Downregulation of phosphoglycerate mutase 5 improves microglial inflammasome activation after traumatic brain injury

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Background
Traumatic brain injury (TBI), a global health challenge, is known to be one of the most common causes of death and disability in young men, and it imposes a burden on patients and their families, as well as a society [1, 2]. Typically, a complex set of pathophysiological processes leads to severe secondary brain injury within hours or days of acute brain injury, which follows the primary injury-mediated neuroinflammation, immune response, oxidative stress, mitochondrial dysfunction, and apoptosis [3, 4]. Neuroinflammation is believed to play an important role in secondary changes [4, 5]. Because in primary brain injury, the opportunity of treatment can be lost within a very short period, the effective intervention of cascade pathology of secondary brain injury promptly will become a potential therapeutic direction for TBI [6]. Thus, it is urgent and important to further study the physiological mechanism of neuroinflammation and nerve injury to alleviate the poor prognosis of TBI patients.

Phosphoglycerate mutase 5 (Pgam5), a mitochondrial protein, localized to the mitochondrial outer–inner membrane contact sites, its activation leads to a linear fracture of string-arranged mitochondria, and this phenomenon mediated mitochondrial and cellular fate [7], including cellular senescence [8] and early stages of necroptosis [9–11]. Some studies have suggested that Pgam5 drives necroptosis through imposing mitochondrial quality control via Drp1 phosphorylation [12, 13]. Pgam5 plays an important role in cardiac microvascular and skin ischemia-reperfusion injury through the Pgam5/CypD/mPTP pathway and Pgam5/Drp1 necrotic pathway [14, 15]. Previously, we demonstrated Pgam5 involved in the progression of neuronal injury following TBI via Drp1 activation-mediated mitochondrial dysfunction [16]. Furthermore, Pgam5 involves regulating IL-1β secretion in mouse bone marrow-derived dendritic cells (BMDCs) [10, 17]. However, whether Pgam5 is involved in IL-1β secretion and neuroinflammation post-TBI and by what pathway is still unclear.

Pyroptosis, a kind of inflammatory cell necrosis, is mainly characterized by the activation of caspase1 and the secretion of mature IL-1β and IL-18 and where Nlrp3 inflammasome acts as the pivotal regulatory signal of pyroptosis, which serves as a potential biomarker and therapeutic target [18]. In our previous studies, we found TBI was accompanied by increased neuroinflammation and pyroptosis during the acute phase post-TBI [19], which were significantly relieved by suppressing Nlrp3 inflammasome-mediated pyroptosis [19–21]. Thus, exploring the regulation mechanism of the NLRP3 inflammasome may provide a new idea

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for regulating the secretion of inflammatory factor II-1β and neuroinflammation after TBI.

This study aims to explore the potential role and molecular mechanism of Pgam5 in the pathogenesis of TBI. Pgam5 depletion was used in vivo controlled cortical impact (CCI) TBI mouse model and in vitro lipopolysaccharide (LPS)-induced microglia models to demonstrate the role of Pgam5 in downstream neuroinflammation post-TBI. Our data indicate that Pgam5 facilitates physiological processes of TBI, which may be presented as a promising therapeutic target post-TBI.

MATERIALS AND METHODS

CCI model

We carried out our experiments on Pgam5-deficient (Pgam5−/−); Cyagen Biosciences Inc., Guangzhou, China; CS7BL/6 background) adult male mice (8–10 weeks) and age-matched wild type (WT) CS7BL/6 male mice. These animal experiments were approved by the Animal Care and Use Committee of First Affiliated Hospital of Xiamen University, China. A schematic diagram of the experimental design in vivo was shown in Fig. S1. Pgam5 and WT mice were numbered by computer and randomly divided into TBI and sham groups. About 60 mice were used in each group.

The CCI device was used to establish a TBI mouse model in vivo [20]. Briefly, a 0.5 mm diameter hole was drilled in the right parietal cortex, 2 mm posterior to the bregma and 2 mm lateral to the sagittal suture. The CCI device (PinPoint™PC3000; Hatteras Instruments Inc., Cary, North Carolina, USA) was inserted into the skull, and the impact site was sent to LC Sciences (Hangzhou, China) for RNA-seq library preparation. After cluster generation, Transcriptome sequencing was carried out on an Illumina Novaseq™ 6000 platform that generated raw reads. The data that support the findings of this study have been deposited in the CNSA (https://db.cngb.org/cnsa/) of CNGBdb with accession number CNP0000970. After removing adaptor sequences, ambiguous N nucleotides (with the ratio of N greater than 5%) and low-quality sequences (with a quality score less than 10), the remaining clean reads were assembled using Trinity software as described for de novo transcriptome assembly with a reference genome. The mapped clean-read number was normalized to RPMK (reads per kilo of per million mapped reads). We used the edgeR package to determine the StringTie genes. Threshold of significant difference was |log2foldchange| ≥ 1, p < 0.05. The Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Heatmap analysis, and VolcanoPlot analysis were conducted at https://www.lc-bio.cn/ (LC Sciences, Hangzhou, China).

II-1β analysis

Commercial enzyme-linked immunosorbent assay (ELISA) kits (Beyotime, Shanghai, China) were used for measuring IL-1β levels in cortical tissue or supernatant as per the manufacturer’s instructions.

Immunofluorescence analysis

Paraffin section of brain tissue was obtained after transcardial perfusion and primary cortical microglia was fixed by 4% paraformaldehyde after LPS + ATP treatment, then followed by overnight incubation with primary antibodies: primary anti-Cd11b antibodies (ab184308, Abcam, Cambridge, UK), anti-Gfap (80788, CST, MA, USA), anti-Nlrp3 antibodies (19771-1-AP, Proteintech, Wuhan, China), anti-caspase1 antibodies (22915-1-AP, Proteintech), anti-caspase6 antibodies (ab185645, Abcam), anti-II-1β (#12242, CST), and anti-a-tubulin (#3873, CST), and then incubated with secondary antibodies. Cell nuclei were stained with DAPI, 6 random high-power fields were chosen and images were obtained using a fluorescence microscope (Leica, Oskar-Barnack, Germany).

Co-immunoprecipitation (CoIP)

Samples of cortical tissue or primary cortical microglia were lysed using a Pierce IP lysis buffer (Thermo Scientific) with a proteinase inhibitor cocktail (Roche, Basel, Switzerland). After centrifugation, suspensions were collected. CoIP was performed using a Dynabeads™ Co-Immunoprecipitation Kit (Thermo Scientific), anti-Nlrp3 (ab263899, Abcam), and anti-Asc (#67824, CST) antibodies were chosen and images were obtained using a fluorescence microscope for electrophoresis.

Western blot analysis

The total protein from cortical tissue or primary cortical microglia was lysed by the lysis buffer (Sigma-Aldrich), and a BCA protein kit (Thermo Scientific) was used to quantify protein concentration. Following SDS-PAGE electrophoresis and western transfer, a PVDF membrane (Millipore, Billerica, MA, USA) was blocked with 5% bovine serum albumin (Sigma-Aldrich) and incubated at 4°C for overnight with the primary antibodies: Pgam5 (ab131552, Abcam), Nlrp3 (ab263899, Abcam), caspase1 (p20) (AG-20B-0042-C100, AdipoGen), Asc, Gsdmd (Gadermin-D) (ab219800, Abcam), caspase8 (#4790, CST), Cleaved caspase8 (#8592, CST), II-1β (#12242, CST), Cleaved II-1β (#63124, CST), and Gapdh (#2118, CST).

For chemical cross-linking, the monomer and oligomer of Asc (#67824, CST) and Pgam5 (ab131552, Abcam) were detected as described by Feuerer-Allgayer and van Hooft (2012). Then crude cell pellets were resuspended in CHAPS buffer and was chemically crosslinked with 4 mM non-cleavable disuccinimidyl suberate (DSS) cross-linker for 30 min. The samples obtained were used for Western blot analysis.

qRT-PCR analysis

The total RNA was isolated using Trizol reagent (Invitrogen, Waltham, MA, USA). A HiFi-MMLV cDNA First-Strand Synthesis Kit (CW Bio, Beijing, China) was used for reverse transcription. GoTaq qPCR Master Mix (Promega, WI, USA) and qPCR primers were used for qRT-PCR analysis. The qPCR reactions were run with an initial denaturation step of 95°C for 15 min and 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s. Each reaction was run in triplicate, and the average Ct value was calculated. The fold changes were calculated relative to the control, which was set as 1.
USA) was used for qRT-PCR analysis on the CFX96TM real-time system (Bio-Rad, CA, USA). The expression of the genes of interest was normalized to the levels of Gapdh. Primer sequences are listed in Table S1:

**Primary cortical microglia culture**

Pure neonatal microglia cultures were obtained from the cortices of neonatal Pgam5−/− or WT mice as previously described in detail [23]. Briefly, cortices were incubated in trypsin/EDTA solution for 15 min at 37 °C, and then the culture medium (DMEM with the addition of 10% fetal calf serum, 1% penicillin/streptomycin, and 2 mM L-glutamine) was added to stop the reaction. After dissociated the cortices, the suspension was centrifuged at 1000 rpm for 2 min. Cells were resuspended in culture medium and grown in 75 ml flasks at 37 °C with 5% CO2, while changed the culture medium every 3 days. After 2 weeks in culture, the cells were isolated by gently shaking of the flask (250 rpm) at 37 °C for 1 h to detach microglia, and collected the medium and immediately centrifuged for 2 min at 1200 rpm, and the obtained pure microglia pellet was resuspended in fresh culture medium and seeded into subcultures. About 95% of these cells were positive for CD11b, a marker for microglia cell types. Experiments were started 24 h after cultivation.

**Transfection in primary cells**
Pgam5-shRNA Lentiviral Particles (sc-152184-V; Santa, CA, USA), Rip3-shRNA Lentiviral Particle (sc-61483-V; Santa), and caspase8-shRNA Lentiviral Particle (sc-37226-V; Santa) were used for transfection following the manufacturer’s instructions in primary cortical neurons or microglia. At 48 h after infection, transfection efficiency was assessed using qRT-PCR analysis (Fig. S2).

**Microglial viability and activation**

10 ng/mL LPS (Sigma-Aldrich) + 5 mM ATP (Sigma-Aldrich) were used in vitro [16]. After LPS treatment for 4 h and ATP for an additional 10, 20, 30, or 45 min, cells and medium supernatant were collected for subsequent detections. Cell Counting Kit-8 (CCK8; Dojindo Laboratories, Tokyo, Japan) was used to determine cell viability. Griess reaction (Solarbio, Beijing, China) to measure the NO production in microglia.

**Asc speck staining**

Cells were blocked with 5% goat serum after fixation and permeabilization, then incubated with anti-Asc antibody (#67824, CST) at 4 °C overnight and washed with PBS. Then, the cells were permeabilized with 0.5% Triton-X-100 in PBS and blocked with 5% goat serum after which Alexa Fluor 594-conjugated secondary antibody (Abcam) was added at 37 °C for 2 min at 1200 rpm, and the obtained pure microglia pellet was resuspended in fresh culture medium and seeded into subcultures. About 95% of these cells were positive for CD11b, a marker for microglia cell types. Experiments were started 24 h after cultivation.

**Statistical analysis**

All the data were presented as the mean ± SEM and were analyzed using SPSS statistical software (version 22.0, IBM, Armonk, NY, USA) and GraphPad Prism 5 software (San Diego, CA, USA). Sample size and animal asks at 37 °C with 5% CO2, while changed the culture medium every 3 days. After 2 weeks in culture, the cells were isolated by gently shaking of the flask (250 rpm) at 37 °C for 1 h to detach microglia, and collected the medium and immediately centrifuged for 2 min at 1200 rpm, and the obtained pure microglia pellet was resuspended in fresh culture medium and seeded into subcultures. About 95% of these cells were positive for CD11b, a marker for microglia cell types. Experiments were started 24 h after cultivation.

**RESULTS**

**Pgam5 depletion relieves neurological deficits and nerve injury after TBI**

In Pgam5−/− mice, the mRNA and protein levels of Pgam5 were near negligible in comparison to WT mice (Fig. 1A). Before the tests, no differences in mNSS, Rotarod, or open field scores were observed between the two groups. 7 days after TBI, Pgam5−/− mice showed lower mNSS scores compared to WT mice (Fig. 1B). In the Rotarod test (Fig. 1C), WT-TBI mice were easy to fall off the stick, but the time of latency to fall of Pgam5−/−/TBI mice was better compared to the WT-TBI mice. In the open field test (Fig. 1D), WT-TBI mice significantly moved less in the perimeter region resulting in less total distance, and Pgam5−/−/TBI mice showed a significant increase in the activity of the perimeter region and the total distance.

TBI induced significant brain tissue edema, the score was maximal at 2 d and gradually decreased, but still with significant difference compared to the sham groups (Fig. 1E). Pgam5 deficiency significantly alleviated this phenomenon compared with WT-TBI mice from day 2 (Fig. 1E). T2-weighted MRI images were also used to evaluate the effect of Pgam5 deletion at 48 h post-TBI (Fig. 1F) and Pgam5 deficiency alleviated histological impairments. The TUNEL/NeuN positive cells were significantly enhanced in WT-TBI mice, but that significantly reduced in Pgam5−/−/TBI mice (Fig. 1G). After TBI, the damaged neuron cells had increased, presenting extensive degenerative changes, including less Nissl body staining. Inversely, severe nerve injury was seen to be significantly reduced in Pgam5−/−/TBI mice (Fig. 1H). Pgam5 depleted blocked the reduction in the arborization of neurites after TBI (Fig. 1I).

**Pgam5 facilitates II-1β activation and microglia activation after TBI**

To classify the biological function of the differentially expressed genes (DEGs), a GO enrichment analysis was carried out. As shown in Fig. 2A, the enriched DEGs were mainly associated with the inflammatory response (GO:0006954), and the extra-cellular region (GO:0005576). The KEGG enrichment analysis showed that the cytokine-cytokine receptor interaction (ko04060), NF-kappa B signaling pathway (ko04064), Tnf signaling pathway (ko04668), and IL-17 signaling pathway (ko04657) were enriched (Fig. 2B), indicating that Pgam5 in regulating inflammatory response through regulating these genes’ expression patterns. To assess the role of Pgam5 on neuroinflammation, 1,076 genes associated with inflammatory response were isolated and analyzed. 3 genes were up-regulated and 37 genes were down-regulated when WT-Ctl were compared to WT-TBI; 6 genes were up-regulated and 80 genes were down-regulated when WT-TBI were compared to Pgam5−/−/TBI (Fig. 2C). Heatmap analysis showed TBI decreased Dsc2, Traf1, and Fzd11 mRNA levels but induced II-1β, Hmox1, Cxcl10, and Nlrp3 expression (Fig. 2D). Interestingly, Pgam5 deletions did not mediate II-1β, Hmox1, Cxcl10, and Nlrp3 mRNA levels (Fig. 2D), that might regulate inflammatory responses by affecting the post-translational modification and protein expression of related genes, including Nlrp3, caspase1, Gsdmd, and II-1β.

As shown in Fig. 3A, pyroptosis and inflammation molecules were still high mRNA levels Pgam5−/−/TBI mice after TBI. II-1β mRNA level in cortex significantly increased within 48 h after TBI, mRNA levels peaked at 12 h, and then it went down (Fig.3A). However, the mRNA level was still 23.7 ± 4.6-fold relative change at 12 h in Pgam5−/−/TBI mice (Fig. 3A). Inversely, II-1β levels peaked 2427 ± 434 pg/mg at 48 h and then gradually declined after TBI, while this was significant in the Pgam5−/−/TBI group (Fig. 3B). However, Pgam5 deficiency did not affect the pyroptosis-related gene expression levels (Fig. 3C), which was consistent with the result of RNA-seq. Pgam5 level was at the same tendency with II-1β level and appeared to peak at 2 days after TBI (Fig. 3D). The data suggested that II-1β might be linked to Pgam5. TBI triggered the recruitment of Pgam5 to Nlrp3 complex, and Pgam5 deficiency suppressed the assembly of Nlrp3 inflammasome, including Nlrp3 interacting with caspase1 and Asc, and II-1β was regulated by Nlrp3-mediated pyroptosis (Fig. 3E). Furthermore, a high level of Cd11b appeared around the damaged cortical tissue after TBI, and TBI increased the interaction with Nlrp3 and caspase1 (Fig. 3F), and induced Cd11b and Gfap positive cells, indicating the activation of microglia and astrocytes (Fig. 3G). In Pgam5−/−/TBI mice, Cd11b/Il-1β positive cells were significantly less and the interaction with Nlrp3 and caspase1 was blocked compared with the WT-TBI group (Fig. 3F-H). The data suggest that Pgam5 facilitates microglia activation-mediated II-1β and...
acute inflammation, which may be involved in the pathology of nerve damage.

**Pgam5 is required for microglia-induced IL-1β secretion via activating Asc oligomerization**

There was a significant decrease in nitrite in LPS-induced Pgam5−/−, Rip3-shRNA, and caspase8-shRNA microglia compared with WT microglia (Fig. 4B). LPS + ATP suppressed cell viability in WT microglia but not in Pgam5−/−, Rip3-shRNA, and caspase8-shRNA microglia (Fig. 4B). As shown in Fig. 4C, LPS + ATP significantly induced IL-1β secretion, while IL-1β secretion was significantly suppressed in Pgam5−/− microglia after stimulation related to LPS + ATP-induced WT microglia but not in Rip3-shRNA and caspase8-shRNA microglia (Fig. 4B). Time-course analysis showed that LPS-induced IL-1β secretion by Pgam5−/− was reduced at all time points tested, but not influenced by Rip3-shRNA and caspase8-shRNA (Fig. 4D). LPS + ATP stimulation activated the Nlrp3 inflammasome in primary microglia, which manifested as caspase1 p20 and IL-1β p17 secretion in an Nlrp3/Asc-dependent manner. Reduced levels of activated caspase1 (p20) and IL-1β p17 were measured in LPS + ATP-induced Pgam5−/− microglia (Fig. 4E). Indeed, caspase8 was activated in Pgam5−/− and WT microglia, and Rip3 or caspase8 downregulation does little to regulate caspase-1 p20 and IL-1β p17 secretion in LPS + ATP-induced microglia (Fig. 4E). Pgam5 downregulation reduced mature IL-1β secretion, but not mRNA levels of IL-1β and other inflammatory cytokines (Fig. 4F). LPS-induced high levels of pyroptosis-related genes were still in Pgam5−/− microglia (Fig. 4G).

CoIP results showed that interaction with Asc and Nlrp3, caspase1, and caspase8 were significantly decreased in Pgam5−/− microglia compared with WT microglia, but caspase8-shRNA did not affect this phenomenon (Fig. 4H). In WT microglia, LPS + ATP-induced Asc oligomerization was measured concomitant with the appearance of caspase1 p20 (Fig. 4I). ASC translocation to an insoluble compartment and Asc oligomerization were significantly reduced in Pgam5−/− microglia, but weak variation was seen in

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**Fig. 1** Pgam5 deficiency improves neurological deficits, neuron damage, and neuronal neurites degeneration after TBI. A Western blot and RT-PCR results showed that Pgam5 expression was abolished in Pgam5−/− mice. Neurological performances were assessed by (B) mNSS, (C) Rotarod test, and (D) open field test, n = 8. E At 1, 2, 3, 5, and 7 days after TBI in WT and Pgam5−/− mice, the brain water content was detected. F At 2 days after TBI, the T2-weighted MRI images showed histological impairments. G Cortical injuries to coronal sections were assessed using TUNEL staining 48 h post-TBI. Nuclei were stained with DAPI and neurons were stained with NeuN. H Nissl staining was performed to determine the morphological changes and damage of cortex neurons after TBI. I Representative pictures from cortical sections show prominent Golgi-Cox staining of degenerating neurites 2 days post-TBI. The images shown are representative of typical images from 6 mice in each group. E–I, n = 6. Data shown are means ± SEM, *p < 0.05 compared to the WT group and #p < 0.05 compared to WT TBI group by two-way ANOVA followed by Tukey’s post hoc multiple comparison test.
Fig. 2  RNA-seq analysis of cortical tissue. Total 55,450 genes of cortical tissues were detected by LC Sciences for RNA-seq analysis. A GO enrichment analysis was carried out to classify the biological function of DEGs. Each box shows the GO term number, the p-value, and GO term. B KEGG pathway enrichment analysis for DEGs. The top 20 KEGG pathways are shown. The box color indicates the level of statistical significance. The dot size means the gene number. C A 1076 genes associated with inflammatory response were isolated and analyzed by VolcanoPlot. D Heatmap analysis of inflammation-related DEGs between WT group, WT TBI group, Pgam5−/− group, and Pgam5−/− TBI group. Only the top 40 genes were included in the DEGs heatmap.
Furthermore, LPS + ATP drove Pgam5 translocation to the insoluble compartment and Pgam5 oligomerization (Fig. 4I). After stimulation of LPS or LPS + ATP in microglia, large intracellular Asc specks were rapidly formed in the cytosol. Asc specks were reduced in Pgam5−/− microglia, but weak variation was seen in caspase8-shRNA microglia (Fig. 4J). Although some reports have shown that caspase8 participates in the processing of pro-IL-1β under certain conditions [24, 25], the data suggest that Pgam5 facilitates the Asc-dependent pro-IL-1β processing via caspase1-associated inflammasome that is independent of Rip3/caspase8 pathway in LPS + ATP-stimulated microglia.

**DISCUSSION**

Microglia are the primary mediators of the innate immune response in CNS, and the proinflammatory cytokines released by microglia, such as IL-1β and TNF-α, are important assessment indices of microglia polarization and pro-inflammatory after TBI [24]. Indeed, inhibiting microglia activation-mediated inflammation is suggested to improve the neurological outcomes post-TBI [25]. In this study, we have investigated the molecular mechanism of Pgam5 after TBI. Pgam5 deletion reduced TBI-induced Nlrp3/caspase1/IL-1β pathway activation, microglial activation, and neurological deficits in vivo. Pgam5 facilitated Asc polymerization...
and IL-1β secretion in microglia, and Pgam5 downregulation alleviated LPS + ATP-induced microglia activation and IL-1β via Asc/caspase1-mediated pyroptosis.

Neuroinflammation is one of the leading causes of secondary brain injury, and its reduction is regarded as an excellent choice to improve the prognosis of TBI [26, 27]. In clinical practice, Helmy et al. have demonstrated that the injection of recombinant IL-1R antibody reduces IL-1-mediated brain injury post-TBI by alleviating neuroinflammation [28]. Moriwaki et al. have verified that Pgam5 promotes inflammasome activation including IL-1β secretion in...
Pgam5 also facilitated pyroptosis via Asc/caspase1 pathway. Phillips et al. have identified and Pgam5 participated in Asc/caspase1-mediated microglia crosstalk and redundancy between caspase1 and caspase8 in Il-1β induced Asc polymerization [33]. The results suggest that Pgam5 deficiency is not required for dsRNA-β-cas-β-caspase8 deactivation after TBI, and TBI-induced neuroinflammation may be exacerbated cardiac ischemia-reperfusion injury through disrupting mitochondrial quality control. Redox Biol. 2021;38:101777.

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CONCLUSION
In this study, we have discussed the mechanism of Pgam5 in nerve injury after TBI. Pgam5 facilitates microglial inflammasome activation to Asc/caspase1-mediated inflammasome activation after TBI, and TBI-induced neuroinflammation may be partly dependent on the interaction between Pgam5 and Nlrp3 inflammasome and Asc oligomerization. Thus, Pgam5 may be a potential therapeutic target to improve neuroinflammation and nerve injury after TBI.
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AUTHOR CONTRIBUTIONS
YHC, WL, and ZXW contributed to conceptualization. YHC and KG contributed to data analysis and graphing. MLM and BCZ performed in vitro experiments, and MLM contributed to RNA-seq data analysis and graphing. KG, BCZ, SFC and ZYL performed animal experiments. YHC contributed to the writing-original draft. WL and ZXW contributed to supervision, and QHX, WL, and ZXW contributed to writing and editing.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
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