Real Time Discrimination of Inflammatory Macrophage Activation Using Agilent Seahorse XF Technology

Application Note

Authors

Yoonseok Kam
Pamela M. Swain
Brian P. Dranka

Agilent Technologies, 121 Hartwell Ave. Lexington, MA 02421

Abstract

Immune cell activation and differentiation is controlled by a complex interaction of cellular signals, environment, and metabolism. This application note demonstrates how XF technology can be used for the detection and mechanistic investigation of the macrophage activation continuum. M1 polarized macrophages are typified by an activation-associated increase in glycolysis after pathogenic stimulation. Glycolysis is required to support high energy and biosynthetic demands of activated macrophages. Using XF technology, glycolytic flux can be quantified by real-time measurement of proton efflux rate (PER). The PER of M1 macrophages derived from human peripheral blood mononuclear cells (PBMC) was elevated within one hour after injection of lipopolysaccharide (LPS). This increase was sustained for hours and correlates with M1 macrophage-specific cytokine release. In contrast, macrophage cell lines exhibited a bi-phasic metabolic response after co-stimulation with LPS and interferon gamma (IFNγ). These results suggest that both short- and long-term XF analysis provide valuable kinetic information and mechanistic details of macrophage activation. The application models described here can be adapted for various studies on pathogenic and phagocytic activation of macrophages.

Agilent Technologies

Introduction

Immunometabolism research has dramatically expanded our understanding of immune cell function in recent years. Now this research area is moving beyond characterization of cell phenotype to controlling cell fate by modulating metabolism[1,2]. This increases the demand for relevant cell-based assays that can measure energy metabolism in living cells. XF technology is at the forefront of this field allowing researchers to measure cellular metabolic activity acutely and continuously as cells are stimulated in a real-time manner.

As the central components of the mononuclear phagocyte system, monocytes, macrophages, and dendritic cells (DCs) show distinctive physiological characteristics and functions depending on the direction and stage of differentiation, as well as the tissue in which they develop and reside[3]. For example, the differentiation and functional activation of PBMCs have typically been studied by characterizing the expression of cell subtype-specific surface markers and cytokine expression[4]. Recent investigations have demonstrated that metabolic reprogramming plays an essential role mediating differentiation and activation of macrophages and DCs[5,6].

Macrophages and DCs exhibit an immediate glycolytic response to pathogenic stimuli. DCs stimulated by LPS rapidly increases the glycolytic rate which is required for activation and signaling function through TBK1-IKKε and Akt[7]. For bone marrow-derived macrophages (BMDMs), the LPS-induced acute activation was potentiated through elevation of glycolytic capacity by granulocyte macrophage colony stimulating factor (GM-CSF) treatment[8].

Using Seahorse XF technology to measure the extracellular acidification rate (ECAR) as a qualitative indicator of glycolytic rate, a correlation has been established between macrophage activation and a rapid increase in ECAR[7,8]. Notably, glycolytic rate changes can now be presented as proton efflux rates (PER) by accounting for the buffer factor of the system and the acidification contribution from the TCA cycle to provide quantitative and accurate glycolytic rate data[9].

In this application note, we present examples of glycolytic responses associated with macrophage activation during a short time frame (< 2 h) or for an extended period (> 6 h). Simultaneously, mitochondrial function was monitored via oxygen consumption rate (OCR). These examples can be adapted for kinetic analysis of macrophages, DCs, and other mononuclear phagocytotic cell types.

Results and Discussion

Immediate early glycolytic response of macrophages

Human CD14+ PBMCs were polarized toward inflammatory M1 macrophages by exposure with GM-CSF for 6 days. Cells were plated in XF96 microplates in growth media containing GM-CSF one day prior to activation. Changes in PER and OCR were measured the following day in response to activation by LPS and IFNγ. As shown in Figure 1A, glycolytic rates increased rapidly upon co-injection of LPS and IFNγ while there was no response with assay medium injected alone. The injection slightly decreased the OCR but the effect was small compared to the changes in PER (Figure 1B). The presence of GM-CSF in the XF assay media during the analysis did not affect the change in PER or OCR (data not shown).

The functional relevance of activation-associated elevation in glycolytic rate was further validated by using fully differentiated M1 macrophages derived from human PBMC. They were stimulated by injection of LPS, IFNγ, or both, and metabolic rates were monitored for 10 h. Changes in metabolism were compared with cytokine accumulation measured at two different time points: upon the completion of XF analysis in the assay medium (0-10 h), and after a further 16 h culture in RPMI-1640 based
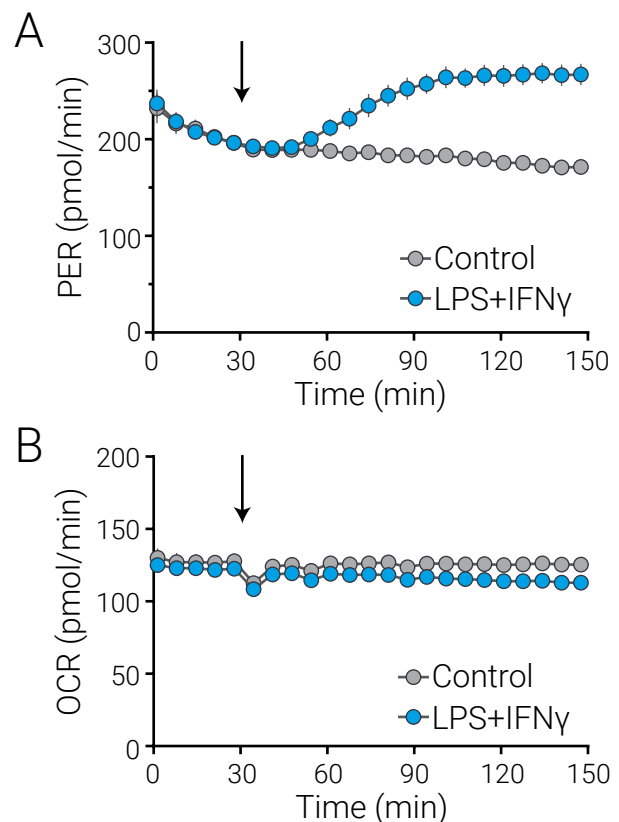

Figure 1. Glycolytic rate response of PBMC polarized by GM-CSF
M1 polarized PBMC were stimulated by injecting (arrow) LPS (100 ng/ml) and IFNγ (20 ng/ml) while monitoring PER (A) and OCR (B) for 2 h. The assay medium (control) or LPS and IFNγ mixture (LPS+IFNγ) was injected after 30 min of background measurements. Data shown are mean ± SD, n=8 technical replicates.
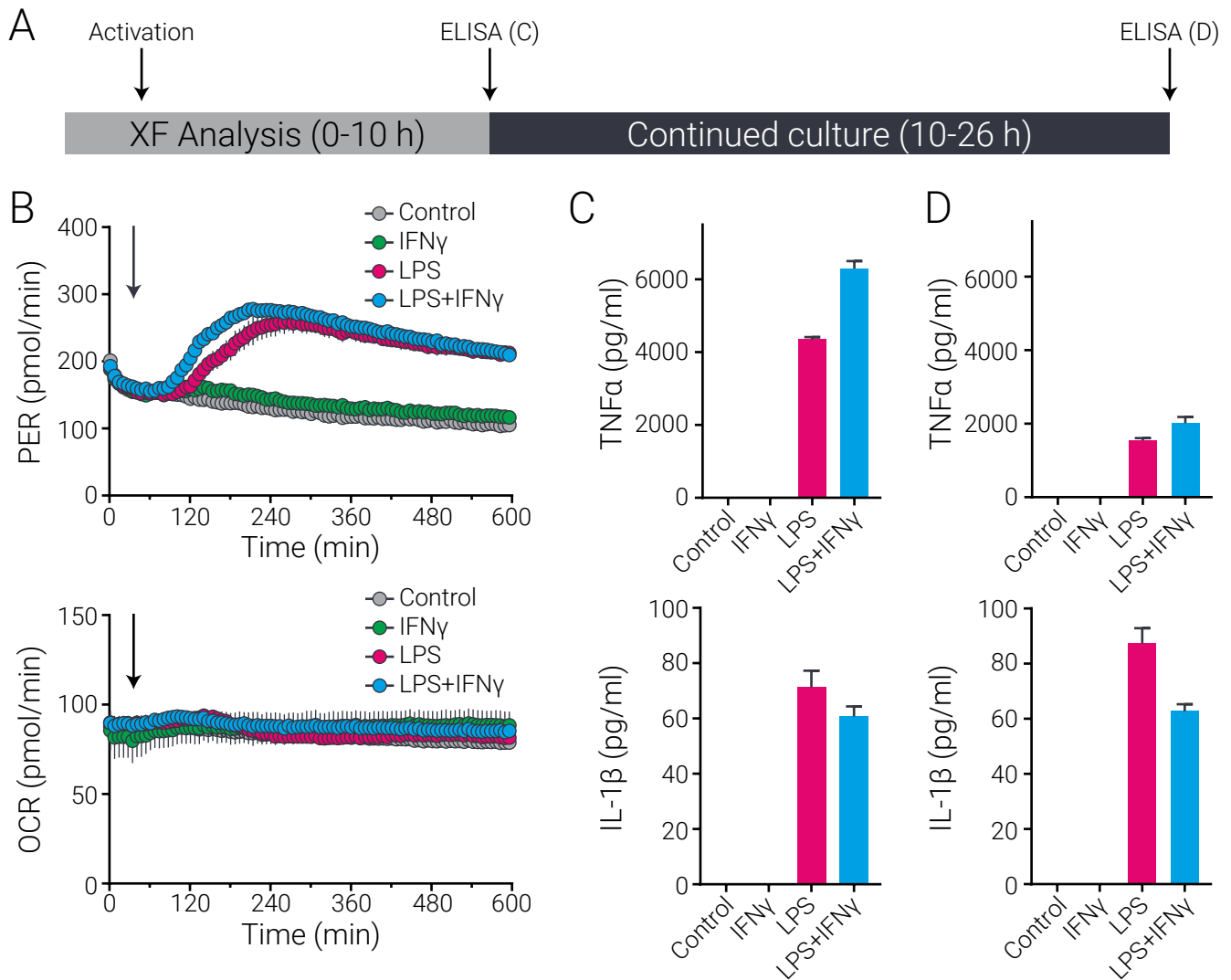
Figure 2. Activation of PBMC-derived M1 macrophages

(A) The experiment design including a long term (> 6 h) XF analysis followed by two consecutive ELISA analyses is schematically described. The assay medium and growth medium were collected at 10 and 26 h respectively for ELISA analysis. (B) The changes in PER and OCR were monitored for 10 h with or without agonist injection as indicated. (C) The accumulation of two cytokines, TNFα and IL-1β for 10 h in the assay medium were measured by ELISA. (D) The cytokine accumulation for 16 h in the growth medium was measured. Data shown are mean ± SD, n=4 technical replicates.

growth medium containing agonist(s) indicated (10-26 h) as described in Figure 2A. Figure 2B shows the immediate increase in glycolytic rate induced by LPS with or without IFNγ, but not by IFNγ alone. Elevated glycolytic rate was sustained until the completion of the assay, gradually decreasing 2 to 3 h after activation.

The immediate increase in glycolytic rate of M1 macrophages corresponded to the production of M1 activation-associated cytokines, tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) (Figure 2C and D). LPS stimulation induced the production of both cytokines in this XF assay condition as well as the continued culture in the growth medium regardless of IFNγ. IFNγ alone did not cause any significant accumulation of either TNFα or IL-1β corresponding to the lack of change in glycolytic rate. Interestingly, however, there was a clear difference in the

production kinetics between TNFα and IL-1β. The accumulation of TNFα diminished after 10 h whereas IL-1β production was sustained.

Activation-associated metabolic changes of RAW264.7 cells

RAW264.7 cells are widely used to understand molecular mechanisms of macrophage activation, typically in an M1-polarized setting. Although not identical to PBMC-derived M1 macrophages, RAW264.7 cells also rapidly increased glycolytic rate upon co-stimulation with LPS and IFNγ without impacting OCR (Figures 3A and B). In contrast to the PBMC-derived macrophages, RAW cells had a bi-phasic response to activation (Figure 3C). In addition to the initial effects described above, glycolytic rates were further increased when cells were treated
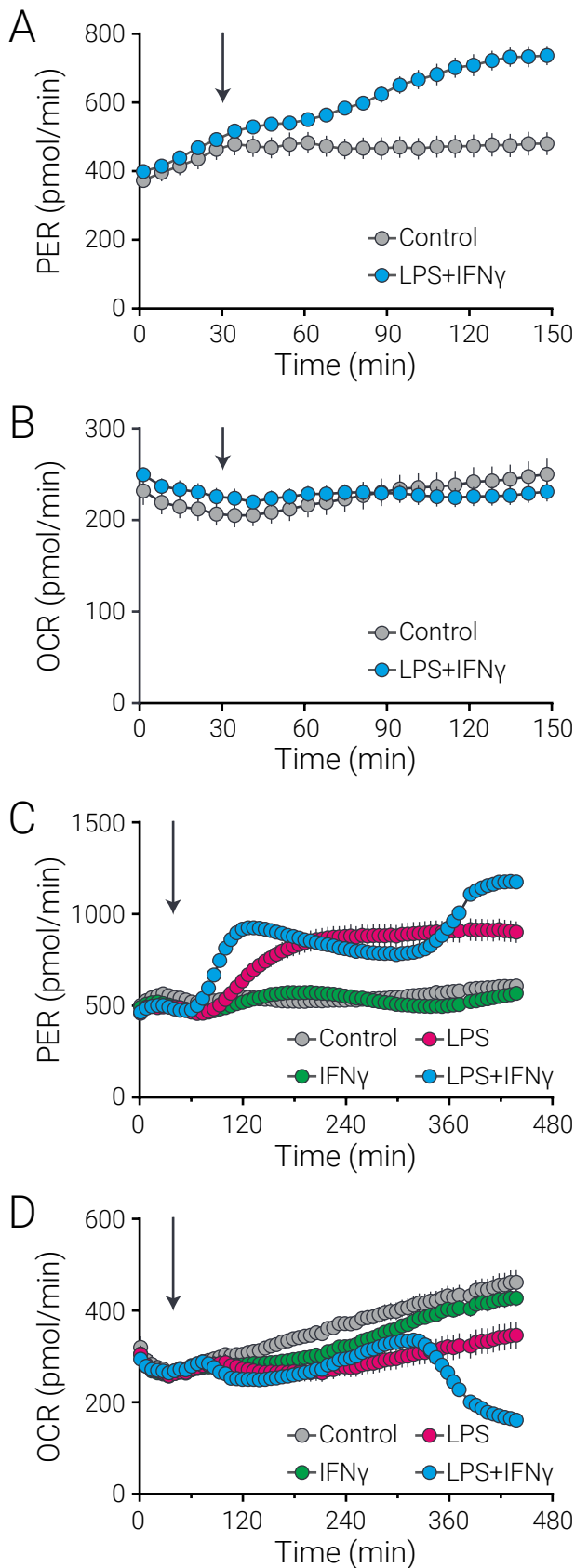
with both LPS and IFNγ. This was accompanied by a significant decrease in mitochondrial respiration (Figure 3D). Interestingly, there was no secondary change in glycolytic or respiration rates with only IFNγ. A similar secondary glycolytic rate increase, along with a rapid decrease in cellular respiration, was observed using another macrophage cell line, J774.A1 (data not shown).

Requirement of IFNγ-iNOS signaling for the secondary metabolic response

To gain better insight into the secondary metabolic response, RAW264.7 cells were stimulated with LPS and varying INFγ concentrations. As shown in Figure 4A, there was no IFNγ effect on the immediate early glycolytic rate increase. However, the secondary increase in glycolytic rate (accompanied with significant decrease in OCR in Figure 4B) appears to be highly dependent on the IFNγ dose.

A suggested mechanism whereby LPS and INFγ stimulation suppresses mitochondrial respiration is through nitric oxide generation following iNOS upregulation. In the next set of experiments, iNOS signaling was suppressed by using two different iNOS inhibitors: 1400W dihydrochloride (1400W) and s-ethylisothiourea hydrobromide (SEITU) As shown in Figure 4C and D, 1400W (gray arrows) and LPS and IFNγ (black arrows) were serially administered, and PER and OCR were monitored. There was no effect on the LPS-dependent PER increase by either inhibitor and a slight decrease in OCR was still observed. However, both secondary PER increase and OCR decrease after 4 h were completely blocked in groups pretreated by 1400W. Similar data were obtained using SEITU (data not shown).

These results imply that IFNγ is responsible for the suppression of respiration in the secondary phase and the IFNγ effect is mediated by iNOS upregulation. This corresponds with previously published data demonstrating iNOS involvement in response of DCs to LPS stimulation[7].

Future applications using real-time activation assays

One of the biggest hurdles in studying macrophages is the large variety of cell types such as mononuclear phagocytes[3]. Although lineage characterization has been established by intensive investigation using surface antigen profiling, the functional mechanism of lineage regulation is still limited. Even defining subtypes such as proinflammatory M1 vs. anti-inflammatory M2 macrophages can be challenging[10]. Although some subtypes and some stimuli of macrophages have been studied relatively well, there are still many stimuli that need to be explored. Real-time activation with XF technology is a simple and direct way to examine macrophage reaction to internal (i.e. cancer cells) or external (i.e. pathogens) cues.
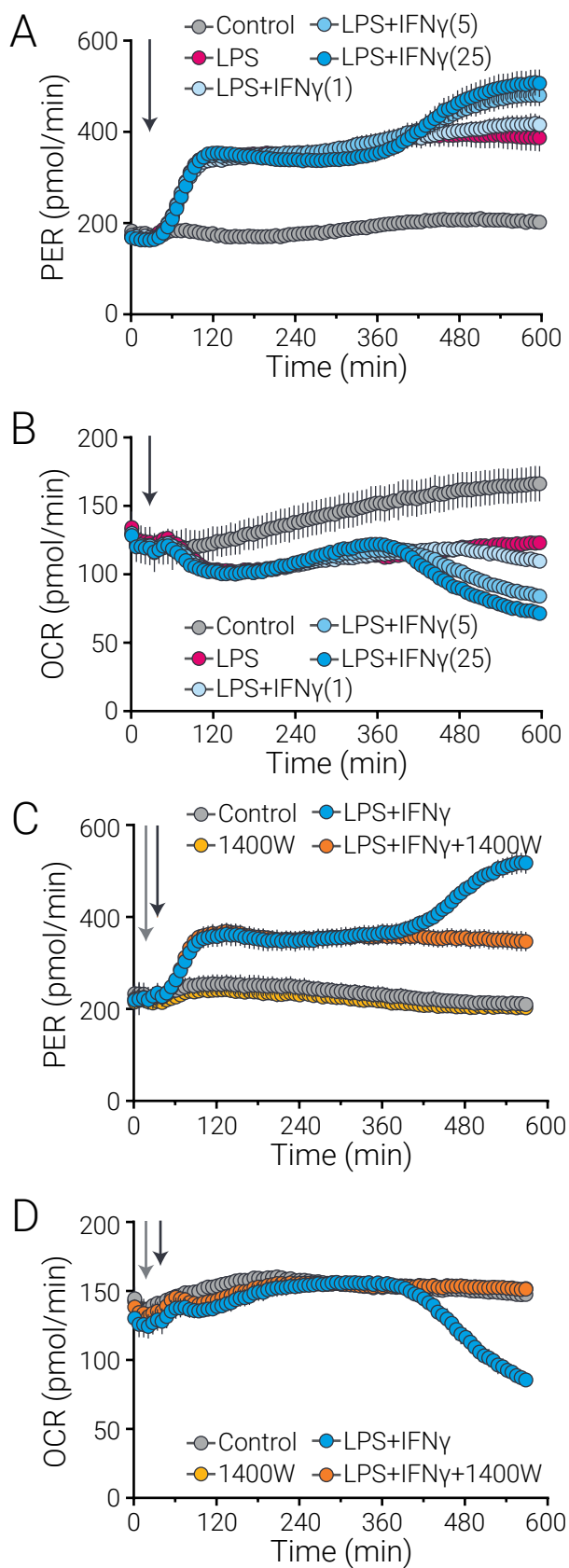
Figure 4. IFNγ-iNOS signaling requirement for the second metabolic response (A and B) RAW264.7 cells were stimulated (black arrow) by 100 ng/ml LPS with various concentrations of INFγ (IFN 1, 1 ng/ml; IFN 5, 5 ng/ml; IFN 25, 25 ng/ml) and the real-time changes in PER and OCR were monitored. (C and D) Cells were activated by co-stimulation with 100 ng/ml LPS plus 20 ng/ml IFNγ (black arrow, LPS+IFN), with or without pretreatment of 100 μM 1400W (gray arrow). Data shown are mean ± SD, n=6 technical replicates.

Cell profiling and functional molecular marker measurements using methods such as flow cytometry and ELISA are well established conventional approaches. However, the limitations of being endpoint assays remain. Specifically, cytokine accumulation detection approaches depend on the expression kinetics and should be designed appropriately to identify proper cytokine sets and timing. As shown in Figure 2, optimum detection timings are different between two representative cytokines for macrophage activation. In contrast, real-time detection of the immediate glycolytic response can be used as a rapid and reliable method for macrophage activation detection. Furthermore, longer term kinetic monitoring after *in vitro* activation can provide time resolved information which enables a better understanding of the dynamic activation mechanism.

Further, the nondestructive nature of this functional assay enables correlation with other downstream biological measurements, as exemplified in Figure 2. This application also can be adapted for evaluating other pathogenic stimuli in addition to mechanistic research on immune cells. The workflow suggested for the metabolic analysis of macrophage activation is outlined schematically in Figure 5A.

Conclusion

Both PBMC-derived M1 macrophages and immortalized murine macrophage cell lines increased glycolytic rate after activation using LPS. The murine cell line showed secondary PER elevation accompanied by a decrease in OCR in a manner that required IFNγ co-stimulation. Notably, this shift was only detectable by long-term (> 6 h) XF analysis. As in these application examples, a real-time activation assay can provide valuable kinetic information on macrophage activation. By correlating this real-time functional information to orthogonal biological data, greater insight is gained with respect to the cause and mechanism of macrophage activation.
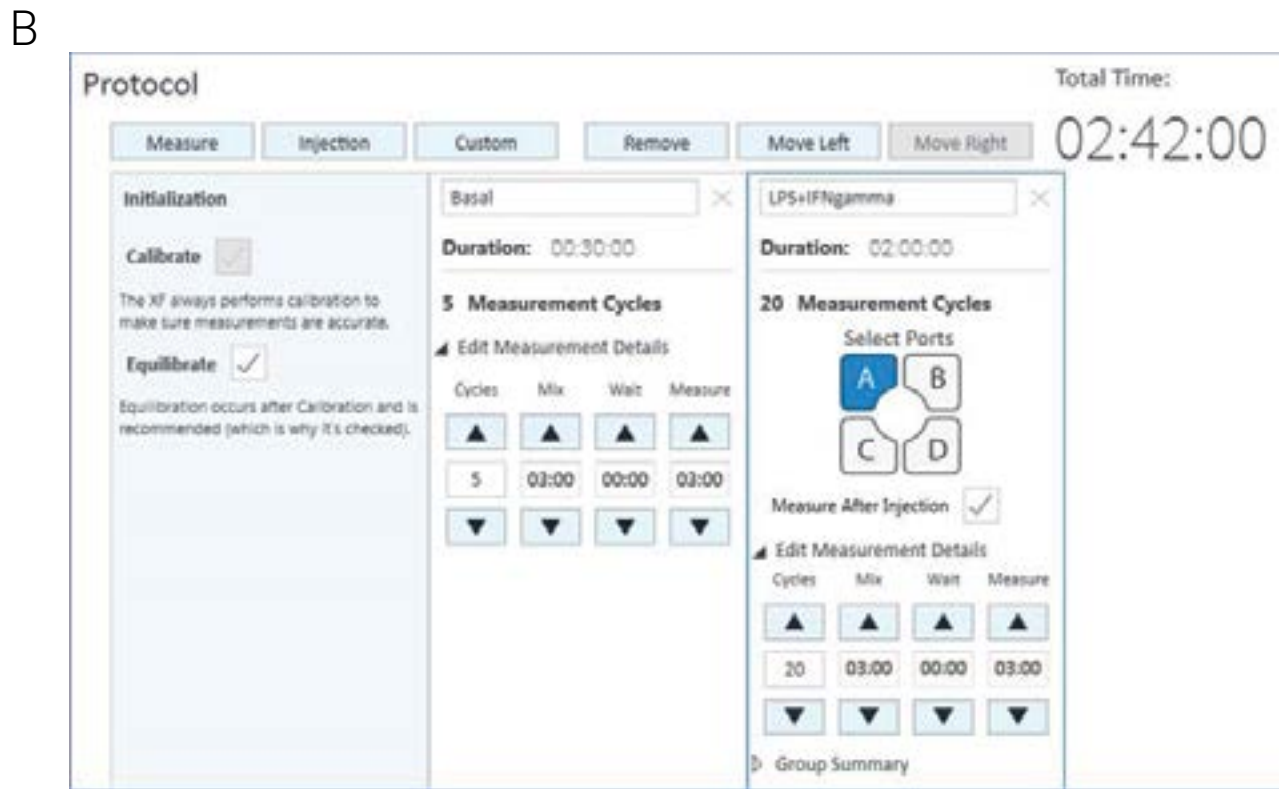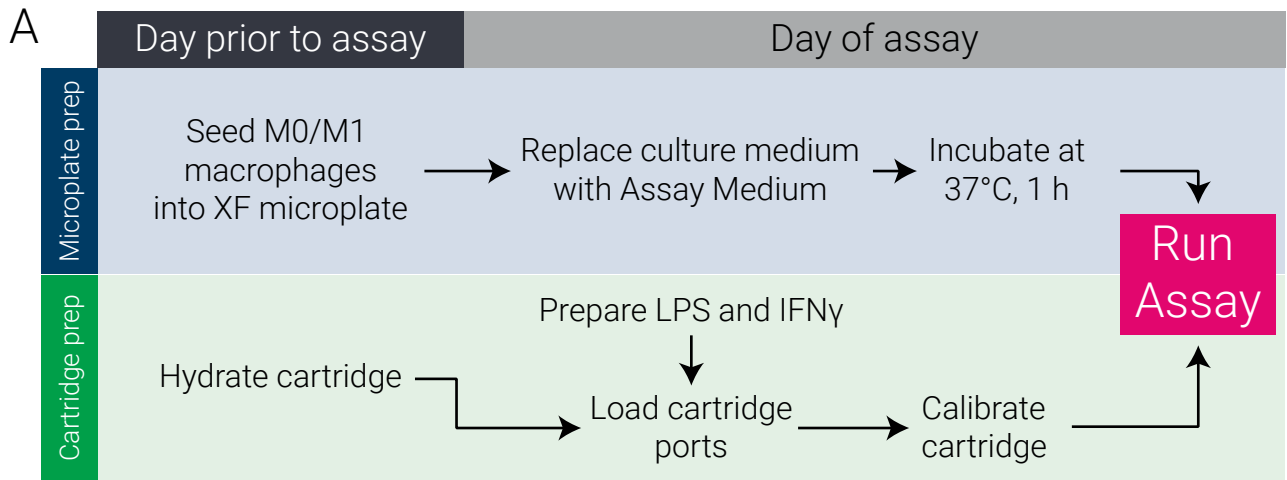
Figure 5. Metabolic activation assay design using XF analyzer
(A) Workflow suggested for real time metabolic activation assay using M0/M1 type macrophages. Upon the completion of the XF assay, the media and cells can be recovered for the further tests, such as ELISA or RT-PCR. (B) An example of a real-time activation instrument protocol designed in WAVE 2.4.

Materials and Methods

Cells and materials

Human PBMC (PB-14NC-1) and fully differentiated M1 macrophages originated from PBMC (PBM1C-MON-1) were purchased from Hemacare and maintained by following the provider's guideline. Briefly, cells were thawed and cultured in RPMI-1640 (Gibco, 21870-076) growth medium supplemented with 10% FBS (HyClone, SH30070.03), 2 mM L-glutamine (Corning, 25-005-CI), 1 mM sodium pyruvate (Corning, 25-000-CI) at 37 °C in a 5% CO_2 atmosphere. For the differentiation of PBMCs, cells were cultured in the presence of 50 ng/ml GM-CSF (R&D Systems, 7954-GM-010) for six days to differentiate to M0/M1 macrophages. RAW264.7 and J744.A1, murine macrophage cell lines were purchased from ATCC and maintained in high glucose DMEM (Gibco, 11960-44)-base growth medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate. 1400W (Tocris, 1415) and SEITU (Sigma, 301310) were used to inhibit iNOS. LPS from *Escherichia coli* O111:B4 was purchased from Sigma (L2630) and recombinant human and mouse IFNγ were from R&D Systems (285-IF-100) and 485-MI-100 respectively) to activate macrophages.

Real time metabolic assay design

For the activation of RAW264.7 or J744.A1 macrophage cell lines, cells were seeded on XF96 plate at the density of 3×10^4 to 5×10^4 cells/well one day prior to the XF assay. On the day of assay, the medium was replaced with assay medium composed of XF Base Medium without Phenol Red (Agilent Technologies, 103335-100) supplemented with 10 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5 mM HEPES, pH 7.4 (Agilent Technologies, 103337-100) and incubated at 37 °C without CO_2 45 min prior to XF assay.

For PBMC-derived macrophages, cells were seeded on XF96 microplate at a density of 3×10^4 cells per well one day prior to the assay. On the day of assay, the media was replaced with assay medium based on XF RPMI media without Phenol Red (Agilent Technologies, 103336-100) containing 10 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 mM HEPES, pH 7.4 (Agilent Technologies, 103337-100) and incubated at 37 °C without CO_2 45 minutes prior to XF assay.

For XFe96 instrument operation and guidelines, please refer to *Basic Procedures to Run an XFe96 Assay* (http://www.agilent. com/en-us/products/cell-analysis-(seahorse)/seahorse-analyzers/seahorse-xfe96-analyzer/basic-procedures-to-run-an-xfe96-assay). Figure 5B shows an example of assay template designs for the short term (2 h) analysis without any inhibitor pretreatment. The basic instrument commands into the assay template file were as follows:

i.   Baseline measurement with 5 cycles; Mix 3 min, Wait 0 min, Measure 3 min

ii. Inject LPS and/or IFNγ through Port A

iii. Measurement with 10 to 99 cycles; Mix 3 min, Wait 0 min, Measure 3 min

The total run time is 2 to 11 h. This allows for 1 to 10 h of real-time monitoring after activation and can be adjusted depending on the time window in interest.

Any inhibitor effect can be assessed by a slight modification in the instrument commands:

iv.   Baseline measurement with 3 cycles; Mix 3 min, Wait 0 min, Measure 3 min

v.   Inject an iNOS inhibitor or assay medium through Port A

vi.   Measurement with 3 cycles; Mix 3 min, Wait 0 min, Measure 3 min

vii. Inject LPS and/or IFNγ through Port B

viii. Measurement with 10 to 99 cycles Mix 3 min, Wait 0 min, Measure 3 min

Cytokine measurement by ELISA

ELISA assay was performed using the Seahorse XF assay medium recovered from cell plates post XF analysis and the growth medium collected after additional culture in the presence of agonist(s) (Figure 2A). The following ELISA kits were used: for RAW 264.7 cells, murine TNFα (R&D Systems, DY410-05) and murine IL-1β (R&D Systems, DY401-05), for human PBMC derived M1 macrophage human TNFα (R&D Systems, DY210-05) and human IL-1β (R&D Systems, DY201-05).

References

1. O'Neill, L. A. J., Kishton, R. J., Rathmell, J. (2016) A guide to immunometabolism for immunologists. *Nat. Rev. Immunol.* 16: 553-565

2. Loftus, R. M., Finlay, D. K. (2016) Immunometabolism: Cellular Metabolism Turns Immune Regulator. *J. Biol. Chem.* 291: 1-10

3. Gordon, S., Taylor P. R. (2005) Monocyte and macrophage heterogeneity. *Nat Rev Immunol.* 5:953-964

4. Chow, A., Brown, B. D., Merad, M. (2011) Studying the mononuclear phagocyte system in the molecular age. *Nat. Rev. Immunol.* 11:788-798

5. Kelly, B., O'Neill, L. A. J. (2015) Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res.* 25:771-784

6. Van den Bossche, J., O'Neill, L. A., Menon, D. (2017) Macrophage immunometabolism: Where are we (going)? *Trends Immunol.* 38:395-406

7. Everts, B., Amiel, E., Huang, S. C-C., Smith, A. M., Chang, C-H., Lam, W. Y., Redmann, V., Freitas, T. C., Blagih, J., van der Windt, G. J. W., Artyomov, M. N., Jones, R. G., Pearce, E. L., Pearce, E. J. (2014) TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. *Nat. Immunol.* 15:323-332

8. Na, Y. R., Gu, G. J., Jung, D., Kim, Y. W., Na, J., Woo, J. S., Cho, J. Y., Youn, H., Seok, S. H. (2016) GM-CSF Induces Inflammatory Macrophages by Regulating Glycolysis and Lipid Metabolism. *J. Immunol.* 197, 4101-4109

9. Romero, N., Swain, P., Neilson, A., Dranka, B (2017) Improving quantification of cellular glycolytic rate using Agilent Seahorse XF technology. White Paper, Agilent Technologies. http://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-00ca/t/page/fm/1

10. Martinez, F. O., Gordon, S. (2014) The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6:13

www.agilent.com/chem/immunology

Agilent Technologies