Neutrophil-Mediated Clinical Nanodrug for Treatment of Residual Tumor After Focused Ultrasound Ablation

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Research Article

Keywords: Neutrophils mediated drug delivery, Inflammation targeting, High intensity focused ultrasound, Residual tumor

DOI: https://doi.org/10.21203/rs.3.rs-779104/v1

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Abstract

Background: The risk of local recurrence of high intensity focused ultrasound (HIFU) is relatively high, resulting in poor prognosis of malignant tumors. Combination of HIFU with traditional chemotherapy still remains the unsatisfactory outcome because of off-site drug uptake.

Results: Herein, we proposed the strategy of inflammation-tendency neutrophil-mediated clinical nanodrug targeting therapy for residual tumor of HIFU ablation. Neutrophils as a carrier, and PEGylated liposome doxorubicin (PLD) as a nanodrug model of chemotherapy, were selected to form an innovative cell therapy drug (PLD@NEs). The targeting performance and therapeutic potential of PLD@NEs were evaluated using hepa1-6 cells and corresponding tumor-bearing mouse model. After HIFU ablation, PLD@NEs were recruited to the tumor site by inflammation, and released PLD with inflammatory stimuli, leading to targeted and localized postoperative chemotherapy.

Conclusion: This effective integration takes full use of the advantages of both HIFU, chemotherapy and neutrophil, attracting more focus on the practice of improving the existing clinical therapeutics.

Introduction

Radiofrequency ablation (RFA), microwave ablation (MWA) and cryo-ablation are the most commonly used ablation techniques for clinic treatment of solid tumor. Thereinto, high intensity focused ultrasound (HIFU) is the only non-invasive hyperthermic ablation modality [1]. Thermal damage and mechanical destruction of tissue are the main principal mechanisms responsible for HIFU-induced tissue necrosis. In the process of ablation, multiple ultrasound beams are incorporated to focus on a target area to produce a temperature up to 60 °C, directly leading to coagulation necrosis of ablated tissue [2]. HIFU has been routinely used in the clinical treatment of prostate disease and gynecological tumors [2–4]. It is worth noting that HIFU, as a promising hyperthermia technology, is under exploring for more potential applications of clinic treatment on solid tumors, such as thyroid tumor, liver cancer, kidney cancer and so on [5–8]. However, in similar to other ablation techniques, incomplete ablation is still the main limitation and challenge of HIFU. There may be several reasons: (a) Poor detection of tumor borders using current imaging techniques, especially for small tumor; (b) Loss of accurate detection caused by respiratory motion of patients; (c) Scattering or absorption of acoustic waves at the gas or bone interface in the sound field; (d) “Heat-sink” effect as known as “cool-down” effect occurred when heat in thematic region absorbed by flowing blood thus decreasing the HIFU efficacy [1, 9]. The survival of residual tumor cells after incomplete ablation will eventually lead to tumor recurrence Therefore, how to eliminate the residual tumor cells to suppress tumor recurrence is of great importance for improving the therapeutic outcomes. In addition, ablation associated inflammatory response can be observed after ablation with HIFU, depicted by infiltration of immune cells around the ablated lesion [10]. It has been reported that the presenting of immune cells such as macrophages can enhance systemic anti-tumor immunity [11]. However, it has also been reported the infiltrating macrophages in tumor environment can polarize to M2-phenotype tumor associated macrophages, contributing to tumor progressing and even tumor metastasis.
Although inflammation after ablation is a double-edged sword, it provided us a new idea for drug delivery, that is, targeting the tumor's inflammatory microenvironment after HIFU ablation for drug delivery, using postoperative response to improve the integrity of HIFU treatment.

Previous studies have provided insights into inflammation mediated drug delivery based on neutrophils loaded with nanodrugs for treatment of a variety of disease. Neutrophils, the most abundant leukocytes in mammalian circulation, which are the first type of leukocytes to migrate to the site of inflammation and can be used for cellular drug delivery [13]. Those treatment strategies are divided into two categories. One is based on the design of nanocarriers, that is, the surface specific modified nanocarriers will target the activated neutrophils in vivo after intravenous injection. Then, anti-inflammatory or anti-cancer agents can hijack neutrophils to cross the vascular barrier and target the inflammatory or tumor site by using the natural characteristics of neutrophils [14–16]. Another strategy is based on the neutrophils themselves, that is, using neutrophils as carriers to load drugs and subsequently inject them into the body. Neutrophils infiltrate into the tumor through the concentration gradient of chemokines, which makes more drugs accumulate in the lesions and significantly improves the therapeutic effect[17, 18]. Primary studies showed surgery, radiotherapy (RT), or photothermal therapy (PTT) was adopted to amplify the inflammatory signal of tumor microenvironment (TME), and then the hitchhiking nanodrugs were delivered to residual lesions through the natural inflammatory chemotaxis of neutrophils. These works provide a promising prospect and laid the foundation of exploiting more combination therapy strategies of clinical applied therapy and the innate biological functions of neutrophils [17–19]. However, patients suffered from cancers, such as unresectable HCC, have limited response to radiotherapy, which may require multiple rounds of treatment and multiple radiations [20, 21]. Although photothermal therapy has been used in clinical practice for more than 40 years to treat a variety of cancers, including superficial skin lesions, esophageal and lung tumors, its therapeutic effect on deep tumors is limited [22]. As the only non-invasive ablation modality, HIFU has been confirmed by FDA to be used in the treatment of many kinds of solid tumors without limitation of the depth of tumor site [23]. Furthermore, patients received HIFU ablation will not have to bear either the trauma of operation or the radiation side effects. Therefore, we anticipated the combination of HIFU and neutrophil mediated drug delivery system may be a more practical anti-cancer strategy.

Herein, based on the changes of tumor microenvironment after HIFU treatment, we innovatively proposed the strategy of inflammation-tendency neutrophil-mediated clinical nanodrug targeted therapy for residual tumor of HIFU ablation. Neutrophils as a natural carrier, and PEGylated liposome doxorubicin (PLD, the first liposome drug approved by U.S. Food Drug Administration (FDA) in 1995) as a nanodrug model of chemotherapy [24, 25], were designed to construct an efficient cell therapy innovative drug (PLD@NEs). For the proof of concept, liver cancer was selected as a tumor model, and neutrophil-mediated drug delivery system (PLD@NEs) was used to inhibit the recurrence of liver cancer after HIFU ablation. This anti-cancer strategy showed the prospect of “triple-win”, that is, (a) direct thermal ablation of tumor to kill most of the lesions; (b) targeting drug delivery through ablation induced inflammation, and (c) reduced the side effects of systemic chemotherapy, which is an urgent problem to be solved in clinical cancer treatment. The in vitro and in vivo results showed that PLD@NEs targeted, infiltrated and
accumulated in residual tumor tissue, and locally release chemotherapeutic drugs via inflammatory stimulation to treat residual tumor and thereby inhibit tumor recurrence (Scheme 1). Furthermore, the combination of clinically recognized treatments shows the great potential of the proposed strategy in clinical transformation.

**Materials And Methods**

### Cell culture

Hepa1-6 and HepG 2 cells were purchased from Stem Cell Bank, Chinese Academy of Sciences. 4T1 cells was kindly provided by Dr. Xue Xie (Shanghai Jiao Tong University Affiliated Sixth People’s Hospital). Hepa1-6 was cultured in DMEM (high glucose) medium. 4T1 cells were cultured in 1640 medium. HepG 2 cells were cultured in MEM medium. All the medium was supplemented with FBS (10%, v: v), penicillin (100 U/ml) and streptomycin (100 µg/ml). All the cells were cultured at 37°C in a humidified environment containing 5% CO2.

The ex vitro 3D tumor spheroids of Hepa1-6 were obtained using a liquid overlay method. Each well of 96-well plates was pre-coated with 100 µl of FBS free medium containing sterile agarose (1.5%, w: v). Subsequently, Hepa1-6 cells (5000 cells) were seeded into each well and cultured in the complete medium containing FBS (10%, v: v). The tumor spheroids were harvest at day 14 with the average diameter measured approximately 400 ~ 500 µm. The formation of hepatoma spheroids was monitored by optical microscope (Nikon, Japan).

### Preparation and characterization of PLD@NEs

PLD@NEs were obtained by incubating PLD with neutrophils. The freshly isolated neutrophils were cultured in a sterile tube with different concentrations of PLD at 37°C in a humidified environment containing 5% CO2. After washing with PBS thrice, PLD@NEs were obtained and used for subsequence research immediately. The amount of doxorubicin in loading NEs was quantified by high performance liquid chromatography (HPLC, Agilent). Cell lysis buffer (Beyotime) were added to PLD@NEs to disrupted the cell and release PLD from cells. Then, the cell lysate was centrifuged at 10,000 g for 5 min. Afterwards, the supernatant (100 µl) was collected and mixed with 400 µl methanol, vortexed for 5 min and centrifuged at 15,000 g for 5 min. The supernatant (20 µl) was injected into HPLC system for quantification. The morphology of loading NEs was observed using same method as previous mentioned.

### Evaluation of physiological functions

In order to achieve the purpose of inflammatory targeted drug deliver, the physiological functions activities of PLD@NEs were evaluated, including the inflammation-responsive expression of the specific CD11b, chemotaxis and superoxide-anion production. The inflammatory-mediated superoxide generation capability of PLD@NEs was determined by dihydroethidium (DHE, Beyotime). NEs or PLD@NEs (1×10⁵
cells) were incubated with fMLP (1 µM) at 37°C for 30 min, washed with PBS thrice and stained with DHE (5 µM) at 37°C for 30 min. The fluorescence intensity was measured by flow cytometry (Beckman).

The chemotaxis of PLD@NEs was investigated by transwell migration assay (transwell polycarbonate membrane: 3 µm pre size, 6.5 mm diameter and 0.33 cm² membrane surface area, Corning). Blank NEs and PLD@NEs (2×10⁵ cells) were added to the upper chamber of the transwell plate. The lower chamber of transwell plate was filled with 600 µL FBS free culture medium containing different concentrations of fMLP. After 30 min of incubation, the cells inside the upper chamber were eliminated, and the centrifuge at 2500 rpm for 5 min to harvest the migrating cells. The chemotaxis index was calculated as (N_{fMLP} - N_{control}) / N_{control}. N_{fMLP} or N_{control} refers to the numbers of neutrophils in the lower chamber under the incubating condition with or without fMLP, respectively.

The expression level of CD11b on the membrane was examined by flow cytometry. Neutrophils and PLD@NEs were incubated with different concentrations of fMLP at 37°C for 30 min. After washing with PBS thrice, cells were incubated with FITC-conjugated CD11b antibody (1 µg/ml, Biolegend) for 30 min. After another cycle of PBS washing, the fluorescence intensity was determined.

**Cytotoxicity of PMA-treated PLD@NEs against tumor cells**

The in vitro cytotoxicity of PLD@NEs after PMA stimulation against Hepa1-6 cells was determined by CCK8 assay. Hepa1-6 cells (1×10⁴ cells) was seeded in 96-well plate and cultured for 24 h. PLD@NEs were prepared and pretreated with PMA (100 nM) for 4 h. Then, both untreated and PMA-treated PLD@NEs were centrifuged at 1500 rpm for 5 min. The supernatant was obtained and incubated with Hepa1-6 cells for different times. Afterward, CCK8 solution was added to a final concentration of 10% (v:v). The absorbance was measured at a test wavelength of 450 nm by microplate reader (Thermo Fisher). The cell viability was calculated as (A_{sample} - A_{blank}) / (A_{control} - A_{blank}) ×100%. The cytotoxicity of PLD@NEs towards HepG 2 and 4T1 cells were using the same methods.

**Inflammatory immediate drug permeability in 3D tumor spheroids**

In vitro drug delivery capability was determined by assessing the drug permeable depth of PLD@NEs in tumor spheroids. 3D tumor spheroids were cultured and harvest as previously mentioned. At predetermined time intervals, culture medium was sampled and tumor spheroids were gently dispersed to single cells, followed by centrifugation at 400 g for 5 min. The level of cytokines CXCL1/KC was detected during 14 days by corresponding enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The tumor spheroids were then transferred in a confocal dish and incubated with 1 ml PLD@NEs (1 × 10⁶ cells equivalent to 5 µg PLD) or PLD for 8 h. The images were obtained in real time at the dixed depth of 50 µm from the surface to the middle of the tumor spheroid using Z-stack tomoscaning (Olympus, Japan).

**Mice and ectopic liver cancer model**
All animals were treated in accordance with the Guide for Care and Use of Laboratory Animals, approved by the Animal Experimentation Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. The Institute of Cancer Research Mice (BALB/c, male, six to eight-week-old and nude mice, male, four-week-old) were provided by Animal Laboratory of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (No. DWLL2021-0677).

To establish a heterotopic tumor model, the mice were given a percutaneous injection of Hepa1-6 cells ($2 \times 10^6$ cells per mouse). The status of tumor growth was monitored daily. When tumor size measured about $100 \text{ mm}^3$, mice were treated according to protocol. Tumor volume was calculated as $\text{Length} \times \text{Width}^2/2$.

**HIFU ablation therapy**

The HY2900 HIFU system (Wuxi Haiying Technology, Wuxi, China) was used in this study. A diagnostic transducer was localized in the center of the therapeutic transducer. The frequency of the diagnostic transducer and therapeutic transducer was 3.5 MHz and 1.5 MHz, respectively. The focal region of the therapeutic transducers was an ellipsoid with dimension of 8 mm along the beam axis and 1.15 mm in the transverse direction, which was calibrated using a PVDF needle hydrophone with a spot diameter of 0.5 mm in a tank filled with degassed water. In order to ensure the survival rate of tumor-bearing mice underwent HIFU, we designed a flexible box to perform the treatment (Fig. 4a). Due to the high energy produced by HIFU, we put an additional water sac between matrix and transducer. And for acoustic coupling, conventional ultrasound gel was applied. The focal spot of HIFU was setting aimed at the lower center of solid tumor. In consider of the tumor was small, treatment was performed in a horizontal point by point mode with one layer. Voltage was applied at $V = 5 \text{ V}$, and pulse duration was 1000 msec with and exposure separation of 3000 msec between each point. The interval distance between each points was approximately 1 mm. B-mode Ultrasound was used to guide HIFU therapy and monitored the entire process of treatment in real time. The therapeutic efficiency was determined using contrast enhanced ultrasound (CEUS) and histology.

**Statistical analysis**

All experiments were repeated at least three times and each condition was analyzed triplicated. Animals were randomly selected to different groups prior to the initiation of the treatment, but were not blinded for outcome assessment and data analysis. Statistical analysis was performed by using Prism 8.0 (Graph Pad) and Excel (Microsoft). All data were expressed with means ± SD as indicated. For comparison between two groups, two-tailed Student's t-tests were applied. In cases of comparison among more than two groups, ANOVA for multiple comparison adjustments was applied. Statistical significance was donated by asterisks in the appropriate figures (defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

**Results**

Preparation and characterization of PLD@NEs
In order to construct an inflammation-response targeting drug delivery system, mature neutrophils were isolated from bone marrow of murine hind limb long bone by using a discontinuous, three-layer Percoll® density gradient as previously described [26]. Based on the expression level determination of mature mouse neutrophil-specific biomarkers (including Ly6G and CD11b), the purity was quantified as more than 90% (Fig. 1a). The cells are round in a donut-shape, mitotic and yield about $8 \times 10^6$ cells per mouse (Fig. 1b). The survival rate of the isolated neutrophils was about 98.7% and kept above 90% within 12 hours. These results revealed that the neutrophils were successfully extracted from mouse bone marrow. The neutrophils are in sufficient quantity and in good condition to be used for encapsulating drugs. The cytotoxicity of PLD against neutrophils was studied by CCK8 assay. Within 12 hours of incubation, PLD showed no obvious toxicity to neutrophils under the tested concentrations. As a control, free doxorubicin (DOX) showed extremely cytotoxicity against neutrophils (Fig. 1c). The result revealed the reason why we chose liposomal drugs (PLD) as model drug, that is, the free chemotherapeutic drugs (DOX) themselves will directly kill neutrophils in short time, and thus they cannot be used for preparing chemo@NEs. The commercial nanodrug PLD was incubated with the purified neutrophils to form PLD@NEs. There was no significant morphological change of neutrophils after PLD loading (Fig. 2b, e). The activity of neutrophils was confirmed during 12 h after loading PLD (Fig. 1d and Figure S1). It is known that neutrophils circulate in the bloodstream with a half-life about 8 hours [27]. The results demonstrated that though the proportion of neutrophil death increased with the prolonged loading time, the viability was above 80%, which proved that PLD@NEs can remain active within the effective time window before it migrating to the tumor site

**Assessment of physiological functions of PLD@NEs**

Considering that the phagocytosis of neutrophils almost suspended after 1 h, we chose 1 h as the co-incubating time for preparation of PLD@NEs. The achieved PLD@NEs had a loading capacity of approximately 5 μg PLD per $10^6$ cells determined by HPLC (Fig. 2d). The achieved PLD@NEs were used for further research immediately. Since the physiological functions of PLD@NEs were essential for in vivo chemotic migration, we explored the physiological functions of PLD@NEs in response of inflammatory signal, including capability of superoxide generation, expression level of specific protein (CD11b) and chemotaxis capability [28, 29]. Formylmethionylleucylphenylalanine (fMLP), a neutrophil chemotactic peptide,[30] was used to stimulate PLD@NEs in vitro. As expected, there was significantly increasing superoxide level of fMLP-stimulated PLD@NEs, which had an equivalent effect on neutrophils (Fig. 2a and Figure S2). Meanwhile, the expression level of CD11b was dramatically increased with stimulation of fMLP in both PLD@NEs and neutrophils (Fig. 2b and Figure S3). In addition, the chemotaxis of PLD@NEs and neutrophils were investigated by a transwell migration assay, and the corresponding results indicated that PLD did not affect chemotic ability of neutrophils in PLD@NEs (Fig. 2c and Figure S4). All of these results, taken together, demonstrated that PLD@NEs maintained the natural physiological function of neutrophils, which can respond positively to the inflammatory signal and migrate to the inflammation sites.
Next, the releasing behavior of PLD from PLD@NEs under three different conditions (normal physiological condition, chemotaxis process and inflammatory environment) was explored. In order to simulate the inflammatory environment in vivo, fMLP and phorbol myristate acetate (PMA) were used as inflammatory factors in blood circulation and inflammatory site, respectively [31]. Results showed that most PLD can keep retaining inside neutrophils within 8 hours under physiological conditions. Additionally, minimal leakage of PLD from PLD@NEs was detected with fMLP stimulation of even 8 h. On the contrary, the PLD released rapidly from PLD@NEs after PMA stimulation of 2 h (Fig. 2e). The rapid response to the inflammatory environment ensures that PLD will be maximum released after reaching the target site.

**Inflammation-directed sequential delivery of PLD@NEs in 3D tumor spheroid model**

Previous studies have shown that in the inflammatory environment, nanodrugs encapsulated in neutrophils are released from neutrophils through the formation of a network structure composed of chromatin and granule protein (known as neutrophil extracellular traps, NETs) [32], and then the released nanodrugs are endocytosed by tumor cells. Thus, we evaluated the formation of NETs in PLD@NEs with PMA stimulation for 4 hours (Fig. 3a, left). After PMA stimulation, the red filiform fluorescence of extracellular DNA stained with Propidium Iodide (PI) were observed obviously in PLD@NEs, indicating the formation of NETs in PLD@NEs (Fig. 3a, right). These results suggest that it is possible to establish a transport cascade involving neutrophils: Neutrophils release PLD through rupture and transfer PLD to target cells (Fig. 3b and Figure S5).

After proving that PLD can be effectively released from PLD@NEs, in vitro anticancer effect of PLD@NEs was verified using CCK8 assay on Hepa 1-6 cells after 24 h or 48 h incubation with the supernatant medium of PLD@NEs after treated with 100 nM PMA (Fig. 3c, d). We additionally verified the cytotoxicity of PLD@NEs against HepG2 cells and 4T1 cells (Figure S6 and S7). Compared with control group, PLD released from PLD@NEs exhibited equally cytotoxic effect against tumor cells. The fluorescence images showed the accumulation of PLD in the cytoplasm after 8h endocytosis by tumor cells (Fig. 3b and Figure S5). Therefore, it is reasonable to believe that the cell-based chemotherapeutics have the capacity to accumulate and release drug at the tumor site as long as HIFU primed inflammatory stimulation. These results suggested that PLD could be released from neutrophils under inflammatory condition and exert a toxic effect on tumor cells, which probably due to the NETs formation.

It is known that the penetration depth of nanodrugs in tumor is an important factor affecting the therapeutic effect of tumor [33]. Based on the verification of the cytotoxicity of the inflammatory cells based chemotherapy system, we established Hepa 1-6 multicellular spheroids to mimic the tumor microenvironment in vivo [34] and investigated the tumor-penetration ability of PLD@NEs. The 3D liver tumor spheroids, with an average size of 400~500 μm, were obtained on day 14 post-seeding. And the level of cytokines CXCL1/KC markedly increased inside spheroid and established a concentration gradient between inside and outside (supernatant medium) tumor sphere during growth monitor (Figure S8). The formation of this inflammatory factor concentration gradient is a necessary condition for
neutrophils to infiltrate into the spheroids. Then, we incubated the 3D tumor spheroids with PLD@NEs and detected the doxorubicin signal inside the tumor models in order to verify tumor permeability of PLD@NEs. The red fluorescence represented DOX was distributed in most areas of the sphere after 8 hours of incubation in the PLD@NEs group. However, DOX signal could be only observed on the periphery of the spheroid in the PLD group (Fig. 3e). The results suggested that the cell-based chemotherapy showed satisfactory tumor spheroids permeability. And the results of the in vitro experiment provided a basis for exploring the permeability of PLD@NEs in tumor in vivo.

**HIFU ablation on Hepa 1-6 tumor-bearing mice**

Hepatocellular carcinoma (HCC) accounts for approximately 80% of primary liver cancer, and is the most lethal form of liver cancer. Its prevalence rate and incidence rate are increasing year by year. The poor prognosis of patients largely results from diagnosis of the disease usually occurs at an advanced stage and failed to meet the criteria of operation or transplantation [21, 35]. With the development of technology, high intensity focused ultrasound (HIFU) has emerged as an effective image-guided, noninvasive therapeutic modality for multiple solid tumors, including unresectable HCC [36]. However, the main limitation and challenge of HIFU, being the same as other thermal ablation treatments, is the risk of local recurrence caused by residual tumor after ablation [9, 37]. In addition, sorafenib, the first-line chemotherapeutic drug for HCC, provides unsatisfactory efficacy in patients due to its margin improvement of survival rate [38]. Until now, neither ablation nor chemotherapy can effectively improve the prognosis of this devastating disease. We chose liver cancer as a tumor model to explore the effectiveness of the proposed HIFU-cytopharmaceutical chemotherapy strategy.

At present, HIFU system based on single spherical focusing transducer has been widely used in small animal research [39]. The intensity ranges from 5 W to 40 W, which is far lower than that of clinical HIFU devices [40-42]. Since HY2900 we used in this study has been used in clinical application, and has a maximum power of 479.2 W, it is necessary to explore an appropriate ablation method for tumor-bearing mice before the experiment. In order to treat tumor bearing mice with HIFU successfully, we made a HIFU box applicable for mice, which was used to immobilize mice and protect mice from excessive ablation damage (Fig. 4a). The framework can prevent the force of big water sac from acting on mice directly and reduce the death of mice caused by stress. In addition, the surface was covered with acoustic absorbing board and the hole on the surface only allowed tumor to be exposed to the acoustic beam, which effectively controlled the ablation range of HIFU ablation and protected mice from death caused by overtreatment. The mice could tolerate the operation with a low mortality rate (<1%) and no normal tissue damage after ablation.

B-mode ultrasound showed that the change of gray scale in tumor was not as obvious as previous studies on rabbits or rats during HIFU [43, 44]. Contrast enhanced Ultrasound (CEUS) was used to show the blood perfusion of tumor before and after ablation. Before ablation, the tumor showed homogenous enhancement and solid appearance. CEUS showed that there were perfusion defects in the tumor, suggesting that HIFU caused partial vascular occlusion (Fig. 4b and Movie. S1). To further confirm the
effectiveness, mice were sacrificed on day 0 and day 14, and tumors were dissected. The excised tumor swelled with hemorrhage on day 0 and showed distinct coagulative necrosis on day 14 (Figure S9). The results of contrast-enhanced ultrasound and pathology showed that HIFU ablation was successfully achieved in tumor bearing mice.

Chemotaxis of PLD@NEs to HIFU ablated tumor

It has been reported that live neutrophils can efficiently target primary tumors [16, 45]. We investigated whether local HIFU could induce the enhancement of inflammatory signal in tumor site and improve the tumor targeting ability of neutrophils in vivo, so as to turn the weakness of HIFU-induced inflammation into advantages. We examined the expression level of inflammatory cytokines in tumor and serum of mice after HIFU treatment. CXCL1/KC is a mouse homolog of pro-inflammatory IL-8, which has a strong neutrophil chemotactic activity equivalent to human IL-8, and can broadcast the location of residual tumor to neutrophils [46]. As shown in Fig. 4c and Fig. 4d, HIFU constructed an inflammatory tumor microenvironment with concentration gradients of chemokines CXCL1/KC and IL-10, which is attributed to the pro-inflammatory response caused by the necrosis of tumor cells and the release of necrotic debris [47]. Such an increase of chemokines effectively elevates the accumulation of neutrophils, because they can actively home to inflammatory site, exudate from the blood vessels, and penetrate into the tumor with the gradient of chemokines.

Afterwards, we labeled PLD and the constructed PLD@NEs with fluorescence dye of 1,1'-diocatadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) respectively to investigated the chemotactic migration in vivo by in vivo imaging system (IVIS). In the PLD@NEs group, a significantly strongest capability of residual tumor targeting in HIFU treated tumor-bearing mice than other groups (Fig. 4e). Fluorescent signal can be observed at tumor site in after 1-hour post-injection in PLD@NEs (+) group and maintained as least 48h during our observation. The results proved that tumor tissue can elicit enough inflammatory response to facilitate chemotactic migration of PLD@NEs to the residual tumor site.

In Vivo Therapeutic Efficacy and Biosafety Evaluation of PLD@NEs on HIFU-treated tumor-bearing mice

Encouraged by the in vitro results, we finally evaluated the therapeutic efficacy of PLD@NEs on HIFU-treated HCC-bearing mice model. After treatment with individualized HIFU ablation, PLD@NEs were subsequently administrated intravenously to mice at a dosage of 5×10^6 cells per mouse (equivalent to 25 μg doxorubicin) (Fig. 5a). In clinical practice, incomplete ablation occurs when the treatment area is limited in order to reduce damage to normal tissue around lesions or special tumor locations (e.g. liver cancer in the diaphragm area). And this is exactly the problem we are trying to solve. As shown in Fig. 5b-d, ablation effect of HIFU alone suppressed tumor growth with 4 days. Tumor regeneration was observed as incomplete ablation only induced part of the tumor undergo apoptosis and necrosis. In contrast, PLD@NEs (+) group displayed a potent tumor inhibition capability compared to all other groups, including PLD (+) group (Fig. 5d). The result suggested that the increasing recruitment of PLD@NEs to the tumor site enhanced the effectiveness of chemotherapy. In addition, there is no significant inhibitory
effect was observed of between NEs and saline group, which suggesting that neutrophils lacked the anti-
tumor capacity and did not interfere with treatment efficacy. Under the upregulation of inflammatory
factors induced by HIFU, PLD@NEs broke down their nuclear contents and released NETs, which
facilitated the release of PLD from NEs for killing tumor cells[48]. We also utilized a histological analysis
to observe the cell morphology of the recurrent tumor. The tumor collected from PLD@NEs (+) group
showed a massive tumor cell death. Furthermore, apparent caspase-3 activation was shown in the
PLD@NEs (+) group compared with other groups. Tunel assay also revealed more apoptosis was
observed in PLD@NEs group (Fig. 5f). The systemic toxicity of PLD@NEs was evaluated by monitoring
the body weight of the mice and a blood biochemical assay after treatment. As shown in Fig. 5e, no body
weight loss was identified following treatment with PLD@NEs (+); and the serum liver-enzyme levels of
mice in PLD@NEs (+) group were similar to those of saline group (Figure S11), which suggests that
PLD@NEs (+) have no serious systematic toxicity. Moreover, major organs were also excised from mice
on day 14. There were no pathological manifestations were observed in the main organs after treatment
with PLD@NEs (Figure S10).

Taken together, these data provide evidence that the therapeutic strategy combining HIFU with
neutrophils-mediated chemotherapy exhibited a good tumor suppression efficacy, which showing a
potential future application in the clinical situation.

Discussion

Here, we developed neutrophil-mediated targeting nanodrug to establish an efficient strategy for
treatment of residual tumor of HIFU ablation. Our approach includes the following consecutive steps
(Scheme 1): (1) HIFU ablation creates an inflammatory environment in tumor site, stimulating
transmigration of neutrophils. (2) Neutrophils carry chemotherapy drug PLD (PLD@NEs) home to the
tumor site, exude endothelial lined blood vessels, and penetrate into the tumor, thus overcoming the
biological barriers encountered by traditional drug delivery approaches. (3) Once PLD@NEs enters tumor
tissue, PLD is released from PLD@NEs through reticular structure, and then internalized by tumor cells.
These administration strategies endowed the clinic drug PLD with a favorable tumor targeting and
permeable capacity, and exert anti-tumor effects at a dose lower than the recommended dose.

Nanotechnology has made immense progress in the last decade, which provides a novel perspective as a
last resort for refractory malignancies. Despite efforts to optimize the physicochemical properties of
nanocarriers, such as size, shape or binding affinity, some stubborn limitations still hinder the application
of nanomaterials in drug delivery [49]. Moreover, the extensive heterogeneity of drug delivery system
between patients and tumors remains a barrier to efficacy and clinical transformation [50]. And the tumor
vascular system is also still the barrier that restricts drug access to deep tumor tissues, especially in solid
tumors [51]. We are committed to build an alternative strategy to integrate and improve the existing
clinical therapies, rather than focusing on new engineered nanotherapies. Previous studies have
confirmed the feasibility of neutrophil based targeted drug delivery system for a variety of diseases,
showing good capability of active targeting and crossing vascular barrier (e.g. brain blood barrier) [17]. In
our research, we prepared the 3D tumor spheroids with the concentration gradient of inflammatory factors inside and outside the tumor to mimic the tumor microenvironment (Figure S8). Further results showed that drug loaded neutrophils were able to deliver the agent inside the tumor (Fig. 3e). Before giving the cytopharmaceuticals chemotherapy, traditional therapy such as surgery, PTT or RT was performed to establish the therapy-associated inflammation for activate neutrophil mediated drug delivery system [17–19]. HIFU, as the only non-invasive ablation therapy for deep tumors, has not yet been explored in this combination therapy.

So far, the clinical applications of focused ultrasound approved by FDA include prostate tumor, Parkinson's disease and bone metastasis. Meanwhile, the application of HIFU in breast cancer, pancreatic cancer, renal cell carcinoma, liver cancer, soft tissue tumors and other malignant tumors has also been explored (Focused Ultrasound Foundation: Http://WWW.fusfoundation.org). Many of the early large sample clinical trials were conducted by Chinese researchers. Typically, Feng et al. introduced the clinical experience of 1038 patients with various kinds of solid tumors treated with HIFU. A data set of 4-year follow-up confirmed the safety and effectiveness of HIFU treatment in multiple carcinomas [8]. In recent years, with the development of technology, HIFU-related clinical trials are being carried out all over the world (NCT04796220, NCT04852367, NCT04573881). HIFU has become a promising alternative treatment to conventional surgical resection or enhanced adjuvant therapy (such as chemotherapy, radiotherapy or immunotherapy) [9]. Our results showed that the inflammatory response at TME after HIFU ablation was sufficient to attract neutrophils to migrated to tumor site and rapidly release the drug (Fig. 4e). Therefore, our proposed neutrophil-based combined therapy strategy is of great significance for the treatment of unresectable tumors, chemo/radio-therapy resistant tumors or deep tumors.

As far as we know, there are four types of PEGylated liposome doxorubicin available clinically, including Doxil, Doxisome, Lipo-Dox, LIBOD and etc [52]. The main difference between them lies in the composition or proportion of synthetic lipids. The reason why we use PLD as a model drug to complete the cytochenmotherapeutics is the free chemotherapy drugs will kill neutrophils, which makes it impossible to be loaded into neutrophils directly for further use. However, the nanodrug showed good biocompatibility and low cytotoxicity during incubation with neutrophils (Fig. 2c, d and Figure S1). In addition, a recent research has revealed that anti-PEG antibodies are present in many healthy individuals as well as in patients receiving PEG-functionalized drugs, which can result in rapid release of encapsulated doxorubicin from liposome. This procedure may alter therapeutic efficacy and safety of patients with high levels of pre-existing antibodies against PEG [52]. In our research, this problem was solved because PLD was loaded into neutrophils. Therefore, with the increase of cytokine concentration gradient, more anticancer drugs can accumulate in the tumor site.

Although our data suggested a promising approach for hepatoma therapy, there are still several limitations. Neither 3D tumor spheroids or subcutaneous xenograft tumors can necessarily reflect the real TME in spontaneously occurring cancer [53]. We will explore the construction of tumor organoids and patient-derived organoid transplantation (PDOX) in further research [54]. In addition, more convincing evidence of efficacy and safety is needed to achieve clinical translation, such as the feasibility of batch
extraction of human neutrophils enough for treatment. But it is well known that autologous bone marrow transplantation (ABMT) is a mature technique. Our philosophy is to further extract and purify neutrophils from bone marrow and make them encapsulate drugs. As HIFU (a clinical technique for tumor ablation) and PLD (an anticancer chemotherapy drug) have been approved by FDA, how to maintain the activity of isolated neutrophils and accurately calculate the dose of drug delivery in the process of drug delivery is the difficulty of clinical transformation.

Conclusion

This study suggested that HIFU ablation combined with neutrophil mediated drug delivery system is a promising therapeutic approach for anticancer treatment. We focused on the therapy-induced inflammation, so as to explore a treatment strategy that can transmigrate the weakness into strength. Our strategy enhanced the accumulation of chemotherapy drug in the tumor, maximized the anti-cancer effects by taking advantage of both methods, as well as overcoming the weakness of HIFU and chemotherapy. It shows the prospect of “triple-wins”. First, direct thermal ablation of the tumor is performed to kill most of the lesions (tumor size is one of the indications for surgical treatment). Second, the active targeted administration of ablation-induced inflammation (The EPR mechanism cited in preclinical researches have not been effectively verified in the clinic). Third, reducing the side effects of systemic chemotherapy (Targeted accumulation can reduce the dosage of chemotherapy drugs). These are urgent problems to be solved in clinical cancer treatment. In general, neutrophil mediated drug delivery system could be an impressive achievement of smart nanoparticle design to cooperate with HIFU in cancer therapy. The platform has established a bridge to effectively combine the two clinical treatment methods, giving it a broad prospect for clinical transformation.

Abbreviations

HIFU: high intensity focused ultrasound; PLD: PEGylated liposome doxorubicin; TME: tumor microenvironment; DOX: doxorubicin; NE: neutrophil; fMLP: formylmethionylleucylphenylalanine; PMA: phorbol myristate acetate; HCC: hepatocellular carcinoma; NET: neutrophil extracellular trap; CEUS: contrast enhanced ultrasound.

Declarations

Acknowledgements

The authors would like to thank prof. Yan Huo (Shanghai Jiao Tong University Affiliated Sixth People’s Hospital) for his assistance with HPLC experiment. We also thank prof. Lixin Jiang (Shanghai Jiao Tong University Medical School Affiliated Renji Hospital) for providing the initial concept of mouse HIFU ablation box.

Authors contributions
XJC developed the concept, provided the overall research direction and reviewed all the original data. JS, JNH and XJC designed the experiments. JS and JNH performed all experiments and compiled all original data. YNC, HRL and JS performed HIFU experiment. JS wrote the original manuscript. JW, BH, XJC, YW and YYZ reviewed the original data and supervised the manuscript. All authors contributed to finalize the manuscript and approved the final version of the manuscript.

**Funding**

This work was supported by National Natural Science Foundation of China (No. 82030050, 81671700, 81801822); NSFC Key Projects of International Cooperation and Exchanges (No. 81720108023); Translational medicine national science and technology infrastructure (Shanghai) open project fund (No. TMSK-2020-004); Shanghai Rising-Star Program (21QA1407100).

**Conflict of Interest**

The authors declare no conflict of interest.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Ethics approval and consent to participate**

All animal experiments were approved by the Animal Experimentation Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (No. DWLL2021-0677).

**Consent for publication**

All authors are consent for publication.

**Competing interests**

The authors declare that they have no competing interests.

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Figures

Figure 1

Preparation and characterization of PLD@NEs. a Flow cytometry analysis of the purity of isolated neutrophils stained with FITC-conjugated Gr-1 and PE-conjugated CD11b antibodies. The average population of cells is shown in the corner of each quadrant. b Morphological images of NEs (left) and PLD@NEs (right) stained with Wright-Giemsa stain. Scale bar: 10 μm. c Cytotoxicity of PLD towards neutrophils. (n=5) d Cell viability of PLD@NEs during 12 h of incubation. (n=5) e Fluorescence
microscope images of PLD@NEs. The nuclei and membrane of neutrophil was stained with DAPI and DiO, respectively. Scale bar: 10 μm.

Figure 2

Physical functions of NEs and PLD@NEs under different conditions. a Superoxide generation after stimulated by fMLP. (n=3) b Change of CD11b expression on the membrane of neutrophils under different concentrations of fMLP. (n=3) c Chemotactic activity after treatment with different concentrations of fMLP. (n=3) d Cellular uptake of PLD by neutrophils (n=3) and color change of neutrophils before and after PLD loading. e Determination of the amount of PLD retained in PLD@NEs under different conditions, including in the absence of fMLP/PMA, in the presence of fMLP (100 nM) and of PMA (100 nM). (n=3)
Figure 3

Inflammatory mediated targeting delivery. a Schematic illustration of the mechanism of PLD release from PLD@NEs (left). The fluorescence images of NETs released from PLD@NEs after incubating with PMA (100 nM) for 4h in comparison with fMLP (100 nM) (right). b Fluorescence image of Hepa 1-6 cells after incubated with supernatant medium of PMA (100 nM) untreated/treated PLD@NEs for 8 h. The nuclei were stained with DAPI. Scale bar: 50 μm. c-d Cytotoxicity of PMA treated PLD@NEs against Hepa1-6 after incubating for 24 h (c) or 48 h (d). Data are shown as mean ± SD. *p<0.05, ** p<0.01, *** p<0.001. (n = 5) e Tumor permeability of PLD@NEs after incubation 3D hepa1-6 tumor spheroids for 8h. CLSM images were obtained from the surface to the middle of tumor spheroids in a Z-stack thickness of 50 μm. Scale bar: 200 μm.
Figure 4

HIFU ablation recruited PLD@NEs to migrate to the tumor site. a Schematic illustration of performing HIFU ablation on tumor-bearing mice. b Ablation efficiency verified by contrast enhanced ultrasound in real time. Before HIFU, the perfusion of tumor was overall enhanced, suggesting that it was a solid tumor with abundant blood flow. After HIFU, a perfusion defect area appeared in tumor, indicating ablation damage. c, d Expression levels of CXCL1/KC and IL-10 in tumor (c) and serum (d) of mice over 48 h after
HIFU ablation (n=3). e In vivo fluorescence images of tumor-bearing mice. DiR-PLD, DiR labeled PLD; DiR-NEs, DiR labeled neutrophils. PLD@DiR-NEs were injected at a dosage of $3 \times 10^6$ cells per mouse.

**Figure 5**

Therapeutic effect mediated by HIFU ablation and neutrophil based chemotherapy. a Schematic illustration of the neutrophil mediated actively chemotherapy drug delivery for suppressing hepatoma recurrence after HIFU. Mice were treated with (+) or without (-) HIFU and subsequently received different
formulations of saline, PLD, bank neutrophils (NEs), or PLD@NEs, respectively. Blood and tumor were harvested at day 7 and day 14 for biotoxicity evaluation. b Representative images of isolated Hepa 1-6 tumors after different treatments on day 14 (n = 3). c Individual tumor growth kinetics of each group (n = 3). d) The mean percentage change in tumor volume relative to initial volume (n = 3). * p<0.05, ** p<0.01, ***p<0.001. e Body weight changes of tumor-bearing mice monitored during the administration of treatment. f Histological observation of tumor excised from hepatoma-bearing mice after treatment at day 14. The tumor sections were stained with H&E (Top row), TUNEL (Middle row) and caspase-3 (Bottom row). Scale bar: 50μm.

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