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Title: Leucine rich repeats and calponin homology domain containing 1 inhibits microglia-mediated neuroinflammation in a rat traumatic spinal cord injury model

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Abstract

Background: Spinal cord injury (SCI) triggers the primary mechanical injury and secondary inflammation-mediated injury. Neuroinflammation-mediated insult causes secondary and extensive neurological damage after SCI. Microglia play a pivotal role in the initiation and progression of post-SCI neuroinflammation.

Methods: To elucidate the significance of LRCH1 to microglial functions, we applied lentivirus-induced LRCH1 knockdown in primary microglia culture, and tested the role of LRCH1 in microglia-mediated inflammatory reaction both *in vitro* and in a rat SCI model.

Results: We found that LRCH1 was down-regulated in microglia after traumatic SCI. LRCH1 knockdown increased the production of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 after in vitro priming with lipopolysaccharide and adenosine triphosphate. Furthermore, LRCH1 knockdown promoted the priming-induced microglial polarization towards the pro-inflammatory M1 type, as demonstrated by increased differentiation into inducible nitric oxide synthase (iNOS)+ microglia. LRCH1 knockdown also enhanced microglia-mediated N27 neuron death after priming. Further analysis revealed that LRCH1 knockdown increased priming-induced activation of p38 mitogen-activated protein kinase (MAPK) and Erk1/2 signaling, which are crucial for M1 polarization of microglia. When LRCH1-knockdown microglia were adoptively injected into rat spinal cords, they enhanced post-SCI production of

pro-inflammatory cytokines, increased SCI-induced recruitment of leukocytes, aggravated SCI-induced tissue damage and neuronal death, and worsened the locomotor function.

Conclusion: Our study reveals for the first time that LRCH1 serves as a negative regulator of microglia-mediated neuroinflammation after SCI, and provides clues for developing novel therapeutic approaches against SCI.

Keywords: Leucine rich repeats and calponin homology domain containing 1; spinal cord injury; microglia; neuroinflammation; mitogen-activated protein kinase

Background

Spinal cord injury (SCI) triggers the primary mechanical injury and secondary inflammation-mediated injury [1]. The mechanical trauma of the spinal cord tissue initiates the primary injury, while the secondary neuroinflammatory reactions, which mediate additional and extensive neurological injury, takes place following the primary injury. Controlling the detrimental acute neuroinflammation could be a therapeutic strategy to confine the injury and promote functional recovery.

Microglia, a spinal cord-resident immune cell type, play a pivotal role in the post-SCI secondary injury. Microglia are usually located in the margins of the lesion core [2]. It is considered that classical activation microglia are neurotoxic and growth inhibitory through producing high levels of pro-inflammatory molecules [2, 3]. However, microglia may exert anti-inflammatory and neuroprotective effects on injured spinal cords [4, 5]. The exact role of microglia might change over time in different stages of SCI. Carefully tuning the microglia function is necessary to treat SCI to achieve better recovery.

Leucine rich repeats and calponin homology domain containing 1 (LRCH1) is a relatively novel gene encodes a protein with a leucine-rich repeat and a calponin homology domain. The significance of LRCH1 protein remains a mystery. Previous studies suggest that a genetic variant in LRCH1 is associated with knee osteoarthritis [6,

7]. A recent study indicates that LRCH1 act to restrain PKCα-dedicator of cytokinesis 8 (DOCK8)-Cdc42 module-mediated T cell migration in experimental autoimmune encephalomyelitis, through competing with Cdc42 for interaction with DOCK8. However, no further study has been conducted to show the functions of LRCH1 in other cell types or on other signal pathways.

In the current research, we found that LRCH1 was down-regulated in microglia after traumatic SCI. To elucidate the significance of LRCH1 in microglial functions, we applied lentivirus-induced LRCH1 knockdown in primary microglia. Our study reveals for the first time that LRCH1 serves as a negative regulator of microglia-mediated neuroinflammation after SCI, and provides clues for developing novel therapeutic approaches against SCI.

Materials and methods

Rat SCI model

The animal study was approved by the Animal Care and Use Committee of the Second Affiliated Hospital of Fujian Medical University and Shenzhen Pingle Orthopedic Hospital. The surgical procedures were conducted in compliance with the institutional guidelines for laboratory animal usage in neuroscience and behavioral research. Male Sprague-Dawley rats (10-week old, 250~300g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were housed in the pathogen-free condition.

The SCI model was established by the following steps. Rats were anesthetized via inhaling 3% of isoflurane at the flow rate of 1 L/min. Midline skin incisions were cut and the T12 spinous processes were exposed. A laminectomy was performed at T12. The compression was conducted by placing the base of a compression platform (area 2×5 mm^2) onto the exposed spinal cord. A 50-g weight was then placed steadily to the platform for 5 min. The platform was then removed and the muscles and skins were sutured. Rats were transferred to the cages after they regained the righting reflex. The urinary retention was relieved by twice-daily bladder expressions. The sham-operated rats received every surgical step except for the spinal cord compression.

Immune cell enrichment from spinal cords

Rats were anesthetized by inhalation of 3% isoflurane. Each rat was transcardially perfused with 200 ml of ice-cold phosphate-buffered saline (PBS). The spinal cord was taken, minced into approximately 1 mm^3 pieces, and treated with RPMI1640 supplemented with 2 mg/ml collagenase IV (Thermo Fisher Scientific), 200 U/ml DNase I (Sigma-Aldrich), 10% fetal bovine serum (FBS) and 2.0 mM CaCl$_2$ for 30 min at 37 ^{o}C in an incubator. Digested spinal cord tissues were then filtered through 70-µm cell strainers and overlaid onto 20% Percoll (GE Healthcare), followed by centrifugation at 250g for 10 min. The cell pellet was resuspended in PBS or culture medium before further experiments. In some experiments, spinal cords of 3 to 5 rats were pooled to collect enough cells.

Flow Cytometry and cell sorting

The following fluorophore-conjugated antibodies were purchased from Biolegend: APC anti-Granulocyte (RP-1), PE anti-CD45 (OX-1), APC anti-TCRαβ (R73), PE anti-TCRγδ (V65) and PE-Cy7 anti-CD11b (OX-42). Polyclonal APC anti-Arginase-1 antibody (IC5868A) was purchased from Novus Biologicals. Unconjugated polyclonal anti-inducible nitric oxide synthase (iNOS) antibody (ab15323) was purchased from Abcam. For cell surface marker staining, cells were stained with 2 µg/ml each antibody on ice for 30 minutes. Dead cells were excluded by staining with 1 µg/ml propidium iodide (PI, ebioscience). For intracellular staining, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 90% ice-cold

methanol for 30 minutes, followed by incubation with 1:100 diluted anti-Arginase-1 antibody and 10 μg/ml anti-iNOS antibody for 1 hour at room temperature. After three washes with PBS, cells were incubated with 1 μg/ml Alexa Fluor® 594 goat anti-rabbit IgG (ab150080, Abcam) for 30 minutes. For apoptosis assay, cells were stained with PI and Annexin V following the instructions of the APC Annexin V Apoptosis Detection Kit with PI (Biolegend). Cells were then washed with PBS twice and loaded either onto either a BD LSR-II flow cytometer (BD Biosciences) for analysis or a BD FACSAria II sorter (BD Biosciences) for sorting.

Quantitative RT-PCR (qRT-PCR)

Cellular RNAs were purified using the ARCTURUS PicoPure RNA Isolation Kit (Thermo Fisher Scientific). Tissue RNAs were extracted using the Trizol reagent (Thermo Fisher Scientific) following the vendor's manual. SuperScript® III First-Strand Synthesis Kit (Thermo Fisher Scientific) was used to prepare cDNA. On a 7300 qPCR thermocycler (Invitrogen), quantitative PCR was achieved using Fast SYBR®Green Master Mix (Thermo Fisher Scientific) at the following steps: $50^{o}C$ for 2 min, then $94^{o}C$ for 10 min, and then 40 cycles of 30 sec at $94^{o}C$ and 1 min at $60^{o}C$. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers are listed in **Supplementary Table 1**.

Primary rat microglia culture

Primary microglia were obtained by isolation from mixed glial cell

cultures of 1–day-old neonatal rat brains, according to previous publications [8-10]. Briefly, after removing the meninges, the cerebral cortices were incubated in 0.25% trypsin-EDTA (Invitrogen) for 30 minutes at 37 °C while shaking at 50 rpm on an orbital shaker. The cortices were then mechanically dissociated in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS and 200 U/ml DNase I until no tissue clump was seen. The whole cortical cells were passed through a 70-µm cell strainer and washed with DMEM once. The cells were cultured in DMEM supplemented with 10% FBS and the medium was replaced every 3 days. Two weeks later, the culture was mildly trypsinized with 1:4 diluted 0.25% trypsin-EDTA for 20 minutes at room temperature. The floating cells were carefully aspirated and microglia which remained attaching to the bottom were kept for further experiments.

Lentiviral packaging and transduction

Lrch1-set siRNA/shRNA/RNAi Lentivector (i050632) and the corresponding control lentivector piLenti-siRNA-GFP were purchased from abmgood Inc. The packaging was conducted using the Ecotropic Lentiviral Packaging System (VPK-205, Cell Biolabs, Inc.) according to the vendor's protocol. The lentiviruses were purified with Lenti-X™ Maxi Purification Kit (Clontech). The viral titer was determined by Neuronbiotech Company. The lentivirus containing the LRCH1 shRNA sequence was termed "LL" while the control virus was termed "LC".

Primary microglia were cultured at the density of 2×10^6 cells/ml in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 50 µg/ml penicillin/streptomycin, and in the presence of 6 µg/ml polybrene (Thermo Fisher Scientific) in 48-well plates. Lentiviral particles were added into microglia culture at the MOI of 20 and incubated overnight. The next morning the medium was then replaced with fresh medium. Cells were incubated in fresh medium for 2 days, followed by incubation with 2 µg/ml puromycin (Sigma-Aldrich) for 4 days.

In vitro treatment of microglia

1×10^6/ml lentivirus-infected microglia were primed with 20 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich) for 6 hours (or 1 hour if specified) followed by treatment with 5 mM adenosine triphosphate (ATP) for additional 1 hour.

The N27 rat dopaminergic neural cell line was purchased from Sigma-Aldrich. N27 cells were cultured in RPMI 1640 medium supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C. N27 cells were differentiated with 2 mM dibutyryl cAMP (D0260, Sigma-Aldrich) for 5 days and then used for the experiment described below. To test microglia-mediate neuron death, 5×10^4 N27 rat dopaminergic neural cell line (SCC048, Sigma-Aldrich) were co-cultured with 1×10^5 unprimed or primed primary microglia in each well of a round-bottom 96-well culture plate (Corning) for 24 hours. All cells were then lifted by 5-min

trypsinization at 37ºC with 0.25% trypsin-EDTA. Cell apoptosis and necrosis were then determined using the APC Annexin V Apoptosis Detection Kit with PI (Biolegend).

ELISA

The culture supernatants were collected and centrifuged at 250g for 5 minutes before storage at -80ºC. Microglia were lysed using the denaturing M-PER Mammalian Protein Extraction Reagent (78503, Thermo Fisher Scientific) supplemented with the protease inhibitor cocktail (S8820, Sigma-Aldrich) for 5 minutes on ice. The cell lysates were then centrifuged at 10000g for 5 minutes before storage at -80ºC. The indicated cytokines were measured with the Rat IL-6 ELISA Set (550319, BD Biosciences), Rat TNF ELISA Set (558535, BD Biosciences), and Rat IL-1 beta ELISA Kit (ab100768, Abcam), respectively.

Immunoblotting

Rabbit anti-LRCH1 antibody (bs-9327R) was purchased from Beijing Bioss Antibodies Inc. The following antibodies were purchased from Cell Signaling Technology: beta-actin antibody (3700), phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182) antibody (9216), phospho-SAPK/Jun N-terminal kinase (JNK) (Thr183/Tyr185) antibody (9255), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (9101), p44/42 MAPK (Erk1/2) antibody (4695), p38 MAPK antibody (8690), SAPK/JNK Antibody (9252).

Adoptive transfer of microglia

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The adoptive transfer procedure was conducted based on established approaches with several modifications [5, 11, 12]. Briefly, immediately before SCI, 1×10^6 lentivirus-infected microglia in 5 µl of 0.9% NaCl solution was injected into the SCI area (epicenter) at a depth of 1 mm at the rate of 0.2 µl/min, using a 5-µl micro-syringe with a 33-gauge Hamilton needle. Rats in the vehicle group received the same amount of saline. After injection, the injectors were removed and muscles and skins were sutured in separate layers.

Hematoxylin and eosin (H&E) staining

Rats were transcardially perfused with ice-cold PBS followed by cold 4% paraformaldehyde (PFA). Spinal cords were then fixed in 4% PFA for 16 hours. The next day, spinal cords were immersed in 30% sucrose-PBS for three days. Spinal cords were then embedded in paraffin. Five-micron thick cross sections were prepared and stained following the standard H&E staining protocol.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

The spinal cord sections were labeled with the DeadEnd™ Fluorometric TUNEL System (Promega) following the manufacturer's manual.

Neurologic evaluation

The hindlimb locomotor function was evaluated before and 1, 3, 7, 14 and 21 days after SCI using the BBB locomotor test developed by

Basso et al. [13]. The hindlimb movements during locomotion were quantified using a scale ranging from 0 to 21. The rats were observed for 5 minutes at each time point by two observers who were blinded to the study.

Statistical analysis

Experiments were independently conducted three times unless specified, with 6 to 10 different samples in each group. Data were shown as mean $\pm$ standard deviation and were analyzed by GraphPad Prism 7.0. Student's t-test or one-way ANOVA was used to compare the mean values among the groups. P-value <0.05 was considered significant.

Results

LRCH1 is down-regulated in post-SCI microglia

To determine LRCH1 expression in microglia after SCI, we enriched Granulocyte-CD45^{low}CD11b$^+$ spinal cord microglia from sham-operated rats and SCI rats (Figure 1A). The LRCH1 mRNA quantity was determined by qRT-PCR and we found that LRCH1 mRNA was decreased in microglia in a time-dependent manner after SCI (Figure 1B). LRCH1 protein underwent similar decreases on day 2, day 4 and day 7 after SCI (Figure 1C).

LRCH1 knockdown elevates the production of pro-inflammatory cytokines

One of the crucial roles of post-SCI microglia is to produce pro-inflammatory cytokines in the acute stage [2]. To ascertain whether LRCH1 is involved in microglia-mediated neuroinflammation, we infected rat primary spinal cord microglia with a lentivirus that expresses both LRCH1 shRNA and GFP, followed by puromycin selection. The infection efficiency reached approximately 90% after puromycin selection, as indicated by the proportion of GFP$^+$ microglia on day 6 after infection (Figure 2A). As shown in Figure 2B, LRCH1 protein was drastically reduced in microglia infected with shRNA-encoding lentivirus (hereinafter LL), in comparison to microglia infected with the control lentivirus (hereinafter LC). LRCH1 knockdown had no remarkable impact on microglia survival or cytokine transcription under the *in vitro* steady state (Figure 2C

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and 2D). These microglia were then primed with LPS plus ATP into a pro-inflammatory state. The priming did not alter LRCH1 transcription in either LC-infected or LL-infected microglia (Figure 2E). The analysis of cytokines in the culture supernatants revealed that without priming, LL-infected microglia and LC-infected microglia secreted comparable traces of IL-1β and TNF-α (Figure 2F and 2G). After priming, both groups had dramatically high levels of IL-1β and TNF-α in the supernatants, whereas LL-infected microglia produced even more IL-1β and TNF-α than LC-infected microglia (Figure 2F and 2G). With regard to supernatant IL-6, no matter the priming was conducted or not, LL-infected microglia always secreted more IL-6 than LC-infected microglia (Figure 2H). To investigate whether the above changes of supernatant cytokines were attributed to an overall down-regulation of pro-inflammatory cytokine expression or deficient cytokine exocytosis, the microglia were lysed in a non-denaturing lysis buffer, and the lysates were mixed with the supernatants. The cytokines, including both mature and immature forms, were determined in the lysate-supernatant mixtures. It turned out that LL-infected microglia produced significantly more pro-inflammatory cytokines than LC-infected microglia (Figure 2I).

LRCH1 knockdown facilitates microglial M1 polarization *in vitro*

To further understand the significance of LRCH1, we analyzed the microglial polarization towards M1 or M2 type by staining the M1

marker iNOS and M2 marker Arginase-1. As indicated in Figure 3, without priming, Arginase-1⁺ population was predominant in both LC-infected microglia and LL-infected microglia. However, there were more iNOS⁺ cells in unprimed LL-infected microglia than that in unprimed LC-infected microglia. After priming, iNOS⁺ cells were increased and Arginase-1⁺ cells were decreased in both LC-infected microglia and LL-infected microglia. However, primed LL-infected microglia possessed more iNOS⁺ cells and less Arginase-1⁺ cells than primed LC-infected microglia. Therefore, LRCH1 is a negative regulator for microglial M1 polarization.

LRCH1 knockdown enhances microglia-mediated neuronal death

Previous research indicates that activated microglia induce neuronal death [14-17]. To test if LRCH1 is crucial for this process, we co-cultured unprimed or primed microglia with the N27 rat dopaminergic neural cell line for 24 hours. As indicated in Figure 4, primed microglia substantially induced N27 cell apoptosis and necrosis, and primed LL-infected microglia induced more apoptosis and necrosis of N27 cells than primed LC-infected microglia.

LRCH1 knockdown promotes the activation of p38 MAPK and Erk1/2

To find out the signal pathways that are responsible for the effect of LRCH1, we analyzed the MAPK pathways because they are critical to LPS-mediated pro-inflammatory activation of microglia [18, 19].

As shown in Figure 5, under unprimed condition, the activating phosphorylation of p38 MAPK, Erk1/2 and JNK was comparable in LC-infected microglia and LL-infected microglia. However, after priming, we observed higher phosphorylation of p38 MAPK and Erk1/2 in LL-infected microglia as compared with LC-infected microglia. The JNK phosphorylation showed no significant difference in LC-infected microglia and LL-infected microglia after priming.

LRCH1 knockdown in microglia increases post-SCI leukocyte recruitment *in vivo*

To elucidate the effect of LRCH1 *in vivo*, we microinjected LC-infected microglia or LL-infected microglia into the T12 spinal cord of each normal rat before SCI was conducted on these recipient rats. At indicated time points after SCI, the T12 spinal cord was taken and the injected microglia and infiltrating leukocytes were isolated. As indicated in Figure 6A and 6C, on day 3 after SCI, GFP$^+$ transferred microglia were present in the spinal cords, and the frequencies of LC-infected microglia and LL-infected microglia were comparable in the recipient spinal cords. After SCI, in the spinal cords injected with LL-infected microglia, there were more infiltrating TCR$_{\alpha\beta}^+$ αβT cells, TCR$_{\gamma\delta}^+$ γδT cells, and Granulocyte$^+$ neutrophils as compared with the spinal cords injected with LC-infected microglia (Figure 6A, 6B and 6D). Furthermore, we retrieved the transferred microglia from the recipient spinal cords and evaluated the expression of pro-inflammatory cytokines. We found that after SCI, LL-infected

microglia expressed higher IL-1β, TNF-α, and IL-6 than LC-infected microglia in the spinal cords (Figure 6E).

LRCH1 knockdown in microglia aggravated spinal cord damage and function

About the post-SCI lesion, the H&E staining displayed more extensive and severe tissue damage in the spinal cords injected with LL-infected microglia, as compared with the spinal cords injected with LC-infected microglia (Figure 7A). After SCI, the spinal cords injected with LL-infected microglia also had more apoptotic neurons than the spinal cords injected with LC-infected microglia (Figure 7B and 7C). Moreover, the rats receiving LL-infected microglia showed a lower BBB score than the rats receiving LC-infected microglia from day 7 to day 21 after SCI, suggesting that LRCH1 knockdown aggravated post-SCI locomotor function impairment (Figure 7D). Furthermore, the overall levels of IL-1β, TNF-α, and IL-6 were remarkably higher in the spinal cords injected with LL-infected microglia, in comparison to those in the spinal cords injected with LC-infected microglia (Figure 7E).

Discussion

Microglia are important effector cells in the injured spinal cord tissue. In the secondary phase of SCI, inflammation is the most important mechanism that directly or indirectly controls the sequelae after SCI. It is reported that microglia turn into M1 type (classical activation) shortly after SCI and secrete pro-inflammatory cytokines and chemokines which mediate secondary damage following mechanical injury [20]. However, depending on the spinal cord microenvironment, microglia can also be protective after SCI, through polarization towards M2 type (alternative activation) to secrete anti-inflammatory cytokines and chemokines lead to the suppression of excessive inflammatory responses [2]. Therefore, unveiling the mechanisms by which microglial polarization is regulated is important for understanding the pathogenesis of SCI, and for developing therapeutic interventions against post-SCI secondary damage.

Our study discloses for the first time the effect of LRCH1 on microglia function. We paid our attention to this molecule because our unpublished microarray data suggest a profound change in the expression of LRCH1 in microglia in the acute phase of SCI. The q-RT-PCR and Immunoblotting analysis confirmed the down-regulation of LRCH1 in post-SCI microglia. However, the exact factors that cause this change are still unknown. To our knowledge, no previous research has suggested any clues of the transcription factor

responsible for the expression of LRCH1. Our lab is using some transcription factor binding sites prediction software to analyze the potential transcription factors that bind to the promoter sequence of *Lrch1* gene locus.

According to the Human Protein Atlas, LRCH1 mRNA is present in almost every organ and tissue including the spinal cord, with the highest expression in the cerebellum (https://www.proteinatlas.org/ENSG00000136141-LRCH1/tissue). The universal expression of LRCH1 suggests its critical role in the functionality of almost all cells. It is therefore surprising that researchers have neglected this molecule for so long. Although the present study focuses on the effect of LRCH1 in microglia, it will be valuable to investigate the expression and functions of LRCH1 in neurons, astrocytes, oligodendrocytes, and macrophages in the spinal cord. Our ongoing studies are using different approaches such as Immunohistochemistry, single-cell RNA sequencing, and genetical modification to ascertain the expression pattern of LRCH1 in these cell types under normal or SCI condition.

Our study demonstrates that LRCH1 is an inhibitory factor of microglia-mediated inflammation both *in vitro* and *in vivo*. Using the RNA interference method, we showed that LRCH1 alleviated the production of pro-inflammatory cytokines in LPS-and-ATP-primed microglia, likely through inhibiting the polarization into M1 type. Interestingly, LRCH1 deactivated LPS-induced p38 MAPK and

Erk1/2 pathway while not interfering with JNK pathway. p38 MAPK, Erk1/2, and JNK signaling are all critical for LSP-induced macrophage or microglial inflammatory response [21-23]. Especially, MAPKs activate activator protein 1 (AP-1) signaling [24, 25], and AP-1 supports the M1 polarization of macrophages and microglia [21, 26]. Hence, LRCH1 might restrain the microglial M1 polarization via deactivating the MAPK-AP-1 signaling. However, LPS also activates NF-κB pathway which plays a significant role in M1 polarization [27-29]. Whether LRCH1 impacts NF-κB pathway remains unknown and will be the target of our future study. Furthermore, how LRCH1 regulates the phosphorylation of p38 MAPK and Erk1/2 remains undetermined.

It was recently reported that LRCH1 competes with Cdc42 for interaction with DOCK8 and restrains T cell migration in experimental autoimmune encephalomyelitis [7]. It is also reported that DOCK8 modulates macrophage migration through Cdc42 activation [30]. Hence, it is very likely that LRCH1 regulates microglia migration or motility under normal or pathological conditions. Although in the present study we did not pay much attention to macroglia migration, it will be tempting to investigate the role of LRCH1 in microglia migration, phagocytosis, and secretion of other neurotoxic or neuroprotective mediators. Meanwhile, we will continue to explore the effect of LRCH1 in the M2 polarization of microglia in the chronic stage of SCI to elucidate the significance of LRCH1 for the resolution of neuroinflammation

and tissue recovery after SCI. Moreover, Cdc42 has been shown to activate MAPK signaling in certain cell types [31, 32], so perhaps Cdc42 exerts the same effect on MAPK activation in microglia, and LRCH1 blocks Cdc42 activation and subsequently inhibits MAPK activation. Our future studies will test this hypothesis.

The *in vivo* experiments confirmed LRCH1-mediated inhibition of microglial inflammatory response after SCI. We guessed that the decrease in LRCH1 expression in post-SCI microglia facilitates microglial M1 polarization and inflammatory reaction. So if there is an approach, either through chemical compounds or genetical modification, to increase LRCH1 expression in microglia, it would ameliorate the outcome of post-SCI neuroinflammation and facilitate spinal cord recovery.

Conclusion

Our study demonstrates that LRCH1 inhibits the production of pro-inflammatory cytokines from activated microglia. In addition,

LRCH1 inhibits the polarization of microglia towards the classically activated M1 type. The effect of LRCH1 is mediated through alleviating the activation of p38 MAPK and Erk1/2 signaling. Adoptive transfer of LRCH1-knockdown microglia worsens spinal cord injury-induced tissue damage and functional impairment. Therefore, LRCH1 serves as a negative regulator of microglia-mediated neuroinflammation after spinal cord injury.

Abbreviations:

SCI: spinal cord injury; LRCH1: leucine rich repeats and calponin homology domain containing 1; DOCK8: dedicator of cytokinesis 8; PBS: phosphate-buffered saline; FBS: fetal bovine serum; iNOS: inducible nitric oxide synthase; MAPK: mitogen-activated protein kinase; JNK: Jun N-terminal kinase; TUNEL : Terminal deoxynucleotidyl transferase dUTP nick end labeling; AP-1: activator protein 1

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WKC, LJF and WPL were involved in the design of the study, carried out the experiments, and participated in the data analysis and manuscript preparation. QDL, QFK, PYC, PRZ, LQC and NLH contributed to the data analysis and interpretation of the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of the Second Affiliated Hospital of Fujian Medical University and Shenzhen Pingle Orthopedic Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Alizadeh A, Dyck SM, Karimi-Abdolrezaee S: Traumatic Spinal Cord Injury: An Overview of Pathophysiology, Models and Acute Injury Mechanisms. Front Neurol. 2019;10:282.

2. Zhou X, He X, Ren Y: Function of microglia and macrophages in secondary damage after spinal cord injury. Neural Regen Res. 2014;9:1787-1795.

3. Tan AM, Zhao P, Waxman SG, Hains BC: Early microglial inhibition preemptively mitigates chronic pain development after experimental spinal cord injury. J Rehabil Res Dev. 2009;46:123-133.

4. Bellver-Landete V, Bretheau F, Mailhot B, Vallieres N, Lessard M, Janelle ME, Vernoux N, Tremblay ME, Fuehrmann T, Shoichet MS, Lacroix S: Microglia are an essential component of the neuroprotective scar that forms after spinal cord injury. Nat Commun. 2019;10:518.

5. Akhmetzyanova ER, Mukhamedshina YO, Zhuravleva MN, Galieva LR, Kostennikov AA, Garanina EE, Rizvanov AA: Transplantation of Microglia in the Area of Spinal Cord Injury in an Acute Period Increases Tissue Sparing, but Not Functional Recovery. Front Cell Neurosci. 2018;12:507.

6. Spector TD, Reneland RH, Mah S, Valdes AM, Hart DJ, Kammerer S, Langdown M, Hoyal CR, Atienza J, Doherty M, et al: Association between a variation in LRCH1 and knee osteoarthritis: a genome-wide single-nucleotide polymorphism

association study using DNA pooling. Arthritis Rheum. 2006;54:524-532.

7. Xu X, Han L, Zhao G, Xue S, Gao Y, Xiao J, Zhang S, Chen P, Wu ZY, Ding J, et al: LRCH1 interferes with DOCK8-Cdc42-induced T cell migration and ameliorates experimental autoimmune encephalomyelitis. J Exp Med. 2017;214:209-226.

8. Lin L, Desai R, Wang X, Lo EH, Xing C: Characteristics of primary rat microglia isolated from mixed cultures using two different methods. J Neuroinflammation. 2017;14:101.

9. Saura J, Tusell JM, Serratosa J: High-yield isolation of murine microglia by mild trypsinization. Glia. 2003;44:183-189.

10. Xing C, Wang X, Cheng C, Montaner J, Mandeville E, Leung W, van Leyen K, Lok J, Lo EH: Neuronal production of lipocalin-2 as a help-me signal for glial activation. Stroke. 2014;45:2085-2092.

11. Kou D, Li T, Liu H, Liu C, Yin Y, Wu X, Yu T: Transplantation of rat-derived microglial cells promotes functional recovery in a rat model of spinal cord injury. Braz J Med Biol Res. 2018;51:e7076.

12. Yu TB, Cheng YS, Zhao P, Kou DW, Sun K, Chen BH, Wang AM: Immune therapy with cultured microglia grafting into the injured spinal cord promoting the recovery of rat's hind limb motor function. Chin J Traumatol. 2009;12:291-295.

13. Basso DM, Beattie MS, Bresnahan JC: A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma. 1995;12:1-21.

14. Verma AK, Ghosh S, Pradhan S, Basu A: Microglial activation

induces neuronal death in Chandipura virus infection. Sci Rep. 2016;6:22544.

15. Yanuck SF: Microglial Phagocytosis of Neurons: Diminishing Neuronal Loss in Traumatic, Infectious, Inflammatory, and Autoimmune CNS Disorders. Front Psychiatry. 2019;10:712.

16. Cho BP, Sugama S, Shin DH, DeGiorgio LA, Kim SS, Kim YS, Lim SY, Park KC, Volpe BT, Cho S, Joh TH: Microglial phagocytosis of dopamine neurons at early phases of apoptosis. Cell Mol Neurobiol. 2003;23:551-560.

17. Neher JJ, Neniskyte U, Zhao JW, Bal-Price A, Tolkovsky AM, Brown GC: Inhibition of microglial phagocytosis is sufficient to prevent inflammatory neuronal death. J Immunol. 2011;186:4973-4983.

18. Voet S, Prinz M, van Loo G: Microglia in Central Nervous System Inflammation and Multiple Sclerosis Pathology. Trends Mol Med. 2019;25:112-123.

19. Lim HS, Kim YJ, Kim BY, Park G, Jeong SJ: The Anti-neuroinflammatory Activity of Tectorigenin Pretreatment via Downregulated NF-kappaB and ERK/JNK Pathways in BV-2 Microglial and Microglia Inactivation in Mice With Lipopolysaccharide. Front Pharmacol. 2018;9:462.

20. Nakajima H, Uchida K, Guerrero AR, Watanabe S, Sugita D, Takeura N, Yoshida A, Long G, Wright KT, Johnson WE, Baba H: Transplantation of mesenchymal stem cells promotes an alternative pathway of macrophage activation and functional

recovery after spinal cord injury. J Neurotrauma. 2012;29:1614-1625.

21. Liu YC, Zou XB, Chai YF, Yao YM: Macrophage polarization in inflammatory diseases. Int J Biol Sci. 2014;10:520-529.

22. Arora S, Dev K, Agarwal B, Das P, Syed MA: Macrophages: Their role, activation and polarization in pulmonary diseases. Immunobiology. 2018;223:383-396.

23. Kaminska B, Mota M, Pizzi M: Signal transduction and epigenetic mechanisms in the control of microglia activation during neuroinflammation. Biochim Biophys Acta. 2016;1862:339-351.

24. Qin H, Roberts KL, Niyongere SA, Cong Y, Elson CO, Benveniste EN: Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia. J Immunol. 2007;179:5966-5976.

25. Zhang Y, Ting AT, Marcu KB, Bliska JB: Inhibition of MAPK and NF-kappa B pathways is necessary for rapid apoptosis in macrophages infected with Yersinia. J Immunol. 2005;174:7939-7949.

26. Park JY, Chung TW, Jeong YJ, Kwak CH, Ha SH, Kwon KM, Abekura F, Cho SH, Lee YC, Ha KT, et al: Ascofuranone inhibits lipopolysaccharide-induced inflammatory response via NF-kappaB and AP-1, p-ERK, TNF-alpha, IL-6 and IL-1beta in RAW 264.7 macrophages. PLoS One. 2017;12:e0171322.

27. Genard G, Lucas S, Michiels C: Reprogramming of Tumor-Associated Macrophages with Anticancer Therapies:

Radiotherapy versus Chemo- and Immunotherapies. Front Immunol. 2017;8:828.

28. Goldmann T, Prinz M: Role of microglia in CNS autoimmunity. Clin Dev Immunol. 2013;2013:208093.

29. Liu T, Zhang L, Joo D, Sun SC: NF-kappaB signaling in inflammation. Signal Transduct Target Ther. 2017;2.

30. Shiraishi A, Uruno T, Sanematsu F, Ushijima M, Sakata D, Hara T, Fukui Y: DOCK8 Protein Regulates Macrophage Migration through Cdc42 Protein Activation and LRAP35a Protein Interaction. J Biol Chem. 2017;292:2191-2202.

31. Zhong B, Jiang K, Gilvary DL, Epling-Burnette PK, Ritchey C, Liu J, Jackson RJ, Hong-Geller E, Wei S: Human neutrophils utilize a Rac/Cdc42-dependent MAPK pathway to direct intracellular granule mobilization toward ingested microbial pathogens. Blood. 2003;101:3240-3248.

32. Chu JY, Dransfield I, Rossi AG, Vermeren S: Non-canonical PI3K-Cdc42-Pak-Mek-Erk Signaling Promotes Immune-Complex-Induced Apoptosis in Human Neutrophils. Cell Rep. 2016;17:374-386.

Figure legends

Fig. 1. LRCH1 expression is reduced in post-SCI microglia. (A) The gating and enrichment of microglia in spinal cord cells. Granulocyte⁻ cells were first gated from the whole intact cells, and $CD45^{low}CD11b^{+}$ microglia were then recognized and sorted. d2~d7: day 2 to day 7 after SCI. **(B)** LRCH1 mRNA levels in microglia at indicated time points after SCI. Sh: sham control. n=5 in 3 independent experiments. **(C)** LRCH1 protein levels in microglia. The data represent two independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig. 2. LRCH1 knockdown enhances the expression of pro-inflammatory cytokines in primed microglia. (A) GFP expression in lentivirus-infected microglia. Non: non-infected microglia. L-P-: lentiviral infection without puromycin selection. L-P+: lentiviral infection followed by puromycin selection. Note that only the cells infected with control lentivirus are shown here. The data are representative of two independent experiments. **(B)** LRCH1 protein in microglia after lentiviral infection and puromycin selection. LC: cells infected with control lentivirus. LL: cells infected with LRCH1 shRNA-encoding lentivirus. The data are representative of two independent experiments. **(C)** Microglia apoptosis and necrosis after lentiviral infection. The data are representative of three independent experiments. **(D)** mRNA levels of indicated cytokines in microglia after lentiviral infection. ns: non-significant. n=3 in 3

independent experiments. **(E)** LRCH1 mRNA in microglia before (0) or after (1 or 6-hour) priming with LPS+ATP. n=4 in 3 independent experiments. **(F to H)** Concentrations of indicated cytokines in the supernatants of lentivirus-infected microglia culture after 6-hour priming. Un: no priming. LPS+ATP: priming. n=6 in 3 independent experiments. **(I)** Concentrations of indicated cytokines in the supernatant-lysate mixtures of lentivirus-infected microglia culture. n=5 in 3 independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001 in comparison to the "LC" group under the same conditions.

Fig. 3. LRCH1 knockdown facilitates the M1 polarization of microglia. (A) Representative dot plots showing the expression of iNOS and Arginase-1 in lentivirus-infected microglia after 6-hour priming. **(B)** Statistics for the frequencies of iNOS$^+$ (upper panel) or Arginase-1$^+$ (lower panel) cells. n=8 in 4 independent experiments. ns: non-significant. *, p<0.05; **, p<0.01; ***, p<0.001.

Fig. 4. LRCH1 knockdown increases microglia-mediated N27 cell death. (A) Representative dot plots showing the apoptosis and necrosis of N27 cells after co-culture with primed lentivirus-infected microglia. N27 cells were distinguished from GFP$^+$ microglia. LC: N27 cells cultured with microglia infected with control lentivirus. LL: N27 cells cultured with microglia infected with LRCH1 shRNA-encoding lentivirus. Un: unprimed microglia. LPS+ATP: primed microglia. **(B)** Statistics for the frequencies of Annexin-V$^+$ cells and PI$^+$ cells. Note that PI$^+$ cells include PI$^+$Annexin-V$^+$ cells and

PI$^+$Annexin-V$^-$ cells. n=6 in 3 independent experiments. ns: non-significant. *, p<0.05; **, p<0.01; ***, p<0.001.

Fig. 5. LRCH1 knockdown promotes priming-induced activation of p38 MAPK and JNK signaling. (A) Representative Immunoblotting images showing the phosphorylation of p-38 MAPK (30 minutes after priming), Erk1/2 phosphorylation (15 minutes after priming), and JNK (30 minutes after priming) in microglia. **(B)** Statistics for the phosphorylation levels (phosphorylated protein of interest : total protein of interest) of each signaling molecule in microglia with or without priming. LC: microglia infected with control lentivirus. LL: microglia infected with LRCH1 shRNA-encoding lentivirus. *, p<0.05.

Fig. 6. LRCH1 knockdown enhances microglia-mediated neuroinflammation after SCI. (A and B) Representative dot plots showing the gating of αβT cells, GFP$^+$ exogenous microglia (on day 6 after SCI), as well as γδT cells and neutrophils (on day 2 after SCI) in isolated spinal cord cells. LC: rats receiving microglia infected with control lentivirus. LL: rats receiving microglia infected with LRCH1 shRNA-encoding lentivirus. **(C and D)** Statistics for the frequencies of GFP$^+$ microglia **(C)** and indicated leukocyte populations **(D)** in spinal cord cells. **(E)** mRNA levels of indicated cytokines in GFP$^+$ microglia sorted from recipient spinal cords. n=5 in 3 independent experiments. ns: non-significant. *, p<0.05; **, p<0.01; ***, p<0.001.

Fig. 7. LRCH1 knockdown worsens tissue damage and locomotor function after SCI. (A) H&E staining of spinal cords on day 7 after SCI. LC: rats receiving microglia infected with control lentivirus. LL: rats receiving microglia infected with LRCH1 shRNA-encoding lentivirus. **(B and C)** TUNEL analysis of spinal cords on day 7 after SCI. Representative fluorescent images are shown in **(B)**. Statistics for labeled cells are shown in **(C)**. n=4 in 3 independent experiments. **(D)** BBB scores. n=8. **(E)** mRNA levels of indicated cytokines in spinal cord tissues on day 7 after SCI. n=6 in 3 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.