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Liver kinase B1 depletion from astrocytes worsens disease in a mouse model of multiple sclerosis

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Abstract

Liver kinase B1 (LKB1) is a ubiquitously expressed kinase involved in the regulation of cell metabolism, growth, and inflammatory activation. We previously reported that a single nucleotide polymorphism in the gene encoding LKB1 is a risk factor for multiple sclerosis (MS). Since astrocyte activation and metabolic function have important roles in regulating neuroinflammation and neuropathology, we examined the serine/threonine kinase LKB1 in astrocytes in a chronic experimental autoimmune encephalomyelitis mouse model of MS. To reduce LKB1, a heterozygous astrocyte-selective conditional knockout (het-cKO) model was used. While disease incidence was similar, disease severity was worsened in het-cKO mice. RNAseq analysis identified Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched in het-cKO mice relating to mitochondrial function, confirmed by alterations in mitochondrial complex proteins and reductions in mRNAs related to astrocyte metabolism. Enriched pathways included major histocompatibility class II genes, confirmed by increases in MHCII protein in spinal cord and cerebellum of het-cKO mice. We observed increased numbers of CD4⁺ Th17 cells and increased neuronal damage in spinal cords of het-cKO mice, associated with reduced expression of choline

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CONFLICT OF INTEREST

The authors declare that they have no competing conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

acetyltransferase, accumulation of immunoglobulin- γ , and reduced expression of factors involved in motor neuron survival. In vitro, LKB1-deficient astrocytes showed reduced metabolic function and increased inflammatory activation. These data suggest that metabolic dysfunction in astrocytes, in this case due to LKB1 deficiency, can exacerbate demyelinating disease by loss of metabolic support and increase in the inflammatory environment.

Keywords

astrocyte; EAE; liver kinase B1; mitochondria; motor neuron; multiple sclerosis

1 | INTRODUCTION

Liver kinase B1 (LKB1) is a serine–threonine kinase (STK11) initially identified as a tumor suppressor gene and commonly found mutated in several cancers including lung and cervical (Katajisto et al., 2007). LKB1 phosphorylates and activates multiple downstream kinases, and regulates metabolism, migration, polarity, and proliferation. LKB1 acts as a metabolic sensor and helps maintain ATP levels under conditions of increased activity, for example, during periods of proliferation or cytokine production (Alexander & Walker, 2011; Sebbagh, Olschwang, Santoni, & Borg, 2011). LKB1 has roles in T cell functions, since LKB1 depletion increases Th1/Th17 cytokine production (MacIver et al., 2011; Tamas et al., 2010) and reduces regulatory T cell (T reg) numbers (Chen et al., 2018). In the nervous system, neuronal LKB1 helps establish cell polarity (Mencarelli et al., 2018), enhances growth (Ohtake et al., 2019), and has roles in migration (Ryan et al., 2017) and myelination (Kuwako & Okano, 2018). In Schwann cells (SCs), depletion of LKB1 caused delayed peripheral myelination and neuropathy (Pooya et al., 2014; Shen et al., 2014) due to loss of SC polarity and reduced mitochondrial production of citrate needed for lipid synthesis.

The functions of LKB1 in astrocytes are not well known. As in other cells, LKB1 regulates metabolic stress (Mukherjee et al., 2008) and under ischemic conditions helps maintain energetic homeostasis (Liang et al., 2015). Astrocytic LKB1 has been implicated in induction of autophagy under stress conditions (Son, Kang, Choi, & Mook-Jung, 2015) which could help reduce energy requirements. Mutations in the C-terminus of LKB1 impair astrocyte migration, implicating a role in astrocyte polarity (Forcet et al., 2005). In both multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE), astrocytes carry out beneficial (production of anti-inflammatory factors, production of lactate as an energy supply, maintenance of homeostatic environment) and damaging (production of pro-inflammatory factors, reactive oxygen, and nitrogen species) functions. Whether LKB1 has roles in astrocyte functions during EAE or MS has not been examined.

We previously described a family where all five siblings were diagnosed with MS or clinically isolated syndrome, which led to identification of a single nucleotide polymorphism (SNP; dbSNP ID: rs9282860) in the STK11 gene (Boullerne et al., 2015), present at heterozygosity in all siblings. We showed that this SNP has higher prevalence in women with MS, with an odds ratio of 1.6 in relapsing remitting patients. The effects of the SNP on LKB1 expression have not yet been determined; however, it is located in a consensus

binding site for several transcription factors (TFs) which is removed by the SNP, suggesting the SNP can influence TF binding and transcription. In view of the roles that LKB1 has in regulating metabolism and inflammation, we hypothesized that deficiencies in astrocytic LKB1 could contribute to increased MS risk, and show that heterozygous conditional knockout (het-cKO) of LKB1 in adult mice exacerbates EAE, associated with reductions in metabolic function and increases in inflammatory responses.

2 | METHODS

2.1 | Animals

All animal studies were approved by both the UIC and the Jesse Brown VA Institutional Animal Care and Use Committees. LKB1^{lox/lox} mice on an FVB/n background (Bardeesy et al., 2002) contain loxP sequences flanking STK11 Exons 3–6 (Figure S1a). Experimental animals were generated by crossing LKB1^{lox/lox} mice with ALDH1L1-CreERT2^{+/-} (Cre^{+/-}) mice on a C57BL/6N background (Winchenbach et al., 2016), and the LKB1^{lox/+}Cre^{+/-} offspring bred to LKB1^{lox/lox}Cre^{-/-} mice to yield 4 genotypes: mice homozygous (LKB1^{lox/lox}) and heterozygous (LKB1^{lox/+}) for the loxP allele; and null (Cre^{-/-}) or heterozygous (Cre^{+/-}) for the Cre allele. The resulting mice contain 75% FVB/n and 25% C57BL/6N. PCR primers were as described (Gan et al., 2010). The loxP allele was detected using LoxPF: 5'-TCT AAC AAT GCG CTC ATC GTC ATC CTC GGC-3' and 39R: 5'-GAG ATG GGT ACC AGG AGT TGG GGC T-3' which yield a 300 bp product when the loxP is present (Figure S1b). The WT allele (lack of loxP allele) was detected using 36F: 5'-GGG CTT CCA CCT GGT GCC AGC CTG T-3' and 39R which yield a 220 bp product when the loxP is absent (Figure S1c); under some conditions these primers generate a 600 bp product from the loxP allele. Cre-mediated deletion was determined using 36F and 37R: 5'-GAT GGA GGA CCT CTT GGC CGG CTC A-3' which yield a 310 bp product when Exons 3–6 are deleted (Figure S1d). PCR primers for ALDH1L1-CreERT2 allele were forward 5'-CAA CTC AGT CAC CCT GTG CTC-3' and reverse 5'-TTC TTG CGA ACC TCA TCA CTC G-3' which yield a 590 bp product from the Cre allele.

2.2 | Induction of EAE

Eight-week LKB1^{lox/+}Cre^{+/-} mice were administered tamoxifen (TAM, 100 mg/kg/day, i.p.) for five consecutive days to obtain het-cKO mice in which one LKB1 allele is deleted. LKB1^{lox/+}Cre^{-/-} mice received identical treatment to generate control mice (WT) in which both LKB1 alleles remain intact. EAE reagents were purchased from Hooke Laboratories (EK-2110). In brief, 10-week old mice (9 days after TAM treatment) were injected with 200 µg of MOG_{35–55} peptide emulsified in CFA (two 100 µl s.c. injections into adjacent areas in one hind limb). Two hours later, mice received an i.p. injection of pertussis toxin (PT; 125 ng in 100 µl phosphate buffered saline [PBS]), then 24 hr later a second PT injection. Clinical signs were scored as: 0, no clinical signs; 1, limp tail; 2, impaired righting (unable to return to upright position after placed on back); 3, paresis of one hind limb; 4, paresis of two hind limbs; 5, death. Scoring was performed every other day at the same time and by the same investigator blinded to allocation. For analysis of variance (ANOVA), if a mouse died or was sacrificed, its last score was carried forward till the end of the study. Mice were excluded from analysis if the clinical score increased from 0 to 4 with no intermediate signs.

2.3 | Immunocytochemical and histological staining

Mice were euthanized with carbon dioxide, then transcardially perfused with ice-cold PBS. Brains were removed, dissected sagittally at midline, and one hemisphere postfixed in 4% PFA for 48 hr, followed by 2 days in 30% sucrose solution for cryoprotection. The other hemisphere was dissected into regions (CB, cerebellum; CTX, cortex) and frozen at -80°C till use. Spinal cords were removed and processed the same way for immunostaining. Primary antibodies were rabbit polyclonal anti-Iba1 (1:1,000, Wako 019–19741); goat polyclonal anti-choline acetyltransferase (ChAT, 1:1,000, Millipore, AB144P), and rat monoclonal B2.210 anti-glial fibrillary acidic protein (GFAP, 1:1,000) (Trojanowski, Walkenstein, & Lee, 1986). Sections (20 μM) were incubated overnight at 4°C in primary antibody, washed $3\times$ in PBS for 5 min each, then incubated in rhodamine red- or fluorescein-conjugated secondary antibodies (1:1,000, Vector Laboratories) in blocking solution. Negative control sections were prepared without primary antibody. Sections were counterstained with DAPI, then mounted with VECTASHIELD H-1000 mounting medium (Vector). Images were collected on a Zeiss Axioplan 2 microscope equipped with an MRm camera. Axiovision version 4.7 software parameters were set to define positive staining versus background values, obtained from the same regions in negative control sections. ChAT+ cell numbers and area were quantified for cells with $>125\text{ }\mu\text{m}^2$ area. Spinal cord neurons were stained with NeuroTrace 530/615 fluorescent Nissl stain (ThermoFisher); and the number and area of neurons $>125\text{ }\mu\text{m}^2$ quantified in the anterior horn.

2.4 | Primary astrocytes

Primary astrocytes were prepared as described (Kalinin et al., 2017). In brief, frontal cortices from p1 pups (both male and female) were dissected, meninges removed, then homogenized into single cell suspension by passing through 1,000 μl then 200 μl pipette tips. Cells were passed through 70 μm nylon mesh then seeded into T75 flasks precoated with poly-D-lysine. Cortices from different pups were kept separate and tail DNA was used for genotyping. Cells were grown in DMEM containing 10% FBS and antibiotics. After 3 days, the cells were treated with 2 μM 4-hydroxy-TAM (Sigma 47904) to induce LKB1 knockout. The cells were grown till 90% confluence then microglia removed by shaking (280 rpm for 30 min at 37°C) adhering oligodendrocyte progenitor cells removed by overnight shaking (180 rpm at room temperature). Resulting cultures were greater than 95% pure determined by staining for GFAP and Iba1. In some studies, LKB1 depletion from WT astrocytes was accomplished by treating cells with siRNA directed to LKB1 (Life Technologies 4390771), or nontargeting control siRNA (Life Technologies 4390843).

2.5 | Adult astrocyte isolation

10-week old LKB1^{lox/+}Cre^{+/-} and LKB1^{lox/+}Cre^{-/-} female mice were treated for 5 days with TAM, then 9 days later astrocytes isolated using magnetic microbeads coated with mouse monoclonal to astrocyte extracellular protein ACSA-2 (Miltenyi Biotec 130–097-678). In brief, whole brains were digested using adult brain dissociation kit (Miltenyi Biotec 130–107-677), dissociated into single cells, extracellular matrix digested, myelin, cell debris, and erythrocytes removed, then resulting cells incubated with ACSA-2 coated magnetic microbeads. The cell suspension was applied to an MACS column, unabsorbed

cells removed by washing with PBS containing 0.5% BSA, then astrocytes eluted from the column in the absence of the magnetic field. Astrocyte enrichment was estimated between 10- and 20-fold based on increased GFAP and ALDH1L1 mRNA expression in RNA prepared from astrocytes versus astrocyte-depleted flow through (Figure S1e).

2.6 | Immunoblot analysis

Tissues were homogenized in RIPA buffer (Sigma-Aldrich R0278) containing protease and phosphatase inhibitors (Roche 11836153001). Lysates were cleared by centrifugation and protein concentration measured by BCA protein assay (Thermo-Scientific 23252). Equal amounts of protein were solubilized in Laemmli buffer (2% SDS) and heated for 5 min at 95°C. Extracts were fractionated through 8% or 10% SDS-polyacrylamide gels, or NuPage 4–12% Bis-Tris Gel (Invitrogen NP0322) then transferred to nitrocellulose membranes (Invitrogen iBlot). Membranes were dried and incubated with TBS blocking buffer (LI-COR 927–50000) for 60 min, washed once with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) then incubated with mouse monoclonal antibody cocktail to 5 OxPhos complexes (1:1,000, Abcam ab110413), rat monoclonal anti- α -tubulin (1:5,000, Millipore clone YL1/2 MAB1864); rabbit monoclonal anti-LKB1 (1:1,000, Cell Signaling D60C5F10); goat polyclonal anti- β -actin (1:5,000, Abcam ab8229), or rat monoclonal GFAP (1:2,000, (Trojanowski et al., 1986) at 4°C for 12 hr. Membranes were washed three times for 10 min in TBST and incubated with a 1:5,000 dilution of IRDye 680LT- or 800CW-conjugated anti-mouse, anti-rabbit or anti-rat antibodies for 2 hr (LI-COR). Blots were washed with TBST, scanned (LI-COR, Odyssey), and bands quantified using Li-COR Image studio Lite. For MHCII, membranes were incubated 1 hr in 5% nonfat milk in wash buffer (20 mM Tris base, 137 mM NaCl and 0.05% Tween-20) at room temperature, then incubated overnight at 4°C with primary anti-GAPDH (1:1,000, Millipore MAB374) and anti-MHCII (I-A/I-E) (1:1,000, Thermo Fisher 14–5321-82) diluted in 5% nonfat milk or 5% BSA. Membranes were washed, then incubated with horseradish peroxidase-conjugated goat anti-mouse (1:1,000, Jackson Laboratories 115–035-003) or anti-rabbit (1:1,000, Jackson Laboratories 111–035-144) IgG for 1 hr then visualized by enhanced chemiluminescence (Thermo Scientific) and a Chemidoc imager (BioRad).

2.7 | RNA isolation

RNA was isolated from tissues and cells using Direct-zol RNA Micro-Prep (Zymo Research) according to instructions. The resulting RNA quality was determined using a 4200 TapeStation Instrument (Agilent, Santa Clara, CA) and all samples had RNA integrity numbers above 8.

2.8 | Library generation

Illumina compatible libraries were prepared from cerebellar RNA using QuantSeq 3' mRNA-seq Library Prep Kit FWD (Lexogen) according to manufacturer instructions. Library generation was initiated by oligo-dT priming and first-strand synthesis, RNA removed, then random-primed second-strand synthesis done. Illumina-specific linker sequences were added and resulting double-stranded cDNA purified with magnetic beads. Then, 12 cycles of PCR amplification were used to introduce barcodes and generate sufficient DNA for cluster generation. After final purification libraries were measured on

TapeStation and Qubit (ThermoFisher). The resulting libraries were averaged 400 bp size with an average insert size of 270 bp. ERCC (External RNA Controls Consortium) RNA Spike-In Mix (Cat 4456740 Thermo Fisher Scientific) was added before library preparation to allow intersample normalization and control for variabilities.

2.9 | RNA-sequencing and analysis

Barcoded libraries were pooled and sequenced on an Illumina NextSeq (Illumina) generating an average of 12 M reads of nonpaired 75 nt per sample. RNA-seq generated FASTQ files were aligned to mouse reference genome with STAR aligner (Dobin et al., 2013) with mismatches set to 14. Differentially expressed (DE) mRNAs were determined in DeSeq2 using negative binomial distribution and false discovery rate of 0.1% (Love, Huber, & Anders, 2014).

2.10 | Quantitative real-time PCR

Total RNA (1 µg) was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher 4368814). The cDNA was amplified with specific primers (Table S1) using FastStart Universal SYBR Green Master mix (Applied Biosystems, 04913914001) in a Corbett RotoGene real time PCR machine (Qiagen). Relative mRNA levels were calculated from threshold take-off cycle number and normalized to values measured for β -actin and α -tubulin in the same samples. In some experiments (Figure 9) cDNA was amplified using Maxima Probe/ROX qPCR master mix (ThermoFisher K0233), probe-based qPCR primers from IDT designed in QuantStudio 3 (Applied Biosystems) and results normalized to levels for HPRT measured in same samples.

2.11 | Fluorescence activated cell sorting

Mononuclear cells were isolated as described (Lutz et al., 2017). Spinal cords from WT and het-cKO female mice 21 days postimmunization were homogenized between frosted glass slides. Mononuclear cells were isolated at the interphase of a 30%–70% Percoll gradient (GE Healthcare). Spleens were mechanically homogenized, and red blood cells lysed. Single-cell suspensions were restimulated for 6 hr in vitro with phorbol 12-myristate 13-acetate, ionomycin, brefeldin, and monensin (eBioscience). After Fc receptor blockade, cells were stained with e780 viability dye (eBioscience) and CD45-BV421 (BD Pharmingen). Cells were fixed, permeabilized, and stained for IL-17 α -PE (phycoerythrin) and IFN- γ -allophycocyanin (APC) (BD Pharmingen). Unstained cells were used for single-channel compensation, isotype controls, and fluorescence-minus-one controls. Compensation and analysis was done with Kaluza software (Beckman Coulter).

For MHC Class II, astrocytes were stimulated with IFN γ for indicated times and doses, then detached using Accutase (Fisher Scientific) for 3 min at 37°C. Cells were centrifuged, resuspended in fluorescence activated cell sorting (FACS) buffer (PBS, 2 mM EDTA and 2 mg/ml BSA), then stained with anti-MHCII-APC (Miltenyi Biotec 130–112-388) for 30 min on ice. Cells were washed twice with FACS buffer then analyzed on a LSR Fortessa (BD Bioscience).

2.12 | Data analysis

Data are presented as mean $\pm$ SEM of at least three independent experiments. Pair-wise comparisons were made using Kruskal–Wallis nonparametric analysis. Clinical scores were compared using two-way repeated measures ANOVA with Griess–Greenhouse correction which does not assume equal variability of differences between groups, followed by Sidak's post hoc analysis. Analyses were done using GraphPad Prism Version 8.0 (GraphPad Software, San Diego, CA). KEGG pathway analyses were done in the STRING platform (Szklarczyk et al., 2019) by permutation based, nonparametric testing (Yu et al., 2017) using as input identified DEs with associated log2-fold change.

3 | RESULTS

3.1 | EAE disease severity is increased in LKB1 heterozygous cKO mice

To examine the effects of LKB1 depletion from astrocytes on EAE, we crossed LKB1^{lox/lox} mice to ALDH1L1-CreERT2 mice (Winchenbach et al., 2016) to generate mice heterozygous for the LKB1 loxP allele (het-cKO) which retain one intact LKB1 allele, and corresponding WT mice which retain both LKB1 alleles after TAM treatment. Studies were carried out using het-cKO mice since all MS patients screened for the STK11 SNP were heterozygous (Boullerne et al., 2015). LKB1 mRNA levels were approximately 30% lower in adult astrocytes isolated 9 days after TAM treatment from het-cKO mice compared to WT mice, but were similar in RNA isolated from cells present in the het-cKO and WT astrocyte-depleted flow through (Figure S1e) confirming astrocyte specific depletion. After 9 days, the mice were immunized with MOG_{35–55} peptide to develop EAE. In female mice, disease incidence increased more rapidly in het-cKO than WT mice but was similar at the end of the study and reached 60% (9/15) in WT, and 76% (16/21) in het-cKO mice (Figure 1a). Comparison of disease severity in female mice (Figure 1b) revealed greater severity in het-cKO than WT mice ($F[15,360] = 1.997$, $p = .0148$). In male mice, disease incidence (Figure 1c) reached 72% (16/22) in WT and 67% (14/21) in het-cKO mice. Disease severity was also increased in het-cKO mice ($F[12,336] = 1.822$, $p = .044$), although post hoc comparisons did not show differences at any single time point, (Figure 1d). Further biochemical studies used tissues prepared from female mice.

LKB1 protein and mRNA levels were reduced in spinal cords of het-cKO compared to WT mice (Figure 2a–c). The modest reduction of LKB1 protein in het-cKO sham compared to WT sham mice did not reach statistical significance, however LKB1 levels were significantly lower in het-cKO EAE versus WT EAE mice. LKB1 protein levels were modified by EAE, with a slight decrease observed in WT mice, and a significant decrease in het-cKO mice. LKB1 mRNA levels in the spinal cord were significantly reduced in het-cKO versus WT mice, in both sham and EAE groups (Figure 2c). Since LKB1 expression is greater in neurons than astrocytes, these changes may reflect alterations in neuronal expression. In cerebellar samples, LKB1 protein levels did not differ between het-cKO sham and WT sham mice, as observed in the spinal cord. Cerebellar LKB1 protein levels were slightly increased by EAE in the WT mice, but not by EAE in the het-cKO mice (Figures 2d,e); however, those changes were not significantly different. As in spinal cord, LKB1 mRNA levels were significantly lower in cerebellum of het-cKO EAE versus WT EAE mice

(Figure 2f). These data suggest that neuropathology and associated changes in LKB1 are greater in the spinal cord than cerebellum.

Since a major target of LKB1 is AMPK, we tested if AMPK depletion from astrocytes replicated effects of LKB1 deficiency. However, homozygous depletion of AMPK α 1/ α 2 from adult mice did not affect progression or severity of EAE disease (Figure S2), suggesting that reduced AMPK activation is not responsible for the increased severity observed in LKB1 het-cKO mice.

3.2 | LKB1 het-cKO alters gene expression

RNAseq analysis was carried out to identify effects of LKB1 depletion on gene expression. We used cerebellar samples from male mice, to maximize identification of DE genes altered by LKB1 het-cKO, and minimize identification of DEs due to subsequent neuropathology (which is less in males than in females). In each of the four groups (WT and het-cKO; sham and EAE), we identified over 13,000 mRNAs having an average FPKM >5 (Table S2). Comparison of sham to EAE samples (Table S3) identified 544 DEs in WT mice, and 1,499 DEs in het-cKO mice. In WT mice, 232 DEs (42%) were increased and 312 DEs (58%) decreased. In het-cKO mice 977 DEs (65%) were increased and 522 (35%) DEs decreased (Table S4). There was considerable overlap of the DEs in het-cKO and WT mice (Figure 3a). Of all DEs that were increased, most (825) were only increased in het-cKO mice. Of the DEs that were decreased, the majority (363) were decreased only in het-cKO mice. Complement pathway transcripts were among the most increased mRNAs in WT mice (Figure 3b) while in het-cKO mice (Figure 3c) mRNAs involved in antigen presentation (H2-Aa, H2-Eb2, H2-Ab1, Ctss, Cd74) were the most increased. In the DEs only increased in het-cKO mice (Figure 3d), MHCII (H2-Eb1, H2-DMa) and mRNAs involved in inflammatory pathways and T cell activation (Bcl2a1b, Chil1, Tnfrsf1b, Irf5, Ccl8, and Vav1) were highly increased.

Pathway analysis identified nine KEGG pathways in WT Des (Table S5a), and 34 in het-cKO DEs (Table S5b) including the same nine found in WT mice. Many of the het-cKO only pathways with highest enrichment include genes related to MHCII signaling and antigen presentation; and several pathways include genes related to mitochondrial function. Analysis of the 594 DEs only found in het-cKO mice (Table S5c) identified nine KEGG pathways containing transcripts related to antigen presentation (H2-D1, H2-DMa, H2-Eb1, H2-Ob, H2-Q4, CD86) increased in EAE versus sham mice. Increased MHCII expression in het-cKO mice was confirmed by immunoblot analysis of cerebellar (Figure 3e,g) and spinal cord samples (Figure 3f,h).

One of the most enriched pathways (Huntington's disease [HD]) includes transcripts for mitochondrial NADH dehydrogenase subunits (Ndufa9, Ndufa10, Ndufa12, Ndufb7, Ndufv3); ATP synthase peripheral stalk (ATP5H); cytochrome c reductase (Cycc); and superoxide dismutase (Sod2); and all were reduced in EAE mice. Immunoblot analysis shows that in the spinal cord, there were significant reductions in Complexes III (60% lower), IV (70% lower), and V (65% lower) comparing het-cKO EAE versus WT EAE samples (Figure 4a,b). Levels of Complex III were slightly increased by EAE in the WT mice; but decreased by EAE in the het-cKO mice. There were no effects of het-cKO on any

complex in the sham mice. Since mitochondrial complex levels were not altered in spinal cords of sham mice, in cerebellar samples we tested if het-cKO caused any changes in EAE mice. In contrast to spinal cords, in the cerebellum (Figure 4e), we observed modest increases in Complexes I, III, and V; although there was a significant decrease (about 30%) in Complex IV.

Measurement of mRNA levels related to astrocyte glucose handling, glycolysis, and glycogenolysis shows that in spinal cord (Figure 4c) levels of GLUT1 (glucose transporter 1) were significantly reduced in het-cKO EAE compared to WT EAE mice; while levels of GLAST (glutamate transporter 1), GYS1 (glycogen synthase), and MCT1 (lactate transporter 1) were lower but that did not reach statistical significance. As found for mitochondrial complex, the levels of these mRNAs were not altered by het-cKO in sham mice. In cerebellar samples (Figure 4f) levels of GLUT1, GLYS1, MCT1, and GLAST were all decreased in the het-cKO EAE compared to WT EAE mice. Together these data suggest that modest decreases in astrocyte-related mRNAs in the spinal cord are sufficient to induce significant mitochondrial dysfunction, while in the cerebellum despite larger decreases in astrocyte mRNAs, overall mitochondrial function is maintained. The magnitude of reductions seen in spinal cord mitochondrial proteins further suggests that LKB1 het-cKO is leading to neuronal, as well as glial damage.

3.3 | LKB1 depletion increases spinal cord neuropathology

FACS analysis of spinal cord leukocytes (Figure 5) revealed that the percentage of CD45^{high} IFN γ ⁺ cells was modestly, but not significantly increased in het-cKO samples ($8.7 \pm 1.5\%$ vs. $7.4 \pm 0.8\%$). In contrast, the percentage of CD45^{high} IL17⁺ cells ($10.9 \pm 0.6\%$ vs. $7.7 \pm 0.5\%$) was significantly greater in het-cKO samples compared to WT samples, as was the percentage of CD45^{high} IL17⁺ IFN γ ⁺ cells ($3.1 \pm 0.4\%$ vs. $2.0 \pm 0.2\%$). There were no differences observed in splenic leukocytes (Figure 5d), suggesting that LKB1 depletion from astrocytes does not affect development of IL17 producing T cells.

In the spinal cord, staining for ChAT for large motor neurons showed reduced staining in het-cKO EAE compared to WT EAE (Figure 6a), with over 50% reduction of the number of ChAT⁺ stained cells (Figure 6b), and an increase (about 25%) in average cell area (Figure 6c). In sham mice, het-cKO did not affect ChAT cell numbers, but led to a small reduction in average cell area. Histological staining of WT and het-cKO EAE spinal cords for Nissl bodies (Figure 6d) showed similar numbers of neurons in the anterior horn (Figure 6e); however, the percentage of large ($>700 \mu\text{m}^2$) motor neurons was increased in het-cKO EAE mice (Figure 6f). We also observed large motor neurons in het-cKO EAE, but not WT EAE mice stained when the primary antibody was omitted, indicating accumulation of IgG (Figure 7a). Such staining was never observed in sham mice (either WT or het-cKO), was not colocalized with Iba1⁺ microglia, and showed some overlap with GFAP stained processes. Consistent with this, RNAseq analysis (Figure 7(b)) shows increased levels of Fc γ R2b, Fc γ R3, and Fc ϵ R1 γ in WT EAE mice, while in het-cKO mice, these were increased to a greater extent; and Fc γ R1, Fc γ R4, and Fc γ Rt, a neonatal Fc γ R also expressed in brain (Stamou, Grodzki, van Oostrum, Wollscheid, & Lein, 2018) were increased. We also found (Figure 7c) that mRNA levels for ChAT, VEGF, Glut1, and

CRMP1 were decreased in het-cKO samples, genes shown to be needed for motor neuron survival (Fakira & Elkabes, 2010; Stanojlovic et al., 2016). In contrast, caspase-3 levels were not altered, indicating absence of apoptotic cells.

3.4 | Metabolic dysfunction and inflammatory activation in LKB1 deficient primary astrocytes

Studies in primary astrocytes generated from WT and het-cKO mice were done to determine if LKB1 deficiency directly influenced astrocyte metabolic function. Treatment with 4-hydroxytamoxifen significantly reduced LKB1 mRNA and protein in these cells (Figures 8a,b). Stimulation with a robust cytokine mixture ("TII," TNF α , IFN γ , and IL1 β) increased nitrite production from het-cKO astrocytes about two-fold greater than from WT cells (Figure 8c). The het-cKO astrocytes released significantly less lactate than WT cells (Figure 8d), suggesting dysregulation of metabolic function. Quantitation of mRNAs involved in astrocyte metabolism (Figure 8e) showed reduced levels of GYS1 in the het-cKO cells. In contrast to tissues, GLAST mRNA levels were increased in het-cKO astrocytes.

Expression of MHCII genes was examined in astrocytes treated with siRNA to LKB1 as an alternate means of depleting LKB1, which achieved about 80% decrease in LKB1 mRNA levels (Figure 9a). In control cells, IFN γ induced expression of CIITA and STAT1, TFs which regulate MHCII expression, and those levels were further increased in LKB1 deficient cells (Figures 9b,c). Flow analysis showed that IFN γ increased MHCII expression measured after 48 hr (Figure 9d), and the average MHCII mean fluorescent intensity was significantly greater in LKB1 deficient astrocytes (Figure 9e).

4 | DISCUSSION

The current findings support a role for LKB1 in astrocytes during development of EAE, and show that partial depletion is sufficient to exacerbate disease severity. Heterozygous LKB1 deficiency from astrocytes led to metabolic disturbances, indicated by alterations in expression of mitochondrial electron transport chain complexes, and reductions in mRNAs related to astrocyte metabolism. The inflammatory milieu was increased in LKB1 het-cKO mice, indicated by increased IL-17 producing Th17 and double positive IFN γ /IL17 producing cells, and increased MHCII expression. We observed an increase of spinal cord neuronal damage, which may be due to the presence of metabolic dysregulation together with an increased inflammatory environment. Metabolic and inflammatory changes were observed in primary astrocytes in which LKB1 expression was reduced, suggesting that astrocyte dysfunction was a primary cause for in vivo findings. Transcriptome profiling identified DE mRNAs and KEGG pathways in het-cKO mice not present in WT mice, including ones related to mitochondrial function and antigen presentation. Together, our results demonstrate that dysregulation of astrocyte metabolic function, in this case due to reduction of LKB1, has multiple downstream consequences which together increase neurological damage.

In contrast to LKB1, deletion of AMPK α 1/ α 2 from astrocytes did not influence EAE (Figure S3). Similarly, neither the effects of LKB1 homozygous cKO from SC (Beirowski et al., 2014) or from spinal cord neurons (Sun, Reynolds, Leclerc, & Rutter, 2011) replicated

by AMPK α 1/ α 2 deficiency. LKB1 phosphorylates at least 12 other AMPK-related kinases (Gan & Li, 2014) including Nuak1 (Courchet et al., 2013) and SIK1, 2 and 3 (Sakamoto, Bultot, & Goransson, 2018) involved in mitochondrial functions, and MARK4 (Li, Thome, et al., 2017), SNRK (Li et al., 2018) and SIK2 (Darling, Toth, Arthur, & Clark, 2017) involved in regulating inflammation. LKB1 also controls levels of the mitochondrial calcium uniporter which regulates the switch from glycolytic to fatty acid oxidation (Natarajaseenivasan et al., 2018). The role of these kinases in astrocyte function during EAE has not been explored.

We used ALDH1L1-CreERT2 mice (Winchenbach et al., 2016) to achieve conditional depletion of LKB1. It was previously shown that this promoter efficiency in S100 β stained astrocytes is about 90% in the fimbria of the hippocampus, cerebral cortex, and cerebellum; and the specificity ranged from 4 to 6% in the hippocampus and cerebellum, to about 12% in the cortex. Our reporter studies show a similar efficiency in the spinal cord where $92 \pm 2\%$ of GFAP stained cells express the tomato reporter (Figure S4). Costaining shows that about 1% of carbonic anhydrase 2 stained oligodendrocytes were tomato positive; and there was no costaining for the neuronal marker NeuN. These results suggest that the present findings are due to depletion of LKB1 from astrocytes and not from other cell types. Furthermore, the similar recombination efficiencies in spinal cord and cerebellum suggest that differences observed between these tissues are not due to differences in Cre-mediated LKB1 depletion.

In adult astrocytes, LKB1 mRNA levels were approximately 70% of those measured in cells present in the astrocyte-depleted flow through. This is consistent with previous RNAseq measurements where the LKB1 mRNA was similar in astrocytes, neurons, and oligodendrocytes (Cahoy et al., 2008). In both male and female mice, EAE incidence as well as initial disease progression was similar in WT and het-cKO mice. Disease incidence in both male and female WT mice was slightly lower than that typically observed in WT C57BL/6 mice which may be due to the genetic background of the mice used. However, while severity plateaued in WT mice at about Day 25, it continued to increase in het-cKO mice. This consistent with the fact that LKB1 deficiency from astrocytes is not expected to affect early events occurring outside of the CNS (e.g., T cell activation, lymphocyte migration). The maximum clinical scores reached in WT mice were lower than those typically observed in MOG₃₅₋₅₅ peptide EAE (Boullerne et al., 2014); we ascribe this to the mixed lineage of the mice used, comprised of 75% of an FVB/n background and 25% of a C57BL6/N background. However, since comparisons were made between mice derived from the same litters, the results indicate that LKB1 deficiency exacerbates disease.

In WT mice, spinal cord LKB1 protein levels (Figure 2b) were not significantly altered by EAE, however levels were significantly reduced by EAE in the het-cKO mice. Since LKB1 is expressed at higher levels in neurons than astrocytes, these findings may reflect greater neuronal damage occurring during EAE in the het-cKO mice, and loss of LKB1 from damaged neurons. This may also be due in part to increased turnover of pre-existing LKB1, or reduced de novo expression of new protein in the EAE, but not the sham mice. In contrast, in the cerebellum (Figure 2e), we did not observe any significant effect of EAE on LKB1 protein levels in either WT or het-cKO mice. The basis for this difference as

compared to spinal cord is not known, but may reflect the fact that neuronal damage is less in cerebellum than spinal cord. We did not see any effect of EAE on LKB1 mRNA levels, either in the SC (Figure 2c) or CB (Figure 2f). In the SC, we detected a significant decrease in LKB1 mRNA comparing WT to het-cKO samples, in both sham and EAE mice. In contrast, in the CB, although LKB1 mRNA levels were lower in het-cKO samples, those reductions did not reach statistical significance by one-way ANOVA.

4.1 | LKB1 as a regulator of inflammatory activation

Recent studies describe critical roles for LKB1 in immune cell function and inflammation. LKB1 is essential for hematopoietic cell metabolic function and survival (Gan et al., 2010; Gurumurthy et al., 2010; Nakada, Saunders, & Morrison, 2010). Disruption of LKB1 in T regulatory cells leads to a fatal inflammatory disease (He et al., 2017; Yang et al., 2017), and LKB1 expression in dendritic cells is needed to prevent excessive expansion of T regulatory cells (Chen et al., 2018). In macrophages, LKB1 suppresses NF- κ B-driven inflammatory responses stimulated by LPS (Liu et al., 2015). The regulation of inflammation extends beyond immune cells, as deletion of LKB1 from renal tubules increased inflammation and immune cell infiltration into the kidney (Viau et al., 2018). Additionally, loss-of-function of LKB1 in tumor cells leads to increased cytokine and chemokine expression and increased recruitment of immune cells into the tumor environment (Koyama et al., 2016). Our data similarly show that LKB1 is essential to constrain excessive inflammation and maintain metabolic function in astrocytes.

IFN γ is a Th1-produced cytokine upregulated and correlated with disease severity in MS (Calabresi, Tranquill, McFarland, & Cowan, 1998). The role of IFN γ in EAE is complex (Ottum, Arellano, Reyes, Iruetagoiena, & Naves, 2015), but importantly disruption of IFN γ signaling in astrocytes, and not microglia, ameliorates EAE (Ding et al., 2015). Our data show that reduced LKB1 in vitro enhances IFN γ -induced gene expression including STAT1, CIITA, and MHCII. In vivo, 6 (H2-DMA, H2-EB1, IRF5, CCL8, Tnfrsf1b, and Chil1) of the top 10 genes induced uniquely in LKB1 het-cKO mice (Figure 3h) are induced by IFN γ (Samarajiwa, Forster, Auchettl, & Hertzog, 2009). As such, the response to the inflammatory milieu may be augmented by increased expression of STAT1 and IRF5. STAT1 is the central mediator of the response to IFN γ but can also amplify toll like receptor and IL-1 β driven inflammation in astrocytes (Chung & Benveniste, 1990). These findings are consistent with previous work showing that IFN γ increases astrocyte MHCII expression (Cornet et al., 2000; Soos et al., 1999; Yang et al., 2012) and that IFN γ -treated astrocytes can present antigen to T cells and promote T cell proliferation (Cornet et al., 2000; Soos et al., 1998; Tompkins et al., 2002). While CNS-resident dendritic cells are likely the main CNS cell type that presents myelin antigen to T cells in EAE (Giles, Duncker, Wilkinson, Washnock-Schmid, & Segal, 2018), elevated MHCII on astrocytes due to reduced LKB1 may amplify this process. Overall, our data suggest a general enhancement of the astrocytic response to IFN γ which may contribute to greater disease severity and increased neuroinflammation observed in het-cKO mice.

4.2 | Possible mechanisms underlying increased Th17 populations

We observed that LKB1 deficiency increased the population of Th17 and double positive IFN γ /IL17 producing cells. This could be due to increased expansion, migration, or survival mediated by astrocyte-derived factors. Astrocytes normally produce cytokines which limit Th17 activation (IL4, IL2, IL27) (Senecal et al., 2016) and several which increase activation including IL15 (Broux et al., 2015; Li, Li, et al., 2017). T cell recruitment can be increased by chemokines including CCL2, CCL6, CCL9, CXCL7, and CXCL10 (Cedile, Wlodarczyk, & Owens, 2017; Kim et al., 2014; Rubio et al., 2014; Yamano & Coler-Reilly, 2017), while astrocyte-derived cytokines (IL23) promote differentiation to the Th17 lineage (Constantinescu et al., 2005).

Although Th17-related KEGG pathways were not detected using ranked analysis (Table S6), analysis of the 594 DEs only detected in het-cKO mice using a nonranked approach identified the Th17 cell differentiation pathway (Figure S4) having 22 DEs (Table S5), all but four increased in EAE versus sham. This includes IL12rb1 a component of the IL23R; proto-oncogene Lck critical for T cell maturation and function; TFs (NFATc1, Runx1, Stat3, Stat6) which contribute to T cell maturation, proliferation, and survival, and Lat-1 (linker for activation of T cells family member 1) required for T cell receptor signaling. Among the DEs decreased is Ppp3ca, a subunit of calcineurin which normally phosphorylates and activates NFAT. Since NFAT deficiency reduces Th17 maturation (Reppert et al., 2015), reduced calcineurin activity could reduce NFAT activation leading to increased Th17 responses and worsened EAE. While the cellular origins remain to be determined, these changes provide a mechanism that can help account for increased Th17 population in spinal cords of het-cKO EAE mice.

4.3 | Motor neuron damage in EAE and MS

Our results point to increased damage occurring to spinal cord anterior horn neurons in the het-cKO EAE mice, as evidenced by neuronal hypertrophy, reduction in ChAT+ stained cells, lower levels of mRNAs implicated in motor neuron health, and increased IgG accumulation. Motor neuron loss has been described in various EAE models including MOG-peptide induced (Bannerman et al., 2005; Vogt et al., 2009), spinal cord homogenate induced (Giardino, Giuliani, Fernandez, & Calza, 2004), adoptive transfer of PLP specific T cells (Vogt et al., 2009), and PLP peptide RR and MOG peptide chronic models (Aharoni et al., 2011). In the latter models, motor neuron loss reached 46% at 50 days after immunization (Aharoni et al., 2011). We also observed decreases in CRMP1 (collapsing response mediator protein 1) expression, a protein involved in neuritogenesis and growth (Fakira & Elkabes, 2010), and whose levels were reduced in motor neurons following knockdown of plasma membrane calcium ATPase (Kurnellas, Nicot, Shull, & Elkabes, 2005). We also found reduced levels of VEGF-A, a neurotrophic growth factor whose levels are reduced in spinal cords during the course of EAE leading to suppression of VEGFR2 signaling and exacerbated motor neuron loss (Stanojlovic et al., 2016). We also found lower levels of GLT1, the astrocyte glutamate transporter which removes excessive glutamate thus limiting excitotoxicity. Motor neuron loss in ALS (amyotrophic lateral sclerosis) is due in part to deficits in glycolytic and mitochondrial pathways needed to maintain energy

requirements. The reductions in mitochondrial complexes and astrocyte metabolic mRNA in the spinal cord likely contributes to motor neuron damage in the het-cKO EAE mice.

We observed IgG accumulation in spinal cord motor neurons in het-cKO EAE mice, as reported previously to occur in EAE (Bucher, Maccioni, Rivero, Riera, & Roth, 1996; Slavin, Bucher, Degano, Soria, & Roth, 1996). IgG accumulation could be due to direct binding to targets located on or in neurons, or to neuronal expressed Fc γ R. Although Fc γ Rs are mainly expressed on immune effector cells, they are also expressed on neurons and may have roles in AD and PD (Fuller, Stavenhagen, & Teeling, 2014). Our RNAseq data showed increased expression of several Fc γ Rs in EAE compared to sham mice, and the increases were greater in het-cKO than WT mice, suggesting that IgG accumulation is due at least in part to receptor occupation, and not to antigen targets. Increased IgG accumulation may also reflect increased IgG leakage across the BBB, and several factors produced by astrocytes that can cause BBB damage were increased in het-cKO mice compared to WT mice, including CCL5 (Patabendige et al., 2018) and CCL2 (Gyoneva et al., 2015; Wang et al., 2014). In addition, in the het-cKO mice, there was an increase in *Irgm1*, an immune-related GTPase that is increased during EAE, and whose deletion led to reduced BBB disruption and lower disease severity (Wang et al., 2013).

4.4 | Astrocyte metabolic dysfunction contribution to disease severity

RNAseq analysis of het-cKO specific DEs identified HD as an enriched pathway, including eight transcripts relating to mitochondrial function. The HD pathway is identified due to the presence of *DLG4*, and *PLCB2* and 4, known risk factors for HD (Chang, Wang, Zhu, & Wu, 2017). Mitochondrial dysfunction was confirmed by decreases of oxidative phosphorylation proteins in the spinal cord and smaller changes in the cerebellum. These reductions are consistent with findings that *LKB1* deficiency reduced mitochondrial proteins in muscle (Brown et al., 2011) and induced defective mitochondria and reduced ATP levels in Tregs (He et al., 2017; Yang et al., 2017). Changes in mitochondrial electron chain complexes occur in MS (Hargreaves, Mody, Land, & Heales, 2018), and in EAE increases in Complex IV occur and thought to help provide energy under inflammatory conditions (Packialakshmi & Zhou, 2018). We also observed lower mRNA levels for *GLUT1* and *GYS1* in the SC and CB; for *MCT1* in the CB; and for *GYS1* and *GLUT1* in primary astrocytes. While not all reductions reached statistical significance, the combined effect of decreasing several could significantly reduce astrocyte glucose uptake and glycogen synthesis, reduce lactate production, and reduce lactate export through *MCT1*. This is consistent with findings that *LKB1* depletion from SC reduced lactate release (Beirowski, 2019), and in *drosophila*, *LKB1* increases lactate transporter trafficking (Jang, Lee, & Chung, 2008). While the importance of lactate transfer from astrocytes to neurons to neuronal function and survival remains controversial (Magistretti & Allaman, 2018; Pellerin & Magistretti, 2012), evidence exists that in inactive MS lesions, a shift to increased glycolysis increases lactate shuttling between astrocytes and neurons, thought to be critical for the survival of demyelinated axons (Nijland et al., 2015). Our results suggest that, together with reduced glutamate uptake through *GLAST1* and an increased inflammatory milieu, deficiencies in the ability of astrocytes to provide lactate to neurons could account for increased neuronal damage.

4.5 | Relevance to MS

Findings that LKB1 deficiency from astrocytes exacerbates EAE suggests that changes in expression or activity of astrocyte LKB1 could also influence disease progression or severity in MS, supported by observations that the STK11 SNP is a risk factor for MS. Although effects of the SNP on LKB1 are not yet known, the fact it is expressed in all tissues and cells makes it likely that LKB1 changes in other cells also contribute to increased MS risk. In this regard, LKB1 homozygous cKO from spinal cord neurons caused axon degeneration, macrophage infiltration, and hind limb paralysis (Sun et al., 2011); mice with LKB1 deletion from Tregs developed an early onset autoimmune disease (Wu et al., 2017); and LKB1 deletion from B cells led to their spontaneous activation (Walsh et al., 2015). Methods to increase astrocyte metabolic and mitochondrial function, under conditions of LKB1 deficiency, may represent a potential adjunct to MS treatment strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ALDH1L1	aldehyde dehydrogenase 1 family member L1
ATPb	ATP synthase subunit beta
ChAT	choline acetyltransferase
cKO	conditional knockout
EAE	experimental autoimmune encephalomyelitis
FcγR	Fc gamma receptor
FPKM	fragments per kilobase of transcript per million mapped reads
GLUT1	glucose transporter 1

GLAST	glutamate aspartate transporter
GYS1	glycogen synthase
het-cKO	heterozygous cKO
LKB1	liver kinase B1
MCT1	monocarboxylate transporter 1
MHCII	major histocompatibility Class II
NT	nontargeting
GLT1	excitatory amino acid transporter 2
STK11	serine–threonine kinase 11
VEGF	vascular endothelial growth factor

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FIGURE 1.

Astrocyte liver kinase B1 (LKB1) heterozygous cKO mice exhibit worsened EAE disease severity. (a) In female mice, maximal disease incidence reached 60% (9/15) in WT (○); and 76% (16/21) in het-cKO mice (●). (b) Disease severity in females that exhibited clinical signs was significantly increased in cKO mice (time × score factor $F[15,360] = 1.997$, $p = .0148$, two-way repeated measures analysis of variance (ANOVA), * $p < .05$ Sidak's multiple comparison test). (c) In male mice, maximal disease incidence reached 72% (16/22) in WT; and 67% (14/21) in het-cKO mice. (d) Disease severity in males that exhibited clinical signs was significantly increased in the cKO mice (time × score factor $F[12,336] = 1.822$, $p = .044$, two-way repeated measures ANOVA. Sidak's multiple comparison test did not identify differences at any single time point, which could be due to reduced sample size when using pair-wise comparisons, or that the ANOVA uses different assumption than pair-wise post hoc tests. Data are combined from two independent experimental autoimmune encephalomyelitis (EAE) studies. §, $p < .05$, two-way repeated measures ANOVA time × score factor. cKO indicates het-cKO mice

FIGURE 2.

Liver kinase B1 (LKB1) expression in spinal cord and cerebellum. Protein and mRNA samples were prepared from spinal cord (SC) and cerebellum (CB) of sham and experimental autoimmune encephalomyelitis (EAE) immunized, WT and het-cKO female mice 35 days after immunization. (a) Representative immunoblot for LKB1 and β -actin in SC; (b) SC LKB1 protein levels; (c) SC LKB1 mRNA levels. (d) Representative immunoblot for LKB1 and GAPDH in CB; (e) CB LKB1 protein expression normalized to GAPDH; and (f) CB LKB1 mRNA levels. Data are mean $\pm$ SE, $n = 4$ per group. * $p < .05$. LKB1 mRNA normalized to α -tubulin and β -actin measured in same samples. cKO indicates het-cKO mice

FIGURE 3.

Overlap of differentially expressed (DE) mRNAs. (a) Venn diagram showing overlap of DE identified by RNAseq of cerebellar samples comparing experimental autoimmune encephalomyelitis (EAE) to sham groups in male WT mice (544 DEs total); and sham to EAE groups in male het-cKO mice (1,499 DEs total). The DEs that were most increased and decreased between EAE and sham in (b) WT mice and in (c) het-cKO mice. (d) The DEs most increased and decreased between EAE and sham groups that are only present in het-cKO mice. Immunoblotting showing increased expression of MHC Class II in (e) cerebellum and (g) spinal cords of het-cKO and WT EAE mice. (f,h) Relative MHCII band intensities normalized to GAPDH measured in the same samples. Data are mean $\pm$ SE, n = 5 or 6 per group. p -Values are indicated. cKO indicates het-cKO mice

FIGURE 4.

Liver kinase B1 (LKB1) depletion alters mitochondrial complex expression. (a) Immunoblot analysis of spinal cord proteins from WT and het-cKO, sham, and experimental autoimmune encephalomyelitis (EAE) female mice, showing changes in mitochondrial Complexes I–V using total OxPhos antibody kit. (b) Band intensities for indicated complexes normalized to β -tubulin measured in same samples, and relative to levels in WT sham samples. Mean $\pm$ SEM, $n = 3$, $**p < .005$; $***p < .0005$; EAE WT versus EAE het-cKO; \$, $p < .05$ WT Sham versus WT EAE; %, $p < .05$, het-cKO Sham versus het-cKO EAE, 1-way ANOVA, Tukey. (d,e) Analysis of cerebellar samples from WT EAE and het-cKO EAE female mice. Data mean $\pm$ SE of band intensity normalized to β -tubulin and relative to WT EAE samples, $n = 5$, $*p < .05$ versus WT EAE. qRT-PCR analysis of indicated mRNAs from (c) spinal cord and (f) cerebellum. $n = 4$ per group. $*p < .05$ versus EAE WT. cKO indicates het-cKO mice

FIGURE 5.

Th17 and double positive IFN γ /IL17 cells are increased in het-cKO spinal cords. Flow cytometry plots for leukocytes isolated from spinal cords of (a) WT and (b) het-cKO experimental autoimmune encephalomyelitis (EAE) female mice shows (c) significant increase of IL17 and IL17/IFN γ expressing cells in het-cKO samples. (d) There was no difference in IL17 or IL17/IFN γ expressing cells in splenic monocytes isolated from the same animals. $N = 6$ per group, * $p < .05$, ** $p < .005$ versus WT. cKO indicates het-cKO mice

FIGURE 6.

Spinal cord neuronal pathology is increased in het-cKO mice. Spinal cord anterior horn neurons from WT and het-cKO female mice were identified by (a) immunostaining for choline acetyl transferase (ChAT) and (d) staining with Neurotrace for Nissl bodies. (b,c) Quantitation of ChAT staining for number of ChAT+ cells and average cell area. Data are mean $\pm$ SE, $n = 6$ per group. * $p < .05$, ** $p < .005$ versus WT EAE; ###, $p < .0005$ versus WT Sham. Quantitation of Nissl staining for (d) neuronal population and (e) size distribution of Nissl stained neurons. Bin size refers to cell body area and covers from value $\pm 50 \mu\text{m}^2$. Data are mean $\pm$ SE, $n = 5$ per group, * $p < .05$ versus WT. Scale bar is 50 μm . cKO indicates het-cKO mice

FIGURE 7.

Spinal cord motor neurons accumulate IgG. (a) Immunostaining of spinal cord sections from WT and het-cKO female mice using secondary anti-mouse IgG shows increased staining in het-cKO sections; partially colocalized with GFAP but not with Iba1. (b) Relative expression (in FPKM) of mRNAs encoding FcγR isoforms, identified by RNAseq comparisons between sham and EAE mice, shows larger increases in het-cKO versus WT mice. (c) qRT-PCR of SC samples from WT and het-cKO EAE mice shows significant reductions in CRMP1, ChAT, VEGF, and Glut1. Data are mean $\pm$ SE, $n = 4$ per group, $*p < .05$ versus WT. cKO indicates het-cKO mice

FIGURE 8.

LKB1 depletion increases astrocyte activation. Primary astrocytes were prepared from $LKB1^{lox/+} Cre^{-/-}$ (WT) and $LKB1^{lox/+} Cre^{+/-}$ (het-cKO) pups, then incubated with 4-hydroxytamoxifen to deplete LKB1. After 3 days, some cells were incubated with a pro-inflammatory cytokine mixture ("TII": $TNF\alpha$, $IL1\beta$, $IFN\gamma$). (a) qRT-PCR analysis of LKB1 mRNA levels. (b) Immunoblotting analysis shows reduced levels of LKB1 protein in het-cKO samples. (c) Nitrite production measured 24 hr after incubation in fresh media with nothing (control) or TII. (d) Extracellular lactate levels measured 24 hr after incubation in fresh media. (e) qRT-PCR analysis of WT and het-cKO astrocytes for indicated transcripts. Data are mean $\pm$ SE, $n = 4-6$ per group, $*p < .05$ versus WT. cKO indicates het-cKO mice

FIGURE 9.

Liver kinase B1 (LKB1) depletion increases astrocyte MHCII expression. Primary astrocytes were prepared from C57BL/6 mice and treated in vitro with siRNA to LKB1 (or NT siRNA, nontargeting siRNA) then treated with IFN- γ (10 ng/ml) for the indicated times. qRT-PCR for (a) LKB1, (b) CIITA, and (c) STAT1 mRNAs normalized to HPRT measured in same samples. * $p < .05$; ** $p < .005$; *** $p < .0005$ versus NT siRNA. (d) Fluorescence activated cell sorting (FACS) analysis for membrane associated MHC Class II on control (NT siRNA) and depleted (LKB1 siRNA) astrocytes at 48 hr after incubation with nothing (UT) or IFN- γ . (e) Mean fluorescence intensity of MHCII expression, $n = 3$ per group. *** $p < .0005$ versus NT siRNA