The Monocarboxylate Transporter 4 Is Required for Glycolytic Reprogramming and Inflammatory Response in Macrophages*

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Background: Glycolysis has an important role in inflammation.

Results: MCT4 is up-regulated in inflammatory activated macrophages and required for innate immune response.

Conclusion: MCT4 up-regulation represents a positive feedback mechanism in inflammation.

Significance: Delineation of the role of MCT4 provides further insight into the regulation of inflammation by glycolysis.

There has been fast growing evidence showing that glycolysis plays a critical role in the activation of immune cells. Enhanced glycolysis leads to increased formation of intracellular lactate that is exported to the extracellular environment by monocarboxylate transporter 4 (MCT4). Although the biological activities of extracellular lactate have been well studied, it is less understood how the lactate export is regulated or whether lactate export affects glycolysis during inflammatory activation. In this study, we found that MCT4 is up-regulated by TLR2 and TLR4, but not TLR3 agonists in a variety of macrophages. The increased expression of MCT4 was mediated by MYD88 in a NF-κB-dependent manner. Furthermore, we found that MCT4 is required for macrophage activation upon TLR2 and TLR4 stimulations, as evidenced by attenuated expression of pro-inflammatory mediators in macrophages with MCT4 knockdown. Mechanistically, we found that MCT4 knockdown leads to enhanced intracellular accumulation of lactate and decreased glycolysis in LPS-treated macrophages. We found that LPS-induced expression of key glycolytic enzymes hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 is diminished in macrophages with MCT4 knockdown. Our data suggest that MCT4 up-regulation represents a positive feedback mechanism in macrophages to maintain a high glycolytic rate that is essential to a fully activated inflammatory response.

Inflammation is one of the first lines of host defense against microbial invasion (1, 2). During the response, inflammatory cells, such as macrophages and neutrophils, are activated to rapidly produce proinflammatory mediators and reactive oxygen species, through which these cells are able to contain and eliminate microbial infection (3–6). However, inflammatory response needs to be tightly controlled because excessive inflammation often causes collateral tissue injury, such as that which occurs to the lungs during sepsis (5, 7, 8). There has been great advance in our understanding of the regulation of inflammatory response (5, 7). However, the large volume of knowledge we attained has only met with limited success in discovering effective treatments for acute inflammation-associated disorders (9).

Glucose is the major nutrient for bioenergy production in cells (10–12). However, glucose metabolism also participates in a broad spectrum of molecular and cellular activities, such as transcription, translation, and biosynthesis (13–16). Glycolysis consists of a sequence of 10 reaction steps that end at pyruvate production (10–12). When oxygen supply is sufficient, pyruvate is converted into acetyl-CoA that enters the TCA cycle. NADH produced from the TCA cycle serves as donors of electrons that flow through the electron transport chain to generate ATP, a process called oxidative phosphorylation (10–12). Under oxygen deprivation, most pyruvate is converted into lactate that is transported by the lactate transporter monocarboxylate transporter 4 (MCT4)2 outward to the extracellular environment (17–20).

Augmented glycolysis not only takes place during hypoxia but also occurs under aerobic conditions (10, 21). It is well known that cancer cells run high rate of glycolysis even when there is plenty of oxygen (10, 21). Most pyruvate produced by cancer cells is converted into lactate. This phenomenon was initially reported by Otto Warburg and has since been named the “Warburg effect” (10, 21).

However, it has been recently shown that Warburg effect is also associated with many other cellular activities. For example, augmented aerobic glycolysis was found in LPS-treated dendritic cells and macrophages and has an important role in reg-
ulating the activation of these cells (13–16). Although the biological activities of extracellular lactate are well studied (22–25), it is less understood how the lactate export is regulated or whether lactate export affects glycolysis during inflammatory activation. In this study, we found that MCT4 is up-regulated in activated macrophages. We found that the increased expression of MCT4 and the resulting lactate export are necessary for the sustainment of high glycolysis and inflammatory response in macrophages.

MATERIALS AND METHODS

Reagents—Ultrapure LPS from Salmonella minnesota R595, PAM3CSK4 (PAM), polyniosine-polyctydial acid (poly(I:C)), heat-killed Pseudomonas aeruginosa (HKPA), heat-killed Listeria monocytogenes (HKLM), and BAY 11-7082 were from Invivogen. α-Cyano-4-hydroxycinnamic acid (CHCA) was from Sigma-Aldrich. Establishment of Mouse Bone Marrow-derived Macrophages (BMDMs), Mouse Peritoneal Macrophages, Mouse Alveolar Macrophages, and Human Peripheral Blood Mononuclear Cell-derived Macrophages—Mouse BMDMs were established from bone marrow cells of C57BL/6 mice (NCR-Fredrick). Briefly, bone marrow cells were cultured in DMEM containing 10% FBS and 50 ng/ml murine macrophage colony-stimulating factor (R&D Systems) for 5 days. The differentiated cells were then split and plated for following experiments. Peritoneal macrophages were elicited from C57BL/6 mice by intraperitoneal injection of 1 ml of sterile 4% Brewer thioglycollate (Sigma-Aldrich). 4 days after injection, cells were harvested by peritoneal lavage and plated. After 1 h at 37 °C, nonadherent cells were removed, and adherent macrophages were used for following experiments. Alveolar macrophages were obtained by bronchoalveolar lavage using 1 ml of PBS containing 5 mM EDTA. The bronchoalveolar lavage fluids were centrifuged, and the pellets were resuspended in DMEM containing 10% FBS and plated. After 1 h at 37 °C, the nonadherent cells were removed, and the adherent macrophages were used as alveolar macrophages for the following experiments. Human peripheral blood mononuclear cells were purchased from ZenBio Inc. Peripheral blood mononuclear cells were cultured in DMEM containing 10% FBS and 50 ng/ml human macrophage colony-stimulating factor (R&D Systems) for 5 days. The cells were then split and plated for following experiments. The animal protocol was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

ELISA for Cytokines—Levels of TNF-α, IL-1β, and IL-6 in cell culture supernatants were determined using DuoSet ELISA development kits (R&D Systems) according to the manufacturer’s instructions.

siRNA Transfection—Macrophages were transfected with control nontargeting siRNAs or specific MCT4 siRNAs using HiperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. Specifically, siRNAs and HiperFect reagents (1:2 ratio, pmol of siRNA:μl of reagents) were dissolved in Opti-MEM medium and incubated for 5 min at room temperature. The siRNA-reagent complexes were then added to macrophages and incubated at 37 °C for 6 h. The transfection complexes were removed, and fresh media were added to the macrophages. The cells were cultured for additional 2 days before treatment. This transfection method was regularly used in our previous studies and confirmed to be highly efficient (26).

Control nontargeting siRNA SMARTpool, mouse MCT4 ON-TARGETplus SMARTpool, human MCT4 ON-TARGETplus SMARTpool, and two individual ON-TARGETplus mouse MCT4 siRNAs were all purchased from Dharmacon and GE Lifesciences.

Quantitative Real Time PCR—Real time PCR was performed using SYBR Green Master Mix kit (Roche). The primer sequences were: mouse GAPDH, sense, 5'-CGACTTCAACAGCACTCCCACCTCTCC-3', and antisense, 5'-TGGGGTGTTCCAGGTTTTCTTACTCCTTT-3'; mouse tubulin, sense, 5'-GGATGTCGCCATAAATCTGCTGGT-3', and antisense, 5'-GGCAAGAAGTGGAAAGAAAG-3'; mouse TNF-α, sense, 5'-AGAGTACCAAGAGATCACCAG-3', and antisense, 5'-TCAGATTTCAGGGTCAACTTCATC-3'; mouse IL-6, sense, 5'-CCCAATTTCATGCTCTCTCA-3', and antisense, 5'-AGGAATGTCCACAAACTGATATC-3'; mouse IL-12 p40, sense, 5'-CCCAATTACTCCGGAGGTTTCAC-3', and antisense, 5'-CAGACAGAGACGGCCTATCCCCATT-3'; mouse MCT4, sense, 5'-GCCACCTCAACGCTGCTA3', and antisense, 5'-TGTCGGGTACACCCATATC-3'; and human MCT4, 5'-CCAAAGCCCGAAGGTTTCAAG-3', and antisense, 5'-CCACCCACCTCCATTAAGTGC-3'.

To calculate fold change in the expression of cytokines, ΔCt values were first obtained: ΔCt = Ct of GAPDH or tubulin – Ct of cytokines. ΔCt values were then obtained: ΔΔCt = ΔCt of treated groups – ΔCt of untreated control groups. Fold change was calculated as 2ΔΔCt, with control groups as 1-fold.

Western Blotting—Western blotting was performed as previously described (26). Anti-MCT4, lamin B, INOS, GAPDH, and p65 antibodies were from Santa Cruz. Anti-HK2, IκB-α, p-IκB-α, ERK, and p-ERK antibodies were from Cell Signaling. Anti-actin and -tubulin antibodies were from Sigma-Aldrich. Anti-HIF-1α antibody was from Novus Biologicals. Anti-PFKFB3 antibody was from Proteintech.

Real Time Cell Metabolism Assay—XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) was used for real time analysis of oxygen consumption rate and extracellular acidification rate (ECAR). BMDMs were seeded in XF-24 cell culture microplates (1.5 × 10^5 cells/well). The cells were treated without or with 100 ng/ml LPS for 6 h, and oxygen consumption rate and ECAR were then determined.

Intracellular Glucose 6-Phosphate (G-6-P), Fructose 6-phosphate (F-6-P), and Lactate Assays—Intracellular levels of G-6-P, F-6-P, and lactate were determined using a glucose 6-phosphate fluorometric assay kit (Cayman Chemical), a PicoProber™™ fructose 6-phosphate fluorometric assay kit (Biovision), and a lactate colorimetric/fluorometric assay kit (BioVision) according to the manufacturers’ instructions.

Glucose Consumption Assay—Cell supernatants were diluted 1:200. Glucose levels in the supernatants were determined using a glucose colorimetric/fluorometric assay kit (Biovision) according to the manufacturer’s instructions. Glucose consum-
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...tion was calculated as (glucose level of fresh medium-glucose levels of supernatants)/incubation hours.

Statistical Analysis—One-way analysis of variance followed by the Bonferroni test was used for multiple group comparisons. The Student’s t test was used for comparison between two groups. p < 0.05 was considered statistically significant.

**RESULTS**

**MCT4 Is Up-regulated in LPS-treated Macrophages**—There has been rapidly accumulating evidence showing that glycolysis plays a crucial role in the activation of dendritic cells, T cells, and macrophages (13–16). Augmented glycolysis, such as that occurring in the activated inflammatory cells, leads to increased lactate production that results in intracellular and extracellular acidification. Extracellular lactate is known to have many biological activities (22–25). However, it is less clear how the export of the intracellular lactate is regulated or what the functional implication of lactate export is during inflammation.

We found that the lactate transporter MCT4 is up-regulated in LPS-treated mouse BMDMs at both mRNA and protein levels (Fig. 1, A and B). To determine whether LPS-induced MCT4 also occurs in other types of macrophages, we examined the expression of MCT4 in mouse peritoneal and alveolar macrophages. Similar to those found in BMDMs, MCT4 was up-regulated in LPS-treated peritoneal and alveolar macrophages (Fig. 1, C–E), indicating that LPS-induced MCT4 is a general phenomenon during macrophage activation. MCT4 was also up-regulated by LPS in human macrophages (Fig. 1F). Taken together, these data suggest that MCT4 may participate in the inflammatory response of macrophages. Additionally, we found that MCT4 is up-regulated in the lungs of mice that were exposed to LPS (Fig. 1G), suggesting a potential role of MCT4 in acute lung injury.

**MCT4 Is Induced by TLR2, but Not TLR3 Activation**—To determine whether MCT4 expression responds to the activation of other Toll-like receptors (TLRs), we treated mouse BMDMs with TLR2 agonist PAM and TLR3 agonist poly(I:C) and found that PAM, but not poly(I:C), induces MCT4 expression (Fig. 2, A and B). To further characterize MCT4 induction in response to TLR2 activation, we performed the same experiments using a more specific TLR2 agonist, lipoteichoic acid, and found that lipoteichoic acid induces MCT4 in BMDMs (Fig. 2C). Additionally, PAM, but not poly(I:C), induced MCT4 expression in mouse peritoneal and human macrophages (Fig. 2, D–F). These data suggest that MCT4 is involved in the macrophage activation by TLR2 and TLR4. Both TLR2 and TLR4 require the adaptor MYD88 to effectively activate NF-κB (27). To determine whether MYD88 mediates LPS-induced MCT4, we examined MCT4 expression in wild-type and MYD88−/− macrophages and found that the induction of MCT4 by LPS is abrogated in MYD88-deficient cells (Fig. 2G). Because the MYD88-dependent pathway leads to NF-κB activation upon LPS stimulation (27), we next determined whether NF-κB activation is required for LPS-induced MCT4 expression. As shown in Fig. 2H, the up-regulation of MCT4 in LPS-treated macrophages was abolished by a specific inhibitor of NF-κB activation, BAY 11-7082.

**Knockdown of MCT4 Diminishes the Response of Macrophages to LPS Stimulation**—Based on the data that MCT4 is induced by LPS, we reasoned that MCT4 may play a role in the inflammatory response of macrophages to TLR4 stimulation. To test this hypothesis, we knocked down MCT4 in mouse BMDMs using a pool of four individual siRNAs and assessed the expression of proinflammatory cytokines after LPS treatment (Fig. 3A–C). To confirm the specificity of MCT4 knockdown, we performed real time PCR and Western blotting to assess the levels of MCT4 mRNA and protein (Fig. 3D–F). As shown in Fig. 3G, MCT4 knockdown significantly attenuated LPS-induced expression of iNOS, TNF-α, and IL-6. To rule out the possibility that the findings were nonspecific effects caused by any of the pooled siRNAs, we repeated the experiments using two individual MCT4 siRNAs with independent sequences. As shown in Fig. 3H, both siRNAs diminished LPS-induced expression of TNF-α and IL-6 (Fig. 3D), consistent with the results found with the MCT4 siRNA pool. Of note, both siRNAs also effectively knocked down...
MCT4 (Fig. 3E). Additionally, we found that LPS-induced TNF-α and IL-6 are also reduced in human macrophages (Fig. 3F) and mouse peritoneal macrophages (data not shown) with MCT4 knockdown. Taken together, these data suggest that MCT4 is required for macrophage activation by TLR4 stimulation.

Blocking MCT4 Decreases the Response of Macrophages to LPS Stimulation—MCT4 transports intracellular lactate to the extracellular environment. This suggests that MCT4 knockdown may impair lactate export from the activated macrophages, thereby resulting in intracellular accumulation of lactate. To determine whether the intracellular accumulation of lactate is responsible for the diminished inflammatory response, we treated macrophages with a MCT4 inhibitor, CHCA, and found that CHCA indeed decreases LPS-induced expression of IL-1β, IL-6, TNF-α, and iNOS (Fig. 4, A and B). Together, these data suggest that lactate export is necessary for macrophage activation by LPS.

Knockdown of MCT4 Diminishes the Response of Macrophages to Bacteria That Signal through TLR2—The requirement of MCT4 for macrophage activation by T2LR2 prompted us to ask whether MCT4 participates in the activation of macrophages by bacteria that mainly signal through TLR2. To address this question, we knocked down MCT4 in mouse BMDMs and treated the cells with heat killed P. aeruginosa. P. aeruginosa is one of the most common pathogens that cause pneumonia and sepsis (28–30). We found that MCT4 knockdown significantly attenuates P. aeruginosa-induced expression of TNF-α, IL-6, and IL-12 (Fig. 6, A and B). Additionally, we examined the effect of MCT4 knockdown on the activation of macrophages by heat killed L. monocytogenes, another common virulent bacteria that induce inflammatory response mediated by interactions between MyD88 and TLR2 (31). As shown in Fig. 6C, MCT4 knockdown significantly attenuated L. monocytogenes-induced expression of TNF-α and IL-6. Taken together, these data suggest that MCT4 may participate in the inflammatory response to infections by some common bacteria, thereby getting involved in infection-associated organ injury, such as acute lung injury.

MCT4 Knockdown Has No Effect on LPS-induced Proximal Cytoplasmic Signaling—Engagements of TLR2 and TLR4 with agonists initiate sequential intracellular signaling events that lead to the expression of proinflammatory cytokines and mediators. To delineate the mechanism of MCT4-regulated inflam-
mation, we first examined the proximal cytoplasmic signaling events upon LPS stimulation. As shown in Fig. 7A, MCT4 knockdown had no effect on LPS-induced ERK phosphorylation. Furthermore, neither IkBα phosphorylation nor degradation upon LPS treatment was affected by MCT4 knockdown (Fig. 7B). Consistent with no change in IkBα degradation, we found that there is no defect in the translocation of the NF-κB subunit p65 into nuclei in LPS-induced macrophages with MCT4 knockdown (Fig. 7C). These data suggest that MCT4 does not participate in the LPS-induced cytoplasmic signaling events.

**MCT4 Knockdown Leads to Intracellular Accumulation of Lactate and Decreased Glycolysis in Macrophages**—There is increasing appreciation of the importance of glycolysis in the activation of immune cells, such as dendritic cells, macrophages, and T cells (13–16). LPS stimulation enhances glycolytic rate, which leads to greater formation of lactate in these cells (14, 32). To determine whether MCT4 expression regulates glycolysis, we first measured ECAR, a surrogate of glycolytic rate in macrophages. As shown in Fig. 8A, LPS-treated macrophages with transfection of control siRNAs demonstrated tremendously enhanced ECAR. Because we have found that MCT4 knockdown attenuates macrophage activation by TLR2 and TLR4 agonists, we reasoned that this may be caused by a diminished glycolysis in macrophages with MCT4 knockdown. To test this hypothesis, we also assessed ECAR in LPS-treated macrophages with MCT4 knockdown. As shown in Fig. 8A, LPS increased ECAR was alleviated by MCT4 knockdown. However, it needs to be pointed out that the diminished ECAR in macrophages with MCT4 knockdown could be a result of reduced export of lactate, attenuated glycolysis, or both. To answer this question, we then measured intracellular lactate in macrophages. As shown in Fig. 8B, LPS treatment significantly increased intracellular lactate levels. Furthermore, and as expected, the increase in intracellular lactate in LPS-treated macrophages was enhanced by MCT4 knockdown (Fig. 8B).
MCT4 Regulates Inflammatory Response

Although there have been many good examples that immune response is regulated at the transcriptional, translational, and epigenetic levels (5, 7), it is less clear how cellular metabolism may contribute to this process. In this study, we found that MCT4 is up-regulated in macrophages that are activated by TLR2 or TLR4 agonists. The increased expression of MCT4 is required for sustaining a high level of glycolysis that is necessary for the activation of macrophages and expression of proinflammatory mediators.

The increased expression of MCT4 was seen in macrophages that were treated with TLR2 or TLR4 but not TLR3 agonists. Both TLR2 and TLR4 signal through MYD88 to activate NF-κB, whereas TLR3 primarily elicits a strong type I interferon response through TRIF-dependent activation of IRF3 (27). Therefore, MCT4 expression appears to be regulated by NF-κB, but not by IRF3. This conclusion is also supported by the findings that the enhanced expression of MCT4 in LPS-treated macrophages is abrogated by the specific inhibitor of NF-κB activation.

Enhanced aerobic glycolysis has been recognized as a metabolic hallmark of cancer (10, 37, 38). However, there is also growing appreciation of its role in the regulation of immune response (14, 15, 32, 33, 39–41). Activated dendritic cells, T cells, and macrophages undertake enhanced glycolysis (14, 15, 8C). Taken together, our data suggest that intracellular accumulation of lactate hinders the glycolytic up-regulation in LPS-treated macrophages, thereby inhibiting macrophage activation.

MCT4 Knockdown Attenuates LPS-induced Expression of Glycolytic Enzymes HK2 and PFKFB3—We found that MCT4 knockdown causes intracellular accumulation of lactate in LPS-treated macrophages. This event was accompanied by the attenuation of LPS-enhanced production of G-6-P and F-6-P and glucose consumption in macrophages with MCT4 knockdown. To determine the mechanisms that might be involved in the diminished glycolysis found in MCT4 knockdown macrophages, we examined the expression of HK2, a key glycolytic enzyme that phosphorylates glucose to produce G-6-P (34), and PFKFB3, a critical activator of the key glycolytic enzyme, 6-phosphofructo-1-kinase (35). As shown in Fig. 9 (A and B), the expression of HK2 and PFKFB3 was increased in LPS-treated macrophages, consistent with the augmented glycolysis in these cells. More importantly, we found that LPS-induced HK2 and PFKFB3 is attenuated by MCT4 knockdown (Fig. 9, A and B), which is also concordant with the diminished LPS-augmented glycolysis. Because the expression of glycolytic enzymes is mainly controlled by the master transcriptional factor HIF-1α (36), we speculated that the attenuation of LPS-induced HK2 and PFKFB3 by MCT4 knockdown may be resulted from a similar decrease in HIF-1α expression. However, we found that LPS-induced HIF-1α remains largely unaltered in macrophages with MCT4 knockdown (Fig. 9, A and B). Regardless, our data suggest that the diminished expression of HK2 and PFKFB3 may contribute to the attenuated glycolysis observed in macrophages with MCT4 knockdown.

DISCUSSION

Consistent with the role of MCT4 as the main transporter of the intracellular lactate and the reduced ECAR in macrophages with MCT4 knockdown.

Because MCT4 knockdown inhibits export of intracellular lactate, ECAR and intracellular lactate are no long suitable indicators of glycolytic rate in macrophages with MCT4 knockdown. To overcome this issue and assess glycolytic rate, we examined the early steps of glycolysis, such as generation of HK2 and PFKFB3—We found that MCT4 knockdown causes intracellular accumulation of lactate in LPS-treated macrophages. This event was accompanied by the attenuation of LPS-enhanced production of G-6-P and F-6-P and glucose consumption in macrophages with MCT4 knockdown. To determine the mechanisms that might be involved in the diminished glycolysis found in MCT4 knockdown macrophages, we examined the expression of HK2, a key glycolytic enzyme that phosphorylates glucose to produce G-6-P (34), and PFKFB3, a critical activator of the key glycolytic enzyme, 6-phosphofructo-1-kinase (35). As shown in Fig. 9 (A and B), the expression of HK2 and PFKFB3 was increased in LPS-treated macrophages, consistent with the augmented glycolysis in these cells. More importantly, we found that LPS-induced HK2 and PFKFB3 is attenuated by MCT4 knockdown (Fig. 9, A and B), which is also concordant with the diminished LPS-augmented glycolysis. Because the expression of glycolytic enzymes is mainly controlled by the master transcriptional factor HIF-1α (36), we speculated that the attenuation of LPS-induced HK2 and PFKFB3 by MCT4 knockdown may be resulted from a similar decrease in HIF-1α expression. However, we found that LPS-induced HIF-1α remains largely unaltered in macrophages with MCT4 knockdown (Fig. 9, A and B). Regardless, our data suggest that the diminished expression of HK2 and PFKFB3 may contribute to the attenuated glycolysis observed in macrophages with MCT4 knockdown.

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The increased expression of MCT4 was seen in macrophages that were treated with TLR2 or TLR4 but not TLR3 agonists. Both TLR2 and TLR4 signal through MYD88 to activate NF-κB, whereas TLR3 primarily elicits a strong type I interferon response through TRIF-dependent activation of IRF3 (27). Therefore, MCT4 expression appears to be regulated by NF-κB, but not by IRF3. This conclusion is also supported by the findings that the enhanced expression of MCT4 in LPS-treated macrophages is abrogated by the specific inhibitor of NF-κB activation.

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The glycolytic up-regulation was found to be necessary to the activation of these immune cells because this process produces more intermediates to participate in the transcription and translation of proinflammatory mediators (14, 15, 32, 33, 39–42). The enhanced glycolysis in LPS-treated macrophages led to higher production of lactate, which was also accompanied by increased lactate export. This activity probably demands a greater expression of the lactate transporter MCT4. Therefore, MCT4 up-regulation in LPS-treated macrophages may represent a positive feedback mechanism through which these cells are able to avoid an intracellular accumulation of lactate and the resulting aggravation of acidification.

However, lactate is nevertheless a byproduct of glycolysis. First, pyruvate conversion into lactate is an essential step to replenish NADH that is consumed at the early steps of glycolysis and thus is necessary for the continuation of the glycolytic flux (43). Second, exported lactate has been shown to have many biological activities through either autocrine or paracrine actions (23–25, 44–46). These previous findings suggest that the increased MCT4 expression may be necessary to the LPS-treated macrophages. Consistent with this conclusion, MCT4 knockdown or blockage by inhibitors decreased LPS or PAM induced proinflammatory cytokines.

Although we did not attempt to examine a potential alteration in the exported lactate in LPS-treated macrophages with MCT4 knockdown, we did find that the increased glycolysis caused by MCT4 knockdown might account for the down-regulated expression of proinflammatory cytokines.

We found that MCT4 knockdown attenuates the augmented glycolysis in LPS-treated macrophages. However, oxidative phosphorylation was largely unaffected (data not shown). These data suggest that oxidative phosphorylation may not have a significant role in LPS-induced macrophage activation. This conclusion is consistent with previous studies that activa-
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Constrasted to play an important role in the transactivation of its transfected with 20 nM control siRNA or MCT4 siRNA. 48 h after transfection, the cells were treated with 100 ng/ml LPS for 6 h. ECAR was then determined.

100 ng/ml LPS for 6 h. ECAR was then determined. **, mouse BMDMs were transfected with 20 nM control siRNA or MCT4 siRNA. 48 h after transfection, the cells were treated with 100 ng/ml LPS for 6 h. The levels of intracellular G-6-P, F-6-P, and glucose consumption were determined. The increase (absolute value (glucose consumption) or percentage (G-6-P and F-6-P)) over the untreated group was plotted. *, p < 0.05; **, p < 0.01; ***, p < 0.001. The experiments were performed three times with similar results obtained. con, control; si, siRNA.

tion of dendritic cells is independent of oxidative phosphorylation (14).

Although intracellular accumulation of lactate caused by MCT4 knockdown may slow down glycolytic flux in macrophages, we also found that LPS-induced HK2 and PFKFB3 is diminished. HK2 is a key rate-limiting glycolytic enzyme (34), and PFKFB3 is a critical activator of the key glycolytic enzyme, 6-phosphofructo-1-kinase (35), indicating that the decreased expression of HK2 and PFKFB3 may also contribute to the attenuated glycolysis. Under hypoxia, the expression of many glycolytic enzymes is controlled by the master transcriptional factor HIF-1α (47–49). However, we did not find a significantly different expression of HIF-1α in LPS-treated macrophages when MCT4 is knocked down. Although how LPS-induced expression of HK2 and PFKFB3 is thwarted by MCT4 knockdown needs further elucidation, there are several potential scenarios that may explain these findings. It has been previously shown that decreased intracellular pH causes activation of HDACs (50), which could lead to deacetylation of HIF-1α or HIF-1α-responsive elements located in the promoters of HIF-1α target genes. In fact, HIF-1α acetylation has been demonstrated to play an important role in the transactivation of its regulated genes (51). Because we have found that there is an increased level of intracellular lactate in LPS-treated macrophages with MCT4 knockdown, the more acidic intracellular environment could result in hypoacetylation of HIF-1α and/or the promoters of HIF-1α target genes and thus compromising the transactivation of these targets.

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FIGURE 8. MCT4 knockdown leads to intracellular accumulation of lactate and decreased glycolysis in macrophages. A, mouse BMDMs were transfected with 20 nM control siRNA or MCT4 siRNA. 24 h after transfection, the cells were trypsinized and seeded in Seahorse XF-24 cell culture microplates (1.5 × 10⁵ cells/well). 24 h after plating, the cells were treated without or with 100 ng/ml LPS for 6 h. ECAR was then determined. B, mouse BMDMs were transfected with 20 nM control siRNA or MCT4 siRNA. 48 h after transfection, the cells were treated with 100 ng/ml LPS for 6 h. The levels of intracellular lactate were determined. C, mouse BMDMs were transfected with 20 nM control siRNA or MCT4 siRNA. 48 h after transfection, the cells were treated with 100 ng/ml LPS for 6 h. The levels of intracellular G-6-P, F-6-P, and glucose consumption were determined. The increase (absolute value (glucose consumption) or percentage (G-6-P and F-6-P)) over the untreated group was plotted. *, p < 0.05; **, p < 0.01; ***, p < 0.001. The experiments were performed three times with similar results obtained. con, control; si, siRNA.

FIGURE 9. MCT4 knockdown attenuates LPS-induced expression of glycolytic enzymes HK2 and PFKFB3. A, mouse BMDMs were transfected with 20 nM control siRNA or MCT4 siRNA. 48 h after transfection, the cells were treated with 100 ng/ml LPS for 6 h. The levels of HK2, PFKFB3, HIF-1α, and MCT4 in A were quantified by ImageJ software. ** p < 0.01, *** p < 0.001. The experiments were performed three times with similar results obtained. con, control; si, siRNA.
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