CD73 alleviates GSDMD-mediated pyroptosis in spinal cord injury through PI3K/AKT/Foxo1 signaling

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Research

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Abstract

Background

Neuroinflammation-induced secondary injury is an important cause of sustained progression of spinal cord injury. Inflammatory programmed cell death pyroptosis executed by the pore-forming protein gasdermin D (GSDMD) is an essential step of neuroinflammation. However, it is unclear whether CD73, a widely accepted immunosuppressive molecule, can inhibit pyroptosis via mediating GSDMD.

Methods

CD73 deficient mice and LPS induced BV2 cells were respectively used to illustrate the effect of CD73 on macrophages/microglia pyroptosis in vivo and in vitro. A combination of molecular and histological methods was performed to assess pyroptosis and explore the mechanism both in vivo and in vitro.

Results

We showed molecular evidence for CD73 suppresses the activation of NLRP3 inflammasome complexes to reduce the maturation of GSDMD, leading to decreased pyroptosis in macrophages/microglia. Further analysis reveals that adenosine- A_{2B} adenosine receptor-PI3K-AKT-Foxo1 cascade is a possible mechanism of CD73 regulation. Importantly, we determine that CD73 inhibits the expression of GSDMD at the transcriptional level through Foxo1. What's more, we confirm the accumulation of HIF-1 α promotes the overexpression of CD73 after SCI, and the increased CD73 in turn upregulates the expression of HIF-1 α , eventually forming a positive feedback regulatory loop.

Conclusion

Our data reveal a novel function of CD73 on macrophages/microglia pyroptosis, suggesting a unique therapeutic opportunity for mitigating the disease process in SCI.

Background

Spinal cord injury (SCI) remains a devastating condition affecting millions of people worldwide, leading to severe dysfunction below injured segment[1]. About 240,000-337,000 people live with SCI in United States, and this figure is thought to grow by 17,000 annually[1]. Nearly 52% of these patients are paraplegic and 47% are considered quadriplegic[2]. Presently, no effective pharmacological therapy exists. The pathogenesis of SCI is complicated and can be categorized into two phases[3]. After a transient initial mechanically inflicted trauma, a long-lasting second phase injury happens characterized in part by secretion of cytokines and chemokines produced at the lesion site, contributing largely to neurological damage[4]. Existing evidence demonstrates neuroinflammation exerts a key role in the secondary phase of SCI[5][6][7].

Traumatic injury to central nervous system (CNS) leads to the disruption of blood spinal barrier, triggering the invasion of cells and other components of immune system cause axonal destruction, neuronal loss and demyelination. Previous studies suggest activation of cytoplasmic inflammasome complex is essential for neuroinflammation post CNS trauma[8][9]. Inflammasomes are cytosolic multiprotein scaffolds assembled by particular pattern recognition receptors (PRRs), sensors of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), enables the activation of pro-inflammatory caspase[10]. Presently, a number of inflammasome-associated sensors have been discovered, such as NLRP1, NLRP3, AIM2 and pyrin[11]. Assembly of sensor proteins, scaffolding protein ASC and pro-inflammatory caspase (caspase-1 and -4/5 in humans and caspase-1 and -11 in mice) into inflammasome promotes autoactivation of caspase and subsequent proteolytic gasdermin D (GSDMD) cleavage, resulting in cell pyroptosis[10].

Pyroptosis is proinflammatory form of programmed cell death that relies on the activity of cytosolic GSDMD driven by inflammasomes[12]. Upon activation, GSDMD transfers to the plasma membrane and binds to the inner membrane lipids, oligomerizing to form membrane pores, resulting in local cell swelling, membrane rupture and extravasation of cytoplasmic DAMPs[13][14][15]. Released DAMPs will further recruit immune cells and aggravate inflammatory cascade[16]. Macrophages/microglia are vital mediators of innate immune responses following CNS injury[17]. These cells are also considered to be the main cells in CNS where pyroptosis occurs[10][18]. Pyroptosis of macrophages/microglia have been implicated in the pathogenesis of multiple CNS disease, including SCI and traumatic brain injury[19][20]. Although pyroptosis of macrophages/microglia is repeatedly mentioned in a variety of neuroinflammatory-related diseases, the mechanism of its occurrence is not well understood.

CD73, also known as ecto-5'-nucleotidase (NT5E), is an AMP hydrolase that is part of an extracellular enzymatic mechanism that regulates the conversion of extracellular ATP to adenosine[21]. Plenty of studies have point to CD73 as a key regulator in various pathophysiological processes, including maintaining immune homeostasis by regulating the balance between pro-inflammatory ATP and immunosuppressive adenosine to prevent excessive immune responses[22][23]. In addition, our previous research determined CD73 has an anti-neuroinflammatory role in SCI, which is attributable to its regulation of macrophages/microglia polarization via adenosine-p38 cascade[24]. An increasing piece of evidence now clearly indicates CD73 is involved in activation of inflammasome[25][26]. Therefore, whether the immunosuppressive mechanism of CD73 after SCI contains regulation of macrophages/microglia pyroptosis is promising.

We hypothesized that CD73 could attenuate inflammasome activation and inhibit pyroptosis of macrophages/microglia, reducing neuroinflammation after spinal cord injury. In the current study, we first confirmed the role of CD73 and GSDMD in spinal cord injury through SCI patient blood samples. Then we explored the mechanism of CD73 regulating macrophages/microglia pyroptosis by in vivo and in vitro experiments. Finally, we investigated the upstream factor regulating the expression of cd73 after spinal cord injury.

Methods

Donor recruitment and blood sample preparation

From January 2019 to June 2019, 20 SCI patients from Huashan Hospital, Fudan University were enrolled. Inclusion criteria included the following: (1) a clear history of trauma, and there were no neurological abnormalities of spinal cord injury before injury; (2) existing neurological abnormalities such as limb sensation, motor abnormality, and dysfunction of the bowel and bladder; (3) MRI examination showed spinal cord compression and spinal cord signal changes. Patients with treatment of methylprednisolone before blood taking, with a history of brain disease, with a history of spinal surgery were excluded. Peripheral blood was collected from 20 patients with SCI and 20 healthy donors with similar gender and age distribution, respectively. The Japanese orthopedic association (JOA) score, cervical dysfunction index (NDI) and American spinal injury association (ASIA) classification were used to assess the severity of SCI. The whole blood RNA was extracted using GeneJET Stabilized and Fresh Whole Blood RNA Kit (ThermoFisher, CA, USA) in accordance with the manufacturer's protocol.

Quantitative real-time PCR (RT-PCR) analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions, and quantification of mRNAs was performed using a 10- μ l final reaction volume using SYBR Green PCR Master Mix (ThermoFisher, CA, USA). GAPDH mRNA was used as a housekeeping gene and relative expression levels of mRNAs were calculated using the comparative $\Delta\Delta$ CT method.

Animals

C57BL/6 CD73 knock out (KO) male mice used in our study were reproduced from those gifted by Prof. Thompson, Oklahoma Medical Research Foundation, Oklahoma City, USA. The wild-type (WT) male C57BL/6 mice were supplied by Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University.

Establishment of SCI model and drugs treatment

Just as described in our previous study[24], mice were anesthetized with pentobarbital by intraperitoneal injection(35mg/kg). Each mouse was inflicted with spinal crush injury at the T8–T9 with Dumont-type forceps with a 0.2 mm spacer. We carried out the vertebrae laminectomies of T8-T9 with a pair of fine rongeurs, and the dura mater of mice was protected. The mice SCI model was made by lateral compression of the spinal cord with a depth of 0.2 mm for 20s. After operation, the mice were given intramuscular injection of penicillin, 20,000 units once to resist infection. For further studied the effect of AKT and HIF-1 α on SCI, SC79 (40mg/kg in DMSO) and BAY87-2243 (0.5mg/kg and 4mg/kg in DMSO) were injected intraperitoneally into mice every day after the establishment of mice SCI model. The mice in the control group were injected with the same volume of DMSO at the same time point.

Locomotion recover assessment

The locomotor behavior after SCI was detected by the Basso, Beattie, and Bresnahan (BBB) locomotor recovery scale. This scale is based on the observation of hindlimb movements, stepping, and coordination in an open field and consists of a 21-point scale. SCI group and SCI+NBP group were assessed on postoperative days 1, 3, 7, 14, 21 and 28. An inspector blinded to the treatments assessed them for 4 min in an open field and got the score from 0 (no spontaneous locomotor activity) to 21 (normal coordinated gait with parallel paw placement).

Cell cultures

BV2 cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA), 50 g/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and 50 U/ml penicillin in a humidified atmosphere of 95% air and 5% CO₂. The plasmid of RNAi-CD73, pcDNA-CD73-RNAi-HIF-1 were constructed by Genechem Co., Ltd. (Shanghai, China) and then transfected into BV2 cells using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad CA, USA) following the protocol stipulated by the manufacturer. Different combinations of LPS (1 µg/ml), adenosine (10µM), MRS1706 (0.3µM), MK2206 (3µM) were added to the media 24h after plasmid transfection, and 8h later, mRNA and protein were harvested.

RNA sequencing and functional enrichment analysis

Total RNA was isolated from cells using the Trizol (Invitrogen-Carlsbad CA, USA) according to the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2200 TapeStation (Agilent Technologies, USA) and each sample had the RIN above 7.0. Subsequently, the purified RNAs were subjected to first strand and second strand cDNA synthesis following by adaptor ligation and enrichment with a low-cycle according to instructions of NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NEB, USA). The purified library products were evaluated using the Agilent 2200 TapeStation and Qubit®2.0(Life Technologies, USA) and then diluted to 10 pM for cluster generation in situ on the pair-end flow cell followed by sequencing (2×150 bp) HiSeq3000. The clean reads were obtained after removal of reads containing adapter, ploy-N and at low quality from raw data. HISAT2 was used to align the clean reads to the mouse reference genome mm10 with default parameters. HTSeq was subsequently employed to convert aligned short reads into read counts for each gene model. Differential expression was assessed by DEseq using read counts as input. The Benjamini-Hochberg multiple test correction method was enabled. Differentially expressed genes were chosen according to the criteria of fold change > 2 and adjusted p-value < 0.05. All the differentially expressed genes were used for heat map analysis and KEGG ontology enrichment analyses. For KEGG enrichment analysis, a P-value < 0.05 was used as the threshold to determine significant enrichment of the gene sets.

Enzyme-linked immunosorbent assay

In cell culture supernatants or mouse spinal cord homogenate, the measurement of protein levels of IL-1 β , IL-6 and TNF- α was taken using the commercial ELISA kits from Sigma (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Cytotoxicity assay

The release of lactate dehydrogenase (LDH) was detected to determine the cytotoxicity using the LDH Cytotoxicity Assay Kit (Beyotime, Shanghai, China) following the manufacturer's instructions.

Western blot analysis

Protein of spinal cord tissue and BV2 cells was homogenized in radioimmunoprecipitation assay (RAPI) lysis buffer, and protein concentrations were determined using the BCA assay. Protein samples were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. Following blocking for 1 h with 5% skimmed milk in TBST, membranes were incubated with antibodies included NLRP3 (1:1000, Abcam, ab214185), PI3K (1:1000, CST, 4228), AKT (1:1000, CST, 2920), p-AKT (1:1000, CST, 4070), Foxo1 (1:1000, CST, 14952), p-Foxo1 (1:1000, CST, 9641), GSDMD (1:1000, Abcam, ab210070), ASC (1:1000, CST, 67824) and GAPDH (1:2000, Abcam, ab8245) overnight at 4°C. After being washed in TBST, membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 h, and protein was detected using an enhanced chemiluminescence (ECL) kit. Signal intensities were quantified by gel imaging system (UVP LLC, Upland, CA, USA).

Immunohistochemical assessment

At the third day after SCI, different groups mice were deeply anesthetized with 10 % chloral hydrate (3.5 ml/kg, i.p.) and perfused with 0.9 % NaCl, followed by 4 % paraformaldehyde in 0.01 M phosphate-buffered saline (PBS, pH= 7.4). Spinal cord segments near the lesion epicenter were collected. Three transverse paraffin sections (25 μ m thick) were mounted on poly-L-lysine-coated slides. For immunohistochemical analysis, deparaffinized sections were incubated with H₂O₂ and methanol for 10 min to block endogenous peroxidase, and then incubated for 30 min with serum-blocking solution. The sections were then incubated with primary antibodies of CD73 (1:100, Abcam, ab175396), GSDMD (1:100, Abcam, ab210070) and CASP-1 (1:100, Abcam, ab1872) for 1 h, followed by incubation with HRP-conjugated anti-rabbit secondary antibodies for 30 min. Bound antibodies were visualized by incubation with DAB for 10 min. All images were captured using a Nikon ECLIPSE Ti microscope (Nikon, Japan).

Immunofluorescence assessment

Spinal cord tissue samples were harvested as described above. BV2 cell samples were fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min after the treatment described in cell cultures. Then all samples were blocked for 1 h with 1 % bovine serum albumin containing 0.3 % Triton X-100, followed by incubation (overnight at 4 °C) with GSDMD (1:100, Abcam, ab210070) and CASP-1

(1:100, Abcam, ab1872) the primary antibodies. Then all the samples were incubated (for 2 h at room temperature) with their respective secondary antibody: Dylight (Dy)488- and Dy594-conjugated secondary antibodies (all 1:1000; Jackson ImmunoResearch, West Grove, PA). All images were acquired using Nikon ECLIPSE Ti microscope (Nikon, Japan).

Promoter cloning and generation of Dual-luciferase reporter assay

A -2000/+200bp mouse GSDMD promoter of C57BL/6J mouse genomic DNA was cloned and inserted in the pGL3 basic vector (Promega, Madison, WI, USA). Further deletion generated -1450/+200bp, -900/+200bp, -300/+200bp, +50/+200bp reporter plasmids. Mutant GSDMD reporter plasmids were generated based on -300/+200bp plasmid. BV2 cells were cotransfected with luciferase reporter plasmids, pRL-TK reporter plasmid (control reporter), and foxo1 plasmid (pc-foxo1). After transfection for 24 hours, cells were harvested and measured using the toolVeritas 9100-002 (Turner BioSystems, Sunnyvale, CA, USA), and luciferase activity was divided by the Renilla luciferase activity to normalize for transfection efficiency.

Chromatin Immunoprecipitation assay

ChIP assay was performed using a ChIP assay kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. In brief, primary antibodies of foxo1 (1:500, CST, 2880) or IgG (1:500, Abcam, ab172730) were used. DNA–protein cross-linking complexes were collected, and purified DNA was subjected to qPCR with SYBR Green PCR Master Mix (ThermoFisher, CA, USA).

Statistical analysis

All results are expressed as mean $\pm$ standard deviation. Student's unpaired t tests and two-way analysis of variance (ANOVA) followed by Dunnett's test were used to analyze data. A p value of less than 0.05 was considered to be statistically significant. All statistical analyses were done with the SPSS 14.0 software.

Results

Expression of NLRP3/GSDMD genes in peripheral blood of patients with SCI is positive correlated with the severity of injury.

To investigate whether NLRP3/GSDMD plays a role in neuroinflammation after SCI, 20 blood samples from SCI patients as well as 20 blood samples from normal people were subjected to RT-PCR assay. Then 20 SCI patients were divided into high-expression and low-expression groups based on whether NLRP3/GSDMD gene expression reached an average level. As showed in Table 1, various clinical parameters in SCI patients were compared between two groups. Specifically, NLRP3/GSDMD high-expression group had a lower JOA score and a higher NDI index compared to NLRP3/GSDMD low-expression group. Strikingly, quantitative analysis revealed that the expression of NLRP3/GSDMD was significantly higher in the SCI patient samples in comparison to normal samples (Fig. 1A-AB). Furthermore, linear regression analysis confirmed a positive correlation between NLRP3/GSDMD

expression and Japanese Orthopaedic Association score (JOA score), while a negative correlation between CD73/GSDMD expression and Neck Disability Index (NDI index) (Fig.1C-1D). ROC curve demonstrated they have a good diagnostic significance for SCI (Fig.1E). Collectively, these results indicate the expression of CD73 and GSDMD in SCI patients correlate with the severity of injury.

CD73 deficiency facilitates NLRP3 inflammasome activation and pyroptosis of macrophages/microglia in vivo

To examine SCI inflammasome expression in a systematic manner, a wider panel of inflammasome genes was assessed in spinal cord tissue samples from mice with SCI or sham surgery. All inflammasome-associated genes examined were detectable in mice spinal cord tissue 3 days after surgery, with increased IL-1 β , IL-18, CASP-1 and GSDMD transcript levels in SCI group (Supplementary Fig.1A). Besides, all inflammasome sensors examined were also detectable, and NLRP3 and AIM2 transcript levels were upregulated (Supplementary Fig.1B) after SCI. To investigate the influence of CD73 on inflammasome activation, the mRNA detection was performed once again in CD73 KO mice with SCI. Importantly, compared with WT mice, various inflammasome-associated genes (IL-1 β , IL-18, CASP-1, NLRP3, ASC and GSDMD) and three inflammasome sensors (NLRP3, NLRP1 and AIM2) showed higher levels in CD73 KO mice 3 days post injury (Fig.2A-2B). The release of proinflammatory factor (IL-1 β , IL-6 and TNF- α) and LDH was also effectively increased in CD73 KO mice after SCI (Fig.2C-2D). Western Bolt and immunohistochemistry revealed similar protein expression patterns of NLRP3, GSDMD, ASC and CASP-1 as their mRNA expression (Fig.2E-2F, 2H). In particular, levels of full length GSDMD and N-terminal GSDMD (GSDMD-N, an active form of GSDMD) were both increased (Fig.2E-2F). Immunofluorescence results showed increased expression of GSDMD coincides with CD68 (a marker of macrophages/microglia) (Fig.2G). Together, these results demonstrate that CD73 deficiency exacerbate macrophages/microglia pyroptosis caused by NLRP3 inflammasome activation in SCI.

CD73 alleviates LPS-induced NLRP3 inflammasome activation and pyroptosis in BV2 cells through A_{2B} adenosine receptor

Our previous studies have showed that CD73 regulates microglia polarization through the A_{2B} adenosine receptor [A_{2B}AR]. To determine whether A_{2B}AR mediates the CD73 ability to alleviates microglia pyroptosis, we constructed BV2 cells that either had downregulated or upregulated CD73 expression, exposed them with adenosine or A_{2B}AR antagonist MRS1706 after LPS pretreatment. Results of RT-PCR and ELISA showed that CD73 downregulation caused a profound induction of pyroptosis genes in transcript levels including NLRP3, ASC, CASP-1 and GSDMD, increased the release of IL-1 β , IL-6, TNF- α and LDH, while CD73 upregulation was counterproductive (Fig.3A-3C). Immunoblot and Immunofluorescence revealed similar changes in protein levels of NLRP3, GSDMD and ASC replying to CD73 change (Fig.3D-3F). Notably, administration of adenosine or MRS1706 offsets CD73 downregulation or upregulation effect respectively in all above experiments (Fig.3A-3F). All these results suggest that A_{2B}AR is necessary for CD73 to regulate microglia pyroptosis.

CD73 attenuates microglia pyroptosis via PI3K/AKT/Foxo1 signal

To further investigate the mechanism by which CD73 regulates macrophage/microglia pyroptosis, mRNA sequencing were performed on CD73-overexpressing BV2 cells after administrated with LPS. A total of 1649 mRNAs were differentially expressed between CD73 overexpressed BV2 cells and normal subjects (Fig.4A); 1081 were upregulated and 568 were downregulated; 55 differentially expressed mRNAs were enriched in PI3K/AKT pathway (Fig.4B). The mRNA and protein expression of PI3K were increased in CD73 over expressed BV2 cells, and the phosphorylated AKT and Foxo1 were also observed (Fig.4C-4E). In addition, after pretreated with LPS, we utilized MRS1706 and an AKT inhibitor MK2206 to try to reverse the effect of CD73 upregulation. We found the mRNA levels of PI3K was decreased after exposed to MRS1706 in CD73 upregulated cells, and the phosphorylated AKT and Foxo1 could be suppressed by MRS1706 and MK2206 (Fig.4C-4E). MK2206 treatment also caused a remarkably increase in transcript levels including NLRP3, ASC, CASP-1 and GSDMD, increased the release of IL-1 β , IL-6, TNF- α and LDH (Fig.4F-4H). Immunoblot demonstrated similar changes in protein levels of NLRP3, GSDMD and ASC replying to MK2206 (Fig.4I-4J). In vivo study, intraperitoneal injection of 40mg/kg SC79 (an AKT activator) was carried out every day after surgery in CD73 KO mice, and the results of immunohistochemistry indicated SC79 could decrease the expression of GSDMD and CASP-1 at the lesion site (Fig.4K). Furthermore, the BBB scoring in SC79 group was significantly superior to DMSO group at 28th day after injury (Fig.4L). Taken together, we conclude that the CD73 inhibitory effect on microglial pyroptosis is mediated via the PI3K/AKT/Foxo1 pathway.

Foxo1 is a transcriptional activator in the promoter region of GSDMD gene

In order to analyze the molecular mechanisms involved in GSDMD regulation, we used the JASPAR database (<http://jaspar.genereg.net>) to identify potential binding sites of GSDMD promoter region for Foxo1. To measure the activity of potential 3 cis-acting elements and determine the minimum sequence required for activity, a series of 5 reporters constructs with progressively larger deletions from the 5' end of the promoter was generated. Luciferase assay revealed an increased promoter activity of the pGL3-2000/+200bp as compared to the control group in BV2 cells, indicating the existence of Foxo1 binding site in the region of GSDMD gene (Fig.5A). Consistently, the ChIP assay confirmed the binding of foxo1 to GSDMD promoter region (Fig.5B). When the promoter was deleted to position +50bp, the promoter activity with pGL3+50/+200bp decreased significantly compared with the pGL3-300/+200 (Fig.5A), this result demonstrated that positive regulatory elements were located in the -300/+50 region in BV2 cells. Further mutation of this binding site blocked the transcription of GSDMD (Fig.5C). Overexpression of Foxo1 in BV2 cells also increased the expression of GSDMD, while cotreatment of Foxo1 plasmid and CD73 plasmid inhibited the elevation of GSDMD mRNA, as well as reduced the release of IL-1 β , IL-6, TNF- α and LDH (Fig.5D-5F). Thus, we conclude Foxo1 regulates the expression of GSMDM at transcriptional level.

HIF-1 α mediates CD73 upregulation in microglia

Several reports have shown that HIF-1 α can regulate CD73 expression. So, in this study, we exposed LPS activated BV2 cells with a HIF-1 α small interference RNA to determine its regulatory role. LPS-induced pyroptosis could simultaneously promote the expression of HIF-1 α and CD73 in BV2 cells, and the overexpression of CD73 was inhibited after interference of HIF-1 α (Fig.6A-C). The results of in vivo experiment indicated SCI could increase the expression of HIF-1 α along with the elevation of CD73 in damaged spinal cord tissue, while BAY87-2243 treatment, a HIF-1 α inhibitor, reversed the expression pattern of CD73 (Fig.6D-G). Surprisingly, BAY87-2243—which was supposed to only affect the HIF-1 α activity, also reduced HIF-1 α expression both in mRNA and protein levels (Fig.6D-F). We suspect that CD73 reduction should be responsible for this phenomenon. As we thought, reduce the expression of CD73 in BV2 cells or knockout CD73 gene in mice blocked the increase of HIF-1 α caused by LPS or SCI, respectively (Fig.7A-F). Moreover, Merighi et al. reported a stimulatory effect of adenosine on HIF-1 α by activation of p38 mitogen-activated protein kinases (MAPKs) phosphorylation via A_{2B} adenosine receptor[27]. Our previous study also determined that CD73 can regulate the polarization of macrophages/microglia through this pathway[24]. This prompted us to verify whether CD73 regulates HIF-1 α expression is associated with this signal. In BV2 cells, the results of RT-PCR and immunoblots showed that both a A_{2B} adenosine receptor inhibitor MRS1706 and a p38 inhibitor SB203580 can reduce the increase in HIF-1 α caused by overexpression of CD73 (Fig.7G-8I). The above studies confirmed that HIF-1 α and CD73 can promote each other to accumulate and jointly regulate microglia pyroptosis.

Discussion

CNS trauma, either SCI or traumatic brain injury (TBI), can be divided into two stages (primary injury and secondary injury) according to its characteristics of pathological process[28]. Part of the secondary injury is characterized by persistence and diffuseness, including delayed glial and neuron cells death, leading to significant expansion of the damage site to higher segments and progressive neurodegeneration[29][30]. Neuroinflammation initiated by the innate immune response plays an important role in the pathology of secondary injury after CNS trauma, and inhibition of inflammation becomes a potential therapy for such injury[2][6]. More recently, the scientific community has determined that the activation of cytoplasmic inflammasome complex leading to pyroptosis was an essential step of neuroinflammation in secondary CNS damage[7]. Although inflammasomes have been proposed in SCI, the effect of the pyroptosis-executing protein GSDMD on SCI was not clear. In addition, whether CD73, a 5'-ecto-nucleotidase which can attenuate neuroinflammation after SCI determined by our previous study, can affect the expression of GSDMD has not been studied. Here, we showed that NLRP3 and GSDMD were significantly upregulated in the blood samples from SCI patients and they were also correlated with the severity of this disease (Fig.1A-1D). Moreover, RCO curve identified these genes have the potential to be a novel target for the diagnosis of human SCI (Fig.1E). These results confirmed our speculation about the involvement of NLRP3 and GSDMD in SCI.

In generally, inflammasome complexes consist of three parts: a cytosolic pattern-recognition receptor, pro-inflammatory caspase and an adaptor protein ASC that facilitates the interaction between the two[11].

[31]. Among the multiple inflammasome complexes, NLRP3 appears to be one of the most relevant components in CNS trauma[32]. Results of the research carried out by Liu et al. indicate NLRP3 inflammasome was overexpressed in microglia, neurons and astrocytes in TBI rats[33]. The experimental results of Wu et al demonstrate that pharmacologic suppression of NLRP3 inflammasome activation controls neuroinflammation, attenuates mitochondrial dysfunction in mice[19]. In addition, Adamczek and colleagues found AIM2 is expressed in cortical neurons and can be activated by TBI[34]. In this study, we systematically examined the expression of inflammasome-associated genes and found NLRP3 and AIM2 were overexpressed in spinal cord tissue after injury. What's more, the increase in NLRP3 was more pronounced, confirming that it may play a more important role after SCI, which was consistent with previous findings (Supplementary Fig.1). To estimate the effects of CD73 on inflammasome pathway in SCI, we compared inflammasome-related gene expression profile between CD73 KO mice and WT mice. We found that CD73 deficiency promoted expression of inflammasome genes, as well as increased the release of pro-inflammatory. Based on these results, we can conclude that CD73 plays a crucial role in the regulation of pyroptosis driven by NLRP3 inflammasome after SCI.

Macrophages/microglia are vital mediators of the innate immune response following CNS trauma and are essential for subsequent inflammatory responses[35][36]. There exists a substantial amount of evidence supporting that pyroptosis mainly occurs in macrophages/microglia in CNS neuroimmune diseases. A possible reason is that these cells express higher levels of PRRs which can recognize PAMPs and DAMPs, and initiate pyroptosis cascade[10][18]. We also observed intense GSDMD immunostaining in CD68-labeled macrophages/microglia and a profound increase in spinal cord lesions of CD73 KO mice (Fig.2G). Taken together, the present results defined a pyroptosis occurring in macrophages/microglia could be inhibited by CD73 in SCI.

CD73, a glycosylphosphatidylinositol (GPI) anchored cell surface protein, has a central role in the adenosinergic system and is considered as the rate-limiting enzyme in the generation of extracellular adenosine[37]. Kuleshkaya et al. found about 85–95% of murine AMP-hydrolyzing capabilities are mediated by CD73[38]. When the concentration of extracellular adenosine increase, it can activate P1 purinergic receptors (adenosine receptors) on target cells and stimulates a myriad of protective cellular responses restoring homeostasis[39][40]. The P1 G-protein-coupled receptor family consists of 4 distinct subtypes: A₁R, A_{2A}R, A_{2B}R and A₃R[41], however, these four receptors have different affinities with adenosine. A₁R, A_{2A}R and A₃R are categorized as high-affinity adenosine receptors because they can be activated by physiologic adenosine concentrations, while A_{2B}R only be activated under pathological conditions which is relevant to inflammatory events[42][43]. Experimental evidence reveal that hypoxia can increase the expression of A_{2B}R [44], resulting in the activation of A_{2B}R signaling to protect rats lung injury[45] as well as myocardial ischemia[46]. Similarly, we confirmed CD73 attenuate SCI in mice by A_{2B}R in our previous research[24]. Taken together, we assumed that adenosine-A_{2B}R cascade may be one of the mechanisms by which CD73 regulates the pyroptosis of macrophage/microglia after SCI. In the subsequent study, our data showed that the effect of CD73 interference or overexpression in BV2 cells

can be counteracted by adenosine or MRS1706 and these results are consistent with our hypothesis (Fig.3).

To further investigate the mechanism by which CD73 regulates macrophage/microglia pyroptosis, mRNA sequencing were performed on CD73-overexpressing BV2 cells after administrated with LPS. KEGG analysis showed a large number of differential genes enriched in the PI3K/AKT pathway. PI3K/AKT signaling pathway play a central role in multiple cellular functions such as cell proliferation and survival[47]. More noteworthy is that the activation of this pathway is closely related to the inflammatory response. For example, the research of Yin et al. demonstrated the expression of pro-inflammatory factors IL-12, TNF- α and IL-6 in human innate immune cells was increased by PI3K or AKT inhibitors, while the expression of anti-inflammatory factor IL-10 was decreased[48]. Another study showed that the activation of AKT inhibits inflammatory responses in LPS-induced sepsis mice and sepsis rabbits[49]. These studies reflect the immunosuppressive function of the PI3K/AKT pathway, which is consistent with the role of CD73. Foxo1 is a vital transcription factor downstream of AKT. When PI3K/AKT pathway is constitutively activated, Foxo1 can be phosphorylated by AKT in the nucleus, resulting in the translocation of Foxo1 to the cytoplasm and inactivate it[50]. A large number of studies have found that inhibition of Foxo1 can reduce inflammation in different cells[51][52]. To date, however, there are no reports about Foxo1 is related to CD73 or pyroptosis. In the present study, our results indicated that overexpression of CD73 alleviates the pyroptosis of macrophages/microglia in a PI3K/AKT dependent way both in vivo and in vitro (Fig.4). Furthermore, the results of dual-luciferase reporter assay in BV2 cells demonstrated that there is a Foxo1 binding site between 200 bp upstream of the GSDMD gene and 50 bp downstream, and this discovery was also verified by ChIP assay (Fig.5). Based on the above findings, we believe that the PI3K/AKT/Foxo1 pathway is essential for CD73 to regulate pyroptosis.

The fact that CD73 was overexpressed after SCI has been confirmed repeatedly in the present study as well as our previous research. However, the biological mechanism behind this is still unclear. HIF-1 is a heterodimeric transcription factor composed of a HIF-1 α subunit and HIF-1 β subunit and functioned as a master regulator of oxygen homeostasis[53]. When in anoxic conditions, the HIF-1 α subunit accumulates and then binds to HIF-1 β ultimately activates HIF-1-target genes, whose products are regulating angiogenesis, glucose metabolism, cell survival, invasion and metastasis[54]. Numerous studies suggested that the pathogenesis of SCI involves the ischemia and hypoxia in lesion site, which increase the expression of HIF-1, enhancing the resilience of neuronal cells under hypoxia[55][56]. Karhausen et al. once reported activation of HIF-1 α promoted CD73 transcription, resulting in attenuated loss of barrier during colitis in vivo[57]. Besides, Synnestvedt et al. examined the CD73 gene promoter and identified a binding site for HIF-1 α , and inhibition of HIF-1 α expression by antisense oligonucleotides resulted in significant decrease of hypoxia-inducible CD73 expression[58]. In this study, we also found that overexpression of CD73 after spinal cord injury is associated with HIF-1 α . Interfering with the expression of HIF-1 α or inhibiting the activity of HIF-1 α can reduce the expression of CD73 after spinal cord injury (Fig.6). Interestingly, we also found that the expression of HIF-1 α in microglia was CD73 dependent. The adenosine-A_{2B}AR-p38 pathway, which has been confirmed regulated the function of

macrophages/microglia after spinal cord injury in our previous study, probably the mechanism by which CD73 regulates HIF-1 α expression (Fig.7). This finding is consistent with that reported by Merighi[27]. Therefore, we believe that a positive feedback regulatory loop can be formed between CD73 and HIF-1 α after spinal cord injury, which is involved in the regulation of macrophages/microglia pyroptosis and inhibits neuroinflammatory response.

Conclusion

In summary, our present study demonstrates for the first time that CD73 has an anti-pyroptosis role in SCI, which is partly attributable to its inhibition of GSDMD via adenosine- A_{2B}AR-PI3K-AKT-Foxo1 signal. Moreover, our results showed that the creation of a positive feedback loop between CD73 and HIF-1 α may an important mechanism for reducing neuroinflammation after SCI (Fig.8). We propose that CD73 may be utilized as a target molecule for the development of novel therapeutic methods for SCI.

Abbreviations

GSDMD: gasdermin D, SCI: Spinal cord injury, CNS: central nervous system, PRRs: pattern recognition receptors, PAMPs: pathogen-associated molecular patterns, DAMPs: damage-associated molecular patterns, JOA: Japanese orthopedic association, NDI: cervical dysfunction index, ASIA: American spinal injury association.

Declarations

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Consent for Publication

Not applicable.

Availability of supporting data

All data generated or analyzed during this study are included in this published article.

Author Contributions

Shun Xu, Wei Zhu, Minghao Shao, Fan Zhang, Haocheng Xu performed the experiments; Feizhou Lu, Wei Zhu, Xiaosheng Ma, and Shun Xu designed the study; Shun Xu analyzed the data and wrote the manuscript. Feizhou Lu, Jianyuan Jiang, Xiaosheng Ma reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All study surgical procedures and experiment protocols were performed in accordance with standard guidelines approved by the Ethics Committee of Experimental Research, Shanghai Medical College, Fudan University (Shanghai, China).

Conflict of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted

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Table

Table 1. Clinical characteristics of patient samples and expression of NLRP3 and GSDMD.

Figures

	Total	NLRP3		p value	GSDMD		p value
		High	Low		High	Low	
Patients(n)	20	9	11	-	10	10	-
Sex(n)	20	9	11	0.49	10	10	1
Age(years)	49.7±10.3	51.3±12.1	48.4±9.1	0.54	51.9±11.5	57.5±9.1	0.36
Weight(kg)	66.9±7.6	69.2±5.8	64.9±8.6	0.22	67.4±7.9	66.3±7.7	0.76
JOA	7.3±2.3	5.67±2.2	8.6±1.1	0.01	5.8±2.2	8.8±1.0	0.01
NDI	35.6±3.9	38.2±3.9	33.6±2.2	0.03	37.9±3.9	33.2±5.3	0.04
ASIA(n)							
a	1	1	0	0.056	1	0	0.053
b	7	1	6		1	6	
c	9	4	5		5	4	
d	3	3	0		3	0	
e	0	0	0		0	0

Figure 1

Expression of NLRP3 and GSDMD genes in peripheral blood of patients with SCI is positive correlated with the severity of injury. A-B. Relative mRNA expression of NLRP3 and GSDMD was assessed by RT-PCR in 20 normal people and 20 SCI patients. C. The linear regression analysis of the relevance between NLRP3 mRNA expression and JOA score ($r = -0.81$, $p < 0.001$), and NDI index ($r = 0.86$, $p < 0.001$). D. The linear regression analysis of the relevance between GSDMD mRNA expression and JOA score ($r = -0.88$, $p < 0.001$), and NDI index ($r = 0.87$, $p < 0.001$). E. The ROC curve analysis the diagnostic significance of NLRP3 (AUC=0.74) and GSDMD (AUC=0.72) for SCI.

Figure 2

CD73 deficiency facilitates NLRP3 inflammasome activation and pyroptosis of macrophages/microglia in vivo. A-B. Relative mRNA expression of pyroptosis related genes on the third day after SCI in WT and CD73 KO mice. C. The release of IL-1β, IL-6 and TNF-α in spinal cord homogenate was measured by ELISA. D. The release of LDH was detected by LDH Assay Kit. E-F. Representative Western blotting and statistical comparison of pyroptosis related protein on the third day after SCI or sham surgery in WT or CD73-deficient mice. G. Representative two-photon excitation images of immunofluorescence of CD68 and GSDMD acquired from WT or CD73 KO mice 3 days post-injury or sham surgery. H. Immunopositive particles for GSDMD and CASP-1 3 days post-injury or sham surgery in WT or CD73 KO mice. *p < 0.05, **p < 0.01, ***p < 0.001. Data are shown as the mean ± SD from 3 independent experiments.

Figure 3

CD73 alleviates LPS-induced NLRP3 inflammasome activation and pyroptosis in BV2 cells through A2BAR. After small interference RNA (RNAi) or pcDNA3.1 transfection for 24 h, BV2 cells were challenged with LPS (1µg/ml) for another 8 h in presence of adenosine (10µM) or MRS1706 (1µM). A. After administration of LPS combined with adenosine or MRS1706, microglial pyroptosis markers (NLRP3, ASC, CASP-1, GSDMD) mRNA expression were identified by RT-PCR in CD73 downregulated or

upregulated BV2 cells. B. The release of IL-1 β , IL-6 and TNF- α in different cultured groups measured by ELISA. C. The release of LDH was detected by LDH Assay Kit in different cultured groups. D-E. The pyroptosis related protein expression and statistical comparison in BV2 cells in different treated groups. F. Representative images of immunofluorescence of GSDMD acquired from different treated BV2 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus RNAi-CD73; & $p < 0.05$, && $p < 0.01$ versus pc-CD73. Data are shown as the mean $\pm$ SD from 3 independent experiments.

Figure 4

CD73 attenuates microglia pyroptosis via PI3K/AKT/Foxo1 signal. After overexpression plasmid transfection for 24 h, BV2 cells were challenged with LPS (1µg/ml) for another 8 h, then 3 independent samples and their controls were RNA sequenced A. The heat map analysis of differentially expressed mRNAs. B. The KEGG ontology enrichment analyses of differentially expressed mRNAs. C. The mRNA expression of PI3K, AKT, Foxo1 in BV2 cells challenged with LPS in presence or absence of pcDNA3.1 or MK2206 (3µM). D-E. The expression phosphorylation of PI3K, AKT, Foxo1 and statistical comparison in BV2 cells in different treated groups. F. The microglial pyroptosis markers (NLRP3, ASC, CASP-1, GSDMD) mRNA expression were identified by RT-PCR in CD73 upregulated BV2 cells in presence of MK2206 (3µM) or not. G. The release of IL-1β, IL-6 and TNF-α in different cultured groups measured by ELISA. H. The release of LDH was detected by LDH Assay Kit in different cultured groups. I-J. The pyroptosis related protein expression and statistical comparison in BV2 cells in different treated groups. *p < 0.05, **p < 0.01, ***p < 0.001 versus control; #p < 0.05, ##p < 0.01, ###p < 0.001 versus pc-CD73. Data are shown as the mean ± SD from 3 independent experiments. Intraperitoneal injection of 40mg/kg SC79 or DMSO was carried out every day after surgery in CD73 KO mice. K. Representative immunohistochemical staining for GSDMD and CASP-1 on the third day after SCI or sham surgery. L. Degree of motor disturbance assessed by the Basso, Beattie, and Bresnahan (BBB) criteria at different time points after SCI with SC79 treatment or not. *p < 0.05, **p < 0.01. Data are shown as the mean ± SD from 3 independent experiments.

Figure 5

Foxo1 is a transcriptional activator in the promoter region of GSDMD gene. A series of plasmids containing 5' unidirectional deletions of the promoter region of the GSDMD gene fused in frame to luciferase gene were transfected into BV2 cell lines as well as pc-Foxo1. Luciferase assay were performance after 48h. A. Dual-luciferase reporter assay of GSDMD and Foxo1. B. Result of ChIP analysis between GSDMD and Foxo1. C. The mutant GSDMD promoter-driven luciferase reporters with mutated

bases shown in red were constructed and relative fluorescence intensity was measured. D. The mRNA level of GSDMD of BV2 cells after incubated with pc-Foxo1 in presence with pc-CD73 or not for 24h. E. The release of LDH was detected by LDH Assay Kit in different cultured groups. F. The release of IL-1 β , IL-6 and TNF- α in different cultured groups measured by ELISA. **p < 0.01, ***p < 0.001 versus control; #p < 0.05, ##p < 0.01, ###p < 0.001 versus pc- Foxo1. Data are shown as the mean $\pm$ SD from 3 independent experiments.

Figure 6

HIF-1 α mediates CD73 upregulation in microglia. A. The mRNA expression of HIF-1 α and CD73 in BV2 cells challenged with LPS in presence or absence of RNAi-HIF-1 α . B-C. The HIF-1 α and CD73 protein expression in BV2 cells challenged with LPS in presence or absence of RNAi-HIF-1 α and statistical comparison. D. The mRNA expression of HIF-1 α and CD73 in WT mice 3 days post SCI, in which intraperitoneal injection of BAY87-22430 or DMSO was carried out every day after surgery. E-F. The HIF-1 α and CD73 protein expression in WT mice of different groups and statistical comparison. G. Immunopositive particles for CD73 3 days post-injury in WT mice with or without BAY87-2243 treatment. ***p < 0.001 versus blank; #p < 0.05, ##p < 0.01, ###p < 0.001 versus LPS group. Data are shown as the mean $\pm$ SD from 3 independent experiments.

Figure 7

CD73 mediates HIF-1α accumulation in microglia. A. The mRNA expression of HIF-1α and CD73 in BV2 cells challenged with LPS in presence or absence of RNAi-CD73. **p < 0.01, ***p < 0.001 versus blank; ##p < 0.01, ###p < 0.001 versus LPS group. B-C. The HIF-1α and CD73 protein expression in BV2 cells challenged with LPS in presence or absence of RNAi-CD73 and statistical comparison., ***p < 0.001 versus blank; #p < 0.05 versus LPS group. D. The mRNA expression of HIF-1α and CD73 in WT or CD73

KO mice 3 days post SCI. **p < 0.01, ***p < 0.001 versus WT-Sham; #p < 0.05 versus WT-SCI. E-F. The HIF-1 α and CD73 protein expression in WT or CD73 KO mice 3 days post SCI. ***p < 0.001 versus WT-Sham; ##p < 0.01 versus WT-SCI. G. After 24h pretreated with pc-CD73, the mRNA expression of HIF-1 α and CD73 in BV2 cells with determined by RT-PCR after challenged with LPS combined with MRS1706 (1 μ M) or SB203580 (10 μ M) for another 8h. *p < 0.05, **p < 0.01 versus control; #p < 0.05 versus pc-CD73. H-I. The HIF-1 α and CD73 protein expression in different treated BV2 cells. ***p < 0.001 versus control; ##p < 0.01 versus pc-CD73. Data are shown as the mean $\pm$ SD from 3 independent experiments.