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# Glycolysis regulates LPS-induced cytokine production in M2 polarized human macrophages

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ABSTRACT

M1 and M2 macrophages are the key players in innate immunity, and are associated with tissue homeostasis and diseases. Although M2 macrophages are known to depend on fatty acid oxidation (FAO) for their activation, how metabolic pathways affect the production of each cytokine induced by pathogen or bacterial components is unclear. Here, we examined the role of the glycolytic pathway in M2 polarized human macrophages in cytokine production induced by lipopolysaccharide (LPS) stimulation. Human monocytes were isolated from peripheral blood by positive selection for CD14 expression and cultured with macrophage colony-stimulating factor (M-CSF), to obtain M-CSF-induced macrophages (M-MΦ). LPS-induced cytokine production by M-MΦ in the presence or absence of metabolic inhibitors was evaluated. M-MΦ showed a M2 macrophage phenotype with a high IL-10 production level. Glycolytic pathway inhibitors reduced IL-6 production by M-MΦ. Meanwhile, an FAO inhibitor suppressed IL-10 production, while it did not suppress IL-6 production. Interestingly, glycolytic pathway inhibitors downregulated extracellular signal-regulated kinase (ERK) phosphorylation, but FAO inhibitor did not. Nuclear factor kappa B (NF-κB) and the other mitogen-activated protein kinases (MAPKs), p38 and c-jun N-terminal kinase (JNK), were not affected by these metabolic inhibitors. These results suggest that M2 polarized human macrophages use the glycolytic pathway in addition to FAO for cytokine production. Furthermore, ERK may be the key molecule that links metabolic pathways to cytokine production, especially the glycolytic pathway.



## 1. Introduction

Macrophages, the major components of innate immunity, are present in a variety of tissues and are associated with tissue homeostasis and many diseases, including infection, obesity, autoimmune diseases and cancer [1]. There are two well-known types of macrophages, M1 and M2 macrophages [2]. M1 macrophages are involved in acute inflammation and produce inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)−1β and IL-6, while M2 macrophages are involved in tissue homeostasis and produce anti-inflammatory cytokines such as IL-10 [3,4]. Recently, it has been demonstrated that the cellular metabolism modulates the function of immune cells [5]. For example, activated T cells upregulate the expression of glucose transporter 1 and augment the glycolysis flux. CD8+ memory T cells enhance mitochondrial fatty acid oxidation (FAO) to prolong cell survival [6,7]. In the case of B cells, glycolysis plays important roles in antibody production and B cell proliferation, and intestinal plasma cells produce

*Abbreviations:* a.u., arbitrary unit; C/EBP, CCAAT-enhancer-binding protein; ChIP, chromatin immunoprecipitation; DCA, dichloroacetate; ECAR, extracellular acidification rate; ERK, extracellular signal-regulated kinase; ETO, etomoxir; FAO, fatty acid oxidation; IFNγ, interferonγ; IL, interleukin; ILC, innate lymphoid cells; JNK, c-jun N-terminal kinase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; M-MΦ, macrophage differentiated with M-CSF; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; Mγ-MΦ, macrophage differentiated with M-CSF and INF-γ; NF-κB, nuclear factor kappa B; n.s., not significant; OCR, oxygen consumption rate; PAMPs, pathogen-associated molecular patterns; TAMs, tumor-associated macrophages; TCA, tricarboxylic acid; TNFα, tumor necrosis factor α; 2-DG, 2-deoxy-D-glucose.

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IgA using vitamin B1 [8,9]. In the case of macrophage activation by pathogen-associated molecular patterns (PAMPs), it has been suggested that M1 macrophages rely mainly on glycolysis to produce reactive oxygen species, nitric oxide and pro-inflammatory cytokines, whereas M2 macrophages use FAO to sustain an alternative anti-inflammatory phenotype [6,10].

Our group has demonstrated that intestinal macrophages show M2 macrophage phenotypes and contribute to sustaining tissue homeostasis [11]. As the intestinal tract is exposed to many exogenous agents such as bacteria and food, macrophages are involved in the maintenance of tissue homeostasis with the anti-inflammatory phenotype. Impaired IL-10 production by macrophages, which means loss of the key anti-inflammatory phenotype of macrophages, resulted in disruption of the intestinal homeostasis and onset of colitis in mice [12]. Also, we have revealed that inhibition of the glycolytic pathway during the differentiation of human monocytes into M2 macrophages resulted in the loss of its characteristic cytokine production pattern [13]. The glycolytic pathway is important for the differentiation of M2 macrophages and may contribute to the maintenance of tissue homeostasis. Although intracellular metabolism or related metabolites are thought to be essential for macrophage activation, their precise roles in cytokine production by M2 macrophages induced by pathogens or bacteria are unclear.

Here, we investigated the role of the glycolytic pathway in M2 polarized human macrophages in cytokine production induced by lipopolysaccharide (LPS) stimulation, which is a component of bacterial cell stimuli. We showed that human M2 polarized human macrophages depend on the glycolytic pathway for the production of the pro-inflammatory cytokine, IL-6, upon LPS stimulation, while it seems that IL-10 production by M2 polarized human macrophages depends on different metabolism from IL-6. Furthermore, ERK may be the key molecule that connects the intracellular metabolism to cytokine production.

## 2. Materials and methods

### 2.1. In vitro macrophage differentiation

This study was approved by the research ethics committees of Keio University School of Medicine (Approval Number: 20090259). Peripheral blood samples were collected from healthy donors after obtaining written informed consent. After separation of mononuclear cells by gradient centrifugation, CD14-positive cells were isolated using a magnetic cell separation system with anti-human CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To obtain human peripheral blood-derived M2 polarized macrophages (M-MΦ) and M1 polarized macrophages (Mγ-MΦ), CD14-positive cells were resuspended in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) containing 10% heat-inactivated fetal bovine serum (BioSource, Camarillo, CA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, La Jolla, CA). Cells were seeded in 6-well plates at $1 \times 10^6$ cells/well and cultured for 6 days with 50 ng/ml macrophage colony-stimulating factor (M-CSF, R&D Systems, Abingdon, UK), or 50 ng/ml M-CSF and 100 ng/ml IFNγ (R&D) to get M-MΦ and Mγ-MΦ, respectively.

### 2.2. Analysis of intracellular metabolites

Cells ($2–4 \times 10^6$) were stimulated with LPS for 3 h, and then washed with 5% mannitol. Intracellular metabolites were extracted by methanol. Intracellular metabolites were detected by capillary electrophoresis-triple quadrupole mass spectrometry (CE-QqQMS) analysis (conducted by Human Metabolome Technologies, Yamagata, Japan).

### 2.3. Measurement of the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR)

ECAR and OCR of macrophages were measured using XF Glycolysis Stress Test Kits (Seahorse Bioscience, North Billerica, MA) according to the manufacturer's instructions. In brief, macrophages were seeded in XF24 plates at $1.6 \times 10^5$ cells/well the night before the experiments. After measurements of the basal ECAR and OCR, test compounds were added to the cells, and ECAR and OCR were measured. Test compounds were used at the following concentrations: 10 mM glucose, 3 mM 2-deoxy-D-glucose (2-DG; Sigma-Aldrich), 20 mM dichloroacetate (DCA; Sigma-Aldrich), 200 μM Etomoxir (ETO; Sigma-Aldrich). 2-DG and DCA are glycolytic pathway inhibitors, and ETO is FAO inhibitor. ECAR and OCR were monitored with an XF24 analyzer (Seahorse Bioscience).

### 2.4. Stimulation of differentiated macrophages

The differentiated macrophages described above were seeded in 96-well culture plates at $1 \times 10^5$ cells/well and cultured overnight. They were stimulated with 100 ng/ml LPS (Sigma) with or without 2-DG (0, 0.3, 3 mM), DCA (0, 4, 20 mM) or ETO (0, 20, 200 μM). After 6 h or 16 h of stimulation, supernatants were collected to evaluate cytokine production.

### 2.5. Lactate dehydrogenase (LDH) release assay

Cell viability was assessed by the release of LDH using the Cyto Tox 96 Non-Radioactive Cytotoxicity assay (Promega, Madison, WI). The assay was performed according to the manufacturer's instructions.

### 2.6. Cytometric beads assay

Production of IL-6, IL-10 and TNFα was measured by the cytometric beads assay (Human Inflammatory Cytokine Kit, BD Biosciences, San Diego, CA) using FACS Calibur (BD Biosciences) according to the manufacturer's instructions.

### 2.7. Quantitative RT-PCR

Total RNA was isolated from macrophages using NucleoSpin RNA kits (Takara Bio Inc., Shiga, Japan). Quantitative RT-PCR was performed using a One Step SYBR PrimeScript RT-PCR Kit (Takara Bio Inc.) with specific primers for *IL-6* (forward, 5′-GCCAGAGCTGTGCAGATGAG-3′; reverse, 5′-TCAGCAGGCTGGCATTTG-3′), *IL-10* (forward, 5′-GAGATGCCTTCAGCAGAGTGAAGA-3′; reverse, 5′-AAGGCTTGGCAACCCAGGTA-3′), *TNFα* (forward, 5′-TGCTTGTTCCTCAGCCTCTT-3′; reverse, 5′-CAGAGGGCTGATTAGAGAGAGGT-3′) and *18SrRNA* (forward, 5′-ACTCAACACGGGAAACCTCA-3′; reverse, 5′-AACCAGACAAATCGCTCCAC-3′). PCR amplifications were performed using a CFX96 Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA). Relative expression was determined by normalization to the amplification of the *18S rRNA* sequence.

### 2.8. Western blot analysis

Macrophages ($3 \times 10^5$ cells) were suspended in 120 μl of M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) containing PhosSTOP (Sigma-Aldrich)