Bioorthogonal Equipping CAR-T Cells with Hyaluronidase and Checkpoint Blocking Antibody for Enhanced Solid Tumor Immunotherapy

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ABSTRACT: Adoptive cellular therapy utilizing chimeric antigen receptor redirected T (CAR-T) cells has shown impressive therapeutic effects on hematological malignancies. In contrast, the efficacy of CAR-T therapies in treating solid tumors is still poor, which is largely due to inefficient penetration into solid tumors and the immunosuppressive tumor microenvironment. Herein, we engineered hyaluronidase (HAase) and the checkpoint blocking antibody α-PDL1 on the CAR-T cell surface via highly efficient and biocompatible bioorthogonal click chemistry to improve their therapeutic effects on solid tumors. The modified HAase degrades hyaluronic acid and destroys the tumor extracellular matrix, allowing CAR-T cells to penetrate deeply into solid tumors, as evidenced by in vitro infiltration experiments and in vivo biodistribution studies. In addition, in vitro cytotoxicity studies showed stronger antitumor activity of α-PDL1-decorated cells than traditional CAR-T cells. Importantly, HAase- and α-PDL1-engineered CAR-T cells showed better therapeutic efficacy on two solid tumor models and did not cause significant systemic side effects. In this work, we provide a simple, efficient, and biologically safe chemical strategy to engineer traditional CAR-T cells for enhanced therapeutic efficacy on solid tumors, which can be extended to other adoptive cellular immunotherapies and holds great potential for clinical application.

INTRODUCTION

Immunotherapy utilizing T cell engineering with chimeric antigen receptors (CARs) has shown promise in cancer therapy, especially for hematological malignancies.1,2 The U.S. Food and Drug Administration (FDA) has approved several CD19-targeted CAR-T products for treating several hematological malignancies.3,4 Although CAR-T therapies have shown exciting clinical efficacy for hematological malignancies, their efficacy for solid tumors is still very limited.5,6 The poor efficacy of CAR-T therapy in the treatment of solid tumors is due to the unique tumor microenvironment of solid tumors.7−9 First, it is known that binding of target antigens on tumor cell surface is the fundamental prerequisite for CAR-T therapy.6 In hematological malignancies, CAR-T cells can easily target tumor cells, as the tumor cells share the same hematopoietic origin and tend to migrate to similar areas.6−9 However, in the case of solid tumors, the dense extracellular matrix (ECM) formed by cancer-associated fibroblasts (CAFs) greatly prevents T cells from infiltrating the deep area of the tumor, thus inhibiting continuous contact between tumor cells and CAR-T cells.10 Second, the immunosuppressive microenvironment also plays an important inhibitory role in the antitumor effect of CAR-T therapy. Solid tumors contain a large number of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg's) and tumor-associated macrophages (TAMs), which cause T cells to lose their antitumor effect through multiple mechanisms, thereby forming an immunosuppressive microenvironment.11,12 Besides, activated T cells secrete multiple cytokines, such as IFN-γ, which in turn increase the expression of programmed cell death 1 ligand 1 (PDL1) on tumor cells.13 PDL1 binds to the programmed death protein 1 (PD1) on T cell surfaces and inhibits their antitumor activity.14 Therefore, enhancing the infiltration capability in combination with modulating tumor immunosuppressive microenvironment is a prerequisite for improving CAR-T therapies for solid tumors.

In this landscape, numerous efforts have been made to improve the efficacy of CAR-T therapies for solid tumors. The degradation of the ECM is an effective approach to breaking through the barrier of CAR-T cell infiltration in solid tumors.

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Many tumor cells express high levels of heparan sulfate proteoglycan in the ECM. Engineering CAR-T that express heparanase, an enzyme that degrades heparan sulfate proteoglycan, has shown enhanced tumor infiltration and antitumor efficacy in solid tumors. Checkpoint blockade immunotherapy, which intervenes in the immunosuppressive pathway by using antibodies to block checkpoint proteins, such as PDL1, and cytotoxic T lymphocyte associated antigen 4 (CTLA4), has also shown great prospects in solid tumor treatment. Thus, checkpoint blockade immunotherapy, including PD1/PDL1 antibodies, engineering cells to secrete checkpoint inhibitors, and knocking out PD1, have been used in combination with CAR-T therapy and have shown an enhanced therapeutic effect on solid tumors. However, these methods require complicated genetic engineering and long-term in vitro culture, which may limit the efficiency of CAR-T therapy. Therefore, it is urgent to develop simple and innovative CAR-T cell modification strategies for solid tumor treatment.

Bioorthogonal reactions can occur in living cells or tissues without interfering with native biochemical reactions and are often used in combination with click chemistry for chemical modification of living cells. In this study, ECM-degrading hyaluronidase (HAase), for enhanced tumor penetration, and checkpoint blocking antibody α-PDL1, for immunosuppressive tumor microenvironment modulation, were simultaneously conjugated on CAR-T cell surface via highly efficient and biocompatible bioorthogonal click chemistry. Specifically, while CAR-T cells were constructed with lentivirus, N-azidoacetylmannosamine tetraacylated (Ac4ManNAz), an azide-containing metabolic glycoprotein labeling reagent, was added to the medium, and the T cell membranes were then modified with azide groups through intrinsic biosynthesis. Then, azide-modified cells were conjugated with dibenzocyclooctyne (DBCO)-modified HAase for enhanced tumor penetration, while DBCO-modified α-PDL1 was conjugated with tumor extracellular acidity-responsive maleic acid amide bonds. α-PDL1 can be released from α-PDL1- and HAase-engineered cells (H-P@CAR-T) under the acidic condition of the tumor microenvironment and reverse the immunosuppression of the PD1−PDL1 pathway to improve the antitumor activity. Furthermore, compared with unmodified cells, H-P@CAR-T cells degraded tumor hyaluronic acid (HA) and showed enhanced tumor infiltration in both in vitro and in vivo experiments. This study provides a simple and efficient strategy to improve the therapeutic effect of CAR-T cells in solid tumors, which may expand their clinical application.

Figure 1. Schematic of HAase- and α-PDL1-engineered CAR-T cells (H-P@CAR-T) for enhanced solid tumor immunotherapy. (a) Tumor extracellular matrix (ECM) degrading enzyme HAase and checkpoint blocking antibody α-PDL1 were engineered on the CAR-T cell surface by metabolic glycan biosynthesis and click reaction. α-PDL1 was conjugated with tumor extracellular acidity-responsive maleic acid amide bonds and could be released in a low pH tumor microenvironment. (b) H-P@CAR-T cells destroy tumor ECM by degrading hyaluronic acid and show enhanced tumor infiltration capability. Furthermore, the checkpoint blocking antibody α-PDL1 releases from H-P@CAR-T cells under the acidic condition of the tumor microenvironment which reverses the immunosuppression of PD1−PDL1 pathway and in turn improves the antitumor activity.
RESULTS

Construction of HAase- and α-PDL1-Engineered CAR-T Cells (H-P@CAR-T).

To construct H-P@CAR-T cells, we first isolated splenic CD3+ T cells from mice using the immunomagnetic bead separation method. Then, purified CD3+ T cells were stimulated by anti-CD3/CD28 antibodies to induce activation and expansion and then transfected with lentivirus containing mouse CD19-targeted CAR. Meanwhile, Ac4ManNAz (50 mM) was added and incubated for 48 h to modify T cell membranes with azide groups through metabolic engineering. Transfection efficiency was assessed by expression of enhanced green fluorescent protein (EGFP) introduced into the CAR vector. As shown in Figure 2a, the percentage of CAR-positive T cells in the Ac4ManNAz-treated group and the Ac4ManNAz-free group were similar and were approximately 40%, indicating that Ac4ManNAz did not affect the transduction efficiency. To detect the azide groups on the membranes, T cells were incubated with DBCO-Cy5 to react with the azide groups and then detected with flow cytometry. As shown in Figure 2b, almost all T cells carried azide groups on their membranes and could react with DBCO for further labeling.

To conjugate α-PDL1 and HAase on CAR-T cells, α-PDL1 and HAase were modified with DBCO (Figures S1 and S2), and the resulting products showed similar activities compared with unmodified α-PDL1 and HAase studied by ELISA and enzyme activity measurement, respectively (Figure S3a,b). To confirm that DBCO-modified α-PDL1 and HAase were conjugated on the cell surface through bioorthogonal click reaction of DBCO and azide groups, α-PDL1 and HAase were labeled with fluorescent dyes of phycoerythrin (PE) and Cy5, respectively. As shown in Figure 2c, both PE (green) and Cy5 (red) fluorescence signals were observed on the surface of H-P@CAR-T cells, demonstrating that α-PDL1 and HAase were successfully conjugated to cell membrane. In addition, to quantify the amounts of HAase and α-PDL1 on the cell surface, we measured the fluorescence intensity of unbound DBCO-modified HAaseCy5 and DBCO-modified α-PDL1PE in the supernatant. The amounts of HAase and α-PDL1 modified on different CAR-T cells were controlled at similar levels, which is necessary for the subsequent studies (Figure S4).

CD3, CD4, and CD8 are important molecules related to T cell functions. As shown in Figure 2d, no obvious changes in the expression of CD3, CD4, and CD8 on CAR-T cells incubated with Ac4ManNAz and modified with HAase and α-PDL1 was observed by flow cytometry, indicating that the metabolic Ac4ManNAz-based glycans biosynthesis and click reaction had little effect on the function of T cells.

We next assessed whether Ac4ManNAz affected the cytotoxicity of CAR-T cells against tumor cells. CAR-T cells (effector) were incubated with A20 cells (target) at a variety of effector/target ratios. To evaluate the cytotoxicity, the level of lactate dehydrogenase (LDH) in the supernatant released from lysed tumor cells was measured after 24 h of incubation. As presented in Figure 2e, Ac4ManNAz-treated CAR-T cells
Figure 3. Hyaluronidase decoration improves the infiltration capability of CAR-T cells. (a) Schematic diagram of the in vitro ECM model using a transwell to evaluate the infiltration capability of different CAR-T cells across the ECM-mimicking gel. (b) Infiltration index of different cells after 12 h of incubation. Infiltration index is defined as the ratio of the number of different CAR-T cells that infiltrated the lower chamber to the number of unmodified cells that infiltrated the lower chamber, which presents the infiltration capability of different CAR-T cells relative to the unmodified cells. The infiltration index was calculated as infiltration index = N/N_\text{not modified} \times 100, where N represents the numbers of different CAR-T cells in the lower chamber and N_\text{not modified} represents the numbers of unmodified cells in the lower chamber. n = 3. (c) Illustration of an ex vivo tumor coculture model to evaluate the infiltration capabilities of different cells. (d) Representative fluorescent images of sectioned ex vivo tumor tissues after 24 h of coculture. CAR-T cells were labeled with DiD (red), and nuclei were stained with DAPI (blue). (e) Quantitative analysis of the infiltration depth of individual T cells in the tumor tissue. n = 10 for I and III groups; n = 30 for II and IV groups. Data are shown as the mean ± SD. Statistical significance was calculated via one-way ANOVA test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Hyaluronidase Improves the Infiltration Capability of CAR-T Cells. Collagen is the most abundant protein in the tumor ECM and forms a scaffold, while HA fills the space between collagen fibers.28 To study the tumor infiltration capability of H-P@CAR-T cells, we prepared a gel that mimicked tumor ECM using HA and rat tail collagen I on the bottom of the upper chamber and added 20 µg/mL CXCL5 to the lower chamber, which held chemotactic T cells (Figure 3a). Different CAR-T cells at an equal amount of 1 × 10^6 were seeded into the upper chamber, and at 12 h post incubation, the numbers of cells that infiltrated the lower chamber were counted using a hemocytometer. T cells equipped with HAase, including HAase@CAR-T and H-P@CAR-T, exhibited significantly higher infiltration capability than those without HAase modification (Figure 3b). In addition, the infiltration capability of α-PDL1@CAR-T was similar to that of unmodified cells, demonstrating that the checkpoint blocking antibody α-PDL1 has no effect on the infiltration of T cells. These results indicated that HAase could improve the infiltration capability of CAR-T cells to penetrate the ECM-mimicking gel.

We further investigated the tumor infiltration capability of H-P@CAR-T in ex vivo A20 tumors. A20 is a CD19-expressing tumor cell line derived from B cells that can be used to establish a solid tumor model subcutaneously (sc).29-31 Flow cytometry analysis confirmed that the A20 cells expressed high levels of CD19 (Figure S7). In addition, histochemical staining showed abundant collagen and HA were present in the ECM of A20 tumors (Figures S8 and S9), which demonstrated that A20 tumors are a suitable solid tumor model for this study. As shown in Figure 3c, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) labeled CAR-T cells were incubated with excised A20 tumor tissue for 24 h, and then the tumor infiltration was evaluated through tissue sections. As presented in Figure 3d, the representative fluorescent images of sectioned tumor tissues showed that HAase-modified cells penetrated more and farther into the tumor tissues compared with those without HAase modification. Quantitative analysis of the infiltration depth of individual T cells in the tumor tissue further demonstrated the enhanced tumor infiltration capability of HAase-modified cells, including HAase@CAR-T and H-P@CAR-T cells (Figure 3e), which is consistent with the above gel infiltration results.

Equipped α-PDL1 Enhances the Cytotoxicity of CAR-T Cells against A20 Tumor Cells. Many cancer cells highly showed similar cytotoxicity against A20 tumor cells compared with untreated controls. In addition, engineered cells exhibited phenotypes similar to those of unmodified CAR-T cells (Figure S5). These results collectively demonstrated that Ac-ManNAz-based bioorthogonal membrane modification has little effect on CAR-T cells.

Next, we investigated the dynamic stability and pH response of HAase^{31}/α-PDL1^{31} decoration on cells using flow cytometry. In pH 7.2 medium, both HAase^{31} and α-PDL1^{31} were relatively stable on CAR-T cells for up to 24 h. In pH 6.5 medium, which mimics the acidic microenvironment of tumors, HAase^{31} was still stable on CAR-T cells, while α-PDL1^{31} was rapidly released as it was linked with tumor extracellular pH-sensitive maleic acid amide bonds (Figure S6 and Figure 2f).

Collagen is the most abundant protein in the tumor ECM and forms a scaffold, while HA fills the space between collagen fibers.28 To study the tumor infiltration capability of H-P@CAR-T cells, we prepared a gel that mimicked tumor ECM using HA and rat tail collagen I on the bottom of the upper chamber and added 20 µg/mL CXCL5 to the lower chamber, which held chemotactic T cells (Figure 3a). Different CAR-T cells at an equal amount of 1 × 10^6 were seeded into the upper chamber, and at 12 h post incubation, the numbers of cells that infiltrated the lower chamber were counted using a hemocytometer. T cells equipped with HAase, including HAase@CAR-T and H-P@CAR-T, exhibited significantly higher infiltration capability than those without HAase modification (Figure 3b). In addition, the infiltration capability of α-PDL1@CAR-T was similar to that of unmodified cells, demonstrating that the checkpoint blocking antibody α-PDL1 has no effect on the infiltration of T cells. These results indicated that HAase could improve the infiltration capability of CAR-T cells to penetrate the ECM-mimicking gel.

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Equipped α-PDL1 Enhances the Cytotoxicity of CAR-T Cells against A20 Tumor Cells. Many cancer cells highly
express PDL1, which binds to PD1 on T cells, leading to T cell dysfunction. Flow cytometry analysis showed that A20 tumor cells express a high level of PDL1 (Figure S10), which may cause exhaustion of T cells. Therefore, equipping CAR-T cells with α-PDL1 was expected to relieve the exhaustion and enhance the antitumor cytotoxicity. To evaluate the effect of α-PDL1 decoration on the cytotoxicity against tumor cells, CAR-T cells (effector) were incubated with A20 tumor cells (target) at a variety of effector/target ratios. After 24 h of incubation under acidic conditions at pH 6.5, the level of LDH released from lysed tumor cells in the supernatant was measured to evaluate the cytotoxicity of CAR-T cells. As presented in Figure 4a, CAR-T cells in each group showed cytotoxicity against A20 tumor cells in a dose-dependent manner. In addition, α-PDL1@CAR-T and H-P@CAR-T exhibited stronger cytotoxicity than those without α-PDL1 modification, demonstrating that the modification of α-PDL1 can enhance the antitumor cytotoxicity of CAR-T cells.

To verify the enhanced cytotoxicity of α-PDL1-modified CAR-T cells to tumor cells, we further studied the apoptosis or necrosis of A20 tumor cells incubated with different CAR-T cells at an effector/target ratio of 2:1. After 24 h of incubation, tumor cells were stained with Annexin V/propidium iodide (PI) and then analyzed by flow cytometry. As shown in Figure 4b,c, more than 96.2% of A20 cells were located in the viable area after incubation with untransfected T cells or without any treatment. When treated with CAR-T or HAase@CAR-T, the percentages of apoptotic and necrotic cells were 49.4 and 54.5%, respectively. More importantly, the percentages of apoptotic and necrotic cells increased to 72.3 and 72.9% when treated with α-PDL1@CAR-T or H-P@CAR-T, respectively. These results further illustrated the role of α-PDL1 in improving the antitumor effect of CAR-T cells.

Figure 4. Modified α-PDL1 enhances the cytotoxicity of CAR-T cells against A20 tumor cells. (a) Lysis of A20 tumor cells after incubation with different CAR-T cells evaluated by measuring released lactate dehydrogenase (LDH) in medium. n = 3. (b) Apoptosis analysis of A20 tumor cells after incubation with T cells as indicated for 24 h. (c) Quantification of apoptosis and necrosis of A20 tumor cells after incubation as indicated. n = 3. (d–f) Secretion of IFN-γ, TNF-α, and IL2 after coincubation for 2 h examined by enzyme-linked immunosorbent assay (ELISA). n = 3. (g) Schematic illustration of IFN-γ secretion from CAR-T cells and checkpoint blockade inducing the upregulation of PDL1 expression in A20 tumor cells. (h) The mRNA level of PDL1 in A20 tumor cells incubated with various CAR-T cells. n = 3. Data are shown as the mean ± SD. Statistical significance was calculated via one-way ANOVA test. *, p < 0.05; **, p < 0.01; ***, p < 0.0001.
In addition, the activities of CAR-T cells in various groups were evaluated by measuring released cytokines. After incubation with A20 tumor cells for 2 h, α-PDL1@CAR-T and H-P@CAR-T that were modified with α-PDL1 showed higher releases of IFN-γ, TNF-α, and IL2 than those without α-PDL1 modification (Figure 4d–f), suggesting that activation of CAR-T can be enhanced by α-PDL1 decoration.

It is reported that the expression of PDL1 on most tumor cells is induced by IFN-γ and is closely related to the success of PD1/PDL1 checkpoint blockade. Therefore, we evaluated the expression levels of PDL1 in A20 tumor cells after coculture with CAR-T by quantitative real-time PCR (Figure 4g). Obviously, the levels of PDL1 mRNA in A20 cells treated with α-PDL1@CAR-T and H-P@CAR-T were significantly upregulated compared with that in A20 cells treated with cells without α-PDL1 modification, demonstrating the PDL1 expression of A20 cells could be upregulated after coculture with α-PDL1-equipped CAR-T cells, likely due to enhanced IFN-γ release and successful checkpoint blockade by α-PDL1 (Figure 4h).

**Modified HAase Enhances Tumor Infiltration of CAR-T Cells In Vivo.** Infiltration of deep tumor tissue is essential for the enhanced therapeutic effect of CAR-T therapy. Thus, we further evaluated the in vivo tumor infiltration capability of CAR-T cells using the A20 solid tumor model. 1,1-Dioctadecyl-3,3,3,3-tetramethylindocarbocyanine iodide (DiR), a near-infrared fluorescent dye, was used to label CAR-T cells, and then the DiR-labeled cells were injected intravenously into A20 tumor bearing mice. At 24 h post injection, the main organs and tumors of mice in each group were excised and examined with an in vivo imaging system. Ex vivo fluorescent images in Figure 5a show that all kinds of CAR-T cells inevitably entered the liver and spleen, possibly because liver and spleen are the main metabolic organs. Moreover, distinct amounts of DiR signals were detected in the tumor tissue after injection of HAase@CAR-T or H-P@CAR-T. The fluorescence intensity in mice treated with HAase@CAR-T or H-P@CAR-T showed enhanced tumor accumulation, which was 4.6-fold or 4.3-fold higher than those without HAase modification, respectively (Figure 5b). The enhanced tumor accumulation of HAase-modified cells should be attributed to the tumor ECM degrading HAase.

To further study the intratumoral distribution, carboxyfluorescein succinimidyl ester (CFSE) labeled CAR-T cells were administrated into A20 tumor bearing mice. Tumors were excised and sectioned at 24 h post injection, followed by immunofluorescence staining of CD31, a biomarker of blood vessels. As shown in Figure 5c, immunofluorescence staining showed that more administrated cells entered the tumor site in mice treated with HAase@CAR-T or H-P@CAR-T than those without HAase modification, which is consistent with the ex vivo fluorescence imaging study. It is worth noting that cells without HAase modification concentrated at the perivascular sites of the tumor, while HAase@CAR-T and H-P@CAR-T cells infiltrated the deep area of the tumor far from blood vessels. On the basis of these fluorescence images, we further quantified the infiltration depth (distance from the nearest blood vessel) and the number of CAR-T cells in tumor tissue. The infiltration depths of HAase@CAR-T and H-P@CAR-T cells were both about 20 μm, which was significantly deeper than that of cells without HAase modification (Figure 5d). By quantifying the cell number in tumor tissue, we found that HAase@CAR-T and H-P@CAR-T cells were about 5 times more abundant than those without HAase modification in tumor tissue (Figure 5e).
In addition, we also quantified the numbers of different CAR-T cells infiltrating tumor tissues by flow cytometry. Cells ($5 \times 10^6$) were injected intravenously into A20 tumor bearing mice. At 24 and 72 h, the tumors in each group were excised, weighed, and prepared in single-cell suspensions, and the number of tumor infiltrated CAR-T cells was quantified with flow cytometry. The numbers of HAase@CAR-T and H-P@CAR-T were much higher than those without HAase modification at both time points (Figure 5f). Besides, the numbers of all kinds of CAR-T cells in the tumor tissue increased at 72 h compared to 24 h, possibly due to their consistent infiltration or proliferation. Collectively, these results demonstrate that the modification of HAase improves the infiltration capability of CAR-T cells, which in turn enhances their accumulation in tumors.

**Modified α-PDL1 Showed Favorable Effects on the Immunophenotype of Engineered CAR-T Cells.** Having demonstrated that engineered CAR-T cells can effectively infiltrate tumor tissue, we next investigated their intratumoral fates in vivo. CAR-T cell phenotypes are critical for effective cancer therapy, and the tumor-killing activity is mainly attributed to CD8$^+$ T cells,34 so we first analyzed the phenotypes of CD8$^+$ CAR-T cells in vivo by flow cytometry 72 h after administration (gating strategy shown in Figure S11). It has been reported that CAR-T cells that are more naive and less differentiated correlate with enhanced therapeutic efficacy.35,36 As shown in Figure 6a,b, α-PDL1-modified α-PDL1@CAR-T and H-P@CAR-T contained more CD44$^-$CD62L$^+$ naive cells but fewer CD44$^+$CD62L$^-$ effector memory cells compared with other groups, indicating α-PDL1 significantly reduced the loss of naive cells and differentiation to CD44$^+$ cells.

We next detected the activation and exhaustion of tumor infiltrating CAR-T cells by flow cytometry. It is obvious from Figure 6c that α-PDL1-modified α-PDL1@CAR-T and H-P@CAR-T exhibited elevated expression of activation marker CD69 than other cells, indicating α-PDL1 enhanced T cell activation. In addition, decreased expression of exhaustion markers LAG3, PD1, and TIM3 demonstrated α-PDL1 alleviated CAR-T cell exhaustion (Figure 6d–f). Collectively, α-PDL1 has a favorable effect on the in vivo immunophenotype of engineered CAR-T cells and is expected to enhance the antitumor activity.

**Antitumor Effect of Engineered CAR-T Cells on A20 Tumors In Vivo.** Encouraged by the enhanced infiltration capability and antitumor cytotoxicity of engineered CAR-T cells, we next studied their therapeutic efficacy on A20 tumors. On the seventh day after A20 tumor cell inoculation, mice were divided into five groups and received various formulations (Figure 7a). Mice in each group received three intravenous injections (i.v) of indicated formulations at an equivalent dose of $5 \times 10^6$ cells on the first, fourth, and seventh days. As shown
in Figure 7b, it is obvious from the tumor growth curves that all kinds of CAR-T cells could suppress tumor growth. Cells without modification showed the lowest tumor growth suppression (42.6% inhibition rate compared to control group). In contrast, H-P@CAR-T cells exhibited the most remarkable antitumor effect (95.1% inhibition rate) compared with other groups, which was attributed to their efficient tumor infiltration and alleviation of the tumor immunosuppressive microenvironment. HAase@CAR-T (76.3% inhibition rate) and α-PDL1@CAR-T (70.0% inhibition rate) showed moderate tumor growth suppression, demonstrating that the synergy of HAase and α-PDL1 plays an important role in the therapy. The photograph and weight of tumors confirmed the superior therapeutic efficacy of H-P@CAR-T (Figure 7c,d). The excised tumors were sectioned for immunohistochemical staining of Ki67, a proliferation marker protein. As shown in Figure S12, obvious positive staining of Ki67 was observed in the tumors treated with PBS or CAR-T, but less positive staining was found in the mice treated with HAase@CAR-T or α-PDL1@CAR-T. Most importantly, almost no positive staining was observed in H-P@CAR-T cell treated tumors.

In vivo biosafety is a strong concern for CAR-T therapy. To evaluate the potential toxicity of H-P@CAR-T cells, body weights of the mice were recorded. Slight body weight loss was observed in all CAR-T treated groups compared with the PBS control group (Figure 7e), properly due to cytokine release syndrome (CRS). However, the weight loss was almost completely recovered on the 18th day, indicating the side effects were tolerable. After ending the tumor treatment experiment, we also harvested main organs for pathological analysis by hematoxylin and eosin (H&E) staining. No obvious organ damage was observed in the mice treated with all kinds of cells (Figure S13). These results demonstrated the biocompatibility and safety of H-P@CAR-T cells. Collectively, we proved the enhanced therapeutic efficacy on solid tumors and in vivo biosafety of H-P@CAR-T cells, which might have promising prospects for clinical applications.
To further investigate whether engineered cells have long-term antitumor activity in vivo, we first measured the pharmacokinetics of different CAR-T cells (Figure S14a). Tumor-bearing mice received single injections (iv) of CAR-T cells, whose number in peripheral blood was monitored with flow cytometry every 2 days. As shown in Figure S14b, various types of CAR-T cells expanded rapidly and reached a peak around day 7, which was due to the stimulation by CD19+ B cells in peripheral blood. Importantly, the levels of α-PDL1-modified α-PDL1@CAR-T and H-P@CAR-T cells in peripheral blood were significantly higher than those of other groups and decreased more slowly, indicating that α-PDL1 modification can enhance the persistence of CAR-T cells.

Next, we evaluated the antitumor effect of a single injection of CAR-T cells on A20 tumor and the survival of the mice was monitored up to 60 days. Tumor growth curves (Figure 7f) and tumor volumes on day 20 (Figure 7g) of mice showed that a single injection had significant inhibitory effects on tumor growth. CAR-T cells without modification showed a 27.7% inhibition rate of tumor growth, which was much lower than that of the treatment with three injections. In contrast, a single injection of H-P@CAR-T cells still exhibited the most significant antitumor effect (91.5% inhibition rate), which was comparable to that with three injections. Similarly, a single injection of both HAase@CAR-T (71.9% inhibition rate) and α-PDL1@CAR-T cells (67.5% inhibition rate) showed tumor growth inhibition rates comparable to those with three injections. These results implied that the engineered cells had more durable antitumor activity than those without modification. Survival curves of tumor-bearing mice in Figure 7h demonstrated that engineered cells including HAase@CAR-T, α-PDL1@CAR-T, and H-P@CAR-T could significantly extend the survival of mice. Most significantly, a single injection of H-P@CAR-T extended the survival of most mice to around 60 days. On the contrary, the mice in the CAR-T group all died before the 40th day, suggesting a long-term antitumor effect of the engineered CAR-T.

In addition, we assessed the in vivo activity of different CAR-T cells by measuring the level of cytokines released in peripheral blood 7 days after CAR-T injection. The levels of IL-2, TNF-α, and IFN-γ in the H-P@CAR-T group were significantly higher than that in other CAR-T groups (Figure 7i), demonstrating that HAase and α-PDL1 synergistically enhanced the antitumor activity of CAR-T cells in vivo.

Although serum cytokines significantly increased after treatment, no mouse died at an early stage of treatment when CAR-T cells were highly activated and possessed obvious cytokine producing ability, which suggested the engineered cells did not induce severe CRS.

Engineered CAR-T Cells Showed Promising Therapeutic Effect on CT26 Colon Tumor In Vivo. To prove the general applicability of our engineered CAR-T in treating different solid tumors, we further prepared CAR-T cells targeting carcinoembryonic antigen (CEA) as well as the CT26 colon cancer cell line expressing CEA (CT26-CEA) (Figure 8a). To examine the specific cytotoxicity, CAR-T cells were
incubated with CT26 or CT26-CEA tumor cells for 24 h. Compared with CT26 cells, CAR-T cells showed obvious cytotoxicity against CT26-CEA cells in a dose-dependent manner, demonstrating that they have specific cytotoxicity to CEA-positive targets (Figure 8b). The modification of HAase and α-PDL1 on CEA-targeted CAR-T is the same as the modification of CD19-targeted CAR-T, and the successful modification of HAase and α-PDL1 was confirmed by laser confocal microscopic observation (Figure S15).

We next studied the therapeutic efficacy of engineered CEA-targeted CAR-T cells on CT26-CEA solid tumors. CT26-CEA tumor bearing mice in each group received a single administration of different CAR-T cells at an equivalent dose of 5 × 10^6 cells on day 0. As the tumor growth curves (Figure 8c) and tumor volume on day 20 (Figure 8d) showed, it is obvious that engineered cells including HAase@CAR-T, α-PDL1@CAR-T, and H-P@CAR-T inhibited the growth of CT26 tumors more effectively than those without modification. Most significantly, H-P@CAR-T exhibited the most remarkable antitumor effect in comparison with other engineered cells. The relatively higher survival rate of tumor-bearing mice treated with H-P@CAR-T also demonstrated a remarkable and long-term antitumor effect (Figure 8e).

Surprisingly, no significant weight loss (less than 10%) was found in all CAR-T cell-treated groups compared with the PBS control group (Figure 8f). In addition, the levels of IL-2, TNF-α, and IFN-γ in peripheral blood 7 days after administration further demonstrated the enhanced antitumor activities of engineered cells, especially H-P@CAR-T cells (Figure 8g). These results demonstrate that our engineered cells can effectively treat the CT26-CEA solid tumor model, illustrating the general applicability of our engineered CAR-T cells for the treatment of solid tumors.

## Discussion

Although CAR-T therapy has achieved exciting results in the treatment of hematological malignancies, the treatment of solid tumors has always been a difficulty needing to be faced and also a hot spot for preclinical research. This undesired efficacy of CAR-T therapy is most likely hindered by multiple factors present inside solid tumors, of which inefficient infiltration of solid tumors and immunosuppressive tumor microenvironment are two important factors. In this landscape, many strategies have been developed to improve the efficacy of CAR-T cells in the treatment of solid tumors: (I) combining checkpoint blockade antibodies with CAR-T therapy,18,19 (II) knocking out PD1 on T cells,21,22 (III) local delivery,16,37 (IV) equipping extracellular matrix (ECM) degrading enzymes or secretory checkpoint inhibitors by genetic engineering.15,20 Although these strategies can improve the effect of CAR-T cells on treating solid tumors, there is still an urgent need for new strategies that can expand the clinical utility.

In this work, we equipped CAR-T cells with ECM-degrading enzyme hyaluronidase (HAase) and checkpoint blockade antibody α-PDL1 via bioorthogonal click chemistry, which were often used for chemical modification of living cells without interfering native biochemical reactions. Compared with the genetic engineering methods, our bioorthogonal click chemistry based strategy for CAR-T engineering does not require extra in vitro cell culture and is simple to perform, thus showing better clinical application potential.

Hyaluronic acid (HA) is the major component of tumor ECM, and hyaluronidase (HAase)-modified nanomedicine has been proven to enhance tumor penetration.26 In this work, CAR-T cells equipped with HAase exhibited enhanced infiltration capability, thereby enhancing their tumor accumulation and allowing them to deeply infiltrate solid tumors. Although cell proliferation could reduce the amount of HAase on the surface, tumor-infiltrating CAR-T cells can expand within the tumor, resulting in a significant increase of cell number within the tumor (Figure 5f). Meanwhile, modified α-PDL1 released from the engineered cells in the acidic tumor microenvironment was expected to reverse tumor-mediated immunosuppression by the intervening PD1/PDL1 pathway. Through in vitro antitumor experiments, we found that CAR-T cells equipped with α-PDL1 showed enhanced cytotoxicity to A20 tumor cells, which demonstrated the successful reversal of tumor-mediated immunosuppression by α-PDL1 (Figure 4). In addition, immunological analyses of engineered cells revealed that α-PDL1 can alleviate T cell exhaustion in vivo (Figure 6d–f). Finally, CAR-T cells equipped with HAase or/and α-PDL1 exhibited an enhanced therapeutic effect on two solid tumor models compared with unmodified cells (Figures 7 and 8). Most significantly, H-P@CAR-T exhibited significantly better antitumor activities than unmodified cells or HAase-/α-PDL1-engineered cells on two solid tumor models, demonstrating that the combination of enhanced infiltration capacity and reversal of tumor-mediated immunosuppression synergistically improved the therapeutic effect. It is worth noting that serum cytokine levels were lower in the CEA-CT26 model compared with the A20 model after H-P@CAR-T treatment (Figures 7i and 8g), indicating that the specificity of CAR-T target influenced CAR-T induced system cytokine release because expression of CEA was limited while CD19+B cells were abundant in circulation. Therefore, improved tumor target specificity might be one critical factor to reduce CRS. Our study did not focus on target specificity, whereas low pH-responsive release of α-PDL1 from engineered cells inhibited off-target tissue damage, which may also help reduce CRS development. The body weight changes of mice (Figures 7e and 8f) and the H&E pictures of main organs after treatment (Figure S13) proved that H-P@CAR-T cells have no obvious side effects compared with unmodified cells, indicating our strategy for CAR-T engineering is safe and has clinical application potential.

Although our strategy for cell engineering has been shown to alleviate the insufficient infiltration and tumor-mediated immunosuppression of CAR-T cells in solid tumors, it cannot address some of the problems of CAR-T therapy in solid tumors, such as limited targetable antigens and heterogeneous expression of tumor cell antigens.38,39 In this work, our engineered CAR-T cells targeted CD19 (naturally expressed on the surface by B cells) and CEA (an artificial antigen) in A20 and CT26-CEA tumors, respectively. Although the result from these two models successfully demonstrated our conclusions, it differs from authentic solid tumors. Nevertheless, we believe that our engineered CAR-T cells could be used to treat authentic solid tumors in the future if equipped with a suitable CAR targeting tumor cell.

In this work, we have provided a highly efficient, biocompatible, and simple strategy to equip CAR-T cells with functional molecules for enhanced solid tumor immunotherapy, which can be easily extended to other adoptive cellular therapies, such as tumor infiltrating lymphocytes, TCR-T cells, and NK cells. Taken together, our strategy for CAR-T engineering has obvious advantages,
which makes us believe that it holds great potential for clinical application.

**ASSOCIATED CONTENT**

- **Supporting Information**
  The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.2c00163.

Materials and methods; synthetic routes of DBCO-PEG2k-HAase and DBCO-CDM-α-PDL1; PDL1 binding capabilities of α-PDL1 and DBCO-CDM-α-PDL1; enzyme activities of HAase and DBCO-PEG2k-HAase; quantification of amounts of HAase and α-PDL1 on engineered cells; phenotypes of CAR-T cells in vitro; representative flow cytometry plots of HAase\(^{Cy5}\) and α-PDL1\(^{PE}\) of engineered CAR-T cells; flow cytometry plot of CD19 of A20 cells; hyaluronic acid collagen and staining in A20 tumor extracellular matrix; expression of PDL1 of A20 cells; gate strategies of flow cytometry; Ki67 staining of tumor tissue; H&E staining of main organs; pharmacokinetics of CAR-T cells; fluorescence images of CEA-targeted CAR-T cells (PDF)

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Y. Z., Y. D., and S. Y. contributed equally to this work. Y.Z. and Y.Y. designed the project. Y.Z., Y.D., S.Y., Y.T., C.W., and J.L. performed the experiments. Y.Z., Y.D., and S.Y. analyzed the data. Z.L. and Y.Y. supervised all personnel. Y.Z., Y.D., and Y.Y. wrote the manuscript. Y.Z., Y.D., S.Y., Y.T., C.W., J.L., Z.L., and Y.Y. revised the manuscript. All authors approved the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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