Deficiency of LRRC4 Accelerates Experimental Autoimmune Encephalomyelitis by Disrupting Th1/Treg Cell Balance

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Research

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Abstract

Background

Leucine rich repeat containing 4 (LRRC4), also known as netrin-G ligand-2 (NGL-2), belongs to the superfamily of LRR proteins and serves as a receptor for netrin-G2. LRRC4 regulates the formation of excitatory synapses and promotes axon differentiation. Mutations in LRRC4 occur in Autism Spectrum Disorder (ASD) and intellectual disability. Multiple sclerosis (MS) is a chronic autoimmune disease characterized by immune-mediated demyelination and neurodegeneration of the central nervous system (CNS). Here, we investigated the role of LRRC4 in the pathological process of experimental autoimmune encephalomyelitis (EAE), a widely used mouse model of MS.

Methods

LRRC4 was detected in the CNS of EAE mice by the use of real-time PCR and western blotting. LRRC4 -/- mice were created and immunized with myelin oligodendrocyte glycoprotein peptide (MOG) 35–55 . Pathological changes in spinal cords of LRRC4 -/- and WT mice 15 days after immunization were examined by using hematoxylin and eosin (H&E), Luxol Fast Blue (LFB) staining and immunohistochemistry. The number of Th1/Th2/Th17/Treg cells in spleens and blood were measured with flow cytometry. Differential gene expression in the spinal cords from WT and LRRC4 -/- mice was analyzed by using RNA sequencing (RNA-seq). Adeno-associated virus (AAV) vectors were used to overexpress LRRC4 (AAV-LRRC4) and were injected into EAE mice to assess the therapeutic effect of AAV-LRRC4 ectopic expression on EAE.

Results

We discovered that the level of LRRC4 decreases in the spinal cords of the EAE mice. Deletion of LRRC4 accelerates infiltration of leukocytes into the spinal cords and disease exacerbation in vivo. We further showed that LRRC4 deletion disrupts the balance between Th1 cells and Treg cells and causes a shift toward Th1 cells and that the disrupted balance may be attributed to up-regulation of IL-6, IFN-γ and down-regulation of TNF-α in EAE mice. At a mechanistic level, we found that deficiency of LRRC4 induces elevated NF-κB p65 expression and does so by up-regulating Rab7b, while ectopic expression of LRRC4 alleviates the clinical symptoms of EAE mice and protects the CNS from immune damages.

Conclusions

We establish a critical role of LRRC4 in the progression of EAE and provide novel mechanistic insights into EAE development. Our findings also suggest that LRRC4 may be used as a potential target for therapeutic treatment of MS.

Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease in which T cells infiltrate the central nervous system (CNS), causing demyelination, neurodegeneration and paralysis[1, 2]. Dysregulation of the immune system is widely considered to be the factor for both initiation and progression of MS. $CD4^+$ T-helper (Th) cells play an important role in MS pathogenesis [3] and have been shown as the causative factor that mediates MS pathogenesis in humans and in the rodent model of experimental autoimmune encephalomyelitis (EAE) [4]. Th1 cells produce cytokines, such as interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-β, which can induce cell-mediated immunity and phagocyte-dependent inflammation [5]. IL-12 is required for differentiation of Th1 cells, while mice with IL-12 deletion (IL-12 $p40^{-/-}$) are resistant to EAE [6]. Furthermore, treatment of MS patients with recombinant IFN-γ causes the disease to exacerbate [7]. In contrast, IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice are more susceptible to EAE induction with disseminated neutrophil invasion [8]. The transcription factors signal transducer and activator of transcription 1 (STAT1) and T-bet control the differentiation of Th1 cells. T-bet-deficient mice are resistant to the development of EAE while $STAT1^{-/-}$ mice are susceptible to EAE and develop more severe disease [9]. Th17 cells have been defined as a distinct subset of $CD4^+$ T cells that produce IL-17A, IL-17F, IL-21, IL-22 and TNF-α, promote inflammation, and are pathogenic in many autoimmune disorders [10]. IL-23 promotes the differentiation of Th17 cells, while the IL-23-induced IL-17^+ T cells are capable of inducing EAE via adoptive cell transfer. T cells lacking retinoid-related orphan receptor-γt (ROR-γt) or STAT3, both of which are required for Th17 cell development, fail to induce EAE [11]. IL-17 has been shown to promote inflammatory cell infiltration into the brain parenchyma, resulting in a clinically atypical EAE. IL-17 also interferes with remyelinating processes, inhibits the maturation of cells of the oligodendrocyte lineages, and reduces their survival [12]. Thus, both Th1 and Th17 cells play complementary roles in the pathogenesis of EAE. Indeed, EAE can be induced by adoptive cell transfer of either Th1 or Th17 cells, which both secrete granulocyte-macrophage colony-stimulating factor (GM-CSF). The cells deficient in GM-CSF fail to induce of EAE [13].

In contrast to Th1 and Th17 cells, Th2 cells secrete IL-4, -10, and − 13 and induce strong antibody responses while inhibiting several functions of phagocytic cells [14]. Th2 cells are reported to have an inhibiting effect on MS/EAE, and mice deficient in IL-4 exhibited more severe EAE clinical diseases [15]. Myelin basic protein (MBP)-activated Th2 cell transfer promotes myelination and preservation of neurons and repairs injured spinal cord in EAE [16]. Regulatory T (Treg) cells are thought to play a central role in the maintenance of peripheral immune tolerance [17]. The function of Treg cells in relapsing-remitting MS (RRMS) patients is severely impaired compared with healthy individuals [18]. Treg cells protect the mice from developing chronic EAE, implying that Treg cells contribute to the protection of individuals against MS [19]. Many treatments which increase Treg cell number lead to significant amelioration of Myelin Oligodendrocyte Glycoprotein (MOG)-induced EAE [20]. Treg cells secret anti-inflammatory cytokines IL-10 and TGF-β to suppress proliferation and cytokine production of other T cells [21]. While in the presence of IL-6, differentiation of Treg cells is switched to the Th17 cells [22]. This skewed pattern of differentiation leads to secretion of the proinflammatory cytokine IL-17 but does not promote differentiation of Treg cells or TGF-β production. IL-6 and TGF-β together induce the differentiation of Th17 cells by inducing IL-17-specific transcription factor RORγt [23]. Thus, the balance among Th1, Th17 and Treg cells appears

critical in MS pathogenesis, and regulation of Th cell differentiation may prove to be a potential strategy for MS diagnosis and treatment [24].

LRRC4 also known as NGL-2, belongs to the superfamily of LRR proteins and serves as a receptor for netrin-G2 [25]. LRRC4 regulates the formation of excitatory synapses by clustering excitatory postsynaptic proteins, participates in the differentiation of neurons, and promotes neurite extension of hippocampal neurons [26, 27]. In addition, LRRC4 associates with N-Methyl-D-aspartate receptors (NMDARs) and is involved in promoting excitatory synapse development in specific dendritic segments [28, 29]. Earlier studies showed that knockout of LRRC4 in mice suppresses NMDAR-dependent synaptic plasticity in the hippocampus and that LRRC4$^{-/-}$ mice display mild autistic-like behaviors, which can be rapidly rescued by pharmacological activation of NMDAR [30]. LRRC4 associates with the polarity-associated partitioning defective (PAR) complex through binding to PAR6 to stabilize axonal microtubules and promote axon differentiation via the aPKCζ/MARK2 pathway [31]. Moreover, LRRC4 acts as a tumor suppressor gene and significantly inhibits glioma cell proliferation by regulating phospho-ERK and phospho-AKT [32]. LRRC4 suppresses the expression of CXCR4, SDF-1α/CXCR4 and cytokines such as VEGF and TGF-β to inhibit glioblastoma cell proliferation, migration and angiogenesis [33, 34]. It has been recently shown that LRRC4 binds to phosphoinositide-dependent protein kinase 1 (PDPK1), facilitates activation of NF-κB of GBM cells, and promotes the secretion of IL-6, CCL2 and IFN-γ, resulting in tumor-infiltrating Treg cell expansion and GBM cell growth suppression [35].

Mutations of LRRC4 gene in humans have been implicated in Autism Spectrum Disorder (ASD), and intellectual disability. Whole-genome sequencing (WGS) analysis revealed a missense mutation in LRRC4 in an individual with ASD [36]. Locomotor training increases synaptic structure with high NGL-2 expression after spinal cord hemisection [37]. A microdeletion in LRRC4 has been shown to be associated with intellectual disability and autism [38]. Moreover, netrin-G2, the receptor for LRRC4, is linked to schizophrenia and bipolar disorder [39]. Because of the difficulties associated with the studies of human patients, it becomes necessary to establish the function of LRRC4 in animal models. However, to date, whether functional perturbations of LRRC4 cause behavioral abnormalities and CNS autoimmune disease in the animal models and whether they are associated with pathogenesis of MS remain undefined.

By using a murine model of EAE, we discovered that the level of LRRC4 decreases in the spinal cords of the EAE mice. Deletion of LRRC4 accelerates infiltration of leukocytes into the spinal cords and disease exacerbation in vivo. At a mechanistic level, we found that deficiency of LRRC4 induces elevated NF-κB p65 expression and does so by up-regulating Rab7b. NF-κB up-regulation further elevates the expression of cytokines and causes changes in the ratio of Th1/Treg cells, ultimately altering the immune responses of CNS and accelerating disease progression of EAE.

Materials And Methods

Mice and EAE induction

C57BL/6 mice were purchased from the Slake Experimental Animal Company. LRRC4-loxP chimeric mice were constructed by Cyagen Biosciences , crossbred with widespread expression cre mice [B6.C-Tg (CMV-cre) 1Cgn/J, Jackson Laboratory], and then hybridized offspring of LRRC4$^{-/-}$ mice were obtained. The genotypes of mice were identified with real-time PCR and western blotting. Wild-type littermate controls with same genetic background were used. All mice were maintained in specific pathogen-free conditions in the Laboratory Animal Department of Central South University. EAE was induced by the use of myelin oligodendrocyte glycoprotein (MOG_{35-55}, H-MEVGWYRSPFSRVVHLYRNGK-OH, GL Biochem) in 8-week-old female mice. Each mouse was immunized by subcutaneously injection with 200 μg of MOG_{35-55} emulsified in Complete Freund's Adjuvant (CFA, Sigma) with 500 μg mycobacterium tuberculosis (strain H37RA, DIFCO). Mice were then intraperitoneally injected with 200 ng of pertussis toxin (List Biological Laboratories) at the time of immunization and 2 days after. Mice were evaluated for clinical scoring and body weight daily after immunization for 30 days. Neurological signs were recorded to evaluate motor deficit as follows: 0, no deficit; 1, dysfunction of tail; 2, dysfunction of tail and one limb; 3, limp tail and dysfunction of two limbs; 4, disturbed function in tail and all limbs; 5, moribund state or death. Scoring was performed in a blinded fashion.

Neuronal culture and electrotransfection

Newborn mice were sacrificed by decapitation and sterilized by using 70% ethanol. Hippocampus was isolated and digested with 0.25% trypsin-EDTA (HyClone) for 30 min at 37°C, followed by trituration with pipetting in DMEM-F12 (HyClone) medium. Dissociated neurons were transfected using electrotransfection. Neurons were plated onto dishes coated with poly-D-lysine (0.1 mg/ml, Sigma). After cultivation for 4 h, the media were replaced with neuronal culture medium (Gibco) containing 1% glutamate (Sigma) and 2% B27 medium (Sigma) at 37 °C and 5% CO_2 in a humidified atmosphere.

RNA interference and adeno-associated virus infection

The target sequences of Rab7b shRNA1 and shRNA2 were 5'- AGTGGACTTGAAACTTATCATTGTTGGTG -3' and 5'- AAGTTAGTGCGAAGAATGACATC

AATGTG -3', respectively. All the DNA segments were synthesized by Sangon Biotech and inserted into the pSuper Vector. LRRC4 and Rab7b were amplified from mouse brains and cloned into pcDNA3.1 plasmids. Transfection of plasmids was conducted by following the manufacturer's instructions. The adeno-associated virus (AAV) vector for overexpressing LRRC4 (AAV-LRRC4) and controls (AAV-CON) were constructed by Vigene Biosciences. AAV-LRRC4 was packaged in HEK293T cells, and the cells were then lysed by the use of freeze-thaw cycles. The viruses were purified using the iodixanol gradient ultracentrifugation method. The viruses for AAV-LRRC4 or AAV-CON were injected intravenously at the tail at a dose of 5×10^{12} vector genome (vg)/kg 7 days before immunization.

Real-time PCR analysis

Total RNA was extracted from tissues or cultured cells by using the TRI reagent (Molecular Research Center, MRC) according to the manufacturer's instructions. 2 μg total RNA was reverse transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) according to the manufacturer's instructions. Real-time PCR analyses were performed using with SYBR Green PCR kits (Bimake) following manufacturer's instructions. The primers used were as described in Suppl. Table 1.

Western blotting

Western blotting analysis was conducted according to standard procedures. Tissues or cultured cells were collected and lysed in lysis buffer (300 mM NaCl, 50 mM Tris pH 8.0, 0.4 % NP-40, 10 mM MgCl2, and 2.5 mM CaCl2) supplemented with protease inhibitors cocktail (Bimake) and phosphatase inhibitors (Bimake). 30 μg protein samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF, Millipore) membrane, blocked by 5% non-fat milk, and incubated with different primary antibodies and secondary antibodies. Signals were detected using chemiluminescent HRP substrate (Millipore). The primary antibodies used were as follows: LRRC4, Rab7b (Abcam), NF-κB p65, ERK1/2, p-ERK1/2 (Thr202/Tyr204), p-AKT (Ser473) (Cell Signaling Technology), AKT and GAPDH (Proteintech).

Immunohistochemistry

Mice were anesthetized using barbital sodium and then intracardially perfused with normal saline and 4% paraformaldehyde for fixation. Spinal cords were dissected, fixed in 4% paraformaldehyde at 4℃ overnight, and sliced in 4 μm while pathological changes were examined with hematoxylin and eosin (H&E) staining and Luxol Fast Blue (LFB) staining. For immunohistochemistry analysis, sections were blocked with 3% hydrogen peroxide for 10 min and normal goat serum for 1 h at room temperature. The sections were then incubated at 4℃ overnight with anti-GFAP (Abcam), Iba1 (Thermo Scientific) antibodies, biotinylated secondary antibody (Maxim Biotechnologies) for 20 min followed by the treatment of streptavidin-conjugated HRP (Maxim Biotechnologies) for 10 min. Detection was enabled with 3,3-diaminobenzidine (DAB; Maxim Biotechnologies) treatment, while hematoxylin was used for counterstaining. The cell number of infiltrated lymphocytes or microglias or astrocytes in spinal cords were counted by Image-pro Plus software.

Flow cytometry

Spleens and blood from the mice were harvested, and a single cell suspension was prepared. For quantification of the number of Th1/Th2/Th17 cells, cells were stimulated with PMA (50 ng/ml, Sigma), activation cocktail (750 ng/ml, BioLegend), and monensin (2 μmol/L, BioLegend). Cells were thereafter stained with FITC-conjugated anti-CD4 antibodies (BioLegend), permeabilized with permeabilization solution, and then stained with PE/Cy7-conjugated anti-IFN-γ, PE-conjugated anti-IL-4 and APC-conjugated anti-IL-17A (BioLegend). For detection of Treg cells, the cells were stained with PE-conjugated anti-CD25 and Alexa FluorR 647-conjugated anti-Foxp3 (BioLegend). Fluorescence was examined with FACS Canto II (BD Biosciences). All flow cytometry data presented herein were gated by the use of $CD4^+$,

while IFN-γ^+ cells represented Th1 cells, IL-4^+ cells represented Th2 cells, IL-$17A^+$ cells represented Th17 cells, and $CD25^+Foxp3^+$ cells represented Treg cells. Data were analyzed with the FlowJo software.

Statistical analysis

All the experiments were repeated at least three times, and the representative data are shown. The statistical analysis was performed using GraphPad Prism 5 and SPSS version 17.0. Data analysis was performed with Student's t test and one-way ANOVA and presented as the mean ± SEM. P values less than 0.05 were considered significant.

Results

Down-regulation of spinal cord LRRC4 during EAE pathogenesis

To determine if LRRC4 has a role in CNS autoimmunity, we induced EAE in C57BL/6 mice using myelin oligodendrocyte glycoprotein (MOG_{35-55}) and subsequently determined the mRNA and protein levels of LRRC4 before (naive) or 15 days after immunization. LRRC4 mRNA expression in the spinal cords of the immunized mice was significantly down-regulated relative to the healthy mice, while little difference was detected in the brains (Figure 1A). We also found that the LRRC4 protein level was significantly reduced during the development of EAE in the spinal cords (Figures 1B, C). The down-regulation of LRRC4 during EAE pathogenesis indicated that LRRC4 may play a role in the process.

LRRC4 deletion leads to exacerbated EAE progression

The down-regulation of LRRC4 during EAE pathogenesis led us to assess the effect of LRRC4 deletion on EAE. We constructed mice with LRRC4 deletion ($LRRC4^{-/-}$) and subsequently induced EAE in $LRRC4^{-/-}$ mice and the wild type (WT) littermates (Figures S1A-C). Consistent with the earlier report by Zhang et al, $LRRC4^{-/-}$ mice showed decreased threshold of auditory brainstem response (ABR) (Figures S1D, E), suggesting reduction in synchronization of auditory neurons in the spiral ganglia [40]. In addition, $LRRC4^{-/-}$ mice exhibited much more exacerbated disease development than WT mice with the difference peaking at day 16 (Figure 2A). Furthermore, $LRRC4^{-/-}$ mice showed accelerated loss of body mass (Figure 2B). Experiments with hematoxylin-eosin (H&E) staining of the spinal cords collected at day 15 after immunization revealed increased lymphocyte infiltration in the spinal cords of $LRRC4^{-/-}$ mice compared with those of WT mice (Figure 2C). We also used luxol fast blue staining and found more severe demyelination in $LRRC4^{-/-}$ mice than in WT mice. Finally, as shown by immunohistochemistry analysis with anti-Iba1 and GFAP antibodies, there was an increased density of microglia and astrocytes around demyelinated lesion sites in $LRRC4^{-/-}$ mice compared with WT mice. Thus, the loss of LRRC4 leads to aggravated demyelination and inflammation in EAE, implying a protective role for LRRC4 in EAE.

LRRC4 deletion disrupts the balance between Th1 and Treg cells

It is well established that helper T cells have a strong influence on the progression of EAE. We therefore examined whether LRRC4 deletion causes any alterations in the cell populations during EAE development. To do so, we measured the proportion of cytokine-producing cells in the spleen and blood from LRRC4$^{-/-}$ and WT mice (both naïve mice and mice with EAE induction) 15 days after immunization by using flow cytometry. Intracellular staining of IL-4, IL-17A and IFN-γ showed that LRRC4 deletion did not change the proportion of Th2 (CD4^{+} IL-4^{+}) cells or Th17 (CD4^{+} IL-17A^{+}) cells in the spleen and blood (Figures 3A, B) whether or not EAE occur. However, although LRRC4 deletion failed to change the proportion of Th1 (CD4^{+} IFN-γ^{+}) cells in the spleen and blood of naïve mice, it increased the proportion of Th1 cells in the spleen and blood of EAE mice (Figure 3C). We next assessed the effect of LRRC4 deletion on regulatory T (Treg) cells, which reportedly play a critical role in the regulation of immune processes during EAE. Little difference in Treg cells (CD4^{+}CD25^{+}FoxP3^{+}) was found between naïve LRRC4$^{-/-}$ and WT mice in the spleen. However, a marked reduction in Treg cells was seen in the spleen of LRRC4$^{-/-}$ EAE mice compared with WT EAE mice (Figure 3D). Thus, LRRC4 deletion disrupts the balance between Th1 and Treg cells in EAE and causes a shift to Th1 cells, which may contribute to EAE progression.

RNA-seq analysis revealed a role of rab7b in EAE

To obtain a more comprehensive understanding of the distinct molecular programs between WT mice and LRRC4$^{-/-}$ mice, we isolated the spinal cords from WT and LRRC4$^{-/-}$ mice before or 15 days after immunization and analyzed them by using RNA sequencing (RNA-seq). The top 50 genes in WT versus LRRC4$^{-/-}$ mice were selected for cluster analysis (Figure 4A). We performed GO and KEGG analyses to investigate the molecular function and biological pathways of the differentially expressed genes (DEGs). The top 10 enriched GO terms and KEGG pathways of the up-regulated and down-regulated DEGs, according to the percentage of genes, were selected (Figure 4). For instance, Figure 4B showed that cellular response to IFN-γ was among the enriched GO terms of up-regulated DEGs, consistent with earlier findings that IFN-γ$^{-/-}$ and IFN-γR$^{-/-}$ mice show more severe and chronic-progressive course of EAE [41]. We also compared common DEGs between the up-regulated and down-regulated DEGs, and as shown in Figure 4D and 4E, the number of shared DEGs in GO terms between up-regulated and down-regulated DEG was small. Among these DEGs between WT and LRRC4$^{-/-}$ mice, Rab7b was of special interest, because it was recently shown that Rab7b is involved in the regulation of phorbol 12-myristate 13-acetate (PMA)-induced activation of NF-κB and enhances the production of IL-6 [42] . We examined Rab7b expression in the spinal cords of WT and LRRC4$^{-/-}$ mice and found that both the mRNA and protein levels were elevated in LRRC4$^{-/-}$ mice compared with WT mice (Figures 4F, G). We also assessed Rab7b mRNA expression in the spinal cords of naïve mice and EAE mice and detected increased Rab7b mRNA expression in the spinal cords of EAE mice (Figure 4H). Furthermore, Rab7b mRNA levels correlated inversely with LRRC4 expression (Figure 4I). Thus, Rab7b expression in the spinal cords of EAE mice is elevated upon LRRC4 deletion, raising the possibility that Rab7b may be involved in EAE pathogenesis.

LRRC4 deletion up-regulates NF-κB in EAE mice

After showing that LRRC4 deletion caused elevated inflammatory responses in the spinal cords and exacerbation of EAE pathogenesis, we sought to dissect the underlying molecular mechanisms. LRRC4 has been reported to inhibit NF-κB activation and regulate the ERK/MAPK and PI3K/AKT pathway [32]. NF-κB plays an important role in controlling expression of genes including pro-inflammatory cytokines, chemokines, nitric oxide synthases and cell adhesion molecules related to the pathogenesis of autoimmunity [43]. The ERK/MAPK and PI3K/AKT signaling pathways reportedly modulate Tregs and Th17 cells differentiation, while the PI3K/AKT signaling pathway promotes oligodendrocyte differentiation and myelination [44]. The well established role of NF-κB, ERK/MAPK and PI3K/AKT signaling in inflammation and EAE pathogenesis led us to investigate whether they might play a role in mediating the function of LRRC4 in EAE.

We first assessed the levels of NF-κB, p-AKT and p-ERK1/2 in LRRC4$^{-/-}$ and WT mice under naïve and EAE conditions. Under naïve conditions, NF-κB p65 was up-regulated, while the ratio of p-AKT/AKT was reduced in the spinal cords of LRRC4$^{-/-}$ mice compared with that of WT mice (Figures 5A). After immunization, LRRC4$^{-/-}$ mice expressed higher levels of NF-κB p65 than WT mice, at clinical score 2 and 3 in the spinal cords (Figures 5B). The ratio of p-AKT/AKT decreased in spinal cords of LRRC4$^{-/-}$ mice at score 2 and 3 compared with that of WT mice. In contrast, the ratio of p-ERK/ERK exhibited little difference between WT and LRRC4$^{-/-}$ mice, while increasing at score 2 and decreasing at score 3 in spinal cords of LRRC4$^{-/-}$ mice. We also examined the levels of various cytokines. We found that the levels of IL-6 mRNA were elevated in the spinal cords of LRRC4$^{-/-}$ mice with different scores of EAE when compared with WT mice (Figures 5C). In addition, IFN-γ mRNA levels were enhanced at score 2 and 3 of EAE in spinal cords of LRRC4$^{-/-}$ mice. In contrast, IL-10 mRNA levels were reduced at the score 1 and 2 of EAE in spinal cords, while increasing at the score 3 in LRRC4$^{-/-}$ mice. Meanwhile, TNF-α mRNA levels were reduced at the score 1 and 2 in spinal cords, while TGF-β levels decreased at the score 2 of EAE spinal cords in LRRC4$^{-/-}$ mice. In contrast, IL-17A mRNA levels exhibited no significant difference in spinal cords between LRRC4$^{-/-}$ and WT mice. Thus, LRRC4 deletion induces an up-regulation of NF-κB, causing alterations in the levels of inflammation-related cytokines in the spinal cords, which may contribute to accelerated progression of EAE.

Rab7b mediates NF-κB up-regulation

The elevated Rab7b expression in LRRC4$^{-/-}$ mice susceptible to EAE led us to determine the molecular link between LRRC4, Rab7b and NF-κB. To do so, we isolated mouse neurons of WT and LRRC4$^{-/-}$ mice and subsequently determined Rab7b and NF-κB p65 expression. As expected, Rab7b and NF-κB p65 were up-regulated in LRRC4$^{-/-}$ neurons compared with LRRC4$^{+/+}$ neurons (Figure 6A). Ectopic expression of LRRC4 with the pcDNA3.1-LRRC4 expression vector induced a reduction of Rab7b and NF-κB p65 expression (Figure 6B). RNAi-mediated knockdown of Rab7b in LRRC4$^{+/+}$ neurons caused NF-κB p65 expression to reduce but not in LRRC4$^{-/-}$ neurons (Figure 6C). Ectopic expression of Rab7b had little effect on NF-κB p65 expression but strongly inhibited NF-κB p65 expression when Rab7b was co-transfected with LRRC4 (Figure 6D). Thus, Rab7b regulates NF-κB in the presence of LRRC4 but plays no such role in LRRC4-

deficient neurons. These results suggest that Rab7b might serve as a downstream effector of LRRC4 in the regulation of NF-κB.

Ectopic LRRC4 expression alleviates EAE progression

Having shown that LRRC4 is down-regulated in EAE mice and that LRRC4 deletion leads to aggravated EAE progression, we asked whether ectopic LRRC4 expression could rescue the pathological defects of EAE. We injected adeno-associated virus (AAV) vector intravenously to ectopically express LRRC4 (AAV-LRRC4) or control virus vector (AAV-CON) in the mice and subsequently induced EAE with MOG_{35-55} 10 days after injection. Clinical scores of EAE were measured and documented daily, showing that AAV-LRRC4 injection alleviated the progression of EAE and body mass loss compared with AAV-CON injection (Figures 7A, B). At the tissue level, AAV-LRRC4 injection caused lymphocyte infiltration into spinal cords to decrease in EAE mice, as revealed by H&E staining (Figure 7C). In addition, experiments with luxol fast blue (LFB) staining showed that overexpression of LRRC4 decreased demyelination in the spinal cords of EAE mice, while the density of microglia and astrocytes were also reduced around demyelinated lesion sites in the spinal cords of mice injected with AAV-LRRC4, as illustrated by immunohistochemical analysis using anti-Iba1 and GFAP antibody (Figure 7C). Thus, LRRC4 ectopic expression alleviates the defects in demyelination and autoimmunity caused by EAE. As such, AAV-LRRC4 virus may be potentially used as a therapeutic tool for treating MS patients.

Consistent with our earlier findings, NF-κB p65 was down-regulated in the spinal cords of mice injected with AAV-LRRC4. In contrast, the levels of p-ERK1/2 and p-AKT were unaltered in the spinal cords with AAV-LRRC4 injection, indicating that the down-regulation of NF-κB p65 was specific (Figure 7D). These results again suggested that NF-κB serves as a key downstream signaling molecule to mediate the function of LRRC4 in protecting mice from CNS autoimmunity.

Discussion

LRRC4, which was first cloned by our laboratory, is specifically expressed in the central nervous system [45]. Our previous studies showed that LRRC4 can inhibit cytokine-induced NF-κB activation in glioma cells and that LRRC4 regulates the ERK/MAPK and the PI3K/AKT signaling pathway and therefore modulates cell proliferation, migration, and invasion [46]. LRRC4 (NGL-2) serves as the receptor for netrin-G2, and LRRC4 (NGL-2) interacts with netrin-G2 to play a role in synapse formation [26]. LRRC4 promotes hippocampal neuron development, while knock-down of LRRC4 reduces dendritic spine density in the hippocampal CA1 region [29]. In this study, we found that LRRC4 is down-regulated in spinal cords of EAE mice, while deletion of LRRC4 accelerates infiltration of leukocytes into the spinal cords and disease exacerbation in vivo. We further showed that LRRC4 deletion disrupts the balance between Th1 cells and Treg cells and causes a shift toward Th1 cells. At a mechanistic level, we found that deficiency of LRRC4 induces elevated NF-κB p65 expression and does so by up-regulating Rab7b, while ectopic expression of

LRRC4 alleviates the clinical symptoms of EAE mice and protects the CNS from immune damages. Together, we establish, for the first time, a critical role of LRRC4 in the progression of EAE and provide novel mechanistic insights into EAE development.

EAE is an inflammatory disorder characterized by demyelination of white matter accompanied by neurodegenerative lesions. Changes in synapse have been found in the CNS of EAE mice including the spinal cord, hippocampus, cerebellum, striatum, and cortex [47]. The inflammatory environment of the CNS may be the main cause of neurological changes and synaptic loss. The oxidative stress, mitochondrial damage and ion channel dysfunction caused by chronic inflammation have a continuous effect on neurons, leading to neuron death [48]. Our experiments with RNA sequencing results showed that LRRC4 deletion mainly affects IFN-γ response, neuropeptide signaling, hippocampal development, learning, memory and cognition, suggesting that LRRC4 may play a role in cognitive function and immune responses in EAE mice. LRRC4 is mainly expressed in neuronal cells and is down-regulated in spinal cords of EAE mice, which is likely caused by neuronal damage and degeneration. We further constructed the EAE model in WT mice and LRRC4$^{-/-}$ mice and discovered that the degree of disease is aggravated in LRRC4$^{-/-}$ mice accompanied by increased demyelination, enhanced lymphocytes infiltration into the spinal cords, and elevated microglia and astrocyte proliferation and activation. Our results strongly suggest that LRRC4 plays a protective role in the pathogenesis of EAE.

We found that NF-κB p65 expression is elevated while the ratio of p-AKT/AKT is reduced in the spinal cords of LRRC4$^{-/-}$ mice, suggesting that NF-κB and PI3K/AKT signaling act as key downstream effectors of LRRC4 in modulating EAE progression. The NF-κB signaling cascade plays a critical role in the regulation of immune and inflammatory responses and the function of resident cells of the CNS that are implicated in the pathogenesis of MS and EAE [49]. NF-κB is activated in the CNS of EAE and persists throughout the development of the disease [50]. Upon activation, NF-κB induces the expression of inflammatory factors and triggers the immune responses during EAE progression [51]. Meanwhile, the PI3K/AKT signaling pathway in oligodendrocytes has a critical function in the myelination process after demyelinating injury in EAE [52]. Our findings suggest the following model: LRRC4 deletion causes up-regulation of NF-κB p65 and down-regulation of p-AKT/AKT, leading to altered secretion of inflammatory factors such as IL-6, IFN-γ, IL-10, TGF-β and TNF-α. The secretion of inflammatory factors in turn disrupts the balance between Th1 and Treg cells, increases lymphocytes infiltration and gliosis in the CNS, decreases formation of myelin, and ultimately accelerates the pathogenesis of EAE.

Our model is supported (direct or indirect) by several lines of evidence from previously published studies. For instance, the level of IL-6 is elevated in the central nervous system of MS patients and EAE mice, while IL-6 inhibitors can inhibit the differentiation of Th1 and Th17 cells and reduce the pathogenesis of EAE [53]. IL-6 also inhibits the function of Treg cells and TGF-β-induced Treg cell differentiation and regulates the balance of Treg/Th17 cells [54]. In addition, it has been shown previously that IFN-γ plays a role in the pathogenesis of MS and EAE [55]. Specifically, IFN-γ is elevated in the serum of MS patients, while administration of IFN-γ to MS patients in a clinical trial aggravates the development of the disease [56]. In addition, IFN-γ promotes development of Th1 cells but inhibits Th17 cell differentiation from naive

precursor cells [57]. Thus, these earlier findings support our notion that the increase in Th1 cells and the decrease in Treg cells in EAE mice with LRRC4 deletion may be attributed to elevated levels of IL-6 and IFN-γ.

Our model is also in agreement with the previously established role of TGF-β, IL-10 and TNF-α, all of which are down-regulated in the EAE mice with LRRC4 deletion. TGF-β reportedly plays a role in regulating T cell differentiation and function [58]. For instance, TGF-β in combination with IL-2 and retinoic acid can induce differentiation of primary $CD4^+$ cells into Treg cells, while TGF-β in combination with IL-6 can promote the differentiation of Th17 cells [59, 60]. In contrast, TGF-β functions as an inhibitor of Th1 cell differentiation [61]. In addition, myelin immune-reactive T cells stimulated by TGF-β are unable to differentiate into effector T cells and cannot induce EAE [62]. IL-10 is an anti-inflammatory cytokine and can inhibit inflammation in autoimmune diseases. IL-10-deficient mice develop more severe EAE, indicating that IL-10 has a protective role in the pathogenesis of EAE [63]. The immunosuppressive function of IL-10 involves the regulation of antigen-presenting cells (APCs), inhibition of T cell proliferation, and maintenance of Treg cell function [64]. As such, the reduction of TGF-β and IL-10 levels in EAE mice with LRRC4 deletion, as shown in our present study, may inhibit Treg cell differentiation and function. It has been shown previously that the level of TNF-α is elevated in the CNS of MS patients and EAE models [65]. Antibodies against TNF-α or TNFRI can inhibit the development of EAE [66]. Deletion of TNF-α in mice delays the onset of EAE without changing in the incidence and severity of EAE [67]. However, TNFR2 deletion in mice causes an increase in inflammatory responses, demyelination, and the severity of EAE. Thus, TNFR2 promotes the function of oligodendrocytes, inhibits lymphocyte infiltration, and plays a protective role in EAE [68]. As such, the reduced TNF-α expression we have observed in EAE mice with LRRC4 deletion may explain the elevated lymphocyte infiltration.

Our experiments with RNA seq showed that Rab7b expression is elevated in mice with LRRC4 deletion, indicating that Rab7b may be involved in the pathogenesis of EAE. Rab7b is a member of small GTPase family and regulates transport between various compartments of the endomembrane system in eukaryotic cells [69]. Earlier findings demonstrated that Rab7b attenuates TLR4 and TLR9 expression and inhibits NF-κB while decreasing the production of TNF-α, IL-6, NO and IFN-β in macrophages [70]. However, a separate study showed that Rab7b promotes PMA-induced NF-κB activation and IL-6 production in megakaryocytes [71]. Rab7b is reportedly up-regulated in the transient middle cerebral artery occlusion (tMCAO) model, while overexpression of Rab7b in the brain can reduce cerebral infarction of tMCAO and improve neurological functions [72]. In the current study, we found that Rab7b is up-regulated in the spinal cords of EAE mice, while overexpression of LRRC4 suppresses Rab7b expression. The effect of LRRC4 on NF-κB p65 is dependent on Rab7b, suggesting that Rab7b might serve as a critical downstream target of LRRC4 to exert LRRC4 protective function from EAE development.

Conclusions

In summary, our data demonstrate the critical role of LRRC4 in EAE progression. LRRC4 decrease in spinal cords of EAE mice. LRRC4 deficiency increases NF-B p65 and decreased p-AKT, leads to up-regulation of IFN-γ and IL-6, and down-regulation of IL-10 and TGF-β, results in increased Th1 cells and decreased Treg cells, and aggravates demyelination and inflammation in EAE mice. LRRC4 regulates Rab7b expression may involve in the regulation of NF-κB and pathology of EAE. Restoring LRRC4 represents a new strategy for preventing autoimmunity in EAE.

Supplementary Information

Additional file 1. Supplementary result. **Figure S1** The auditory brainstem response (ABR) of LRRC4$^{-/-}$ mice compared with that of the WT control. **A** Schemeatic diagrams showing construction of LRRC4$^{-/-}$ mice. **B** Real-time PCR analysis of LRRC4 mRNA levels in brains and spinal cords of WT or LRRC4$^{-/-}$ mice. **C** Western blotting analysis of LRRC4 protein levels in brains and spinal cords of WT or LRRC4$^{-/-}$ mice. **D** Representative diagrams of ABR of WT or LRRC4$^{-/-}$ mice showing waves at different decibel levels of click stimuli. **E** The threshold of ABR of WT or LRRC4$^{-/-}$ mice. Results are shown as means ± SEM (n=8). $^{**}p < 0.01$. **Table S1**. A list of primers used in real-time PCR analysis.

Abbreviations

LRRC4: leucine rich repeat containing 4; NGL-2: netrin-G ligand-2; ASD: autism spectrum disorder; MS: multiple sclerosis; CNS: central nervous system; EAE: experimental autoimmune encephalomyelitis; MOG: oligodendrocyte glycoprotein peptide; LFB: luxol fast blue; AAV: adeno-associated virus; TNF: tumor necrosis factor; ROR-γ: tretinoid-related orphan receptor-γt; GM-CSF：granulocyte-macrophage colony-stimulating factor; MBP：myelin basic protein; NMDARs: N-Methyl-D-aspartate receptors; PDPK1: phosphoinositide-dependent protein kinase 1; ABR: auditory brainstem response.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

YZ mainly performed the project and wrote the manuscript. DL, QMZ, JBF and HJF assisted in the experiments. ZHL and BX assisted in approving the final version of the manuscript. HY and MHW developed the experimental design and revised the manuscript. All authors have read and approved the final submitted version of the manuscript.

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Availability of data and materials

The data used in this article are available to researchers subject to confidentiality if necessary.

Ethics approval and consent to participate

The animal experiments were approved by the Joint Ethics Committee of the Central South University Health Authority. All protocols were performed in accordance with the guidelines for the care of laboratory animals and the Animal Care and Use Committee of Central South University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

LRRC4 is down-regulated in spinal cords during EAE. A Real-time PCR analysis of LRRC4 mRNA levels in the brains and spinal cords of healthy (naive) and EAE mice. All values are normalized to the level (=1) of one of healthy mice. Results are shown as means ± SEM (n=4). B Western blotting analysis of LRRC4 protein levels in the brains and spinal cords of healthy and EAE mice. C Quantification of the Western blotting images in (B). All values are represented as the values of LRRC4 protein levels divided by those of GAPDH. Results are shown as means ± SEM (n=4). **p < 0.01.

Figure 2

LRRC4 deletion leads to exacerbated EAE progression. A Clinical scores of MOG-immunized WT and LRRC4−/− mice. Data represent the mean clinical scores of 10 mice per group ± SEM. B Body mass of WT and LRRC4−/− mice. Data represent the mean body mass of 10 mice per group ± SEM. C Histopathological analysis of neuroinflammation and demyelination in spinal cords of WT and LRRC4−/

− mice 15 days after EAE induction. Sections were stained by the use of H&E staining, LFP staining and immumohistochemical staining with anti-IBA1 and -GFAP antibody. *p < 0.05, **p < 0.01.

Figure 3

LRRC4 deletion disrupts the balance between Th1 and Treg cells. A Flow cytometry of Th2 (CD4+ IL-4+) cells of WT and LRRC4−/− mice before or 15 days after EAE induction in spleens (left) or blood (right). B Flow cytometry of Th17 (CD4+ IL-17A+) cells of WT and LRRC4−/− mice before or 15 days after EAE induction in spleens (left) or blood (right). C Flow cytometry of Th1 (CD4+ IFN-γ+) cells of WT and LRRC4−/− mice before or 15 days after EAE induction in spleens (left) or blood (right). D Flow cytometry of Treg (CD4+ CD25+ Foxp3+) cells of WT and LRRC4−/− mice before or 15 days after EAE induction in spleens. *p < 0.05, **p < 0.01.

Figure 4

RNA seq analysis revealed a role of Rab7b in EAE. A A heatmap showing DEGs in spinal cord of WT mice versus LRRC4-/- mice from cluster analysis of RNA-seq. B The top 10 GO terms of up-regulated and down-regulated DEGs between WT and LRRC4-/- mice. C The top 10 KEGG pathways of up-regulated and down-regulated DEGs between WT and LRRC4-/- mice. D The comparison of GO terms between up-regulated DEGs and down-regulated DEGs. E Comparison of KEGG pathways between up-regulated DEGs and down-regulated DEGs. F Rab7b mRNA levels of spinal cords in WT and LRRC4-/- mice as determined with real-time PCR. Results are shown as means ± SEM (n=4). G Rab7b protein levels of spinal cords in WT and LRRC4-/- mice as assessed with Western blotting. H Rab7b mRNA levels of the spinal cords in healthy and EAE mice assessed with real-time PCR. Results are shown as means ± SEM (n=4). I Correlation analysis of Rab7b and LRRC4 mRNA levels in healthy and EAE mice. *p < 0.05, **p < 0.01.

Figure 5

LRRC4 deletion up-regulates NF-κB in EAE mice. A Western blotting analysis of spinal cord lysates from WT and LRRC4−/− mice and quantification of the Western blotting images. Each lane represents samples from an individual mouse. B Western blotting analysis of spinal cord lysates in WT and LRRC4−/− mice that are healthy or with different clinical scores and qantification of the Western blotting images. C Real-time PCR analysis of cytokine mRNA levels of spinal cords in WT and LRRC4−/− mice that are healthy or with different clinical scores. Results are shown as means ± SEM (n=3). *p < 0.05, **p < 0.01.

Figure 6

Rab7b mediates NF-κB up-regulation. A Western blotting analysis of various proteins in LRRC4+/+ and LRRC4-/- neurons. B Western blotting analysis of various proteins in LRRC4+/+ and LRRC4-/- neurons after ectopic expression of LRRC4. C Western blotting analysis of various proteins in LRRC4+/+ and LRRC4-/- neurons after knockdown of Rab7b. D Western blotting analysis of various proteins in LRRC4+/+ and LRRC4-/- neurons co-transfected with Rab7b and LRRC4.

Figure 7

Ectopic LRRC4 expression alleviates EAE progression. A Clinical scores of MOG-immunized WT mice after intravenously injected with adeno-associated virus (AAV) vector containing LRRC4 (AAV-LRRC4) or control virus vector (AAV-CON). Data represent the mean clinical scores of 10 mice per group ± SEM. B Body mass of EAE mice after intravenously injected with AAV-LRRC4 and AAV-CON. Data represent the mean body mass of 10 mice per group ± SEM. C Histopathological analysis of neuroinflammation and demyelination in spinal cords of mice 15 days after EAE induction. Sections were subjected to H&E staining, LFP staining and immumohistochemical staining with anti-IBA1 and anti-GFAP antibody. D Western blotting analysis of various spinal cords proteins in EAE mice after intravenously injected AAV-LRRC4 and AAV-CON.

Supplementary Files

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