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Costunolide inhibits proinflammatory cytokines and iNOS in activated murine BV2 microglia

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1. ABSTRACT

Costunolide, a sesquiterpene lactone present in *Costus speciosus* root exerts a variety of pharmacological activity but its effects on neuroinflammation have not been studied. Microglia, the resident phagocytic cells in the central nervous system respond to neuroinflammation and their overwhelming response in turn aggravate brain damage during infection, ischemia and neurodegenerative diseases. In this study, we report the effect of Costunolide on the production of proinflammatory mediators and mechanisms involved in BV2 microglial cells stimulated with LPS. Costunolide attenuated the expression of tumour necrosis factor-alpha, interleukin-1,6, inducible nitric oxide synthase, monocyte chemotactic protein 1 and cyclooxygenase 2 in activated microglia. This Costunolide-mediated inhibition was correspondent with the inhibition of NFkappaB activation. It has been further shown that Costunolide suppressed MAPK pathway activation by inducing MKP-1 production. Collectively our results suggest that Costunolide shows an ability to inhibit expression of multiple neuroinflammatory mediators and this is attributable to the compounds inhibition of NFkappaB and MAPK activation. This novel role of Costunolide upon investigation may aid in developing better therapeutic strategies for treatment of neuroinflammatory diseases.

2. INTRODUCTION

Microglia, the resident immune cells in the central nervous system (CNS) contribute to about 10% of the total glial cell population (1). Microglia activation is a protective mechanism wherein the activated cells rapidly proliferate, become hypertrophic and express a variety of proinflammatory cytokines such as tumor necrosis factor-alpha(TNF-alpha), interleukins- IL-1,IL-6 (2), and chemokines such as monocyte chemotactic protein-1 (MCP-1) that regulate inflammation and migration of microglia to the injury sites in CNS thereby amplifying neuroinflammation (3,4). These responses regulate tissue repair and recovery in the early phase of neural damage. Adding to this immunological cascade, activated microglia also release cytotoxic free radicals such as superoxide and nitric oxide which are products of the enzymes NADPH oxidase and inducible nitric oxide synthase (iNOS) respectively. These molecules are well known to be involved as regulatory mediators in pathological reactions and inflammatory response pathways in the CNS.

The excessive release of the wide array of inflammatory mediators and cytotoxic molecules resulting from the chronic activation of microglia is implicated for the neurotoxicity underlying many neurodegenerative diseases including Parkinson's disease, Alzheimer's,

Multiple sclerosis and HIV dementia (5-8). Hence it is of paramount importance and an important strategy in neurodegenerative disease therapy to identify and study the mechanism of the molecules which can curtail the neuroinflammation mediated by activated microglia. Though several anti-inflammatory drugs have been shown to diminish neuroinflammation, a very few have direct functional effects on microglial activity (9). Moreover there is a surging interest in naturally occurring plants and plant derived compounds which prove to be highly efficous drugs with less adverse side effects.

Costunolide (Cos), a well known sesquiterpene lactone present in many medicinal plants, exhibits a variety of biological activities including antifungal (10), antiviral (11), anticarcinogenic (12) and also inhibits telomerase activity in human breast carcinoma cells (13). Although Costunolide from several plant resources have been reported for a variety of actions, the effect of Costunolide on neuroinflammation remains unknown. The anti-inflammatory activity of Costunolide isolated from the bark of *Magnolia sieboldii* mediating NOS inhibition has been reported in murine macrophage J774.1 (14) and similar functional activity of Costunolide isolated from *Saussurea lappa* has been studied in Raw 264.7 (15). But these information provide little insight on the compound's activity on microglial cells, which by their localization in the neuronal environment and morphological traits differ from other peripheral macrophages (16). Therefore this study investigated the effect of Costunolide isolated from the hexane extract of *Costus speciosus (Koen ex.Retz).Sm* roots on the activated BV2 microglial cells. Our findings indicate for the first time, Costunolide's influence on the release of aforementioned inflammatory mediators from BV2 microglia stimulated with LPS, a bacterial endotoxin.

3. MATERIAL AND METHODS

3.1. Murine BV-2 Cell Culture

Murine BV-2 microglia cells were cultured in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic, at 37°C in humidified 5% CO2 atmosphere. The confluent BV-2 cells were plated in six-well plates with poly-L-lysine coated cover slips at a density of 1.0 $3x10^6$ cells/ml cells. For immunocytochemistry, cells were plated at 2.5 $x10^5$per well on a 24 multi-well culture dish and incubated at 37ºC in a humidified incubator for 24h. For immunoblotting and RNA isolation, cells were plated at $1x10^6$ cells per $75cm^2$ flask. The following day, the cells were subjected to different treatments. The purity of microglia was assessed histochemically using *Lycopersicon esculentum* (tomato) lectin (1:100; Sigma, St. Louis, MO; Cat. No. L0401),a widely used marker for rat microglia. Glass coverslips (12mm) coated with L-lysine were used for histochemical analysis. The cellular morphology was examined under a phase-contrast microscope.

3.2. Treatment of BV2 cells

All experiments were started 24 hours after plating. In order to study the effects of LPS and Costunolide on microglial cells, the cells were washed with PBS and incubated either with LPS (1µg/ml), Costunolide (1µM) or LPS plus Costunolide. Cells were pretreated with 1uM Costunolide 30 minutes prior to LPS (1µg/ml) treatment. DMSO diluted in DMEM was used to dissolve Cos at its various concentrations (0.1-3 µM). In all the experiments, control cells were treated with DMSO as the vehicle at final concentrations used in experimental cultures. The final concentration of DMSO in all assays did not exceed 0.1%.

3.3. Cytotoxicity Assay

Cytotoxicity was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTS) assay. BV-2 cells were seeded in 96-well plates and treated with various concentration of Cos (0.1µM – 10µM) and were cultured in a CO_2 incubator for 3 and 6hrs. After removing the media, MTS was added and incubated at 37ºC for 4h. DMSO (100**µ**l) was added to each well to dissolve the blue crystal. After incubating at room temperature for 30 min, the reactants were measured by a microplate reader (UV max, Molecular Devices, USA) at 540 nm.

3.4. Immunofluorescence labeling

For immunofluorescence labeling, microglial cells seeded on a 24-multi-well culture dish at a density of 2.5 $x10^5$cells/well, were treated with LPS (1ug/ml) in the presence or absence of Cos (1µM) for 3h. After the incubation, the cells were fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 30 min, blocked in 1% bovine serum albumin (BSA) in 0.1M PBS containing 0.1% Triton X-100 for 30 min, and subsequently incubated with the specific primary antibodies overnight. Double immunofluorescence labeling study was carried out between DAPI and different primary antibodies directed against TNF-alpha (1:100; Chemicon, CA; Cat. No. AB1837P), NF-kappaB, (p65 subunit, 1:100; Santa Cruz, CA; Cat. No. SC-109) iNOS (1:400; Chemicon, Temecula,CA; Cat. No. AB5382,) MKP-1 (Santa Cruz Biotechnology; Cat No.sc-1102) and MCP-1 (1 : 100; Santa Cruz Biotechnology, USA; Cat. No sc-1785) in BV2 cultures. Subsequently cells were incubated with fluorochrome-conjugated secondary antibodies and examined under a Carl Zeiss LSM 410 confocal microscope. Quantitative analysis of the extent of NF-kappaB translocation was carried out by counting 8 fields of 50 cells in the control, LPS treated microglia cultures in the presence or absence of 1µM Cos. The cells with blue nuclei were counted as NF-kappaB translocation- negative; the cells with pink were counted as NF-kappaB translocation-positive. Each experiment was done in duplicate. The number of cells positively stained for NF-kappaB in the nucleus in 3 randomly selected microscopic fields (400x) in each section were counted, and the mean positive cell number of 3 fields was determined.

3.5. RNA isolation and real-time RT-PCR

Microglial cells were treated with LPS (1µg/ml), LPS + Cos (1µM), Cos(1µM) for 3 h. Total RNA was extracted from these cultures using RNAeasy mini kit (Qiagen, Hilden, Germany; Cat. No. 75161) according to the manufacturer's instructions and quantified spectrophotometrically. The reaction mixture containing

Table 1. Primer sequences used for RT–PCR

	Sense	Antisense	Size (bp)
TNF-alpha	CGTCAGCCGATTTGCTATC	CGGACTCCGCAAAGTCTAAG	205
IL-1	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG	229
IL-6	AACGATGATGCACTTGCAGA	GGAAATTGGGGTAGGAAGGA	276
iNOS	GCTTGTCTCTGGGTCCTCTG	CTCACTGGGACAGCACAGAA	217
NFκB	CTGACCTGAGCCTTCTGGAC	GCAGGCTATTGCTCATCACA	177
Cox2	CCCCCACAGTCAAAGACACT	CTCATCACCCCACTCAGGAT	191
MCP-1	TCTCTTCCTCCACCACTATGCA	GGCTGAGACAGCACGTGGAT	91
β-actin	TCACCCACACTGTGCCCATCTACGA	GGATGCCACAGGATTCCATACCCA	314

2µg of RNA, 2.5 µmol/L of oligo (dT) primer (Promega, Madison, WI, USA; C1101), and 200U of Molony Murine leukemia Virus Reverse Transcriptase (Promega) in a total volume of 25µl was incubated for 1h at 42ºC for reverse transcription, and the reaction stopped by heating for 10min at 70ºC. Aliquot (0.5 µl) of the each reverse transcription product was added to the reaction mixture (20µl) containing LightCycler- FastStart DNA Master SYBR Green I, 0.5µmol/L of each primer corresponding to the mouse TNF-alpha, IL-1,6, iNOS , NF-kappaB, Cox2, MCP-1, and β-actin, and 4 mmol/L $MgCl_2$ to amplify each of the genes in a LightCycler (Roche, Mannheim, Germany). The oligonucleotide primer sequences used for TNF-alpha, IL-1,6, iNOS, NF-kappaB,Cox2, MCP-1, and β-actin are listed in Table 1. After pre-incubation at 95ºC for 10 min, the PCR was performed as follows: 35 cycles of denaturation at 95ºC for 15s, annealing at 60/62 ºC depending on the primer sets, and elongation at 72ºC for PCR product size per 25s. The expression level of each gene was quantified, expressed as Ct, the cycle number at which the LightCycler system detected the upstroke of the exponential phase of PCR product accumulation, and normalized by the level of expression of β-actin in each individual sample. The mRNA expression levels of various genes studied in the experimental groups and control groups were compared using the 2-[DDCt] method (17)**.** The fold changes in the mRNA expression were calculated in comparison to that of control.

3.6. Nitrite assay

The nitric oxide production was quantified colorimetrically using the nitric oxide colorimetric bioassay kit (Cat No. N2577-01,US Biological, MA, USA). NUNC Maxisorp 96 well plate was coated with 200 µl/well of diluted assay buffer. The supernatant of each (80 µl) collected from cultures of BV2 cells treated differentially (LPS, COS,LPS+COS) for 3h was added to each well and mixed with 10µl of the reconstituted enzyme Cofactor mixture and 10µl of the reconstituted nitrate reductase mixture sequentially. The plate was covered and incubated at room temperature for 1–4 h. Griess Reagent R1 (50µl/well) and Griess Reagent R2 (50 µl) were added to each well immediately. Color was allowed to develop for 10 min at room temperature and the absorbance was read using a microplate spectrophotometer at 540 nm. The standard curve of Nitrate standards was used to calculate the nitric oxide production of the samples. Bacground nitrite was subtracted from experimental values.

3.7. NF-kappaB Transcription factor assay

Nuclear extraction was done from the cultures of BV-2 cells differentially treated (Cos, LPS, LPS+Cos) for 3h, using Nuclear Extraction kit(Chemicon, Cat.No.2900) as per manufacturer's protocol. NF-kappaB p65 DNA binding activity was specifically assayed using the Transcription Factor colorimetric kit (Millipore Cat.No. 70-520) according to the recommended protocol.

3.8. Western Immunoblot Assay

BV2 microglial cells were seeded on a 75-cm^2 flask at a density of 1.2 x 10^6 cells/ml and treated with LPS(1ug/ml) in the presence or absence of Cos (1µM). Protein extracts from microglial cultures subjected to various treatments for different periods were prepared using the protein extraction kit (Pierce; Cat. No. 78501) and protease inhibitor cocktail kit (Halt; Cat. No. Prod#78410), and were quantified using a protein assay kit (Bio-Rad, Hercules, CA; Cat. No. 500- 0007). Aliquots of protein extracts (20 µg each) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride transfer membranes. Membranes were incubated with MKP-1 primary antibody (Santa Cruz Biotechnology; Cat. No. sc-1102) and mouse anti-actin (Abcam, Cambridge, UK, Cat. No. ab18061) overnight at 4°C. The blots were subsequently incubated for 1h with secondary antibodies conjugated with horseradish peroxidase, visualized by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ) and analyzed with Quantity One software, version 4.4.1.

3.9. Statistical analysis

Data used for statistical analysis were derived from three to four independent experiments and presented as means +/- SD. Statistical significance was evaluated by either the Student's test or one-way ANOVA analysis of variance. Results were considered as significant at $p < 0.05$

4. RESULTS

The murine microglial cell line, BV-2 cells were stained with lectin, a known marker for microglia (Figure 1A-D). Since it is generally accepted that microglial cells are activated to certain extent during the preparation procedure for culture (18), microglia *in vitro* exhibit reactive phenotype and produce some amount of cytokines that are specific for reactive microglia. In this study, the BV2 microglia was stimulated by LPS for 3h and 30 min pre-treatment with Cos. The microglial cells treated with LPS appeared with abundant cytoplasmic inclusions (Figure 1C) whereas those treated with Cos alone or Cos and LPS did not show any morphological change, in comparison to the control cells (Figure 1B, D).

4.1. Effect of Costunolide on BV2 microglial cell viability

To verify the toxicity of Cos in the BV2 cultures, an MTS assay was performed to determine the viability of

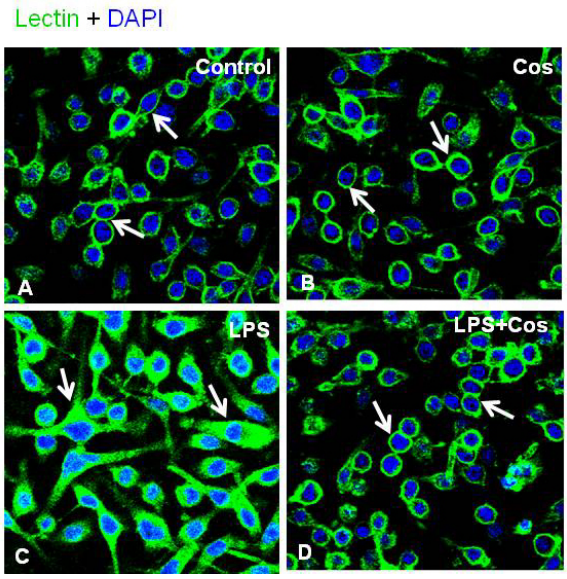

Figure 1. Confocal images of BV-2 microglia stained with lectin (green) and DAPI(blue), a nuclear marker from untreated culture (A), and cultures treated with Cos (B), LPS for 3hrs (C), and Cos+LPS (D). A majority of the cells treated with LPS appear to be activated and exhibit abundant cytoplasmic inclusions. Scale bar = 50 uM.

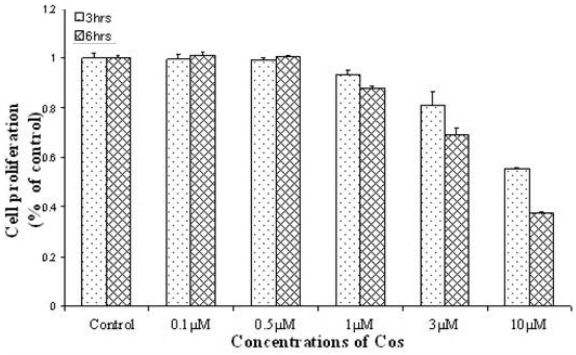

Figure 2. Effect of Cos on viability of BV2 cells. The MTT assay of BV-2 cells after incubation with indicated concentrations of Costunolide for 3 and 6 hours, showed that Cos did not effect cytotoxicity up to 1µM concentration and with further increase in the concentrations, the viability was slightly affected. The data represents the mean +/- SD of 4 independent experiments. Significant differences between Control *vs.* LPS; LPS vs. LPS+Cos groups are indicated by * $p<0.05$, ** $p<0.01$

cells after treatment with various concentrations of Cos (0.1µM – 10µM) for 3 h and 6 h. The viability of cells treated with up to 1µM concentration of Cos was near normal levels compared to untreated controls (Figure 2). However cell viability appeared to be decreased at 3µM and 10µM concentrations of Cos. Further, Cos treatment up to 3 h showed markedly less cell toxicity and hence this time point was chosen for 1µm Cos treatment for all further studies.

4.2. Inhibitory effects of Costunolide on LPS-induced TNF-alpha, IL-1, IL-6 mRNA expression in BV2 cells

To examine the effects of Cos on the LPS-induced production of inflammatory mediators we analyzed Cos mediated changes in LPS-induced TNF-alpha, IL-1, IL-6 expression. The effect of 1µM Cos on TNF-alpha mRNA expression in microglia treated with LPS have been analyzed by the real time RT-PCR (Figure 3A). LPS induced TNF-alpha mRNA expression level in BV2 cells to 18 folds and interestingly this increase in expression was reduced (10 folds) significantly by preincubation of cells with Cos. In line with this, LPS treatment markedly induced the TNF-immunoreactivity in the majority of microglial cells as detected by immunocytochemistry (Figure 3D). The TNF-alpha positive cells exhibit increased size and abundant cytoplasm with cytoplasmic inclusions which are a phenotypic characteristic of reactive cells. TNF-alpha immunoreactivity was hardly detectable in the untreated control and Cos treated microglial cells (Figure 3B, C). Cos markedly reduced the intensity of TNF-alpha immunostaining in LPS treated cells (Figure 3E). It is also noted that these cells displayed non-reactive phenotypic features as in the untreated cultures. The quantitative real time RT-PCR analysis (Figure 3F,G) further showed that pretreatment of microglia with Cos for 30min significantly reduced the LPS-induced expression of IL-1 (by 200 fold) and IL-6 (by 50 fold). It is to be noted that Cos alone had no any significant effect on the expression of these cytokines in BV2 cells.

4.3. Effect of Costunolide on the expression of iNOS and NO release in LPS-stimulated BV2 microglia

Next we examined the potential effects of Cos on LPS-mediated iNOS and NO release. The inhibitory effect of Cos on LPS-induced gene expression of iNOS in microglia has been analyzed by the real-time RT-PCR (Figure 4A). LPS induced the iNOS mRNA expression level to 15 folds in BV2 microglia and pretreatment of cells with Cos suppressed the induction of iNOS expression in LPS treated microglial cells by 50%. The immunohistochemical analysis revealed that iNOS immunoreactivity was hardly detectable in the untreated and Cos treated microglial cells (Figure 4B,C). In cells treated with LPS for 3h, the number of intensely stained iNOS positive cells was found to be increased markedly (Figure 4D). However, pretreatment of cells with 1µM Cos for 30min reduced the intensity of iNOS positive staining and number of iNOS positive cells in the LPS-treated microglial cells (Figure 4E) Nitric oxide assay (Figure 4F) showed that LPS treatment led to the increase in production of NO in BV2 cells. In untreated cells and Cos treated cells, the NO levels remained at baseline levels and incubation of BV2 cells with Cos prior to LPS treatment brought back the elevated NO production to basal level.

4.4. Attenuating role of Costunolide on Cox gene expression and NF-kappaB translocation from cytoplasm to nucleus in microglia exposed to LPS

Since there are significant upregulation of gene expression of proinflammatory cytokines and increased NO release in LPS-activated microglia and a marked inhibition of this by Cos treatment, we decided to investigate the

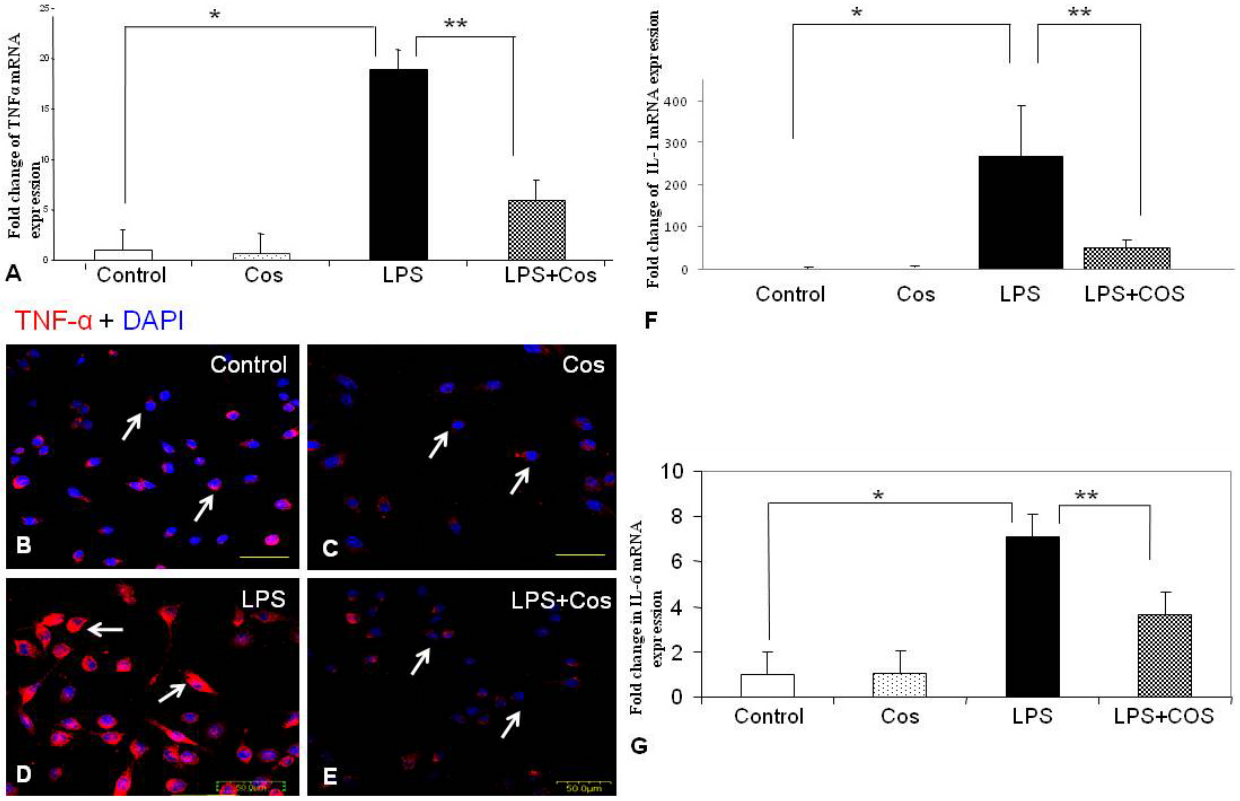

Figure 3. Cos inhibits the TNF-a, IL-1 and IL-6 expression in microglia stimulated by LPS. A) Real time PCR analysis demonstrates that the TNF-alpha expression level in microglial cells treated with LPS is elevated in comparison to that of control. Cos significantly decreased the mRNA expression of TNF-alpha in microglial cells treated with LPS. B-E) Confocal images of microglial showing TNF-alpha immunoreactivity (red) and DAPI (blue), the nuclear marker. Note that TNF-alpha immunoreactivity is hardly detectable in both untreated and Cost treated BV-2 microglial cells (B,C respectively), while majority of the LPS-induced cells exhibit intense TNF-alpha immunoreactivity (D). Cos decreased the staining intensity of TNF-alpha immunoreactivity in the microglial cells treated with LPS (E). F-G) Real time RT-PCR analysis demonstrating the inhibitory effects of Cos on IL-1, IL-6 mRNA expression induced by LPS for 3h in BV2 microglia cells. After the treatment with LPS, the mRNA expression levels of IL-1 (3F) and IL-6(3G) were elevated in comparison to that of control. Pretreatment of cells with Cos before LPS induction caused a 200 and 50 fold reduction in gene expression of the IL-1 and IL-6, respectively. Data represent mean +/- SD (n=4).Significant differences between Control *vs.* LPS; LPS vs. LPS+Cos groups are indicated by * $p<0.05$, ** $p<0.01$

effects of Cos on the expression of NF-kappaB,as NF-kappaB is an essential transcription factor regulating the induction of iNOS and cytokines like TNF-alpha, IL-1 and IL-6. We have also examined the effect of Cos on the NF-kappaB translocation in LPS-treated microglia. In the untreated culture, NF-kappaB was localized in the cytoplasm but not in the nucleus (Figure 5A). LPS treatment induced NF-kappaB translocation from cytoplasm to the nucleus of the microglia (Figure 5C), however this translocation of the transcription factor was reversed in the stimulated cells in the presence of 1µM Cos (Figure 5D). The number of cells with translocated NFkB in their nucleus was highest in the LPS treatment, whereas significant reduction in the nuclear translocation was seen with LPS+Cos treatment (Figure 5E). The transcription factor assay further confirmed that the highest amount of p65, the active subunit of NFkappaB was found in the nuclear extract of LPS treated cells while the Cos pretreatment before induction by LPS decreased the p65 translocation to nucleus (Figure 5F). Quantitative mRNA expression analysis by real time RT-PCR revealed that the LPS-induced NF-kappaB mRNA expression level was suppressed to near normal levels in the cells pretreated with Cos (Figure 5G).

Previous studies have well established that the expression of the COX-2, the key prostaglandin E2 synthesising enzyme at inflammatory sites, is transcriptionally regulated by NFkappaB (19). Our next aim was to see if the Cos mediated inhibition of NFkB transcription activity also affected Cox-2 expression. Our results show that Cos inhibited the LPS-induced Cox-2 expression in BV2 microglia (Figure 5H) following the similar trend of NF-kappaB repression. Taken together, the results obtained demonstrate that Cos inhibited the proinflammatory pathway in association with NF-kappaB activity.

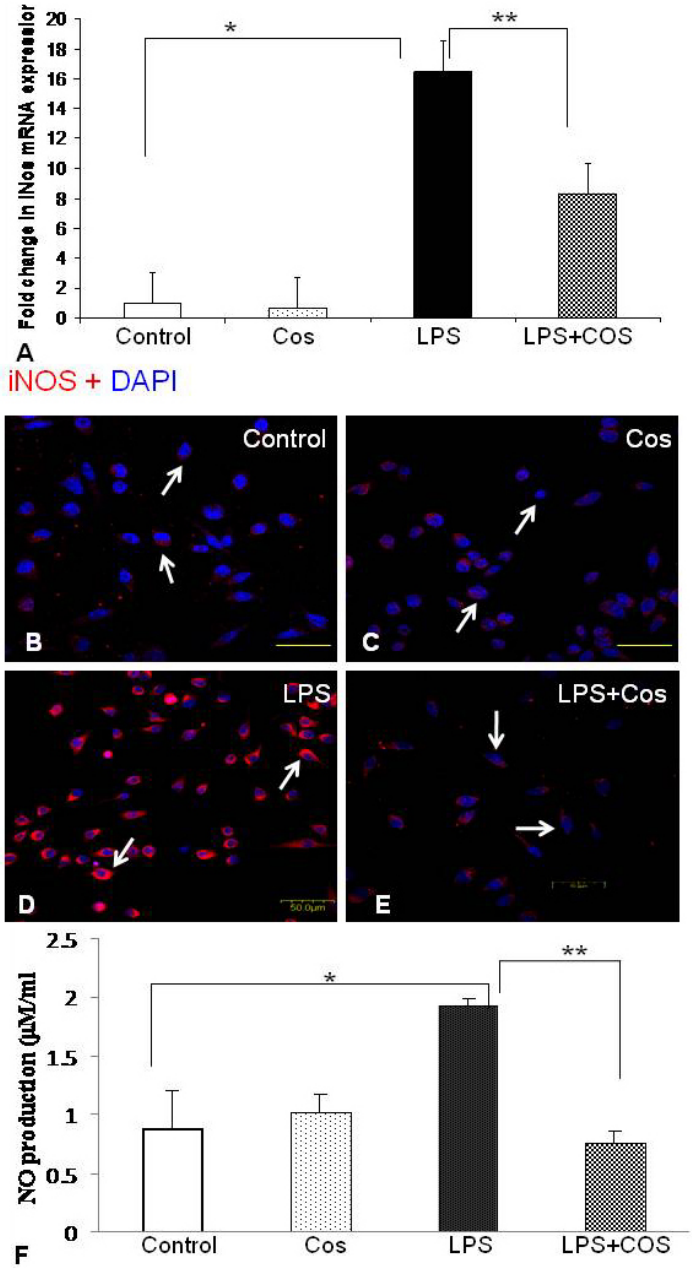

Figure 4. Inhibition of iNOS expression and NO production by Cos in LPS stimulated BV2 cells. The mRNA expression of iNOS was remarkably less in untreated and Cos-treated BV-2 microglial cells but substantially increased after 3 h LPS treatment. However Cos inhibited the iNOS expression significantly in LPS stimulated BV-2 cellsm in comparison to that of LPS-treated cells (4A). Data represent mean +/- SD (n=4). Significant differences between Control vs. LPS; LPS vs. LPS+Cos groups are indicated by * P<0.05, ** P<0.01. B-E) Immunostaining of microglia with iNOS (red) and DAPI (blue). Note the enhanced expression of iNOS in LPS treated cells (4D).Cos treatment 30 min prior to LPS decreases iNOS expression markedly (4E). 4F) The nitrite assay analysis indicates that the significantly increased level of nitrite as a consequence of LPS induction was brought down to near normal levels by Cos.

4.5. Costunolide mediated inhibition of MCP-1 through induction of MKP-1 in activated BV2 microglia

Considering our findings that Cos inhibited the NF-kappaB mediated inflammation, we were prompted to investigate the effect of Cos on MAPK pathway, which has been shown to regulate the expression of inflammatory genes in activated microglia(20-22). Thus the effect of Cos on the mRNA expression level of MCP-1, the chemokine was analyzed by the real time RT-PCR. Treatment of BV2 cells with LPS caused a 19 fold induction of transcriptional activity of MCP-1; whereas the cells treated with LPS+ Cos showed 14 folds downregulation of MCP-1 expression. The

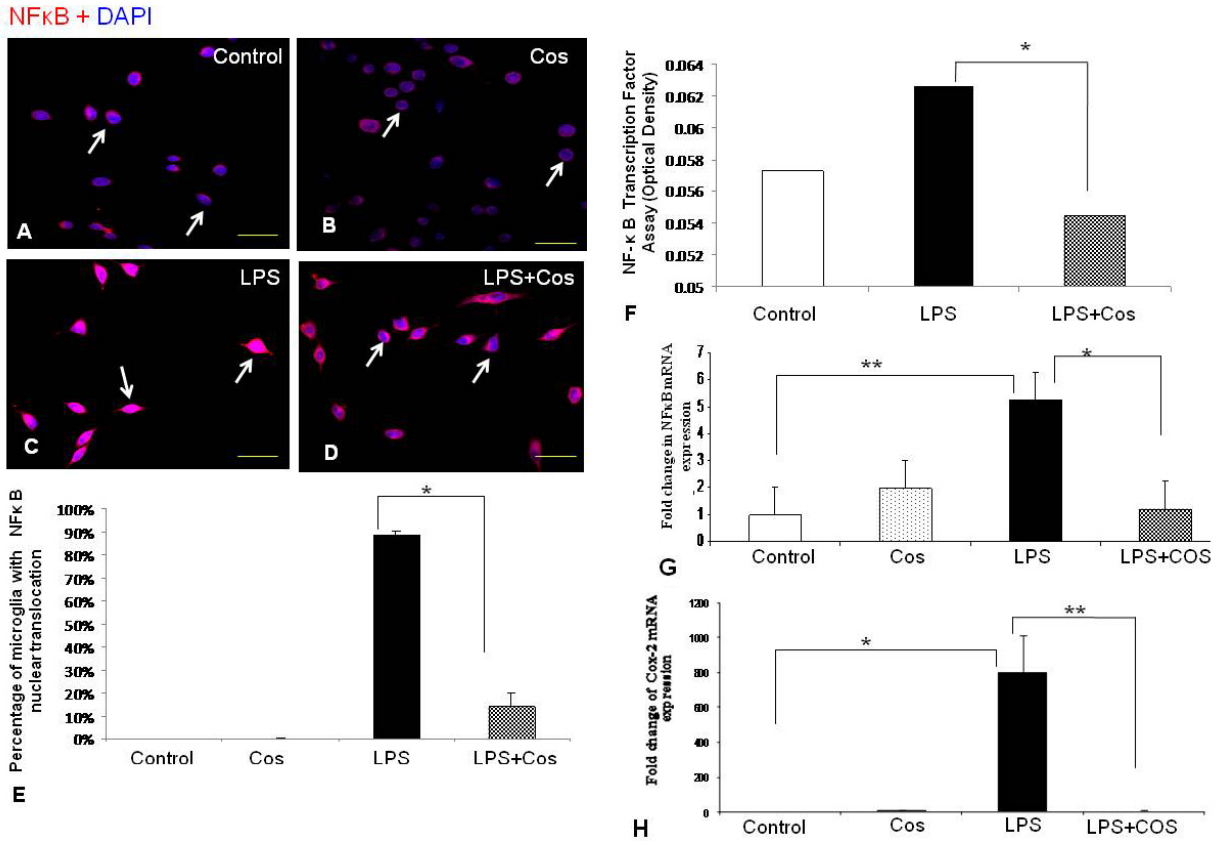

Figure 5. Repression of NF-kappaB nuclear translocation and Cox2 expression by Cos. A-D) Confocal images of microglia immunostained for NF-kappaB (red) and DAPI (blue). In the control and Cos treated groups (A,B) NF-kappaB expression is restricted to the cytoplasm of microglia (arrows). Translocation of NF-kappaB into the nuclei (stained with DAPI) is evident in microglia treated with LPS (C). Cos (1uM) decreased the translocation of NF-kappaB into the nucleus in the LPS stimulated cells(D). The percentage of NF-kappaB positive cells counted within a defined area clearly denotes the nuclear translocation trend(E) Real time RT-PCR analysis shows that Cos decreased the mRNA expression of NF-kappaB (G) and Cox-2 (H) in BV-2 microglia stimulated with LPS when compared to the higher expression levels in cells stimulated by LPS alone for 3 h. (F) The EZ-TFA kit was used to measure the DNA binding activity of the transcription factor, NFkappaB p65, in LPS-stimulated BV-2 nuclear extract. The assay detects NF-kappaB p65 factor in the nuclear extract by binding it to a positive control DNA probe, LPS activated cells shows maximum binding while minimal signal was observed in LPS+Cos treatment. Data represent mean +/- SD (n=4). Significant differences between Control vs. LPS; LPS vs. LPS+Cos groups are indicated by * $p<0.05$, ** $p<0.01$. Scale bar (A-D), 50uM.

MCP-1 mRNA expression level appeared to be unaltered in Cos-treated BV2 cells in comparison to that of untreated cells (Figure 6A). Immunocytochemical analysis revealed that the MCP-1 expression was weak and hardly detectable in control and Cos-treated cells. However, the staining intensity of MCP-1 was increased remarkably in BV-2 cells treated with LPS and that the increase in staining intensity was substantially decreased in cells treated with both LPS and Cos (Figure 6B-E).

Since several reports (20-22) have demonstrated that the proinflammatory response is largely mediated by the MAPK pathways besides NF-kappaB dependent pathways, we investigated whether Costunolide suppressed the release of proinflammotory mediators *via* induction of MAP kinase phosphatase-1(MKP-1) which is a negative regulator of the MAPK pathway. The immunohistochemical analysis revealed that the number of intensely stained MKP-1 immunopositive cells were more in culture treated with Cos+ LPS than that in the LPS treated and untreated microglia (Figure 7A-D). The induction of MKP-1 expression by Cos in activated BV2 microglia was further confirmed by Western blot analysis (Figure 7 E,F).However, there is no significant change in the level of MKP-1 protein expression in the LPS+Cos microglial cells compared to cells treated only with LPS.

5. DISCUSSION

Microglial cells are the primary immune effector cells responsible for the inflammation of the neurological diseases (23). Microglial activation and associated induction of proinflammatory cytokines and chemokines have been well documented in various acute and chronic

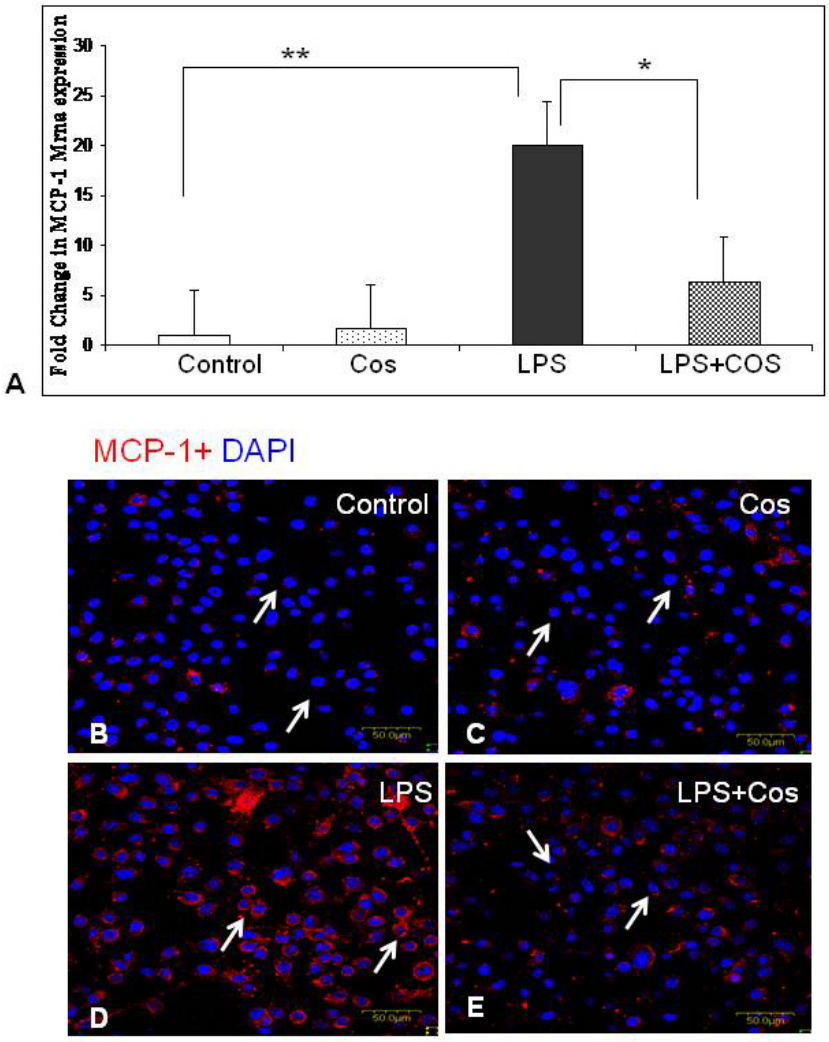

Figure 6. Inhibition of MCP-1 and induction of MKP-1 in BV-2 microglial cells by Costunolide. A) Real time RT-PCR analysis demonstrates that MCP-1 mRNA expression level in microglial cells treated with LPS is clearly increased to that of control. Cos decreased the expression of MCP-1 significantly in microglial cells treated with LPS. B-E) Immunofluorescence images show that MCP-1 expression is weak and hardly detectable in untreated (B) and Cos treated groups(c) but is increased upon LPS treatment (D). However, Cos treatment limited the increase in MCP-1 immunoreactivity in LPS-activated microglia cells (E). Data represent mean ± SD (n=4).Significant differences between Control vs. LPS; LPS vs. LPS+Cos groups are indicated by * $p<0.05$, ** $p<0.01$.

neuropathological conditions (24). The chemotaxis of microglial cells mediated by the chemokines is responsible for amplifying the CNS inflammation (25) since such inappropriately or persistently activated microglia could aggravate the neuronal damage. Therefore it is important to develop a novel class of neuroprotective agents and understanding mechanisms that modulate inhibition of the neurotoxic effect of microglial cells would offer new specific therapeutic avenue for immune mediated brain disorders. Several mediators such as Vitamin E (21), retinoic acid (3), dexamethasone (4), minocycline (26) have been shown to be involved in the inhibition of microglial activation. In the present study, we investigated the potential involvement of a natural plant compound, Costunolide in the inhibition of proinflammatory and neurotoxic effects of activated microglia stimulated by LPS.

Costunolide has been widely used to treat skin diseases, asthma, worm infection, diabetes, bronchitis and inflammation. We have previously reported the hypoglycemic and hypolipidemic effect of Costunolide isolated from the hexane extracts of Costus roots (27). Costunolide, has also been reported to inhibit the basic inflammatory signaling pathway in peripheral macrophages (28,15). However, there were no reports on the potential benefit of this compound in controlling microglia-mediated neuroinflmmation. The present study for the first time demonstrate the Cos-mediated inhibition of inflammatory response of activated BV2 microglia. Of great interest is

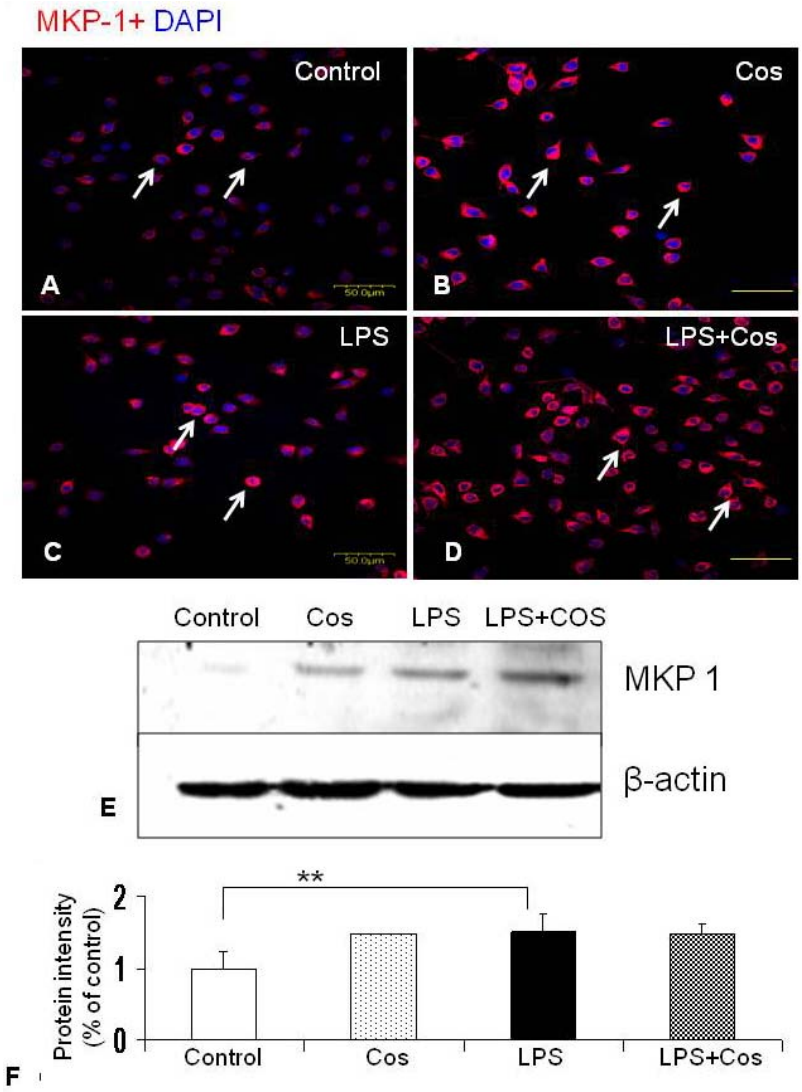

Figure 7. A-F) Confocal images of microglia showing MKP-1 immunostaining. Microglial cells treated with Cos (B), LPS(C) and the Cos+LPS (D) shows an increased number of MKP-1 expressing cells compared to that of control (A). E, F) Western blot analysis also showed that LPS stimulated MKP-1 protein expression in microglial cells. Pre-incubation of cells with Cos 30min prior to LPs treatment further enhanced MKP-1 protein expression. Data represents the mean signal intensity of MKP-1 relative to control (mean ± SD n=3 independent experiments). Significant difference between Control vs. LPS is indicated by ** $p<0.01$. There was no significant difference between LPS vs. LPS+Cos.

our finding that Cos negatively regulates the expression of TNF-alpha, IL-1, IL-6, MCP-1, iNOS, and Cox2 and the,NF-κB nuclear translocation in activated microglia. Further, Cos suppresses the MAPK signaling pathway by upregulating the expression of MKP-1 .

LPS has been widely used for the activation of microglia, which in response release excessive amount of neurotoxic and proinflammotory mediators such as TNF-alpha and IL-1, 6 (29,30,23). The present study shows that Cos significantly inhibits this LPS induced TNF-alpha and IL-1, 6 production in activated microglia. TNF-alpha has been shown to be responsible for the secondary damages of inflammatory disorders (31) and it has been found that TNF-alpha and cytokines exacerbate the neurotoxic environment in Alzheimer's disease (32). Given the fact that high levels of TNF-alpha and IL-6 in the CNS correlate with acute neurodegenerative pathology, it is of promising interest that Cos reversed these proinflammatory toxic effects of microglia. Besides the compound did not exhibit toxicity to the BV2 cells, as the viability was found to be 90% by the MTS assay indicating potential safe usage

Another cytotoxic molecule released by the activated microglia is nitric oxide (NO) synthesis of which is catalyzed by the inducible nitric oxide synthase (iNOS) (33). NO is a major regulator of acute inflammatory responses and the excessive release of NO from the

activated microglia has been shown to cause the oligodendrocyte degeneration in demyelinating disorders like multiple sclerosis and neuronal death in Azheimer's disease (34). Several proinflammotory agents and cytokines in circulation increase the production of NO in macrophages through activation of iNOS (35-37). It is noteworthy that Costunolide decreased the iNOS protein and mRNA expression, eventually leading to attenuation of NO production in BV2 cells.

The plausible mechanism behind the downregulation of these proinflammatory agents such as TNF-alpha, IL-1, IL-6 and iNOS by Cos could be due to its repressive action on NFkappaB, as demonstrated in this study. Upon stimulation the NFkB transcription factor which exist in the cytoplasm as an inactive form(p65/100 heterodimer), forms the active p50/p65 heteroduplex and translocates to the nucleus where it activates gene transcription. (38). This translocation of NFkappaB favors DNA binding and tightly regulates the transcriptional activation of proinflammatory cytokines (39). It has been widely shown that inhibition of NFkappaB pathway further potentiates the suppression of inflammatory responses (40). The observed inhibitory effect of Cos on NFkappaB translocation is concordant with the report describing that the Sesquiterpene lactones like Costunolide functions as the inhibitor of the NFkappaB transcription pathway (41). The mechanistic link could be that terpenoids such as Costunolide induce the expression of heat shock proteins especially the hsp70 chaperone (42) which blocks I kappa B kinase (IKK) activation (43). The activation of IKK is known to cause inhibition of the phosphorylation and subsequent degradation of IkappaB, which is generally bound to NFkappaB in untreated conditions. Thus degradation of IkappaB releases NFkappaB, facilitating its nuclear translocation and ultimately its proinflammatory transcriptional pathway. Our *in vitro* analyses suggest that Cos suppressed the induction of proinflammatory cytokines such as TNF-alpha, IL-1, IL-6 through inhibition of nuclear translocation of NFkB. In light of this, the downregulation of iNOS can also be associated with NFkappaB as iNOS promoter is known to have several NFkappaB binding sites (44).

The NF-kB pathway, besides its proven transactivation of iNOS is also known to activate expression of inducible isoform of cycloxygenase, COX-2 which is a critical enzyme in the inflammatory response responsible for the production of prostaglandins (45,46). We showed here that Cos not only decreased iNOS production but also inhibited Cox-2 expression in BV-2 microglial cells, effectively controlling the two common NFkB dependent inflammatory pathways.

To add to this our studies also demonstrate that Costunolide appeared to interfere with the MAPKinase pathway by inducing the expression of MAPKinase Phosphatase-1(MKP-1), which antagonizes the activation of MAPK pathway. In response to proinflammatory stimuli, the MAPKinases such as p38 and JNK become activated and mediate transcriptional and post-transcriptional changes in gene expression of proinflammatory mediators. The inhibition of the MAPK pathway by glucocorticoids has been reported to be mediated *via* induction of the MKP-1 (47-49). MKP-1 is also induced by proinflammatory stimuli such as LPS, and this activates a negative feedback loop downregulating the production of proinflammatory cytokines such as TNF-alpha, IL-1 and IL-6 (47,50,51).It has been reported that cannabinoids, the active component of Cannabis sativa, suppresses microglial activation during CNS inflammation by inducing MKP-1 gene expression and subsequent ERK1/ 2 MAPK pathway dephosphorylation, which in turn abolishes NO release resulting in neuroprotection (52). In view of these facts it is possible that Cos suppressed proinflammatory cytokines and NO in activated microglia by inducing MKP-1 expression.

MCP-1, a chemokine released in response to activation of MAPK pathway is known to bind with its receptor CCR2 and induces the transient mobilization of intracellular calcium and subsequent changes in actin polymerization which contributes to the migration of microglia (53). Excessive release of MCP-1 during inflammation may lead to overcrowding of microglia around the injury site and exacerbates the inflammation (24). In the present study, the downregulation of MCP-1 mRNA expression in activated microglia by Costunolide treatment suggests that that Costunolide through the inhibition of MCP-1 could also block the development and progression of the neurological disorders caused by microglial recruitment to the injury sites. Our results also suggest that this effect of Costunolide was mediated by the inhibition of the activity of NFkappaB and MCP-1 through the induction of MKP-1

6. CONCLUSION

This present study demonstrated that the Costunolide inhibits the expression of proinflammatory cytokines such as TNF-alpha, IL-1,6 gene expression, MCP-1 expression and NO release in activated BV2 microglial cells. This inhibition was associated with the suppression of iNOS and Cox-2 mRNA expression. Our results also suggest that this effect of Costunolide was mediated by the inhibition of the activity of NFkappaB and the induction of MKP-1. The current data provide a new insight on Cos as a potential regulator of microglia-mediated neuroinflammation and further studies would establish the feasibility of usage in treating neuroinflammation and neurodegenerative disorders.

7. ACKNOWLEDGEMENT

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Abbreviations: BSA: Bovine serum albumin, Cos: Costunolide, Cox-2: Cycloxygenase, DMEM: Dulbecco's Modified Eagle Medium, DMSO: Dimethyl sulfoxide, FBS: Foetal bovine serum, I?B: Inhibitor of kappa B, IKK: I kappa B kinase, IL-1: Interleukin - 1, IL-6: Interleukin - 6, iNOS: Inducible nitric oxide synthase, LPS: Lipolysaccharide , MAPK pathway, Mitogen activated protein kinase pathway, MCP-1: Monocyte chemotactic protein-1, MKP-1: MAP kinase phosphatase-1, MTS: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, NADPH : Nicotinamide adenine dinucleotide phosphate, NF?B: Nuclear factor kappa B, NO: Nitric oxide, PBS: Phosphate buffered saline, TNF a: Tumor necrosis factor-alpha

Key Words: Costunolide, inflammation, BV2 microglia, interleukins, nitrite release, MAPK pathway

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