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Combination of Imipramine, a sphingomyelinase inhibitor, and β-caryophyllene improve their therapeutic effects on experimental autoimmune encephalomyelitis (EAE)

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ABSTRACT

Multiple Sclerosis (MS) is one of the most common inflammatory diseases with the essential role of immune system in the demyelination, damage and inflammation of the central nervous system neurons (CNS). β-Caryophyllene (BCP), a natural and selective CB_2 agonist, possesses several protective effects. In the present study, we evaluated the protective effects of low dose of BCP (5 mg/kg), sphingomyelinase (SMase) inhibitor imipramine (IMP, 10 mg/kg), and the combination of BCP (2.5 and 5 mg/kg) with IMP in the treatment of experimental autoimmune encephalomyelitis (EAE) mice as a known model of chronic MS. These effects were assessed on the levels of pro- or anti-inflammatory cytokines as well as the polarization of spleen lymphocytes and microglia, in EAE mice. Our results indicated that low dose of BCP, IMP and BCP combined with a SMase inhibitor IMP exert protective effects in treatment of EAE mice. We also found that they reduced the clinical and pathological defects in EAE mice through modulation of both local (microglia) and systemic (lymphocytes and blood) immunity from inflammatory ($Th_1/Th_{17}/M_1$) towards anti-inflammatory ($Th_2/T_{reg}/M_2$) phenotypes. Therefore, it can be suggested that a low dose of BCP alone or combined with IMP as a known SMase inhibitor deserve a therapeutic position for treatment of MS.

1. Introduction

Inflammation plays an important role to protect the body against different endogenous and exogenous life threatening stimuli. However, the chronicity of inflammation turns the protective role of immune system towards a deteriorative agent, leading to further several damages to our body conversely [1–5]. Contextually, several studies show that any disturbance or fault in the immune system changes its target to specific parts of the body instead of hazardous stimuli, resulting in several autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), celiac disease, diabetes mellitus type 1, Graves' disease and inflammatory bowel disease [1–4].

MS is one of the most common immune-mediated diseases affecting the central nervous system (CNS). In fact, during this disease, the immune system targets myelin sheath of neurons and myelin compartments, which leads to demyelination and inflammation of neurons as well as axonal loss [6]. Consequently, the communication and neural transmission in the CNS are disrupted, resulting in a wide range of disability including double vision, blindness in one eye, muscle weakness, and trouble with sensation and coordination [6–8]. World Health Organization (WHO) classified the MS as a debilitating disease affecting more than one million people in the world especially females (female/male ratio = 3/1) [6,7]. Although the exact cause and pathophysiology mechanism of MS disease are not clearly understood; however, several studies suggested that it may be caused by a complex of genetic and environmental factors such as infectious agents. Moreover, there is a strong consensus about pathophysiology of MS that this disease is an organ-specific T-lymphocytes-mediated autoimmune disease affecting the brain and spinal cord neurons [6–8]. Additionally, it has been shown that $CD4^+$ T helper (Th) cells targeting myelin are the main culprit cells to the initiation, exacerbation and progression of in patients with MS [9,10]. Indeed, several studies indicate that T helper type 1 (Th_1), producing interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and type 17 (Th_{17}), producing (IL)-17 and IL-22,

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cells are the main population of Th-cells in pathophysiology of MS [1–4]. These cells mainly produce inflammatory cytokines which cause the activation of microglia and development of neuroinflammation in the CNS [4]. In contrast, Th_2 and regulatory T (T_{reg}) cells mainly produce and liberate the anti-inflammatory cytokines including interleukin (IL)-4, IL-10 and transforming growth factor beta-1 (TGF-β_1) [1–4], resulting in the resolution of inflammation, inactivation of microglia, and also attenuation of MS disease progression [4,9,10].

The activation of cannabinoid receptor type 2 (CB_2) is recently paid more attention due to its lake of the psychological and CNS stimulant effects, and also its numerous protective properties against diverse endogenous and exogenous noxious stimuli through decreasing the levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-17, and IFN-γ) and increasing the level of anti-inflammatory cytokine (IL-10) and neuronal survival rates [5,11–13]. β-Caryophyllene (BCP) is a natural and selective CB_2 agonist, founding in different medicinal plants including *Syzygium aromaticum*, *Origanum vulgare L.* and *Piper nigrum L.* [14,15]. Many studies indicated that BCP has promising protective, anti-inflammatory and anti-oxidant effects at both low and high doses in a CB_2 receptor-dependent manner but with different mechanisms [16–18]. Contextually, it has been demonstrated that low doses and concentrations of BCP (10 mg/kg or ≤2 μM, respectively) provide healing and protective effects including analgesic [14], anti-inflammatory [14], reducing the neuropathic pain [19], and anti-apoptogenic effects against divers noxious stimuli comprising the MPP^+ (1-methyl-4-phenylpyridinium)-induced Parkinson model [16] and glutamate-induced excitotoxicity [20], in a CB_2 receptor dependent fashion. On the contrary, second group of studies have indicated that the use of high doses and concentrations of BCP (≥25 mg/kg and 10 μM, respectively) also have protective properties including healing effects in different *in vivo* and *in vitro* models of inflammation, including multiple sclerosis [21,22], ulcerative colitis [23], neuropathic pain [15,22], and β Amyloid (Aβ)-induced cytotoxicity and inflammation in BV-2 murine microglia cell line [17].

Recently, the high doses of BCP (25–100 mg/kg) have been investigated for their protection on the experimental autoimmune encephalomyelitis (EAE) as a chronic and proper model of MS [21,22]. However, in our previous studies, we revealed that the protective effect of BCP at low concentrations is considerably more than its effects at high concentrations [18,24]. We also showed that the protective effect of BCP is augmented when combined with imipramine (IMP), a sphingomyelinase (SMase) inhibitor, in a synergistic manner [18,24]. Interestingly, we demonstrated that the protective effects of BCP at both low and high concentrations are mediated through CB_2 receptor using cyclic adenosine monophosphate (cAMP) functional-selectivity assay [18,24]. Therefore, in the present study, we hypothesized that the use of low dose of BCP possibly possesses therapeutic effects in the treatment of MS and this effect may be potentiated by a SMase inhibitor such as IMP in the EAE model of chronic MS. Experimentally, we also evaluated the effects of BCP, IMP and their combination on polarizations of lymphocytes (Th_1/Th_2 and Th_{17}/T_{reg}) and microglia (M_1/M_2), and the levels of inflammatory and anti-inflammatory cytokines profile in EAE mice.

2. Materials and methods

2.1. Chemicals and kits

β-Caryophyllene (BCP, C9653 SIGMA), RPMI-1640 and DMEM culture media, penicillin plus streptomycin (pen/strep), amphotericin B, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), Ficoll®, DNAse I, dispase II, Bradford reagent (B6916 SIGMA), 2′, 7′-dichlorofluorescin diacetate (DCFH DA, code D6883), Griess reagent (G4410 SIGMA), sulfanilamide, n-(1-naphthyl) ethylenediamine, triton-X, sulfosalicylic acid, potassium phosphate buffer, EDTA disodium salt, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), potassium chloride (KCl), dithiothreitol (DTT), Nonidet P40, phenylmethanesulfonyl fluoride (PMSF), protease inhibitor cocktail, complete Freund's adjuvant (CFA, H37Ra strain), pertussis toxin, Hank's Balanced Salt Solution (HBSS) and other cell culture materials were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Imipramine hydrochloride (IMP, sc-207753A) was purchased from Santa Cruz Biotechnology Inc. Enzyme-linked immunosorbent assay (ELISA) kits including TNF-α (BMS607-3, 3.7 pg/mL), IL-6 (BMS603-2, 6.5 pg/mL), IL10 (BMS614INST, 5.28 pg/mL), IL-17 (BMS6001, 1.6 pg/mL), IL-4 (BMS613HS, 0.32 pg/mL), IFN-γ (BMS606, 5.3 pg/mL), and TGF-β_1 (BMS608-4, 7.8 pg/mL) were obtained from eBioscience (San Diego, CA, USA). The transcription factors forkhead box P3 (Foxp3, MBS731757, 0.1 ng/mL), retinoic acid receptor (RAR)-related orphan receptor gamma (ROR-γt, MBS751809, 0.1 ng/mL), GATA transcription factor 3 (GATA3, MBS728318, 0.1 ng/mL), T-box transcription factor TBX21 (T-bet, MBS763917, 0.094 ng/ml), arginase-1 (Arg-1, MBS1601158, 0.28 ng/ml) and iNOS (MBS764477, 0.188 ng/ml) mouse ELISA kits were from MyBiosource (CA, USA). Mouse prostaglandin E2 (PGE_2, CSB-E07966m, 0.2 pg/mL) ELISA kit was also purchased from CUSABIO (Wuhan, Hubei, China). Myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}, sequence: MEVGWYRSPFSRVVHLYRNGK) was from SBS Genetech Co. Ltd. (Beijing, China). BrdU kit (colorimetric) was purchased from Roche Applied Science (Indianapolis, IN, USA). RBC Lysis Buffer (10x, Cat No:420301) was from Biolegend (San Diego, CA, USA).

2.2. Animals and husbandry

Female C57BL/6 mice (8–10 week old, weighing 18–20 g) were purchased from the Pasteur Institute of Iran. The animals were maintained at a controlled room temperature (22–25 °C) with a 12/12 h light/dark cycle and 50% relative humidity. All animals allowed to a clean and safe filtered tap water and commercial standard rodent chow *ad libitum*. All experiments and procedures were carried out according to the *NIH's Guideline for the Care and Use of Laboratory Animals*, and accompanying the approval from the *Animal Ethics Committee of Mashhad University of Medical Sciences* (approval no. 960037, IR.MUMS.fm.REC.1396.200).

2.3. Experimental autoimmune encephalomyelitis (EAE) induction and clinical evaluation

Female C57BL/6 mice were actively immunized using two subcutaneous injections with an emulsion containing myelin MOG_{35-55} (250 μg) and an equal volume of CFA (250 μg) into different sites of each hind flank. Moreover, mice received two intraperitoneal (i.p.) injections of pertussis toxin (250 ng) on the day zero (day of immunization) and 48 h later (day 2) of MOG + CFA injection. The procedure of EAE induction was summarized in Fig. 1A. Animals were daily weighed and clinically scored from day zero to day 37 (the last day of the experiment). The scaling of EAE's clinical score and its description were in compliance with the explanation of Bittner *et al.* [25], which illustrated in Table 1. Mice were also assessed for the incidence (sick/total), mean onset day of disease (MDO, days after immunization), maximal mean clinical score (MMCS, during the entire course of treatment), mean clinical score (MCS, at the peak day) and cumulative disease index (CDI, total disease score over experiment duration), which were indicated in Table 3.

2.4. Study protocols

2.4.1. Protocol 1

The protocol was done to evaluate the protective effects of low dose of BCP (5 mg/kg) in the EAE model (Table 2). BCP (5 mg/kg, dissolved in corn oil) was orally given once a day from days 10–37 after immunization (Fig. 1A, n = 8/each group). The selection of BCP dose was

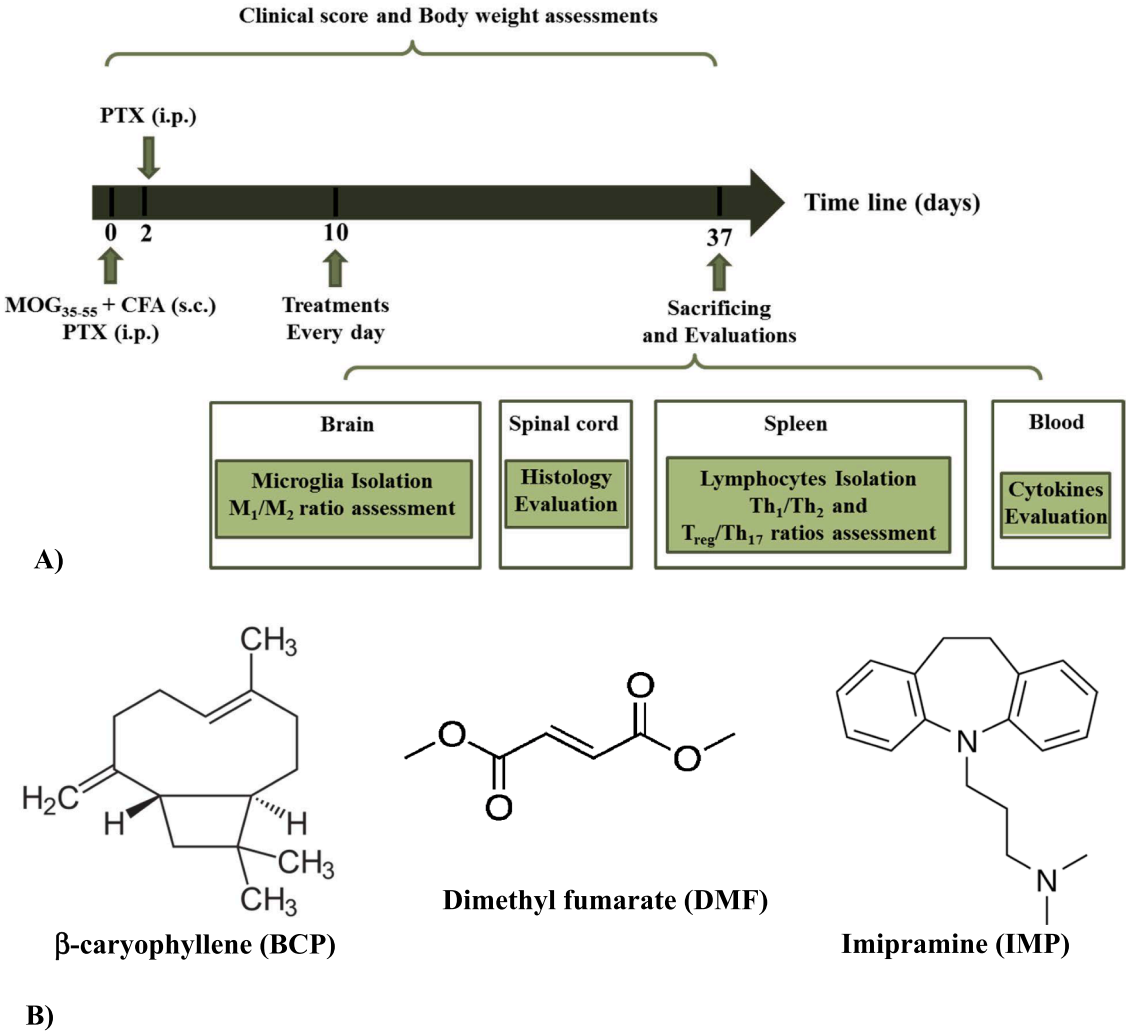

Fig. 1. Protocol of treatment and chemical structures; (A) the summarized protocols of induction and treatments, (B) chemical structures of the used compounds in the present study.

based on our previous *in vitro* experiments regarding the BCP (microglia [24] and oligodendrocytes [18]) and previous *in vivo* studies about the protective effects of BCP (25 and 50 mg/kg/day, [21,22]) on EAE. In fact, we performed the present study by one-tenth of previous reported doses of BCP in treatment of EAE. In this study, dimethyl fumarate (DMF) was considered a positive control group as a standard, orally available and approved medication by the US food and drug administration (FDA) for patients with MS. DMF-treated group received 30 mg/kg DMF (dispersed in 0.08% w/v methylcellulose) twice a day per oral gavage (n = 8) [26,27]. Control group was EAE mice receiving the same volume of vehicle (n = 8). In this study, the sham group comprised healthy mice receiving the same volume of PBS injections instead of MOG_{35-55}-induced EAE.

2.4.2. Protocol 2

This protocol was also done to investigate the protective effects of IMP, a SMase inhibitor, and its interactive effects with BCP (Table 2). In brief, IMP (dissolved in PBS, 10 mg/kg/day; p.o. [28,29]) was administrated either alone or in the combination with BCP (2.5 and 5 mg/kg/day; p.o.). Combination groups received IMP one hour before the

Table 1
Clinical scoring of EAE mice [25].

Score	Clinical manifestations (important comment)
0	No clinical signs
1	Partially limp tail (Normal gait, a tip of the tail droops)
2	Paralyzed tail (Normal gait, tail droops)
3	Hind limb paresis, uncoordinated movement (Uncoordinated gait, tail limps, hind limbs respond to pinching)
4	One hind limb paralyzed (Uncoordinated gait with one hind limb dragging, tail limps, one hind limb does not respond to pinch)
5	Both hind limbs paralyzed (Uncoordinated gait with both hind limbs dragging, tail limps, both hind limbs do not respond to pinch)
6	Hind limbs paralyzed, weakness in forelimbs (Uncoordinated gait with forelimbs struggle to pull body, forelimbs reflex after pinching, tail limps)
7	Hind limbs paralyzed, one forelimb paralyzed (Mouse cannot move, one forelimb responds to toe pinch, tail limps)
8	Hind limbs paralyzed, both forelimbs paralyzed (Mouse cannot move, both forelimbs do not respond to toe pinch, tail limps)
9	Moribund (No movement, worsen breathing)
10	Death

Table 2
The summarized protocols were performed in the present study.

Protocol	Groups	EAE	From day 10 to 37 (every day)	
1 (n = 8/each group)	Sham	–	–	
	Control	✓	Vehicle	
	BCP (5 mg/kg)	✓	BCP 5 mg/kg/day; p.o.	
	DMF (60 mg/kg)	✓	DMF 30 mg/kg twice a day; p.o.	
2 (n = 8/each group)				One hour later
	Sham	–	–	–
	Control	✓	IMP vehicle; p.o.	+ BCP vehicle; p.o.
	DMF (60 mg/kg)	✓	IMP vehicle; p.o.	DMF 30 mg/kg twice a day; p.o.
	BCP (5 mg/kg)	✓	IMP vehicle; p.o.	+ BCP 5 mg/kg/day; p.o.
	IMP (10 mg/kg)		IMP 10 mg/kg/day; p.o.	+ BCP vehicle; p.o.
	IMP (10 mg/kg) + BCP (2.5 mg/kg)	✓	IMP 10 mg/kg/day; p.o.	+ BCP 2.5 mg/kg/day; p.o.
	IMP (10 mg/kg) + BCP (5 mg/kg)	✓	IMP 10 mg/kg/day; p.o.	+ BCP 5 mg/kg/day; p.o.

BCP medication, (n = 8/each group). Control group was also EAE mice receiving the same volume of the vehicle (n = 8).

In all experiments, we found no statistical differences in the clinical score of control groups receiving different vehicles.

2.5. Preparation of serum and examination of the cytokines profile

On the last day of the experiment (day 37), following the deep anesthesia, 1.5 ml of blood was drawn by cardiac puncture and then collected in 2 ml EDTA-coated microtube containing 140 μg aprotinin. Immediately, the samples were centrifuged at 2500 g, 4 °C for 10 min. Subsequently, the plasma was collected and stored at −20 °C for further evaluations. The levels of inflammatory (TNF-α, IL-6, and IL-17) and anti-inflammatory (IL-10) cytokines were determined by the commercially available ELISA kits according to the manufacturer's instruction and expressed as pg per ml of plasma.

2.6. Histological evaluation

On day 37 (the time of sacrifice), mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and then sacrificed [30,31]. Afterward, for evaluating the level of CNS inflammation, the spinal cord was immediately isolated and maintained in 4% (v/v) paraformaldehyde. Longitudinal sections (5 μm thick) of the spinal cords paraffin-embedded were stained with Hematoxylin and Eosin (H&E) for evaluation of inflammation. Sections scoring were then performed using light microscopy in a blinded manner (by analysis of three fields) at 40 × magnification (Olympus, Tokyo, Japan). The pathological scores were determined according to the previous published studies [32] as follows;

0- No inflammation,
1- Low level of inflammatory cells,
2- Perivascular infiltrates, and
3- Extension in the intensity of perivascular cuffing with expansion into contiguous tissue.

2.7. Isolation of lymphocyte from spleen, and cell culture

Following the mice euthanasia, the spleen was aseptically removed and placed into a 15 ml tube containing 5 ml ice-cold RPMI-1640/FBS (RPMI-1640, 100 U/mL penicillin, 100 μg/mL streptomycin and 2% v/v FBS). Preparation of spleen lymphocytes was performed according to the previously describe methods using Ficoll® density gradients [1]. For excluding monocytes, pellets were suspended into an enriched RPMI-1640 (10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate and HEPES buffer) and cultured in 250 ml flasks for an overnight. Afterward, the supernatant including lymphocytes was transferred to 50 ml sterilized conical tube [1,2]. Isolated lymphocytes were cultured in RPMI-1640 supplemented with 1% v/v of pen/strep (100X), 10% v/v of heat-inactivated FBS, 0.5 μg/mL amphotericin B and 2 mM L-glutamine. Cells were maintained at 37 °C and 5% v/v CO_2, in a humidified incubator.

2.7.1. Proliferation assay

Lymphocytes (5×10^4 cells/well) were cultured in 96-well plates and treated with either medium alone or medium + $MOG_{35–55}$ peptides (30 μg/mL), for 48 h at 37 °C and 5% v/v CO_2. For the last 24 h, BrdU labeling solution (0.1% v/v) was added into the plates. Cell proliferation was assessed by a cell proliferation ELISA, BrdU kit (colorimetric) according to the manufacturer's manual. The absorbance was read out at 450 nm using the microplate reader (Stat Fax 2100 Awareness, Phoenix, Arizona, USA). The mean of cell proliferation level of the $MOG_{35–55}$-treated shame group was considered 100%.

2.7.2. Assessment of the cytokines profile

To have a better insight on the effects of different treatments on the polarizations of Th_1/Th_2 and Th_{17}/T_{reg} in EAE, we evaluated the levels of Th_1 (IFN-γ), Th_{17} (IL-17), Th_2 and T_{reg} (IL-4, IL-10 and TGF-β_1) related cytokines, as well as inflammatory cytokines IL-6 and TNF-α, in the supernatant of MOG_{35-55},-stimulated lymphocytes. For this aim, the isolated lymphocytes (2×10^6 cells/well) were cultured in 12-well plates and treated with either medium alone or medium + $MOG_{35–55}$ peptides (30 μg/mL) for 48 h at 37 °C and 5% v/v CO_2. Afterward, the supernatants were collected and stored at −20 °C for evaluation of the levels of cytokines using the ELISA method according to the manufacturer's manual [33,34]. The levels of cytokines were standardized and reported as pg/mg protein. In each experiment, the total protein contents were measured according to Bradford's method [35].

2.7.3. Assessments of intracellular levels of transcription factors

For confirmation of lymphocytes polarization, the levels of specific and valid transcription factors for Th_1 (T-bet), Th_2 (GATA3), Th_{17} (ROR-γt) and T_{reg} (Foxp3) cells were measured [1,10,36]. To this end, the isolated lymphocytes (2×10^6 cells/well) were cultured in 12-well plates and treated with either medium alone or medium + $MOG_{35–55}$ peptides (30 μg/mL) for 48 h at 37 °C and 5% v/v CO_2. After that, lymphocytes were centrifuged to provide the pellets and washed with PBS for three times. Then, the pellets were re-suspended into the cell lysis buffer (10 mM HEPES; pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Nonidet-P40 and 0.5 mM PMSF along with the protease inhibitor cocktail) and maintained on the icy water for 15-20 min with intermittent vortexing. In this stage, total protein contents were also measured according to Bradford's method [35]. Samples were stored at −20 °C for evaluation of the levels of transcription factors using the ELISA method according to the manufacturer's manual. The transcription factors levels were expressed as ng or pg/mg protein.

2.8. Microglia isolation

Microglial cells were isolated using a described method with Lee and Tansey [24,37,38]. Isolated microglia cells were cultured in the enriched DMEM (1% v/v of pen/strep (100X), 10% v/v of heat-inactivated FBS, 0.5 μg/mL amphotericin B and 2 mM L-glutamine), at 37 °C and 5% v/v CO_2, in a humidified incubator.

2.8.1. Proliferation assay

Microglia cells (8×10^3 cells/well) were cultured in 96-well plates and treated with either medium alone or medium + $MOG_{35–55}$ peptides (30 μg/ml) for 48 h, at 37 °C and 5% v/v CO_2. For the last 24 h, BrdU labeling solution (0.1% v/v) was added into the plates. Cell proliferation was assessed by a cell proliferation ELISA-based BrdU kit (colorimetric) according to the manufacturer's manual. The absorbance was read out at 450 nm using the microplate reader (Stat Fax 2100 Awareness, Phoenix, Arizona, USA). The mean of cell proliferation level of the $MOG_{35–55}$-treated shame group was considered 100%.

2.8.2. Assessment of the cytokines levels

The levels of anti-inflammatory (IL-10) and inflammatory cytokines (TNF-α and PGE_2) were measured by the ELISA-based method according to the manufacturer's instructions. The cells were cultured in 6-well plates (10^6 cells/each well) and incubated with either medium alone or medium + $MOG_{35–55}$ peptides (30 μg/ml) for 48 h, at 37 °C in 5% v/v CO_2 incubator. Finally, the supernatants were collected for measuring the levels of cytokines. The levels of cytokines were reported as pg/mg protein.

2.8.3. Assessment of the intracellular levels of iNOS and Arg-1

Intra-cellular levels of iNOS and Arg-1 (as indicators of M_1 and M_2 cells, respectively) were assessed using a commercially available ELISA-based kit according to the manufacturer's manual. The cells were cultured in 6-well plate (2×10^6 cells/each well) and incubated with either medium alone or medium + $MOG_{35–55}$ peptides (30 μg/ml) for 48 h, at 37 °C in 5% v/v CO_2 incubator. After that, the cells were collected, and lysed using lysis buffer and then homogenized (DIAX 100, Heidolph, Schwabach, Germany) on the cold water (0–4 °C) for 2–3 min along with vortexing (every 30 sec). The samples were centrifuged at 12,000 g for 10 min at 4 °C, and 50 μL of supernatants were then undergone of assessment. The levels of iNOS and Arg-1 were reported as ng/mg protein.

2.8.4. Assessment of the levels of nitric oxide metabolites (NO) and urea

The levels of nitric oxide metabolites (NO, produced by M_1 cells) and urea (produced by M_2 cells) were measured in the supernatants of cultured microglia. The NO production level was examined using the Griess method which described previously [39]. In brief, 50 μL of the supernatant was incubated with an equal volume of sulfanilamide and *n*-(1-naphthyl)-ethylenediamine in 2N hydrochloric acid at controlled room temperature for 10 min. The absorbance was read out at 540 nm using the microplate reader (Stat Fax 2100 Awareness, Phoenix, Arizona, USA). The concentration of NO was determined using sodium nitrite standard curve [38].

The level of urea was determined using a commercially available urea assay kit according to the manufacturer's instructions. Briefly, 50 μL of supernatant was mixed with equal volumes of medium (blank) and 50 μL urea-solution (5 mg/dl or 850 μM), and then incubated with the 200 μL working reagent at controlled room temperature for 50 min. The absorbance was read out at 430 nm using the Stat Fax 2100 Awareness microplate reader. The level of NO and urea were expressed as nmol/mg protein.

2.9. In vitro evaluation of the effects of BCP, IMP and their combination on lymphocyte

This set of experiment was carried out to evaluate the direct effects of BCP and IMP, and also their interactive combination on lymphocytes' cell proliferation and polarizations (Th_1/Th_2 and Th_{17}/T_{reg} ratios). Isolation of spleen lymphocytes were performed according to Section 2.7 and were from the vehicle-treated EAE mice. Lymphocytes were cultured either in 96-well plates (5×10^4 cells/well) or in 12-well plates (2×10^6 cells/well) for assessment of cell proliferation or cytokines profile, respectively. Then, the cells were simultaneously incubated with different concentration of BCP, IMP or their combination in the presence of $MOG_{35–55}$ peptides (30 μg/mL), for 48 h at 37 °C and 5% v/v CO_2. Experimental protocols were as follows.

Group 1, Sham: spleen lymphocytes isolated from the vehicle-treated EAE mice receiving no treatments, and no $MOG_{35–55}$ stimulation (30 μg/mL)

Group 2, Vehicle: spleen lymphocytes isolated from the vehicle-treated EAE mice receiving no treatments but receiving $MOG_{35–55}$ stimulation (30 μg/mL)

Groups 3 & 4, BCP treated: spleen lymphocytes isolated from the vehicle-treated EAE mice receiving BCP treatment (0.2 or 1 μM), and $MOG_{35–55}$ stimulation (30 μg/mL)

Group 5, IMP treated: spleen lymphocytes isolated from the vehicle-treated EAE mice receiving IMP treatment (1 μM), and $MOG_{35–55}$ stimulation (30 μg/mL)

Groups 6 & 7, BCP + IMP treated: spleen lymphocytes isolated from the vehicle-treated EAE mice receiving of combination of IMP treatment (1 μM) and BCP treatments (0.2 or 1 μM), and $MOG_{35–55}$ stimulation (30 μg/mL)

2.9.1. Proliferation assay

For cell proliferation assay, BrdU labeling solution (0.1% v/v) was added into the plates 24 h before the assessment. Cell proliferation was measured by a cell proliferation ELISA, BrdU kit (colorimetric) according to the manufacturer's manual. The mean of cell proliferation level of sham group, received neither BCP, IMP, their combination nor the $MOG_{35–55}$, was considered 100%.

2.9.2. Assessment of the cytokines profile

The supernatants were collected and stored at −20 °C for subsequent assay of the levels of cytokines (IFN-γ, IL-4, IL-17, IL-10) using the ELISA method according to the manufacturer's manual. The levels of cytokines were standardized and reported as pg/mg protein. In each experiment, the total protein contents were measured according to Bradford's method [35].

2.10. Combination index assessment

To assess the pharmacological interaction between BCP and IMP, the combination index (CI) was calculated based on Chou equation using the CompuSyn® software (ComboSyn, NJ[Q]) as described previously [18,40]. In this study, cell proliferation (for *in vitro* study) was considered as the endpoints for the calculation of CI. Synergistic, additive and antagonistic effects are concluded when the CI is lesser, equal or greater than 1.0, respectively.

2.11. Statistical analysis

Data were analyzed using GraphPad Prism ® 6 (GraphPad Software, San Diego, CA) software and expressed according to the nature of parametric or none-parametric as means ± SEM or median ± range (for pathological score), respectively. For parametric data, normality test was performed based on Kolmogorov–Smirnov and Bartlett's tests that assess the homogeneity of variances; afterwards, comparisons between groups were done using two-way analysis of variance (ANOVA)

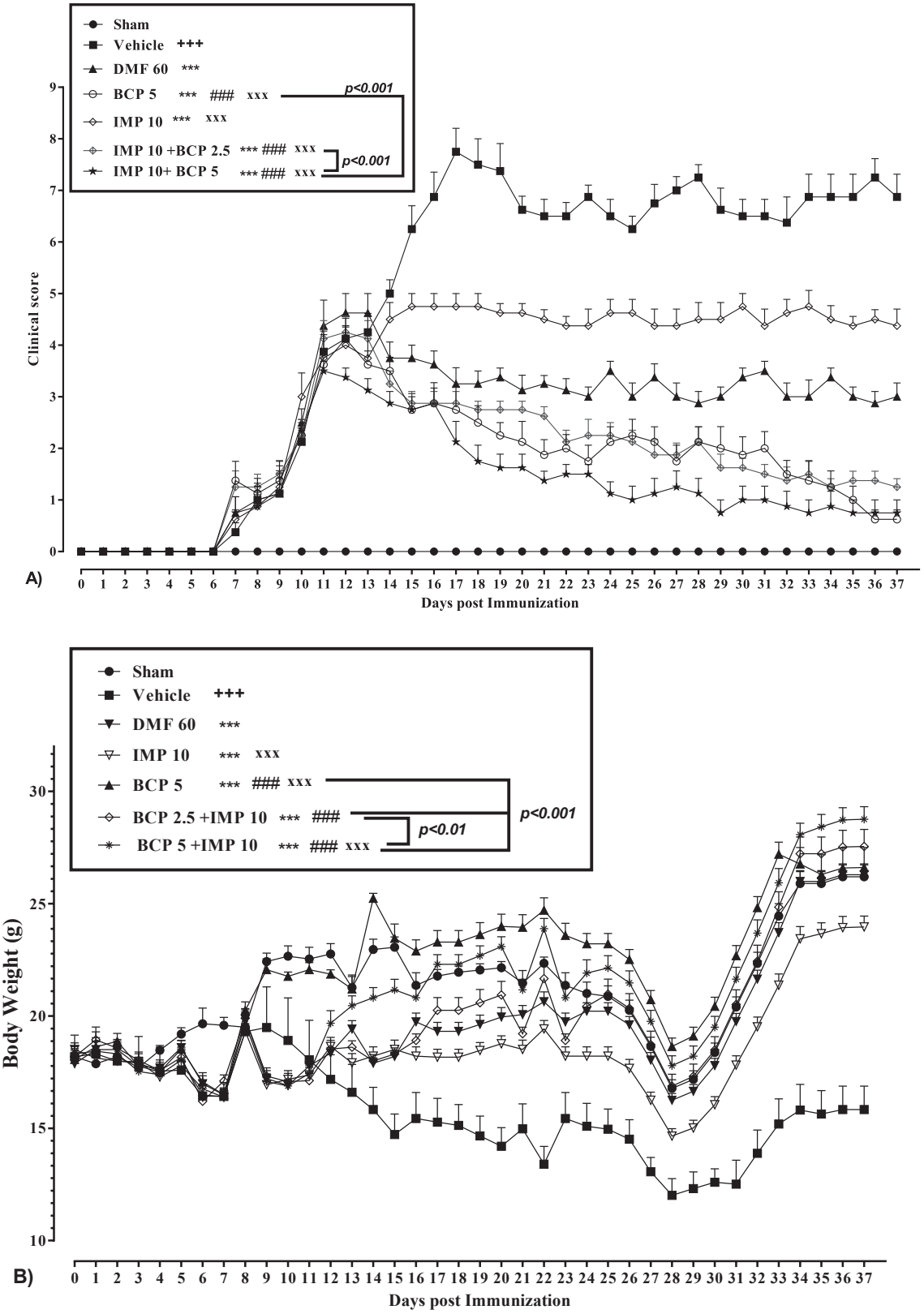

Fig. 2. The effects of treatment with BCP, IMP and their combinations on clinical score and body weight of EAE mice; (A) The effects of BCP (5 mg/kg/day; p.o.), DMF (60 mg/kg; 30 mg/kg twice a day; p.o.), IMP (10 mg/kg; p.o.), and the combination of BCP (5 mg/kg/day; p.o.) with IMP (10 mg/kg; p.o.) on the clinical score of EAE mice. (B) The effects of BCP (5 mg/kg/day; p.o.), DMF (60 mg/kg; 30 mg/kg twice a day; p.o.), IMP (10 mg/kg; p.o.), and the combination of BCP (5 mg/kg/day; p.o.) with IMP (10 mg/kg; p.o.) on the body weight of EAE mice. Data were presented as mean ± SEM, n = 8 for each protocol of experiment. Repeated measures two-way ANOVA test was carried out with the following Tukey's multiple comparisons test. ($^{+}$) compares vehicle-treated group with the sham group, $^{+++}$: $p < 0.001$.; (*) shows a comparison with the vehicle group, ***: $p < 0.001$; ($^{\#}$) represents a comparison with the IMP group in each graph, $^{\#\#\#}$: $p < 0.001$; (^{x}) indicates a comparison with the DMF treated group in each graph, ^{xxx}: $p < 0.001$. Abbreviations: BCP: β-caryophyllene, IMP: Imipramine, DMF: Dimethyl fumarate, ANOVA: analysis of variance.

with the following Dunnett's *post-hoc* multiple comparisons test. For clinical score and body weight, repeated measures two-way ANOVA was done with the following Tukey's multiple comparisons test. None parametric data were analyzed using the Kruskal-Wallis' test with Dunn's *post-hoc* multiple comparisons test. *P* values (p) were considered statistically significant when $p \leq 0.05$, 0.01 and 0.001. The data and statistical analysis complied with the recommendations on experimental design, analysis [41] and data sharing and presentation in preclinical pharmacology [42,43].

3. Results

3.1. The effects of BCP, IMP and their combinations on the clinical score

Our results showed that the level of clinical score in vehicle-treated EAE group was significantly increased in comparison with the sham group ($p < 0.001$, Fig. 2A). In contrast, the level of clinical score was notably diminished in DMF (60 mg/kg; p.o.), BCP (5 mg/kg; p.o.), IMP (10 mg/kg; p.o.), and combinations of BCP (2.5 and 5 mg/kg; p.o.) with IMP (10 mg/kg; p.o.) groups in comparison to the vehicle group ($p < 0.001$ for all cases, Fig. 2A). Moreover, we revealed that the clinical score of BCP (5 mg/kg) and the combination of BCP (2.5 and 5 mg/kg) with IMP groups were markedly lower than both IMP and DMF groups ($p < 0.001$ for all cases, Fig. 2A). Of note, the clinical score of IMP (10 mg/kg)-treated group was notably lower than DMF group ($p < 0.001$ Fig. 2A). Moreover, we observed that the level of clinical score in groups receiving the combinations of BCP (2.5 and 5 mg/kg) with IMP was significantly reduced in comparison to the BCP (5 mg/kg)-treated group ($p < 0.001$ for both cases, Fig. 2A).

As illustrated in Table 3, all vehicle-treated mice (control group) elaborated EAE and showed the first signs of disease onset with partially limp tail on day 6.42 ± 0.82 achieving an MCS of 7.35 ± 0.77 on day 17 (EAE peak day). Our data indicated that the disease incidence in BCP (5 mg/kg), and BCP (2.5 and 5 mg/kg) combined with IMP (10 mg/kg) groups was significantly attenuated in comparison to the vehicle group ($p < 0.001$ for all cases, Table 3). Furthermore, treatment with DMF (60 mg/kg), IMP (10 mg/kg), BCP (5 mg/kg), and the combination of BCP (2.5 and 5 mg/kg) with IMP meaningfully reduced the MCS at EAE peak day, MMSC and CDI in comparison to the vehicle group ($p < 0.001$ for all cases, Table 3). We also observed that the levels of MCS at EAE peak day, MMCS and CDI in groups receiving BCP (5 mg/kg) alone and the combination of BCP (2.5 and 5 mg/kg) with IMP (10 mg/kg) were significantly less than DMF and IMP alone treated groups ($p < 0.001$ for all case, Table 3). In addition, the levels of MCS at EAE peak day and CDI in BCP (5 mg/kg) + IMP (10 mg/kg)-treated group was notably lower than BCP (5 mg/kg)-treated group ($p < 0.001$ for both cases, Table 3).

3.2. The effects of BCP, IMP and their combinations on the body weight

In the vehicle-treated EAE group, the level of body weight was significantly decreased in comparison to sham group ($p < 0.001$, Fig. 2B). On the contrary, we observed that treatment with DMF (60 mg/kg; p.o.), IMP (10 mg/kg; p.o.), BCP (5 mg/kg; p.o.) and the combination of IMP with BCP (2.5 and 5 mg/kg) significantly increased the body weight of EAE mice in comparison to the vehicle-treated group ($p < 0.001$ for all cases, Fig. 2B). Our records also indicated that Our records also indicated that the body weight level was significantly lower in IMP-treated group in comparison to the DMF-treated group ($p < 0.001$, Fig. 2B). The level of body weight was also markedly greater in groups receiving BCP (5 mg/kg) alone and the combination of BCP (2.5 and 5 mg/kg) with IMP compared to both IMP- and DMF-treated groups ($p < 0.001$ for all cases, Fig. 2B). Interestingly, we found that the combination of IMP with BCP (2.5 and 5 mg/kg) meaningfully increased the body weight of EAE mice in comparison to the groups receiving either BCP (5 mg/kg) alone or IMP alone ($p < 0.001$–0.01 for all cases, Fig. 2B)

3.3. The effects of BCP, IMP and their combinations on the level of pathological score

The pathological investigation showed that the vehicle-treated EAE group has greater pathological score including massive leukocyte infiltrations with several foci of inflammation and vacuolization in comparison to the sham group (Fig. 3A, B and C). In contrast, the levels of pathological scores were significantly diminished in groups treated with DMF (60 mg/kg, $p < 0.05$), IMP (10 mg/kg, $p < 0.05$), BCP (5 mg/kg, $p < 0.001$), and the combination of BCP (2.5 mg/kg, $p < 0.01$, and 5 mg/kg, $p < 0.001$) with IMP (10 mg/kg) in comparison to the vehicle-treated group (Fig. 3A-H). Our results also demonstrated that the pathological scores were dramatically decreased in BCP (5 mg/kg) alone and its combination with IMP (10 mg/kg) comparing to both DMF and IMP groups ($p < 0.05$ for all cases, Fig. 3A and D-H).

Table 3
The effects of BCP, IMP and their combinations on different clinical parameters of EAE mice.

Groups	Incidence (sick/total)	MOD[a]	MMCS[a]	MCS at peak EAE (Day 17) [a]	CDI[a,b]	Day presents significant changes[c]
Sham	0/16 (0%)	–	0 ± 0	0 ± 0	0	0
Vehicle	16/16 (100%)$^{+++}$	6.42 ± 0.82^{+++}	7.35 ± 0.77^{+++}	7.35 ± 0.77^{+++}	29.20 ± 1.45^{+++}	9[d]
BCP 5	9/16 (56.25%)$^{***, ###, xxx}$	6.72 ± 0.92	3.32 ± 0.28$^{***, ###, xxx}$	2.85 ± 0.53$^{***, ###, xxx}$	11.20 ± 0.93$^{***, ###, xxx}$	15[e]
DMF 60	15/16 (93.75%)	6.62 ± 0.85	4.85 ± 0.21***	3.49 ± 0.61***	15.38 ± 1.62***	15[e]
IMP 10	8/8 (100%)	7.54 ± 0.95	4.78 ± 0.26***	4.75 ± 0.26***	20.72 ± 1.71***	17[e]
BCP 2.5 + IMP 10	6/8 (75%)$^{*, #, xx}$	6.22 ± 0.62	3.52 ± 0.19$^{***, ##, xx}$	2.87 ± 0.22$^{***, ###, xx}$	11.40 ± 1.06$^{***, ###, xx}$	15[e]
BCP 5 + IMP 10	4/8 (50%)$^{***, ###, xxx}$	7.20 ± 0.43	2.90 ± 0.16$^{***, ###, xxx}$	1.96 ± 0.22$^{***, ###, xxx, †††}$	7.98 ± 0.93$^{***, ###, xxx, †††}$	13[e]

Mean onset day of disease (MOD, days after immunization), maximum mean clinical score (MMCS), mean clinical score (MCS), and cumulative disease index (CDI; total disease score over experiment duration). $^{+}$ compared with the sham group; $^{+++}p < 0.001$. * compared with the vehicle-treated group; $^{*}p < 0.5$ and $^{***}p < 0.001$. $^{\#}$ compared to IMP-treated alone group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$. ^{x} compared to DMF-treated group; $^{xxx}p < 0.001$. † compared with BCP-treated group; ††† $p < 0.001$.

[a] Data were expressed as mean ± SEM.

[b] Sum of clinical scores over the entire period.

[c] Shows days after immunization with MOG_{35-55}.

[d] Indicates a significant changes in comparison to sham group.

[e] Represents a significant changes in comparison to the vehicle group.

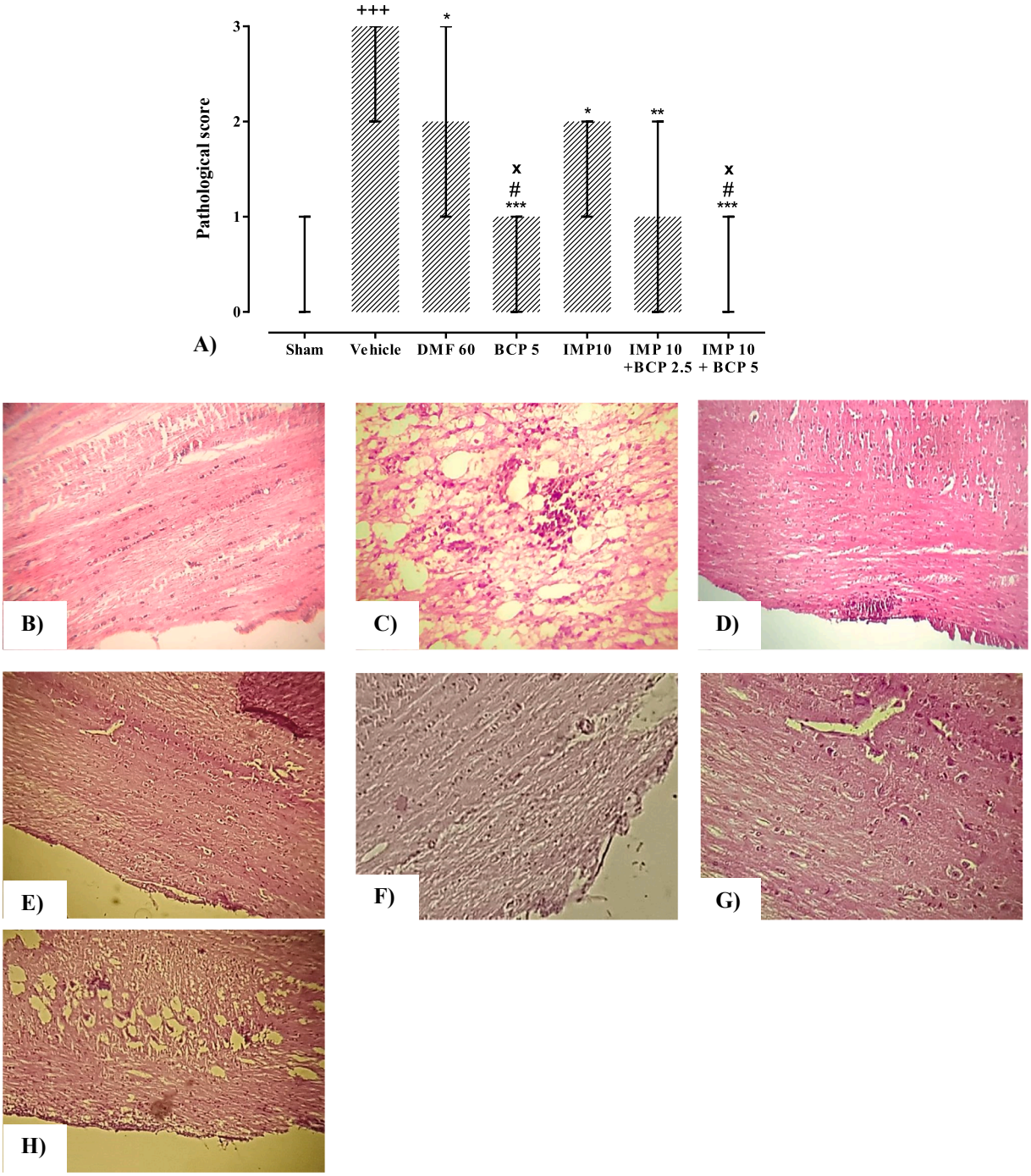

Fig. 3. The effects of different treatments on the inflammatory cell infiltration and inflammation into the spinal cords with H&E to enumerate infiltrating leukocytes; (A) The effects of BCP (5 mg/kg/day; p.o.), DMF (60 mg/kg; 30 mg/kg twice a day; p.o.), IMP (10 mg/kg; p.o.), and the combination of BCP (2.5 and 5 mg/kg/day; p.o.) with IMP (10 mg/kg; p.o.) on the pathological score in EAE mice. (B) Sham group, (C) Vehicle, (D) DMF 60 mg/kg, (E) BCP 5 mg/kg, (F) BCP 2.5 mg/kg + IMP 10 mg/kg, (G) BCP 5 mg/kg + IMP 10 mg/kg, and (H) IMP 10 mg/kg; show lumber spinal cord sections of H&E staining (40 ×); 0- No inflammation; 1- low level of inflammatory cells, 2- perivascular infiltrates, and 3- extension in the intensity of perivascular cuffing with expansion into contiguous tissue; Data were expressed as mean ± range, n = 7 animals per group, three to four fields of view per animal for each protocol of experiment. Given the non-parametric nature of data, data were analyzed by the Kruskal-Wallis test and followed by Dunn's *post-hoc* multiple comparisons test. (*) shows the comparison between different doses BCP, DMF, IMP and BCP + IMP –treated groups and vehicle group, *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.; ($^{+}$) compares vehicle-treated group to the sham group, $^{+++}$: $p < 0.001$.; $^{\#}$ compares to the IMP group, $^{\#}$: $p < 0.05$. ^{x} compares to the DMF group, ^{x}: $p < 0.05$. Abbreviations: BCP: β-caryophyllene, IMP: Imipramine, DMF: Dimethyl fumarate.

3.4. The effects of BCP, IMP and their combinations on serum cytokines levels

In the vehicle-treated group, the levels of TNF-α ($p < 0.001$, Fig. 4A), IL-6 ($p < 0.001$, Fig. 4B), and IL-17 ($p < 0.001$, Fig. 4C), and IL-17/IL-10 ratio ($p < 0.001$, Fig. 4E) were significantly propagated comparing to the sham group. In contrast, the level of IL-10 ($p < 0.001$, Fig. 4D) in the vehicle group was significantly lower than the sham group. However, treatment with DMF (60 mg/kg), IMP (10 mg/kg), BCP (5 mg/kg) and the combination of BCP (2.5 and 5 mg/

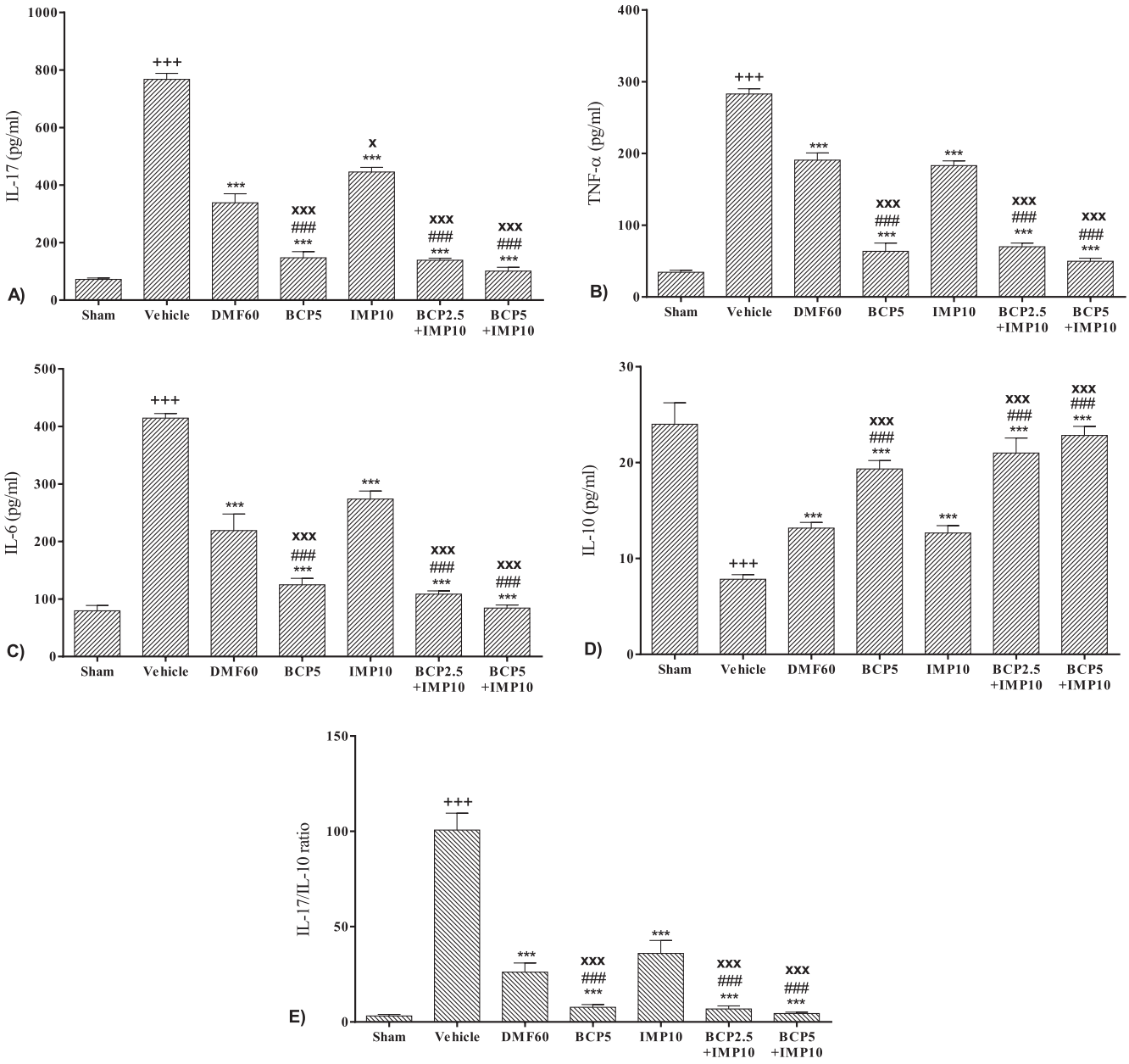

Fig. 4. The effects of BCP (5 mg/kg/day; p.o.), DMF (60 mg/kg; 30 mg/kg twice a day; p.o.), IMP (10 mg/kg; p.o.) and the combination of BCP (5 mg/kg/day; p.o.) with IMP (10 mg/kg; p.o.) on the levels of pro-inflammatory and anti-inflammatory cytokines in the serum of EAE mice; (A) TNF-α, (B) IL-6, (C) IL-17, (D) IL-10, (E) IL-17/IL-10 ratio; Data were expressed as Mean ± SEM, n = 6 animals per group for each protocol of experiment. Repeated measures two-way ANOVA was done with the following Tukey's multiple comparisons test. (*) shows the comparison to the vehicle group, ***: $p < 0.001$.; ($^{+}$) compares vehicle-treated group to the sham group, $^{+++}$: $p < 0.001$.; $^{\#}$ represents a comparison to the IMP group in each graph, $^{\#\#\#}$: $p < 0.001$; ^{x} shows a comparison to the DMF group in each graph, ^{x}: $p < 0.05$, and ^{xxx}: $p < 0.001$. Abbreviations: BCP: β-caryophyllene, IMP: Imipramine, DMF: Dimethyl fumarate, ANOVA: analysis of variance.

kg) with IMP considerably reduced the levels of TNF-α (Fig. 4A), IL-6 (Fig. 4B), and IL-17 (Fig. 4C), and IL-17/IL-10 ratio (Fig. 4E), while notably increased the level of IL-10 (Fig. 4D), compared to the vehicle group ($p < 0.001$ for all cases, Fig. 4A-E). Additionally, we observed that BCP (5 mg/kg) alone and the combination of BCP (2.5 and 5 mg/kg) with IMP (10 mg/kg) significantly reduced the levels of TNF-α (Fig. 4A), IL-6 (Fig. 4B), and IL-17 (Fig. 4C), and IL-17/IL-10 ratio (Fig. 4E), and markedly increased the level of IL-10 (Fig. 4D) in comparison with both IMP- and DMF-treated alone groups ($p < 0.001$ for all cases, Fig. 4A-E).

3.5. The effects of BCP, IMP and their combinations on spleen lymphocytes

To have a better understanding on the protective effects of BCP, IMP and their combinations in the treatment of EAE, the levels of Th_1/Th_2- and Th_{17}/T_{reg}- produced cytokines in spleen lymphocytes of EAE and their polarizations from Th_1 and Th_{17} towards Th_2 and T_{reg} cells, respectively, were assessed. To this end, we measured the levels of cell proliferation, inflammatory (TNF-α, IL-6, IFN-γ, and IL-17) and anti-inflammatory (IL-4, IL-10 and TGF-β_1) cytokines, and the ratios of IFN-γ/IL-4 (Th_1/Th_2) and both IL-17/IL-10 and IL-17/TGF-β_1 (Th_{17}/T_{reg}) ratios as well as the intracellular levels of the specific transcription factors including T-bet (Th_1), GATA3 (Th_2), ROR-γt (Th_{17}) and Foxp3 (T_{reg}), and their ratios T-bet/GATA3 (Th_1/Th_2) and ROR-γt/Foxp3 (Th_{17}/T_{reg}) in spleen lymphocytes of treated mice in the presence and absence of MOG_{35-55} stimulation (30 μg/ml) [1–3,10].

3.5.1. In the absence of MOG stimulation

Without the MOG stimulation, in vehicle-treated group, the levels of cell proliferation ($p < 0.001$, Fig. 5A), inflammatory cytokines including TNF-α ($p < 0.001$, Fig. 5B), IL-6 ($p < 0.001$, Fig. 5C), IFN-γ ($p < 0.001$, Fig. 5D) and IL-17 ($p < 0.001$, Fig. 5E), ratios of IFN-γ/IL-4 ($p < 0.001$, Fig. 5I), IL-17/IL-10 ($p < 0.001$, Fig. 5J) and IL-17/

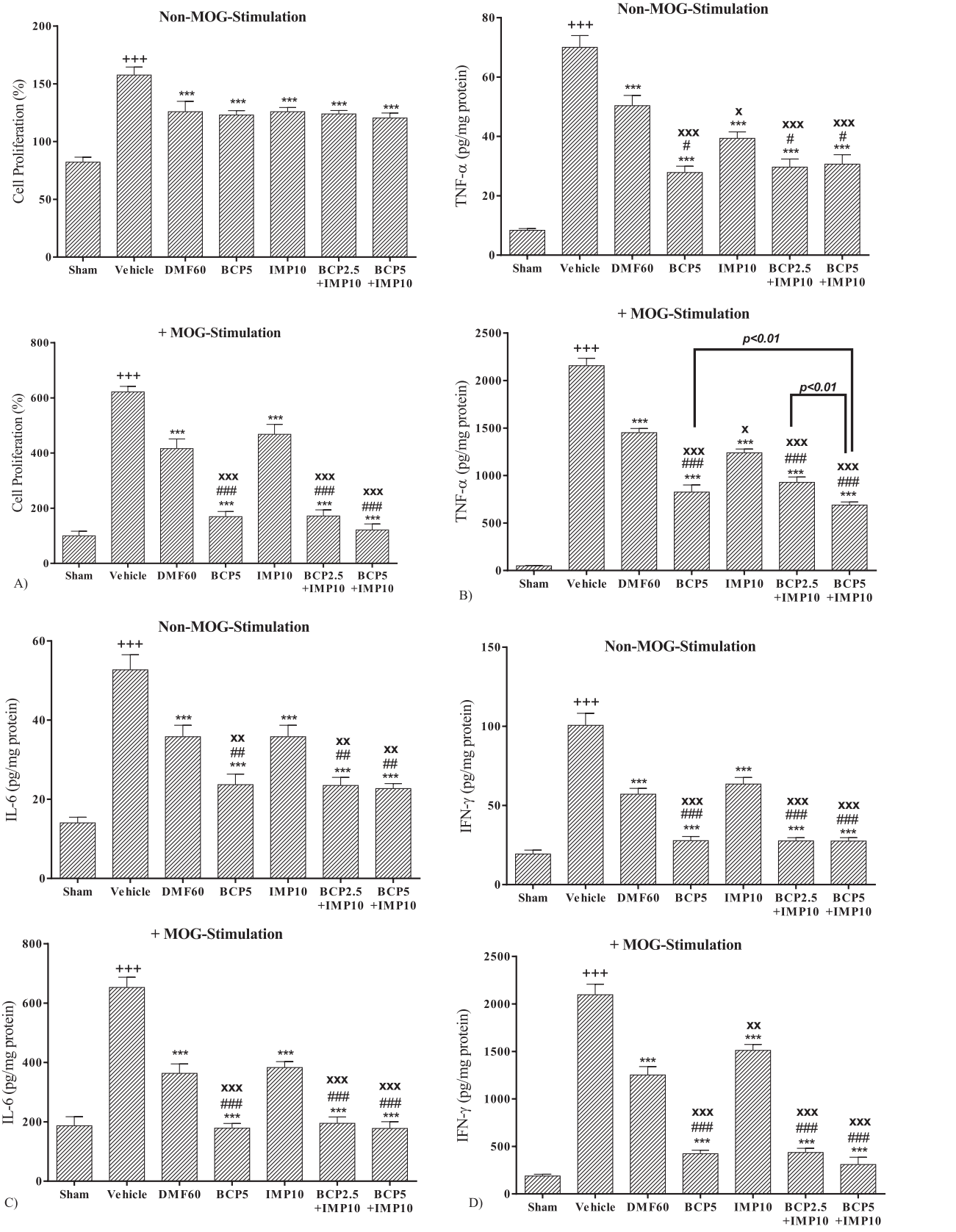

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Fig. 5. The effects of BCP (5 mg/kg/day; p.o.), DMF60 (30 mg/kg twice a day; p.o.) IMP (10 mg/kg; p.o.), and the combination of BCP (2.5 and 5 mg/kg/day; p.o.) with IMP (10 mg/kg; p.o.) on cell proliferation, pro- and anti-inflammatory cytokines levels of isolated lymphocytes from EAE mice; (A) Cell proliferation, (B) TNF-α, (C) IL-6, (D) IFN-γ, (E) IL-17, (F) IL-4, (G) IL-10, (H) TGF-β_1, (I) IFN-γ/IL-4 ratio, (J) IL-17/IL-10 ratio (K) IL-17/TGF-β_1 ratio; Data were expressed as Mean ± SEM, n = 6 animals per group for each protocol of experiment. Repeated measures two-way ANOVA was done with the following Tukey's multiple comparisons test. (*) shows the comparison between different doses BCP, IMP or their combinations –treated groups, and vehicle group, *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.; ($^{+}$) compares vehicle-treated group to the sham group, $^{+++}$: $p < 0.001$.; $^{\#}$ compared to IMP group in each graph, $^{\#}$: $p < 0.05$, $^{\#\#}$: $p < 0.01$, and $^{\#\#\#}$: $p < 0.001$. ^{x} compared to the DMF group in each graph, ^{x}: $p < 0.05$, ^{xx}: $p < 0.01$, and ^{xxx}: $p < 0.001$. Abbreviations: BCP: β-caryophyllene, IMP: Imipramine, ANOVA: analysis of variance.

TGF-β_1 ($p < 0.001$, Fig. 5K), T-bet ($p < 0.001$, Fig. 6A), ROR-γt ($p < 0.001$, Fig. 6C), and ratios of T-bet/GATA3 (Th_1/Th_2, $p < 0.001$, Fig. 6E) and ROR-γt/Foxp3 (Th_{17}/T_{reg}, $p < 0.001$, Fig. 6F) were significantly increased in comparison to the sham group.

Treatment with DMF (60 mg/kg), IMP (10 mg/kg), BCP (5 mg/kg) and the combination of BCP (2.5 and 5 mg/kg) with IMP (10 mg/kg) remarkably decreased the levels of cell proliferation ($p < 0.001$, for all cases, Fig. 5A), inflammatory cytokines including TNF-α ($p < 0.001$, for all cases, Fig. 5B), IL-6 ($p < 0.001$-$p < 0.01$ for all cases, Fig. 5C), IFN-γ ($p < 0.001$, for all cases, Fig. 5D) and IL-17 ($p < 0.001$, for all cases, Fig. 5E), and the ratios of IFN-γ/IL-4 ($p < 0.001$, for all cases, Fig. 5I), IL-17/IL-10 ($p < 0.001$, for all cases, Fig. 5J) and IL-17/TGF-β_1 ($p < 0.001$, for all cases, Fig. 5K), in comparison to the vehicle group. Treatment with BCP (5 mg/kg) and IMP (10 mg/kg) combined with BCP (2.5 and 5 mg/kg) significantly augmented the levels of IL-4 ($p < 0.001$ for all cases, Fig. 5F), IL-10 ($p < 0.001$ for all cases, Fig. 5G) and TGF-β_1 ($p < 0.001$ for all cases, Fig. 5H) compared to the vehicle group. IMP (10 mg/kg) and DMF (60 mg/kg) also significantly increased the level of IL-4 in comparison to the vehicle group ($p < 0.01$, Fig. 5F).

It was further found that treatment with DMF (60 mg/kg), BCP (5 mg/kg) alone, and BCP (2.5 and 5 mg/kg) combined with IMP (10 mg/kg) significantly decreased the levels of T-bet ($p < 0.01$ for all cases, Fig. 6A), ROR-γt ($p < 0.001$ for all cases, Fig. 6C) and the ratios of T-bet/GATA3 ($p < 0.001$ for all cases, Fig. 6E) and ROR-γt/Foxp3 ($p < 0.001$ for all cases, Fig. 6F) in spleen lymphocytes in comparison to the vehicle group. We also demonstrated that BCP (5 mg/kg) alone and BCP (2.5 and 5 mg/kg) combined with IMP (10 mg/kg) markedly increased the levels of GATA3 ($p < 0.001$ for both cases, Fig. 6B) and Foxp3 ($p < 0.001$ for both cases, Fig. 6D) compared to the vehicle group; however, DMF (60 mg/kg) significantly elevated the level of Foxp3 only ($p < 0.001$, Fig. 6D) compared to vehicle group. IMP (10 mg/kg) also notably attenuated the levels of ROR-γt ($p < 0.001$, Fig. 6D), and also the ratios of T-bet/GATA3 ($p < 0.05$, Fig. 6E) and ROR-γt/Foxp3 ($p < 0.001$, Fig. 6F) in comparison to the vehicle group.

3.5.2. In the presence of MOG stimulation

In the presence of MOG_{35-55} stimulation, in vehicle-treated EAE mice, the levels of cell proliferation ($p < 0.001$, Fig. 5A), inflammatory cytokines including TNF-α ($p < 0.001$, Fig. 5B), IL-6 ($p < 0.001$, Fig. 5C), IFN-γ ($p < 0.001$, Fig. 5D) and IL-17 ($p < 0.001$, Fig. 5E), and the ratios of IFN-γ/IL-4 ($p < 0.001$, Fig. 5I), IL-17/IL-10 ($p < 0.001$, Fig. 5J) and IL-17/TGF-β_1 ($p < 0.001$, Fig. 5K) ratios as well as the levels of T-bet (Th_1, $p < 0.001$, Fig. 6A), GATA3 (Th_2, $p < 0.001$, Fig. 6B), ROR-γt (Th_{17}, $p < 0.001$, Fig. 6C), and the ratios of T-bet/GATA3 (Th_1/Th_2 ratio, $p < 0.001$, Fig. 6E) and ROR-γt/Foxp3 (Th_{17}/T_{reg} ratio, $p < 0.001$, Fig. 6F) were considerably elevated in comparison to the sham group. On the contrary, in this group, the levels of anti-inflammatory cytokines including IL-4 ($p < 0.001$, Fig. 5F), IL-10 ($p < 0.001$, Fig. 5G), TGF-β_1 ($p < 0.001$, Fig. 5H) and transcription factor Foxp3 (T_{reg}, $p < 0.001$, Fig. 6D) were meaningfully more than the sham group.

Our results showed that treatment with DMF (60 mg/kg), IMP (10 mg/kg), BCP (5 mg/kg) and the combination of IMP (10 mg/kg) with BCP (2.5 and 5 mg/kg) significantly decreased the levels of cell proliferation ($p < 0.001$ for all cases, Fig. 5A), inflammatory cytokines including TNF-α ($p < 0.001$ for all cases, Fig. 5B), IL-6 ($p < 0.001$ for all cases, Fig. 5C), IFN-γ ($p < 0.001$ for all cases, Fig. 5D) and IL-17 ($p < 0.001$ for all cases, Fig. 5E), and the ratios of IFN-γ/IL-4 ($p < 0.001$ for all cases, Fig. 5I), IL-17/IL-10 ($p < 0.001$ for all cases, Fig. 5J) and IL-17/TGF-β_1 ($p < 0.001$ for all cases, Fig. 5K) as well as transcription factors including T-bet ($p < 0.001$ for all cases, Fig. 6A), ROR-γt ($p < 0.001$ for all cases, Fig. 6C), and the ratios of T-bet/GATA3 ($p < 0.001$ for all cases, Fig. 6E) and ROR-γt/Foxp3 ($p < 0.001$ for all cases, Fig. 6F).

However, it was found that treatment with DMF (60 mg/kg), IMP (10 mg/kg), BCP (5 mg/kg) and the combination of IMP (10 mg/kg) with BCP (2.5 and 5 mg/kg) markedly increased the levels of anti-inflammatory cytokines including IL-4 ($p < 0.001$–0.05 for all cases, but not for IMP, Fig. 5F), IL-10 ($p < 0.001$–0.05 for all cases, but not for IMP, Fig. 5G), TGF-β_1 ($p < 0.001$ for all cases, but not for IMP, Fig. 5H), GATA3 ($p < 0.001$ for all cases, Fig. 6B) and Foxp3 ($p < 0.001$ for all cases, Fig. 6D), compared to the vehicle group. The effects of BCP (5 mg/kg) and the combination of IMP (10 mg/kg) with BCP (2.5 and 5 mg/kg) on the levels of all measured parameters were notably greater than DMF (60 mg/kg) and IMP (10 mg/kg) groups ($p < 0.001$–0.05 for all case, Figs. 5A–K and 6A-F).

3.6. The effects of BCP, IMP and their combinations on microglia

3.6.1. In the absence of MOG stimulation

In the absence of MOG stimulation, the levels of TNF-α ($p < 0.001$, Fig. 7B), PGE_2 ($p < 0.001$, Fig. 7C), iNOS ($p < 0.001$, Fig. 8A), NO ($p < 0.001$, Fig. 8D), and the ratios of iNOS/Arg-1 ($p < 0.001$, Fig. 8C) and NO/urea ($p < 0.001$, Fig. 8F) were remarkably increased in the vehicle group comparing with the sham group. In contrast, in the vehicle-treated group, the levels of IL-10 ($p < 0.05$, Fig. 7D), Arg-1 ($p < 0.001$, Fig. 8B) and urea ($p < 0.001$, Fig. 8E) were significantly reduced in comparison to the sham group.

Treatment of EAE mice with DMF (60 mg/kg) showed a significant reduction in the levels of TNF-α ($p < 0.001$, Fig. 7B), PGE_2 ($p < 0.001$, Fig. 7C), iNOS ($p < 0.05$, Fig. 8A), NO ($p < 0.001$, Fig. 8D), and the ratio of NO/urea ($p < 0.001$, Fig. 8F), while indicated a meaningful increment in the level of urea only ($p < 0.01$, Fig. 8E), compared to the vehicle group.

The treatment of EAE mice with BCP (5 mg/kg) dramatically decreased the levels of TNF-α ($p < 0.001$, Fig. 7B), PGE_2 ($p < 0.001$, Fig. 7C), iNOS ($p < 0.001$, Fig. 8A), NO ($p < 0.001$, Fig. 8D), and the ratios of iNOS/Arg-1 ($p < 0.001$, Fig. 8C) and NO/urea ($p < 0.001$, Fig. 8F), whereas significantly elevated the levels of IL-10 ($p < 0.001$, Fig. 7D), Arg-1 ($p < 0.001$, Fig. 8B) and urea ($p < 0.001$, Fig. 8E), in comparison to the vehicle group.

In the group receiving IMP (10 mg/kg), it was observed that the levels of TNF-α ($p < 0.01$, Fig. 7B), PGE_2 ($p < 0.01$, Fig. 7C), NO ($p < 0.001$, Fig. 8D) and NO/urea ratio ($p < 0.001$, Fig. 8F) were significantly lower than the vehicle group.

The combination therapy of IMP with BCP (2.5 and 5 mg/kg) meaningfully reduced the levels of TNF-α ($p < 0.001$ for both cases, Fig. 7B), PGE_2 ($p < 0.001$ for both cases, Fig. 7C), iNOS ($p < 0.001$ for both cases, Fig. 8A), NO ($p < 0.001$ for both cases, Fig. 8D), and the ratios of iNOS/Arg-1 ($p < 0.001$ for both cases, Fig. 8C) and NO/urea ($p < 0.001$ for both cases, Fig. 8F), whereas these combinations

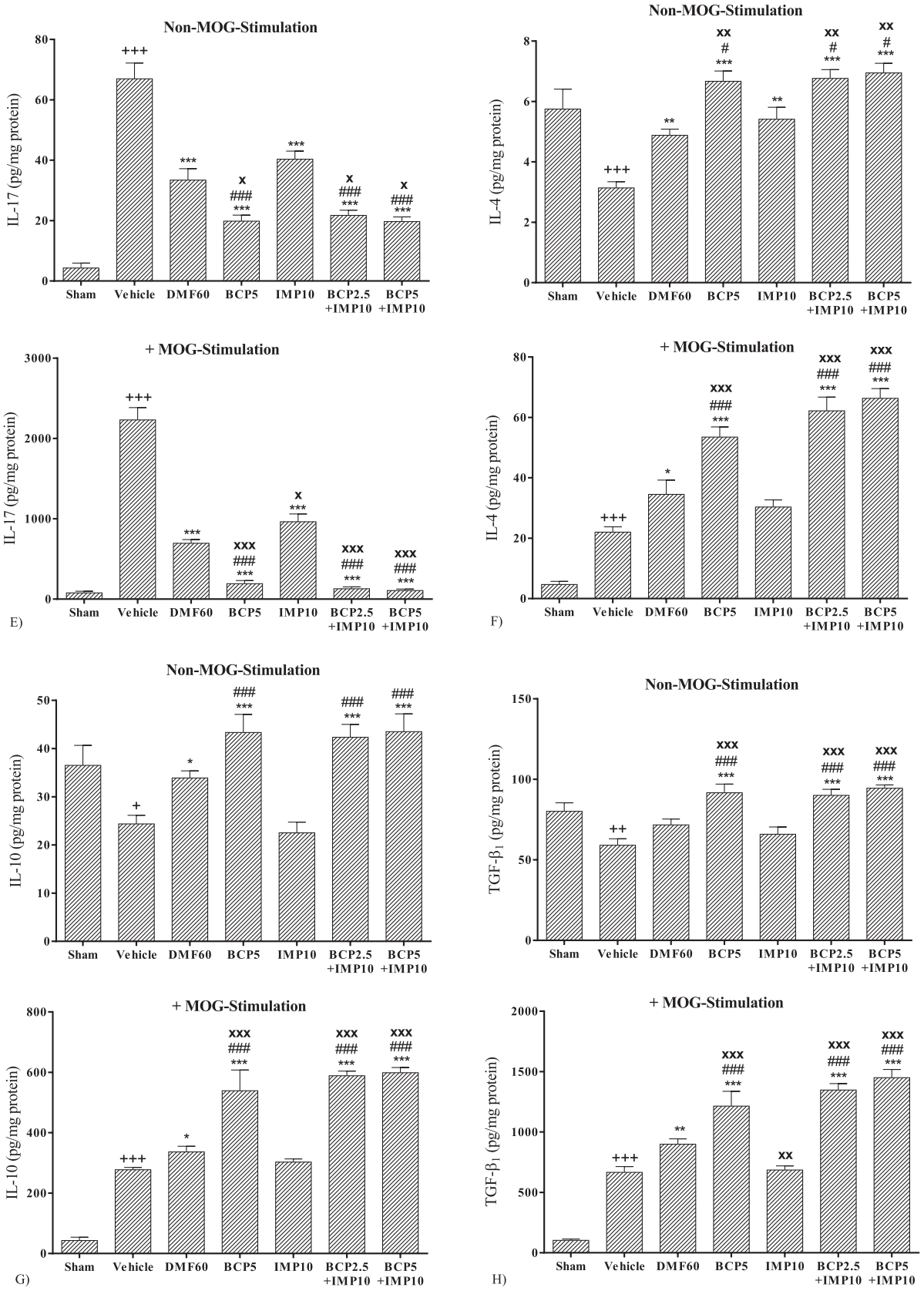

Fig. 5. (*continued*)

significantly increased the levels of IL-10 ($p < 0.001$ for both cases, Fig. 7D), Arg-1 ($p < 0.001$ for both cases, Fig. 8B) and urea ($p < 0.001$ for both cases, Fig. 8E), in comparison to the vehicle group.

3.6.2. *In the presence of MOG stimulation*

In the vehicle group, MOG stimulation markedly increased the levels of cell proliferation ($p < 0.001$, Fig. 7A), TNF-α ($p < 0.001$,

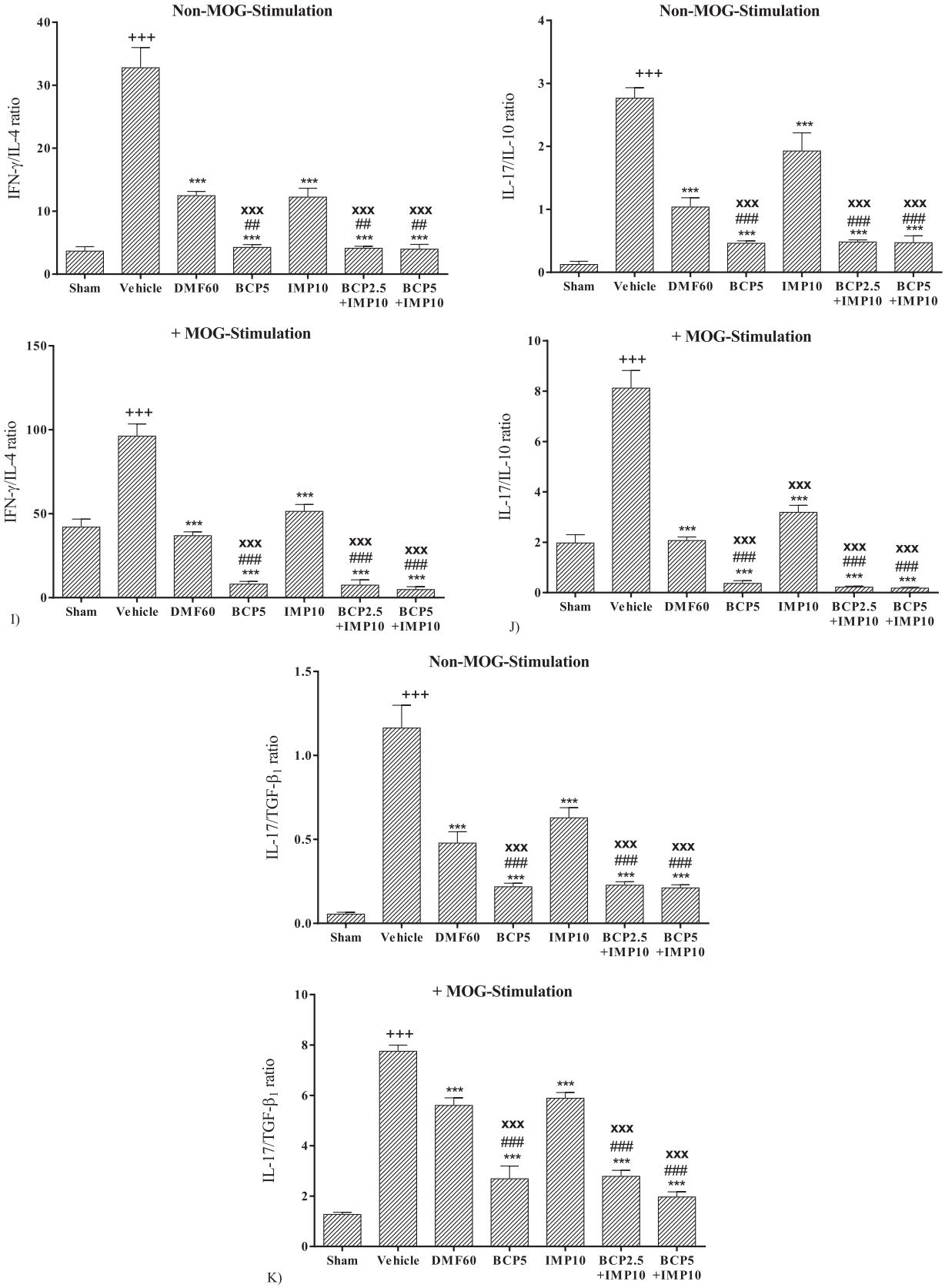

Fig. 5. (*continued*)

Fig. 7B), PGE_2 ($p < 0.001$, Fig. 7C), IL-10 ($p < 0.001$, Fig. 7D), iNOS ($p < 0.001$, Fig. 8A), NO ($p < 0.001$, Fig. 8D), and the ratios of iNOS/Arg-1 ($p < 0.001$, Fig. 8C), and NO/urea ($p < 0.001$, Fig. 8F) in comparison to the sham group, while significantly decreased the levels of Arg-1 ($p < 0.001$, Fig. 8B), and urea ($p < 0.001$, Fig. 8E) in comparison with the sham group.

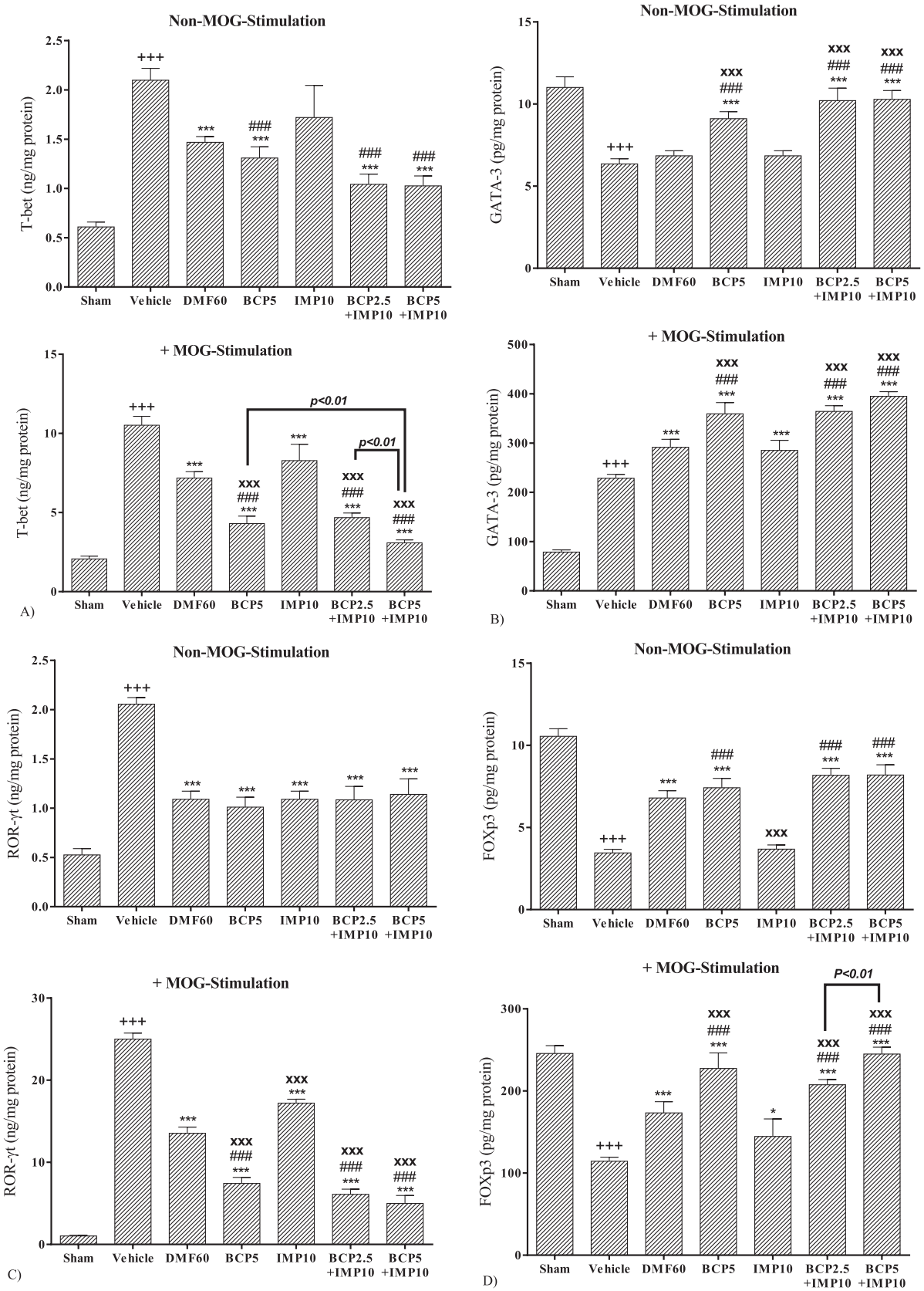

Fig. 6. The effects of BCP (5 mg/kg/day; p.o.), DMF60 (30 mg/kg twice a day; p.o.), IMP (10 mg/kg; p.o.), and the combination of BCP (2.5 and 5 mg/kg/day; p.o.) with IMP (10 mg/kg; p.o.) on the levels of transcription factors T-bet (Th_1), GATA3 (Th_2), ROR-γt (Th_{17}) and Foxp3 (T_{reg}) of isolated lymphocytes from EAE mice; (A) T-bet, (B) GATA3, (C) ROR- γt, (D) Foxp3, (E) T-bet/GATA3 (Th_1/Th_2) ratio, (F) ROR- γt/Foxp3 (Th_{17}/T_{reg}) ratio; Data were expressed as Mean ± SEM, n = 6 animals per group for each protocol of experiment. Repeated measures two-way ANOVA was done with the following Tukey's multiple comparisons test. (*) shows the comparison between different doses BCP, IMP or their combinations –treated groups, and vehicle group, *: $p < 0.05$, and ***: $p < 0.001$.; ($^{+}$) compares vehicle-treated group to the sham group, $^{+++}$: $p < 0.001$.; $^{\#}$ compared to IMP group in each graph, $^{\#\#\#}$: $p < 0.001$. ^{x} compared to DMF group in each graph, ^{xxx}: $p < 0.001$. Abbreviations: BCP: β-caryophyllene, IMP: Imipramine, ANOVA: analysis of variance.

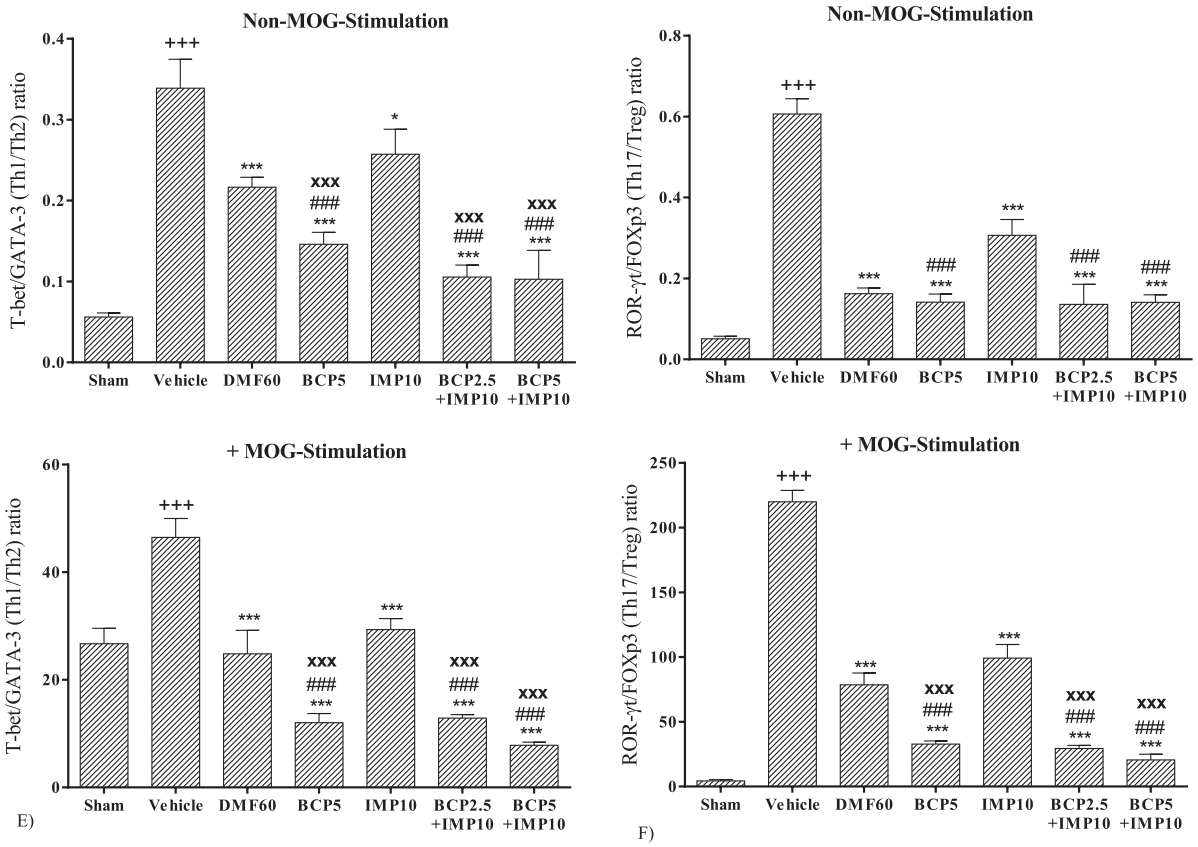

Fig. 6. (*continued*)

On the contrary, treatment with DMF (60 mg/kg) and BCP (5 mg/kg) notably decreased the levels of cell proliferation ($p < 0.001$ for all cases, Fig. 7A), TNF-α ($p < 0.001$ for all cases, Fig. 7B), PGE_2 ($p < 0.001$ for all cases, Fig. 7C), iNOS ($p < 0.001$ for all cases, Fig. 8A), iNOS/Arg-1 ratio ($p < 0.001$ for all cases, Fig. 8C), NO ($p < 0.001$ for all cases, Fig. 8D), and NO/urea ratio ($p < 0.001$ for all cases, Fig. 8F), while significantly elevated the levels of IL-10 ($p < 0.001$ for BCP, and $p < 0.01$ for DMF, Fig. 7D), Arg-1 ($p < 0.001$ for all cases, Fig. 8B) and urea ($p < 0.001$ for all cases, Fig. 8E), in comparison to the vehicle group. Treatment with IMP (10 mg/kg) significantly attenuated the levels of cell proliferation ($p < 0.001$, Fig. 7A), TNF-α ($p < 0.001$, Fig. 7B), PGE_2 ($p < 0.001$, Fig. 7C), iNOS ($p < 0.001$, Fig. 8A), iNOS/Arg-1 ratio ($p < 0.001$, Fig. 8C), NO ($p < 0.001$, Fig. 8D), and NO/urea ratio ($p < 0.001$, Fig. 8F), while significantly increased the level of urea only ($p < 0.001$, Fig. 8E), compared to the vehicle group. The combination of IMP (10 mg/kg) with BCP (2.5 and 5 mg/kg) led to a significant decrease in the levels of cell proliferation ($p < 0.001$ for both cases, Fig. 7A), TNF-α ($p < 0.001$ for both cases, Fig. 7B), PGE_2 ($p < 0.001$ for both cases, Fig. 7C), iNOS ($p < 0.001$ for both cases, Fig. 8A), iNOS/Arg-1 ratio ($p < 0.001$ for both cases, Fig. 8C), NO ($p < 0.001$ for both cases, Fig. 8D) and NO/urea ratio ($p < 0.001$ for both cases, Fig. 8F) in comparison to the groups receiving vehicle, DMF (60 mg/kg) or IMP (10 mg/kg). Besides, these combinations resulted in meaningful increases in the levels of IL-10 ($p < 0.001$ for both case, Fig. 7D), Arg-1 ($p < 0.001$ for both cases, Fig. 8B) and urea ($p < 0.001$ for both cases, Fig. 8E) compared to the groups receiving vehicle, DMF (60 mg/kg) or IMP (10 mg/kg). Additionally, we found significant differences between the effects of BCP (5 mg/kg) and DMF (60 mg/kg) or IMP (10 mg/kg) ($p < 0.001$-$p < 0.05$ for all cases, Fig. 7A-D and 8A-F).

3.7. The direct effects of BCP, IMP and their combinations on spleen lymphocytes from EAE mice

For better insights on the effect of BCP, IMP and their interactive pharmacological effects, we examined their direct effects on spleen lymphocytes isolated from non-treated EAE mice. Our findings demonstrated that the addition of MOG_{35-55} (30 μg/ml) to lymphocytes led to a significant increase in the levels of cell proliferation, IFN-γ, IL-17, IL-4, IL-10, and the ratios of IFN-γ/IL-4 (Th_1/Th_2) and IL-17/IL-10 (Th_{17}/T_{reg}) in comparison to the absence of MOG_{35-55} stimulation ($p < 0.001$ for all cases, Fig. 9A–G).

In contrast, the medication of lymphocytes with BCP (0.2 and 1 μM), IMP (1 μM) and the combination of IMP (1 μM) with BCP (0.2 and 1 μM) resulted in a significant reduction in the levels of cell proliferation ($p < 0.001$ for all cases, Fig. 9A), IFN-γ ($p < 0.001$ for all cases, Fig. 9B), IL-17 ($p < 0.001$ for all cases, Fig. 9C), and the ratios of IFN-γ/IL-4 ($p < 0.001$ for all cases, Fig. 9F) and IL-17/IL-10 ($p < 0.001$ for all cases, Fig. 9G), in comparison to the vehicle group. However, the level of IL-4 was increased in groups treated with BCP (1 μM), and the combination of IMP with BCP (0.2 and 1 μM), compared to the vehicle group ($p < 0.001$ for all cases, Fig. 9D). Moreover, in all groups receiving BCP alone (0.2 and 1 μM) and its combination with IMP, the level of IL-10 was significantly increased in comparison to the vehicle group ($p < 0.001$ for all cases, Fig. 9E).

Our results also indicated significant differences between groups treated with different concentrations of BCP alone and its combination with IMP. The levels of cell proliferation ($p < 0.001$ for all cases, Fig. 9A), IFN-γ ($p < 0.001$ for all cases, Fig. 9B), IL-17 ($p < 0.001$ for all cases, Fig. 9C), and the ratios of IFN-γ/IL-4 ($p < 0.001$ for all cases, Fig. 9F) and IL-17/IL-10 ($p < 0.001$ for all cases, Fig. 9G) were significantly attenuated in lymphocytes treated with either BCP (1 μM) or the combination of IMP (1 μM) with BCP (0.2 and 1 μM), in comparison to the IMP-treated alone group. Furthermore, we found that the levels

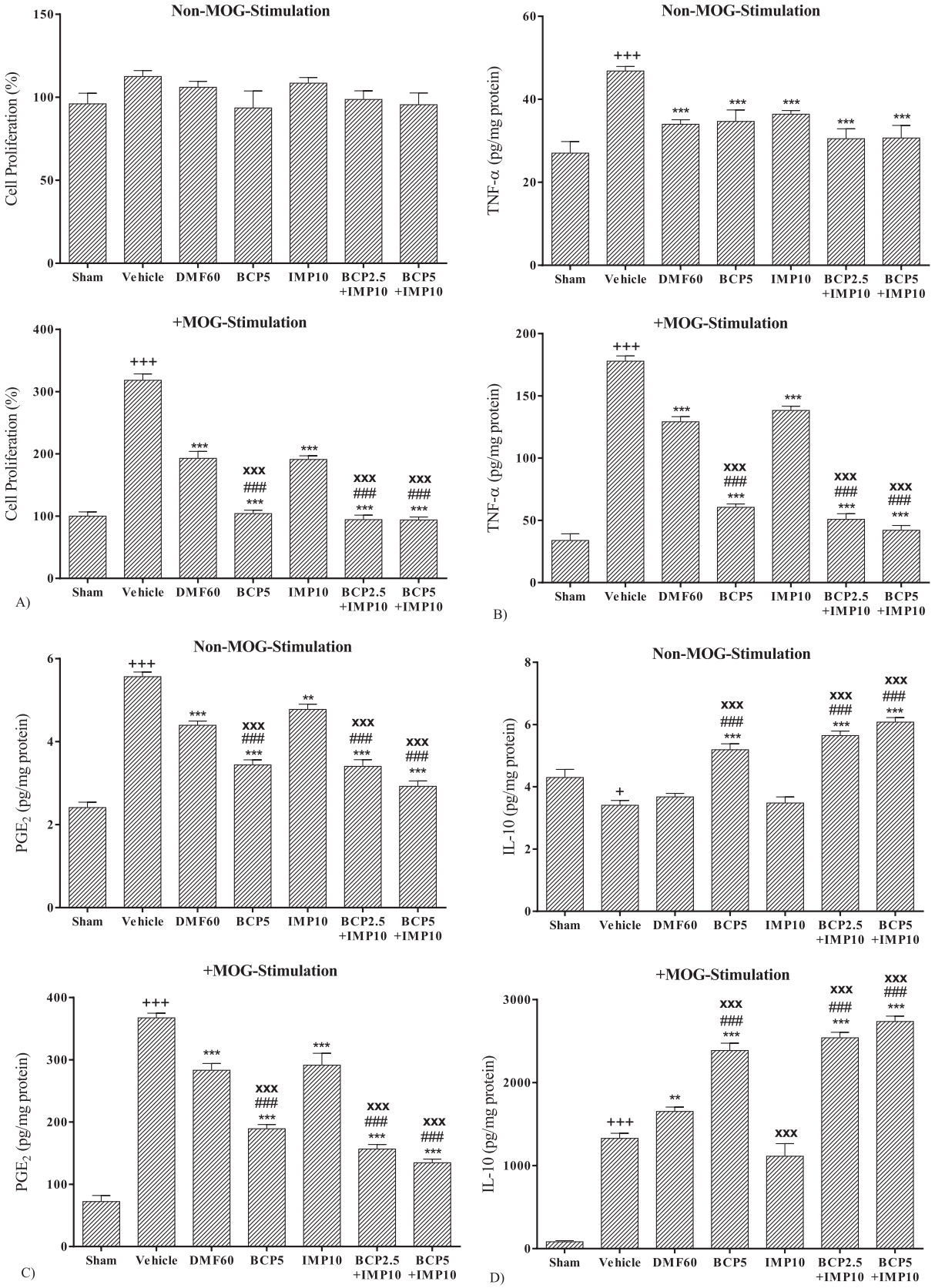

Fig. 7. The effects of BCP (5 mg/kg/day; p.o.), DMF60 (30 mg/kg twice a day; p.o.), IMP (10 mg/kg; p.o.), and the combination of BCP (2.5 and 5 mg/kg/day; p.o.) with IMP (10 mg/kg; p.o.) on the levels of cell proliferation, inflammatory and anti-inflammatory cytokines of isolated microglia from EAE mice; (A) Cell proliferation, (B) TNF-α, (C) PGE_2, (D) IL-10; Data were expressed as Mean ± SEM, n = 6 animals per group for each protocol of experiment. Repeated measures two-way ANOVA was done with the following Tukey's multiple comparisons test. (*) shows the comparison between different doses BCP or DMF–treated groups and vehicle group, **: $p < 0.01$, and ***: $p < 0.001$.; ($^{+}$) compares vehicle-treated group to the sham group, $^{+++}$: $p < 0.001$.; $^{\#}$ compared to IMP group in each graph, $^{\#\#\#}$: $p < 0.001$. ^{x} compared to the DMF group in each graph, ^{xxx}: $p < 0.001$. Abbreviations: BCP: β-caryophyllene, DMF: Dimethyl fumarate, ANOVA: analysis of variance.

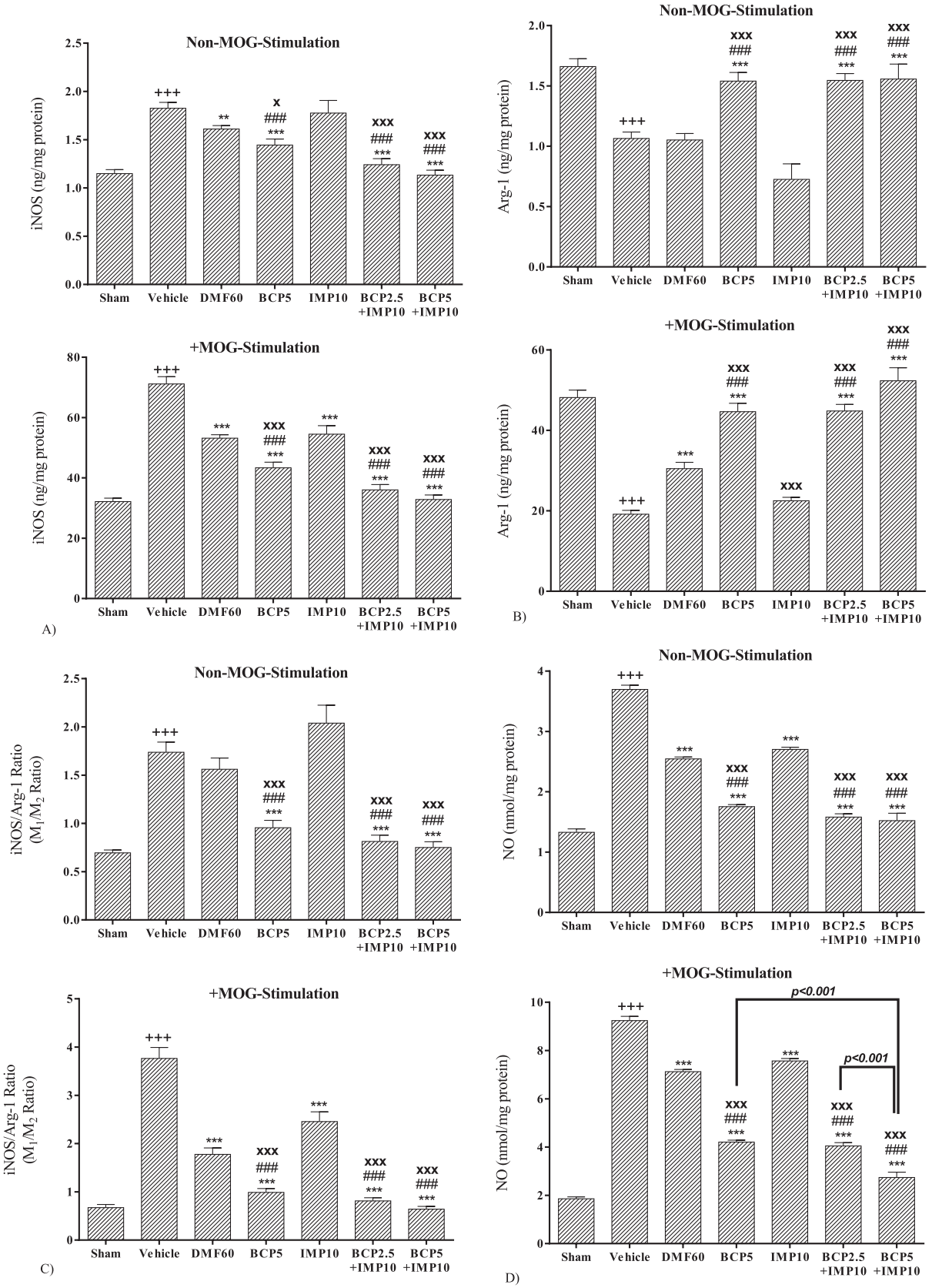

Fig. 8. The effects of BCP (5 mg/kg/day; p.o.), DMF60 (30 mg/kg twice a day; p.o.), IMP (10 mg/kg; p.o.), and the combination of BCP (2.5 and 5 mg/kg/day; p.o.) with IMP (10 mg/kg; p.o.) on intracellular levels of iNOS and Arg-1 and their consequent products NO and urea, respectively, of isolated microglia from EAE mice; (A) iNOS, (B) Arg-1, (C) iNOS/Arg-1 (M_1/M_2) ratio, (D) NO, (E) Urea, (F) NO/Urea (M_1/M_2) ratio; Data were expressed as Mean ± SEM, n = 6 animals per group for each protocol of experiment. Repeated measures two-way ANOVA was done with the following Tukey's multiple comparisons test. (*) shows the comparison between different doses BCP, IMP or their combinations –treated groups, and vehicle group, ***: $p < 0.001$.; ($^{+}$) compares vehicle-treated group to the sham group, $^{+++}$: $p < 0.001$.; $^{\#}$ compared to IMP group in each graph, $^{\#\#\#}$: $p < 0.001$. ^{x} compared to DMF group in each graph, ^{x}: $p < 0.05$, and ^{xxx}: $p < 0.001$. Abbreviations: BCP: β-caryophyllene, IMP: Imipramine, ANOVA: analysis of variance.

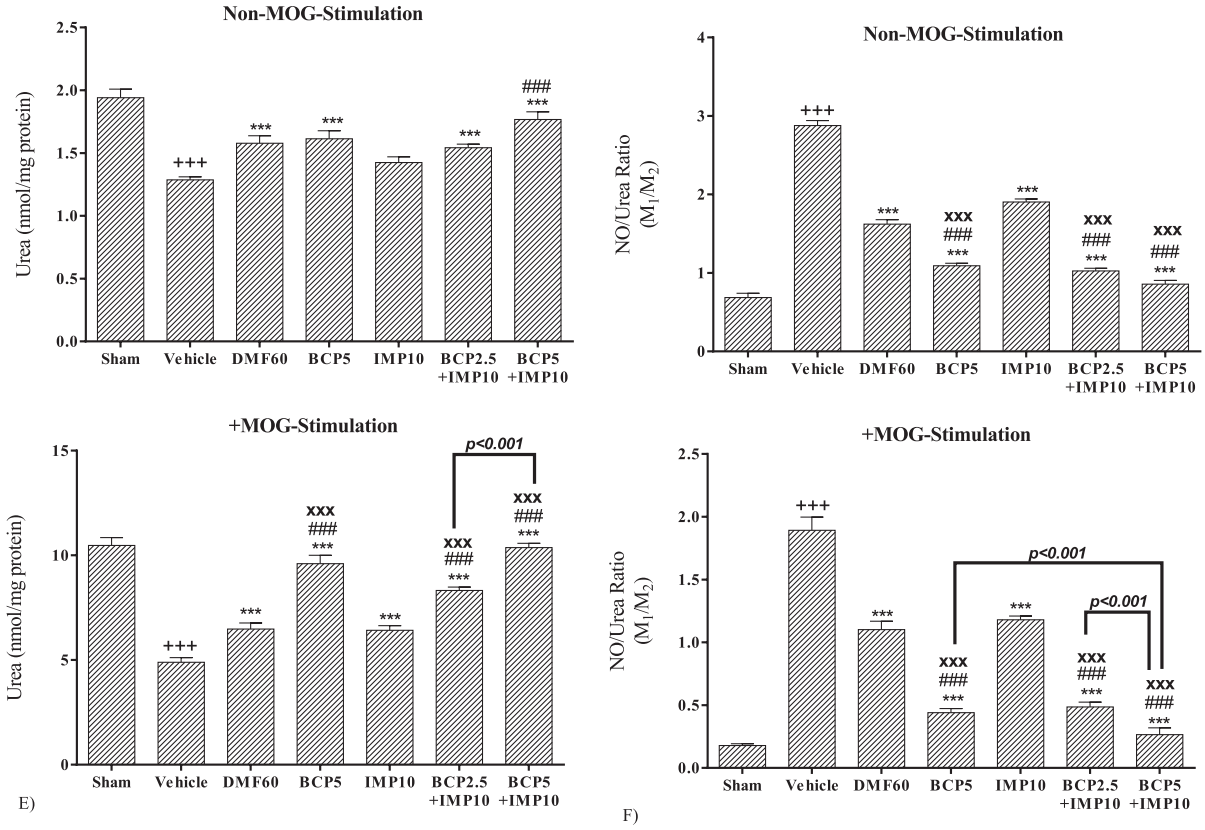

Fig. 8. (*continued*)

of IL-17 ($p < 0.01$, Fig. 9C) and IL-17/IL-10 ratio ($p < 0.001$, Fig. 9G) were also significantly lower in BCP (0.2 μM)-treated group than the IMP-treated alone group. The levels of IL-4 ($p < 0.001$ for all cases, Fig. 9D) and IL-10 ($p < 0.001$ for all cases, Fig. 9E) were also significantly higher in groups treated with BCP (1 μM) alone and the combination of BCP (0.2 and 1 μM) with IMP, than the IMP-treated alone group. Nevertheless, BCP (0.2 μM) alone markedly increased the levels of IL-10 more than IMP ($p < 0.001$, Fig. 9E).

Additionally, we observed that the levels of cell proliferation ($p < 0.05$ and 0.01, respectively, Fig. 9A), IFN-γ ($p < 0.001$ and 0.05, respectively, Fig. 9B), IL-17 ($p < 0.001$ for both cases, Fig. 9C), and the ratios of IFN-γ/IL-4 ($p < 0.001$ for both cases, Fig. 9F) and IL-17/IL-10 ($p < 0.01$ for both cases, Fig. 9G) were meaningfully more in lymphocytes treated with 0.2 μM of BCP than cells treated with BCP (1 μM) alone and the combination of BCP (0.2 μM) with IMP. However, the levels of IL-4 ($p < 0.01$ for both cases, Fig. 9D) and IL-10 ($p < 0.05$ for both cases, Fig. 9E) were observed significantly greater in groups treated with BCP (1 μM) alone and the combination of BCP (0.2 μM) with IMP, than the group reviving BCP (0.2 μM)-alone group.

Calculated combination index (CI) for groups receiving BCP (0.2 μM) + IMP (1 μM) and BCP (1 μM) + IMP (1 μM), was 0.247 and 0.689, respectively (Table 4). In fact, the CI < 1 indicates synergistic interactions between BCP (0.2 or 1 μM) and IMP (1 μM).

4. Discussion

For the first time, in the present study, we demonstrated the protective effects of BCP low dose (5 mg/kg/d; p.o.), IMP (as a pharmacological sphingomyelinase inhibitor, 10 mg/kg/d; p.o.) and their interaction in the treatment of EAE as animal model of chronic MS. In this study, we showed that daily administration of BCP low dose significantly reduced the levels of clinical and pathological score of EAE mice through reducing the levels of inflammation and polarization of spleen lymphocytes and brain microglia from inflammatory states $Th_1/Th_{17}/M_1$ towards anti-inflammatory and healing states $Th_2/T_{reg}/M_2$. As results, we found that the use of IMP alone is capable to reduce the clinical and pathological score of EAE by reducing the inflammatory cytokines, increasing the anti-inflammatory cytokine, and modulating spleen lymphocytes and brain microglia towards their anti-inflammatory phenotypes ($Th_2/T_{reg}/M_2$). Interestingly, we also observed that in groups receiving both IMP and BCP (2.5 and 5 mg/kg) concomitantly the clinical and pathological scores were dramatically lower than vehicle, BCP and IMP alone–treated groups. Additionally, we showed that BCP, IMP and their combinations can directly attenuate the level of inflammation and auto-reactivity of spleen lymphocytes isolated from non-treated EAE mice in the presence of MOG_{35-55} stimulation. In this regard, we revealed that the low concentrations of BCP combined with IMP can provide a synergistic protective effect with CI < 1 (Table 4).

There are several studies regarding the EAE model as a proper and autoimmune animal model imitating inabilities and inflammations those seen in patient with chronic MS. In the pathogenesis of EAE, Th_1 (producing IFN-γ and expressing T-bet) and Th_{17} cells (producing IL-17 and expressing ROR-γt) are the main players leading to the initiation and progression of demyelination, axonal loss and paralysis similar to those happened in patient with MS [10]. On the other hand, anti-inflammatory population of T cells including Th_2 (producing IL-4, and expressing GATA3) and T_{reg} (producing IL-10 and TGF-β, and expressing Foxp3) lead to the resolution of inflammation conversely and antagonized the effects of Th_1 and Th_{17} cells [10,44]. In this study, we indicated that the clinical and pathological scores were increased in the vehicle-treated mice. It was also found that the levels of inflammatory cytokines in blood and spleen lymphocytes (as systemic immunity) and microglia (as CNS immunity) are notably increased following the EAE induction comparing to the sham group. We also obviously observed that both spleen lymphocytes and brain microglia isolated from EAE mice significantly responded to the stimulation with MOG_{35-55} and showed the higher levels of cell proliferation and secretion of

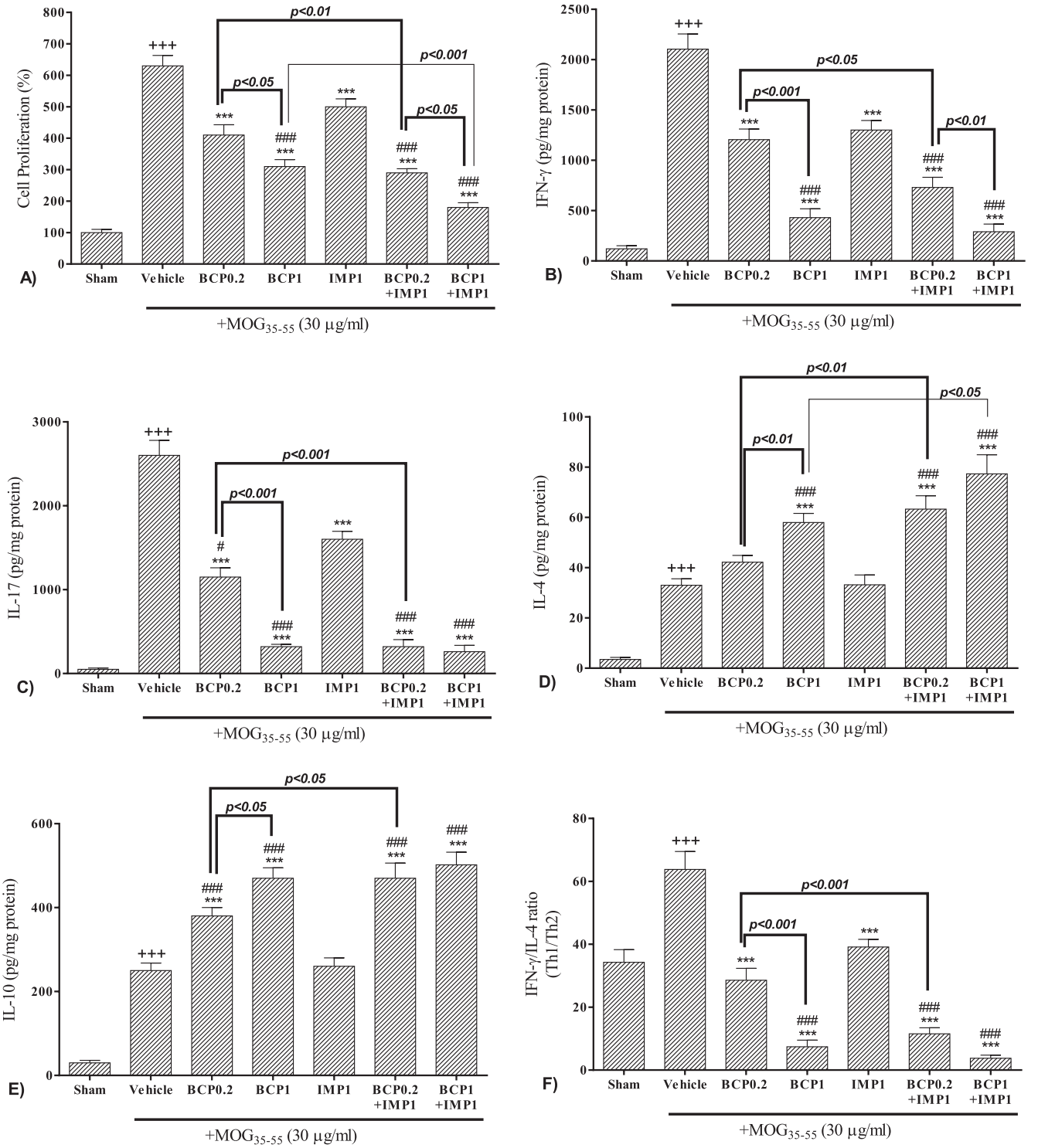

Fig. 9. The effects of BCP (0.2 or 1 µM), IMP (1 µM), and the combination of BCP (0.2 or 1 µM) with IMP (1 µM) on cell proliferation, pro- and anti-inflammatory cytokines levels of isolated lymphocytes from EAE mice; (A) Cell proliferation, (B) IFN- γ, (C) IL-17, (D) IL-4, (E) IL-10, (F) IFN-γ/IL-4 ratio, (G) IL-17/IL-10 ratio; Data were expressed as Mean ± SEM, n = 6. One-way ANOVA was done with the following Tukey's multiple comparisons test. (*) shows the comparison between different doses BCP, IMP or their combinations –treated groups, and vehicle group, ***: $p < 0.001$.; ($^{+}$) compares vehicle-treated group to the sham group, $^{+++}$: $p < 0.001$.; $^{\#}$ compared to IMP group in each graph, $^{\#}$: $p < 0.05$ and $^{\#\#\#}$: $p < 0.001$. Abbreviations: BCP: β-caryophyllene, IMP: Imipramine, ANOVA: analysis of variance.

inflammatory cytokines. Indeed, this result also provides a proof that these cells are highly sensitive and specific to the MOG_{35-55} peptide stimulation. By measuring the secretory cytokine and specific transcription factors, our findings demonstrated that the ratios of Th_1/Th_2, Th_{17}/T_{reg} and M_1/M_2 in EAE model were perturbed and shifted towards inflammatory phenotypes Th_1, Th_{17} and M_1 cells, respectively. Our results were consistent to other researcher's findings regarding EAE model [45–47]. In this regard, many studies indicated that the phenotype of Th_1, Th_{17}, and M_1 cells are dominant in the EAE model [48].

In the current study, we considered another group receiving DMF,

Table 4
The combination index (CI) values report the interactive effects between BCP and IMP.

BCP (μM)	IMP (μM)	Effect	CI	Interpretation
0.2	1	0.641	0.247	Synergism
1	1	0.661	0.689	Synergism

A CI of less than, equal to, and more than one shows synergy, additive, and antagonism, respectively. CI was computed according to the direct effects of BCP, IMP and their combinations on the level of lymphocytes proliferation.

an oral and FDA-approved treatment for MS patients as positive control, and showed that DMF decreased the intensity level of clinical and pathological scores in EAE mice. Moreover, systemic and CNS levels of inflammation were considerably lower in the DMF-treated group in comparison to the vehicle-treated group. In fact, DMF modulated and diminished the inflammation through rebalanced Th_1/Th_2, Th_{17}/T_{reg} and M_1/M_2 ratios from inflammatory phenotypes towards healing and anti-inflammatory phenotypes Th_2, T_{reg} and M_2 cells, respectively. In consistent with the current findings, several studies reported the effectiveness of DMF in different animal models of inflammation and oxidative stress. In this context, DMF is capable to suppress auto-reactive Th_1 and Th_{17} cells by reducing the expression and secretion of pro-inflammatory cytokines including IFN-γ, TNF-α, and IL-17 [49,50], and increasing the subpopulation of T cells including T_{reg} and Th_2 cells and the levels of IL-4, IL-5, and IL-10 cytokines antagonizing Th1 and Th17 cells [50,51].

Recently, we have shown that the low concentrations of BCP had protective effects against inflammatory models of oligodendrocytes and microglia in a CB_2 receptor dependent manner [18,24]. Indeed, in our previous studies, we determined that not only low concentrations of BCP could be considered an effective treatment against inflammation but also these concentrations are more potent than its higher concentrations. On the other hands, in our previous studies, we revealed that both low and high concentrations of BCP provide protective effects through either Nrf2 or PPAR-γ receptors signaling pathways depending on the CB_2 receptor activation [18,24]. Moreover, we found that this controversy can be explained by the CB_2-mediated SMase activation because the use of SMase inhibitors, including IMP and FLX, dramatically compensates and increases the protective effects of BCP and provides a leftward shift in dose-response of BCP [18,24]. On this basis, in the present study, we assessed our hypothesis regarding the effectiveness of low dose of BCP alone and in the combination of a SMase inhibitor IMP in the treatment of EAE.

Interestingly, in the present study, we demonstrated that the low dose of BCP (5 mg/kg; p.o.) attenuates the levels of clinical and pathological scores, and both systemic (blood and lymphocytes) and CNS (microglia) inflammations, and conversely increased the levels of anti-inflammatory cytokine IL-10 and body weight in EAE mice. Our data showed that low dose of BCP provides its protective effects through reduction of the main culprit cells (Th_1 and Th_{17}) involving in the pathogenesis of EAE, and also through recrudescence of anti-inflammatory cells (Th_2, T_{reg} and M_2) leading to the resolution of inflammatory responses. Furthermore, we demonstrated that BCP (5 mg/kg) simultaneously implies its effects on both systemic (blood and lymphocytes) and CNS (microglia) immune systems. In line with the results, there are several in-vitro and in-vivo studies reporting the capability of BCP in reducing the different inflammation conditions. Recently, we indicated that both low and high concentrations of BCP significantly diminished the levels of inflammatory mediators (IL-1β, TNF-α, PGE_2, iNOS, NO and ROS), and markedly increased the levels of anti-inflammatory parameters (IL-10, Arg-1, and urea) polarizing microglia to M_2 anti-inflammatory phenotype [24].

Experimentally, BCP (1, 5 and 10 mg/kg) exerts significant analgesic effects in animal models of inflammatory and neuropathic pain through CB_2 receptor, because the use of either a CB_2 receptor antagonist or genetically deletion of CB_2 declined the protective responses of BCP [15]. They also explained that the use of BCP reduces the activity and infiltration of macrophage and astrocytes in the dorsal horn of the spinal cord so that all these effects were CB_2 receptor-mediated [15]. Additionally, it was indicated that BCP (10 mg/kg) provides liver protection and prevents over-activation of hepatic macrophages, namely Kupffer cells, by switching them towards M_2 cells. These effects were through the CB_2 receptor, because the ablation of CB_2 receptor completely reversed the protective effects of BCP against chronic and binge ethanol-induced liver injury and inflammation [52]. Taken together, these studies could support the effectiveness of low dose of BCP in the reduction of inflammation and demyelination in the spinal cord by preventing the infiltration of inflammatory cells into the area and polarization of inflammatory macrophages, in a CB_2 receptor-dependent fashion.

As one of the important target of CB_2-dependent G-protein coupling receptors signaling pathway, the activation of sphingomyelinase (SMase) increases ceramide overproductions. It has been shown that ceramides may interfere with Nrf2/PPARγ signaling pathways and trigger a different signaling pathway [53,54]. Furthermore, SMase increases the levels of sphingosine-1 phosphate (S1P) and its regulation considered an emerging pharmacological target for inflammatory diseases such as MS [55,56]. On the other hand, a higher level of phospholipid and lower content of sphingolipid were reported in the white and gray matter of patients with MS in comparison to controls, respectively [57], whereas sphingosine content was intensified in the white matter of patients with MS [58]. Furthermore, it has been well established that the anti-depressant activities of tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRI) such as amitriptyline and fluoxetine (FLX), respectively, are associated with the inhibition of acid SMase–ceramides system [59]. In this context, in mouse models of stress-induced depression, therapeutic concentrations of amitriptyline and FLX abrogate SMase activity and subsequently reduced the levels of ceramide in the hippocampus, and lead to increases in neuronal proliferation, maturation and survival levels and improvement in behavior. Experimentally, they have shown that the overexpression of SMase or directly injection of C16 ceramide in the hippocampus provides lower rates of neuronal proliferation, maturation, and survival, and represents depression-like behaviors even in the absence of stress, in comparison with controls [59].

Interestingly, we observed that IMP (10 mg/kg) significantly ameliorates the levels of the clinical and pathological score, inflammation and cell infiltrations into the CNS. Furthermore, IMP decreased the levels of inflammatory cytokines and IL-17/IL-10 ratios and increases anti-inflammatory cytokine IL-10. We also showed that IMP exerts its protective and immunomodulatory properties through polarization of Th_1/Th_2, Th_{17}/T_{reg} and M_1/M_2 ratios towards anti-inflammatory and healing phenotypes Th_2, T_{reg} and M_2, respectively. In agreement with our findings, it has been reported that IMP (20 mg/kg; i.p.) was an effective treatment in EAE [60]. However, their experimental aims were not associated to directly elucidate the protective effects of IMP as a SMase inhibitor in EAE. Moreover, in 2002, the protective effects of IMP (10 mg/kg) in EAE was reported, but the authors mainly focused on the anti-depressant activity of IMP and neglected to evaluate the effects of IMP in the treatment of EAE as a chronic model of MS [61]. Moreover, IMP showed anti-inflammatory properties on lymphocytes and microglial cells by switching to M_2, in both in-vivo and in-vitro models [18,24,62,63]. In fact, IMP significantly reduces the levels of inflammatory cytokines IL-17, IL-6, TNF-α, and IFN-γ, and increases the levels of anti-inflammatory cytokines IL-4 and IL-10. Also, it has been indicated that IMP blocks the increases of IL-6, TNF-α, and IL-1β peripherally (blood and lymphocytes) and centrally (brain tissue and microglia) [63]. There are further reports showing the protective properties of SMase inhibitors such as FLX (10 and 20 mg/kg) [64,65] in the treatment of EAE mice. In line with our results, they showed that FLX

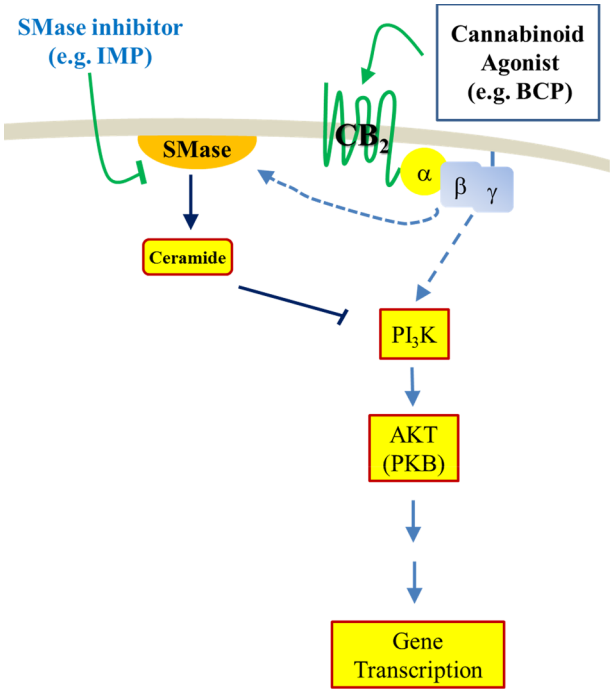

Fig. 10. A graphical schematic illustrated the possible interactive pathways involved in CB_2 receptor and SMase.

reduces the clinical and pathological scores, and also decreases the levels of inflammatory cytokines TNF-α and IFN-γ in EAE mice [64]. Recently, it has been demonstrated that FLX reduced the levels of lymphocytes proliferation, inflammatory cytokines (IL-17, IL-6, TNF-α, and IFN-γ), and increases the level of anti-inflammatory cytokine IL-10, in both *in-vivo* and *in-vitro* (lymphocyte and dendritic cell) studies [65]. These studies may support our findings on the protective effects of IMP as a known functional SMase inhibitor in EAE.

Overproduction of ceramides due to CB_2 receptor-mediated activation of SMase stimulates serine/threonine protein phosphatases (PP). In this regard, Stimulation of PP_{2A} results in the dephosphorylation and inactivation of Akt (PKB) as a downstream target of phosphatidylinositol 3-kinase (PI_3K) (Fig. 10) [66,67], which is a crucial pathway involving in the promotion and protection of neuronal survival [68]. In fact, the SMase-derived ceramides play a key role in various inflammatory diseases by inhibition of the PI_3K/Akt axis. Particularly, it has been demonstrated once ceramides generated, they lead to the activation and overexpression of pro-inflammatory transcription factors (NF-κB), cytokines (IL-1β, IL-6, and IL-8), and chemokines (monocyte chemoattractant protein-1, MCP-1) [69,70]. Reciprocally, pro-inflammatory cytokines (IFN-γ, IL-1β, and TNF-α) also activate SMase and increase the levels of ceramides [71]. Furthermore, the activation of PI_3K/Akt signaling pathway is considered necessary for CB_2 receptor to provide its full protective effects including differentiation and development of oligodendrocyte [68,72], anti-inflammatory and immunomodulatory effects [73–75]. With the consideration that the inhibition of SMase is associated with the activation of PI_3K and given the nature of CB_2 receptor leading to the activation of SMase, in this study, we assessed the combination of BCP (a CB_2 receptor agonist) with IMP (a functional SMase inhibitor) in the treatment of EAE mice. Interestingly, we found putative and protective effects between BCP (2.5 and 5 mg/kg) and IMP (10 mg/kg) in the treatment of EAE. In fact, we observed that the levels of clinical score, pathological defects, and inflammatory cytokines and transcription factors related to Th_1, Th_{17}, and M_1 cells were markedly lower in the combination treatment groups. On the other hand, the levels of anti-inflammatory cytokines and transcription factors related to Th_1, T_{reg} and M_2 cells were dramatically increased in the combination groups. Furthermore, in another set of experiment, we evaluated the direct effects of BCP (low concentrations), IMP (a pharmacological SMase inhibitor) and their interactions on the levels of cell proliferation, inflammatory (IL-17 and IFN-γ) and anti-inflammatory (IL-10 and IL-4) cytokines, and the ratios of IFN-γ/IL-4 (Th_1/Th_2) and IL-17/IL-10 (Th_{17}/T_{reg}), in spleen lymphocytes isolated from EAE mice and in the presence of MOG_{35-55} stimulation. Our data showed that the combination of low concentrations of BCP (0.2 or 1 μM) with IMP (1 μM) produce synergistic effects (CI $<$ 1).

In conclusion, our results indicated that low doses of BCP were also effective in the treatment of EAE as a chronic model of MS. In fact, by *in-vivo*, *ex-vivo* and *in-vitro* evaluations, we demonstrated that BCP reduces the clinical and pathological score in EAE, by modulation of both innate (microglia) and adaptive (lymphocytes) immune systems from the inflammatory state ($Th_1/Th_{17}/M_1$) toward anti-inflammatory and healing state ($Th_2/T_{reg}/M_2$). As another interesting finding of the present study, we showed the protective effects of IMP as functional SMase inhibitor, alone or in combination with BCP in EAE. Eventually, we also indicated that the addition of IMP to BCP low doses can increase the effects of BCP in-vivo; however, their interactive effects were estimated synergism by our in-vitro study (lymphocytes). With regards to the combination therapy of low doses of BCP as a selective CB_2 agonist, with IMP as a SMase inhibitor, we assume that this study may open a new window and also give a better and more in-depth insight into the concurrent application of CB_2 receptor agonists and the functional inhibitors of SMase.

Declaration of transparency and scientific rigor

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105923.

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