An immune cocktail therapy to realize multiple boosting of the cancer-immunity cycle by combination of drug/gene delivery nanoparticles

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Immune checkpoint blockade therapy (ICT) has shown potential in the treatment of multiple tumors, but suffers poor response rate in clinic. We found that even combining ICT with chemotherapy, which was wildly used in clinical trials, failed to achieve satisfactory tumor inhibition in the B16F10 model. Thus, we further constructed a previously unexplored immune cocktail therapy and realized multiple boosting of the cancer-immunity cycle. Cocktail therapy consisted of two kinds of tumor microenvironment-responsive drug and gene delivery nanoparticles to achieve specific delivery of doxorubicin and codelivery of plasmids expressed small hairpin RNA of PD-L1 (pshPD-L1) and hyaluronidase (pSpam1) in the tumor area. Experimental evidences proved that any component in the cocktail therapy was indispensable, and the cocktail therapy exhibited excellent antitumor effects against different types of tumors. The cocktail therapy presented here offers a searching strategy for more synergistic units with ICT and is meaningful for developing more efficient antitumor immunotherapy.

INTRODUCTION

Immune checkpoint blockade therapy (ICT), which interferes with the interactions of immune checkpoints and their receptors, has achieved inspiring therapeutic effects in many tumor types (1). However, the poor response rate of ICT has largely restricted its further development (2). Previous findings have shown that inducing tumor cell immunogenic cell death (ICD) could largely increase the therapeutic effect of ICT (3). Specific chemotherapeutic agents, such as doxorubicin (DOX) and oxaliplatin, have been proven to effectively induce tumor ICD (4). Through exposing calreticulin (CRT) on the cell surface, ICD promotes the tumor antigen uptake by dendritic cells (DCs). Meanwhile, the release of danger signal molecules, including high-mobility group box 1 protein (HMGB1) and adenosine triphosphate (ATP), facilitates DC recruitment and activation (5). ICD is associated with the subsequent activation of tumor-specific T cells, thus exerting a synergistic effect with immune checkpoint antibodies (the most widely used ICT agent in the clinic).

However, the intravenous injection of DOX and checkpoint antibodies induces undesirable adverse effects (6, 7). Using nanodelivery systems could partially solve this problem (8). Compared with normal tissues, tumor areas usually have a lower pH value, ranging from 6.5 to 7.2 (9). Acid-responsive nanodelivery systems could increase the accumulation of nanoparticles via the enhanced permeability and retention (EPR) effect and release more cargo when stimulated by the tumor-specific acid environment (10). Furthermore, using small interfering RNA or small hairpin RNA to silence checkpoints locally in the tumor area would induce negligible immune-related adverse effects (irAEs) compared to the systemic administration of checkpoint antibodies (11).

On the basis of these observations, we developed two kinds of acid-responsive drug and gene delivery systems to achieve combined therapy with immunogenic chemotherapy and ICT (binary therapy). The as-prepared drug delivery system consisted of DOX-loaded poly(l-glutamic acid)-g-methoxy poly(ethylene glycol) (abbreviated DOX NPs), and the as-prepared gene delivery system consisted of pshPD-L1–loaded aldehyde-modified polyethylene glycol (OHC-PEG-CHO), poly(l-glutamic acid) (PLG), and polyethylenimine (PEI) (abbreviated shPD-L1 NPs). Unexpectedly, this combined therapy of immunogenic chemotherapy and ICT was insufficient to control the tumor growth in a highly malignant B16F10 mouse model. It was speculated that the dense extracellular matrix (ECM) in the tumor area restricted the therapeutic effect in two ways. First, the immunosuppressive tumor microenvironment (TME) induced by the ECM continuously instigated T cells into a dysfunctional state that was weakly responsive to ICT. Second, ECM acted as a physical barrier, preventing the infiltration of functionally normal peripheral T cells. Therefore, eliminating the tumor ECM would have the potential to adjust the immunosuppressive TME and promote peripheral T cell infiltration simultaneously.

In this work, an immune cocktail therapy was proposed to improve the above binary therapy by introducing an ICT destroyer as a third component. A sperm adhesion molecule 1 plasmid (pSpam1), expressing hyaluronidase (HAase), was chosen to perform the role of ECM elimination. pSpam1 can effectively degrade hyaluronic acid (HA), which is a critical component of the tumor ECM and overexpressed in 25 to 30% of the tumor types (12). Cocktail therapy was accomplished by exploiting two kinds of nanoparticles, DOX NPs and (shPD-L1 + Spam1) dual-gene codelivery NPs, both of which exhibited acid-responsive behavior (Fig. 1). The cocktail therapy has the following superiorities: (i) facilitating T cell priming via inducing tumor ICD, (ii) polarizing an immunosuppressive TME to an immune-active phenotype, (iii) notably increasing the amount of peripheral CD8+ T cell infiltration, and (iv) inducing strong immune memory effects and effectively preventing tumor metastasis. Benefiting from these advantages, outstanding immunotherapeutic effects were achieved in different murine tumor types.
In summary, we reported an innovative immune cocktail therapy for antitumor treatment by combining immunogenic chemotherapy, immune checkpoint blockade, and ECM elimination. In addition, a set of drug/gene nanodelivery systems matching the cocktail therapy was developed. This work presents a promising comprehensive immunotherapy strategy that integrates multiple aspects in regulating the cancer-immunity cycle. We expect this could be an instructive study for other cancer immunotherapies.

**RESULTS**

**Synthesis and characterization of acid-responsive DOX NPs**

Although DOX is a widely used drug for chemotherapy in clinical trials, it still has some side effects, especially cardiotoxicity, which makes many patients suffer from unexpected pain (6). To solve this problem, nano-scaled drug delivery systems were developed in the last few decades (13). In this work, the biocompatible poly(l-glutamic acid)-g-methoxy poly(ethylene glycol) (PLG-g-mPEG) was used to encapsulate DOX (fig. S1, A and C). The drug-loading efficiency (DLE) and the drug-loading content (DLC) of the DOX NPs was 80.3 and 28.6%, respectively, calculated by the ultraviolet-visible (UV-Vis) absorbance of the DOX-encapsulated nanoparticles (fig. S1B). The DOX NPs exhibited a uniform particle size around 95 nm and zeta potential of −23.2 mV, which could enhance tumor accumulation via the EPR effect and achieve longer blood circulation (fig. S1, D and E). A pH-responsive drug-releasing characteristic of DOX NPs was observed in our experiments (fig. S1F). Lower pH could reduce interactions between PLG-g-mPEG and DOX via changing the hydrophobicity and charges of PLG, causing faster DOX release in the tumor area (14). The DOX NPs exhibited comparable cytotoxicity with free DOX in the B16F10 cells (fig. S1G).

**DOX NPs induce ICD in vitro and in vivo**

Effective antigen presentation is one of the most important steps in T cell activation. Immunologic cell death can help DCs maturation and enhance their antigen presentation ability (15). CRT, ATP, and HMGB1 are three representative markers of ICD (16). To provide the evidence that DOX NPs worked as immunogenic chemotherapy,
For more efficient and precise gene delivery to the tumor site, we chose OHC-PEG-CHO/PEI/PLG as the gene delivery system. OHC-PEG-CHO/PEI/PLG nanoparticles (P[(GP)D] NPs) were prepared (fig. S2, A to C). The amino groups of PEI could quickly form Schiff bases with the aldehyde groups of OHC-PEG-CHO in neutral aqueous solution, whereas the Schiff bases were labile at pH 6.8 (fig. S2D). The P[(GP)D] NPs exhibited pH-triggered size/charge dual-rebound characteristics (fig. S2E) and higher cellular uptake efficiency at pH 6.8 than at pH 7.4 in our experiment (fig. S2F). Last, an optimized mass ratio of PEG:PLG:PEI:DNA = 5:1:2.5:1 was chosen for the P[(GP)D] NPs according to an in vitro transfection experiment to achieve relatively higher transfection efficiency in the tumor and avoid undesirable gene expression in the normal tissues (fig. S2G).

**Synthesis and characterization of an acid-responsive nanocarrier for gene delivery**

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**Combination therapy by using DOX NPs and shPD-L1 NPs**

For the first-stage antitumor treatment, we combined DOX NPs with shPD-L1 NPs for treating a B16F10 model in C57BL/6 mice (Fig. 2A). The results showed that immunogenic chemotherapy could generate synergic effects with ICT when we compared the DOX NPs + shPD-L1 NPs group with the shPD-L1 NPs group (Fig. 2B), which could be attributed to the increased numbers of CD8+ T cells (Fig. 2, C and D). Unexpectedly, the number of intratumoral CD8+ T cells was not significantly different between the DOX NPs + shPD-L1 NPs group and the DOX NPs group, which induced a similar therapeutic effect. As a result, the DOX NPs + shPD-L1 NPs only achieved 60.6% tumor inhibition (Fig. 2B). In a normal situation, combining immunogenic chemotherapy with ICT should achieve much higher CD8+ T cell numbers compared with chemotherapy (17). There might be some special immunosuppressive factors in the B16F10 tumor that restricted further increases in CD8+ T cell numbers in the DOX NPs + shPD-L1 NPs treatment.

Recent reports have shown that most preexisting CD8+ T cells in the immunosuppressive TME were dysfunctional T cells, which could only partially improve their function after ICB treatment but had nearly no proliferative capacity (18). Recruiting functional normal T cells from the periphery would be a wise strategy for improving the antitumor efficiency of the current DOX NPs + shPD-L1 NPs treatment. In addition, reversing the immunosuppressive TME is also important because it would continuously exhaust the intratumoral T cells, whether they were preexisting or newly infiltrated (19). Considering that B16F10 is a high-grade malignant tumor type, dense ECM during the growing process might generate an immunosuppressive TME and largely hinder the attraction of peripheral CD8+ T cells (20). We supposed that introducing extra therapy targeting the ECM would increase the therapeutic effect of immunogenic chemotherapy and ICT.

**Immune cocktail therapy for antitumor treatment**

HA is an important component of the tumor ECM and performs multiple immunosuppressive functions (21). In this work, pSpam1 was chosen to generate mouse HAase, codelivered with shPD-L1 via our gene delivery system. We first designed an experiment to observe the antitumor effect of immune cocktail therapy, which consisted of DOX NPs and (shPD-L1 + Spam1) NPs (Fig. 3A). As we expected, the cocktail therapy group exhibited the most effective tumor inhibition, demonstrating 97.3% tumor shrinkage (Fig. 3B). This was a marked improvement in the therapeutic effect when we compared to 60.3% tumor shrinkage in the DOX NPs + shPD-L1 NPs group (Fig. 3B) and looked back to the 60.6% tumor shrinkage for the DOX NPs + shPD-L1 NPs group in the previous experiment.

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**Fig. 2. Combination of immunogenic chemotherapy and ICT on the subcutaneous B16F10 model.** (A) Combinational treatment scheme of DOX NPs and shPD-L1 NPs. Mice received shPD-L1 NPs on days 12 and 16 via intravenous injection. Mice received DOX NPs on days 14 and 18 via intravenous injection. (B) Tumor growth curves in phosphate-buffered saline (PBS), DOX NPs, shPD-L1 NPs, and DOX NPs + shPD-L1 NPs treated groups. (C) CD45+CD3+CD8+ T cell ratios in the tumor of different treatment groups on day 21. (D) Immunofluorescence staining for the P[(GP)D] NPs according to an in vitro transfection experiment to achieve relatively higher transfection efficiency in the tumor and avoid undesirable gene expression in the normal tissues (fig. S2G).
Further analyses of the tumor inhibition showed that any other formulations exhibited poorer antitumor efficiency compared to the cocktail therapy, indicating the indispensability of any component in the cocktail therapy. The tumor weight in the different treatment groups also exhibited the same tendencies (Fig. 3C). Furthermore, in the cocktail therapy group, five of six mice had a partial response (PR), indicating that cocktail therapy could increase the overall response rate and present a potential benefit to more patients (Fig. 3D). The photos and hematoxylin and eosin (H&E) staining of the excised tumors further proved the superior treatment effect of the cocktail therapy (Fig. 3E and fig. S3A). The weight of the mice did not change obviously during the therapeutic process (fig. S3B). The H&E results of other major organs indicated that all treatments had only negligible side effects (fig. S3C). In addition, we evaluated the systemic cytotoxicity of different treatment groups by measuring uric acid, blood urea nitrogen, creatinine, alkaline phosphatase, glutamate pyruvic transaminase, and aspartate aminotransferase in the serum (fig. S3D). No abnormal indicator was observed in our cocktail therapy when compared with the phosphate-buffered saline (PBS) group.

**Cocktail therapy reprograms an immunosuppressive TME to an active immune phenotype**

To further interrogate the mechanisms of how the cocktail therapy worked, immunohistochemistry was used to evaluate related biological markers in the tumor sections. As clearly shown in fig. S4A, shPD-L1 NPs significantly reduced PD-L1 expression, whether in monotherapy or in combinational therapies. At the same time, we monitored the level of FoxP3 in the tumor sections, which could reflect the amounts of regulatory T (T_{reg}) cells. Excess T_{reg} cells could inhibit the activity of CD8^{+} T cells (22). In the DOX NPs + shPD-L1 NPs group, the amount of FoxP3 was increased compared to the PBS group, probably because the immune system tried to achieve immune homeostasis to balance the active immune state. After combined therapy with pSpam1, the immunostained area of FoxP3 was reduced in the cocktail therapy group because degrading HA could deactivate the T_{reg} cells (23). Furthermore, HAase generated by pSpam1 remarkably reduced the immunostained area of hypoxia-inducible factor 1-α, which could help more immune cells function better (24).

For a comprehensive understanding of the changes in the immune cell populations, flow cytometry analyses were carried out.
DOX-induced tumor IC1 successfully increased the percentage of CD80$^+$ major histocompatibility complex (MHC) II$^+$ mature DCs in the tumor-draining lymph nodes (TDLNs) (Fig. 4A and fig. S4B). The cocktail therapy group showed a marked increase in intratumoral T cells (Fig. 4B). The intratumoral number of CD8$^+$ T cells in the cocktail therapy group was 7.9-fold higher than that in the PBS group and 2.4-fold higher than that in the DOX NPs + shPD-L1 NPs group (Fig. 4C). The cocktail therapy group also showed an increase in the number of CD4$^+$ T cells compared to the PBS group (Fig. 4D). Further calculations showed that the cocktail therapy group exhibited high CD8$^+$/CD4$^+$ T cell ratios (Fig. 4E and fig. S4C), indicating an active immune status in the tumor. Besides, cocktail therapy significantly reduced the percentage of M2 macrophages compared to the PBS group or DOX NPs + shPD-L1 NPs group, which would help CD8$^+$ T cells function normally (Fig. 4F) (25).

Next, the immune status of the TME was further investigated by enzyme-linked immunosorbent assay (ELISA) and reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR) analysis of the protein and gene levels. The ELISA results showed that the cocktail therapy decreased PD-L1 expression (Fig. 4G) and increased the amount of HAase (Fig. 4H) in the tumors, indicating that the therapeutic gene-loaded NPs could function as intended. Interferon-γ (IFN-γ) and granzyme B are two key elements in antitumor efficiency (26). The cocktail therapy generated 1.9-fold more intratumoral IFN-γ than the PBS group and 1.3-fold more than the DOX NPs + shPD-L1 NPs group (Fig. 4I).
Therapy with HA degradation

Therapy without HA degradation

Fig. 5. Cocktail therapy increased peripheral T cell infiltration. (A) Treatment scheme for peripheral T cells depletion experiment. (B) Left: Tumor growth curves in different treatment groups. Right: Amplified region of tumor growth curves. (C) Relative fold change of peripheral CD45⁺CD3⁺CD8⁺ T cells in blood compared with the PBS group. (D) CD45⁺CD3⁺CD8⁺ T cells in tumor compared with the PBS group. (E) Immunofluorescence staining in tumor tissues for CD8 (green) and granzyme B (purple) of different treatment groups. Scale bars, 50 μm. (F) Immunofluorescence staining in tumor tissues for α-SMA (green) and CD31 (purple). Scale bars, 100 μm. (G) RT-qPCR results of Cxcl9, Cxcl10, Icam1, and Vcam1 mRNA expression level in tumor tissues. (H) Quantitative measurement of ICAM1 and VCAM1 in tumor tissues. (I) Immunofluorescence staining in tumor tissues for HA (green). Scale bars, 100 μm. (J) The schematic diagram for suggested mechanisms that could affect peripheral T cell infiltration. We concluded four crucial steps: vessel normalization, chemokines level, adhesion molecules level, and ECM elimination. The score table for the comprehensive contributions to peripheral T cell infiltration was also shown in the figure. We supposed that the cocktail therapy group gained 3.5 "points," whereas the DOX NPs + shPD-L1 NPs group gained 0.5 points in the score table. Significant differences in (B) to (D), (G), and (H) were assessed using t test (ns represented not significant, *P < 0.05, **P < 0.01, ***P < 0.001). Results were expressed as means ± SD (n = 5 or 3).
Furthermore, the amount of granzyme B in the cocktail therapy group was 3.8-fold higher than in the PBS group and 1.6-fold higher than the DOX NPs + shPD-L1 NPs group (Fig. 4J). The higher levels of CD8 and granzyme B in the cocktail therapy group were further confirmed by immunofluorescence (Fig. 4M and fig. S5A). These results proved that the cocktail therapy exerted much stronger killing ability against the tumors, indicated by the up-regulation of IFN-γ and granzyme B. In addition, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are two typical proinflammatory factors that can facilitate immune cell recruitment, maturation, or activation (27). The cocktail therapy further increased these two cytokines compared to the PBS group and the DOX NPs + shPD-L1 NPs group (Fig. 4, K and L). Furthermore, the heatmap of related genes showed that the cocktail therapy could down-regulate the expression of immunosuppressive genes (Pd-l1 and Tgf-β) and up-regulate the expression of chemokines (Cxc10, Cxcl9, Ccl5, and Ccl3), adhesion molecules (Icam1 and Vcam1), proinflammatory factors (Il-1α, Il-1β, Il-6, and Tnf-α), and nuclear transcription factor κB compared to the PBS group (Fig. 4N). Together, with pSpam1 assistance, cocktail therapy demonstrated a powerful capacity for TME regulation at the protein and gene levels.

**Cocktail therapy increases peripheral T cell infiltration**

In the previous experiment, we speculated that most of the T cells in the DOX NPs + shPD-L1 NPs group were dysfunctional T cells and were weakly responsive to ICT. Peripheral functionally normal CD8+ T cells could have a better synergistic effect with ICT, continuing proliferation and activation. To study whether pSpam1 could promote peripheral CD8+ T cell infiltration in the tumors, FTY720, a widely used peripheral T cell depletion agent (28), was used to establish a peripheral CD8+ T cell depletion model (Fig. 5A). The tumor growth curves were recorded and the CD8+ T cells in the peripheral blood and tumors were analyzed (Fig. 5, B to D).

First, the CD8+ T cells in peripheral blood clearly showed that FTY720 decreased the numbers of peripheral CD8+ T cells by 80 to 90% in the related groups, indicating that this model was successfully established (Fig. 5C). The tumor growth curves showed that the PBS + FTY720 group was not obviously different from the PBS group (Fig. 5B). This could be attributed to the poor infiltration of the peripheral CD8+ T cells into the tumor area (Fig. 5D). In the B16F10 tumor–bearing mice, it was difficult for the tumor-specific peripheral CD8+ T cells to infiltrate the tumor because of the high ECM density. Thus, the peripheral CD8+ T cells contributed little to the tumor inhibition in the PBS group. It was understandable that the depletion of peripheral CD8+ T cells by FTY720 in the PBS group would not cause an obvious difference in the tumor growth curves. In contrast, an extra FTY720 injection significantly increased the tumor growth rate in mice treated with Spam1 NPs (Fig. 5B), indicating that peripheral CD8+ T cells played an important role in the pSpam1 NP group. pSpam1 NPs could effectively destroy the ECM by the in situ expression of HAase in the tumors, thus increasing the infiltration of peripheral CD8+ T cells into the tumors. The tumor inhibition ability of the pSpam1 group largely relied on the infiltration of peripheral CD8+ T cells. Therefore, it was obvious that FTY720 injections would significantly eliminate the possibility of peripheral CD8+ T cell infiltration into the tumors from the source (Fig. 5D), greatly decreasing the tumor-inhibiting ability of the pSpam1 NPs. Similarly, FTY720 injections could badly damage the tumor-inhibiting ability of the (shPD-L1 + Spam1) NPs (Fig. 5B). Originally, the (shPD-L1 + Spam1) NPs demonstrated the best therapeutic effect among all the treatment groups, which was attributed to the following aspects. First, the infiltration of peripheral CD8+ T cells into the tumors was improved. The second was the activation and proliferation of CD8+ T cells, which was boosted by PD-L1 silencing. Therefore, the depletion of CD8+ T cells by FTY720 injection would inevitably decrease the number of CD8+ T cells (Fig. 5, D and E) and the amount of granzyme B (Fig. 5E) in the tumors in the (shPD-L1 + Spam1) NPs group, further decreasing the antitumor efficiency. In conclusion, the ICT for the tumors with dense ECM was heavily dependent on the infiltration of peripheral CD8+ T cells into the tumors, which could be boosted by HA degradation. This was the critical reason why we included a functional HA degradation Spam1 gene in our cocktail therapy.

After confirming that pSpam1 could promote peripheral CD8+ T cell infiltration, we further explored the mechanism that could cause this phenomenon. Several aspects that could influence the T cell infiltration capacity were assessed, respectively. First, the degree of vessel normalization was evaluated by immunofluorescence (Fig. 5F). The colocalization of alpha-smooth muscle actin (α-SMA) and CD31 was seen as a biomarker of a normalized vessel (29). Second, Fig. 5G showed the gene expression levels of chemokines (Cxc10 and Cxcl10) and adhesion molecules (Icam1 and Vcam1), which were measured by RT-qPCR. ELISA was further carried out to confirm the amount of ICAM1 and VCAM1 protein in the tumors (Fig. 5H and fig. S5, B and C). Last, HA degradation in the tumor was visualized by immunofluorescence (Fig. 5I). On the basis of the above results, we summarized the contribution of different factors that affected peripheral T cell infiltration and compared the tumors with dense HA to tumors after HA degradation (Fig. 5J). Although the gene expression level of the chemokines in the cocktail group (with HA degradation) was slightly lower than that in the DOX NPs + shPD-L1 NPs group (without HA degradation), the cocktail group gained more points in vessel normalization, adhesion molecule expression, and ECM physical barrier destruction in the TME. Therefore, introducing pSpam1 in the cocktail group could induce effective HA degradation in the tumor and act as a trigger for a series of cascading effects, and lastly, greatly improving the infiltration of peripheral CD8+ T cells. Besides promoting immune cell infiltration, HA degradation has also been proven to increase nanoparticle accumulation in tumors (30). The fluorescence imaging experiment result showed that Spam1 NPs induced a higher DOX NPs accumulation in the B16F10 tumor at different times (fig. S6, A to C). In addition, the photoacoustic (PA) imaging experiment also showed that Spam1 NPs could increase the average signal of contrast agent in the 4T1 tumor when compared with PBS-treated mice (fig. S6, D and E). We proved that Spam1 NPs increased the accumulation of nanoparticles in different tumor types, which were mainly attributed to the destruction of tumor ECM. This would also increase the antitumor efficiency.

**Cocktail therapy induces strong immune memory effects**

It was reported that effector T cells newly infiltrating into the tumor site, not preexisting dysfunctional T cells in the tumor site, might be transformed into memory T cells (31, 32). Therefore, the cocktail therapy was also expected to enhance the immune memory effect. Because the spleen is an important immune organ that provides a place for immune cell homing and differentiation (33), T cells in the spleen were carefully analyzed after the treatments. The cocktail therapy group showed increased numbers of CD8+ T and CD4+ T cells.
compared to the PBS group but no significant differences compared to the DOX NPs + shPD-L1 NP group (Fig. 6, A and B). However, as shown in Fig. 6C, the cocktail therapy group exhibited significantly increased numbers of CD3+CD8+CD44+CD62L− effector memory T (T_{EM}) cells in the spleen compared to the DOX NPs + shPD-L1 NP group (1.6-fold) and the PBS group (2.5-fold). Considering that a higher number of T_{EM} cells indicate a profound and durable antitumor efficiency, the cocktail therapy group might prolong the survival and enhance the resistance to lung metastasis. As shown in Fig. 6 (D and E), the median survival in the cocktail therapy group

Fig. 6. Cocktail therapy largely increased immune memory cells in the spleen, prolonged medium survival, and could inhibit lung metastasis. Flow cytometry results for (A) CD3+CD4+ T cell ratios, (B) CD3+CD8+ T cell ratios, and (C) CD3+CD8+CD44+CD62L− effective memory T cell ratios of different treatment groups in the spleen (n = 5). (D) Treatment scheme of survival experiment. (E) Mouse survival curves of different treatment groups (n = 6). (F) Treatment scheme of lung metastasis experiment. (G) H&E results for lungs of naive mouse group (control) and cocktail therapy group. Scale bars, 500 μm. Significant differences in (A) to (C) were assessed using t test (ns represents not significant, **P < 0.01, ***P < 0.001). Significant differences in (E) were assessed using log-rank (Mantel-Cox) test (*P < 0.05, ***P < 0.001).
was markedly increased to 38.5 days, compared with the PBS group (17 days) and the DOX NPs + shPD-L1 NP group (21.5 days). Next, a lung metastasis model was established to verify whether the cocktail therapy could protect mice from tumor metastasis (Fig. 6F). The H&E staining results clearly showed that the B16F10 tumors developed rapidly in the lungs of naive mice, but there was no metastasis in the cocktail therapy group (Fig. 6G). Another wound-healing experiment also proved that pSpam1 could impede tumor migration in vitro (fig. S7).

**Cocktail therapy shows enhanced therapeutic effect in other tumor types**

Considering that the cocktail therapy has achieved superior antitumor efficiency in the B16F10 tumor, we further explored whether this strategy could be applied to other tumor types. Recently published studies have shown that combining chemotherapy with ICB could greatly increase the therapeutic effect in both breast cancer or colorectal cancer when compared with single ICB therapy (3, 17). More effective antitumor results were expected to be achieved in these kinds of tumors by the cocktail therapy. We then established subcutaneous 4T1 and CT26 models on BALB/c mice and performed the treatments according to fig. S8A. The results showed that the cocktail therapy group exhibited slowest 4T1 tumor growth rate and minimized tumor weight when compared with other treatment groups, including DOX NPs + shPD-L1 NPs group (fig. S8, B and C). Tumor photos of different treatment groups also showed that the cocktail therapy had the best antitumor efficiency (fig. S8F). The intratumoral immune cell analyses provided the evidence that the cocktail therapy could further elevate the CD3+ and CD8+ T cell amounts when compared with DOX NPs + shPD-L1 NPs group (fig. S8, D and E). Similar tendencies of therapeutic outcomes and immune cell analyses were also observed in the CT26 model (fig. S8, G to K).

In summary, the cocktail therapy could effectively inhibit the growth of several subcutaneously tumor models and could promote a strong immune memory effect with prolonged median survival and protection against tumor metastasis in the B16F10 model. It is worth mentioning that the cocktail therapy could further increase the intratumoral CD8+ T cells infiltration in multiple tumor types when compared with traditional combination therapy that consists of immunogenic chemotherapy and ICB. This could be a pivotal factor to produce a better therapeutic effect. We supposed that the cocktail therapy has the potential to be used in a variety of tumor types.

**DISCUSSION**

Although ICT has achieved great success in treating a variety of tumors, the effectiveness of ICT depends on patient characteristics (34). Even in melanoma, one of the most sensitive tumor types to ICT, nivolumab (a U.S. Food and Drug Administration–approved PD-1 antibody) could only achieve an objective response rate of 20.3 to 43.6% (35). Recent reports found that immunogenic chemotherapy could help increase the therapeutic effects of ICT by promoting immune activation. For example, short-term DOX treatment remarkably increased the PD-1 blockade response rate in patients with triple-negative breast cancer (3).

However, systemic administration of DOX and checkpoint antibodies could induce undesirable side effects. Cardiotoxicity is the main side effect of DOX treatment, using nanotechnology to develop a tumor-specific drug delivery system could minimize the damage to normal tissues. TMEs have a lower pH value ranged from 6.5 to 7.2 compared with normal tissues. Developing acid-responsive nanodelivery systems with good biocompatibility could release cargos specifically in the TME and improve the therapeutic effect. For checkpoint antibodies, irAEs such as fatigue, skin disorders, and hematologic adverse effects are a major concern. Recently, reports have shown that using gene technology to hinder the generation of checkpoint molecules locally at tumor area may induce lower irAEs compared with the systemic administration of checkpoint antibodies (11, 36).

Thus, in a preliminary study, we synthesized acid-responsive DOX NPs and shPD-L1 NPs, mirroring the common combinational therapy of immunogenic chemotherapy and ICT in clinical use and expected that the nanodelivery systems would have fewer side effects.

Unexpectedly, the shPD-L1 + DOX NP group only achieved 60.6% tumor inhibition in the mouse B16F10 model, which was insufficient for effective antitumor therapy (Fig. 2B). Dense tumor ECM facilitated the formation of an immunosuppressive TME, which continuously exhausted the T cells inside the tumor, making them insensitive to ICT. In addition, the ECM could also prevent peripherally functionally normal T cells from infiltrating the tumor. As a result, most T cells in the TME were dysfunctional T cells that were weakly responsive to ICT, eventually failing to inhibit tumor growth.

To further improve the therapeutic effect, an immune cocktail therapy that consisted of DOX NPs and (shPD-L1 + Spam1) NPs was created. The cocktail therapy induced 97.3% tumor shrinkage compared to the PBS group, and five of six mice in this group achieved PRs (Fig. 3, B and D). The mice that received cocktail therapy achieved longer median survival and better inhibition of lung metastasis. Cocktail therapy had the following superiorities: (i) promoting T cell priming via inducing tumor ICD, (ii) reprogramming the immunosuppressive TME into an active immune phenotype, (iii) facilitating peripheral T cell infiltration into the tumor, and (iv) inducing a vigorous immune memory effect. In addition, we proved that the cocktail was also suitable for other tumor types, such as the 4T1 model and CT26 model (fig. S8).

Previously reported immunotherapies related to ECM clearance usually observed an increase of CD8+ T cells into the tumor after treatment, but few of them studied the source of these T cells (4). Whether increased T cells came from the proliferation of originally resident T cells or the infiltration and proliferation of peripheral T cells remained unknown. In this study, we established a peripheral T cell depletion model and successfully demonstrated that peripheral T cell infiltration was an important factor in increasing the number of CD8+ T cells in the tumor (Fig. 5, B and D). pSpam1, the key component of cocktail therapy, could promote peripheral T cell infiltration via vascular normalization, chemokine up-regulation, adhesion molecule up-regulation, and physical barrier (ECM) degradation (Fig. 5). It was worth noting that the antitumor effect obtained with pSpam1 treatment alone was limited. Combining pSpam1 with DOX and shPD-L1 was necessary because the combination could effectively increase T cell priming and avoid T cell exhaustion.

There were some potential safety concerns in this work. One was whether the acid-responsive carriers could reduce the toxicity to normal organs and minimize irAEs. Throughout the treatments, there were no obvious mouse weight changes or irAEs in any treatment group (fig. S4B). The H&E results of the major organs and measurements for liver and kidney functions proved that our cocktail therapy had little damage to normal organs (fig. S4, C and D). However, ECM elimination might be a double-edged sword in immunotherapy.
Some reports pointed out that endogenous HAase could promote tumor growth and cause faster tumor metastasis (37). Actually, HAase as a tumor promoter or suppressor depends on its intratumoral concentration (38). In this study, no identifiable metastasis could be observed in any treatment group according to the H&E results in the major organs (fig. S4C). There might be another problem regarding whether the normalization of blood vessels caused by ECM elimination would increase the number of some immunosuppressive cells in the tumor. Our immunohistochemistry and flow cytometry results showed that both the ratio of Treg cells and M2 macrophages were decreased in the cocktail treatment group compared to the PBS group (Fig. 4F and fig. S5A). Together, our cocktail therapy was a safe and effective antitumor strategy.

We believe that in a sturdy cancer-immunity cycle, some steps are crucial (39). These steps include but not limited to (1) tumor antigen release, (2) DC maturation and antigen uptake, (3) antigen presentation and T cell priming, (4) T cells trafficking from the periphery to the tumor, (5) successful recognition of the tumor cells by tumor-specific T cells, (6) killing of tumor cells, and (7) the generation of immune memory T cells. ICT could be helpful for restoring the cytotoxic effect of tumor-specific T cells but could only achieve a limited promotion of the abovementioned steps. In contrast, our cocktail therapy expanded the range of regulation, and at least, affected steps (1), (2), (4), (6), and (7). In view of the excellent antitumor efficiency of our cocktail therapy, any strategy that could promote a positive shift in the cancer-immunity cycle would be a possible cornerstone for new cocktail therapy. We look forward to the further innovation of “super cocktail therapies” with pleiotropic regulations of the cancer-immunity cycle, which would help people fight tumors more effectively.

**MATERIALS AND METHODS**

**Materials**

Branched PEI (molecular weight 25,000 Da) and 4′,6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DOX hydrochloride (DOX-HCl) was purchased from Meilun Biotechnology Corporation (Dalian, China). Fingolinol hydrochloride (FTY720) was purchased from Sunsurechem Biotechnology Corporation (Nanjing, China). DNA labeled with Cyanine 5 (Cy5-DNA) was purchased from RiboBio (Guangzhou, China). A luciferase reporter gene assay kit was purchased from Promega (Mannheim, Germany). SYBR Green qPCR Mix for qPCR was purchased from TIANGEN Corporation (Beijing, China). The plasmid expressed shPD-L1, and all the primers for qPCR were synthesized by Sangon (Shanghai, China). The plasmid DNA-expressed mouse Spam1 was synthesized by GenePharma (Shanghai, China). An ATP assay kit was purchased from Beyotime Biotechnology Corporation (Shanghai, China). The flow cytometry antibodies were purchased from Thermo Fisher Scientific or Abcam. Immunohistochemistry or immunofluorescence antibodies were purchased from Thermo Fisher Scientific, Abcam, R&D Systems, or Servicebio Biotechnology Corporation (Wuhan, China). A PV-6000 two-step immunohistochemistry kit was purchased from Zhongshan Goldbridge Biotechnology (Beijing, China). ELISA kits were purchased from Thermo Fisher Scientific or Anoric Bio-technology Corporation (Tianjin, China). The other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd., China. Antibodies used in this study were listed in table S1. Primers sequences used in this study were listed in table S2.

**Synthesis and characterization of PLG**

PLG was prepared according to the previously reported method (39). Bruker AV-300 nuclear magnetic resonance (NMR) spectrometer (Bruker, Ettlingen, Germany) was used to characterize the final product. D2O was used as the solvent.

**Synthesis and characterizations of PLG-g-mPEG**

PLG-g-mPEG was prepared according to the previously reported method (40). The final product was characterized by $^1$H NMR spectra through a Bruker AV-300 NMR spectrometer (Bruker, Ettlingen, Germany) in D2O.

**Synthesis and characterizations of aldehyde group modified PEG (OHC-PEG-CHO)**

OHC-PEG-CHO was prepared according to the previously reported method (10). Bruker AV-300 NMR spectrometer (Bruker, Ettlingen, Germany) was used to characterize the final product. CDCl3 was used as the solvent. To verify the pH sensitivity of the reaction between PEI and OHC-PEG-CHO, they were dissolved in D2O, adjusted pH of the solution by DCl and NaOD and reacted for 10 min. Then, the $^1$H NMR spectra were detected.

**Preparation of the pH-responsive NPs for gene delivery system**

For the preparation of pH-responsive NPs for in vivo gene delivery, the method was described in the following steps. First, PEI 25k aqueous solution (0.25 mg/ml) was mixed with PLG aqueous solution (0.1 mg/ml) in equal volume. After 15-s vortex and 10-min incubation at room temperature, DNA aqueous solution (0.1 mg/ml) was added into the previous solution for 15-s vortex and 10-min incubation. Last, different concentration of OHC-PEG-CHO aqueous solution was added into the above prepared solution and kept at room temperature for 5 min. PEG[(PLG/PEI)/DNA] ([P(GP)]D) complexes with mass ratio of (2.5 ~ 10):1:2.5:1 for PEG[(PLG/PEI)/DNA] were obtained.

**Preparation of the pH-responsive NPs for drug delivery system**

For the preparation of pH-responsive NPs for immunogenic chemotherapy, the method was described in the following steps. First, PLG-g-mPEG (100 mg) was dissolved in Milli-Q water (0.5 ml). After completely dissolved, dimethyl sulfoxide (DMSO) (4.5 ml) was added into the solution. The final concentration of PLG-g-mPEG was 20 mg/ml. DOX-HCl (50 mg) was dissolved in DMSO (5.0 ml) to form 10 mg/ml solution. Next, PLG-g-mPEG solution was mixed with DOX-HCl solution in equal volume. After 15-s vortex, the mixture solution was added into distilled water drop by drop and stirred for 0.5 hours in the dark to form stable micelle NPs. The excess drug was removed by dialysis [molecular weight cut off (MWCO 2000)] against deionized water for 48 hours. For determination of DLE and DLC, the solution after dialysis was diluted with 50 volumes of DMSO and measured by UV-Vis spectrometer at 480 nm. DLE and DLC were calculated according to the following equation

\[
\text{DLE (wt%)} = \frac{\text{weight of loaded drug}}{\text{weight of feeding drug}} \times 100\%
\]

\[
\text{DLC (wt%)} = \frac{\text{weight of loaded drug}}{\text{weight of NPs}} \times 100\%
\]
Zeta potential and particle size
The zeta potential and particle size of PEG[(PLG/PEI)/DNA] NPs and PLG-g-mPEG/DOX NPs (DOX NPs) were measured at room temperature by using a zeta potential/BI-90Plus particle size analyzer (Brookhaven, USA). Data were shown as means ± SD based on triplicate independent experiments. The morphological characteristic of the PLG-g-mPEG/DOX NPs was observed by field emission scanning electron microscope (Zeiss Merlin FE-SEM).

pH-responsive drug release behavior of DOX NPs
The pH-responsive drug release behavior of DOX NPs was evaluated in PBS of pH 7.4 or pH 6.8 or pH 5.5 at 37°C by a dialysis method. Briefly, DOX NPs were added in dialysis bag at a concentration of 2 mg/ml (4 ml) and set in a 50-ml beaker, 36 ml of PBS at different pH was added into the beaker. Then, put the beaker into a 37°C constant temperature shaking box, take out the liquid in 1 ml of the beaker at intervals and measure the UV absorption at 480 nm, and replenish the lost liquid in the beaker.

Cell lines and animals
Murine B16F10 melanoma cancer cells, murine 4T1 breast cancer cells and murine CT26 colorectal cancer cells were obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) contains 10% fetal bovine serum. Cells were incubated at 37°C for 2 hours. After 2 hours of incubation, the cells were washed twice with PBS and fixed with 4.0% paraformaldehyde for 18 min at room temperature. After washed four times with PBS, the cell nuclei were stained with DAPI for 10 min. Last, the coverslips were carefully taken out and placed on the slides, enclosed with glycerol. The samples were observed by confocal laser scanning microscopy (CLSM) (ZEISS LSM780, Germany).

Cell uptake
Cell uptake experiment was tested by flow cytometry. Briefly, B16F10 cells were seeded in six-well plates at a density of 1.5 × 10⁵ cells per well. After 24 hours of incubation in 37°C, DOX and DOX NPs were added to each well, the cells were incubated for another 4 hours. After incubation, the cells were washed twice with cold PBS and digested with trypsin for 30 s. DMEM was added and the cells were collected after washed twice with cold PBS. The cells were tested with a Guava easyCyte flow cytometer (Guava Technologies).

In vitro DNA transfection
The DNA transfection experiment of the PEG[(PLG/PEI)/DNA] NPs was carried out in a murine B16F10 melanoma cancer cells. The luciferase plasmid DNA was used as the reporter gene. B16F10 cells were seeded in 96-well plates at a density of 1.0 × 10⁴ cells per well and then cultured at 37°C for 12 hours. The culture medium in the plates was replaced with new DMEM with different pH values (7.4 and 6.8) before transfection, the PEG[(PLG/PEI)/DNA] NPs at various mass ratios were prepared and added into the plates and incubated for 2 hours. After that, the culture medium was replaced by fresh DMEM and then incubated for another 46 hours. The luciferase level in each well was measured according to the manufacturer’s instruction.

Cytotoxicity assay
The cytotoxicity of the NPs was measured by MTT assay. Briefly, B16F10 cells were seeded in 96-well plates at a density of 8000 cells per well and then cultured at 37°C for 12 hours. Different concentration of DOX and DOX NPs were added to each well, and the plates were returned back to the incubator for 48 hours. MTT (20 μl, 5 mg/ml) was then added to each well. After 4 hours of incubation, the medium was removed and 150-μl DMSO was added to each well for dissolving the formazan crystals. The samples were measured by using a TECAN Infinite F50 at 492 nm.

Confocal laser scanning microscopy
B16F10 cells were seeded in six-well plates at a density of 1.0 × 10⁵ cells per well. After 24 hours of incubation in 37°C, DOX NPs were added to each well. After 2 hours of incubation, the cells were washed twice with PBS and fixed with 4.0% paraformaldehyde for 18 min at room temperature. After washed four times with PBS, the cell nuclei were stained with DAPI for 10 min. Last, the coverslips were carefully taken out and placed on the slides, enclosed with glycerol. The samples were observed by confocal laser scanning microscopy (CLSM) (ZEISS LSM780, Germany).

Induction of ICD with the DOX NPs in vitro and in vivo
To determine chemotherapy-induced ICD of the B16F10 tumor cells in vitro, surface expression of CRT, extracellular release of HMGB1, and ATP secretion were examined via different methods. For flow cytometry detection of CRT expression, B16F10 cells were seeded in six-well plates at a density of 3.0 × 10⁵ cells per well. After 12 hours of incubation in 37°C, DOX and DOX NPs were added into each well at the identical DOX concentration of 10 μM and incubated for 6 hours. The cells were then washed twice with PBS and incubated with anti-CRT Alexa Fluor 647 for 45 min. The cells were washed twice with PBS and tested with a Guava easyCyte flow cytometer.

Intracellular HMGB1 release was visualized using immunofluorescence analysis. B16F10 cells were seeded in six-well plates at a density of 1.5 × 10⁵ cells per well. After 12 hours of incubation in 37°C, DOX NPs were added into each well at the identical DOX concentration of 2 μM and incubated for 24 hours. The cells were then washed, fixed with 4% paraformaldehyde for 18 min, and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with goat serum for 30 min, the cells were incubated with the anti-HMGB1 antibody for 1 hour and then incubated with Cy5-conjugated secondary antibody for 30 min. The cells were then stained with DAPI for 20 min and observed using CLSM.

Extracellular release of ATP was tested using an ATP assay kit. B16F10 cells were seeded in six-well plates at a density of 1.5 × 10⁵ cells per well. After 12 hours of incubation in 37°C, DOX and DOX NPs were added into each well at the identical DOX concentration of 2 μM and incubated for 24 hours. Next day, the supernatants were collected and measured with an ATP assay kit according to the manufacturer’s instruction.

To demonstrate the DOX NPs could induce ICD in vivo via intravenous injection, C57BL/6 mice were injected by 1.0 × 10⁶ B16F10 cells at the right flank. When the tumor volume reached 200 mm³, mice were intravenously injected with DOX NPs at a DOX dose of 5 mg/kg. Treatment was given twice every 4 days. Twenty-four hours after the last treatment, mice were euthanized and the tumors were harvested and fixed to make paraffin section. CRT and HMGB1 in tumor tissues were visualized via a typical immunohistochemistry (IHC) procedure. Anti-CRT antibody and anti-HMGB1 antibody were used as primary antibodies, respectively.
Establishment of animal models for in vivo tumor inhibition experiments

Subcutaneous B16F10 melanoma model was established by injection of 1.0 × 10⁶ B16F10 cells in 100-μl PBS into the right dorsal flank of female C57BL/6 mice. Subcutaneous 4T1 or CT26 models for tumor inhibition were established by injection of 1.5 × 10⁴ 4T1 or CT26 cells into the right dorsal flank of female BALB/c mice. Subcutaneous 4T1 or CT26 models for PA imaging were established by injection of 2.0 × 10⁵ 4T1 or CT26 cells into the right dorsal flank of female BALB/c nude mice.

B16F10 tumor-bearing C57BL/6 mice, 4T1 tumor-bearing BALB/c mice, or CT26 tumor-bearing BALB/c mice were randomized distribute into different treatment groups, and the investigator was blinded to each group during the whole experiments. When the tumor volume reached 80 to 120 mm³, mice in different treatment groups were intravenously injected with PBS, DOX NPs, shPD-L1 NPs, DOX NPs + shPD-L1 NPs, Spam1 NPs, DOX NPs + Spam1 NPs, (shPD-L1 + Spam1) NPs, and DOX NPs + (shPD-L1 + Spam1) NPs (cocktail therapy group). We used PBS solution during injection to ensure a pH range around 7.4. The dosage for immunogenic chemotherapy was 5 mg/kg body weight on a DOX basis. The dosage for gene therapy was 1.2 mg/kg body weight on a total pDNA basis. The tumor volume and body weight were monitored every other day. Tumor volume was calculated by the formula: \( L \times S^2/2 \), where \( L \) referred to the longer diameter and \( S \) referred to the shorter diameter. PR represented tumors' larger diameter was reduced by more than 30% compared with their shorter diameter. Maturation DCs were marked as (CD11c + CD80 + MHC II + ) using F4/80 staining.

PA imaging experiment

To investigate whether Spam1 NPs could increase the accumulation and permeation of nanoparticles in tumor, PA imaging was carried out. Our previously reported FeT NPs, which composed of PLG-g-mPEG, ferric iron, and tannic acid, were used as contrast agent (41). For the in vivo PA imaging, FeT NPs (100 μl, 250 μg ml⁻¹) were administered into 4T1 or CT26 bearing BALB/c nude mice via intravenous injection. Mice received PBS or Spam1 NPs via intravenous injection 2 days before FeT NPs injection. The PA signals of tumors were continuously recorded by the MSOT InVision 128 system (iThera Medical, Germany) at different time points after injection.

Flow cytometry assay

After treatment, tumor tissues, TDLNs, and spleen were made into single-cell suspensions. Lymphocytes were quantitatively analyzed by flow cytometry (BD Canto II) after staining. Briefly, tissues were harvested and ground with 1-ml PBS in 12-well plates. The suspension was filtered through a 200-mesh sieve and then washed with PBS. Cells were collected and dispersed with 100 μl of PBS and stained by the addition of fluorescence-conjugated antibodies. Markers are as follows: CD3⁺ T cells (CD45⁺CD3⁺), CD8⁺ T cells (CD45⁺CD3⁺CD8⁺), and CD4⁺ T cells (CD45⁺CD3⁺CD4⁺CD8⁺). Another tube was stained to investigate M2 macrophages (CD11b⁺CD206⁺F4/80⁻) in tumor tissues. Maturation DCs were marked as (CD11c⁺CD80⁻MHC II⁺) in TDLNs. Cells in spleen were stained to investigate T lymphocyte subsets including CD4⁺ T cells, CD8⁺ T cells, and the TEM cells (CD3⁺CD8⁻CD4⁺CD62L⁻). Antibodies used in flow cytometry were listed in table S1.

Immunohistochemistry or immunofluorescence analyses

To estimate the safety and therapeutic effect of NPs, heart, liver, spleen, lung, kidney, and tumors were stored in 4% paraformaldehyde and paraffin sections were made. H&E and HE were used as dye for pathological analysis. For the IHC or immunofluorescence experiment, the already prepared paraffin sections were put in a 65°C drying oven for 1 hour, followed by deparaffination and rehydration in xylene and graded alcohols (100, 95, and 75%). Citrate was used to retrieve antigen epitopes via high-pressure thermal repair. Primary antibodies used in IHC or IF were listed in table S1.

RT-qPCR assay

Tumor tissues were grinded in liquid nitrogen treated mortar, and total RNA was extracted by TRizol according to the manufacturer’s instruction. The RNA was reversely transcribed to cDNA. RT-qPCR experiment was performed using SYBR Green qPCR Mix according to the manufacturer's instruction on Roche LightCycler 96 instrument. Primers sequences used in this study were listed in table S2.

Enzyme-linked immunosorbent assay

Different kinds of proteins in tumor tissues were measured by mouse ELISA kits for tumor tissue homogenate. Tumor tissues were chipped and homogenized in cold PBS with ice bath. After centrifugation, the supernatants were collected and measured according to the manufacturer’s instruction.

Statistics and data analysis

GraphPad Prism 8.0 software was used for constructing graphs and analyzing statistical significance. FlowJo v10 software was used for flow cytometry analyses. Student’s t test and log-rank (Mantel-Cox) test were used to calculate statistical significance.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/40/eabc7828/DC1

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

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