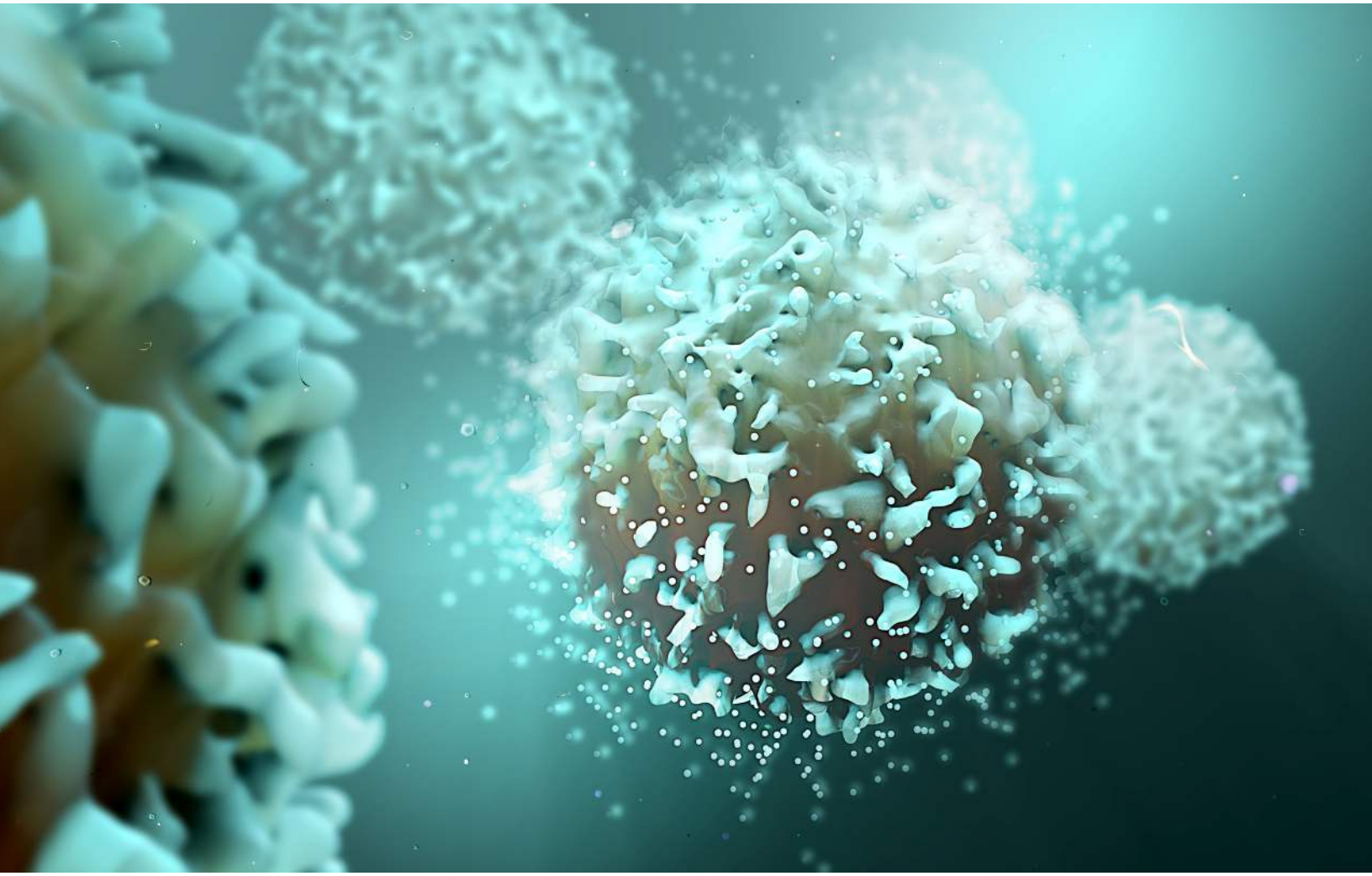
E BOOK

Optimizing Cell Therapy Products with IsoPlexis® Proteomics and Excellos: Starting Material and Manufacturing Characterization



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EB-1 REV 1.0

Optimizing Cell Therapy Products with IsoPlexis® Proteomics and Excellos: Starting Material and Manufacturing Characterization

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Introduction

Chimeric antigen receptor (CAR)-T and tumor infiltrating lymphocyte (TIL) cell therapies have been breakthroughs for patients with specific types of malignancies. Efforts are underway to broaden the use of TIL- and CAR-based immunotherapeutics to other cancer types. While the clinical outcomes are promising, the overall response rates (ORR) and recurrence rates are variable. Recent data on CAR-T therapy outcomes show that relapse can occur, ranging from 20-50% relapse/recurrence rates within one year for patients who receive CAR-T therapy [1], indicating that there are opportunities to enable greater clinical benefit for patients. The majority of current TIL and CAR-T cell therapies use autologous cells, but there is a growing shift to allogeneic applications, as well as NK- and iPSC-based approaches, requiring access to healthy donors and starting materials to supply these demands.

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Factors Contributing to Clinical Outcome Variability

Part of the variability in clinical response rates and relapse is due to the patient's health status, disease stage, and tumor resistance. These patient- and disease-related characteristics are independent variables that are not easily overcome. Still, there are dependent variables that can be addressed to assure greater clinical benefit for the patient, particularly as they relate to donor and starting material characterization, analysis of intermediates, and final product evaluation.

While the mechanisms of action for cellular therapies differ from molecular therapies, cell therapeutics must adhere to basic principles of pharmacology for clinical benefit. Final product purity, potency, and safety, as well as dose and schedule optimization must be considered to assure clinical benefit. There are manufacturing and testing strategies for cell therapies that can be improved, potentially resulting in clinical benefits for patients. These properties include effector function, durability of pharmacodynamic effect, and target exposure to the therapeutic.

CAR-T Immunotherapy Progression

CAR-T cell therapies have matured over the past decade with the intent of improving clinical outcomes. First generation CAR constructs were focused on effector function transactivation motifs (Figure 1 [2]), and while antitumor efficacy was noted, a second-generation series of CAR constructs including cell proliferation signaling were developed to further improve the efficacy of CAR-T therapies. Third-generation CAR motifs now include pro-survival transactivation elements to further improve clinical responses. While these improvements in the CAR constructs have led to better clinical outcomes, these aspects are still dependent on the inherent downstream functionality of the signal transduction and gene expression pathways that are present in the parent T cell. If these pathways are not functioning optimally, the later generation CAR constructs will not give the degree of benefit they are intended to deliver. Therefore, it is important to more fully understand the status of the starting material T cell physiology to assure optimal performance of CAR-based therapies.

CAR-T Therapy Progression

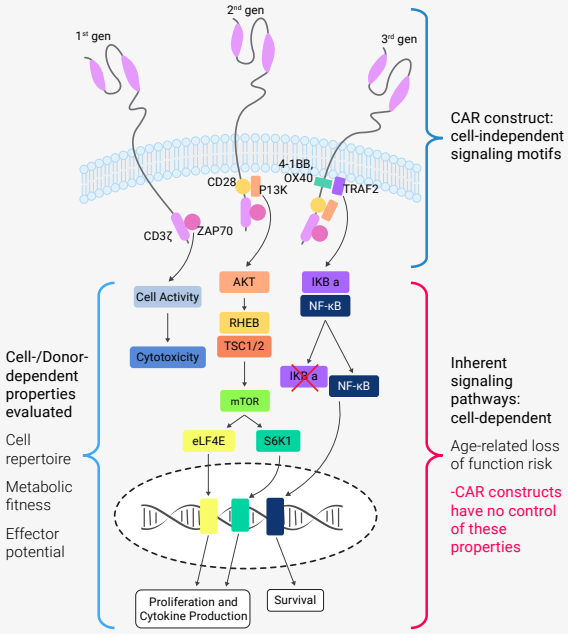


Figure 1 | Initial CAR-T cells had clinical responses but still had low complete response (CR) versus partial response (PR) (1st gen). CARs were designed to increase proliferation to address this (2nd gen). Durability of responses were poor, so CARs were modified to improve CAR-T cell survival (3rd gen). *(Figure adapted from [2])*

Effector/Memory Function-API Considerations

Final product (FP) refers to a well characterized therapeutic preparation containing minimal unrelated or inactive/interfering contaminants and a consistent dosage of the therapeutic agent. For molecular-based therapies, this is termed the active pharmaceutical ingredient (API). The FP should be produced in a consistent manner across all clinical manufacturing campaigns to assure consistent control of dosage administered. API variability in the final product can result in variability in therapeutic effect, as well as having safety implications related to required dosing. This can confound clinical outcomes and should be minimized. Control of final product API is a straightforward process for most molecular therapies, assuring that not only are the active pharmaceutical ingredients uniform in their potency and composition, but that unrelated/ineffective contaminants are minimized in the dose administered. Utilizing the API concept to denote and characterize the cellular active pharmaceutical ingredient (cAPI) to produce consistent therapeutics has been lagging for cell therapies and should be addressed.

In regard to cAPI, approaches have been implemented with cell therapies, such as using standard starting material collection and processing procedures including donor screening, enrichment of specific cell subsets, generation of master cell banks, cloning, controlled expansion/enrichment protocols, and testing of the manufactured products. In particular, T cell expansion/activation *in vitro* typically uses media formulations containing IL-2 or a combination of cytokines and factors to facilitate proliferation and activation of these cells as part of the manufacturing process for CAR- and TIL-based therapies. Still, better characterization of the cAPI to minimize the content of ineffective or low potency cells present in the final product is lacking. This can impact the % cAPI present in the final product, which can cause efficacy and safety variability by interfering with the targeting of tumor cells or tissues, resulting in decreased potency and increased toxicity risk per dose [1][3]. A potential solution to address this problem is to carry out deeper characterization of the donor starting material, manufacturing intermediates, and FP to minimize loss of potency, impact on safety, and cAPI content present in the infused therapy.

While standard characterization practices can help incrementally in manufacturing robust final product, there is a clear need for greater efforts in this area. Better characterization of effector, memory, and metabolic function of cell samples from donors, manufacturing intermediate and FP testing will enhance the production of cell therapies with greater consistency and potency, resulting in greater efficacy and reduced safety issues, as well as potentially reducing the cost of manufacturing.

cAPI Manufacturing Challenges

An aspect of manufacturing that has been challenging is balancing the proportion of cells in a final product that retain the desired effector and memory functions, such as tumor cell killing mediated by CAR-T cell therapies along with T_{memory} cells, which are necessary for durable clinical responses. Cytokine release is one of the most important metrics to assess T cell functional activity in response to antigenic stimulation. The IsoPlexis single-cell functional proteomics platform and highly multiplexed IsoCode® chip enables researchers to dissect cellular functional heterogeneity and identify highly polyfunctional cell subsets – cells that are capable of co-secreting 2 or more cytokines simultaneously. Polyfunctional cells possess various biological functions such as effector, regulatory, inflammatory, or stimulatory properties and are capable of exerting coordinated responses against cancer cells or infected cells. Increasing evidence has shown significant correlations relating the presence of polyfunctional cells with clinical response and/or drug efficacy. As such, percent polyfunctionality is a useful metric to describe the polyfunctional potential of a donor's cells. Furthermore, multiplying the frequency of polyfunctional single cells by the average intensity of each individual cytokine can be used to create a unique metric called the Polyfunctional Strength Index (PSI®) to provide deep functional assessments for samples. Proteomic studies using IsoPlexis technology can identify effector function-related cytokine release from CAR-T, TCR-T, and TIL cell products, PBMC-derived T cells, and other cell types. These results have shown that the percentage of cells retaining the desired effector phenotypes can vary. The percentage of highly polyfunctional cells to be administered can be lower than anticipated, resulting in a product with less potency and greater variability. This

may affect the efficacy and safety of the treatment for the patient. While CAR construct transduction efficiencies can be as high as 90%, if a significant fraction of the transduced T cells are deficient in effector potential, they serve as competitive inhibitors, blocking the access of high polyfunctional CAR-T cells that produce many effector cytokines from binding to the cancer target, compromising antitumor effects. Additionally, while these low polyfunctionality CAR-T cells may not be effective, they can still contribute to safety risks, such as cytokine storm (CSC) or other CSC-related adverse events [4]. Figure 2 depicts two key characteristics of PBMC-derived CD8+T cell functional properties and heterogeneity across healthy individuals in response to CD3/CD28 stimulation. The left panel demonstrates the percentage of polyfunctional cells from healthy donors in terms of the fraction of cells capable of polyfunctional cytokine secretion, while the right panel depicts the variability of the PSI metric comprised of various functional classes of cytokines across different donors. This type of analysis facilitates the identification of preferred donors for starting materials such as PBMCs to better optimize the production of allogeneic T cell therapeutics or assessing the therapeutic potential of autologous T cell therapies. By implementing this type of analysis at early stages of manufacturing, one can better assure the production of cell immunotherapies with potent effector function and a better cAPI content.

Donor Cell Variability

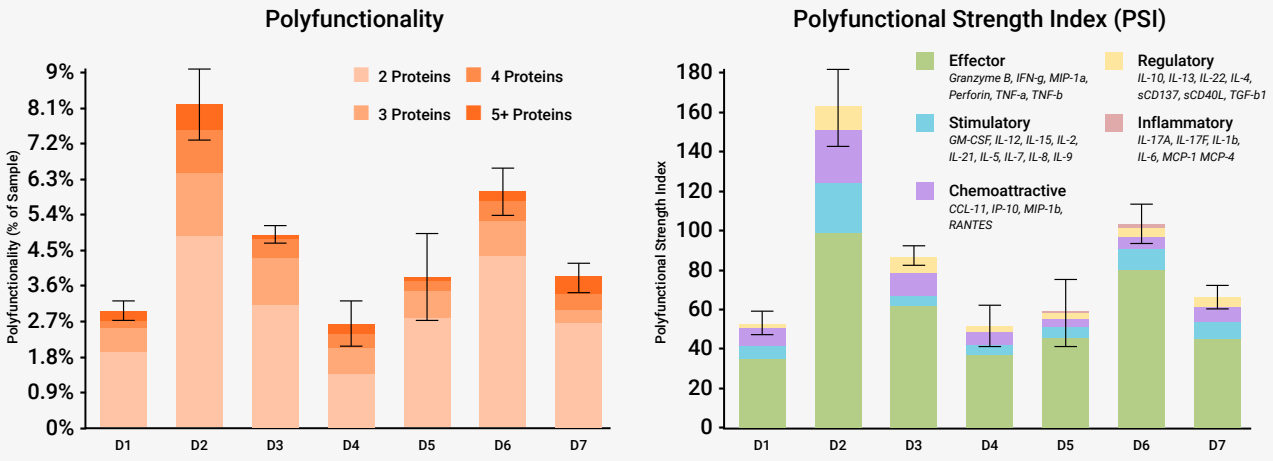
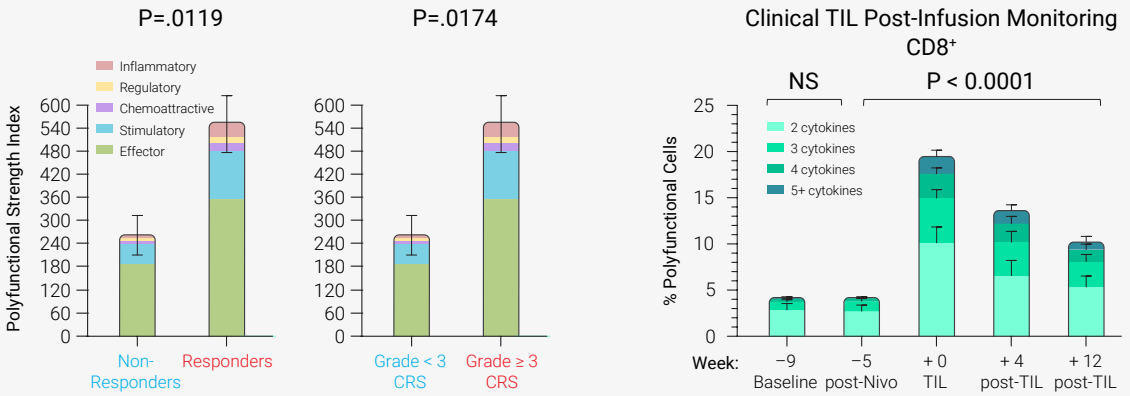


Figure 2 | **IsoPlexis Polyfunctional Strength Index (PSI)® Proteomics-Based Characterization of PBMC Effector Factors in Donors** Donor to donor variability exists for "quality" of PBMC source material, PBMC PSI assessment can identify "preferred" donors for generation of starting material

PSI/Clinical Outcomes Correlates

Effector Function Assessment IsoPlexis Polyfunctional Strength Index (PSI)
Proteomics-Based Analysis of T Cell Effector Factors



Preinfusion polyfunctional anti -CD19 chimeric antigen receptor T cells are associated with clinical outcomes in NHL.
Data from [4]

Tumor-infiltrating lympphocytes treatment for anti-PD-1-resistant metastatic lung cancer: a phase 1 trial.
Data from [5]

Figure 3 | Demonstrated correlation with clinical outcomes in CAR-T trials

In Figure 3, the left panel demonstrates the correlation between PSI of pre-infusion CD19 CAR-T products and clinical responders or non-responders in NHL patients treated with a CD19 CAR-T cell therapy while the middle panel shows the PSI in predicting severe cytokine release syndrome (CRS) for the same patient cohort [4]. The right panel illustrates the polyfunctionality kinetics of CD8+ T cells of PBMCs from patients with metastatic lung cancer in the evaluation of post-anti-PD-1 or TILs treatment, indicating that the cAPI content for highly competent, increased polyfunctional cells comprises ~20% of the total cell fraction in patients that receive TIL infusions compared to those with less than 5% after anti-PD-1 therapy [5].

Strategies to Monitor and Improve T_{eff} Markers of cAPI Content in Cellular Therapy Products

T_{eff} PSI

A variety of assays have been used to evaluate the effector function of immunotherapeutics. These include cell killing assays, ELISpot assays and cytokine and cell marker expression analyses. Typically, these have not demonstrated a strong correlation with clinical outcomes but have been used to demonstrate effector potential of the cell therapy. New assays to confirm the effector potency of T cell immunotherapies that also have a clinical response correlate are being developed. In this context, the IsoPlexis functional proteomics analysis platform has demonstrated a significant correlation with pre-clinical and clinical outcomes. PSI and/or polyfunctionality together with functional visualizations have been shown to correlate with multiple outcomes, such as differentiating responder and non-responder endpoints, identifying relapse mechanisms, characterizing functional attributes of cell products, and validating manufacturing optimizations (Table 1). Notably, the proprietary PSI metric from IsoPlexis generates comparisons based on the functional secretion of cytokines by the immunotherapeutic cells to identify high-performing donors. PSI or related single-cell proteomics parameters should be considered for donor screening, testing starting materials, manufacturing intermediates and final product characterization as part of the manufacturing process to assure better potency and cAPI content of the infusion product.

Table 1| IsoPlexis Applications in Cell Therapy

Author	Year	Journal	Keywords	Product	Main Findings
Gatla	2022	Frontiers in Medical Technology	Manufacturing optimization	PBMC	Polyfunctionality and PSI measurements showed that stirred-tank bioreactors enhance the functional performance of CD8+ and CD4+ T cells
Bai	2022	Science Advances	Clinical relapse versus complete response	CD19 CAR-T	Th2 cytokine functional strength index is an effective biomarker to distinguish complete response versus relapse
Kim	2022	Nature Communications	Manufacturing optimization	CD19 Universal CAR-T	Universal CD19 CAR-Ts expanded with a modified long-lasting IL-7 can retain polyfunctional cytokine secretion capabilities
Diorio	2022	Blood	Manufacturing optimization	CD7 Allogeneic CAR-T	Polyfunctionality improved in allogeneic CAR-Ts with PD1 knockout cytosine base editing
Zurko	2022	Cytotherapy	Product characterization; clinical response, relapse, and toxicity	CD19/CD20 Bispecific CAR-T	IsoPlexis metrics revealed key cytokines involved in preventing relapse and cytokine release syndrome
Chockley	2021	Cytotherapy	Product characterization	HER2 CAR-NK	Polyfunctionality and PSI correlated with *in vitro* tumor killing potential and revealed the need for further optimization of the CAR's co-stimulatory domain
Roselli	2021	JITC	Product characterization	CD19 CAR-T	Polyfunctionality and PSI were important metrics for optimizing CAR co-stimulatory domains containing 4-1BB and modified CD28
Spiegel	2021	Nature Medicine	Product characterization; potency and relapse mechanism	CD19/CD22 Bispecific CAR-T	Stimulation of the bispecific CAR-T revealed weaker activation through CD22 which directly related to patients experiencing CD22-mediated relapse
Creelan	2021	Nature Medicine	Immunomonitoring	Non-Small Cell Lung Cancer TIL	TIL infusion induced dynamic changes in peripheral T cell polyfunctionality that could be used to track TIL performance
Fousek	2020	Leukemia	Product characterization	CD19/ CD20/CD22 Multispecific CAR-T	IsoPlexis metrics revealed the polyfunctional profiles of multispecific CAR-Ts and functional efficacy against target cells lacking CD19
Li	2020	Gastroenterology	Product characterization	GPC3 CAR-T	IsoPlexis technology was used to compare 2 different GPC3 CAR-T designs to identify the best performing candidate that correlated with *in vitro* and *in vivo* results
Schmidts	2019	Blood Advances	Product characterization	TriPRIL CAR	Comparisons between BCMA, APRIL, and TriPRIL CAR designs revealed improved polyfunctional performance from TriPRIL CAR-Ts which correlated with *in vitro* and *in vivo* results
Rossi	2018	Blood Advances	Clinical responders versus non-responders; toxicity	CD19 CAR-T	CAR-T PSI directly correlated with patient response and risk of cytokine release syndrome
Xue	2017	JITC	Donor heterogeneity	CD19 CAR-T	IsoPlexis metrics revealed measurable stratification between different donors

Immune Cell Metabolism and Function

T Cell Effector Function

The metabolic state of immune cells is also a critical component of their effector function. Specifically, T cell immune responses are dependent on a metabolic shift from oxidative phosphorylation (oxphos) to glycolysis (Figure 4 [6]). Quiescent naïve T cells primarily rely on oxphos for energy needs. Upon pathogen-mediated activation by T cell receptor (TCR) stimulation, quiescent T cells are activated to become T effector cells (T_{eff}) and shift to glycolysis for energy production and effector function. Studies have shown that not only does this shift to glycolysis supply additional energy to T_{eff} cells, it also functions as a modulator of effector molecule expression (e.g. IFN-γ) via GAPDH-mediated regulation of effector protein expression [7]. Hence, optimal T_{eff} function for the eradication of tumors is dependent on the metabolic shift of $T_{naive/mem}$ cells to T_{eff} cells, which in part is mediated by a glycolytic shift in metabolism by these cells. These data demonstrate the importance of assessing the glycolytic shift capabilities of the immunotherapeutic cells intended for administration to patients, including the cells serving as starting materials, manufacturing intermediates, and the final product.

T Cell Memory and Metabolism

While significant tumor reduction can occur with CAR-T and TIL therapies, tumor recurrence post-treatment can still be unacceptably high, and rates of complete responses compared to partial responses should be improved. Studies have shown that one of the factors correlated with the lack of durable tumor responses is related to a lack of T_{mem} cells post-immunotherapeutic intervention and addressing this is needed to assure durable responses in patients.

As T_{eff} cells become quiescent after tumor debulking/eradication, the shift from glycolysis back to oxphos supports T_{mem} formation and maintenance. This illustrates the need for two metabolic phases that are required for optimal clinical benefit. As mentioned previously, the first phase is upregulation of glycolysis for the production of T_{eff} cells for tumor killing. The second phase, once the tumor burden has been decreased or eradicated, is a metabolic shift of the T_{eff} cells from glycolysis back to oxphos. This second metabolic shift facilitates the generation and maintenance of T_{mem} cells and is critical to assuring durable antitumor responses because upon tumor recurrence and T_{mem} TCR-mediated restimulation, upregulation of glycolysis enables the shift of T_{mem} cells back to T_{eff} cells for further tumor eradication, resulting in a prolonged clinical response and control of tumor burden and recurrence. Ensuring maintenance of these metabolic characteristics or minimizing T_{mem} attrition by characterization of memory potential and metabolic status of starting materials can assist in assuring durable clinical responses.

Metabolic Dependency on T_{eff} and T_{mem} Generation

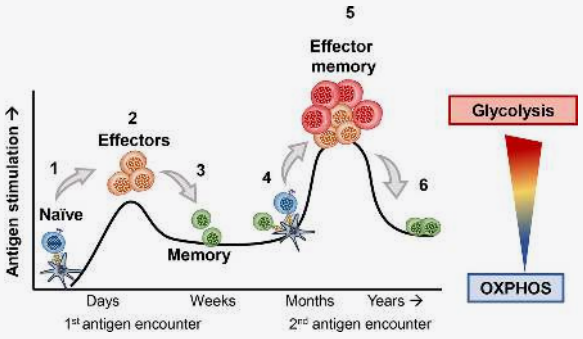


Figure 4 | Immune cell metabolism and glycolysis essential for effector cell function and memory cell production

Strategies to Monitor and Improve Metabolic Markers of cAPI Content in Cellular Therapy Products

T_{eff} Potential

Confirming the glycolytic shift potential of starting materials, as well as monitoring this parameter throughout the manufacturing process, is an important consideration for assuring a potent final product in terms of T_{eff} content, which is essential for tumor debulking.

T_{mem} Potential

While robust tumor burden reduction by T_{eff} is essential, without durability of these responses, the utility of the treatment regimen is of limited benefit. Assessing the metabolic shift of activated T_{eff} cells from glycolysis to oxphos, which is necessary for T_{mem} production, is another key feature to monitor during manufacturing.

Technology exists to carry out these types of analyses and this, or other analytical methods, should be considered for implementation as part of the manufacturing process for cellular therapies that require T_{eff} and T_{mem} for optimal disease impact.

Conclusion

Cell therapies are a paradigm shift in medicine, already demonstrating remarkable results in a variety of indications to date with numerous clinical trials ongoing or planned in the near term. Despite these promising results, this class of therapeutics can be further optimized to garner even greater clinical benefit. Some key aspects for further improvement include better characterization of the potency and durability of cells from donors, starting materials, manufacturing intermediates, and final products manufactured. The technologies to enable this, using metabolic and proteomics-based functional evaluations, can be leveraged to improve the cAPI composition of the infused product, resulting in greater clinical benefit to the patient.

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