

Liver-specific paraoxonase-1 alleviates regulatory T cell-driven immunosuppression via metabolic reprogramming in hepatocellular carcinoma

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Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

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)

In this manuscript, Lu, Shi, and Gao et al explore the role of paraoxonase-1 in hepatocellular carcinoma disease progression. Their key findings are as follows:

- PON1 is expressed in the liver and expression is downregulated in HCC tumors compared with normal tissue across multiple public datasets. The authors validate this finding by immunohistochemistry in an internal dataset.
- Both in publicly available and in internal datasets, the authors show that PON1 expression correlates with prognosis in HCC.
- To directly test the role of PON1 in HCC progression, the authors primarily utilize Hepa1-6 cells in which PON1 is either overexpressed or suppressed via shRNA. They show that PON1 overexpression reduces subcutaneous tumor growth while knockdown of PON1 accelerates growth. Interestingly, manipulation of PON1 expression did not alter tumor cell proliferation in vitro, suggesting a non tumor cell autonomous effect.
- They subsequently identify regulatory T cell infiltration as inversely correlating with PON1 expression in patient tumors. Moreover, PON1 suppression increased intratumoral abundance of FoxP3+ cells while PON1 overexpression blunted FoxP3+ regulatory T cell abundance. Depleting CD25+ T cells using a monoclonal antibody abrogated the negative prognosis of control Hepa1-6 cells but not PON1-overexpressing cells.
- Mechanistically, the authors find that PON1 suppressed several metabolic pathways including those related to glycolysis and lactate production, and that co-administration of lactate was sufficient to reverse the impact of PON1 overexpression on anti-tumor immunity. Furthermore, they showed that PON1 promotes HIF-1a ubiquitylation and degradation, in part by binding and putatively enhancing the activity of the prolyl hydroxylase VHL.

Generally speaking, I found the work to be fairly comprehensive and impressive in scope and I find the authors' argument that PON1 suppresses regulatory T cell infiltration by attenuating HIF1a-dependent glucose uptake and lactate production to be compelling. I have a few suggestions which would provide some additional clarity to the study.

1. The methodology for experiments is sparse in many locations. I would recommend that the authors provide more detail on how the experiments are conducted within the main text rather than having those details exclusively in the methods. Just one example of this is when the authors state that "exogenous and endogenous interaction between PON1, VHL and HIF-1a were confirmed in Hepa1-6 cells" but they do not provide additional details as to which proteins were exogenous, whether they were FLAG or HA tagged, and/or how they were introduced into cells.
2. The authors suggest that the impact of PON1 manipulation is tumor cell autonomous because it has no impact on proliferation in vitro. Can the authors demonstrate that there is no difference in tumor growth when implanted in Rag KO mice?
3. The PON1 manipulation experiments are all performed using a single cell line and shRNA for knockdown. The authors should do at least one proof of principle experiment using CRISPR to edit the PON1 locus and it would be nice to see at least one alternate cell line in which this phenotype is observed.
4. The authors show that targeting CD25+ cells reduces tumor growth in WT, but not PON1-overexpressing cells. But I think

a better experiment is whether targeting CD25 reverses the negative impact of PON1 loss on tumor growth.
5. Finally, the authors show that PON1 interacts with VHL – I would be interested to hear any thoughts on how this interaction might alter VHL function. How might the enzymatic activity of PON1 activity?

Reviewer #2

(Remarks to the Author)

The manuscript by Lu et al. proposed that liver-specific paraoxonase-1 alleviates regulatory T cell-driven immunosuppression via reducing lactic acid production in hepatocellular carcinoma. They found that PON1 was downregulated during HCC progression and PON1 overexpression suppressed tumor growth through reducing Treg cell infiltration. They also proved that lactic acid was a key metabolic player of PON1 in regulating Treg cell infiltration in HCC. Mechanistically, it was shown that PON1 decreased lactic acid production through the recruitment of Von Hippel-Lindau protein which mediated Hypoxia-inducible factor alpha ubiquitination. Finally, therapeutic effects of PON1 were confirmed using recombinant PON1 protein and Quercetin. This work is truly interesting. The authors are addressing an important area of research. The role of PON1 in HCC progression and immunotherapy is not well understood. The experiments were well designed and the majority of the experiments support the findings. I have listed some of my concerns and suggestions.

1. How is PON1 expressed in CD4⁺T cells? Whether PON1 can directly affect CD4⁺T cell differentiation ?
2. Figure 3j-k mentioned that lactic acid promoted Treg cell differentiation in vitro. Does lactic acid have an effect on the proliferation and apoptosis of Treg cells?
3. In Figure 3i, lactic acid undermines the tumor-suppressive effect of PON1. What is the effect of lactic acid treatment alone on the proportion of Treg cells and tumor growth?
4. According to Figure 3a, the metabolism is significantly affected by the overexpression of PON1. I'd recommend incorporating a discussion about the relationship between PON1 and metabolism.
5. There are some typos in the manuscript, it may be useful to check the manuscript by a professorial English editing service agency.

Reviewer #3

(Remarks to the Author)

In this study, Lu et al. have uncovered the role of paraoxonase-1 (PON1) in shaping the immune landscape of hepatocellular carcinoma (HCC). They demonstrate that low expression of PON1 correlates with elevated Treg numbers and exacerbated tumor growth, while PON1 overexpression shows the inverse, accompanied by the expansion of IFN- γ ⁺ CD8 T cells. The authors suggest that PON1 loss induces lactic acid production in HCC, which promotes Treg-mediated immune suppression. Mechanistically, PON1 targets HIF1 α for degradation, and PON1 loss stabilizes HIF1 α , leading to increased lactic acid production in HCC. This is a well-designed study with logical experimental setups and high-quality data. However, several questions remain.

1. While the authors claim that exacerbated HCC growth is linked to lactic acid-mediated Treg expansion, they should exclude the possibility that PON1 may have a tumour-intrinsic function regulating growth. To address this, the authors should compare the growth of PON1-OV and PON1-KD cells in both B6 and Rag1^{-/-} (T cell-deficient) mice.
2. The authors show that depleting Tregs in the PON1-OV setting did not make a difference compared to the Treg undepleted cohort. However, an ideal experiment would involve anti-CD25 treatment of mice bearing PON1-KD tumours and comparing the results with PON1-NC tumours.
3. Lactic acid accumulates in highly proliferating tumours. The authors should assess the in vitro proliferation rates of PON1-NC and PON1-OV groups.
4. While HIF1 α regulates lactic acid, the authors could further strengthen the lactic acid-Treg link by deleting LDHA in Hpa1-6 tumours and by deleting MCT1 in Tregs, preventing them from sensing lactic acid.
5. How does lactic acid expand Tregs? Do existing Tregs expand, or is there recruitment of new Tregs to PON1-KD tumors? The authors also seem to suggest that CD4 T cells upregulate Foxp3. The authors should test the origin of Tregs in PON1-KD tumours and perform TCR-seq on Tregs and CD4 T cells in this setting to assess overlap between these subsets.
6. Could the authors provide the method for the co-culture experiment of CD4 T cells with PON1-OV tumour cells?
7. In the lactic acid treatment experiment, the authors observe elevated tumour growth. However, in this condition, Tregs are lower in frequency compared to the NC group, which contradicts the argument that lactic acid induces Tregs. Could the authors clarify this discrepancy?
8. In the lactic acid-treated condition, tumour growth is exacerbated, which the authors propose is mediated by Treg expansion and dampened anti-tumour immunity. However, lactic acid is known to directly promote CD8 T cell dysfunction, which could also contribute to tumor growth. Please address this possibility.

9. How was the experiment demonstrating lactic acid dose-dependent induction of Foxp3 performed? Was it a co-culture of lactic acid, TGF- β , and CD4 T cells? Was anti-CD3/28 or IL-2 used? Please provide the experimental method.

10. The authors should induce Hpa1-6 tumours in both PON1^{-/-} and wild-type mice to assess whether tumour growth is faster in the knockout mice, which have more Tregs.

Reviewer #4

(Remarks to the Author)

In the present manuscript, the authors explore a novel role for liver-specific paraoxonase-1 (PON1) in modulating regulatory T cell (Treg)-mediated immunosuppression in hepatocellular carcinoma (HCC), primarily using murine subcutaneous hepatoma tumor model. They propose an intriguing mechanism by which PON1 suppresses lactic acid production via VHL-mediated ubiquitination and degradation of HIF-1 α , leading to reduced Treg infiltration. Clinical associations further suggest a potential therapeutic advantage in modulating PON1 expression to enhance anti-tumor immunity.

This is an interesting and novel conceptual framework that highlights the immunometabolic interplay between PON1 and Treg cells in the tumor microenvironment. The therapeutic potential of modulating PON1 via Quercetin to enhance anti-PD-1 responses in HCC is particularly compelling. However, the manuscript lacks important PON1 biology and function in their proposed model.

Major comments:

1. The authors heavily relied on using the murine hepatoma cell line Hepa1-6 and derived subcutaneous tumors in B6 mice. Hepa1-6 is derived from chemically-induced HCC which heavily lacks hallmark human HCC-associated oncogenes (e.g., TP53, CTNNB1, TERT promoter mutations) and implicated signaling because of differences in the nature of tumor induction. This limits the translatability of their major findings in human HCC, especially how PON1 is metabolically implicated in these tumors and their microenvironment. This is not to argue that Hepa1-6 model is not an appropriate HCC model but rather is lacking of components important to contextualize the conclusions in this manuscript in clinically-relevant HCC biology. Therefore, it is my opinion that the main findings should be tested as well in relevant human HCC models (e.g., PDX mice, patient-derived or PDX-derived organoids) or, at the very least, implicate the immunosuppression findings in a spontaneous HCC mouse model.

2. Considering the crude evidence on how PON1 is differentially expressed in different malignancies and how PON1 can be differentially regulated to induce pro-tumorigenic or tumor suppressive effects, depending on its localization along the secretory route (Golgi/ER, cytoplasm, nucleus) (Aldonza et al., 2023, eLife; Aldonza et al., 2017, Oncotarget), the authors should address potential localization-specific signature of PON1 in HCC and how its localization contextualizes the VHL binding and HIF-1 α regulation and other functional data. The authors present a subcellular localization of PON1 in Hepa1-6 cells, which I think is derived from their proteomics data, but was not in any way validated via subcompartment fractionation or implicated functionally. This is going to be a critical advance in its molecular biology in cancer.

3. The authors suggest a direct mechanism involving PON1 interaction with VHL to degrade HIF-1 α (Fig. 4 and Fig. 5). However, the co-immunoprecipitation and immunofluorescence assays provided (Fig. 5a-b) do not adequately confirm direct physical interactions or functional causality. To address this, additional biochemical validations, such as proximity ligation assays, should be performed to explicitly validate this molecular interaction. Further, the authors should involve how PON1 enzyme activity (paraoxonase activity) and PON1 secretion are impacted by this VHL binding. At this point in the PON1 literature, this should be clarified, especially in the TME context. I am also curious how VHL binding affects PON1 protein stability while inside the cells and the subsequent effect on PON1 secretion. Lastly, and perhaps the most important for me, the authors need to leverage a proper KO model of PON1 (or VHL) specifically in vivo to unambiguously confirm the involvement of the PON1-VHL-HIF-1 α -lactic acid pathway.

4. In the circulation, PON1 is highly bound to HDL. How do this biology factor in the lactate-driven immunoregulation of HCC? In secretome models, diminished antioxidant capacity due to lower secreted PON1 or PON1-HDL in the blood leads to increased oxidative stress and inflammation exacerbating glycolysis and lactate accumulation within the TME. The manuscript largely ignores the importance of this secretory aspect in PON1 biology considering that in host physiology, PON1 is reliably synthesized and secreted from the liver. Confirmatory experiments on how secreted PON1 directly influence immune microenvironment of HCC is important.

5. The authors strongly associates lactic acid levels with Treg infiltration (Fig. 3h, Fig. 2c-e). However, functional causality through direct manipulation of lactic acid production pathways (e.g., using genetic LDHA knockdown or pharmacological inhibitors) independent of PON1 should be experimentally demonstrated to substantiate this claim definitively.

6. The authors suggest Quercetin enhances anti-PD-1 therapy primarily through increased PON1 expression, yet no direct mechanistic validation (such as promoter-reporter assays, ChIP assays for transcription factors regulating PON1, or epigenetic profiling following Quercetin treatment) is provided.

7. The rPON1 and anti-PD-1 experiments are too important in this manuscript to have this preliminary-level data. The manuscript would greatly benefit from dose-response curves, time-dependent studies, and comparative effectiveness studies (rPON1 alone vs. combination with Quercetin or anti-PD-1). Validate whether Quercetin and rPON1 administration

specifically alter the tumor microenvironment independently of the direct effect on cancer cell proliferation. It would be beneficial to include experiments employing conditional depletion of Tregs or lactic acid production pathway inhibitors.

8. PON1 is a premier example of a highly glycosylated protein in normal and disease/cancer states. Aside from mentioning that PON1 is a glycoprotein, the authors appeared to have neglected the growing literature of PON1 glycosylation in cancer. This is an important discussion as to how PON1 biology is functionally implicated in the TME and how this impacts response to therapy, especially as it has been preliminarily demonstrated by others that PON1 glycosylation in the secretion affects immunoregulatory cytokines and inflammation (Aldonza et al., 2023, eLife; Ahn et al., 2014, MCP).

9. The technical description of each data panel across all figures and equally important, the methods section, suffer from severe lack of information, especially on their key data such as proteomics, PLS-DA, FACS, and in vivo experiments. This was supposed to be a minor comment but has forced me to include this as a major issue since it has impaired my ability to understand each assay performed, the experimental design, and what exactly is the data being presented. For example, the authors mentioned in the Methods and supplementary figure that they generated PON1-KO mouse model (as opposed to PON1-KO or KD in Hepa1-6 cells or SC tumors). Is this indeed the case?

Minor comments:

- The overall quality of data visualization requires substantial improvement, and figure panel arrangement needs to be significantly revised, addressing the following issues:
- Differences in text font sizes in every figure are distracting. The figure label and technical texts (e.g., n size, axis labels) have different sizes, even in many X-Y axes in many panels have different text sizes. The authors need to revise them so all figure panels in one figure have the similar text sizes and text font styling.
- Protein size (kDa) should be included in all blots, per conventional practice. The authors should provide raw full blots in their imaged full or cut membranes.
- ICC images should have size (um) labels (Fig. 5b).
- Kaplan-Meier survival curves and clinical correlations must explicitly provide patient cohort details (stage, grade, treatment history). Data visualization lacks proper annotations regarding sample sizes and statistical methods used.
- Fig. 4: Inadequately represent the proposed mechanistic interactions (PON1-VHL-HIF-1 α axis). Including detailed graphical diagrams or schematic illustrations demonstrating the proposed molecular interactions and validation experiments would significantly enhance clarity, this aside from their overall summary in Fig. 7.
- Incomplete or inconsistent labeling. Many figure panels lack "assay" labels, such as in Fig. 4a, PON1 subcompartment cell localization. Fig 4b, DEPs from their proteomics, and many more across all figures.
- Sizing. The FACS data are presented poorly. Their overall size is small, and hard to decipher any information from it, even when the gating boxes and % cell information are included. The KEGG pathway in Fig. 3a is so small I can only read the text "metabolic pathways". In Fig. 3e, the legend labels are unreadable even if you zoom the image. This sizing issue goes on across all figures.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have sufficiently addressed all of my concerns and I would recommend the manuscript for publication.

Reviewer #2

(Remarks to the Author)

My concerns has been answered by the authors.

Reviewer #3

(Remarks to the Author)

The authors have addressed all of my comments satisfactorily. I have no further concerns and congratulate them on this excellent study!

Reviewer #4

(Remarks to the Author)

The authors have addressed the majority of the main concerns using robust experimental strategies and have significantly

improved the overall presentation of the data and manuscript. Specifically, they substantially strengthened the mechanistic case for a PON1–VHL–HIF-1 α –lactate axis and convincingly tied lactate to Treg accrual using LDHA loss and MCT1 inhibition. The VHL-dependence and secretion/catalytic independence of the anti-tumor effect are shown, and rPON1 is effective with Treg-dependence in vivo.

However, I have some remaining concerns:

- Most of the subcutaneous tumor data shown (not the liver tumors) in the main figures only include end-point tumor weights. Did the authors perform multiple time-point tumor volume measurements for all of the mouse tumor experiments, especially those involving Treg-depleted mice? If so, please present them along with the endpoint tumor data.

- While it is greatly appreciated that the authors have included the murine liver hepatoma cell line (Hep-53.4) and generated subcutaneous tumors, assuming AKT/NRAS co-activation-driven, the authors must still acknowledge the limitation of these murine models when no patient-derived model has been used. Specifically, all the murine HCC cell lines used are originally derived from chemically-induced HCC transformation, not typical of human disease (common TP53 LOF, CTNNB1 activating mutations, TERT, etc).

- Please add a minimal HDL-context control: either compare rHDL–PON1 versus free rPON1 in a single readout already used (lactate or Treg induction) or document that administered rPON1 partitions into HDL and remains active by simple lipoprotein fractionation. This is a brief addition but will pre-empt questions about the physiological carrier state.

- There are sweeping generalizations in the Discussion that should be reworded or better cautioned. For example, certain phrasing implies that the PON1–VHL–HIF-1 α –lactate axis is a broadly applicable mechanism across all HCC contexts, whereas the work is based entirely on murine-derived HCC cell lines and mouse models lacking hallmark human oncogenic lesions.

Similarly, conclusions about therapeutic potential (quercetin as an ICB sensitizer) would benefit from language that clearly distinguishes proof-of-principle in controlled models from clinical generalizability. This will prevent overextension of claims beyond what the data can directly support. A few carefully placed qualifiers (“in our models...”, “in murine HCC...”, “potentially relevant to...”) would preserve the strength of the findings while aligning interpretation with the actual evidence.

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The point-by-point responses are presented in blue, as follows.

Reviewer #1

In this manuscript, Lu, Shi, and Gao et al explore the role of paraoxonase-1 in hepatocellular carcinoma disease progression. Their key findings are as follows:

- PON1 is expressed in the liver and expression is downregulated in HCC tumors compared with normal tissue across multiple public datasets. The authors validate this finding by immunohistochemistry in an internal dataset.

- Both in publicly available and in internal datasets, the authors show that PON1 expression correlates with prognosis in HCC.

- To directly test the role of PON1 in HCC progression, the authors primarily utilize Hepa1-6 cells in which PON1 is either overexpressed or suppressed via shRNA. They show that PON1 overexpression reduces subcutaneous tumor growth while knockdown of PON1 accelerates growth. Interestingly, manipulation of PON1 expression did not alter tumor cell proliferation in vitro, suggesting a non tumor cell autonomous effect.

- They subsequently identify regulatory T cell infiltration as inversely correlating with PON1 expression in patient tumors. Moreover, PON1 suppression increased intratumoral abundance of FoxP3⁺ cells while PON1 overexpression blunted FoxP3⁺ regulatory T cell abundance. Depleting CD25⁺ T cells using a monoclonal antibody abrogated the negative prognosis of control Hepa1-6 cells but not PON1-overexpressing cells.

- Mechanistically, the authors find that PON1 suppressed several metabolic pathways including those related to glycolysis and lactate production, and that co-administration of lactate was sufficient to reverse the impact of PON1 overexpression on anti-tumor immunity. Furthermore, they showed that PON1 promotes HIF-1 α ubiquitylation and degradation, in part by binding and putatively enhancing the activity of the prolyl hydroxylase VHL.

Generally speaking, I found the work to be fairly comprehensive and impressive in scope and I find the authors' argument that PON1 suppresses regulatory T cell infiltration by attenuating HIF1 α -dependent glucose uptake and lactate production to be compelling. I have a few suggestions which would provide some additional clarity to the study.

Response: Thank you for your comments. As requested, we validated the anti-tumor effects of PON1 using an additional cell line (Hep-53.4) and a hydrodynamic tail vein injection plasmid model. Collective evidence demonstrated that the anti-tumor efficacy of PON1 was mediated exclusively by Treg cells, with no direct impact on tumor cell autonomy. Details are as follows.

Q1. The methodology for experiments is sparse in many locations. I would recommend that the authors provide more detail on how the experiments are conducted within the main text rather than having those details exclusively in the methods. Just one example of this is when the authors state that “exogenous and endogenous interaction between PON1, VHL and HIF-1 α were confirmed in Hepa1-6 cells” but they do not provide additional details as to which proteins were exogenous, whether they were FLAG or HA tagged, and/or how they were introduced into cells.

Response: Thank you for this constructive suggestion. As specified in **Extended Data Fig. S4a** and **Fig. 5b**, we have augmented the experimental details in the revised manuscript.

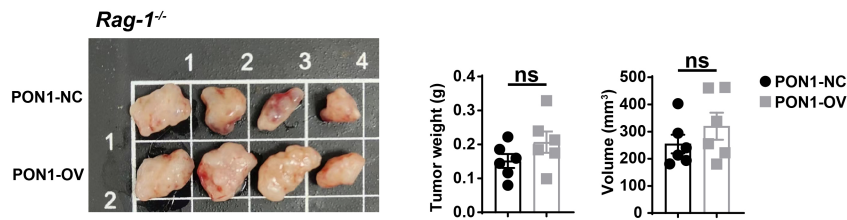
Revised description in the text

Subsequently, we co-transfected Flag-tagged HIF-1 α plasmids and VHL plasmids into PON1-overexpressing Hepa1-6 cells using Lipofectamine 2000. After 48 h, protein interactions were analyzed using co-immunoprecipitation (co-IP). As shown in **Extended Data Fig. S4a**, there were pairwise interactions among PON1, VHL, and HIF-1 α . Additionally, co-IP assays directly performed in Hepa1-6 cells revealed endogenous interactions among HIF-1 α , VHL, and PON1 (**Fig. 5b**).

Q2. The authors suggest that the impact of PON1 manipulation is tumor cell autonomous because it has no impact on proliferation in vitro. Can the authors demonstrate that there is no difference in tumor growth when implanted in Rag KO mice?

Response: Thank you for your comment. In *Rag-1*^{-/-} mice subcutaneously inoculated with PON1-NC and PON1-OV Hep1-6 cells, no significant difference in tumor size was observed between groups (**Extended Data Fig. S11**)

Added Extended Data Fig. S11

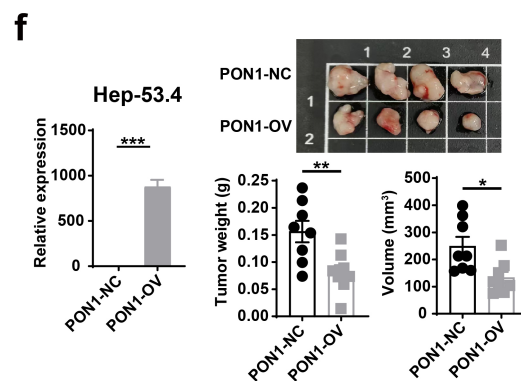


(I) Representative images, weights, and volumes of subcutaneous tumors in *Rag-1*^{-/-} mice implanted with PON1-NC and PON1-OV Hepa1-6 cells (n=6). Data are shown as mean ± SEM and represent at least two independent experiments. ns (not significant).

Q3. The PON1 manipulation experiments are all performed using a single cell line and shRNA for knockdown. The authors should do at least one proof of principle experiment using CRISPR to edit the PON1 locus and it would be nice to see at least one alternate cell line in which this phenotype is observed.

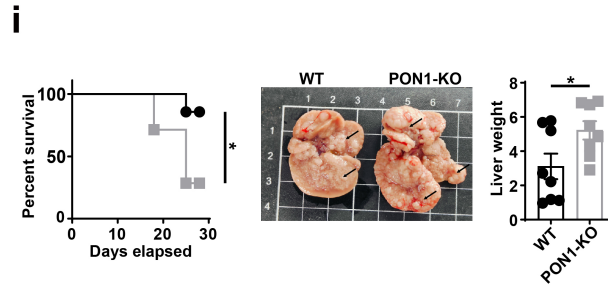
Response: Thank you for your comments. We validated the anti-tumor effects of PON1 in the Hep-53.4 cell line, which recapitulated the tumor-suppressive phenotype observed in Hepa1-6 cells (Extended Data Fig. S1f). Moreover, using hydrodynamic tail vein injection of oncogenic plasmids (pT3-myr-AKT-HA, pT/Caggs-NRASV12 and pCMV(CAT)T7-SB100) to induce autochthonous HCC, we observed accelerated tumor growth and reduced overall survival in PON1-KO mice compared to that in WT controls (Extended Data Fig. S1i).

Added Extended Data Fig. S1f



(f) PON1 expression in Hep-53.4 cells (left). Representative images, tumor weights, and volumes of subcutaneous tumors in the PON1-OV and PON1-NC groups (n=8, right). Data are shown as mean ± SEM and represent at least two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Added Extended Data Fig. S1i

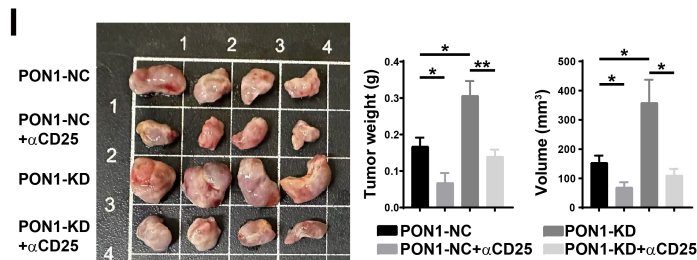


(i) Survival curves (n=7), representative liver tumor images, and liver weights (n=8) in WT and PON1-KO mice with spontaneous HCC induced by hydrodynamic tail vein injection of oncogenic plasmids. Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05.

Q4. The authors show that targeting CD25⁺ cells reduces tumor growth in WT, but not PON1-overexpressing cells. But I think a better experiment is whether targeting CD25 reverses the negative impact of PON1 loss on tumor growth.

Response: Thank you for your comment. By establishing subcutaneous tumor models using PON1-KD Hepa1-6 cells and in combination with anti-CD25-mediated Treg depletion, we demonstrated that Treg ablation reversed the pro-tumorigenic effects of PON1 knockdown (Fig.21).

Added Fig.21



(l) Representative images, weights, and volumes of subcutaneous tumors from the different groups (n=5). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05, **P < 0.01.

Q5. Finally, the authors show that PON1 interacts with VHL –I would be interested to hear any thoughts on how this interaction might alter VHL function. How might the enzymatic activity of PON1 activity?

Response: This an interesting question. Lentiviral shRNA-mediated VHL knockdown models were established to probe PON1 regulation. *In vitro* protease sensitivity assays revealed that PON1's stability was unaltered upon VHL binding (as follows). Consistently, *in vivo* analyses revealed comparable serum PON1 levels and enzymatic activity in VHL-NC and VHL-KD tumors (Extended

Data Fig. S4h). These data collectively establish that the VHL-PON1 interaction did not modulate PON1 secretion or activity. Regarding the functional impact of VHL, we used HDOCK server to predict binding sites for the PON1-VHL interaction and identified the top four sites ranked by binding free energy (**as follows**). However, to date, no functional studies have been conducted on these sites. Our findings establish that PON1 recruits VHL to facilitate HIF-1 α degradation, confirming the integrity of VHL's E3 ubiquitin ligase function within the complex. However, the potential modulation of non-canonical VHL activity (e.g., extracellular matrix assembly or microtubule stabilization) requires further investigation [1-2].

Unpublished Data Figure Redacted

Immunoblot analysis of PON1 expression in VHL-NC and VHL-KD Hepa1-6 cells. Lysates were exogenously treated with or without indicated trypsin concentration.

Added Extended Data Fig. S4g

Unpublished Data Figure Redacted

(h) Serum PON1 levels and activity in tumor-bearing mice from the different groups (n=4). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05, **P < 0.01.

Unpublished Data Figure Redacted

The binding sites of PON1 and VHL predicted by HDOCK server.

1. Kurban, G., et al., Characterization of a von Hippel Lindau pathway involved in extracellular matrix remodeling, cell invasion, and angiogenesis. *Cancer Res*, 2006. 66(3): p. 1313-9.

2. Pu, Y., et al., Microtubule dynamics is a therapeutic vulnerability in VHL-deficient renal cell carcinoma. *Int J Biol Sci*, 2025. 21(7): p. 3286-3305.

Reviewer #2

The manuscript by Lu et al. proposed that liver-specific paraoxonase-1 alleviates regulatory T cell-driven immunosuppression via reducing lactic acid production in hepatocellular carcinoma. They found that PON1 was downregulated during HCC progression and PON1 overexpression suppressed tumor growth through reducing Treg cell infiltration. They also proved that lactic acid was a key metabolic player of PON1 in regulating Treg cell infiltration in HCC. Mechanistically, it was shown that PON1 decreased lactic acid production through the recruitment of Von Hippel-Lindau protein which mediated Hypoxia-inducible factor alpha ubiquitination. Finally, therapeutic effects of PON1 were confirmed using recombinant PON1 protein and Quercetin. This work is truly interesting. The authors are addressing an important area of research. The role of PON1 in HCC progression and immunotherapy is not well understood. The experiments were well designed and the majority of the experiments support the findings. I have listed some of my concerns and suggestions.

Response: Thank you for your comments. In the revised manuscript, we have added content on the regulation of Treg cell differentiation by PON1 and the effect of lactic acid on tumor growth and Treg infiltration. Details are as follows.

Q1. How is PON1 expressed in CD4⁺T cells? Whether PON1 can directly affect CD4⁺T cell differentiation?

Response: Thank you for your comments. PON1 is expressed specifically in the liver. We detected PON1 expression in CD4⁺ T cells and Hepa1-6 cells and found that PON1 levels were significantly higher in Hepa1-6 cells than in CD4⁺ T cells (**as follows**). To investigate the effect of PON1 on Treg cell differentiation, an *in vitro* Treg cell differentiation model was treated with various concentrations of recombinant PON1 (rPON1). The results demonstrated that rPON1 did not alter Treg cell differentiation (**Extended Data Fig. S3m**).

Unpublished Data Figure Redacted

143

144 *PON1* expression in CD4⁺T cells and Hepa1-6 cells. **P < 0.01.

145 **Added Extended Data Fig. S3m**

Unpublished Data Figure Redacted

146

147 (m) Treg cell percentages in groups treated with different rPON1 concentrations (n=4). Data are shown
148 as mean ± SEM and represent at least two independent experiments. ns (not significant).

149 Q2. Figure 3j-k mentioned that lactic acid promoted Treg cell differentiation in vitro. Does lactic acid
150 have an effect on the proliferation and apoptosis of Treg cells?

151 **Response:** Thank you for your comments. *In vitro*, we found that lactic acid promoted Treg cell
152 differentiation in a dose-dependent manner. When the isolated Treg cells were treated with lactic acid
153 at an equivalent concentration (5 mM), lactate did not affect Treg cell proliferation or apoptosis
154 (Dye⁺7-AAD⁺ population) but slightly decreased Treg cell death (Dye⁺7AAD⁻ population) (**Extended**
155 **Data Fig. S3e AND S3g**).

156 **Added Extended Data Fig. S3e**

Unpublished Data Figure Redacted

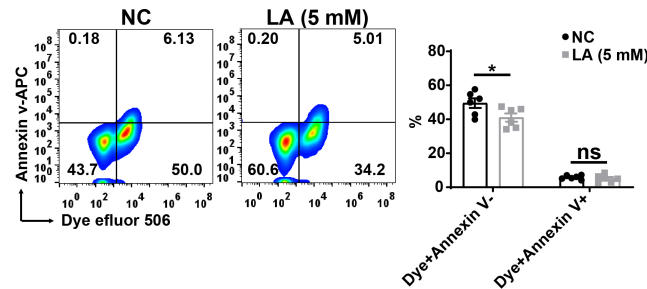
157

158 (e) Treg cell proliferation (n=6) with or without lactic acid treatment. Data are shown as mean ± SEM
159 and represent at least two independent experiments. ns (not significant).

160

Added Extended Data Fig. S3g

g



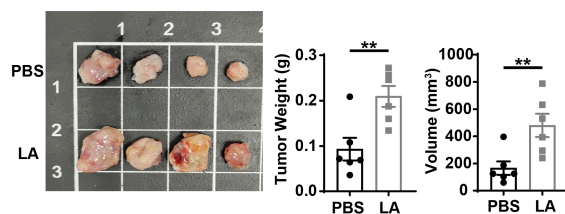
(g) Treg cell apoptosis (n=6) with or without lactic acid treatment. Data are shown as mean $\pm$ SEM and represent at least two independent experiments. * $P < 0.05$, ns (not significant).

Q3. In Figure 3i, lactic acid undermines the tumor-suppressive effect of PON1. What is the effect of lactic acid treatment alone on the proportion of Treg cells and tumor growth?

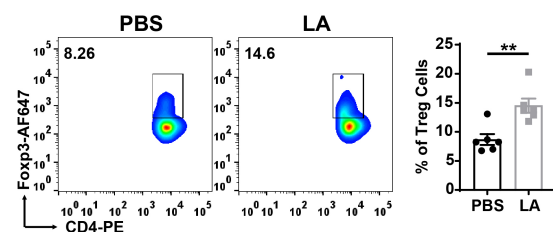
Response: Thank you for your comments. In the revised manuscript, we established subcutaneous tumor-bearing models using Hepa1-6 cells and treated them with or without lactic acid. As expected, intratumoral lactic acid administration increased Treg cell infiltration and promoted tumor growth (Extended Data Fig. S3n-o).

Added Extended Data Fig. S3n-o

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o



(n) Representative images, weights, and volumes of subcutaneous tumors treated with or without lactic acid (n=6). (o) Treg cell percentages in subcutaneous tumors treated with or without lactic acid (n=6). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. ** $P < 0.01$.

Q4. According to Figure 3a, the metabolism is significantly affected by the overexpression of PON1. I'd recommend incorporating a discussion about the relationship between PON1 and metabolism.

Response: Thank you for your comment. We have incorporated a discussion on the relationship between PON1 and metabolic reprogramming in the revised manuscript (as follows).

Addition to the Discussion

PON1 has been reported to be critical for maintaining normal physiological levels of crucial lipid metabolic intermediates. PON1 deficiency significantly reduces circulating glycerol and β -hydroxybutyrate, while enhancing macrophage cholesterol synthesis [41]. Although PON1 has been implicated in glucose homeostasis and glycolytic regulation, its mechanistic basis remains elusive [42]. Here, we demonstrated that PON1 overexpression reprograms tumor metabolism, suppresses multiple intermediates, and mechanistically delineates its role in lactic acid biogenesis. These findings provide novel insights into PON1's function as an important metabolic regulator.

Q5. There are some typos in the manuscript, it may be useful to check the manuscript by a professorial English editing service agency.

Response: Thank you for your suggestions. The manuscript was proofread by professional editor from Wiley Editing Services (<https://en.wileyeditingservices.com/>, MBTRK_1).

Reviewer #3

In this study, Lu et al. have uncovered the role of paraoxonase-1 (PON1) in shaping the immune landscape of hepatocellular carcinoma (HCC). They demonstrate that low expression of PON1 correlates with elevated Treg numbers and exacerbated tumor growth, while PON1 overexpression shows the inverse, accompanied by the expansion of IFN- γ ⁺ CD8 T cells. The authors suggest that PON1 loss induces lactic acid production in HCC, which promotes Treg-mediated immune suppression. Mechanistically, PON1 targets HIF1 α for degradation, and PON1 loss stabilizes HIF1 α , leading to increased lactic acid production in HCC. This is a well-designed study with logical experimental setups and high-quality data. However, several questions remain.

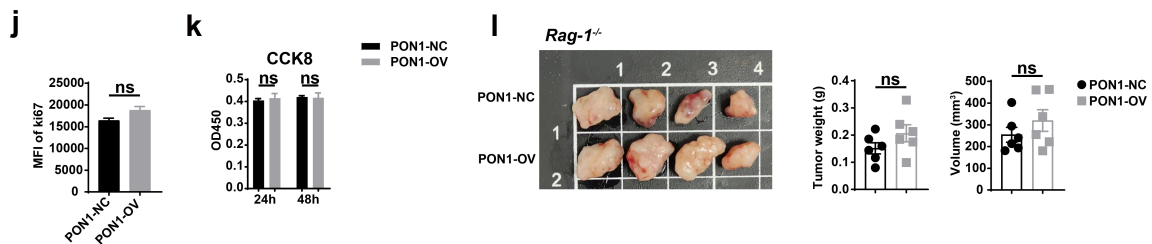
Response: In the revised manuscript, we inoculated *Rag-1*^{-/-} mice with PON1-NC and PON1-OV Hepa1-6 cells. Tumor growth analysis revealed no significant differences, ruling out cell-intrinsic tumorigenic effects for PON1. By combining PON1 knockdown in Hepa1-6 cells with *in vivo* Treg depletion, we confirmed that the anti-tumor effect of PON1 was mediated by Treg cells. Additionally, we further consolidated the evidence that lactic acid regulated Treg cell differentiation.

Q1. While the authors claim that exacerbated HCC growth is linked to lactic acid-mediated Treg expansion, they should exclude the possibility that PON1 may have a tumour-intrinsic function regulating growth. To address this, the authors should compare the growth of PON1-OV and PON1-

KD cells in both B6 and Rag1^{-/-} (T cell-deficient) mice.

Response: Thank you for your comment. *In vitro*, PON1 overexpression did not affect the proliferation of Hepa1-6 cells (Extended Data Fig. S1j-k). To definitively rule out the cell-intrinsic tumorigenic effects for PON1, we inoculated Rag1^{-/-} mice with PON1-NC and PON1-OV Hepa1-6 cells. The results showed comparable tumor growth between groups (Extended Data Fig. S1l).

Extended Data Fig. S1j-l

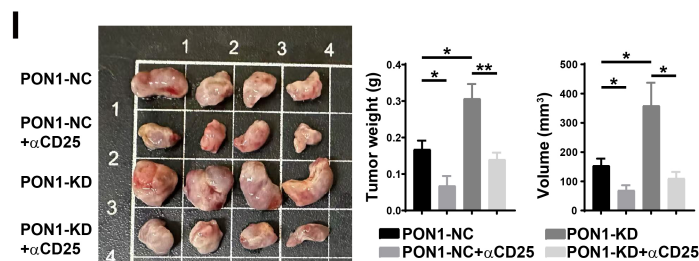


(j) Ki67 expression in PON1-NC and PON1-OV Hepa1-6 cells (n=3). (k) Cell viability of PON1-NC and PON1-OV Hepa1-6 cells was determined via CCK-8 assay (n=4). (l) Representative images, weights, and volumes of subcutaneous tumors in Rag1^{-/-} mice implanted with PON1-NC and PON1-OV Hepa1-6 cells (n=6). Data are shown as mean ± SEM and represent at least two independent experiments. ns (not significant).

Q2. The authors show that depleting Tregs in the PON1-OV setting did not make a difference compared to the Treg un-depleted cohort. However, an ideal experiment would involve anti-CD25 treatment of mice bearing PON1-KD tumours and comparing the results with PON1-NC tumours.

Response: Thank you for your comment. By establishing subcutaneous tumor models using PON1-KD Hepa1-6 cells and in combination with anti-CD25-mediated Treg depletion, we demonstrated that Treg ablation reversed the pro-tumorigenic effects of PON1 knockdown (Fig.2l).

Added Fig.2l

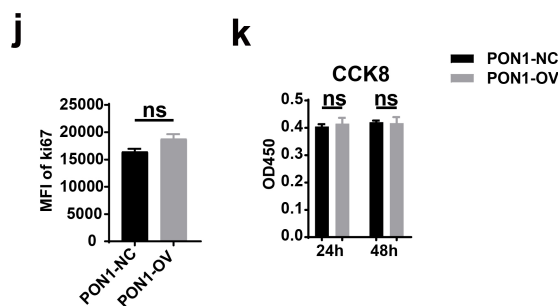


(l) Representative images, weights, and volumes of subcutaneous tumors from the different groups (n=5). Data are shown as mean ± SEM and represent at least two independent experiments. *P < 0.05,

****P < 0.01.**

Q3. Lactic acid accumulates in highly proliferating tumours. The authors should assess the in vitro proliferation rates of PON1-NC and PON1-OV groups.

Response: Thank you for the comment. In **Extended Data Fig. S1j-k**, the proliferation assays of PON1-NC and PON1-OV Hepa1-6 cells showed no significant differences in either Ki67 staining or CCK-8 analysis. Based on this phenomenon, we hypothesize that beyond lactic acid alterations, PON1 overexpression induces changes in other metabolites, potentially involving compensatory mechanisms in the regulation of cellular proliferation.

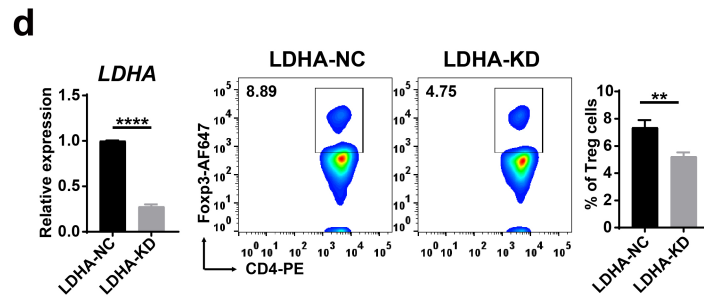


(j) Ki67 expression in PON1-NC and PON1-OV Hepa1-6 cells (n=3). (k) Cell viability of PON1-NC and PON1-OV Hepa1-6 cells was determined via CCK-8 assay (n=4). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. ns (not significant).

Q4. While HIF1 α regulates lactic acid, the authors could further strengthen the lactic acid-Treg link by deleting LDHA in Hpa1-6 tumours and by deleting MCT1 in Tregs, preventing them from sensing lactic acid.

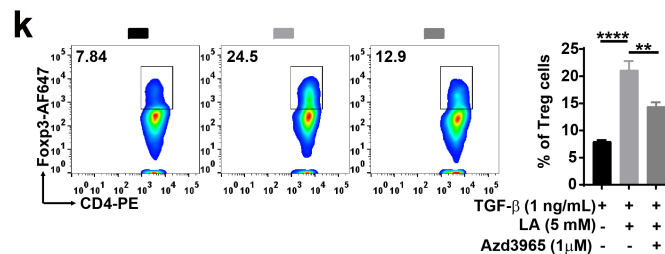
Response: Thank you for your suggestions. In the revised manuscript, co-culture experiments showed that CD4⁺ T cells incubated with LDHA knockdown (LDHA-KD) Hepa1-6 cells exhibited significantly reduced Treg cell differentiation compared to LDHA-NC controls (**Extended Data Fig. S3d**). Furthermore, we demonstrated that lactic acid promoted differentiation of CD4⁺ T cells into Treg cells, an effect abolished by the MCT1 inhibitor AZD3965 through blockade of lactic acid transport (**Fig.3k**). These results provide evidence for lactic acid-dependent Treg regulation.

Added Extended Data Fig. S3d



(d) The expression of LDHA in Hepa1-6 cells, and its effect on Treg cell differentiation (n=6). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. **P<0.01, ****P < 0.0001.

Revised Fig. 3k



(k) Proportion of Treg cells treated with lactic acid or MCT1 inhibitor (AZD3965, 1 μ M) (n=5). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. **P<0.01, ****P < 0.0001.

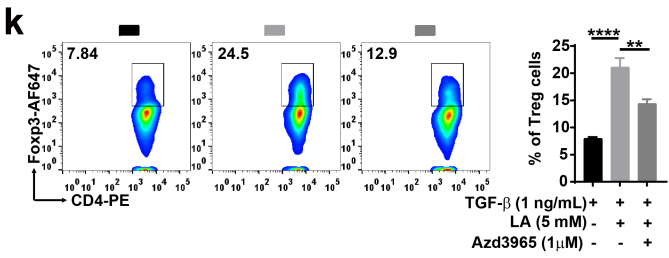
Q5. How does lactic acid expand Tregs? Do existing Tregs expand, or is there recruitment of new Tregs to PON1-KD tumors? The authors also seem to suggest that CD4 T cells upregulate Foxp3. The authors should test the origin of Tregs in PON1-KD tumours and perform TCR-seq on Tregs and CD4 T cells in this setting to assess overlap between these subsets.

Response: Thank you for your comments.

(1) Flow cytometry results showed that 5 mM lactic acid promoted the differentiation of total CD4⁺ T cells isolated from the spleen into Treg cells (Fig. 3k). *In vitro*, we used TGF- β to induce Treg cell differentiation and then purified the Treg cells using isolation Kit. Purified Treg cells were treated with the same concentration of lactic acid (5 mM), and their proliferation and migration were detected. As shown in (Extended Data Fig. S3e-f), lactic acid did not affect Treg cell proliferation or migration. Lactic acid slightly reduced Treg cell death but not apoptosis (Extended Data Fig. S3g).

Revised Fig. 3k

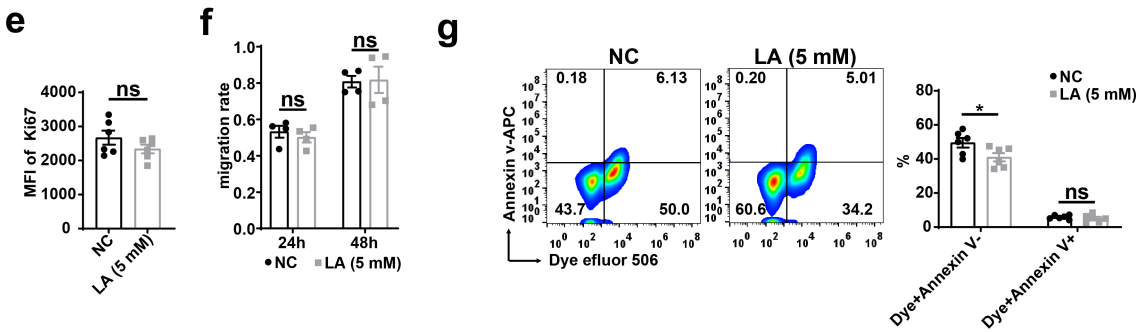
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277 (k) Proportion of Treg cells treated with lactic acid or MCT1 inhibitor (AZD3965, 1 μM) (n=5). Data
278 are shown as mean ± SEM and represent at least two independent experiments. **P<0.01, ****P <
279 0.0001.

280 Added Extended Data Fig. S3e-g



281

282 (e-g) Treg cell proliferation (n=6), migration(n=4), and apoptosis (n=6) with or without lactic acid
283 treatment. Data are shown as mean ± SEM and represent at least two independent experiments. *P <
284 0.05 and ns (not significant).

285 (2) Tan et al. reported that the expanded Treg population in tumors consists not of thymus-derived
286 Tregs (nTregs) but of peripherally induced Tregs (pTregs), which are predominantly derived from T
287 helper type 1 cell (Th1) cells [1]. Intriguingly, our single-cell analysis of PON1-NC and PON1-KD
288 subcutaneous tumors revealed two distinct Treg cell subsets, exhibiting phenotypic characteristics
289 consistent with previously published data (Extended Data Fig. S3h). In PON1-KD tumors, the
290 dominant expanded Treg subset is comprised of pTregs (Extended Data Fig. S3i). These pTregs
291 displayed genomic signatures strikingly similar to those of Th1 cells, including elevated expression of
292 the Th1-specific transcription factor Tbx21(Extended Data Fig. S3j). Pseudotime trajectory analysis
293 further suggested a potential conversion trajectory from Th1 cells to pTregs (Extended Data Fig. S3k).
294 By integrating *in vivo* and *in vitro* evidence, we propose that lactic acid primarily promotes local Treg

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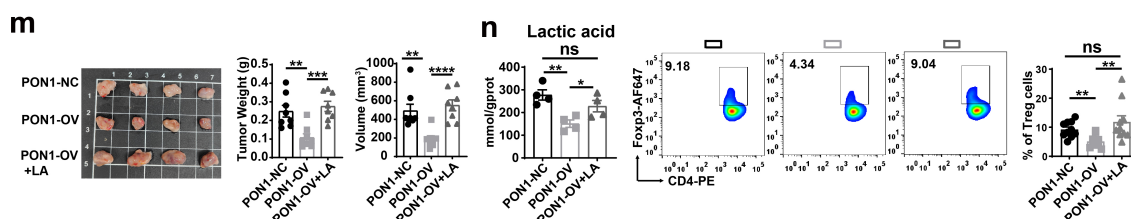
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soluble anti-CD28 antibody (2 $\mu\text{g/mL}$) in RPMI-1640/10% FBS medium. After 48 h incubation under standard conditions (37 $^{\circ}\text{C}$, 5% CO_2), Foxp3 expression was analyzed by flow cytometry.

Q7. In the lactic acid treatment experiment, the authors observe elevated tumour growth. However, in this condition, Tregs are lower in frequency compared to the NC group, which contradicts the argument that lactic acid induces Tregs. Could the authors clarify this discrepancy?

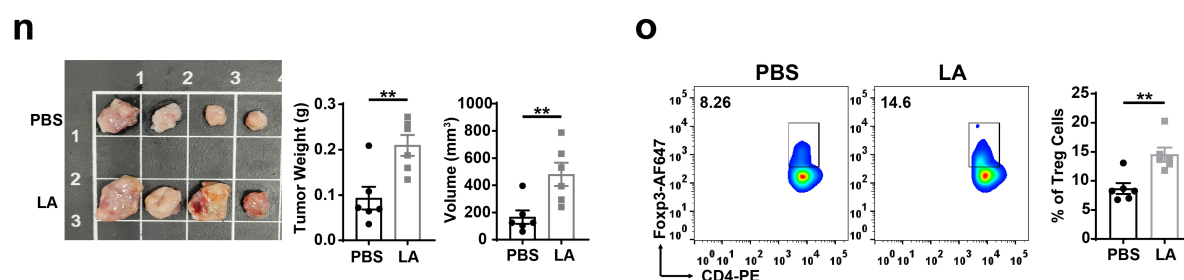
Response: We apologize for this confusion. In the revised manuscript, **Fig. 3m-n** demonstrate that compared to lactic-acid-untreated PON1-OV tumors, intratumoral lactic acid administration significantly increased Treg cell infiltration and accelerated tumor growth. Tumors in the lactic-acid-treated PON1-OV group, however, showed no significant differences in either tumor size or Treg cell proportions when compared with those in the PON1-NC group. In the revised manuscript, we introduced an additional experimental group treated with lactic acid alone in mice bearing subcutaneous Hepa1-6 tumors. As shown in **Extended Data Fig. S3n-o**, lactic acid treatment of Hepa1-6 subcutaneous tumors increased the proportion of Treg cells and accelerated tumor growth, which confirms the role of lactic acid in promoting Treg infiltration."

Revised Fig. 3m-n



(m) Representative images, weights, and volumes of subcutaneous tumors of the different groups (right, n=8). (n) Lactic acid content (n=4) and Treg cell percentages in subcutaneous tumors from the different groups (n=10). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. **P<0.01, ***P<0.001, ****P<0.0001, and ns (not significant)

Added Extended Data Fig. S3n-o



(n) Representative images, weights, and volumes of subcutaneous tumors treated with or without lactic acid (n=6). (o) Treg cell percentages in subcutaneous tumors treated with or without lactic acid (n=6). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. **P < 0.01.

Q8. In the lactic acid-treated condition, tumour growth is exacerbated, which the authors propose is mediated by Treg expansion and dampened anti-tumour immunity. However, lactic acid is known to directly promote CD8 T cell dysfunction, which could also contribute to tumor growth. Please address this possibility.

Response: Thank you for your comments.

We agree with your perspective that lactic acid promotes CD8⁺ T-cell dysfunction. In the lactic acid-treated groups, we observed CD8⁺ T cell dysfunction characterized by reduced secretion of IFN- γ and TNF- α , along with elevated expression of PD-1 (**as follows**). Consistently, *in vitro* lactic acid treatment suppressed IFN- γ and TNF- α production in CD8⁺ T cells (**as follows**).

In vivo

Unpublished Data Figure Redacted

Expression of IFN- γ , TNF- α , PD-1, Lag3, and Tim3 in tumor-infiltrating CD8⁺ T cells from mice treated with or without lactic acid (n=5). **P < 0.01, ****P < 0.0001, and ns (not significant)

In vitro

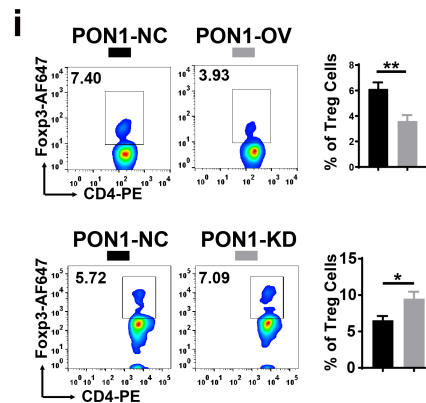
Unpublished Data Figure Redacted

Expression of IFN- γ , TNF- α , PD-1, Lag3, and Tim3 in CD8⁺ T cells treated with or without lactic acid (n=6). **P < 0.01, ***P < 0.001, and ns (not significant)

However, our *in vitro* co-culture experiments demonstrated that compared to PON1-NC controls, PON1-OV Hepa1-6 cells suppress Treg cell generation while exhibiting no measurable impact on

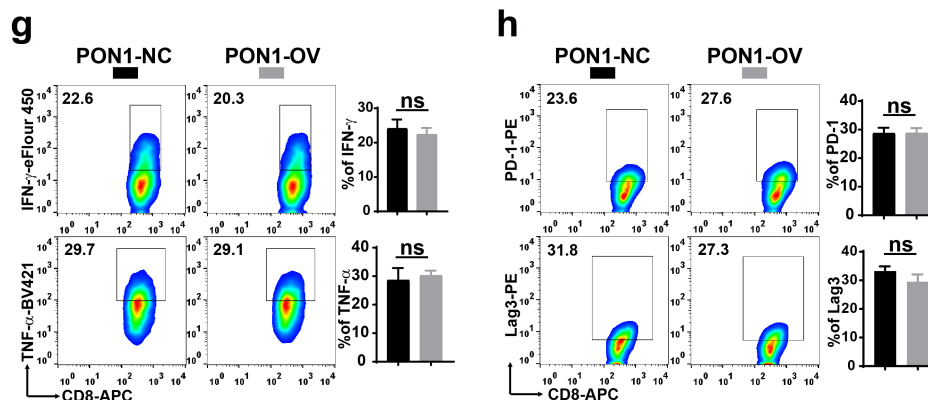
CD8⁺ T cell function (**Fig.2i** and **Extended Data Fig. S2g-h**). Given that PON1 overexpression activates multiple metabolic pathways, compensatory mechanisms are likely to regulate CD8⁺ T cells in this setting, which may explain the absence of functional modulation *in vitro*. *In vivo* intratumoral lactic acid injection significantly enhanced Treg cell infiltration (**Extended Data Fig. S3o**). Crucially, Treg depletion experiments in both the PON1-KD and PON1-OV models established the Treg-dependent nature of PON1-mediated anti-tumor effects (**Fig. 2k-l**). Collectively, these data identify that the PON1-lactic acid-Tregs axis is a pivotal regulatory mechanism in tumors, with *in vivo* CD8⁺ T cell functional alterations predominantly resulting from Treg cell reduction. We have incorporated this discussion on CD8⁺ T cell functionality into the revised manuscript.

Fig.2i



(i) Impact of PON1 expression in Hepa1-6 cells on CD4⁺ T cell differentiation into Treg cells (n=4-5). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05, **P<0.01.

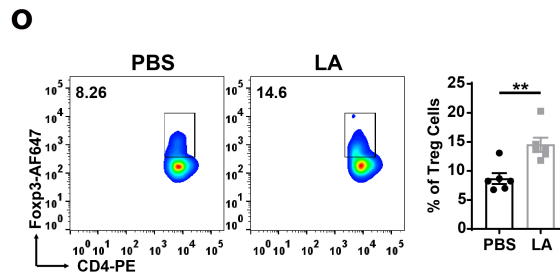
Extended Data Fig. S2g-h



(g) CD8⁺ T cells isolated from spleens by MACS were co-cultured with either PON1-NC or PON1-OV Hepa1-6 cells in 48-well plates, supplemented with anti-CD3 (2 μ g/mL) and anti-CD28 (2 μ g/mL).

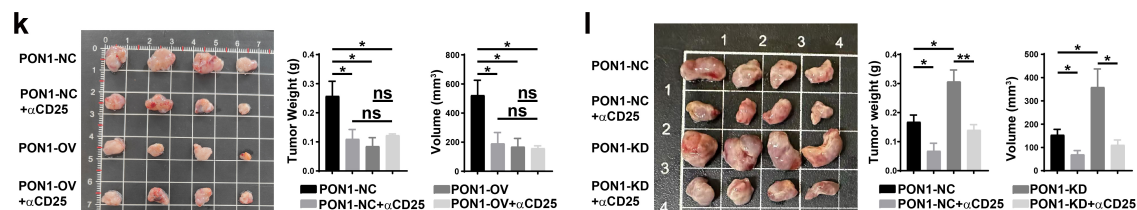
IFN- γ and TNF- α expression in CD8⁺ T cells was measured 48 h later (n=4). (h) PD-1 and Lag3 expression on CD8⁺ T cells after 48 h of co-culture (n=5). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. ns (not significant).

Added Extended Data Fig. S3o



(o) Treg cell percentages in subcutaneous tumors treated with or without lactic acid (n=6). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. **P < 0.01.

Added Fig. 2k-l



(k-l) Representative images, weights, and volumes of subcutaneous tumors from the different groups (n=4-5). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05, **P < 0.01, and ns (not significant).

Q9. How was the experiment demonstrating lactic acid dose-dependent induction of Foxp3 performed? Was it a co-culture of lactic acid, TGF- β , and CD4 T cells? Was anti-CD3/28 or IL-2 used? Please provide the experimental method.

Response: Thank you for your comment. We have added the method to the Supplementary Materials (as follows).

Addition in the Supplementary Methods

In Vitro Treg differentiation Assay

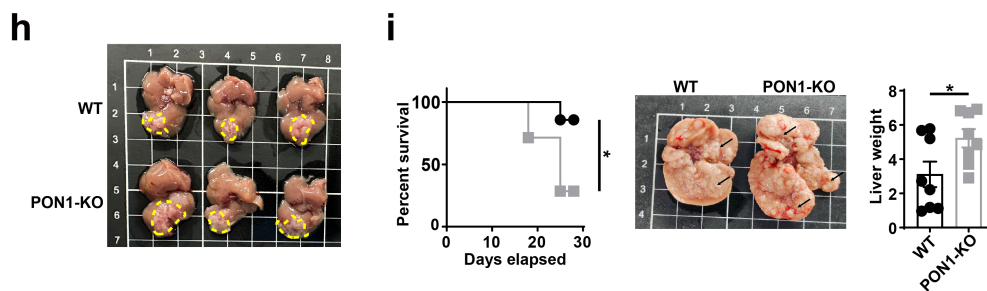
Following overnight coating of 48-well plates with anti-CD3 antibody (2 μ g/mL) at 4°C, total CD4⁺ T cells were isolated from C57BL/6 mouse spleens using the EasySep™ Mouse CD4⁺ T Cell Isolation Kit (STEMCELL, 19852). CD4⁺ T cells (5×10^5 per well) were stimulated with anti-CD28 (2 μ g/mL) and TGF- β (1 ng/mL), treated with varying lactic acid concentrations, and harvested after 48 h or 72 h

for Foxp3 expression analysis.

Q10. The authors should induce Hepa1-6 tumours in both PON1^{-/-} and wild-type mice to assess whether tumour growth is faster in the knockout mice, which have more Tregs.

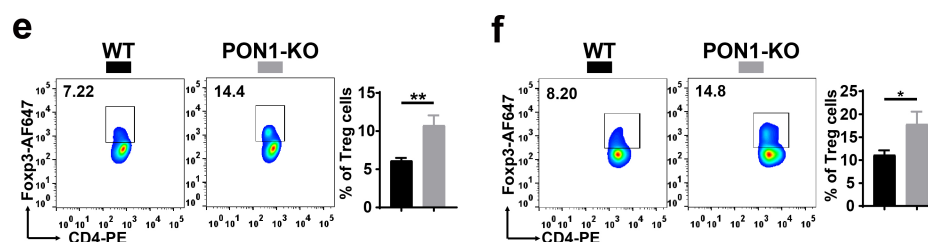
Response: Thank you for the suggestion. As shown in **Extended Data Fig. S1h** and **Extended Data Fig. S2e**, orthotopic implantation of Hepa1-6 cells in WT and PON1-KO mice revealed a significant increase in tumor-infiltrating Treg cells and accelerated tumor growth in KO mice. To further validate PON1's role in tumorigenesis, we established an oncogene-induced spontaneous HCC model via hydrodynamic tail vein injection of oncogenic plasmids (pT3-myr-AKT-HA, pT/Caggs-NRASV12 and pCMV(CAT)T7-SB100). Results demonstrated that PON1-KO mice exhibited exacerbated tumor progression, reduced survival rates, and enhanced intratumoral Treg cell infiltration (**Extended Data Fig. S1i** and **Extended Data Fig. S2f**).

Revised Extended Data Fig. S1h-i



(h) Tumor growth in livers of WT and PON1-KO mice after orthotopic inoculation with Hepa1-6 cells (n=3). (i) Survival curves (n=7), representative liver tumor images, and liver weights (n=8) in WT and PON1-KO mice with spontaneous HCC induced by hydrodynamic tail vein injection of oncogenic plasmids. Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05.

Revised Extended Data Fig. S2e-f



(e) Treg cell percentages in orthotopic Hepa1-6 tumors implanted in WT and PON1-KO mice (n=5). (f) Treg cell percentages in WT and PON1-KO mice with orthotopic HCC induced by hydrodynamic tail-

vein injection (n=6). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05, **P < 0.01.

Reviewer #4

In the present manuscript, the authors explore a novel role for liver-specific paraoxonase-1 (PON1) in modulating regulatory T cell (Treg)-mediated immunosuppression in hepatocellular carcinoma (HCC), primarily using murine subcutaneous hepatoma tumor model. They propose an intriguing mechanism by which PON1 suppresses lactic acid production via VHL-mediated ubiquitination and degradation of HIF-1 α , leading to reduced Treg infiltration. Clinical associations further suggest a potential therapeutic advantage in modulating PON1 expression to enhance anti-tumor immunity.

This is an interesting and novel conceptual framework that highlights the immunometabolic interplay between PON1 and Treg cells in the tumor microenvironment. The therapeutic potential of modulating PON1 via Quercetin to enhance anti-PD-1 responses in HCC is particularly compelling. However, the manuscript lacks important PON1 biology and function in their proposed model.

Response: Using immunofluorescence staining and nucleocytoplasmic fractionation assays, we determined that PON1 was primarily localized to the cytoplasm. By generating VHL-knockdown and VHL-knockdown/PON1-overexpressing Hepa1-6 cells, we established that PON1's anti-tumor effects depend on its interaction with VHL. Additionally, we restructured the figure panels to enhance data clarity.

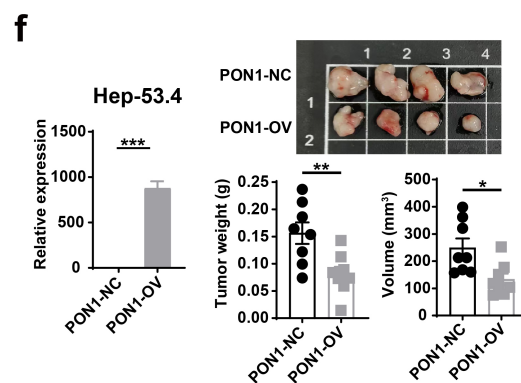
Major comments:

Q1. The authors heavily relied on using the murine hepatoma cell line Hepa1-6 and derived subcutaneous tumors in B6 mice. Hepa1-6 is derived from chemically-induced HCC which heavily lacks hallmark human HCC-associated oncodrivers (e.g., TP53, CTNNB1, TERT promoter mutations) and implicated signaling because of differences in the nature of tumor induction. This limits the translatability of their major findings in human HCC, especially how PON1 is metabolically implicated in these tumors and their microenvironment. This is not to argue that Hepa1-6 model is not an appropriate HCC model but rather is lacking of components important to contextualize the conclusions in this manuscript in clinically-relevant HCC biology. Therefore, it is my opinion that the

main findings should be tested as well in relevant human HCC models (e.g., PDX mice, patient-derived or PDX-derived organoids) or, at the very least, implicate the immunosuppression findings in a spontaneous HCC mouse model.

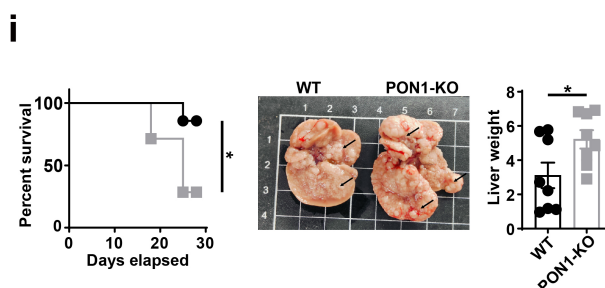
Response: Thank you for the suggestion. To further validate PON1's role in tumor immunosuppression and to enhance its clinical relevance in HCC, we overexpressed PON1 in Hep-53.4 cells and established subcutaneous xenografts. Consistent with the Hepa1-6 cell results, PON1-OV Hep-53.4 tumors showed a significant reduction in growth (**Extended Data Fig. S1f**). Complementarily, using hydrodynamic tail vein injection of oncogenic plasmids to induce spontaneous HCC, we observed that PON1-KO mice showed reduced survival, increased tumor burden, and enhanced intratumoral Treg cell accumulation (**Extended Data Fig. S1i and Extended Data Fig. S2f**).

Added Extended Data Fig. S1f



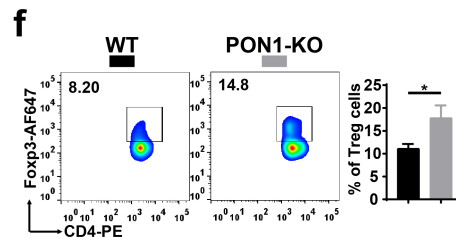
(f) PON1 expression in Hep-53.4 cells (left). Representative images, tumor weights, and volumes of subcutaneous tumors in the PON1-OV and PON1-NC groups (n=8, right). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Added Extended Data Fig. S1i



(i) Survival curves (n=7), representative liver tumor images, and liver weights (n=8) in WT and PON1-KO mice with spontaneous HCC induced by hydrodynamic tail vein injection of oncogenic plasmids. Data are shown as mean $\pm$ SEM and represent at least two independent experiments. *P < 0.05.

470 **Added Extended Data Fig. S2f**

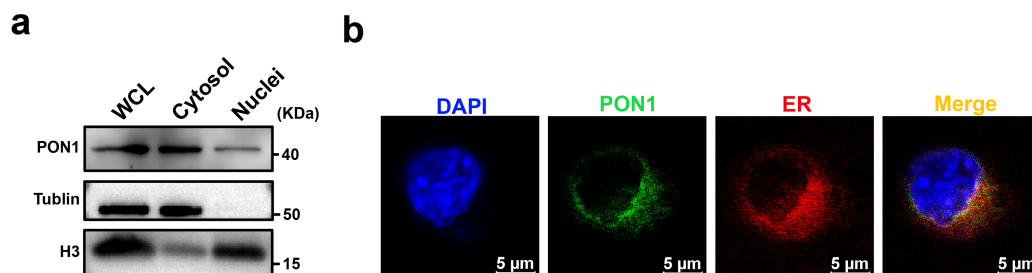


475 (f) Treg cell percentages in WT and PON1-KO mice with orthotopic HCC induced by hydrodynamic
476 tail-vein injection (n=6). Data are shown as mean $\pm$ SEM and represent at least two independent
477 experiments. *P < 0.05.

478 Q2. Considering the crude evidence on how PON1 is differentially expressed in different malignancies
479 and how PON1 can be differentially regulated to induce pro-tumorigenic or tumor suppressive effects,
480 depending on its localization along the secretory route (Golgi/ER, cytoplasm, nucleus) (Aldonza et al.,
481 2023, eLife; Aldonza et al., 2017, Oncotarget), the authors should address potential localization-
482 specific signature of PON1 in HCC and how its localization contextualizes the VHL binding and HIF-
483 a regulation and other functional data. The authors present a subcellular localization of PON1 in
484 Hepa1-6 cells, which I think is derived from their proteomics data, but was not in any way validated
485 via subcompartment fractionation or implicated functionally. This is going to be a critical advance in
486 its molecular biology in cancer.

487 **Response:** Thank you for the suggestion. To determine the subcellular localization of PON1, nuclear-
488 cytoplasmic fractionation assays was performed. As shown in **Fig. 4a**, PON1 was predominantly
489 localized in the cytoplasm. Immunofluorescence staining indicated that PON1 is primarily localized in
490 the cytoplasm **Fig. 4b**.

491 **Added Fig. 4a-b**



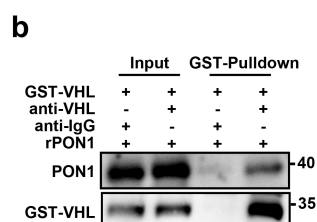
marker Calnexin (red) in Hepa1-6 cells. Scale bar: 5 μ m.

Q3. The authors suggest a direct mechanism involving PON1 interaction with VHL to degrade HIF-1 α (Fig. 4 and Fig. 5). However, the co-immunoprecipitation and immunofluorescence assays provided (Fig. 5a-b) do not adequately confirm direct physical interactions or functional causality. To address this, additional biochemical validations, such as proximity ligation assays, should be performed to explicitly validate this molecular interaction. Further, the authors should involve how PON1 enzyme activity (paraoxonase activity) and PON1 secretion are impacted by this VHL binding. At this point in the PON1 literature, this should be clarified, especially in the TME context. I am also curious how VHL binding affects PON1 protein stability while inside the cells and the subsequent effect on PON1 secretion. Lastly, and perhaps the most important for me, the authors need to leverage a proper KO model of PON1 (or VHL) specifically *in vivo* to unambiguously confirm the involvement of the PON1-VHL-HIF-1 α -lactic acid pathway.

Response: Thank you for your comments.

(1) To demonstrate the direct interaction between PON1 and VHL, we performed a GST pull-down assay. First, we constructed a GST-VHL plasmid and purified the fusion protein. However, owing to the low solubility of the purified protein, we conducted an *in vitro* co-IP experiment by co-incubating the anti-VHL antibody with the GST-VHL protein, followed by the addition of the recombinant PON1 protein. As shown in **Extended Data Fig. S4b**, PON1 interacted directly with VHL.

Added Extended Data Fig. S4b



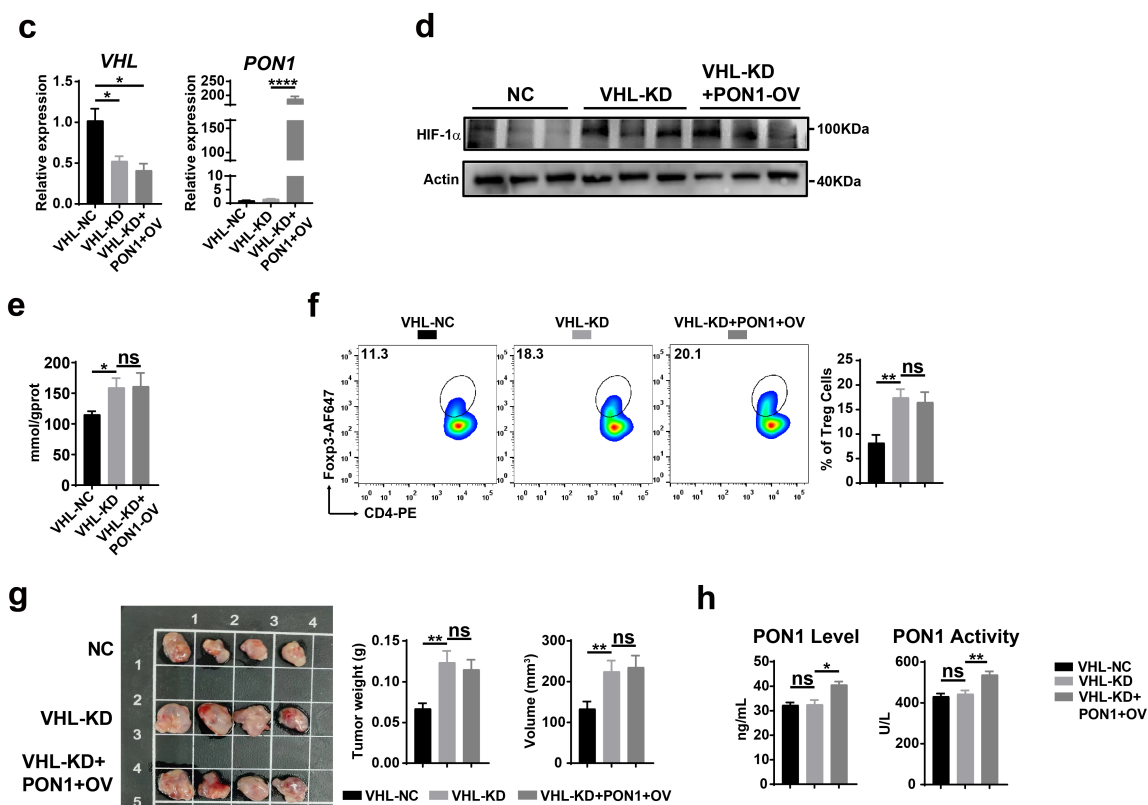
(b) GST pull-down results of the interaction between PON1 and VHL.

(2) To assess whether VHL binding regulates PON1 stability, we performed trypsin sensitivity assays in VHL-knockdown (VHL-KD) Hepa1-6 cells. The results showed that the VHL knockdown did not alter PON1's sensitivity to trypsin (**as follows**).

Immunoblot analysis of PON1 expression in VHL-NC and VHL-KD Hepa1-6 cells. Lysates were exogenously treated with or without indicated trypsin concentration.

(3) To investigate the role of the PON1-VHL interaction in tumorigenesis, we established subcutaneous xenograft models using Hepa1-6 cells with VHL-KD or VHL-KD combined with PON1-overexpression (VHL-KD+PON1-OV) (**Extended Data Fig. S4c**). Compared to controls, the VHL-KD group exhibited increased HIF-1 α protein levels, lactic acid accumulation, Treg cell infiltration and accelerated tumor growth (**Extended Data Fig. S4d-g**). Analysis of serum PON1 levels and enzymatic lactonase activity across the three experimental groups revealed that VHL knockdown did not affect PON1 secretion or activity compared to the controls, whereas the VHL-KD+PON1-OV group exhibited significantly increased PON1 secretion and activity relative to the VHL-KD group (**Extended Data Fig. S4h**). Critically, PON1 overexpression in VHL-KD cells did not alter the HIF-1 α -lactic acid-Treg axis and failed to reverse the pro-tumor effects of VHL knockdown (**Extended Data Fig. S4d-g**). These findings demonstrate that the anti-tumor activity of PON1 was independent of its secreted enzymatic function but strictly dependent on its binding to VHL.

Added Extended Data Fig. S4c-h



(c) VHL and PON1 expression in Hep1-6 cells with different treatments (n=3). Subcutaneous tumors established using Hepa1-6 cells with VHL knockdown, with or without PON1 overexpression (d-f) HIF-1 α protein levels (n=3), lactic acid concentrations (n=4), and Treg cell proportions (n=4) in subcutaneous tumors from the different groups. (g) Representative images, weights, and volumes of subcutaneous tumors from the different groups (n=12). (h) Serum PON1 levels and activity in tumor-bearing mice from the different groups (n=4). Data are representative of at least two independent experiments (mean $\pm$ SEM). *P<0.05, **P<0.01, ****P < 0.0001, and ns (not significant).

Q4. In the circulation, PON1 is highly bound to HDL. How do this biology factor in the lactate-driven immunoregulation of HCC? In secretome models, diminished antioxidant capacity due to lower secreted PON1 or PON1-HDL in the blood leads to increased oxidative stress and inflammation exacerbating glycolysis and lactate accumulation within the TME. The manuscript largely ignores the importance of this secretory aspect in PON1 biology considering that in host physiology, PON1 is reliably synthesized and secreted from the liver. Confirmatory experiments on how secreted PON1 directly influence immune microenvironment of HCC is important.

Response: Thank you for your comments.

(1) Resistance to oxidative stress is a classical critical function of PON1, and our investigation initially

focused on this antioxidant capacity. *In vitro* experiments demonstrated that the modulation of PON1 expression did not alter intracellular ROS levels (**Extended Data Fig. S6a-b**).

Revised Extended Data Fig. S6a-b

Unpublished Data Figure Redacted

(a) ROS levels in control, PON1-OV, and PON1-KD Hepa1-6 cells (n=4). (b) Mitochondrial structures of PON1-NC and PON1-OV Hepa1-6 cells. Data in a are representative of at least three independent experiments (mean $\pm$ SEM). ns (not significant).

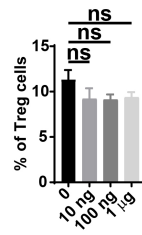
(2) In *in vitro* investigations, hydrogen peroxide (H₂O₂) treatment did not enhance lactic acid production in Hepa1-6 cells (**as follows**). In addition, *In vitro* Treg cell differentiation assays using CD4⁺ T cells treated with graded concentrations of recombinant PON1 confirmed the absence of a direct effect on Treg cell differentiation (**Extended Data Fig. S3m**). More significantly, as demonstrated in **Extended Data Fig. S4g**, PON1 overexpression failed to reverse the protumorigenic effects induced by VHL-KD. Collectively, these findings indicate that PON1's anti-tumor activity operates independently of both its secretion and enzymatic catalysis.

Unpublished Data Figure Redacted

Lactic acid concentration in intracellular compartments and supernatants of Hepa1-6 cells treated with different concentrations of H₂O₂. Data are representative of at least three independent experiments (mean $\pm$ SEM). ns (not significant).

Added Extended Data Fig. S3m

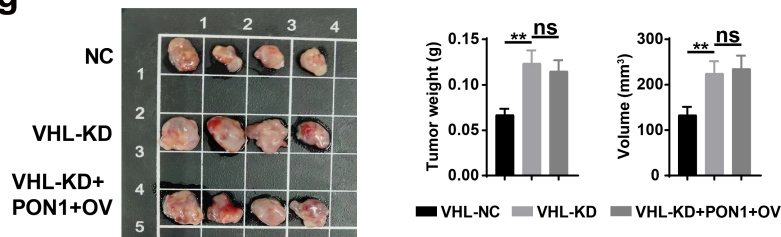
m



(m) Treg cell percentages in groups treated with different rPON1 concentrations (n=4). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. ns (not significant).

Added Extended Data Fig. S4g

g

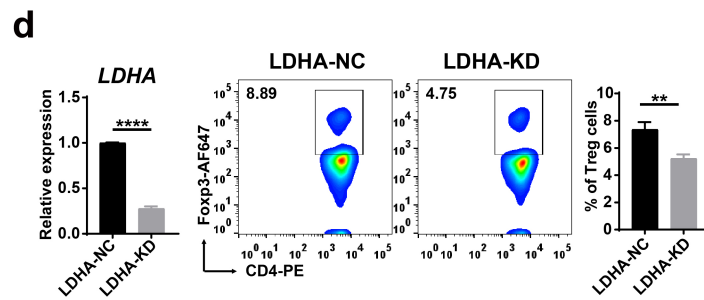


(g) Representative images, weights, and volumes of subcutaneous tumors from the different groups (n=12). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. **P<0.01, and ns (not significant).

Q5. The authors strongly associates lactic acid levels with Treg infiltration (Fig. 3h, Fig. 2c-e). However, functional causality through direct manipulation of lactic acid production pathways (e.g., using genetic LDHA knockdown or pharmacological inhibitors) independent of PON1 should be experimentally demonstrated to substantiate this claim definitively.

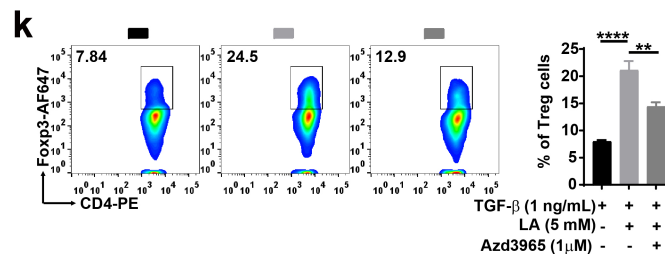
Response: Thank you for your comments. LDHA-knockdown (LDHA-KD) in Hepa1-6 cells suppressed Treg cell differentiation in co-culture systems compared to LDHA-NC cells (**Extended Data Fig. S3d**). Lactic acid has been reported to promote Treg cell differentiation. In Treg differentiation assays, lactic acid enhanced Treg cell generation, whereas the MCT1 inhibitor AZD3965, which blocks lactic acid transport significantly reversed this pro-differentiation effect (**Fig. 3k**). These results establish the critical role of lactic acid in Treg cell differentiation.

Added Extended Data Fig. S3d



(d) The expression of LDHA in Hepa1-6 cells, and its effect on Treg cell differentiation (n=6). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. **P<0.01, ****P < 0.0001.

Revised Fig. 3k

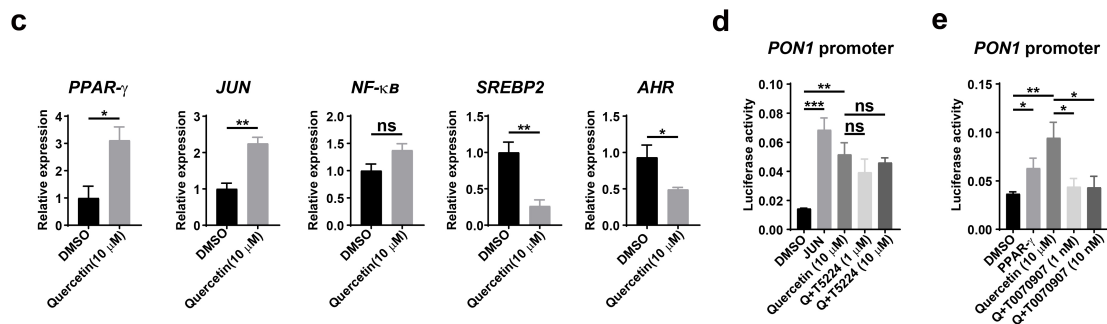


(k) Proportion of Treg cells treated with lactic acid or MCT1 inhibitor (AZD3965, 1 μ M) (n=5). Data are shown as mean $\pm$ SEM and represent at least two independent experiments. **P<0.01, ****P < 0.0001.

Q6. The authors suggest Quercetin enhances anti-PD-1 therapy primarily through increased PON1 expression, yet no direct mechanistic validation (such as promoter-reporter assays, ChIP assays for transcription factors regulating PON1, or epigenetic profiling following Quercetin treatment) is provided.

Response: Thank you for your comments. We examined transcription factors previously reported to regulate PON1 expression (*PPAR- γ* , *JUN*, *NF- κ B*, *SP1*, *SREBP2*, and *AHR*) in quercetin-treated Hepa1-6 cells (10 μ M). Quercetin significantly upregulated expression of *PPAR- γ* and *JUN* (**Extended Data Fig. S5c**). Dual-luciferase reporter assays demonstrated that both *JUN* and *PPAR- γ* expression plasmids activated PON1 transcription (**Extended Data Fig. S5d-e**). However, only the *PPAR- γ* antagonist T0070907 abolished the upregulation of PON1 induced by quercetin (**Extended Data Fig. S5d-e**).

Added Extended Data Fig. S5c-e



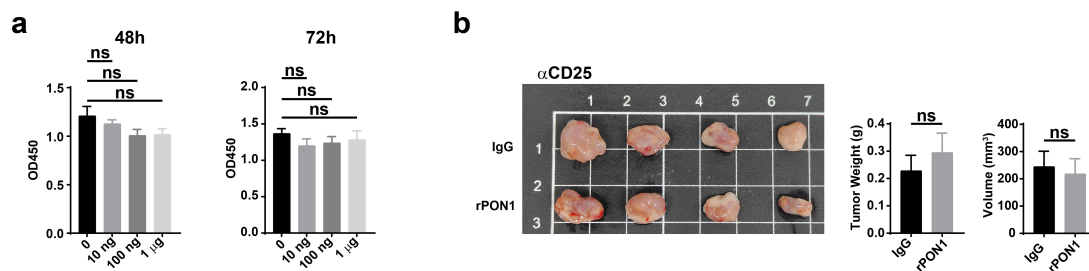
(c) Expression of PPAR- γ , JUN, NF- κ B, SREBP2, AHR and SP1 in Hepal-6 cells treated with quercetin (10 μ M, n=4). SP1 was undetectable in our assays and excluded from data reporting. (d) Luciferase activity of the PON1 promoter in Hepal-6 cells treated with JUN plasmid, quercetin, or quercetin combined with the JUN antagonist (T5224, n=5). (e) Luciferase activity of the PON1 promoter in Hepal-6 cells treated with PPAR- γ plasmid, quercetin, or quercetin combined with the PPAR- γ antagonist (T0070907, n=5). Data are representative of at least two independent experiments (mean $\pm$ SEM). *P<0.05, **P<0.01, ***P < 0.001, and ns (not significant).

Q7. The rPON1 and anti-PD-1 experiments are too important in this manuscript to have this preliminary-level data. The manuscript would greatly benefit from dose-response curves, time-dependent studies, and comparative effectiveness studies (rPON1 alone vs. combination with Quercetin or anti-PD-1). Validate whether Quercetin and rPON1 administration specifically alter the tumor microenvironment independently of the direct effect on cancer cell proliferation. It would be beneficial to include experiments employing conditional depletion of Tregs or lactic acid production pathway inhibitors.

Response: Thank you for your suggestions.

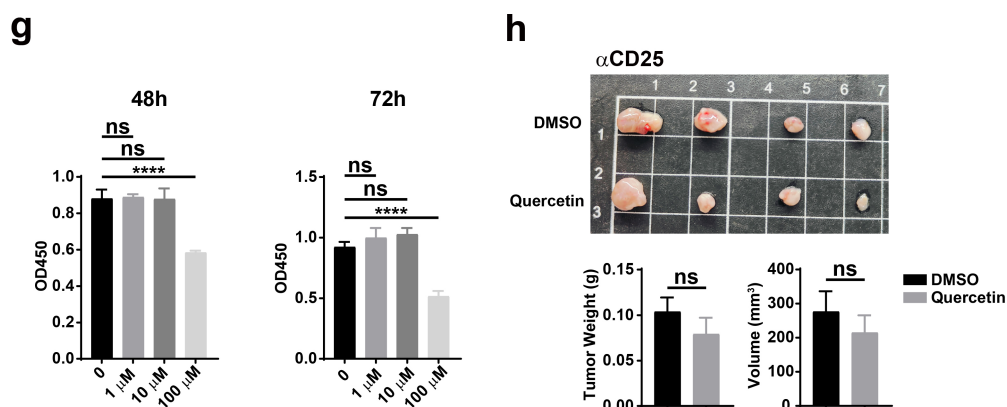
(1) Hepal-6 cells were treated with graded concentrations of recombinant PON1 protein *in vitro*. The results showed that rPON1 had no effect on cell proliferation, as determined by the CCK-8 assay (Extended Data Fig. S5a). More significantly, Treg depletion using anti-CD25 antibodies abolished the anti-tumor efficacy of rPON1(Extended Data Fig. S5b).

Added Extended Data Fig. S5a-b



(a) Cell viability of Hepal-6 cells treated with different rPON1 concentrations (n=7). (b) Representative images, weights, and volumes of subcutaneous tumors from Treg-depleted mice treated with or without rPON1 (n=6). Data are representative of at least two independent experiments (mean ± SEM). ns (not significant).

(2) *In vitro* dose-response assays showed that high concentrations of quercetin suppressed Hepal-6 cell proliferation (Extended Data Fig. S5g). Correspondingly, in Treg-depleted mouse models, the anti-tumor efficacy of quercetin (100 μg) was completely abrogated *in vivo* (Extended Data Fig. S5h). Added Extended Data Fig. S5h and Extended Data Fig. S5h



(g) Cell viability of Hepal-6 cells treated with different quercetin concentrations (n=7). (h) Representative images, weights, and volumes of subcutaneous tumors from Treg-depleted mice treated with or without quercetin (n=8). Data are representative of at least two independent experiments (mean ± SEM). ****P < 0.0001, and ns (not significant).

Collectively, these data demonstrate that quercetin and rPON1 administration specifically altered the tumor microenvironment, independent of their direct effect on cancer cell proliferation.

Q8. PON1 is a premier example of a highly glycosylated protein in normal and disease/cancer states. Aside from mentioning that PON1 is a glycoprotein, the authors appeared to have neglected the growing literature of PON1 glycosylation in cancer. This is an important discussion as to how PON1 biology is functionally implicated in the TME and how this impacts response to therapy, especially as

it has been preliminarily demonstrated by others that PON1 glycosylation in the secretion affects immunoregulatory cytokines and inflammation (Aldonza et al., 2023, eLife; Ahn et al., 2014, MCP).

Response: Thank you for your suggestion. In alignment with the established significance of PON1 fucosylation across diverse pathologies, we have substantially augmented the discussion section on PON1 fucosylation in the revised manuscript (**as follows**).

Addition to Discussion

Fucosylation is a critical post-translational modification of PON1. Published evidence indicates that secreted fucosylated PON1 modulates the levels of immunoregulatory cytokines and inflammation [45]. The diagnostic significance of PON1 fucosylation has been established in both lung cancer and HCC [46-47]. Our previous work identified serum PON1 levels as a robust biomarker of microvascular invasion [13-14]. This study aimed to elucidate the metabolo-immunological mechanisms underpinning PON1's role in HCC pathogenesis. However, the effect of PON1 fucosylation on hepatocarcinogenesis remain unclear, representing a novel and mechanistically significant research direction that warrants further investigation.

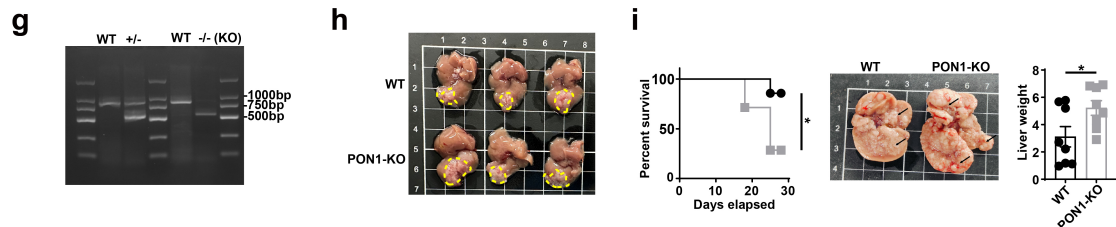
Q9. The technical description of each data panel across all figures and equally important, the methods section, suffer from severe lack of information, especially on their key data such as proteomics, PLS-DA, FACS, and in vivo experiments. This was supposed to be a minor comment but has forced me to include this as a major issue since it has impaired my ability to understand each assay performed, the experimental design, and what exactly is the data being presented. For example, the authors mentioned in the Methods and supplementary figure that they generated PON1-KO mouse model (as opposed to PON1-KO or KD in Hepa1-6 cells or SC tumors). Is this indeed the case?

Response: We apologize for the lack of experimental details in our initial manuscript. Comprehensive methodological descriptions of key techniques, including flow cytometry, proteomic profiling, co-culture systems, and Treg cell differentiation protocols, are now provided in the **Supplementary Materials**.

In the orthotopic model presented in **Extended Data Fig. S1g-h**, the implantation of Hepa1-6 cells into the liver parenchyma of WT and PON1-KO mice demonstrated significantly accelerated tumor growth in PON1-KO mice compared to that in WT controls. To strengthen these findings, we employed a hydrodynamic tail vein injection model to deliver an oncogene plasmid cocktail (pT3-myr-

AKT-HA, pT/Caggs-NRASV12 and pCMV(CAT)T7-SB100) in the revised manuscript. Consistently, PON1-KO mice exhibited a significant increase in liver tumor burden and a marked survival disadvantage (Extended Data Fig. S1i).

Added Extended Data Fig. S1g-i



(g) Genotype verification of PON1-KO mice. (h) Tumor growth in livers of WT and PON1-KO mice after orthotopic inoculation with Hepa1-6 cells (n=3). (i) Survival curves (n=7), representative liver tumor images, and liver weights (n=8) in WT and PON1-KO mice with spontaneous HCC induced by hydrodynamic tail vein injection of oncogenic plasmids. Data in f and h-i are representative of at least two independent experiments (mean $\pm$ SEM). *P < 0.05.

Regarding the PON1-KO mouse HCC model, we have revised the description in the results section to make the statements clearer (as follows).

Revised Results

Additionally, we generated PON1-knockout (PON1-KO) mice and induced HCC using two distinct methods: orthotopic inoculation of Hepa1-6 cells and hydrodynamic tail vein injection of oncogenic plasmids (pT3-myr-AKT-HA: pT/Caggs-NRASV12: pCMV (CAT)T7-SB100=1:1:0.04), as previously described [32] (Extended Data Fig. S1g). Notably, PON1 deficiency markedly accelerated tumor progression in both models compared to that in wild-type (WT) controls (Extended Data Fig. S1h-i).

Minor comments:

- The overall quality of data visualization requires substantial improvement, and figure panel arrangement needs to be significantly revised, addressing the following issues:

- Differences in text font sizes in every figure are distracting. The figure label and technical texts (e.g., n size, axis labels) have different sizes, even in many X-Y axes in many panels have different text sizes. The authors need to revise them so all figure panels in one figure have the similar text sizes and text font styling.

Response: We apologize and appreciate your suggestions. The figures have been comprehensively revised to enhance readability, including the standardization of font sizes and styles.

- Protein size (kDa) should be included in all blots, per conventional practice. The authors should provide raw blots of the imaged full or cut membranes.

Response: Thank you for your suggestion. In the revised manuscript, we have indicated the protein size and provided the raw blots in their imaged full or cut membranes.

- ICC images should have size (um) labels (Fig. 5b).

Response: Thank you for your suggestions. We have added size (μm) labels to the ICC images (**Fig. 4b and Fig. 5a**)

- Kaplan-Meier survival curves and clinical correlations must explicitly provide patient cohort details (stage, grade, treatment history). Data visualization lacks proper annotations regarding sample sizes and statistical methods used.

Response: Thank you for your suggestion. Detailed clinical information of patients used in the analyses is presented in **Table S1-3**. Patient information corresponding to the survival curves is provided in **Table S2**. We have labeled **Fig. 1f** with the statistical methods used and the number of patients.

- Fig. 4: Inadequately represent the proposed mechanistic interactions (PON1-VHL-HIF-1α axis). Including detailed graphical diagrams or schematic illustrations demonstrating the proposed molecular interactions and validation experiments would significantly enhance clarity, this aside from their overall summary in Fig. 7.

Response: Thank you for your suggestion. We added a schematic diagram of PON1 interaction with VHL and HIF-1α to **Fig. 5c**.

- Incomplete or inconsistent labeling. Many figure panels lack "assay" labels, such as in Fig. 4a, PON1 subcompartment cell localization. Fig 4b, DEPs from their proteomics, and many more across all figures.

Response: We apologize for this oversight. In the revised manuscript, we have carefully modified and completed all the required labels.

- Sizing. The FACS data are presented poorly. Their overall size is small, and hard to decipher any information from it, even when the gating boxes and % cell information are included. The KEGG

pathway in Fig. 3a is so small I can only read the text "metaoblic pathways". In Fig. 3e, the legend labels are unreadable even if you zoom the image. This sizing issue goes on across all figures.

Response: We apologize for the deficiencies in the original figures. The FACS data were comprehensively revised with refined annotations and the dimensions of the key panels (including **Fig. 3a and 3e**) were maximized to ensure optimal readability.

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed all of my concerns and I would recommend the manuscript for publication.

Reviewer #2 (Remarks to the Author):

My concerns has been answered by the authors.

Reviewer #3 (Remarks to the Author):

The authors have addressed all of my comments satisfactorily. I have no further concerns and congratulate them on this excellent study!

Reviewer #4 (Remarks to the Author):

The authors have addressed the majority of the main concerns using robust experimental strategies and have significantly improved the overall presentation of the data and manuscript. Specifically, they substantially strengthened the mechanistic case for a PON1–VHL–HIF-1 α –lactate axis and convincingly tied lactate to Treg accrual using LDHA loss and MCT1 inhibition. The VHL-dependence and secretion/catalytic independence of the anti-tumor effect are shown, and rPON1 is effective with Treg-dependence in vivo.

We appreciate the reviewers' thoughtful questions regarding our manuscript and their endorsement of the revised data. Below we address their remaining points point-by-point:

- Most of the subcutaneous tumor data shown (not the liver tumors) in the main figures only include end-point tumor weights. Did the authors perform multiple time-point tumor volume measurements for all of the mouse tumor experiments, especially those involving Treg-depleted mice? If so, please present them along with the endpoint tumor data.

Response: Thank you for your comments. We acknowledge that tumor volume measurements were not recorded at every time point for all mouse tumor experiments. As illustrated in our model schematic, the tumor endpoint was set at 14 days post-modeling. In this study, we employed the murine Hepa1-6 cell line. Existing literature suggests that common sampling time points for this model are typically at 14 and 21 days after inoculation (Kimura et al., 2018, Cancer Sci; Ye et al., 2019, Cancer Res; Wei et al., 2019, Gastroenterology; Du et al., 2021, Theranostics; Sung et al., 2022, J Immunother Cancer; Lu et al., 2023, Hepatology; Li et al., 2024, Phytomedicine; Zhang et al., 2024, Theranostics; Gan et al., 2024, J Immunother Cancer).

We selected day 14 as the tumor endpoint based on initial monitoring of tumor growth, which revealed measurable differences between experimental groups by this time. By day 21, the majority of tumors had reached 1000 mm³, with a subset exceeding 1500 mm³ (**as shown below**). Another consideration was the necessity to accurately assess the tumor immune microenvironment.

Excessively large tumors have been shown to develop necrosis and nutrient depletion, leading to impaired immune cell distribution and function, thereby distorting the true state of the immune microenvironment (Yu et al., 2018, PLoS One; Nessler et al., 2020, Cancers; Kim et al., 2021, Front Immunol; De Vleeschauwer et al., 2024, Nat Protoc). Several research groups utilizing Hepa1-6 cell-derived tumor models have chosen to evaluate the intratumoral immune environment approximately 14 days post-inoculation (Wei et al., 2019, Gastroenterology; Kwong et al., 2020, JHEP Rep; Lu et al., 2022, Gut; Xie et al., 2023, J Hepatol). For these reasons, day 14 was established as the experimental endpoint.

To ensure consistency and comparability across assays, all subsequent experiments used day 14 as the standardized endpoint. At this time point, tumors were excised from the mice for volume measurement and weighing. During the period between inoculation and excision, tumors were palpated and measured with calipers three to four times to confirm inoculation success and monitor growth trends. We have provided growth curves documenting four time points, and the trends observed are consistent with the terminal tumor volume (**Fig. 2k, Fig. 2l, Fig. 6a, and Fig. 6e**). Throughout the monitoring process in mice, the observed growth trend of the tumors correlated with the volume measured at the endpoint. The suggestion for growth curves provides essential guidance for future studies, and we will implement rigorous time-point tracking in subsequent work.

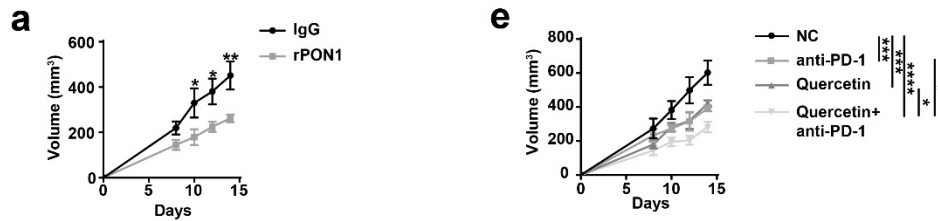
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The growth curve PON1-NC and PON1-OV tumors.
Revised **Fig. 2k-l**

Unpublished Data Figure Redacted

(k) The growth curves for PON1-NC and PON1-OV groups, with or without α CD25 treatment (n = 4 per group). (l) The growth curves for PON1-NC and PON1-KD groups, with or without α CD25 treatment (n = 4 per group) (n = 5 per group).

Revised **Fig. 6a and 6e**



(a) The growth curves of Hepa1-6 tumors from mice intraperitoneally injected with IgG or rPON1 (n=4 mice per group). (e) The growth curves for each group (n=4 per group).

- While it is greatly appreciated that the authors have included the murine liver hepatoma cell line (Hep-53.4) and generated subcutaneous tumors, assuming AKT/NRAS co-activation-driven, the authors must still acknowledge the limitation of these murine models when no patient-derived model has been used. Specifically, all the murine HCC cell lines used are originally derived from chemically-induced HCC transformation, not typical of human disease (common TP53 LOF, CTNNB1 activating mutations, TERT, etc).

Response: We acknowledge the limitations of our model system raised in your review, particularly regarding the applicability of PON1 mechanisms to human HCC. This statement will be added to the Discussion section of the manuscript.

Discussion

In summary, our study reveals a previously unrecognized metabolic function of PON1 in alleviating immunosuppression. PON1 reduces Treg cell infiltration in HCC by destroying lactic acid production, thereby suppressing tumor growth. **We also elucidate the mechanism of the PON1-VHL-HIF-1 α axis in lactic acid production in murine HCC cell lines and mouse models. However, given the constraints of our models, the function of the PON1-VHL-HIF-1 α axis in human HCC warrants further validation through clinically relevant humanized platforms, notably dual-humanized patient-derived xenograft models and patient-derived organoids.** Based on the data presented here, we suggest that increasing PON1 levels can abolish lactic acid-induced immunosuppression, which is a potentially effective therapeutic option for HCC and one of the most favored strategies for ICB combination therapy.

- Please add a minimal HDL-context control: either compare rHDL-PON1 versus free rPON1 in a single readout already used (lactate or Treg induction) or document that administered rPON1 partitions into HDL and remains active by simple lipoprotein fractionation. This is a brief addition but will pre-empt questions about the physiological carrier state.

Response: We acknowledge the value of investigating rPON1-HDL binding dynamics as raised in your comments. Although we are prepared to pursue this direction, the technical feasibility is currently limited by the commercial unavailability of HDL-PON1 complexes. While HDL association primarily modulates PON1's enzymatic activity and antioxidant functions, our results demonstrate that PON1's anti-tumor effects are independent of both its antioxidant capacity and enzymatic properties. We propose to incorporate the following targeted discussion in the manuscript.

Discussion

To investigate the therapeutic potential of PON1, we generated rPON1. Treatment with rPON1 recapitulated the anti-tumor efficacy of PON1 overexpression, significantly suppressing tumor growth, reducing lactic acid production, and decreasing Treg cell infiltration in murine models. While hepatic secretion of PON1 into circulation requires HDL for enzymatic stabilization and antioxidant functionality [6], our data demonstrate that PON1-mediated tumor suppression operates independently of enzymatic activity (**Supplementary Fig. 4g-h**). PON1 exerts its anti-tumor effect through VHL interaction. The role of HDL in this process remains incompletely characterized. Critically, whether exogenous rPON1 associates with HDL *in vivo* and how this interaction modulates rPON1's enzymatic activity constitute key determinants for clinical translation, warranting further investigation.

-There are sweeping generalizations in the Discussion that should be reworded or better cautioned. For example, certain phrasing implies that the PON1–VHL–HIF-1 α –lactate axis is a broadly applicable mechanism across all HCC contexts, whereas the work is based entirely on murine-derived HCC cell lines and mouse models lacking hallmark human oncogenic lesions.

Response: Thank you for your suggestions. We have refined the description of the PON1-VHL-HIF-1 α regulatory axis mechanisms in the Discussion section to enhance precision.

Discussion

In summary, our study reveals a previously unrecognized metabolic function of PON1 in alleviating immunosuppression. PON1 reduces Treg cell infiltration in HCC by destroying lactic acid production, thereby suppressing tumor growth. **We also elucidate the mechanism of the PON1-VHL-HIF-1 α axis in lactic acid production in murine HCC cell lines and mouse models. However, given the constraints of our models, the function of the PON1-VHL-HIF-1 α axis in human HCC warrants further validation through clinically relevant humanized platforms, notably dual-humanized patient-derived xenograft models and patient-derived organoids.** Based on the data presented here, we suggest that increasing PON1 levels can abolish lactic acid-induced immunosuppression, which is a **potentially** effective therapeutic option for HCC and one of the most favored strategies for ICB combination therapy.

Similarly, conclusions about therapeutic potential (quercetin as an ICB sensitizer) would benefit from language that clearly distinguishes proof-of-principle in controlled models from clinical generalizability. This will prevent overextension of claims beyond what the data can directly support. A few carefully placed qualifiers (“in our models...”, “in murine HCC...”, “potentially relevant to...”) would preserve the strength of the findings while aligning interpretation with the actual evidence.

Response: We appreciate your suggestions. To preserve the strength of our findings while aligning interpretation with the actual evidence, explicit caveats regarding the limitations of mouse models have been incorporated throughout all therapeutic potential discussions.

Recombinant PON1 protein (rPON1) effectively impeded tumor growth. Furthermore, enhancing PON1 expression with quercetin sensitized HCC to anti-programmed death-1(PD-1) therapy **in**

murine HCC.

Furthermore, enhancing PON1 expression with quercetin sensitized HCC to anti-programmed death-1(PD-1) therapy **in murine HCC.**

Furthermore, upregulation of PON1 levels by quercetin sensitized HCC to anti-programmed death-1 (PD-1) therapy **in mice.**

These results demonstrate that quercetin inhibits HCC growth and act synergistically with immune checkpoint blockade (ICB) **in our models.**

Furthermore, the combination of quercetin and an anti-PD-1 blockade synergistically inhibited HCC progression compared to single-agent treatments **in murine HCC.**

Based on the data presented here, we suggest that increasing PON1 levels can abolish lactic acid-induced immunosuppression, which is a **potentially** effective therapeutic option for HCC and one of the most favored strategies for ICB combination therapy.