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# *Engineered Claudin18.2-Targeting CAR-Macrophages with shSIRPα Silencing and IFN-γ Receptor Overexpression for Enhanced Antitumor Response in Gastric Cancer*

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***Abstract.*** Gastric cancer remains a lethal malignancy with limited treatment options for advanced-stage patients. While Claudin18.2-targeting chimeric antigen receptor (CAR) -T therapies show promise, their efficacy is hampered by poor solid tumor infiltration and T cell exhaustion. Considering these limitations, chimeric antigen receptor-macrophage (CAR-M) therapy may offer an alternative. In this study, we propose to develop an engineered CAR-M targeting the gastric cancer-specific molecule Claudin18.2 (CLDN18.2), with an FcRIIA transmembrane domain and modular intracellular domains incorporating SIRPα silencing (shSIRPα) and IFN-γ receptor (IFNGR) overexpression to boost antitumor responses. By introducing CARs into human induced pluripotent stem cells (iPSCs), we anticipate that shSIRPα could effectively disrupt the CD47-SIRPα "don't eat me" signal and enhance phagocytosis, while IFNGR upregulation could activate NF-κB signaling and M1 polarization, increasing pro-inflammatory cytokine secretion and antigen presentation. In vivo results from genetically engineered mice models (GEMM) are also expected to reveal improved tumor suppression, prolonged survival, and enhanced T cell infiltration. Mechanistically, we aim to verify that sustained NF-κB/p65 activation is the key driver of M1 polarization, though additional pathways (IRF5, STAT1) may also contribute. To conclude, we highlight here a next-generation CAR-M strategy with improved tumor targeting, immune modulation, and translational potential for gastric cancer treatment, offering evidence and insights for future allogeneic "off-the-shelf" CAR-M manufacturing.

***Keywords:*** Chimeric antigen receptor macrophage, Gastric cancer, M1 polarization, Claudin18.2

## 1. Introduction

Gastric cancer (GC) is the third among leading causes of cancer-related mortality worldwide [1]. While surgery offers the only potentially curative treatment, many patients present with unresectable disease at diagnosis or experience recurrence after surgery [2]. Despite advances in systemic

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therapies, the prognosis for advanced-stage disease remains poor, with a median overall survival (OS) of only approximately one year under standard first-line chemotherapy [3]. With the advent of targeted therapies (e.g., trastuzumab, an antibody targeting HER2-positive tumors) and immune checkpoint inhibitors (e.g., nivolumab, an antibody against PD 1, effective in PD-L1-high/MSI-H microsatellite instability-high tumors), recent years have witnessed improved outcomes in selected subgroups [4]. However, molecularly defined populations such as patients with HER2 overexpression and PD-L1 represent about 18% [5] and 60% [6] of cases respectively. Consequently, patients with HER2-negative, PD-L1-low, and microsatellite-stable (MSS) tumors have few effective options, which underscores an urgent need for innovative therapeutic strategies.

Given these limitations, immunotherapy approaches that can reactivate antitumor immune responses while overcoming tumor immune-evasion mechanisms have gained increasing attention [7]. Among these therapies, chimeric antigen receptor (CAR)-engineered immune cell therapies emerge as promising candidates. While CAR-T cell therapy has portrayed remarkable success in the field of hematological malignancies [8], it has also showed promising results in gastric cancer by targeting Claudin18.2 (CLDN18.2) in recent studies [9,10]. However, significant challenges remain, cytokine release syndrome (CRS) has been reported in approximately 94.6% of patients, and on-target off-tumor toxicity remains a concern [11]. Therefore, exploring alternative therapeutic approaches are needed to overcome these obstacles while maintaining potent anti-tumor efficacy. The development of CAR-based innate immune cell therapies might offer a promising new direction [12].

In GC, hypoxia induces tumor-associated macrophages (TAMs) to adopt an immunosuppressive M2 phenotype that drives angiogenesis, metastasis, and immune evasion. This occurs through dual mechanisms: upregulated PD-L1, which inhibits $CD8^+$ T cells activitiy via JAK/STAT1 signaling, and IL-10 secretion that further promotes M2 polarization— thereby establishing a self-reinforcing immunosuppressive loop [13-15]. Other studies have demonstrated that GC-derived exosomes play an important role inducing M2 polarization via miR-519a-3p/MAPK, circATP8A1/STAT6, and SERPINE1/let-7g-5p/STAT3 pathways [16,17], while TIM4+ macrophages have been illustrated to impair CD8+ T cell function in peritoneal metastases [18]. In contrast, M1 macrophages exert pro-inflammatory and anti-tumor effects by secreting IFN-γ and TNF-α [19]. Therefore, remodeling tumor microenvironment (TME) by reversing M2 polarization while restoring the pro-inflammatory and anti-tumor functions of M1 macrophages may display a promising strategy to enhance the efficacy of immunotherapy for GC.

Building upon existing CLDN18.2-targeted CAR-T therapies [20], we plan to engineer a novel CAR-M platform that harnesses the intrinsic tumor-homing ability of macrophages to overcome stromal barriers and to enhance infiltration [21,22]. This design integrates two key functional modules: (1) an intracellular shSIRPα domain to block the "don't-eat-me" signal and enhance phagocytosis [23], and (2) an IFNGR module to drive M1 polarization, thereby reshaping the immunosuppressive TME [24,25]. Notably, inspired by recent advances in localized cytokine receptor engineering (e.g., an in situ chimeric IL-2 receptors in renal cell carcinoma) [26], we propose an IFNGR module rather than an IFN-γ secreting module to achieve localized M1 polarization while minimizing systemic toxicity, thereby balancing antitumor efficacy and safety. To conclude, this dual-modular strategy has the potential not only to circumvent CAR-T limitations but also actively reprogram the TME toward an immunostimulatory state, offering a promising therapeutic avenue for advanced gastric cancer.

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## 2. Working model

The hypothesis is CLDN18.2-targeting-CAR-shSIRPα-IFNGR-M can more effectively eliminate gastric cancer and prevent metastases with controlled safety. To validate this hypothesis, we designed dual-functional CAR-macrophages targeting Claudin18.2 with SIRPα silencing and IFNγ secretion capabilities for gastric cancer therapy. The working model of this approach is illustrated below (Figure 1a-b).

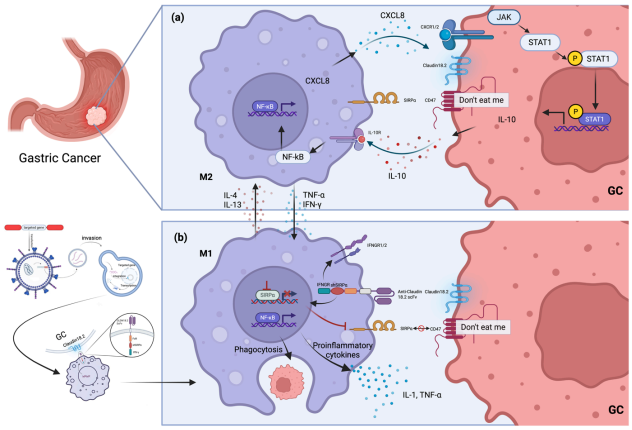


Figure 1. Working model of this study. (a) Untreated state. CLDN18.2 is highly expressed on gastric cancer tumor cell membranes. TAMs (M2) secrete CXCL8, which activates the JAK/STAT1 signaling pathway in tumor cells, leading to increased IL-10 production and reinforcing M2 polarization. In addition, CD47 on tumor cells engages SIRPα on macrophages, whose intracellular ITIM motif deliver inhibitory signals that suppress phagocytosis. (b) CAR-M therapy. CLDN18.2-targeted CAR-macrophages (CAR-Ms) are engineered with intracellular SIRPα silencing domains to restore phagocytosis and IFNGR modules to promote M1-type polarization and pro-inflammatory cytokine release. These M1 CAR-M not only directly eliminate tumor cells through phagocytosis but also secrete cytokines that recruit and activate $CD8^+$ T cells and NK cells, amplifying anti-tumor immunity

Macrophage cell-based immunotherapy represents a promising frontier in the treatment of solid tumors due to its intrinsic immunological safety and potential for off-the-shelf applicability. In this study, we aim to develop a next-generation, rationally engineered CAR-M targeting gastric cancer cells, with a focus on enhancing anti-tumor efficacy through phenotypic reprogramming and functional augmentation.

Given that CLDN18.2 is specifically expressed in gastric cancer cells, we design CAR-Ms targeting Claudin 18.2-positive tumors through engineered fusion proteins. The fusion protein binds humanized single-chain variable fragment (scFv) CLDN18.2 to the Fc receptor FcγRIIa, together with a targeted short hairpin RNA (shRNA) cassette to downregulate macrophages surface SIRPα expression, thereby disrupting the CD47-SIRPα signal axis and "Don't Eat Me" signal. In addition, the design includes modules to enhance IFNGR expression of macrophages, thereby promoting M1-like polarization.

As illustrated in Figure 2, we will use FCM to confirm that re-engineered CAR-Ms promote M1 macrophage polarization. Additionally, we will assess enhanced phagocytic activity via luciferase-based assays and validate increased proinflammatory effects using RT-qPCR and ELISA. In vivo, we will utilize a genetically engineered mouse model (GEMM) to assess the therapeutic potential of

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re-engineered CAR-M cells. This model will allow us to evaluate tumor regression, remodeling of the TME, enhanced T cell infiltration, reactivation of exhausted T cells, and suppression of metastasis. Mechanistically, we hypothesize that M1 polarization and enhanced phagocytosis are mediated by the NF-kB signaling, which will be further verified with immunofluorescent (IF) staining, RNA-seq analysis, and FCM approaches.

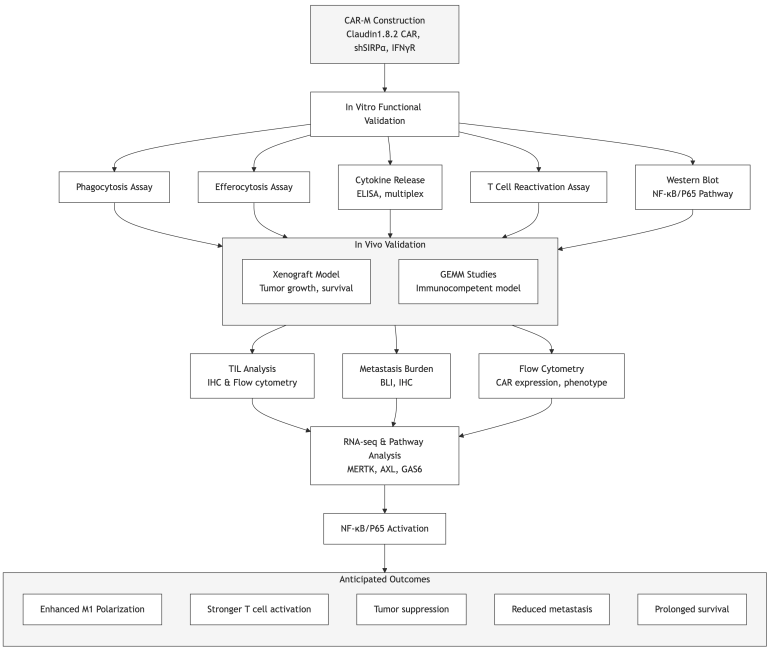


Figure 2. Workflow for CAR-M engineering and functional assays

In summary, we aim to achieve more effective elimination of gastric cancer and to prevent metastases while ensuring controlled safety by developing such a CAR-M. This study seeks to establish a mechanistically in driven CAR-M platform with improved therapeutic performance and translational potential, offering a novel approach for eliminating solid tumor cells and restraining metastasis, without compromising safety.

## 3. Methods

### 3.1. Cell culture and generation of CAR macrophages

Human monocyte-derived macrophages were generated from peripheral blood mononuclear cells (PBMCs) obtained from healthy donors through density gradient centrifugation. Monocytes were purified by positive selection with anti-CD14 MicroBeads and differentiated into macrophages with recombinant human M-CSF (50 ng/mL) for 7 days. For CAR engineering, macrophages were transduced with a lentiviral vector encoding Claudin18.2-specific CAR containing a CD8 hinge, a CD28 transmembrane domain, and a CD3ζ intracellular signaling domain. Transduction efficiency was determined by flow cytometry following staining with an anti-F(ab')2 antibody, which specifically binds to the immunoglobulin-derived scFv domain of the Claudin18.2-CAR on the macrophage surface, thereby serving as a surrogate marker for CAR expression.

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### 3.2. Mice

Genetically engineered MYC-sgp53-sgMsh2 EPO GEMM mice, which spontaneously develop Claudin18.2$^+$ gastric tumors, were employed to evaluate the in vivo efficacy of CAR macrophages. In parallel, NOD/SCID mice bearing subcutaneous xenografts of Claudin18.2$^+$ NUGC3 or HGC27 cells were used for validation. Tumor-bearing mice were randomized into treatment cohorts once tumors reached ~100 mm³ (xenografts) or upon confirmed tumor onset (GEMM), and received intratumoral or intravenous administration of CAR-modified macrophages or control macrophages (Table 1).

Table 1. Treatment protocols for in vivo evaluation of CAR-M therapy

| Treatment group | Description / composition | Function |
|---|---|---|
| M | Unmodified macrophages | Negative control (baseline macrophage effect) |
| CAR-M | Macrophages engineered with Claudin18.2-scFv-FcRIIA-CAR (no additional domains) | Antigen-specific cytotoxicity control |
| CAR-shSIRPα | CAR-M with SIRPα knockdown module | To test effect of CD47/SIRPα checkpoint blockade |
| CAR-IFNGR | CAR-M with IFNGR signaling module | To test IFN-γ–enhanced M1 polarization |
| CAR-shSIRPα-IFNGR-M | CAR-M simultaneously carrying shSIRPα and IFNGR domains | Full construct; to test synergistic therapeutic efficacy |

### 3.3. Flow cytometry analysis

CAR expression and macrophage phenotype were assessed by flow cytometry. Surface markers including CD14, CD68, CD163, SIRPα, IFNGR, PD-L1, CD86, and HLA-DR were analyzed using fluorochrome-conjugated monoclonal antibodies. For T cell co-culture experiments, expression of CD3, CD4, CD8, CD25, CD69, PD-1, TIM-3, granzyme B, and intracellular IFN-γ were measured. Data were acquired on a BD LSRFortessa and analyzed using FlowJo software.

### 3.4. Cytokine release assay

Cytokine secretion by CAR macrophages was quantified using ELISA and multiplex bead-based assays. Culture supernatants were collected after 24 h co-culture with Claudin18.2$^+$ tumor cells (NUGC3, HGC27) or control cells. Levels of IL-6, TNF-α, IL-1β, IL-12p70, and IFN-γ were measured and normalized to cell numbers.

### 3.5. Phagocytosis assay

Phagocytic activity of CAR macrophages was assessed using CFSE-labeled Claudin18.2$^+$ tumor cells. Macrophages were co-cultured with target cells at indicated effector-to-target (E:T) ratios for 4 h at 37 °C. Phagocytosis was quantified by flow cytometry as the percentage of CFSE$^+$ macrophages and further validated by confocal microscopy.

### 3.6. Western blot analysis

Immunoblotting was employed to assess the protein expression of SIRPα, IFNGR, and components of the NF-κB pathway (p-P65, IκBα). Following cell lysis in a RIPA buffer system containing protease and phosphatase inhibitors, protein concentrations were normalized by BCA assay. Equal protein aliquots were then resolved by SDS-PAGE, transferred to PVDF membranes, and blocked

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with 5% BSA. Incubation with specific primary antibodies and subsequent HRP-linked secondary antibodies enabled the detection of target proteins via enhanced chemiluminescence (ECL), with quantification performed using ImageJ software.

### 3.7. Binding and internalization assays

To confirm the binding specificity of Claudin18.2-targeting CAR macrophages, recombinant His-tagged Claudin18.2 protein or Claudin18.2$^+$ gastric cancer cells (NUGC3, HGC27) were incubated with CAR-Ms at defined E:T ratios. Binding was detected by flow cytometry using anti-His antibodies, while internalization was assessed by incubating CAR-Ms with labeled Claudin18.2 protein at 37 °C and measuring fluorescence uptake by flow cytometry and confocal microscopy.

### 3.8. T cell reactivation assay

Jurkat TCR$^+$ cells specific for NY-ESO-1 were co-cultured with CAR macrophages in the presence of NY-ESO-1 peptide-pulsed antigen-presenting cells. After 24 h, T cell activation was measured by flow cytometric analysis of surface expression of CD69, CD25, and PD-1. Additionally, functional cytotoxicity was assessed by intracellular staining of granzyme B (GZMB) and IFN-γ in CD8$^+$ T cells.

### 3.9. Tumor-Infiltrating Lymphocyte (TIL) analysis

Tumor tissues were harvested, formalin-fixed, and paraffin-embedded for immunohistochemistry (IHC) and immunofluorescence (IF). Sections were stained with monoclonal antibodies against CD3, CD4, CD8, PD-1, TIM-3, CD103, and Ki67 to evaluate the density and functional status of TILs. Quantification was performed using digital pathology software, with results expressed as the number of positive cells per mm$^2$ of tumor area.

### 3.10. Metastasis burden assessment

Ex vivo bioluminescence imaging (BLI) of excised organs (liver, lung, spleen, peritoneum) was performed to quantify metastatic dissemination. Hematoxylin and eosin (H&E) staining and IHC for CD68 and CD163 were performed to validate macrophage infiltration and metastatic foci. The number, size, and anatomical distribution of metastatic nodules were quantified under microscopy.

### 3.11. RNA sequencing and pathway analysis

Total RNA from CAR-Ms and control macrophages was extracted using TRIzol, and libraries were prepared for bulk RNA-seq. Sequencing data were aligned to the human reference genome (hg38), and differential expression analysis was performed using DESeq2. Pathway enrichment analyses (KEGG, GSEA) was conducted to identify changes in immune signaling and apoptotic clearance pathways, with particular emphasis on MERTK, AXL, and GAS6 signaling.

### 3.12. Efferocytosis assay

To evaluate apoptotic cell clearance, CAR-Ms were co-cultured with CFSE-labeled apoptotic tumor cells at a 1:1 ratio. After 4 h incubation, uptake of apoptotic cells was analyzed by flow cytometry and confocal microscopy. For super-resolution imaging, highly inclined and laminated optical sheet

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(HILO-SIM) microscopy was used. Pharmacological inhibition of MERTK and AXL was used to confirm pathway dependency.

### 3.13. Statistical analysis

All experiments were performed in at least triplicate. Data were analyzed with GraphPad Prism (version 8.2.1). We expressed the results as mean ± s.e.m. and performed statistical analyses using the two-tailed Student's t-test, one-way ANOVA, or Mann-Whitney U test, as appropriate. In all analyses, a threshold of $P < 0.05$ was used to define statistical significance.

## 4. Anticipated results

### 4.1. Constructed CAR structure targeting Claudin18.2

The designed lentiviral vectors will be transduced into iPSCs within five groups: including M, CAR-M, CAR-shSIRPα, CAR-IFNGR, and CAR-shSIRPα-IFNGR-M for subsequent experiments (Figure 3a-b). It is anticipated that successful expression of CAR in transduced cells will be validated by flow cytometry (FCM) and that the transduction efficiency is stable and reproducible. It is further expected that the results of qRT-PCR and Western blot will demonstrate SIRPa expression is significantly down-regulated in CAR-M containing the shSIRPα structural domain, whereas IFNGR expression is significantly up-regulated in CAR-M containing the IFNGR structural domain. After co-culture, FCM and confocal microscopy are expected to show that CAR-M is able to specifically recognize and engage Claudin18.2-positive tumor cells.

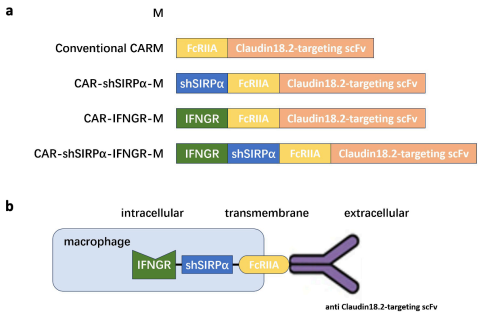


Figure 3. Constructed CAR structure targeting Claudin18.2 (a) Five experimental groups of CAR-M will be designed: M, CAR-M, CAR-shSIRPα, CAR-IFNGR, and CAR-shSIRPα-IFNGR-M for subsequent experiments. (b) The CAR structure. The extracellular domain consists of a Claudin18.2-targeting scFv. For the transmembrane domain, we select FcRIIA as a proper candidate 23. For the intracellular domain, CARs containing the shSIRPα domain, the IFNGR domain, both shSIRPα and IFNGR domain are designed

### 4.2. SIRPα silencing and IFNGR overexpression enhance macrophage antitumor function

The assessment of tumor cell killing is expected to demonstrate that CAR-shSIRPα-IFNGR-M exhibits stronger tumor killing activity under different E/T ratio conditions compared to the control group (Figure 4a), suggesting that these modifications augment the anti-tumor efficacy of macrophages. Analysis of macrophage polarization status is anticipated to reveal that CAR-shSIRPα-IFNGR-M significantly increases the proportion of $CD80^{+}$/CD206- M1-type macrophages,

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while reducing the proportion of CD163+ M2-type macrophages, thereby promoting pro-inflammatory (M1) activation and suppressing the anti-inflammatory (M2) phenotype (Figure 4b). We anticipate that gene expression profiling (RT-qPCR) and cytokine secretion analysis (ELISA) will reveal significant upregulation of pro-inflammatory genes and elevated secretion of inflammatory cytokines in CAR-shSIRPα-IFNGR-M relative to the control group, suggesting that the design might enhance the inflammatory response capacity of macrophages, thereby further improving tumor clearance.

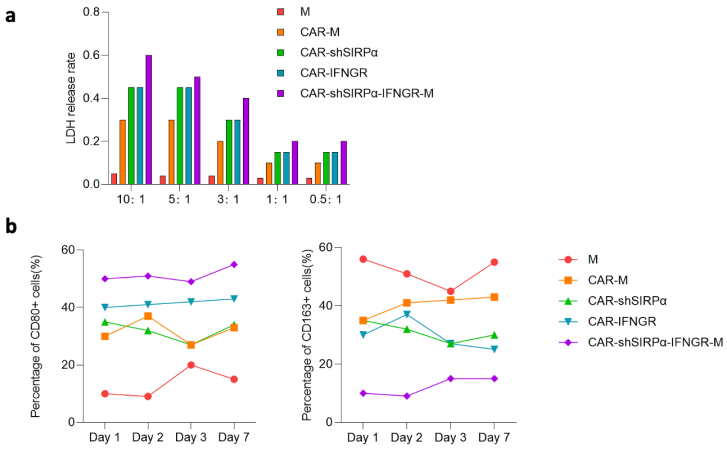


Figure 4. shSIRPα and IFNGR overexpression enhance the anti-tumor function of macrophages. (a) Target cell lysis at varying effector-to-target (E:T) ratios by CAR-M cells (CAR) versus negative control (NC). (b) Quantification of M1, Use flow cytometry gated population indicates CD80+ cells. Quantification of M2 inhibition, Use flow cytometry gated population indicates CD163+ cells

## 4.3. T-cell immunity activated by SIRPα/IFNGR-modified CAR-Ms

The results of in vitro T cell activation assay are expected to show that CAR-shSIRPα-IFNGR-M treatment significantly increases CD69 expression of Jurkat T cells compared with the untreated or unmodified CAR-M group, indicating that its capacity to promote T cell reactivation in vitro. In the mouse model, the results of flow cytometry assay of in vivo T cells are is anticipated to show that mice treated with CAR-shSIRPα-IFNGR-M have significantly higher levels of T-cell activation, suggesting functional restoration of T-cells with enhanced anti-tumor capacity. Immunostaining and IHC analysis of the tumor tissues are expected to show that mice treated with CAR-shSIRPα-IFNGR-M showed a significant increase in T-cell infiltration, in particular the $CD8^+$ and $CD103^+$ subsets, suggesting an enhanced T-cell activity with the potential to establish durable tumor-specific immune memory.

## 4.4. The therapeutic efficacy of Claudin18.2-targeting-CAR-shSIRPα-IFNGR-M in vivo

By monitoring body weight changes and tumor growth kinetics, we anticipate that treated with CAR-shSIRPα-IFNGR-M will exhibit significantly delayed tumor progression and reduced tumor volume compared with controls during the treatment period (Figure 5a). Survival curves are expected to show that the CAR-shSIRPα-IFNGR-M-treated group exhibits a marked extension of survival than the control group (Figure 5b). The results of the experiments testing anti-metastatic ability are expected to show that the frequency and size of tumor foci in common metastatic sites

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such as liver and peritoneum were significantly lower in the CAR-shSIRPα-IFNGR-M treatment group than in the control group, which would be demonstrated by organ specimen dissected from mice model and HE staining. Collectively, we anticipate that treatment with the CAR-shSIRPα-IFNGR-M will significantly inhibit distal tumor metastasis, indicating robust systemic anti-metastatic ability.

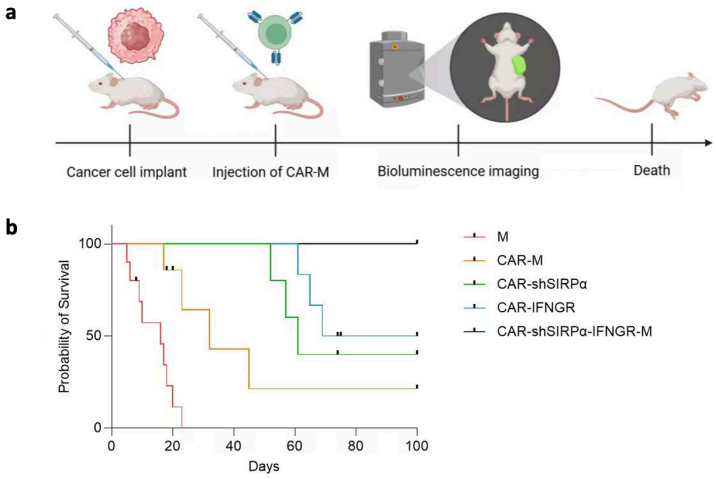


Figure 5. Evaluating the therapeutic efficacy of Claudin18.2-targeting-CAR-shSIRPα-IFNGR-M in vivo. (a) Mouse experiment procedure. (b) After construction of the mice model, weigh the tumor every several days and calculate the survival rate of different groups

### 4.5. NF-κB/P65 activation drive enhanced M1 polarization and antitumor activity in modified CAR-Ms

Detection of the NF-κB/P65 signaling pathway are expected to reveal, via IF staining with significant activation of the NF-κB signaling pathway in shSIRPα-IFNGR-modified CAR-M. This will be evidenced by elevated expression of pro-inflammatory factors, increased phosphorylated P65, decreased expression of IκBα protein, and accumulation of P65 in the nucleus. Confocal microscopy and high-speed structured illumination microscopy (HIS-SIM) image are anticipated to visualize the process of CAR-M phagocytosis of apoptotic cells. Furthermore, functional intervention experiments using efferocytosis inhibitors will confirm that inhibition of this process significantly impairs the anti-tumor effect of CAR-M, suggesting that efferocytosis plays an important role in enhancing macrophage immune function.

## 5. Discussion

The main challenges of current CAR-T cell therapies targeting CLDN18.2 include limited infiltration of T cells into tumor tissue and a high incidence of treatment-related adverse events due to excessive immune activation associated with the high toxicity burden. The advantage of CAR-M is that macrophages have the natural ability to infiltrate and reside in solid tumor tissues and can function in unfavourable environments such as low oxygen/high lactate/dense matrix. In addition, CAR-M has a dual effect of phagocytosis and antigen presentation coupled with the secretion of pro-inflammatory factors such as IL-12/TNF-α that recruit immune effector cells, which can initiate a specific T-cell immune response and help to transform 'cold' tumors into 'hot' tumors. In this study,

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we proposed to generate CAR-M with both tumor-targeted recognition and phagocytosis/inflammation amplification to target the gastric cancer-specific molecule CLDN18.2. shSIRPα deregulates tumor cell CD47-SIRPα inhibition and enhances phagocytosis; IFNGR potentiation further drives upregulation of NF-κB/M1 and co-stimulatory molecules, increasing antigen presentation efficiency and pro-inflammatory cytokine secretion. These make CLDN18.2-targeting-CAR-shSIRPα-IFNGR-M more adaptable in dealing with solid tumor barriers, antigenic heterogeneity, and immunosuppression.

Previous studies have shown that CAR-M therapy synergizes with PD-1 inhibitors enhances tumor regression by concurrently modulating TME, promoting the infiltration of tumor-specific T and B cells, and activating innate immune responses. Our research suggest that CAR-M already reshapes the TME through reinforced M1 polarization and enhanced phagocytosis. Future integration with PD-1 blockade may further boost therapeutic efficacy. Current most therapeutic strategies rely on ICI injections, while alternative approaches have developed macrophage-based drug delivery systems capable of transporting various small molecules. Harnessing this delivery potential, next-generation CAR-M platforms could be designed to co-deliver PD-1 inhibitors, opening avenues for highly coordinated dual-target therapies that may transform future treatment paradigms.

Our results demonstrate that the engineered CAR-Ms exhibit a sustained M1-like phenotype, characterized by elevated IL-12 production, alongside reduced expression of M2 markers such as Arg1 and IL-10. Mechanistically, we observed robust activation of the NF-κB signaling pathway, suggesting that NF-κB may play a central role in mediating CAR-induced M1 polarization. This finding is consistent with previous reports implicating NF-κB as a key regulator of pro-inflammatory macrophage activation [27]. However, it remains plausible that additional signaling cascades contribute to this phenotype. For instance, IRF5 or STAT1 signaling pathways—both established modulators of macrophage polarization—could be co-activated in response to CAR engagement or cytokine milieu, warranting further investigation. Future studies using pathway-specific inhibitors or knockdown models will be essential to delineate the relative contribution of these alternative pathways. Furthermore, while maintaining an M1 phenotype is desirable for anti-tumor efficacy, sustained M1 polarization also poses potential risks, including cytokine storm [28].

To further refine the in vivo validation system for CAR-M therapy, future researchers can also employ an NSG-SGM3 mice as the better alternative model for constructing a humanized gastric cancer mouse model. By transplanting human umbilical cord blood derived $CD34^+$ hematopoietic stem cells into these immunodeficient mice, we can establish a stable and functional human immune environment [29]. The NSG-SGM3 strain lacks murine cytokine signaling, which facilitates the long-term engraftment and survival of human immune cells. This feature supports extended in vivo monitoring and functional evaluation of therapeutic efficacy in a physiologically relevant immune context.

Autologous methods are generally regarded as safer in clinical application, as they minimize the risk of strong immune rejection. But they cannot be mass-produced, require individualized manufacturing for each patient, and involve a time-consuming and technically complex preparation process. That is why off-the-shelf approaches represent a particularly promising therapeutic approach. Our CAR-M technology leverages the unique properties of macrophages, including their relatively low propensity to trigger strong alloimmune responses. This renders them promising potential for allogeneic application and large-scale production. If successful, it could greatly reduce the time and cost associated with therapeutic preparation. However, heterogenous MHC-I on the

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surface of transplanted macrophages might cause unwanted immune activation. Therefore, further investigation will be required to optimize these strategies and minimize potential adverse effects.

## 6. Conclusion

In summary, our study highlights the potential of CLDN18.2-targeting-CAR-shSIRPα-IFNGR-M as a next-generation cellular therapy for gastric cancer, offering advantages in tumor infiltration, antigen presentation, and inflammatory amplification compared with current CAR-T strategies. By combining enhanced phagocytic capacity with sustained M1 polarization and robust NF-κB activation, CAR-M effectively remodels the immunosuppressive tumor microenvironment, thereby augmenting antitumor immunity. Importantly, the versatility of CAR-M platforms opens avenues for synergistic strategies, including integration with immune checkpoint blockade or co-delivery of therapeutic payloads, to achieve more durable and comprehensive responses. Future refinement of preclinical models, such as the adoption of NSG-SGM3 humanized mice, and optimization of allogeneic manufacturing protocols will be critical steps toward clinical translation. Collectively, these findings position CAR-M as a promising immunotherapeutic modality with the potential to overcome key barriers in solid tumor treatment and pave the way for more accessible, effective, and scalable therapeutic options.

## Author contributions

J.F. and Y.B.: conceived, designed and developed the methodology of the study. J.F., Y.B., Z.W., and T.W: wrote the original draft, reviewed and edited the manuscript. J.F., Y.B., Z.W., and T.W.: designed and prepared the graphical materials and visualizations. All authors have read and agreed to the published version of the manuscript.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. We confirm that all figures and tables are original. No third-party material is reproduced.

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