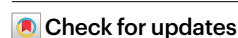


Nanorobots hold PD-L1 and break membrane of colorectal cancer cells for immunotherapy

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Limited immune cell infiltration is the main reason for poor immunotherapeutic efficacy in colorectal cancer patients. Here we design a peptide-based nanorobot that recognizes PD-L1 and breaks cancer cell membranes by in situ forming fibrils through a pH-responsive module. The nanorobot shows long retention in targeted tumours (>120 h) through interaction with PD-L1 and blocks PD-1/PD-L1 to activate the T cell killing effect. At the same time, in the tumour microenvironment (pH 6.5), it forms fibrils that break the cancer cell membrane, inducing immunogenic cell death with the release of damage-associated molecular patterns and the subsequent infiltration of T cells. The nanorobot shows higher therapeutic efficacy than the regimen of α PD-L1+oxaliplatin in a variety of colorectal-cancer-tumour-bearing mouse models and has good biocompatibility due to the targeted breakage of cancer cells, exhibiting great potential for colorectal cancer immunotherapy in clinic.

Currently, the blockade of programmed cell death protein 1 (PD-1) and its receptor programmed death ligand 1 (PD-L1) provides a tremendous prospect in cancer therapy^{1–5}. Despite the exciting progress of PD-1/PD-L1 blockade therapy of many cancers in clinic^{6–8}, over 95% colorectal cancer (CRC) patients are proficient mismatch repair (pMMR) cold tumour and fail to respond to PD-1/PD-L1 blockade^{9,10}, where immune cells can hardly infiltrate into the immunosuppressive tumour microenvironment (TME)^{11–13}. Strategies like chemotherapy¹⁴, radiotherapy¹⁵ and photothermal therapy¹⁶ can potentially reverse pMMR-type ‘cold’ tumours to ‘hot’ tumours through immunogenic cell death (ICD) and the release of damage-associated molecular patterns (DAMPs)^{17,18}, thereby remodelling the TME^{19–21} to sensitize cancer cells to immune checkpoint inhibitors^{22–26}. However, combining these

therapeutic methods may cause serious side effects, such as myelosuppression, diarrhoea or immune-related adverse events^{18,26}. There is, thus, a pressing need to develop effective immunotherapies with fewer side effects, specifically for pMMR CRC. DAMPs released through a molecular-targeted way combined with immune checkpoint inhibition are promising for pMMR-type CRC.

In this paper, we propose a peptide-grafted polymer-based nanorobot (NRb) that concurrently blocks PD-1/PD-L1 and releases DAMPs by disrupting the targeted cancer cell membranes. The NRb comprises three modules: (1) d-type ^dCNYSKPTDRQYHF (assigned as P) for PD-L1 binding and inhibition^{27,28}, (2) CFFVLKK-(PEG)_{1,000}-H₆G (assigned as K) for fibrillogenesis behaviour under the tumour pH microenvironment (6.5–6.8) due to the fibrillar FFVLK sequence^{29–31} and pH-responsive

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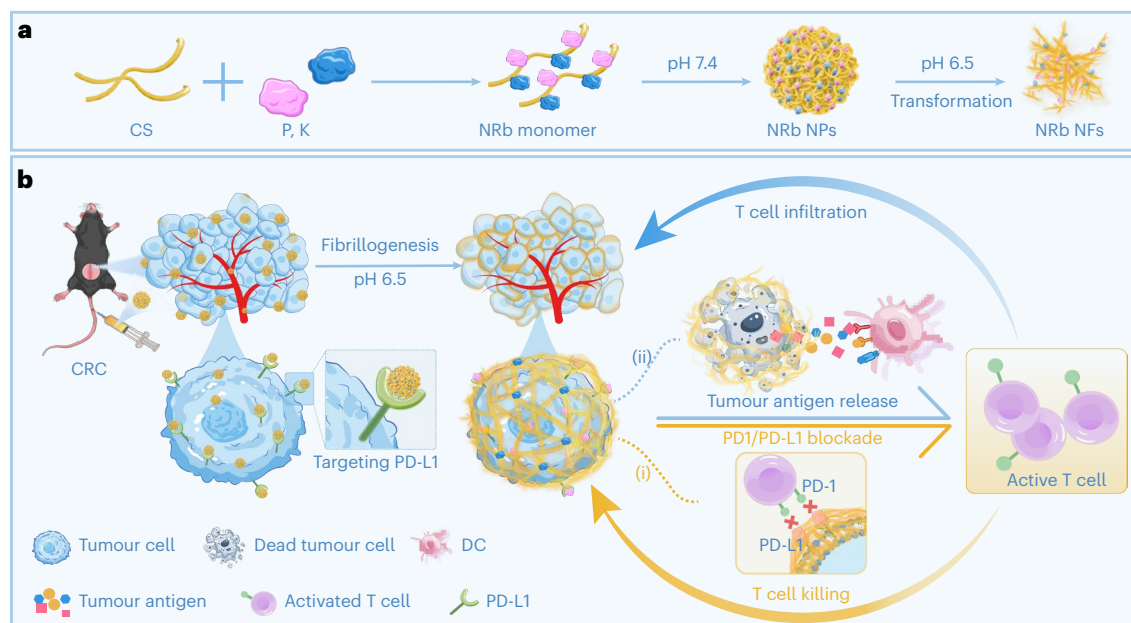


Fig. 1 | NRb anchors on tumour cells and transforms for PD-1/PD-L1 blockade and DAMP release. a, Schematic of the preparation of NRb with peptide P, PD-L1-targeting sequence; K, pH-responsive transformation unit; and CS unit. The NRb transformed from NPs into NFs under pH 6.5. **b**, NRb targeted tumour

accumulation through binding PD-L1 and in situ transforming at pH 6.5, followed by (i) long-term PD-1/PD-L1 blockade to rehabilitate T cell cytotoxicity and (ii) release tumour antigens to promote T cell infiltration in tumour through membrane breakage for enhanced immunotherapy.

(6.5) H_6 (refs. 32–34) with hydrophilic polyethylene glycol ($PEG_{1,000}$, $M_w = 1,000$ Da) as a spacer and (3) chitosan (CS) core capable of activating natural killer (NK) cells^{35,36} (Fig. 1a). NRbs are nanoparticles (NPs) at pH 7.4. NRbs bind to PD-L1 and transform into nanofibres (NFs)^{37–39} on the membrane of CT26/MC38 cells at pH 6.5, enabling stable PD-L1 blockade by forming a fibrous network, effectively restoring T cell cytotoxicity (Fig. 1b(i)). Concurrently, the transformation process mechanically breaks the cancer cell membrane, inducing targeted ICD and the release of DAMPs, which, in turn, recruit T cells (for example, $CD4^+/CD8^+$; Fig. 1b(ii)). In brief, one NRb combines two key points for immunotherapy, exhibiting both superior PD-1/PD-L1 blockade and T cell infiltration with good biocompatibility, providing an opportunity for pMMR CRC treatment in clinic.

Fibrillogenesis behaviour in solution and on PD-L1+ cell surface

NRb was synthesized by conjugating K and P to CS (Fig. 2a). Importantly, the PD-L1 binding and in situ targeted fibrillogenesis of NRb on cancer cells might break cell membranes^{40,41}, releasing tumour-related antigens to home T cells and boost immunotherapy. For comparison, the controls including C-NRb ($K' = CGGAACK(PEG)_{1,000}-H_6G$ and scrambled $P' = ^dCDNKHTPFYYQSR$), C-NRb1 (K:P = 0:1) and C-NRb2 (K:P = 1:0) were prepared. Acrylate double bonds in acryl-CS were calculated through ultraviolet–visible spectrophotometry (Supplementary Fig. 1 and Supplementary Table 1). The structures of acryl-CS, NRb, C-NRb and C-NRb1/C-NRb2 were established by ^1H nuclear magnetic resonance. Acrylate double bonds at 5.7–6.2 ppm in acryl-CS²⁹ disappeared along with the peptide peaks at around 6.6–7.8 ppm (Fig. 2b and Supplementary Figs. 2 and 3). NRb was purified through dialysing away excess K and P using dialysis tubing with a molecular weight cut-off of 3,500 Da (Supplementary Fig. 4).

NRb NPs transform into NFs at pH 6.5 due to the protonation of histidine (Fig. 2c). The transmission electron microscopy (TEM) images showed that NRb NPs (23.0 ± 1.7 nm at pH 7.4) fibrillated into NFs ($d = 10.09 \pm 2.58$ nm) after 24 h of incubation at pH 6.5, whereas C-NRb remained as NPs (20.8 ± 1.0 nm; Fig. 2d). Dynamic light scattering

revealed a hydrodynamic diameter (larger than the TEM diameter⁴²) of NRb from single size to multidisperse on cultivating NRb at pH 6.5, unlike C-NRb (Fig. 2e,f). Fourier transform infrared spectroscopy showed a characteristic C=O stretching vibration shifting from 1,642 to 1,631 cm^{-1} under pH 6.5 (Fig. 2g), implying the hydrogen-bonded β -sheet structure of NRb^{43,44}, which was absent in C-NRb groups (Fig. 2h). Cy5-labelled NRb (NRb-Cy5; Supplementary Fig. 5) incubating with thioflavin T (ThT) at pH 6.5 showed that the fibres (red) were well merged with green ThT (Pearson coefficient, 0.578), confirming the β -sheet structure in NRb NFs^{44–46}, not for NRb (pH 7.4) and other controls (Fig. 2i and Supplementary Fig. 6). NRb NFs gradually grew longer over time, further implying the transformation ability of NRb at pH 6.5 (Supplementary Fig. 7). For C-NRb1 and C-NRb2 controls, C-NRb1 maintained NPs, whereas C-NRb2 could fibrillate into NFs at pH 6.5 in solution as NRb (Supplementary Figs. 8 and 9).

For the transformation of NRb in cellular levels, PD-L1+ CT26/MC38 cells and PD-L1– human colon epithelial cells (HCoEpiCs) were chosen as the experimental cell lines. Compared with C-NRb-Cy5, NRb-Cy5 gradually accumulated on the PD-L1+ CT26/MC38 cell membrane from 1 to 4 h at pH 6.5 (Fig. 2j and Supplementary Figs. 10 and 11a). Minimal binding occurred for NRb-Cy5 at pH 7.4 or C-NRb-Cy5 groups. PD-L1– HCoEpiCs also showed no notable NRb binding on the membrane (Supplementary Fig. 11b). The quantification of the fluorescence signal (Fig. 2j, white dotted line) demonstrated the NRb anchoring on the CT26 cell membrane (Supplementary Fig. 12). Scanning electron microscopy (SEM) confirmed that many dense NRb NFs (orange arrow) and a few NRb NPs (white dotted circle) anchored on the CT26/MC38 cell membrane at pH 6.5 (Fig. 2k and Supplementary Fig. 13). By contrast, few NPs and no NFs were found on the cell membrane of NRb at pH 7.4, C-NRb or phosphate-buffered saline (PBS)-treated CT26/MC38 cells. Notably, the energy-dispersive X-ray spectroscopy of iodine (I)-labelled NRb-treated CT26 cells displayed a 2.38% of I-element composition in the fibrous structures on the membrane (Supplementary Fig. 14). The HCoEpiCs groups showed neither NFs nor NPs on the membrane (Supplementary Fig. 15). It was found that either C-NRb1 or C-NRb2 seldom accumulated on the membrane

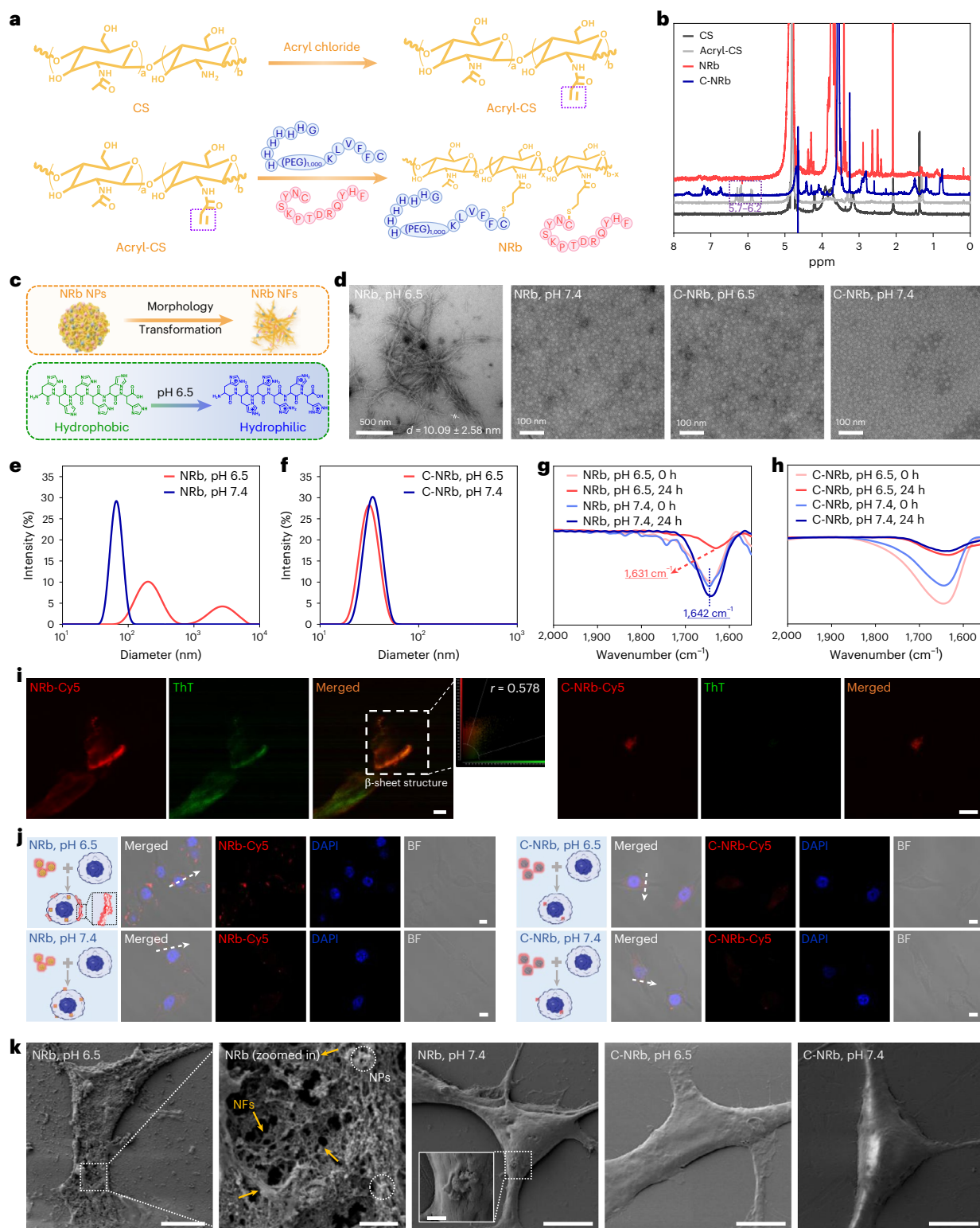


Fig. 2 | Preparation and pH-induced transformation of NRb. **a**, Synthesis route of acryl-CS and NRb. NRb was synthesized through the Michael addition reaction between the olefinic bond in acryl-CS and excessive thiol in K and P. The molar ratio of K:P was 1:1. The control group C-NRb was prepared using the same method by the reaction between acryl-CS, K' and P'. **b**, ^1H nuclear magnetic resonance of CS, acryl-CS, NRb and C-NRb in D_2O . **c**, Schematic of NRb NPs transforming into NRb NFs induced by the protonation of histidine under an acidic environment. **d**, TEM images of NRb (1.0 mg ml^{-1}) and C-NRb (1.0 mg ml^{-1}) incubated at pH 6.5 and pH 7.4 for 24 h, respectively. $n = 3$ independent samples. **e, f**, Size distribution of NRb (**e**) and C-NRb (**f**) cultured at pH 6.5 and 7.4 for 24 h.

g, h, Fourier transform infrared spectra of NRb (**g**) and C-NRb (**h**) at pH 6.5 and pH 7.4 for 0 and 24 h. **i**, CLSM images of Cy5-labelled NRb and C-NRb incubating with ThT ($20 \mu\text{M}$, green) for 24 h at pH 6.5. Scale bar, $5 \mu\text{m}$. $n = 3$ independent samples. **j**, Scheme and CLSM images of CT26 cells cultivated with NRb or C-NRb for 4 h at pH 6.5 or pH 7.4. Scale bar, $10 \mu\text{m}$. $n = 3$ independent samples. **k**, SEM images of CT26 cells incubated with NRb or C-NRb at pH 6.5 or pH 7.4, respectively ($n = 3$ biological independent samples). Scale bars, $10 \mu\text{m}$, $1 \mu\text{m}$ (zoomed-in images). Red channel, $\lambda_{\text{ex}} = 647 \text{ nm}$, $\lambda_{\text{em}} = 649\text{--}693 \text{ nm}$; green channel, $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{--}550 \text{ nm}$; blue channel, $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 415\text{--}465 \text{ nm}$.

(Supplementary Fig. 16a–d). Also, although C-NRb2 was capable of fibrillating into NFs in solution under pH 6.5, it failed to observe NFs on CT26 cell surface (Supplementary Fig. 16e). The above results validated that NRb accumulated on the cell membrane by recognizing PD-L1 via P module, and this binding, in cooperation with the K module, induces *in situ* fibrillogenesis under acidic pH. Both P and K components were indispensable for this process. Interestingly, it was easily found that NRb-treated CT26 cells exhibited notable membrane disruption (Fig. 2k).

Break membrane and release antigens to activate immune *in vitro*

It is conceivable that NRb probably disrupted the cellular membrane. NRb treatment at pH 6.5 caused calcein (white arrow) leakage, whereas calcein was retained in the cytoplasm in other control groups (Fig. 3a and Supplementary Figs. 17 and 18). Strikingly, obvious gaps (white arrow) were found on NRb-treated cell membranes (red, Dil staining) at pH 6.5 (Fig. 3b and Supplementary Figs. 19 and 20). The cytoplasmic green calcein fluorescence in the NRb-treated group at pH 6.5 was much lower than those in controls (Supplementary Fig. 21). Also, lactate dehydrogenase (LDH; a biomarker of membrane breakage^{47,48}) leakage in the NRb-treated medium was significantly enhanced at pH 6.5 (Fig. 3c). Furthermore, cryogenic TEM detection demonstrated membrane breakage with some cellular content release of CT26 cells by NRb at pH 6.5, whereas the PBS or NRb (pH 7.4) group showed a clearly intact membrane structure (Supplementary Fig. 22), validating the breakage of the cell membrane.

The interaction of NRb and CT26 cells was also studied by atomic force microscopy by detecting live or paraformaldehyde-fixed CT26 cells. Obvious NRb NFs (white arrow) adhered to cell membranes after NRb NPs or preformed NF treatment for 24 h, compared with other controls (Fig. 3d and Supplementary Figs. 23 and 24a–c). The stiffness and Young's modulus of cells treated with NRb NPs or preformed NFs for 24 h were also significantly higher than other controls (Fig. 3e and Supplementary Fig. 24d). The cantilever force versus indentation distance curves showed a notably increased slope in NRb NP (24 h) and NRb NF (Fig. 3f) groups. Similar results were found in paraformaldehyde-fixed CT26 cells of NRb NP and NRb NF groups at 24 h (Supplementary Fig. 25). These results indicated that *in situ*-formed or *in situ*-preformed NRb NFs could anchor on the cell surface through binding PD-L1 and increase the stiffness of cells.

Membrane-breakage-induced cancer cell death by NRb was then studied by Cell Counting Kit-8 (CCK-8) assays. It revealed that

NRb of $>1.0 \text{ mg ml}^{-1}$ at pH 6.5 exhibited significant cytotoxicity on CT26 cells, with a cell viability of $58.8 \pm 4.8\%$, but non-cytotoxicity under pH 7.4 (Fig. 3g). Other three control molecules showed good biosafety to CT26 cells under both pH 6.5 and pH 7.4 (Fig. 3g and Supplementary Fig. 26). NRb and C-NRb exhibited a similar tendency of cytotoxicity for MC38 cells with CT26 cells, but were always non-toxic to HCoEpiCs (Supplementary Figs. 27 and 28). The time-dependent CCK-8 assays of NRb (1.0 mg ml^{-1}) showed that the CT26 cell death rate gradually increased over time (Supplementary Fig. 29). The live–dead staining measurements exhibited that more than half of dead CT26/MC38 cells (red) were in NRb groups at pH 6.5, whereas most of the cells were alive (green) in other groups (Supplementary Fig. 30). These results suggested that NRb could break the cell membrane and even selectively kill PD-L1+ tumour cells at a concentration over 1.0 mg ml^{-1} at pH 6.5.

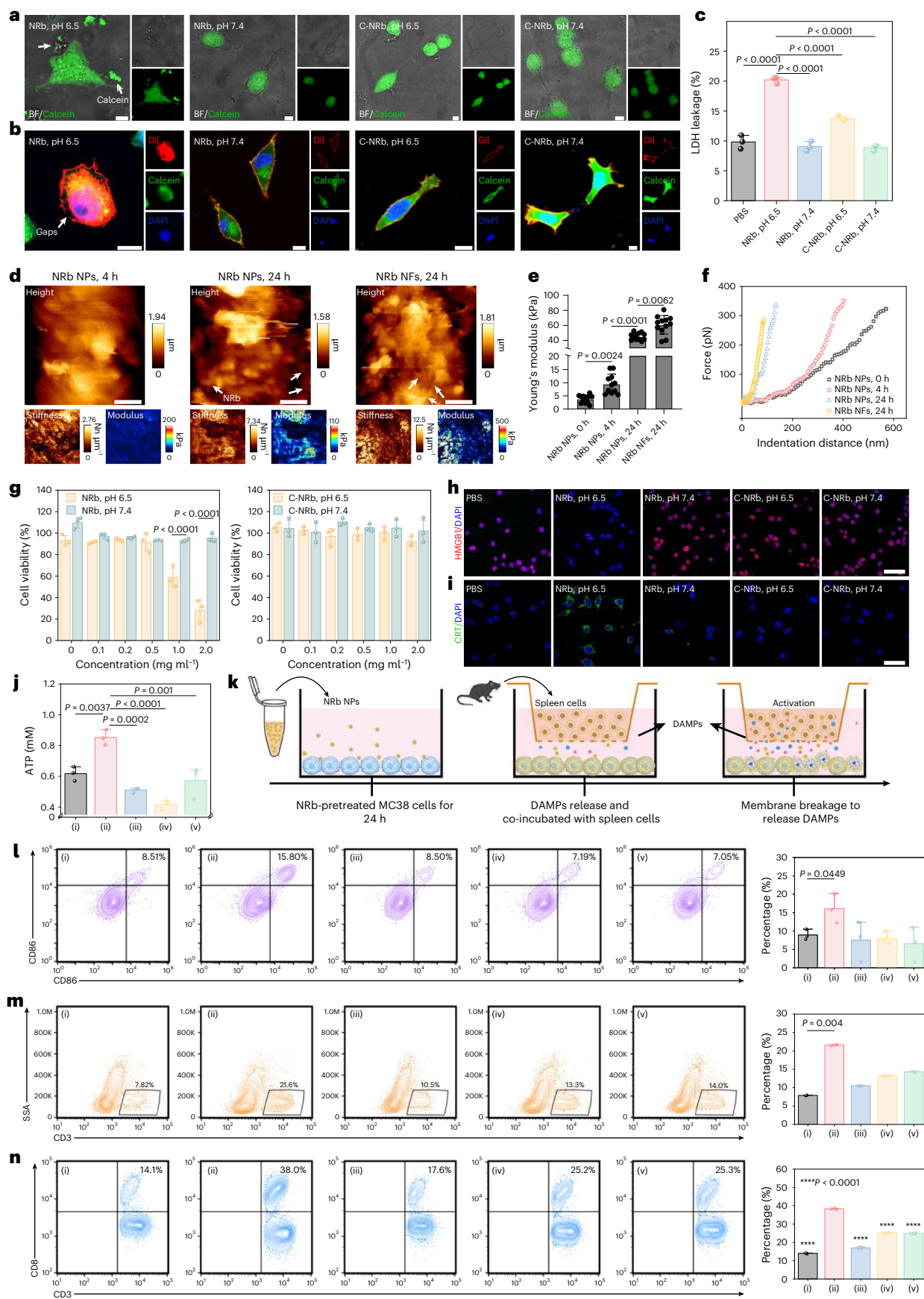
Interestingly, preformed NRb NFs (1.0 mg ml^{-1}) showed negligible toxicity to PD-L1+ CT26/MC38 cells over time (Supplementary Fig. 31). Additionally, there was much more green calcein in the cytoplasm in NRb-NF-treated cells than the NRb NP group at pH 6.5 (Supplementary Fig. 32a,b). The cell membrane breakage rate in the NRb NP group was calculated to be $51.2 \pm 8.2\%$, significantly higher than the preformed NRb NF group ($16.4 \pm 4.1\%$; Supplementary Fig. 32c), consistent with the CCK-8 results. This stark contrast in the potent cytotoxicity of *in situ*-transformed NRbs clearly indicates that membrane disruption is not caused by the mere physical presence of stiff fibres. Instead, it must result from the dynamic mechanical forces generated during the process of PD-L1 binding and the subsequent *in situ* transformation from NPs to NFs.

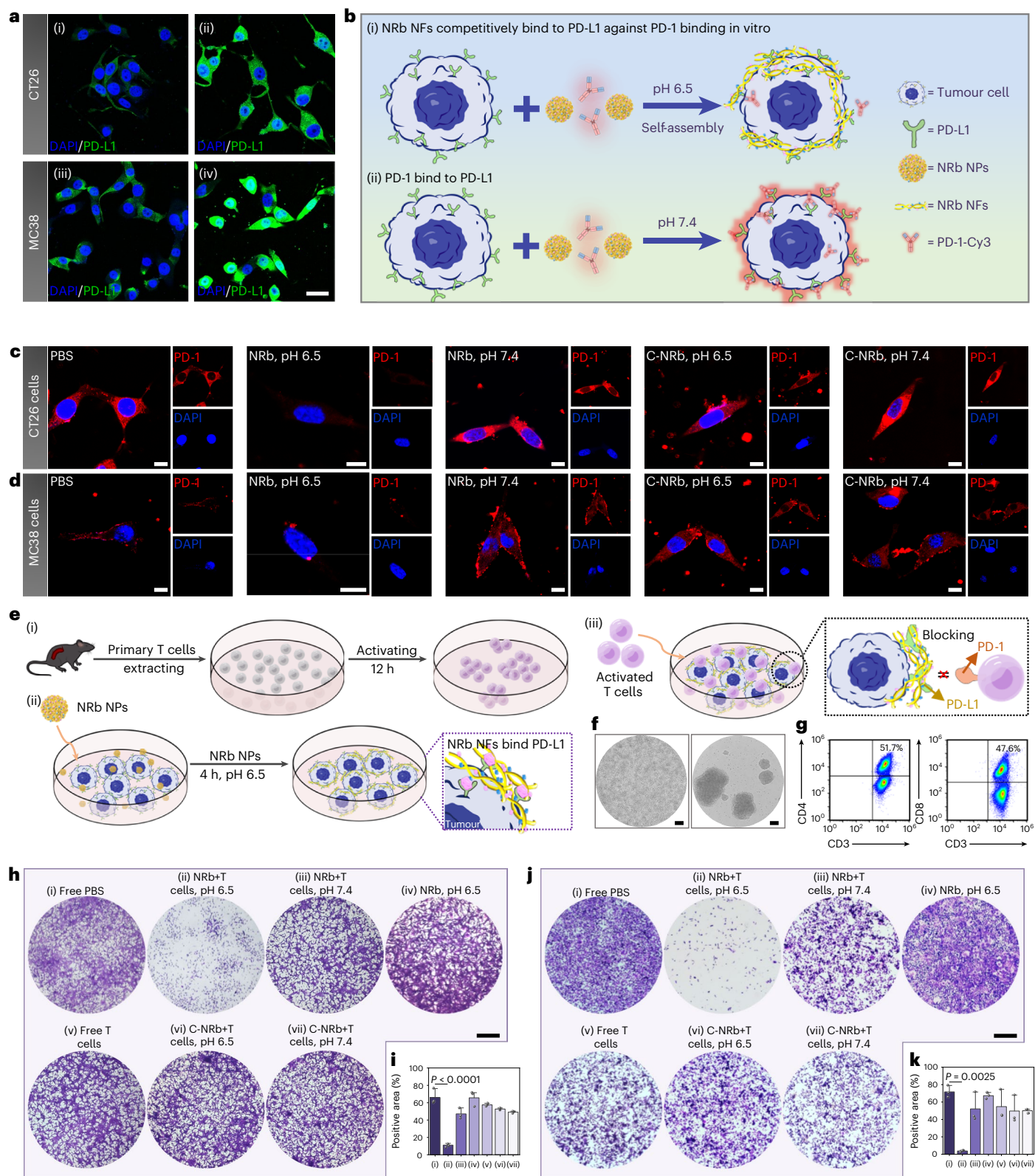
The time-dependent confocal laser scanning microscopy (CLSM) showed that red NRb-Cy5 (white arrow) gradually aggregated on the cell membrane within 4 h at pH 6.5, but did not cause cell death (Supplementary Video 1). When extending the incubation time, time-dependent CLSM (Supplementary Fig. 33 and Supplementary Videos 2 and 3) and three-dimensional holographic tomography showed that NRb at pH 6.5 could induce cell rounding, shrinkage, collapse and death, which was fairly different from controls including preformed NRb NFs (Supplementary Fig. 34 and Supplementary Videos 4–7), further indicating that NRb fibrillogenesis *in situ* disrupted the targeted cell membrane.

To study tumour antigen release through membrane breakage, DAMPs such as high mobility group box-1 (HMGB1), calreticulin (CRT) and ATP of CT26 cells after incubation with NRb NPs were detected. Compared with other controls, NRb-treated cells at pH 6.5 revealed

Fig. 3 | NRb breaks cell membrane by *in situ* fibrillogenesis behaviour to release DAMPs. **a**, CLSM images of CT26 cells co-culturing with $0.2 \mu\text{M}$ of calcein (green) for 0.5 h, followed by the treatment of NRb or C-NRb at pH 6.5 or pH 7.4 for another 24 h. Scale bar, $7.5 \mu\text{m}$. $n = 3$ independent samples. **b**, CLSM images of CT26 cells treated the same way as in **a**, plus staining the cell membrane with Dil and nucleus with DAPI. Scale bar, $10 \mu\text{m}$. $n = 3$ independent samples. **c**, LDH leakage of CT26 cells in the culture medium after treating with PBS, NRb or C-NRb at pH 6.5 and pH 7.4. Data are presented as mean $\pm$ s.d., $n = 3$ biological independent samples. Statistical significance was calculated through one-way ANOVA with Tukey's post hoc test. **d**, Height, stiffness and Young's modulus of CT26 cells incubated with NRb NPs for 4 h and 24 h, as well as NRb NFs for 24 h at pH 6.5 measured by atomic force microscopy. Scale bar, $2 \mu\text{m}$. $n = 3$ independent samples. **e**, Quantification of Young's modulus of CT26 cells in **d**. Data are presented as mean $\pm$ s.d., $n = 12$ per group ($n = 11$ for the 4-h group), and $n = 3$ independent samples. Statistical significance was measured by Welch's ANOVA test. **f**, Cantilever force versus indentation distance on the CT26 cells shown in **d**. The force curves were registered with respect to the onset of force increase. **g**, Cell viability of CT26 cells treated by NRb (left) and C-NRb (right) with different concentrations at pH 6.5 and pH 7.4, respectively. Data are presented as mean $\pm$ s.d., $n = 3$ independent experiments. Statistical significance was calculated by one-way ANOVA with Tukey's post hoc test. **h,i**, HMGB1 (**h**) and CRT (**i**) immunofluorescence

assay of CT26 cells treated with NRb under pH 6.5. Scale bar, $50 \mu\text{m}$. $n = 3$ independent samples. **j**, Released ATP of CT26 cells in the culture medium after treating with PBS (**i**), NRb at pH 6.5 (**ii**) and pH 7.4 (**iii**) and C-NRb at pH 6.5 (**iv**) and pH 7.4 (**v**). Data are presented as mean $\pm$ s.d., $n = 3$ biological independent samples. Statistical significance was calculated by one-way ANOVA with Tukey's post hoc test. **k**, Schematic of the transwell model for studying the T cell activation through DAMPs released by NRb at pH 6.5 *in vitro*. **l**, Representative flow cytometry plots and the quantitative analysis of DC maturation (CD80⁺CD86⁺) gating on CD11c⁺ cells. Data are presented as mean $\pm$ s.d., $n = 3$ biological independent samples. Statistical significance was calculated by one-way ANOVA test. **m**, Flow cytometric analysis images and the corresponding quantitative analysis of CD3⁺ T cells from the spleen of mice after incubation with the transwell system. Data are presented as mean $\pm$ s.d., $n = 3$ biological independent samples. Statistical significance was measured through a Kruskal–Wallis test. **n**, Representative results and the quantitative analysis of CD8⁺ T cells gating on CD3⁺ cells from the spleen of mice. (**i**), PBS; (**ii**), NRb, pH 6.5; (**iii**), NRb, pH 7.4; (**iv**), C-NRb, pH 6.5; (**v**), C-NRb, pH 7.4). Data are presented as mean $\pm$ s.d., $n = 3$ independent experiments. *P* values were determined by one-way ANOVA with Tukey's post hoc test. Red channel, $\lambda_{\text{ex}} = 555 \text{ nm}$, $\lambda_{\text{em}} = 570\text{--}620 \text{ nm}$; green channel, $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{--}550 \text{ nm}$; blue channel, $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 415\text{--}465 \text{ nm}$.





lower HMGB1 in the nucleus (Fig. 3h) and higher CRT expression in the cytoplasm (Fig. 3i), showing promise in boosting the immune response^{49,50}. ATP that can abundantly secrete by dying cells and bind with the P_2X_7 receptor of dendritic cells (DCs)^{14,51} was significantly increased in the NRb medium at pH 6.5 than controls (Fig. 3j), promising to promote DC maturation in immunotherapy. In a transwell system (Fig. 3k), DAMPs from NRb-treated MC38 cells at pH 6.5 promoted

DC maturation ($CD80^+CD86^+$; Fig. 3l) and significantly activated $CD3^+$ T cells (21.6% versus 7.82% (PBS); Fig. 3m), as well as $CD8^+$ T cells (38.0% versus other controls (14.1%–25.3%); Fig. 3n). Also, NRb could enhance interferon- γ (INF- γ) and CD107a in NK-92MI cells compared with the PBS group (Supplementary Fig. 35), surpassing equivalent CS, further implying capacity to activate NK cells by NRb. Taken together, NRb could evoke the immune response, thereby warming

Fig. 4 | NRb competitively binds PD-L1 to improve T cell cytotoxicity in vitro. **a**, CLSM images of immunofluorescence staining of PD-L1 (green) on the membrane of CT26 cells ((i) and (ii)) and MC38 cells ((iii) and (iv)) treated with ((ii) and (iv)) or without ((i) and (iii)) INF- γ . Scale bar, 25 μm . $n = 3$ independent samples. **b**, Scheme of NRb transformation on the membrane for competitively binding and blocking PD-L1 of tumour cells. **c, d**, The CLSM tests of CT26 cells (**c**) and MC38 cells (**d**) co-cultured with PD-1-Cy3 protein ($10 \mu\text{g ml}^{-1}$) and PBS, NRb or C-NRb at pH 6.5 or pH 7.4 for 4 h. Scale bar, 10 μm . $n = 3$ independent samples. **e**, Sketch route of NRb NFs blocked the interaction between PD-1 on T cells and PD-L1 on tumour cells in vitro. The primary T cells were first extracted from the spleen of C57BL/6 mice, followed by 12 h of activation (i). Tumour cells were pretreated by NRb at pH 6.5 for 4 h (ii). Subsequently, the activated T cells were added into the NRb-treated tumour cells for another 24 h (iii). **f**, Images of unactivated (left) and activated (right) T cells under a microscope. $n = 3$ independent samples. Scale bars, 1 mm (left), 100 μm (right). **g**, Flow cytometry

analysis of CD4 $^{+}$ T cells (left) and CD8 $^{+}$ T cells (right) after activation. Experiments were repeated three times. **h**, Crystal violet staining of CT26 cells with or without T cells for 24 h pretreated by NRb or C-NRb at pH 6.5 and pH 7.4 for 4 h. Scale bar, 200 μm . $n = 3$ independent samples. **i**, Quantification of live cells in each group by counting the relative positive areas of CT26 cells in **h**. Data are presented as mean $\pm$ s.d., $n = 3$ biological independent experiments. The statistical analysis was measured through one-way ANOVA with Tukey's post hoc test. **j**, Crystal violet staining of MC38 cells in different treatments. Scale bar, 200 μm . $n = 3$ independent samples. **k**, Quantification of live cells in each group by counting the relative positive areas of MC38 cells in **j**. Data are presented as mean $\pm$ s.d., $n = 3$ biological independent samples. The P value was measured through a Kruskal–Wallis test. In **h–k**, cells treated with free PBS (i), NRb+T cells at pH 6.5 (ii) or pH 7.4 (iii), free NRb at pH 6.5 (iv), free T cells (v) and C-NRb+T cells at pH 6.5 (vi) or pH 7.4 (vii). Red channel, $\lambda_{\text{ex}} = 555 \text{ nm}$, $\lambda_{\text{em}} = 570\text{--}620 \text{ nm}$; green channel, $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{--}550 \text{ nm}$; blue channel, $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 415\text{--}465 \text{ nm}$.

up the cold immune microenvironment of pMMR tumour to boost the antitumour effect.

Improve T cell cytotoxicity in vitro by PD-L1 blockade

PD-L1 on CT26/MC38 cells was firstly upregulated through INF- γ (Fig. 4a). NRb competitively binding PD-L1 was characterized by co-culturing with Cy3-labelled PD-1 (PD-1-Cy3) and INF- γ treated CT26/MC38 cells (Fig. 4b). Different from controls, a little red PD-1-Cy3 signal was observed on NRb-treated CT26/MC38 cell membrane at pH 6.5 (Fig. 4c,d), suggesting an effective blockade of PD-1/PD-L1 by NRb fibrillogenesis in situ.

To study the T cell killing effect in vitro through NRb blocking PD-1/PD-L1, primary T cells from C57BL/6 mice were activated in vitro, followed by co-incubating with NRb-pretreated cancer cells (Fig. 4e). Activated primary T cells including CD4 $^{+}$ and CD8 $^{+}$ T cells were finally obtained (Fig. 4f,g). The crystal violet staining results showed that co-culturing with NRb-pretreated (4 h, pH 6.5) CT26/MC38 cells resulted in significant tumour cell killing (83.3% CT26 and 95.3% MC38) by T cells, whereas NRb pretreatment alone (without T cells) showed low cytotoxicity (Fig. 4h–k). The direct cytotoxicity by NRb was excluded through $\sim 100\%$ cell viability of CT26/MC38 cells in the absence of T cells by CCK-8 tests (Supplementary Fig. 36). In addition, no HMGB1/CRT translocation occurred after 4-h incubation of NRb (Supplementary Fig. 37), indicating no ICD by NRb and no subsequent T cell activation. siRNA knockdown of PD-L1 on CT26 cells were developed to study T cell-mediated killing by PD-1/PD-L1 blockade (Supplementary Fig. 38). Significantly enhanced T cell killing effect was observed in siRNA-treated cells compared with wild-type cells treated by free T cells (Supplementary Fig. 39a), with a positive area of $10.03 \pm 0.06\%$ versus $55.14 \pm 4.82\%$ (Supplementary Fig. 39b). Also, a positive area of $11.07 \pm 2.23\%$ in NRb+T cell-treated siRNA CT26 cells further implied that the T cell activity was boosted through PD-1/PD-L1 blockade by NRb.

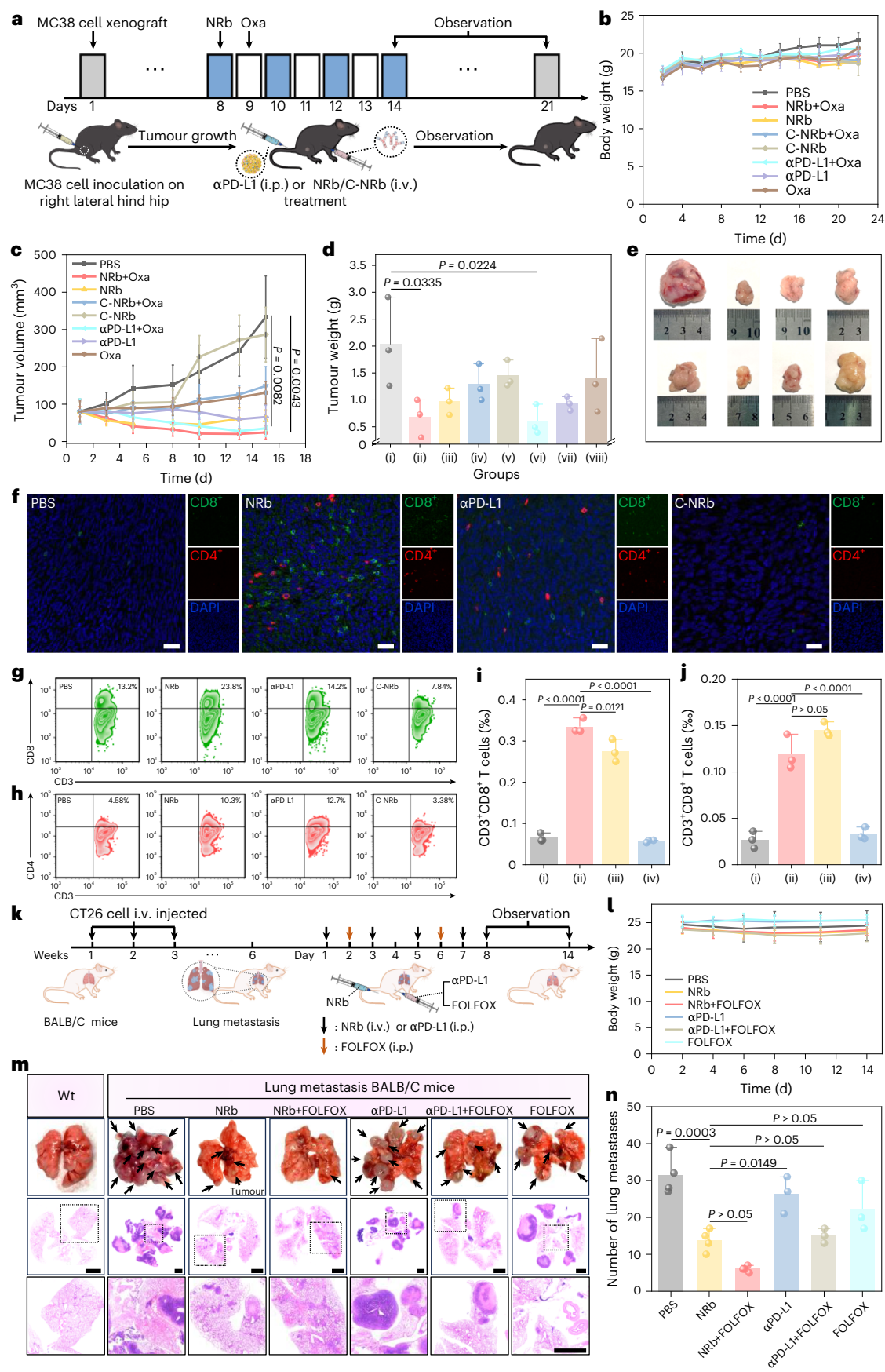
Fig. 5 | Anticancer effect of NRb in MC38-xenografted C57BL/6 mice or CT26 lung metastasis BALB/C mice. **a**, Schematic of MC38 mice building, treatment with different administration regimens (PBS (i), NRb+Oxa (ii), NRb (iii), C-NRb+Oxa (iv), C-NRb (v), $\alpha\text{PD-L1}$ +Oxa (vi), $\alpha\text{PD-L1}$ (vii) and Oxa (viii)) and observation in three weeks. **b**, Body weights of mice with different treatments. Data are presented as mean $\pm$ s.d., $n = 4$ mice ($n = 5$ for C-NRb with and without Oxa and $\alpha\text{PD-L1}$ +Oxa group). **c**, Tumour volumes of different treated mice. Data are presented as mean $\pm$ s.d., $n = 4$ mice ($n = 5$ for C-NRb with and without Oxa and $\alpha\text{PD-L1}$ +Oxa group; $n = 3$ for PBS, Oxa group). The statistical analysis was measured through a Kruskal–Wallis test. **d**, Ex vivo tumour weights of mice after in vivo evaluation. Data are presented as mean $\pm$ s.d., $n = 3$ mice per group. The P values were measured via one-way ANOVA with Tukey's post hoc test. **e**, Images of ex vivo tumours after in vivo evaluation ($n = 3$ mice per group). **f**, Representative immunofluorescence images of CD8 $^{+}$ (green) and CD4 $^{+}$ (red) T cells in tumour tissues after treatment. Scale bar, 25 μm . $n = 3$ independent samples. **g, h**, Flow

In vivo evaluation of fibrillar-transformable behaviour

In MC38-tumour-bearing mice, NRb-Cy5 showed obviously improved tumour accumulation and retention ($>120 \text{ h}$) compared with controls (Extended Data Fig. 1 and Supplementary Fig. 40). NRb-Cy5 targeted to accumulate in tumours and reached the maximum intensity at 24 h, then gradually decreased over time, and was detectable at 120 h with a 19.7% fluorescence signal residue, 4.4-/4.6-fold higher than the PBS/C-NRb groups (Extended Data Fig. 2). Ex vivo images of tumours and main organs confirmed NRb retention in tumours at 120 h (20.8-/13.4-fold C-NRb/PBS; Extended Data Fig. 3), and clearance from kidney slowly compared with other controls (Supplementary Figs. 41 and 42). The NRb retention effect was proven by the immunofluorescence staining analysis of ex vivo tumours, where NRb-Cy5 (white dotted circle) co-localized with PD-L1 (green) and the red fluorescence signal was 6.3-/4.8-fold higher than that of C-NRb-Cy5/PBS-treated groups (Extended Data Fig. 4). Bio-TEM revealed that abundant NFs (white arrows) between the intercellular space of tumours in NRb mice, but not in other controls (Extended Data Fig. 5a and Supplementary Fig. 43), demonstrating NRb in situ fibrillating into NFs in tumours. This was further confirmed by I-labelled NRb (0.44% I; Extended Data Fig. 5b, black dotted rectangle). These results implied active targeting to PD-L1 and in situ fibrillogenesis behaviour improved the retention of NRb, benefitting the therapeutic efficacy.

Therapeutic efficacy of NRb was performed in MC38-cell-xenografted C57BL/6 mice (Fig. 5a). Almost no dehydration symptoms and notable body weight loss occurred in all mice (Fig. 5b). Compared with the PBS group, NRb monotherapy exhibited potent antitumour efficacy (82.8% volume inhibition), comparable with $\alpha\text{PD-L1}$ (80.2%), where NRb+oxaliplatin (Oxa) and $\alpha\text{PD-L1}$ +Oxa combined groups achieved 92.7% and 89.4% tumour volume inhibition, respectively (Fig. 5c). Ex vivo tumour weights and images also demonstrated the good antitumour ability of NRb or NRb+Oxa (Fig. 5d,e). As a comparison, C-NRb1 and C-NRb2 with and without Oxa had a poor

cytometry analysis of CD8 $^{+}$ T cells (**g**) and CD4 $^{+}$ T cells (**h**) in immune cells of tumours after treating with PBS, NRb, C-NRb or $\alpha\text{PD-L1}$. **i, j**, Percentages of CD8 $^{+}$ (i) and CD4 $^{+}$ (j) T cells in the entire tumour tissue after administering PBS (i), NRb (ii), $\alpha\text{PD-L1}$ (iii) or C-NRb (iv). Data are presented as mean $\pm$ s.d. ($n = 3$ biological independent samples). The statistical analysis was measured through one-way ANOVA with Tukey's post hoc test. **k**, Schematic of CT26-tumour-bearing mice showing the establishment, treatment with different administration regimens and observation in three weeks. **l–n**, Body weight (l), representative lung tissue images and its corresponding haematoxylin and eosin staining (m) and average number of tumour metastatic foci in lung tissues (n) of mice in each group. Scale bar, 2 mm. $n = 4$ mice ($n = 3$ mice for $\alpha\text{PD-L1}$, $\alpha\text{PD-L1}$ +FOLFOX and FOLFOX groups), and data for l and n are presented as mean $\pm$ s.d. The statistical analysis was measured through one-way ANOVA with Tukey's post hoc test. Red channel, $\lambda_{\text{ex}} = 555 \text{ nm}$, $\lambda_{\text{em}} = 570\text{--}620 \text{ nm}$; green channel, $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{--}550 \text{ nm}$; blue channel, $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 415\text{--}465 \text{ nm}$. Wt, wild type.



therapeutic effect (Supplementary Fig. 44). Considering that Oxa monotherapy lacks in a substantial therapeutic effect in CRC patients in clinic, standard-of-care chemotherapeutic regimen (FOLFOX: (3.0 mg kg⁻¹ of Oxa, 90.0 mg kg⁻¹ of leucovorin and 50.0 mg kg⁻¹ of 5-fluorouracil) was employed for comparison⁵². Consistent with clinical expectations, NRb+FOLFOX also showed high efficacy (93.9% volume inhibition) comparable with α PD-L1+FOLFOX (93.5%) and superior to FOLFOX alone (73.5%; Supplementary Fig. 45).

The immunofluorescence revealed significantly enhanced infiltration of CD8⁺ and CD4⁺ T cells in NRb-treated tumours, which was 39.6/48.23-, 1.68-/1.40- and 28.3-/16.3-fold that of the PBS, α PD-L1 and C-NRb groups, respectively (Fig. 5f and Supplementary Figs. 46 and 47). Increased T cell infiltration was also demonstrated by flow cytometry (Fig. 5g,h and Supplementary Fig. 48). The total contents of CD8⁺ and CD4⁺ T cells in NRb-treated tumours were calculated to about 0.335% and 0.119%, respectively, significantly higher than the PBS group (CD8⁺, 0.065%; CD4⁺, 0.027%; Fig. 5i,j). Additionally, NRb treatment increased the IFN- γ expression, HMGB1 release, CRT exposure and DC infiltration compared with negative controls (Supplementary Figs. 49–51), implying that NRb effectively converted the immunosuppressive environment. For C-NRb1/C-NRb2 with and without the Oxa-treated groups, the CD4⁺/CD8⁺ T cells, INF- γ , DCs and HMGB1/CRT were all negligible (Supplementary Fig. 52). Moreover, the immunosuppressive environment was also improved by NRb+FOLFOX and superior to the α PD-L1+FOLFOX or FOLFOX groups (Supplementary Figs. 53–56), further implying the great potential of NRb with good biocompatibility (Supplementary Figs. 57 and 58) in upregulating the immune activity. In a distant tumour model of C57BL/6 mice, the NRb group showed obviously reduced tumour than PBS/C-NRb groups, outperforming α PD-L1, indicating an improved immunologic memory and a trend towards superior efficacy by NRb (2/4 tumours completely regressed versus 1/4 for α PD-L1; Supplementary Fig. 59).

NRb could not currently compete with commercially successful α PD-L1 in therapeutic efficacy through MC38-subcutaneous-tumour-bearing mouse, which is a mismatch repair deficiency (dMMR)-type CRC and effectively responds to immunotherapy. We reasoned that the unique strength of NRb lies in its ability to simultaneously disrupt the immunosuppressive microenvironment and kill cancer cells, a combination particularly crucial for treating immunologically ‘cold’ tumours. Since over 95% of CRC patients in the clinic are pMMR-type cold tumours⁵³, we next evaluated the anticancer capability of NRbs in pMMR-type CT26-bearing BALB/C mice⁵⁴ (Supplementary Fig. 60a). Notably, NRb monotherapy with good biocompatibility exhibited superior antitumour efficacy (96.1% tumour volume inhibition) and comparability as NRb+FOLFOX (98.2%), outperforming the FOLFOX regimen (85.3%) and combination therapy (α PD-L1+FOLFOX, 82.8%; Supplementary Fig. 60b,c). Survival analysis demonstrated significant extension of survival time in NRb-treated mice (Supplementary Fig. 60d). NRb treatment resulted in substantial CD4⁺/CD8⁺ T cells and DCs infiltration in tumours, which were

8.5-/0.8-/3.5-/1.9-/2.6-, 7.3-/1.1-/5.3-/2.9-/2.7- and 2.3-/0.7-/1.4-/0.9-/1.6-fold that in the PBS/NRb+FOLFOX/ α PD-L1/ α PD-L1+FOLFOX/FOLFOX groups, respectively (Supplementary Figs. 61 and 62). Similarly, the HMGB1/CRT levels were notably increased in NRb-treated tumours (Supplementary Fig. 63). Collectively, these findings demonstrated that NRb exhibited great therapeutic efficacy against dMMR and pMMR CRC, supporting its potential as a generalized therapeutic strategy.

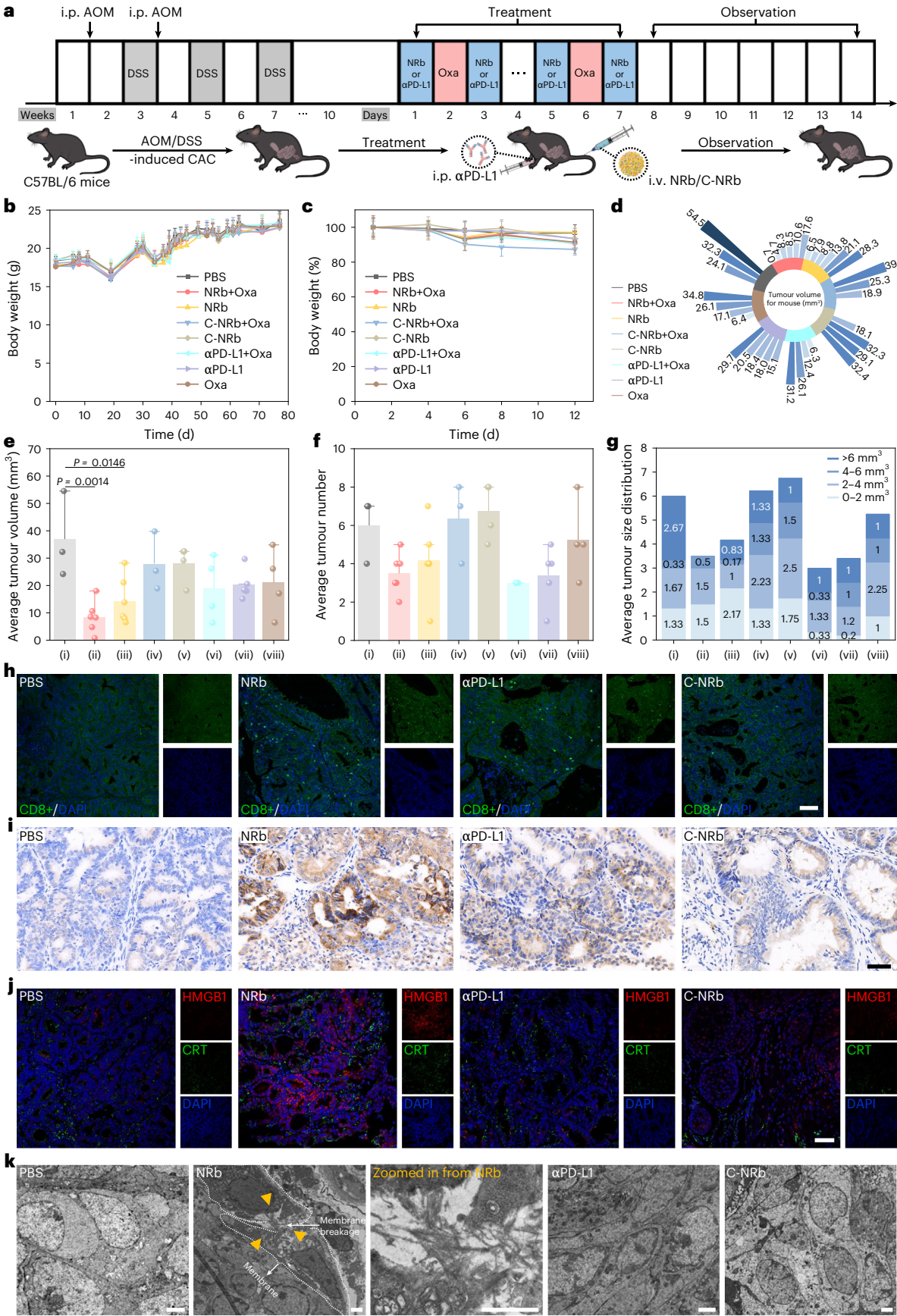
Subsequently, a late-stage lung metastasis BALB/C mice model was constructed through intravenously (i.v.) injected CT26 cells⁵⁵ (Fig. 5k) for further evaluating the NRb therapeutic efficacy. The negligible body weight loss suggested the low toxicity of NRb (Fig. 5l). After treatment, the images and histopathological stains of ex vivo lung tissues showed that there was an obvious morphological difference between wild-type mice and lung metastatic tumour model mice, where there were obvious tumour invasions or metastasis foci (Fig. 5m and Supplementary Fig. 64). Compared with PBS, α PD-L1, FOLFOX and α PD-L1+FOLFOX, NRb administration notably decreased lung metastatic nodules (Fig. 5m and Supplementary Fig. 64, black arrows). The metastatic tumour foci number in NRb/NRb+FOLFOX-treated lung tissues was significantly impaired compared with the negative control groups (Fig. 5n), suggesting the antitumour potential of NRb in advanced CRC. The good biosafety of NRb was also confirmed by the haematoxylin and eosin staining of other organs (heart, liver, spleen and kidneys; Supplementary Fig. 65). The above results demonstrated the potential therapeutic efficacy of NRb in advanced CRC, exhibiting great potential in clinic.

The azoxymethane (AOM)/dextran sulfate sodium (DSS) primary CRC model mouse was built (Fig. 6a), which is currently one of the most established CRC models through simulating the inflammatory-bowel-disease-induced pathogenesis of colitis-associated cancer^{56–58} (Fig. 6b). Six mice were randomly assigned to each treatment group, except for the NRb+Oxa and NRb groups; 3, 2, 3, 1, 2, and 2 mice died in the PBS, C-NRb, C-NRb+Oxa, α PD-L1, α PD-L1+Oxa and Oxa groups, respectively. No obvious dehydration and weight loss symptoms happened during treatment (Fig. 6c). After treatment, all mice were euthanized and the colorectal tissues were isolated (Supplementary Fig. 66). The MLH1, PMS2, MSH2 and MSH6 concurrently highly expressed in the tumour tissues of AOM/DSS mice (Supplementary Fig. 67), indicated that AOM/DSS mice were pMMR-type CRC⁵⁹. NRb+Oxa/NRb effectively inhibited the tumour volume to 0.7–17.6 and 6.5–28.2 mm³, respectively, outperforming the α PD-L1+Oxa group ($V_{\text{tumour}} = 6.3\text{--}31.2\text{ mm}^3$) and α PD-L1 group ($V_{\text{tumour}} = 15.1\text{--}29.7\text{ mm}^3$; Fig. 6d). The average tumour volume of NRb+Oxa mice ($8.47 \pm 3.36\text{ mm}^3$) was about 4.36-/1.70-/3.30-/3.31-/2.24-/2.40-/2.49 times smaller than that of the PBS/NRb/C-NRb+Oxa/C-NRb/ α PD-L1+Oxa/ α PD-L1/Oxa-treated mice, respectively (Fig. 6e). Additionally, NRb/NRb+Oxa effectively reduced the tumour number compared with negligible controls (Fig. 6f). Moreover, 42.8% and 52.0% tumours in the NRb+Oxa/NRb groups were 0–2 mm³, but less than 25.9% in the other groups in which most tumours were distributed in 2–4, 4–6 or

Fig. 6 | Antitumour activity of NRb in AOM/DSS-induced primary tumour models.

a, Schematic for the process of model establishment and antitumour treatments. AOM/DSS was used to establish a mimic of colitis-associated CRC (CAC); after 70 days of moulding, the mice were randomly divided into 8 treatment groups (PBS (i), NRb+Oxa (ii), NRb (iii), C-NRb+Oxa (iv), C-NRb (v), α PD-L1+Oxa (vi), α PD-L1 (vii) or Oxa (viii)). **b**, Body weight trend in each group of mice during model establishment. Data are presented as mean $\pm$ s.d., $n = 6$ mice per group. **c–g**, Body weight maintenance (normalizing the body weight with that on the first day (c)), radial bar chart of tumour volume for each mouse (**d**), average tumour volume (**e**), average tumour number (**f**) and average tumour size distribution with different sizes (0–2, 2–4, 4–6 and >6 mm³; **g**) of mice in each group. $n = 3, 6, 3, 4, 4, 5$ and 4 in the PBS, NRb+Oxa, NRb, C-NRb+Oxa, C-NRb, α PD-L1+Oxa, α PD-L1 and Oxa group, respectively, and data for **c**, **e** and

f are presented as mean $\pm$ s.d. Statistical significance was calculated through a one-way ANOVA test. **h**, Immunofluorescence staining of CD8⁺ T cells in tumour sections ($n = 3$ biological independent samples). Scale bar, 75 μ m. **i**, Immunohistochemistry analysis of IFN- γ (brown) in tumour tissues after treatment ($n = 3$ biological independent samples). Scale bar, 40 μ m. **j**, HMGB1 (red) and CRT (green) immunofluorescence staining of tumour tissues after receiving PBS, NRb, α PD-L1 or C-NRb injection ($n = 3$ biological independent experiments). Scale bar, 50 μ m. **k**, Bio-TEM images of tumours of PBS-, NRb-, α PD-L1- and C-NRb-treated mice, revealing the breakage of cell membrane in the NRb-treated group ($n = 3$ biological independent samples). Scale bar, 2 μ m. Red channel, $\lambda_{\text{ex}} = 555\text{ nm}$, $\lambda_{\text{em}} = 570\text{--}620\text{ nm}$; green channel, $\lambda_{\text{ex}} = 488\text{ nm}$, $\lambda_{\text{em}} = 500\text{--}550\text{ nm}$; blue channel, $\lambda_{\text{ex}} = 405\text{ nm}$, $\lambda_{\text{em}} = 415\text{--}465\text{ nm}$.



>6 mm³ (Fig. 6g). The average number of tumours with a volume of >6 mm³ was from 1.0 to 2.7 in all groups except for the NRb+Oxa/NRb groups (0/0.8).

NRb treatment enhanced CD8⁺ T cell infiltration, which was 4.05-, 1.34- and 6.12-fold that of PBS, α PD-L1 and C-NRb groups, respectively (Fig. 6h and Supplementary Figs. 68 and 69). Subsequently, single-cell RNA sequencing confirmed obviously higher T cell proportions (45.8%) in NRb-treated tumours versus PBS (0.6%) and α PD-L1 (3.2%), with increased resident/memory T cells (Supplementary Fig. 70 and Supplementary Table 2). Notably, both resident and memory CD4⁺/CD8⁺ resident T cells in NRb-treated tumour tissues (resident versus memory CD4⁺/CD8⁺: 1.6%/8.6% versus 7.4%/1.3%) were notably upregulated, compared with PBS or α PD-L1 treatment, implying a substantial improvement in the tumour immune microenvironment.

NRb also dramatically upregulated IFN- γ compared with the PBS, α PD-L1 and C-NRb groups (Fig. 6i and Supplementary Fig. 71), suggesting the improved activity from T/NK cells in tumours. The enhanced HMGB1 release and CRT exposure by NRb implied an increased CD8⁺ T cell infiltration in tumours (Fig. 6j). Meanwhile, bio-TEM revealed obvious NFs and membrane breakage in NRb-treated tumours, giving evidence that NRb fibrillogenesis in situ induced membrane disruption in vivo (Fig. 6k). Terminal deoxynucleotidyl transferase dUTP nick-end labelling indicated upregulated apoptosis level in NRb-treated tumours (Supplementary Fig. 72). Haematoxylin and eosin staining demonstrated the good biocompatibility of NRb (Supplementary Fig. 73). Mild lesions (white arrow) in α PD-L1+Oxa-, C-NRb+Oxa- and Oxa-treated livers were mainly caused by the non-specific uptake of Oxa. Additionally, NRb+FOLFOX showed effective antitumour efficacy with markedly reduced tumour volume and number in AOM/DSS mice (Supplementary Fig. 74a–e). Notably enhanced CD8⁺ T cell infiltration occurred in NRb+FOLFOX mice might imply the outperformance of therapeutic efficacy (Supplementary Fig. 75). The recovered body weight after treatments and pathological findings suggested favourable biocompatibility by NRb+FOLFOX (Supplementary Figs. 74f and 76). These in vivo/ex vivo data demonstrated that NRb is able to compete with commercially successful α PD-L1 and FOLFOX therapy in pMMR CRC.

Conclusion

We have developed an NRb that represents a ‘transformable’ therapeutic modality. The NRb performs dynamic, in situ fibrillogenesis on the cancer cell surface, triggered by the TME. This process actively generates mechanical force for membrane disruption, unlike static drug carriers. The NRb targets to bind PD-L1 and transforms into NFs on cancer cells to break the membrane of tumour cells and release DAMPs, sensitizing cancer cells to immune checkpoint inhibitors with enhanced therapeutic efficacy. The NRb effectively addresses the dual challenges of immune evasion and poor T cell infiltration in pMMR CRC, exhibiting high therapeutic efficacy, better than commercial α PD-L1. This NRb monotherapy strategy offers several advantages over the conventional combination of radiotherapy/chemotherapy and immunotherapy, including improved drug retention, targeted ICD, fewer side effects and effective therapeutic effect for pMMR-type cold CRC.

Although this work offers a promising strategy for immunotherapy-resistant pMMR CRC, several challenges have to be addressed before practical clinical application. For instance, although the pH-triggered targeted ICD improved the therapeutic efficacy in model mice, its antitumour performance and mechanism should also be studied in human beings. Also, although no acute toxicity was observed in the model mice of this study, the long-term biodegradation and biosafety of nanofibrous NRbs should be developed in higher species. We believe that the NRb-based monotherapy strategy will serve as a broadly impactful nanomedicine-based immunotherapy method for pMMR-type CRC, encouraging diverse basic and clinical application studies.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41565-025-02071-3>.

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Methods

Synthesis of acryl-CS

Acryl-CS was prepared according to our previous work²⁹. Here 0.8 g of CS (including 5 mmol of $-NH_2$ groups) were dissolved in 10 ml of deionized water, followed by heating to 50 °C under stirring for 30 min. Subsequently, the mixed solution was cooled to room temperature, followed by adding Et_3N (2.95 g, about 30 mmol) into the solution. Then, acryloyl chloride (1.35 g, 15 mmol) was added dropwise into the above mixed liquids under stirring in an ice water bath. Finally, the reaction mixtures were kept at room temperature in the dark for more than 24 h, followed by dialysis against deionized water (molecular weight cut-off, 5,000) for 72 h. The above solution with a $-C=C-$ concentration of 0.1 mmol ml^{-1} was obtained through volume stabilization and was used for the subsequent synthesis. Thereafter, to verify the structure of the product, a little solution was harvested and lyophilized to obtain a yellow solid, which was used to determine the by ^1H nuclear magnetic resonance spectra (600 MHz, Agilent).

Preparation of NRb

The NRb was synthesized by the Michael addition reaction between the thiol group ($-SH$) and alkenyl group ($-C=C-$) in PBS buffer with pH 8.0 (ref. 29). First, 0.8 ml of the above acryl-CS (0.08 mmol of $-C=C-$ in total) was added in the flask for standby. Then, 0.044 mmol of peptide CFFVLKK(PEG_{1,000})-H₆G and 0.044 mmol of peptide ^{d} CNYSKPTDRQYHF were co-dissolved in 2.0 ml of dimethyl sulfoxide, followed by addition into acryl-CS drop by drop. Subsequently, the above mixtures were bubbled to remove air in the solution by N_2 blowing for 0.5 h. Immediately, the solutions were reacted at 37 °C for 72 h in the dark. Next, the resultant solutions were then dialysed against deionized water (molecular weight cut-off, 3,500 Da) for 48 h to obtain the final NRb production with a concentration of 50 mg ml^{-1} by determining the solvent volume. The unreacted material was effectively removed through dialysis (Supplementary Fig. 4). Similarly, the control group C-NRb was obtained using the same methods by replacing peptide CFFVLKK(PEG_{1,000})-H₆G and peptide ^{d} CNYSKPTDRQYHF with K' (CGGAACK (PEG_{1,000}-H₆G) and P' (^{d} CDNKHTPFYYQSR, a random sequence of P) at the same time. Furthermore, C-NRb1 only containing P and C-NRb2 only containing K were also synthesized using the same method.

TEM and dynamic light scattering tests

Here 1.0 mg ml^{-1} of NRb/C-NRb solutions with pH 6.5 or pH 7.4 were cultured on a shaking table at 37 °C for 24 h. Then, uniformly dispersed NRb/C-NRb samples were obtained by ultrasonic treatment, followed by dynamic light scattering measurement (BeNano 180Zeta). In addition, 10 μ l of NRb/C-NRb samples was dropped on a copper mesh and precipitated for 5 min. Then, the sample solutions were removed and stained with uranyl acetate for another 5 min. Subsequently, the samples were washed with deionized water and observed by TEM (Talos F200S, Thermo Fisher Scientific).

CLSM observation of cells

First, HCoEpiCs, CT26 and MC38 cells were seeded in confocal dishes and cultured at 37 °C in a humidified environment containing 5% CO_2 overnight. Then, 1.0 mg ml^{-1} of NRb-Cy5 was prepared by diluting with serum-free Dulbecco's modified Eagle's medium with pH 6.5 or pH 7.4, followed by co-incubating cells for additional 4 h. Thereafter, the solutions were removed away and washed with PBS for three times and observed through CLSM (Dmi8, Leica or LSM880, ZEISS).

SEM observation of NRb on cell surface

SEM was utilized to explore the fibrillogenesis behaviour of NRb on cell membrane under pH 6.5. First, HCoEpiCs, CT26 and MC38 cells were incubated on coverslips for 24 h. Then, the medium was removed and replaced with 1.0 mg ml^{-1} of NRb/C-NRb diluted by serum-free Dulbecco's modified Eagle's medium, followed by co-culturing for

another 24 h. To simulate the weak acidity of the TME, the pH of the medium was modulated to 6.5 with NaOH and HCl, which was close to the TME value (pH 6.5–6.8). Subsequently, the pretreated cells were progressively dehydrated by ethanol and *tert*-butyl alcohol. Finally, the cells on the coverslips were sprayed with gold and detected through focused-ion-beam SEM (Crossbeam 340, ZEISS).

Cell membrane destruction and cytoplasm leakage analysis

To explore NRb-induced membrane destruction and subsequent cytotoxicity release, CT26 cells were co-cultured with 0.2 μ M of calcein with or without Dil for 0.5 h. Then, the media were removed and washed by PBS for three times. Thereafter, the cells were incubated with NRb/C-NRb (1.0 mg ml^{-1}) at pH 6.5 or pH 7.4 for another 24 h, followed by CLSM detection (Dmi8, Leica).

Atomic force microscopy characterization

First, the CT26 cells were incubated on coverslips for 24 h, and then, the medium was removed and replaced by 1.0 mg ml^{-1} of NRb NPs in serum-free Dulbecco's modified Eagle's medium co-cultured at pH 6.5 for 0, 4 and 24 h. Also, preformed NRb NFs were chosen to culture CT26 cells for comparison. The above cell samples with or without fixation were detected using an atomic force microscope (Bruker, JPK NanoRacer).

Cryo-TEM characterization of CT26 cells

First, CT26 cells were seeded at a density of 1×10^5 cells per well into six-well plates equipped with hydrophilically treated gold grids and incubated overnight. Then, the culture medium was replaced with a serum-free medium containing PBS or 1.0 mg ml^{-1} of NRb NPs (pH 6.5/7.4), followed by another 24 h of incubation. The gold grids with CT26 cells were subsequently carefully washed with PBS for three times, and the sample platform was quickly placed on a quick-freezing instrument (Vitrobot, FEI). The excess water on the gold grid was moved away from the edges of the grid by filter paper, followed by quickly freezing with liquid ethane cooled by liquid nitrogen through the Vitrobot. Thereafter, the freezing cells were immediately used for cryo-TEM observation (Titan Krios G3i).

HMGB1, CRT, ATP and LDH release tests

CT26 cells were first treated by NRb or C-NRb at different pH values for 24 h. Then, HMGB1 and CRT were immunofluorescence stained and observed by CLSM (LSM880, ZEISS). The ATP and LDH were detected by luminescent ATP Detection Assay Kit and LDH Cytotoxicity Assay Kit through a microplate reader (SpectraMax M2), respectively. The LDH leakage was qualified as follows:

$$LDH \text{ leakage (\%)} = [(OD_{\text{sample}} - OD_{\text{negative}})/(OD_{\text{positive}} - OD_{\text{negative}})] \times 100\%$$

NK cell activation in vitro

Here 1×10^6 NK-92MI cells were planted on a six-well plate and treated with NRb (1.0 mg ml^{-1}) or an equivalent dose of CS (0.05 mg ml^{-1}) for 24 h. Then, the NK-92MI cells were stained with fluorescein-isothiocyanate-conjugated anti-human CD56, phycoerythrin (PE)-conjugated anti-human CD107a and phycoerythrin-cyanin e7-conjugated anti-human IFN- γ . After 30 min of incubation at 4 °C, cells were washed with a cell stain buffer, followed by flow cytometry analysis (Gallios, Beckman Coulter).

Cell flow cytometry detection of DCs and T cells in vitro

First, MC38 cells were planted on a 24-well plate at a density of 1.5×10^4 cells per well on the lower transwell insert overnight, and treated with NRb for 24 h. Then, the female C57BL/6 mice (6–8 weeks old, six mice) were killed, and the spleen was extracted and ground into a single-cell suspension. Thereafter, 1×10^6 spleen cells were added into the upper transwell insert to co-incubate with MC38 cells for

another 24 h. Subsequently, the spleen cells were collected to stain by antigen-presenting-cell-conjugated anti-mouse CD11c, PE-conjugated anti-mouse CD80 and fluorescein-isothiocyanate-conjugated anti-mouse CD86. For CD3⁺ and CD8⁺ cell detection, the above spleen cells were treated with antigen-presenting-cell-conjugated anti-mouse CD3 and PE-conjugated anti-mouse CD8. Finally, the cells were measured through flow cytometry (Gallios, Beckman Coulter).

NRb NF blockade of PD-1/PD-L1 interaction in vitro

First, 1.5×10^5 CT26 cells or MC38 cells were seeded in confocal dishes for 24 h, and were pretreated with 20 ng ml⁻¹ of IFN- γ . Then, NRb (1.0 mg ml⁻¹) and Cy3-labelled recombinant mouse PD-1 protein (PD-1-Cy3) were co-incubated with tumour cells for 4 h, followed by staining with DAPI (10 μ g ml⁻¹) and washing with PBS for three times. Finally, the samples were observed by CLSM (Dmi8, Leica or LSM880, ZEISS).

T cell-mediated tumour cell killing assay

First, female C57BL/6 mice (6–8 weeks old, 12 mice) were euthanized to collect the spleens. Then, the isolated spleens were carefully and quickly ground in a sterile environment and the red blood cells were moved away to obtain a lymphocyte suspension. Thereafter, the above lymphocyte suspension was treated with EasyStep Mouse T Cell Isolation Kit for obtaining the primary T cells. Next, the mouse primary T cells were stimulated with phorbol 12-myristate 13-acetate (50 ng ml⁻¹) and ionomycin (500 ng ml⁻¹) for 12 h, and proliferated in ImmunoCult-XF T cell expansion medium containing mouse recombinant IL-2 (10 ng ml⁻¹) for standby, which was confirmed by flow cytometry analysis. Then, CT26 or MC38 cells were pretreated with NRb (1.0 mg ml⁻¹) for 4 h, followed by co-culturing with T cells (in a ratio of 1:10) in RPMI 1640 medium containing anti-mouse CD3e antibody (10 ng ml⁻¹) and IL-2 (10 ng ml⁻¹) for another 24 h. Finally, the samples were washed by PBS for three times, followed by staining with crystal violet for 10 min and photographed by an inverted fluorescence microscope (IX83, Olympus).

The CT26 cells were treated by siRNA (sequences CCAGAGAATT TCCGTGGAT, GTGCAGATTCCTGTAGAA and GCCACAGCGAAT GATGTTT, synthesized by RiboBio) to knockdown PD-L1, followed by detecting with western blot and quantitative polymerase chain reaction. The primers for the quantitative polymerase chain reaction were obtained from Sangon and listed as below: PD-L1-mouse-F: 5'-GCTCCAAAGGACTTGTACGTG-3'; PD-L1-mouse-R: 5'-TGATCTGAAG GGCAGCATTTTC-3'; GAPDH-mouse-F: 5'-AATGGATTTGGACGC ATTGGT-3'; GAPDH-mouse-R: 5'-TTTGCACCTGGTACGTGTTGAT-3'. Then, the obtained PD-L1- CT26 cells were used for the same experiment as above for validating the PD-1/PD-L1-blockage-mediated T cell killing by NRb.

NRb targeted tumour accumulation and retention

Female C57BL/6 mice (6–8 weeks old, three mice per group; total, 54 mice) were purchased from Charles Rivers Laboratories, and housed under specific pathogen-free conditions under controlled temperature (21–23 °C), humidity (30%–70%) and a 12-h light/dark cycle. The C57BL/6 mice were subcutaneously implanted with 1×10^6 MC38 cells into the right lateral hind hip to build the subcutaneous transplant model mice. Then, 200 μ l of PBS, NRb-Cy5 (1.0 mg ml⁻¹) or C-NRb-Cy5 (1.0 mg ml⁻¹) was i.v. injected, followed by acquiring images at 4–120 h post-administration by IVIS Lumina III (PerkinElmer; excitation filter, 620 nm; emission filter, 670 nm). The tumours of mice at 4, 24 and 120 h were collected for ex vivo fluorescence images, immunofluorescence analysis and bio-TEM observation (HT7700, Hitachi).

In vivo therapeutic effect of NRb on MC38-implanted model mice

The MC38-implanted C57BL/6 mice (female, 6–8 weeks old; total, 67 mice) received different treatments: (1) PBS, (2) NRb (200 μ l,

1.0 mg ml⁻¹)+Oxa (oxaliplatin, 10 mg kg⁻¹), (3) NRb (200 μ l, 1.0 mg ml⁻¹, 0.7), (4) C-NRb (200 μ l, 1.0 mg ml⁻¹)+Oxa (10 mg kg⁻¹), (5) C-NRb (200 μ l, 1.0 mg ml⁻¹), (6) α PD-L1 (100 μ l, 5 mg kg⁻¹)+Oxa (10 mg kg⁻¹), (7) α PD-L1 (100 μ l, 5 mg kg⁻¹) or (8) Oxa (10 mg kg⁻¹). The dose of NRb/C-NRb (i.v. injected) was 0.7 times equivalent to α PD-L1 drug (intraperitoneally (i.p.) injected), which were administered on days 1, 3, 5 and 7, whereas Oxa was i.p. injected into mice on days 2 and 6. The body weight of the mice was recorded every other day for 2 weeks, as well as the volume of the tumour being measured by an electronic digital caliper. The tumour volume was calculated as follows:

$$V = AB^2/2, \quad (1)$$

where A is the longest diameter (mm) and B is the minimum diameter (mm).

Additionally, NRb-combined FOLFOX (3.0 mg kg⁻¹ of Oxa, 90.0 mg kg⁻¹ of leucovorin and 50.0 mg kg⁻¹ of 5-fluorouracil) was used for evaluating this model. Also, the tumours after the final observation were divested and weighed for further evaluating the therapeutic effect of NRb.

CD4⁺/CD8⁺ T cell infiltration in tumours

The tumours after treatment were collected for the preparation of paraffin sectioning, followed by immunofluorescence staining of CD4⁺ and CD8⁺ T cells and detecting through CLSM (Dmi8, Leica or LSM880, ZEISS). Also, the CD45⁺, CD3⁺, CD4⁺ and CD8⁺ T cells in tumours were characterized by flow cytometry analysis.

Immunologic memory by NRb treatment

A week after treatment, 1×10^6 MC38 cells were further subcutaneously implanted on the left lateral hind hip of C57BL/6 mice (female, 6–8 weeks before treatments; total, 16 mice). Subsequently, the distal tumours at 14 days post-implantation were isolated, followed by weighing and measuring volume.

In vivo therapeutic effect of NRb on CT26-implanted model mice

Female BALB/C mice (6–8 weeks old, five mice per group; total, 30 mice) were purchased from Charles Rivers Laboratories, and housed under specific pathogen-free conditions with controlled temperature (21–23 °C), humidity (30%–70%) and a 12 h light/dark cycle. Here 100 μ l ($V_{\text{medium}}:V_{\text{matrix gel}} = 1:1$) containing 1.2×10^7 CT26 cells were subcutaneously implanted to female BALB/C mice for pMMR CRC model mice. Then, the CT26 mice were administered (1) PBS, (2) NRb (200 μ l, 1.0 mg ml⁻¹), (3) NRb+FOLFOX, (4) α PD-L1 (5 mg kg⁻¹), (5) α PD-L1+FOLFOX or (6) FOLFOX. NRb (i.v. injected) and α PD-L1 (i.p. injected) were administered on days 1, 3, 5 and 7, whereas FOLFOX was i.p. injected into mice on days 2 and 6. The body weight of mice was recorded every other day for 3 weeks, as well as the volume of the tumour being measured by an electronic digital caliper. The tumour volume was calculated using equation (1).

Also, after the treatment, the tumour volume of the mice was continually monitored for 70 days. Mice were recorded as dead when the tumour volume reached or exceeded 1,000 mm³. A survival curve was plotted based on these observations to evaluate the survival outcomes by NRb.

In vivo therapy assays of NRb in AOM/DSS model mice

Female C57BL/6 mice (6–8 weeks; total, 64 mice) were obtained from Charles Rivers Laboratories for establishing the AOM/DSS model mice according to previous literature^{56,57}. Generally, 10 mg kg⁻¹ of AOM was i.p. injected in mice twice every other week, followed by 7 days of routine feeding and care. Then, the mice were administered with 2% DSS dissolved in drinking water for 7 consecutive days, subsequently followed by another 7 days of regular diet for recovery. The cycle was

repeated for another two times, and then the mice were fed with a routine diet for another 4 weeks. About 70 days in total, AOM/DSS mice were substantially established. Then, the AOM/DSS mice were administered as the MC38-implanted experiments and always housed under specific pathogen-free conditions with controlled temperature (21–23 °C), humidity (30%–70%) and a 12 h light/dark cycle. After the treatment, the mice were killed and the ex vivo colorectal tissues were expanded on the desktop, followed by photographing and recording the numbers and volumes of tumours on colorectal tissues. Furthermore, the tumour tissues of AOM/DSS mice were immunofluorescence stained to observe MLH1, PMS2, MSH2 and MSH6 expressions to assess whether it belongs to the pMMR or dMMR tumour.

RNA sequencing analysis

RNA sequencing was performed at the tissue levels to elucidate the infiltration of CD4⁺/CD8⁺ T cells by NRb in tumours. Total RNA was extracted from tumour tissues of AOM/DSS mice (female, 6–8 weeks old before administration; total, 9 mice) in the end of PBS, NRb or α PD-L1 treatment through TRIzol, of which a total of 6,870, 11,001 and 13,082 cells were collected, respectively. The RNA concentration and quality were analysed through a Nanodrop ND-1000 spectrophotometer (Gene Company) and a 2100 Bioanalyser (Agilent), respectively. The purified RNA samples were subsequently utilized for library preparation and sequencing through the Illumina HiSeq X10 platform. Bioinformatics data analysis was developed using the NovoMagic Cloud Platform (Novogene) and the Majorbio platform.

In vivo therapeutic effect of NRb on CT26 lung metastasis model mice

Female BALB/C mice (6–8 weeks; total, 28 mice) were chosen for CT26 lung metastasis model mice experiments. First, 200 μ l of CT26 cell suspension (1×10^7 cells per ml) was administered via tail-vein injection to female BALB/C mice aged 6–8 weeks (ref. 55), which was repeated once per week for 3 consecutive weeks. The CT26 lung metastasis model mice could be successfully established through ex vivo lung tumour observation at the fifth week. Then, the lung metastasis mice were treated by PBS, NRb, NRb+FOLFOX, α PD-L1, α PD-L1+FOLFOX or free FOLFOX. The dosages and concentrations of NRb (i.v.), α PD-L1 (i.p.) and FOLFOX (i.p.) were consistent with those used in CT26-implanted mice. Also, NRb and α PD-L1 were administered on days 1, 3, 5 and 7, whereas FOLFOX was i.p. injected on days 2 and 6. Body weight was recorded every other day for 2 weeks. Finally, the mice were euthanized and the lung tissues were extracted for observation and pathological staining.

Statistics and reproducibility

Intergroup comparison was analysed by a paired-sample *t*-test (two tailed). One-way analysis of variance (ANOVA) was used for multiple-group analysis. The level of significance was defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$. All statistical tests were two sided. Data are presented as mean $\pm$ standard deviation (s.d.). All experiments were repeated for more than or equal to three times with similar results. No statistical methods were used to predetermine the sample sizes, but our sample sizes are more than or equal to three to ensure the reproducibility and reliability of our findings. The sample sizes in our study are similar to those reported in previous publications^{25,60–63}.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the protocols approved by the Laboratory Animal Welfare and Ethics Committee of the Army Medical University (Animal Ethical Statement Number AMUWEC20210257), which specifies a maximum permitted tumour burden of $<2,000 \text{ mm}^3$. The study was conducted in strict compliance with this requirement and did not exceed the stipulated tumour size/burden at any point. Mice with a tumour burden of $>1,000 \text{ mm}^3$ were determined to be dead.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data supporting the findings of this study are available within the article and its Supplementary Information. The single-cell sequencing data generated for this study are deposited in the NCBI Sequence Read Archive under accession number [PRJNA1337589](https://doi.org/10.1038/s41565-025-02071-3). All raw datasets provided with this paper are also available for research purposes from the corresponding authors upon reasonable request. Source data are provided with this paper.

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Author contributions

Q.L., H.W., S.Y. and M.X. contributed to the conception and design of the experiment. W.Y., C.Z., C.G., J.Y. and G.X. conducted the production, purification and characterization of the material. J.L., Y.H., R.D., X.L. and W.C. participated in the cellular experiments. Q.L. and W.Y. conducted the animal experiments. L.W., H.W., M.X., W.Y., J.Z., C.Z., K.Z., C.G., G.X., C.F., Y.Z. and J.L. participated in the data analysis. Q.L., W.Y., C.Z., L.W., K.Z., H.W., S.Y. and M.X. prepared the paper. Q.L. and S.Y. supervised the entire project. All authors took part in the writing and reviewing of the paper and accepted the final paper.

Competing interests

The authors declare no competing interests.

Additional information

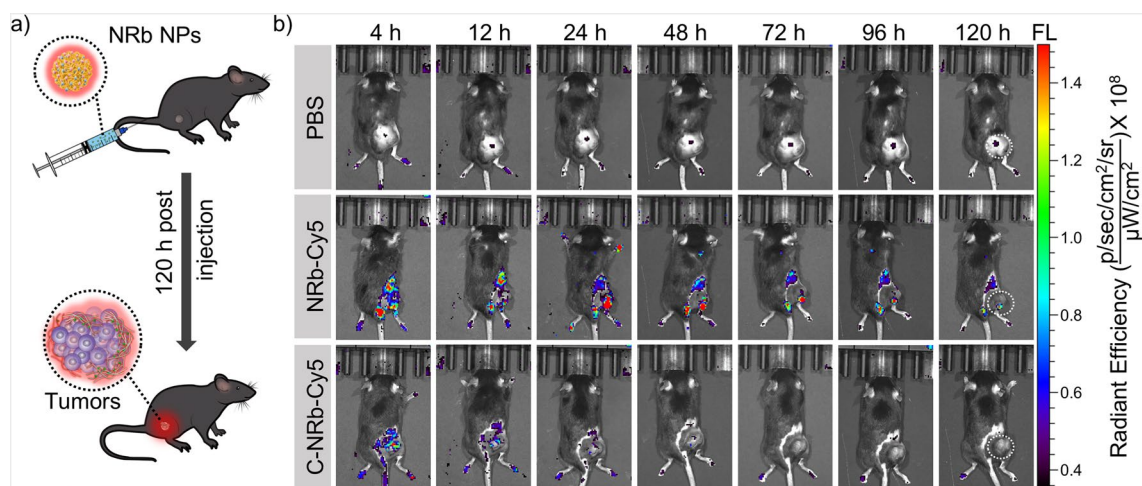
Extended data is available for this paper at <https://doi.org/10.1038/s41565-025-02071-3>.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41565-025-02071-3>.

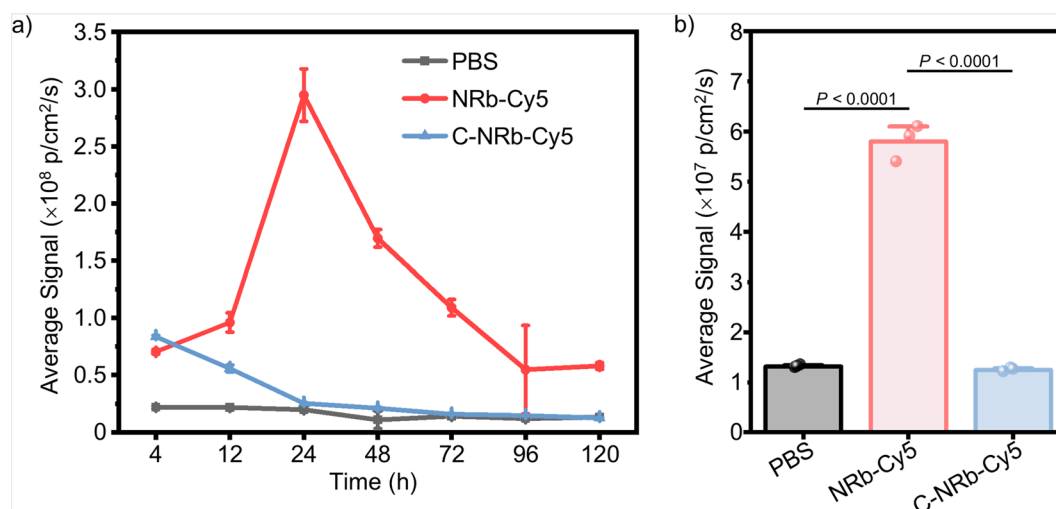
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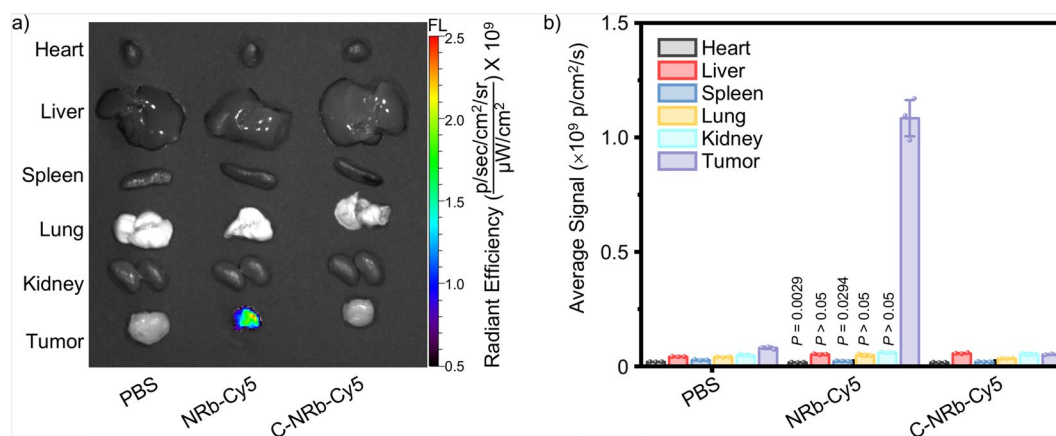


Extended Data Fig. 1 | NRb targeted accumulation and retention in tumours. a) Schematic illustration of NRb targeted to tumours with high accumulation and long-term retention in vivo. b) The representative in vivo images of Cy5-labeled NRb (1.0 mg/mL), C-NRb (1.0 mg/mL) and PBS treated MC38-bearing C57BL/6 mice from 4–120 h, $n = 3$ mice in each group.



Extended Data Fig. 2 | Fluorescence intensity of NRb at tumours. a) The quantification of fluorescence intensity of tumour tissues in (Extended Data Fig. 1b) with time from 4–120 h. Data are presented as mean $\pm$ SD, $n = 3$ biological independent samples. **b)** The fluorescence signal intensity at tumour tissue in

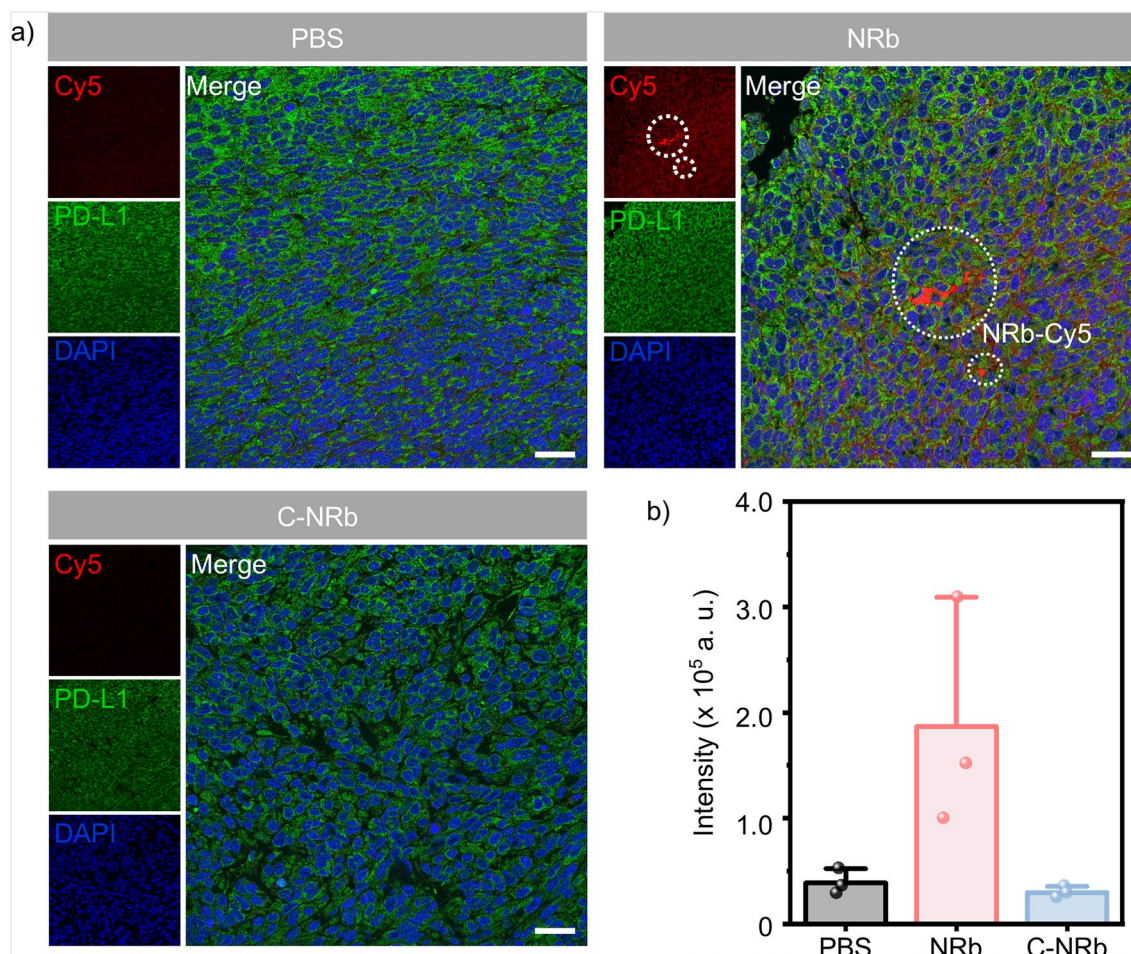
Extended Data Fig. 1b) at 120 h. Data are presented as mean $\pm$ SD, $n = 3$ biological independent experiments. The statistical analysis was measured through one-way ANOVA with a Tukey post hoc test.



Extended Data Fig. 3 | Ex vivo fluorescence imaging and quantification.

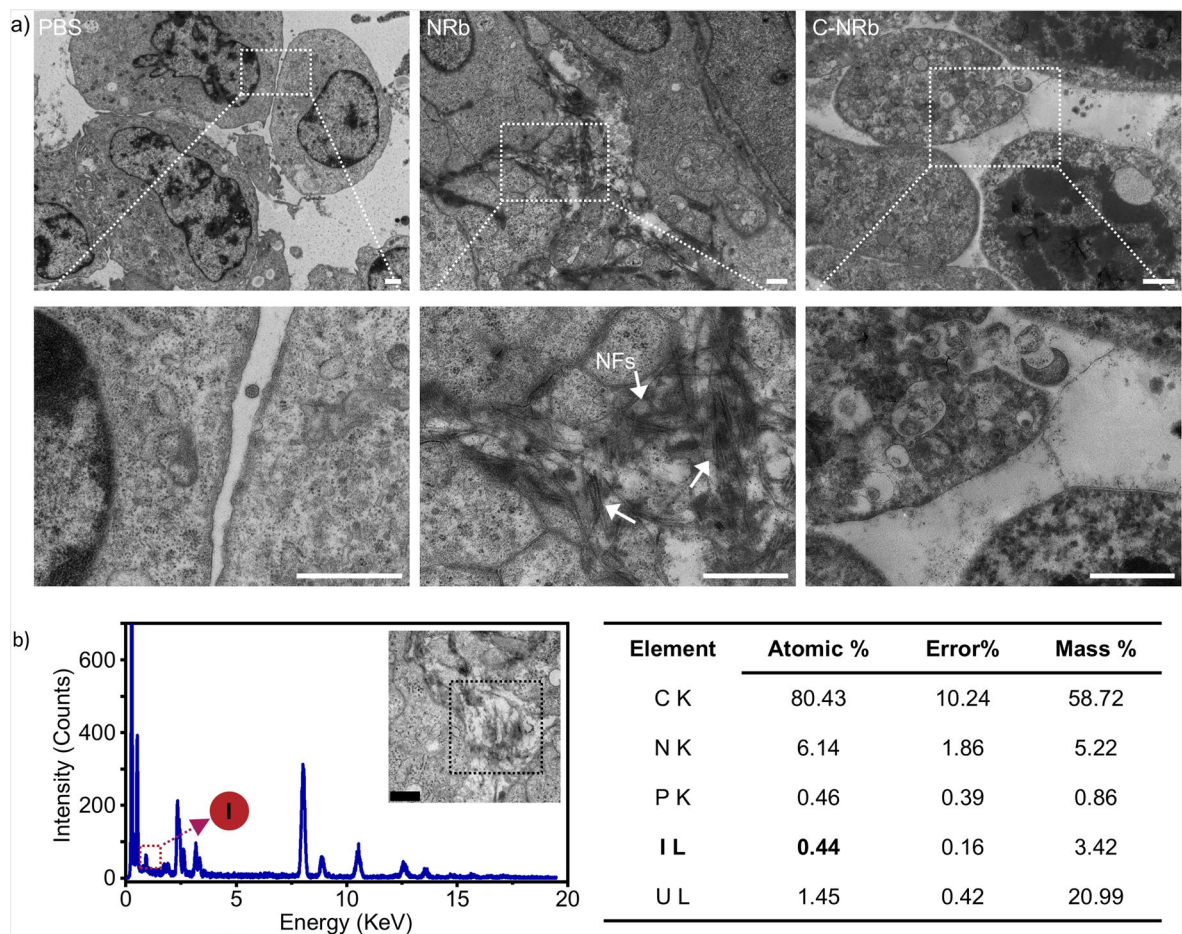
a) Ex vivo fluorescence images of tumour tissue, heart, liver, spleen, lung and kidney collected at 120 h post-administration of Cy5-labeled NRb (1.0 mg/mL), C-NRb (1.0 mg/mL) and PBS of MC38-bearing C57BL/6 mice, respectively (3 mice

per group). **b)** Average fluorescence intensity of ex vivo tumours and main organs from (a). Data are presented as mean $\pm$ SD, $n = 3$ biological independent samples. The statistical analysis between tumor and other organs was measured through Kruskal-Wallis test.



Extended Data Fig. 4 | NRb retention at tumour tissues. a) Immunofluorescence staining images of PD-L1 of ex vivo tumour tissue slides for analyzing the accumulation and retention of NRb by CLSM. Scale bar: 25 μ m. n = 3 independent samples. **b)** The quantification of red NRb-Cy5 fluorescence signal intensity in

(a). Data are presented as mean $\pm$ SD, n = 3 biological independent samples. Red channel, λ_{ex} = 647 nm, λ_{em} = 651-721 nm; green channel, λ_{ex} = 488 nm, λ_{em} = 500-550 nm; blue channel, λ_{ex} = 405 nm, λ_{em} = 415-465 nm.



Extended Data Fig. 5 | NRb in situ fibrillogenesis in tumours. **a)** Bio-TEM images of tumour tissues of MC38-xenografted mice. Scale bar: 1 μ m. $n = 3$ independent samples. **b)** The EDS spectrum analysis of fibers at tumours (dotted rectangle in the illustration) of the mice after injecting iodine (I) labeled-NRb for 120 h. Scale bar: 1 μ m. $n = 3$ independent samples.

Reporting Summary

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n/a	Confirmed
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☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated

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Software and code

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Data collection	600MHz010601 (Agilent), Agilent 6546 (Agilent), Agilent Cary60 (Agilent), BeNano 180Zeta, TALOS F200S (Thermo Fisher Scientific), Spectrum100 (PerkinElmer), Dmi8 (Leica), LSM880 (Zeiss), Crossbeam 340 (Zeiss), Tomocube HT-X1 (Daejeon), JPK NanoRacer (Bruker), Titan Krios™ G3i (Darwin), Gallios (Beckman Coulter), IVIS Spectrum In Vivo Imaging System (PerkinElmer), HT7700 (HITACHI), Nanodrop ND-1000 (Gene Company), 2100 Bioanalyzer (Agilent), Illumina HiSeq X10 platform (Illumina), NovoMagic Cloud Platform (Novogene), VS200 SLIDEVIEW (Olympus), IX83 inverted microscope (Olympus), SpectraMax M2, C300 (Azura), FQD-96C (BIOER), FEI Neitherland (Vitrobot).
Data analysis	MestReNova (v12.0.0-20080), ChemDraw (v19.0.0.22), OriginPro 2021 (v9.8.0.200), Graphad Prism (v9.5.1), Zeiss ZEN (v3.8.99), LAS X (v3.7.6), TA Viewer (v2.1.5), JPK Data Processing (v8.0.144), FlowJo (v10.8.1), Image J (v1.53), Living Image (v4.5.5), OLYMPUS OlyVIA (v4.1), AzureSpot (v2.1.097) and K-viewer (v1.7.1) were used for data analysis.

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Data Availability:

The authors declare that all data supporting the findings of this study are available within the Article and its Supplementary Information. The single-cell sequencing data generated for this study are deposited in the NCBI Sequence Read Archive under accession PRJNA1337589. All raw dataset provided with this paper and are also available for research purposes from the corresponding author upon reasonable request.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design; whether sex and/or gender was determined based on self-reporting or assigned and methods used. Provide in the source data disaggregated sex and gender data, where this information has been collected, and if consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected. Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.

Reporting on race, ethnicity, or other socially relevant groupings

Please specify the socially constructed or socially relevant categorization variable(s) used in your manuscript and explain why they were used. Please note that such variables should not be used as proxies for other socially constructed/relevant variables (for example, race or ethnicity should not be used as a proxy for socioeconomic status). Provide clear definitions of the relevant terms used, how they were provided (by the participants/respondents, the researchers, or third parties), and the method(s) used to classify people into the different categories (e.g. self-report, census or administrative data, social media data, etc.) Please provide details about how you controlled for confounding variables in your analyses.

Population characteristics

Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

Recruitment

Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

Ethics oversight

Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No statistical methods were used to determine sample size. For in vitro study, n=3 biologically independent replicates. This sample size is often reported in previous publications to be sufficient for a confident data analysis. For in vivo study, n=3 or n=4 or n=5 or n=6 or n=10 animals per group were used are based on the need for proper statistical evaluation.

Data exclusions

No data were excluded.

Replication

All data were collected from at least three biological independent replications and analyzed as mean $\pm$ SD to verify the reproducibility of the experimental findings. All attempts at replication were successful.

Randomization

The experimental groups were allocated randomly.

No formal blinding was used. Investigators were not blinded to the group allocation due to the data analysis was objectively based on measurable data.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems		Methods	
n/a	Involved in the study	n/a	Involved in the study
☐	☒ Antibodies	☒	☐ ChIP-seq
☐	☒ Eukaryotic cell lines	☐	☒ Flow cytometry
☒	☐ Palaeontology and archaeology	☒	☐ MRI-based neuroimaging
☐	☒ Animals and other organisms		
☒	☐ Clinical data		
☒	☐ Dual use research of concern		
☒	☐ Plants		

Antibodies

Antibodies used	Flow cytometry Experiments: APC Anti-Mouse CD11c Antibody (Elabscience, clone N418, E-AB-F0991E, dilution: 1/40), PE Anti-Mouse CD80 Antibody (Elabscience, clone 16-10A1, E-AB-F0992D, dilution: 1/40), FITC Anti-Mouse CD86 Antibody (Elabscience, clone GL-1, E-AB-F0994C, dilution: 1/40), APC Anti-Mouse CD3 Antibody (Elabscience, clone N17A2, E-AB-F1013E, dilution: 1/40), PE/Elab Fluor® 594 Anti-Mouse CD8 Antibody (Elabscience, clone YTS-169, AN00576P, dilution: 1/40), APC Anti-Mouse CD45 Antibody (BD Biosciences, clone 30-F11, 559864, dilution: 1/200), BV510 Anti-Mouse CD3e Antibody (BD Biosciences, clone 145-2C11, 563024, dilution: 1/200), FITC Anti-Mouse CD4 Antibody (BD Biosciences, clone RM4-5, 553046, dilution: 1/200), PerCP-Cy5.5 Anti-Mouse CD8a Antibody (BD Biosciences, clone 53-6.7, 551162, dilution: 1/200), FITC Anti-human CD56 (Biolegend, clone HCD56, 318303, dilution: 1/200), PE Anti-human CD107a Antibody (BD Biosciences, clone H4A3, 555801, dilution: 1/200), PE-Cy7 Anti-human IFN-γ Antibody (BD Biosciences, clone B27, 560924, dilution: 1/200). Immunofluorescence Experiments: Recombinant PD-L1 antibody (Servicebio, clone 2H8E3, GB12339, dilution: 1/400), Recombinant Anti-CD4 antibody (Servicebio, clone HEK293, GB300601, dilution: 1/400), Recombinant Anti-CD8 antibody (Servicebio, clone 5C11B7-2, GB12068, dilution: 1/400), Recombinant Anti-CD11c antibody (Servicebio, clone 8H7F2, GB12059, dilution: 1/200), Anti-HMGB1 Rabbit pAb (Servicebio, GB11103, dilution: 1/1000), Anti-Calreticulin Rabbit pAb (Servicebio, GB112134, dilution: 1/1000). Immunohistochemistry Experiments: Recombinant IFN-γ antibody (Servicebio, GB11107, dilution: 1/200). Western blotting Experiments: Vinculin Polyclonal antibody (Proteintech, 66305-1-Ig, dilution: 1/10000), Anti-PD-L1 antibody (Abcam, clone 28-8, ab205921, dilution: 1/1000) Animal Experiments: InVivoMAb anti-mouse PD-L1 (B7-H1) (BioXcell, clone 10F.9G2™ BE0101, dilution: 1/10).
Validation	All antibodies were verified by the supplier's websites and each lot has received quality tested. APC Anti-Mouse CD11c Antibody, Elabscience, http://wbm.elabscience.cn/p-apc-anti-mouse-cd11c-antibody-n418-e-ab-f0991e-35563 PE Anti-Mouse CD80 Antibody, Elabscience, http://wbm.elabscience.cn/p-apc-anti-mouse-cd80-antibody-16-10a1-e-ab-f0992e-34994 FITC Anti-Mouse CD86 Antibody, Elabscience, https://www.elabscience.cn/p-pe-anti-mouse-cd86-antibody-gl-1-e-ab-f0994d-34620 APC Anti-Mouse CD3 Antibody, Elabscience, http://wbm.elabscience.cn/p-apc-anti-mouse-cd3-antibody-17a2-e-ab-f1013e-36316 PE/Elab Fluor® 594 Anti-Mouse CD8 Antibody, Elabscience, http://wbm.elabscience.cn/p-pe-elab-fluor-594-anti-mouse-cd8-antibody-yts-169-an00576p-94184 APC Anti-Mouse CD45 Antibody, BD Biosciences, https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/apc-rat-anti-mouse-cd45.559864 BV510 Anti-Mouse CD3e Antibody, BD Biosciences, https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/bv510-hamster-anti-mouse-cd3e.563024 FITC Anti-Mouse CD4 Antibody (BD Biosciences, 553046, https://www.bdbiosciences.com/zh-cn/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/fits-rat-anti-mouse-cd4.553046

PerCP-Cy5.5 Anti-Mouse CD8a Antibody, BD Biosciences, https://www.bdbiosciences.com/zh-cn/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/percp-cy-5-5-rat-anti-mouse-cd8a.551162?tab=product_details
 FITC Anti-human CD56, Biolegend, <https://www.biolegend.com/en-gb/products/fits-anti-human-cd56-ncam-antibody-3795>
 PE Anti-human CD107a Antibody, BD Biosciences, <https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-mouse-anti-human-cd107a.555801>
 PE-Cy7 Anti-human IFN-γ Antibody, BD Biosciences, <https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-cy-7-mouse-anti-human-ifn.560924>
 Recombinant PD-L1 antibody, Servicebio, <https://www.servicebio.cn/goodsdetail?id=12840>
 Recombinant Anti-CD4 antibody, Servicebio, <https://www.servicebio.cn/goodsdetail?id=14083>
 Recombinant Anti-CD8 antibody, Servicebio, <https://www.servicebio.cn/goodsdetail?id=18498>
 Recombinant Anti-CD11c antibody, Servicebio, <https://www.servicebio.cn/goodsdetail?id=23084>
 Anti-HMGB1 Rabbit pAb, Servicebio, <https://www.servicebio.cn/goodsdetail?id=15532>
 Anti-Calreticulin Rabbit pAb, Servicebio, <https://www.servicebio.cn/goodsdetail?id=5108>
 Recombinant IFN-γ antibody, Servicebio, <https://www.servicebio.cn/goodsdetail?id=15536>
 Vinculin Polyclonal antibody, Proteintech, <https://www.ptgcn.com/products/Vinculin-Antibody-66305-1-Ig.htm>
 Anti-PD-L1 antibody, Abcam, <https://www.abcam.cn/products/primary-antibodies/pd-l1-antibody-28-8-ab205921>
 InVivoMAb anti-mouse PD-L1 (B7-H1), BioXcell, <https://bioxccl.com/invivomab-anti-mouse-pd-l1-b7-h1-be0101>

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	The CT26 (Catalog No. CRL-2638) and NK-92MI (Catalog No. CRL-2408) cell lines were obtained from ATCC. The MC38 cell line (Catalog No. SCSP-5431) was sourced from the National Collection of Authenticated Cell Cultures (NCACC), and the HCoEpiC cell line (Catalog No. 2950) was from ScienCell Research Laboratories.
Authentication	All cell lines authenticated with STR profiling or morphological conforming through the information provided by culture collections.
Mycoplasma contamination	All cell lines were negative for mycoplasma.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	6-8 weeks old, female Balb/C and C57BL/6 mice were purchased from Charles Rivers laboratories (China) to establish colorectal cancer (CRC) models. The Balb/C mice were utilized to establish the CT26 subcutaneous tumor-bearing mice model and CT26 cell lung metastatic mice model. The C57BL/6 mice were used to establish MC38 subcutaneous tumor-bearing mice model and AOM/DSS induced primary tumor model. All mice were housed under specific pathogen-free conditions with controlled temperature (21-23 °C), humidity (30% to 70%), and a 12 h light/dark cycle. They were fed a standard laboratory diet and had ad libitum access to drinking water.
Wild animals	The study did not involve wild animals.
Reporting on sex	Balb/C and C57BL/6 mice were female. Sex were not considered in study design.
Field-collected samples	None field-collected samples were utilized in this study.
Ethics oversight	All animal experiments were performed in accordance with protocols approved by the Laboratory Animal Welfare and Ethics Committee of the Army Medical University (Animal Ethical Statement Number: AMUWEC20210257).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	<i>Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.</i>
Novel plant genotypes	<i>Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.</i>
Authentication	<i>Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosaicism, off-target gene editing) were examined.</i>

Flow Cytometry

Plots

Confirm that:

- ☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cell flow cytometry detection of activated dendritic cells (DCs) and T cells by NRb in vitro:

The spleen tissues were extracted and grounded into single cell suspension. The obtained spleen cells were seeded into the upper trans-well insert (1×10^6 cells/well) to co-incubate with pretreated MC38 cells for 24 h. Subsequently, the spleen cells were collected and stained with antigen presenting cell (APC)-conjugated anti-mouse CD11c, PE-conjugated anti-mouse CD80, and FITC-conjugated anti-mouse CD86. For CD3+ and CD8+ cell detection, spleen cells were stained with APC-conjugated anti-mouse CD3 and PE-conjugated anti-mouse CD8.

Cell flow cytometry detection of NK cells activation:

The NK-92MI cells pretreated with chitosan or NRb were collected and stained with PE-conjugated anti-human CD107a, PE-Cy7 anti-human IFN- γ and FITC-conjugated anti-human CD56.

Cell flow cytometry detection of CD4+/CD8+ T cells in tumor tissues:

Freshly harvested tumor tissues were digested into single cell suspensions in PBS with Collagenase I (2 mg/mL, Acme), Collagenase IV (5 mg/mL, Acme) and DNase I (5 mg/mL, Acme) at 37 °C for 1 h, the resulting single-cell suspensions were filtered through a 70- μ m strainer. The cell suspension was centrifuged and stained with the following antibodies: APC-conjugated anti-mouse CD45, BV510-conjugated anti-mouse CD3, FITC-conjugated anti-mouse CD4 and PerCP/Cy5.5-conjugated anti-mouse CD8a. The above cells were fixed with 4% PFA and analyzed by flow cytometry.

Instrument

Gallios (Beckman Coulter)

Software

Flowjo 10.8.1

Cell population abundance

All cells were used for analysis of a relevant cell population. Cell count of at least 10,000 events were collected of a relevant cell population after the initial gating.

Gating strategy

Preliminary cell populations were gated for singlets using FS-A/SS-A or FS-H/SS-H. Control stains were used to distinguish between specific antibody staining and background staining. Specific immune cell populations were gated based on the specific antibody staining as described in each experiment.

- ☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.