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Graphical abstract

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Enhanced glycolytic metabolism supports transmigration of brain-infiltrating macrophages in multiple sclerosis

Deepak Kumar Kaushik,1,2 Anindita Bhattacharya,1,2 Reza Mirzaei,1,2 Khalil S. Rawji,1,2 Younghee Ahn,3,4 Jong M. Rho,3,4,5 and V. Wee Yong1,2

1Hotchkiss Brain Institute, 2Department of Clinical Neurosciences, 3Alberta Children’s Hospital Research Institute, 4Department of Pediatrics, and 5Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada.

Introduction

Multiple sclerosis (MS) is a demyelinating and neurodegenerative condition associated with the migration of activated lymphocytes and macrophages into the CNS. These leukocytes enter the white matter by crossing the blood-brain barrier (BBB) in areas of inflammation called the perivascular cuffs (1–3), as well as through the CNS-meningeal barrier (4, 5). The accumulation and activation of leukocytes in areas of infiltration initiates a cascade of events leading to the loss of BBB integrity, which further aids the transmigration of leukocytes into the brain parenchyma (3, 6, 7). Recent studies highlight the existence of a metabolic switch within leukocytes that intricately regulates their activation states by changing their metabolic profiles. For example, proinflammatory macrophages and T cells rely heavily on glycolysis, with a strong resemblance to the metabolic states of tumor cells (8, 9). In contrast, antiinflammatory macrophage subsets predominantly utilize oxidative phosphorylation for their functions (9, 10). In this regard, the metabolic requirement of leukocytes and its relevance to MS pathogenesis are not well understood.

During glycolysis, a molecule of glucose generates 2 molecules of pyruvate, which enters the TCA cycle (oxidative phosphorylation) or is reduced under anaerobic conditions to lactate. Generation of lactate from pyruvate is referred to as anaerobic glycolysis or the Warburg phenomenon, which is a well-known feature of tumor cells. The generation of lactate from pyruvate is driven by lactate dehydrogenase (LDH), which works at near-equilibrium thermodynamics (11). LDH functions as a tetramer of LDHA and LDHB subunits. While LDHB favors lactate-to-pyruvate conversion, LDHA is a rate-limiting enzyme with higher affinity for pyruvate, which it converts to lactate. This step is crucial for recycling of NAD+, a coenzyme important for the functioning of glyceraldehyde 3-phosphate dehydrogenase, another glycolytic enzyme. LDHA is strongly associated with aerobic glycolysis and is implicated in the initiation and maintenance of tumor cells (8, 12). It is crucial in the production of lactate, a monocarboxylic acid, that has the capacity to modulate innate and adaptive immune responses (13–15). Thus, interference with lactate machinery presents an attractive opportunity to treat immune-mediated disorders. Since LDH activity is not critical for cells for glucose oxidation to CO2 through the TCA cycle, interfering with its activity should specifically target cells that resort to aerobic glycolysis to meet their metabolic demands and spare nonproliferative/nonlactate-generating cells (16).

The migration of leukocytes into the CNS drives the neuropathology of multiple sclerosis (MS). It is likely that this penetration utilizes energy resources that remain to be defined. Using the experimental autoimmune encephalomyelitis (EAE) model of MS, we determined that macrophages within the perivascular cuff of postcapillary venules are highly glycolytic, as manifested by strong expression of lactate dehydrogenase A (LDHA), which converts pyruvate to lactate. These macrophages expressed prominent levels of monocarboxylate transporter-4 (MCT-4), which is specialized in the secretion of lactate from glycolytic cells. The functional relevance of glycolysis was confirmed by siRNA-mediated knockdown of LDHA and MCT-4, which decreased lactate secretion and macrophage transmigration. MCT-4 was in turn regulated by EMMPRIN (also known as CD147), as determined through coexpression and co-IP studies and siRNA-mediated EMMPRIN silencing. The functional relevance of MCT-4–EMMPRIN interaction was confirmed by lower macrophage transmigration in culture using the MCT-4 inhibitor α-cyano-4-hydroxy-cinnamic acid (CHCA), a cinnamon derivative. CHCA also reduced leukocyte infiltration and the clinical severity of EAE. Relevance to MS was corroborated by the strong expression of MCT-4, EMMPRIN, and LDHA in perivascular macrophages in MS brains. These results detail the metabolism of macrophages for transmigration from perivascular cuffs into the CNS parenchyma and identify CHCA and diet as potential modulators of neuroinflammation in MS.
Monocarboxylate transporters (MCTs) play a crucial role in the sustained production of lactate by transporting excess lactate across cell membranes; MCTs such as MCT-1 and MCT-4, which have a low affinity for lactate, are enriched in cells with high rates of glycolysis (17, 18). Studies in cancer cells suggest that MCTs rely on their chaperone extracellular matrix metalloproteinase inducer (EMMPRIN, also known as basigin and CD147) (19, 20) for their localization on plasma membranes (21). We have previously described EMMPRIN as an important mediator in the transmigration of leukocytes across the perivascular cuff in MS and its inflammatory model, experimental autoimmune encephalomyelitis (EAE) (1, 2, 22). Since glycolytic reprogramming favors migration in leukocytes (23), we tested the hypothesis that macrophages rely on EMMPRIN–MCT-4 interaction to meet their metabolic demands in order to cross the BBB into the CNS parenchyma. In particular, we have investigated the relevance of LDHA and the mechanisms of energy utilization in perivascular cuffs in EAE and MS. Moreover, we have evaluated whether perturbation of energy utilization inhibits the transmigration of macrophages, the predominant leukocyte population in perivascular cuffs. Our results

Figure 1. Perivascular cuffs in EAE cerebellum and cervical spinal cord harbor LDHA-expressing leukocytes. (A) Low-magnification image shows a lateral section of an EAE mouse cerebellum (arrows point to perivascular cuffs) along with enlarged images of a perivascular cuff labeled with pan-laminin (green) and the pan-leukocyte marker CD45 (red). Scale bars: 50 μm. (B) Expression of LDHA and CD45 in a low-magnification cerebellar section of EAE depicts detectable LDHA expression exclusively in CD45⁺ cells within the white matter. Scale bar: 100 μm. (C) Representative images of individual cuffs (demarcated by a dotted line) at higher magnification (scale bars: 50 μm) show the expression of LDHA in CD45⁺ cells in D16 EAE cerebella and spinal cords. Images are representative of 8 mice from 3 independent experiments. 3D reconstruction using Imaris confirmed the presence of LDHA within the CD45⁺ cells in the inflammatory cuffs (scale bars: 20 μm). (D) Representative images show LDHA expression within F4/80⁺ macrophages; images are representative of 8 mice from 3 independent experiments (scale bar: 50 μm), with a corresponding 3D reconstruction of this image (scale bar: 20 μm). Insets in C and D show magnified cells (original magnification of insets, ×120). (E) Percentage of LDHA⁺CD45⁺ cells within the perivascular cuffs of D16 EAE mice (peak EAE; n = 3 mice; 2 sections per mouse were analyzed). (F) Lactate levels measured in spinal cord homogenates from 3 D16 EAE mice and 3 naive mice using the L-lactate assay kit. Graphs show the mean ± SD. Data were compared using a 2-tailed Student’s t-test. *P < 0.05.
describe mechanisms of inflammation in EAE and MS and highlight glycolytic macrophages and MCT-4–EMMPRIN interactions that are amenable to perturbation, including by dietary factors, in order to alleviate signs of EAE in mice.

Results

Macrophages within the perivascular cuffs in EAE mice have high expression of LDHA. In the inflamed postcapillary venules of EAE-afflicted mice, leukocytes that have migrated out of the vessel lumen across the endothelial cell layer accumulate within the perivascular space between the endothelial and parenchymal basement membranes (2, 3); the collective structure is referred to as a perivascular cuff (Figure 1A). Previous studies have emphasized that leukocytes within the perivascular space have remarkable functions, including antigen presentation (6) and secretion of MMPs (24), and these actions probably have high metabolic demands. In support of this, we observed that the expression of LDHA, a key glycolytic enzyme involved in the conversion of pyruvate to lactate, was strongly upregulated in the perivascular cuff on day 16 (D16, a period of prominent clinical disability) in the white matter of the cerebellum, a CNS region where perivascular cuffs are clearly demarcated in EAE (Figure 1B). Higher-magnification micrographs showed that LDHA in perivascular cuffs was expressed by a majority of leukocytes and other cells, probably reactive astrocytes, in the cerebellar white matter (Figure 1C, upper panel) as well as in the spinal cord (Figure 1C, lower panel). We noted that the leukocytes within the parenchyma were largely devoid of LDHA staining, suggesting that these cells may have different metabolic requirements after they have invaded the parenchyma. A 3D rendering using the image reconstruction software Imaris confirmed the expression of LDHA in more than 60% of CD45+ cells within the perivascular cuffs (Figure 1, C and E). Further, staining with the F4/80 macrophage marker revealed that macrophages within cuffs were highly immune reactive for LDHA (Figure 1D). When we examined lactate, a functional measure of LDHA activity, EAE spinal cord lysates had significantly higher lactate levels compared with levels in control lysates (Figure 1F).

Inflammatory macrophages exhibit stabilization of HIF-1α (25), a master regulator of LDHA and thus glycolysis. We therefore confirmed the previously published findings on HIF-1α expression in EAE (26) in the perivascular cuffs of EAE cerebellum and spinal cords (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI124012DS1). The cytoplasmic as well as nuclear expression of HIF-1α was confirmed with 3D reconstruction and colocalization using Imaris software (Supplemental Figure 1B). It is worth mentioning that the role of HIF-1α in mediating glycolytic reprogramming in macrophages is not well understood. Although a recently published study by Le Moan et al. refuted its involvement in driving the EAE disease course (26), mice with knocked out HIF-1α (23) in myeloid cells showed impaired macrophage migration. This suggests important yet unexplained roles for HIF-1α in EAE pathogenesis.

In EAE, CNS infiltrates may manifest first as subpial and then as parenchymal perivascular cuffs (5). To study the relevance of LDHA expression in a time-dependent manner, we examined leukocytes during the onset of clinical signs of EAE (D10) and focused on the meninges, since the formation of perivascular cuffs in white matter is not consistent at this time point. We found that more than 50% of leukocytes expressed LDHA within the meninges on D10 in myelin oligodendrocyte glycoprotein–immunized (MOG-immunized) mice as compared with expression in mice subjected to CFA control, which had fewer leukocytes in the subpial spaces, where only about 10% of the leukocytes expressed LDHA (Supplemental Figure 2, A and C). Once EAE was established, more than 70% of leukocytes expressed LDHA in perivascular cuffs, even at a later time point of post-peak disease severity (D21; Supplemental Figure 2, B and D).

LDHA activity is important for the proinflammatory phenotype of macrophages. Proliferation and differentiation are key features of activated leukocytes during inflammation. Since leukocyte proliferation can lead to lactate accumulation in the extracellular compartment (27), we examined the abundance of proliferative leukocytes within cuffs. According to the literature, approximately 20% of leukocytes proliferate in the subarachnoid space (28) of meninges in EAE-afflicted mice, whereas fewer proliferate within the parenchyma (29). Consistent with these findings, we observed that a modest 10%-15% of CD45+ cells were weakly positive for the proliferation marker Ki67 in perivascular cuffs (Supplemental Figure 3A) in comparison with CD45+ leukocytes in meninges, where approximately 25% to 30% of these cells had proliferated (Supplemental Figure 3B). Since proliferation is halted in macrophages during inflammation, which implicates HIF-1α-mediated enhancement of glycolysis (30), the nonproliferative macrophages that utilize glycolysis may bear proinflammatory traits.

We tested whether LDHA modulates proinflammatory activities and aids leukocyte infiltration across the BBB. We resorted to macrophages in culture, as these cells typically constitute more than 80% of leukocytes within perivascular cuffs at peak EAE (24). When bone marrow–derived macrophages (BMDMs) were exposed to a nontoxic (20 μM; Figure 2A) concentration of a small-molecule–specific LDHA inhibitor, 3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propynaphthalene-1-carboxylic acid (FX11) (12, 31), we detected a significant decrease in secreted lactate levels as compared with LPS-treated cells (Figure 2B), indicating a decrease in LDHA function. Intracellular lactate accumulation and/or its conversion to pyruvate can regulate glycolysis (11); therefore, when we measured glycolysis by the extracellular acidification rate (ECAR) in FX11-treated, LPS-stimulated cells, we found a significant decrease in both glycolysis (measured by the generation of lactate upon glucose addition) and glycolytic capacity (the maximum capacity of lactate generation upon inhibition of oxidative phosphorylation) in these BMDMs (Figure 2, C and D). Importantly, inhibiting LDHA activity reduced the migratory capacity of macrophages across a Boyden chamber model of the BBB in culture (Figure 2, E and F). Glycolysis feeds anabolic pathways such as the pentose phosphate pathway, which provides necessary precursors for the production of proinflammatory cytokines, and therefore we assessed TNF-α expression upon treatment with FX11. We found a significant decrease in the production of TNF-α upon FX11 treatment in LPS-activated cells (Figure 2G). Further, we tested whether the inhibitory effects of FX11 on macrophage migration were mediated by chemotactic signals. Indeed, FX11 decreased the production of MCP-1 (also known as CCL2) and IP-10 (also known as CXCL10) chemokines in macrophages...
mice (Figure 3, A, B, and D). As with LDHA, we observed that several leukocytes in the parenchyma did not have detectable MCT-4 expression, suggesting a switch in metabolic programming in these cells upon infiltration into the parenchyma. A 3D Imaris rendering confirmed the expression of MCT-4 on CD45+ leukocytes including in F4/80+ macrophages (Figure 3, B and C).

As seen with LDHA, MCT-4 was expressed in meningeal leukocytes as early as D10 (Supplemental Figure 4A), with more than 50% of the leukocytes found positive for MCT-4 as compared with CFA control leukocytes (Supplemental Figure 4C). Similarly, more than 60% of leukocytes expressed MCT-4 in post-peak D21 perivascular cuffs (Supplemental Figure 4B), suggesting that these cells express this lactate transporter even at later stages of the disease. Notably, we also detected MCT-4 expression in reac-
tive astrocytes abutting the perivascular cuffs in the EAE cerebellum (Supplemental Figure 5A). For a thorough analysis, we also assessed the expression of MCT-1 in CD45+ leukocytes and reactive astrocytes in perivascular cuffs, which suggested the presence of MCT-1 in astrocytes as well as in a small subset of leukocytes (Supplemental Figure 5B).

In order to corroborate the significance of LDHA and establish a role for MCT-4 in aiding proinflammatory activities in BMDMs, we performed siRNA-mediated knockdown of LDHA and MCT-4 in BMDMs (Figure 4, A and C). Notably, knockdown of MCT-4 and, expectedly, LDHA, resulted in decreased LDHA expression in BMDMs treated with LPS (Figure 4, B, D, and E). Further, knockdown of LDHA and MCT-4 significantly reduced lactate levels in LPS-treated BMDM supernatant (Figure 4F). We then sought to study the relevance of LDHA and MCT-4 in mediating proinflammatory activities in knockdown cells (Figure 4G). We found a modest yet significant reduction in TNF-α production after 6 hours of LPS treatment in LDHA- and MCT-4–knockdown cells (Figure 4G). However, this reduction was not observed after 24 hours of stimulation (data not shown), possibly due to a lack of a complete reduction of either gene. Also, the transmigration assay confirmed that migration of LDHA- and MCT-4–knockdown BMDMs was significantly reduced when compared with scRNA-transfected cells in response to LPS treatment (Figure 4H).

EMMPRIN, a molecular chaperone for MCT-4, governs proinflammatory functions in macrophages. We addressed the mechanisms by which MCT-4 is regulated in leukocytes; specifically, we evaluated EMMPRIN, as it is known to chaperone MCTs in cancer cells (21). We found EMMPRIN to be strongly associated with MCT-4 in cells within perivascular cuffs (Figure 5A). Western blot analysis of EMMPRIN in EAE spinal cords revealed elevated expression of various glycosylated forms of this protein, ranging from 40 kDa to more than 80 kDa (Figure 5B). To further assess EMMPRIN–MCT-4 interactions in both inflammatory and noninflammatory conditions, we treated macrophages with LPS and found that MCT-4 increased within 24 and 48 hours of treatment (Supplemental Figure 6A). Using co-IP with MCT-4 antibody to “pull” for interacting partners, we found that an approximately 50-kDa form of EMMPRIN...
PRIN interacted with MCT-4 in LPS-treated as well as untreated macrophages (Figure 5C), indicating that the 2 proteins interact in macrophages during both their resting and activated states.

To further our understanding of the functions of EMMPRIN, we performed siRNA-mediated knockdown of EMMPRIN in macrophages and isolated their membrane fractions. Upon knockdown of EMMPRIN (Figure 5D), MCT-4 expression on membranes was significantly reduced (Figure 5E). This was also supported by immunofluorescence staining of MCT-4 on BMDMs, which primarily localized on the plasma membrane, suggesting that knockdown of EMMPRIN as well as LDHA and MCT-4 significantly reduced membrane MCT-4 expression levels in BMDMs (Supplemental Figure 6B). These results suggest that EMMPRIN regulates the expression of MCT-4 in BMDMs by chaperoning it to macrophage membranes. Relevant to this observation, we found that an LPS-mediated increase in glycolysis and glycolytic capacity was significantly abrogated in EMMPRIN-knockdown cells (Figure 6A), as was also confirmed by decreased lactate secretion from LPS-stimulated BMDMs. EMMPRIN knockdown as well as LDHA and MCT-4 significantly reduced membrane MCT-4 expression levels in BMDMs and the migration of EMMPRIN-knockdown BMDMs was significantly retarded across the Boyden chamber (Figure 6E). These results highlight the critical role of the EMMPRIN-MCT-4 interaction in lactate metabolism during inflammation.

Inhibition of EMMPRIN-MCT-4 interaction reduces macrophage activation. In order to further interrogate the relevance of MCT-4 and its interaction with EMMPRIN in macrophages, we used α-cyano-4-hydroxy-cinnamic acid (CHCA), a reversible and relatively specific inhibitor of MCT-4, albeit through unknown mechanisms (33, 34). We determined that CHCA was not toxic to macrophages, even at 1 mM concentration (Figure 7A). Notably, 400 μM CHCA significantly reduced the secretion of lactate in activated macrophages (Figure 7B). Further, CHCA-treated macrophages had a reduced ECAR (Figure 7C), in which both the glycolysis and glycolytic capacity of BMDMs were reduced (Figure 7D, E, and F). These observations were similar to the effects observed with either EMMPRIN knockdown or FX11-mediated LDHA inhibition described earlier. It is known that knockdown of MCT-4 reduces glycolytic enzymes in macrophages (34). Also, as described earlier, siRNA-mediated knockdown of MCT-4 produced a decrease in the expression of LDHA and secretion of lactate in BMDMs (Figure 4, E and F). In this regard, we observed that LPS-induced expression of EMMPRIN, LDHA, and HIF-1α in BMDMs was reduced in the presence of CHCA (Figure 7C), possibly due to a feedback inhibition by accumulation of lactate intracellularly. Since no changes were observed in MCT-4 interaction with
the 50-kDa EMMPRIN in untreated and LPS-treated macrophages (Figure 5C), we studied the higher glycosylated forms of EMMPRIN, which govern proinflammatory functions of macrophages (35). Using a different EMMPRIN antibody, we found that, whereas the 42-kDa EMMPRIN form did not interact with MCT-4, a higher glycosylated EMMPRIN (~50–56 kDa) associated with MCT-4 (Figure 7G). However, no significant differences were observed in response to either LPS or CHCA conditions in the approximately 50-kDa EMMPRIN form. Nonetheless, another glycosylated form of EMMPRIN (>100 kDa) increased upon LPS activation and was reduced with CHCA treatment (Figure 7, G and H). These results suggest that high-molecular-weight glycosylated forms of EMMPRIN may play a crucial role in guiding MCT-4 to macrophage membranes. These findings also show that functional MCT-4 inhibition reduced EMMPRIN–MCT-4 interaction, which could also be a reflection of decreased EMMPRIN expression upon treatment with CHCA.

Finally, we studied the consequence of pharmacological perturbation of MCT-4 function by evaluating the transmigration of macrophages in the Boyden chamber model of the BBB. We found that the elevated transmigration of macrophages upon LPS activation was significantly retarded by CHCA treatment (Figure 7, I and J), as was observed with MCT-4 knockdown in BMDMs (Figure 4H), further supporting the hypothesis that an intact lactate machinery, regulated by EMMPRIN–MCT-4 interaction, is crucial for inflammatory activities of macrophages.

CHCA treatment decreases disease severity in EAE mice. Mice immunized for EAE were injected i.p. daily with 25 mg/kg (data not shown) or 50 mg/kg CHCA from D4 after immunization (Figure 8A) while asymptomatic. We noted that the weight drop in EAE mice during severe clinical disability (D14–D20) was prevented by CHCA (data not shown). While mice in the vehicle- and CHCA-treated groups succumbed at comparable rates to EAE between D10 and D12, the subsequent clinical disability was dampened by CHCA compared with vehicle (Figure 8B). When vehicle-treated mice had an average maximum score of 2.5 on D12 (limp tail and paresis of hind limbs and forelimbs) on a 5-point scale, CHCA-treated mice had a score of 1 (limp tail only).

We analyzed the formation of cuffs in spinal cords on D16 and found a significant reduction in the number of cuffs in CHCA-treated mice as compared with vehicle-treated animals.

**P < 0.01.
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spinal cords is affected (Supplemental Figure 8). We found that infiltration of both CD3+ T cells and F4/80+ myeloid cells was significantly reduced in CHCA-treated spinal cords compared with vehicle-treated controls (Supplemental Figure 8, A and B), suggesting that CHCA, either directly or indirectly via its effect on chemokine production by macrophages, may affect T cell migration into EAE spinal cords. Further, when we analyzed the levels of LDHA on either CD45+Ly6G–CD11b+ monocytes (Supplemental Figure 8C) or CD3+ T cells (Supplemental Figure 8E) in blood from D16 EAE mice, we found that the expression of LDHA was significantly elevated on monocytes (Supplemental Figure 8D), but not on T cells (Supplemental Figure 8F), when compared with the naive condition.

To assess the direct effects of CHCA on T cells, we treated either the polyclonally activated splenocytes (Supplemental Figure 9, A–D) or the Th1 (Supplemental Figure 9, E and F) or Th17-polarized cells (Supplemental Figure 9, G–H) in vitro. We found that CHCA did not affect the proliferation of splenocytes (Supplemental Figure 9B). However, it significantly decreased IFN-γ+T–bet+ Th1 cells in splenocytes (Supplemental Figure 9C), but increased the Th1 polarization in pure T cell cultures (Supplemental Figure 9, E and F), without affecting the polarization of Th17 cells in either polarization paradigm (Supplemental Figure 9, D, G, and H). This further reinforces the observation that the presence of other modulatory cells, probably monocytes in splenocytes, may significantly affect pathogenic Th1–polarized cells in the presence of CHCA in vivo. However, further experiments are needed to assess the effects of CHCA on T cell polarization in vivo.

Figure 6. EMMPRIN knockdown results in decreased glycolysis and reduced proinflammatory functions in macrophages. (A) ECAR measurement for cells transfected with EMMPRIN siRNA. Graphs depict glycolysis and glycolytic capacity in EMMPRIN-knockdown cells. Final values were normalized to micrograms of protein (n = 3). (B) Graph depicts lactate levels in supernatants from EMMPRIN-knockdown cells either in the presence or absence of LPS. (C) Flow plot and representative graph show LDHA MFI in EMMPRIN-knockdown cells. (D) TNF-α ELISA and (E) transmigration assay suggested a relevance for EMMPRIN in regulating these aspects of BMDM inflammation. All assays were performed in triplicate or quadruplicate for 3 independent experiments unless otherwise indicated. All graphs show the mean ± SD. Means were compared by 1-way ANOVA with Tukey’s post hoc test. **P < 0.01.
Inflammatory lesions within MS brains harbor leukocytes with glycolytic traits. We assessed the relevance of the EAE results by examining autopsied brains from deceased subjects with MS. At the sites of perivascular cuffs (Figure 9A) containing CD68+ macrophages (Figure 9) or CD45+ leukocytes (Supplemental Figure 10), we could clearly detect LDHA, MCT-4, and HIF-1α, corresponding with CD68+ macrophages (Figure 9, B–D) or CD45+ leukocytes (Supplemental Figures 10 and 11). EMMPRIN-expressing cells were immunoreactive for MCT-4 in perivascular cuffs (Figure 9E), corroborating the EAE data indicating the important role of EMMPRIN in the maintenance of MCT-4 levels in infiltrating leukocytes. To assess whether the age of the MS subjects determined the glycolytic profile of leukocytes in perivascular cuffs, we analyzed LDHA expression in the brains of 4 MS subjects of different ages (39–82 years; Supplemental Figure 11, A–D). Notably, we found that approximately 70% of the leukocytes within the active cuffs of all the brains analyzed were LDHA+ (Supplemental Figure 11E), suggesting that infiltration into active lesions has high metabolic demands, irrespec-
myelin-reactive T cells are considered to be the major contributor to MS pathology and depend on aerobic glycolysis for their differentiation (37), macrophages constitute the predominant population of leukocytes at perivascular cuffs with the potential to aid T cell migration and activation (24, 38). Recent evidence suggests that proinflammatory macrophages utilize glycolysis and not oxidative phosphorylation as a major source of energy (9). Indeed, we have now determined that macrophages within inflamed perivascular cuffs are enriched in MCT-4 and contribute to CNS infiltration of leukocytes and CNS pathology in EAE. (A) Schematic shows the timeline of CHCA injections in EAE mice. (B) Clinical disease scores for CHCA- or vehicle-treated EAE mice. Results are representative of 3 independent EAE experiments (from D16 and D21 EAE mice). Plot represents 2 similarly designed independent EAE experiments combined (D21 data sets). n = 17 mice per group, and the means of each group were compared using a 2-way ANOVA with Dunnett’s post hoc multiple comparisons test. Data represent the mean ± SEM. (C) Total number of perivascular cuffs counted across 2 sections of at least 6 EAE D16 spinal cords. (D) Images depict the distance transformation (Imaris) of representative perivascular cuffs and infiltrating CD45+ cells (spheres) in vehicle- or CHCA-treated D16 spinal cords. Cuffs were across 2 spinal cord sections of at least 3 mice analyzed. Scale bars: 20 μm. (E and F) Graphs show the (E) number of cells within 100 μm or (F) percentage of cells within 20 μm of the cuff. (G–I) Meningeal inflammation studied by immunofluorescence using laminin (green) and CD45 (red) markers or H&E staining. Insets depict a magnified inflamed region within the EAE spinal cords. Scale bars: 100 μm (G) and 75 μm (H); original magnification, ×20 (enlarged insets). (J) Flow cytometric plots of CD11b+Ly6G− monocytes (gated on CD45+ cells) in blood. (K) Significant increase in the proportion of monocytes in blood of CHCA-treated EAE mice; n = 7–8 mice per group. (L) Graph shows the overlay of LDHA in CD45+ leukocytes in the EAE and EAE plus CHCA treatment groups (n = 8–9 mice per group). (M) Percentage of LDHA+CD45+ cells and (N) MFI of LDHA in CD45+ cells. Graphs show the mean ± SD. Data were compared with a 2-tailed Student’s t test unless otherwise indicated. **P < 0.01.

Discussion

The perivascular cuffs of postcapillary venules are one of the major routes for leukocytes such as T cells and macrophages to enter the CNS during EAE and MS pathogenesis. Although...
It is noteworthy that the leukocytes that have migrated into the parenchyma seemed to be devoid of either LDHA or MCT-4 expression, suggesting that their metabolic needs may differ from those of leukocytes that accumulate in areas of cuffs. We also found a subset of leukocytes in the perivascular cuffs that expressed MCT-1 (Supplemental Figure 5B), which is another lactate exporter on macrophages. However, MCT-4 is suggested to be crucial for the secretion of large amounts of lactate from highly glycolytic cells (32, 33), particularly due to its lower affinity for lactate over MCT-1 (46). Furthermore, it is also understood that the switch to glycolysis by EMMPRIN involves MCT-4 and not MCT-1 (47). The expression of MCT-4 in both the EAE and MS brains affirms the importance of this transporter in regulating lactate levels in macrophages. Recently, neuroimaging using hyperpolarized 13C magnetic resonance spectroscopy in the cuprizone mouse model of MS demonstrated that areas rich in infiltrating macrophages had higher rates of lactate production (48), thus corroborating our findings of the importance of lactate metabolism for macrophage transmigration into the CNS. Knocking down MCT-4 has been described cular cuffs express high levels of LDHA, indicating the generation of lactate within these cells. Our studies using FX11 confirmed that LDHA activity is indeed important for the proinflammatory functions of macrophages. The Ldha gene bears hypoxia regulatory elements (HREs) in its promoter sequence (39, 40), suggesting that it is probably regulated by HIF-1α. This transcription factor in turn can also influence the expression of MCT-4 (41, 42), potentially by its effects on EMMPRIN (43), and it is intrinsically linked with metabolic reprogramming during inflammation (44). On this point, we found that the infiltrating leukocytes expressed HIF-1α along with MCT-4 and its ancillary protein, EMMPRIN. The stabilization of HIF-1α in autoimmune diseases has been attributed to mild hypoxia that is generated as a result of increased oxygen demand in the tissues locally (45). However, it is also noteworthy that a glycolytic shift occurs in the tissues even in normoxic conditions, an example of which is LPS-induced HIF-1α stabilization (inflammatory hypoxia) without apparent hypoxia. Therefore, the relevance of hypoxia-induced HIF-1α stabilization to the underlying glycolytic shift in macrophages requires further investigation.

Figure 9. Infiltrating macrophages in active MS cuffs express LDHA and other glycolytic markers including HIF-1α and MCT-4 and EMMPRIN. (A) Low-magnification micrographs of an active lesion of an MS brain; 2 different active lesions per MS brain were analyzed. Arrows in the merged image point to perivascular cuffs, one of which is represented with laminin (green) and CD45 (red). The merged image identifies different regions: EBM, endothelial basement membrane; PBM, parenchymal basement membrane; PS, perivascular space within a cuff (arrows). (B–D) CD68 macrophages (red) expressing (B) LDHA (green), (C) HIF-1α (green), and (D) MCT-4 (green) within an active MS lesion. (E) EMMPRIN is colocalized with MCT-4 in one such active lesion. Scale bars: 50 μm.
in the literature to reduce glycolysis via reduction of hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 in activated macrophages (34). In addition, we found that MCT-4 knockdown resulted in a reduction of LDHA expression, and thus lactate generation, in LPS-stimulated BMDMs. This impaired the production of TNF-α and migration of BMDMs in vitro, the key proinflammatory features of macrophages.

Our current observations implicate EMMPRIN as a chaperone to MCT-4 within macrophages that enter the perivascular cuffs of EAE and MS brains. Moreover, the EMMPRIN/MCT-4 axis plays a crucial role in the maintenance of LDHA-dependent lactate production in inflammatory macrophages. In support of these observations, we found that genetic knockdown of MCT-4 as well as functional inhibition of MCT-4 with CHCA reduced the migratory function of macrophages, and this was underscored by decreased EMMPRIN–MCT-4 interaction. The molecular forms of EMMPRIN range from 27 kDa (unglycosylated protein) to higher than 80 kDa, depending on the complexity of glycosylation (49, 50). Notably, we observed changes in several glycosylated forms of EMMPRIN (up to 80 kDa) in EAE spinal cords; enrichment of protein fractions from macrophages upon co-IP yielded other relevant glycosylated EMMPRIN forms. Our findings suggest that while a glycosylated form of EMMPRIN of approximately 50 kDa interacted with MCT-4, a higher glycosylated form of EMMPRIN (>100 kDa; likely a multimeric form of glycosylated EMMPRIN) was particularly sensitive to the inflammatory status of macrophages. The observations that knockdown of EMMPRIN exerted effects similar to those of functional inhibition of MCT-4 on decreased glycolytic functions and impaired TNF-α production as well as BMDM migration in activated macrophages suggest the importance of EMMPRIN in maintaining key biological functions, much like its effects in other cell types. Indeed, EMMPRIN-null mice have reduced survival rates (51) and impaired sensory and memory functions (52, 53), suggesting the crucial role of EMMPRIN in the maintenance of homeostasis.

Diet plays an important role in the outcome of inflammatory disorders (54, 55). It is noteworthy that cinnamon acid is a major ingredient of cinnamon and turmeric, which are major dietary components in several parts of the world. Both cinnamon and turmeric are widely used as antiinflammatory agents in the form of flavoring agents and spices (56, 57). Of relevance to MS, ground cinnamon and cinnamon derivatives may influence MS outcomes.

Given that blocking MCT-4 functions on macrophages alleviated their activation and modulated the severity of EAE disease, interfering with the glycolytic functions is an attractive therapy to treat inflammatory conditions such as MS. A recent study by Kornberg et al. (64) showed that the MS drug dimethyl fumarate and its metabolite monomethyl fumarate act by inactivating the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase in peritoneal macrophages and CD4+ T cells in both mice and humans. Similarly, glatiramer acetate (Copaxone) modulates immune infiltration in part by decreasing glycolysis within the T cells of patients with relapsing-remitting MS (RRMS) (65). Thus, targeting glycolysis in immune subsets may represent a viable therapy to treat MS. However, it is important to keep in mind the role lactate may serve centrally during an ongoing pathology, as the lactate produced by CNS cells such as astrocytes and oligodendrocytes can serve as an additional energy source for neurons and oligodendrocytes during stress. Therefore, further validation and studies examining the feasibility of long-term lactate/glycolysis targeting are necessary to unravel the true benefits of this approach.
Methods

EAE induction. Eight- to ten-week-old C57BL/6 female mice (Charles River Laboratories) were immunized s.c. with 50 μg/100 μL MOG 35–55 peptide in CFA supplemented with 4 mg/mL heat-inactivated Mycobacterium tuberculosis H37Ra (Thermo Fisher Scientific); 50 μL emulsion was deposited on either side of the tail base. Pertussis toxin (PTX) (300 ng/200 μL; 180, List Biological Laboratories) was injected i.p. on days 0 and 2 after MOG immunization. CHCA (C2020, MilliporeSigma) was dissolved in PBS, and the pH was adjusted to approximately 7.4 with 1N NaOH and administered i.p. (in 100-μL volumes) starting on D4 after MOG immunization. CHCA was given once daily at a dose of 25 or 50 μg/kg until the experiment was terminated. A similar volume of PBS was administered i.p. as control injections. Three EAE experiments were conducted independently. Daily monitoring of EAE mice was performed, and the mice were scored on a scale of 0 to 5.

Tissue harvesting and flow cytometry. Peripheral monocytes from EAE and healthy mice that were anesthetized with a lethal dose of ketamine and xylazine were harvested from whole blood isolated by cardiac puncture. Approximately 0.5 mL heparinized whole blood was collected from each mouse, and RBC lysis was carried out using RBC lysis solution (555899, BD Biosciences) before proceeding with flow cytometry.

For the myelin removal protocol, myelin from spinal cord homogenates was isolated using a Percoll gradient as described earlier (I). Flow cytometry was performed using fluorescence-conjugated antibodies against CD45-PerCP (553310, BD Biosciences, 1:50); LDHA monoclonal antibody (AF14A11; MA5-17247, Thermo Fisher Scientific, 1:50); or anti–MCT-4 antibody (sc-50329; Santa Cruz Biotechnology) or EMMPRIN siRNA (sc-35299, Santa Cruz Biotechnology) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. Transfection was carried out for at least 36 hours before membrane isolation or measurement of ECAR activity and for at least 48 to 72 hours for measurement of lactate.

BMDM isolation and treatment. BM from 6- to 8-week-old C57Bl/6 mice was harvested by flushing the femur and tibia, and the cells were seeded at a density of 10^6 cells per bacterial Petri dish and grown in DMEM in the presence of 10% L929 conditioned supernatant and 10% FBS along with 2% penicillin-streptomycin for 1 week as described previously (66). The BMDMs were treated with 100 ng/mL LPS in the presence or absence of 200 or 400 μM CHCA and 20 μM FX11 (427218, MilliporeSigma). Control cells were treated with a similar volume of DMSO.

For knockdown studies, BMDMs were transfected with either 50 nM scrambled RNA (sc-37007); LDHA siRNA (ID 156092, Thermo Fisher Scientific); MCT-4 siRNA (ID 174958, Thermo Fisher Scientific); or EMMPRIN siRNA (sc-35299, Santa Cruz Biotechnology) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. Transfection was carried out for at least 36 hours before membrane isolation or measurement of ECAR activity and for at least 48 to 72 hours for measurement of lactate.
Western blot analysis. The BMDM lysates (30 μg per lane) and both the unbound fractions and the eluate from co-IP were loaded onto a 10% polyacrylamide gel and transferred onto PVDF membranes. The membranes were then blocked with either 5% milk or Starblock T20 buffer (Thermo Fisher Scientific; for co-IP blots) for 1 hour and then incubated with the following antibodies overnight at 4°C: rabbit anti-EMMPRIN (42–58 kDa, ab108317, Abcam, 1:1000); rat anti-EMMPRIN (50 kDa, OXI14, MCA2283, 1:500, Bio-Rad Laboratories); rabbit anti–MCT-4 (SC-50329, Santa Cruz Biotechnology, 1:500); rabbit anti–HIF-1α (NB100-134, Novus Biologicals, 1:1000); anti-rabbit LDHA (NBPI-48336, Novus Biologicals, 1:1000); anti-rabbit–MCT-4 (clone OX114, MCA2283, Bio-Rad Laboratories, 1:30); rabbit anti–HIF-1α (NB100-134, Novus Biologicals, 1:100); mouse anti–human CD68 (V1/824, 556059, BD Pharmingen, 1:100); rabbit anti–MCT-1 (ab90582, Abcam, 1:100); anti-F4/80 (MCA497EL; BD Biosciences); rabbit anti–MCT-4 (sc-50329, Santa Cruz Biotechnology, 1:500); and α rabbit anti–MCT-4 (SC-50329, Santa Cruz Biotechnology, 1:500); rabbit anti-EMMPRIN (42–58 kDa, ab108317, Abcam, 1:1000); rat anti-EMMPRIN (SC-50329, Santa Cruz Biotechnology, 1:500); rabbit anti–HIF-1α (NB100-134, Novus Biologicals, 1:1000); anti-rabbit LDHA (NBPI-48336, Novus Biologicals, 1:1000); anti-rabbit–MCT-4 (sc-50329, Santa Cruz Biotechnology, 1:500); and rabbit anti–HIF-1α (NB100-134, Novus Biologicals, 1:100); mouse anti–human CD68 (V1/824, 556059, BD Pharmingen, 1:100); anti–CD3 (ab11089, Abcam, 1:100); anti-F4/80 (MCA497EL; BD Biosciences); rabbit anti–GAPDH (G3893, MilliporeSigma, 1:100); mouse anti–human CD68 (V1/824, 556059, BD Pharmingen, 1:100); mouse anti–human CD68 (V1/824, 556059, BD Pharmingen, 1:100); anti–CD3 (ab11089, Abcam, 1:100); anti–F4/80 (MCA497EL; BD Biosciences); rabbit anti–GAPDH (G3893, MilliporeSigma, 1:100); or mouse anti–MCT-1 (ab90582, Abcam, 1:100) antibodies overnight at 4°C. After additional washes, sections were incubated with Alexa Fluor 488– and Alexa Fluor 546–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, 1:500). The nuclei were stained with nuclear yellow (Hoechst), and MS brains were incubated for an additional 30 seconds with Sudan black dye to quench signals from endogenous lipofuscin granules. Images were captured on a Fluoview FV10i confocal microscope (Olympus).

3D reconstruction of the Z-stacks from static confocal images was performed using Imaris 3D reconstruction software (Bitplane), in which surfaces were created for each channel and then a merged surface was reconstructed. For colocalization analysis, a colocalization channel was created by setting thresholds on the merged images, and the percentage of colocalization between different channels was reported.

Real-time PCR. BMDMs were homogenized in 1 mL TRIzol Reagent (Thermo Fisher Scientific), and RNA was isolated using the RNeasy Mini Kit (74104, Qiagen) following the manufacturer’s instructions. Real-time PCR (RT-PCR) was performed on the QuantStudio 6 Flex (Applied Biosystems) using Fast SYBR Green (Thermo Fisher Scientific), transcripts were analyzed by the ΔΔCt method, and the data were normalized to Gapdh or Actb. Primers for Gapdh (Qiagen ref. QTO16568692; NCBI RefSeq: NM_008084); Ldha (Qiagen ref. QT02325414; NCBI RefSeq: NM_010699); Mct-4 (Qiagen ref. QT000116039; NCBI RefSeq: NM_030696); and Actb (Qiagen ref. QT00095242; NCBI RefSeq: NM_007393) were obtained from Qiagen. The primer sequences for EMMPRIN (basigin isoform 2; NCBI RefSeq: NM_001077184.1) were as follows: forward, 5′-GCGGCGGGGACCTATCCAAACG-3′; reverse, 5′-ATGCTATTCTGTATGACGTCG-3′.

Live/dead assay and ImageXpress. BMDMs were seeded in a 96-well plate at a density of 50,000 cells per well in a black-bottomed plate (BD Biosciences). After BMDMs were treated for 24 hours with 100 ng/mL LPS, either in the presence or absence of different concentrations of CHCA and FX11, 20 μL of a mixture containing nuclear yellow dye (NucBlue, Thermo Fisher Scientific), 5 μM calcein AM (Invitrogen, Thermo Fisher Scientific), and 5 μg/mL propidium iodide (PI) (MiliiporeSigma) was added directly to the media for 20 minutes. Images of calcein AM– (green; live cells) and PI– cells (red; dead cells) were captured using the live image module of ImageXpress (Molecular Devices), and the percentage of live and dead cells was calculated using MetaXpress acquisition and analysis software (Molecular Devices). MCT-4–stained BMDMs were also captured using ImageXpress, and the fluorescence per cell was quantified using Fiji software (ImageJ, NIH).

Lactate measurement. BMDMs were treated or siRNA transfected in 0.5% FBS DMEM (without L929 supernatant supplementation and without sodium pyruvate), and 48 hours or 72 hours later, the BMDMs were treated with LPS for another 18 to 24 hours, followed by collection of the supernatant. Lactate from cell culture supernatant was measured using the fluorescence-based lactate measurement kit according to the manufacturer’s protocol (ab65330, Abcam). Similarly, lactate from spinal cord lysates was measured and normalized to the protein concentration. Briefly, cell culture supernatants (1:200 primary dilution) and spinal cord lysates (1:500 primary dilution) and the standards were incubated in the presence of the lactate probe and enzyme mix at room temperature for 30 minutes and measured at 535/587 nm excitation/emission. Lactate levels were calculated from the standard curve and are represented in millimolars.

Luminex assay and ELISA. TNF-α was measured from the supernatants of BMDMs conditioned with different treatments using an ELISA kit (BMS607-3, Thermo Fisher Scientific) according to the manufacturer’s protocol. For the Luminex assay, 50 μL cell culture supernatant was analyzed using the 31-Plex Mouse Cytochemistry Array (MD31, Eve Technologies). For inhibitor experiments, BMDMs were stimulated with 100 ng/mL LPS in the presence of inhibitors for 24 hours. However, for measurement of TNF-α in knockdown conditions, BMDMs were stimulated for 6 hours with 50 ng/mL LPS.

ECAR measurement. ECAR was determined using the Seahorse XF24 Flux Analyzer (Agilent Technologies). Briefly, the cells were seeded at a density of 2.5 × 10^4 per well the day before the assay and treated with LPS in the presence or absence of CHCA or FX11, or subjected to transfection. The media were changed to the XF assay media (Agilent Technologies) either after 12 hours (for CHCA) or 18 hours (for FX11 and siRNA transfection) of LPS treatment and then incubated with media for 20 minutes. Images of calcein AM + (green; live cells) and ImageXpress. BMDMs were also stained using ImageXpress, and the fluorescence per cell was quantified using Fiji software (ImageJ, NIH).
gomycin (Enzo Life Sciences), and 100 μM 2-deoxyglucose (2DG) (MilliporeSigma), in ports A, B and C, respectively. Seahorse Wave software was used to analyze the data, which were normalized to the amount of protein in micrograms. The parameter equations used for the calculation of glycolysis and glycolytic capacity were as follows: \[(\text{measurement before oligomycin injection}) - (\text{measurement after 2DG injection})\] and \[(\text{measurement after oligomycin}) - (\text{measurement after 2DG injection})\], respectively.

**Statistics.** All results are expressed as the mean ± SD unless otherwise stated. Statistical analysis was performed with GraphPad Prism 7.0 (GraphPad Software). Comparisons of 2 groups were done using a 2-tailed Student’s \(t\) test, and multiple means were compared using either a 1-way ANOVA with Tukey’s post hoc test or a 2-way ANOVA with Dunnett’s post hoc multiple comparisons test (EAE time course experiments). \(P\) values of less than 0.05 were considered significant.

**Study approval.** All experiments were conducted in accordance with guidelines of the Canadian Council on Animal Care and with ethics approval from the Animal Care Committee at the University of Calgary. All MS tissues were obtained and used with approval from the institutional ethics committee of the University of Calgary.

**Author contributions**

DKK and VWY conceived the study and drafted the manuscript. DKK performed experiments and analyzed data. AB, KSR, RM, and YA carried out experiments and helped with the editing of the manuscript. JMR provided resources for the Seahorse experiments and edited the manuscript. All the authors read and approved the final version of this manuscript.

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Address correspondence to: V. Wee Yong, University of Calgary, 3330 Hospital Drive, Calgary, Alberta T2N 4N1, Canada. Phone: 403.220.3544; Email: vyong@ucalgary.ca.