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GITR domain inside CAR co-stimulates activity of CAR-T cells against cancer

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1. ABSTRACT

T cells expressing Chimeric antigen receptors or CAR-T cells are used as a novel treatment against hematological and solid cancers. In this report, we designed CAR with glucocorticoid-induced TNFR-related protein (GITR) co-stimulatory domain to study its ability to co-activate CAR-T cells. EGFR-GITR-CD3 CAR-T cells were cytotoxic against EGFR-positive: pancreatic and ovarian cancer cells but not against EGFR-negative cancer cells. The cytotoxic activity of EGFR-GITR-CD3 CAR-T cells was comparable or better than EGFR-28-CD3 or EGFR-41BB-CD3 CAR-T cells. We designed also EGFR-CD3-GITR-CAR and EGFR-ΔGITR-CD3 with deleted 184-192 amino-acids of co-stimulatory GITR domain, and showed that EGFR-GITR-CD3 had significantly higher cytotoxic activity against EGFR-positive cells. The EGFR-GITR-CD3 cells secreted significantly higher levels of IFN-gamma than EGFR-CD3-GITR and EGFR-ΔGITR-CD3 cells. In addition, Mesothelin-GITR-CD3 CAR-T cells also killed mesothelin-positive ovarian cancer cell lines, and pancreatic cancer cells. Moreover, CD19-GITR-CD3 CAR-T cells had significant cytotoxic activity against CD19-positive cancer cells *in vitro* and in Raji

xenograft tumors *in vivo*. Thus, our results clearly show that GITR co-stimulatory domain can be used as a novel co-stimulatory domain in CAR-T cells.

2. INTRODUCTION

Chimeric Antigen Receptor (CAR) T cell therapy using autologous T cells with CAR against tumor cell surface antigen demonstrated remarkable results against hematological cancers (1), (2), (3, 4). The CAR consists of single-chain variable antibody fragment (scFv) detecting tumor-associated cell surface antigen; then hinge (from CD8 alpha, CD28 proteins or other proteins); transmembrane domain; co-stimulatory domains: CD28, 41-BB or CD27 and CD3 zeta (CD3 zeta activating domain) (1-3). The CAR-T cells recognize tumor-associated antigen and stimulate secretion of cytokines leading to cancer cell death (5). Several challenges remain to make CAR-T cell effective against solid tumors due to suppressive tumor microenvironment, suppressive checkpoint inhibitor signaling or inhibitory regulatory T cell (Treg signaling), angiogenesis, vasculogenesis, tumor associated macrophages (TAM) and other pathways (6).

GITR or glucocorticoid-induced tumor necrosis factor receptor (TNFRSF18 or AITR, CD357) is a member of TNF superfamily proteins (such as 4-1BB, OX-40, CD40, CD27 and other) that is expressed at high levels on T reg cells, and also expressed at a lower level on effector T cells, NK (natural killer) cells and other cells of the immune system (7, 8). GITR expression can be up-regulated upon activation of T cells with up-regulation of NF-kappa B and p38, JNK and ERK pathways (9). GITR can co-stimulate both CD4+ and CD8+ T cells (9). In addition, GITR can stimulate both Treg with inhibitory functions and effector T cells with stimulatory functions, thus mediating dual signaling in immune response that should be balanced (10).

In this report, we designed CAR with GITR co-stimulatory domain and compared it with CAR containing CD28 and 4-1BB co-stimulatory domains to mediate CAR-T killing functions against different cancer cells. We show that GITR domain as is effective as CD28 domain and in several cell lines is better than 41-BB domains. In addition, we compared CAR with GITR-CD3, delta-GITR-CD3 and CD3-GITR and found that GITR-CD3 better stimulated CAR-T cells and secreted more cytokines than other domains. Moreover, we demonstrate that EGFR, Mesothelin-GITR-CD3 CAR-T cells killed Mesothein-positive cells. Moreover, we show that CD-19-GITR-CD3 CAR-T were effective against Hela-CD19 cells and Raji cells using *in vivo* mouse xenograft model. Thus, GITR domain can be used for co-stimulation of CAR-T cells with similar or better functional activity.

3. MATERIALS AND METHODS

3.1. Cell lines

Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained in the Stanford Hospital Blood Center, Stanford, CA according to IRB-approved protocol using Ficoll-Paque solution (*GE Healthcare*). HEK293FT cells from *AlStem* (Richmond, CA) were cultured in RPMI containing 10% FBS and penicillin/streptomycin. SKOV-3, A1847 ovarian, pancreatic BxPC3 and MCF-7 breast cancer cell lines were obtained from ATCC and cultured in DMEM plus 10% FBS and penicillin/streptomycin. The cell lines were authenticated by flow cytometry (FACS) using cell-specific surface markers.

3.2. CAR constructs

Human EGFR scFv using low affinity antibody C10 (KD=264 nM) (11), (12) were designed with CD8 alpha signaling peptide, CD8 hinge, CD28 transmembrane domain and either CD28, 4-1-BB, GITR (184-193 amino acids) co-stimulatory domain; delta GITR or ΔGITR with deleted 184-193 amino acids; and CD3 zeta (z) or CD3 activation domain. Human Mesothelin ScFv of P4 antibody (13) was used for generation of Mesothelin (Meso)-28-CD3 or Meso-GITR-CD3 CAR. Mouse CD19 ScFv (FMC63) (14) was used for generation of CD19-GITR-CD3 CAR using either CD8 alpha or CD28 trans-membrane domains. The EGFR-CAR were synthesized and sequenced in both directions by *Syno Biological* (Beijing, China). The Meso and CD19-CAR were designed by re-cloning Mesothelin and CD19 scFv into lentiviral CAR using Xho I and Nhe I sites flanking EGFR ScFv. All CAR were inserted into Xba I and EcoR I sites of lentiviral vector from *System Biosciences.*

3.3. Lentivirus preparation

293 FT cells, Lentivirus Packaging Mix and transfection agent (ALSTEM) were used with CAR DNA to prepare lentivirus as described previously (15), (16). The virus particles were collected by centrifugation at 112,000 *g* for 100 min, suspended in AIM V medium, aliquoted and frozen at -80 °C. The titers of the virus were determined by quantitative RT-PCR using the Lenti-X qRT-PCR kit (*Takara*) according to the manufacturer's protocol and the 7900HT thermal cycler (*Thermo Fisher*). The lentiviral titers were expressed in pfu/ml. The lentivirus experiments were performed according to the approved Biosafety level-2 regulations.

3.4. CAR-T cell expansion

PBMC (samples (provided by Stanford University, California according to approved IRB

protocol) were suspended at 1-2 x 10^6 cells/ml in AIM V-AlbuMAX medium (*Thermo Fisher*) containing 10% FBS with 300 U/ml IL-2 (*Thermo Fisher*). PBMC were activated with CD3/CD28 Dynabeads (*Invitrogen*) at cell to bead ratio 1:1, and cultured in non-treated 24-well plates. Lentivirus was added at 24 and 48 hours to the cultures at a multiplicity of infection (MOI) of 5 with 1 ml of TransPlus transduction enhancer (*AIStem*). The CAR-T cells were counted every 3 days with fresh medium added to maintain the cell density at 1-2 x 10^6 cells/ml.

3.5. Flow cytometry

To measure CAR expression, 5x10^5 cells were suspended in 100 ml of buffer (1xPBS with 0.5.% BSA) and incubated on ice with 1 ml of human serum (*Jackson Immunoresearch*, West Grove, PA) for 10 min. Then 1 ml of allophycocyanin (APC)-labeled anti-CD3 (*eBioscience*, San Diego, CA), 2 ml of 7-aminoactinomycin D (7-AAD, *BioLegend*, San Diego, CA), and 2 ml of anti-human or mouse F(ab)2 or its isotype control was added for detecting EGFR scFv, Mesothelin or CD19 CAR, respectively, and the cells were incubated on ice for 30 min. The cells were rinsed with buffer, and acquired on a FACSCalibur *(BD Biosciences*). Cells were analyzed first for light scatter versus 7-AAD staining, then the 7-AAD$^-$ live gated cells were plotted for CD3 staining versus F(ab)2 staining or isotype control staining.

3.6. Real-time cytotoxicity assay (RTCA)

Adherent target cells were seeded into 96-well E-plates in triplicate (*Acea Biosciences,* San Diego, CA) at 1 x 10^4 cells per well and monitored in culture overnight with the impedance-based real-time cell analysis (RTCA) iCELLigence system (*Acea Biosciences*). The next day, the medium was removed and replaced with AIM V-AlbuMAX medium containing 10% FBS ± 1 x 10^5 effector cells (CAR-T cells or non-transduced T cells). The cells were monitored for another 48 hours with the RTCA system, and impedance converted to the cell index was plotted over time. Cytolysis was calculated as (impedance of target cells without effector cells – impedance of target cells with effector cells) x100 / impedance of target cells without effector cells.

3.7. Human IFN-gamma ELISA assay

The target cells were cultured with the effector cells (CAR-T cells or non-transduced T cells) at a 1:1 ratio (1 x 10^4 cells each) in U-bottom 96-well plates with 200 ml of AIM V-AlbuMAX medium containing 10% FBS, in triplicate. After 16 h the top 150 ml of medium was transferred to V-bottom 96-well plates and centrifuged at 300 g for 5 min to pellet any residual cells. The supernatant was transferred to a new 96-well plate and analyzed by ELISA for human IFN-gamma using *R&D Systems* kit according to the manufacturer's protocol.

3.8. Mouse xenograft tumor growth

Six-week old male NSG mice (*Jackson Laboratories*, Bar Harbor, ME) were used in accord with the Institutional Animal Care and Use Committee (IACUC). Each mouse was injected subcutaneously with 3 x 10^6 Raji-luciferase-positive cells in sterile 1x PBS with Matrigel. The 1x10^7 CD19-GITR-CD3 CAR-T cells were injected intravenously at day 12, and imaging was performed at days 12, 18, 35, 32 and 39 using Xenogen IVIS Spectrum (*PerkinElmer*, Waltham, MA) system. The percent of increased BLI Bioluminescence (photon/sec) was plotted over time.

3.9. Statistical analysis

Student's t test, ANOVA and Tukey's t-tests were used for analyses between groups, and the difference was considered significant with p-value<0.0.5.

4. RESULTS

4.1. EGFR-GITR-CD3 CAR-T cells expand *in vitro* similarly to EGFR-CD28-CD3 and EGFR-41BB-CD3 CAR-T cells

Three EGFR scFv (C10 low affinity antibody)-containing CAR were designed with CD28, 41BB or GITR co-stimulatory domains (Figure 1A). The CAR lentiviruses were used for transduction of T cells, and CAR-T cells expanded *in vitro*. All CAR-T cells expressed CAR+ (Figure 1B), and expanded *in vitro* >50-fold (Figure 1C). The expansion of EGFR-GITR-CD3-CAR-T cell was similar to EGFR-28-CD3 and EGFR-41BB-CD3 cells, suggesting that GITR co-stimulatory domain leads to effective expansion of CAR-T cells.

4.2. EGFR-GITR-CD3 CAR-T cells kill EGFR-positive cancer cells similarly or better than EGFR-CD28-CD3 or EGFR-4-1BB-CD3 CAR-T cells

EGFR-GITR-CD3-CAR-T cells were tested by Real-time cytotoxicity assay (RTCA) to compare with EGFR-CD28-CD3 and EGFR-4-1BB-CD3-CAR-T cells (Figure 2). EGFR-GITR-CD3 CAR-T cells killed EGFR-positive BxPC3 pancreatic cancer cells similarly to EGFR-CD28- and EGFR-41BB-CD3 CAR-T cells (Figure 2A). The EGFR-GITR-CD3-CAR-T cells better killed SKOV-3 ovarian cancer cells than EGFR-41BB-CD3 CAR-T cells and similarly to EGFR-CD28-CD3 CAR-T cells (Figure 2B). EGFR-GITR-CD3-CAR-T cells also better killed A1847 ovarian cancer cells than EGFR-CD28-CD3 CAR-T cells (Figure 2 C).

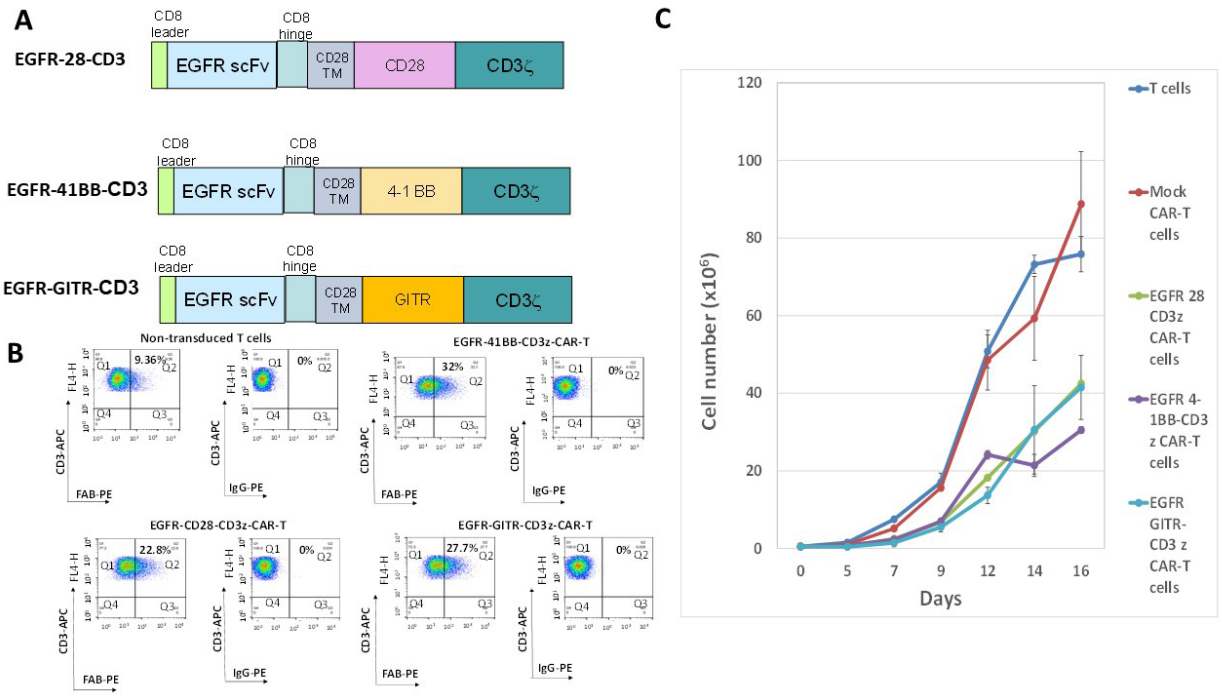

Figure 1. CAR structures, expression and CAR-T cell expansion. A. EGFR-GITR, -CD28, -4-1BB-CD3 CAR structure. B. Expression of CAR after EGFR-CAR lentiviral transduction. Expression was detected by FACS with anti-human FAB antibody. C. CAR-T cell growth and expansion. 0.5.x10^6 cells were used for starting CAR-T expansion. All EGFR-CAR-T cells expanded similarly. The expansion of all CAR-T cells was >50-fold at day 16.

There was no killing of EGFR-negative MCF-7 cells (Figure 2C). The quantification of killing in EGFR-positive and EGFR-negative cells is shown in (Figure 2D). Thus, EGFR-GITR-CD3 CAR-T cells specifically killed EGFR-positive cancer cells similarly or better than EGFR-CAR-T cells with CD28 and 41BB co-stimulatory domains.

4.3. EGFR-GITR-CD3 CAR-T cells kill cancer cells more significantly and secrete more IFN0gamma than EGFR-ΔGITR-CD3 or EGFR-CD3-GITR-CAR-T cells

To test that the full length GITR-co-stimulatory domain is important for stimulation of CAR-T cells, we deleted 9 amino-acids from the start of the GITR co-stimulatory domain (deleted 184-192 aa acids of GITR protein), and this designed CAR called EGFR-ΔGITR-CAR, and also designed CAR with reverse orientation of GITR and CD3 domains, calling it EGFR-CD3-GITR CAR (Figure 3 A). After transducing T cells with these CAR lentiviruses, at 14 days expansion of CAR-T cells, we confirmed CAR+ expression by FACS (Figure 3B) and performed RTCA assay (Figure 3C). The EGFR-GITR-CD3 CAR-T cells had significantly higher cytotoxic activity than EGFR-ΔGITR-CD3 (with shorter GITR domain) or EGFR-CD3-GITR (with reversed CD3 and GITR) CAR-T cells (Figure 3D). Moreover, EGFR-GITR-CD3 CAR-T cells also secreted a significantly higher level of IFN-gamma than EGFR-ΔGITR-CD3 and EGFR-CD3-GITR CAR-T cells (Figure 3 E).

Thus, EGFR-GITR-CD3-CAR-T cells with full length GITR co-stimulatory domain, EGFR-GITR-CD3 CAR-T cells more significantly killed EGFR-positive cells and secreted more IFN-gamma than EGFR-ΔGITR-CD3 and EGFR-CD3-GITR CAR-T cells.

4.4. Mesothelin-GITR-CD3 CAR-T cells kill Mesothelin-positive cancer cells and secrete IFN-gamma

To test that other ScFv can be used with GITR domain for generation of CAR-T cells killing cancer cells, we designed Mesothelin-GITR-CD3 CAR-T cells and compared them with Mesothelin-CD28-CD3 (2^{nd} generation) and Mesothelin-CD28-4-1BB-CD3 (3^{d} generation) CAR-T cells (Figure 4). The Mesothelin-GITR-CD3 CAR-T cells killed Mesothelin-positive cancer cells similarly to Mesothelin-CD28-CD3 and Mesothelin-CD28-4-1BB-CD3 CAR-T cells (Figure 4 A, B, C). The Mesothelin-GITR-CAR-T cells secreted significantly more IFN-gamma than non-transduced T cells and Mock-CAR-T cells, and similarly to Mesothelin-CD28-CD3 and Mesothelin-CD28-4-1BB-CD3 CAR-T cells (Figure 4 D).

4.5. CD19-GITR-CD3 CAR-T cells kill Hela-CD19 cancer cells *in vitro* and significantly block Raji xenograft tumor growth *in vivo*

To test the efficacy of GITR co-stimulatory domain against hematological cancer targets, we

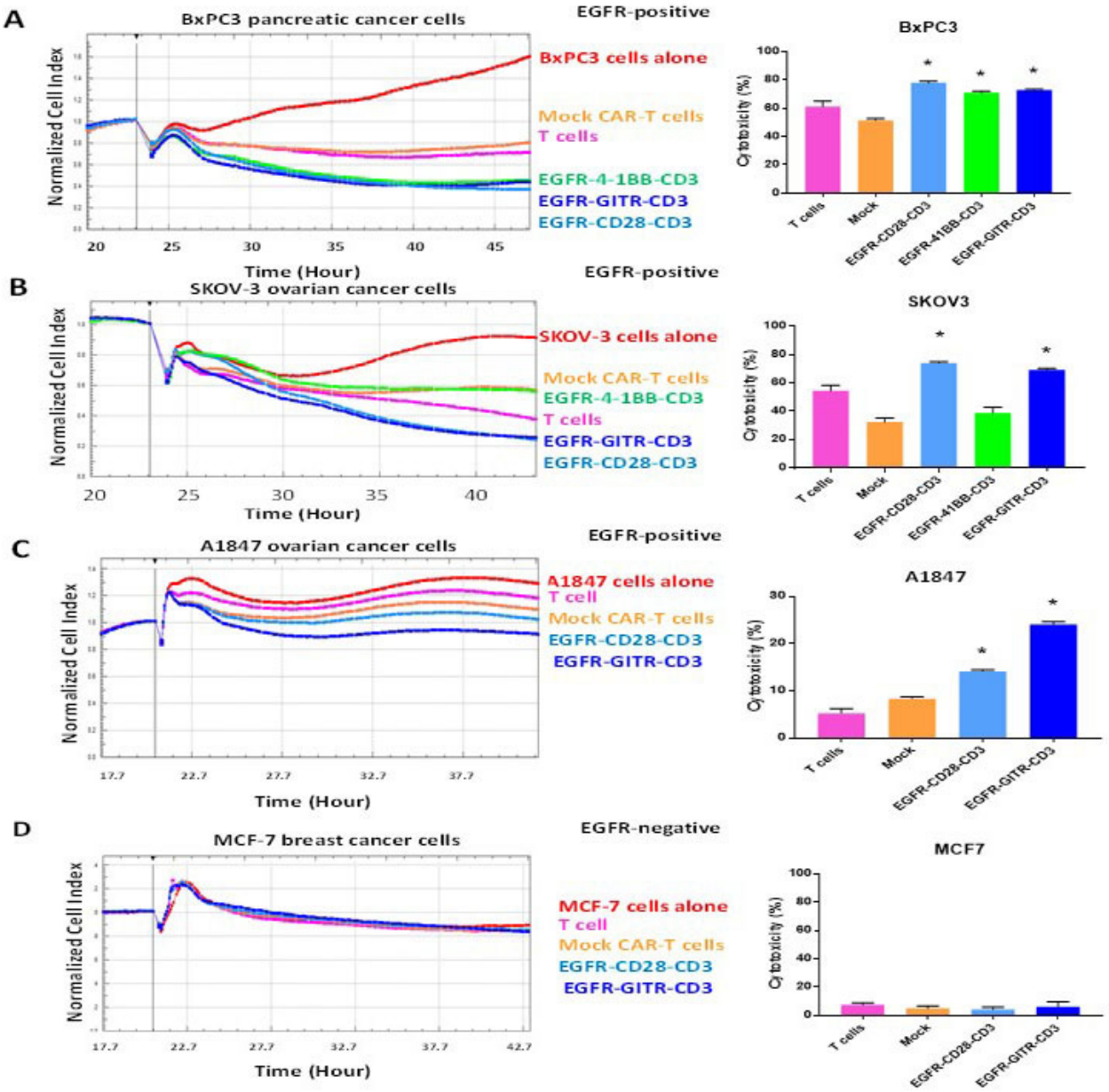

Figure 2. EGFR-GITR-CD3 CAR-T cells kill EGFR-positive cells but not EGFR-negative cells similarly or better to EGFR-CD28-CD3 or EGFR-41BB-CD3 cells. A, B, C EGFR-positive target cell lines. *p<0.0.5, ANOVA and Tukey's t-test CAR-T cells versus T or Mock CAR-T cells. D, EGFR-negative target cell line.

designed two CD19-GITR-CD3 CARs with different transmembrane domains. One construct contained CD28 trans-membrane domain-CD19-GITR-CD3 (called CD19-GITR-CD3-1) and another contained CD8 alpha trans-membrane domain CD19-GITR-CD3 (called CD19-GITR-CD3-2) and was used to generate CAR-T cells (Figure 5 A). We tested both CD19-GITR-CD3-CAR-T cells by RTCA with CD19-postive Hela-CD19 target cells (stably expressing CD19 antigen) (Figure 5B). Both CD19-GITR-CAR-T cells killed target Hela-CD19 cells (Figure 5B).

We also tested CD19-GITR-CAR-T cells in Raji-luciferase positive xenograft *in vivo* model (Figure 5C). We performed imaging of Raji-luc+ xenograft growth. CD19-GITR-CAR-T cells significantly less increased bioluminescence signal in xenograft Raji tumors compared with tumors from control mice (Figure 5B). Thus, CD19-GITR-CD3-CAR-T cells significantly decrease Hela-CD19 cells growth *in vitro* and Raji xenograft growth *in vivo*.

5. DISCUSSION

This report compared EGFR-GITR-CD3 CAR-T cells with EGFR-CD28-CD3 and EGFR-4-1BB-CD3 cells and demonstrated the high efficacy of EGFR-GITR-CD3 cells--similar or better than EGFR-CD28-

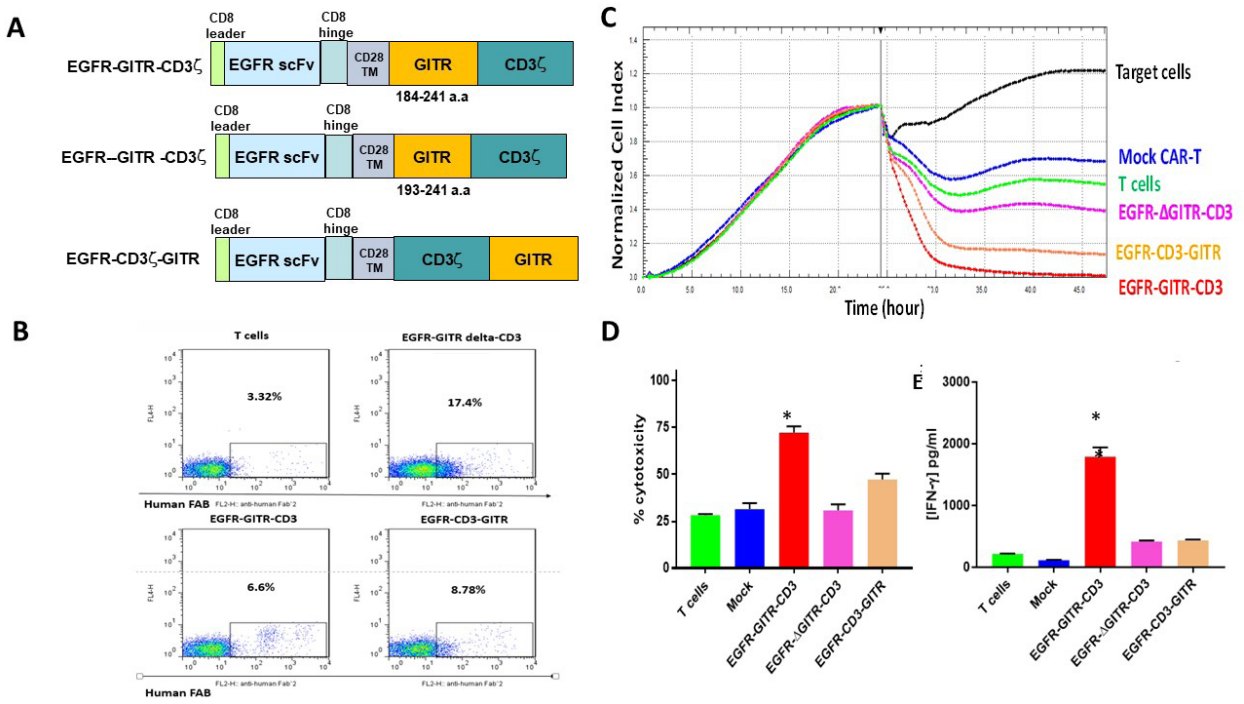

Figure 3. EGFR-GITR-CD3-CAR-T cells kill EGFR-positive cells and secrete cytokines more significantly than EGFR-CD3-GITR and EGFR-ΔGITR-CD3. A. The structure of CAR constructs with EGFR-CD3-GITR and EGFR-ΔGITR (deletion of 184-192 amino-acids). B. Expression of CAR in EGFR-CAR-T cells by FACS; C. RTCA assay with EGFR-CAR-T cells and EGFR-positive BxPC3 pancreatic cancer cells; D. Quantification of cytotoxicity of CAR-T cells in RTCA assay from C.*p<0.05, Tukey's test EGFR-GITR-CD3 vs EGFR-ΔGITR and EGFR-CD3-GITR and negative control T cells. E. IFN-gamma ELISA assay, p<0.05, CAR-T cells vs T cells or Mock CAR-T cells, Student's t-test

CD3 or EGFR-4-1BB-CD3 CAR-T cells. In addition, deletion of the first N-terminal several amino-acids (184-192 aa) of GITR co-stimulatory domain resulted in less activity of CAR-T cells. And GITR-CD3 orientation was better than CD3-GITR reverse orientation for co-stimulation of CAR-T cells. In addition, Mesothelin-GITR-CAR-T cells also effectively killed mesothelin-positive cancer cells. Moreover, CD19-GITR-CAR-T cells killed Hela-CD19 positive cells and decreased tumor growth *in vivo* using the Raji xenograft NSG mice model. This study demonstrates that GITR can be used for stimulation of CAR-T cells.

GITR belongs to the TNF superfamily proteins (such as 4-1BB, OX-40, CD27 and others) that modulate immune response (10). GITR is activated by GITR ligand (GITRL) and can co-stimulate T cells (10). GITR is highly expressed in Treg cells with suppressive activity, although GITR is activated in effector cells to levels similar to the levels on T reg cells (10). Thus, GITR has a dual function associated with Treg and effector T cells and there should be a balance between these two functions in the immune response (17).

In several CAR-T cells that we analyzed the co-stimulatory function of GITR domain was significantly higher than 4-1BB or CD28 co-stimulatory domain, suggesting that GITR domain co-stimulatory function and signaling differ from other domains. This was observed with EGFR, CD19 and Mesothelin scFv-containing CAR-T cells, suggesting its antigen-independent co-stimulatory function. In addition, we have shown that Mesothelin-GITR-CD3 CAR-T cells had a lower level of IFN-gamma secretion than Mesothelin-CD28-CD3 and Mesothelin-CD-4-1BB-CD3 CAR-T cells, which can be advantageous in clinical studies to reduce cytokine release syndrome (CRS).

It has been shown that signaling mediated by CD28 and 4-1BB co-stimulatory domains differed in regulation of metabolic pathways in CAR-T cells (18, 5), where CD28 domain increased glycolytic metabolism and 4-1BB domain enhanced oxidative metabolism (18). In addition, CAR-T cells with CD28 domain characterized by less persistence and increased effector memory phenotype, while CAR-T cells with 4-1BB domain characterized by increased persistence and increased T central memory phenotype (18). Future study can compare the effect of CD28, 4-1BB and GITR co-stimulatory domains on regulating metabolic functions of CAR-T cells and also on T cell memory functions and phenotype, gene signature and proteomics profiles.

We deleted several amino-acids from the N-terminal part of the GITR cytoplasmic domain, which resulted in decreased activation of CAR-T cells,

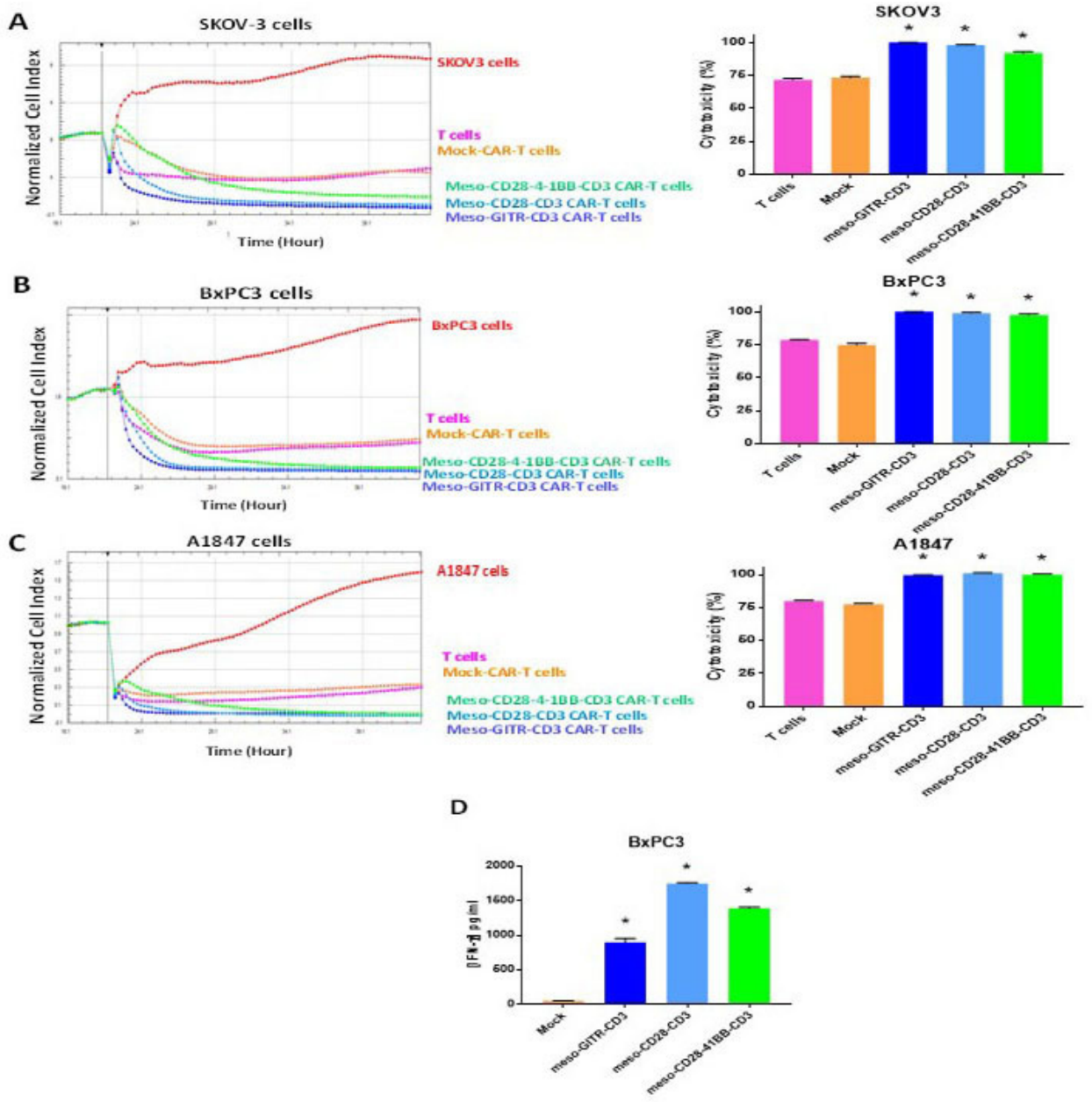

Figure 4. Mesothelin-GITR-CAR-T cells kill mesothelin-positive cells similarly to Mesothelin-CD28-CAR-T cells. Mesothelin-CD28-CD3 (2nd generation) and Mesothelin-CD28-41BB-CD3 (third generation) CAR-T cells were used for RTCA analysis. SKOV-3 (A), A1847 (B) and BxPC3 (C) cells are shown. D. ELISA assay demonstrate significant increase of IFN-gamma by Mesothelin-GITR-CD3 and Mesothelin-CD28-CD3 or Mesothelin-CD28-4-1BB-CD3 cells against Mesothelin-positive BxPC3 cells. *p<0.05, Meso-CAR-T cells vs T cells. Student's t-test.

suggesting that these amino-acids are critical for the function of GITR in activating CAR-T cells. In fact, these amino-acids were unique and differed in other members of TNF superfamily such as 4-1BB, OX-40 and CD27 domains (10). The cartoon of GITR domain with deleted 184-192 amino-acids that differ in other co-stimulatory domains is shown in (Figure 6). Future studies on the importance of these and other amino-acids in co-stimulation of CAR-T cells and down-stream signaling will lead to detailed analysis of GITR domain function in CAR-T cell biology. In addition, the full length GITR co-stimulatory domain should be in front of the CD3 activation domain for maximal co-stimulation of CAR-T cells.

We also detected the high efficacy of GITR-CAR-T cells against CD19-positive cells. Since the CAR structure can affect the activity of CAR-T cells (2), we used either different CD28 or CD8 hinge alpha trans-membrane regions in CD19-CAR-T cells. Both

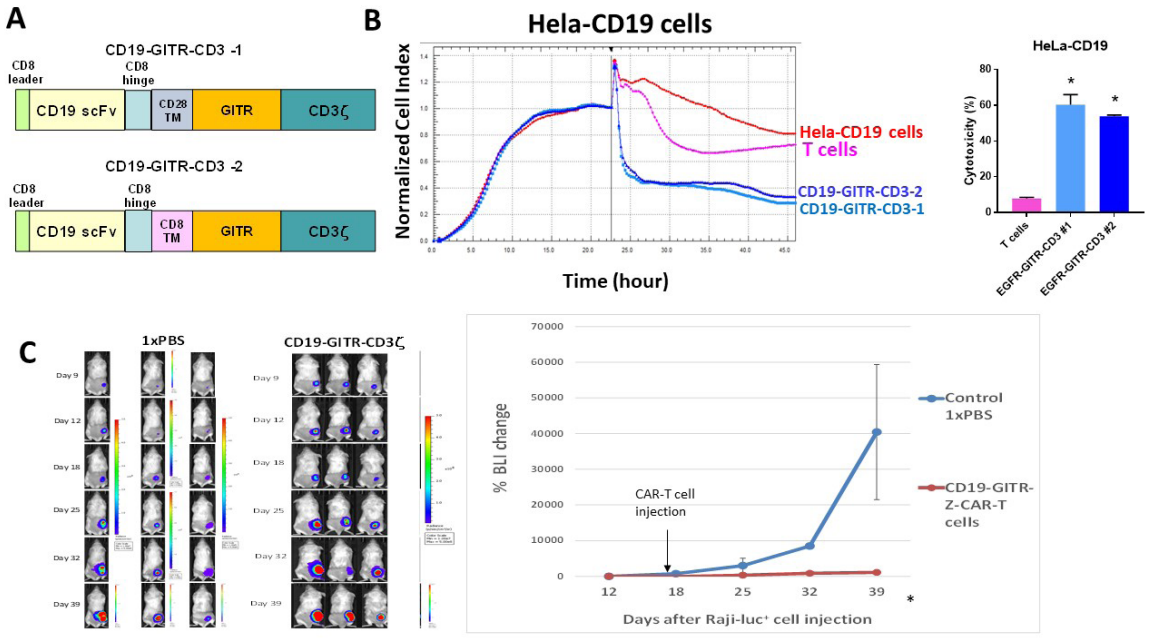

Figure 5. CD19-GITR-CD3 CAR-T cells kill Hela-CD19 cells *in vitro*. A. The structure of CD19-GITR-CD3 constructs. B. RTCA assay with CD19-GITR-CD3 CAR-T cells and Hela-CD19-positive cells. *p<0.0.001, CD19-GITR-CD3 (1) CAR-T cells versus T cells. * p<0.0002, CD19-GITR-CD3 (2) CAR-T cells versus T cells. C. CD19-GITR-CD3-CAR-T cells block Raji tumor growth *in vivo*. The IVIS imaging system was used for analysis of Raji tumor growth. *p<0.05, Student's t-test.

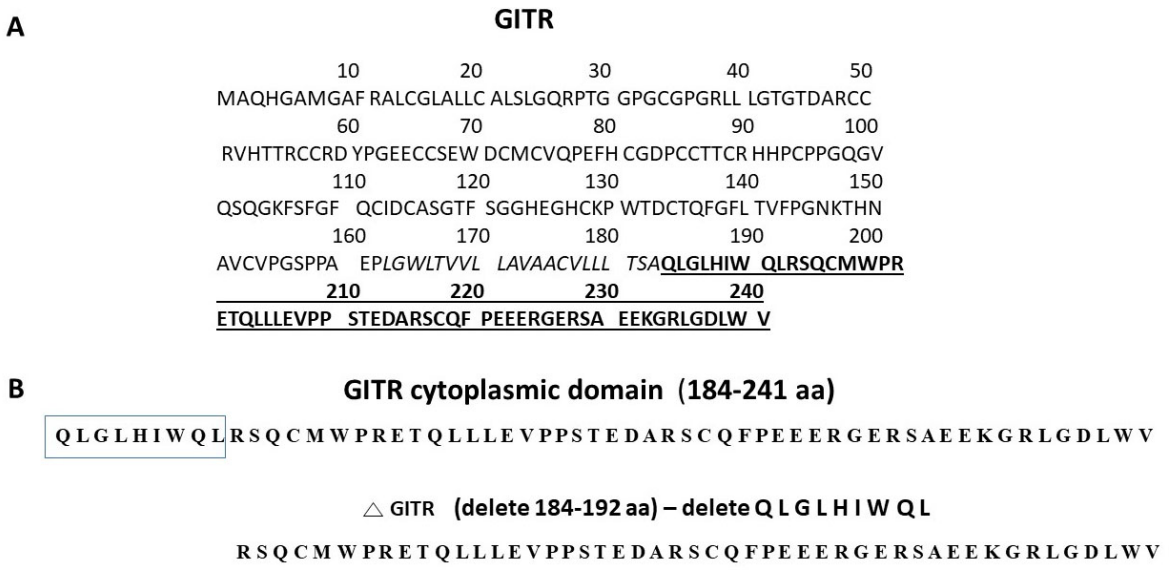

Figure 6. The structure of GITR and ΔGITR domains. A. The structure of full length GITR domain. The cytoplasmic domain is underlined and the trans-membrane domains is marked in italics. The structure of GITR domains are obtained from Uniprot software and database. B. The sequence of deleted amino-acids and ΔGITR domain. The deleted sequence differed in CD27, OX-40 and 4-1BB cytoplasmic domains (by Uniprot software).

CD19-GITR-CD3 CAR-T cells had a similar killing activity with either CD28 or CD8 alpha trans-membrane regions against CD-19-positive cancer cells. These CD19-CAR-T cells were effective in the Raji mouse model, suggesting their use in future clinical trials.

Thus, this study shows the novel function of the GITR co-stimulatory domain in regulating CAR-T cell activities against cancer cells that is important for developing future cellular therapeutics.

6. ACKNOWLEDGEMENTS

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7. REFERENCES

1. MV Maus, SA Grupp, DL Porter, CH June, C.H. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. *Blood* 123, 2625-2635 (2014)
DOI: 10.1182/blood-2013-11-492231
PMid:24578504 PMCid:PMC3999751

2. Z Eshhar, T Waks, G Gross. The emergence of T-bodies/CAR T cells. *Cancer J* 20, 123-126 (2014)
DOI: 10.1097/PPO.0000000000000027
PMid:24667957

3. CA Klebanoff, TN Yamamoto, NP Restifo. Immunotherapy: Treatment of aggressive lymphomas with anti-CD19 CAR T cells. *Nat Rev Clin Oncol* 11, 685-686 (2014)
DOI: 10.1038/nrclinonc.2014.190
PMid:25384948

4. V Golubovskaya, CAR-T Cell Therapy: From the Bench to the Bedside. *Cancers* (Basel) *9* (2017)

5. V Golubovskaya, L Wu. Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy. *Cancers* (Basel) 8 (2016)

6. DE Gilham, R Debets, M Pule, RE Hawkins, H Abken. CAR-T cells and solid tumors: tuning T cells to challenge an inveterate foe. *Trends Mol Med* 18, 377-384 (2012)
DOI: 10.1016/j.molmed.2012.04.009
PMid:22613370

7. HY Wang, DA Lee, G Peng, Z Guo, Y Li, Y Kiniwa, EM Shevach, RF Wang. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. *Immunity* 20, 107-118 (2004)
DOI: 10.1016/S1074-7613(03)00359-5

8. G Nocentini, L Giunchi, S Ronchetti, LT Krausz, A Bartoli, R Moraca, G Migliorati, C Riccardi. A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. *Proc Natl Acad Sci U S A* 94, 6216-6221 (1997)
DOI: 10.1073/pnas.94.12.6216
PMid:9177197 PMCid:PMC21029

9. LM Snell, GH Lin, AJ McPherson, TJ Moraes, TH Watts. T-cell intrinsic effects of GITR and 4-1BB during viral infection and cancer immunotherapy. *Immunol Rev* 244, 197-217 (2011)
DOI: 10.1111/j.1600-065X.2011.01063.x
PMid:22017440

10. LT Krausz, R Bianchini, S Ronchetti, K Fettucciari, G Nocentini, C Riccardi. GITR-GITRL system, a novel player in shock and inflammation. *Scientific World Journal* 7, 533-566 (2007)
DOI: 10.1100/tsw.2007.106
PMid:17525820

11. Y Zhou, DC Drummond, H Zou, ME Hayes, GP Adams, DB Kirpotin, JD Marks. Impact of single-chain Fv antibody fragment affinity on nanoparticle targeting of epidermal growth factor receptor-expressing tumor cells. *J Mol Biol* 371, 934-947 (2007)
DOI: 10.1016/j.jmb.2007.05.011
PMid:17602702 PMCid:PMC4198021

12. X Liu, S Jiang, C Fang, S Yang, D Olalere, EC Pequignot, AP Cogdill, N Li, M Ramones, B Granda, L Zhou, A Loew, RM Young, CH June, Y Zhao. Affinity-Tuned ErbB2 or EGFR Chimeric Antigen Receptor T Cells Exhibit an Increased Therapeutic Index against Tumors in Mice. *Cancer Res* 75, 3596-3607 (2015)
DOI: 10.1158/0008-5472.CAN-15-0159
PMid:26330166 PMCid:PMC4560113

13. E Lanitis, M Poussin, IS Hagemann, G Coukos, R Sandaltzopoulos, N Scholler, DJ Powell. Redirected antitumor activity of primary human lymphocytes transduced with a fully human anti-mesothelin chimeric receptor. *Mol Ther* 20, 633-643 (2012)
DOI: 10.1038/mt.2011.256
PMid:22127019 PMCid:PMC3293635

14. JN Kochenderfer, SA Feldman, Y Zhao, H Xu, MA Black, RA Morgan, WH Wilson, SA Rosenberg. Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. *J Immunother* 32, 689-702 (2009)
DOI: 10.1097/CJI.0b013e3181ac6138
PMid:19561539 PMCid:PMC2747302

15. R Berahovich, S Xu, H Zhou, H Harto, Q Xu, A Garcia, F Liu, VM Golubovskaya, L Wu. FLAG-tagged CD19-specific CAR-T cells eliminate CD19-bearing solid tumor cells *in vitro* and *in vivo*. *Front Biosci (Landmark Ed)* 22, 1644-1654 (2017)
DOI: 10.2741/4563

16. V Golubovskaya, R Berahovich, H Zhou, S Xu, H Harto, L Li, CC Chao, MM Mao, L Wu. CD47-CAR-T Cells Effectively Kill Target Cancer Cells and Block Pancreatic Tumor Growth. *Cancers* (Basel) 9 (2017)

17. S Cuzzocrea, E Ayroldi, R Di Paola, M Agostini, E Mazzon, S Bruscoli, T Genovese, S Ronchetti, AP Caputi, A.P, C Riccardi. Role of glucocorticoid-induced TNF receptor family gene (GITR) in collagen-induced arthritis. *FASEB J 19*, 1253-1265, (2005)

18. OU Kawalekar, RS O'Connor, JA Fraietta, L Guo, SE McGettigan, AD Posey, PR Patel, S Guedan, J Scholler, B Keith, N Snyder, J Blair, MC Milone, CH June. Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells. *Immunity*, 44, 380-90 (2016)
DOI: 10.1016/j.immuni.2016.01.021
DOI: 10.1016/j.immuni.2016.02.023

Abbreviations: CAR, Chimeric Antigen Receptor; scFv, Single Chain Variable Fragment; PBS, Phosphate-Buffered ; GITR, glucocorticoid-induced TNFR-related protein

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