nature portfolio

Peer Review File

Transferrin-Phosphatidylserine Liposomes Target TDP-43 and Neuroinflammation in Male Mice with Neuropathic Pain

Corresponding Author: Dr Jiamin Miao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study has executed sound methodology, utilized various techniques, overall presented the data effectively, is soundly written and generally provides an interesting perspective regarding the specific targeting of glial cells using TF/PS/TDP-43-LPs. Research that repurposes and/or refines the utility of specific protein inhibitors such as TDP-43 aggregation inhibitors is a promising area of investigation and relevant for various neurological conditions. While this study holds significant potential to contribute to the field by identifying therapeutic alternatives and targets for neuropathic pain, in its present format, this manuscript presents major concerns that need to be addressed in order for it to fully realize its impact and make a more substantial contribution. Major revisions are necessary for this manuscript be considered for the high standard of publication in Nature Communications.

Major concerns:

This study focuses on the aberrant actions/interactions of microglia and astrocytes in driving neuropathic pain. While aberrant interactions between microglia and astrocytes are critical drivers of neuropathic pain, they act within a broader, interconnected system that includes neurons and peripheral components. These glial interactions may amplify and sustain pain states, but they cannot fully account for the initiation or all mechanisms of neuropathic pain. Thus, a comprehensive understanding requires considering all cellular players and their dynamic interplay, critically neurons.

In its present format this study lacks critical insights into the bi-directional interactions of neurons and glia in the context of neuropathic pain and neuroinflammation. This is of particular significance given neurons are central to the development and perpetuation of neuropathic pain and neuron-glial interactions underlie neuroinflammatory processes, as neurons can signal glial cells to release inflammatory factors and vice versa in response to insults. How can an assessment of neuroinflammation be made without consideration of neurons and what about neurotransmitter release which underlies neuropathic pain?

While it's appreciated that the specific role of non-neuronal cells- glia is focused on throughout the paper to optimize drug delivery and targeting cellular mechanisms of pain regulation rather than merely blocking pain signals- particularly in the cell culture models, it is not possible to draw conclusions as this study does regarding an alleviation of neuropathic pain (and subsequent damage/prevention of damage to neurons/implications for synaptic pruning of neurons) without assessment of the neuron populations themselves. This could have been achieved by staining for neuronal markers (e.g. MAP2, NeuN, B-II-tubulin) in the CCI mouse model brain and spinal cord tissue, or even assessment of the gene enrichment of the neuron population identified using scRNAseq of the spinal cord or assessment of neuronal integrity/synaptic markers/neurotransmitters using qPCR. It is confusing as to why these rather obvious and yet straightforward assessments were not conducted for a more in depth understanding of TF/PS/TDP-43-LPs in alleviating aberrant mechanisms in neuropathic pain specifically. In its current format, this study provides a very simplistic interpretation and modelling of treatment of neuropathic pain using TF/PS/TDP-43-LPs and largely ignoring neurons, the central population of interest, is a significant flaw of this study. This study risks misinterpreting glial contributions and in its current format is not translatable providing an incomplete picture of the assessment of therapeutics for neuroinflammation underlying neuropathic pain.

While the study identifies new concepts regarding TF/PS/TDP-43-LPs inhibition of the activation of cGAS-STING pathways in astrocytes and microglia in the CCI mouse model (spinal cord scSeq), assessed in isolation without the consideration of

neurons and neuron-glia interactions restricts the interpretation of this data in the context of neuroinflammatory mechanisms underlying neuropathic pain specifically. Without neuronal assessment, it is unclear whether observed pain modulation stems from direct effects on neurons, indirect effects via glial modulation, or a combination of both. Furthermore, neurons may express components of the cGAS-STING pathway. Inhibiting this pathway in glia alone does not rule out neuronal involvement or contributions to pain signaling, which might operate independently or synergistically with glial mechanisms. Targeting glial pathways alone with TF/PS/TDP-43-LPs might not account for pain persistence driven by neuronal maladaptation, leading to incomplete therapeutic efficacy. Without evaluating neuronal changes, the relevance of cGAS-STING pathway inhibition to clinical outcomes remains uncertain.

Furthermore, TDP-43, the therapeutic target in this study, has significant roles within the CNS, shuttling between the nucleus and cytoplasm and is vital for the regulation of RNA processes that support neuronal integrity and synaptic function. In addition, TDP-43 expression in glial cells ensures their proper functioning which is essential for maintaining neuronal health and in regulating immune responses in the CNS. This study utilizes TDP-43 aggregation inhibitors, with promising data shown in glial cells, however, the study fails to provide assessment of homeostatic levels of TDP-43 or the effects of TF/PS/TDP-43-LPs on 'normal' TDP-43 expression within the CNS, particularly the effects of targeting TDP-43 in neurons and the implications of this for the modulation of the cGAS-STING pathway. Staining or western blot analysis of pan-TDP-43/wild-type TDP-43 protein expression is required to elucidate further the mechanisms of action of TF/PS/TDP-43-LPs in the context of neuroinflammation and neuropathic pain.

Minor revisions
• Introduction: lines 72-74, ‘TDP-43 aggregation inhibition shown promise in several ND diseases’ this statement requires references
• It is unclear throughout, which brain region specifically is being stained for in the CCI mouse model and how this brain/tissue region is relevant for the investigation of neuropathic pain?
• The figure legends are somewhat vague and often do not explain/provide enough information to fully interpret the data in many instances. While it is appreciated there is a limit to figure legends, they should be detailed enough so that each figure and caption can, as far as possible, be understood in isolation from the main text- at present this is not the case for many of the figure legends.
Fig 3E & F: Treatment/group labels missing from histological images
Supp 4A: left panel is incorrectly labelled/order is wrong, DAPI is labelled GFAP and GFAP is labelled DAPI
Figure 4D or a supplementary figure should show staining of controls (sham/CCI) alongside treated for more accurate interpretation of results
Fig 4D: Why was IBA1 used here as opposed to CD68/CD206 as used to demonstrate microglial expression in Fig 3F? The IBA1 staining in Fig 4D appears to have more of a neuronal phenotype and does not resemble the typical staining morphology for microglia stained with IBA1. To address this, counterstaining with neuronal markers (e.g. NeuN, MAPT) is advised.
Fig 6A+C staining panels would benefit from the addition of DAPI staining in each panel to better show nuclei especially when trying to interpret ‘merge’ representative images
Fig 7A+B staining panels would benefit from the addition of DAPI staining in each panel to better show nuclei especially when trying to interpret ‘merge’ representative images
Fig 7B: still not convinced about the IBA1 staining being 'microglial' specific, morphology appears neuronal-like as mentioned previously. For further context and interpretation of results, this figure would significantly benefit from NeuN/MAP2 counter-staining to determine effects of treatment on neurons and also to confirm no overlap of microglial staining with neuronal staining.
Fig 7C: Western blot should also include quantification of IBA1 and GFAP protein levels to confirm expression across treatment groups and further support staining data. The study would also benefit from assessment of wild-type/pan-TDP43 protein expression via western blot across treatment groups.

Reviewer #2

(Remarks to the Author)
The authors present an interesting study utilizing phosphatidylserine (PS) and transferrin (TF)-modified liposomes (TF/PS/TDP-43-LPs) loaded with a TDP-43 aggregation inhibitor as a potential treatment for neuropathic pain (NP) in a chronic constriction injury (CCI) mouse model. While the study demonstrates promising results, including effective blood-brain barrier (BBB) penetration and NP alleviation linked to cGAS-STING pathway inhibition and microglial-astrocytic crosstalk modulation, several critical limitations undermine its impact and clarity.

Major Concerns:
1. Unclear Mechanistic Insights into TDP-43 Inhibition:
The manuscript lacks evidence directly linking TDP-43 inhibition to the observed therapeutic effects. While behavioral and anti-inflammatory outcomes are reported, no data confirms whether these are specifically mediated by TDP-43 inhibition or are broader anti-inflammatory responses. Experiments to measure TDP-43 protein levels or aggregation (e.g., Western blot, immunohistochemistry) in treated tissues are essential. Furthermore, testing alternative TDP-43 inhibitors or non-specific anti-inflammatory agents would help determine if the effects are TDP-43-specific. This lack of mechanistic clarity significantly weakens the study's claims.

2. Role of TDP-43 in cGAS-STING Pathway Activation:
The study identifies the cGAS-STING pathway as a key target but does not sufficiently explore the role of TDP-43 in

activating this pathway. Previous studies suggest a link between TDP-43 and cGAS-STING activation. Knockdown experiments using siRNA to silence TDP-43 in microglia or astrocytes could provide direct evidence for its involvement. Without this, the connection between TDP-43 inhibition and cGAS-STING suppression remains speculative.

3. Lack of Analysis on Nerve Repair and Immune Modulation:
The manuscript does not investigate whether the nanoparticles directly promote nerve repair or primarily modulate immune responses. This omission limits understanding of the therapeutic mechanism. Nerve tissue analysis should be conducted. Additionally, quantifying inflammatory cytokines and immune cell infiltration in the injured nerves would offer a more comprehensive evaluation of the nanoparticles' effects.

4. Ambiguity in Nanoparticle Naming and Terminology:
The naming of the TDP-43 inhibitor and nanoparticles is confusing. For example, "CCI+TF/PS/TDP-43-LPs" could mislead readers into thinking the nanoparticles contain TDP-43 protein rather than an inhibitor. Moreover, referring to the TDP-43 inhibitor as "TDP-43-IN-1," its established name in the literature, would ensure consistency and clarity. The current terminology detracts from the study's focus and accessibility.

5. Limited Insights into Nanoparticle Uptake and Release Mechanisms:
The manuscript does not clarify how nanoparticles are internalized by cells or how they release their cargo. Without this information, the therapeutic efficacy cannot be fully understood. Investigations using endocytosis inhibitors, fluorescence imaging to trace uptake, and assays like LysoTracker to study endosomal escape are necessary. Linking in vitro drug release profiles to in vivo pharmacokinetics would further validate the proposed delivery mechanism.

6. Missing and incorrect References:
The potential roles of TDP-43 aggregation inhibitor are not property cited in the manuscript.

Minor Concerns:
1. Behavioral Assays:
Behavioral recovery conclusions rely on a single test. Additional tests, such as those for thermal hyperalgesia (Hargreaves' test), cold allodynia (acetone test), and motor functon(rotarod test), would provide a more robust assessment of neuropathic pain and recovery.

2. Short-Lived Therapeutic Effects:
In Figure 5, the therapeutic effects appear short-lived (~4–7 days). This raises concerns about the nanoparticles' stability in the brain. The authors should discuss this limitation and consider investigating nanoparticle stability and sustained release.

3. Image-Data Discrepancy:
The cGAS immunofluorescence images in Figure 7A show minimal signal reduction in the treatment group, conflicting with the reported statistical significance. This inconsistency raises questions about the reliability of the results. Additional representative images and clarification of the fluorescence quantification methodology are needed to ensure reproducibility.

Reviewer #3

(Remarks to the Author)
Thank you for inviting me to review this manuscript. This study presents an innovative approach by using transferrin (TF) and phosphatidylserine (PS) modified liposomes (TF/PS/TDP-43-LPs) to encapsulate TDP-43 aggregation inhibitors, exploring their therapeutic potential in neuropathic pain (NP). This work provides a novel perspective on NP treatment strategies. However, I have the following concerns and suggestions for improvement:

1.The manuscript does not include a detailed evaluation of different doses of TF/PS/TDP-43-LPs to determine the optimal therapeutic dose. Dose-response studies are critical to establish the most effective and safe dose for preclinical applications.
2.While the study qualitatively demonstrates the ability of TF/PS/TDP-43-LPs to cross the blood-brain barrier (BBB), it lacks quantitative data to confirm the extent of brain penetration and drug concentration. Moreover, although the study demonstrates that TF/PS/TDP-43-LPs can cross the BBB, the exact molecular mechanisms behind the targeted delivery remain unclear.
3.While the study adopts a rigorous preclinical design, it does not sufficiently discuss the challenges associated with translating TF/PS/TDP-43-LPs into clinical practice. Key issues such as the scalability of the liposome formulation process, cost-effectiveness of production, and the safety profile of liposomal delivery systems in humans should be addressed.
4.The study focuses predominantly on the cGAS-STING pathway in the context of NP. However, NP is a complex condition involving multiple molecular and cellular pathways. Exploring or discussing alternative mechanisms of action could enhance the manuscript and provide a more comprehensive understanding of the therapeutic potential of TF/PS/TDP-43-LPs.
5.The potential off-target effects of TF/PS/TDP-43-LPs are not addressed in the manuscript.
6. The study does not investigate potential sex differences in response to TF/PS/TDP-43-LPs. Given the known biological and hormonal differences in NP between males and females, it is important to evaluate whether treatment efficacy varies by sex.
7.The statistical methods used for some analyses are not described in sufficient detail.
8.The manuscript lacks a detailed characterization of the liposome formulation process.
9.The discussion section could be expanded to provide a more thorough analysis of the potential clinical implications of the findings.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The rebuttal by the authors to the reviewer comments—both major and minor—has been sufficiently addressed. The additional experiments conducted, predominantly included as supplementary figures, have enhanced the scientific rigor of the study, while the minor revisions have improved clarity for the reader. I am now confident this manuscript is suitable for publication with some further minor edits as outlined below.

All western blots included in the supplementary figures should be properly formatted as figures, labelled with the protein names and size of protein bands with standard blot scale. In their present format these blots are not suitable for publication.
A minor remaining critique is that when the authors state 'future studies will address neurotransmitter regulation and synaptic function', they should provide more specific detail in the discussion on how these investigations will be conducted. Additionally, outlining how their current model could be leveraged for this purpose would strengthen the perceived feasibility and translational potential of their approach, supporting its broader application in the field of chronic pain research.

Overall I commend the authors for their diligence in addressing the reviewer comments and presenting a substantially improved manuscript. Once revised to address these minor edits, the updated manuscript will meet the academic standards expected for Nature Communications and offers valuable scientific insights that will benefit the broader field.
Recommendation: Accept with minor revisions.

Reviewer #2

(Remarks to the Author)
Comments:

The authors have made significant revisions that address many of the concerns raised in the initial review. In particular, the inclusion of additional data linking TDP-43 expression to cGAS-STING pathway activation, as well as the siRNA-mediated knockdown results showing suppression of inflammatory markers, has strengthened the mechanistic framework of the study. The revised manuscript represents a clear improvement; however, some points still require clarification or refinement. A summary of major and remaining concerns is provided below:

1. TDP-43 specificity: The addition of western blot data and NSAID control experiments supports the specificity of TDP-43 involvement. However, the absence of direct evidence for TDP-43 aggregation remains a limitation. The authors are encouraged to acknowledge this explicitly when interpreting mechanistic conclusions.
2. cGAS-STING pathway: The siRNA knockdown data effectively demonstrate a functional connection between TDP-43 and cGAS-STING activation. This concern has been satisfactorily addressed.
3. Neuroprotection vs. immunomodulation: The data on neuronal markers and apoptosis confirm the platform’s safety. However, the lack of investigation into peripheral nerve repair should be noted as a limitation of the current study.
4. Nanoparticle uptake and release: The authors have provided improved pharmacokinetic data and uptake analyses. This aspect is now well supported.
5. References: Previously missing citations have been appropriately added.

In addition to the above points, the following issues remain and should be addressed through minor revisions to enhance clarity and data presentation:

• Figure 9: Terminology should be unified for consistency. The illustration of the inhibitory pathway is currently unclear and may be misleading. The role of TDP-43 should be explicitly included.
• Terminology: The abbreviation “LPs” may be easily confused with “LPS.” Replacing it with “liposomes” or a clearer alternative would improve clarity.
• Figure 4F: The figure would benefit from more detailed channel labeling, visual indicators (e.g., arrows), and quantitative analysis to support claims about perinuclear localization.
• Figure S9C (TDP-43 localization): The resolution of the imaging is insufficient to support the stated conclusions. Higher-resolution images and/or quantification would be beneficial. If not available, a more cautious interpretation is recommended.

Reviewer #3

(Remarks to the Author)
I have no further comments.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The study has executed sound methodology, utilized various techniques, overall presented the data effectively, is soundly written and generally provides an interesting perspective regarding the specific targeting of glial cells using TF/PS/TDP-43-IN-1. Research that repurposes and/or refines the utility of specific protein inhibitors such as TDP-43 aggregation inhibitors is a promising area of investigation and relevant for various neurological conditions. While this study holds significant potential to contribute to the field by identifying therapeutic alternatives and targets for neuropathic pain, in its present format, this manuscript presents major concerns that need to be addressed in order for it to fully realize its impact and make a more substantial contribution. Major revisions are necessary for this manuscript be considered for the high standard of publication in Nature Communications.

Major concerns:

This study focuses on the aberrant actions/interactions of microglia and astrocytes in driving neuropathic pain. While aberrant interactions between microglia and astrocytes are critical drivers of neuropathic pain, they act within a broader, interconnected system that includes neurons and peripheral components. These glial interactions may amplify and sustain pain states, but they cannot fully account for the initiation or all mechanisms of neuropathic pain. Thus, a comprehensive understanding requires considering all cellular players and their dynamic interplay, critically neurons.

In its present format this study lacks critical insights into the bi-directional interactions of neurons and glia in the context of neuropathic pain and neuroinflammation. This is of particular significance given neurons are central to the development and perpetuation of neuropathic pain and neuron-glial interactions underlie neuroinflammatory processes, as neurons can signal glial cells to release inflammatory factors and vice versa in response to insults. How can an assessment of

neuroinflammation be made without consideration of neurons and what about neurotransmitter release which underlies neuropathic pain?

Response: Thank you for your insightful comments on the study design. We have added additional experiments to evaluate the potential effects of the nanoparticles on neurons.

Immunofluorescence and Western blot analyses of neuronal markers (NeuN, MAP2, and β-III-tubulin) showed no significant differences between groups. TUNEL and Nissl staining also revealed no apparent neuronal apoptosis or functional alteration (Figure S9A–F). Furthermore, TDP-43 expression and its nuclear-to-cytoplasmic ratio, as well as neuronal cGAS-STING pathway activation, showed no significant changes (Figure S9C–D, S9G). These findings indicate that TF/PS/TDP-43-IN-1 exerts its therapeutic effect primarily through targeting microglia rather than neurons.

Previous studies have demonstrated that modulation of glial cell activity can suppress neuroinflammation and reduce neuronal overstimulation without directly inhibiting neuronal function (PMID: 33239064; PMID: 32709045). Our results suggest that this nanoplatform alleviates neuropathic pain by indirectly influencing neuron-glia interactions. The direct impact on neurotransmitter systems will be explored in future work.

While it's appreciated that the specific role of non-neuronal cells- glia is focused on throughout the paper to optimize drug delivery and targeting cellular mechanisms of pain regulation rather than merely blocking pain signals- particularly in the cell culture models, it is not possible to draw conclusions as this study does regarding an alleviation of neuropathic pain (and subsequent damage/prevention of damage to neurons/implications for synaptic pruning of neurons) without assessment of the neuron populations themselves. This could have been achieved by staining for neuronal markers (e.g. MAP2, NeuN, B-II-tubulin) in the CCI mouse model brain and spinal cord tissue, or even assessment of the gene enrichment of the neuron population identified using scRNAseq of the spinal cord or assessment of neuronal integrity/synaptic markers/neurotransmitters

using qPCR. It is confusing as to why these rather obvious and yet straightforward assessments were not conducted for a more in depth understanding of TF/PS/TDP-43-IN-1 in alleviating aberrant mechanisms in neuropathic pain specifically. In its current format, this study provides a very simplistic interpretation and modelling of treatment of neuropathic pain using TF/PS/TDP-43-IN-1 and largely ignoring neurons, the central population of interest, is a significant flaw of this study. This study risks misinterpreting glial contributions and in its current format is not translatable providing an incomplete picture of the assessment of therapeutics for neuroinflammation underlying neuropathic pain.

Response: We appreciate your concern regarding the limited assessment of neurons in this study. In response, we have conducted additional experiments to evaluate neuronal status.

Immunofluorescence and Western blot analyses showed no significant changes in neuronal markers (NeuN, MAP2, and β-III-tubulin) among groups in the CCI model. TUNEL and Nissl staining also revealed no neuronal apoptosis or functional abnormalities (Figure S9A–F). Additionally, no changes were observed in TDP-43 expression or its nuclear/cytoplasmic distribution in neurons, nor in the activation of the cGAS-STING pathway (Figure S9C–D, G). These data suggest that TF/PS/TDP-43-IN-1 does not directly act on neurons but exerts its therapeutic effect predominantly through modulation of microglial activation.

We agree that neuron-glia interactions are critical in neuropathic pain. This study emphasizes that regulating glial inflammation can alleviate neuronal hyperexcitability indirectly (PMID: 33239064; PMID: 32709045). Further investigation into neurotransmitter regulation and synaptic function will be addressed in future studies.

While the study identifies new concepts regarding TF/PS/TDP-43-IN-1 inhibition of the activation of cGAS-STING pathways in astrocytes and microglia in the CCI mouse model (spinal cord scSeq), assessed in isolation without the consideration of neurons and neuron-glia interactions restricts the interpretation of this data in the context of neuroinflammatory mechanisms underlying neuropathic

pain specifically. Without neuronal assessment, it is unclear whether observed pain modulation stems from direct effects on neurons, indirect effects via glial modulation, or a combination of both. Furthermore, neurons may express components of the cGAS-STING pathway. Inhibiting this pathway in glia alone does not rule out neuronal involvement or contributions to pain signaling, which might operate independently or synergistically with glial mechanisms. Targeting glial pathways alone with TF/PS/TDP-43-IN-1 might not account for pain persistence driven by neuronal maladaptation, leading to incomplete therapeutic efficacy. Without evaluating neuronal changes, the relevance of cGAS-STING pathway inhibition to clinical outcomes remains uncertain.

Response: We sincerely thank the reviewer for this important comment. We fully agree that understanding the bidirectional interactions between neurons and glial cells is essential to interpret the neuroinflammatory mechanisms underlying neuropathic pain.

In response, we have performed additional experiments to evaluate the neuronal response in the CCI mouse model. Immunofluorescence and Western blot analyses showed no significant changes in neuronal markers (NeuN, MAP2, β-III-tubulin), and TUNEL and Nissl staining revealed no neuronal apoptosis or structural damage (Figure S9A–F). Moreover, TDP-43 expression and nuclear/cytoplasmic distribution in neurons remained unchanged, and there was no detectable activation of the cGAS-STING pathway in neuronal populations (Figure S9C–D, G). These findings suggest that TF/PS/TDP-43-IN-1 does not exert direct effects on neurons.

Therefore, we conclude that the therapeutic effects of TF/PS/TDP-43-IN-1 are primarily mediated via glial modulation, particularly by suppressing microglial and astrocytic activation of the cGAS-STING signaling cascade, which indirectly mitigates neuronal hyperexcitability and neuropathic pain (as detailed in the Discussion). Nevertheless, we recognize the value of further investigations, such as neuronal subtype-specific scRNA-seq analyses or neurotransmitter profiling, to provide a more complete picture of neuronal involvement.

Furthermore, TDP-43, the therapeutic target in this study, has significant roles within the CNS,

shuttling between the nucleus and cytoplasm and is vital for the regulation of RNA processes that support neuronal integrity and synaptic function. In addition, TDP-43 expression in glial cells ensures their proper functioning which is essential for maintaining neuronal health and in regulating immune responses in the CNS. This study utilizes TDP-43 aggregation inhibitors, with promising data shown in glial cells, however, the study fails to provide assessment of homeostatic levels of TDP-43 or the effects of TF/PS/TDP-43-IN-1 on 'normal' TDP-43 expression within the CNS, particularly the effects of targeting TDP-43 in neurons and the implications of this for the modulation of the cGAS-STING pathway. Staining or western blot analysis of pan-TDP-43/wild-type TDP-43 protein expression is required to elucidate further the mechanisms of action of TF/PS/TDP-43-IN-1 in the context of neuroinflammation and neuropathic pain.

Response: Thank you for your valuable comments on the study design.

We conducted additional experiments to evaluate potential neuronal effects of TF/PS/TDP-43-IN-1. Immunofluorescence staining of brain sections revealed no significant changes in the nuclear/cytoplasmic distribution of TDP-43 in neurons (Figure S9C). Western blot analysis also showed that total TDP-43 expression levels remained unchanged (Figure S9D). Furthermore, immunofluorescence and Western blot analysis of MAP2, NeuN, and β-III-tubulin revealed no significant differences across groups, indicating stable neuronal structural markers. Nissl staining further confirmed no functional impairment of neurons across groups (Figure S9E-F). We also analyzed cGAS-STING pathway proteins by Western blot and found no significant changes in neuronal expression levels (Figure S9G), suggesting that the inhibitory effect of TF/PS/TDP-43-IN-1 in the CCI model primarily occurs in microglia..

Minor revisions

• Introduction: lines 72-74, ‘TDP-43 aggregation inhibition shown promise in several ND diseases’ this statement requires references

Response: We apologize for the omission of relevant citations in the previous version. We have

now added appropriate references (PMID: 35499795； PMID: 35499795; PMID: 34916658) to the revised manuscript to support the newly added content.

• It is unclear throughout, which brain region specifically is being stained for in the CCI mouse model and how this brain/tissue region is relevant for the investigation of neuropathic pain?

Response: Thank you for your question regarding the staining region in brain slices. All immunofluorescence staining and molecular analyses targeting neurons and glial markers were conducted in the anterior cingulate cortex (ACC). The ACC is a critical hub for affective pain processing and central sensitization. In the CCI model, astrocytes and microglia in the ACC release pro-inflammatory cytokines (e.g., IL-6, TNF-α), exacerbating NMDA receptor phosphorylation and synaptic plasticity abnormalities, ultimately promoting chronic pain (PMID: 35658977). As highlighted in the revised discussion, the ACC not only functions as the "emotional center" of pain but also represents a key site of glial-mediated modulation in neuropathic pain (PMID: 27307118; PMID: 31097621). Targeting glial polarization and receptor signaling in the ACC thus offers a promising multi-dimensional intervention strategy for neuropathic pain—ranging from analgesia to affective-cognitive restoration (PMID: 29886177). This context has been integrated into the corresponding section of the manuscript.

• The figure legends are somewhat vague and often do not explain/provide enough information to fully interpret the data in many instances. While it is appreciated there is a limit to figure legends, they should be detailed enough so that each figure and caption can, as far as possible, be understood in isolation from the main text- at present this is not the case for many of the figure legends.

Response: Thank you for your suggestion regarding manuscript presentation.

We have added detailed figure legends to improve clarity and aid the reader’s understanding of the experimental results..

Fig 3E & F: Treatment/group labels missing from histological images

Response: We apologize for the lack of annotations in the previous version. The revised manuscript now includes the necessary annotations to enhance interpretability.

Supp 4A: left panel is incorrectly labelled/order is wrong, DAPI is labelled GFAP and GFAP is labelled DAPI

Response: We sincerely apologize for the labeling errors. The order of the relevant images has been corrected and the figure labels have been updated accordingly in the revised manuscript.

Figure 4D or a supplementary figure should show staining of controls (sham/CCI) alongside treated for more accurate interpretation of results

Response: Thank you for your suggestions on result presentation.

We have included immunofluorescence staining of the sham group in Figure 4E. In the sham-operated mice, astrocytes and microglia did not show co-localization with DiR signals, confirming that TF/PS/TDP-43-IN-1 selectively targets activated astrocytes and microglia in the CCI model.

Fig 4D: Why was IBA1 used here as opposed to CD68/CD206 as used to demonstrate microglial expression in Fig 3F? The IBA1 staining in Fig 4D appears to have more of a neuronal phenotype and does not resemble the typical staining morphology for microglia stained with IBA1. To address this, counterstaining with neuronal markers (e.g. NeuN, MAPT) is advised.

Response: Thank you for your constructive input on experimental design.

In the revised study, we replaced IBA1 with CD86 to better visualize microglial morphology. CD86 provided a more prominent signal, and clear co-localization was observed in the experimental groups. These updated results are presented in the revised Figure 4E.

Additionally, we performed immunofluorescence staining of brain sections using NeuN and

TUNEL to assess neuronal apoptosis. No significant apoptotic changes were observed across groups (Figure S9A).

Fig 6A+C staining panels would benefit from the addition of DAPI staining in each panel to better show nuclei especially when trying to interpret ‘merge’ representative images

Response: Thank you for your suggestion regarding figure presentation. We have added DAPI staining to the relevant images to enhance the visualization of nuclei.

Fig 7A+B staining panels would benefit from the addition of DAPI staining in each panel to better show nuclei especially when trying to interpret ‘merge’ representative images

Response: Thank you again for your suggestion.

DAPI staining has been included in the figures as suggested, which improves the clarity and accuracy of data interpretation.

Fig 7B: still not convinced about the IBA1 staining being 'microglial' specific, morphology appears neuronal-like as mentioned previously. For further context and interpretation of results, this figure would significantly benefit from NeuN/MAP2 counter-staining to determine effects of treatment on neurons and also to confirm no overlap of microglial staining with neuronal staining.

Response: Thank you for your valuable suggestions on the experimental design.

We replaced IBA1 with CD86 to better visualize microglial morphology. CD86 staining revealed more prominent microglial features, and clear co-localization with DiR signals was observed in the treatment group. These updated results are now presented in the revised Figure 7B.

In addition, we performed immunofluorescence staining using NeuN (mature neurons) and TUNEL (apoptotic cells). The results showed no significant neuronal apoptosis across groups (Figure S9A).

Fig 7C: Western blot should also include quantification of IBA1 and GFAP protein levels to

confirm expression across treatment groups and further support staining data. The study would also benefit from assessment of wild-type/pan-TDP43 protein expression via western blot across treatment groups.

Response: Thank you for your suggestions regarding data presentation.

We have supplemented Figure 7C with Western blot quantification of GFAP and CD86 protein levels. The results showed that both GFAP and CD86 were significantly upregulated in the CCI group compared to the TF/PS/TDP-43-IN-1 group.

Additionally, we assessed TDP-43 protein expression by Western blot. Compared to the sham group, the CCI group showed significantly increased TDP-43 expression, whereas treatment with si-TDP-43 significantly reduced its expression compared to the CCI group.

Reviewer #2 (Remarks to the Author):

The authors present an interesting study utilizing phosphatidylserine (PS) and transferrin (TF)-modified liposomes (TF/PS/TDP-43-IN-1) loaded with a TDP-43 aggregation inhibitor as a potential treatment for neuropathic pain (NP) in a chronic constriction injury (CCI) mouse model. While the study demonstrates promising results, including effective blood-brain barrier (BBB) penetration and NP alleviation linked to cGAS-STING pathway inhibition and microglial-astrocytic crosstalk modulation, several critical limitations undermine its impact and clarity.

Major Concerns:

1. Unclear Mechanistic Insights into TDP-43 Inhibition:

The manuscript lacks evidence directly linking TDP-43 inhibition to the observed therapeutic effects. While behavioral and anti-inflammatory outcomes are reported, no data confirms whether these are specifically mediated by TDP-43 inhibition or are broader anti-inflammatory responses. Experiments to measure TDP-43 protein levels or aggregation (e.g., Western blot, immunohistochemistry) in treated tissues are essential. Furthermore, testing alternative TDP-43

inhibitors or non-specific anti-inflammatory agents would help determine if the effects are TDP-43-specific. This lack of mechanistic clarity significantly weakens the study's claims.

Response: Thank you for your insightful feedback.

To confirm the relationship between therapeutic efficacy and TDP-43 expression, we analyzed TDP-43 levels via Western blot. Results demonstrated that TDP-43 was significantly upregulated in the CCI group compared to the sham group and significantly decreased in the si-TDP-43-treated group (Figure 7D), indicating that TDP-43 expression is associated with cGAS-STING pathway activation in CCI.

Furthermore, to determine whether the therapeutic effect was specifically mediated by TDP-43 modulation, we conducted a supplementary experiment using a non-specific anti-inflammatory drug (NSAID). Results showed that, compared to the sham group, the expression of cGAS/GFAP and STING/GFAP was markedly elevated in the CCI group. TF/PS/TDP-43-IN-1 treatment significantly suppressed this elevation, whereas NSAID administration failed to inhibit cGAS/GFAP and STING/GFAP expression. These findings further support the TDP-43 specificity of the therapeutic effect (Figure 7A-B).

2. Role of TDP-43 in cGAS-STING Pathway Activation:

The study identifies the cGAS-STING pathway as a key target but does not sufficiently explore the role of TDP-43 in activating this pathway. Previous studies suggest a link between TDP-43 and cGAS-STING activation. Knockdown experiments using siRNA to silence TDP-43 in microglia or astrocytes could provide direct evidence for its involvement. Without this, the connection between TDP-43 inhibition and cGAS-STING suppression remains speculative.

Response: Thank you for your comments on the study design.

We performed Western blot analysis of cGAS-STING pathway-related proteins. Compared with the sham group, the CCI group showed significantly increased expression of cGAS, STING, p-TBK1/TBK1, p-IRF3/IRF3, and p-STAT1/STAT1. Treatment with si-TDP-43 significantly

reduced the expression of these proteins, indicating that TDP-43 contributes to the activation of the cGAS-STING pathway in CCI (Figure 7D).

3. Lack of Analysis on Nerve Repair and Immune Modulation:

The manuscript does not investigate whether the nanoparticles directly promote nerve repair or primarily modulate immune responses. This omission limits understanding of the therapeutic mechanism. Nerve tissue analysis should be conducted. Additionally, quantifying inflammatory cytokines and immune cell infiltration in the injured nerves would offer a more comprehensive evaluation of the nanoparticles' effects.

Response: Thank you for this insightful suggestion. To clarify whether TF/PS/TDP-43-IN-1 exerts direct neuroprotective effects or acts mainly via immune modulation, we performed complementary in vivo experiments. Immunofluorescence staining of brain sections using NeuN and TUNEL showed no significant neuronal apoptosis in any group (Figure S9A). Western blot analysis of apoptosis markers (Bax, Bcl-2, Cleaved Caspase-3) also revealed no notable differences among groups (Figure S9B). Neuronal TDP-43 nuclear/cytoplasmic ratios and total expression levels were unchanged (Figure S9C-D), and expression of neuronal markers MAP2, NeuN, and β-III-tubulin remained stable (Figure S9E-F). Nissl staining confirmed preserved neuronal morphology and function. Moreover, cGAS and STING expression in neurons was unaffected (Figure S9G), suggesting that TF/PS/TDP-43-IN-1 primarily targets microglia in the CCI model rather than neurons directly.

This mechanism aligns with prior findings that microglia and astrocytes play central roles in neuropathic pain via cytokine-mediated neuron-glia interactions (PMID: 33239064; PMID: 32709045). While TF/PS/TDP-43-IN-1 likely alleviates NP by modulating glial inflammation and cytokine profiles, its direct effect on neurotransmission warrants further study. We have incorporated these results into the revised manuscript and clarified that immune modulation is the main therapeutic mechanism.

4. Ambiguity in Nanoparticle Naming and Terminology:

The naming of the TDP-43 inhibitor and nanoparticles is confusing. For example, "CCI+TF/PS/TDP-43-IN-1" could mislead readers into thinking the nanoparticles contain TDP-43 protein rather than an inhibitor. Moreover, referring to the TDP-43 inhibitor as "TDP-43-IN-1," its established name in the literature, would ensure consistency and clarity. The current terminology detracts from the study's focus and accessibility.

Response: Thank you for your suggestion regarding manuscript writing.

We have revised the terminology throughout the manuscript by replacing "TF/PS/TDP-43-IN-1" with the more accurate "TF/PS/TDP-43-IN-1" to reflect the formulation containing the TDP-43 aggregation inhibitor.

5. Limited Insights into Nanoparticle Uptake and Release Mechanisms:

The manuscript does not clarify how nanoparticles are internalized by cells or how they release their cargo. Without this information, the therapeutic efficacy cannot be fully understood. Investigations using endocytosis inhibitors, fluorescence imaging to trace uptake, and assays like LysoTracker to study endosomal escape are necessary. Linking in vitro drug release profiles to in vivo pharmacokinetics would further validate the proposed delivery mechanism.

Response: Thank you for your helpful suggestions regarding experimental design.

We performed fluorescence imaging to track the uptake of nanoparticles. Confocal microscopy revealed that TF/PS/TDP-43-IN-1 was efficiently absorbed by cells within 1 to 24 hours. Large clusters of nanoparticles were observed adhering to live cells after 1 hour of incubation, and the signal intensity peaked around the perinuclear region after 24 hours. These results have been added to the revised Figure 4F. We also measured drug concentrations in mouse plasma and brain tissue. At all time points, the brain concentration of TF/PS/TDP-43-IN-1 was significantly higher than that of TDP-43-IN-1 alone. The in vitro release profiles were correlated with in vivo pharmacokinetics,

and the relevant data have been added to Figure 4C.

6. Missing and incorrect References:

The potential roles of TDP-43 aggregation inhibitor are not property cited in the manuscript.

Response: Thank you for your suggestion. We have revised the Discussion section to clarify the mechanisms of TDP-43 aggregation inhibitors and included appropriate citations. These inhibitors mitigate pathology by disrupting C-terminal LCD-mediated interactions that drive pathological phase separation and aggregation (PMID: 33335017; PMID: 32976566). They also reduce glial activation and downstream proinflammatory cytokine release (e.g., IL-1β, TNF-α) (PMID: 37450244; PMID: 33803845), and attenuate TLR4/MyD88/NF-κB signaling. These references have now been incorporated to support the therapeutic rationale.

Minor Concerns:

1. Behavioral Assays:

Behavioral recovery conclusions rely on a single test. Additional tests, such as those for thermal hyperalgesia (Hargreaves' test), cold allodynia (acetone test), and motor functon(rotarod test), would provide a more robust assessment of neuropathic pain and recovery.

Response: Thank you for your suggestions regarding behavioral assessments.

In the thermal hyperalgesia test, CCI mice exhibited a significantly reduced paw withdrawal latency compared to the sham group from day 1 to day 14. Pretreatment with TDP-43-IN, TDP-43-IN-1, or TF/PS/TDP-43-IN-1 effectively reversed the thermal hypersensitivity. On postoperative days 1, 3, and 5, mice in all treatment groups showed increased thermal withdrawal latency, with TF/PS/TDP-43-IN-1 exhibiting the most pronounced effect (Figure 5B).

We also included assessments of cold allodynia and motor function. In the cold allodynia test (acetone evaporation), CCI mice displayed significantly reduced paw withdrawal responses and shortened withdrawal latency from day 1 to 14. Pretreatment with TDP-43-IN, TDP-43-IN-1, or

TF/PS/TDP-43-IN-1 increased cold withdrawal responses, with TF/PS/TDP-43-IN-1 showing the strongest effect (Figure 5C).

In the rotarod test, which evaluates motor coordination and fatigue resistance, CCI mice showed delayed response times compared to the sham group. Treatment groups displayed reduced latency times on days 1, 3, and 5, with the TF/PS/TDP-43-IN-1 group showing superior recovery (Figure 5G).

2. Short-Lived Therapeutic Effects:

In Figure 5, the therapeutic effects appear short-lived (~4–7 days). This raises concerns about the nanoparticles' stability in the brain. The authors should discuss this limitation and consider investigating nanoparticle stability and sustained release.

Response: Thank you for your insightful comments.

Indeed, the transient nature of the therapeutic effect underscores key challenges in CNS drug delivery, including nanoparticle stability, targeting durability, and the complexity of pathological mechanisms. Future research integrating material engineering, combinatorial therapy, and real-time monitoring may overcome current limitations and support the translation of TF/PS/TDP-43-IN-1 into a long-acting clinical analgesic strategy. We have added a discussion of these limitations and future directions in the revised discussion section.

3. Image-Data Discrepancy:

The cGAS immunofluorescence images in Figure 7A show minimal signal reduction in the treatment group, conflicting with the reported statistical significance. This inconsistency raises questions about the reliability of the results. Additional representative images and clarification of the fluorescence quantification methodology are needed to ensure reproducibility.

Response: Thank you for your attention to detail.

We have replaced the previously included data with more representative images to better illustrate

the experimental results.

Reviewer #3 (Remarks to the Author):

Thank you for inviting me to review this manuscript. This study presents an innovative approach by using transferrin (TF) and phosphatidylserine (PS) modified liposomes (TF/PS/TDP-43-IN-1) to encapsulate TDP-43 aggregation inhibitors, exploring their therapeutic potential in neuropathic pain (NP). This work provides a novel perspective on NP treatment strategies. However, I have the following concerns and suggestions for improvement:

1.The manuscript does not include a detailed evaluation of different doses of TF/PS/TDP-43-IN-1 to determine the optimal therapeutic dose. Dose-response studies are critical to establish the most effective and safe dose for preclinical applications.

Response: Thank you for your suggestions regarding safety evaluation.

We assessed the hemolytic activity of different concentrations of TDP-43 (3.125 – 100 μM) on mouse red blood cells to determine a safe dosage range. As shown in Figure S3B, hemolysis increased from 0.25% at 3.125 μM to 4.95% at 100 μM. Based on standard hemolysis index classifications, these values fall within the "non-hemolytic" (<2%) and "slightly hemolytic" (2 – 5%) ranges, indicating acceptable safety.

We also evaluated cytotoxicity of TDP-43-IN-1 and TF/PS/TDP-43-IN-1 in primary astrocytes and microglia using the CCK-8 assay. Cells were incubated with a concentration range of 0 – 200 μg/mL for 48 hours. As shown in Figure S3C, there were no significant differences in cell viability between groups, confirming that both formulations are non-toxic to glial cells.

2.While the study qualitatively demonstrates the ability of TF/PS/TDP-43-IN-1 to cross the blood-brain barrier (BBB), it lacks quantitative data to confirm the extent of brain penetration and drug concentration. Moreover, although the study demonstrates that TF/PS/TDP-43-IN-1 can cross

the BBB, the exact molecular mechanisms behind the targeted delivery remain unclear.

Response: Thank you for your insightful comments on the study design.

We conducted additional experiments to track nanoparticle uptake using fluorescence imaging. Confocal microscopy revealed that TF/PS/TDP-43-IN-1 nanoparticles were fully internalized within 1 to 24 hours. As early as 1 hour post-incubation, large aggregates were observed attaching to live cells, and by 24 hours, fluorescence signals were predominantly concentrated in the perinuclear region. These results have been added to the revised Figure 4F.

Furthermore, we measured drug concentrations in mouse plasma and brain tissues. At all time points, the brain concentrations of TF/PS/TDP-43-IN-1 were significantly higher than those of TDP-43-IN-1 alone (Figure 4C).

The underlying targeting mechanism of TF/PS/TDP-43-IN-1 may involve ligand–receptor interactions, such as transferrin receptor (TFRC) on endothelial cells and TREM2 on microglia (PMID: 34978008), as well as lysosomal-associated membrane protein 1 (LAMP1)-mediated endocytosis (PMID: 35266854). These targeting mechanisms are essential to understanding the pharmacodynamics of TF/PS/TDP-43-IN-1 and will be the focus of our future studies. A corresponding discussion has been added to the revised manuscript.

3.While the study adopts a rigorous preclinical design, it does not sufficiently discuss the challenges associated with translating TF/PS/TDP-43-IN-1 into clinical practice. Key issues such as the scalability of the liposome formulation process, cost-effectiveness of production, and the safety profile of liposomal delivery systems in humans should be addressed.

Response: Thank you for your suggestion regarding manuscript writing.

TF/PS/TDP-43-IN-1 requires the integration of transferrin (TF), phosphatidylserine (PS), and the TDP-43 aggregation inhibitor, involving multi-step conjugation and encapsulation processes. These steps may result in variable coupling efficiency and limitations in drug loading capacity. Moreover, due to the thrombogenic potential of TF and PS modifications, preclinical safety assessments

should include multi-species studies, especially long-term repeated-dose toxicity evaluation (⩾6 months) in non-human primates. We have included these considerations in the discussion section.

4.The study focuses predominantly on the cGAS-STING pathway in the context of NP. However, NP is a complex condition involving multiple molecular and cellular pathways. Exploring or discussing alternative mechanisms of action could enhance the manuscript and provide a more comprehensive understanding of the therapeutic potential of TF/PS/TDP-43-IN-1.

Response: Thank you for your valuable suggestions.

One of the hallmarks of NP is aberrant neuronal firing, involving upregulation of various ion channels (e.g., Nav1.3, Nav1.7, Cav3.2) and receptors (e.g., TRPV1, P2X3) (PMID: 35926582). TDP-43 aggregation can impair RNA metabolism and disrupt mRNA splicing/stability of ion channels (e.g., Kv1.2), exacerbating neuronal hyperexcitability. By reducing TDP-43 aggregation, TF/PS/TDP-43-IN-1 may restore ion channel homeostasis and suppress ectopic firing.

Additionally, glia–neuron communication via CX3CL1-CX3CR1 and CCL2-CCR2 axes promotes chronic pain (PMID: 37071481). TDP-43 aggregation activates the TLR4/MyD88 pathway in microglia, inducing CX3CL1 and CCL2 release. TF/PS/TDP-43-IN-1 may block this pathological aggregation, thereby reducing chemokine release and abnormal neuron–glia crosstalk. We have added this mechanistic insight to the discussion section.

5.The potential off-target effects of TF/PS/TDP-43-IN-1 are not addressed in the manuscript.

Response: Thank you for raising this important issue.

TDP-43 is an RNA-binding protein involved in pre-mRNA splicing, transport, and stability regulation (PMID: 35101542). Inhibiting TDP-43 aggregation may lead to off-target interactions with other RNAs (e.g., miRNA precursors, lncRNAs), resulting in aberrant gene expression such as oncogene activation or tumor suppressor silencing. Moreover, potential cross-reactivity with other TDP family members (e.g., TDP-35) or RNA-binding proteins (e.g., FUS, hnRNPA1) may interfere

with their physiological roles. We have added a discussion of these possible off-target effects in the revised manuscript.

6. The study does not investigate potential sex differences in response to TF/PS/TDP-43-IN-1. Given the known biological and hormonal differences in NP between males and females, it is important to evaluate whether treatment efficacy varies by sex.

Response: Thank you for your suggestions regarding experimental design.

Given the sex-based hormonal differences, the efficacy of TF/PS/TDP-43-IN-1 may vary by sex. Exploring the therapeutic effects in both male and female subjects will be a critical focus in our future studies. We have addressed this limitation in the discussion.

7.The statistical methods used for some analyses are not described in sufficient detail.

Response: Thank you for your suggestion.

We have supplemented the "Statistical Analysis" section in the Methods part of the manuscript accordingly.

8.The manuscript lacks a detailed characterization of the liposome formulation process.

Response: Thank you for your attention to formulation details.

The formulation of TF/PS/TDP-43-IN-1 consists of phosphatidylserine (PS), 1,2-dipalmitoyl-sn-glycerol-3-phosphate (DPPG), transferrin (TF), lecithin, cholesterol (Chol), and TDP-43 inhibitor (TDP-43-in), in the ratio of PS:DPPG:TF:lecithin:Chol:TDP-43-in = 1:4:2:12:1:8 (w/w). This information has been added to the "Preparation of LPs" section in the Methods.

9.The discussion section could be expanded to provide a more thorough analysis of the potential clinical implications of the findings.

Response: Thank you for your helpful suggestions.

Currently, clinical treatment of NP relies on antidepressants (e.g., amitriptyline), antiepileptics (e.g., gabapentin), and opioids, which are often limited by insufficient efficacy and significant side effects (PMID: 20650402; PMID: 32584191).

This study proposes a “nanocarrier–targeted delivery–glial polarization regulation” strategy. By modifying liposomes with PS and TF, TF/PS/TDP-43-IN-1 can efficiently cross the BBB and selectively accumulate in astrocytes and microglia, significantly increasing drug concentration at the lesion site. The TDP-43 inhibitor acts via multiple mechanisms—including suppression of aggregation and regulation of the cGAS-STING pathway—modulating both inflammation and glial phenotypes, offering multi-target efficacy superior to single-pathway inhibitors.

This targeted nanocarrier design reduces systemic exposure and minimizes hepatorenal toxicity, which is especially important for chronic pain patients requiring long-term therapy.

Traditional NP treatments primarily target neuronal hyperexcitability (e.g., sodium channel blockers), while often neglecting the key role of glial cells in pain maintenance (PMID: 29500312; PMID: 28205574). Our study demonstrates that promoting A2 astrocyte and M2 microglial polarization leads to secretion of neurotrophic factors (e.g., BDNF, GDNF), supporting neuronal survival and axonal regeneration. Since glial polarization imbalance is a key driver of chronic pain progression, targeting glial phenotype may reverse its pathophysiology.

Future clinical translation may incorporate biomarkers (e.g., C3, Arg1) from CSF or blood to stratify patients for individualized therapy. Additionally, PET imaging with TSPO ligands could monitor glial activation in real time, guiding treatment timing and dosing. We have added these insights into the revised discussion section.

Response to Reviewers

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The rebuttal by the authors to the reviewer comments—both major and minor—has been sufficiently addressed. The additional experiments conducted, predominantly included as supplementary figures, have enhanced the scientific rigor of the study, while the minor revisions have improved clarity for the reader. I am now confident this manuscript is suitable for publication with some further minor edits as outlined below.

All western blots included in the supplementary figures should be properly formatted as figures, labelled with the protein names and size of protein bands with standard blot scale. In their present format these blots are not suitable for publication.

Response: We thank the reviewer for this suggestion. All Western blot images in the source data have now been reformatted to meet publication standards. Specifically, we have added molecular weight markers and clearly labeled all target proteins (e.g., TDP-43, cGAS, STING, GFAP, CD86, etc.) using standardized annotation. The updated figures include accurate scale bars and improved image contrast.

Figures and Source data

A minor remaining critique is that when the authors state 'future studies will address neurotransmitter regulation and synaptic function', they should provide more specific detail in the discussion on how these investigations will be conducted. Additionally, outlining how their current model could be leveraged for this purpose would strengthen the perceived feasibility and translational potential of their approach, supporting its broader application in the field of chronic pain research.

Overall I commend the authors for their diligence in addressing the reviewer comments and presenting a substantially improved manuscript. Once revised to address these minor edits, the updated manuscript will meet the academic standards expected for Nature Communications and offers valuable scientific insights that will benefit the broader field. Recommendation: Accept with minor revisions.

Response: We thank the reviewer for the insightful suggestions. We have added specific directions for future research in the Discussion section. Future studies will investigate how TF/PS/TDP-43-IN-1 modulates synaptic neurotransmission (such as Glu/GABA balance) and plasticity (such as spine

density) in NP. Our CCI model allows direct measurement of spinal LTP and mEPSCs, while the blood-brain barrier–penetrating lipid nanoparticles can be targeted to the synaptic microenvironment. These findings may help identify novel synaptic biomarkers for pain staging or inform combination therapies with neuromodulators such as pregabalin.

Line 438-443

Reviewer #2 (Remarks to the Author):

Comments:

The authors have made significant revisions that address many of the concerns raised in the initial review. In particular, the inclusion of additional data linking TDP-43 expression to cGAS-STING pathway activation, as well as the siRNA-mediated knockdown results showing suppression of inflammatory markers, has strengthened the mechanistic framework of the study. The revised manuscript represents a clear improvement; however, some points still require clarification or refinement. A summary of major and remaining concerns is provided below:

1. TDP-43 specificity: The addition of western blot data and NSAID control experiments supports the specificity of TDP-43 involvement. However, the absence of direct evidence for TDP-43 aggregation remains a limitation. The authors are encouraged to acknowledge this explicitly when interpreting mechanistic conclusions.

Response: We thank the reviewer for this important comment. In the revised manuscript, we have explicitly acknowledged the lack of direct experimental data on TDP-43 aggregation as a limitation of the current study. We have revised the Discussion section accordingly and proposed future work to further validate TDP-43 aggregation–related mechanisms using the techniques mentioned. Although our data indicate that TF/PS/TDP-43-IN-1 reduced TDP-43 protein levels and downstream cGAS-STING signaling (Figure 7C–D), this study lacks direct evidence (e.g., immunofluorescence or filter-trap assays) to visualize TDP-43 aggregation in CCI-activated glial cells. This limitation precludes a definitive conclusion on whether the therapeutic effect is mediated by disrupting pre-existing aggregates or preventing the formation of new ones. Future studies will employ methods such as sarkosyl-insoluble fractionation or thioflavin T staining to quantify TDP-43 aggregation in this model.

Line 417-424

2. cGAS-STING pathway: The siRNA knockdown data effectively demonstrate a functional connection between TDP-43 and cGAS-STING activation. This concern has been satisfactorily addressed.

Response: We thank the reviewer for the positive evaluation.

3. Neuroprotection vs. immunomodulation: The data on neuronal markers and apoptosis confirm the platform's safety. However, the lack of investigation into peripheral nerve repair should be noted as a limitation of the current study.

Response: We appreciate the reviewer's insightful suggestion. We acknowledge that this study primarily focused on immune regulation and neuroinflammation of central glial cells in neuropathic pain, without directly evaluating peripheral nerve regeneration. In response, we have added a new paragraph in the Discussion section. This study mainly focused on the role of glial cell–neuron interactions within the central nervous system (spinal cord and brain) in neuropathic pain. Although we confirmed that TF/PS/TDP-43-IN-1 had no significant impact on neuronal apoptosis or functional markers (Figure S9), we did not systematically assess its effects on peripheral nerve repair following sciatic nerve injury. This limitation arises from the CCI model, which primarily simulates nerve compression rather than transection, posing challenges for peripheral regeneration assessment. Future studies will utilize sciatic nerve crush or transection models, combined with morphological (nerve fiber density) and functional (nerve conduction velocity) evaluations, to comprehensively assess the peripheral repair potential of the nanomedicine.

Line 463-471

4. Nanoparticle uptake and release: The authors have provided improved pharmacokinetic data and uptake analyses. This aspect is now well supported.

Response: We thank the reviewer for recognizing the improvements in our pharmacokinetic and cellular uptake data. We are pleased that the provided results sufficiently support the efficiency and targeting properties of the TF/PS/TDP-43-IN-1 formulation. These data are critical in demonstrating the system's BBB-penetration, cell-specific uptake, and sustained release behavior.

5. References: Previously missing citations have been appropriately added.

Response: We thank the reviewer for acknowledging our corrections.

In addition to the above points, the following issues remain and should be addressed through minor revisions to enhance clarity and data presentation:

• Figure 9: Terminology should be unified for consistency. The illustration of the inhibitory pathway is currently unclear and may be misleading. The role of TDP-43 should be explicitly included.

Response: We thank the reviewer for pointing out this issue. We have revised Figure 9 by changing "TF/PS/TDP-43-LPs" to "TF/PS/TDP-43-IN-1" and adding a schematic illustration of TF/PS/TDP-43-IN-1 inhibition of the cGAS-STING pathway. Please kindly review the updated figure.

Figure 9

• Terminology: The abbreviation “LPs” may be easily confused with “LPS.” Replacing it with “liposomes” or a clearer alternative would improve clarity.

Response: We appreciate the reviewer’s helpful suggestion regarding terminology. To avoid confusion between “LPs” and “LPS,” we have revised the manuscript by replacing all instances of “LPs” with “liposomes.”

The Whole manuscript

• Figure 4F: The figure would benefit from more detailed channel labeling, visual indicators (e.g., arrows), and quantitative analysis to support claims about perinuclear localization.

Response: We thank the reviewer for the constructive suggestion. We have revised Figure 4F by adding arrow indicators to highlight the perinuclear accumulation of nanoparticles. These improvements strengthen the visual support for the conclusion regarding their perinuclear localization.

Figure 4F

• Figure S9C (TDP-43 localization): The resolution of the imaging is insufficient to support the stated conclusions. Higher-resolution images and/or quantification would be beneficial. If not available, a more cautious interpretation is recommended.

Response: We appreciate the reviewer’s insightful comment. We acknowledge that the resolution of Figure S9C was insufficient to fully support the stated conclusions. Due to limitations in the available tissue samples, it is currently not feasible to provide higher-resolution images. To address this concern, we have supplemented Figure S9C with quantitative statistical analysis of the nuclear-to-cytoplasmic ratio of TDP-43, which provides additional support for the observed localization trend. At the same time, we have revised the text in the Discussion section to interpret these findings more cautiously, noting that while the statistical data support the localization trend, the limited image resolution warrants careful consideration. We also added that future studies will employ higher-resolution imaging techniques to further validate these observations.

Figure 9; Line 471-477

Reviewer #3 (Remarks to the Author):

I have no further comments.

Response: We thank the reviewer for their time and positive evaluation.