Application Note

Cell Analysis

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Assessing T Cell Bioenergetic Poise and Spare Respiratory Capacity Using Extracellular Flux Analysis

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Abstract

The Agilent Seahorse XF T Cell Metabolic Profiling kit is a robust solution for the complete assessment of T cell metabolism in real time. Metabolism has emerged as a key driver of T cell fate and function. Indeed, it has been demonstrated that metabolic reprogramming of T cells can be used as a strategy to improve the antitumor efficacy of adoptive T cell therapies. The XF T Cell Metabolic Profiling kit allows the simultaneous measurement of glycolytic and mitochondrial activity in T cell populations combined with the measurement of mitochondrial respiratory capacity. These parameters have been linked with optimal function and improved persistence of T cell therapies. Previously, measurement of mitochondrial respiratory capacity using FCCP required concentration optimization between cell types, differentiation stage, donor and healthy or disease state. The XF T Cell Metabolic Profiling kit uses an improved uncoupler (BAM15) for more consistent and accurate measurements of T cell bioenergetic capacity with less concentration optimization required than FCCP. In addition, this application note highlights the use of the kit in assessing the impact of the medium composition during T cell expansion following the XF T Cell Persistence Assay workflow. This workflow allows for evaluation of glycolysis and mitochondrial activity and capacity simultaneously, providing a comprehensive metabolic profile of T cells that can be incorporated into monitoring and improving the design and development of T cell therapies.

Introduction

The development of cell-mediated immunotherapies has revolutionized cancer research as well as the study of the immune system. One of the most promising types of cell therapies involves the genetic engineering of novel chimeric antigen receptor (CAR) T cells to target cancer cells. There is strong evidence suggesting that metabolic properties of T cells – that is, how T cells sustain bioenergetic demands – play an essential role in regulating their antitumor function and dictating the effectiveness of T cell-based immunotherapies.[1]

T cells undergo a series of changes in their metabolic phenotype during the activation and differentiation into effector and memory cells, which are critical to maintaining T cell function.[2] Naïve T cells are in a quiescent state, with low metabolic demands sustained mainly by mitochondrial respiration. Antigen stimulation induces the exit of the quiescent state, a rapid increase in nutrient uptake, increased anabolic metabolism, and reprogramming of mitochondrial metabolism. These metabolic changes are critical to support rapid T cell proliferation and differentiation to produce cytokines or molecules that trigger cytotoxicity. After successfully clearing the antigenic stimulus, remaining differentiated memory T cells revert to a more quiescent phenotype supported primarily by mitochondrial activity and high spare respiratory capacity (SRC). Under the conditions of chronic stimulation or in a metabolically restricted environment, T cells can become metabolically dysfunctional – a state known as T cell exhaustion – where T cells exhibit decreased mitochondrial bioenergetic capacity and effector function (Figure 1).

Due to the strong impact of metabolic modulation in T cell fate and function, determining the metabolic signatures of CAR-T and other adoptive T cell therapies can play a crucial role in defining T cell persistence and antitumor function. Moreover, modulation or reprogramming of the metabolic pathways of T cells can be used as a strategy to improve the antitumor efficiency of T cells.

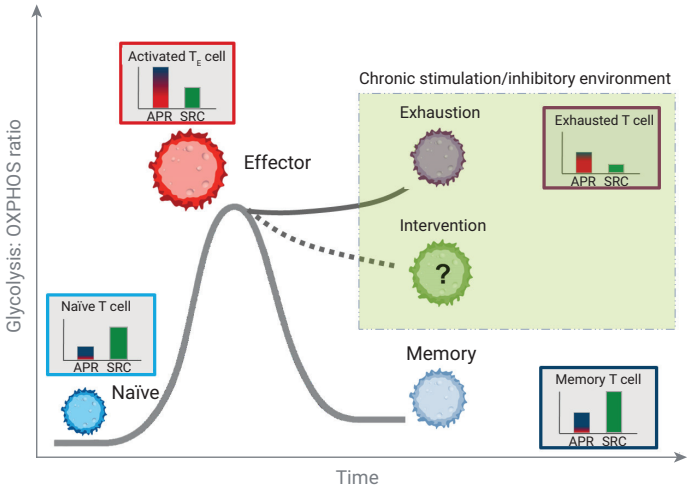

Figure 1. The fundamentals of T cell energy metabolism with measurements of T cell metabolic phenotypes, illustrating a relationship between glycolysis/OXPHOS ratio and cell fate, fitness, and function. Under chronic stimulation or in the presence of an inhibitory environment such as the tumor microenvironment, T cells can develop into an exhausted phenotype with impaired mitochondrial function.

The complete characterization of both glycolytic and mitochondrial bioenergetic pathways in T cells at different stages of T cell lifespan, as well as information about metabolic adaptations to different cellular environments or stress signals, are critical outputs to optimize T cell therapies and improve the antitumor potency of immunotherapy products. Designed to simultaneously interrogate glycolytic and mitochondrial function in live cells, Agilent Seahorse XF technology is one of the leading platforms used to study immune cell metabolism and is a key contributor to the current understanding of immunometabolism and the recognition of the fundamental role of the metabolic changes that occur during T cell activation and differentiation.[3,4]

This application note presents the new Agilent Seahorse XF T Cell Metabolic Profiling Kit, designed to enable the simultaneous acquisition of robust measurements of glycolytic and mitochondrial activity in T cell populations combined with mitochondrial respiratory capacity. These measurements provide complete characterization of the T cell metabolic profile from a single assay and can be used to correlate with increased or decreased antitumor function of T cell therapy products. Obtaining these measurements can be especially valuable during the therapy development processes targeted at improving T cell persistence or avoid metabolic exhaustion postactivation in the tumor microenvironment.

Performance review of mitochondrial uncoupler used in T cells

The study of mitochondrial function in T cells using the Agilent Seahorse XF Cell Mito Stress Test combined with Agilent Seahorse XF analyzers has provided the foundational knowledge about T cell energy metabolism and its role in directing T cell fate and function.[3,4] The Agilent Seahorse XF Cell Mito Stress Test Kit combines a series of reagents that allow a complete characterization of mitochondrial function. In particular, sequential injections of an ATP synthase inhibitor (oligomycin), a mitochondrial uncoupler (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone, FCCP) and a mitochondrial inhibitor (mixture of rotenone and antimycin A (rot/AA)) allow calculation in intact cells of the oxygen consumption rate coupled to mitochondrial ATP production (ATP-linked respiration), the uncoupled maximal respiration as well as the SRC, i.e, the difference between basal respiration and maximal respiratory capacity. These parameters have been widely used to characterize the mitochondrial function of different T cell populations and to describe processes that improve antitumor potency of T cells.[5-7] However, despite the wide adoption of this assay in the immunometabolism field, its use in T cells presents some challenges, primarily due to the use of the protonophore uncoupler FCCP.

There are several reasons that the use of FCCP with T cells (and potentially other immune cells) is not ideal. First, the optimal concentration of FCCP varies depending on many factors, including immune cell type, differentiation stage, donor, and disease state. If its concentration is not titrated or optimized for each individual experiment, it can lead to underestimation of maximal respiratory capacity. Second, in naïve T cells and some other differentiated T cells, the oxygen consumption rate (OCR) after FCCP exposure is not stable, resulting in high variation in the measurements, and potentially under-reporting the maximal respiratory capacity.

In addition, the use of FCCP in the XF Cell Mito Stress Test limits the ability of the assay to provide quantitative measurements of the glycolytic activity which is the glycolytic ATP (glycoATP) production rate. Calculation of glycoATP production rate using Seahorse XF ECAR (extracellular acidification rate) measurements requires correction from CO_2 contribution which is estimated using basal OCR measurements and OCR after addition of rot/AA.[8] However, when FCCP is used before rot/AA in the assay, it can result in underestimation of CO_2 contribution and impacts quantification of glycolytic activity. This is especially important in cells that are highly oxidative, i.e., cells that rely primarily on mitochondrial oxidative phosphorylation (OXPHOS) for energy, where CO_2 contribution to ECAR is not negligible.

Simultaneous assessment of mitochondrial ATP (mitoATP) production rate and glycoATP production rates allows characterizing basal cellular energetic demand (i.e., total cellular ATP production rate) and basal metabolic poise (i.e., the ratio between glycoATP and mitoATP production rates). These are critical parameters for determining T cell fate and function. Yet, they are not provided by the XF Cell Mito Stress Test, resulting in an incomplete characterization of T cell bioenergetics.

Development of the new Agilent Seahorse XF T Cell Metabolic Profiling Kit

To enable the simultaneous acquisition of accurate, consistent, and quantitative bioenergetic measurements for both glycolytic and mitochondrial activity in T cell populations, the Agilent Seahorse XF T Cell Metabolic Profiling Kit was developed. This kit allows accurate measurements of the maximal respiratory capacity in T cells by using the uncoupler BAM15 ((2-fluorophenyl){6-[(2-fluorophenyl)amino] (1,2,5-oxadiazolo[3,4-e]pyrazin-5-yl)}amine). BAM15 is a novel uncoupler reported to have similar potency as FCCP but with less cytotoxicity and lower affinity for the plasma membrane, resulting in a broader effective range.[9]

As shown in Figures 2A to 2C (red lines), when naïve T cells are tested with XF Cell Mito Stress Test Kit, the rate of change in O_2 level during the three minutes of the instrument measurement after FCCP injection is not constant or linear, resulting in variable OCR calculations and underestimation of maximal respiration (Figures 2D to 2F, red lines). In addition, there could be a significant overestimation of OCR after rot/AA injection, as indicated by the comparison of the kinetic traces obtained with FCCP injection or with vehicle injection (Figure 2D, red or blue trace). However, when the uncoupler BAM15 is used instead of FCCP, a stable increase in OCR is obtained after BAM15 injection (Figures 2A to 2C, green lines), resulting in a more accurate and precise determination of maximal respiration (Figures 2D to 2F, green lines).

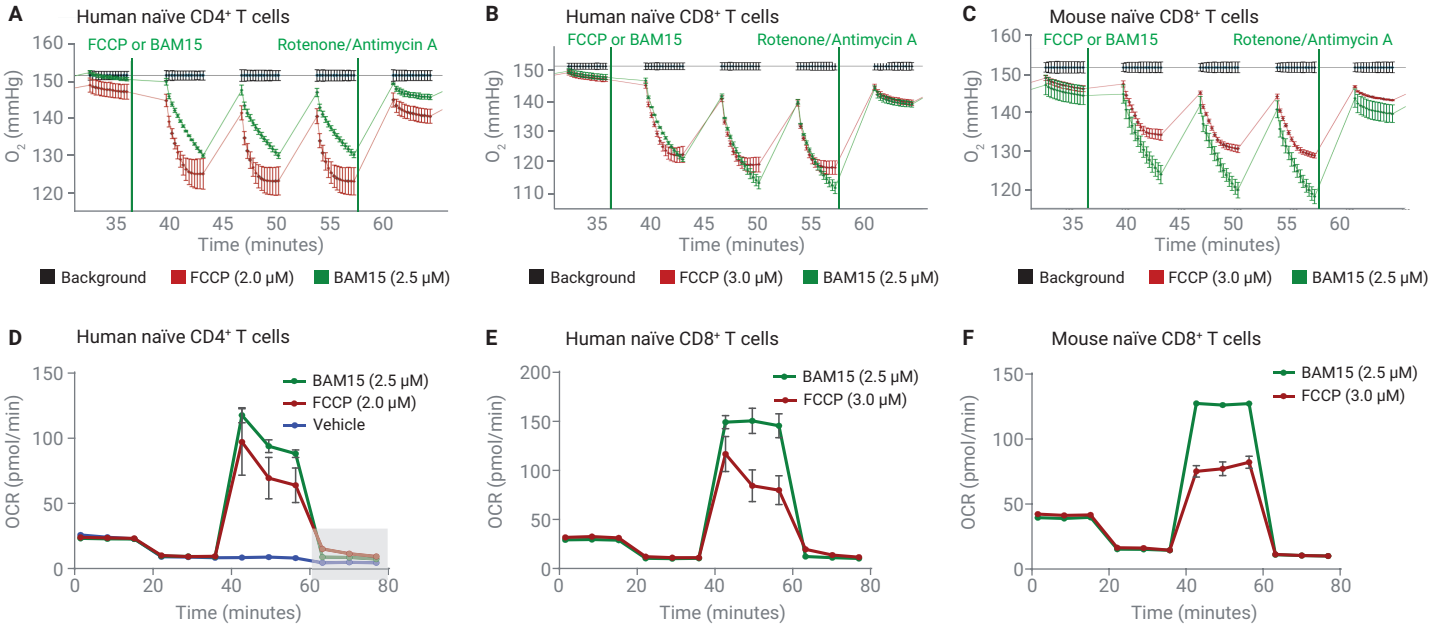

Figure 2. Comparison of Oxygen consumption measurements using uncoupler FCCP and BAM15. Human and mouse naïve T cells were seeded at 2×10^5 and 1.5×10^5 cells/well, respectively, in Seahorse XF RPMI assay medium, pH 7.4 supplemented with 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate. Changes in extracellular oxygen level in naïve human CD4+ (A), naïve human CD8+ (B), and spleen-derived mouse CD8+ T cells (C) after addition of optimal concentrations of the uncouplers FCCP (red lines) or BAM15 (green lines). Oxygen consumption rate (OCR) obtained from the XF Cell Mito Stress Test (red lines) or T Cell Metabolic Profiling Kit assay (green lines) in naïve human CD4+ (D), naïve human CD8+ (E), and spleen-derived mouse CD8+ T cells (F). Blue line in 2D corresponds to the condition where assay medium was injected instead of uncoupler.

These preliminary tests were followed with additional experiments using human naïve CD4⁺, human naïve CD8⁺, human PBMC, and spleen-derived mouse CD8⁺ T cells from at least three different donors per cell type. In all the cases, side-by-side titrations using FCCP or BAM15 were performed. Higher maximal respiration and lower standard deviations were consistently obtained with BAM15 as the uncoupler, when compared to those obtained at the optimal FCCP concentrations (Figures 3A to 3C). Titration experiments also demonstrated that the range of optimal BAM15 concentration is wider than the optimal FCCP range, indicating minimal need to optimize uncoupler concentration for each sample when BAM15 is used (Figures 3A and 3B, middle bar graphs).

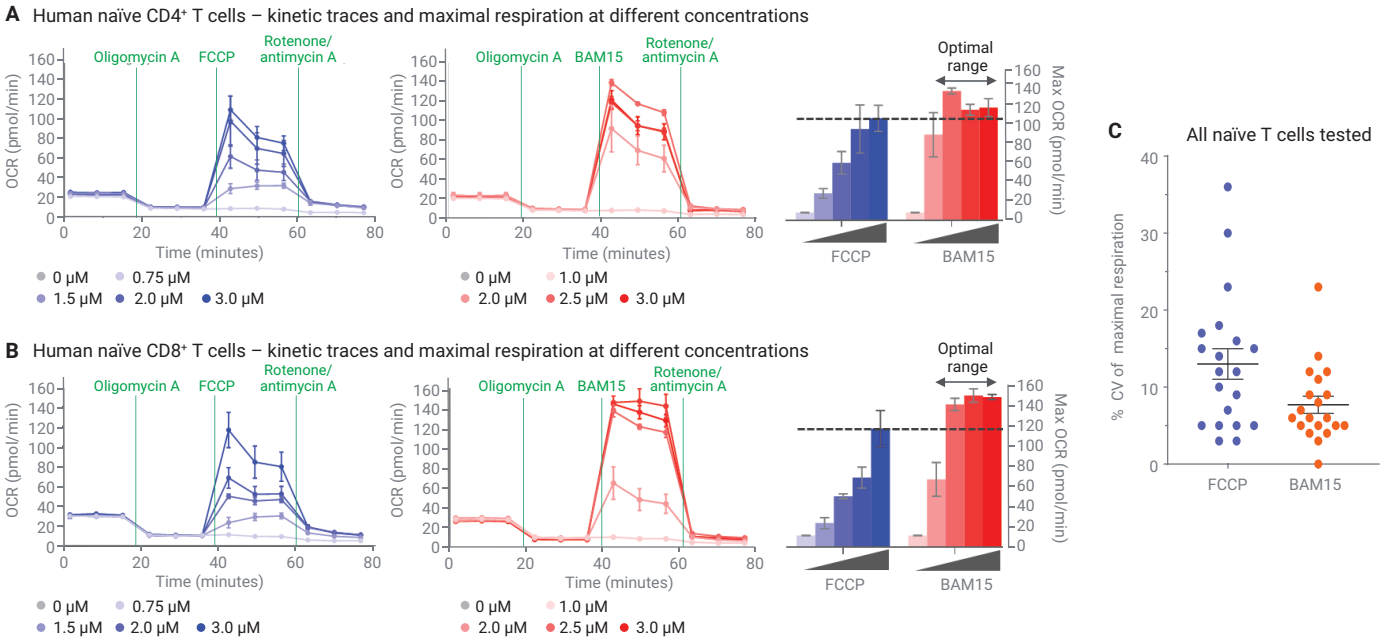

Figure 3. Comparison of maximal respiration measurements from FCCP and BAM15 titration experiments using human naïve CD4⁺ T cells (A) and CD8⁺ T cells (B). Bar graphs in the middle represents the maximal respiration obtained at different uncoupler concentration. (C) % CV of maximal respiration obtained for the panel of naïve T cells tested when optimal concentrations of FCCP or 2.5 µM BAM15 were used.

The glycoATP production rates calculated from using the XF T Cell Metabolic Profiling Kit (with BAM15 uncoupler) were also compared with those obtained in parallel experiments using the Agilent Seahorse XF Real-Time ATP Rate Assay Kit. The results show that there are no significant differences in the basal glycoATP production rates obtained from these two kits or assays (Figure 4).

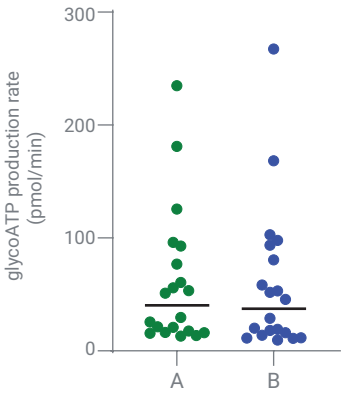

Figure 4. Comparison of basal glycoATP production rates in naïve T cells, calculated using the Agilent Seahorse XF Real Time ATP Rate Assay Kit (B) or the XF T Cell Metabolic Profiling Kit (A) with BAM15 injected between oligomycin and rot/AA injections.

Finally, a broad panel of T cells was selected to further evaluate the XF T Cell Metabolic Profiling Kit, from humans to mice and including different donors and differentiation states. Titration experiments were performed and maximal OCR values obtained at optimal FCCP concentration were compared with the values obtained at a single concentration of BAM15 (2.5 µM) for the T cell panel (Figure 5). In all the cases, the maximal OCR obtained with 2.5 µM BAM15 was at least 90% of the maximal OCR obtained at the optimal FCCP concentration within the same cell type. Maximal OCR obtained with 2.5 µM BAM15 was, on average, 20% higher than the value obtained with the optimal FCCP concentration, demonstrating that the BAM15 reagent from the kit can be used at a fixed concentration of 2.5 µM for all T cell types or requires minimal optimization.

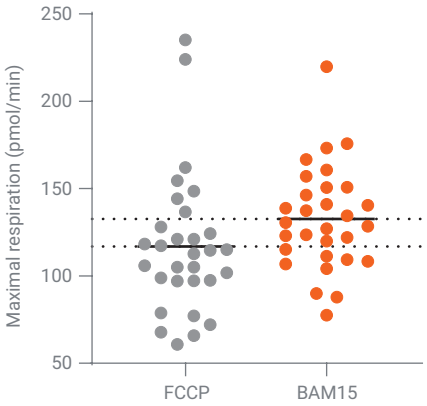

Figure 5. Comparison of maximal respiration obtained at optimal FCCP concentration or 2.5 µM BAM15 from a broad panel of T Cells, including human PBMC, naïve $CD4^+$, naïve $CD8^+$, activated $CD4^+$, activated $CD8^+$, effector $CD8^+$, memory $CD8^+$, and mouse naïve and activated $CD8^+$ T cells (n = 3 per cell type).

Application of XF T Cell Metabolic Profiling Kit in optimizing persistence for T cell therapy products

One critical attribute to consider when evaluating CAR-T and other adoptive T cell therapies is the metabolic signature of the cells, since it plays a crucial role in defining T cell persistence and antitumor function. Indeed, CAR-T cells that acquire an effector phenotype with high metabolic activity during *in vitro* expansion have been reported to display poor persistence and antitumor activity *in vivo*. However, CAR-T cells that show low-to-medium metabolic activity during *in vitro* expansion and high spare respiratory capacity confer good antitumor immunity, characterized by enhanced proliferation capabilities, higher rates of tumor cell killing, and cytokine production.[10] Several publications indicate that expansion conditions during manufacturing could produce T cell products with an undesired metabolic phenotype that results in reduced *in vivo* potency. These publications also point out that metabolic conditioning of T cells during expansion can induce metabolic reprogramming that results in extended *in vivo* persistence and improved antitumor function.[5,11,12]

Designed to assist T cell therapy development, the XF T Cell Metabolic Profiling Kit delivers a complete picture of T cell metabolic profile from a single assay which includes total basal energetic demand, basal metabolic poise, and spare respiratory capacity – all parameters were previously used to describe T cell metabolic states with increased or reduced persistence.[10] Therefore, it is ideally suited for assessing and optimizing T cell expansion conditions that result in the desired metabolic phenotype for increased T cell persistence.

Here, the XF T Cell Metabolic Profiling Kit was used to evaluated how cell culture medium composition (RPMI containing 10 mM glucose + 10% FBS or ImmunoCult XF Medium (STEMCELL Technologies)), as well as the addition of different interleukins (IL-2 or IL-15), can impact metabolic profile of T cell products. Previous studies indicated that cells expanded in IL-15 present a less differentiated phenotype than cells cultured in IL-2.[13] In this study, pan T cells were activated from different healthy human donors with magnetic beads conjugated with CD3/CD28 antibodies. After three days, the magnetic beads were removed, and cells expanded in the indicated medium conditions (Figure 6). Cells were maintained in culture at a cell density of 1 × 10^6 cells/mL, with medium refreshed and volume adjusted every three days. At day 7, 14, and 22, samples were removed and analyzed using the XF T Cell Persistence assay supported by the XF T Cell Metabolic Profiling Kit following the recommended assay conditions.[14]

First, SRC in the course of cell expansion was compared. As shown in Figure 6A, at day 7 a difference in the SRC of cells expanded in IL-15 or IL-2 is observed, independent of the cell culture media used during expansion. The increased SRC of cells expanded in IL-15 is accentuated at day 22, particularly in the cells cultured in the optimized ImmunoCult XF medium (Figure 6C). Increased SRC is characteristic of memory-like phenotype and has been previously associated with increased persistence.

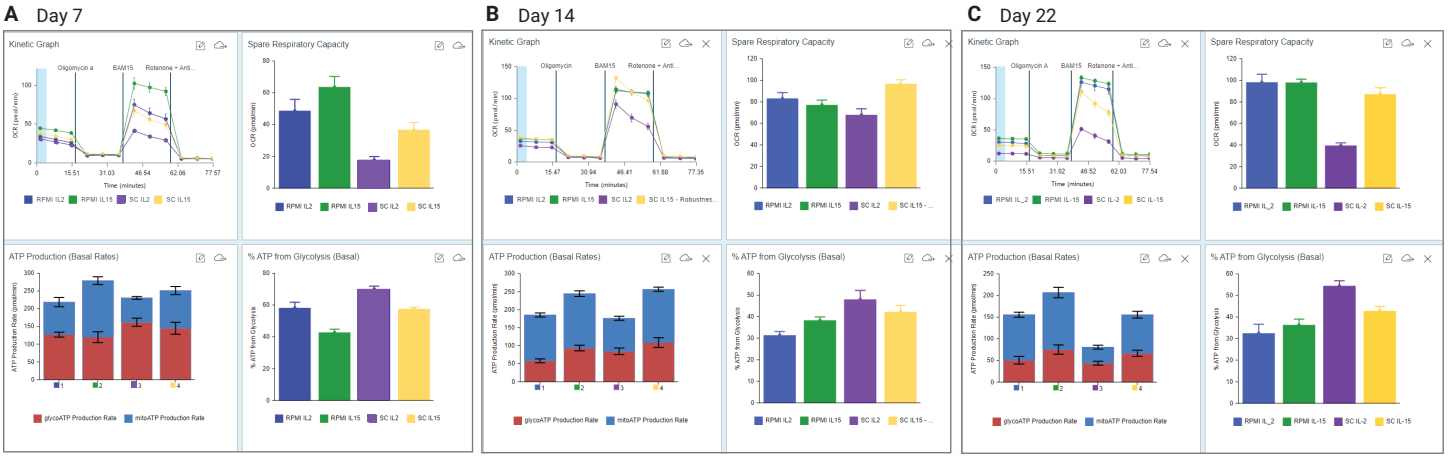

Figure 6. Impact of cell expansion condition on T cell metabolic measurements. Human peripheral blood pan T cells were activated with Dynabeads Human Activator CD3/CD28 in ImmunoCult XF T Cell Expansion Medium and cultured at 37 °C in a 5% CO_2 incubator. Two days after activation, Dynabeads were removed and cells were split in 4 groups and resuspended at 1 × 10^6 cells/mL in the following 4 medium conditions: Blue – RPMI supplemented with 2 mM glutamine, 10% FBS and IL-2 (300 U/mL); Green – RPMI supplemented with 2 mM glutamine, 10% FBS and IL-15 (10 ng/mL); Purple – ImmunoCult XF Medium supplemented with IL-2 (300 U/mL); Yellow – ImmunoCult XF Medium supplemented with IL-15 (10 ng/mL). Samples were taken and analyzed at day 7, day 14, and day 22 post activation. Data reported for each day include OCR kinetic traces (top left), SRC (top right), ATP production rates (bottom left), and percent of ATP from glycolysis (bottom right).

The next step was examining the additional outputs enabled by this assay for better characterizing and improving expansion conditions, which are the basal ATP production rates from glycolysis and mitochondria (Figure 6, lower two graphs in each panel). Cells expanded in ImmunoCult XF medium containing IL-15 have a more oxidative metabolic poise (lower % ATP from glycolysis) compared to that containing IL-2 (Figure 6, lower right graph in each panel, yellow versus purple bars). In addition, cells expanded and differentiated in RPMI medium present higher metabolic demand (higher total basal ATP production rate) than cells expanded and differentiated in ImmunoCult XF medium, regardless which interleukin was used (Figure 6, lower left graph in each panel). Increased metabolic demand is associated with effector T cell phenotype. In fact, when the expression of the CCR7-A and CD45RO-A surface markers was analyzed using the Agilent NovoCyte Advanteon flow cytometer, it was observed that cells expanded in ImmunoCult XF medium maintained a higher ratio of CCR7-A$^+$/CD45RO-A$^+$ central memory population compared to cells expanded in RPMI that were enriched in effector memory phenotype (Figure 7).

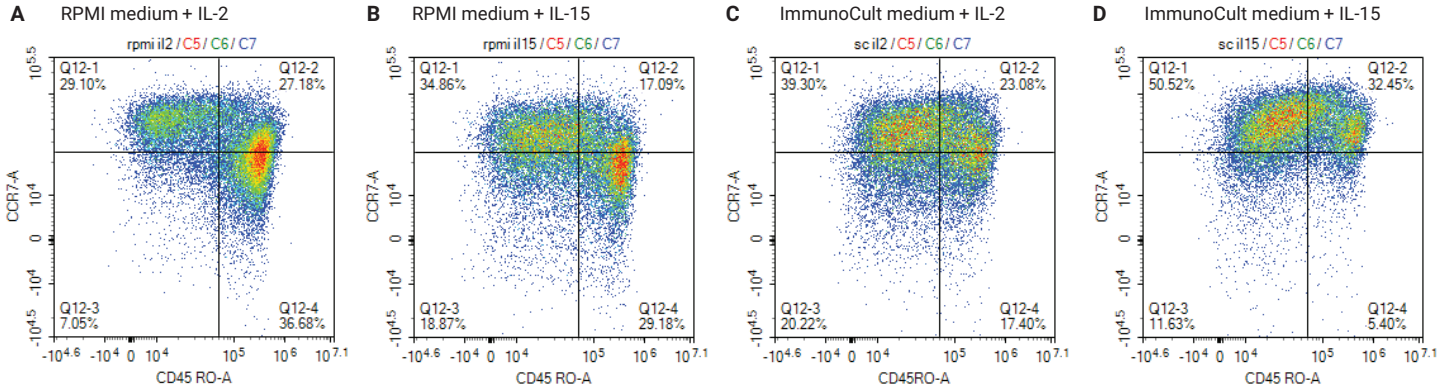

Figure 7. Flow cytometry analysis of the cell surface markers CCR7-A and CD45RO-A with day 22 samples expanded in different medium conditions as indicated in the graphs. RPMI medium is also supplemented with 2 mM glutamine and 10% FBS.

Conclusion

This document presents an optimized assay for the complete characterization of the metabolic profile of T cells. The assay combines the use of the XF analyzer with the XF T Cell Metabolic Profile Kit, providing an optimized uncoupler that allows for robust measurements of maximal respiration and spare respiratory capacity in T cells and minimal uncoupler concentration optimization. In addition, this assay allows users to obtain quantitative information about glycolytic activity in the same cell sample, together with a unique measurement of basal cell bioenergetic demand.

To improve the ability to develop and predict T cell therapy efficiency, it is required to use a combination of tools and orthogonal assays that provide a comprehensive data set to characterize CAR-T cell therapeutic products. It is clear that metabolic characterization of T cells is one of the critical attributes that need to be analyzed to improve the persistence and antitumor potency of T cell products. The XF T Cell Persistence Assay delivers a multiparametric outputs that provide a complete characterization of T cell metabolic profile from the same sample and can be incorporated as a routine assay to optimize the design and manufacture of T cell-derived therapeutics.

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RA44573.6858796296

Printed in the USA, March 15, 2022
5994-4494EN

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