Application Note

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Developing a Multimodal Workflow for the Comprehensive Assessment of Repeatedly Challenged CAR T Cells

Interrogating serial killing using Agilent analytical platforms

Author

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Abstract

Adoptive cell therapy with chimeric antigen receptor T cells (CAR T cells) has been highly successful in individuals with hematological malignancies. However, its efficacy against most solid tumors remains elusive due to short-term persistence and inadequate expansion of CAR T cells within the tumor microenvironment (TME). Traditional in vitro cytolysis assays often fail to reflect the true antitumor potential of CAR T cells, as they typically involve brief coculture periods and assess CAR T cell performance solely from cytotoxic capacity perspectives. To address these limitations, a prolonged multimodal workflow that can robustly and comprehensively assess CAR T cells throughout multiple rechallenges, mimicking a large tumor burden in vivo to a certain extent, was developed.

In this study, the EpCAM CAR T cells were challenged with an EpCAM-expressing breast cancer cell line, T47D cells, over seven consecutive rounds. During each challenge, the cytolytic activity of CAR T was measured and evaluated in real time using an Agilent xCELLigence RTCA eSight system. Additionally, at the end of each challenge, the metabolic profile of CAR T cells was measured using an Agilent Seahorse XF Pro analyzer. Cell proliferation, differentiation, exhaustion, and percentage of CAR T cells were determined using an Agilent NovoCyte flow cytometer. Using these approaches, it was found that repeated antigen exposure accelerates CAR T cell differentiation, promotes exhaustion, compromises metabolic fitness, reduces proliferative capacity, and ultimately impairs cytotoxic function. Thus, the integrated strategy provides a valuable framework for gaining deeper insights into CAR T cell dynamics under high tumor burden, guiding CAR construct optimization and supporting the advancement of preclinical and clinical efforts in solid tumor therapy.

Introduction

CAR T cells are genetically engineered T cells that express tumor-specific antigen receptor fragments on their surface, allowing for targeted antigen recognition and precise activation of T cells. Consequently, the activated CAR T cells selectively attack antigen-expressing tumor cells. CAR T has been used as a novel form of immunotherapy and shows promising clinical outcomes for hematological malignancies. However, its therapeutic benefits have been limited in solid tumor cases, mainly due to the lack of highly selective antigens, high tumor antigen heterogeneity, the immunosuppressive TME, and limited infiltration and persistence of CAR T cells.[1]

To date, human tumor xenografted immunodeficient mice are used to assess the therapeutic potential of CAR T cells, including their antitumor efficacy and optimal dosing. However, these in vivo murine studies are low-throughput, time-consuming, and labor-intensive. Various in vitro CAR T potency assays have been developed to overcome these limitations, such as the cytokine release assay and the cell-based cytotoxicity assay.

One of the most widely adopted in vitro cytotoxicity assays is using the impedance readout of Agilent xCELLigence RTCA systems to measure and quantify the cytolytic potency of immune cells through a specialized biosensor microplate, the Agilent xCELLigence RTCA E-Plate.[2,3] Impedance measurement, a label-free and noninvasive technology, can detect subtle cellular changes of live cells adhering to the biosensors of the E-Plate, including cell-substrate attachment, proliferation, viability, and morphology, in real time. In an effector and tumor cell coculture impedance-based cytotoxicity assay, changes in impedance primarily arise from the growth/proliferation of adherent tumor cells and the death of adherent tumor cells induced by the suspended effectors, as suspended effector cells minimally contribute to the impedance signal.

In addition to the degree of initial CAR T cell killing potency, the persistence of their cytolytic activity is another critical factor in determining their therapeutic efficacy. Even when millions or billions of CAR T cells are infused, during in vivo tumor eradication, they often encounter substantial tumor burdens. As a result, they must be persistent and take multiple rounds of cytolytic action to achieve complete tumor elimination. Throughout this prolonged process, these living therapies adapt, proliferate, differentiate, and eventually become exhausted. These dynamics pose significant challenges to assessing and predicting CAR T therapeutic outcomes. Therefore, a short-term, single-readout in vitro assay, such as one that solely measures T cell cytokine release, killing potency, differentiation, or metabolism, cannot fully capture the dynamic changes of CAR T cells and thoroughly characterize a CAR T product.

This application note presents a multimodal workflow for the comprehensive characterization of CAR T cells over consecutive tumor cell rechallenges (Figure 1). Several CAR T cell critical quality attributes (CQA) were evaluated in a single workflow, including CAR T killing potency using the xCELLigence RTCA eSight system, which simultaneously monitors impedance and acquires images from the same well; proliferation capability, percentage of CAR T cells, T cell immunophenotyping, and expression of exhaustion-associated markers using NovoCyte flow cytometers; and CAR T cell metabolic fitness using the Seahorse XF Analyzer, after each round of exposure to the tumor cells.

Our results, which capture the dynamic changes in CQAs throughout the serial killing process, demonstrate the utility of multiplexing metrics derived from this multimodal workflow to guide CAR construct design and optimize the manufacturing process. Moreover, this approach provides insights into CAR T cell behavior in vivo, leading to enhanced predictivity for clinical outcomes of CAR T cell products.

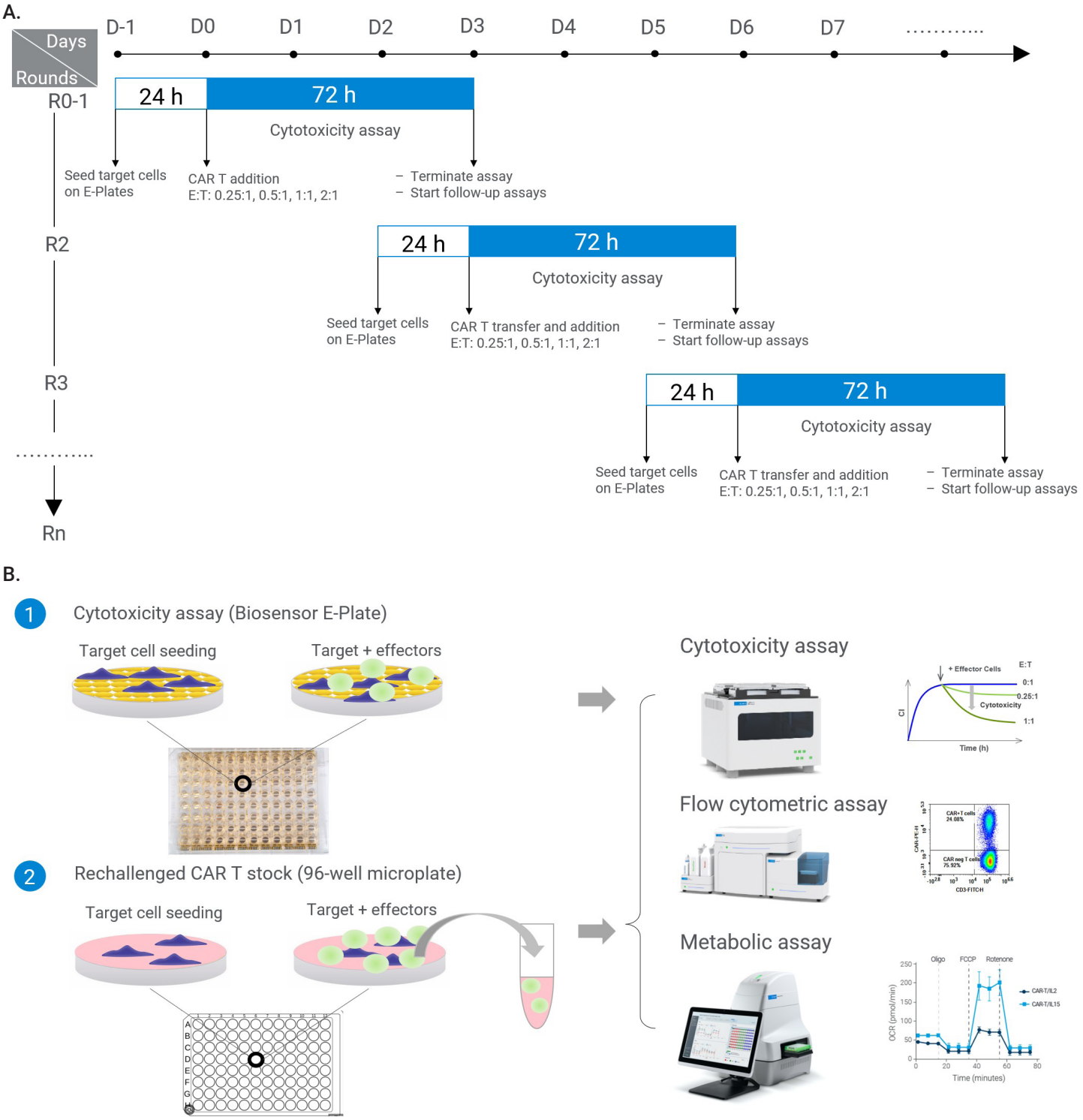

Figure 1. The multimodal workflow for characterization of effector cells across serial killing rounds. A. Overview of the workflow. B. Schematics of the multiple analytical tools used for each round of target cell rechallenge. D: day; R: round; R0: after effector cell revival.

Experimental

Cells and reagents

Target cells: T47D, a human breast cancer cell line expressing the EpCAM antigen, was purchased from ATCC (part number HTB-133). The cells were transduced with lentiviral particles harboring the gene encoding for red fluorescent protein (eLenti Red, Agilent, part number 8711011) and subsequently selected by puromycin to generate T47D-red cells. T47D-red cells were maintained in RPMI 1640 medium (Cytiva, part number SH3025501), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) at 37 °C in a 5% CO_2 incubator, and subcultured at 80 to 90% confluency.

EpCAM CAR T and untransduced T cells: EpCAM CAR T cells were produced in a Lonza Cocoon platform. Briefly, $CD4^+/CD8^+$ cells provided by Lonza were thawed and rested overnight at a density of 0.325×10^6 per mL in X-VIVO 15 medium (Lonza, part number 02-053Q) supplemented with 5% human serum (Sigma, part number H4522). The next day (defined as day zero), the T cells were centrifuged and loaded to a disposable Cocoon Cassette in the expansion medium, consisting of X-VIVO 15 medium, 5% human serum, 200 U/mL IL2 (StemCell Technologies, part number 78036.3) and supplemented with TransAct activator (Miltenyi Biotec, part number 130-111-160) for activation. The following day (day one), the cells were transduced at a multiplicity of infection (MOI) equal to 0.35 in the expansion medium for EpCAM CAR production. No transduction was performed for untransduced T cells (UT). After 24 hours (day two), transduction was stopped by completely replacing the viral vector-containing media with fresh expansion media. To maintain and expand CAR T cells, the expansion media was recirculated daily and exchanged every one to two days afterward until cell harvest on day 10. The cells were stored in liquid nitrogen in a CytoStorCS10 freezing medium (StemCell Technologies, part number 100-1061).

Cell count

After homogeneously resuspending cells by pipetting up and down, 50 µL of the cell suspension was transferred to a 96-well U-bottom plate, and 50 µL of a solution containing the cell-staining buffer (Biolegend, part number 420201) and 1 µL of 7-amino-actinomycin D (7-AAD) solution (Biolegend, part number 420404) was added. After incubation for 5 minutes at room temperature in the dark, the absolute count of each sample was acquired in duplicates using an Agilent NovoCyte Quanteon flow cytometer (Agilent, part number 2010102). Following sample acquisition, proper gating was applied, and the absolute count of 7AAD-negative cells was reported as the total number of live cells.

CAR T cytotoxicity assay

Before seeding target cells to the Agilent xCELLigence E-Plate VIEW 96 (E-Plate) (Agilent, part number 300601030), 50 µL of assay medium (RPMI 1640, 10% FBS, and 1% pen/strep) was added to the wells of the E-Plates for plate background recording on an RTCA eSight system (Agilent, part number 380601600). Dissociated T47D-red cells were then seeded at a density of 8,000 cells/well in a volume of 50 µL in a biosafety hood. After a 30-minute room temperature (RT) incubation, allowing even distribution of cells on the well, the target cell plate was placed in the RTCA eSight system and continuously recorded at 15-minute intervals until the addition of effector cells the following day. On the day of the assay, the number of effector cells required for each effector-to-target ratio (E:T) was calculated according to the different E:T ratios (2:1, 1:1, 0.5:1, and 0.25:1). The E:T ratios were determined based on the number of CAR-positive T cells. The effector cells were added to the wells of the E-Plates in a volume of 100 µL. Several controls were included on the same E-Plate. The effector cell-only wells served as the Cell Index (CI) background control. The target plus UT T cell wells at various E:T ratios were used to determine nonspecific cytotoxicity. The target cell-only wells were used as a reference for the percentage of cytolysis calculation. Given that including the appropriate controls and adequate replicates for treatment on the same plate is essential for accurate data interpretation and reliable potency measurement, we set up at least three replicates for each E:T ratio and at least six replicates for the target-only control. After adding effector cells, the E-Plate was returned to the RTCA eSight system, and recording resumed immediately at 15-minute intervals for three days.

Repetitive cytotoxicity assay

For the detailed assay protocol, refer to the serial killing assay protocol.[4] Briefly, after the three-day cytotoxicity assay was completed, CAR T cells were collected from the E-Plate by gently pipetting the media in each well up and down three times using a multichannel pipette, ensuring the removal of all the effector cells while leaving the live target cells on the plate. The effector cells from the same E:T ratio wells were pooled into a 5 mL centrifuge tube and centrifuged at 300 x *g* for five minutes. The supernatant containing cell debris was discarded. The cell pellet was resuspended in the cytotoxicity assay medium, followed by the cell count using a NovoCyte Quanteon flow cytometer. The desired number of effector cells was then added to the new E-Plate of T47D-red cells, seeded 24 hours in advance, for the next round of killing assay.

Table 1. Antibodies used to characterize CAR T cell populations.

Marker	Panel	Fluorochrome	Part Number	Manufacturer	Clone	Antibody (µL) (per 100 µL)
CAR detection antibody	Common	PE	115-116-072	Jackson ImmunoResearch	$F(ab')_2$ Fragment Goat Anti-Mouse IgG, $F(ab')_2$	2
CD3	Common	FITC	344804	BioLegend	SK7	3
CD4	Common	BV650	317436	BioLegend	OKT4	3
CD8	Common	APC CY7	344714	BioLegend	SK1	3
CCR7	Differentiation	BV421	353208	BioLegend	G043H7	5
CD45RA	Differentiation	PECY7	304126	BioLegend	HI100	3
CD95	Differentiation	BV605	305627	BioLegend	DX2	5
CD45RO	Differentiation	APC	304210	BioLegend	UCHL1	5
CD39	Exhaustion	PECY7	328211	BioLegend	A1	5
PD1	Exhaustion	BV421	329920	BioLegend	EH12.2H7	5
LAG3	Exhaustion	BV605	369323	BioLegend	11C365	5
TIM3	Exhaustion	APC	364804	BioLegend	A18087E	3

Rechallenged CAR T cell stock preparation

In parallel to each round of the cytotoxicity assay performed using E-Plates on the xCELLigence RTCA eSight system, two regular 96-well tissue culture plates were used to prepare tumor-challenged CAR T cells by coculturing CAR T and T47D-red cells at an E:T ratio of 2:1 for three days, ensuring enough effector cells for the following cytotoxicity, metabolism, and immunophenotyping assays.

Metabolic profiling of CAR T cells

The day before the metabolic assay, the cartridge (Agilent, part number 103792-100) was hydrated in Agilent Seahorse XF calibrant solution, and the Agilent Seahorse XFe96/XF Pro PDL plates (Agilent, part number 103799-100) were placed overnight at 37 °C in a non-CO_2 incubator. On the day of the assay, the immune cells were centrifuged at 300 x *g* for five minutes, and resuspended in an appropriate volume of prewarmed Agilent Seahorse XF RPMI assay media pH 7.4 (Agilent, part number 103576-100) supplemented with 10 mM glucose (Agilent, part number 103577-100), 2 mM glutamine (part number 103579-100), and 1 mM pyruvate (Agilent, part number 103578-100) to reach the recommended concentration of total cells, 1.5 to 2 x 10^6 cells/mL. A cell suspension of 50 µL was added in triplicate to quintuplicates to the PDL plate. The cell plate was then centrifuged at 200 x *g* for one minute to ensure cell attachment to the plate. After gently adding 150 µL of assay media per well, the cell plate was incubated at 37 °C in a non-CO_2 incubator for 45 minutes. The Agilent Seahorse XF T cell Metabolic Profiling kit (Agilent, part number 103772-100) reagents were loaded into the injection ports of the XF cartridge. The assay was performed using the Agilent Seahorse XF Pro Analyzer (Agilent, part number S7855A). For the detailed assay protocol, refer to the Agilent Seahorse XF T Cell Metabolic Profiling kit user guide.[5] Seahorse Analytics (SHA), a web-based software platform, was used to analyze XF result files.

CAR T immunophenotyping and measurement percentage

The CAR T cell populations postrevival from frozen stocks (R0) and after each round of killing were characterized using flow cytometry. The antibody panel is listed in Table 1. T cell differentiation and exhaustion were studied using separate multicolor flow cytometry panels. The viability dye 7-AAD was included in all panels for gating live cells. Single-stained compensation controls, fluorescence minus one (FMO) controls, and unstained controls were included to determine the fluorescence spread and the gating boundaries.

Briefly, an average number of 1 x 10^6 cells per sample was resuspended in a cell staining buffer (BioLegend, part number 420201). Fc receptors were blocked by adding Human TruStain FcX blocking buffer (BioLegend, part number 422302) and incubating at RT for seven minutes. Normal goat serum (Vector Laboratories, part number S1000-20) was added and incubated for another 10 minutes.

Washing steps for this assay were performed by adding 1 mL of staining buffer and centrifuging at 300 *x g* for five minutes to collect the cell pellet and discard the buffer. Cells were washed and stained with the EpCAM CAR detection antibody for 20 minutes at RT. Cells were washed twice before blocking with mouse serum (Sigma-Aldrich, part number S25) for 15 minutes at RT. Finally, after a washing step, cells were resuspended in cell staining buffer and stained with the surface antibodies of interest, then washed once and analyzed on a NovoCyte Quanteon flow cytometer and NovoExpress software.

Results and discussion

The multimodal workflow for characterizing CAR T cells during the serial killing process

To gain a deeper insight into CAR T cell dynamics during rechallenges, we developed a multimodal workflow to characterize T cells from multiple perspectives (Figure 1A and B). The day before the cytotoxicity assay, T47D-red cells were seeded at 8,000 cells/well in both an E-Plate for in vitro cytotoxicity assay using an RTCA eSight system and regular 96-well plates to serve as rechallenged CAR T stock plates, ensuring sufficient cells for the sequential multiple assays. The target cells were incubated at 37 °C and 5% CO_2 overnight. The next day, EpCAM CAR T cells were added to the T47D-containing E-Plate at various E:T ratios (0.25:1, 0.5:1, 1:1, and 2:1) and to the regular 96-well plates (cell stock plates) at the E:T ratio of 2:1. Of note, the E:T ratios were calculated using the number of CAR-positive T cells. CAR T cytolytic activity was continuously monitored and assessed for 72 hours on the E-Plate using the RTCA eSight system. The day before the next round of the killing assay (approximately 48 hours after adding effector cells), new target cell plates were prepared by seeding T47D cells and letting them grow overnight. The following day, CAR T cells were collected from the original plates, resuspended in fresh media, and counted using a NovoCyte flow cytometer (Figure 1A). The CAR T cells were further aliquoted into three groups for concurrent assays, including the repeated cytotoxicity assay, flow cytometric assay, and metabolic assay. (Figure 1B). The percentage of CAR T cells must be determined before the next round of the cytotoxicity assay, as it may change over time. This change can consequently affect the E:T ratio calculation, as the number of effector cells was based on CAR-positive cells. This cycle was repeated until a decreasing trend in the killing activity of CAR T cells was observed. Notably, since substantial cell death occurred during the seventh round of rechallenge, the remaining cell number was insufficient for flow cytometric analysis and metabolic assessment.

CAR T cell proliferation and survival dynamics across sequential killing assays

The clinical outcomes of CAR T cell therapy have been associated with in vivo expansion and prolonged antitumor activity.[6] Growing evidence suggests that successful responses to CAR T cell treatments depend on the survival, expansion, and persistence of CAR T cells after infusion.[7,8] Some studies have shown that sustained detection of CAR T cells in peripheral blood is associated with superior responses, even in patients with high-grade diseases.[9]

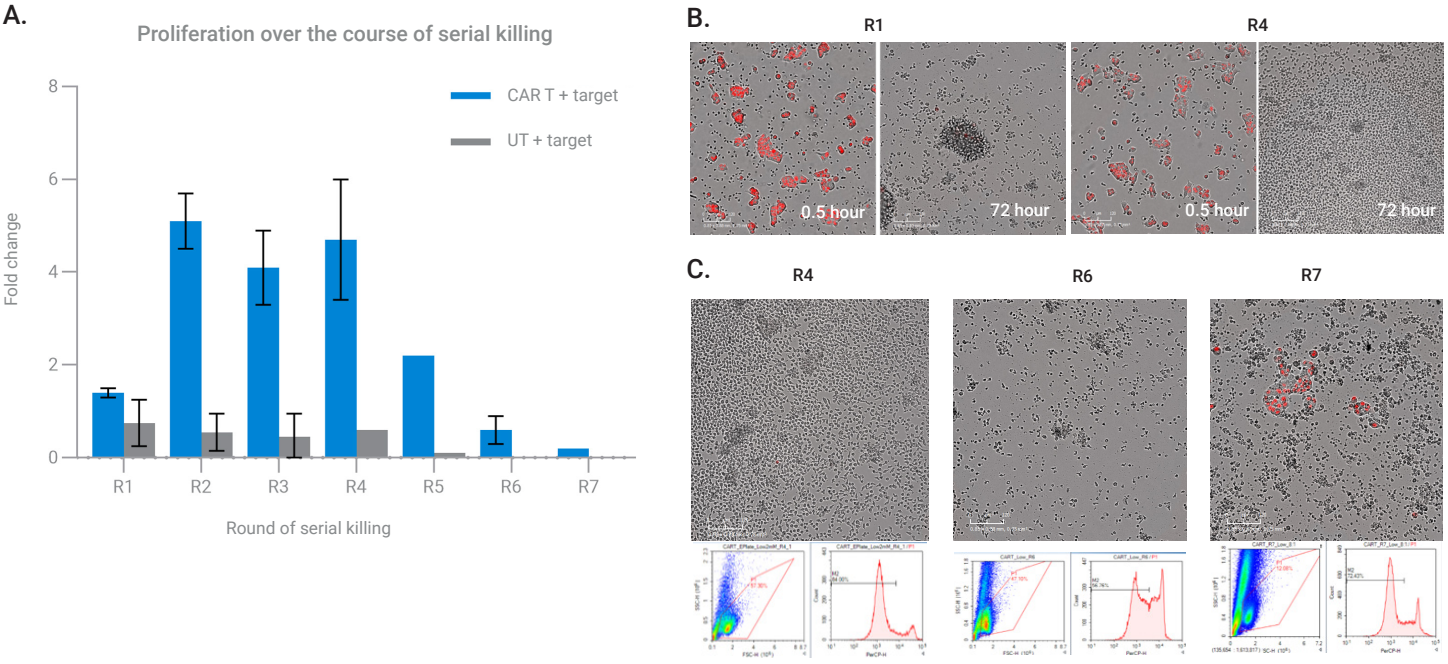

Figure 2. Proliferative capacity of CAR T cells and untransduced (UT) T cells during the serial killing process. A. Fold changes in cell number obtained from the coculture of target cells and either CAR T cells or UT T cells. Data were presented as mean ± SD, N ≥ 2. B. Representative images of the coculture of T-47D-red and EpCAM CAR T cells captured using brightfield and red channel at 0.5 and 72 hours after effector addition, during the first round (R1) and the fourth round (R4) of killing. The nonfluorescent cells are the T cells. C. Images of CAR T cells in the wells of E-Plate and flow cytometric analysis of CAR T cell viability at the beginning of the fourth (R4), sixth (R6), and seventh (R7) killing rounds.

Therefore, we first evaluated cell expansion ability over serial rounds of killing against tumor cells. The fold change in cell number after each round of killing was determined by dividing the number of cells present immediately after the cytotoxicity assay by the initial number of cells added to the target wells. The change in cell number collected from the E:T ratio of the 2:1 treatment was used to assess cell expansion.

Figure 2A shows that upon initial exposure to target cells, EpCAM CAR T cells were activated and began to proliferate, leading to an approximately 1.5-fold increase in cell number. Subsequent rounds of rechallenge resulted in even greater increases in cell number, with a 4 to 5 fold change observed from the second to the fourth round. This result aligns with previous studies.[7] While the initial exposure led to CAR T expansion, subsequent challenges can further enhance proliferation.

In our study, in the first challenge, the EpCAM CAR T cells comprised only 26% CAR-positive cells, with the remaining population being UT cells. The smaller increase in cell number after the first round may also be partially attributed to a higher proportion of nonproliferative UT cells compared to proliferative CAR-positive cells. As the percentage of CAR-positive T cells increased and the percentage of UT cells decreased throughout rechallenges (Figure 3), the fold change in cell numbers became more reflective of CAR T cell proliferation rather than UT cell contribution during the later rounds. Additionally, the imaging data recorded using the RTCA eSight system over rechallenges also confirm that more T cells were detected during the fourth round compared to the first round after a three-day killing (Figure 2B). However, a significant drop in the proliferation rate was observed during the fifth round. From the sixth to the seventh rechallenges, the CAR T cells struggled to survive, reflected by a fold change in cell numbers of less than one, low viability determined by flow cytometer, and unhealthy cell morphology observed in imaging data (Figure 2C). As anticipated, UT cells failed to survive and expand over multiple rechallenges, likely due to the absence of antigen activation and lack of IL2 supplementation in the assay media. Using UT cells as nonspecific CTRL had to be terminated after the fifth round due to the inadequate number of cells for the sequential cytotoxicity assay.

Dynamic changes in the percentage of CAR T cells during the serial killing process

Throughout the entire serial killing process, the percentage of EpCAM CAR-positive cells was determined by flow cytometry (Figure 3A). The initial percentage of EpCAM-positive T product (R0) was only 26% due to the low MOI (0.35) used for transduction during cell production. However, a progressive increase in CAR-positive cells from 26% to 86% was observed between the first and third rounds of rechallenges. The high percentage of CAR T-positive cells remained stable until the fifth round of killing (Figure 3A and 3B), indicating that rechallenging with target cancer cells enriched the CAR T population. This enrichment was potentially due to the differing proliferative and survival capabilities of CAR-positive and CAR-negative T cells. Figure 2A demonstrates that the CAR T cells continued proliferating up to the fifth round, whereas the UT cells died off. Comparable percentages of CAR T cells were observed in both the cytotoxicity assay plate (96-well E-Plate) and the regular 96-well plate (data not shown), indicating that cell culture vessels have minimal impact on cell performance and responses when using the same format. This suggests that regular 96-well plates are suitable for preparing sufficient rechallenged CAR T cells for multiple parallel assays and closely represent the cells in E-Plate 96 for cytotoxicity assays.

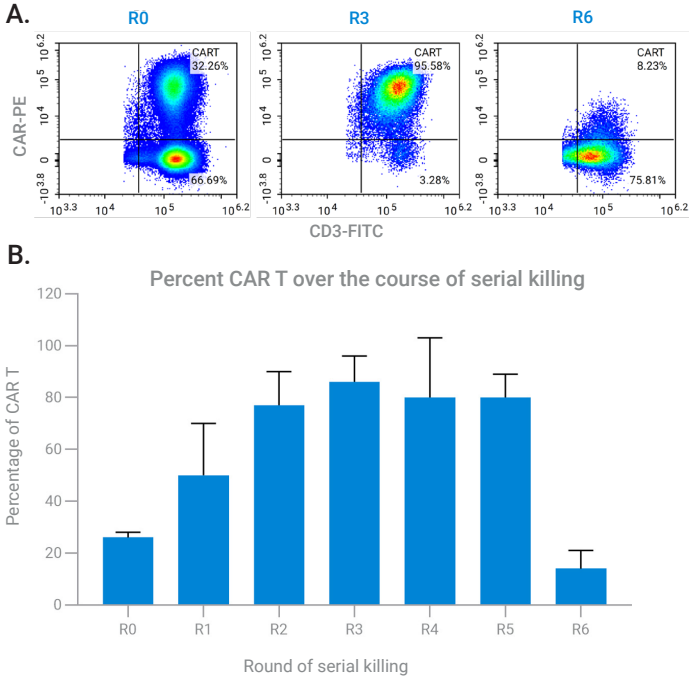

Figure 3. The percentage of EpCAM CAR T cells was determined using a flow cytometer immediately after each round of challenge with T-47D-red cells. A. Representative cytograms of CAR T cells rested overnight after thawing (R0) or immediately after the third (R3) and sixth (R6) rounds of rechallenges. B. The percentage of CAR T cells within the surviving $CD3^+$ T cell populations was quantified after each round.

The cytolytic potency of CAR T cells declined over sequential killing assays

CAR T cells may have reduced cytotoxic activity as they became exhausted after chronic stimulation.[10] Thus, CAR T cell titer doesn't necessarily imply clinical responses. The durability of their cytolytic activity was assesed using the xCELLigence RTCA eSight system, given that clinical to CAR T cell therapy are also determined by the long-term persistence of functional CAR T cells.

The EpCAM CAR T cells were added to the wells containing T47D-red cells at E:T ratios of 0.25:1, 0.5:1, 1:1, and 2:1. All the E:T ratios used in the application were calculated based on pure CAR T cells. The resulting impedance traces determined by the killing kinetics of the CAR T against target cells were measured in real time during the assay. The impedance measurement was converted to percent cytolysis using the immunotherapy module of Agilent xCELLigence RTCA software, enabling the comparison of the relative efficacy between different effector cells. To reflect the real-time killing potency of CAR T cells over a 72-hour cytotoxicity assay, the time course of percent cytolysis was further transformed into an endpoint output, the Area Under the Curve (AUC) of percent cytolysis in 72 hours. Figure 4A demonstrates that, except for the highest E:T ratio of 2:1, for the remaining E:T ratios, the AUC of percent cytolysis declines throughout rechallenges. The onset of AUC reduction appears to be effector cell number-dependent, as evidenced by the fact that lower E:T ratios lead to an earlier decline in AUC. To have a better appreciation of the killing potency reduction over serial killing, the percent cytolysis obtained from E:T ratio of 0.5:1 was plotted over approximately 500 hours of seven rechallenges. From the fourth round and beyond, CAR T cell-mediated cytolysis no longer reached 100% by the end of the 72-hour assay. Instead, it progressively declined, reaching approximately 50% by the seventh rechallenge. These results indicate that the CAR T cells slowly lost their effector potency during the serial killing process. This may potentially be caused by T cell exhaustion and/or cell death (Figure 2A).

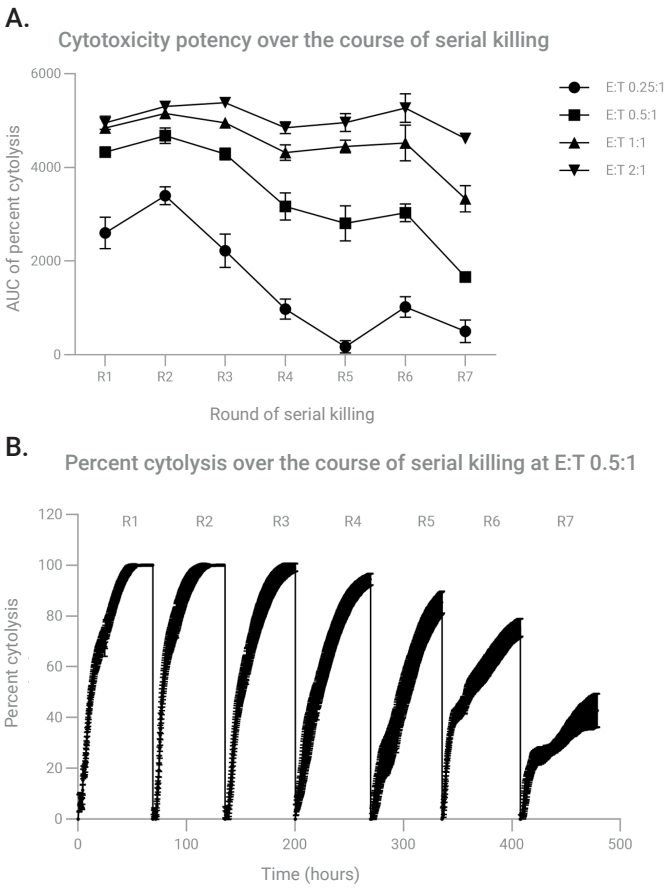

Figure 4. Quantification of CAR T cells' cytolytic potency throughout the serial killing process. A. The Area Under the Curve (AUC) of percent cytolysis at various E:T ratios, 0.25:1, 0.5:1, 1:1, and 2:1. B. The percent cytolysis measured from the E:T ratio of 0.5:1 sample was plotted as a function of time throughout seven rechallenges.

Differentiation phenotyping and cell exhaustion status assessment throughout the serial killing process

After revival and each round of killing, approximately 1 x 10^6 CAR T cells were washed and stained with the corresponding antibodies for differentiation phenotyping. Phenotypic exhaustion was assessed by flow cytometry.

Studies on CAR T persistence, potency, and clinical efficacy have shown a progressive loss of undifferentiated memory phenotypes accompanied by an accumulation of terminally differentiated phenotypes.[11-13] Before being challenged with target cells (R0, Figures 5A to 5C), the CAR T cell product was predominantly composed of T memory stem cell (TSCM), a less differentiated subset associated with long-term persistence and therapeutic potency.[14,15] Notably, among the CAR-positive T cells, over 80% of $CD8^+$ cells exhibited the TSCM phenotype, while the $CD4^+$ cells were more heterogeneous, comprising TSCM, T central memory (TCM), and T effector memory (TEM) subsets. The TSCM subset accounted for nearly half of the $CD4^+$ CAR T population at the baseline (R0). The progression from round two to round three marked a notable transition in both $CD4^+$ and $CD8^+$ CAR T cells towards the more differentiated phenotypes. By the sixth round, the surviving populations were enriched in terminally differentiated effector T cells, or TEMRA cells. The observed phenotypic shift paralleled a decline in cytotoxic activity over successive rounds (Figure 4B), suggesting functional exhaustion of the CAR T cells. This trajectory is consistent with prior studies in which repeated antigen exposure drove differentiation from TSCM to short-lived effector populations,

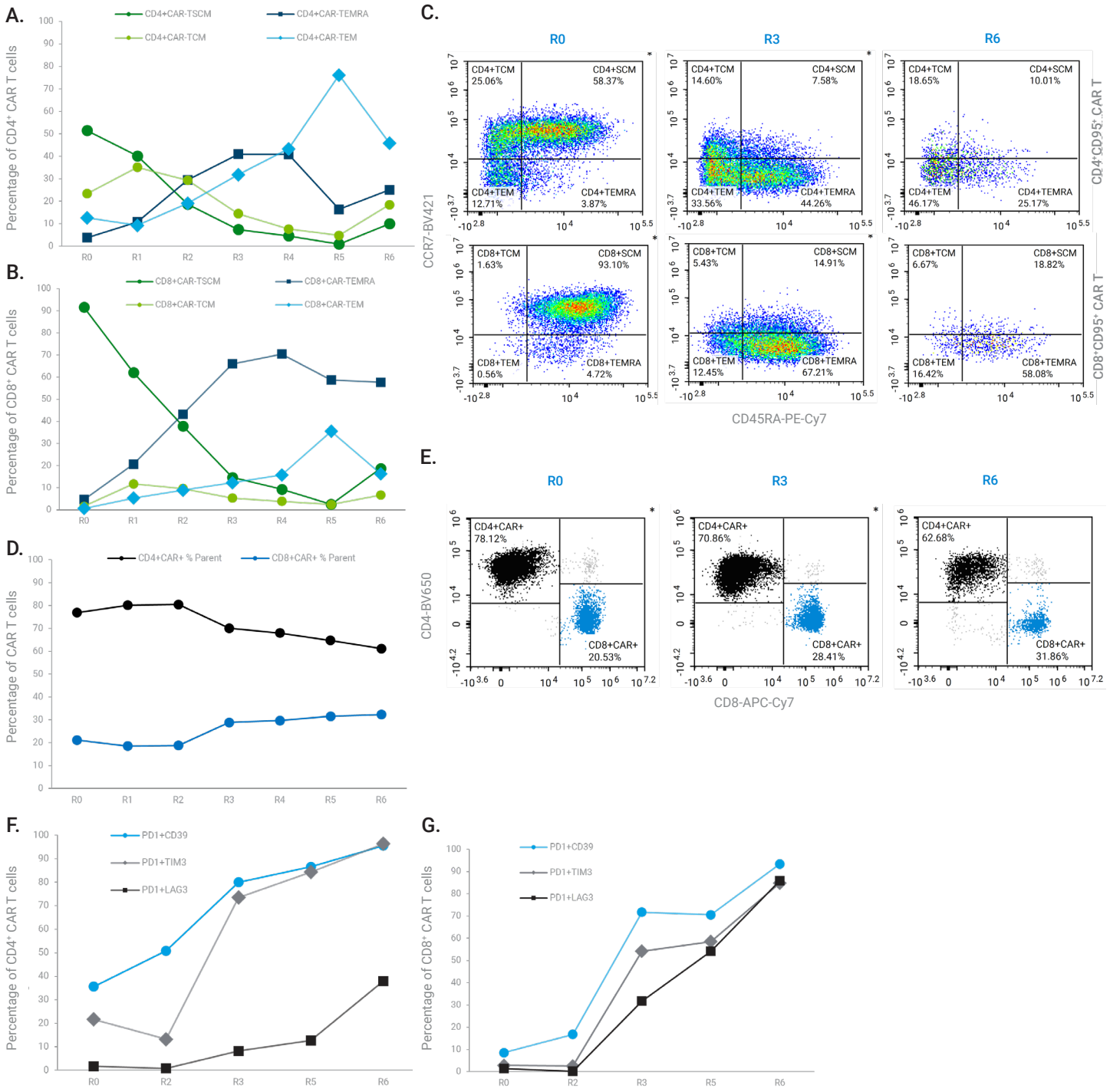

Figure 5. Changes in differentiation and exhaustion status of CD4⁺ and CD8⁺ subsets within the surviving EpCAM CAR T population were determined on the Agilent Novocyte Quanteon flow cytometer after each round of challenge with T-47D-red cells. A. and B. The plots show changes in memory and terminally differentiated phenotypes within CD4⁺ and CD8⁺ CAR T cells, respectively. C. Representative plots show CD4⁺ and CD8⁺ CAR T cells expressing CD95, were subsequently gated on the expression of CD45RA and CCR7 to identify stem cell memory (SCM), central memory (CM), effector memory (EM) and terminally differentiated effectors re-expressing CD45RA (TEMRA). D. Changes in CD4⁺ and CD8⁺ composition. E. Representative cytograms of CAR T cells after overnight rest following thawing (R0) or immediately after the third (R3) and sixth (R6) rounds of rechallenges. F. and G. The expression of exhaustion markers, CD39, TIM3 and LAG3 increased within the PD1-expressing CD4⁺ and CD8⁺ subsets of CAR T cells.

reducing therapeutic efficacy.[13,15]The higher TSCM content in the CD8⁺ subset, along with the greater heterogeneity and more differentiated state of CD4⁺ CAR T cells observed at baseline (Figure 5A to 5C), may explain the temporal changes in CD4⁺ and CD8⁺ CAR T cell proportions (Figure 5D and 5E), characterized by a decline in CD4⁺ T cells and relative persistence of CD8⁺ T cells. Importantly, PD-1 expression increased in both CD4⁺ and CD8⁺ CAR T cells, suggesting T cell activation and exhaustion driven by sustained antigen stimulation.[14, 16, 17] Consistent with current models, a progressive increase in PD1+ CAR-positive T cells (Figure 5F and 5G) co-expressing the markers of exhaustion, TIM3, LAG3, and CD39, indicated T cell exhaustion with repeated tumor challenge.

Our findings are consistent with the literature on the relevance of CAR T cell differentiation status in determining persistence and function. In this product, CD8⁺ CAR T cells enriched in TSCM phenotype demonstrated greater resilience to repeated tumor exposure compared to their CD4⁺ counterparts. These results support strategies to preserve or enrich less-differentiated T cell subsets during CAR T cell manufacturing to enhance long-term efficacy.[18] Additionally, they raise the possibility that modulating the differentiation status of CD4⁺ T cell phenotype could further improve CAR T functionality and therapeutic outcomes.

Metabolic reprogramming of CAR T cells over serial killing

The metabolism of CAR T cells is strongly associated with T cell fate and function. In the context of CAR T cell therapy, oxidative metabolism is associated with enhanced persistence and memory-like phenotypes, whereas a reliance on sustained glycolysis supports immediate effector functions but may contribute to T cell exhaustion and reduced long-term efficacy.[19-21] In addition to evaluating the sustained cytolytic activity of the cells and differentiation phenotype shifts, we were also interested in the impacts of rechallenges on the metabolic fitness of CAR T cells. For that, CAR T cells recovered after each killing assay were resuspended in XF assay medium before performing the XF T cell Metabolic Profiling assay. The real-time live cell metabolic profiles were then acquired using the Seahorse XF Pro analyzer.

Compared to the CAR T cells after revival (R0), an abrupt increase in the basal energy production, measured as ATP production rate, was observed after the initial antigen challenge (R1), which remained up to the second round of rechallenge and progressively declined to below the initial energetic activity (R0) (Figure 6A). The alteration of basal energy production reflects the change in ATP demand driven by activation, expansion, tumor eradication, and

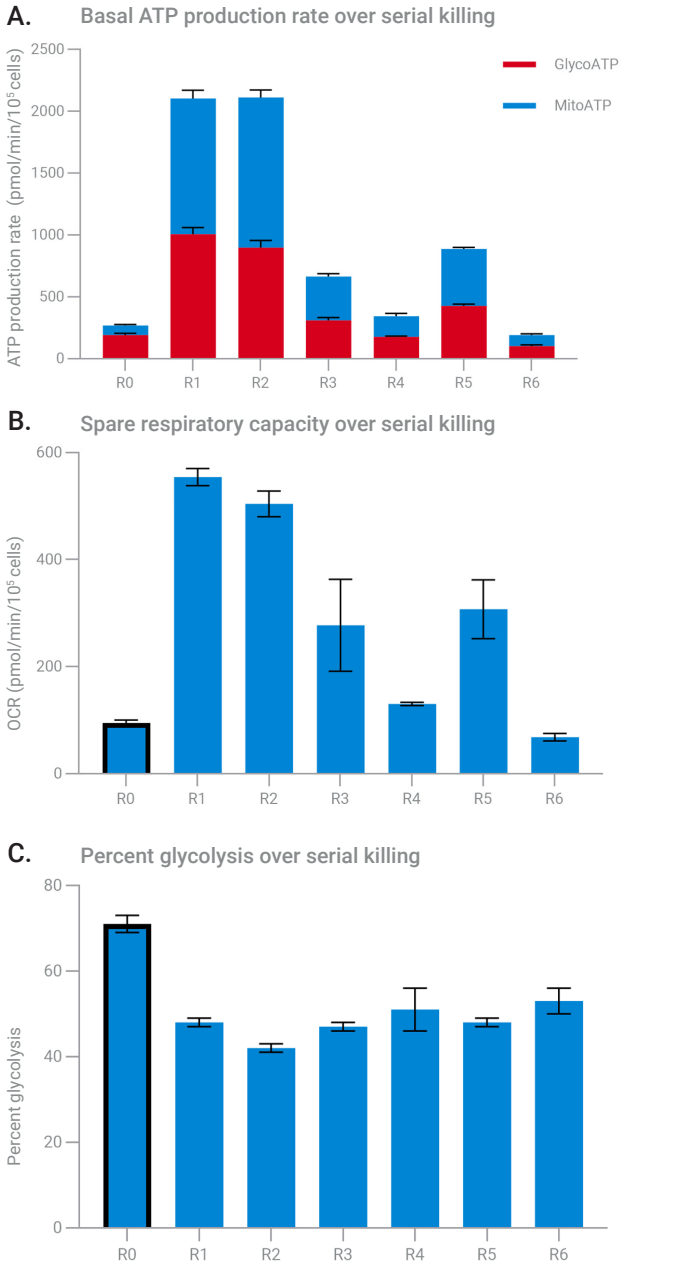

Figure 6. Dynamic changes in metabolic profile of CAR T cells across serial killing rounds. Basal ATP production rate (A) and spare respiratory capacity (B) normalized to the number of live T cells. C. Metabolic poise (measured as percent of glycolysis over total ATP Production rate).

differentiation throughout antigen rechallenges. The balance between oxidative phosphorylation and glycolytic metabolic pathways, referred to as metabolic poise, shifted from a glycolysis-dominant state (71% of total ATP production) after CAR T cell revival to an equal contribution from both glycolysis and mitochondrial oxidative phosphorylation immediately after initial antigen encounter. This balance was maintained

thereafter (Figure 6A and 6C). Analysis of mitochondrial spare respiratory capacity (SRC), defined as the difference between maximal and basal respiration, is often associated with T cell persistence and longevity.[22] Similarly to the trend in basal ATP production rate, SRC increased sharply after initial antigen exposure (R1), followed by a gradual decrease starting with the third round of rechallenge, except for a transient increase in SRC in round five (Figure 6B). The SRC decrease may reflect metabolic exhaustion of the CAR T cells, which was correlated to their reduced cytolytic ability (Figure 4).

Conclusion

Repetitive challenge of CAR T cells with tumor targets in vitro has proven to be a valuable model for evaluating differentiation status, persistence, and susceptibility to exhaustion.[23-26] Differentiation status determines the persistence of CAR T cells, which is relevant for therapeutic efficacy.[12,17]

In this study, we performed seven rounds of tumor cell rechallenges over three weeks to simulate the high tumor burden associated with in vivo tumor models. The Critical Quality Attributes of CAR T cells, including proliferative capacity, percentage of CAR expression, cell subsets, exhaustion, metabolic fitness, and durability of cytolytic activity, were concurrently monitored and determined using multiple Agilent analytical platforms throughout the entire process. Our results reveal a gradual enrichment of CAR-positive T cells from 26% to 86% over the first three rounds (Figure 3B), associated with fast CAR T cell expansion and senescence of untransduced T cells within the total population (Figure 2A).

In the meantime, progressive changes in differentiation and phenotypic exhaustion of the CAR T population occurred over successive tumor encounters. During the first three rounds of killing, the CAR T product was predominantly composed of highly persistent and proliferative TSCM cells. Over time, we observed a gradual contraction of the TSCM pool, coinciding with the increase in terminally differentiated TEMRA, a less sustainable and persistent subset than memory T cells. Over successive rounds, increasing expression of PD-1 coexpressed with other exhaustion markers, TIM3, LAG3, and CD39, in both $CD4^+$ CAR T and $CD8^+$ CAR T cells, suggested progressive phenotypic exhaustion. Along with the dynamic changes of T cell differentiation, a decrease in basal ATP production rate and spare respiratory capacity was also observed using the Agilent Seahorse XF analyzer, indicating a potential mitochondrial dysfunction associated with exhaustion. Consistent with this, the Agilent xCELLigence RTCA system captured a progressive decline in the killing potency of CAR T cells over successive antigen encounters, further illustrating the functional consequences of exhaustion.

Our results demonstrate that unique insights into distinct aspects of CAR T cell biology, including immunophenotype, metabolic state, and functional potency, can be gained through the use of each Agilent analytical system individually. These cellular properties are closely interconnected: less differentiated subsets, such as naive and memory T cells, exhibit greater mitochondrial fitness, supporting longer-term persistence and sustained cytotoxic function. In contrast, more differentiated effector T cells rely more on glycolysis, typically displaying short-term cytolytic activity and reduced durability. This interplay between phenotype, metabolism, and function highlights the need for integrated approaches when evaluating CAR T cell products. Therefore, a multimodal workflow was employed in this serial killing study. Integrating data from the Agilent NovoCyte flow cytometer, xCELLigence RTCA system, and Seahorse XF analyzer revealed complementary insights that together enabled a comprehensive characterization of CAR T cell immunophenotype, metabolism, and function throughout the serial killing process. This multimodal workflow not only holds potential for predicting clinical outcomes of CAR T cell products but also serves as a valuable tool for guiding CAR construct design and optimizing the cell manufacturing process.

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