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Anti-PD1 antibody and not anti-LAG-3 antibody improves the antitumor effect of photodynamic therapy for treating metastatic breast cancer

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Photodynamic therapy (PDT) has limited effects in treating metastatic breast cancer. Immune checkpoints can deplete the function of immune cells; however, the expression of immune checkpoints after PDT is unclear. This study investigates whether the limited efficacy of PDT is due to upregulated immune checkpoints and tries to combine the PDT and immune checkpoint inhibitor to observe the efficacy. A metastatic breast cancer model was treated by PDT mediated by hematoporphyrin derivatives (HpD-PDT). The anti-tumor effect of HpD-PDT was observed, as well as $CD4^+$T, $CD8^+$T and calreticulin (CRT) by immunohistochemistry and immunofluorescence. Immune checkpoints on T cells were analyzed by flow cytometry after HpD-PDT. When

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combining PDT with immune checkpoint inhibitors, the antitumor effect and immune effect were assessed. For HpD-PDT at 100 mW/cm^2 and 40, 60 and 80 J/cm^2, primary tumors were suppressed and CD4$^+$T, CD8$^+$T and CRT were elevated; however, distant tumors couldn't be inhibited and survival could not be prolonged. Immune checkpoints on T cells, especially PD1 and LAG-3 after HpD-PDT, were upregulated, which may explain the reason for the limited HpD-PDT effect. After PDT combined with anti-PD1 antibody, but not with anti-LAG-3 antibody, both the primary and distant tumors were significantly inhibited and the survival time was prolonged, additionally, CD4$^+$T, CD8$^+$T, IFN-γ^+CD4$^+$T and TNF-α^+CD4$^+$T cells were significantly increased compared with HpD-PDT. HpD-PDT could not combat metastatic breast cancer. PD1 and LAG-3 were upregulated after HpD-PDT. Anti-PD1 antibody, but not anti-LAG-3 antibody, could augment the antitumor effect of HpD-PDT for treating metastatic breast cancer.

Keywords: Photodynamic therapy; anti-PD1 antibody; anti-LAG-3 antibody; anti-tumor immune effects; metastatic breast cancer.

1. Introduction

Breast cancer is the most common malignant tumor and is the leading cause of tumor death in women.[1] The five-year survival rate of metastatic breast cancer is very low.[2] Photodynamic therapy (PDT) is a potentially anti-tumor treatment modality that utilizes the combination of a photosensitizer, light and oxygen to generate therapeutic cytotoxic molecules.[3] PDT is mainly used for tumors in the cavity and superficial lesions. PDT applications have been broadened to breast cancer and the breast area is easily accessible using the PDT equipment. Hematoporphyrin injection is a kind of hematoporphyrin derivative (HpD) and is currently the only photosensitizer approved by the China Food and Drug Administration for PDT of tumors.[4] HpD-PDT has been used for metastatic breast cancer in the clinic.

Tumors can be destroyed by PDT in three ways.[5] First, PDT directly destroys tumor cells, resulting in apoptosis, necrosis or autophagy. Second, PDT damages tumor blood vessels, blocking tumor blood and nutrient supply. Third, PDT causes an inflammatory response, eliciting anti-tumor immune effects.[6] Tumor antigens and damage-associated molecular patterns (DAMPs) can be exposed and released from dying tumor cells after PDT.[6–8] DAMPs are recognized by pattern receptors on immune cells and ultimately lead to the activation of the anti-tumor immune response.[9,10] DAMPs include calreticulin (CRT), heat shock protein 70 (HSP70), HSP90, etc. CRT is an important DAMP. The exposure of CRT on the surface of tumor cells facilitates their engulfment by dendritic cells (DCs), which leads to tumor antigen presentation and activating effector T cells, including CD4$^+$ and CD8$^+$T cells.[11,12] Activated T cells disseminate into systemic circulation through blood vessels and infiltrate into tumor tissue, which could help to eliminate primary and distant tumor cells. Antitumor immune effects can be induced by low doses of PDT,[13] but the immune effects induced by different energy densities of PDT at a power density of 100 mW/cm^2 that are commonly used in clinical practices are not clear.

Studies have reported that distant tumors can be suppressed after PDT treatment of primary tumors.[14,15] However, this phenomenon is only a case, not a rule.[16] There are many reasons to attenuate the PDT-induced immune effect, one of which is the elevated expression of immune checkpoint molecules.[16] Tumor treatment can cause immune checkpoints on immune cells to be elevated. Takaya *et al.*[17] found that the expression of PD1 on T cells was elevated after stomach cancer surgery. Davern *et al.*[18] reported that first-line chemotherapy regimens significantly altered the immune checkpoint expression profile of T cells, increasing the expression of PD-1, A2aR, KLRG-1, PD-L1, PD-L2 and CD160 and decreasing the expression of TIM-3 and LAG-3. The binding of immune checkpoint molecules and ligands can suppress the function of immune cells.[19] Therefore, PDT treatment may also alter the expression of immune checkpoints on T cells. However, the expression of immune checkpoints on T cells after PDT treatment is not clear.

In this study, a metastatic breast cancer model was established, and primary tumors were treated with HpD-PDT at a fixed power density of

$100\,mW/cm^2$ with different energy densities to observe the tumor suppressive effect and detect the immune effect, while the expression of immune checkpoint molecules on T cells was investigated after HpD-PDT. Additionally, the therapeutic effect of HpD-PDT combined with immune checkpoint inhibitors for metastatic breast cancer was also investigated.

2. Materials and Methods

2.1. *Materials*

HpD was purchased from Chongqing Huading Modern Biomedical Co., Ltd. Mouse monoclonal antibodies such as anti-CD4 (GB13064-2, Servicebio), anti-CD8 (GB13429, Servicebio) and goat anti-rabbit immunoglobulin G secondary antibodies (GB23303, Servicebio) that were applied for immunohistochemical staining. Mouse monoclonal anti-CRT (Abcam, Cambridge, UK) was used for immunofluorescence staining. PerCP-7-AAD, BV510-CD45, PE-Cy7-CD3, APC-Cy7-CD4, APC-CD8, FITC-PD-1, BV421-LAG-3, PE-TIM-3, BV421-CTLA-4, PE-TIGIT, PE-Cy7-IFN-γ and BV510-TNF-α were utilized for flow cytometry (Biolegend, San Diego, CA, USA). Anti-IgG1, anti-PD-1 antibody and anti-LAG-3 antibody were purchased from Bioxcell. The 630 nm laser was supported by Leimai Technology Co., Ltd (Shenzhen, China).

2.2. *Animals*

Balb/c female mice, 6–8 weeks old and approximately 20 g, were purchased from the First Medical Animal Center of PLA General Hospital. Metastatic breast cancer model: 1×10^6 4T1 cells were inoculated subcutaneously into the right flank of the mice and 3×10^5 4T1 cells were inoculated subcutaneously into the left flank of the mice. PDT treatment was performed 5–6 days after inoculation. Mice were housed and treated in compliance with the approval of the Animal Ethics Committee of The First Medical Animal Center of PLA General Hospital (approval 2019-X15-68).

2.3. *PDT administration*

Five or six days after tumor implantation, HpD (5 mg/kg) was administered intravenously via the tail vein. After 60–72 h, mice were anesthetized by inhaled isoflurane (Jiangsu Heng Feng Qiang Biotechnology Co. Ltd), and primary tumors were illuminated with 630 nm light delivered via a diode laser. During illumination, the normal skin around the primary tumor was covered with a cloth for protection. PDT was administered when the primary tumor volume was approximately $90\,mm^3$ and the distant tumor was approximately $40\,mm^3$.

2.4. *Assessment of tumor growth and survival*

Primary tumors and distant tumors were measured by Vernier calipers and the weights of mice were quantified. The survival time of the mice was recorded and the mice were euthanized by cervical dislocation when the tumor size reached $15\,mm^3$. Tumor volume was calculated by the formula: $V = lwh/2$. l represents the longest axis of the tumor, w represents the longest axis perpendicular to l and h represents the height of the tumor. When l reached 15 mm, mice were sacrificed by cervical dislocation.

2.5. *Immunohistochemistry*

After treatment, tumors were harvested and fixed in formalin for 48 h and then processed according to the immunohistochemistry protocol as described in the previous research.[20] The 4 μm section was stained with anti-CD4 (GB13064-2, 1:800; Servicebio), anti-CD8 (GB13429, 1:200; Servicebio) and anti-granzyme B (PA1-26616, Abcam, UK). The positive area of immune cells was analyzed in 12 random fields of view.

2.6. *Immunofluorescence analysis of CRT*

Standard immunofluorescence methods were described in a previous article.[20] Sections of tumor tissues were stained with anti-CRT (ab92516, 1:500; Abcam) antibody followed by incubation with a secondary anti-rat IgG (GB21303, 1:300; Servicebio). Images (×200) were captured using a Nikon microscope (Nikon Digital Eclipse C1 and Nikon DS-U3; Nikon, Tokyo, Japan). Nine fields of view (×200) were randomly collected and analyzed using ImageJ software. The ratio of the positive area of immunofluorescence images was statistically analyzed using the GraphPad Prism 8 software.

2.7. *Flow cytometry analyses*

The primary tumors were isolated from the metastatic breast cancer mouse model. The tumor tissues were cut into pieces with a volume of approximately $1\,mm^3$, and then tumor tissue dissociation enzyme was added and dissociated for 45 min. The dissociated cell suspension was filtered through a $30\,\mu m$ strainer to prepare a tumor tissue single-cell suspension. Tumor single cells were stained with 7-AAD, anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-PD1, anti-LAG-3, anti-TIM-3, anti-CTLA-4, anti-TIGIT, anti-IFN-γ and TNF-α for flow cytometry.

2.8. *Statistical analysis*

Repeated measures ANOVA was used to analyze the tumor growth and the survival time of mice was analyzed by log-rank analysis. A t test was used to compare the two groups. One-way ANOVA was applied to analyze the data between the groups. GraphPad Prism and SPSS were applied for statistical analyses. $P < 0.05$ was defined as the significant.

3. Results

3.1. *The antitumor effect induced by HpD-PDT at different energy densities*

The antitumor effects were triggered by HpD-PDT at a fixed power density of $100\,mW/cm^2$ with different energy densities of 40, 60, 80, 100, 120 and $140\,J/cm^2$ were investigated (Fig. 1). As shown in Fig. 1(a), a metastatic breast cancer model was established. The primary tumor was treated by HpD-PDT at different energy densities, while the distant tumor was served as the observation index without any treatment. HpD-PDT with different energy densities significantly delayed the primary tumor growth. Notably, with increasing energy density, the primary tumor suppressive effect tended to become strong and HpD-PDT at $140\,J/cm^2$ had the greatest suppressive effect (Fig. 1(b)). However, the distant tumor growth was not inhibited, and the survival time was not prolonged after HpD-PDT at the light dose used in this work (Figs. 1(c) and 1(d)).

The immune effect on the seventh day after HpD-PDT with different energy densities was evaluated. The $CD4^+T$ and $CD8^+T$ cells in the primary tumor were stained with immunohistochemistry (Figs. 1(e)–1(h)). The percentages of $CD4^+T$ and $CD8^+T$ cells were significantly increased after HpD-PDT at energy densities of 40, 60 and $80\,J/cm^2$ ($P < 0.05$); however, those immune cells were not significantly increased after HpD-PDT at energy densities of 100, 120 and $140\,J/cm^2$.

To further investigate DAMP, which is related to the activation of immune effects, CRT was detected in the primary tumor. Figures 1(i) and 1(j) show that the expression of CRT increased after HpD-PDT at energy densities of 40, 60 and $80\,J/cm^2$. The CRT significantly increased after HpD-PDT at an energy density of $80\,J/cm^2$ ($P < 0.05$). These results indicated that the primary tumor could be inhibited and that the antitumor immune response could be induced after HpD-PDT at energy densities of 40, 60 and $80\,J/cm^2$. However, the distant tumor could not be inhibited.

3.2. *Immune response activated by HpD-PDT at different times*

The immune response activated by PDT treatment in tumor tissues is time-dependent.[21] To investigate the immune response at different times induced by HpD-PDT, we analyzed the $CD4^+T$ and $CD8^+T$ cells in primary tumors at different times after HpD-PDT (energy density: $80\,J/cm^2$, power density: $100\,mW/cm^2$) (Fig. 2). Figure 2(a) shows that the primary tumors were isolated at days 1, 3, 7, 10, 14 and 18 after HpD-PDT. Figures 2(d) and 2(e) show that the number of $CD4^+T$ and $CD8^+T$ cells first increased and then decreased after HpD-PDT. The numbers of $CD4^+T$ and $CD8^+T$ cells reached a maximum on day 10. On day 10, the numbers of $CD4^+T$ cells and $CD8^+T$ cells in the HpD-PDT group were 2.9 times ($P < 0.0001$) and 3.4 times ($P < 0.0001$) higher than those in the control group, respectively.

3.3. *The immune checkpoint molecules on T cells in primary tumors on day 10 after HpD-PDT*

After PDT treatment, the immune response in primary tumors could be induced, but distant tumors could not be inhibited and survival could not be prolonged. To explore the reason for the limited immune effect induced by PDT, the expression of

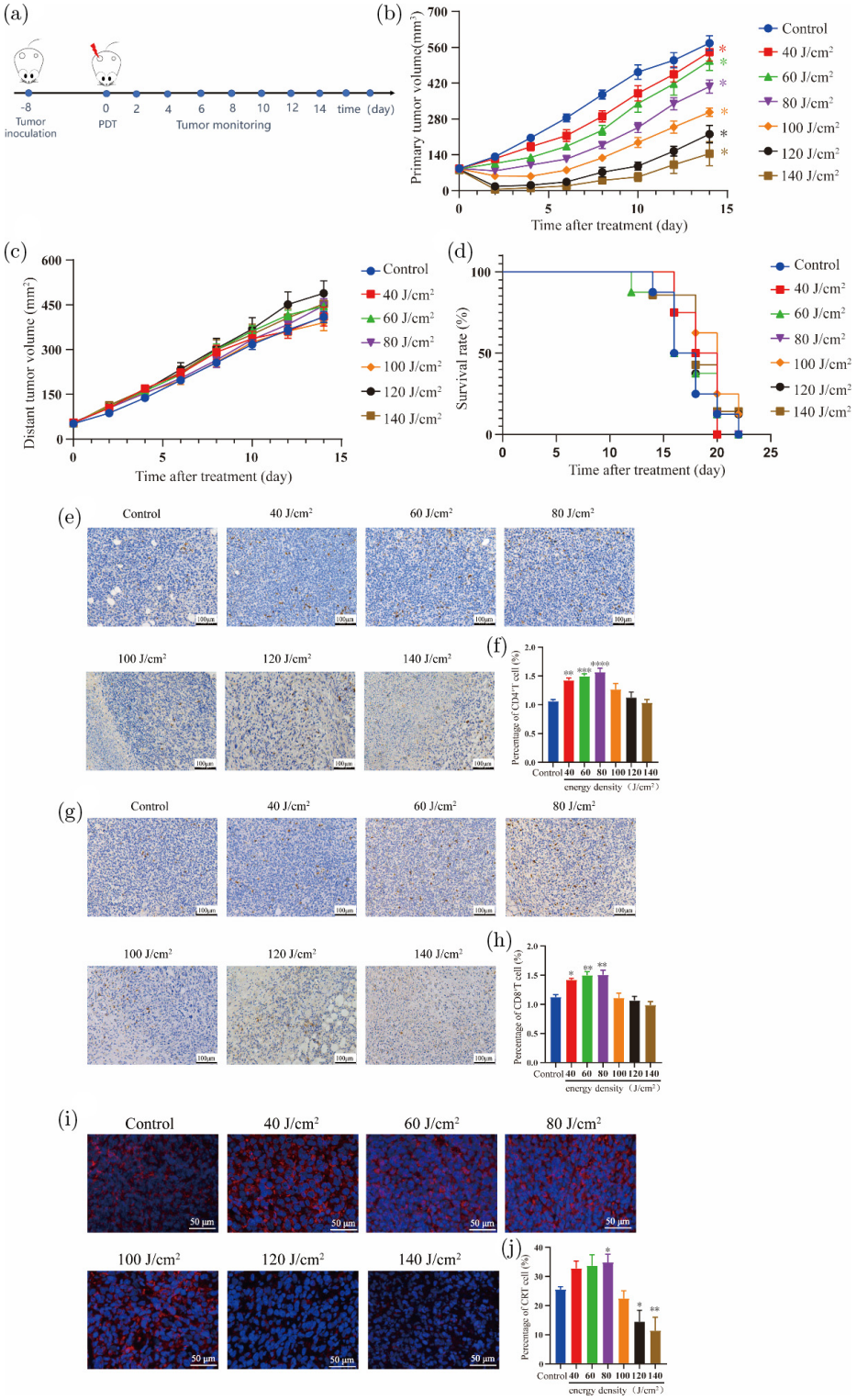
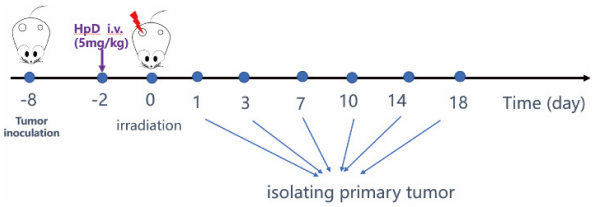
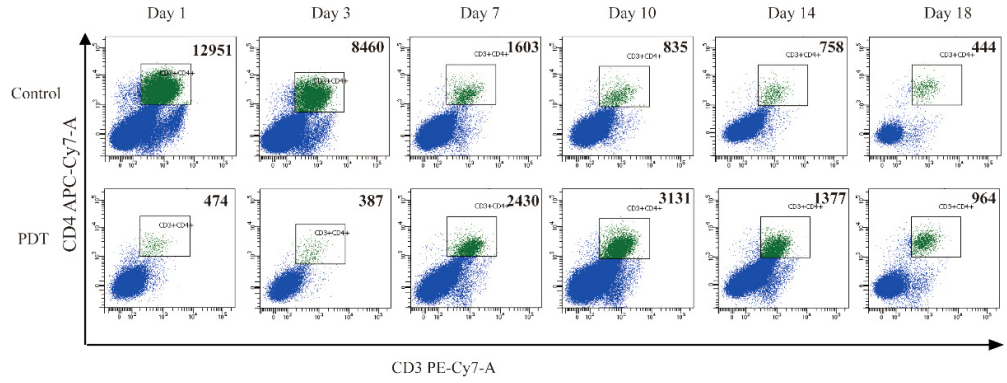


Fig. 1. Antitumor effect of HpD-PDT with different energy densities. (a) Flow chart of PDT treatment of mice bearing metastatic tumors. (b) Primary tumor volume after HpD-PDT. (c) Distant tumor volume after HpD-PDT. (d) Survival time of mice bearing metastatic tumors after HpD-PDT. (e) Representative immunohistochemical profile of $CD4^+$T cells. (f) Pooled data of $CD4^+$T cells in primary tumors. (g) Representative immunohistochemical profile of $CD8^+$T cells. (h) Pooled data of $CD8^+$T cells in primary tumors. (i) Representative immunofluorescence staining of CRT in primary tumors. (j) Pooled data of CRT in primary tumor. Dates are representative of 6–8 animals per group. Error bars represent standard error of measurement (SEM). (Compared with the control group, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).



(a)



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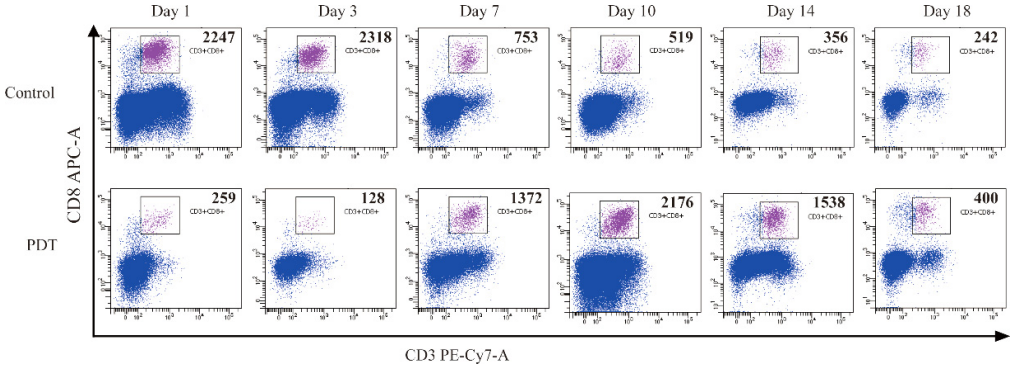

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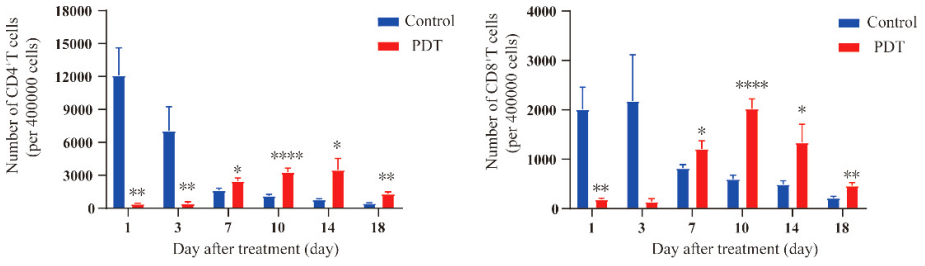

(d) (e)

Fig. 2. The immune effect induced by HpD-PDT at different times after HpD-PDT. (a) Flow chart of HpD-PDT treatment and immune effect detected in primary tumors isolated at days 1, 3, 7, 10, 14 and 18 after HpD-PDT. (b) Representative flow cytometry profile of $CD4^+$T cells. (c) Representative flow cytometry profile of $CD8^+$T cells. (d) Quantification data of $CD4^+$T cells. (e) Quantification data of $CD8^+$T cells. Dates are representative of 6–8 animals per group. Error bars represent the SEM. $^*P < 0.05$ versus control, $^{**}P < 0.01$ versus control and $^{****}P < 0.0001$ versus control.

immune checkpoints on T cells after HpD-PDT (energy density: 80 J/cm^2, power density: 100 mW/cm^2) on day 10 was investigated. As shown in Fig. 3, the immune checkpoint molecules PD-1, LAG-3, TIM-3, CTLA-4 and TIGIT were upregulated after HpD-PDT (Figs. 3(c) and 3(d)). It is important to note that the expression level of PD-1 on CD4$^+$T cells and CD8$^+$T cells was the highest, followed by the expression of LAG-3. PD-1 on tumor-infiltrating CD4$^+$T cells in the HpD-PDT group was 3.44 times higher than that in the control group ($P < 0.05$) and LAG-3 on tumor-infiltrating CD4$^+$T cells in the HpD-PDT group was 2.73 times higher than that in the control group ($P < 0.05$). PD1 on tumor-infiltrating CD8$^+$T cells in the HpD-PDT group was 4.41 times higher than that in the control group ($P < 0.05$) and LAG-3 on tumor-infiltrating CD8$^+$T cells in the HpD-PDT group was

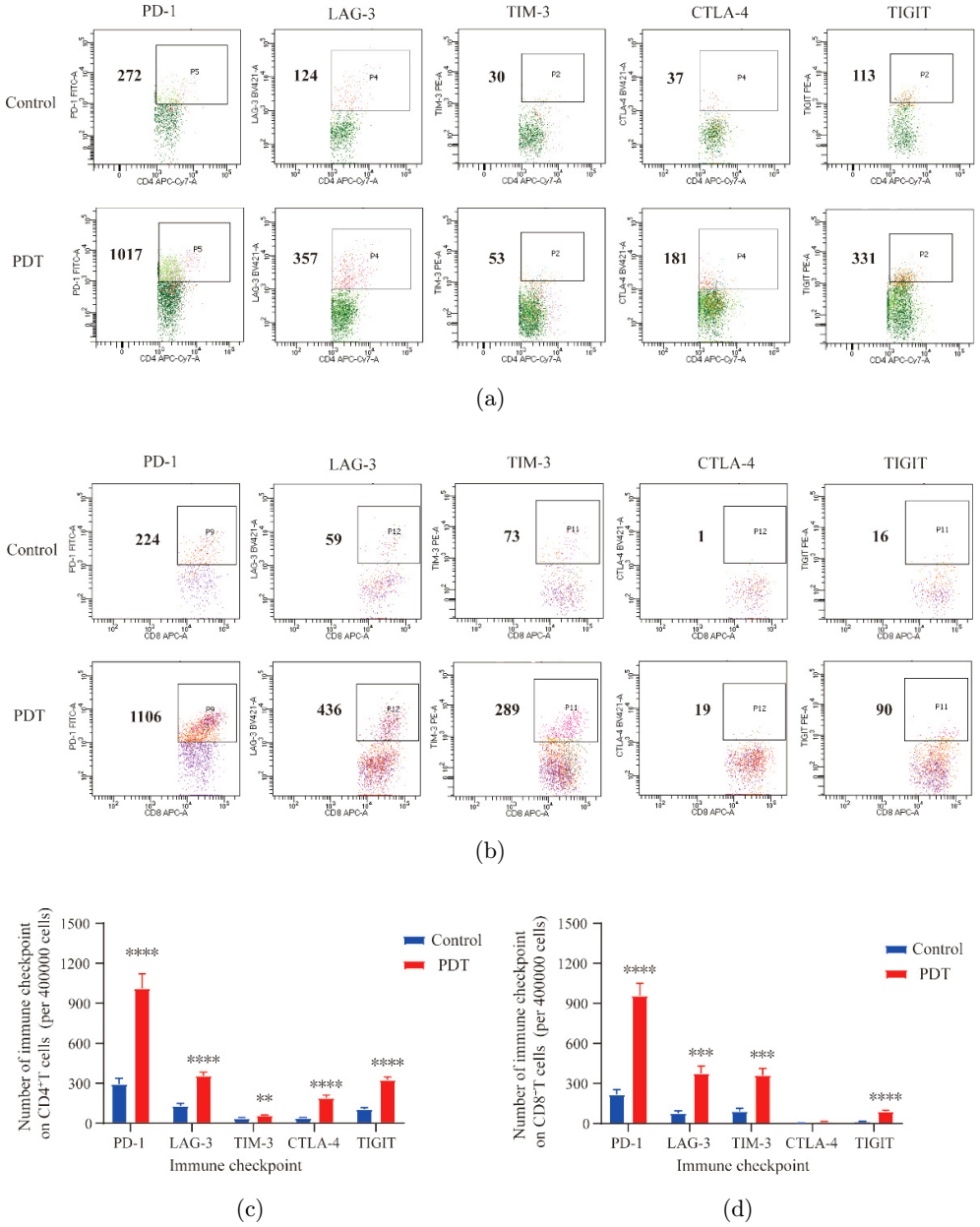

Fig. 3. The immune checkpoint molecules on T cells in primary tumors at day 10 after HpD-PDT. (a) Representative flow cytometry profile of immune checkpoint molecules on CD4$^+$T cells. (b) Representative flow cytometry profile of immune checkpoint molecules on CD8$^+$T cells. (c) Statistical plots of the number of immune checkpoint molecules positive CD4$^+$T cells. (d) Statistical plots of the number of immune checkpoint molecules on positive CD8$^+$T cells. Dates are representative of eight animals per group. Error bars represent the SEM. $^{**}P < 0.01$ versus control, $^{***}P < 0.01$ versus control and $^{****}P < 0.01$ versus control.

4.8 times higher than that in the control group ($P < 0.05$).

3.4. *Antitumor effect induced by HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody*

To augment the antitumor immune effect of HpD-PDT, PDT combined with anti-PD1 antibody or anti-LAG-3 antibody were used to treat mice with metastatic breast cancer. Anti-PD1 antibody or anti-LAG-3 antibody was injected intraperitoneally on days 1, 3, 5, 7 and 9 after HpD-PDT treatment, as shown in Fig. 4(a). The HpD-PDT combined with anti-PD1 antibody group and HpD-PDT combined with anti-LAG-3 antibody group exhibited significant effects on tumor growth compared with the anti-PD1 antibody, anti-LAG-3 antibody, IgG1 (isotype control) and control groups. It is worth noting that HpD-PDT combined with anti-PD1 antibody showed a significant effect on tumor growth compared with that in the HpD-PDT group ($P < 0.05$); however, HpD-PDT combined with anti-LAG-3 antibody did not significantly delay the tumor growth compared with that in the HpD-PDT group ($P > 0.05$) (Fig. 4(b)). Importantly, HpD-PDT combined with anti-PD1 antibody, but not with anti-LAG-3 antibody, significantly inhibited distant tumors and prolonged the survival of mice ($P < 0.05$) (Figs. 4(c) and 4(d)). These results suggested that HpD-PDT combined with anti-PD1 antibody had better antitumor effect than HpD-PDT combined with anti-LAG-3 antibody for treating metastatic breast cancer. There was no significant change in the body weight of mice after treatment with each treatment regimen ($P > 0.05$) (Fig. 4(e)), indicating that there were no noticeable systemic toxic side effects induced by the combination treatment regimen.

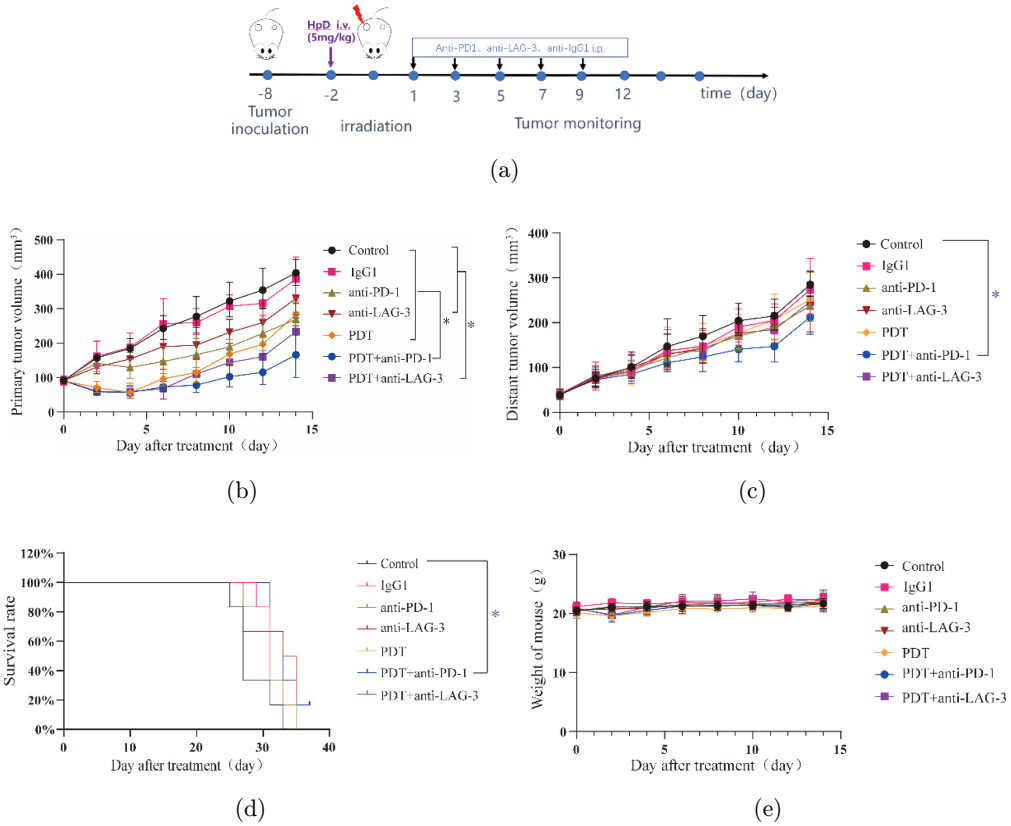

Fig. 4. Antitumor effect of PDT combined with anti-PD1 antibody or anti-LAG-3 antibody. (a) Flow chart of HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody. (b) Primary tumor volume after HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody. (c) Distant tumor volume after HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody. (d) Survival time of mice bearing metastatic tumors after HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody. (e) Weight of mice after HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody. Data are representative of six animals per group. $^*P < 0.05$.

3.5. *Immune effect induced by HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody*

On the 12th day after treatment, the immune effects induced by different treatment regimens were analyzed. The number of $CD4^+T$ and $CD8^+T$ cells in the primary tumor was detected by flow cytometry and the results are shown in Fig. 5. The number of $CD4^+T$ cells in the HpD-PDT combined with anti-PD1 antibody group and the HpD-PDT combined with anti-LAG-3 antibody group was significantly ($P < 0.05$) increased compared with that in the control group, the IgG1 group, the anti-PD1 antibody group and the anti-LAG-3 antibody group. It is critical to note that the number of $CD4^+T$ cells in the HpD-PDT combined with anti-PD1 antibody group was significantly increased compared with that in the HpD-PDT group ($P < 0.05$); however, there was no significant difference in the HpD-PDT combined with anti-LAG-3 antibody group compared with that in the anti-HpD-PDT group ($P > 0.05$).

The number of $CD8^+T$ cells in the HpD-PDT combined with anti-PD1 antibody group significantly increased compared with that in the HpD-PDT group, anti-PD1 antibody group, anti-LAG-3 antibody group, IgG1 group and control group ($P < 0.05$). However, the number of $CD8^+T$ cells in the HpD-PDT combined with anti-LAG-3 antibody group was elevated compared with that in the control group, IgG1 group, anti-PD1 antibody group, anti-LAG-3 antibody group and HpD-PDT group, but there was no significant difference.

The ratio of IFN-γ and TNF-α was detected by flow cytometry (Fig. 6). The proportion of IFN-γ^+ $CD4^+T$ cells significantly increased in the HpD-PDT combined with anti-PD1 antibody group compared with that in the HpD-PDT group, anti-PD1 antibody group, anti-LAG-3 antibody group, IgG1 group and control group ($P < 0.05$); however, the proportion of IFN-γ^+ $CD4^+T$ cells did not significantly increase in the HpD-PDT combined with anti-LAG-3 antibody group compared with the other groups (Fig. 6(b)).

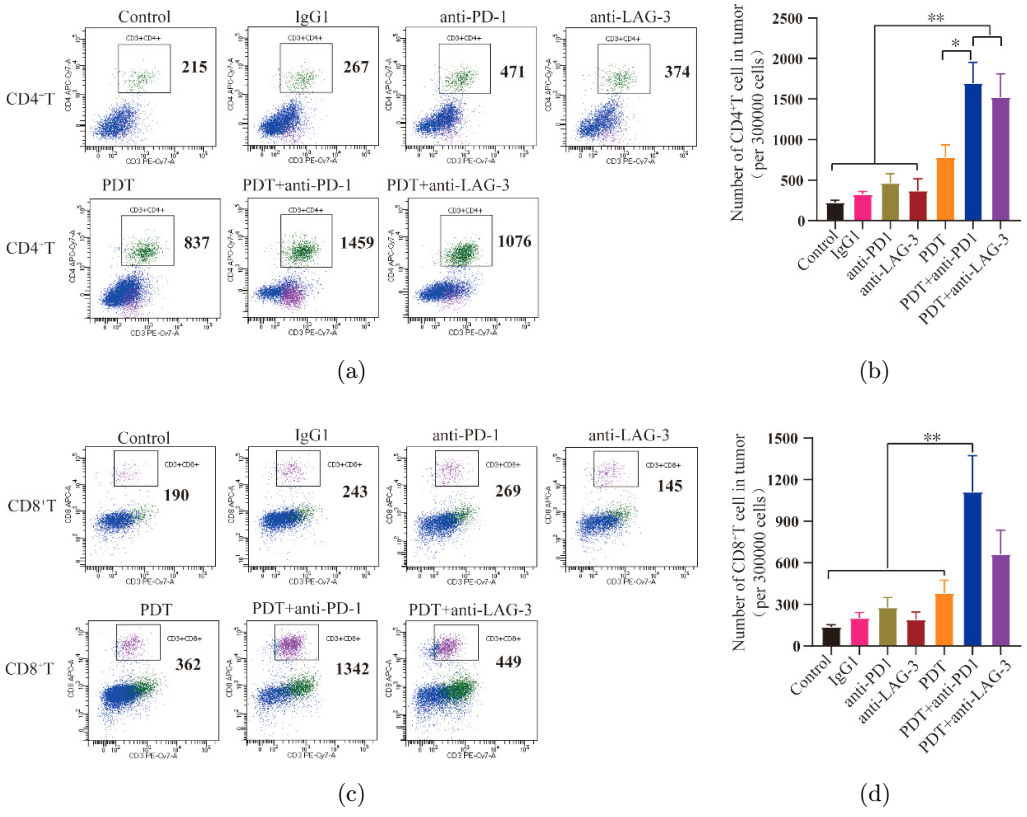

Fig. 5. Number of $CD4^+T$ and $CD8^+T$ cells in primary tumors induced after HpD-PDT combined with anti-PD1 or anti-LAG-3 antibody. (a) Representative flow cytometry profile of $CD4^+T$ cells in primary tumors. (b) Statistical plots of $CD4^+T$ cells in primary tumors. (c) Representative flow cytometry profile of $CD8^+T$ cells in primary tumors. (d) Statistical plots of $CD8^+T$ cells in primary tumors. Dates are representative of six animals per group. Error bars represent the SEM. $^*P < 0.05$ and $^{**}P < 0.01$.

As shown in Fig. 6(d), the proportion of TNF-α^+ CD4$^+$T cells in the HpD-PDT combined with anti-PD1 antibody group significantly increased compared with that in the HpD-PDT combined with anti-LAG-3 group, HpD-PDT group, anti-PD1 antibody group, anti-LAG-3 antibody group, IgG1 group and control group ($P < 0.05$). However, the proportion of TNF-α^+ CD4$^+$T cells in the HpD-PDT combined with anti-LAG-3 antibody group does not noticeably increased when compared with that in the other groups ($P > 0.05$).

The proportions of IFN-γ^+ CD8$^+$T cells and TNF-α^+ CD8$^+$T cells in the HpD-PDT combined with anti-PD1 antibody group significantly

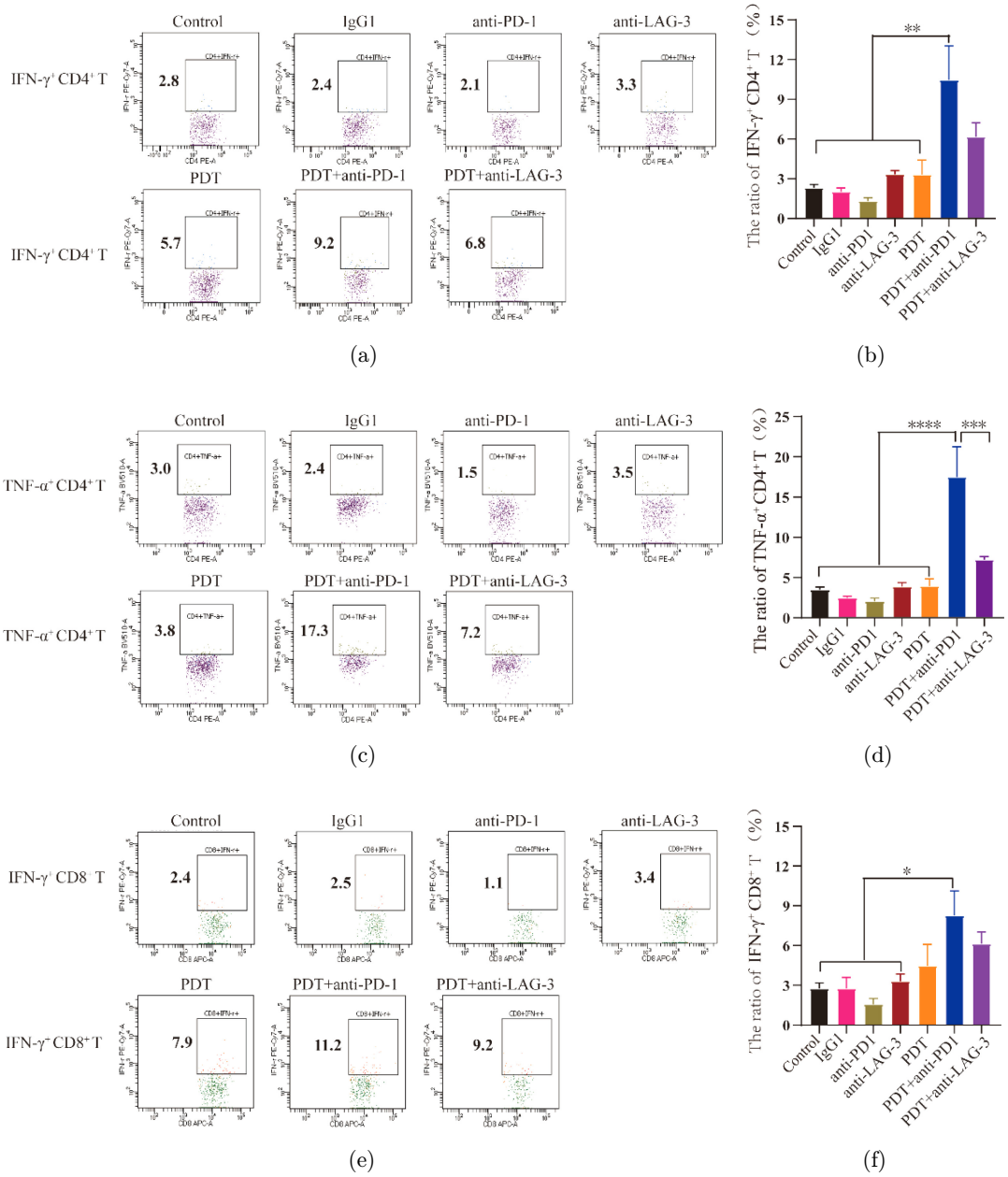

Fig. 6. Cytokines IFN-γ and TNF-α are induced by HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody in primary tumors. (a) Representative flow cytometry profile of IFN-γ^+ CD4$^+$T cells. (b) Statistical plots of IFN-γ^+ CD4$^+$T cells. (c) Representative flow cytometry profile of TNF-α^+ CD4$^+$T cells. (d) Statistical plots of TNF-α^+ CD4$^+$T cells. (e) Representative flow cytometry profile of IFN-γ^+ CD8$^+$T cells. (f) Statistical plots of T IFN-γ^+ CD8$^+$T cells. (g) Representative flow cytometry profile of TNF-α^+ CD8$^+$T cells. (h) Statistical plots of TNF-α^+ CD8$^+$T cells. Dates are representative of six animals per group. Error bars represent the SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

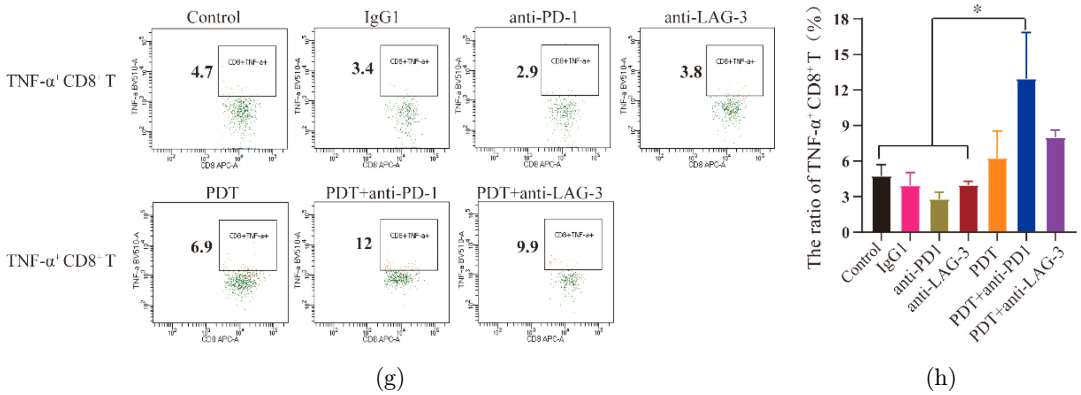

Fig. 6. (*Continued*)

increased when compared with those in the anti-PD1 antibody group, anti-LAG-3 antibody group, IgG1 group and control group ($P < 0.05$). The IFN-γ^+ CD8$^+$T cells and TNF-α^+ CD8$^+$T cells in the HpD-PDT combined with anti-PD1 antibody group were 1.85-fold and 2.07-fold higher ($P > 0.05$) than those in the HpD-PDT group, respectively (Figs. 6(f) and 6(h)). However, the proportions of IFN-γ^+ CD8$^+$T cells and TNF-α^+ CD8$^+$T cells in the HpD-PDT combined with anti-LAG-3 antibody group weren't significantly different from those in the other groups (Figs. 6(f) and 6(h)). These results suggested that HpD-PDT combined with anti-PD1 antibody could improve the immune effect of HpD-PDT, and HpD-PDT combined with anti-PD1 antibody has better efficacy than HpD-PDT combined with anti-LAG-3 antibody for treating metastatic breast cancer.

To explore the reason why the therapeutic effect of HpD-PDT combined with anti-LAG-3 antibody was less effective than that of HpD-PDT combined with anti-PD1 antibody, we further investigated the expression of immune checkpoint molecules on T

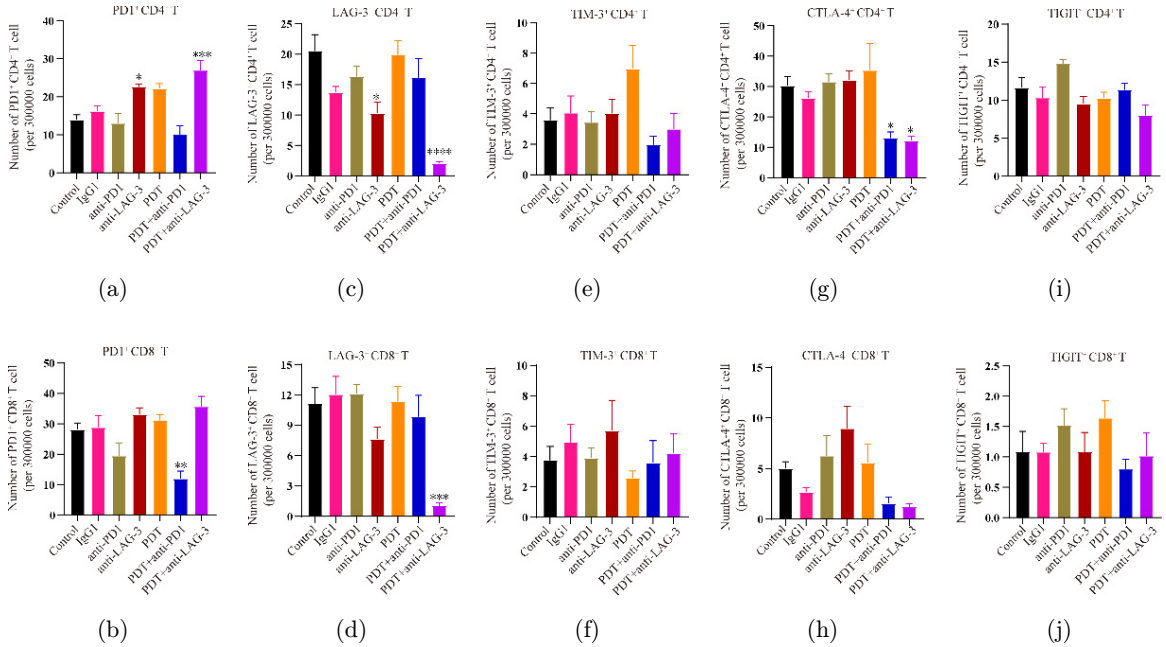

Fig. 7. The expression of PD1, LAG-3, TIM-3, CTLA-4 and TIGIT on CD4$^+$T and CD8$^+$T cells after HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibody. (a)–(j) Pooled data of PD1$^+$ CD4$^+$T cells, PD1$^+$ CD8$^+$T cells, LAG-3$^+$ CD4$^+$T cells, LAG-3$^+$ CD8$^+$T cells, TIM-3$^+$ CD4$^+$T cells, TIM-3$^+$ CD8$^+$T cells, CTLA-4$^+$ CD4$^+$T cells, CTLA-4$^+$ CD8$^+$T, TIGIT$^+$ CD4$^+$T cells and TIGIT$^+$ CD8$^+$T cells. Dates are representative of six animals per group. Error bars represent the SEM. Compared with the control, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

cells after combination therapy (Fig. 7). The results showed that the number of $PD1^+$ $CD4^+T$ cells significantly increased after PDT combined with anti-LAG-3 antibody compared with the control group (Fig. 7(a)), while the immune checkpoint molecules were not significantly upregulated after PDT combined with PD1 antibody (Figs. 7(a)–7(j)). The increasing number of $PD1^+CD4^+T$ cells may be one of the reasons for the attenuated effect of PDT combined with anti-LAG-3 antibody.

By further evaluating the antitumor immune effect in distant tumors (Fig. 8), we found that the proportions of infiltrating $CD4^+T$ and $CD8^+T$ cells significantly increased in the HpD-PDT groups combined with anti-PD1 antibody group than that in the control and HpD-PDT groups. Besides, Granzyme B could be produced by T cells and has a function of kill tumor cells. Granzyme B significantly increased in HpD-PDT combined with anti-PD1 antibody group than that in control.

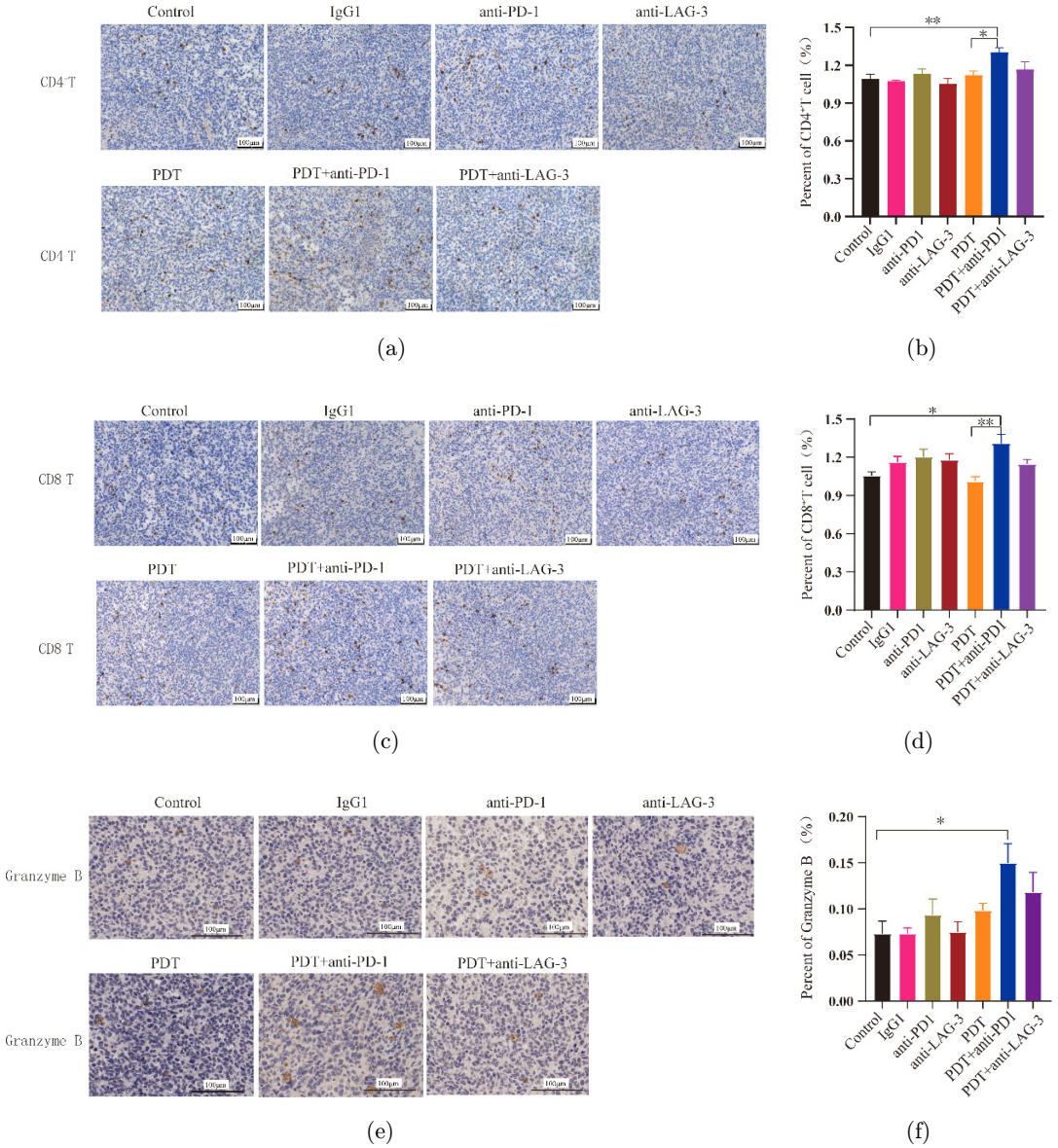

Fig. 8. $CD4^+T$, $CD8^+T$ cells and Granzyme B in distant tumors after HpD-PDT combined with anti-PD1 antibody or anti-LAG-3 antibodies. (a) Representative immunohistochemical profile of $CD4^+T$ cells. (b) Pooled data of $CD4^+T$ cells. (c) Representative immunohistochemical profile of $CD8^+T$ cells. (d) Pooled data of $CD8^+T$ cells. (e) Representative immunohistochemical profile of Granzyme B. (f) Pooled data of Granzyme B. Data are representatives of six animals per group. Error bars represent the SEM. $^*P < 0.05$ and $^{**}P < 0.01$.

4. Discussion

PDT is a promising candidate for breast cancer treatment.[22] It has been reported in the literature that the immune effect can be induced by PDT treatment, which can suppress distant tumors, but this is only an individual case, not a general rule.[23] One of the reasons for limiting the immune effect of PDT may be the elevated expression of immune checkpoint molecules. Therefore, this experiment investigated the therapeutic effect of HpD-PDT with different energy densities on metastatic tumor model mice and the effect on immune checkpoint molecules. The therapeutic effect of the combination of immune checkpoint inhibitors and HpD-PDT was also studied for treating metastatic tumors.

In this study, we found that with HpD-PDT at the same power density, the primary tumor-inhibitory effects were enhanced with an increase in the energy density. The principle of PDT for tumor treatment is that the photosensitizer is transformed into the excited state by laser irradiation, and the latter reacts with oxygen photodynamically to form reactive oxygen species, which can destroy tumor cells.[24] Therefore, at the same power density, the more reactive oxygen species are produced with increasing energy density (longer irradiation time), the greater the killing effect on tumors. Henderson *et al.*[25] also found that during PDT treatment, the effects of tumor inhibition were enhanced with increasing energy density.

It has been reported in the literature that PDT at low doses can induce anti-tumor immune effects,[13] but the power density commonly used in clinical practice is 100–250 mW/cm^2. Therefore, this study was the first to investigate the immune effects induced by HpD-PDT at different energy densities, when the power density was fixed at 100 mW/cm^2. In this experiment, we found that the infiltration of immune cells increased by HpD-PDT in primary tumors at low- energy densities of 40, 60 and 80 J/cm^2 suggested that HpD-PDT can convert "cold tumor" to "hot tumor", which is important for immune therapy. Similar results have been reported in the previous literature[26] finding that PDT with 14 mW/cm^2 and 48 J/cm^2 excited stronger immune effects than PDT with 14 mW/cm^2 and 132 J/cm^2. The immune effects induced by PDT with different energy densities may be related to the vascular damage caused by PDT. The degree of vascular damage by PDT with low-energy density is weaker than that by PDT with high- energy density[27] and the immune cells induced by PDT infiltrate into the tumor tissue through the vasculature.[28] We further detected CRT in primary tumors. The expression of CRT increased after HpD-PDT at energy densities of 40, 60 and 80 J/cm^2 but did not increase after HpD-PDT at energy densities of 100, 120 and 140 J/cm^2. The expression of CRT is important for the activation of the immune response.[29]

Although infiltrating $CD4^+$T cells and $CD8^+$T cells in primary tumors were increased after HpD-PDT, it had no inhibitory effect on distant tumors. There are many factors limiting the immune effect, one of which is the upregulation of immune checkpoint molecules.[30,31] Tumor treatments, such as chemotherapy and radiotherapy, can induce the upregulation of immune checkpoint molecule expression on immune cells.[18,32] The interactions between immune checkpoints and their ligands negatively regulate T cell activation pathways.[33] Therefore, we hypothesize that the immune checkpoints may be upregulated after PDT. This study first found that PD1 and LAG-3 on T cells in primary tumors were upregulated after HpD-PDT. The mechanism underlying PDT-induced PD-1 and other immune checkpoints expression on the surface of T cells are related to several factors: First, in this study, we found that T cells could be activated by HpD-PDT (80 J/cm^2 and 100 mW/cm^2). In the process of T cell activation, T cell receptor (TCR) binds to MHC to activate the TCR signaling pathway and costimulatory molecules bind their ligands to activate the costimulatory signaling pathway. To maintain immune balance, the body can induce upregulation of inhibitory immune checkpoint molecules on T cells.[34] Besides, Fourcade *et al.*[35] reported that tumor antigen stimulation can promote upregulation of PD-1 expression on T cells. PDT can promote the exposure of tumor antigens.[6] Therefore, this study found that the increased expression levels of multiple immune checkpoints may be related to antigen exposure. It was found that cytokines such as IL-2, IL-7, IL-15 and IL-21, as well as the transcription factor FoxO1, could induce PD-1 upregulation.[36,37] Austin *et al.*[38] found that IL-6 enhanced the PD-1

expression through STAT3. Multiple inflammatory factors, such as CXCL2, IL-6, IL-1b and TNF-α, can be upregulated after PDT.[39–41] In addition, it has been reported in the literature that hypoxia could induce upregulation of CTLA-4, TIM-3 and PD-L1 on tumor-associated macrophages, bone marrow-derived macrophages and Treg cells.[42] 4T1 breast cancer is a hypoxic tumor and requires oxygen consumption during PDT; therefore, the elevation of multiple immune checkpoints observed in this experiment may be hypoxia-related.

To improve the antitumor immune effect of HpD-PDT more precisely, we chose anti-PD1 antibody and anti-LAG-3 antibody to combine with PDT for metastatic breast cancer, respectively. After HpD-PDT is combined with anti-PD-1, the primary and distant tumor could be significantly inhibited and the survival time of mice could be significantly prolonged in treating metastatic breast cancer. However, the combination of HpD-PDT and anti-LAG-3 antibody could not inhibit the distant tumor and prolong the survival time. The increased number of $PD1^+CD4^+$T cells after PDT combined with anti-LAG-3 antibody may attenuate the antitumor effect for treating metastatic breast cancer. In addition, the higher expression of PD1 than LAG-3 on T cells after HpD-PDT may also contribute to the lower efficacy of PDT combined with anti-LAG-3 than PDT combined with anti-PD1. There are several reasons for the effective treatment of HpD-PDT combined with anti-PD1 antibody. First, tumor antigens that exposed and released DAMPs after HpD-PDT could recruit $CD8^+$T cells to tumor tissues and increase the immunogenicity of tumors. The infiltration of T cells in tumor tissues is one of the predictors of effective anti-PD1/PD-L1 antibody treatment.[43,44] Second, anti-PD1 antibodies can relieve the inhibitory effect of PD1 on T cells, thus enhancing the T cell immune effect.[45]

There are many inhibitor molecules that could be upregulated after PDT. Gao *et al.*[46] demonstrated that PDT could upregulate the indoleamine 2,3-dioxygenase 1(IDO-1). Increased IDO can inhibit the anti-tumor immune effects of T cells and natural killer cells enhancing the inhibitory effect of Treg cells.[47] O'Shaughnessy *et al.* reported that the expression of PD-L1 on tumor cells could be upregulated after PDT.[48] The PD-L1 on tumor cells could be regulated by IFN-γ-JAK-STAT3 pathway.[49] To the best of our knowledge, this is the first time that the expression of immune checkpoint molecules on T cells after PDT has been investigated. The upregulated immune checkpoints on T cells not only explain the reason for the limited immune effect induced by HpD-PDT but can also provide guidance for the choice of appropriate immune checkpoint inhibitors to augment the antitumor effect of HpD-PDT. PDT in combination with anti-PD1/PD-L1 for breast cancer had also been reported in previous studies.[50,51] Consistent with the previous reports, we also found that PDT in combination with an anti-PD1 antibody can achieve a promising therapeutic result. In addition, we further found that PDT combined with PD1 did not lead to the upregulation of other immune checkpoint molecules on T cells. Notably, we explored the therapeutic effect of PDT combined with anti-LAG-3 antibodies for the first time and found that it could not improve the antitumor immune effect of HpD-PDT, which might be due to the upregulation of PD1 on T cells after PDT combined with anti-LAG-3 antibody. These results suggest that according to the effect of PDT on immune checkpoints, a better therapeutic effect can be achieved after choosing the appropriate immune checkpoint inhibitor in combination with PDT.

5. Conclusions

HpD-PDT alone was not effective in treating metastatic breast cancer. The upregulation of immune checkpoint molecules on T cells, especially PD1 and LAG-3,may be one of the reasons for the limited effect of HpD-PDT. HpD-PDT combined with anti-PD-1 antibody suppressed both primary and distant tumors and prolonged the survival. However, HpD-PDT combined with anti-LAG-3 antibody could not augment the effect of HpD-PDT, which may be due to the elevated PD1 on T cells after HpD-PDT combined with anti-LAG-3 antibody. This study provides more information about immune checkpoints for HpD-PDT and a protocol for augmenting the antitumor effect of HpD-PDT.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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