 91.8 ± 12.8 % (Santa Cruz Bio Ab) at their highest doses (Fig. 2A–F). The Santa Cruz Bio Ab was most effective and its ED50 obtained from the dose-response data was 0.83 μg (95 % confidence interval: 0.63–1.09 μg). As for Santa Cruz Bio Ab, additional experiment was performed because its effect showed a plateau at 180 min, and we found that the effect continued until 8 h, and no effect was observed at 24-h post-administration (see Supplementary Data 1).

Intrathecal administration of TLR4 antagonist, TAK-242 significantly increased the PWT and MPE compared to vehicle in a dose-dependent manner (Fig. 2G and H). The MPE of TAK-242 reached 88.7 ± 11.3 % at the highest dose, comparable to that of InvivoGen Ab or Santa Cruz Bio Ab. The ED50 of TAK-242 was 17.5 μg (95 % confidence interval: 6.19–49.71 μg). The anti-allodynic effect started as early as 15 min and dissipated in 1 h after the treatment.

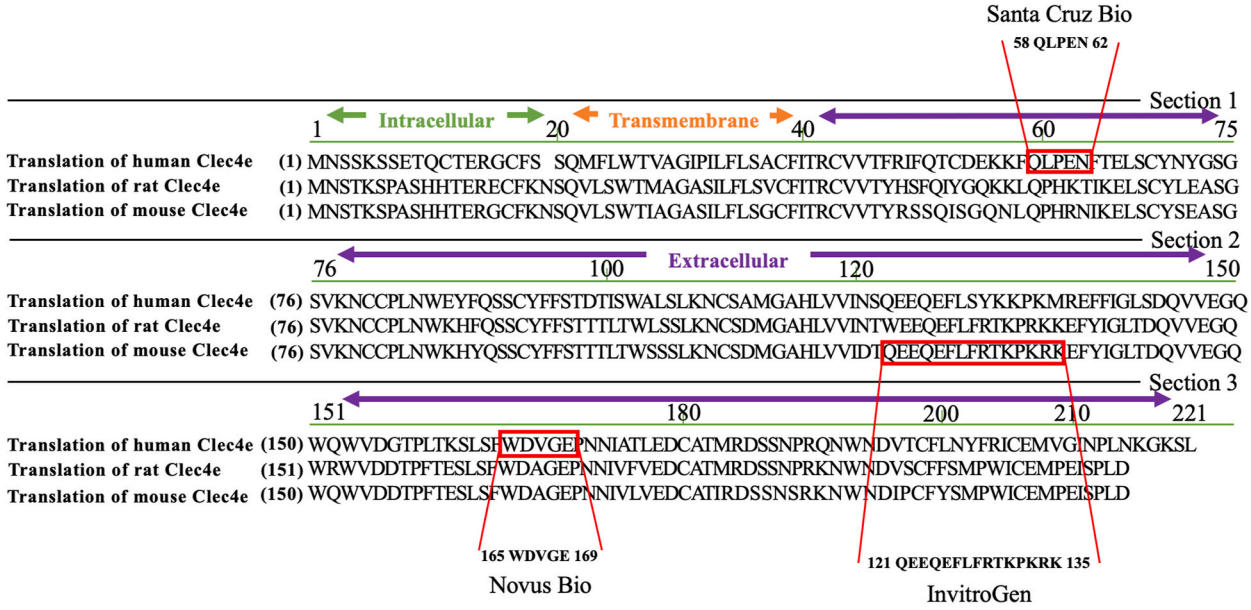

Fig. 1. Clustered distribution of Mincle (Clec4e) epitopes bound by three different Mincle antibodies. Each antibody has different binding site of amino acid sequences, which is in the extracellular portion, but not overlapped with each other. Specifically, binding sites of the InvivoGen Ab (mouse), Novus Bio Ab (human), and Santa Cruz Bio Ab (human) are identified in the epitope mapping at '58 QLPEN 62′, '165 WDVGE 169′ and '121 QEEQEFLFRTKPKRK 135′, respectively. Santa Cruz Bio Ab: Mincle antibody from Santa Cruz Biotechnology; TAK-242: a specific TLR4 inhibitor.

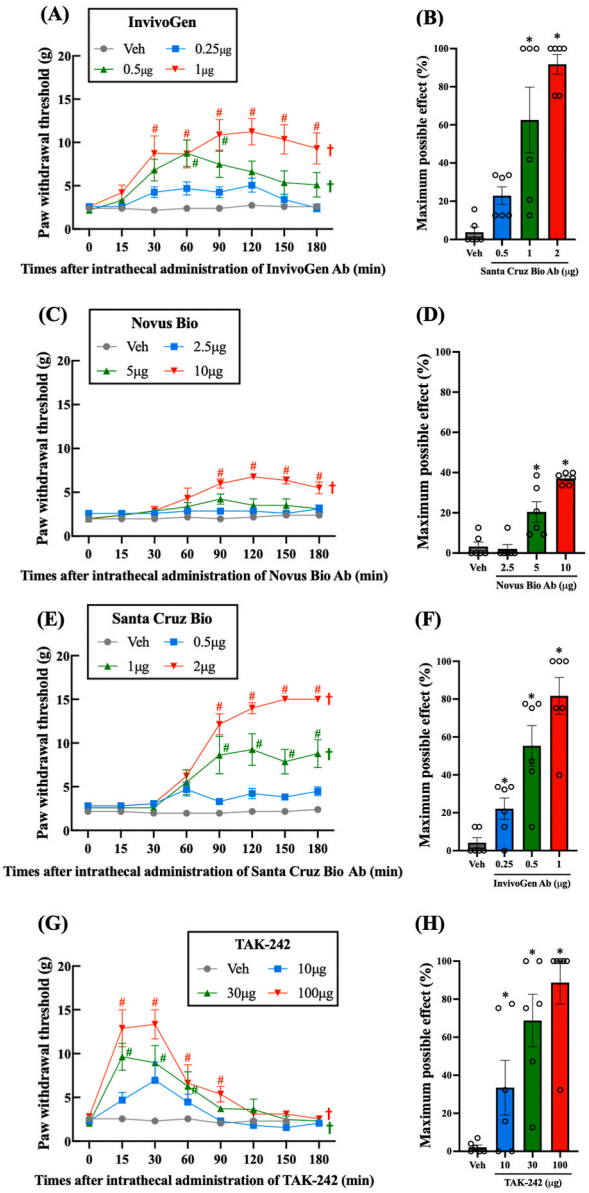

Fig. 2. Time course of paw withdrawal thresholds (PWT) and maximal possible effects (MPE) produced by intrathecal administration of Mincle antibodies or TAK-242. All the animals underwent the spinal nerve ligation (SNL) 7 days before this experiment. The PWT (A, C, E, G) increases significantly after i. t treatment of Mincle antibodies or TAK-242 (†$P < 0.05$ vs. Veh). In addition, the PWT on each time point is significantly different from that of Veh ($^{\#}P < 0.05$ vs. Veh). The MPE (B, D, F, H) also significantly increases compared to vehicle in a dose-dependent manner (*$P < 0.05$ vs. Veh). The value of MPE is the lowest with Novus Bio Ab, and the highest with the Santa Cruz Bio Ab at the highest dose of each antibody. Data are expressed as mean ± SEM and $N = 7$ per group. Novus Bio Ab: Mincle antibody from Novus Biologicals, InvivoGen Ab: from InvivoGen, Santa Cruz Bio Ab: from Santa Cruz Biotechnology.

Combination of TAK-242 and Santa Cruz Bio Ab also produced a significant and dose-dependent anti-allodynic effect, with an MPE of 91.0 ± 5.5 %. The anti-allodynic effect appeared as early as 15 min and lasted for 3 h after intrathecal injection of the mixture. In isobolographic analysis, the experimental ED50 of SC or TAK-242 in mixture did not differ from the theoretical additive ED50 calculated from the ED50 of each agent when administered alone, as shown in Fig. 3. Additionally, TFV was 0.95 [=(8.37/17.54) + (0.40/0.83)], indicating an additive interaction for the anti-allodynic effect between TAK-242 and Santa Cruz Bio Ab.

3. Discussion

A substantial body of evidence underscores the critical role of neuroinflammation in the pathogenesis of CNS disorders such as

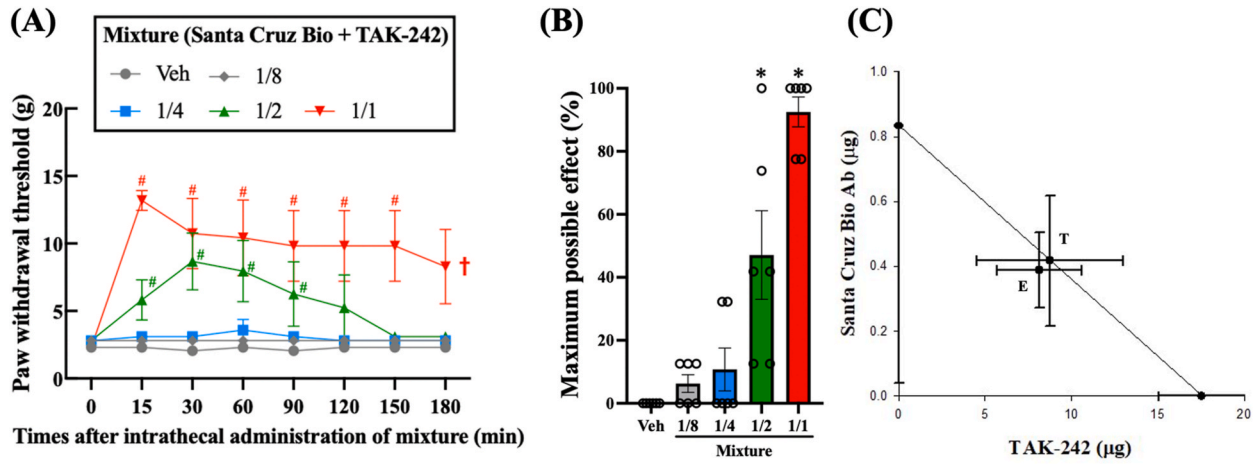

Fig. 3. Isobologrphic analysis for the interaction between Santa Cruz Bio Ab and TAK-242 for anti-allodynic effect in the spinal nerve ligation (SNL) of rat. Time course of paw withdrawal threshold (A) and changes in the maximal possible effect (B) according to the dose after intrathecal administration of the mixture of Santa Cruz Bio Ab and TAK-242 is depicted. The mixture was administered via intrathecal catheter 10 min before von Frey test. The doses of mixture were the fractions of the ED50 of each agent with fixed 1:1 proportion (1/1, 1/2, 1/4 and 1/8; N = 7, each dose). In the isobologram (C), the theoretical ED50 values of Santa Cruz Bio Ab and TAK-242 are marked in the X- or Y-axis. The straight line connecting the two points is the theoretical additive line, representing the combination of doses that could be predicted to produce a 50 % of maximal possible effect. The horizontal and vertical bars represent the SEM of experimental (E) and theoretical ED50 (T) of Santa Cruz Bio Ab or TAK-242 in the mixture. Data are expressed as mean ± SEM and n = 7 per group. †$P < 0.05$ vs. Veh, $^{\#}P < 0.05$ vs. Veh, *$P < 0.05$ vs. Veh. Santa Cruz Bio Ab: Mincle antibody from Santa Cruz Biotechnology; TAK-242: a specific TLR4 inhibitor.

Alzheimer's disease, multiple sclerosis, Parkinson's disease, and traumatic and ischemic brain injury [16,17]. Neuropathic pain is likewise regarded as a manifestation of neuroinflammation in both the peripheral and central nervous system, triggered by various insults including peripheral nerve injury, intense inflammation, or chemotherapy. Among the significant contributors to neuroinflammation are the pattern recognition receptors (PRRs), with Toll-like receptors (TLR) being the most studied and characterized. TLRs play a pivotal role in the development of pathological pain [6]. In the context of neuropathic pain, the activation of TLR4 is associated with neuroinflammation, glial activation, and neuronal sensitization, all of which are crucial processes in its development [4,18]. Conversely, there have been relatively few studies exploring the role of other membrane-bound PRR including C-type lectin receptor (CLR) in neuropathic pain. The present study shows that blocking Mincle with its antibody can elicit a significant anti-allodynic effect comparable to TLR inhibition. This finding suggests an essential role of Mincle in neuropathic pain induced by peripheral nerve injury.

Mincle, a member of CLRs, has an important role in the innate immunity and has been implicated in various inflammatory responses [14]. Inhibition of Mincle/spleen tyrosine kinase (Syk) signaling with Syk inhibitors has been shown to ameliorate the neuroinflammatory responses or produce beneficial effect in several CNS disease models [8,9,19]. A recent study also suggests that Mincle may contribute to the development of neuroinflammation in the spinal cord, leading to spinal sensitization and pain behavior. Intrathecal injection of the Mincle ligand induces microglial activation and an increase of NF-kappa B and TNF-alpha levels in the spinal cord, accompanied by pain behavior in rats [11]. Similarly, an important role of Mincle in peripheral nerve-induced neuropathic pain was reported in a model of spinal nerve injury in Mincle-deficient mice [5]. However, in this study, while the expression of Mincle mRNA was increased in the injured spinal nerve, it was not observed in the dorsal root ganglion and spinal dorsal horn, suggesting no direct involvement of Mincle at the spinal level. In contrast, the current study highlights a significant role of Mincle signaling in the spinal cord, although the precise molecular mechanism remains to be elucidated.

While the InvivoGen Ab is designed as a neutralizing Ab, the others are primarily manufactured for western blotting or immunofluorescence, and their neutralizing activity has not been conclusively determined. However, both the Santa Cruz Bio Ab and InvivoGen Ab demonstrate comparable effectiveness in alleviating mechanical allodynia, with a similar MPE around 90 %. Moreover, the MPE induced by intrathecal TAK-242 was also comparable to that of Santa Cruz Bio or InvivoGen Ab. A similar approach was applied in a previous study that investigated the role of Mincle in traumatic brain injury using a monoclonal antibody (a product of Millipore) designed for immunoblotting. The Millipore Ab was applied to rat cortical neurons to neutralize and inhibit Mincle signaling, resulting in reduced levels of Mincle downstream, including phospho-Syk and TNF-alpha [9].

In contrast, the MPE produced by the Novus Bio Ab was less than 50 % of that observed with Santa Cruz Bio or InvivoGen Ab, indicating relatively lower neutralizing activity. Interestingly, the epitope for Novus Bio Ab includes part of EPN motif (amino acids 169–171 of Mincle), which is known to be well-conserved in mannose-recognizing C-type lectins and crucial for ligand recognition by Mincle [20]. However, the Novus Bio Ab was primarily designed for Mincle detection, and its neutralizing activity has not been investigated. Additionally, its anti-allodynic effect was the weakest compared to the Santa Cruz Bio Ab, which does not bind to the EPN motif. These complex findings suggest the need for further studies to identify key epitopes that enable the highest blocking activity.

Association between Mincle and TLR in their expression and responsiveness has been observed in myeloid cells. While it is found

that TLR is required for Mincle expression in myeloid and dendritic cells [13], Mincle has also been shown to suppress TLR4 signaling stimulated with LPS in cultured splenocyte [12]. Additionally, it is also suggested that Mincle is not merely proinflammatory activator but rather a modulator for immune response [14,15]. However, to our knowledge, the association has not been studied in neuron or glial cells in the central nervous system. The current study found that TLR4 inhibitor and Mincle antibody did not exhibit a significant interaction for the anti-allodynic effect. Furthermore, the timing for the peak and duration of their anti-allodynic effect differed significantly, suggesting that the roles of TLR and Mincle signaling are not closely related to each other in spinal nerve ligation model.

Several weaknesses can be identified in this study. First, the non-specific effect should have been controlled using isotype IgG antibodies to eliminate the possibility of non-specific effect from each Mincle antibody. In additional experiments, we confirmed that intrathecal administration of isotype IgG2b antibodies had no significant impact on pain behavior in animals with spinal nerve ligation (see Supplementary Data 2). Furthermore, epitope analysis revealed that the epitope size of the InvivoGen Ab is longer than the typical range of 5–10 amino acids. However, further conformational mapping would be essential to identify a more accurate and specific epitope for the InvivoGen Ab.

We utilized the rat L5/6 spinal nerve ligation model to induce neuropathic pain, considering the reactivity of the Mincle antibodies. While the Santa Cruz Bio Ab and Novus Bio Ab target human Mincle and exhibit reactivity for both rat and human Mincle, the InvivoGen Ab is derived from rats and designed to specifically interact with murine Mincle, without cross-reactivity with human Mincle. The observed anti-allodynic effect with the InvivoGen Ab in the current study may be attributed to the functional similarity of Mincle among human and rodent species and high level of sequence identity [21]. However, further investigations using a mouse model of neuropathic pain are essential to thoroughly assess the impact of the InvivoGen Ab.

Additionally, the direct relationship between anti-allodynic effect and neutralizing activity of the Mincle antibodies was not established in this study. Nevertheless, we tested the anti-allodynic effect of the three Mincle antibodies in rats with Mincle ligand-induced mechanical allodynia [11], demonstrating significant anti-allodynic effects (see Supplementary Data 3). These additional results provide indirect evidence supporting the mechanism underlying the anti-allodynic effects of the Mincle antibodies.

In conclusion, Mincle antibodies demonstrates a significant anti-allodynic effect and show an additive interaction with TLR4 antagonist in the rat spinal nerve ligation model. This suggest that Mincle plays a pivotal role of Mincle in neuropathic pain and its targeted inhibition with specific antibodies appears to be an effective strategy for managing neuropathic pain.

4. Material and methods

4.1. Experiment animals

Male Sprague–Dawley rats weighing 200–230 g was housed in a room constant temperature of 22–23 °C under an alternating 12 h light/dark cycle with unrestricted access to food and water. All experiments were approved by the Chonnam National University Hospital Animal Care and Ethical Use Committee (CNUHIACUC-18008) and performed according to the International Association for the Study of Pain guidelines for the use of animals in research. The results of this preclinical animal study are described in accordance with the ARRIVE guideline.

4.2. Intrathecal catheter implantation and spinal nerve ligation model

All experimental agents were administered using an intrathecal catheter. A polyethylene-5 catheter was implanted into the intrathecal space following the method described by Yaksh and Rudy for the administration of experimental drugs [22]. Under general anesthesia induced by sevoflurane, the catheter was inserted through the atlanto-occipital membrane and advanced caudally by 8.5 cm to the level of the lumbar enlargement. Any rats displaying neurological deficits after catheter implantation were immediately euthanized with an overdose of inhalational anesthetic. Following surgery, all rats were individually placed in cages. After a recovery period of 5 days, spinal nerve ligation (SNL) was performed to establish a neuropathic pain model [23]. In brief, during sevoflurane anesthesia, the left L5 and L6 spinal nerves were isolated adjacent to the vertebral column and tightly ligated with a 6-0 silk suture distal to the dorsal root ganglia. The surgical procedure for the sham group was identical to that of the SNL group, except that the L5 and L6 spinal nerves were not ligated.

4.3. Administration of Mincle antibodies and assessment of mechanical allodynia

Intrathecal administration of Mincle antibodies and von Frey test were conducted on day 7 following the spinal nerve ligation. The antibodies utilized were purchased from three different suppliers: 1) InvivoGen (rat antibody anti-mMincle-IgG, Cat. # mabg-mmcl), 2) Santa Cruz Biotechnology (mouse antibody Clec4e (B-7), Cat. # sc-390,806), and 3) Novus Biologicals (mouse antibody Clec4e (16E3), Cat. # NBP1-49311). Mincle antibodies were administered using the stock solution of each antibody or its 1/2 or 1/4 saline-diluted solution with an injection volume of 10 μL. The amount of each antibody was as follows: InvivoGen Ab (1.0, 0.5, 0.25 μg/10 μL), Novus Bio Ab (10, 5, 2.5 μg/10 μL), and Santa Cruz Bio Ab (2.0, 1.0, 0.5 μg/10 μL). Additional 10 μL of normal saline was administered to flush the catheter.

Mechanical allodynia was evaluated by applying von Frey filaments to the hind paws of the rats. The paw withdrawal threshold (PWT) was measured using von Frey filaments of various sizes ranging from 0.4 g to 15 g pressure. Each filament was applied to the sole of the paw until it bent slightly and maintained for approximately 5 s. A positive avoidance response, indicated by pawing or licking behavior during filament stimulation, was recorded. The up-down method was utilized to calculate thresholds, with a cutoff

value of 15 g of PWT recorded if no withdrawal or licking response was observed. Assessment of mechanical allodynia was conducted at 15, 30, 60, 90, 120, 150, and 180 min following the intrathecal administration of each antibody. All behavioral experiments were performed by the same researcher, who was blind to the nature of the injectate.

The effect of the TLR4 antagonist, TAK-242, was also examined using the same protocol as described above for the Mincle antibodies. The does for TAK-242 (10, 30, 100 μg) was chosen based on the results of our pilot study.

4.4. Isobolographic analysis for interaction between Mincle Ab and TLR4 antagonist

Isobolographic analysis was conducted to evaluate the pharmacological interaction between Santa Cruz Bio Ab and TAK-242 in their anti-allodynic effect [24–26]. This method involves comparing the doses that equi-effective. The Santa Cruz Bio Ab was chosen for its highest MPE. Initially, the individual ED50 value of Santa Cruz Bio Ab and TAK-242 for anti-allodynic effect were determined using dose-response (MPE) data obtained from time vs PWT measurement. Following this, different groups of animals were received a mixture of various fractions (1/1, 1/2, 1/4 and 1/8) of the ED50 of TAK-242 and Santa Cruz Bio Ab. The anti-allodynic effect of the mixture was assessed using the von Frey test to evaluate the mode of pharmacologic interaction.

To construct the isobologram, the ED50 values of each single agent were plotted on the X and Y axes, respectively, as shown in Fig. 3. The theoretical (additive) dose for combination of Santa Cruz Bio Ab and TAK-242 (theoretical ED50) was calculated based on the individual ED50 of Santa Cruz Bio Ab or TAK-242. The ED50 value of the mixture (experimental ED50) was determined from the dose-response curves of the mixture treatments. Furthermore, total fraction value (TFV) was calculated to describe the magnitude of the interaction. TFV is calculated as follows: TFV = (ED50 of TAK-242 combined with Santa Cruz Bio Ab)/(ED50 for TAK-242 given alone) + (ED50 of Santa Cruz Bio Ab combined with TAK-242)/(ED50 for Santa Cruz Bio Ab given alone). The fractional values indicate the proportion of the single ED50 value that was represented by the corresponding ED50 value for the combination of treatments. TFV near 1 indicate an additive interaction, values greater than 1 imply an antagonistic interaction, and values less than 1 indicate a synergistic interaction.

4.5. Epitope mapping for Mincle antibodies

Epitope mapping via microarray analysis was performed by AbClon Inc (Seoul, Korea). Human and mouse Clec4e sequences were utilized for epitope analysis. Neutral GSGSGSG linkers were added at the C- and N-terminus of the antigen sequences of human and mouse Clec4e to prevent truncated peptide formation. Subsequently, the elongated antigen sequences were converted into linear 15 amino acid peptides, with a peptide-peptide overlap of 14 amino acids. The resulting human and mouse Clec4e peptide microarrays comprised 219 (human Clec4e) or 214 (mouse Clec4e) different peptides, with each peptide printed in duplicate (438 spots for human Clec4e and 428 for mouse Clec4e). The microarrays were framed by additional HA control peptides.

The human and a mouse Clec4e peptide microarray were pre-stained with the different secondary and/or control antibodies in incubation buffer to investigate background interactions with the antigen-derived peptides that could interfere with the main assays. The secondary antibodies were goat anti-rat IgG or goat anti-mouse IgG (H + L) DyLight680, and control antibody was mouse monoclonal anti-HA (12CA5) DyLight800). Subsequent incubation of further human and mouse Clec4e peptide microarrays with the antibodies (Santa Cruz Bio, Novus Bio, InvivoGen Ab) at concentrations of 1 μg/ml, 10 μg/ml and 100 μg/ml was followed by staining with the respective secondary and control antibodies as well as microarray read-out with an Innopsys InnoScan 710-IR Microarray Scanner. The analysis of microarray images was done with PepSlide® Analyzer.

4.6. Statistical analysis

All data are presented as means ± standard error of the mean (SEM). Dose-response data are expressed as a percentage of the maximum possible effect (MPE), calculated using the formula: [(post-drug PWT – post-injury baseline PWT)/(cut-off PWT – post-injury baseline PWT)] × 100(%). A Repeated-measures analysis of variance (ANOVA) with Bonferroni post hoc test was conducted to compare the time course data of PWT among vehicle and Mincle antibodies or TAK-242. Differences in PWT on each time point among groups were compared using one-way ANOVA. The MPE data were analyzed by one-way ANOVA followed by Bonferroni post hoc test.

The experimental ED50 values for Santa Cruz Bio Ab and TAK-242 combinations were determined by least-squares linear regression method, and compared to their theoretical additive ED50 values using *t*-test. A P-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (version 29.0.1.0).

Summary: This study demonstrates the anti-allodynic effect of intrathecal Mincle antibodies in a rat spinal nerve ligation model and highlights the potential of targeting Mincle signaling with specific antibodies, in combination with TLR4 inhibition, as an effective treatment strategy for neuropathic pain.

CRediT authorship contribution statement

Dong Ho Kang: Writing – original draft, Investigation, Conceptualization. **Woong Mo Kim:** Visualization, Formal analysis, Resources. **Hong Beom Bae:** Software, Resources, Formal analysis. **Jihoon Yang:** Writing – review & editing, Project administration, Investigation, Conceptualization. **Jeong Il Choi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Data availability statement

Data will be made available on request.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT4o in order to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Declaration of competing interest

Authors declare that we have no conflict of interest regarding this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40694.

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