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ARTICLEInterleukin-1 $\beta$  orchestrates underlying  
inflammatory responses in microglia via Krüppel-  
like factor 4Deepak K. Kaushik, Menaka C. Thounaojam, Kanhaiya L. Kumawat,  
Malvika Gupta and Anirban Basu*National Brain Research Centre, Manesar, Haryana, India***Abstract**

Microglia are the resident macrophages of the CNS, which secrete several pro- and anti-inflammatory cyto-chemokines including interleukin-1 $\beta$  (IL-1 $\beta$ ), in response to pathogenic stimuli. Once secreted, IL-1 $\beta$  binds to IL-1 receptor present on microglia and initiates the production of inflammatory cytokines in microglia. However, the detailed information regarding the molecular mechanisms of IL-1 $\beta$  triggered inflammatory pathways in microglia is lacking. Our studies focused on the role of Krüppel-like factor 4 (Klf4) in mediating the regulation of pro-inflammatory gene expression upon IL-1 $\beta$  stimulation in

microglia. Our studies show that stimulation of microglia with IL-1 $\beta$  robustly induces Klf4 via PI3K/Akt pathway which positively regulates the production of endogenous IL-1 $\beta$  as well as other pro-inflammatory markers, cyclooxygenase-2, monocyte chemoattractant protein-1 and interleukin-6 (IL-6). In addition, we report that Klf4 negatively regulates the expression of inducible nitric oxide synthase, thereby playing a key role in regulating the immunomodulatory activities of microglia.

**Keywords:** brain, cytokines, iNOS, Krüppel-like factor 4, neuroinflammation.

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Interleukin-1 $\beta$  (IL-1 $\beta$ ) is a prominent pro-inflammatory cytokine, which is expressed at low levels in healthy CNS, but increases rapidly early in response to stress or pathogenic invasion of the CNS (Rothwell and Luheshi 2000). IL-1 $\beta$  is implicated in the progression of chronic neurodegenerative diseases including Alzheimer's disease, and Parkinson's disease as well as acute neuroinflammatory conditions such as traumatic brain injury and stroke (Meda *et al.* 1999; Rothwell and Luheshi 2000; Halle *et al.* 2008). In addition, circulating IL-1 $\beta$  can up-regulate the production of prostaglandins and other toxic mediators in the CNS and is therefore considered as master regulator of neuroinflammation (Saper and Breder 1992; Basu *et al.* 2004). Microglia and astrocytes are the primary source of IL-1 $\beta$  in the CNS (Davies *et al.* 1999; Toda *et al.* 2002), and once secreted, it can further stimulate its own production in an autocrine/paracrine fashion by binding to its cognate IL-1 receptors (IL-1Rs), primarily IL-1RI, as membrane bound receptor that belongs to the IL-1 superfamily (McMahan *et al.* 1991; Rothwell and Luheshi 2000; Toda *et al.* 2002). This ensures a constitutive production of IL-1 $\beta$  by activated microglia which further amplifies injury signals (Basu *et al.* 2004). However, the mechanisms of endogenous IL-1 $\beta$  production in microglia in response to

IL-1 $\beta$  are complex and not fully understood. Also, the production of pro-inflammatory cytokines and pro-inflammatory enzymes including cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS) by microglia in response to IL-1 $\beta$  has been long debated and existing literature presents contradictory evidences (Basu *et al.* 2002; Pinteaux *et al.* 2002). Therefore, this study was undertaken to investigate the detailed signatures of IL-1 $\beta$  induced microglial activation.

Previous studies from our lab have shown that microglia up-regulate the production of Krüppel like factor 4 (Klf4), a zinc finger transcription factor, in response to lipopolysaccharide (LPS) (Kaushik *et al.* 2010). In response to LPS,

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*Abbreviations used:* CBA, cytokine bead array; CNS, central nervous system; Cox-2, cyclooxygenase-2; DAPI, 4,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; IL-1RI, type I IL-1 receptor; IL, interleukin; iNOS, inducible nitric oxide synthase; Klf4, Krüppel like factor-4; LPS, lipopolysaccharide; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PI3K, phosphoinositide 3-kinase.

Klf4 co-activates iNOS promoter by potentially interacting with nuclear factor- $\kappa$ B (NF- $\kappa$ B) (p65) in macrophages and microglia (Feinberg *et al.* 2005; Kaushik *et al.* 2010). Effect of IL-1 $\beta$  has also been studied on the expression of Klf4 in rat cardiac myocytes where it was reported that IL-1 $\beta$  regulates Klf2, but it had no effects on the expression of Klf4 (Cullingford *et al.* 2008). However, literature is lacking on the role of Klf4 in IL-1 $\beta$  mediated microglial activation. Therefore we focused on IL-1 $\beta$  mediated Klf4 production in murine microglia and sought to understand the role of Klf4 on downstream production of key inflammatory markers. Although, a myeloid specific Klf4 knockdown model has been described recently (Liao *et al.* 2011), there are no known commercially available Klf4 genetic knockout models as Klf4<sup>-/-</sup> mice do not survive and die shortly after birth because of defect in epithelial differentiation and barrier formation (Segre *et al.* 1999; Katz *et al.* 2002). Therefore, Klf4 knockdown and other transfection studies are carried out mostly in *in vitro* models of inflammation.

## Materials and methods

### Cell culture and reagents

Mouse microglial cell line BV-2 was gifted by Dr Steve Levison, University of Medicine and Dentistry, New Jersey, USA and maintained as described previously (Kaushik *et al.* 2010). Primary microglia were prepared from primary mixed glial cell cultures that were isolated from P0-P2 mouse brains as described in our previous studies (Kaushik *et al.* 2012). Pathway specific inhibitors, U0126 [Extracellular signal-regulated kinase (ERK) inhibitor], SB203580 (p38 pathway inhibitor), SP600125 (JNK inhibitor), and LY294002 (PI3K/Akt pathway inhibitors) were procured from Calbiochem (Millipore, Billerica, MA, USA) and used at a final concentration of 10  $\mu$ M for 30 min prior to IL-1 $\beta$  treatment. Mouse recombinant IL-1 $\beta$  (R&D Systems, Minneapolis, MN, USA) was used at a final concentration of 5 ng/mL for different time points in accordance with the current literature (Basu *et al.* 2002).

### Animal experiments

Six- to eight-week-old BALB/c mice of either sex were injected intraperitoneally (i.p.) with 100  $\mu$ L of 10 ng/g body weight of IL-1 $\beta$  dissolved in 1X phosphate-buffered saline (PBS) every 24 h for different durations as described elsewhere (Ogimoto *et al.* 2006). Mock-treatment group received the same volume of the carrier (1X PBS). Groups of three mice were sacrificed at each time point either for tissue or protein. 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 vide protocol no. NBRC/IAEC/2013/77 and NBRC/IAEC/2011/62. All the animal research was done in compliance with the ARRIVE guidelines.

### Immunoblotting

Mock-treated and IL-1 $\beta$  treated BV-2 cells were harvested for the isolation of total, cytoplasmic and nuclear extracts as described previously (Kaushik *et al.* 2010). Whole brain homogenates from

IL-1 $\beta$  and mock-treated mice were prepared for total protein for *in vivo* studies. The proteins were subjected to western blot analysis to measure their levels and primary antibodies against Klf4 (1 : 500), iNOS and Cox-2 (1 : 1000; Millipore); pNF- $\kappa$ B (Ser536), pAkt (Ser473), total Akt, pERK (Thr202/Tyr204), total ERK (1 : 1000; Cell Signaling technology, Beverly, MA, USA), total NF- $\kappa$ B (1 : 1000; Santa Cruz biotechnology, Santa Cruz, CA, USA) and IL-1 $\beta$  (1 : 500; R&D Systems) were used. The images were captured using the Genesnap software from Syngene (Syngene, Cambridge, UK). The blots were stripped and re-probed either with anti  $\beta$ -actin (1 : 10 000; Sigma Aldrich, St. Louis, MO, USA) or C23 (nucleolin) antibodies (1 : 1000; Santa Cruz biotechnology) and the protein levels were normalized to  $\beta$ -actin or C23.

### Immunocytochemistry

Primary mouse microglia cells were stained with rabbit anti-Klf4 antibody (1 : 250; Millipore) overnight at 4°C. After PBS washes, the anti-rabbit fluorescein isothiocyanate (FITC) conjugated secondary antibody (1 : 250; Vector Laboratories, Burlingame, CA, USA) was added for 1.5 h and then mounted with mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories). Images were captured using Zeiss apotome microscope (Carl Zeiss MicroImaging GmbH, Göttingen Germany; magnification  $\times$ 20; Scale bar 20  $\mu$ m).

### Immunohistochemistry

IL-1 $\beta$  treated and age-matched mock-treated BALB/c mice were perfused, and the brains were fixed and processed for cryostat sectioning as described elsewhere (Kaushik *et al.* 2010). Anti-Iba1 antibody (1 : 250; cat. MABN92; Millipore) and rabbit anti-Klf4 (1 : 100; Millipore) were used for double labeling of microglia. Similarly, astrocytes and neurons were also double labeled with Klf4 along with mouse anti-gial fibrillary acidic protein (GFAP) (1 : 200; Millipore), and mouse anti- $\beta$ III-tubulin antibody (1 : 200; Cell signaling technologies), respectively. After five washes with 1X PBS, the sections were incubated with anti-mouse Alexa Fluor 594 (1 : 1000; Molecular Probes, Eugene, OR, USA) for Iba1, GFAP and  $\beta$ III-tubulin and anti-rabbit FITC (1 : 250; Vector Laboratories) for Klf4 for additional 1.5 h. The slides were then mounted with medium containing 4,6-diamidino-2-phenylindole (Vector laboratories). Images were captured using Zeiss apotome fluorescence microscope (Zeiss, Germany; magnification  $\times$ 40; Scale bar 50  $\mu$ m).

### Quantitative real-time PCR (qRT-PCR)

qRT-PCR using PowerSYBR Green (Applied Biosystems, Foster city, CA, USA) was performed on ABI Prism 7500 sequence detection system (Applied Biosystems) as described previously (Kaushik *et al.* 2012). Oligonucleotide primers specific for different genes were procured from Sigma (Sigma Aldrich) and listed in Table S1. The conditions used for real-time PCR were as follows: 95°C for 3 min (1 cycle), 40 cycles of (94°C for 20 s, 60°C for 30 s, and 72°C for 40 s). The results were analyzed using the iCycler Thermal Cycler Software (Applied Biosystems) and normalized with those from 18S rRNA internal control and quantified using comparative C<sub>t</sub> (2<sup>-[ $\Delta$ ] $\Delta$ C<sub>t</sub>) method.</sup>

### Cytokine bead array

The cytokine bead array kit (mouse inflammation cytokine bead array kit; BD Biosciences, Franklin Lakes, NJ, USA) was used to

quantitatively measure cytokine levels in the control and IL-1 $\beta$  treated BV-2 cells. The assay was performed according to the manufacturer's instructions and analyzed on the FACS Calibur (BD Biosciences) as described previously (Ghoshal *et al.* 2007).

#### Site-directed mutagenesis

Site-directed mutagenesis (SDM) was carried out using QuikChange SDM kit procured from Stratagene (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The sequence of double stranded primer designed for site-directed mutagenesis at -212 bp on iNOS promoter in iNOS/pGL2 luciferase construct is as follows: 5'-CTA CGT GCT GCC TAG TGT CCA CTG CCT TGG ACG-3'.

#### Generation of K-10 (pcDNA3.1-Klf4) construct

Oligonucleotide primers were designed to amplify the coding sequence of full length mouse Klf4 cDNA that yielded a 1452 bp (1.4 kb) product. The sequences of the oligonucleotide primers used for cloning is as follows: forward primer: 5'-ATG AGG CAG CCA CCT GGC GA-3'; reverse primer: 5'-TTA AAA GTG CCT CTT CAT GTG TAA-3'. The amplified product was inserted into the pcDNA3.1/V5 His TopoTA expression vector (kindly gifted by Dr Nihar Ranjan Jana, National Brain Research Centre, Manesar, Haryana, India) and ligated to generate pcDNA3.1-Klf4 (K-10) construct. The construct was then sequenced commercially (ILS Bioservices, Manesar, Haryana, India).

#### Transfection studies

Short interfering RNA (siRNA) against mouse Klf4, siKlf401 (sense: 5'-CGG AGU UGG ACC CAG UAU A-3') was procured from Sigma Aldrich, siKlf402 (sense: 5'-UCC AAA GAA GAA GGA UCU CUU-3') and scrambled siRNA (scRNA) (sense: 5'-GUG CAC AUG AGU GAG AU UU-3') were procured from Dharmacon RNAi technologies (Thermo fisher Scientific, Waltham, MA, USA). 100 nM of siRNA and scRNA were used for transfection using lipofectamine RNAi max (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For over-expression studies, BV-2 cells were transfected with K-10 (pcDNA3.1-Klf4) and pcDNA3.1-LacZ constructs using lipofectamine 2000 (Invitrogen) as described previously (Kaushik *et al.* 2012).

#### Luciferase assay

Luciferase reporter gene constructs, iNOS/pGL2 (kindly provided by Dr Mark W. Feinberg and Dr Mukesh K. Jain, Harvard Medical School, Boston, Massachusetts, USA; originally constructed by Dr Mark Perrella) (Perrella *et al.* 1996), -212 $\Delta$ iNOS/pGL2 (SDM mutated iNOS/pGL2) and pCOX301 (a kind gift from Dr Manikuntala Kundu, Bose Institute, Kolkata, India) (Pathak *et al.* 2004), were used for promoter binding assays. After 24 h of transfection of BV-2 cells with either Klf4-SiRNA or K-10 construct (pcDNA3.1-Klf4), 1  $\mu$ g each of luciferase reporter constructs were transfected using 5  $\mu$ L of lipofectamine 2000 (Invitrogen) in Opti-MEM for additional 24 h before IL-1 $\beta$  treatment.

#### Chromatin immunoprecipitation (ChIP)

ChIP was performed using Chip-IT express kit according to manufacturer's protocol (Active motif, Carlsbad, CA, USA) as described earlier (Caretta *et al.* 2003). Sheared chromatin was incubated with 2  $\mu$ g of mouse anti-Klf4 Transcruze antibody (Santa

Cruz biotechnology) or 2  $\mu$ g of rabbit IgG (Abcam, Cambridge, MA, USA). Input (1 : 10 dilution) and ChIP DNA were analyzed using a thermocycler (Applied biosystems) with the following program: 94°C for 3 min; 36 cycles of (94°C for 20 s, 59°C for 30 s and 72°C for 30 s); hold cycle at 10°C. DEPC H<sub>2</sub>O was added to water only control. The primers on IL-1 $\beta$  promoter used for ChIP reactions which generated 111 bp amplicon (-99 bp to +10 bp) are as follows: forward: 5'- TCA GTT TTG TTG TGA AAT CAG T - 3', reverse: 5'- CAG GGT TTG TTG TCC AAC TT- 3'.

#### Statistical analysis

All the experiments were performed in sets of three unless otherwise mentioned and the data generated were analyzed statistically by paired two-tailed Student's *t*-test. A statistical *p* value < 0.01 and 0.05 were considered significant.

## Results

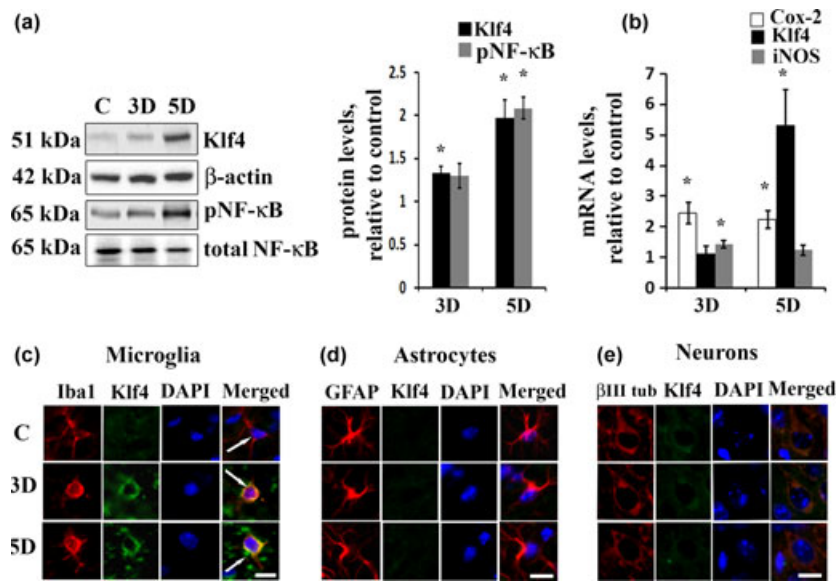
### IL-1 $\beta$ induces neuroinflammation and Klf4 up-regulation in microglia *in vivo*

To find out if IL-1 $\beta$  increased the levels of Klf4 in murine brains, we performed immunoblotting of the brain homogenates from cytokine treated and mock-treated animals. We observed a significant increase in the levels of Klf4 by about 1.5-fold and 2-fold after 3 and 5 days of IL-1 $\beta$  injections, respectively as compared to mock-treated mice brain (Fig. 1a). We also observed twofold increase in the phosphorylation of NF- $\kappa$ B in the brains of mice after 5 days of IL-1 $\beta$  injection (Fig. 1a). In addition, qRT-PCR analysis of Klf4 showed a significant increase in the transcripts of Klf4, iNOS and Cox-2 gene within 5 days of IL-1 $\beta$  treatment as compared to mock-treated group (Fig. 1b).

Furthermore, using double-immunostaining, we observed that within 3 days after injection of the cytokine, microglial cells exhibited a transformation from 'resting' state, with basal levels of Iba1 expression (control, upper panel; arrow) to an 'activated' (ameboid) state with increased Iba1 expression (lower panels, 3 day and 5 day treatment groups; arrows) (Fig. 1c; Figure S1a). In addition, expression of Klf4 increased significantly after 3 and 5 days of IL-1 $\beta$  injection in these cells as witnessed by co-localization of Klf4 (green) with Iba1 (red) (merged images; Fig. 1c; Figure S1a). Furthermore, we observed that while astrocytes (GFAP +ve cells) did not express Klf4 (Fig. 1d; Figure S1b), neurons ( $\beta$ III-tubulin +ve cells) expressed Klf4 at basal levels (Fig. 1e; Figure S1c). However, no changes in the levels of Klf4 were observed either in astrocytes or neurons even after 5 days of IL-1 $\beta$  injection. Overall, our findings show for the first time that IL-1 $\beta$  stimulates microglial activation along with an up-regulation of Klf4 in microglia.

### IL-1 $\beta$ stimulates Klf4 up-regulation and nuclear translocation in microglia *in vitro*

In primary microglia, we observed that with respect to mock-treated cells (upper panel), Klf4 staining intensity increased



**Fig. 1** IL-1 $\beta$  induces Krüppel like factor-4 (Klf4) expression in mice brain. 6- to 8-week-old BALB/c mice were injected with 10 ng/kg body weight of IL-1 $\beta$  i.p. for different days. (a) Immunoblot analysis of Klf4 and pNF- $\kappa$ B from mice brains after 3 and 5 days of IL-1 $\beta$  treatment. Graph represents fold change in the levels of Klf4 and pNF- $\kappa$ B with respect to control. The levels of pNF- $\kappa$ B were normalized to total NF- $\kappa$ B and Klf4 levels were normalized to  $\beta$ -actin. (b) qRT-PCR analysis of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox-2) and Klf4 transcript levels relative to mock-treated condition after different

time durations. Values were normalized to 18S rRNA levels. (c) Double staining with anti-Klf4 (green) and anti-Iba1 (red) antibody. (d and e) Double staining for the expression of Klf4 (green) in (d) GFAP +ve (red) astrocytes and (e)  $\beta$ III-tubulin +ve (red) neurons. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (blue) and images were captured using Zeiss apotome fluorescence microscope (Scale bar-50  $\mu$ m; magnification-40 $\times$ ). Data represent mean  $\pm$  SEM from three animals in each group. \*Statistical difference in comparison to mock-treated condition ( $p < 0.05$ ).

within 1 h of IL-1 $\beta$  treatment, and that it is localized within the nuclei as shown by the arrows (lower panel; FITC, green) (Fig. 2a). We also carried out time-dependent stimulation of BV-2 microglia with IL-1 $\beta$  and found that it resulted in increased expression of total Klf4 protein within 1 h of stimulation (Fig. 2b). In addition, we measured the levels of Klf4 from cytoplasmic/nuclear extractions isolated from BV-2 microglia that were stimulated with IL-1 $\beta$  for different durations of 5, 15, 30, 45 or 60 min. Expression of Klf4 increased gradually in the cytoplasmic extracts after 30 min of IL-1 $\beta$  stimulation and reached about 2-fold with respect to untreated control condition after 60 min of treatment (Fig. 2c; CE). However, Klf4 translocated to nuclei as early as 5 min and remained at significantly higher levels after 15 min of IL-1 $\beta$  stimulation (Fig. 2c; NE). We also observed a considerable twofold increase in phosphorylation of NF- $\kappa$ B within 1 h of IL-1 $\beta$  stimulation of BV-2 cells (Fig. 2d).

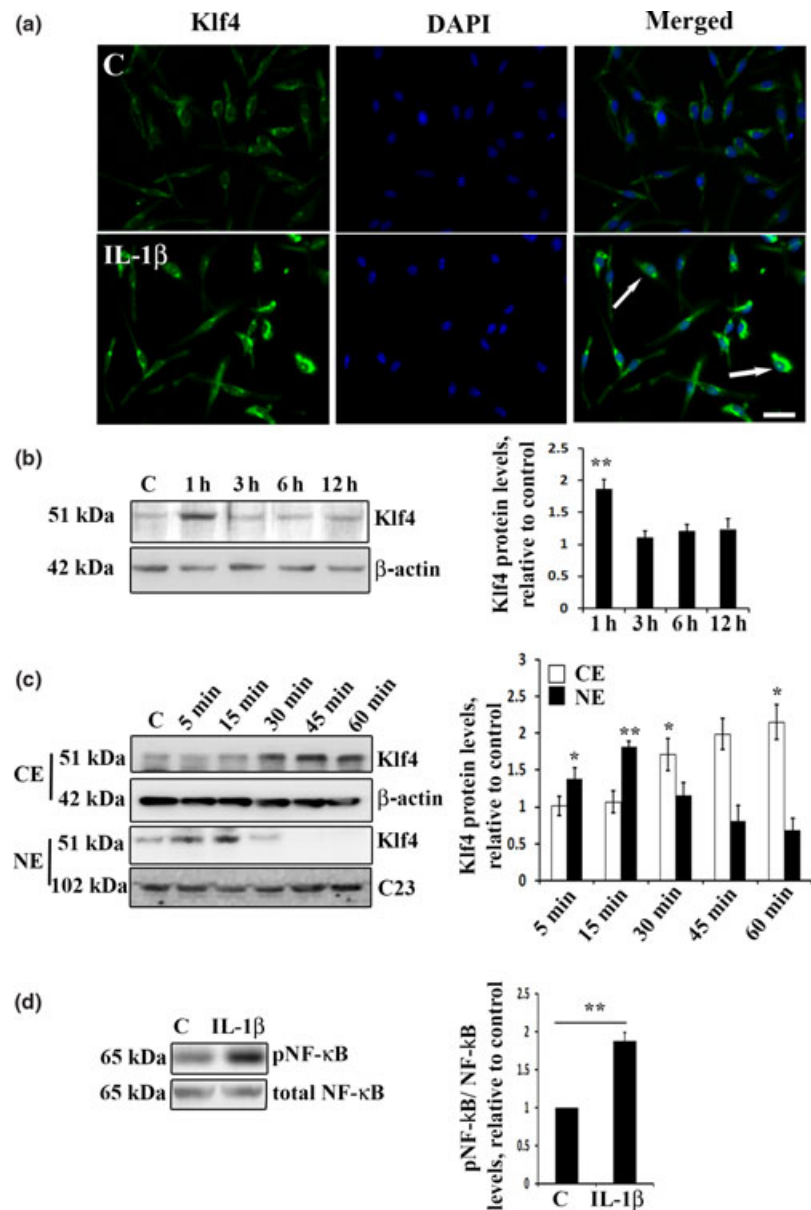
#### IL-1 $\beta$ induces the phosphorylation of ERK and Akt, and PI3K/Akt pathway plays a crucial role in determining Klf4 expression

Although there are reports of IL-1 $\beta$  induced ERK activation in mixed glial cells (McNamee *et al.* 2010), there is conflicting information on the role of interleukin-1 in

mediating the activation of ERK or PI3K/Akt pathways in microglia. However, we observed significant phosphorylation of both Akt and ERK pathways in IL-1 $\beta$  stimulated microglia (Fig. 3a and b). Also, we found that within 45 min of IL-1 $\beta$  stimulation, the levels of ERK decreased significantly which may be because of the negative feedback by several mechanisms including direct activation of MAPK phosphatases by p42 and p44 MAPK (Brondello *et al.* 1999; Peng *et al.* 2010). The levels of ERK phosphorylation were again found to be increased after 60 min of IL-1 $\beta$  stimulation. Similar observations regarding ERK phosphorylation were made by another study on BV-2 cells in response to a mixture of different cytokines (Sheng *et al.* 2011).

Furthermore, to find out if either of these pathways is crucial for Klf4 up-regulation in IL-1 $\beta$  mediated microglial activation, we incubated microglia with specific inhibitors of ERK or PI3K/Akt pathways for 30 min prior to 1 h of IL-1 $\beta$  stimulation. The specificity of inhibition was confirmed by significant decrease in phosphorylation of ERK and Akt in unstimulated and IL-1 $\beta$  stimulated cells in presence of ERK (U0126) and PI3K/Akt (LY294002) inhibitors, respectively (Figure S2). Interestingly, we observed a significant up-regulation of Akt phosphorylation upon ERK inhibition in both unstimulated and IL-1 $\beta$  stimulated conditions. This

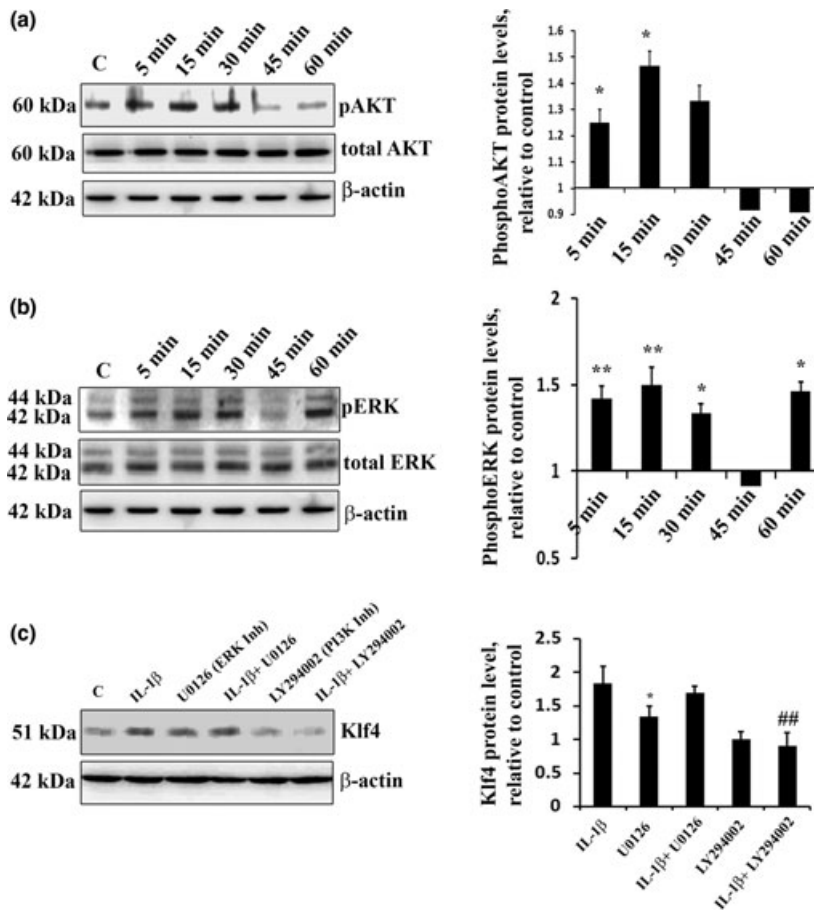
**Fig. 2** IL-1 $\beta$  up-regulates the production of total Krüppel like factor-4 (Klf4) and increases its nuclear translocation *in vitro*. Primary microglia and BV-2 murine microglia cells were treated with IL-1 $\beta$  at a concentration of 5 ng/mL for different durations. (a) Images showing the staining for Klf4 [fluorescein isothiocyanate(FITC); green], 4,6-diamidino-2-phenylindole (DAPI) (blue) and merged images (Klf4 + DAPI). Klf4 expression increases in IL-1 $\beta$  treated primary microglia as compared to untreated control cells as shown by the arrows. Images were captured using Zeiss apotome fluorescence microscope (Scale bar-20  $\mu$ m; magnification-20X). (b) Immunoblotting for Klf4 from total protein isolated from BV-2 cells treated with IL-1 $\beta$ . (c) Immunoblotting for cytoplasmic (CE) and nuclear (NE) Klf4 from BV-2 cells treated with IL-1 $\beta$ . (d) Immunoblot analysis of pNF- $\kappa$ B after 1 h of IL-1 $\beta$  treatment. Klf4 protein levels were normalized to  $\beta$ -actin and C23; pNF- $\kappa$ B levels were normalized to total NF- $\kappa$ B levels. Klf4 mRNA was normalized to 18S rRNA. The graphs represent fold change in normalized Klf4 mRNA and protein levels at various time points with respect to mock-treated cells. Data represent mean  $\pm$  SEM from three independent experiments. \*, \*\* Statistical differences in comparison to mock-treated condition (\* $p$  < 0.05; \*\* $p$  < 0.01).



phenomenon has already been reported in a number of studies which suggest that inhibition of ERK leads to the phosphorylation of Akt at Ser473 by a possible feedback regulation in different types of cancer cells and macrophages (Luyendyk *et al.* 2008; Chen *et al.* 2012; Turke *et al.* 2012). We observed that IL-1 $\beta$  failed to increase Klf4 levels in cells pre-treated with LY294002, as compared to cells treated with IL-1 $\beta$  alone, highlighting the role of PI3K/Akt signaling in IL-1 $\beta$  mediated Klf4 up-regulation (Fig. 3c). In contrast, ERK inhibition by U0126 did not lead to any significant changes in Klf4 levels in response to IL-1 $\beta$  stimulation. We also observed a modest increase in Klf4 levels in control cells treated with U0126, which might be a consequence of enhanced Akt signaling with ERK inhibition.

#### Klf4 is crucial for Cox-2 up-regulation in IL-1 $\beta$ stimulated microglia

We also measured a significant increase in Cox-2 expression in response to IL-1 $\beta$  *in vitro* (Fig. 4a and b). To study if Klf4 regulated the production of Cox-2, we carried out knock-down of Klf4. To assess transfection efficiency and specificity of knockdown, we used two different siRNAs, siKlf401 and siKlf402 that targeted Klf4 transcript at different regions, and carried out qRT-PCR for Klf4 as well as for Klf2, a closely related transcription factor. We observed that while siKlf401 offered more than 50% knockdown, siKlf402 showed about 35% knockdown of Klf4 mRNA in unstimulated as well as IL-1 $\beta$  stimulated BV-2 cells. Also, none of the siRNAs showed any off-target



**Fig. 3** PI3K/Akt pathway is crucial for the expression of Krüppel like factor-4 (Klf4) in IL-1 $\beta$  stimulated microglia. (a) Immunoblot analysis of phospho and total Akt upon IL-1 $\beta$  stimulation. Graph represents fold change in pAkt/Akt levels over mock-treated cells. (b) Immunoblot analysis of phospho and total ERK after IL-1 $\beta$  treatment. Graph represents fold change in pERK/ERK levels over mock-treated condition. Values for pERK and pAkt were normalized with total ERK and total Akt, respectively. (c) Immunoblot analysis of Klf4 from BV-2 cells incubated for 30 mins with pathway specific inhibitors (U0126, ERK inh; LY294002, PI3K inh), prior to 1 h of treatment with IL-1 $\beta$ . Graph represents fold change in Klf4 protein levels with respect to mock-treated cells. Klf4 values were normalized to  $\beta$ -actin; Data represent mean  $\pm$  SEM from three independent experiments. \*, \*\* Statistical differences in comparison to mock-treated condition (\* $p$  < 0.05; \*\* $p$  < 0.01); ## statistical difference with respect to IL-1 $\beta$  treated condition (## $p$  < 0.01).

effects as measured by the levels of Klf2, which remained unaltered (Fig. 4c). Therefore, we used siKlf401 (hereafter referred to as 'siKlf4') for the rest of the experiments, owing to its better knockdown efficiency over siKlf402. We found that siRNA mediated knockdown of Klf4 resulted in significant reduction in Cox-2 protein levels in IL-1 $\beta$  stimulated BV-2 microglia suggesting that Klf4 is crucial for Cox-2 production (Fig. 4d).

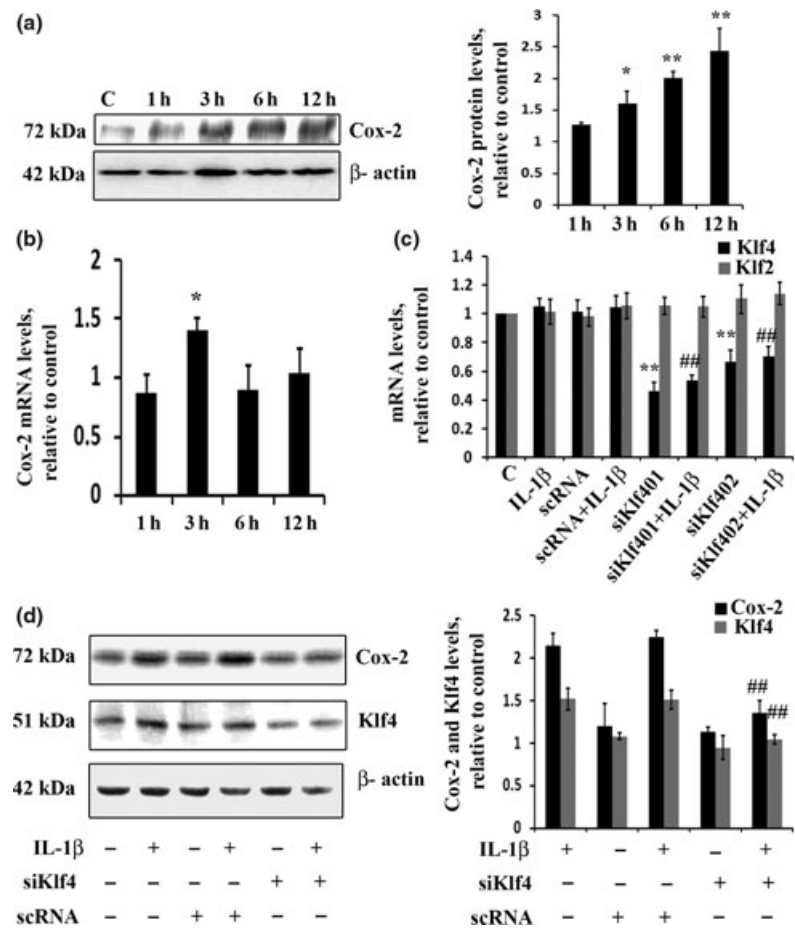
#### Klf4 negatively regulates iNOS expression in microglia

We then wanted to find if IL-1 $\beta$  up-regulated iNOS, and whether Klf4 was responsible for its up-regulation. In contrast to many existing studies (Tsujino *et al.* 1994; Wong *et al.* 1996; Ravichandran *et al.* 2011), we found that in response to IL-1 $\beta$ , iNOS protein levels were not altered when compared with mock-treated condition (Fig. 5a). Furthermore, there were no changes in iNOS mRNA levels even after 12 h of IL-1 $\beta$  treatment in microglia *in vitro* (Fig. 5b), suggesting that transcriptional regulation of iNOS is lacking. We then analyzed iNOS mRNA and protein levels upon Klf4 knockdown in BV-2 cells after IL-1 $\beta$  treatment. Interestingly, we observed that both iNOS transcript and protein levels were significantly up-regulated in siKlf4 alone as well

as in siKlf4 + IL-1 $\beta$  treatment condition with respect to IL-1 $\beta$  alone condition (Fig. 5c and d), suggesting that Klf4 is highly critical for regulating both, basal expression of iNOS as well as its levels in response to IL-1 $\beta$ .

To further validate the role of Klf4 in down-regulation of iNOS, we transfected BV-2 cells with K-10 construct which over-expressed Klf4 (Klf4-pcDNA3.1). We also transfected the cells with LacZ construct (pcDNA3.1), which was used as a control. We observed that Klf4 over-expression was associated with a significant decrease in iNOS protein levels in both, mock-treated cells and in response to IL-1 $\beta$  treatment as compared to LacZ + IL-1 $\beta$  condition (Fig. 6a). Furthermore, to assess iNOS promoter activity upon Klf4 over-expression, we carried out transfection with iNOS promoter-luciferase construct (iNOS/pGL2) and co-transfected the cells with either K-10 or LacZ constructs. We observed that upon Klf4 over-expression in unstimulated microglial cells (K-10 alone), iNOS promoter activity was significantly reduced by more than twofold, as measured by decrease in relative luciferase activity with respect to LacZ condition (Fig. 6b). IL-1 $\beta$  stimulation in K-10 transfected cells potentiated this decrease in iNOS promoter activity, further strengthening the role of Klf4 in negative regulation

**Fig. 4** Krüppel like factor-4 (Klf4) is critical for IL-1 $\beta$  mediated induction of cyclooxygenase-2 (Cox-2) *in vitro*. (a) Immunoblot analysis of Cox-2 protein in IL-1 $\beta$  treated and mock-treated conditions. Graph represents fold change in Cox-2 protein levels with respect to control. (b) Graph showing qRT-PCR analysis of Cox-2 mRNA in response to IL-1 $\beta$  treatment and is represented as fold change over control. (c) Graph representing Klf4 and Klf2 mRNA levels showing knockdown efficiency and specificity of different siRNAs (siKlf401 and siKlf402). (d) Immunoblot analysis of Cox-2 and Klf4 expression in IL-1 $\beta$  stimulated and unstimulated BV-2 cells transfected with either 100 nM of siKlf4 (siKlf401) or scRNA. Graph represents fold change in Cox-2 and Klf4 levels over mock-treated condition. Values were normalized to  $\beta$ -actin. Data represent mean  $\pm$  SEM from three independent experiments. \*, \*\* Statistical differences in comparison to mock-treated condition (\* $p$  < 0.05, \*\* $p$  < 0.01); ## statistical difference with respect to IL-1 $\beta$  treated condition (## $p$  < 0.01).



of basal iNOS expression by probably interacting with its promoter element.

#### Klf4 decreases iNOS promoter activity in response to IL-1 $\beta$

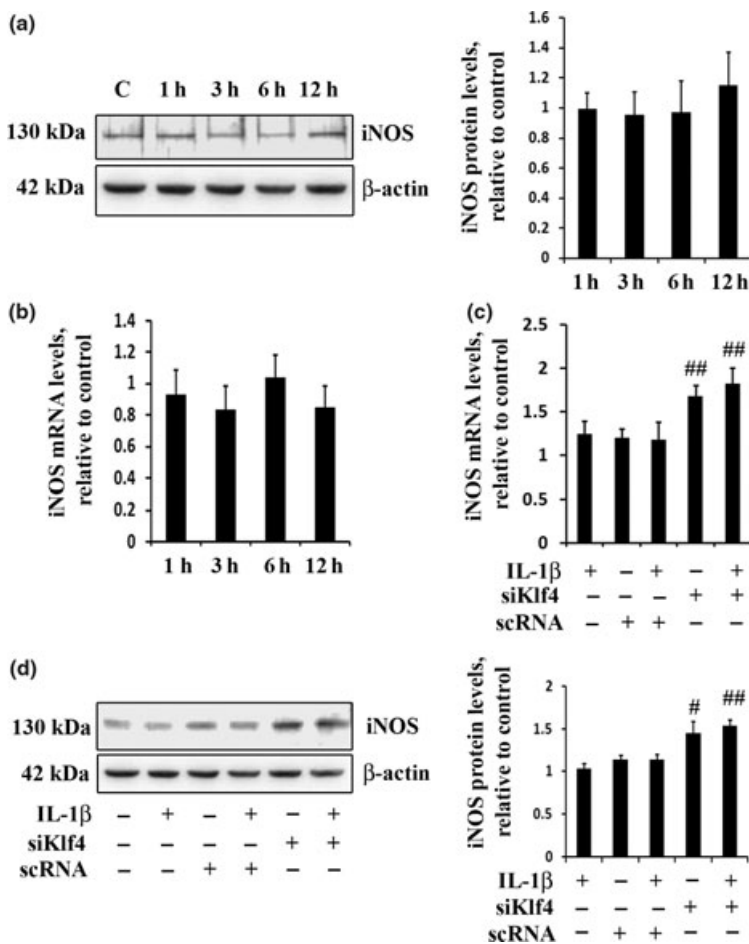
To confirm if direct Klf4 binding was required for down-regulating iNOS promoter activity, we mutated the Klf4 binding site at  $-212$  bp position by carrying out site-directed mutagenesis of iNOS/pGL2 to make a mutated construct,  $-212\Delta$ iNOS/pGL2 and transfected BV-2 microglia using these constructs. We found that while iNOS/pGL2 promoter activity did not increase upon IL-1 $\beta$  stimulation, transfection with  $-212\Delta$ iNOS/pGL2 construct significantly increased the promoter activity in response to 3 h of IL-1 $\beta$  treatment (Fig. 6c). We observed more than twofold increase in iNOS promoter activities in BV-2 cells in  $-212\Delta$ iNOS/pGL2 + IL-1 $\beta$  condition with respect to iNOS/pGL2 transfected cells or iNOS/pGL2 + IL-1 $\beta$  condition. These findings confirmed that Klf4 negatively regulated iNOS promoter activity.

#### IL-1 $\beta$ stimulates the production of MCP-1 and IL-6 via Klf4

We also sought to confirm whether IL-1 $\beta$  up-regulated pro-inflammatory cyto-chemokines in BV-2 murine microglia.

We observed considerable increase in the levels of both, monocyte chemoattractant protein-1 (MCP-1) and IL-6 upon IL-1 $\beta$  stimulation with respect to control untreated conditions (Figure S3a and b). In addition, we observed remarkable decrease in the levels of both these cytokines after 3 h of IL-1 $\beta$  stimulation (Figure S3a and b). These findings suggest that while IL-1 $\beta$  potentially up-regulated both MCP-1 and IL-6 in microglia, this pathway is tightly regulated, especially during the early response phase.

Since our earlier studies showed that Klf4 played an important role in mediating the up-regulation of these cyto-chemokines in response to LPS (Kaushik *et al.* 2010), we were interested in understanding the role of Klf4 in regulating these cytokines in response to IL-1 $\beta$ . We found that after 6 h of IL-1 $\beta$  stimulation, knockdown of Klf4 resulted in a significant decrease in the levels of both these cytokines by approximately 2-fold with respect to IL-1 $\beta$  treatment group (Figure S3c). Similarly, after 12 h of IL-1 $\beta$  stimulation, IL-6 levels were found to be significantly reduced in Klf4 knockdown condition as compared to IL-1 $\beta$  condition (Figure S3d). Also, we observed a significant reduction in the levels of MCP-1 in siKlf4 + IL-1 $\beta$  condition with respect to IL-1 $\beta$  alone condition, as well as in



**Fig. 5** Inducible nitric oxide synthase (iNOS) expression is negatively regulated by Krüppel like factor-4 (Klf4). (a) Immunoblot analysis of iNOS from total cellular extracts of IL-1 $\beta$  treated BV-2 cells at different time points. (b) Graph showing qRT-PCR analysis of iNOS mRNA at different time points in response to IL-1 $\beta$ , and represents fold change over control. (c) qRT-PCR analysis of iNOS transcript levels from IL-1 $\beta$  stimulated microglia in siKlf4 and scRNA transfected and mock-treated conditions. Graph represents fold change in Klf4 mRNA levels in treated conditions over control. (d) iNOS protein levels from IL-1 $\beta$  stimulated microglia in siKlf4 and scRNA transfected and mock-treated conditions. Values were normalized to  $\beta$ -actin. Data represent mean  $\pm$  SEM from three independent experiments. #, ## Statistical difference with respect to IL-1 $\beta$  treated condition (# $p$  < 0.05; ## $p$  < 0.01).

siKlf4-alone condition with respect to mock-treated conditions (Figure S3d). This suggested that Klf4 potentially regulated the basal level expression of pro-inflammatory cytokines and also significantly influenced their up-regulation in response to IL-1 $\beta$ .

#### IL-1 $\beta$ stimulates production of endogenous IL-1 $\beta$ by interaction of Klf4 with IL-1 $\beta$ promoter

The evidences in favor of endogenous IL-1 $\beta$  production in response to IL-1 $\beta$  treatment are contradictory. We observed that while mature IL-1 $\beta$  is increased in total cellular extracts after 6 h of IL-1 $\beta$  treatment, significant amounts of mature IL-1 $\beta$  were detected in supernatants after 1 h of cytokine stimulation which continued to be significant until 6 h after the treatment (Fig. 7a). Similarly, IL-1 $\beta$  mRNA levels were also found to be significantly up-regulated after 1 and 3 h of IL-1 $\beta$  treatment (Fig. 7b). In one of the studies published recently, it was proposed that Klf4 negatively regulated IL-1 $\beta$  production in murine macrophage cell line by directly interacting with its promoter elements (Liu *et al.* 2012). To find out if Klf4 negatively regulated IL-1 $\beta$  expression, we measured IL-1 $\beta$  mRNA in Klf4 knockdown conditions. In contrast, we found that upon Klf4 knockdown, IL-1 $\beta$  mRNA

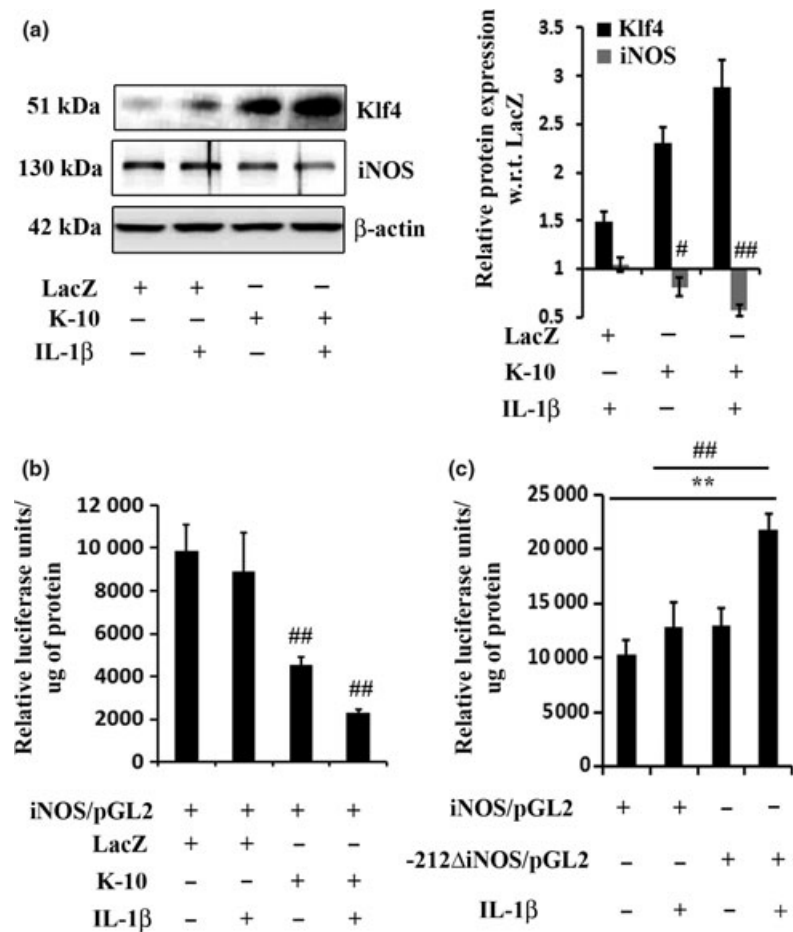
levels reduced significantly over that of external IL-1 $\beta$  treatment (Fig. 7c). Furthermore, ChIP analysis of IL-1 $\beta$  promoter using Klf4 antibody confirmed that Klf4 directly interacted with IL-1 $\beta$  promoter element (Fig. 7d).

#### Discussion

In this study, we investigated the role played by Klf4 in regulating inflammatory processes in microglia upon IL-1 $\beta$  stimulation. We show that microglia and not astrocytes or neurons exhibit increased levels of Klf4 in response to IL-1 $\beta$  *in vivo*. *In vitro* studies have shown that the levels of Klf4 did not increase after 1 h of IL-1 $\beta$  stimulation of microglia, which may define its role in early gene response. Our studies show that Klf4 plays a crucial role for the up-regulation of Cox-2 in microglia in response to IL-1 $\beta$ , an observation similar to our findings from previous studies on LPS. Another interesting finding of our study is the negative regulation of iNOS by Klf4, which is in contrast to studies that have shown that LPS and IL-1 $\beta$  strongly induce iNOS expression in different cell types (Tsujino *et al.* 1994; Kaushik *et al.* 2010). However, according to a study, stimulation of BV-2 microglia with higher doses of IL-1 $\beta$



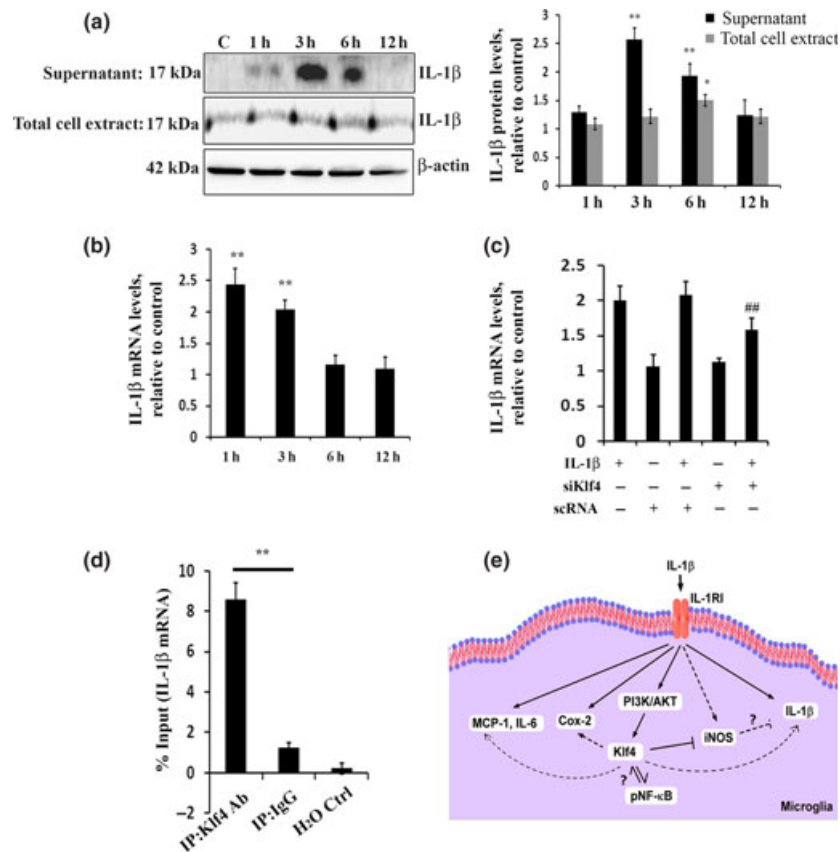
**Fig. 6** Krüppel like factor-4 (Klf4) negatively regulates inducible nitric oxide synthase (iNOS) expression by down-regulating its promoter activity. (a) Immunoblot analysis for the expression levels of iNOS and Klf4 upon Klf4 over-expression in BV-2 cells. Graph represents relative expression of iNOS and Klf4 in different conditions with respect to LacZ alone transfected cells. (b) Luciferase activity measured in iNOS/pGL2 transfected cells co-transfected with either K-10 or LacZ in unstimulated BV-2 cells and after 3 h of IL-1 $\beta$  treatment. (c) Luciferase activity of iNOS promoter from BV-2 and transfected with either iNOS/pGL2 or -212 $\Delta$ iNOS/pGL2 in presence or absence of IL-1 $\beta$ . While protein levels were normalized to  $\beta$ -actin, arbitrary luciferase units were normalized to amount ( $\mu$ g) of protein present in the samples. Data represent mean  $\pm$  SEM from three independent experiments. \*\*, Statistical difference with respect to mock-treated condition (\*\*  $p < 0.01$ ). #, ## Statistical difference with respect to IL-1 $\beta$  treated condition in LacZ transfected cells (\* $p < 0.05$ ; ## $p < 0.01$ ).



was unable to induce iNOS activity as measured by nitric oxide (NO) production even after 24 h of stimulation (Kim *et al.* 2006). Similar observations were made by Ryu *et al.*, who report that IL-1 $\beta$  does not induce iNOS expression and subsequent NO production in macrophages (Ryu *et al.* 2000). In the light of the role played by Klf4 in response to LPS, the current data present contrasting observation and emphasize on the pleiotropic properties of Klf4 as a transcription factor.

Furthermore, the finding that PI3K/Akt pathway is involved in Klf4 mediated iNOS regulation in response to IL-1 $\beta$ , in part, reinforces the immunomodulatory role of PI3K/Akt pathway. Our study also revisited the production of endogenous IL-1 $\beta$  in the light of Klf4 regulation. According to a study on mouse macrophages, NO produced by iNOS activity can down-regulate the endogenous production of IL-1 $\beta$  suggesting that IL-1 $\beta$  gene expression may be controlled in a feedback fashion (Obermeier *et al.* 1999). In support of this finding, we found that during earlier time points in response to IL-1 $\beta$ , endogenous IL-1 $\beta$  levels were robustly elevated in culture supernatants. It is already known that NF- $\kappa$ B plays a significant role in production of pro-IL-1 $\beta$  (Mariathasan and Monack 2007), and since knockdown

of Klf4 resulted in decreased pro-IL-1 $\beta$  mRNA, we hypothesize that Klf4 may be another crucial factor involved in the production of IL-1 $\beta$  by binding to its promoter elements. Furthermore, in accordance with the existing literature, our studies show that the levels MCP-1 and IL-6 were found to be significantly increased in response to IL-1 $\beta$  in BV-2 microglial cells. However, it is interesting to note that the cytokine levels in response to IL-1 $\beta$  are significantly less when compared to LPS stimulated microglia (Kaushik *et al.* 2010). Similar observations were made by Sheng *et al.* in their studies when NO production in BV-2 cells was found to be lower in response to a mixture of Tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  cytokines when compared with LPS + IFN- $\gamma$  treatment condition (Sheng *et al.* 2011), suggesting that cytokines may have immunomodulatory properties. In addition, a role of Klf4 is established in regulating the expression of these cyto-chemokines in response to IL-1 $\beta$ . It has already been shown that Klf4 interacts with p65 in our previous studies in response to LPS (Kaushik *et al.* 2010), therefore, in response to IL-1 $\beta$ , whether the two transcription factors interact with each other, requires further investigation. The schematic representation of our key observations and hypotheses is outlined in Fig. 7e.



**Fig. 7** Endogenous IL-1 $\beta$  production is enhanced and positively regulated by Krüppel like factor-4 (Klf4) in response to IL-1 $\beta$  treatment *in vitro*. (a) Immunoblot analysis of IL-1 $\beta$  protein levels from supernatant (upper panel) and total cellular extracts (lower panel) at different time points. Graph represents normalized densitometry analysis of IL-1 $\beta$  protein levels with respect to mock-treated condition. (b) qRT-PCR analysis of IL-1 $\beta$  transcript levels upon IL-1 $\beta$  treatment of BV-2 cells at each time point. (c) qRT-PCR analysis of IL-1 $\beta$  transcript levels upon siRNA mediated knockdown of Klf4. Graph represents fold change in IL-1 $\beta$  transcript levels in knockdown conditions with respect to mock-

treated condition. (d) qRT-PCR analysis of Klf4 binding to IL-1 $\beta$  promoter, carried out by chromatin immunoprecipitation (ChIP) after 1 h of IL-1 $\beta$  stimulation. Anti-IgG and H<sub>2</sub>O were used as negative controls. Input DNA was used as a positive control for immunoprecipitation and data are represented as % input. Data represent mean  $\pm$  SEM from three independent experiments. \*, \*\* Statistical differences in comparison to either mock-treated condition (\* $p$  < 0.05, \*\* $p$  < 0.01). ## statistical difference with respect to IL-1 $\beta$  treated condition (## $p$  < 0.01). (e) Schematics showing the role of Klf4 in IL-1 $\beta$  stimulated microglia.

In summary, the premise for an inflammatory role of Klf4 in microglia is encouraged by current observations documenting Klf4 up-regulation and subsequent orchestration of inflammatory genes in response to IL-1 $\beta$ . Although, detailed studies are further required to understand the implications underlying our findings, our study reveals a potential molecular target for the development of therapeutic agents for the management of inflammatory conditions of CNS.

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## Competing interests

The authors declare no competing financial interests.

## Supporting information

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

**Table S1.** Primers used for quantitative real time-PCR reactions.

**Figure S1.** Lower magnification images showing co-localization of Klf4 in different cell types *in vivo*.

**Figure S2.** Specificity of inhibition using ERK and PI3K inhibitors.

**Figure S3.** Klf4 is required for MCP-1 and IL-6 production in microglia.

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