Dendritic cells (DCs) are first responders of the innate immune system that integrate signals from external stimuli to direct context-specific immune responses. Current models suggest that an active switch from mitochondrial metabolism to glycolysis accompanies DC activation to support the anabolic requirements of DC function. We show that early glycolytic activation is a common program for both strong and weak stimuli, but that weakly activated DCs lack long-term HIF-1α-dependent glycolytic reprogramming and retain mitochondrial oxidative metabolism. Early induction of glycolysis is associated with activation of AKT, TBK, and mTOR, and sustained activation of these pathways is associated with long-term glycolytic reprogramming. We show that inhibition of glycolysis impaired maintenance of elongated cell shape, DC motility, CCR7 oligomerization, and DC migration to draining lymph nodes. Together, our results indicate that early induction of glycolysis occurs independent of pro-inflammatory phenotype, and that glycolysis supports DC migratory ability regardless of mitochondrial bioenergetics.
Dendritic cells (DCs) are among the first responding immune cells to any infection, injury, or threat. DCs express a wide variety of pattern recognition receptors (PRRs), which are germline-encoded receptors that recognize conserved moieties such as non-self microbe/pathogen-associated molecular patterns and danger-associated molecular patterns, often released during cell death or injury. Membrane-bound PRRs include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). DCs adopt different activation phenotypes depending on the combination of receptors engaged and the context in which activation occurs; this process is known as differential activation. Differential activation enables DCs to transmit context-specific information to other cells of the immune system and consequently direct the nature of inflammatory responses.

Regardless of their activation phenotype, DCs migrate from peripheral tissues to the draining lymph node (LN) where they interact with other cells of the immune system. For example, the activation phenotype of DCs directly determines T helper (Th) cell differentiation. DCs that are stimulated with Toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS) stimulate a pro-inflammatory phenotype characterized by production of cytokines such as interleukin (IL)-12, IL-6, tumor necrosis factor (TNF)-α, and moderate amounts of IL-10, and promote the differentiation of naive Th cells into interferon (IFN)-γ-producing Th1 cells. DCs stimulated by the yeast component zymosan (Zym), which engages TLR2, TLR6, and the CLR Dectin-1, produce less IL-12 and more IL-10 relative to LPS-stimulated DCs, and promote the induction of Th17 cells. DCs that encounter the allergen house dust mite (HDM) engage TLR2, TLR4, and Dectin-2, and produce very little pro-inflammatory cytokines, but are able to promote the activation and differentiation of Th2 and Th17 cells.

In recent years, cellular metabolism has become recognized as an important determinant of immune cell inflammatory phenotype and function. Glycolysis and oxidative phosphorylation (OXPHOS) are the main bioenergetic catabolic pathways, taking place in the cytosol and mitochondria, respectively. Glucose-derived pyruvate can either be converted to lactic acid and expelled from the cell, or completely oxidized in the mitochondria via the tricarboxylic acid (TCA) cycle. The TCA cycle fuels OXPHOS by providing reducing agents to drive the electron transport chain, consuming oxygen as the final electron acceptor. Although OXPHOS supports a higher yield of ATP per molecule of glucose, induction of glycolytic metabolism despite the presence of oxygen (i.e., the Warburg effect) is observed in many cell types. We and others have shown that TLR-mediated DC activation results in a striking Warburg-like metabolic shift to glycolysis, which is necessary to support their pro-inflammatory phenotype. The immediate increase in glycolytic activity in TLR-activated DCs was found to be required for de novo fatty acid synthesis to expand the endoplasmic reticulum and Golgi to accommodate de novo fatty acid synthesis, as well as expression and cytokine secretion compared to LPS-stimulated DCs. LPS-stimulated DCs are not capable of intracellular fatty acid synthesis to expand the endoplasmic reticulum and Golgi to accommodate de novo fatty acid synthesis, as well as expression and cytokine secretion.

In TLR-activated DCs, by measuring the extracellular acidification rate (ECAR), a measure of glycolysis, we found that glycolytic metabolism is also necessary for CCR7 oligomerization, which is essential for efficient DC migration. These findings reveal that glycolysis induction occurs independent of acquisition of a pro-inflammatory phenotype and supports the migratory capacity of DCs.

Results
Differentially activated DCs display distinct bioenergetic profiles. Stimulation of DCs with strong TLR agonists such as LPS promotes metabolic reprogramming to glycolysis with concomitant loss of ATP-coupled mitochondrial respiration. To assess whether this is a universal effect of PRR stimulation, we examined the bioenergetic profiles of DCs stimulated with a range of activators that engage different PRRs (Table 1), resulting in different inflammatory phenotypes. We measured surface expression of the co-stimulatory molecules CD80, CD86, CD40, and major histocompatibility complex (MHC) II (Fig. 1a, Supplementary Fig. 1a), as well as the production of cytokines IL-12p40, TNF-α, IL-6, and IL-10 (Fig. 1b, Supplementary Fig. 1b) following stimulation with these activators. Strong activators (i.e., LPS, Zym, and curdlan) induced much greater surface marker expression and cytokine secretion compared to “weak” activators (i.e., HDM and Zym depleted of its TLR ligands (ZymD)).

To assess metabolic reprogramming induced by these activators, we examined glucose consumption and lactate production in DCs 18 h following stimulation with either strong or weak DC activators. All DC activators stimulated increased glucose consumption and lactate production relative to resting DCs, with strong DC activators generally displaying higher levels of glucose metabolism than weaker activators (Fig. 1c). We next used Seahorse profiling, measuring the extracellular acidification rate (ECAR, a measure of glycolysis) and oxygen consumption rate (OCR, Fig. 1e), to determine the bioenergetic profiles of differentially activated DCs. Consistent with previously published reports, resting DCs engaged both OXPHOS and glycolysis, while LPS-treated DCs no longer displayed ATP-coupled mitochondrial respiration and exclusively used glycolysis for ATP production (Fig. 1d, f, g). Similar to LPS stimulation.

Table 1 DC activators

| Activator           | PRRs engaged | Concentration |
|---------------------|--------------|---------------|
| Lipopolysaccharide  | TLR4         | 10 or 100 ng/mL |
| LPS                 | TLR2/4, Dectin-2 | 50 μg/mL |
| House dust mite     | TLR2/4, Dectin-2 | 25 or 50 μg/mL |
| (HDM)               |              |               |
| Curdian             | Dectin-1     | 5 or 10 μg/mL  |
| Zymosan             | TLR2/6, Dectin-1 | 10 or 25 μg/mL |
| Zymosan-depleted     | Dectin-1     | 10 or 25 μg/mL |
| (ZymD)              |              |               |
Fig. 1 Bioenergetic profiles of differentially activated DCs. a, b Activation marker expression (CD80, CD86, CD40, and MHC II) and cytokine production (IL-12p40, TNF-α, IL-6, and IL-10) were measured following 18 h activation with indicated activators. c Glucose consumption (left) and lactate increase compared to the unstimulated condition (right) were measured 18 h following stimulation. d, f–h ECAR and OCR were measured using a Seahorse Bioanalyzer following an 18 h stimulation. e Coupled respiration is represented by the decrease in OCR from baseline after oligomycin treatment, and is therefore the OCR attributed to ATP production. Spare respiratory capacity (SRC) is represented by the increase in OCR from baseline after FCCP treatment. f Coupled respiration as a percentage of total respiration. g Mean coupled respiration versus mean ECAR. h SRC. Data shown in a-c are from one experiment representative of at least three independent experiments (mean and s.d. of triplicates (a, c) or duplicates (b)). Data shown in d, f–h are from three to seven experiments (depending on condition) (mean and s.e.m.). LPS: lipopolysaccharide, HDM: house dust mite, ZymD: zymosan depleted of TLR ligands, Zym: zymosan. ECAR: extracellular acidification rate, OCR: oxygen consumption rate. Data were analyzed using t-test, comparing each condition relative to the control. **p < 0.001, *p < 0.01, *p < 0.05
with the highly pro-inflammatory activators curdlan and Zym increased ECAR and decreased coupled respiration (Fig. 1d, f). Interestingly, weak DCs agonists (HDM and ZymD) induced slight but consistent increases in ECAR while retaining coupled respiration (Fig. 1d, f).

The difference in maximal mitochondrial respiratory capacity, measured after fluoro-carbonyl cyanide phenylhydrazone (FCCP) treatment, and basal respiration is known as the spare respiratory capacity (SRC) (Fig. 1e, h). DCs stimulated with strong activators (LPS, curdlan, and Zym) most often displayed negligible SRC, although in some experiments DCs stimulated with curdlan and Zym retained SRC, while the SRC of weakly activated DCs was consistent with literature demonstrating that Zym be largely non-mitochondrial (Supplementary Fig. 2), which is consumption observed with these stimuli was determined to be largely non-mitochondrial (Supplementary Fig. 2), which is consistent with literature demonstrating that Zym stimulates NADPH oxidase activity and the consequent production of reactive oxygen species3, 18. The serine/threonine protein kinase AKT is known to be required for glycolytic activity in TLR-activated DCs14. Analysis of phospho-AKT (Ser473) levels following stimulation for 1 h revealed increased, albeit variable, AKT activation in differentially activated DCs regardless of inflammatory stimulus (Fig. 2b). Thus, the downregulation of OXPHOS observed in strongly stimulated DCs is a long-term adaptation to PRR stimulation, while AKT activation and increased glycolysis is an early event following activation and is independent of a pro-inflammatory phenotype.

To further characterize glucose metabolism following DC activation, we investigated the fate of 13C-labeled glucose in activated DCs using stable isotope tracer analysis (Fig. 2c)19. DCs were activated with LPS, HDM, or Zym for 2 h and pulsed with U-[13C]-glucose for an additional 2 h (Fig. 2c). Zym- and LPS-stimulated DCs displayed hallmark profiles of Warburg metabolism, characterized by increased conversion of 13C-glucose to 13C-pyruvate and 13C-lactate (Fig. 2c). Consistent with their bioenergetic profile (Fig. 1), HDM-stimulated DCs displayed increased 13C-lactate production from 13C-glucose, although at lower levels than observed in LPS- or Zym-stimulated DCs (Fig. 2c). Moderate but consistent increases in 13C-glucose-derived TCA cycle intermediates were observed in activated DCs (Fig. 2c), suggesting evidence of glucose conversion to acetyl-CoA. Of note

**Early glycolytic flux is a hallmark of both weak and strong DC activators.** Given that both strongly and weakly activated DCs increase glycolysis, regardless of their pro-inflammatory nature, we monitored the bioenergetic states (ECAR and OCR) of differentially activated DCs in real-time following PRR stimulation. All differentially activated DCs, including those weakly activated by HDM or ZymD, displayed increased ECAR immediately after stimulation (Fig. 2a, left). Short-term activation did not change the OCR of differentially activated DCs, except those stimulated with Zym and ZymD (Fig. 2a, right). The increase in oxygen consumption observed with these stimuli was determined to be largely non-mitochondrial (Supplementary Fig. 2), which is consistent with literature demonstrating that Zym stimulates NADPH oxidase activity and the consequent production of reactive oxygen species3, 18. The serine/threonine protein kinase AKT is known to be required for glycolytic activity in TLR-activated DCs14. Analysis of phospho-AKT (Ser473) levels following stimulation for 1 h revealed increased, albeit variable, AKT activation in differentially activated DCs regardless of inflammatory stimulus (Fig. 2b). Thus, the downregulation of OXPHOS observed in strongly stimulated DCs is a long-term adaptation to PRR stimulation, while AKT activation and increased glycolysis is an early event following activation and is independent of a pro-inflammatory phenotype.

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was an increased abundance of unlabeled α-ketoglutarate and succinate in strongly activated DCs (Fig. 2c), suggesting an alternative carbon source is contributing to the increased production of these metabolites. DCs also converted a significant amount (~30%) of 13C-glucose to alanine, indicating that glucose-derived pyruvate has several metabolic fates—lactate, alanine, and acetyl-CoA—which are all produced independent of pro-inflammatory phenotype.

**Loss of coupled respiration directly correlates with iNOS expression and NO production.** Increased glycolytic metabolism in LPS-stimulated DCs has been reported to be required for energy production due to the inhibition of mitochondrial respiration by NO15. To determine whether loss of mitochondrial metabolism also correlates with NO production in differentially activated DCs, iNOS expression and NO production were measured in DCs activated by various stimuli. iNOS expression increased in a dose-dependent manner for curdlan and Zym (levels plateaued after 10 µg/mL for Zym) (Fig. 3a), with corresponding increases in nitrite levels (Fig. 3b). In these strongly activated DCs, the increase in iNOS expression and NO production correlated with the loss of coupled respiration (Fig. 3c). Inhibition of mitochondrial oxidative metabolism was further reflected by the little to no SRC remaining in the strongly activated DCs (Fig. 3d). No appreciable levels of iNOS or NO were induced by HDM or ZymD stimulation of DCs (Fig. 3a, b), and both coupled respiration and SRC were maintained in these activated DCs (Fig. 3c, d). Together, these data show that iNOS expression and NO production correlates with the loss of coupled respiration in differentially stimulated DCs and that an early increase in glycolysis can occur regardless of whether iNOS is later induced.

**Weakly inflammatory DCs lack long-term glycolytic reprogramming.** Metabolic reprogramming in immune cells is reinforced by changes in expression of key metabolic pathway genes that underlie changes at the biochemical level. To assess the impact of different TLR agonists on DC metabolic reprogramming, we examined the kinetics of gene expression of key enzymes in glycolysis following activation. During early stages of DC activation (4 h post stimulation), minor changes in the expression of Glut1 and Hk2 were observed, but expression of distal components of glycolysis (Pkm2, Gapdh, and Ldha) were unchanged (Fig. 4a, left). However, at late time points (18 h post stimulation), DCs activated with pro-inflammatory stimuli (LPS, curdlan, and Zym) showed increased expression of glycolytic genes, whereas DCs stimulated with "weak" activators HDM and ZymD showed little or no change in the expression of these enzymes relative to resting DCs (Fig. 4a, right). These results suggest that increased early glycolysis induced by stimulation occurs independent of large-scale changes in metabolic gene expression. Metabolic reprogramming at the level of transcription occurs following long-term DC activation in strongly pro-inflammatory DCs, but not in weakly activated DCs.

The TBK-IKKα signaling pathways have been identified as key kinases important for early induction of glycolysis in DCs14. We found that early activation of TBK, AKT, and mTORC1 occurred regardless of the activation phenotype (Fig. 4b). However long-term activation of both mTORC1 (measured by phospho-S6K and phospho-S6 levels) and mTORC2 (measured by phospho-AKT Ser473 levels) was associated only with the presence of a pro-inflammatory phenotype (Fig. 4b). Analysis of the time course revealed that AKT and mTORC1 activity was progressively lost in HDM-stimulated DCs around 8–12 h following activation (Fig. 4c). Early glycolysis induction by HDM, like LPS, could be prevented by AKT inhibition (Supplementary Fig. 3). Interestingly, TBK phosphorylation levels were maintained in HDM-stimulated cells (Fig. 4c). Stable HIF-1α protein expression was observed in LPS-stimulated DCs between 8–18 h post activation, but absent in HDM-stimulated DCs at all time points (Fig. 4c). Consistent with the role of HIF-1α in glycolytic reprogramming, the absence of detectable HIF-1α protein levels in DCs stimulated with weak agonists correlated with the lack of significant long-term induction of HIF-1α-dependent glycolytic gene expression (Hk2 and Ldha)30 in these cells (Fig. 4a). Induction of iNOS was also absent in HDM-stimulated DCs (Fig. 4d). These data are consistent with evidence linking mTORC1 to long-term commitment to glycolysis by induction of iNOS and HIF-1α21.
Induction of glycolysis is required for DC motility and migration. Our results indicate that early glycolytic flux is necessary for an aspect of DC function beyond sustaining a pro-inflammatory phenotype. Activated DCs, regardless of activation phenotype, must migrate to the draining LN to stimulate immune responses. Therefore, we examined the impact of glucose availability on DC migration. Using live imaging via confocal microscopy, we determined the cell velocity and displacement (distance traveled) in the presence or absence of glucose in culture medium. Under glucose-limiting conditions, DCs exhibited dramatic reductions in motility, characterized by both reduced velocity and shorter overall distance traveled (Fig. 5a, Supplementary Movie 1). To ensure that reduced DC motility was not due to reduced cell viability, we cultured DCs in glucose-free medium and measured their motility upon the addition of glucose (10 mM). DCs immediately increased both velocity and displacement upon addition of glucose, displaying motility equal to that of DCs initially cultured in the presence of glucose (Fig. 5b, c, Supplementary Movie 2). Adding the glycolytic inhibitor 2-deoxyglucose (2-DG) significantly decreased DC velocity and displacement (Fig. 5c), implicating glycolysis in this process. Culturing DCs without glucose or with 2-DG also impacted DC morphology, stimulating the cells to become more round with retraction of dendrites (Fig. 5d, Supplementary Fig. 4). When glycolytic activity was reduced, either by glucose withdrawal or culture with 2-DG, DCs displayed metabolic compensation to mitochondrial OXPHOS (Fig. 5e), allowing DCs to maintain cellular ATP levels (Fig. 5f). These data demonstrate that DCs are capable of metabolic plasticity (shifting between glycolysis and OXPHOS) to maintain ATP production. However, increased OXPHOS is not sufficient to restore DC motility, and thus glycolysis is essential for DC motility. These data suggest that glycolysis is necessary for cytoskeletal changes that support cell shape and motility.

In vivo, upregulation of CCR7, the receptor for the chemokines CCL21 and CCL19, in response to activating stimuli is necessary for...
DC migration to the draining LN. We found that in vitro, glucose was essential for DC migration toward CCL21 (Fig. 6a). Glucose limitation had little effect on overall CCR7 expression on either resting or activated DC populations, however decreased CCR7 expression on CCR7⁺ DCs was observed (Fig. 6b, Supplementary Fig. 5). Oligomerization of CCR7 monomers enables efficient DC migration more so than expression levels, and is necessary to guide DCs to the draining LN²². By fluorescence resonance energy transfer (FRET), we determined that CCR7 oligomerization on DCs was significantly impaired under conditions of reduced glucose availability, both in the absence and presence of activating stimuli (Fig. 6c). Finally, we examined DC migration toward CCL21 by splenic DCs, and found that, like bone marrow-derived DCs (BMDCs), splenic DC migration toward CCL21 was reduced upon glucose limitation or when glycolysis was blocked by 2-DG (Fig. 6d).

To determine the impact of glycolysis on DC migration in vivo, we first examined the ability of differentially activated DCs injected in the footpad to migrate to draining LNs. Carboxyfluorescein succinimidyil ester (CFSE)-labeled DCs were left unstimulated or stimulated with either LPS or HDM, and then injected subcutaneously into the footpads of mice. The size of the draining LNs increased following injection of DCs compared to the phosphate-buffered saline (PBS)-injected control mice, but activated DCs migrated to a greater extent to the LN than unstimulated DCs (Fig. 6e). Notably, LPS and HDM promoted DC migration to a similar extent (Fig. 6e), indicating that migratory ability was not entirely dependent on a pro-inflammatory phenotype. HDM-activated DCs treated with 2-DG prior to injection displayed reduced uptake of HDM by the DCs (Supplementary Fig. 6).

Finally, to examine the migration of endogenous DCs in vivo, we administered HDM intranasally and monitored DC migration from the lung to the mediastinal LN²³. At the time of stimulation, animals were treated with or without intraperitoneal

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**Fig. 5** Glycolysis is required for DC motility and morphology. Images of DCs were captured over time using confocal microscopy. a Mean cell velocity (left) and cell displacement (right) of DCs in the presence or absence of 10 mM glucose after 4 h. b Mean cell velocity (left) and cell displacement (right) of DCs cultured in the absence of glucose following the addition of media with or without 10 mM glucose. c Mean cell velocity (left) and cell displacement (right) of DCs cultured in the presence of glucose after the addition of media with or without 10 mM 2-deoxyglucose (2-DG). d Roundness (4 × A/π × d²); top left) and aspect ratio (d/π; bottom left) of DCs cultured without glucose after the addition of media with or without 10 mM glucose, and roundness (top right) and aspect ratio (bottom right) of DCs cultured in the presence of glucose after the addition of media with or without 10 mM 2-DG. e Coupled respiration as a percentage of total respiration of DCs cultured without glucose and following the addition of glucose (left), and of DCs cultured in the presence of glucose and following the addition of 2-DG (right). f Fold change of ATP levels in DCs cultured with or without glucose relative to glucose condition. Data in a represent 164-210 cells. Data in b-d represent 179-268 cells from one experiment representative of two experiments. Data in e are three to five experiments (depending on condition) (mean and s.e.m.). Data in f are fold changes of three experiments (mean and s.e.m.). Data were analyzed using unpaired t-tests. ***p < 0.001, **p < 0.01, *p < 0.05.
administration of 2-DG\textsuperscript{24}. Administration of 2-DG during allergic inflammation induced by HDM did not significantly impair the migration and accumulation of total immune cells (Fig. 6g). However, consistent with our adoptive transfer experiment (Fig. 6f), 2-DG administration reduced the migration of endogenous CD11c\textsuperscript{+}MHCII\textsuperscript{high} DCs to the lung in response to HDM (Fig. 6g). Together these data indicate that induction of glycolysis in DCs in vivo is essential to support DC motility and CCR7 oligomerization necessary for DC migration, regardless of PRR stimulus and activation phenotype.

**Discussion**

Reprogramming of cellular metabolism following immune cell activation is integral to support their activation and function. The majority of studies examining cellular metabolism in DCs use TLR agonists that induce strongly pro-inflammatory activation states\textsuperscript{7,14,15,25}, which collapses mitochondrial respiration and promotes aerobic glycolysis. By examining DCs stimulated with differential TLR agonists, we demonstrate that the bioenergetic profile of DCs is far more heterogeneous than previously reported. While strong pro-inflammatory activators, such as LPS, Zym, and curdlan, collapse ATP-coupled mitochondrial respiration and promote a prominent switch to glycolysis, DCs activated with TLR agonists that promote alternative DC activation with low inflammatory potential retain mitochondrial respiration. Using \textsuperscript{13}C-glucose-tracing techniques, we demonstrate differential use of glucose-derived carbon in DCs stimulated with differential DC agonists, indicating diverse metabolic pathways usage by DCs depending on activation stimulus. The common metabolic link
between highly and weakly inflammatory DCs is the immediate induction of glycolysis. We demonstrate that glycolysis is required for DCs to efficiently migrate in vitro, to retain their characteristic dendritic morphology, and to home to secondary lymphoid organs in vivo. Mechanistically, CCR7 oligomerization, which is essential for efficient DC migration\textsuperscript{24}, was found to be highly dependent on glycolysis. Together our data implicate the early upregulation of glycolysis as a key mechanism supporting DC migration, in addition to supporting a pro-inflammatory phenotype of strongly activated DCs.

Previous work has established that inflammatory signals promote a metabolic shift to glycolysis in DCs\textsuperscript{7, 14}. Our data here indicate that induction of glycolysis is not limited to DCs that acquire a highly pro-inflammatory phenotype. DCs treated with HDM, which induce Th2 differentiation\textsuperscript{25}, did not exhibit a detectable inflammatory phenotype, but strongly induced the activation of TBK, AKT, and mTORC1/2, and showed an early increase in glycolysis (Figs. 2 and 4)\textsuperscript{14}. In contrast to LPS stimulation, HDM-activated DCs maintained OXPHOS and SRC, similar to unstimulated DCs. SRC is linked to the total mitochondrial capacity available to cells, and can be used under conditions of stress to produce energy and maintain cell viability.\textsuperscript{26} The lack of SRC in LPS-stimulated DCs limits their adaptability when glucose levels are limiting\textsuperscript{15}. Thus, while early glycolysis stimulated by all TLR agonists supports DC migration, the greater SRC in alternatively activated DCs argues that they can engage mitochondrial respiration for ATP production and are more metabolically flexible than pro-inflammatory DCs. In fact, in many experiments, Zym- and curdlan-activated DCs retained some coupled respiration while increasing glycolysis (Fig. 1c) suggesting that complete loss of OXPHOS may be unique to inflammatory DCs stimulated with certain agonists such as LPS. Of note, results obtained using ZymD were highly variable depending on the batch of ZymD. When ZymD was weakly activating, DCs acquired inflammatory and metabolic phenotypes similar to HDM-activated DCs; however, more stimulatory batches of ZymD sometimes led to a stronger pro-inflammatory phenotype with metabolic characteristics more similar to Zym-activated DCs.

Despite the fact that HDM induces no appreciable pro-inflammatory phenotype in DCs (as measured by upregulation of MHC, co-stimulatory molecules, and cytokine production), HDM stimulated the activation of TBK, AKT, and mTORC1/2. Whether these pathways regulate cell shape and motility in DCs beyond their involvement in glycolysis will be the subject of future study. Despite the activation of mTORC1 in HDM-activated DCs, we did not observe significant stabilization of HIF-1α or induction of iNOS. Interestingly, TBK phosphorylation levels were maintained in HDM-stimulated cells even after AKT and mTORC1/2 activity had subsided suggesting that TBK may have another role in HDM-activated DCs at later time points.

We confirm that long-term metabolic reprogramming is modulated in part by HIF-1α in pro-inflammatory DCs\textsuperscript{26, 27}. HIF-1α is stabilized under conditions of hypoxia and promotes expression of glycolytic genes to allow cells to generate more energy by glycolysis in the absence of oxygen. In several immune cell types, including DCs, HIF-1α can be stabilized by LPS stimulation even under normoxic conditions, consequently promoting aerobic glycolysis.\textsuperscript{28–30} Jantsch et al.\textsuperscript{25} demonstrated that HIF-1α regulates DC activation by LPS, and our results here demonstrate that HIF-1α protein also accumulates when DCs are stimulated by other strong activators like LPS, Zym, and curdlan. These results are in line with a recent report demonstrating that HIF-1α is responsible for sustained glycolytic reprogramming in DCs.\textsuperscript{27} Weakly activated DCs, such as those stimulated by HDM or ZymD, did not significantly increase HIF-1α protein expression, in agreement with the lack of glycolytic reprogramming we observed in these DCs.

The induction of NOS triggered by TLR agonists and subsequent NO production is known to inhibit mitochondrial respiration\textsuperscript{15}, and NO promotes HIF-1α stabilization under normoxic conditions\textsuperscript{21, 31}. We show here that the expression of iNOS and production of NO induced by a variety of DC activators, including those that engage C1Rs, is directly correlated with the loss of coupled respiration. Pro-inflammatory DCs reduced coupled respiration in a dose-dependent manner, while DCs stimulated by weak activators HDM and ZymD did not appreciably increase iNOS or NO levels even at higher doses, thus maintaining coupled respiration. Therefore, we hypothesize that NO simultaneously inhibits mitochondrial function and stabilizes HIF-1α, reducing mitochondrial metabolism and promoting glycolysis, respectively. The differential usage of these metabolic pathways in DCs was dependent on the strength of activation, which was based on the nature and dose of the particular stimuli. Long-term metabolic programming at the transcriptional level stimulated by strong agonists likely depends on the presence of NO. DCs that do not express iNOS have been shown to similarly alter their metabolic activity in the presence of exogenous NO, which can be derived from other cells such as inflammatory macrophages\textsuperscript{17, 21}, indicating that environmental factors beyond TLR agonists can help shape DC metabolism.

Here we show that glycolysis is important for the elongated shape of DCs and their ability to migrate. Migration is a crucial aspect of DC function; DCs that encounter foreign antigen must migrate to the draining LN to alert the adaptive immune system regardless of the strength or type of activation. One of our key observations is that mitochondrial bioenergetics is not sufficient to support these cellular features, but rather early activation of glycolysis is essential. Following activation, DCs upregulate CCR7, the receptor for CCL19 and CCL21, which enables them to be guided to the LNs by CCL21 produced by lymphatic endothelial cells. Recently, CCR7 oligomerization has been shown to be essential for efficient DC migration by forming a signaling hub to optimize signal transduction\textsuperscript{22}. We found that CCR7 oligomerization was specifically dependent on glucose availability in DCs. Accordingly, blocking glycolysis was sufficient to disrupt optimal DC migration to the draining LN. However, this defect in migration is not solely due to CCR7 oligomerization but also due to a defect in motility. In vitro, we observed that DCs possess a rounded morphology in the absence of glucose, while adopting a more dynamic and elongated phenotype when glucose is present, implying strongly that glycolysis may play a crucial role in cytoskeletal remodeling.

In human prostate and breast cancer cell lines, cell migration is exploited to facilitate metastasis, and increased glycolytic activity has been shown to be associated with greater cytoskeletal rearrangements and therefore cell motility\textsuperscript{32}. Interestingly, the ATP generated from OXPHOS was not sufficient to support cell shape and migration. A possible explanation for this requirement for glucose-derived ATP is that local ATP is used for actin polymerization at specific areas of the cytoskeleton such as for the formation of actin-rich structures lamellipodia, filopodia, and podosomes. This compartmentalization of glycolytic activity allows for rapid ATP production locally at the cytoskeleton rather than throughout the cell for the energetically demanding remodeling processes\textsuperscript{33}. Vessel branching via migrating endothelial cells has been shown to be regulated by glycolysis, with glycolytic activator PFKFB3 playing a major role\textsuperscript{34}. PFKFB3 was found to be enriched in lamellipodia, promoting local glycolytic activity to generate ATP at these actin-rich structures\textsuperscript{34}. In addition to bioenergetic requirements, another mechanism for glycolytic control of cytoskeletal remodeling involves the association of the glycolytic enzyme aldolase to the cytoskeleton. A recent study describes the regulation of glycolysis by
phosphoinosside 3-kinase, which activates the GTPase RAC to mobilize the cytoskeleton, and consequently releasing the actin-bound aldolase. Our data show that without glycolysis, there is little motility of the cytoskeleton, suggesting a feed-forward loop may exist where glycolytic activity is needed for cytoskeletal reorganization necessary to release aldolase to further increase glycolysis. Additional work will be necessary to reveal how glycolysis can support the cytoarchitecture and motility of DCs.

Inhibiting glucose availability may also alter signaling pathways in DCs that affect their migratory responses. AMPK, a cellular energy sensor that is activated in response to increased AMP:ATP or ADP:ATP ratios, can antagonize mTORC1 activity. mTORC1 inhibition can affect cytoskeletal dynamics by preventing the protein expression of small GTPases. mTORC2 also regulates cytoskeletal reorganization via protein kinase C activity. Therefore, altered AMPK and/or mTOR activity could contribute to the observed defects in cell shape, motility, and migration following glucose deprivation or 2-DG treatment. Additionally, since in vivo administration of 2-DG can affect multiple cell types, it is possible that defects in other cells are contributing to the migratory defect observed in these experiments.

Defined metabolic pathways are increasingly being shown to direct specific cellular functions. Previously, induction of glycolysis was thought to primarily support the bioenergetic and catabolic functions. Previously, induction of glycolysis with DC migratory capacity, limiting their ability to migrate to lymph nodes may exist where glycolytic activity is needed for cytoskeletal reorganization. Our data suggest that nutrient competition demonstrative of nutrient competition may exist where glycolytic activity is needed for cytoskeletal reorganization.

Methods

Mice. Female wild-type C57BL/6 mice or BALB/c were purchased from Charles River Laboratories at 6–8 weeks of age (Montreal, QC, Canada). Animals were maintained in a specific pathogen-free environment. All experiments were conducted following the guidelines of the Canadian Council of Animal Care, as approved by the McGill University Animal Care Committee. Animals were randomly assigned to the different treatment groups. Investigators were not blinded.

In vitro BMDC generation and stimulation. Bone marrow was extracted from C57BL/6 mice in the presence of 20 ng/mL granulocyte-macrophage colony-stimulating factor (PeproTech) in “complete DC medium” (CDCM) containing RPMI-1640 (Corning) medium with 10% fetal calf serum (FCS) (Hyclone), 2 mM L-glutamine, 100 µM β-mercaptoethanol (Gibco). After 8–10 days, DCs were collected, seeded at 2 million cells/mL, and stimulated with indicated LPS (O111:B4 Sigma-Aldrich), HD (low endotoxin from Greer Laboratories, curdlin (InvivoGen), Zym (InvivoGen), or Zym-depleted (InvivoGen). HD was labeled with Alexa Fluor 647 in indicated experiments with the Alexa Fluor 647 Protein Labeling Kit (Invitrogen). Glucose withdrawal experiments used glucose-free RPMI-1640 (Wisent) supplemented with 10% dialyzed FCS (Wisent), 2 mM L-glutamine, 100 µM penicillin-streptomycin, and 0.01% β-mercaptoethanol.

Splenic DC isolation. Flt3L-expressing B16 cells were subcutaneously injected into C57BL/6 mice (3.5 × 10^5 cells/mouse) in order to expand the DC population. After approximately 2 weeks, the tumor-bearing mice were sacrificed and the spleens were harvested for DCs. Spleens were digested with 1 mg/mL collagenase and 10 µg/mL Dnase I (Roche), and red blood cells lysed by ACK lysis buffer. Splenic DCs were purified using the Pan DC Isolation Kit (Miltenyi), according to the manufacturer’s protocol.

Seahorse assay. An XF-96 Extracellular Flux Analyzer (Seahorse Bioscience) was used to analyze real-time changes of the ECAR and OCR. DCs were seeded in XF-96 cell culture plates at 7.5 × 10^4 cells/well in “Seahorse medium”, and the stimuli were injected into the wells by the XF analyzer.

Metabolite analysis. Nova Bioanalyzer Supernatant was collected from BMDCs stimulated for 18 h, and spun down to remove any cells and debris. Supernatants were then run on the Nova Bioanalyzer to measure the levels of various metabolites, including lactate and glucose.

Gas chromatography-mass spectrometry analysis of 13C metabolites: Gas chromatography-mass spectrometry (GC-MS) metabolite analysis was conducted as previously described. Briefly, 5 × 10^6 BMDCs were cultured with indicated activators in CDM for 2 h. Following activation, the medium was changed to glucose-free RPMI (with 10% dialyzed FCS, Wisent) containing 11 mM U-L-[13C6]-glucose (Cambridge Isotope Laboratories) for 2 h. Metabolites were extracted from cells using dry ice-cold 80% methanol, followed by sonication and removal of cellular debris by centrifugation at 4 °C. Metabolite extracts were dried, derivatized as tert-butylmethylsilyl esters, and analyzed via GC-MS as previously described. Uniformly deuterated myristic acid (750 ng/sample) was added as an internal standard following cellular metabolites extraction, and metabolite abundance was expressed relative to the internal standard and normalized to cell number. Mass isotopomer distribution was determined using a custom algorithm developed at McGill University.

Liquid chromatography-mass spectrometry analysis of nucleotides: Nucleotide levels were determined by liquid chromatography-mass spectrometry (LC-MS), as previously described. BMDCs were cultured with or without glucose for 4 h, and then washed with cold 150 mM ammonium formate solution pH 7.4 and extracted with 600 µL of 31.6% MeOH/36.3% acetonitrile (ACN in H2O (v/v)). Cells were lysed and homogenized by bead-beating for 2 min at 30 Hz using a 5 mm metal bead (Tissuelyser II—Quagen). Cellular extracts were partitioned into aqueous and organic layers following dimethyl chloride treatment and centrifugation. Aqueous supernatants were dried by vacuum centrifugation with sample temperature maintained at −4 °C (Labconco, Kansas City, MO, USA). Pellets were subsequently resuspended in 25 µL of H2O as the injection buffer. For semiquantitative targeted metabolite analysis of mono-, di-, and tri-phosphate nucleoside, samples were injected onto an Agilent 6430 Triple Quadrupole (Agilent Technologies, Santa Clara, CA, USA). Chromatography was achieved using a 1290 Infinity ultra-performance LC system (Agilent Technologies) consisting of vacuum degasser, autosampler, and a binary pump. Separation was performed on a Schermo SM-C18 column (3 μm, 3 × 150 mm) (Intakt Corp, JAPAN) maintained at 10 °C. The chromatographic gradient started at 100% mobile phase A (5 mM ammonium acetate in water) with a 5 min gradient to 100% B (200 mM ammonium acetate in 20% ACN/80% water) at a flow rate of 0.4 μL/min. This was followed by a 5 min hold time at 100% mobile phase B and a subsequent re-equilibration time (6 min) before next injection. A sample volume of 5 μL of sample was injected for analysis. Sample temperature was maintained at 4 °C before injection.

The mass spectrometer was equipped with an electrospray ionization source and samples were analyzed in positive mode. Multiple reaction monitoring (MRM) transitions were optimized on standards for each metabolite quantitated. Transitions for qualifier and quantifier ions are described in Table 2 below. Gas chromatography-mass spectrometry analysis of13C metabolites: Gas chromatography-mass spectrometry (GC-MS) metabolite analysis was conducted as previously described. Briefly, 5 × 10^6 BMDCs were cultured with indicated activators in CDM for 2 h. Following activation, the medium was changed to glucose-free RPMI (with 10% dialyzed FCS, Wisent) containing 11 mM U-L-[13C6]-glucose (Cambridge Isotope Laboratories) for 2 h. Metabolites were extracted from cells using dry ice-cold 80% methanol, followed by sonication and removal of cellular debris by centrifugation at 4 °C. Metabolite extracts were dried, derivatized as tert-butylmethylsilyl esters, and analyzed via GC-MS as previously described. Uniformly deuterated myristic acid (750 ng/sample) was added as an internal standard following cellular metabolites extraction, and metabolite abundance was expressed relative to the internal standard and normalized to cell number. Mass isotopomer distribution was determined using a custom algorithm developed at McGill University.

| Table 2 Agilent 6430 Triple Quadrupole MRM transitions of nucleosides |
|-----------------------------|-----------------------------|-----------------------------|
| Compound | Precursor ion (m/z) | Quantifier ion (m/z) | Qualifier ion (m/z) |
|-----------------------------|-----------------------------|-----------------------------|
| ATP | 508.0 | 136.0 | 410.1 |
| ADP | 428.0 | 136.0 | 348.1 |
| AMP | 348.0 | 136.0 | 118.9 |
| CTP | 484.0 | 112.1 | 97.1 |
| GDP | 404.0 | 112.0 | 69.0 |
| CMP | 324.0 | 112.0 | 69.0 |
| TTP | 483.0 | 81.1 | 53.0 |
| TDP | 403.0 | 81.1 | 207.0 |
| TMP | 323.0 | 207.1 | 126.9 |
| GDP | 524.0 | 152.0 | 134.9 |
| GDP | 444.0 | 152.0 | 97.0 |
| GMP | 364.0 | 152.0 | 97.0 |
| ITP | 509.0 | 152.0 | 97.0 |
| IDP | 429.0 | 152.0 | 97.0 |
| IMP | 349.0 | 152.0 | 110.0 |
| UDP | 485.0 | 114.0 | 110.0 |
| GDP | 405.0 | 97.0 | 69.2 |
| UDP | 325.0 | 97.0 | 113.0 |
Relative gene expression was normalized to that of hypoxanthine-guanine phosphoribosyltransferase (HPRT).

| Table 3 Sequences of primers for quantitative RT-PCR |  |
|---|---|
| Gene | Sequence |
| Glut1 | Forward: CTGGACCTCAACACTTTAGTGGG <br> Reverse: GGGGTGCTGTCTACCTTTGCTGG |
| Hk2 | Forward: CCGTGTTGGGAAAGAAGTGAAGAC <br> Reverse: GGACAGCTCAGATCCGAGCAGC |
| Ptkm2 | Forward: GGTACGCAAGAGAAAGGATAC <br> Reverse: GCTGGGTCTGAATGAAGGCAGTC |
| Gapdh | Forward: GTCTGGTGAAAGGATTT <br> Reverse: TAGATCCCTGACAGATTACAGCA |
| Ldh | Forward: TGTCTGCCTGAGATAGCTACTGT <br> Reverse: GAGTGTTCTGACAAGTTGGGA |

4 × 10^5 cells/insert. CCL21 (250 ng/mL) in media with or without glucose was added to the bottom chambers. Cells were allowed to migrate for 2 h at 37 °C. Cells that migrated onto the bottom chambers and cells remaining in the insert were collected and counted on the BD LSR Fortessa using counting beads (123count eBeads, eBioscience). Results are expressed as the percentage of cells that migrated of total cells.

In vivo migration experiments. Footpad injection: BMDCs were stained with CFSE and stimulated for 6 h with the indicated activators. After 6 h, cells were collected and resuspend in PBS at 20 × 10^6 cells/mL. In all, 5 × 10^5 (25 μL) cells were injected subcutaneously into each rear footpad of C57BL/6 mice. After 48 h, the draining popliteal LNs were isolated (popliteal LNs from each mouse were pooled) and digested with DNase 1 (10 μg/mL) and collagenase D (1 mg/mL). CFSE⁺ cells in the dLNs were detected by flow cytometry and cells were counted using 123count eBeads (eBioscience).

HDM-induced allergic asthma model: To investigate the effects of 2-DC on DC activation and migration, female Balb/c (7–8 weeks) were briefly anaesthetized with isoflurane and treated intranasally with 40 μg of low-endotoxin HDM (LE-HDM: Greer) in a volume of 30 μL. Mice were injected intraperitoneally with 500 mg/kg body weight 2-DC or control saline in a volume of 200 μL daily, starting 1 day prior to LE-HDM delivery. Mice were sacrificed 72 h after application of LE-HDM, and mediastinal LNs were collected for flow cytometry analysis. CD11c⁺ MHCII⁺ cells were gated on viable CD45⁺ cells from which cells expressing CD19, CD3ε, and F4/80 were excluded.

Fluorescence resonance energy transfer. CCR7 oligomerization was determined by FRET. BMDCs were stimulated in the presence or absence of glucose (10 mM) or 2-DC (10 mM) for 6 h. Cells were stained for 45 min with phycoerythrin (PE)-labeled CCR7 and allophycocyanin (APC)-labeled CCR7 (clone 4B12; eBioscience) at a 1:200 dilution in the presence of Fc block at 37 °C. Samples were washed and acquired on the BD LSR Fortessa without compensation. FRET was calculated as previously described⁴⁴, using the following formula:

\[
\text{FRET unit} = \frac{(E_{365} - E_{330})}{(E_{365} - E_{330}) \times (E_{260} - E_{260})}
\]

where E1 is the fluorescence detected at 580 nm upon excitation at 561 nm, E2 is the fluorescence detected at 670 nm upon excitation at 640 nm, and E3 is the fluorescence detected at 670 nm upon excitation at 641 nm. The positive population percentage was used for E stained with neither PE nor APC (Enone), PE only (EPE), APC only (EAPC), or both PE and APC (EAPC). SEPAPC.

Statistical analysis. Data were analyzed using GraphPad Prism software (version 6). An unpaired Student’s t-test was performed to determine statistical significance between two samples and significance was considered to be when p values were < 0.05. Stars without lines indicate significance when compared to control. A one-way analysis of variance was performed to determine statistical significance between multiple groups.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files.

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