Regulation of IFN-Is by MEF2D Promotes Inflammatory Homeostasis in Microglia

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Research

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Abstract

Background

Microglia play an essential role in the host defense of central nervous system. Transcription factor MEF2D is known to participate in stress regulation of various cells and is tightly triggered in microglia in vivo and in vitro. MEF2D is shown to bind at the promoter region of several cytokine genes in immune cells, and directly regulates inflammatory response, suggesting that MEF2D may act as a key stimulus response regulator of microglia and is involved in the regulation of brain micro-homeostasis. In order to uncover the molecular mechanism of MEF2D in inflammatory system, in the present study, we investigated the global effect of MEF2D in activated microglia, and explored its potential regulatory network.

Methods

Experiments of recombinant lentivirus vector of shRNA and specific MEF2D over-expression were performed in BV2 cells. Transcriptome sequencing and the global gene expression patterns were analyzed in lipopolysaccharide-stimulated shMEF2D BV2 cells. The pro- and anti-inflammatory factors were assessed by western blot, qPCR or ELISA, and microglia activity by phagocytosis and morphologic analysis. The directly binding of MEF2D to the promoter regions of IRF7 were tested by ChIP-qPCR and PCR. The ISGs were tested by qPCR.

Results

MEF2D was shown to actively participate in the inflammatory response of BV2 microglial cells. RNAi induced stable silence of MEF2D broke the immune balance of microglia, in two ways: (1) promoted the expression of pro-inflammatory factors, such as NLRP3, IL-1β, iNOS; and (2) markedly inhibited the type-I interferon signaling pathway by directly modulating the transcription of IRF7. On the contrary, overexpression of MEF2D significantly reduced the expression of NLRP3 and iNOS under LPS stimulation, and alleviated the level of immune stress in microglia.

Conclusions

These findings demonstrate that MEF2D plays an important role in the regulation of inflammatory homeostasis partly through transcriptional regulation of the IFN-Is response signaling pathway.

Background

Microglia, as the specific resident immune cells of central nervous system (CNS), are the forefront mechanism participating in host defense and immune surveillance in CNS (1). Microglia play an
important role in brain development and the maintenance of the neural environment in aging (2), as well as in neurodegeneration (3-5). Modest activating microglia is necessary and beneficial for CNS homeostasis, but the excessive or persistent activated microglia-induced uncontrolled neuroinflammation is a salient feature in almost all neuron system diseases, such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis (6-8). Therefore, keeping the hierarchical and balanced immune response of microglia is the key for healthy brain. Although considerable researches have focused on the microglial inflammatory homeostasis, the convergent regulatory mechanisms are still not fully understood.

Myocyte enhancer factor-2 (MEF2) family, initially identified as transcription factors in muscle lineage (9), has four mammalian isoforms, MEF2A, B, C and D. MEF2s are important for the processes of development, proliferation, differentiation and immune as a sensor of stimulators for various cell types. In neurons, MEF2s function as converging factors to regulate neuronal proliferation, differentiation, survival, as well as synapse development (10, 11). MEF2s transcriptional activity is tightly regulated by extracellular stimuli, such as neurotrophic stimulation as well as calcium influx (12, 13). Dysregulation of MEF2s by toxic signals contributes to many neurodegenerative diseases (14-16). Our previous study demonstrated that MEF2D promotes the survival of dopamine neurons in the SNc of Parkinson's mouse model (11, 14). MEF2 proteins are also present in cells of the immune system, such as T-cell, B-cell and microglia. In mammals, MEF2C could be phosphorylated by p38 mitogen-activated protein kinases (p38 MAPK) in myeloid lineage cells resulting in increased transcription of c-jun (17), and MEF2D could directly regulate the transcription of Interleukin-2 (IL-2) and Interleukin-10 (IL-10) genes relatively in T-cell and microglia (13, 18). In Drosophila, MEF2s have been identified as a critical transcriptional switch between metabolism and immunity, playing an important role of the innate immune response (19). All these pioneering studies about immune regulation of MEF2s in various species or cell types suggest that MEF2s may also be involved in microglial immune response to regulate the neuroinflammatory response in CNS.

In previous study we found that MEF2D was induced by lipopolysaccharide (LPS) in microglia (18), and participated in the regulation of several cytokines. While as an important stress responder, the function of MEF2D in microglia needs to be further explored in details. Now we applied RNA interference (RNAi) technology, chromatin immunoprecipitation quantitative real-time PCR (ChIP-qPCR) and transcriptome sequencing to identify the regulatory role of MEF2D in activated microglia. Our data showed that compared to negative control, RNAi-induced silence of MEF2D promoted the expression of inducible nitric oxide synthase (iNOS), NACHT Leucine-rich repeat Protein 3 (NLRP3), and the downstream mature IL-1β, and significantly blocked the interferon signaling pathway; and that MEF2D could directly bind to MEF2 consensus sites in the promoter region of interferon regulatory factor 7 (IRF7) to regulate its transcription. These results indicate that MEF2D is an integrate sensor to regulate the innate immune response in activated microglia.

Methods
Cell culture

BV2 cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12) (HyClone, Logan City, Utah, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), and incubated with 5% CO2 at 37°C.

Lentivirus infection

Recombinant lentivirus vectors to silence (MEF2D knockdown by shRNA (shMEF2D)) or enhance (overexpression of MEF2D (OE-MEF2D)) MEF2D gene expression were obtained commercially from Hanbio, Shanghai, China. RNAi sequence of MEF2D: GCTGGATACTTGGACATTAAA. BV2 cells were seeded in a 6-well plate and were infected with lentivirus in 1 ml medium for 12 h before replaced with fresh medium. Then, after 72 h incubation, puromycin (5 μg/ml) was used to select for shMEF2D or OE-MEF2D stable cell lines for at least 3 generations.

Luciferase assay

The MEF2-Firefly reporter vector was assessed from Mao laboratory (Department of Pharmacology and Neurology, Emory University School of Medicine, 615 Michael Street, Atlanta, GA 30322, USA). The shMEF2D and NC stable BV2 cells were transfected with vectors of MEF2-Firefly reporter and the reference Renilla reporter. Then the cells were seeded in 48-well plate, and stimulated with 500 ng/ml LPS for 24 h. The luciferase assay was done using Dual-Luciferase® Reporter Assay Kit (Promega, Madison, WI, USA) following procedures provided by the manufacturer.

Immunoblotting

Nuclear and cytoplasmic protein were extracted using a kit (Sango Biotech, Shanghai, China) as manual. The whole cell protein was harvested with ice-cold lysis buffer containing protease inhibitors. The protein concentration was tested using a BCA protein assay kit (Thermo, Rockford, USA). Equal amount of protein was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membranes. After 2-h blocking with 5% fat-extracted milk at room temperature, the membranes were incubated with primary antibodies MEF2D (BD, #610774), NLRP3 (Cell Signaling, Danvers, USA (CST), #13158), Nrf2 (CST, #12721), iNOS (Abcam, Cambridge, UK, ab178945) and β-actin (Proteintech, Chicago, USA #66009-1-lg) overnight at 4°C, washed with the buffer of tris buffered saline tween 20 (TBST) three times, and then incubated with horseradish peroxidase (HRP) secondary antibody for 2 h at room temperature. Protein bands were visualized using electrochemiluminescence (ECL) and analyzed by ImageJ software.

Quantitative real-time PCR (qPCR)

Total RNA was extracted using TRizol reagent (Roche, Basel, Switzerland). cDNA was reverse transcribed by First Strand cDNA Synthesis Kit (Roche). Quantification of mRNA was tested using qPCR SYBR Green
Master Mix (Yeasen, Shanghai, China). All target mRNA levels were normalized by β-actin as a standard. The primers are listed in Table S1.

**ChIP-qPCR**

ChIP assay was using a ChIP Assay Kit (Millipore, Massachusetts, USA). The quantity of DNA was analyzed by qPCR, gene-specific primers of promoter used as Table S1.

**Enzyme-linked immunosorbent assay (ELISA)**

Quantity of IL-1β in the supernatant of NC and shMEF2D stable BV2 cells were tested using mouse enzyme-linked immunosorbent assay kits (R&D Systems, Minnesota, USA) according to manufacturer procedures.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

NC and shMEF2D stable BV2 cells were seeded in 96-well plates (5 × 10^3 cells/well), then treated with LPS (500 ng/ml) for 24 and 48 h. 20 μl of MTT (5 mg/ml) (Millipore) were added to each well 4 hours before the very detection time. Then, after removing supernatant, 150 μl of dimethylsulfoxide (DMSO) (Millipore) was added to each well and mixed thoroughly for 10 min. The optical density was measured at 490 nm.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining**

For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, FragEL™ DNA Fragmentation Detection Kit (Millipore) was used according to manufacturer’s instructions.

**Transcriptome sequencing (RNA-seq)**

BV2 stable cell lines of negative control and shMEF2D were stimulated with LPS (500ng/ml) for 12 h, the whole lysates were collected in 1 ml TRIzol reagent. The total RNA was extracted, and TruSeq® RNA LT Sample Prep Kit v2 (Illumine, San Diego, USA) was used to construct cDNA libraries. The cDNA fragments were sequenced using an Illumina HiSeq2500. Biological triplicate RNA sequencing was performed on 12 independent RNA samples of BV2 cell lines.

**Phagocytosis**

Fluorescent beads (AAT Bioquest, Protonex Red 600 latex beads) were administered to NC, shMEF2D, OE-MEF2D stable BV2 cells for 4 h. After washing, uptake of fluorescent beads was analyzed by laser scanning confocal microscope (Nikon, Minato, Japan).

**Statistical analyses**
Data were expressed as mean ± standard error of the mean (SEM) from at least three independent experiments. Data were analyzed by either one-way ANOVA or two-way ANOVA as appropriate. Statistical analyses were carried out using SPSS 19.0. A value of $P < 0.05$ was considered statistically significant.

## Results

**MEF2D participated in the inflammatory response of BV2 cells**

Previous studies showed MEF2D participated in MPTP-induced microglial activity. This prompted us to explore the transcriptional regulation network of MEF2D. We chose the model of LPS-induced BV2 microglia cell line, a widely used model of inflammation. After LPS treatment, the level of MEF2D was gradually increased, and reaching a significantly higher level at 24 h (Figure 1A left). As MEF2D is a transcript factor, the nuclear and cytoplasm were separated to confirm the nuclear distribution of MEF2D (Figure 1A right). The elevated MEF2D after LPS treatment was contributed only in nuclear, while the cytoplasmic level didn’t change, indicating that MEF2D may play a transcript role in inflammation.

To verify the regulating effect of MEF2D in inflammation, we used lentivirus vector to get stable BV2 cells of shMEF2D and OE-MEF2D. After LPS stimulation, the expressions of proinflammatory factors NLRP3 and iNOS were significantly promoted by MEF2D knockdown, and diminished by overexpression of MEF2D compared with NC (Figure 1B). As IL-4 could inhibit macrophage production of proinflammatory cytokines, we also administered IL-4 and found that the MEF2D level did not change significantly compared to untreated groups in different stable cell lines (Figure 1B). These data indicate that MEF2D is more inclined to participate in the proinflammatory reaction process.

Morphology is also an important indicator of the activation state of microglia. In the resting state, BV2 microglia cells were round with small cell bodies, while under LPS stimulation, the morphology changed significantly: the cell body became larger and the protrusions became more and thicker. It is interesting to note that knockdown MEF2D made BV2 cells turbulent on the baseline. The rate of activated shMEF2D BV2 cells was significantly higher than NC at 12 h after LPS treatment. On the other hand, overexpression of MEF2D mitigated the activation state even under LPS treatment (Figure 1C). Immune phagocytosis is another main feature of activated microglia. The data showed consistent results that knockdown MEF2D enhanced immune phagocytic function of BV2 cells, while overexpression of MEF2D alleviated the immunophagocytic activity to certain extent (Figure 1D). These data suggest that MEF2D might act an inhibitor of excessive inflammation to be tightly involved in the regulation of inflammatory homeostasis.

**MEF2D knockdown promoted pro-inflammatory response of BV2 cells**

Our above data showed that MEF2D may participate in the inflammatory response of microglia. To further test this proposal, we used steady shMEF2D BV2 cells, of which the knockdown efficiency was about 80% of the background compared to NC. Even with LPS stimulation for 24 h, the expression of MEF2D in shMEF2D BV2 cells was significantly lower (Figure 2B and E). The MTT and TUNEL assays
were performed, and the data showed that knockdown MEF2D didn’t affect the survival and proliferation of BV2 cells, even under stress condition of LPS (Figure S1A and B).

To role out the exact influences of MEF2D in activated BV2 cells, we performed time course studies of several key inducible inflammatory factors in shMEF2D BV2 cells under LPS treatment. NLRP3 is an innate immune pro-inflammatory factor to mediate the cleave-mature process of IL-1β. Its function is crucial for the regulation of neuroinflammation mediated by microglia. Knockdown MEF2D significantly promoted the expression of NLRP3 after LPS treatment for 8 h, and the trend lasted for at least to 24 h in BV2 cells (Figure 2A and B). The secretion level of downstream cytokine IL-1β of shMEF2D was significantly higher after 12 h of LPS stimulation compared to NC (Figure 2C). The mRNA and protein levels of another pro-inflammatory factor iNOS were also increased markedly by knockdown MEF2D compared with NC after 8 h treatment with LPS (Figure 2D and E). On the contrary, the protein but not the mRNA level of the anti-inflammatory factor nuclear factor-erythroid 2-p45 derived factor 2 (Nrf2) was deceased (Figure 2D and E). These results strongly support the fact that knockdown MEF2D markedly enhances the susceptibility and the pro-inflammatory response of BV2 microglia cells.

Differently expressed genes in response to MEF2D knockdown enriched in the immune system in BV2 cells

To investigate the regulatory function of MEF2D in microglia, we used RNA-seq and analyzed the global gene expression patterns in LPS-stimulated BV2 cells with or without steady MEF2D knockdown. To monitor the changes of inflammatory factors in the acute stage, we chose the 12 h after LPS treatment to perform transcriptome sequencing. The knockdown efficiency and the transcriptional activity were tested for the quality control (Figure 3A and B). The RNA-seq transcriptional analysis was performed using three independent samples (biological replicates) of each treatment: NC, NC + LPS stimulation, shMEF2D, shMEF2D + LPS stimulation. The principal components analysis (PCA) showed a good separation and a high level of consistency between biological replicates of the same population in BV2 cells (Figure 3C). Using a false discovery rate, P≤0.01, and fold change≥1.5 log₂ as the cutoff values, we identified 310 differently expressed genes in knockdown MEF2D cells relative to controls without LPS treatment (Figure 3E). The genes were enriched in the gene ontology (GO) annotations of “tissue development”, “cell adhesion”, and “cell differentiation” (Figure 3F), participated in the top kyoto encyclopaedia of genes and genomes (KEGG) pathway classes “infectious diseases”, “cancers”, “digestive system”, “neurodegenerative diseases” and “cellular community” (Table S2). While under the LPS stimulation for 12 h, 242 genes altered between shMEF2D and NC stable BV2 cells (Figure 3E), enriched in the GO annotations of “response to stimulus”, “immune system process” and “innate immune response” (Figure. 3F), involved in the top KEGG pathway classes of “immune system”, “infectious diseases” “cancers” and “signal transduction” (Table S2). These results strongly support the fact that MEF2D could impact the immune response and that knockdown MEF2D could change the immunoregulatory function of BV2 microglia cells.

Knockdown MEF2D significantly blocked the interferon response system
Interferon signal pathway is an essential part of the innate immune response in microglia by participating in the immune response to a variety of stimuli from endogenous and exogenous, triggering the phenotypical plasticity and the cascade of interferon-stimulated genes (ISGs). Interferons (IFNs) have been implicated in the dysregulation of immune responses in autoimmune diseases (20) and neurodegeneration (21, 22). Strikingly, we found that part of the down regulated genes of shMEF2D cells compared to NC, after LPS treatment for 12 h, were clustering to the interferon signal pathway, such as Cxcl10, Ifit1, Il12b, Il6, IRF7, Isg15, Mx2, etc. (Figure 4A). And most of the suppressed ISGs were induced mainly by IFN-Is pathway. These data well-documented that knockdown MEF2D obviously restrained the interferon response system. In other words, MEF2D is an indispensable element for the fully activation of interferon signaling pathway.

IFN-Is are transcriptionally regulated (23), and induced following recognition of pathogen components during infection by various host pattern recognition receptors. IRF7 and IRF3 are the most important transcriptional factors of the initiation of IFN-Is (24). The Genes network indicated that IRF7 is the key transcriptional factor of the significantly and differently expressed genes in shMEF2D and NC stable BV2 cells with LPS treatment (Figure 4B). The transcript abundance (in Read count) of IRF7 and IRF3 were evaluated, and the data showed that the mRNA level of IRF7 was suppressed, while IRF3 was not affected (Figure 4C). Also, the transcript abundances of IFN-Is such as IL-6, IL12B and CXCL10, were significantly suppressed by shMEF2D under LPS stimulation (Figure 4D). As IRF7 is a master regulator not only in the stage of initiation, but also in the second wave of cascade (24, 25), the data of RNA-seq make us to speculate that IRF7 may be the key factor for MEF2D to regulate the IFN-Is.

Transcriptional regulation of RSAD2 and IRF7 by MEF2D

To readout the key regulatory point of MEF2D, the down regulated genes in shMEF2D cells were screened with a putative MEF2 binding motif in the promoter sequences. Several genes including IRF7 were predicted to possess the potential sites (Figure 5A). To assess the direct binding of MEF2D, we carried out ChIP-PCR assay. The data showed that MEF2D bound specifically to the regions of the promoters of IRF7 that contains the putative sites by PCR. And the binding quantity was significantly induced in response to LPS treatment in BV2 cells (Figure 5B and C). We also performed qPCR to conform the subsequent IFN-Is. The data showed that LPS stimulation could significantly trigger IFN-I signaling pathway, and knockdown MEF2D markedly inhibited the elevation of IRF7, IFNA1, MX2, IFIT1, IFI27, ISG15, RSAD2, ZBP1 and DDX58 (Figure 5D). Together, these findings demonstrated that MEF2D directly regulate the transcriptional activity of IRF7, the key regulatory factor of IFN-Is (26, 27). These results strongly support the conclusion that MEF2D is a powerful regulator of the immune system, mainly through the IFN-I signaling pathway in BV2 microglia cells.

Discussion

Microglia are the native macrophages of brain. As highly plastic cells, microglia adopt diverse phenotypes and play seemingly paradoxical roles ranging from neurotoxic to neuroprotective effects, to
maintain micro homeostasis of the brain, under pathological conditions. Obviously, the high plasticity of microglia requires a set of the precise regulatory systems to control a wide range of functional activities in various conditions. Extensive studies have proved that the transcriptional regulation, especially some key transcriptional factors, would be an important part of these controlling systems. Our study revealed that MEF2D, the sensitive responser of diverse stresses, was strongly induced in microglia under LPS stimulation, associated with the changes of microglia phenotypes and functions. Although MEF2D is well-studied as a key converger of several survival and death signals to promote neuronal survival in several model systems in neurons (28, 29), its role in immune inflammation system needs to be further clarified. For the purpose to uncover the specific regulatory function of MEF2D, we constructed the MEF2D stable knockdown and overexpressed BV2 microglia cell lines. We found that compared to NC, knockdown MEF2D didn't affect the proliferation or apoptosis of BV2 cells, but significantly increased the expressions of pro-inflammatory factors, such as NLRP3, IL-1β and iNOS, with LPS treatment for 12 h. This is different from our previous study in DA neuron (11). This may be due to the much lower background content of MEF2D in microglia compared with DA neurons. And the inducible characteristic indicates that MEF2D is more likely to act as a stress-regulator involved in inflammatory response in microglia, rather than a nutritional factor. Inhibiting the expression of MEF2D could break the balance of inflammatory cytokines, leading to hyper-reactivation of the pro-inflammation. Our further data also indicate that knockdown MEF2D markedly and specifically inhibits the IFN-I signaling pathway. IFN-I signals were tightly involved in the process of inflammation in microglia. They are a group of related anti-microbial cytokines, critical for effective host defense against viruses and other pathogens (30). Associating with the elevated levels of pro-inflammatory factors NLRP3, IL-1β, and iNOS, we can reasonably speculate that the inhibition of IFN-I signals by MEF2D knockdown contribute to the incompetent of immune responses, which compulsively leads to over-activations of other pro-inflammatory signaling pathways. But the exact mechanism of MEF2D involved in the cross-talk between the classical pro-inflammatory signaling pathway such as p38 MAPK and the IFN signaling pathway needs further investigation.

IFNs can be divided in three classes based on sequence homology: types I, II, and III (31). The well-known IFN-Is are essential in initiating and regulating innate and adaptive immunity (30). In vitro studies proved that microglia are the major IFN-I responder cell population in the CNS (32). The chronic production of IFN-Is partly contributes to the dysfunction of microglia, and implicates in the development of age-related neuropathological disease and chronic neurodegeneration, such as age-associated cognitive decline (33), prion disease (34), and Alzheimer's disease (35). IFN-Is exert the antiviral and immunomodulatory activities by the induction of ISGs. IRF7 is the master transcriptional regulator of IFN-Is depended immune response (26, 36). The regulatory mechanism of IRF7 in activated microglia is unclear. Using targeted resequencing of 215 candidate genes involved in autoimmunity, a rare regulatory variant rs200395694: G>T located in intron 4 of the MEF2D gene was identified associated with SLE in Swedish cohorts. The region has properties of an active cell-specific enhancer (37). In current study we proved that the key regulator IRF7 of IFN-Is response is directly transcribed by MEF2D, explaining how IRF7 works in stress-induced microglia.
Conclusions

The imbalance of IFN-Is and inflammatory response are often synchronizingly accompanied by aging and neurodegenerative diseases. Properly controllable inflammatory response is the key to brain homeostasis. IFN-Is are pleiotropic cytokines with a critical role in the initiation, blossom and cooling down of the pro-inflammatory response in CNS (38, 39). Our data indicate that MEF2D, as an endogenous inducible factor, participates in the regulation of inflammatory homeostasis mainly by regulating the IFN-I signaling pathway. Our findings reveal that MEF2D, as a stress-sensing and regulatory molecule, implicitly and powerfully regulates IFN-I signaling pathways and is involved in the regulation of inflammatory homeostasis. It appears that MEF2D is rare and standstill at the initial stage of inflammation, but leaps to an inhibitory role in the blossom of pro-inflammatory response. This leads us to speculate that MEF2D may be an important fulcrum in regulating the balance between effective immune responses and excessive inflammation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and 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

FL, QY and JL conceived and supervised the study; FL, RW, TN, FG, SY, LH and LX performed the experiments; FL, KS and QY analyzed the data and wrote the manuscript, and JL edited the manuscript. All authors have read and approved the final version of the manuscript.
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Footnotes

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Abbreviations

ChIP-qPCR: Chromatin Immunoprecipitation quantitative real-time PCR

CNS: central nervous system

CXCL10: C-X-C motif chemokine 10

DDX58: DExD/H-Box Helicase 58

DMEM/F12: Dulbecco’s modified Eagle’s medium/F12

DMSO: dimethylsulfoxide
ECL: electrochemiluminescence

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

GO: gene ontology

HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

HRP: horseradish peroxidase

IFI27: Interferon Alpha Inducible Protein 27

IFIT1: Interferon Induced Protein With Tetratricopeptide Repeats 1

IFNA1: Interferon Alpha 1

IFN-Is: type-I interferons

IL-10: Interleukin-10

IL-1β: Interleukin-1β

IL-2: Interleukin-2

IL-4: Interleukin-4

IL-6: Interleukin-6

IL12B: Interleukin 12B

iNOS: inducible nitric oxide synthase

IRF3: interferon regulatory factor 3

IRF7: interferon regulatory factor 7

ISGs: interferon-stimulated genes

ISG15: interferon-stimulated gene 15

KEGG: kyoto encyclopaedia of genes and genomes

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinases
MEF2: Myocyte enhancer factor-2

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MX2: MX Dynamin Like GTPase 2

NC: negative control

NLRP3: NAcht Leucine-rich repeat Protein 3

Nrf2: nuclear factor-erythroid 2-p45 derived factor 2

OE-MEF2D: overexpression MEF2D

PAGE: polyacrylamide gel electrophoresis

PCA: principal components analysis

PVDF: polyvinylidene fluoride

qPCR: quantitative real-time PCR

RNAi: RNA interference

RNA-seq: Transcriptome sequencing

RSAD2: Radical S-Adenosyl Methionine Domain Containing 2

shMEF2D: MEF2D knockdown by shRNA

TBST: tris buffered saline tween 20

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

ZBP1: Z-DNA-binding protein 1

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Figures

Figure 1
MEF2D participated in the inflammatory response of BV2 cells. (A) LPS-induced increase in MEF2D protein levels and mainly distributed in nucleus in BV2 cells. BV2 cells were exposed to 500 ng/ml LPS for 6, 12 or 24 h, nuclear and cytoplasm were separated for MEF2D quantity by immunoblotting. (A right graph) Relative quantification of MEF2D. (B) BV2 cells were transfected with recombinant lentivirus expressing either control shRNA, shRNA-MEF2D (shMEF2D), or overexpression-MEF2D (OE-MEF2D), and selected for stable cell lines, then treated with LPS for 12 h or IL-4 for 18 h. The protein level of pro-inflammatory factors NLRP3 and iNOS were tested by immunoblotting. (B right graphs) Relative quantifications. Data of A and B from three independent experiments were expressed as the mean ± SEM and analyzed by one-way ANOVA (**P ≤ 0.01). (C) The morphological effect of MEF2D in BV2 cells. NC, shMEF2D, OE-MEF2D stable cells were treated with LPS for 12 h, and the morphological changes was analyzed by optical microscopy. Scale bars represent 50 μm. (C bottom graph) The active cells were defined as the ratio of long axis to short axis greater than 1.5. (D) The phagocytosis capacity effect of MEF2D in BV2 cells. Fluorescent beads were added to the cells for 4 h. Immunofluorescence were executed to measure phagocytosis capacity. Scale bars represent 50 μm. (D bottom chat) The ratio of yellow fluorescence to green fluorescence reflects the phagocytic activity of microglia. Data of C and D from at least 300 cells of 5 vision field in three independent experiments.
Knockdown MEF2D lead to hyperactivity of BV2 cells. BV2 stable cell lines of NC and shMEF2D treated with LPS. (A and B) The mRNA and protein levels of pre-inflammatory factors NLRP3 were tested by qPCR and western blot. (B bottom graph) Relative quantification. (C) The effect of shMEF2D on the secretion of pre-inflammatory cytokines IL-1β under LPS treatment. The secreted levels of IL-1β were tested by ELISA. (D and E) The mRNA and protein levels of pre-inflammatory factors iNOS, and oxidative...
stress regulator Nrf2 were tested by qPCR and immunoblotting. (E bottom graph) Relative quantification. All of the data from three independent experiments were expressed as the mean ± SEM and analyzed by two-way ANOVA (**$P \leq 0.01$).

**Figure 3**

Functional annotation and canonical pathways of knockdown MEF2D effected in BV2 cells. (A and B) The knockdown efficiency of shRNA-MEF2D were tested by western blot and luciferase reporter. (C) The
first principal component (PC1) separates the NC, NC + LPS, shMEF2D and LPS + shMEF2D samples, while the second principal component (PC2) separates the biological replicates of the same population in BV2 cells. (D) A heat map representing of RNA-Seq shared differentially expressed genes between shMEF2D and NC with LPS stimulation. Heat maps were generated with the Multi Experiment Viewer (version 4.8) software. (E) The number of differently expressed genes between different groups. (P ≤ 0.01, and log2-fold change ≥ 1.5). (F) Gene ontology analysis of the functional annotations of differently expressed genes of knockdown MEF2D compared to NC with (right) or without (left) LPS treatment in the BV2 cells. (P ≤ 0.01, and log2-fold change ≥ 1.5).
Figure 4

Knockdown MEF2D significantly inhibited interferon signaling pathway. (A) A heat map representation of the interferon-stimulated genes differently expressed between shMEF2D and NC in BV2 cells with LPS stimulation. Data is from three independent experiments (P ≤ 0.01, and log2-fold change ≥ 1.5). Heat maps were generated with the Multi Experiment Viewer (version 4.8) software. (B) Genes network of significantly different expressed genes evaluated by RNA-seq in shMEF2D and NC stable BV2 cells with
LPS treatment (P ≤ 0.01, and log2-fold change ≥ 1.5). (C) Transcript abundance (in Read count) was evaluated using RNA-seq in the key regulators (IRF7 and IRF3) and effectors (IL-6, IL12B and CXCL10) of the type I interferon signaling pathway in LPS-treated different stable BV2 cells. (**)P ≤ 0.01)

Figure 5

Regulation of interferon signaling pathway by MEF2D. (A) Identification of MEF2 binding sites in the promoter of IRF7. The underlined sequences in red indicate the MEF2 binding site, in blue indicate the...
auxiliary sequence TATA box. (B and C) Binding of MEF2D to the promoter region of IRF7 in BV2 cells. BV2 cells treated with LPS for 12 h were analyzed by ChIP-qPCR and PCR. (D) Knockdown MEF2D significantly reduced the key ISGs level. BV2 stable cell lines of NC and shMEF2D were stimulated by LPS, the mRNA levels of IRF7 and several ISGs, the key inducible factors of interferon signaling pathway, were quantified by qPCR. All of the data from three independent experiments were expressed as the mean ± SEM and analyzed by two-way ANOVA (**P ≤ 0.01).

Supplementary Files

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