Introduction

- Macrophages show dynamic metabolic changes during the polarization and activation as a crucial tumor microenvironment factor affecting tumor cell survival and progression.
- The metabolic switching from oxidative to glycolytic phenotype is an essential process for inflammatory macrophage activation, and this change can be quantitatively compared by measuring ATP production rates.
- Since macrophage activation is accompanied with proliferation rate changes, the metabolic rates measured by Seahorse XF system should be normalized. A newly developed Agilent Seahorse XF Imaging and Normalization System enables cell number based data normalization.
- Cancer cell-derived exosomes is a newly emerging tumor microenvironmental factor enabling cancer cells to communicate with macrophages. Recent researches suggest that exosomes from cancer cells can promote inflammatory macrophage activation.
- In vitro activation of RAW264.7 macrophages by injecting lipopolysaccharide (LPS) and/or interferon γ (IFNγ) in a Seahorse XF analyzer enables a real-time monitoring of cellular metabolic changes. The exosome effect on the glycolytic activation of macrophage was examined by using the real-time activation assay protocol.

Approach

XF Data Normalization using Seahorse XF Imaging and Cell Counting Software

Cellular metabolic rates (oxygen consumption rate, OCR; proton efflux rate, PER; and ATP production rates) were normalized by the Agilent Seahorse XF Imaging and Normalization System which counts fluorescently-labeled cells by Seahorse XF Imaging and Cell Counting software linked to CytoTome 1 (Bi泰K). Cell permeable Hoechst 33342 was co-injected with the test injection medium (e.g. 2DG in Glycolytic Rate Assay) to stain nuclei. Fluorescently-labeled cells were imaged and counted using offline XF analysis by the software. Captured images and cell counts were imported to normalize XF data in Wave software.

ATP Production Rate Comparison

Total ATP production rate is the sum of ATP production rate from glycolysis and mitochondrial oxidative phosphorylation. Using calculations previously validated for the Seahorse XF glycolytic rate assay and based on known reaction stoichiometry, ECAR data can be converted to glycolytic ATP production rate. During oxidative phosphorylation, OCR that drives mitochondrial ATP synthesis can be calculated by addition of oxygen consumption and CO₂ production. An average I/O value of 2.75 was validated that accurately represents cell experimental conditions.

Exosome preparation

Exosome-enriched fractions were isolated from HT-29-MCF7 and SKOV3 cell lines by using ExoQuickTM Exosome Precipitation System (System Biosciences).

Results and Discussion

Bi-phasic activation of macrophages

A. 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. B. 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.

Activation-associated changes in cellular energy metabolism

A. Glycolysis (normalized) B. OxPhos (normalized) C. ATP Production Rate

ATP Production Rate (normalized)

D. Changes in glycolysis and mitochondrial respiration upon macrophage activation were measured by Seahorse XF glycolytic rate assay and by XF cell mito stress test (B) and C. ATP production rates were calculated using data from A and B. Normalized data show metabolic switch to highly glycolytic phenotype upon the activation. The total ATP production rates per cell were maintained stably, even with severe suppression of mitochondrial respiration. Data shown are mean ± SD, n=6 technical replicates.

HT29 cell-derived exosome-enriched fraction potentiates macrophage activation

A. RAW264.7 macrophages were activated by LPS/IFNγ (arrow) with or without co-injection with exosome-enriched fraction isolated from conditioned medium of HT-29 cells. The activated exosome-associated glycolytic rate increase was accelerated by exosome fraction co-injection. B. Mitochondria respiration was slightly downregulated but the change was less significant compared to glycolytic activation. Data shown are mean ± SD, n=6 technical replicates.

Conclusions

- In vitro macrophage activation is tightly associated with the oxidative-to-glycolytic switching in metabolic phenotype mediated by bi-phasic responses; immediate early (< 1 h) and prolonged (> 2 h), which makes cellular ATP energy production more depend on glycolysis.
- LPS/IFNγ co-stimulation suppresses mitochondrial function severely through iNOS signaling and cells turn to rely on glycolysis fully for the ATP energy production.
- Data normalization is critical for quantitative companion of metabolic phenotype changes. Normalized results showed that the total ATP production rate per cell is stably maintained even with significant downregulation of mitochondria.
- Exosome-enriched fraction derived from cancer cells potentiates LPS-induced glycolytic activation by accelerating activation-associated early metabolic responses. More study is required for the target and mechanism (e.g. mitochondrial downregulation).

References

- www.agilent.com/chem/normalization
- www.agilent.com/chem/immunology
- www.agilent.com/chem/discoverEx

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