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Membrane-bound IL-7 immobilized by the CD8 transmembrane region improves efficacy of CD19 CAR-T cell therapy

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Abstract

Enhancing the efficacy of CD19 CAR-T cell therapy can significantly improve patient outcomes by reducing relapse rates in CD19 + B cell malignancies. Exogenous or transgenic cytokines are often used to boost the expansion and durability of CAR-T cells but pose risks of severe toxicities. A promising approach to address these limitations is to immobilize cytokines on the surface of CAR-T cells using transmembrane (TM) anchor domains. Given IL-7 can enhance T-cell proliferation and antitumor activity, our study developed membrane-bound IL-7 constructs using different TM anchor domains (CD8, CD28 and B7-1). We primarily found that the CD8 TM provided superior anchoring for IL-7 compared to CD28 and B7-1. Moreover, the IL-7 construct with a CD8 TM (IL7/CD8) enhanced naïve T cell proliferation and effector functions, and improved the in vitro and in vivo antitumor activity of CD19 CAR-T cells. Importantly, although IL7/CD8 could promote T-cell proliferation, it did not sustain long-term autonomous expansion, which could ensure the safety of CD19 CAR-T cells expressing IL7/CD8 in clinical applications. Collectively, the IL7/CD8 construct represents a promising strategy for enhancing the therapeutic potential of CD19 CAR-T cell therapy.

Keywords Membrane-bound IL-7, CD19 CAR-T, Hematologic malignancy

To the editor,

Cytokines have the ability to improve the proliferative and antitumor functions of CD19 CAR-T cells [1, 2]. However, there have been cases of toxicities resulting from uncontrolled systemic administration and release of cytokines from genetically modified T cells [3–5]. A previous conference abstract reported that membrane-bound IL-7 could enhance CAR-T proliferation without the need for additional cytokine support [6]. However, previous research reported that different transmembrane domains can vary in their ability to anchor cytokines to the cell membrane [7]. Our study attempted to develop membrane-bound IL-7 using different transmembrane (TM) anchor domains to enhance proliferation and

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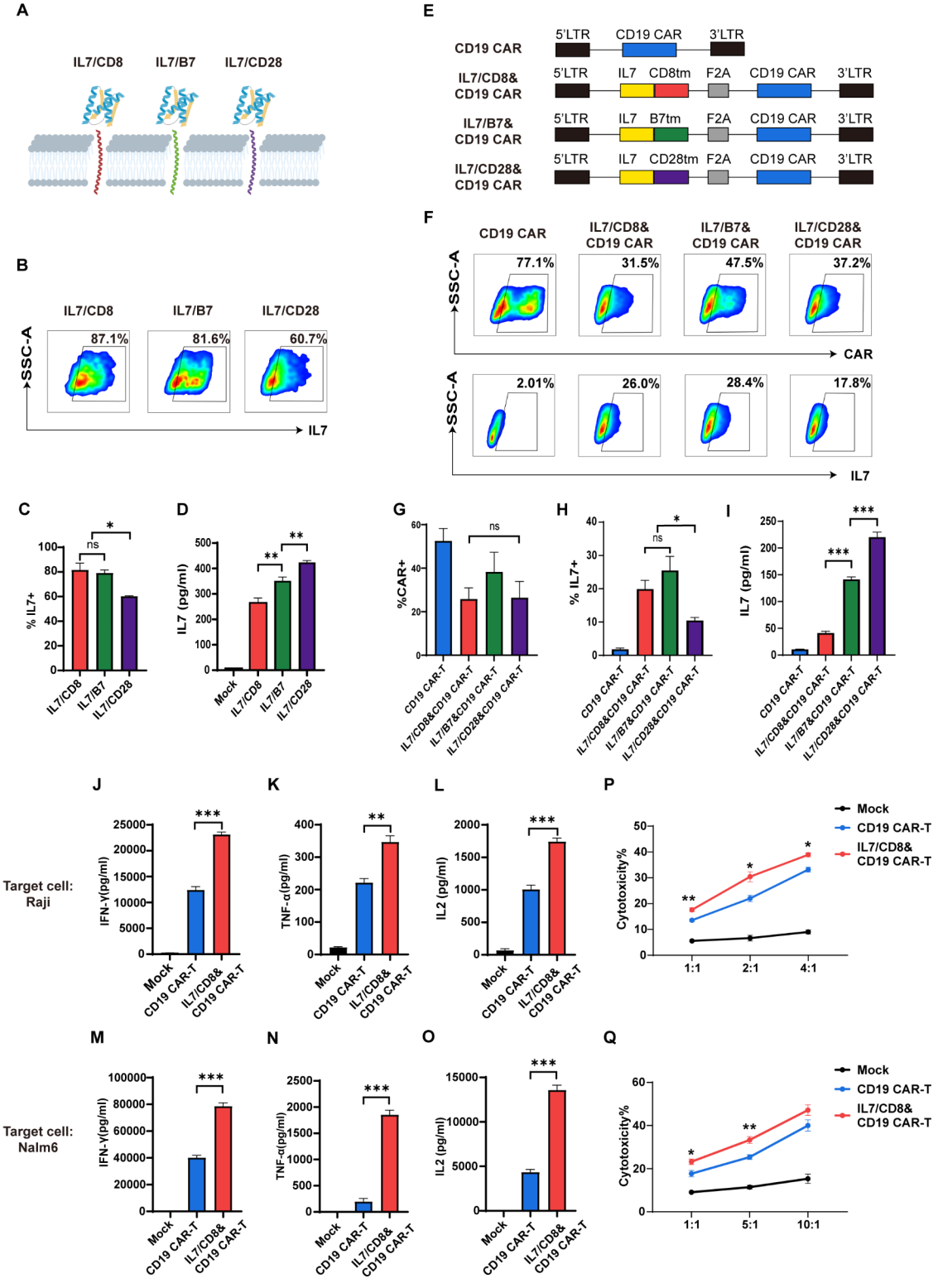

Fig. 1 (See legend on next page.)

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Fig. 1 Construct, optimization and effect function of membrane-bound IL-7 **A**, Schematic representations of membrane-bound IL-7 using different TM anchor domains (CD8, CD28, and B7-1). **B-C**, Representative flow cytometric results showing membrane-bound IL-7 with different TM anchor domains on the cell surface **(B)** and corresponding quantitative analysis **(C)**. **D**, IL-7 concentrations in the supernatant of T cells expressing membrane-bound IL-7 with different TM anchor domains, measured by ELISA. **E**, Schematic representations of CD19 CAR and membrane-bound IL-7 with different TM anchor domains. **F-H**, Representative flow cytometric results showing expression level of CD19 CAR and membrane-bound IL-7 with different TM anchor domains on the surface of T cells **(F)** and corresponding quantitative analysis of CD19 CAR expression level **(G)**, and membrane-bound IL-7 expression level **(H)**. **I**, IL-7 concentrations in the supernatant of CD19 CAR-T cells expressing membrane-bound IL-7 with different TM anchor domains, measured by ELISA **J-L**, IFN-γ **(J)**, TNF-α **(K)**, and IL-2 **(L)** secreted by T cells 24 h after coculture with Raji tumor cells, determined by ELISA. **M-O**, IFN-γ **(M)**, TNF-α **(N)**, and IL-2 **(O)** secreted by T cells 24 h after coculture with Nalm6 tumor cells, determined by ELISA. **P-Q**, Lysis of Raji cells **(P)** and Nalm6 cells **(Q)** by T cells. Data are representative of at least three independent experiments with more than three different donors. Mean values from each group are plotted. Error bars represent SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, analyzed by one-way ANOVA with Bonferroni posttest)

anti-tumor activity of CD19 CAR-T cells and meanwhile minimize unexpected systemic toxicity.

To construct membrane-bound IL-7, we tested three TM anchors: CD8, B7-1, and CD28 (Fig. 1A and Table S1). The cell surface expression levels of IL-7 constructs with the CD8 TM and B7-1 TM were similar and significantly higher than those of the IL-7 construct with the CD28 TM (Fig. 1B and C). The culture supernatant of T cells expressing the CD8 TM construct had significantly lower soluble IL-7 compared to the culture supernatant of T cells expressing the B7-1 or CD28 TM constructs (Fig. 1D). To further evaluate the cell surface expression levels of different membrane-bound IL-7 constructs in CD19 CAR-T cells, we generated multi-cistronic lentiviral vectors to express either a conventional anti-CD19 targeting CAR with 4-1BB and CD3ζ signaling domains (CD19 CAR) or CD19 CAR also expressing membrane-bound IL-7 following a ribosome skipping 2 A element (Fig. 1E). As expected, we found that the cell surface expression levels of IL-7 constructs with the CD8 TM and B7-1 TM were comparable and markedly exceeded those of the IL-7 construct with the CD28 TM and yet expression levels of CD19 CAR were similar in different constructs (Fig. 1F-H). Meanwhile, the culture supernatant of CD19 CAR-T cells expressing the CD8 TM construct had significantly lower soluble IL-7 compared to the culture supernatant of T cells expressing the B7-1 or CD28 TM constructs (Fig. 1I). In summary, due to its relatively high membrane expression and relatively low shedding, the IL-7 construct with a CD8 TM (IL7/CD8) was selected for further study.

Greater amounts of IFN-γ, IL-2, and TNF-α were produced by IL7/CD8&CD19 CAR-T cells compared to CD19 CAR-T cells when cocultured with Raji and Nalm6 cells (Fig. 1J-O). Compared with CD19 CAR-T cells, IL7/CD8&CD19 CAR-T cells exhibited more potent cytotoxicity activity against Raji and Nalm6 cells (Fig. 1P and Q). Collectively, IL7/CD8 enhances in vitro cytokine production and cytotoxicity of CD19 CAR-T cells.

After infusion into patients, CAR-T cells lack the support of anti-CD3 and anti-CD28 antibodies and IL-2, making it essential to evaluate the effect of IL7/CD8 on CD19 CAR-T cells following withdrawal of these antibodies and IL-2. Following a 4-day withdrawal, we found that the viability, absolute number, and proliferative capacity of CD19 CAR-T cells expressing IL7/CD8 were higher than those of CD19 CAR-T cells (Fig. 2A-E). Meanwhile, we found that IL7/CD8&CD19 CAR-T cells retained a significantly higher naïve phenotype and lower central memory phenotype compared with CD19 CAR-T cells (Fig. 2F and G). To evaluate whether IL7/CD8 could promote antigen-independent long-term expansion, we cultured CD19 CAR-T cells and IL7/CD8&CD19 CAR-T cells without the supplementation of anti-CD3 and anti-CD28 antibodies and IL-2. Although IL7/CD8&CD19 CAR-T cells persisted significantly longer than CD19 CAR-T cells in vitro, the IL7/CD8&CD19 CAR-T population began to contract by day 5 to 10, with all cells dying by day 25 (Fig. 2H). This confirmed that IL7/CD8 did not sustain long-term autonomous T-cell expansion, which is a crucial aspect of safety.

After CAR-T cells are infused into patients, they not only lack necessary support from anti-CD3 and anti-CD28 antibodies and IL-2, but could repeatedly encounter tumor cells. Therefore, it is essential to evaluate the impact of IL7/CD8 on CD19 CAR-T cells during consecutive in vitro tumor cell challenges following withdrawal of anti-CD3 and anti-CD28 antibodies and IL-2. IL7/CD8&CD19 CAR-T cells and CD19 CAR-T cells were then continuously stimulated with Raji cells twice at an E: T ratio of 1:2, with a three-day interval between each stimulation (Figure S1A). After each round of stimulation, CAR-T cells were recollected and cocultured with Raji cells at an E: T ratio of 1:1 for 24 h. During the initial round of coculture, both IL7/CD8&CD19 CAR-T cells and CD19 CAR-T cells effectively eliminated Raji cells (Figure S1B). However, after the second stimulation, IL7/CD8&CD19 CAR-T cells exhibited more potent cytotoxic activity against Raji compared to CD19 CAR-T cells (Figure S1B). Analysis of the phenotypes of CAR-T cells at the end of each round of stimulation was also conducted. Compared with CD19 CAR-T cells, IL7/CD8&CD19 CAR-T cells exhibited higher proliferative capacity and a more naïve phenotype after the second round of stimulation (Figure S1C-F). However, expression levels of exhausted makers, such as PD1, TIM3 and

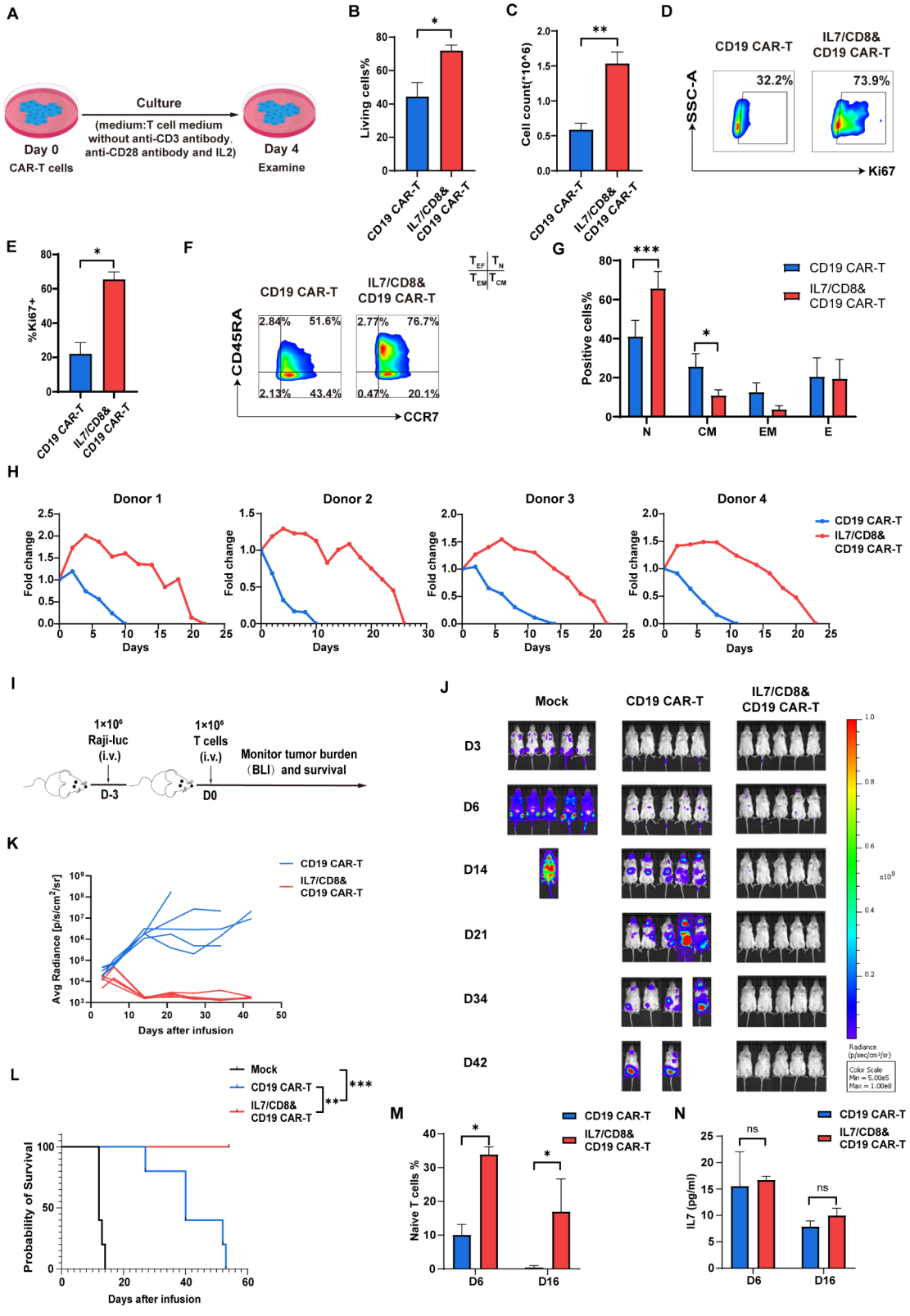

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Fig. 2 Phenotypes and in vivo antitumor activity of CD19 CAR-T cells expressing IL7/CD8 **A**, Schematic representation of T cell culture following a 4-day withdrawal of anti-CD3 and anti-CD28 antibodies and IL-2. **B-C**, Percentages **(B)** and counts **(C)** of living T cells. **D-E**, Representative flow cytometric plots showing percentages of Ki67 + T cells **(D)** and corresponding quantitative analysis **(E)**. **F-G**, Representative flow cytometric plots showing CD45RA and CCR7 expression **(F)** and corresponding quantitative analysis **(G)**. **H**, Quantitative analysis of in vitro persistence of T cells following withdrawal of anti-CD3 and anti-CD28 antibodies and IL-2. Live cells were counted using trypan-blue exclusion. The X-axis denotes the number of days after anti-CD3 and anti-CD28 antibodies and IL-2 were withdrawn from the culture media. **I**, Schematic representation of the Raji xenograft model. NCG mice were intravenously inoculated via tail injection with 1×10^6 Raji cells labeled with Firefly-luciferase and three days later received T cells intravenously. **J-K**, Representative bioluminescent images **(J)** and quantitated bioluminescent signals **(K)** showing tumor growth over time. **L**, Kaplan–Meier survival curve showing the survival of mice. Significance was determined by the log-rank test. ** $p < 0.01$, *** $p < 0.001$. **M-N**, Quantification of naïve T cells **(M)** and IL-7 **(N)** in the peripheral blood of treated mice at days 6 and 16 after T-cell infusions. Significance was determined by a two-tailed t test. * $p < 0.05$, ns, not significant. Data are representative of at least three independent experiments with more than three different donors. Mean values from each group are plotted. Error bars represent SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, analyzed by two-tailed t test)

Lag3, were similar between CD19 CAR-T cells and IL7/CD8&CD19 CAR-T cells (Figure S1G-H). Overall, IL7/CD8 improves antitumor activity and expansion of CD19 CAR-T cells, but could not affect exhaustion levels during sequential encounters with tumor cells.

To evaluate the in vivo antitumor activity of IL7/CD8&CD19 CAR-T cells and CD19 CAR-T cells, we intravenously delivered a suboptimal dose (1×10^6) of CAR-T cells into NCG mice engrafted with the Raji tumor cell line (Fig. 2I). Tumors grew rapidly in mice treated with mock cells, while both CD19 CAR-T cells and IL7/CD8&CD19 CAR-T cells controlled tumor growth (Fig. 2J and K). However, only IL7/CD8&CD19 CAR-T cells succeeded in controlling tumor growth and eliminating all tumor cells (Fig. 2J and K). Additionally, IL7/CD8&CD19 CAR-T cells induced long-term survival in all five mice, while all five mice receiving CD19 CAR-T cells died before day 54 (Fig. 2L). Consistent with the in vitro data, IL7/CD8&CD19 CAR-T cells were enriched in naïve T cells in the peripheral blood at days 6 and 16 after CAR-T cell infusion, compared to CD19 CAR-T cells (Fig. 2M). Additionally, the concentration of soluble IL7 were similar and relatively low in the serum of mice in both groups at days 6 and 16 after CAR-T cell infusion (Fig. 2N). To assess whether IL7/CD8&CD19 CAR-T cells remained present and active against tumor cells, we rechallenged three mice on day 54 that did not have detectable residual tumors after treatment with IL7/CD8&CD19 CAR-T cells (Figure S2A). These mice were not fully protected against tumor outgrowth, but tumor growth was delayed compared to that in naive age-matched control mice, suggesting residual presence and activity of tumor-reactive T cells (Figure S2B and S2C).

Having observed improved tumor control of CD19 CAR-T with the addition of IL7/CD8, we attempted to evaluate how IL7/CD8 acted on CAR-T cells: in cis, trans or even both? Since phosphorylation of STAT5, a signaling mediator downstream of IL-7R, is a marker of pathway activity, phosphorylation of STAT5 could be used to evaluate how IL7/CD8 acted on CAR-T cells. We found the IL7/CD8 positive T cells had more STAT5 phosphorylation than the IL7/CD8 negative T cells, implying that membrane-bound IL-7 not only acts on its own T cells but can also affect other T cells (Figure S3A and S3B).

In conclusion, we have successfully developed a technology that incorporates CD8 transmembrane region-anchored IL-7 into CD19 CAR-T cells. This technology represents a promising avenue for enhancing the therapeutic potential of CD19 CAR-T cell therapy and warrants further exploration in clinical trials.

Abbreviations

CAR-T	Chimeric antigen receptor T cell
TM	transmembrane
ELISA	Enzyme-linked immunosorbent assay
IFN-γ	Interferon-γ
TNF-α	tumor necrosis factor-α
PBMC	Peripheral blood mononuclear cell
PBS	phosphate buffer saline

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12943-024-02154-0.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

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Not applicable.

Author contributions

Z. L, W. Y and C. Z designed the research; T. L, C. Z, S. L, X. T, Y. Z, G. Z, H. X, K. S and J. T conducted experiments; C. Z, T. L and Z. L analyzed data; and C. Z, Z. L, and T. L wrote the paper.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal experiments were approved by Experimental Animal Ethics Committee of Peking university Cancer Hospital (EAEC 2018-05).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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