Reviwers 1:

Major Comments:

1.A search in one database indicated that the short title so presented had been published already… find below:
https://www.sciencedirect.com/science/article/pii/S0300483X24001458#:~:text=Mlkl%20deficiency%20can%20alleviate%20acute,NF%2D%CE%BAB%20p65's%20nuclear%20entry.

Thus, it is important for authors to bring out the novelty in their research. Authors said nothing on data availability. Find below statements provided by the authors that actually did not indicate data availability: Data Availability A data availability statement is compulsory for all research articles. This statement describes whether and how others can access the data supporting the findings of the paper, including 1) what the nature of the data is, 2) where the data can be accessed, and 3) any restrictions on data access and why. If data are in an archive, include the accession number or a placeholder for it. Also include any materials that must be obtained through a Material Transfer Agreements (MTA).

☞ We thank you for your time and consideration on our submission. Thank you for the reviews and recommendations for improving the quality of the manuscript.

Reviewer raised a good point and we agree with your suggestion. As you pointed out, the data supporting the findings of this study can be requested directly from the corresponding author, at the email address [noshin@hanyang.ac.kr].

The structure of CPD4 is detailed in the paper titled "ATP-Competitive MLKL Binders Have No Functional Impact on Necroptosis." You can synthesize CPD4 by following the synthetic methods outlined in this publication. If you have any specific questions about the synthesis process or require clarification on certain steps, please feel free to email me for assistance.

☞Additionally, in response to the reviewers' comments regarding the novelty of our research, we wholeheartedly agree with your perspective. As a result, we have explicitly highlighted the unique contributions of this study in the discussion section (Page 22, Line No. 463~474).

"In this study, we present a novel mechanism for CPD4 as an ATP-competitive binder of MLKL, providing critical insights into its role in necroptosis. Our findings reveal that, contrary to prior research, CPD4 does not alter the functional pathway of necroptotic cell death, thus challenging established beliefs about the interactions of MLKL. Specifically, we demonstrate through biochemical assays that CPD4 binding does not inhibit MLKL oligomerization or membrane translocation, key processes in necroptosis.

Moreover, our research highlights the potential of CPD4 as a tool for investigating MLKL's regulatory pathways, suggesting that it may serve as a framework for developing targeted thera

pies for conditions associated with dysregulated necroptosis, such as inflammatory disorders. T his work lays the groundwork for further studies aimed at elucidating the complex mechanis ms governing MLKL activity and its implications in disease."

Abstract: Well noted… but what is the gap the authors are addressing since there seem to be other published articles Introduction: Stating hypothesis and refencing it as such makes readers wonder if the working hypothesis is actually an original hypothesis from the others or a borrowed hypothesis from other published work. See below: "So, our hypothesis is whether ATP pocket inhibitor can regulate inflammation via necroptosis independent pathway.

☞ Thank you for your good points. Thank you for the reviews and recommendations for improving the quality of the manuscript.

Our explanation of the hypothesis presented in this study has been incorporated into the introduction section. Specifically, we propose that the ATP-binding pocket of Mixed Lineage Kinase Domain-like Protein (MLKL) can serve as a novel therapeutic target for regulating inflammation in liver diseas es, independent of necroptosis. This hypothesis is grounded in the observation that MLKL is involved in inflammatory pathways beyond its role in cell death. By focusing on the ATP-binding pocket, we aim to investigate how inhibiting this region can mitigate inflammatory responses without triggering necroptosis, thus providing a safer therapeutic avenue for managing liver conditio ns. This approach not only broadens the understanding of MLKL's functions but also seeks to uncove r new strategies for treating liver diseases that are often associated with. (Page 6~7, Line No. 129~141)

"This study presents several significant advancements compared to previous research on MLK L: **Exploration of Necroptosis-Independent Pathways**: In contrast to earlier studies that primar ily focused on the role of MLKL in mediating necroptosis and resulting cell death, this resear ch investigates the ATP-binding pocket of MLKL as a potential target for regulating inflammat ion without triggering necroptosis. This shift highlights the investigation of MLKL's functions beyond cell death, suggesting that it may play a critical role in inflammatory processes indepe ndently of its traditional necroptotic functions. **Comprehensive Use of Animal Models**: Furthe rmore, we employed a variety of animal models, including both alcoholic and non-alcoholic fa tty liver disease models, to rigorously test and validate the anti-inflammatory effects of the M LKL ATP-binding inhibitor, CPD4. This approach allows for a thorough examination of the co mpound's efficacy across different pathological contexts, providing stronger evidence for its pot ential therapeutic applications in liver diseases."

2. In this study, we investigated the role of the ATP-binding pocket of MLKL as a possible novel target of MLKL." Method: Just wondering why I still cannot locate ethical clearance statement; study design; were archival samples used or... What is CDHFD? Find below: 'Animal models of non-alcoholic fatty liver disease were fed a CDHFD for six weeks.'

☞ Thank you for your good points. Thank you for the reviews and recommendations for improving the quality of the manuscript.

The design of the animal experiment is outlined in the submitted animal experiment plan, which comprehensively details the overall experimental process. This includes specific objectives, animal selection criteria, experimental groups, treatment protocols, and methods for data collection and analysis. The plan ensures ethical considerations are met, such as appropriate housing, care, and humane endpoints for the animals involved. In the Method section, we provide a step-by-step description of the experimental procedures, including how the MLKL ATP-binding pocket inhibitor (CPD4) is administered, the monitoring of animal health and behavior, and the techniques used to assess outcomes related to liver inflammation and injury. This structured approach aims to validate the efficacy of the treatment while maintaining high standards of animal welfare. (Page 12~13, Line No. 252~272)

"**Non-alcoholic liver disease experimental animal models**

Six-week-old C57BL/6 mice (n=8) were sourced from Central Lab Animal Inc. in Seoul, South Korea, and housed in the animal laboratory at Hanyang University School of Medicine. Prior to the start of the experiment, all mice underwent a one-week acclimatization period to minimize stress and ensure a stable environment. During this period, the mice were kept at a controlled temperature of 23 ± 2°C with 55 ± 5% relative humidity. The facility maintained a specific pathogen-free environment, and a consistent light-dark cycle (12 hours light/12 hours dark) was implemented. All experimental procedures were approved by the Animal Committee of Hanyang University (approval numbers: HY-IACUC-2022-0217A and HY-IACUC-2023-0276A). To model non-alcoholic fatty liver disease (NAFLD), the mice were fed a choline-deficient high-fat diet (CDHFD) for six weeks, which is known to induce hepatic steatosis and inflammation. Following the diet period, the MLKL ATP pocket-binding inhibitor (Compound-4), which was dissolved in a solution of 10% dimethyl sulfoxide (DMSO) and 90% corn oil, was administered via intraperitoneal injection at a dosage of 10 mg/kg. This treatment was given three times a week for a duration of seven weeks. Throughout the study, body weight was monitored weekly to assess overall health and response to treatment. At the conclusion of the experiment (Week 13), all mice were humanely euthanized. Liver tissues and serum samples were collected fo

r further analysis of inflammatory markers and histological examination, allowing for a compr ehensive evaluation of the effects of the MLKL ATP pocket-binding inhibitor on liver patholog y."

3. Results: What is 5AD? See below: 'We initially conducted a toxicity test using 5AD, a methylation inhibitor, based on a study indicating that hepatocyte hypermethylation does not induce necroptosis. Results showed that 5AD exhibited no significant cytotoxicity, even at 10 μM, compared to the untreated group' Discussion: Start discussion with main finding rather. Conclusion: I could not locate the conclusion in the main text after discussion. Authors should kindly rectify this.

☞Thank you for the reviews and recommendations for improving the quality of the manuscript. We apologize for the confusing description. In order to express it correctly, it has been modified as follows. (Page 15~16, Line No. 330~342)

"We initially conducted toxicity tests using the methylation inhibitor 5AD (5-aza-2'-deoxycytidi ne, also known as decitabine), an epigenetic drug that inhibits DNA methylation. This choice was based on a previous study indicating that high levels of methylation in liver cells do not lead to necroptosis. Our findings suggest that RAW 264.7 cells do not undergo cell death whe n stimulated for necroptosis, which we speculate is due to their high methylation status. The results demonstrated that, compared to the untreated group, 5AD did not show significant cyt otoxicity at concentrations up to 10 μM (Figure 3A). Following this, RAW 264.7 and HT29 cell s were treated with 5AD for 72 hours to induce demethylation, after which necroptosis was i nduced. Post-treatment, HT29 cells exhibited no changes in necroptosis-induced cell death, whi le RAW 264.7 cells showed a dose-dependent increase in cell death in response to necroptosis stimulation. This indicates that the induction of cell death in RAW 264.7 cells following necro ptosis stimulation is attributed to the inhibition of DNA methylation (Figures 3B and 3C)."

Additionally, we have included the full name, catalog number, and company of 5AD in the Materials and Methods section. The details are as follows (Page 8, Line No. 161~163): "and the cells were treated with 5-aza-2'-deoxycytidine (5AD, #189825, Sigma-Aldrich; 20, 10, 5, 2.5, or 1 μM) for 48 h for demethylation."

Reviwers 2:

In the present study, the authors investigate the possibility of inhibition of the ATP-binding pocket of MLKL for the treatment of necroptosis-associated liver diseases. To confirm the hypotheses, cell death analysis following necroptosis stimuli was evaluated using cell proliferation assays, flow cytometry, and electron microscopy in various cells including HT29, RAW264.7, U937, and LX-2 cells. Both alcoholic and non-alcoholic fatty liver disease animal models were used to determine the possibility that MLKL ATP pocket inhibitors could attenuate liver injury. In addition, the human liver organoid system was also used to evaluate the potency to attenuate inflammation with the MLKL ATP pocket-binding inhibitor. The introduction is well written and provides a clear background on the topic. The methodology is sound and the experimental design is appropriate for the research question. The results are presented clearly and are supported by appropriate statistical analysis. The discussion is insightful and provides a good interpretation of the results, but lacks a critical evaluation of the study's findings in the context of existing literature. The references are relevant and up-to-date. However, the author could benefit from providing a more detailed literature review to demonstrate the novelty of the study. The manuscript is generally well written, but there are a few instances of awkward phrasing and grammatical errors. A thorough proofreading would improve the overall quality of the paper. Overall, the manuscript is well written and presents interesting findings, which may present a valuable contribution to the field. Besides, the manuscript is rich in data but a few issues need to be addressed.

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1. Where is the MLKL ATP-binding inhibitor from and what are the differences among Compound-4, CPD4, and CPD30?

☞Thank you for the reviews and recommendations for improving the quality of the manuscript. We apologize for the confusing description.

The MLKL ATP-binding inhibitors include several compounds, with CPD-4 being the most representative. To avoid confusion, I will ensure that all references to MLKL ATP-binding inhibitors are unified and consistently use the term "MLKL ATP-binding inhibitors" throughout the document, specifically highlighting CPD-4 as the primary example.

2. Why are HT29 (human colon cancer cells), RAW264.7, U937 cells (human monocytes), and LX-2 (Human hepatic stellate cells) cells used here? What's the relationship between these cells and necroptosis-associated liver diseases?

☞ We appreciate the reviewer for this important comment.

It is well established that the response to necroptosis stimulation varies significantly across different cell types. For instance, in the liver, the effects of necroptosis differ between hepatocytes and stellate cells. Typically, necroptosis induces cell death; however, stellate cells tend to activate rather than undergo cell death in response to similar stimuli. Furthermore, MLKL, a key mediator of necroptosis, exhibits substantial structural differences between humans and mice. This variation suggests that

necroptosis inhibitors might elicit different responses in human-derived cells compared to mouse-derived cells. To address these differences, our study incorporated both human-derived and mouse-derived cell lines. We aimed to include a variety of cell types, including inflammatory cells that play a critical role in the development of liver disease, ensuring a comprehensive assessment of necroptosis across these different cellular contexts.

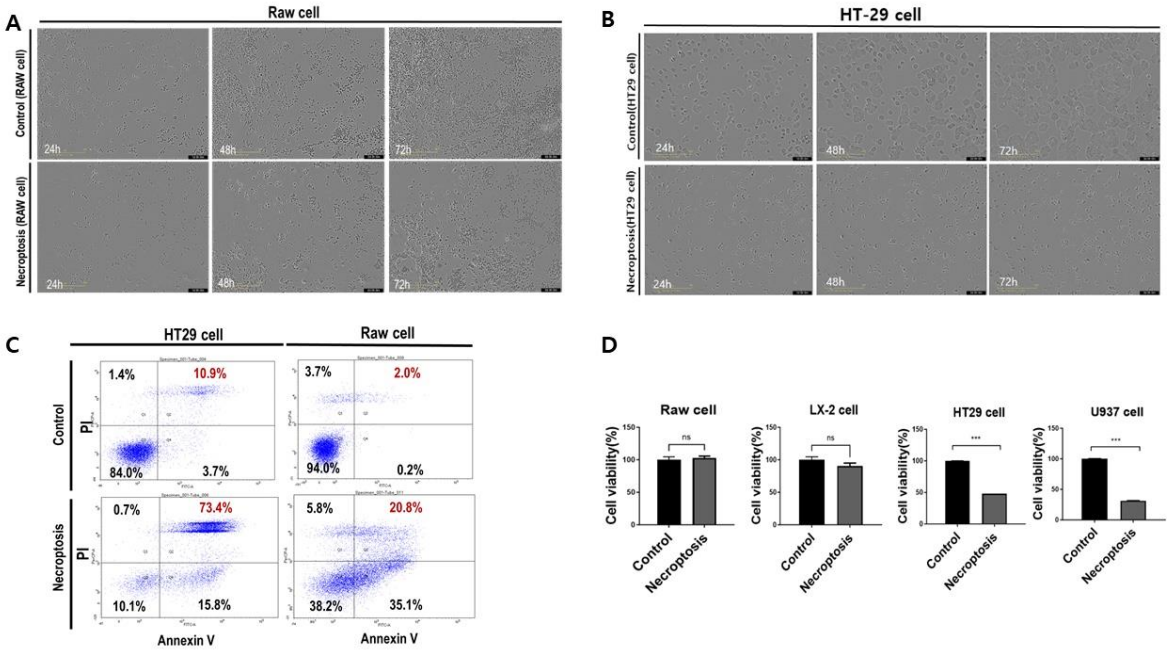
3. Statistical analysis should be performed for all results. For example, there is no statistical analysis for some results in Figure 1D.

☞ Thank you for your careful review and we apologize for the error on Figure 1D.

We carefully reviewed the raw data and performed comprehensive statistical analysis. As a result, the corresponding section of Figure 1D has been updated to reflect these changes.

☞ Additionally, the figure legend section has been updated to include detailed explanations of the meanings represented by the asterisks (Page 28, Line No. 590~591). Each asterisk now corresponds to specific statistical significance levels or experimental conditions, providing clarity on the results presented in the figures.

"Results are shown as mean. The asterisk denotes $p < 0.05$ and double asterisks denote $p < 0.01$."



4. "Figure 4. MLKL ATP-binding inhibitor cannot evade cell death after necroptosis stimuli but increase inflammation." Here should be inhibition of inflammation.

☞ Thank you for your comment and we agree. we are sorry for the confusion.

To ensure accurate representation of our findings, the following modifications have been made (Page 31, Line No. 620~621): "Figure 4. MLKL ATP-binding inhibitor cannot evade cell death after necroptosis stimuli but inhibition of inflammation".

5. When describing the results of Figure 4A-4D, the type of cells involved should be specified.

☞ Thank you for your careful review and we apologize for presenting of inconsiderable results. In order to express it correctly, it has been modified as follows. (Page 16~17, Line No. 346~358)

"After inducing necroptosis in RAW 264.7 cells, MLKL ATP-binding inhibitor (Compound 4) was administered at concentrations of 0.01, 0.1, and 1 μM. While the MLKL ATP-binding inhibitor (Compound 4) did not prevent cell death following necroptosis stimulation, MLKL ATP-binding inhibitor (Compound 4) significantly reduced the expression of key inflammatory markers. A similar downward trend in these markers was also observed in liver tissue from mice (Figure 4A-4B). Additionally, treatment with MLKL ATP-binding inhibitor (Compound 4) led to decreased levels of NF-κB, c-Jun, and IκB following necroptosis stimulation in RAW 264.7 cells. These results suggest that MLKL ATP-binding inhibitors exert anti-inflammatory effects through modulation of the NF-κB signaling pathway (Figure 4C-4D). In summary, while MLKL ATP-binding inhibitors may not directly inhibit necroptosis-induced cell death, they effectively regulate inflammation, highlighting their potential as therapeutic agents in inflammatory conditions."

6. "Data Availability A data availability statement is compulsory for all research articles. This statement describes whether and how others can access the data supporting the findings of the paper, including 1) what the nature of the data is, 2) where the data can be accessed, and 3) any restrictions on data access and why. If data are in an archive, include the accession number or a placeholder for it. Also include any materials that must be obtained through a Material Transfer Agreements (MTA)." There is no substantial content in this section.

☞ Thank you for your comment and we agree with your point out.

Reviewer raised a good point and we agree with your suggestion. As you pointed out, the data supporting the findings of this study can be requested directly from the corresponding author, at the email address [noshin@hanyang.ac.kr].

7. Some sentences should be modified to be more clarified. E.g., “At the same time MLKL also involved cell death pathway as well as cell activation via NFkB-mediated inflammatory pathway at the same time, and it depends on the type of cell and situations.”, “Recent studies have suggested many various alternative pathways of MLKL.”.

☞ We appreciate the reviewer for this important comment. To enhance clarity, the following modifications have been made to the sentence (Page 6, Line No. 117~123): MLKL has been considered a key molecule in necroptosis-induced cell death triggered by TNF-alpha stimulation. However, recent studies have revealed that the outcomes of TNF-alpha-induced necroptosis stimulation vary significantly depending on the cell type. According to the study by Oh et al., among the cells that constitute the liver, necroptosis stimuli induce cell death in hepatocytes and inflammatory cells, while stellate cells evade cell death and instead undergo activation.[3] This mechanism of cell death evasion is thought to be associated with NF-κB activation.