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Melatonin synergistically potentiates the effect of methylprednisolone on reducing neuroinflammation in the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is an autoimmune neurodegenerative disease of unknown etiology characterized by infiltration of encephalitogenic cells in the central nervous system (CNS) resulting in the presence of multifocal areas of demyelination leading to neurodegeneration. The infiltrated immune cells population is composed mainly of effector $CD4^+$ and $CD8^+$ T lymphocytes, B cells, macrophages, and dendritic cells that secrete pro-inflammatory factors that eventually damage myelin leading to axonal damage. The most common clinical form of MS is relapsing-remitting (RR), characterized by neuroinflammatory episodes followed by partial or total recovery of neurological deficits. The first-line treatment for RRMS relapses is a high dose of glucocorticoids, especially methylprednisolone, for three to five consecutive days. Several studies have reported the beneficial effects of melatonin in the context of neuroinflammation associated with MS or experimental autoimmune encephalomyelitis (EAE), the preclinical model for MS. Therefore, the objective of this study was to evaluate the effect of the combined treatment of melatonin and methylprednisolone on the neuroinflammatory response associated with the EAE development. This study shows for the first time the protective synergistic effect of co-treatment with melatonin and methylprednisolone on reducing the severity of EAE by decreasing CD4 lymphocytes, B cells, macrophages and dendritic cells in the CNS, as well as modulating the population of infiltrated T and B cells toward regulatory phenotypes to the detriment of pro-inflammatory effector functions. In addition to the potentiation of the protective role of methylprednisolone, treatment with melatonin from the clinical onset of EAE improves the natural course of the EAE and the response to a subsequent treatment with methylprednisolone in a later relapse of the disease, pointing melatonin as potential therapeutic tool in combination with methylprednisolone for the treatment of relapses in MS.

1. Introduction

Multiple sclerosis (MS) is an autoimmune neurodegenerative pathology that affects 2.8 million people worldwide [1]. Although MS is a multifactorial disease [2,3] of unknown etiology, the potential pathological mechanism is related to an immunological attack against axonal myelin, leading to neuroinflammation and neurodegeneration of the central nervous system (CNS) [4]. The most common clinical form of MS

Abbreviations: APCs, antigen presenting cells; BBB, blood-brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; GC, glucocorticoids; MS, multiple sclerosis; p.i., post-induction; RR-MS, relapsing-remitting multiple sclerosis; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_n, naïve T cells; Treg, T regulatory cell.

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is relapsing-remitting (RR), characterized by neuroinflammatory episodes followed by partial or total recovery of neurological deficits [5]. Preclinical studies conducted in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS [6] and clinical studies have shown that the onset of the disease involves a pathogenic presentation of myelin peptides to encephalitogenic $CD4^+$ T cells by antigen-presenting cells (APCs) in secondary lymphoid tissues, resulting in T cells with an effector phenotype, which escapes the control of regulatory cells in the periphery and reaches the CNS by crossing the blood-brain barrier (BBB), the integrity of which is compromised by the pertussis toxin used in the EAE induction protocol [7].

These cells, mainly $CD4^+$ T cells, are reactivated in the CNS by APCs, releasing pro-inflammatory cytokines, including TNF and IFN-γ [8,9], which compromise the integrity of the BBB again and favor the entry of additional immune cells. Thus, the population of infiltrated immune cells is mainly composed of $CD4^+$ and, to a lesser extent, macrophages [10], $CD8^+$ T lymphocytes [11] and dendritic cells [12]. B cells, although minor, have also been shown to play a possible role in the immunopathology of EAE [13,14]. These infiltrating cells finally secrete mediators such as pro-inflammatory cytokines and antibodies, among others, that eventually can damage myelin leading to axonal damage [15].

Effector Th1 $CD4^+$ T cells, represented by the production of TNF and IFN-γ cytokines, play a pathological role in the neuroinflammatory responses associated with MS [16,17]. In particular, effector memory T cells (T_{EM}, $CD4^+$ $CD44^+$ $CD62L^-$), after encountering antigen and activation, are prompt to migrate to inflamed sites to develop effector functions [18]. In this line, the pharmacological reduction in T_{EM} cells improves the clinical course of EAE [19]. The T cell activation signal is also modulated by co-stimulatory and co-inhibitory signals being the programmed death 1 (PD-1) and the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) immune checkpoints, negative regulators of T cell immune function [20] whose blocking exacerbates EAE [21,22]. Unlike T effector cells, regulatory T cells (Treg; $CD4^+$ $CD25^+$ $FoxP3^+$) suppress excessive inflammation in MS and EAE [23–25] and IL-10, one of the main anti-inflammatory cytokines, has been associated with protection in the context of MS and EAE [26,27].

Effector $CD8^+$ T lymphocytes ($CD8^+$ $PD\text{-}1^-$ $IL\text{-}10^-$) have also been described to produce pro-inflammatory cytokines such as TNF and IFN-γ [28]. In fact, $CD8^+$ T cells specific for myelin proteins that produce TNF and IFN-γ have been identified in MS patients [29–31]. In addition, regulatory $CD8^+$ T cells ($CD8^+$ Treg), characterized by the expression of PD-1 and the production of IL-10 ($CD8^+$ $PD\text{-}1^+$ $IL\text{-}10^+$), have been shown to limit exacerbated innate and adaptive immune responses [32]. Accordingly, the expression of PD-1 in $CD8^+$ Treg is reduced in patients with RRMS [33] and increases in the stable phase of the disease compared to the acute one [34].

Plasmablasts ($CD19^+$ $B220^{low}$ $CD138^+$) and plasma cells ($CD19^-$ $B220^{low}$ $CD138^+$), have also been identified to contribute to parenchymal inflammation [35] and the production of antibodies against axonal myelin in brain tissue and cerebrospinal fluid (CSF) of MS patients [36]. Furthermore, numerous studies have reported an imbalance of the cytokine profile produced by B cells in MS. In particular, effector B cells ($CD19^+$ TNF^+) of patients have been shown to produce higher levels of TNF compared to normal individuals [37]. On the contrary, knocking out IL-10 produced by Breg cells ($CD19^+$ $IL\text{-}10^+$) increased the severity of EAE [38].

Currently, more than 20 disease-modifying therapies (DMT) have been approved for the treatment of MS, mostly consisting of immunomodulators aimed at controlling the pathogenic cells responsible for the neuroinflammation episodes [39]. However, none of these therapies can completely prevent relapses. First-line treatment for RRMS relapses is a high dose (1g/day) of intravenous glucocorticoids (GC) for three to five consecutive days (commonly methylprednisolone) [40]. During therapy, patients may experience immediate adverse effects such as insomnia, mood changes, gastrointestinal disorders, or palpitations, among others. Serious events, such as psychosis, depression, and mania, have also occasionally occurred [40], with acute hepatitis frequently reported [37,41,42]. Although there is some controversy regarding the impact of relapses on long-term patient disability, several studies have shown that the relapse rate of the disease can contribute to long-term disability [43–45]. Given that recovery is incomplete in approximately half of the patient [46], therapies that reduce the severity of relapses remain a cornerstone of MS care.

Melatonin is a compound with powerful antioxidant and anti-inflammatory properties in acute inflammation [47,48]. In the context of neuroinflammation associated with MS or EAE, several studies have reported the beneficial effects of melatonin [49–53]. In particular, melatonin decreases the inflammatory infiltrate in the CNS [19,54], and reduces Th1 response, while increasing the production of IL-10 by $CD4^+$ T lymphocytes [19,55,56].

Therefore, the objective of this study was to evaluate the effect of combined treatment of melatonin and methylprednisolone on the neuroinflammatory response associated with the development of EAE.

2. Material and methods

2.1. Animals and EAE induction

Eight-week-old female C57BL/6N mice were obtained from the University of Seville Animal Facility and housed at the IBiS Animal Facility under a 12 h light/dark schedule (lights on at 8:00 a.m.) and ad libitum access to water and food. EAE was induced in the animals by subcutaneous immunization in both hind legs with 100 μg of MOG35–55 (Cambridge Research Biochemicals, Cleveland) emulsified in CFA (Sigma) containing 50 μg of heat-killed Mycobacterium tuberculosis (H37Ra, ATCC 25177) and two doses of intraperitoneal pertussis toxin (200 ng/day) (List Labs, California) on days 0 and 2 post-induction. Mice were daily evaluated and scored for clinical signs using a 0 to 6 points scale, as follows: 0, without signs; 1, flaccid tail; 2, impaired righting reflex and/or gait; 3, partial hind-limb paralysis; 4, total hind-limb paralysis; 5, hind limb paralysis with partial front-limb paralysis; and 6, moribund or dead. The mice were housed and maintained under pathogen-free conditions. All experiments were approved by the Ethic Committee of the Virgen Macarena-Virgen del Rocío University Hospital (reference number 24-11-15-368) and were carried out under Spanish legislation and the EU Directive 2010/63/EU for animal experiments.

The immunized animals were randomly divided into 6 groups of: Control, Mel, MPD40, Mel + MPD40, MPD160, and Mel + MPD160. Melatonin (Mel) was always administered at 80 mg/kg/day, while methylprednisolone (MPD) was administered at 40 and 160 mg/kg/day. At the beginning of the experiment, day 0 (immunization day), the animals were divided equally into two experimental groups: animals treated with placebo (Control) and animals treated with melatonin. When any animal in each group started to show clinical signs (days 8–10, depending on the experiment; show in Supplementary Table 1), they were distributed homogeneously according to score and weight in the different experimental groups: animals treated with placebo for the Control, MPD40 and MPD160 groups and animals treated with melatonin for the Mel, Mel + MPD40 and Mel + MPD160 groups, and methylprednisolone treatment was started, where appropriate, for 5 days. Regarding the re-exposure experiment of Fig. 7, once all animals were recovered from the first immunization, the disease was induced following the same protocol. All treatments were administered intraperitoneally.

2.2. Isolation of CNS mononuclear cells

Mice were sacrificed at the peak of the disease (day 15 after induction) and subjected to perfusion with ice-cold sterile PBS through cardiac puncture. The CNS (brain and spinal cord) was collected, homogenized and enzymatically dissociated with 1.87 mg/ml of

collagenase IV (Worthington) and 0.25 mg/ml of DNase I (AppliChem) for 35 min at 37 °C to obtain a suspension of single cells. Subsequently, a 37 %:70 % discontinous percoll gradient was carried out to isolate CNS-infiltrating mononuclear cells.

2.3. Flow cytometry

To assess the profile of infiltrated immune cells in the CNS, cells were stained with the following antibodies against surface markers: CD45, CD4, CD8α, CD19, CD11b, CD11c, CD44, CD62L, B220, CD138, PD-1 (CD279), CTLA-4 (CD152), FAS (CD95), and CD25. To identify Treg and analyze intracellular production of TNF, IFN-γ and IL-10, after surface staining, cells were fixed and permeabilized using the FoxP3/transcription factor staining buffer set (eBioscience) and stained with anti-FoxP3 or anti-TNF, -IFN-γ and -IL-10, respectively. The intracellular production of cytokines was carried out in cells cultured at 2.5×10^6 cells/ml with RPMI 1640 supplemented with 5 % fetal bovine serum, 1 % L-glutamine and 1 % of penicillin/streptomycin incubated in the presence or absence of Phorbol-Myristate-Acetate (PMA) and Ionomycin (Sigma) with brefeldin A (eBioscience) for 5 h. To assess the effects of melatonin on co-stimulatory/co-inhibitory signals, adhesion molecules and chemokine receptors in $CD4^+$ cells from draining lymph nodes (inguinal and para-aortic lymph nodes) and CNS (brain and spinal cord) on days 5 (priming), 10 (onset of the symptoms) and 15 (peak of the disease) after EAE induction, cells were stained with the following antibodies against surface markers: CD4, CD28, CD40L, PD-1, VLA-4, LFA-1 and CCR7. Dead cells were excluded from analyses using the LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen). FACS analysis was performed using a Cytek Aurora spectral cytometer, and data were analyzed using FlowJo software (Treestar). Supplementary Table S2 shows antibody characteristics.

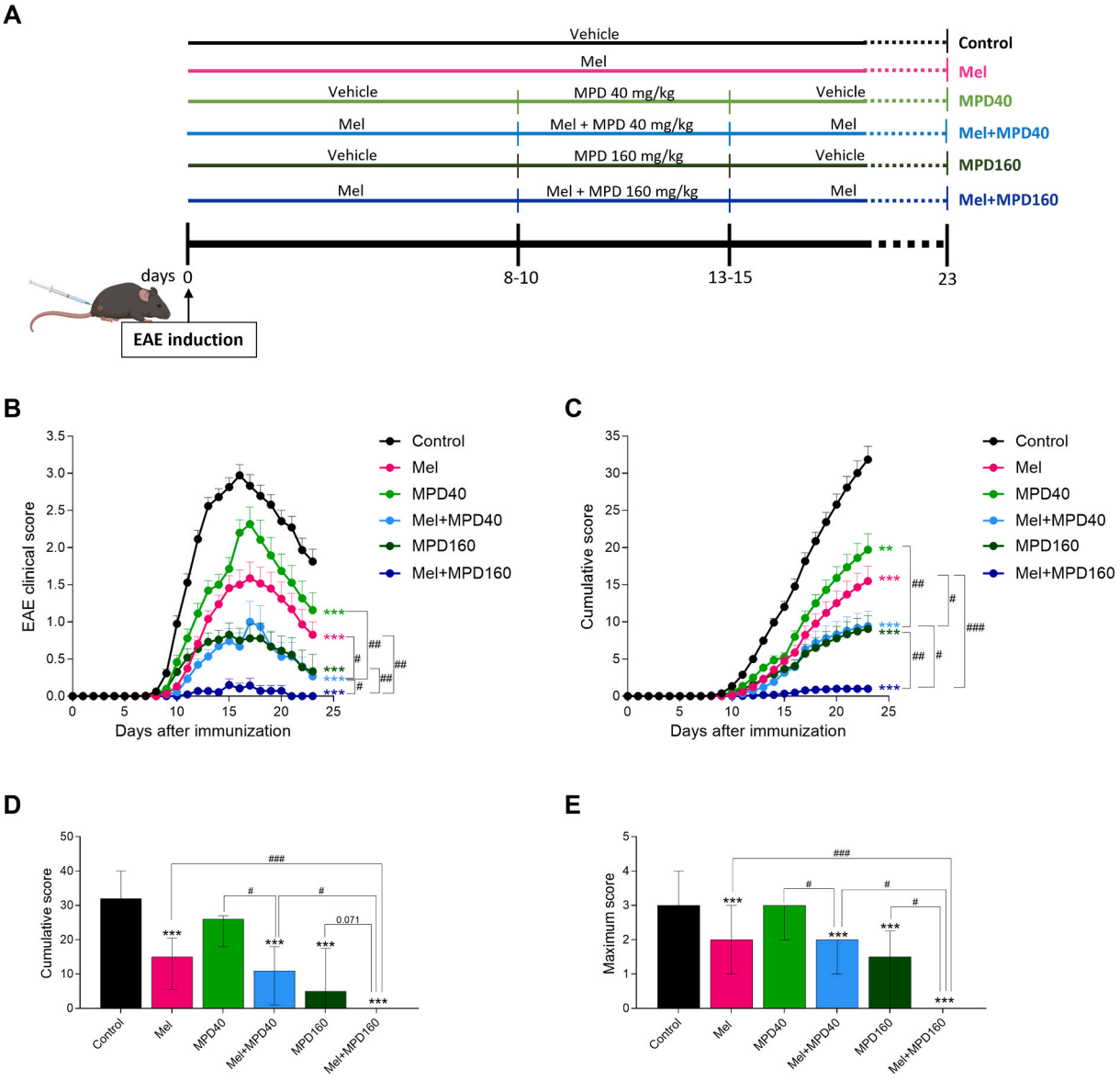

Fig. 1. Melatonin (Mel) and combined treatment of melatonin and methylprednisolone (Mel + MPD40 and Mel + MPD160) ameliorate EAE in a dose-dependent manner of methylprednisolone. Treatment paradigm scheme used in these experiments (A). Mean clinical EAE score (B), cumulative score curve (C), cumulative score (D), and maximum score (E) reached by each animal in the different experimental groups. Representative data from eight independent experiments with n = 5 mice per group (B and C represented as mean ± SEM and D and E as median and IQR). **, $p \leq 0.01$; ***, $p \leq 0.001$ with respect to the control group. #, $p \leq 0.05$; ##, $p \leq 0.01$; ###, $p \leq 0.001$.

2.4. Statistics

Statistical analysis was performed using SPSS v24.0 software (IBM) and all results were expressed as median and IQR (except for Fig. 1A–B and 7B, which are presented as mean ± SEM because the data are discrete). Kruskal-Wallis followed by Dunn's post hoc test was used for multiple comparisons. Mann-Whitney *U* test was used for comparison between two groups. A P value of less than 0.05 was considered significant. Exact P values for all comparisons are given in Supplementary Tables 6–12, which refer to Figs. 1–7, respectively.

3. Results

3.1. The combination of melatonin and methylprednisolone treatment synergistically reduces the severity of EAE

Both monotherapies with melatonin, from the initial immunization with MOG_{35-55} until sacrifice, or with methylprednisolone, for 5

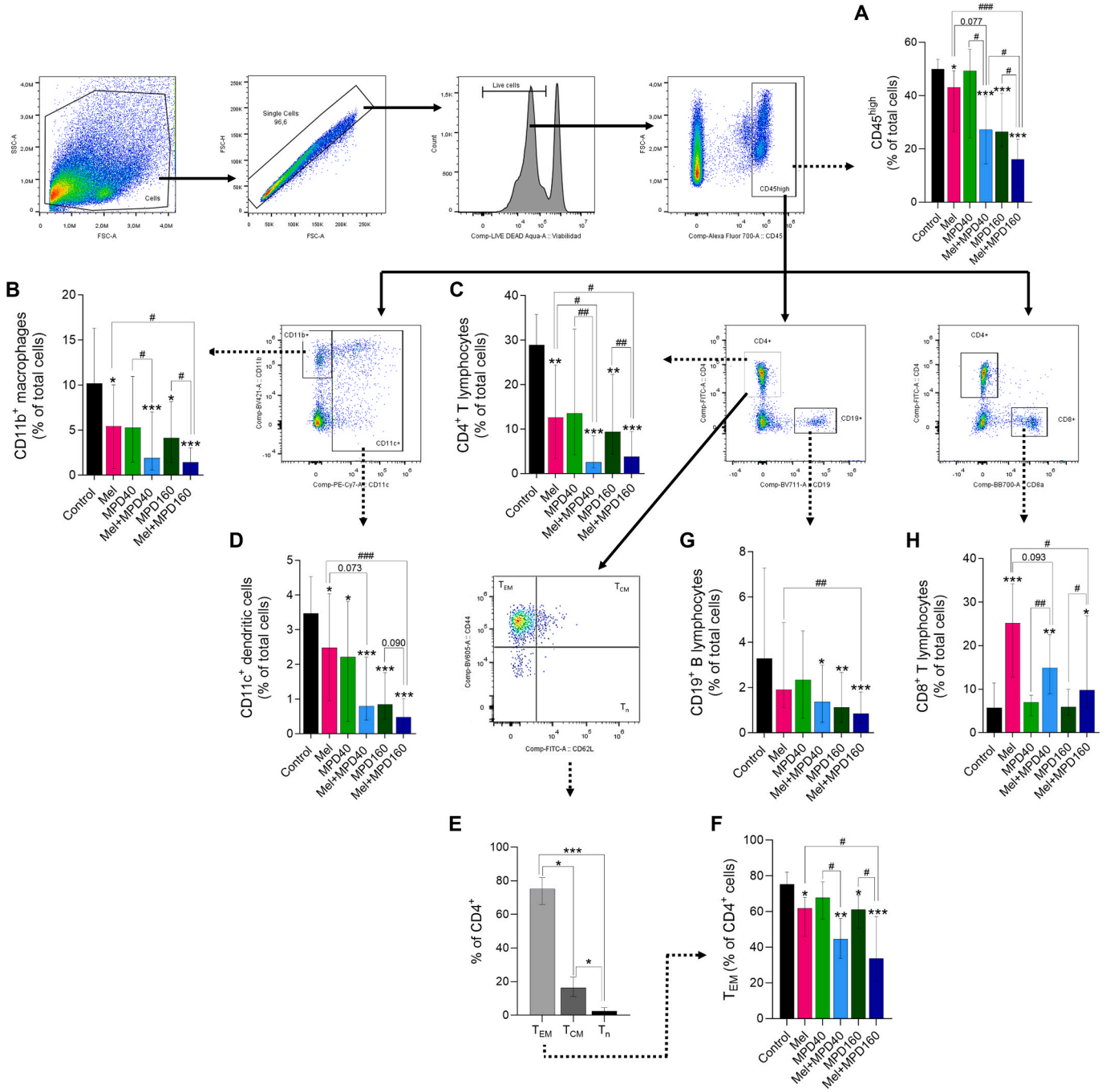

Fig. 2. Melatonin (Mel) and combined treatment with melatonin and methylprednisolone (Mel + MPD40 and Mel + MPD160) modulate the access of infiltrating immune cells into the CNS of EAE mice. Schematic representation of the gate-strategy carried out for data analysis and percentages of total inflammatory infiltrate (A), $CD11b^+$ macrophages (B), $CD4^+$ T lymphocytes (C) and $CD11c^+$ dendritic cells (D). Frequencies of T_{EM} ($CD44^+$ $CD62L^-$), T_{CM} ($CD44^+$ $CD62L^+$) and T_n ($CD44^-$ $CD62L^+$) in the control group (E) and effect of treatments on T_{EM} (F). Percentages of $CD19^+$ B cells (G) and $CD8^+$ T lymphocytes (H). Representative data from five independent experiments with n = 5 mice per group (median and IQR). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ with respect to the control group. #, $p \leq 0.05$; ##, $p \leq 0.01$; ###, $p \leq 0.001$. T_{EM}: effector memory T cell; T_{CM}: central memory T cell; T_n: naïve T cells.

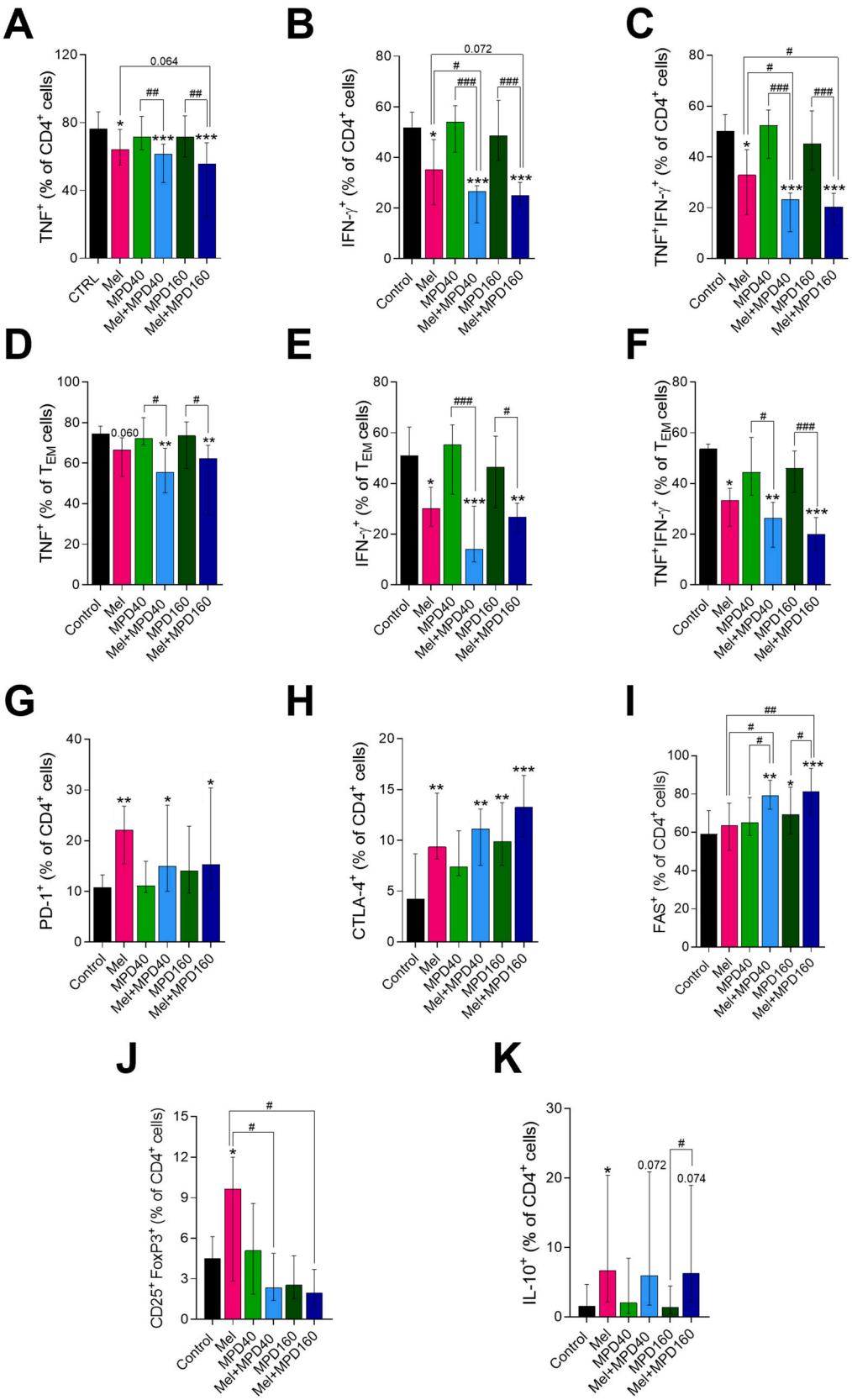

Fig. 3. Melatonin (Mel) and combined treatment of melatonin and methylprednisolone (Mel + MPD40 and Mel + MPD160) decrease the effector and increase the regulatory response mediated by $CD4^+$ T cells. Percentages of TNF^+ (A), IFN-γ^+ (B) and TNF^+ IFN-γ^+ (C) cells in $CD4^+$ T cells. Percentages of TNF^+ (D), IFN-γ^+ (E) and TNF^+ IFN-γ^+ (F) cells in T_{EM}. Percentage of $CD4^+$ T cells expressing PD-1 (G), CTLA-4 (H) and FAS (I). Percentage of T regulatory population ($CD25^+$ $FoxP3^+$) (J) and IL-10-producing T $CD4^+$ lymphocytes (K). Representative data from three independent experiments with n = 5 mice per group (median and IQR). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ with respect to the control group. #, $p \leq 0.05$; ##, $p \leq 0.01$; ###, $p \leq 0.001$. Representative plots of Fig. 3 have been included in the supplementary material (Supplementary Fig. S2).

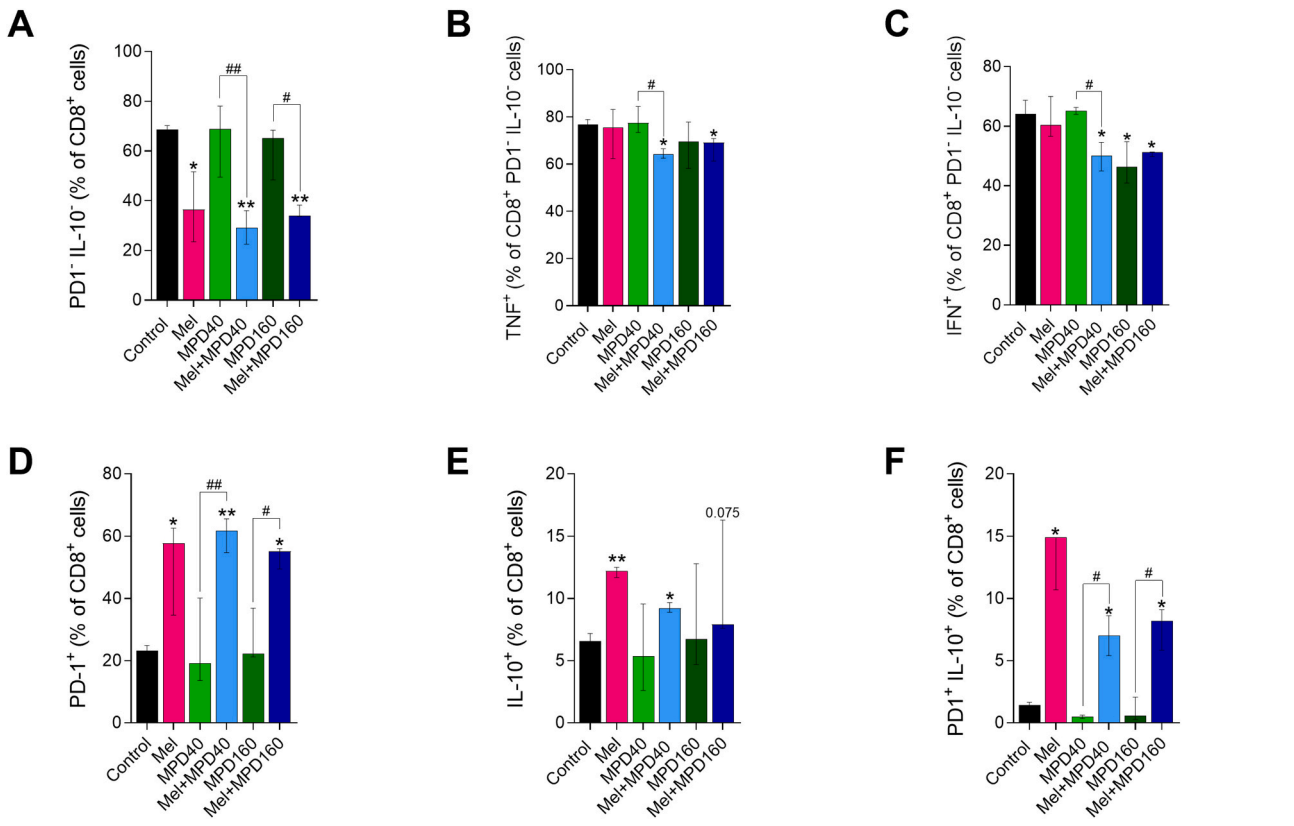

Fig. 4. Melatonin (Mel) and combined treatment of melatonin and methylprednisolone (Mel + MPD40 and Mel + MPD160) decrease the effector and increase the regulatory $CD8^+$ T response. Percentage of $CD8^+$ effector lymphocytes ($CD8^+$ PD-1^- IL-10^-) (A), and TNF (B) and IFN-γ (C) cytokine-producing $CD8^+$ effector cells. Percentage of $CD8^+$ T lymphocytes expressing PD-1 (D) and producing IL-10 (E). Percentage of $CD8^+$ Treg cells ($CD8^+$ PD-1^+ IL-10^+) (F). Representative data from two independent experiments with n = 5 mice per group (median and IQR). *, $p \leq 0.05$; ***, $p \leq 0.001$ with respect to the control group. #, $p \leq 0.05$; ##, $p \leq 0.01$. Representative plots of Fig. 4 have been included in the supplementary material (Supplementary Fig. S3).

consecutive days from the onset of the symptoms, attenuated the clinical score of EAE (Fig. 1B) and decreased both the cumulative score (Fig. 1C and D) and the maximum score (Fig. 1E) compared to the control group. The combined treatment of methylprednisolone and melatonin significantly decreased the development of the disease compared to both melatonin and methylprednisolone monotherapies (Fig. 1B–E). Interestingly, the combination of melatonin at 80 mg/kg and methylprednisolone at 40 mg/kg protected animals in the same range as the monotherapy with 160 mg/kg methylprednisolone. Moreover, the combination of melatonin (80 mg/kg) and methylprednisolone at 160 mg/kg almost completely abrogated EAE (Fig. 1B–E).

3.2. Melatonin treatment potentiates the effect of methylprednisolone on reducing immune infiltrating cells into the CNS of EAE mice and modifies the cell composition of the infiltrated cells

Mice treated with monotherapy of melatonin or methylprednisolone at 160 mg/kg showed a significant decrease in the percentage of inflammatory infiltrate (CD45high cells) in the CNS compared to the control group (Fig. 2A). The combined treatment of methylprednisolone and melatonin significantly decreased the infiltration of cells in the CNS compared to the monotherapies. The Mel + MPD160 group showed approximately one third as many $CD45^{high}$ inflammatory infiltrating cells as the control group. A similar pattern was shown in the decrease of macrophages (Fig. 2B), CD4 T cells (Fig. 2C) and dendritic cells (Fig. 2D). Regarding CD4 T cells, the frequency of effector memory T cells (T_{EM}), which was the most frequent population in the CNS compared to central memory T cells (T_{CM}) and naïve T cells (T_n) (Fig. 2E), was significantly reduced, revealing the synergistic effect of the combined treatment (Fig. 2F). Melatonin also acted as a modulator of priming in the draining lymph nodes, trafficking and reactivation of $CD4^+$ cells in the CNS, resulting in a reduced number of $CD4^+$ cells in the lymph nodes on days 5 and 10 after induction of EAE and a subsequent reduction in the number of infiltrating $CD4^+$ cells in the CNS (Supplementary Fig. S1A). In particular, melatonin decreased VLA-4 expression while increasing PD-1 levels in $CD4^+$ cells in the lymph nodes during the priming phase (Supplementary Fig. S1B). Furthermore, $CD4^+$ cells from the CNS of melatonin-treated mice had significantly lower levels of CCR7, LFA-1, CD28 and CD40L compared to control animals, both on days 10 and 15 post-EAE induction (Supplementary Fig. S1C). The mRNA levels of CCL19, VCAM-1 and ICAM-1 were also down-regulated in the CNS of the melatonin group (Supplementary Fig. S1D). The percentage of CD19 cells decreased only in the Mel + MPD40, MPD160 and Mel + MPD160 groups compared to the control group. The synergistic effect of melatonin and methylprednisolone was also observed in the Mel + MPD160 group compared to the MPD160 and Mel groups (Fig. 2G). Interestingly, neither melatonin nor methylprednisolone treatments in monotherapy or in combination were able to prevent access of CD8 T cells to the CNS (Fig. 2H).

3.3. The combination of melatonin and methylprednisolone skews the Th1/Treg balance to suppressive $CD4^+$ T lymphocytes in the CNS of EAE mice

The frequency of cells producing TNF (Fig. 3A) and IFN-γ (Fig. 3B) within $CD4^+$ T lymphocytes was synergistically reduced after the combined treatment with melatonin and methylprednisolone compared to the monotherapies. A significant decline in the TNF and IFN-γ double positive population was also observed (Fig. 3C). Furthermore, the combined effect of melatonin and methylprednisolone on decreasing

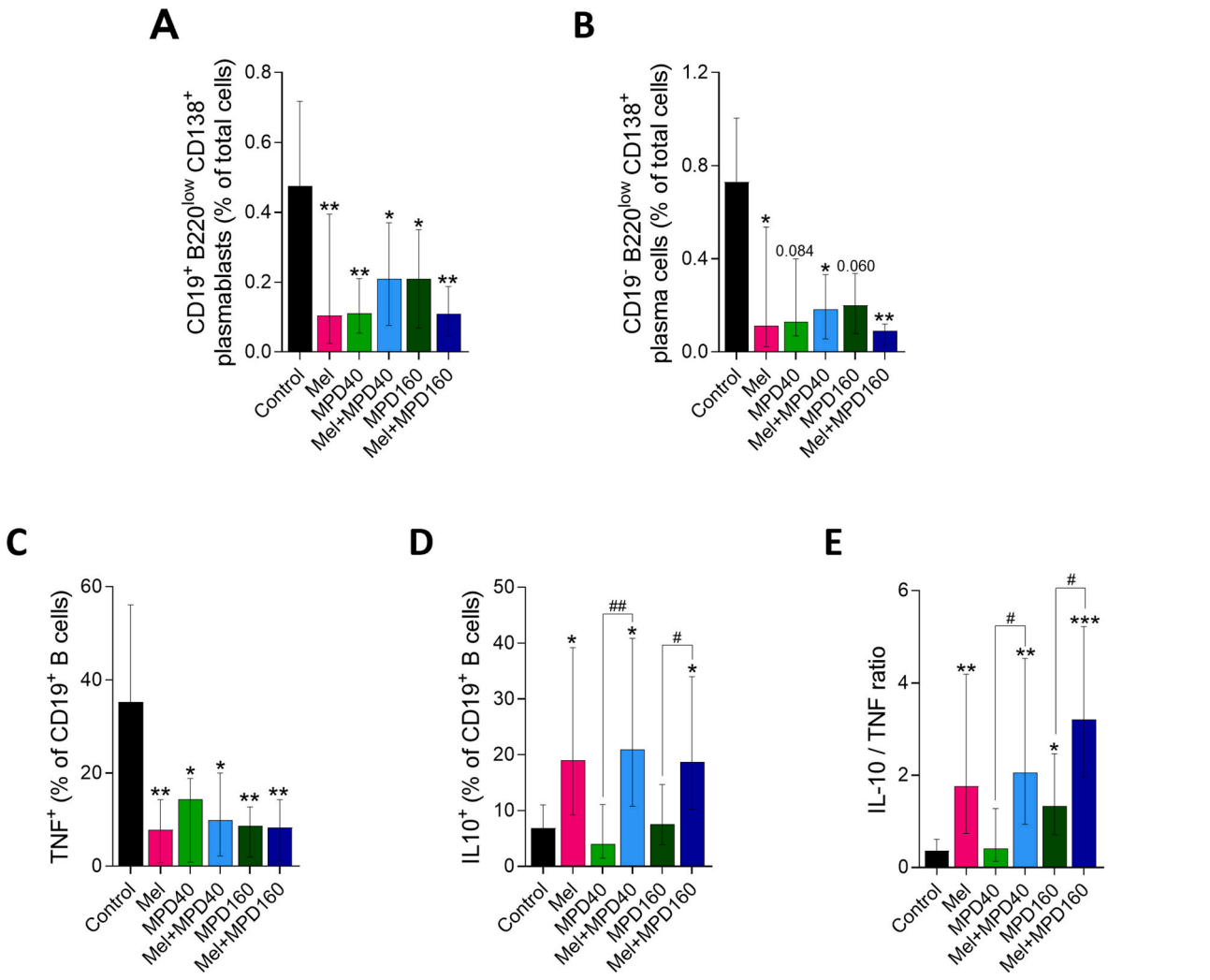

Fig. 5. Melatonin (Mel) and combined treatment of melatonin and methylprednisolone (Mel + MPD40 and Mel + MPD160) restore the anti-/pro-inflammatory balance in CNS infiltrated B cells. Percentage of plasmablasts ($CD19^+$ $B220^{low}$ $CD138^+$) (A), plasma cells ($CD19^+$ $B220^{low}$ $CD138^+$) (B), and TNF^+ B lymphocytes (C). Percentage of IL-10-producing B lymphocytes (D), and ratio of $IL\text{-}10^+$ and TNF^+ cells in B cells (D). Representative data from three independent experiments with n = 5 mice per group (median and IQR). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ vs. control group. #, $p \leq 0.05$; ##, $p \leq 0.01$. Representative plots of Fig. 5 have been included in the supplementary material (Supplementary Fig. S4).

IFN-γ^+ and TNF^+ cells within the population of CD4 T_{EM} cells was significantly higher compared to methylprednisolone monotherapy (Fig. 3D–F). The suppressive markers PD-1 and CTLA-4 were up-regulated in $CD4^+$ T cells treated with melatonin both in monotherapy and in combination with methylprednisolone (Fig. 3G and H). Furthermore, a synergistic effect of melatonin and methylprednisolone treatment was observed in the increase in Fas within $CD4^+$ T cells (Fig. 3I). Interestingly, only melatonin treatment was capable of increasing the percentage of Treg cells (Fig. 3J). In addition, the frequency of cells producing IL-10 within the $CD4^+$ T lymphocytes was significantly increased after monotherapy with melatonin or combined therapy with methylprednisolone (Fig. 3K).

3.4. *Both melatonin monotherapy and in combination with methylprednisolone reduce the effector response and increase regulatory $CD8^+$ population in the CNS*

Effector $CD8^+$ T lymphocytes ($CD8^+PD\text{-}1^-IL10\text{-}$) decreased significantly after melatonin monotherapy or in combination with methylprednisolone treatments (Fig. 4A). The pro-inflammatory capacity of effector CD8 cells, in terms of IFN-γ and TNF production, was significantly reduced by the combination of melatonin and methylprednisolone therapy compared to the control group (Fig. 4B and C). Moreover, the percentage of $CD8^+$ T cells that express PD-1 (Fig. 4D) or produce IL-10 Fig. 4E) increased with melatonin alone or in combination with methylprednisolone. In particular, melatonin monotherapy or in combination with methylprednisolone significantly increased the frequency of CD8 T cells expressing PD-1 and producing IL-10 (Fig. 4F).

3.5. *Melatonin monotherapy and in combination with methylprednisolone skew the response of B cells in the CNS towards suppressive functions*

The percentage of plasmablasts ($CD19^+B220^{low}CD138^+$) and plasma cells ($CD19^+B220^{low}CD138^+$) decreased significantly after treatments (Fig. 5A and B). Within the CD19 cells population of mice treated with melatonin (without or with methylprednisolone), a lower percentage of cells producing TNF (Fig. 5C) and an increase in cells producing IL-10 were shown (Fig. 5D). Consequently, combined melatonin and methylprednisolone treatments synergistically increased the ratio of CD19 cells producing IL-10/TNF (Fig. 5E).

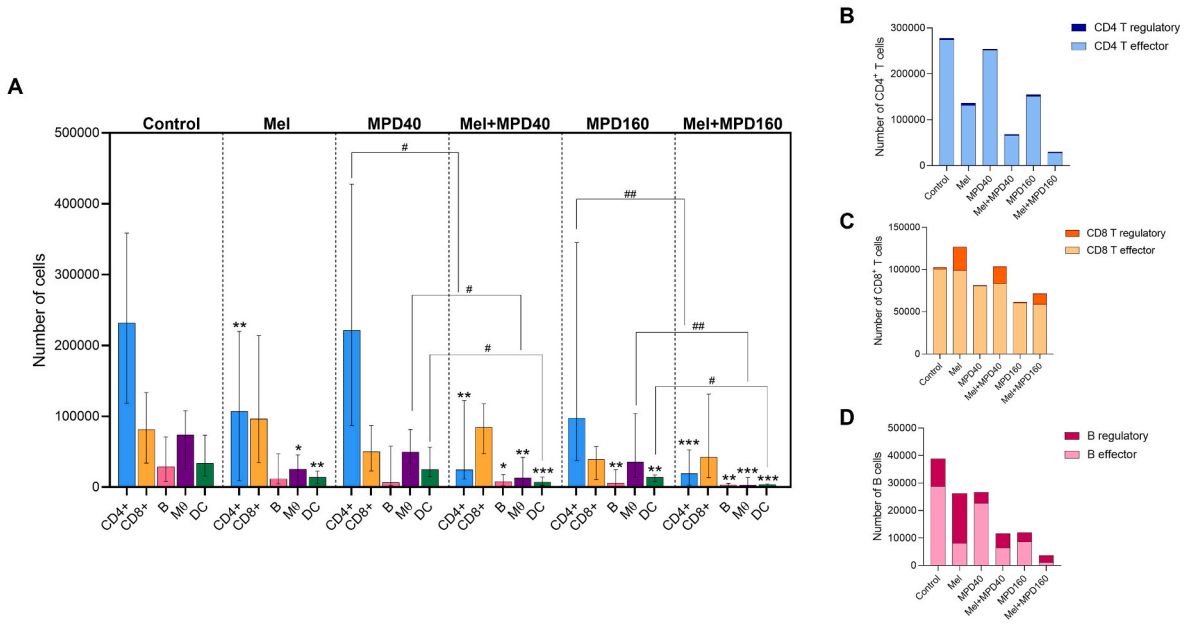

Fig. 6. Melatonin (Mel) and the combined treatment with melatonin and methylprednisolone (Mel + MPD40 and Mel + MPD160) restrict the infiltration of immune cells into the CNS while reducing effector profiles and/or increasing regulatory ones. Number of total $CD4^+$ and $CD8^+$ T lymphocytes, B cells, macrophages, and dendritic cells (A) in each experimental group. Number of effector ($CD4^+$ TNF^+ IFN^+) and regulatory ($CD4^+$ $CD25^+$ $FoxP3^+$) cells in $CD4^+$ T lymphocytes (B). Number of effector ($CD8^+$ $PD\text{-}1^-$ $IL10^-$) and regulatory ($CD8^+$ $PD\text{-}1^+$ $IL10^+$) cells in $CD8^+$ T lymphocytes (C). Number of effector B cells ($CD19^+$ TNF^+) and regulatory B cells ($CD19^+$ $IL\text{-}10^+$) (D). Representative data from two independent experiments with n = 5 mice per group (median and IQR). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ vs. the same population in the control group. #, $p \leq 0.05$; ##, $p \leq 0.01$; ###, $p \leq 0.001$.

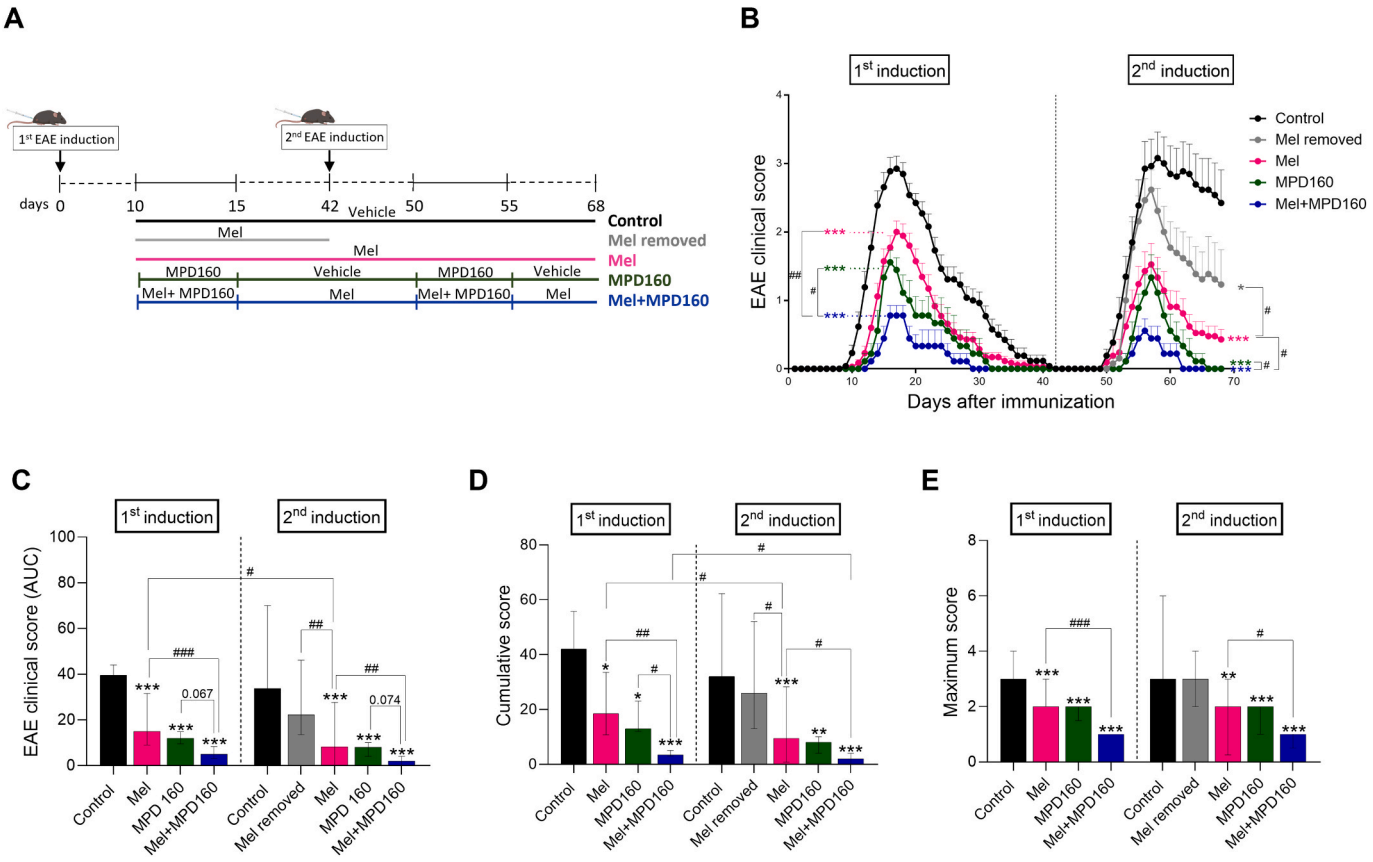

Fig. 7. Melatonin (Mel) treatment from the clinical onset of EAE improves the natural course of the disease and the response to subsequent treatment with methylprednisolone in a following relapse of the disease. Treatment scheme (A). Mean (B) and AUC (C) of clinical scores, cumulative score (D), and maximum score (E). Representative data from two independent experiments with n = 5 mice per group (B represented as mean ± SEM and C-E as median and IQR). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ vs. control group. #, $p \leq 0.05$; ##, $p \leq 0.01$; ###, $p \leq 0.001$.

3.6. The combined treatment of melatonin and methylprednisolone not only decreased the number of CD4 T cells, B cells, macrophages and dendritic cells infiltrated in the CNS, but also skew the response of the T and B cells in the CNS to suppressive phenotypes

Analysis of the CNS compartment showed a significant decrease in the total number of CD4 T cells, B cells, macrophages and dendritic cells after treatments (Fig. 6A). The decline was particularly evident in the CNS of animals treated with the combination of melatonin and methylprednisolone that showed a synergistic effect compared to the monotherapies. In fact, the number of CD4 T lymphocytes, B cells, macrophages and dendritic cells in the CNS of animals in the Mel + MPD160 group was marginal compared to the control group. Although the total number of CD8 T lymphocytes was not significantly modified by the treatments, a bias toward a CD8 regulatory response was observed in mice treated with melatonin alone or in combination with methylprednisolone (Fig. 6C). In the same line, the regulatory response of CD4 T and B cells were increased after treatments (Fig. 6B and D) compared to the effector responses.

3.7. Melatonin treatment from the onset of clinical signs improves the disability of animals in a subsequent relapse

Melatonin not only potentiated the effect of methylprednisolone on reducing the severity of EAE but also the chronic melatonin treatment from the onset of EAE significantly reduced the disability of mice after a subsequent relapse compared to the first neuroinflammatory episode (Fig. 7A–E). In fact, animals treated with melatonin from the onset of clinical signs in a first immunization had significantly less disability in a second immunization compared to the control group and the group in which melatonin was removed from the second induction of the disease (Mel removed). This effect was particularly pronounced in the Mel + MPD160 group (Fig. 7B–E).

4. Discussion

This study demonstrates for the first time the protective synergistic effect of co-treatment with melatonin and methylprednisolone on reducing the severity of EAE neuroinflammation by decreasing CD4 lymphocytes, B cells, macrophages and dendritic cells in the CNS, as well as modulating the infiltrated population of T and B cells toward regulatory phenotypes to the detriment of pro-inflammatory effector functions. In addition to the potentiation of the protective role of methylprednisolone, melatonin treatment from the clinical onset of EAE improves the natural history of the disease and the response to a subsequent treatment with methylprednisolone in a later neuroinflammatory relapse of the disease.

In the present study, we show that mice treated with melatonin from the day of immunization have milder disease than control animals. This result agrees with previous studies carried out by our group [19] and others [50,53,57,58]. Methylprednisolone treatment administered for 5 consecutive days from the onset of symptoms also reduced the severity of EAE in a dose-dependent manner with the most potent effect at 160 mg/kg/day, equivalent to 1g/day in humans, according to Ref. [59]. This treatment regimen resembles the standard first-line treatment for MS relapses consisting of 1g/day of intravenous or oral methylprednisolone for 3–5 consecutive days [40]. Interestingly, co-treatment with melatonin and methylprednisolone at 40 mg/kg reduced the severity of EAE signs in the same range as monotherapy with methylprednisolone at 160 mg/kg while the combination of melatonin and methylprednisolone (160 mg/kg) almost completely ameliorated the disease. To our knowledge, this is the first study to show that melatonin synergizes with methylprednisolone to alleviate EAE, pointing to the potential use of melatonin in combination with methylprednisolone for the treatment of relapses associated with MS.

Melatonin monotherapy or in combination with methylprednisolone reduced the infiltration of $CD45^{high}$ leukocytes into the CNS in a dose-dependent and synergistic manner. Although the protective role of melatonin associated with a decrease in inflammatory infiltrating cells in the CNS has previously been described in EAE [19] and rats [60], this is the first time that the potentiation of melatonin on the action of methylprednisolone on the control of leukocyte infiltration associated with relapses is described. The infiltrating cells were greatly reduced by the combination of melatonin and methylprednisolone, and of particular interest, the dose of methylprednisolone used was equivalent to that used in the clinical setting. Treatments not only reduced the frequency of $CD45^{high}$ cells in the CNS but also the percentage of $CD4^+$ T lymphocytes, macrophages, dendritic cells and B cells within the population of $CD45^{high}$ cells in a dose-dependent and synergistic way. In fact, co-treatment with melatonin and methylpredinisolone at a dose of 160 mg/kg reduced the frequency of $CD4^+$ T lymphocytes, macrophages, dendritic cells and B cells by more than 75 % compared to the control group.

In addition to the blockage that treatments exert on the infiltration of immune cells into the CNS, the combination of melatonin and methylprednisolone synergistically modulated the effector and regulatory capacity of infiltrating cells. Regarding $CD4^+$ cells, there is evidence of the restriction that melatonin exerts on the Th1 response both in mononuclear cells from patients with RR-MS [55] and in the EAE model [19,54,60]. A decrease in T_{EM} frequency in the CNS of EAE has also been described after melatonin treatment [19]. The present study not only agrees with these previous results, but also describes for the first time the synergistic effect of the combination of melatonin and methylprednisolone on the inhibition of Th1 cells (cells producing TNF and IFN-γ) both within the $CD4^+$ cells population and $CD4^+$ T_{EM} cells. Given that Th1 are the main population of T cells involved in MS [61,62], while T_{EM} are the most encephalitogenic subset when transferred to naïve animals [63], these results indicate that combined treatment targets key populations involved in the pathogenesis of MS and EAE. In addition to cytokines production, numerous immune checkpoint pathways regulate the activity of T cells. In this line, co-inhibitory molecules such as CTLA-4 and PD-1 have been shown to be essential for the control of autoimmune responses in MS [20,64]. In fact, RRMS patients have significantly lower expression of CTLA-4 and PD-1 compared to healthy controls [65,66]. Our results show for the first time an increase in the frequency of $CD4^+$ T lymphocytes expressing CTLA-4 and PD-1, in particular in the CNS of animals treated with the combination of melatonin and methylprednisolone, which could in turn be related to the increase in $CD4^+$ cells producing IL-10 observed both after monotherapy with melatonin and the combination of melatonin and methylprednisolone. This idea is supported by He et al. who showed that deletion of PD-1 in $CD4^+$ T cells induces decreased production of IL-10 in a murine model of spinal cord injury [67].

A synergistic effect of melatonin and methylprednisolone on the increase of $CD4^+$ cells expressing the pro-apoptotic factor Fas was also observed. The role of apoptotic processes in MS has been well documented, being responsible for the removal of autoreactive lymphocytes [68]. In fact, increased expression of Fas and FasL mRNA in PBMC of MS patients is associated with slower progression of the disease [69]. In addition, $CD4^+Fas^+$ cells could contribute to the increase in IL-10 production given that cells induced to undergo Fas-mediated apoptosis produce a significant amount of IL-10 [70].

Interestingly, significant enrichment in Treg cells was shown in animals treated with melatonin, but not in co-treated animals. Although previous studies have shown the positive influence of melatonin on the Treg response in the context of EAE [19,57] and MS [55], the effect of glucocorticoids on Treg cells is unclear. On the one hand, a significant increase in Treg from PBMC of MS patients has been demonstrated after intravenous methylprednisolone administration for two days [71]. On the other hand, the number of Treg decreased in PBMC after five days of intravenous methylprednisolone treatment [72] and in EAE mice splenocytes by three consecutive days of treatment with glucocorticoids

[73]. Although further studies are needed to clarify the effects of glucocorticoids on Treg cells in MS, our data show a positive influence of co-treatment with melatonin and methylprednisolone on the regulatory response mediated by $CD4^+IL10^+$ cells.

$CD8^+$ T lymphocytes can also be found in MS lesions and trigger the death of oligodendrocytes altering the myelin repair process [74]. Although several DMT have previously been reported to modulate effector $CD8^+$ T cells [75,76], here we describe for the first time an effect of the combination of melatonin and methylprednisolone on the decrease of TNF- and IFN-γ-producing $CD8^+$ effector cells in EAE while co-treatment synergistically increases the regulatory capacity of $CD8^+$ cells by enhancing PD1 expression and IL-10 production. In the context of MS, the proportion of PD-1^+ $CD8^+$ T cells in the CSF correlates with a good response to steroids during the acute phase of the disease whereas IFN-β treatment induces PD-1 expression and IL-10 production [77]. Although there is no evidence on the effect of melatonin on $CD8^+$ T cells related to MS, melatonin increases the frequency of $CD8^+IL\text{-}10^+$ cells in the spleen of rats during Trypanosoma cruzi infection [78].

Here we report for the first time that both melatonin and methylprednisolone decrease the percentage of plasmablasts and plasma cells in the CNS. To the best of our knowledge, no study has reported the effect of melatonin on B cells in the context of MS/EAE while only one study showed that acute administration of corticosteroids reduces the rate of CNS IgG synthesis without modifications in CSF IgG oligoclonal bands [79]. Moreover, melatonin and methylprednisolone not only decreased the frequency of TNF-producing B cells but also act synergistically to enhance the proportion of Breg cells ($CD19^+IL\text{-}10^+$), which supports the beneficial effect of the combined treatment, since B cells from MS patients produce abnormally higher levels of TNF [80] and Breg are involved in axonal repair tasks and neuroinflammation control [81,82] promoting the resolution of the disease by inducing an anti-inflammatory environment such as IL-10 production [38,83]. Although a previous study has described the role of plasma cells in the production of anti-inflammatory cytokines in various contexts in mice [84], the literature shows that the greatest contribution of these cells to EAE or MS is due to their ability to secrete antibodies directed to myelin components [35,85,86]. In fact, a previous study has shown that antibodies again MOG are highly effective in the opsonisation process of mouse myelin debris [87] and have been associated with the relapse phase of EAE [88]. Furthermore, antigen presentation and activation of cognate T cells by B cells may also be important for pathogenesis [14].

Taking into account the total number of cells infiltrating the CNS, the main population is $CD4^+$ T lymphocytes, followed by $CD8^+$ T lymphocytes and macrophages, while B and dendritic cells are the minority. These results are consistent with previous results showing the leading role of $CD4^+$ T lymphocytes in the development of EAE [89]. This study shows for the first time the synergistic effect of melatonin in combination with methylprednisolone on decreasing the number of effector $CD4^+$ and B cells, as well as macrophages and dendritic cells that infiltrate the CNS of EAE animals. The main differences are found in $CD4^+$ cells and, in particular, in their effector function, significantly reduced by treatments, especially Mel + MPD160. Furthermore, regulatory populations increased in the context of CD4, CD8 and B cells in the CNS of mice treated with melatonin, both monotherapy and in combination with methylprednisolone. Note that although the Treg/Teffector ratio in CD4+T cells is higher after treatments, it is not as high as in the CD8 and B cell compartments. Further studies are needed to elucidate the mechanisms by which melatonin does not significantly affect CD8 effector cells, but increases CD8 regulatory cells.

Therefore, combined therapy not only restricts the entry of effector cells into the CNS but also promotes an anti-inflammatory environment in the CNS that supports the almost complete abrogation of signs of EAE in animals treated with melatonin and methylprednisolone at 160 mg/kg.

To evaluate the combined therapy in a more clinical context, melatonin was administered from the onset of clinical signs (rather than from the day of immunization) and once the animals were fully recovered from the first neuroinflammatory episode, the animals were subjected to a second immunization to study the long-term effect of treatments. Interestingly, melatonin monotherapy and in combination with methylprednisolone significantly improved the clinical score of EAE of the second induction compared to the first one. Although our previous results have shown the beneficial effects on EAE of melatonin administration from the onset of clinical signs [27,50,52,53,56,58], this is the first description of the effect of melatonin (alone and in combination with methylprednisolone) on the natural history of the disease, which is of special relevance as a potential therapeutic tool.

Even though the exact mechanism by which melatonin ameliorates EAE remains to be further elucidated, previous studies have shown that the draining lymph node cells of melatonin-treated mice have a reduced proliferative response after the recall response to $MOG_{35–55}$ [27]. Splenic $CD4^+$ T cells from melatonin-treated mice have also shown decreased proliferation to $MOG_{35–55}$ [52,56]. Fewer Th1 and Th17 encephalitogenic T cells have also been described in the draining lymphoid nodes and spleen, as well as an increase in Treg and Tr1 regulatory cells, in EAE mice treated with melatonin [27,52,56]. In addition, draining $CD4^+$ T effector memory cells from melatonin-treated mice have a reduced surface expression of CD44 on day 10 after immunization [27]. Note that the most studied function of CD44 is the recruitment of T lymphocytes to inflamed sites, including a special role in the adhesion of T cells to the endothelium [90]. Therefore, melatonin could prevent T cells from accessing the brain parenchyma. This fact is also in line with histological studies showing a mild infiltration in the spinal cord of melatonin-treated animals, confined to the subarachnoid space, compared to the extensive infiltration of the group without treatment [27]. To clarify these aspects and given the importance of $CD4^+$ cells in the immunopathology of EAE, the effects of melatonin on the priming of $CD4^+$ T cells in the periphery, trafficking, and/or reactivation in the CNS were interrogated. Melatonin reduced priming in the periphery, as evidenced by the significant decrease in the number of $CD4^+$ cells in the draining lymph nodes on days 5 and 10 after EAE induction. In line with this, melatonin reduced $CD4^+$ cell activation by upregulating PD-1 levels on days 5 and 10 after induction, while it impaired migration by downregulating VLA-4 levels on day 5 after induction in the lymph nodes. According to the reduced trafficking capacity, $CD4^+$ cells from melatonin-treated mice had reduced levels of CCR7 and LFA-1 on both days 10 and 15 after induction. Additionally, $CD4^+$ cells from the melatonin group were less prompt for reactivation in the CNS due to reduced levels of CD28 and CD40L, key molecules in the immune synapse. Interestingly, mRNA levels of CCL19, VCAM-1 and ICAM-1, ligands of CCR7, VLA-4 and LFA-1, respectively, were significantly down-regulated in the CNS of animals treated with melatonin. Taken together, these data suggest that melatonin acts as a modulator of priming, trafficking and reactivation of $CD4^+$ cells in the CNS, resulting in reduced infiltration of $CD4^+$ cells both at the onset of the symptoms (day 10) and at the peak of the disease (day 15). The fact that CCL19 plays a role in the trafficking of immune cells into MS lesions [91] and in neuroinflammation in EAE [92] also suggests that melatonin affects the trafficking of effector cells into the CNS. These data are consistent with known reductions of CCL20 and ICAM-1 in the CNS of EAE mice treated with melatonin [52,60].

In addition, the in vitro effect of melatonin on $CD4^+$ T cell priming has previously been described. On the one hand, melatonin modulates the in vitro differentiation of EAE mice and human Th17 and Tr1 cells. On the other hand, melatonin suppresses the in vitro activation of naive 2D2+ transgenic T cells with $MOG_{35–55}$ both by dendritic cells and by anti-CD3 and anti-CD28 [56]. Moreover, the in vitro anti-inflammatory effects of melatonin have also been described in peripheral lymphocytes of MS and systemic lupus erythematous patients [93,94].

Although the effect of melatonin on $CD4^+$ T cell priming as responsible for reduced immune cell infiltration into the CNS cannot be ruled out, the direct role of melatonin in the CNS of EAE mice has also been

described in the present and other studies [27,52,56]. In fact, a previous study has shown that daily treatment with melatonin would reflect the additive effect of the prophylactic actions of melatonin (administered from the day of immunization, which primarily acts on the periphery) and the therapeutic actions of melatonin (administered from the clinical onset of EAE, with more emphasis on the direct effects on the CNS) [27]. In this line, the present study also shows that animals treated with melatonin from the onset of clinical signs, when infiltrated cells are already found in the CNS [27], have significantly less disability, also indicating a direct role of melatonin in the pathogenic infiltrated cells.

5. Conclusions

This study is the first to show the protective effect of co-treatment with melatonin and methylprednisolone on reducing the severity of EAE by preventing access to the CNS of CD4 lymphocytes, B cells, macrophages and dendritic cells, as well as modulating infiltrated T and B cell populations toward regulatory phenotypes to the detriment of pro-inflammatory effector functions. Moreover, treatment with melatonin from the clinical onset of EAE improves the natural course of the disease and the response to a subsequent treatment with methylprednisolone in a later relapse of the disease, highlighting melatonin as a potential therapeutic tool in combination with methylprednisolone for the treatment of relapses in MS and a possible strategy to reduce the dose of methylprednisolone in patients who have a certain resistance or toxicity to the use of corticosteroids.

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Declaration of competing interest

The authors have declared that no competing interests exist.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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