Bi-phasic Metabolic Responses to *In Situ* Macrophage Activation

Yoonseok Kam, Pamela M. Swain, Brian P. Dranka

Agilent Technologies, MA, USA

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Introduction

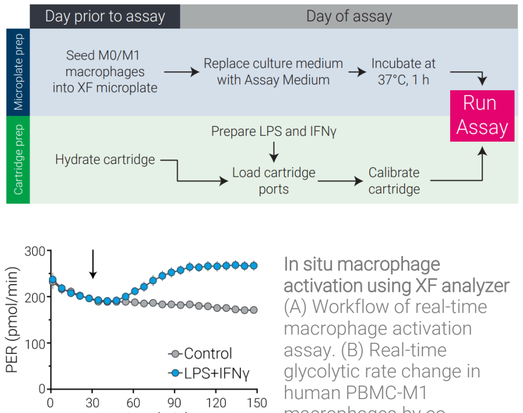
Effective activation of macrophages is critical for their functions in innate immunity, and antigen presentation for cells of the adaptive immune system. Timing of this activation appears to be important in influencing the polarized phenotype.

However, most approaches for quantifying this activation only measure cells at a single point in time.

Dynamic metabolic reprogramming during macrophage polarization and activation has been reported. This results in an immediate increase in glycolysis upon activation of macrophages as well as dendritic cells.

We sought to develop a standardized approach to quantifying macrophage activation. The non-invasive real-time assay presented here discriminates macrophage activation within 2 hours.

Approach



In situ macrophage activation using XF analyzer (A) Workflow of real-time macrophage activation assay. (B) Real-time glycolytic rate change in human PBMC-M1 macrophages by co-stimulation with LPS and IFNγ.

Real time Macrophage Activation Assay

Human PBMC-M1 macrophages (Hemacare) or RAW264.7 (ATCC) murine macrophage cell lines were seeded in XF96 microplate at a density of 3 to 5x10^{4}cells per well one day prior to the assay.

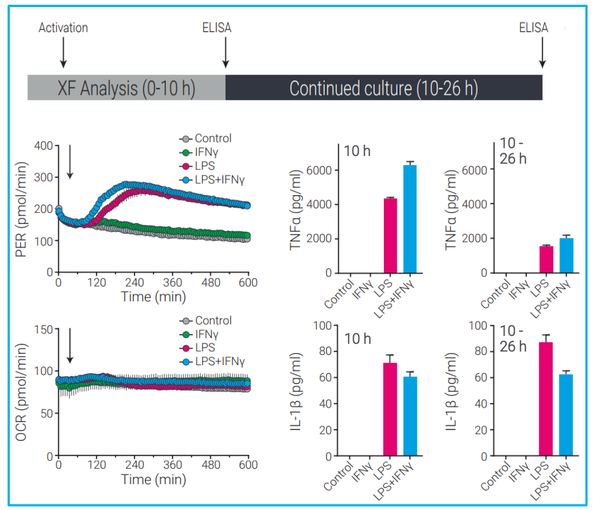
On the day of assay, PER and OCR were monitored in a real-time manner in XF analyzer with in situ injection of activators and/or inhibitors.

XF RPMI medium with 1 mM HEPES (pH 7.4) or XF Base Medium with 5 mM HEPES (pH 7.4) was used in analyzing metabolic rates of hPBMC-M1 or RAW264.7 respectively. Both media were supplemented with 10 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate.

Upon the completion of XF analysis, the assay medium was collected for cytokine analysis using ELISA and cells were fixed and stained for immunofluorescence image analysis.

Results and Discussion

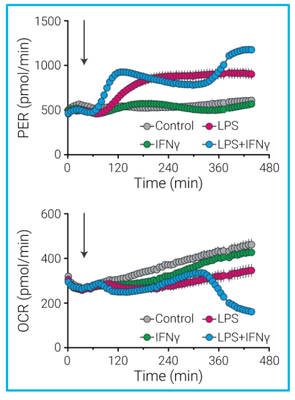
Long-term Glycolytic Rate Changes in PBMC-M1 and Corresponding Cytokine Production



LPS (100 ng/ml), IFNγ (20 ng/ml) or both was injected after 5 basal measurements and the changes in PER and OCR monitored continuously.

The long term XF analysis was followed by two consecutive ELISA analyses as schematically described. The assay medium and growth medium were collected at 10 and 26 h respectively for ELISA analysis. The PER was elevated by LPS with or without IFNγ and sustained up to 10 h. The accumulation of two cytokines, TNFα and IL-1β for the first 10 h and the later 16 h corresponded well with PER changes. Data shown are mean ± SD, n=4 technical replicates.

Bi-phasic Metabolic Response of RAW264.7 Macrophages

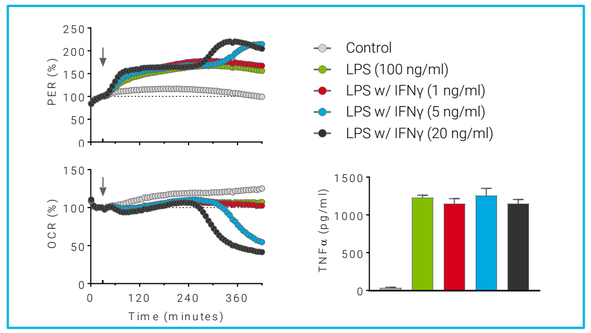


RAW264.7 cells were stimulated by injection of LPS, IFNγ or both, and metabolic responses were measured for an extended period (> 6 h) after the stimulation. Data shown are mean ± SD, n=4 technical replicates.

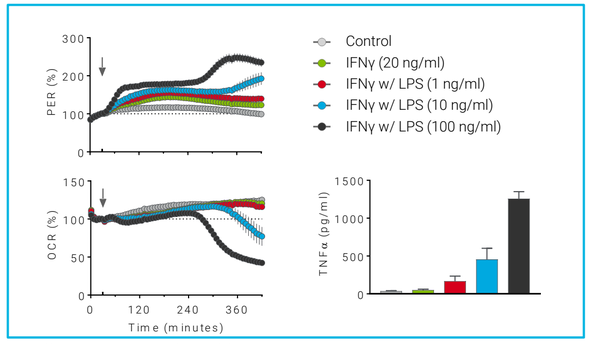
The short-term response was similar to hPBMC-M1, and the secondary increase in PER accompanied with rapid decrease in OCR was observed 4 h after the activation.

Results and Discussion

IFNγ Requirement of Secondary Metabolic Response RAW264.7 Macrophages

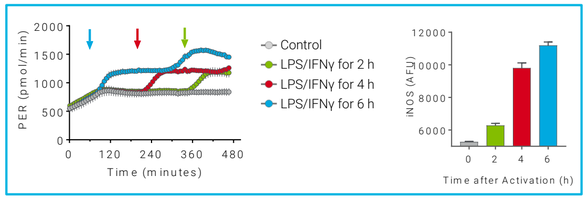


Long term metabolic response was monitored by varying IFNγ injection concentration as indicated. The secondary glycolytic response along with OCR decrease is highly dependent on IFNγ doses. Neither the immediate early PER elevation nor TNFα production was not affected significantly by IFNγ dose variation. Data shown are mean ± SD, n=6 technical replicates.



Cells were stimulated by LPS at various concentration as indicated together with 20 ng/ml IFNγ. LPS is required for the early and the secondary metabolic responses. The immediately early PER elevation corresponded with TNFα for 6 h. Data shown are mean ± SD, n=6 technical replicates.

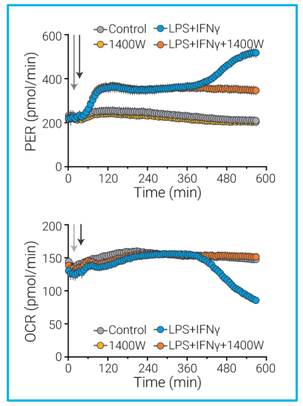
iNOS Signaling Involvement in Secondary Metabolic Response



Cells were stimulated by LPS and IFNγ at three different time points indicated by arrows, and the metabolic rate changes were monitored (left). Upon the completion of the assay, cells were fixed and stained for iNOS. The average fluorescence intensities measured by Cytation 3 (BioTek) are shown (right). Data shown are mean ± SD, n=6 technical replicates. The secondary glycolytic elevation is closely correlated to iNOS level change.

Results and Discussion

iNOS Inhibitor (1400W) blocks the Secondary Metabolic Response in RAW264.7



Assay medium with or without iNOS inhibitor (1400W, gray arrow) was injected prior to LPS and IFNγ (black arrow). Only the secondary changes in PER and OCR were completely blocked by the inhibitor. Data shown are mean ± SD, n=6 technical replicates.

Conclusions

Both human PBMC-derived M1 macrophages and murine macrophage cell lines increase glycolytic rate after activation using LPS. The immediate early elevation in glycolytic rate closely corresponds to M1 specific cytokine production (i.e. TNFα and IL-1β).

A murine macrophage cell line RAW264.7 shows a bi-phasic glycolytic response to the activation in contrast to human PBMC-M1 which maintains the elevated glycolytic rate stably.

The secondary up-regulation of glycolysis (PER) requires the cooperative action of IFNγ with LPS and tightly corresponds to down-regulation of mitochondrial respiration (OCR).

Increased iNOS activity is required for the secondary metabolic change. This activity is correlated with an increase in iNOS protein level.

Future studies will examine the function and impact of this biphasic response in other macrophage subpopulations.

References

1. Romero, N. Swain., P.; Neilson, A.; Dranka, B Improving quantification of cellular glycolytic rate using Agilent Seahorse XF technology. http://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-00ca/t/page/fm/1.
2. Kam, Y., Swain, P. M., Dranka, B. P. Real time discrimination of inflammatory macrophage activation using Agilent Seahorse XF technology http://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-010e/t/page/fm/0