nature portfolio

Peer Review File

Polymeric Nanocarrier via Metabolism Regulation Mediates Immunogenic Cell Death with Spatiotemporal Orchestration for Effective Cancer Immunotherapy

REVIEWER COMMENTS

Reviewer #1 (Cancer immunotherapy, melanoma, agonist):

Specifics:

Figure 3:

A – very small

D – labels small and hard to read

All figures very busy and hard to read. I would move some of the primary data to supplementals and focus on those of relevance. The work is comprehensive but the extent of details make the images lack a message or focus.

Figure 2 screens compounds for ICD, but then 2f and g focus on dose response and timing of pRNCThiorther+DEA only. This is a confusing transition and figure labels are unclear.

What is reason for difference between CRT and HMGB1 between the last 3 conditions? Patterns not the same and little discussion or clarification? Figure panel C (right summary figure) should be more promimantly displayed. There is a discussion in the "results" section, but is still very technical and hard to interpret.

Figure 3 is dense and hard to read. In 3d, need a label which are PBS vs pRNA (they blur into one another). C and e have lots of data, but very little "take home" information. What is the point you are trying to convey. Just listing pathways and genes is not particularly helpful. Similar comment for f and g panels (network maps). I find the data presentation overly detailed with little clarity about what the data contributes to the larger story.

Fig 3h, i, j a little more easy to interpret, but again the panels are a bit fragmented in messaging.

Figure 4 data and technically dense, but hard to interpret. Would be nice to simplify data presentation and improve "message" delivery. Figure 4h, i are important panels, but they are lost in the way the data is currently presented. Same with j,k. Would simplify figures or present the data differently.

Figure 5 has an explanation in the top panel and is much easier to interpret.

Figure 6 ha a top panel, but a large amount of data packed into the figures, again making interpretation more challenging. A page of flow data could be presented in a better way or perhaps not all conditions need to be represented in main figures (could go to supplementals) to allow more streamlined messaging.

Also despite all the data, unclear if any peripheral "vaccine" work done. For example, using tetramers/dextramers to look at Ag-specific T cell responses and durability of these responses.

Overall, interesting concept. The manuscript is too technical and too much data presented with a lack of clear messaging. When thinking about clinical translation, one would need to know how this performs compared to existing vaccination approaches (mRNA, peptide) and how successful are the vaccine related immune responses (see comment re: tetramer staining from PBMC). It would be nice to see this approach compared to a more translational vaccination approach to see if it outperforms and/or elicits a similar or distinct immunologic response.

Reviewer #2 (Polymeric NPs, drug delivery, cancer therapy):

The authors reported a nano-strategy for melanoma immunotherapy by developing a B16F10 cell-targeted nanoparticle and a TAM-targeted nanoparticle, which both based on a same pH sensitive tri-block copolymer that encapsulated an immune adjuvant R848. After combination with anti-PD-1 and anti-CD47, the two nanoparticles were expected to target B16F110 for cancer vaccination by mediating ICD, and to target TAM for TME modulation by TAM polarization, respectively. The study is interesting. Below are some comments and suggestions.

1. Besides hydrodynamic size, other characterization of cRGD-mix Man-pRNCThioether+DEA@R848 should also be provided.

2. What is the stability of the nanocarriers? Would pRNCThioether+DEA and pRNCDEA degrade in the TME?

3. How was the mixing ratio of cRGD-mix Man-pRNCThioether+DEA@R848 determined?

4. The data of cRGD-mix Man-pRNCThioether+DEA@R848 are suggested to be supplemented in Figure 4.

5. Did the time and concentration dependence of ICD mediated by pRNCThioether+DEA reach statistical significance?

6. Did Man-pRNCThioether+DEA@DIR in G5 promote the cRGD-based tumor targeting, compared with G4, in Figure 5? What is the reason?

7. Why was there an obvious difference between the therapeutic efficacy after treatments of cRGD-mix Man-pRNCThioether+DEA@R848 in Figure 7 and 8? Was there any difference between the tumor sizes in the two Figures?

Reviewer #3 (ICD, cancer therapy):

In this manuscript, the authors present the development of a novel nanoplatform fabricated using a pH-sensitive tri-block copolymer synthesized through reversible addition-fragmentation chain transfer polymerization. The authors assert that these nanostructures can induce immunogenic cell death (ICD). The article offers some interesting technological advancements and explores attractive scientific hypotheses. However, in its current state, the manuscript is quite premature and can not be suitable for publication in Nature Communications.

Major Comments:

1. First, there are serious concerns regarding the measurement of DAMPs (Damage-Associated Molecular Patterns) (Fig. 2 and Fig. 4). The methods employed by the authors are not accurate and may yield false-positive data, which would hinder the correct characterization of ICD.

- CRT should be measured in combination with Sytox dye or PI to exclude data with internal staining, which does not represent the pool of surface-exposed CRT (lines 633-639 and Fig. 4). The data should be thoroughly re-done and re-analyzed.

- The authors mentioned that the measurement of ATP release was performed using ELISA. This is not accurate. ATP is typically measured using the luminescence assay, which does not involve the use of antibodies (lines 647-653). Please review and update accordingly.

- The release of HMGB1 should be measured in the supernatant using ELISA (lines 640-646). Therefore, the experiments must be redone, and the data should be re-analyzed.

- The number of experimental repeats should be indicated in Figure 2 and in other figures.

2. The authors have stated that “by pRNCThioether+DEA treatment compared with that of PBS, indicating that the nanocarrier also elicited ER stress.” This conclusion is drawn from the observed upregulation of CHOP. Importantly, one of the typical features of classical ICD inducers is the

induction of ER stress, characterized by eIF2α and PERK phosphorylation. Therefore, it is advisable for the authors to include these additional data as they are a key element of ICD.

3. The intracellular ROS level should be quantified and statistically compared to the control groups (Fig 3h, i, j).

4. The authors should provide clear data on the kinetics and dose-response of cell death induction by pRNCThioether+DEA. These data should be shown in the first figures of the manuscript.

5. Analysis of maturation/activation of DCs: It is unclear which groups correspond to G1-G4. A clear description of these groups should be provided in the legend for Figure 4. Additionally, it would be helpful to indicate the ratio of DCs to dead cells, along with the rate of cell death before adding them to the DCs. Important negative and positive controls are missing, such as co-culture with viable B16 cells, LPS-treated DCs, etc

6. The data presented in the Fig 4 should be quantified and statistically analyzed. The viability (□ cell death) of the cells should be provided for accumulation studies on cRGD-pRNCThioether+DEA@FITC; pRNCThioether+DEA@FITC; Man-pRNCThioether+DEA@FITC and pRNCThioether+DEA@FITC.

7. The data on the specific targeting of melanoma B16 and macrophages RAW264.7 by cRGD-pRNCThioether+DEA and Man-pRNCThioether+DEA, respectively, are not convincing. These data require a substantial justification with additional experimental data. As it is now it is not clear at all.

8. The authors claimed that there is an induction of pyroptosis in the target cells, but did not provide firm qualitative data. The type of regulated cell death (i.e., apoptosis, necroptosis, ferroptosis, pyroptosis) should be experimental identified, and confirmed and it is needed to demonstrate that this technology can target also other cancer cell types and thus it is not cell line specific.

9. The article should be rewritten to increase clarity and proofread.

Point-by-point response to reviewers' comments:
Note: Reviewers' comments are in black, and our response is highlighted in blue.

Reviewer #1 (Cancer immunotherapy, melanoma, agonist):
1. Specifics: Figure 3: A- very small; D-labels small and hard to read
Response: Thanks for the reviewer's comment. As suggested, the images and fonts in **Fig. 3**A&D have been adjusted into proper sizes.

2. All figures very busy and hard to read. I would move some of the primary data to supplementals and focus on those of relevance. The work is comprehensive but the extent of details make the images lack a message or focus.
Response: Thanks for the reviewer's comment. As suggested, we have re-adjusted Figures layouts and font sizes, and moved some data to supplementals for a better focus. Please see the revised version.

3. Figure 2 screens compounds for ICD, but then 2f and g focus on dose response and timing of pRNC$_{Thiorther+DEA}$ only. This is a confusing transition and figure labels are unclear. What is reason for difference between CRT and HMGB1 between the last 3 conditions? Patterns not the same and little discussion or clarification? Figure panel C (right summary figure) should be more promimantly displayed. There is a discussion in the "results" section, but is still very technical and hard to interpret.
Response: Thanks for the reviewer's questions.

For the first question, the reviewer is right that **Fig. 2** mainly screens ICD-inducible compounds. We investigated their ability to induce CRT exposure, HMGB1 and ATP release *via* flow cytometry, CLSM, ELISA and ATP Assay Kit (**Fig. 2**d-f, Supplementary Fig. 15). Then, we explored influences of nanocarrier concentration and incubation time on the best compound pRNC$_{Thioether+DEA}$ mediated ICD to select the optimal conditions for the following experiments. Thus, **Fig. 2**g-h results were added to the main text. To better and more clearly present the results, we re-constructed the layouts as shown in the following:

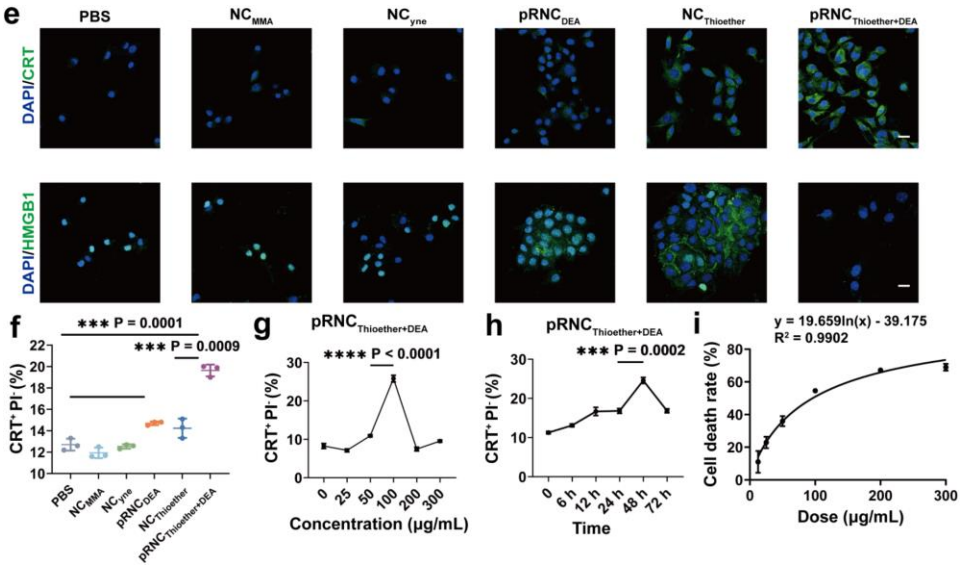
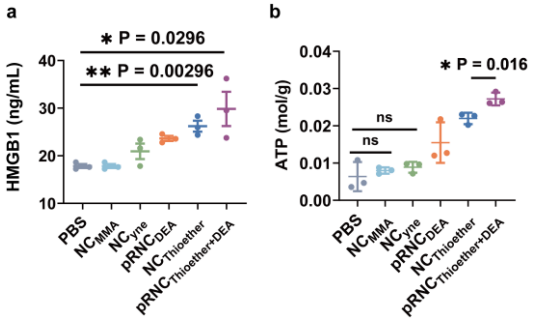


Fig. 2 e CRT exposure and HMGB1 release of B16F10 cells after different treatments characterized by

CLSM. **f** Semi-quantification analysis of CRT exposure after different treatments. **g** $pRNC_{Thiorther+DEA}$ with different concentrations induced CRT exposure in B16F10 cells. **h** CRT exposure of B16F10 cells was induced by $pRNC_{Thioether+DEA}$ after different incubation time. **i** The kinetics and dose-response of cell death induction by $pRNC_{Thioether+DEA}$.



Supplementary Fig. 15. ICD inducibility of $pRNC_{Thiother+DEA}$ *via* detecting HMGB1 and ATP release.

For the second and third questions, $pRNC_{Thioether+DEA}$ mediated the most CRT exposure and HMGB1 release compared with those of $NC_{Thiother}$ and $pRNC_{DEA}$, probably attributing to the dual ICD effects of both DEA and thioether groups (**Fig. 2**d-f, Supplementary Fig. 15). The intensive green fluorescence intensity was observed after $pRNC_{Thioether+DEA}$ treatment indicating its potent ability to induce CRT exposure (**Fig. 2**e). HMGB1 can be released from nuclear to cytoplasm even extracellular medium after ICD inducer treatment. Thus, the weakest HMGB1 fluorescence intensity (green) was observed after $pRNC_{Thioether+DEA}$ treatment (**Fig. 2**e) due to its ICD inducibility resulting in HMGB1 release to extracellular medium. To further investigate HMGB1 release, we then used the classical ELISA technique to detect HMGB1 levels in supernatant after different treatments as shown in the above Supplementary Fig. 15a.

As for the last comment, we think that the reviewer may want to mention Figure panel d (right summary figure) not c. To better present, it has been updated as **Fig. 2**f as shown in the above Figure results. The updated discussions have been highlighted in red color in the revised version and also can be seen in the following:

"FCM results in Fig. 2d, f indicated that NC_{MMA} and NC_{yne} couldn't induce CRT exposure both of which displayed similar CRT positive ratios with that of PBS. Slightly elevated ratios were detected in $pRNC_{DEA}$ (14.70 ± 0.17) and $NC_{Thioether}$ (14.23 ± 0.90) groups and the highest ratios (19.63 ± 0.55) was measured in $pRNC_{Thioether+DEA}$ group, indicating that both tertiary amine and thioether were able to induce ICD. Compared with $pRNC_{DEA}$ and $NC_{Thioether}$, $pRNC_{Thioether+DEA}$ induced the most CRT exposure which was 1.33-1.37 fold higher than those two groups, probably attributing to the dual ICD effects of both DEA and thioether groups (Fig. 2d, f, Supplementary Fig. 14)."

4. Figure 3 is dense and hard to read. In 3d, need a label which are PBS vs pRNA (they blur into one another). C and e have lots of data, but very little "take home" information. What is the point you are trying to convey. Just listing pathways and genes is not particularly helpful. Similar comment for f and g panels (network maps). I find the data presentation overly detailed with little clarity about what the data contributes to the larger story.

Response: Thanks for the reviewer's comments.

As suggested, **Fig. 3** has been adjusted for a better understanding where only very related

signaling pathways and genes were presented (See data in the following). The most important ones were highlighted. Labels to differentiate PBS and pRNC have been added in **Fig. 3**d.

As for **Fig. 3**c, e, f and g, some not well related data have been removed, and some very important genes and signaling pathways were highlighted. The related discussions were highlighted in the revised version when we added some key information to display more clearly what we want to convey.

"As shown in scRNA-seq analysis result, mitochondrial cytochrome c oxidase subunit 3 gene (mt-co3) and GSDMD displayed significant differences according to the volcano image for B16F10 cells with $pRNC_{Thioether+DEA}$ treatment (**Fig. 3**c). The obvious mt-co3 down regulation and perp (a mediator of p53 dependent apoptosis) up-regulation were detected demonstrating that $pRNC_{Thioether+DEA}$ could also induce tumor apoptosis (**Fig.** 3c, d). Otherwise, in **Fig.** 3c, we found that multiple genes related to chemokines (*e.g.*, chemokine (C-X-C motif) ligand 11 (CXCL11)) and GSDMD were upregulated after $pRNC_{Thioether+DEA}$ treatment, which would activate multiple downstream immune related signaling pathways as well as pyroptosis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that the dominant pathways enriched by $pRNC_{Thioether+DEA}$ were NOD-like receptor, p53, and TNF signaling pathway as well as oxidative phosphorylation, which would mediate a series of downstream immune and inflammatory cascades, apoptosis and metabolism regulation (**Fig.** 3e). According to KEGG regulatory network diagram results, the metabolic pathways, especially the genes related to oxidative phosphorylation, were significantly different (**Fig.** 3g). Multiple immune-related regulatory pathways including NOD-like receptor, chemokines and IL-17 signaling pathways were activated after $pRNC_{Thioether+DEA}$ treatment (**Fig.** 3f)."

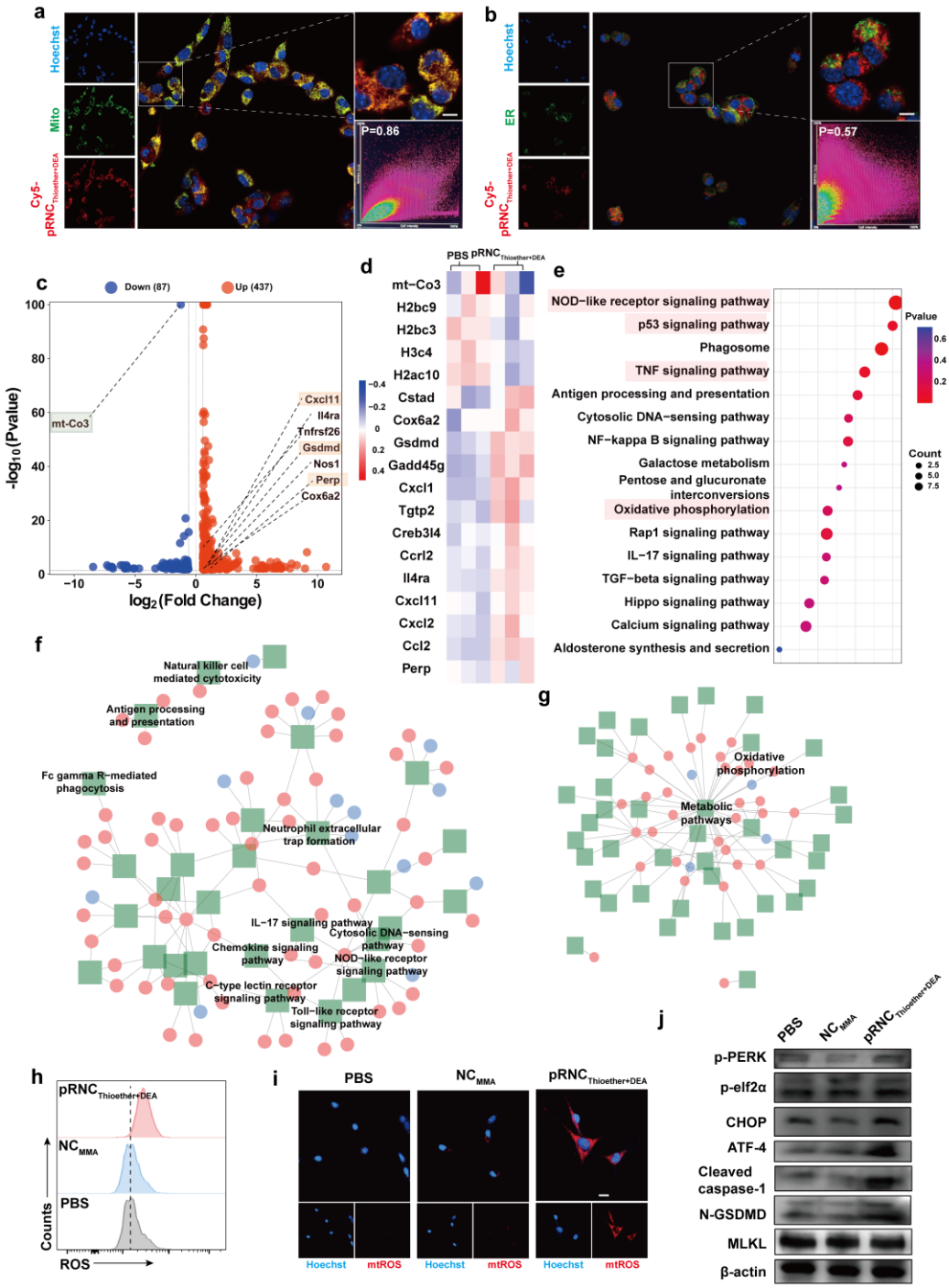


Fig. 3 Mechanism investigation of $pRNC_{Thioether+DEA}$ mediated B16F10 ICD.

5. Fig 3h, i, j a little more easy to interpret, but again the panels are a bit fragmented in messaging.

Response: Thanks for the reviewer's comments. As we mentioned in the main text, both metabolism regulation and redox homeostasis disruption in mitochondria can mediate tumor ICD. We firstly investigated the mechanism of $pRNC_{Thioether+DEA}$ mediated ICD *via* single-cell RNA sequencing analysis and found that mitochondria metabolism regulation *via* upregulating oxidative phosphorylation with ER-stress and GSDMD-mediated pyroptosis induced tumor ICD (**Fig. 3**c-g). Then, we explored the redox homeostasis disruption *via* detecting the

intracellular ROS and mitochondria ROS levels after different treatments (**Fig. 3**h and i), which would induce ER stress mediated ICD. **Fig. 3**j was then used to characterize the classical ER stress involved pathway recombinant DNA damage-inducing transcript 3 (CHOP) expression. For more comprehensive investigation of ER stress, phosphorylation of protein kinase R-like ER kinase (PERK) and eukaryotic translation initiation factor 2α (elf2α), as well as activating transcription factor 4 (ATF4) expression were also added in the revised version. Therefore, **Fig. 3**h-j in fact have close relationships which are also well related with **Fig. 3**c-g. The whole **Fig. 3** acts to investigate the mechanism of $pRNC_{Thioether+DEA}$ mediated ICD.

6. Figure 4 data and technically dense, but hard to interpret. Would be nice to simplify data presentation and improve "message"delivery. Figure 4h, i are important panels, but they are lost in the way the data is currently presented. Same with j,k. Would simplify figures or present the data differently.

Response: Thanks for the reviewer's suggestions. As suggested, some data have been moved into supplementary parts to simplify presentation only with the important ones kept in the main text. **Fig. 4** has been adjusted as shown in the following:

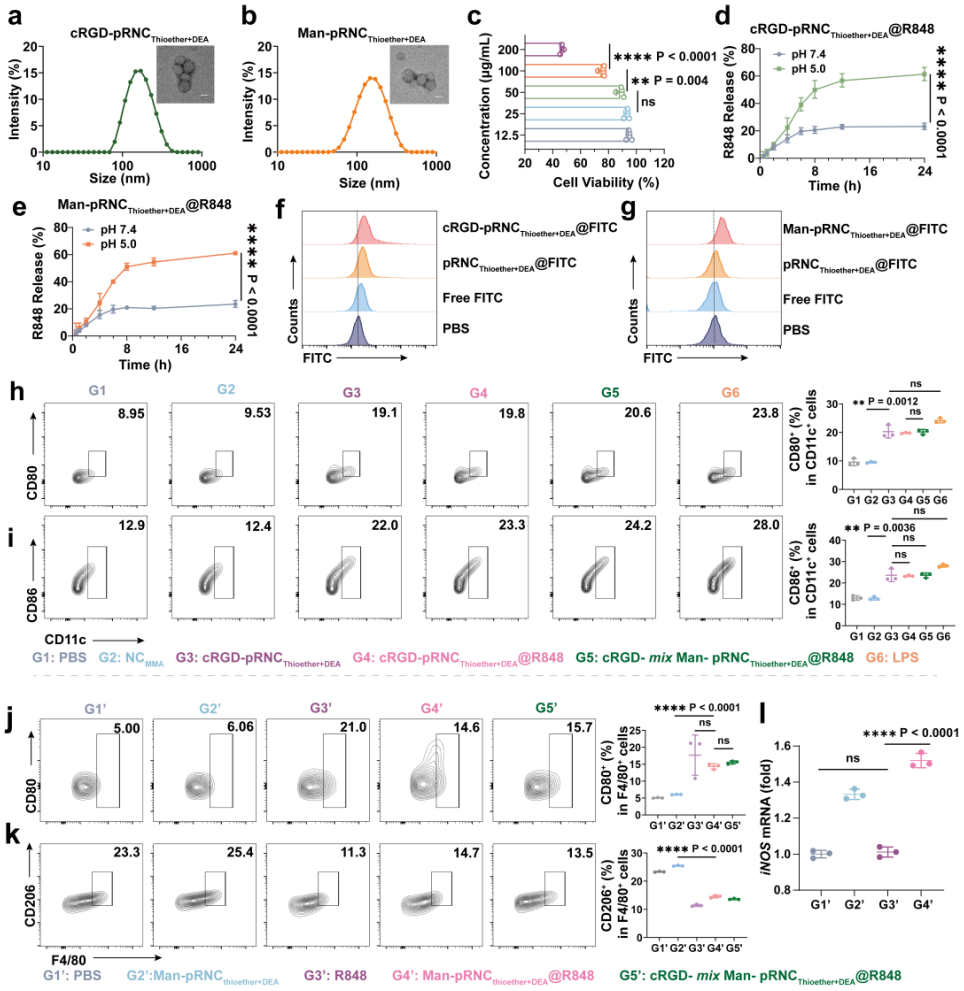


Fig. 4 Preparation and characterization of targeted nanoformulations.

7. Figure 5 has an explanation in the top panel and is much easier to interpret.
Response: We really appreciate the reviewer's positive comments.

8. Figure 6 has a top panel, but a large amount of data packed into the figures, again making interpretation more challenging. A page of flow data could be presented in a better way or perhaps not all conditions need to be represented in main figures (could go to supplementals) to allow more streamlined messaging.
Response: Thanks for the reviewer's suggestions. As suggested, we have adjusted **Fig. 6** in the revised version and moved some data to supplementary parts for a clearer presentation. Please see the updated **Fig. 6** in the following:

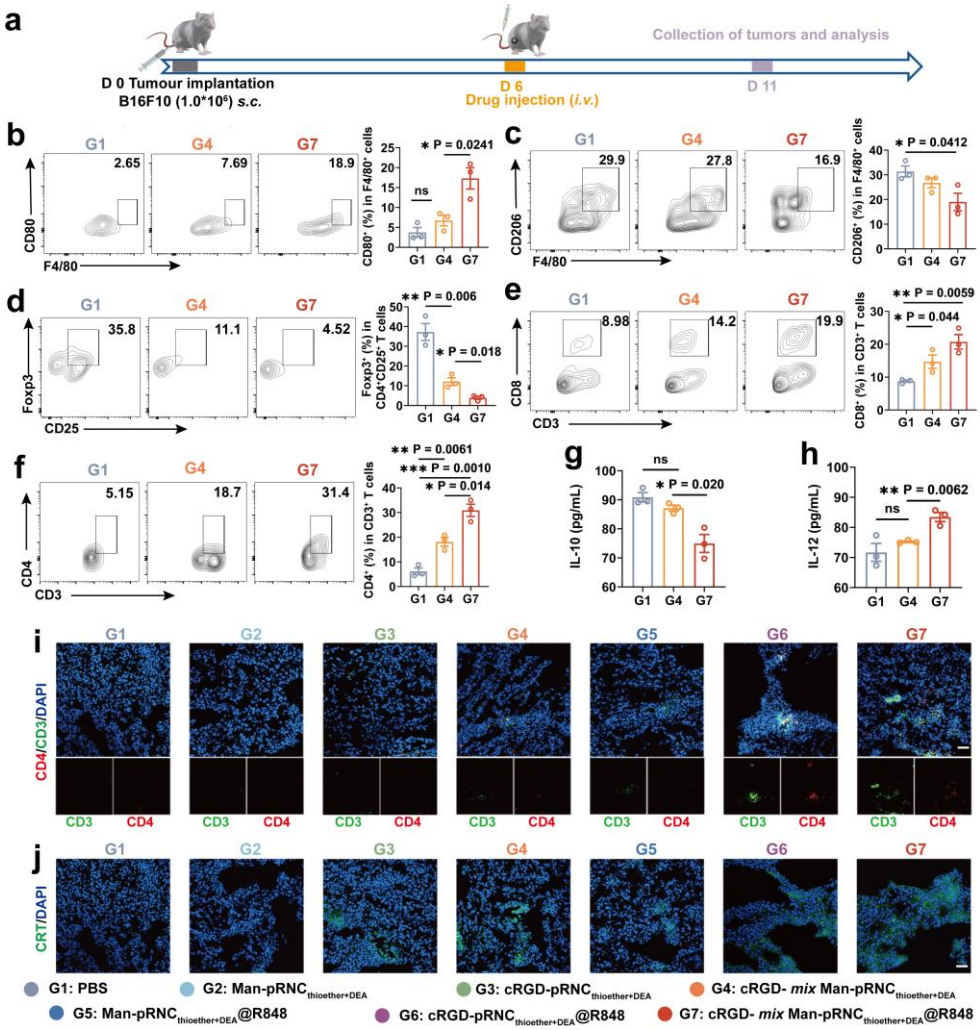
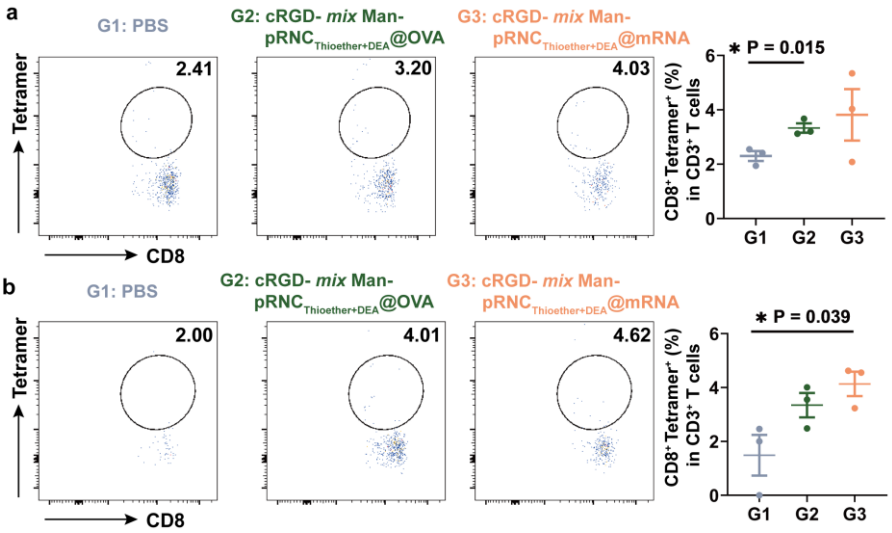


Fig. 6 *In vivo* antitumor immune response of cRGD- *mix* Man-pRNC$_{Thioether+DEA}$@R848 with ICD inducement, TAMs polarization, Tregs decrement and $CD8^+/CD4^+$ proliferation.

9. Also despite all the data, unclear if any peripheral "vaccine" work done. For example, using tetramers/dextramers to look at Ag specific T cell responses and durability of these responses.
Response: Thanks for the reviewer's constructive suggestions. To investigate the peripheral "vaccine" work, OVA$_{257-264}$ peptide and mRNA were separately encapsulated into cRGD- *mix* Man-pRNC$_{Thioether+DEA}$ to self-assemble into nanovaccines for Ag specific T cell and durability

responses exploration. According to our results, the OVA peptide and mRNA "vaccines" could induce Ag-specific T cell responses when notably elevated $CD8^+Tetramer^+$ T cell ratios were detected after $pRNC_{Thioether+DEA}$@OVA and $pRNC_{Thioether+DEA}$@mRNA treatments at day-7 compared with that of PBS. They also displayed durable responses when still high $CD8^+Tetramer^+$ T cell ratios were observed at day-14 post-treatments. Please see the following data:



Supplementary Fig. 46. Representative flow dot plots and statistics of $CD8^+Tetramer^+$ ratios in $CD3^+$ T cells in peripheral blood mononuclear cells of mice at day-7 a) and day-14 b) post immunization.

10. Overall, interesting concept. The manuscript is too technical and too much data presented with a lack of clear messaging. When thinking about clinical translation, one would need to know how this performs compared to existing vaccination approaches (mRNA, peptide) and how successful are the vaccine related immune responses (see comment re: tetramer staining from PBMC). It would be nice to see this approach compared to a more translational vaccination approach to see if it outperforms and/or elicits a similar or distinct immunologic response.

Response: Thanks for the reviewer's positive and comprehensive comments. Some data have been moved into supplementary parts for a clearer presentation and focus. As suggested, the vaccination approaches including mRNA and peptide have been added in the revised version the data of which can also be found in the above comment 9 part. According to our results, the nanomedicine we used revealed a similar immunological response with the vaccination approaches, which proved its potent translation potential.

Reviewer #2 (Polymeric NPs, drug delivery, cancer therapy):

The authors reported a nano-strategy for melanoma immunotherapy by developing a B16F10 cell-targeted nanoparticle and a TAM-targeted nanoparticle, which both based on a same pH sensitive tri-block copolymer that encapsulated an immune adjuvant R848. After combination with anti-PD-1 and anti-CD47, the two nanoparticles were expected to target B16F10 for cancer vaccination by mediating ICD, and to target TAM for TME modulation by TAM polarization, respectively. The study is interesting. Below are some comments and suggestions.

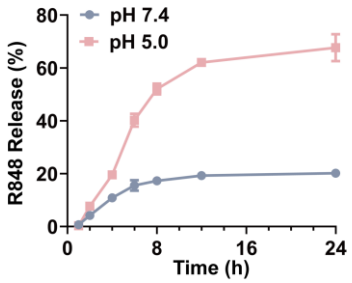
Response: We really appreciate the reviewer's positive comments.

1. Besides hydrodynamic size, other characterization of cRGD-mix Man-pRNC$_{Thioether+DEA}$@R848 should also be provided.

Response: Thanks for the reviewer's suggestions. DLC, DLE and *in vitro* drug release behavior of cRGD *mix* Man-pRNC$_{Thioether+DEA}$@R848 have been investigated and provided in Supplementary Table 3 and Supplementary Fig. 29. Please see the data in the following:

Supplementary Table 3. Drug loading content and efficiency characterization.

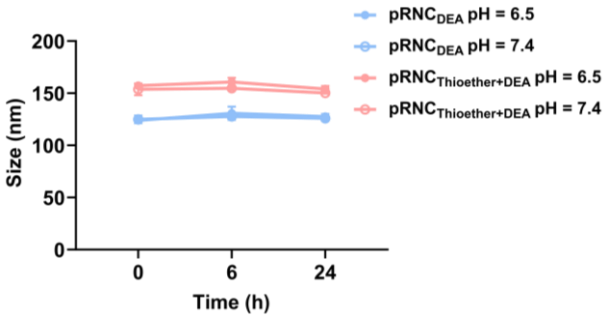
Polymersomes	Thero. DLC (wt.%)	Size [a] (nm)	PDI [a]	DLC [b] (wt.%)	DLE [b] (%)
cRGD-NP$_{Thioether+DEA}$ @R848	5%	168.0 ± 4.8	0.22 ± 0.02	4.78	95.6
	10%	236.4 ± 2.5	0.12 ± 0.10	8.15	81.3
	20%	246.5 ± 5.9	0.18 ± 0.02	11.64	58.2
Man-NP$_{Thioether+DEA}$ @R848	5%	143.3 ± 5.5	0.16 ± 0.01	4.84	96.8
	10%	143.5 ± 1.0	0.17 ± 0.01	8.32	83.2
	20%	171.4 ± 4.0	0.06 ± 0.02	12.27	61.3
cRGD- *mix* Man-pRNC$_{Thioether+DEA}$@R848	5%	169.9 ± 22.7	0.21 ± 0.06	4.63	92.6
	10%	183.3 ± 4.6	0.16 ± 0.04	7.99	79.9
	20%	176.2 ± 3.9	0.28 ± 0.03	11.49	57.4



Supplementary Fig. 29. *In vitro* R848 release from cRGD *mix* Man- pRNC$_{Thioether+DEA}$ within 24 h at different pH values (7.4 or 5.0).

2. What is the stability of the nanocarriers? Would pRNC$_{Thioether+DEA}$ and pRNC$_{DEA}$ degrade in the TME?

Response: Thanks for the reviewer's questions. According to results in Supplementary Fig. 13, the nanocarriers kept good stability for at least 24 h at both pH 7.4 and 6.5 with negligible size changes observed. Neither pRNC$_{Thioether+DEA}$ nor pRNC$_{DEA}$ degraded in the TME. Please see the following data:



Supplementary Fig. 13. Hydrodynamic size of pRNC$_{Thioether+DEA}$ and pRNC$_{DEA}$ in acetate buffer solution (pH 6.5, 10 mM, 150 mM NaCl).

3. How was the mixing ratio of cRGD- mix Man-pRNC$_{Thioether+DEA}$@R848 determined?

Response: Thanks for the reviewer's question. The mixing ratio was determined by cRGD-pRNC$_{Thioether+DEA}$ mediated B16F10 ICD and Man-pRNC$_{Thioether+DEA}$ mediated RAW264.7 cell viabilities. According to **Fig. 2**g and **Fig. 4**c, cRGD-pRNC$_{Thioether+DEA}$ induced the most CRT exposure in B16F10 cells at a concentration of 100 μg/mL, and Man-pRNC$_{Thioether+DEA}$ showed good safety in RAW264.7 cells at a concentration of 50 μg/mL. Thus, the two concentrations were used in the mixed nanoformulation where the molar ratio of cRGD- and Man-pRNC$_{Thioether+DEA}$@R848 was chosen as 2 to 1.

4. The data of cRGD- mix Man-pRNC$_{Thioether+DEA}$@R848 are suggested to be supplemented in Figure 4.

Response: Thanks for the reviewer's suggestion. The data of cRGD- *mix* Man-pRNC$_{Thioether+DEA}$ have been added in **Fig. 4** when the mixing of the two nanoformulations didn't affect their targetabilities, ICD inducibility cascade with DC maturation, and macrophoage polarization ability. Please see the results in the following:

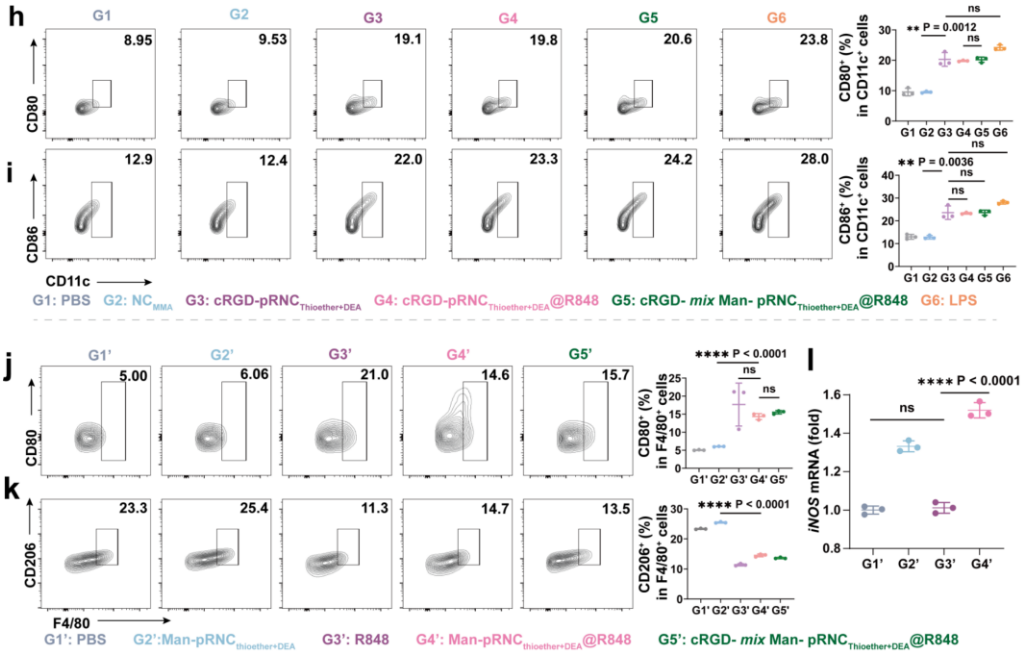


Fig. 4 h, i Representative flow cytometric images and quantification of mature DCs. **j, k** Representative flow cytometric images and quantification of polarization of macrophages. **l** Expression of iNOS in cells after

different treatments.

5. Did the time and concentration dependence of ICD mediated by $pRNC_{Thioether+DEA}$ reach statistical significance?

Response: Thanks for the reviewer's questions. To know whether the time and concentration dependence of ICD by $pRNC_{Thioether+DEA}$ have statistical significance, we then re-investigated them *via* detecting the CRT^+ ratios after different treatments (n = 3). According to our results (**Fig. 2**g, h), $pRNC_{Thioether+DEA}$ at a concentration of 100 μg/mL and incubation time of 48 h revealed the most CRT^+ ratios as 25.80 ± 0.85% and 24.57 ± 0.76%, 2.37-3.61 and 1.46-2.18 fold higher than those of other concentrations and time. Both time and concentration dependence revealed statistical significance in $pRNC_{Thioether+DEA}$ medaited ICD.

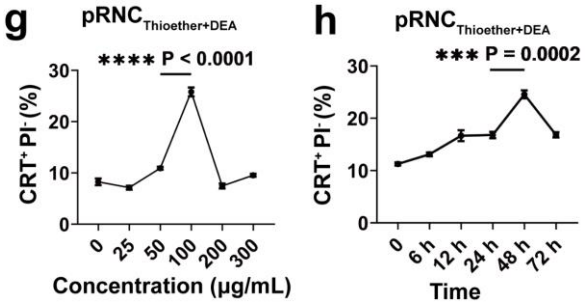


Fig. 2 g $pRNC_{Thiorther+DEA}$ with different concentrations induced CRT exposure in B16F10 cells. **h** CRT exposure of B16F10 cells was induced by $pRNC_{Thioether+DEA}$ after different incubation time.

6. Did Man-$pRNC_{Thioether+DEA}$@DIR in G5 promote the cRGD-based tumor targeting, compared with G4, in Figure 5? What is the reason?

Response: Thanks for the reviewer's question. According to the semi-quantitative analysis in **Fig. 5**c, the tumor accumulation in G5 and G4 had negligible differences over time, therefore Man-$pRNC_{Thioether+DEA}$@DIR did not promote cRGD-based tumor targeting.

7. Why was there an obvious difference between the therapeutic efficacy after treatments of cRGD- mix Man-$pRNC_{Thioether+DEA}$@R848 in Figure 7 and 8? Was there any difference between the tumor sizes in the two Figures?

Response: Thanks for the reviewer's questions. In fact, there were differences in the inoculation density and the initial treatment day between **Fig. 7** and **8**. In **Fig. 7**, B16F10 cells were inoculated at a density of 1.0×10^6 cells per mouse and then the initial treatment was executed at day-6 post-inoculation (initial tumor size: ~50 mm^3) when cRGD- *mix* Man-$pRNC_{Thioether+DEA}$@R848 treated mice showed a robust antitumor activity. We then investigated whether cRGD- *mix* Man-$pRNC_{Thioether+DEA}$@R848 had good antitumor efficacy in a relatively large initial tumor size (~120 mm^3) and also wondered how about CD47 and PD-1 antibodies combination therapy efficacy. Therefore, B16F10 cells were inoculated at a density of 1.5×10^6 cells per mouse and the initial treatment was executed at day-8 post-inoculation in **Fig. 8**. According to the results in **Fig. 8**, cRGD- *mix* Man-$pRNC_{Thioether+DEA}$@R848 couldn't inhibit tumor growth with a relatively large tumor volume at the therapeutic ending point compared with that of **Fig. 7**. However, after combination with CD47 and PD-1 antibodies, the tumor volume growth in **Fig. 8** was notably inhibited.

Reviewer #3 (ICD, cancer therapy):
In this manuscript, the authors present the development of a novel nanoplatform fabricated using a pH-sensitive tri-block copolymer synthesized through reversible addition-fragmentation chain transfer polymerization. The authors assert that these nanostructures can induce immunogenic cell death (ICD). The article offers some interesting technological advancements and explores attractive scientific hypotheses. However, in its current state, the manuscript is quite premature and can not be suitable for publication in Nature Communications.
Response: We sincerely thank the reviewer for the valuable feedback that helped us to improve the quality of our manuscript.

Major Comments:
1. First, there are serious concerns regarding the measurement of DAMPs (Damage-Associated Molecular Patterns) (Fig. 2 and Fig. 4). The methods employed by the authors are not accurate and may yield false-positive data, which would hinder the correct characterization of ICD.
Response: Thanks for the reviewer's suggestion. We have re-done the experiments and re-analyzed the data related to DAMPs in **Fig. 2** and **Fig. 4** as suggested by the reviewer.

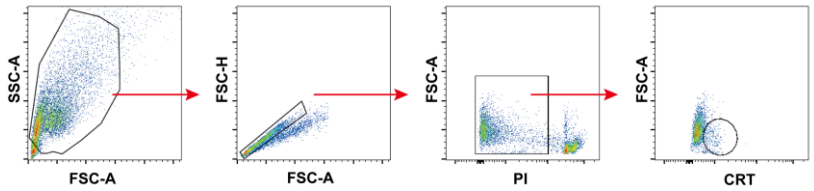
- CRT should be measured in combination with Sytox dye or PI to exclude data with internal staining, which does not represent the pool of surface-exposed CRT (lines 633-639 and Fig. 4). The data should be thoroughly re-done and re-analyzed.
Response: Thanks for the reviewer's constructive suggestion. We have re-done the experiments of CRT exposure with co-staining of PI and CRT antibodies and re-analyzed the results (**Fig. 2**d, f, g, h and Supplementary Fig. 14, 34). The updated discussions have been added to the revised version and were highlighted in red color. Please see the following:

"FCM results in Fig. 2d, f indicated that NC_{MMA} and NC_{yne} couldn't induce CRT exposure both of which displayed similar CRT positive ratios with that of PBS. Slightly elevated ratios were detected in $pRNC_{DEA}$ (14.70 ± 0.17%) and $NC_{Thioether}$ (14.23 ± 0.90%) groups and the highest ratios (19.63 ± 0.55%) was measured in $pRNC_{Thioether+DEA}$ group, indicating that both tertiary amine and thioether were able to induce ICD. Compared with $pRNC_{DEA}$ and $NC_{Thioether}$, $pRNC_{Thioether+DEA}$ induced the most CRT exposure which was 1.33-1.37 fold higher than those of two groups, probably attributing to the dual ICD effects of both DEA and thioether groups (Fig. 2d, f, Supplementary Fig. 14)."

"We found that CRT exposure increased with $pRNC_{Thioether+DEA}$ concentration increment at the same time point, when the most CRT exposure was detected at 100 μg/mL, 2.37-3.61 fold higher than that of others (Fig. 2g). Thus, $pRNC_{Thioether+DEA}$ at 100 μg/mL was chosen as the ICD inducer for the following experiments. As shown in Fig. 2h, the CRT exposure increased with incubation time prolonging and the maximum exposure was observed at 48 h, 1.46-2.18 fold higher than that of other time points. The aforementioned results indicated that $pRNC_{Thioether+DEA}$ mediated ICD was dependent on both concentration and incubation time."

"Obvious Aleax 488-anti-CRT fluorescence shift was observed for cells treated with cRGD-$pRNC_{Thioether+DEA}$ or cRGD-$pRNC_{Thioether+DEA}$@R848 than that of NC_{MMA} and PBS. Negligible differences were observed among cRGD-$pRNC_{Thioether+DEA}$, cRGD-

pRNC$_{Thioether+DE}$@R848 and cRGD- *mix* Man-pRNC$_{Thioether+DEA}$@R848 treated groups, indicating cRGD-pRNC$_{Thioether+DEA}$ induced ICD while R848 not, as well as the mixing of cRGD- and Man-nanoformulations didn't affect the ICD inducibility of cRGD-pRNC$_{Thioether+DEA}$ (Supplementary Fig. 34). When B16F10 cells supernatant was added into DCs, it was found that both cRGD-pRNC$_{Thioether+DEA}$ ($CD11c^+CD80^+$: 20.30 ± 2.25%; $CD11c^+CD86^+$: 23.60 ± 2.95%) and cRGD-pRNC$_{Thioether+DEA}$@R848 ($CD11c^+CD80^+$: 19.80 ± 0.20%; $CD11c^+CD86^+$: 23.30 ± 0.36%) treated cancer cells displayed a notable DC maruration than that of NC$_{MMA}$ ($CD11c^+CD80^+$: 9.53 ± 0.27%; $CD11c^+CD86^+$: 12.80 ± 0.78%) and PBS ($CD11c^+CD80^+$: 9.56 ± 1.24%; $CD11c^+CD86^+$: 13.00 ± 1.05%), similar with the positive control LPS ($CD11c^+CD80^+$: 24.20 ± 0.87%; $CD11c^+CD86^+$: 28.07 ± 0.70%) (Fig. 4h, i, Supplementary Fig. 35). It suggests that tumor ICD mediated by cRGD-pRNC$_{Thioether+DEA}$ with DAMP secretion can facilitate DCs maturation. In addition, cRGD- *mix* Man-pRNC$_{Thioether+DEA}$ treated group had similar ratios of $CD11c^+CD80^+$ (20.20 ± 0.87%) and $CD11c^+CD86^+$ (23.60 ± 1.13%) with cRGD-pRNC$_{Thioether+DEA}$, demonstrating that the mixing of cRGD and Man nanoformulations didn't affect cRGD-pRNC$^{Thioether+DEA}$ mediated ICD cascade with DC maturation."



Supplementary Fig. 14. Scatter plots of the gating strategies for CRT^+PI^- cells.

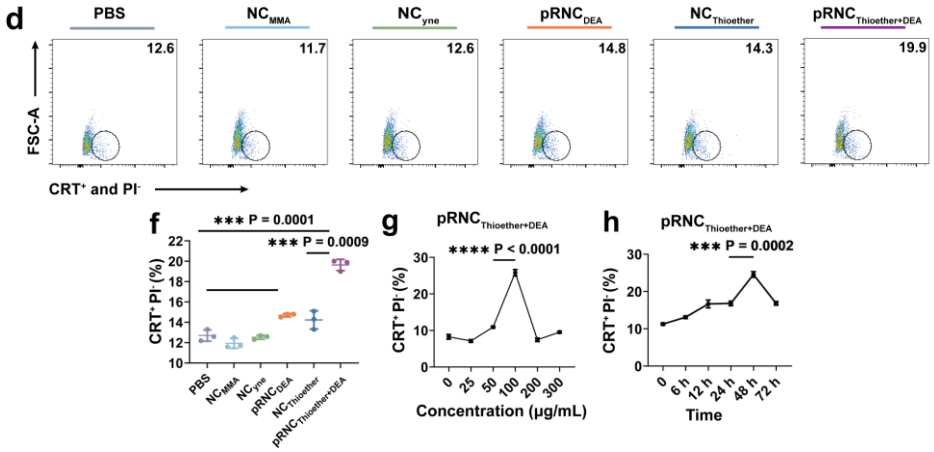
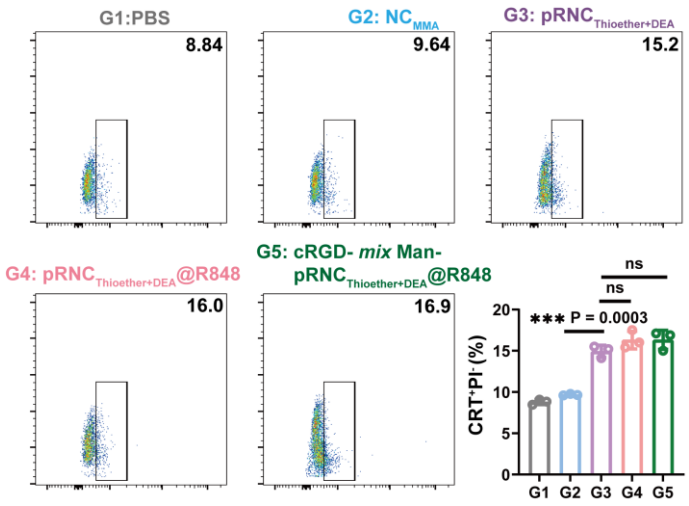


Fig. 2 d CRT exposure after different treatments *via* flow cytometry characterization. **f** Quantification analysis of CRT exposure after different treatments. **g** pRNC$_{Thiorther+DEA}$ with different concentrations induced CRT exposure in B16F10 cells. **h** CRT exposure of B16F10 cells was induced by pRNC$_{Thioether+DEA}$ after different incubation time treatment.



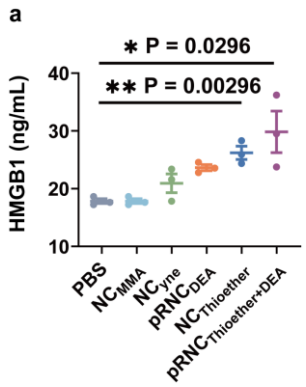
Supplementary Fig. 34. Representative flow cytometric images and quantification analysis of CRT exposure in B16F10 cells after different treatments *via* flow cytometry characterization.

- The authors mentioned that the measurement of ATP release was performed using ELISA. This is not accurate. ATP is typically measured using the luminescence assay, which does not involve the use of antibodies (lines 647-653). Please review and update accordingly.

Response: Thanks for the reviewer's suggestion. We are sorry that we made a mistake in the writing part about ATP release measurement. In fact, we used the ATP Assay Kit (S0026, Beyotime, Shanghai, China) [not via ELISA] as shown in the materials part to detect ATP levels in supernatant. The correct writing has been updated in the revised version.

- The release of HMGB1 should be measured in the supernatant using ELISA (lines 640-646). Therefore, the experiments must be redone, and the data should be re-analyzed.

Response: Thanks for the reviewer's suggestion. The HMGB1 release has been investigated *via* ELISA as shown in the following:



Supplementary Fig. 15. a Release of HMGB1 in supernatant for cells after different treatments *via* ELISA characterization.

The related discussion has also been added in the revised version "In addition, after different treatments, $pRNC_{Thioether+DEA}$ induced the most HMGB1 release in the cell supernatant where the highest level was detected *via* ELISA characterization (Supplementary Fig. 15a).

- The number of experimental repeats should be indicated in Figure 2 and in other figures.
Response: Thanks for the reviewer's suggestion. The number of experimental repeats have been added in the revised version.

2. The authors have stated that "by $pRNC_{Thioether+DEA}$ treatment compared with that of PBS, indicating that the nanocarrier also elicited ER stress." This conclusion is drawn from the observed upregulation of CHOP. Importantly, one of the typical features of classical ICD inducers is the induction of ER stress, characterized by eIF2α and PERK phosphorylation. Therefore, it is advisable for the authors to include these additional data as they are a key element of ICD.
Response: Thanks for the reviewer's constructive suggestion. Additional data about the phosphorylation of eIF2α and PERK have been added to the revised version. The related discussions also have been added in the main text and highlighted as red color. We can also see them in the following:

"To further investigate how $pRNC_{Thioether+DEA}$ induces ICD in B16F10 cells, we used western blot to examine ER stress involved pathways. The phosphorylation of PERK and eukaryotic translation initiation factor 2α (elf2α) enhanced after $pRNC_{Thioether+DEA}$ treatment at 48 h compared with those of PBS and NC_{MMA}. The downstream activating transcription factor 4 (ATF4) and recombinant DNA damage-inducing transcript 3 (CHOP) also increased. The aforementioned data indicated that ER stress was triggered by $pRNC_{Thioether+DEA}$ (Fig. 3j, Supplementary Fig. 20)."

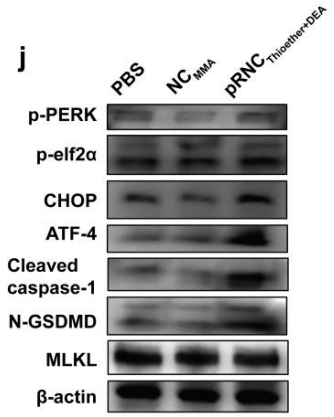
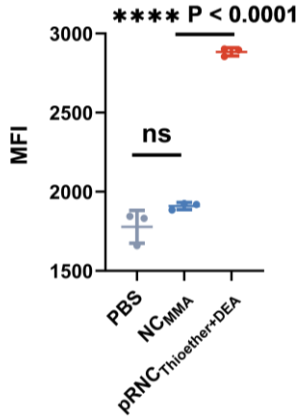
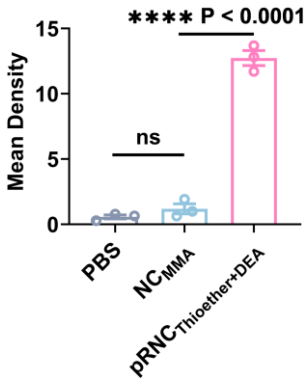


Fig. 3 j Western blot of p-PERK, p-elf2α, ATF4, cleaved caspase-1, N-GSDMD, MLKL expression after different treatments. β-actin was used as loading control.

3. The intracellular ROS level should be quantified and statistically compared to the control groups (Fig 3h, i, j).
Response: Thanks for the reviewer's suggestion. The semi-quantitative and statistical analysis of intracellular ROS levels have been added to Supplementary Fig. 18, 19.



Supplementary Fig. 18. ROS flow cytometry quantification.



Supplementary Fig. 19. Intracellular mtROS level quantification.

4. The authors should provide clear data on the kinetics and dose-response of cell death induction by pRNC$_{Thioether+DEA}$. These data should be shown in the first figures of the manuscript.

Response: Thanks for the reviewer's constructive suggestion. The data on the kinetics and dose response of cell death induction by pRNC$_{Thioether+DEA}$ have been investigated and added to the revised version. Please see them in the following (**Fig. 2**i). According to the results, with pRNC$_{Thioether+DEA}$ concentarion increasing from 12.5 to 300 μg/mL, cell death ratio increased in logarithm the IC$_{50}$ of which was 93.04 μg/mL. According to the result in **Fig. 2**g, its ICD inducibility also emerged concentration dependence when pRNC$_{Thioether+DEA}$ at 100 μg/mL had the highest CRT ratios (25.80 ± 0.85%), 2.37-3.61 fold higher than others. Therefore, the concentration 100 μg/mL of pRNC$_{Thioether+DEA}$ was chosen as the optimal one for the further studies in this manuscript. Please see the related results in the following:

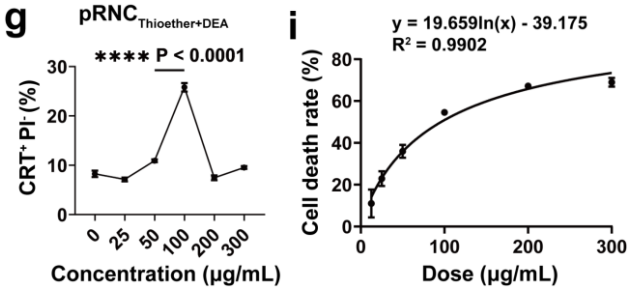


Fig. 2 g $pRNC_{Thiorther+DEA}$ with different concentrations induced CRT exposure in B16F10 cells. **i** The kinetics and dose-response of cell death induction by $pRNC_{Thioether+DEA}$.

5. Analysis of maturation/activation of DCs: It is unclear which groups correspond to G1-G4. A clear description of these groups should be provided in the legend for Figure 4. Additionally, it would be helpful to indicate the ratio of DCs to dead cells, along with the rate of cell death before adding them to the DCs. Important negative and positive controls are missing, such as coculture with viable B16 cells, LPS-treated DCs, etc

Response: Thanks for the reviewer's suggestions. As suggested, the related descriptions of G1-G4 have been added in **Fig. 4**. The ratio of DCs to dead cells has been calculated as shown in Supplementary Fig. 36. According to the result, both $pRNC_{Thioether+DEA}$ and $pRNC_{Thioether+DEA}$@R848 groups had lower ratios than the other groups, indicating that the two groups induced more dead cells for DAMPs generation with DC maturation. Also, the negative and positive controls as suggested have been added as shown in Supplementary Fig. 35.

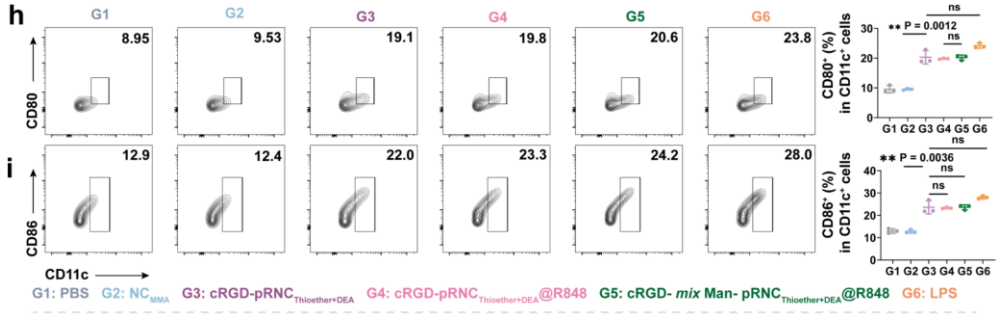
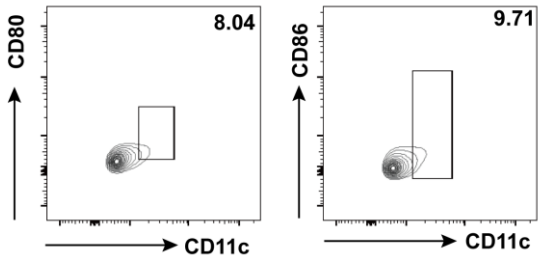
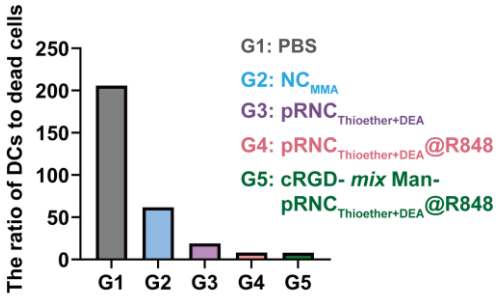


Fig. 4 h, i Representative flow cytometric images and quantification of mature DCs.



Supplementary Fig. 35 Negative control of DC maturation investigation using viable B16F10 cells directly to incubate with DCs**.**



Supplementary Fig. 36 The ratio of DCs to dead cells after different treatments.

6. The data presented in the Fig. 4 should be quantified and statistically analyzed. The viability (cell death) of the cells should be provided for accumulation studies on cRGD-pRNCThioether+DEA@FITC;pRNCThioether+DEA@FITC; Man-pRNCThioether+DEA@ FITC and pRNCThioether+DEA@FITC.

Response: Thanks for the reviewer's suggestions. The quantification and statistical analyses have been added in **Fig. 4**.

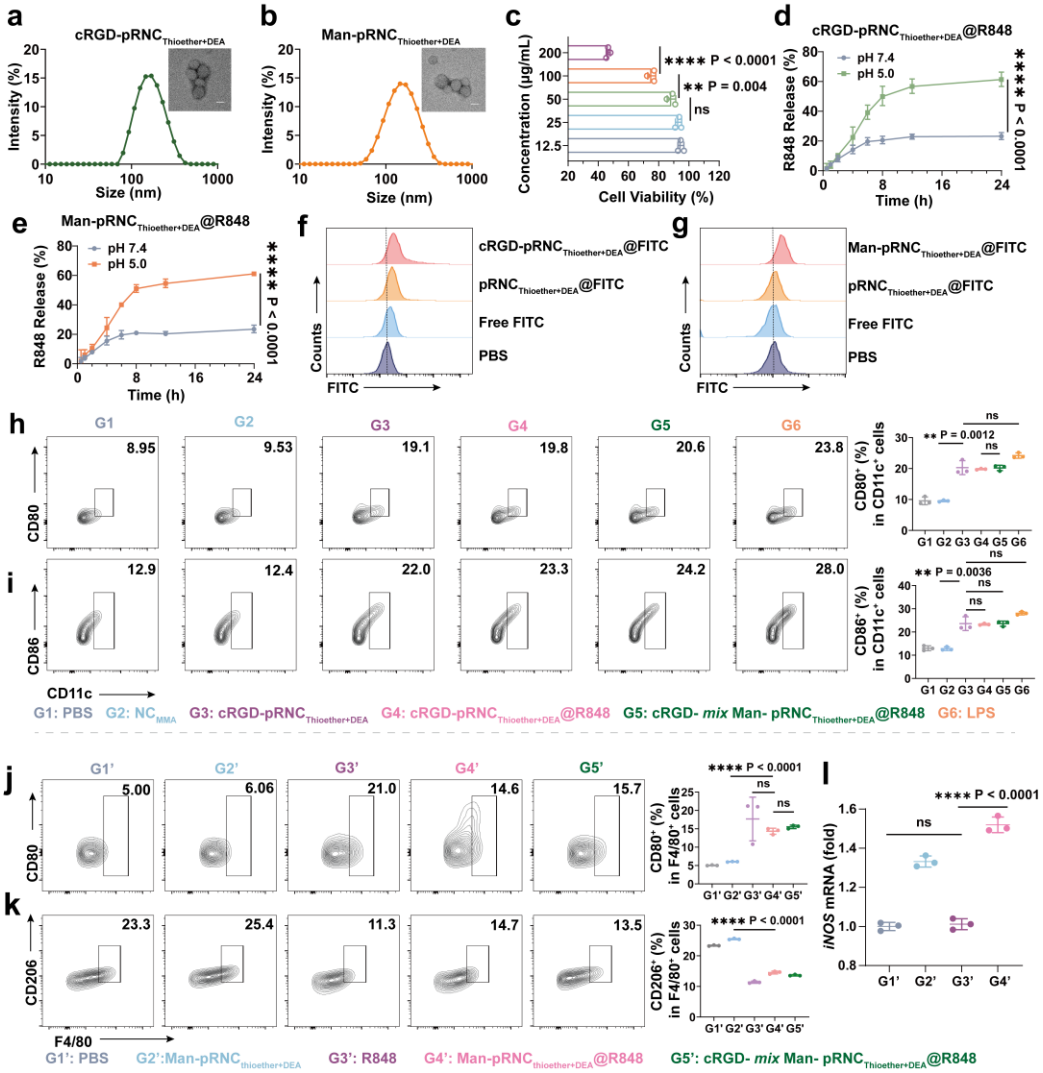
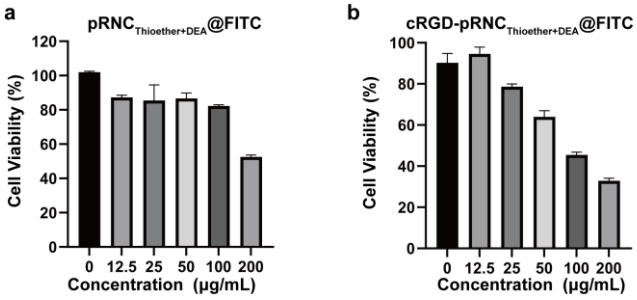
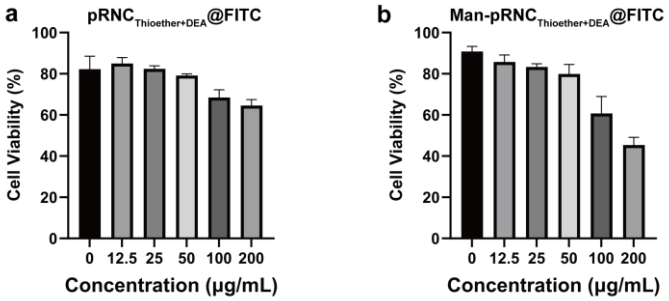


Fig. 4 Preparation and characterization of targeted nanoformulations.

As suggested, the cell viability was also explored and added in Supplementary Fig. 30 and 31.



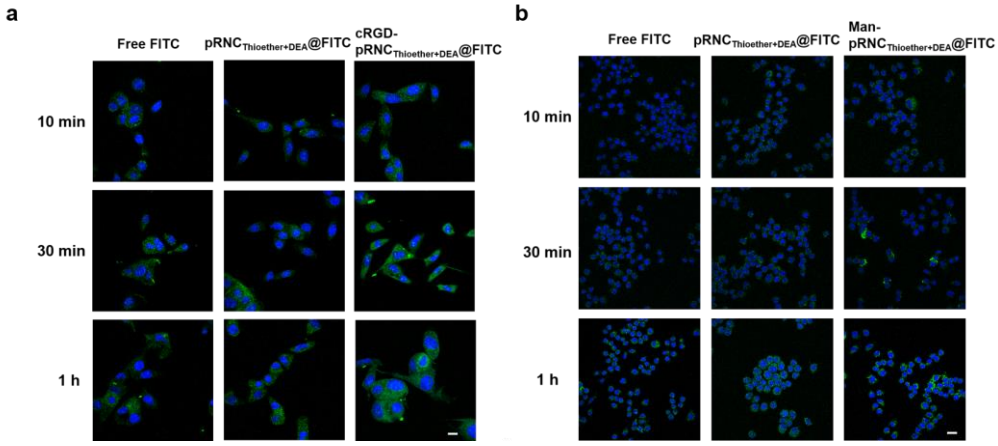
Supplementary Fig. 30. Dosage dependent cytotoxicity of a) $pRNC_{Thioether+DEA}$@FITC and b) cRGD-$pRNC_{Thioether+DEA}$@FITC in B16F10 cells by MTT assays.



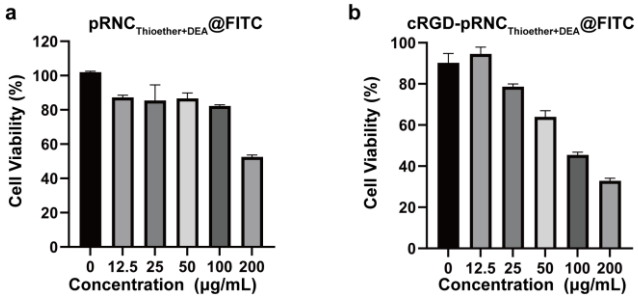
Supplementary Fig. 31. Dosage dependent cytotoxicity of a) $pRNC_{Thioether+DEA}$@FITC and b) Man-$pRNC_{Thioether+DEA}$@FITC in RAW264.7 cells by MTT assays.

7. The data on the specific targeting of melanoma B16 and macrophages RAW264.7 by cRGD-$pRNC_{Thioether+DEA}$ and Man-$pRNC_{Thioether+DEA}$, respectively, are not convincing. These data require a substantial justification with additional experimental data. As it is now it is not clear at all.

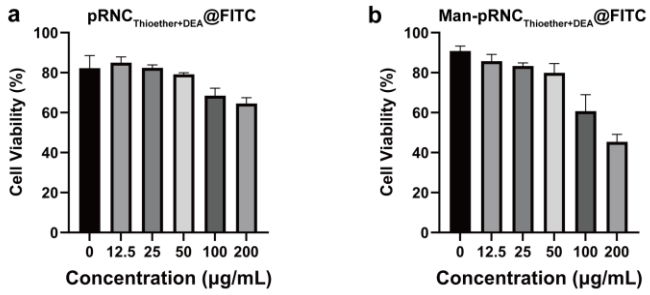
Response: Thanks for the reviewer's suggestion. To further illustrate this issue, we have re-photographed cellular internalization by CLSM and investigated the cytotoxicity for cells after different treatments by MTT assays as shown in the following. According to the CLSM results, notable cRGD and Man targetabilities were observed when intensive green fluorescence intensity was seen in targeted groups compared with no decoration ones (Supplementary Fig. 33). In addition, cell viabilities decreased in targeted groups than the other one which also proved the targetabilities of cRGD and Man (Supplementary Fig. 30 and 31).



Supplementary Fig. 33. Cellular internalization of different nanoformulations *via* CLSM characterization.



Supplementary Fig. 30. Dosage dependent cytotoxicity of a) $pRNC_{Thioether+DEA}$@FITC and b) cRGD-$pRNC_{Thioether+DEA}$@FITC in B16F10 cells by MTT assays.



Supplementary Fig. 31. Dosage dependent cytotoxicity of a) $pRNC_{Thioether+DEA}$@FITC and b) Man-$pRNC_{Thioether+DEA}$@FITC in RAW264.7 cells by MTT assays.

8. The authors claimed that there is an induction of pyroptosis in the target cells, but did not provide firm qualitative data. The type of regulated cell death (*i.e.*, apoptosis, necroptosis, ferroptosis, pyroptosis) should be experimental identified, and confirmed and it is needed to demonstrate that this technology can target also other cancer cell types and thus it is not cell line specific.

Response: Thanks for the reviewer's constructive suggestions. We investigated the induction of pyroptosis *via* WB to characterize cleaved caspase-1 and N-GSDMD expression, as well as using kit to detect LDH activity as shown in the following **Fig. 3**j and Supplementary Fig. 21. The induction of apoptosis, necroptosis and ferroptosis was explored by flow cytometry, WB and CLSM characterization as shown in the following. The related discussions were added in the revised version and highlighted as red color.

"In addition, the expression of N-GSDMD and cleaved caspase-1 also up-regulated after $pRNC_{Thioether+DEA}$ treatment, and lactate dehydrogenase (LDH) activity was 2.4-fold higher than that of PBS, indicating that $pRNC_{Thioether+DEA}$ induced pyroptosis through GSDMD pathway (Fig. 3j, Supplementary Fig. 21). According to Supplementary Fig. 22, $pRNC_{Thioether+DEA}$ also induced apotosis when the elevated apoptosis ratio (72.72 ± 3.94%) was detected compared with that of PBS (22.48 ± 0.43%) and NC_{MMA} (25.06 ± 0.97%) groups (Supplementary Fig. 22). In addition, we found that oxidized lipid peroxides (LPO) level elevated after $pRNC_{Thioether+DEA}$ treatment indicating its ability to induce cancer ferroptosis, and the ratio of oxidized to reduced LPO increased to 40.01 ± 0.12% compared with that of PBS (30.97 ± 0.32%) and NC_{MMA} (29.60 ± 0.24%) (Supplementary Fig. 23). Moreover, negligible mixed lineage kinase domain-like protein (MLKL) expression changes was observed after different treatments, indicating that $pRNC_{Thioether+DEA}$ cannot induce cell necrosis (Fig. 3j)."

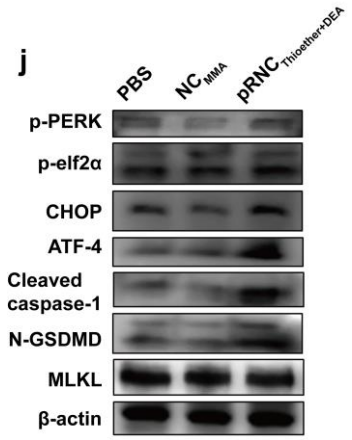
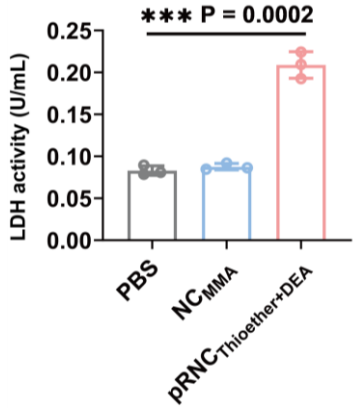
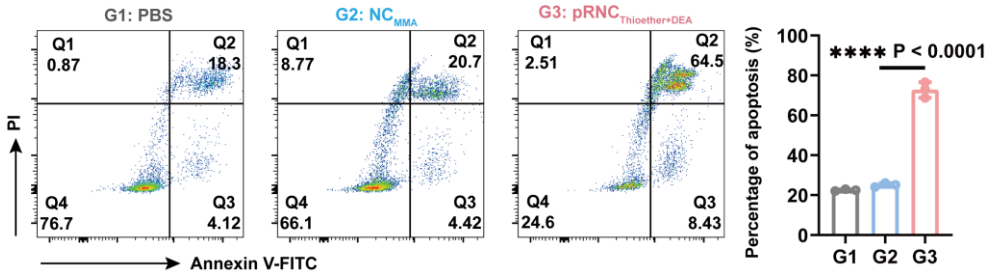


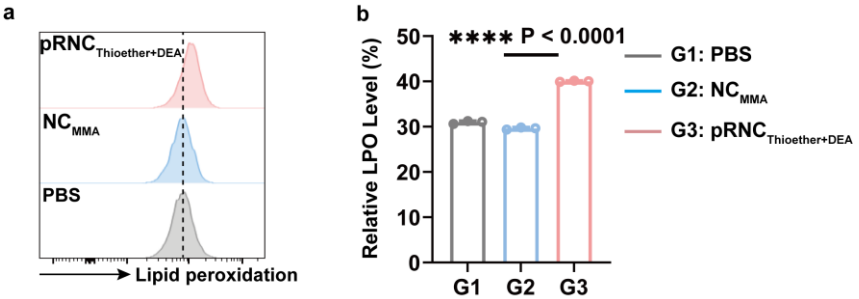
Fig. 3 j Western blot of PERK, p-PERK, p-elf2α, ATF4, Cleaved caspase-1, N-GSDMD, MLKL expression after treatments, β-actin was used as loading control.



Supplementary Fig. 19. LDH activity in the supernatant after different treatments *via* Microplate plate test kit characterization.



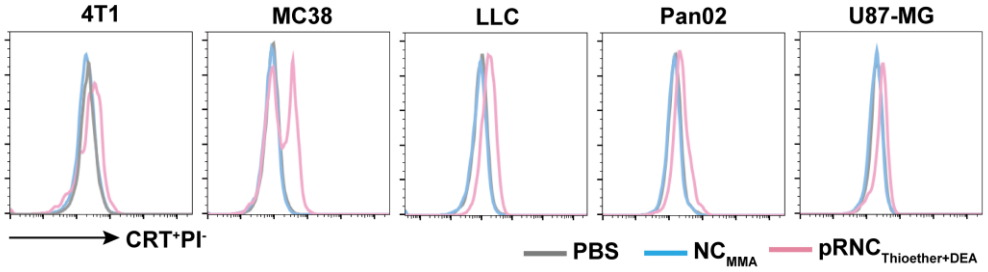
Supplementary Fig. 22. Representative flow cytometric images and semi-quantitative analysis to show the annexin V-FITC/PI apoptosis detection analysis of B16F10 cells.



Supplementary Fig. 23. a Representative flow cytometric image and b) the semi-quantitative analysis of flow cytometric to show the LPO generation for B16F10 cells treated with different groups using C11-BODIPY$^{581/591}$.

As suggested, we then investigated the ICD inducibility of pRNC$_{Thioether+DEA}$ in other cancer cell types. As shown in Supplementary Fig. 16, pRNC$_{Thioether+DEA}$ could induce ICD in multiple cancer cells. Related discussions have been added in the revised version and highlighted as red color.

"Besides B16F10 cells, pRNC$_{Thioether+DEA}$ induced ICD in multiple cancer cells such as colorectal carcinoma MC38, Lewis lung cancer (LLC) cells, pancreatic carcinoma Pan02 with notable CRT fluorescence shift observation, when slight CRT exposure was observed in breast cancer 4T1 and glioma U87-MG cells (Supplementary Fig. 16). The above results revealed that pRNC$_{Thioether+DEA}$ can be widely applied into a variety of tumors for immunotherapy *via* inducing ICD."



Supplementary Fig. 16. ICD effect of pRNC$_{Thioether+DEA}$ on 4T1, MC38, LLC, Pan02 and U87-MG after 48 h incubation, respectively.

9. The article should be rewritten to increase clarity and proofread.

Response: Thanks for the reviewer's suggestion. The revised version has been carefully proofread and polished.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I find the response to reviewer comments thoughtful and comprehensive. The manuscript is much clearer, data easier to interpret, and has been contextualized into existing clinical context much more deeply.

Reviewer #2 (Remarks to the Author):

The authors have significantly improved the current manuscript, with a clearer logic and flow. This reviewer has no further comments from the aspects of nanomedicine.

Reviewer #3 (Remarks to the Author):

The authors have provided an improved version of the manuscript. However, there are still important elements that need to be addressed before it can be accepted for publication.

1. The analysis of apoptosis using Annexin-V/PI staining was used. However, this technique only detects the stage of cell death and not the type, as phosphatidylserine (PS) exposure can also occur during other cell death modalities such as necroptosis.

It is strongly recommended to revise the data, possibly by employing specific cell death inhibitors like zVAD-fmk for apoptosis, Nec-1s for necroptosis, and Fer-1, DFO, and Vit-E for ferroptosis. This should be tested on the main cell lines used in the manuscript.

2. Additionally, in Supplementary Figure 22, the authors present quantitative data without clearly indicating which population it represents; this should be clarified.

3. The statement "Moreover, negligible mixed lineage kinase domain-like protein (MLKL) expression changes were observed after different treatments, indicating that pRNCThioether+DEA cannot

induce cell necrosis (Fig. 3j)," needs revision. Given the advice to use cell death inhibitors (as mentioned above).

The term "necrosis" should be corrected to "necroptosis" in this context.

4. Depending on the outcomes of experiments with inhibitors, the authors should also revise the abstract to accurately reflect the involvement of cell death modalities other than pyroptosis.

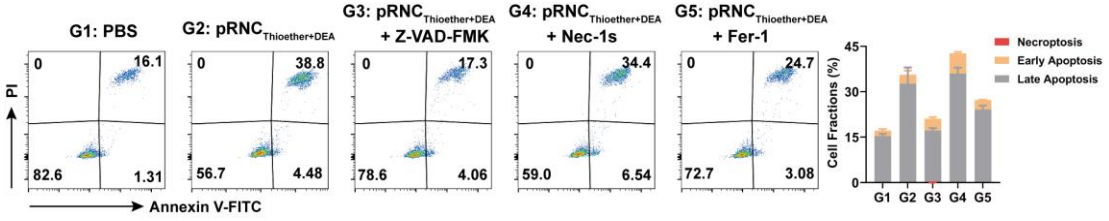
Point-by-point response to reviewers' comments:
Note: Reviewers' comments are in black, and our response is highlighted in blue.

Reviewer #1 (Remarks to the Author):
I find the response to reviewer comments thoughtful and comprehensive. The manuscript is much clearer, data easier to interpret, and has been contextualized into existing clinical context much more deeply.
Response: We really appreciate the reviewer's positive comments.

Reviewer #2 (Remarks to the Author):
The authors have significantly improved the current manuscript, with a clearer logic and flow. This reviewer has no further comments from the aspects of nanomedicine.
Response: Thanks for the positive comments.

Reviewer #3 (Remarks to the Author):
The authors have provided an improved version of the manuscript. However, there are still important elements that need to be addressed before it can be accepted for publication.
Response: We appreciate the reviewer's insightful and constructive comments and suggestions, and we have made a proper revision of the manuscript.

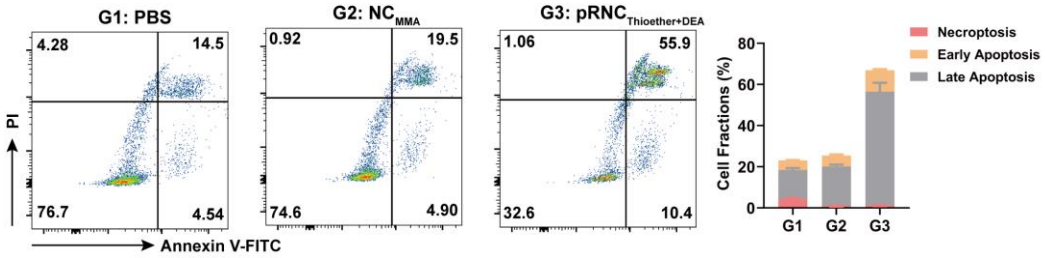
1. The analysis of apoptosis using Annexin-V/PI staining was used. However, this technique only detects the stage of cell death and not the type, as phosphatidylserine (PS) exposure can also occur during other cell death modalities such as necroptosis. It is strongly recommended to revise the data, possibly by employing specific cell death inhibitors like zVAD-fmk for apoptosis, Nec-1s for necroptosis, and Fer-1, DFO, and Vit-E for ferroptosis. This should be tested on the main cell lines used in the manuscript.
Response: Thanks for the reviewer's comments. As suggested, we have re-done $pRNC_{Thioether+DEA}$ induced apoptosis with Z-VAD-FMK, Nec-1s and Fer-1 treatment. The updated results and discussions have been added in the revised version and highlighted as blue color. Please see in the following:
"As shown in Supplementary Fig. 25, the ratio of apoptotic cells induced by $pRNC_{Thioether+DEA}$ was highly suppressed after an apoptosis inhibitor Z-VAD-FMK treatment. After treatment with the ferroptosis inhibitor Ferrostatin-1 (Fer-1), the apoptosis ratio slightly reduced, while negligible changes were observed in cells treated with the necroptosis inhibitor necrostatin 2 racemate (Nec-1s). The data indicated that $pRNC_{Thioether+DEA}$ induced a series of cell death modalities including pyroptosis, ferroptosis and apoptosis."



Supplementary Fig. 25. Representative flow cytometric images and semi-quantitative analyses to show the apoptosis populations in B16F10 cells after treatments with PBS, $pRNC_{Thioether+DEA}$ with or without different cell death inhibitors.

2. Additionally, in Supplementary Figure 22, the authors present quantitative data without clearly indicating which population it represents; this should be clarified.

Response: Thanks for the reviewer's comments. As suggested, we have analyzed more detailed data to distinguish the populations in the revised version as shown in the following:

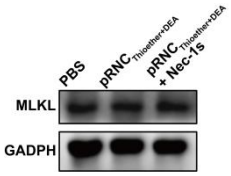


Supplementary Fig. 22. Representative flow cytometric images and semi-quantitative analyses to show the apoptosis populations in B16F10 cells after different treatments.

3. The statement "Moreover, negligible mixed lineage kinase domain-like protein (MLKL) expression changes were observed after different treatments, indicating that $pRNC_{Thioether+DEA}$ cannot induce cell necrosis (Fig. 3j)," needs revision. Given the advice to use cell death inhibitors (as mentioned above). The term "necrosis" should be corrected to "necroptosis" in this context.

Response: Thanks for the reviewer's comments. As suggested, we have added $pRNC_{Thioether+DEA}$ + Nec-1s group to further clarify whether $pRNC_{Thioether+DEA}$ could induce cell necroptosis. Please see the following:

"Moreover, negligible mixed lineage kinase domain-like protein (MLKL) expression changes were observed after treatment with PBS, NC_{MMA}, $pRNC_{Thioether+DEA}$. The presence of Nec-1s didn't affect the expression of MLKL, further confirming that $pRNC_{Thioether+DEA}$ cannot induce cell necroptosis (Fig. 3j, Supplementary Fig. 25)."



Supplementary Fig. 24. Western blot of MLKL expression after treatments with PBS, $pRNC_{Thioether+DEA}$ and $pRNC_{Thioether+DEA}$ + Nec-1s.

4. Depending on the outcomes of experiments with inhibitors, the authors should also revise the abstract to accurately reflect the involvement of cell death modalities other than pyroptosis.

Response: Thanks for the reviewer's comments. As suggested, the abstract has been updated as shown in the following:

"The nanocarrier itself can induce melanoma immunogenic cell death (ICD) *via* tertiary amine and thioether concentrating on mitochondria to regulate metabolism in triggering endoplasmic

reticulum stress and upregulate gasdermin D for pyroptosis as well as induce ferroptosis and apoptosis.”

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The authors indeed answered the comments, however, there are several important inconsistencies in the terminology that should be corrected before the publication of this manuscript in the journal.

In the text:

“As shown in Supplementary Fig. 25, the ratio of DEAD/DYING cells induced by pRNCThioether+DEA was highly suppressed after an apoptosis inhibitor Z-VAD-FMK treatment. After treatment with the ferroptosis inhibitor Ferrostatin-1 (Fer-1), the CELL DEATH ratio slightly reduced, while negligible changes were observed in cells treated with the necroptosis inhibitor necrostatin 2 racemate (Nec-1s). The data indicated that pRNCThioether+DEA induced MIXED cell death modalities WITH SOME FEATURES OF pyroptosis, ferroptosis, and apoptosis. ADDITIONAL STUDIES ARE NEEDED TO ANALYZE IN DETAIL THE VARIOUS TYPES OF CELL DEATH MODALITIES”

--> The changed parts are re-written in the capital letters.

--> To make such strong conclusions about the involvement of apoptosis and ferroptosis the authors should repeat these experiments at least three times (in the current version it is not mentioned how much time it was repeated (in the figure legend) and the authors should make a proper statistical analysis. Now it is not done. As such these data can not be published.

The authors have written: "Supplementary Fig. 25. Representative flow cytometric images and semi-quantitative analyses to show the apoptosis populations in B16F10 cells after treatments with PBS, pRNCThioether+DEA with or without different cell death inhibitors."-

-> Apoptosis should be changed to cell death everywhere because in the results above the authors indicate that there is also a contribution of other cell death modalities. In the same way, the figure legend to suppl. fig. 25 should be changed to "early cell death" and "late cell death." Please carefully address this terminology in the manuscript.

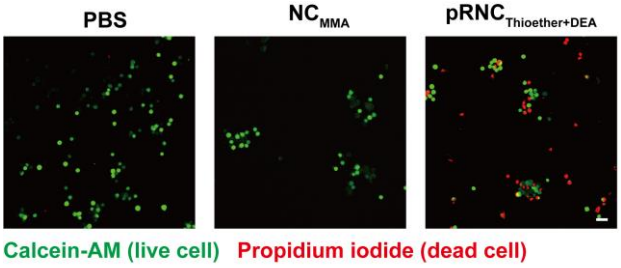
The authors have written in the. abstract- I have adapted in the capital letters:

“The nanocarrier itself can induce melanoma immunogenic cell death (ICD) via tertiary amine and thioether concentrating on mitochondria to regulate metabolism in triggering endoplasmic reticulum stress and upregulate gasdermin D for pyroptosis as well as SOME FEATURES OF ferroptosis and apoptosis.”

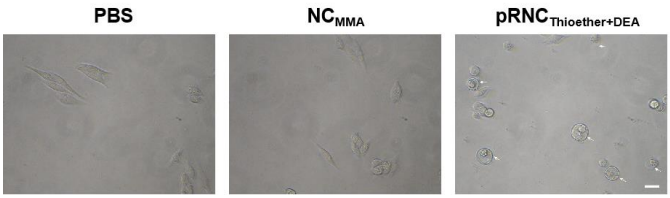
Point-by-point response to reviewers' comments:
Note: Reviewers' comments are in black, and our response is highlighted in blue.

Reviewer #3 (Remarks to the Author):
The authors indeed answered the comments, however, there are several important inconsistencies in the terminology that should be corrected before the publication of this manuscript in the journal.
Response: We appreciate the reviewer's insightful and constructive comments and suggestions, and we have made a proper revision to the manuscript.

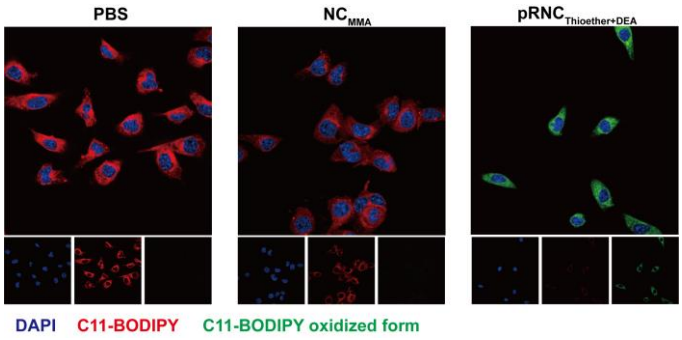
1. In the text:
"As shown in Supplementary Fig. 25, the ratio of DEAD/DYING cells induced by $pRNC_{Thioether+DEA}$ was highly suppressed after an apoptosis inhibitor Z-VAD-FMK treatment. After treatment with the ferroptosis inhibitor Ferrostatin-1 (Fer-1), the CELL DEATH ratio slightly reduced, while negligible changes were observed in cells treated with the necroptosis inhibitor necrostatin 2 racemate (Nec-1s). The data indicated that pRNCThioether+DEA induced MIXED cell death modalities WITH SOME FEATURES OF pyroptosis, ferroptosis, and apoptosis. ADDITIONAL STUDIES ARE NEEDED TO ANALYZE IN DETAIL THE VARIOUS TYPES OF CELL DEATH MODALITIES"
--> The changed parts are re-written in capital letters.
Response: Thanks for the reviewer's comments. The related descriptions have been revised in the main text as suggested by the reviewer. The additional studies were also added including the characterization of live/dead cell death, pyroptosis, and ferroptosis as shown in the following. The related descriptions were updated in the revised version highlighted as red colors.



Supplementary Fig. 21. Imaging of Calcein-AM (green channel, living cells) and PI (red channel, dead cells) staining of cells after different treatments. Scale bar, 50 μm.



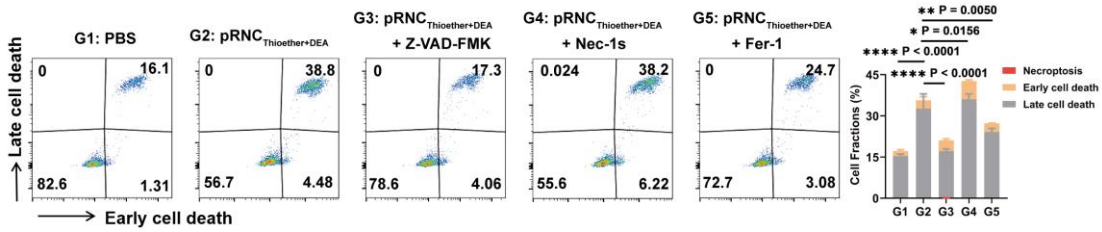
Supplementary Fig. 22. Morphological features of cells after various treatments. The white arrows represent pyroptotic cells. Scale bar, 30 μm.



Supplementary Fig. 26. LPO generation for B16F10 cells treated with different groups using C11-BODIPY$^{581/591}$ as a probe. Scale bar, 20 μm.

--> To make such strong conclusions about the involvement of apoptosis and ferroptosis the authors should repeat these experiments at least three times (in the current version it is not mentioned how much time it was repeated (in the figure legend) and the authors should make a proper statistical analysis. Now it is not done. As such these data can not be published.

Response: Thanks for the reviewer's comments. The results in Supplementary Fig. 28 were repeated three times as shown in the Figure Caption "Supplementary Fig. 28. Representative flow cytometric images and the semi-quantitative analysis to show the cell death populations in B16F10 cells after treatment with PBS, pRNC$_{Thioether+DEA}$ with or without cell death inhibitors. Data are shown as mean ± SD (n = 3 independent experiments), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$." The statistical analysis was added in the revised version and can be seen in the following:

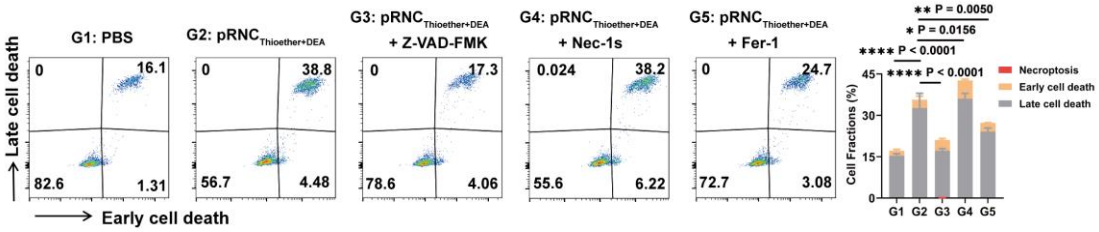


Supplementary Fig. 28. Representative flow cytometric images and the semi-quantitative analysis to show the cell death populations in B16F10 cells after treatment with PBS, pRNC$_{Thioether+DEA}$ with or without different cell death inhibitors. Data are shown as mean ± SD (n = 3 independent experiments), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

2. The authors have written: "Supplementary Fig. 25. Representative flow cytometric images and semi-quantitative analyses to show the apoptosis populations in B16F10 cells after treatments with PBS, pRNC$_{Thioether+DEA}$ with or without different cell death inhibitors."-

-> Apoptosis should be changed to cell death everywhere because in the results above the authors indicate that there is also a contribution of other cell death modalities. In the same way, the figure legend to suppl. fig. 25 should be changed to "early cell death" and "late cell death." Please carefully address this terminology in the manuscript.

Response: Thanks for the reviewer's comments. The word "apoptosis" has been replaced by "cell death" in the main text. The figure legend in Supplementary Fig. 28 has been changed to "early cell death" and "late cell death" as suggested. Please see the following:



Supplementary Fig. 28. Representative flow cytometric images and the semi-quantitative analysis to show the cell death populations in B16F10 cells after treatment with PBS, $pRNC_{Thioether+DEA}$ with or without different cell death inhibitors. Data are shown as mean ± SD (n = 3 independent experiments), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

3. The authors have written in the abstract- I have adapted in the capital letters:

"The nanocarrier itself can induce melanoma immunogenic cell death (ICD) *via* tertiary amine and thioether concentrating on mitochondria to regulate metabolism in triggering endoplasmic reticulum stress and upregulate gasdermin D for pyroptosis as well as SOME FEATURES OF ferroptosis and apoptosis."

Response: Thanks for the reviewer's comments. As suggested, we have corrected the related descriptions in the abstract.