Allogeneic Polyclonal CD38KO/CD38-CAR γδT Cells For The Treatment Of T Cell Malignancies

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Abstract

Relapsed and refractory T cell malignancies are associated with poor clinical outcomes. Although autologous CAR-αβT cells have been employed in the treatment of several cancers, generating CAR-T cells for T cell malignancies remains challenging. The need to obtain enough healthy αβT cells from patients to generate CAR-αβT cells, combined with the problem of shared antigens such as the pan-cancer target CD38 expressed on both malignant and normal T cells, which can lead to fratricide of healthy CAR-αβT has hindered the application of CAR-T therapy for T-ALL. The use of allogeneic αβT and NK cells has also been explored for treating T-ALL. However deletion of the TCRαβ/CD3 complex to avoid graft-versus-host disease (GvHD) for αβT and multiple infusions of NK cells, can increase the cost of manufacturing and logistics.

In this study, we genetically engineered polyclonal gamma delta T (γδT) cells as an alternative allogeneic cell source for cancer immunotherapy. γδT share innate immune properties of NK cells and adaptive immunity of αβT cells. Generating CAR γδT would allow us to minimize the cost of generating allogenic *TRAC*-KO CAR αβT cells and lower the number of infusions needed when NK cells are used. Utilizing a novel expansion protocol in combination with CRISPR/AAV gene editing, we developed CD38 knockout (CD38KO)/CD38-CAR polyclonal γδT cells that target T-ALL. Our editing strategy enabled site-directed, on-target insertion of the CD38-CAR transgene into the CD38 locus, with no evidence of significant random CAR DNA integration (as commonly seen with lentiviral CAR transduction) or chromatin abnormalities resulting from CRISPR editing. This approach effectively mitigated fratricide through simultaneous CD38 disruption and CAR expression. We evaluated the edited γδT cells in vitro across multiple patient-derived T-ALL samples and demonstrated the efficacy of the CD38KO/CD38-CAR γδT cells against both baseline and relapsed samples. In vivo, a single injection of CD38KO/CD38-CAR γδT cells without cytokine support resulted in potent anti-leukemic efficacy

Fratricide-resistant CD38KO/CD38-CAR polyclonal γδT cells thus represent a promising off-the-shelf therapeutic platform. They may serve as a bridge to allogeneic hematopoietic stem cell transplantation (allo-HSCT), potentially enabling molecular remission in T cell malignancies and other CD38-expressing cancers such as AML.

Introduction

Treating patients with relapsed and refractory T-cell acute lymphoblastic leukemia (T-ALL) remains a significant clinical challenge. While conventional therapies including allogeneic hematopoietic stem cell transplantation (HSCT) are widely used, long-term outcomes remain poor, with 5-year survival rates of approximately 25% in children and 50% in adults[1,2,3-5].

Novel treatment strategies such as chimeric antigen receptor (CAR) T cells have shown remarkable success in malignancies expressing surface antigens like CD19, CD20, and BCMA[6]. FDA-approved CAR-T cell therapies typically utilize autologous αβT cells from the patient. However, this approach is not optimal for T cell malignancies due to the high burden of malignant T cells within the patient's own healthy T cell compartment [3,4]. Additionally, shared surface antigens such as CD70, CD7 and CD38 between malignant and healthy T cells lead to fratricide during CAR-T cell manufacturing, thereby compromising yield and functionality [1,3,7].

While the use of allogeneic donor-derived αβT cells is being explored including CD38CAR αβT cells[8], it necessitates genetic disruption of the TCRαβ/CD3 complex to prevent graft-versus-host disease (GvHD), adding complexity and cost to CAR-T production [9,10]. Alternatively, the use of CD38CAR NK cells, which have been shown by us and others to be effective against CD38-expressing malignancies, requires multiple infusions [11,12].

γδT cells have garnered increasing interest in cancer immunotherapy owing to studies demonstrating their prognostic value in various cancers [13,14]. These cells act as a bridge between innate and adaptive immunity and can recognize transformed cells in a major histocompatibility complex (MHC)-independent manner. However, their intrinsic antitumor activity has shown limited clinical efficacy when used as an adoptive cell therapy [13,15-17]. Engineering γδT cells with CARs has been proposed to enhance their antitumor potential [13,18,19]. Lentiviral-based CAR transduction of γδT cells has proven technically challenging and is typically restricted to specific subtypes such as Vδ1 or Vδ2 due to their preferential expansion, despite evidence that multiple γδT cell subpopulations play crucial roles in immune surveillance across different tissues [18,20,21].

To address this, we developed a method enabling robust expansion of diverse γδT cell subsets, including Vδ1+, Vδ2+, and Vδ1−/Vδ2− populations. In this method we used a hybrid

system starting with a short-term antibody-based pan γδTCR stimulation followed by expansion of these stimulated cells using mbIL21 and 41BBL expressing feeder cells (referred as FC) allowing the expansion of polyclonal γδT cells. Building upon our previously reported isatuximab-based CD38-targeting CAR, which was successfully implemented in αβT and natural killer (NK cells) using a simultaneous CD38 knockout and CAR knock-in strategy to avoid fratricide, we now apply a similar genome editing approach to γδT cells [11]. This would allow us to minimize the cost of generating *TRAC*^{KO} CAR αβT cells to avoid GvHD and lower the number of infusions needed when NK cells are used due to the lower ability of NK cells to expand in vivo compared to αβT cells. To enable site-specific insertion of the CD38-CAR construct, we utilized CRISPR/AAV-mediated knock-in at the endogenous CD38 locus in expanded polyclonal γδT cells. Unlike lentiviral vectors which integrate randomly and have been associated with variable expression and clinical efficacy [22], our targeted editing approach resulted in a high rate of precise on-target insertion and no significant chromatin abnormalities compared to non-edited γδT cells. This strategy also disrupts native CD38 expression, minimizing fratricide and enhancing therapeutic potential. Functionally, CD38 KO/CD38-CAR γδT cells demonstrated the capacity to eradicate CD38-expressing T-ALL cells and may facilitate a programmed allogeneic HSCT approach to induce molecular remission in relapsed/refractory T-ALL and other CD38+ malignancies.

Results

Expansion of γδT Cells using hybrid pan-γδTCR stimulation and coculture with mbIL21/41BBL feeder cells preserves the polyclonal nature of the γδT cell and improves cell yield

Expansion of polyclonal γδT cells using mbIL21/41BBL feeder cells (FC) has been described before [20]. However, the expansion rate remained modest. Therefore, we hypothesized that using a hybrid expansion system initiating with a short-term pan-γδTCR antibody stimulation followed by FC co-culture can induce γδT cells expansion (Figure 1A). Our results demonstrated that the hybrid system significantly improved expansion (~10-fold) in two weeks in compared to FC-only expansion system (Figure 1B). We next evaluated the purity of isolated and expanded γδT cells. Flow cytometry analysis from three healthy donors confirmed high purity of γδT post-expansion. We saw an increase in CD3^{pos} cells and a

decrease in $CD3^{neg}$ in both expansion systems. There was a marked reduction in $CD3^{pos}$αβ T cells (from 64.4% to 0.3%) and an in increased in the frequency of γδT cells detected as $CD3^{pos}$αβ T^{neg} cell) in three donors from 3% in PBMCs to 82% in FC-expanded cells, and a reduction in $CD3^{-}CD56^{+}$ NK cells (from 19% to 1.8%) (Figure 1C). Both expansion methods maintained subtype diversity over three weeks (Figure 1D and 1E). These results confirmed that regardless of prior pan-γδTCR antibody activation, FCs supported the expansion of polyclonal γδ T cells. Mass cytometry (CyTOF) further confirmed product purity and preserved polyclonality in expanded γδT cells. Importantly, no $CD3^{+}$αβ T cells were detected, supporting potential clinical-grade manufacturing devoid of GvHD-inducing contaminants (Figure 1F).

Generation of CRISPR/AAV-edited CD38KO/CD38CAR γδT cells

To generate CD38KO/CD38CAR γδT cells, we next electroporated γδT cells expanded either with the FC-only system or the hybrid system using a Cas9/RNP complex targeting CD38 with the gRNA we reported before[11,23-26]. Electroporation resulted in a substantial reduction in CD38 expression: from 99% in WT to 9% in CD38KO in FC-only expanded γδT cells, and from 71% in WT to 14% in CD38KO in hybrid-expanded γδT cells. Notably, we observed a significant decrease in CD38 expression in WT γδT cells expanded with the hybrid system compared to those expanded with the FC-only system (Figure 2A). Following electroporation, γδT cells were transduced using two different multiplicities of infection (MOIs), 75K and 150K of AAV6 to generate CD38KO/CD38CAR γδT cells. We used our previously reported isatuximab-based CD38CARv3 construct[11]. CD38CAR-T cells were generated at two different scales: a small scale (3×10^6 cells) and a clinically relevant large scale (15×10^6 cells). On average, CAR expression reached 38% with the hybrid system and 22% with the FC-only system, measured 20 days after CAR generation. Although not statically significant, CAR expression was higher in γδT cells expanded in hybrid system. There were no significant differences in CAR expression between the two scales, highlighting the clinical feasibility of this protocol (Figure 2B-D). Furthermore, CAR expression remained stable between the first and second expansion rounds addressing a known limitation of other γδT cell CAR generation methods such as lentiviral transduction[27] .

Cytogenetic analysis using the Kromatid Kromasure assays showed successful site directed insertion of CAR and no significant CRISPR/AAV mediated chromosol abnormalities

We have previously shown the low off-target effect of the CD38 targeting gRNA used in this study [26]. Here, we further studied the off-target insertion and chromosomal abnormalities post CRISPR/AAV gene editing. Across all three assays, K-Band, SCREEN, and InSite, both the edited CD38KO/CD38CAR γδT and WT γδT-cell populations displayed overall diploid karyotypes with low levels of structural variation. K-Band analysis detected no recurrent abnormalities involving the CD38 target chromosome (Chr 4) in either sample. Aneuploidy was significantly lower in the edited sample compared to wildtype (5% vs. 17%, p= 0.011), driven entirely by fewer whole-chromosome losses; other structural variants occurred at low frequencies in both samples with no statistically significant differences.

SCREEN results were consistent with these findings, showing no significant differences in event rates for Chr 4, including no enrichment of inversions, SCEs or translocations at the target locus. Both samples exhibited a recurrent small inversion on Chr 8p in 100% of cells, homozygous in most, which is likely a germline variant in the donor. Inversions and SCEs were the most frequent event types, with the edited sample showing slightly fewer total events (301 vs. 317 in wildtype), slightly lower aneuploidy (8% vs. 10%), a single insertion not present in the wildtype, modestly more translocations (3 vs. 2 cells), and more complex events (4 vs. 1 cell), (Figure 2E).

InSite analysis confirmed on-target integration of the CAR transgene in 110 of 200 γδT cells, with 29 showing biallelic integration, and no structural rearrangements, translocations, truncations, or complex events involving the CD38 locus in either sample. Off-target insertions were observed in 44 CD38KO/CD38CAR γδT cells, appeared randomly distributed, were non-clonal, and did not exceed two integrations per cell, consistent with expectations for a safe drug product profile, particularly when contrasted with lentiviral approaches, where random integration is inherent (Figure 2F and 2G).

Together, these results indicate that the editing process did not introduce detectable clonal structural variation at the target locus and that the overall genomic integrity of the edited cells was comparable to the wildtype control.

Effect of gene engineering on γδT cells metabolism

We next studied the effect of gene editing and the deletion of CD38 as an NADase enzyme on the metabolism of γδT cells. We measured the expression of nutrient receptors and the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of CD38KO γδT cells and CD38KO/CD38CAR γδT cells compared to WT γδT cells. We observed no difference in the percent surface expression of Glut1 (glucose transporter 1) and CD71 (iron transporter), or MFI of CD98 (amino acid transporter) on γδT cells lacking CD38 expression compared to WT controls. Further, both CD38KO and CD38KO/CD38 CAR γδT cells had similar basal glycolysis, as well as glycolytic capacity and reserve as WT γδT cells as measured through their ECAR. When assessing oxidative phosphorylation (OxPhos) through OCR, we observed that CD38KO γδT cells had similar basal and maximal OxPhos compared to WT γδT cells. Lastly, the spare respiratory capacity and mitochondrial linked-ATP production of CD38KO γδT cells was comparable to WT γδT cells (Supplementary Figure 1). Overall, these results demonstrate that gene editing does not affect the metabolic capacity of γδT cells. Although we had previously shown that CD38KO can enhance metabolism of NK and αβT cells[11,26] deletion of CD38 did not improve the glycolytic or oxidative metabolism of γδT cells.

CD38KO/CD38CAR γδT Cells Exhibit Potent In Vitro Antitumor Activity Against Jurkat Cell Line

To evaluate the antitumor activity of CD38KO/CD38CAR γδT cells against T-ALL, we performed a long-term CyTOF-based cytotoxicity assay by co-culturing Jurkat cells (αβTCR$^+$ T-ALL) with either WT or CAR γδT cells for 24 and 48 hours. CyTOF analysis revealed a significant reduction in viability of the αβTCR$^+$ Jurkat cell indicated by pRB$^+$ expression within 48 hours, in the CAR γδT-treated group (Figure 3A and 3C). Among the few surviving Jurkat cells, the lowest CD38 expression was observed in the CAR γδT-treated group at 48 hours post co-culture (Figure 3B and 3D). These findings demonstrate both the potency and specificity of CD38KO/CD38CAR γδT cells.

CD38KO/CD38CAR γδT cells show potent in vitro antitumor activity against patient-derived baseline and relapsed T-ALL cells

To assess the clinical relevance of CD38KO/CD38CAR γδT cells, we evaluated their antitumor activity against primary T-ALL cells obtained from patients at both baseline and relapse stages using a 3-hour Calcein-based cytotoxicity assay. Despite the technical challenges of culturing thawed primary samples, we successfully performed the assay on three baseline and two relapsed patient samples using WT or CD38KO/CD38CAR γδT cells. Across all samples, CD38KO/CD38CAR γδT cells consistently exhibited superior anti–T-ALL activity compared with WT γδT cells across multiple effector-to-target (E:T) ratios (Figure 3E). The data demonstrate the feasibility of using off-the-shelf polyclonal CD38KO/CD38CAR γδT cells as a potential treatment to reduce tumor burden and serve as a bridge to allogeneic HSCT.

CD38KO/CD38CAR γδT Cells Exhibit Potent Antitumor Activity Against T-ALL In Vivo

To assess the in vivo potency of CD38KO/CD38CAR γδT cells, we infused luciferase-expressing Jurkat T cells (Luc^+ Jurkat) into NOD/SCID/$\gamma c^{-}/^{-}$ (NSG) mice (n = 15, 10×10^6 Jurkat/mouse). Three days post-injection, tumor engraftment was confirmed via bioluminescent imaging. Mice were then treated intravenously (day 3) with one of the following: non-edited γδT cells (WT, 10×10^6 /mouse, n = 5), CAR γδT cells (n = 5, 10×10^6 /mouse, n = 5), or PBS (n = 5). Tumor progression was monitored weekly using bioluminescent imaging (Figure 4A). By day 24, mice treated with PBS or WT γδT cells reached humane endpoints due to weight loss, consistent with previously reported findings using Jurkat xenografts[3]. The weight difference between groups was statistically significant: PBS vs. CAR-T (adjusted p = 0.0024) and WT γδT vs. CAR γδT (adjusted p = 0.01) (Figure 4J). All mice were euthanized for flow cytometry analysis. Mice treated with CAR γδT cells exhibited a significant reduction in tumor bioluminescence. Dorsal-side bioluminescence further confirmed reduced tumor burden in WT γδT treated mice compared to PBS-treated mice (adjusted p = 0.02), with the most profound tumor control seen in the CAR γδT-treated group vs PBS (adjusted p <0.0001) and vs WT γδT (adjusted p = 0.0008), similar trend was seen in ventral-side bioluminescence in CAR γδT-treated group vs PBS (adjusted p <0.0001) (Figure 4B-D). These imaging findings were further confirmed by flow cytometry, which revealed a substantial clearance of Jurkat cells (live $CD3^+/\alpha\beta TCR^+$) from peripheral blood,

bone marrow, and spleen in the CAR γδT treated mice. The average frequency of live $CD3^+/\alpha\beta TCR^+$ cells in peripheral blood was 7.4% in PBS-treated mice, 9.8% in WT-treated mice, and only 0.1% in CAR-T-treated mice. Bone marrow aspirates from CAR γδT treated mice contained significantly fewer Jurkat cells compared to the PBS group (adjusted p = 0.0017). A similar trend was observed in spleen samples: PBS vs. CAR γδT (adjusted p = 0.0009), and WT γδT vs. CAR γδT (adjusted p = 0.02) (Figure 4E-4G). A significant difference was detected in the average frequency of live human $CD3^+/\alpha\beta TCR^-$ (γδT) cells between the PBS group (45%) and the CAR γδT group (91%) (adjusted p = 0.001) (Figure 4H and 4I).

Methods

γδT isolation and expansion

We conducted a comprehensive optimization to isolate and expand γδT cells for the generation of CAR γδT cells; here, we report only the most optimized protocol. Human primary γδT cells were isolated from PBMCs obtained from healthy donor buffy coats using density gradient centrifugation[11,26], followed by isolation with the EasySep™ Human γδT Cell Isolation Kit (STEMCELL Technologies, Catalog #19225). The isolated γδT cells were expanded using two systems: the FC-only system and the hybrid system.

In the FC-only system, freshly isolated γδT cells were stimulated by co-culturing with genetically engineered K562 cells expressing mbIL21 and 4-1BBL (referred to as FC) at a 1:1 ratio on days 0 and 10. On day 20, the cells were used for CAR-T production as described below.

In the hybrid system, isolated γδT cells on day 0 were stimulated with 10 μg/mL plate-bound αPan-γδ TCR antibody (Miltenyi Biotec, Catalog #130-122-291) and 2 μg/mL soluble αCD28 antibody (Tonbo Bioscience, In Vivo Ready™ Anti-Human CD28 (CD28.2), Catalog #400289U100) for 7 days in the presence of 100 IU/mL human recombinant IL-2[15]. For the first 2 days of stimulation, the cells were cultured in standard plates at a concentration of 1 × 10^6 cells/mL, then transferred to G-Rex 6-well plates for an additional 5 days (total of 7 days). The day 7–stimulated cells were further expanded by FC stimulation for 10 days. On day 17, the cells were used for CAR-T production as described below.

In all conditions, γδT cells were cultured in Human T Cell Media (Bio-Techne, R&D Systems, Catalog #CCM038-GMP-1L) supplemented with 5% CTS™ Immune Cell Serum Replacement

(Gibco™, Catalog #A2596101), antibiotics (penicillin–streptomycin), and 100 IU/mL human recombinant IL-2, which was replenished every 2–3 days. To expand the γδT cells, 2.5×10^6 γδT cells were co-cultured with 2.5×10^6 FC cells at a density of 0.5×10^6 cells/cm^2 in 6-well G-Rex culture plates (Wilson Wolf, Catalog #80660M) for 10 days per stimulation cycle in total volume of 100ml. Expanded γδT cells could be frozen and subsequently re-expanded. Freezing was performed at the end of each expansion period (day 7 or 17) using CryoStor® CS10 (STEMCELL Technologies, Catalog #100-1061).

Generation of CAR-γδT Cells Using CRISPR and AAV

We conducted a comprehensive optimization to generate CAR γδT cells; here, we report only the most optimized protocol. FC-only expanded and hybrid-expanded γδT cells at the end of the second expansion as described above were used for the generation of CD38KO/CD38 CAR γδT cells. After three washes with PBS, cells were pelleted and resuspended in 20 µl for small-scale electroporation of 3×10^6 cells or 100 µl for large-scale electroporation of 1.5×10^6 cells in Lonza P3 buffer. The Cas9/RNP complex was prepared as previously described[11]. Briefly, HiFi CRISPR-associated protein 9 (Cas9) Nuclease V3 (Integrated DNA Technologies, Coralville, IA) was combined with guide RNA targeting exon 1 of the CD38 gene (5'-CTGAACTCGCAGTTGGCCAT-3'). Cells were electroporated using program EN-138 on a Lonza 4D nucleofector. Electroporated cells were then returned to culture media and transduced within 30 minutes with 75,000 or 150,000 (MOI) of adeno-associated virus type 6 (AAV6; Andelyn Biosciences) carrying the CD38 CAR expression cassette flanked by CD38 homology arms, as previously described[11]. 75,000 MOI was the preferred method since we did not see any difference in CAR expression as described in the results section.

The electroporated and transduced cells were rested in complete media for 1 day in a 6-well plate at a concentration of 1×10^6 cells/ml. On day 2 post-transduction, the media volume was doubled. On day 3 post-transduction, the cells were transferred to a 6-well G-Rex flask seeded with 3×10^6 feeder cells for small scale or at 1:1 ratio for the large scale in 100 ml media supplemented with IL-2. During the first week, fratricide may result in significant cell death due to remaining CD38 protein on surface of γδT Cells; however, by day 10 post-transduction, a second round of stimulation at a 1:1 ratio was performed, and cells grew at normal rate. Additional expansion with FC was performed every 10 days, as described above.

Flow cytometry for studying the efficiency of gene editing, invitro and in vivo studies

To study the purity of the isolated and expanded γδT cells and for in vivo studies we used flowcytometry with following antibodies, Viability (Zombie NIR, Biolegend), APC-CD3 (Biolegend, UCHT1, 62405S), PE-αβTCR (Biolegend, mouse IgG1, clone IP26, #306708), BV421-γδTCR (BD Horizon, clone#B1, cataloge#562560), FITC-Vδ1 (Invitrogen, clone#TS8.2, caraloge#130-122-285) and PE/Cy7-Vδ2 (Biolegen, clone#B6, caraloge#331421). 7 days after transduction CD38KO and CAR expression were measured by flow cytometry in the CAR γδT cells. Cells were stained in phosphate-buffered saline containing 2% FBS using anti-CD38/PE (Miltenyi Biotec; clone REA572) and anti-whitlow (Cell Sigbaling Whitlow/218 Linker (E3U7Q) Rabbit mAb (PE Conjugate) #62405).

Sample preparation for cytogenetic analysis

Sample preparation for cytogenetic analysis with the KROMASURE™ K Band, SCREEN, and InSite assays was using γδT Cells generated from one donor. At the end of 10 days expansion with FC stimulation (culture conditions described above), 2.5×10^6 γδ T cells were plated in two T25 flask at 0.5×10^6cell/mL concentration, one flask designated for K Band and the other for SCREEN and InSite assays.

For the SCREEN and InSite assays, culture medium was replaced with a solution containing DNA analogs from the directional genomic hybridization (dGH) Cell Prep Kit (KROMATID, Cat # dGH 0001). Cells were arrested in first mitosis via a four hours Colcemid block (KROMATID, Cat # COL 001), harvested, and fixed in freshly prepared 3:1 methanol:acetic acid fixative (Fisher Scientific, Waltham, MA, USA). The K-Band samples were prepared identically, except that no DNA analogs were added. All fixed samples were then shipped to KROMATID for metaphase spread preparation.

G-banded metaphases for K-Band were generated using the standard G-banding protocol, trypsin digestion followed by Giemsa staining, which produces characteristic dark (AT-rich) and light (GC-rich) bands that allow detection of structural and numerical chromosomal abnormalities[28].

Metaphase preparations for the SCREEN and InSite assays followed the dGH protocol which includes UV- and exonuclease-mediated removal of the analog-incorporated daughter strand from metaphase chromosomes prior to probe hybridization[29].

K-Band Assay

In this study, the K-Band assay encompassed culture of cryopreserved samples, staining and imaging of prepared slides, scoring and analysis of metaphase images, and generation of Genomic Integrity reports for each sample. Unlike conventional diagnostic G-banding, which is often used to assess constitutional or acquired cytogenetic abnormalities in a clinical context, KROMATID's K-Band assay is designed for genotoxicity assessment. Each cell is evaluated for the presence of structural variants, and results are aggregated to provide karyotype-level interpretation and quantitative variant frequency that enables detection of rare or low-frequency genomic alterations that can occur in engineered cell populations. Chromosome analysis follows the International System for Human Cytogenomic Nomenclature (ISCN) guidelines, and its recommendations cover the description of numerical and structural chromosomal changes detected using microscopic and cytogenetic techniques.

Custom InSite Assay

A custom InSite assay was developed to detect targeted integration of the CD38CAR transgene and to assess structural variation at the integration site. The assay employs three custom-designed probes: one targeting a 3.5 kb unique region of the CD38CAR sequence, labeled with ATTO-643, and two probes targeting approximately 2 Mb of genomic sequence immediately flanking the CD38 edit site on Chromosome 4 (999 kb telomeric and 958 kb centromeric). The telomeric flanking probe, labeled with ATTO-550, spans Chr 4 coordinates 14,809,769–15,768,219 (GRCh38), while the centromeric flanking probe, labeled with 6-FAM, spans Chr 4 coordinates 15,863,887–16,863,163. Probes were designed using a unidirectional tiled oligonucleotide strategy restricted to unique genomic sequences. Repetitive elements were masked, and probe specificity was validated via BLAST alignment against the human reference genome. Quality control testing for the flanking chromosome 4 probes was performed on metaphase spreads from unedited control samples to confirm

accurate localization. The CD38CAR-specific probe was tested on edited samples known to contain the transgene to verify performance.

Following qualification, the InSite assay was applied to two experimental samples: S028178 (WT γδT Cells) and S028182 (CD38KO/CD38CAR γδT Cells). Metaphase spreads were prepared and imaged using the same methods as for the SCREEN assay. Spreads were pre-qualified to ensure intact chromosome morphology and successful hybridization. Imaging was performed with appropriate excitation and emission filters for each fluorophore, allowing independent resolution of each probe signal. Analysis focused on probe localization and signal configuration to determine on-target versus off-target integration and to detect structural rearrangements at the edit site. Each metaphase was assessed for chromosomal abnormalities at or near the CD38 integration locus, including translocations, truncations, inversions, sister chromatid exchanges, deletions, and insertions.

Screen Assay

For whole-genome analysis, KROMATID's SCREEN assay was hybridized to prepared metaphase spreads, counterstained with DAPI (Vectashield, Vector Laboratories, Newark, CA, USA), and imaged on an Applied Spectral Imaging Harmony system (Applied Spectral Imaging, Carlsbad, CA, USA) using a 100× objective. The assay consists of single-stranded, unidirectional tiled oligos designed to hybridize to unique sequences in each of the 24 human chromosomes. Each chromosome's oligos are end-labeled with one of five spectrally distinct fluorophores, combined such that chromosomes sharing the same color can still be differentiated by size, shape, and centromere position.

Expected KROMASURE™ SCREEN signal patterns for the diploid human reference genome were used to construct preliminary karyograms for 50 cells[30]. These karyograms enabled per-chromosome attribution of inter- and intra-chromosomal structural variation events, including inversions, translocations, aneuploidy (gain and loss), insertions, centromere abnormalities, and complex rearrangements for each sample. The assay detects translocations, insertions, and inversions at a much finer resolution than possible with K-Band (~20 kb vs. 5-10 Mb), although it does not provide band-level attribution of breakpoints.

Metabolic assays

Extracellular flux assays

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of γδT cells was assessed with the Seahorse XFe96 Extracellular Flux Analyzer. To measure ECAR, γδT cells were washed and resuspended in Seahorse XF RPMI media containing 2 mM L-Glutamine. To measure OCR, γδT cells were washed and resuspended in Seahorse XF RPMI media containing 11.1 mM glucose, 1 mM sodium pyruvate, and 2 mM L-glutamine. γδT cells were plated at 2×10^5 cells per well in at least triplicates on Poly-L-Lysine (Sigma Aldrich) coated XF96 culture microplates. ECAR was measured after sequential addition of 11.1 mM Glucose, 2 μM oligomycin, and 50 mM of 2-deoxy-D-glucose. OCR was measured after sequential addition of 2 μM oligomycin, 0.5 μM carbonyl cyanide p-trifluoro methoxyphenyl hydrazone (FCCP), and 0.5 μM rotenone/antimycin A.

Nutrient receptor expression

Extracellular expression of nutrient receptors was assessed with the following antibodies: Glut1 FITC (R&D Systems, clone 202915), CD71 BV711 (BD Biosciences, clone M-A712), and CD98 PE (BD Biosciences, clone UM7F8).

Mass cytometry assay for purity and lysis assay

To study the purity of the expanded γδT, expanded cells were stained and events were acquired on the Helios CyTOF system (Fluidigm, Toronto, Canada) as previously described[11]. For lysis assay, T-ALL (Jurkat) cells were cultured in a 6-well plate (precoated with rat tail collagen [Corning]) in serum-free media. The Jurkat cells were cultured for 24. After 24 hours, WT or CD38KO/CD38CAR γδT cells were added at a ratio of 1:1 and cocultured for 24 and 48 hours. Control wells were cultured in parallel. Cells were stained and events were acquired on the Helios CyTOF system (Fluidigm, Toronto, Canada) as previously described. In both assays, cells were identified using markers and analyzed using SPADE. We used antibodies that we previously described[11].

Cytotoxicity assay on primary samples

Three-hour calcein AM (Fisher Scientific, Hampton, NH) cytotoxicity assays were performed on primary samples as previously described[11]. In brief, frozen primary samples (baseline and

relapse) were acquired from The Ohio State University Comprehensive Cancer Center Hematology Tissue Bank. The samples were thawed and immediately stained with calcein AM at a concentration of 2μg/mL and co-cultured with effector cells at the indicated effector-to-target (E:T) ratios. Culture supernatant was collected after 3 hours and assessed by fluorimetry. Percent specific lysis was then calculated using Triton X-100 4% for the maximum control and untreated for the spontaneous control. Cytotoxicity is plotted as percent specific lysis.

in vivo studies

Jurkat cell line was transduced with luciferase expressing lentiviral vectors. 8 to 10 week old female NSG mice were obtained from Jackson laboratory and inoculated by intravenous tail vein injection with 10×10^6 Jurkat cells in 200 ul of PBS. Mice were treated 3 days later with non-edited γδT cells (WT, 10×10^6), CAR γδT cells (10×10^6), or PBS (n = 5) in 200ul of PBS by tail vein injection. Tumor engraftment and burden was monitored by bioluminescent imaging (IVIS Spectrum imaging system) following injection of D-luciferin[3]. Mice were monitored every 7 days for weight loss (20% cutt-off), decreased activity, or hindlimb paralysis and humanely euthanized when control cohorts met endpoint criteria. Blood, spleen, and bone marrow were collected for analysis by flow cytometry. Animal studies were performed in accordance the Institutional Animal Care and Use Committee (IACUC) at Nationwide Children's Hospital approved protocols (IACUC Protocol # AR24-00097).

Discussion

FDA-approved CAR-T cell therapies with enhanced specificity and efficacy for relapsed/refractory cancers rely on autologous αβT cells from patients. However, this approach presents major limitations, particularly for patients with T cell malignancies. Key challenges include: 1. the presence of malignant T cells (e.g., T-ALL), which makes isolation of healthy autologous T cells difficult for CAR-T manufacturing, 2. the risk of severe graft-versus-host disease (GvHD) associated with allogeneic αβT cells, 3. manufacturing delays, 4. the high production costs of bespoke manufacturing (>$400,000/patient), which limit accessibility and affordability, and 5. fratricide caused by shared antigens, leading to CAR-T cells attacking one another. These barriers highlight the urgent need for alternative T cell

sources. Although allogeneic CAR-T cells targeting other T-ALL antigens such as CD70, CD5, CD7, and CD2 have been developed and tested, CD38 is recognized as a pan-targeting antigen across multiple hematological malignancies, not just T-ALL[4]. Importantly, CD38 expression is consistently high in T-ALL across different disease stages and subtypes, including precursor T-ALL, thymic T-ALL, and mature T-ALL[31]. Moreover, CD38 expression is significantly higher on T-ALL cells than on normal T cells[31,32]. It has been reported that CD38 is expressed on more than 80% of T-ALL cells at MRD (88.7%) and in relapsed cases (82.9%)[31]. We previously demonstrated that an isatuximab-based CD38CAR, inserted into the CD38 gene, can overcome fratricide in both αβT cells and NK cells. Our method enables robust expansion of all γδT cell subsets and establishes optimal conditions for gene editing and large-scale clinical manufacturing. Here, we describe a highly efficient method for genetically engineering polyclonal γδT cells, including Vδ1$^+$, Vδ2$^+$, and Vδ1$^-$/Vδ2$^-$ subsets. The subtypes of γδT cells play distinct roles in responding to cancer and infection. The prevalence of different subtypes across tissues may represent unique biological properties that contribute to controlling T-ALL in both peripheral blood and tissues. This was evident in our mouse model, where we observed a significant reduction of T-ALL burden in the blood, bone marrow, and spleen. Our technology represents a promising alternative because of their inherent anti-tumor activity, lack of GvHD induction, suitability for off-the-shelf use, and enhanced in vivo persistence. These cells share many innate-like immune properties with NK cells while also providing adaptive immunity similar to αβT cells. Overall, we demonstrate the efficacy of a novel off-the-shelf CD38CAR γδT cell product that is effective against multiple T-ALL subtypes, with activity both in vitro and in vivo. Beyond T-ALL, our off-the-shelf CD38CAR γδT cells also have potential therapeutic applications in other CD38$^+$ malignancies, such as AML and MM.

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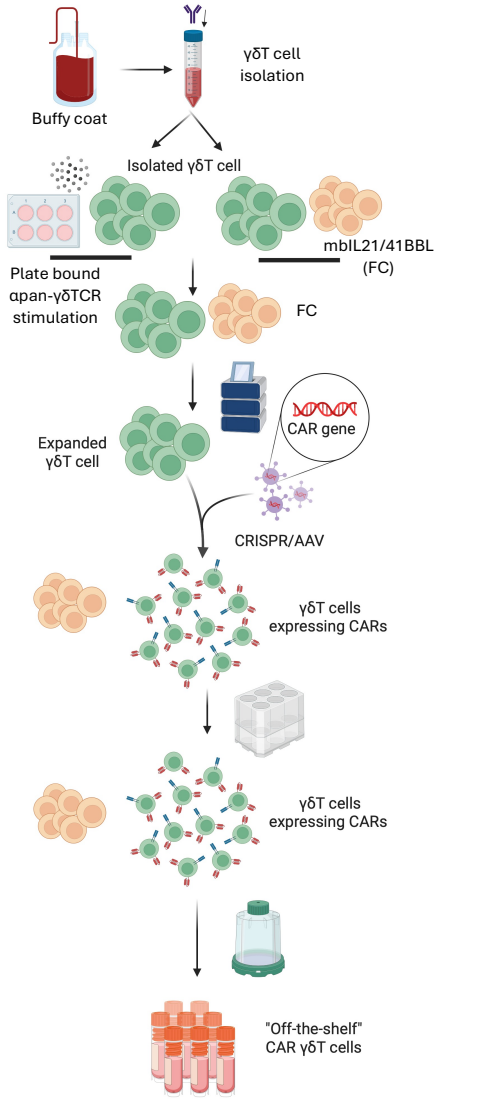
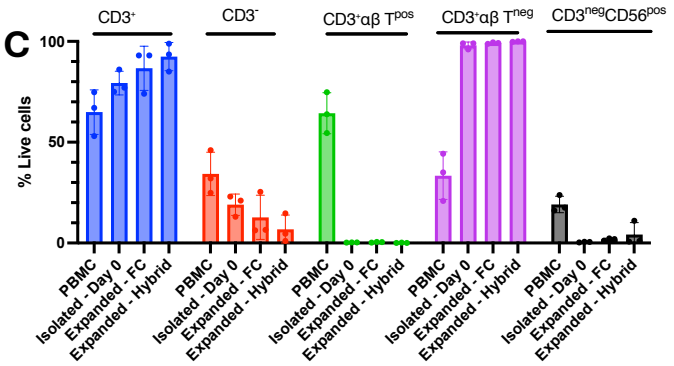
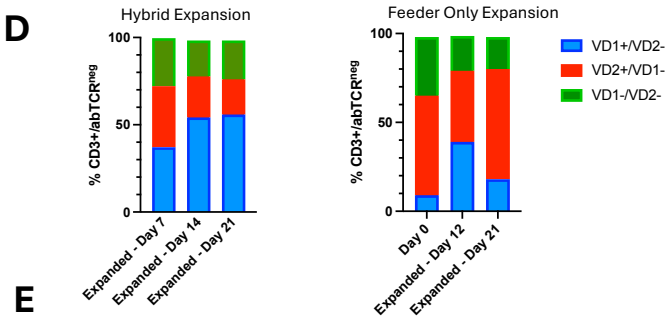
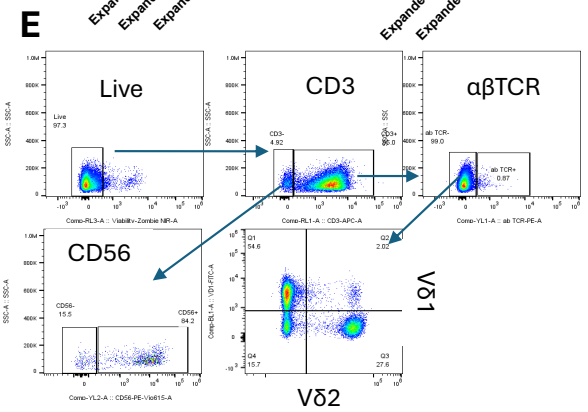
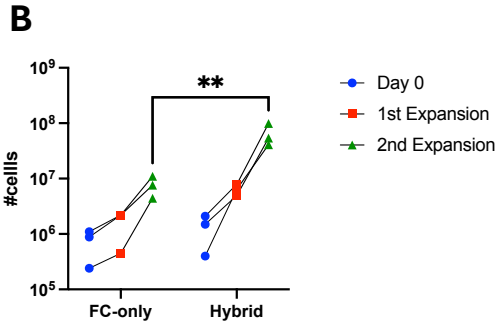
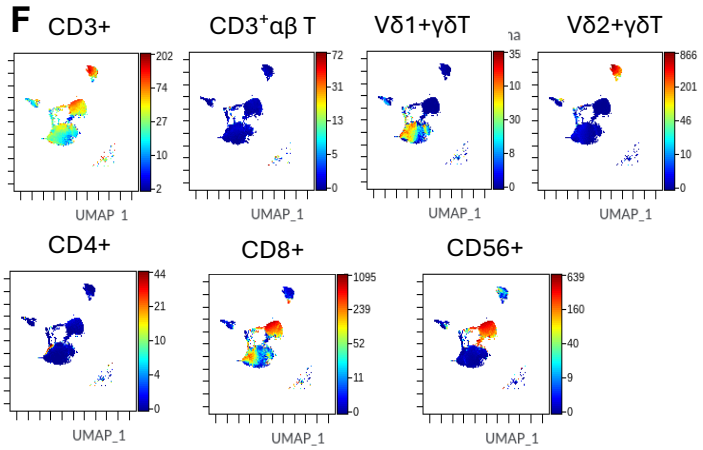

Buffy coat
γδT cell isolation
Isolated γδT cell
Plate bound αpan-γδTCR stimulation
mbIL21/41BBL (FC)
FC
Expanded γδT cell
CAR gene
CRISPR/AAV
γδT cells expressing CARs
γδT cells expressing CARs
"Off-the-shelf" CAR γδT cells


C
CD3+
CD3-
CD3+αβ Tpos
CD3+αβ Tneg
CD3negCD56pos
% Live cells
100
50
0
PBMC
Isolated - Day 0
Expanded - FC
Expanded - Hybrid


D
Hybrid Expansion
Feeder Only Expansion
% CD3+/abTCRneg
100
50
0
Expanded - Day 7
Expanded - Day 14
Expanded - Day 21
Day 0
Expanded - Day 12
Expanded - Day 21
VD1+/VD2-
VD2+/VD1-
VD1-/VD2-


E
Live
CD3
αβTCR
CD56
Vδ1
Vδ2


B
#cellls
10^9
10^8
10^7
10^6
10^5
**
FC-only
Hybrid
Day 0
1st Expansion
2nd Expansion


F
CD3+
CD3+αβ T
Vδ1+γδT
Vδ2+γδT
CD4+
CD8+
CD56+
UMAP_1

- **Figure 1. Expansion and Characterization of Polyclonal γδT Cells**

(A) Schematic overview of γδT cell expansion protocols using hybrid system (αPan-γδ TCR antibody stimulation followed by FC expansion) or FC expansion-only, along with subsequent CAR γδT cell manufacturing and expansion steps. **(B)** Hybrid system resulted in higher yield of γδT cell compared to FC-only expansion; p values were calculated using a 2-way analysis of variance (ANOVA); $**p= 0.0013$. **(C and D)** Flow cytometry analysis confirms high purity of γδT cells post-expansion, with minimal contamination from $CD3^{+}$αβ T cells or $CD3^{-}CD56^{+}$ NK cells (n = 3 healthy donors). Both expansion platforms maintained γδT cell subtype diversity over a three-week culture period, preserving the polyclonal nature of the population. **(E)** Representative flow cytometry plots showing the gating strategy on the γδT cells to study their purity. **(F)** Mass cytometry (CyTOF) profiling revealed a preserved polyclonal composition and validated the immunophenotypic purity of the expanded γδ T cell product.

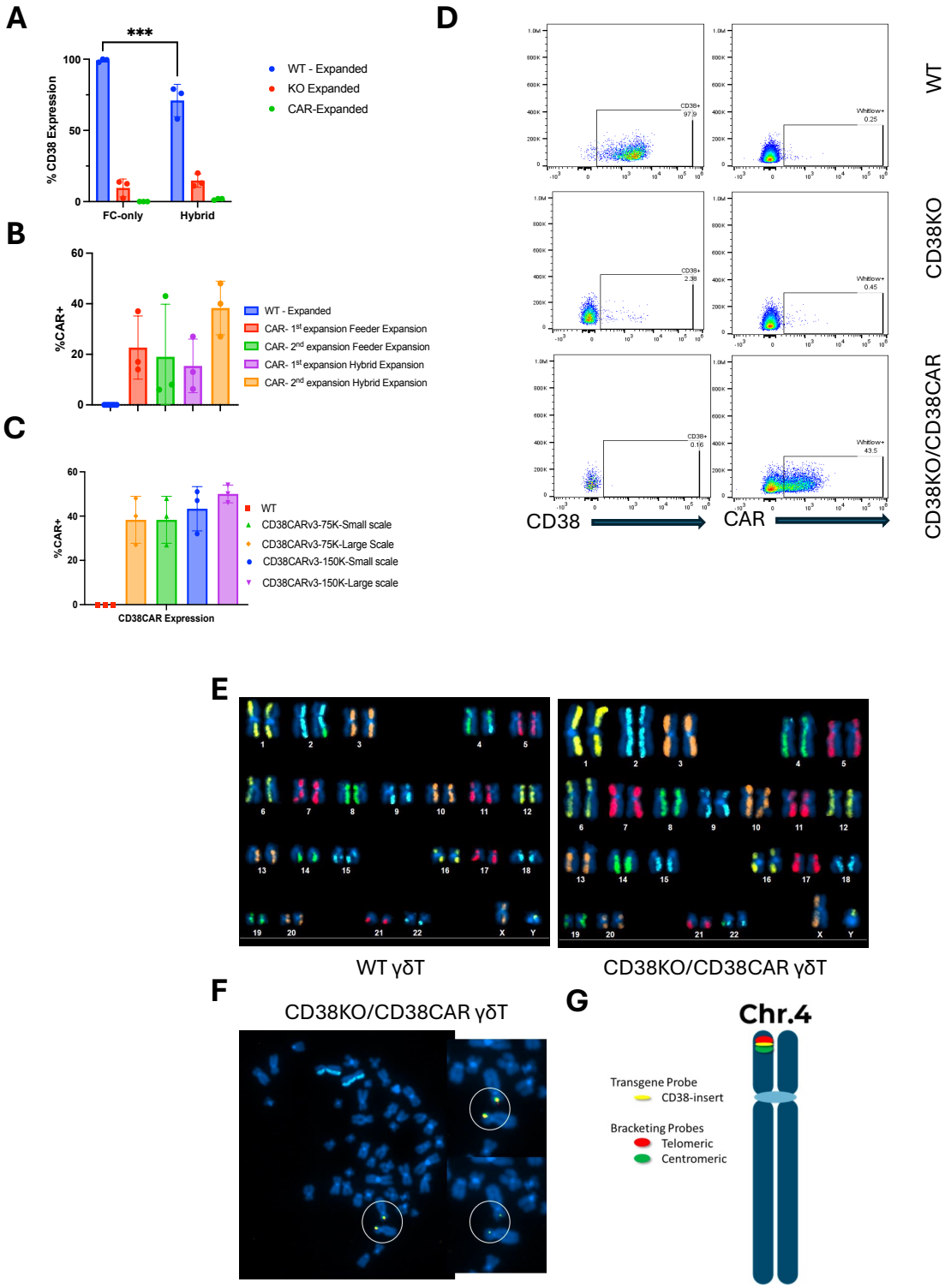

A
***
% CD38 Expression
100
50
0
FC-only
Hybrid
WT - Expanded
KO Expanded
CAR-Expanded
B
%CAR+
60
40
20
0
WT - Expanded
CAR- 1st expansion Feeder Expansion
CAR- 2nd expansion Feeder Expansion
CAR- 1st expansion Hybrid Expansion
CAR- 2nd expansion Hybrid Expansion
C
%CAR+
60
40
20
0
CD38CAR Expression
WT
CD38CARv3-75K-Small scale
CD38CARv3-75K-Large Scale
CD38CARv3-150K-Small scale
CD38CARv3-150K-Large scale
D
WT
CD38KO
CD38KO/CD38CAR
CD38
CAR
E
WT γδT
CD38KO/CD38CAR γδT
F
CD38KO/CD38CAR γδT
G
Chr.4
Transgene Probe
CD38-insert
Bracketing Probes
Telomeric
Centromeric

Figure 2. Successful generation of CD38KO/CD38CAR γδT Cells

(A) CRISPR-mediated targeting of CD38 in γδT cells significantly reduced CD38 expression, as assessed by flow cytometry. Residual $CD38^+$ γδT cells underwent fratricide, resulting in complete loss of detectable CD38 on CD38CAR γδT cells ($p = 0.0002$; two-way ANOVA; n = 3 healthy donors). **(B)** The CRISPR/AAV approach achieved highly efficient surface expression of CD38CAR on γδT cells. CAR expression remained stable after expansion on feeder cells across two expansion systems (n = 3 healthy donors). **(C)** Both small- and large-scale manufacturing of CD38CAR γδT cells yielded efficient CAR generation, with no significant differences in CAR expression between two multiplicities of infection (MOIs) of AAV6 (75,000 vs. 150,000). **(D)** Representative flow cytometry plots showing the gating strategy and expression of CD38 and CD38CAR in γδT cells. **(E**) Karyotype (K-band) analysis revealed no recurrent abnormalities involving chromosome 4 (the CD38 locus) in either wild-type or CD38KO/CD38CAR γδT cells. **(F)** InSite analysis confirmed precise, on-target integration of the CAR transgene, demonstrating biallelic integration without structural rearrangements, translocations, truncations, or complex genomic events at the CD38 locus. Cells with CAR integration at both CD38 alleles are circled. Enlarged images (right) display transgene signals in a separate channel (bottom right). **(G)** Integration was detected using probes specifically designed for the CD38CAR transgene, as shown in the schematic.

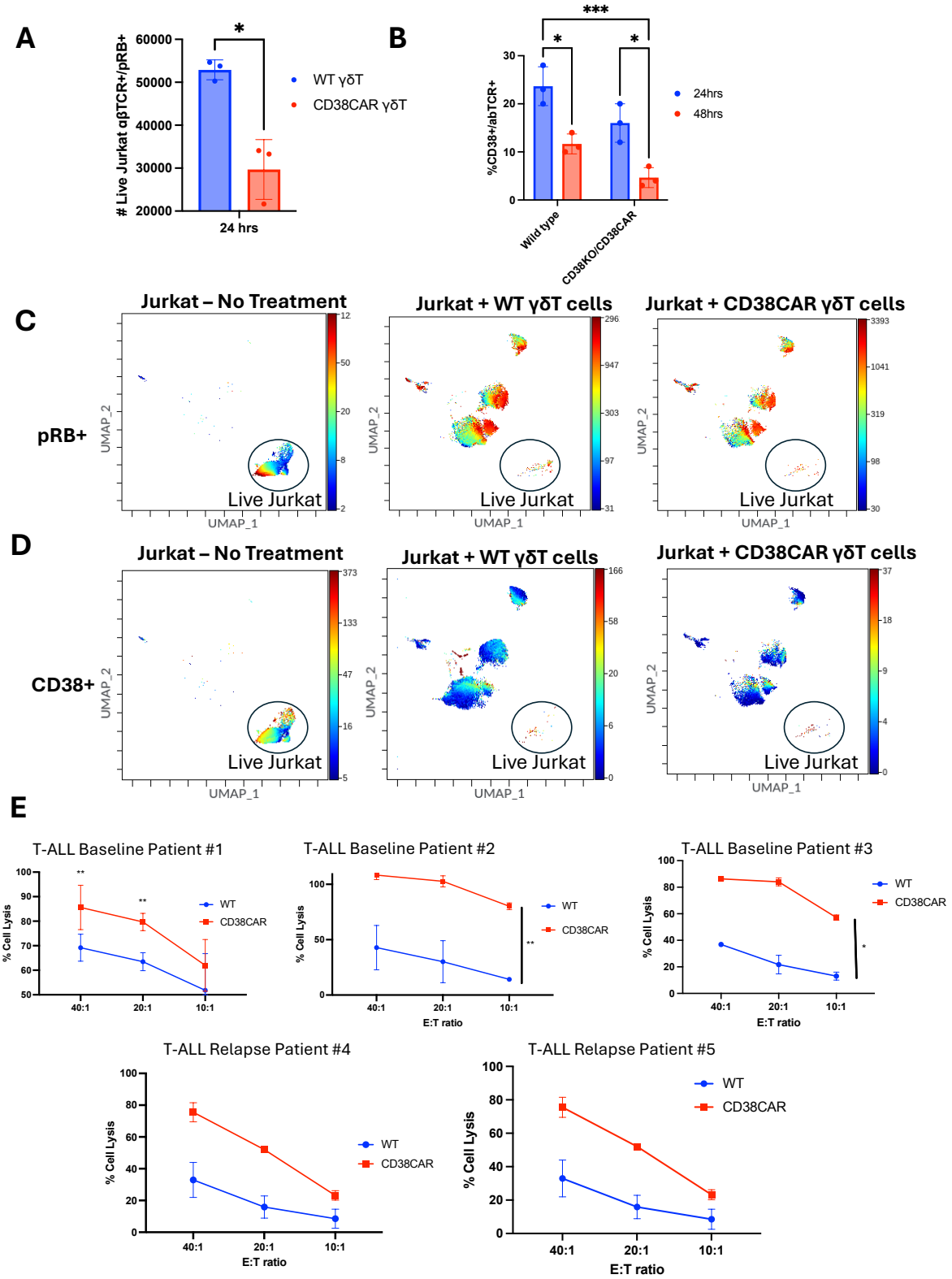

A
Live Jurkat αβTCR+/pRB+
60000
50000
40000
30000
20000
*
24 hrs
WT γδT
CD38CAR γδT
B
%CD38+/abTCR+
30
20
10
0
***
*
*
Wild type
CD38KO/CD38CAR
24hrs
48hrs
C
pRB+
Jurkat – No Treatment
Jurkat + WT γδT cells
Jurkat + CD38CAR γδT cells
UMAP_2
UMAP_1
Live Jurkat
D
CD38+
Jurkat – No Treatment
Jurkat + WT γδT cells
Jurkat + CD38CAR γδT cells
UMAP_2
UMAP_1
Live Jurkat
E
T-ALL Baseline Patient #1
T-ALL Baseline Patient #2
T-ALL Baseline Patient #3
T-ALL Relapse Patient #4
T-ALL Relapse Patient #5
% Cell Lysis
E:T ratio
40:1
20:1
10:1
WT
CD38CAR

Figure 3. Antitumor activity of CD38KO/CD38CAR γδT cells against Jurkat and primary T-ALL samples.

(A) Long-term CyTOF-based cytotoxicity assay in which Jurkat cells (αβTCR$^+$ T-ALL) were co-cultured with either wild-type (WT) in blue or CD38KO/CD38CAR γδT cells in red for 24 hours. CAR γδT cells significantly reduced the number of live Jurkat cells, measured as pRB$^+$ αβTCR$^+$ cells (paired *t*-test; $p = 0.0133$; $n = 3$ donors). **(B)** CAR γδT treatment also significantly decreased the number of CD38 expressing αβTCR$^+$ Jurkat cells, confirming CAR specificity ($p = 0.01$; $*p = 0.0005$; two-way ANOVA; $n = 3$ donors). **(C-D)** Representative CyTOF plots showing marked reduction of live (pRB+) CD38$^+$ Jurkat cells following γδT cell treatment. **(E)** CD38KO/CD38CAR γδT cells (red) exhibited enhanced antitumor activity against both baseline and relapsed primary T-ALL samples relative to WT γδT cell (blue). For each assay, one donor-derived γδT cell (WT and CAR) was tested in triplicate. P value was calculated using unpaired *t*-test (t-test; * $p = 0.01$; ** $p = 0.004$).

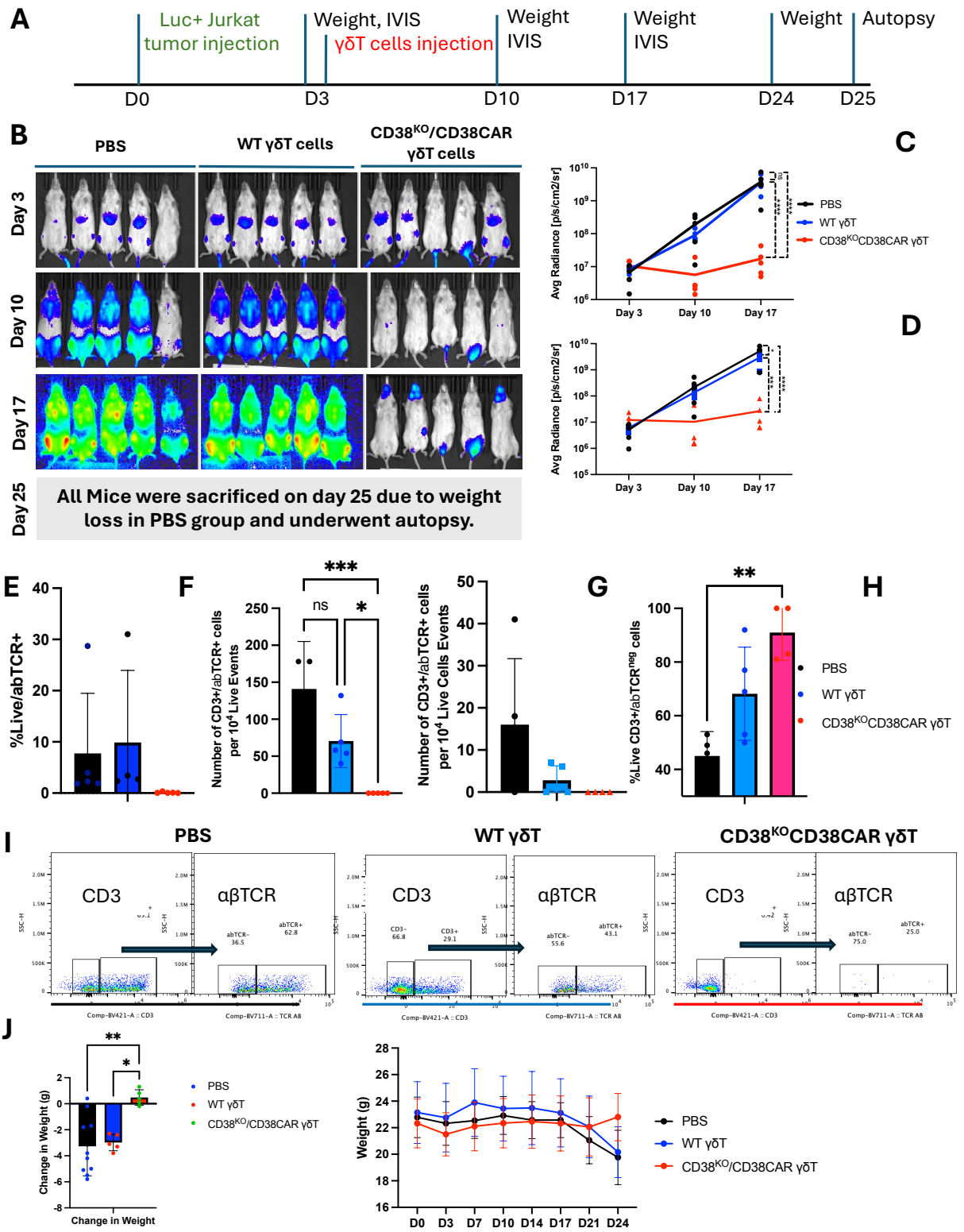
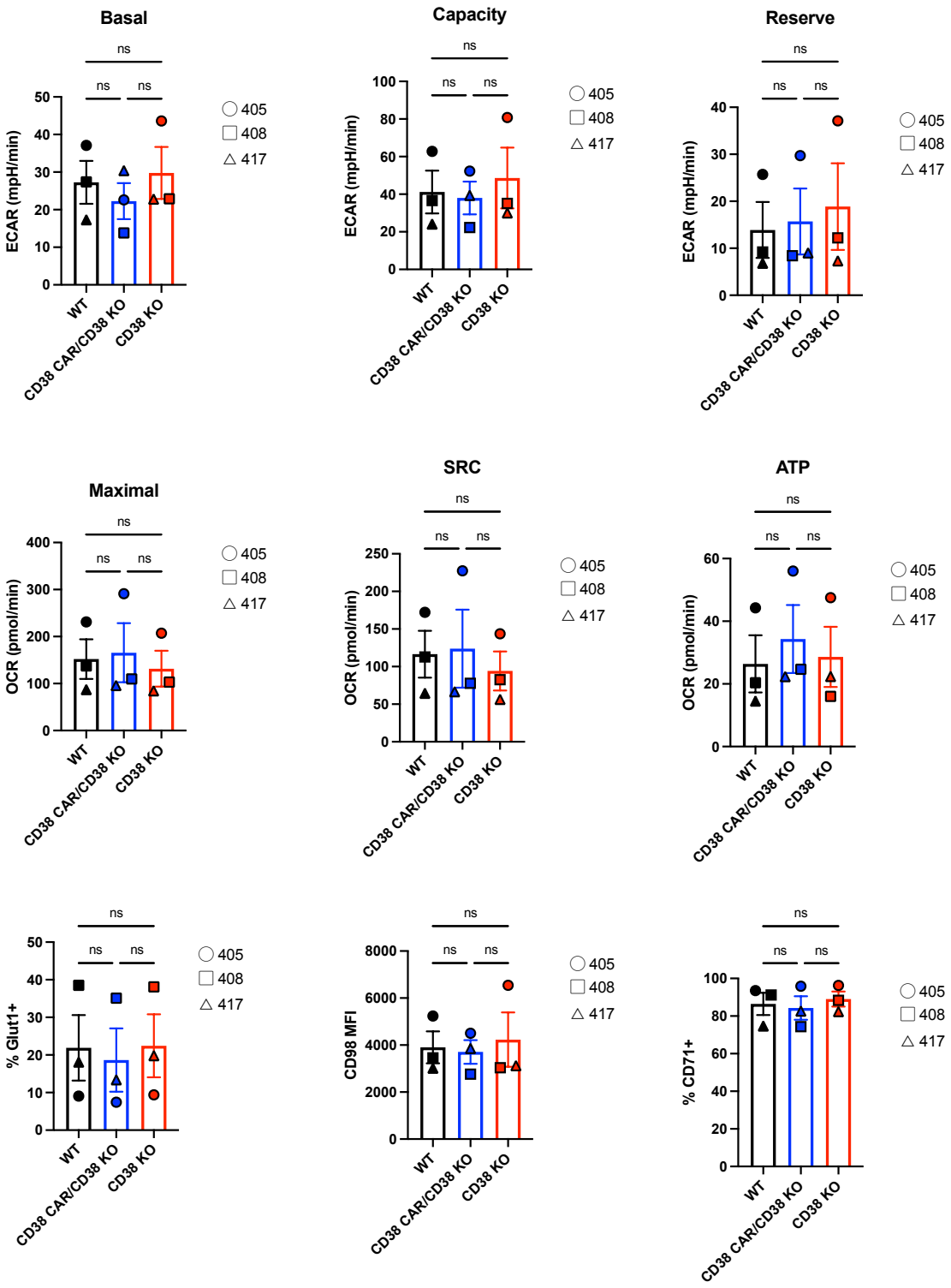

A
Luc+ Jurkat tumor injection
Weight, IVIS
γδT cells injection
Weight IVIS
Weight IVIS
Weight
Autopsy
D0
D3
D10
D17
D24
D25
B
PBS
WT γδT cells
CD38KO/CD38CAR γδT cells
Day 3
Day 10
Day 17
Day 25
All Mice were sacrificed on day 25 due to weight loss in PBS group and underwent autopsy.
C
Avg Radiance [p/s/cm2/sr]
Day 3
Day 10
Day 17
PBS
WT γδT
CD38KOCD38CAR γδT
D
E
%Live/abTCR+
F
Number of CD3+/abTCR+ cells per 10^4 Live Events
ns
*
***
G
Number of CD3+/abTCR+ cells per 10^4 Live Cells Events
H
%Live CD3+/abTCRneg cells
**
I
PBS
WT γδT
CD38KOCD38CAR γδT
CD3
αβTCR
J
Change in Weight (g)
Change in Weight
Weight (g)
D0
D3
D7
D10
D14
D17
D21
D24

Figure 4. CD38KO/CD38CAR γδT Cells Exhibit Potent Antitumor Activity Against T-ALL In Vivo

NSG mice engrafted with Luc^+ Jurkat cells were treated with PBS, WT γδT, or CD38KO/CD38CAR γδT cells **(A and B)**. PBS (black) and WT γδT cells (blue) treated mice showed significant increase in tumor burden compared to CAR γδT (red) treated mice based on average radiance detected by bioluminescence imaging from ventral **(C)** and dorsal **(D)** side of the mice (**** $p = 0.0001$; *** $p = 0.0008$; two-way ANOVA). CAR γδT cells significantly reduced tumor burden ($\alpha\beta TCR^+$ T-ALL) measured in samples from blood **(E)** , bone marrow **(F)**, and spleen **(G),** for all figures % in live cells**. (H)** In addition human $CD3^+$ $\alpha\beta TCR^-$ cells as an indicator of γδT cells were found to be the most prevalence in the bone marrow of the CD38KO/CD38CAR γδT cells treated (* $p = 0.02$; ** $p = 0.001$, *** $p = 0.0009$; two-way ANOVA); **(I)** The representative flow cytometry gating strategy used to study the samples); **(J)** The mice in CD38CAR γδT treated group had the lowest wight loss in compared to WT γδT and PBS treated groups at Day 24 (* $p = 0.01$; ** $p = 0.002$; one-way ANOVA)**.**



Supplementary Figure 1.

Metabolic assays showed no significant changes in γδT cells post editing and CD38 deletion (n=3 healthy donors).