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ORIGINAL ARTICLE

MicroRNA-29b modulates Japanese encephalitis virus-induced microglia activation by targeting tumor necrosis factor alpha-induced protein 3

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Abstract

Japanese encephalitis virus (JEV), a single-stranded RNA (ssRNA) virus, is the leading cause of encephalitis in Asia. Microglial activation is one of the key events in JEV-induced neuroinflammation. Although the various microRNAs (miRNAs) has been shown to regulate microglia activation during pathological conditions including neuroviral infections, till date, the involvement of miRNAs in JEV infection has not been evaluated. Hence, we sought to evaluate the possible role of miRNAs in mediating JEV-induced microglia activation. Initial screening revealed significant up-regulation of miR-29b in JEV-infected mouse microglial cell line (BV-2) and primary microglial cells. Furthermore, using bioinformatics tools, we identified tumor necrosis factor alpha-induced protein 3, a negative regulator of nuclear factor-kappa B signaling as a potential target of miR-29b. Interestingly, *in vitro* knockdown of miR-29b resulted in significant over-expression of tumor necrosis factor alpha-induced protein 3, and subsequent decrease in nuclear translocation of pNF-κB. JEV infection in BV-2 cell line elevated inducible nitric oxide synthase, cyclooxygenase-2, and pro-inflammatory cytokine expression levels, which diminished after miR-29b knockdown. Collectively, our study demonstrates involvement of miR-29b in regulating JEV- induced microglial activation.

Keywords: Japanese encephalitis, microglia, miRNA, neuroinflammation.

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Japanese encephalitis virus (JEV), a single-stranded RNA (ssRNA) is the leading cause of encephalitis in Asiatic region (Ghosh and Basu 2009; Tu *et al.* 2012; Upadhyay 2013). The incidences of Japanese encephalitis (JE) is of endemic proportion in India, China, Japan and most of South East Asia with collectively reported ~ 50 000 cases per year (Sehgal *et al.* 2012). The clinical manifestations of JE includes fever, headache, vomiting, signs of meningeal irritation, and altered consciousness (Solomon *et al.* 2003; Ghosh and Basu 2009). JEV-infected neuronal cells secrete a number of pro-inflammatory cytokines and chemokines such as IL-12, tumor necrosis factor alpha (TNF-α), MCP-1 and IL-6 (interleukin-12, tumor necrosis factor-α, monocyte chemo attractant protein-1, and interleukin-6, respectively) that are capable of directly activating microglia (Kaushik *et al.* 2010; Nazmi *et al.* 2011, 2012). In response, microglial cells subsequently produce a number of pro and anti-inflammatory cytokines (Kaushik *et al.* 2010), both of which induce neuronal cell death (Ghoshal *et al.* 2007). Pattern recognition receptors, such as retinoic acid-inducible gene 1 (RIG-I) and NOD-like receptor family, pyrin domain containing 3 have been implicated in JEV-induced microglia activation, and subsequent secretion of interferon-β, IL-1 and IL-18 (Chang *et al.* 2006; Kaushik *et al.* 2012). In a previous study, it has been reported that microglia cells can be directly infected by JEV, and acts as a long-lasting reservoir of this virus (Thongtan *et al.* 2010). Owing to the direct and indirect involvement of microglia in JEV-induced neuroinflammation, microglial activation is a key event.

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Abbreviations used: CBA, cytokine bead array; Cox-2, cyclooxygenase-2; IL, interleukin; iNOS, inducible nitric oxide synthase; JE, Japanese encephalitis; JEV, Japanese encephalitis virus; microRNAs, miRNAs; NF-κB, nuclear factor-kappa B; TNFAIP3, tumor necrosis factor alpha-induced protein 3; TNF-α, tumor necrosis factor alpha.

MicroRNAs (miRNAs) are single-stranded non-coding regions of approximately 21 nucleotides that regulate protein synthesis by targeting mRNAs for translational repression or degradation at the post-transcriptional level (He and Hannon 2004; Pillai 2005). miRNAs have been implicated in various neurodegenerative diseases (Sonntag 2010; Abe and Bonini 2013), including viral encephalitis (Hill *et al.* 2009; Mishra *et al.* 2012). Several studies have reported pro-inflammatory role of various miRNAs in microglia activation (Ponomarev *et al.* 2013; Thounaojam *et al.* 2013). One of the extensively studied miRNA, miR-155 has been shown to regulate lipopolysaccharide (LPS)-induced microglia activation (Cardoso *et al.* 2012). However, miR-124 has been reported to play crucial role in maintaining quiescent state of microglia (Ponomarev *et al.* 2011). In a recent study, miR-32 has been shown to play a pivotal role in human microglia activation following HIV-1 Tat-C treatment (Mishra *et al.* 2012).

Our research group and others have reported molecular mechanisms of JEV-induced neuroinflammation. However, there are no reports on the involvement of miRNAs in regulating JEV-induced microglia activation. In this study, we identified miR-29b as one of the significantly up-regulated miRNA in JEV-infected mouse microglia (BV-2) and primary microglia cells. Further investigations revealed that miR-29b governs microglia activation by targeting tumor necrosis factor alpha-induced protein 3 (TNFAIP3), a negative regulator of nuclear factor-kappa B (NF-κB) activity.

Materials and methods

Ethical statement

The animals were procured from the animal facility of National Brain Research Centre, and all the experiments were performed according to the protocol approved by the institutional animal ethics committee. All the animal research was done in compliance with the ARRIVE (animal research: reporting *in vivo* experiments) guidelines.

Virus isolation and titration

Post-natal 3- to 4-day-old suckling BALB/C mice of either sex were infected with GP78 strain of JEV. Upon onset of clinical manifestation of JEV (limb paralysis, poor pain response, and whole body tremor), animals were killed and brains were excised after repeated transcardial perfusion with ice-cold 1X phosphate-buffered saline (PBS). Brain homogenate prepared in minimum essential medium (MEM) was centrifuged at 10 000 *g* to remove cellular debris, and resultant suspension was filtered through 0.22 μm sterile filter to obtain viral suspension. Immediately, aliquots of filtered virus suspension were stored at −80°C until further use (Ariff *et al.* 2013).

Plaque formation assay was employed to titrate JEV using PS (Porcine stable Kidney) cell line as reported earlier (Vrati *et al.* 1999). PS cells incubated for 1 h at 37°C post-JEV infection was washed with 1X PBS solution and overlaid with MEM containing 4% fetal bovine serum (FBS), 1% low melting agarose and a cocktail of antibiotic-antimyotic solution. Culture plates were incubated at 37°C for 72–96 h until appearance of visible plaques, and henceforth fixed in 10% formaldehyde. Later, plaques were stained with 0.1% crystal violet and counted manually (Mishra and Basu 2008).

Inactivation of virus

To inactivate JEV, virus suspension was exposed to a short wavelength ultraviolet radiation (UVC, 254 nm) in a UV cross-linker (UVC 500, Hoefer scientific, Holliston, MA, USA) by maintaining a distance of 5 cm for 10 min on ice (Kaushik *et al.* 2012).

BV-2 cell culture

Mouse microglia cell line (BV-2) was obtained from Dr Steve Levison (University of Medicine and Dentistry, New Jersey, USA) and maintained in our laboratory. BV-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% sodium bicarbonate (NaHCO3), 10% (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% carbon dioxide (Kaushik *et al.* 2010).

Primary microglia culture

Primary microglia cells were isolated from BALB/C mouse pups as reported previously (Kaushik *et al.* 2010). Briefly, whole brain cortex was carefully extracted from P0-P2 mouse cleared off meninges under dissecting microscope. After mincing the cortex mechanically, was enzymatically digested using tissue trypsin-DNAse to obtain a cell suspension. In order to obtain cell pellet, cell suspension passed through 100-mm cell strainers and centrifuged at 400 *g* for 7.5 min. Cells were plated onto 75-cm^2 tissue culture flasks at a density of 2×10^5 viable cells/cm^2 using complete MEM (supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin 0.6% glucose, and 2 mM glutamine). Complete MEM was replaced every 2 days until the mixed glia culture became confluent. On day 12, microglia cells were dislodge by gentle shaking on an orbital shaker (New Brunswick Scientific, NJ, USA) at 250 rpm for 60–75 min and plated onto culture plates. When the microglial cells became completely adherent they were treated with accutase followed by gentle scrapping, and centrifugation to obtain microglia cell pellet. Later cells were plated onto 2-well chamber slides (Nunc, Denmark) at a density of 8×10^4 viable cells/cm^2 using complete MEM, and maintained at 37°C for further experiments.

Infection of BV-2 and primary microglia cells with JEV

BV-2 cells were plated onto 90 mm cell culture dishes at a density of 1.5×10^6 cells using Dulbecco's modified Eagle's medium containing 10% FBS for 15–18 h followed by incubation in serum free media for additional 4–5 h before virus treatment. Then BV-2 cells were infected with either JEV or UV inactivated JEV at a multiplicity of infection (MOI) 5 for 90 min. The unbound virus particles were removed by gentle washing with 1 × PBS, and cells were further incubated in serum free MEM for different time intervals. Similarly, primary microglia cells were plated onto chamber slides as mentioned above and treated with JEV for 12 or 24 h.

MicroRNA array

miRNA isolation from BV-2 cells was performed as per the manufacturer's instructions (miRNeasy Mini Kit; Qiagen, Valencia, CA, USA). cDNA was prepared using miRNA miScript II RT Kit (Qiagen), using following conditions: 37°C for 60 min, and 95°C for 5 min. miRNA PCR array was performed using miScript SYBR green PCR kit (miRNA PCR array; Qiagen). The following thermal cycling profile was used for qPCR analysis (ABI 7900; Applied Biosystems, Foster City, CA, USA): 95°C for 15 min, 40 cycles at 94°C for 15 s, and 55°C for 30 s.

Over-expression of miR-29b in BV-2 cells

BV-2 cells were seeded onto 60 mm cell culture dishes at a density of 6×10^5 cells, and transfected with 100 pmol of miScript miR-29b mimic (Qiagen) or miRNA negative control (Ambion, Grand Island, NY, USA) using commercial medium (Opti-MEM; Invitrogen, Carlsbad, CA, USA). After 24 h of transfection, cells were collected and miR-29b expression was performed using qPCR. In another set of experiment, TNFAIP3 expression was evaluated following over-expression of miR-29b using immunoblot.

Inhibition of miR-29b in BV-2 cells

BV-2 cells were seeded onto 60 mm or 90 mm cell culture dishes at a density of 6×10^5 cells or 1.5×10^6 cells respectively, and transfected (Lipofectamine 2000; Invitrogen) with 100 pmol of miScript anti-miR-29b inhibitor (Qiagen) or Cye3-labeled control anti-miR (Ambion). After 24 h of transfection, cells were infected with JEV for different time intervals, and used for subsequent protein lysate preparation or RNA isolation. Transfection efficiency was assessed by visualizing the fluorescence of Cye3-labeled control anti-miR.

Cloning of 3 UTR/TNFAIP3 luciferase reporter construct and luciferase assay

TNFAIP3 3 UTR luciferase reporter construct was generated by amplifying 1794 bp sequence using the following primers against 3 UTR of the TNFAIP3 gene from a mouse cDNA library: (forward): 5′-AAGACTAGTGTGCGAACACATTGACAGT-3′and (reverse): 5′-AAGACGCGTTATTGACTTTTGTGAAGTTACAG-3′. The amplicon was then inserted in pMIR-REPORT vector (Ambion) using *Spe*I and *Mlu*I sites in the 5′ and 3′ positions of the luciferase gene, respectively. Henceforth the construct commercially sequenced at Xcelris Labs Ltd., Ahmedabad, Gujarat, India.

For luciferase assay, BV-2 cells were transfected either either with pMIR-REPORT 3′ UTR/TNFAIP3 luciferase reporter, pMIR-REPORT plasmid, or pMIR-REPORT β-galactosidase, and co-transfected with 100 pmol of anti-miR-29b. β-galactosidase was used for normalizing transfection efficiency. After 24 h of transfection, the cells were infected with JEV for additional 24 h and luciferase assays were performed using a luciferase assay system (Promega, Madison,USA). Briefly, the cells were washed with 1X PBS and lysed in reporter lysis buffer provided by the kit and centrifuged at 12 000 *g* for 5 min at 4°C and supernatant was collected. 100 μL of luciferase assay reagent was dispensed into three sets of luminometer tubes, and 20 μL of the collected supernatant was added to each tube and the reading was taken using Sirius single tube luminometer (Berthold detection systems GmBH, Pforzheim, Germany). The luciferase units were measured as Relative Luciferase Units (RLU) and these values were normalized to the amount of protein present in the sample.

Immunoblotting

Post-treatment, control, and treated BV-2 cells were harvested using ice-cold 1X PBS, and lysed using a lysis buffer (containing 1% Triton-X-100, 10 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P, 1 mM EDTA, 0.2% EGTA, 0.2% sodium orthovanadate, and protease inhibitor cocktail). The protein concentration was estimated by bicinchoninic method. Thirty to fifty microgram of each protein sample was electrophoresed on 7.5 or 10% sodium dodecyl sulfate–polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was then blocked using 5% skimmed milk prepared in 1X PBS-Tween-20 (1X PBST) for 4 h at 25°C on a shaker. After blocking, the blots were incubated with rabbit anti-mouse primary antibodies for TNFAIP3, phospho NF-κB, phospho IKKα/β, phospho Ikβα (Cell Signaling technology, Billerica, MA, USA), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox-2) (Millipore, MA, USA), total NF-κB, IKKα/β, Ikβα (Santa Cruz Biotechnology, Dallas, TX, USA) at a dilution of 1 : 1000 overnight at 4°C with gentle shaking. After five washes of 5 min each with 1X PBST, blots were incubated with goat anti-rabbit horseradish peroxidase secondary antibody (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1 : 5000 (prepared in 1X PBST) for 90 min with gentle shaking. Then, blots were rinsed with 1X PBST and developed using chemiluminescence reagent (Millipore) in Chemigenius, Bioimaging System (Syngene, Cambridge, UK). The images were captured and analyzed using the Genesnap, and Genetools softwares. Later, blots were stripped and re-probed with anti-β-tubulin (1 : 1000; Santa Cruz Biotechnology) or anti-β-actin (1 : 10 000; Sigma Aldrich, St. Louis, MO, USA) to determine equivalent loading. The protein levels were normalized with either β-tubulin or β-actin levels.

miRNA qPCR

For detection of mature miRNA, cDNA was prepared using miScript II RT Kit (Qiagen). Later, qPCR was performed using miScript SYBR Green PCR Kit, (which contains the miScript Universal reverse primer) on ViiA 7™ Real-Time PCR system (Applied Biosystems). The following thermal cycling profile was used for qPCR analysis: 95°C for 15 min (1 cycle), 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s (40 cycles). The dissociation curves were generated to check the specificity of primer annealing to the template. The q PCR results were normalized to SNORD-68 (Qiagen) internal control and quantified using comparative Ct method (2-[Δ][Δ]Ct).

mRNAqPCR

For detection of iNOS (forward): 5′-CCC CCG AAG TTT CTG GCA GCA GC-3′, (reverse): 5′- GGC TGT CAG AGC CTC GTG GCT TTG G -3′, COX-2: (forward): 5′-AAG GCC TCC ATT GAC CAG -3′, (reverse): 5′- TCT TAC AGC TCA GTT GAA CGC -3′, interleukin-1β (IL-1β) (forward): 5′-TGG AAA AGC GGT TTG TCT -3′, (reverse): 5′- ATA AAT AGG TAA GTG GTT GCC -3′) and GP78 (forward) 5′-TTG ACA ATC ATG GCA AAC GA-3′,(reverse): 5′-CCC AAC TTG CGC TGA ATA AT-3′ mRNA levels by q PCR, cDNA was synthesized using advantage RT-for-PCR kit (Clontech laboratories, Mountain View, CA, USA). A 500 ng of cDNA was amplified using power SYBR green PCR master mix (Applied

Biosystems) on a ViiA 7™ Real-Time PCR (Applied Biosystems) system. The following thermal cycling profile was used for qPCR analysis: 95°C for 3 min (1 cycle), 94°C for 20 s, 55°C for 30 s, and 72°C for 40 s (40 cycles). The dissociation curves were generated to check for the specificity of primer annealing to the template. The qPCR results were then normalized to 18S rRNA internal control and quantified using comparative Ct method (2-[Δ][Δ]Ct).

Cytokine bead array

The cytokines levels in culture supernatants obtained from control and treated BV2 cells were quantified using mouse cytokine bead array kit (BD Biosciences, San Diego, CA, USA) as per instructions of the manufacturer. Briefly, 50 μL of bead mix (beads coated with IL-6, TNF-α and MCP-1) was mixed with test supernatants or standards and fluorescent dye, and incubated for 2 h at 25°C in dark. Later, the beads were washed and re-suspended in 300 μL of wash buffer, and analyzed using cell quest pro software in FACS Calibur (Becton Dickinson, San Diego, CA, USA). Data were analyzed using cytokine bead array software (Becton Dickinson), and concentration of various cytokines was expressed as pg/mL (Kaushik and Basu 2013).

Immunocytochemistry

For immunocytochemistry study, BV-2 cells were seeded onto 2-well chamber slides (Nunc, Roskilde, Denmark). The cells were transfected with anti-miR-29b for 24 h followed by JEV infection (MOI = 1) for additional 24 h at 37°C. At the end of incubation, cells were fixed in 4% paraformaldehyde for 20 min followed by washing in 1 X PBS. Then non-specific binding sites were blocked by incubating cells in blocking solution (containing 5% goat serum in 1X PBS with 0.03% Triton-X) for 90 min at 25°C. Later, the cells were incubated with primary antibodies (1 : 250) against mouse TNFAIP3 or phospho-NF-κB (Cell signaling) (prepared in 2% goat serum with 0.01% Triton-X) overnight at 4°C in a humidified chamber. Following overnight incubation, cells were washed with 1X PBS and incubated for 90 min with either FITC (Vector Labs) or Alexa 594-conjugated (Molecular Probes, Oregon, USA) secondary antibodies (1 : 500), and then mounted using vectashield mounting solution containing 4',6-diamidino-2-phenylindole (Vector Labs). Images were captured using Zeiss AxioObserver Z1 Microscope and ZEN 2011 Microscope Software (40X magnification, Carl Zeiss, Dublin, CA, USA) at the same exposure time for each filter (to allow comparison of fluorescence intensities between different fields and conditions).

Statistical analysis

All the experiments were performed in sets of three unless otherwise mentioned. All the data generated were analyzed statistically by one-way ANOVA following Bonferroni's multiple comparison tests unless otherwise stated. The results are expressed as mean ± SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego, CA, USA.

Results

miR-29b levels up-regulate in JEV-infected BV-2 cells and primary microglia

First, the effect of JEV infection on miRNA profile in BV-2 using miRNA PCR array was evaluated. Results of miRNA array revealed significant up-regulation of few miRNAs (more than 10 folds) at 12 and 24 h following JEV infection, where miR-29b showed highest fold change (Fig. 1a). miR-29b has been reported to play pro-inflammatory role and therefore its possible role in modulating JEV-induced microglia activation was speculated. Later, expression of miR-29b in JEV-infected BV-2 cells using specific primer assay at different time intervals was evaluated. As expected, there was up-regulation of miR-29b expression in a time-dependent manner with 12 and 24 h showing plateau (Fig. 1b). Furthermore, the expression of miR-29b in JEV-infected primary microglial cells was analyzed which also showed significant up-regulation at 12 and 24 h (Fig. 1c), thus supporting the miRNA array findings. Contrary to these findings, UV- inactivated JEV infection failed to induce miR-29b up-regulation in BV-2 cells (Fig. 1b), thus providing further supporting evidence on up-regulation of miR-29b under active JEV infection. To figure out the efficacy of miR-29 inhibitor and mimic, BV-2 cells were transfected with anti-miR-29b or miR-29b mimic for 24 h, and the expression level of miR-29b was analyzed. Significant decrease or increase was observed in miR-29b level in BV-2 cells transfected with anti-miR-29b or miR29b mimic respectively, compared to cells transfected with scrambled or anti-miR negative control (Figure S1).

miR-29b modulates TNFAIP3 expression in JEV-infected BV-2 cells

Various studies have reported TNFAIP3 as a potential target for miR-29b (Graff *et al.* 2012). Therefore, time-dependent expression pattern of TNFAIP3 protein (Fig. 2a) and mRNA (Fig. 2b) in BV-2 cells following JEV infection was studied. Significant down-regulation in TNFAIP3 mRNA and protein expression at 12 and 24-h post-JEV infection was observed. In another experiment, miR-29b was over-expressed by transfecting BV-2 cells with miR-29b mimic, and the protein expression of TNFAIP3 was determined after 24 h. A decrease in TNFAIP3 expression after miR-29b mimic transfection was observed (Fig. 2c), which indicates that miR-29b can modulate TNFAIP3 expression. Furthermore, the expression of TNFAIP3 in JEV-infected BV-2 cells following anti-miR-29b oligo-mediated knock down of miR-29b was analyzed. Inhibition of miR-29b resulted in enhanced expression of TNFAIP3 protein (as observed in both immunoblot and immunocytochemistry) compared to JEV-infected group (Fig. 2d and e). It was thus evident from the results that miR-29b can modulate TNFAIP3 expression during JEV infection.

Induction of miR-29b elevates the levels of inflammatory markers

Elevated expression of iNOS and COX-2 is a phenotype associated with microglial activation. Therefore, effect of miR-29b inhibition on expression of these microglia

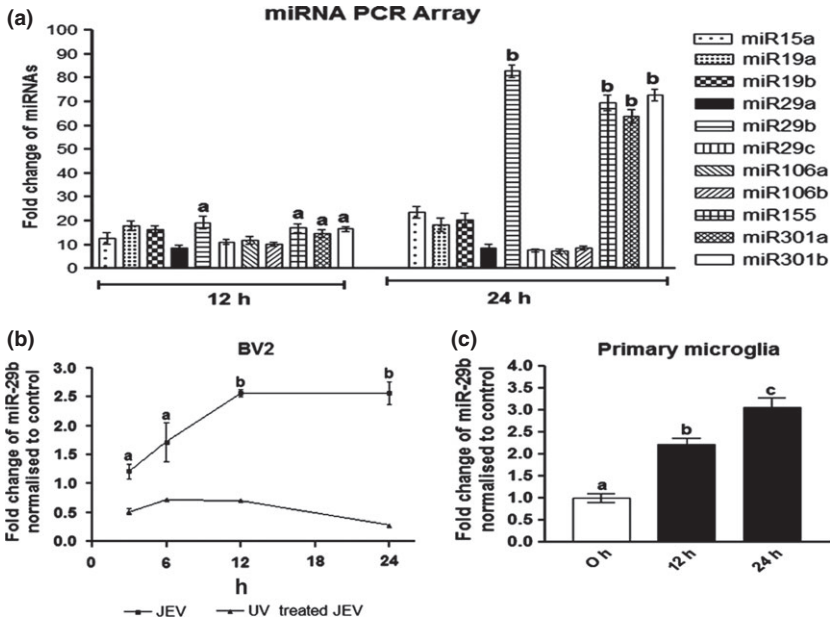

Fig. 1 Expression of miR-29b in Japanese encephalitis virus (JEV) infected BV-2 cells and primary microglia. (a) Effect of JEV infection on miRNA profile of BV-2 Cells. Results represent significantly (> 10 fold) up-regulated miRNAs selected from total 84 miRNA PCR array. Data represent mean ± SEM from three independent experiments and was statistically analyzed using two-way ANOVA with variables of time and miRNA identity. Where time points not sharing common letter indicate statistically significant differences ($p < 0.05$). (b) Time-dependent expression of miR-29b in JEV and/or UV irradiated JEV-infected BV-2 cells for 3, 6, 12, or 24 h. Total miRNA was isolated from treated cells and expression of miR-29b was evaluated using qPCR. Data represent mean ± SEM from three independent experiments. Where time points not sharing common letter indicate statistically significant differences ($p < 0.05$). (c) Expression of miR-29b in JEV-infected primary microglia cells. Primary microglia cells were isolated from mixed glia culture obtained from P0–P2 mouse brain pups and, cultured for 12 days before being infected with JEV for 12 or 24 h. Total miRNA was isolated from treated cells and expression of miR-29b was evaluated using qPCR. Data represent mean ± SEM from three independent experiments. Where columns not sharing common letter indicate statistically significant differences ($p < 0.05$).

activation markers was evaluated. JEV infection of BV-2 cells resulted in higher expression of iNOS and COX-2 mRNA, and protein compared to un-infected cells (Fig. 3a–c). However, *in vitro* inhibition of miR-29b resulted in significant decline in the levels of iNOS and COX-2 mRNA (Fig. 3a and b) and protein compared to JEV-infected cells (Fig. 3c). The results clearly indicate that miR-29b modulates the expression of inflammatory markers in JEV infection.

miR-29b is necessary for JEV-induced expression of pro-inflammatory cytokines in microglia cells

Activated microglia produce various pro-inflammatory cytokines and chemokines, which in turn results in significant neural death. Therefore, targeting these pro-inflammatory factors can have a positive influence on the overall neural health. We hypothesized that inhibition of miR-29b may reduce the generation of pro-inflammatory factors. Infection of BV-2 cells with JEV resulted in significant higher expression of IL-6, TNF-α, MCP-1 and IL1β as compared to un-infected cells (Fig. 4a–d). However, transfection of BV-2 cells with anti-miR-29b significantly reduced levels of these pro-inflammatory markers compared to JEV-infected cells (Fig. 4a–d). Hence the results corroborate the claim that miR-29b expression is imperative for the induction of pro-inflammatory cytokines in BV-2 cells following JEV infection.

miR-29b expression activates NF-κB pathway in JEV-infected BV-2 cells

It has been well established that phosphorylation of NF-κB, IKKα/β and Ikβα proteins is a key determinant of NF-κB activation. Therefore, it was of interest to evaluate the effect of miR-29b knockdown on the expression of pNF-κB, phosphoinositide three-kinase-related kinase (pIKK)α/β and pIkβα proteins under JEV infection of BV-2 cells. JEV infection to BV-2 cells resulted in significant upsurge in pNF-κB (12 and 24 h), pIKKα/β and pIkβα (24 h) expresion levels. As shown in Fig. 5a, transfection of BV-2 cells with anti-miR-29b resulted in decreased levels of pNF-κB, pIKKα/β and pIkβα proteins as compared to JEV-infected BV-2 cells. However, change in pIkβα was not statistically significant (Fig. 5a). In another set of experiment it was observed that a significant increase in nuclear translocation of

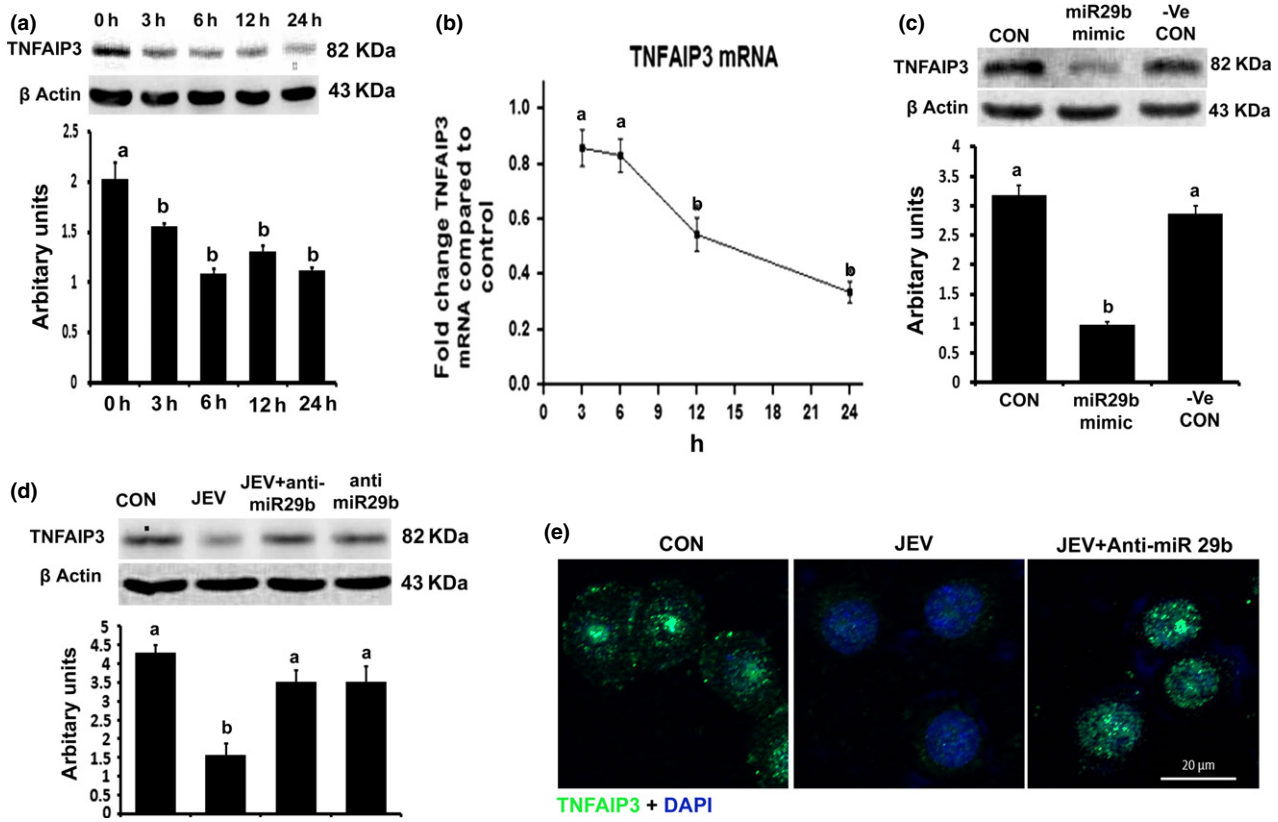

Fig. 2 Expression of tumor necrosis factor alpha-induced protein 3 (TNFAIP3) in Japanese encephalitis virus (JEV) infected BV-2 cells. Time-dependent expression of TNFAIP3 protein (a) and mRNA (b) in JEV-infected BV-2 cells. BV-2 cells were infected with JEV for 0, 3, 6, 12, or 24 h. Protein and mRNA expression of TNFAIP3 was analyzed by immunoblot and qPCR respectively. Column Graph (a) represents densitometric quantification of TNFAIP3 normalized to β-actin. Data represent mean ± SEM from three independent experiments. Where time points not sharing common letter indicate statistically significant differences ($p < 0.05$). (c) Effect of miR-29b over-expression on TNFAIP3 protein expression in JEV-infected BV-2 cells. BV-2 cells were transfected with miR-29b mimic for 24 h and further infected with JEV for 24 h to analyze TNFAIP3 expression by immunoblot. Data represent mean ± SEM from three independent experiments. Where columns not sharing common letter indicate statistically significant differences ($p < 0.05$). (d) Effect of miR-29b knock down on TNFAIP3 protein expression in JEV-infected or un-infected BV-2 cells. BV-2 cells were transfected with anti-miR-29b oligo for 24 h and further infected with JEV or mock- infected [treated with phosphate-buffered saline (PBS)] for 24 h and TNFAIP3 protein expression was analyzed by immunoblot. Column graph (d) represents densitometric quantification of TNFAIP3 normalized to β-actin. Data represent mean ± SEM from three independent experiments. Where columns not sharing common letter indicate statistically significant differences ($p < 0.05$). (e) In another similar set of experiment, cells were fixed and immunofluorescence staining for TNFAIP3 was performed. Florescent microscopy images showed significant expression of TNFAIP3 at 24 h time point in JEV-infected BV-2 cells as compared to mock infected cells while knockdown of miR-29b significantly decreased the expression of TNFAIP3 as compare to JEV-infected cells. Scale bar: 20 μm.

pNF-κB at 24 h in JEV-infected BV-2 cells as compared to un-infected cells while, knockdown of miR-29b significantly decreased pNF-κB nuclear translocation (Fig. 5b). Thus, JEV-induced miR-29b plays crucial role in phosphorylation of NF-κB, its nuclear translocation and activation of downstream pathway.

TNFAIP3 is a potential target of miR-29b

Luciferase assay was performed in order to establish interaction between miR-29b and TNFAIP3. In this experiment, BV-2 cells were transfected with luciferase reporter construct, pMIR-REPORT 3 UTR/TNFAIP3 and/or co-transfected with anti-miR-29b oligo for 24 h followed by JEV infection for 24 h. The empty construct pMIR-REPORT was simultaneously transfected with anti-miR-29b oligo as a negative control. The luciferase activity of the reporter containing the 3 UTR of TNFAIP3 decreased significantly in cells treated with JEV as compared to cells treated with 3 UTR/TNFAIP3 alone. While, inhibition of miR-29b showed a significant increase in luciferase activity as compared to JEV group (Fig. 6). pMIR-REPORT β-galactosidase was used as normalization control. The results clearly indicate that miR-29b interact with TNFAIP3 by binding on the 3 UTR region of TNFAIP3.

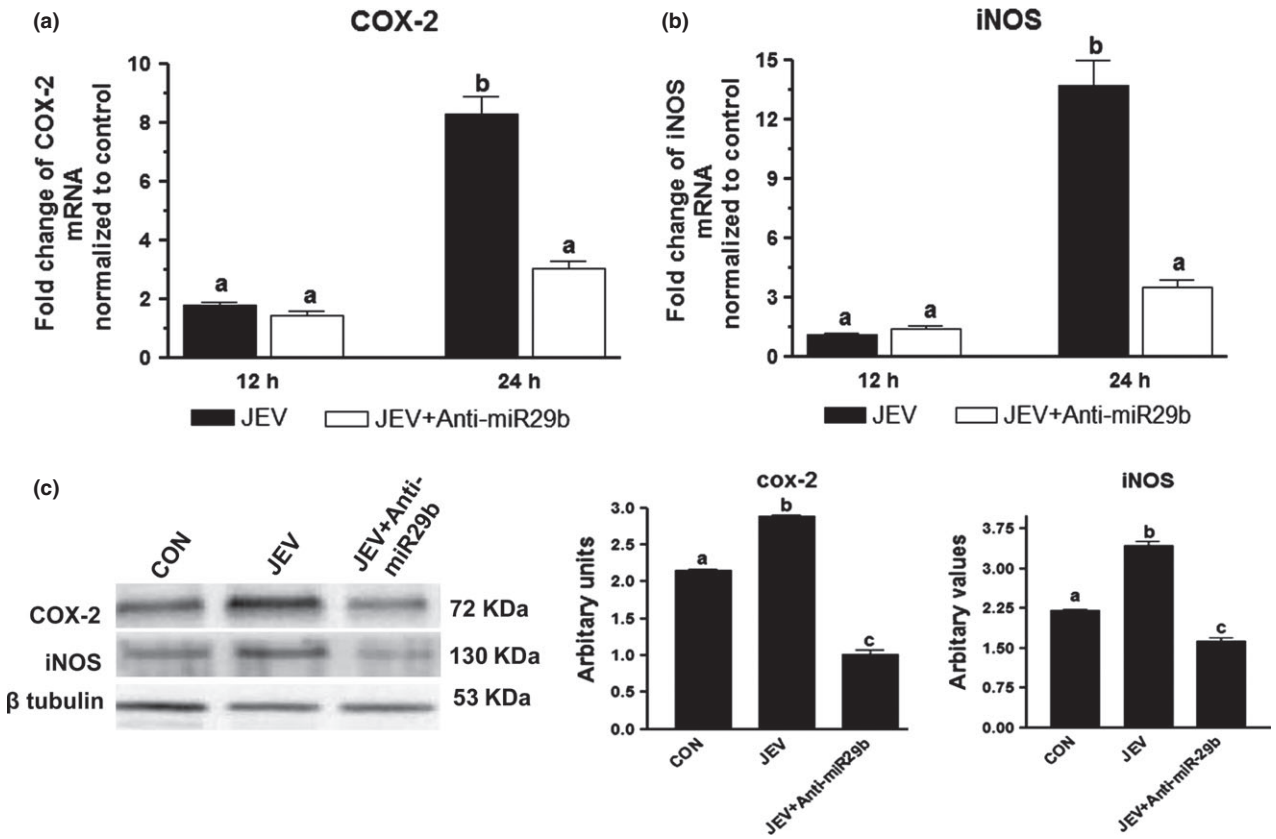

Fig. 3 Anti-miR-29b down-regulates expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in Japanese encephalitis virus (JEV) infected BV-2 cells. (a and b) BV-2 cells were transfected with anti-miR-29b for 24 h followed by infection with JEV for 12 or 24 h. At the end of each time point, RNA was isolated and subsequent expression of IL-1β, iNOS and COX-2 mRNA was evaluated using qPCR. Data represent mean ± SEM from three independent experiments. Where columns not sharing common letter indicate statistically significant differences ($p < 0.05$). (c) Expression of iNOS and Cox-2 proteins in JEV-infected BV-2 cells. BV-2 cells were transfected with anti-miR-29b for 24 h followed by JEV infection for 24 h. At the end of each time point, the cells were lysed for protein extraction and levels of iNOS and Cox-2 proteins were determined by immunoblot. The graphs represent densitometric quantification of iNOS and COX-2 normalized to β-tubulin. Data represent mean ± SEM from three independent experiments. Where columns not sharing common letter indicate statistically significant differences ($p < 0.05$).

Discussion

Microglia plays a key role in both innate and adaptive immune responses of central nervous system (CNS), and its activation is a hallmark of neuroinflammation (Guedes 2013; Thounaojam *et al.* 2013). Recently, potential role of miRNAs in regulating microglial activation and immune responses has been reported (Guedes 2013). Cardoso *et al.*, demonstrated that miR-155 targets suppressor of cytokine signaling 1, leading to the up-regulation of several inflammatory mediators characteristic of the M1 phenotype (Cardoso *et al.* 2012). Till date, there are no reports on possible involvement of miRNA in regulating JEV-induced microglia activation. Therefore, we sought to evaluate possible involvement of miRNAs in regulating JEV-induced microglia activation.

In this study, miRNA array (of 84 miRNAs) analysis from JEV-infected BV-2 cells showed significant up-regulation (more than 10 folds) in expression of miR-29 (a,b, c), miR-155, miR-301(a, b), miR-106 (a, b), miR-19(a, b), and miR-15a. On the basis of the results of fold up-regulation and literature survey on relevant miRNAs, we selected miR-29b for further evaluation. miR-29 family has three mature members, miR-29a, miR-29b, and miR-29c (Kriegel *et al.* 2012), and all members have been shown to regulate various facets of inflammation (Chen *et al.* 2011; Ma *et al.* 2011; Graff *et al.* 2012). In this study, we observed up-regulation in all three members of miR-29 family. However, in terms of fold change, miR-29b recorded more robust change following JEV infection compare to other two. Furthermore, miR-29b has been shown to suppress immune responses to intracellular pathogens by targeting interferon- λ (IFN-λ) (Ma *et al.* 2011). In fact, viral infections have been shown to induce miR-29b expression (Fang *et al.* 2012). A recent study by Joel *et al.* reported that miR-29b regulates macrophage M1 polarization by targeting TNFAIP3, and thereby regulating NF-κB

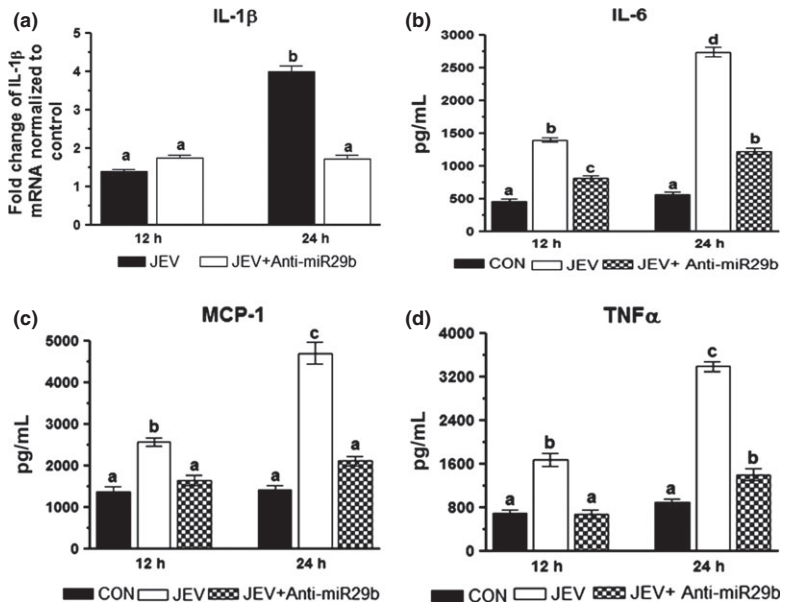

Fig. 4 Anti-miR-29b reduces expression of pro-inflammatory cytokines in Japanese encephalitis virus (JEV) infected cells. BV-2 cells were transfected with anti-miR-29b for 24 h followed by 12 or 24 h of JEV infection and mRNA level of IL-1β (a) and protein levels of IL-6, tumor necrosis factor alpha (TNF-α) and MCP-1 (b–d) were analyzed using qPCR and cytokine bead array respectively. Data represent mean ± SEM from three independent experiments performed in duplicate. Data represent mean ± SEM from three independent experiments. Where columns not sharing common letter indicate statistically significant differences ($p < 0.05$).

expression (Graff *et al.* 2012). TNFAIP3 (earlier identified as negative regulator of TNF induced apoptosis) has been shown to negatively regulate NF-κB activity (Coornaert *et al.* 2009). TNFAIP3 expression is induced by pro-inflammatory cytokines (TNF-α and IL-1) and microbial products (LPS) that trigger pathogen recognition receptors, such as Toll-like receptors (Verstrepen *et al.* 2010).

Time kinetic studies on expression of miR-29b and its target protein TNFAIP3 revealed an inverse relationship with respect to each other's expression till 12-h time point. Up-regulation of miR29b following JEV infection showed plateau at 12 and 24 h however, TNFAIP3 mRNA levels showed decrement at 24 h post-infection compared to 12 h time point. It could be possible that degree of miR-29b induction at 12 and 24 h is sufficient to even further reduce its target mRNA (TNFAIP3). However, further studies are required to resolve this issue of differential kinetics of miR-29b and its target. Reportedly, miRNAs induce degradation or translational repression of their target by binding to their 3′ UTR region (Lytle *et al.* 2007; Cannell *et al.* 2008; Thounaojam *et al.* 2013). In this study, we used luciferase reporter assay to evaluate interaction of miR-29b with 3′UTR of TNFAIP3. It was evident from luciferase reporter assay that miR-29b binds to this region of TNFAIP3 and suppresses its expression. These observation is in accordance with previous study on inhibition of TNFAIP3 in macrophage cells by miR-29b (Graff *et al.* 2012). In agreement with changes in TNFAIP3 protein, we observed significant decrement in the TNFAIP3 mRNA at 12 and 24 h after JEV infection. These observations indicate that miR-29b produced in response to JEV infection targets TNFAIP3 RNA, leading to its degradation and resulting in reduced TNFAIP3 protein. In a recent report by Balkhi *et al.* (2013), it was shown that miR-29b can stabilize TNFAIP3 RNA in tumor cells. It was demonstrated that miR-29b interacts with the RNA binding protein HuR (human antigen R; also known as ELAVL1), and prevents its binding to 3′ UTR of TNFAIP3, and subsequent recruitment of the RNA degradation complex RISC (RNA-induced silencing complex) preventing degradation of TNFAIP3 transcripts. Various studies have reported up-regulation of HuR in tumor cells and its important role in cancer (Wang *et al.* 2013). However, till date involvement of HuR in microglia activation has not been reported and hence, we assume that the differences in the regulation of TNFAIP3 transcript stability in our study and a study by Balkhi *et al.* (2013) could be because of involvement of HuR protein.

Microglial activation is marked by elevated expression of iNOS, COX-2 and pro-inflammatory mediators (such as IL-1β) (Lau *et al.* 2009; Olajide *et al.* 2013). Previous studies from our laboratory have shown augmented expression of iNOS and COX-2 in JEV-infected BV-2 cells (Ghoshal *et al.* 2007; Das *et al.* 2011). Also, induction of COX-2 expression during viral infection was shown to be mediated by miR-29 (Fang *et al.* 2012). We evaluated iNOS, COX-2 and IL-1β mRNA, and iNOS and COX-2 protein expression in anti-miR-29b transfected BV-2 cells followed by JEV infection. Interestingly, inhibition of miR-29b significantly reduced expression of iNOS, COX-2 and IL-1β at 12 and 24-h post-infection. We have previously shown that supernatant from JEV-infected microglia cell culture is capable of inducing neuronal cell death (Ghoshal *et al.* 2007). In this context, pro-inflammatory mediators such as TNF-α, IL1β, IL-6, and MCP-1 secreted by activated microglia are responsible for

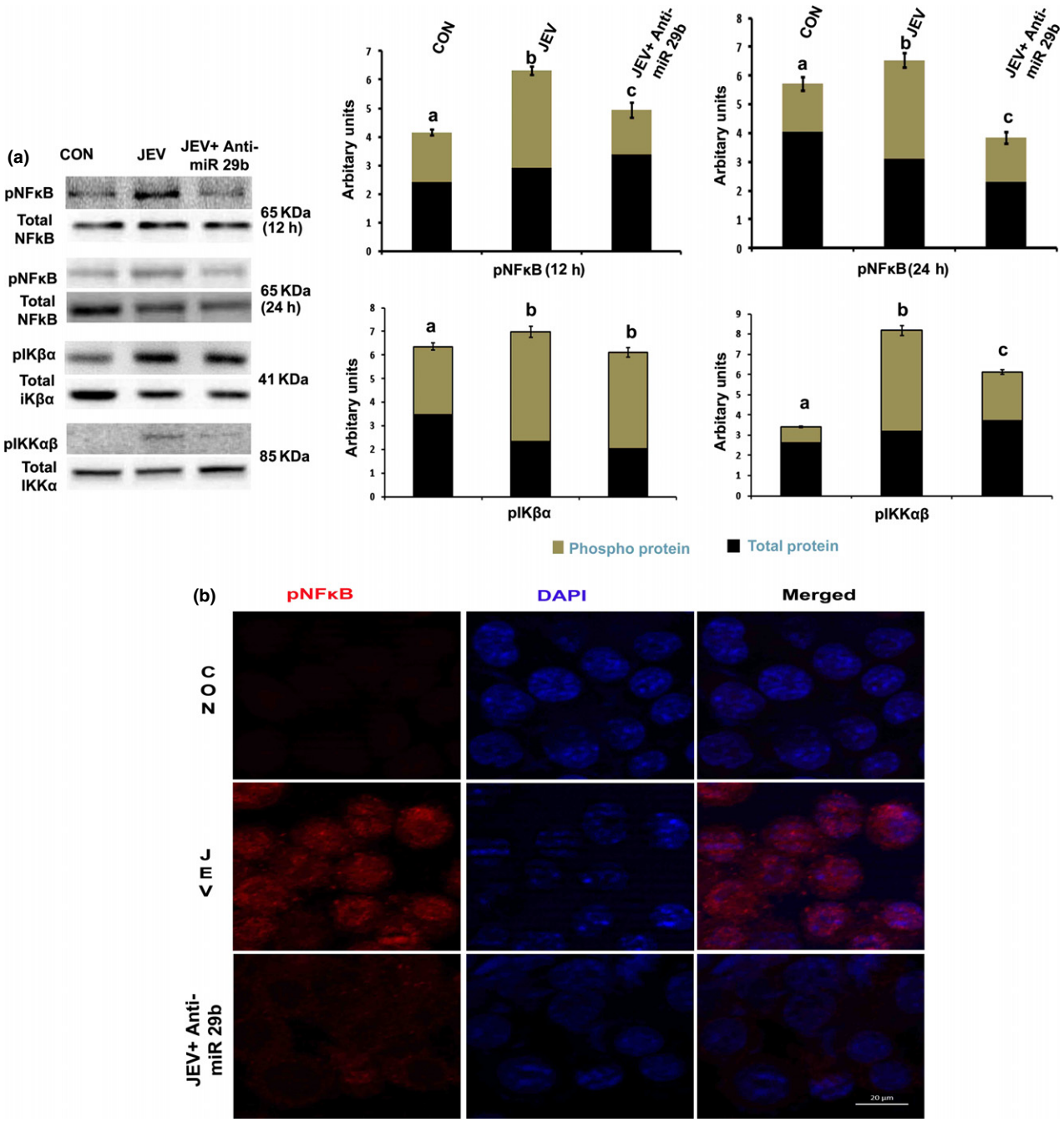

Fig. 5 Effect of miR-29b knockdown on expression pNF-κB, pIKKα/β and pIkβα protein levels in Japanese encephalitis virus (JEV) infected BV-2 cells. (a) Expression of pNF-κB, pIKKα/β and pIkβα proteins in JEV-infected BV-2 cells. BV-2 cells were transfected with anti-miR-29b for 12 or 24 h followed by JEV infection for 24 h. Expression levels of pNF-κB (12 and 24 h), pIKKα/β and pIkβα (24 h) were determined by immunoblotting. Graphs represent the densitometric analysis of the immunoblots where the lightly shaded parts of the blots denote the phosphorylated fraction and the darkly shaded parts represent the total protein of nuclear factor-kappa B (NF-κB), IKKα/β and Ikβα. Data represent mean ± SEM from three independent experiments. Where columns not sharing common letter indicate statistically significant differences among phosphorylated form of proteins ($p < 0.05$). (b) In another similar set of experiment, cells were fixed and immunofluorescence staining for pNF-κB was performed. Florescent microscopy images clearly indicate increased in phosphorylation of NF-κB at 24 h time point in JEV-infected BV-2 cells as compared to mock infected cells while knockdown of miR-29b significantly decreased the phosphorylation. Scale bar: 20 μm.

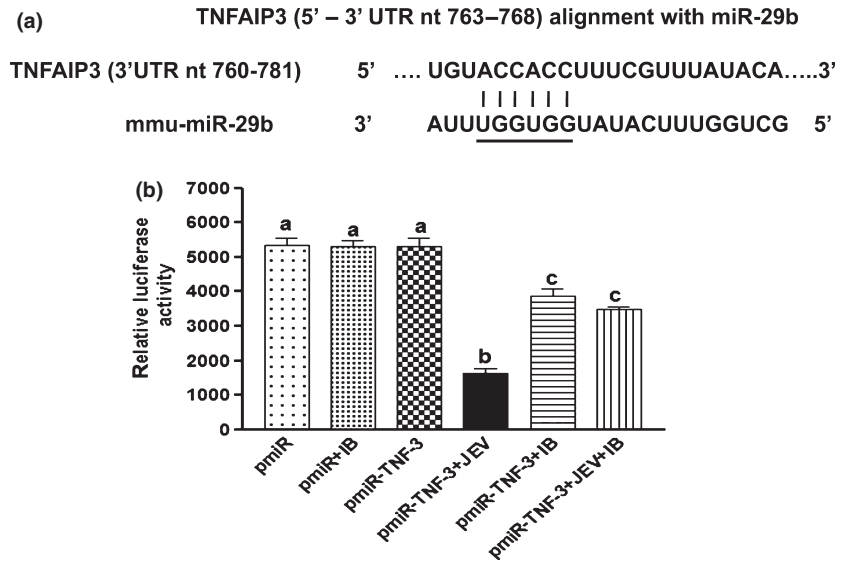

Fig. 6 miR-29b targets the 3′-UTR tumor necrosis factor α-induced protein 3 (TNFAIP3). (a) Seed sequence in miR-29b and complementary sequence in the 3′ UTR of TNFAIP3 mRNA showing six-mer binding in TNFAIP3 3′ UTR. (b) BV-2 cells were transfected with luciferase reporter construct pMIR-REPORT 3 UTR/TNFAIP3 and/or co-transfected with anti-miR-29b oligo for 24 h and were followed by Japanese encephalitis virus (JEV) infection for 24 h. The luciferase reporter assay showed that TNFAIP3 was a direct target of miR-29b, with luciferase activity of the reporter containing 3′-UTR of TNFAIP3 treated with JEV showed significant decreased when compared with pMIR-REPORT 3 UTR/TNFAIP3 alone. While inhibition of miR-29b expression with or without JEV infection showed significant increase in the luciferase activity as compared to pMIR-REPORT 3 UTR/TNFAIP3 treated with JEV group. Data represent mean ± SEM from three independent experiments performed in duplicate. Data represent mean ± SEM from three independent experiments. Where columns not sharing common letter indicate statistically significant differences ($p < 0.05$). pmiR represents pMIR-REPORT, pmiR-TNF-3 represents luciferase reporter construct pMIR-REPORT 3 UTR/TNFAIP3 and IB represents anti-miR-29b oligo.

neuronal death under JEV infection. Consistent with previous reports from our laboratory, JEV infection significantly elevated expression of TNF-α, IL1β, IL-6, and MCP-1 in BV-2 cells. However, inhibition of miR-29b minimized JEV-induced elevation of these inflammatory mediators indicating potential role of miR-29b in regulating microglia activation.

NF-κB is a key regulator of inflammatory responses in various cell types (Tak and Firestein 2001; Yamamoto and Gaynor 2001). Recently, extensive details on the mechanisms that control the dynamics of NF-κB activation have been published by various research groups (Oeckinghaus *et al.* 2011; Lai *et al.* 2013). In this context, TNFAIP3 has been described as a key player in the termination of NF-κB signaling. TNFAIP3 blocks the interactions between E3 ligases and E2 ubiquitin conjugating enzymes, thus inhibiting the "positive" K63-linked ubiquitination, while promoting K48-linked ubiquitination and proteasome-dependent degradation (Vereecke *et al.* 2009; Hymowitz and Wertz 2010; Verstrepen *et al.* 2010). Under various neuroinflammatory conditions, activation of NF-κB and subsequent inflammatory response has been demonstrated in microglial cells (Dalal *et al.* 2012). Under JEV infection, inflammatory response of microglia cells is mediated by NF-κB activation (Chen *et al.* 2011). Furthermore, molecules such as IKK and IαBα tightly regulate NF-κB activity. It is known that upon stimulation by various ligands, IαBα is phosphorylated and subsequently polyubiquitinated and degraded by proteasome, releasing NF-κB, which then accumulates in the nucleus and activates transcription of its target genes (Israël 2010). In most cell types, basal TNFAIP3 expression is very low but its transcription is rapidly induced upon NF-κB activation (Vereecke *et al.* 2009). Recently, Lai *et al.* 2013 demonstrated that transcription of TNFAIP3 is regulated by both NF-kB and p38-dependent C/EBPb in LPS treated macrophages (Lai *et al.* 2013). Once expressed, TNFAIP3 functions as a negative feedback regulator of NF-κB activation. Our immunocytochemical studies clearly demonstrated phosphorylation of NF-κB in JEV-infected cells along with lower expression of TNFAIP3 compared to anti-miR-29b transfected JEV-infected BV-2 cells. To evaluate activation of NF-κB, we estimated expression of pNF-κB, p-IKKαβ and pIkβα proteins in anti-miR-29b transfected JEV-infected BV-2 cells. In agreement with immunocytochemical staining results, we observed decreased expression of pNF-κB and p-IKKαβ. However, pIkβα showed non-significant change when compared to JEV treated condition. This observation

indicates that miR-29b mediated regulation of NF-κB activity possibly be regulated by p-IKKαβ only. Hence, it is hypothesized that miR-29b produced in response to JEV infection degrades TNFAIP3 RNA and in turn prevents the normal negative feedback control that would serve to shut-off NF-κB signaling.

In a study by Bandyopadhyay *et al.* 2011; miR-29b over-expression had been shown to reduce Hepatitis C viral abundance *in vitro* (Bandyopadhyay *et al.* 2011). Similarly, miR-29b had been shown to directly inhibit simian immunodeficiency virus replication (Sisk *et al.* 2013). Hence, we were interested in evaluating whether presently observed positive effects with anti-miR-29b transfection are because of suppressed viral replication or not. Therefore, we measured viral loads (GP78 mRNA levels) in JEV-infected BV-2 cells transfected with either anti-miR-29b or miR-29b mimic. Interestingly, we observed non-significant changes in viral load after transfection with anti-miR-29b or miR-29b mimic compared to JEV alone (Figure S1c). This result clearly indicates that in BV-2 cells, transfection of miR-29b/miR-29b mimic does not alter viral replication. Therefore, presently observed reduction in the JEV-induced inflammatory changes by anti-miR-29b transfection cannot be because of suppressed JEV replication.

To conclude, the results clearly demonstrate pro-inflammatory role of miR-29b during JEV-induced microglial activation. The same is mediated via inhibition of anti-inflammatory protein, TNFAIP3, resulting in sustained activation of NF-κB, and subsequent secretion of pro-inflammatory cytokines. JEV-induced neuroinflammation and pathological changes involve regulation of multiple events occurring in the brain, and in this study, we identified that a key event, microglial activation is regulated by miR-29b. Therefore, further studies may venture to assess the effects of other miRNAs in regulating overall inflammation under JEV infection.

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Competing interests

The authors declare that they have no competing interests with respect to the authorship and/or publication of the article.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Efficacy of anti-miR-29b and miR-29b mimic transfection.

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