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# Metabolic Preconditioning Improves Engineered T Cell Fitness and Function

Using real-time cell potency and bioenergetic assays to optimize critical process parameters for manufacturing of engineered T cells

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## Introduction

Adoptive transfer of Chimeric antigen receptor (CAR) T cell-based therapies has shown promising results in multiple cancers. Decisive features needed to achieve improved clinical outcomes in CAR/engineered T cell receptor (TCR) T cell-based therapy are clonal stability, persistence, and metabolic fitness. A recently published, decade-long follow-up study by Melenhorst *et al.* on chronic lymphocytic leukemia (CLL) remission demonstrates this fact.[1] Optimal CAR/TCR T cell metabolic fitness enables their extravasation to the tumor[2] physical remodeling of the extracellular, matrix in the TME[3], avoidance of suppressive signaling[4,5], coping with nutrient depletion[6], tolerance of hypoxia[7], and overcoming oxidative stress[8] in addition to execution of cytolytic activity, particularly in the context of solid tumors. Augmented T cell phenotypes are, in turn, associated with increased mitochondrial capacity, ATP production, and spare respiratory capacity (SRC).[9] Several studies have aimed at improving CAR T cell metabolic fitness using CAR engineering approaches or by introduction of supplemental genes that enhance mitochondrial respiration.[10]

Beyond introduction of genetic modifications, growing evidence demonstrates that simple preconditioning protocols can also improve T cell fitness/function by enhancing mitochondrial bioenergetic capacity. Preincubating TCR/CAR T cells *in vitro* under reduced glucose[12] or elevated arginine[13] conditions improves potency and ability to clear tumors in xenograft-bearing mice. Indeed, preconditioning in either low glucose or high arginine alters bioenergetics (glycolysis and oxidative phosphorylation), which affects differentiation, persistence, tumor infiltration, and resistance to exhaustion.[13] Collectively, these preconditioning results highlight the diverse means by which T cell fitness/function can be enhanced. Because preconditioning is both inexpensive and technically facile, it is likely to become integral to CAR/TCR T cell manufacturing in the future.

This study presents a comprehensive, robust, real-time workflow using the xCELLigence eSight, where T cell potency as measured by immune cell killing is combined with biogenetics and persistence parameters. This workflow provides two independent perspectives from the same experiment that can replace several time-consuming assays performed in series during cell therapy process development.

As proof of concept, this study used eSight to examine the impact of preconditioning in elevated concentrations of arginine on the killing efficacy of MART-1-specific TCR T cells. In parallel, the Seahorse XF Pro Analyzer was used to assess T cell metabolic fitness. Supplementing the growth medium with 6 mM arginine increased killing efficacy dramatically (more than 5-fold). Concurrent Seahorse XF assays demonstrated that these high arginine-stimulated gains in killing efficacy were also associated with an increase in maximal and basal oxygen consumption rates (OCR) and SRC of the T cells.

## Materials and methods

Please refer to the application note Metabolic Preconditioning Improves Engineered T Cell Fitness and Function for information about methods and materials.

## Results and discussion

### Preliminary validation of TCR activity and specificity

Before interrogating the impact of varying preconditioning parameters, it is important to validate the function and specificity of the transduced receptor. Towards this end, E-Plate wells were seeded with 8,000 Mel-624.38 melanoma target cells. After allowing cells to adhere and proliferate for 24 hours, a T cell cytotoxicity assay was performed using an E:T ratio of 5:1. Addition of mock transduced T cells had minimal impact on the growth and proliferation of Mel-624.38 target cells over the 75-hour window (black trace in Figure 1A). In contrast, MART-1 TCR T cells led to an immediate and sustained decrease in impedance, consistent with target cell death (aqua trace in Figure 1A). The killing observed in this study was expected considering the previously characterized activity of the DMF5 receptor[11], and the fact that Mel-624.38 cells express HLA-A*02:01, which is required for displaying the MART-127-35 peptide that DMF5 recognizes (Figure 1A). When the same assay is repeated with Mel-624.28 cells, which express significantly less HLA-A*02:01 and therefore present less MART-127-35 peptide on their surface, no killing is observed (Figure 1B). This conclusion is corroborated by the real-time imaging time course shown in Figures 1C and 1D. Specifically, this data set demonstrates that when seeding 8,000 melanoma target cells, using an E:T of 5 yields robust killing within a reasonable time frame.
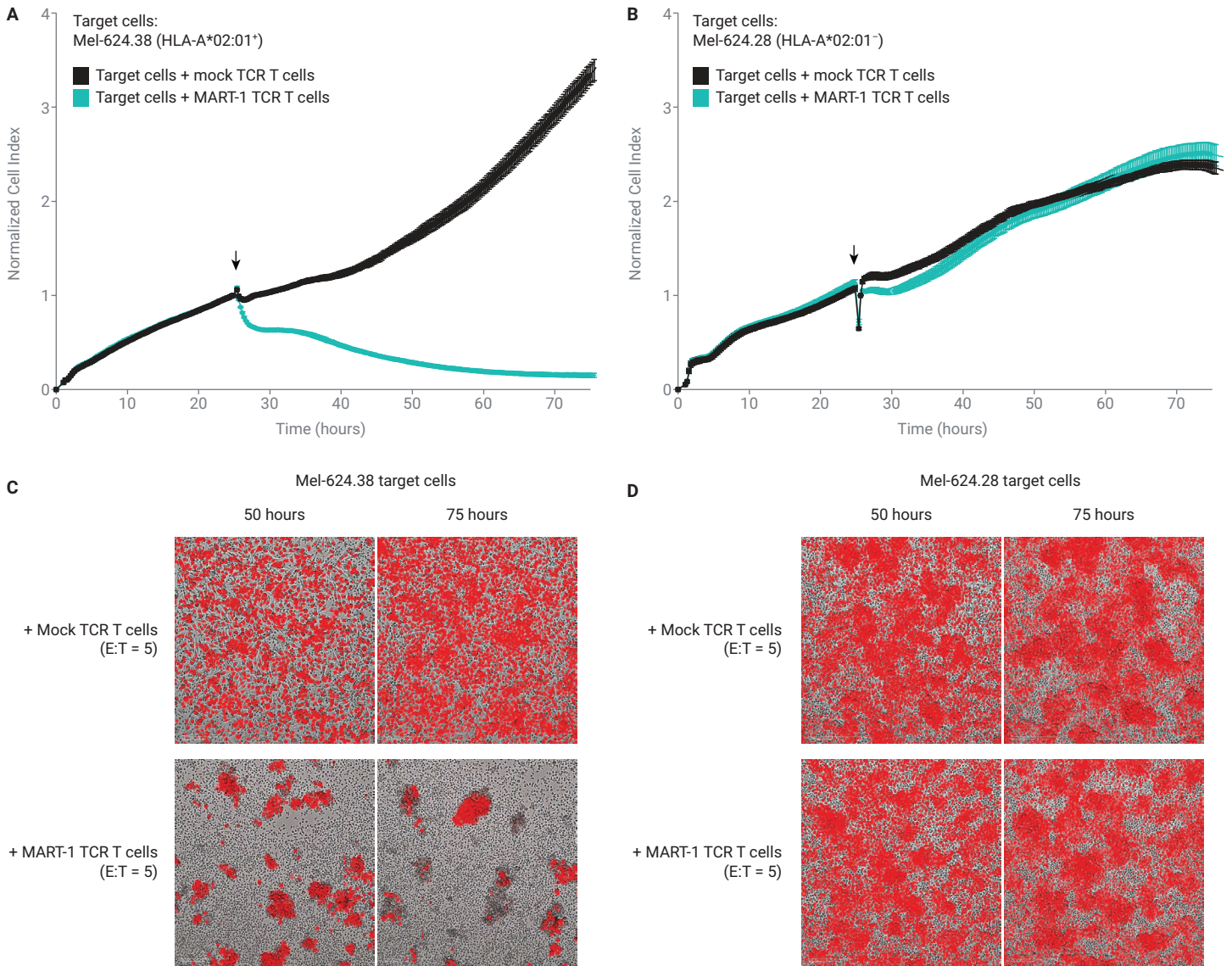
**Figure 1.** Preliminary validation of DMF5 TCR activity and specificity. (A,B) Using impedance to track MART-1 TCR T cell-mediated killing of Mel-624.38 (A) and Mel-624.28 (B) target cells that display the MART-1 peptide on their surface. The black arrow at 24 hours denotes the time when T cells were added. (C,D) Composite brightfield + red fluorescence photos corroborate killing kinetics observed in impedance traces.

## Arginine preconditioning enhances T cell cytotoxicity and mitochondrial respiration

It is well established that differentiation of T cell fitness and function are intimately linked to their metabolism. Preconditioning TCR T cells *in vitro* in an elevated concentration of arginine shifted bioenergetics (increasing oxidative phosphorylation), and was associated with increased killing efficacy once T cells were transfused into tumor bearing mice.[10] Unfortunately, screening a broad array of T cell preconditioning parameters using mouse xenograft models is both costly and time-consuming. For this reason, an *in vitro* assay to evaluate the functional impact of diverse preconditioning parameters would be advantageous. As a demonstration of eSight's ability to address these types of questions, the killing efficacy of MART-1 TCR T cells was evaluated after being preconditioned in three different concentrations of arginine. A Seahorse assay was performed simultaneously to asses the relationship between cytoxicity and bioenergetics under different conditions.

After transduction, T cells were metabolically preconditioned for 7 days in RPMI supplemented with 6 mM arginine, and base RPMI media. The killing assay was set up at an E:T ratio of 5:1, to demonstrate the eSight's capability to distinguish differences in potency using impedance curves (Figure 2A). All killing assays were performed at a 5:1 E:T ratio. While preconditioning, MART-1 TCR T cells in RPMI supplemented with 6 mM arginine stimulated killing efficacy substantially (Figure 2A). Geiger and coworkers demonstrated that high arginine stimulated gains for *in vivo* killing efficacy were coupled with a shift towards use of oxidative phosphorylation as the ATP source.[10] To confirm whether this is also true for MART-1 TCR T cells, the XF Cell Mito Stress Test was performed in the XF Analyzer in parallel with the eSight cytotoxicity assays. To perform this assay, MART-1 TCR transduced 6 mM arginine preconditioned T cells, RPMI T cells, and non-transduced T cells were used. The result shows that spare respiratory capacity (SRC) and maximal respiration of arginine preconditioned T cells was significantly higher than RPMI-preconditioned T cells (Figures 2B and 2C), a parameter previously correlated with increased T cell persistence.[13]
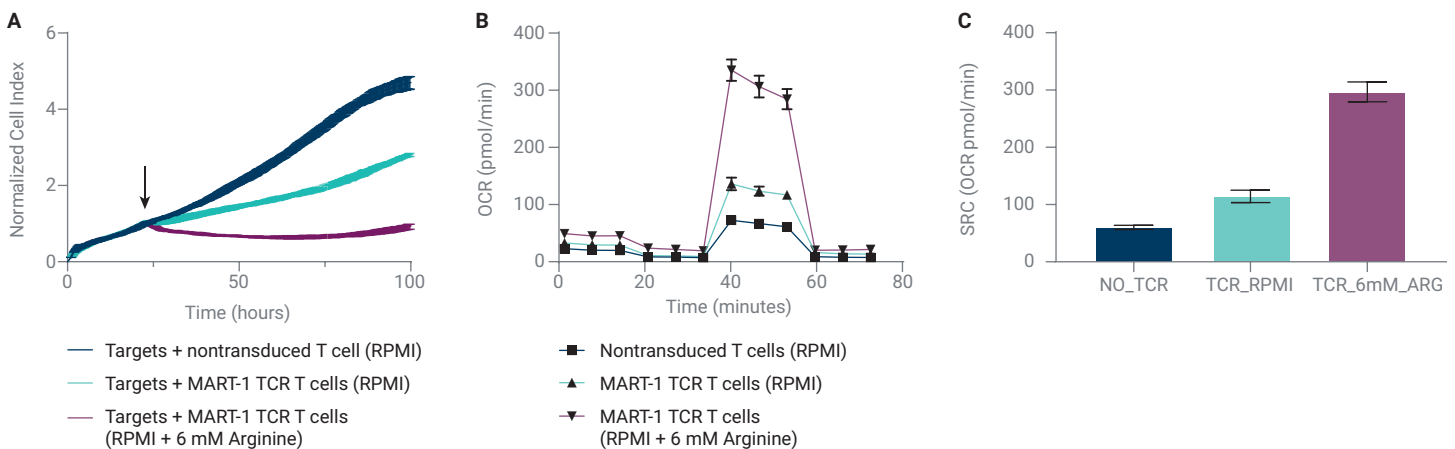


**Figure 2.** (A) Impedance traces for Mel-624.38 target cell cytotoxicity assay with MART-1 TCR T cells preconditioned for 7 days in RPMI medium supplemented with an additional 0- and 6-mM arginine. The E:T ratio was 5. Black arrow denotes time of T cell addition (24 hours). (B) Kinetic trace of OCR in basal conditions and after injection of oligomycin, uncoupler, and rotenone/antimycin A. (C) SRC of non-transduced, expanded in RPMI, or preconditioned in 6 mM arginine for 7 days.

## Discussion

The maximal therapeutic efficacy of T cells depends on cell potency and bioenergetics status. This study shows a unique workflow that combines two Agilent T cell analysis platforms, eSight and XF Seahorse Analyzers, to provide a comprehensive picture of T cell potency and metabolic fitness. This study demonstrates eSight's ability to screen in real time for T cells with high potency after preconditioning with 6 mM arginine. If one wishes to evaluate the impact preconditioning has on T cell killing capacity, the eSight assay shown here can do so efficiently. Using real-time XF Seahorse measurements in parallel, the metabolic fitness of these potent arginine preconditioned T cells was associated with increased oxidative phosphorylation (Figures 2B and 2C). T cell persistence and memory phenotypes have previously been associated with higher SRC.[15,16] SRC provides valuable information about the bioenergetics of a T cell, which can be correlated with T cell potency using the Agilent XA eSight. The combination of these two techniques supplies valuable information about the critical parameters of T cell function relevant to cell therapy. The data from both eSight and XF Seahorse assays provide a comprehensive view of potency and metabolic fitness that can enable testing critical process parameters during CAR T cell manufacturing.

As the field of engineered T cell therapy continues to evolve, it seems increasingly clear that maximal therapeutic efficacy will be achieved not simply by transducing T cells with a single receptor, but by making multifaceted adjustments to their phenotype before transfusion. While supplementing TCR/CAR T cells with additional genes (for cytokines, metabolic enzymes, etc.) holds great promise, it is evident from past publications and this study that substantial gains in fitness can be achieved simply by changing the composition of the growth medium. Thus, it would seem that a huge array of preconditioning parameters have yet to be examined. Combining two or more preconditioning parameters previously proven to be beneficial will likely bring additive or even synergistic gains.

By eliminating the need for tedious, less informative endpoint analyses, the eSight real-time killing assay significantly lowers the barrier to systematically evaluate different preconditioning protocols.

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