nature portfolio

Peer Review File

A plug-and-play monofunctional platform for targeted degradation of extracellular proteins and vesicles

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors present a paper on the targeted degradation of specific proteins using an interesting method that exploits the natural ability of nanoparticles being trafficked to lysosomes. The paper is of general interest, however I think for publication in a journal such as Nature Communications, a comparison to conventional lysosomal targeting agents is required, so it is possible to benchmark this approach to the exisiting agents. In addition to the need for a comparison to lysosomal targeting agents, a number of specific points should be addressed before the paper is suitable for publication.

Chemical inhibitors are used to block cell uptake. These inhibitors are non-specific and a discussion around how these inhibitors inhibit multiple pathways should be included.

In Figure 1b & d, the MFI rather than the % position should be used to measure the particle signal. In 1d and the SI, some of the data points show >100% positive cells. This is not possible. The gating scheme of how the % positive cells are determined should be included in the SI.

Flow cytometry data shows association, not uptake. The paper should be reworded to reflect this.

The doubling of particle size when the particles are functionalised with antibodies (extended data 3a), strongly suggests aggregation of the particles. This should be noted and discussed.

The authors say uptake (should be association) is increased with Ab functionalisation, but all the numbers in extended data 3b look similar and I don't think there is any significant difference in any of these numbers.

Figure 2g. Is there a statistical significance in the increase of the markers? The text says all markers were increased, but RAB7 shows no increase.

I found the data in Fig4b to be a bit confusing. I don't understand why the APC-antiPD-L1 shows no signal in figure 4b, but in figure 4f, when the cells are stained with Cy5-antiPD-L1, the cells show strong signal.

To be able to observe the hook affect described for 4i, densitometry should be performed and the relative densities of each band plotted. This comment applies to all the gel data where trends are being described. I assume full gel images would be made

available for the final manuscript.

When describing the increase in particle size for the data in extended data 6a, it should be referred to as particle aggregation.

In figure 5d, have the authors taken into account photobleaching of the eGFP? How many total images were acquired over the 21 hour period?

Reviewer #2:
Remarks to the Author:

In this paper, Yao et al. developed a platform technology for targeted degradation of extracellular proteins and vesicles. They showed that nanoparticles can bind and carry various molecules to lysosome and help their degradation. However, in my opinion, the overall strategy and data are not sufficient to demonstrate that this study is suitable for publication in 'Nature Communication' because its novelty is not strong, and overall data are not enough to demonstrate the usefulness of this study. Personally, I think that the other journals (more specific) would be more suitable. Critical points are as below.

1. Most important thing. In my opinion, at least one animal test would be essential to demonstrate the feasibility and potential of this strategy considering the quality of 'Nature Communication'.

2. Polystyrene NP is maybe non-degradable. It would be accumulated in body. There are so many biodegradable nanoparticles available. The authors commented about it in discussion, it needs to be considered before starting experiments.

3. Because the NPs have no active targeting ligands or cationic charges, maybe the cellular uptake would not be efficient, and many NPs would be still localized outside of the cells. Considering the amounts of NPs treated, the efficiency looks low.

4. In the application to EV, the change in the concentration of EVs outside of the cells should be analyzed, not inside.

5. Lysosomal colocalization needs to be analyzed quantitatively. For example, using Pearson coefficient.

6. In my opinion, there are some typos and sentences not fluent. Careful revision of overall sentences needs to be performed by authors. After that, English editing by

natives is also needed.

Reviewer #3:
Remarks to the Author:
In the manuscript “a plug-and play monofunctional platform for targeted degradation of extracellular proteins and vesicles”, the authors developed modified nanoparticle with targeting binders (MONOTAB) using a nanoparticle that has inherent lysosome trafficking ability. By fusing antibodies or binders onto these nanoparticles, they showed degradation of membrane proteins such as PD-L1, secreted proteins like MMP2, and extracellular vesicles. The platform presented here seems versatile, but some of the mechanisms and experiments need to be solidified.

1. The authors claim that the MONOTABs are not dependent on specific receptors, and showed that similar uptake efficiency was observed with different cell types that presumably have different surface receptor patterns. I’m not convinced from this statement that the platform is truly receptor independent. They show that these MONOTABs go through clathrin-mediated endocytosis, which makes me think there could be some surface receptor involved. Could the authors show the receptor independnece somehow, by using a KO library of cell surface receptors or showing that the nanoparticles do not bind to anything on the cell surface without the antibody binders? This claim is crucial as it distinguishes this paper from other papers that use nanoparticles to degrade proteins via lysosomes (https://doi.org/10.1021/acs.nanolett.3c03148).
2. Can the authors elaborate a little bit more on the nature of the polystyrene nanoparticle they are using? They highlight how their system is much easier to synthesize than previous technologies. Are these nanoparticles commercially available/how accessible are they? These need to be discussed in order claim their superiority over “multi-step chemical synthesis or protein recombination” required for the other approaches.
3. The authors showed that using IgG:antiFc ratios of 2:1 resulted in more efficient NP uptake. Is this because at higher ratios, there are more IgGs coated on the nanoparticle? Can they quantify the number of IgGs in their nanoparticles with different ratios? And I’m a little confused as to why adding more IgG would affect internalization, if the internalization is only mediated by NP. Perhaps the IgGs are interacting with Fc receptors on the cell surface? To eliminate that possibility, Fc receptors KO would be helpful.
4. I’m not convinced of the lysosome biogenesis argument based on data in Fig2. Increase in LysoTracker signal and endo/lysosomal markers could indicate a lot of things. First, LysoTracker is not specific for lysosome, as it stains any acidic

compartments (including Golgi). Often, increase in Lysotracker staining or lysosome markers are observed when there is some dysfunction in lysosomes due to lack of lysosomal hydrolases or activity. I think there needs to be further explanation for why lysosomal biogenesis would be happening. Is it possible that the presence of the nanoparticles is affecting lysosomal health/composition that contribute to lysosome biogenesis as a way to compensate? Probably a further dive into what is happening to the lysosomal composition/activity in response to the nanoparticles is needed.
5. In Figure 4, how do the authors explain the difference internalization vs. degradation kinetics? They see internalization of their MONOTABs within a few hours, but to see PD-L1 degrdation, they only see significant degradation starting at 24h. Are some of the nanoparticles internalizing before they get a chance to bind to PD-L1?
6. The EV degradation is not very convincing. First, GFP is unstable in low pH, so using it as a readout for degradation is not very accurate, as it would lose fluorescence in endosomes as well. In addition, some EVs can exocytose upon internalization – can the authors show that most of the EVs do not exocytose?

Response to Reviewers' Comments

Reviewer #1

Comment #1. The authors present a paper on the targeted degradation of specific proteins using an interesting method that exploits the natural ability of nanoparticles being trafficked to lysosomes. The paper is of general interest, however I think for publication in a journal such as Nature Communications, a comparison to conventional lysosomal targeting agents is required, so it is possible to benchmark this approach to the exisiting agents. In addition to the need for a comparison to lysosomal targeting agents, a number of specific points should be addressed before the paper is suitable for publication.

- We thank the reviewer for this positive comment. As per the suggestion, we performed additional experiments to benchmark MONOTAB against two bifunctional chimera-based methods (IFLD and GalNAc-LYTAC) in terms of PD-L1 degradation (Line 330-343 in the Revision). Because these degraders are not commercially available, we first synthesized BMS-L1-RGD (IFLD type) and αPD-L1-GalNAc (GalNAc-LYTAC type) for targeting PD-L1 (Page 33-39 in the revised Supplementary Information). Compared to the near-complete degradation of PD-L1 with 6.7 nM of αPD-L1-NP (Fig. 3g), treatment with 50 nM of BMS-L1-RGD for the same duration led to ~46% PD-L1 degradation only (Fig. R1 or Supplementary Fig. 8c).
  In addition, we found that both αPD-L1-NP and αPD-L1-GalNAc induced similar levels of PD-L1 degradation at a low concentration of 1.3 nM. However, at higher concentrations, αPD-L1-GalNAc exhibited the hook effect, while αPD-L1-NP caused even more substantial degradation (Fig. R2 or Supplementary Fig. 8d).

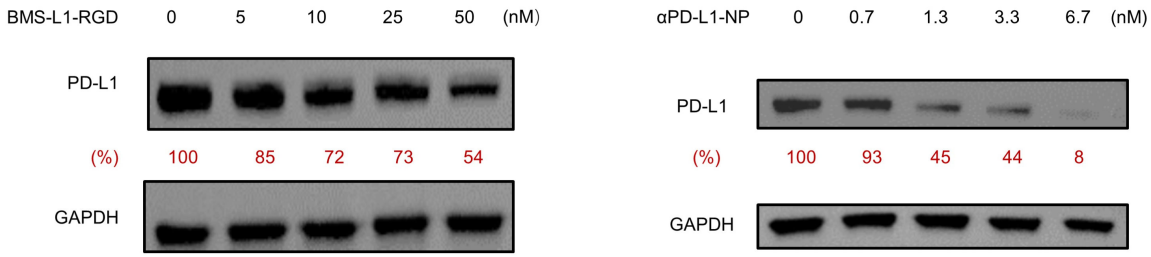

Fig. R1. Western blot analysis of PD-L1 degradation in B16F10 cells after co-incubation with BMS-L1-RGD at the indicated concentrations for 24 hours.

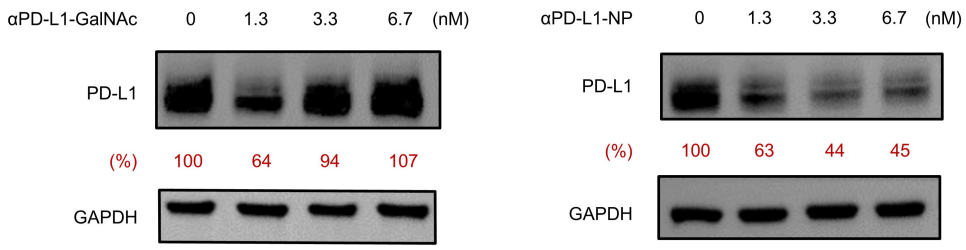

Fig. R2. Western blot analysis of PD-L1 degradation in Hepa1-6 cells after co-incubation with αPD-L1-GalNAc or αPD-L1-NP at the indicated concentrations for 24 hours.

Comment #2. Chemical inhibitors are used to block cell uptake. These inhibitors are non-specific and a discussion around how these inhibitors inhibit multiple pathways should be included.

- The detailed discussion on how these inhibitors worked was included in the Revision (Line 143-155).

  "Flow cytometry analysis showed that low-temperature treatment significantly inhibited ^{RB}NP uptake in all cell lines, pointing to an energy-dependent uptake process. Inhibition of caveolae-dependent endocytosis through cholesterol sequestration (filipin) did not influence NP uptake, while tyrosine kinase inhibition (genistein) showed a mild inhibitory effect in a cell line-specific manner. The observation is not surprising since genistein, albeit an established inhibitor of caveolae-dependent endocytosis, acts as a tyrosine kinase inhibitor that also affects molecules involved in other endocytic pathways. Similarly, inhibition of actin polymerization (cytochalasin D) or PI3K signaling (wortmannin), which target macropinocytosis, resulted in a slight reduction in NP uptake in certain cell lines. Interestingly, inhibitors of clathrin-mediated endocytosis (chlorpromazine and Pitstop 2) exhibited conflicting effects, with Pitstop 2 showing no impact, while chlorpromazine effectively reduced NP uptake across all cell lines."

Comment #3. In Figure 1b & d, the MFI rather than the % position should be used to measure the particle signal. In 1d and the SI, some of the data points show >100% positive cells. This is not possible. The gating scheme of how the % positive cells are determined should be included in the SI.

- Per the reviewer's suggestion, these figures were revised with the MFI value used (Fig. R3 and R4 or Figure 1b and 1d).

  We apologize for not presenting the data clearly. The original value referred to the relative positive ratio which was derived by normalizing the positive cell ratio against that of the control treatment. In the Revision, the true positive cell ratio was used instead (Fig. R5 or Supplementary Fig. 2a). The gating strategies for determining the positive cell ratio was included in the revised figure (Fig. R5).

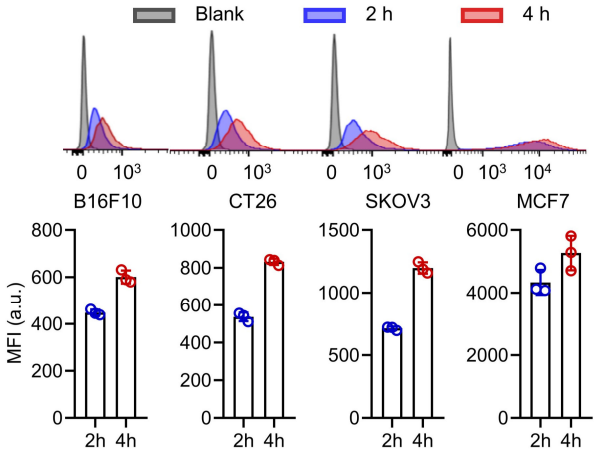

Fig. R3. Cellular uptake of ^{RB}NPs after co-incubation with B16F10 cells for 2 or 4 hours.

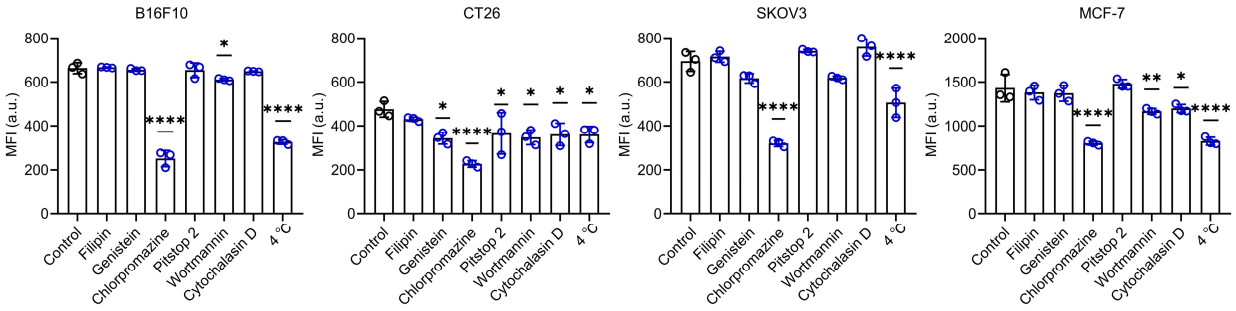

Fig. R4. Effects of endocytic inhibitors on cellular uptake of ^{RB}NPs in different cell lines.

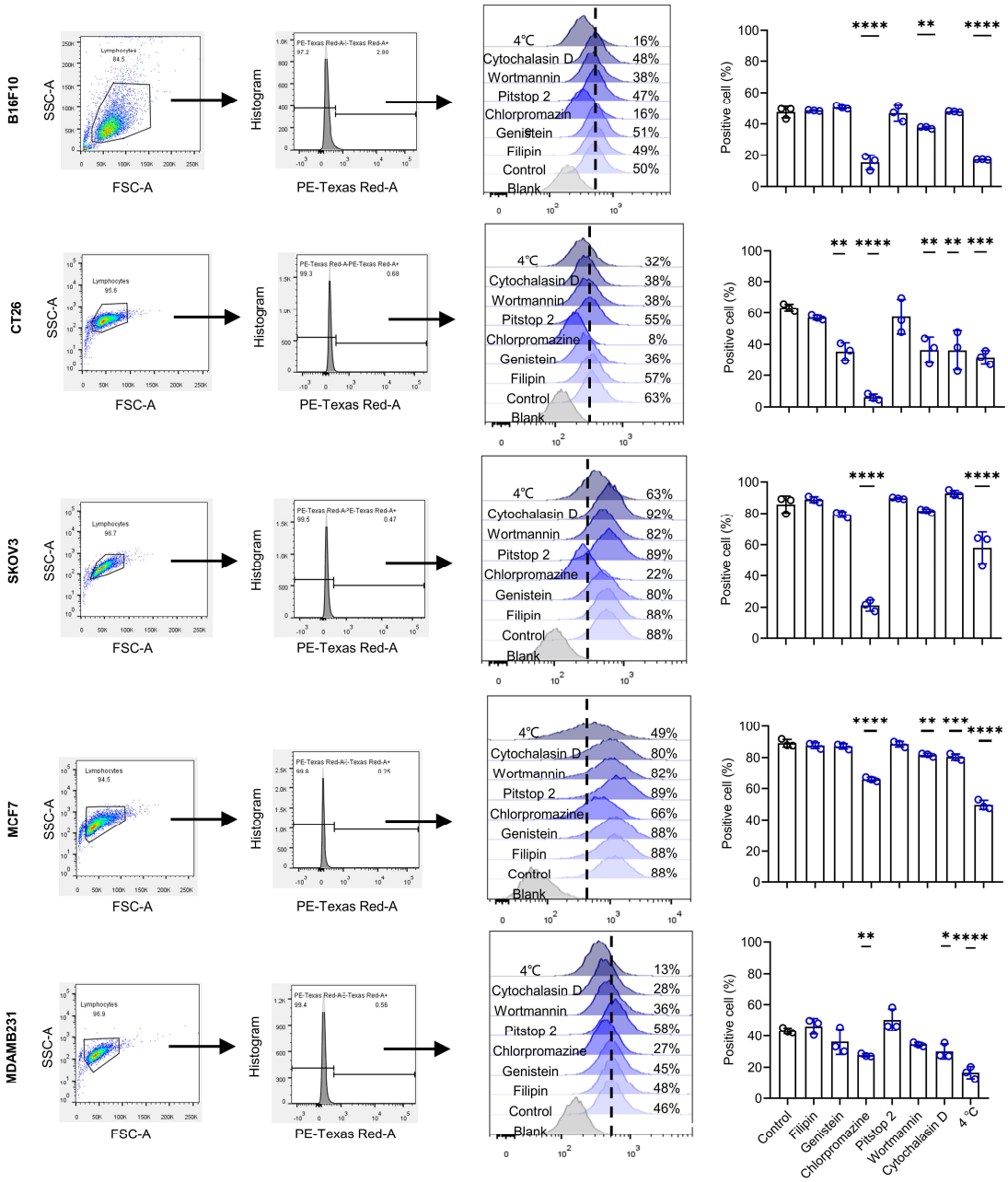

Fig. R5. Flow cytometry gating strategy for analyzing RB-positive cells.

Comment #4. Flow cytometry data shows association, not uptake. The paper should be reworded to reflect this.

- We performed the trypan blue quenching assay to distinguish cellular association or uptake. The result showed that nearly all the detected NPs were internalized into cells but not absorbed on the membrane (Fig. R6 or Supplementary Fig. 1b).

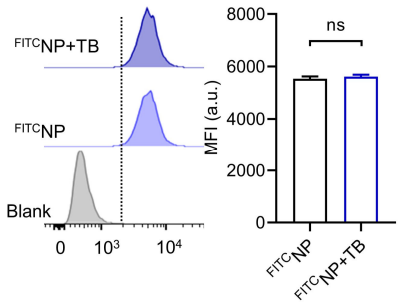

Fig. R6. Flow cytometry analysis of cellular association and uptake of ^{FITC}NP by B16F10 cells *via* trypan blue quenching. The fluorescence of surface-bound ^{FITC}NPs would be quenched by trypan blue.

Comment #5. The doubling of particle size when the particles are functionalised with antibodies (extended data 3a), strongly suggests aggregation of the particles. This should be noted and discussed.

- We measured the hydrodynamic size of particles prepared from multiple batches. As shown in Fig. R7 (Supplementary Fig. 5a and b), the average particle size of αFc-NPs was ~120 nm, approximately 20 nm larger than that of NPs, suggesting no aggregation of αFc-NP. In addition, we also used Cryo-TEM to further confirm the size and well-dispersed state of αFc-NP (Fig. R8 or Supplementary Fig. 6c and d)

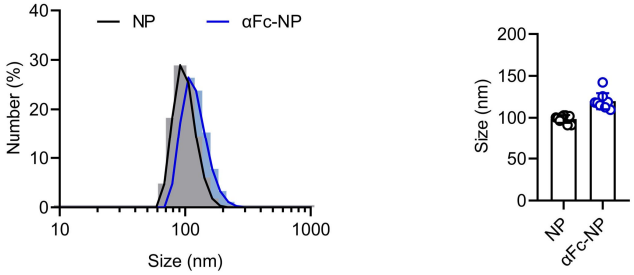

Fig. R7. Left, DLS measurements of NPs and αFc-NPs. Right, Average size of NPs and αFc-NPs.

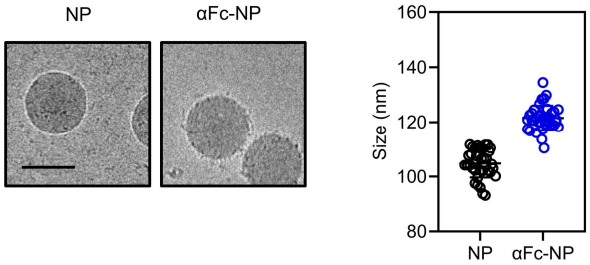

Fig. R8. Left, Representative Cryo-TEM images of NP and αFc-NP. Scale bar, 100 nm. Right, Particle size distribution analysis of Cryo-TEM images.

Comment #6. The authors say uptake (should be association) is increased with Ab functionalisation, but all the numbers in extended data 3b look similar and I don't think there is any significant difference in any of these numbers.

- To address the reviewer's concern, we carried out additional flow cytometry experiments. Indeed, there's no significant difference across all the αFc:IgG ratios (Fig. R9 or Supplementary Fig. 6f). The corresponding discussion was revised in the Revision (Line 234-235).

  In addition, we confirmed that the Ab-functionalized NPs were also mostly internalized into cells but not absorbed on the membrane via trypan blue quenching (Fig. R10).

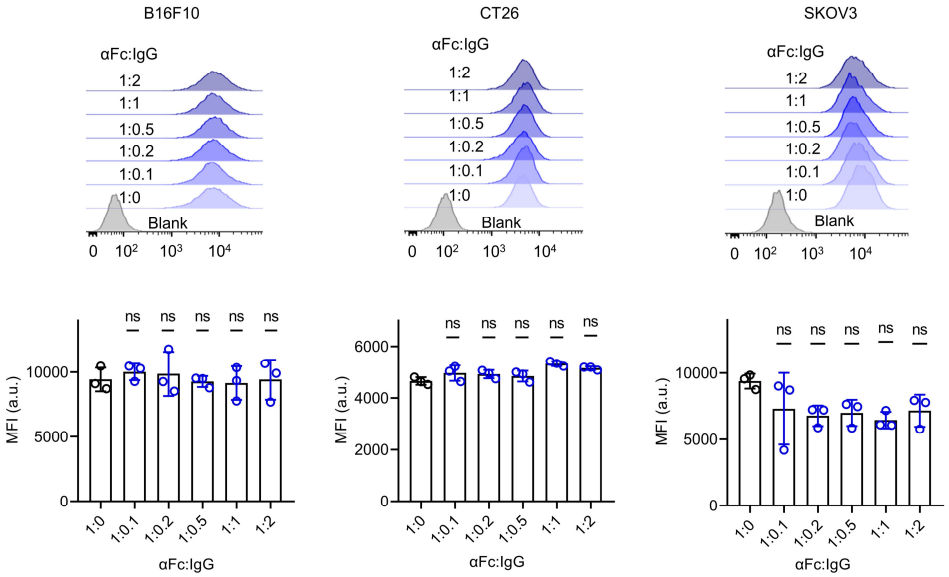

Fig. R9. Cellular uptake of CTRL-^{RB}NPs with varying αFc:IgG molar ratios in B16F10, CT26, and SKOV3 cells within 4 hours.

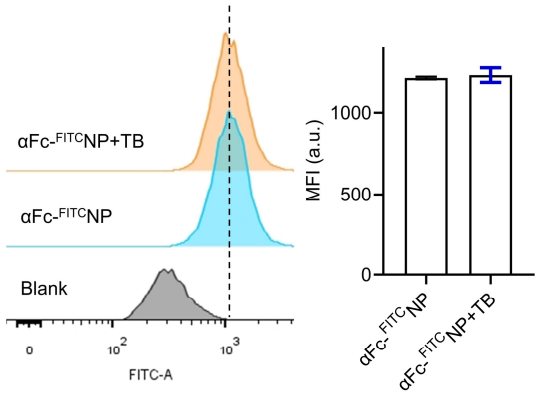

Fig. R10. Flow cytometry analysis of cellular association and uptake of αFc-^{FITC}NP by B16F10 cells *via* trypan blue quenching. The fluorescence of surface-bound αFc-^{FITC}NPs would be quenched by trypan blue.

Comment #7. Figure 2g. Is there a statistical significance in the increase of the markers? The text says all markers were increased, but RAB7 shows no increase.

- We carried out statistical analysis of the band densities. The results showed that all the markers were significantly increased after the CTRL-NP treatment (Fig. R11 or Fig. 2g).

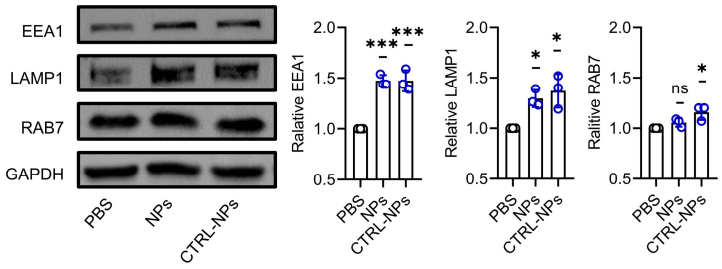

Fig. R11. Western blots (left) and quantitative analysis (right) of endo-lysosome markers after incubation with CTRL-NPs or NPs for 10 hours.

Comment #8. I found the data in Fig4b to be a bit confusing. I don't understand why the APC-antiPD-L1 shows no signal in figure 4b, but in figure 4f, when the cells are stained with Cy5-antiPD-L1, the cells show strong signal.

- We thank the reviewer for this valuable observation. In the original Fig. 4b, the cells were incubated with APC-antiPD-L1 (Biolegend, #124311), in which the APC dye is highly vulnerable to photobleaching so that this antibody is only suitable for flow cytometry but not confocal imaging. To address this, we re-performed this experiment by using FITC-labeled antibody (FITC-anti-mouse CD274/PD-L1 antibody, Elabscience, E-AB-F1132UC) instead. As shown in Fig. R12 (Fig. 3b in the Revision), the FITC signal was observed on cell surface following the ^{FITC}αPD-L1 treatment.

  As to Fig. 4f (Fig. 4e in the Revision), the cells were actually stained with unlabeled anti-PD-L1 (Proteintech, #66248-1) and then Alexa Fluor 647-labelled secondary antibody (Abcam, #ab150115). Therefore, the signal would be much brighter due to the signal amplification from multiple secondary antibodies binding.

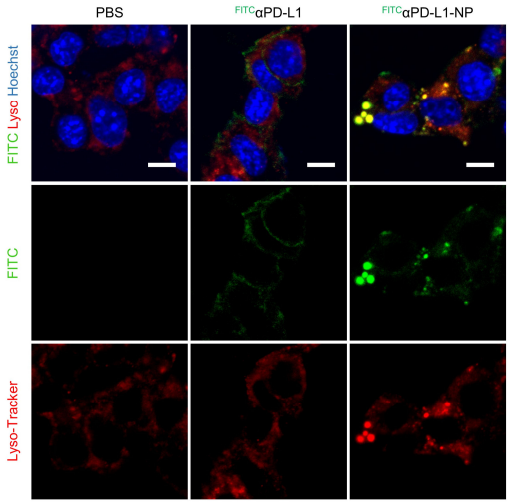

Fig. R12. Live-cell images of B16F10 cells treated with ^{FITC}αPD-L1 (3.3 nM) or ^{FITC}αPD-L1-NP (^{FITC}PD-L1-equiv., 3.3 nM) for 4 hours. Scale bar, 10 μm.

Comment #9. To be able to observe the hook affect described for 4i, densitometry should be performed and the relative densities of each band plotted. This comment applies to all the gel data where trends are being described. I assume full gel images would be made available for the final manuscript.

➢ Per the reviewer's suggestion, densitometric analysis of blots and gels was performed. The relative density values (Fig. R13) were included in the Revision. In addition, unprocessed blot and gel images (Fig. R14) were included in the revised Supplementary Information (Page 19-20).

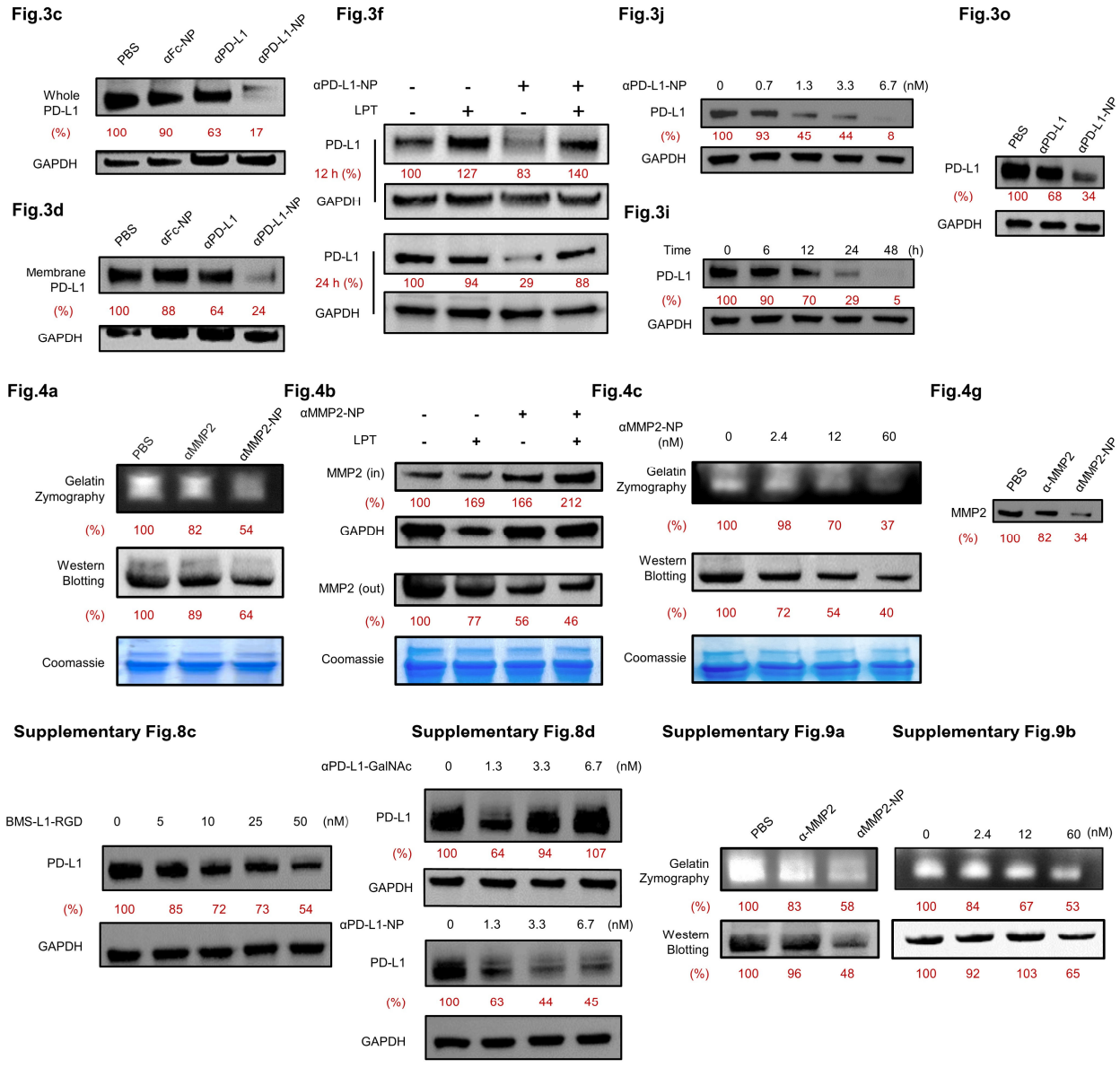

Fig. R13. Densitometric analysis of blots and gels.

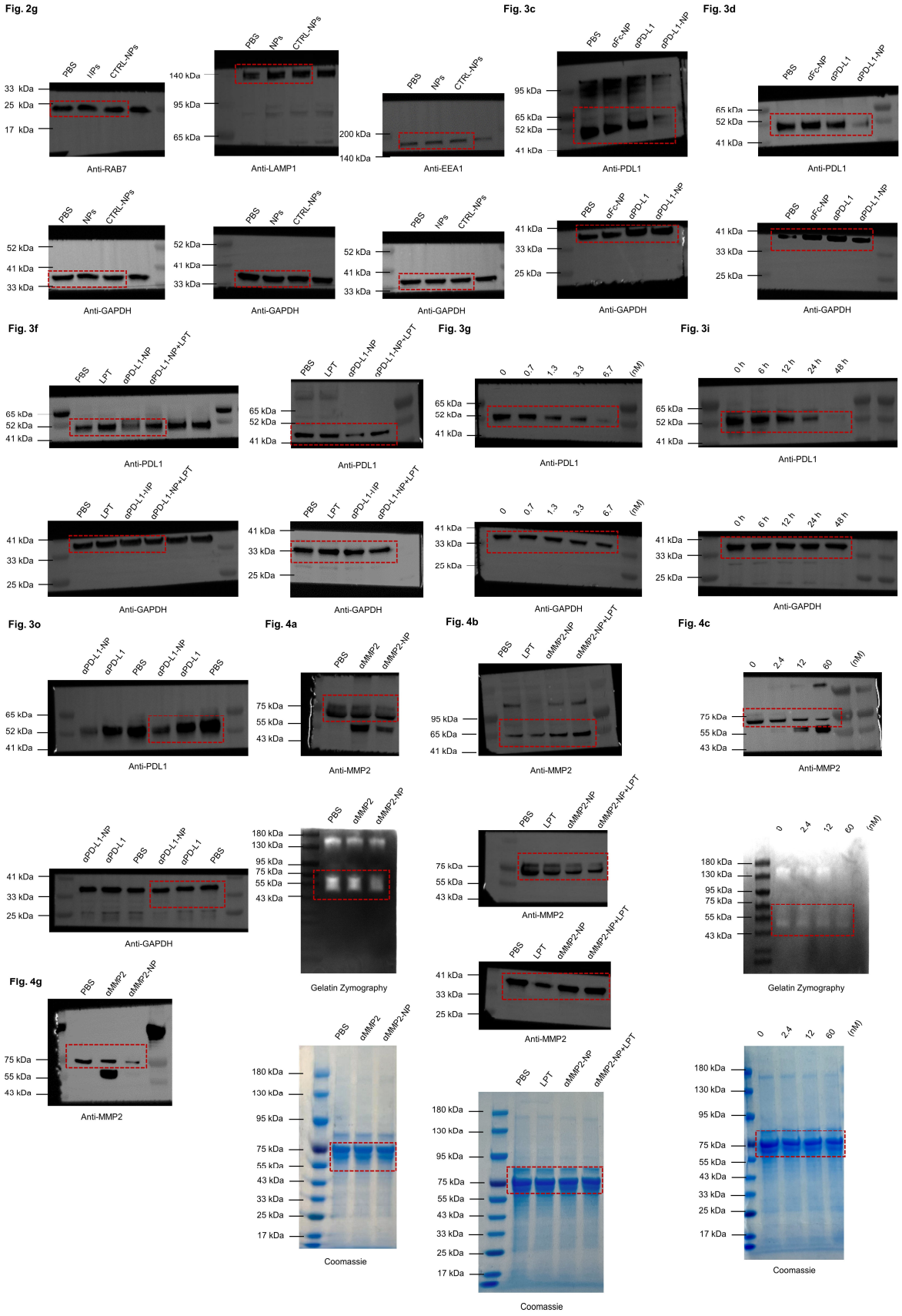
Fig. 2g
PBS
IPs
CTRL-NPs
33 kDa
25 kDa
17 kDa
Anti-RAB7
PBS
NPs
CTRL-NPs
140 kDa
95 kDa
65 kDa
Anti-LAMP1
PBS
NPs
CTRL-NPs
200 kDa
140 kDa
Anti-EEA1
PBS
NPs
CTRL-NPs
52 kDa
41 kDa
33 kDa
Anti-GAPDH
PBS
NPs
CTRL-NPs
52 kDa
41 kDa
33 kDa
Anti-GAPDH
PBS
NPs
CTRL-NPs
52 kDa
41 kDa
33 kDa
Anti-GAPDH
Fig. 3c
PBS
αFc-NP
αPD-L1
αPD-L1-NP
95 kDa
65 kDa
52 kDa
41 kDa
Anti-PDL1
PBS
αFc-NP
αPD-L1
αPD-L1-NP
41 kDa
33 kDa
25 kDa
Anti-GAPDH
Fig. 3d
PBS
αFc-NP
αPD-L1
αPD-L1-NP
65 kDa
52 kDa
41 kDa
Anti-PDL1
PBS
αFc-NP
αPD-L1
αPD-L1-NP
41 kDa
33 kDa
25 kDa
Anti-GAPDH
Fig. 3f
PBS
LPT
αPD-L1-NP
αPD-L1-NP+LPT
65 kDa
52 kDa
41 kDa
Anti-PDL1
PBS
LPT
αPD-L1-NP
αPD-L1-NP+LPT
65 kDa
52 kDa
41 kDa
Anti-PDL1
PBS
LPT
αPD-L1-HP
αPD-L1-NP+LPT
41 kDa
33 kDa
25 kDa
Anti-GAPDH
PBS
LPT
αPD-L1-HP
αPD-L1-NP+LPT
41 kDa
33 kDa
25 kDa
Anti-GAPDH
Fig. 3g
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0.7
1.3
3.3
6.7
(nM)
65 kDa
52 kDa
41 kDa
Anti-PDL1
0
0.7
1.3
3.3
6.7
(nM)
41 kDa
33 kDa
25 kDa
Anti-GAPDH
Fig. 3i
0 h
6 h
12 h
24 h
48 h
65 kDa
52 kDa
41 kDa
Anti-PDL1
0 h
6 h
12 h
24 h
48 h
41 kDa
33 kDa
25 kDa
Anti-GAPDH
Fig. 3o
αPD-L1-NP
αPD-L1
PBS
αPD-L1-NP
αPD-L1
PBS
65 kDa
52 kDa
41 kDa
Anti-PDL1
αPD-L1-NP
αPD-L1
PBS
αPD-L1-NP
αPD-L1
PBS
41 kDa
33 kDa
25 kDa
Anti-GAPDH
Fig. 4g
PBS
αMMP2
αMMP2-NP
75 kDa
55 kDa
43 kDa
Anti-MMP2
Fig. 4a
PBS
αMMP2
αMMP2-NP
75 kDa
55 kDa
43 kDa
Anti-MMP2
PBS
αMMP2
αMMP2-NP
180 kDa
130 kDa
95 kDa
75 kDa
55 kDa
43 kDa
Gelatin Zymography
PBS
αMMP2
αMMP2-NP
180 kDa
130 kDa
95 kDa
75 kDa
55 kDa
43 kDa
33 kDa
25 kDa
17 kDa
Coomassie
Fig. 4b
PBS
LPT
αMMP2-NP
αMMP2-NP+LPT
95 kDa
65 kDa
41 kDa
Anti-MMP2
PBS
LPT
αMMP2-NP
αMMP2-NP+LPT
75 kDa
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Anti-MMP2
PBS
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αMMP2-NP
αMMP2-NP+LPT
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Anti-MMP2
PBS
LPT
αMMP2-NP
αMMP2-NP+LPT
180 kDa
130 kDa
95 kDa
75 kDa
55 kDa
43 kDa
33 kDa
25 kDa
17 kDa
Coomassie
Fig. 4c
0
2.4
12
60
(nM)
75 kDa
55 kDa
43 kDa
Anti-MMP2
0
2.4
12
60
(nM)
180 kDa
130 kDa
95 kDa
75 kDa
55 kDa
43 kDa
Gelatin Zymography
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55 kDa
43 kDa
33 kDa
25 kDa
17 kDa
Coomassie

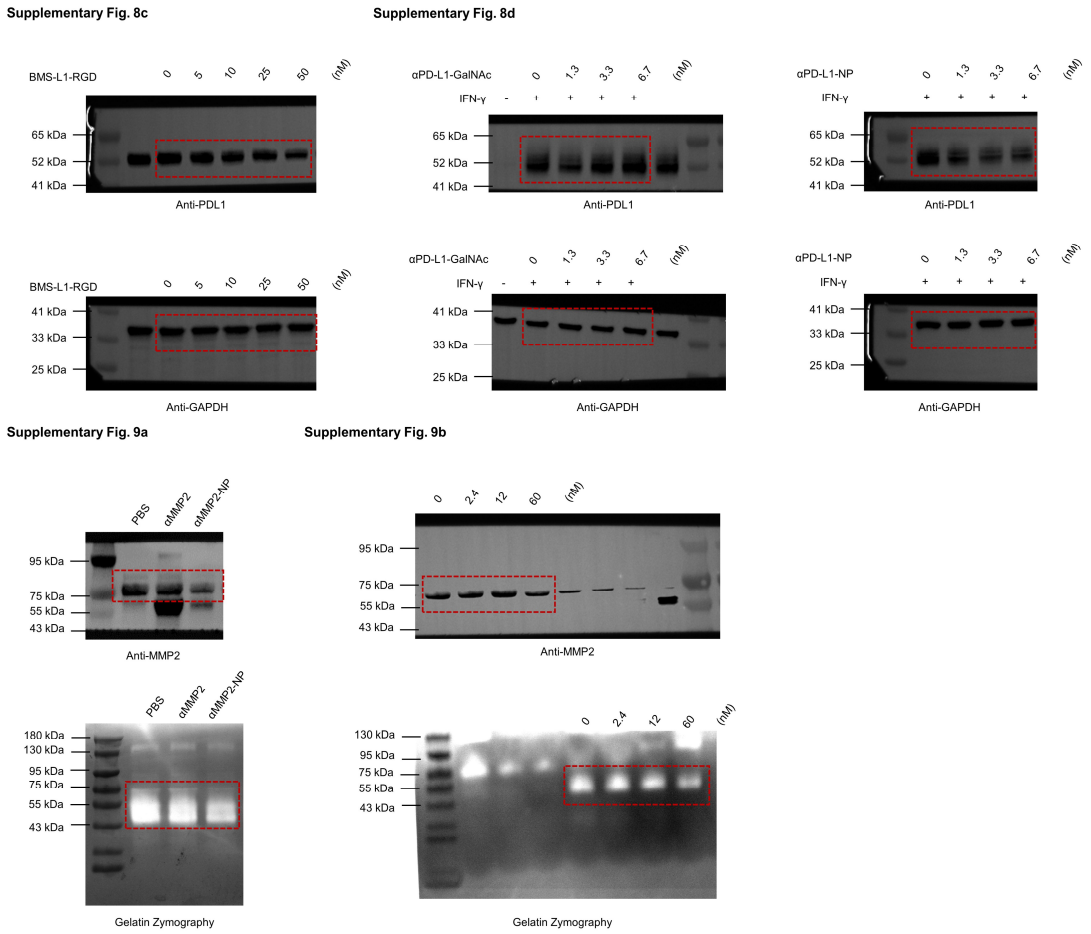

Fig. R14. Unprocessed blot and gel images.

Comment #10. When describing the increase in particle size for the data in extended data 6a, it should be referred to as particle aggregation.

➢ As mentioned in the response to Comment #5, we used DLS and Cryo-TEM to determine the size and dispersion state of CTRL-NPs. The DLS data showed that the average particle size of CTRL-NPs was 150 nm, approximately 30 nm larger than that of αFc-NP, suggesting no aggregation of CTRL-NP. Cryo-TEM further confirmed the size and well-dispersed state of CTRL-NPs (Fig. R15 or Supplementary Fig. 6c and d).

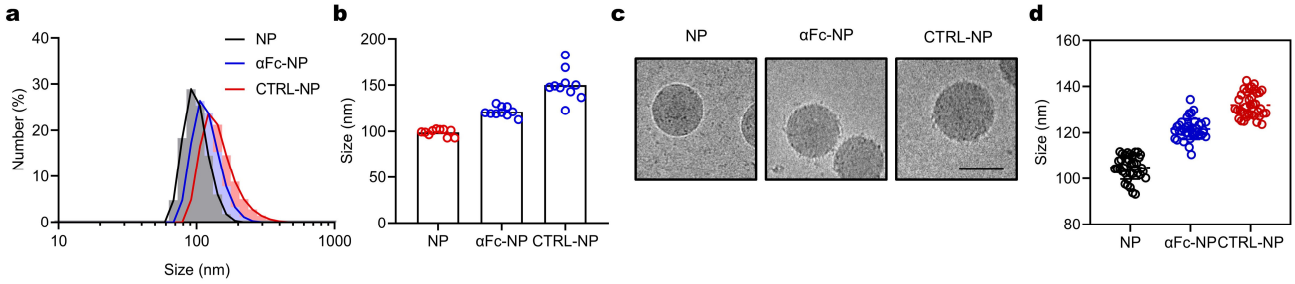

Fig. R15. a, b, Size distribution (**a**) and average size (**b**) of NP, αFc-NP, and CTRL-NP determined by DLS. **c,** Representative Cryo-TEM images of NP, αFc-NP, and CTRL-NP. Scale bar, 100 nm. **d,** Particle size distribution analysis of Cryo-TEM images.

Comment #11. In figure 5d, have the authors taken into account photobleaching of the eGFP? How many total images were acquired over the 21 hour period?

- Indeed, we have taken the photobleaching issue of EGFP into account then. During the fluorescence tracking experiment, only 4 images were captured for each field.
  To further rule out the influence of photobleaching, we tracked the EGFP signal in ECDHCC1-PalmGRET cell debris with 20-round confocal scanning. The results showed that only minimal change was detected, confirming the negligible effect of photobleaching on EGFP loss (Fig. R16 or Supplementary Fig. 10e and f).

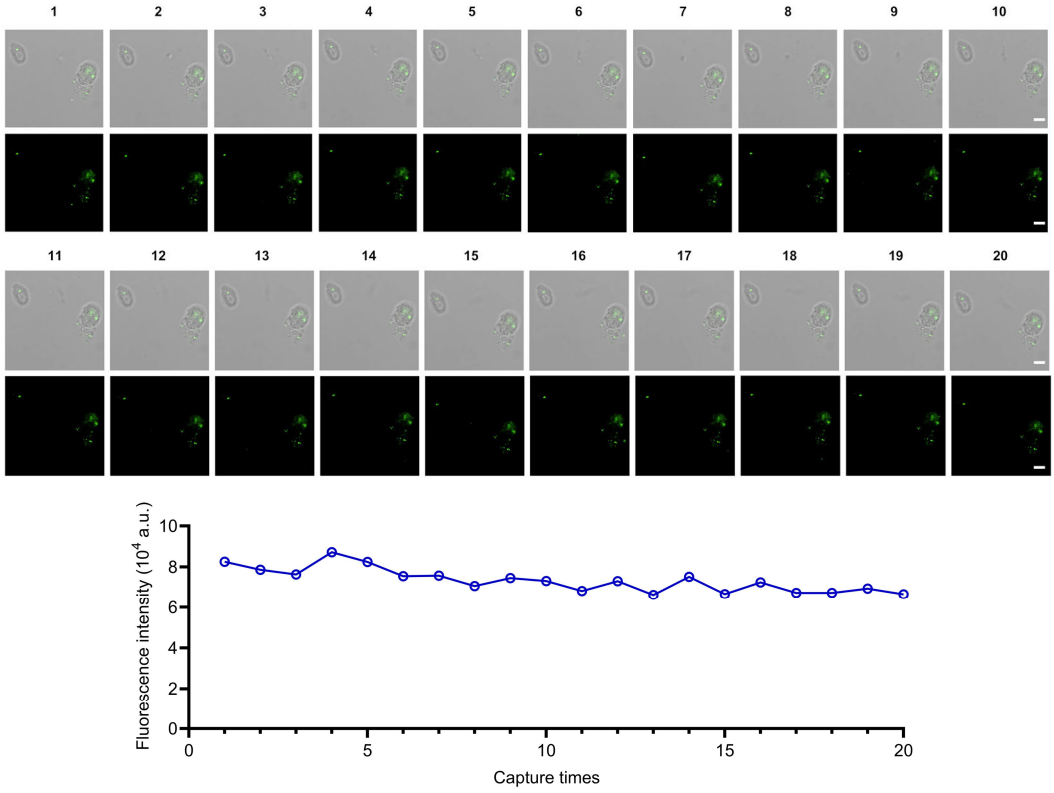

Fig. R16. Upper, Confocal tracking of the EGFP signal in ECDHCC1-PalmGRET cell debris with 20 rounds of image capture. Bottom, Quantitative image analysis of the EGFP signal.

Reviewer #2

Comment #1. Most important thing. In my opinion, at least one animal test would be essential to demonstrate the feasibility and potential of this strategy considering the quality of 'Nature Communication'.

- Per the reviewer's suggestion, we conducted an *in vivo* antitumor study of αPD-L1-NP. The new data (Fig. R17 or Fig. 3k-o) were included in the Revision (Line 344-353).

  "Next, we evaluated the in vivo antitumor effects of αPD-L1-NP. C57BL/6 mice bearing subcutaneous B16F10 tumors were treated with PBS, αPD-L1, or αPD-L1-NP, respectively, and tumor size was measured (Fig. 3k). Compared with the control and αPD-L1 groups, tumor growth was significantly inhibited by the treatment with αPD-L1-NP (Fig. 3l, m and Supplementary Fig. 8e) and no body weight loss was observed during the experiment (Supplementary Fig. 8f). Immunofluorescence analysis of PD-L1 expression in tumor sections revealed a markedly reduced PD-L1 level in the αPD-L1-NP group (Fig. 3n and Supplementary Fig. 8g), which was further corroborated by Western blot analysis (Fig. 3o and Supplementary Fig. 8h). These results underscore the therapeutic potential of MONOTAB in vivo."

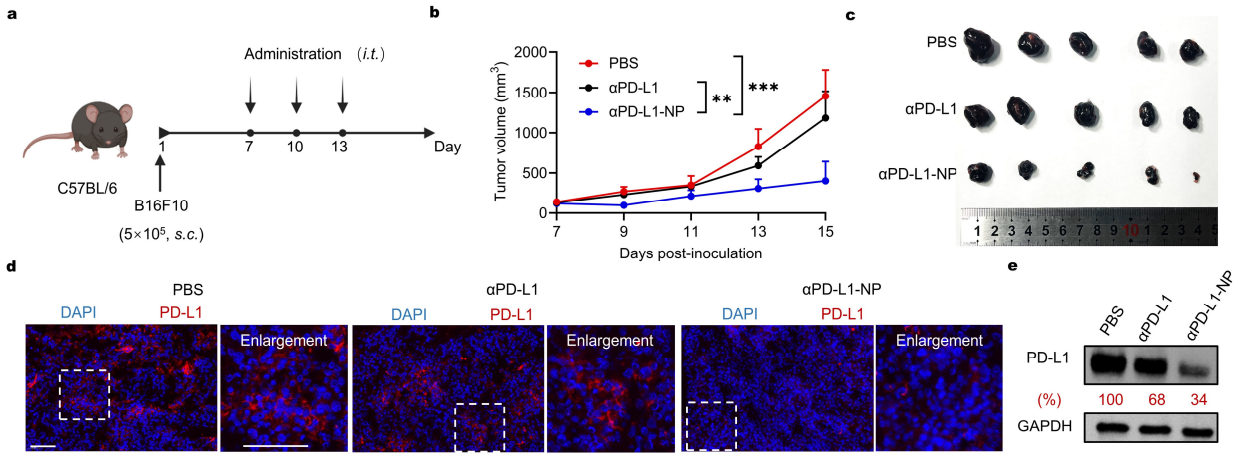

Fig. R17. *In vivo* antitumor study. **a,** Schematic diagram outlining the experimental design. **b,** Tumor growth curves of mice receiving different treatments. **c,** Image of tumors resected after animal euthanasia. **d,** Immunofluorescence staining of PD-L1 in tumor sections. **e,** Western blot of PD-L1 in tumor lysates.

Comment #2. Polystyrene NP is maybe non-degradable. It would be accumulated in body. There are so many biodegradable nanoparticles available. The authors commented about it in discussion, it needs to be considered before starting experiments.

- We thank the reviewer for this critical comment. Polystyrene NP is indeed non-degradable. However, in this work, these NPs were just used to validate the MONOTAB concept. In our ongoing work, we have further validated the potential of MONOTAB using biodegradable nanoparticles such as PLGA.

Comment #3. Because the NPs have no active targeting ligands or cationic charges, maybe the cellular uptake would not be efficient, and many NPs would be still localized outside of the cells. Considering the amounts of NPs treated, the efficiency looks low.

- We measured the fluorescence intensity in the medium following co-incubation with ^{RB}NPs. The remaining concentration of ^{RB}NP was calculated from a standard curve for fluorescence intensity versus ^{RB}NP concentration. The results showed that ~22% of the given NPs were internalized within 2 hours and ~28% within 4 hours (Fig. R18 or Supplementary Fig. 1c and d). This efficiency is already rather considerable.

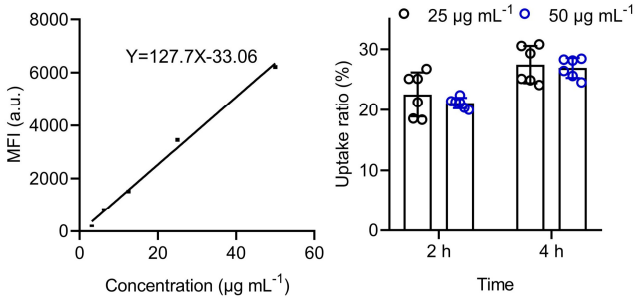

Fig. R18. Left, Standard curve for fluorescence intensity versus ^{RB}NP concentration. Right, Ratios of internalized ^{RB}NPs after co-incubation with B16F10 cells for 2 or 4 hours.

Comment #4. In the application to EV, the change in the concentration of EVs outside of the cells should be analyzed, not inside.

- We thank the reviewer for this suggestion. We measured the fluorescence intensity of ^{EGFP}EVs outside of the cells after each treatment. The results showed that co-incubation with Annexin V-NPs significantly decreased the fluorescence intensity of ^{EGFP}EVs in the medium, which has no significant difference from the blank level (Fig. R19 or Fig. 5c).

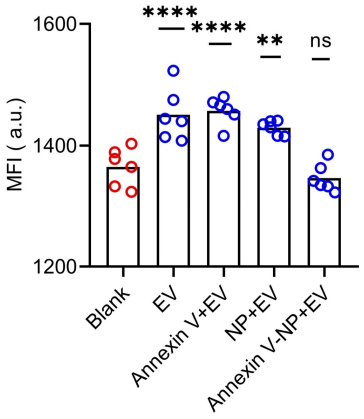

Fig. R19. Fluorescence intensity of ^{EGFP}EVs in the medium after 8-hour co-incubation with B16F10 cells.

Comment #5. Lysosomal colocalization needs to be analyzed quantitatively. For example, using Pearson coefficient.

- Given that the intensities of the NP signal and the LysoTracker signal are not correlated in principle, we did not use Pearson's coefficient as the metric because this function measures only linear relationships between variables. Instead, we used Manders' colocalization coefficient that describes the amount of overlap between two channels to evaluate the degree of lysosomal colocalization. The new data were included in the revised Supplementary Information (Fig. R20 or Supplementary Fig. 1f and 5j).

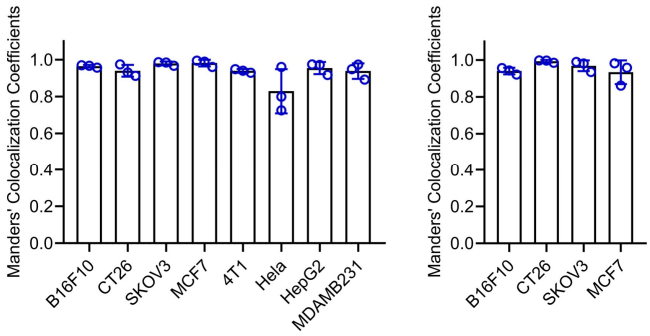

Fig. R20. Manders' colocalization coefficients measuring the colocalization of ^{RB}NPs (Left) or αFc-^{RB}NPs (Right) within lysosomes.

Comment #6. In my opinion, there are some typos and sentences not fluent. Careful revision of overall sentences needs to be performed by authors. After that, English editing by natives is also needed.

- We thank the reviewer for this comment. We have proofread the manuscript carefully.

Reviewer #3

Comment #1. The authors claim that the MONOTABs are not dependent on specific receptors, and showed that similar uptake efficiency was observed with different cell types that presumably have different surface receptor patterns. I'm not convinced from this statement that the platform is truly receptor independent. They show that these MONOTABs go through clathrin-mediated endocytosis, which makes me think there could be some surface receptor involved. Could the authors show the receptor independence somehow, by using a KO library of cell surface receptors or showing that the nanoparticles do not bind to anything on the cell surface without the antibody binders? This claim is crucial as it distinguishes this paper from other papers that use nanoparticles to degrade proteins via lysosomes (https://doi.org/10.1021/acs.nanolett.3c03148).

- We thank the reviewer for this important comment. To ascertain the independence of NP uptake from specific surface receptors, we screened a panel of typical receptors associated with lysosomal trafficking and found

that targeted gene silencing via CRISPRi or RNAi did not affect NP uptake (Fig. R21 or Fig. 1e and f). In addition, we treated B16F10 cells with trypsin for 3 hours to digest extracellular domains of membrane proteins and then used the cells to incubate with ^{RB}NPs. The results showed only a mild decrease in the MFI, with no change in the ratio of RB-positive cells (Fig. R22 or Fig. 1g). However, when co-incubated with ^{Cy3}Avidin and Biotin-cRGD, which are internalized via integrin-mediated endocytosis, the Cy3-positive cell ratio was decreased significantly (Fig. R22 or Supplementary Fig. 4). Collectively, these results suggest that NP uptake does not rely on specific surface receptors.

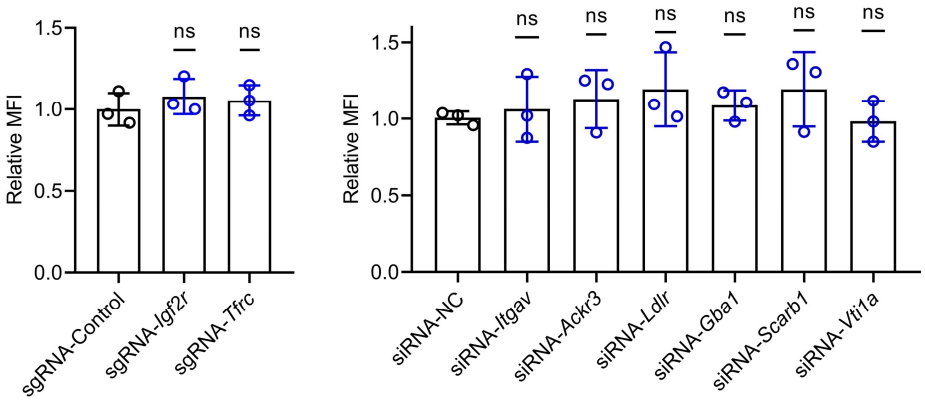

Fig. R21. Cellular uptake of ^{RB}NPs after co-incubation with *Igf2r*- or *Tfrc*-KO B16F10 cells, or *Itgav*-, *Ackr3*-, *Ldlr*-, *Gba1*-, *Scarb1*- or *Vti1a*-silencing B16F10 cells for 2 hours.

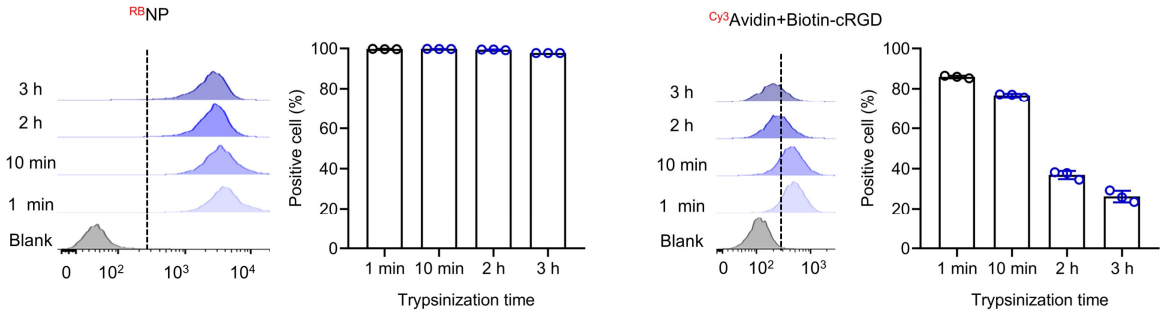

Fig. R22. Cellular uptake of ^{RB}NPs (Left) or ^{Cy3}Avidin+Biotin-cRGD (Right) after co-incubation with B16F10 cells pre-treated with trypsin within 3 hours.

Comment #2. Can the authors elaborate a little bit more on the nature of the polystyrene nanoparticle they are using? They highlight how their system is much easier to synthesize than previous technologies. Are these nanoparticles commercially available/how accessible are they? These need to be discussed in order claim their superiority over "multi-step chemical synthesis or protein recombination" required for the other approaches.

- Polystyrene nanoparticles are commercially available with various surface modifications. Those we used are modified with streptavidin with a grafting ratio of 2~3%, which makes them particularly suitable for validating the concept of our approach. This point was mentioned in the Revision (Line 187).
  "Commercially available streptavidin-conjugated NPs were chosen as the chassis"

Comment #3. The authors showed that using IgG:antiFc ratios of 2:1 resulted in more efficient NP uptake. Is this because at higher ratios, there are more IgGs coated on the nanoparticle? Can they quantify the number of IgGs in their nanoparticles with different ratios? And I'm a little confused as to why adding, if the internalization is only mediated by NP. Perhaps the IgGs are interacting with Fc receptors on the cell surface? To eliminate that possibility, Fc receptors KO would be helpful.

- We used the Dot blot assay to quantify the IgG content on CTRL-NPs with different αFc:IgG ratios and found that more IgGs were coated on the NPs at higher ratios (Fig. R23 or Supplementary Fig. 6e).
  We also carried out additional flow cytometry experiments to confirm whether more IgG would affect internalization. The results revealed that the varying αFc:IgG ratios did not impact NP uptake efficiency (Fig. R24 or Supplementary Fig. 6f), echoing that the internalization is only mediated by NPs.
  To rule out the involvement of Fc receptors (FcRs), we used FcR-preblocked cells to incubate with CTRL-NPs. As shown in Fig. R25 (Supplementary Fig. 6g), no change in the NP uptake was observed.

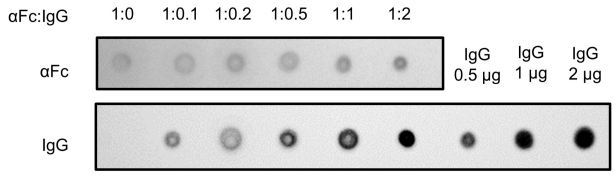

Fig. R23. Dot blot assay for quantifying the αFc and IgG contents in CTRL-NPs with varying αFc:IgG ratios.

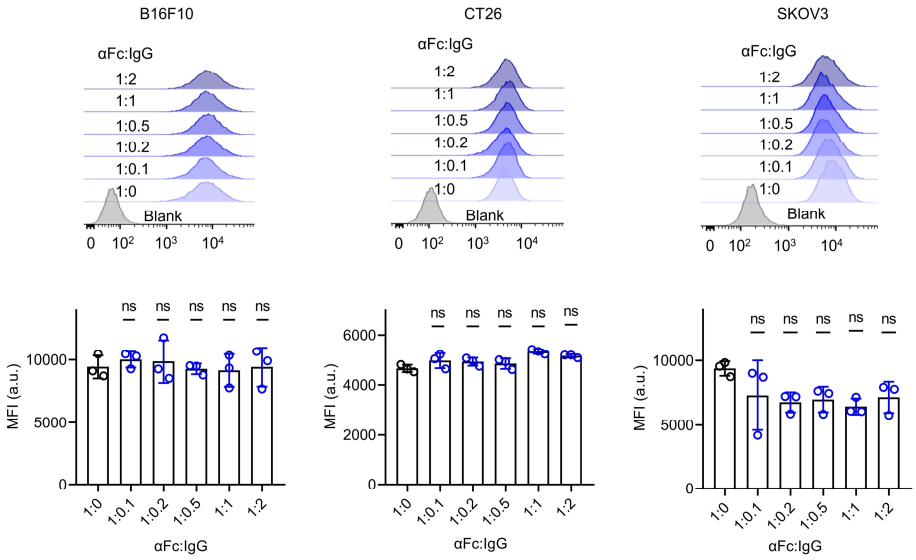

Fig. R24. Cellular uptake of CTRL-^{RB}NPs with varying αFc:IgG molar ratios after 4-hour co-incubation.

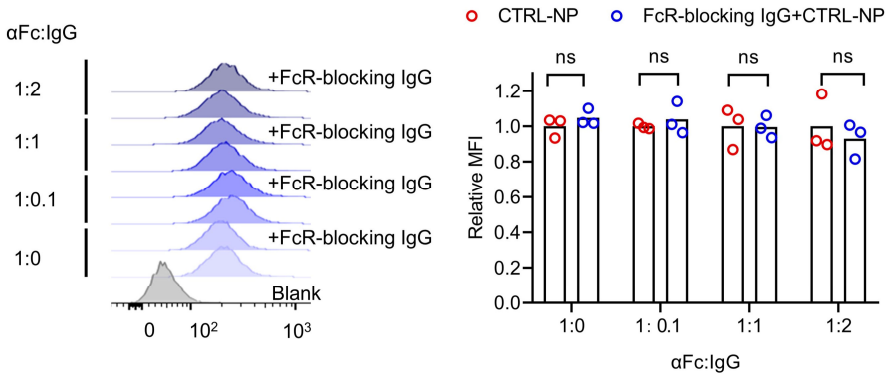

Fig. R25. Cellular uptake of CTRL-^{RB}NPs with varying αFc:IgG molar ratios in FcR-preblocked B16F10 cells within 4 hours.

Comment #4. I'm not convinced of the lysosome biogenesis argument based on data in Fig2. Increase in LysoTracker signal and endo/lysosomal markers could indicate a lot of things. First, LysoTracker is not specific for lysosome, as it stains any acidic compartments (including Golgi). Often, increase in Lysotracker staining or lysosome markers are observed when there is some dysfunction in lysosomes due to lack of lysosomal hydrolases or activity. I think there needs to be further explanation for why lysosomal biogenesis would be happening. Is it possible that the presence of the nanoparticles is affecting lysosomal health/composition that contribute to lysosome biogenesis as a way to compensate? Probably a further dive into what is happening to the lysosomal composition/activity in response to the nanoparticles is needed.

- We thank the reviewer for this important comment. First of all, statistical analysis of the endo/lysosomal markers indeed showed a significant increase of all the markers after the CTRL-NP treatment (Fig. R11 or Fig. 2g). Second, to clarify whether the nanoparticles could potentially affect the activity of lysosomal proteases, we performed the DQ Green BSA assay. DQ green BSA is a BSA labelled with a self-quenching fluorescent dye. Upon hydrolysis of DQ Green BSA into single, dye-labelled peptides by proteases, this quenching is relieved, producing brightly fluorescent products. As shown in Fig. R26 (Supplementary Fig. 7f and g), untreated B16F10 cells and cells treated with NPs, αFc-NPs, or CTRL-NPs exhibited bright green fluorescence, while cells treated with Bafilomycin A1, an established lysosomal inhibitor exhibited no fluorescence.

  In addition, we also performed the acridine orange (AO) assay to examine the permeability/integrity of lysosomal membranes. AO emits red fluorescence when protonated in intact lysosomes and green fluorescence when deprotonated in cytoplasm. We observed only red fluorescence in untreated cells and cells treated with NPs, αFc-NPs, or CTRL-NPs, but strong green fluorescence in cells treated with chlorquine (CQ), a lysosome-permeability enhancer (Fig. R27 or Supplementary Fig. 7h and i). Collectively, these results demonstrate that MONOTAB may promote lysosome biogenesis without affecting lysosomal health.

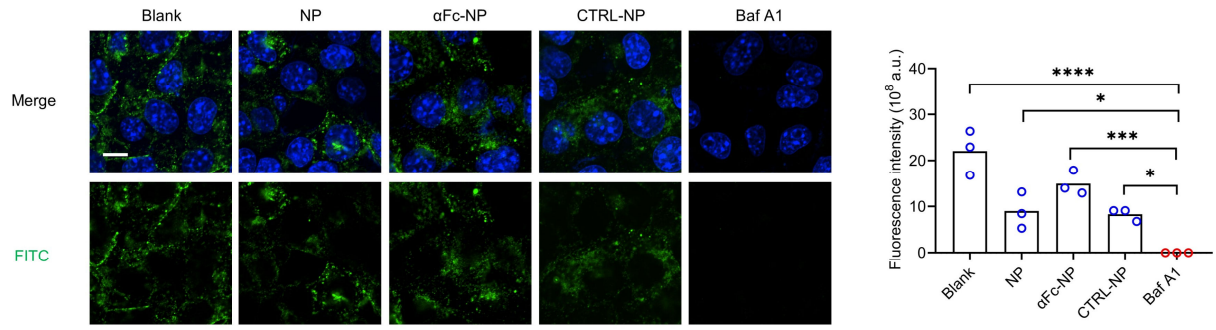

Fig. R26. Left, Live-cell images of B16F10 cells treated with NP, αFc-NP, or CTRL-NP for 10 hours or Bafilomycin A1 (Baf A1) for 1 hour, then loaded with DQ Green BSA for 10 hours. Right, Quantification of the green fluorescence.

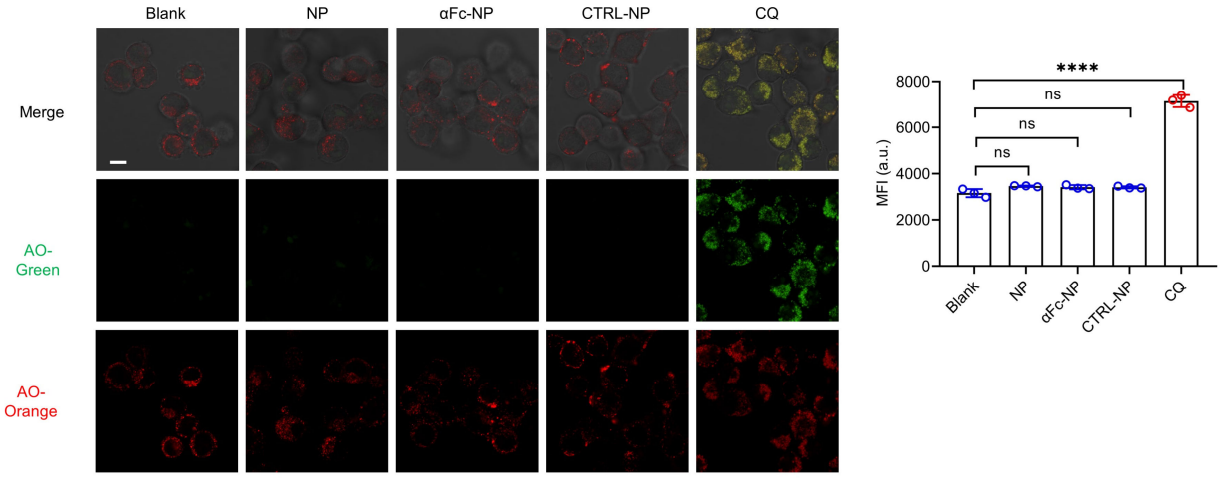

Fig. R27. Live-cell images (Left) and flow cytometry analysis (Right) of B16F10 cells treated with NP, αFc-NP, or CTRL-NP for 10 hours or 10 μM of chloroquine (CQ) plus 50 μM of $ZnCl_2$ for 30 minutes, then stained with AO for 15 minutes.

Comment #5. In Figure 4, how do the authors explain the difference internalization vs. degradation kinetics? They see internalization of their MONOTABs within a few hours, but to see PD-L1 degradation, they only see significant degradation starting at 24h. Are some of the nanoparticles internalizing before they get a chance to bind to PD-L1?

- We thank the reviewer for this comment. The internalized cargoes are first delivered to early endosomes, which can further mature to late endosomes and finally fuse with lysosomes. The entire process may take varying times, typically several hours. In addition, protein degradation in lysosomes also takes time, depending on factors such as protein turnover rates, protein folding and stability, lysosomal enzyme activity, etc.. These two aspects may both account for the different kinetics between internalization and degradation.

Yes, we do think some of the nanoparticles would be internalized before they can bind to PD-L1 because the internalization does not need the interactions between nanoparticles and PD-L1. This proportion may vary with the PD-L1 expression level as well as the nanoparticle concentration.

Comment #6. The EV degradation is not very convincing. First, GFP is unstable in low pH, so using it as a readout for degradation is not very accurate, as it would lose fluorescence in endosomes as well. In addition, some EVs can exocytose upon internalization – can the authors show that most of the EVs do not exocytose?

- We thank the reviewer for this critical comment. Actually, the GFP variant we used is a pH-stable one. We also validated its pH stability and found that ^{EGFP}EV could emit stable fluorescence at pH as low as 5.0 after incubation for even 48 hours (Fig. R28 or Supplementary Fig. 10b).

  To determine whether the internalized EVs could be exocytosed again, we carried out another experiment. B16F10 cells treated with ^{EGFP}EVs, Annexin-V + ^{EGFP}EVs, NPs + ^{EGFP}EVs, or Annexin-V-NPs + ^{EGFP}EVs were further cultured in fresh serum-free medium. The fluorescence intensity of EGFP in the medium was measured after 10-hour incubation. As shown in Fig. R29 (Fig. 5e in the Revision), no significant difference was observed across all groups compared to the ^{EGFP}EV-free group. Therefore, the internalized EVs may not undergo exocytosis.

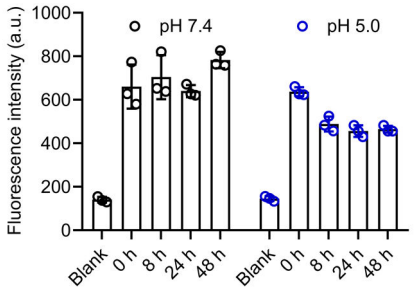

Fig. R28. Fluorescence intensity change of ^{EGFP}EVs incubated at pH 7.4 or pH 5.0 for different durations. Blank refers to the background intensity in the buffer.

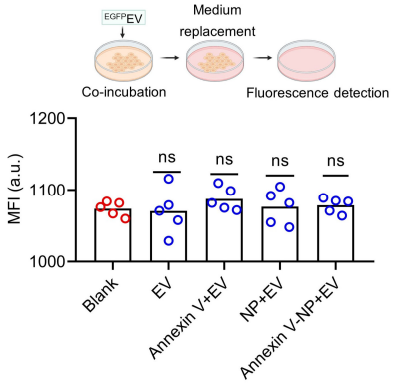

Fig. R29. Fluorescence intensity measurement of exocytosed ^{EGFP}EVs in the medium.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have addressed a number of the comments that I made and have conducted additional experiments and included more detailed data analysis that addresses a number of my original concerns. However a number of my comments were not addressed fully and I think the paper is suitable for publication in Nature Communications only after these comments are fully addressed.

The section on the use of chemical inhibitors needs further discussion and the conclusions need to be qualified. It is quite well established in the literature that the inhibitors the authors have used are not specific for the pathways that they claim (i.e. the inhibit multiple pathways). This needs to be stated and discussed. Additionally, there is little evidence for caveolin mediated endocytosis being a major uptake pathway for nanoparticles so references to this should be revised. Filipin inhibits this CLIC pathway as well as having off target inhibiton of a number of other pathways. Chlorpromazine inhibits the FEME, Cav and CLIC pathways, so it isn't surprising that the inhibition behaviour differs from pitstop.

Trypan Blue can be used to quench FITC fluoresce, but it is highly dependant on the concentration of Trypan Blue used. The authors should include a control (4ºC treatment) that demonstrates that the Trypan Blue is efficiently quenching the fluorescence of the FITC when it is conjugated to the nanoparticles. Without this data, the flow cytometry data should be referred to as association and not uptake/internalisation.

FITC is an unusual choice as a fluorophore as it has pH dependant fluorescence, so if material is trafficked to acidic compartments the fluoresence will change. This should be noted.

I disagree with the authors interpretation of their DLS data. DLS is measuring brownian motion, therefore if two 100nm particles aggregate, the size measured will not be 200nm but ~125nm. TEM analysis is not a good measure of particle aggregation. The image shown for aFC-NP is likely showing two aggregated nanoparticles.

Reviewer #2:
Remarks to the Author:
The manuscript was revised and improved well. All the responses and changes are OK.

Reviewer #3:
Remarks to the Author:
Overall, I think the authors addressed most of my concerns and performed a lot of additional experiments to make the manuscript stronger. I have a few minor points:

For SI Fig 8 on comparing anti-PD-L1 GalNAc, is there an evidence that the conjugation of GalNAc was successful? I see that the authors included a native blot, but normally, after GalNAc conjugation, an upward shift is observed instead of the downward shift. Perhaps proteomics data or other methods to confirm the successful conjugation? It's just a bit strange that the hook effect was observed for anti-PD-L1 GalNAc at such a low concentration (nM) when in the original paper, there was no such effect even at 1 uM.

I'm still not fully convinced about the lysosomal biogenesis, although the authors have conducted the DQ Green BSA assay and AO assay. These assays did indeed show that lysosomes remained functional/membranes are intact. But there are many other factors that could affect lysosomal number as shown by Lysotracker. I think the subtitlte statement of "MONOTAB promotes lysosomal biogenesis" is a bit too strong. The authors need to demonstrate that genes that are normally upregulated for lysosomal biogenesis are upregulated to make such a statement. Alternatively, the authors could change the text to not overclaim and present it as an implication/suggestion. Mechanistic explanation of how this may be happening might be also helpful.

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Response to Reviewers' Comments

Reviewer #1

Comment #1. The section on the use of chemical inhibitors needs further discussion and the conclusions need to be qualified. It is quite well established in the literature that the inhibitors the authors have used are not specific for the pathways that they claim (i.e. the inhibit multiple pathways). This needs to be stated and discussed. Additionally, there is little evidence for caveolin mediated endocytosis being a major uptake pathway for nanoparticles so references to this should be revised. Filipin inhibits this CLIC pathway as well as having off target inhibiton of a number of other pathways. Chlorpromazine inhibits the FEME, Cav and CLIC pathways, so it isn't surprising that the inhibition behaviour differs from pitstop.

- In response to the reviewer's comment, we revised the discussion on how these inhibitors worked (Line 125-140) and updated the references accordingly (Ref #24 – 31).

  "To identify the critical pathways involved in NP internalization, we next performed a set of endocytosis inhibition experiments. Cells were pre-treated with chemical endocytosis inhibitors or exposed to low temperature (4 °C), followed by incubation with ^{RB}NPs for 1.5 hours. Flow cytometry analysis showed that low-temperature treatment significantly inhibited ^{RB}NP uptake in all cell lines, pointing to an energy-dependent uptake process. Cholesterol sequestration (filipin) or inhibition of clathrin-coated pit (CCP) dynamics (Pitstop 2) did not influence NP uptake, while the tyrosine kinase inhibitor (genistein), actin polymerization inhibitor (cytochalasin D), and PI3K signaling inhibitor (wortmannin) showed mild inhibitory effects in a cell line-specific manner. Notably, chlorpromazine significantly reduced NP uptake in all cell lines. Since chlorpromazine inhibits clathrin-mediated endocytosis but not specifically, we used siRNA (small interfering RNA) to knock down clathrin to confirm the involvement of clathrin in the internalization process. This knockdown resulted in a significant decrease in NP uptake by ~60%. These results suggest a major role of clathrin-mediated endocytosis in NP internalization."

Comment #2. Trypan Blue can be used to quench FITC fluoresce, but it is highly dependant on the concentration of Trypan Blue used. The authors should include a control (4ºC treatment) that demonstrates that the Trypan Blue is efficiently quenching the fluorescence of the FITC when it is conjugated to the nanoparticles. Without this data, the flow cytometry data should be referred to as association and not uptake/internalisation.

- We thank the reviewer for this important comment. The concentration of Trypan Blue (TB) we used was 0.01% (v/v). This concentration or even a lower one (0.005%) has been used to quench the surface fluorescence in other reports (***Adv Funct Mater*** 2019, 29, 1903686; ***Int J Nanomedicine*** 2016, 11, 3049-3063).

To validate if 0.01% TB could indeed quench FITC fluorescence effectively, we performed two additional experiments. In the first experiment, we measured the fluorescence of ^{FITC}NP (50 μg mL^{-1}) in the presence or absence of 0.01% TB. As shown in Fig. R1 (Supplementary Fig. 1b), the FITC fluorescence was substantially quenched (~94.8%) by TB. Per the reviewer's suggestion, we also performed the TB quenching assays at 37 ^{o}C and 4 ^{o}C, respectively. TB significantly quenched the FITC fluorescence when the cells were incubated at 4 ^{o}C, while no fluorescence quenching effect was observed with the cells incubated at 37 ^{o}C (Fig. R2 or Supplementary Fig. 1c). These results demonstrate that 0.01% TB is effective enough to quench the surface FITC fluorescence of cells.

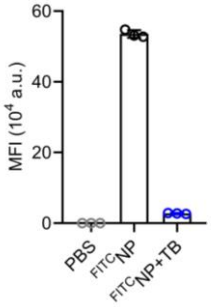

Fig. R1. Fluorescence intensity of ^{FITC}NP (50 μg mL^{-1}) in the presence or absence of 0.01% TB. Ex: 494 nm; Em: 525 nm.

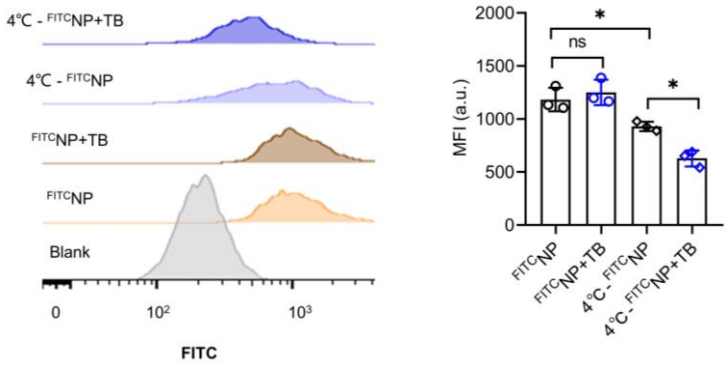

Fig. R2. Flow cytometry analysis of cellular association and uptake of ^{FITC}NP by B16F10 cells *via* Trypan Blue quenching. The fluorescence of surface-bound ^{FITC}NPs would be quenched by Trypan Blue.

Comment #3. FITC is an unusual choice as a fluorophore as it has pH dependant fluorescence, so if material is trafficked to acidic compartments the fluoresence will change. This should be noted.

- We agree with the reviewer's comment. The fluorescence of FITC decreases at acidic pH, but the intensity was still strong enough for flow cytometry measurement. We did not use ^{RB}NPs in the TB quenching assay because the complex formed by TB and proteins emits red fluorescence (***Cell Physiol Biochem*** 2021, 55, 171-184), which would interfere with the signal of RB (Fig. R3).

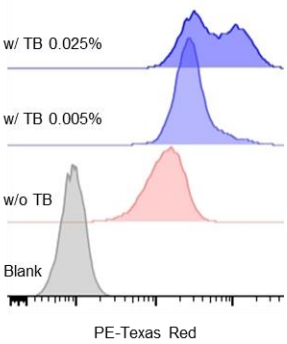

Fig. R3. Flow cytometry analysis of cellular association and uptake of ^{RB}NP by B16F10 cells *via* Trypan Blue quenching. Enhanced fluorescence signals were detected upon TB treatments.

Comment #4. I disagree with the authors interpretation of their DLS data. DLS is measuring brownian motion, therefore if two 100nm particles aggregate, the size measured will not be 200nm but ~125nm. TEM analysis is not a good measure of particle aggregation. The image shown for aFC-NP is likely showing two aggregated nanoparticles.

- In response to the reviewer's comment, we re-performed TEM of NPs and αFc-NPs at the same concentration as used for DLS (50 μg mL^{-1}). The new TEM images showed well-dispersed NPs and αFc-NPs (Fig. R4 or Supplementary Fig. 5c). The particle sizes of NPs and αFc-NPs determined from the TEM images were consistent with the DLS results. Particle size analysis of Cyro-TEM images also showed similar size distributions (Fig. R5 or Supplementary Fig. 6d).

  To further address the reviewer's concern, we have revised the interpretation of the DLS data as follows: "*the average hydrodynamic diameter of CTRL-NPs was ~30 nm larger than that of αFc-NP, suggesting the successful immobilization of the IgG control onto αFc-NPs*" (Line 206-207).

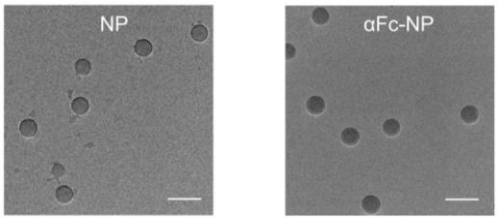

Fig. R4. TEM images of NPs and αFc-NPs. Scale bar, 200 nm.

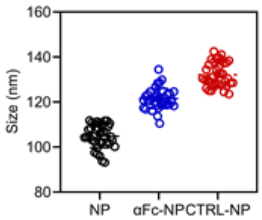

Fig. R5. Particle size distribution analysis of Cryo-TEM images with ImageJ.

Reviewer #2

Comment #1. The manuscript was revised and improved well. All the responses and changes are OK.

- We thank the reviewer for the positive comment.

Reviewer #3

Comment #1. For SI Fig 8 on comparing anti-PD-L1 GalNAc, is there an evidence that the conjugation of GalNAc was successful? I see that the authors included a native blot, but normally, after GalNAc conjugation, an upward shift is observed instead of the downward shift. Perhaps proteomics data or other methods to confirm the successful conjugation? It's just a bit strange that the hook effect was observed for anti-PD-L1 GalNAc at such a low concentration (nM) when in the original paper, there was no such effect even at 1 uM.

- The resolution of proteins in a native gel depends not only on their molecular weight but also on their surface charge. In the original paper (***Nat Chem Biol*** 2021, 17, 937-946), the antibodies also showed a downward shift after GalNAc conjugation (Fig. R6), which was consistent with our result.

  Per the reviewer's suggestion, we used MALDI-TOF MS to further validate the conjugation. A mass shift of 25327.90 between αPD-L1 and αPD-L1-GalNAc was observed (Fig. R7 or Supplementary Information Page 39). As the expected mass shift per a single conjugation of NHS-$(PEG)_4$-azide and Tri-GalNAc-DBCO is 2006.31, the calculated number of GalNAc ligands per antibody was 12.6.

  Regarding the hook effect issue, the original study (***Nat Chem Biol*** 2021, 17, 937-946) did not involve the degradation of PD-L1 or the use of Hepa1-6 cells, so we think this comparison lacks relevance without considering the specific target protein or cell background.

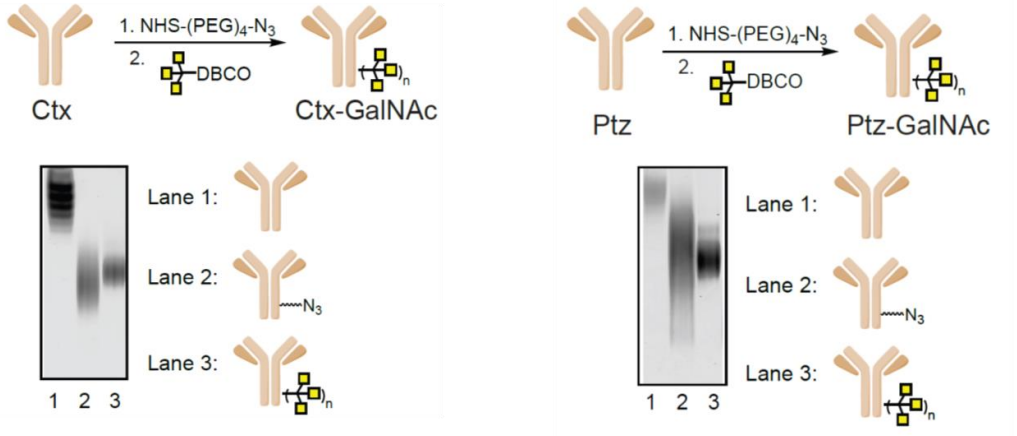

Fig. R6. Native gel electrophoresis showing the synthesis of Ctx-GalNAc (left) and Ptz-GalNAc (right) in the original paper (***Nat Chem Biol*** 2021, 17, 937-946).

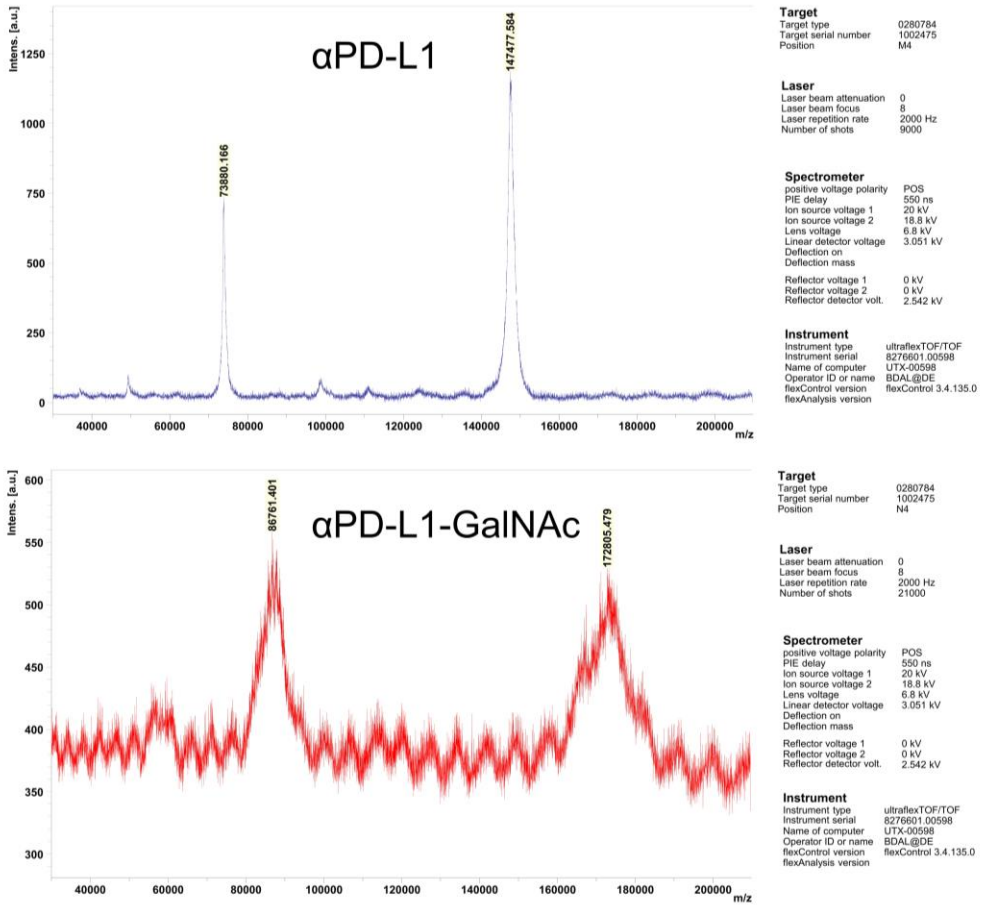

Fig. R7. MALDI-TOF MS spectra of αPD-L1 and αPD-L1-GalNAc.

Comment #2. I'm still not fully convinced about the lysosomal biogenesis, although the authors have conducted the DQ Green BSA assay and AO assay. These assays did indeed show that lysosomes remained functional/membranes are intact. But there are many other factors that could affect lysosomal number as shown by Lysotracker. I think the subtitlte statement of "MONOTAB promotes lysosomal biogenesis" is a bit too strong. The authors need to demonstrate that genes that are normally upregulated for lysosomal biogenesis are upregulated to make such a statement. Alternatively, the authors could change the text to not overclaim and present it as an implication/suggestion. Mechanistic explanation of how this may be happening might be also helpful.

- Per the reviewer's suggestion, the subtitle and context were changed in the Revision (Line 248-276). A mechanistic explanation for the potential lysosomal biogenesis was also included in the Revision.

  "Effects of MONOTAB on lysosomal function

  After co-incubation with ^{Cy5}CTRL-NPs, we observed an unexpected increase in the LysoTracker signal. This observation raises the possibility that MONOTAB might promote lysosomal biogenesis. To test this hypothesis, we examined the expression levels of endo-lysosome markers, including LAMP1 (lysosome), EEA1 (early

endosome), and RAB7 (late endosome), and found that all the tested markers were upregulated after the CTRL-NP treatment. Immunofluorescence assay further confirmed the increase of LAMP1. These results are consistent with a previous report indicating that internalization of anionic polystyrene nanoparticles results in activation of the transcription factor EB, a master regulator of lysosome biogenesis, and increased lysosomal degradation capacity.

As an increase in LysoTracker staining or lysosome markers may be also observed upon lysosomal dysfunction, one may question if the nanoparticles could potentially impair lysosomal health, thereby activating lysosome biogenesis as a compensatory response. To clarify this question, we performed the DQ Green BSA assay to evaluate lysosomal degradation capacity. Untreated B16F10 cells and cells treated with NPs, αFc-NPs, or CTRL-NPs exhibited bright green fluorescence, indicating the effective hydrolysis of the DQ Green BSA into single, dye-labelled peptides by lysosomal proteases. In contrast, no fluorescent signal was observed in cells treated with Bafilomycin A1 (BafA1), an established lysosomal inhibitor. We also examined lysosome membrane stability with acridine orange (AO), a fluorescent dye that emits red fluorescence when protonated in intact lysosomes and green fluorescence when deprotonated in cytoplasm. Strong green fluorescence was detected in cells treated with chlorquine (CQ, a lysosome-permeability enhancer), while untreated cells and cells treated with NPs, αFc-NPs, or CTRL-NPs exhibited red fluorescence only. These results imply that MONOTAB may promote lysosomal biogenesis without affecting lysosomal health, which promises higher protein degradation potential."

Reviewers' Comments:

Reviewer #3:
Remarks to the Author:
The manuscript has been revised sufficiently.