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Peng Zhang, Zhi Li, Weiling Cao, Jingjie Tang, Yi Xia, et al.. A PD-L1 Antibody-Conjugated PAMAM Dendrimer Nanosystem for Simultaneously Inhibiting Glycolysis and Promoting Immune Response in Fighting Breast Cancer. *Advanced Materials*, In press, <10.1002/adma.202305215>. <hal-04193228>

HAL Id: hal-04193228

<https://hal.science/hal-04193228v1>

Submitted on 1 Sep 2023

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A PD-L1 Antibody-Conjugated PAMAM Dendrimer Nanosystem for Simultaneously Inhibiting Glycolysis and Promoting Immune Response in Fighting Breast Cancer

Peng Zhang, Zhi Li, Weiling Cao, Jingjie Tang, Yi Xia, Ling Peng,* and Jing Ma*

Breast cancer is the most frequent malignancy affecting women, yet current therapeutic strategies remain ineffective for patients with late-stage or metastatic disease. Here an effective strategy is reported for treating metastatic breast cancer. Specifically, a self-assembling dendrimer nanosystem decorated with an antibody against programmed cell death ligand 1 (PD-L1) is established for delivering a small interfering RNA (siRNA) to target 3-phosphoinositide-dependent protein kinase-1 (PDK1), a kinase involved in cancer metabolism and metastasis. This nanosystem, named PPD, is designed to target PD-L1 for cancer-specific delivery of the siRNA to inhibit PDK1 and modulate cancer metabolism while promoting programmed cell death 1 (PD-1)/PD-L1 pathway-based immunotherapy. Indeed, PPD effectively generates simultaneous inhibition of PDK1-induced glycolysis and the PD-1/PD-L1 pathway-related immune response, leading to potent inhibition of tumor growth and metastasis without any notable toxicity in tumor-bearing mouse models. Collectively, these results highlight the potential use of PPD as an effective and safe tumor-targeting therapy for breast cancer. This study constitutes a successful proof of principle exploiting the intrinsic features of the tumor microenvironment and metabolism alongside a unique self-assembling dendrimer platform to achieve specific tumor targeting and siRNA-based gene silencing in combined and precision cancer therapy.

1. Introduction

Cancer is one of the leading causes of death worldwide and will likely become the first in 2060.^[1] Among the various types, breast cancer is the most frequent malignancy in women and the second most frequent cancer.^[2] The current therapeutic approach for treating breast cancer involves a multidisciplinary strategy comprising surgery, radiotherapy, chemotherapy, neoadjuvant therapy, and adjuvant therapy.^[3] While effective for early-stage patients, this strategy falls short in cases of late-stage disease and/or metastasis for which an efficacious treatment is thus urgently required.

Immunotherapy aiming to improve antitumor immune responses has recently shifted the paradigm for cancer treatment.^[4] Therapeutic agents capable of activating or boosting immune surveillance mechanisms to eliminate cancer cells constitute a powerful arsenal in cancer immunotherapy that can effectively treat or even eradicate cancer.^[5–7] Targeting programmed cell death 1 (PD-1) expressed


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DOI: 10.1002/adma.202305215

on the surface of T cells and the programmed cell death 1 ligand 1 (PD-L1) expressed on cancer cells using immune checkpoint molecules is an important strategy in this field.^[8] Specifically, monoclonal antibodies (mAbs) that target either PD-1 or PD-L1 can block the interaction between them, hence promoting T-cell activation and enabling T-cell-mediated tumor cell death.^[9] However, PD-1/PD-L1-based immunotherapy proved unsatisfactory in solid tumors due to the compact and hypoxic tumor microenvironment,^[10,11] thereby hindering its broad implementation in cancer treatment.^[10,11]

Metabolism alteration is another key strategy of regulating cancer pathogenesis and tumor development, particularly for solid tumors.^[12] Tumor proliferation, invasion, and metastasis have a relentless energy requirement because of the very high proliferation rate of cancer cells. Glycolysis, found particularly elevated in metastatic cancer, provides the most energy for cancer cells and can promote tumor invasion, metastasis, and immune escape.^[13,14] Therefore, downregulating glycolysis is a viable approach for treating metastatic cancer via dual antitumor and immune boosting effects. Specifically, 3-phosphoinositide-dependent protein kinase-1 (PDK1) regulates metabolic reprogramming and metastatic potential in metastatic breast cancer cells through early glycolytic shift.^[15,16] PDK1 is directly targeted by the oncoprotein hypoxia-inducible factor-1 α (HIF-1 α), found overexpressed in breast cancer cells and specimens, and of which the expression levels are closely associated with breast cancer patient survival rates.^[17,18] PDK1 thus represents a promising target in treating advanced and metastatic breast cancer.^[19] While no therapy is currently clinically available, several PDK1 inhibitors are in the preclinical or clinical development stage. Interestingly, a recent study has demonstrated that combination therapy trials of PDK1-targeted therapy and other agents have provided some benefits.^[20]

Considering all these findings, we reasoned that simultaneously inhibiting glycolysis by targeting PDK1 and inducing immune activation by blocking the PD-1/PD-L1 interaction could offer combined and potent antitumor and antimetastasis effects for treating advanced and metastatic breast cancer. Specifically, we aimed to inhibit glycolysis by targeting PDK1 using a small interfering RNA (siRNA; referred to as siPDK1 hereafter). RNA interference (RNAi)-based gene silencing can offer the unique advantage of knocking down a gene of interest with high efficiency and specificity.^[21] However, successful gene silencing first requires effective and safe delivery of the siRNA molecules, which are not only unstable due to their chemo- and enzyme ability^[22] but also challenging with regard to entry into cells due to their highly charged phosphate backbones.

Recently, we developed amphiphilic dendrimers as precision vectors for siRNA delivery by virtue of their well-defined dendritic structure and cooperative multivalence.^[23–28] These amphiphilic dendrimers also boast combined delivery advantages of both lipid and polymer vectors, the two most advanced vectors for siRNA delivery. Notably, cationic amphiphilic dendrimers strongly bind to negatively charged siRNAs, forming stable nanoparticles that protect the RNA molecules from degradation and prolong its half-life, leading to enhanced siRNA accumulation in tumor lesions via the enhanced permeation and retention (EPR) effect, also known as passive tumor targeting.^[23]

Furthermore, actively targeted delivery to cancer cells poses a formidable challenge for the clinical application of siRNA therapeutics in cancer treatment.^[22] In breast cancer, high PD-L1 expression levels have been associated with large tumor size, high grade, high proliferation, estrogen receptor-negative (ER⁻) status, and HER2-positive (HER2⁺) status, and are inversely correlated with survival rate in breast cancer.^[29] Considering both the high PD-L1 expression levels and capacity to induce immune escape, we wanted to target PD-L1 with a monoclonal antibody (named as PD-L1 mAb thereafter) for actively targeted delivery of siPDK1. This strategy aimed to simultaneously inhibit glycolysis and the PD-1/PD-L1 interaction to achieve both anticancer effects and immune activation for treating breast cancer.

In this study, we specifically designed and constructed a dendrimer nanosystem decorated with an anti-PD-L1 antibody for targeted delivery of siPDK1 with a view to treating aggressive and metastatic breast cancer (Figure 1a). The resulting assembled siPDK1-PD-L1 mAb-dendrimer complex (named as PPD) effectively protected siPDK1 from degradation, delivered siPDK1 into tumor cells, thereby allowing downregulation of PDK1 expression resulting in significant inhibition of cancer cell proliferation, adhesion, migration, and invasion without any notable toxicity. Compared with the nontargeted delivery system for siPDK1, the targeting system decorated with the PD-L1 antibody had much elevated cellular uptake and tumor accumulation, highlighting the effective targeting ability of PPD. Most importantly, PPD could successfully inhibit both PDK1-induced glycolysis and the PD-1/PD-L1 axis for an increased immune response, leading to effective and combined antitumor and antimetastasis effects in metastatic breast cancer. This study therefore offers a new and efficacious treatment option against recalcified and metastatic breast cancer.

2. Results

2.1. PPD Protected siPDK1 and Delivered It Specifically to Cancer Cells via Targeting PD-L1

We previously reported that the amphiphilic dendrimer AD (Figure 1b), which is composed of two hydrophobic chains and one hydrophilic poly(amidoamine) (PAMAM) dendron bearing eight primary amine terminals, is effective for siRNA delivery and potent gene silencing.^[26–28] We also described the amphiphilic dendrimer ADZ (Figure 1c) carrying four azido terminals for drug conjugation via click chemistry.^[30] In this study, we have demonstrated that the dendrimer ADZ was able to conjugate with the dibenzocyclooctyne (DBCO)-modified PD-L1 antibody via click chemistry. We therefore co-assembled AD and ADZ for AD-mediated siRNA delivery alongside ADZ-mediated click reaction with the DBCO-modified PD-L1 antibody (DBCO-PD-L1 mAb) via the accessible azido terminals aimed at targeting PD-L1 (Figure 1a). Specifically, siPDK1 was first added to the mixture of AD/ADZ (ratio 5:1) allowing electrostatic interaction between the negatively charged siPDK1 and the positively charged AD. Then, the DBCO-PD-L1 mAb was added to the mixture to couple with the azido terminals in ADZ via click reaction, yielding the targeted delivery system PPD (Figure 1a).

With the above constructed PPD in hand, we first assessed its ability to retain siPDK1 at different N/P ratios. The N/P

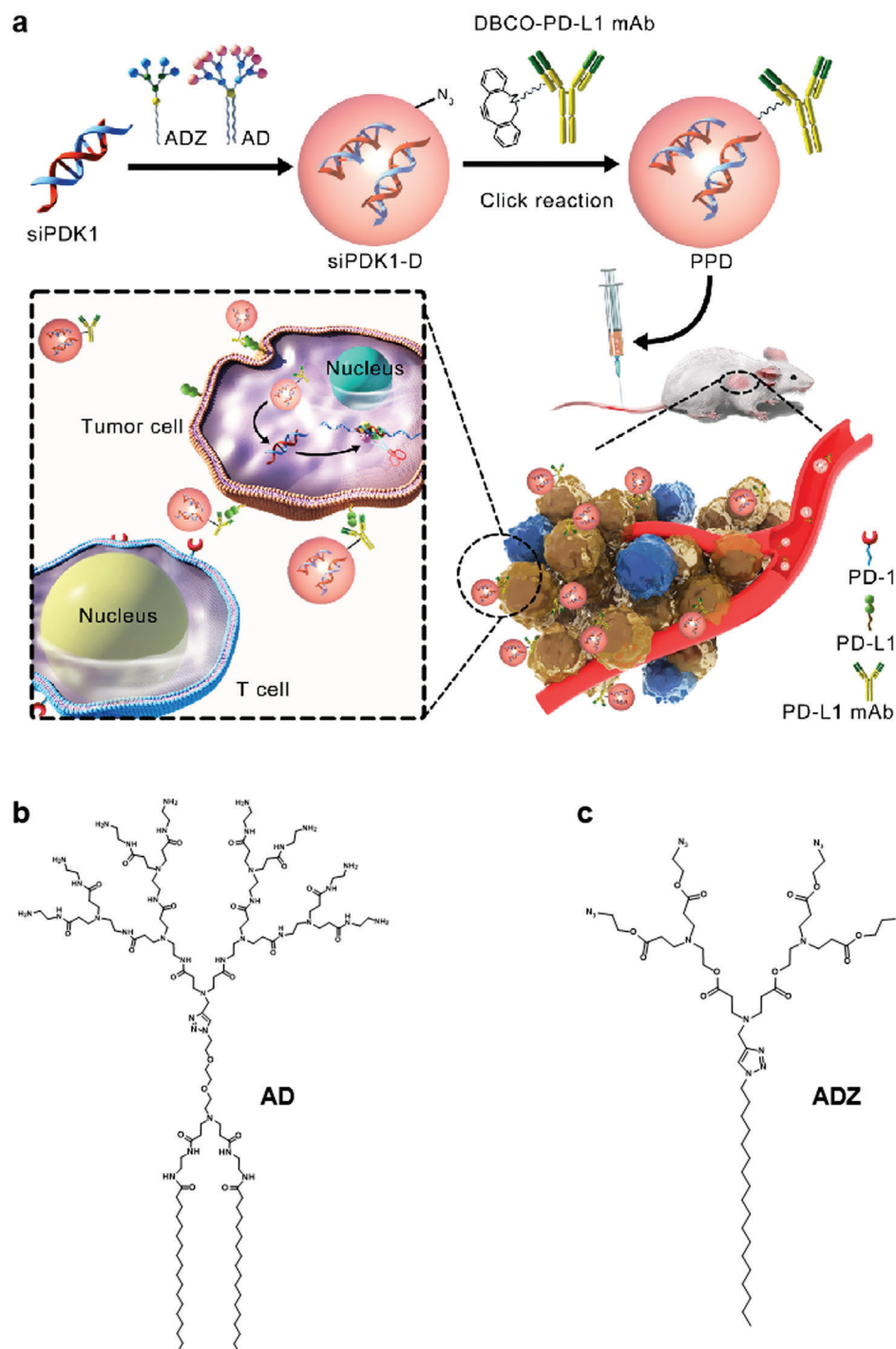


Figure 1. Schematic illustration of the construction of the siPDK1-PD-L1 mAb-Dendrimer complex (PPD). a) Schematic illustration of the construction of the PPD, and the targeted delivery to silence PDK1 for anticancer activity. b,c) Chemical structures of the amiphilic dendrimer AD bearing amine terminals for siRNA delivery (b) and the amiphilic dendrimer ADZ carrying azido terminals for click reaction to conjugate with the dibenzocyclooctyne (DBCO)-modified PD-L1 antibody (c).

ratio refers to the total number of amine terminals in AD divided by the total number of phosphate groups in siPDK1. As shown in the gel mobility shift assay results (Figure 2a), siPDK1 showed significantly reduced migration within PPD at an N/P ratio of 2 or above, highlighting the fully complex and stable

containment of siRNA molecules within PPD. Dynamic light scattering (DLS) analysis revealed that PPD at an N/P ratio of 2 had an average size of ≈ 90 nm (Figure 2b). Further transmission electronic microscopy (TEM) imaging allowing a visualization of PPD revealed as small, uniform, spherical nanoparticles

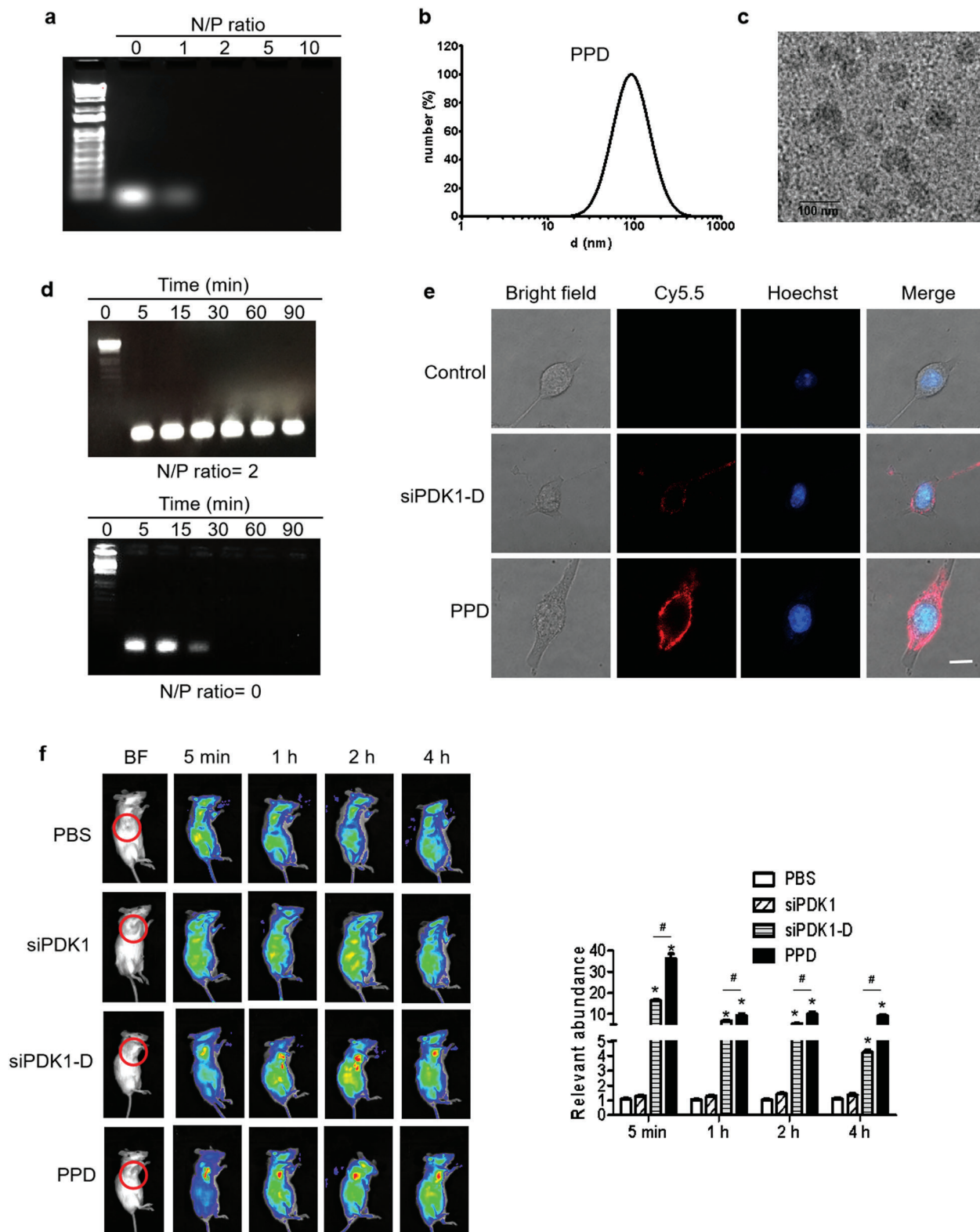


Figure 2. The siPDK1-PD-L1 mAb-dendrimer complex (PPD) is composed of small, uniform, spherical nanoparticles that can protect small interfering RNA (siRNA) from degradation, accumulate in tumor lesion, and deliver siRNA specifically to 4T1 breast cancer cells. a) The ability of PPD to bind with siPDK1 at different N/P ratios (0–10) was assessed using an agarose gel electrophoresis. Naked siPDK1 (N/P ratio = 0) served as a control. b) Dynamic light scattering (DLS) analysis and c) transmission electronic microscopy (TEM) imaging of PPD at an N/P ratio of 2. d) siPDK1 within (upper panel) and without (lower panel) PPD was incubated with 0.01 mg mL⁻¹ RNase A at room temperature for 0–90 min, then run on a 1.2% sodium dodecyl

(Figure 2c). Importantly, PPD protected siPDK1 from RNase A digestion (Figure 2d, upper panel), whereas naked siPDK1 was degraded rapidly within 5 min (Figure 2d, lower panel). Collectively, these results confirm the formation of a stable PPD able to encapsulate siPDK1 and protect it from degradation.

Considering the importance of targeted delivery to specific cells for siRNA therapeutics, we next assessed the targeting capacity of PPD by evaluating its cellular uptake in mouse breast cancer 4T1 cells, which have high expression levels of PD-L1.^[31] Using confocal microscopy with siRNA molecules labeled with a Cy5.5 fluorescent tag, we observed a strong fluorescent signal throughout the cytosol in cells treated with PPD (Figure 2e). However, there was only a weak fluorescent signal in the cytosol when cells were treated with siPDK1-D, the siPDK1/dendrimer nanosystem without PD-L1 mAb decoration. This finding illustrates that PD-L1 mAb decoration imparted PPD with the effective targeting ability, leading to significantly enhanced cellular uptake.

We further examined the *in vivo* biodistribution of PPD in mice bearing subcutaneous 4T1 tumors to verify if the PD-L1 mAb-decorated dendrimer nanosystem could indeed improve the targeting ability in tumors in a mouse model. As shown in Figure 2f, the Cy5.5 fluorescent signal accumulated at the tumor site 5 min after injection and was retained at a higher level in the PPD group compared with the siPDK1-D, siPDK1, and phosphate-buffered saline (PBS) groups. Additionally, the fluorescent signal was much weaker in tumors in the PBS and siPDK1 groups compared with the siPDK1-D group. Collectively, these results demonstrate the successful accumulation of PPD at the tumor site via both PD-L1/PD-L1 mAb-mediated active tumor targeting and nanoparticle-mediated passive tumor targeting.

2.2. PPD Decreased PDK1 Protein Expression Levels and Inhibited Cell Proliferation and Metastasis

Considering the elevated expression levels of PDK1 observed in breast cancer cells and tumors^[17] and the association between PDK1 overexpression and increased tumor growth,^[32] we next evaluated the ability of PPD to downregulate PDK1 expression and thereby inhibit cancer cell proliferation and metastasis using mouse breast cancer 4T1 cells. As shown in Figure 3a, PPD effectively decreased PDK1 expression levels in 4T1 cells in a dose-dependent manner, with $\approx 80\%$ gene knockdown achieved at a concentration of 20 nM siPDK1 and an N/P ratio of 2. In contrast, no PDK1 downregulation was observed using a nonspecific (NS) siRNA encapsulated within the PD-L1 mAb-decorated dendrimer nanosystem. These results indicate that siPDK1 indeed led to an effective and specific downregulation of PDK1.

Further cell proliferation evaluation using 3-(4,5)-dimethylthiazol-2-yl-5-(3,4-dimethyl-5-phenyl-1H-tetrazolium)methyl (MTT) assay revealed that compared with the control group, PPD was able to significantly inhibit 4T1 cell proliferation 96 h post-

treatment (Figure 3b), whereas no inhibition was observed in the HC11 mouse breast epithelial cells (Figure 3c), which have low PD-L1 expression levels and hence almost no response to PPD. The robust and specific antiproliferation effect observed specifically in cancer cells (4T1; high response to PPD), and not in normal cells (HC11; low response to PPD), highlights the effective targeting ability of PPD allowing specific and safe delivery of siPDK1 to cancer cells wherein its anticancer activity can be unleashed.

Additional experiments on the effects of PPD on cancer metastasis showed significant inhibition at key stages in breast cancer metastasis, including adhesion (Figure 3d), migration (Figure 3e), and invasion (Figure 3f), with $73.9 \pm 5.5\%$, $62.9 \pm 5.8\%$, and $72.9 \pm 10.4\%$ inhibition, respectively. In addition, the wound area of 4T1 cell monolayers showed less healing in the presence of PPD than in the absence of PPD (Figure 3g). Collectively, these results demonstrate that PDK1 is indeed a promising therapeutic target for fighting metastatic breast cancer, and that siPDK1 could be effectively and specifically delivered by PPD to breast cancer 4T1 cells to silence PDK1 expression and hence inhibit cancer cell proliferation and metastasis.

2.3. PPD Inhibited Glucose Uptake and ATP Generation, and Impeded Glycolysis

PDK1 is known as one of the key enzymes in glycolysis, and PDK1 overexpression correlates with excessive metabolic activation.^[33] Considering glucose uptake as the very first step of glycolysis, we therefore measured glucose uptake using the Glucose Uptake Fluorometric Assay Kit. As shown in Figure 3h, a significant reduction in glucose uptake ($46.6 \pm 4.7\%$ inhibition) was observed when 4T1 cells were treated with PPD (Figure 3h). This finding indicates the effective attenuation of metabolic activation following PDK1 silencing.

Additionally, PDK1, as the downstream target of HIF-1 α , can phosphorylate the pyruvate dehydrogenase (PDH) E1 α subunit and inactivate the PDH enzyme complex that otherwise converts pyruvate to acetyl-coenzyme A, hence resulting in inhibited pyruvate oxidation via the tricarboxylic acid cycle to generate energy.^[34] We therefore further assessed the total ATP generation. As shown in Figure 3i, following treatment with PPD, ATP generation was significantly decreased ($71.5 \pm 2.5\%$ inhibition) when compared with the control group. Collectively, these results demonstrate that PPD effectively inhibited glycolysis and ATP generation, supporting the potential of siPDK1 to alter cancer metabolism for anticancer activity.

2.4. PPD Inhibited Tumor Growth and Activated the Immune System *In Vivo*

Satisfied with the results supporting the ability of PPD to simultaneously target PD-L1 and inhibit PDK1-induced glycolysis, we

sulfate (SDS) agarose gel. e) Microscopic study on the cellular uptake of PPD labeled with Cy5.5 in 4T1 cells. 4T1 cells were treated with PBS control, siPDK1-D (no PD-L1 mAb decoration), or PPD, then imaged with bright-field or fluorescent microscopy. Scale bar = 10 μm . f) BALB/c mice bearing 4T1 tumors were intravenously injected with PBS, siPDK1 alone, siPDK1-D, or PPD using siRNA labeled with Cy5.5, and fluorescence images were taken at indicated time intervals. The red circles indicate the tumor location. Data are expressed as mean \pm standard deviation. * indicates $P < 0.05$ vs PBS group, # indicates $P < 0.01$ vs siPDK1-D group.

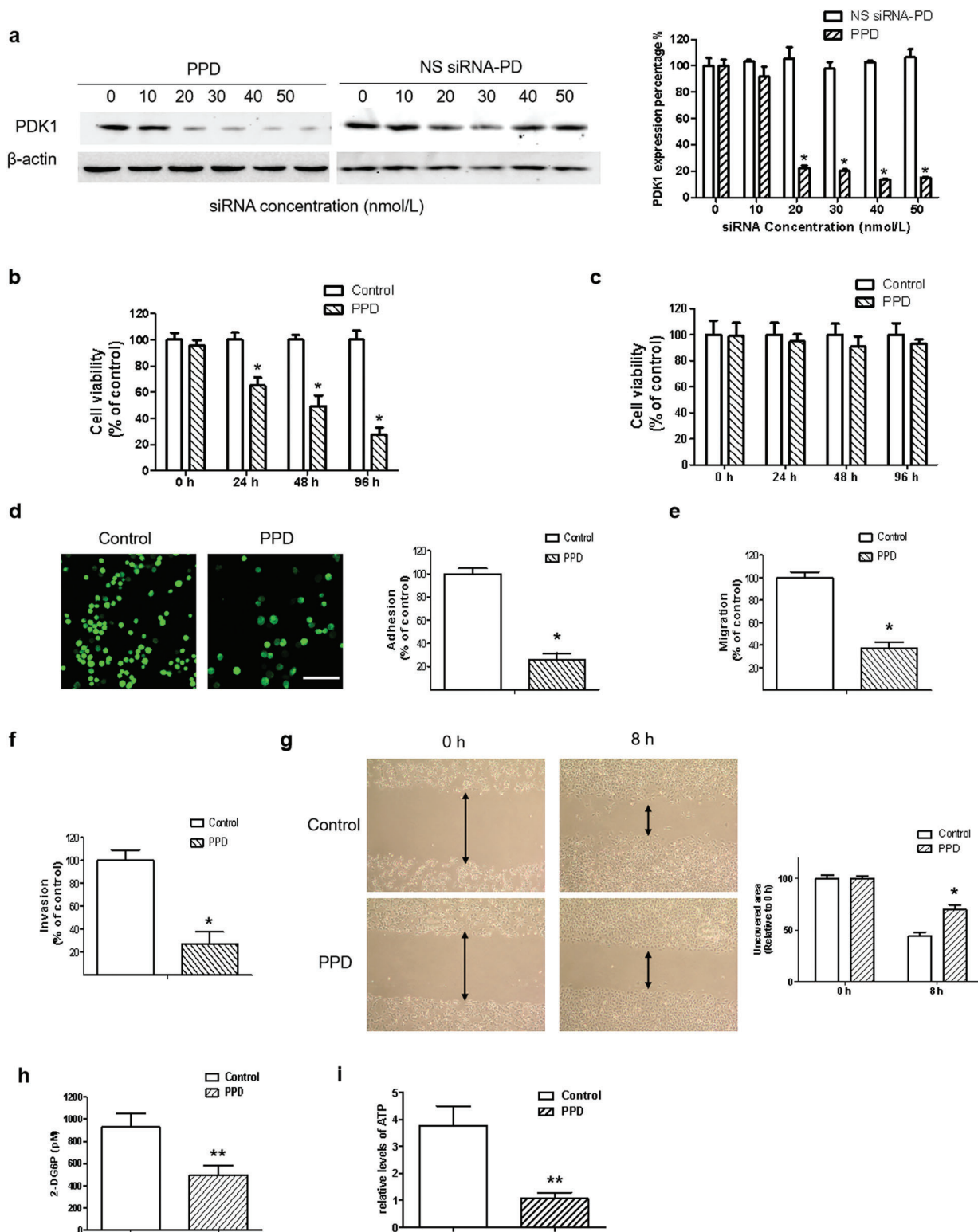


Figure 3. The siPDK1-PD-L1 mAb-dendrimer complex (PPD) can effectively reduce PDK1 expression levels and inhibit 4T1 breast cancer cell proliferation, adhesion, migration, and invasion rates. a) 4T1 cells were treated with PPD and nonspecific (NS) siRNA-PD. PDK1 protein expression levels were assessed using western blot analysis. β -actin served as the internal control. b,c) Proliferation of 4T1 cells (b) and HC11 cells (c) after treatment with PPD or NS siRNA-PD. Cell viability was evaluated using MTT assay for cell proliferation. d) PPD-treated 4T1 cells were plated onto a confluent HC11 cell monolayer and were allowed to adhere for 30 min. Adherent 4T1 cells were fixed and counted. Scale bar = 100 μ m. e,f) PPD-treated 4T1 cells were seeded in a noncoated (e) or Matrigel-coated (f) transwell filter for 24 h. After removing the cells in the upper chamber, the remaining cells were stained with crystal violet and counted. g) Confluent monolayers of 4T1 cells were wounded with a uniform scratch, washed to remove cell debris, and cultured

next evaluated its antitumor activity *in vivo* using a mouse model with subcutaneous 4T1 tumors. PPD significantly inhibited tumor growth compared with the PBS, siPDK1-D, and PD-L1 mAb groups (Figure 4a; Figure S2, Supporting Information). At the end of the experimental period, mice treated with PPD showed considerably reduced tumor mass compared with mice in the PBS, siPDK1-D, and PD-L1 mAb groups (Figure 4b). Although the groups treated with siPDK1-D and PD-L1 mAb showed inhibited tumor growth rates, the PPD-treated group showed a much greater reduction in tumor size and weight. In addition, PDK1 protein expression in the tumor tissues collected from mice treated with PPD was reduced compared to that in the other groups (Figure 4c). Altogether, these data demonstrate the successful delivery of siPDK1 via both active and passive tumor targeting, and siPDK1-mediated gene knockdown for effective anticancer activity.

Further immunohistochemistry (IHC) analysis (Figure 4d) confirmed decreased PDK1 protein levels accompanied by fewer Ki-67-positive cells, but many more caspase 3-positive cells, in the tumor tissues from mice treated with PPD compared with those in the PBS, siPDK1-D, and PD-L1 mAb groups (Figure 4d). These findings indicate that apoptosis induction is the mechanism behind the observed anticancer effects following PDK1 knockdown. Importantly, IHC analysis also revealed increased CD8 levels, demonstrating activation of the immune system (Figure 4d).

Notably, we observed no loss in body weight (Figure 4e) or any changes to liver and kidney function index levels (aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK) or blood urea nitrogen (BUN)) in mice in the PPD treatment group indicating no liver or kidney toxicity (Figure 4f–j). This was further supported by the hematoxylin and eosin (H&E) staining results of the major organs (heart, liver, kidney, lung, and spleen) isolated from mice following the treatment (Figure 4k). Collectively, these results demonstrate that PPD could successfully knock down PDK1 expression levels and activate the immune system, achieving significant inhibition of tumor growth without any acute toxicity.

2.5. PPD Inhibited Tumor Metastasis In Vivo

To further demonstrate the functional role of PPD in inhibiting breast cancer metastasis, we used a metastatic breast cancer mouse model. Specifically, BALB/c mice were intravenously injected with 1.0×10^5 4T1 cells for 10 days and then treated with PPD. When compared with the control group treated with PBS, PPD treatment considerably inhibited tumor metastasis (Figure 5a,b). Remarkably, the metastatic tumor number in mice treated with PPD was reduced to 4.0% relative to the control group (Figure 5c), demonstrating its effective inhibition of metastasis. Additionally, there was no obvious body weight loss compared with the control group (Figure 5d), further supporting the absence of any notable acute toxicity. Collectively, our results in-

dicate that PPD exhibited excellent potency against tumor metastasis, without any adverse effects.

3. Discussion

Breast cancer is the second most frequent cancer worldwide, and there is no efficacious treatment for advanced and metastatic breast cancer. The current therapeutic approach consists of surgery, radiotherapy, chemotherapy, neoadjuvant therapy, and adjuvant therapy, which is only effective for early-stage patients.^[3] In this study, we established a dendrimer nanosystem PPD decorated with PD-L1 mAb for specific delivery of siRNA to target PDK1 for antitumor and antimetastasis effects in breast cancer via inhibition of both glycolysis and the PD-1/PD-L1 pathway using the siRNA and anti-PD-L1 antibody simultaneously. Indeed, PPD effectively protected siPDK1 from degradation and supported its accumulation in tumor lesions for specific delivery of siPDK1 via both active and passive tumor targeting, leading to siRNA-mediated knockdown of PDK1 and significant inhibition of breast cancer cell proliferation and metastasis without notable toxicity. The potent antitumor and antimetastasis effects of PPD can be ascribed to the inhibition of PDK1-induced glycolysis and the PD-1/PD-L1 pathway-related immune response. The PPD nanosystem described and tested in this study, hence, constitutes a promising option for fighting breast cancer.

Metastasis is the leading cause of breast-cancer-related deaths,^[3] and PDK1 actively regulates metastatic potential and metabolic reprogramming in breast cancer.^[16,35] PDK1 depletion in breast cancer cells can inhibit metastasis and suppress tumorigenesis and lung colonization,^[36] whereas increased PDK1 expression levels were correlated with excessive metabolic activation.^[34] In addition, inactivation of PDK1 could reprogram the metabolism of tumor-infiltrating macrophages and stimulate M1 macrophage polarization, thus inhibiting tumor growth and suppressing tumor metastasis.^[37] Therefore, PDK1 is a promising target for treating advanced and metastatic breast cancer.^[38] While several PDK1 inhibitors for treating breast cancer are currently in the preclinical or clinical stage, no treatments are clinically available. In this study, we developed an siRNA (siPDK1) as an effective agent to target PDK1, which allowed significant downregulation of PDK1 expression levels in breast cancer and potent anticancer and antimetastasis effects.

Interestingly, siRNA-based therapeutic agents are widely expected to bring new hope for cancer therapy by specifically and effectively downregulating genes involved in malignancy, especially cancers which are otherwise difficult to target pharmacologically.^[21] Nevertheless, successful implementation of siRNA therapeutics requires safe and effective delivery systems^[22] to protect the siRNA from degradation and mask its highly negative charge to enable its cell membrane penetration and effective delivery into specific cancer cells. We have previously developed an amphiphilic dendrimer AD as a safe and

for the indicated times. Images of cell cultures were captured at 0 and 8 h after scratching; representative pictures are shown in the left panel. The arrow indicates the wound edge. The amount of wound repair was expressed as the uncovered area at the indicated time compared with initial uncovered area at time zero (right panel). h) Glucose uptake and i) ATP generation in 4T1 cells upon treatment with and without PPD. Glucose uptake and ATP generation were assessed using the Glucose Uptake Fluorometric Assay Kit and ATPlite Luminescence Assay System, 300 Assay Kit, respectively. Data are expressed as mean \pm standard deviation. * indicates $P < 0.05$ vs control group, ** indicates $P < 0.01$ vs control group.

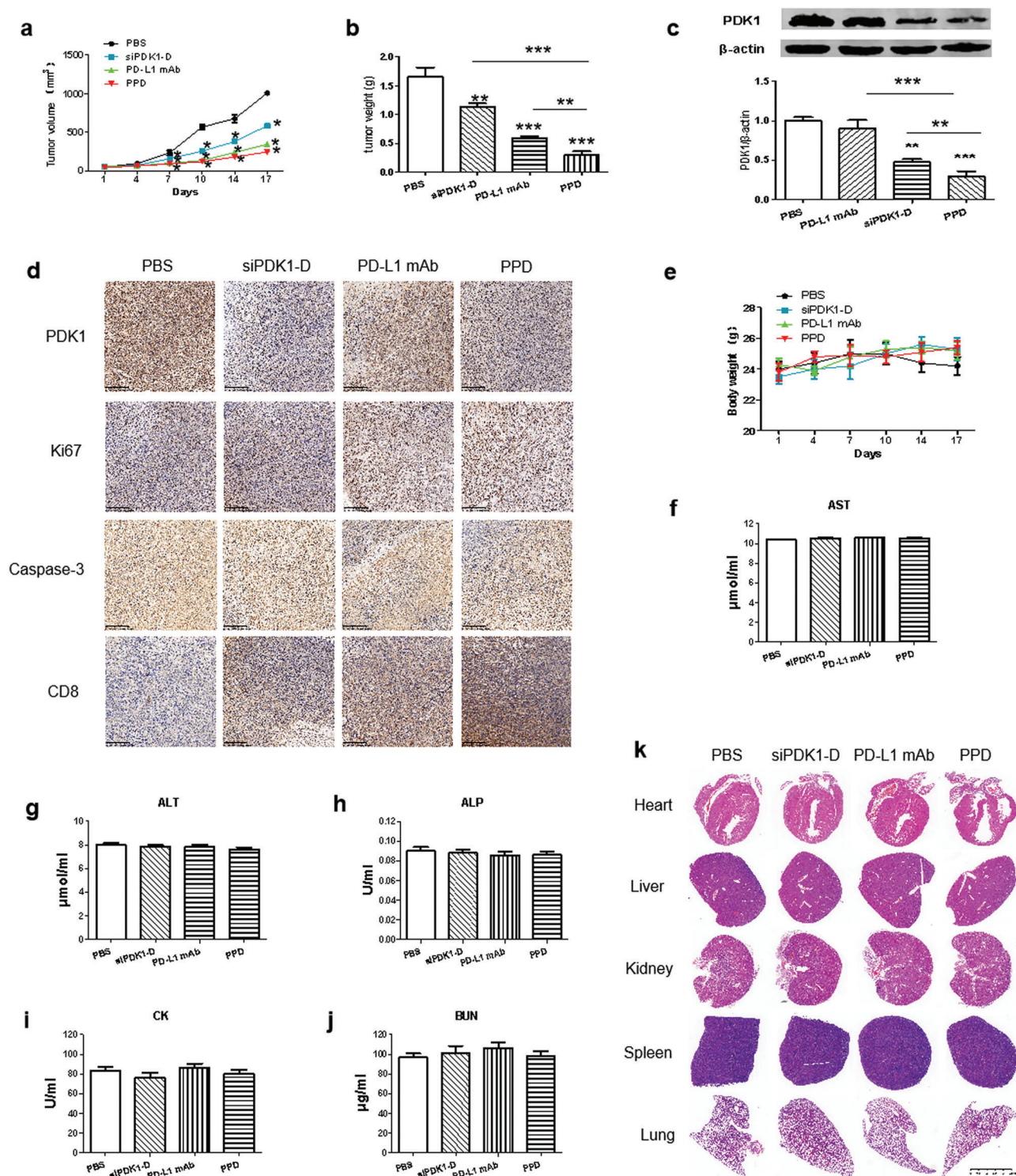


Figure 4. In vivo antitumor effects of the siPDK1-PD-L1 mAb-dendrimer complex (PPD). BALB/c mice bearing 4T1 tumors were intravenously injected with PBS, siPDK1-D, PD-L1 mAb, or PPD (siRNA: 1.0 mg kg⁻¹, PD-L1 mAb: 75 μg per mouse; two injections per week). a) Tumor volume and b) tumor weight data from the mice. c) Western blot data of PDK1 protein expression levels. β-actin served as the internal control. d) Immunohistochemistry images of the tumors for cell proliferation (Ki-67 staining), caspase-3 expression, PDK1 expression, and CD8 levels. Scale bar = 200 μm. e) Mouse body weight data over the whole treatment period of 17 days. f–j) Blood biochemistry analysis of aspartate aminotransferase (AST) (f), alanine transaminase (ALT) (g), alkaline phosphatase (ALP) (h), creatine kinase (CK) (i), and blood urea nitrogen (BUN) (j) levels. k) Hematoxylin and eosin (H&E) staining images of organs (heart, liver, lung, kidney, and spleen). Data are expressed as mean ± standard error of the mean. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$. All data were compared with the PBS group, unless otherwise indicated.

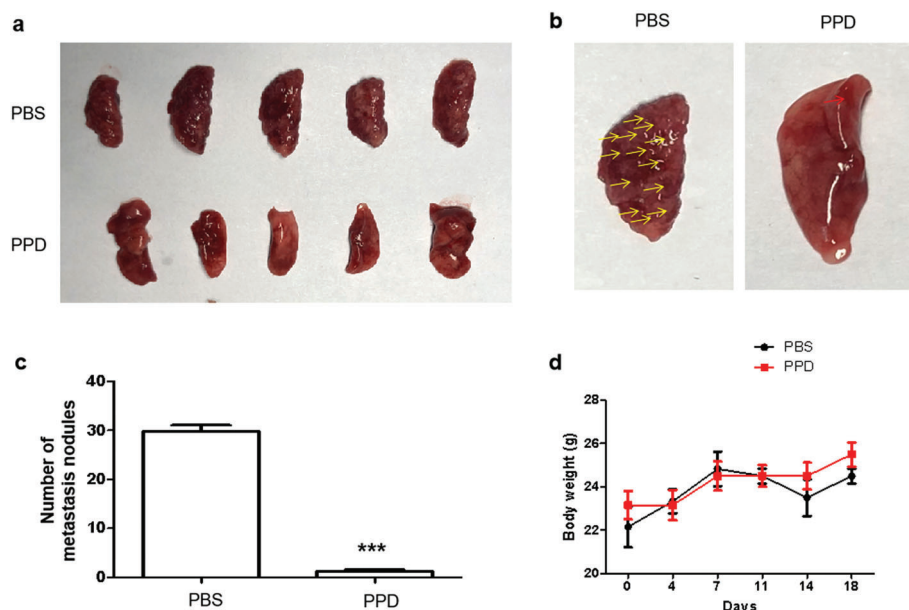


Figure 5. Antimetastasis effect of the siPDK1-PD-L1 mAb-dendrimer complex (PPD). BALB/c mice were intravenously injected with 1.0×10^5 4T1 cells for 10 days, then intravenously injected with PBS or PPD (siRNA: 1.0 mg kg^{-1} , PD-L1 mAb: $75 \mu\text{g}$ per mouse; two injections per week). a) Image of representative mouse lungs and b) zoomed-in representative images of mouse lungs following PBS and PPD treatment, respectively. The yellow arrows indicate metastatic tumors. c) Number of lung metastasis nodules in each group. d) Mouse body weight data. Data are expressed as mean \pm standard error of the mean. *** indicates $P < 0.001$ vs the PBS group.

effective vector for siRNA delivery and gene knockdown in various cells, including cancer cells, stem cells, and primary immune cells, as well as in animal models of different diseases.^[25–28] AD can complex siRNA molecules into small and uniform nanoparticles that protect them from degradation and promote cell uptake. In the present work, we employed AD for siPDK1 delivery.

In addition to AD, we also included another amphiphilic dendrimer ADZ that bears azido terminals for conjugation with the antibody of PD-L1 via click chemistry. Using this approach, we aimed to target PD-L1 on the breast cancer cells to achieve specific delivery of siPDK1 via active tumor targeting, as well as simultaneously inhibit the PD-1/PD-L1 interaction to promote the immune response. Elevated PD-L1 expression levels in breast cancer are often associated with large tumor size, high grade, and high proliferation rates, and are inversely correlated with breast cancer patient survival.^[30] In addition, PD-L1 increases tumorigenesis and invasiveness, and makes tumor cells less susceptible to specific CD8⁺ T cells.^[39] The PPD developed in this study contained an antibody specifically targeting PD-L1 and hence effectively delivered siPDK1 to breast cancer cells for PDK1 knockdown and glycolysis attenuation. In addition, it simultaneously inhibited PD-1/PD-L1-induced immune escape for treating breast cancer. Consequently, PPD showed potent inhibition of tumor growth and metastasis.

PD-1 plays a vital role in inhibiting immune responses and promoting self-tolerance through regulating T-cell activation. In normal tissues, PD-1/PD-L1 ligation plays important roles in maintaining homeostasis of the immune system and preventing autoimmunity during infection or inflammation. In the tumor microenvironment, however, PD-1/PD-L1 ligation provides an immune escape mechanism for tumor cells by turning off cy-

totoxic T cells.^[40] Blocking the PD-1/PD-L1 interaction with antibodies that target either PD-1 or PD-L1 enables T-cell-mediated tumor cell death. Respective antibody-induced immune checkpoint blockade has been demonstrated to trigger efficient anti-tumor responses not only in “immunogenic” tumors such as melanoma and renal cell carcinoma, but also in solid tumors, including lung, colorectal, ovarian, bladder, and breast cancers. However, the efficacy of PD-1 blockade in metastatic breast cancer is not very high.^[41,42] Therefore, effective and safe strategies that synergize PD-1 blockade with other treatments aiming to enhance antitumor and antimetastasis effects are urgently needed. The PPD established in this study simultaneously inhibited PD-1/PD-L1-induced immune escape and PDK1-induced glycolysis, leading to significantly reduced tumor growth and metastasis without notable side effects in an aggressive and metastatic breast cancer model. Therefore, PPD can be further developed as a promising option for breast cancer immunotherapy.

4. Conclusion

The PPD established in this study maximized on targeting PD-L1 for both specific siRNA delivery to inhibit PDK1-induced glycolysis and effective blockade of PD-1/PD-L1 axis to activate the immune system. This generated potent antitumor and antimetastasis effects in breast cancer, yet avoided notable toxicity. Therefore, this study offers a new and efficacious treatment option against recalcitrant and metastatic breast cancer. Furthermore, the PPD developed in this study can be applied to the targeted treatment of other cancer types, thus providing an alternative avenue to explore for the development of effective and safely targeted cancer therapies.

5. Experimental Section

In Vivo Animal Study: All the animal experiments were authorized by the Institutional Research Ethics Committee, the 8th Affiliated Hospital of Sun Yat-Sen University (2020-066-01). BALB/c mice (5–6 week old) were purchased from Guangdong Yaokang Biotechnology Co., Ltd. A full description of the materials and methods as well as all experimental details is provided in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos. 82003251 and 82102118), Shenzhen Excellent Scientific and Technological Innovation Talent Training Project (Doctoral Basic Research Launched, RCB20210706092256079), Sanming Project of Medicine in Shenzhen (Project No. SZSM201801060), the Ligue Nationale Contre le Cancer (LP), the French National Research Agency as part of the Era-Net EURONANOMED European Research projects “TABRAINFECC”, “NANOGLIO”, and “iNanoGUN”.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

P.Z. and Z.L. contributed equally to this work. P.Z. performed cellular experiments. Z.L. performed cellular experiments and animal experiments. W.C. conceived and supervised the project. J.T. performed dendrimer synthesis. Y.X. provided dendrimers, wrote, and edited the manuscript. L.P. provided dendrimers, wrote, and edited the manuscript, supervised the project. J.M. contributed in conceptualization and methodology, and performed the animal experiments, wrote the original draft, reviewed, and edited the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

dendrimers, glycolysis, immune response, PD-L1 antibody, siPDK1

Received: June 1, 2023

Revised: July 26, 2023

Published online:

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