

# 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.<sup>1</sup> Optimal CAR/TCR T cell metabolic fitness enables their extravasation to the tumor<sup>2</sup>, physical remodeling of the extracellular matrix in the TME<sup>3</sup>, avoidance of suppressive signaling<sup>4,5</sup>, coping with nutrient depletion<sup>6</sup>, tolerance of hypoxia<sup>7</sup>, and overcoming oxidative stress<sup>8</sup> 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).<sup>9</sup> 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.<sup>10</sup>

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<sup>12</sup> or elevated arginine<sup>13</sup> 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.<sup>13,15</sup> 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.

Two important questions arise regarding T cell preconditioning: Firstly, what preconditioning protocols can steer engineered T cells towards maximal potency and persistence? Secondly, how does it correlate with T cell bioenergetic status? While these questions must ultimately be addressed using *in vivo* models, first interrogating the functional impact and bioenergetics of preconditioning using simple *in vitro* assays would be informative. Metabolism has a strong influence on T cell fate and potency.<sup>10</sup> Furthermore, characterizing the metabolic profile of CAR/TCR T cells for clinical cell therapies can play a crucial role in identifying T cell persistence and tumor resolution. Unfortunately, there are no clinical processes in place to directly assess the metabolic fitness of a CAR/TCR T cell. This application note reports a workflow to evaluate T cell potency and metabolic status after preconditioning by combining the Agilent xCELLigence RTCA eSight and XF Seahorse Analyzer platforms.

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. Using gold biosensors integrated into the bottom of microplate wells, eSight uses cellular impedance to continuously monitor health and behavior as target cancer cells are subjected to different therapies. While simple to set up, requiring zero hands-on time once cells have been added to the plate, this assay is highly sensitive and provides nuanced killing kinetics. Within the same wells that impedance is measured, eSight also collects live cell images in both

brightfield and fluorescent (red, green, blue) channels – thereby providing both a primary and confirmatory result from a single simple workflow.

As proof of concept, this study used eSight to examine the impact of preconditioning in elevated concentrations of leucine, glutamine, and arginine has 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. In addition to examining multiple amino acid concentrations, the duration of the preconditioning protocol was also varied. Whereas elevated leucine and glutamine concentrations were found to have minimal impact on MART-1 TCR T cell killing of melanoma cells, supplementing the growth medium with 6 mM arginine increased killing efficacy dramatically (more than 5-fold). Interestingly, killing efficacy not only increased after 2 days, but increased even further upon lengthening the protocol to 4 and 7 days. 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

### Cells

Human melanoma target cells Mel-624.38 (HLA-A\*0201<sup>+</sup>) and Mel-624.28 (HLA-A\*0201<sup>-</sup>), as well as PG13 retrovirus producing cells, were kind gifts from Michael Nishimura (Loyola University Chicago). Each of these cell lines were grown at 37 °C/5% CO<sub>2</sub> in DMEM medium (GIBCO, part number 11995040) containing 10% heat inactivated FBS (Sigma, part number 12106C-500ML) and 1% Pen/Strep (Hyclone, part number SV30010). The Mel-624.38-Red and Mel-624.28-Red cell lines, which

stably expresses nuclear-localized red fluorescent protein (RFP), were produced by transduction with Agilent eLenti Red (part number 8711011) at a multiplicity of infection of 1. Cells were subsequently grown in the presence of puromycin (2 µg/mL) for 14 days to select for stable clones expressing RFP. The PG13 cell line used in this study produces the SAMEN-DMF5 retrovirus, which encodes a T cell receptor (TCR) that recognizes a fragment of MART-1 (27-AAGIGILTV-35) when it is displayed on cells' surface by HLA-A\*0201. To characterize transduction efficiency, this TCR is fused to a CD34 tag. Frozen CD3<sup>+</sup> T cells from three different donors (514, 1057, and 508) were purchased from Charles River. After thawing, T cells were added to 10 mL of RPMI 1640 (Cytiva, part number SH30027.FS) containing 10% heat inactivated FBS and 1% Pen/Strep. Cells were immediately pelleted at 37 °C and 300 × g for 5 minutes, then resuspended in 10 mL of the same medium supplemented with 300 IU of rhIL-2 (StemCell Technologies, part number 78036) and 25 µL/mL of ImmunoCult Human CD3/CD28 T cell Activator (StemCell Technologies, part number 10971). T cells were then incubated in a 6-well plate for 3 or 5 days. Although cells were not counted during this incubation period, fresh media were added as needed to maintain neutral pH. Cells were then subjected to the transduction protocol described below.

### T cell transduction

3 × 10<sup>6</sup> PG13 retrovirus-producing cells in complete growth medium (DMEM + 10% FBS + 1% Pen/Strep) were seeded in a T75 flask. After 24 hours, the growth medium was supplemented with 10 mM sodium butyrate (which aids retrovirus production). After 8 to 10 hours of incubation with sodium butyrate, this medium was replaced with fresh DMEM + 10% FBS + 1% Pen/Strep. The following day, the retrovirus-containing medium

was collected and passed through a 0.45 µm cellulose acetate filter. Then, 2 mL of the retrovirus-containing medium was centrifuged onto the retronectin-coated (13 µg/cm<sup>2</sup>; Takara Bio, part number T100B) wells of a 24-well plate at 37 °C and 2,000 × g for 2 hours. Subsequently, media were aspirated from these wells and 1 × 10<sup>6</sup> activated T cells were added along with 1 mL of the retrovirus-containing medium. After centrifugation at 37 °C and 2,000 × g for 10 minutes, the medium was supplemented with 600 IU of rhIL2/mL. The T cells were then incubated at 37 °C overnight. The following day, T cells were collected and centrifuged at 37 °C and 300 × g for 5 minutes and were then resuspended in fresh RPMI + 10% FBS +1% Pen/Strep supplemented with 600 IU of rhIL2/mL. After a 2-day rest/recovery period, the T cells were subjected to a second round of the same transduction protocol to increase transduction efficiency.

### **Metabolic preconditioning**

Immediately after the second round of transduction, T cells were transferred to RPMI + 10% FBS + 1% Pen/Strep supplemented with varying concentrations (0, 1.5, 3, or 6 mM) of arginine, glutamine, or leucine. Because the concentrations of arginine, glutamine, and leucine in the base RPMI medium are 1.15, 2.06, and 0.38 mM, respectively, the final amino acid concentrations evaluated during this preincubation step were: 1.15/2.65/4.15/7.15 mM (arginine), 2.06/3.56/5.06/8.06 (glutamine), and 0.38/1.88/3.38/6.38 (leucine). T cells were cultured in supplemented media at a density of 1 × 10<sup>6</sup> cell/mL for 7 days and base RPMI groups were considered a baseline control sample.

The cells were counted and volume was adjusted daily to maintain the cell density. After the seventh day, the killing capacity and bioenergetics of the T cells were evaluated, as described in the next section.

### **Cytotoxicity assay**

T cell killing efficacy was evaluated using the xCELLigence RTCA eSight. To each well of an Agilent E-plate VIEW microplate (part number 300601010), 50 µL of complete growth medium (DMEM + 10% FBS; no antibiotics) was added. After recording the background impedance signal, 8,000 target cells (Mel-624.38-Red or Mel-624.28-Red) were added to each well in a volume of 50 µL (bringing the total volume to 100 µL/well). After allowing the cells to settle for 30 minutes at room temperature, the plate was transferred to the eSight and data acquisition was initiated. While impedance readings were collected every 15 minutes, photos were taken every 60 minutes. In each well, four fields of view were captured for each channel (brightfield and red fluorescence). Exposure times were as follows: brightfield = automatically adjusted by the eSight software, and red = 400 ms. T cells subjected to metabolic preconditioning were prepared by pelleting at 37 °C and 300 × g for 5 minutes, then resuspending them in DMEM + 10% FBS. After 24 hours, 100 µL of preconditioned T cells were added to each well (bringing the total volume to 200 µL/well). T cell densities were varied to achieve E:T ratios of 5:1 or 10:1. Data acquisition was then resumed for an additional 4 days.

### **Flow cytometry**

Validation of HLA-A\*2:01 expression levels in the Mel-624.38 and Mel-624.28 cell lines was performed using a NovoCyte flow cytometer and APC-antihuman HLA-A2 Clone BB 7.2 (Biolegend, part number 343307). Transduction efficiency for the DMF5 receptor in T cells was assessed using an APC-conjugated anti-CD34 antibody (Biolegend, part number 343607).

### **Seahorse XF assay**

T cells subjected to the above preconditioning protocol were resuspended in Agilent Seahorse XF RPMI, pH7.4 medium (part number 103576-100) supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine. T cells (150,000 cells) were then seeded into each well of an Agilent XF96 PDL-coated microplate (part number 103730-100 or 103798-100). T cell bioenergetics were analyzed in six replicate wells using an Agilent Seahorse XF Cell Mito Stress Test kit (part number 103015-100) on an Agilent Seahorse XFe96 Analyzer. A new assay kit is also available to assess complete metabolic profile of T cells, which is the XF T cell Metabolic profiling kit (part number 103772-100). This improved kit uses an optimized uncoupled reagent for T cells BAM15 instead of FCCP, which provides a more robust response and allows for obtaining additional information about the glycolytic activity of T cells in one assay.

## 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<sup>11</sup>, 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 (Figures 1B). This conclusion is corroborated by the real-time imaging time course shown in Figures 1C and 1D. Transduction efficiency was measured using anti-CD34 APC on the Novocyte. Figure 1E shows CD34 staining of T cells after transduction to be approximately 30%. Besides confirming the efficacy and specificity of the DMF5 TCR, these data provide a reference point to inform setup of future assay iterations. 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.

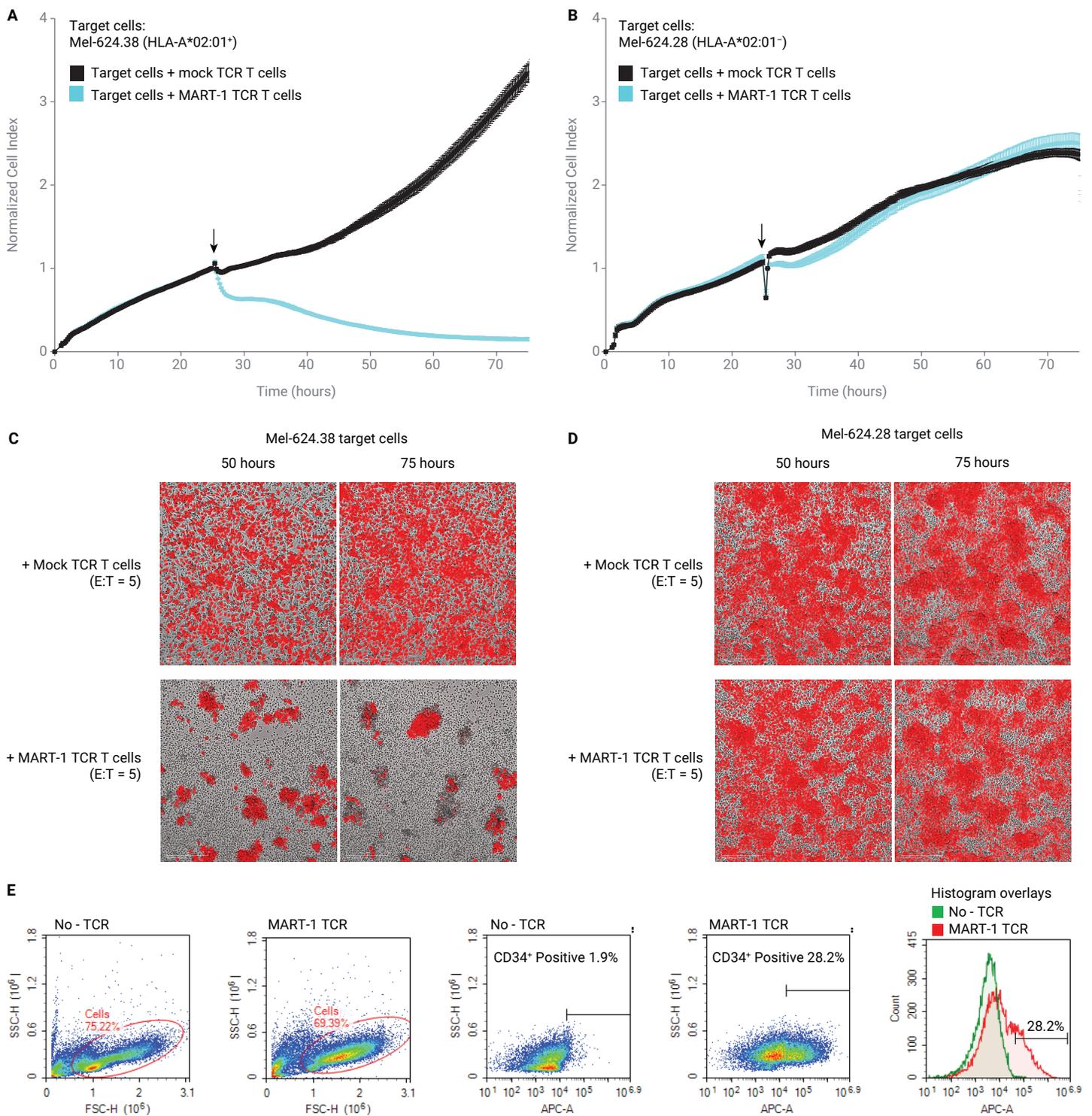
### Arginine preconditioning enhances T cell cytotoxicity and mitochondrial respiration

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.<sup>10</sup> 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, glutamine, and leucine. Since glutamine and leucine feed into the TCA cycle and have a large impact on T cell metabolism and activation<sup>16</sup>, their concentration was also titrated. A Seahorse assay was performed simultaneously to assess the relationship between cytotoxicity and bioenergetics under different conditions.

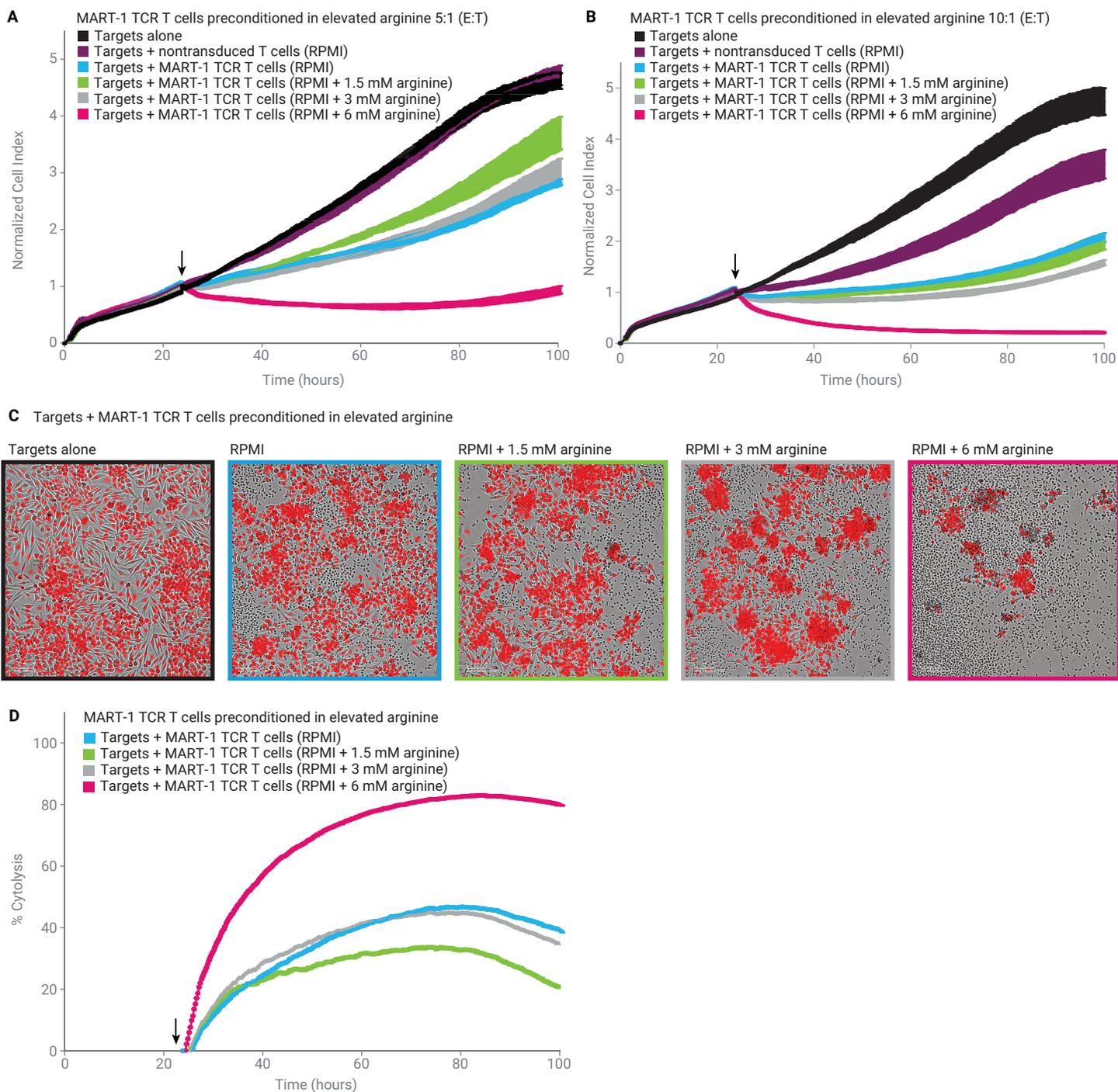
After transduction, T cells were metabolically preconditioned for 7 days in RPMI supplemented with 1.5, 3, and 6 mM arginine, and base RPMI media. The killing assay was set up at two E:T ratios, 5:1 and 10:1, to demonstrate the eSight's capability to distinguish differences in potency using impedance curves (Figures 2A and B). To distinctly monitor killing kinetics during a more

extended period of time and between samples, the 5:1 E:T ratio was better than 10:1 (Figures 2A and B). All further killing assays were performed at a 5:1 E:T ratio. While preconditioning MART-1 TCR T cells in RPMI supplemented with 1.5 or 3 mM arginine had minimal impact, 6 mM arginine stimulated killing efficacy substantially (Figures 2A and 2B). This is highlighted in the real-time % cytotoxicity plots of Figure 2D, and is corroborated using eSight's imaging capabilities in Figure 2C. At the 60-hour time point, target cells with no T cells added proliferated to confluence and displayed a spread out morphology. In contrast, in the presence of MART-1 TCR T cells preconditioned in regular RPMI, the melanoma target cells were rounded and displayed significant clustering (Figure 2C). This death phenotype was more robust when using MART-1 TCR T cells preincubated in RPMI supplemented with 6 mM arginine.

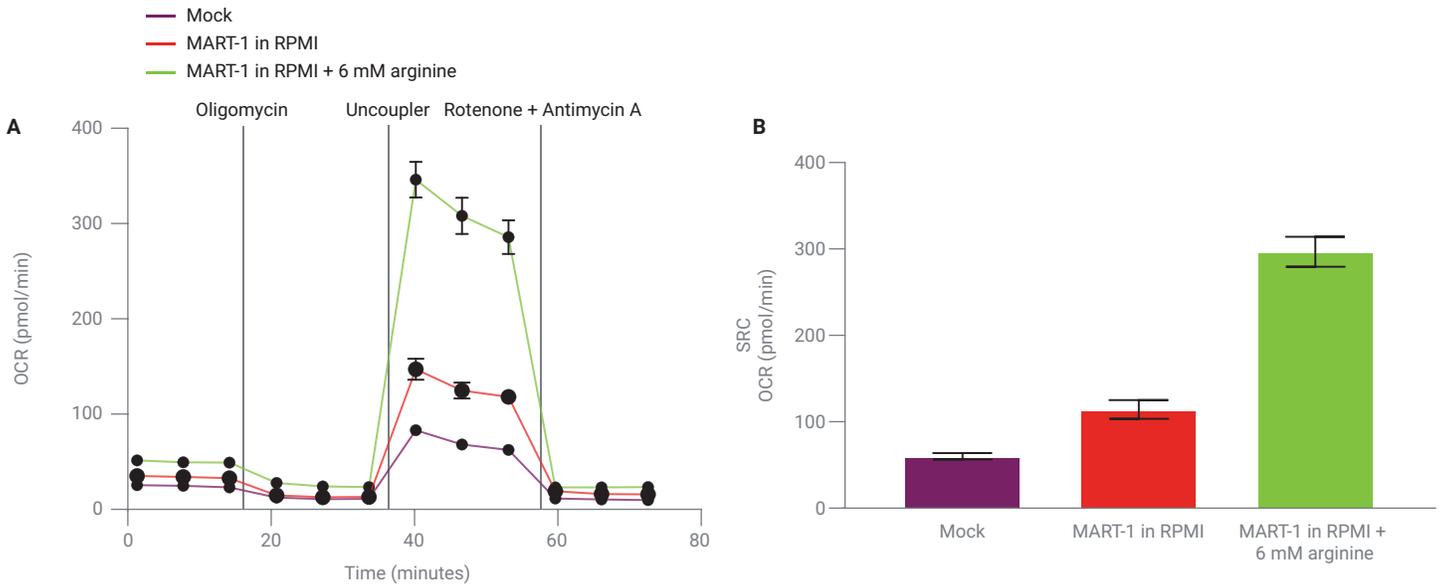
Geiger and coworkers demonstrated that high arginine-stimulated gains for *in vivo* killing efficacy were coupled with a shift towards use of oxidative phosphorylation as the ATP source.<sup>10</sup> To confirm whether this is also true for MART-1 TCR T cells, the XF Cell Mito Stress Test was performed 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) of arginine-preconditioned T cells was significantly higher than RPMI-preconditioned T cells (Figure 3B), a parameter previously correlated with increased T cell persistence.<sup>17</sup>



**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. (E) Flow-based validation of transduction efficiency using APC labeled anti-CD34 antibody. Transduction efficiency varied between 15 and 30%.



**Figure 2.** Examining the impact elevated concentrations of arginine have on MART-1 TCR T cell killing efficacy. (A,B) Impedance traces for Mel-624.38 target cell cytotoxicity assays treated with MART-1 TCR T cells preconditioned for 7 days in RPMI medium supplemented with an additional 0, 1.5, 3, or 6 mM arginine. The E:T ratio was 5 (A) and 10 (B). Black arrow denotes time of T cell addition (24 hours). (C) Images from the 60-hour time point, highlighting the stimulatory effect 6 mM arginine preconditioning has on killing efficacy. Scale bars = 200  $\mu$ m. (D) Real-time % cytotoxicity was calculated from impedance data in panel A.



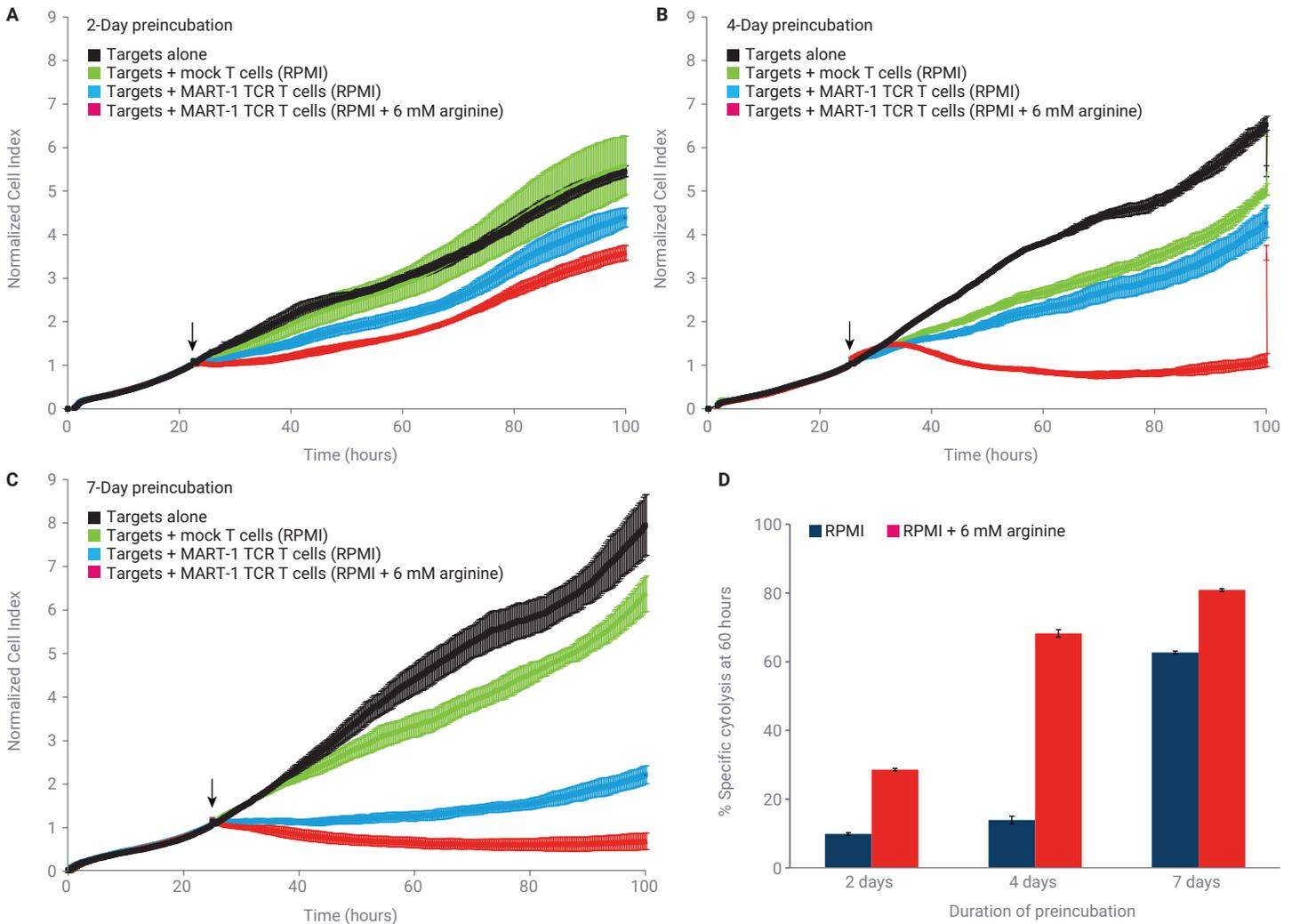
**Figure 3.** Examining impact of arginine preconditioning using Agilent Seahorse technology. (A) Kinetic trace of oxygen consumption rate (OCR) in basal conditions and after injection of oligomycin, uncoupler, and rotenone/antimycin A. (B) Spare respiratory capacity (SRC) of nontransduced, expanded in RPMI, or preconditioned in 6 mM arginine for 7 days.

**Screening preconditioning parameters: temporal duration**

For autologous T cell therapies, a significant emphasis is currently placed on reducing processing time between T cell isolation and patient reinfusion. The benefits of 6 mM arginine shown above were observed after a 7-day preconditioning step after T cell transduction. An obvious question is raised: can similar gains be achieved using protocols of shorter duration? To address this, MART-1 TCR T cells were preconditioned in RPMI supplemented with 6 mM arginine for either 2, 4, or

7 days before being used in a killing assay (Figures 4A to 4C). The differences in killing efficacy evident in these real-time plots are brought into greater focus by plotting the % specific cytolysis at the 60-hour time point (Figure 4D). After a preconditioning step of only 2 days, T cells grown in elevated arginine have a killing efficacy that is >2x higher compared to their counterparts grown in regular RPMI. Extending the duration of preconditioning from 2 to 4 days has minimal impact on the RPMI control cells but more than doubles the killing efficacy of the high arginine cells. Upon

increasing the preconditioning duration from 4 to 7 days, something interesting happens; the RPMI control T cells display a ~6x increase in killing efficacy while the high arginine T cells improve only minimally. It is well known that the length of time T cells are allowed to recover post transduction can have an impact on their fitness/function. This data set highlights that the gains achieved via a preconditioning step are most likely attributable to multiple parameters, including both the medium composition and the protocol's duration.



**Figure 4.** Duration of preconditioning in elevated arginine has a significant impact on MART-1 TCR T cell killing efficacy. 24 hours after seeding Mel-624.38 target cells, the cytotoxicity assay was performed using the MART-1 TCR T cells (E:T = 5) preconditioned in RPMI or RPMI + 6 mM arginine for either 2 days (A), 4 days (B), or 7 days (C). Black arrows denote time of T cell addition. (D) Using impedance data from panels A, B, and C, % specific cytotoxicity was calculated for the 60-hour time point.

### Glutamine and leucine preconditioning suppressed T cell mediated killing of 624.38 HLA-A\*2:01+ melanoma cells compared to RPMI preconditioned T cells

Glutamine and leucine feed into the TCA cycle and are important amino acids involved in T cell activation and proliferation.<sup>16</sup> Figures 5A and 5B show impedance killing curves for Mel-624.38 target cells using MART-1 TCR T cells that had been preconditioned for 7 days in RPMI supplemented with 0, 1.5,

3, and 6 mM leucine and glutamine. Preconditioning with leucine and glutamine were found to decrease killing efficacy. In Figures 4A and 4B, while the T cells preconditioned in unsupplemented RPMI (aqua trace) do indeed cause a drop in impedance relative to the target cell control (black trace), the extent of killing is less substantial than what was observed in Figure 1A. This interassay variability is a reflection of the biology, not the instrumentation. Although this MART-1 TCR construct always affects a killing

response, the magnitude varies when using T cells from different donors (expected), and even between rounds of transduction using T cells from the same donor. This highlights the necessity of examining the impact of different preconditioning parameters in parallel rather than across different eSight assays. Because the basal killing efficacy of the MART-1 TCR T cells can vary from one preparation to the next, an additional assay was conducted with all three amino acids at 6 mM to ensure that the impact of

6 mM arginine supplementation is reproducible (Figure 5C). Similar to the previous results, preconditioning with 6 mM arginine was found to stimulate killing efficacy while preconditioning with 6 mM of leucine and glutamine were found to decrease killing efficacy. These conclusions are corroborated by quantifying the total integrated intensity of target cells' red fluorescent signal (Figure 5D). Note that although the impedance and imaging data lead to similar conclusions, they do so with differing degrees of sensitivity. For the 6 mM arginine-supplemented sample, target cell death is detectable by the ~35-hour time point using impedance (Figure 5C) but is not detectable until the ~50-hour time point using imaging (Figure 5D). This is a reflection of what each readout is detecting. Whereas impedance detects even subtle changes in attachment strength occurring in the very early stages of the target cell death cascade, the image-based readout does not register target cell death until much later when the nuclear-localized red fluorescent protein is degraded or target cells are lysed. This emphasizes the importance of using both impedance and imaging methods for information richness, validation, and mechanism of action.

## 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 amino acids under a range of concentrations. 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 (Figure 3). T cell persistence and memory phenotypes have previously been associated with higher SRC.<sup>17,18</sup> 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.

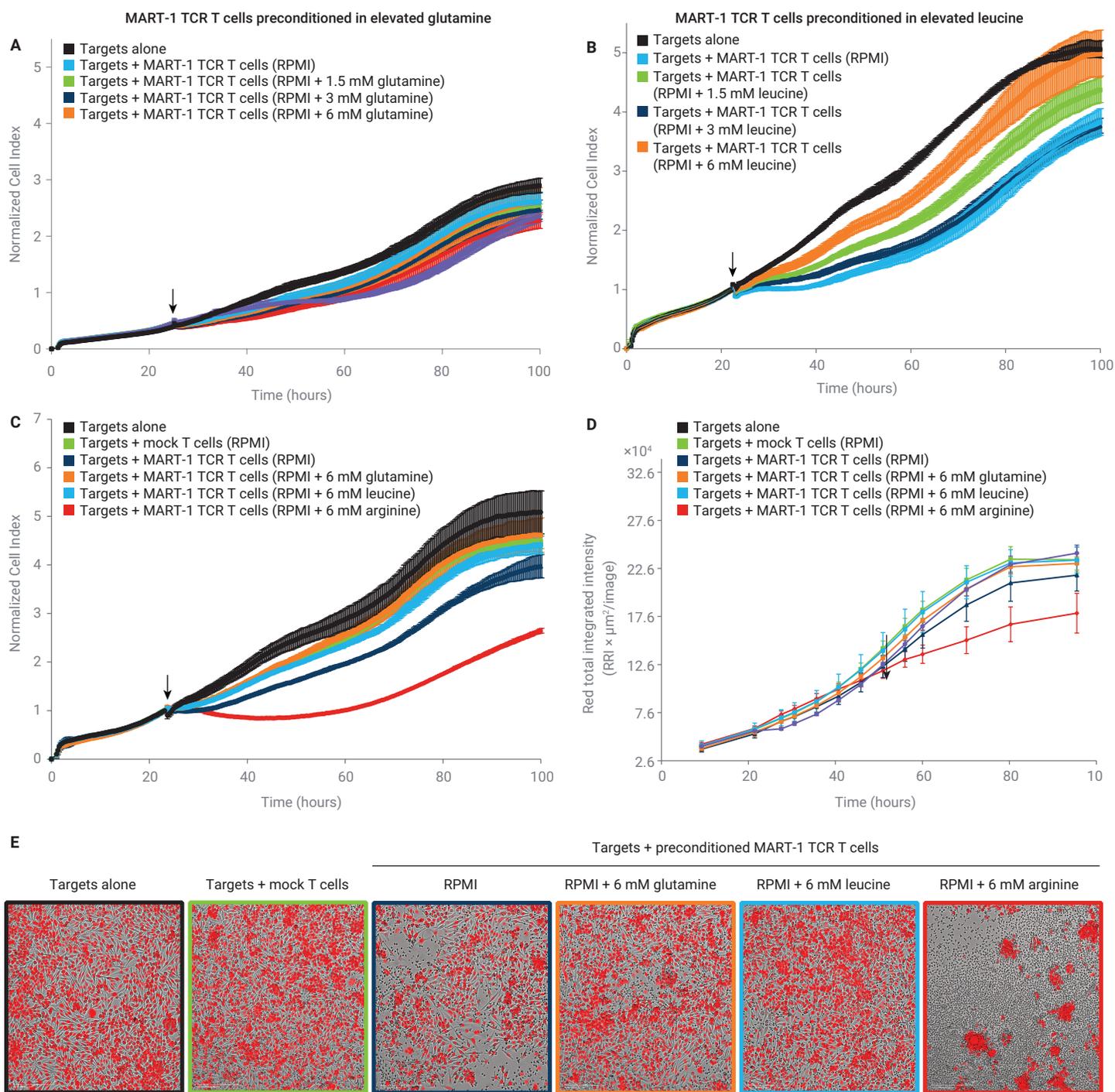
This study demonstrates a potency evaluation of preconditioned T cells using eSight. Despite minimal hands-on time required for assay set up, the wealth of kinetic information generated would be difficult to acquire using traditional methods. Figure 2D illustrates this fact, where the % cytotoxicity traces increase up to ~80 hours before plateauing or, in some cases, decrease in value. This could either indicate T cell exhaustion or death not confirmed experimentally. While these conclusions are obvious in real-time impedance and imaging data, they are less so when using endpoint assays.

There are several mechanisms by which T cells can augment cytotoxic potential, through increased production of perforin/granzyme/cytokines or proliferation. The enhanced performance of arginine-preconditioned T cells due to enhanced cytolytic capacity or proliferation was not tested. T cells grown in 6 mM arginine-supplemented media consistently grew more slowly than their counterparts grown in regular RPMI measured at the end of 7-day

preconditioning by total live cell count (MART-1 TCR T cells in RPMI = 6.5 million and MART-1 TCR T cells in 6 mM arginine = 1.9 million).

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.

Finally, it is important to reiterate the importance of making comparisons within a single assay versus between assays run on different days. Table 1 shows % cytotoxicity data for MART-1 TCR T cells produced from different donors and for different transductions of cells from the same donor. While the % cytotoxicity can vary significantly for assays run on different days, by comparing regular versus supplemented samples within the same assay it is clear that preconditioning in high arginine always results in a stimulation of killing efficacy. Access to this type of information was much more difficult in the past. 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.



**Figure 5.** Glutamine and leucine preconditioning suppresses T cell mediated killing. (A) Impedance data show the effect of glutamine concentration (0 to 6 mM) after 7 days of preconditioning with 5:1 E:T ratio. (B) Impedance data show the effect of leucine concentration (0 to 6 mM) after 7 days of preconditioning with 5:1 E:T ratio. (C) Impedance graph showing simultaneous assessment of three amino acids together in one assay. (D) Image analysis using eSight software where red total integrated intensity is plotted against time. (E) Representative images from each group. The color of each photo's outline corresponds to the plot colors in panel C. Scale bars = 200  $\mu\text{m}$ .

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**Table 1.** Comparison of % cytotoxicity, calculated using impedance data at the 60-hour time point, when using MART-1 TCR T cells subjected to 7 days of preconditioning. Data are shown for multiple donors (described in materials and methods), and multiple transductions of the same donor.

	% Cytotoxicity						
	Donor 1,057	Donor 1,057	Donor 514	Donor 514	Donor 514	Donor 508	Donor 508
MART-1 in RPMI	37	63	21	31	21	38	57
MART-1 in RPMI + 6 mM Arginine	76	81	59	88	60	75	83
Ratio for RPMI + Arginine/RPMI	2	1.3	2.8	2.8	2.9	2	1.5

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