An NIR-Fluorophore-Based Theranostic for Selective Initiation of Tumor Pyroptosis-Induced Immunotherapy

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2 **Tumor Pyroptosis-Induced Immunotherapy**

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15

16 **Abstract:**

17 Pyroptosis is an inflammatory form of programmed cell death that can effectively eliminate
18 malignant cells and boost anticancer immunity. However, most of the current pyroptosis inducers
19 lack cell selectivity, which may cause severe side effects for cancer therapy. In this work, for the
20 first time, we discovered that the commonly used near-infrared (NIR) fluorogenic hemicyanine
21 (CyNH₂) induces pyrolysis to kill cancer cells and boost antitumor immunity. Cancer cells
22 overexpressing the NAD(P)H: quinone oxidoreductase isozyme 1 (NQO1)-responsive theranostic
23 (NCyNH₂) were designed for selective cell pyroptosis and were nonfluorescent with low toxicity
24 before activation. In the presence of NQO1, the fluorescence of CyNH₂ was restored and could
25 selectively initiate pyroptosis of cancer cells and further lead to systemic antitumor immunity
26 activation for solid tumor therapy. Thus, this fluorogenic NIR dye may represent a novel theranostic
27 agent for the selective initiation of tumor pyroptosis.

28

29 **KEYWORDS:** Fluorophore, hemicyanine, pyroptosis, NQO1, immunotherapy

30

31 **Introduction**

32 An important therapeutic method to kill cancer cells is programmed cell death (PCD), which
33 primarily includes autophagy, apoptosis, pyroptosis and ferroptosis ¹⁻³. Pyroptosis is a unique form
34 of PCD characterized by DNA fragmentation, chromatin condensation, cellular swelling with large
35 bubbles and cell content leakage and has been shown to be related to malignant tumors and
36 inflammatory diseases ⁴⁻⁹. Pyroptosis critically depends on the formation of plasma membrane
37 pores by members of the gasdermin protein family, often (but not always) as a consequence of
38 inflammatory caspase activation. After caspase cleavage, the gasdermin protein family (GSDMA,
39 GSDMB, GSDMC, GSDMD and GSDME) releases gasdermin-N domains that combine with
40 membrane phospholipids and perforate the cell membrane with a pore size of ~18 nm, disrupting
41 osmotic potential and leading to cell swelling and large bubbles ¹⁰⁻¹².

42 Compared to apoptosis, pyroptosis may represent a more relevant pathway for cancer therapy
43 due to its ability to prime antitumor immune responses. Immunotherapies using the patient's own
44 immune system to specifically recognize and kill tumor cells have been very successful in the clinic
45 and have the potential to cure cancer ^{13,14}. Tumors that are highly immunogenic (hot) are key to
46 effective immunotherapy ¹⁵. Unfortunately, most human tumors are immunogenically cold and lack
47 tumor-infiltrating lymphocytes, which limits the success of current immunotherapy strategies ¹⁶.
48 Recent studies have shown that inducing tumor cell pyrolysis improves the immune
49 microenvironment in solid tumors, which induces tumor regression to increase CD4⁺ T helper cells,
50 cytotoxic T cells and natural killer cells and decreases the percentage of T regulatory cells ¹⁷. Cell
51 pyroptosis is characterized by cell membrane rupture, and many proinflammatory cytokines and
52 antigens of tumor cells are released into the extracellular environment through pores, which activate
53 the immune system and lead to inflammation ¹⁸⁻²⁰. Based on these findings, intense inflammation
54 induced by proinflammatory cytokines and activation of antigen-specific T cells presented with
55 antigens can cause powerful immune responses ^{21,22}. In addition, the pyroptosis-induced

inflammatory microenvironment in tumors enhances effective lymphocyte infiltration, and activation can improve the response rate of checkpoint blockade in inducing antitumor immunity¹⁷. Therefore, the initiation of pyroptosis death through various strategies brings new opportunities for cancer therapy.

Several studies have shown that certain molecules, chemotherapeutic drugs or ions, such as iron^{23,24}, NaCl nanoparticles²⁵, Na₂S₂O₈ nanoparticles²⁶, metformin²⁷, and glucose oxidase (GOD)²⁸, induce cell pyroptosis in a GSDMD/GSDME-mediated manner. In addition, Liu et al. designed a series of membrane anchoring photosensitizers (TBD-R PSs) that produce cytotoxic ROS in situ can induce cell pyroptosis upon light irradiation.²⁹ Pretreatment of cancer cells with decitabine to reverse GSDME silencing and in combination with the chemotherapeutic drug cisplatin also triggers pyroptosis for tumor immunotherapy³⁰. Although pyroptosis has been extensively studied in various inflammatory diseases in recent years, its application in cancer therapy is still in early stages, not to mention selectively initiating pyroptosis in cancer cells.

Recent development of cancer theranostics that integrate the function of imaging and therapy has allowed real-time monitoring of drug distribution, assessment of therapeutic outcomes, and individualized selection of treatment modalities, which holds great promise for personalized medicine³¹. Herein, for the first time, we discovered that an amino (-NH₂)-containing hemicyanine (Cy) fluorophore (denoted as CyNH₂) has the potential to induce pyrolysis to kill tumor cells. We demonstrated that CyNH₂ selectively accumulates within energized mitochondria, which causes mitochondrial membrane damage, leading to cytochrome c (Cyt c) release into the cytoplasm and activating caspase-3. Then, activated caspase-3 cleaves GSDME, generating a GSDME-N fragment that perforates membranes and induces pyroptosis. To improve the tumor selectivity of CyNH₂, an NQO1-responsive CyNH₂ (denoted as NCyNH₂) was designed because NQO1 is overexpressed by up to 100-fold in different cancer cells³². Before activation, fluorescence of CyNH₂ is in the off state due to the intramolecular charge transfer (ICT) of CyNH₂. More importantly, the cytotoxicity

of NCyNH₂ is low. Upon encountering overexpressed intracellular NQO1 in cancer cells, the NIR fluorescent signal of CyNH₂ is turned on, and the synchronous cytotoxicity of CyNH₂ is restored to initiate selective pyroptosis in cancer cells. In addition, cancer cell pyroptosis induced by NCyNH₂ induces systemic antitumor immunity for tumor therapy. Furthermore, NCyNH₂ was encapsulated in the Food and Drug Administration (FDA)-approved amphiphilic polymer poly(ethylene glycol)-*block*-poly(lactic-*co*-glycolic acid) (PEG-*b*-PLGA) as a nanocarrier for systemic administration and combined with the immune checkpoint inhibitor αPD-1 to effectively inhibit tumor growth and prolong survival in mice. Furthermore, combination therapy could induce powerful memory efficacy to prevent tumor recurrence, suggesting the potential of NIR fluorophore-based CyNH₂ for selective initiation of tumor pyroptosis-induced immunotherapy.

Results and discussion

The synthesis of CyNH₂ is shown in Scheme S1. Compound **1** was synthesized by an N alkylation reaction of trimethylindolenine and iodoethane. Compound **2** was synthesized by a Vilsmeier-Haack reaction of phosphorus oxychloride and cyclohexanone. In the presence of sodium acetate, compound **3** was synthesized through a condensation reaction. CyNH₂ was synthesized through a nucleophilic substitute reaction of compound **3** and 3-nitrophenol in the presence of triethylamine, followed by nitro reduction and cyclization in the presence of SnCl₂ and concentrated HCl. The purified products were characterized by NMR spectroscopy to confirm their structures (Fig. S1–S6 in the Supporting Information). The chemical structure and photophysical properties of CyNH₂ are shown in Fig. 1A, which shows an absorption maximum at 670 nm and an emission maximum at 730 nm, indicating that CyNH₂ has the potential to be an NIR dye for fluorescence imaging.

The mechanism of CyNH₂-induced cell pyrolysis. We used flow cytometry analysis and confocal imaging to evaluate the internalization of CyNH₂ by mouse breast cancer cells (4T1) and mouse colon cancer cells (CT26). As shown in Fig. S7, we observed a time-dependent increase in

intracellular fluorescence signals of CyNH₂, indicating that CyNH₂ can be efficiently taken up by cells and can be used as an NIR dye for fluorescence imaging. Intracellular localization of CyNH₂ in 4T1 or CT26 cells was further studied using confocal microscopy. Cells were exposed to CyNH₂ (1 µg mL⁻¹) for 4 h and costained with MitoTracker Green. As shown in Fig. 1B and Fig. S8, CyNH₂ (red fluorescence) overlapped well with MitoTracker (green fluorescence). Quantification of the fluorescence overlaps of CyNH₂ and MitoTracker by Pearson correlation coefficients was calculated to be 0.81. These results indicate that CyNH₂ exhibits selective mitochondrial localization in 4T1 and CT26 cells.

To evaluate the effect of CyNH₂ on mitochondrial function, the MitoProbe™ JC-1 Assay Kit was used to detect changes in mitochondrial membrane potential in 4T1 cells after exposure to CyNH₂ at different concentrations. As shown in Fig. S9A, cells without any treatments showed bright-red fluorescence, while the red fluorescence in cells treated with CyNH₂ was significantly decreased, and green fluorescence was significantly increased. These results indicate that CyNH₂ depolarizes the mitochondrial membrane potential in 4T1 cells, which was further quantitatively confirmed by flow cytometry analysis. As shown in Fig. 1C and S9B, compared to the high red fluorescence intensity and low green fluorescence intensity of PBS-treated cells, CyNH₂-treated cells showed a concentration-dependent decrease in red fluorescence intensity and an increase in green fluorescence intensity. These results indicate that CyNH₂ at low concentrations of 1 µg mL⁻¹ decreases mitochondrial membrane potential and damages the mitochondrial membrane.

CyNH₂ aggregation in mitochondria causes mitochondrial membrane damage, which leads to mitochondrial dysfunction. As shown in Fig. S10A-S10C, the mitochondrial oxygen consumption rate (OCR) and mitochondrial respiration rate (MRR) were reduced by 53.2% and 89.6%, respectively, after cells were pretreated with 6 µg mL⁻¹ CyNH₂ for 8 h. The decreased OCR and MRR in turn affect adenosine triphosphate (ATP) and reactive oxygen species production at complexes I and III of the mitochondrial respiratory chain¹⁰. Levels of intracellular ROS after

131 treatment with different concentrations of CyNH₂ were evaluated using the cell-permeable ROS
132 sensor 2',7'-dichlorofluorescein diacetate (DCFH-DA). As shown in Fig. S11, cells treated with
133 CyNH₂ exhibited bright green fluorescence, demonstrating increased ROS generation in 4T1 cells.
134 Flow cytometry was used to quantify ROS levels, which showed that CyNH₂ at a concentration of
135 1 µg mL⁻¹ was sufficient to maximize ROS generation (Fig. 1D). A firefly luciferase-based ATP
136 assay kit was used to measure intracellular ATP levels. As shown in Fig. 1E, 4T1 cells treated with
137 CyNH₂ showed a concentration-dependent decrease in levels of intracellular ATP. Relative
138 intracellular ATP levels in 4T1 cells treated with 4 µg mL⁻¹ CyNH₂ decreased to 13.2% of that in
139 PBS-treated cells.

140 To confirm that CyNH₂ induces pyrolysis, we first observed the morphological changes in 4T1
141 cells after treatment with different concentrations of CyNH₂. We observed that 4T1 cells clearly
142 exhibited pyroptosis with cell swelling and large bubbles on the plasma membrane in response to
143 CyNH₂ treatment (Fig. 1F). Next, the leakage of lactate dehydrogenase (LDH) in the cell
144 supernatant was examined. As shown in Fig. 1G, 4T1 cells showed a concentration-dependent
145 release of LDH upon incubation with CyNH₂. Considering that Cyt c is a heme protein anchored to
146 the inner membrane of mitochondria, when CyNH₂ aggregates in mitochondria, it causes
147 mitochondrial membrane damage, Cyt c release into the cytoplasm and activates caspase-3. Thus,
148 Cyt c levels in the cytoplasm and caspase-3 activity in 4T1 cells were analyzed after treatment with
149 different concentrations of CyNH₂. As shown in Fig. 1H, the green fluorescence signal of Cyt c
150 aggregates in untreated cells, but after treatment with different concentrations of CyNH₂, the
151 fluorescence signal of Cyt c began to diffuse. When cells were exposed to 8 µg mL⁻¹ CyNH₂ for 8
152 h, the green fluorescent signal was distributed throughout the cells. These results confirmed that
153 cells treated with CyNH₂ induce Cyt c release into the cytoplasm. Next, intracellular caspase-3
154 activity in 4T1 cells treated with different concentrations of CyNH₂ was studied. Intracellular
155 caspase-3 activity (Fig. 1I) exhibited a concentration-dependent increase. Compared to the negative

control, intracellular caspase-3 activity increased by 13.84-fold in response to treatment with 10 μ g mL⁻¹ CyNH₂ for 8 h. Recent studies claim that chemotherapeutic drug-induced activation of caspase-3 cleaves GSDME to produce a GSDME-N fragment that perforates membranes and induces pyroptosis³³. To further confirm CyNH₂-induced pyroptosis, intracellular levels of GSDME-FL and GSDME-N were detected by western blotting analysis. As shown in Fig. 1J and Fig. S12, GSDME-N was observed in CyNH₂-treated cells at high levels. Additionally, cells treated with CyNH₂ were stained with propidium iodide (PI) and annexin V-FITC to analyze apoptosis and necrosis. As shown in Fig. 1K and Fig. S13, compared to untreated cells, the percentages of necrotic cells significantly increased with increasing concentrations of CyNH₂ for 12 h. In summary, we conclude that when CyNH₂ aggregation in mitochondria causes mitochondrial membrane damage, Cyt c is released into the cytoplasm and activates caspase-3, which cleaves GSDME, generating a GSDME-N fragment that perforates membranes and finally induces pyroptosis (Fig. 1L).

We next examined the cytotoxicity of CyNH₂ in different cell lines. As shown in Fig. S14, the cancer cell lines 4T1 and CT26, human lung adenocarcinoma (A549), human cervical cancer cells (HeLa), human breast cancer cells (MCF-7), mouse melanoma cells (B16), and mouse pancreatic cancer (Panco2)) were effectively killed by CyNH₂, with half maximal inhibitory concentration (IC₅₀) values ranging from 1.91 to 9.58 μ M. The live/dead assay (Fig. S15) was consistent with the cytotoxicity study. Normal cells, such as mouse embryonic fibroblasts (MEFs), human embryonic kidney cells (293T) and human umbilical vein endothelial cells (HUVECs), were also killed by CyNH₂ with IC₅₀ values of 3.08, 1.86 and 3.19 μ M, respectively. The high toxicity and poor cell selectivity seriously hampers the application of CyNH₂ in cancer treatment.

Tumor cell selectivity of NCyNH₂. In recent decades, chemotherapy use has been severely restricted due to the severe side effects caused by high toxicity and poor tumor selectivity. To improve the tumor selectivity of chemotherapy and selectively release anticancer drugs in response to stimuli in tumor microenvironments, such as enzymes, pH, ROS, etc., extensive efforts have

181 been devoted to developing stimuli-responsive prodrugs³⁴. Since enzymatic reactions are selective,
182 effective, and involved in all physiological, metabolic, and pathological processes, they have been
183 increasingly used as a trigger for responsive nanostructures. Extensive studies have shown that
184 NQO1 is upregulated up to 100-fold in pancreatic, breast, lung, cervical, and colorectal cancers³⁵.
185 Thus, an NQO1-responsive CyNH₂ theranostic (denoted as NCyNH₂) was designed and
186 synthesized according to Scheme S2 and characterized in Fig. S16-S18.

187 NCyNH₂ is initially weakly fluorescent, as CyNH₂ is in a caged state with diminished electron-
188 donating capacity of the nitrogen atoms³⁶. In this state, the toxicity of NCyNH₂ is also low. The
189 fluorescence and cytotoxicity of CyNH₂ is recovered when NCyNH₂ is degraded by NQO1 to
190 release free CyNH₂ (Fig. 2A). We first studied the fluorescence change in NCyNH₂ (2 µg mL⁻¹)
191 after incubation with NQO1 (40 µg mL⁻¹) at different time points. The fluorescence intensity of
192 NCyNH₂ (Fig. 2B) significantly increased with incubation time, indicating that CyNH₂ is released
193 in free form from NCyNH₂ upon incubation with NQO1. Then, the intracellular fluorescence of
194 CyNH₂ was studied by incubating NCyNH₂ (1 µg mL⁻¹) with 4T1 cells and MEFs. Cells were
195 incubated with NCyNH₂ or CyNH₂ (1 µg mL⁻¹) for 4 h and further incubated for 12 h before being
196 observed by CLSM. For some of the groups, cells were pretreated with an NQO1 inhibitor
197 (dicoumarol (DIC), 50 µM) for 1 h to inhibit intracellular NQO1 activity. As shown in Fig. 2C,
198 cells coincubated with CyNH₂ showed a strong red fluorescence signal with or without NQO1
199 inhibitor pretreatment. In contrast, cells coincubated with NCyNH₂ showed a strong red
200 fluorescence signal, while cells pretreated with the NQO1 inhibitor showed a very weak
201 fluorescence signal. MEFs with low NQO1 levels were incubated for an additional 20 h after 4 h
202 of coincubation with NCyNH₂ or CyNH₂ (1 µg mL⁻¹). Only cells coincubated with CyNH₂ showed
203 a strong red fluorescence signal, while cells treated with NCyNH₂ exhibited a diminished
204 fluorescence signal. These results indicate that NCyNH₂ has good selectivity for tumor cells
205 overexpressing NQO1.

206 To further confirm whether NCyNH₂ induces pyroptosis, the morphology of 4T1 cells after
207 treatment with NCyNH₂ was observed by confocal microscopy. We observed that 4T1 cells clearly
208 presented cell membrane ballooning after treatment with NCyNH₂ (Fig. 2D). However, pyroptotic
209 cells were significantly reduced when intracellular NQO1 activity was inhibited by the NQO1
210 inhibitor in the NCyNH₂ group, while the inhibitor had no effect on cells treated with CyNH₂. These
211 cells were further stained with propidium iodide (PI) and annexin V-FITC to analyze apoptosis and
212 necrosis. As presented in Fig. S19, the percentages of necrotic cells were significantly reduced
213 when intracellular NQO1 activity was inhibited by the NQO1 inhibitor in the NCyNH₂ group,
214 while there were no differences in cells treated with CyNH₂. The cell morphology changes of MEFs
215 after treatment with CyNH₂ or NCyNH₂ were also studied, and the results are shown in Fig. 2E.
216 Most of the cells clearly showed membrane ballooning after CyNH₂ treatment, while cells treated
217 with NCyNH₂ showed little pyroptosis. These results indicate that NCyNH₂ has good selectivity to
218 induce pyroptosis in cancer cells overexpressing NQO1. To further confirm this phenomenon, the
219 cytotoxicity of NCyNH₂ to different cell lines was examined. As shown in Fig. S20 and Fig. 2F,
220 cancer cells (4T1, CT26, A549, HeLa, MCF-7, B16, Panco2) were effectively killed by NCyNH₂,
221 with IC₅₀ (48 h) values between 3.21 and 10.4 μ M, which is comparable to CyNH₂. In contrast, the
222 IC₅₀ (48 h) values of NCyNH₂ for MEFs, 293T cells and HUVECs increased 4.3-fold, 7.8-fold and
223 5.4-fold compared to that of CyNH₂. These results confirm that NCyNH₂ has high selectivity for
224 cancer cells overexpressing NQO1.

225 **Mature mouse bone marrow-derived dendritic cells (BMDCs) and *in vivo* prophylactic**
226 **tumor vaccination study.** Recent studies have demonstrated that tumor cell pyroptosis can boost
227 antitumor immunity¹⁷. To further verify whether NCyNH₂-induced tumor cell pyrolysis initiates
228 antitumor immunity, ATP and high mobility group box 1 (HMGB1) were monitored in the cell
229 supernatant after treatment with different concentrations of NCyNH₂. Fig. 3A shows that HMGB1
230 was primarily localized in the nucleus of 4T1 cells without any treatments and that treatment with

NCyNH₂ induced HMGB1 release from the nucleus. The concentration of HMGB1 in the supernatant of cells treated with NCyNH₂ was analyzed through enzyme-linked immune sorbent assay (ELISA). As shown in Fig. 3B, a large amount of HMGB 1 was released from the nucleus of 4T1 cells after treatment with NCyNH₂. Moreover, 4T1 cells treated with NCyNH₂ exhibited increased ATP secretion (Fig. 3C). Furthermore, we investigated NCyNH₂-induced antitumor immunity by testing DC maturation. Bone marrow-derived dendritic cells (BMDCs) were separated from BALB/c mice and incubated with 4T1 cells pretreated with different concentrations of NCyNH₂ (Fig. 3D). Upregulated levels of costimulatory molecules, such as CD80 and CD86, were detected to evaluate DC maturation *in vitro*. As shown in Fig. 3E and 3F, the results showed negligible DC maturation after incubation with untreated 4T1 cells for 24 h because the tumor cells had low immunogenicity. The frequency of mature DCs significantly increased when DCs were incubated with NCyNH₂-pretreated 4T1 cells. 4T1 cells pretreated with NCyNH₂ (6 μg mL⁻¹) exhibited a 3.1-fold higher DC maturation rate (approximately 58.6%) than cells without any treatments. These results indicate that tumor cell pyroptosis induced by NCyNH₂ has high immunogenicity.

Furthermore, *in vivo* vaccination was performed to verify whether NCyNH₂-induced tumor cell pyrolysis induced antitumor immunity. 4T1 tumor cells pretreated with NCyNH₂ were subcutaneously injected into the left armpit of male BALB/c mice at 0 and 7 d, and mice were injected with 4T1 cells without any treatment into the contralateral flank 14 days after immunization (Fig. 3G). Doxorubicin (DOX), a typical chemodrug that induces immunogenic death of tumor cells and causes antitumor immunity, was used as a positive control³⁷. As shown in Fig. 3H, the effect of vaccination with PBS-treated cells on contralateral tumor growth was negligible, while both DOX- and CyNH₂-treated cells prevented (or delayed) challenged tumor growth. It is worth noting that through the end of the study (17 days), 5 of 6 mice in the NCyNH₂-treated group were tumor-

free. These results indicate that 4T1 cell death induced by NCyNH₂ boosts antitumor immunity to prevent tumorigenesis.

***In vivo* antitumor effects of localized delivery of NCyNH₂.** Localized therapies, such as regional radiotherapy, photodynamic therapy and photothermal therapy, can induce immunogenic cell death to activate antitumor immunity and thereby inhibit the growth of distant tumors, which is known as the “abscopal effect”³⁸⁻⁴¹. We further studied whether localized administration of NCyNH₂ also elicits an “abscopal effect” through a bilateral subcutaneous tumor model. DOX induces immunogenic death of cancer cells and activates immune systems to inhibit the growth of distant tumors and was used as a positive control⁴². In this respect, we established a bilateral subcutaneous tumor model in immunocompetent mice whose left (termed “local tumor”) and right (termed “distant tumor”) second breast pads were subcutaneously inoculated with 4T1 cells. When the local tumor reached approximately 100 mm³, therapeutic agents were injected into this tumor four times, while the distant tumor was deliberately left untreated (Fig. 4A). As shown in Fig. 4B, little body weight change occurred during the treatments, indicating negligible side effects. Tumor volume growth curves are shown in Fig. 4C. Compared to the PBS group, the tumor volumes of both local and distant tumors exhibited a slight increase after treatment with NCyNH₂ or DOX. The tumor weight and corresponding tumor images are shown in Fig. 4D and 4E, which were consistent with the tumor inhibition results. To further evaluate the therapeutic effect *in vivo*, histological analysis by hematoxylin and eosin (H&E) staining, apoptosis detection by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining and cell proliferation activity detection by Ki67 staining of tumor sections were performed, and results are shown in Fig. S21A. The results clearly demonstrated that tumors treated with DOX or NCyNH₂ presented severe fragmentation and nuclear shrinkage in the tumor area, decreased tumor cell proliferation and an increased percentage of tumor cell apoptosis, consistent with the results of the therapeutic study. At the end of treatment, the lungs of all mice after different treatments were collected and analyzed

by H&E staining. H&E staining of lung tissue is shown in Fig. S21B. The mice treated with PBS showed obvious lung metastasis, while mice treated with NCyNH₂ or DOX showed little or no lung metastases. These results indicate that localized delivery of NCyNH₂ effectively suppresses the growth of local and distant tumors and inhibits tumor metastasis, confirming that localized treatments with NCyNH₂ elicit abscopal antitumor effects. Next, we studied whether local and distant tumor growth- and metastasis-related inhibition is attributable to enhanced antitumor immunity. CD8⁺ cytotoxic T lymphocytes (CTLs) actively eliminate tumor cells after being activated by DCs presenting tumor antigens, which play the most important role in the antitumor immune response⁴³, while regulatory T cells (Tregs) interfere with effector T cells and DC function to suppress antitumor immunity⁴⁴. CD11c⁺ differentiation was detected to evaluate levels of infiltrating DCs in primary tumors in response to different treatments. As shown in Fig. S22A and B, the presence of DCs (CD45⁺ CD11c⁺) in tumor tissues was significantly increased after treatment with NCyNH₂ or DOX, and the percentage of mature DCs (CD45⁺CD11c⁺CD80⁺, Fig. S22C and D) was high in the primary tumor, indicating that this treatment significantly promoted tumor antigen presentation. Flow cytometry analysis revealed a significantly increased presence of CTLs (CD45⁺CD3⁺CD8⁺) and decreased Tregs (CD45⁺CD3⁺CD4⁺Foxp3⁺) in both local and distant tumors within the tumor microenvironment after treatment with NCyNH₂ and DOX (Fig. 4A-4D). These results indicate that NCyNH₂ represents a good strategy to induce selective tumor cell pyroptosis and boost the antitumor immune response. Tumor draining lymph nodes (TDLNs) are key sites where mature DCs present captured antigens to T cells for functionally priming CD8⁺ T cells towards antitumor immunity⁴⁵. CTLs in TDLNs were also analyzed. As shown in Fig. S23A-23B, the percentage of CTLs in TDLNs significantly increased. TNF-α and IFN-γ in the serum after different treatments were quantitatively detected by ELISA. Mice treated with DOX or NCyNH₂ exhibited significantly elevated concentrations of TNF-α (Fig. 4J) and IFN-γ (Fig. 4K) compared to those treated with PBS, indicating that treatment caused a stronger antitumor immune response.

305 These results demonstrate that tumor cell pyroptosis induced by NCyNH₂ induces strong antitumor
306 immunity.

307 **Preparation and characterization of NCyNP.** Although NCyNH₂ has a satisfactory
308 therapeutic effect, poor water solubility hinders its systemic delivery. In this case, we selected the
309 FDA-approved amphiphilic polymer PEG_{5k}-*b*-PLGA_{8k} to encapsulate NCyNH₂, and the obtained
310 nanoparticles were denoted NCyNP. As shown in Fig. S24, the average hydrodynamic particle size
311 of NCyNPs was approximately 150 nm. The loading ratio of NCyNH₂ was calculated as 6%
312 according to the standard curve of NCyNH₂. Confocal images in Fig. S25A show that CyNH₂ can
313 also be released and activated in tumor cells overexpressing NQO1. As shown in Fig. S25B, we
314 observed that 4T1 cells clearly presented cell membrane ballooning after treatment with NCyNP.
315 The cytotoxicity test demonstrated that NCyNP elicited higher cytotoxicity in tumor cells than in
316 normal cells, as shown in Fig. S25C. These results indicate that NCyNP also has good selectivity
317 for tumor cells overexpressing NQO1. Encouraged by these promising imaging results *in vitro*, we
318 further examined the feasibility of NCyNP as an *in vivo* NIR fluorescence-tracked prodrug
319 activation system utilizing the NIR CyNH₂ channel. The results are shown in Fig. S26A. NIR
320 fluorescence was clearly observable 4 h after intravenous injection, and fluorescence intensity
321 gradually increased over time. When NQO1 activity in the tumor microenvironment was inhibited
322 by intratumoral injection of DIC, the NIR fluorescence intensity significantly weakened, indicating
323 that less CyNH₂ was released from NCyNPs. The tumors and major organs were collected from
324 mice for *ex vivo* imaging 36 h after intravenous injection, as shown in Fig. S26B, and quantitative
325 analysis of the NIR fluorescence intensity is shown in Fig. S26C. The results clearly show that the
326 fluorescence intensity in tumors is significantly higher than in non-tumor tissues, indicating that
327 NCyNPs have good selectivity for tumors. These results suggest that NCyNPs can be used as
328 theranostics to allow real-time monitoring of drug activation, which holds great promise for
329 personalized medicine.

***In vivo* antitumor activity of NCyNP plus α PD-1.**

Immune checkpoint blockade has been very successful in clinical cancer treatment, but the response rate is low^{15,46}. The primary reason for this low response rate is ineffective infiltration and/or activation of T lymphocytes¹⁵. The inflammation caused by pyroptosis in the tumor microenvironment can recruit and activate large numbers of T lymphocytes to improve the response rate of immune checkpoint blockade. Encouraged by the robust antitumor immune response induced by NCyNH₂, we surmised that this strategy might enhance the anticancer efficacy of checkpoint inhibitors, such as α PD1. Therefore, we established a 4T1 tumor-bearing Balb/c mouse model, which is known to have a low response rate to α PD1 treatment¹⁷, to test the treatment effect of NCyNP combined with the immune checkpoint inhibitor α PD1. Briefly, tumor-bearing mice were randomly divided into four groups (n = 6) and treated with saline, α PD1, NCyNP or NCyNP plus α PD1 (6 mg kg⁻¹ NCyNH₂, 100 μ g α PD1 per mouse). As shown in Fig. 5A, NCyNP therapy was administered by i.v. injection on days 0, 3, 5 and 7, and α PD-1 therapy was administered by i.v. injection on days 6, 8, 10 and 12. Tumor volume (Fig. 5B) and tumor weight (Fig. 5C) in the NCyNP plus α PD1 group were significantly lower than in the other groups, and the NCyNP plus α PD1 group presented the highest tumor inhibition rate, reaching 71.5% (Fig. 5E). The corresponding tumor images are shown in Fig. 5D, which was highly consistent with the tumor inhibition study. H&E staining, Ki67 staining and TUNEL staining of tumor sections (Fig. S27) clearly demonstrated that NCyNP plus α PD1 is an effective strategy to reduce tumor cell proliferation and increase the percentage of tumor cell apoptosis, consistent with the results of the therapeutic study. In addition, no significant weight loss or tissue damage in mice was observed through all treatments (Fig. S28 and S29), indicating that NCyNP-induced antitumor therapy is highly biocompatible. All of the above results suggest that NCyNPs can synergize with α PD1 to further enhance the antitumor effect.

To understand the effect of immune cells in combination therapy, tumor tissues and TDLNs were separated at the end of the antitumor study to detect the immune cell populations. As shown

in Fig. 5F-L, significant enrichment of CTLs in tumor tissue and mature DCs in TDLNs was observed in the NCyNP and NCyNP plus α PD1 groups. Moreover, CTLs and mature DCs in mice treated with NCyNP plus α PD1 were significantly higher than in mice treated with NCyNP. Further flow cytometry analysis showed a significantly increased presence of CTLs and decreased Tregs in tumors within the tumor microenvironment in mice treated with NCyNP and NCyNP plus α PD1. Compared to the PBS groups, mice treated with NCyNP or NCyNP plus α PD1 exhibited significantly increased concentrations of IFN- γ (Fig. S30A) and TNF- α (Fig. S30B) in the serum, indicating that NCyNP plus α PD1 induced a stronger antitumor immune response. These results indicate that NCyNH₂ improves the response rate of α PD1 and causes a strong immune response to enhance solid tumor therapy.

The above results confirmed that pyroptosis (initiated by NCyNP) promotes the infiltration and activation of T lymphocytes in the tumor microenvironment and synergizes with α PD1 to induce antitumor immunity. We further verified whether this antitumor strategy is applicable in other tumor models. To this end, CT26 tumor-bearing Balb/c mice were randomly divided into four groups (n = 6) and treated as described in the 4T1 tumor model. As shown in Fig. S31A, the antitumor efficacy was similar to that in the 4T1 tumor model, and NCyNP plus α PD1 treatment exhibited the most effective inhibition of tumor growth. No significant body weight changes, shown in Fig. S31B, indicate negligible side effects of this treatment. Additionally, while all mice in the PBS and α PD1 groups died within 32 days, mice in the NCyNP group took 44 days to die, and the NCyNP plus α PD1 group exhibited 33.3% animal survival within 50 days (Fig. S31C). Therefore, NCyNP plus α PD1 could be an effective antitumor agent and prolongs survival in tumor-bearing mice.

Encouraged by NCyNP plus α PD-1 administration, which effectively activated the antitumor immune response, we surmised that it could generate an immune memory effect to prevent tumor recurrence. To this end, the spleen was separated to measure the proportions of memory T cells 7

380 days after the final treatment by flow cytometry. As shown in Fig. 6A-6D and Fig. S32, the
381 percentages of T_{CM} and T_{EM} in mice treated with NCyNP plus α PD-1 were significantly higher than
382 the other groups. In addition, a lung metastatic tumor model was established as previously reported.
383 Briefly, primary tumors were surgically removed from CT26 tumor-bearing mice 7 days after the
384 final treatment, and then CT26 tumor cells were injected into mice by intravenous infusion (Fig.
385 6E). Compared to the other groups, mice treated with NCyNP plus α PD-1 had the fewest metastatic
386 foci in the whole lung. Furthermore, H&E staining confirmed that the number and size of metastatic
387 nodules in the combined group were significantly reduced (Fig. 6F). The lung wet weight in the
388 NCyNP plus α PD-1 groups was significantly lower than in the other groups (Fig. 6G). These results
389 indicate that mice treated with NCyNP plus α PD-1 generate an immune memory effect to prevent
390 tumor recurrence.

391 In summary, we first reported that the commonly used NIR fluorogenic hemicyanine CyNH₂
392 induces pyrolysis to kill cancer cells and studied its mechanism to induce cell pyrolysis.
393 Furthermore, a tumor cell-overexpressing NQO1-responsive theranostic (NCyNH₂) was designed
394 for selective cell pyroptosis. NCyNH₂ has high selectivity for cancer cells overexpressing NQO1,
395 and changes in the NIR fluorescence signal of CyNH₂ allow real-time monitoring of CyNH₂
396 activation. More importantly, cancer cell pyroptosis induced by NCyNH₂ can boost antitumor
397 immunity responses for solid tumor immunotherapy. NCyNH₂ was also encapsulated in PEG-*b*-
398 PLGA as a theranostic nanocarrier (NCyNP) for systemic administration and real-time detection of
399 CyNH₂ activation by an *in vivo* fluorescence imaging system. NCyNPs synergized with α PD1 to
400 further enhance the antitumor effect and produce long-term memory efficacy.

401

402 **METHODS**

403 2,3,3-Trimethylindolenine, iodoethane, phosphorus oxychloride (POCl₃), cyclohexanone, tin
404 chloride (SnCl₂), triethylamine (TEA), *p*-nitrophenyl chloroformate (NPC), 3-nitrophenylboronic

acid, meta-chloroperbenzoic acid (m-CPBA), 2,2'-dithiodiethanol, 1,6-hexanediol, triphosgene were purchased from Energy Chemical (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Dalian Meilun Biotechnology Co. (Dalian, China). All other chemicals used in the experiments were of analytical grade and used without further purification. Hoechst 33342 were purchased from Thermo Fisher Scientific (Waltham, USA). RPMI 1640, trypsin-EDTA and penicillin–streptomycin were obtained from Gibco BRL (Eggenstein, Germany). Fetal bovine serum (FBS) was obtained from ExCell Biology, Inc (Shanghai, China). Recombinant human DT diaphorase (NQO1), dicoumarol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. Calcein-AM/PI Double Stain Kit was purchased from Bestbio (Shanghai, China). Flow cytometric antibodies for immune cell labeling were purchased from biolegend. Anti-mouse-PD1(CD279) was purchased from BioXcell.

NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. The absorption spectra were measured on a UV-3802 (UNICO, Shanghai, China) spectrophotometer. Photoluminescence spectra were measured on a Shimadzu RF-6000 spectrofluorometer (Shimadzu UV-2600, Japan). Confocal images were acquired by confocal microscope (CLSM, Nikon Ti-E A1, Japan). Absorbance and fluorescence intensity were measured by a multifunctional micropore detection board analysis system (Biotek Cytation5, BioTek, United States).

Synthesis of compound 1. 2,3,3-Trimethylindolenine (1.6 g, 10.0 mmol) and iodoethane (2.34 g, 15.0 mmol) were dissolved in CH₃CN (30 mL). The mixture was refluxed for 24 h. After the removal of solvent under reduced pressure, the resulting crude product was washed with n-hexane three times to obtain a white solid product of compound **1** (2.8 mg, 89.7%).

Synthesis of compound 2. The CH₂Cl₂ solution of phosphorus oxychloride (5.3 mL, 34.5 mmol) and cyclohexanone (1.48 g, 15.0 mmol) were added dropwise into the mixture of *N,N*-dimethylformamide (DMF, 6.0 mL) and CH₂Cl₂ (10 mL) in an ice bath under N₂ atmosphere. The

reaction mixture was stirred and refluxed for 5 h. Then the mixture was poured into ice and stay overnight. The mixture was filtered to collect a yellow solid product of compound **2** (2.0 g, 78%).

Synthesis of compound 3. A mixture of compound **1** (2.5 g, 8.0 mmol), compound **2** (0.7 g, 4.0 mmol) and sodium acetate (0.67 g, 8.0 mmol) in acetic anhydride (40 mL) was stirred at 130 °C for 1 h. After the removal of solvent under reduced pressure, the crude product was washed with sodium bicarbonate solution and extracted with dichloromethane. The solvent was removed under reduced pressure and the crude product was purified by silica gel flash chromatography with dichloromethane/methanol in a volume ratio of 20:1 to afford compound **3** as a green powder (2.04 g, 80%).

Synthesis of 3-nitrophenol (compound 4). 3-Nitrophenylboronic acid (0.8 g, 5.0 mmol) and m-CPBA (1.0 g, 6mmol) were added into the flask at room temperature was dissolved in a H₂O: EtOH (1:2) solution (10 mL) in a round-bottom flask. Sodium bicarbonate solution (50 mL) was added to the mixture after 3-nitrophenylboronic acid was completely consumed. The mixture was then extracted with ethyl acetate (2×50 mL). The organic layer was collected and washed with sodium chloride solution (50 mL), dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and then the crude product was purified by silica gel flash chromatography with n-hexane/ethyl acetate in a volume ratio of 4:1 to afford 3-nitrophenol as a colorless solid of compound **4** (0.57 g, 86%).

Synthesis of compound 5. A mixture of compound **3** (640 mg, 1.0 mmol), 3-nitrophenol (167 mg, 1.2 mmol) and triethylamine (121 mg, 1.2 mmol) and in DMF (10 mL) was stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure and the precipitate was dissolved in CH₂Cl₂, followed by washing with sodium bicarbonate solution for three times and dried over anhydrous sodium sulfate. The crude product was purified by silica gel flash chromatography with dichloromethane/methanol in a volume ratio of 20:1 to afford compound **5** as a golden powder (606 mg, 85%).

Synthesis of CyNH₂. Compound **5** (374 mg, 0.5 mmol) was dissolved in 10 mL CH₃OH. SnCl₂ (2.4 g, 12.5 mmol) dissolved in concentrated HCl (2.0 mL) was added slowly to the above solution and refluxed overnight. Then, the mixture was neutralized by saturated NaHCO₃. The precipitate was obtained by filtration and washed with CH₂Cl₂. The collected filtrate was washed with water and dried over anhydrous magnesium sulfate. The solvent was removed by evaporation under reduced pressure, and the crude product was purified by silica gel flash chromatography with dichloromethane/methanol in a volume ratio of 50:1 to afford CyNH₂ as a green powder (189 mg, 72%).

Synthesis of compound 6. 2,3,5-Trimethylhydroquinone (4.0 g, 26.2 mmol) and 3,3-dimethacrylic acid (2.9 g, 29.0 mmol) were dissolved in methanesulfonic acid (20 mL), then the reaction mixture was stirred at 85°C for 3 h under nitrogen atmosphere. After the mixture was cooled to room temperature, 700 mL of water was added and stirred for 20 mins, then the mixture was extracted with ethyl acetate (3×50 mL). The collected organic layer was successively washed with sodium saturated sodium bicarbonate solution (3×100 mL) and water (3×100 mL). After dried over anhydrous magnesium sulfate, the organic phase was concentrated and recrystallized with n-hexane/ethyl acetate (1:1) to obtain a white solid (4.9 g, 80%).

Synthesis of compound 7. Compound **6** (3.16 g, 13.48 mmol) and NBS (2.52 g, 14.16 mmol) were dissolved in a mixed solvent (30 mL acetonitrile and 6 mL water), and stirred for 30 min at room temperature. After the reaction, the collected organic layer was extracted with dichloromethane (3×50 mL). The organic layer was collected and dried with anhydrous magnesium sulfate, and the product was obtained after vacuum distillation (3.2 g, 94.8%).

Synthesis of NCyNH₂. Compound **7** (0.8 g, 3.2 mmol), 2-(7-azabenzotriazole)-*N,N,N',N'*-tetramethylurea hexafluorophosphate (HATU) (1.52 g, 4.0 mmol) and *N,N*-diisopropylethylamine (DIPEA) (500 μL) was dissolved in dichloromethane (60 mL). After stirred at 0°C for 30 min, CyNH₂ (524 mg, 1.0 mmol) was dissolved in dichloromethane (10 mL) and added dropwise to the

above reaction solution, then the reaction mixture was stirred 24 h at 35°C. The reaction mixture was precipitated into a large amount of methanol, collected the white solid and dissolve in *N, N*-dimethylformamide, then precipitated into methanol again, and the precipitation was repeated three times to obtain NCyHN₂ (295 mg, 39%).

Cell line and animals. Human breast cancer cells (MCF-7), human umbilical vein endothelial cells (HUVECs) and mouse embryonic fibroblast (MEF) were cultured in DMEM medium with 10% FBS, 1% penicillin-streptomycin. Mouse breast cancer cells (4T1), mouse colon cancer cells (CT26), human pancreatic cancer cells (Panc-2), human embryonic kidney cells (293T), human cervical cancer cells (HeLa), human lung adenocarcinoma (A549) and mouse melanoma cells (B16) were cultured in 1640 medium with 10% FBS, 1% penicillin-streptomycin. Cells were cultured in 5% CO₂ and 21% O₂ incubator at 37 °C. Female BALB/c mice (20 ± 2 g, 5–6 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China). Animal studies were performed according to a protocol approved by the Ethics Committee of the South China University of Technology.

***In vitro* cellular uptake.** The cellular uptake of CyNH₂ was investigated by using a confocal laser scanning microscope (CLSM, Zeiss LSM 410, Germany). Briefly, 4T1 cells or CT26 cells were seeded into a 35 mm glass-bottom dishes at a density of 1×10⁵ cells and incubated overnight. The cells were treated with 1 µg mL⁻¹ CyNH₂ for 0.5, 1, 2 or 4 h, respectively. After incubation, the cells were then washed with PBS and stained with Hoechst 33342 and studied by CLSM. The quantitative evaluation of cellular uptake was further evaluated by flow cytometry analysis. Briefly, 4T1 cells or CT26 cells were seeded in 24-well plates at a density of 1×10⁵ cells per well. After 24 h incubation, the culture medium was replaced with fresh medium containing 1 µg mL⁻¹ CyNH₂ for 0.5, 1, 2 or 4 h, respectively. After incubating, the medium was removed, and the cells were washed with PBS for three times. The cells were trypsinized and 0.5 mL of cell suspension was taken to quantify the internalization by flow cytometry (BD Biosciences, USA).

Intracellular localization of CyNH₂. 4T1 or CT26 cells were seeded into 35 mm glass-bottom dishes at a density of 5×10^4 cells and incubated overnight. Cells were incubated with $1 \mu\text{g mL}^{-1}$ CyNH₂ for 4 h. For colocalization experiments, cells were washed with PBS, and then 200 nM MitoTracker Green (Thermo Fisher, USA) was added and incubated at 37°C for 45 min. After staining with Hoechst 33342, the medium was removed, and cells were rinsed with PBS and imaged immediately by CLSM.

Mitochondrial membrane potential. Mitochondrial membrane potential was measured by MitoProbe™ JC-1 Assay Kit (Beyotime, China). 4T1 cells were seeded into a 35 mm glass-bottom dishes at a density of 5×10^4 cells and incubated overnight. Then the cells were treated with different concentrations (0, 1, 2, 4 and $6 \mu\text{g mL}^{-1}$) of CyNH₂ for 6 h at 37 °C, cells were washed three times with PBS and incubated with 50 nM JC-1 for 30 min. After stained with Hoechst 33342, the cells were washed, incubated with fresh medium, and imaged immediately by CLSM. For quantitative analysis, 4T1 cells were seeded into a 24-well plate at a density of 1×10^5 cells and incubated overnight. Then the cells were treated with different concentrations (0, 1, 2, 4 and $6 \mu\text{g mL}^{-1}$) of CyNH₂ for 6 h at 37 °C, cells were washed three times with PBS and incubated with 50 nM JC-1 for 30 min. After washed with PBS, the cells were collected, and the fluorescence intensity of cells were analyzed by flow cytometry.

OCR and MRR measurements. The OCR was measured using an Oxygen Consumption Rate Assay Kit (BestBio, China), according to the manufacturer's instructions. It is based an oxygen sensitive fluorescent probe with a maximum excitation wavelength at 468 nm and a maximum emission wavelength at 603 nm. Its fluorescence reacts with oxygen through molecular collision and is quenched by O₂. Therefore, the amount of fluorescent signal is inversely proportional to the amount of extracellular O₂ in the sample. Thus, it can be used to monitor changes in extracellular oxygen consumption. Briefly, 4T1 cells were seeded into an opaque-walled 96-well at a density of 1×10^4 cells each well and incubated overnight. Then the cells were treated with different

529 concentrations (0, 1, 2, 4 and 6 $\mu\text{g mL}^{-1}$) of CyNH₂ for 4 h at 37 °C. The cells were washed with
530 PBS and incubated with 150 μL fresh medium containing 10 μL oxygen sensitive fluorescent probe,
531 then 100 μL oxygen blocking fluid was seeded into each well. The change in the fluorescence signal
532 over time were reader by the microplate reader, the excitation was 468 nm, and the emission filter
533 was 603 nm. The OCR was calculated from the change in the fluorescence signal over time.

534 The MRR were measured according to previously reported methods. Briefly, 4T1 cells were
535 seeded into a 24-well at a density of 1×10^5 cells each well and incubated overnight. Then the cells
536 were treated with different concentrations (0, 1, 2, 4 and 6 $\mu\text{g mL}^{-1}$) of CyNH₂ for 4 h at 37 °C. The
537 cells were washed with PBS buffer and incubated with fresh medium for 4 h, and half of the cells
538 were treated with 2 μM of oligomycin A (Sigma, USA) to inhibit the synthesis of ATP. After that,
539 the cells in each group were harvested and lysed. The level of intracellular ATP was quantitatively
540 determined by ATP bioluminescent assay kit according to manufacturer's instruction (Beyotime).
541 The MRR in support of ATP production was calculated before/after the oligomycin treatment.

542 **Intracellular ROS levels.** To study oxidative stress caused by CyNH₂ treatment, levels of
543 intracellular ROS were evaluated using the cell-permeable ROS sensor 2',7'-dichlorofluorescein
544 diacetate (DCFH-DA, Sigma, USA), which can be oxidized by ROS to form fluorescent
545 dichlorofluorescein (DCF). 4T1 cells were seeded into 35 mm glass-bottom dishes at a density of
546 5×10^4 cells and incubated for 24 h. Afterward, the medium was discarded, and cells were incubated
547 with fresh medium containing different concentrations (0, 1, 2, 4 and 6 $\mu\text{g mL}^{-1}$) of CyNH₂ for 8 h.
548 Cells were then washed with PBS followed by DCFH-DA staining at 37°C for 20 min. DCF
549 fluorescence images were acquired by confocal microscopy and quantitatively measured by a
550 multifunctional micropore detection board analysis system.

551 **Cellular ATP activity assay.** Cellular ATP levels were measured using a firefly luciferase-
552 based ATP assay kit (Beyotime, China) according to the manufacturer's instructions. Briefly, 4T1
553 cells were seeded in an opaque-walled 96-well at a density of 1×10^4 cells each well and incubated

overnight. After the cells were incubated with fresh medium containing different concentrations (0, 1, 2, 4 and 6 $\mu\text{g mL}^{-1}$) of CyNH₂ for 8 h, the cells in each group were harvested and lysed. The level of intracellular ATP was quantitatively determined by ATP bioluminescent assay kit according to manufacturer's instruction.

Cell morphology change. The morphology change of 4T1 cells were monitored by taking bright-field images every 10 minutes between 30 min and 130 min after incubation with CyNH₂ (8 $\mu\text{g mL}^{-1}$) on a CLSM. A time-lapse video was generated using the bright-field images to show the morphology changes.

Lactate dehydrogenase (LDH) release study. The amount of LDH in cell cultural medium was determined by colorimetry based on an 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride chromogenic reaction catalyzed by diaphorase with LDH Release Assay Kit (Beyotime, China). 4T1 cells was seeded in 96-well plates at a density of 1×10^4 per well. After overnight incubation, CyNH₂ at different concentrations (0, 2, 4, 6, 8 and 10 $\mu\text{g mL}^{-1}$) were added into the wells for 12 h incubation. LDH release reagents was added to the maximum enzyme activity sample control wells (untreated cell wells for subsequent lysis) by 10% of the volume of original medium 1h before the measurement. Later the cells were centrifuged at 400 g for 5min to obtain the supernatant. Subsequently, 120 μL of the supernatants were added into corresponding wells of a new 96-wells plate. Then 60 μL of LDH detection reagents was added to each well and incubated in the dark for 30 min after blending. The absorbance (A) was measured at 490 nm. The rates of LDH release were calculated as follows: $(A_{\text{drug-treated sample}} - A_{\text{sample control}}) / (A_{\text{the maximum enzyme activity sample control}} - A_{\text{sample control}}) \times 100 \%$.

Cyt c release and caspase-3 activation. For Cyt c release, 4T1 cells were seeded into a 35 mm glass-bottom dishes at a density of 5×10^4 cells per well for overnight. The cells were incubated with CyNH₂ at a concentration of 0, 2, 4, 6 or 8 $\mu\text{g mL}^{-1}$ for 6 h. Then the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed three times with PBS. After fixation,

the cells were permeabilized by 0.1% Triton X-100 in PBS and incubated with a FITC anti cytochrome c antibody (Thermo Fisher, USA) at 4 °C overnight. After stained with Hoechst 33342, the cells were washed with PBS for 3 times and imaged by confocal laser scanning microscope. For caspase-3 activity study, 4T1 cells was seeded in 96-well plates at a density of 1×10^4 per well. After overnight incubation, CyNH₂ at different concentrations (0, 2, 4, 6, 8 and 10 $\mu\text{g mL}^{-1}$) were added into the wells for 8 h incubation. Intracellular caspase-3 activation was monitored by Caspase-Glo[®] 3/7 Assay (Beyotime, China) according to the manufacturer's instructions.

Western blot analysis. 4T1 cells were seeded into 24-well plates at a density of 1×10^5 per well. After overnight incubation, CyNH₂ at different concentrations (0, 3, 5, 10 $\mu\text{g mL}^{-1}$) was added to the wells for 8 h incubation. Then, cells were lysed in buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, and protease inhibitors and clarified by spinning at 14,000 RPM for 10 min at 4°C. Cell lysates were fractionated by 12% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes with anti-GSDME (Abcam, ab215191) used as the primary antibody.

***In vitro* Annexin V-FITC/PI assay.** Apoptosis assays against 4T1 cells were performed to evaluate the death mechanism of CyNH₂. 4T1 cells were seeded into 24-well plates at a density of 1×10^5 per well and incubated overnight. Then, cells were incubated with different concentrations of CyNH₂ (2, 4, 6, 8, or 10 $\mu\text{g mL}^{-1}$) for 12 h. Cells were stained with Annexin V-FITC/PI (Beyotime, China) according to the instructions of the kit and then analyzed by FACS.

***In vitro* cytotoxicity assay.** *In vitro* cell cytotoxicity was evaluated in MCF-7, 4T1, CT26, A549, B16, HeLa, Panc-2, MEF, HUVECs and 293T cells using a standard MTT assay. Briefly, cells at a density of 1×10^4 per well were seeded into 96-well plates. After overnight incubation, CyNH₂ was added at different concentrations to the wells for 24 or 48 h incubation. Afterward, MTT solution (10 μL , 5 mg mL^{-1}) was added to each well for an additional 4 h. The medium containing MTT solution was discarded, and dimethyl sulfoxide (DMSO, 200 μL per well) was

604 added to dissolve formazan crystals for 30 min. Finally, a microplate reader was used to measure
605 absorbance at 490 nm.

606 ***In vitro* live/dead assay.** 4T1 cells were seeded into 35 mm glass-bottom dishes with a density
607 of 1×10^5 per well. After treated with different concentrations (0, 2, 4, 6, 8 and $10 \mu\text{g mL}^{-1}$) of
608 CyNH₂ for 24 h, the Live/Dead kit (BestBio, China) was used for imaging cell viability. The dyes
609 Calcein AM and Propidium Iodide (PI) in the kit stains live and dead cells, respectively. The
610 fluorescence of Calcein AM and PI was observed by CLSM.

611 **Fluorescence recovery of NCyNH₂.** Fluorescence changes of NCyNH₂ were investigated by
612 fluorescence spectrophotometry after incubation with NQO1. Briefly, NCyNH₂ ($2 \mu\text{g mL}^{-1}$, 10%
613 DMSO in PBS) treated with NQO1 ($40 \mu\text{g mL}^{-1}$) and the changes in fluorescence over time were
614 studied. The fluorescence changes of NCyNH₂ were further investigated in 4T1 cells. Briefly, 4T1
615 cells were seeded into 35 mm glass-bottom dishes at a density of 1×10^5 cells and incubated
616 overnight. 4T1 cells were incubated for an additional 12 h after 4 h of coincubation with NCyNH₂
617 or CyNH₂ ($1 \mu\text{g mL}^{-1}$), and cells were pretreated with or without an NQO1 inhibitor (DIC, $50 \mu\text{M}$)
618 for 1 h to inhibit intracellular NQO1 activity. Afterward, cells were washed with PBS, stained with
619 Hoechst 33342, and observed by CLSM.

620 ***In vitro* cytotoxicity of NCyNH₂.** *In vitro* cytotoxicity of NCyNH₂ to MCF-7, 4T1, CT26,
621 A549, B16, HeLa, Panc-2, MEF, HUVECs and 293T cells were evaluated using a standard MTT
622 assay. Briefly, cells at a density of 1×10^4 per well were seeded into 96-well plates. After overnight
623 incubation, NCyNH₂ was added at different concentrations to the wells for 24 or 48 h incubation.
624 Afterward, MTT solution ($10 \mu\text{L}$, 5 mg mL^{-1}) was added to each well for an additional 4 h. The
625 medium containing MTT solution was discarded and DMSO ($200 \mu\text{L}$ per well) was added to
626 dissolve formazan crystals for 30 min. Finally, a microplate reader was used to measure absorbance
627 at 490 nm.

ATP and HMGB-1 release. Extracellular secretion of ATP was tested using an ATP assay kit. Briefly, 4T1 cells were seeded into a 24-well at a density of 1×10^5 cells each well and incubated overnight. Afterward, the medium was discarded, and cells were incubated with fresh medium containing different concentrations (0, 2, 4 and $6 \mu\text{g mL}^{-1}$) of NCyNH₂. After 12 h, the supernatants were collected and then conducted with centrifugation using 12,000 rpm for 10 min at 4 °C. The level of secreted ATP was quantitatively determined by ATP bioluminescent assay kit according to manufacturer's instruction. Intracellular HMGB1 distribution was tested using immunofluorescence analysis. Briefly, 4T1 cells were seeded in a 35 mm glass-bottom dishes at a density of 1×10^5 cells and incubated overnight. Afterward, the medium was discarded and cells were incubated with fresh medium containing different concentrations (0, 2, 4 and $6 \mu\text{g mL}^{-1}$) of NCyNH₂ for 12 h. The supernatants were collected and then conducted with centrifugation using 12,000 rpm for 10 min at 4 °C, the extracellular levels of HMGB1 were quantitatively determined by mouse high mobility group protein B1ELISA kit (CUSBIO, CSB-E08225m) according to manufacturer's instruction. The cells were washed with PBS for three times, then fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 4% BSA, the cells were incubated with anti-HMGB1 antibody (Proteintech, 10829-1-AP) for 1 h at room temperature, and then incubated with Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher, R37116) for 30 min. The cells were then stained with Hoechst 33342 for 20 min and observed using CLSM.

BMDC maturation *in vitro*. To investigate DC maturation *in vitro*, BMDCs were generated from the bone marrow of 5- to 6-week-old BALB/c mice. 4T1 cells were pretreated with different concentrations of NCyNH₂ (0, 2, 4, $6 \mu\text{g mL}^{-1}$). Cells pretreated with PBS were used as a negative control. Afterwards, 1×10^6 immature DCs were cocultured with 1×10^5 4T1 cells pretreated with NCyNH₂. After staining with anti-CD11c-FITC (Clone: N418), anti-CD80-PE (Clone: 16-10A1)

652 and anti-CD86-APC (Clone:GL-1) antibodies, the maturation of DC cells was examined by using
653 flow cytometry.

654 ***In vivo* prophylactic tumor vaccination study.** The female Balb/c mice were randomly
655 divided into three group with each group containing 6 mice. The 4T1 cells pretreated with CyNH₂
656 or DOX (positive control) were used as cancer vaccines for *in vivo* experiment. The healthy mice
657 were immunized with the corresponding cancer vaccines (containing 2×10⁶ cells in 100 μL sterile
658 PBS) twice on day 0 and day 7, respectively, by subcutaneous injection into mouse right axilla. As
659 a control, sterile PBS instead of cancer cell vaccine was subcutaneously injected into the mice in
660 control group on day 0 and day 7, respectively. On day 14, all the mice in 4 groups were inoculated
661 with 1×10⁶ CT26 cells into the left axilla. After that, the tumor growth of 4 groups were monitored
662 by measuring the tumor volumes. The tumor volume (V) was calculated as $V = W^2 \times L/2$, where
663 W and L are the width and length of the tumor, respectively.

664 ***In vivo* antitumor activity assessment.** Female BALB/c mice were subcutaneously injected
665 with 4T1 cell suspension in the bilateral infra-axillary dermis (5×10⁵ cells on the right side per
666 mouse and 2×10⁵ cells on the left side per mouse). When the tumor volume on the right side reached
667 approximately 100 mm³, mice were randomly divided into three groups and intratumorally injected
668 with PBS, NCyNH₂ and DOX (3 mg kg⁻¹ DOX and 2 mg kg⁻¹ NCyNH₂) twice in the right tumor.
669 The tumor volumes and body weights were measured every other day. The tumor volume (V) was
670 calculated as $V = W^2 \times L/2$, where W and L are the width and length of the tumor, respectively.
671 The tumor weight and pulmonary metastasis in the lung were examined at day 27 after tumor
672 implantation. Lungs were fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining
673 followed by observation under an inverted microscope (Olympus IX71, Japan) equipped with a
674 DP73 digital camera.

675 **Preparation of NCyNP.** The NCyNP was prepared by nanoprecipitation method. Briefly, a
676 mixture containing PEG_{5k}-b-PLGA_{8k} (10 mg) and NCyNP (2 mg) were dissolved in 1.0 mL of

677 DMSO, and then gradually added into 10 mL of ultrapure water under stirring. After additional
678 stirring for 2 h, the solution was transferred into dialysis bag (MWCO 3500) to remove DMSO
679 against ultrapure water for 24 h, and the solution was filtered through a 0.45 μm filter to obtain
680 NCyNP.

681 **Characterization of NCyNP.** The size distribution was performed in triplicate using a zeta
682 sizer (Nano ZS, Malvern Instruments) at 25°C. The samples were diluted using ultrapure water.
683 The drug loading capacity of NCyNH₂ into PEG_{5k}-*b*-PLGA_{8k} was determined through UV
684 spectrophotometer based on the standard curve of NCyNH₂.

685 ***In vivo* combination therapy.** For breast tumor-bearing mouse model, Female BALB/c mice
686 were inoculated with 4T1 cells (5×10^5 cells per mouse) in right mammary pad. When the tumor
687 volume reached to approximately 60 mm³, the mice were randomly divided into four groups and
688 intravenously injected with PBS, $\alpha\text{PD-1}$, NCyNP and NCyNP plus $\alpha\text{PD-1}$ (6 mg kg⁻¹ of NCyNH₂,
689 100 μg per mouse of $\alpha\text{PD-1}$) for four times, respectively. The tumor volumes and body weights
690 were measured every other day. The mice were sacrificed 18 days. The histological analysis was
691 carried out after treatments. The tissues of each representative organs of the heart, liver, spleen,
692 lung, kidney, and tumor tissues of the mice in five groups were excised, fixed in 4%
693 paraformaldehyde, embedded in paraffin, sliced and stained with H&E. Tumor sections were also
694 stained with TUNEL apoptosis detection kit assay and then observed by CLSM.

695 For colon tumor-bearing mouse model, Female BALB/c mice were implanted with colon
696 tumor cells (5×10^5 cells per mouse) and received different treatments as described above (“*In vivo*
697 antitumor activity assessment” section). The tumor volumes and body weights were measured every
698 other day. Moreover, the survival rates were also monitored throughout the whole study.

699 **Anti-metastasis effect.** Subcutaneous colorectal tumors were established and treated with
700 different formulations as the methods described previously. The tumors were removed by surgery
701 7 days post last treatment, and 2×10^6 CT26 cells were administered intravenously *via* tail vein

infusion into each BALB/c mouse. 20 d later, after sacrificed mice, the lungs were photographed and weighted. The tissues were fixed in 4% formaldehyde and embedded in paraffin. Paraffin-embedded 8 µm lung sections stained with H&E were prepared for immunohistochemical analysis.

Analysis of immune cells. In order to understand the underlying mechanism of the antitumor effect aroused by NCyNH₂, a series of immune cells were analyzed by flow cytometry after staining with antibodies. For tumor tissue, tumors were excised, minced, and incubated in 1640 containing 1 mg mL⁻¹ collagenase type I (Sigma, USA), 1 mg mL⁻¹ collagenase type IV (Biosharp, China), 100 µg mL⁻¹ hyaluronidase (Sigma, USA) and 100 µg mL⁻¹ DNase I (Biofroxx, Germany) for 40 min at 37 °C. Tumor cell suspensions were passed through a 200-mesh nylon mesh and resuspended in 40% percoll after centrifugation to collect lymphocytes by centrifugation. Fc receptors were blocked using anti-CD16/32 mAb (Clone: 93). For the analysis of T cells, cells were stained by anti-CD45-APC-Cy7 (Clone: 104), anti-CD3-FITC (Clone: 17A2), anti-CD4-BV510 (Clone: RM4-5) anti-CD8-BV785 (Clone:53-6.7) and DAPI according to the supplier's protocol. For the analysis of regulatory cells (Tregs), cells were stained by anti-CD45-APC-Cy7 (Clone: 104), anti-CD3-FITC (Clone: 145-2C11), anti-CD4-BV510 (Clone: RM4-5), anti-Foxp3-PE (Clone: 150D) and DAPI according to the supplier's protocol. For the analysis of mature DCs, cells were stained by anti-CD45-APC-Cy7 (Clone: 104), anti-CD11c-PE-Cy7 (Clone: N418), anti-CD80-PE (Clone: 16-10A1) and DAPI according to the supplier's protocol. The axillary lymph nodes were harvested from the mice and prepared to single cell suspensions by mechanical grinding. Fc receptors were blocked using anti-CD16/32 mAb (Clone: 93). For the analysis of T cells, cells were stained by anti-CD45-APC-Cy7 (Clone: 104), anti-CD3-FITC (Clone: 17A2), anti-CD4-BV510 (Clone: RM4-5) anti-CD8-BV785 (Clone:53-6.7) and DAPI according to the supplier's protocol. For the analysis of mature DCs, cells were stained by anti-CD45-APC-Cy7 (Clone: 104), anti-CD11c-PE-Cy7 (Clone: N418), anti-CD80-PE (Clone: 16-10A1) and DAPI according to the supplier's protocol. Furthermore, the spleens were also collected from the mice, which were then prepared to single cell

suspensions by mechanical grinding. After that, the cell suspensions were harvested by centrifugation at 1,500 rpm for 5 min, followed by removal of red blood cells (RBCs) using modified RBC lysis buffer at 4 °C for 5 min and washing twice using 1×PBS. Fc receptors were blocked using anti-CD16/32 mAb (Clone: 93). For the analysis of T cells, cells were stained by anti-CD45-APC-Cy7 (Clone: 104), anti-CD3-FITC (Clone: 17A2), anti-CD4-BV510 (Clone: RM4–5) anti-CD8-BV785 (Clone:53–6.7) and DAPI according to the supplier’s protocol. For the analysis of memory T cells, cells were stained by anti-CD45-APC-Cy7 (Clone: 104), anti-CD3-BV610 (Clone: 17A2), anti-CD4-BV510 (Clone: RM4–5) anti-CD8-BV785 (Clone:53–6.7), anti-CD62L-FITC (Clone: MEL-14), anti-CD44-APC (Clone: IM7) and DAPI according to the supplier’s protocol. Flow cytometry was performed by BD FACS Celesta (BD Biosciences, USA).

Statistical analysis. All the obtained data were expressed as mean ± standard deviation. Statistical significance of the treatment groups was evaluated using Student’s t test or one-way analysis of variance (ANOVA) with the least significant difference post hoc test. ns, nonsignificant. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 were considered statistically significant in analyses.

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Competing interests: The authors declare that they have no competing interests.

Data availability: All the data supporting the findings of this study are available within the article and its Supplementary information files and from the corresponding author upon reasonable request.

Fig. 1. CyNH₂ induces cell pyroptosis. (A) The chemical structure, absorption and emission spectra of CyNH₂. (B) The intracellular localization of CyNH₂ in 4T1 cells. (C) Flow cytometry analysis of changes in mitochondrial membrane potential after incubation with different concentrations of CyNH₂. (D, E) The levels of intracellular reactive oxygen species (D) and intracellular ATP (E) after incubation with different concentrations of CyNH₂. (F) Cellular morphology changes over time in 4T1 cells pretreated with CyNH₂ (8 μg mL⁻¹) for 2 h; the white arrows indicate pyroptotic cells. (G-J) LDH release (G), intracellular localization of cytoplasmic cyto c (H), intracellular caspase-3 activation (I) and western blotting analysis of pyroptosis-related protein expression (GSDME-FL, GSDME-N) (J) in 4T1 cells after treatment with different concentrations of CyNH₂. (K) Flow cytometry analysis of apoptosis and necrosis in 4T1 cells treated with different concentrations of CyNH₂. (L) Schematic diagram of the mechanism of CyNH₂-induced cell pyroptosis. Data are presented as the mean ± SD. Statistical analysis was performed using the one-way ANOVA test (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

Fig. 2. Tumor cell selectivity of NCyNH₂. (A) Schematic of intracellular NQO1-triggered cleavage of quinone trimethyl lock moieties of NCyNH₂, accompanied by NIR emission turn-on and restored cytotoxicity due to release of CyNH₂. (B) Evolution of fluorescence emission spectra ($\lambda_{\text{ex}} = 670 \text{ nm}$) of NCyNH₂ ($2 \mu\text{g mL}^{-1}$) upon incubation with NQO1 ($40 \mu\text{g mL}^{-1}$, 0.1 mM NADH, 0.007% BSA; pH 7.4 PBS, 100 mM KCl) for different lengths of time. (C) Confocal images of 4T1 cells and MEFs treated with CyNH₂ or NCyNH₂ ($1 \mu\text{g mL}^{-1}$) for 24 h in the absence or presence of

an NQO1 inhibitor (50 μ M DIC, 1 h pretreatment). (D) Representative cell morphology of 4T1 cells after treatment with CyNH₂ or NCyNH₂ (8 μ g mL⁻¹) for 8 h in the absence or presence of an NQO1 inhibitor (50 μ M DIC, 1 h pretreatment). (E) Representative cell morphology of MEFs after treatment with CyNH₂ or NCyNH₂ (8 μ g mL⁻¹) for 8 h. (F) Comparison of IC₅₀ values of CyNH₂ and NCyNH₂ in different cell lines.

Fig. 3. Mature mouse bone marrow-derived dendritic cells (BMDCs) and *in vivo* prophylactic tumor vaccination study. (A) CLSM examination of HMGB1 release in 4T1 cells after treatment with different concentrations of NCyNH₂. Quantitative examination of HMGB1 release (B) and ATP secretion (C) in 4T1 tumor cells after treatment with different concentrations of NCyNH₂. (D) Schematics showing the experimental procedure of stimulating BMDCs with cell lysates from cancer cells after being treated with different concentrations of NCyNH₂. Representative flow cytometry images (E) and corresponding quantification (F) of mature DCs (CD11c⁺CD80⁺CD86⁺) after stimulation with 4T1 cell lysates and treatment with different concentrations of NCyNH₂. (G) Vaccination approach *in vivo* of NCyNH₂- or DOX-treated dying 4T1 cells incubated subcutaneously at 0 and 7 d, followed by injection of untreated cells in the opposite flank 14 days later. (H) Growth of second tumors after vaccination. Data are presented as the mean $\pm$ SD. Statistical analysis was performed using the one-way ANOVA test (* P < 0.05, ** P < 0.01, and *** P < 0.001).

Fig. 4. *In vivo* antitumor effects of localized delivery of NCyNH₂ in 4T1 tumor-bearing mice. (A) Schematic illustration of 4T1 tumor-bearing mice where only the local tumor (right side) received intratumoral injection (i.t.) of PBS, DOX (3 mg kg⁻¹) or NCyNH₂ (2 mg kg⁻¹) four times (7, 9, 11, 13 days after tumor implantation). (B) Body weight changes over the course of treatment. (C) Growth curves of local (right side) tumors and distant (left side) tumors in 4T1 tumor-bearing mice with local tumors intratumorally injected with PBS, NCyNH₂ or DOX as described in (A). (D, E) Tumor images (D) and relative tumor weights (E) of bilateral tumors (local and distant) isolated

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904 in tumor tissues at the end of antitumor therapy. (H, I) Corresponding quantification of the
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910 0.001).

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Fig. 5. *In vivo* antitumor activity of NCyNP plus αPD-1 in a breast tumor-bearing mouse model. (A) Schematic illustration showing *in vivo* treatment details. (B) Tumor growth curves after treatments with PBS, αPD-1, NCyNP, or NCyNP plus αPD-1. (C) The relative tumor weight of tumors from each group. (D) Tumor images from each group after animals were sacrificed. (E) Inhibition rates of tumor growth (IRT) in response to different treatments. (F, G) Representative flow cytometry images (F) and corresponding quantification (G) of the percentage of CD8⁺ T cells in the CD45⁺CD3⁺ tumor infiltrating leukocytes examined by flow cytometry. (H, I) Representative flow cytometry images (H) and corresponding quantification (I) of the percentage of Treg cells

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Fig. 6. Metastasis prevention via combination therapy-induced long-term immune memory effects. (A, B) The proportions of CD8⁺ central memory T cells (CD44^{hi}CD62 L^{hi}) (A) and CD8⁺ effector memory T cells (CD44^{hi}CD62 L^{low}) (B) in the spleen. (C, D) The proportions of CD4⁺ central memory T cells (CD44^{hi}CD62 L^{hi}) (C) and CD4⁺ effector memory T cells (CD44^{hi}CD62 L^{low}) (D) in the spleen. (E) Schematic illustration of NCyNP plus αPD-1 combination therapy to inhibit tumor metastasis. (F) The image shows lung metastatic nodules 20 d post i.v. infusion of CT26 cells and H&E staining of lung tissues collected at the end of experiments. (G) The lung wet weight 20 d post i.v. infusion of CT26 cells. Data are presented as the mean ± SD. Statistical analysis was performed using the Student's t test (ns, nonsignificant. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

Figures

Figure 1

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