The Modulatory Role of 2-Methoxyestradiol in M1/M2 Polarization of Glial Cells

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ABSTRACT

Activation of the M1 microglial phenotype plays a key role in the progression of central nervous system (CNS) disorders and is a significant contributor to neuroinflammatory diseases. Contrariwise, M2 macrophages exhibit anti-inflammatory activities that promote tissue repair and reduce inflammation in the CNS. Thus, converting pro-inflammatory M1 to the anti-inflammatory M2 has emerged as a potential therapeutic approach to prevent the progression of neuroinflammatory diseases. 2-Methoxyestradiol (2-ME), a metabolite of estradiol, has gained interest for its remarkable anti-inflammatory properties. This study aimed to evaluate the modulatory effects of 2-ME on M1 polarisation in lipopolysaccharide (LPS) stimulated-IMG microglial cells. Firstly, the cells were treated with different concentrations of 2-ME to assess its cytotoxicity. Then, the modulatory effects of 2-ME on inflammatory markers were evaluated. Quantitative PCR (qPCR) was conducted to measure mRNA expression levels of IL-6, IL-1β, TNF-α, and TGF-β1. The protein concentrations of these cytokines were determined via enzyme-linked immunosorbent assays (ELISA). Additionally, flow cytometry was used to determine cell surface markers (CD86 and CD206) in IMG cells subjected to LPS or IL-4. The results revealed that 2-ME exhibited no cytotoxicity towards IMG cells. In addition, 2-ME significantly inhibited the upregulation of typical M1 markers including IL-6, IL-1β, and TNF-α both at the gene and protein levels. Flow cytometry analysis showed that 2-ME effectively decreased the expression of M1 marker (CD86) in LPS-stimulated IMG cells, while IL-4-stimulated cells treated with 2-ME displayed a marked increase in M2 marker (CD206) expression. In conclusion, 2-ME plays a modulatory role in microglial polarization, enhances the shift towards the M2 phenotype, and emphasizes its potential therapeutic value in the treatment of neuroinflammatory disorders.

Keywords: 2-Methoxyestradiol; Macrophages; Microglia; Lipopolysaccharides

INTRODUCTION

Microglia represent the intrinsic macrophages of the central nervous system (CNS). They have key factors in the maintenance of CNS homeostasis and the pathogenesis of neuroinflammatory conditions[1]. In recent years, the dynamic nature of microglia has gained considerable attention, as their roles extend far beyond immune surveillance to encompass the development, maintenance, and repair of neural circuity[2]. In addition to their roles in in CNS health, microglia are also involved in the pathogenesis of several neuroinflammatory disorders[3]. A basic function of a microglial cells is its ability to shift between different activation states in response to environmental cues[4]. This phenomenon is called microglial polarization, which to resemble the M1/M2 polarisation in peripheral macrophages. M1 microglia are ideal for immune defense; however, their prolonged activation can result in neuroinflammation and the exacerbation of CNS disorders[4]. M1 microglia play a pivotal role in Parkinson's disease by releasing pro-inflammatory cytokine, including interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α)[5]. In Alzheimer's disease, M1 microglia complicate neuroinflammation near amyloid-beta plaques and contribute to blood-brain barrier disruption[6,7]. In multiple sclerosis, M1 microglia and infiltrating macrophages contributes to the destruction of myelin sheaths and axonal damage[8].

Considering the detrimental effects of M1 microglial activation in neuroinflammatory disorders, targeting microglial polarisation represents a premising therapeutic approach. One such effective molecule is 2-Methoxy estradiol (2-ME), an endogenous metabolite of estradiol that is widely acknowledged for its anti-inflammatory properties and neuroprotective properties. Several reports have indicated the appropriateness of estrogen and its related compounds to suppress neuroinflammation[9,10]. 2-ME is an endogenous estradiol metabolite with a low binding affinity to estrogenic receptors[11]. 2-ME has shown protective properties against several inflammation-related diseases including rheumatoid arthritis, and experimentally-induced autoimmune encephalomyelitis[12-14].

Additionally, 2-ME has demonstrated a protective role against ischemia/reperfusion injuries by modulating inflammatory responses[15]. According to Liao et al. (2021), 2-ME alleviates lung I/R injury by upregulating the expression of annexin A1 (a protein renowned for its anti-inflammatory and tissue-protective properties) to reduce inflammation and enhance cellular regeneration in injured tissues. Similarly, Chen et al. (2014) demonstrated that 2-ME protects against renal injury by suppressing the expression of pro-inflammatory cytokines (including IL-6, TNF-α, and IL-1β)[16]. The promising protective benefits of 2-ME have been confirmed in animal models of traumatic brain injury[17] and

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it has been proven to combat oxidative stress which is a key contributor to neuroinflammatory and neurodegenerative processes[9].

Experimentally, treatment with 2-ME markedly decreased macrophage infiltration in adipose tissue by shifting polarisation from pro-inflammatory M1 to anti-inflammatory M2 markers. This was supported by enhancement of arginase 1 and IL-10, as well as inhibition of IL-6, IL-1β. This change ameliorates high fat diet-induced obesity and glucose intolerance by decreasing inflammation in adipose tissue. Therefore, the aim of this study was to evaluate the modulatory role of 2-ME on the production of inflammatory cytokines and M1 polarisation in lipopolysaccharide (LPS)-stimulated IMG microglial cells.

MATERIALS AND METHODS

Chemicals and kits: 2-ME (>98%) was obtained from Fraken Biochem Co., Ltd., (Qingdao, China). Lipopolysaccharides (LPS) from Escherichia coli, Trypan blue and Thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Merck (Darmstadt, Germany). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and trypsin were bought from Gibco (Grand Island, NY, USA).

Cell culture: Immortalized Microglial Cell Line (IMG) obtained from Kerafast (Boston, MA, USA) were routinely cultured in DMEM with 4.5 g/L glucose and glutamate supplemented with 1% penicillin/streptomycin and 10% heat-inactivated FBS in a humidified, 5% (v/v) CO_2 atmosphere at 37 ºC. In this study, cells were used passages between 10 and 20.

Evaluating of 2-ME cytotoxicity in IMG cell lines: 2-ME dissolved in dimethylsulfoxide (DMSO) was used to prepare a 10 mg/ml stock solution. The Cytotoxic effect of 2-ME was assessed against IMG cell line using a Thiazolyl Blue Tetrazolium Bromide (MTT). A solution of 0.25% trypsin-EDTA was used to collect cells which were then plated in 96-well plates at 4000 cells/well. Cells were then exposed to 2-ME (10-1000 μM/mL) for 24 h and subsequently stained with MTT solution for 4 h at 37 °C and incubated in 5% CO_2. This was followed by the addition of DMSO for 10 min at room temperature in dark place. DMSO was used to dissolve the MTT stained cells, and the color intensity was assessed at 570 nm using SPECTRAmax microplate spectrophotometer (Synergy HT, BioTek, Winooski, VT, USA).

Quantitative polymerase chain reaction (RT-PCR): Cellular RNA was extracted exploiting Qiagen's RNeasy Mini Kit (Qiagen, Manchester, UK) as per the provided instructions. Purity and concentration of RNA were assessed using a Nanodrop spectrophotometer (ND-2000C, ThermoFisher Scientific, Waltham, MA, USA). A ratio of A260 nm/A230 nm of 1.8 and A260 nm/A280 nm ratio of 1.9 were confirmed in all RNA samples.

For cDNA synthesis, 2 μg RNA was used for reverse transcription using SuperScript III cDNA Synthesis system (Invitrogen, UK) following the manufacturer's manual. Real-time (qRT)–PCR was performed using the SYBR select Master Mix (Applied Biosystems, Waltham, MT, US), as per the manufacturer's recommendation. The primers used throughout this study were designed to bind to the selected target and were checked using the National Centre for Biotechnology Information (NCBI) Genome Browser (BLAST). The primers were purchased from Macrogen (Macrogen, Korea). Primer nucleotide sequences for the genes used in the study are shown in Table 1. The thermal cycling and detection was performed on a Step One Plus real-time PCR system (Applied Biosystems, Waltham, MT, US).

The thermal cycle consisted of an initial uracil-DNA glycosylase activation of 2 min at 50 °C to prevent product ontamination, the DNA polymerase activation of 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C. A final extension of 5 min at 72°C followed and the reaction was halted by extended incubation and cooling down to 4°C. Primes; sequence was validated in our laboratory and were consistent with those in the literature [18,19]. The quantification method was based on the relative quantification (ΔΔCt) method [20]. Results were normalized to GAPDH.

Table 1. Nucleotide sequences of the used primers

Gene	Primer Name	Sequence
Il6	F	TTGGTCCTTAGCCACTCCTT
	R	TAGTCCTCCTACCCCAATT
Il1b	F	ATCTTTTGGGGTCCGTCAACT
	R	GCAACTGTTCCTGAACTCAACT
Tnfa	F	TTGGTGGTTTGTGAGTGTGAG
	R	GACGTGGAACTGGCAGAAGAG
Tgf1b	F	GCCTTAGTTTGGACAGGATCTG
	R	CTCCCGTGGCTTCTAGTGC
Gapdh	F	GCACCGTCAAGGCTGAGAAC
	R	TGGTGAAGACGCCAGTGGA

Assessment of IL-6, IL-1β, TNFα and TGF-β1 concentration: The conditioned media collected from LPS- treated IMG, were assessed for release of IL-6 using ELISA MAX™ Standard Set Mouse Interleukin-6 purchased from BioLegend (Cat. No. 431304, CA, USA). IL-1β, TNF-α and TGF-β1 were determined using the commercial ELISA kits (Cat. No. SEKM-0002, SEKM-0034 and SEKM-0035 respectively, Solarbio, Beijing, China).

Fluorescence-activated cell sorting (FACS): Cells (10^6) were washed with FACS-PBS that contained (PBS, 0.1% BSA, 0.1% NaN3) and incubated for 10 min with 2 μl of a CD16/32-Fc receptor blocker (Cat. No. 14-0161-82) to reduce nonspecific antibody binding. For surface staining of IMG cells, samples were incubated in the dark at 4°C for 40 min with the specific mouse monoclonal antibodies: CD206 (an M2 maker) (Cat. No. 17-2061-80) or CD86 (an M1 marker) (Cat. No. 12-0862-81) (Invitrogen, Carlsbad, CA, USA). After that, cells were washed again and analyzed on (FACS) flowcytometer (FACSCalibur, BD Biosciences, San Diego, CA, USA) equipped BD FACSDiva v9.0 Software.

Statistical analysis: Data are shown as mean±SD. One-way analysis of variance (ANOVA) with Tukey's post *hoc* test was used to examine statistical significance. At a *p* value less than 0.05, differences between samples were considered significant. The GraphPad Prism program, version 8.0.2 (GraphPad program Inc., San Diego, CA, USA), was used for all analyses.

RESULTS

Evaluation of cell viability

MTT assay was performed to evaluate the viability of IMG cells incubated with different concentrations of 2-ME ranged from 10 to 1000 μM. The data in Figure 1 indicate that there were no significant differences in cell viability observed in the different groups and 2-ME has no significant cytotoxicity on IMG cells. Therefore, 2-ME at concentrations of 10 & 20 μM was chosen for subsequent experimentation.
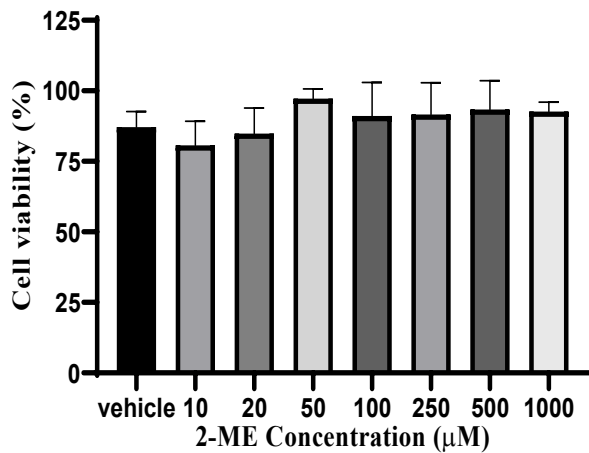
Figure 1. Impact of 2-ME on the viability of IMG cells

The effect of 2-ME in mRNA expression of Il6, Il1β and Tnfα

Microglial cells subjected to LPS treatment showed a significant increase in the expression of *Tnfα* (3.59 ± 0.15) compared to control. Then, treating these cells with 2-ME (10 μM) significantly prevented the rise in M1 markers Il6, Il1β and Tnfα mRNA expression by about 28%, 88% and 72% respectively, compared to cells subjected to LPS treatment. Similarly, cells treated with a higher dose of 2-ME (20 μM) significantly prevented up-regulation of *Il1β* and *Tnfα* by 171% and 72% respectively when compared to LPS group (Figure 2A, 2B and 2C). This suggests that 2-ME effectively suppresses the pro-inflammatory responses induced by LPS treatment in a dose-dependent manner. By significantly reducing the expression of key M1 markers such as *Il6, Il1β, and Tnf-α,* 2-ME demonstrates its ability to modulate microglial polarization and excessive inflammatory responses.
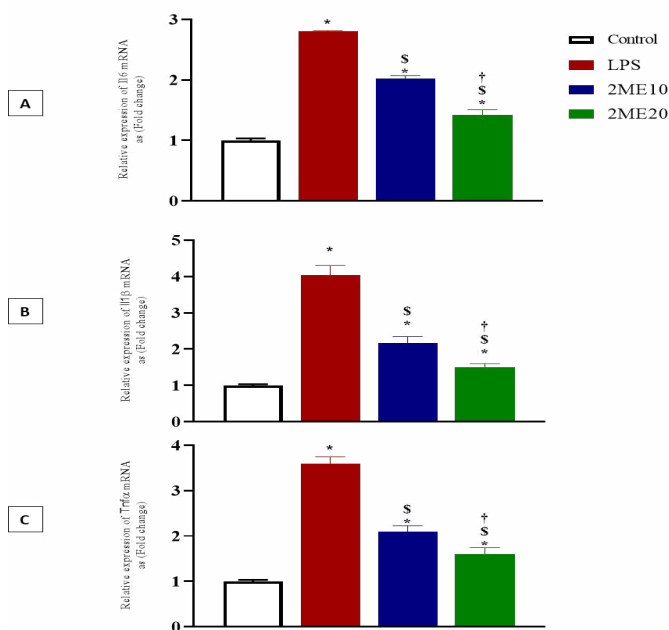

Figure 2. Graphical presentation of changes in mRNA expression of Il6 (A), Il1β (B) and Tnfα (C)in IMG cells stimulated with LPS. Data are represented as mean ± SD. *, $, † denote statistically significant differences from control, LPS and 2-ME 10 μM respectively at p < 0.05.

The effect of 2-ME on the release of IL-6, IL-1β and TNF-α

The data in Figure 3A illustrate that cells challenged with LPS showed an almost 20-fold release of IL-6 concentration as compared to control cells. However, pre-treatment of IMG cells with 2-ME prevented the rise in IL-6 release by 33.3% as compared to LPS-treated cells. As shown in Figure 3B, cells stimulated with LPS alone showed a dramatic increase in IL-1β amounting to 586 % as compared to control cells. However, pre-treatment with 2-ME 10 or 20 μM significantly ameliorated the rise in IL-1β concentration to almost control values. Likewise, 2-ME (10 or 20 μM) significantly attenuated the LPS-induced rise in TNF-α concentration by 32.5% and 53.9% respectively (Figure 3C).
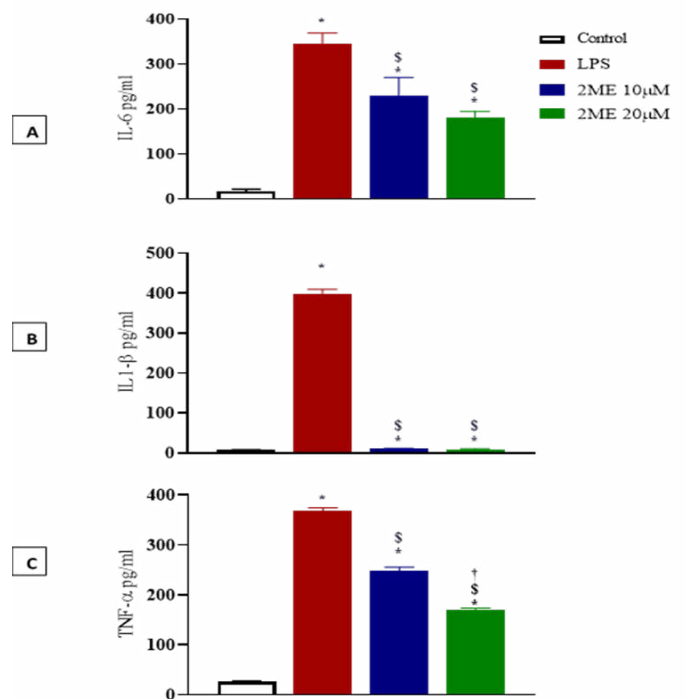

Figure 3. Effect of 2-ME on IL-6 (A), IL-1β (B) and TNF-α (C) release from IMG cells stimulated with LPS. Data are represented as mean ± SD. *, $, † denote statistically significant differences from control, LPS and 2-ME 10 μM respectively at p < 0.05.

Evaluation of surface marker CD86 and CD206 by flow cytometry

Flow cytometric analysis of CD86 in IMG cells challenged with LPS confirmed a polarization toward M1 as the cellular fraction with positive reactivity was 41.2%. However, pre-treatment of the cells with 2-ME (10 μM) decreased the cellular fraction to 25.6% (Figure 4). Interestingly, cells showed an observable increase in CD 206 expression upon stimulation with IL4 when compared to the controlled cells. Treating the cells with 2-ME 10 μM significantly increased CD 206 positive cells to 81.9 % (Figure 5). This suggests that 2-ME effectively modulates microglial polarization by suppressing pro-inflammatory M1 activation while promoting the anti-inflammatory M2 phenotype.

DISCUSSION

Inflammation has been linked to almost all neurodegenerative diseases[21]. Modulating the interaction between the nervous system and immune system to prevent inflammation can be a crucial approach to intervene in neurodegenerative diseases[22]. Microglia are the immune-
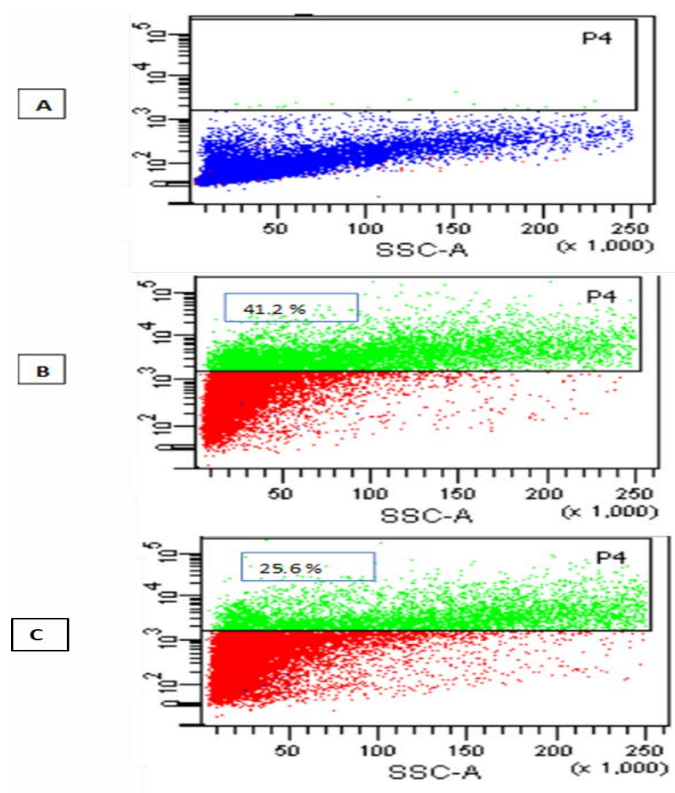
Figure 4. The effect 2-ME on CD86 expression of LPS-treated IMG cells. Data represent (A) control (B) cells stimulated with LPS (C) cells treated with 2-ME (10 µM)
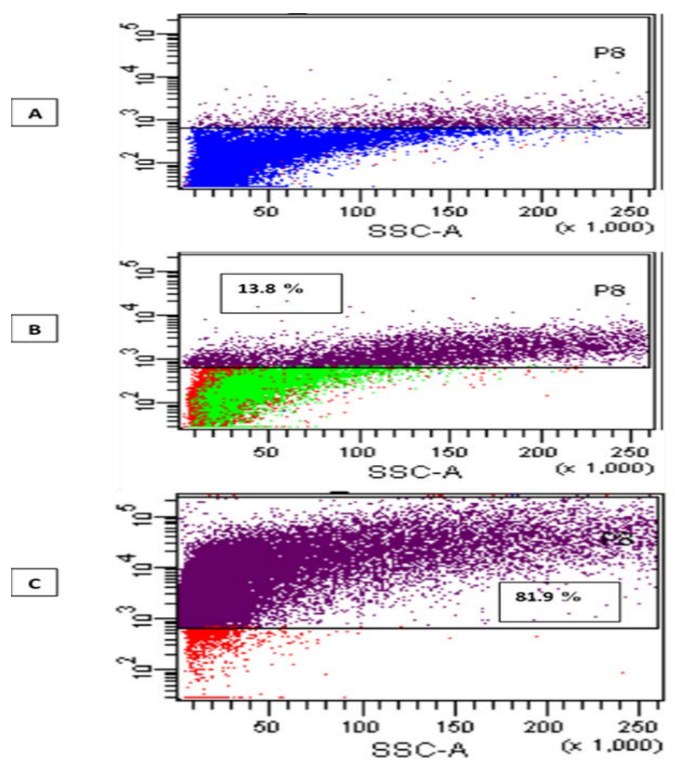

Figure 5. The effect of IL-4 and 2-ME (10 µM) on CD206 expression. Data represent (A) control (B) cells stimulated with IL4 (C) cells treated with 2-ME 10µM.

competent cells of the brain. They play a key role in maintaining normal brain functions via orchestrating immunological responses[23]. Microglia have two types of activation states; the M1 state (pro-inflammatory) and the M2 state (anti-inflammatory)[24]. The concept of M1/M2 polarization and mitigating the inflammatory environment by modulating the activation state of microglia has become an important strategy for the management of neurodegenerative diseases[25]. 2-ME has a plethora of pharmacological activities including inhibition of neuro-inflammation[26] and microglial activation[27]. Therefore, this study aimed at exploring the potential 2-ME to positively influence M1/M2 polarization in LPS-challenged microglial cells.

IMG cells were used in the current study as they retain several advantages. They exhibit a robust response to inflammatory signals (M1/M2 polarization) and can thus be used to examine mechanisms of action and efficacy of drugs with regard to M1/M2 polarization[28]. An initial dose-response experiment was performed using 2-ME in a wide range of concentrations. 2-ME had no obvious cytotoxicity on IMG cells and thus was safe on cell growth. This is in harmony with the reported non-toxic effects of 2-ME against non-cancerous cells and tissues[29-31]. The two concentrations of 2-ME 10 and 20 µM were chosen for subsequent experimentation. In the current work, IMG cells were challenged with LPS. The M1 markers IL6, IL1β and TNFα[32] were evaluated. In response to activation, IMG cells showed significant increases in the all assessed M1 markers as mRNA expression.

These observations were confirmed by the detected increased cellular concentrations of the same markers. Several experimental studies give support to these data[33,34]. However, pretreatment of the cells with 2-ME ameliorated the rise in IL6, IL1β and TNFα mRNA expression and concentration. In line with these findings, 2-ME was previously reported to prevent LPS-induced activation as evidenced via amelioration of iNOS and COX-2 production[27]. Further, 2-ME was reported to inhibit microglial activation and decrease concentrations of IL6, IL1β and TNFα in rat brains subjected to subarachnoid hemorrhage[26]. On the other hand, microglia alternative activation was developed largely based on work showing a role for IL-4 in the activation of M2 status[35] inducing expression of the anti-inflammatory cytokine TGF-β[36]. Previous research indicated that TGF-β plays an important role in IL4-induced alternative activation of microglia[37]. However, our data indicate that incubating IMG cells with IL-4 resulted in increased content of TGFβ1 as well as mRNA. However, addition of 2-ME, in either concentration, didn't further increase TGFβ1. This may be due to an insufficient time of incubation or concentration of 2-ME.

To further substantiate the impact of 2-ME on microglia M1/M2 polarization, the M1 surface marker CD86 was assessed in LPS-activated IMG cells by flow cytometry. The obtained data highlighted an increase in CD86 expression. This gains support by a previous study on macrophages treated with LPS and interferon (IFN) and displayed an increase of M1 cell surface markers CCR7 and CD80/CD86[38]. Treatment of the cells with 2-ME ameliorated the expression of CD86. This is in harmony with the ability of 2-ME to inhibit expression of CD86 by hypoxic tonsil epithelial cells[39]. On the other side, incubating the cells with IL-4 caused an increase in the expression of CD206 expression. An earlier report indicated that IL4/IL13-induced microglia activation in mouse brain was associated with enhanced expression of CD206[40].

CONCLUSION
Our results highlighted the ability of 2-ME to enhance expression of CD206. Collectively, the observed shift from M1 to M2 phenotype in IMG cells by 2-ME is in line with the activity in other tissues41,42.

In conclusion, 2-ME ameliorates activation of IMG cells and shift microglia polarization from pro-inflammatory M1 to M2 anti-inflammatory phenotypes. One limitation of the study is the use on of in vitro model only using IMG microglial cells which, does not fully cover the complexity of the in vivo CNS microenvironment where multiple cell types and factors are involved. To address this gap, future studies should investigate the effects of 2-ME in animals' models of CNS disorders, such as multiple sclerosis and Alzheimer's disease. Moreover, exploring the molecular signaling pathways underlying the immunomodulatory actions of 2-Me e.g. NF-κB will be essential to elucidate its mechanism of action and therapeutic potential in neuroinflammatory conditions.

Authorship Contribution: All authors share equal effort contribution towards (1) substantial contributions to conception and design, acquisition, analysis and interpretation of data; (2) drafting the article and revising it critically for important intellectual content; and (3) final approval of the manuscript version to be published. Yes.

Potential Conflicts of Interest: None

Competing Interest: None

Acceptance Date: 22 May 2025

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