Allergy and Immunology

AIMS Allergy and Immunology, 10(2): 83–107.
DOI: 10.3934/Allergy.2026008
Received: 15 February 2026
Revised: 19 May 2026
Accepted: 26 May 2026
Published: 11 June 2026

https://www.aimspress.com/journal/Allergy

Research article

An effective approach for targeting lymphoma and leukemia cell lines with a novel Tan-CAR (CD30/CD20) T cell

Rimjhim Mohanty* and Niladri Ganguly

School of Biotechnology, Kalinga Institute of Industrial Technology (KIIT), Deemed to be University, Bhubaneswar, 751024, India

* **Correspondence**: Email: rimjhim.mohanty09@gmail.com.

Abstract: Despite the success of single-target antigen CART therapy against CD19, there are certain limitations that need to be investigated properly to build a universal treatment for hematologic cancer. Other than CD19, there have been many reports on CD30 and CD20, which are also clinically relevant in different types of hematologic malignancies, but no reports have been published yet that target both antigens as bispecific targets on a single cell. Therefore, we developed a tandem receptor that targets dual antigens with a single receptor and likely reduces tumor marker escape mutations. In this study, we employed a gene editing strategy to disrupt TCRs and a target-specific integration of CAR receptors into T cells to develop a novel CAR model to address tumor-off-target limitations.

Keywords: CAR T cell therapy; third-generation CAR receptor; tandem-CAR; bispecific targets; CD20; CD30; CRISPR/CasS9

Abbreviations: CAR: Chimeric antigen receptor; CTLA-4: Cytotoxic T Lymphocyte-associated protein 4; SHP-1: Src homology domain containing tyrosine phosphatase 1; GM-CSF: Granulocyte-macrophage colony stimulating factor; HPSC: Hematopoietic stem cell; FasR: Fas receptor; LAG-3: Lymphocyte activation gene 3; iPSC: Induced pluripotent stem cell; PD-1: Programmed death-1; TGF-βR2: Tumor growth factor β receptor 2; TCR: T-cell receptor

1. Introduction

T cells are in charge of locating and combating diseases all over the body with the aid of TCRs,

but in some conditions, such as cancer, the immune system is unable to identify abnormal cells by their antigens, which prevents T cells from killing cancer cells [1].

An innovative method of cancer immunotherapy known as chimeric antigen receptors allows T cells to recognize cancerous cells by their antigenic markers. This revolutionary therapy was first introduced by an Israeli immunologist, Zelig Eshhar, from the Weizmann Institute of Science. These synthetic receptors can be used as a weapon in our immune system to hunt down cancer cells [2]. In 1993, Eshhar and colleagues presented the prototype of a chimeric antigen receptor (CAR), which specifically activates and targets T cells by using molecules with an antibody domain that targets an antigen and a signal-generating subunit from either the Fc epsilon receptor or the T-cell receptor, respectively [3]. The genetically engineered receptors in T cells are those that are able to recognize tumor antigens with a new receptor that can bind and kill particular types of cancer cells. These cells are called CAR T cells, making it a targeted therapy for each type of cancer. Therefore, CAR T-cell therapy designed for one type of cancer will not work against another type of cancer [4]. Although synthetic CARs play a role similar to that of TCRs, they do not rely on MHC molecules to identify and capture their targets. As a result, tumors' primary defense mechanisms against MHC-restricted T-cell recognition, such as down-regulating human leukocyte antigen (HLA) class I molecules and improper antigen processing, have no effect on the recognition of target cells by CAR T cells. Hence, CART cell therapy is an MHC-independent process [5].

Anti-CD19 CAR T cells have been shown to be clinically effective against relapsed B-cell cancers in a number of studies. However, these trials have uncovered significant shortcomings in the CAR technology, such as tumor cell's propensity for antigen escape [6]. According to a recent clinical trial, 90% of patients who received CD19 CAR T-cell therapy achieved a complete response, while 10% eventually relapsed with CD19-negative tumors [7]. Antigens that are frequently downregulated or mutated in cancer cells produce antigen-loss escape variants hence, dual-antigen-target T cells could be a possible way to stop tumor cells from escaping [8]. It is also possible that such bispecificity could enhance T-cell activation and function by enhancing avidity and by extending the therapeutic range of these cells [9]. Hence, targeting dual tumor antigens is likely to have substantial therapeutic benefits associated with making an individual T cell bispecific. Moreover, finding out bispecific antigens and constructing CAR T cells that can target various hematologic malignancies will take this therapy to a new horizon [10,11].

CD30 was originally recognized as the marker for Hodgkin, non-Hodgkin lymphoma, and Reed–Sternberg lymphomas. Recent studies have also revealed that peripheral T-cell lymphomas (PTCLs) express CD30 [12]. Besides that, lymphocytes that have been exposed to a pathogenic virus and various leukemia cells overexpress CD30 antigen on the cell surface [13,14]. CD20 expression was first discovered in classical Hodgkin lymphoma and also in anaplastic large cell lymphoma (ALCL) however, numerous studies have asserted that CD20 is aberrantly expressed in T-cell lymphoma and leukemia as well [15–17]. Because CD20 and CD30 are expressed specifically on lymphomatous cells, they are the most promising targets for immunotherapy [18,19].

In order to achieve the bispecific ability of T cells, we constructed CAR-containing antigen recognition domains for CD20 and CD30 on a single transgenic receptor. This first-of-its-kind CAR-T receptor may be promising for both B-cell and T-cell malignancies. So far, we have conducted an extended computerized study of this novel Tan-CAR (CD30/CD20) to evaluate its binding affinity to target antigens and physical properties to be stable inside cells by molecular modeling, docking, and a dynamic approach.

2. Materials and methods

2.1. Isolation and culture of CD8 T cells

Fresh whole blood was collected from healthy donors through an EDTA-coated vacutainer. CD8 T cells were harvested using the CD8 T-cell enrichment cocktail (Stemcell Technology) and Hisep TM density gradient media (Himedia) from the intermediate layer of PBMC, followed by centrifugation at 300 × g for 20 min (Figure S1). Harvested CD8 T cells were stimulated in Lymphogrow (CYTOGEN) at a cell density of 1×10^7 for 24 h at 37 ℃ in a 5% CO_2 incubator and then activated with ImmunoCult Hu CD3/CD28 T-cell activator for 48 h prior to performing any cellular assay (10 μg/mL, Stemcell Technology). A cryopreservative was used to store cells for future use (Stemcell Technologies, CryoStor CSB).

2.2. Cell lines and cell culture

Human leukemia cell lines (K562, Jurkat) and human lymphoma cell lines (Raji, Ramos) were procured from NCCS, Pune. Cells were cultured in IMDM medium (Gibco, Life Technologies, California, USA) supplemented with 10% FBS (Gibco, Life Technologies, California, USA) and 1% antibiotics (100 U/mL penicillin and 10 mg/mL streptomycin) at 37 ℃ in a humidified incubator containing 5% CO_2 (Figure S2). Cells were stored for further use in a freezing medium containing FBS and DMSO in a ratio of 9:1 in a 1 ℃ cooler in a −80 ℃ freezer.

2.3. Knockout of TCR using the CRISPR/Cas9 technique

The CRISPR/Cas9 technique was used to knock out TCR in primary T cells. Top and bottom sgRNA oligo inserts for the TCR gene were designed using the CRISPR Design Tool (http://crispr.mit.edu/) (Table S1. The pSpCas9 (BB)-2A-Puro vector was procured from Addgene (plasmid ID: 48139) and used for cloning the sgRNA oligos (Figure S3). The top and bottom strands of sgRNAs were phosphorylated before annealing them at 37 ℃ for 30 min, 95 ℃ for 5 min, and 25 ℃ at 5 ℃/min. The pSpCas9 (BB)-puro-2A vector was digested with BbsI restriction endonuclease for 1–16 h at 37 ℃. T4 DNA ligase was used to ligate sgRNAs into pSpCas9 (BB)-puro-2A overnight at 16 ℃ prior to heat-inactivation for 10 min at 65 ℃ (Figures S4). During the transformation process, 5 μL of the above mixture was added to 50 μL of chemically competent *E. coli* DH5 cells. The Qiagen Midi-Prep Plasmid Isolation Kit was used to isolate and purify plasmids from positive cloned cells.

For transfection, cells were seeded in a 24-well plate at a density of 1×10^5 cells per well overnight. Lipofectamine LTX was used to transfect 500 ng of total sgRNA at equimolar ratios. In order to screen for positive transfected cells, the cells were treated with 2 μg/mL of the mammalian selective antibiotic Puromycin after 3 days of incubation. Puromycin was reduced to 1 μg/mL after 3–4 days to maintain the cells. The DNA was extracted, and gene-specific primers were used to perform PCR after stable transfection. Moreover, anti-TCR alpha/beta antibodies (BioLegend) were used to confirm gene expression in the stable cells by FACS.

2.4. CAR gene construction, transfection, and expansion

A novel tandem chimeric gene was constructed against CD20 and CD30 tumor antigens. As described in our previous manuscript, the entire gene construct contains an extracellular domain and an internal domain. The extracellular domain contains scFv sequences composed of variable heavy and light chains from CD30 [20,21] and CD20 [22,23] monoclonal antibody sequences, allowing them to target both tumor antigens at the same time. Adjacent to the external domain, there is a hinge and transmembrane region from CD8A (P01732), followed by the intracellular domain, which consists of a few costimulatory molecules like CD28 (P10747) and CD137 (Q07011) and a signaling molecule, i.e., CD3z (P20963). The scFv sequence was separated using a general linker, i.e., 3 (GGGS). GenScript Company synthesized and cloned the CAR gene into the pcDNA3.1-6his vector. LTX Lipofectamine (Invitrogen) was used to transfect 500 ng of plasmid cloned with the CAR gene for 24 h. Following 72 h of incubation, a selective antibiotic (G418) was added to the cells to screen for positively transfected cells. G418 antibiotic (Sigma-Aldrich) was reduced to 50 μg/mL after 3 days and maintained thereafter.

CAR-gene-transfected CD8 T cells were cultured and maintained in T-cell expansion media. CST OpTmizer T Cell Expansion SFM (Gibco) was used to expand the T-cell population. We cryopreserved CAR T cells in CryoStore R CSB (STEMCELL Technologies) for long-term use, and before performing any assay, they were thawed and cultured in lymphogrowth medium for 24 h.

2.5. CAR T-cell proliferation assay

Primary T cells were isolated, further cultured, and maintained with supplements like IL2 and CD28/CD3 activators for their steady growth. To check the proliferative rate of CAR-gene-transfected T cells with or without additive activators, only T cells (untransfected T cells) were analyzed together in an experimental plate (Figure S5). Different numbers of CAR T-cell suspension were inoculated into 96-well plates, and the plate was then incubated for 96 h to observe the proliferation rate using the CCK8 Assay Kit (Figure S6). The experiment was also carried out in a time-dependent manner, from 24 to 96 h with 2000 cells incubated in every well to check the proliferation rate (Figure S7). Each well was treated with 10 μL of CCK8 solution, a highly water-soluble tetrazolium salt named WST-8 that reduces in cells to form a yellow-colored substance called formazan. The number of living cells directly correlates with the amount of formazan dye released in the cell medium. After 4 h of incubation, the absorbance at 450 nm was measured using a microplate reader.

2.6. Cell cycle analysis of CAR T cells

CAR T cells were collected and washed twice with 1 mL of cold 1× PBS containing 0.05% RNase-A (Himedia, 10 mg/mL). Cells were pelleted and resuspended in a fixation solution dropwise in the tube (300 μL of chilled 70% ethanol). After incubation at 4 ℃ for 4 h, cells were centrifuged at 200 rpm for 5 minutes at room temperature to remove ethanol. Cells are rinsed again with 1× PBS, pelleted, resuspended with 0.5 mL of propidium iodide staining solution (Invitrogen, 50 μg/mL PI, 20 μg/mL RNase A, and 0.02% Triton X-100) and incubated in the dark for 30 min (Figure S8). Cell suspensions were analyzed through flow cytometry. A BD FACS Canto II (Becton & Dickinson, CA, USA) flow cytometry device was used to analyze stained samples.

2.7. Genomic DNA extraction and confirmation of CAR gene by polymerase chain reaction (PCR)

After stable cell preparation, genomic DNA was extracted using the DNA Isolation Kit (QIAamp DNA Mini Kit, Germany) based on the manufacturer's protocol to determine the gene expression of the CAR-transfected cells. The PCR reaction was performed as follows: 5 min of initial denaturation at 94 ℃, then 40 cycles of denaturation at 94 ℃ for 1 min, then annealing at 52 ℃ for 1 min, and extension at 72 ℃ for 5 min. The final extension was performed for 10 min at 72 ℃. After examining the final PCR product on a 1% agarose gel electrophoresis, the CAR gene (2280 bp) was confirmed using a 1 kb ladder. The primer sequences are mentioned in Table S1.

2.8. RNA extraction and real-time qPCR (RT-qPCR)

To validate the PCR result, stable cells from both transfected and non-transfected T cells were harvested and subjected to total RNA extraction with TRIZOL (Invitrogen). The SuperScriptTM IV First-Strand Synthesis System Kit (Invitrogen, Thermo Scientific) was used to create the cDNA in the first step. The RNA sample was reverse-transcribed into cDNA using a random hexamer primer and the SuperScriptTM IV First Strand Synthesis System (Invitrogen, Thermo Fisher Scientific) as instructed by the manufacturer. Real-time qPCR was performed using the Fast SYBR Green PCR Master Mix (Invitrogen, Life Technologies) and the Bio-Rad CFX96 Real Time PCR Detection System (Bio-Rad, USA). All primers are listed in Table S1. Each sample was examined twice. Using the 2-ΔΔCT method, the mRNA expression levels were then calculated after being normalized to the internal control GAPDH. For statistical analysis, a Student's t-test was used, and $p < 0.05$ was considered statistically significant.

2.9. Neutral red uptake assay

The cytotoxicity of target cells was analyzed by a neutral red uptake assay. The NRU stock solution (15 mL) was prepared by dissolving 0.12 g of neutral red powder (Sigma-Aldrich) in 12 mL of water and 0.11 g of NaCl in 2.5 mL of water. The two solutions were mixed well and sterilized. 96-well plates were plated with 200 µL of cell suspension in each well, each containing 10,000 target cells with an increasing number of effector cells in an effector-to-target cell ratios of 1:1, 5:1, and 10:1, and incubated for 24 h at 37 ℃ in 5% CO_2. Following the incubation period, a microplate-compatible centrifuge machine was used to spin the plate at 2000 rpm at 4 ℃ to carefully aspirate the media from the plate. The plate was then supplemented with 150 µL of 1× neutral red solution (50 µg/mL) and incubated for an additional 3 h. Cells were spun down by the above process to discard neutral red solution, and 150 µL of 1× dissolving solution (5.5 g of SDS + 25 mL of 0.02 M HCl + 25 mL of isopropanol) was added and kept for 20–30 min on a rocker at room temperature. The absorbance of the solution in each well was measured on a microplate reader at 540 nm.

2.10. Immunophenotyping of CAR T cells and cancer cells

In order to identify all cell surface antigens, whether the target tumor antigens or the early activation markers on cocultured cells, 1 million cell samples of different target cell lines and coculture cell aliquots were centrifuged at 1000 × g for 5 min and then washed twice in PBS (pH 7.2). According to the

manufacturer's protocol, 1 million cells were mixed with 5 μL of conjugated primary antibodies and incubated on ice for 30 min. The antibodies used to perform different assays were CD8-APC, CD25-PE, and CD69-FITC, anti-TCR alpha/beta, CD20-PE, and CD30-APC monoclonal antibodies (BioLegend, USA). After incubation, the cells were centrifuged twice with PBS at 300 × g for 5 min to remove extra antibodies, and flow cytometry was performed on the resulting cells. The experiment was conducted in parallel with untreated, fluorochrome-matched, and nonspecific isotype-labeled controls. BD FACS Canto II (Becton & Dickinson, CA, USA) was used to analyze the stained samples. Dead cells and debris were excluded from the FACS acquisition. Data from stained cells acquired over ten thousand times were analyzed using BD's FACS Diva software.

2.11. Cytokine analysis

The expression of cytokines was determined using an indirect ELISA. Protein antigens and coupling buffer were coated on a 96-well plate (model number 3679, Corning, NY, USA), and extra binding sites were blocked with a blocking solution. Antigens were detected using particular primary antibodies, which were then followed by secondary antibodies that were HRP-conjugated. Microplate readers (Berthold, Germany) were used to detect the absorbance of the colored substance at 405 nm after adding the ABTS substrate solution. Each purified cytokine and growth factor for the experiments was purchased from ProSpec-Tany Techno Gene Ltd., USA. Each purified protein (cytokines and growth factors) was quantified using standard curves created from various concentrations of a sub-stock (1 ng/mL). The concentrations of each protein were calculated by plotting concentrations (pg/mL) versus sample concentrations and comparing the test absorbance value discovered from our experiments with the relevant standard curves.

2.12. Protein extraction and western blot analysis

A western blot experiment was conducted to check the expression of CD20 and CD30 in all the target cell lines and to check the expression of signaling molecules in a cocultured cell sample. Cells (two million) were harvested and washed in cold PBS buffer and then lysed with 100 μL of cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, 1% Triton-X-100, Milli-Q water) containing a protease inhibitor cocktail (Thermo Fisher). The lysate was incubated on ice for 30 min, followed by sonication for 30 s, and pelleted at 12,000 rpm in a tabletop centrifuge at 4 ℃ for 10 min. The supernatant was collected and stored at −20 ℃. 50 μg of protein sample was separated by 10% SDS-PAGE gel and then transferred to the PVDF membrane. The membranes were blocked in 7% skimmed milk solution for 45 min and then probed with primary antibodies (CD30 and CD20 polyclonal antibodies; Proteintech, USA, 1:1000) and anti-His-Tag antibodies (AKT, NF-kb, and GAPDH; Cell Signaling Technology, USA, 1:1000). After overnight incubation at 4 ℃, secondary antibodies were added for 2 h at room temperature (Cell Signaling Technology, USA, 1:1000). Finally, protein bands were visualized by ImageQuant LAS 500 chemiluminiscence (GE Healthcare, USA).

2.13. Statistical analysis

All experiments were conducted in triplicate. GraphPad Prism 6 software was used to perform

statistical analysis. Two-way ANOVA and the Student's t-test were used to assess statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1. TCR knockout by CRISPR/Cas9

TCR is a major receptor of T cells, playing a major role in recognizing the MHC ligand of antigen-presenting cells and destroying infected cells [1]. Despite the fact that chimeric receptors are widely believed to identify and kill tumor cells without the involvement of the MHC complex, the decision to test the hypothesis by removing the TCR receptor was made by the application of the CRISPR/Cas9 method. To enable editing of the TCR gene, we prepared gRNA specific to TRAC and TRBC locations of the TCR receptor. T cells were isolated and transfected with CRISPR/Cas9-mediated disruption to make a population of TCR-KO-T cells. Genomic DNA was isolated from the transfected cells, and 50 ng/μL of template was used for the PCR reaction, clearly showing no sign of TRAC or TRBC in knockout cells, while two distinct bands were identified at around 900 and 1200 bp (Figure 1A). Furthermore, to quantify the expression of endogenous TCR, we checked untransfected and CRISPR/Cas9-treated cells in FACS (Figure 1B). More than 85% of control cells expressed TCR, whereas 60%–70% of transfected cells were found to be TCR-disrupted (Figure 1C). Representative results shown in Figure 1 confirm that TCR is significantly knocked out of the transfected T cells by the application of the CRISPR/Cas9 method.

3.2. Successful transfection of the CAR gene in CD8 T cells

Figure 2A shows a schematic representation of the recombinant receptor we have designed and synthesized. We constructed a bispecific tandem third-generation CAR gene sequence that encodes a fusion protein composed of single-chain variable fragments (scFv) of CD30 and CD20 mAb, as well as some costimulatory domains like CD28, 41-BB, and a signaling domain, CD3, that was synthesized and cloned into pcDNA3.1-(6-His tag). To get a clear picture of the novel CAR protein, we put the amino acid sequence of the whole CAR protein in a computerized protein modeling server, i.e., I-Tasser, and found the result matching exactly the design of our constructed CAR protein (Figure 2B). Healthy, growing primary T cells (1×10^5 cells/well in 500 μL media w/o antibiotics) were inoculated in a 24-well plate and transfected with the CAR vector. Successfully transfected, stable cells were screened by G418 antibiotic selection. Then, transfected stable cells were grown and maintained in T-cell expansion media. The confirmatory CAR gene band was identified by PCR reaction in the range 2000–3000 bp (Figure 2C). We also checked the mRNA expression of the CAR gene by qRT-PCR, keeping GAPDH as a control, which confirmed a significant expression of the CAR gene (Figure 2F). Furthermore, protein expression of CAR was analyzed by a western blot experiment, and a protein band was detected at 90 kDa using an anti-His tag antibody against the 6-Histidine tag in the CAR vector (Figure 2D). Moreover, to check the expression of both antigen-binding sites of the CAR protein, we also checked the CAR-gene-transfected cells in FACS. According to this analysis, approximately 60% of cells were found to have CD30 scFv, and approximately 40% of cells were found to have CD20 scFv in an individual binder test, whereas approximately 50% of transfected T cells showed the expression of both antibodies from the combined antibody test. All the

above experiments were conducted in triplicate tests and are presented in a bar graph in Figure 2E. From the in silico study, it is evident that the constructed CAR receptor accurately depicts its original structure in the required manner. Additionally, western blot and PCR results further demonstrated the successful transfection of the CAR gene and expression of the CAR protein.

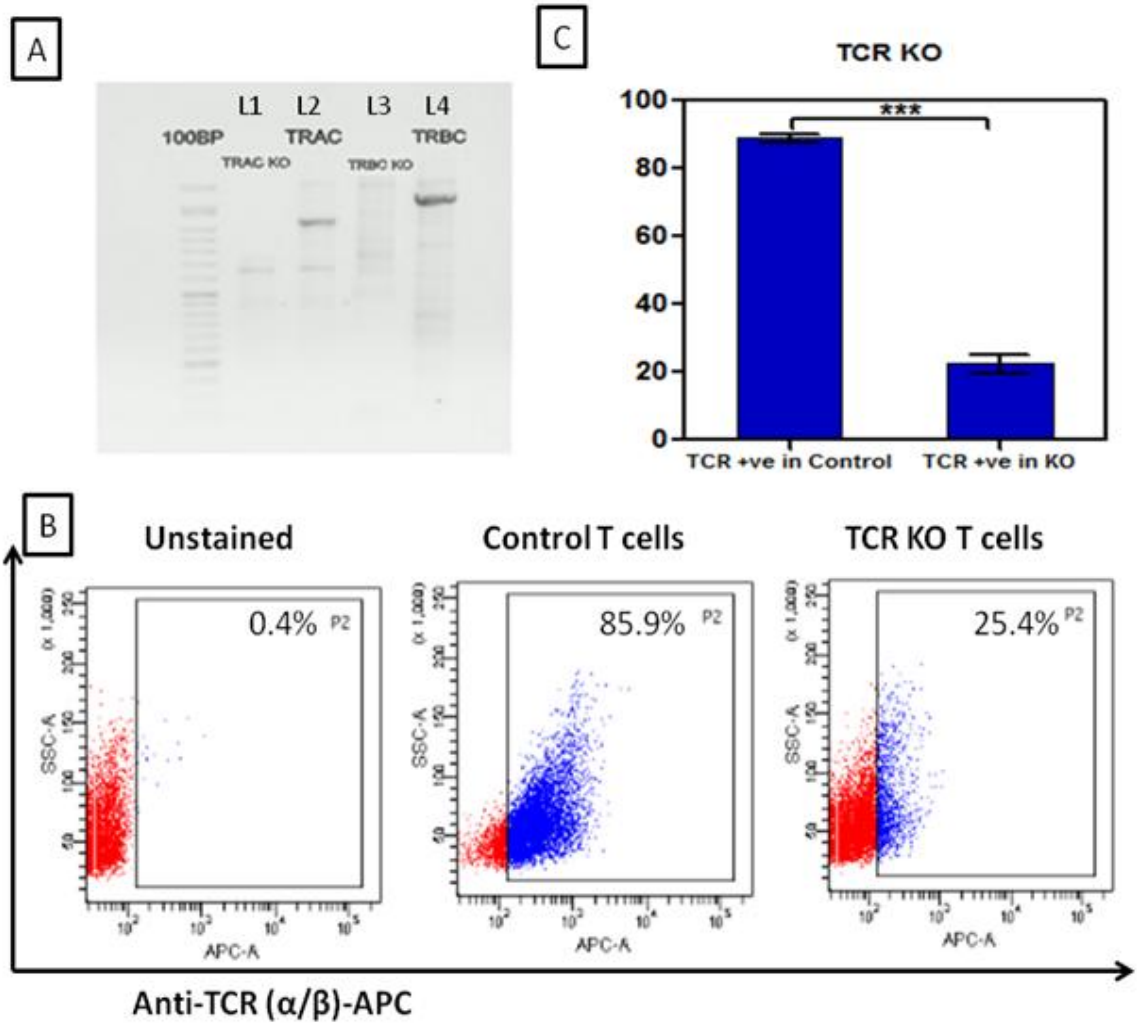

Figure 1. Confirmation of TCR gene knockout by the CRISPR/Cas9 gene editing process. (A) 1.5% agarose gel image of PCR products of genomic DNA isolated from TCR-gene edited and non-edited T cells to check the knockout of the TRAC (alpha) and TRBC (beta) genes. L1 and L3 confirm the knockout of TRAC and TRBC of the TCR gene. (B) Scatterplot representation of anti-TCR (alpha/beta) antibody expression in normal T cells and TCR knockout T cells. (C) Graphical representation of FACS result, showing percentage of TCR-positive T cells in control and edited T cells.

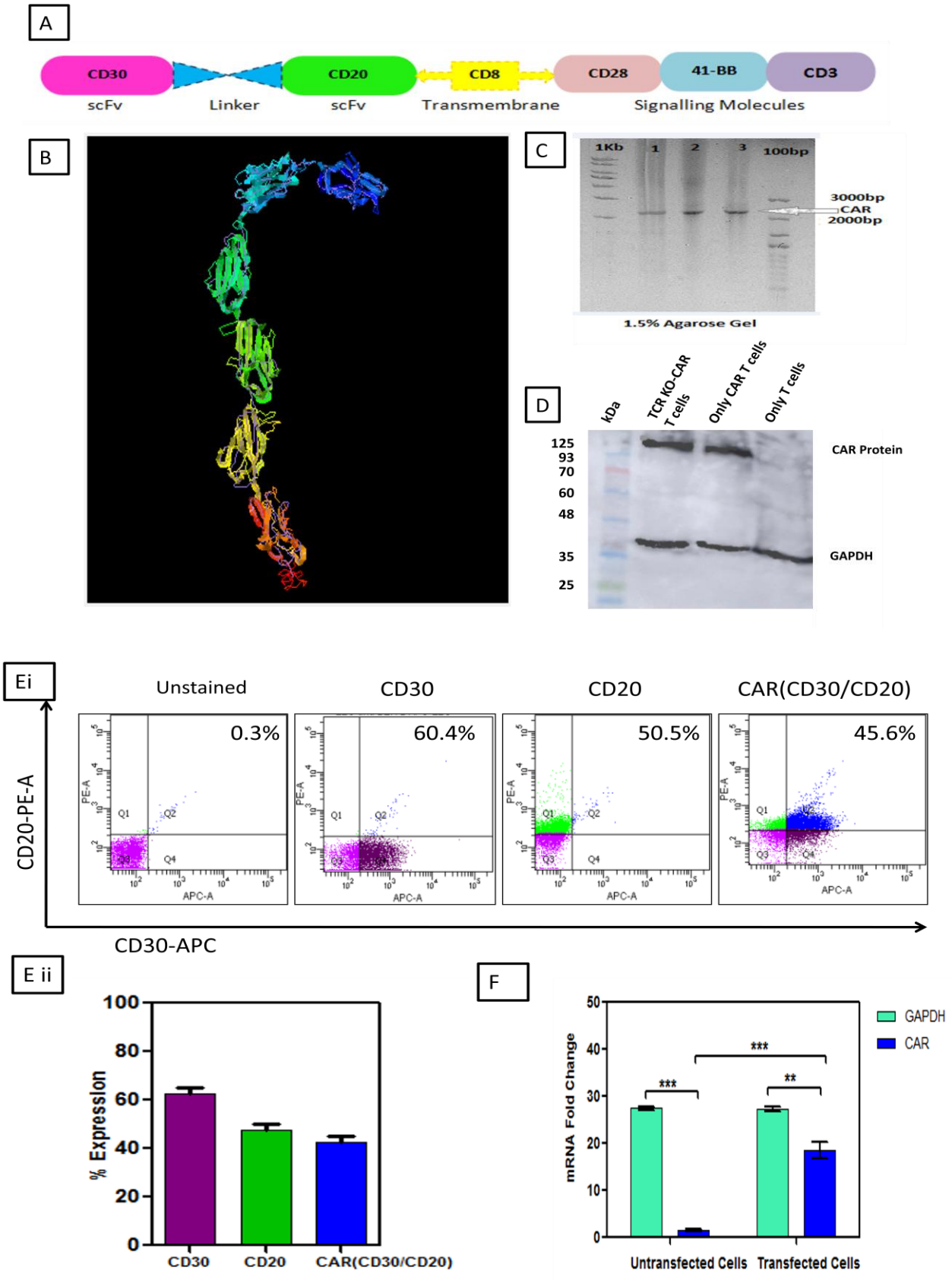

Figure 2. Confirmation of CAR gene expression. (A) Schematic representation of CAR gene design and construction. (B) The in silico study represents the structure of the CAR

protein from I-TASSER. (C) 1.5% agarose gel image of the PCR product of genomic DNA isolated from CAR-gene-transfected T cells in all three lanes in triplicate, with a 1 kb and 100 bp DNA ladder in both side lanes. (D) The SDS-PAGE gel image represents CAR protein expression by using the anti-His-tag antibody, and GAPDH serves as the loading control. (E) (i) Expression of CAR (CD30/CD20) was accessed by FACS analysis. (ii) Graphical representation of FACS data. (F) mRNA fold change shows a clear expression of CAR in transfected T cells, whereas no significant expression was found in untransfected T cells. GAPDH was used as a housekeeping gene control. The above results confirm successful transfection of the CAR gene into T cells. All the above experiments were carried out in three biological replicates.

3.3. Identification of target tumor antigens on target cells

According to various studies on tumor antigens, CD20 and CD30 were found to be associated with both lymphoma and leukemia. Finding dual-target antigens that coexist in both malignancies will be way more promising than targeting single antigens on either cell lineage or different dual targets for T as well as B-cell lineages [24]. Hence, we hypothesized that both CD20 and CD30 coexist in T cells and B cells, allowing us to address both malignancies with one treatment. Thus, in order to test our hypothesis, we have taken some leukemia cell lines for this analysis, such as K562, Jurkat (T cell lines), and lymphoma cell lines such as Raji and Ramos (B cell lines). According to various reports, CD30 and CD20 are marker antigens for hematologic malignancies [19,25]. For this analysis, we have checked all the target cell lines for the presence of both the tumor marker by FACS (Figure 3A) and western blot analysis. We found that T cells show 25%–30% of CD20, whereas B cells show 50%–60%. T cells show 60%–80% of CD30 expression, whereas B cells show 30%–40% in FACS analysis. According to FACS analysis, the CD30 expression in T-cell lines was found to be 30%–60%, while it was 30%–40% in B cells (Figure 3B). Likewise, T-cell lines showed 25%–30% CD20 expression, while B-cell lines showed 50%–60% (Figure 3C). Moreover, we also checked its protein expression in a western blot using RIPA lysis buffer; 50 μg/μL of protein sample was used, and GAPDH was used as a loading control to validate the previous results (Figure 3D).

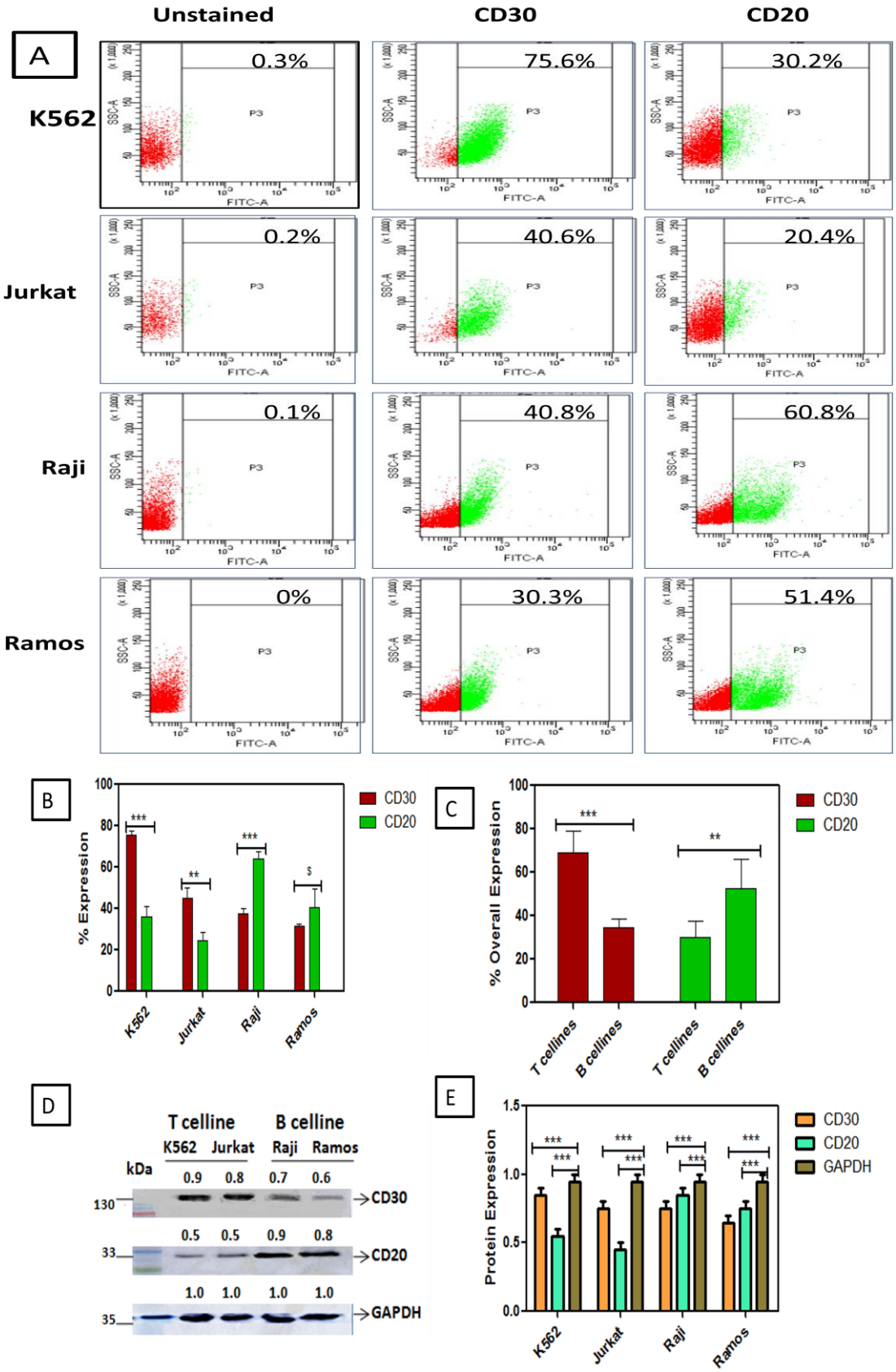

Figure 3. Confirmation of tumor markers (CD30 and CD20) on target cell lines. (A) FACS analysis was performed to check the expression of tumor markers in all four

targeted cell lines. (B) Graphical representation of FACS data. (C) Bar graph showing the overall expression of CD20 and CD30 T cells and B-cell lineage. (D) Expression of CD30 and CD20 in T-cell lines and B-cell lines in a western blot; GAPDH was used as a loading control. (E) Graphical presentation of the western blot experiment. The experimental images shown above are representative of three different sets of experiments. Statistical significance was determined by two-way ANOVA; *P < 0.05, **P < 0.01, and ***P < 0.001 represent statistically significant differences, whereas $ indicates non-significant.

3.4. TAN-CAR-mediated cell killing

We have performed a coculture experiment to examine whether expression of the TAN-CAR (CD30/CD20) can be effective in killing cells that are expressing lymphoblastic targets like CD20 and CD30. We tested the cytotoxicity activity of TAN-CAR (CD30-CD20) T cells against a panel of human T cell and B-cell leukemia lines (K562, Jurkat, Raji, and Ramos). The flow cytometry analysis revealed homogenous weak CD20 expression in T cells, but high levels of CD30 antigen, while B cells express low levels of CD30 and high levels of CD20 antigen. The lysis rate by untransfected primary CD8 T cells was found to be 10% at a E:T ratio of 1:1 and <20% at a 10:1 ratio. In contrast, lysis by TAN-CAR CD30-CD20 significantly increased to 15%–30% at a E:T ratio of 1:1 and reached 35%–60% at a 10:1 ratio (Figure 4A). To further validate the cytolytic function of CAR T cells, we conducted a serial killing experiment, in which 1000 target cells were taken in each of five wells of a 96-well plate. A serial addition of CART cells was performed every 2 days up to a period of 10 days. As a result, the first well received 5× the amount of 2000 CAR T cells (E:T ratio 10:1). For the second well, 4× 2500 CART cells were given, while 3× 3333 CART cells were given to the third well, 2× 5000 CART cells were given to the fourth well, and 10,000 CART T cells were given to the fifth well to maintain the predetermined E:T ratio of 10:1. We observed a clear shift in the rate of cell death in the first well of all four target cells experiment, indicating that fresh addition of CAR T cells in a specific interval was more effective than bulk addition. It is important to note that the cytotoxicity levels of the CART and TCR KO CART groups were nearly comparable at low E:T ratios (1:1 and 5:1), but at 10:1, the cytotoxicity of the CAR groups was significantly higher than that of untransfected T cells. In addition, the overall expression of both target antigens in T and B cells was seen in the previous findings (Figure 3), thus confirming from that study that a higher level of target cell clearance was only seen in cells expressing higher levels of both target antigens. Hence, CART cells seem to be cytotoxic in a dose-dependent and antigen-specific manner, while maintaining stable expression of chimeric receptor on their cell surface.

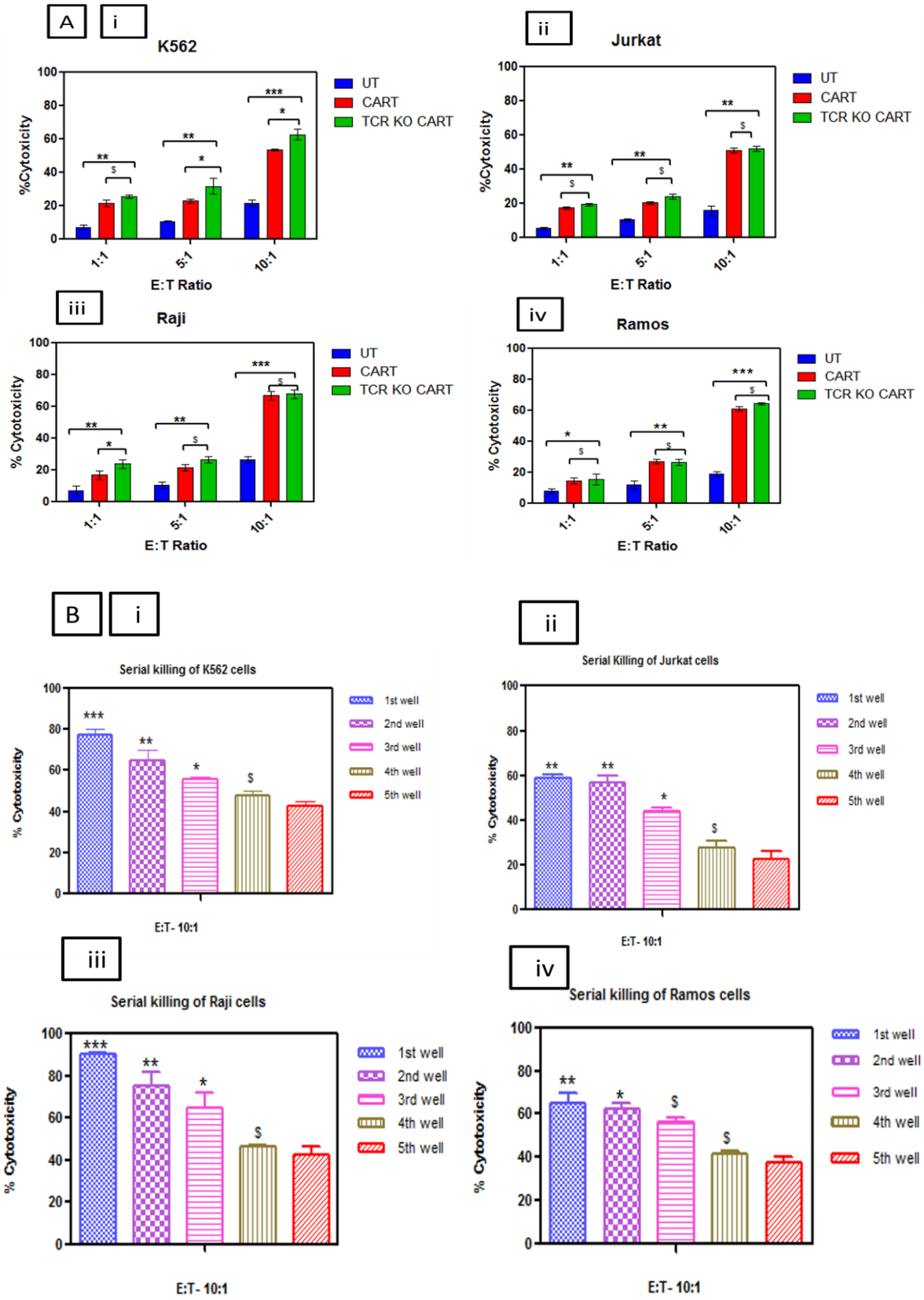

Figure 4. Cytolytic activity of TAN-CAR (CD30/CD20). (A) Targeted cell killing activity of CART cells upon coculturing with (i) K562, (ii) Jurkat, (iii) Raji, and (iv)

Ramos, individually incubated for up to 96 h at different effector-to-target ratios (E:T) (1:1, 5:1, and 10:1). (B) Coculture of target cells with serial addition of CART cells up to 5 days. In both experiments, with increasing CART cell numbers, target cell lysis increases significantly at a 10:1 E:T ratio. All results are representative of three independent experiments with SEM (n = 4). Student's t-test was used to find out statistical significance (ns or $, $P > 0.05$), *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$.

3.5. Expression of the CAR T-cell activation marker upon binding to target cells

T cells express some early activation markers within 48 h of binding to target cells (CD69 and CD25). Panels of lymphoma and leukemia cells were cocultured with prepared TAN-CAR-T cells for 48 h to determine their activation and functionality. To confirm the activation of CAR-T cells upon coculture, flow cytometry analysis was performed. For this, we used all three coculture cell samples with untransfected T cells, CART cells, and TCR-edited CART cells for analyzing the expression of CD25 and CD69 activation markers. We combined anti-CD8, anti-CD25, and anti-CD69 in each test sample and checked the expression of CD25 and CD69 from the CD8-gated cells on CART cells. All coculture cell samples with UT, CAR-T, and TCR-edited CART cells showed high levels of $CD8^+$ surface markers (85%–90%), as shown in Figure 5. A significant increase in CD25 expression was observed in both TCR-CAR T cell and CAR T gene-transfected cells, compared to untransfected T cells cocultured with K562 and Raji cell lines (30% and 27.6% vs. 10.5%) (Figure 5A–D). Both CD25 and CD69 expression levels were comparatively lower in coculture with Jurkat and Ramos cell lines. Although there is no significant change in the expression of both CD25 and CD69 between CAR-T cells and TCR-CAR T-cell coculture with all four target cells, TCR-edited CAR T cell coculture samples showed a slightly higher expression than the coculture with CAR T cells (30.6% vs. 27.6%). Figure 5E shows FACS results, where it is clear that K562 and Raji cell lines show significantly higher expression of both activation markers than the other two target cells. As a result of coculturing all target cell lines with both TCR-non-edited and TCR-edited CART cells, a considerable change in the expression of both CD25 and CD69 is evident in Figure 5. Raji and K562 cells showed 30% and 25% expression, respectively.

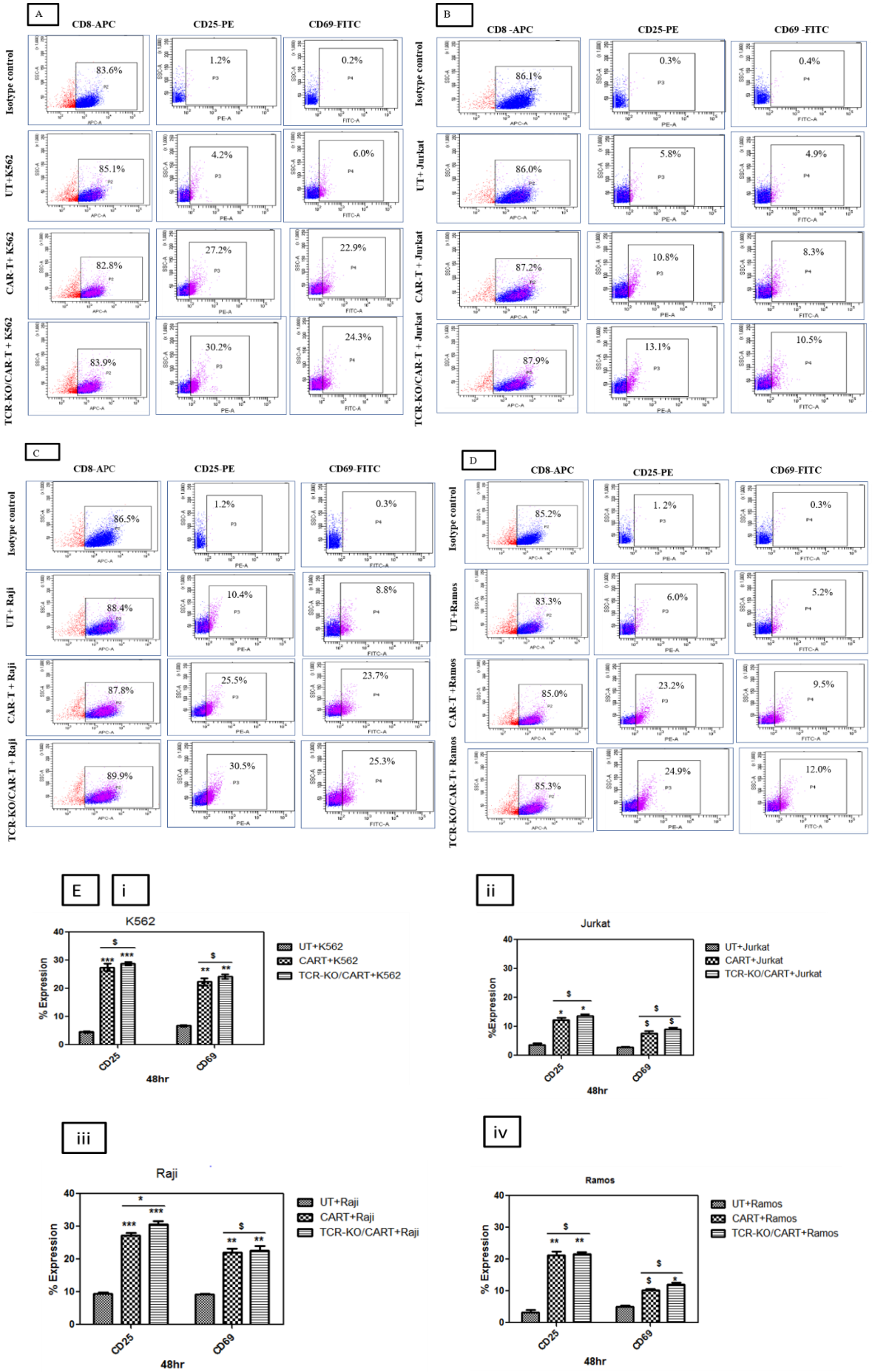
A
CD8-APC
CD25-PE
CD69-FITC
Isotype control
UT+K562
CAR-T+ K562
TCR-KO/CAR-T + K562
83.6%
1.2%
0.2%
85.1%
4.2%
6.0%
82.8%
27.2%
22.9%
83.9%
30.2%
24.3%
B
CD8 -APC
CD25-PE
CD69 -FITC
Isotype control
UT+ Jurkat
CAR-T + Jurkat
TCR-KO/CAR-T + Jurkat
86.1%
0.3%
0.4%
86.0%
5.8%
4.9%
87.2%
10.8%
8.3%
87.9%
13.1%
10.5%
C
CD8-APC
CD25-PE
CD69-FITC
Isotype control
UT+ Raji
CAR-T + Raji
TCR-KO/CAR-T + Raji
86.5%
1.2%
0.3%
88.4%
10.4%
8.8%
87.8%
25.5%
23.7%
89.9%
30.5%
25.3%
D
CD8-APC
CD25-PE
CD69-FITC
Isotype control
UT+Ramos
CAR-T +Ramos
TCR-KO/CAR-T+ Ramos
85.2%
1. 2%
0.3%
83.3%
6.0%
5.2%
85.0%
23.2%
9.5%
85.3%
24.9%
12.0%
E
i
K562
% Expression
UT+K562
CART+K562
TCR-KO/CART+K562
CD25
CD69
48hr
ii
Jurkat
%Expression
UT+Jurkat
CART+Jurkat
TCR-KO/CART+Jurkat
CD25
CD69
48hr
iii
Raji
% Expression
UT+Raji
CART+Raji
TCR-KO/CART+Raji
CD25
CD69
48hr
iv
Ramos
% Expression
UT+Ramos
CART+Ramos
TCR-KO/CART+Ramos
CD25
CD69
48hr

Figure 5. Early activation marker expression upon coculture and binding with target cells. (A) FACS experiment was performed to verify the expression of the early activation marker on K562 coculture cell samples. CD25 and CD69 expression was tested on T cells used in coculture experiments; all three antibodies were mixed into each test sample, and CD8 T cells were gated in each experiment; CD25 and CD69 expression were measured on the gated CD8 T cells. The same experiment was performed individually in the remaining three cell lines, as shown in panels B–D. (E) Graphical representation of FACS results, clearly showing that CD25 expression was higher than CD69 expression in all four cell lines. All experiments were conducted in triplicate. Statistical analysis conducted through a two-way ANOVA, where *P < 0.05, **P < 0.01, and ***P < 0.001.

3.6. CAR T-cell stimulation induces the secretion of some major signaling proteins, which subsequently cause death to the target cell line

When CAR-T cells bind to cancer cells, they release a variety of cytokines and chemokines, which are cytotoxic in nature. The activated T cells and the secreted signaling proteins, like cytokines and chemokines, then potentially cause inflammation in the cancer cell, thus allowing those cells to die in the surrounding environment. We have tried to investigate whether any molecular changes occur upon binding to the target cell. Coculture supernatants were used in an indirect ELISA kit to identify the molecular mechanism underlying TAN-CAR (CD30-CD20) T-cell activation upon binding to target cancer cells and resulting death of target cells in coculture. We investigated the molecular mechanism behind the signaling cascade that actually occurs in this CAR by analyzing the secretion of different cytokines. According to Figure 6, the expression of all four cytokines (IFN-γ, TNF-α, IL-2, and GNZM-B) in both CART + target and TCR-CART + target cultures increased in comparison to UT + target in all the target cell line cultures. In more detail, we could observe the highest level of INF-γ in K562 cell lines, followed by Jurkat, where, similarly to Raji and Ramos, INF-γ secretion was found to be less than in T-cell lines (K562, Jurkat). INF-γ secretion acts as a cytotoxic cytokine and, together with granzyme-B and perforin, can initiate apoptosis in cancer cells. In the cases of K562, Jurkat, Raji, and Ramos, fold induction in INF-γ expression was 5.8, 4.2, 3.5, and 3.2, respectively, compared to INF-γ secretion from the effector alone (Figure 6A). TNF-α secretion is thought to trigger caspase-dependent cell death or the NF-kb pathway. TNF-α is expressed significantly less in Jurkat cells compared to Raji and K562 cells, and more so in Ramos cells. Effector cells showed 3.8, 2, 4, and 3.2-fold increases in TNF-α expression. In addition, CART + target and TCR-CART + target cells showed 3.1, 1, 3.5, and 2.5-fold increases in TNF-α expression compared to UT + target cells. The release of IL-2 is necessary to activate the immune system and drive cancer cells out of the body. All four cell lines were found to have significantly higher levels of IL-2 than effector cells (4.5, 3.5, 2.6, and 2.5-fold). The secretion levels in all four cell lines cocultured with UT cells were 3.2, 2.4, 2.5, and 2-fold higher than in each cell line alone.

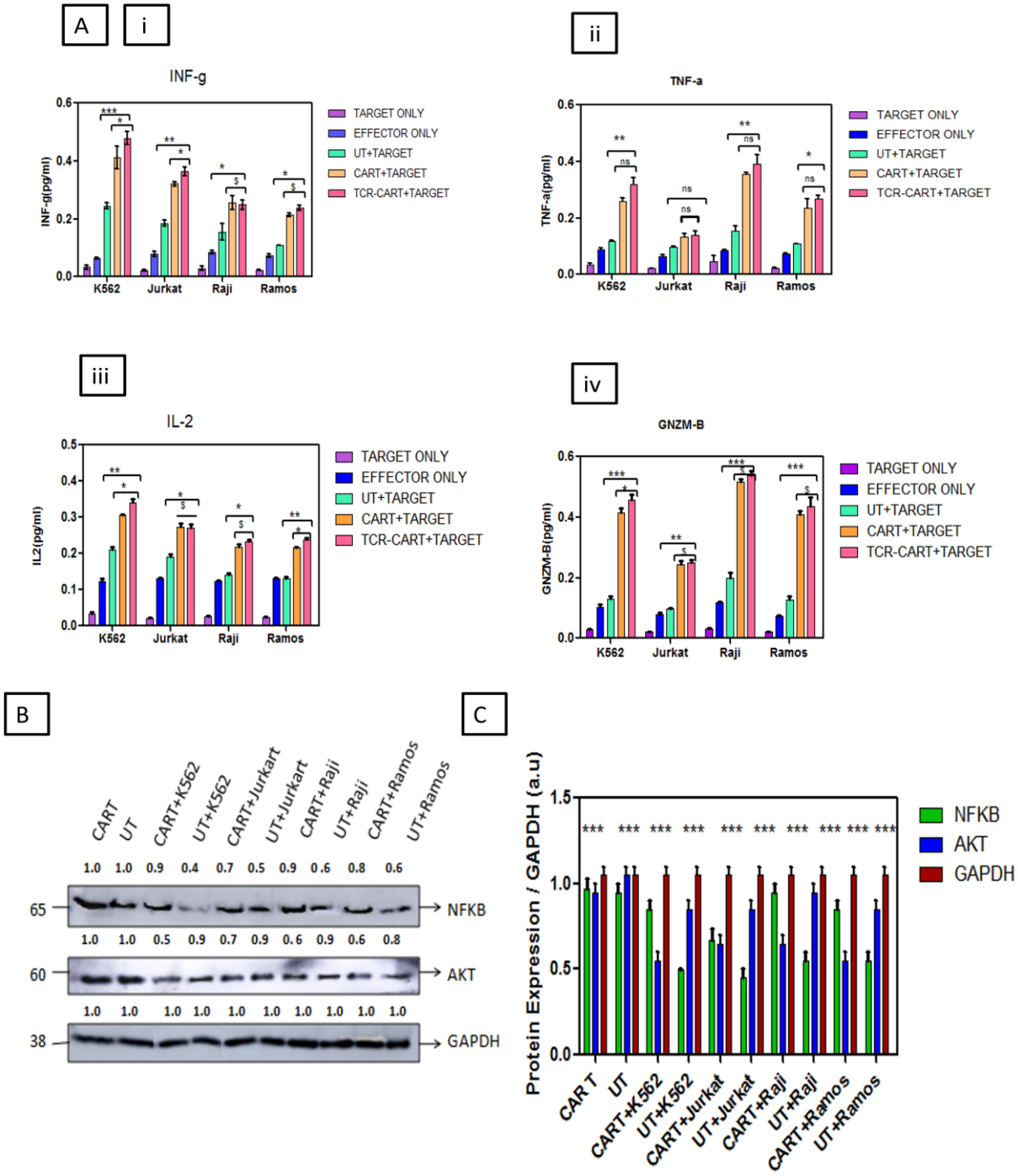

Figure 6. Analysis of CART cell-mediated cytotoxic factors in terms of cytokines and signaling proteins. (A) Coculture supernatant media was used to check the level of cytokines secreted by all three T cells (UT, CART, TCR-KO-CART cells) cocultured with target cell lines individually at an E:T ratio of 10:1 for 96 h. The levels of cytokines IFN-γ (i), TNF-α (ii), IL-6 (iii), and Granzyme-B (iv) were measured by indirect ELISA. (B) To investigate the signaling pathways, the expression of NF-kB and AKT was checked in a western blot, and GAPDH was used as a loading control. (C) Graphical presentation of the western blot result. A two-way ANOVA was used to find out statistical significance, with *P < 0.05, **P < 0.01, ***P < 0.001, and $ or ns, non-significant.

Granzyme-B is a significant chemokine that is released when CAR T cells bind to a cancer cell. It damages the cell membrane and eventually results in the death of the cancer cell. It was evident from the experiment results that granzyme-B expression increased considerably higher in all target + effector cell cocultures than in the effector cell alone, with values of 6.3, 3.2, 8.3, and 5.4, respectively, compared with 5.2, 3, 6.5, and 5 in UT + target culture. Based on the above observation, we conclude that the CAR receptor's internal CD3 signaling domain 4-1BB and CD28 costimulatory domains and their secretion are likely interlinked.

For further validation and confirmation, we carried out a western blot experiment to check the expression of a signaling pathway protein linked to the above cytokine secretion. Moreover, as per our in silico study [26], other signaling pathways were also investigated by the western blot experiment, but did not show any significant results, except AKT and NF-kB. NF-kB is upregulated, while AKT is downregulated, in CART cell coculture samples, compared with UT cell coculture samples. Thus, it is concluded that the cytotoxic activity of this Tan-CAR T cell is primarily mediated by the AKT and NF-kB signaling pathways.

4. Discussion

Over the last five years, the FDA has approved CART cell therapy as a mainstay treatment modality for liquid cancer treatment. The latest CAR T-cell therapies, namely ABECMA, which targets BCMA-expressing multiple myeloma cells, and Carvykti (ciltacabtagene autoleucel), were approved by the FDA on February 28, 2022, for the treatment of adult patients suffering from multiple myeloma who have relapsed or presented refractory disease after receiving at least four previous treatments such as immunomodulating agents, proteasome inhibitors, and monoclonal antibodies against CD38 [27]. CART cell therapy is expected to receive FDA approval for treating chronic lymphocytic leukemia. Solid tumors and autoimmune diseases are being tested with CART cell therapy [28,29]. Hematologic cancers are associated with poor clinical outcomes and high mortality rates, which have proven difficult to improve in current clinical trials of CART cell therapy. Besides CD19, we have previously witnessed many therapies targeting single antigens, such as CD19, CD20, CD22, BCMA40, PSMA42, and mesothelin for the treatment of B-cell lymphoma [30–32]. Despite the effectiveness of that single-antigen targeting CAR T-cell therapy, clonal cancer cells may have other positive targets for which the therapy may relapse. Thus, to overcome such a clinical impediment, two or three tumor antigens have been taken into consideration to develop bispecific and trispecific CART cell therapy. It was found that dual antigen pairs like CD19/CD20, CD19/CD22, CD20/CD22, and CD19/CD20/CD22 were more effective than single targets, but they were only effective against B-cell lymphoma [33–36].

One of the biggest challenges in expanding CAR T-cell therapy is the limited effectiveness and tumor microenvironment suppression. The CRISPR/Cas9 system has been developed as a strategy to overcome the limitations of CART cells [37]. Allogeneic CART cells can be created by disrupting the TRAC and B2M loci, and the expansion and persistence of CART cells can be improved by modifying inhibitory or apoptotic receptors [38]. Other target sites have been investigated for CRISPR/Cas9 editing tools, such as GM-CSF, FasR, HPSC, LAG-3, iPSC, PD-1, SHP-1, TCR, and TGF-βR2. In order to produce autologous products using CRISPR, there is a high level of complexity, expense, and labor involved in the process [39]. There are also a number of toxicities, including CRS, neuro-inflammation, and on-target effects on tumor cells.

A new generation of engineered cellular products is emerging with the rapid development of CRISPR gene editing and delivery technologies. As a result of innovations in sgRNA homology arms and the development of newer generations of CRISPR, gene editing has become more precise and efficient. By doing so, CART cells have been modified in a secondary way in order to produce more efficient inhibitor-resistant off-the-shelf or non-viral products [40]. Despite our experimental outcome that TCR-disrupted CAR T cells do not show very significant improvements in cell lysis, there is a noticeable increase in the percentage of cell lysis in the TCR-disrupted CAR T cells compared to the normal CAR T cells. As we move forward with this innovative technology in the clinic, it will be extremely important to monitor unwanted genomic editing effects or chromosomal breakage as a result of CRISPR gene editing [41]. CRISPR-modified products should be tested both in the final product and in successive doses following treatment in patients. Conventional cytogenetics and whole-genome sequencing are two strategies that can be used. Innovations in the CRISPR field are expected to lead to a rise in CRISPR-modified CART cell applications in the coming decade [42].

Even though CART cell immunotherapy has been extensively studied in B-cell lymphoma, the world of hematologic malignancies remains in need of a reliable universal cure. Thus, our goal was to find dual targets expressed in both T-cell and B-cell malignancies that could also be effective against leukemia and lymphoma. So far, CD30 has not been studied extensively, and no bispecific target studies have been presented that take CD30 and any other target antigens together. This is the first study to target both CD30 and CD20 simultaneously. Hence, the current study demonstrated the therapeutic efficacy of targeting two major tumor antigens on a single type of cancer. To support our hypothesis, one piece of evidence was found in a recent clinical investigation on clinical Hodgkin lymphoma. In that study, out of 15 patients, 13 were found to be positive for both CD20 and CD30 antigens [43]. Targeting both CD20 and CD30 antigens in the treatment of leukemia and lymphoma presents an advantage, as both are expressed exclusively on the lymphocyte lineage, which is a major reason why they are promising as universal therapies for hematologic malignancies. Dual tumor antigen targeting prevents antigen loss relapse after CAR T-cell immunotherapy. In some recently published clinical studies, NCT02690545 and NCT0917083, CART cell expansion in the body led to high rates of durable responses with safety profiles among patients with relapsed and refractory Hodgkin lymphoma treated with CD30 CART cells [12,44,45]. Moreover, there has been evidence that anti-CD30 CAR T-cell therapy from the second and third generations mediates anti-tumor activity in phase I/II studies in HL and ALCL patients who are refractory or relapsed [46,47].

The cytotoxicity activity of CD30-20 CAR-engineered CD8 T cells was assessed using a neutral red uptake assay by coculturing with some lymphoma and leukemia cell lines as tumor targets. First, we verified the existence of CD20 and CD30 markers on both target cell lines. We found both T and B cells, and then we checked for the coculture assay. Each CAR T cell contains a scFv, a spacer domain, a transmembrane domain, and a cytoplasmic domain, all of which contribute to its maximal antitumor activity [48]. While first- to third-generation of CARs are categorized by the number of their intracellular signaling domains, most clinical applications focus on second-generation CARs utilising either CD28 or 4-BB, as well as third-generation CARs that combines both CD28 and 4-1BB [49]. The present study demonstrates that 4-1BB- and CD28-containing CARs exhibit superior antitumor activity and tumor homing in comparison to second-generation CD28-containing CARs by recruiting an antitumor signaling cascade. The CAR receptor recruits some downstream signaling molecules upon binding to target cells [50]. As per our in silico study, a few major signaling pathways are

identified as interlinked. The findings indicate that cells with both antigens had higher levels of cytotoxicity and better anti-tumor activity than cells with less of each antigen. In comparison to CARs with a single costimulatory domain, CARs targeting PSMA and mesothelin consistently demonstrated superior tumor eradication and increased persistence in solid tumor models.

We developed the first CART cell with a dual target for lymphocyte carcinoma (CD30-CD20) and discovered it to be effective against four different cancer cell types in vitro, but additional preclinical research is needed to assess the therapeutic gene's efficacy in terms of how it responds to severe disease burden and long-lasting persistence in the body (Figure 7).

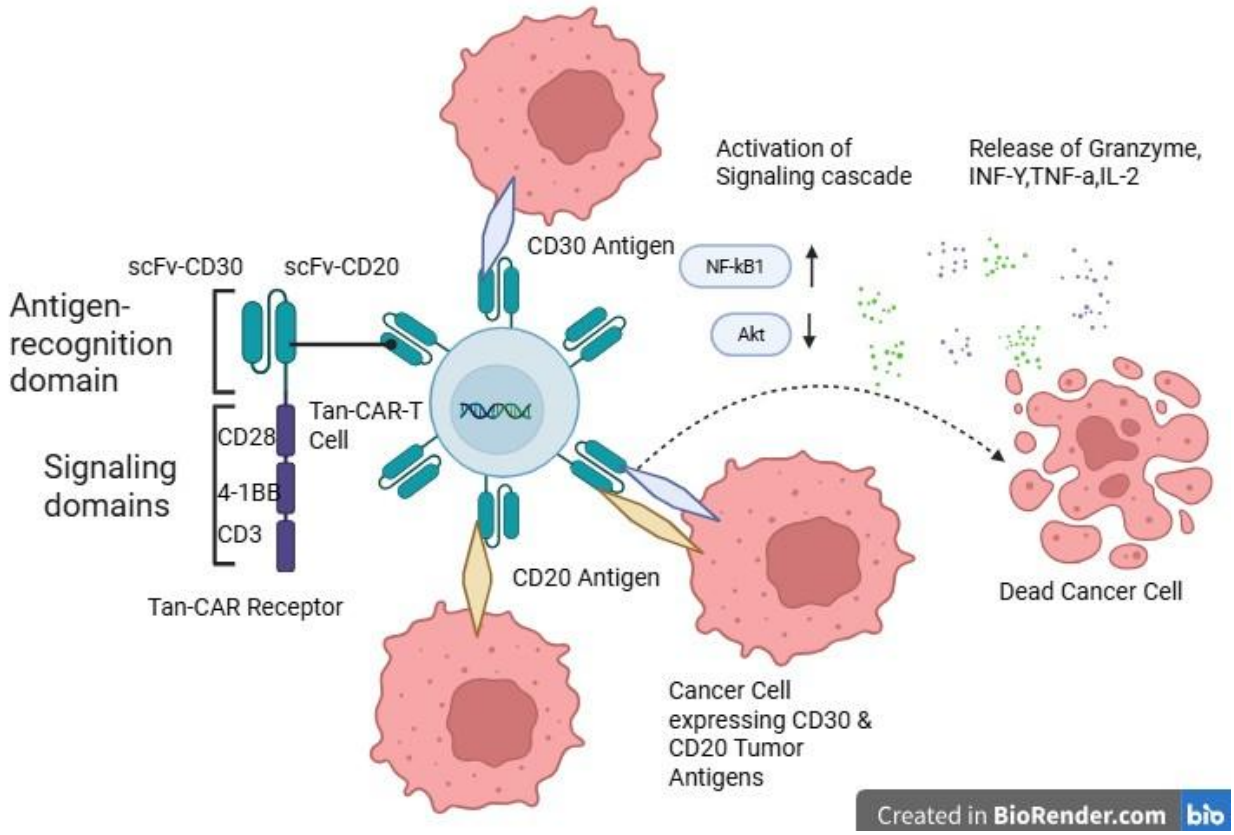

Figure 7. Action of Tan-CAR T cell on targeted cancer cells. Tan-CAR T cells are activated when they recognize and bind to target cancer cells expressing tumor antigens (such as CD30 and CD20) on their surface. Upon binding, the T cell forms an immune synapse with the target cancer cell, prompting the internal signaling domain of the Tan-CAR T cell to initiate a signaling cascade. This triggers the release of cytokines and cytolytic proteins to destroy the cancer cells. The extent of Tan-CAR T-cell activation and subsequent action is determined by the density of target tumor antigens present on the cancer cell surface. Image created by BioRender.

5. Conclusion

Despite CART cells having been investigated against both antigens individually, CARs against CD20 and CD30 have not been compared side by side to assess their therapeutic effectiveness

against different types of hematologic cancers. For more convincing proof of its efficacy, additional in vivo research needs to be conducted. It is important to note that, apart from this study, the analysis of tumor antigens, which are uniformly present in liquid cancers, can pave a promising path for this therapy. To address the variability of tumor antigens and the complex tumor microenvironment, CAR T-cell design should be improved.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Author contributions

RM—Conceived the project idea, Grant Writing, Funding Acquisition, Conceptualization, Experimental Design, Investigation and Data Curation, Writing- Original draft.

NG—Methodology Preparation, Project Administration, Validation, Data Interpretation, Review and Editing and Approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgement

I would like to express my gratitude to the funding agency DST, New Delhi, INDIA for approving me DST Women Scientist Grant and also KIIT University for providing me the scientific facilities and infrastructure to carry out the research work. I would also like to give special thanks to my colleague Dr. Deepak ku Ojha for drawing blood samples from healthy donors and all the generous persons had came forward to donate their blood samples required for this study.

Ethics approval status

This study has been approved by the institutional ethical committee of KIIT School of Biotechnology (KSBT/IEC/2019/MEET-1/A7). A written consent was obtained from all participants who voluntarily involve for this study and a small compensation was provided to all participants for their contribution.

References

1. Masopust D, Schenkel JM (2013) The integration of T cell migration, differentiation and function. *Nat Rev Immunol* 13: 309–320. https://doi.org/10.1038/nri3442
2. Eshhar Z, Waks T, Gross G (2014) The emergence of T-bodies/CAR T cells. *Cancer J* 20: 123–126. https://doi.org/10.1097/PPO.0000000000000027

3. Eshhar Z, Waks T, Bendavid A, et al. (2001) Functional expression of chimeric receptor genes in human T cells. *J Immunol Methods* 248: 67–76. https://doi.org/10.1016/S0022-1759(00)00343-4
4. Posa MK, Singh J, Parveen S, et al. (2026) CAR T- cell therapy: A promising novel approach for treatment of cancer. *Cancer Treat Res Commun* 47: 101125. https://doi.org/10.1016/j.ctarc.2026.101125
5. June CH, O'Connor RS, Kawalekar OU, et al. (2018) CAR T cell immunotherapy for human cancer. *Science* 359: 1361–1365. https://doi.org/10.1126/science.aar6711
6. McGuirk J, Waller EK, Qayed M, et al. (2017) Building blocks for institutional preparation of CTL019 delivery. *Cytotherapy* 19: 1015–1024. https://doi.org/10.1016/j.jcyt.2017.06.001
7. Tumaini B, Lee DW, Lin T, et al. (2013) Simplified process for the production of anti–CD19-CAR–engineered T cells. *Cytotherapy* 15: 14061415. https://doi.org/10.1016/j.jcyt.2013.06.003
8. Qi Y, Zhao MF, Hu YX, et al. (2022) Efficacy and safety of CD19-specific CAR T cell–based therapy in B-cell acute lymphoblastic leukemia patients with CNSL. *Blood* 139: 3376–3386. https://doi.org/10.1182/blood.2021013733
9. Maude SL, Laetsch TW, Buechner J, et al. (2018) Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med* 378: 439–448. https://doi.org/10.1056/NEJMoa1709866
10. Zhao J, Song Y, Liu D (2019) Clinical trials of dual-target CAR T cells, donor-derived CAR T cells, and universal CAR T cells for acute lymphoid leukemia. *J Hematol Oncol* 12: 1–11. https://doi.org/10.1186/s13045-019-0705-x
11. Grada Z, Hegde M, Byrd T, et al. (2013) TanCAR: A novel bispecific chimeric antigen receptor for cancer immunotherapy. *Mol Ther Nucleic Acids* 2: e105. https://doi.org/10.1038/mtna.2013.32
12. Ramos CA, Grover NS, Beaven AW, et al. (2020) Anti-CD30 CAR-T cell therapy in relapsed and refractory Hodgkin lymphoma. *J Clin Oncol* 38: 3794. https://doi.org/10.1200/JCO.20.01342
13. Ramos CA, Ballard B, Zhang H, et al. (2017) Clinical and immunological responses after CD30-specific chimeric antigen receptor–redirected lymphocytes. *J Clin Invest* 127: 3462–3471. https://doi.org/10.1172/JCI94306
14. Bhatt G, Maddocks K, Christian B (2016) CD30 and CD30-targeted therapies in Hodgkin lymphoma and other B cell lymphomas. *Curr Hematol Malig Rep* 11: 480–491. https://doi.org/10.1007/s11899-016-0345-y
15. Mark E, Sutton M, Gru A (2022) Primary cutaneous anaplastic large-cell lymphoma with aberrant CD20 expression: Case report and review of the literature. *Am Jo Dermatopathol* 44: 971–978. https://doi.org/10.1097/DAD.0000000000002281
16. Huang Y, Chen S, Wei R, et al. (2020) CD20-positive extranodal NK/T cell lymphoma: clinicopathologic and prognostic features. *Virchows Arch* 477: 873–883. https://doi.org/10.1007/s00428-020-02776-x
17. Imai M, Jiang JG, Wu Z, et al. (2013) CD20-positive T-cell lymphoma involving bone marrow: Report of four cases. *J Hematopathol* 6: 201–205. https://doi.org/10.1007/s12308-013-0177-9
18. Karube K, Kakimoto Y, Tonozuka Y, et al. (2021) The expression of CD30 and its clinico-pathologic significance in peripheral T-cell lymphomas. *Expert Rev Hematol* 14: 777–787. https://doi.org/10.1080/17474086.2021.1955344

19. Onaindia A, Martínez N, Montes-Moreno S, et al. (2016) CD30 expression by B and T cells. *Am J Sur Pathol* 40: 378–385. https://doi.org/10.1097/PAS.0000000000000571
20. Klimka A, Barth S, Matthey B, et al. (1999) An anti-CD30 single-chain Fv selected by phage display and fused to *Pseudomonas* exotoxin A (Ki-4 (scFv)-ETA') is a potent immunotoxin against a Hodgkin-derived cell line. *Br J Cancer* 80: 1214–1222. https://doi.org/10.1038/sj.bjc.6690488
21. Guercio M, Orlando D, Di Cecca S, et al. (2021) CD28.OX40 co-stimulatory combination is associated with long *in vivo* persistence and high activity of CAR. CD30 T cells. *Haematologica* 106: 987. https://doi.org/10.3324/haematol.2019.231183
22. Shah NN, Johnson BD, Schneide D, et al. (2020) Bispecific anti-CD20, anti-CD19 CAR T cells for relapsed B cell malignancies: A phase 1 dose escalation and expansion trial. *Nat Med* 26: 1569–1575. https://doi.org/10.1038/s41591-020-1081-3
23. Wu AM, Tan GJ, Sherman MA, et al. (2001) Multimerization of a chimeric anti-CD20 single-chain Fv-Fc fusion protein is mediated through variable domain exchange. *Protein Eng* 14: 1025–1033. https://doi.org/10.1093/protein/14.12.1025
24. Guo Z, Tu S, Yu S, et al. (2021) Preclinical and clinical advances in dual-target chimeric antigen receptor therapy for hematological malignancies. *Cancer Sci* 112: 1357–1368. https://doi.org/10.1111/cas.14799
25. Zhang WY, Wang W, Guo YL, et al. (2016) Treatment of CD20-directed chimeric antigen receptor-modified T cells in patients with relapsed or refractory B-cell non-Hodgkin lymphoma: An early phase IIa trial report. *Signal Transduction Targeted Ther* 1: 1–9. https://doi.org/10.1038/sigtrans.2016.2
26. Mohanty R, Manoswini M, Dhal AK, et al. (2022) *In silico* analysis of a novel protein in CAR T-cell therapy for the treatment of hematologic cancer through molecular modelling, docking, and dynamics approach. *Comput Biol Med* 151: 106285. https://doi.org/10.1016/j.compbiomed.2022.106285
27. Jagannath S, Joseph N, Crivera C, et al. (2023) Component costs of CAR-T therapy in addition to treatment acquisition costs in patients with multiple myeloma. *Oncol Ther* 11: 263–275. https://doi.org/10.1007/s40487-023-00228-5
28. Oh S, Mao X, Manfredo-Vieira S, et al. (2023) Precision targeting of autoantigen-specific B cells in muscle-specific tyrosine kinase myasthenia gravis with chimeric autoantibody receptor T cells. *Nat Biotechnol* 41: 1229–1238. https://doi.org/10.1038/s41587-022-01637-z
29. Shen CJ, Yang YX, Han EQ, et al. (2013) Chimeric antigen receptor containing ICOS signaling domain mediates specific and efficient antitumor effect of T cells against EGFRvIII expressing glioma. *J Hematol Oncol* 6: 1–7. https://doi.org/10.1186/1756-8722-6-33
30. Gagelmann N, Ayuk F, Atanackovic D, et al. (2020) B cell maturation antigen-specific chimeric antigen receptor T cells for relapsed or refractory multiple myeloma: A meta-analysis. *Eur J Haematol* 104: 318–327.https://doi.org/10.1111/ejh.13380
31. Pan J, Niu Q, Deng B, et al. (2019) CD22 CAR T-cell therapy in refractory or relapsed B acute lymphoblastic leukemia. *Leukemia* 33: 2854–2866. https://doi.org/10.1038/s41375-019-0488-7
32. O'Hara M, Stashwick C, Haas AR, et al. (2016) Mesothelin as a target for chimeric antigen receptor-modified T cells as anticancer therapy. *Immunotherapy* 8: 449–460. https://doi.org/10.2217/imt.16.4

33. Lam N, Finney R, Yang S, et al. (2021) Development of a Bicistronic anti-CD19/CD20 CAR construct including optimization to abrogate retroviral recombination events. *Blood* 138: 4808. https://doi.org/10.1016/j.omto.2023.07.001
34. Wu J, Meng F, Cao Y, et al. (2021) Sequential CD19/22 CAR T-cell immunotherapy following autologous stem cell transplantation for central nervous system lymphoma. *Blood Cancer J* 11: 131. https://doi.org/10.1038/s41408-021-00523-2
35. Schneider D, Xiong Y, Wu D, et al. (2021) Trispecific CD19-CD20-CD22–targeting duoCAR-T cells eliminate antigen-heterogeneous B cell tumors in preclinical models. *Sci Transl Med* 13: eabc6401. https://doi.org/10.1126/scitranslmed.abc6401
36. Xue Y, Lai X, Li R, et al. (2021) CD19 and CD30 CAR T-cell immunotherapy for high-risk classical Hodgkin's lymphoma. *Front Oncol* 10: 607362. https://doi.org/10.3389/fonc.2020.607362
37. Uddin F, Rudin CM, Sen T (2020) CRISPR gene therapy: Applications, limitations, and implications for the future. *Front Oncol* 10: 1387. https://doi.org/10.3389/fonc.2020.01387
38. Eyquem J, Mansilla-Soto J, Giavridis T, et al. (2017) Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* 543: 113–117. https://doi.org/10.1038/nature21405
39. Sadeqi Nezhad M, Yazdanifar M, Abdollahpour-Alitappeh M, et al. (2021) Strengthening the CAR-T cell therapeutic application using CRISPR/Cas9 technology. *Biotechnol Bioeng* 118: 3691–3705. https://doi.org/10.1002/bit.27882
40. Manriquez-Roman C, Siegler EL, Kenderian SS (2021) CRISPR takes the front seat in CART-cell development. *BioDrugs* 35: 113–124. https://doi.org/10.1007/s40259-021-00473-y
41. Ren J, Zhao Y (2017) Advancing chimeric antigen receptor T cell therapy with CRISPR/Cas9. *Protein cell* 8: 634–643. https://doi.org/10.1007/s13238-017-0410-x
42. Xia AL, He QF, Wang JC, et al. (2019) Applications and advances of CRISPR-Cas9 in cancer immunotherapy. *J Med Genet* 56: 4–9. https://doi.org/10.1136/jmedgenet-2018-105422
43. Hartmann S, Goncharova O, Portyanko A, et al. (2019) CD30 expression in neoplastic T cells of follicular T cell lymphoma is a helpful diagnostic tool in the differential diagnosis of Hodgkin lymphoma. *Modern Pathology* 32: 37–47. https://doi.org/10.1038/s41379-018-0108-5
44. Greenbaum U, Mahadeo KM, Kebriaei P, et al. (2020) Chimeric antigen receptor T-cells in B-acute lymphoblastic leukemia: State of the art and future directions. *Front Oncol* 10. https://doi.org/10.3389/fonc.2020.01594
45. Zhang S, Gu C, Huang L, et al. (2022) The third-generation anti-CD30 CAR T-cells specifically homing to the tumor and mediating powerful antitumor activity. *Sci Rep* 12: 10488. https://doi.org/10.1038/s41598-022-14523-0
46. Iżykowska K, Rassek K, Korsak D, et al. (2020) Novel targeted therapies of T cell lymphomas. *J Hematol Oncol* 13: 1–38. https://doi.org/10.1186/s13045-020-01006-w
47. Rafiq S, Hackett CS, Brentjens RJ (2020) Engineering strategies to overcome the current roadblocks in CAR T cell therapy. *Nat Rev Clin Oncol* 17: 147–167. https://doi.org/10.1038/s41571-019-0297-y
48. Cheng Z, Wei R, Ma Q, et al. (2018) *In vivo* expansion and antitumor activity of coinfused CD28-and 4-1BB-engineered CAR-T cells in patients with B cell leukemia. *Mol Ther* 26: 976–985. https://doi.org/10.1016/j.ymthe.2018.01.022

49. Weinkove R, George P, Dasyam N, et al. (2019) Selecting costimulatory domains for chimeric antigen receptors: Functional and clinical considerations. *Clin Transl Immunol* 8: e1049. https://doi.org/10.1002/cti2.1049
50. Shirasu N, Kuroki M (2012) Functional design of chimeric T-cell antigen receptors for adoptive immunotherapy of cancer: Architecture and outcomes. *Anticancer Res* 32: 2377–2383.

AIMS Press