

USP2 inhibition unleashes CD47-restrained phagocytosis and enhances anti-tumor immunity

Corresponding Author: Dr Jinfang Zhang

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 work, the authors used an arrayed siRNA screen in model cell lines expressing an exogenous CD47-GFP fusion to identify a DUB, USP2, whose knockdown resulted in reduced CD47 expression. The authors conducted a fairly robust set of assays to confirm that USP2 binds to CD47 and regulates CD47 protein levels by preventing its ubiquitination.

In vivo, the authors utilized syngeneic mouse tumor models to demonstrate the chemical inhibition or knockout of USP2 enhanced to anti-tumor immunity, reduced tumor M2 macrophages, increased M1 macrophages and CD8 T cells in TME, while having no observed toxicity.

Clinically, the authors also looked patient samples and found samples with low USP2 shows more M1 macrophage infiltration than M2 and that USP2 expression is higher in non-responders of anti-PD1 therapy. Overall this is a very nice study, and while there are a few issues that could use clarification and remaining open questions, I think it will be a solid contribution to the field.

Major points:

1. How specific is the interaction between USP2 and CD47, and how essential is this for the phenotypes observed? e.g. what else is regulated by USP2? The authors convincingly show that body weight, liver toxicity, and blood cell count are not different btw treated and untreated groups. Would there have been an equivalent effect equivalent effect w/CD47 ko in these cells? what else could ML364 be doing? Are some compensatory mechanisms between different DUBs that the authors could briefly comment on? Overall I think the findings in this study are solid enough and interesting as presented (regardless of potential off-targets), but there remain some fundamental questions about whether there are other effects of the drug they use, and other targets beyond CD47 for USP2.
2. Reference 44 is a study about how USP2 inhibition increased PD-L1 expression in breast cancer cells, sensitizing cancer cells to anti-PD-1; how much does this pathway contribute to the synergistic effect of the USP2 inhibitor and anti-PD1 mAb in treating lung cancer here? The authors might consider measuring PD1 expression in their models.
3. The synergistic effect of ML364 and anti-PD1 is very impressive. the phagocytosis images are a bit hard to discern in Fig 4i, and it's probably hard to really tell if those are cancer cells inside a macrophage? but I don't think this is a critical problem, there is plenty of other supportive data to say there are more active M1-like macrophages present.
4. Fig. 3A: are the macrophages or target cells dying in response to ML364? if so, dead cells definitely are phagocytosed more rapidly. controls in ext data fig 2b suggest KO of CD47 is enough to increase phagocytosis, but still would be nice to see whether ML364 is toxic alone.

Minor points

1. In the siRNA screen, cells with decreased GFP signal were selected; can the authors comment on the specificity of the effect for USP2 (rather than GFP itself), or whether the c-terminal fusion was essential?
2. There seem to be other DUBs whose knockdown also stabilizes expression of CD47, some even more strongly than USP2; if these DUBs are also expressed in cancer cells, how cell type dependent is their expression? What is the rationale behind the screen data presentation (what are A-J and 1-10 in extended figure 1a? this figure needs clearer labeling.)
3. An introduction of the small molecule inhibitor and USP2 would be nice.
4. Figure 1t: how are the scores calculated?
5. Figure 2h: legend off set.

6. Would be nice to show PD1 expression in USP2 inhibited groups.

Reviewer #2

(Remarks to the Author)

Dai et al. demonstrated that USP2 functions as a deubiquitinase for CD47, and inhibiting USP2 can modulate the tumor microenvironment, thereby sensitizing anti-PD-1 immunotherapy. The authors employed multiple in vivo models, clinical data, and patient samples to support their findings and explore potential strategies for enhancing the efficacy of tumor immunotherapy. However, there are several issues that need to be addressed in order to further strengthen the conclusions.

1. The authors did not investigate the endogenous interaction between USP2 and CD47 in tumor cells and lung adenocarcinoma tissues.
2. The authors should utilize antibodies targeting specific ubiquitin chains for the Ub IP experiment.
3. The combinational treatment of ML364 and PD-1 mAb shows promising results. However, the treatment time window appears to be limited. It would be meaningful for the authors to examine whether long-term treatment can exert a sustained inhibitory effect on tumors and to what extent it can prolong the survival of the mice. Additionally, it is important to investigate whether resistance develops during extended treatment periods.
4. The authors claimed that the lower mRNA level of Usp2 contributes to the reduced hematologic cytotoxicity of ML364. However, they did not investigate whether ML364 treatment negatively impacts the physiological function of organs such as the lung, colon, kidney, and stomach, which exhibit higher Usp2 mRNA expression.
5. In Fig. 5b-d, ML364 or PD-1 mAb monotherapy demonstrates efficacy, while in Fig. 5f-i, knocking out USP2 or PD-1 mAb monotherapy appears to have little influence on tumor growth. The reasons for this difference should be elucidated. Additionally, the authors chose a different frequency of PD-1 mAb administration in Fig. 5e compared to that in Fig. 5a. An explanation for this discrepancy is warranted.
6. The authors validated their findings using clinical samples and public databases. Did they observe any patient-derived mutations in USP2 or CD47 within the regions involved in their interaction? Do these mutations impact CD47 expression? Providing such data would further enhance the significance of their findings.
7. It remains unclear whether the alterations in USP2 expression in clinical samples are attributable to changes at the transcriptional or protein levels.
8. Quantifications for Fig. 1f-g need to be provided.
9. The authors measured tumor sizes in terms of area units (mm²) in both Figure 4m and Figure 5h. The authors should explain how this parameter was calculated.

Reviewer #3

(Remarks to the Author)

In this manuscript authors identify the deubiquitinase USP2 as a regulator of CD47 and demonstrate that it can be therapeutically targeted to inhibit tumoral CD47, increase phagocytosis of macrophages by tumor cells, and enhance response to anti-PD-1 immunotherapy. Recent studies have identified USP2 as a therapeutic target for cancer, however this is the first report that it is involved in stabilizing CD47 and that its inhibition can reduce CD47 levels and exert an antitumor immune effect. Interestingly, the lack of USP2 expression in blood cells means that unlike other therapies targeting CD47 directly, USP2 inhibition does not induce anemia or thrombocytopenia. The authors demonstrate that ML364, a small molecule inhibitor of CD47, can be used in combination with anti-PD-1 antibodies to control syngeneic subcutaneous, orthotopic, and spontaneous tumors in mice.

The manuscript is well written and of significant novelty and impact. While it presents compelling data regarding the relationship between USP2 and CD47, where it is less clear is in how anti-USP2 therapies remodel the tumor immune microenvironment. A central question that is not addressed is whether the effect of ML364 is solely through CD47. Other literature cited by the authors demonstrates that USP2 has effects on other signaling pathways of relevance to cancer, for example p53 and PD-L1. This calls into question the extent to which the effects observed in vivo are attributable to USP2's effects on CD47. Indeed, the combination of ML364 with anti-PD-1 would appear to be dependent on all of these effects. In this respect the manuscript is underdeveloped mechanistically. Evaluation of the effects of ML364 +/- anti-PD-1 on tumors in which CD47 has been knocked down or out as well as on hosts lacking macrophages and/or T cells are conspicuously absent.

Specific issues:

Figure 3f: It would be instructive to compare ML364 treatment in this model with anti-CD47 antibody treatment and/or treatment in tumors in which CD47 has been knocked down or out to gauge how USP2-targeted treatment performs in vivo

relative to conventional anti-CD47 therapy, and whether its effects are dependent on CD47 expression as seen in previous figures.

Figure 3k: Other work (Zhang PLoS One 2016) has suggested that CD47 blockade increases M1 polarization but does not necessarily reduce M2 polarization. Again, it would be helpful to benchmark this therapeutic approach against one that acts directly against CD47 (antibody, genetic) to assess whether the effects seen here are solely attributable to how USP2 modulates CD47.

Figure 4: The mechanism of the combined therapy is not clearly developed. It is unclear whether anti-USP2 and anti-PD-1 therapy functions by inducing macrophage phagocytosis of tumor cells (as suggested in panel i), by inducing CD8 T cell-mediated killing of tumor cells (as would be expected from anti-PD-1 therapy), or a combination of the two. PD-1 has been implicated as a don't eat me signal similar to CD47 (Gordon Nature 2017) suggesting this therapy may function solely through macrophage phagocytosis, but this is not clearly demonstrated.

Figure 5f: The microCT scans are difficult to interpret and compare tumor progression with the histologic data. Annotation and/or quantitation of tumor burden in these scans is needed.

Extended data figure 6d: The authors present a model of the effects of USP2 but have not elucidated the specifics of this process. For example, they indicate that USP2 inhibition via ML364 inhibits M1 macrophages and suggest that this is independent of CD47, however this has not been shown.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have satisfactorily addressed my concerns. The updated combinational therapy experiment is impressive.

Reviewer #3

(Remarks to the Author)

The authors have made a thorough revision to the manuscript and thoughtfully responded to all queries raised by the reviewers in the previous round of critiques. The results are significant, innovative, and derived from rigorous science.

As a mediator for Reviewer #1

In the previous round of reviews, reviewer #1 recognized the manuscript as a solid contribution to the field and mentioned it only needed a few areas of clarification. One of the major points raised by reviewers #1 and #3 was whether the effects of ML364 were solely through inhibition of CD47. They used a CD47 knockout tumors model to show that ML364 did not further reduce tumor growth beyond the effects of PD-1 blockade, in contrast to tumors expressing CD47. They further investigated the effects of their dosing regimen on expression of CD47 and PD-L1, showing that their lower doses (relative to other published work) of ML364 reduced CD47 levels but not PD-L1 levels in vitro and in vivo. Studies using higher doses replicated the published results. The additional data provided directly address reviewer #1's concerns. The new data does raise subsequent questions about the precise mechanism of tumor cell death for low and high dose ML364 and PD-1 blockade, specifically the role of individual immune cells in this effect, but these were addressed in the response to reviewer #3. Overall the revised manuscript is viewed as highly responsive to the previous critique and all issues raised have been adequately addressed.

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Point-by-Point Responses to Reviewers' Comments
(NCOMMS-24-19566-T/ NCOMMS-24-19566-A)

We would like to sincerely thank the three reviewers for their thorough evaluations and invaluable suggestions, which have greatly enhanced the quality of our study. Over the past five months, we have gathered substantial new experimental data, including 8 new main figure panels and 61 new supplementary figure panels, further strengthening our findings. We hope that, upon reviewing our detailed point-by-point responses below, both the editor and reviewers will find that we have adequately addressed all concerns raised. We believe that the revised manuscript is now suitable for publication as a research article in *Nature Communications*.

In the document that follows, reviewer comments are presented in italicized blue text, with our responses in regular font.

Reviewers' Comments:

Reviewer #1 Comments (Remarks to the Author):

In this work, the authors used an arrayed siRNA screen in model cell lines expressing an exogenous CD47-GFP fusion to identify a DUB, USP2, whose knockdown resulted in reduced CD47 expression. The authors conducted a fairly robust set of assays to confirm that USP2 binds to CD47 and regulates CD47 protein levels by preventing its ubiquitination.

In vivo, the authors utilized syngeneic mouse tumor models to demonstrate the chemical inhibition or knockout of USP2 enhanced to anti-tumor immunity, reduced tumor M2 macrophages, increased M1 macrophages and CD8 T cells in TME, while having no observed toxicity.

Clinically, the authors also looked patient samples and found samples with low USP2 shows more M1 macrophage infiltration than M2 and that USP2 expression is higher in non-responders of anti-PD1 therapy. Overall this is a very nice study, and while there are a few issues that could use clarification and remaining open questions, I think it will be a solid contribution to the field.

Response: We sincerely thank the reviewer for acknowledging the key conclusions of our study and the contribution to the field. We greatly appreciate the constructive feedback, which has significantly enhanced the quality of our work. We fully agree with the reviewer's suggestion that additional evidence is needed to further clarify and strengthen our conclusions. In the following, we provide a detailed, point-by-point response to each of the reviewer's concerns, and we hope the reviewer will find that we have made every effort to address the comments in this revised version.

Major points:

1. How specific is the interaction between USP2 and CD47, and how essential is this for the phenotypes observed? e.g. what else is regulated by USP2? The authors convincingly show that body weight, liver toxicity, and blood cell count are not different btw treated and untreated groups. Would there have been an equivalent effect w/CD47 ko in these cells? what else could ML364 be doing? Are some compensatory mechanisms between different DUBs that the authors could briefly comment on? Overall I think the findings in this study are solid enough and interesting as presented (regardless of potential off-targets), but there remain some fundamental questions about whether there are other effects of the drug they use, and other targets beyond CD47 for USP2.

Response: We sincerely thank the reviewer for their insightful comments. To address the reviewer's question regarding the specificity of USP2's regulation of CD47 and its impact on combination therapy, we conducted experiments involving the knockout of *Cd47* in LLC lung cancer cells and subsequent subcutaneous tumor implantation in immunocompetent C57BL/6J mice. The mice bearing tumors were randomly assigned to six treatment groups, as follows: 1) sgControl & Vehicle, 2) sgControl & 5 mg/kg ML364, 3) sgControl & 5 mg/kg ML364 & PD-1 mAb, 4) sgCd47 & Vehicle, 5) sgCd47 & 5 mg/kg ML364, and 6) sgCd47 & 5 mg/kg ML364 & PD-1 mAb.

The results demonstrate that in the sgControl group, treatment with ML364 alone reduced tumor growth (**Revised Supplementary Fig. 8a, b**). However, combination therapy with ML364 and PD-1 mAb resulted in even greater tumor

inhibition compared to single-agent treatment, thus validating our findings. In the sg*Cd47* group, while knockout of *Cd47* led to reduced tumor growth, ML364 treatment did not further inhibit tumor growth significantly (**Revised Supplementary Fig. 8a, b**), suggesting that the anti-tumor effect of ML364 may primarily depend on CD47 expression, at least under our experimental conditions. Moreover, we observed no significant difference in the anti-tumor effects of the combination therapy (ML364 & PD-1 mAb) between the sg*Control* and sg*Cd47* groups (**Revised Supplementary Fig. 8a, b**). This suggests that in our mouse lung cancer model, the anti-tumor effect of USP2 inhibition by ML364, when combined with PD-1 mAb, is likely driven primarily by the USP2-CD47 axis.

A recent study reported that USP2 inhibitor (ML364 at 30 mg/kg) combined with PD-1/PD-L1 blockade significantly suppressed tumor growth, largely through regulation of the USP2 downstream target PD-L1 protein in a mouse breast cancer model (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941). This suggests that ML364 may also regulate other USP2 downstream targets beyond CD47 under different experimental conditions. In our study, we used a lower dose of ML364 (5 mg/kg for *in vivo* treatments and 2 μ M for *in vitro* cell treatments), while Dr. Yi's study employed higher doses (30 mg/kg for *in vivo* treatments and 20 μ M for *in vitro* treatments). We hypothesized that varying doses of ML364 might differently affect the expression of USP2 downstream targets.

To test this hypothesis, we treated lung cancer cell lines with increasing doses of ML364 (1, 2, 5, 10, 20 μ M). Our results showed that low doses of ML364 (1 and 2 μ M) dramatically reduced CD47 protein levels without affecting PD-L1 and p53 expression in PC9 cells. In contrast, high doses of ML364 (10 and 20 μ M) significantly increased PD-L1 expression, consistent with Dr. Yi's findings (**Revised Supplementary Fig. 1q**). Similar results were observed in the H460 cell line, which was also used in Dr. Yi's study: low doses of ML364 reduced CD47 levels without affecting PD-L1 and p53, whereas high doses elevated PD-L1 expression (**Revised Supplementary Fig. 1r**). The differential response to ML364 treatment may be attributed to the fact that CD47 is a direct deubiquitinase substrate of USP2, while PD-

L1 and p53 are indirect downstream targets of USP2, regulated through the VPRBP pathway.

Next, we explored the effects of different ML364 doses *in vivo*. Immunohistochemical (IHC) staining of subcutaneous LLC tumor tissues treated with low-dose ML364 (5 mg/kg) revealed a significant reduction in CD47 levels, but no significant change in PD-L1 levels, whether ML364 was used alone or in combination with PD-1 mAb (**Revised Supplementary Fig. 7e, f, i**). Given that higher doses of ML364 (30 mg/kg) have been shown to improve therapeutic outcomes in breast cancer models (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941), we applied a high-dose regimen of ML364 in our lung cancer model to assess CD47 and PD-L1 expression, as well as therapeutic efficacy. The treatments were as follows: 1) Vehicle, 2) ML364 (30 mg/kg), 3) PD-1 mAb (200 µg/mouse), and 4) ML364 (30 mg/kg) & PD-1 mAb (200 µg/mouse).

Indeed, the high-dose ML364 treatment yielded improved therapeutic efficacy compared to low-dose treatment (5 mg/kg), with a significant extension in survival (**Revised Supplementary Fig. 8l-n**). Notably, two mice in the combination therapy group exhibited complete tumor regression and were considered cured (**Revised Supplementary Fig. 8l-n**), a result not observed in the low-dose treatment group. IHC staining showed that high-dose ML364 significantly decreased CD47 levels while increasing PD-L1 protein abundance (**Revised Supplementary Fig. 8o-q**). These findings suggest that in the highly malignant LLC mouse tumor model, high-dose ML364 treatment not only dramatically reduces CD47 expression but also increases PD-L1 expression, enhancing the effectiveness of PD-1/PD-L1 blockade therapy, a result consistent with findings in a breast cancer tumor model (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941).

Additionally, we observed no significant blood or liver toxicity, nor any noticeable changes in body weight, in the sg*Cd47* group treated with ML364 or the ML364 & PD-1 mAb combination (**Revised Supplementary Fig. 8c-h**). In this study, we primarily identified USP2 as the deubiquitinase (DUB) responsible for regulating CD47 in lung cancer. However, whether other DUBs also contribute to the regulation

of CD47 in lung or other cancer types remains an important area for future investigation.

In conclusion, our newly obtained *in vitro* and *in vivo* results suggest that in our low-dose ML364-treated mouse lung cancer model (5 mg/kg), the observed therapeutic effects of the combination therapy primarily depend on the USP2-CD47 axis. However, consistent with recent report, high-dose ML364 treatment may offer enhanced therapeutic efficacy, likely through effects on both CD47 and other downstream targets such as PD-L1. We have incorporated these new results and further discussion in the updated manuscript.

2. Reference 44 is a study about how USP2 inhibition increased PD-L1 expression in breast cancer cells, sensitizing cancer cells to anti-PD-1; how much does this pathway contribute to the synergistic effect of the USP2 inhibitor and anti-PD1 mAb in treating lung cancer here? The authors might consider measuring PD-L1 expression in their models.

Response: We sincerely appreciate the reviewer's insightful comments and suggestions. We agree with the reviewer that a recent study reported USP2 inhibitor (ML364 at 30 mg/kg) sensitizes tumors to PD-1/PD-L1 blockade, primarily through the upregulation of the USP2 downstream target PD-L1 in a mouse breast cancer model (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941). Together with our findings, this study suggests that ML364 may regulate different USP2 downstream targets depending on the experimental conditions as our response in major point #1.

In our study, we used a lower dose of ML364 (5 mg/kg for *in vivo* treatments and 2 μ M for *in vitro* cell treatments), whereas Dr. Yi's study employed higher doses (30 mg/kg for *in vivo* treatments and 20 μ M for *in vitro* treatments). We hypothesized that varying doses of ML364 might differentially affect the expression of USP2 downstream targets.

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The high-dose ML364 treatment (30 mg/kg) led to improved therapeutic efficacy compared to the low-dose treatment (5 mg/kg), with a significant extension of survival (**Revised Supplementary Fig. 8l-n**). Notably, two mice in the combination therapy group with high-dose treatment exhibited complete tumor regression and were considered cured (**Revised Supplementary Fig. 8l-n**), a result not observed in the low-dose treatment group. IHC staining revealed that high-dose ML364 significantly decreased CD47 levels while increasing PD-L1 protein abundance (**Revised Supplementary Fig. 8o-q**). These findings suggest that in the highly malignant LLC mouse tumor model, high-dose ML364 treatment not only dramatically reduces CD47 expression but also increases PD-L1 expression, which may enhance the effectiveness of PD-1/PD-L1 blockade therapy. This result is consistent with findings in a breast cancer tumor model (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941).

Collectively, our newly obtained *in vitro* and *in vivo* results suggest that in our low-dose ML364-treated mouse lung cancer model (5 mg/kg), the observed therapeutic effects of the combination therapy may primarily depend on the USP2-CD47 axis. However, consistent with a recent report, high-dose ML364 treatment may offer enhanced therapeutic efficacy, likely through its effects on both CD47 and other downstream targets such as PD-L1. We have incorporated these new results and further discussion into the updated manuscript.

3. The synergistic effect of ML364 and anti-PD1 is very impressive. the phagocytosis images are a bit hard to discern in Fig 4i, and it's probably hard to really tell if those are cancer cells inside a macrophage? but I don't think this is a critical problem, there is plenty of other supportive data to say there are more active M1-like macrophages present.

Response: In our original Figure 4i, we used the Akoya tissue section automatic quantitative analysis system to scan the tumor tissue at a magnification of 20 x. To address the reviewer's concern, we repeated the staining experiment and used a confocal microscope with a 60 x oil immersion objective to obtain higher resolution images. As shown in the revised figure (**Revised Fig. 4i**), we were able to more clearly observe that in the combination therapy group, a greater number of macrophages (red) were engulfing tumor cells (green). Additionally, we zoomed in on representative phagocytosis areas within the combination therapy group and performed layer scanning to generate a 3D view. By anchoring a representative cell, we were able to observe along the X, Y, and Z axes that the red macrophage fully encapsulated the green tumor cell, providing clear evidence of tumor cell phagocytosis by macrophages in the combination therapy group.

4. Fig. 3A: are the macrophages or target cells dying in response to ML364? if so, dead cells definitely are phagocytosed more rapidly. controls in ext data fig 2b suggest KO of CD47 is enough to increase phagocytosis, but still would be nice to see whether ML364 is toxic alone.

Response: We greatly appreciate the reviewer for providing constructive suggestion. In our study (original Fig. 3a and Extended Data Fig. 2b), lung cancer cells (PC9 and H1975) were pre-treated with ML364 for 16 hours before co-culturing with macrophages. To evaluate the impact of ML364 on cancer cell viability, we applied the same treatment conditions to the lung cancer cells and compared cancer cell death between the DMSO and ML364 groups. Flow cytometry analysis of 7-AAD staining showed that low dose of ML364 treatment did not significantly increase lung cancer cell death compared to the DMSO group (**Revised Supplementary Fig. 2i-n**). Therefore, the enhanced phagocytosis of lung cancer cells with ML364 pre-treatment is likely due to ML364's inhibitory effect on CD47.

Minor points

1. In the siRNA screen, cells with decreased GFP signal were selected; can the authors comment on the specificity of the effect for USP2 (rather than GFP itself), or whether the c-terminal fusion was essential?

Response: We appreciate the reviewer for raising this concern. To address it, we transfected siUSP2 into H1975 and HEK293T cells stably expressing either the pLenti-GFP empty vector or pLenti-CD47-GFP, and measured GFP (FITC) mean fluorescence intensity (MFI) by flow cytometry 72 hours post-transfection. The results showed that GFP (FITC) MFI was only reduced in cells expressing pLenti-CD47-GFP following siUSP2 transfection, while no significant change was observed in cells expressing the pLenti-GFP empty vector (**Revised Supplementary Fig. 1e-h**). These findings indicate that siUSP2 specifically downregulated CD47 signaling, rather than affecting GFP expression itself.

Additionally, our immunofluorescence results demonstrate that the fusion of GFP to the C-terminal of CD47 does not disrupt its normal localization on the cell membrane, in contrast to the single GFP protein, which is widely distributed throughout the cell. This suggests that the normal subcellular localization of C-terminal fusion with GFP may not affect the function of CD47 in cells (**Revised Supplementary Fig. 1a**)).

2. There seem to be other DUBs whose knockdown also stabilizes expression of CD47, some even more strongly than USP2; if these DUBs are also expressed in cancer cells, how cell type dependent is their expression? What is the rationale behind the screen data presentation (what are A-J and 1-10 in extended figure 1a? this figure needs clearer labeling.)

Response: We sincerely thank the reviewer for their insightful comments. To identify deubiquitinating enzymes that may positively regulate CD47 expression, we performed an siRNA screen in H1975 and 293T cells, which revealed that USP2 was the only gene consistently ranked among the top ten hits in both cell lines. This finding, observed in two different cell lines, suggests that USP2 is a relatively universal regulator of CD47 protein expression. To further validate this, we performed co-immunoprecipitation assays to analyze the interactions of other identified candidates, such as USP51, UBR1, ZRANB1, YOD1, OTUD5, USP32, and JOSD1, with CD47. The results showed that USP2 exhibited a stronger interaction with CD47 compared to the other deubiquitinating enzymes we tested, suggesting that USP2 may directly interact with and stabilize CD47 in cells (**Revised Supplementary Fig. 1d**). However, we agree with the reviewer that, while our results support the role of USP2, we cannot exclude the possibility that other deubiquitinating enzymes may also contribute to the regulation of CD47 protein homeostasis, either directly or indirectly, under different conditions or in different cancer types.

Additionally, the **Revised Supplementary Fig. 1b** and **c** show the MFI values of GFP-CD47 in H1975 and HEK293T cells following siRNA transfection, as assessed by flow cytometry. The MFI values for GFP-CD47 across all samples, including siControl and siDUBs, are displayed in a heatmap, with colors ranging from blue to red to represent the MFI values. In response to the reviewer's suggestion, we have now provided a more detailed description of the figure creation process in the figure legend. The DUB gene names represented by A-J and 1-10 in the original extended Figure 1a (**Revised Supplementary Fig. 1b, c**) are provided in detail in the original data, with the USP2 gene indicated in row 5, column E.

Collectively, we have incorporated these new results, updated figure legends,

and expanded discussion in the revised manuscript.

3. An introduction of the small molecule inhibitor and USP2 would be nice.

Response: We sincerely appreciate the reviewer for their valuable and helpful suggestions. We have added a brief introduction to the deubiquitinase USP2 and its small-molecule inhibitor in the Introduction of main text.

4. Figure 1t: how are the scores calculated?

Response: We sincerely appreciate the reviewer's constructive suggestions. The IHC scoring was based on the average optical density (AOD) value in Figure 1t. Following IHC staining, the AOD for each spot on the tissue microarray was quantified using the Halo v3.0.311.314 analysis software with the Indica Labs - Area Quantification v2.1.3 module, calculated as $[AOD = \text{Integrated Optical Density (IOD) SUM} / \text{Area SUM}]$. In accordance with the reviewer's recommendations, we have provided a detailed description of the calculation method for IHC scoring in both the figure legend and the Materials and Methods section.

5. Figure 2h: legend off set.

Response: We appreciate the reviewer for pointing out this error, and we have corrected and revised the legend in the figure accordingly.

6. Would be nice to show PD-L1 expression in USP2 inhibited groups.

Response: As kindly instructed, we have included the PD-L1 results in updated files. Please see our detailed response to major concerns #1 and #2 from Reviewer #1.

Reviewer #2 (Remarks to the Author):

Dai et al. demonstrated that USP2 functions as a deubiquitinase for CD47, and inhibiting USP2 can modulate the tumor microenvironment, thereby sensitizing anti-PD-1 immunotherapy. The authors employed multiple in vivo models, clinical data, and patient samples to support their findings and explore potential strategies for enhancing the efficacy of tumor immunotherapy. However, there are several issues that need to be addressed in order to further strengthen the conclusions.

Response: We appreciate the reviewer's insightful comments, which have guided us in further improving our manuscript. Below, we provide detailed responses to each of the reviewer's comments and concerns.

1. The authors did not investigate the endogenous interaction between USP2 and CD47 in tumor cells and lung adenocarcinoma tissues.

Response: We thank the reviewer for the valuable suggestions. As recommended, we conducted endogenous co-immunoprecipitation experiments in the human lung cancer cell lines H1975 and PC9, which revealed a strong interaction between USP2 and CD47 in both cell lines (**Revised Fig. 2j, k**). Additionally, we performed immunofluorescence staining in human lung cancer tissues, where we observed colocalization of USP2 and CD47 on the cellular membrane (**Revised Supplementary Fig. 2a**). These results provide further evidence of a robust interaction between USP2 and CD47, both in lung cancer cell lines and tissues.

2. The authors should utilize antibodies targeting specific ubiquitin chains for the Ub IP experiment.

Response: We appreciate the reviewer for their excellent comments. To determine the specific type of ubiquitin linkage(s) assembled on CD47, we co-transfected CD47 with each of seven K-only ubiquitin constructs, where each construct retained only the specified lysine, with the remaining six lysine residues mutated to arginine. The results revealed that CD47 is predominantly modified by K48-linked ubiquitination (**Revised Supplementary Fig. 2b-d**).

Following the reviewer's helpful suggestions, we utilized a K48-linked ubiquitin chain-specific antibody to detect endogenous CD47 ubiquitination. Our results showed strong K48-linked ubiquitination of CD47 in both H1975 and PC9 lung cancer cell lines (**Revised Fig. 2o, p**). Notably, knocking down *USP2* with two different shRNAs resulted in an increase in endogenous K48-linked ubiquitination of CD47, suggesting that *USP2* specifically regulates K48-linked ubiquitination on CD47 (**Revised Fig. 2q, r**).

Taken together, these findings indicate that CD47 primarily undergoes K48-linked ubiquitination, and that *USP2* plays a key role in regulating this modification.

3. The combinational treatment of ML364 and PD-1 mAb shows promising results. However, the treatment time window appears to be limited. It would be meaningful for the authors to examine whether long-term treatment can exert a sustained inhibitory effect on tumors and to what extent it can prolong the survival of the mice. Additionally, it is important to investigate whether resistance develops during extended treatment periods.

Response: We sincerely appreciate the reviewer's valuable comments and suggestions. In our initial survival analysis, we observed that combination therapy significantly prolonged the survival of mice compared to either ML364 monotherapy or PD-1 mAb monotherapy. To better understand the effects of combination therapy on survival, we extended treatment from 12 to 18 doses of ML364 and from 4 to 6 doses of PD-1 mAb in mice with subcutaneous tumors. The treatment groups included: 1) Vehicle, 2) ML364 (5 mg/kg), 3) PD-1 mAb (100 µg/mice), and 4) ML364 (5 mg/kg) & PD-1 mAb (100 µg/mice). The results indicated that, while combination therapy substantially extended survival, all mice in the combination group eventually succumbed around day 35 (**Revised Supplementary Fig. 8i-k**). However, even in the combination therapy group, we did not observe complete tumor elimination or cure (**Revised Supplementary Fig. 8i-k**). This outcome is likely due to the high invasiveness and malignancy of LLC-derived tumors or the use of low-dose treatments.

Given that a recent study has reported improved therapeutic outcomes with

higher doses of ML364 (30 mg/kg) combined with PD-1 mAb in a breast cancer model (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941), we applied a high-dose ML364 regimen in our lung cancer model to evaluate long-term survival. Following treatment with: 1) Vehicle, 2) ML364 (30 mg/kg), 3) PD-1 mAb (200 µg/mouse), and 4) ML364 (30 mg/kg) & PD-1 mAb (200 µg/mouse), the combination therapy group showed a significant extension of survival, with mice surviving until approximately day 45 before substantial mortality occurred (**Revised Supplementary Fig. 8I-n**). Notably, two mice in the combination therapy group exhibited complete tumor regression, remaining tumor-free until day 70, and were considered cured (**Revised Supplementary Fig. 8I-n**). These results suggest that, in the highly malignant LLC mouse tumor model, high-dose ML364 combined with PD-1 mAb therapy effectively inhibits tumor growth and significantly prolongs survival.

With the exception of the mice that were completely cured, the remaining mice exhibited rapid tumor growth after day 30 post-tumor implantation, with tumors reaching approximately 1500 mm³ or leading to death around day 45. Tumor size was not effectively controlled in these cases. However, the reasons why some mice develop resistance to combination therapy remain unclear and warrant further investigation in future studies.

4. The authors claimed that the lower mRNA level of Usp2 contributes to the reduced hematologic cytotoxicity of ML364. However, they did not investigate whether ML364 treatment negatively impacts the physiological function of organs such as the lung, colon, kidney, and stomach, which exhibit higher Usp2 mRNA expression.

Response: We appreciate the reviewer's insightful comments. To address the reviewer's concern, we conducted histological analysis via H&E staining on various organs, including the lung, colon, kidney, and stomach, following ML364 treatment in the mouse model. The results revealed that, in comparison to the control group, ML364 treatment did not induce significant toxic effects in these organs (**Revised Supplementary Fig. 5o**). Although mRNA levels of ML364 target Usp2 in these organs may be relatively higher than in hematologic cells, they are still likely lower

than those observed in cancer cells. Furthermore, the low-dose ML364 regimen used in these experiments could also contribute to the absence of significant toxicity. These factors may help explain why ML364 treatment did not result in notable toxic effects in our study. In keeping with these results, *Usp2* KO mice are viable and treatment with USP2 inhibitor, ML364, does not show obvious signs of toxicity in mice (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941; Daniel Pouly et al., *Am J Physiol Renal Physiol.* 2013 Jul 1;305(1): F21-30), further supporting the notion that USP2 may be an ideal target for cancer treatment.

5. In Fig. 5b-d, ML364 or PD-1 mAb monotherapy demonstrates efficacy, while in Fig. 5f-i, knocking out USP2 or PD-1 mAb monotherapy appears to have little influence on tumor growth. The reasons for this difference should be elucidated. Additionally, the authors chose a different frequency of PD-1 mAb administration in Fig. 5e compared to that in Fig. 5a. An explanation for this discrepancy is warranted.

Response: We sincerely thank the reviewer for the insightful comments. In our original Figures 5b-d and 5f-i, the sample sizes were relatively small (n=5 and n=4 mice per group, respectively). As you know, it is challenging to gather a larger number of *Kras*^{LSL-G12D/+}; *Trp53*^{fl/fl} (KP) or *Kras*^{LSL-G12D/+}; *Trp53*^{fl/fl}; *Usp2*^{-/-} (KPU) mice for treatment experiments in a single batch, which may account for the intra-group variability and some of the differences observed in our initial results. We also noticed this discrepancy during the initial submission and subsequently made efforts to increase the number of mice for treatment. In this revised version, we have increased the sample size for both KP and KPU mice to n=8 per group. The updated statistical analysis now shows that ML364 or PD-1 mAb monotherapy can slightly inhibit lung cancer progression in KP mice, whereas the combination therapy significantly inhibited tumor growth compared to single-agent treatments (**Revised Fig. 5c, d and i, j**).

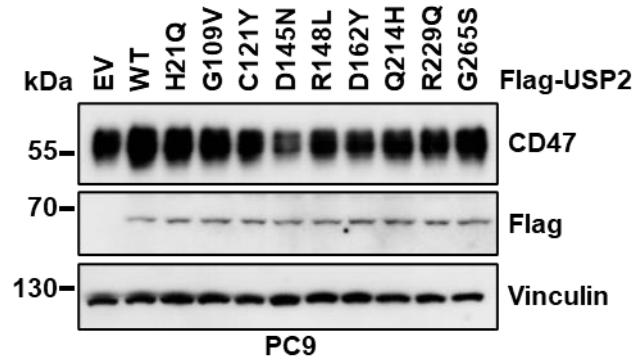
Additionally, the discrepancy in PD-1 mAb administration frequency between **Figures 5e and 5a** was due to a typographical error. In both experiments, PD-1 mAb was administered every three days via intraperitoneal injection. We have corrected this inconsistency in the revised version to ensure clarity and accuracy in the experimental

details.

We once again thank the reviewer for their thoughtful suggestions, which have significantly contributed to the improvement of our study.

6. The authors validated their findings using clinical samples and public databases. Did they observe any patient-derived mutations in USP2 or CD47 within the regions involved in their interaction? Do these mutations impact CD47 expression? Providing such data would further enhance the significance of their findings.

Response: We thank the reviewers for their insightful suggestions. To address the question, we examined potential mutations in the USP2 and CD47 genes in clinical non-small cell lung cancer (NSCLC) patients using the cBioPortal database (<https://www.cbioportal.org/>). Our analysis identified nine mutation sites within the 1-266 domain of USP2, which is essential for its interaction with CD47. In contrast, no mutations were found in the 290-323 domain of CD47, which interacts with USP2, in clinical patient samples. Based on these findings, we focused on mutations within the 1-266 region of USP2 and generated corresponding USP2 mutant constructs. We then overexpressed wild-type (WT) USP2 and its mutants in the PC9 lung cancer cell line and assessed the endogenous expression of CD47. Our results showed that, similar to USP2 WT, most USP2 mutants also enhanced CD47 expression in PC9 cells (**shown in the figure below**). However, the USP2 D145N mutant dramatically reduced CD47 protein levels (**shown in the figure below**). Whether and how these USP2 mutants affect CD47 protein levels in other lung cancer cell lines and their function *in vivo* remains unclear. These questions may warrant a separate study to explore the underlying mechanisms.



Revision Figure: Immunoblotting analysis of whole-cell lysates derived from PC9 cells co-transfected with indicated constructs.

7. It remains unclear whether the alterations in USP2 expression in clinical samples are attributable to changes at the transcriptional or protein levels.

Response: We sincerely thank the reviewers for their insightful suggestions. In our study, USP2 expression in clinical patient tissue samples was assessed solely by immunohistochemical (IHC) staining, which did not clarify the underlying mechanisms driving changes in USP2 expression—whether at the transcriptional or protein level. To address this, we collected an additional five pairs of fresh tumor and adjacent non-tumor tissues from lung cancer patients. RNA was extracted from a subset of these samples to measure *USP2* mRNA levels. Our results revealed that *USP2* mRNA levels were significantly higher in tumor tissues compared to matched adjacent non-tumor tissues in four of the five pairs we examined (**Revised Supplementary Fig. 10a**). Consistent with this, IHC staining on the same patient tissue samples demonstrated similar patterns of USP2 protein expression (**Revised Supplementary Fig. 10b, c**), correlating with the observed mRNA levels.

These findings suggest that alterations in USP2 expression may primarily occur at the transcriptional level, potentially driven by regulatory factors that modulate USP2 transcription. However, we cannot exclude the possibility of post-translational dysregulation of USP2 in certain cancer patient tissues. Validation in larger patient cohorts will be essential to confirm the regulatory mechanisms underlying changes in USP2 expression.

We hope these additional data help clarify the potential mechanisms regulating USP2 expression in lung cancer and we look forward to further exploring these findings in future studies.

8. Quantifications for Fig. 1f-g need to be provided.

Response: We appreciate the reviewers' valuable suggestions. In response, we have included the quantification of Mean Fluorescence Intensity (MFI) values of CD47 protein following immunofluorescence scanning (**Revised Supplementary Fig. 1o, p**), which has further strengthened our results.

9. The authors measured tumor sizes in terms of area units (mm²) in both Figure 4m and Figure 5h. The authors should explain how this parameter was calculated.

Response: We sincerely appreciate the reviewer's insightful suggestions. To quantify tumor areas in KP and KPU mice, three nonconsecutive whole-lung sections were selected from each mouse and subjected to HE staining. The stained sections were scanned using the Leica Aperio VERSA 8 multifunctional scanner, and tumor sizes were analyzed using ImageScope (Leica). The results, presented in the **Revised Fig. 4l** and **Fig. 5i (original Fig. 4m and Fig. 5h)**, reflect the average tumor area calculated from the three nonconsecutive sections per mouse. In accordance with the reviewer's recommendation, a more detailed description of the calculation method will be included in the figure legend.

Reviewer #3 (Remarks to the Author):

In this manuscript authors identify the deubiquitinase USP2 as a regulator of CD47 and demonstrate that it can be therapeutically targeted to inhibit tumoral CD47, increase phagocytosis of macrophages by tumor cells, and enhance response to anti-PD-1 immunotherapy. Recent studies have identified USP2 as a therapeutic target for cancer, however this is the first report that it is involved in stabilizing CD47 and that its inhibition can reduce CD47 levels and exert an antitumor immune effect. Interestingly, the lack of USP2 expression in blood cells means that unlike other therapies targeting CD47 directly, USP2 inhibition does not induce anemia or thrombocytopenia. The authors demonstrate that ML364, a small molecule inhibitor of CD47, can be used in combination with anti-PD-1 antibodies to control syngeneic subcutaneous, orthotopic, and spontaneous tumors in mice.

The manuscript is well written and of significant novelty and impact. While it presents compelling data regarding the relationship between USP2 and CD47, where it is less clear is in how anti-USP2 therapies remodel the tumor immune microenvironment. A central question that is not addressed is whether the effect of ML364 is solely through CD47. Other literature cited by the authors demonstrates that USP2 has effects on other signaling pathways of relevance to cancer, for example p53 and PD-L1. This calls into question the extent to which the effects observed in vivo are attributable to USP2's effects on CD47. Indeed, the combination of ML364 with anti-PD-1 would appear to be dependent on all of these effects. In this respect the manuscript is underdeveloped mechanistically. Evaluation of the effects of ML364 +/- anti-PD-1 on tumors in which CD47 has been knocked down or out as well as on hosts lacking macrophages and/or T cells are conspicuously absent.

Response: We sincerely appreciate the reviewers for recognizing the novelty and impact of our study and for providing valuable and insightful feedback, which have greatly contributed to the improvement of our work. Below, we provide a detailed point-by-point response, demonstrating that we have conducted additional experiments to thoroughly address the reviewers' concerns.

1. Figure 3f: It would be instructive to compare ML364 treatment in this model with anti-CD47 antibody treatment and/or treatment in tumors in which CD47 has been knocked down or out to gauge how USP2-targeted treatment performs in vivo relative to conventional anti-CD47 therapy, and whether its effects are dependent on CD47 expression as seen in previous figures.

Response: We sincerely appreciate the reviewer's critical concerns and insightful suggestions. In response, we established a subcutaneous lung cancer model using LLC cells, both with and without *Cd47* knockout. The experimental groups were as follows: 1) sgControl & Vehicle, 2) sgControl & ML364, 3) sgControl & CD47 mAb, 4) sgCd47 & Vehicle, 5) sgCd47 & ML364, and 6) sgCd47 & CD47 mAb. Mice with similarly sized tumors were randomly assigned to one of the six groups, and treatments were administered as follows: ML364 (5 mg/kg daily for 12 days) and CD47 mAb (200 µg/mouse every three days for four doses), with tumor growth monitored throughout the study.

Our results showed that in the sgControl group, both ML364 and CD47 mAb significantly inhibited tumor growth compared to the vehicle treatment, with no significant difference between the ML364 and CD47 mAb treatments (**Revised Supplementary Fig. 6b-d**). We found that *Cd47* knockout using sgCd47 significantly retarded tumor growth compared to the sgControl group with vehicle treatment, consistent with previous report showing that *Cd47* knockout inhibits tumor growth (Kipp Weiskopf et al., *J Clin Invest.* 2016 Jul 1;126(7):2610-20). However, in the sgCd47 group, neither ML364 nor CD47 mAb treatment further significantly inhibited tumor growth compared to the vehicle treatment (**Revised Supplementary Fig. 6b-d**). These findings suggest that the anti-tumor effect of ML364 at the low dose of 5 mg/kg may primarily depends on the USP2-CD47 axis.

A recent study by Yi et al. (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941) demonstrated that the USP2 inhibitor ML364 (at 30 mg/kg) sensitizes tumors to PD-1/PD-L1 blockade, primarily through the upregulation of the USP2 downstream target PD-L1 in a mouse breast cancer model. Together with our findings, these studies suggest that the anti-tumor immune effects of ML364 may depend on

distinct USP2 downstream targets, which could vary under different experimental conditions.

In our study, we used a lower dose of ML364 (5 mg/kg for *in vivo* treatments and 2 μ M for *in vitro* treatments), whereas Yi et al. employed higher doses (30 mg/kg for *in vivo* treatments and 20 μ M for *in vitro* treatments). We hypothesized that the differential doses of ML364 might affect the expression of USP2 downstream targets in distinct ways.

To test this hypothesis, we treated lung cancer cell lines with increasing doses of ML364 (1, 2, 5, 10, and 20 μ M). Our results showed that low doses of ML364 (1 and 2 μ M) dramatically reduced CD47 protein levels without affecting PD-L1 and p53 expression in PC9 cells. In contrast, higher doses of ML364 (10 and 20 μ M) significantly increased PD-L1 and p53 expression, consistent with Yi et al.'s findings (**Revised Supplementary Fig. 1q**). Similar results were observed in the H460 cell line, which was also used in Yi's study: low doses of ML364 reduced CD47 levels without altering PD-L1 and p53 expression, whereas high doses increased PD-L1 and p53 expression (**Revised Supplementary Fig. 1r**). These differential responses may be attributed to CD47 being a direct deubiquitinase substrate of USP2, while PD-L1 and p53 are indirect downstream targets of USP2, regulated through the VPRBP pathway.

Next, we explored the effects of different ML364 doses *in vivo*. Immunohistochemical (IHC) staining of subcutaneous LLC tumor tissues treated with low-dose ML364 (5 mg/kg) revealed a significant reduction in CD47 levels, but no significant change in PD-L1 levels, whether ML364 was used alone or in combination with PD-1 mAb (**Revised Supplementary Fig. 7e, f, i**). Given that higher doses of ML364 (30 mg/kg) have been shown to improve therapeutic outcomes in breast cancer models (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941), we applied a high-dose regimen of ML364 in our lung cancer model to assess CD47 and PD-L1 expression, as well as therapeutic efficacy. The treatments were as follows: 1) Vehicle, 2) ML364 (30 mg/kg), 3) PD-1 mAb (200 μ g/mouse), and 4) ML364 (30 mg/kg) & PD-1 mAb (200 μ g/mouse).

Indeed, the high-dose ML364 treatment yielded improved therapeutic

efficacy compared to low-dose treatment (5 mg/kg), with a significant extension in survival (**Revised Supplementary Fig. 8l-n**). Notably, two mice in the combination therapy group exhibited complete tumor regression and were considered cured (**Revised Supplementary Fig. 8l-n**), a result not observed in the low-dose treatment group. IHC staining showed that high-dose ML364 significantly decreased CD47 levels while increasing PD-L1 protein abundance (**Revised Supplementary Fig. 8o-q**). These findings suggest that in the highly malignant LLC mouse tumor model, high-dose ML364 treatment not only dramatically reduces CD47 expression but also increases PD-L1 expression, enhancing the effectiveness of PD-1/PD-L1 blockade therapy, a result consistent with findings in a breast cancer tumor model (Jingjie Yi et al., *Nat Commun.* 2023 Apr 6;14(1):1941).

Taken together, our *in vivo* and *in vitro* findings suggest that in our low-dose ML364-treated mouse lung cancer model (5 mg/kg), the therapeutic effects observed with combination therapy may primarily depend on the USP2-CD47 axis. However, high-dose ML364 (30 mg/kg) treatment may offer enhanced therapeutic efficacy, likely through its effects on both CD47 and other downstream targets such as PD-L1. We have incorporated these new results and further discussion into the updated manuscript.

2.Figure 3k: Other work (Zhang PLoS One 2016) has suggested that CD47 blockade increases M1 polarization but does not necessarily reduce M2 polarization. Again, it would be helpful to benchmark this therapeutic approach against one that acts directly against CD47 (antibody, genetic) to assess whether the effects seen here are solely attributable to how USP2 modulates CD47.

Response: We sincerely appreciate the constructive suggestions provided by the reviewers. According to the study by Zhang et al. (*PLoS One*, 2016), CD47 blockade using anti-CD47 antibody promoted an increase in M1 macrophages without affecting the number of M2 macrophages in glioblastoma xenografts in NSG mice. To determine whether the effect of ML364 on macrophage reprogramming is dependent on CD47 expression in tumor cells, we performed flow cytometric analysis of M1 and M2 macrophages in our subcutaneous mouse lung cancer LLC tumors, both with and

without *Cd47* knockout, in C57BL/6J background mice. In WT *sgcontrol* LLC tumors, we observed ML364 or anti-CD47 treatment significantly increased the tumor-infiltrated M1 macrophages, accompanying with decreased M2 macrophages (**Revised Supplementary Fig. 6e, f**). Notably, knockout of *Cd47* using *sgCd47* significantly increased the tumor-infiltrating M1 macrophages and decreased the M2 macrophages (**Revised Supplementary Fig. 6e, f**). However, the ML364 or anti-CD47 treatment didn't further significantly affect the proportion of M1 and M2 macrophages in *Cd47* KO tumors (**Revised Supplementary Fig. 6e, f**). These results together demonstrate that inhibition of USP2 using ML364 increases the proportion of M1 and reduces M2 in LLC tumors derived from C57BL/6J background mice largely through regulation of CD47.

A recent study also demonstrated that anti-CD47 treatment also significantly increased the M1 macrophages and decreased the M2 macrophages in carcinogen-induced orthotopic HCC C57BL/6J mice (Shulan Han et al., *Sci Adv.* 2023 Jul 21;9(29): eadg2697). The discrepancy for the changes of M2 macrophages with anti-CD47 treatments is not consistent with previous report (Zhang et al. (*PLoS One*, 2016)) might be due to using the different cancer model, mouse strain, and different CD47 antibodies. We have incorporated these results and discussion in our updated manuscript.

3.Figure 4: The mechanism of the combined therapy is not clearly developed. It is unclear whether anti-USP2 and anti-PD-1 therapy functions by inducing macrophage phagocytosis of tumor cells (as suggested in panel i), by inducing CD8 T cell-mediated killing of tumor cells (as would be expected from anti-PD-1 therapy), or a combination of the two. PD-1 has been implicated as a don't eat me signal similar to CD47 (Gordon Nature 2017) suggesting this therapy may function solely through macrophage phagocytosis, but this is not clearly demonstrated.

Response: We sincerely appreciate the insightful questions raised by the reviewers. In response, we established a subcutaneous mouse lung LLC cancer model. Mice with similarly sized tumors were randomly assigned to one of five treatment groups: 1) Vehicle, 2) ML364 & PD-1 mAb (MP), 3) MP & CD8 blockade (α CD8), 4) MP &

macrophage blockade (clodronate liposomes, CL), and 5) MP & α CD8 & CL. Tumor growth analysis revealed that blockade of either CD8 T cells or macrophages partially mitigated the tumor-suppressive effects of ML364 & PD-1 mAb (MP) therapy (**Revised Supplementary Fig. 9d-h**). However, when both CD8 T cells and macrophages were blocked, the tumor-suppressive effect of the MP combination therapy was completely abolished (**Revised Supplementary Fig. 9d-h**). These results indicate that ML364 & PD-1 mAb combination therapy inhibits tumor growth through the cooperative actions of both macrophage-mediated phagocytosis and CD8 T cell-mediated tumor cell killing. We also agree with the reviewer that PD-1 expression on tumor-associated macrophages may play a role in macrophage phagocytosis (Sydney R Gordon et al., *Nature*. 2017 May 25;545(7655):495-499). We have cited this relevant study and expanded on this discussion in the updated manuscript.

4.Figure 5f: The microCT scans are difficult to interpret and compare tumor progression with the histologic data. Annotation and/or quantitation of tumor burden in these scans is needed.

Response: We sincerely appreciate the reviewer's insightful suggestions. In our mouse micro-CT imaging analysis, we performed CT whole-lung scans on mice from each treatment group and employed 3D reconstruction to visualize the overall lung morphology. Due to the difficulty in precisely defining tumor boundaries outside the lung stroma, accurate 3D reconstruction of the tumor proved challenging. As tumor invasion into normal lung tissue increased, the defects in the reconstructed lung stroma became more pronounced, with greater volumes of lung tissue damage. Consequently, the volume of the reconstructed healthy lung stroma was used as a measure of spontaneous lung cancer progression. In response to the reviewer's suggestion, we quantified the volume of the 3D reconstructed lung stroma using the recently reported method (Yuedong Guo et al., *Nat Commun*. 2023 Nov 11;14(1):7306). We have increased the sample size for each treatment group of KP and KPU to gather more quantitative data and provide representative 3D reconstructions of the entire lung for each group (**Revised Fig. 5f**). The quantification results revealed that the lung volume

in the KPU group treated with PD-1 mAb was significantly larger, with markedly reduced tumor progression, compared to the remaining groups (**Revised Fig. 5g**).

5.Extended data figure 6d: The authors present a model of the effects of USP2 but have not elucidated the specifics of this process. For example, they indicate that USP2 inhibition via ML364 inhibits M1 macrophages and suggest that this is independent of CD47, however this has not been shown.

Response: We sincerely appreciate the reviewer's constructive suggestions. To assess whether the effect of ML364 on macrophage reprogramming depends on CD47 expression in tumor cells, we performed flow cytometric analysis of M1 and M2 macrophages in subcutaneous LLC lung cancer tumors, both with and without CD47 knockout, in C57BL/6J background mice. In wild-type (WT) sgControl LLC tumors, we observed that treatment with ML364 or anti-CD47 significantly increased the proportion of tumor-infiltrating M1 macrophages, accompanied by a decrease in M2 macrophages (**Revised Supplementary Fig. 6e, f**). Notably, knockout of CD47 using sgCD47 also led to a significant increase in M1 macrophages and a decrease in M2 macrophages (**Revised Supplementary Fig. 6e, f**). However, ML364 or anti-CD47 treatment did not further significantly affect the proportions of M1 and M2 macrophages in CD47 knockout tumors (**Revised Supplementary Fig. 6e, f**). Consistently, our single-cell sequencing analysis of LLC tumors also showed that ML364 treatment increased the proportion of M1 macrophages and decreased M2 macrophages (**Revised Fig. 3k**). Together, these results demonstrate that inhibition of USP2 by ML364 increases the proportion of M1 macrophages and reduces M2 macrophages in LLC tumors derived from C57BL/6J mice, primarily through regulation of CD47.

However, we also observed an increase in tumor-infiltrating CD8 effector T cells in LLC tumors treated with ML364 by single-cell sequencing analysis (**Revised Supplementary Fig. 3h**). Flow cytometry analysis confirmed that ML364 treatment significantly increased the proportion of cytotoxic CD8 T cells compared to the vehicle group (**Revised Fig. 3o**). Collectively, these findings suggest that ML364 treatment not

only modulates macrophage polarization but also recruits CD8 T cells to the tumor microenvironment to exert anti-tumor effects.

Overall, the impact of ML364 targeting USP2 on macrophage M1/M2 polarization is likely mediated primarily through CD47 expression. Additionally, ML364 treatment appears to promote the recruitment of CD8 T cells to the tumor immune microenvironment. The precise mechanism underlying this recruitment requires further investigation. Based on these findings and the reviewer's suggestions, we have revised and updated the model diagram accordingly (**Revised Supplementary Fig. 10d**) and discussed this in the updated manuscript.