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Characterization of Metabolic Gene Expression in the RAW 264.7 Monocyte Cell Line

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Abstract

Immune cells undergo changes in gene expression and metabolism when activated by a foreign molecule. Although the changes are short-lived in response to an acute infection, they persist in disorders with underlying chronic inflammation. To understand how changes in gene expression relate to changes in metabolism, the expression of metabolic transporter proteins was analyzed in response to chronic inflammation in a mouse monocyte cell line. Specifically, Glucose Transporter-1 (GLUT1), Monocarboxylate Transporter-1 (MCT1), Monocarboxylate Transporter-4 (MCT4), and Glutamate Aspartate Transporter (GLAST) were analyzed, and it was hypothesized that all the transporters would increase in expression in response to a prolonged inflammatory stimulus when compared to control conditions. RAW 264.7 mouse monocytes were treated with lipopolysaccharide (LPS, 1 mg/mL) or a similar volume of phosphate buffered saline (PBS) for 24 hours. The cells were fixed and subjected to immunocytochemical analyses. Relative fluorescence intensity was measured for the different treatment groups and compared statistically. It was determined that expression of GLUT1 and MCT4 increased in response to LPS, as compared to PBS, whereas MCT1 and GLAST decreased in response to LPS, as compared to PBS. Although the hypothesis was not supported, the data do reflect those of a study using human immune cells that were derived from diabetic patients. The data suggest that this cell-based system could represent the conditions of immune cells derived from humans with chronic inflammation and therefore be used for studies and aimed at finding treatments for those conditions.

Introduction

Humans are exposed to millions of foreign molecules every day. While some of those molecules are benign, others can cause infections and diseases. To avoid infection and maintain good health, the human body relies on the immune system to protect against the foreign molecules that are pathogenic or disease-causing.

There are two different arms of the immune response: the adaptive and the innate. The adaptive immune system uses specific interactions between molecules and receptors on lymphocytes to initiate a response that is remembered such that a more robust response is generated when the foreign molecule is encountered again (Marshall et al., 2018). The innate immune system is the body's first line of defense against invading pathogens and relies on the recognition of patterns commonly found in microbes and other foreign entities (Marshall et al., 2018). The innate immune system relies on leukocytes to engulf the foreign particles and produce small molecules to fight the infection (Marshall et al., 2018).

When a pathogen invades, the innate immune system elicits an inflammatory response. At the site of infection, a series of blood vessel defense responses facilitate the attachment and action of phagocytic leukocytes such as monocytes. The action carried out by monocytes is mediated by a cell-surface protein that detects pathogens and initiates an immune response. The inflammatory response is initiated by activation of pattern recognition receptors, or PRRs, and mediated by intracellular signaling molecules that cause the monocyte to become activated (Janssens and Bayaert, 2003). The molecule lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a known mediator of inflammation through activation of a PRR (Janssens and Bayaert, 2003).

Monocytes, like all cells, rely on the production of cellular energy to maintain their cell functions. In a non-activated state, basic cellular needs are carried out using cellular respiration, in which organic molecules are oxidized through a series of steps for the ultimate production of adenosine triphosphate (ATP; Ratter et al., 2018). Once that monocyte becomes activated through the stimulation of a cell-surface receptor by a foreign molecule, the amount of ATP required increases. Therefore, the cellular metabolism of the monocyte increases. Typically, ATP is produced through aerobic respiration. In this process, glucose is brought into the cell using specific transporters. It is then processed through a pathway known as glycolysis to generate the molecule pyruvate, which is then transformed into acetyl coA and enters the citric acid cycle (Li et al., 2014). Reduced high energy electron carriers generated during glycolysis, acetyl coA production, and the citric acid cycle fuel oxidative phosphorylation in mitochondria to produce large amounts of ATP. However, in monocytes that are chronically activated, through persistent infections, auto-immune disorders, and diseases like diabetes, ATP production occurs through

aerobic glycolysis (Sun et al., 2020). After pyruvate is generated from glucose through the process of glycolysis, it is reduced to lactate through the process of fermentation. This produces less ATP per reaction yet an increased amount overall because of the increased speed of performing fewer reactions (Li et al., 2014). The lactate is then removed from the cell using membrane-associated transporters.

The ability to study how monocytes respond to chronic inflammation is critical for the future development of treatments and therapies that can minimize the effects of diseases including COVID-19, cancer, diabetes, heart disease, and neuroinflammation. Therefore, the purpose of this study was to analyze transporter expression in the RAW 264.7 mouse monocyte cell line to generate a cell-based assay system for studying chronic inflammation in monocytes.

The expression of transporters for glucose, lactate, and amino acids in RAW 264.7 cells subjected to chronic inflammation or control conditions was measured using biochemical assays. It was hypothesized that the expression of transporters for glucose, lactate, and amino acids would increase in response to chronic inflammation, when compared to the control conditions. Immunohistochemical analyses indicate that expression of the glucose transporter GLUT1 and the lactate transporter MCT4 increased in response to chronic inflammation, when compared to the control. However, expression of the lactate transporter MCT1 and the amino acid transporter GLAST decreased in response to chronic inflammation when compared to the control. Although the results did not fully support the hypothesis, they do align with published reports of the metabolic gene expression profile of monocytes recovered from diabetic patients (Ratter et al., 2018). Overall, it appears that the cell-based system will serve as an appropriate model system to study chronic inflammation in monocytes.

Methods

Cell culture

RAW 264.7 monocytes (American Type Culture Collection) were grown at 37°C in 5% CO_2 in RPMI 1640 medium (Gibco) containing 10% FBS (Hyclone). For the assays, the RAW 264.7 cells were plated on chamber slides (Corning) in serum-free RPMI 1640 medium and allowed to attach overnight.

The cells were treated with LPS (1 µg/mL; InvivoGen) or Dulbecco's phosphate buffered saline (D-PBS) for 24 hours at 37°C in 5% CO_2. The cells were fixed for subsequent analyses by removing the cell media and incubating in 4%

paraformaldehyde in phosphate buffered saline (PFA) for 15 minutes at room temperature.

Immunocytochemistry

For immunohistochemistry, the cells were treated with blocking buffer (tris buffered saline [TBS] containing 0.1% Tween 20 and 2% normal goat serum [Pierce/Thermo Scientific]) and incubated at 4°C overnight. The cells were incubated in the presence of antibodies specific for MCT1 (Millipore), MCT4 (Millipore), GLUT1 (Millipore), or GLAST (Millipore), diluted to 1 µg/mL in blocking solution for one hour at 37°C and then at 4°C overnight. After washing with at least 10 changes of TBS, the cells were treated with Alexa488-conjugated goat anti-rabbit (GAR) secondary antibody, diluted 1:250 in blocking solution, for 30 minutes at 37°C. The cells were washed 10 times with TBS, with the first wash containing 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) diluted to 1:1000. Cover slips were mounted with 30% glycerol containing p-phenylenediamine (Sigma Chemical Company) and the cells were visualized and imaged on an Olympus FV1000 microscope. Subsequently, fluorescence was quantified using the accompanying software and specifying regions of interest (ROIs). Expression in LPS-treated cells versus D-PBS-treated cells was compared using a paired, one-tailed T test. Significance was measured at $p < 0.05$.

Results

The use of cell lines to test cellular responses to various stimuli is an important first step in designing treatments for human disorders. The RAW 264.7 cell line is a mouse monocyte primary cell line that may serve as a useful system for studying the cellular effects of chronic inflammation, like that observed in patients with auto-immune disorders and diabetes. Therefore, the purpose of the present study was to analyze the expression of nutrient transporters in RAW 264.7 cells in response to stimuli that mimic chronic inflammatory conditions compared to saline control.

The expression of transporters used to move monocarboxylates like pyruvate, lactate, and ketone bodies across the plasma membrane was compared in chronically inflamed monocytes and control cells. It was determined that expression of Monocarboxylate Transporter-1 (MCT1) was significantly decreased in response to 24 hours of incubation with lipopolysaccharide (LPS), a known inducer of inflammation

in monocytes, when compared to saline control (Figure 1). Conversely, expression of a related transporter, MCT4, significantly increased in expression in response to 24 hours of incubation with LPS, when compared to control conditions (Figure 2).

The expression of a transporter used to move the monosaccharide glucose across the plasma membrane was compared in chronically inflamed monocytes and control cells. Expression of the transporter Glucose Transporter-1 (GLUT1) significantly increased in expression in response to 24 hours of exposure to LPS, when compared to treatment with saline (Figure 3).

The expression of a transporter used to move the amino acids across the plasma membrane was compared in chronic inflammatory versus control conditions. Expression of the Glutamate Aspartate Transporter (GLAST) was determined to decrease in response to LPS, when compared to control conditions (Figure 4).

Discussion

The purpose of this investigation was to analyze transporter expression in the RAW 264.7 mouse monocyte cell line to generate a cell-based assay system for studying chronic inflammation in monocytes. Glucose, lactate, and amino acid transporters were tested using immunohistochemical assays. It was hypothesized that each of the transporters analyzed would have increased expression in response to a chronic inflammatory stimulus because of the increased need for cellular metabolism under those conditions. Results from immunohistochemical assays showed that this hypothesis was not fully supported. Expression of the glucose transporter GLUT1 and the lactate transporter MCT4 increased under chronic conditions compared to control conditions, whereas expression of the lactate transporter MCT1 and the amino acid transporter GLAST decreased in response to chronic conditions compared to control conditions. The data suggest that transporters respond to an increased need for glucose and for removal of lactate from the cells, via MCT4, and that the cell decreases metabolism related to amino acids.

It is well established that the immune cells reprogram their metabolism in response to an inflammatory stimulus. Cells of the innate and adaptive arms of the immune system, which typically produce ATP via aerobic respiration or oxidative phosphorylation, switch to the production of ATP via aerobic glycolysis for a rapid host defense mechanism (Sun et al., 2020). Like the observation in the present study, the cells increase the expression of GLUT1 to increase delivery of glucose to the cells.

The mechanism of aerobic glycolysis produces lactate, which results in the increased expression of MCT4, to ensure that the lactate is removed from the cell so that glycolysis can continue. That reprogramming reverts to the homeostatic mechanism after the inflammatory insult has been removed from the system. However, in cases of chronic inflammation, the pendulum swings toward glycolysis, with the immune cells remaining in that heightened state of activation (Sun et al., 2020).

A recent study of peripheral blood mononuclear cells (PBMCs), which include monocytes, isolated from diabetic and healthy patients found that transporter expression changed in response to chronic inflammation resulting from diabetes. Microarray analyses of RNA isolated from diabetic and healthy PBMCs indicated that expression of MCT4 is increased, whereas expression of MCT1 is not affected (Ratter et al., 2018). Increased extracellular lactate concentrations were observed to decrease the production of pro-inflammatory cytokines in PBMCs from healthy individuals, which suggests a feedback mechanism for regulation of the immune response in acutely activated monocytes (Ratter et al., 2018). Unfortunately, the study did not determine the effects of increased extracellular lactate on chronically activated PBMCs (Ratter et al., 2018).

The observations from the present study align with known metabolic changes in activated immune cells. The metabolic transporter expression patterns suggested that a switch from oxidative phosphorylation to aerobic glycolysis occurred, like that seen for chronically activated monocytes and PBMCs of diabetic patients. This suggests that the RAW 264.7 cells challenged with LPS for 24 hours represent the conditions observed in chronically inflamed humans and can therefore be used as a cell-based model for studying diseases including COVID-19, cancer, diabetes, heart disease, and neuroinflammation.

Acknowledgements

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Figures

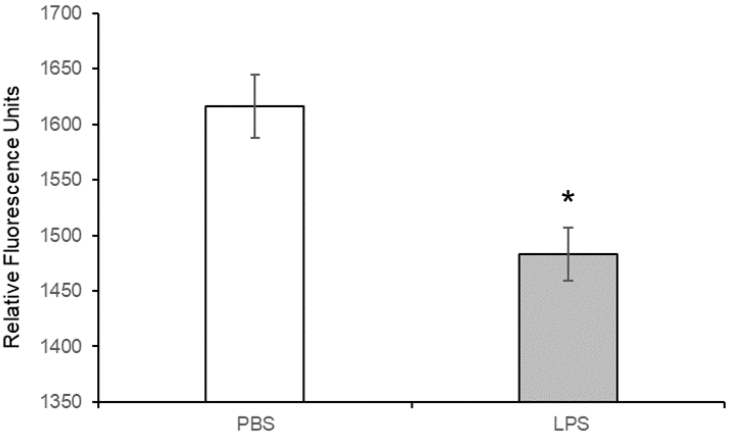

Figure 1. Expression of Monocarboxylate Transporter-1 (MCT1) in response to chronic inflammation. The white bars represent the average relative fluorescence of regions of interest (ROI) in RAW 264.7 cells in response to phosphate buffered saline (PBS) treatment for 24 hours. The gray bars represent the average relative fluorescence of ROI in RAW 264.7 cells in response to lipopolysaccharide (LPS) treatment for 24 hours. The error bars represent the standard error. * = $p<0.05$ via a paired, one-tailed T-test.

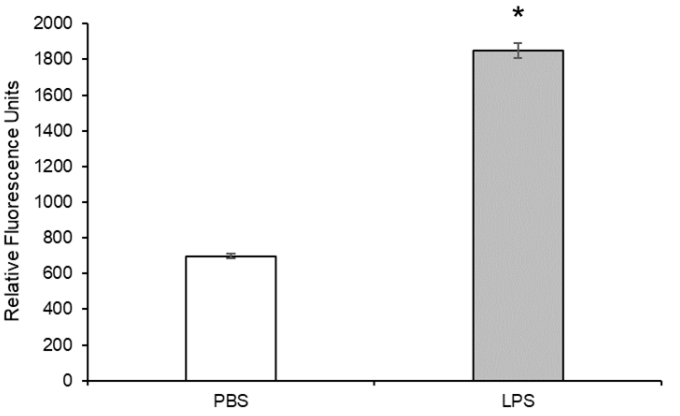

Figure 2. Expression of Monocarboxylate Transporter-4 (MCT4) in response to chronic inflammation. The white bars represent the average relative fluorescence of regions of interest (ROI) in RAW 264.7 cells in response to phosphate buffered saline (PBS) treatment for 24 hours. The gray bars represent the average relative fluorescence of ROI in RAW 264.7 cells in response to lipopolysaccharide (LPS) treatment for 24 hours. The error bars represent the standard error. * = $p<0.05$ via a paired, one-tailed T-test.

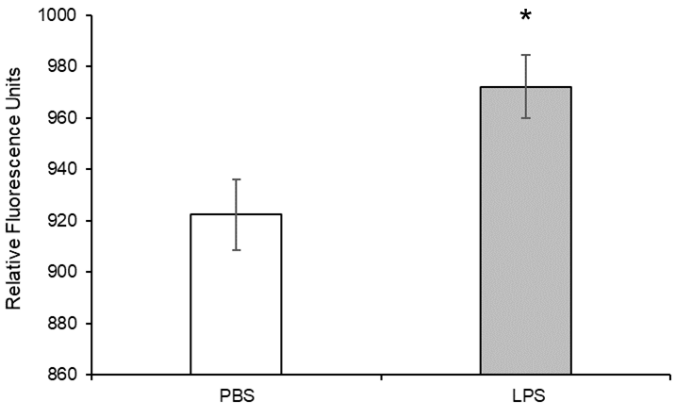

Figure 3. Expression of Glucose Transporter-1 (GLUT1) in response to chronic inflammation. The white bars represent the average relative fluorescence of regions of interest (ROI) in RAW 264.7 cells in response to phosphate buffered saline (PBS) treatment for 24 hours. The gray bars represent the average relative fluorescence of ROI in RAW 264.7 cells in response to lipopolysaccharide (LPS) treatment for 24 hours. The error bars represent the standard error. * = $p<0.05$ via a paired, one-tailed T-test.

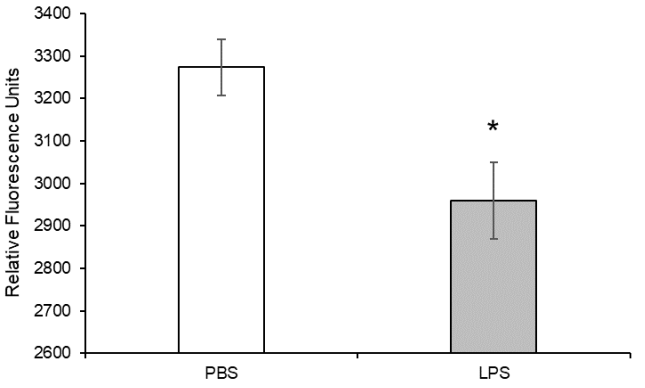

Figure 4. Expression of Glutamate Aspartate Transporter (GLAST) in response to chronic inflammation. The white bars represent the average relative fluorescence of regions of interest (ROI) in RAW 264.7 cells in response to phosphate buffered saline (PBS) treatment for 24 hours. The gray bars represent the average relative fluorescence of ROI in RAW 264.7 cells in response to lipopolysaccharide (LPS) treatment for 24 hours. The error bars represent the standard error. * = $p<0.05$ via a paired, one-tailed T-test.