Application Note

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Real-Time Detection and Modulation of Human T Cell Activation Using Agilent Seahorse XF Hu T Cell Activation Assay Kit

Authors

Yoonseok Kam, Lisa P. Winer, George W. Rogers, and James Hynes

Agilent Technologies, Inc.

Abstract

Agilent Seahorse XF technology delivers real-time insights into cellular energy metabolism, and is relevant to the study of T cell activation and function. This utility is now extended by the Agilent Seahorse XF Hu T Cell Activation Assay, which provides a rapid and standardized method to measure early activation-associated metabolic responses in human T cells, offering important insights into both the early dynamics of activation and its broader metabolic underpinning. The XF Hu T Cell Activation Assay workflow incorporates a soluble CD3/CD28 activator, precoated poly-D-lysine (PDL) XF Cell Culture Microplates, and Agilent Seahorse Analytics, an intuitive, cloud-based data analysis tool. The XF T Cell Activation Assay delivers a convenient, consistent solution to study T cell activation and associated metabolic reprogramming, and supports two primary experimental designs: a standard assay for testing T cell activation, and a modulation assay for investigating acute modulatory effects of test compounds on T cell activation in naïve or pre-activated T cells. This application note provides an overview of these discrete assay designs as well as application examples illustrating how to apply these methods in the context of T cell immunology and metabolism, which are relevant for immune cell therapies and drug discovery.

Introduction

Agilent Seahorse XF technology is a label-free, integrated platform that seamlessly combines an XF Analyzer, sensor cartridge, assay kits, and intuitive software to deliver functional cellular metabolic data with real-time kinetics. Built on this platform, Agilent offers a variety of XF assay kits providing a full spectrum of information, from a broad assessment of cellular function, to specific details of metabolic characteristics. In particular, the glycolytic activity of live cells can be conveniently assessed in XF assays via measuring the extracellular acidification rate (ECAR) and subsequently calculating a proton efflux rate (PER), which correlates with the lactate production rate.[1,2]

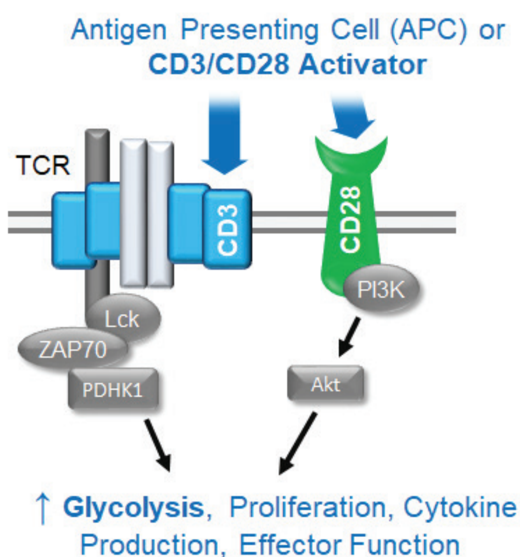

Figure 1. APC or CD3/CD28 activators induce a glycolytic increase in T cells. Upon the activation by APC or CD3/CD28 activator, the signal from T cell receptors (TCRs) and CD28 ligands are transmitted by signaling cascades including various kinases, resulting in an immediate increase in glycolysis and subsequent proliferation, cytokine production, associated effector functions, and prolonged metabolic alterations.

This ability to measure glycolytic changes in real-time is particularly powerful in the context of T cell activation, where a rapid early increase in glycolytic activity is recognized as a signature activation event and is critical for subsequent T cell proliferation and differentiation. This immediate increase in glycolysis can be captured in real time through *in situ* injections of the soluble T cell activators co-stimulating CD3 and CD28 ligands commonly used for *in vitro* T cell activation (Figure 1).[3-6] This accessible, streamlined assay enables real-time monitoring and modulating of T cell activation in live cells, offering an early window into the activation responses across different T cell subtypes or functional responses to specific pharmaceutical or genetic interventions that typically cannot be obtained by orthogonal methods measuring gene expression, cell proliferation, or cytokine production.

The XF Hu T Cell Activation Assay Kit is designed to measure human T cell activation via a simple and reliable workflow. For real-time XF T cell activation assays, there are several choices of activators mimicking an APC, including anti-CD3 and/or anti-CD28 antibodies[3-5] or beads conjugated with relevant antibodies.[6] The XF Hu T Cell Activation Assay Kit exploits the soluble ImmunoCult Human CD3/CD28 T Cell Activator (STEMCELL Technologies), a tetrameric complex of human CD3/CD28 T cell ligand activators, as it offers a distinct advantage over bead-based approaches, both in terms of reduced preparation time and improved injection performance, thereby increasing the convenience, reliability, and consistency of T cell activation measurements on the XF platform. The XF Hu T Cell Activation Assay Kit also incorporates two further assay components that facilitate additional assay performance and convenience: ready-to-use PDL-coated XF Cell Culture Microplates decrease assay preparation time and ensure consistent PDL coating across wells and plates. Seahorse Analytics, a versatile, cloud-based XF data analysis tool, provides convenient, flexible data transformation to aid analysis and interpretation of T cell activation assay data. This application note provides an overview of the assay design, performance, and application of the XF Hu T Cell Activation Assay Kit in combination with XF PDL Cell Culture Microplates and Seahorse Analytics through examples using naïve and pre-activated human T cells.

Experimental

Materials

Materials	Vendor
Primary CD4+ and CD8+ T cells, PBMC	STEMCELL Technologies or Hemacare
ImmunoCult-XF T Cell Expansion Medium	STEMCELL Technologies
Interleukin 2 (IL-2)	
Agilent Seahorse XF Hu T Cell Activation Kit (includes ImmunoCult Human CD3/CD28 T Cell Activator and 2-DG)	Agilent Technologies
XFe96 PDL Cell Culture Microplates	
XFe96 Sensor Cartridges	
XF Calibrant	
XF RPMI Medium, pH 7.4	
XF Glucose	
XF Pyruvate	
XF Glutamine	
Seahorse XFe96 Analyzer	
Human IL-2 DuoSet ELISA (DY202)	R&D Systems
Dasatinib	Millipore Sigma
Lck inhibitor	
96-well cell culture plates (for ELISA)	Corning
Microplate Reader	Tecan Group Ltd

Cells

For naïve T cell activation experiments, frozen naïve CD4+ or CD8+ T cells isolated from human PBMCs (STEMCELL Technologies) were thawed and cultured in ImmunoCult-XF T Cell Expansion Medium following the manufacturer's instructions. For the reactivation assay, a portion of naïve T cells was activated on six-well plates using Dynabeads or ImmunoCult human CD3/CD28 T cell activator in ImmunoCult-XF T Cell Expansion Medium. The expansion medium was supplemented with 300 U/mL IL-2 starting the fourth day after activation, and activated cells were continually cultured up to 20 days.

ELISA

To measure IL-2 production, parallel sets of naïve T cells were prepared in conventional 96-well microplates for interrogation by ELISA. Briefly, naïve T cells were placed in suspension at a density of 1×10^5 cells/well in ImmunoCult-XF T Cell Expansion Medium without supplement. For CD3/CD28 activator titration (Figure 5), activator volumes were adjusted to 2.5, 5, 10, and 20 µL per 200 µL, which correspond to the activator concentration per well used in the corresponding XF T Cell Activation Assay. For pharmaceutical modulation (Figure 8D), cells were pretreated with dasatinib for 30 minutes followed by addition of 10 µL of the CD3/CD28 activator. After the activations, cells were then cultured for 72 hours followed by media collection. Media samples from each well were collected after microplate centrifugation (200 × g for 1 minute). IL-2 levels were detected using a Human IL-2 DuoSet ELISA (DY202, R&D Systems) as per manufacturer's instructions with absorbance measured at 450, 540, and 570 nm (Spark, Tecan).

Cell imaging

Bright-field images were acquired using the Agilent Seahorse XF Imaging and Normalization System immediately after cell seeding.[7]

The XF T Cell Activation Assays

All XF T Cell Activation Assays were performed as described in the XF T Cell Activation Assay User Guide[8] including sensor cartridge preparation, PDL-coated microplate preparation, cell seeding, and compound dilution. The overall assay procedure is summarized in Figure 2.

XF T Cell Activation Assay strategy and design

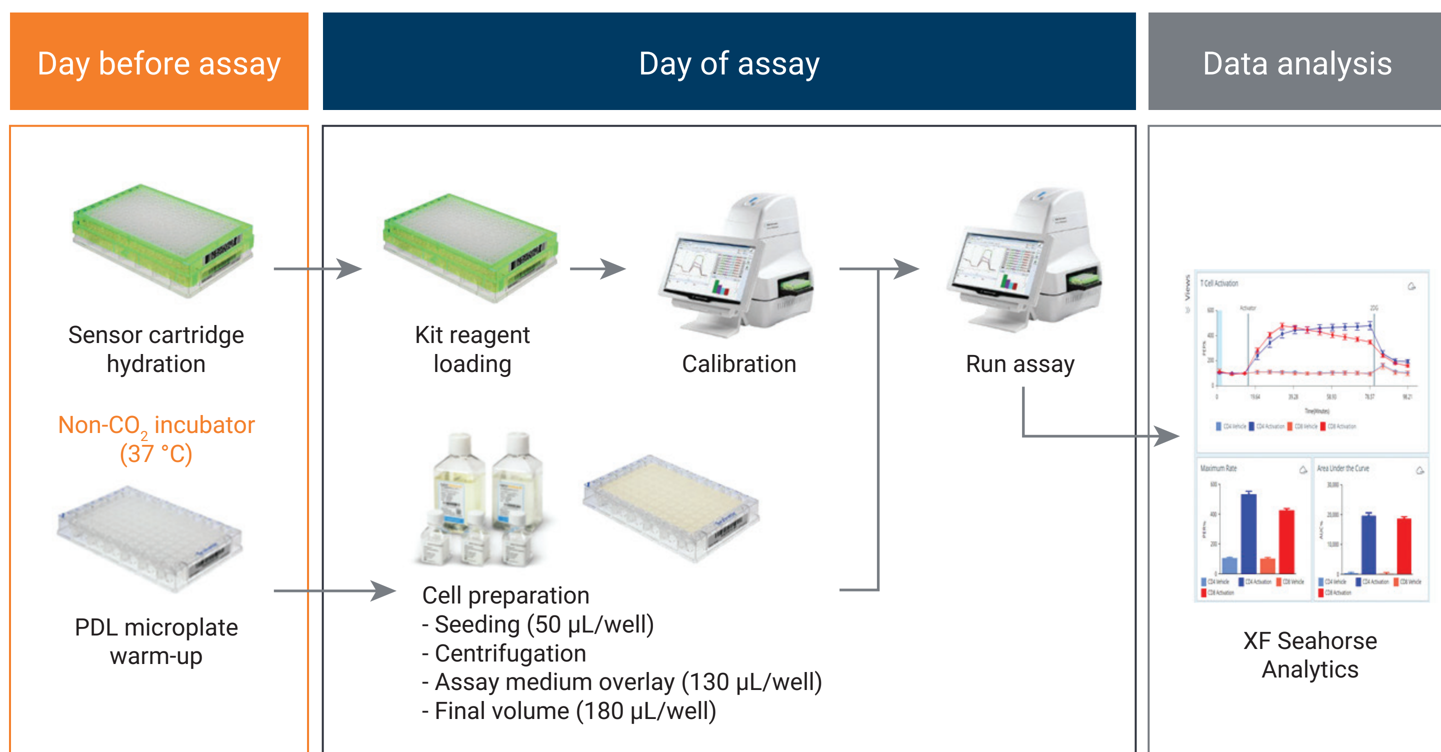

Figure 2. Summary of T cell activation assay workflow.

The schematic illustration in Figure 3 summarizes specific XF Hu T Cell Activation Assay designs and associated data outputs. Basal ECAR is first established, followed by injection of the CD3/CD28 Activator. The increase in ECAR induced by T cell activation is monitored over a period of time, typically 10 measurement cycles over 60 minutes, followed by an injection of 2-DG, which abrogates the activation-associated glycolytic increase. The resulting ECAR data is then automatically transformed to proton efflux rate (PER, in pmol/min) via Wave or Seahorse Analytics (Figure 3).

The XF T Cell Activation Assay supports two experimental designs: the standard assay for testing T cell activation (Figure 3A) and the modulation assay for investigating acute modulatory effects of test compounds on T cell activation (Figure 3B). The standard assay includes two injections: an injection of CD3/CD28 activator is followed by an injection of 2-DG and can be used to compare activation potential among T cells from different donors or T cells with applied interventions (e.g., genetic and/or pharmaceutical intervention). The modulation assay incorporates an additional injection of test compound(s) prior to the CD3/CD28 activator injection, thereby interrogating acute modulatory effects of test compound(s) on T cell activation potential.

XF T Cell Activation Assay data analysis using XF Seahorse Analytics

XF Seahorse Analytics is a web-based software platform that provides a simple streamlined data analysis tool for the XF T Cell Activation Assay. The T Cell Activation Assay View within Seahorse Analytics provides a summary table and three graphic views: a kinetic graph (PER versus time), a bar chart of maximum PER (pmol/min or %), and a bar chart of area under the curve (AUC, pmol or %) calculated for the activation period. The kinetic graph is used for real-time assessment of T cell activation dynamics and provides a comprehensive overview of the T cell response. The maximum rate, calculated using a least square best-fit, is useful for comparing the magnitude of activation among different groups/samples (Figure 4A). The AUC provides

cumulative information with respect to T cell activation potential (Figure 4B). For additional analysis or graphing needs, the output data can be exported to a Microsoft Excel or GraphPad Prism file format using the data export function included in Seahorse Analytics.

In general, it is recommended that data evaluation begin with an analysis of absolute rate data (PER in pmol/min) minimizing potential loss of critical information. However, within Seahorse Analytics, kinetic trace and maximal rate data can be transformed using the *Baseline* feature.[9] This transformation converts absolute XF rate data to a relative (%) scale based typically on the measured rate immediately before activator injection, thereby enabling relative comparisons across a series of experiments and minimizing well-to-well variation due to minor differences in cell seeding.

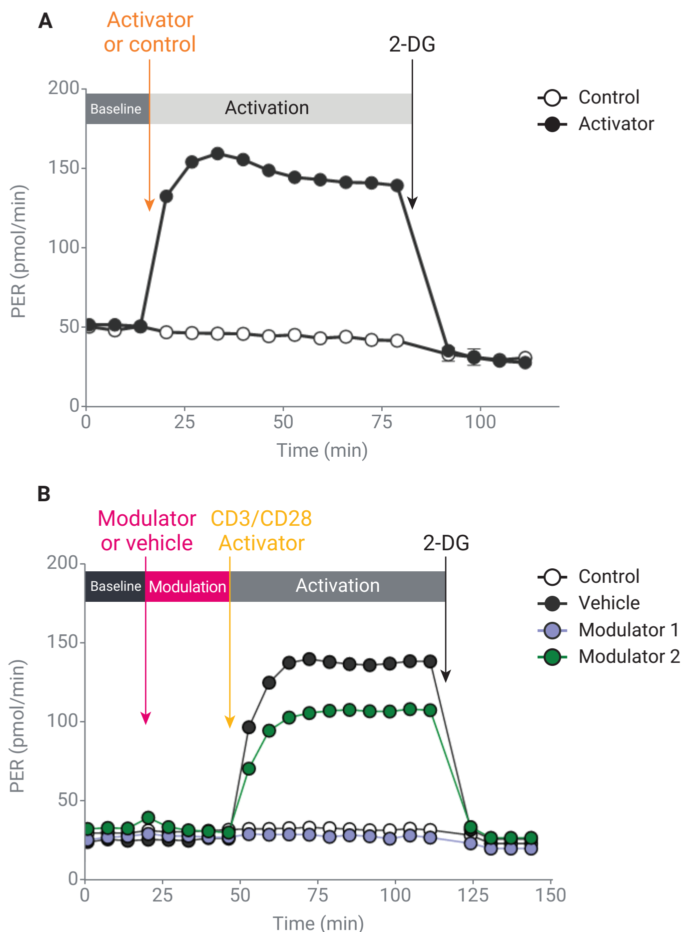

Figure 3. Agilent Seahorse XF Hu T Cell Activation Assays measuring the glycolytic response of T cells upon the injection of CD3/CD28 activator in the standard assay design (A) and the modulation assay design (B). The glycolytic rate can be analyzed by the absolute PER value (pmol/min) or by the relative increase to the baseline, PER (%).

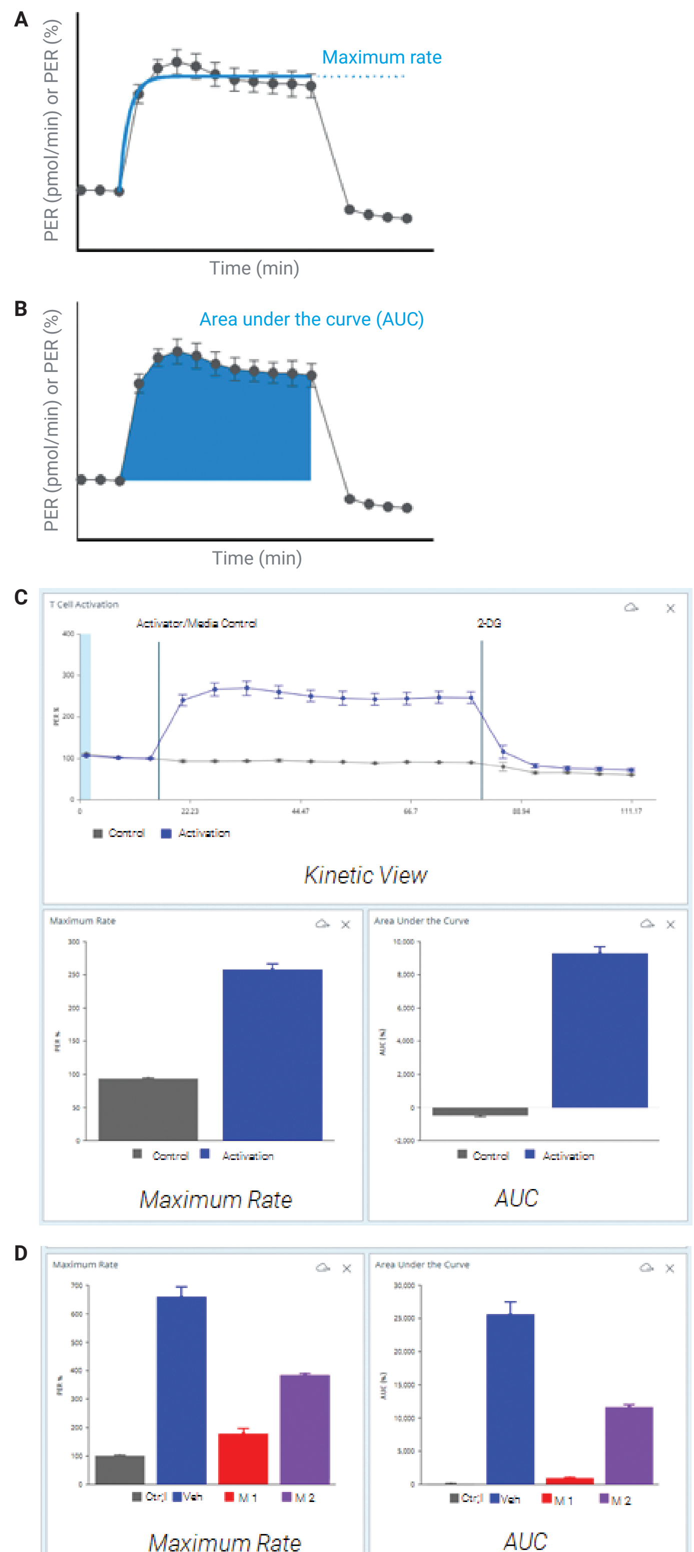

Figure 4. Data analysis of Agilent Seahorse XF Hu T Cell Activation Assay using XF Seahorse Analytics. (A) and (B) are schematic illustrations for the methods calculating the maximum rate and the AUC, respectively. (C) and (D) are example views of kinetic graph and bar charts for maximum rate and AUC for the standard assay data and the modulation assay data, respectively.

Despite its utility, it should not be considered a substitute for interpretation of absolute rates, as valuable information may be lost upon transformation, and consideration should also be given to enabling interlaboratory data comparisons, therefore reporting of absolute values is encouraged.

Results and discussion

Activator evaluation and optimization

As an initial demonstration of principle and to establish optimal assay conditions, the volume of ImmunoCult CD3/CD28 activator reagent was evaluated using naïve CD4+ T cells, ranging from 2.5 to 20 µL/well (total injection volume of 20 µL/well). Kinetic data in Figure 5A show that the glycolytic response increases as the activator dose increases. The data also indicate that activator at 10 µL/well is sufficient to achieve maximal sustained activation.

T cell activation was confirmed by an orthogonal ELISA, measuring IL-2 production in parallel sets of cells using the same activator concentrations (Figure 5D). These ELISA data show little difference in IL-2 accumulated for 72 hours among cells stimulated with activator amounts from 5 to 20 µL/well. In contrast, the XF data acquired immediately post-activation showed activation patterns that correlate to the activator dose as observed in the kinetic PER traces (Figure 5A) and in both maximum rate and AUC values (Figure 5B and 5C, respectively). The XF real-time assay can therefore offer a unique way to measure and delineate immune modulation and efficacy that is not possible using other end-point assays, such as ELISA.

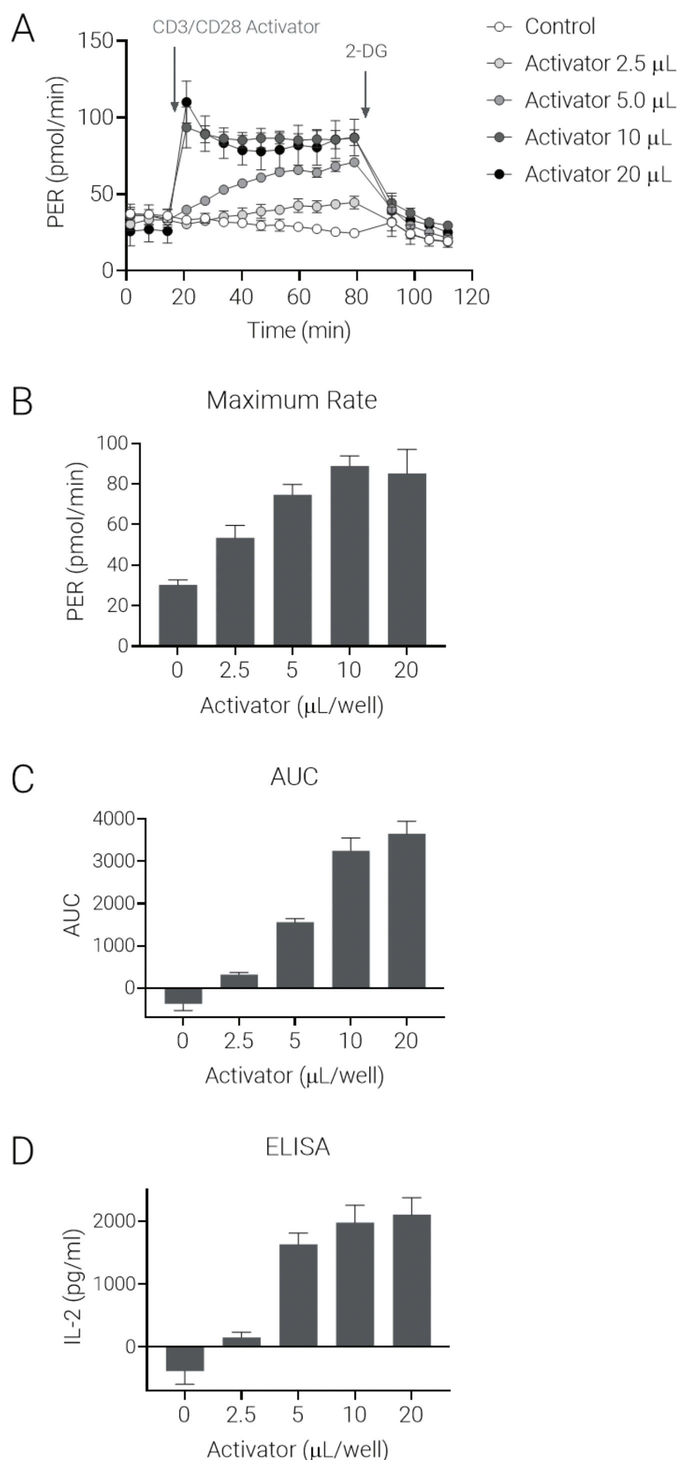

Figure 5. Activation of naïve CD4+ T cells by varying amounts of the CD3/CD28 activator. The total injection volume of activator was fixed at 20 µL/well for all groups, which was achieved by diluting the activators in XF assay medium for groups with less than 20 µL volumes prior to loading to the injection ports. (A) Kinetic graph exhibiting real-time changes in PER upon injection of the activator at different doses. (B) and (C) Maximum PER and AUC values at different activator doses. (D) IL-2 production at different activator doses as measured by ELISA with culture medium collected at 72 hours post-cell activation.

Application for assessing activation potential of T cells

T cell activation, as well as subsequent functions of proliferation and differentiation, are metabolically regulated and can be pharmacologically modulated. The XF Hu T Cell Activation Assay can be used to compare the activation potential and other activation characteristics among T cells from different subjects or T cells with applied interventions (e.g., a genetic manipulation and/or chronic compound exposure). Figure 6 shows a simple example comparing the activation between CD4+ and CD8+ T cells. Each cell type was from a different, unrelated subject, and T cell activation kinetics, as well as maximal rate and AUC values, were compared using absolute PER (pmol/min) and relative (%) values. Both CD4+ and CD8+ cells showed increases in PER upon injection of CD3/CD28 activator and decreases in PER with subsequent injection of 2-DG.

Analysis of absolute PER suggest that CD4+ cells exhibited both higher basal rates and higher activation-induced maxima than CD8+ cells (Figures 6A and B); however, phase contrast images acquired using the XF Seahorse Imaging and Normalization System (Figure 6C) illustrate that these differences in absolute PER are due largely to differences in cell number per well. This is further supported by the observation that when absolute PER values are transformed to a relative (%) scale, the relative differences in maximal rate are absent, suggesting that the CD4+ and CD8+ cells have similar activation potentials, thereby underscoring the utility of baseline normalization. In addition, both absolute value an % data suggest different kinetic behavior, as CD4+ cells maintained elevated glycolytic activity for the duration of measurement, while elevated CD8+ cell PER was slightly attenuated 20 to 30 minutes post-activation.

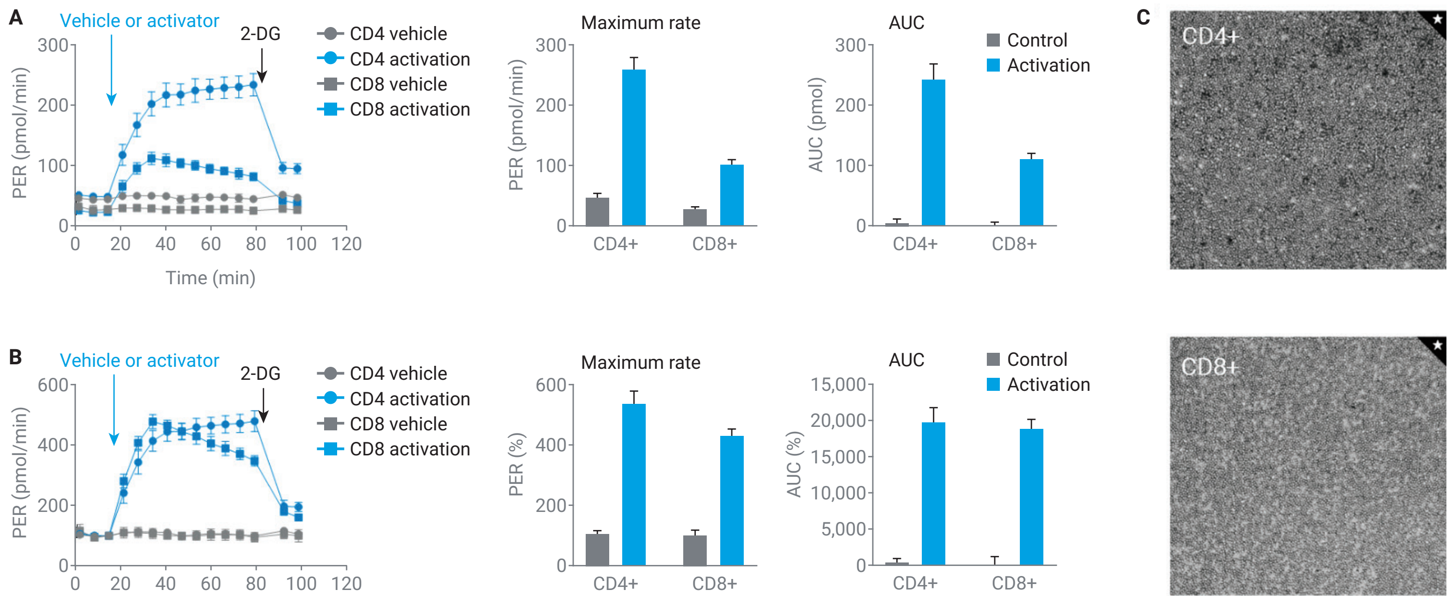

Figure 6. XF T cell activation assay data with or without baseline normalization. Naïve CD4+ and CD8+ T cells were activated by the standard assay protocol and the data were plotted as absolute PER (pmol/min) (A) and as relative PER (% change relative to the last basal rate measurement) (B). The cell seeding conditions were compared by using the brightfield imaging function of the Agilent Seahorse XF Imaging and Normalization System (C).

Applications for measuring effects of T cell modulators

The XF Hu T Cell Activation Assay can also be used to interrogate the acute effect of pharmacological agents on T cell activation using either the standard or modulation assay design. Lck is a well-known tyrosine kinase included in the T cell receptor complex. It transmits the early activation signals from the receptors to downstream cascades (Figure 1). Recently, dasatinib, a Bcr-Abl kinase inhibitor originally developed as an anticancer agent, was reported as a potential CAR-T cell activation modulator through Lck suppression.[10] To exemplify the utility of the XF Hu T Cell Activation Assay in assessing the pharmacological modulation of activation, the effects of dasatinib and Lck-inhibitor on primary T cell activation were measured. To determine the effective drug concentration, previously activated CD4+ T cells were pretreated with increasing amounts of compound for 30 minutes prior to the T cell activation assay. Figure 7 shows that T cell activation was suppressed in a dose-dependent manner by both dasatinib and Lck-inhibitor. Kinetic data can be conveniently transformed to maximum rates and AUC values using Seahorse Analytics, which can be exported to Microsoft Excel or GraphPad Prizm for additional analysis. Figure 7 shows examples of dose-dependent curves with IC_{50} value generation by GraphPad Prizm software using the maximum rate and AUC values exported from Seahorse Analytics.

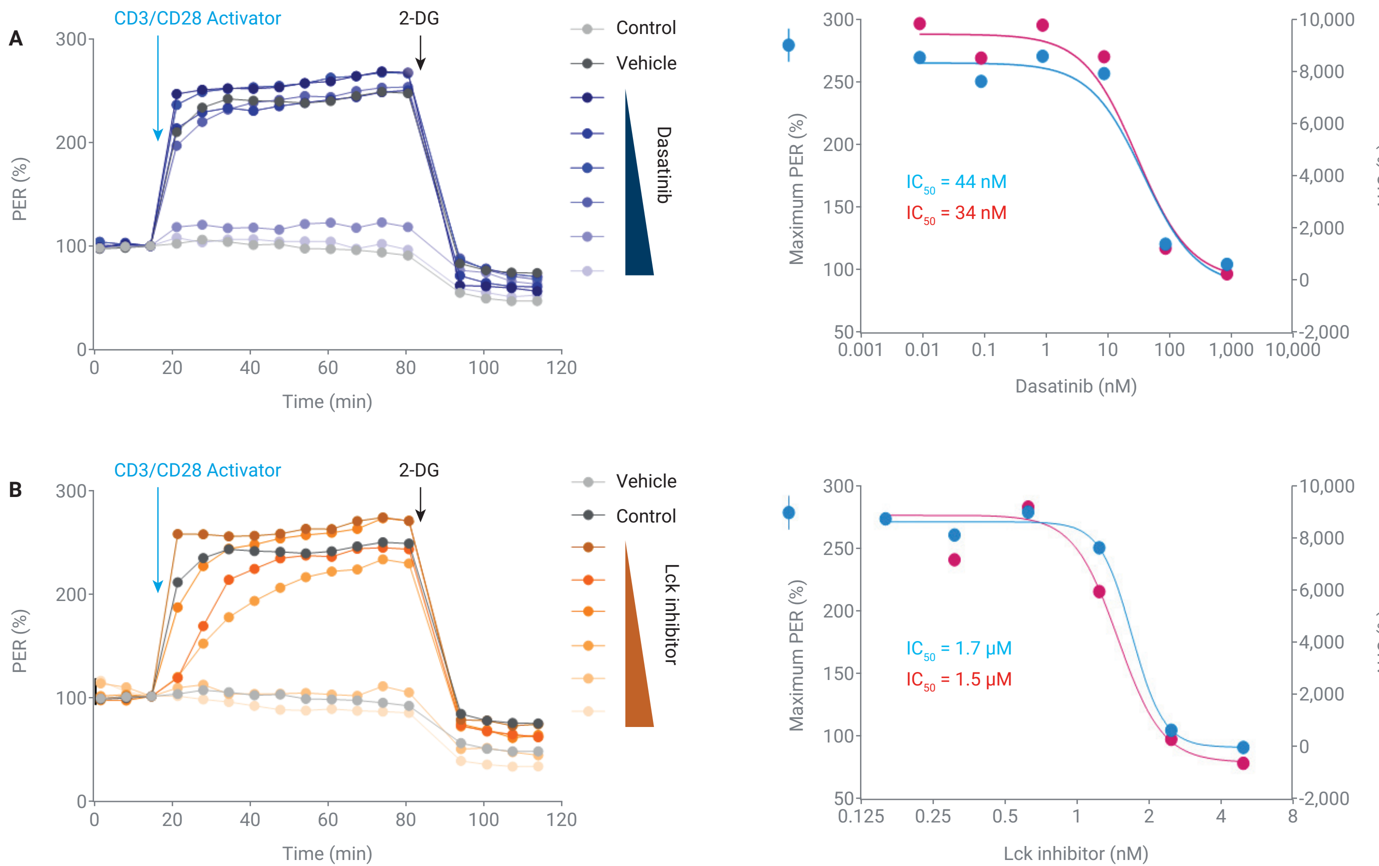

Figure 7. Characterization of T cell activation suppressors. Pre-activated CD4+ T cells were pretreated with dasatinib (A) or Lck inhibitor (B) at various concentrations for 30 minutes before the activation assay. The % maximum PER and % AUC values (left panels) were calculated with Agilent Seahorse Analytics and plotted against the drug concentrations. Dose curves as well as IC_{50} values (right panels) were derived using GraphPad Prism software.

Pharmacological modulation can also be assessed acutely using *in situ* injections of test compounds prior to activator injection. Figure 8 shows the acute effect of dasatinib on naïve T cell activation. Two different concentrations of dasatinib were injected (final well concentrations of 50 and 100 nM), and the impact on PER were monitored for five measurement cycles (~30 minutes). This was followed by CD3/CD28 activator to initiate activation. The T cell activation-associated PER increase was partially suppressed by 50 nM dasatinib, while it was almost completely suppressed by 100 nM dasatinib. These observations are consistent with the IC_{50} values observed in Figure 7. The transformed data in Figure 8B and 8C show the maximum activation potential in % PER and % AUC for the four conditions tested. The effect of dasatinib on T cell activation was also tested by measuring IL-2 production. The results corresponded well to the XF Hu T cell Activation Assay data, showing significantly decreased levels of IL-2 suppression in the presence of dasatinib (Figure 8D).

Conclusion

This study describes the assay design and proof of concept for the XF Hu T Cell Activation Assay, which provides a rapid method to visualize T cells activation by monitoring increases in PER in real-time. This assay allows for early detection of T cell activation and comparison of activation kinetics. It also facilitates analysis of modulation by pharmacological or genetic interventions. The XF Hu T Cell Activation Assay Kit employs a soluble CD3/CD28 activator to maximize assay performance, and when used in conjunction with precoated PDL XF Cell Culture Microplates, provides a standardized and convenient workflow. In addition, Seahorse Analytics delivers a streamlined and accessible analytical tool that automatically provides key assay parameters for data interpretation. This turnkey solution for real-time analysis of T cell activation facilitates investigation of chronic and/or acute immune modulators and can therefore be of significant utility in cellular and molecular immunotherapy discovery for various diseases including cancer, immune dysfunction, and various metabolic diseases.

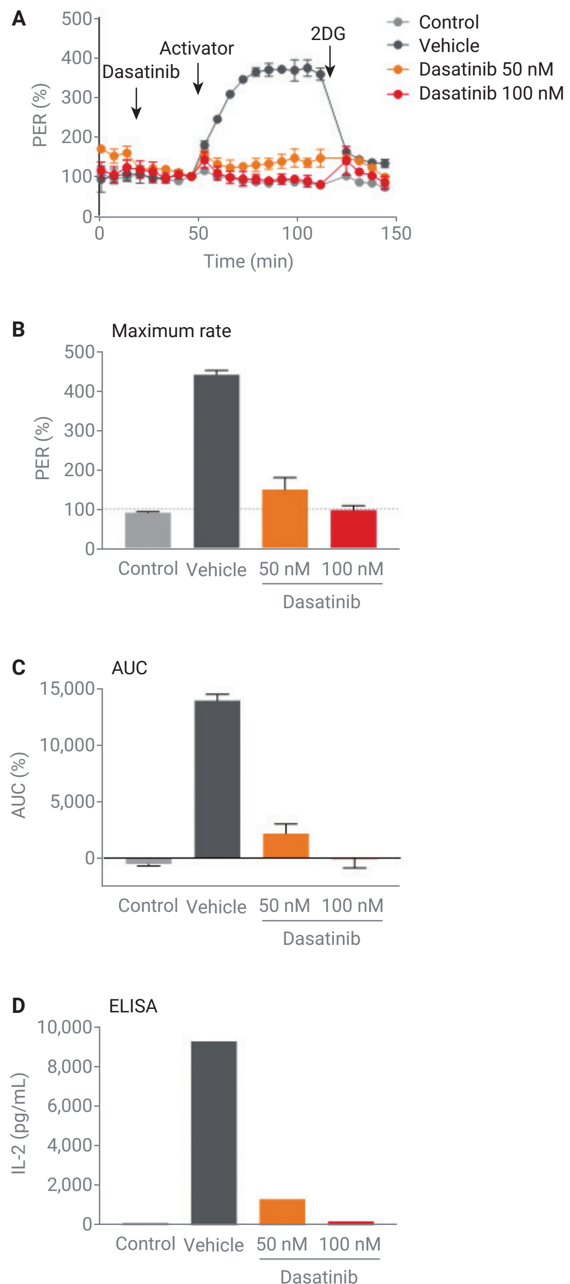

Figure 8. Evaluation of the acute effect of dasatinib on CD4+ T cell activation using the modulation assay protocol. Dasatinib was injected at two different concentrations (50 and 100 nM) prior to the activator injection. (A) Kinetic graph of real-time changes in % PER; (B) Maximum rates in % PER; (C) AUC values in % PER; (D) IL-2 production measured by ELISA with culture medium collected at 72 hours post-cell activation.

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