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TRAIL-R1-targeted chimeric antigen receptor T cells exhibit dual antitumor effects

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) has limited expression in normal tissues but highly expression in a broad range of tumors, making it an attractive target for cancer immunotherapy. We have previously prepared a fully human monoclonal antibody targeting TRAIL-R1 (TR1419), which can specifically induce apoptosis in antigen-positive tumor cells. Here, we prepared the TR1419CAR-T cells using the single chain variable fragment (scFv) from TR1419, which were evaluated for the phenotypes and function. The TR1419CAR-T cells induced cytolysis of TRAIL-R1-positive tumor cells not only via activation of the death receptor-dependent apoptotic pathway, but also via T-cell mediated cytotoxicity. Furthermore, compared to the second-generation TR1419-28ζ and TR1419-BBζ CAR-T cells, the third-generation TR1419-28BBζ CAR-T cells had greater sensitivity to target antigen, exhibited a better proliferative ability, but showed slightly higher PD-1 expression after antigen stimulation. Altogether, TR1419CAR-T cells, especially TR1419-28BBζCAR-T cells could be a promising treatment strategy for TRAIL-R1 positive tumors.

TRAIL-R1-targeted chimeric antigen receptor T cells exhibit dual antitumor effects

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) has limited expression in normal tissues but highly expression in a broad range of tumors, making it an attractive target for cancer immunotherapy. We have previously prepared a fully human monoclonal antibody targeting TRAIL-R1 ($TR1^{419}$), which can specifically induce apoptosis in antigen-positive tumor cells. Here, we prepared the $TR1^{419}$CAR-T cells using the single chain variable fragment (scFv) from $TR1^{419}$, which were evaluated

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for the phenotypes and function. The TR1^{419}CAR-T cells induced cytolysis of TRAIL-R1-positive tumor cells not only via activation of the death receptor-dependent apoptotic pathway, but also via T-cell mediated cytotoxicity. Furthermore, compared to the second-generation TR1^{419}-28ζ and TR1^{419}-BBζ CAR-T cells, the third-generation TR1^{419}-28BBζ CAR-T cells had greater sensitivity to target antigen, exhibited a better proliferative ability, but showed slightly higher PD-1 expression after antigen stimulation. Altogether, TR1^{419}CAR-T cells, especially TR1^{419}-28BBζCAR-T cells could be a promising treatment strategy for TRAIL-R1 positive tumors.

Key words: TRAIL-R1, CAR-T, Apoptosis, Cytotoxicity, Third-generation

Introduction

Chimeric antigen receptors (CARs) are synthetic molecules composed of an antigen recognition domain of single chain variable fragment (scFv), a hinge region, a transmembrane domain, intracellular signaling region, costimulatory domains and a CD3ζ chain [1-3]. CAR-T cells have been considered as promising new modalities for adoptive cellular therapy [4]. Multiple clinical trials reported the CD19-target in the treatment of patient with malignancies [5,6]. However, there are still many encumbered challenges in solid tumor [7]. The key to the success of CAR-T cells therapy is the selection of an ideal target antigen, which should be expressed on the surface of cancer cells and shouldn't or little be expressed on normal cells [8,9]. Therefore, finding an ideal target for CAR-T cells is one of the most important tasks for successful CAR-T therapy in solid tumors [10]. Additionally, CAR co-stimulation domains play an essential role in promoting the expansion and antitumor of CAR-T cells [11-13]. It has been reported that the phenotypic and functional differences of the second and third generation CAR-T cells are related to the selection of target antigen and the design of scFv structure [14].

TRAIL is a member of the tumor necrosis family (TNF) superfamily that interacts with its death receptors (TRAIL-R1/TR1/DR4) and induces apoptosis in a wide range of cancer cell types but not in normal cells [15]. However, some clinical trials have shown that TRAIL has little antitumor efficacy [16]. Some cancer cells are resistant to TRAIL based therapies [17]. One reason is that TRAIL appeared not to induce effective aggregation of its cognate death receptor, leading to insufficient apoptosis of some tumor cells [18]. To overcome this shortcoming, agonistic antibodies that target TRAIL-R1 have been developed for clinical application. Previously, we developed a fully human monoclonal antibody (TR1^{419}) using the ISAAC (immunospot array assay on a chip) technology and chimeric TransChromo (TC) mice with human immunoglobulin g and ϰ loci [19,20]. TR1^{419} could strongly induce apoptosis in TR1 positive cancer cell lines in the presence of crosslinking antibodies [21]. Moreover, TR1^{419}can enhance TRAIL-induced apoptosis by activation of caspase-8 and upregulated expression of TR1 and increased phosphorylation of JNK [22,23].

In the present study, we first generated third-generation TR1^{419}-28BBζ CAR-T cells, which were shown to induce cytolysis of target tumor cells not only via TR1-mediated apoptosis but also via CAR signal-induced cytolysis. Furthermore, we compared which CARs structure was the most appropriate for scFv from TR1^{419}. The results showed that the third-generation TR1^{419}-28BBζ CAR-T cells had higher sensitivity to target antigen, exhibited a better proliferative ability, but had a higher level of PD-1 expression after target antigen stimulation, compared to the second-generations TR1^{419}-28ζ and TR1^{419}-BBζ CAR-T cells. This study suggests that TR1^{419}CAR-T cells could be a promising treatment strategy for TRAIL-R1 positive tumors, and the dual killing mechanism provides a novel optimizing strategy for other CARs design.

Materials and methods

2.1. Plasmid construction and lentivirus preparation

The target TR1^{419}CAR consisted of TR1^{419} scFv, CD28 or 4-1BB costimulatory domains, and CD3-ζ signaling domain [24,25]. They were obtained by overlap PCR amplification, PCR products were clone into lentivirus vector pWPXL and verified by enzymatic digestion and sequencing. To produce the lentivirus supernatant, 8×10^5 HEK-293T cells were plated in 6-well. After 24h, 293T cells were transfected with pMD.2G encoding VSV-G envelope, pSPAX2 lentivirus plasmid, and the CAR-pWPXL plasmids by using

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xfect Transfection Reagent (Takara), according to the manufacturer's instructions. The lentivirus supernatants were collected and filtered with 0.45 μM filter at 48h after transfection.

2.2. Cell lines and cell culture conditions

SW480, HCT116, K562 and huh7 were obtained from ATCC. Human Jurkat cell line was maintained in our laboratory. These cells were cultured in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, ISR). Human breast cancer cell lines MDA-MB-231 and HS578T were obtained from ATCC and cultured in Dulbecco's modified Eagle medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, ISR). The HEK-293T cell lines were purchased from ATCC and maintained in complete growth medium (Gibco invitrogen), containing with 10% fetal bovine serum (FBS) (Biological Industries, ISR), 2mM L-glutamine (Gibco, Invitrogen) and 1mM sodium pyruvate solution (Gibco, Invitrogen). All above cells were cultured in a humidified atmosphere containing 5% CO_2 at 37.

2.3. Human CAR-T cells generation

Human peripheral blood mononuclear cells (PBMCs) samples were obtained from healthy donors according to the manufacturer's protocol. Written informed consents were obtained from all donors. PBMC were stimulated with anti-CD3/CD28 for 48 h. Then, the activated T cells were infected with the CAR-encoding lentiviral supernatant. 24h after lentiviral infection, the lentiviral supernatant was replaced with fresh medium containing RPMI-1640 and supplemented with 10% FBS (Gibco), 200U/ml IL-2, 25 mM HEPPES, 55 μM 2-M, 100 U/ml penicillin and 100 μg/ml streptomycin. The infected T cells were cultured for 7 days.

2.4. Flow cytometry

For cell-surface staining, the lentivirus-infected T cells were harvested and washed with phosphate buffered saline (PBS) followed by incubation with 1ug/ml recombinant protein TRIL-R1-Fc (R&D Systems, Minneapolis, MN) at room temperature for 20 min. After being washed, the cells were incubation with 1ug/ml APC conjugated anti-human IgG secondary antibodies and other cocktail antibodies (Biolegend) at room temperature for 15 min in the dark. After being washed with PBS, the cells were analyzed with a BD celesta (BD Biosciences) software by flow cytometry. The following monoclonal antibodies were used with the indicated subtypes: APC-lableled human IgG-Fc (clone HP6017), Percy5.5-lableled anti-CD4 (clone RPA-T4), Alex700-lableled anti-CD8 (clone HIT8a), BV421-lableled anti-CCR7 (clone G043H7), PE-lableled anti-CD45RO (clone UCHL1), AF488-lableled anti PD-1(clone EH12.2H7), BV605-lableled anti-LAG-3 (clone 11C3C65), BV785-lableled anti-TIM-3 (clone F38-2E2). All sample data analyzed was done on[?]10,000 events using the FlowJo V 10 data analysis software.

2.5. Killing activity assay

The ability of CAR-T cells to kill tumor cells was determined via Calcein AM (CAM, Dojindo) release-based cytotoxic cell assay [26]. Briefly, target cells were stained with 10μM CAM for 30 minutes at 37, followed by terminating the reaction with addition of Durbe phosphate buffered saline without calcium (DPBS) buffer. The target tumor cells were washed five times with DPBS. Non-transduced T cells were used to normalize the percentage of CAR-positive cells. Then, CAR-T cells and target cells were plated in a 96-well microplate at various effector/target (E:T) ratios. After incubation for 6h at 37 °C, the 50ul supernatant were transferred to 96-black plates to measure fluorescence intensity (FI) at 485 nm excitation and 520 nm emission wavelengths. The percentage of cytotxicity was calculated according to the following formula: lysis% = (test release–spontaneous release)/(maximal release–spontaneous release)x100%.

The ability of CAR-T cells to kill 293T cells was detected via Real Time Cell Analysis (RTCA, ACEA). One prior day to seeded approximately $1*10^4$ 293T cells on 16 E-plate, the cells were grown to mid-logarithmic growth phase. Non-transduced T cells were used to normalize the percentage of CAR-positive cells, and CAR-T cells were added to the cultures at indicated effector-to-target ratio. RTCA DP analyzer was used to monitor real-time target cell growth, and the results were analyzed by RTCA software [27]. The percentage

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of cytotxicity was calculated according to the following formula: lysis% = [1 – (experiments/empty culture)] x100%.

2.6. Enayme-linked immunosorbent assay

The CAR-T cells and target tumor cells were co-cultured at various effector/target (E:T) ratios in a 96-well plate for 24hr at 37. The culture supernatants were harvested and subjected to ELISA to detect INF-γ and Granzyme B, according to the manufacturer's instructions. Every value represented the mean of triplicate wells.

2.7. Tumor cells apoptosis assay

CAR-T cells and target cells were incubated for 2h at 37°C, and target cell apoptosis was assayed using an Annexin V/7-AAD Apoptosis Detection Kit (Biolegend), according to the manufacturer's instructions. Target cell caspase-3 activity was detected used a caspase-3 activity detection assay kit (BD). In brief, the cells were washed twice with cold PBS, followed by fixing and permeabilizing in Cytofix/CytoPerm for 20 min on ice (BD Biosciences). Subsequent staining was performed with 1×PermWash as the staining and wash buffer and incubated for 15 min at room temperature. Washed cells were analyzed with a BD celesta (BD Biosciences) software by flow cytometry.

2.8. Statistical analysis

Statistical analyses in this study were performed with Graph Pad Prism software 8.0.2 version. Data are presented as the mean ± SEM. When there were two experiment groups, Student's t-test was used or ANOVA analysis for multiple comparisons. $P<0.05$ were considered statistically significance.

Results

3.1. TP1^{419}-28BBζ ΤAP-T ςελλς εξηιβιτεδ συπεριορ κιλλινγ εφφιςαςψ αγαινστ TPAIΛ-P1 ποσιτιε τυμορ ςελλς

In order to generate TR1^{419} CAR-T cells, we first constructed the lentivirus vector incorporating a CD28 and CD137 co-stimulatory domain, and CD3ζ activation domain. The single-chain variable fragment (scFv) was derived from a fully human monoclonal antibody (TR1^{419}), which has high affinity and specificity for TRAIL-R1. We generated human TR1^{419}-28BBζ CAR-T or mock-T cells by lentiviral transduction of PBMCs derived from healthy donors. The surface expression of CAR on the T cells was measured by flow cytometry. As shown in Fig. 1A, flow cytometric analysis confirmed that the frequency of CAR expression was 39.3%. To characterize the phenotypes of the CAR-T cells, the cells were harvested for flow cytometric detection of CD45RA, CCR7, PD-1, LAG-3 and TIM-3 by day 7 after transfection. 45%-55% T cells were naïve T cells (CCD45RA$^+$CCR7$^+$), and the proportions of the other three subtypes (central memory T cells, effector memory T cells, and effector T cells) were comparable (Fig.1B). No significant differences of immunoinhibitory molecule expression, PD-1, LAG-3 and TIM-3 were observed between TR1^{419}-28BBζ CAR-T cells and mock T cells, indicating the phenotypes of T cells irrespective of gene transfection (Fig. 1C)

To determine whether TRAIL-R1 positive cancer cell lines were susceptible to TR1^{419}-28BBζ CAR-T-cell-mediated lysis in vitro, we performed cytotoxicity assays using engineered T cells as effector cells, and different cancer cells as target cells. The TRAIL-R1 expression in different cancer cells was validated by flow cytometry (Fig. S1). Compared with mock-transduced T cells, TR1^{419}-28BBζ CAR-T cells displayed significantly higher specific killing activity against TRAIL-R1 positive SW480, HCT116 and MDA-MB-231 cells at all E:T ratios, and accompanied by an increased IFN-γ and Granzyme B secretion (Fig. 1D-F). As for the TRAIL-R1 negative 293T cells, TR1^{419}-28BBζ CAR-T cells had no significant killing effect and secretion of effector cytokines, compared with the mock T cells (Fig. 1D-F).

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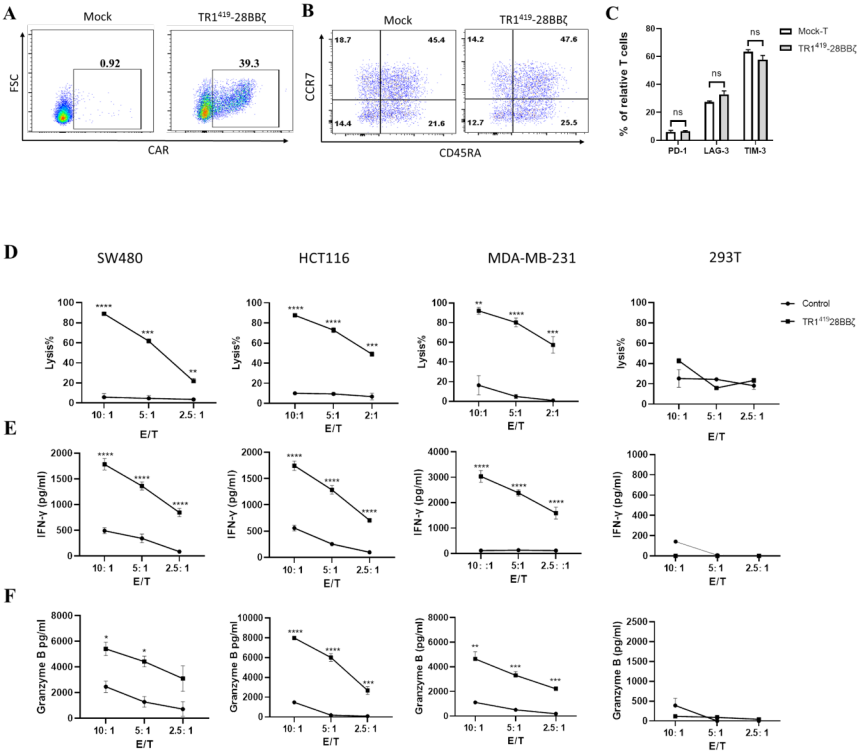

Fig.1.TR1^{419}-28BBζ CAR-T cells had specific killing to tumor cells. (A) CAR construct with humanized anti-TRAIL-R1 scFv, CD8a hinge, CD28 transmembrane domain and intracellular co-stimulatory domain, CD137 intracellular co-stimulatory domain, and intracellular CD3ζ activation domain. (B) Expression rate of the CAR on T cells was confirmed by flow cytometry on day 7 following lentiviral transfection. Mock T cells were served as a negative control. (C-D) Expression of inhibitory receptors and subtypes on CAR-T cells was examined by flow cytometry, CD45RA^{+}CCR7^{+}: naïve T cells, CD45RA^{-}CCR7^{+}: central memory T cells, CD45RA^{-}CCR7^{-}: effector memory T cells, CD45RA^{+}CCR7^{-}: effector T cells. (E) Cytotoxic activity of TR1^{419}-28BBζ CAR- or mock transduced T cells against TRAIL-R1 positive tumor cell lines. The effector cells were co-cultured for 6 h with target cells at E:T ratio of 10:1, 5:1 and 2.5:1, respectively. Cytotoxicity was determined by Calcein AM release-based cytotoxic cell assay. (F-G) IFN-γ and Granzyme B production by TR1^{419}-28BBζ CAR-T cells or mock-transduced T cells was detected by ELISA when co-cultured with the indicated cells for 24 h. The analyses were performed using Students' t tests. Data reflected the mean ± SEM of three separate experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3.2. ScFv from TR1^{419} CAR was proved to mediate tumor apoptosis in Jurkat cells

To explore whether scFv from TR1^{419} CAR could mediate tumor apoptosis through activation of the death receptor-dependent apoptotic pathway, we first constructed TR1^{419}[?]ζ lentivirus vector, which was composed of scFv from TR1^{419} fused to a CD8α hinge-transmembrane region, and truncated CD3ζ region (Fig. 2A). We generated TR1^{419}-28BBζ or TR1^{419}[?]ζ-jurkat cells by lentiviral transduction, and the surface expression of scFv on the jurkat cells was measured by flow cytometry (Fig. 2B). Then, the xCELLigence RTCA DP was adopted to determine whether TR1^{419}-28BBζ or TR1^{419}[?]ζ-jurkat cells could mediate tumor apoptosis. We have previously demonstrated with CD19-CAR that CAR-Jurkat cells have no ability to mediate the death of target antigen-positive tumor cells (Fig. S2). However, our results showed that TRAIL-R1-positive tumor cells were lysed by TR1^{419}-28BBζ-jurkat cells and TR1^{419}[?]ζ-jurkat cells, and the specific cytotoxic effect can be blocked by the soluble target antigen TRAIL-R1 (Fig. 2C).

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Next, TR1^{419}-28BBζ-jurkat cells and TR1^{419}[?]ζ-jurkat cells were co-incubated separately with SW480 cell lines for 2 h at a 1:1 ratio, then the expression of AnnexinV/7-AAD and caspase3 in SW480 cells was detected by flow cytometry. The percentages of 7-AAD$^-$Annexin V$^+$ early (25.3%) and 7-AAD$^+$Annexin V$^+$ late (24.9%) apoptotic SW480 cells mediated by TR1^{419}-28BBζ-jurkat cells and TR1^{419}[?]ζ-jurkat cells were significantly higher than the control (Fig. 2D). The percentages of Caspase3$^+$ in SW480 cells mediated by TR1^{419}-28BBζ-jurkat cells and TR1^{419}[?]ζ-jurkat cells were also significantly higher than the control. Meanwhile, the soluble target antigen blocked 100% of the above killing activity. Collectively, these results suggested scFv from TR1^{419} CAR can mediate TRAIL-R1-positive tumor apoptosis through activation of the death receptor-dependent apoptotic pathway.

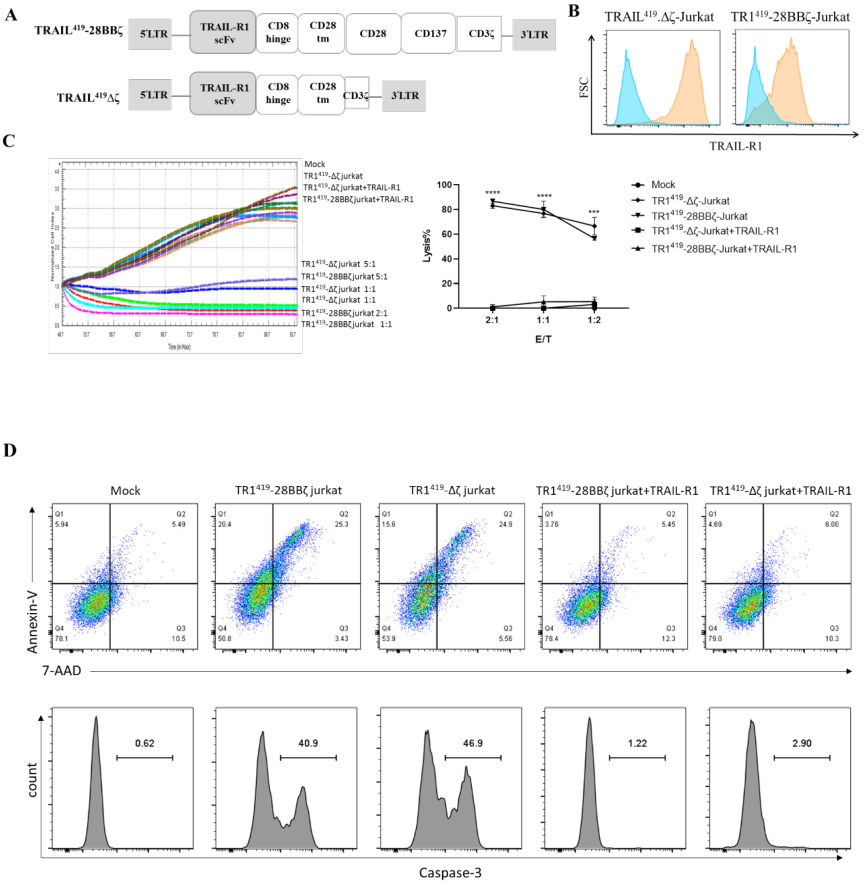

Fig.2. scFv from TR1^{419} CAR mediated tumor cell apoptosis by binding with TRAIL-R1. (A) Schematic representation of TR1^{419}[?]ζ and the lentiviral vector. The construct consisted of the extracellular portion of anti-TRAIL-R1 scFv, linking via a CD8α hinge-transmembrane domains to the truncated CD3ζ molecule. (B) The expression of TR1^{419}-28BBζ and TR1^{419}[?]ζ in Jurkat cells was detected by flow cytometry. (C) Cytotoxicity of TR1^{419}-28BBζ- and TR1^{419}[?]ζ -Jurkat cells against SW480 in the absence or in the presence of soluble TRAIL-R1 protein was determined by RTCA. The analysis was performed using Students' t tests. Data reflected the mean ± SEM of triplicate wells, $***p < 0.01$. (D) SW480 were fixed and labeled with annexin V and 7-AAD and then analyzed by flow cytometry to identify apoptotic cells (annexin V$^+$/7-AAD$^-$ and annexin V$^+$/7-AAD$^+$), after incubation with effector cells for 2h. (E) The expression of caspase3 protein in SW480 analyzed by flow cytometry, after incubation with effector cells for 2h.

3.3. TP1^{419}-28BBζ ΆAP-T ςελλς εξηιβιτεδ δυαλ αντιτυμορ εφφεςτς

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To further investigate whether TR1^{419}-28BBζ CAR-T cells can mediate tumor apoptosis through binding to death receptor TRAIL-R1, we first generated TRAIL-R1 (full length) and TRAIL-R1 (truncation) lentivirus vectors. The TRAIL-R1 (full length) was composed of full TRAIL-R1, and the death domain of TRAIL-R1 was truncated in the TRAIL-R1 (truncation) [28] (Fig. 3A). We produced 293T (TRAIL-R1 full length) and 293T (TRAIL-R1 trunc) cells by lentiviral transfecting the associated plasmids (Fig. 3B).

Next, we generated TR1^{419}-28BBζ CAR-T cells and TR1^{419}[?]ζ-T cells by lentiviral transduction, which transfection rates were detected by flow cytometry (Fig. 3C). Then, we performed cytotoxicity assays using TR1^{419}-28BBζ CAR-T cells and TR1^{419}[?]ζ-T cells as effector cells, and different 293T cells as target cells. As for the TRAIL-R1 negative 293T cells, TR1^{419}-28BBζ CAR-T cells and TR1^{419}[?]ζ-T cells had no significant killing effect and secretion of effector cytokines, compared with the mock T cells (Fig. 3D-F). The TR1^{419}-28BBζ CAR-T cells and TR1^{419}[?]ζ-T cells effectively killed the 293T (TRAIL-R1 full length) cells, whereas TR1^{419}-28BBζ CAR-T cells displayed significantly higher specific killing compared to TR1^{419}[?]ζ-T cells (Fig. 3D). Meanwhile, the data showed that much higher levels of IFN-γ and Granzyme B were produced by TR1^{419}-28BBζ CAR-T cells compared to TR1^{419}[?]ζ-T cells when co-culturing with 293T (TRAIL-R1 full length) cells. However, no significant difference was found in cytokine secretion between TR1^{419}[?]ζ-T cells and mock T cells (Fig. 3E-F). As for the 293T (TRAIL-R1 trunc) cells, TR1^{419}[?]ζ-T cells had no significant killing effect and secretion of effector cytokines compared with the mock T cells, but TR1^{419}-28BBζ CAR-T cells displayed significantly killing effect and IFN-γ and Granzyme B production (Fig. 3D-F). The results above suggested that TR1^{419}-CAR-T cells can not only effectively induce T-cell mediated cytotoxicity toward tumor cells, but also mediate tumor cell apoptosis through activation of the death receptor-dependent apoptotic pathway, indicating dual anti-tumor activity.

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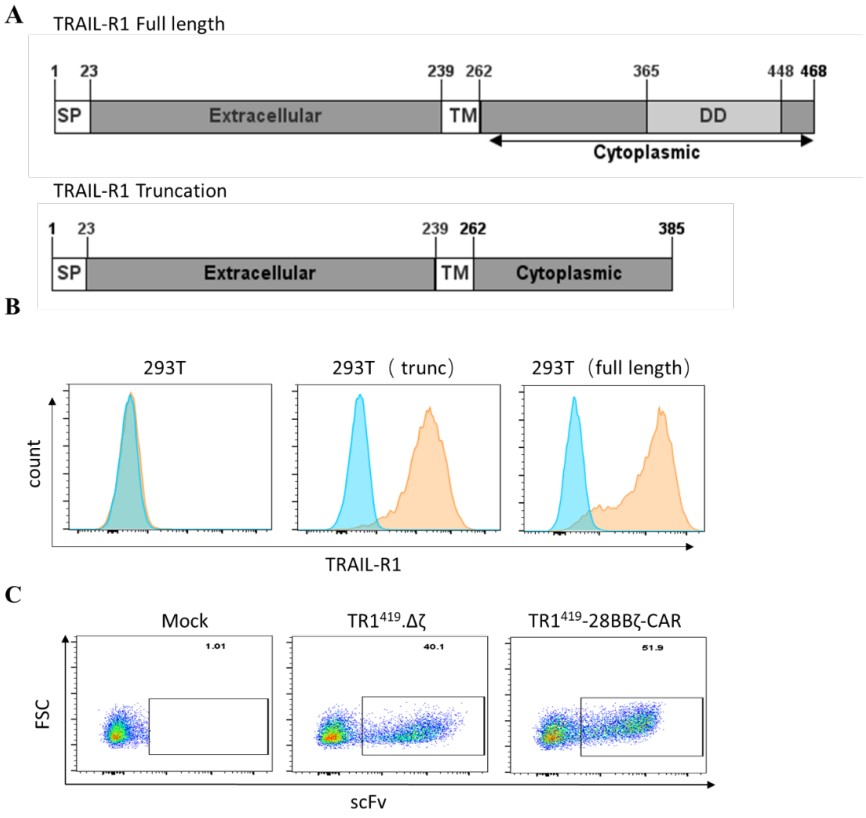

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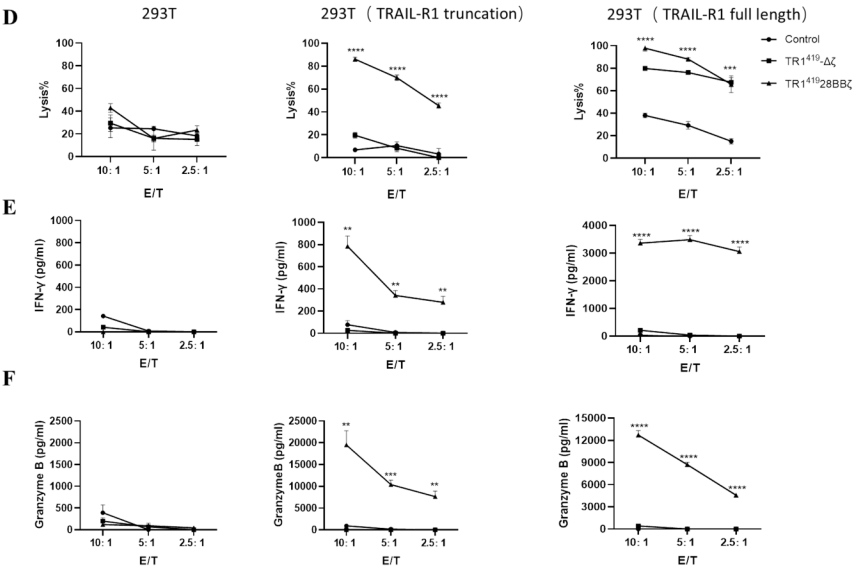

Fig.3.TR1^{419}-28BBζ CAR-T cells mediated dual tumour cell killing. (A) Schematic representation of TRAIL-R1 (full length) and TRAIL-R1 (truncation). TRAIL-R1 (full length) comprised the extracellular region, the transmembrane domain, and the intracellular region with death domain. TRAIL-R1 (truncation) comprised the extracellular region, the transmembrane domain, and the intracellular region without death domain, SP: signal peptide, TM: transmembrane domain, DD: death domain. (B) 293T cells were transfected with the lentiviral expression TRAIL-R1 trunc and TRAIL-R1 full length plasmids, respectively. Then, the expression of TRAIL-R1 in 293T cells was determined by flow cytometry. (C) Mock (untransduced), TR1^{419}-28BBζ CAR-T cells or TR1^{419}[?]ζ-T cells were evaluated by flow cytometry for scFv expression. (D) Cytotoxic activity of TR1^{419}-28BBζ CAR- and TR1^{419}[?]ζ-T cells against 293T, 293T (TRAIL-R1 trunc) and 293T (TRAIL-R1 full length) was determined by RTCA assay. (E, F) IFN-γ and Granzyme B production by TR1^{419}-28BBζ CAR-T cells or TR1^{419}[?]ζ-T cells was detected by ELISA when co-cultured with the indicated cells for 24 h. The analyses were performed using Students' t tests. Data reflected the mean ± SEM of three triplicate wells. $*p < 0.05$, $**p< 0.01$ and $***p < 0.001$.

3.4. The costimulatory domains did not influence the phenotypes of TR1^{419}-CAR-T cells

It has been reported that the phenotypic and functional differences of the second and third generation CAR-T cells are related to the selection of target antigen and the design of scFv structure [29,30], which suggested that we should select the most appropriate CARs based on our own scFv structure. To explore which CARs structure is the most appropriate for scFv from TR1^{419}, we first constructed CD28-containing (TR1^{419}-28ζ) and 4-1BB-containing (TR1^{419}-BBζ) TR1^{419}-CARs (Fig. 4A). For the following studies, we generated TR1^{419}-28ζ, TR1^{419}-BBζ and TR1^{419}-28BBζ CAR-T cells by lentiviral transduction. The surface expression of CAR on the T cells was measured by flow cytometry. Three TR1^{419}-CARs showed comparable transfection rates and mean fluorescence intensity (Fig. 4B). Meanwhile, TR1^{419}-28ζ, TR1^{419}-BBζ and TR1^{419}-28BBζ CAR-T cells exhibited similar cell surface T cell phenotypes, of which approximately 60% were Tn (Fig. 4C). CD8$^+$TR1^{419}-28BBζ CAR-T cells showed higher levels of PD-1 and LAG-3 expression than CD8$^+$TR1^{419}-28ζ CAR-T cells. However, CD4$^+$ TR1^{419}-28ζ CAR-T cells showed higher levels of TIM-3 expression than CD4$^+$TR1^{419}-28BBζ CAR-T cells (Figure 4D). (Fig. 4D).

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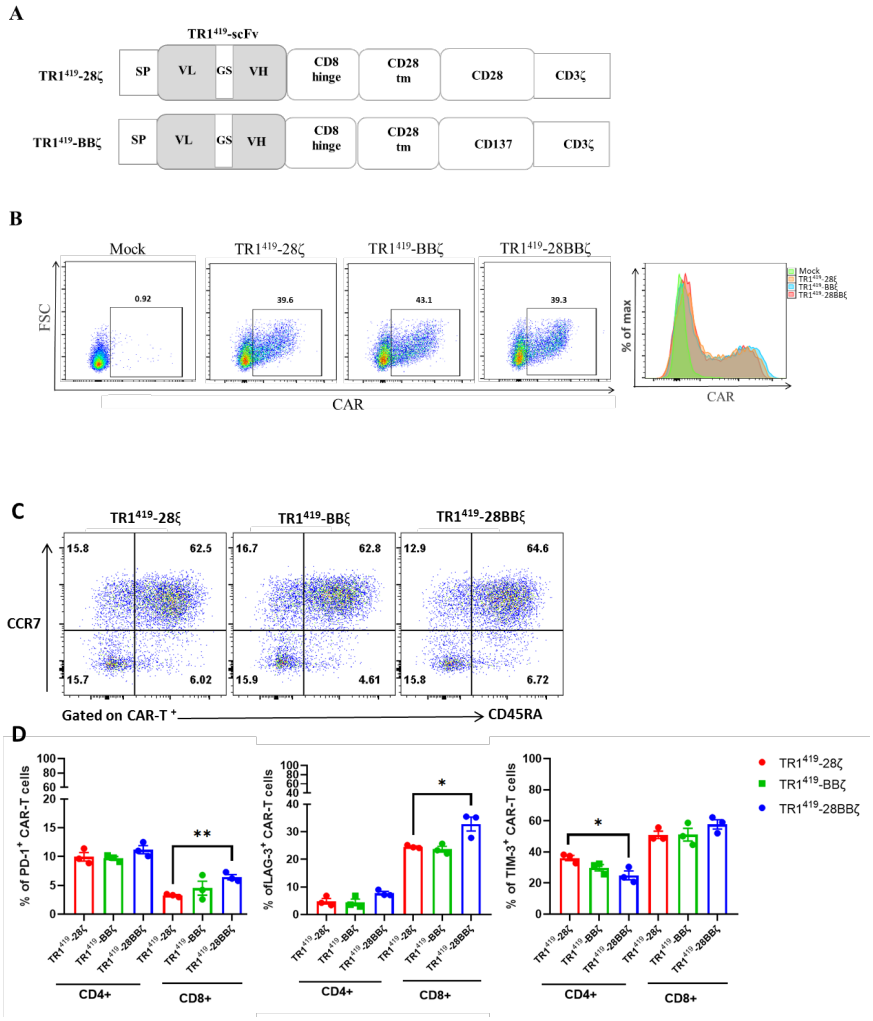

Fig.4. The costimulatory domains did not impact TR1^{419}CAR-T phenotypes. (A) Diagram of the lentiviral expression cassette with the second generation TR1^{419}-CARs containing the humanized scFv (TR1^{419}) targeting TRAIL-R1, with a CD8α hinge domain, a CD28 transmembrane domain, a cytoplasmic CD28 or 4-1BB costimulatory domain, and a cytolytic CD3ζ domain. (B) Mock (untransduced), TR1^{419}-28ζ, TR1^{419}-BBζ or TR1^{419}-28BBζ CAR T cells were evaluated by flow cytometry for scFv expression to detect lentiviral transduction of CARs. (C) Representative CAR-T cell phenotyping plot based on CD45RA and CCR7. CD45RA^{+}CCR7^{+}: naïve T cells, CD45RA^{-}CCR7^{+}: central memory T cells, CD45RA^{-}CCR7^{-}: effector memory T cells, CD45RA^{+}CCR7^{-}: effector T cells. (D) Quantification of inhibitory molecules (PD-1, LAG-3 and TIM-3) expression on CD4^{+}CAR and CD8^{+}CAR T cells on day 7 following lentiviral transfection. The analyses were performed using Students' t tests. Data reflected the mean ± SEM of three independent experiments, $*p< 0.05$, $**p < 0.01$.

3.5. TP1^{419}-28BBζ ΤΑΡ-Τ ςελλς προλιφερατεδ μορε ραπιδλψ τηαν TP1^{419}-28ζ ΤΑΡ-Τ ςελλς ανδ TP1^{419}-BBζ ΤΑΡ-Τ ςελλς υπον αντιγεν στιμυλατιον

To explore the functional differences among TR1^{419}-28ζ, TR1^{419}-BBζ and TR1^{419}-28BBζ CAR-T cells, we first examined the expression of CD137 in TR1^{419}CAR-T cells after 24 hours of stimulation with different concentrations of plate-bound recombinant human TRAIL-R1 protein. While TR1^{419}-28ζ, TR1^{419}-BBζ and TR1^{419}-28BBζ CAR-T cells exhibited similar CD137 expression against 0.5ug/ml, 0.01ug/ml and

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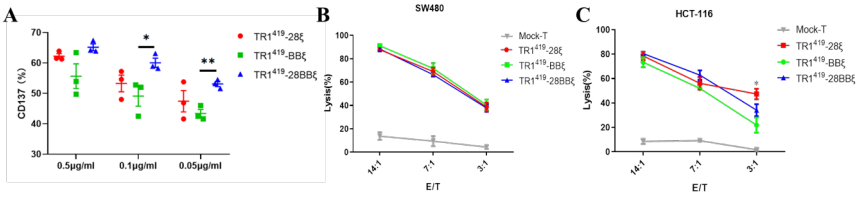
0.005ug/ml antigen protein, TR1^{419}-28BBζ CAR-T cells induced higher CD137 expression against 0.1ug/ml and 0.05ug/ml antigen protein (Fig. 5A). Then, we performed in vitro tumor killing assays. TR1^{419}-28ζ, TR1^{419}-BBζ and TR1^{419}-28BBζ CAR-T cells were co-cultured with various tumor targets, and Calcein AM release-based cytotoxic cell assay was used to quantify tumor cell killing. The results showed that TR1^{419}-28ζ, TR1^{419}-BBζ and TR1^{419}-28BBζ CAR-T cells killed TRAIL-R1-expressing tumor cells with similar efficiency (Fig. 5B).

The proliferation of TR1^{419}-CAR-T cells upon antigen stimulation was assessed using CFSE staining, and the CFSE fluorescence declined following each cell division. The expression CFSE of TR1^{419}-CAR-T cells was detected after co-cultured with TRAIL-R1 positive SW480 cells for 7 days. TR1^{419}-28BBζ CAR-T cells showed superior expansion compared with TR1^{419}-28ζ and TR1^{419}-BBζ CAR-T cells against TRAIL-R1 positive tumors, and no significant difference was found in cell proliferation between TR1^{419}-28ζ and TR1^{419}-BBζCAR-T cells (Fig. 5C). Meanwhile, we also detected the expression of PD-1 in TR1^{419}CAR-T cells after co-incubation with SW480. The results indicated that PD-1 was more frequently expressed by TR1^{419}-28BBζCAR-T cells than TR1^{419}-28ζ and TR1^{419}-BBζCAR-T cells. However, the frequencies of PD-1^{+} CAR-T cells between TR1^{419}-28ζ and TR1^{419}-BBζ groups were similar (Fig. 5D). There has shown that the highest level of PD-1 expression reflects T-cell exhaustion status [31]. Thus, based on positive PD-1 expression, PD-1^{+}TR1^{419}CAR-T cells were divided into two subgroups: high PD-1 expressing cells (PD-1^{high}) and low PD-1 positive cells (PD-1^{low}). There was no significant difference in the expression of PD-1^{low} among the TR1^{419}-28ζ, TR1^{419}-BBζ and TR1^{419}-28BBζ CAR-T cells. However, PD-1^{high} was more frequently expressed by TR1^{419}-28BBζCAR-T cells than TR1^{419}-BBζCAR-T cells (Fig. 5E). In summary, TR1^{419}-28BBζ CAR-T cells proliferated more rapidly than TR1^{419}-28ζ CAR-T cells and TR1^{419}-BBζ CAR-T cells upon antigen stimulation, but expressed higher levels of PD-1.



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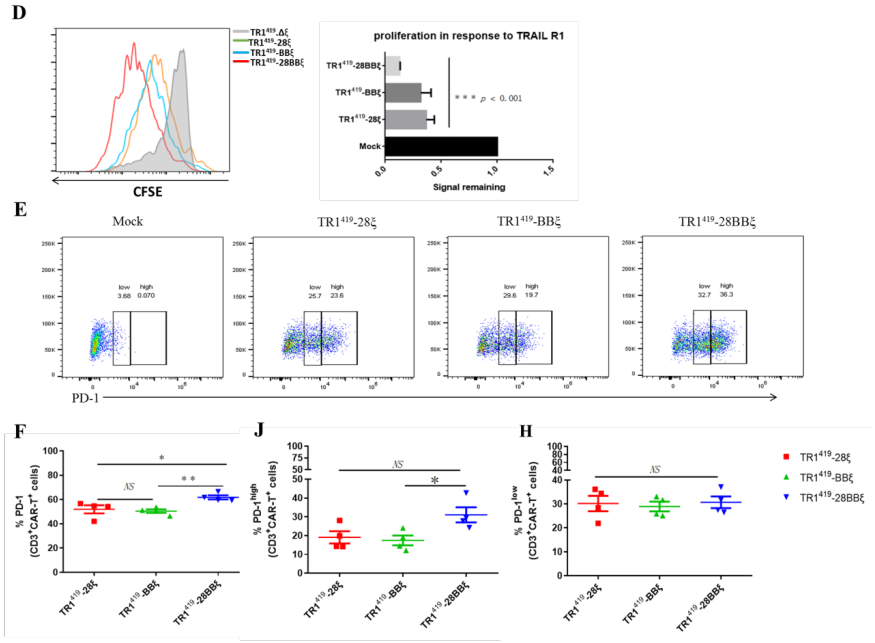

Fig.5.TR1^{419}-28BBζ CAR-T cells showed greater antigen-specific expansion compared with TR1^{419}-28ζ CAR-T cells and TR1^{419}-BBζ CAR-T cells. (A) Expression of CD137 on TR1^{419}CAR-T cells was detected by flow cytometry cultured overnight on plate-bound recombinant human TRAIL-R1 at varying protein concentrations. (B, C) Cytotoxic activity of TR1^{419}-28ζ CAR, TR1^{419}-BBζ CAR or TR1^{419}-28BBζ CAR T cells against TRAIL-R1 positive tumor cell lines (SW480 and HCT116). The effector cells were co-cultured for 6 h with target cells at E:T ratio of 14:1, 7:1 and 3:1, respectively. Cytotoxicity was determined by Calcein AM release-based cytotoxic cell assay. The analysis was performed using Students' t tests. Data reflected the mean ± SEM of three triplicate wells, $^*p < 0.05$. (D) TR1^{419}CAR T cell proliferation indicated by division of CFSE was assessed by flow cytometry. TR1^{419}-28ζ CAR, TR1^{419}-BBζ CAR and TR1^{419}-28BBζ CAR T cells were stained with CFSE, respectively. Then, TR1^{419}CAR T cells were cultured with SW480 for 7 d, and analyzed by flow cytometry. (E-H) Expression of PD-1 on TR1^{419}CAR T cells was analyzed by FACS after 7 days of co-culture with tumor cells. TR1^{419}CAR T cells were classified as PD-1 negative, PD-1low and PD-1high expressing sub-populations. The analyses were performed using Students' t tests. Data reflected the mean ± SEM of three independent experiments, $^*p < 0.05$, $^{**}p < 0.01$.

Discussion

In the present work, we demonstrated that TR1^{419}-based CAR-T cells had potent dual anti-tumor activities in vitro. On the one hand, TR1^{419}CAR can mediate tumor cell apoptosis through activation of the death receptor-dependent apoptotic pathway. On the other hand, TR1^{419}CAR can effectively induce T-cell mediated cytotoxicity by inducing multiple signals with the binding of TRAIL-R1. Meanwhile, by comparing phenotype and function between the second- and third-generation TR1^{419}CAR-T cells, the third-generation TR1^{419}-28BBζ CAR-T cells showed higher sensitivity to target antigen, proliferated more rapidly and expressed higher levels of PD-1 upon antigen stimulation.

Previously, we developed fully human TR1^{419}-IgG with tumoricidal activity, but this antibody required additional crosslinking antibodies to induce apoptosis in the target cells [32]. We demonstrated that the scFv from TR1^{419} designed into CAR structure can directly mediate tumor apoptosis by binding to TRAIL-R1 in the absence of additional crosslinking antibodies. The underlying mechanism of cell apoptosis induced by scFv from TR1^{419} may be related to the spatial structure of the TR1^{419}CAR. The specific mechanism still requires further research and investigation.

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Currently, the clinical efficacy of CAR-T cells in hematological malignancies is rarely achieved in solid tumors, and the factors necessary for improving its efficacy are currently being determined [33,34]. Combinations with other treatments will be a promising strategy to improve the efficacy of CAR-T cells on solid tumors [35]. In our study, $TR1^{419}$-based CAR-T cells had potent dual anti-tumor activities. The induction of apoptotic signals from TR1 on the target cells was equivalent to that of other therapies in this case. However, the apoptotic signals from TR1 are targeted, unlike chemotherapy and radiotherapy, without inducing localized immune cell damage [36]. Moreover, TR1-mediated tumor cell apoptosis can induce immunogenic cell death of tumor cells which enhances antigen presentation and antitumor immune responses.

Although, the role of costimulatory signals in CAR-T development is well-established, the optimal costimulatory domains for CAR-T cells remains to be defined, and should be evaluated case-by-case in order to fine-tune immunotherapy approaches [37,38]. Literature reported that the choice of 4-1BB signaling domain in CARs conferred improved selectivity for higher tumor antigen density, reduced T cell exhaustion phenotype and reduced basal T-cell activation [39,40]. In our research, there was no significant difference in phenotypes and function between the $TR1^{419}$-28ζ and $TR1^{419}$-28BBζ CAR-T cells. However, the third-generation $TR1^{419}$-28BBζ CAR-T cells showed higher sensitivity to target antigen and proliferated more rapidly upon antigen stimulation. The incongruent results may have the following reasons: 1) the various preparation methods and experimental conditions, 2) the various scFv domains, 3) the various other components of CARs.

There are some limitations in our study that requires further acknowledgement. Further and more comprehensive studies are required to confirm these findings. And additional in vivo experiments are needed to verify the results of the above in vitro. Lastly, the safety issues related to off-tumor toxicity also needs further studies to address. In conclusion, we designed and demonstrated that $TR1^{419}$CAR-T cells can mediate dual tumor cell killing, and the third-generation $TR1^{419}$28BBCAR-T cells exhibited a higher sensitivity at low antigen, and proliferated more rapidly upon antigen stimulation, compared to the second-generation $TR1^{419}$CAR-T cells. This dual killing mechanism provides a novel strategy for other CARs design.

Author Contributions:

Aishun Jin: Conceptualization, Supervision, Project administration. **YN** and **LD:** Investigation, Methodology, Writing—original draft preparation**: MS** and**JH:** Methodology, Project administration **TL:** Software, Methodology. **FL** : Software, Data curation**,**Bioinformatics analysis. **XH:** Writing-review and Editing.**LD:** Formal analysis, Investigation, Resources. **MS:**Investigation, Formal analysis. **DP:** Supervision.

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Conflict of Interests

The authors declare no competing interests.

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