The Journal of Immunology

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J Immunol 2015; 194:6082-6089; Prepublished online 11 May 2015;
doi: 10.4049/jimmunol.1402469
http://www.jimmunol.org/content/194/12/6082

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Pyruvate Dehydrogenase Kinase 1 Participates in Macrophage Polarization via Regulating Glucose Metabolism

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The M1 and M2 polarized phenotypes dictate distinctive roles for macrophages as they participate in inflammatory disorders. There has been growing interest in the role of cellular metabolism in macrophage polarization. However, it is currently unclear whether different aspects of a specific metabolic program coordinately regulate this cellular process. In this study, we found that pyruvate dehydrogenase kinase 1 (PDK1), a key regulatory enzyme in glucose metabolism, plays an important role in the differential activation of macrophages. Knockdown of PDK1 diminished M1, whereas it enhanced M2 activation of macrophages. Mechanistically, PDK1 knockdown led to diminished aerobic glycolysis in M1 macrophages, which likely accounts for the attenuated inflammatory response in these cells. Furthermore, we found that mitochondrial respiration is enhanced during and required by the early activation of M2 macrophages. Suppression of glucose oxidation, but not that of fatty acids, inhibits this process. Consistent with its inhibitory role in early M2 activation, knockdown of PDK1 enhanced mitochondrial respiration in macrophages. Our data suggest that two arms of the glucose metabolism synergistically regulate the differential activation of macrophages. Our findings also highlight the central role of PDK1 in this event via controlling glycolysis and glucose oxidation. ***The Journal of Immunology*****, 2015, 194: 6082–6089.**

Macrophages are an essential component of innate immunity and well recognized to play a critical role in inflammation, systemic metabolism, tissue repair, and tumor surveillance (1–3). Bearing such diverse functions necessitates macrophages to assume a wide spectrum of phenotypes that are currently divided into two subgroups, namely, M1 and M2, a specification emulating the Th1/Th2 classification of T cells (1–6).

Macrophages activated by INF-γ and/or TLR agonists are called M1 macrophages (3, 4, 7, 8), whereas macrophages differentiated with Th2 cytokines, immunocomplexes, TGF-β, and IL-10 are named M2 macrophages (3, 4, 7, 8). M1 macrophages produce high levels of proinflammatory cytokines and reactive oxygen and nitrogen species, which are crucial to their microbicidal activities (1, 3, 4). M2 macrophages have high expression of markers of alternative activation, such as arginase-1 (Arg1), chitinase 3–like 3 (also called YM-1), and found in inflammatory zone 1 (FIZZ-1), and also demonstrate high levels of scavenger, mannose, and galactose-type receptors (1, 2, 9). M2 macrophages have an important role in parasite infection, tissue remodeling, angiogenesis, and tumor progression (10, 11).

The regulation of macrophage polarization is under extensive study at both the transcriptional and the posttranscriptional levels. A number of transcriptional factors, such as IFN-regulatory factors and Kruppel-like factors, have been found to participate in the differential activation of macrophages (4). Recently, a class of small noncoding RNAs, microRNAs, also began to emerge as major players in macrophage polarization (8).

There has been growing interest in understanding the role of cellular metabolism in macrophage activation. It has been demonstrated that aerobic glycolysis is essential to the activation of many types of immune cells, such as dendritic cells, T cells, and macrophages (12–18). In addition, M2 macrophage polarization requires fatty acid oxidation (19–21). These studies have provided compelling evidence that macrophage polarization can be regulated by the different aspects of cellular metabolism.

Pyruvate dehydrogenase kinase 1 (PDK1) is a key regulatory enzyme in glucose metabolism (22–24). PDK1 incites inhibitory phosphorylation on components of the pyruvate dehydrogenase (PDH) complex that converts pyruvate produced from glycolytic flux to acetyl-CoA (22–24). Pyruvate-derived acetyl-CoA enters the tricarboxylic acid (TCA) cycle that generates NADH that fuels the electron transport chain for oxidative phosphorylation (25–27). The importance of the regulatory role of PDK1 in glucose metabolic homeostasis has been well studied in cancer cells (22). Amplified expression of PDK1 frequently found in many types of cancers is believed to be responsible for the aerobic glycolysis undertaken by these malignant cells (22). Despite the rapid progress made in other areas, the role of PDK1, as well as its regulated glucose metabolism in macrophage polarization, has not been described.

In this study, we found that PDK1 promotes M1, whereas it inhibits M2 macrophage polarization. Our data therefore suggest

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Received for publication September 26, 2014. Accepted for publication April 7, 2015.

This work was supported by National Institutes of Health Grants HL105473 (to G.L.), HL076206 (to G.L.), and HL114470 (to V.J.T.). This work was also supported by University of Alabama at Birmingham Diabetes Research Center Bio-Analytical Redoux Biology Core Grant P30 DK079626 (to D.R.M.).

G.L. designed and supervised the study; Z.T., N.X., H.C., and D.R.M. performed the experiments and analyzed the data; E.A. and V.J.T. contributed intellectual inputs; and Z.T., E.A., V.J.T., and G.L. wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Arg1, arginase-1; BMDM, bone marrow–derived macrophage; Ct, cycle threshold; 2-DG, 2-deoxy-D-glucose; ETO, etomoxir; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; FIZZ-1, found in inflammatory zone 1; GM-BMM, GM-CSF–induced macrophage; HKPA, heat-killed *Pseudomonas aeruginosa*; OCR, oxygen consumption rate; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; siRNA, small interfering RNA; TCA, tricarboxylic acid.

that two arms of the glucose metabolism synergistically regulate the differential activation of macrophages.

Materials and Methods

Reagents

Ultrapure LPS from *Salmonella minnesota* R595, PAM3CSK4, heat-killed *Listeria monocytogenes*, and heat-killed *Pseudomonas aeruginosa* (HKPA) were purchased from Invivogen.

Establishment of mouse bone marrow–derived macrophages and mouse peritoneal macrophages

Mouse bone marrow–derived macrophages (BMDMs) were derived from bone marrow cells of C57BL/6 mice (National Cancer Institute-Fredrick). In brief, bone marrow cells were cultured for 5 d in DMEM media containing 10% FBS and 50 ng/ml murine M-CSF (R&D Systems). The established BMDMs were then plated for the following experiments. Peritoneal macrophages were elicited by 4% thioglycolate. Four 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 the 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 and IL-6 in cell culture supernatants were determined using DuoSet ELISA Development kits (R&D Systems) according to the manufacturer's instructions.

Small interfering RNA transfection

ON-TARGETplus negative control small interfering RNA (siRNA) pool and specific PDK1 siRNA pool were purchased from Dharmacon. A total of 20 nM control siRNA or PDK1 siRNA was mixed with Hiperfect (Qiagen) transfection reagents for 5 min. The complexes were incubated with macrophages at 37˚C for 6 h and then removed. The cells were cultured in fresh media and respective experiments performed 2 d after transfection.

Quantitative real-time PCR

Real-time PCR was performed using SYBR Green Master Mix kit (Roche). Primer sequences were: mouse GAPDH: sense, 5′-GACTTCAACAGCAACTCCCACTCTTCC-3′; antisense, 5′-TGGGTGGTCCAGGGTTTCTTACTCCTT-3′; mouse β-Tubulin: sense, 5′-GGATGCTGCCAATAACTATGCTCGT-3′; antisense, 5′-GCCAAAGCTGTGGAAAACCAAGAAG-3′; mouse IL-6: sense, 5′-CCCAATTTCCAATGCTCTCCTA-3′; antisense, 5′-AGGAATGTCCACAAACTGATATGCT-3′; mouse IL-12 p40: sense, 5′-CCAAATTACTCCGGACGGTTCAC-3′; antisense, 5′-CAGACAGAGACGCCATTCCACAT-3′; mouse IL-1β: sense, 5′-AAGGAGAACCAAGCAACGACAAAATA-3′; antisense, 5′-TTTCCATCTTCTTCTTTGGGTATTGC-3′; mouse iNOS: sense, 5′-ATCTTTGCCACCAAGATGGCCTGG-3′; antisense, 5′-TTCCTGTGCTGTGCTACAGTTCCG-3′; mouse Arg1: sense, 5′-TGACTGAAGTAGACAAGCTGGGGAT-3′; antisense, 5′-CGACATCAAAGCTCAGGTGAATCGG-3′; mouse YM-1: sense, 5′-ATGAAGCATTGAATGGTCTGAAAG-3′; antisense, 5′-TGAATATCTGACGGTTCTGAGGAG-3′; mouse FIZZ-1: sense, 5′-AGGTCAAGGAACTTCTTGCCAATCC-3′; antisense, 5′-AAGCACACCCAGTAGCAGTCATCCC-3′; mouse mannose receptor, C type 1 (MRC1): sense, 5′-GGGCAGTCACCATATTTTATTGGC-3′; antisense, 5′-GCAAAGTTGGGTTCTCCTGTAGCC-3′.

To calculate fold change in the expression of cytokines, Δ cycle threshold (Ct) values were first obtained: ΔCt = Ct of GAPDH or Tubulin − Ct of cytokines. ΔΔCt values were then obtained: ΔΔCt = ΔCt of treated groups − ΔCt of untreated control groups. Fold change was calculated as $2^{-\Delta\Delta Ct}$, with control groups as 1-fold.

Western blotting

Western blotting was performed as previously described (28). Anti-Cox2, PDK1, and PDH-E1α Abs were from Cell Signaling. Anti–p-PDH-E1α Ab was from EMD Chemicals. Anti-Arg1 and iNOS Abs were from Santa Cruz Biotechnology.

NO measurement

NO release by macrophages was determined by measuring nitrite concentration in culture media with NO Fluorometric Assay Kit (BioVision) according to the manufacturer's manual.

Real-time cell metabolism assay

XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) was used for real-time analysis of oxygen consumption rate (OCR). BMDMs were seeded in Seahorse XF-24 cell culture microplates (1.5×10^5 cells/well). The cells were treated without or with 2 ng/ml IL-4 for 6 h, followed by sequential treatment with 1.5 μg/ml oligomycin (Oligo), 4.5 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 1 μM rotenone plus 4 μM antimycin (Rot + Ant). Real-time OCR was recorded according to the manufacturer's manual.

Intracellular and extracellular lactate assays

Intracellular and extracellular levels of lactate were determined using lactate assay kit (BioVision) according to the manufacturer's instructions.

Statistical analysis

One-way ANOVA followed by the Bonferroni test was used for multiple-group comparisons. The Student *t* test was used for comparison between two groups. A p value <0.05 was considered statistically significant.

Results

Knockdown of PDK1 diminishes LPS-induced M1 macrophage activation

Although there has been growing evidence showing that glucose metabolism is involved in innate immune responses (12–16, 18), it

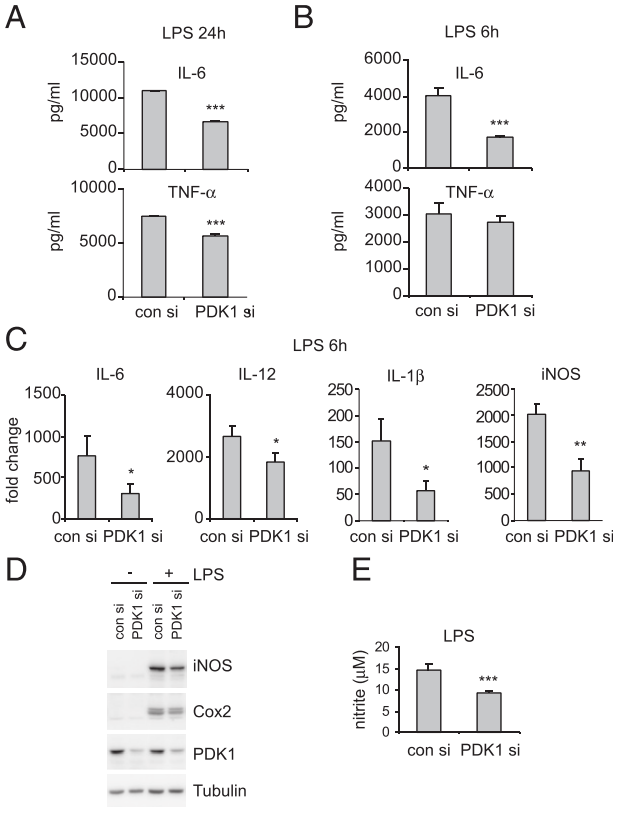

FIGURE 1. Knockdown of PDK1 diminishes LPS-induced M1 macrophage activation. (**A** and **B**) Mouse BMDMs were transfected with 20 nM control siRNA (con si) or PDK1 siRNA (PDK1 si). Forty-eight hours after transfection, the cells were treated without or with 100 ng/ml LPS for 24 (A) or 6 h (B). Levels of IL-6 and TNF-α in the cell culture supernatants were determined by ELISA assays. ***$p < 0.001$ compared with con si group. (**C**) Mouse BMDMs were transfected with 20 nM con si or PDK1 si. Forty-eight hours after transfection, the cells were treated without or with 100 ng/ml LPS for 6 h. mRNA levels of IL-6, IL-12, IL-1β, and iNOS were determined by real-time PCR. *$p < 0.05$, **$p < 0.01$ compared with con si group. (**D**) The experiments were performed as in (B), and protein levels of iNOS, Cox2, PDK1, and Tubulin were determined by Western blotting. (**E**) The experiments were performed as in (B) and NO production represented by nitrite concentrations in culture media was determined. ***$p < 0.001$ compared with con si. The experiments were performed three times with similar results obtained.

is less clear whether this metabolic event participates in the differential activation of macrophages. PDK1 is a key regulatory enzyme involved in glucose metabolism (22, 23). PDK1 limits the conversion of pyruvate to acetyl-CoA that enters the TCA cycle to produce NADH for the ensuing oxidative phosphorylation in the mitochondria (22, 23). Functionally, PDK1 sits at a pivotal point in glucose metabolism where it controls the bifurcation of the metabolic events leading to lactate production or oxidative phosphorylation (22, 23). To determine the role of PDK1 and glucose metabolism in macrophage activation, we first examined the effect of PDK1 knockdown in the inflammatory response of macrophages to LPS stimulation. As shown in Fig. 1A and 1B, we found that PDK1 knockdown attenuates the expression of proinflammatory cytokines, such as TNF-α and IL-6, in LPS-treated macrophages. The diminished expression of proinflammatory cytokines or mediators in LPS-treated macrophages with PDK1 knockdown occurred at the transcriptional level because mRNA levels of IL-6, IL-12, IL-1β, and iNOS were all decreased in these cells (Fig. 1C). Consistent with the reduced mRNA levels of iNOS, protein levels of iNOS, as well as Cox2, were markedly decreased in macrophages with PDK1 knockdown (Fig. 1D). Furthermore, LPS-induced NO production was also diminished by PDK1 knockdown (Fig. 1E). Of note, although PDK1 was effectively knocked down by specific PDK1 siRNAs, the expression of other key rate-limiting glycolytic enzymes, that is, hexokinase II, PFK1, and PKM2, was not affected (Supplemental Fig. 1A). These data suggest that the observed effects are more likely caused by PDK1 knockdown. To further confirm that PDK1 is required for LPS-stimulated cytokine production in macrophages, we performed similar experiments in peritoneal macrophages and the macrophage cell line RAW264.7 cells, and found that knockdown of PDK1 markedly decreases LPS-induced expression of TNF-α and IL-6 (Supplemental Fig. 2B, 2C). Collectively, these data suggest that PDK1 is required for M1 macrophage activation.

PDK1 is required for M1 macrophage activation by TLR2 activation

To determine whether PDK1 participates only in the response to LPS or in general M1 macrophage activation, we next examined whether PDK1 knockdown affects macrophage responses to TLR2 stimulation. As shown in Fig. 2A and 2B and similarly to results found with LPS treatment, PDK1 knockdown significantly attenuated PAM-induced expression of TNF-α and IL-6. Furthermore, the attenuated expression of these proinflammatory mediators also

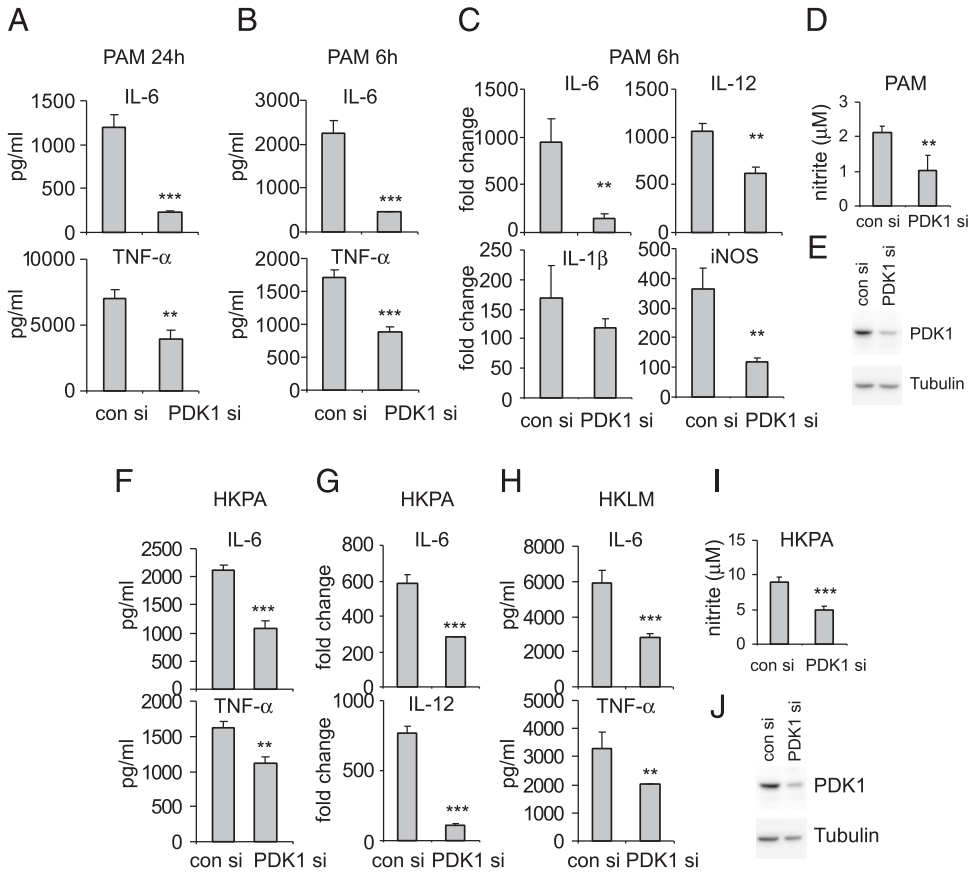

FIGURE 2. PDK1 is required for M1 macrophage activation by TLR2 activation. (**A** and **B**) Mouse BMDMs were transfected with 20 nM control siRNA (con si) or PDK1 siRNA (PDK1 si). Forty-eight hours after transfection, the cells were treated without or with 1 μg/ml PAM for 24 (A) or 6 h (B). Levels of IL-6 and TNF-α in the cell culture supernatants were determined by ELISA assays. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with con si group. (**C**) Mouse BMDMs were transfected with 20 nM con si or PDK1 si. Forty-eight hours after transfection, the cells were treated without or with 1 μg/ml PAM for 6 h. mRNA levels of IL-6, IL-12, IL-1β, and iNOS were determined by real-time PCR. $^{**}p < 0.01$ compared with con si group. The experiments were performed three times with similar results obtained. (**D**) Experiments were performed as in (B). NO production was determined. $^{**}p < 0.01$ compared with con si. (**E**) Representative blot of PDK1 knockdown in (A)–(D) is shown. (**F**–**H**) Mouse BMDMs were transfected with 20 nM con si or PDK1 si. Forty-eight hours after transfection, the cells were treated without or with 1×10^7/ml HKPA (F and G) or heat-killed *L. monocytogenes* (H) for 6 h. Protein (F and H) and mRNA (G) levels of TNF-α, IL-6, and IL-12 were determined by ELISA assays or real-time PCR. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with con si. (**I**) Experiments were performed as in (F). NO production was determined. $^{***}p < 0.001$ compared with con si. (**J**) Representative blot of PDK1 knockdown in (F)–(I) is shown. The experiments were repeated twice.

took place at the transcriptional level (Fig. 2C). In addition, PAM-induced NO production was also diminished by PDK1 knockdown (Fig. 2D) Taken together, these data suggest that PDK1 is required for M1 macrophage activation by general stimuli.

Knockdown of PDK1 diminishes the response of macrophages to bacteria that signal through TLR2

The finding that PDK1 is required for M1 macrophage activation by TLR2 intrigued us to determine whether PDK1 participates in the response of macrophages to bacteria that primarily activate TLR2. To do this, we treated macrophages with HKPA, a common bacteria frequently found to cause pneumonia and sepsis (29–31). We found that PDK1 knockdown considerably diminishes *P. aeruginosa*–induced expression of IL-6, IL-12, and TNF-α (Fig. 2F and 2G). In addition, we examined the effect of PDK1 knockdown on the activation of macrophages by *L. monocytogenes*, another common virulent bacterium that induces inflammation mediated by interactions of MyD88 and TLR2 (32). As shown in Fig. 2H, PDK1 knockdown significantly attenuated *L. monocytogenes*–induced expression of TNF-α and IL-6. In addition, HKPA-induced NO production was also diminished by PDK1 knockdown (Fig. 2I) Collectively, these data suggest that PDK1 is involved in the inflammatory response to infections by some common bacteria and may participate in the infection-associated organ injury, such as acute respiratory distress syndrome.

M-CSF–differentiated macrophages are primed for IL-10 production upon TLR stimulation, and the induced IL-10 suppresses the expression of other proinflammatory cytokines in both autocrine and paracrine manners (33, 34). To determine whether IL-10 is involved in the attenuated response of macrophages with PDK1 knockdown to LPS, PAM, or HKPA, we directly measured IL-10 levels from the same set of experiments. As shown in Supplemental Fig. 1B, the induction of IL-10 by LPS, PAM, or HKPA in macrophages with PDK1 knockdown was all diminished, similar to the decreased expression of other proinflammatory cytokines. To further define whether there is a potential role of IL-10 in PDK1-regulated M1 macrophage activation, we performed the same

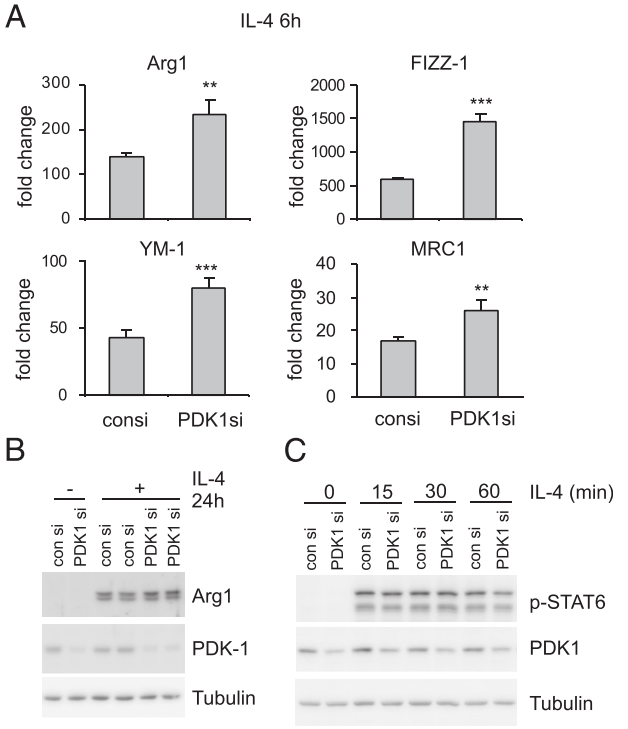

FIGURE 4. PDK1 knockdown enhances M2 macrophage activation. (**A**) Mouse BMDMs were transfected with 20 nM control siRNA (con si) or PDK1 siRNA (PDK1 si). Forty-eight hours after transfection, the cells were treated without or with 2 ng/ml IL-4 for 6 h. Levels of Arg1, YM-1, FIZZ-1, and MRC1 were determined by real-time PCR. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with con si group. (**B**) Mouse BMDMs were transfected with 20 nM con si or PDK1 si. Forty-eight hours after transfection, the cells were treated without or with 2 ng/ml IL-4 for 24 h. Protein levels of Arg1 and Tubulin were determined by Western blotting. (**C**) Mouse BMDMs were transfected with 20 nM con si or PDK1 si. Forty-eight hours after transfection, the cells were treated with 2 ng/ml IL-4 for the indicated periods. Levels of p-STAT6, PDK1, and Tubulin were determined by Western blotting. Data are representative of three experiments.

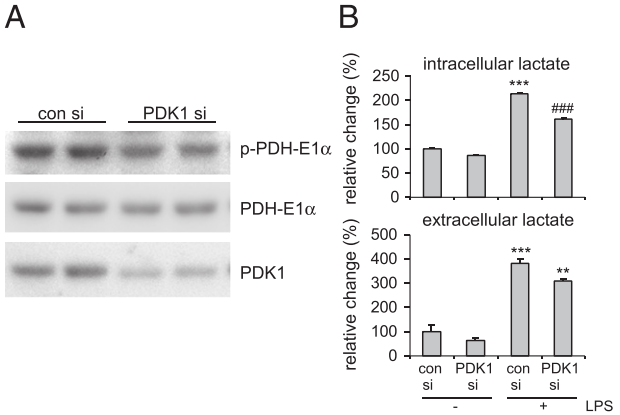

FIGURE 3. PDK1 knockdown inhibits glycolysis in macrophages. (**A**) Mouse BMDMs were transfected with 20 nM control siRNA (con si) or PDK1 siRNA (PDK1 si). Forty-eight hours after transfection, cellular extracts were prepared and levels of p-PDH-E1α, PDH-E1α, and PDK1 were determined by Western blotting. (**B**) BMDMs were transfected with 20 nM control siRNA or PDK1 siRNA. Forty-eight hours after transfection, the cells were treated without or with 100 ng/ml LPS for 6 h. The cell supernatants were collected. Cells were lysed in lactate assay buffer. Levels of lactate in the supernatants and in the cellular extracts were determined by lactate assay kit. $^{***}p < 0.001$ compared with con si−, $^{**}p < 0.01$ compared with con si+, $^{\#\#\#}p < 0.001$ compared with con si+. The experiments were performed three times with similar results obtained.

experiments in GM-CSF–induced macrophages (GM-BMMs), which have low expression of IL-10 upon TLR stimulations. As shown in Supplemental Fig. 2A, the expression of TNF-α was significantly diminished in PAM- or HKPA-treated GM-BMM with PDK1 knockdown. Taken together, these data suggest that the regulation of PDK1 on M1 macrophage activation is independent of IL-10. However, we found that PDK1 knockdown has little effect on LPS-activated cytokine production in GM-BMMs (data not shown). The different regulation of the responses to TLR2 and TLR4 agonists by PDK1 in GM-BMMs suggests that LPS-induced glycolytic augmentation in this type of cell involves more than PDK1-controlled pathways. Notably, GM-BMMs are often used as dendritic cells (35).

PDK1 knockdown inhibits glycolysis in macrophages

Glycolysis has been recently shown to be required for the optimal activation of many types of inflammatory cells, including monocytes, dendritic cells, and macrophages (12–16, 18). To determine the mechanism by which PDK1 regulates M1 macrophage activation, we investigated the effect of PDK1 on glycolytic flux in macrophages. PDK1 phosphorylates components of the PDH complex in the mitochondria (22, 23). This inhibitory phosphorylation suppresses pyruvate conversion to acetyl-CoA, thereby augmenting lactate production (22–24). We first confirmed that PDK1 knockdown reduces the phosphorylation of PDH-E1α (Fig. 3A). We then

directly measured the intracellular and extracellular levels of lactate, the glycolytic end product. As shown in Fig. 3B, LPS treatment enhanced lactate production. However, such an increase was significantly weakened by PDK1 knockdown (Fig. 3B). Taken together, these data indicate that augmented glycolysis is critical to the inflammatory response. These data also suggest that PDK1 is required for the enhanced glycolysis in LPS-treated macrophages, thereby promoting the full activation of M1 macrophages.

PDK1 knockdown enhances M2 macrophage activation

Although there is a well-recognized role of fatty acid oxidation in M2 macrophage establishment, that is, macrophages treated with IL-4 for ≥24 h (19, 20), it is unclear whether glucose metabolism also participates in this cellular process. Because we have found that PDK1 is required for the enhanced glycolysis and M1 macrophage activation, we next asked whether PDK1 regulates macrophage polarization to M2. To address this question, we examined the effect of PDK1 knockdown on IL-4–induced expression of M2 macrophage phonotypical markers, such as Arg1, YM-1, FIZZ-1, and MRC1. As shown in Fig. 4A, PDK1 knockdown remarkably enhanced the expression of M2 markers at the early time point (6 h) of IL-4 stimulation. Consistent with the increased mRNA levels of Arg1, the protein levels of this M2 marker were enhanced in IL-4–treated macrophages with PDK1 knockdown (Fig. 4B). These data suggest that PDK1 is a negative regulator of M2 macrophage differentiation, in contrast with its promoting role in M1 macrophage activation. To delineate the mechanism by which PDK1 regulates M2 differentiation, we examined the levels of IL-4–induced STAT6 phosphorylation and found that it is not affected by PDK1 knockdown (Fig. 4C). These data suggest that the augmented glucose oxidation does not affect the signaling events proximal to the activation of the IL-4R. Therefore, the mechanism by which oxidative phosphorylation promotes M2 differentiation needs further delineation and remains a focus of future studies.

Mitochondrial respiration is increased during and required for the early differentiation of M2 macrophages

There is enhanced mitochondrial respiration in the established M2 macrophages, that is, 24 h after IL-4 treatment (19, 20). However, it is not clear whether oxidative phosphorylation at the early time points of IL-4 treatment regulates M2 differentiation. To address this question, we measured mitochondrial OCR in macrophages at 6 h after IL-4 treatment. As shown in Fig. 5A, there was a significant increase in basal and maximal OCR at this time point. These data suggest that mitochondrial respiration may also be involved in the early activation of M2 macrophages.

To determine whether mitochondrial respiration is required for the early differentiation of M2 macrophages, we pretreated macrophages with the specific mitochondrial respiratory inhibitor, oligomycin. As shown in Fig. 5B, oligomycin diminished the expression of M2 phenotypic markers at 6 h after IL-4 treatment. These data suggest that mitochondrial respiration is required for the early differentiation of M2 macrophages.

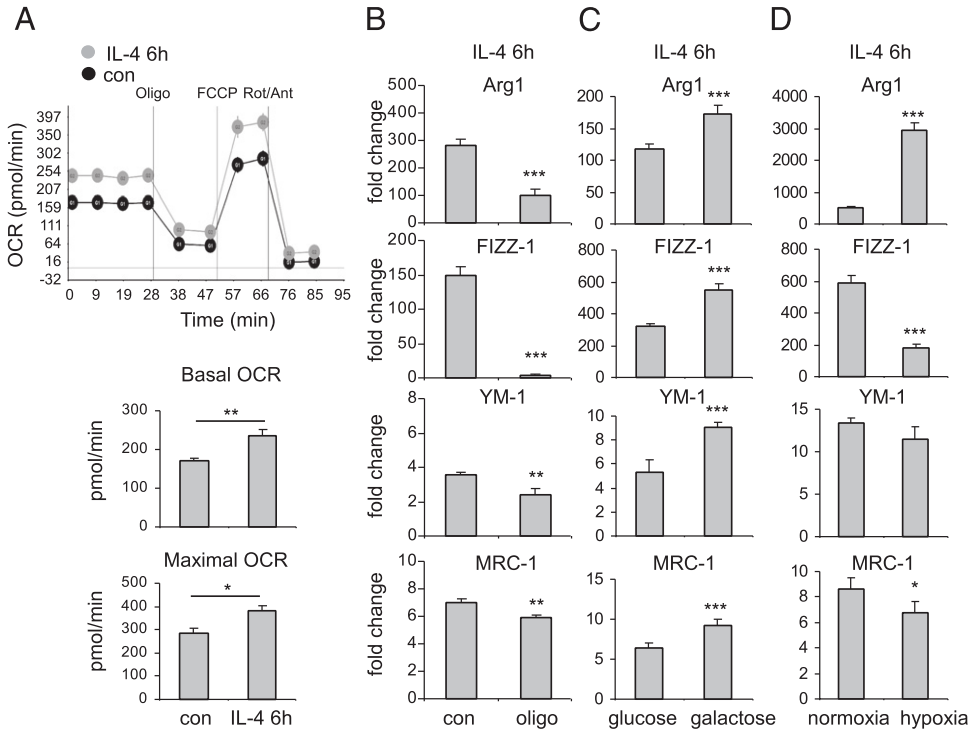

FIGURE 5. Mitochondrial respiration is increased during and required for the early differentiation of M2 macrophages. (**A**) BMDMs were seeded in Seahorse XF-24 cell culture microplates (1.5×10^5 cells/well). The cells were treated without or with 2 ng/ml IL-4 for 6 h, followed by sequential treatment (separated by vertical lines) with oligomycin (Oligo), FCCP, and rotenone plus antimycin (Rot + Ant). OCR (basal, nonmitochondrial [after treatment with Oligo], and reserve [after treatment with FCCP]) were then determined. OCR at basal and maximal levels of the indicated conditions was plotted in bar graphs. $*p < 0.05$, $**p < 0.01$. (**B**) Mouse BMDMs were pretreated without or with 0.5 μg/ml oligomycin for 1 h. The cells were then treated without or with 2 ng/ml IL-4 for 6 h. Levels of Arg1, YM-1, FIZZ-1, and MRC1 were determined by real-time PCR. $**p < 0.01$, $***p < 0.001$ compared with "con" group. (**C**) Mouse BMDMs were cultured in DMEM containing 25 mM glucose or 25 mM galactose for 24 h. The cells were then treated without or with 2 ng/ml IL-4 for 6 h. Levels of Arg1, YM-1, FIZZ-1, and MRC1 were determined by real-time PCR. $***p < 0.001$ compared with "con" group. (**D**) Mouse BMDMs were pre-exposed to normoxia or hypoxia (1% O_2) for 90 min, followed by treatment with 2 ng/ml IL-4 for 6 h. Levels of Arg1, YM-1, FIZZ-1, and MRC1 were determined by real-time PCR. $*p < 0.05$, $***p < 0.001$ compared with mock treatment (con) group. Data are representative of three experiments.

To further define the role of mitochondrial respiration in the early differentiation of M2 macrophages, we replaced glucose with galactose in the culture media. Galactose enters glycolysis through the Leloir pathway that causes a negative net production of ATP at the end of glycolytic flux, and thus forcing cells to respire (36, 37). As shown in Fig. 5C, the expression of IL-4–induced M2 markers was significantly greater in macrophages cultured with galactose than that in cells cultured with glucose, consistent with the findings that augmented oxidative phosphorylation promotes M2 macrophage differentiation. We next blunted mitochondrial respiration by exposing to hypoxia and found that there is less expression of IL-4–induced M2 markers, except for Arg1, in macrophages under hypoxic conditions (Fig. 5D). The exceptional increase in IL-4–induced Arg1 in macrophages exposed to hypoxia could be caused by HIF-2α stabilized under hypoxic conditions. HIF-2α has been previously shown to activate Arg1 expression (38). Nevertheless, these data suggest that enhanced glycolysis is in general pro-M1 but anti-M2 macrophage differentiation, consistent with the effects of PDK1 knockdown on the differential activation of macrophages.

Fatty acid oxidation is dispensable to the early differentiation of M2 macrophages

Increased fatty acid oxidation is present in established M2 macrophages and is required for optimal activation of M2 macrophages (19, 20). Because we found augmented mitochondrial respiration during the early stage of M2 activation, we asked whether fatty acid oxidation is involved in this event. To test this hypothesis, we pretreated macrophages with etomoxir (ETO), a specific inhibitor of carnitine palmitoyltransferase I that is an essential enzyme in fatty acid oxidation (39). We found that inhibition of fatty acid oxidation has only marginal effects on mitochondrial respiration (Fig. 6A), suggesting that the elevated mitochondrial respiration during the early activation of M2 macrophages is not derived from fatty acid oxidation. Consistently, ETO treatment only slightly diminished the expression of one of the M2 markers during early M2 macrophage activation (Fig. 6B), in contrast with what has been reported in established M2 macrophages (19, 20). These data suggest that, although fatty acid oxidation is necessary for M2 polarization, it does not impose its role until relatively late in the process. These data also allude to a critical role for glucose oxidation in the early activation of M2 macrophages.

Glucose oxidation is required for the early differentiation of M2 macrophages

Because we have found that fatty acid oxidation is largely dispensable to the early activation of M2 macrophages, we asked whether glucose oxidation is required for this early event. To test this hypothesis, we pretreated macrophages with 2-deoxy-D-glucose (2-DG) for 3 h and included 2-DG in the media during the IL-4 stimulation. 2-DG is an inhibitor of a key glycolytic enzyme, hexokinase II, and suppresses glycolytic flux to fuel the TCA cycle and possibly the ensuing oxidative phosphorylation (40). As shown in Fig. 7A, 2-DG did inhibited oxidative phosphorylation during the early differentiation of M2 macrophages. More importantly, we found that 2-DG diminishes the early expression of M2 markers (Fig. 7B). Together with the findings with ETO, these data suggest that glucose oxidation, but not that of fatty acids, is necessary for the early differentiation of M2 macrophages.

PDK1 knockdown enhances mitochondrial respiration in macrophage

Now we have demonstrated that PDK1 knockdown enhances the early differentiation of M2 macrophages. We have also shown that glucose oxidation is likely required for the M2 activation at the early time points. To determine the mechanism by which PDK1

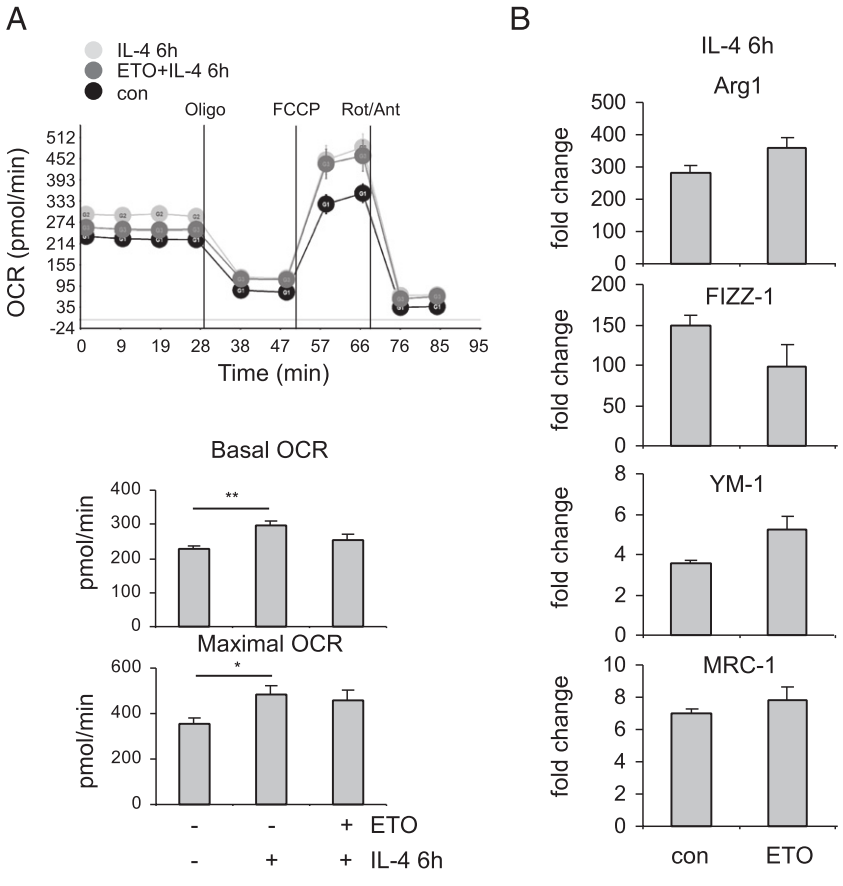

FIGURE 6. Fatty acid oxidation is dispensable to the early differentiation of M2 macrophages. (**A**) BMDMs were seeded in Seahorse XF-24 cell culture microplates (1.5×10^5 cells/well). The cells were pretreated without or with 100 μM ETO for 1 h and then treated without or with 2 ng/ml IL-4 for 6 h, followed by sequential treatment with Oligo, FCCP, and Rot plus Ant. OCR at basal and maximal levels of the indicated conditions were plotted in bar graphs. $*p < 0.05$, $**p < 0.01$. (**B**) Mouse BMDMs were pretreated without or with 100 μM ETO for 1 h. The cells were then treated without or with 2 ng/ml IL-4 for 6 h. Levels of Arg1, YM-1, FIZZ-1, and MRC1 were determined by real-time PCR. The experiments were performed three times with similar results obtained. con, mock treatment.

knockdown enhances M2 macrophage differentiation, we investigated whether PDK1 knockdown affects glucose oxidation. As shown in Fig. 8, PDK1 knockdown augmented mitochondrial oxidative phosphorylation in macrophages. These data suggest that PDK1 regulates M2 macrophage differentiation via controlling glucose oxidation during the early differentiation of M2 macrophages.

Discussion

Several recent studies showed that M1 macrophages, as well as other inflammatory cells, such as dendritic cells, are dependent on glycolysis for their full activation (12–16, 18). In addition, it has been also established that M2 macrophage differentiation requires fatty acid oxidation (19–21). Such studies clearly suggest that metabolic reprogramming is an indispensable step during the activation of macrophages. However, these previous findings have also portrayed a picture that the differential states of macrophage activation seem to be controlled by relatively independent aspects of cellular metabolism, mostly glycolysis versus fatty acid oxidation. Evidence regarding whether there is a unified and synergistic action during macrophage polarization is lacking. In this study, we showed that such a synergy does exist in these processes. We found that one of the key enzymes in glucose metabolism, PDK1, regulates both M1 and M2 macrophage activations.

Upregulation of PDK1, concurrent with increased phosphorylation of the PDH complex, is frequently found in cancer cells (22–24). Such dysregulation leads to a decline in the entry of glycolytic flux-derived pyruvate into the TCA cycle, which is believed to contribute to the "Warburg" effect, a metabolic hallmark of cancers (25–27, 41–43). In our study, we found that M1 macrophages direct glucose metabolism to aerobic glycolysis. These data are consistent with previous studies and lend further support to the notion that glycolysis is essential to the inflammatory activation of these cells (12–16). More importantly, we found that knockdown of PDK1 substantially attenuates glycolytic flux in macrophages, which is likely responsible for the diminished M1 activation observed in PDK1 knockdown cells. Our data suggest that PDK1, as a critical component of glucose metabolism, participates in M1 macrophage activation. Our data also indicate a potential therapeutic target in treating inflammatory disorders that involve M1 macrophage activation.

Although it is unclear whether glucose metabolism is involved in M2 macrophages, there has been ample evidence showing that fatty acid oxidation is essential to this process (19–21). This conclusion is supported by findings that M2 activation can be blocked by suppression of the activity of carnitine palmitoyltransferase I, an essential enzyme in fatty acid oxidation, and inhibition of mitochondrial oxidative respiration (19–21). Furthermore, a recent study demonstrated that fatty acids fueling M2 activation are mainly from an extracellular source (19). Compared with the compelling evidence showing the crucial role of fatty acid metabolism in the established M2 macrophages, that is, resting macrophages treated with IL-4 for ≥24 h, there is little knowledge regarding the metabolic demand in the early differentiation of M2 macrophages. We found that mitochondrial oxidative respiration is required early in the M2 activation as well. More importantly, these oxidative events

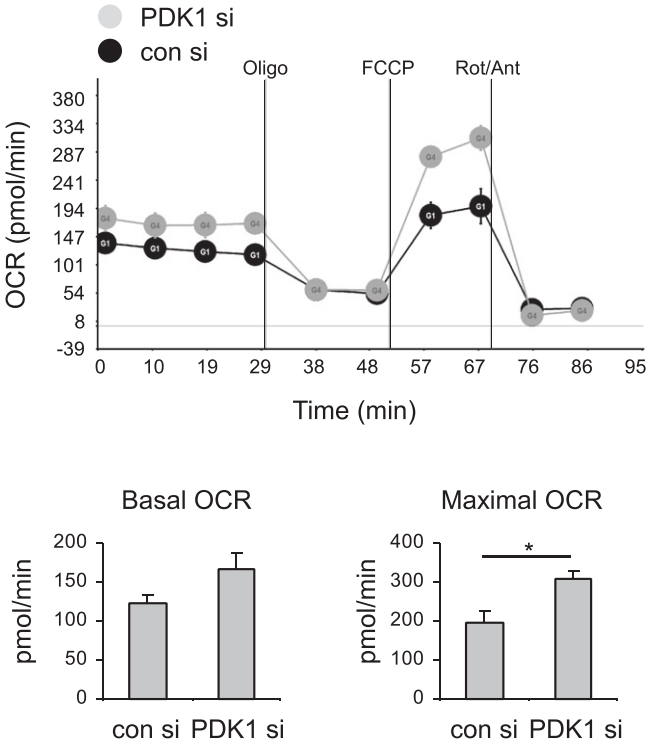

FIGURE 8. PDK1 knockdown enhances mitochondrial respiration in macrophages. Mouse BMDMs were transfected with 20 nM control siRNA (con si) or PDK1 siRNA (PDK1 si). Forty-eight hours after transfection, the cells were seeded in Seahorse XF-24 cell culture microplates (1.5×10^5 cells/well) for overnight. The cells were then treated sequentially with Oligo, FCCP, and Rot plus Ant. OCR at basal and maximal levels of the indicated conditions were plotted in bar graphs. The experiments were performed three times with similar results obtained. $*p < 0.05$.

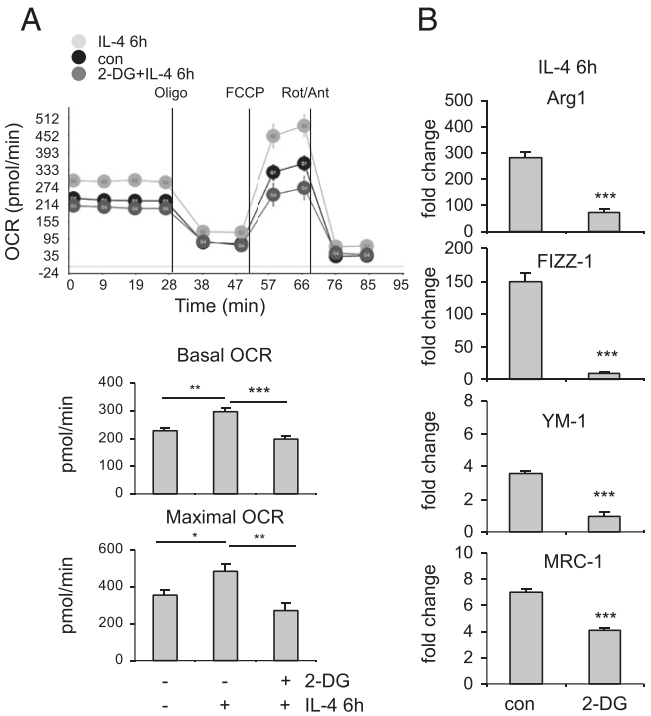

FIGURE 7. Glucose oxidation is required for the early differentiation of M2 macrophages. (**A**) BMDMs were seeded in Seahorse XF-24 cell culture microplates (1.5×10^5 cells/well). The cells were pretreated without or with 1 mM 2-DG for 3 h and then treated without or with 2 ng/ml IL-4 for 6 h, followed by sequential treatment with Oligo, FCCP, and Rot plus Ant. OCR at basal and maximal levels of the indicated conditions were plotted in bar graphs. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**B**) Mouse BMDMs were pretreated without or with 1 mM 2-DG for 3 h. The cells were then treated without or with 2 ng/ml IL-4 for 6 h. Levels of Arg1, FIZZ-1, YM-1, and MRC1 were determined by real-time PCR. $***p < 0.001$ compared with mock treatment (con) group. The experiments were performed three times with similar results obtained.

involve no fatty acids, but rather are dependent on glucose. This conclusion was based on our findings that inhibition of mitochondrial oxidative respiration, but not suppression of fatty acid cross-mitochondrial transportation, diminishes the early activation of M2 macrophages. Collectively, these data suggest that the early activation of M2 macrophages is largely independent of fatty acid oxidation.

However, we found that mitochondrial respiratory activity is increased during the early activation of M2. We believe that it is possibly caused by a spurt in glucose oxidation during this process. Consistent with this inference, 2-DG, a glycolytic inhibitor that suppresses glucose metabolism, attenuated the augmented mitochondrial respiration during early M2 differentiation. Likely because of the affected oxidative phosphorylation, 2-DG–treated macrophages demonstrated impaired early M2 activation. Being further in line with the important role of mitochondrial respiration and PDK1 during the early activation of M2, PDK1 knockdown macrophages demonstrated enhanced mitochondrial respiration. Taken together, these data suggest that PDK1-regulated glucose oxidation plays an important role in the early activation of M2 macrophages.

Our data suggest that there is a transition in the demand for mitochondrial respiration during M2 macrophage activation, with glucose oxidation of the early phase gradually being switched to fatty acid oxidation at later time points. These findings indicate the importance of the synergistic actions from the two key metabolic programs that fuel the mitochondrial respiration during M2 macrophage activation. Our data also highlight the central role of PDK1 in the differential activation of macrophages via controlling glycolysis and glucose oxidation.

Disclosures

The authors have no financial conflicts of interest.

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