

CD36 promotes diabetic brain injury by activating the NLRP3 inflammasome via ERK/NF- κ B signaling

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CD36 promotes diabetic brain injury by activating the NLRP3

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Subtitle: CD36 promotes DBI via NLRP3

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Abstract

Objective: To investigate the role and mechanism of Cluster of Differentiation 36 (CD36) in diabetic brain injury.

Methods: Diabetic (db/db) mice were injected with CD36 shRNA or NC lentivirus every 2 weeks for 8 weeks. Spatial learning and memory were evaluated by Morris water maze. Hippocampal pathology, apoptosis, and mRNA/protein levels of CD36, NLRP3, IL-1 β , and Caspase-1 were assessed by H&E, Nissl, TUNEL staining, RT-qPCR, and western blotting, respectively. In vitro, high glucose (30 mM)-stimulated astrocytes were transfected with CD36 or NLRP3 shRNA. Expression of inflammasome components, apoptosis-related proteins (Bax, Bcl-2), and ERK/NF- κ B signaling molecules was detected.

Results: Diabetic mice exhibited impaired spatial learning and memory, hippocampal neuronal damage, and increased CD36/NLRP3 inflammasome activation. CD36 knockdown alleviated these pathological changes and reduced NLRP3, Caspase-1, and IL-1 β expression ($P < 0.05$). In high glucose-stimulated astrocytes, CD36 knockdown reversed NLRP3 inflammasome activation, Bax/Bcl-2 imbalance, and ERK/NF- κ B phosphorylation. NLRP3 silencing attenuated inflammation and apoptosis without affecting CD36, confirming CD36 acts upstream of NLRP3.

Conclusion: Our data suggest that CD36 is associated with diabetic brain injury and NLRP3 inflammasome activation, potentially involving ERK/NF- κ B signaling. CD36 knockdown attenuated neuroinflammation and neuronal apoptosis; however, the causal hierarchy remain to be determined. These preliminary findings

preliminarily implicate CD36 as a potential therapeutic target.

Key words: Diabetic brain injury; Inflammation; Cluster of differentiation 36; NOD-like receptor protein 3; Apoptosis

Introduction

Currently, with the modernization of lifestyles and changes in dietary structures, diabetes mellitus (DM) has become a major disease that seriously threatens human life and health. In past decades, the prevalence and incidence of DM have increased rapidly. The results of the ninth edition of the Global Diabetes Map published by the International Diabetes Federation in 2022 showed that the number of global diabetes in 20–79 years was estimated to rise to 783.2 million(12.2%) in 2045. The number of diabetes in China ranks first in the world[1]. Central nervous system (CNS) complications caused by type 2 diabetes mainly include cerebrovascular diseases and cognitive impairment. Diabetic Encephalopathy (DE) is the complication of DM in the central nervous system. The clinical symptom of DE is mainly cognitive decline, and severe cases can lead to dementia. At present, mainstream research proposes that DE refers to cognitive impairment and brain structure and neurophysiological changes caused by diabetes [2]; however, little is known about how diabetes damages brain structure and function. The pathogenesis of DE is still unclear. Currently, it is believed that cerebrovascular diseases; blood-brain barrier destruction; amyloid beta (A β) deposition; long-term metabolic disorders such as hyperglycemia, hyperlipidemia, and insulin resistance; oxidative stress; inflammatory reactions; neurotransmitter changes; nerve cell degeneration; and apoptosis are all involved in

67 DE [3]. Among these pathological mechanisms, neuroinflammation has been the
68 focus of growing attention[4].

69 CD36 is a single-chain transmembrane glycoprotein on the cell surface,
70 belonging to scavenger receptor family B. To date, the scavenger receptor function of
71 CD36 has been widely studied. In the CNS, CD36 is expressed in many types of cells,
72 including cerebral vascular endothelial cells, astrocytes, microglia, and neurons [5, 6].
73 Its main functions include promoting the uptake of specific lipid molecules,
74 participating in the inflammatory response and oxidative stress, and participating in
75 clearing apoptotic cells. CD36 is also involved in regulating inflammation and
76 oxidative stress. CD36 interacting with β -amyloid can trigger a Src kinase-dependent
77 production of inflammatory mediators in macrophage cells.[7]. In atherosclerosis and
78 Alzheimer's disease, CD36 ligands facilitate sterile inflammation via the assembly of
79 a Toll-like receptor 4 and 6 heterodimer[8]. In patients with diabetes, a hyperglycemic
80 environment can induce increased CD36 expression, and then activate platelets to
81 aggravate the platelet-mediated inflammatory reaction. CD36 can also interact with
82 transforming growth factor beta 1(TGF β 1) and activate its biological activity of
83 fibrosis. The binding of CD36 to A β in microglia can lead to phosphorylation of
84 downstream mitogen-activated protein kinase (MAPK) p44/42, which eventually
85 leads to increased expression of TNF- α and IL- β , and increased levels of reactive
86 oxygen species (ROS)[9]. Recent pharmacological and genetic experiments have
87 shown that CD36 is also intimately involved in the pathological and
88 pathophysiological processes of many CNS diseases, such as stroke, Alzheimer's

disease (AD), and Parkinson's disease (PD) [10]. Microglial activation in AD involves β -amyloid – mediated engagement of surface immune receptors (e.g., CD36, CD40, CD45, integrins) and downstream recruitment of Src-family kinases, thereby activating the ERK signaling cascade and driving pro-inflammatory gene expression[11]. CD36 can regulate inflammatory reactions and mediate oxidative stress in ischemic stroke, atherosclerosis, chronic kidney disease, metabolic disorder, and cancer, and thus might be a therapeutic target in these diseases [12]. CD36-dependent mechanisms play crucial roles in the development of diabetic complications[13].

An inflammasome is defined by its sensor protein (a pattern recognition receptor (PRR)), and is a critical component of the innate immune system. There are five PRRs that have been confirmed to form inflammasomes: the nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing proteins family members NLRP1, NLRP3, and NLRC4; absent in melanoma 2 (AIM2); and pyrin [14]. The functional structure of NLRP3 is the most clear [15]. It consists of ASC (apoptosis-associated speck-like protein containing a caspase reciprocity domain), NLRP3, and pro-caspase-1 [16], which can mediate apoptosis and the secretion of IL-18, IL-1 β , and other pro-inflammatory factors [17, 18]. The number of NLRP3 inflammasomes extracted from neurons in rats after craniocerebral injury was upregulated, and associated gene expression also increased [19]. Thus, the NLRP3 inflammasome is implicated in the pathogenesis of many diseases, including obesity, diabetes, and AD [14, 20]. The activation of NLRP3, NLRP1, NLRC4 or AIM2

inflammasomes is associated with various neurological diseases[21].

In recent years, more attention has been paid to the relationship between CD36, NLRP3 inflammasomes, and IL-1 β [22, 23]. Sheedy et al. found a CD36-mediated endocytosis pathway that coordinates the transformation of soluble ligands into crystals or fibers in cells, resulting in lysosome destruction and activation of NLRP3 inflammasomes. They found that CD36-mediated uptake of ox-LDL caused the formation of cholesterol crystals in cells, which led to lysosome instability and NLRP3 activation. Through the recognition of sterile ligands by CD36, the soluble ligands are transformed into their pathogenic granule forms, thereby activating NLRP3 inflammasomes, which are related to type 2 diabetes, atherosclerosis, and AD. This finding emphasized the importance of CD36 in the production and nucleation of NLRP3 ligands in macrophages, and locates CD36 as a central regulator of inflammatory body activation in aseptic inflammation[24]. Recent studies have shown that CD36-mediated activation of NLRP3 inflammasomes might be one of the potential causes of podocyte injury [25].

Despite research progress, the role of CD36 in DE remains unreported. Elucidating whether CD36 modulates the NLRP3 pathway in promoting diabetic brain injury is crucial for deepening our understanding of inflammation - induced DE and uncovering novel DE therapeutic targets. Thus, we hypothesize that CD36 plays a role in contributing to DE development by affecting the NLRP3 inflammatory pathway.

Experimental materials and methods

Animals and treatments

We used C57BL/ks db/db mice as a genetic model of type 2 diabetes, which exhibit natural disease progression due to leptin receptor mutation. Eighteen healthy, 8-week-old, male, SPF-grade C57BL/ks db/db diabetic mice (40 ± 2.5 g) and six male C57BL/ks db/m control mice (20 ± 2.5 g) were purchased from Jiangsu Jicui Yaokang Biotechnology Co., Ltd. (Jiangsu, China), and were routinely fed in the experimental animal room at 22 ± 2 ° C and $55 \pm 2\%$ relative humidity. The C57BL/ks db/db diabetic mice were randomly divided into three groups: Model, Model+sh-NC, and Model+sh-CD36. The Model+sh-NC and Model+sh-CD36 groups (n = 6 each) were injected with negative control (NC) lentivirus vectors and CD36 knockdown lentivirus vectors, respectively, into the brain every two weeks for a total of eight weeks. Following Morris water maze test evaluation, all mice were sacrificed at the end of the 8th week. All experimental protocols were approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (Approval No. DW2022079), and all procedures were in accordance with the Guide for the Care and Use of Laboratory Animals[26].

Behavioral testing

The Morris water maze test was used to evaluate the spatial learning and memory ability of the mice. Experiments included spatial exploration experiments and positioning navigation experiments. The first day comprised adaptive training. The positioning navigation experiment was carried out on days 2 to 6, with a platform placed in four quadrants, and each group of mice were placed in the E, S, W, and NE

directions against the wall of the pool slowly. On the first day after the adaptive training, the training direction was S-E-WS-NE; the next day it was WS-S-NE-E; on the third day it was NE-WS-E-S; on the fourth day it was E-NE-S-WS; and on the fifth day it was S-E-WS-NE. The actual time (i.e., escape latency) taken by the mice to find the escape platform after entering the water was taken as the observation index, and if no platform was found within 60 seconds, it was recorded as 60 seconds. For the spatial exploration experiment: On the 6th day, the platform was removed, and the mice were put into the water maze from the SE direction. KEmaze Behavioral Software (KW-MWM, Nanjing Calvin Biotechnology Co., LTD, China) was used to measure the animals' performance (swim velocity, total distance, latency to reach the platform, and the number of times crossing the platform).

Cell culture and transfection

C8-D1A cells (ATCC CRL-2541), a murine astrocyte cell line derived from the cerebellum of postnatal day 8 C57BL/6 mice, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 1 g/L glucose, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cells were divided into six experimental groups: (i) normal control (NG): 5.5 mmol/L glucose; (ii) osmotic control (NG+Man): 5.5 mmol/L glucose plus 24.5 mmol/L mannitol; (iii) high glucose (HG): 30 mmol/L glucose; (iv) HG + control shRNA; (v) HG + CD36 shRNA; and (vi) HG + NLRP3 shRNA. For HG treatment, additional

glucose was added to low-glucose DMEM to achieve a final concentration of 30 mmol/L. Cells were collected after 48 h of stimulation.

For gene silencing, C8-D1A cells were infected with lentiviral particles carrying CD36 shRNA, NLRP3 shRNA, or control shRNA (Genechem, Shanghai, China) at a multiplicity of infection (MOI) of 10 in the presence of 8 µg/mL polybrene. Stable knockdown cells were established by puromycin selection prior to high glucose treatment.

Haematoxylin and eosin (HE) staining

Paraffin sections of mouse hippocampal tissues were prepared, followed by baking, dewaxing, and hydration. The sections were then stained with hematoxylin dye solution(ZLI-9610, Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 3 to 5 min, washed with running water, differentiated with 1% hydrochloric acid alcohol(1ml hydrochloric acid +64ml alcohol), dyed with anti-blue solution(G1040, Servicebio, Wuhan, China) and eosin(G1100, Solarbio, Beijing, China) for 3 to 5 min, dehydrated, sealed, and observed under a microscope (BX43, Olympus, Tokyo, Japan). Normal neurons are characterized by a large cell body with abundant cytoplasm and one or two large, round nuclei. In contrast, damaged neurons exhibit shrunken cell bodies, pyknotic nuclei, dark cytoplasm, and numerous empty vesicles. Hippocampal neuronal damage was assessed using a standardized semi-quantitative scale by a blinded manner[27, 28], as follows: grade 0, no damage in any hippocampal subregion; grade 1, scattered neuronal damage in the CA1 subregion; grade 2, moderate neuronal damage in the CA1 subregion (<50% of neurons affected);

grade 3, severe damage (>50% of neurons affected) in the CA1 pyramidal cell layer;
grade 4, extensive damage across all hippocampal regions. Four random high-power
($\times 400$) fields per brain section were evaluated.

Nissl staining

After baking, dewaxing, and hydration, the paraffin sections of mouse hippocampal
tissues were dewaxed with xylene I, II, and III (33535, Xilong Scientific Co., Ltd.,
Shantou, China) for 10 min each, soaked in 100% ethanol for 5 min, followed by 70%
and 90% ethanol (32061, Xilong Scientific Co., Ltd., Shantou, China) for 2 min each,
and finally soaked in distilled water for 2 min. The sections were stained using Nissl
dyeing solution (G1036, Servicebio, Wuhan, China) for 10 min and then washed with
distilled water twice. The sections were dehydrated using 95% ethanol I and II (32061,
Xilong Scientific Co., Ltd., Shantou, China) for 2 min each, and xylene I and II were
applied to render the sections transparent for 5 min each. The slides were sealed with
neutral gum and dried before observing the changes in Nissl bodies under a scanning
electron microscope (BX43, Olympus, Tokyo, Japan). Quantitative analysis was
performed using ImageJ 9.0 software.

TUNEL staining

TUNEL staining was used to detect the apoptosis level of cells in brain tissue.
Mouse hippocampuses were sectioned, baked, dewaxed, and hydrated, and then
treated with proteinase K for repair. The sections were incubated with TUNEL
solution (Beyotime Biotechnology, Shanghai, China) for 1 h at 42 °C, stained with
4,6-diamidino-2-phenylindole (DAPI) for 5 min, and observed under a microscope

(BX43, Olympus). TUNEL positive expression was detected as fluorescein-labeled red fluorescence. And the results were quantified using ImageJ 9.0 software.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from hippocampal tissue and C8-D1A cells using TRIzol reagent (CW0580S, CWBIO, Jiangsu, China). RNA concentration and purity were assessed using a NanoPhotometer spectrophotometer (NP80, Implen, Germany). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a reverse transcription kit (Q711-02, Vazyme, Nanjing, China) according to the manufacturer's instructions. qPCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Shanghai, China) using ChamQ SYBR qPCR Master Mix (Q331-02, Vazyme, Nanjing, China). The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Melting curve analysis was performed to confirm primer specificity. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [29] and normalized to Tbp as the reference gene.

Primer sequences used for hippocampal tissue

Cd36: 5'-CCAAGCTATTGCGACATGATT-3' (sense) and 5'-

CCGAACACAGCGTAGATAGACC-3' (antisense)

Nlrp3: 5'-GAGTGGATGGGTTTGCTGG-3' (sense) and 5'-

GCGTTCCTGTCCTTGATAGAGT-3' (antisense)

242 *Casp1*: 5'-ACAGAACAAGAAGATGGCACA-3' (sense) and 5'-

243 CAGATAATGAGGGCAAGACG-3' (antisense)

244 *Il1b*: 5'-GAAATGCCACCTTTTGACAGTG-3' (sense) and 5'-

245 TGGATGCTCTCATCAGGACAG-3' (antisense)

246 **Primer sequences used for C8-D1A cells**

247 *Cd36*: 5'-CTTTTCTTCTTCACAGCTGCC-3' (sense) and 5'-

248 CTCAAGGACAACCTCCCTTTTG-3' (antisense)

249 *Nlrp3*: 5'-TGTACCCAAGGCTGCTATCTGG-3' (sense) and 5'-

250 GGGGCTTAGGTCCACACAGAAA-3' (antisense)

251 *Casp1*: 5'-TCCAGGAGGGAATATGTGGGA-3' (sense) and 5'-

252 ACTCCTTGTTTCTCTCCACGG-3' (antisense)

253 *Il1b*: 5'-GCCACCTTTTGACAGTGATGAG-3' (sense) and 5'-

254 AGCTTCTCCACAGCCACAAT-3' (antisense)

255 *Bax*: 5'-AAACTGGTGCTCAAGGCC-3' (sense) and 5'-

256 GGTCCCGAAGTAGGAGAGGA-3' (antisense)

257 *Bcl2*: 5'-GACTGAGTACCTGAACCGGC-3' (sense) and 5'-

258 AGTTCCACAAAGGCATCCCAG-3' (antisense)

259 *Rela* (p65): 5'-GCCAAAGAAGGACACGACA-3' (sense) and 5'-

260 TCCCGGAGTTCATCTCATAGT-3' (antisense)

261 **Western blotting analysis**

262 Proteins were extracted from hippocampal tissues and C8-D1A cells using RIPA

263 lysis buffer supplemented with protease and phosphatase inhibitor cocktails. Protein

concentrations were determined using a BCA protein assay kit. Equal amounts of protein (30 µg per lane) were separated by 10–12% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature and then incubated overnight at 4 °C with the following primary antibodies: anti-IL-1 β , anti-Caspase-1, anti-cleaved Caspase-1 (p20), anti-NF- κ B p65, anti-ERK, anti-p-ERK, and anti-Bcl-2 (1:1000; Cell Signaling Technology, Danvers, MA, USA); anti-CD36 and anti-NLRP3 (1:1000; Abcam, Cambridge, UK); anti-Bax (1:1000; Proteintech, Chicago, IL, USA); and anti- β -actin (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing three times with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (1:10,000; Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) substrate and detected using an Odyssey Fc Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analysis and graph generation were performed using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). All experiments were independently repeated three times. Data are presented as mean $\pm$ standard deviation (SD). Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc tests. A P-value < 0.05 was

considered statistically significant.

Results

CD36 knockdown ameliorates cognitive impairment in diabetic mice

To assess the effect of CD36 knockdown on cognitive function, a Morris water maze test was performed. Compared with the non-diabetic Control group, db/db mice injected with negative control (NC) lentivirus (Model+sh-NC group) exhibited significantly decreased swimming velocity and total distance traveled ($P < 0.05$). The platform distance and number of platform crossings showed downward trends, although these parameters did not reach statistical significance. Notably, CD36 knockdown (Model+sh-CD36 group) reversed these alterations, as evidenced by upward trends in swimming velocity and total distance compared with the Model+sh-NC group, indicating that CD36 silencing improves spatial learning and memory abilities in diabetic mice (Figure 1).

CD36 knockdown attenuates hippocampal pathological damage and neuronal apoptosis in diabetic mice

Histopathological examination of hippocampal CA1 regions was conducted to evaluate neuronal integrity. Hematoxylin and eosin (H&E) staining revealed densely packed, morphologically intact neurons in the Control group. In contrast, db/db mice (Model group) displayed reduced neuronal density and enlarged intercellular spaces. The Model+sh-NC group exhibited more severe damage, including disordered neuronal arrangement, deeply stained cytoplasm and nucleoli, cytoplasmic keratinization, nuclear pyknosis, and extensive inflammatory cell infiltration. CD36

knockdown markedly alleviated these pathological changes, with reduced inflammatory infiltration, attenuated neuronal damage, and improved nuclear pyknosis compared with the Model+sh-NC group ($P < 0.05$; Figure 2A).

Consistent with these findings, Nissl staining demonstrated reduced or absent Nissl bodies in the hippocampal CA1 region of the Model group. The Model+sh-NC group showed significantly decreased, disordered, and elongated Nissl bodies. Importantly, the number of Nissl bodies was significantly higher in the Model+sh-CD36 group than in the Model+sh-NC group ($P < 0.05$), indicating attenuated neuronal damage following CD36 knockdown (Figure 2B).

Furthermore, TUNEL staining revealed an upward trend in hippocampal apoptosis in the Model group compared with the Control group. The apoptosis rate was significantly increased in the Model+sh-NC group ($P < 0.05$). CD36 silencing significantly reduced the apoptosis rate compared with the Model+sh-NC group ($P < 0.05$), suggesting a protective effect against diabetes-induced hippocampal cell apoptosis (Figure 3).

CD36 knockdown suppresses NLRP3 inflammasome activation in the hippocampus of diabetic mice

To investigate the molecular mechanism underlying CD36-mediated neuroprotection, the expression levels of inflammation-related genes were quantified. Quantitative real-time PCR (qRT-PCR) analysis showed that, compared with the Control group, the mRNA levels of interleukin-1 β (IL-1 β) and Caspase-1 were significantly elevated in the Model group ($P < 0.05$), whereas CD36 and NLRP3

mRNA expression did not change significantly. In the Model+sh-NC group, mRNA expression of CD36, NLRP3, IL-1 β , and Caspase-1 was significantly increased compared with the Model group ($P < 0.05$). Following CD36 knockdown, the mRNA levels of CD36, NLRP3, IL-1 β , and Caspase-1 were significantly decreased compared with the Model+sh-NC group ($P < 0.05$; Figure 4).

Western blot analysis further confirmed these findings at the protein level. Compared with the Control group, protein levels of CD36, NLRP3, Caspase-1, and IL-1 β were significantly increased in both the Model and Model+sh-NC groups ($P < 0.05$). CD36 knockdown significantly downregulated the expression of all four proteins compared with the Model+sh-NC group ($P < 0.05$; Figure 5). Collectively, these results demonstrate that CD36 knockdown alleviates diabetic brain injury by suppressing NLRP3 inflammasome activation in the hippocampus.

High glucose upregulates CD36/NLRP3 axis and pro-apoptotic gene expression in astrocytes

To validate the *in vivo* findings *in vitro*, primary astrocytes were exposed to high glucose (HG, 30 mM). RT-qPCR analysis showed that HG stimulation significantly upregulated CD36 mRNA expression compared with the normal glucose (NG, 5.5 mM) group ($P < 0.001$; Fig. 6A). Concurrently, mRNA levels of NLRP3 inflammasome components, including NLRP3, IL-1 β , and Caspase-1, were markedly elevated (all $P < 0.001$; Fig. 6B – D). No significant differences were observed between the mannitol osmotic control (NG+Man) and NG groups ($P > 0.05$), excluding hyperosmotic stress as a confounding factor (Fig. 6A – D).

Apoptosis-related gene analysis further revealed that HG stimulation significantly increased the pro-apoptotic gene Bax ($P < 0.001$; Fig. 6E) and decreased the anti-apoptotic gene Bcl-2 ($P < 0.001$; Fig. 6F), resulting in a significantly elevated Bax/Bcl-2 ratio ($P < 0.001$; Fig. 6G). In contrast, NF- κ B p65 mRNA expression did not differ significantly among any groups ($P > 0.05$; Fig. 6H), suggesting that NF- κ B activation, if present, is primarily regulated at the post-translational level.

CD36 knockdown attenuates high glucose-induced NLRP3 inflammasome activation and apoptosis

To investigate the regulatory role of CD36, astrocytes were transfected with CD36-targeting shRNA lentivirus. RT-qPCR confirmed efficient CD36 knockdown, with mRNA expression reduced by more than 80% in the HG+shCD36 group compared with the HG group ($P < 0.001$; Fig. 6A). CD36 silencing significantly attenuated HG-induced upregulation of NLRP3, IL-1 β , and Caspase-1 mRNA (all $P < 0.001$; Fig. 6B – D). Moreover, CD36 knockdown partially restored the apoptotic balance, as evidenced by significantly decreased Bax ($P < 0.001$; Fig. 6E) and increased Bcl-2 ($P < 0.05$; Fig. 6F) expression, thereby reducing the Bax/Bcl-2 ratio ($P < 0.001$; Fig. 6G). No significant differences were detected between the HG+GFP and HG groups ($P > 0.05$; Fig. 6), confirming that lentiviral transfection per se did not confound the results. At the protein level, Western blot analysis revealed that HG stimulation significantly upregulated CD36 ($P < 0.05$), NLRP3, pro-IL-1 β , cleaved Caspase-1, and mature IL-1 β protein levels compared with the NG group ($P < 0.001$; Fig. 7A – F). CD36 knockdown significantly suppressed HG-induced NLRP3 expression ($P < 0.05$) and

reduced pro-IL-1 β protein levels ($P < 0.05$), alongside decreased cleaved Caspase-1 ($P < 0.05$) and mature IL-1 β ($P < 0.05$; Fig. 7B – F). Furthermore, CD36 silencing partially reversed HG-induced Bax upregulation ($P < 0.001$) and Bcl-2 downregulation ($P < 0.05$), thereby reducing the Bax/Bcl-2 ratio ($P < 0.001$; Fig. 8A – D). No significant differences were observed between the osmotic control (NG+Man) and NG groups, or between the empty vector control (HG+GFP) and HG groups for any target protein ($P > 0.05$; Figs. 7 and 8), confirming that the observed effects were attributable to high glucose itself.

NLRP3 silencing attenuates high glucose-induced inflammation and apoptosis without affecting CD36

To determine the hierarchical relationship between CD36 and NLRP3, astrocytes were transfected with NLRP3 shRNA. RT-qPCR confirmed efficient NLRP3 knockdown, with mRNA expression reduced by more than 90% in the HG+shNLRP3 group compared with the HG group ($P < 0.01$; Fig. 6B). NLRP3 silencing significantly attenuated HG-induced upregulation of IL-1 β and Caspase-1 mRNA (all $P < 0.001$) and reversed the dysregulated Bax/Bcl-2 ratio ($P < 0.001$; Fig. 6C, 6D, 6G). However, NLRP3 knockdown had no significant effect on CD36 mRNA expression ($P > 0.05$; Fig. 6A), suggesting that CD36 acts upstream of NLRP3 in this pathway.

Western blot analysis corroborated these findings. NLRP3 knockdown markedly blocked inflammasome activation, as evidenced by significantly reduced NLRP3 protein ($P < 0.001$), decreased mature IL-1 β ($P < 0.001$), and nearly abolished

cleaved Caspase-1 ($P < 0.001$; Fig. 7A – F). Although pro-IL-1 β remained elevated relative to the NG group, its expression in the HG+shNLRP3 group was significantly lower than that in the HG group ($P < 0.05$; Fig. 7E). Importantly, NLRP3 silencing did not significantly affect CD36 protein expression ($P > 0.05$; Fig. 7A, 7B). NLRP3 knockdown also partially reversed HG-induced Bax upregulation ($P < 0.001$) and Bcl-2 downregulation ($P < 0.05$), consequently lowering the Bax/Bcl-2 ratio ($P < 0.001$; Fig. 8A – D).

ERK and NF- κ B pathways are activated in high glucose-stimulated astrocytes and differentially modulated by CD36 and NLRP3

To elucidate the signaling mechanisms linking CD36 to NLRP3 activation, the activation status of ERK and NF- κ B pathways was examined by Western blot. Total ERK protein levels remained unchanged across all experimental groups ($P > 0.05$; Fig. 9B). However, HG stimulation significantly increased ERK phosphorylation (p-ERK) compared with the NG group ($P < 0.001$; Fig. 9C), and this effect was markedly attenuated by both CD36 knockdown ($P < 0.001$) and NLRP3 knockdown ($P < 0.001$; Fig. 9C).

Similarly, total p65 expression did not differ significantly among groups ($P > 0.05$; Fig. 9E). In contrast, HG stimulation significantly elevated phosphorylated p65 (p-p65) levels ($P < 0.001$; Fig. 9F) and reduced total I κ B α expression ($P < 0.001$; Fig. 9D), indicative of NF- κ B pathway activation. CD36 knockdown partially reversed these changes, significantly decreasing p-p65 phosphorylation ($P < 0.01$; Fig. 9F) and restoring I κ B α levels ($P < 0.01$; Fig. 9D). Notably, NLRP3 knockdown did not

significantly affect p-p65 expression ($P > 0.05$; Fig. 9F), despite significantly increasing total I κ B α ($P < 0.001$; Fig. 9D). No significant differences were observed between the osmotic control (NG+Man) and NG groups, or between the empty vector control (HG+GFP) and HG groups for any target protein ($P > 0.05$; Fig. 9), confirming that the observed effects were attributable to high glucose itself.

Collectively, these findings indicate that ERK and NF- κ B pathways serve as critical signaling bridges connecting CD36 to downstream NLRP3 inflammasome activation under high-glucose conditions.

Discussion:

The present study provides convergent in vivo and in vitro evidence establishing CD36 as a causal upstream mediator of diabetic brain injury via NLRP3 inflammasome activation. In db/db mice, hippocampal neuronal injury, apoptosis, and cognitive deficits were accompanied by elevated CD36 and NLRP3 inflammasome expression. Lentivirus-mediated CD36 knockdown attenuated NLRP3 activation, reduced neuroinflammation and apoptosis, and ameliorated cognitive impairment. Complementary experiments in high glucose (HG)-stimulated astrocytes confirmed that CD36 drives NLRP3 inflammasome assembly, IL-1 β maturation, and Bax/Bcl-2-mediated apoptosis. Mechanistically, CD36 engages ERK and NF- κ B signaling to prime and activate the NLRP3 inflammasome. Collectively, these findings identify the CD36/NLRP3 axis as a core pathogenic pathway in diabetic encephalopathy (DE).

CD36 functions upstream of the NLRP3 inflammasome

A critical finding of this study is the hierarchical positioning of CD36 upstream of NLRP3. Reciprocal knockdown experiments revealed that CD36 silencing suppressed NLRP3 inflammasome activation and reduced ERK/NF- κ B signaling, whereas NLRP3 knockdown — while effectively blocking inflammasome activation and apoptosis—failed to alter CD36 expression. This asymmetry demonstrates that CD36 is not merely a correlative marker but an initiating sensor that triggers downstream inflammatory cascades[30]. This positioning is consistent with the established role of CD36 as a pattern recognition receptor capable of sensing metabolic stress and engaging pro-inflammatory signaling[30].

Astrocytes as active participants in diabetic neuroinflammation

Our in vitro findings further indicate that astrocytes are active participants rather than passive bystanders in diabetic neuroinflammation[4, 31]. HG exposure upregulated CD36 in astrocytes, promoting NLRP3 inflammasome assembly and Caspase-1-dependent IL-1 β maturation, which subsequently triggered apoptotic signaling. CD36 knockdown in astrocytes reversed these alterations, confirming the cell-autonomous role of CD36 in initiating neuroinflammatory responses under metabolic stress.

CD36 as a convergent node linking metabolic stress to neuroinflammation

CD36 is widely recognized as a convergent receptor linking metabolic dysregulation to inflammation across diverse pathologies. In Alzheimer's disease (AD), amyloid- β binding to CD36 elicits robust pro-inflammatory responses[32], and CD36 deficiency confers protection against atherosclerotic plaque formation and

amyloid deposition[33, 34]. Age- and sex-related variations in CD36 distribution further modulate neuroinflammatory processes in cerebral amyloid angiopathy and AD[35]. Similarly, CD36-mediated uptake of ox-LDL stimulates monocytes to release IL-1 β , IL-6, and TNF- α [36], and ox-LDL-induced cholesterol crystal formation via NF- κ B-dependent pathways activates NLRP3 in atherosclerosis[22]. Recent studies have extended this paradigm to renal tubular epithelial cells, where CD36 facilitates NLRP3 activation through mitochondrial ROS[37], and to diabetic myocardial remodeling, where CD36 translocation, NLRP3 activation, and AGE/RAGE signaling operate synergistically[38]. Our study provides direct evidence that this CD36-NLRP3 axis is operational in diabetic brain injury, bridging a critical gap in the understanding of DE pathogenesis.

The NLRP3 inflammasome in diabetes-associated cognitive decline

The NLRP3 inflammasome, comprising NLRP3, ASC, and Caspase-1, is increasingly implicated in diabetes-associated neurological complications [39-41]. Upon activation, Caspase-1 cleaves pro-IL-1 β into its biologically active form, driving neuroinflammation[40, 41]. Previous studies have demonstrated that high glucose activates hippocampal NLRP3 inflammasomes in diabetic mice with cognitive impairment, and that pharmacological NLRP3 inhibition ameliorates cognitive dysfunction [42, 43]. Ward et al. further reported that high glucose increases NLRP3 expression and activation in diabetic vascular complications[44]. Consistent with these reports, our data demonstrate that diabetic mice exhibit hippocampal neuronal injury with elevated NLRP3, IL-1 β , and Caspase-1 expression, and that

NLRP3 suppression alleviates brain damage. Our findings further suggest the existence of an NLRP3/IL-1 β -dependent inflammatory amplification loop that contributes to spatial learning and memory deficits.

ERK and NF- κ B serve as signaling bridges linking CD36 to NLRP3

The present study identifies ERK and NF- κ B as critical signaling bridges connecting CD36 to NLRP3 activation. HG stimulation significantly increased ERK phosphorylation and NF- κ B p65 phosphorylation, accompanied by I κ B α degradation—hallmarks of canonical NF- κ B activation. CD36 knockdown markedly attenuated both pathways, indicating that CD36 engagement triggers a kinase cascade that primes the inflammasome. Notably, NLRP3 knockdown reduced ERK phosphorylation but did not affect p-p65 levels, while significantly elevating total I κ B α . This dissociation suggests that ERK may function within a feedback or parallel regulatory circuit, whereas NF- κ B activation lies specifically downstream of CD36 and upstream of NLRP3 transcriptional priming. The ERK pathway, operating through the MAPKKK-MAPKK-MAPK tiered kinase hierarchy[45], is context-dependent and capable of modulating both inflammatory and apoptotic responses[46, 47]. ERK-mediated phosphorylation of BIM regulates its interaction with Bax [48], and ERK1/2 signaling modulates Bcl-2/Bax ratios in angiotensin II-induced endothelial apoptosis [49]. Thus, ERK may serve as a convergent node integrating inflammatory and apoptotic signals downstream of CD36.

Interplay between NLRP3 inflammasome activation and apoptosis

Bax and Bcl-2 are central regulators of the mitochondria-mediated intrinsic apoptotic pathway[50, 51] . In the present study, HG treatment upregulated Bax and downregulated Bcl-2, resulting in an elevated Bax/Bcl-2 ratio indicative of apoptotic priming. Both CD36 and NLRP3 knockdown partially restored the Bax/Bcl-2 balance, attenuating apoptosis. These findings indicate that blocking either the upstream sensor (CD36) or the downstream effector (NLRP3) can interrupt the inflammatory-apoptotic axis, underscoring the intimate interplay between these pathways in diabetic brain injury. The NLRP3/IL-1 β cascade likely amplifies this process through a positive feedback mechanism, further driving Bax/Bcl-2 dysregulation and neuronal cell death.

Therapeutic implications

From a translational perspective, CD36 represents a promising therapeutic target for diabetic encephalopathy. Current management of diabetes-associated cognitive decline remains largely symptomatic, and anti-inflammatory strategies targeting IL-1 β or NLRP3 are under active investigation. Intervening at the level of CD36 may offer broader efficacy by blocking the initiating signal upstream of the inflammasome, thereby preventing both the inflammatory and apoptotic sequelae. CD36-mediated NLRP3 inflammasome activation represents an early pathogenic pathway that links metabolic stress to neuroinflammation, supporting the development of CD36-targeted therapeutics for DE[8, 52].

Limitations

This study has several limitations. First, cognitive assessment was limited to the Morris water maze, and hippocampal analysis precludes generalization to other brain regions affected by diabetic brain injury. Second, genetic interventions were restricted to shRNA-mediated knockdown without germline knockout or overexpression. Third, the proposed CD36 – ERK/NF- κ B – NLRP3 causal hierarchy remains incompletely validated. We did not employ pharmacological inhibitors to acutely block kinase activity, nor did we perform rescue experiments using constitutively active ERK/NF- κ B mutants in CD36-silenced cells to determine whether forced activation of these pathways restores NLRP3 inflammasome activity. Consequently, we cannot establish whether ERK/NF- κ B phosphorylation is necessary or sufficient for CD36-driven NLRP3 priming, or whether CD36 regulates NLRP3 through parallel, ERK/NF- κ B-independent pathways. Additionally, off-target effects of shRNA cannot be fully excluded without complementary pharmacological approaches. Fourth, canonical inflammasome components, including ASC speck formation, GSDMD cleavage, and pyroptosis markers, were not assessed. Fifth, in vitro experiments were confined to astrocytes, and CD36/NLRP3 signaling was not examined in neuronal cells or other neurovascular unit components (e.g., microglia, endothelial cells). Future studies should employ pharmacological and genetic strategies to validate this signaling axis, investigate ASC/GSDMD-mediated pyroptosis, extend the analysis to neurons and other CNS cell types, and explore pharmacological CD36 inhibitors in diabetic animal models.

Conclusions:

In conclusion, our findings suggest an association between CD36 and NLRP3 inflammasome activation in diabetic brain injury, with potential involvement of ERK/NF- κ B signaling. However, the proposed causal hierarchy remains to be validated using pharmacological inhibitors and genetic rescue strategies. Furthermore, the roles of ASC and GSDMD-mediated pyroptosis, as well as the conservation of this pathway in neuronal cells and other neurovascular unit components, were not addressed in the present study. While CD36 knockdown attenuated neuroinflammation and neuronal apoptosis in db/db mice and HG-stimulated astrocytes, these results should be interpreted as associative rather than causative. Collectively, these findings identify the CD36/NLRP3 axis as a candidate mechanism in diabetic brain injury and warrant further investigation into CD36-targeted therapeutic strategies.

List of abbreviations

DBI: Diabetic brain injury; Inflammation; CD36: Cluster of differentiation 36; NLRP3: NOD-like receptor protein 3; DM: diabetes mellitus; DE: Diabetic Encephalopathy; NC: negative control; CNS: Central nervous system; TGF β 1: transforming growth factor beta 1; MAPK: mitogen-activated protein kinase ; ROS: reactive oxygen species; AD: Alzheimer's disease; PD: Parkinson's disease; PRR: pattern recognition receptor; NOD: nucleotide-binding oligomerization domain; LRR: leucine-rich repeat; AIM2: absent in melanoma 2; ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain; qRT-PCR: quantitative real-time reverse transcription; RIPA: radioimmunoprecipitation assay; SDS-PAGE:

sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride

Declarations

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (Approval No. DW2022079), and all procedures were in accordance with the Guide for the Care and Use of Laboratory Animals.

Consent for publication:

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Clinical trial number

Not applicable.

Author Contributions

Chunyan Zhang and Xueqin Cao contributed to the study design, conducted the animal experiments, and participated in writing the manuscript. Pengfei Meng and Yanying Wu assisted with the animal experiments. Bo Bai and Beibei Gao contributed to the preliminary experiments and data analysis. Chunyan Zhang and Cunshui Xue conceived and supervised the study. All authors approved the final manuscript.

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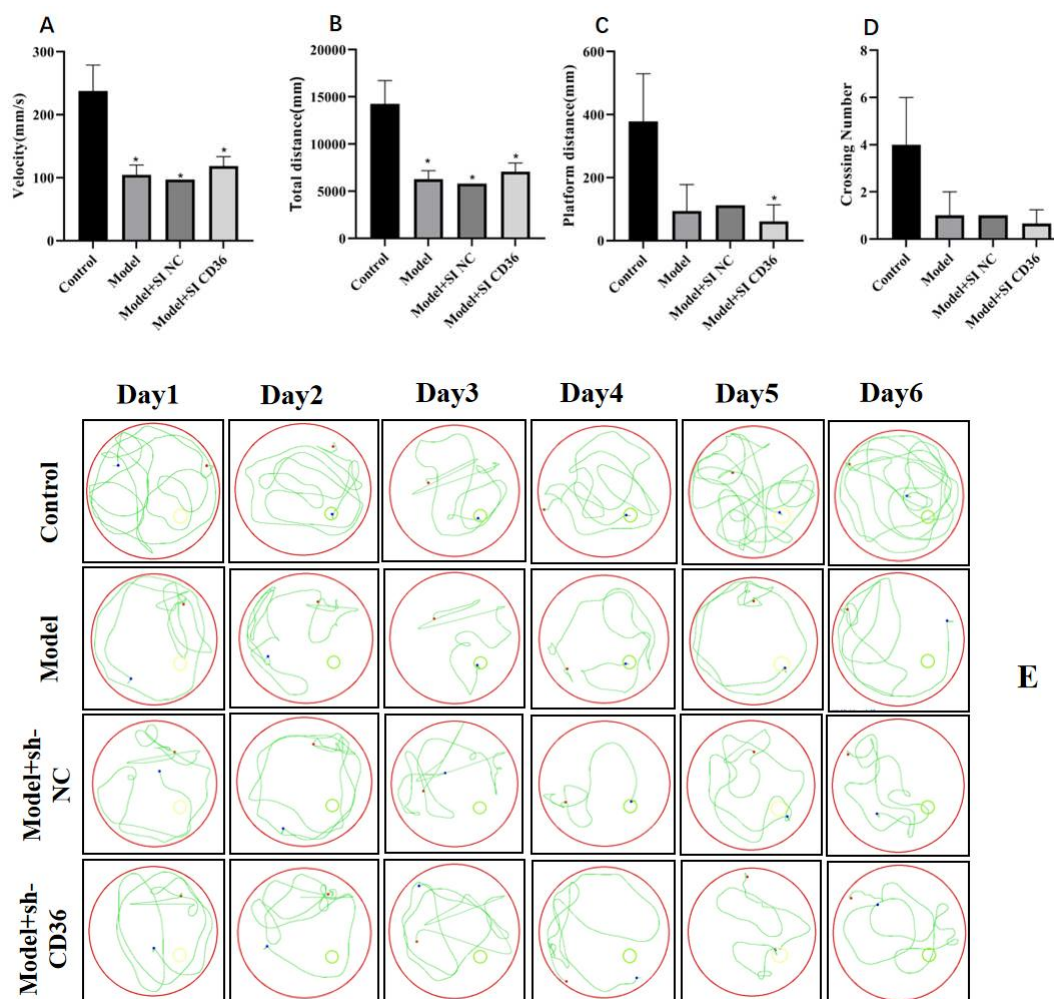


Fig 1. Morris Water Maze test results; (A) The velocities of the different groups of mice in the probe tests. (B) The total distance travelled of the different groups of mice in the probe tests. (C) The platform distances of the different groups of mice in the probe tests. (D) The crossing numbers of the different groups of mice in the probe tests. (E) Representative swim paths of different groups of mice in visible platform task trials. (The red dot represents the starting point, and the blue dot represents the endpoint). Data are presented as mean $\pm$ SD. * $P < 0.05$ compared with the control group by ANOVA. Model, mouse db/db diabetes model; sh-NC, negative control short hairpin RNA; sh-CD36 short hairpin RNA targeting mouse CD36.

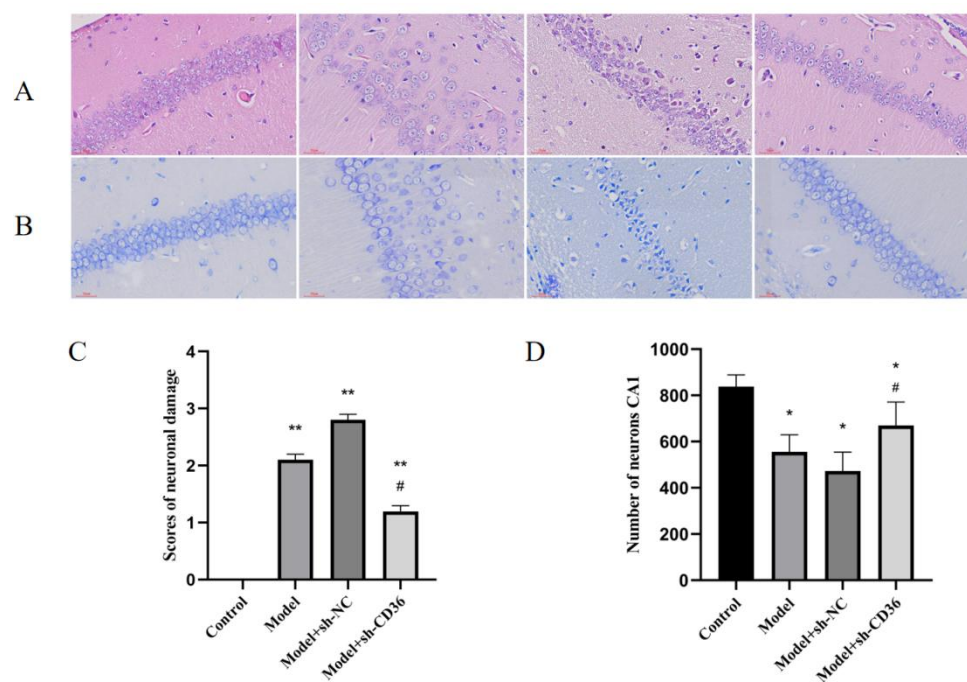


Fig 2. Effect of CD36 knockdown on diabetes-induced hippocampal damage in model mice. Histological assessment via HE and Nissl staining ($\times 400$ magnification). A: Representative pictures of HE staining in the hippocampus of mice from different groups are shown. B: Representative Nissl staining in the hippocampus of mice from different groups are shown. C: Semiquantitative analysis in the hippocampal neuronal damage scores. D: Quantitative Nissl staining analysis according to signal intensity using ImageJ 9.0 software. Scale bar = $30 \mu\text{m}$. * $P < 0.05$ vs. Control group, ** $P < 0.01$ vs. Control group by ANOVA. # $P < 0.05$ vs. Model+sh-NC group by ANOVA. Model, mouse db/db diabetes model; Abbreviations: HE, hematoxylin and eosin; sh-NC, negative control short hairpin RNA; sh-CD36, short hairpin RNA targeting mouse CD36.

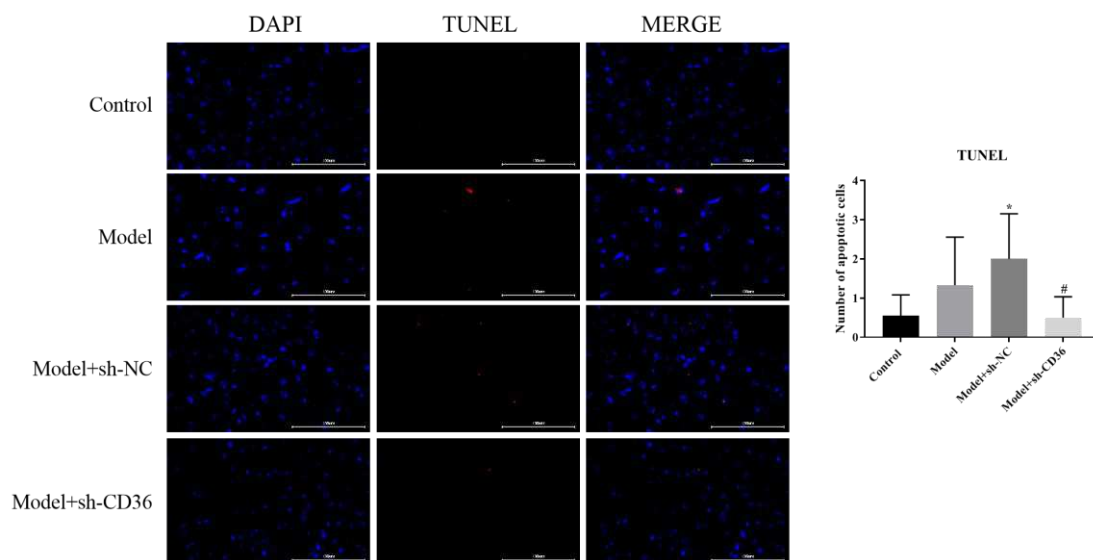


Fig 3. CD36 silencing reduced the apoptosis of hippocampal cells induced by diabetes. TUNEL staining images (magnification: $\times 400$) and quantification of TUNEL-positive cells per field are shown. Data are presented as mean $\pm$ SD. * $P < 0.05$ vs. Control group; # $P < 0.05$ vs. Model+sh-NC group by ANOVA. TUNEL, terminal deoxynucleotidyl transferase nick-end-labeling; DAPI, 4,6-diamidino-2-phenylindole; Model, mouse db/db diabetes model; sh-NC, negative control short hairpin RNA; sh-CD36, short hairpin RNA targeting mouse CD36.

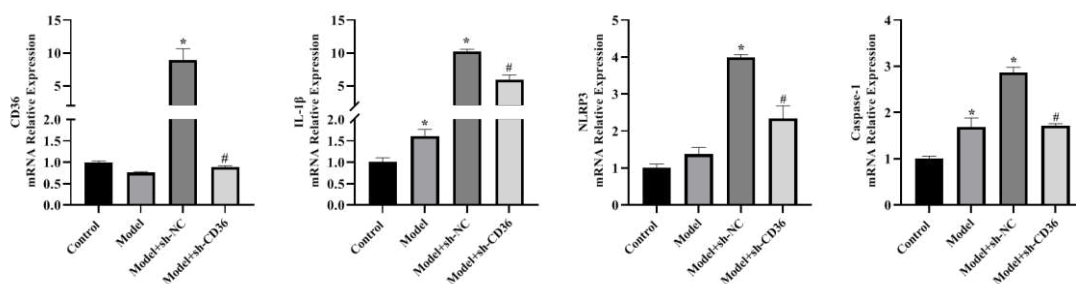


Fig 4. CD36 silencing reversed the diabetes-induced increase in inflammatory factor mRNA expression. qRT-PCR analysis of CD36, NLRP3, Caspase-1, and IL-1 β gene expression in mouse hippocampal tissues. Data are presented as mean $\pm$ SD. * $P < 0.05$ vs. Control group; # $P < 0.05$ vs. Model+sh-NC group by ANOVA.

0.05 vs. Model+sh-NC group by ANOVA. CD36, cluster of differentiation 36; IL-1 β , interleukin 1 β ; NLRP3, NLR family pyrin domain containing 3; Model, mouse db/db diabetes model; sh-NC, negative control short hairpin RNA; sh-CD36, short hairpin RNA targeting mouse CD36.

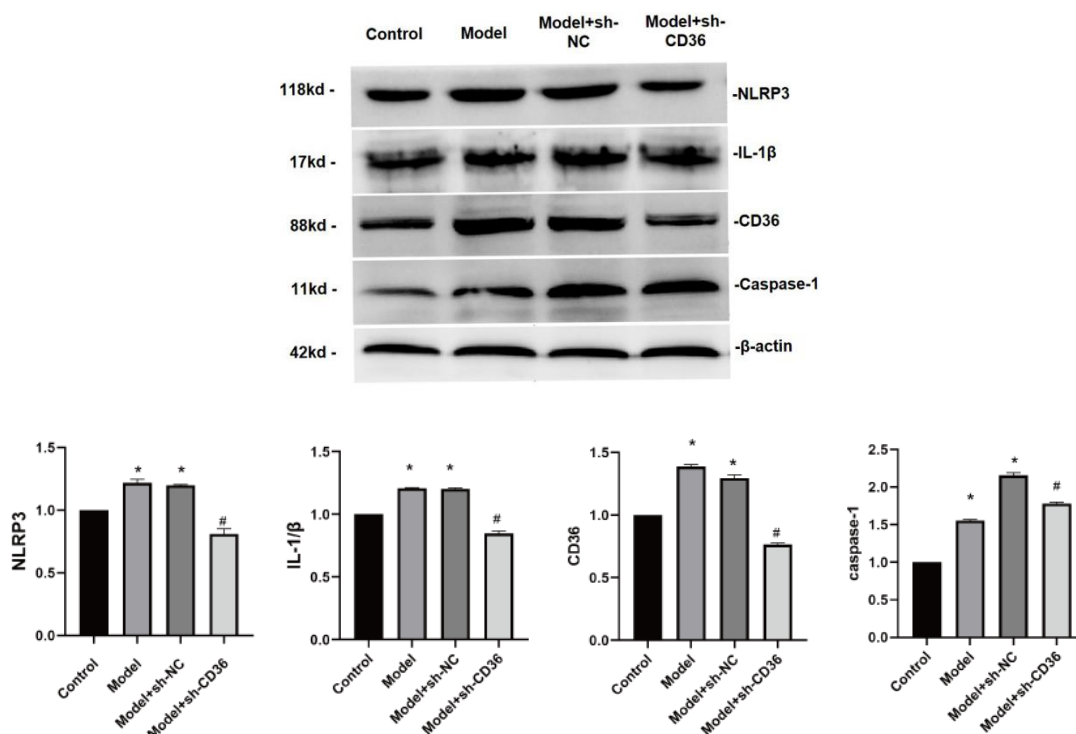


Fig 5. CD36 knockdown reversed the diabetes-induced increase in inflammatory factors in mice.

Western blotting analysis of CD36, NLRP3, Caspase-1, and IL-1 β protein levels in hippocampal tissues. Data are presented as mean $\pm$ SD. *P < 0.05 vs. control group; #P < 0.05 vs. Model+sh-NC group by ANOVA. CD36, cluster of differentiation 36; IL-1 β , interleukin 1 β ; NLRP3, NLR family pyrin domain containing 3; Model, mouse db/db diabetes model; sh-NC, negative control short hairpin RNA; sh-CD36, short hairpin RNA targeting mouse CD36.

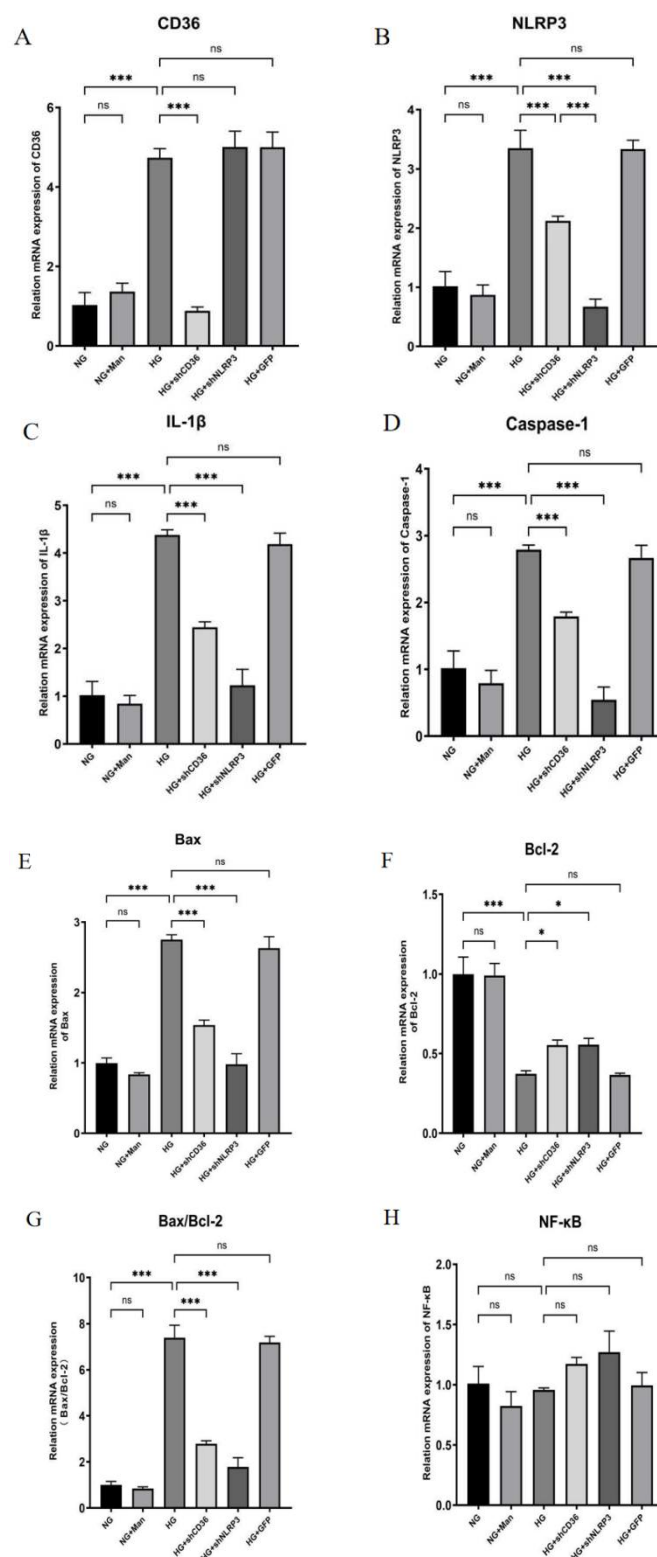
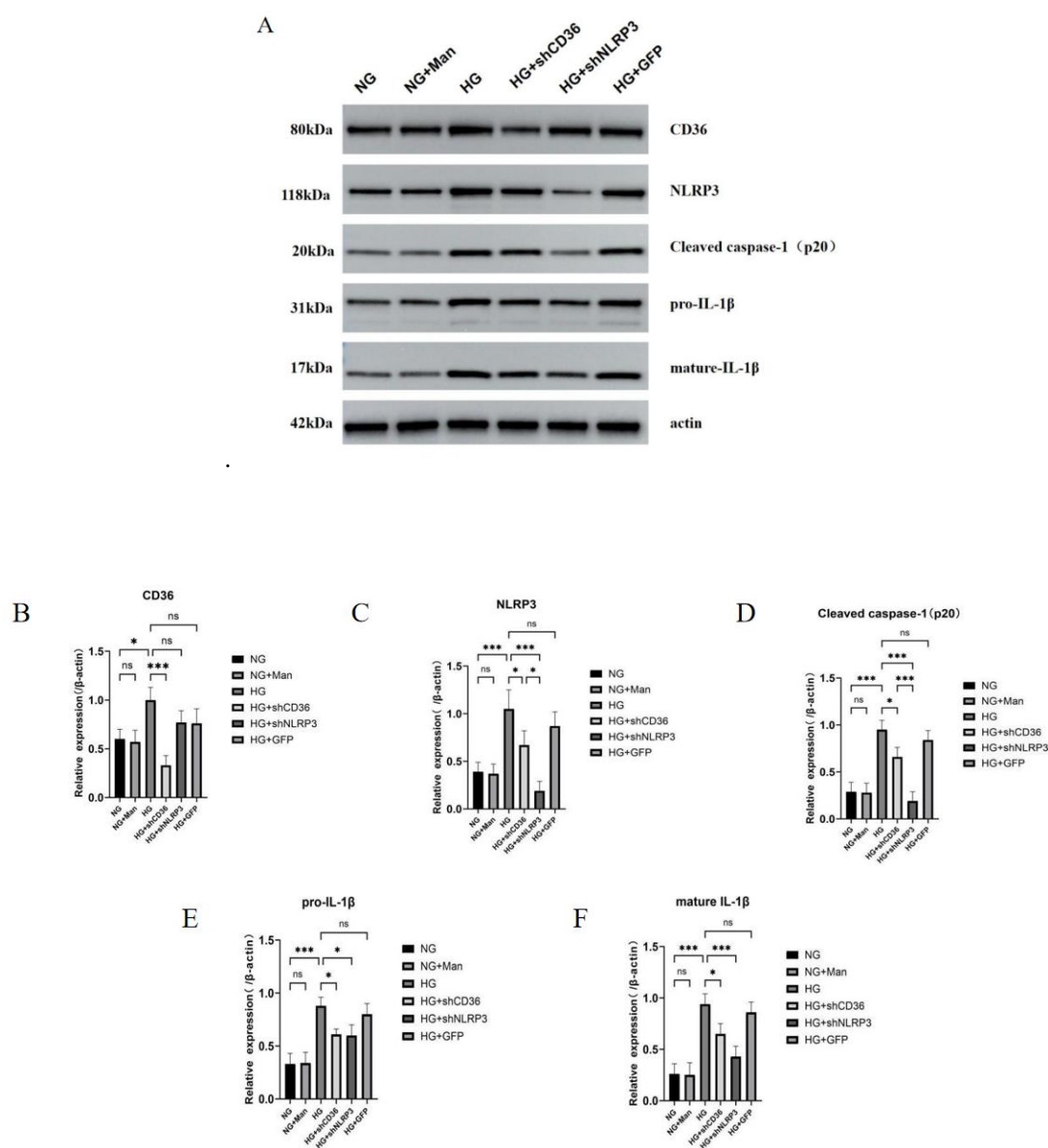


Figure 6. qPCR analysis of mRNA expression of inflammation- and apoptosis-related genes in astrocytes under high-glucose stimulation and following CD36 or NLRP3 knockdown. (A) Relative CD36 mRNA expression. (B – D) NLRP3 inflammasome pathway-related genes: NLRP3

808 (B), IL-1 β (C), and Caspase-1 (D). (E and F) Apoptosis-related genes: Bax (E) and Bcl-2 (F). (G)
 809 Bax/Bcl-2 ratio. (H) Relative NF- κ B p65 mRNA expression. Data are presented as mean $\pm$
 810 standard deviation (n = 3). *P < 0.05, ***P < 0.001; ns, P > 0.05. Tbp was used as the reference
 811 gene for normalization



814
 815 Figure 7. Activation of key molecules in the CD36/NLRP3 inflammasome pathway under high-
 816 glucose stimulation and the effects of CD36 or NLRP3 knockdown. (A) Representative Western
 817 blot images showing protein expression of CD36, NLRP3, Caspase-1, and pro- and mature IL-1 β
 818 in C8-D1A astrocytes. Cells were treated as follows: normal glucose control (NG), normal glucose

plus mannitol osmotic control (NG+Man), high glucose (HG), high glucose plus CD36 knockdown (HG+shCD36), high glucose plus NLRP3 knockdown (HG+shNLRP3), and high glucose plus empty vector control (HG+GFP). (B – F) Relative densitometric quantification of CD36 (B), NLRP3 (C), Caspase-1 (D), and pro- and mature IL-1 β (E, F). Data are presented as mean $\pm$ standard deviation (n = 3 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001; ns, P > 0.05.

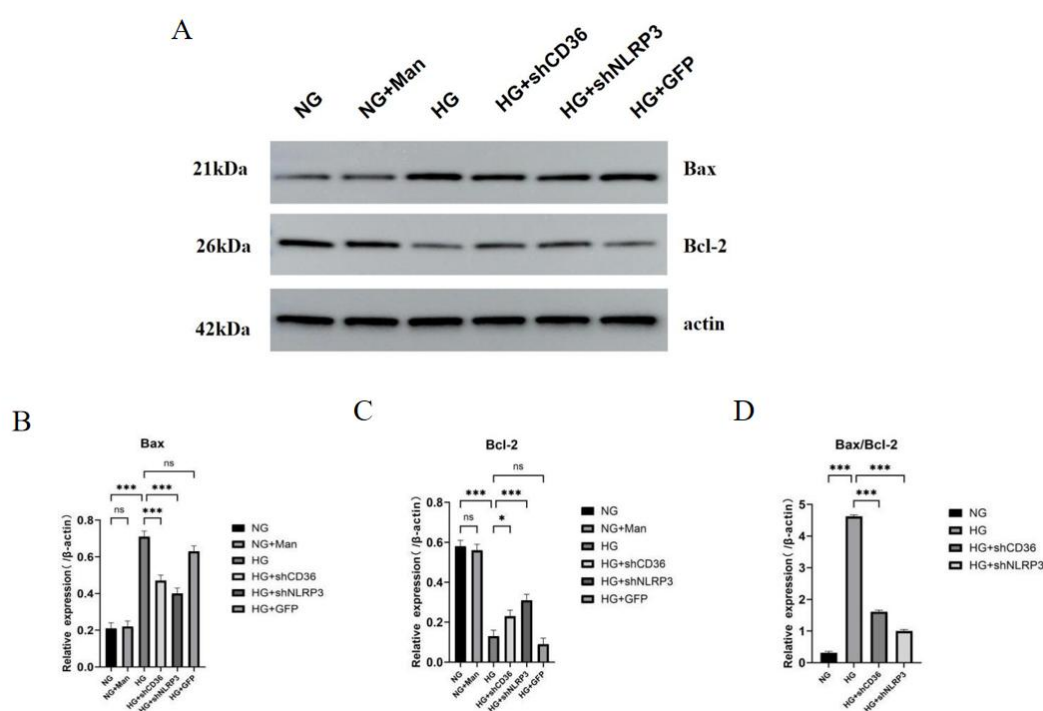


Figure 8. Expression of apoptosis-related proteins under high-glucose stimulation and the effects of CD36 or NLRP3 knockdown. (A) Representative Western blot images showing protein expression of Bax and Bcl-2 in C8-D1A astrocytes. Cells were treated as follows: normal glucose control (NG), normal glucose plus mannitol osmotic control (NG+Man), high glucose (HG), high glucose plus CD36 knockdown (HG+shCD36), high glucose plus NLRP3 knockdown (HG+shNLRP3), and high glucose plus empty vector control (HG+GFP). (B and C) Relative densitometric quantification of Bax (B) and Bcl-2 (C). (D) Bax/Bcl-2 ratio. Data are presented as

mean $\pm$ standard deviation (n = 3 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001;
ns, P > 0.05.

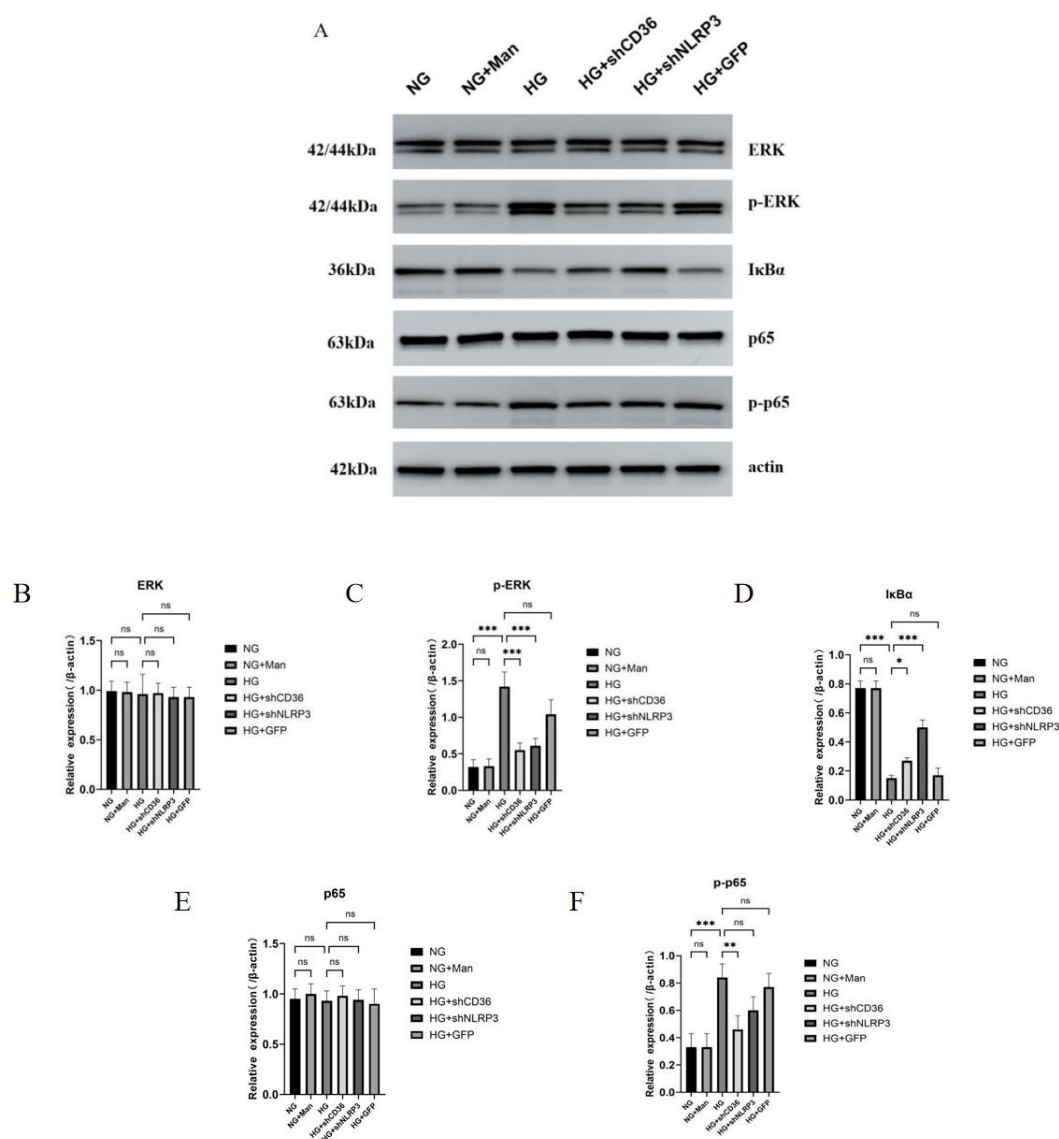


Figure 9. Activation of ERK and NF- κ B signaling molecules under high-glucose stimulation and the effects of CD36 or NLRP3 knockdown. (A) Representative Western blot images showing protein expression of total ERK, phosphorylated ERK (p-ERK), I κ B α , p65, and phosphorylated p65 (p-p65) in C8-D1A astrocytes. Cells were treated as follows: normal glucose control (NG), normal glucose plus mannitol osmotic control (NG+Man), high glucose (HG), high glucose plus CD36 knockdown (HG+shCD36), high glucose plus NLRP3 knockdown (HG+shNLRP3), and

843 high glucose plus empty vector control (HG+GFP). (B – F) Relative densitometric quantification
844 of total ERK (B), p-ERK (C), I κ B α (D), p65 (E), and p-p65 (F). Data are presented as mean $\pm$
845 standard deviation (n = 3 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001; ns, P >
846 0.05.