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ORIGINAL ARTICLE

CRISPR/Cas9 Editing of Glia Maturation Factor Regulates Mitochondrial Dynamics by Attenuation of the NRF2/HO-1 Dependent Ferritin Activation in Glial Cells

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Abstract

Microglial cells are brain specific professional phagocytic immune cells that play a crucial role in the inflammation- mediated neurodegeneration especially in Parkinson's disease (PD) and Alzheimer's disease. Glia maturation factor (GMF) is a neuroinflammatory protein abundantly expressed in the brain. We have previously shown that GMF expression is significantly upregulated in the substantia nigra (SN) of PD brains. However, its possible role in PD progression is still not fully understood. The Clustered-Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR Associated (Cas) protein9 (CRISPR/Cas9) system is a simple, rapid and often extremely efficient gene editing tool at desired loci, enabling complete gene knockout or homology directed repair. In this study, we examined the effect of GMF editing by using the CRISPR/Cas9 technique in BV2 microglial cells (hereafter referred to as BV2-G) on oxidative stress and nuclear factor erythroid 2-related factor 2 (NRF2)/Hemeoxygenase1 (HO-1)-dependent ferritin activation after treatment with (1-methyl-4-phenylpyridinium) MPP^+. Knockout of GMF in BV2-G cells significantly attenuated oxidative stress via reduced ROS production and calcium flux. Furthermore, deficiency of GMF significantly reduced nuclear translocation of NRF2, which modulates HO-1 and ferritin activation, cyclooxygenase 2 (COX2) and nitric oxide synthase 2 (NOS2) expression in BV2 microglial cells. Lack of GMF significantly improved CD11b and CD68 positive microglial cells as compared with untreated cells. Our results also suggest that pharmacological and genetic intervention targeting GMF may represent a promising and a novel therapeutic strategy in controlling Parkinsonism by regulating microglial functions. Targeted regulation of GMF possibly mediates protein aggregation in microglial homeostasis associated with PD progression through regulation of iron metabolism by modulating NRF2-HO1 and ferritin expression.

Keywords Parkinson's disease · Microglial cells · Glia maturation factor · CRISPR/Cas9

Introduction

Parkinson's disease (PD) is a common age dependent neurodegenerative disease, characterized by progressive loss of melanin pigmented dopaminergic neurons in the nigrostriatum of midbrain (Goldman 2014; Reeve et al. 2014). Continuous loss of dopaminergic neurons in the brain is mainly associated with chronic neuroinflammation (Jenner et al. 1992; Dawson and Dawson 2003; Block et al. 2007) probably due to excessive activation and expression of neuroinflammatory mediators from microglial cell activation (Perry et al. 2010; Perry and Teeling 2013). Microglial cells are the brain resident immune cells, functionally similar to macrophages. Microglia plays a central role in the neuroinflammation and implements neuroinflammatory sequences under neurodegenerative condition by producing variety of inflammatory molecules (Cunningham 2013; Perry and Teeling 2013). Microglial cells protect the brain parenchyma

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cells from the intra and extra-cellular pathogenic stimuli through activation of the lysosomal compartment, that bears critical proteases and exhibits bactericidal action (Kim and Joh 2006; Wirenfeldt et al. 2007). Microglial cells remove potentially toxic microbes and dead cells by phagocytosis to maintain the brain homeostatic condition. In addition, microglia are involved in shaping and maintaining the neuronal activity and synaptic network, throughout life (Wu et al. 2015; Yin et al. 2017; Soto and Sibson 2018). Under physiological conditions, activated microglia can perform diverse beneficial functions essential for neurons survival including cellular maintenance and innate immunity. At least two different phenotypes have been associated with these functions. On one side, the classical activation of phenotypic microglial cells participates in protection against pathogens and endogenous toxins and leads to the release of inflammatory products. In contrast, the alternative activation of phenotypic microglial cells is involved in neuro inflammation and wound healing (Perry et al. 2010; Morris et al. 2013; Xu et al. 2016). The balance between both phenotypes of microglial cells is crucial to ensure a protective function in the brain. In brief, accumulating findings acknowledge that damaged neurons release range of molecules that expand classical activation of phenotypic microglial cells and drive inflammatory stress propagating a cycle of neuronal death and chronic neuroinflammation (Block et al. 2007; Gao and Hong 2008).

Peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α) initiates a diverse set of metabolic programs through its interaction with, nuclear factor erythroid 2-related factor 1 (NRF1) and 2 (De Nuccio et al. 2011; Lin et al. 2005). Additionally, NRF1, NRF2, and PGC-1α are primarily responsible for regulating the expression of nuclear-encoded mitochondrial genes, including the components of respiratory complexes I-V, cytochrome-c and mitochondrial transcription factor A (TFAM; Kelly and Scarpulla 2004). Furthermore, those nuclear-encoded mitochondrial genes regulate the energy metabolism of the brain. Recent advances demonstrate that increased mitochondrial stress, loss of neuromelanin and trophic factors along with calcium (Ca^{2+}) binding protein deficiency, iron accumulation and Ca^{2+} influx leads to dopaminergic cell death. In addition, induction of heme oxygenase (HO) dependent ferritin expression and nitric oxide synthase (iNOS) along with release of cytokines have been implicated in the pathogenesis of PD (Prasad et al. 1999; Fernandez-Gonzalez et al. 2000; Ebadi et al. 2002). However, the exact cause of nigrostriatal degeneration in PD has not been identified with certainty. The Clustered-Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR Associated (Cas) protein9 (CRISPR/Cas9)-mediated gene insertion or deletion has shown significant impact in both in vitro as well as in vivo models due to far more cost-effective and easier method than any other gene editing tools currently available in the field of research (Khadempar et al. 2018; Raikwar et al. 2018b). Recently, we showed that targeted gene editing of GMF in microglial BV2 cells showed significantly less p38 MAPK phosphorylation and its dependent adverse effects (Raikwar et al. 2018a; Raikwar et al. 2018b).

Glia maturation factor (GMF), a neuroinflammatory acidic protein abundant in the brain was discovered, isolated, sequenced, and cloned in our laboratory (Lim et al. 1990; Kaplan et al. 1991; Zaheer et al. 1993). GMF is highly expressed in the brain during cellular stress conditions and has been implicated in PD pathology. The cellular localization of GMF leaves room for the possibility that GMF might have a dual function; an intracellular function in intact cells and an extracellular activity when released after injury. The extracellular release of structurally diverse compounds that is produced by the cell or compounds derived from exogenous sources can perturb the repression of the transcription factor NRF2, leading to increased translocation and subsequent transcriptional activation of NRF2-dependent genes. Recently, special attention has been drawn to the beneficial aspects of NRF2-mediated HO-1induction, the enzyme that degrades heme to generate CO, biliverdin and free iron (Abraham and Kappas 2008; Scapagnini et al. 2011). In the present study, we found that the genetic knockout of GMF by using the technique of CRISPR/Cas9 editing could inhibit MPP^{+}-induced oxidative stress, stimulated NRF2 nuclear translocation that lead to increased HO-1 expression and ferritin activation in BV-2 microglial cells. Our present study further implicates the effect of GMF on mitochondrial stress probably through COX2 and NOS2 in regulating the NRF2/HO-1 pathway.

Materials and Methods

Cell Culture

The murine BV2 microglial cell line was obtained from American Type Culture Collection (Manassas, VA), and cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 100 mg/ml penicillin/streptomycin (P/S) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37 C in 5% CO_2 incubator.

Lentiviral Vector Production for GMF Gene Editing

GMF gene editing was achieved by utilizing two separate VSV envelope pseudo typed third generation integration competent lentiviral vectors as described earlier (Raikwar et al. 2018b). The lentiviral expression vectors encoding EF1α-promoter driven *Streptococcus pyogenes* Cas9 (SpCas9), SV40 promoter driven eGFP and neomycin (CP-LvC9NU-09) and U6 promoter driven GMF sgRNAs and SV40 promoter driven mCherry and puromycin (MCP232778-LvSG03–3-B) or U6 promoter driven scrambled sgRNA control sequence and SV40 promoter driven mCherry and

puromycin (CCPCTR01-LvSG03) were purchased as glycerol stocks (Genecopoeia, Rockville, MD).

The nucleotide sequence of the GMF-specific sgRNA is GMF sgRNA1: ACTTATAATAGCAGCATTGT and of the control scrambled sequence is GCTTCGCGCCGTAG TCTTAG, respectively.

Generation of GMF-Edited BV2-G Cell Line

As described earlier (Raikwar et al. 2018b), proliferating BV2 cells were plated at a cell density of 1×10^5 cells per well of the 6-well plate. For lentiviral transduction, the cells were washed with PBS and maintained in Opti-Mem. Viral transductions were performed at a low multiplicity of infection (MOI) of 10 in the presence of 8 μg/ml polybrene (Cat No. H9268, Millipore-Sigma, St. Louis, MO) for 8 h. The cells were washed and maintained in regular growth medium for 72 h. The lentiviral vector LV-EF1α-SpCas9-eGFP-transduced BV2 cells were trypsinized and plated in growth medium containing neomycin (750 μg/ml) for the generation of stable cell line BV2-CRISPR/Cas9. The stable BV2-CRISPR/Cas9 cell line was transduced with the second lentiviral vector LV-GMF-sgRNA-mCherry. A GMF-edited BV2 cell line (here after referred to as BV2-G), using GMF-specific sgRNAs was generated and used in this study. In some experiments, GMF gene editing by lentiviral transduction was performed using sub-confluent BV2 cells maintained in Opti-Mem medium. Lentiviral co-transductions were performed at a MOI of 10 each of LV-EF1α-Cas9-eGFP and LV-GMF-sgRNA in the presence of 8 μg/ml polybrene (Cat No. H9268, Millipore-Sigma, St. Louis, MO) for 8 h. The lentiviral vector LV-EF1α-Cas9-eGFP and LV-GMF-sgRNA co-transduced BV2 cells were washed with 1XPBS trypsinized and plated in regular growth medium for further experiments.

MPP^+ Treatments

After the viral transduction for 8 h, the cells were changed into regular cell culture media supplemented with 10% of FBS along with 1% P/S and incubated for 72 h to reach the confluency (~75%–85%) and these cells were used for further experiments in this study. The BV2 and BV2-G microglial cells were grown to maximum confluency and then treated with MPP^+ (0.1 mM) for 24 h under standard culture conditions (Zhou et al. 2016).

Determination of Intracellular ROS by DCFH-DA Staining Method

The levels of intracellular ROS was determined by using 2,7-diacetyl dichloro-fluorescein-diacetate (DCFH-DA) staining kit according to manufacturer's instruction (Abcam, Cambridge, MA; ab139476) using a cell-permeant fluorogenic DCFH-DA. The dye after entering into the cell is deacetylated by cellular esterases, then oxidized by ROS and converted into fluorescent dichlorofluorescein (DCF). After the experimental period, ROS formation was determined by fluorescence microscopy. We determined the green fluorescence intensity on the whole field (not on individual cells) of the image acquired from three different experiments by using ImageJ quantification software (NIH domain), and have represented the MFI as arbitrary units(AU). (Khan et al. 2014a; Lunov et al. 2014; Selvakumar et al. 2018). In addition, we performed background correction on the images obtained from each group.

Determination of Apoptosis by Ethidium Bromide/Acridine (EtBr/AO) Orange Staining

Condensed nuclear morphology of the cells was studied by fluorescence microscopy after staining with ethidium bromide/acridine orange (EtBr/AO). In brief, both the BV2 and BV2G cells were cultured in a 24 well plate containing poly-D-lysine coated cover glass at the final concentration of 1×10^5 cells/ml. Cells were left untreated or treated with MPP^+ (0.1 mM) for 24 h at 37 °C. The samples in a 24-well plate were divided into 4 groups, with 6 well samples in each group corresponding to different type of cells and treatment. After 24 h, dual EtBr/AO (Sigma) fluorescent staining solution (1 μl) containing EtBr/AO (1:1 *v*/v; 100 μg/ml AO and 100 μg/ml EtBr was added to each well. They were incubated at room temperature for 5 min. The apoptotic morphological changes in the cells were examined within 20 min using a fluorescent confocal microscope. The dual EtBr/AO staining method was repeated at least 3 times (Ribble et al. 2005; Liu et al. 2015). Confocal imaging was performed on a Leica TCP SP8 laser scanning confocal microscope with a 405-nm diode laser and tunable super continuum white light laser using 63X oil immersion objective. The following excitation/emission band-pass wavelengths were used: 405/420–480 nm (DAPI), 495/505–550 nm (Alexa Fluor 488) and 570/580–630 nm (Alexa Fluor 568).

Calcium Flux Assay

The BV2 and BV2-G microglial cells were seeded into a 96-well plates at 1×10^5 cells/well were cultured in serum-free medium for 24 h. MPP^+ induced kinetic changes in intracellular Ca^{2+} concentration were measured with the Fura-2 QBT Calcium Assay kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. The plates were transferred to a FLEX Station3 multi-mode microplate reader (Molecular Devices). The cells were excited at 340 nm and 380 nm and Ca^{2+}-bound Fura-2 emission was recorded at 510 nm. The emission signals with the Fura-2 QBT kit was

measured as relative fluorescence units (RFU) at 510 nm and results expressed from ratiometric analysis.

Western Blotting

Western blot analysis was used to determine the protein expression of NRF2 (Cat No. sc-365949; 1:600); HO-1 (Cat No. sc-136960; 1:500); COX2 (Cat No. sc-376861; 1:600); NOS2 (Cat No. sc-7271; 1:600; Santa Cruz Biotech), Ferritin (Cat No. ab75973; 1:500; abcam, Inc). Briefly, after termination of incubation with MPP^+ cells were harvested and washed with PBS. Cells were lysed in 100 μl lysis buffer (20 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 30 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) followed by centrifugation at 1000 g for 5 min at 4 C. The total lysates were preserved on ice and vortexed for 20 min followed by pelleting at 13,000 g for 10 min at 4 C. To analyze NRF2 translocation from cytosol to nuclear region, we followed Kang et al. (Kang et al. 2002) as reported previously and protein samples were subjected to 4–12% poly acrylamide gel electrophoresis. A total volume of 25–30 μg of protein was loaded per lane and separated proteins were blotted onto a PVDF membrane. After blocking with 5% bovine serum albumin, the membranes were incubated with the above mentioned primary antibodies. The membranes were then incubated with HRP conjugated secondary antibodies at a concentration of 1:2000, washed thrice with TBST for 5 min each. The bands were visualized by treating the membranes with ECL prime detection reagent (SuperSignal West Pico PLUS Substrate, Cat No. 34580; ThermoScientific). Blots were stripped and re-probed for β-actin as a loading control. Densitometry was performed using ChemiDoc-It2 Imaging System (UVP LLC, Upland, CA) analysis software.

Immunocytochemistry and Confocal Imaging

Briefly, after the treatment with neurotoxin MPP^+, BV2 and BV2-G microglial cells were washed with PBS and incubated with fixative (4% paraformaldehyde) for 20 min at 4 °C. Cells were permeabilized by incubation with PBST (1XPBS + TritonX100) for 15 min. Then, the cells were rinsed three times in PBS, blocked with 5% normal goat serum for 30 min and finally incubated for 2 h at room temperature with the respective antibodies [NRF2, Ferritin (1:400 dilution), HO-1 (1:500), (Cat No. MAB1387Z; EMD Millipore, MA) and CD68 (Cat No. ab31630; Abcam Inc., Cambridge, MA) (1:200–1:400)]. After that, cells were washed three times with PBS and incubated with appropriate secondary goat anti-rabbit IgG conjugated with green fluorescent dye Alexa Fluor 488 or goat anti-mouse IgG conjugated with red fluorescent dye Alexa Fluor 568. Finally, the cells were counterstained with VECTASHIELD Antifade Mounting Medium with DAPI. Confocal imaging was performed on a Leica TCP SP8 laser scanning confocal microscope as detailed above.

Flow Cytometry Detection of CD11b and CD68

BV2 and BV2-G cells were trypsinized using 0.25% trypsin, resuspended in the growth medium, and counted. Approximately 2×10^6 cells were used for each staining reaction. The cells were washed with 1XPBS, fixed in 4% paraformaldehyde for 15 min, washed with PBS, and permeabilized using PBST (PBS with 0.01% Tween20) for 15 min. The cells were washed three times with PBS and blocked with PBS containing 3% BSA. Subsequently, the cells were subjected to staining with an anti-CD11B and CD68 at a 1:100–1:400 dilution for 1 h at 4 C. The cells were washed thrice with PBS and stained with Alexa Fluor 488 (Cat: A11008; ThermoFisher Scientific, Waltham, MA) and Alexa Fluor 568 (Cat No. A10042; ThermoFisher Scientific) conjugated secondary antibody at a dilution of 1:300–1:500 for 30 min. The cells were washed three times to remove unbound antibodies and analyzed using the Beckman Coulter-CytoFlex Flow cytometer (6 lasers & 21 fluorescent detectors with 488 nm, 638 nm, 405 nm, 375 nm, 561 nm, 808 nm). CytExpert 2.1 software was used to analyze and display the flow cytometry data.

Statistical Analysis

All the results were expressed as mean ± SEM and statistical analysis of the data was done by using GraphPad InStat Prism 7 software. Statistical analysis of the data was done by independent student *t*-test and the *p* value less than <0.05 was considered as statistically significant in all the experiments.

Results

Effect of GMF on MPP^+ Induced Oxidative Stress in BV2 Microglial Cells

We determined the effect of GMF on MPP^+ induced ROS accumulation in BV2 microglial cells using the DCFH-DA staining method. BV2 and BV2-G microglial cells were subjected to MPP^+ (0.1 mM) treatment for 24 h. Results show that cells incubated with MPP^+ showed elevated ROS intensity in both BV2 and BV2-G microglial cells as compared with untreated cells (Fig. 1a and b). However, BV2-G cells showed ROS intensity that was significantly lower when compared with BV2 cells treated with MPP^+. There are no significant changes between untreated cells of both BV2 and BV2-G microglial cells.

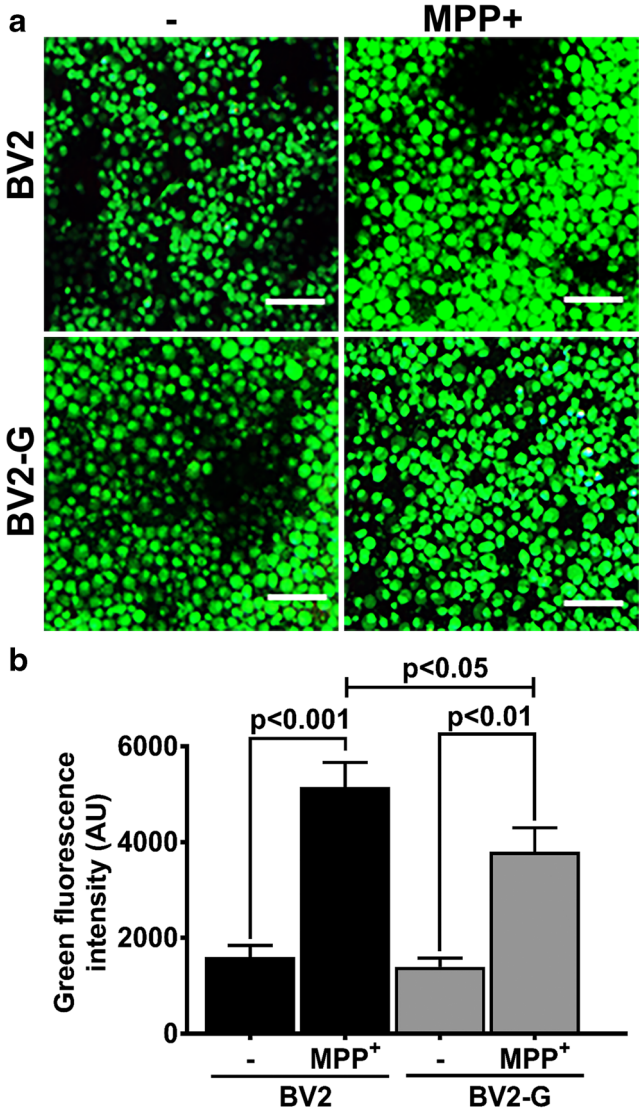

Fig. 1 Effect of GMF on MPP^+ induced oxidative stress in BV2 microglial cells. The BV2 and BV2-G microglial cells were seeded (3×10^6) in a 96-well plate. Then the cells were pre-incubated with DCFH-DA dye for 45 min and the cells were treated with MPP^+ (0.1 mM) for 24 h. After the incubation period, cells were used to measure ROS intensity on a fluorescence microscope. Representative images show the toxic effect of MPP^+ induced ROS intensity (green fluorescence; **a**). Bar graphs show the green fluorescence intensity significantly reduced in GMF deficient BV2-G cells as compared with BV2 microglial cells (**b**). Statistical significance was assessed by independent student *t*-test using GraphPad InStat Prism-7 software. The *p* value less than <0.05 was considered as statistically significant in all the experiments; Values are given as mean ± SEM of three experiments in each group. $p < 0.001$ and $p < 0.01$ untreated controls vs MPP^+-treated cells; $p < 0.05$ MPP^+-treated BV2 cells vs MPP^+-treated BV2-G cells. Scale bar 100 μm. AU arbitrary unit

GMF Promotes MPP^+ Induced Apoptotic Cell Death in BV2 Microglial Cells

Further to determine, whether knockout of GMF can prevent the apoptosis induced by MPP^+ in BV2 cells, we used the EtBr/AO staining method. The bright green color represents healthy viable cells while the red and dark orange color represents apoptotic body formation due to apoptosis. Results show that, MPP^+ (0.1 mM) treatment increased apoptosis in both BV2 as well as in BV2-G when compared with untreated cells (Fig. 2a and b). However, BV2-GMF cells show significantly reduced number of apoptotic cells when compared

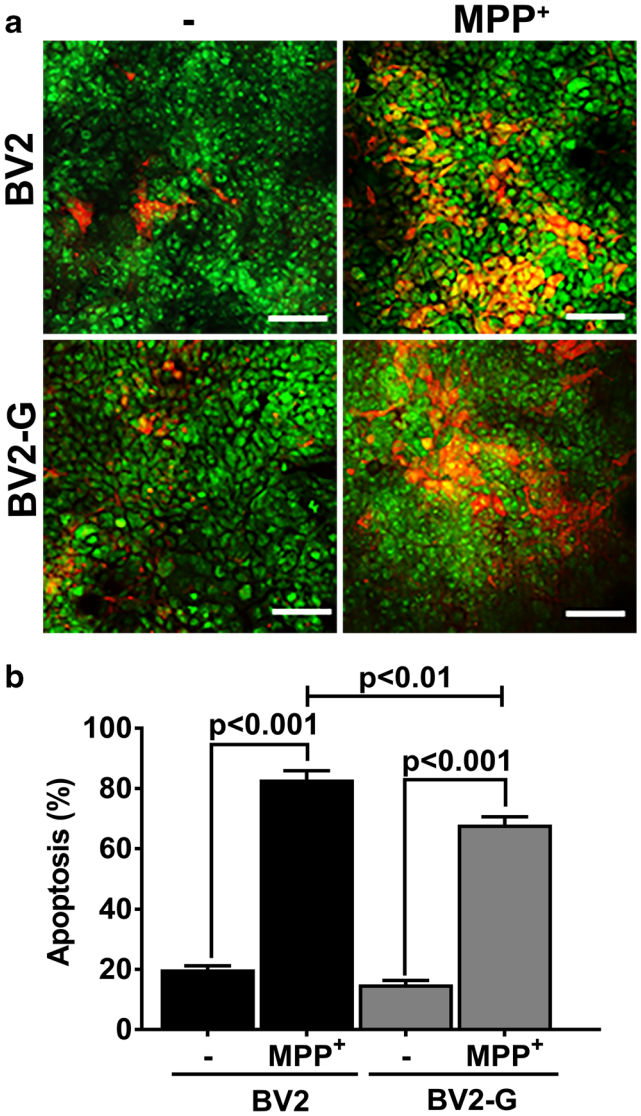

Fig. 2 Effect of GMF on MPP^+ induced apoptosis in BV2 microglial cells. The BV2 and BV2-G cells were seeded (3×10^6) in a 96-well plate. The cells were treated with MPP^+ (0.1 mM) for 24 h. After the incubation period, cells were stained with EtBr/AO solution (1:1 *v*/v) at a final concentration of 100 μg/ml for 5 min and finally imaged under fluorescence microscope. Images represents the toxic effect of MPP^+ – induced apoptotic cell death (dark orange red; Fig. 2a). Bar graphs show that apoptotic changes were significantly reduced in BV2-G microglial cells as compared with BV2 cells (B). Values are given as mean ± SEM of three experiments in each group. Statistical significance was assessed by independent student *t*-test using GraphPad InStat Prism-7 software. $p < 0.001$ untreated controls vs MPP^+-treated cells; $p < 0.01$ MPP^+-treated BV2 cells vs MPP^+-treated BV2-G cells. The *p* value less than <0.05 was considered as statistically significant in all the experiments; Scale bar 100 μm

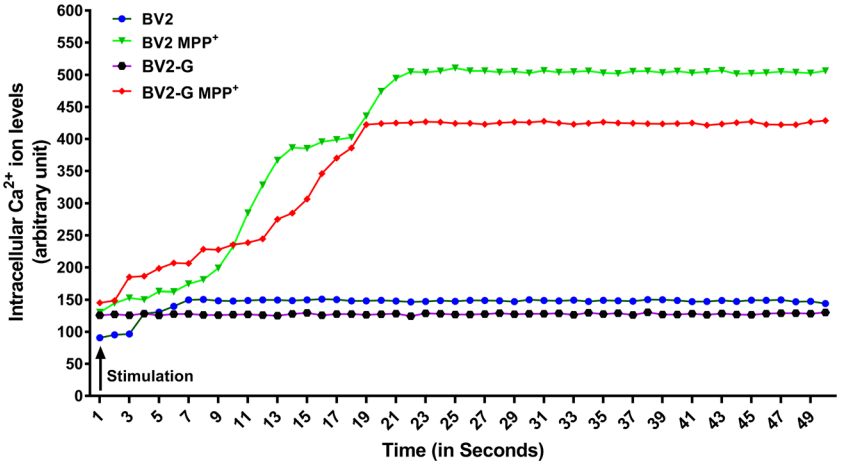

Fig. 3 Effect of GMF on MPP^+ induced Ca^{2+} influx in BV2 microglial cells. The BV2 and BV2-G cells were seeded (3×10^6) in a 96-well plate. Then the cells were pre-stained using Fura-2 QBT Calcium Assay kit according to the kit manufacturer's instruction. The cells were treated MPP^+ (0.1 mM) and plates were transferred to a FLEX Station3 multi-mode benchtop scanning fluorometer chamber (Molecular Devices). Representative results show the effect of MPP^+ induced Ca^{2+} mobilization (flux) which is significantly reduced in BV2-G cells as compared with BV2 microglial cells. Values are given as mean ± SEM of three experiments in each group and represented as arbitrary unit

with MPP^+ treated BV2 cells. There was no significant difference in apoptotic cell death between both the untreated groups.

Effect of GMF on MPP^+ Induced Ca^{2+} Flux in BV2 Microglial Cells

MPP^+ induced Ca^{2+} was determined using the Fura-2 QBT Calcium Assay kit and the FlexStation 3 benchtop multi-mode microplate reader. Results show that MPP^+ treatment significantly elevated the Ca^{2+} flux in BV2 microglial cells as compared with untreated cells (Fig. 3). We also found that BV2-G microglial cells show significantly reduced Ca^{2+} increase as compared with MPP^+ treated BV2 cells. The results indicate that GMF may regulate Ca^{2+} dependent microglial events such as M1 to M2 activation. No significant Ca^{2+} flux found between untreated cells of both BV2 and BV2-G microglial cells.

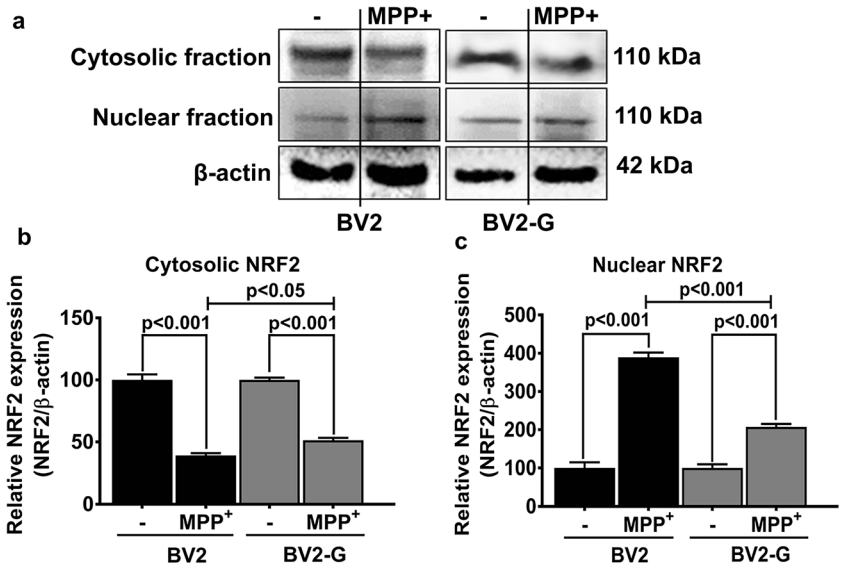

Fig. 4 Effect of GMF on MPP^+ induced NRF2 translocation in BV2 microglial cells. The BV2 and BV2-G cells were seeded in a T25 culture flask and cultured under standard conditions. Cells were incubated with MPP^+ (0.1 mM) for 24 h. After the incubation period, cells were washed with PBS lysed and cytosolic and nuclear fractions separated. Aliquots were subjected to western blot analysis (**a**). Decreased nuclear and increased cytosolic expression level of NRF2 was found in BV2-G cells as compared with BV2 cells. Bar graphs shows the mean densitometry analysis of bands after normalizing with β-actin as a loading control of each group (**b** and **c**). Values are given as mean ± SEM of three experiments in each group. Statistical significance was assessed by independent student *t*-test using GraphPad InStat Prism-7 software. $p < 0.001$ untreated controls vs MPP^+-treated cells; $p < 0.05$ and $p < 0.001$ MPP^+-treated BV2 cells vs MPP^+-treated BV2-G cells. The *p* value less than <0.05 was considered as statistically significant in all the experiments

GMF Mediates MPP+ Induced NRF2 Nuclear Translocation in BV2 Microglial Cells

Transcriptional response to oxidative stress is mediated by NRF2 and ROS increases nuclear translocation of NRF2. Immunoblotting (Fig. 4a, b and c) results demonstrate that cells incubated with MPP+ show translocation of NRF2 from cytoplasm to the nuclei. BV2 microglial cells treated with MPP+ shows significant NRF2 expression in nuclear region when compared with untreated cells. Whereas, BV2-G microglial cells treated with MPP+ shows decreased NRF2 translocation between cytoplasmic and nuclear regions indicating that GMF contributes to oxidative stress resulting in increased nuclear translocation of NRF2.

Effect of GMF on MPP+ Induced HO-1 and Ferritin Expression in BV2 Microglial Cells

To examine the downstream effects of NRF2 activation mediated by MPP+, we studied HO-1 expression and its combined effect on ferritin activation in BV2 and BV2-G microglial cells. Previously, we reported that MPP+ enhances ROS intensity dependent oxidative stress in astrocytes and neuronal cells (Khan et al. 2014a; Selvakumar et al. 2018), and that lesser oxidative stress induces less NRF2 translocation into the nuclear site, which further leads to less HO-1 expression in BV2 cells. In addition, it has been demonstrated that shear stress induced nuclear translocation of NRF2 (Hsieh et al. 2009). Using immunoblotting (Fig. 5a, b and c) and immunocytochemistry (Fig. 6a, b and c), we demonstrate that NRF2 activated HO-1/ferritin and also show the co-localization of these proteins in both types of BV2 cells. Results show that MPP+ treatment significantly increased the expression of HO-1 and ferritin as compared with untreated cells (Fig. 5a). We found that BV2-G cells showed significantly lesser expression of HO1 and ferritin as compared with BV2 cells.

Representative immnunocytochemical images show the expression of cytosolic and nuclear NRF2 expression along with HO-1(Fig. 6a) and ferritin expression (Fig. 6d) in BV2-G microglial cells treated with MPP+ for 24 h. Results show quantitatively increased total positive area (B) and total average intensity (C) of NRF2 and HO-1 expression when compared with MPP+ treated BV2-G cells (Fig. 6a). In addition, we found that administration of MPP+ quantitatively increased total positive area (E) and total average intensity (F) of NRF2 translocation to nuclear region and ferritin expression as compared with untreated BV2 cells and this increase was reduced in the BV2-G microglial cells. These results demonstrate that GMF regulates ROS dependent oxidative stress and mediates NRF2/HO-1 antioxidant signaling in microglial cells.

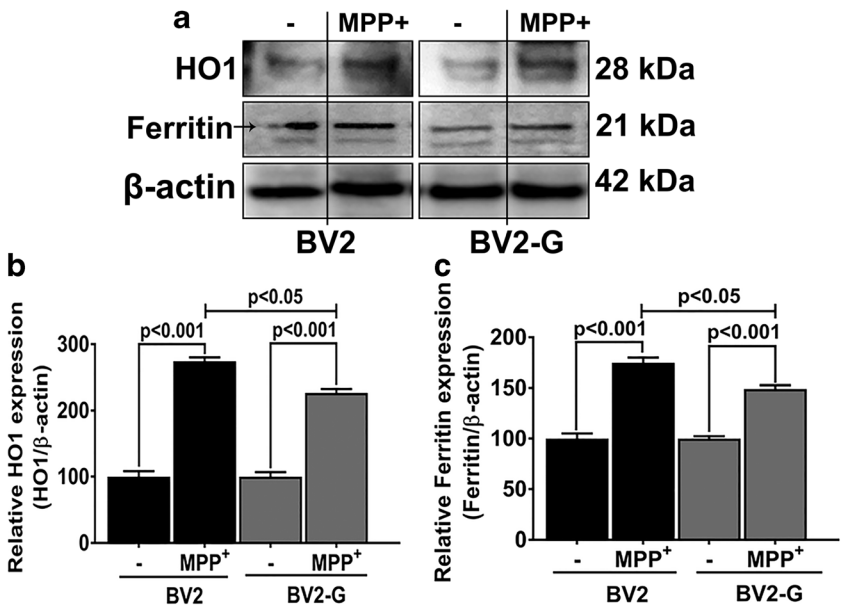

Fig. 5 Effect of GMF on MPP+ induced HO-1 and ferritin expression in BV2 microglial cells. The BV2 and BV2-G cells were seeded in a T25 culture flask and cultured under standard conditions. The cells were incubated with MPP+ (0.1 mM) for 24 h. After the incubation period, cells were washed with PBS and prepared for western blot analysis. Decreased HO-1 and ferritin expressions were found in BV2-G cells as compared with BV2 cells (**a**). Bar graphs show the mean densitometry analysis of bands after normalizing with β-actin as a loading control of each group (**b** and **c**). Values are given as mean ± SEM of three experiments in each group. Statistical significance was assessed by independent student *t*-test using GraphPad InStat Prism-7 software. $p < 0.001$ untreated controls vs MPP+-treated cells; $p < 0.05$ MPP+-treated BV2 cells vs MPP+-treated BV2-G cells. The p value less than <0.05 was considered as statistically significant in all the experiments

Effect of GMF Deficiency on MPP$^+$ Induced COX2/NOS2 Expression in BV2 Microglial Cells

To determine whether knockout of GMF prevents the expression of COX2/NOS2 in BV2 microglial cells we used immunoblotting assay (Fig. 7a, b and c). Our immunoblotting results show that MPP$^+$ treatment significantly increased the expression of COX2/NOS2 in BV2 microglial cells as compared with untreated cells. We found thatBV2-G cells show significantly decreased COX2/NOS2 expression when compared with BV2 cells following MPP$^+$ treatment. No significant changes were observed between both the BV2 and BV2-G microglial cells. Consequently, our results indicate that knockout of GMF suppresses the production of inflammatory mediators in BV2 microglia.

Effect of GMF on MPP$^+$ Induced Markers of Microglial Activation in BV2 Microglial Cells

To investigate whether the absence of GMF affected the surface expression of CD11b and CD68 in activated microglia, we used immunocytochemistry (Fig. 8a) and FACS analysis (Fig. 8b). FACS analysis shows the expression of CD68-APC-A750-A (red fluorescence) versus CD11b-FITC-A (green fluorescence) in BV2 and BV2-G cells. Results show that MPP$^+$ treatment for 24 h significantly increased cell surface markers CD11b and CD68 in BV2 microglial cells when compared with untreated cells. Knockout of GMF in microglial cells, significantly attenuated CD11b and CD68 as compared with MPP$^+$ treated BV2-G cells as shown by FACS analysis. No significant changes were observed between both the untreated BV2 and BV2-G microglial cells. Taken together,

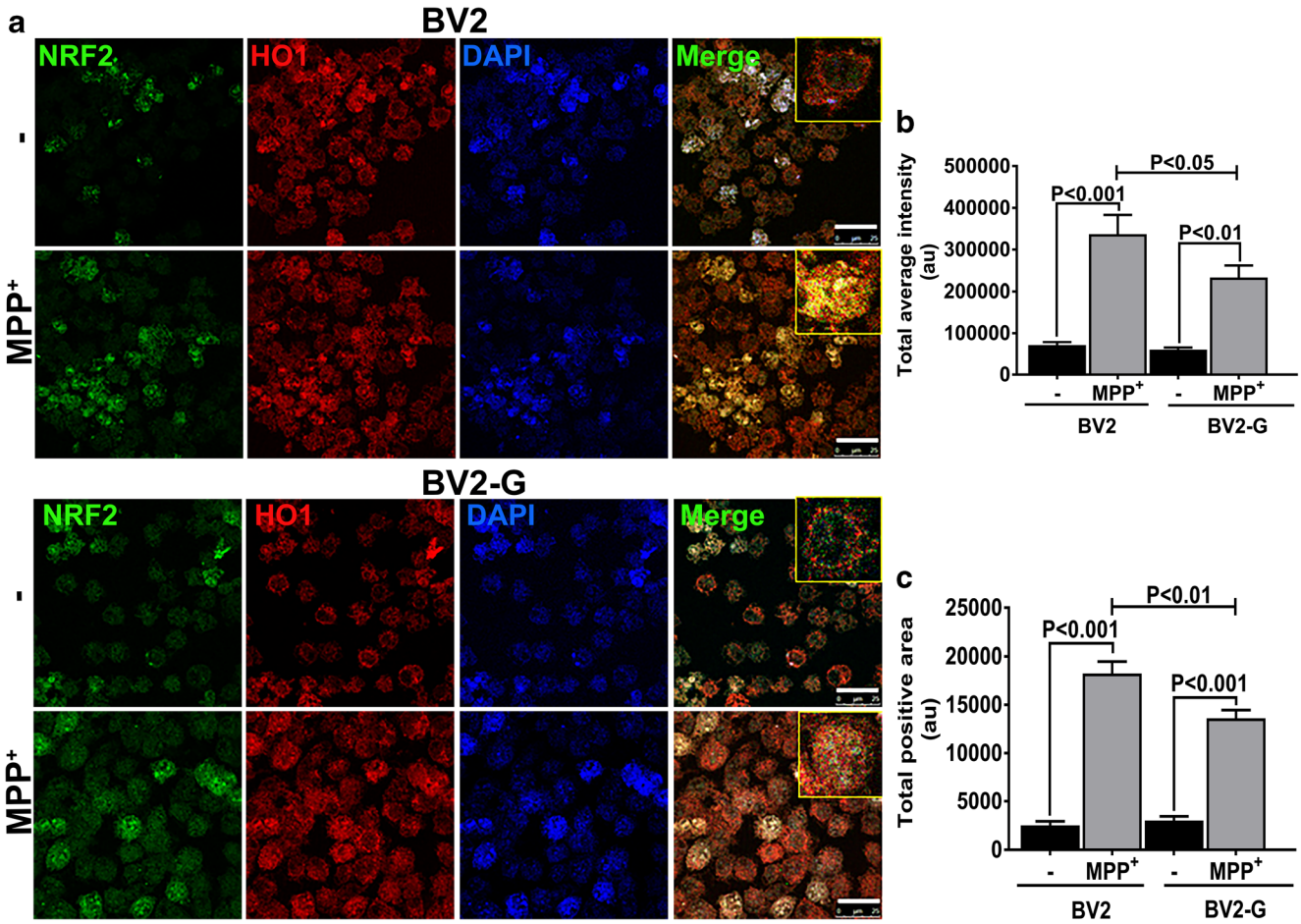

Fig. 6 Effect of GMF on MPP$^+$ induced HO-1 and ferritin expression in BV2 microglial cells. The BV2 and BV2-G microglial cells were seeded on poly-D-lysine coated coverslips. The cells were incubated with MPP$^+$ (0.1 mM) for 24 h. After the incubation period, cells were washed with PBS and processed for immunocytochemistry to detect HO-(**a**) and ferritin (**d**) co-localization along with NRF2 expression. Quantitatively decreased total positive area and total average intensity of HO-1 (**b** and **c**) and ferritin (E and F) along with NRF2 translocation to nuclear site were found in BV2-G cells as compared with BV2 microglial cells. Statistical significance was assessed by independent student *t*-test using GraphPad InStat Prism-7 software. $p < 0.05$ untreated controls vs MPP$^+$-treated cells; $p < 0.05$ MPP$^+$-treated BV2 cells vs MPP$^+$-treated BV2-G cells. The p value less than <0.05 was considered as statistically significant in all the experiments; Scale bar 100 μm; au arbitrary units

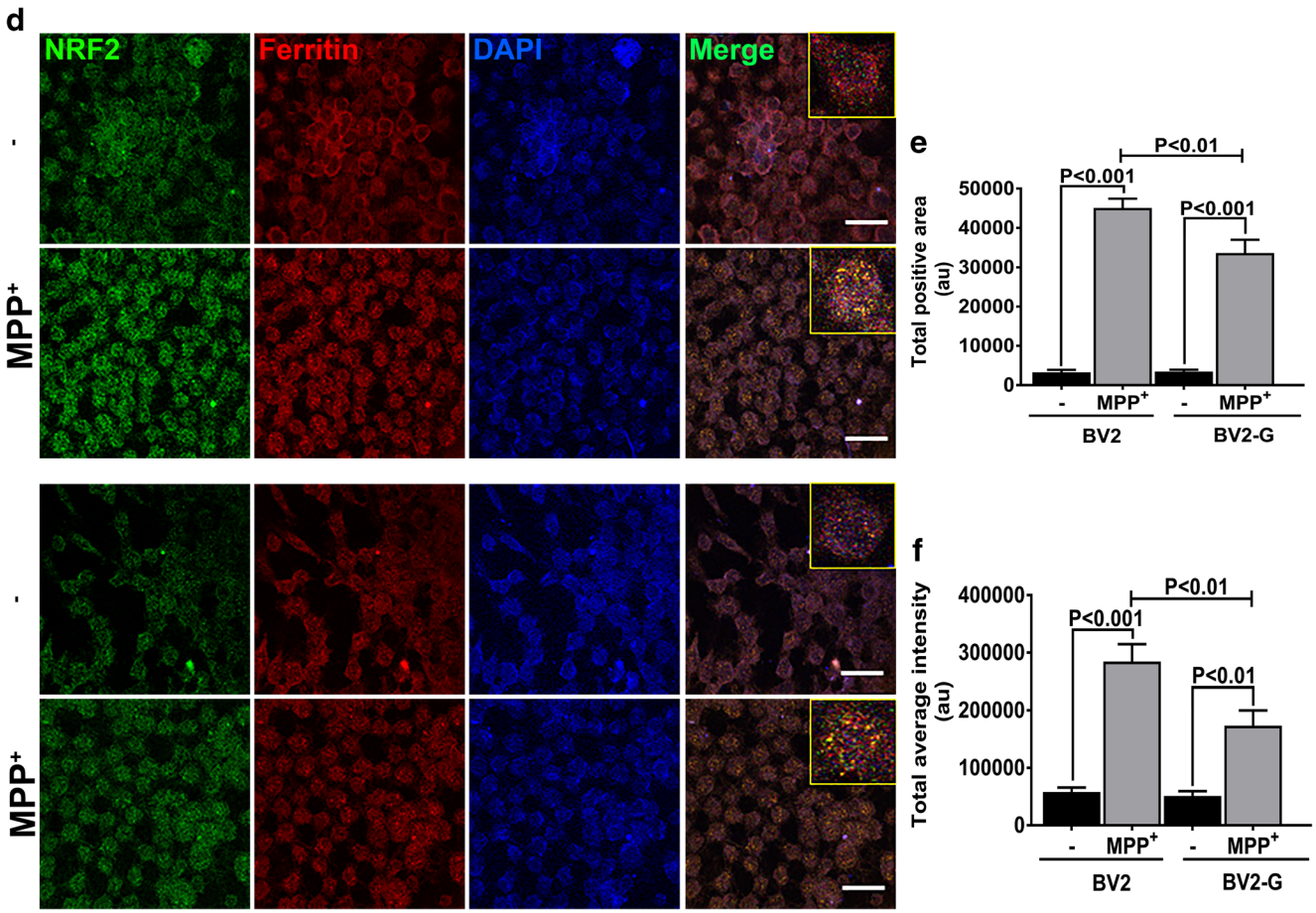

Fig. 6 (continued)

these results indicate that knockout of GMF is capable of suppressing the activation of microglia from M1 to M2 state. The level of CD11b was also elevated along with CD68 in MPP^+ treated cells.

Discussion

PD is a common and prominent neurodegenerative disease in elder population that produces multiple signs of motor and non-motor symptoms due to dopaminergic neuronal loss in SN of the midbrain (Miklya et al. 2014; Chen et al. 2015). PD pathogenesis is mainly associated with oxidative stress and neuroinflammation (Jenner et al. 1992; Dawson and Dawson 2003). Innate immune cell activations such as microgliosis leads to neuroinflammatory changes ultimately causing the pathological development of PD (Wilms et al. 2007), although the exact etiology of PD remains unclear. Several mechanisms have been proposed to explain the pathogenesis of PD, including the production of ROS brought about by dopamine auto-oxidation and mitochondrial dysfunction, and α-synuclein deposition (Valente et al. 2004; Olanow and Schapira 2013). Accumulating evidence indicate that oxidative stress and its related pathological modifications in the microglial cells leads to morphological and functional plasticity of microglial cells responsible for the pathogenesis of PD. In the present study, we demonstrate that the genetic knockout of GMF (an inflammatory modifier) using the CRISPR/Cas9 method ameliorates microgliosis by improving mitochondrial dynamics and oxidative stress dependent ferritin activation via NRF2/HO-1 in BV2 microglial cells.

Microglia are the brain resident macrophages. Activation of microglial cells initiates a complex response that includes the activation and release of extracellular and intracellular ROS, in response to pathogens or neurotoxins (Block et al. 2007; Hung et al. 2008; Innamorato et al. 2008). The cellular production of ROS ultimately enhances oxidative stress mediated neuroinflammation and cell death resulting in pathogenesis of PD (Andersen 2004; Valente et al. 2004; Park et al. 2012). Further, microglial cells act as a major source for ROS generation and cellular reservoir during neuroinflammation (Colton and Gilbert 1987; Colton et al. 2000).

Increases in the intracellular Ca^{2+} level affects mitochondrial membrane permeablization and mitochondrial stress that

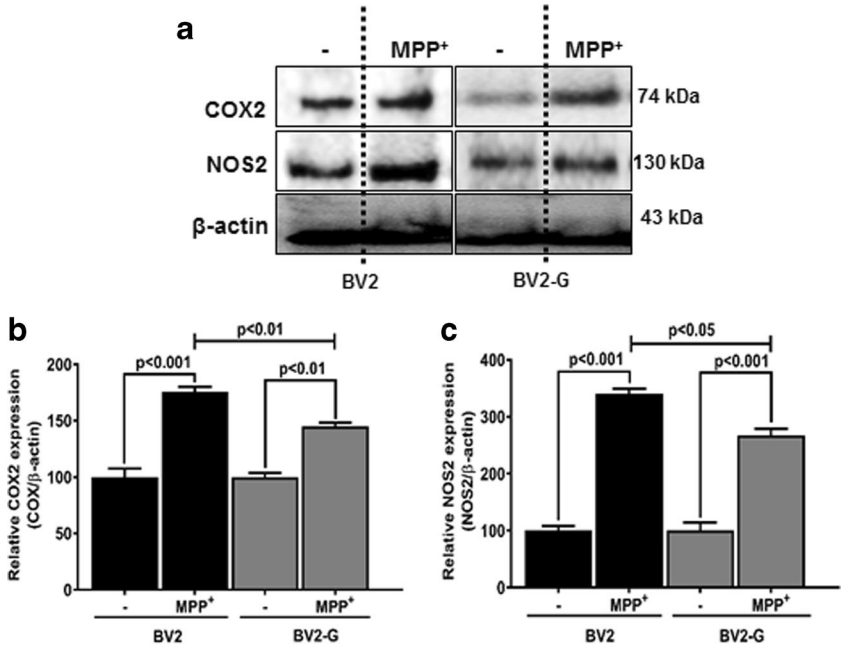

Fig. 7 Effect of GMF on MPP^+ induced COX2 and NOS2 expression in BV2 microglial cells. The BV2 and BV2-G microglial cells were seeded in a T25 culture flask. The cells were incubated with MPP^+ (0.1 mM) for 24 h. After the incubation period, cells were washed with PBS and cell lysates were prepared from these cells for western blot studies. Decreased expression of COX2 and NOS2 were found in BV2-G cells when compared with BV2 microglial cells (**a**). Bar graphs show the mean densitometry analysis of bands after normalizing with β-actin as a loading control of each group (**b** and **c**). Values are given as mean ± SEM of three experiments in each group. Statistical significance was assessed by independent student *t*-test using GraphPad InStat Prism-7 software. $p < 0.001$ and $p < 0.01$ untreated controls vs MPP^+-treated cells; $p < 0.01$ and $p < 0.05$ MPP^+-treated BV2 cells vs MPP^+-treated BV2-G cells. The p value less than <0.05 was considered as statistically significant in all the experiments

leads to activation of calpain and subsequently classical caspase dependent cell death pathway (Sareen et al. 2007). Previously we reported that absence of GMF reduced ROS mediated astrocyte activation and enhances the resistance against MPP^+ neurotoxicity (Khan et al. 2014a). In the present study, administration of MPP^+ significantly increases ROS production and Ca^{2+} flux in microglial BV2 cells, which further leads to cell death. The GMF-edited BV2-G microglial cells show significant reduction in ROS intensity and Ca^{2+} level compared to BV2 cells. These results indicate that absence of GMF could potentiate microglial protection from apoptotic cell death, which corroborate previous studies (Park et al. 2008; Khan et al. 2014a; Khan et al. 2014e; Fan et al. 2015; Sun et al. 2015).

NRF2 is known to regulate the expression of antioxidant and detoxification responsive genes (Strom et al. 2016). Recently it was reported that changes in the NRF2 expression can modulate mitochondrial metabolism- dependent homeostasis function (Holmstrom et al. 2016). Previously we reported that GMF is an essential cofactor for GSK-3β activation (Zaheer et al. 2008), a well-known inhibitor of NRF2, which protects ROS dependent cell death (Salazar et al. 2006; Chen et al. 2009). We also indicated that neutralization or specific knockdown of GMF expression could attenuate the GSK-3β dependent NRF2 activation that leads to prevention of oxidative stress induced neuronal loss (Salazar et al. 2006; Chen et al. 2009; Zaheer et al. 2011). Furthermore, in this study, we found that genetic knockout of GMF potentiates the protective effect of BV2 cells against MPP^+ -induced GMF -dependent NRF2 translocation in BV2-G microglial cells. These results also suggest that MPP^+ implements its toxicity by activating GMF and NRF2 dependent oxidative stress in BV2 microglial cells. We also found that BV2-G cells showed decreased NRF2 translocation from cytosolic to nuclear fractions and also HO-1 expression was significantly less than in BV2 cells, due to decreased ROS dependent oxidative stress.

In contrast, during oxidative stress condition NRF2 mediates the induction of HO-1, the enzyme that degrades heme to generate CO_2, biliverdin and free iron molecules and finally prevents cell death (Abraham and Kappas 2008; de Vries et al. 2008). It was reported that HO-1 expression specifically increased (4-fold higher) in SN apart from other brain regions of PD specimens, suggesting that the SN is the region of brain that bears oxidative stress (Schipper and Song 2015). Ferritin is a classical iron storage and sequestration molecule, commonly found in cytoplasm of the cell that mainly supplies the iron depending on cellular iron status and need. It was assumed that increased expression of HO-1 is mainly linked with increased expression of the iron-sequestering protein ferritin (Vile and Tyrrell 1993). Iron released by HO-1 activation also regulates ferritin as well as NO synthase gene expression (Weiss et al. 1994).

It has been reported that increased HO-1 expression in microglial cells correlates with its anti-inflammatory actions. The upregulated expression of HO-1 is found to inhibit the pro-inflammatory mediators and supreses COX2 and iNOS expression (Petrache et al. 2000). The up-regulation of inflammatory genes such as COX2 and iNOS in the immunocompetent cells such as in microglial cells may be a compensatory and prosurvival or preconditioning mechanism during the apoptotic cell death. It has been reported that during oxidative condition the increased H_2O_2 level activates the JAK-STAT pathway leading to modulation in COX2 and iNOS expression (Yu et al. 2012). Our earlier findings suggest that deficiency of GMF significantly attenuates the different STSTs such as STAT1, STAT2, STAT3, STAT4, STAT5, and STAT6 genes in autoimmune encephalomyelitis (EAE) (Zaheer et al. 2007). In our current study, we found that significant upregulation of COX2 and iNOS in BV2 cells correlated with increased apoptotic cell death, that may be due to activation of the JAK-STAT pathway. Genetically targeted knockdown of certain genes such as FoxO (FoxO3) impairs the release of inflammatory mediators including interferon-γ (IFN- γ), interleukin-10 (IL-10)

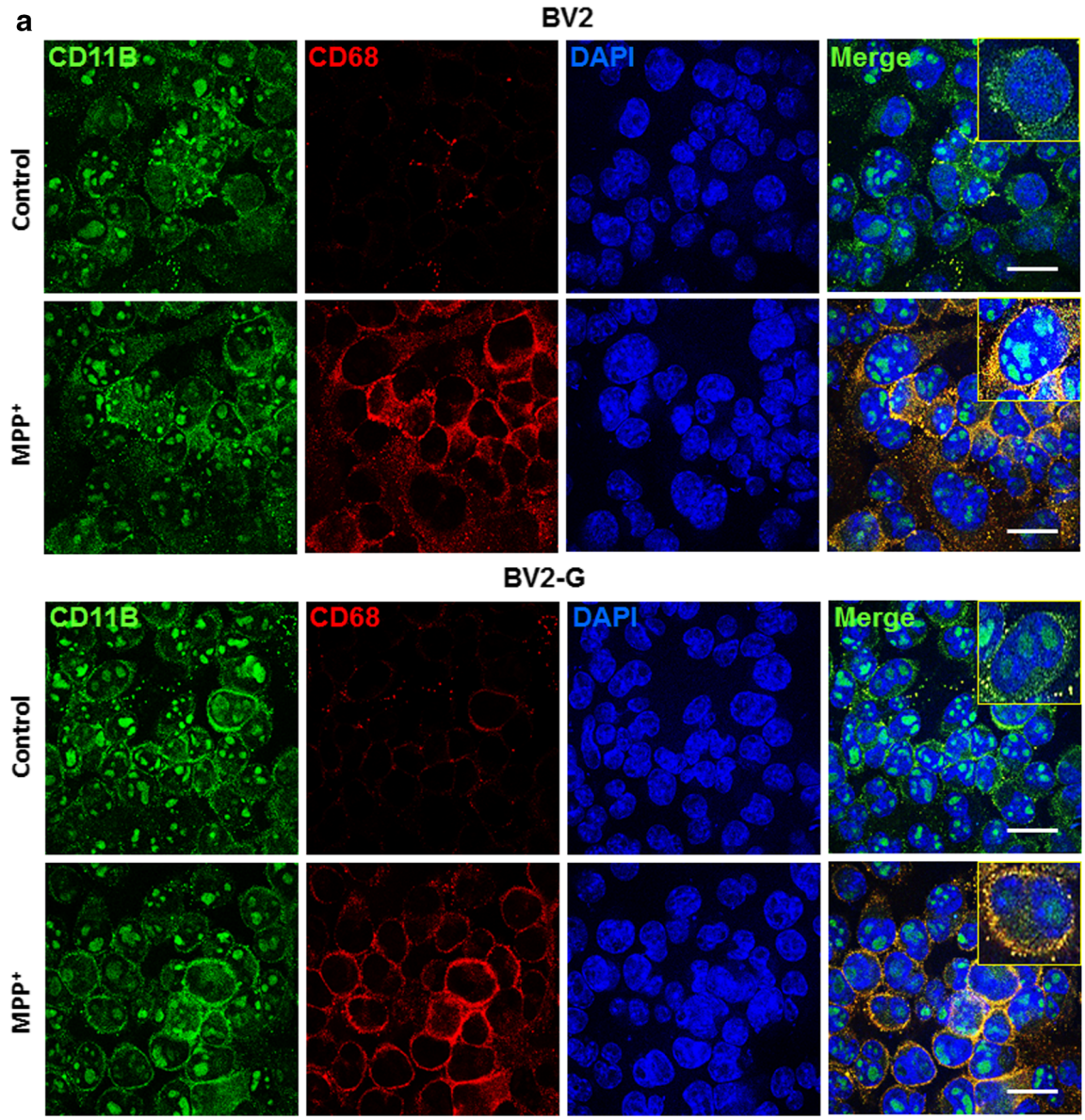

Fig. 8 Effect of GMF on MPP^+ induced activated microglial markers CD11b and CD68 expression in BV2 microglial cells. The BV2 and BV2-G microglial cells were seeded in T25 culture flask and poly-D-lysine coated coverslips. Then the cells were incubated with MPP^+ (0.1 mM) for 24 h. After the incubation period, cells were washed with PBS and prepared for immunocytochemistry (**a**) and FACS analysis (**b**) of CD11b and CD68. Reduced expression levels of CD11b and CD68 were found in BV2-G cells when compared with BV2 microglial cells. FACS results shows the CD68-APC-A750-A (red fluorescent) versus CD11B-FITC-A (green fluorescent) derived fluorescence of BV2 and GMF deficient BV2-G cells in the plot panels (**b**). All data represent one of at least three separate experiments

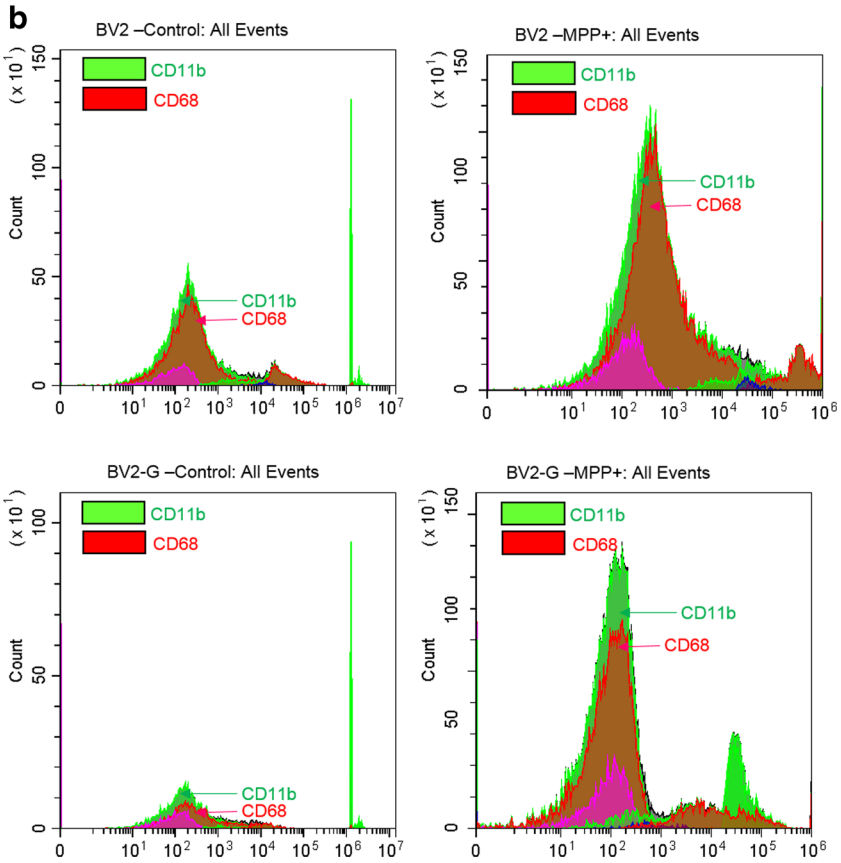

Fig. 8 (continued)

monocyte chemoattractant protein-1 (MCP-1) of the innate immune cells (Depaolo et al. 2005; Hwang et al. 2011; MacNamara et al. 2011). Previously, we reported that absence of GMF reduced MPP^+ induced proinflammatory protein expression such as TNF-α, interleukin-1β (IL-1β), IL-17, IL-33, and chemokine such as (C-C motif) ligand 2 (CCL2) in GMF deficient murine mast cells, as well as in neuro-glial cells (Kempuraj et al. 2013; Kempuraj et al. 2018). Recently, we reported that genetically trageted knockout of GMF by CRISPR/Cas9 gene editing technique in BV2 microglial cells reversed the AD associated pathogenesis via p38MAPK pathway (Raikwar et al. 2018b). In this study, we found that HO-1, COX2 and NOS2 expressions were significantly increased in MPP^+ treated BV2 microglial cells. In addtion, CD68 and CD11b markers were enhanced in MPP^+ treated BV2 microglial cells. However, absence of GMF causes reduction in expression of those proteins thus enhancing the anti-inflammtory actions in BV2 microglial cells, in concurrence with previuos reports (Valente et al. 2004; Aggarwal and Shishodia 2006; Zaheer et al. 2008; Hwang et al. 2011; Zaheer et al. 2011). The current experiments were designed to investigate the possible mechanism of action of GMF-dependent PD pathogenesis via oxidative stress and mitochondrial biogenesis. Using the specific microglial markers CD11b and CD68, and NRF2/HO-1 - dependent ferritin activation pathway we found that genetic knockoutof GMF by CRISPR/Cas9 prevented microglial activation by reducing the oxidative stress- dependent mitochondrial stress with augmented NRF2/HO-1 dependent ferritin activation.

Conclusion

In the present study, we demonstrate that targeted knockout of GMF through CRISPR/Cas9 inhibits microglial cell activation by reducing oxidative stress, Ca^{2+} flux by limiting NRF2 translocation. Further, absence of GMF reduces NRF2 translocation -dependent HO-1and ferritin activation ultimately leading to limiting the CD68 and CD11b positive cells. Absence of GMF suppresses the MPP^+ induced NRF2 dependent HO-1/ferritin activation. These results highlight the beneficial properties of GMF-gene editing by CRISPR/Cas9 and also indicate that GMF targeted drug could be used for therapeutic intervention in neurodegenerative disorders. The present study highlights that combinational regulation of GMF may potentiate microglial cells-mediated neuroprotection in PD.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there are no conflicts of interest.

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