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ABSTRACT

The therapy of solid tumors is often hindered by the compact and rigid tumoral extracellular matrix (TECM). Precise reduction of TECM by hyaluronidase (HAase) in combination with nanotechnology is promising for solid tumor therapeutics, yet remains an enormous challenge. Inspired by the treatment of iron poisoning, here we propose an “on-site” unwrapping strategy of metal-polyphenol-packaged HAase (named PPFH) by sequentially injecting PPFH and a clinically used iron-chelator deferoxamine (DFO). The in situ dynamic disassembly of PPFH could be triggered by the intravenously injected DFO, resulting in the release, reactivation and deep penetration of encapsulated HAase inside tumors. Such a cost-effective HAase delivery strategy memorably improved the subsequent photothermal and photodynamic therapy (PTT/PDT)-induced intratumoral infiltration of CD8\(^+\) T cells and the cross-talk between tumor and tumor-draining lymph nodes (TDLN), thereby decreasing the immunosuppression and optimizing tumoricidal immune response that can efficiently protect mice from tumor growth, metastasis and recurrence in multiple mouse cancer models. Overall, this work presents a proof-of-concept of the dynamic disassembly of metal-polyphenol nanoparticles for extracellular drug delivery as well as the modulation of TECM and immunosuppressive TME.

Keywords: Tumoral extracellular matrix, dynamic disassembly, hyaluronidase, photothermal/photodynamic therapy, tumor immune microenvironment.
Solid cancers have become one of the major diseases that seriously threaten human health, and cancer recurrence or metastasis after treatment is the leading cause of cancer-related deaths\textsuperscript{1,2}. Although the therapeutic paradigm of solid cancer has moved from monotherapies toward combinatorial therapeutics\textsuperscript{3,4,5}, their efficacy in ablating solid tumors and reshaping tumor microenvironment has been greatly hindered by the condensed tumoral extracellular matrix (TECM)\textsuperscript{6,7}. In particular, the uncontrolled deposition of TECM caused by the cancer-associated fibroblasts (CAFs) and elevated mechanical forces by the fast tumor growth would increase the stiffness of solid tumors and compress the blood and lymphatic vasculature inside tumors\textsuperscript{8,9,10}, thereby leading to limited blood perfusion and largely aggravated tumor hypoxia state\textsuperscript{11,12}.

It has been widely acknowledged that the TECM acts as a tumoral physical barrier is intimately associated with therapeutic inefficiency and immunosuppression. One major caveat is that such a tumoral physical barrier facilitates the functions of cancer-associated immune cells, such as M2-like macrophages, while severely impairing the intratumoral infiltration of CD8$^+$ T cells\textsuperscript{13,14,15}, therefore suppressing the immunogenicity of tumors and anti-tumor response, which is one of the critical contributors to the establishment of immunosuppressive tumor microenvironment (TME) or even immune deserts\textsuperscript{16,17}. The finite infiltration of immune cells is largely related to the limited response of solid cancer patients to immune checkpoint inhibitors, such as programmed cell death protein 1 (PD-1), which aim to restore the tumoricidal function of pre-existing lymphocytes inside tumors\textsuperscript{18,19}. In yet another setting, reprogramming the immune cell composition by immune factors or chimeric antigen receptor (CAR)-T cell can partly relieve immunosuppressive TME\textsuperscript{20,21}, whereas the TECM is still presence and will cause the dysfunction of infiltrated T cells again\textsuperscript{22}. From the other side, the distribution of TECM in perivascular areas inside tumors preferentially excludes therapeutic agents or nanoformulations that intend to enter
the tumor cores, resulting in incomplete tumor destruction and recurrence post-treatment\textsuperscript{23,24,25}. As a result of this, enhanced tumor growth inhibition is readily to achieve if higher drug doses are used, while serious toxic side effects also increase. Therefore, a groundswell of nanocarriers (of size-switchable\textsuperscript{26,27} or charge-switchable\textsuperscript{28,29}) have been engineered and witnessed substantial progress in improving interstitial diffusion and deep penetration of therapeutic agents inside tumors for enhanced antitumor efficacy, but most of them do not specifically tackle the tumoral physical barrier and immunosuppressive TME, thus have limitations for potential clinical application. Therefore, a promising therapeutic strategy is to break down the rigid tumoral physical barrier, which will not directly reprogram the TEM toward antitumoral features but promote the penetration of therapeutic nanoparticles and and the infiltration of activated CD8\textsuperscript{+} T cells inside tumors, improving the objective response rate in a two-pronged approach.

Based on the understanding towards the composition of TECM, hydrolysis enzymes\textsuperscript{30,31,32}, such as hyaluronidase (HAase)\textsuperscript{33,34,35}, have been preliminarily voted as a “scissor” for disrupting the tumoral physical barrier to improve the therapeutic efficacy of solid cancers. As a promising therapeutic strategy, PEGylated recombinant human hyaluronidase PH20 (rHuPH20), has been clinically trialed with a focus on prolonging blood circulation times and enhancing drug penetration within tumors\textsuperscript{36,37}. However, the enzyme activity might be compromised by the chemical modification of non-cleavable PEG chains. Alternatively, researchers have developed a biocompatible polymer-modified HAase via a pH-responsive traceless linker and enabled them to self-assemble into nanoparticles, which is expected to release native HAase in response to the acidic TME\textsuperscript{38}. Unfortunately, the predominant distribution of nanoparticles around the tumor vasculatures usually limits the interaction of these stimuli-responsive nanoparticles with the intricate physiological environment, such as pH and reactive oxygen species (ROS) present, in
Scheme 1. Schematic illustration of the (A) preparation of PPFH based on the coordination between Fe$^{3+}$ and polyphenol, and the dynamic disassembly process of these nanoparticles triggered by DFO; (B) modulations of TECM by sequentially injecting PPFH and DFO via tail vein, and (C) the effects of triplet-combination of PPFH, DFO and PTT/PDT on ablating primary tumors and boosting anti-tumor immune responses via enhancing the
tumoral infiltration of CD8$^+$ T cells and the cross-talk between tumor and tumor-draining lymph node (TDLN). After PPFH reaching tumor tissues, the subsequently injected DFO could rapidly chelate iron ions in PPFH that enables \textit{in situ} dynamic disassembly of PPFH and HAase release for TECM digestion. Owing to the loosened TECM and tumoral blood perfusion, further injected therapeutic liposomes, IR780@LNPs, could effectively accumulate and penetrate inside the solid tumors. Upon 808 nm laser irradiation, the IR780@LNPs-based PTT/PDT could activate the adaptive anti-tumor immune responses. Importantly, the loosened TECM can not only facilitate tumoral infiltration of CD8$^+$ T cells to form tumoricidal “T cells hive”, but improve the cross-talk between tumor and TDLN, leading to the remodeled immune microenvironment in both tumor and TDLN and amplified antitumor immune responses.

the tumors, thus compromising their intratumoral delivery efficiency$^{39,40}$. Moreover, the delicate pH-responsive process is difficult to precisely monitor and control in different types of solid cancer patients. Consequently, we looked to engineer an alternative drug delivery strategy with simple and stable structure of nanocarriers and artificially controllable drug release capacity to deliver HAase to the tumoral extracellular sites for TECM digestion, making the solid tumor more sensitive to cancer therapies. Metal-polyphenol networks (MPN), automatic assembly by the coordination of metal ions and polyphenols, have emerged as multifunctional drug delivery platforms for cancer therapeutics$^{41,42,43}$. Considering the extremely simple composition and stability of MPN and the reversible inhibition effect of Fe$^{3+}$ against HAase, the Fe$^{3+}$-based MPNs would be promising candidates for HAase encapsulation and tumor-targeting delivery.

Given the inspiration of treatment regimes of iron poisoning$^{44,45}$, we envisioned that the tumor-accumulated Fe$^{3+}$ based MPNs could be controllably disassembled by iron chelator, thus
triggering structure collapse of MPNs and the in situ release and reactivation of encapsulated
HAase in TME. To verify our hypothesis, we employed a simple “one-pot” method to prepare a
type of Fe\(^{3+}\)-based MPNs using paonia lactiflora pall extracted pentagalloylglucose (PGG),
poly(vinylpyrrolidone) (PVP) and Fe\(^{3+}\) ions, and leveraged its network property to encapsulate
HAase, and obtain the nanoformulation (named PPFH), which acts as both a tumor-targeting
carrier and a scaffold for \textit{in situ} disassembly. During blood circulation, the PPFH nanoparticles
displayed blocked enzyme activity, negligible immunogenicity and superior tumor-targeting
ability. After it arriving the tumor tissues, deferoxamine (DFO), a US Food and Drug
Administration approved iron-chelating agent, was introduced to initiate the \textit{in vivo} dynamic
disassembling process of PPFH by chelating Fe\(^{3+}\) ions within PPFH and destroying the
coordination bond between Fe\(^{3+}\) and PGG. Remarkably, the sequential injection of PPFH and
DFO can not only boost the release of HAase from PPFH in TME but also realize the deep-
penetration of native free HAase to tumor central, resulting in obvious TECM degradation of
whole tumor tissues, and then relieving tumor hypoxia and enhancing blood perfusion (Scheme
1).

In order to cooperate with therapeutic nanoparticles-mediated photothermal and
photodynamic therapy (PTT/PDT), we constructed tumor-targeting liposomes loaded with NIR
photosensitizer IR780 iodide, namely IR780@LNPs, as model therapeutic nanoparticles for
intravenous injection. As a result of loosened TECM, the IR780@LNPs exhibit improved tumor
accumulation and penetration, empowering more potent PTT/PDT efficiency and immunogenic
cell death (ICD) of tumor cells upon laser irradiation to provoke the adaptive antitumor immune
response. More importantly, the PTT/PDT-caused tumoral damage guaranteed the long-lasting
TECM destruction caused by the activated HAase, which contributes to the tumoral infiltration
of activated CD8+ T cells. Moreover, the released tumor antigens can easily pass through the
tumor tissues and be taken by DCs to enhance the cross-talk between tumor and tumor-draining
lymph node (TDLN), further amplifying tumoricidal immune response and reprogramming
immunosuppressive TME. We systematically verified the therapeutic effects of the ternary
combination of PPFH, DFO and PTT/PDT in inhibiting tumor growth, recurrence and metastasis
and prolonging survival time in both the mouse breast cancer model and the B16F10 melanoma
model. Overall, our study provides a proof-of-concept of the dynamic disassembly of metal-
polyphenol-packaged HAase for breaking-down TECM of solid cancers. In this way, improving
the efficiency of therapeutic nanoparticles and optimizing tumoricidal immune response against
solid tumors can be simultaneously achieved, which demonstrates promising application
potential in other combination cancer therapies.

Results

Construction and characterization of PPFH nanoparticles and the dynamic disassembly
process of PPFH triggered by DFO in vitro and in vivo

In this work, HAase was encapsulated by the biocompatible MPN composed of PGG, Fe3+, and PVP through a “one pot” method for potential clinically transferable applications (Fig. 1a
and Supplementary Fig. 1). According to our design, Fe3+ ions in the preparation system of
PPFH can directly bind to the active sites of HAase to inhibit the enzyme activity of HAase, thus
avoiding the unwanted HA degradation of PPFH before arriving tumor tissues. The relative
enzyme activity of HAase after Fe3+ or Fe3+/PVP incubation (room temperature, 30 min) was
investigated using the 3,5-dinitrosalicylic acid (DNS) method. The hyaluronic acid hydrolysis
produced reducing sugar could react with DNS, leading to increased absorbance at 540 nm. As
the Fe\textsuperscript{3+} concentration increased, the solution color changed from dark orange to orange, corresponding with the decreased catalytic ability of HAase (Fig. 1b). The introduction of PVP can sterically stabilize the nanocomposite and promote the nucleation owing to the weak coordination between Fe\textsuperscript{3+} and amide moieties of PVP. The morphology and size of the as-prepared PPFH were analyzed by transmission electron microscopy (TEM) and dynamic light scattering (DLS), which showed a net-like structure with an average size of \( \sim 255.3 \) nm in diameter and zeta-potential of around -44.3 mV, respectively (Fig. 1c and Supplementary Fig. 2). Moreover, PPFH showed excellent colloidal stability at 4 °C, with no obvious changes in the particle size (Supplementary Fig. 3). The HAase loading efficiency calculated based on BCA test is around 85.5 %. The Fourier transform infrared (FTIR) spectrum of PPFH presents a band around 1670 cm\textsuperscript{-1} (attributed to C=O stretching of the amide unit) and a shouter peak around 1600 cm\textsuperscript{-1}, reflecting the successful integration of PVP and interactions of PVP with Fe\textsuperscript{3+}, respectively (Fig. 1d).

Then the DFO triggered dynamic disassembling properties of PPFH was evaluated \textit{in vitro}. Dark-field TEM and elemental mapping images of PPFH show that iron element is uniformly distributed in the network of PPFH (Fig. 1e, f and Supplementary Fig. 4). As a comparison, the intensity of iron element in the precipitate of DFO-treated PPFH solutions was largely decreased, suggesting that DFO can efficiently chelate the Fe\textsuperscript{3+} ions in PPFH (Fig. 1e, f and Supplementary Fig. 4). The UV-vis absorption spectrum of the PPFH solution exhibited significant change after DFO addition, in which the specific absorption peak of coordination between Fe\textsuperscript{3+} and PGG at 596 nm disappeared (Fig. 1g). Additionally, the dynamic changes of absorption of DFO-treated PPFH solution at 596 nm was recorded. As shown in Fig. 1h, the DFO addition led to a gradually reduced UV-vis absorption at 596 nm within 1 min, implying that the coordination between Fe\textsuperscript{3+}
and PGG and PVP can be rapidly destroyed by DFO. Moreover, the DFO incubation caused the obvious release of PGG from PPFH relative to the PBS incubation (Supplementary Fig. 5). These results collectively confirmed the DFO-triggered dynamic disassembly of PPFH. It is interesting to notice that the addition of DFO into the PPFH solution initiated a reassembly process of nanoparticles, produced a solution containing micro-sized composites, as confirmed by DLS, TEM and atomic force microscopy (AFM) of the collected milk-white precipitate (Fig. i, j and Supplementary Fig. 6), which may be caused by the new hydrogen bond formation between PGG, PVP and unreleased HAase after Fe$^{3+}$ ions disappear (Fig. 1k). For validation, HAase was labeled with FITC to prepare PPFH-FITC and then treated with DFO. As shown in the confocal images, the supernate of DFO-treated PPFH solution displayed widespread green fluorescence intensity corresponding to the released native-free HAase, while only micro-sized green fluorescence dots were observed in the precipitate of DFO-treated PPFH solution, thus confirming our hypothesis that part of HAase cannot released from PPFH after the disassembly process occurred (Fig. 1l). Such biodegradable micro-sized composites containing HAase would probably be a desired “enzyme warehouse” to enable long-term tumor accumulation and release of HAase.

As for the DFO-triggered release of native-free HAase from PPFH, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was applied to compare the protein profile of free HAase and the precipitate or supernate of DFO-treated PPFH solution (Fig. 1m, n). As shown in Fig. 1n, the protein profile in the supernate of DFO-treated PPFH solution was in correspondence with that of free HAase, revealing that HAase can release from the PPFH in a free state after DFO treatment. What’s more, SDS-PAGE result also verified the presence of HAase in the precipitate, which showed a dispersive protein profile (Fig. 1n). Different from
typical drug delivery systems targeting tumor cells or intracellular components, the cell internalization of HAase by cancer cells impairs the degradation efficiency of HAase against TECM. Therefore, the *in vitro* uptake behavior of PPFH-FITC by 4T1 mouse breast cancer cell line was evaluated by flow cytometry. The results showed that the fluorescence of intracellular HAase-FITC in the PPFH-FITC+DFO group was lower than that of the PPFH-FITC group, demonstrating that the dynamic disassembly process of PPFH could markedly inhibit the uptake of loaded-HAase by cancer cells (Supplementary Fig. 7). Based on the above results, we next explored if the dynamic disassembly process of PPFH can reactivate the HAase activity. For this experiment and further *in vivo* studies, two analogs of PPFH with different enzyme activity (namely PPFH\(_{300}\) and PPFH\(_{3000}\)) were prepared from HAase (300 U mg\(^{-1}\) or 3000 U mg\(^{-1}\)) using the same method. Inductively coupled plasma-mass (ICP-MS) results showed that the similar iron chelation efficiency of DFO against against PPFH\(_{300}\) and PPFH\(_{3000}\) (Fig. 1o). As shown in the DNS results, the enzyme activity of the PPFH\(_{3000}\) solution was undetectable and almost the same with PPFH\(_{300}\), implying the blocked enzyme activity after encapsulation (Fig. 1p). Notably, the enzyme activity of the supernate of PPFH\(_{3000}\)+DFO solution was significantly higher than other samples, with a deeper color of DNS solution than others (Fig. 1p). These results collectively demonstrated that the dynamic disassembly process of PPFH can be triggered by DFO and the encapsulated HAase could be successfully reactivated.

After that, the bio-safety of PPFH *in vivo* was evaluated by hemolysis assay and hepatorenal function test. As could be seen, neither PPFH\(_{300}\) nor PPFH\(_{3000}\) caused obvious hemolysis, and the main parameters of hepatorenal function in the PPFH\(_{3000}\) group and PBS group were at the normal range, suggesting the good biocompatibility of PPFH (Fig. 1q, r). Besides, the physical encapsulation of HAase by the nanocomposite resulted in much lower immunogenicity of
exogenous HAase, along with a decreased generation of TNF-α in plasma compared to that of mice injected with free HAase (Fig. 1s). Then the tumor accumulation and biodistribution of PPFH were investigated in mice bearing 4T1 breast tumor, following the intravenous administration of IR780 co-loaded PPFH\textsubscript{3000}. The \textit{in vivo} fluorescence images showed the massive accumulation of PPFH\textsubscript{3000} at the tumor region at 8 h post-injection and gradually increased fluorescence intensity over time during 48 h (Fig. 1t). Additionally, the fluorescence image of major organs and tumor tissues at 48 h post-injection further confirmed the great tumor-targeting ability of PPFH (Fig 1t). Note that, though a slightly higher tumor accumulation was achieved at 48 h post-injection of PPFH relative to 24 h, a more prolonged accumulation time might lead to more cell internalization by tumor cells, thus impeding the dynamic disassembly of PPFH by DFO in TME. Hence, we choose the time point of 24 h after PPFH administration to intravenously inject DFO solution to initiate the dynamic disassembly process of tumor-accumulated PPFH.

The DFO-triggered dynamic disassembly of the tumor-accumulated PPFH was evaluated by monitoring the iron intensity in the blood and tumor tissues at different time points. As shown in Fig. 1u, the iron intensity in the tumor was decreased over time in 168 h following systemic administration of DFO, which was more significant than that of mice without DFO injection. Additionally, the iron intensity in the blood was markedly increased after DFO injection, and then gradually decreased from 48 h (Fig. 1u). We also evaluated the fluctuations of iron intensity in the liver, kidney, urine and faces of injected mice. As we known, part of intravenously injected nanoparticles could be captured by macrophages and endothelial cells in the liver and be secreted into the intestine, finally eliminated through feces. We found that the iron intensity in the liver and feces of mice could be significantly decreased by the intravenous injection of DFO.
Fig 1. Construction and characterization of PPFH nanoparticles and the dynamic disassembly process of PPFH triggered by DFO in vitro and in vivo. The enzyme activity of HAase used in these experiments is 300 U mg⁻¹ if not otherwise described. (a) Schematic illustrating the preparation of PPFH nanoparticles by a “one-pot” method. (b) DNS assay showing the HAase activity at different reactive solutions. Photographs of DNS solutions
(left) and relative quantitative results (right). (c) Transmission electron microscopy (TEM) image of PPFH at different magnifications. Scale bar: 500 nm (left), 100 nm (right). (d) FTIR spectra of PVP, HAase, and PPFH. (e, f) Representative element mapping, (g, h) UV-vis spectra, and (i) size measurement of PPFH solution with or without DFO treatment. (The changes in UV-vis absorption values were recorded at 596 nm). (j) Atomic force microscope (AFM) topography images of the sediment of PPFH solution with DFO treatment. Scale bar: 2 µm (k) 3D confocal images of the supernate and sediment of PPFH-FITC solution after DFO treatment. The loaded-HAase was labeled with FITC. Scale bar: 2 µm. (l) Schematic illustrating the dynamic disassembly and reassembling of PPFH nanoparticles after DFO treatment. (m, n) Photographs and the SDS-PAGE analysis of the supernate and sediment of PPFH solution with or without DFO treatment. (1), free HAase; (2), sediment of PPFH solution without DFO treatment; (3) supernate of PPFH-FITC solution without DFO treatment; (4) sediment of PPFH solution with DFO treatment; (5) supernate of PPFH-FITC solution with DFO treatment. (o) Chelation efficiency of DFO against iron ions within PPFH_{300} and PPFH_{3000} detected by ICP-MS. (p) HAase activity of different samples detected by DNS methods and representative UV-vis spectra of DNS solutions. (Sed and sup indicate sediment and supernate of corresponding solutions, respectively). (q) Hemolysis assay of PPFH_{300} and PPFH_{3000} at different concentrations ranging over 10-200 µg ml^{-1}. Deionized water and PBS were used as positive and negative control, respectively. (r) Hepatorenal function of mice after PBS (black) or PPFH_{3000} (violet) treatment. (s) TNF-α concentration in plasma of mice after various treatments. (t) Representative in vivo and ex vivo imaging of 4T1 tumor-bearing mice at different time points after intravenous injection of PPFH_{3000}. The NPs were co-loaded with IR780 as the
fluorescence probe. (u) The intensity of iron element in the tumor, blood, liver, kidney, feces and urine from the PPFH$_{3000}$ injected mice with or without DFO injection. (v, w) Confocal images and relative quantification of the mean fluorescence intensity (MFI) of HAase-FITC at the exterior, middle, and interior regions of tumors. Scale bar: 200 µm. (x) The intratumor diffusion of Evans Blue in tumors. Mice were intravenously injected with 1% Evans blue after 24 h of different treatments. (y) Laser speckle imaging of tumor blood perfusion of the PPFH$_{3000}$ injected mice with or without DFO injection. All data are shown as mean ± SD. Statistical significance between two groups was calculated via two-tailed Student’s t-test. Error bars are based on SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$.

compared with that of mice without DFO injection (Fig. 1u). In comparison, a gradually increased iron intensity in the kidney and urine of mice was recorded from 24 h to 48 h after DFO injection (Fig. 1u). These results collectively suggested that iron ions in the PPFH that accumulated in the tumor could be effectively chelated by the injected DFO and excreted through renal clearance pathway.

It has been reported that various nanoparticles are usually trapped by the condensed TECM around the tumor vasculatures, and cause a limited therapeutic efficiency towards deep tumors as a result of inhibited intratumor diffusion$^{46,47}$. Thus, it could be of great significance to investigate whether DFO could initiate the release and deep penetration of the encapsulated HAase from PPFH in the tumor. For validation, the distribution of HAase in the tumor from mice at 24 h after DFO injection, i.e., 48 h after PPFH injection was analyzed by using fluorescence HAase-FITC as a payload. From the confocal images, rather weak HAase-FITC signals were observed in different depths of tumor sections from mice only injected with PPFH-FITC, and showed
markedly colocalization with anti-CD31 labeled blood vessels (red signals) (Fig. 1v, w). Importantly, in the tumor sections of mice sequentially injected with PPFH-FITC and DFO, strong and widespread HAase-FITC fluorescence signals were recorded even in the tumor center and the regions far from blood vessels, indicating that the encapsulated HAase could release from PPFH in response to the injected DFO to achieve efficient tumor penetration and distribution (Fig. 1v,w). The high interstitial flow pressure (IFP) caused by compact TECM would result in decreased tumor perfusion and hypoxic condition inside the tumor [11-12]. Inspired by the successful release and deeper penetration of HAase encapsulated in the PPFH after DFO injection, it is reasonable to forecast that tumor perfusion would be greatly enhanced after the combinatorial treatment of PPFH\textsuperscript{3000} and DFO. For validation, Evans blue was used as an indicator of tumoral perfusion by intravenously injecting 1 % Evans blue solution into mice at 24 h after different treatments. At 2 h after Evans Blue injection, the tumor tissues were harvested and cut open for camera imaging, and the result showed that abundant and homogeneous perfusion of Evans Blue was noticed in the exterior, middle, and interior regions of PPFH\textsuperscript{3000}+DFO treated tumor than that of PBS group and PPFH\textsuperscript{3000} group (Fig.1x). Besides, laser speckle contrast imaging (LSCI) was applied to analysis the blood perfusion of tumors at different time points after different treatments. In the PPFH\textsuperscript{3000} treated tumor, only negligible changes were observed in the surrounding and central regions of the tumor (Fig. 1y). By contrast, the blood signals were gradually enhanced at 16 h after injection of DFO in periphery regions of tumor of mice pre-injected with PPFH\textsuperscript{3000} (Fig. 1y). To sum up, the \textit{in vivo} disassembly process of tumor-accumulated PPFH could be triggered by DFO, and resulted in the \textit{in situ} release and reactivation of encapsulated HAase and enhanced tumor blood perfusion.
Destruction of TECM by the released HAase resulted in the alleviated tumoral hypoxic state and enhanced EPR effect of therapeutic nanoparticles

We then wondered whether the enhanced tumoral perfusion after the combinatorial treatment of PPFH_{3000} and DFO was ascribed to the degradation capacity of the released HAase towards TECM instead of the disassembling process of nanoparticles. For this purpose, PPFH_{300} or PPFH_{3000} was intravenously injected into orthotopic 4T1 tumor-bearing mice, followed by the injection of DFO. The tumor was collected at 24 h after DFO injection for further experiments (Fig. 2a). We used scanning electron microscope (SEM) to characterize the destruction of TECM by the released HAase. Encouragingly, a large number of small holes and microcracks were observed in the tumor section in the PPFH_{3000}+DFO group as compared with the PBS group and PPFH_{300}+DFO group (Fig. 2b). Additionally, Hematoxylin & Eosin (H&E) staining images of tumor sections also displayed many circular gaps in the interior regions of tumor section from the mice in the PPFH_{3000}+DFO group (Fig. 2c). Considering that the formation of TECM is closely related to the collagen fibers and CAFs, the impact of PPFH_{3000}+DFO on the expression of collagen and α-SMA was evaluated by immunofluorescence staining. As shown in Fig 2d, no significant difference was observed in the distribution of collagen fibers in the tumor sections between the PBS group and the PPFH_{300}+DFO group. By contrast, more sporadic collagen was observed in the PPFH_{3000}+DFO group, implying the degraded and destroyed collagen fiber networks inside tumors (Fig. 2d, e). Masson staining of tumor sections revealed similar results (Fig. 2c and Supplementary Fig. 8). Concurrently, the fluorescence intensity and expression areas of α-SMA in the PPFH_{3000}+DFO treated tumor was markedly decreased as compared to the PBS group and PPFH_{300}+DFO group (Fig. 2d, e). Thus, it could be inferred that the destruction of TECM and the TME modulation after PPFH_{3000}+DFO treatment originated from the
cooperation of the dynamic disassembly of PPFH and the degradation capacity of the released HAase towards TECM. We next evaluated how the combinatorial treatment of PPFH<sub>3000</sub> and DFO would affect the physiologic state of blood vessels. Compared to tumors in the PBS group and PPFH<sub>300</sub>+DFO group in which only limited blood vessels were observed, the dilated blood vessel was dramatically enhanced at 24 h after PPFH<sub>3000</sub>+DFO treatment (Supplementary Fig. 9). Altogether, it could be concluded that the DFO-triggered in situ release of HAase with high-enzymatic activity could effectively digest the major extracellular matrix component, suppress the activation of CAFs, and increase the effective vasculature area within tumors, which would legitimately explain the enhanced tumor blood perfusion after PPFH<sub>3000</sub>+DFO treatment.

On the basis of above results, the influences of loosened TECM and enhanced tumor perfusion on the tumoral hypoxic state and immune cells residing in tumors were further excavated. Hypoxia-inducible factor 1 alpha (HIF-1α) is an oxygen-regulated protein, and the hypoxic state leads to enhanced expression of HIF-1α. Therefore, immunofluorescence staining towards the tumor sections was conducted for evaluation of the tumor hypoxia relief after different treatments, showing dramatically decreased expression levels of HIF-1α in the PPFH<sub>3000</sub>+DFO group than that of the PBS group and the PPFH<sub>300</sub>+DFO group (Fig. 2d, e). Moreover, pimonidazole, as a hypoxia probe, was intravenously injected into mice at 24 h after different treatments to detect the tumor hypoxia condition using the FITC-labelled anti-pimonidazole antibody. Accordantly, the tumoral hypoxic probe signals were obviously dimed by the PPFH<sub>3000</sub>+DFO treatment, implying the greatly relieved hypoxia state (Fig. 2f). The tumoral oxygen concentrations and physical microenvironment are important factors for immune cells composition inside tumor tissues before and after PTT/PDT and are closely related to TME immunosuppression and the therapeutic efficiency. To uncover the effect of PPFH<sub>3000</sub>+DFO
treatment on immune cells, tumors were harvested after 24 h of different treatments for flow cytometric analysis. As shown in Fig. 2g, PPFH\textsubscript{3000}+DFO treatment slightly increased the populations of CD8\textsuperscript{+} T cells, which showed a 2.5-fold increase compared to that in the PBS group and a 2.0-fold increase compared to that in the PPFH\textsubscript{300}+DFO treatment, confirming the potential influence on tumoral immune cell composition (Fig. 2g, h). Besides, the population of MDSCs was slightly decreased in the PPFH\textsubscript{3000}+DFO group (Fig. 2g, h). While no statistically significant difference was detected in the populations of M2-like macrophages between three groups (Fig. 2g, h).

We next hypothesized that the loosened TECM and improved blood perfusion by the PPFH\textsubscript{3000}+DFO treatment might contribute to the accumulation and penetration of intravenously injected therapeutic nanoparticles inside tumors. Considering that IR-780 is a typical photosensitizer, while polyethylene glycol (PEG)-modified liposomes (LNPs) is a widely applied drug delivery system for increasing the tumor-targeting ability of therapeutic agents, we first used a solvent displacement method to prepare LNPs and load IR-780, yielding IR780@LNPs nanoparticles for \textit{in vivo} PTT/PDT (Fig. 2i). Spherical morphology with uniform diameter of the as-prepared IR780@LNPs were observed under TEM. According to the DLS measurement, the hydrodynamic size of IR780@LNPs was around 130.5 nm and the zeta potential was around -22.1 mV (Fig. 2i and Supplementary Fig. 10). Besides, the as-prepared IR780@LNPs show good colloidal stability in the feta bovine serum, as their hydrodynamic diameters has no significant changes compared to the PBS dispersed IR780@LNPs for at 3 days (Supplementary Fig. 11), suggesting that IR780@LNPs could keep relatively stable in blood circulations and accumulated in the tumor tissues through EPR effect. Next, to quantitatively evaluated the tumor accumulation behavior of IR780@LNPs after the PPFH\textsubscript{3000}+DFO treatment, tumor-bearing mice
Fig 2. Destruction of TECM by the released HAase alleviated the tumoral hypoxic state and enhanced the EPR effect of therapeutic nanoparticles. (a) Schematic diagram of the PPFH and DFO treated mice for evaluating TECM destruction. Female BALB/c mice bearing orthotopic 4T1 subcutaneous tumors with a tumor volume of around 250 mm$^3$ were used in this experiment. (G1), PBS; (G2) PPFH$_{300}$+DFO; (G3), PPFH$_{3000}$+DFO. (n = 3 biologically independent mice per group). (b) Environment scanning electron microscope images of the tumor sections from mice in different groups. Scale bars for 200 and 500 $\times$ magnification are 100 and 50 $\mu$m, respectively. (c) H&E and Masson staining images of the obtained tumor sections. Scale bars for H&E and Masson staining images is 200 and 50 $\mu$m, respectively. (d, e) Immunofluorescence images and relative quantification of $\alpha$-SMA, collagen and HIF-α of the obtained tumor sections. $\alpha$-SMA (red fluorescence), collagen (green fluorescence), HIF-α (red fluorescence) and DAPI (blue fluorescence). Scale bar for the upper panel is 500 $\mu$m. Scale bar for the bottom panel is 200 $\mu$m. (f) Immune fluorescence staining of the hypoxic area of frozen tumor sections. Hypoxic area (green fluorescence). Tumor-bearing mice were intravenously injected with hypoxia probe after 24 h of different treatments. Scale bar: 200 $\mu$m. (g) Flow cytometric results and relative quantification of the percentages of DCs maturation (gating on CD11c$^+$CD80$^+$CD86$^+$), CD8$^+$ T cells (gating on CD3$^+$CD8$^+$), M2-like macrophages (gating on CD45$^+$CD11b$^+$F4/80$^+$) and MDSCs (gating on CD45$^+$CD11b$^+$Gr-1$^+$) after 24 h of different treatments. (i) Schematic illustration of the preparation of IR780@LNPs, and the photograph and transmission electron microscopy (TEM) image of as-prepared IR780@LNPs. Scale bar: 50 nm. (j) Schematic diagram of the PPFH, DFO and IR780@LNPs treated mice for evaluating tumor accumulations of nanoparticles. (G1), IR780@LNPs; (G2)
PPFH\textsubscript{300}+DFO+IR780@LNPs; (G3), PPFH\textsubscript{3000}+DFO+IR780@LNPs. (n = 3 biologically independent mice per group). BALB/c mice bearing 4T1 orthotopic breast cancer with tumor volume of around 250 mm\textsuperscript{3} were used in this experiment. (k) IVIS imaging of 4T1 tumor-bearing mice at different time points after intravenous injection of IR780@LNP, and (l) the quantified fluorescence intensity at tumor regions. Excitation wavelength: 780 nm, emission wavelength: 800 nm. (m, o) Ex vivo fluorescence imaging of collected tumors and major organs, and (n) the corresponding quantified analysis of the fluorescence intensity of collected tumors. (p, q) Confocal images of the frozen tumor sections and the quantitative results of MFI. IR780@LNPs nanoparticles (green fluorescence). Scale bar: 500 μm. (r) The thermal imaging of mice after injection of IR780@LNP for 12 h and laser irradiation (808 nm, 1 W cm\textsuperscript{-2}). (s) Representative H&E and CRT staining images of tumor sections collected from mice after 24 h of treatment. CRT (green fluorescence). Scale bar: 50 μm. All data are shown as mean ± SD. Statistical significance between two groups was calculated via two-tailed Student’s t-test. Error bars are based on SD. *P< 0.05, **P< 0.01, ***P<0.001 and ****P<0.0001.

were intravenously injected with IR780@LNP and imaged by the IVIS system following the workflow (Fig. 2j). In vivo fluorescence imaging of mice demonstrated that IR780@LNP accumulated in the tumor region at 4 h post-injection, with the fluorescence signals maintained at 24 h. Compared to the mice treated with PBS or PPFH\textsubscript{300}+DFO, mice injected with PPFH\textsubscript{3000}+DFO displayed the most significant tumoral accumulation of IR780@LNPs, especially at 12 h after injection of IR780@LNPs (Fig. 2k, l). Meanwhile, mice were sacrificed at 24 h after injection of IR780@LNPs with both tumors and major organs harvested for ex vivo fluorescence imaging, displaying the strongest fluorescence signal of IR780@LNPs in the tumors in the
PPFH<sub>3000</sub>+DFO group (Fig. 2m). Quantitatively, the tumoral fluorescence intensity in the PPFH<sub>3000</sub>+DFO group was nearly 1.6-fold higher than that of PBS group and PPFH<sub>300</sub>+DFO group (Fig. 2n). Moreover, ex vivo imaging of major organs at 24 h post-injection indicated that the PPFH<sub>3000</sub>+DFO treatment did not alter the accumulation intensity of IR780@LNPs at other organs, further ensuring the bio-safety of subsequent PTT/PDT (Fig. 2o). To spatially visualize the enhanced EPR effect of IR780@LNPs by the PPFH<sub>3000</sub>+DFO treatment, we used confocal microscopy for tumoral distribution measurement. As shown in the volume images of tumor sections, there was a dim fluorescence signal of IR780@LNPs in the tumor of mice treated with PBS or PPFH<sub>300</sub>+DFO due to the restriction by the compact TECM, consistent with the ex vivo fluorescence images (Fig. 2p, q). Notably, a significantly higher fluorescence signal of IR780@LNPs was observed in the tumor sections in the PPFH<sub>3000</sub>+DFO group (Fig. 2p, q).

As for PTT/PDT treatment, the tumor region of mice was exposed to laser irradiation (808nm, 1 W cm<sup>-2</sup>) for 3 min at 12 h after IR780@LNPs injection, and the temperature of tumors during laser irradiation was monitored through an infrared thermal camera. The enhanced tumor accumulation of IR780@LNPs after PPFH<sub>3000</sub>+DFO treatment was further confirmed by the robust in vivo photothermal effect, as confirmed by the higher temperature of tumor tissues upon laser irradiation than that of PTT/PDT alone (Fig. 2r). These results collectively substantiated that PPFH<sub>3000</sub>+DFO treatment by digesting TECM could greatly enhance the EPR effect of IR780@LNPs in tumor-bearing mice. Encouraged by the better-distributed IR780@LNPs inside tumors and relieved hypoxic state after PPFH<sub>3000</sub>+DFO treatment, we speculated that the directly tumor-killing effect of IR780@LNPs-mediated PTT/PDT could be amplified with the aid of PPFH<sub>3000</sub>+DFO treatment. At 24 hours after laser irradiation, the tumors were collected for section and analysis. Hematoxylin and eosin (H&E) stained tumor sections suggested the most
severe damage of tumor tissues in the PPFH\textsubscript{3000}+DFO treatment plus PTT/PDT group, whereas modest and similar damages were observed for tumors in the other two PTT/PDT-based groups (Fig. 2s). In parallel, tumor tissues in the PPFH\textsubscript{3000}+DFO treatment plus PTT/PDT group showed the most significant release of damage-associated molecular patterns (DAMPs), as revealed by the immunofluorescence staining against calreticulin (CRT) (Fig. 2s), providing more danger signals for activation of adaptive anti-tumor immune responses.

**Therapeutic efficiency in orthotopic breast tumor-bearing mice**

Subsequently, to evaluate the long-term therapeutic earnings of PPFH\textsubscript{3000}+DFO treatment plus PTT/PDT against solid cancers, orthotopic 4T1 breast tumor-bearing mice were established and randomly divided into six groups for receiving different treatments: (G1): PBS, (G2): PTT/PDT, (G3): PPFH\textsubscript{300}+DFO treatment plus PTT/PDT, (G4): PPFH\textsubscript{3000}+DFO treatment, (G5) PPFH\textsubscript{3000} treatment plus PTT/PDT and (G6): PPFH\textsubscript{3000}+DFO treatment plus PTT/PDT (Fig. 3a). The PPFH\textsubscript{300}+DFO treatment plus PTT/PDT can be a control to exclude the potential anti-tumor effect of DFO-triggered \textit{in vivo} disassembling process of metal-phenol nanocomposite owing to the negligible effect of PPFH\textsubscript{300}+DFO treatment at the experiment dose on TECM degradation. The tumor volume and body weight of mice were recorded every two days until the PBS-treated mice reached the criterion for euthanization or day 14. As shown in Fig. 3b-f, PPFH\textsubscript{3000}+DFO treatment did not show substantial inhibition on the tumor growth curves compared to the PBS-treated mice, indicating that \textit{in situ} reactivation of HAase with high enzyme activity induced no therapeutic effect against tumors. Both PTT/PDT-based therapy including (G2), (G3), (G5) and (G6) group displayed obvious delayed tumor growth (Fig. 3b-f). Besides, either PPFH\textsubscript{300}+DFO treatment plus PTT/PDT or PPFH\textsubscript{3000} treatment plus PTT/PDT at the current dose showed no significant difference in the tumor growth inhibition effect compared to the single PTT/PDT
Fig. 3 Therapeutic efficiency in orthotopic breast cancer bearing mice. (a) Treatment schedule of the combination therapy. Female BALB/c mice bearing orthotopic 4T1 subcutaneous tumors with tumor volume of around 100 mm$^3$ were used in this experiment.
(G1), PBS; (G2), PTT/PDT; (G3), PPFH_{300}+DFO plus PTT/PDT; (G4), PPFH_{3000}+DFO; (G5), PPFH_{3000} plus PTT/PDT; (G6), PPFH_{3000}+DFO plus PTT/PDT. (b) Individual body weight fluctuation of tumor bearing mice during different treatment. (c) Average tumor growth curves and (d) average tumour growth curves of tumor bearing mice during different treatment. (e) Photographs of mice on day 14 after different treatments. Three representative mice per group are shown. (f) Photographs of excised tumor tissues from mice at the end of different treatments. (g) Tumor inhibition rates in different groups. (h) H&E, TUNEL, PCNA, α-SMA and collagen staining images of the obtained tumor sections. Scale bars for H&E and enlarged H&E is 2000 and 100 μm, respectively. Scale bars for TUNEL, PCNA, α-SMA and collagen staining images is 500 μm. (i-l) The corresponding quantitative analysis of MFI of TUNEL, PCNA, α-SMA and collagen staining images. All data are shown as mean ± SD. Statistical significance between two groups was calculated via two-tailed Student’s t-test. Error bars are based on SD. *P< 0.05, **P< 0.01, ***P< 0.001 and ****P< 0.0001.

treatment, while tumors on mice subjected to PPFH_{3000}+DFO treatment plus PTT/PDT were effectively ablated (Fig. 3b-f). Notably, tumors on 4 out of 6 after the PPFH_{3000}+DFO treatment plus PTT/PDT showed complete eradication on day 14, with the tumor inhibition rate achieved 97.1% (Fig. 3g). The most robust inhibition effect on tumor growth in PPFH_{3000}+DFO treatment plus PTT/PDT revealed that the DFO-triggered in situ disassembly of PPFH and sufficient enzyme activity are two crucial factors for enhancing the anti-tumor efficacy of IR780@LNPs mediated PTT/PDT.

Consistently, PPFH_{3000}+DFO treatment plus PTT/PDT displayed the best efficacy in inducing apoptosis and necrosis of tumor cells, as confirmed by H&E and terminal
doxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (Fig. 3h, i). Moreover, by using immunofluorescence staining against proliferating cell nuclear antigen (PCNA) in tumor sections, we further verified the superior tumor inhibition of PPFH$_{3000}$+DFO treatment plus PTT/PDT against breast cancer (Fig. 3h, j). Considering that PPFH$_{3000}$+DFO treatment could modulate TECM at an early stage while PTT/PDT therapy also has a certain destructive effect against CAFs within tumors, we sought to verify whether the combination of PPFH$_{3000}$+DFO and PTT/PDT caused long-term TECM destruction. The representative confocal images suggested considerable collagen fibers and α-SMA re-expression in the tumors in the PPFH$_{3000}$+DFO group by comparison with PBS group, probably owing to the re-construction of TECM by the activated-CAFsduring tumor growth (Fig. 3h-l). In sharp contrast, PPFH$_{3000}$+DFO treatment plus PTT/PDT could significantly destroy and inhibit the re-construction of TECM even at day 14 after treatment, benefiting from the synergistic effect of loosened TECM at the early stage and the potential killing effect of PTT/PDT on CAFs in tumors (Fig. 3h-l).

Establishment of tumoricidal TME and immunological memory in orthotopic breast tumor-bearing mice.

It has been reported that the TECM destruction would facilitate the infiltration of activated CD8$^+$ T cells into solid tumors [32]. Therefore, we were in the situation to evaluate the local immune activation performance of PPFH$_{3000}$+DFO treatment plus PTT/PDT. To this end, the tumors of treated mice in all groups were harvested on day 14 for immune cell composition analysis. PTT/PDT alone partially improved the proportions of matured DCs and CD8$^+$ T cells inside tumors by approximately 3.4 % and 9.4 % compared with that of the PBS group, because PTT/PDT could induce robust danger singles and tumor antigens release of tumor cells, thus triggering the anti-tumor immune responses (Fig. 4a-d). As expected, PPFH$_{3000}$+DFO treatment
had a minor effect on promoting the DCs maturation and CD8\(^+\) T cells infiltration due to the limited tumor antigen release without PTT/PDT stimulation (Fig. 4a-d). Additionally, the DCs maturation and CD8\(^+\) T cells infiltration in other PTT/PDT-based groups was similar to that of the PTT/PDT group, owing to the negligible degradation of TECM and thus ineffective tumor infiltration of immune cells (Fig. 4a-d). With the synergism of loosened TECM and PTT/PDT-induced immune activation, PPFH_{3000}+DFO treatment plus PTT/PDT treatment potently enhanced the immune activation inside tumors, which displayed the best efficacy among all groups and improved the proportions of matured DCs and CD8\(^+\) T cells by more than 15.4 % and 17.8 % compared with that of the PBS group, respectively (Fig. 4a-d). The compact TECM and limited T cells infiltration are closely related to the immune-suppressive TME. Accordingly, we further tested whether the immunosuppressive cell populations could be affected by the PPFH_{3000}+DFO treatment plus PTT/PDT. As could be seen, although the ratios of CD8\(^+\) T cells and DCs maturation in mice in the PTT/PDT-based group, including (G2), (G3), and (G5), were significantly increased compared with the PBS group, these group also showed remarkably increased infiltration of M2-like macrophages in the tumors compared to that of the PBS group, as well as the significantly increased proportions of myeloid-derived suppressor cells (MDSCs), which might be caused by the PTT/PDT-induced immunosuppression and limited T cells infiltration\(^{48}\) (Fig. 4e-h). What’s exciting is that PPFH\(_{3000}\)+DFO treatment plus PTT/PDT group dramatically decreased the proportions of M2-like macrophages in the tumor tissues by more than 6.2 % compared with that of PBS group, and more than 29.1 % compared with that of PTT/PDT group. Similarly with that, the proportions of MDSCs in the PPFH\(_{3000}\)+DFO treatment plus PTT/PDT group were decreased by more than 11.8 % compared with that of the PBS group, and more than 19.6 % compared with that of the PTT/PDT group (Fig. 4e-h). Moreover, the
Fig 4. Establishment of tumoricidal TME and immunological memory in orthotopic breast cancer model. 4T1 tumors were harvested from mice at 14 days after different treatments. (a-d) Representative flow cytometric results and (e-h) the quantification results of DCs.
maturation, T cells infiltration, M2-like macrophages and MDSCs within the tumor. (i)
Representative immunofluorescence staining images of tumors showing the intratumoral
infiltration of M2-like macrophages and CD8⁺ T cells after different treatment. Scale bars:
500 μm. (j) Representative immunofluorescence staining images of tumors showing the
cytokine production for PBS, PTT/PDT and PPFH₃₀₀₀ + DFO plus PTT/PDT group, and (k)
the corresponding quantification results of MFI. (l) Schematic illustrating the mechanism
of ternary combination of PPFH₃₀₀₀ and PTT/PDT in promoting T cells infiltration. (m, n)
Representative flow cytometric results and (o) the quantification results of memory CD4⁺ T
cells (gating on CD3⁺ CD4⁺ CD62L⁺) and memory CD8⁺ T cells (gating on CD3⁺ CD8⁺
CD44⁺) in spleens of cured mice in the PPFH₃₀₀₀ + DFO plus PTT/PDT group. Spleens were
harvested from cured mice 30 days after treatment. 4T1 tumor bearing mice without any
treatment were designated as naive mice. (p) Proliferation of the T cells from the spleen
with the stimulation of 4T1 cells antigens. All data are shown as mean ± SD. Statistical
significance between two groups was calculated via two-tailed Student’s t-test. Error bars
are based on SD. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Tumoral infiltrations of the CD8⁺ and M2-like macrophages in different groups were visualized
by immunofluorescence staining, which showed similar trends with the flow cytometric results
(Fig. 4i). It was worth noting that, the PPFH₃₀₀₀+DFO treatment plus PTT/PDT group resulted in
a number of highly recognizable “CD8⁺ T cells hives”, which distributed in most regions of the
tumor section (Fig. 4i). This phenomenon can be well explained by the cooperation of loosened
TECM and PTT/PDT-invoked anti-tumor immune responses (Fig. 4l). Secreting pro-
inflammatory cytokines including tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) is a
critical function for tumoral infiltrated effector CD8⁺ T, we thus performed immunofluorescence
staining to evaluate the levels of these cytokines inside tumors. As shown in Fig 4j, k, the positive areas and expression intensity of TNF-α and IFN-γ in the PPFH3000+DFO treatment plus PTT/PDT group were larger and stronger than those of PTT/PDT groups, emphasizing that the loosened TECM not only facilitated the T cell infiltration after PTT/PDT but also enhanced the secretion of antitumoral cytokines.

Considering the robust effector CD8^+ T-cell response inside tumors and the excellent tumor inhibition ratio achieved by PPFH3000+DFO treatment plus PTT/PDT group, we further investigated the establishment of long-term immunological memory in the cured mice. As expected, cured mice that received PPFH3000+DFO treatment plus PTT/PDT displayed an approximately 1.5-fold of increase in the proportions of memory CD4^+ T cells and approximately 1.3-fold of increase in the proportions of memory CD8^+ T cells in the spleen compared with that of PBS group at 30 days after treatment (Fig. 4m-o). To further test the antigen-specificity of the established immunological memory, the T cells isolated from the spleens of cure mice were labeled by cell tracker (CFDA-SE) and incubated with tumor-antigens obtained from the supernatant of 4T1 cells after PTT/PDT treatment. As a result, nearly 28.9 % of CD8^+ T cells had proliferated in the cured group, while the frequency of proliferation in the naive group was only 3.7 % (Fig. 4p and Supplementary Fig. 12), indicating the antigen-specific of the established immunological memory that ensured the immunotherapy performance.

4.4 Enhanced tumor antigens translocation and the cross-talk between tumor and TDLN, and the inhibition against lung metastasis

The TDLN constituted of massive resident lymphocytes is known to be the primary immune organ for presenting tumor antigens to the naive CD8^+ T cells and activating robust and long-
lasting anti-tumor immunity. We next evaluated whether the initiated efficient immunological response in mice after the PPFH$_{3000}$+DFO treatment plus PTT/PDT was originated from the enhanced DCs modulation and T cells priming within TDLN. TDLN of mice was collected at 2 days after different treatments for immunofluorescence and flow cytometry analysis. As shown in Fig. 5a, all PTT/PDT-based treatments increased the percentages of DCs maturation within TDLN compared with the PBS group (Fig. 5a). In particular, PPFH$_{3000}$+DFO treatment plus PTT/PDT displayed the best efficiency in inducing the percentages of matured DCs among all treatment groups, which caused a 2.0-fold increase compared to that in the PBS group and a 1.5-fold increase compared to that in the PTT/PDT group (Fig. 5a). Similarly, mice that received PTT/PDT based treatment exhibited expansion of CD8$^+$ T cells within TDLN beyond the level in the PBS group, whereas PPFH$_{3000}$+DFO treatment plus PTT/PDT group further increased the expansion of CD8$^+$ T cells compared with that in the PTT/PDT group and PPFH$_{3000}$+DFO treatment plus PTT/PDT group (Fig. 5b). As expected, the percentages of these immunostimulatory cells were not further enhanced in PPFH$_{300}$ plus PTT/PDT treatment group compared with PTT/PDT group (Fig. 5a, b). In the meantime, PTT/PDT group and PPFH$_{300}$+DFO treatment plus PTT/PDT group generated an approximately 5.0-fold increase in the percentages of M2-like macrophage and a 2.3-fold increase in the percentages of MDSCs within the TDLN compared with PBS group, which could be explained by the tumor-induced immune suppression following PTT/PDT (Fig. 5c, d). In contrast, the mice treated with PPFH$_{3000}$+DFO treatment plus PTT/PDT group showed a notable decrease in the percentages of M2-like macrophage and MDSCs within the TDLN, comparable to those in the PTT/PDT group and PPFH$_{300}$+DFO treatment plus PTT/PDT group (Fig. 5c, d). Correspondingly, the confocal images visually indicated the highly infiltrated CD8$^+$ and DCs and largely decreased M2-like
macrophages and MDSCs in the TDLN of the mice after PPFH$_{3000}$+DFO treatment plus PTT/PDT (Fig. 5e). These results suggested that loosening the TECM by dynamic disassembling PPFH can efficiently enhance DCs modulation and T cells priming and reprogram the immunosuppression within TDLN after PTT/PDT.

In light of the excellent performance of PPFH$_{3000}$+DFO treatment plus PTT/PDT in these experiments, we next explored how this approach amplified the PTT/PDT induced-antitumor immune response. It has been reported that DCs with normal functions are located around the marginal regions of tumor tissues, which is critical for the cross-talk between TDLN and tumors$^{49}$. Unfortunately, since the restriction of compact TECM on the expansion of tumor antigens, these DCs cannot capture enough tumor antigens to trigger an effective anti-tumor immune response$^{50}$. On the other side, uncaptured tumor antigens may be rapidly removed from the tumor stroma. We hypothesized that the loosened TECM may facilitate the expansion of tumor antigens within tumors, thus enhancing tumor antigens uptake by DCs and transfer to TDLN, finally remodeling the immune microenvironment within TDLN after the PTT/PDT treatment of neighbor tumors (Fig. 5f). For verification, FITC-labeled BSA (BSA-FITC) as a model protein antigen was intratumorally injected at 2 h after laser irradiation to mimic the release of tumor antigens after PTT/PDT and track the capture efficiency by DCs within tumors (Fig. 5g). It was found that there were more BSA$^+$MHC-II$^+$ DCs in the tumors after PPFH$_{3000}$+DFO treatment plus PTT/PDT than in other treatment groups, which caused a 5.2-fold increase compared to that in the PBS group and a 2.6-fold increase compared to that in the PTT/PDT group (Fig. 5h, j). Therefore, loosening TECM by PPFH$_{3000}$+DFO treatment could enhance tumor antigens expansion within tumor tissues for ameliorated antigens capture and presentation by DCs, which is an effective way to awaken the host’s immune system. After
The image contains various scientific illustrations and data, likely from a biological or medical research context. The diagrams show results from experiments, possibly involving tumor models and treatment outcomes. The tables and graphs display quantitative data, with some statistical significance markers such as *p < 0.05* and *p < 0.001*. The text labels indicate comparisons between different groups or treatments over time. The figures are accompanied by legends and annotations that detail the experimental setup and results.
Fig 5. Enhanced cross-talk between tumor and TDLN and the therapeutic effect against lung metastasis. (a, b, c, d) Flow cytometric results and relative quantification of the percentages of DCs maturation, CD8+ T cells, M2-like macrophages and MDSCs in the TDLN from mice. (e) Representative immunofluorescence images of immune cells composition in the collected TDLN from mice after different treatments. Scale bars: 500 μm. (f) Schematic diagram of the potential mechanism of remodeled antitumor immune response after PPFH3000+DFO plus PTT/PDT treatment. The loosened TECM promotes tumor antigens translocation within tumor tissues, thus promoting the DCs maturation and migration to TDLN. (g) Schematic diagram of the experimental design for 4T1 tumor-bearing mice receiving various treatments follow by intratumoral injection of FITC-BSA solution. FITC-BSA injection was performed at 2 h after the laser irradiation of the tumor regions. BALB/c mice bearing 4T1 orthotopic breast cancer with tumor volume of around 250 mm³ were used in this experiment. (h-j) The determination of BSA-FITC positive DCs cells (gating on CD11c⁺MHC-II⁺BSA-FITC⁺) within tumor and TDLN by flow cytometry. Tumor and TDLN of mice were harvested at 12 h after the FITC-BSA injection for flow cytometric analysis. (k) Schematic diagram of the experimental design for tumor lung metastasis treatment on orthotopic 4T1 tumor models. BALB/c mice bearing 4T1 orthotopic breast cancer with tumor volume of around 100 mm³ were used in this experiment. (l) In vivo bioluminescence images to track and evaluate the growth of intravenously injected Luc-4T1 cells in the tumor bearing mice at different time points. (m) Representative ex vivo bioluminescence images and relative quantification of luminescence intensity of the collected lung tissues from mice on day 28. (n) Representative photographs of the collected lung tissues, and the quantification of the metastatic nodules per lung. (o)
representative H&E staining images of the collected lung slices. The metastatic nodules were indicated with orange circles. The scale bar for the upper panel is 2000 μm. The scale bar for the bottom panel is 200 μm. (p) Flow cytometry analysis and relative quantitation of CD8^+ T cells in blood collected from mice after different treatments. All data are shown as mean ± SD. Statistical significance between two groups was calculated via two-tailed Student’s t-test. Error bars are based on SD. *P< 0.05, **P< 0.01, ***P< 0.001 and ****P< 0.0001.

capturing tumor antigens, these migratory DCs will migrate to TDLN for T-cell priming. We thus extracted the TDLN around the tumors to evaluate the percentage of BSA^+MHC-II^+ DCs. We found that PPFH_{3000}+DFO treatment plus PTT/PDT effectively increased the percentage of BSA^+MHC-II^+ DCs in TDLN compared with other treatment groups, which caused a 2.3-fold increase compared to that in the PBS group and a 1.5-fold increase compared to that in the PTT/PDT group, indicating its superior T-cell priming activity (Fig. 5i, j). It is worth noting that unlike delivering immune activators for which restores the functions of DCs in the immunosuppressive TME, there is more opportunities for activating anti-tumor immunity by transferring the released tumor antigens from tumors to TDLN by loosening TECM^51. Collectively, our data support that the HAase-synergistic therapy may activate a stronger anti-tumor immunity through enhancing the cross-talk between tumor and TDLN.

Other than robust tumor ablation after PTT/PDT, long-lasting inhibition against tumor metastasis and recurrence, which mainly depends on immunological memory, is meaningful for improving the prognosis of solid tumors. To evaluate the therapeutic potential of PPFH_{3000}+DFO treatment plus PTT/PDT in preventing tumor metastasis after treatment, orthotopic 4T1 breast cancer-bearing mice were randomly divided into three groups and subjected to indicated
administrations. Typically, seven days post the laser irradiation, mice in each group were intravenously rechallenged with $2 \times 10^5$ Luc-4T1 cells to mimic the hematogenous metastasis mode of breast cancer, and the development of the lung metastases could be directly monitored by detecting the bioluminescence signals (Fig. 5k). Compared to the PBS group with progressive luciferase signals at the lung tissues, the mice in PPFH$_{3000} +$DFO treatment plus PTT/PDT group showed decreased bioluminescence signals (Fig. 5l, m). Notably, no bioluminescence signal at the lung tissue could be detected in the PPFH$_{3000} +$DFO treatment plus PTT/PDT group on day 21 and longer time point, thus indicating the eradication of metastatic tumor cells (Fig. 5l, m). Correspondingly, obvious metastatic tumor nodules were observed on the surface of lung tissues from PBS, PTT/PDT as well as PPFH$_{300} +$DFO treatment plus PTT/PDT groups, whereas PPFH$_{3000} +$DFO treatment plus PTT/PDT significantly reduced the number of metastatic tumor nodules nearly 60% compared with other groups (Fig. 5n). Meanwhile, no obvious pulmonary fibrosis was observed in the H&E staining images of lung tissue of mice treated with PPFH$_{3000} +$DFO treatment plus PTT/PDT, further indicating its superior potential in suppressing tumor metastasis (Fig. 5o). Moreover, circulating CD$^8^+$ T cells in the blood of these mice was evaluated using flow cytometry. As expected, the PPFH$_{3000} +$DFO treatment plus PTT/PDT significantly outperformed PTT/PDT and PPFH$_{300} +$DFO treatment plus PTT/PDT to enhance the systemic T cell anti-tumour immune responses, eliciting a 2.0-fold increase in the percentage of circulating CD$^8^+$ T cells compared with the PTT/PDT group, which further explained the above anti-metastasis effect (Fig. 5p). These results collectively demonstrated that the PPFH$_{300} +$DFO treatment could reprogram the PTT/PDT-induced tumoricidal immune response through enhancing the tumor antigen expands and the cross-talk between tumor and TDLN after...
loosening TECM, which in turn efficiently amplified the PTT/PDT-induced anti-tumor immune responses to prevent tumor metastasis.

**PPFH<sub>3000</sub>+DFO treatment plus PTT/PDT prevented tumor recurrence and proliferation of advanced-stage breast cancer.**

Encouraged by the robust local and systemic immune responses by PPFH<sub>3000</sub>+DFO treatment plus PTT/PDT, we next evaluated the potential of PPFH<sub>3000</sub>+DFO treatment plus PTT/PDT in preventing tumor recurrence. The establishment and treatment procedures of the dual-tumor model were shown in Fig. 6a. At three days post-PDT treatment of primary 4T1 tumors, $2 \times 10^6$ Luc-4T1 cells were subcutaneously injected into the right flank of mice to mimic tumor recurrence, excluding the influence of the drug itself on the distant tumor. As a result, for mice treated with PTT/PDT, obvious solid recurrent tumors were recorded seven days after inoculation, similar to that of PBS-treated mice (Fig. 6b-d). Noticeably, PPFH<sub>3000</sub>+DFO plus PTT/PDT treatment substantially regressed the growth of primary tumors, and remarkably completely inhibited five of five recurrent tumors, as proven by the tumor growth curves and bioluminescence imaging, suggesting the robust immunotherapy of recurrent tumors with abscopal effect could effectively inhibit tumor growth and recurrence (Fig. 6b-d). Besides, spleens collected from PBS and PTT/PDT groups exhibited compensatory splenomegaly owing to the large-tumor burden (Fig. 6i). In contrast, normal morphology and weight of spleens were observed in the PPFH<sub>3000</sub>+DFO plus PTT/PDT treatment group (Fig. 6i).

Next, stroma-rich advanced-stage breast cancer model was available to further evaluate the synergistic advantages of the combination of dynamic disassembly strategy of PPFH<sub>3000</sub> and PTT/PDT. We started the treatment 20 days after the Luc-4T1 cells inoculation when the tumor
Fig. 6 PPFH$_{3000}$+DFO plus PTT/PDT treatment-triggered inhibition on tumor recurrence and advanced-stage 4T1 orthotopic tumor model. Schematic illustration for the establishment and treatment of tumor recurrence of 4T1 orthotopic tumor bearing mice. BALB/c mice bearing 4T1 orthotopic breast cancer with tumor volume of around 100 mm$^3$ were used in this experiment. (n = 5 biologically independent mice per group). (G1), PBS; (G2), PTT/PDT; (G3), PPFH$_{3000}$+DFO plus PTT/PDT. Parameters of laser irradiation: 1.0 W cm$^{-2}$, 5 min. (b) Whole-animal in vivo bioluminescence images of mice after different treatment. (c) Individual primary 4T1 tumour growth curves and individual distant 4T1 tumour growth curves of mice after after different treatments. (e) Representative images of mice on day 28. Black and blue circles indicate the primary and recurrent tumor (distant tumor), respectively. (f) Representative photographs of primary and distant tumors collected from the mice on day 14. (g, h) Average growth curves of primary and distant tumor of mice. (i) Weight of spleens collected from mice in different groups at 14 days. (j) Whole-animal in vivo bioluminescence images of advanced-stage 4T1 orthotopic tumor bearing mice after different treatment. BALB/c mice bearing 4T1 orthotopic breast cancer with tumor volume of around 500 mm$^3$ were used in this experiment. (n = 5 biologically independent mice per group). (k, l) Individual and average 4T1 tumour growth curves of mice after after different treatments. (m) Representative photographs of representative tumors from advanced-stage tumor-bearing mice on day 14 after different treatments. (n) Survival curve of mice after different treatments. (o, p) Flow cytometric results and relative quantification of the percentages of DCs maturation, CD8$^+$ T cells, M2-like macrophages and MDSCs in the treated tumors from mice. (q, r) Flow cytometric results and relative quantification of the percentages of DCs maturation, CD8$^+$ T cells, M2-like
macrophages and MDSCs in the TDLN from mice. All data are shown as mean ± SD. Statistical significance between two groups was calculated via two-tailed Student’s t-test. Error bars are based on SD. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

volume of mice was rather huge (~500mm$^3$). Mice were treated with PBS (G1), PTT/PDT (G2) or PPFH$_{3000}$+DFO treatment plus PTT/PDT (G3), respectively. In view of the larger tumor size, the laser irradiation time at the tumor sites was extended to 10 min. As presented in Fig 6k, the tumor volumes of the PBS group gradually reached 1000 mm$^3$ within 5 days, and all mice died within 8 days. Besides, PTT/PDT alone only caused tumor regression at the initial stage, and the residual tumor grew rapidly after 6 days, probably due to the limited tumor penetration of intravenously injected IR780@LNPs and insufficient antigens translocation within tumors, collectively resulting in feeble immune response for inhibiting tumor proliferation (Fig. 6j-m). In sharp contrast, PPFH$_{3000}$+DFO treatment plus PTT/PDT strongly suppressed the tumor growth, which exhibited the highest tumor inhibition efficiency and gradually smaller tumor size over the 14-day time course (Fig. 6j-m). Besides, no compensatory splenomegaly was observed in the PPFH$_{3000}$+DFO treatment plus PTT/PDT group on day 14 (Supplementary Fig. 13). As a result, the mice in PPFH$_{3000}$+DFO treatment plus PTT/PDT group acquired the longest survival time among all group (Fig. 6n).

We also dissect the advantages of PPFH$_{3000}$+DFO plus PTT/PDT treatment in modulating the immune microenvironment of tumors and TDLN in the advanced-stage breast cancer model. Importantly, the strongest antitumor effect of PPFH$_{3000}$+DFO treatment plus PTT/PDT was associated with substantially increased DCs maturation (1.6-folds than PTT/PDT groups) and CD8$^+$ T cells infiltration (2.0-folds than PTT/PDT groups) in the tumors (Fig. 6o, p). Moreover, the percentages of immunosuppressive M2-like macrophage and MDSCs significantly decreased
to 13.0 % and 16.8 % in the tumors, which were 1.5 and 1.6-fold lower than those of the PTT/PDT group, respectively (Fig. 6o, p). Analysis of immune cell composition in the TDLN yielded analogous tendency (Fig. 6q, r). These data indicate that the dynamic disassembly strategy of PPFH$_{3000}$ was efficacious for optimizing the therapeutic efficiency of PTT/PDT against advanced-stage breast cancer.

4.6 Therapeutic outcomes in B16F10 melanoma and immune microenvironment modulation.

The encouraging outcomes of the PPFH$_{3000}$+DFO plus conventional PTT/PDT in treating breast cancer motivated us to further explore its anti-tumor effect on the highly aggressive and poorly immunogenic B16F10 melanoma model in C57BL/6 mice, in which the relatively soft TECM also play a critical role in tumor progression and restricting CD8$^+$ T-cells recruitment to tumor. When the tumor volumes of mice reached around 100 mm$^3$, the mice were treated with PBS (G1), PTT/PDT (G2), or PPFH$_{3000}$+DFO treatment plus PTT/PDT (G3) (Fig. 7a). Notably, PPFH$_{3000}$+DFO treatment plus PTT/PDT triggered notable tumor regression and long-term tumor-free survival time at least 30 days in about 50% of mice, with negligible changes of body weight, a better result than PTT/PDT alone (Fig. 7b-d). Of note, PTT/PDT alone did not cause complete tumor ablation, and all mice died within 20 days, owing to the fast progression of the B16F10 melanoma model and limited infiltration of CD8$^+$ T cell in the immunosuppressive TME (Fig. 7b-d). The images of mice at 12 days further suggested the higher anti-tumor efficacy of PPFH$_{3000}$+DFO treatment plus PTT/PDT relative to PTT/PDT (Fig. 7e). To elucidate the activated immune response in tumors and TDLN, mice were dissected at 2 days after different treatments, and the immune cell composition was analyzed using flow cytometry. As a result, the PPFH$_{3000}$+DFO treatment plus PTT/PDT group showed the highest percentage of DCs
Fig 7. Immune microenvironment modulation in tumor and TDLN and therapeutic outcomes in large B16F10 melanoma. (a) Schematic depicting the treatment protocol of mouse B16F10 melanoma model. C57 mice bearing B16F10 melanoma with tumor volume of 100 mm$^3$ were used in this experiment. (n = 6 biologically independent mice per group).
(G1), PBS; (G2), PTT/PDT; (G3), PPFH$_{3000}$+DFO plus PTT/PDT. (b) Body weight changes of mice after different treatments. (c) Individual and (d) average tumor growth curves of mice in different groups. (e) Photographs of tumor bearing mice on day 12 after different treatments. (f) Survival curve of mice after different treatments. (g, h) Flow cytometric results and relative quantification of the percentages of DCs maturation, CD8$^+$ T cells, M2-like macrophages and MDSCs in the treated tumors from mice. (i, j) Flow cytometric results and relative quantification of the percentages of DCs maturation, CD8$^+$ T cells, M2-like macrophages and MDSCs in the TDLN from mice. (k) Representative immune fluorescence staining images of DCs maturation, CD8$^+$ T cells, M2-like macrophages and MDSCs in the TDLN from mice, and (l) the corresponding quantification of positive cells area per TDLN. Scale bars: 500 μm. (m) Flow cytometric analysis and relative quantification of memory CD4$^+$ T cells (gating on CD3$^+$ CD4$^+$ CD62L$^+$) and memory CD8$^+$ T cells (gating on CD3$^+$ CD8$^+$ CD44$^+$) in the spleen of cured mice after PPFH$_{3000}$+DFO plus PTT/PDT treatment. (n) Schematic depicting the treatment protocol of PPFH$_{3000}$+DFO plus PTT/PDT in the B16F10 metastasis model. (n = 3 biologically independent mice per group). (o) Representative photograph and (p) H&E staining images of the collected lung slices on day 21 after different treatments. The metastatic nodules were indicated with orange circles. The scale bar for the upper panel is 2000 μm. The scale bar for the bottom panel is 200 μm. All data are shown as mean ± SD. Statistical significance between two groups was calculated via two-tailed Student’s t-test. Error bars are based on SD. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

maturation and CD8$^+$ T cell infiltration among the three groups in both the tumor tissues and TDLN (Fig. 7g-j). Nevertheless, the PTT/PDT treatment only slightly increased these
immunostimulatory immune cells, and the percentages of M2-like macrophage and MDSCs in the tumor in the PTT/PDT group were 1.3- and 4.8-fold higher than that of the PBS group, respectively, reflecting the typical PTT/PDT-induced immunosuppressive TME and impeded DCs functions (Fig. 7g-h). By contrast, PPFH$_{3000}$+DFO treatment plus PTT/PDT did not significantly increase the percentages of M2-like macrophages and MDSCs in the tumors (Fig. 7g, h). Compared with the PBS group, the enhanced percentages of M2-like macrophages and MDSCs were also recorded in the TDLN of the PTT/PDT-treated mice, whereas the opposite tendency was detected in the TDLN of mice treated with PPFH$_{3000}$+DFO treatment plus PTT/PDT (Fig. 7i, j). The modulated immune cell composition in TDLN by PPFH$_{3000}$+DFO treatment plus PTT/PDT was further confirmed by immunofluorescence staining (Fig. 7k, l).

Further, the percentages of memory CD4$^+$ and memory CD8$^+$ T cells in the spleen of the cured mice in the PPFH$_{3000}$+DFO treatment plus PTT/PDT group was 2.9- and 1.6-fold higher than that in the naive mice, respectively, indicating the establishment of immunological memory (Fig. 7m).

These results manifested that PPFH$_{3000}$+DFO treatment can also potent the therapeutic efficacy of PTT/PDT and induce effective and long-lasting anti-tumor immune responses in the melanoma model via enhancing cross-talk between tumor and TDLN. We next tested the potential of PPFH$_{3000}$+DFO treatment plus PTT/PDT in preventing the lung metastasis of the B16F10 melanoma model. B16F10 tumor-bearing mice were grouped and treated as exhibited in Fig 7n. Three days after different treatments, mice were intravenously inoculated with $3 \times 10^5$ B16F10 cells without further treatment. After another 14 days, the lung tissues of mice were excised, and many lung metastatic foci and pulmonary metastasis nodules were viewed in the PBS as well as PTT/PDT groups (Fig. 7n-p). Impressively, nearly no lung metastasis could be observed in the lung tissues of mice in the PPFH$_{3000}$+DFO treatment plus PTT/PDT group,
further confirming the superior effects of PPFH3000+DFO mediated TECM destruction in boosting the local and systemic anti-tumor effects of PTT/PDT (Fig. 7n-p).

**Discussion**

Mounting evidence has confirmed that the tumoral physical barrier, consisting of a large amount of CAFs and condensed TECM, not only causes insufficient accumulation of therapeutic nanoparticles in solid tumors but also compromises antigen-specific CD8+ T cell infiltration to deep tumor parenchyma, thereby limiting the therapeutic efficacy of loaded drugs and antitumor immunity. Although the capability of HAase to digest TECM has brought distinct assistance to cancer therapies, such as enhancing the EPR effect of therapeutic nanoparticles, relieving tumoral hypoxia state, and reprogramming TME, it remains a formidable challenge for HAase to significantly accumulate and penetrate tumor tissues for successful TECM digestion. Nowadays, intratumoral injection of HAase or stimuli-responsive polymers encapsulation for intravenously administration are two effective routes to guarantee the therapeutic effect of HAase in animal-based cancer models, while the difficulties in the application of intratumoral injection to deep-site tumors and the complexity of large-scale organic synthesis impede the implementation of HAase-based strategy in the clinic. In this work, we employed an iron chelator DFO to initiate the *in situ* dynamic disassembly process of iron-rich nanoparticles PPFH and the reactivation of encapsulated-HAase at the tumor site, which offers several key merits relative to other polymer-based delivery nanocarriers in terms of super-simple preparation procedure and artificially controllable drug release. This strategy is beneficial to systematically enhance the immune activation of T cells in the tumor after PTT/PDT treatment.
Specifically, we synthesized PPFH\textsubscript{3000} nanoparticles by integrating HAase (3000 U mg\textsuperscript{-1}) with the coordination assembly of PGG, PVP, and Fe\textsuperscript{3+}, which displayed significantly blocked enzyme activity without DFO treatment owing to the Fe\textsuperscript{3+} binding and physical encapsulation and would preferentially accumulate in perivascular areas inside tumors via EPR effect. Besides, the undesired immunogenicity of HAase in blood circulation is largely reduced after encapsulation. After intravenous administration of DFO, iron ions within the tumor-accumulated PPFH would be promptly chelated, and those encapsulated native-free HAase would be released around the tumor vasculatures, leading to intratumoral deep-penetration of HAase and destruction of TECM. During the first stage of PTT/PDT, the intravenously injected liposomal photosensitizers (named IR780@LNPs) significantly accumulated inside tumors owing to the loosened TECM as well as increased tumoral blood perfusion. After local laser irradiation of primary tumors, the therapeutic effect of PTT/PDT was largely amplified by the relieved tumor hypoxia and enhanced accumulation of IR780@LNPs, with the great potential to completely eradicate the established primary orthotopic 4T1 breast tumor or B16F10 melanoma in about 80 % of mice within seven days. Importantly, the combination of PPFH\textsubscript{3000} and DFO could strengthen the PTT/PDT-induced tumoricidal CD\textsuperscript{8+} T cell immune responses while decreasing the immune suppression within both tumors and TDLN, which is ascribed to the enhanced T cells infiltration and tumor antigens translocation by DCs after TECM reduction, thus evoking robust antitumor immunity to prevent tumor growth in multiple types of solid tumors. Worth noting, single-dose of PPFH\textsubscript{3000}+DFO treatment plus PTT/PDT completely regressed the distant tumors and lung metastasis owing to the powerful abscopal effect and durable immunological memory. Our data suggest that the ternary-combination of PPFH\textsubscript{3000}, DFO and PTT/PDT fully acted on the TECM regulation and photothermal effects to obtain synergistic antitumor efficacy.
As a prediction, the dual-combination of PPFH\textsubscript{3000} and DFO holds great potential in combination with various therapeutic regimens, such as radiotherapy and chemotherapy, since all those therapeutic regimens are also impeded by the immunosuppressive TME caused by TECM. Besides, we believe that this strategy possesses substantial potential in the clinic translation for the combinatorial treatment of solid cancers. Particularly, key reagents used in this combinatorial therapy, including PVP, DFO and IR780 iodide, are all FDA-approved, and the formed iron-amine complex is nontoxic and can be readily eliminated through renal clearance. In the future, the therapeutic payload of this delivery system, such as HAase and PGG, can be easily replaced by recombinant human hyaluronidase PH20 (rHuPH20) and other FDA-approved polyphenols to further fulfill the rigorous safety requirement. As for the dual-combination of PPFH\textsubscript{3000} and DFO, using magnetic resonance (MR) imaging to monitor the \textit{in vivo} distribution of PPFH in real-time provides a more feasible flexible route for selecting the relatively suitable time for DFO administration. One limitation of this study is the PPFH\textsubscript{3000} has not been further surface modified to increase the tumor-targeting ability, which is meaningful to further ensure the bio-safety of the dual combination of PPFH\textsubscript{3000} and DFO.

More importantly, with the help of DFO, the presented drug delivery strategy does not depend on the specific physiological environment inside tumors, thus is less restricted by individual differences and heterogeneity, and is promising for treating other solid tumor types facing such TECM barriers. Although we mainly referred to the modulation of tumoral physical barrier, our proposed drug delivery strategy could be fruitful to realize the \textit{in situ} controlled release of immune checkpoint inhibitors, such as PD-L1 antibody and CD47 antibody, for potent cancer immunotherapy. In summary, this study pioneers the implementation of the combination of metal-polyphenol-packaged HAase and DFO to modulate TECM before the PTT/PDT of solid
tumors, opening up a novel dimension of drug delivery pattern and adjuvant nanomedicine for cost-effective combinatorial therapy to optimize the tumoricidal immune responses.

Methods

Materials and reagents

HAase with low (300U mg\(^{-1}\)) or high (3000U mg\(^{-1}\)) enzyme activity, 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol)-2000 (DSPE-PEG\(_{2000}\)), lecithin, cholesterin and Evans Blue were obtained from Yuanye Biotechnology Co., Ltd (China). Iron(III) chloride hexahydrate (FeCl\(_3\).6H\(_2\)O), PVP (molecular weight = 8,000 g mol\(^{-1}\)), Deferoxamine (DFO), egg yolk lecithin and IR780 iodide was purchased from Sigma-Aldrich (USA). Roswell Park Memorial Institute (RPMI) 1640 Medium, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and Cell Counting Kit-8 (CCK-8) and bicinchoninic acid (BCA) Protein Assay Kit, Color-enhanced protein molecular marker (EC0019) and 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) preparation kit (EC0023) were purchased from sparkejade Biotechnology Co., Ltd (China). TdT-mediated DUTP nick end labeling (TUNEL) assay kit were purchased from Beyotime Biotechnology Co., Ltd (China). Anti-mouse calreticulin antibody, anti-mouse α-SMA and anti-mouse collagen were purchased from ZEN-BIOSCIENCE (China). Anti-mouse tumor necrosis factor-α (TNF-α) antibody and anti-mouse , and interferon-γ (IFN-γ) were purchased from Abbkine Biotechnology Co., Ltd (China). FITC-conjugated anti-mouse CD3 (catalog no. 100203), PE-conjugated anti-mouse CD4 (catalog no. 100407), Percp-conjugated anti-mouse CD8 (catalog no.100731), FITC-conjugated anti-mouse CD45 (catalog no.103107), PE-conjugated anti-mouse CD11b (catalog no.101207), APC-conjugated anti-mouse F4/80 (catalog no.123115), APC-conjugated anti-mouse Ly-6G/Ly-6C
(Gr-1) (catalog no.108411) and APC-conjugated anti-mouse CD62L (catalog no.104412) were purchased from BioLegend (USA). APC-conjugated anti-mouse CD11c, FITC-conjugated anti-mouse CD80 and PE-conjugated anti-mouse CD86 were purchased from MULTISCIENCES Biotechnology Co., Ltd (China). Collagenase Type IV and 3,5-dinitrosalicylic acid (DNS) agent were purchased from Solarbio Biotechnology Co., Ltd (China). Mouse TNF-α ELISA kit for was purchased from Jianglai Biotechnology Co., Ltd (China).

**Cell lines and animals**

All cell lines, including 4T1 (mouse breast cancer cell line), Luc-4T1 (mouse breast cancer cell line expressing firefly luciferase) and B16F10 (mouse melanoma cancer cell line) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences and cultured in an RPMI-1640 cell culture medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The single cell suspension harvested from spleens of mice were maintained in RPMI-1640 cell culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines was cultured in a humidified 37 °C incubator with 5% CO$_2$. Six-weeks old female BALB/c mice (18-22 g) and C57BL/6 mice (18-22 g) were ordered from Ziyuan Laboratory Animal Technology Co. Ltd (Hangzhou, China). All Mice were housed and cared in a room with a temperature around 20–22 °C and a 12-h light/12-h dark cycle. All animal experiments were performed according to animal care regulations and the Ethics Committee of the Anhui University of Chinese Medicine (approval ID: AHUCM-mouse-2023065).

**Preparation and characterization of PPFH**
To obtain PPFH, HAase with low (300U mg\(^{-1}\)) or high (3000U mg\(^{-1}\)) enzyme activity was encapsulated into the network formed by PGG, Fe and PVP through a simple “one pot” method. In brief, 80 µL FeCl\(_3\) aqueous solution (10 mg ml\(^{-1}\)), 1 ml PVP aqueous solution (10 mg ml\(^{-1}\)) and 2 ml HAase aqueous solution (1.5 mg ml\(^{-1}\)) were added into 2 ml ultrapure water and stirred at room temperature for 30 min. After that, 80 µL PGG alcoholic solution (32 mg ml\(^{-1}\)) and the above mixture were sequentially added into 5 ml ultrapure water with vigorously stirring for 15 min. Then, the resultant large-size PPFH product was collected by centrifugation (7500 rpm, 3 min) and washed with ultrapure water two times to remove the unloaded component. After that, the small-size PPFH was obtained by repeatedly extrusion by a 1 ml injector. The loading efficiency of HAase was tested by analyzing the protein concentration in the supernatants after centrifugation by the BCA method.

The nanoparticle diameter and zeta-potential of the prepared PPFH\(_{3000}\) were tested by dynamic light scattering, and the morphology of PPFH\(_{3000}\) was observed by transmission electron microscope (TEM). The nanoparticle diameter, zeta-potential and morphology of PPFH\(_{3000}\) were not influenced by the enzyme activity of the encapsulated HAase. To evaluate the DFO-triggered dynamic disassembly of PPFH, 100 µL DFO aqueous solution (20 mg mL\(^{-1}\)) was added into the PPFH solution (2 mL, 0.5 mg mL\(^{-1}\)) and incubated at room temperature. Meanwhile, the changes of the color and the ultraviolet-visible (UV-vis) absorption spectra of the mixture were recorded by a camera and a UV-vis spectrophotometer. As a test of the dynamic destruction of coordination bond within the PPFH\(_{3000}\) structure, the UV-vis absorption values at 596 nm was recorded at indicated time points within 4 min. The chelation behavior of DFO against Fe\(^{3+}\) irons in the PPFH\(_{3000}\) was investigated by element-mapping analysis and inductively coupled plasma–mass spectrometry (ICP–MS). In brief, the PPFH\(_{3000}\) solution (2 mL, 0.5 mg ml\(^{-1}\)) was treated
with 100 µL DFO aqueous solution (20 mg mL\(^{-1}\)) for 10 min of reaction at room temperature, then the sediments were collected by centrifugation (7500 rpm, 3 min) for element-mapping analysis, and the supernatants collected by centrifugation were analyzed by ICP-MS to quantitatively evaluate the chelation efficiency. The protein profiles and enzyme activity in the obtained sediments and supernatants were tested by SDS-PAGE experiment and 3,5-dinitrosalicylic acid (DNS) method, respectively, to evaluate the influence of DFO treatment on the release and enzyme activity of encapsulated HAase.

**Tumor accumulation and biodistribution of PPFH**

In these experiments, PPFH\(_{3000}\) co-loaded with near-infrared fluorescence dye IR780 was used. To establish orthotopic 4T1 cancer-bearing mice, female BALB/c mice (six-weeks old, 18-22 g of body weight) were subcutaneously injected 2×10\(^5\) 4T1 cells into the mammary fat pad of mice. When the tumor volume reached around 250 mm\(^3\) the mice were intravenously injected with PPFH\(_{3000}\) at a dose of 600 U HAase per mouse. At predetermined time points, the mice were anaesthetized and imaged by IVIS spectrum system. At 48 h post-injection, the mice were sacrificed, and the tumor and major organs were taken out for fluorescence imaging by the IVIS spectrum system.

**DFO-triggered dynamic disassembly process of PPFH in vivo.**

To evaluate the influence of DFO injection on the dynamic disassembly process of PPFH and *in situ* reactivation of loaded HAase *in vivo*, we selected PPFH\(_{3000}\) for some related experiments. Orthotopic 4T1 tumor-bearing mice were established as described above. For evaluating the DFO-triggered iron chelation and clearance in vivo, 4T1 tumor-bearing mice were intravenously injected with 100 µL of PPFH\(_{3000}\) (600 U per mouse), followed by the intravenous
injection of DFO (10 mg kg\(^{-1}\)) or PBS at 24 h post PPFO\(_{3000}\) injection. Then, the tumor, blood, urine, feces, liver and kidney of injected mice were collected at predetermined time points after DFO injection. All collected samples were digested with aqua regia at 70 °C for 1 h. Finally, iron intensity in different samples was measured by using ICP-MS.

For evaluating the DFO-triggered release and deep penetration of HAase inside tumors, HAase-FITC (HAase with FITC labelling) loaded PPFO (PPF-FITC) was prepared and intravenously injected into the 4T1 tumor-bearing mice, followed by the intravenous injection of DFO (10 mg kg\(^{-1}\)) or PBS at 24 h post PPFO-FITC injection. After another 24 h, mice were sacrificed, and the tumor tissues were harvested and fixed with liquid nitrogen for preparing frozen sections. Afterward, the distribution of HAase in the frozen sections was directly observed by confocal imaging. For evaluating DFO-triggered \textit{in situ} reactivation of HAase and its influence on tumor perfusion, 4T1 tumor-bearing mice were intravenously injected with 100 µL of PPFO\(_{3000}\) (600 U per mouse), followed by the intravenous injection of DFO (10 mg kg\(^{-1}\)) or PBS at 24 h post PPFO\(_{3000}\) injection. Then, the blood perfusion at the tumor site of tumor-bearing mice was imaged by a laser speckle imaging system at predetermined time points after DFO injection. 150 µL 1 % Evans Blue was intravenously injected into the mice at 24 h post DFO injection to visually demonstrate the blood perfusion.

**The degradation of the released HAase against TECM**

To evaluate whether the enhanced tumor perfusion after PPFO\(_{3000}\)+DFO treatment was ascribed to the degradation ability of HAase against TECM, we selected PPFO\(_{300}\) and PPFO\(_{3000}\) for some related experiments. The morphology of the tumor sections of mice after different treatments was measured by scanning electron microscopy (SEM). Specifically, 4T1 tumor-
bearing mice were randomly divided into three groups (PBS group, PPFH\textsubscript{300}+DFO and PPFH\textsubscript{300}+DFO) and intravenously injected with PBS, PPFH\textsubscript{300} (60 U per mouse) or PPFH\textsubscript{3000} (600 U per mouse), respectively. 24 h later, mice in PPFH\textsubscript{300}+DFO and PPFH\textsubscript{3000}+DFO groups were intravenously injected with DFO (10 mg kg\textsuperscript{-1}). At 48 h post DFO injection, the mice were killed, and tumors of mice in three groups were collected and fixed with 4% formaldehyde for further investigation. Then, the tumor tissues were cut into small pieces and subjected to gradient dehydration, critical point drying and sprayed gold for SEM observation.

In order to evaluate the TECM degradation and hypoxia relief after different treatments, the collected tumor tissues above were subjected to paraffin section and immunofluorescence staining against collagen I, α-SMA and HIF-α. Specifically, paraffin sections were treated with xylene and gradient ethanol of 95%, 85% and 75% ethanol to deparaffinize and rehydrate, respectively. Then, the slides were treated with improved antigen retrieval buffer (pH 6.0) for 15 min at 100 °C. After cooling, the slides were washed three times with PBS (pH 7.4). After remove clear liquid on slides, the tumor sections were circled by liquid blocker pen and covered with PBS containing 1% BSA for 2 h at room temperature. After that, tumor sections were incubated with primary antibody (diluted with PBS containing 1% BSA appropriately) and appropriate secondary antibody (respond to the species of primary antibody) according to the specifications. Finally, the tumor sections were observed by a confocal imaging system (Olympus FV3000).

The tumor hypoxia state after different treatments was detected by a hypoxic probe. Specifically, 4T1 tumor-bearing mice were randomly divided into three groups (PBS group, PPFH\textsubscript{300}+DFO and PPFH\textsubscript{3000}+DFO) and treated as mentioned above. At 24 h post DFO injection, mice were injected with 100 µL pimonidazole hydrochloride (60 mg kg\textsuperscript{-1}) according to the
specification. After another 2 h, mice were sacrificed, and tumor tissues were harvested for preparing frozen sections. Then, FITC-conjugated mouse anti-pimonidazole monoclonal antibody was applied to detect the intratumoral pimonidazole hydrochloride. Images were detected by fluorescence microscopy. The other mice in each group that subjected to indicated treatments for 24 h while not intravenously injected with pimonidazole hydrochloride were executed, and the tumor tissues were harvested to analyze the influence of on tumoral infiltration of CD8\(^+\) T cells and immune-suppressive cells (M2-like macrophages and MDSCs) by flow cytometry. Typically, the tumor tissues were cut into ~1mm\(^3\) small pieces and digested in a dissociation buffer (RPMI-1640 containing 2 mg mL\(^{-1}\) collagenase IV) for 1 h at 37 °C to form a single-cell suspension. The obtained single-cell suspension was filtrated through a cell strainer (pore diameter: 70 μm), washed with PBS containing 1% bovine serum albumin in PBS, and incubated with anti-mouse CD16/32 antibody for 10 min at 4 °C. Then the single-cell suspension was divided into three tubes to detect different immune cell. For detecting CD8\(^+\) T cells, the single-cell suspension was incubated with antibodies against CD3, CD4, and CD8. For detecting M2-like macrophages, the single-cell suspension was incubated with antibodies against CD45, CD11b, and F4/80. For detecting MDSCs, the single-cell suspension was incubated with antibodies against CD45, CD11b, and Gr-1. After washed with PBS for three times, the stained cells were detected by flow cytometer.

**Evaluation of the EPR effect and PTT/PDT efficiency**

IR780 iodide-loaded PEGylated liposomal nanoparticles (IR780@LNPs) were prepared by solvent displacement technique. In brief, 10 mg of IR780 iodide, 22.8 mg of cholesterin, 12 mg of DSPE-PEG\(_{2000}\) and 160 mg of egg yolk lecithin were fully dissolved in 1.5 mL of ethyl alcohol at 55 °C. Then, the mixture was slowly injected into 10 mL of PBS with stirring and
stirred for 3 h at 55 °C to volatilize ethyl alcohol. IR780@LNPs were obtained and purified by extrusion and ultrafiltration for further use. The encapsulation efficiency of IR780 was detected by a fluorescence microplate reader. To investigate the influence of loosened TECM on the EPR effect of IR780@LNPs, 4T1 tumor-bearing mice (tumor volume around 250 mm³) were randomly divided into three groups (PBS group, PPFH₃₀₀+DFO and PPFH₃₀₀₀+DFO) and treated as mentioned above. At 24 h post DFO injection, the mice were intravenously injected with 100 µL IR780@LNPs at an IR780 dose of 12 mg kg⁻¹ body weight. The fluorescence imaging of tumor-bearing mice was captured at different time points after IR780@LNPs injection to evaluate the tumor-targeting and accumulation behavior of IR780@LNPs. At 24 h post IR780@LNPs injection, mice were euthanized, and major organs and tumors were taken out for fluorescence imaging. Then the tumor tissues were collected for preparing frozen sections and observing the intratumoral distribution of IR780@LNPs by a confocal imaging system.

For evaluating the PTT/PDT efficiency of IR780@LNPs after various treatments, 4T1 tumor-bearing mice (tumor volume around 250 mm³) were randomly divided into four groups and treated with PBS only, IR780@LNPs only, PPFH₃₀₀+DFO plus PTT/PDT, and PPFH₃₀₀₀+DFO plus PTT/PDT. For PTT/PDT based groups, the tumor region of mice was exposed to laser irradiation (808 nm, 1 W cm⁻²) for 3 min at 12 h post IR780@LNPs injection. 24 h later, mice in each group were euthanized, and tumor tissues were harvested and fixed with 4% formaldehyde. Then the tumor sections were subjected to H&E staining and immunofluorescence staining against CRT.

*In vivo anti-tumour activity*
For the treatment of 4T1 breast cancer model, orthotopic 4T1 tumor-bearing mice were established in female BALB/c mice as mentioned above. When the tumor volume reached around 100 mm³, the mice were randomly divided into 6 groups and treated with PBS (G1), PTT/PDT (G2), PPFH_{300}^{+}DFO plus PTT/PDT (G3), PPFH_{3000}^{+}DFO (G4), PPFH_{3000} plus PTT/PDT (G5) and PPFH_{3000}^{+}DFO plus PTT/PDT (G6). Each group consisted of six mice. On day 0, mice in (G1) were injected intravenously with 150 µL PBS, mice in (G3) were injected intravenously with 100 µL PPFH_{300} (60 U HAase per moue), mice in (G4), (G5) and (G6) were injected intravenously with 100 µL PPFH_{3000} (600 U HAase per mouse). At 24 h after injection of indicated formulations, mice in (G3), (G4) and (G6) were injected intravenously with 100 µL DFO (15 mg kg⁻¹). At 24 h after DFO injection, mice in (G2), (G3), (G5) and (G6) were injected intravenously with 100 µL IR780@LNPs at a IR780 dose of 12 mg kg⁻¹ body weight. The tumor region of mice in (G2), (G3), (G5) and (G6) were exposed to laser irradiation (808 nm, 1 W cm⁻²) for 5 min at 12 h post IR780@LNPs injection. The tumor volumes and body weights of mice were measured every two days until day 14. Tumor volume was calculated by the following formula: Tumor volume = tumor length × tumor width²/2. The tumor inhibition rate was calculated by the following formula: Tumor weight of (G1)-Tumor weight of experiment group/Tumor weight of (G1) × 100%. For evaluating the anti-metastasis effect of different treatments, orthotopic Luc-4T1 tumor (expressing firefly luciferase)-bearing mice were grouped and treated as described in ‘the treatment of 4T1 breast cancer model’. On day 6, mice in each group were intravenously challenged with 2×10⁵ Luc-4T1 tumor cells without further treatment. For evaluating the anti-recurrence effect of different treatments, orthotopic 4T1 tumor-bearing mice were grouped and treated as described in ‘the treatment of 4T1 breast cancer model’.
day 6, mice in each group were subcutaneously inoculated with $2 \times 10^5$ Luc-4T1 tumor cells on the right flank.

For the treatment of the advanced-stage 4T1 breast cancer model, orthotopic 4T1 tumor-bearing mice were established in female BALB/c mice as mentioned above. When the tumor volume reached around 500 mm$^3$, the mice were randomly divided into 3 groups and treated with PBS (G1), PTT/PDT (G2) and PPFH$_{3000}$+DFO plus PTT/PDT (G3). On day 0, mice in (G1) were injected intravenously with 150 µL PBS, mice in (G3) were injected intravenously with 100 µL PPFH$_{3000}$ (600U HAase per mouse). At 24 h after injection of PPFH$_{3000}$, mice were injected intravenously with 100 µL DFO (15 mg kg$^{-1}$). At 24 h after DFO injection, mice in (G2) and (G3) were injected intravenously with 100 µL IR780@LNPs at a IR780 dose of 12 mg kg$^{-1}$ body weight. The tumor region of mice in (G2) and (G3) were exposed to laser irradiation (808 nm, 1 W cm$^{-2}$) for 10 min at 12 h post IR780@LNPs injection. For the treatment of the melanoma tumor model, female C57BL/6J mice (six-weeks old, 18-22g) were subcutaneously inoculated with $3 \times 10^5$ B16F10 tumor cells on the right flank of mice. When the tumor volume reached around 100 mm$^3$, the mice were randomly divided into 3 groups and treated with PBS (G1), PTT/PDT (G2) and PPFH$_{3000}$+DFO plus PTT/PDT (G3) as described in ‘advanced-stage 4T1 breast cancer model’.

**Statistical analysis**

All data were expressed as means ± standard deviation. Statistical analysis between two groups was performed by using two-tailed Student’s t test. P values < 0.05 represented statistically significant, P values: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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

**Data availability**

The main data supporting the findings of this study are available within the Article and its Supplementary Information. Extra data are available from the corresponding authors upon reasonable request.

**References**


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Author contributions

Q.S., Y.L., and W. S. contributed equally to this work. Q.S., and D.Y. conceived this work Q.S., Y.L., W.S., and Y.X. designed, prepared, and characterized the materials. Q.S., Y.L., J.Y., J.C., and W.G. carried out the in vitro, ex vivo, and in vivo experiments. Q.S., Y.Y., D.Y., F.X., and Q.W. analyzed the data and discussed the results. Q.S., Y.Y., D.Y., and W.S. wrote and edited the manuscript. D.Y., and Y.Y. supervised the project. The manuscript was written through contributions of all authors. All authors have given approval for the final version of the manuscript.

Competing interests
The authors declare no competing interests.

**Additional information**

**Supplementary information** is available for this paper at xxxxx

**Correspondence** and requests for materials should be addressed to F.X., Y.Y., or D.Y.

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