Pore forming–mediated intracellular protein delivery for enhanced cancer immunotherapy

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Directly delivering therapeutic proteins to their intracellular targets remains a great challenge. Here, we apply CD8⁺ T cells to form pores on the tumor cells’ plasma membranes, enabling perfusion of ribonuclease A (RNase A) and granzyme B into cells, therefore effectively inducing tumor apoptosis and pyroptosis by activating caspase 3 and gасdermin E pathways to potentiate the CD8⁺ T cell–mediated immunotherapy. Then, RNase A, programmed cell death ligand 1 antibody, and a photothermal agent were further loaded into an injectable hydrogel to treat the low immunogenic murine breast cancer. Notably, three courses of laser irradiation induced efficient cell apoptosis and immune activation, resulting in a notable therapeutic efficacy that 75% of the tumors were ablated without relapse.

INTRODUCTION
Protein drugs have been applied for treating a variety of diseases (1), including diabetes (2), inflammation (3), and cancers (4). The commercialized protein therapeutics mainly target extracellular space due to internalization challenges (5, 6). So far, several strategies such as forming lipid or polymeric delivery systems (7, 8) or applying the electroporation technology (9, 10) have been developed to address these issues. Whereas the assembly of protein drugs with carriers could result in protein denaturation (11), the specific devices required for electroporation technology are limited for in vivo employment (12). Therefore, it remains urgent to develop improved clinically translational approaches for efficient intracellular protein delivery (6).

CD8⁺ T cells or natural killer (NK) cells secrete perforin (PRF) to exert their cytotoxicity (13, 14). PRF could insert into the membranes and form multiple transmembrane pores on the target cells (15). The size of these pores is generally larger than 10 nm (15), whereas the usually applied protein drugs are between 1 and 10 nm, so the proteins could directly pass through the pores to intracellular space. CD8⁺ T cells are the major mediators of the antitumor immune response, and many approaches have been developed to recruit CD8⁺ T cells to tumors, including vaccines (16), photothermal therapy (PTT) (17), photodynamic therapy (18), chemotherapy (17), or radiotherapy (19). Among these approaches, PTT was able to trigger a strong antitumor immune response by inducing immunogenic cell death (ICD) of tumor cells, turning the “cold” tumor “hot” (20), and promoting CD8⁺ T cells priming and infiltration in the tumor tissues (21).

RESULTS

Pore forming by PRF1 for perfusion of protein into cells
The hydrodynamic size of the commonly used protein therapeutics, including RNase A, granzyme B (GrB), ovalbumin, deoxyribonuclease I, saporin, horseradish peroxidase (HRP), and bovine serum albumin (BSA) was detected to be in the range of 1 to 10 nm. Meanwhile, the complement-like transmembrane pores arising from PRF polymerization are 10 to 20 nm (23), larger than the diameter of protein therapeutics. The cell toxicity of PRF1 was first evaluated on mouse breast cancer cell lines (4T1) by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylethrazolium bromide (MTT) assay. The pore formation by PRF1 could interfere with the matter exchange between the inner and the outer space of cell membrane, which thus exhibited concentration-dependent toxicity on 4T1 cells. The cell viability decreased to 79.8 and 63.2% separately after incubation with PRF1 (2.5 or 5 μg/ml) (fig. S1). When the concentration was higher than 10 μg/ml, nearly all of the cells were dead. Therefore, we chose PRF1 (2.5 μg/ml) with moderate toxicity for pore-forming application.

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The cytotoxic protein RNase A and GrB were used as the model proteins for combination therapy with PRF1, both of which could induce cell apoptosis after endocytosis (24, 25). The morphology had no obvious change after treatment with proteins only, while many fragments were generated after treatment with PRF1, which was a typical morphology feature of apoptosis (fig. S2). Furthermore, after cotreatment with RNase A or GrB, many cells exhibited pyroptosis feature with cell membrane ballooning (26).

We next evaluated whether the PRF1 could promote the uptake of proteins. The RNase A was labeled with fluorescein isothiocyanate (RNase A–FITC). The intracellular green fluorescence was significantly weak when 4T1 cells were incubated with RNase A–FITC only (Fig. 1B). In contrast, strong fluorescence signal was observed in the cells after incubating RNase A with PRF1 simultaneously, validating that PRF1 treatment could promote the perfusion of proteins into cells. This conclusion was also confirmed by flow cytometry (FCM) analysis (Fig. 1, C and D) where the mean fluorescence intensity of the PRF1(+) group was around 12-fold compared to the PRF1(−). Apart from RNase A, enhanced protein perfusion by PRF1 was also checked on GrB by FCM analysis. The GrB expression after transduction in 4T1 cells was detected after GrB antibody staining (Fig. 1, C and D). Of note, the GrB+ cell ratios increased sharply from 3.95% of PRF1(−) to 38.6% of PRF1(+).

Furthermore, we evaluated whether the enhanced uptake of RNase A/GrB could generate stronger cytotoxicity to cancer cells. As illustrated in Fig. 1E, RNase A alone had no toxicity on 4T1 cells even when the concentrations reached as high as 20 μg/ml. Consistent with the improved cell uptake, the RNase A coincubation with PRF1 could significantly enhance the cytotoxicity on 4T1 cells. The cell viability decreased to 40% versus control after incubation with RNase A (20 μg/ml) + PRF1 (2.5 μg/ml). The combination index (CI) was calculated as 0.3129 (<1), indicating a strong synergistic effect between PRF1 and RNase A. The same conclusion could also be obtained on GrB protein (Fig. 1F).

**CD8⁺ T cell–mediated intracellular protein perfusion and synergistic tumor cell killing**

During immunotherapy, the activated CD8⁺ T cells could secrete PRF to mediate the tumor cell death by forming pores on the cell membrane (15, 27). However, the pore-repair process may be a critical reason for the failure of immunotherapy, especially in cold tumors where the infiltrated CD8⁺ T cells were insufficient (28). Thus,
supplementing exogenous GrB or other cytotoxic proteins (RNase A) before the pore closed may enhance the CD8+ T cell–mediated tumor cell killing (Fig. 2A).

The CD8+ T cell was activated in vivo by the tumor PTT and separated by magnetic bead sorting technique [magnetic-activated cell sorting (MACS)], where the interferon-γ (IFN-γ) CD8+ effector T cells were detected to be 32.8% (fig. S3). The cell viability of tumor cells after incubation with CD8+ T was detected by using luciferase-overexpressing 4T1 cells (4T1-Luc) (29). The 4T1-Luc cell viability decreased with the increasing ratio of CD8+ T/4T1-Luc. Specifically, the cell viability was 80% at a ratio of 5 to 1, and it further decreased to 60% when the ratio reached 10 to 1 (Fig. 2B). It was indicated that a large amount of CD8+ T cells was needed for efficient antitumor therapy. As expected, the combination with RNase A or GrB could significantly enhance the tumor cell–killing activity of CD8+ T cells. Figure 2C and fig. S4 indicated that RNase A or GrB alone had no toxicity on 4T1 cells, while the combination with CD8+ T could generate a strong synergistic killing effect where the cell viability decreased from 80.6% of CD8+ T group to 47.8% of CD8+ T + RNase A group and 34.1% of CD8+ T + GrB group. It means that the nontoxic RNase A or GrB could amplify the toxicity of CD8+ T cells on 4T1 tumor cells, holding the potential to be applied for achieving synergistic immunotherapy.

The synergistic effect could be ascribed to the cell uptake enhancement of protein by CD8+ T–mediated pore formation; thus, we further evaluated the cell uptake of RNase A or GrB in the presence or absence of CD8+ T. As shown in Fig. 2 (D and E), the RNase A–FITC–positive cells were only 6.87% for RNase A–FITC treatment group while significantly increased to 70.4% after cotreatment with CD8+ T cells. As for GrB+ cell detection (Fig. 2, F and G), similar to RNase A, the GrB+ cell ratio was as low as 1.35% after treatment with GrB only. Of note, the activated CD8+ T cells itself could only up-regulate the GrB+ cell ratio to 37.1%, which was further markedly increased to 87.1% after coinoculation with GrB protein. It validated our hypothesis that, although the CD8+ T cells could secrete PRF and GrB directly, the amount is not sufficient for killing the cancer cells. It may thus lead to the failure of CD8+ T cell–mediated immunotherapy, especially in cold tumors with less immune cell infiltration (30). The combination therapy with cytotoxic proteins (RNase A or GrB) was able to enhance their tumor cell–killing outcome.

Cell apoptosis, pyroptosis, and immune activation in vitro

We next investigated the synergistic killing mechanism between CD8+ T cells and proteins. The RNase A could induce the degradation of intracellular RNA and thus trigger the activation of Cas 3 and cell apoptosis (31). In addition, apart from inducing cell apoptosis, the
cleaved Cas 3 (C-Cas 3) was capable of cleaving the gasdermin E (GSDME) to generate N-terminal GSDME (N-GSDME) fragment. N-GSDME was able to insert into the cell membrane and form pores to induce cell pyroptosis (32). Besides, the GrB could not only activate the Cas 3 to induce cell apoptosis and pyroptosis but also directly cleave the GSDME for inducing the pyroptosis (33). We therefore further studied the cell-killing mechanism of combination therapy. The cell apoptosis was evaluated by annexin V/propidium iodide (PI) double staining. The annexin V+/PI− indicates the late apoptosis or necrosis cells. As depicted in Fig. 3 (A and B), consistent with the cell viability assay, the RNase A or the GrB could not induce annexin V+/PI− cells owing to the poor cell uptake. Besides, the CD8+ T only induced 14.1% annexin V+/PI− cells. The CD8+ T combination with RNase A or GrB induced 26.5 and 34.5% annexin V+/PI− cell, respectively, further substantiating the synergistic effect.

The key protein expression of apoptosis and pyroptosis was evaluated by Western blot. After incubation with CD8+ T cells (CD8+ T/4T1 = 5:1), both the C-Cas 3 and N-GSDME were not effectively activated (Fig. 3C), which could be ascribed to the weak killing effect on 4T1 cells under relatively low CD8+ T/4T1 ratio. However, when the CD8+ T cells were combined with the RNase A or GrB, the expression of C-Cas 3 and N-GSDME were markedly up-regulated. The combined data of Fig. 3 (A and C) indicated that the cells were under apoptosis. In addition, the up-regulated N-GSDME reflected the successful induction of pyroptosis, which was further confirmed by the high level of lactate dehydrogenase [LDH; the biomarker of pyroptosis (26)] in the supernatants (Fig. 3D). The LDH released ratios were 29.8 and 55.8% after treatment with CD8+ T plus RNase A or GrB, respectively. Moreover, the secretion of high-mobility group box 1 (HMGB1) (Fig. 3E), as the biomarker of ICD (34), increased from 0.45 to 1.15 and 1.82 ng/ml after treatment with CD8+ T + RNase A or CD8+ T + GrB. In addition, the adenosine triphosphate (ATP) secretion (Fig. 3F), as the “find me” signal in damage-associated molecular patterns (35), was also significantly promoted after combination treatment, where the ATP level in the supernatant was three- or fivefold higher compared to the untreated cells.

The supernatant of each group was incubated with dendritic cell lines (DC 2.4) to evaluate their ability to trigger the DC maturation. As shown in Fig. 3G and fig. S5, the DC maturation ratio increased from 3.79% of the untreated group to 8.15% for CD8+ T–treated group, indicating a weak immune response to sole treatment of CD8+ T cells. Nevertheless, DC maturation ratio sharply increased to 18.5% (CD8+ T + RNase A) and 30% (CD8+ T + GrB), respectively, attributed to the effective induction ICD by the combination strategy. In addition, the RNase A or GrB was demonstrated to have no negative influence on
the activation state of CD8+ T cells by detecting the IFN-γ level in the medium with or without the presence of RNase A/GrB (fig. S6). These results demonstrated that the combination with RNase A or GrB was a convincing strategy to amplify the CD8+ T cell–mediated immune response and tumor cell growth inhibition.

**In vivo antitumor therapy by the combination of PTT with RNase A**

The in vitro study has validated the synergistic therapy efficacy between CD8+ T cells and RNase A/GrB. Therefore, we investigated the in vivo application potential of this strategy by taking RNase A as the model protein drug. The therapeutic schedule was displayed in Fig. 4A, where the tumor-specific CD8+ T cell was activated by PTT after injecting with IR780 and giving laser irradiation. Meanwhile, the RNase A was administrated by intratumoral injection. The photothermal curve indicated that the tumoral temperature of untreated mice could only rise to 36°C after laser irradiation, while the temperature of IR780 administrated tumor could reach as high as 45°C within 2 min and maintained for 3 min (Fig. 4B). The tumor volume was monitored every 3 days. As shown in Fig. 4 (C and D),

![Image](image_url)
after three times of PTT, the tumor still grew fast with moderate anti-tumor efficacy. In contrast, the combination of PTT with RNase A could significantly inhibit the tumor growth, which was much stronger than the single PTT treatment, indicating that RNase A could not inhibit the tumor growth but could strengthen the therapeutic efficacy of PTT. On day 18, the excised tumors of mice with PTT + RNase A treatment were obviously smaller than those in other groups (Fig. 4E). The tumor weight of PTT group and PTT + RNase A group was 66% and 30% for the saline group, respectively (Fig. 4F). In addition, no significant weight loss in the mice was observed during monitoring (fig. S7). Moreover, the survival time of each group was also monitored. Without treatment, the median survival time was only 30 days, which was prolonged to 44 days after PTT (Fig. 4G). Whereas, the survival ratio was still above 50% until day 90 for the PTT + RNase A combination--treated mice.

The hematoxylin and eosin (H&E) staining of the tumor sections indicated clear apoptosis morphology in PTT-treated tumor and serious necrosis in PTT + RNase A--treated tumors (Fig. 4H). The cell nuclei and cytoplasm were shown as dark blue and pink, respectively, in the saline treatment group. However, after mild PTT + RNase A treatment, obvious shrinking and breaking of nuclei occurred, shown as the disappearance of dark blue and the enhancement of pink. The tumor infiltration of CD8\(^+\) T cells was analyzed by immunofluorescence (IF) staining and FCM. The red fluorescence signal of CD8\(^+\) T cells was weak in both saline and RNase A--treated tumor, while it was very strong in PTT + RNase A--treated tumor, reflecting the highly efficient infiltration of CD8\(^+\) T cells in the tumor tissues. Besides, the IFN-\(\gamma\) production (effector cytokines) after different treatments was detected by an enzyme-linked immunosorbent assay (ELISA) kit. As shown in fig. S8, the mild PTT and RNase A combination could significantly up-regulate the generation of IFN-\(\gamma\), much higher than mild PTT group or RNase A group. It reflected that more CD8\(^+\) T cells were activated after mild PTT + RNase A treatment. The FCM analysis also indicated that the infiltrated CD8\(^+\) T cell ratios increased from 3.65% of the saline group to 16.5% of the PTT group and 40.3% of the PTT + RNase A group, respectively (Fig. 4I; gating strategy shown in fig. S9).

The preparation and characterization of HIR780, RNase A, and PD-L1--coloaded hydrogels (IRA/\(\alpha\)PD-L1 gel)

Previous studies have reported that the mild PTT could induce the up-regulation of programmed cell death ligand 1 (PD-L1) on tumor cells, protecting the tumor cells from attacking by CD8\(^+\) T cells (20). Hence, the \(\alpha\)PD-L1 antibody (PD-L1 blocking antibody) was desired to be coadministered for immune checkpoint blocking to prevent the side effects of mild PTT and further amplify the immune effect (36).

Hydrogel formulation was used for the coadministration of the three components (HIR780, RNase A, and \(\alpha\)PD-L1), defined as IRA/\(\alpha\)PD-L1 gel. In this hydrogels, IR780 (dye) was encapsulated in HSA to form HSA@IR780 (HIR780) nanoparticles as the photothermal agents for PTT to induce CD8\(^+\) T cell activation. RNase A was used as the model protein therapeutics for the combination therapy with CD8\(^+\) T cells. In addition, \(\alpha\)PD-L1 antibody was applied as immune checkpoint blocker to enhance the immunotherapy. Then, nonionic materials of P407 and \(\alpha\)-CD were used to prepare the protein-loaded hydrogels (Fig. 5A), both of which did not interfere with the secondary structures of proteins as measured by the circular dichroism spectrum (Fig. 5B). With laser irradiation, the temperature could quickly rise to 45°C and maintain for 3 to 4 min for both the HIR780 solution and IRA/\(\alpha\)PD-L1 gels (Fig. 5C). Of note, the IRA/\(\alpha\)PD-L1 gels exhibited better stability than HIR780 solutions, which could achieve three heat/cool cycles without obvious attenuation (Fig. 5D).

In addition, the IRA/\(\alpha\)PD-L1 gel showed thermo-responsive mechanical strength change (Fig. 5E) and irreversibly switched within three circles (Fig. 5F). The IRA/\(\alpha\)PD-L1 was in gel state under 37°C but turns to solution state under 45°C (Fig. 5G). Consistently, the thermo-induced phase transition accelerates the drug release after each laser irradiation (Fig. 5H) (37).

In addition, the hydrogel formulations act as the drug depot for prolonged tumor retention and sustained release of drugs (38). The free HIR780 solutions underwent fast metabolism within several days, while the IRA/\(\alpha\)PD-L1 gels could maintain strong retention at tumor site over 15 days (Fig. 5I). Furthermore, the RNase A was labeled with FITC to track its in vivo distribution by detecting the fluorescence intensity of FITC in the lysate of main organs [heart, liver, spleen, lung, kidney, and lymph node (LN)]. Similar to HIR780, the results also confirmed that RNase A had negligible distribution in these organs (fig. S10). Of note, the PTT induced infiltration of tumor cell--specific CD8\(^+\) T cells, which only form pores on tumor cells rather than normal cells. Thus, although RNase A partly distributed to normal tissues, it could not generate toxicity effectively. To further confirm that, the alanine transaminase (ALT), aspartate transaminase (AST), and blood urea nitrogen (BUN), as the biomarkers of the liver and kidney functions, were detected to have no significant change after various treatments (fig. S11).

**Antitumor efficacy of IRA/\(\alpha\)PD-L1 hydrogels**

The HIR780, RNase A, and \(\alpha\)PD-L1 solutions (IRA/\(\alpha\)PD-L1) or coloaded hydrogels (IRA/\(\alpha\)PD-L1 gel) were intratumorally injected on day 0 with single dose, followed by laser treatment on days 0, 3, and 6 (Fig. 6A). The tumor growth curve in Fig. 6B indicated that the drug solution administration of IRA/\(\alpha\)PD-L1 + L only generate slightly tumor inhibition effect. Although the \(\alpha\)PD-L1 was further combined with PTT + RNase A treatment, it did not obviously enhance the therapeutic efficacy, which could mainly be ascribed to the poor retention of free therapeutics at tumor site. Therefore, the therapeutic efficacy was obviously enhanced after treatment with gel (IRA/\(\alpha\)PD-L1 gel + L), which could totally inhibit the tumor growth and even lead to most of the tumor ablation after three times of laser irradiation. Of note, the therapeutic efficacy of the IRA gel + L (without \(\alpha\)PD-L1) was much weaker than IRA/\(\alpha\)PD-L1 gel + L, confirming the necessity of the combination with \(\alpha\)PD-L1. It overcame the shortcomings of PTT-induced PD-L1 up-regulation for enhancing the CD8\(^+\) T--mediated immunotherapy. The ex-tumor images shown in Fig. 6C indicated that, although two tumors remained after IRA/\(\alpha\)PD-L1 gel + L treatment, they were much smaller than those in the saline group. The excellent therapeutic efficacy significantly prolonged the survival time of 4T1 tumor--bearing mice, where the survival ratio was still above 75% until day 100 (Fig. 6D). For the saline group, all of the mice were dead within 35 days. In addition, after treatment with IRA/\(\alpha\)PD-L1 solution + L or IRA gel + L, the medium survival prolonged from 27 to 49 and 61 days separately.

The immune activation in vivo was analyzed. After IRA/\(\alpha\)PD-L1 gel + L treatment, the ratio of tumor infiltration CD8\(^+\) T cells could reach as high as 74.6 versus 9.37% of saline, much higher than IRA/\(\alpha\)PD-L1 + L or IRA gel + L group of 27.8 and 43.7% (Fig. 6, E and F). Furthermore, for the IRA/\(\alpha\)PD-L1 gel + L treatment group, the ratio of mature DC in the LN was 14-fold higher than that of the saline.
group (Fig. 6, G and H). In addition, the IRA/αPD-L1 gel + L treatment also markedly up-regulated the contents of tumor necrosis factor–α (TNF-α) and IFN-γ in plasma (fig. S12).

**DISCUSSION**

In this work, an approach for intracellular protein delivery was developed by using CD8⁺ T cells to generate pores on cell membrane. The intracellular delivery of RNase A or GrB was significantly enhanced after coincubation with the PRF1 or the CD8⁺ T cells, consequently working synergistically to achieve enhanced tumor cell killing ability. The perfusion of RNase A activated the Cas 3 and GSDME pathways, which was proved to induce tumor cell apoptosis and pyroptosis for further immune activation. Moreover, mild PTT (45°C) was applied for CD8⁺ T cells priming in vivo on xenografted 4T1 tumors. To enhance the drug retention and control the protein release, we prepared P407/α-CD thermosensitive hydrogels to load photosensitizer HIR780, RNase A, and αPD-L1, obtaining IRA/αPD-L1 gel. A single dose of the hydrogels plus three times of laser irradiation could cure 75% of the mouse breast cancer without relapse. These results validated that the supplementary of exogenous cytotoxic protein drugs was an efficient strategy for enhancing cancer immunotherapy. Apart from PTT, other therapeutic agents could induce CD8⁺ T cells priming in vivo, which may also hold the potential to be applied for synergistic therapy with protein drugs targeted toward intracellular destination. Besides, Chimeric Antigen Receptor T (CAR-T) cell (33) or NK cell (39) immunotherapy may create pores on the cell membrane, which could share the similar potential to be coapplied with protein drugs.
MATERIALS AND METHODS

Materials

RNase A was purchased from Macklin Biochemical Co. Ltd. (Shanghai, China). Recombinant mouse GrB was purchased from Prospector Technogen Ltd (Ness Ziona, Israel). PRF1 was purchased from Cloud-Clone Corp (Wuhan, China). FITC was purchased from Aladdin Chemical Regent Co. (Shanghai, China). HSA and IR780 were purchased from Sigma-Aldrich (USA). αPD-L1 for in vivo application was purchased from Bio X Cell. Annexin V–FITC/PI cell apoptosis assay kits and ATP detection kits were purchased from Solarbio (Beijing, China). LDH assay kits, Luciferase Reporter Gene Assay Kit, Dulbecco’s modified Eagle’s medium (DMEM), phosphate-buffered saline (PBS), and MTT were purchased from KeyGEN BioTECH (Nanjing, China). Enhanced chemiluminescence (ECL) solution was purchased from Vazyme Biotech Co. Ltd. (Nanjing, China). GSDME, Cas 3, and β-tubulin antibodies were purchased from Abcam (Shanghai, China). Brilliant Violet 421 anti-mouse CD45 (catalog number, 103134), phycoerythrin (PE) anti-mouse CD3 (catalog number, 100206), FITC anti-mouse CD4 (catalog number, 100206), PE anti-mouse CD11c (catalog number, 104714), and APC anti-mouse CD80 (catalog number, 104714) were purchased from BioLegend.
Cell lines
The murine breast cancer cell lines (4T1) were purchased from the American Type Culture Collection and cultured in complete DMEM (Gibco, Invitrogen) with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), and 1% l-glutamine.

Animals model
BALB/c mice (6 to 8 weeks old, 18 to 20 g) were purchased from the Yangzhou University (Yangzhou, China). All of the animal experiments were complied with the protocol approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University. The mouse breast cancer model was constructed by subcutaneously injecting 4T1 cells on BALB/c female mice (10⁶ cells per mouse).

CD8⁺ T cells priming in vivo and isolation
The 4T1-specific CD8⁺ T cells were primed in vivo by inducing photothermal immune response and sorted by MACS. The 4T1-bearing mice were intratumorally injected with IR780 and given laser irradiation for 5 min (1 W/cm², 808 nm) when the tumor volume reached 200 mm³. After 5 days, the 4T1-specific CD8⁺ T cells were isolated from the spleen or LNs of the mice by MACS (Mouse CD8⁺ T Cell Isolation Kit, BD Biosciences) and were cultured in DMEM containing interleukin-2 (100 U/ml) with activation by the anti-CD3/CD28 beads for further use. The IFN-γ CD8⁺ T cell ratio was detected by FCM.

Cytotoxicity test in vitro
To evaluate the cytotoxicity of PRF1, protein drugs (RNase A and GrB) or the combination of PRF1 with protein drugs, the 4T1 cells were seeded into 96-well plates and grown to 50% confluence. The drugs were diluted with DMEM to the intended concentrations and incubated with the cells for 24 hours. Then, each well was added with 20 µl of MTT (5 mg/ml in PBS) and incubated for additional 4 hours under 37°C. The generated formazan was dissolved by dimethyl sulfoxide (DMSO) and detected by microplate reader (492 nm). The CI was calculated by the following formula

$$CI = \frac{D_1}{(D_m)_1} + \frac{D_2}{(D_m)_2}$$

(D_m)_1 and (D_m)_2 represent the median inhibitory concentration (IC50) of treatments RNase A and PRF1 applied separately, while D_1 and D_2 are IC50 of treatments RNase A and PRF1 applied as a combination.

The tumor cell–killing effect of CD8⁺ T cells combined with protein drugs was evaluated by using luciferase expression 4T1 cells (4T1-Luc). The 4T1-Luc cells were seeded into 96-well plates (2000 per well) and grown to 50% confluence. The CD8⁺ T cells were incubated with 4T1-Luc cells with different ratios (CD8⁺ T/4T1-Luc: 2:1, 5:1, 10:1, 20:1, and 40:1), and protein therapeutics or the combination was diluted with DMEM and incubated with 4T1-Luc cells for 24 hours. Afterward, the old medium was removed. The luciferase intensity of each well was detected by using an enhanced luciferase assay kit.

Perfusion of protein therapeutics into cells
The RNase A was first labeled with fluorescence dye FITC for cell perfusion study. Briefly, 10 mg of RNase A was dissolved in Na₂CO₃/NaHCO₃ buffer (pH 9). Then, the FITC dissolved in DMSO was added and reacted with RNase A for 24 hours under room temperature (RT). The reaction solution was dialyzed against purified water, and it was lyophilized to obtain RNase A–FITC powder for further application.

The perfusion of RNase A in the absence or presence of PRF1/CD8⁺ T cells was observed by confocal laser scanning microscopy (CLSM) and FCM. For CLSM experiment, the 4T1 cells were seeded into confocal dishes and were treated with RNase A–FITC (20 µg/ml) in the absence or presence of PRF1 (2.5 µg/ml) for 6 hours, followed by washing twice with PBS and fixing with paraformaldehyde. The nuclei were stained with 4',6-diamidino-2-phenylindole solution for 15 min before observation. For FCM analysis of RNase A–FITC perfusion, the 4T1 cells were seeded into a 24-well plate, which were given the same treatment as mentioned in CLSM experiment and collected after treatment for 6 hours for FCM analysis.

The perfusion of GrB in the absence or presence of PRF1/CD8⁺ T cells was evaluated by FCM. The 4T1 cells were seeded into a 24-well plate and incubated with GrB (5 µg/ml) in the absence or presence of PRF1 (2.5 µg/ml) for 6 hours. For detection of intracellular GrB, the cells were treated with Intracellular Fixation and Permeabilization Buffer (eBiosciences) and then incubated with anti-mouse GrB-PE antibody for 1 hour under 4°C environments.

Tumor cell apoptosis detection
The 4T1 tumor cell apoptosis after CD8⁺ T cells and protein drugs treatment was detected by annexin V-FITC/PI double staining. 4T1 cells were seeded into a 12-well plate and grown to 50% confluence. They were separately treated with CD8⁺ T (CD8⁺ T/4T1-Luc = 5:1), protein therapeutics (10 µg/ml), or the combination for 24 hours, followed by washing twice. The cells were collected for annexin V-FITC/PI staining for 15 min before FCM analysis.

Western blot analysis of the protein expression
The main protein expressions of apoptosis and pyroptosis were investigated by Western blot. The 4T1 cells were seeded into a six-well plate and given the same treatment as mentioned in cell apoptosis experiment. Subsequently, the cells were lysed by radioimmunoprecipitation assay lysis buffer and centrifuged to collect the proteins. Protein concentrations were determined by a bicinchoninic acid assay kit. It was then run on SDS–polyacrylamide gel electrophoresis to separate the different molecular weight proteins and then transferred to polyvinylidene difluoride membranes. The membranes were blocked by 5% BSA and incubated with the primary antibodies overnight, including Cas 3, C-Cas 3, GSDME, N-GSDME, and β-tubulin. HRP-conjugated secondary antibody was then incubated with the membrane for 1 hour after washing for five times with Tris Buffer Solution-Tween (TBST) buffer (5 min per wash). The bands were observed by ECL.

DC maturation detection
The 4T1 cells were given the same treatment as mentioned in cell apoptosis experiment. The supernatants were collected and centrifuged to remove the suspended CD8⁺ T cells, followed by incubation with DC 2.4 for additional 24 hours. DC 2.4 cells were stained with anti-mouse CD80-FITC and anti-mouse CD86-APC antibodies for 1 hour under 4°C. The CD80⁺ CD86⁺–positive cell ratios were analyzed by FCM.

Hydrogel preparation and characterization
The IR780-loaded HSA nanoparticles (HSA@IR780, HIR780) were fabricated as reported. Briefly, 100 mg of HSA was dissolved in
50 ml of water and added with dithiothreitol (20 mM) to reduce the disulfide bond. Then, 10 mg of IR780 dissolved in 5 ml of ethanol was dropwise added into the solutions and stirred at RT for 6 hours. The products were dialyzed against water for 24 hours and freeze-dried to obtain HIR780 nanoparticles for further use. The drug loading efficiency of IR780 was determined to be 8.2%.

The P407 was dissolved in water by stirring under 4°C environment, and α-CD was dissolved in water under RT. For preparing hydrogels, the therapeutics (RNase A, HIR780, and αPD-L1 antibody) dispersed in sterile water were mixed with P407 and α-CD together. The content of each compound was P407 (100 mg/ml), α-CD (70 mg/ml), IR780 (400 µg/ml), RNase A (800 µg/ml), and αPD-L1 (400 µg/ml). The solutions would gradually turn to gel state under 37°C. The storage modulus ($G'$) and loss modulus ($G''$) of the gels were detected by using a dynamic shear rheometer (Kinexus Rotational Rheometer, Malvern, UK) with a temperature range from 30° to 60°C. Besides, the cyclic temperature–dependent modulus change was also detected between 37° and 45°C for three circles. The in vitro release behavior of RNase A was investigated by labeling RNase A with FITC. The HIR780 and RNase A–FITC–colloidal gels were immersed in PBS (pH 7.4) and incubated under 37°C water bath for 15 days. To further evaluate the laser-triggered drug release, the gels were given laser irradiation for 5 min (1 W/cm², 808 nm) on days 1, 3, and 5.

For in vivo gel retention test, the HIR780 or IRA@αPD-L1 gels (IR780 dose, 1 mg/kg) were intratumorally injected into 4T1-bearing mice. The fluorescence photographs of the mice were taken on days 1, 2, 4, 6, 8, 12, and 15. To evaluate the biodistribution of RNase A, FITC-labeled RNase A–FITC was coloaded in IRA@αPD-L1 gels and administrated by intratumoral injection. The tumor tissues and main organs (heart, liver, spleen, lung, kidney, and LN) were collected. The FITC fluorescence intensity in each tissue was measured and normalized with tissue weight.

In vivo anti-tumor study

The 4T1-bearing mice were constructed as mentioned above. The mice were randomly divided into four groups ($n = 16$): saline, mild PTT, RNase A, or mild PTT + RNase A. Among the 16 mice for each group, three mice were used for immune cell analysis on day 10 and five mice for tumor growth curve monitoring until day 18. The residue mice were used for survival monitoring to day 90. On days 0, 1, and 3, the mice were intratumorally injected with saline, IR780 (1 mg/kg), RNase A (2 mg/kg), or IR780 + RNase A. The laser irradiation (1 W/cm², 808 nm) was performed immediately after administration for 5 min on days 0, 1, and 3. The tumor volume and body weight were recorded every 3 days. On day 10, a part of the mice was harvested for immune analysis. The tumor tissues were sheared into small fragments and digested with collagenase for 30 min under 37°C. The tumor-infiltrated lymphocyte cells underwent enrichment by Percoll buffer and stained with Brilliant Violet 421 anti-mouse I-A/I-E, FITC anti-mouse CD86, and APC anti-mouse CD80 antibodies. The survival was monitored until day 100.

Cytokines detection and blood biochemical test

For cellular study, the CD8⁺ T cells were incubated with 4T1 cells in the presence or absence of RNase A/GrB. After incubation for 24 hours, the culture medium was collected, and the IFN-γ secretion was detected by a mouse IFN-γ ELISA kit.

For animal study, the mice were given the treatment as mentioned above in antitumor study, and the serum was collected on day 10. TNF-α and IFN-γ in plasma were detected by a mouse TNF-α or IFN-γ ELISA kit. ALT, AST, and BUN were detected by the corresponding kits under the guidance of the standard protocol.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6 software. Data from the experiments were performed for three times or more than three times. The results were expressed as means ± SD. A two-tailed Student’s $t$ test was performed for statistical analysis of the difference between the two groups. $P$ value < 0.05 was considered statistically significant between the datasets, where all significant values were indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.sciencemag.org/content/58/11/eaab4659.

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES

1. C. Krejza, M. Rogge, W. Sadee, Protein therapeutics: New applications for pharmacogenetics. Nat. Rev. Drug Discov. 5, 507–521 (2006).
2. J. Yu, J. Wang, Y. Zhang, G. Chen, W. Mao, Y. Ye, A. R. Kakhoska, J. B. Buse, R. Langer, Z. Gu, Glucose-responsive insulin patch for the regulation of blood glucose in mice and minipigs. Nat. Biomed. Eng. 4, 499–506 (2020).
3. J. Xu, Z. Li, Q. Fan, J. Lv, Y. Li, Y. Cheng, Dynamic polymer amphiphiles for efficient intracellular and in vivo protein delivery. Adv. Mater. 33, 2104355 (2021).
4. Y. Rui, D. R. Wilson, J. Choi, M. Varanasi, K. Sanders, J. Karlsson, M. Lim, J. Green, Carboxylated branched poly (l-amino ester) nanoparticles enable robust cytosolic protein delivery and CRISPR-Cas9 gene editing. Sci. Adv. 5, eaay3255 (2019).
5. J. Lv, Q. Fan, H. Wang, Y. Cheng, Polymers for cytosolic protein delivery. Biomaterials 218, 119358 (2019).
6. Z. Gu, A. Biswas, M. Zhao, Y. Tang, Tailoring nanocarriers for intracellular protein delivery. Chem. Soc. Rev. 40, 3638–3655 (2011).
7. T. D. Brown, K. A. Whitehead, S. Mitragotri, Materials for oral delivery of drugs and peptides. Nat. Rev. Mater. 5, 127–148 (2020).
8. S. Mitragotri, P. A. Burke, R. Langer, Overcoming the challenges in administering biopharmaceuticals: Formulation and delivery strategies. Nat. Rev. Drug Discov. 13, 655–672 (2014).
9. Y. Cao, E. Ma, S. Castellanos-Blanco, B. Zhang, R. Qiu, Y. Su, J. A. Doudna, P. Yang, Nontoxic nanopore electroporation for effective intracellular delivery of biological macromolecules. Proc. Natl. Acad. Sci. 116, 7899–7904 (2019).
10. Y. Qu, Y. Zhang, Q. Yu, H. Chen, Surface-mediated intracellular delivery by physical membrane disruption. ACS Appl. Mater. Interfaces 12, 31054–31078 (2020).
11. Z. Zhang, W. Shen, J. Ling, Y. Yan, J. Hu, Y. Cheng, The fluorination effect of fluoroamphiphiles in cytosolic protein delivery. Nat. Commun. 9, 1377 (2018).
12. P. Mukherjee, S. S. P. Nathamgari, J. A. Kessler, H. D. Espinosa, Combined numerical and experimental investigation of localized electroporation-based cell transfection and sampling. *Sci. Adv.* 12, 12118–12128 (2018).

13. H. Raskov, A. Orhan, J. P. Christensen, J. Gøgenur, Cytotoxic CD8⁺ T cells in cancer and cancer immunotherapy. *Br. J. Cancer* 124, 359–367 (2021).

14. T. Bald, M. F. Krummel, M. J. Smyth, K. C. Barry, The NK cell–cancer cycle: Advances and new challenges in NK cell–based immunotherapies. *Nat. Immunol.* 21, 835–847 (2020).

15. I. Voskoboinik, J. C. Whistock, I. A. Trapani, Perforin and granzymes: Function, dysfunction and human pathology. *Nat. Rev. Immunol.* 15, 388–400 (2015).

16. J. Xu, J. Lv, Q. Zhang, Z. Yang, Z. Cao, L. Xu, P. Pei, C. Wang, H. Wu, Z. Dong, A general strategy towards personalized nanovaccines based on fluoropolymers for post-surgical cancer immunotherapy. *Nat. Nanotechnol.* 15, 1043–1052 (2020).

17. Y. Jiang, J. Huang, C. Xu, K. Pu, Activatable polymer nanoaonogist for second near-infrared photothermal phototherional therapy of cancer. *Nat. Commun.* 12, 742 (2021).

18. W. Li, J. Yang, M. Jiang, B. Qin, H. Yin, C. Zhu, X. Yuan, J. Zhang, Z. Luo, Targeting photodynamic and photothermal therapy to the endoplasmic reticulum enhances immunogenic cancer cell death. *Nat. Commun.* 10, 3349 (2019).

19. R. B. Patel, R. Hernandez, P. Carlson, J. Grudzinski, A. M. Bates, J. C. Jagodinski, A. Erbe, I. R. Marsh, I. Arthur, R. Alucio-Sardi, Low-dose targeted radionucleide therapy renders immunologically cold tumors responsive to immune checkpoint blockade. *Sci. Transl. Med.* 13, eabb3631 (2021).

20. L. Huang, Y. Li, Y. Du, Y. Zhang, X. Wang, Y. Ding, X. Yang, F. Meng, J. Tu, L. Luo, Mild photothermal therapy potentiates anti-PD-1 treatment for immunologically cold tumors via an all-in-one and all-in-control strategy. *Nat. Commun.* 10, 4871 (2019).

21. Z. Zhou, H. Wu, R. Yang, A. Xu, Q. Zhang, J. Dong, C. Qian, M. Sun, GSH depletion liposome adjuvant for augmenting the photothermal immunotherapy of breast cancer. *Sci. Adv.* 6, eabo4373 (2020).

22. X. Zhang, J. Du, Z. Guo, Y. Yu, Q. Gao, W. Yin, S. Zhu, Z. Gu, Y. Zhao, Efficient near infrared light triggered nitric oxide release nanocomposites for sensitizing mild photothermal therapy. *Adv. Sci.* 6, 1801122 (2019).

23. I. Voskoboinik, M. J. Smyth, J. A. Trapani, Perforin-mediated target-cell death and immune homeostasis. *Nat. Rev. Immunol.* 6, 940–952 (2006).

24. X. Qian, Z. Shi, H. Qi, M. Zhao, K. Huang, D. Han, J. Zhou, C. Liu, Y. Liu, Y. Lu, A novel Granzyme B nanoparticle delivery system simulates immune cell functions for suppression of solid tumors. *Theranostics* 9, 7616–7627 (2019).

25. X. Si, S. Ma, Y. Xu, D. Zhang, N. Shen, H. Yu, Y. Zhang, W. Song, Z. Tang, X. Chen, Hypoxia-sensitive supramolecular nanogels for the cytosolic delivery of ribonuclease A as a breast cancer therapeutic. *J. Control. Release* 320, 83–95 (2020).

26. J. Ding, K. Wang, W. Liu, Y. She, Q. Sun, J. Shi, H. Sun, D.-C. Wang, F. Shao, Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature* 535, 111–116 (2016).

27. Z. Zhang, Y. Zhang, S. Xia, Q. Kong, S. Li, X. Liu, C. Junqueira, K. F. Meza-Sosa, T. M. Y. Mok, J. Ansara, Gasdermin E suppresses tumour growth by activating anti-tumour immunity. *Nature* 579, 415–420 (2020).

28. M. Binnewies, E. W. Roberts, K. Kersten, V. Chan, D. F. Fearon, M. Merad, L. M. Coussens, D. I. Gabrilovich, S. Ostrand-Rosenberg, C. C. Hedrick, Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat. Med.* 26, 541–550 (2018).

29. V. O. Pimentel, A. Yaromina, D. Marcus, L. J. Dubois, P. Lambin, J. Ansara, Gasdermin E suppresses tumour growth by activating anti-tumour immunity. *Nat. Med.* 26, 541–550 (2018).

30. B. Bockorny, V. Semenisty, T. Macarulla, E. Borazanci, B. M. Wolpin, S. M. Stemmer, T. Golan, R. Geva, M. J. Borad, K. S. Pedersen, BL-8040, a CXCR4 antagonist, in combination with pembrolizumab and chemotherapy for pancreatic cancer: The COMBAT trial. *Nat. Med.* 26, 878–885 (2020).

31. J. E. Lee, R. T. Raines, Ribonucleases as novel chemotherapeutics: The raniprase example. *Biodrugs* 22, 53–58 (2008).

32. Y. Wang, W. Gao, X. Shi, J. Ding, W. Liu, H. He, K. Wang, F. Shao, Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. *Nature* 547, 99–103 (2017).

33. Y. Liu, Y. Fang, X. Chen, Z. Wang, X. Liang, T. Zhang, M. Liu, N. Zhou, J. Lv, K. Tang, Gasdermin E–mediated target cell pyroptosis by CAR T cells triggers cytokine release syndrome. *Sci. Immunol.* 5, eaax7969 (2020).

34. F. Zhou, B. Feng, H. Yu, D. Wang, T. Wang, Y. Ma, S. Wang, Y. Li, Tumor microenvironment-activatable prodrug vesicles for nonencoded cancer chemoinmunotherapy combining immunogenic cell death induction and CD47 blockade. *Adv. Sci.* 31, 1805888 (2019).

35. Z. Chen, L. Liu, R. Liang, Z. Luo, H. He, Z. Wu, H. Tian, M. Zheng, Y. Ma, L. Cai, Biosynspired hybrid protein oxygen nanocarrier amplified photodynamic therapy for eliciting anti-tumor immunity and abscopal effect. *ACS Nano* 12, 8633–8645 (2018).

36. P. Sharma, J. P. Allison, The future of immune checkpoint therapy. *Science* 348, 56–61 (2015).

37. D. Cao, X. Chen, F. Cao, W. Guo, J. Tang, C. Cai, S. Cui, X. Yang, L. Yu, Y. Su, An intelligent transdermal formulation of ALA-loaded copolymer thermogel with spontaneous asymmetry by using temperature-induced sol–gel transition and gel–sol (suspension) transition on different sides. *Adv. Funct. Mater.* 31, 2100349 (2021).

38. Y. Chao, Q. Chen, Z. Liu, Smart injectable hydrogels for cancer immunotherapy. *Adv. Funct. Mater.* 30, 1902785 (2020).

39. F. Fang, W. Xiao, Z. Tian, NK cell-based immunotherapy for cancer. *Semin. Immunol.* 31, 37–54 (2017).

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