CD47 monoclonal antibody enhances the inhibitory effect of anti-HER2 chimeric antigen receptor macrophages on ovarian cancer

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Abstract. Macrophages have garnered notable interest as therapeutic vehicles due to their innate phagocytic ability, tumor tropism, and pivotal role in linking innate and adaptive immunity. These attributes have led to the development of various macrophage-centered treatments. Among them, chimeric antigen receptor macrophages (CAR-Ms) have emerged as a promising adoptive cell therapy, demonstrating potential for clinical application in multiple solid tumors. However, their effector functions remain amenable to further enhancement. In the present study, anti-HER2 CAR-Ms were constructed using an adenoviral vector system. The specificity and antitumor efficacy against $HER2^+$ ovarian cancer cells were assessed using flow cytometry. The effects of a CD47 monoclonal antibody (mAb) on CAR-M-mediated killing and phenotypic polarization were subsequently investigated. Additionally, the combined therapeutic benefit of CD47 mAb and anti-HER2 CAR-Ms was evaluated in a murine subcutaneous xenograft model of ovarian cancer. Anti-HER2 CAR-Ms specifically recognized and killed HER2-expressing ovarian cancer cells. The addition of CD47 mAb enhanced the phagocytic capacity, promoted a pro-inflammatory phenotype and inflammatory cytokine secretion in CAR-Ms. *In vivo*, combination therapy significantly reduced tumor burden in xenograft-bearing mice. Collectively, these findings indicate that the combination of CAR-Ms with CD47 blockade offers a robust and promising strategy for personalized targeted therapy in solid tumors.

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Abbreviations: CAR, chimeric antigen receptor; CAR-M, chimeric antigen receptor macrophage; CRS, cytokine release syndrome; FBS, fetal bovine serum; ICIs, immune checkpoint inhibitors; monoclonal antibody, mAb; scFv, single-chain variable fragment; TME, tumor microenvironment

Key words: chimeric antigen receptor, macrophage, CD47 monoclonal antibody, immunotherapy

Introduction

Ovarian cancer is the most lethal gynecological malignancy, characterized by extensive peritoneal metastasis and the frequent development of chemoresistance, which necessitates the exploration of novel therapeutic strategies (1). Amplification or upregulation of HER2 has been documented in a subset of ovarian cancer, particularly in the aggressive subtypes lacking effective targeted therapies, making it a compelling candidate for targeted intervention (2). Despite the marked success of chimeric antigen receptor (CAR)-T cell therapy in hematological malignancies, its clinical application in solid tumors such as ovarian cancer has been hampered by the immunosuppressive tumor microenvironment (TME) and the physical barriers posed by the fibrous stroma that restrict T-cell infiltration (3). Against this backdrop, novel cell therapies based on CAR engineering platforms have been proposed and are undergoing continuous optimization (4).

Developed based on the innate biological properties of macrophages, CAR-macrophage (CAR-M) technology represents an innovative approach in the field of immunotherapy (5-7). This strategy involves genetically modifying macrophages to express specific CARs, thereby enabling targeted recognition of tumor-associated antigens and enhancing their phagocytic activity (1). In contrast to the clinically established CAR-T cell therapy, CAR-M therapy exhibits distinct antitumor mechanisms: Engineered macrophages are capable of not only phagocytosing and presenting antigens, but also potently initiating immune cascade responses (8). This innovative methodology markedly augments the capacity of the immune system to identify and eliminate malignant cells, offering novel pathways for cancer treatment. However, the antitumor efficacy of first-generation CAR-Ms remains

limited, and its sustained *in vivo* performance lacks robust support from extensive preclinical and clinical evidence. In light of these limitations, novel engineering strategies are being explored to enhance the functionality and potency of CAR-Ms (9).

During the development of CAR immune cell therapies, such as CAR-T and CAR-natural killer (NK) cells, researchers have demonstrated that co-expression of cytokines alongside the CAR construct notably enhances therapeutic efficacy (10,11). This strategy not only improves the biological functions and *in vivo* persistence of CAR immune cells, but also concurrently activates both innate and adaptive immune responses. It facilitates the release of multiple inflammatory mediators and chemokines, promotes immune cell infiltration into solid tumors, and enhances tumor recognition and clearance (12).

In the context of CAR-M therapy, co-expression of IFN-γ has been shown to augment phagocytic activity and promote a pro-inflammatory polarization, thereby converting immunologically 'cold' tumors into 'hot' tumors and strengthening antitumor responses (13). Moreover, key regulatory genes have been identified that modulate these processes. For example, aconitate decarboxylase 1 (ACOD1) in macrophages acts as a crucial metabolic regulator involved in inflammatory regulation. Wang *et al* (14) demonstrated that ACOD1 knockout increased reactive oxygen species production, enhanced phagocytosis, improved tumor-killing capacity and prolonged survival in ovarian tumor-bearing mice. In addition, optimization of CAR structure remains a central strategy for enhancing immune cell function. In CAR-M research, including several products currently undergoing clinical studies (such as CT-0508 and SY001), the intracellular domain design based on CD3ζ is still employed. This signaling molecule, originally applied in CAR-T cell therapy, can still function in macrophages by inducing spleen associated tyrosine kinase activation. However, to fully realize the therapeutic potential of CAR-Ms, it is essential to move beyond the constraints of conventional T cell signaling and integrate macrophage-specific intracellular domains to achieve optimal efficacy. For example, tandem Toll/interleukin-1 receptor/resistance protein and CD3ζ domains have been shown to induce potent phagocytosis and inflammatory polarization (15). Beyond direct CAR modification and genetic regulation, combination strategies integrating cell therapies with immune checkpoint inhibitors (ICIs) represent another highly promising approach (16).

The combination of ICIs with cell-based therapies has been increasingly validated in clinical practice, markedly improving the durability and efficacy of cellular treatments. In CAR-T cell therapy for hematological malignancies, the incorporation of ICIs [programmed cell death protein 1 (PD-1) or programmed death ligand 1 (PD-L1) monoclonal antibodies (mAbs] has demonstrated notable benefits, including higher response rates and manageable toxicity profiles, supported by robust data from multiple clinical trials (17,18). By contrast, clinical outcomes in solid tumors remain modest despite ongoing investigations, with some studies reporting conflicting results (19,20). While CAR-M therapy has emerged as a promising modality for solid tumors, its combination with ICIs, particularly those targeting phagocytosis checkpoints, has been underexplored. Due to the dependence of CAR-Ms on phagocytic function and the limited antitumor activity of first-generation constructs, blockade of phagocytosis checkpoints may represent a viable strategy to enhance CAR-M efficacy. The present study focused on evaluating the combined use of a CD47 mAb and CAR-M therapy, with the aim of developing a novel augmentation strategy based on phagocytosis checkpoint inhibition.

Materials and methods

Cells and animals. The cell lines and their culture media used in the present study, namely SKOV3, A2780 and THP-1, were obtained from Procell Life Science & Technology Co., Ltd. THP-1 and A2780 cells were cultured in RPMI-1640 medium (cat. no. 01-100-1ACS; Shanghai Basalmedia Technologies Co., Ltd.) supplemented with 10% fetal bovine serum (FBS; cat. no. 087-150; Wisent Biotechnology), whereas SKOV3 cells were maintained in McCoy's 5A medium (cat. no. L630KJ; Shanghai Basalmedia Technologies Co., Ltd.) containing 10% FBS. All cell lines were incubated at 37˚C under a humidified atmosphere containing 5% CO_2. The identity of each cell line was confirmed by short tandem repeat profiling, and regular testing was conducted to ensure they were free from mycoplasma contamination.

Wild-type female BALB/c nude mice (age, 6-8 weeks) were acquired from GemPharmatech Co., Ltd. and housed under specific pathogen-free (SPF) conditions. The animals were provided with SPF-grade water and diet, and maintained under a 12-h light/dark cycle. All experimental procedures were approved by the Animal Ethics Committee of Anhui Medical University (Hefei, China; approval no. PZ-2025-029).

In vivo tumor model. To establish a xenograft tumor model, tumor cells in the logarithmic growth phase were harvested, resuspended in pre-cooled PBS and inoculated subcutaneously into nude mice. Specifically, $1x10^6$ SKOV3 cells were injected into the subcutaneous space of the flank region. A total of 4 h after inoculation, $2x10^6$ anti-HER2 CAR-Ms or empty vector-transduced macrophages were administered via subcutaneous injection. Once the average tumor volume reached 50 mm^3, the mice treated with anti-HER2 CAR-Ms were randomly divided into two groups, one of which received an intravenous injection of CD47 mAb (cat. no. E16HU047; EnoGene Biotech Co, Ltd.) via the tail vein. Tumor growth was monitored regularly, with measurements taken every 3 days. When the maximum tumor volume approached 1,500 mm^3, euthanasia was performed on the tumor-bearing mice using inhalational anesthetics. For euthanasia, animals were deeply anesthetized with 5% isoflurane for 15 min in a closed chamber until respiration ceased completely. Death was confirmed by absence of spontaneous breathing for >5 min and absence of heartbeat. No additional drugs were used.

CAR-M production. The CAR construct was cloned into a replication-deficient type 5 adenoviral vector backbone under the control of a CMV promoter, and high-titer adenovirus was subsequently packaged by OBiO Technology (Shanghai) Corp., Ltd. Anti-HER2 CAR-Ms and vector control cells were generated by transducing THP-1-derived macrophages with the adenovirus, according to the manufacturer's

instructions. Transduced cells were digested with Accutase (cat. no. A6964-100ML; Sigma-Aldrich; Merck KGaA) cell dissociation solution and collected. Subsequently, the expression levels of green fluorescent protein (GFP) in the cells were analyzed using flow cytometry to determine CAR expression.

Flow cytometry. After collection, the cells were resuspended in PBS and labeled with corresponding flow cytometry antibodies. The mixture was incubated at 4˚C for 30 min, followed by washing and processing on a flow cytometer. For intracellular proteins requiring fixation and permeabilization, the cells were first incubated with fixation buffer (cat. no. 00-5223-57; Thermo Fisher Scientific, Inc.) at 20˚C for 10-20 min, followed by permeabilization buffer (cat. no. 00-5223-57; Thermo Fisher Scientific, Inc.) at 20˚C for 10-15 min before antibody labeling. The antibodies used included anti-HER2 (cat. no. MA5-60199; Invitrogen; Thermo Fisher Scientific, Inc.), anti-CD86 (cat. no. 374207; BioLegend, Inc.), anti-CD206 (cat. no. 321125; BioLegend, Inc.) and anti-Ki-67 (cat. no. 350514; BioLegend, Inc.). The experiment was conducted using a flow cytometer (CytoFLEX; Beckman Coulter, Inc.) and the results were analyzed with CytExpert software (version 2.6.0.105; Beckman Coulter, Inc.).

Analysis of phagocytosis by flow cytometry. Briefly, 1 μl CMPTX dye (cat. no. C34552; Invitrogen; Thermo Fisher Scientific, Inc.) was added to a 1-ml/1x10^6 SKOV3 or A2780 cell suspension and incubated in a cell culture incubator at 37˚C for 20-30 min, after which, the cells were centrifuged at 1,500 rpm (125.7 x g) for 5 min at 4˚C, the supernatant was discarded and the cells were washed with PBS to remove residual dye. THP-1M cells or anti-HER2 CAR-Ms, each expressing GFP, were co-cultured with tumor cells (SKOV3 or A2780) labeled with CMPTX (detected via the ECD channel) dye at a 1:1 ratio (1x10^5 cells each) in a 37˚C incubator for 4 h. After co-culture, all cells were collected, washed with PBS and subjected to flow cytometry. None of the macrophages received prior M1 stimulation. The percentage of $CMPTX^+$ cells within the GFP^+ macrophage population was used to quantify phagocytosis of target cells by macrophages. Flow cytometry was performed on a CytoFLEX instrument (CytoFLEX; Beckman Coulter, Inc.) and the data were analyzed using CytExpert software.

Analysis of the effects of CD47 mAb on the phagocytic and cytotoxic activities of CAR-Ms. Co-culture systems were established in three experimental groups: Empty control + SKOV3; anti-HER2 CAR-M + SKOV3; and anti-HER2 CAR-M + SKOV3 + CD47 mAb. For the phagocytosis assay, 1x10^5 GFP^+ THP-1Ms or anti-HER2 CAR-Ms were co-cultured with 1x10^5 CMPTX-labeled SKOV3 cells (pretreated with CD47 mAb) at 37˚C for 4 h. Following co-culture, all cells were collected, washed with PBS and subjected to flow cytometric analysis. Please note that none of the macrophages received prior M1 stimulation. The percentage of $CMPTX^+$ cells within the GFP^+ macrophage population was quantified to evaluate phagocytic activity.

In the cytotoxicity assay, unlabeled tumor cells were co-cultured with GFP^+ CAR-Ms for 24 h. The residual tumor cells were then identified as the GFP^- cell population and normalized to the control group. All flow cytometry data were acquired using a CytoFLEX flow cytometer and analyzed with CytExpert software.

ELISA. The concentrations of IFN-γ (cat. no. ELH-IFNg; RayBio, Inc.), TNF-α (cat. no. ELH-TNFα; RayBio, Inc.), IL-6 (cat. no. KIT10395A; Sino Biological, Inc.) and IL-1β (cat. no. EL-H0149; Wuhan Elabscience Biotechnology Co., Ltd.) in the supernatant of the co-culture system were detected by ELISA kits, according to the manufacturer's instructions. The results were analyzed using a microplate reader (Infinite M1000 Pro; Tecan Group, Ltd.).

H&E staining and immunohistochemistry. Immunohistochemical experiments were performed according to standard protocols (21), and antigen retrieval was performed as per the requirements of the primary antibodies, including proliferating cell nuclear antigen (PCNA; cat. no. ab92552; Abcam) and α-smooth muscle actin (SMA; cat. no. ab5694; Abcam). Briefly, isolated tumor tissues were fixed with 4% paraformaldehyde (cat. no. BL539A; Biosharp Life Sciences) at room temperature for 24 h, then embedded in paraffin and cut into 5-μm sections. After dewaxing, the sections were subjected to antigen retrieval by heating in citrate buffer (pH 6.0) at 100˚C for 15 min, followed by rehydration with 0.5% Triton X-100 (cat. no. X100-100ML; MilliporeSigma), followed by blocking with 1.5% BSA (from a two-step detection kit; cat. no. PV-9000; OriGene Technologies, Inc.) at 37˚C for 30 min. The primary antibody was diluted at 1:200 and incubated with the sections overnight at 4˚C. Subsequently, a horseradish peroxidase-conjugated secondary antibody (from a two-step detection kit; cat. no. PV-9000; OriGene Technologies, Inc.) was diluted at 1:500 and incubated with the sections at 37˚C for 30 min, and staining was developed with DAB (cat. no. BB-3503; BestBio, Inc.). Images of the sections were then captured using a brightfield microscope (Leica Microsystems, Inc.).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8.0 (Dotmatics). Data normality and homogeneity of variances were assessed using the Shapiro-Wilk test and Levene's test, respectively. For comparisons between two independent groups, an unpaired two-tailed Student's t-test was applied. For comparisons among three or more groups, one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons was used. All experiments were performed independently, at least in triplicate, and data are presented as the mean ± standard error. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Anti-HER2 CAR-Ms can specifically target HER2-positive ovarian cancer cells. An anti-HER2 CAR was constructed (Fig. 1A), in which the extracellular antigen-binding domain consisted of an anti-HER2 single-chain variable fragment (scFv) that specifically recognizes HER2. CD8α was utilized as a hinge region to connect the antigen-binding domain to the transmembrane domain derived from CD28. The intracellular signaling domain was composed of CD3ζ, which

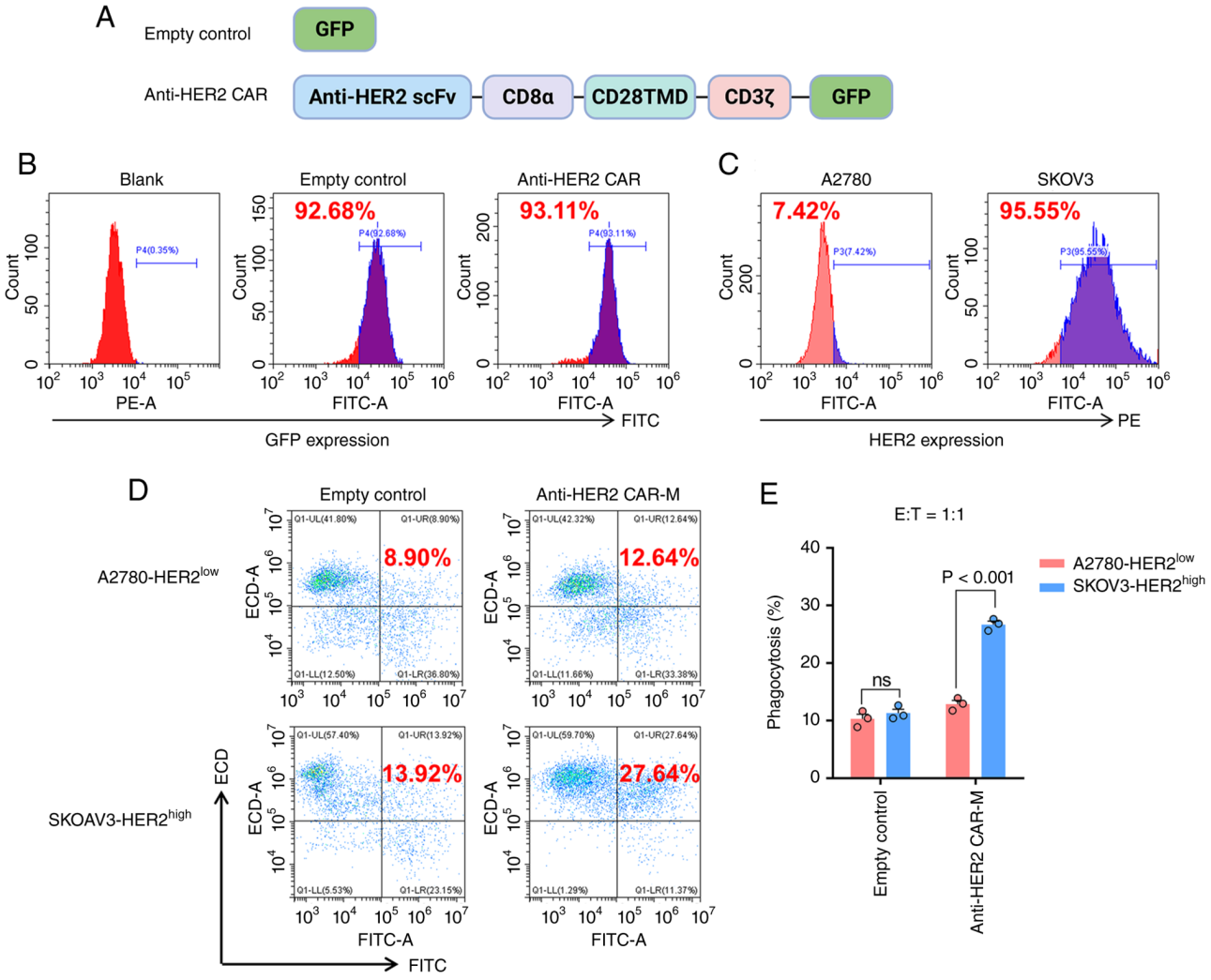

Figure 1. Construction and validation of anti-HER2 CAR-M. (A) Schematic depiction of the anti-HER2 CAR construct. (B) Transduction efficiency of the anti-HER2 CAR lentivirus in macrophages, as assessed using flow cytometry. (C) Flow cytometric analysis of HER2 expression on the surface of SKOV3 and A2780 cell lines. (D and E) Phagocytosis of A2780 or SKOV3 tumor cells by CAR-Ms following a 4-h co-culture was assessed using flow cytometry (n=3). Double positivity for FITC and ECD indicates CAR-M cells that have undergone phagocytosis. In the representative flow cytometry plot, the first quadrant (upper left) shows ECD^+ tumor cells; the second quadrant (upper right) shows CAR-Ms or control macrophages that underwent phagocytosis (ECD^+ $FITC^-$); the third quadrant (lower left) shows macrophages that did not express GFP (ECD^- $FITC^-$); the fourth quadrant (lower right) shows CAR-Ms or control macrophages that did not undergo phagocytosis (ECD^- $FITC^+$). CAR-M, chimeric antigen receptor macrophage; ns, not significant.

contains multiple immunoreceptor tyrosine-based activation motifs responsible for activating macrophage phagocytosis. Additionally, GFP was incorporated as a reporter gene to monitor CAR expression and subcellular localization. Control macrophages were transduced with an empty vector.

The GFP tag was used to indicate the transduction of the CAR molecule. Flow cytometric analysis demonstrated high GFP expression in both the control and anti-HER2 CAR-Ms (Figs. 1B and S1). To evaluate the target specificity of anti-HER2 CAR-Ms, two ovarian cancer cell lines were selected: SKOV3, which exhibits high HER2 expression; and A2780, with low HER2 expression, as confirmed by flow cytometry (Fig. 1C). Upon co-culture with CAR-Ms, significant phagocytosis of SKOV3 cells was observed, whereas only baseline phagocytic activity was detected against A2780 cells (Fig. 1D and E). These results indicate that anti-HER2 CAR-Ms were successfully constructed and can specifically target and phagocytose HER2-positive ovarian cancer cells.

CD47 mAb enhances the anti-ovarian cancer activity of anti-HER2 CAR-Ms in vitro. To investigate the potential synergistic effect between CD47 mAb and CAR-Ms, anti-HER2 CAR-Ms were co-cultured with tumor cells in the presence or absence of CD47 mAb. Given that the targeting specificity of the CAR molecule has been validated by co-culture experiments, here co-culture was performed only with HER2-positive SKOV3 cells. Flow cytometric analysis revealed that the presence of the CD47 mAb enhanced the phagocytic ability of anti-HER2 CAR-Ms against SKOV3 cells (Figs. 2A and B, S1). Furthermore, the effect of CD47 mAb was evaluated on the cytotoxic function of anti-HER2 CAR-Ms. The results demonstrated that CD47 mAb potentiated the elimination of HER2-positive ovarian cancer cells by anti-HER2 CAR-Ms (Fig. 2C and D). Collectively, these findings indicated that CD47 mAb significantly augments the antitumor activity of anti-HER2 CAR-Ms against target antigen-positive ovarian cancer cells.

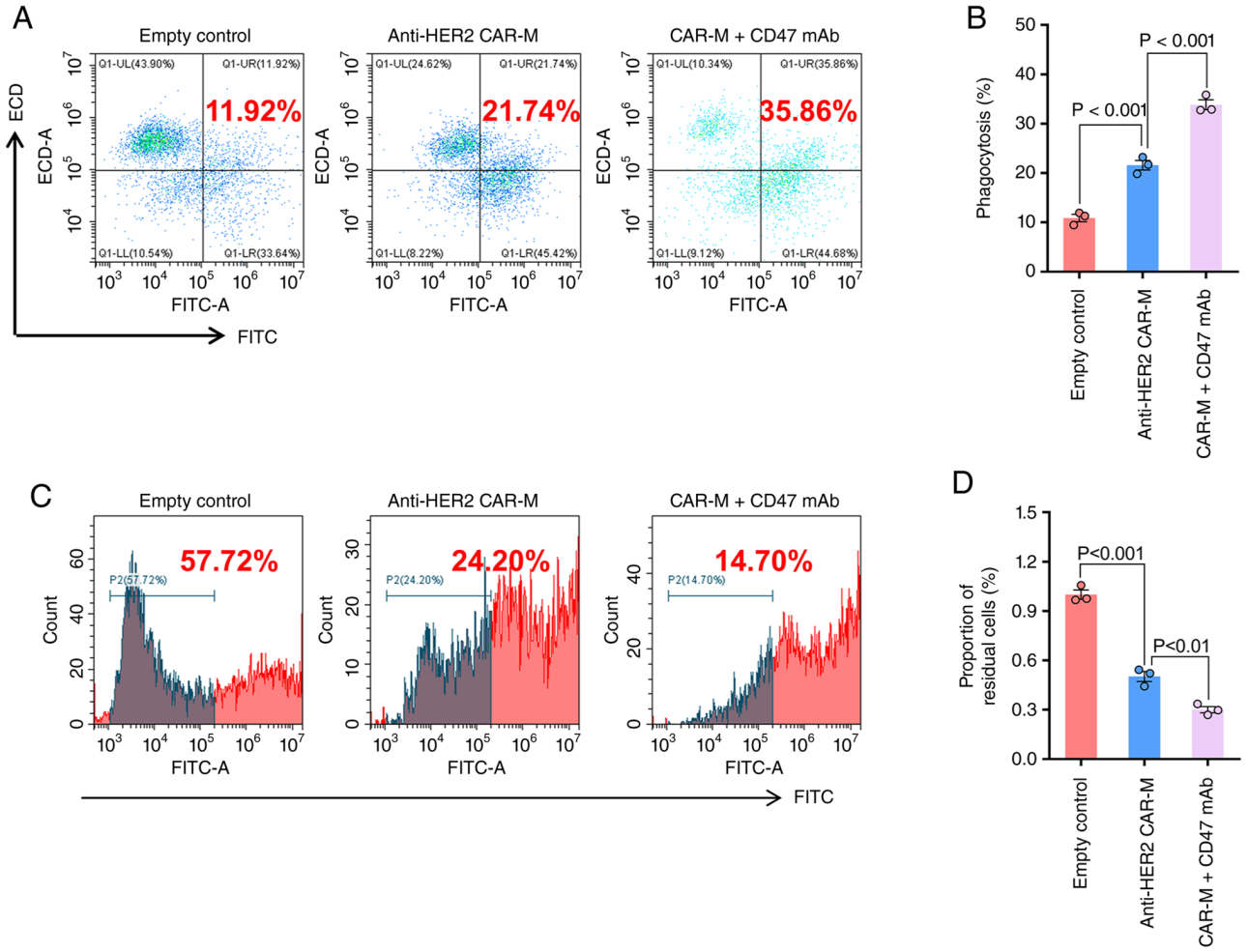

Figure 2. Anti-CD47 mAb potentiates the antitumor activity of anti-HER2 CAR-Ms against ovarian cancer. (A) Representative images of flow cytometric detection of phagocytic activity in anti-HER2 CAR-Ms treated with CD47 mAb. (B) Quantitative analysis of (A) (n=3). (C) Representative images of flow cytometric analysis of cytotoxic effects mediated by anti-HER2 CAR-Ms in the presence of anti-CD47 mAb. (D) Quantitative analysis of (C) (n=3). CAR-M, chimeric antigen receptor macrophage; mAb, monoclonal antibody.

CD47 mAb enhances the pro-inflammatory polarization of anti-HER2 CAR-Ms in vitro. Beyond their phagocytic capability, the inflammatory polarization of CAR-Ms represents a key mechanism through which they counteract the immunosuppressive TME of solid malignancies and is regarded as a critical determinant of their antitumor efficacy (22). Using flow cytometry, the expression levels of the surface markers CD206 and CD86 were evaluated on macrophages following co-culture with SKOV3 cells. The results indicated that, relative to the empty vector control group, CAR-Ms exhibited upregulation of CD86 and downregulation of CD206 (Figs. 3A-D, S1). This shift toward a pro-inflammatory phenotype was further enhanced in the presence of a CD47 mAb. Subsequent analysis of inflammatory cytokines in the co-culture supernatant revealed increased secretion of IFN-γ, TNF-α, IL-6 and IL-1β compared with in CAR-Ms alone (Fig. 3E-H), supporting the conclusion that CD47 mAb augments CAR-M-mediated inflammatory activation.

CD47 mAb enhances the antitumor activity of CAR-Ms in a subcutaneous tumor model in vivo. To further evaluate whether CD47 mAb augments the antitumor efficacy of CAR-Ms *in vivo*, a subcutaneous tumor model was established and CAR-Ms were administered in combination with CD47 mAb. A total of 4 h after subcutaneous implantation of SKOV3 cells, a single dose of macrophages was injected subcutaneously, followed by intravenous injection of CD47 mAb (Fig. 4A). The results demonstrated that anti-HER2 CAR-Ms significantly suppressed ovarian tumor growth compared with that in the control group, and this inhibition was further enhanced by the addition of CD47 mAb (Fig. 4B-E). Moreover, flow cytometric analysis revealed that the combination treatment group exhibited the lowest levels of Ki-67 expression in the tumor tissues among all groups (Fig. 4F). Similarly, immunohistochemical staining showed comparable trends for PCNA and α-SMA (Fig. 4G), indicating a synergistic antitumor effect between CD47 mAb and CAR-Ms in suppressing ovarian cancer progression.

Discussion

HER2 is a proto-oncogene that exhibits amplification or upregulation in various solid tumors, including ovarian, breast, gastric and lung cancer (23-25), making it a valuable prognostic and predictive biomarker (26). In recent decades, HER2 has emerged as a key target for drug development,

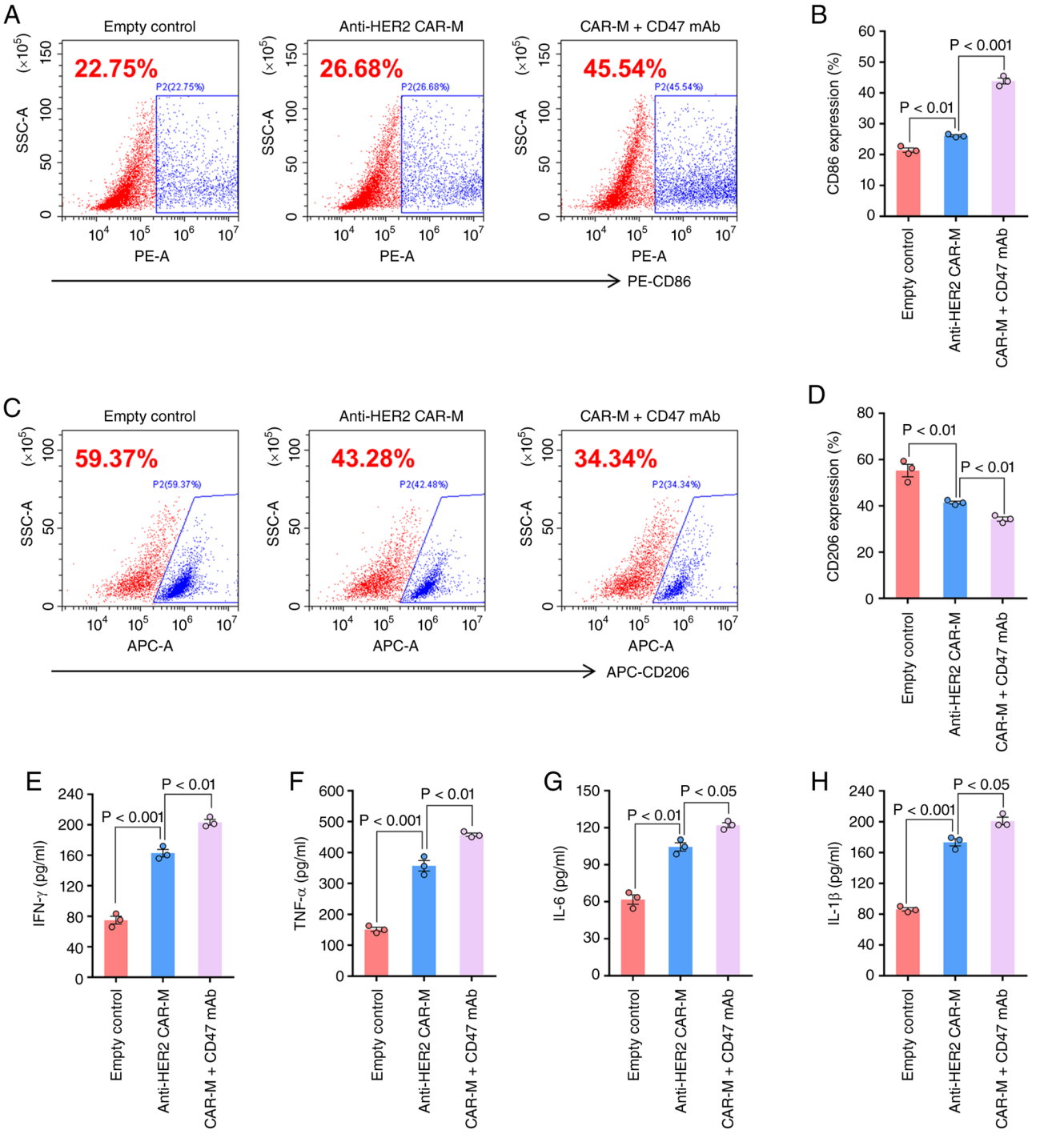

Figure 3. CD47 mAb enhances the pro-inflammatory polarization of anti-HER2 CAR-Ms. (A) Flow cytometry analysis and (B) quantification of the effect of CD47 mAb on CD86 expression on the surface of anti-HER2 CAR-Ms (n=3). PE-CD86 indicates that a PE-channel flow cytometry antibody was used to detect the CD86-positive cell population. (C) Flow cytometry analysis and (D) quantification of the effect of CD47 mAb on CD206 expression of anti-HER2 CAR-Ms (n=3). APC-CD206, indicates that an APC-channel flow cytometry antibody was used to detect the CD206-positive cell population. ELISA results showing the effect of CD47 antibody on the secretion of inflammatory cytokines (E) IFN-γ, (F) TNF-α, (G) IL-6 and (H) IL-1β by CAR-Ms (n=3). CAR-M, chimeric antigen receptor macrophage; mAb, monoclonal antibody.

involving therapeutic modalities such as tyrosine kinase inhibitors, antibody-drug conjugates, bispecific antibodies and cell therapy (27,28). However, successful treatments remain limited, with trastuzumab being the first approved first-line targeted therapy for cancer, demonstrating favorable efficacy in the treatment of advanced-stage malignancies (29).

CAR-Ms represent an emerging therapeutic modality within the field of adoptive cell therapy. Leveraging the established CAR technology platform, this approach provides a novel strategy to selectively activate macrophages for targeting a broad spectrum of malignancies. Furthermore, CAR-M therapy demonstrates distinct advantages over other CAR immune therapies, particularly in the context of solid tumor treatment (30,31). One rationale for developing macrophages as a novel cellular therapy is their inherent tumor-homing capacity. Unlike hematological malignancies,

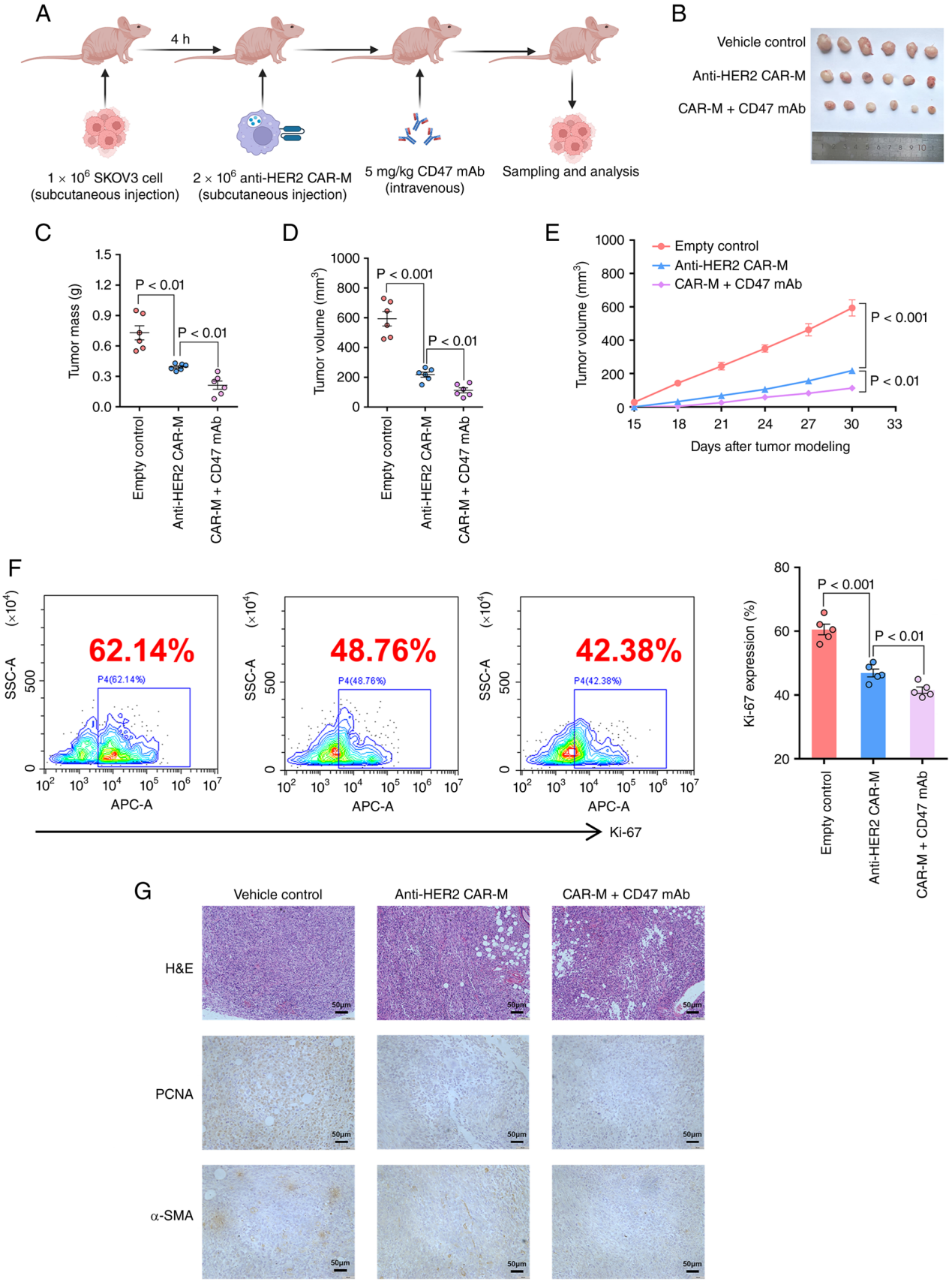

Figure 4. CD47 mAb enhances the antitumor efficacy of anti-HER2 CAR-Ms *in vivo* (n=6). (A) Schematic diagram of the *in vivo* treatment protocol. (B) Images of resected tumors from each experimental group; in the present study, the maximum tumor volume measured *in vivo* was 707.28 mm^3, and the maximum diameter was 12.15 mm. (C) Analysis of tumor weight in each group. (D) Analysis of tumor volume in each group. (E) Tumor growth curves in the *in vivo* model. (F) Flow cytometric quantification of Ki-67-positive cells within tumor tissues. (G) H&E and immunohistochemical staining for PCNA and α-SMA expression in tumor sections. CAR-M, chimeric antigen receptor macrophage; mAb, monoclonal antibody; PCNA, proliferating cell nuclear antigen; α-SMA, α-smooth muscle actin.

which are characterized by fluid dispersion, solid tumors present dense physical barriers composed of fibroblasts and stromal matrix that restrict immune cell infiltration. This limitation often leads to the failure of T cell or NK cell therapies (3). By contrast, macrophages efficiently penetrate solid tumor tissues, enabling CAR-Ms to directly engage with and eliminate malignant cells. The antitumor mechanisms of CAR-M platforms involve phagocytosis and subsequent downstream effects, such as potent activation of adaptive immunity and macrophage-specific cytokine secretion. A previous study has demonstrated that CAR-Ms can secrete TNF-α to induce tumor cell apoptosis, and enhance the infiltration of $CD8^+$ T cells and NK cells (15).

An initial Phase I clinical trial of a CAR-M therapy (NCT04660929) has reported safety and clinical efficacy outcomes. Among 14 treated patients, no dose-limiting toxicities, severe cytokine release syndrome (CRS) or immune effector cell-associated neurotoxicity syndrome were observed. Some patients developed grade 1-2 CRS, which resolved within 3 days. Sequential tumor biopsies revealed that adoptively transferred anti-HER2 CAR-Ms migrated into the TME, prompting its remodeling, enhancing $CD8^+$ T-cell expansion and boosting broad antitumor immunity. To the best of our knowledge, these data provide the first clinical evidence supporting the efficacy, safety and tolerability of CAR-M therapy (3). Nevertheless, the long-term effects of CAR-Ms remain unassessed. Strategies to improve *in vivo* persistence and antitumor activity include further engineering and combination therapies, such as with PD-1/PD-L1 inhibitors. Due to the dependence of CAR-Ms on phagocytic function, targeting phagocytosis checkpoints may represent a more effective and suitable approach (32). For example, Zeller *et al* (33) demonstrated that dual blockade of CD47 and LILRB1 enhances CD20 antibody-dependent phagocytosis of lymphoma cells by macrophages. Furthermore, Li *et al* (34) revealed that targeting the CD200R1-CD200 axis represents a promising macrophage immune checkpoint blockade strategy, where blocking or eliminating CD200R1 in macrophages or CD200 in tumor cells enhances phagocytosis and suppresses tumor growth.

Another pivotal issue is whether CAR-Ms can elicit sustained antitumor responses, which hinges upon their controlled elimination and interactions within the TME. Available evidence has indicated that adoptively transferred macrophages exhibit tropism *in vivo* (35). The majority of infused CAR-Ms accumulate in the liver, with a smaller proportion infiltrating tumor tissues (36). Current investigations have focused primarily on the persistence of CAR-Ms within tumors. In animal models, these cells demonstrate extended survival; however, their numbers diminish rapidly within the first few days post-administration. This decline suggests that non-proliferative CAR-Ms undergo regulated elimination *in vivo*, thereby supporting long-term safety profiles (7).

A major challenge in CAR-M therapy lies in the immunosuppressive nature of the solid TME. Circulating monocytes and macrophages recruited into tumor sites are often educated by immunosuppressive signals to adopt a pro-tumor M2 phenotype (37). Although CAR-M activation promotes an M1-like phenotype, the sustainability of this polarization remains unclear. A previous study employed engineered adenoviruses or exogenous cytokine stimulation to induce M1 polarization, while others propose that first-generation CAR constructs alone may suffice (38). Nevertheless, the durability of these interventions remains compromised by the immunosuppressive tumor milieu. Theoretically, in addition to enhancing the phagocytic activity of CAR-Ms and lowering their activation threshold, blocking phagocytosis checkpoints can reprogram tumor-associated macrophages (TAMs). This reprogramming not only eliminates the functional impairments that TAMs impose on CAR-M therapy but also converts this major immunosuppressive cell population into antitumor effector cells. Through this dual-pronged mechanism, a sustained innate immune response can be established by alleviating immunosuppression while enhancing cytotoxic activity (39). Nevertheless, further studies are required to determine whether phagocytosis checkpoint blockade can consistently achieve CAR-M-mediated reprogramming of TAMs within the complex TME, and whether the potential safety risks associated with excessive macrophage activation are controllable (40).

The present study demonstrated that blockade of CD47 using a mAb enhanced CAR-M-mediated phagocytosis and tumor cell killing while concurrently promoting inflammatory polarization. Mechanistically, CD47 functions as a 'don't eat me' signal by engaging signal regulatory protein α (SIRPα) on the macrophage surface, which recruits SHP-1 and SHP-2 phosphatases to the intracellular domain of SIRPα, thereby inhibiting phagocytic synapse formation and suppressing cytoskeletal rearrangement. Blockade of CD47 with a monoclonal antibody disrupts this CD47-SIRPα interaction, relieving the inhibitory constraint on macrophage phagocytosis (41). In the context of CAR-M therapy, this disinhibition likely lowers the threshold for CAR-mediated activation, permitting more robust ITAM-dependent signaling through the CD3ζ intracellular domain and consequently enhancing both phagocytic and cytotoxic effector functions. Furthermore, the enhanced phagocytosis of tumor cells may provide additional pro-inflammatory stimuli through the exposure of damage-associated molecular patterns, thereby promoting the M1-like polarization and increased secretion of inflammatory cytokines observed in the current study. These mechanistic insights support the synergistic potential of combining phagocytosis checkpoint inhibitors with CAR-based macrophage therapy.

Several limitations of the present study should be acknowledged. First, the *in vivo* experiments were conducted in immunodeficient BALB/c nude mice, which lack functional T cells and NK cells. This model does not recapitulate the intact immune system and therefore precludes assessment of the secondary effects of CD47 blockade on adaptive immune activation, such as antigen cross-presentation by CAR-Ms and subsequent T-cell priming (42). Future studies employing syngeneic tumor models or humanized immune system mice are warranted to fully evaluate the immunomodulatory consequences of combining CAR-Ms with CD47 mAb. In addition, only a subcutaneous xenograft model was used, which does not reflect the peritoneal dissemination pattern typical of clinical ovarian cancer. Orthotopic or patient-derived xenograft models would provide more clinically relevant insights. Moreover,

the CAR-Ms were derived from the THP-1 cell line, and it remains to be determined whether primary human monocytes or induced pluripotent stem cell-derived macrophages with a similar engineering strategy could achieve comparable efficacy and safety profiles. The long-term persistence, biodistribution and eventual fate of adoptively transferred CAR-Ms with or without CD47 mAb were not investigated. Longitudinal imaging and terminal tissue analysis are needed to assess these pharmacokinetic parameters. Finally, only a single dose of CD47 mAb was administered; optimization of dosing schedule, duration of treatment and potential biomarkers of response requires further exploration. Addressing these limitations through comprehensive preclinical studies will be essential to advance this combination strategy toward clinical translation.

In summary, to the best of our knowledge, the present study was the first to evaluate the combination of CAR-M therapy with phagocytic checkpoint inhibitors *in vitro* and *in vivo*. The results demonstrated that an anti-CD47 mAb enhanced the antitumor efficacy of CAR-Ms by promoting phagocytosis and modulating their inflammatory activation. Given that CAR-M therapy elicits broad innate and adaptive immune responses, combining CAR-Ms with other types of ICIs represents a promising strategy (43). In the future, next-generation CAR-M designs may incorporate intracellular CD47 scFv domains, thereby integrating phagocytic checkpoint blockade directly into the CAR-M construct (44). Such an approach could facilitate localized inhibitor delivery via tumor-infiltrating CAR-Ms or through *in vivo* CAR-M programming, potentially mitigating the systemic toxicity associated with checkpoint inhibition (45).

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JQia, QK and JQiu designed the study. JQia and QK performed the experiments. JQia drafted the manuscript. QK and JQiu revised the manuscript. JQia and JQiu confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Animal Ethics Committee of Anhui Medical University (approval no. PZ-2025-029).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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