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Original Article

Inhibition of RIPK1-driven necroptosis ameliorates inflammatory hyperalgesia caused by lipopolysaccharide: involvement of TLR-, NLRP3-, and caspase-11-mediated signaling pathways

Seda Kurt[1], Sefika Pinar Senol[2], Dilsah Ezgi Yilmaz[1], Omer Bahceli[1], Beyza Ozgen[1], Zainab Sabrie[1], Muhammed Ahmed-Reda Elosman[1], Bahar Tunctan[1*]

[1] *Department of Pharmacology, Faculty of Pharmacy, Mersin University, Mersin, Türkiye*
[2] *Department of Pharmacy Services, Health Services Vocational School, Tarsus University, Tarsus, Mersin, Türkiye*

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Abstract

Increasing evidence suggests that inhibition of receptor-interacting serine/threonine-protein kinase (RIPK) 1/RIPK3/mixed lineage kinase domain-like pseudokinase (MLKL) necrosome has protective effects *in vivo* models of painful conditions seen in humans associated with inflammation and demyelination in the central nervous system. However, the contribution of RIPK1-driven necroptosis to inflammatory pain remains unknown. Therefore, this study aims to determine the effect of necrostatin (Nec) -1s, a selective RIPK1 inhibitor, on lipopolysaccharide (LPS)-induced inflammatory pain and related underlying mechanisms. In the saline-, LPS-, and/or Nec-1s-injected male mice, thermal hyperalgesia was evaluated by hot plate test. Alterations in the expression of proteins involved in the RIPK1, toll-like receptor (TLR) 4, myeloid differentiation factor (MyD) 88/toll-interleukin (IL)-1 receptor domain-containing adapter-inducing interferon-β (TRIF)/nuclear factor (NF)-κB, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing (NLRP) 3/apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)/pro-caspase-1, and caspase-11/gasdermin D (GSDMD) signaling pathways, as well as proteins related to demyelination and remyelination in the brain and spinal cord were determined by the immunoblotting method. The LPS-induced alleviation of thermal hyperalgesia was prevented by necrostatin-1s. Necrostatin-1s reversed (1) increased activity of RIPK1, RIPK3, MLKL, and NF-κB p65, (2) enhanced expression of TLR4, MyD88, TRIF, NF-κB p65, HMGB1, NLRP3, ASC, caspase-1 p20, IL-1β, caspase-11 p20, p30-GSDMD, and semaphorin 3A, and (3) diminished myelin PLP expression induced by LPS. These findings suggest that the use of RIPK1 inhibitors could be a therapeutic approach in the management of inflammatory pain associated with necroptosis, pyroptosis, and demyelination.

Keywords: Necrostatin-1s, Necroptosis, Pyroptosis, İnflammatory hyperalgesia, Demyelination

1. Introduction

Necroptosis is initiated by numerous pro-inflammatory stimuli that require the activation of receptor-interacting serine/threonine-protein kinase (RIPK) 1, RIPK3, and mixed lineage kinase domain-like pseudokinase (MLKL) necrosome complex [1-3]. Among the components of this complex, RIPK1 has been considered an important target for the management of several diseases [1-3]. During necroptotic signaling, RIPK1 is activated by stimulation of specialized cell receptors such as toll-like receptor (TLR) 3/4, tumor necrosis factor (TNF) receptor (TNFR) 1 and Fas receptor [4]. Following the phosphorylation of RIPK1, the necrosome complex is formed between RIPK1, RIPK3, and MLKL (4) Further activation of MLKL by RIPK3 results in the release of high-mobility-group-box (HMGB1) and interleukin (IL)-1 family of cytokines thereby promoting inflammation [1,4].

Studies have also shown that TLRs recognize lipopolysaccharide (LPS) and activate down-stream inflammatory pathways (i.e., the nuclear factor [NF]-κB and pathway) with or without the mediation of myeloid differentiation factor (MyD) 88 [5]. RIPK1 has been reported to be required for the MyD88-independent pathway activity [6]. As demonstrated by Kaiser et al. [7], TLRs, with the adapter protein MyD88, also stimulate activation of RIPK1/RIPK3 indirectly by inducing TNF that initiates necroptosis via TNFR1. However, TLR3 and TLR4 activate RIPK3 directly through toll-IL-1 receptor domain-containing adapter-inducing interferon-β (TRIF). In recent years, RIPK1-mediated necroptosis, initiated by TLR4 ligands (e.g., LPS) has been implicated in several degenerative neuroinflammatory states [1,2,4].

Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing (NLRP) 3, adapter protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1 are

* Corresponding author.
E-mail address: btunctan@gmail.com (B. Tunctan).
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components of the canonical NLRP3 inflammasome that responds to pro-inflammatory stimuli, bacterial toxins, urea, β-amyloid, and adenosine triphosphate [8]. NLRP3 inflammasome activity is also regulated by the RIPK1-mediated necroptotic pathway [9]. In preclinical studies, inhibition of NLRP3 has been proven to exert significant therapeutic effects on in central nervous system (CNS) disorders including diverse inflammatory pain conditions in addition to neurodegenerative diseases [8,10].

Bacterial endotoxins such as lipid A are detected by the caspase-11 inflammasome in rodents [11,12]. Following activation by mainly Gram-negative bacteria, oligomerization of caspase-11 leads to cleaving gasdermin D (GSDMD) into peptides (i.e., p30-GSDMD, GSDMD-N; the catalytic N-terminal domain of GSDMD) which are responsible for pore-formation [13]. Pore formation mediated by GSDMD which results in pyroptosis increased formation of mainly HMGB1 and IL-1β leads to inflammation [14]. In addition, a number of studies highlighted that the NLRP3 inflammasome activation by MLKL and caspase-11/GSDMD pathway results in pyroptosis [10,15].

Findings from numerous studies suggest that inhibition of RIPK1/RIPK3/MLKL necrosome, TLR4/MyD88/NF-κB pathway, or NLRP3/ASC/pro-caspase-1 inflammasome has protective effects *in vivo* models of inflammatory painful conditions such as neuropathic pain [16]. Previous studies showed that hyperalgesia caused by LPS is also associated with enhanced activity of TLR4/MyD88/transforming growth factor-activated kinase (TAK) 1/NF-κB/TNFR-associated factor (TRAF) 6/inhibitor of κB (IκB) kinase (IKK)/IκB-α/NF-κB and NLRC4/ASC/pro-caspase-1 and caspase-11 inflammasomes, and NLRP3/ASC/pro-caspase-1 pathways, as well as increased production of IL-1β in the CNS during endotoxemia [17-22]. According to the results of our recent study, we obtained evidence that not only a reduction in the markers of pyroptosis mediated by caspase-11, necroptosis mediated by RIPK1, and semaphorin (SEMA) 3A but also enhanced expression of myelin proteolipid protein (PLP) in the mice CNS, may involve in the protective effect of necrosulfonamide, a GSDMD and MLKL inhibitor, against hyperalgesia caused by LPS [21]. However, to our knowledge, the effects and the underlying mechanisms of selective pharmacological inhibition of RIPK1-driven necroptosis have not been investigated in inflammatory pain induced by LPS. Therefore, this study aimed to investigate whether necrostatin (Nec)-1s, a selective RIPK1 inhibitor [23], ameliorates hyperalgesia induced by LPS mediated by RIPK1, TLR4, NLRP3, and caspase-11 signaling pathways, in an inflammatory pain model in mice.

2. Materials and Methods

2.1. Chemicals

Lipopolysaccharide (*Escherichia coli* LPS; O111:B4; L4130), DMSO (A1584), and Nec-1s (17802) were from Sigma-Aldrich Co. (St. Louis, MO, USA), Applichem GmbH (Darmstadt, Germany), and Cell Signaling Technology (Massachusetts, MA, USA), respectively. Primary antibodies used include TLR4 (sc-293072), MyD88 (sc-74532), NF-κB p65 (sc-8008); phosphorylated NF-κB p65 (p-NF-κB p65) (sc-33020), HMGB1 (sc-56698), NLRP3 (sc-134306), ASC (sc-22514-R), caspase-1 p20 (sc-398715), IL-1β (sc-52012), caspase-11 p20 (sc-374615), p30-GSDMD (sc-393656), SEMA3A (sc-74554), and β-tubulin (sc-5274) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); myelin PLP (bs-11093R-HRP) from Bioss Antibodies Inc. (Woburn, MA, USA); TRIF (PA5-106871), RIPK3 (PA5-19956), phosphorylated RIPK3 (p-RIPK3) (PA5-105701); MLKL (PA5-102810), and phosphorylated MLKL (p-MLKL) (PA5-105678) from Thermo Fisher Scientific (Waltham, MA USA), RIPK1 (ARG55746) and phosphorylated RIPK1 (p-RIPK1) (ARG66476) from Arigo Biolaboratories (Hsinchu City, Taiwan). Rainbow marker (RPN 800E), goat anti-rabbit IgG-horseradish peroxidase (RPN4301), sheep anti-mouse (RPN4201), and ECL Prime Western Blotting Detection Reagent (RPN2232) were purchased from Amersham Life Sciences (Cleveland, OH, USA).

2.2. Animals

Male mice (BALB/c; weighing 20-30 g) (n=80) were used in this study. The animals provided by the Mersin University Research Center of Experimental Animals were fed on standard chow and housed under a 12-hour light/dark cycle. The study and all experimental protocols involving animals were approved by the Mersin University Experimental Animals Local Ethics Committee (Approval date: June 29, 2021; Protocol number: 2021/30). The experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Inflammatory pain model

The pain model was induced by a single intraperitoneal (i.p.) injection of LPS and pain behavior given through nociceptors was examined by the hot plate test as described previously [18-25]. Results of *in vivo* studies performed in mice suggest that Nec-1s, when administered at doses of 0.3, 1.65, 2, 4, or 10 mg/kg (i.p.) or 0.6, 4, or 6 mg/kg (intravenous), may have beneficial effects on various conditions associated with inflammation and necroptosis, such as TNF-induced systemic inflammatory response syndrome and mortality, age-related neuroinflammation, D-galactosamine (DGal)-, DGal/LPS-, or DGal/TNF-induced hepatic inflammation, and collagen-induced arthritis [26-29]. On the other hand, according to our best knowledge, there is no information in the literature regarding the dose at which Nec-1s can prevent LPS-induced inflammatory hyperalgesia when administered to mice. Based on information found in the literature, the dose-response relationship studies were performed using four doses of Nec-1s in the (1) saline- (control group), (2) 10 mg/kg LPS- (inflammatory hyperalgesia group), (3) saline- plus dimethyl sulfoxide (DMSO)-, (4) saline plus 0.01 mg/kg Nec-1s-, (5) LPS plus 0.001 mg/kg Nec-1s-, (6) LPS plus 0.01 mg/kg Nec-1s-, (7) LPS plus 0.1 mg/kg Nec-1s-, and (8) LPS plus 1 mg/kg Nec-1s-injected mice groups [23]. DMSO or Nec-1s was injected into the mice at 10 ml/kg (i.p.) with saline or LPS. LPS and Nec-1s were prepared in saline and DMSO (10%; 0.11 mg/ml final concentration in saline), respectively. The animals were placed individually on a pre-heated plate (Commat Ltd., Ankara, Türkiye; AHP 9601) at 55 ± 0.2C. The hot plate latency of pain within 1 minute was recorded after the animals showed the first sign of licking the paw 6 hours following the injection of these agents. After the test was completed, the animals were euthanized and the brain and spinal cord tissues were removed for immunoblotting studies.

2.4. Immunoblotting studies

In immunoblotting studies, the tissue homogenate supernatants prepared from brain and spinal cord tissues (30 µg of total protein) were loaded with sodium dodecyl sulfate-polyacrylamide gel (10%) together with a molecular weight marker and subjected to electrophoresis [17-22]. Afterward, the membranes were incubated with primary antibodies. As secondary antibodies, goat anti-rabbit IgG-horseradish peroxidase or sheep anti-mouse were used in the experiments. The membranes were incubated with western blotting detection reagent and immunoreactive bands were visualized using a gel imaging system. To determine the relative densities of immunoreactive bands, Image J densitometry analysis software was used. The intensities of immunoreactive bands for specific proteins were calculated as a ratio to that of β-tubulin.

2.5. Statistical analysis

The data were given as mean ± standard error of the mean (SEM). To determine statistical differences from the saline- or LPS-injected groups, Student's *t*-test was used. A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of Nec-1s on the hyperalgesia caused by LPS

In order to investigate whether RIPK1-driven necroptosis contributes to LPS-induced hyperalgesia, mice were treated with a selective RIPK1 inhibitor, Nec-1s. LPS administration caused a reduction in the hot plate latency of pain compared with saline-injected group values (Figure 1) (P<0.05). The LPS-induced reduction in the hot plate latency of pain was ameliorated by Nec-1s at a dose of 0.01 mg/kg (P<0.05). The other doses of Nec-1s did not significantly affect the decrease in latency observed in the LPS-injected mice (P>0.05). No mortality was observed throughout the experiments. Hereby, tissues of mice treated with 0.01 mg/kg dose of Nec-1s were used for further experiments.

3.2. Effect of Nec-1s on the RIPK1/RIPK3/MLKL necrosome activity

The effects of Nec-1s on alterations caused by LPS administration in RIPK1/RIPK3/MLKL necrosome activity induced by LPS were evaluated by determining expression of unphosphorylated and/or phosphorylated RIPK1, RIPK3, MLKL, and HMGB1 in the tissues. Nec-1s prevented the increased expression of phosphorylated RIPK1, RIPK3, and MLKL in addition to HMGB1 in the brain (Figure 2A-D) and spinal cord (Figure 2E-H) tissues of LPS-injected animals (P<0.05).

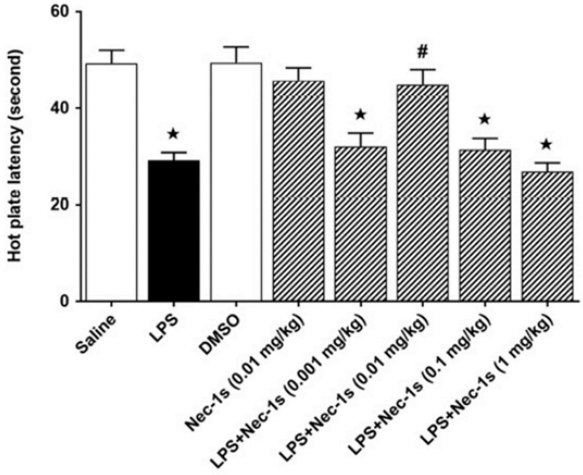

Fig. 1. Nec-1s treatment ameliorated LPS-induced hyperalgesia. Data represent mean ± SEM (n=6-10). *P<0.05 against saline-injected group; #P<0.05 against LPS-injected group.

3.3. Effect of Nec-1s on the TLR4/MyD88/TRIF/NF-κB pathway activity

The effects of Nec-1s on alterations caused by LPS administration in TLR4/MyD88/TRIF/NF-B pathway were evaluated by determining expression of unphosphorylated and/or phosphorylated TLR4, MyD88, TRIF, and NF-κB p65 in the tissues. Nec-1s prevented the increased expression of TLR4, MyD88, TRIF, NF-κB p65, and p-NF-κB p65 in the brain (Figure 3A-D) and spinal cord (Figure 3E-H) tissues of LPS-treated animals (P<0.05).

3.4. Effect of Nec-1s on the activity of NLRP3/ASC/pro-caspase-1- and caspase-11/GSDMD-mediated pathways

To further investigate whether Nec-1s affects the alterations induced by LPS in the components of NLRP3/ASC/pro-caspase-1 inflammasome and caspase-11/GSDMD expression, protein levels of NLRP3, ASC, caspase-1 p20, IL-1β, caspase-11 p20, and p30-GSDMD were evaluated in the tissues. Nec-1s prevented the increased expression of NLRP3, ASC, caspase-1 p20, IL-1β, caspase-11 p20, and

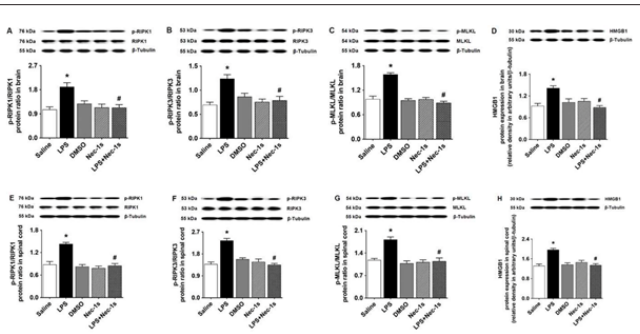

Fig. 2. Nec-1s treatment reversed the enhanced activity of RIPK1/RIPK3/MLKL necrosome induced by LPS in the (A), (B), (C), and (D) brain and (E), (F), (G), and (H) spinal cord of mice. Data represent mean ± SEM (n=4). *P<0.05 against saline-injected group; #P<0.05 against LPS-injected group.

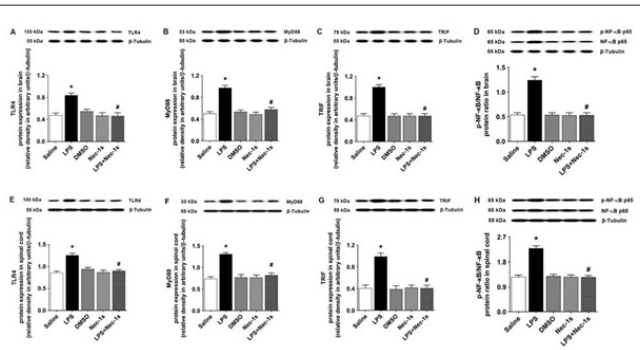

Fig. 3. Nec-1s treatment reversed the increased activity of the TLR4/MyD88/TRIF/NF-κB signaling pathway induced by LPS in the (A), (B), (C), and (D) brain and (E), (F), (G) and (H) spinal cord of mice. Data represent mean ± SEM (n=4). *P<0.05 against saline-injected group; #P<0.05 against LPS-injected group.

p30-GSDMD in the brain (Figure 4A-F) and spinal cord (Figure 4G-L) tissues of LPS-injected animals (P<0.05).

3.5. Effect of Nec-1s on the SEMA3A expression

To determine whether Nec-1s affects the LPS-induced alterations in the SEMA3A expression, protein levels of SEMA3A were evaluated in the tissues. Nec-1s prevented the increased expression of SEMA3A in the brain (Figure 5A) and spinal cord (Figure 5B) tissues of LPS-injected animals (P<0.05).

3.6. Effect of Nec-1s treatment on the myelin PLP expression

To further whether Nec-1s affects the LPS-induced alterations in the myelin PLP expression, protein levels of myelin PLP were evaluated in the tissues. Nec-1s prevented the reduction in myelin PLP expression in the brain (Figure 6A) and spinal cord (Figure 6B) tissues of LPS-treated animals (P<0.05).

4. Discussion

The results of this study demonstrate that selective RIPK1 inhibitor, Nec-1s, ameliorates LPS-induced hyperalgesia by inhibiting enhanced expression/activity of RIPK1, RIPK3, MLKL, TLR4, MyD88, TRIF, NF-κB p65, HMGB1, NLRP3, ASC, caspase-1 p20, IL-1β, caspase-11 p20, p30- gasdermin D, and SEMA 3A expression in addition to diminished expression of myelin PLP. The findings of this study provide evidence that Nec-1s ameliorates inflammatory hyperalgesia induced by LPS both suppressing RIPK1/RIPK3/MLKL, TLR4/MyD88/TRIF/NF-κB, NLRP3/ASC/pro-caspase-1, and caspase-11/GSDMD pathways and preventing demyelination as well as promoting remyelination in the mice CNS (Figure 7).

In response to thermal stimuli, LPS is reported to enhance sensation of pain at spinal and supraspinal levels in rodent models of pain [30]. Increasing evidence demonstrates that RIPK1/RIPK3/MLKL-, TLR4/MyD88/TRIF/NF-κB-, NLRP3/ASC/pro-caspase-1-, and caspase-11/GSDMD-mediated necroptotic, inflammatory, and pyroptotic pathways play a critical role in the pathophysiology of models of CNS diseases caused by LPS together with neuroinflammatory and neurodegenerative alterations [1-4,8,30]. In addition, studies are reporting that selective RIPK1 inhibitors such as Nec-1s can prevent necroptosis-mediated neuroinflammation by entering the CNS when administered systemically [28]. Nevertheless, the effects of selective RIPK1 inhibition on the changes in the signaling pathways triggered by LPS during inflammatory hyperalgesia in the CNS have not been investigated. Our findings demonstrate that systemically Nec-1s administration into mice ameliorates hyperalgesia induced by LPS. Therefore, the results are also consistent with the findings of aforementioned *in vivo* studies showing that Nec-1s can prevent inflammatory hyperalgesia induced by LPS and perceived at the supraspinal level. Furthermore, increased phosphorylation of RIPK1, RIPK3, MLKL, and NF-κB p65 proteins were reversed by Nec-1s in ad-

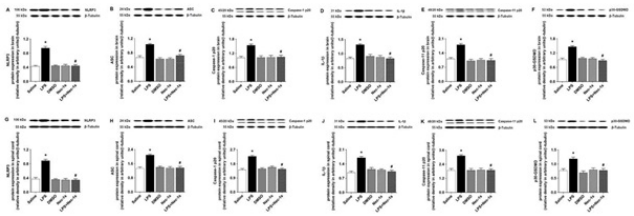

Fig. 4. Nec-1s treatment reversed the LPS-induced increase in the expression of the components of NLRP3/ASC/pro-caspase-1- and caspase-11/GSDMD-mediated pathways induced by LPS in the (A), (B), (C), (D), (E) and (F) brain and (G), (H), (I), (J), (K), and (L) spinal cord of mice. Data represent mean ± SEM (n=4). *P<0.05 against saline-injected group; #P<0.05 against LPS-injected group.

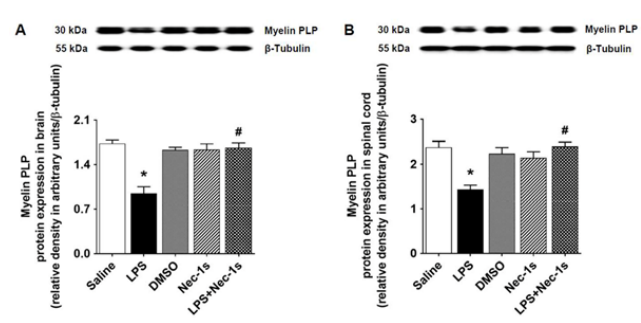

Fig. 5. Nec-1s treatment reversed the LPS-induced decrease in the expression of SEMA3A induced by LPS in the (A) brain and (B) spinal cord of mice. Data represent mean ± SEM (n=4). *P<0.05 against saline-injected group; #P<0.05 against LPS-injected group.

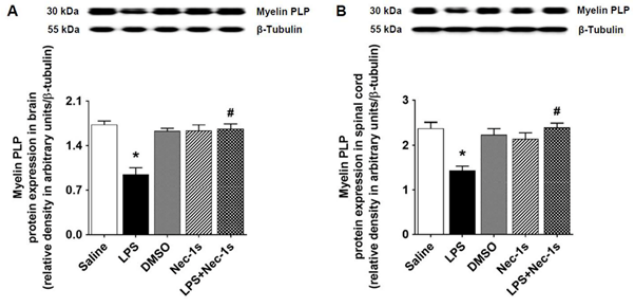

Fig. 6. Nec-1s treatment reversed the LPS-induced decrease in the expression of myelin PLP in the (A) brain and (B) spinal cord of mice. Data represent mean ± SEM (n=4). *P<0.05 against saline-injected group; #P<0.05 against LPS-injected group.

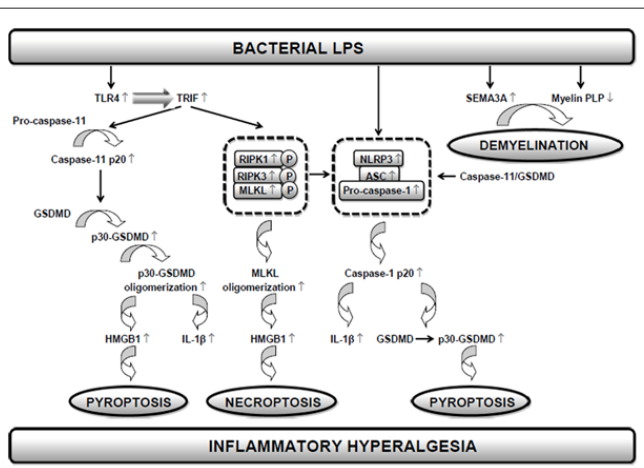

Fig. 7. Diagram showing the effect of RIPK1 inhibitor, Nec-1s, on LPS-induced alterations in the RIPK1/RIPK3/MLKL-, TLR4/MyD88/TRIF/NF-κB-, NLRP3/ASC/pro-caspase-1-, and caspase-11/GSDMD-mediated necroptotic and pyroptotic pathways in the CNS of mice during inflammatory hyperalgesia. (↑) Increased by LPS; (↓) decreased by LPS.

dition to TLR4, MyD88, TRIF, NLRP3, ASC, caspase-1 p20, caspase-11 p20, and GSDMD protein expression in the CNS of LPS-treated mice. As indicators of the activity of RIPK1/RIPK3/MLKL, TLR4/MyD88/TRIF/NF-κB, NLRP3/ASC/pro-caspase-1, and caspase-11/GSDMD signaling pathways, increased expression of IL-1β and HMGB1 induced by LPS was also reversed by Nec-1s in these tissues. Thus, the results of this study provide novel findings suggesting that Nec-1s ameliorates hyperalgesia via the reduced formation and release of pro-inflammatory mediators (e.g., IL-1β and HMGB1) by necroptotic and pyroptotic cells through suppression of the RIPK1/RIPK3/MLKL, TLR4/MyD88/TRIF/NF-κB, NLRP3/ASC/pro-caspase-1, and caspase-11/GSDMD signaling pathways [17-22]. It is also possible that binding to MLKL directly to prevent oligomerization of MLKL, as well as, indirectly or directly reducing the RIPK1-driven necrosome activity in the mice CNS treated with LPS participates in the protecting effect of Nec-1s. Moreover, Nec-1s may directly suppress the increased expression and/or activity of proteins involved in the TLR4/MyD88/TRIF/NF-κB, NLRP3/ASC/pro-caspase-1-, and/or caspase-11/GSDMD signaling pathways. Considering the interactions between these signaling pathways have been demonstrated in numerous studies, the proposed mechanisms do not seem surprising [15]. On the other hand, detailed studies are necessary to verify these conjectures.

SEMA3A, an endogenous remyelination inhibitor, is reported to have enhanced levels in the brain of LPS-injected mice [31]. A reduction of myelin proteins (e.g., PLP) associated with neuroinflammatory alterations in the CNS in response to LPS administration in rats has also been reported [32]. Nevertheless, the effect of Nec-1s on the LPS-induced alterations in the SEMA3A and myelin PLP expression is currently unknown. In agreement with the findings of previous studies [32], we also demonstrated that treatment of mice LPS results in increased SEMA3A protein expression as well as reduced myelin PLP expression in the mice CNS. Furthermore, Nec-1s reversed the alterations in not only the expression of SEMA3A but also myelin PLP induced by LPS. Thus, the findings suggest that Nec-1s reduces demyelination and facilitates remyelination caused by inflammatory hyperalgesia.

An important point to emphasize is that Nec-1s, which is a more specific RIPK1 inhibitor and has a superior safety profile compared to other necrostatin derivatives such as Nec-1, also have less *in vivo* and *in vitro* toxicity [23]. Nevertheless, according to our best knowledge, there are no studies in the literature investigating the side and/or toxic effects of Nec-1s treatment, especially in the LPS-induced inflammatory hyperalgesia model in mice. On the other hand, in a recent study, we observed that following LPS (10 mg/kg; i.p.) injection into mice, the mortality rates were 10%, 50%, and 60% at the 24th, 36th, and 48th hours, respectively [33]. When endotoxemic mice were treated with Nec-1s (0.01 mg/kg; i.p.), mortality rates were 60%, 90%, and 100% at the 18th, 30th, and 42nd hours, respectively. Mice injected with saline, DMSO (10%; 10 ml/kg), or Nec-1s survived during the experiments. The results of the study also indicated that moderate to serious injury regarding histopathological changes related to hemorrhage, interstitial edema, vascular congestion, inflammatory cell infiltration, and necrosis in the heart and kidney tissues also occurred 6 hours after injection of LPS into mice. The scores regarding histopathological changes determined in the tissues of rats administered DMSO or Nec-1s with saline or LPS were higher than the control group values, indicating a mild degree of injury. Nec-1s attenuated these LPS-induced histopathological changes only in the kidney tissue. Furthermore, in another study performed in a rodent model of septic shock, we observed a fall in blood pressure and a rise in heart rate associated with increased systemic and arterial RIPK1/RIPK3/MLKL necrosome activity in addition to histopathological changes in the heart, kidney, brain, and lung tissues isolated 4 hours after LPS (10 mg/kg; i.p.) administration to rats [34]. In this study, Nec-1s (1 mg/kg; i.p.) not only prevented hypotension, tachycardia, and systemic and arterial RIPK1/RIPK3/MLKL necrosome activity but also reduced the elevated scores regarding histopathological changes only in the kidney tissue isolated from rats in the LPS-treated group. Systemic administration of DMSO (1%; 4 ml/kg) or Nec-1s to healthy rats also resulted in histopathological changes similar to LPS-induced heart, kidney, brain, and lung injury. In the present study, we did not observe mortality or changes in animal welfare during the *in vivo* experiments performed with LPS, DMSO, or Nec-1s. Therefore, we suggest that the treatment of healthy and endotoxemic mice with Nec-1s, especially at its effective dose that prevents LPS-induced inflammatory hyperalgesia, has no negative side effects. It is also essential to emphasize that the therapeutic application of Nec-1s requires extreme caution because itself and/or its solvent also cause tissue damage. On the other hand, to make Nec-1s suitable for clinical use, studies need to be carried out to develop an appropriate pharmaceutical formulation using a solvent that will not cause organ damage.

5. Conclusion

The novel observation that selective RIPK1 inhibitor, Nec-1s, not only ameliorates hyperalgesia but also decreases the activity of RIPK1/RIPK3/MLKL, TLR4/MyD88/TRIF/NF-κB, NLRP3/ASC/pro-caspase-1, and caspase-11/GSDMD pathways triggered by LPS in the mice CNS. The results of the current study suggest that the use of systemically applicable selective RIPK1 inhibitors such as Nec-1s could be a promising approach for the treatment of inflammatory pain-related diseases. Our data also suggest that inhibition of additional nonspecific targets other than RIPK1-dependent necroptosis in the CNS also contributes to the preventive effect of Nec-1s on the inflammatory hyperalgesia induced by LPS. Therefore, effects of Nec-1s on the nonspecific targets and/or pathways should be investigated further. While RIPK1 inhibition can prevent LPS-induced hyperalgesia, exploring the long-term effects of Nec-1s treatment on inflammatory pain and demyelination in the CNS would also provide important information regarding its sustained therapeutic benefits. Moreover, further detailed *in vitro*, *in vivo*, and *ex vivo* studies regarding the possible side and toxic effects of Nec-1s in vital organs such as the brain, heart, kidney, lung, and liver will provide scientific support for the clinical use of selective RIPK1 inhibitors in the treatment of inflammatory pain. In addition, since the number of preclinical and clinical studies on the treatment approaches using RIPK1 inhibitors like Nec-1s, especially for the treatment of necroinflammatory diseases, has been increasing in recent years, it can be suggested that the translatio-

nal potential of these inhibitors in clinical settings for the management of inflammatory pain-related diseases.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed Consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Interest of conflict
The authors declare no conflict of interest/competing interests.

Author' contribution
BT conceptualized and conceived the research design, analyzed the data, and drafted the manuscript. BT, SK, SPS, DEY, OB, BO, ZS, and MAE carried out the experiments. All authors read and approved the final manuscript.

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References

1. Gardner CR, Davies KA, Zhang Y, Brzozowski M, Czabotar PE, Murphy JM, Lessene G (2023) From (tool) bench to bedside: the potential of necroptosis inhibitors. J Med Chem 66:2361-2385. doi:10.1021/acs.jmedchem.2c01621
2. Li W, Yuan J (2023) Targeting RIPK1 kinase for modulating inflammation in human diseases. Front Immunol 14:1159743. doi:10.3389/fimmu.2023.1159743
3. Xu L, Zhang W, Zhuang C (2023) Receptor-interacting protein kinase 1 (RIPK1) inhibitor: a review of the patent literature (2018-present). Expert Opin Ther Pat 33:101-124. doi:10.1080/13543776.2023.2195548
4. Mitroshina EV, Saviuk M, Vedunova MV (2023) Necroptosis in CNS diseases: focus on astrocytes. Front Aging Neurosci 14:1016053. doi:10.3389/fnagi.2022.1016053
5. Murad S (2014) Toll-like receptor 4 in inflammation and angiogenesis: a double-edged sword. Front Immunol 5:313. doi:10.3389/fimmu.2014.00313
6. Bohannon JK, Hernandez A, Enkhbaatar P, Adams WL, Sherwood ER (2013) The immunobiology of toll-like receptor 4 agonists: from endotoxin tolerance to immunoadjuvants. Shock 2013; 40: 451-462. doi:10.1097/SHK.0000000000000042
7. Kaiser WJ, Sridharan H, Huang C, Mandal P, Upton JW, Gough PJ, Sehon CA, Marquis RW, Bertin J, Mocarski ES (2013) Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. J Biol Chem 288:31268-31279. doi:10.1074/jbc.M113.462341
8. Chen C, Smith MT (2023) The NLRP3 inflammasome: role in the pathobiology of chronic pain. Inflammopharmacology 31:1589-1603. doi:10.1007/s10787-023-01235-8
9. Prasad Panda S, Kesharwani A, Prasanna Mallick S, Prasanth D, Kumar Pasala P, Bharadwaj Tatipamula V (2023) Viral-induced neuronal necroptosis: detrimental to brain function and regulation by necroptosis inhibitors. Biochem Pharmacol 213:115591. doi:10.1016/j.bcp.2023.115591
10. Speir M, Lawlor KE (2021) RIP-roaring inflammation: RIPK1 and RIPK3 driven NLRP3 inflammasome activation and autoinflammatory disease. Semin Cell Dev Biol 109:114-124. doi:10.1016/j.semcdb.2020.07.011
11. Garcia-Vello P, Di Lorenzo F, Zucchetta D, Zamyatina A, De Castro C, Molinaro A (2022) LPS lipid A: a promising molecule for new immunity-based therapies and antibiotics. Pharmacol Ther 230:107970. doi:10.1016/j.pharmthera.2021.107970
12. Wright SS, Vasudevan SO, Rathinam VA (2022) Mechanisms and consequences of noncanonical inflammasome-mediated pyroptosis. J Mol Biol 434:167245. doi:10.1016/j.jmb.2021.167245
13. Rathkey JK, Zhao J, Liu Z, Chen Y, Yang J, Kondolf HC, Benson BL, Chirieleison SM, Huang AY, Dubyak GR, Xiao TS, Li X, Abbott DW (2018) Chemical disruption of the pyroptotic pore-forming protein gasdermin D inhibits inflammatory cell death and sepsis. Sci Immunol 3:eaat2738. doi:10.1126/sciimmunol.aat2738
14. Heilig R, Dick MS, Sborgi L, Meunier E, Hiller S, Broz P (2018) The gasdermin-D pore acts as a conduit for IL-1β secretion in mice. Eur J Immunol 48:584-92. doi:10.1002/eji.201747404
15. Gullett JM, Tweedell RE, Kanneganti TD (2022) It's all in the PAN: crosstalk, plasticity, redundancies, switches, and interconnectedness encompassed by PANoptosis underlying the totality of cell death-associated biological effects. Cells 11:1495. doi:10.3390/cells11091495
16. Fang P, Sun G, Wang J (2021) RIP3-mediated necroptosis increases neuropathic pain via microglia activation: necrostatin-1 has therapeutic potential. FEBS Open Bio 11:2858-2865. doi:10.1002/2211-5463.13258
17. Bahceli O, Senol SP, Temiz-Resitoglu M, Horat MF, Sahan-Firat S, Tunctan B (2022) Bexarotene ameliorates LPS-induced hyperalgesia: contribution of TLR4/MyD88-dependent pro-inflammatory, anti-apoptotic, and anti-inflammatory signaling pathways. Int J Pharmacol 18:1171-1188. doi:10.3923/ijp.2022.1171.1188
18. Cagli A, Senol SP, Temiz-Resitoglu M, Guden DS, Sari AN, Sahan-Firat S, Tunctan B (2021) Soluble epoxide hydrolase inhibitor trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea prevents hyperalgesia through regulating NLRC4 inflammasome-related pro-inflammatory and anti-inflammatory signaling pathways in the lipopolysaccharide-induced pain mouse model. Drug Dev Res 82:815-825. doi:10.1002/ddr.21786
19. Dolunay A, Senol SP, Temiz-Resitoglu M, Guden DS, Sari AN, Sahan-Firat S, Tunctan B (2017) Inhibition of NLRP3 inflammasome prevents LPS-Induced inflammatory hyperalgesia in mice: contribution of NF-κB, caspase-1/11, ASC, NOX, and NOS isoforms. Inflammation 40:366-386. doi:10.1007/s10753-016-0483-3
20. Senol SP, Temiz-Resitoglu M, Guden DS, Sari AN, Sahan-Firat S, Tunctan B (2021) Suppression of TLR4/MyD88/TAK1/NF-kB/COX-2 signaling pathway in the central nervous system by bexarotene, a selective RXR agonist, prevents hyperalgesia in the lipopolysaccharide-induced pain mouse model. Neurochem Res 46:624-637. doi:10.1007/s11064-020-03197-7
21. Ozgen B, Senol SP, Yilmaz DE, Temiz-Resitogu M, Bahceli O (2023) Pyroptosis and necroptosis inhibitor necrosulfonamide ameliorates LPS-induced inflammatory hyperalgesia in mice. Pharmacia 70:1345-1354. doi:10.3897/pharmacia.70.e108995
22. Yilmaz DE, Senol SP, Temiz-Resitoglu M, Sahan-Firat S, Tunctan B (2023) NLRX1 ligand, docosahexaenoic acid, ameliorates LPS-induced inflammatory hyperalgesia by decreasing TRAF6/IKK/IkB-a/NF-kB signaling pathway activity. Cell Mol Biol 69:15-23. doi:10.14715/cmb/2023.69.9.3
23. Takahashi N, Duprez L, Grootjans S, Cauwels A, Nerinckx W, DuHadaway JB, Goossens V, Roelandt R, Van Hauwermeiren F, Libert C, Declercq W, Callewaert N, Prendergast GC, Degterev A, Yuan J, Vandenabeele P (2012) Necrostatin-1 analogues:

critical issues on the specificity, activity and in vivo use in experimental disease models. Cell Death Dis 3:e437. doi:10.1038/cddis.2012.176

24. Deuis JR, Dvorakova LS, Vetter I (2017) Methods used to evaluate pain behaviors in rodents. Front Mol Neurosci 10:284. doi:10.3389/fnmol.2017.00284
25. Tunctan B, Ozveren E, Korkmaz B, Buharalioglu CK, Tamer L, Degirmenci U, Atik U (2006) Nitric oxide reverses endotoxin-induced inflammatory hyperalgesia via inhibition of prostacyclin production in mice. Pharmacol Res 53:177-192. doi:10.1016/j.phrs.2005.10.009
26. Jhun J, Lee SH, Kim SY, Ryu J, Kwon JY, Na HS, Jung K, Moon SJ, Cho ML, Min JK (2019) RIPK1 inhibition attenuates experimental autoimmune arthritis via suppression of osteoclastogenesis. J Transl Med 17:84. doi:10.1186/s12967-019-1809-3
27. Koschel J, Nishanth G, Just S, Harit K, Kröger A, Deckert M, Naumann M, Schlüter D (2022) OTUB1 prevents lethal hepatocyte necroptosis through stabilization of c-IAP1 during murine liver inflammation. Cell Death Differ 29:891. doi: 10.1038/s41418-021-00819-7
28. Thadathil N, Nicklas EH, Mohammed S, Lewis TL Jr, Richardson A, Deepa SS (2021) Necroptosis increases with age in the brain and contributes to age-related neuroinflammation. Geroscience 43:2345-2361. doi:10.1007/s11357-021-00448-5
29. Zhuang Y, Xu HC, Shinde PV, Warfsmann J, Vasilevska J, Sundaram B, Behnke K, Huang J, Hoell JI, Borkhardt A, Pfeffer K, Taha MS, Herebian D, Mayatepek E, Brenner D, Ahmadian MR, Keitel V, Wieczorek D, Häussinger D, Pandyra AA, Lang KS, Lang PA (2020) Fragile X mental retardation protein protects against tumour necrosis factor-mediated cell death and liver injury. Gut 69:133-145. doi:10.1136/gutjnl-2019-318215
30. Kalyan M, Tousif AH, Sonali S, Vichitra C, Sunanda T, Praveenraj SS, Ray B, Gorantla VR, Rungratanawanich W, Mahalakshmi AM, Qoronfleh MW, Monaghan TM, Song BJ, Essa MM, Chidambaram SB (2022) Role of endogenous lipopolysaccharides in neurological disorders. Cells 11:4038. doi:10.3390/cells11244038
31. Ito T, Yoshida K, Negishi T, Miyajima M, Takamatsu H, Kikutani H, Kumanogoh A, Yukawa K (2014) Plexin-A1 is required for toll-like receptor-mediated microglial activation in the development of LPS-induced encephalopathy. Int J Mol Med 33:1122-1130. doi:10.3892/ijmm.2014.1690
32. Huang P, Chen X, Hu X, Zhou Q, Lin L, Jiang S, Fu H, Xiong Y, Zeng H, Fang M, Chen C, Deng Y (2020) Experimentally induced sepsis causes extensive hypomyelination in the prefrontal cortex and hippocampus in neonatal rats. Neuromolecular Med 22:420-436. doi:10.1007/s12017-020-08602-6
33. Tunctan B, Senol SP, Yilmaz DE, Bahceli O, Kara T, Elosman MA (2024) RIPK1/RIPK3/MLKL necrosome contributes to the nitrosative stress-mediated cardiorenal injury and mortality in lipopolysaccharide-induced sepsis model in mice. Paper presented at the 16th International Medical and Health Sciences Researches Congress, Ankara, Türkiye, ISBN: 978-625-6671-29-4, Article ID: 27.
34. Tunctan B, Senol SP, Yilmaz DE, Bahceli O, Kara T, Ahmed-Reda Elosman M (2023) Activation of vascular RIPK1/RIPK3/MLKL necrosome contributes to the iNOS-mediated hypotension in lipopolysaccharide-induced septic shock model in rats. Paper presented at the 27th National and 2st International Pharmacology Congress, Antalya, Türkiye, ISBN: 978-625-6671-29-4, Article ID: O1.