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Article

A tetramethylpyrazine releasing hydrogel can potentiate CAR-T cell therapy against triple negative breast cancer by reprogramming tumor vasculatures

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ABSTRACT

Irregular vasculature of solid tumors has proven to be a pivotal factor restricting their response to chimeric antigen receptor-T (CAR-T) cell therapy because it is tightly associated with hypoxia and other biological barriers. Herein, an injectable hydrogel composed of poly (ethylene glycol) dimethacrylate (PEGDMA) and ferrous chloride ($FeCl_2$) responding to endogenous hydrogen peroxides (H_2O_2) is developed to enable sustained intratumoral release of Chinese herbal extracts tetramethylpyrazine (TMP). TMP is selected due to its potency in activating vascular endothelial growth factor (VEGF) expression and the endothelial nitric oxide synthase/nitric oxide (eNOS/NO) axis inside vascular endothelial cells. Upon being fixed inside tumors with the PEGDMA based hydrogel, TMP can remodel tumor vasculature by simultaneously promoting angiogenesis and dilating tumor vasculature and thus attenuate tumor hypoxia in two murine xenografts bearing human triple negative breast cancer (TNBC). Resultantly, treatment with TMP fixation potentiates the tumor suppression effect of intravenously injected epidermal growth factor receptor expressing CAR-T (HER1-CAR-T) cells toward two TNBC tumor xenografts by promoting their tumor infiltration, survival, and effector function. This study highlights a concise yet effective approach to reinforce the therapeutic potency of CAR-T cells towards targeted solid tumors by simply remodeling tumor vasculature.

1. Introduction

Triple-negative breast cancer (TNBC) lacking of the expression of progesterone receptor, estrogen receptor and human epidermal growth factor receptor 2 (HER2) is the most aggressive subtype of breast cancers [1,2]. It has been reported that 45%–70% of TNBC patients exhibit epidermal growth factor receptor (EGFR, also known as HER1) overexpression, which has been utilized as the target for inventing new therapeutics to expand our arsenal against TNBC [3]. Unlike small molecule EGFR inhibitors (*e.g.*, Erlotinib, Icotinib, Gefitinib etc.) that are prone to suffer from unwanted drug resistance [4,5], engineering HER1-CAR-T cells represents a promising treatment modality for HER1 positive TNBC tumors because they hold great potential to elicit persistent and even complete anti-tumor response [6–9]. However, similar to other CAR-T cell therapies in treating corresponding solid tumors, the therapeutic efficacy of HER1-CAR-T cells still remains far from satisfactory in treating TNBC tumors due to the complex tumor microenvironment (TME) and many other factors [10–14]. For example, tumor hypoxia, a general feature of solid tumors resulting from irregular blood vasculature and elevated metabolism, has shown the capacity to inhibit the viability and function of CAR-T cells [12]. Recent progress has demonstrated that concurrent delivery of CAR-T cells together with stimulatory cytokines or TME remodeling formulations could effectively potentiate these CAR-T cells against corresponding solid tumors by removing tumor immunosuppression [15–19]. Therefore, development of effective TME remodeling strategies is of great

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practical promise to enhance the therapeutic potency of HER1-CAR-T cells [20].

In the past decades, intensive efforts have been devoted to finding out efficient TME remodeling strategies in both clinical and preclinical explorations [21]. Normalization of tumor vasculature through different mechanisms has attracted tremendous research interest since the abnormal tumor vasculature is tightly associated with tumor hypoxia, tumor acidity, tumor immunosuppression and many others [22–24]. It is therefore shown to be versatile in directly suppressing tumor growth and benefiting other cancer treatment modalities by promoting the tumor accumulation and intratumoral diffusion of distinct cancer therapeutics (*e.g.*, small molecule chemotherapeutics and nanotherapeutics) [25–27]. Recently, we uncovered that tumor targeted delivery of Erlotinib with a pH-responsive nanocarrier could effectively remodel tumor vasculatures through inhibiting the HER1/extracellular regulated protein kinases (ERK)/AKT serine/threonine kinase 1 (AKT) axis, and thus attenuate tumor hypoxia for enhanced photodynamic treatment of TNBC tumors [28]. However, till now, there is no study on exploring the potency of tumor vasculature reprogramming in potentiating CAR-T cell therapy and other adoptive cell transferring therapies [29].

In addition, increased intratumoral levels of nitrogen oxide (NO) and vascular endothelial growth factor (VEGF) through distinct mechanisms could effectively remodel tumor vasculature due to their potency in dilating blood vessels and in regulating angiogenesis, respectively [21,30–32]. Tetramethylpyrazine (TMP), an alkylpyrazine isolated from traditional Chinese medicine, Ligusticum wallichii (Chuan Xiong), has been used for the treatment of varying vascular diseases in China for ~40 years, probably ascribed to its potency in promoting VEGF expression and NO production in vascular endothelial cells [33]. Therefore, in this study, water soluble TMP molecules were *in situ* fixed with endogenous hydrogen peroxide (H_2O_2) responsive hydrogels composed of PEGDMA and $FeCl_2$ for long-term tumor vasculature remolding. It is found that the obtained TMP loaded PEGDMA hydrogel (coined as TMP@PEGgel) upon intratumoral fixation could effectively remodel tumor vasculature through activating the VEGF expression and the eNOS/NO axis of vascular endothelial cells and thus enable sustained tumor hypoxia relief. Furthermore, such TMP@PEGgel treatment was shown to be capable of enhancing the infiltration of intravenously infused HER1-CAR-T cells and thus promoting their survival and effector functions inside MDA-MB-468 and MDA-MB-231 TNBC tumors inoculated on Balb/c nude mice. It was further shown that our TMP@PEGgel assisted HER1-CAR-T cell therapy could synergically suppress the growth of these two TNBC tumors. This study therefore highlights that remodeling of tumor vasculature with TMP treatment is a promising strategy to reinforce the therapeutic potency of HER1-CAR-T cell therapies toward TNBC tumors.

2. Materials and methods

2.1. Materials and reagents

Poly(ethylene glycol) dimethacrylate (PEGDA), $FeCl_2$, rhodamine B were purchased from Sigma-Aldrich. Tetramethylpyrazine (TMP) was purchased from MCE medchemexpress. The LDH cytotoxicity assay kit and MTT assay kit were purchased from Beyotime Biotechnology. The ELISA kits were purchased from Dakewe. Anti-CD3/CD28 beads were purchased from Invitrogen. Fetal bovine serum (FBS) and all the cell cultural medium were purchased from Thermo Fisher Scientific Inc. CCK-8 assay was purchased from Dojindo Laboratories. CAR-related plasmids were purchased from Icareab.

2.2. Preparation and characterization of PEGgel

PEGDMA (10 wt%) was mixed with $FeCl_2$ (1.6 mM) and H_2O_2 (0.5 mM) using a vortex mixer for 10 s, then incubated at room temperature till the gel formation. The photographs were obtained at 1 min post the mixing. The microscopic structure of the as-prepared hydrogel was observed by a scanning electron microscope (SEM, Zeiss Gemini 500). TMP release was evaluated at 37 ˚C in PBS. The released TMP was analyzed with an ultraviolet-visible spectrophotometer (PerkinElmer).

2.3. Cell experiments

MDA-MB-468, MDA-MB-231, HUVEC and HAEC cells were obtained from ATCC, and maintained following the standard procedures.

T cells were obtained from PBMCs from healthy adult donors with signed agreements. In brief, PBMCs isolated from whole blood using ficoll density gradient centrifugation were stimulated in AIM V^{TM} complete medium supplemented with anti-CD3/CD28 beads (2.5μL/mL) with 5% CO_2 at 37 ˚C. Two weeks later, T cells were transduced with lentivirus which contained CD8 signal, CD8 Hinge, CD28, 4–1BB, CD3ζ and HER1 specific single-chain fragment variable (scFv) for 1 day. Afterwards, CAR-T cells were collected after being stimulated with appropriate antibodies and antigens according to the standard methods [34]. Then, the CD3ζ expression levels on these obtained con-CAR-T and HER1-CAR-T cells were evaluated by using real-time PCR and western blotting assays to identify their successful construction according to the previously used method.

Then, the effects of hypoxia incubation on the cell viability of these two CAR-T cells were investigated by incubating these CAR-T cells seeded in 96-well plates (5×10^4 cells per well) at both hypoxia condition (<2% O_2) and normoxic condition (21% O_2) for 24 h. The cell viability of these cells post corresponding treatments were determined by the standard MTT assay.

To evaluate the influence of hypoxia incubation on the specific cell killing of HER1-CAR-T cells, TNBC cells pre-seeded in the 96-well plate (1×10^4 cells per well) for 12 h were co-cultured with HER1-CAR-T cells at varying feeding ratios under hypoxic condition or normoxic conditions for 24 h. Later, the concentration of LDH, released by dead TNBC cells, in the supernatant of each well was quantified by using commercial LDH cytotoxicity assay, while the secretion levels of IL-2, TNF-α and IFN-γ were determined by using corresponding ELISA kits.

To assess the cytotoxicity of TMP on endothelial cells, both HUVEC and HAEC pre-seeded in the 96-well plate (1×10^4 cells per well) were incubated with fresh medium supplemented with different concentrations of TMP at 37 ˚C for another 24 h. Then, their cell viability was measured by the Cell Counting Kit-8 (CCK-8) method.

To assess the potency of TMP treatment in activating VEGF expression and eNOS/NO axis, both HUVEC and HAEC were seeded in the 6-well plates and incubated for 12 h before being incubated with TMP (500 nM) at 37 ˚C for another 24 h. Then, the expression levels of VEGF, p-eNOS, eNOS, β-Actin in these cells was measured by western blotting according to the manufacturers' instruction. Another batch of cells with same treatments were used to evaluate the effect of TMP treatment on the measure of NO production using commercial NO assay kit.

2.4. In vivo *animal experiments*

Female Balb/c nude mice (4-week-old) was purchased from Nanjing University's model animal research center. Nanjing Normal University's medical ethics committee approved the animal experiments in this study (IACUC——20220228).

TNBC cells (5×10^6) were subcutaneously injected into the mammary fat pad of each mouse to generate TNBC cell-line-derived tumor model.

For *in vivo* sustained release study, Rhodamine B@PEGgel and free rhodamine B solution (rhodamine B = 1 mg/kg) were intratumorally

injected into the TNBC tumors. Then, 15 min, 3 days, and 10 days post corresponding injection, tumors of treated mice were excised, cryosectioned, counterstained with DAPI to be observed on a confocal microscopy for recording the rhodamine B fluorescence according to the standard method.

For *in vivo* tumor hypoxia evaluation related study, TNBC tumor bearing mice with intratumoral injection of TMP@PEGgel, PEGgel and TMP (TMP = 1 mg/kg) were sacrificed in 3 days, 10 days post corresponding injection for preparing tumor slices. Then, these tumors were stained with anti-HIF-1a and anti-CD31 primary antibodies and corresponding secondary antibodies by following the procedures provided by the manufacturers, followed by being observed using confocal microscopy.

For pimonidazole staining, another batch of mice with same treatments were intraperitoneally injected with commercial pimonidazole 90 min before they were euthanized. Later, tumors were collected for hypoxia staining by using an hypoxyprobe-1 plus kit.

For evaluating the *in vivo* tumor infiltration, survival and effector functions of HER1-CAR-T cells, 30 mice bearing TNBC tumors were randomly divided into six groups and received the following treatments: 1) PBS, 2) con-CAR-T injection, 3) HER1-CAR-T injection, 4) PEGgel fixation + HER1-CAR-T injection, 5) TMP injection + HER1-CAR-T injection, 6) TMP@PEGgel fixation + HER1-CAR-T injection. And then mice received i.v. injection of 5×10^6 con-CAR-T cells, HER1-CAR-T cells, both with intrinsic expression of RFP on day 1. In 3 days and 10 days post TMP@PEGgel injection, tumors were cryosectioned or homogenized to evaluate the tumor infiltration and survival of both CAR-T cells by recording the RFP fluorescence by using confocal microscopy and flow cytometry, respectively. In addition, tumors were wet-weighted and homogenized in lysis buffer for measuring the secretions of IL-2, TNF-α and IFN-γ by using corresponding ELISA kits according to manufacturers' instruction.

For *in vivo* combined treatment, 6 groups of TNBC tumor bearing mice (~100 mm^3) received the same intratumoral injections of TMP@PEGgel, PEGgel and TMP on day 0 as aforementioned, but received 3 times the amount of i.v. injection of con-CAR-T cells or HER1-CAR-T cells (5×10^6 cells per injection) in 1, 2, 3 days. Digital caliper was used to record the length and width of each tumor every week for calculating tumor volumes following the formula of Volume=1/2 × Length × Width × Width. Digital balance was used to monitor the body weight of each mouse every week.

2.5. Statistical analysis

Statistical analysis was performed by GraphPad Prism software. All results are presented as the mean ± standard error, and the statistic quantification was analyzed by *t*-test.

3. Results and discussion

3.1. Hypoxia inhibits the effector functions of HER1-CAR-T cells

To construct HER1-CAR-T cells, a third-generation CAR expressing plasmid containing CD8 signal, CD8 Hinge, CD28, 4–1BB, and CD3ζ expressing sequences was first fused with the sequence encoding HER1 specific single-chain fragment variable (scFv) (Fig. 1a-b). Then, the obtained HER1-CAR expressing plasmid was encapsulated with lentivirus by using 293T/17 cells and was then utilized to transduce T cells to isolate and differentiate from healthy donor's human peripheral blood mononuclear cells (PBMCs) according to our previously used method [35]. Meanwhile, non-specific control CAR T (con-CAR-T) cells were obtained by transducing T cells with the plasmid encoding the non-specific third-generation CAR through the aforementioned method. The successful construction of these two CAR-T cells were confirmed by the emergence of CD3ζ signals at both the detected transcription and protein levels by using real-time polymerase chain reaction (PCR) and western blot analysis, respectively (Fig. 1c-d). The larger molecular weight of CD3ζ of HER1-CAR-T cells compared to that of con-CAR-T cells could further validate the successful fusion of CAR with anti-HER1 scFv.

The impacts of hypoxia incubation on the cell viabilities and effector functions of these newly engineered HER1-CAR-T cells were then carefully investigated. By using the commercial MTT assay kit, it was found that the cell viabilities of con-CAR-T and HER1-CAR-T cells after being incubated under the hypoxia condition (<2% O_2) for 24 h were only ~67% and ~72% compared with the corresponding cells incubated under the normoxic condition (21% O_2) (Fig. 1e). Besides, it was shown that co-incubation of HER1-CAR-T cells with HER1 overexpressing human MDA-MB-468 and MDA-MB-231 TNBC cells under the normoxic condition for 24 h would lead to severe TNBC cell death as indicated by the cytotoxicity assay (Figs. 1f-g and S1). Meanwhile, it was shown that such HER1-CAR-T cells exhibited negligible toxicity to MCF-10A cells with minimal HER1 expression, indicating the superior specific cell killing capacity of HER1-CAR-T cells toward HER1 overexpressing TNBC tumor cells (Fig. S2).

In addition, it was shown that the hypoxia incubation condition remarkably diminished the specific cell killing capacity of HER1-CAR-T cells toward co-cultured MDA-MB-468 and MDA-MB-231 cells. Meanwhile, it was shown that con-CAR-T cells exhibited negligible disturbance on the cell viability of these co-cultured TNBC cells (Fig. 1f-g). Furthermore, it was uncovered that HER1-CAR-T cells co-incubated with MDA-MB-468 and MDA-MB-231 cells at varying feeding ratios exhibited significant secretion of interleukin 2 (IL-2) (Fig. 1h), which in turn is reported to be able to promote the expansion of T cells [36]. In addition, such treatment was also capable of promoting the secretion of tumor suppression cytokines of tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) (Fig. 1h-m). Yet HER1-CAR-T cells incubated under the hypoxic condition exhibited significantly reduced secretion of these tumor suppression cytokines. Consistently, con-CAR-T cells with same treatments showed limited cytokine secretion. In short, these results demonstrate that hypoxia condition could do suppress the cell viability, specific cell killing and effector cytokine secretion capabilities of HER1-CAR-T cells as reported in previous study [37].

3.2. TMP can activate VEGF expression and eNOS/NO axis

Inspired by the high potency of TMP in remodeling blood vessels through activating the VEGF expression and the eNOS/NO axis inside vascular endothelial cells [38], we therefore explored the possibility of using TMP to attenuate hypoxia and thus benefit HER1-CAR-T therapy against TNBC. Firstly, via the CCK-8 assay, it was shown that commercial TMP molecules at a high incubation concentration of up to ~500 nM imposed insignificant cytotoxicity toward human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC), both of which were utilized as the model target cells of TMP molecules (Fig. 2a-b). Then, the potency of TMP treatment in activating the VEGF expression and the eNOS/NO axis inside vascular endothelial cells was carefully investigated via a series of standard assays. Via Western blotting assay, it was shown that treatment of TMP incubation (500 nM, 24 h) resulted in significantly increased expression of VEGF inside both HUVEC and HAEC cells (Fig. 2c-d). It was further uncovered that such TMP incubation promoted the phosphorylation of eNOS, but not obviously impaired the expression of total eNOS (Fig. 2e-f). In addition, such TMP treatment was shown to be capable of promoting the release of NO from HUVEC and HAEC cells by using a commercial NO detection kit (Fig. 2g). These results collectively demonstrate that TMP treatment can not only enhance VEGF expression, but also promote NO production from vascular endothelial cells (Fig. 2h).

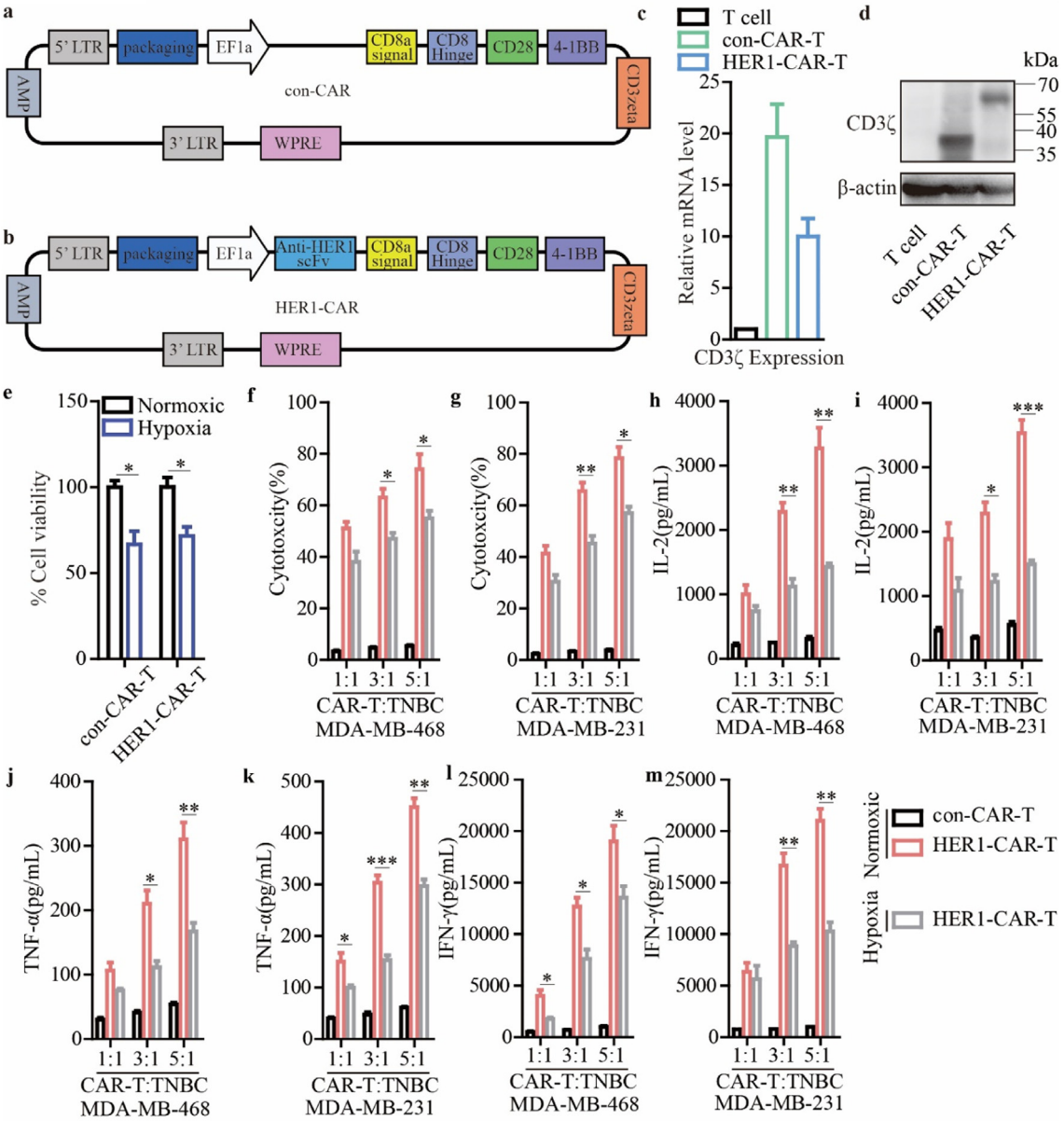

Fig. 1. Construction of HER1-CAR-T cells and their oxygen-dependent effector effects. (a-b) Schematic diagrams showing the genomic organizations of third generation con-CAR (a) and HER1-CAR (b). (c-d) CD3ζ expression levels in the con-CAR-T cells and HER1-CAR-T cells detected by real-time PCR (c) and western blotting (d). (e) Cell viabilities of con-CAR-T and HER1-CAR-T incubated under normoxic condition or hypoxia condition for 24 h. (f-g) Oxygen dependent specific cytotoxicity of HER1-CAR-T cells against MDA-MB-468 (f) and MDA-MB-231 cells (g) at varying effector cells: target cells ratios as indicated. (h-m) Oxygen dependent secretions of IL-2 (h-i), TNF-α (j-k), and IFN-γ (l-m) by HER1-CAR-T cells activated by incubated with MDA-MB-468 and MDA-MB-231 cells at varying feeding ratios as indicated. ($^{***}p < 0.001$, $^{**}p < 0.01$, or $^{*}p < 0.05$).

3.3. Preparation of TMP@PEGgel and its TME modulation effect

The potency of TMP treatment in promoting *in vivo* NO production was carefully investigated (Fig. 3a). To enable sustained release of TMP inside tumors, a type of endogenous H_2O_2 responsive PEGgel was first developed for intratumoral fixation of TMP. It was found that PEGDMA monomer could form hydrogel within one minute upon the addition of H_2O_2 and $FeCl_2$ at optimal feeding ratios (Fig. S3a), both of which can undergo Fenton reaction to generate highly reactive hydroxyl group (·OH), a widely used initiator of polymerization [39,40]. As revealed by scanning electron microscopy, the obtained PEGgel exhibited microscopic porous network structure (Fig. S3b). By recording the absorbance of TMP peaked at 294 nm, it was shown that the TMP loaded PEGgel (TMP@PEGgel) would enable sustained release of TMP (Fig. S3c-e). Via living cell detect assay, such PEGgel exhibited negligible cytotoxicity to both con-CAR-T cells and HER1-CAR-T cells (Fig. S4). Then, rhodamine B was used as a model fluorophore for tracking the *in vivo* behavior of non-fluorescent TMP. As visualized under confocal microscopy, obvious rhodamine B fluorescence was observed on the tumor slices collected from mice with intratumoral fixation of rhodamine B with such PEGgel in 3 days and 10 days post injection (p.i.) (Fig. 3b and S5). In marked contrast, only obvious rhodamine B fluorescence was observed on the tumors slices for mice collected in 3 days p.i. Therefore, these results indicate that PEGgel could enable prolonged retention of loaded small molecules inside tumors.

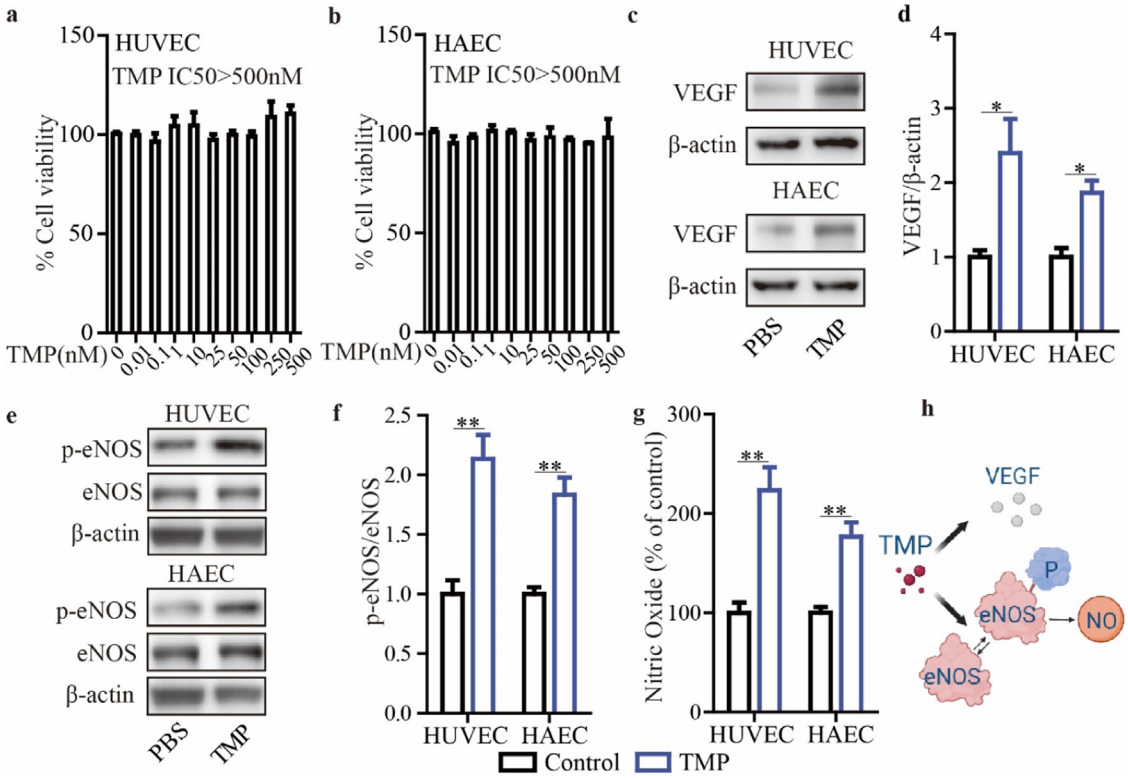

Fig. 2. TMP activates VEGF expression and eNOS/NO axis in vascular endothelial cells. (a-b) Cell viability of HUVEC and HAEC cells incuvated with different concentrations of TMP. (c-d) Western blot analysis (c) and corresponding semiquantitative analysis (d) of VEGF expression levels in HUVEC and HAEC cells with TMP treatment. (e-f) Western blot analysis (e) and corresponding semiquantitative analysis (f) of p-eNOS and eNOS expression levels in HUVEC and HAEC cells with TMP treatment. (g) NO production levels of HUVEC and HAEC cells with TMP treatment. (h) A schematic diagram of TMP activated VEGF expression and eNOS/NO axis. ($^{**}p < 0.01$, or $^{*}p < 0.05$).

Then, the potency of intratumoral TMP fixation on NO production was carefully evaluated on mice bearing MDA-MB-468 and MDA-MB-231 tumors. It was shown that treatment with TMP@PEGgel (TMP = 1 mg/kg) contributed to significantly increased NO production in 3 days and 10 days p.i. compared to these tumor bearing mice with intratumoral injection of saline and plain PEGgel (Fig. 3c-d). In marked contrast, the tumor bearing mice with free TMP treatment only led to increased intratumoral NO production in 3 days p.i. Inspired by the potency of VEGF and NO in promoting angiogenesis and maturation of tumor blood vessels [41,42], we carefully investigated the capacity of TMP@PEGgel treatment in remodeling tumor vasculature. It was shown that treatments with free TMP and TMP@PEGgel were effective in increasing tumor blood vessel intensities in both TNBC xenografts in 3 days p.i., as indicated by the increased expression of CD31, a biomarker of blood vessel endothelial cells, on their tumor slices through the immunofluorescence assay. Furthermore, these two treatments were also able to increase the percentage of effective blood vessels as indicated by measuring the complete margin of blood vessels in 3 days p.i. (Fig. 3e-j). However, only TMP@PEGgel treatment was able to increase intratumoral blood vessel densities and effective vessels percentages in 10 days p.i. In was further shown that plain PEGgel treatment exhibited negligible disturbance on the tumor vasculature. Therefore, these results collectively demonstrate that TMP@PEGgel treatment is capable of remodeling tumor vasculature.

Considering tumor vasculature normalization can enhance tumor blood perfusion and thus tumor oxygenation status, the potency of TMP@PEGgel treatment in relieving tumor hypoxia was therefore carefully evaluated on aforementioned two TNBC tumors xenografts. By using commercial pimonidazole as an exogenous hypoxia-specific probe, we found that treatment with TMP@PEGgel led to dramatically suppressed pimonidazole specific fluorescence signals on tumor slices collected from these two TNBC tumor bearing mice in 3 days and 10 days p.i. under the microscopic observation (Figs. 3k-l and S6a-b). Meanwhile, treatment with free TMP only led to suppressed pimonidazole specific fluorescence on tumor slices collected in 3 days p.i., while other treatments negligibly disturbed the pimonidazole specific fluorescence on tumor slices collected at both time intervals. Moreover, tumor slices of mice with free TMP and TMP@PEGgel treatments showed remarkably reduced expression of hypoxia-inducible factor (HIF)−1α, following similar evolution trends to the results of aforementioned pimonidazole staining assay, via the standard immunofluorescence staining assay (Figs. 3m-n and S6c-d). These results indicate that treatment of TMP@PEGgel enabling sustained release of TMP can effectively attenuate tumor hypoxia by remodeling tumor vasculature in these TNBC tumor xenografts.

3.4. TMP@PEGgel enhances tumor infiltration, survival time and effector functions of HER1-CAR-Tcells

We then investigated the potency of TMP@PEGgel treatment in promoting the tumor infiltration and survival of sequentially administrated HER1-CAR-T cells in both MDA-MB-468 and MDA-MB-231 tumors. HER1-CAR-T cells were first genetically engineered with red fluorescent protein (RFP) for tracking their *in vivo* behaviors post different treatments. We found that tumor slices collected from mice with intratumoral fixation of TMP (TMP = 1 mg/kg, on day 0) and sequential RFP expressing HER1-CAR-T cells injection (5×10^6 cells, on day 1) exhibited obvious RFP fluorescence signals on day 3 and 10 under the microscopic observation (Fig. 4a-c). Consistently, treat-

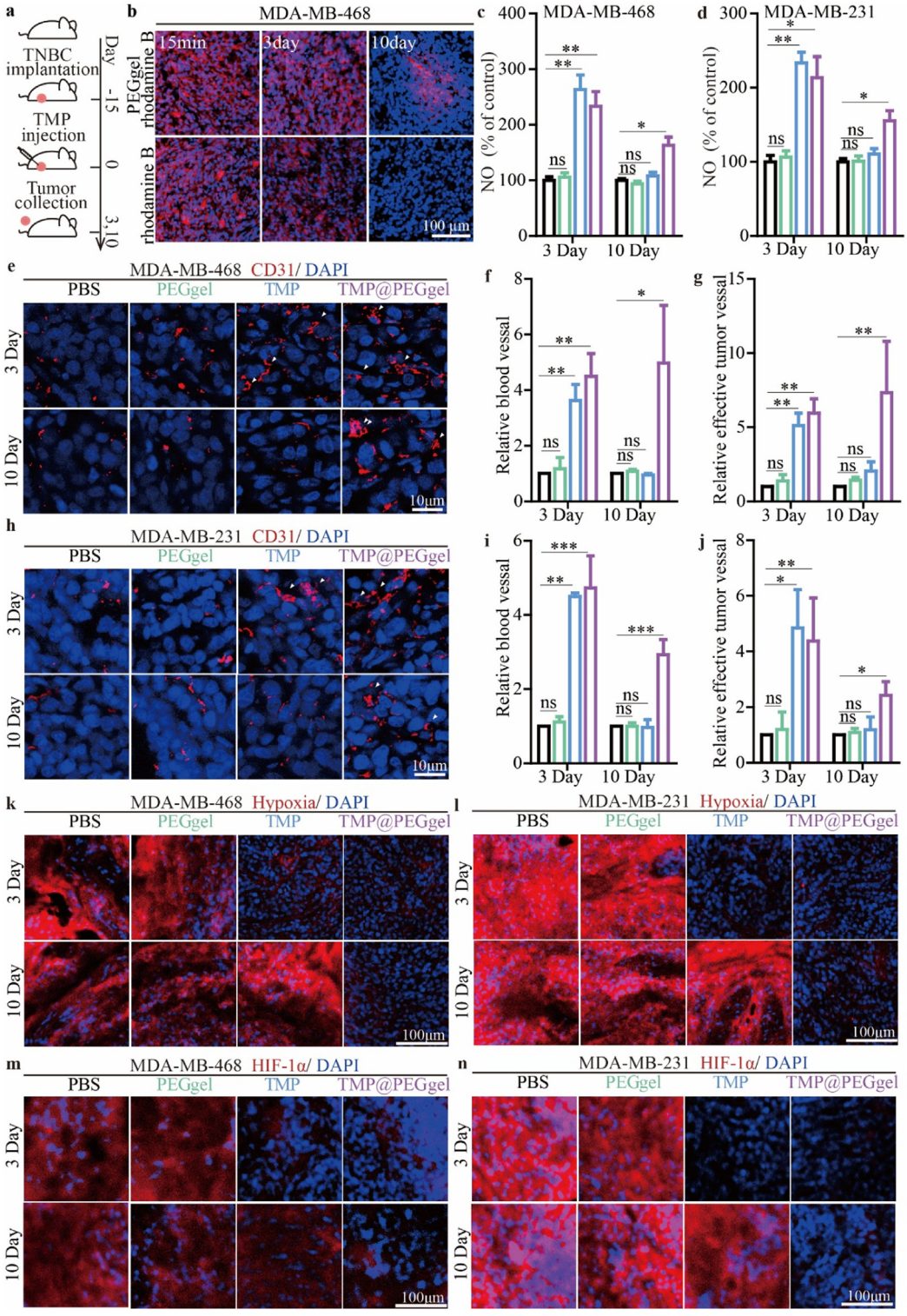

Fig. 3. TMP@PEGgel mediated remodeling of tumor vasculature and tumor hypoxia relief. (a) A schematic diagram of the experimental plan. (b) Fluorescence imaging of tumor slices collected from mice with intratumoral injections of rhodamine B in the presence and absence of PEGgel at 3 days or 10 days p.i. (c-d) NO production levels of MDA-MB-468 tumors (c) and MDA-MB-231 tumors (d) with varying treatments as indicated. (e) Confocal microscopic imaging of blood vessels on MDA-MB-468 tumor slices stained by anti-CD31 and corresponding secondary antibodies. (f-g) Semiquantitative statistical analysis of total blood vessel density (f) and effective vascular area (g) based on the images shown in e. (h) Confocal microscopic imaging of blood vessels on MDA-MB-231 tumor slices stained by anti-CD31 and corresponding secondary antibodies. (i-j) Semiquantitative statistical analysis of total blood vessel density (i) and effective vascular area (j) based on the images shown in h. Color codes in Figure e-j were used to represent corresponding treatments for simplicity. (k-n) Confocal microscopic imaging of different tumor slices with pimonidazole specific (k-l) and HIF-1α specific staining (m-n) post varying treatments as indicated. ($^{***}p < 0.001$, $^{**}p < 0.01$, or $^{*}p < 0.05$).

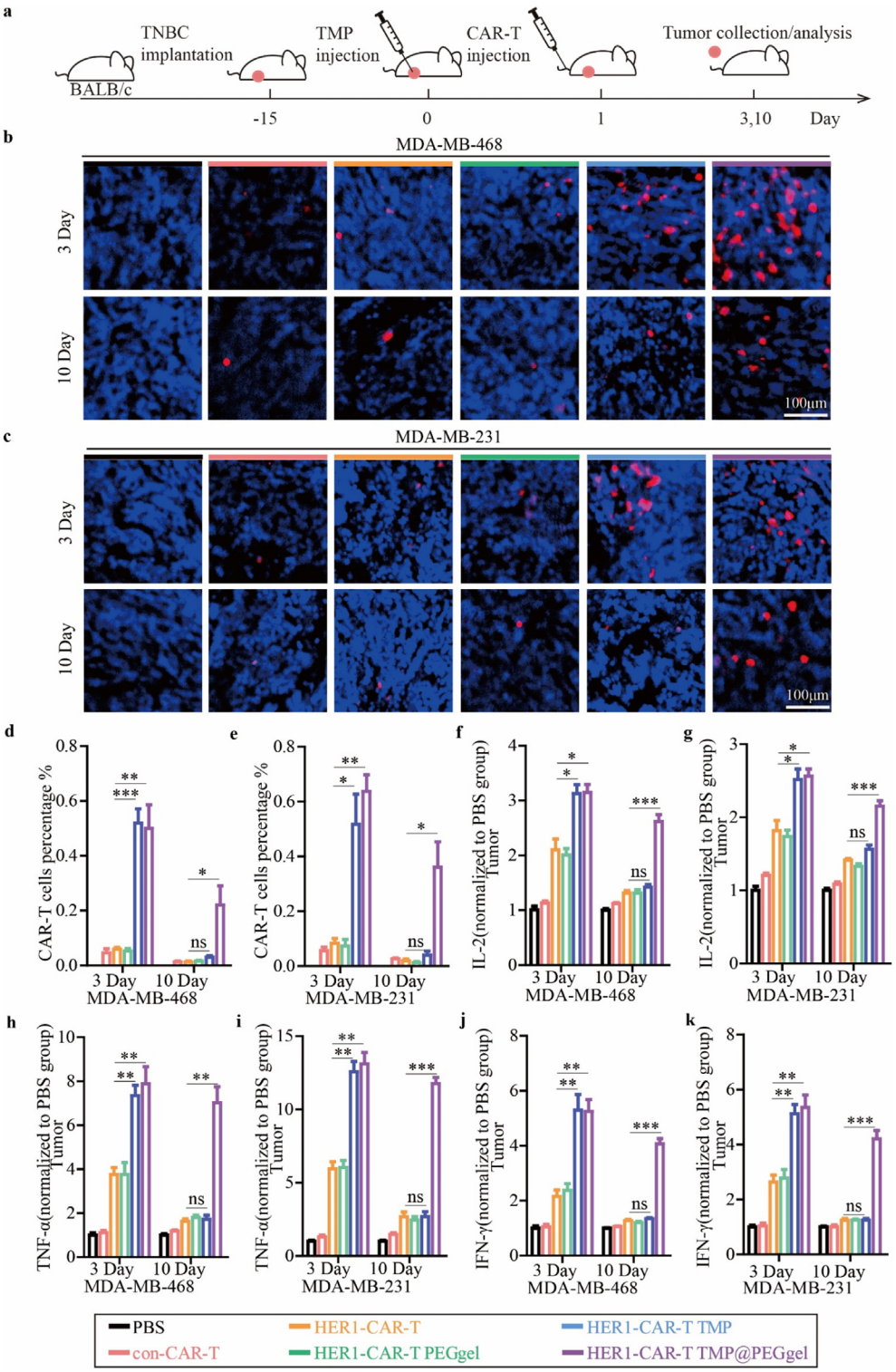

Fig. 4. TMP@PEGgel promoted infiltration, survival and effector functions of HER1-CAR-T cells inside TNBC tumors. (a) A schematic diagram of the experimental schedule. (b-c) Confocal microscopic imaging of RFP positive HER1-CAR-T cells in MDA-MB-468 (b) and MDA-MB-231 tumors (c) with varying treatments as indicated. (d-e) Flow cytometric analysis of the percentages of RFP positive HER1-CAR-T cells in MDA-MB-468 (d) and MDA-MB-231 tumors (e) with varying treatments as indicated. (f-k) Intratumoral secretion levels of IL-2 (f-g), TNF-α (h-i), and IFN-γ (j-k) in MDA-MB-468 and MDA-MB-231 tumors with various treatments as indicated. ($^{***}p < 0.0\,01$, $^{**}p < 0.01$, or $^{*}p < 0.05$).

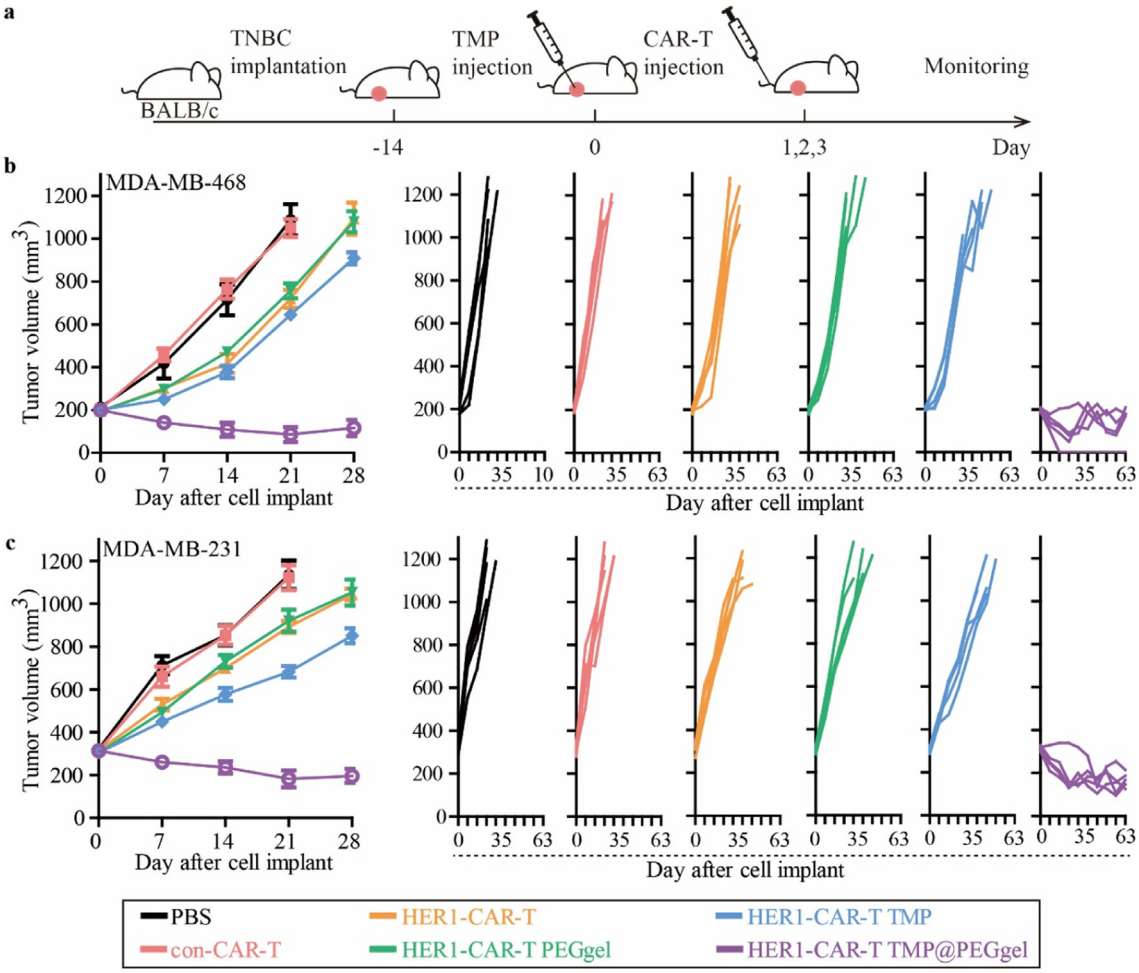

Fig. 5. TMP@PEGgel enhanced HER1-CAR-T cell therapy toward TNBC tumors. (a) A schematic diagram of the experiment schedule. (b, c) Average and individual tumor growth curves of MDA-MB-468 (b) and MDA-MB-231 (c) tumors in mice with different treatments as indicated. (n = 5). Color codes were used to represent corresponding treatments.

ment with free TMP only led to obviously increased tumor infiltration of RFP expressing HER1-CAR-T cells on day 3, while other treatments exhibited minimal effects on the tumor infiltration capacity of these systemically administrated RFP expressing HER1-CAR-T. Furthermore, the potency of TMP@PEGgel in promoting tumor infiltration and survival of HER1-CAR-T cells were confirmed using flow cytometry (Fig. 4d-e).

We then investigated the effects of TMP@PEGgel treatment on the capacity of tumor-infiltrating HER1-CAR-T cells in secreting effector cytokines (*e.g.*, TNFα, IFN-γ, IL-2), which are tightly associated with the activation of CAR-T cells, by using corresponding enzyme linked immunosorbent assay (ELISA) kits. By following the aforementioned therapeutic regime, it was shown that both TNBC tumors on mice with TMP@PEGgel fixation and sequential HER1-CAR-T cells administration exhibited significantly increased secretion levels of IL-2, TNF-α and IFN-γ within 10 days p.i. (Fig. 4f-k). Meanwhile, sequential treatment with free TMP injection and HER1-CAR-T cells administration only led to remarkably increased intratumoral secretions of IL-2, TNF-α and IFN-γ on day 3, while minimally disturbed their secretion on day 10. In sharp contrast, other treatments showed limited impacts on the secretion of these proinflammatory cytokines. These results collectively suggest that TMP@PEGgel treatment can improve the effector functions of HER1-CAR-T cells by enhancing their capacity in secreting proinflammatory cytokines.

3.5. TMP@PEGgel enhances the tumor suppression effect of HER1-CAR-T cells

We then assessed the efficacy of TMP@PEGgel treatment in enhancing the *in vivo* tumor suppression effects of HER1-CAR-T cells on TNBC tumor bearing mice as aforementioned. 30 mice bearing MDA-MB-468 tumors were randomly divided into six groups and received following treatments: 1) PBS, 2) con-CAR-T injection, 3) HER1-CAR-T injection, 4) PEGgel fixation + HER1-CAR-T injection, 5) TMP injection + HER1-CAR-T injection, 6) TMP@PEGgel fixation + HER1-CAR-T injection (Fig. 5a). TMP (1 mg/kg) in the presence and absence of PEGgel were intratumorally injected on day 0, while HER-1-CAR-T cells or non-CAR-T cells (5×10^6 cells per injection) were intravenous injected for three times on day 1, 2 and 3. By measuring tumor sizes, treatment with sequential TMP@PEGgel fixation and HER1-CAR-T injection exhibited the highest potency in regressing the growth of MDA-MB-468 tumors, 1 tumor completely disappeared while the other four tumors showed no obvious growth within 63 days post the treatment tumors (Fig. 5b). In marked contrast, other treatments only slightly delayed tumor growth, and all of these mice died within 56 days post the corresponding treatments. In addition, body weights of mice with varying treatments were negligible disturbed throughout the whole monitoring process (Fig. S7a). Moreover, the excellent therapeutic potency of such TMP@PEGgel assisted HER1-CAR-T treatment was further confirmed

on mice bearing MDA-MB-231 tumors (Figs. 5c and S7b). These results collectively indicate that sequential treatment of TMP@PEGgel fixation and HER1-CAR-T cells is effective in suppressing the growth of these HER1 positive TNBC tumors.

4. Conclusion

In conclusion, we prepared an endogenous H_2O_2 responsive hydrogels composed of PEGDMA and $FeCl_2$ to enable sustained release of Chinese herbal extracts TMP. Upon intratumoral fixation, the obtained TMP@PEGgel promoted angiogenesis and tumor vasculature maturation through activating the VEGF expression and the eNOS/NO axis of vascular endothelial cells. Resultantly, such TMP@PEGgel treatment enabled effective sustained tumor hypoxia relief and thus was capable of enhancing the tumor infiltration, survival and effector functions of intravenously infused HER1-CAR-T cells inside TNBC tumors inoculated on Balb/c nude mice. It was further shown that our TMP@PEGgel assisted HER1-CAR-T cell therapy could thereby synergically suppress the growth of both MDA-MB-468 and MDA-MB-231 TNBC tumors. In this study, it is highlighted that TME remodeling through reprogramming tumor vasculature with TMP@PEGgel treatment is a promising and robust strategy to reinforce the therapeutic potency of CAR-T cell therapies toward solid tumors.

CRediT authorship contribution statement

Yan Liu: Conceptualization, Investigation, Writing – review & editing. **Yu Hao:** Investigation. **Xiang Lv:** Investigation. **Yefei Zhang:** Investigation. **Jiahui Chen:** Investigation. **Jia Tian:** Investigation. **Xinxing Ma:** Investigation. **Yehui Zhou:** Investigation. **Liangzhu Feng:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest in this work.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fmre.2023.05.016.

References

[1] G. Bianchini, C. De Angelis, L. Licata, et al., Treatment landscape of triple-negative breast cancer - expanded options, evolving needs, Nat. Rev. Clin. Oncol. 19 (2022) 91–113.
[2] A.C. Garrido-Castro, N.U. Lin, K. Polyak, Insights into molecular classifications of triple-negative breast cancer: Improving patient selection for treatment, Cancer Discov. 9 (2019) 176–198.
[3] M. Shen, Y.Z. Jiang, Y. Wei, et al., Tinagl1 suppresses triple-negative breast cancer progression and metastasis by simultaneously inhibiting Integrin/FAK and EGFR signaling, Cancer Cell 35 (2019) 64–80 e7.
[4] Q. Liu, S. Yu, W. Zhao, et al., EGFR-TKIs resistance via EGFR-independent signaling pathways, Mol. Cancer 17 (2018) 53.
[5] C.R. Chong, P.A. Janne, The quest to overcome resistance to EGFR-targeted therapies in cancer, Nat. Med. 19 (2013) 1389–1400.
[6] M. Hong, J.D. Clubb, Y.Y. Chen, Engineering CAR-T cells for next-generation cancer therapy, Cancer Cell 38 (2020) 473–488.
[7] L. Xia, Z. Zheng, J.Y. Liu, et al., Targeting triple-negative breast cancer with combination therapy of EGFR CAR T cells and CDK7 inhibition, Cancer Immunol. Res. 9 (2021) 707–722.
[8] C. Kantari-Mimoun, S. Barrin, L. Vimeux, et al., CAR T-cell entry into tumor islets is a two-step process dependent on IFNgamma and ICAM-1, Cancer Immunol. Res. 9 (2021) 1425–1438.
[9] H. Li, Y. Huang, D.Q. Jiang, et al., Antitumor activity of EGFR-specific CAR T cells against non-small-cell lung cancer cells *in vitro* and in mice, Cell Death Dis. 9 (2018) 177.
[10] J. Wagner, E. Wickman, C. DeRenzo, et al., CAR T cell therapy for solid tumors: Bright future or dark reality? Mol. Ther. 28 (2020) 2320–2339.
[11] R. Huang, X. Li, Y. He, et al., Recent advances in CAR-T cell engineering, J. Hematol. Oncol. 13 (2020) 86.
[12] L. Labanieh, R.G. Majzner, C.L. Mackall, Programming CAR-T cells to kill cancer, Nat. Biomed. Eng. 2 (2018) 377–391.
[13] A.K. Singh, J.P. McGuirk, CAR T cells: Continuation in a revolution of immunotherapy, Lancet Oncol. 21 (2020) e168–e178.
[14] E. Roselli, R. Faramand, M.L. Davila, Insight into next-generation CAR therapeutics: Designing CAR T cells to improve clinical outcomes, J. Clin. Invest. 131 (2021).
[15] Q. Hu, H. Li, E. Archibong, et al., Inhibition of post-surgery tumour recurrence via a hydrogel releasing CAR-T cells and anti-PDL1-conjugated platelets, Nat. Biomed. Eng. 5 (2021) 1038–1047.
[16] M.C. van Schalkwyk, S.E. Papa, J.P. Jeannon, et al., Design of a phase I clinical trial to evaluate intratumoral delivery of ErbB-targeted chimeric antigen receptor T-cells in locally advanced or recurrent head and neck cancer, Hum. Gene. Ther. Clin. Dev. 24 (2013) 134–142.
[17] J. Lemoine, M. Ruella, R. Houot, Overcoming intrinsic resistance of cancer cells to CAR T-cell killing, Clin. Cancer Res. 27 (2021) 6298–6306.
[18] Y. Tian, Y. Li, Y. Shao, et al., Gene modification strategies for next-generation CAR T cells against solid cancers, J. Hematol. Oncol. 13 (2020) 54.
[19] J. Li, W. Li, K. Huang, et al., Chimeric antigen receptor T cell (CAR-T) immunotherapy for solid tumors: Lessons learned and strategies for moving forward, J. Hematol. Oncol. 11 (2018) 22.
[20] D.L. Wagner, E. Fritsche, M.A. Pulsipher, et al., Immunogenicity of CAR T cells in cancer therapy, Nat. Rev. Clin. Oncol. 18 (2021) 379–393.
[21] L. Bejarano, M.J.C. Jordao, J.A. Joyce, Therapeutic targeting of the tumor microenvironment, Cancer Discov. 11 (2021) 933–959.
[22] C. Viallard, B. Larrivee, Tumor angiogenesis and vascular normalization: Alternative therapeutic targets, Angiogenesis 20 (2017) 409–426.
[23] Y. Sun, W. Chen, R.J. Torphy, et al., Blockade of the CD93 pathway normalizes tumor vasculature to facilitate drug delivery and immunotherapy, Sci. Transl. Med. 13 (604) (2021) eabc8922.
[24] D. Fukumura, J. Kloepper, Z. Amoozgar, et al., Enhancing cancer immunotherapy using antiangiogenics: Opportunities and challenges, Nat. Rev. Clin. Oncol. 15 (2018) 325–340.
[25] Y. Su, W. Xu, Q. Wei, et al., Chiral polypeptide nanoparticles as nanoadjuvants of nanovaccines for efficient cancer prevention and therapy, Sci. Bull. 68 (2023) 284–294.
[26] J. Zhang, Y. Lin, Z. Lin, et al., Stimuli-responsive nanoparticles for controlled drug delivery in synergistic cancer immunotherapy, Adv. Sci. 9 (2022) e2103444.
[27] W.G. Xu, Y.Z. Su, Y. Ma, et al., Immunologically effective poly(D-lactic acid) nanoparticle enhances anticancer immune response, Sci. China Chem. 66 (2023) 1150–1160.
[28] Y. Liu, X.X. Ma, Y.J. Zhu, et al., pH-responsive nanomedicine co-encapsulated with Erlotinib and chlorin e6 can enable effective treatment of triple negative breast cancer via reprogramming tumor vasculature, Chem. Eng. J. 437 (2022) 135305.
[29] J. Wei, X. Han, J. Bo, et al., Target selection for CAR-T therapy, J. Hematol. Oncol. 12 (2019) 62.
[30] C. Yang, G. Mu, Y. Zhang, et al., Supramolecular nitric oxide depot for hypoxic tumor vessel normalization and radiosensitization, Adv. Mater. 34 (37) (2022) e2202625.
[31] D.C. Singleton, A. Macann, W.R. Wilson, Therapeutic targeting of the hypoxic tumour microenvironment, Nat. Rev. Clin. Oncol. 18 (2021) 751–772.
[32] Y.C. Sung, P.R. Jin, L.A. Chu, et al., Delivery of nitric oxide with a nanocarrier promotes tumour vessel normalization and potentiates anti-cancer therapies, Nat. Nanotechnol. 14 (2019) 1160–1169.

[33] B. Gao, X. Lin, H. Jing, et al., Local delivery of tetramethylpyrazine eliminates the senescent phenotype of bone marrow mesenchymal stromal cells and creates an anti-inflammatory and angiogenic environment in aging mice, Aging Cell 17 (2018) e12741.
[34] L. Xia, Z.Z. Zheng, J.Y. Liu, et al., EGFR-targeted CAR-T cells are potent and specific in suppressing triple-negative breast cancer both *in vitro* and *in vivo*, Clin. Transl. Immunol. 9 (2020) e01135.
[35] Y. Liu, Y. Zhou, K.H. Huang, et al., EGFR-specific CAR-T cells trigger cell lysis in EGFR-positive TNBC, Aging 11 (2019) 11054–11072.
[36] O. Boyman, J. Sprent, The role of interleukin-2 during homeostasis and activation of the immune system, Nat. Rev. Immunol. 12 (2012) 180–190.
[37] G. Liu, W. Rui, X. Zhao, et al., Enhancing CAR-T cell efficacy in solid tumors by targeting the tumor microenvironment, Cell Mol. Immunol. 18 (2021) 1085–1095.
[38] M. Guo, Y. Liu, D. Shi, Cardiovascular actions and therapeutic potential of tetramethylpyrazine (Active Component Isolated from Rhizoma Chuanxiong): Roles and mechanisms, Biomed. Res. Int. 2016 (2016) 2430329.
[39] W.E. Hennink, C.F. van Nostrum, Novel crosslinking methods to design hydrogels, Adv. Drug. Deliv. Rev. 54 (2002) 13–36.
[40] A. Reyhani, T.G. McKenzie, Q. Fu, et al., Fenton-chemistry-mediated radical polymerization, Macromol. Rapid Commun. 40 (2019) e1900220.
[41] X. Zhang, C. Li, F. Liu, et al., High-throughput production of functional prototissues capable of producing NO for vasodilation, Nat. Commun. 13 (2022) 2148.
[42] R.S. Apte, D.S. Chen, N. Ferrara, VEGF in signaling and disease: Beyond discovery and development, Cell 176 (2019) 1248–1264.

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