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Dimethoxycurcumin, a metabolically stable analogue of curcumin, exhibits anti-inflammatory activities in murine and human lymphocytes

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ABSTRACT

The aim of this study was to investigate whether dimethoxycurcumin (DiMC), a synthetic curcumin analogue having higher metabolic stability over curcumin, could exhibit anti-inflammatory activity in murine and human lymphocytes. Both curcumin and DiMC suppressed mitogen as well as antigen driven proliferation of murine splenic lymphocytes. Further, mitogen and antigen-stimulated cytokine (IL-2, IL-4, IL-6 and IFN-γ) secretion by T cells was also abrogated by curcumin and DiMC. Interestingly, curcumin and DiMC suppressed B cell proliferation induced by lipopolysaccharide. Curcumin and DiMC also inhibited Con A-induced activation of early and late T cell activation markers. They scavenged basal reactive oxygen species and depleted GSH levels in lymphocytes. The suppression of mitogen-induced T cell proliferation and cytokine secretion by curcumin and DiMC was significantly abrogated by thiol containing antioxidants suggesting a role for redox in their anti-inflammatory activity. Further, the possibility of curcumin and DiMC directly interacting with thiol-containing antioxidant GSH was monitored by changes in absorbance. Both curcumin and DiMC inhibited Con A induced activation of NF-κB and MAPK. More importantly, curcumin and DiMC inhibited phytohaemagglutinin induced proliferation and cytokine secretion by human peripheral blood mononuclear cells. To explore their therapeutic efficacy, they were added to lymphocytes post-Con A stimulation and we observed a significant suppression of IL-2, IL-6 and IFN-γ. The present study for the first time demonstrates the potent anti-inflammatory activity of DiMC. Further, DiMC could find application as an alternative to curcumin, which is currently used in several clinical studies, due to its superior bioavailability and comparable efficacy.

1. Introduction

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], a polyphenolic compound derived from rhizome (turmeric) of the herb *Curcuma longa*, possesses diverse pharmacological activities including anti-carcinogenic, anti-inflammatory, antioxidant, antiproliferative and antiangiogenic activities [1–3]. In general, curcumin mediates its effects by modulating the functions and expressions of several important molecular targets, including transcription factors (e.g., NF-κB, AP-1, Egr-1, β-catenin, and PPAR-γ), pro-inflammatory molecules (e.g., COX-2, 5-LOX, iNOS, and hemeoxygenase-1), cell cycle proteins (cyclin D1 and p21), cytokines (TNF-α, IL-1, IL-6, and chemokines), receptors (e.g., EGFR and HER2) and cell surface adhesion molecules [4]. This desirable modulation of cellular processes has now lead to curcumin being employed for clinical trials in treating patients suffering from various diseases like cancer, rheumatoid arthritis, atherosclerosis, Alzheimer's disease, psoriasis, renal transplantation, ulcerative colitis, Familial adenomatous polyposis, oral leukoplakia and Irritable bowel syndrome [5–11].

However, the utility of curcumin is limited by its color, lack of water solubility, and relatively low bioavailability in vivo [12]. Oral curcumin shows poor systemic bioavailability in rodents and humans and undergoes extensive metabolic reduction and conjugation in the gastrointestinal tract [13–17]. These studies indicated that curcumin is transformed during absorption in the gut epithelium, and the transformed product(s), which is more

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polar and color-less than curcumin, enters the serosal side. Oetari et al. (1996) also found that curcumin is unstable in phosphate buffer at pH 7.4 [18]. When administered orally at a dose of 1 g/kg (suspension in arachis oil) in rats, about 75% of curcumin was excreted in the feces whereas only traces appeared in the urine [19]. Oral administration of [3H]-curcumin (0.6 mg/rat) led to about 89% of radioactivity being excreted in the feces and 6% being excreted in the urine. After intraperitoneal administration, fecal excretion accounted for 73% of the radioactivity, whereas 11% was found in the bile [20]. The poor absorption from the intestine, coupled with the high degree of metabolism of curcumin in the liver and its rapid elimination in the bile, makes it unlikely that high concentrations of the substance would be found in the body for long periods of time after ingestion. It has been shown to be bio-transformed to dihydrocurcumin and tetrahydrocurcumin. Most of the curcumin administered was reduced by an endogenous reductase system in a stepwise manner and subsequently glucuronidated by UDP glucuronosyl transferases. Only traces of hexahydrocurcumin-glucuronoside were observed in plasma.

To improve the bioavailability of curcumin, numerous approaches have been undertaken. These approaches involve the use of adjuvant like piperine that interferes with glucuronidation, use of liposomal curcumin or curcumin nanoparticles [21,22], use of curcumin phospholipid complex and using structural analogues of curcumin. Dimethoxycurcumin (DiMC) is a structural analogue of curcumin where the phenolic-OH groups in curcumin are replaced with methoxy groups. It has a symmetric structure and is chemically more stable than curcumin [23,24]. In search of curcumin analogues with increased apoptotic efficacy against human cancer cells and/or less toxicity in normal cells and increased metabolic stability (bioavailability), dimethylcurcumin and dimethoxycurcumin were synthesized [1,25]. They have shown antiproliferative activity against several human cancer cell lines. Plasma levels for dimethoxycurcumin were overall higher (3-fold) in comparison with curcumin at same injected dose of 5 mg/kg bw in mice. DiMC is more effective than curcumin in inhibiting cell proliferation and inducing apoptosis in vitro in RAW264.7 cells. Specifically, DiMC is about 2–4 times more potent than curcumin when both compounds are compared at concentrations of 5–15 μmol/L against human colon cancer HCT116 cells [1]. In comparison with curcumin, metabolism of DiMC is less extensive hence it is possible that DiMC exhibits increased apoptotic activity over curcumin. However, whether DiMC could exert similar biological effects on lymphocytes to those of curcumin remains to be investigated.

The present study was aimed to investigate the relative anti-inflammatory and immunosuppressive properties of DiMC and curcumin in murine lymphocytes and human peripheral blood mononuclear cells (PBMC). The molecular mechanism of action of DiMC for the observed anti-inflammatory activity was also studied.

2. Materials and methods

2.1. Chemicals

Curcumin, RPMI-1640, HEPES, EDTA, EGTA, PMSF, leupeptin, aprotinin, benzamidine, dithiothreitol (DTT), glutathione (GSH), N-acetyl cysteine (NAC), NP40, propidium iodide (PI), lipopoysaccharide (LPS) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (MO, USA). Fetal calf serum (FCS) was obtained from GIBCO BRL (MD, USA). DiMC was synthesized according to the previously reported method and characterized by ^{1}H NMR, ^{13}C NMR spectroscopy and mass spectral analysis (data not shown) [23]. Concanavalin A (Con A) and trolox were purchased from Calbiochem (CA, USA). ELISA sets for detection of cytokines (IL-2, IL-4, IL-6 and IFN-γ) and monoclonal antibodies against CD25, CD69, phospho-ERK and phospho-P38 labelled with PE were procured from BD Pharmingen (CA, USA). Antibodies against IκB-α, p-JNK, P65, PARP and α-tubulin were obtained from Cell Signaling Technologies (CA, USA). Trizol reagent, DNaseI amplification grade, cDNA synthesis kit and N-Amp SYBR green PCR mix were purchased from Sigma Chemical Co. (MO, USA).

2.2. Animal maintenance

Six to eight week old inbred Swiss male mice, weighing approximately 20–25 g, reared in the animal house of Bhabha Atomic Research Centre were used. They were housed at constant temperature (23 °C) with a 12/12 h light/dark cycle and were given mouse chow and water ad libitum. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding the maintenance and dissections of small animals were strictly followed.

2.3. Institutional ethics committee

Informed, signed consent was taken from all the registered healthy volunteers before undertaking the studies. The guidelines issued by the Medical Ethics Committee of Bhabha Atomic Research Centre, Government of India, were strictly followed during these studies.

2.4. Cell culture

Jurkat cells (Human T cell leukemia) were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere in a CO_2 incubator.

2.5. Treatment with curcumin or DiMC

A 20 mM solution of curcumin or DiMC was prepared in dimethyl sulfoxide, stored as small aliquots at −20 °C and diluted as needed in cell culture medium. In all in vitro experiments, cells were treated with different doses of curcumin or DiMC for 4 h before the initiation of culture. DMSO (0.1%) was used as vehicle control in vitro.

2.6. Uptake study

Splenocytes were treated with curcumin and DiMC (10 μM) for 4 h at 37 °C and cells were adhered to coverslip, washed with 1XPBS and fixed with 4% paraformaldehyde. Cells were mounted on glass slide in DABCO and analyzed by using Carl–Zeiss confocal microscope with excitation 488 nm and emission in 550 nm range.

2.7. Proliferation assay

Splenocytes were obtained by squeezing the spleen through a nylon mesh in a petri plate containing RPMI medium. The RBCs were lysed by brief hypotonic shock. Splenocytes were stained with carboxyfluorescein succinimidyl ester (CFSE) (20 μM, 5 min, 37 °C) and washed three times using ice-cold RPMI medium containing 10% FCS, 100 IU/ml penicillin and 100 mg/ml streptomycin. Two million splenocytes were treated with different concentrations of curcumin or DiMC (1–10 μM, 4 h) and were stimulated with Con A (10 μg/ml) or LPS (50 μg/ml) or

α-CD3/α-CD28 (1 μg/ml) for 72 h at 37 °C in 2 ml RPMI with 10% FCS in a 95% air/5% CO_2 atmosphere. Vehicle treated cells served as a control. Cell proliferation was measured by dye dilution in a flowcytometer (Partec CyFlow). Percent daughter cells that showed a decrease in CFSE fluorescence intensity were calculated using Flowmax® software and were expressed as daughter cells [26].

2.8. CD4+ and CD8+ T cell isolation and proliferation assay

CD4+ and CD8+ T cells were isolated by using EasySep immunomagnetic cell sorting kit from Stem Cell Technologies, with PE labelled anti-CD4/CD8 antibody conjugated to magnetic nanoparticles through dextran and separation using magnetic field. For cell proliferation analysis, total splenocytes were first

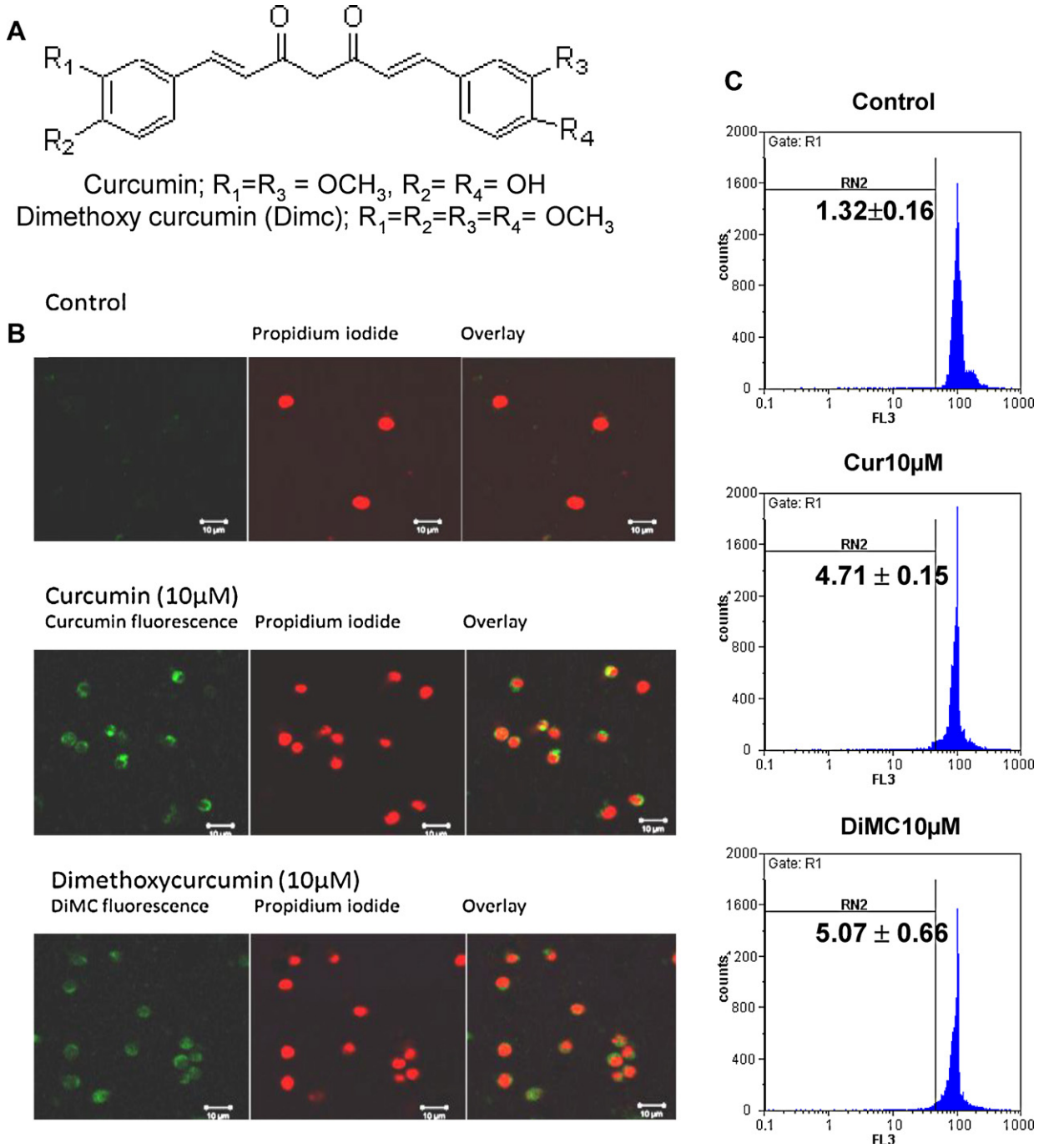

Fig. 1. Curcumin and DiMC inhibit lymphocyte proliferation in vitro: (A) Chemical structure of curcumin and DiMC. (B) Cellular uptake of curcumin and DiMC. (C) Evaluation of the potential cytotoxicity of curcumin and DiMC to lymphocytes. Lymphocytes were cultured with curcumin or DiMC (10 μM) for 24 h and cytotoxicity was measured by PI staining. Vehicle treated cells served as control. (D) Curcumin and DiMC inhibit Con A induced lymphocyte proliferation. For cell proliferation analysis CFSE labelled lymphocytes were treated with curcumin and DiMC (1–10 μM, 4 h) and stimulated with the Con A (10 μg/ml) at 37 °C for 72 h. Twenty thousand cells were acquired in a flowcytometer. Vehicle treated cells served as control. Percent daughter cells were calculated from decrease in mean fluorescence intensity. (E) Each bar represents percentage of daughter cells in each treatment group. (F&G) Curcumin and DiMC inhibit anti-CD3/CD28 induced T cell proliferation. CFSE labelled lymphocytes were treated with curcumin and DiMC (1, 5 and 10 μM, 4 h) and stimulated with coated anti-CD3mAb (1 μg/ml) and soluble anti CD28mAb (1 μg/ml) at 37 °C for 72 h. Percent daughter cells were estimated by CFSE dye dilution. Each bar represents percentage daughter cells in each treatment group. Data points represent mean ± S.E.M. from three replicates and three such independent experiments were carried out. $^{*}p < 0.01$, as compared to vehicle treated cells and $^{\#}p < 0.05$, as compared to Con A or antiCD3/CD28 stimulated cells.

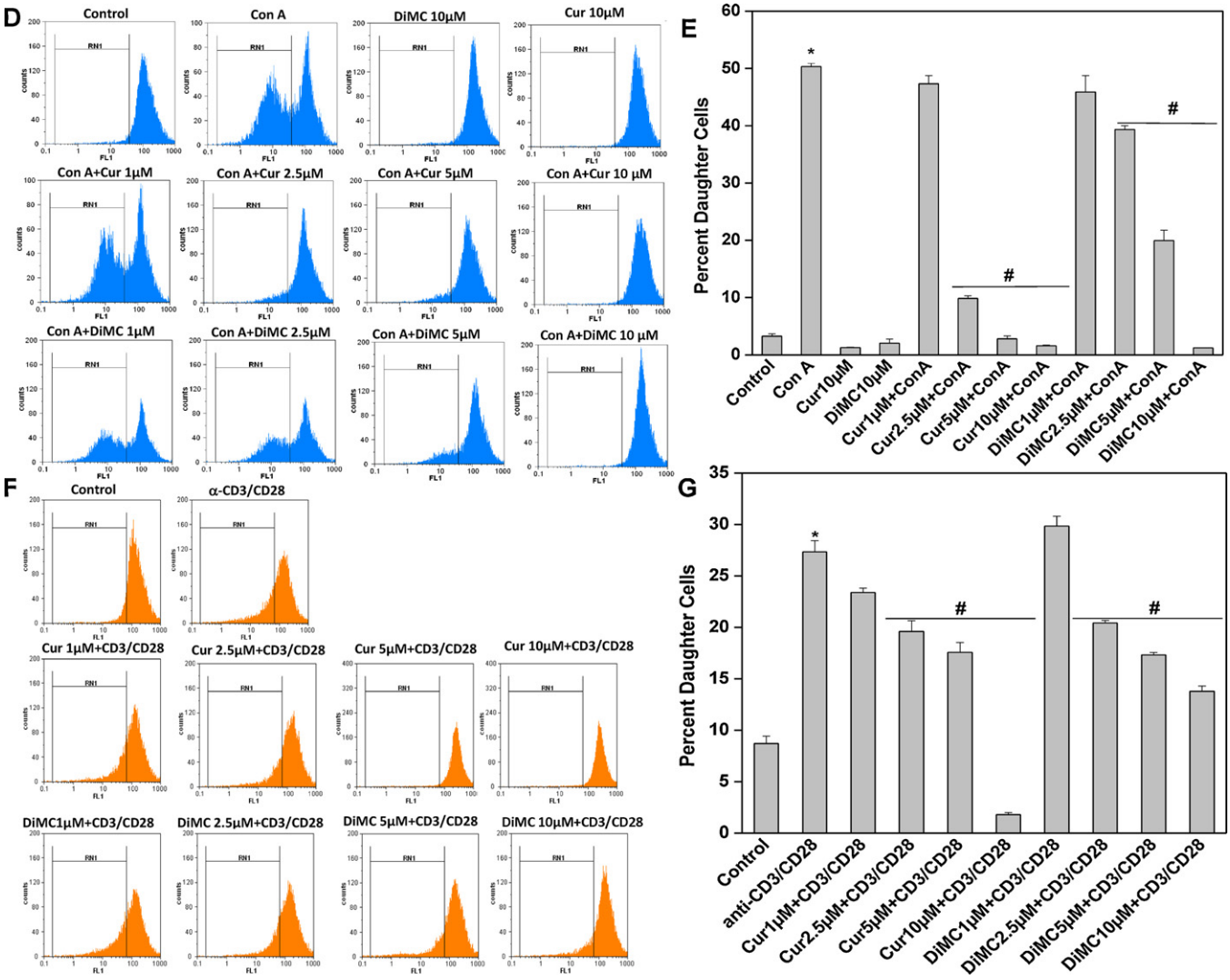

Fig. 1. (*Continued*).

labelled with CFSE and then sorted and cultured for 24 or 72 h, respectively, for cytokine or proliferation assay.

2.9. Human lymphocyte proliferation and cytokine assay

Peripheral venous blood (10 ml) from healthy volunteers was collected from cubital vein and was immediately transferred into heparinized vacutainer tubes (BD Pharmingen). The blood was diluted 1:1 with sterile RPMI medium and gently layered on to 10 ml Histopaque 1077 in a 50 ml centrifuge tube. The tubes were centrifuged at 400 × *g* for 20 min and PBMC were collected from buffy coat [27]. The PBMC were washed three times with RPMI medium and viable cells were counted using trypan blue dye exclusion. PBMC were stained with CFSE (20 μM, 5 min, 37 °C) and washed three times using ice-cold RPMI medium containing 10% FCS, 100 IU/ml penicillin and 100 mg/ml streptomycin. PBMC (1×10^6/ml) were stimulated with phytohaemagglutinin (PHA) (10 μg/ml) in presence or absence of curcumin or DiMC (1–10 μM) and cultured for 24 or 72 h at 37 °C in 2 ml RPMI with 10% FCS in a 95% air/5% CO_2 atmosphere. Vehicle treated cells served as a control. At the end of 72 h, cell proliferation was measured by dye dilution in a flowcytometer. The culture supernatants were collected after 24 h and used for estimation of different cytokines. The supernatants obtained from unstimulated cells and PHA stimulated cells were used as negative and positive control, respectively.

Jurkat cells were pretreated with curcumin or DiMC or vehicle for 4 h and stimulated with PHA for 24 h in RPMI 1640 medium supplemented with 10% FCS in 95% air/5% CO_2 atmosphere at 37 °C. The concentration of IL-2 was estimated in the culture supernatants using cytokine ELISA sets (BD Pharmingen, USA). DMSO was used as vehicle control.

2.10. Estimation of apoptosis

The percentage of apoptotic cells was estimated using a flowcytometer. One million splenocytes were treated with curcumin or DiMC (10 μM) for 24 h in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a 5% CO_2 atmosphere. Vehicle treated cells served as control. The cells were washed with PBS and incubated with 1 ml of staining solution containing 0.5 μg/ml propidium iodide, 0.1% sodium citrate and 0.1% triton X-100 overnight [28]. A total of 20,000 cells were acquired in a flowcytometer and analyzed using FloMax® software. The pre G_1 population represented the apoptotic cells.

2.11. Measurement of cytokine secretion

The concentration of IL-2, IL-4, IL-6 and IFN-γ in the supernatant of control unstimulated cells and cells stimulated with Con A or anti-CD3/CD28 antibodies for 24 h after curcumin or

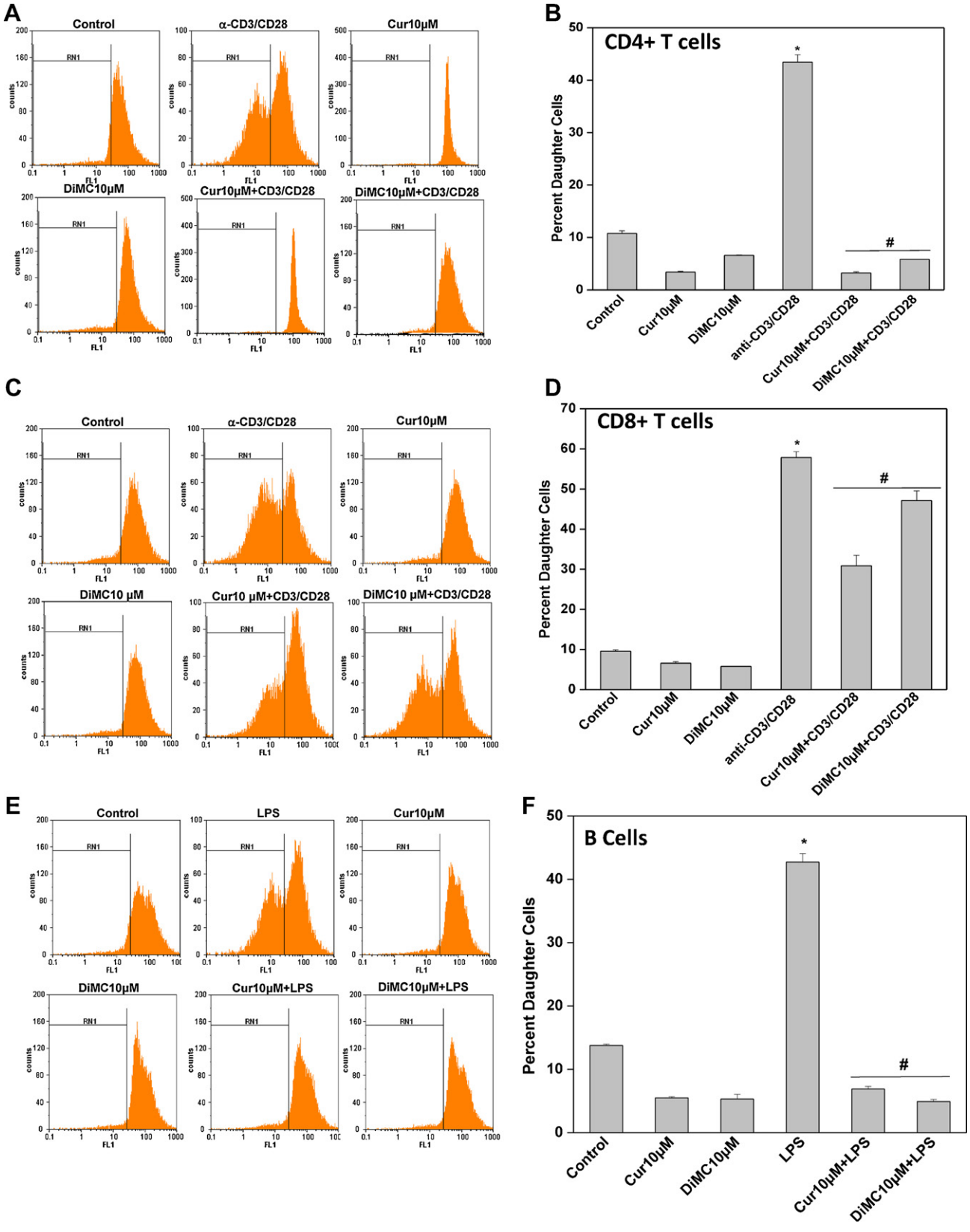

Fig. 2. Curcumin and DiMC inhibit mitogen induced proliferation of CD4+ T cells, CD8+ T cells and B cells: CFSE labelled purified CD4+ T cells (A) or CD8+ Tcells (C) were pre-treated with curcumin or DiMC (10 μM, 4 h) before stimulation with coated anti-CD3mAb (1 μg/ml) and soluble anti CD28mAb (1 μg/ml) for 72 h at 37 °C. Percent daughter cells were calculated from decrease in mean fluorescence intensity. (B and D) Each bar represents percentage of daughter cells in different treatment groups. (E and F) Curcumin and DiMC inhibit LPS induced proliferation of B cells. CFSE labelled lymphocytes were treated with curcumin or DiMC (10 μM, 4 h) and stimulated with the LPS (50 μg/ml) at 37 °C for 72 h. Vehicle treated cells served as control. Percent daughter cells were calculated from decrease in mean fluorescence intensity. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. $^{*}p < 0.01$, as compared to vehicle treated cells and $^{\#}p < 0.05$, as compared to anti-CD3/anti-CD28 or LPS stimulated cells.

DiMC treatment (4 h) was estimated using cytokine ELISA sets [29]. The supernatant obtained from Con A or anti-CD3/CD28 stimulated cells was used as positive control. Cytokines induced by LPS was estimated in the culture supernatant of splenic adherent macrophage. Spleen cells (5×10^6 cells/well) were incubated in a 24-well cell culture plate for 4 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The non-adherent cells were removed by aspiration. The adherent cells (macrophages) were incubated with curcumin or DiMC (10 μM for 4 h) and then stimulated with LPS (50 μg/ml) and further cultured for 24 h at 37 °C. The concentration of IL-6 in the supernatant of cells stimulated with LPS was estimated using cytokine ELISA sets [29].

2.12. RNA isolation, cDNA synthesis and quantitative real time PCR

mRNA levels in the samples were quantified by quantitative real-time polymerase chain reaction (qPCR) as described previously [30]. Briefly, total RNA was isolated from the samples using Trizol reagent following the manufacturer's instructions (Sigma) and was dissolved in deionised DEPC-treated water. From this RNA 5 μg was converted to cDNA by reverse transcription following the manufacturer's instruction (Sigma). qPCR was carried out using the Rotor Gene 3000 machine (Corbett Research, USA). PCR was setup by mixing 2× SYBR green PCR mix (sigma) with 5 μl cDNA, 10 picomoles each of forward and reverse primers (primer sequence given below) and PCR-grade water in 20 μl reaction mixture. The above reaction mixtures were amplified in the following steps: step 1 – denaturation at 95 °C for 5 min; step 2 – denaturation at 95 °C for 15 s; step 3 – annealing at 58 °C for 15 s; step 4 – extension at 72 °C for 20 s; step 5 – melting curve analysis. Steps from 2 to 4 were repeated for 40 cycles. The specificity of respective amplicons was confirmed from the melting curve analysis. The threshold cycle (the cycle at which the amplification enters into exponential phase) values obtained from above runs were used for calculating the expression levels of genes by REST-384 version 2 software [31]. The expressions of genes were normalized against that of a housekeeping gene, β-actin, and plotted as relative change in the expression with respect to control.

Gene	Sequence
IL-2	Forward: GTGCTCCTTGTCAACAGCG
	Reverse: GGGGAGTTTCAGGTTCCTGTA
IFN-gamma	Forward: TGGAGGAACTGGCAAAAGGATGGT
	Reverse: TTGGGACAATCTCTTCCCCAC
β-Actin	Forward: GCGGGAAATCGTGCGTGACATT
	Reverse: GATGGAGTTGAAGGTAGTTTCGTG

2.13. Intracellular ROS measurements

To detect intracellular ROS, lymphocytes were incubated with 20 μM oxidation-sensitive dichlorofluorescein diacetate (DCF-DA)

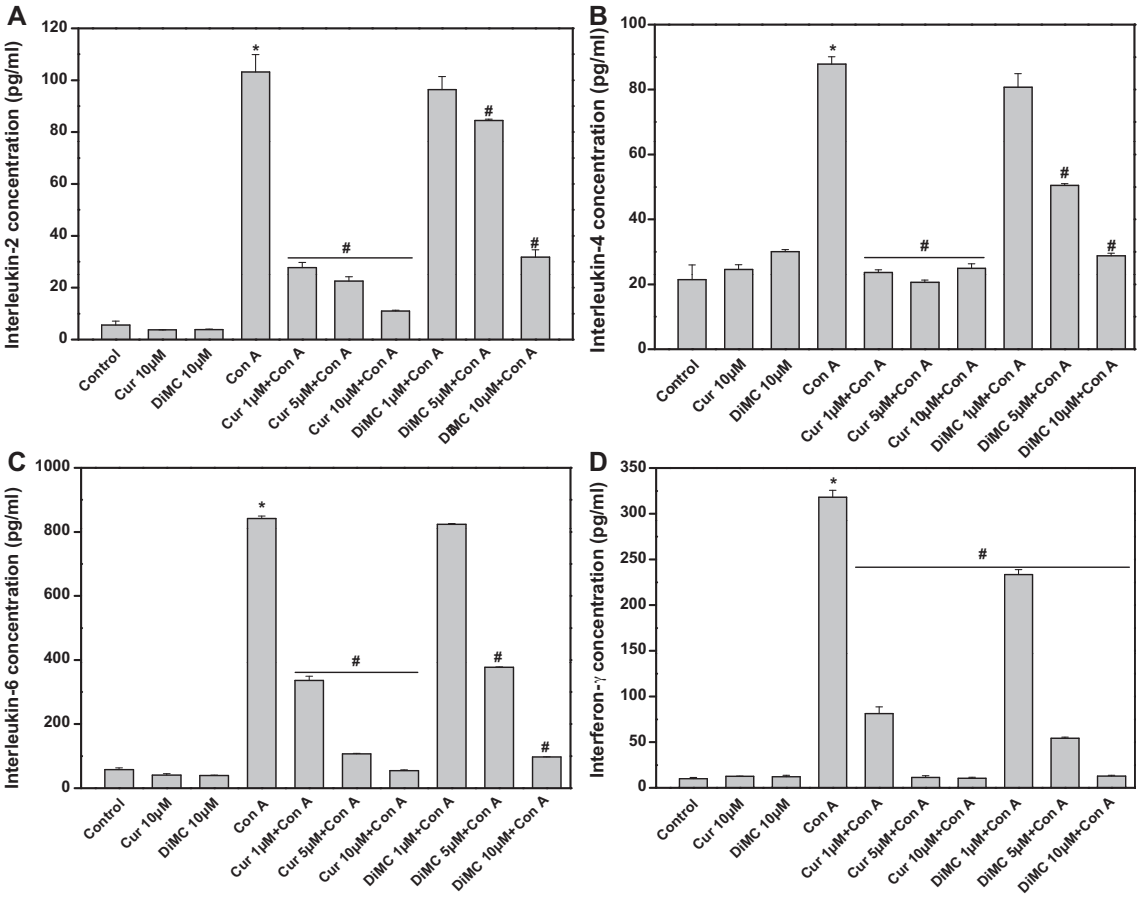

Fig. 3. Curcumin and DiMC inhibit cytokine secretion by lymphocytes at both protein and mRNA level: Lymphocytes were pre-treated with the indicated concentrations of curcumin or DiMC for 4 h before stimulation with Con A (A–D) or anti-CD3/anti-CD28 mAb (E–H) for 24 h at 37 °C. Vehicle treated cells served as control. The concentration of cytokines IL-2, IL-4, IL-6 and IFN-γ in the culture supernatant was estimated using ELISA. (I and J) Relative mRNA copy number of IL-2 and IFN-γ were determined by qRT-PCR. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out.*$p < 0.01$, as compared to vehicle treated cells and $^{\#}p < 0.01$, as compared to Con A or anti-CD3/anti-CD28 stimulated cells.

for 25 min at 37 °C before being treated with various concentrations of curcumin or DiMC. After 1 h of incubation, the increase in fluorescence resulting from oxidation of H_2DCF to DCF was measured using a spectrofluorimeter [32].

2.14. Intracellular GSH assay

The GSH content in cells was determined using total glutathione kit (Abcam) using manufacturer's protocol.

2.15. Absorption spectroscopy

Solutions of curcumin or DiMC were mixed with 10 mM of GSH in a total volume of 1 ml and incubated at 37 °C for 1 h. Absorption spectra of the samples were recorded using a plate reader.

2.16. Intracellular antibody and surface staining by flow cytometry

Three million lymphocytes were cultured in presence or absence of curcumin and DiMC for 4 h and then stimulated with Con A for 1 h at 37 °C. Cultured cells were fixed with 4% paraformaldehyde for 10 min at room temp and excess of paraformaldehyde was removed by washing once with wash buffer (PBS containing 1% BSA). Before staining with monoclonal antibody against pERK & pP38, cells were permeabilized with PBST (PBS containing 0.02% Tween 20) thrice for 5 min each at room temp followed by 2 washes with wash buffer and then incubated with the indicated mAbs for 30 min at room temp, washed twice and analyzed using a Partec Cyflow flowcytometer [33].

Surface staining with PE labelled mAbs CD25/CD69 was done as described earlier [29]. In brief, splenocytes were treated with curcumin and DiMC (10 μM, 4 h) and were further stimulated with

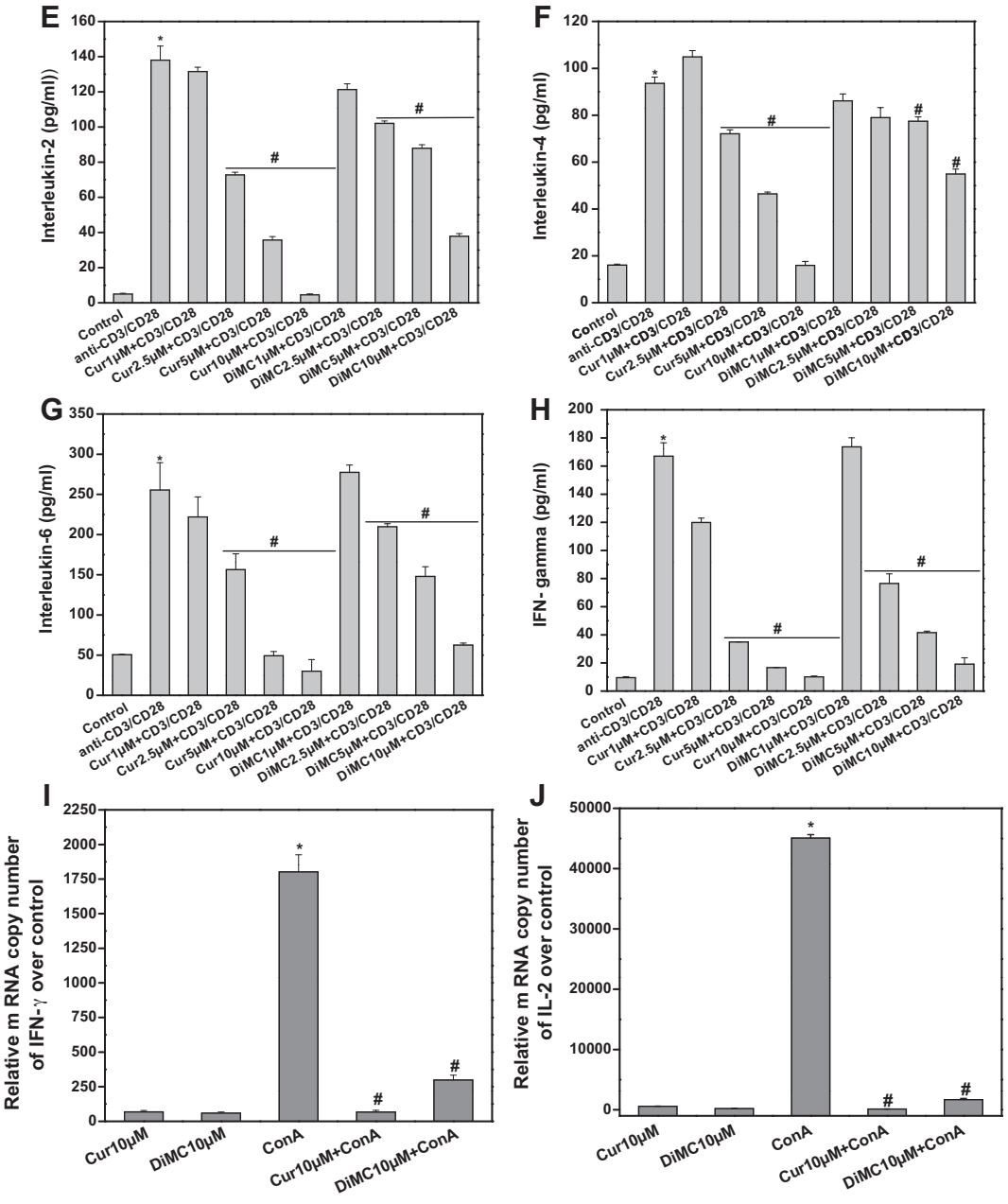

Fig. 3. (*Continued*).

Con A (10 μg/ml) for 24 h. A total 20,000 cells in each group were acquired and analyzed in a Partec Cyflow flowcytometer [29].

2.17. Western blot analysis

Splenocytes (40×10^6 cells/group) were treated with curcumin or DiMC (10 μM, 4 h) and were stimulated with Con A (10 μg/ml) for 1 h at 37 °C and cytosolic or nuclear extract was prepared as described earlier [32]. Vehicle treated cells served as a control. Briefly, cells were washed with ice-cold phosphate buffered saline and suspended in 0.1 ml lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine). Then cells were allowed to swell on ice for 15 min, after which 25 μl of 10% nonidet P-40 was added and tubes were vortexed. The supernatants containing proteins from cytosolic fraction were collected by centrifuging the cells at 8000 rpm for 6 min at 4 °C. The pellet was suspended in nuclear extraction buffer to measure nuclear P65 levels. Protein estimation was carried out by Bradford method using Bio-Rad Protein Assay Kit (Cat No. 500-0006). Equal amounts of protein (50 μg) were resolved by SDS-PAGE (10%) and transferred to nitro cellulose membrane. After the membrane was blocked in 5% non-fat powdered milk, the membrane was incubated overnight with the primary antibodies specific to IκB-α or p-JNK or P65 and washed three times with Tris-buffer saline containing 0.05% Tween 20 (TBST) and further incubated with horseradish peroxidase-labelled secondary antibody for 1 h. The membranes were washed, and specific bands were visualized on X-ray films using enhanced chemiluminiscence kit (Roche, Germany). The membrane was stripped and reprobed with α-tubulin or PARP antibody.

2.18. Statistical analysis

Data are presented as mean ± SEM. The statistical analysis was done using ANOVA with Microcal Origin 6.0 software. * Refers to $p < 0.01$, as compared to vehicle treated control and # refers to $p < 0.01$, as compared to Con A or LPS stimulated cells.

3. Results

3.1. Curcumin and DiMC entered lymphocytes and inhibited Con A and anti-CD3/CD28 mAb induced proliferation of lymphocytes in vitro

Fig. 1A shows the structure of curcumin and DiMC. Fig. 1B shows the confocal images of lymphocytes treated with curcumin or DiMC for 4 h and stained with propidium iodide. Both curcumin and DiMC entered lymphocytes and were localized mostly in the cytoplasm (Fig. 1B). The immunomodulatory effects of curcumin and DiMC were evaluated by stimulating murine splenic lymphocytes with Con A or with bound anti-CD3 plus soluble anti-CD28 mAb in their presence or absence. Con A or anti-CD3/CD28 mAb induced proliferation of splenic T cells was assessed by CFSE dye dilution using a flowcytometer. Both curcumin and DiMC inhibited

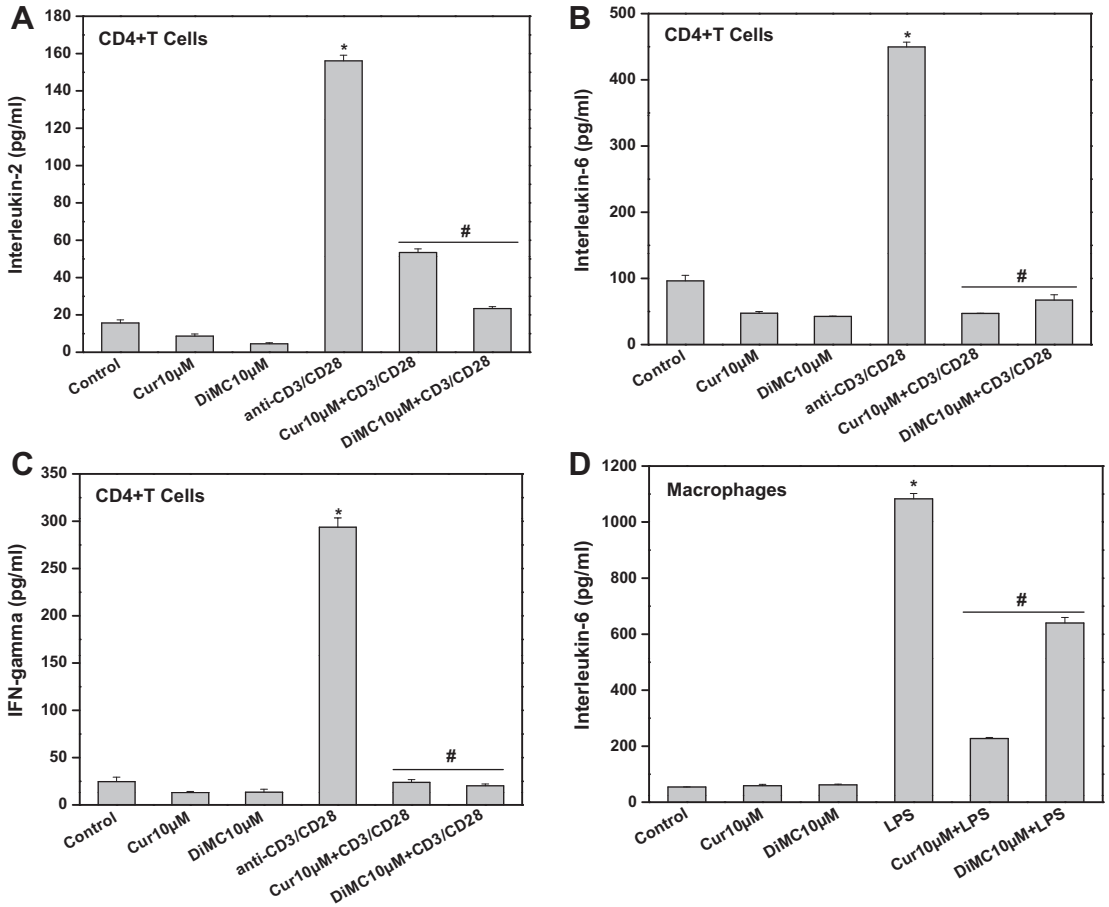

Fig. 4. Curcumin and DiMC inhibit anti-CD3/anti-CD28 induced cytokine secretion by CD4+ T cells (A–C) and macrophages (D). Purified CD4+ T cells and macrophages were pre-treated with different concentrations of curcumin or DiMC for 4 h before stimulation with anti-CD3/anti-CD28 mAb or LPS (50 μg/ml) for 24 h at 37 °C. The concentration of IL-2, IL-6 and IFN-γ cytokines for CD4+ T cells & IL-6 for macrophages in the supernatant was estimated using ELISA. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out.*$p < 0.01$, as compared to vehicle treated cells and #$p < 0.05$, as compared to anti-CD3/anti-CD28 or LPS stimulated cells.

Con A as well as anti-CD3/CD28 mAb induced lymphocyte proliferation in a dose dependent manner in vitro (Fig. 1D–G). This inhibition of mitogen and anti-CD3/CD28 mAb induced T cell proliferation by curcumin and DiMC was not due to induction of cell death as at 10 μM both curcumin and DiMC were found to be non-toxic to lymphocytes as assessed by PI staining (Fig. 1C).

3.2. Curcumin and DiMC inhibited proliferation of CD4+ T cells, CD8+ T cells and B cells

Immune response to any antigenic exposure is mediated via activation and proliferation of T cells (CD4+ and CD8+) and B cells. Therefore, we studied whether curcumin and DiMC act on both these cell types or is specific to any particular cell type. As shown in Fig. 2A–F, both curcumin and DiMC inhibited anti-CD3/CD28 mAb induced proliferation of CD4+ T cells (Fig. 2A and B), CD8+ T cells (Fig. 2C and D) and LPS stimulated proliferation of B cells (Fig. 2E and F).

3.3. Curcumin and DiMC inhibited Con A, anti-CD3/CD28 mAb and LPS induced cytokine secretion by lymphocytes, CD4+ T cells and macrophages in vitro

The secretion of IL-2, IL-4, IL-6 and IFN-γ cytokines by lymphocytes stimulated with Con A or anti-CD3/CD28 mAb in the presence or absence of curcumin or DiMC was measured. Lymphocytes stimulated with Con A or anti-CD3/CD28 mAb produced significantly higher levels of IL-2, IL-4, IL-6 and IFN-γ cytokines (Fig. 3A–H). Pretreatment of cells with curcumin or DiMC completely inhibited both Con A (Fig. 3A–D) and anti-CD3/CD28 mAb (Fig. 3E–H) induced secretion of IL-2, IL-4, IL-6 and IFN-γ cytokines. Further, the effect of curcumin and DiMC on the mRNA levels of IL-2 and IFN-γ was also studied in activated lymphocytes using quantitative real time PCR. It was also observed that pre-treatment of cells with curcumin or DiMC resulted in significant inhibition of Con A induced increase in mRNA copy number of IL-2 and IFN-γ (Fig. 3I and J). Similar anti-inflammatory effects were observed on CD4+ T cells when they were stimulated with anti-CD3/CD28 mAb in the presence of curcumin or DiMC. Treatment of purified CD4+ T cells with curcumin or DiMC prior to stimulation with anti-CD3/CD28 mAb led to complete inhibition of secretion of IL-2, IL-6 and IFN-γ (Fig. 4A–C). It was also observed that treatment of splenic adherent macrophages with curcumin or DiMC prior to stimulation with LPS completely inhibited the secretion of IL-6 cytokine (Fig. 4D).

3.4. Curcumin and DiMC inhibited expression of Con A induced T cell activation markers CD69 and CD25

Fig. 5 shows the expression of early and late T cell activation markers CD69 and CD25 (IL-2R-α) respectively in lymphocytes

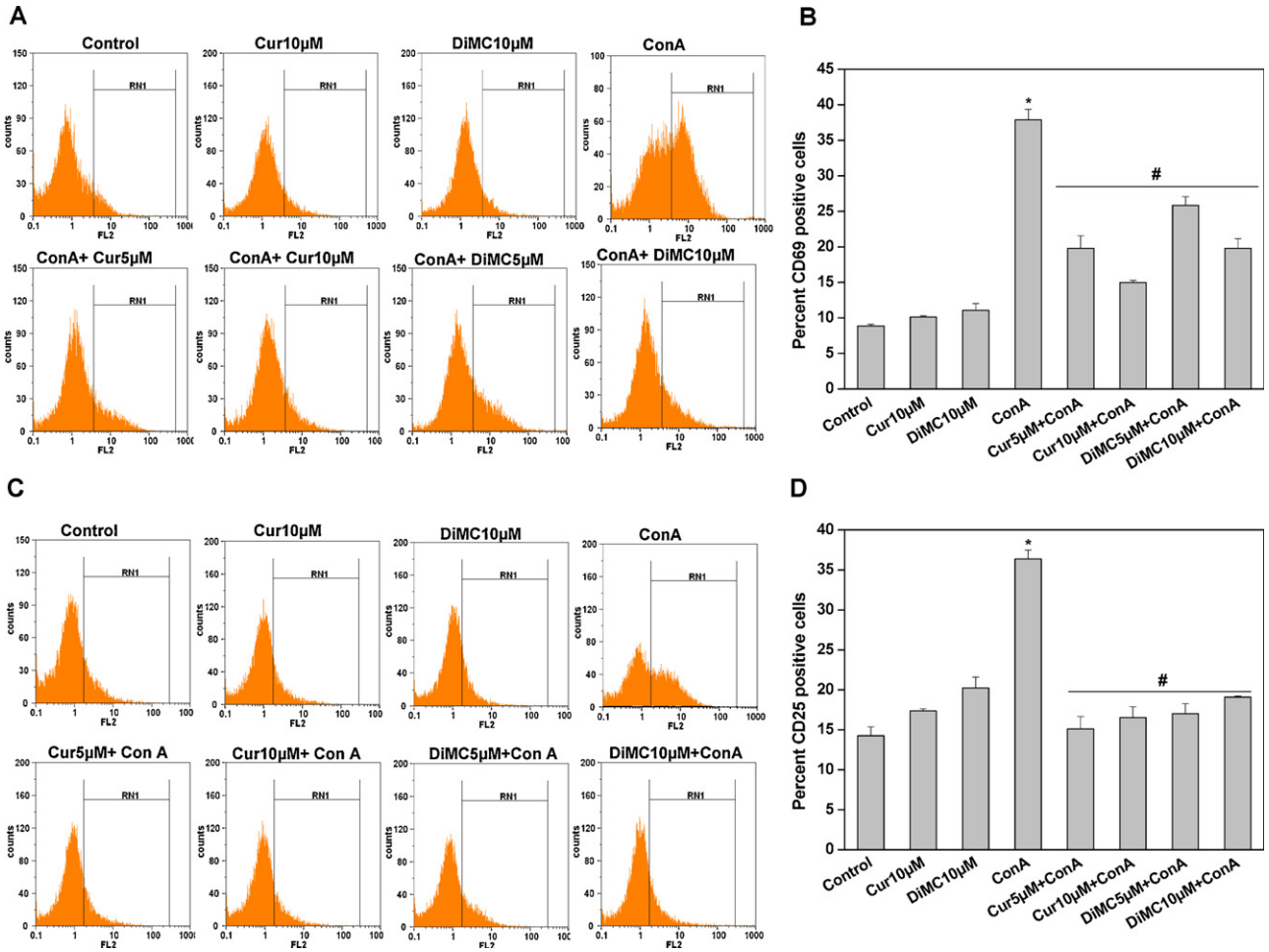

Fig. 5. Curcumin and DiMC suppress inducible expression of T cell activation markers. (A–D) Lymphocytes were treated with curcumin or DiMC (10 μM, 4 h) and then stimulated with Con A (10 μg/ml) for 24 h at 37 °C. In each group, 1×10^6 cells were stained with PE conjugated anti-CD69 (A) or anti-CD25 (C) mAbs. Representative flowcytometric histogram (A and C) and corresponding bar diagrams (B&D) are shown. Data points represent mean ± S.E.M. from three replicates and two such independent experiments were carried out. $^{*}p < 0.01$, as compared to vehicle treated cells and $^{\#}p < 0.05$, as compared to Con A stimulated cells.

treated with curcumin or DiMC (4 h) and stimulated with Con A (10 μg/ml) as compared to that in the cells stimulated with Con A alone. Con A activated cells showed significantly higher expression of CD69 and CD25 as compared to that in control vehicle treated cells. Curcumin or DiMC treatment prior to Con A stimulation led to a complete inhibition of both CD69 and CD25 expression (Fig. 5A–D).

3.5. Modulation of intracellular redox status by curcumin and DiMC

Immune response to antigen stimulation is known to be sensitive to intracellular redox balance and several molecules have been shown to exhibit their biological activity via a redox dependent manner. To ascertain whether curcumin or DiMC also

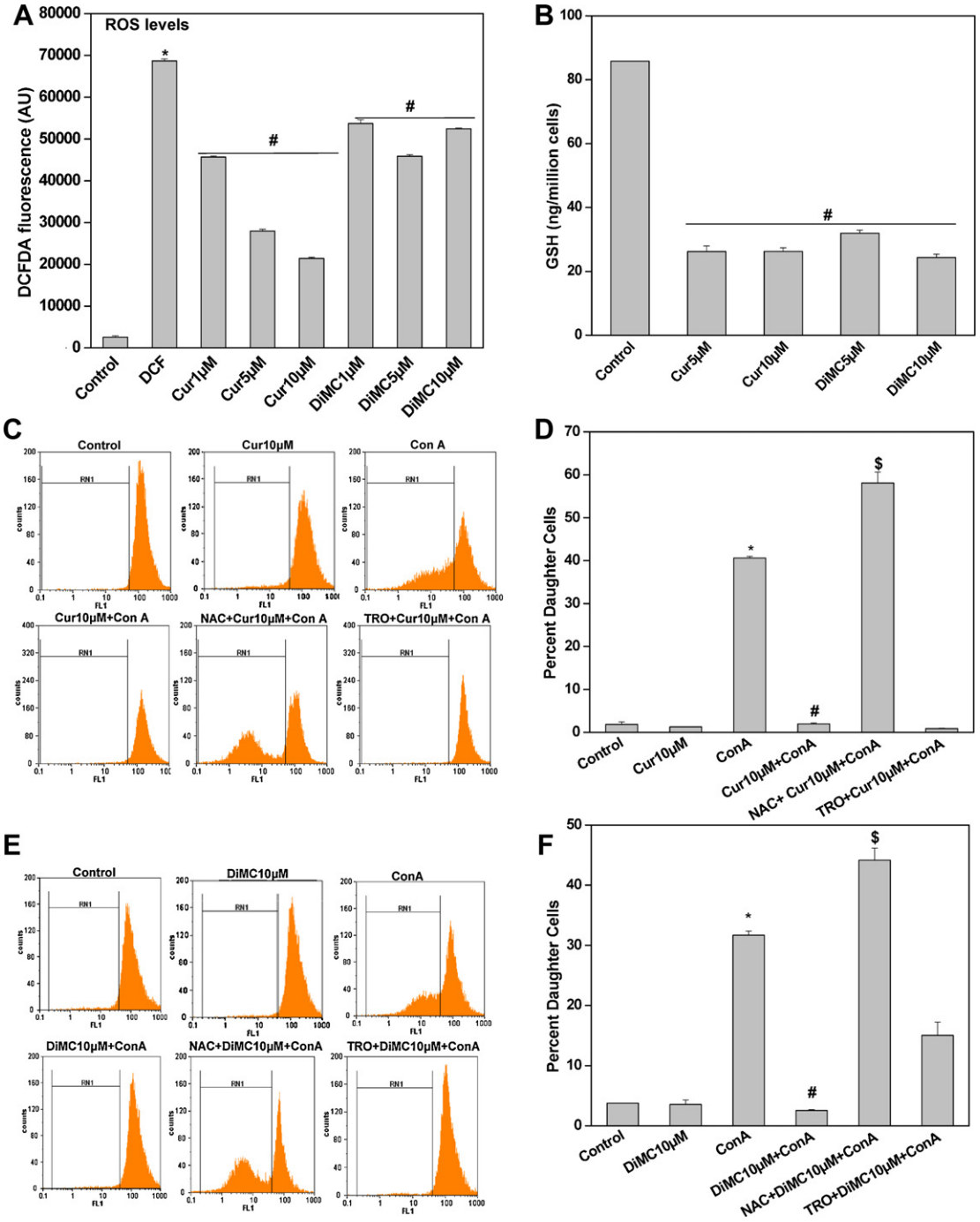

Fig. 6. Curcumin and DiMC modulate cellular ROS and GSH levels: (A) Curcumin and DiMC decreased ROS levels in lymphocytes. Lymphocytes were stained with DCF-DA (20 μM, 30 min at 37 °C), treated with curcumin or DiMC (1–10 μM) for 1 h and fluorescence emission was measured at 535 nm. (B) Effect of curcumin and DiMC on intracellular GSH levels. Lymphocytes were treated with curcumin or DiMC for 4 h at 37 °C and GSH was estimated using Abcam kit. (C–F) Immunosuppressive effects of curcumin and DiMC were dependent on cellular redox status. Lymphocytes were stained with CFSE and were incubated with different antioxidants (NAC 10 mM or trolox 100 μM) for 2 h. The cells were then stimulated with Con A in presence or absence of curcumin (C and D) or DiMC (E and F) for 72 h at 37 °C. Cell proliferation was measured from CFSE dye dilution using a flowcytometer. Representative flowcytometric histograms and corresponding bar diagram are shown. (G and H) Lymphocytes were incubated with different antioxidants (NAC 10 mM or trolox 100 μM) for 2 h. These cells were then stimulated with Con A in presence or absence of curcumin and DiMC for 24 h at 37 °C .The concentration of IL-2 in the culture supernatant was estimated by ELISA. (I and J) Direct interaction of curcumin and DiMC with GSH. To monitor the interaction, changes in absorption spectra of curcumin and DiMC were observed in presence or absence of GSH. Each bar shows mean ± S.E.M. from three replicates and two such independent experiments were carried out. $^{*}p < 0.01$, as compared to vehicle treated cells and $^{\#}p < 0.01$, as compared to Con A stimulated cells, $^{\$}p < 0.01$as compared to compound treated and Con A stimulated cells.

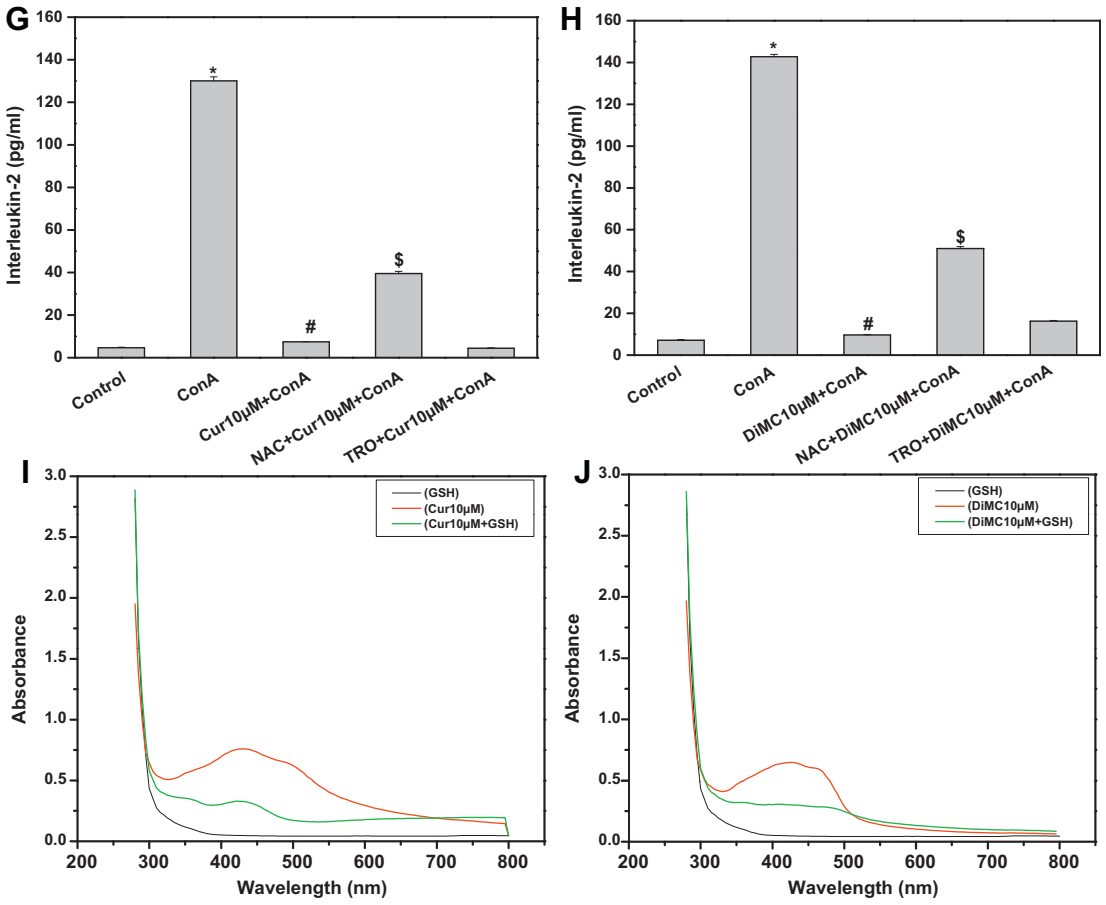

Fig. 6. (*Continued*).

act via a similar manner and modulated the cellular redox status, we studied their effect on intracellular ROS and GSH levels. Treatment of lymphocytes with curcumin or DiMC significantly decreased DCF (Fig. 6A) fluorescence in a concentration dependent manner. The levels of intracellular GSH in lymphocytes following treatment with curcumin or DiMC were measured and we observed a significant decrease at 4 h (Fig. 6B). To determine if ROS and GSH play a role in the observed anti-inflammatory effects of curcumin and DiMC, we studied whether antioxidants could abrogate the suppressive effects of curcumin or DiMC. The suppression of Con A induced lymphocyte proliferation and cytokine secretion by curcumin and DiMC could be abrogated by thiol (N-acetyl cysteine) but not by non-thiol antioxidant (trolox) suggesting that the effects of both curcumin and DiMC are dependent of cellular redox status (Fig. 6C–H).

3.6. Interaction of curcumin and DiMC with thiol group

Since anti-proliferative and anti-inflammatory effects of curcumin and DiMC were sensitive to presence of thiol antioxidants, experiments were carried out to determine whether they can interact directly with GSH. Both curcumin and DiMC interacted with GSH as seen by changes in absorption spectra of curcumin and DiMC in presence of GSH (Fig. 6I and J).

3.7. Curcumin and DiMC suppressed mitogen induced MAPKs and NF-κB activation in lymphocytes

Interaction of TCR on T cells with MHC present on antigen presenting cells leads to the activation of a large number of signalling molecules and transcription factors like MAP kinases and NF-κB that regulate the fate of the ensuing immune response. To study the molecular mechanism of action of DiMC, we studied its ability to modulate the activation of signalling events that are involved in T cell activation and compared them with curcumin. Fig. 7A–E shows the effect of curcumin and DiMC on Con A induced NF-κB activation and MAP Kinase in lymphocytes. Treatment of lymphocytes with curcumin or DiMC inhibited Con A induced ERK, P38 and JNK phosphorylation (Fig. 7A–E). Stimulation of lymphocytes with Con A (10 μg/ml) for 1 h showed degradation of IκB-α in the cytosolic fraction and NF-κB activation in the nuclear fraction as compared to that in vehicle treated control cells (Fig. 7A). However, treatment of cells with curcumin or DiMC and then stimulated with Con A (10 μg/ml, 1 h) inhibited degradation of IκB-α (Fig. 7A) as well as NF-κB activation (Fig. 7A).

3.8. Curcumin and DiMC inhibited proliferation and cytokine secretion by activated human PBMCs and exhibited therapeutic potential by acting post-mitogenic stimulation

To ascertain the effectiveness of curcumin and DiMC in human PBMC, they were incubated in the presence or absence of curcumin or DiMC prior to stimulation with PHA and studied for proliferation and cytokine secretion. We observed that both curcumin and DiMC were equally effective in inhibiting PHA induced proliferation and cytokines secretion (IL-2 & IFN-γ) by human PBMC and Jurkat cells in vitro (Fig. 8A–E). To explore its therapeutic potential, curcumin and DiMC were added to lymphocytes post-mitogenic stimulation at different time points. It was observed that both curcumin and DiMC were able to completely suppress Con A induced cytokine

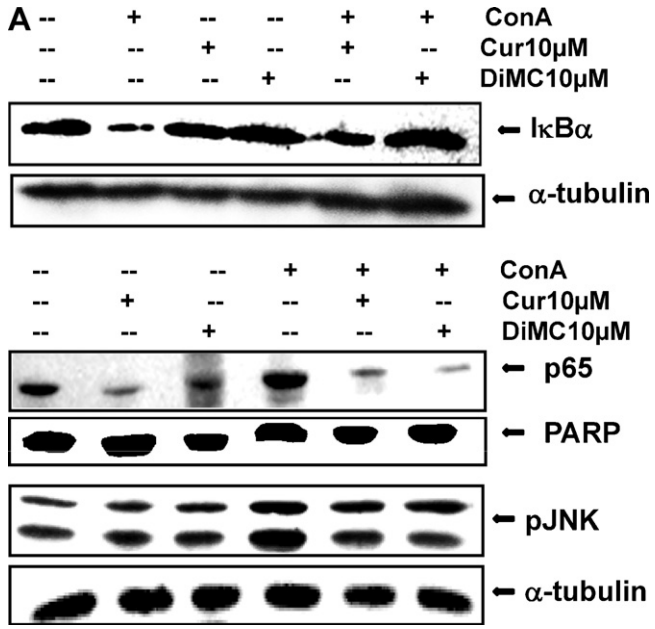

Fig. 7. Inhibition of proliferation/survival associated signaling molecules by curcumin and DiMC in activated T cells. (A) Lymphocytes were incubated with curcumin or DiMC (10 μM, 4 h) and stimulated with Con A (10 μg/ml) for 1 h. Cytosolic and nuclear extracts were prepared, fractionated on 10% SDS–PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using different antibodies specific for p-JNK, IκB-α, P65, PARP and α-tubulin (loading control). (B–E) Lymphocytes were incubated with curcumin or DiMC (10 μM, 4 h) and were stimulated with Con A (10 μg/ml) for 1 h, harvested, fixed, permeablized and stained with PE labelled pERK & pP38 antibody. Representative flowcytometric histograms (A&C) and the corresponding bar diagram (B and D) are shown. Each bar shows mean ± S.E.M. from three replicates and two such independent experiments were carried out. $^{*}p < 0.01$, as compared to vehicle treated cells and $^{\#}p < 0.01$, as compared to Con A stimulated cells.

secretion in murine lymphocytes even when added up to 6 h after mitogenic stimulation (Fig. 8F–H).

Since curcumin and DiMC inhibited the expression of co-stimulatory molecules on activated T cells, we studied whether curcumin and DiMC treatment render these cells anergic and unresponsive to mitogen stimulation. We observed that splenocytes treated with either curcumin or DiMC for 4 h did not respond to mitogenic stimuli when activated with Con A even though they were washed following treatment. This shows that curcumin and DiMC treated cells are hyporesponsive to antigenic stimulation which might be due to a possible induction of anergy (Fig. 8I–K).

4. Discussion

In the past two decades several investigators have shown that, apart from its cardioprotective, neuroprotective, hepatoprotective, anti-HIV, and anti-Alzheimer's activity, curcumin also possesses potent anti-inflammatory effects. It can modulate immune responses by altering the activation of T cells, B cells, macrophages, neutrophils, natural killer cells and dendritic cells [34]. Curcumin has been shown to inhibit the proliferation of lymphocytes induced by different mitogens like Con A, phytohaemagglutinin, and phorbol-12-myristate-13-acetate [35]. However, curcumin has low solubility, and curcumin crystals are not well dispersed in the intestine following oral administration. Most of the absorbed curcumin is metabolized via glucuronidation to glucuronide and glucuronide/sulfate metabolites (di-, tetra-, and hexahydrocurcumin and hexahydrocurcuminol) in the intestinal mucosa and liver and only trace amounts of curcumin (or its metabolites) appear in blood [13,15,17]. Studies on humans have shown that doses of up to 180 mg curcumin fail to produce detectable plasma levels [14] and very high doses (up to 8 g) yield curcumin peak levels of only 0.5–2 μM [36]. Several investigators have prepared curcumin loaded nanoparticles to enhance its bioavailability. In a recent report, curcumin-encapsulated nanoparticles were prepared by emulsion, and a particle size of 264 nm showed enhanced in vivo pharmacokinetics with a 9-fold increase in oral bioavailability compared to curcumin administered with piperine as an absorption enhancer [37]. However, further studies are needed to fully develop strategies for improving the bioavailability of curcumin and to determine whether these methods translate into increased biological activity. Dimethoxycurcumin (chemical structure shown in Fig. 1A), one of several synthetic curcumin analogues, has been reported to exert anticancer activity comparable to curcumin and to have increased metabolic stability in comparison with curcumin [1]. The present data demonstrates for the first time that DiMC possesses potent anti-inflammatory properties in both murine and human lymphocytes.

To explore the anti-inflammatory properties of DiMC, we studied its effect on T cell activation, proliferation and effector responses to different activating stimuli. We observed that DiMC was able to inhibit T cell proliferation in response to both polyclonal and antigen specific activation in a dose dependent manner (Fig. 1D–G). These anti-proliferative effects of DiMC were similar to that of curcumin. A concentration of 10 μM of both curcumin and DiMC could completely suppress activation and proliferation of splenic lymphocytes (Fig. 1D–G). Similar immunosuppressive effects of DiMC were observed in CD4+ T cells, CD8+ T cells and B cells when they were treated with DiMC prior to activation with anti-CD3/CD28 mAbs and LPS, respectively (Fig. 2A–F). Curcumin is known to exert its anti-inflammatory effects via altering the expression and activity of a variety of interleukins, especially IL-1, IL-2, IL-6, IL-8, IL-10, and IL-12 [38–40]. These cytokines which are secreted by different cells participating in the immune response are known to play a critical role in the manifestation of successful pathogen clearance. We observed that, like curcumin, DiMC was also able to completely suppress secretion of IL-2, IL-4, IL-6 and IFN-γ cytokines by activated splenic lymphocytes (Fig. 3A–D). It was also observed, using quantitative RT-PCR, that both curcumin and DiMC inhibited Con A induced increase in expression of IL-2 & IFN-γ genes (Fig. 3I and J). Further, DiMC was also able to suppress anti-CD3/CD28 induced secretion of IL-2, IL-6 and IFN-γ cytokines by CD4+ T cells (Fig. 4A–C). Curcumin has earlier been shown to differentially activate the host macrophages and downregulate Th1 and NO production [41]. In our studies we observed that DiMC was able to suppress LPS induced secretion IL-6 in splenic adherent macrophages (Fig. 4D). Detailed studies on the mechanism of anti-inflammatory effects of DiMC revealed that it also inhibits both early and late events in T cell activation which are dependent on coordinated activation of NFATc and NF-κB [42]. We observed that lymphocytes treated with DiMC or curcumin failed to upregulate T cell activation markers CD69 and CD25 (IL-2 receptor) upon mitogenic stimulation suggesting that, like curcumin, DiMC also affects early as well as late events of the T cell activation pathway (Fig. 5A–D).

Sandur et al., have earlier reported that the anti-inflammatory and antitumor activities assigned to curcumin are mediated through its prooxidant/antioxidant mechanism. They demonstrated that inhibition of NF-κB and NF-κB regulated gene products by curcumin was abrogated by GSH [32]. Further, several studies advocate an important role of cellular redox status in the

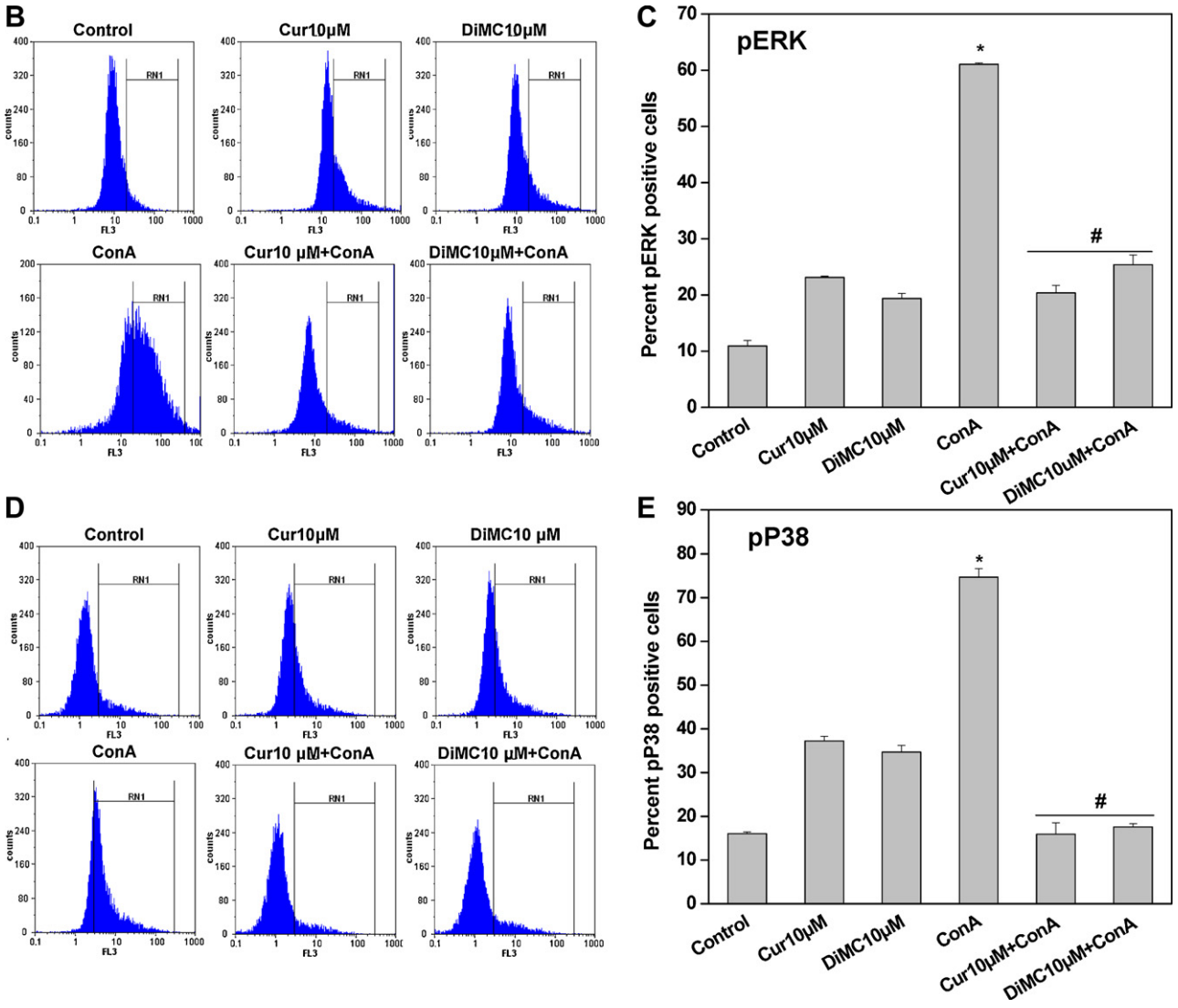

Fig. 7. (*Continued*).

regulation of immune responses [43,44] and addition of antioxidants has been shown to modulate T cell responses as measured in terms of proliferation and cytokine secretion implicating the importance of ROS in antigen mediated T cell activation [44,45]. We recently demonstrated that perturbation of cellular redox status by plumbagin can lead to immunosuppression [46]. Studies were carried out to examine the modulation of cellular redox status by curcumin and DiMC and whether their immunosuppressive effects could be abrogated by antioxidants. We observed that both curcumin and DiMC significantly decreased the basal ROS & GSH levels in lymphocytes (Fig. 6A and B). Further, thiol antioxidant (N-acetyl cysteine) was able to abrogate the suppression of T cell proliferation and cytokine secretion by curcumin and DiMC whereas non-thiol anti-oxidant (trolox) had no effect. Awasthi et al. had earlier shown that curcumin interacts with GSH under in vitro conditions in presence of glutathione-S-transferase which leads to Michael adduct formation [47]. This interaction of curcumin with thiol group present on N-acetyl cysteine could be responsible for the observed abrogation of the immunosuppressive effects of curcumin. Since, DiMC also exhibited a similar mechanism of action we checked for its interaction with GSH and observed that, similar to curcumin, DiMC also interacts with GSH under in vitro conditions (Fig. 6I and J). These results clearly indicate that the mechanism of action of DiMC is same as that of curcumin.

Curcumin primarily exerts its therapeutic effects by inhibiting the degradation of IκB-α resulting in suppression of NF-κB, thus initiating a cascade of downstream inflammatory and immunogenic events [48–50]. The activation of NF-κB is crucial to innate and adaptive immunity and it plays an important role in the activation, differentiation and apoptosis of immune cells. Curcumin mediated inhibition of NF-κB activation, in turn, suppresses the expression of a number of proinflammatory cytokines (e.g., TNF, IL-1, IL-2, IL-6, IL-8, and IL-12) and downregulation of the mRNA expression of several proinflammatory enzymes (e.g., COX-2, LOX-5, MMPs, and iNOS) which are under direct regulation of NF-κB [51–53]. A recent report by Pae et al., showed that DiMC, curcumin and bis-demethoxycurcumin inhibited NO production, iNOS expression and NF-κB activation, with DiMC being the most effective, followed by curcumin and bisdemethoxycurcumin [54]. We also observed that both DiMC and curcumin were equally effective in

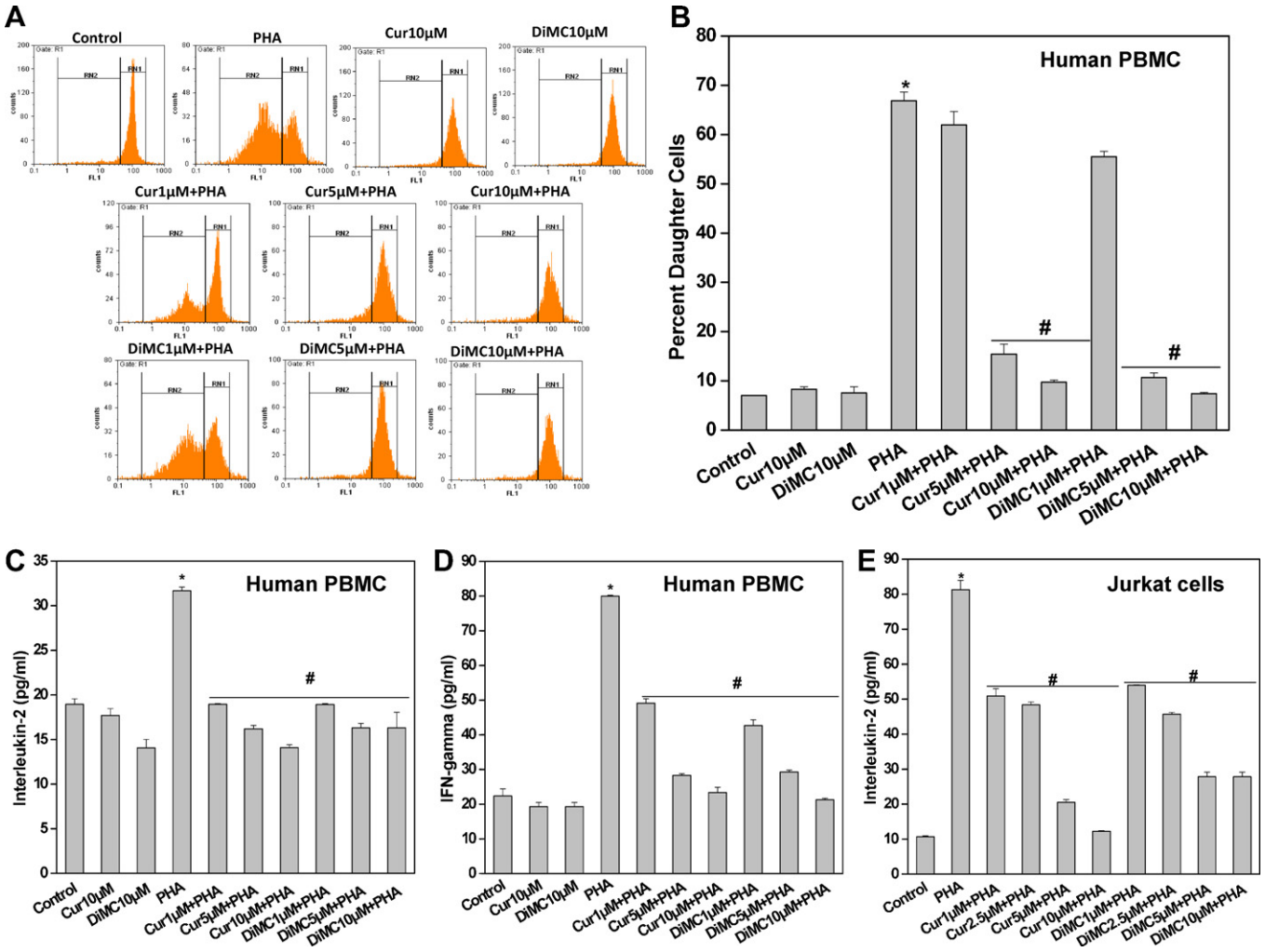

Fig. 8. Curcumin and DiMC inhibit mitogen induced proliferation of human PBMC. (A and B) For cell proliferation analysis CFSE labelled human lymphocytes were pre-treated with different concentrations of curcumin or DiMC (1–10 μM) for 4 h before stimulation with PHA (10 μg/ml) for 72 h at 37 °C. Vehicle treated cells served as control. Twenty thousand cells were acquired in a flowcytometer. Percent daughter cells were calculated from decrease in mean fluorescence intensity. (B) Each bar represents percentage of daughter cells in each treatment group. (C–E) Curcumin and DiMC inhibit PHA induced cytokine secretion in human PBMCs & Jurkat cells. Human PBMCs/Jurkat cells were treated with curcumin or DiMC (1–10 μM, 4 h) and stimulated with the PHA (10 μg/ml) at 37 °C for 24 h. The concentration of IL-2 & IFN-γ (human PBMCs) & IL-2 (Jurkat) in the supernatant was estimated using ELISA. (F–H) Curcumin and DiMC inhibit cytokine production in activated lymphocytes. Lymphocytes were stimulated with Con A (10 μg/ml) following which curcumin or DiMC (10 μM) were added at the indicated time points and the cells were further cultured for 24 h at 37 °C. The concentration of IL-2, IL-6 & IFN-γ in the culture supernatant was estimated using ELISA. (I–K) Curcumin and DiMC inhibit Con A induced cytokine production even after washing. Lymphocytes were treated with curcumin and DiMC (10 μM) for 4 h and washed with normal RPMI twice and then stimulated with Con A (10 μg/ml) for 24 h at 37 °C. The concentration of IL-2, IL-6 and IFN-γ in the culture supernatant was estimated using ELISA. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out.*$p < 0.01$, as compared to vehicle treated cells and $^{\#}p < 0.05$, as compared to PHA or Con A stimulated cells.

inhibiting Con A induced degradation of IκB-α and nuclear translocation and activation of NF-κB in lymphocytes (Fig. 7A). We also checked the effects of DiMC on mitogen induced activation of ERK, JNK and P38 MAPK, since they are important mediators of a number of cellular processes and play a crucial role in complete activation of T cells. We observed that both DiMC and curcumin inhibited mitogen induced phosphorylation of ERK, JNK and p38 MAPkinases indicating that these molecules have multiple targets in exhibiting anti-inflammatory effects (Fig. 7B–E).

In the present report, we have also studied the immunosuppressive activity of curcumin and DiMC in activated human PBMC. Human PBMC & Jurkat cells treated with DiMC or curcumin prior to PHA stimulation showed inhibition of PHA induced proliferation and secretion of cytokines in vitro (Fig. 8A–E). Interestingly, DiMC was able to inhibit Con A induced cytokine secretion in murine lymphocytes even when added up to 6 h post-mitogenic stimulation (Fig. 8F–H) which demonstrated its potential as a therapeutic agent. Further, lymphocytes treated with DiMC or curcumin for 4 h and washed prior to stimulation also showed decreased secretion of IL-2 in response to Con A stimulation indicating that a transient exposure of cells to DiMC or curcumin is sufficient to induce immunosuppression (Fig. 8I–K).

In conclusion, we have shown that the curcumin analogue DiMC exhibited potent anti-inflammatory effects via inhibition of NF-κB and MAPkinases in Con A stimulated lymphocytes. We also show that, like curcumin, DiMC also interacts with thiol groups and that its activity can be abrogated only by thiol containing antioxidants. Further, DiMC and curcumin were also found to be equally effective in suppressing the activation of human PBMC.

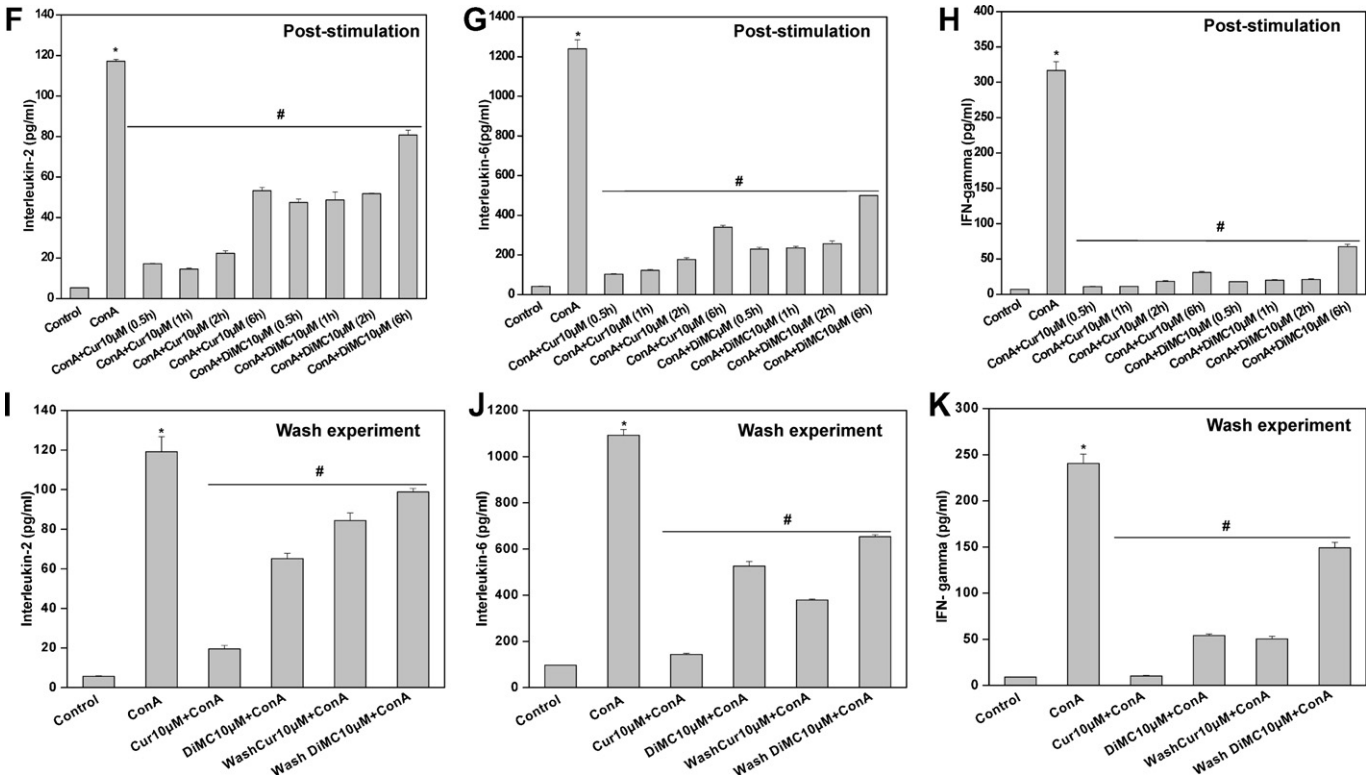

Fig. 8. (*Continued*).

Conflict of interest statement

The authors confirm that there are no conflicts of interest.

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