FGF21 alleviates neuroinflammation following ischemic stroke by modulating the temporal and spatial dynamics of microglia/macrophages

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Abstract

Background: Resident microglia and macrophages are the predominant contributors to neuroinflammation and immune reactions, which play a critical role in the pathogenesis of ischemic brain injury. Controlling inflammatory responses is considered a promising therapeutic approach for stroke. Recombinant human fibroblast growth factor 21 (rhFGF21) has anti-inflammatory properties by modulating microglia and macrophages, but our knowledge of the inflammatory modulation of rhFGF21 in focal cerebral ischemia is lacking. Therefore, we investigated whether rhFGF21 improves ischemic outcomes in experimental stroke by targeting microglia and macrophages. Methods: C57BL/6 mice were subjected to transient middle cerebral artery occlusion (tMCAO) and randomly divided into groups that received intraperitoneal rhFGF21 or vehicle daily starting at 6 h after reperfusion. Behavior assessments were monitored for 14 d after tMACO and the gene expression levels of inflammatory cytokines were analyzed with qPCR. The phenotypic variation of microglia/macrophages and the presence of infiltrated immune cells were examined by flow cytometry and immunostaining. Additionally, magnetic cell sorting (MACS) in combination with fluorescence-activated cell sorting (FACS) was used to purify microglia and macrophages. Results: rhFGF21 administration ameliorated neurological deficits in behavioral tests by regulating the secretion of pro-inflammatory and anti-inflammatory cytokines. rhFGF21 also attenuated the polarization of microglia/macrophages toward the M1 phenotype and the accumulation of peripheral immune cells after stroke, accompanied by a temporal evolution of the phenotype of microglia/macrophages and infiltration of peripheral immune cells. Furthermore, rhFGF21 treatment through its actions on FGF receptor 1 (FGFR1) inhibited M1 polarization of microglia and pro-inflammatory cytokine expression by suppressing nuclear factor-kappa B (NF-κB) and upregulating peroxisome proliferator-activated receptor PPAR-γ. Conclusion...
In summary, rhFGF21 treatment promoted functional recovery in experimental stroke by modulating microglia/macrophage-mediated neuroinflammation via the NF-κB and PPAR-γ signaling pathways, making it a potential anti-inflammatory agent for stroke treatment.

Keywords: rhFGF21, stroke, neuroinflammation, microglia/macrophage, NF-κB, PPAR-γ

Introduction

Ischemic stroke, reduced cerebral blood flow caused by an arterial thrombus, which afflicts approximately 795,000 individuals worldwide each year, and the number of patients suffering stroke is rising [1, 2]. However, the therapeutic options for stroke are desperately limited. Slow and incomplete recovery is compounded by limited drug treatments that facilitate the recovery process. Acute care mainly depends on thrombolytic treatment by administrating tissue plasminogen activator (tPA), but the narrow therapeutic time window of within 6 h ensure that only a small fraction of patients benefit [3]. Furthermore, reperfusion of the ischemic brain is considered a secondary injury, and distinguishing individuals who will benefit from tPA treatment is challenging. Consequently, novel and effective drug treatments that improve the symptoms and sequelae of stroke, especially in acute phases, are urgently needed [4].

Inflammation is a critical component of the secondary injury resolution process under ischemic brain insult. In the event of stroke, central nervous system (CNS)-resident microglia are first responders to cerebral ischemia and are activated within several minutes [5, 6]. Subsequently, infiltrating immune cells, including monocytes, macrophages, neutrophils, lymphocytes and natural killer (NK) cells, pass through the disrupted blood-brain barrier (BBB) and secrete a plethora of cytokines to promote the progression of inflammation [7] [8]. Therefore, understanding the contribution of those immune cells to the immunomodulation reaction is a prerequisite for therapeutic intervention. In particular, resident microglia, as well as invading macrophages, are
commonly recognized as vital contributors to inflammatory circumstances under the pathophysiology of ischemic stroke [9]. Similarly, morphological transformation and common antigens expressed on both cell types give them certain overlapping functions, such as phagocytosis and analogous ability of polarization toward M1- or M2-like phenotypes [6, 10]. The diverse phenotype distinctively impacts on the expression of inflammatory cytokines, which are correlated with neuronal function. Generally, the M1 microglia/macrophage, marked CD16/32 and CD68, are commonly characterized by proinflammatory effects accompanied by the release of proinflammatory cytokines including tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), and interleukin-6 (IL-6), whereas microglia with the M2 phenotype marked by CD206, secrete transforming growth factor beta (TGF-β), insulin-like growth factor 1 (IGF-1), interleukin (IL)-10 and IL-4 to rescue local inflammation and favor tissue repair [11, 12]. Furthermore, the M2 phenotype is divided into three subsets, M2a marked by CD206 and arginase-1 is associated with anti-inflammation and immunity against parasites, M2b marked by CD86 and SOCS3 is related to adaptive immunity, and M2c marked by TGF-β and IL-10 that facilitate tissue regeneration [13]. Notably, the unique changes in microglia and macrophages temporally and spatially under pathophysiological conditions indicate that each cell type has indispensable and complementary roles in the context of ischemic stroke. Thus far, a growing number of studies have focused on the phenotypic moderation of microglia/macrophages rather than on exclusive suppression of their activation. However, the participation of invading immune cells are usually obscuring.

Fibroblast growth factor 21 (FGF21), as a novel and potent regulator of glucose uptake and lipid metabolism, is predominantly expressed in both the rodent and human liver and thymus [14]. In contrast to other FGFs, FGF21 scarcely has mitogenic effects and may be
the only FGF that can across the BBB due to its weak binding affinity with heparin [15, 16]. To date, accumulated evidence has indicated that FGF21 exhibited therapeutic effects in multiple disease models, such as atherosclerosis [17], diabetic cardiomyopathy [18], age-related disorders [19] and enhanced neurite outgrowth [20]. Although its pharmacologic actions mechanism remains elusive, the therapeutic mechanism of FGF21 primarily involves anti-inflammation [21], energy metabolism and vascular homeostasis [22], oxidative stress [23] and tissue repair [24]. FGF21 mediates these effects by interacting with FGF receptors (mainly FGFR1 and FGFR2) via binding a cofactor, β-klotho, a single-pass transmembrane protein from the klotho-family[25]. FGFR1 and its coreceptor β-klotho have also been reported to be widely observed in brain tissue, including microglia [26]. Therefore, FGF21 may have a potential impact on microglia. Additionally, our previous study demonstrated that FGF21 effectively upregulated the downstream effector peroxisome proliferator-activated receptor (PPAR)-γ in human bone marrow endothelial cells [27], while activated PPAR-γ was closely associated with microglial phenotype and inflammatory regulation in the CNS. Moreover, a recent study confirmed that FGF21 suppressed macrophage-mediated inflammation by Nrf2 and the nuclear factor (NF)-κB signaling pathway in a collagen-induced arthritis model [28].

Therefore, FGF21 is likely to regulates the stroke-induced immune-inflammatory response by modulating microglia and macrophages both in the brain and in peripheral tissue in favor of functional recovery. Recently, recombinant human FGF21(rhFGF21) has been reported to modulate the shift of microglia from M1 activation to M2 activation at the subacute and chronic stages of db/db mice with middle cerebral artery occlusion (MCAO), accompanied by the activation of PPAR-γ in the peri-infarct area [29]. However, the potential mechanism by which FGF21 acts on microglia/macrophages and the dynamic alteration of microglia/macrophages and their phenotype have not been elucidated. In the
current study, we investigated the neuroprotective effect and promising mechanism by which FGF21 ameliorates inflammatory responses and microglia/macrophage polarization in a mouse model of MCAO.

Materials And Methods

Regents and Antibodies

rhFGF21 supported by the laboratory of Biotechnology Pharmaceutical Engineering at Wenzhou medical university and synthesized on the basis of the study previously reported [30]. Antibodies in Flow cytometry analysis involving CD3-PE (17A2, 100206), CD8-FITC (53-5.8, 140404), CD4-APC (GK1.5, 100412), F4/80-FITC (BM8, 123108), NK1.1-APC (PK136, 180710), Ly6G-PE (1A8, 127608), Ly6C-APC (HK1.4, 128016), CD45-APC (103112), CD11b-PE (101208), CD206-FITC (C068C2, 141704), CD68-PE (FA-11, 137014), CD86-PE (GL-1, 105008), CD45-PE/Cy7 (30-F11, 103114), CD11b-PE/Cy7 (M1/70, 101216) purchased from BD Biosciences (San Jose, CA, USA) and CD45-APC (OX33, 17046280), CD11b-PE (OX42, 12011080), CD86-FITC (24F, 11086081) purchased from eBioscience (San Diego, CA, USA).

Antibodies applied in Immunofluorescence including CD16/32(AF1460), CD206 (AF2535), purchased from R&D Systems (Minneapolis, MN, USA) and Iba1(019-19741) purchased from wako pure chemical corporation (Japan).

The primary antibodies applied in Western Blot including anti-NF-κB (3033T), anti-FGFR1 (ab824), anti-p-FGFR1 (ab59194), anti-PPAR-γ (ab28364) and anti-β-Actin (ab8227) were purchased from Cell Signaling Technology (Danvers, MA, USA) or Abcam (Cambridge, MA, USA). The secondary antibody used were donkey anti-rabbit IgG H&L (HRP) (ab150075) or goat anti-mouse IgG H&L (HRP) (ab150115), which were commercially purchased from Abcam (Cambridge, MA, USA).

Corresponding reagent or kit applied in this study include Trizol reagent (Qiagen,
Duesseldorf, Germany), PrimeScript™ RT Reagent Kit (TaKaRa, Shiga, Japan), iQ™ SYBR Green supermix (Bio-Rad, Hercules, CA, USA), miRNeasy Micro Kit (Qiagen, Duesseldorf, Germany), QuantiTect reverse Transcription kit (Qiagen, Duesseldorf, Germany), TaqMan® Gene Expression Assays (ThermoFisher Scientific, Fremont, CA, USA), Neural Tissue Dissociation Kits (Miltenyi Biotech, Bergisch Gladbach Germany), Fluoroshield mounting medium with DIPI (Abcam, Cambridge, MA, USA)

Animals Group and Drug Administration

C57BL/6 mice (20-25 g) were purchased from the Animal Center of the Chinese Academy of Science (Beijing, China) and all surgical procedures and experimental protocols were approved by the Animal Care and Use Committee of Wenzhou medical university. All animals were blindly and randomly assigned to three groups: Sham, MCAO, MCAO+rhFGF21 group. Sham-operated mice under anesthesia and surgical procedure identically but without filament inserted. rhFGF21 (1.5mg/kg) were given to animals intraperitoneally beginning at 6 h after reperfusion for consecutive 7 days.

Transient Focal Cerebral Ischemia and Reperfusion Model Preparation

The surgical procedures to establish MCAO model was based on the method induced by intraluminal filament technique [31]. Briefly, the mice were anesthetized by isoflurane and then placed on a heating blanket to maintain body temperature at 37±0.5°C. A midline incision of mice was made to expose common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA). The CCA was temporarily closed and a monofilament was insert into ICA through the ECA until reached the middle cerebral artery and stay for 60 min, laser doppler flowmetry was used to monitor cerebral flow that lower than 20% of pre-ischemic. The occluding filament was returned to ICA to achieve reperfusion after 60 min occlusion.

Neurological function assessment
The modified Neurological Severity Score (mNSS), rotarod test, corner-turning test and adhesive move test were performed to assess neurodeficits, motor coordination, sensorimotor asymmetry and feeling functions at multiple time points after surgery. All animals received training before suffering ischemia reperfusion injury. The procedure of assessment was performed by the same investigator who were blinded to surgical groups in order to reduce the variance in experiment.

**Quantitative real-time PCR**

Total mRNA was isolated from cortex samples around infarcted zone using trizol reagent according to the manufacturer’s instructions. The cDNA was synthesized by PrimeScript™ RT Reagent Kit. PCR were performed on CFX Connect Real-time System (Bio-Rad, Hercules, CA, USA) using SYBR Green. Primers used in this study were involved in table 1. Additionally, total RNA was extracted from sorted microglia using the miRNeasy Micro Kit according to the manufacturer’s protocol. cDNA was transcribed with Reverse Transcription kit and was amplified in stepone using Gene Expression Assays of TNF-α, IL-6, IL-1β and TGF-β. the volume of reaction was set in 20 µl and performed at 50°C for 2 min, 95°C for 20 s, followed by 40 cycles of 1s at 95°C and 20s at 60°C. Data were analyzed using the $2^{\Delta\Delta\text{Ct}}$ method and expression level of relative mRNA were then reported as fold difference.

**Flow cytometry**

After mice were euthanized, fresh brain, spleen and blood tissues were harvested for single-cell suspension preparation for subsequent single-cell analysis using fluorochrome-conjugated antibodies. Spleen and blood tissues were dissociated into single-cell suspensions as previously described [32, 33]. Splenocytes were dissociated by sieving through a 70-µm filter, then lysing solution (BD Bioscience, CA, USA) was used to deplete
red blood cells in the spleen and blood. Brain mononuclear cells were prepared by Neural Tissue Dissociation Kits according to the protocol. Briefly, the ipsilateral hemisphere of the brain was collected and dissected into small pieces. The pieces were pipetted back into an appropriate-sized conical tube, rinsed with cold Hank's balanced salt solution (HBSS), and then centrifuged at room temperature. After the supernatant was carefully aspirated, a preheated enzyme mix was added to digest tissue pieces for cell suspension. Single pellets were isolated by passing through a 30-µm cell strainer. Cell pellets obtained from the spleen, blood and brain were washed and incubated with antibodies targeting the following: CD3, CD8, CD4, F4/80, NK1.1, Ly6G, Ly6C, CD45, CD11b, CD206, CD68 and CD86, and tagged with phycoerythrin (PE), fluorescein isothiocyanate (FTIC), allophycocyanin (APC), PerCP-Cy5.5 or PE-Cy7. Antibody staining was performed following the manufacturer's protocol. Fluorescence-minus-one (FMO) controls were used to determine the gate of each antibody. Flow cytometry analysis was conducted using a FACS Aria flow cytometer (BD Bioscience, CA, USA), and data were analyzed by FlowJo software (Informer Technologies, USA).

**Sorting of microglia and macrophages**

Microglia of the mouse brain tissue were sorted by magnetic cell sorting (MACS) in combination with fluorescence-activated cell sorting (FACS). Cell pellets were prepared as described above. Cells were stained with PE-Cy7-conjugated anti-mouse CD45 antibody and PE-conjugated anti-mouse CD11b, followed by incubation with anti-PE microbeads. Cells labeled with primary antibody conjugated to PE were enriched by using MACS columns, and then targeted microglia were gathered using the FACS Aria cell sorting system. Resident microglia were identified as the CD45^{int}CD11b^{+} population, whereas infiltrated immune cells in the CNS were identified as the CD45^{high}CD11b^{+} population.
Isolated cells were collected in Trizol reagent, vortexed and kept at -80°C for further experiments.

**Isolation of primary microglia**

Primary rat microglia culture was isolated as previously reported [34]. In brief, the cerebral cortices separated from neonatally 1-day-old rats and meninges were removed. Trypsinization was used to digest the striped cortical tissues for 30 min and 70 µm nylon mesh cell strainer was used to obtain the mixed cortical cells. Cells were maintained in DMEM/F12 with fetal bovine serum (FBS), penicillin and streptomycin (Gibco, Grand Island, NY). Culture media were changed every three days until achieving a confluent monolayer at about 15 days. For the isolation of primary microglia, mild trypsinization was added to isolate microglia from the mixed glial cells. Purified microglia were cultured at 37°C in atmosphere condition for future experiment.

**Oxygen-glucose deprivation (OGD)**

To establish ischemic-like condition *in vitro*, primary microglia subjected to OGD as previously reported [35]. Briefly, microglia cultured with serum-glucose-deprived cultures and placed in hypoxic chamber with 95% nitrogen and 5%CO₂ for 5min and sealed tightly. Subsequently, the chamber moved to an incubator under 5% CO₂/37°Cfor 3 h. After OGD treatment, serum and glucose-free medium were exchanged by glucose-containing medium with or without rhFGF21, followed by incubating with 95% air and 5%CO₂ for 5 hours and then analyzed by qRT-PCR.

**Cell culture and treatment**

Primary cultured microglia and BV2 cell line were used to characterize the effect of rhFGF21 on microglial polarization, inflammation cytokine release and activation of NF-κB and PPARγ signaling. Cells exposure to lipopolysaccharide (LPS) to induced polarised
microglia and inflammatory secretion[36]. Corresponding gene assays (IL-1β, iNOS, TNF-α, IL-6, CD86, CD206, Arg-1, IGF-1 and IL-10) were detected in LPS-stimulated or OGD-treated primary microglia by qRT-PCR and the effects of rhFGF21 on transcriptional activity of NF-κB in primary microglia were detected using Immunofluorescence.

Additionally, the polarization of microglia was analyzed by assessing the expression of the M1 maker CD86, identified by FACS staining.

**Western blot**

Total proteins of LPS-treated BV2 cells were purified by RIPA lysis supplemented with a protease and phosphatase inhibitor mixture. Protein concentrations were measured with a Bradford Protein Detection Kit. 60 µg protein of samples and positive controls were separated on a Sodium Dodecyl Sulfate (SDS) polyacrylamide by electrophoresis. Subsequently, proteins were transferred onto PVDF membranes followed by blocking with primary antibodies FGFR1 (1:1000), p-FGFR1 (1:1000), NF-κB (1:1000), PPAR-γ (1:400), and β-Actin (1:500) overnight at 4°C. Then the membranes were incubated with second antibody donkey anti-rabbit IgG or goat anti-mouse IgG with a 1:10000 dilution for 1 hour at room temperature. Finally, the protein bands were detected with Image Lab software using Gel Doc Imager (Bio-Rad, Hercules, CA, USA) and the expression of target proteins were normalized against β-Actin.

**Immunofluorescence analysis**

Immunofluorescence staining was performed on paraffin brain sections as previously described [37]. Briefly, non-specific binding of antibodies were blocked with 5% BAS for 1 h at 37°C and sections were then incubated with one or more primary antibodies against CD16/32, CD206, Iba1 or NF-κB with a dilution following their instruction at 4°C overnight. After washing, secondary antibody conjugated with adequate fluorochrome were added to visualise the expressions of corresponding protein and DAPI were used to stain nuclei.
Images were captured using confocal laser scanning microscope (Laika, Japan) located in peri-infarct cortex of ipsilateral hemisphere. Data were analyzed with Image J for calculating fluorescence intensity or counting numbers of recognized cells per field.

**Statistical analysis**

All statistical analyses of data were processed with prism 7.0 software (GraphPad, San Diego, CA, USA) in a blinded manner. Data from individual groups are expressed as mean ± SEM and characterized by ordinary one-way ANOVA for multiple comparisons or student's t-test (and nonparametric tests) with exception of behavior assessment. Statistical analyses based on behavioral data were determined by two-way ANOVA for multiple comparisons. Statistical significance is considered at $P<0.05$ level.

**Results**

**rhFGF21 protects against brain injury in MCAO mice**

Compared with vehicle treatment, treatment with rhFGF21 has previously been reported to significantly reduce infarct volumes at 24 h after focal ischemia in rats [38]. A similar result was observed in mice at 72 h using 2,3,5-triphenyltetrazolium chloride (TTC) staining (Fig. 1a, b). However, few studies have reported the effects of rhFGF21 on long-term functional recovery. Behavioral assessments were evaluated at 1, 3, 7 and 14 d after MCAO. Neurological deficits and feeling function in the rhFGF21 group, assessed by the modified neurological severity score (mNSS) test (Fig. 1c) and removal test (Fig. 1d,e) respectively, were significantly better than those in the MCAO group. Similarly, the rhFGF21-treated group exhibited a significant improvement in sensorimotor function, demonstrated by fewer right turns in the corner-turning test (Fig. 1f), and enhanced motor coordination, indicated by an increased latency to fall off the rotarod (Fig. 1g), compared to the vehicle-treated group at 14 d after MCAO. Together, these behavioral data suggest that post-stroke treatment with rhFGF21 facilitates functional recovery after MCAO.
rhFGF21 inhibits the inflammatory response in the cortex of the ischemic brain

The secretion of inflammatory cytokines plays a unique role in the inflammatory cascade reaction and neuronal injury following stroke. In this study, an array of inflammatory cytokines including IL-1β, TNF-α, IL-6, cyclooxygenase (COX)-2, monocyte chemoattractant protein (MCP)-1, and chemokine (C-X-C motif) ligand 1 (CXCL1) at 6, 24, 48 and 72 h after stroke were analyzed by qRT-PCR. mRNA expression of pro-inflammatory cytokines, including IL-6, TNF-α and CXCL1 were quickly increased after stroke and peaked at 24 h (Fig. 2b,d), whereas IL-1β, COX-2 and MCP-1 levels peaked at 48 h (Fig. 2a, c, e) after stroke. However, rhFGF21 administration markedly suppressed the stroke-evoked enhancement of cytokine levels beginning at 24 h, especially at 24 and 48 h after stroke (Fig. 2a-f). Interestingly, level of IL-10, generally regarded as an anti-inflammatory cytokine, were robustly but transiently increased at 6 h after stroke, however, this high level of expression continued to 48 h in the rhFGF21 treatment groups (Fig. 2g).

Furthermore, compared to vehicle, rhFGF21 significantly elevated the level of TGF-β at 48 h (Fig. 2h). These results suggest that rhFGF21 ameliorates the MCAO-induced inflammatory response at the acute stage.

Additionally, IL-10 as an M2 marker, is commonly considered to be a mediators of microglial phenotype polarization [37], and TNF-α, IL-1β, IL-6 and MCP-1 are secreted by M1 microglia. Therefore, we speculate that rhFGF21 may affect microglia and their polarization.

**rhFGF21 modulates the polarization of microglia in the ischemic brain and temporal presence of microglial phenotype and numbers**

To investigate the potential impact of rhFGF21 on microglia in the brain after focal ischemic stroke, we first measured the number and phenotypes of resident microglia in the ischemic hemisphere by Flow cytometry analysis at 3 d after stroke. Resident
microglia were defined as CD11b+CD45\textsuperscript{int} cells and the population of CD68, CD86 and CD206 microglia gated using an FMO control (Fig. 3a). The MCAO group had significantly fewer microglia than the sham group, but there were no significant difference in microglia count between the vehicle-treated group and rhFGF21-treated group. Moreover, rhFGF21 obviously suppressed the expression of CD68 and CD86 in microglia evoked by MCAO, but did not markedly affect CD206 expression (Fig. 3b).

The distribution of the microglial phenotype undergoes dynamic changes depending on timing and context; therefore, we further analyzed the variation in microglial phenotype at 1, 3 and 7 d after MCAO. The number of resident microglia was markedly lower than that in the sham group from 1 d to 3 d after stroke; however, this returned to a similar level as that in the sham group by day 7 (Fig. 3c). Furthermore, the expression of CD68 was significantly upregulated beginning at 1 d following the ischemic event and continuing until 7 d post stroke. Importantly, rhFGF21 intervention significantly reversed the elevated CD68 expression, most strongly at 3 d and 7 d after stroke (Fig. 3d). CD86 has been classified as an M1 and M2b microglia marker that reduces the polarization of microglia to the M2a phenotype[36]. CD86 expression was significantly higher than that in the sham group at 24 h after MCAO but then decreased at day 3. However, the protein level of CD86 peaked at 7 d after MCAO at a level higher than that expressed at the other time points. Moreover, exposure to rhFGF21 treatment markedly suppressed the elevation of CD86 at 3 and 7 d after MCAO (Fig. 3e). Intriguingly, the number of CD206\textsuperscript{+} microglia transiently increased in the injured brain as early as 1 d after MCAO and then gradually decreased, consistent with a previous report [11]. However, compared with vehicle treatment, rhFGF21 did not significantly affect the expression of CD206 in microglia (Fig. 3f).

In addition, an immunohistochemistry approach was used to assess the expression of
CD16/32, another marker of M1 microglia, in the lesion border and corresponding cortical tissue of animals. Consistently, rhFGF21 treatment obviously attenuated the high expression of CD16/32 (Fig. 3g). Collectively, these findings suggest that post-stroke treatment with rhFGF21 inhibits the polarization of M1 microglia but does not facilitate the polarization of microglia toward the M2 phenotype.

**rhFGF21 reduces lymphocyte infiltration in the CNS and inhibits macrophage toward M1 phenotype.**

Subsequently, we analyzed the accumulation of infiltrating leukocytes defined as CD11b⁺CD45<sup>high</sup> cells (Fig. 4a) and the phenotypic transformation of infiltrating macrophages gated on CD11b⁺CD45<sup>high</sup>F4/80<sup>+</sup> (Fig. 4b). Flow cytometry analysis revealed that rhFGF21 administration did not significantly affect the numbers of NK (CD11b⁺CD45<sup>high</sup>CD3⁻NK1.1<sup>+</sup>), CD4<sup>+</sup>T (CD11b⁺CD45<sup>high</sup>CD3<sup>+</sup>CD4<sup>+</sup>), or CD8<sup>+</sup>T (CD11b⁺CD45<sup>high</sup>CD3<sup>+</sup>CD8<sup>+</sup>) cells that infiltrated the CNS. However, rhFGF21 treatment of MCAO animals strikingly reduced the number of macrophages (CD11b⁺CD45<sup>high</sup>F4/80<sup>+</sup>) compared with vehicle treatment (Fig. 4c). Meanwhile, the numbers of neutrophils (CD11b⁺CD45<sup>high</sup>Ly-6G<sup>+</sup>) and monocyte cells (CD11b⁺CD45<sup>high</sup>Ly-6G⁻Ly-6C<sup>+</sup>) in the rhFGF21-treated MCAO group were slightly lower than those in the vehicle-treated MCAO group. In addition, the counts of CD68<sup>+</sup> and CD86<sup>+</sup> microglia were significantly lower in rhFGF21-treated MCAO mice than in vehicle-treated MCAO mice at 3 d and 7 d following stroke. Finally, there were no significant differences in CD206<sup>+</sup> microglial counts between the vehicle-treated and rhFGF21-treated groups (Fig. 4d).

Moreover, the absolute counts of infiltrated immune cells (Fig. 4e), macrophages (Fig. 4f) and CD68<sup>+</sup>, CD86<sup>+</sup>, and CD206<sup>+</sup> macrophages (Fig. 4g-i) were dramatically increased in
the CNS of rhFGF21-treated MCAO mice, peaking at 3 d post injury and subsequently returning to baseline levels by 7 d. Together, these results together suggest that rhFGF21 alleviates the accumulation of infiltrated immune cells (particularly macrophages) in the ischemic brain, and might contribute to the inhibition of the macrophage-mediated inflammatory response.

**rhFGF21 suppresses the phenotypic alteration of microphages toward the M1 in the spleen and blood**

Excessive stroke-induced immune responses lead to disturbances in peripheral immunity, which in turn interfere with immune cell infiltration in the injured brain[13]. rhFGF21 has been reported to inhibit macrophage-mediated inflammation by suppressing NF-κB in RAW 264.7 cells [28]. To determine whether rhFGF21 alleviates CNS inflammation mediated by macrophage-mediated peripheral inflammation, we performed flow cytometry analysis to detect alterations in peripheral immune cell subsets in the spleen and blood at 3 d post stroke. The gating strategies of neutrophils (CD11b+Ly6G+), monocytes (CD11b+Ly6C+), CD8+ T cells, CD4+ cells, NK1.1+ cells and macrophages (CD11b+F4/80+) in single-cell suspensions from the spleen (Fig. 5A) and blood (Fig. 6a) were set by FMO. The gate setting of CD68+, CD86+, CD206+ macrophages were set by FMO control in spleen (Fig. 5c) and blood (Fig. 6c). After MCAO, the numbers of macrophages in peripheral spleen organs were substantially diminished (Fig. 5b), but there was no significant difference in the blood (Fig. 6b). Notably, variation in other cell subsets was not observed in either the spleen or blood. In contrast, the depletion of macrophages induced by MCAO was effectively alleviated by rhFGF21. Moreover, rhFGF21 significantly rescued the increased expression of CD68 and CD86 in macrophage cells residing in the spleen (Fig. 5d) and blood (Fig. 6d) following MCAO. However, there was no considerable difference in the
expression of CD206 in macrophages between the vehicle- and rhFGF21- treated groups. These results suggest that rhFGF21 reduces macrophage activation in peripheral tissue, which is associated with the inflammatory processes in the CNS.

**rhFGF21 regulates the secretion of IL-1β, TNF-α, IL-6 and TGF-β cytokines in sorted microglia and macrophages at 3 d after stroke**

To further detect the potential effects of rhFGF21 on the release of inflammatory cytokines in microglia and macrophages, we sorted microglia and infiltrated macrophages from the damaged hemisphere, and macrophages from the spleen to a purity above 90% and assessed gene expression by real-time PCR (Fig. 7a). Compared with the levels in the sham group, the expression levels of IL-1β (5.33-fold) and TNF-α (1.42-fold) in microglia in the MCAO group were dramatically increased, but those of IL-6 (0.09-fold) and TGF-β (0.28-fold) were significantly reduced. However, both the enhancement in IL-1β and TNF-α expression and the decline in IL-6 expression were hindered by the administration of rhFGF21, but TGF-β expression was not significantly affected (Fig. 7b). In addition, upon induction of MCAO, the mRNA levels of IL-1β (11.70-fold), TNF-α (15.47-fold) and IL-6 (2.87-fold) in infiltrated macrophages were dramatically higher than those in macrophages from the spleen. In the presence of rhFGF21, IL-1β expression was remarkably inhibited, but TNF-α and IL-6 expression levels were not significantly affected. In contrast, there was no differences in TGF-β expression among all groups (Fig. 7c). Moreover, administration of rhFGF21 effectively attenuated the MCAO-induced increase in the levels of IL-1β (1.40-fold) and TNF-α (0.93-fold) in macrophages from the spleen but did not remarkably affect the levels of IL-6 (2.44-fold) or TGF-β (1.08-fold) following MCAO (Fig. 7d). In summary, these outcomes further indicate that rhFGF21 mediates anti-inflammatory effects by modulating microglia and macrophages.

**rhFGF21 reduces M1 Marker expression and pro-inflammatory cytokine secretion**
in primary microglia treated with oxygen-glucose deprivation (OGD) or lipopolysaccharide (LPS).

To evaluate the effects of rhFGF21 on the polarization of microglia and pro-inflammatory cytokine production, we examined primary microglia stimulated by OGD or LPS. Similar results were observed, the administration of rhFGF21 markedly suppressed the expression of M1-type genes (IL-1β, iNOS, TNF-α, IL-6 and CD86), but did not affect M2-type genes (CD206, Arg-1, IGF-1 and IL-10) in OGD-treated primary microglia (Fig. 8a). Similarly, rhFGF21 markedly inhibited the mRNA level of iNOS and TNF-α in LPS-stimulated primary microglia (Fig. 8b), and the protein expression of CD86 were detected in protein level using flow cytometry assays (Fig. 8c, d). Moreover, microglial activation during ischemic injury along with the activation of NF-κB is associated with the secretion of inflammatory cytokines. As shown in Fig. 8E, immunofluorescent staining demonstrated that rhFGF21 suppressed nuclear translocation of NF-κB indicating that the transphosphorylation activity of NF-κB was inhibited by rhFGF21. However, co-administration with PD173074, a selective inhibitor of FGFR1, reversed this effect of rhFGF21. These data together demonstrate that rhFGF21 ameliorates microglia-mediated neuroinflammation by inhibiting NF-κB signaling via the FGFR1 receptor.

rhFGF21 upregulates PPAR-γ and inhibites the activation of NF-κB via FGFR1 in LPS-stimulated BV2 cells.

NF-κB activation in microglia is associated with pro-inflammation, whereas the activity of PPAR-γ is positively related to anti-inflammation. To evaluate whether rhFGF21 activated PPAR-γ, and inhibited NF-κB, we performed western blot experiments in BV2 cells exposed to LPS. As expected, rhFGF21 significantly upregulated the phosphorylation level of FGFR1, however, PD173074 obviously reserved this upregulation (Fig. 9a, c). In addition, similar to observations in primary microglia, rhFGF21 markedly suppressed the
transcriptional activity of NF-κB, which was reversed by PD173070 (Fig. 9b, d). Notably, rhFGF21 administration enhanced the expression of PPAR-γ (Fig. 9b, e), which commonly alters M2 gene expression. Additionally, the effect of rhFGF21 was reversed by PD173074. These data indicated that rhFGF21 modulates microglial polarization via the NF-κB and PPAR-γ pathways.

Discussion
The inflammatory response evoked by focal ischemia stroke is a complex and pleiotropic process [13]. The complexity is emphasized by the immunomodulation progression associated with multiple immune cells resident microglia and an influx of hematogenous cells, that changes with time, space and stage-specific milieu. The heterogeneity is highlighted by detrimental and protective immune effects that mediated by those immune cells. Regulation of neuroinflammation has been recognized as an attractive approach for promising therapies in stroke. rhFGF21, as a safe and effective endocrine regulator, has been demonstrated to have strong anti-inflammatory effects and is a promising candidate for microglia/macrophage-based therapy in acute stroke.

In the current study, we demonstrated neuroprotective effects of rhFGF21 and highlighted its immunomodulatory effects by regulating resident microglia and hematogenous macrophages in acute ischemic stroke. Although this study is not first to show that rhFGF21 may protect against cerebral ischemic injury in rats [38], it confirmed that rhFGF21 significantly reduced the infarct size and ameliorated the neurological deficit in mice affected by stroke through a set of experiments (including TTC and behavior assessment). Moreover, our study also revealed that rhFGF21 remarkably dampened the upregulation of pro-inflammatory gene expression. Therefore, our study provides a better understanding that these outcomes associated with the neuroprotective effects of rhFGF21 are tightly linked with its anti-inflammatory function.
Inflammation-associated conditions regulated by pro-inflammatory and anti-inflammatory cytokines are associated with impaired neurogenesis and neuronal survival [39]. Our findings are in agreement with a previous report [40], which showed upregulation of almost all inflammatory cytokines immediately following stroke, with levels peaking at 24 or 48 h after stroke. However, the increase in IL-10 expression appears strongly but transiently as early as 6 h post stroke. Among the cytokines detected, TNF-α has both neurotoxic and neuroprotective effects, whereas the effects of IL-1β are characteristically neurotoxic. Both TNF-α and IL-1β are mainly produced by microglia and macrophages [41] and synthesized by segregated subsets [42]. The cellular source of IL-6 remains controversial and the almost concede stated is IL-6 predominantly expressed in threatened neurons and activated microglia around the infarct region, target at neuron or microglia, and contributes to both the damage and repair processes [43]. TGF-β, as an anti-inflammatory cytokine, is associated with tissue repair. We next investigated the effect of rhFGF21 on inflammatory gene (TNF-α, IL-1β, IL-6, TGF-β) expression in microglia and invading peripheral macrophages, which are defined as the major cellular contributors to neuroinflammation [44, 45]. Although there are analogous phenotypic and functional characteristics among these inflammatory genes, numerous studies have revealed that they may play unique roles under pathological condition [46]. [47] detected higher expression of TNF-α in microglia than in macrophages of LysM-EGFP knock-in mice, while IL-1β and Arg-1 expression levels were higher in macrophages than in microglia in a permanent MCAO model. In our model, rhFGF21 significantly reduced the level of IL-1β expression not only in microglia and infiltrated macrophages but also in splenic macrophages. Surprisingly, the mRNA level of IL-6 in resident microglia isolated from MCAO mice was far lower than that in sham mice. Although, we have no suitable explanation for this phenomenon, investigating the temporal pattern of the source of IL-6
may provide a reasonable explanation. Microglia, the major cellular contributor to postinjury inflammation, have the potential to act as a key factor of disease onset and progression and to contribute to the neurological outcome of acute brain injury [48]. Under pathological conditions, microglia are rapidly activated and undergo dramatic morphological and phenotypic changes accompanied by the induction of inflammatory cytokines. The classical activation phenotype (M1) is an inflammatory phenotype that produces pro-inflammatory cytokines, while the alternative activation phenotype (M2) is an anti-inflammatory phenotype that is characterized by the secretion of anti-inflammatory cytokines [49]. Our findings further demonstrated that rhFGF21 attenuated the polarization of microglia toward M1 but did not encourage an M1-to-M2 shift in the acute phase of the MCAO model. Consistently, our in vitro results demonstrated that rhFGF21 hampered the expression of proinflammatory cytokines in LPS- or OGD-subjected microglia but had no influence on anti-inflammatory genes (IL-10, CD206 and IGF-1). Concomitantly, mice who underwent experimental MCAO exhibited a gradual decrease in the ipsilateral hemisphere from day 1 to 3 after reperfusion that subsequently returned to preinjury levels by day 7. This phenomenon was also described by a previous study [50] in which microglia from the ischemic hemisphere were remarkably reduced at 3 d after stroke. Furthermore, our results revealed the temporal profile of microglial polarization. In accordance with previous research [51], we observed that the levels of M1-type marker (CD68) significantly increased beginning from day 1 onward. Notably, the expression of the M2 marker CD206 was also increased at 1 d after MCAO, but this increase was no longer observed within 7 d of MCAO injury, in accordance with the finding of [52]. Taking into consideration the earlier upregulation of IL-10 gene expression, which mediates the shift of microglia to the M2 phenotype, there may be a temporary increase in M2-like microglia at 1 to 3 d post stroke, and this notion is
supported by the results of [4].

Moreover, our study focused on the temporal and spatial presence of migrated immune cells in the acute phase of stroke and highlighted the participation of peripheral macrophages. [53] showed that the different immune cell types in the postischemic area had distinct temporal profiles, but the majority of immune cells dramatically accumulated in the ischemic hemisphere at 3 d after stroke. Similarly, in our findings, there was a massive accumulation of immune cells at 3 d after reperfusion, and levels were restored back to baseline by day 7. Importantly, rhFGF21 effectively eliminated the invasion of peripheral immune cells. Additionally, rhFGF21 suppressed the activation of peripheral macrophages in the spleen and blood of mice subjected to MCAO, consistent with previous reports that FGF21 reduced macrophage-mediated inflammation by NF-κB in RWA264.7 macrophages [28]. These findings suggest that the anti-inflammatory effects of rhFGF21 are mediated not only by resident microglia in the brain but also by hematogenous macrophages.

NF-κB, is a key transcription factor in the progression of inflammation, and its activation is accompanied by the release of a panel of inflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6 and Cox-2 [54, 55]. Indeed, microglia polarization has been proposed to induced multiple mechanisms, including NF-κB signaling pathways [56]. Previous literature demonstrated that the beneficial effects of rhFGF21 on macrophages occur through inhibition of NF-κB, and our study further validated that rhFGF21 suppresses the activity of NF-κB via FGFR1 in LPS-stimulated murine microglia. In addition, PPAR-γ is a nuclear transcriptional factor [57], whose activation effects not only peripheral systems in ischemia reperfusion-induced kidney injury and trinitrobenzenesulfonic acid (TNBS)-induced inflammatory bowel disease but also the CNS due to its anti-inflammatory ability[40]. In the present study, rhFGF21 significantly elevated the transcriptional
activities of PPAR-γ in LPS-stimulated BV2 cells, which further contributed to anti-inflammation. To recapitulate, rhFGF21, through its actions on FGFR1, suppresses the inflammatory response by modulating the activation of microglia via inhibiting NF-κB and elevating PPAR-γ.

Conclusions

In summary, our studies demonstrate that the anti-inflammatory effect of rhFGF21 on focal cerebral ischemia occurs through regulation of both central microglia/macrophages and peripheral macrophages through NF-κB and PPAR-γ signaling, indicating that rhFGF21 is a promising candidate for the treatment of ischemic stroke.

Abbreviations

MCAO: transient middle cerebral artery occlusion; BBB: Blood-brain barrier; MACS: Magnetic cell sorting; CCA: common carotid artery; ECA: external carotid artery; ICA: internal carotid artery; FACS: Fluorescence activated cell sorting; tPA: tissue plasminogen activator; TNF-α: Tumor necrosis factor-α; iNOS: inducible nitric oxide synthase; IL-1β: Interleukin-1β; IL-6: Interleukin-6; TGF-β: Transforming growth factor beta; IGF-1: Insulin-like growth factor 1; IL-10: Interleukin-10; IL-4: Interleukin-4; MCP-1: Monocyte chemoattractant protein-1; CXCL1: Chemokine C-X-C motif ligand 1; HBSS: Hank's balanced salt solution; PBS: phosphate buffered saline; ANOVA: One-way analysis of variance; CNS: Central nervous system; rhFGF21: recombinant human Fibroblast growth factor 21; NF-κB: Nuclear factor-kappa B; PPAR-γ: Peroxisome roliterator-activated receptor-γ.

Declarations

Ethics approval and consent to participate

All surgical procedures and experimental protocols were approved by the Animal Care and
Use Committee of Wenzhou medical university in Wenzhou (IACUC no:2018-242)

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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Authors’ contributions
DXW performed the experiments, analyzed the results and wrote the manuscript. FL did the tMCAO surgery and flow analysis performed by PL. LYZ and FYH were major contributors in cell culture. LL, YX, XXT and XW conceived of the study, commented on the results, and revised the manuscript. The final manuscript were approved by All authors.

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Table 1

Table 1
| Gene | Primer sequences (5’ to 3’) |
|------|-----------------------------|
| Actin | Forward CACTGCAAACGGGGAAATGG  
         Reverse TGAGATGGACTGCGGATGG |
| IL-1β  | Forward GCG CTG CTC AAC TTC ATC TTG  
             Reverse GTG ACA CAT TAA GCG GCT TCA C |
| IL-6   | Forward CTC CCA ACA GAC CTG TCT ATA C  
             Reverse CCA TTG CAC AAC TCT TTT CTC A |
| TNF-α  | Forward GTG ACA AGC CTG TAG CCC A  
             Reverse ACT CGG CAA AGT CGA GAT AG |
| Cox-2  | Forward CCCCCGGGTGCAAAAGTAA  
             Reverse GCCCTCGCTTATGATCTGTC |
| MCP-1  | Forward ATAGCAGCCACCTTCATTCC  
             Reverse TCCCCAAAGTCTGTATCT |
| CXCL1  | Forward ACC GAA GTG ATA GCC ACA CCTC AAG  
             Reverse TTG TCA GAA GCC AGC GTT CAC C |
| IL-10  | Forward TTC TTT CAA ACA AAG GAC CAG C  
             Reverse GCA ACC CAA GTA ACC CTT AAA G |
| TGF-β  | Forward TGTCTTGTAGGCTCATCCAAGGAG  
             Reverse TGGTTGTAGGCGAAGGAC |

**Figures**
Effects of rhFGF21 on infarct volume and neurodeficits in the MCAO model. a Representative sample of brain slices with 2% TTC staining at 72 h after MCAO and the quantification of the significant difference in Fig. (b), n=8, Values are mean±SEM by one-way ANOVA. c mNSS assessed at 1, 3, 7 and 14 d after MCAO. d-g Cumulative data illustrate the indicated neurobehavioral tests, including the time to contact (d) and the time to removal in the adhesive test (e) and results from the corner-turning test (f) and rotarod test (g) from day 1 to 14 after MCAO. n=4 in sham group, n=10 in MCAO and rhFGF21-treated group. Values are mean ±SEM by two-way ANOVA.
Figure 2

Effects of rhFGF21 on inflammatory cytokines after MCAO. mRNA expression level of IL-1β (a), IL-6 (b), COX-2 (c), TNF-α (d), MCP-1 (e), CXCL1 (f), IL-10 (g) and TGF-β (h) in ipsilateral cortex were detected with qRT-PCR at 6, 24, 48, and 72 h after MCAO. Values are mean±SEM by one-way ANOVA, n=6 per group.
Effects of rhFGF21 on microglial polarization. a Representative pseudocolor and histograms of flow cytometry show the gating strategy for microglia (CD11b+CD45int) and CD68+, CD86+, and CD206+ expressing microglia in cell suspensions from ischemic hemispheres. All gates were set using FMO control samples. b Bar graph summarizing the cell counts of microglia (CD11b+CD45int) and CD68+, CD86+, and CD206+ expressing microglia in the brain 3 d after MCAO. c-f Quantification of flow cytometry shows the number of microglia (c) and their expression of CD68 (d), CD86 (e), and CD206 (f) at 1, 3, and 7 d. g
Immunofluorescence staining shows CD16/32 expression by microglia (Iba-1) in the peri-infarct area at 3 d after MCAO under confocal observation. n=6 in sham group, n=12 in MCAO and rhFGF21-treated group. Values are mean±SEM.

Figure 4

Effects of rhFGF21 on migrated peripheral immune cell infiltration in the CNS. a Representative flow cytometry analysis shows the gating strategy for NK cells (CD11b+CD45highCD3-NK1.1+), CD4+T cells (CD11b+CD45highCD3+CD4+), CD8+T cells (CD11b+CD45highCD3+CD8+), macrophages (CD11b+CD45highF4/80+), neutrophils (CD11b+CD45highLy-6G+) and monocyte cells (CD11b+CD45highLy-6G-Ly-6C+) using FMO. b Gating strategy of CD68,
CD86, and CD206 from the subsets of microglia using FMO control. c Quantification analysis shows the cumulative data for migrated peripheral immune cells at 3 d post stroke. d Protein levels of CD68, CD86, and CD206 expressed in macrophages. e-f Line graphs summarize the flow cytometry data showing the dynamic distribution of infiltrated lymphocytes (e) and macrophages (f) in the brain at 1, 3, and 7 d after stroke. g-i Temporal presence of CD68+ (g), CD86+ (h), and CD206+ (i) macrophages at 1, 3, and 7 days after stroke among the three groups. n=6 in sham group, n = 12 in MCAO and rhFGF21-treated group. Values are mean±SEM by one-way ANOVA.

Figure 5
Effects of rhFGF21 on peripheral lymphocyte subsets in the spleen at day 3 after MCAO in mice. a Representative gating strategy of neutrophils (CD11b+Ly6G+), monocytes (CD11b+Ly6C+), CD8+ T cells, CD4+ cells, NK1.1+ cells and macrophages (CD11b+F4/80+) in a single-cell suspension from the spleen using FMO control samples. b Cumulative data for quantifying the percentage of the above immune cell subsets. c Gating strategy of CD68, CD86 and CD206 expressed in macrophages from the spleen 3 d after stroke. d Bar graph shows the percentage of CD68+, CD86+ and CD206+ cells in macrophages. n=6 in sham group, n=10 in MCAO and rhFGF21-treated group. Values are mean±SEM by one-way ANOVA.
Effect of rhFGF21 on immune cells from blood at 3 d after MCAO. a Representative dot plot showing the gating strategy of immune cell subsets from the blood. b Gating strategy of CD68, CD86 and CD206 in macrophages. c Quantification analysis shows the percentage of NK cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils and monocytes in the blood. d Summarized flow cytometry data for quantifying CD68 and CD86 and CD206 expression in macrophages from the blood. n=6 in sham group, n=10 in MCAO and rhFGF21-treated group. Values are mean±SEM by one-way ANOVA.
Effects of FGF21 on inflammatory cytokines (IL-1β, TNF-α, IL-6 and TGF-β) in sorted microglia and macrophages. a) Microglia (CD11b+CD45int) from the brain, and macrophages (CD11b+CD45highF4/80+) from the brain or spleen sorted by FACS coupled with MACS, producing a purity of above 90%. b-d qRT-PCR shows the gene expression of IL-1β, TNF-α, IL-6 and TGF-β in microglia (b) and macrophages from the brain (c) and macrophages from the spleen (d). n=6 per group. Values are mean±SEM by one-way ANOVA.
Effect of rhFGF21 on the inflammatory response in primary microglia in vitro. a-b Primary cultured microglia were exposed to OGD (a) or LPS (b) plus vehicle or rhFGF21 and subsequently subjected to qRT-PCR to detect the gene expression of pro-inflammatory molecules iNOS, CD86, TNF-α, IL-1β and IL-6 and anti-inflammatory molecules CD206, Arg-1, IL-10 and IGF-1. c-d Dot plots (c) and summarized graph (d) in flow cytometry show the expression of CD86 in primary microglia evoked by LPS. e Colocalization of NF-κB (green) with nuclei (blue) in Iba1 (red)-marked microglia revealed that the nuclear translocation of NF-κB was blocked by rhFGF21 but that the influence of rhFGF21 was reversed by PD173074. n=4 per group. Values are mean±SEM by one-way ANOVA.
Effects of rhFGF21 on PPAR-γ and NF-κB signaling in LPS- stimulated BV2 cell line.

a Representative band of p-FGFR1 and FGFR1 by western blot assay use an internal control of β-actin and quantitated in graph (c). b Amount of NF-κB and PPAR-γ in nuclear control with H3 and quantitated in bar graph d (NF-κB) and e (PPAR-γ). n=4 per group. Values are mean±SEM by one-way ANOVA.