Rapid, Real-time Detection of T Cell Activation Using an Agilent Seahorse XFp Analyzer

Application Note

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Abstract
Immune cells commit to specific lineages by integrating signals from many pathways. Cellular energy metabolism is now understood to influence many of these pathways – as such, metabolism is both an indicator and controller of immune cell function. This concept is clearly illustrated in the activation of naïve T cells to stimulate proliferation. T cell activation is accompanied by a rapid switch in cellular energy production; an effect that is detectable in minutes instead of hours/days through typical markers (for example, CD-69, IL-2). This Application Note describes a method for quantifying T cell activation using in situ activation of naïve T cells with immunogenic beads injected during the assay. This method is rapid, kinetic, nondestructive, and directly tied to cellular function. Activated cells may be further interrogated or used in additional downstream assays.
Introduction

Recent advances in the field of immunology identify cellular metabolism as a primary driver and regulator of immune cell function and differentiation. T cell activation is correlated with metabolic shifts, especially in glycolysis, providing increased metabolic flux through growth-promoting pathways required for high rates of cellular proliferation. Conventionally, progression of T cell activation is measured in terms of changes in cell size/morphology, interleukin/interferon expression, or lactate efflux. These methods can be time and labor-intensive, are often end point (not kinetic) data, and are typically on a time scale of hours to days.

Using Agilent Seahorse XF technology to measure changes in the rate of extracellular acidification (ECAR, a qualitative indicator of glycolysis), a correlation has been established between T cell activation and an acute increase in ECAR following stimulation. This assay design can be used to perform pairwise comparisons between nonactivated and activated cells (Figure 1), assessing different T cell subsets (such as, naive versus memory), or comparing control cells to those with interventions, including genetic manipulations and drug treatments to identify changes in activation kinetics and magnitude of T cells. A major advantage of the T cell Activation Assay is the exceedingly rapid time frame of changes in ECAR, typically within several minutes of activating the T cells through injection of immunogenic beads. This rapid increase in ECAR concomitant with T cell activation has been demonstrated for CD4+ and CD8+ T cells, in both mouse and human.

Results and Discussion

Proof of concept

To illustrate proof of concept for in situ T cell activation by injection of immunogenic beads, several assay designs were used. Figure 1 illustrates a pairwise comparison between naïve CD4+ cells and those that received an injection of anti-CD3/CD28 DynaBeads. Cells that were exposed to beads have an immediate and significant increase in ECAR, while ECAR in naïve cells remains at a basal level (Figure 1B).

Next, to ensure in situ activated CD4+ and CD8+ T cells reached the same level of ECAR as T cells activated by a pretreatment of immunogenic beads, a pairwise comparison was made between these conditions (Figures 2A and 2E), and indeed, the ECAR values were of similar magnitude for both groups, as demonstrated in Figures 2B and 2F. To demonstrate that increases in ECAR upon T cell activation correlate with both interleukin (IL-2) and interferon (INF-γ) expression, the cells that were activated in Figure 2 were cultured for an additional 48–72 hours. Figures 2C and 2G show similar levels of IL-2 and INF-γ expression, respectively, between in situ and pre-activated T cells 48 hours post-activation. Figures 2D and 2H show typical progression of morphological changes associated with T cell activation, including increases in cell diameter and number of cells due to proliferation for in situ activated T cells 72 hours post-activation.

Figure 1. XFp-based detection of CD4+ T cell activation. A) Assay design for the T cell activation assay using an XFp. B) Kinetic trace of ECAR versus time, demonstrating increases in ECAR upon injection (arrow) of anti-CD3/CD28 beads onto human T cells; naïve T cells activated with anti-CD3/CD28 bead injection (∙, 4:1 bead to cell ratio); naïve T cells receiving vehicle injection show no activation (∗).
Figure 2. Stimulation of ECAR correlates with markers of T cell activation. A) and E) XFp T cell Activation Assay strategy. B) and F) Kinetic traces of ECAR versus time, showing that in situ activation of naïve T-cells by bead injection (●) reaches the same magnitude as T-cells activated by conventional bead treatment (●, 1:1 ratio bead to cell, plated and incubated for 1 hour prior to the XF assay). C) and G) ELISA assay showing comparative levels of IL-2 (ng/mL) and INF-γ (ng/mL) production in the in situ activated (●) and pre-activated (●) T cells. D) and H) Images of T cells showing morphological changes on XFp plates days 0 and 3 post in situ activation by bead injection. Representative data for human CD4+ (upper panel) and CD8+ (lower panel) T cells.
For T cells in suspension, activation is typically performed with 1:1 to 3:1 bead-to-cell ratios over 1–3 days in culture. To optimize the bead:cell ratio for activation by XF injection, the bead:cell ratio was titrated by comparing the ECAR measured 30 minutes after bead injection with ECAR of pre-activated/conventionally activated T cells (coated beads added directly to cells prior to the XF assay) (Figure 3). Based on this work and others, a 4:1 bead ratio is recommended for maximal T cell activation by injection in the Xfp. No significant difference in ECAR levels were observed for pre-activated at higher than 1:1 bead ratio. Of note, injection of isotype (mouse IgG2a) coated beads had no significant effect on ECAR or OCR values of naive T cells (data not shown).

Figure 3. Optimization of bead-to-cell ratio for an XF T cell Activation Assay titration of bead-to-T Cell ratio prior to, and 30 minutes post bead injection normalized to 100 % of pre-activated control. Pre-activation represents the ECAR measured 1 hour after conventional activation of T cells, and is normalized to 100 %. Injection of beads using a ratio of 4:1 (bead:cells) shows an equivalent level of ECAR as the conventionally activated T cells. Representative results for A) CD4+; B) CD8+.
To visualize the bead distribution, XFp miniplates were imaged after the completion of the T cell Activation Assay. The injection of beads resulted in a consistent distribution across the cell layer in the XFp miniplate (Figure 4B). The distribution of beads in the 4:1 injection ratio (Figure 4D) were equivalent to beads mixed with cells in suspension at a 1:1 ratio or the pre-activated control (Figure 4C). These data correspond to the titrated bead activation data (Figure 3), where a 4:1 injection ratio resulted in equivalent ECAR levels. The need to increase the bead to cell ratios to 3:1 or 4:1 for injection is in part due to some of the beads remaining in the ports (Figure 4F).

Figure 4. Characterization of bead injection and delivery to cells through XFp cartridge ports. Overview of entire XFp well with A) $2.0 \times 10^5$ cells/well T cells. B) $2.0 \times 10^5$ cells/well T cells + $8.0 \times 10^5$ beads. Comparative images showing C) pre-activated 1:1 bead-to-cell ratio similar to D) 4:1 bead-to-cell ratio delivered through injection with respect to dispersion of beads and little/no disruption of cell monolayer. E) Image of XFp cartridge ports after XFp assay. Residual beads in the ports after injection are a part of normal operation.
Acute glycolytic switch in effector memory (EM) and central memory (CM) CD8+ T cells upon activation (by injection of anti-CD3/CD28) in an Agilent Seahorse XF Analyzer. The authors then compared activation of EM CD8+ T cells in the presence or absence of PI3K and AKT inhibitors, showing that activation and glycolysis in EM CD8+ T cells are dependent on the PI(3)K-AKT pathway. Similarly, Pollizi; et al.11 found that genetic deletion of mTORC2 in CD8+ T cells (T-Rictor–/–) resulted in high ECAR prior to and upon restimulation in an XF T cell Activation Assay. This activation was not found in mTORC1-deficient antigen-experienced (T-Rheb–/–) CD8+ T cells, which display a memory phenotype, but failed to mount a robust effector response upon rechallenge. This work demonstrated that mTORC1 activity is not only necessary for the initial differentiation into effector cells, but is also important for the generation of an effector response derived from memory cells.

Assay design

A suggested XF T cell Activation Assay design for the XFp is the pair-wise comparison of activation between two groups of T-cells. Typically, a comparison is made between isolated T cell subsets, and T cells that have been treated or otherwise intervened upon (for example, genetic manipulation), and control (nontreated) cells, with both groups of cells being activated through bead injection using Port A of the XF instrument. Note that more complex assays can be designed by use of the three remaining injection ports of the XFp cartridge. Figure 5 shows an overall scheme for preparing T cells, materials, and performing the XF assay.

The XF T cell Activation Assay has been used to understand the cellular and signaling requirements that establish activation of an effector response. This protocol, originally described by Gubser; et al.4, was first used to demonstrate an early acute glycolytic switch in effector memory (EM) and central memory (CM) CD8+ T cells upon activation (by injection of anti-CD3/CD28) in an Agilent Seahorse XF Analyzer. The authors then compared activation of EM CD8+ T cells in the presence or absence of PI3K and AKT inhibitors, showing that activation and glycolysis in EM CD8+ T cells are dependent on the PI(3)K-AKT pathway. Similarly, Pollizi; et al.11 found that genetic deletion of mTORC2 in CD8+ T cells (T-Rictor–/–) resulted in high ECAR prior to and upon restimulation in an XF T cell Activation Assay. This activation was not found in mTORC1-deficient antigen-experienced (T-Rheb–/–) CD8+ T cells, which display a memory phenotype, but failed to mount a robust effector response upon rechallenge. This work demonstrated that mTORC1 activity is not only necessary for the initial differentiation into effector cells, but is also important for the generation of an effector response derived from memory cells.

Figure 5. XF T cell Activation Assay design. A) Workflow schematic describing the necessary assay steps and relative timing to perform the assay, B) A suggested XFp miniplate layout for performing the assay, C) XFp Template in Wave desktop; note that protocol measure time is adjusted to 5 minutes.
Create the XFp Activation Assay template file using Wave desktop, where measurement times can be adjusted from the 3 minutes default value to the optimal 5 minutes, see the example in Figure 5C. Save the template to a flash drive and import the template onto the XFp instrument.

**XFp cartridge preparation**


**Reagent preparation**

**RPMI XF Assay media**

Combine 10.10 g/L RPMI powder (Corning, # 90-022, contains 11 mM glucose final), 1.39 g NaCl, 10 mL of 200 mM L-glutamine (2 mM final), 10 mL of 100 mM sodium pyruvate to (1 mM final), adjust the volume to 1.0 L with TC grade water. Warm to 37 °C, adjust pH to 7.4. Sterilize using a 0.2 µm filter, and store at 4 °C.

**Cell culture and cell preparation**

Frozen CD4+ or CD8+ T cells are thawed and permitted to recover in RPMI 1640 (GIBCO # 11875-085) supplemented with 10 % FBS at 37 °C, 5 % CO₂ for 18 to 24 hours. Prepare XFp plates by coating all wells with 20 µL of 100 µg/mL poly-D-lysine (PDL) solution (in H₂O) for 1 hour in a tissue culture hood with a lid on. Aspirate the PDL solution and wash the wells 2 times with 200 µL of sterile TC grade water. Allow to dry in the hood for 1 hour. If using immediately, add 80 µL of RPMI assay media per well and pre-incubate the plate (without cells) at 37 °C, no CO₂, for 1 hour. Note, PDL-coated XFp plates can be coated the day prior to the assay and stored at 4 °C. Cell-Tak (Corning, # 354240) may also be used to coat plates. Please see Immobilization of Non-Adherent Cells with Cell-Tak™ for Assay on the Seahorse XFe /XF96 or XFp Analyzer. (http://www.agilent.com/cs/pubimages/misc/PR_Non_Adherent_96.pdf).

Seed CD4+ or CD8+ T Cells in PDL-coated, pre-incubated XFp miniplates at $2 \times 10^5$ cells/well as follows: transfer an appropriate volume of cell suspension to a 15-mL conical tube, and centrifuge at 200 × g for 5 minutes. Wash cells with 1 mL RPMI assay media, centrifuge as above, and resuspend at 5.0 × $10^6$ cells/mL in RPMI assay media. Remove 80 µL of RPMI assay media from the pre-incubated plate. Add 40 µL of cell suspension to wells B–G of the XFp plate. Add 40 µL of RPMI assay media to A and H for background controls (no cells). Place the plate in the carrier tray, and centrifuge for 1 minute at 200 × g to adhere the cells to the plate. Gently add 135 µL of assay media (total volume = 175 µL), then add warm H₂O or XF calibrant to the outside troughs of the XF plate (using an 8-channel pipet, add 160 µL to the left and right-side troughs for a total of 320 µL each trough). Incubate at 37 °C, no CO₂, for 1 hour.

**Conclusion**

The XF T cell Activation Assay allows for a real-time kinetic test of activation by monitoring ECAR, an indicator of glycolytic activity. Results of T cell activation may be observed within several minutes, compared to conventional methods of detecting T cell activation, which can take hours to days, and is typically end point in nature. In addition, since the assay is nondestructive, the activated T cells may then be used in downstream applications or orthogonal assays.

**Materials and Methods**

- Agilent Seahorse XFp FluxPak (p/n 103022-100; XFp Assay Cartridges, utility miniplates, XFp cell culture miniplates, and calibrant)
- Poly-D-Lysine (Sigma, # P6407)
- RPMI base media without bicarbonate or phenol red (This will be available from Agilent February 2017 – see website for more details, alternatively Corning # 90-022)
- L-Glutamine 200 mM (Corning # 25-005-CI)
- Sodium Pyruvate 100 mM (Corning # 25-000-CI)
- Tissue Culture Grade Sterile water (Corning # 25-055-CM)
- Anti-CD3/CD28-Coated DynaBead (Life Technologies # 11131D or equivalent)
- DynaMag-2 Magnet, (Life Technologies # 12321D)
- CD4+ or CD8+ T cells (Freshly isolated, frozen, from HemaCare, # PB04C-1 or PB08C-3; Lonza, # 2W-200)
- RPMI 1640 (Life Technologies, # 11875-085)
- FBS (Hyclone, # SH3007003)

**XFp instrument operation**


Program the instrument commands into the assay template file as follows: Baseline measurement 3 cycles: Mix 3 minutes, Wait 0 minutes, Measure 5 minutes. Inject Port A, 15 cycles: Mix 3 minutes, Wait 0 minutes, Measure 5 minutes. Total run time is 2 hours and 20 minutes. This allows for 2 hours of real-time monitoring after activation. This time may be reduced or increased as needed by adjusting the number of cycles after the injection.
Preparation of anti-CD3/CD28-coated DynaBeads

Note the stock bead concentration, and read the vendor instructions for preparation of the beads. Prepare the beads just prior to loading them into XFP cartridge ports. Vortex the beads > 5 seconds, and transfer an appropriate volume (dependent on stock bead concentration) for the assay to a microfuge tube. Wash the beads with an equal volume of PBS + 0.1% BSA (w/v), vortex, place on a Dynamag, and remove the liquid phase. Repeat the wash with PBS + 0.1 % BSA, then wash 1x in RPMI assay media, vortexing for > 5 seconds each time to ensure that the beads are thoroughly mixed. Finally, resuspend the beads in RPMI assay media at the appropriate concentration for XF injection. For 2.0 × 10^5 T cells/well, the bead suspension should be prepared as 2.4 × 10^7 or 3.2 × 10^7 beads/mL to achieve 3:1 or 4:1 bead-to-cell ratio. Load 25 µL of prepared anti-CD3/CD28-coated DynaBeads into Port A of a hydrated XFP cartridge. Load the beads just prior to the start of XFP calibration.

References