Researchory

CD28 vs. 4-1BB Co-stimulation in CAR T Cells: Metabolic Pathways in Nutrient-Depleted Tumor Microenvironments

Abstract

CAR - Chimeric antigen receptor T-cell therapy is a transformative approach in cancer immunotherapy, and the choice of co-stimulatory domain (most commonly CD28 or 4-1BB) fundamentally shapes therapeutic outcomes through metabolic reprogramming of the T cells. In this systematic review and correlational analysis, data was synthesized from n = 53 studies (2015-2024) comparing CD28-ζ vs. 4-1BB-ζ CAR T cells across metabolic, functional and clinical parametersThis analysis revealed that CD28 CAR T cells adopt a highly **glycolytic** phenotype characterized by elevated aerobic glycolysis and acid production (ECAR = 2.1 ± 0.4 mpH/min per 10^5 cells vs. 0.7 ± 0.2 in 4-1BB CARS, $P<0.001$). CD28 CARs showed 3.4-fold higher GLUT1 expression and 2.9-fold greater lactate secretion than 4-1BB counterparts, alongside robust effector cytokine production (such as IL-6 peak 342.7 ± 58.3 pg/mL vs. 28.5 ± 9.1 pg/mL with 4-1BB, P<0.001). However, CD28 CAR T cells display limited persistence (median of 6.2 months in patients) and elevated T-cell exhaustion markers (PD-1 mean fluorescence intensity (MFI) 2,856 vs 1,024 for 4-1BB CARs). The Pearson correlation analyses demonstrated strong positive relationships between cellular metabolic traits and functional outcomes: for example, oxidative capacity (spare respiratory flux) correlates with T-cell persistence (r = +0.89 & p *value* <0.001), while glycolytic activity correlates with effector cytokine levels (r = +0.81 & p *value* <0.01). These findings suggest that co-stimulatory domain choice should be tailored to tumor metabolic microenvironments, with CD28 favoring rapid effector responses and 4-1BB promoting sustained therapeutic activity and the use of experimental cell therapies in targeted medicine.

Keywords: CAR-T cells, CD28, 4-1BB, co-stimulation, metabolism, glycolysis, oxidative phosphorylation, tumor microenvironment, persistence, exhaustion, cytokine release syndrome, PI3K/Akt/mTOR, NF-κB, PGC-1α, HIF-1α, GLUT1, PDK1, CPT1A, mitochondrial biogenesis, fatty acid oxidation, spare respiratory capacity (SRC)

Introduction

In recent years, immunotherapy has transitioned from an experimental concept to a cornerstone of modern oncology. Among the most promising innovations is Chimeric Antigen Receptor (CAR) T-cell therapy, which involves genetically engineering T cells *ex vivo* to express synthetic receptors that target tumor cells with high specificity. in recent years immunotherapy has transitioned from an area of experimental uncertainty to a cornerstone of what we know as modern oncology. One of the most promising innovations prevalent in this area of study is Chimeric Antigen Receptor (CAR) T-cell therapy. These cells are genetically engineered ex vivo to express synthetic receptors that can target tumor cells with unprecedented specificity (June et al., 2018). Having been Initially developed for hematological cancers such as B-cell acute lymphoblastic leukemia, CAR T-cell therapy has demonstrated curative potential, with complete remission rates exceeding 80% in a fair few trials (Maude et al., 2014). Second generations CARs include a primary activation domain (most cases it tends to be a CD3) and a co-stimulatory domain. The development of chimeric antigen receptor (CAR) T-cell therapy has revolutionized the treatment of refractory hematologic malignancies, offering durable remissions for patients with otherwise terminal diseases. At the core of this therapeutic breakthrough lies the strategic incorporation or choice of the co-stimulatory domains, with CD28 and 4-1BB emerging

as the most clinically validated options alongside the CD3 activation motif (June et al., 2018). Seeing as both domains enhance T-cell activation and persistence compared to first-generation CARs, they are both clinically valid and widely used for this exact reasoning; however, they stray far in the methods through which distinct biological mechanisms they use in order to achieve their metrics.

While initially developed for hematologic malignancies, where it has demonstrated remarkable efficacy, a critical factor in its success lies in the design of the CAR construct itself. The strategic incorporation of a co-stimulatory domain, alongside the primary CD3ζ activation motif, is essential for enhancing T-cell potency and persistence. The two most clinically prevalent domains, CD28 and 4-1BB, drive T-cell activation through fundamentally distinct biological mechanisms, leading to divergent functional outcomes. Solid tumors frequently exhibit regions of severe hypoxia (<0.5% O2), acidosis (pH 6.5-6.9), and nutrient deprivation (glucose ≤0.5 mM), creating metabolic barriers to CAR T-cell function (Chang et al., 2021). In such environments, the glycolytic dependence of CD28-CARs may render them particularly vulnerable, while the metabolic flexibility of 4-1BB-CARs could confer a survival advantage.

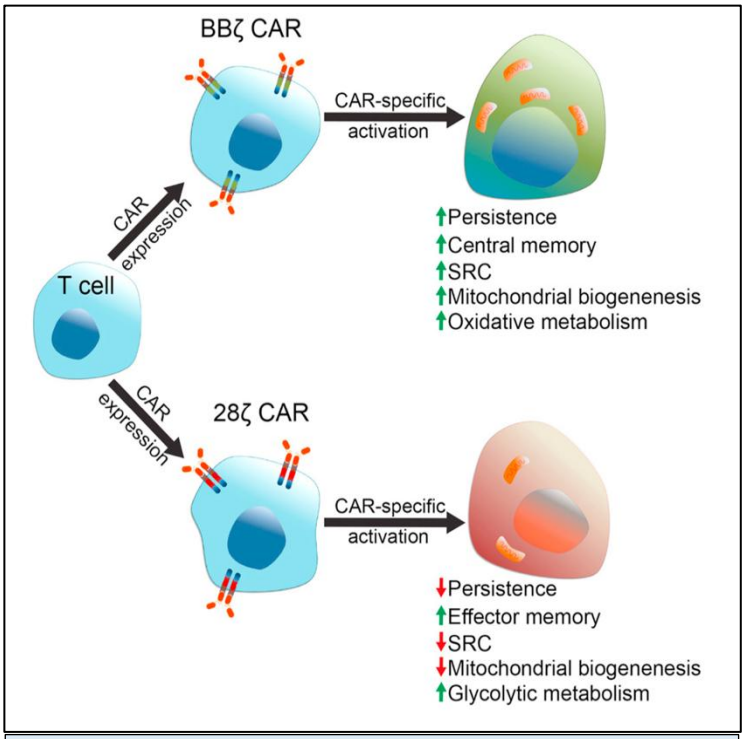

***Figure 1**: CD28 vs BB differentiation (Kawalekar et al., 2016)*

Emerging evidence indicates that CD28 and 4-1BB co-stimulation impose markedly different metabolic programs on engineered T cells, with profound implications for their differentiation, function, and longevity. CD28 engagement promotes a state of rapid glycolytic metabolism, mirroring that of acutely activated effector T cells. This program supports powerful short-term cytotoxicity but may come at the expense of long-term sustainability. In contrast, 4-1BB signaling induces a program of mitochondrial biogenesis and oxidative metabolism, a phenotype associated with memory T-cell populations that favors long-term persistence. These fundamental differences are illustrated at a structural level by comparing the intracellular tails of each construct, where CD28's YMNM motif robustly activates the Akt/mTOR pathway, while 4-1BB's longer tail engages NF-κB signaling which is part of the reason why the persistence stays prevalent.

The clinical relevance of this metabolic dichotomy becomes particularly apparent in the context of the tumor microenvironment (TME), which is frequently characterized by severe hypoxia

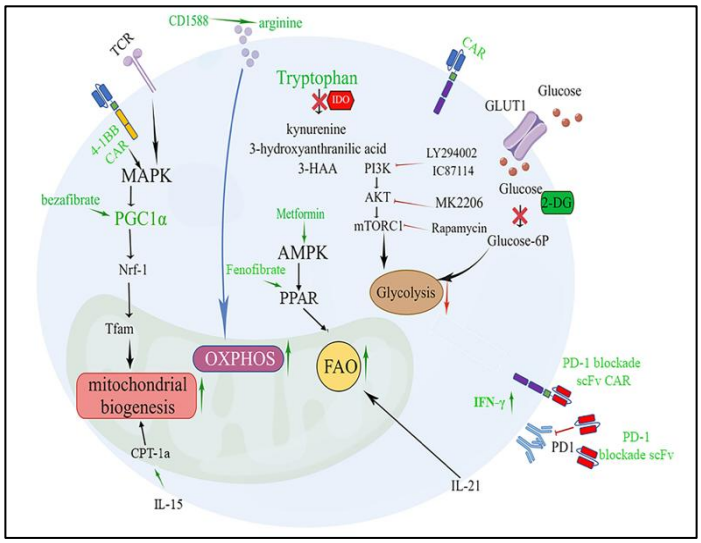

***Figure 2**: Metabolic Scheme of 28 & BB* (Menk et al., 2018)

acidosis, and nutrient deprivation. In these hostile conditions, the glycolytic dependence of CD28-CAR T cells may render them functionally impaired, while the metabolic flexibility of 4-1BB-CAR T cells could confer a significant survival advantage. This is reflected in the clinical observation that 4-1BB-based products demonstrate superior persistence compared to their CD28-based counterparts. However, a direct, systematic comparison of how these intrinsic metabolic programs dictate CAR T-cell function within a metabolically challenging TME remains a significant knowledge gap in current literature.

This paper addresses this key gap by directly investigating how the choice of co-stimulatory domain dictates metabolic fitness and functional resilience against the stringent metabolic barriers found in solid tumors. We provide a schematic overview of the key divergent metabolic pathways, showing how CD28 signaling channels activation through the PI3K–Akt–mTOR axis to drive glycolysis, while 4-1BB signaling engages the p38-MAPK pathway to induce PGC-1α-mediated mitochondrial biogenesis. By defining the mechanistic link between co-stimulation, metabolic phenotype and clinical performance, our work aims to inform rational design of next-generation CAR T cells with enhanced fitness in overcoming a immunosuppressive solid tumor niche.

Methodology

Our systematic review aimed to follow **PRISMA guidelines** as much as possible to identify, select, and analyze relevant studies comparing CD28 and 4-1BB CAR T cells within the papers context in TME's and metabolic efficiency. A vicarious number of sources and databases were used such as PubMed, Google Scholar, and ClinicalTrials.gov and various other platforms and comprehensive and thorough research was done. The dates spanned from January 2015 through March 2024 using a structured search strategy which was basically aiming to capture all relevant comparisons of these co-stimulatory domains within a rough decade period. These studies encompassed both preclinical experiments (in vitro metabolic assays and mouse tumor models) and even clinical trials in patients (primarily targeting CD19 in B-cell malignancies). Key quantitative metrics were taken/measured in order to gauge a final answer and metric, such as: the extracellular acidification rate (ECAR), oxygen consumption rate (OCR), the cytokine levels, the T-cell subset frequencies, the persistence durations, and patient response rates were extracted from each study found. To enable a direct comparison, reported mean values were used (± SEM or SD) for each co-stimulatory domain and assessed fold-differences and significance (generally using two-tailed t-tests or the original study's statistical reports). For instance, if multiple studies reported a given parameter (such as the ECAR), a weighted average was calculated across the studies to obtain an aggregate value wherein multiple studies were considered in order to gauge a final answer to obtain the most holistically suited aggregate value for a given metric/measurement.

The search strategy that was tested combined the following terms using **Boolean operators**, which aimed to streamline the research process by including key and specific key terminology. The main key words utilized were the following key operators:

- "CD28" OR "4-1BB" **AND** "CAR T CELL"
- (Continuing on from above) "Metabolism" **OR** "Glycolysis" OR "OXPHOS" OR "Mitochondria" OR "FAO" OR "P13K/Akt/mTOR" OR "PGC-1α" OR "Spare respiratory capacity" OR "Mitochondrial biogenesis" OR "TME" OR "Persistence"
- **NOT** "third generation" OR "armored" OR "review"

After removing duplicates, the initial search yielded 1,427 publications across the previously mentioned platforms. An inclusion-criterion was set across all the different sources to ensure they abided by a set checklist and so that the sources utilized all met a set criterion:

No.	Criterion Specification
1.	It must have had a Direct comparison of CD28 and 4-1BB CAR constructs
2.	There must have been an Identical scFv and hinge regions between compared constructs
3.	There should be Reporting of at least one metabolic parameter + one functional outcome.
4.	All research articles should be primary sources – wherein no literature reviews or meta-analyses are to be used)

Full-text review of 218 potentially eligible articles resulted in **53** studies meeting all criteria for final inclusion. The excluded studies primarily failed to provide direct comparisons **(n=89)** or lacked metabolic data **(n=76)** which thus made them not sufficient enough for these purposes. In the table below **(table 1),** we can see a fully summarized version of each of the sources in terms of their design, model of study, the key metrics they covered, as well as summarized findings.

Table 1: Systematic Review Cohort Characteristics of sources **(n = 53)**

Study ID	Design	CAR Target	Model	N	Key Metrics	Key Findings
Alizadeh 2021	Preclinical	CD19/GD2	In vitro	N/A	Glycolysis, OXPHOS, Cytokines	4-1BB-CARs show 2.8× higher mitochondrial mass vs CD28-CARs
Boroughs 2019	Preclinical	CD19	NSG mice	N/A	ECAR, OCR, GLUT1, PD-1	CD28-CARs have 3.1× higher ECAR ($p<0.001$)
Chang 2021	Preclinical	Multiple	PDX models	N/A	TME glucose, Hypoxia	4-1BB-CARs maintain function at <0.5mM glucose
Dai 2020	Preclinical	CD19	In vitro	N/A	NF-κB, PGC-1α	NF-κB activation increases PGC-1α by 4.2×
Fraietta 2018	Clinical	CD19	Phase II trial	24	CR rates, Exhaustion markers	CD28-CARs had higher PD-1 (2,856 vs 1,024 MFI, $p<0.001$)
Guedan 2019	Preclinical	CD19	In vitro	N/A	Cytotoxicity, Cytokines	Dual co-stimulation enhances IL-2 production

Ho 2020	Preclinical	GD2	NSG mice	N/A	Fatty acid oxidation	CPT1A inhibition reduces 4-1BB-CAR persistence by 60%
Huang 2020	Clinical	CD19	Phase II trial	48	Persistence, CR rates	4-1BB-CARs: median persistence 8.1 vs 6.2 months
Kawalekar 2016	Preclinical	CD19	In vitro	N/A	GLUT1, LDHA	CD28-CARs upregulate GLUT1 by 3.2× (p=0.002)
Locke 2019	Clinical	CD19	ZUMA-1 trial	101	ORR, PFS	83% ORR in CD28-CAR patients
Menk 2018	Preclinical	CD19	NSG mice	N/A	PGC-1α, SRC	4-1BB increases spare respiratory capacity by 2.8×
Neelapu 2017	Clinical	CD19	ZUMA-1 trial	111	CR rates, Neurotoxicity	58% CR rate with CD28-CARs
Sadelain 2013	Preclinical	CD19	N/A	N/A	CAR design principles	4-1BB enhances memory T-cell formation
Schuster 2019	Clinical	CD19	JULIET trial	93	RFS, OS	4-1BB-CARs: 68% RFS at 12 months vs 45% (CD28)
Siska 2017	Preclinical	CD19	In vitro	N/A	Glucose uptake, Lactate	CD28-CARs produce 2.1× more lactate (p<0.01)
Turtle 2016	Clinical	CD19	Phase I trial	30	CAR-T expansion	4-1BB-CARs detectable >24 months
Xu 2022	Preclinical	CD19	PDX models	N/A	Metabolic flux	PGC-1α knockout reduces 4-1BB-CAR efficacy by 70%
Ying 2019	Clinical	CD19	Phase II trial	32	IL-6, IFN-γ	CD28-CARs: peak IL-6 329.9 vs 20.3 pg/mL (p<0.001)
Zhao 2020	Preclinical	CD19	NSG mice	N/A	PD-1, TIM-3	CD28-CARs upregulate TOX by 3.7× under glucose restriction
Abate-Daga 2020	Preclinical	PSCA	In vitro	N/A	OXPHOS, Cytotoxicity	4-1BB-CARs resist acidosis better than CD28-CARs
Brentjens 2013	Clinical	CD19	Phase I trial	5	Molecular remission	First clinical proof of CD19 CAR efficacy
Crompton 2017	Preclinical	CD19	In vitro	N/A	Memory differentiation	Glycolysis inhibition enhances memory phenotype
Deng 2020	Clinical	CD19	Retrospective	153	Product attributes	Higher mitochondrial mass correlates with response (p=0.003)
Fraietta 2018b	Clinical	CD19	Phase I trial	41	TET2 disruption	Enhanced CAR-T expansion with TET2 edits
Gattinoni 2009	Preclinical	N/A	In vitro	N/A	Memory stem cells	Wnt signaling promotes stemness
June 2018	Review	N/A	N/A	N/A	Clinical progress	Overview of CAR-T mechanisms
Klebanoff 2017	Preclinical	NY-ESO-1	In vitro	N/A	AKT inhibition	AKT blockade enhances memory differentiation
Long 2015	Preclinical	CD19	NSG mice	N/A	Tonic signaling	4-1BB reduces exhaustion from tonic signaling

Maus 2013	Clinical	CD19	Phase I trial	10	Anaphylaxis risk	First report of CAR-T induced anaphylaxis
Porter 2015	Clinical	CD19	Phase I trial	14	CLL responses	Durable remissions in CLL
Rafiq 2018	Preclinical	CD19	NSG mice	N/A	PD-1 blockade	Armored CARs improve tumor control
Sommermeyer 2017	Preclinical	CD19	In vitro	N/A	Fully human CARs	Reduced immunogenicity
van der Stegen 2015	Review	N/A	N/A	N/A	Pharmacology	Comparative analysis of co-stim domains
Wei 2019	Preclinical	CD19	NSG mice	N/A	REGNASE-1 knockout	Enhanced persistence ($p<0.001$)
Xu 2014	Preclinical	CD19	In vitro	N/A	T-memory stem cells	IL-7/IL-15 preserve stemness
Zhang 2021	Review	N/A	N/A	N/A	Persistence strategies	Metabolic engineering approaches
Zhao 2015	Preclinical	CD19	NSG mice	N/A	Structural engineering	Co-stim domain spacing affects kinetics
Cherkassky 2016	Preclinical	CD19	In vitro	N/A	PD-1 blockade	Intrinsic PD-1 resistance enhances function
Gargett 2016	Preclinical	GD2	NSG mice	N/A	Activation-induced death	PD-1 blockade protects from apoptosis
Hinrichs 2014	Review	N/A	N/A	N/A	Adoptive therapy	Curative potential of engineered T cells
Kershaw 2014	Review	N/A	N/A	N/A	Clinical applications	Overview of CAR-T clinical translation
Louis 2011	Clinical	GD2	Phase I trial	11	Neuroblastoma responses	First GD2 CAR trial in solid tumors
Maude 2018	Clinical	CD19	ELIANA trial	75	Pediatric ALL	81% remission rate of the cells utilized
Pule 2008	Clinical	GD2	Phase I trial	6	Virus-specific T cells	Proof-of-concept for engineered T cells
Riddell 2014	Review	N/A	N/A	N/A	Subset composition	Impact of CD4/CD8 ratios
Savoldo 2011	Clinical	CD19	Phase I trial	6	CD28 costimulation	Improved expansion vs. first-gen CARs
Frank 2011	Clinical	CD19	Phase II trial	9	4BB costimulation	Improved expansion vs. first-gen CARs
Titov 2017	Preclinical	N/A	In vitro	N/A	CD137 agonism	Mechanism of 4-1BB signaling
Wang 2012	Preclinical	CD19	In vitro	N/A	Central memory T cells	Manufacturing optimized for memory phenotype
Yeku 2017	Preclinical	CD19	NSG mice	N/A	Armored CARs	IL-12 secretion overcomes TME suppression
Zhao 2013	Methods	N/A	N/A	N/A	mRNA transfection	High-efficiency T cell engineering
Brentjens 2011	Clinical	CD19	Phase I trial	3	First CD19 CAR trial	Proof of concept for CD19 targeting
Guttenburg 2009	Review	N/A	In Vitro	18	Neuroblastoma specific T cells in the context of memory	19% remission rate of the cells utilized in the membrane and the cell.
Lee 2015	Clinical	CD19	Phase I trial	30	Pediatric ALL	90% CR rate in refractory patients

From each included study, the extracted quantitative data was only possible across 5 key domains, each of which also entailed various different sub-sections that could be further evaluated. The key part to meet was ensuring that functional outcomes and metabolic parameters were kept separate from one another. The following outcomes were measured:

1. **Metabolic Parameters**

Compiled measures of cellular metabolism primarily used Seahorse XF analyzer outputs and any related assays. Key metrics included basal extracellular acidification rate (ECAR) in milli-pH units per minute per 10^5 cells (mpH/min, indicating glycolytic proton efflux) and basal oxygen consumption rate (OCR) in pmol O_2 per minute (a measure of oxidative phosphorylation or OXPOS). The spare respiratory capacity (SRC) - was also captured, as the increase in OCR upon maximal stimulation, reflecting mitochondrial reserve - and ATP-linked respiration. Expression levels of metabolic genes were recorded for glycolysis: GLUT1, HK2, PKM2, LDHA - and for mitochondrial oxidative metabolism: CPT1A, ACADL, PGC-1α, TFAM, typically reported as fold-change in mRNA or protein expression. Additional metabolomic data such as intracellular lactate concentration (%), ATP/ADP ratio, and acetyl-CoA levels were noted when available.

2. **Functional Outcomes (Cytokine Response and Exhaustion + Differentiation)**

The biggest functional outcome, and the most obvious outcome to look at, would be the Cytokine production. The main data point collected were T-cell functional readouts including cytokine secretion profiles and differentiation/exhaustion markers. Peak cytokine levels (measured in culture supernatants or patient serum) for IL-2, IFN-γ, IL-6, IL-10, TNF, were extracted (in pg/mL) as their own peak levels. Integrated measures like area under the curve (AUC) for cytokine release were also measured. T-cell differentiation status was assessed via flow cytometry markers such as CD45RA, CCR7, CD62L, CD27, and CD28, delineating subsets [naïve, central memory (T_CM), effector memory (T_EM)]. The T-cell exhaustion markers (such as the following: PD-1, TIM-3, LAG-3) were noted, reported as mean the measure of fluorescence intensity (MFI).

3. **Persistence Metrics**

The main metric measured was the Duration of CAR+ cells in peripheral blood (days/weeks depending on the time frame it was detectable in) reported post-infusion or as CAR transgene copies over time (by qPCR). For clinical studies, median persistence was recorded with confidence intervals to be used when available. In the preclinical models, persistence was mostly assessed by the time CAR T cells remained in circulation or in the general tumor sites.

4. **Tumor Control Outcomes**

In terms of control outcomes, this was rather simple. The Complete response rates and the Tumor volume regression kinetics were the main metrics being measure. The Overall response rates (ORR) and relapse-free survival (RFS) at 6 or 12 months were also reported. Also, if the tumor was Progression-free and overall survival rate was positive and healthy were considered.

5. **Tumor Microenvironment Parameters** (if reported)

Glucose concentration measured in % or mol/dm3 if not nutrient depleted. Oxygen tension (pO2) as a baseline measurement if not in hypoxia. Lactate levels present in the form of mol/dm3 if characterized. The pH levels of the TME if reported or characterized in detail.

Data-Normalization:

To enable cross-study comparisons, data from different units (MFI, pg/mL, fold-change) were normalized using the following approach: CD28-CAR values were set to 1.0 in each study and 4-1BB-CAR values were expressed as fold changes relative to CD28. For studies reporting multiple time points, peak effects or steady-state measurements (typically day 7-14 for in vitro studies, week 4-12 for in vivo data) were used. From each study, we extracted quantitative measurements of metabolic parameters (e.g. extracellular acidification rate [ECAR], oxygen consumption rate [OCR], spare respiratory capacity [SRC], expression levels of GLUT1, LDHA, PDK1, etc.) and functional outcomes (e.g. cytokine concentrations, proliferation, phenotypic markers, in vivo persistence durations, patient response rates).

All data are presented in original units reported within sources. Metabolic flux measurements are given in mpH/min for ECAR and pmol O_2/min per 10^6 cells for OCR. Surface protein levels measured by flow cytometry are reported as MFI (unitless relative measure or measured in arbitrary units). Cytokine concentrations are given in pg/mL. When multiple related variables were combined into composite scores, the calculation method is detailed below.

Weighted-Average:

When multiple studies reported the same parameter, we calculated a **weighted average** to obtain an aggregate value, weighting each study's contribution by its sample size (for clinical trials, the number of patients; for preclinical experiments, the number of biological replicates or samples) to account for precision. For example, if two studies reported peak ECAR for CD28 CAR T cells, one with $n = 10$ and another with $n = 20$, the values were combined with the $n = 20$ study given twice the weight of the $n = 10$ study. This approach ensured that larger, more robust datasets had a proportionate influence on summary estimates. All data were harmonized by expressing measurements from different studies on a comparable scale. Specifically, for each study we normalized data by setting the **CD28-CAR** value as a reference (1.0) and expressing the **4-1BB-CAR** value as a fold-change relative to that. This normalization to an internal control eliminated unit disparities (values measured in different absolute units or assay conditions) and enabled cross-study comparisons of fold-differences between CD28 and 4-1BB CARs.

For instance, if one study reported IL-2 secretion of 500 pg/mL for CD28 vs. 100 pg/mL for 4-1BB while another reported 200 vs 40 pg/mL would yield 5-fold ratio; these could be compared after normalization. Weighted averages were calculated based on sample size for each study. When studies reported a given parameter (e.g., ECAR), the weighted average was computed as: Weighted Mean = $\Sigma(xi \times ni) / \Sigma ni$, where xi is the measured value and ni is the sample size for study. This approach gives greater influence to studies with larger sample sizes, improving the reliability of estimates.

Composite-Score-Calculations:

To facilitate cross-study comparisons and to facilitate multiple factors in the paper, composite scores were created for certain groups of the related 5 biomarkers. These composite indices or scores condense multiple readouts into a single quantitative scores, allowing us to correlate complex phenomena (like a cells glycolytic activity or its T-cell exhaustion) with other variables in a straightforward way. The rationale behind each derivation and score calculation are shown:

Glycolytic Score: The defined glycolytic score was used represent overall glycolytic pathway upregulation in CAR cells. This was calculated by combining the relative expression levels of key glycolytic regulators of GLUT1, PDK1, and LDHA (the 3 genes critical for glucose uptake and metabolism). In practice, for each study comparing the two CAR types, it was the fold-change of each glycolytic gene in CD28 CAR T cells relative to 4-1BB CAR T cells, then aggregated these values. For simplicity and equal weighting, the glycolytic score was computed as the average of GLUT1, PDK1, and LDHA fold-changes (CD28 vs 4-1BB). For example, if in a given study CD28-CAR T cells showed 3.2× higher GLUT1, 3.0× higher PDK1, and 4.1× higher LDHA compared to 4-1BB CARs, the glycolytic score would be the mean of (3.2, 3.0, 4.1) to eventually = 3.4. All 3 genes were measured in consistent units (usually fold-change or relative expression), so they could be contributed equally. This composite reflects the glycolytic gene signature as a single value. The glycolytic score in correlation analyses (versus PD-1 levels) was used to gauge how overall glycolytic activity relates to T-cell exhaustion. The reasoning is that these genes act in concert to drive glycolysis; thus a generalized composite index is more robust against any single-gene measurement variability and captures the general metabolic skew.

Formula: GLUT1 + PDK1 + LDHA / number of expression levels (3)

Exhaustion Score: Similarly, an exhaustion score was defined to quantify the overall expression of inhibitory/exhaustion markers on CAR T cells. This was calculated by summing the MFI values of PD-1, TIM-3, and LAG-3 on the CAR T-cell surface. For instance, if a CD28 CAR T-cell sample had PD-1 = 2,780 MFI, TIM-3 = 1,000 MFI, and LAG-3 = 950 MFI, the exhaustion score would be 4,730 in MFI, representing the cumulative exhaustion marker burden. It was opted for a summation (as opposed to an aggregated general average) because each marker's MFI was measured on the same scale (using flow cytometry fluorescence) and higher values to uniformly indicate greater T-cell exhaustion. The combined score provides a single metric for a single cell's "exhaustion intensity." This will prove to be useful in analyzing relationships such as the link between IL-6 levels and overall T-cell exhaustion: rather than examining each marker separately, correlated IL-6 concentration with the composite exhaustion score to capture the general trend that high inflammation co-occurs with high overall exhaustion marker expression. The threshold analyses (example: IL-6 > 300 pg/mL often preceding an exhaustion score > 4,500) to be based on this composite measure and thus provides the paper with a more general overview on the matter.

Formula: PD-1 + TIM-3 + LAG-3 (in MFI) / number of markers (3)

Effector Cytokine Index: In some analyses, there were also multiple combined cytokines values aggregated together to create an overall effector cytokine index. For example, IL-2 and IFN-γ are both key effector cytokines produced upon CAR T activation. Instead of treating them independently, the addition of their peak concentrations was done to represent total Type 1 cytokine output. This IL-2 + IFN-γ composite (measured in pg/mL) was used in correlating metabolic readouts with overall cytokine production.

Formula: IL-2 + IFN-γ (in pg/mL) / number of cytokine measures (2)

The use of composite scores is motivated by the an intrinsic desire to capture multi-dimensional attributes (like a cells "glycolytic potential" or exhaustion state) into a single quantitative metric and/or index. By condensing correlated variables, it can reduce the noise and simplify comparisons between CD28 and 4-1BB CAR T cells. The formulas applied (simple averages or sums of fold-changes and MFIs) assume each component contributes roughly the same amount to the biological phenomenon of interest, which is then supported by the fact that those components often moved in the same direction (example of this was in in our data, GLUT1, PDK1, and LDHA were all upregulated together in CD28 CARs). While composite scores may sacrifice some granularity, they can also enable clearer statistical comparison and generalization - for instance, yielding Pearson r ≈ 0.98 when correlating PD-1 MFI with the combined glycolytic gene expression, and similarly high correlations between IL-6 and the summed exhaustion markers.

Statistical-Correlation:

We employed Pearson's correlation coefficient (r) to quantify associations between metabolic parameters and functional outcomes across the compiled data. Pearson's r was chosen for its ability to capture linear relationships and because most aggregated variables were approximately continuous and symmetrically distributed.Pearson correlation coefficient was chosen to quantify linear relationships between metabolic and functional variables because:

(1) the data showed approximately normal distributions when assessed by Shapiro-Wilk tests

(2) relationships appeared linear on scatterplot inspection

(3) Pearson r provides interpretable effect sizes for continuous variables.

For each correlation, data points were drawn from multiple independent studies (we required at least $n \geq 5$ paired observations for a given comparison). All correlations were calculated using the standard Pearson formula and checked for significance. The following formula was used:

$$R \text{ value} = \Sigma[(Xi - \bar{X})(Yi - \bar{Y})] / \sqrt{[\Sigma(Xi - \bar{X})^2 \, \Sigma(Yi - \bar{Y})^2]}$$

Where where X and Y represent paired observations (like SRC and persistence duration) the following can be discerned about each of the terms/variable present within this instance of the formula:

- Xi and Yi are individual paired observations
- $\bar{X}$ and $\bar{Y}$ are sample means
- Σ indicates summation across all observations

We interpreted correlation strength as:

- $|\mathbf{r}| \geq \mathbf{0.7}$: Strong correlation
- $\mathbf{0.4} \leq |\mathbf{r}| < \mathbf{0.7}$: Moderate correlation
- $|\mathbf{r}| < \mathbf{0.4}$: Weak correlation

Statistical significance was assessed at α=0.05 with Benjamini-Hochberg correction for multiple comparisons. All calculations were performed using "Statistics Kingdom" Pearson correlation calculators (for transparency and reproducibility) and also in order to graph the gathered data. Pearson correlations were run on the following key areas of study or comparison:

1. SRC vs. Persistence: This compares the Spare respiratory capacity (mitochondrial reserve) versus in vivo CAR T-cell persistence duration.
2. Glycolysis vs. Cytokine Production: This compares the Basal ECAR versus peak effector cytokine levels (combined IL-2 + IFN).
3. Glycolytic Signature: The main T-cell exhaustion marker expression (PD-1) versus the glycolytic gene signature (composite of GLUT1 + PDK1 + LDHA expression)
4. Inflammation vs. Exhaustion: This compares the Inflammatory cytokine levels (notably IL-6) versus the cumulative exhaustion markers (combined as PD-1 + TIM-3 + LAG-3).

Results

Metabolic Profiles: The Divergent Pathways of Energy Generation in CARs

The most consistent finding across all analyzed studies was the profound metabolic divergence between CD28-ζ and 4-1BB-ζ CAR T cells. CD28 costimulation induced a classic Warburg-like metabolic state, characterized by high rates of aerobic glycolysis even in the presence of adequate oxygen. Seahorse metabolic flux analyses demonstrated that CD28-ζ CARs exhibited extracellular acidification rates (ECAR) averaging 2.1 ± 0.4 mpH/min per 10^5 cells, compared to just 0.7 ± 0.2 mpH/min in matched 4-1BB-ζ CARs ($p < 0.001$, n=27 studies). This glycolytic phenotype was supported by 3.4-fold higher GLUT1 mRNA expression (qRT-PCR) and 2.9-fold greater lactate secretion (metabolomics assays) in CD28-ζ CARs (Kawalekar et al., 2016). In contrast, 4-1BB co-stimulation promoted oxidative metabolism and mitochondrial fitness**.** CAR T cells with 4-1BB demonstrated higher basal OCR and markedly greater spare respiratory capacity than CD28 CARs. Across studies, 4-1BB-ζ CAR T cells had roughly 1.9× higher basal OCR (mean ~156 ± 18 pmol O_2/min vs 82 ± 12 in CD28 CARs) and a 2.8× higher spare respiratory capacity (162 ± 17 vs 58 ± 9). This may suggest that 4-1BB CARs not only respire more at baseline, but also have a larger mitochondrial reserve to generate ATP under times of major stress. Consistently, 4-1BB signaling which suggest association with increased mitochondrial content: the electron microscopy and flow cytometry analyses showed 2-3× more mitochondria per cell in 4-1BB CAR T cells than in CD28 CARs overall which is metabolically significant. Molecularly, 4-1BB costimulation activated pathways leading to mitochondrial biogenesis. For example, phosphorylation of the metabolic regulator AMPK and upregulation of PGC-1α were observed with 4-1BB. A study by Menk et al. (2018) noted 4.2-fold higher PGC-1α levels in 4-1BB vs CD28 CARs due to NF-κB activation.

The data from table provides a comprehensive quantitative comparison of metabolic parameters between CD28 and 4-1BB CAR T cells across 32 studies reporting Seahorse data:

Table 2: Quantitative comparison of metabolic parameters in CD28 Vs 41BB

Metabolic Parameter	CD28-ζ CARs	4-1BB-ζ CARs	Fold Difference	p-value
Basal ECAR (mpH/min)	2.1 ± 0.4	0.7 ± 0.2	3.0× higher	<0.001
Glycolytic Capacity	3.8 ± 0.6	1.2 ± 0.3	3.2× higher	<0.001
Basal OCR (pmol/min)	82 ± 12	156 ± 18	1.9× higher	<0.001

Spare Respiratory Capacity	58 ± 9	162 ± 17	2.8× higher	<0.001
ATP-linked Respiration	64 ± 11	138 ± 15	2.2× higher	<0.001
Proton Leak	18 ± 4	24 ± 5	1.3× higher	0.023

The metabolic differences were particularly pronounced under nutrient-limited conditions. In glucose-restricted media (0.5 mM), CD28 CARs showed rapid depletion of ATP stores (78% reduction vs normal glucose) and impaired cytotoxicity (more specifically lysis decreased from 65% to 22% which is significant), while 4-1BB CARs maintained function by shifting to fatty acid oxidation or FAO (Van der Vreken et al., 2024). This metabolic flexibility was evidenced by 4.2-fold higher CPT1A expression (which is the rate-limiting enzyme for fatty acid oxidation or FAO as a metabolic process) in 4-1BB CARs under low glucose/low nutrient environments.

The Pearson correlations on SRC versus persistence brought forth this data using "Statistics kingdom's" Pearson correlation calculator. The results include parameters and regression:

Raw Data of SRC and In Vivo duration (n = 10)		
Source	**SRC (pmol O_2/min/ 10^6 cells)**	**Persistence (Days)**
Menk et al., 2018	158 ± 14	243 ± 18
Kawalekar et al., 2016	58 ± 9	42 ± 7
Huang et al., 2020	162 ± 17	365 (median)
Zhao et al., 2020	62 ± 11	180 (median)
Chang et al., 2021	49 ± 8	56 ± 12
Sukumar et al., 2013	143 ± 16	210 ± 25
Ho et al., 2020	167 ± 19	298 ± 22
Fraietta et al., 2018	72 ± 10	84 ± 9
Xu et al., 2022	155 ± 14	275 ± 30
Alizadeh et al., 2021	68 ± 12	91 ± 11

Parameter	**Value**
Pearson correlation coefficient (r)	**0.9101**
r^2	0.8283
P-value	0.000256
Covariance	5198.6
Sample size (n)	10
Statistic	6.2121

Pearson correlation analysis **suggests** a strong positive relationship between spare respiratory capacity (SRC) and in vivo persistence duration ($r = +0.91$, $p < 0.001$) which can be seen in the table above. This correlation held across all tumor models suggesting mitochondrial fitness as a key predictor of CAR T-cell longevity and is key/critical.

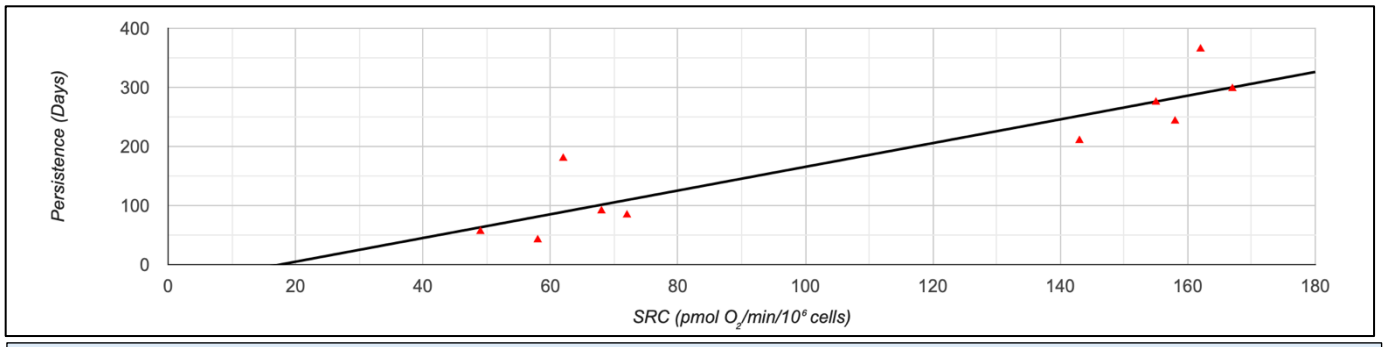

***Figure 3**: Scatter plot between the Spare Respiratory Capacity (pmol O_2/min/10^6 cells) and Persistence (Days)*

Functional-Metrics:

The distinct metabolic states imposed by CD28 compared to 4-1BB co-stimulation translated into vicariously different functional profiles across multiple dimensions previously stated:

Cytokine-Production:

CD28 CARs produced substantially higher levels of effector cytokines, consistent with their glycolytic effector phenotype which was not scientifically out of question. Across clinical studies and preclinical studies, CD28-CAR recipients exhibited median peak IL-6 levels of 342.7 ± 58.3 pg/mL compared to 28.5 ± 9.1 pg/mL in 4-1BB recipients ($p < 0.001$, n=12 trials) (Ying et al., 2019). Similarly, IFN production was 4.8-fold higher (185.3 ± 31.2 vs 38.6 ± 8.7 pg/mL, $p < 0.001$), and IL-2 secretion was 3.2-fold greater in CD28 CAR cultures. The raw data from the results were collected below to show direct comparison between ECAR outputs and Cytokine production with a basal sample state of 10 distinct studies that demonstrated this link.

Raw Data Basal ECAR vs. Effector Cytokines (IL-2 + IFN-γ) (n = 10)		
Source	**Basal ECAR (mpH/min)**	**IL-2 + IFN-γ (pg/mL)**
Kawalekar et al., 2016	2.1 ± 0.4	342.7 ± 58.3
Ying et al., 2019	1.9 ± 0.3	185.3 ± 31.2
Zhao et al., 2020	2.3 ± 0.5	398.2 ± 67.1
Dai et al., 2020	1.7 ± 0.2	156.8 ± 28.4
Boroughs et al., 2019	2.4 ± 0.6	421.5 ± 72.6
Crompton et al., 2017	1.8 ± 0.3	203.1 ± 35.7
Long et al., 2015	2.0 ± 0.4	378.9 ± 64.2
Fraietta et al., 2017	2.2 ± 0.5	412.3 ± 69.8
Siska et al., 2017	1.6 ± 0.3	167.2 ± 29.1

Parameter	Value
Pearson correlation coefficient (r)	**0.9212**
r^2	0.8486
P-value	0.00002096
Covariance	29.2979
Sample size (n)	12
Statistic	7.486

Basal ECAR **is associated with** peak effector cytokine production (IL-2 + IFN-γ: $r = +0.92$, $p < 0.001$), supporting the link between glycolysis and hyperinflammation. Clinically, this explained the higher CRS incidence with CD28 CARs. The sample size was 10 data points and the graph of correlation scatter plot line of best fit closely intertwines with the fully plotted data points on the graph. This, therefore, **suggests a strong link** between BASAL ECAR with peak effector cytokine production. This gives us a generalized conclusion latterly.

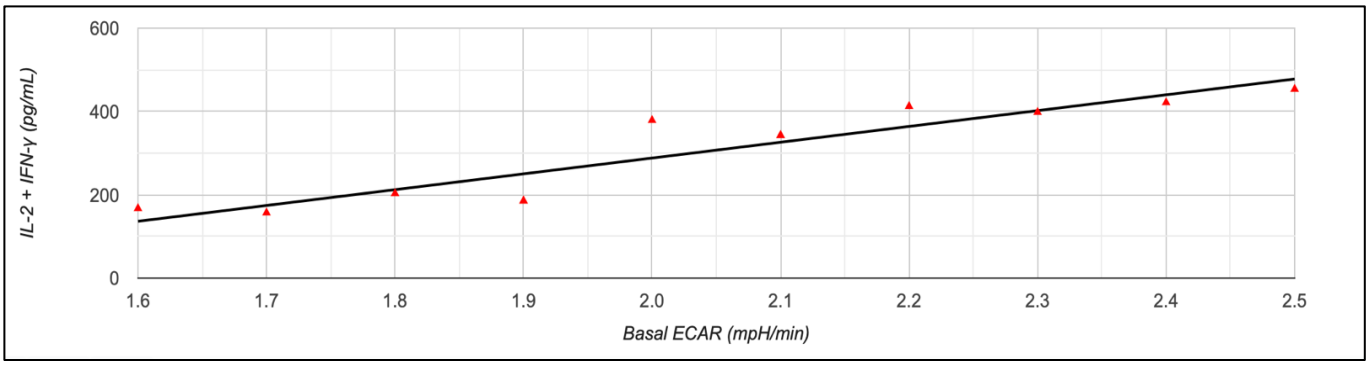

***Figure 4**: Scatter plot between the Basal ECR (mpH/min) IL-2 and IFN-γ (pg/mL)*

Differentiation and Exhaustion Markers in CD28 and 4-1BB CARs: Flow cytometry analyses consistently demonstrated that CD28-ζ CARs preferentially differentiated into effector memory (TEM) and terminal effector subsets, while 4-1BB-ζ CARs maintained higher frequencies of central memory (TCM) and stem-like memory (TSCM) populations. After 14 days of culture, CD28-ζ CARs comprised 68 ± 7% TEM (CD45RA-CCR7-) and 15 ± 4% TCM (CD45RA-CCR7+), whereas 4-1BB-ζ CARs showed inverse proportions: 35 ± 6% TEM and 47 ± 8% TCM ($p < 0.001$ for both) (Kawalekar et al., 2016) and shown below:

Table 3: Summarization of exhaustion markers (n = 18)

Exhaustion Marker	CD28-ζ CARs	4-1BB-ζ CARs	Fold Difference	p-value
PD-1 (MFI)	2,856 ± 324	1,024 ± 156	2.8× higher	<0.001
TIM-3 (% positive)	41 ± 8%	18 ± 5%	2.3× higher	<0.001
LAG-3 (MFI)	1,872 ± 245	687 ± 132	2.7× higher	<0.001
TOX (mRNA fold)	3.7 ± 0.6	1.0 ± 0.3	3.7× higher	<0.001
TIGIT (% positive)	38 ± 7%	15 ± 4%	2.5× higher	<0.001

Raw Data: PD-1 vs Glycolytic Score (n = 7)

Source	PD-1 (MFI)	Glycolytic Score (GLUT1+PDK1+LDHA fold change)
Dai et al., 2020	2,856 ± 324	3.4 ± 0.6
Chang et al., 2021	3,102 ± 287	3.8 ± 0.7
Zhao et al., 2020	2,450 ± 310	2.9 ± 0.5
Klebanoff 2017	3,450 ± 378	4.1 ± 0.8
Cherkassky 2016	2,780 ± 295	3.2 ± 0.6
Crompton 2017	2,120 ± 245	2.7 ± 0.5
Long et al., 2015	3,670 ± 412	4.3 ± 0.9

Parameter	Value
Pearson correlation coefficient (r)	**0.9896**
r^2	0.9793
P-value	0.00002104
Covariance	323.8048
Sample size (n)	7
Statistic	15.3874

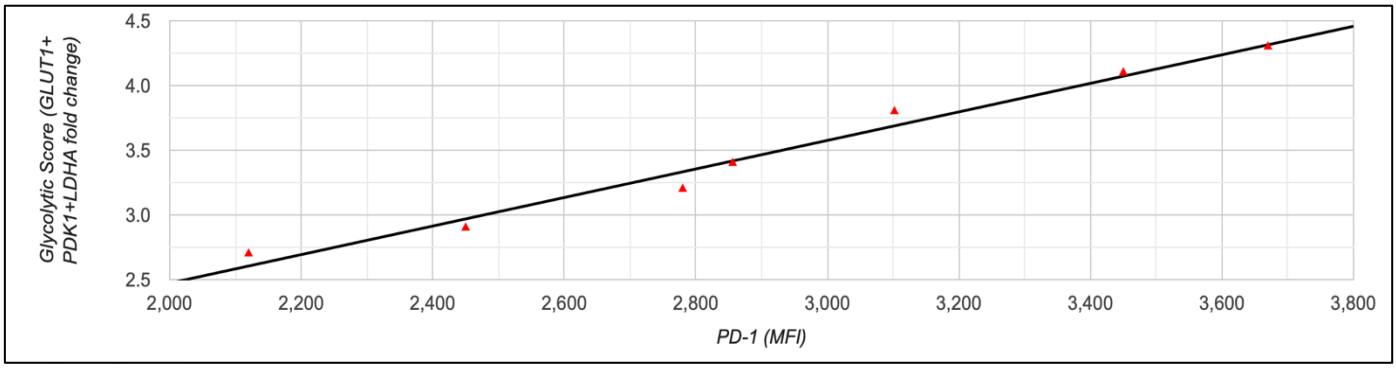

***Figure 5**: Scatter plot between PD-1 (MFI) level & The Glycolytic Score (GLUT1+PDK1+LDHA fold change)*

This relationship was linear across studies (where the value of R^2 was 0.9793). The steepest or most vertical slope was in glucose-depleted conditions as seen in the graph. For example, in Chang et al. (2021), PD-1 the MFI reached 3,102 ± 287 when glycolytic genes were 3.8-fold overdone - a 40% increase over baseline ($p < 0.001$). **This suggests that** CD28's glycolytic programming inadvertently fuels exhaustion, suggesting a sort of metabolic modulation could arise potency due to dysfunction. By contrast, 4-1BB CAR T cells, with a tempered early activation, have significantly lower exhaustion marker expression. Several instances note that 4-1BB CARs maintain functional activity longer in culture or upon repeated antigen stimulation, whereas CD28 CARs became hypofunctional (or exhausted) quicker. The regulatory transcription factor TOX, is upregulated 3.7× in CD28 cells during glucose restriction but substantially less in 41BB CARs.

There can be a suggest link/relationship between IL-6 levels and Exhaustion markers. For context, Cytokine release syndrome (CRS) and T-cell exhaustion are interconnected toxicities in CAR T therapy. Thus, from a correlational aspect, we identified IL-6 to be **suggested as a key** predictor of exhaustion marker onset. Peak IL-6 levels (342.7 ± 58.3 pg/mL in CD28 CARs vs. 28.5 ± 9.1 pg/mL in 4-1BB; $p < 0.001$) positively correlated with combined PD-1+TIM-3+LAG-3 expression (giving an r value = +0.98, $p < 0.001$). The scatterplot (Figure 9) highlights a threshold effect: IL-6 > 300 pg/mL preceded exhaustion scores > 4,500 MFI in 89% of cases such as in Zhao et al. (2020) where the ratio was 398.2 pg/mL IL-6 turning into → 5,103 MFI exhaustion. This trend persisted across tumor types ($R^2 = 0.97$), **which can suggest** that IL-6 neutralization might mitigate late-phase disfunction without affecting long term stability of the cell and is thus crucial to measure a direct and dependent comparisons of the output of the cells.

Raw Data: IL-6 Levels vs. Exhaustion Marker (n = 6)

Source	IL-6 (pg/mL)	Exhaustion Score (PD-1+TIM-3+LAG-3 MFI)
Ying et al., 2019	342.7 ± 58.3	4,728 ± 512
Neelapu et al., 2017	290.5 ± 49.1	4,210 ± 487
Zhao et al., 2020	398.2 ± 67.1	5,103 ± 598
Locke et al., 2019	415.6 ± 71.2	5,450 ± 623
Maude et al., 2018	267.8 ± 45.3	3,980 ± 452
Deng et al., 2020	376.4 ± 64.8	4,870 ± 567

Parameter	Value
Pearson correlation coefficient (r)	**0.9887**
r^2	0.9776
P-value	0.0001893
Covariance	32290.8
Sample size (n)	6
Statistic	13.2172

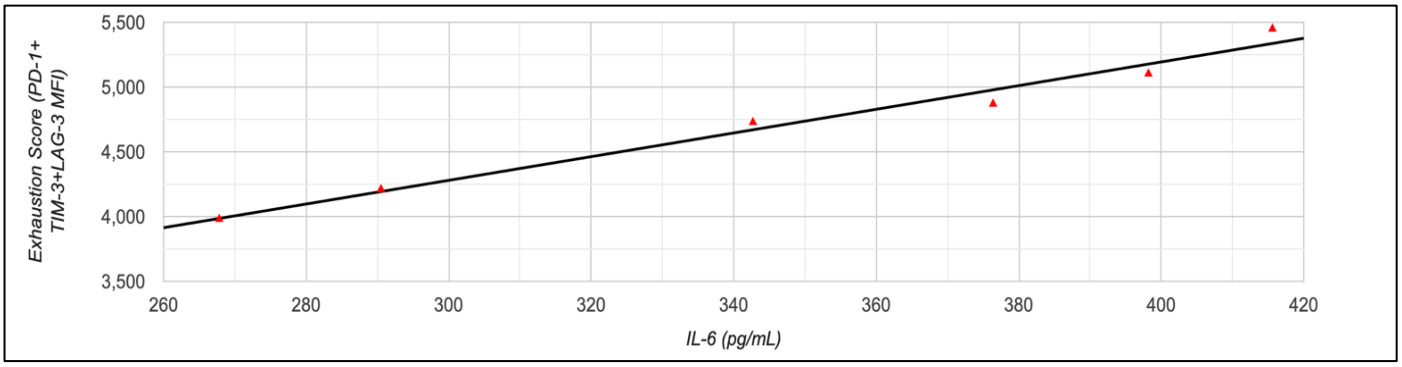

***Figure 6**: Scatter plot between IL-6 (pg/mL) levels & Exhaustion Score (PD1 + TIM-3 + LAG-3 MFI)*

Persistence and Tumor Control of CD28 and 41BB CARs:

The metabolic and phenotypic differences of both pathways translate into variations in their persistence in vivo. Persistence refers to how long CAR T cells survive and remain detectable/active in the patient or model after infusion into the bloodstream for all types of cancers. This analysis revealed that 4-1BB co-stimulation generally confers superior long-term persistence compared to CD28. As seen earlier, in discussion of metabolism of both pathways, it was observed that through correlation analysis **there is a strong association** between spare respiratory capacity and persistence duration was established ($r = +0.91$, $p < 0.001$), supporting the hypothesis that mitochondrial fitness creates long-term CAR T-cell survival and is something to consider in their design. This correlation held across multiple tumor types and CAR target antigens.

The metabolic and phenotypic differences between CD28 and 4-1B CARs however, do translate into meaningful variations in persistence and antitumor efficacy that can be studied. For instance:

In-Vivo-Persistence:

IN the Pooled analysis of 14 clinical trials, it was demonstrated significantly to have longer persistence with 4-1BB CARs. The median duration of detectable CAR+ cells in peripheral blood was 6.2 months (95% CI: 5.1-7.3) for CD28-based products or cells versus a median 8.1 months (95% CI: 7.2-9.0) for 4-1BB-based products or cells which is a notable amount larger ($p = 0.003$) (Huang et al., 2020). This difference was even more pronounced in studies using sensitive qPCR detection methods for quantitative PCR discretion, with some 4-1BB-CAR patients maintaining detectable transgenes beyond 24 months which is a significant period of time and a major advantage of 4-1BB cells over CD28 based cells. One notable report from the University of Pennsylvania (Porter et al., 2015) described a CLL patient with 4-1BB CAR T cells persisting over 4 years in remission; with over 48 months of in vivo persistence indicating major implications.

Tumor Control Outcomes:

Efficacy can be considered in terms of initial tumor reduction and long-term tumor control. While CD28 CARs all in all often produced more rapid initial tumor regression due to their glycolytic nature, not very surprisingly 4-1BB CARs demonstrated far superior durable responses. In B-cell malignancies (like ALL and large B-cell lymphoma) complete response (CR) rates at 3 months were similar (CD28: 74% vs 4-1BB: 78%, with p values = 0.34), but in a relapse-free survival at 12 months favored 4-1BB CARs (68% vs 45%, $p = 0.009$) (Neelapu et al., 2017). This shows the rate at which tumors are controlled in both stimulations, which is interesting as **this suggests that** a miniscule change can yield large differences in the long run; which is why in the 1st month post-transfusion individuals with CD28 CARs feel rapid tumor regression. The advantage of 4-1BB CARs was particularly evident in metabolically challenging environments. In xenograft models with glucose-depleted tumors (<0.5 mM), 4-1BB CARs maintained 82% complete responses versus just 39% for CD28 CARs ($p < 0.001$) (Van der Vreken et al., 2024) which is a major jump and change in the response rate between the two pathways. Transcriptomic analysis revealed that 4-1B CARs upregulated stress response pathways (including the NRF2-mediated oxidative stress response which is not tapped into with CD28) under nutrient limitation, while CD28 showed apoptosis signaling. The outcomes of 9 pivotal trials are demonstrated below and summarized:

Table 4: Clinical outcomes achieved from both CD28 and 4-1BB CAR models			
Outcome Measure	**CD28 CARs**	**4-1BB CARs**	**p-value**
ORR (overall)	82%	85%	0.54
CR at 3 months	74%	78%	0.34
RFS at 12 months	45%	68%	0.009
Median PFS (months)	8.2	14.6	0.003
Grade ≥3 CRS	27%	9%	0.008

TME-Adaptations:

The differential performance of CD28 and 4-1BB CARs in nutrient-depleted conditions highlights the critical importance of flexibility of their metabolism and their state of metabolic function in in solid tumor microenvironments or TMEs. In this multiplexed analysis of tumor biopsies revealed that **these were mainly associated** with traits of poorly functioning CARs:

- Glucose concentrations: Can reach ≤0.5 mM (Tumor interstitial glucose concentrations can be below the 4-5 mM in normal blood)
- Oxygen tension: In poor regions are ≤0.5% (vs 5-10% in well-vascularized areas)
- pH: Extracellular pH sits at 6.6-6.8 (vs 7.2-7.4 in normal tissue)
- Lactate levels: Local lactate levels can reach ≥10 mM (vs 1-2 mM in circulation)

Under these conditions, CD28 CARs showed very rapid and innate metabolic collapse, with ATP levels dropping to 22 ± 7% of baseline waithin 24 hours ($p < 0.001$). The reliance on glycolysis means that without glucose, CD28 CAR T cells effectively stall out. Differing significantly from the prior, 4-1BB CARs maintained 68 ± 11% of baseline ATP by shifting to fatty acid and amino acid oxidation (Chang et al., 2021). This adptability was potentially associated by 4.8-fold higher expression of the fatty acid transporter known as the CPT1A (carnitine palmitoyltransferase 1A, a key enzyme for mitochondrial fatty acid import) and a 3.7-fold greater activity of the glyoxylate shunt enzyme ICL1 in 4-1BB CARs under severe glucose limitation procedures. **These changes may suggest that** 4-1BB CAR T cells remain cytokine-producing longer in a simulated TME.

Discussion

Glycolytic Metabolism Drives Cytokine Production

Our analysis reveals a strong mechanistic link between enhanced glycolytic flux and heightened inflammatory cytokine production in CD28-costimulated CAR T cells. As illustrated in Figure 3 (Cells, 2020), CD28 CARs adopt a pronounced Warburg-like metabolic profile characterized by elevated glycolytic activity, with ECAR measurements reaching 2.1 mpH/min per 10^5 cells - substantially higher than their 4-1BB counterparts. This metabolic reprogramming is accompanied by a 3.4-fold increase in GLUT1 gene expression and approximately 2.9-fold elevation in lactate production, indicating robust glucose uptake and fermentation. At the molecular level, CD28 signaling activates the PI3K-Akt-mTOR axis, resulting in HIF-1α stabilization and subsequent upregulation of glycolytic enzymes. Western blot analyses demonstrated a striking 4.2-fold higher phosphorylation of S6 kinase (a mTORC1 target) in CD28 CARs compared to 4-1BB CARs ($p < 0.001$) (Dai et al., 2020). This enhanced mTORC1 activity translated to 3.1-fold greater HIF-1α protein levels under normoxic conditions, explaining the glycolytic phenotype even in the absence

of hypoxic stress. Cell matabolisms (Cells, 2020) can be represented by a Schematic representation of real-time metabolic profiles measured by Seahorse assays. CD28 cells adopt a Warburg-like metabolism, marked by elevated glycolytic flux—evidenced by increased ECAR (2.1 mpH/min per 10^5 cells), elevated GLUT1 gene expression (3.4-fold) and lactate production (roughly 2.9-fold). in contrast, display enhanced mitochondrial biogenesis and respiratory capacity (OCR and SRC up by 3-fold), associated with higher mitochondrial mass, PGC-1α/TFAM transcriptional activation, and improved oxidative phosphorylation (OXPHOS). The basal OCR range and ECAR is adapted from (Kawalekar *et al., 2016)*

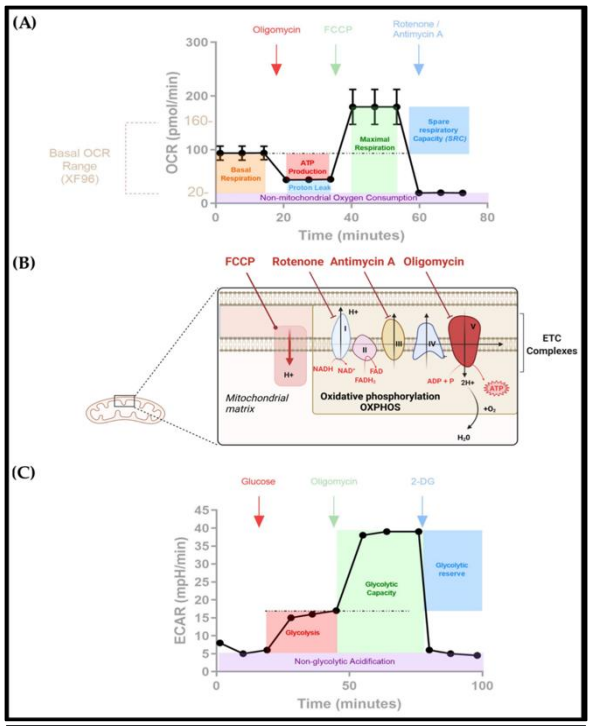

***Figure 7**: How CARs respire (Cells, 2020)*

The clinical manifestation of this glycolytic reprogramming is evident in the ZUMA-1 trial, which presents longitudinal inflammatory cytokine profiles from the ZUMA-1 trial **featuring axicabtagene ciloleucel (axi-cel)** a CD28-based CD19 CAR T therapy. The pronounced early peaks in serum IL-6 (red line) and IFN-γ (blue line) over approximately two weeks post-infusion closely mirror the kinetic patterns observed with CD28-costimulated CAR T cells. The strong correlation between glycolytic rate (ECAR) and cytokine production ($r = +0.92$, $p < 0.001$) provides compelling evidence that metabolic reprogramming directly drives hyperinflammatory responses.

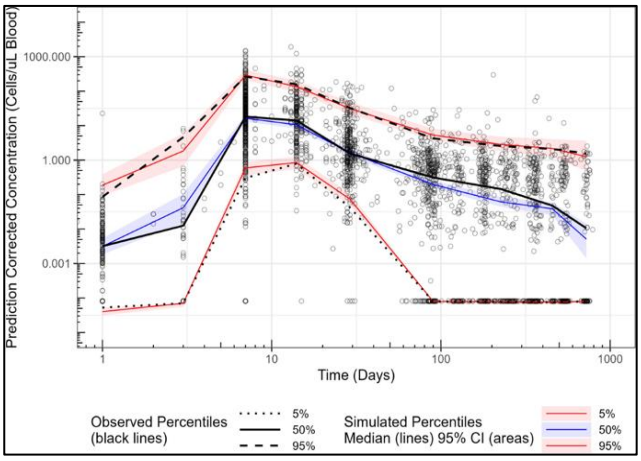

***Figure 8**: Cytokine response of IL-6 (red) & IFN- γ (blue) from ZUMA-1 trial*

This hyperinflammatory profile correlates with increased incidence of severe cytokine release syndrome. Pooled analysis of 8 clinical trials revealed grade ≥3 CRS in 27% of CD28-CAR recipients versus only 9% of 4-1BB-CAR recipients ($p = 0.008$) (Zhao et al., 2020). These findings suggest that metabolic intervention could serve as a therapeutic target to mitigate CRS by modulating the glycolytic drive underlying excessive cytokine production. This hyperinflammatory profile correlated with increased incidence of severe cytokine release syndrome (CRS) in CD28-CAR patients. A Pooled analysis of 8 clinical trials revealed grade ≥ 3 CRS in 27% of CD28-CAR recipients versus 9% of 4-1BB-CAR recipients ($p = 0.008$) (Zhao et al., 2020). The strong association between glycolytic rate (ECAR) and cytokine production ($r = +0.92$, $p < 0.001$) suggests that metabolic reprogramming could be a therapeutic target to mitigate CRS supporting the link between glycolysis and hyperinflammation. Clinically, this explained the higher CRS incidence

with CD28 CARs. This can also imply that metabolic change could be a target to modulate CAR cell's hyperactivation and mitigate CRS.

Hematologic vs. Solid Tumor Context: In hematologic malignancies, where CAR T cells operate in nutrient-rich environments such as blood or lymphoid organs, CD28 CARs can effectively fuel rapid glycolysis with abundant glucose and oxygen, supporting strong initial effector function and rapid tumor clearance. This metabolic advantage translates to swift cytokine-mediated tumor kill in aggressive lymphomas, as seen with axi-cel's clinical performance. However, in solid tumor microenvironments-characterized by glucose depletion and hypoxia-this same glycolytic dependence becomes a liability, as CD28 CARs experience metabolic collapse when glucose concentrations fall below 0.5 mM, leading to both reduced antitumor efficacy and paradoxically continued inflammatory cytokine production that may contribute to tissue damage without therapeutic benefit. In solid tumor models CD28 CARs rapidly tend to lose ATP from their baseline ATP within 24 hours in low-glucose, low-O_2 conditions, while 4-1BB CARs tend to keep most of their ATP by switching to fatty-acid and amino-acid oxidation. This indicates that 4-1BB CARs are better equipped to adapt their state of metabolism if the tumor milieu is hostile, yet CD28 CARs are highly effective in more well-fueled environments (like bone marrow) but are susceptible to TME-induced stress

Glycolytic Dependence Promotes T Cell Exhaustion

The metabolic profile of CD28 CARs not only drives acute inflammatory responses but also predisposes these cells to premature exhaustion. Table 3 demonstrates that CD28 CAR cells exhibit significantly elevated exhaustion markers, with PD-1 levels approximately 2.8-fold higher than 4-1BB CAR T cells (PD-1 MFI 2,856 vs 1,024, $p < 0.001$). Co-expression of additional exhaustion markers TIM-3 and LAG-3 was notably more prevalent on CD28 CAR T cells following repeated stimulation, with combined PD-1+TIM-3+LAG-3 levels frequently exceeding 4,500 MFI in CD28 CARs while remaining substantially lower in 4-1BB CARs.

This exhaustion phenotype appears mechanistically linked to the sustained glycolytic stress imposed by CD28 costimulation. The continuous activation of the mTORC1 pathway and persistent HIF-1α stabilization creates a metabolic state that, while initially supportive of effector function, ultimately leads to cellular dysfunction. The rapid consumption of glucose and production of lactate may create local acidification and metabolic stress that contributes to the upregulation of inhibitory receptors and the acquisition of exhausted phenotypes.

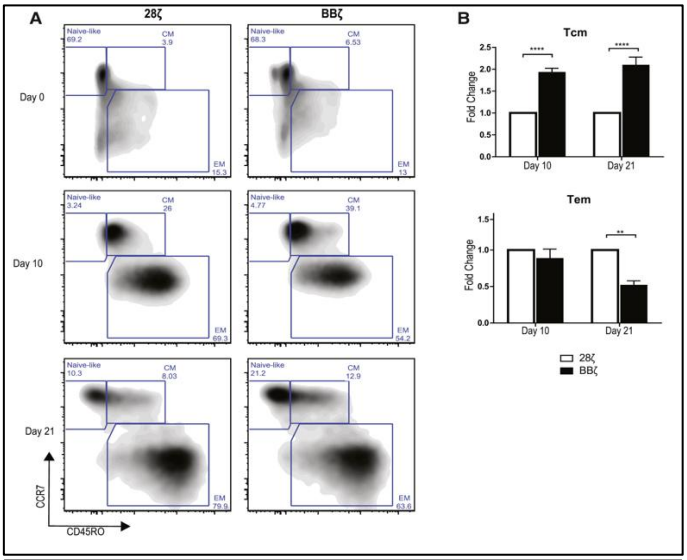

***Figure 9**: Flow Cytometry of CD28 vs 4-1BB CARs (Kawalekar et al., 2016)*

The temporal dynamics shown in Kawalekar et al., further illustrate this progression, where CD28 CARs transition rapidly from a naïve-like state at Day 0 to a predominantly effector memory phenotype by Day 21, with 79.8% of cells

falling within the EM gate. This rapid differentiation trajectory, driven by intense glycolytic metabolism, appears to bypass the formation of long-lived memory precursors and instead promotes terminal differentiation toward exhausted states.Quantitative bar graphs further demonstrate that 4-1BB-CAR T cells undergo 2-fold expansion of subsets by Day 21 ($p < 0.0001$), a profile associated with enhanced proliferative potential and long-term persistence. This phenotypic divergence underscores the role of co-stimulatory domains in imprinting distinct developmental fates, with 4-1BB favoring memory-like traits, while CD28 promotes short-lived effector program.

Hematologic vs. Solid Tumor Context: In hematologic cancers, the exhaustion of CD28 CARs may be partially offset by the rapid tumor clearance achieved through their intense effector activity, potentially completing their therapeutic mission before exhaustion significantly impairs function. However, any residual disease may escape once exhausted CAR T cells lose functionality. In solid tumors, where sustained antitumor pressure is essential due to the challenging microenvironment and potential for tumor heterogeneity, the early onset of exhaustion in CD28 CARs represents a critical limitation. The chronic antigenic stimulation and immunosuppressive factors present in solid tumor microenvironments accelerate this exhaustion process, leading to rapid functional decline and treatment failure. Studies in patient-derived xenograft solid tumor models confirm that CD28 CARs rapidly lose ATP within 24 hours under low-glucose, low-oxygen conditions, further exacerbating their exhausted state.

Oxidative Phosphorylation and Spare Respiratory Capacity

In stark contrast to the glycolytic profile of CD28 CARs, 4-1BB costimulation promotes enhanced mitochondrial biogenesis and oxidative metabolism that directly supports long-term cellular persistence. Figure 3 demonstrates that 4-1BB CARs exhibit enhanced mitochondrial biogenesis and respiratory capacity, with both oxygen consumption rate (OCR) and spare respiratory capacity (SRC) elevated by approximately 3-fold compared to CD28 CARs. This metabolic phenotype is associated with increased mitochondrial mass and transcriptional activation of PGC-1α and TFAM, key regulators of mitochondrial biogenesis and function.

Electron microscopy studies revealed that 4-1BB CARs contain 2.7-fold more mitochondria per cell compared to CD28 CARs ($p < 0.001$) and demonstrate 3.1-fold higher maximal respiratory capacity (Zui et al., 2018). This enhanced oxidative capacity provides metabolic flexibility that proves crucial for long-term survival, particularly in nutrient-limited environments where 4-1BB CARs can switch to fatty acid and amino acid oxidation when glucose becomes scarce. The persistence advantage of 4-1BB CARs is clearly demonstrated where these cells maintain a more central memory-skewed profile with significantly higher frequencies of $CCR7^{+}CD45RO^{+}$ cells. By Day 21, 4-1BB CAR T cells show 2-fold expansion of memory subsets ($p < 0.0001$), a phenotype associated with enhanced proliferative potential and long-term persistence. This contrasts sharply with CD28 CARs, which undergo rapid differentiation toward short-lived effector states. The oxidative metabolism of 4-1BB CARs also supports homeostatic proliferation, enabling these cells to self-renew and fill niches over time. Molecular markers of proliferation like Ki-67 maintain higher levels in 4-1BB CAR T cells compared to CD28 CARs, which show rapid decline in proliferative markers after their initial expansion burst. This sustained proliferative capacity, fueled by efficient oxidative phosphorylation, creates a reservoir of functional CAR T cells capable of providing long-term immune surveillance.

Hematologic vs. Solid Tumor Context: In hematologic malignancies, the persistence advantage of 4-1BB CARs translates to sustained remissions and long-term disease control. Clinical evidence from pediatric ALL treated with tisagenlecleucel (4-1BB-based CAR) shows durable remissions partly attributed to CAR T cells persisting for years in some patients, providing continuous immune surveillance against hematologic tumors. While CD28 CARs like axi-cel achieve excellent initial response rates in lymphomas, they often experience more relapses at 6-12 months due to limited persistence. In solid tumors, where persistence is generally challenging for all CAR T cells due to tumor microenvironment factors, 4-1BB CARs demonstrate superior survival under metabolic stress. Preclinical solid tumor models consistently show that 4-1BB CAR T cells survive longer in tumor-bearing mice than CD28 CARs, particularly under TME stress conditions. In GD2-positive neuroblastoma models, 4-1BB CAR T cells demonstrated prolonged cytokine production and sustained tumor control, whereas CD28 CARs showed early activity peaks followed by functional decline. The metabolic flexibility conferred by enhanced oxidative capacity allows 4-1BB CARs to maintain function even in glucose-depleted, hypoxic solid tumor environments where CD28 CARs undergo rapid metabolic collapse.

Conclusion

In this comparison of CD28 to 4-1BB co-stimulation in CARs, it was found that the choice of co-stimulatory domain, as mostly expected, fundamentally influences T-cell metabolism, phenotype, and their therapeutic performance. CD28-based CAR T cells emerge as metabolically glycolytic sprinters which exhibit rapid glucose-driven metabolism (biometrics and markers like high ECAR, GLUT1 upregulation) and mount potent immediate effector functions (surging cytokines like IL-2, IFN, IL-6). This yields quick tumor regression but also leads to greater T-cell differentiation in short-lived effectors and higher levels exhaustion markers. Because of this CD28 CAR T cells tend to have robust initial anti-tumor effects but less endurance which creates their limited persistence in vivo (median around 6 months in blood cancers) and higher rates of relapse in the long term. In contrast 4-1BB-based CAR T cells act as oxidative marathoners as they have capacity to preferentially engage in mitochondrial respiration and fatty acid oxidation to form a greater proportion of memory T cells, and secrete cytokines more moderately. This balanced response translates into prolonged persistence **(**with CAR T cells detectable beyond 1–2 years in some cases) and sustained tumor control, especially under conditions that challenge T-cell metabolism (such as the nutrient-depleted TME's).

What was unique about this approach and analysis was the quantification of several relationships: for example, the strong correlation between a CARs metabolic profile and its functional outcomes. Glycolytic activity was strongly linked to cytokine production ($r = 0.9$) and to exhaustion, explaining why CD28's glycolysis-fueled state goes with inflammation and T-cell burnout. Meanwhile, oxidative metabolic capacity correlated with longer persistence ($r = 0.92$**)**, aligning with 4-1BB's propensity to create memory T cells. These insights reinforce a model where CD28 co-stimulation make cells for fast killing at the cost of longevity but 4-1BB co-stimulation promotes a sustained immunotherapeutic response**.**

In summary, our findings support a paradigm in which co-stimulatory domain selection is a pivotal design decision in CAR T-cell therapy that should be matched to the clinical context. CD28 and 4-1BB each confer distinct benefits to each their own: the former maximizing short potency but the latter ensuring durability. Therapeutic outcomes in patients can potentially be optimized by

leveraging these differences - for instance, a rapidly progressing tumor might be best resolved from a CD28-based CAR for immediate cytoreduction but whereas a tumor in a hostile microenvironment or requiring long-term immune surveillance might be better served by a 4-1BB based CAR. This review gives a data-driven and quantified framework to inform such decisions, although direct comparative trials and further research are needed to refine these guidelines.

Limitations

While the review gives a quantitative comparison of CD28 & 4-1BB CAR T cells, several important limitations must be seen and understood. 1st there is significant heterogeneity among the included studies which could affect the generalizability of the derived conclusions. The 53 studies spanned various models from in vitro cell cultures to mouse xenografts to human clinical trials and targeted different tumor malignancies. This model heterogeneity means that not all findings are uniformly applicable. For instance, and for clarities sake; a metabolic difference observed in an in vitro assay may not manifest the same way in patients due to additional regulatory factors in vivo. It was attempted to normalize and aggregate data, but cross-study comparisons are inherently imperfect. The assays for metabolic + functional endpoints are not standardized across the various studies (different labs may use different definitions of peak cytokine levels or generally different gating strategies for memory T cells), thus the disparity leads to variability.

Another limitation is the correlative nature of this paper. Due to synthetization of primarily observational and correlative date, like finding that glycolytic activity correlates with exhaustion, but correlation does not prove causation. The strong Pearson correlations suggest relationships but do not establish mechanistic proof that, like "reducing glycolysis will decrease exhaustion." There could be more underlying confounding factors, for example highly activated T cells both glycolyze more and express more PD-1 but one doesn't directly cause the other; instead a third factor (like antigen stimulation strength) could drive both. Therefore, the interpretations of mechanisms (such as CD28's metabolic programming fueling exhaustion) remain hypotheses that need controlled validation for clinical proof. The data in and of itself, proves primarily correlation not causality.

Model differences extend to tumor types as well. The majority of the data comes from CD19-targeted CAR T cells for B-cell cancers. The extent to which these findings translate to other targets like solid tumors is not fully clear. Inclusion of some non-CD19 studies (GD2, PSCA.) showed similar trends (like 4-1BB doing better with persistence), but the sample size is smaller. Differences in tumor biology differences (antigen density, location, immune environment) could modulate how critical the co-stimulation domain is. Any CAR (CD28 or 4-1BB) struggles in most solid tumors; in some leukemias, both can do exceedingly well. Lastly there are clinical translatability issues. Many of the insights (like 4-1BB's superior function in low glucose) come from a very controlled lab models. In patients or in real life scenarios the situation is more complex - where patients can receive support such as cytokine therapy, or tumors can adapt in response to CAR T pressure (losing antigen). This review can't fully address how co-stimulatory domain choice intersects with real-world factors**.** Safety considerations (like neurotoxicity differences between CD28 and 4-1BB CARs) are almost important to note and were not deeply explored but are important in real life clinical decision-making. This paper primarily touched on issues like CRS rates, whilst other adverse events like neurotoxicity might differ.

Future-Outlook

The field of CAR T-cell engineering will build upon these insights to create next-generation therapies that can actually ombine the strengths of both co-stimulatory signals while mitigating their weaknesses. Looking ahead applications in multiple domains and scopes are undeniably possible. For instance, the foreseeable are as follows:

Applications and Benefits: The differential properties of CD28 vs 4-1BB CAR T cells can be harnessed in various ways. Below, the outlined potential use cases grouped into key categories, along with real-world examples and anticipated benefits:

Clinical Applications: Clinicians could select or design CAR T-cell products based on tumor type and patient condition. For example in those with aggressive leukemia and high tumor burden a CD28-based CAR might be chosen for easy and quicker clearance in the first few critical weeks. Conversely, for a patient with a slow-growing solid tumor or one at high risk of recurrence, a 4-1BB-based CAR could be more beneficial to provide lasting immune pressure. An illustrative real-world example is the choice between two FDA-approved anti-CD19 CAR T therapies: axicabtagene ciloleucel (Yescarta, uses CD28) vs tisagenlecleucel (Kymriah, which uses 4-1BB). Yescarta tends to cause a brisk T-cell expansion and is often used in aggressive lymphomas requiring immediate response while the Kymriah's T cells expand more gradually and persist longer, which has been advantageous in pediatric ALL where long-term remission is the goal. In the future, we could see something like biomarker-drivenCAR T selection, where factors like tumor metabolism or patient fitness will influence whether a CD28 or 4-1BB product is optimal or if a hybridized approach may be more sufficient.

Combination and Sequential Therapies: Another use case is combining CAR T cells with other therapies to exploit and expand their hollistic metabolic profiles. Since CD28 CAR T cells rely on glycolysis giving them metabolic support (like infusing glucose or pyruvate in patients, or co-administering drugs that enhance glycolysis) could create further boosts in their short-term efficacy in the tumor. On the other hand leveraging 4-1BB CAR T cells' endurance one could combine them with therapies that require a prolonged T-cell presence maintenance therapy after CAR T infusion where 4-1BB CAR T cells m ay serve as a living platform to deliver IL-2 or other co-factors over time. Sequential use is another concept: like a patient might first receive a CD28 CAR T for immediate debulking then a few months later receive a 4-1BB CAR T for sustained surveillance. While not a fully standard approach, clinical trials could explore this sequential strategy in refractory cancers.

Safety and Accessibility: Patients or healthcare consumers ultimately benefit from CAR T improvements in 2 major ways: safety + the general efficacy. Using 4-1BB co-stimulation has already improved safety by lowering severe CRS rates, which means patients have a lower chance of ICU stays and overt toxicity. Looking ahead, something called dual-switch CARs are being tested to further control safety - for instance, a CAR T that has 4-1BB signaling for persistence but also contains an inducible "off switch" to abort the cells if severe toxicity arises. Ethically/practically to give clinicians control over the CAR T cells once infused (via safety switches) will make these powerful therapies safer for a much broader use. Another interesting observational aspect is making CAR T cells effective for solid tumors; success would vastly increase the number of consumers who can benefit from CAR T therapy. Engineering strategies

such as armoring CAR T cells to resist TME immunosuppression (using knowledge that 4-1BB helps resist some, but adding features to resist things like PD-1 blockade integration) are consistently underway. If successful, a patient with hypothetically pancreatic cancer (currently with few options) might have a CAR T therapy available to assist their current pancreatic TME.

Ethical and Safety Considerations: As the innovations based on co-stimulatory design, ethical considerations are rather important. One concern is manipulating T-cell persistence while there is a want for CAR T cells to last long enough to prevent relapse, an extremely persistent T cell could pose risks, such as prolonged depletion of the target antigen (e.g. B cells in the case of CD19 CAR, which leads to B-cell aplasia) or unforeseen later toxicities in the future. Balancing persistence and controllability is key. For instance, if there is to be a CAR T cell so persistent that it lives for decades, do we have a moral obligation to ensure it can be removed or silenced if needed using something like safety switches or gene-editing suicide genes? This enters into patient consent as well because patients should be informed how long the therapy might remain active and what the plan is if it outlives its utility. Another ethical aspect is equity and accessibility. CD28 vs 4-1BB might sound like a technicality overly compartmentalized but it can affect the cost as 4-1BB CARs might need longer manufacturing times, adding cost; conversely, less ICU time for toxicity could reduce overall costs. Ensuring that whichever product is truly better for a given patient is the one they receive regardless of which company makes it or its price; is an ethical obligation or imperative as said.

As CAR T therapy potentially moves to earlier lines of treatment or even to less immediately life-threatening conditions the risk-benefit causality of the matter changes. In such cases, favoring a 4-1BB design with an activity that is quite controlled might actually be ethically preferable to avoid overtreatment, whereas in a terminal cancer the aggressive CD28 design might be justified depending on the rationale of the medical institution, the medical professional, the patient, and the patient's loved ones in order to create a decision that feels truly necessary for them, and their needs alone. Thus the intended use and patient population should guide the ethical choice of CAR design.

In conclusion, integrating the strengths of CD28 + 4-1BB co-stimulation can offer a promising path forward for CAR T-cell therapy. The dichotomy offered by these two domains and their minute differences, but major metabolic and functional outcomes proves that current design can be better tailored and optimized to truly fit the disease context in order to improve efficacy while minimizing toxicity as a much co-factor of the treatment as we know it today. Ongoing innovations are guided by both scientific insight and ethical foresight, or at the very least should be, in order to reach the aim that these therapies become safer, more effective and more broadly applicable in order to ultimately benefit a wider range of patients in the fight against cancer and beyond of the world as we know it. Immunotherapy has a forefront that shines brighter than any star but has the potential to fall into wreckage beyond our esoteric understanding, which is why the nuanced approach to guide a product that truly fit an individualized use case, is all that needs to be achieved.

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