PSD-93 mediates the crosstalk between neuron and microglia and facilitates acute ischemic stroke injury by binding to CX3CL1

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Abstract

Background: Postsynaptic density 93 (PSD-93) mediates glutamate excitotoxicity induced by ischemic brain injury, which then induces microglial inflammatory response. However, the underlying mechanisms of how PSD-93 mediates the crosstalk between neurons and microglia in the postsynaptic dense region remain elusive. CX3 chemokine ligand 1 (CX3CL1) is a chemokine specifically expressed in neurons while its receptor CX3CR1 is highly expressed in microglia. In this study, we aimed to investigate the role of PSD-93 and CX3CL1 interaction in the crosstalk between neuron and microglia in acute ischemic stroke.

Methods: Male C57BL/6 mice were used to establish middle cerebral artery occlusion model and co-immunoprecipitation and immunoblotting were used to detect the binding of PSD-93 and CX3CL1 at different time points following cerebral ischemic/reperfusion (I/R). ELISA was used to detect soluble CX3CL1. Yeast two-hybrid and co-immunoprecipitation were used to identify special amino acid sequences responsible for the interaction between PSD-93 and CX3CL1. Finally, a fusion small peptide Tat-CX3CL1 was designed to inhibit PSD-93 and CX3CL1 interaction.

Results: The binding of PSD-93 and CX3CL1 peaked at 6 h after I/R. The binding sites were located in the 420-535 amino acid sequence of PSD-93 and 357-395 amino acid sequence of CX3CL1. Tat-CX3CL1 (357-395aa) could inhibit the interaction of PSD-93 and CX3CL1 and inhibited the pro-inflammatory cytokine IL-1β and TNF-α expression and provided neuroprotection following reperfusion.

Conclusions: PSD-93 binds CX3CL1 to activate microglia and initiate neuroinflammation. Specific blockade of PSD-93-CX3CL1 interaction reduces I/R induced neuronal cell death, and provides a new therapeutic target for ischemic stroke.

Introduction

Stroke is the second leading cause of death and the first leading cause of disability worldwide [1]. Neuronal glutamate excitatory toxin and microglia mediated neuroinflammatory response are important pathological mechanisms underlying brain injury after ischemia [2-4]. Therefore, better understanding of the relationship between excitotoxicity and neuroinflammation will provide important guidance for clinical development of new target drugs for stroke.

Accumulating studies in recent years have indicated that ionic glutamic acid receptor agonists can promote the proliferation and activation of microglia cells, and promote the release of pro-inflammatory factors that aggravate the damage of neurons and form a vicious cycle in the process of ischemic brain injury [5-6]. On the other hand, neurons bind to microglia cells through specific chemokines and their receptors, such as CD22/CD45, CD47/CD172a, CD200/CD200R and CX3CL1/CX3CR1, to communicate and activate a series of downstream signaling pathways, ultimately regulating neuron damage [7-12].

Postsynaptic density 93 (PSD-93) is one of PSDs scaffold proteins composed of the PDZ domain, the src homology domain SH3 and the GUK domain [13, 14]. PSD-93 binds directly to the carboxyl terminus of N-

methyl-D-aspartate (NMDA) receptor subunits NR2A and NR2B via the PDZ domain and transports it to the postsynaptic membrane as a major regulator of synaptic maturation [15]. Our recent study showed that the loss of PSD-93 inhibited pro-inflammatory factors and promoted the expression of anti-inflammatory factors, while the application of NMDA receptor (NMDAR) inhibitors provided brain protection [16]. These results suggest that PSD-93 promotes a series of responses to cerebral ischemia-reperfusion injury through the activation of NMDAR, including the regulation of inflammatory factor release in early ischemic cells.

As mentioned above, excitatory amino acid toxicity of neurons interacts with microglial-induced inflammatory responses in cerebral ischemia-reperfusion injury [17, 18]. After ischemic stroke, neurons activate microglia through a variety of regulatory factors, and the activation of NMDAR is closely related to the activation of microglia [3]. CX3 chemokine ligand 1 (CX3CL1) is a chemokine specifically expressed in neurons, while its receptor CX3CR1 is highly expressed in microglia. When neurons are damaged or exposed to glutamate, soluble CX3CL1 disintegrates from its structural domain and binds to CX3CR1 on microglia to activate microglia [19-20]. Therefore, neurons and microglia can communicate through CX3CL1/CX3CR1 signals.

Based on the above results, we hypothesized that PSD-93 might interact with CX3CL1, allowing it to be recruited to the surface of microglia via binding CX3CR1, which then activates microglia and initiates inflammatory response. In this study, we used yeast double hybrid and co-immunoprecipitation to identify the binding sequences of PSD-93 and CX3CL1, and constructed a small peptide to disturb the interaction between PSD-93 and CX3CL1 to attenuate ischemic brain injury.

Material And Method

Antibodies and Reagents

The peptide was synthesized by Qiangyao Biological Tech (Shanghai, China) with following sequence: 5-FITC-(Acp) MFAYQSLQGCPRKMAGEMVEGLRYVPRSCGSNSYVLVPV (purity >95%). Rabbit polyclonal anti-CX3CL1 (ab25091), rabbit monoclonal anti-PSD-93 (ab151721) and mouse monoclonal anti-NMDAR2B (ab93610) were purchased from Abcam. The secondary goat anti-rabbit IgG antibody was purchased from Sigma (St. Louis, MO). ELISA kits were purchased from R&D.

Animals

Eighty-four male C57BL/6 mice (22-26 g weight) were purchased from Jinan pengyue experimental animal breeding co. and maintained in our animal facility in a temperature-controlled room (22-25 °C) with a 12-h dark-light cycle. All animal procedures were reviewed and approved by Institutional Animal Use and Care Committee.

Middle Cerebral Artery Occlusion Model (MCAO)

Mice were exposed to transient middle cerebral artery occlusion (tMCAO) by suture-occlusion method [21]. Mice were anesthetized and the right side of the common carotid artery was exposed and isolated. The right internal carotid artery was clipped by microartery clip. The middle cerebral artery (MCA) was occluded by inserting a suture into the internal carotid artery, which was advanced further until it closed the origin of the MCA and fixed line bolt. After occlusion for 60 min, the aneurysm clips were removed for reperfusion. Rectal temperature was maintained at 37 ± 0.5 °C throughout the surgery. Sham-operated control mice received the same surgical procedures except that the carotid arteries were not occluded.

Immunoprecipitation

Immunoprecipitation was performed following the procedure described previously [22]. Tissue homogenates (1,000 μg of protein) were diluted with 50 mM HEPES buffer (pH 7.4) containing 10% glycerol, 150 mM NaCl, EDTA, EGTA, PMSF, Na3VO4 (1 mM each) and 1% Triton X-100, 0.5% NP-40. The protein samples were incubated with 1-2 μg of primary antibodies overnight at 4 °C, and then incubated with Protein A/G PLUS-Agarose (20 μL, SANTA CRUZ) for 2 h. The pellets were washed with immunoprecipitation buffer three times. Bound proteins were eluted by boiling at 100 °C for 5 min in SDS-PAGE loading buffer and then isolated on 10 % SDS polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon Transfer Membrane, Millipore, USA). After being blocked for 3 h in Tris-buffered saline with 0.1% Tween 20 (TBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4°C with primary antibodies in TBST containing 1% BSA. Membranes were then washed and incubated with alkaline phosphatase conjugate secondary antibodies in TBST for 2 h and developed using ECL luminescent solution kit (BioRad). The densities of the bands on the membrane were scanned with Bio-rad gel imager and analyzed.

ELISA

Soluble CX3CL1 levels were analyzed by ELISA kit (R&D Systems, Stillwater, MN, USA) according to the manufacturer's instructions.

Lateral ventricular injection

The peptides were administered to the mice by injection into the right cerebral ventricle (from the bregma: anteroposterior-1 mm; lateral 1 mm; depth 2 mm) 30 min before ischemia on the first day [24]. The sham group and I/R group rats were injected with same volume of DMSO. The mice were randomly divided into five groups: I/R (ischemia/reperfusion and normal saline treatment), 1 μg/μl (I/R 7 day and 1 μg/μl peptide treatment), 5 μg/μl (I/R 7 day and 5 μg/μl peptide treatment), 10 μg/μl (I/R 7 day and 10 μg/μl peptide treatment) and DMSO (I/R 7 day and DMSO treatment).

Triphenyl tetrazolium chloride (TTC) staining

TTC staining was performed as described previously [24]. Mice were sacrificed, the brains were rapidly stored at -80°C and sectioned into 2-mm thick slices along the coronal plane. The slices were stained with

2% TTC (Sigma, USA) at 37°C for 15 min in the dark. The infarct area ratio was measured by using Image J (version 1.8.0).

Plasmid construction and transfection

To construct pSos-PSD-93-full length, pSos-CX3CL1-full length, pMyr-PSD-93-mut1, pMyr-PSD-93-mut2, pMyr-PSD-93-mut3, pMyr-PSD-93-mut4, pMyr-CX3CL1-full length and pMyr-CX3CL1-mut, the cDNAs encoding PSD-93 or CX3CL1 fragments were amplified by PCR and cloned into respective vectors. The primers were listed in Table 2. 293T cells were transfected with plasmids using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher, USA), according to the manufacturer's instructions.

Yeast double hybridization

After we confirm that PSD-93 does not have self-activating activity and is not toxic to yeast strain cdc25Hα, the bait PSD-93 was transformed into cdc25Hα and hybridized with the CX3CL1 using the Yeast Two-Hybrid System. Gently mix the contents of the tube, add 2μl of β-mercaptoethanol to each tube, and incubate for 30 min at room temperature. The mixture table 3 was shocked for 20 min in an environment of 42 ° C, and then placed on ice for 3 min, and the cells were collected. They were centrifuged at 14000 rpm for 30 s at room temperature. The supernatant was removed, and the cells were resuspended in sorbitol. Each mixture was plated on a 100 mm SD (glucose) plate and incubated at room temperature until the clone appeared about 4-6 days, at least 3 clones were selected and transferred to SD (glucose) (-UL) and SD (galactose) (-UL) plates at 37 °C for the detection of protein-protein interactions. The clones to be screened were transferred to wells, resuspended in high pressure water and 2.5 μl of the yeast/H2O suspension was dropped onto two SD (galactose) (-UL) and two SD (glucose) (-UL) plates respectively. One placed at 37 C and the other at 22-25 C, counting growth clones after 5 days of culture, SD (galactose) (-UL) plates may take 7-10 days.

Plasmid transfection

293T cells were transfected with plasmids using Lipofectamine™ 3000 Transfection Reagent (L3000015; Thermo Fisher, USA), according to the manufacturer's instructions. The mixtures were mixed well and placed in the cell culture box for incubation with 5% CO_2, 37°C. After 6h, they were replaced with 2ml DMEM medium containing 10% fetal bovine serum. Cell culture supernatants were harvested at 24 h post infection. The transfected cells were analyzed by immunoprecipitation and western blotting.

Statistical analysis

Data were presented as mean± standard deviation (SD) from at least three experiments and analyzed using SPSS software version 18.0 (SPSS Inc., Chicago, China). One-way ANOVA and Bonferroni correction were used to analyze the data. The results were considered significant if $P<0.05$.

Results

Interaction of PSD-93 with CX3CL1 and NR2B during reperfusion after ischemia

As a scaffold protein in the postsynaptic membrane, PSD-93 mediates the release of inflammatory factors in early cerebral ischemia through NMDA receptor [15], and CX3CL1 is a specific transmembrane protein expressed on neurons [12]. Therefore, we first investigated the interaction of PSD-93 with NR2B and CX3CL1 during reperfusion after transient (60 min) cerebral ischemia using immunoprecipitation and immunoblotting. As shown in Fig 1A and 1C, the interaction of CX3CL1 with PSD93 increased significantly at I/R 6h ($P<0.01$, n=5) and 72h ($P<0.05$, n=5) groups compared with sham group. In addition, the interaction of CX3CL1 with NR2B increased at I/R 6h group ($P<0.001$, n=5), I/R 48h and I/R 72h group ($P<0.01$, n=5), respectively. Furthermore, we found that the peak of the interaction of NR2B with PSD-93 was at 6h ($P<0.001$, n=5). Taken together, these results suggest that CX3CL1, PSD-93 and NR2B can form macromolecular complexes after I/R and the binding peak is at I/R 6h. Thus we selected I/R 6h point to elucidate the function of PSD-93/NR2B/CX3CL1 complex.

The release of soluble CX3CL1 after I/R

When neurons are damaged or exposed to glutamate, membrane-bound CX3CL1 is cleaved into soluble CX3CL1 by integrin and metalloproteinase [19]. Next, we detected soluble CX3CL1 at different time points after I/R. As shown in Fig. 2, ELISA analysis showed that soluble CX3CL1 level reached the maximum after reperfusion for 6h ($P<0.001$, n=5, I/R 6h vs. Sham), coinciding with the formation of PSD-93/NR2B/CX3CL1 complex. These data suggested that the interaction of PSD-93 with CX3CL1 might play significant role in promoting the release of soluble CX3CL1 in brain ischemia reperfusion injury.

PSD-93 interacts with CX3CL1 in Yeast double-hybrid system

To confirm the interaction between PSD-93 and CX3CL1, pSos-PSD-93-full length with pMyr-CX3CL1-full length and pMyr-CX3CL1-mut, pSos-CX3CL1-full length with pMyr-PSD-93-mut1, mut2, mut3, and mut4 were co-transformed into yeast strain cdc25Hα (Table1) and grown on synthetically defined medium (Supplemental file 1 and 2). Colony growth was observed in SD (glucose)(-ul) and SD (galactose)(-ul) solid medium at 22-25°C (Supplemental file 3). No colonies grew in 37°C SD (glucose)(-ul) solid medium (Supplemental file 4). However, in 37°C SD (galactose)(-ul) solid medium, yeast cells co-transformed with pSos-PSD-93-full length and pMyr-CX3CL1-full length, pSos-CX3CL1-full length with pMyr-PSD-93-mut3 and mut4, and the positive control pSos MAFB+pMyr MAFB grew (Fig. 3A, C, G, H and Table 4), which indicated that PSD-93 and CX3CL1 could bind to each other. Moreover, the sequence (420-535aa) on PSD-93 and the sequence (357-395aa) on CX3CL1 are important for the binding between PSD-93 and CX3CL1.

PSD-93 interacts with CX3CL1 in 293T cells

To further verify the interaction between PSD-93 and CX3CL1 in vitro, we performed co-immunoprecipitation assay. While mut1 and mut2 of PSD-93 could not co-precipitate with CX3CL1 (Fig. 4A, B), PSD-93-mut3 and PSD-93-mut4 co-precipitated with the full-length CX3CL1 (Fig. 4C, D). In

addition, CX3CL1 mut (25-357aa) could not co-precipitate with PSD93. These data are consistent with yeast double-hybrid experiments and suggest that amino acid sequences of (420-535aa) in PSD-93 and (357-395aa) in CX3CL1 are crucial for the interaction between PSD-93 and CX3CL1.

Peptide blocking PSD-93 and CX3CL1 interaction reduced cerebral infarction volume

To investigate whether inhibiting PSD-93 and CX3CL1 interaction can reduce neuron death, we designed a small peptide that inhibited PSD-93 and CX3CL1 interaction. Three peptide concentrations were designed to observe the effects on cerebral infarction volume after 7 days of reperfusion. As shown in Fig. 5A and B, compared with MCAO group, both 5 µg/µl and 10 µg/µl peptide groups significantly improved neurological impairment caused by ischemia-reperfusion (P<0.000). However, the reduction in infarct volume in 10 µg/µl peptide group was more pronounced. Furthermore, to clarify the effect of peptide on microglia activation, we selected pro-inflammatory cytokine IL-1β and TNF-α to detect and found that the small peptide could inhibit the expression of IL-1β (P<0.01) and TNF-α significantly (P<0.05). These results suggest that peptide blocking PSD-93 and CX3CL1 interaction has neuroprotective effects.

Discussion

PSD-93 is a scaffold protein in the postsynaptic membrane that mediates the release of inflammatory factors in the early stage of cerebral ischemia through NMDA receptors [16]. In addition, PSD-93 can interact with other proteins with its function structure domain and change the structure and function of other proteins [25-26]. As a transmembrane protein specifically expressed in neurons, CX3CL1 can be cut into soluble CX3CL1 and reach the surface of the microglia to bind CX3CR1 and activate microglia when neurons are damaged or exposed to glutamate [19-20]. In this study, we found that PSD-93 could bind NR2B and CX3CL1 to form a complex and promote the release of soluble CX3CL1. Moreover, we identified the amino acid binding sequences of PSD-93 and CX3CL1 through yeast double hybrid. Based on the sequences, we designed a small peptide to disturb the interaction of PSD-93 and CX3CL1 and found that the peptide exhibited neuroprotective effect following ischemic brain injury.

In recent years, the relationship between PSDs and early post-ischemic inflammatory response has received increasing attention. Christopherson et al [27] showed that PSD-95, NMDAR and nNOS can assemble into a macromolecular signal complex, and disrupting ischemia-induced interaction of nNOS with PSD-95 improved regenerative repair after stroke [28-30]. In addition, PSD-93 can bind to NR2A and nNOS and facilitate ischemic brain injury [16, 31]. On the other hand, neuronal excitatory toxicity interacted with microglia-induced inflammatory response in ischemic brain injury [32-33]. After ischemic stroke, neurons activate microglia through a variety of neurotransmitters or regulators such as glutamate, fractalkine (FKN, CX3CL1), and NO. Activated microglia then promote neuronal injury by releasing pro-inflammatory factors such as NO, oxygen free radicals, prostaglandin E2, and cytokines such as TNF-, IL-1, and IL-6 [3,34].

CX3CL1 is a unique membrane-bound protein that regulates cell-to-cell communication and adhesion by binding to CX3CR1 [35-37]. CX3CL1/CX3CR1 signaling pathway has been shown to play an important role in regulating communication between neurons and microglia. In addition, neurotoxic pathways are affected by CX3CL1/CX3CR1 signaling through synergistic cooperation of adenosine systems [38]. CX3CL1 exists in two different forms, the full-length membrane-bound and soluble forms. When neuron is damaged or exposed to glutamate, soluble form of CX3CL1 is released to bind to CX3CR1 receptor expressed on microglia [39-43]. Soluble CX3CL1 enhances microglial phagocytosis of neuronal fragments via phosphatidylserine (PS) [44-46]. Based on these studies, promoting the release of soluble CX3CL1 is the key to illuminate the crosstalk between neurons and microglia.

Our findings provide a basis for PSD-93 to regulate CX3CL1/CX3CR1 signaling and mediate acute ischemic stroke injury. In this study, we first examined the interaction of PSD-93 with CX3CL1 after I/R by Co-IP assay. The results showed the interaction of CX3CL1 with PSD-93 increased after reperfusion 6 h and then decreased gradually. We further identified amino acid sequences responsible for CX3CL1 and PSD-93 interaction using yeast two-hybrid system. In addition, we validated CX3CL1 and PSD-93 interaction by co-immunoprecipitation in 293T cells.

To further investigate whether inhibiting PSD-93 and CX3CL1 interaction has neuroprotective effect against ischemia-reperfusion, we designed small peptide to antagonize PSD-93 and CX3CL1 interaction. In vivo experiments showed that inhibiting PSD-93 and CX3CL1 interaction reduced neurons death. Therefore, the small peptide has neuroprotective effect, and could be a potential candidate for drug development for stroke therapy.

Conclusion

In summary, our study revealed the interaction between PSD-93 and CX3CL1 and further explored the mechanism of PSD-93 regulating CX3CL1/CX3CR1 signaling during acute ischemic stroke. The complex PSD-93/NR2B/CX3CL1 promotes the production of soluble CX3CL1 and mediates the crosstalk between neurons and microglia during acute cerebral ischemia. This study sheds new light on the role of the crosstalk between CX3CL1 and PSD-93 after brain ischemia-reperfusion and provides new therapeutic targets against ischemic neuron death.

Declarations

Ethics approval and consent to participate

All animal experiments were performed at Xuzhou Medical University according to Animal Care and Use Committee of Xuzhou Medical University. Mice were euthanized by cervical dislocation.

Consent for publication

Not applicable.

Availability of data and materials

All data are available upon reasonable request to correspondence author.

Competing interests

The authors declare that they have no competing interests.

Author contributions

QZ, LH, HY, MC, XL, XC, and XL performed the experiments, QH, ZC, and TL validated the experiments, XW analyzed data, QZ and LH wrote the paper, QZ and LR designed and supervised the study.

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Tables

Table 1   PCR primers

Name	Primer
pSos-CX3CL1-full-length	CX3CL1-S-BamHI: 5' CGCGGATCCGCATGGCTCCCTCGCCGCTC 3'
	CX3CL1-AS-SalⅠ: 5' ACGCGTCGACTCACACTGGCACCAGGAC 3'
pMyr-PSD-93-mut1	PSD-93-mut1-S-EcoRI: 5' CGGAATTCATGTTCTTTGCATGTTAT 3'
	PSD-93-mut1-AS-SalⅠ: 5' GCGTCGACCACAACAGTCTCCAATAT 3'
pMyr-PSD-93-mut2	PSD-93-mut2-S-EcoRI: 5' CGGAATTCATGTTCTTTGCATGTTAT 3'
	PSD-93-mut2-AS-SalⅠ: 5' G GTCGACGCGGGGCTCCCCTTCCAG 3'
pMyr-PSD-93-mut3	PSD-93-mut3-S-EcoRI: 5' CGGAATTCATGTTCTTTGCATGTTAT 3'
	PSD-93-mut3-AS-SalⅠ: 5' GCGTCGACCTGATTGGTTCGAAGGGA 3'
pMyr-PSD-93-mut4	PSD-93-mut4-S-EcoRI: 5' CGGAATTCATGTTCTTTGCATGTTAT 3'
	PSD-93-mut4-AS-SalⅠ: 5' GCGTCGACGTAGTTTATTTCCTGCCT 3'
CX3CL1-mut	CX3CL1-mut-S-EcoRI: 5' CGGAATTCCAGCACCTCGGCATGACG 3'
	CX3CL1-mut-AS-SalⅠ: 5' GCGTCGACCATGGCCACCCCTAGGCA 3'

Table 2 Mutant Plasmid

| Plasmid	Number	Length
pMyr-PSD-93-mut1	Q91XM9, NM_011807.3	1-192aa
pMyr-PSD-93-mut2		1-420aa
pMyr-PSD-93-mut3		1-535aa
pMyr-PSD-93-mut4		1-661aa
pMyr-CX3CL1-full length	O35188ⅠNM_009142	1-395aa
pMyr-CX3CL1-mut		25-357aa

Table 3　Yeast Conversion Mixture

Number	Plasmid	Weight	Volume	Medium
1	pSos MAFB+pMyr MAFB（阳性对照）	300ng	100μl	SD/glucose (UL)
2	pSos MAFB+pMyr Lamin C（阴性对照）	300ng	100μl	SD/glucose (UL)
3	pSos-PSD-93-full length+pMyr-CX3CL1-mut	300ng	100μl	SD/glucose (UL)
4	pSos-PSD-93-full length+pMyr-CX3CL1-full length	300ng	100μl	SD/glucose (UL)
5	pSos-CX3CL1-full length+ pMyr-PSD-93-mut1	300ng	100μl	SD/glucose (UL)
6	pSos-CX3CL1-full length+ pMyr-PSD-93-mut2	300ng	100μl	SD/glucose (UL)
7	pSos-CX3CL1-full length+ pMyr-PSD-93-mut3	300ng	100μl	SD/glucose (UL)
8	pSos-CX3CL1-full length+ pMyr-PSD-93-mut4	300ng	100μl	SD/glucose (UL)
9	pSos MAFB+ pMyr -CX3CL1-mut	300ng	100μl	SD/glucose (UL)
10	pSos MAFB+ pMyr-CX3CL1-full length	300ng	100μl	SD/glucose (UL)
11	pSos MAFB+ pMyr - PSD-93-mut1	300ng	100μl	SD/glucose (UL)
12	pSos MAFB+ pMyr - PSD-93-mut2	300ng	100μl	SD/glucose (UL)
13	pSos MAFB+ pMyr - PSD-93-mut3	300ng	100μl	SD/glucose (UL)
14	pSos MAFB+ pMyr - PSD-93-mut4	300ng	100μl	SD/glucose (UL)

Table 4　Yeast Transformation results

Yeast transformation	(-UL)/37°C	(after patching)
	Glucose	Galactose
pSos MAFB+pMyr MAFB		
pSos MAFB+pMyr Lamin C		
pSos-PSD-93-full length+ pMyr-CX3CL1-full length		
pSos-PSD-93-full length+pMyr-CX3CL1-mut		
pSos-CX3CL1-full length+ pMyr-PSD-93-mut1		
pSos-CX3CL1-full length+pMyr-PSD-93-mut2		
pSos-CX3CL1-full length+ pMyr-PSD-93-mut3		
pSos-CX3CL1-full length+ pMyr-PSD-93-mut4		
pSos MAFB+ pMyr -CX3CL1-mut		
pSos MAFB+ pMyr-CX3CL1-full length		
pSos MAFB+ pMyr - PSD-93-mut1		
pSos MAFB+ pMyr - PSD-93-mut2		
pSos MAFB+ pMyr - PSD-93-mut3		
pSos MAFB+ pMyr - PSD-93-mut4		
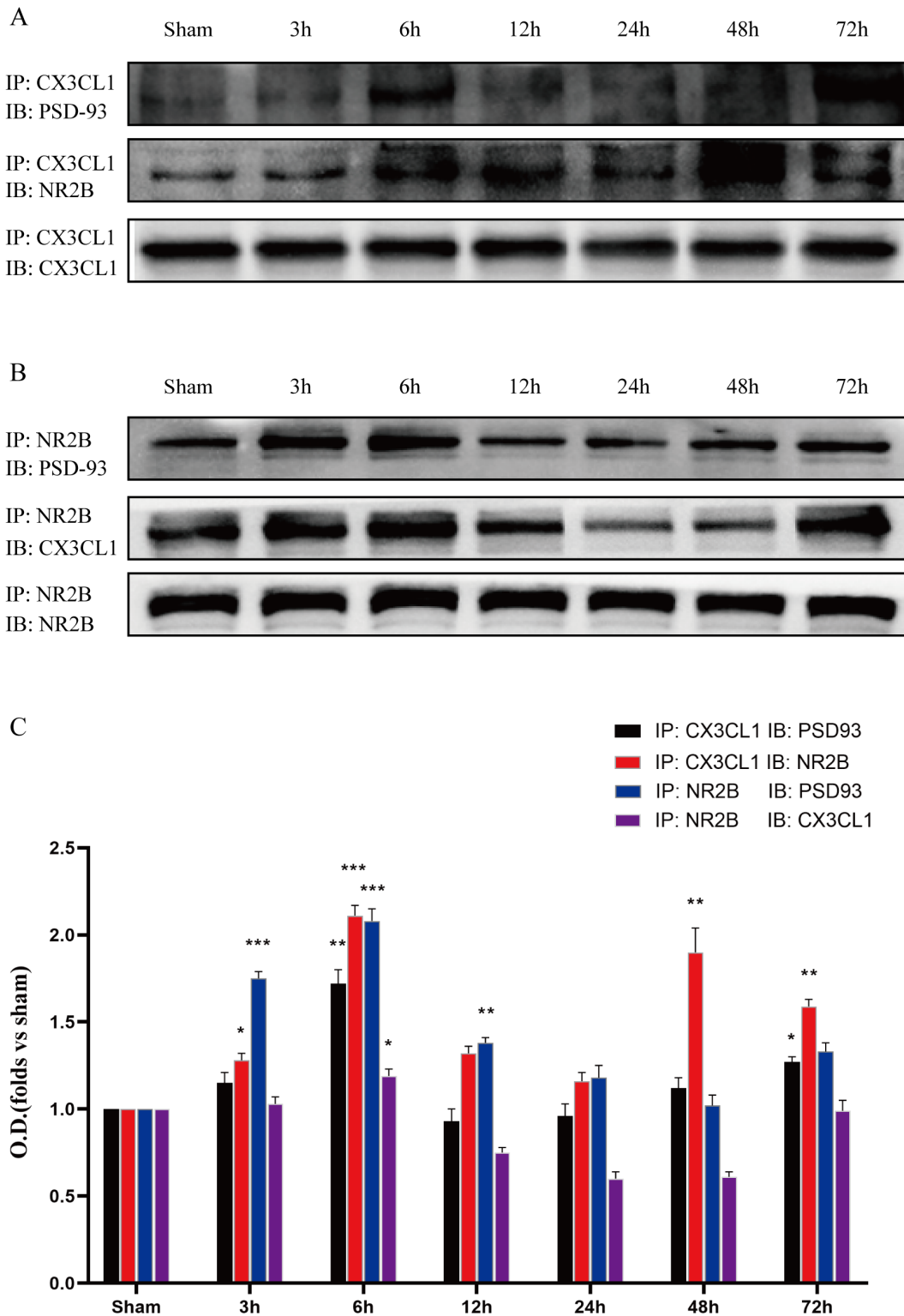
Figure 1

Interaction of PSD-93 with CX3CL1 and NR2B during reperfusion after ischemia. A and B showed representative protein bands. C and D showed CX3CL1 binding to PSD-93 and NR2B. E and F showed NR2B binding to PSD-93 and CX3CL1. Immunoprecipitation and immunoblotting analysis indicated the interaction peak of PSD-93 with CX3CL1 and NR2B at 6h after reperfusion. Results are expressed as means ± SEM (n =5). *P▯0.05, ** P▯0.01, *** P▯0.001 versus sham. O.D. stands for optical density.
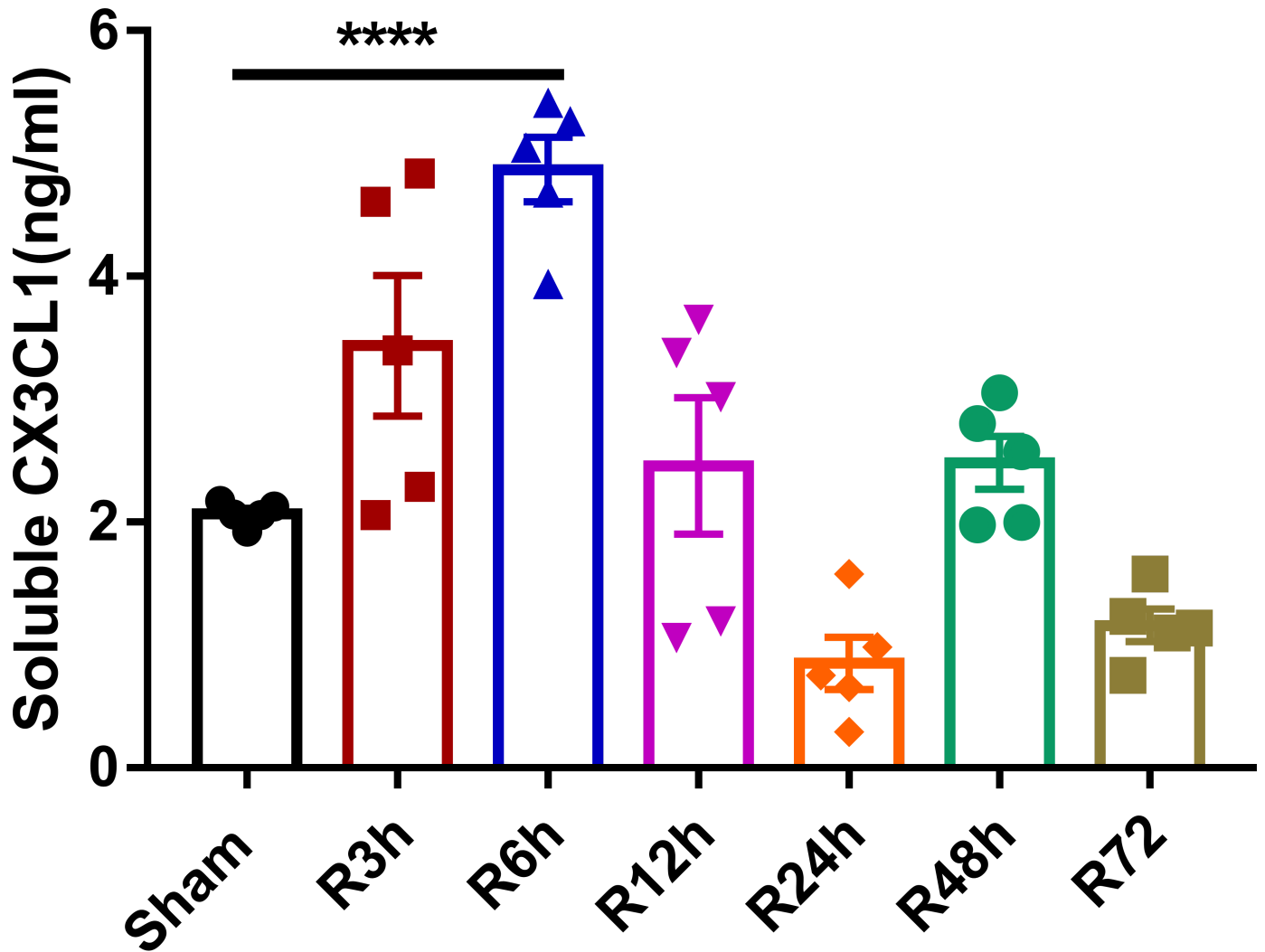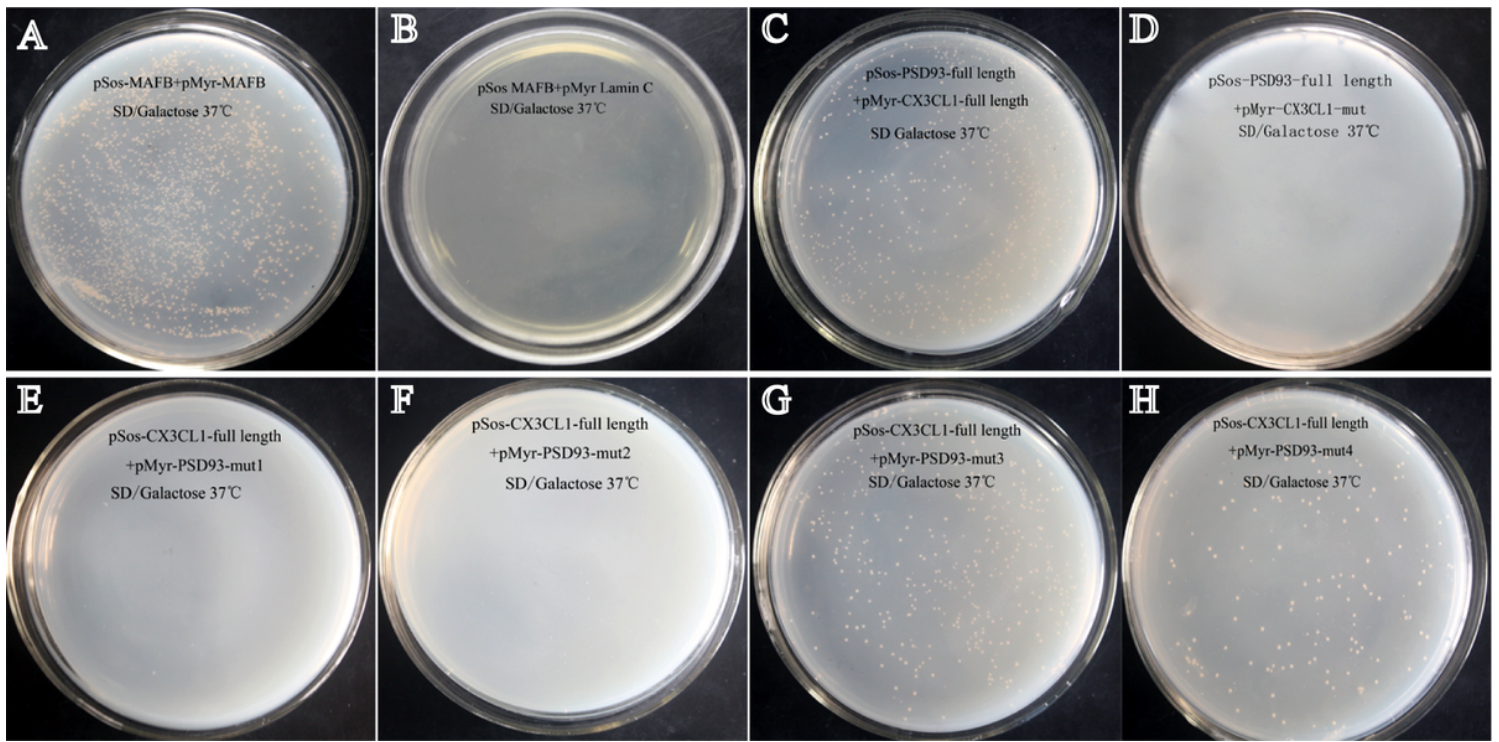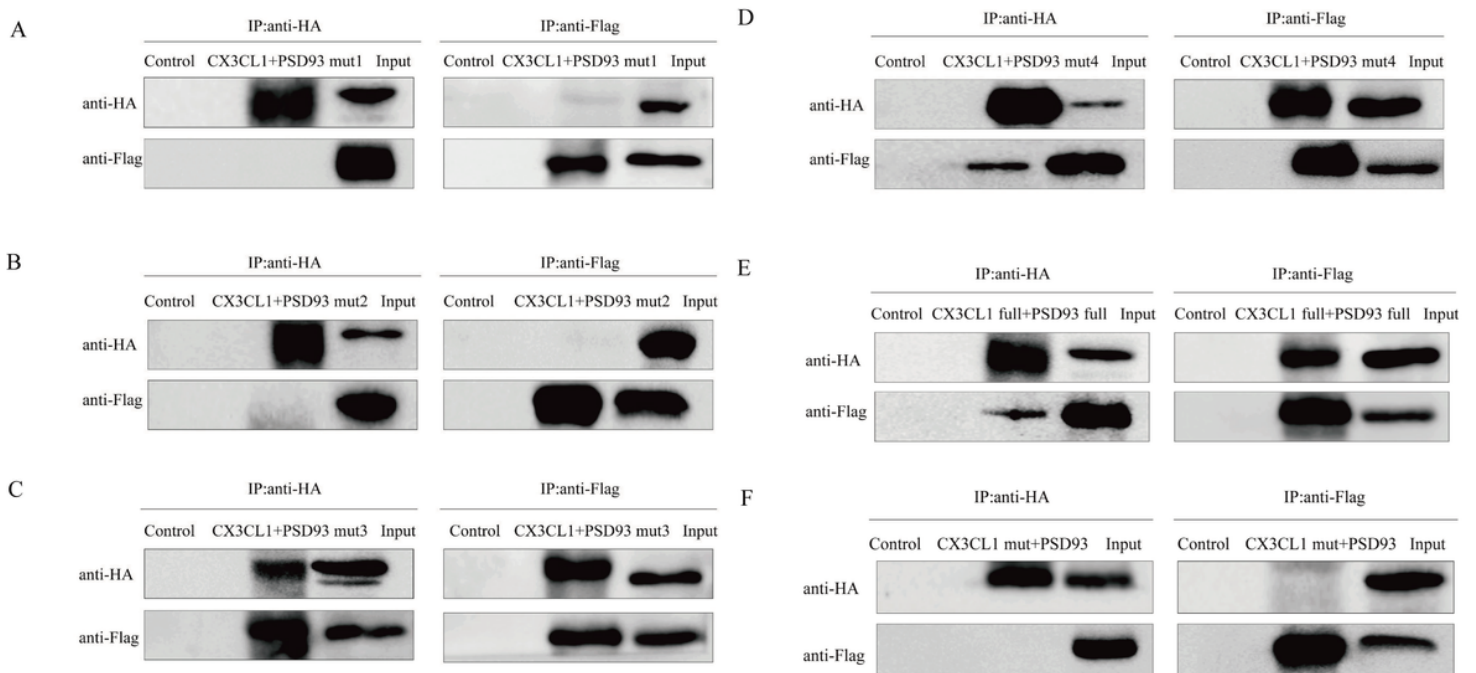
Figure 2

The release of soluble CX3CL1. ELISA analysis shows that the release of soluble CX3CL1 increased remarkably after reperfusion 6h and elevated slightly after I/R 3h compared to the sham group. Results are expressed as means ± SEM (n =5). **** P⊠0.000 versus sham.
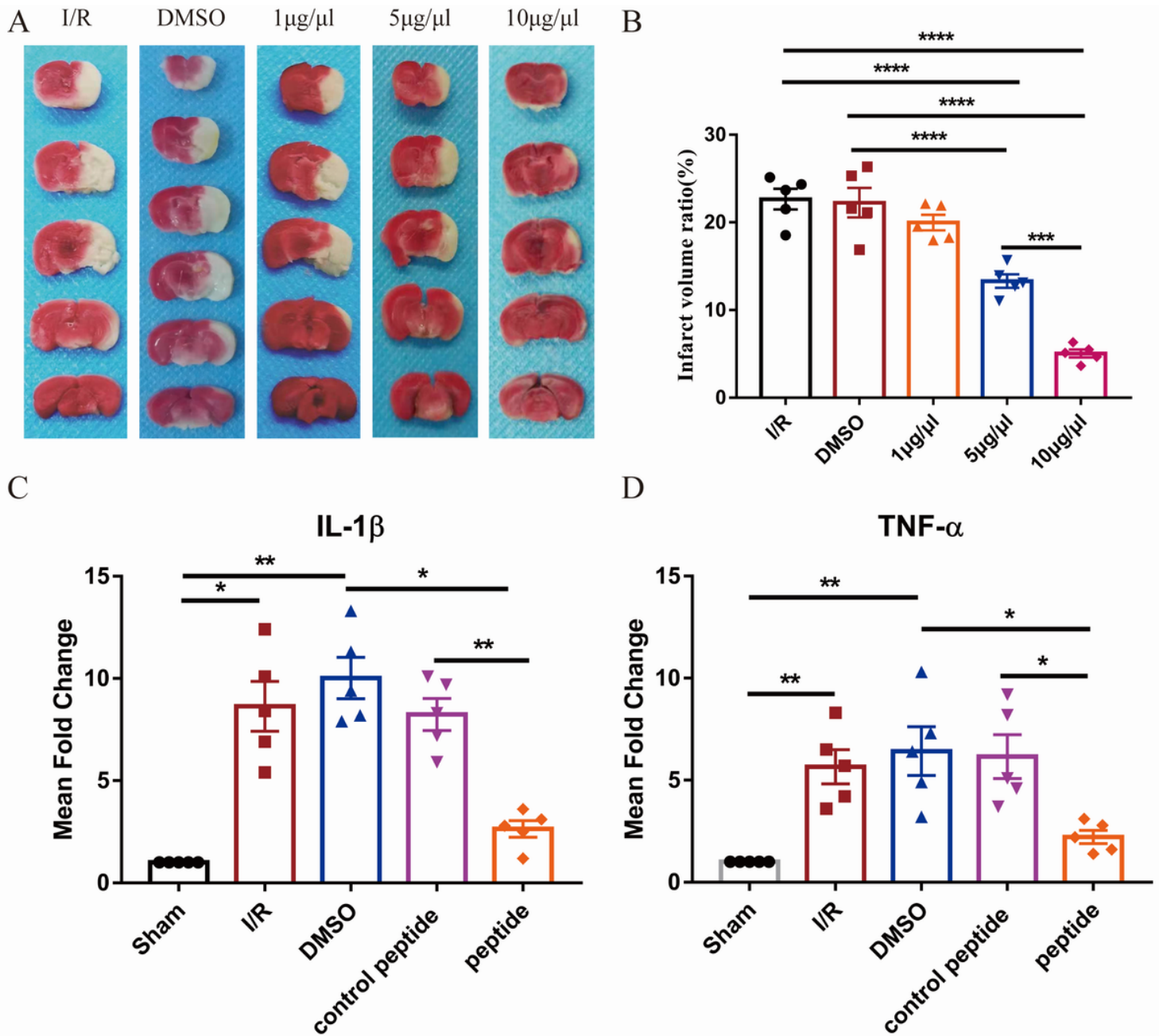
Figure 3

PSD-93 interacts with CX3CL1 in yeast double-hybrid system. A: the positive control group; B: negative control group; C-H: the binding group of mutant plasmids and bait plasmids. The growth conditions of each group on the SD (galactose) medium showed colony growth in group A, C, G and H.

Figure 4

PSD-93 interacts with CX3CL1 in 293T cells. Immunoprecipitation and immunoblotting analysis indicated the interaction of different fragments of PSD-93 and CX3CL1. We repeat every experiment at least 3 times (n=3).

Figure 5

The effect of peptide on infarct sizes at 7 days and pro-inflammatory cytokine IL-1β and TNF-α expression at 6h after ischemia-reperfusion. A, B, Pale unstained sections were considered to be indicative of infarct regions, whereas red-stained sections were indicative of normal tissues. Cerebral infarction volume of mice given different peptide concentrations based on TTC staining. Results are expressed as means ± SEM (n=5). C and D showed pro-inflammatory cytokine IL-1β and TNF-α expression at 6h following cerebral ischemia. *P≤0.05，**P≤0.01，***P≤0.001, **** P≤0.000 versus sham.

Supplementary Files

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