International Immunopharmacology 75 (2019) 105809

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Therapeutic treatment with Modafinil decreases the severity of experimental autoimmune encephalomyelitis in mice

Wesley Nogueira Brandão[a], Monica Levy Andersen[b], João Palermo-Neto[c], Jean Pierre Peron[a], Adriano Zager[c,d,*]

[a] Neuroimmune Interactions Laboratory, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil
[b] Department of Psychobiology, Federal University of São Paulo (UNIFESP-EPM), São Paulo, Brazil
[c] Neuroimmunomodulation Research Group, Department of Pathology, School of Veterinary Medicine, University of São Paulo (USP), São Paulo, Brazil
[d] Division of Neurobiology, Department of Clinical and Experimental Medicine, Faculty of Medicine and Health Sciences, Linköping University, Linköping, Sweden

ARTICLE INFO

Keywords:
Modafinil
Experimental autoimmune encephalomyelitis (EAE)
Th1 immunity
Dopamine
Multiple sclerosis
Inflammation

ABSTRACT

The psychostimulant drug modafinil has been used for many years for the treatment of sleep disorders. Recent studies have indicated that modafinil has immunomodulatory properties in the central nervous system (CNS) and peripheral immune cells. Thus, our aim was to determine the effects of *in vivo* therapeutic treatment with modafinil on the severity of clinical symptoms and immune response during the acute phase of experimental autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis. Modafinil treatment, given after the onset of symptoms, resulted in an improvement of EAE symptoms and motor impairment, which was correlated with reduced cellular infiltrate and a decreased percentage of T helper (Th) 1 cells in the CNS. The spinal cord analysis revealed that modafinil treatment decreased interferon (IFN)-γ and interleukin (IL)-6 protein levels and down regulated genes related to Th1 immunity, such as IFN-γ and TBX21, without affecting Th17-related genes. Our research indicates that therapeutic modafinil treatment has anti-inflammatory properties in an EAE model by inhibiting brain Th1 response, and may be useful as adjuvant treatment for multiple sclerosis.

1. Introduction

Modafinil is a psychostimulant drug prescribed for the treatment of narcolepsy, excessive daytime sleepiness and obstructive sleep apnea [1,2]. Clinical and pre-clinical trials indicate that modafinil antagonizes the dopamine transporter (DAT), consequently increasing extracellular dopamine levels in the striatum [3] and nucleus accumbens [4]. Recent accumulating evidence suggests that modafinil has an immunomodulatory effect following *in vitro* [5] and *in vivo* treatment [6,7], as well as neuroprotective effects in an animal model of Parkinson's disease [8–10].

Our research group has conducted a series of experiments to further study the immunomodulatory effect of modafinil in *in vivo* and *in vitro* models. *In vivo* modafinil treatment prevented the motor impairment and sickness behaviors induced by systemic lipopolysaccharide (LPS) administration in mice, which are partially reversed by blockage of the dopaminergic D1 receptor, indicating a role for dopamine in these effects [11]. *In vitro* and *in vivo* modafinil treatment was shown to increase interferon (IFN)-mediated immunity in peripheral immune cells of mice and humans [12]. Collectively, the data suggest that modafinil treatment has a dual immunomodulatory effect depending on the target organ, being anti-inflammatory in the central nervous system (CNS), and pro-inflammatory in peripheral immune cells.

Modafinil has also been shown to reduce the fatigue and motor impairment related to multiple sclerosis (MS) [13,14]. However, no study has yet addressed the question of whether this improvement in motor function in MS is caused by the psychostimulant effect *per se*, or is related to modafinil's immunomodulatory properties. Given that current treatments for MS are mainly immunosuppressive drugs, which have a number of side effects, it is essential to find new therapeutic strategies that are limited to the CNS, which is the primary target of MS autoimmunity. Thus, the aim of the present study is to determine the effects of therapeutic *in vivo* treatment with modafinil on the severity of clinical symptoms and immune response during the acute phase of experimental autoimmune encephalomyelitis (EAE), an experimental model of MS.

* Corresponding author at: Division of Neurobiology, Department of Clinical and Experimental Medicine, Faculty of Medicine and Health Sciences, Linköping University, 581 85 Linköping, Sweden.
E-mail addresses: adrianozager@hotmail.com, adriano.zager@liu.se (A. Zager).

https://doi.org/10.1016/j.intimp.2019.105809
Received 14 November 2018; Received in revised form 9 July 2019; Accepted 4 August 2019
Available online 16 August 2019

2. Methods

2.1. Animals

Adult (8 weeks old) female C57BL/6J mice from our own colony, weighing 20–30 g each, were used. The animals were housed in standard polypropylene cages at a controlled room temperature (22 ± 2 °C) and humidity level (65–70%), with artificial lighting (12 h light/12 h dark cycle) and with free access to Nuvilab® rodent chow (Nuvital, São Paulo, Brazil) and filtered water. Sterilized and residue-free wood shavings were used as animal bedding.

2.2. Induction of EAE and observation of symptoms

Mice were immunized subcutaneously at the base of the tail with 150 μg of $MOG_{35–55}$ (Proteimax, Brazil) emulsified in complete Freund's adjuvant (CFA *v/v*; Sigma) containing 1 mg of *M. Tuberculosis* (BD Biosciences). The mice also received 2 doses of 200 ng of *Bordetella pertussis* toxin intraperitoneally, 0 and 48 h after immunization. Clinical signs and symptoms of EAE were scored daily in all mice, according to the following scale: 0- no disease, 1- limp tail, 2- weak/partially paralyzed hind legs, 3- completely paralyzed hind legs, 4- complete hind and partial front leg paralysis, 5- complete paralysis/death.

2.3. Treatments

Modafinil (Stavigile, Libbs) tablets were freshly macerated, diluted in β-cyclodextrin solution (2:1 drug:β-cyclodextrin), and intraperitoneally administered at the dose of 90 mg/kg. Animals were treated with vehicle or modafinil for 5 consecutive days, after the onset of EAE symptoms, from D12 to D16 post immunization (p.i.). The dose and treatment regimen were based on previous studies [6,7,11,12].

2.4. Open field test

On D15 p.i., mice were subjected to the open field test 30 min after treatment. Each mouse was individually placed in the center of the apparatus, and the total locomotor activity (distance traveled in centimeters) and mean velocity were automatically measured over a period of 5 min, as previously described [11]. Naive mice were used to determine the baseline locomotion.

2.5. Collection of immune cells from CNS and spleen and flow cytometry

At the peak of EAE clinical symptoms and after the last modafinil administration, 16 days after EAE immunization, a different set of mice were deeply anesthetized by intraperitoneal administration of ketamine/xylazine, and perfused transcardially with 10 ml of 0.9% saline. Their brains and spinal cords were excised, macerated and maintained in 4 ml of DMEM supplemented with 2.5% collagenase D (Roche) at 37 °C in a 5% CO_2 incubator. 45 min later, suspensions of cells were rinsed in DMEM, passed through a cell strainer (70 μm) and centrifuged at 450*g* for 5 min at 4 °C. Then, the cells were resuspended in Percoll 25% and gently laid over Percoll 75% in tubes of 15 ml. The tubes were centrifuged at 950*g* for 20 min with the centrifuge breaks turned off. The ring containing mononuclear cells was collected, rinsed in DMEM and centrifuged at 450*g* for 5 min. The cells were then incubated with the antibodies CD86 (FITC), CD45 (PercP) and CD11b (PE) (Biolegend, CA, USA). An additional set of brain mononuclear cells were stimulated *ex vivo* with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) and incubated for 72 h, followed by phorbol myristate acetate (PMA; 50 ng/ml), Ionomycin (1 μg/ml) and brefeldin (10 μg/ml) for 4 h. Cells were rinsed, fixed and permeabilized (Cytofix/Cytoperm kit, BD Biosciences), and then incubated with the antibodies CD4 (FITC) and IFN-γ (APC) (Biolegend, CA, USA).

Each spleen was collected, mechanically dissociated using a cell strainer (70 μm) and homogenized in 5 ml of sterile DMEM. This suspension was centrifuged at 450*g* for 5 min and resuspended in 9 ml of sterile ammonium chloride to lyse erythrocytes, following which the solution was centrifuged again. The cells were then rinsed in DMEM and incubated with the antibodies CD86 (PercP), CD11b (PE), Ia^b (MHC II, FITC), or with CD4 (FITC) and CD8 (PE) (Biolegend, CA, USA).

The single cells were immunophenotyped by analyzing the frequency and intensity of fluorescence markers in a FACS Accuri flow cytometer (BD Immunocytometry Systems, San Jose, USA). Microglia were differentiated from macrophages based on the levels of CD45 surface expression, from a gated population of $CD11b^+$ cells, based on previous studies [11,15,16]. Microglia were $CD11b^+/CD45^{low}$ and macrophages were $CD11b^+/CD45^{high}$.

2.6. Quantification of cytokines

The distal third part of the spinal cord was collected on D16 p.i. from a different set of EAE mice, and homogenized in Tris HCL buffer (5 μl/mg of tissue; 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0,02% NaN_3, 1% Triton X, and protease inhibitor), and then centrifuged at 17000 *g* for 30 min. Supernatants were analyzed for the concentrations of interleukin (IL)-2, IL-4, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF), IL-17 and IL-10 by flow cytometry using a mouse cytometric bead array (CBA) Th1/Th2/Th17 kit (BD Biosciences, USA) according to the manufacturer's instructions.

2.7. qPCR analysis of spinal cord

On D16 p.i., the distal third part of the spinal cord was collected and total RNA was isolated using the TRIzol® reagent (Invitrogen, USA). RNA (2 μg) was reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Invitrogen, USA). Real-time PCR was performed using the QuantStudio™ 3 (Applied Biosystems, USA) and the TaqMan Gene Expression Master Mix (Applied Biosystems, USA). The Actb primer was used to detect the housekeeping gene. Specific primers were used with Taqman Gene Expression Assay (Invitrogen, USA) and the genes and locus are indicated in Table S1. The relative gene expression data were analyzed by the 2-ΔΔCt.

2.8. Histology

The distal third part of the spinal cord was collected on D16 p.i., fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Spinal cord sections were cut at 5 μm on a microtome, and later processed for hematoxylin and eosin staining.

2.9. Statistical analysis

Data were analyzed using either unpaired Student *t*-test, one or two-way analysis of variance (ANOVA) followed by Tukey's correction for multiple comparisons. The results are expressed as the mean ± SEM and were analyzed using GraphPad Prism 6.0 software, and the level of statistical significance was set at $p \leq 0.05$.

3. Results

A previous study from our laboratory showed that modafinil treatment prevents inflammation-induced motor impairment and sickness symptoms, an effect that is correlated with the reduced activation of brain-derived monocytes and brain IL1-β expression [11]. We then decided to test whether therapeutic modafinil treatment affects the severity of symptoms of EAE, a functional disease model of multiple sclerosis. The results revealed a significant decrease in EAE symptoms in modafinil-treated mice from day 12 to 21 post immunization ($p \leq 0.05$ for D12; $p \leq 0.001$ from D13 to D21), as shown in Fig. 1A

[treatment factor F (1, 1073) = 176,3, $P < 0,0001$; time factor F (21, 1073) = 117,3, $p \leq 0.001$;

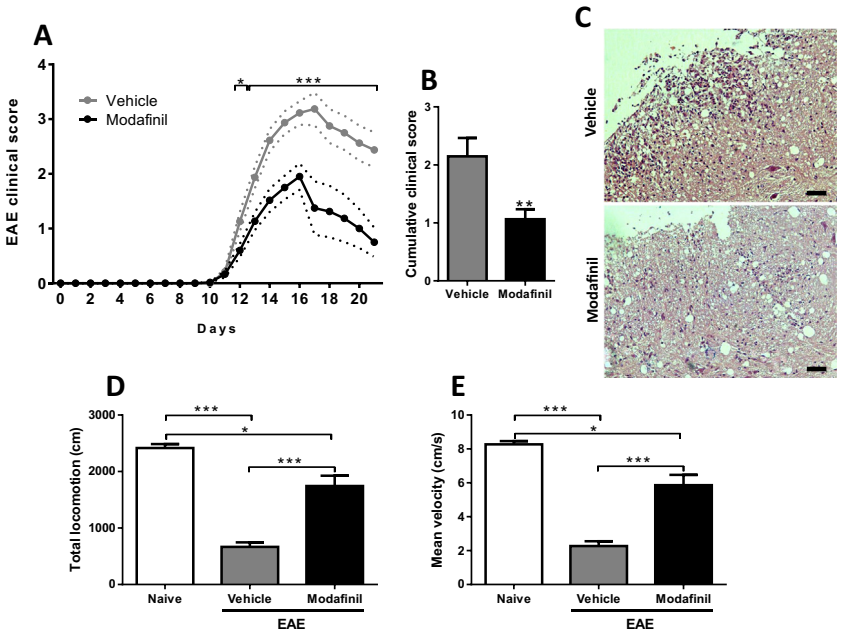

Fig. 1. Therapeutic modafinil treatment ameliorates the severity of clinical symptoms and motor impairment of EAE. Adult female mice were immunized with $MOG_{35–55}$ and, after the onset of EAE symptoms, treated with modafinil for 5 consecutive days. The figure presents the mean values (± SEM) of (A) EAE clinical score and (B) cumulative score from D10 to D21 (n = 31/group, derived from 4 independent experiments). (C) H&E staining in spinal cords shows reduced cell infiltrate in modafinil-treated mice (scale bar = 50 μm). In addition, the figure shows mean values of (D) total locomotion and (E) mean velocity in the open field test on D15 (n = 10/group). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

treatment x time F (21, 1073) = 10,36, p ≤ 0.001]. The cumulative disease score from D10 to D21 further shows the effect of modafinil in reducing EAE clinical symptoms (Fig. 1B; $p \leq 0.01$). The hematoxylin and eosin staining of spinal cords of EAE mice revealed a reduction in the cellular infiltrate in modafinil-treated mice compared to mice treated with vehicle (Fig. 1C).

Given the preventive effect of modafinil on limb paralysis induced by EAE, we next studied motor impairment using a behavioral task, the open field test, during the peak of EAE symptoms, on D15 post EAE immunization. The open field data revealed a decrease in locomotion (Fig. 1D) and mean velocity (Fig. 1E) in EAE mice treated with vehicle ($p \leq 0.001$), compared to naive mice, which was partially prevented by modafinil treatment ($p \leq 0.001$) [Locomotion F (2, 19) = 26.45, $p \leq 0.001$; and Mean velocity F (2, 19) = 26.92, $p \leq 0.001$].

We next aimed to study whether the decrease in EAE symptoms caused by modafinil treatment was related to an improvement in fatigue and general motor stimulation or was caused by a diminished CNS immune response. To do this, we analyzed the CNS-derived immune cells and how they are affected by modafinil treatment in EAE mice. The flow cytometry data revealed a significant reduction in the absolute number of immune cells in the CNS of mice treated with modafinil, compared to vehicle-treated animals ($p \leq 0.05$; Fig. 2B). Additional analysis revealed that modafinil treatment resulted in a significant reduction in the percentage of $CD4^+IFN\text{-}\gamma^+$ cells ($p \leq 0.01$, Fig. 2C) and decreased intracellular expression of IFN-γ by $CD4^+$ cells ($p \leq 0.001$; Fig. 2D). However, we found no significant differences in the percentages of $CD11b^+$, $CD11b^+CD86^+$ cells, and for the mean fluorescence intensity of CD86 in $CD11b^+$ cells ($p > 0.05$), as shown in Fig. 2E to 2G. In addition, modafinil treatment did not affect the percentages of $CD11b^+CD45^{high}$ (Fig. 2H) and $CD11b^+CD45^{low}$ cells (Fig. 2I), as well as the percentage of $CD11b^+CD45^{high}$ and $CD11b^+CD45^{low}$ cells expressing CD86 (Fig. 2J) and the mean fluorescence intensity of CD86 in each of these subsets (Fig. 2K).

Modafinil treatment also decreased the levels of IFN-γ ($p \leq 0.05$) and IL-6 ($p \leq 0.05$); and showed a tendency to reduce TNF levels ($p = 0.06$), as revealed by the cytokine analysis in spinal cord homogenates (Fig. 3A to 3C). The levels of IL-2, IL-4, IL-17 and IL-10 were not detectable by the current methodology.

We then studied the expression of immune-related genes in the spinal cord of EAE mice. The qPCR analysis revealed that modafinil treatment significantly reduced mRNA levels of genes related to Th1 immunity, such as IFN-γ ($p \leq 0.01$; Fig. 4A) and T-box transcription factor 21 (TBX21; $p \leq 0.001$; Fig. 4B). Regarding genes related to Th17 immunity, modafinil treatment did not affect mRNA levels of IL-17 (Fig. 4C) and RAR-related orphan receptor gamma t (ROR-γt; Fig. 4D), whereas it increased mRNA levels of IL-23 ($p \leq 0.01$; Fig. 4E). Modafinil treatment also decreased mRNA levels of nitric oxide synthase 2 (Nos2; $p \leq 0.05$; Fig. 4F), without affecting other innate immunity genes, such as conserved helix-loop-helix ubiquitous kinase (Chuk; Fig. 4G) and IL-6 (Fig. 4H). Additionally, regulatory T cell genes, such as IL-10 ($p \leq 0.01$; Fig. 4I), TGF-β ($p \leq 0.001$; Fig. 4J) and FoxP3 ($p \leq 0.01$; Fig. 4K) were reduced by modafinil treatment.

Considering the stimulatory effect of modafinil treatment in spleen cells reported previously [12], we also tested how modafinil treatment would affect splenic immune cells during EAE. Modafinil treatment resulted in an increase in the absolute number of immune cells (Fig. 5B), although it was not statistically significant ($p = 0.07$), as well as a decrease in the percentage of splenic T $CD4^+$ cells ($p \leq 0.05$; Fig. 5C), without changing the percentage of T $CD8^+$ cells ($p > 0.05$; Fig. 5D). Modafinil treatment did not affect the absolute number of $CD11b^+$ cells ($p > 0.05$; Fig. 5E) and the percentage of $CD11b^+$ cells ($p > 0.05$; Fig. 5F). In addition, modafinil increased the percentages of $CD11b^+CD86^+$ cells ($p \leq 0.05$; Fig. 5G) and $CD11b^+$MHC II^+ cells ($p \leq 0.01$; Fig. 5H), as well as the mean fluorescence intensity of CD86 and MHC II in $CD11b^+$ cells ($p \leq 0.001$; Fig. 5I and J) in the spleen of EAE mice.

4. Discussion

We conducted *in vivo* treatment with modafinil after the onset of EAE symptoms to study the therapeutic potential of the drug. Our results showed that therapeutic modafinil treatment decreased the severity of symptoms and motor impairment in experimental autoimmune encephalomyelitis (EAE). This improvement is related to a reduction in Th1 immunity in the CNS during the acute phase of the disease. MS and its experimental model EAE are characterized by an autoimmune response against myelin driven by autoreactive T cells, causing demyelination, axon degradation and motor impairment [15,17–20]. The disease is initiated in the peripheral immune organs, such as the spleen and draining lymph nodes, but it culminates in the CNS, the target organ, with infiltration of monocytes and autoreactive T cells and subsequent glial activation. We have previously shown that

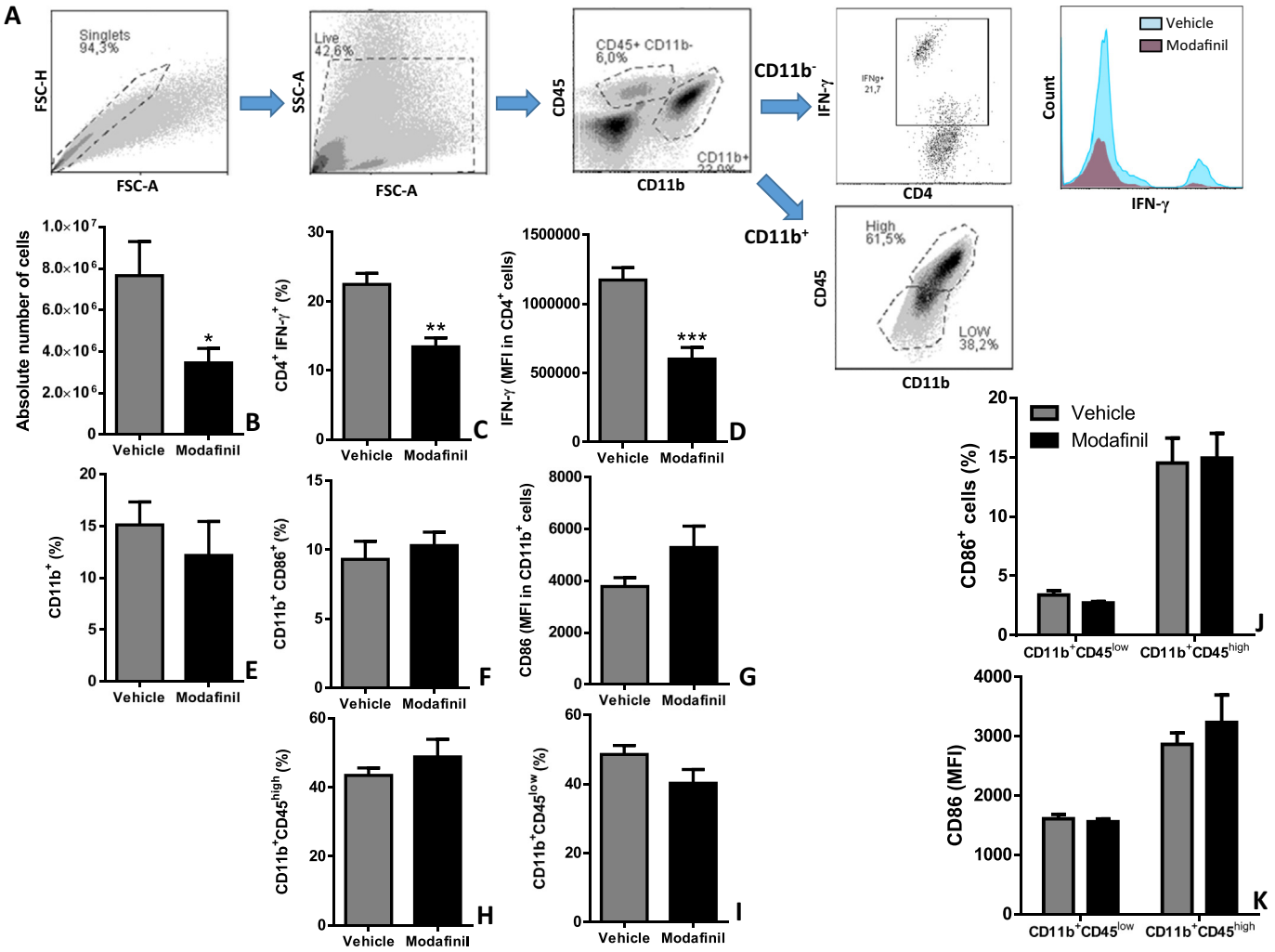

Fig. 2. Therapeutic modafinil treatment reduces inflammatory cell infiltrate and Th1 cells in the CNS of EAE mice. Adult female mice (n = 8/group) were immunized with $MOG_{35–55}$ and, after the onset of EAE symptoms, treated with modafinil for 5 consecutive days. Following the last drug treatment, all mice were subjected to collection of brain and spinal cord and isolation of mononuclear cells, followed by flow cytometry. The figure presents (A) the gating strategy and the mean values (± SEM) of: (B) absolute number of CNS immune cells, (C) percentage of $CD4^+IFN\text{-}\gamma^+$ cells and (D) mean fluorescence intensity of IFN-γ by $CD4^+$ cells, as well as percentages of (E) $CD11b^+$ cells, (F) $CD11b^+CD86^+$ cells, and (G) mean fluorescence intensity of CD86 by $CD11b^+$ cells. In addition, the figure shows the percentages of (H) $CD11b^+CD45^{high}$ cells and (I) $CD11b^+CD45^{low}$ cells; as well as (J) the percentages of $CD11b^+CD45^{high}$ and $CD11b^+CD45^{low}$ cells expressing CD86 and (K) the mean fluorescence intensity of CD86 in each of these subsets. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

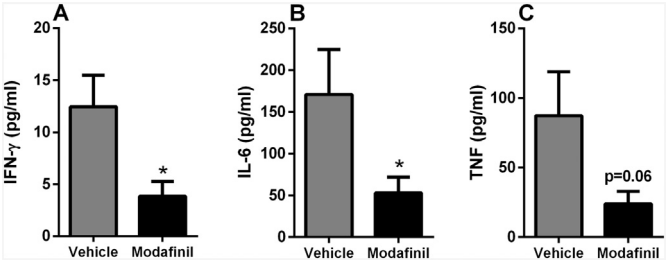

Fig. 3. Therapeutic modafinil treatment decreases IFN-γ and IL-6 protein levels in the spinal cord of EAE mice. Adult female mice (n = 7/group) were immunized with $MOG_{35–55}$ and, after the onset of EAE symptoms, treated with modafinil for 5 consecutive days. At the end of treatment, all mice were subjected to collection of spinal cord and further cytokine analysis. The figure presents the mean values (± SEM) of (A) IFN-γ, (B) IL-6 and (C) TNF. * $p \leq 0.05$.

modafinil has a dual effect on immune-inflammatory response: it is anti-inflammatory in neuroinflammation models, such as those induced by LPS [11] and methamphetamine [6,7], whereas it increases interferon-mediated immunity in peripheral immune cells of mice and humans [12]. For these reasons, we decided to study the effects of modafinil in a functional disease model, EAE.

Our data revealed that modafinil administered daily from 12 to 16 days post immunization significantly reduced the severity of symptoms of EAE. This effect was evident from D12 p.i. to the end of the observation, despite the fact that the daily treatment had been terminated on D16. The open field analysis indicated that the motor impairment caused by EAE was partially prevented by modafinil treatment, revealing that modafinil improves not only limb paralysis, but also the impairment of motor function and exploratory behavior caused by EAE. These findings are in line with a recent study which revealed that a single modafinil treatment in rats improved catalepsy, or tonic immobility, in a 6-hydroxydopamine (6-OHDA)-induced rat model of Parkinson's disease. [21].

The histological analysis showed that modafinil treatment drastically reduced the cell infiltrate in the spinal cord of EAE mice. The characterization of CNS immune cells by flow cytometry further confirmed that modafinil reduced the number of immune cells in the CNS of EAE animals, comprising infiltrating monocytes, neutrophils, T cells, perivascular macrophages, and microglia. Interestingly, the percentage

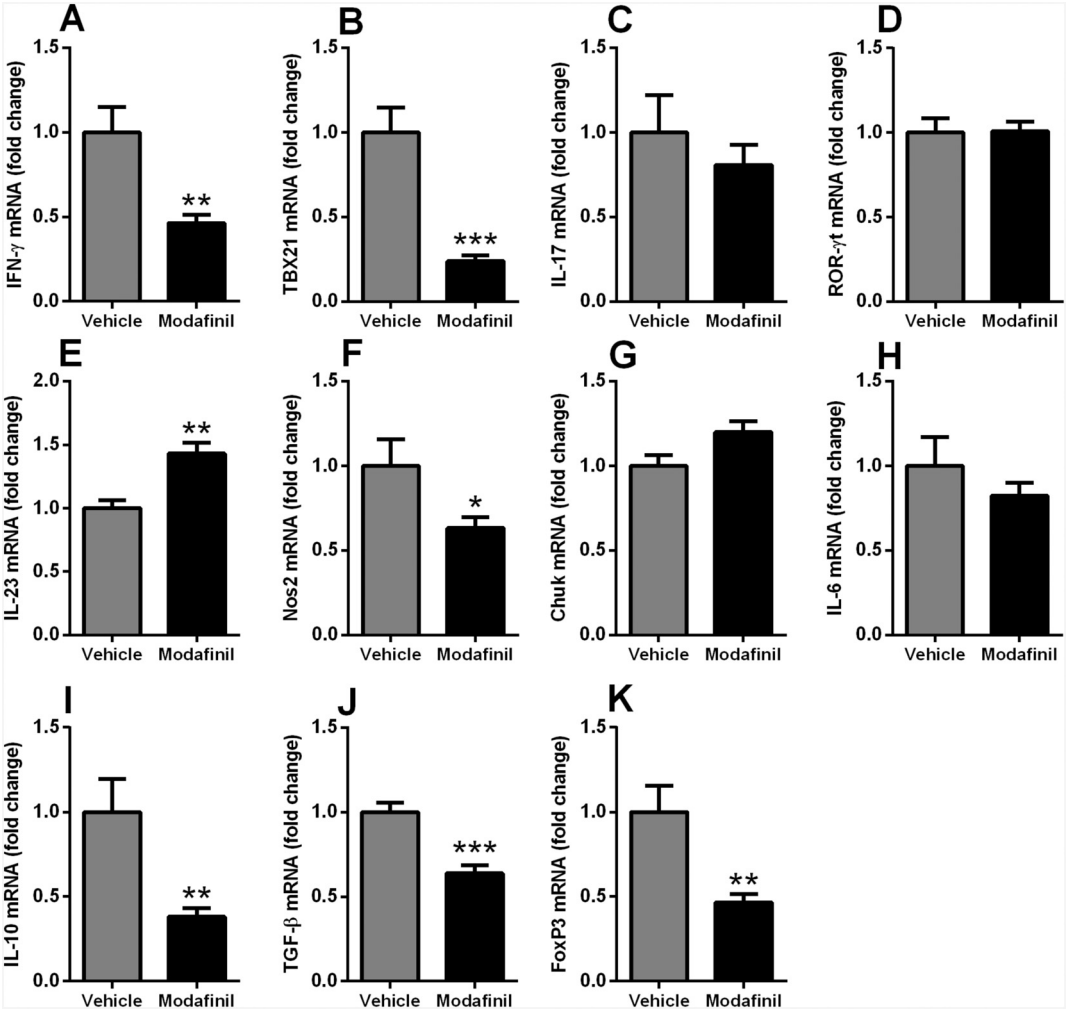

Fig. 4. Therapeutic modafinil treatment affects T helper-related genes in the spinal cord of EAE mice. Adult female mice (n = 5–7/group) were immunized with $MOG_{35–55}$ and, after the onset of EAE symptoms, treated with modafinil for 5 consecutive days. At the end of treatment, all mice were subjected to collection of spinal cord and further qPCR analysis. The figure presents the mean values (± SEM) of (A) IFN-γ, (B) TBX21, (C) IL-17, (D) ROR-γt, (E) IL-23, (F) Nos2, (G) Chuk, (H) IL-6, (I) IL-10, (J) TGF-β and (K) FoxP3 mRNA levels. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

of T helper cells ($CD4^+$ cells) expressing IFN-γ, namely Th1 cells, as well as the intracellular expression of IFN-γ by $CD4^+$ cells, were decreased in the CNS of modafinil-treated animals. This reduction was not accompanied by changes in the percentages of $CD11b^+$ cells, $CD11b^+CD86^+$ cells, $CD11b^+CD45^{high}$ cells (infiltrating monocytes and neutrophils, resident macrophages) and $CD11b^+CD45^{low}$ cells (microglia), as well as in $CD11b^+CD45^{high}$ and $CD11b^+CD45^{low}$ cells expressing the co-stimulatory molecule CD86.

The spinal cord levels of cytokines further demonstrated that modafinil treatment drastically decreased IFN-γ and IL-6 and showed a tendency, although not statistically significant, to decrease TNF levels, corroborating the data on Th1 cells. Our gene expression assay confirmed that modafinil specifically decreased expression of Th1-related genes such as IFN-γ and TBX21, in the spinal cord. The gene expression of IL-23, a cytokine pivotal to T cell differentiation into Th17 cells, was increased in the spinal cord following modafinil treatment. However, this increase seems to have no impact on the other Th17-related genes, IL-17 and ROR-γt, as well as on IL-17 protein levels, which were unaffected following modafinil treatment. Intriguingly, genes related to regulatory T cells were also down-regulated after modafinil treatment, suggesting that Treg cells are not responsible for the anti-inflammatory effect of modafinil. The collective data suggest that modafinil's effects in ameliorating EAE symptoms are mediated by T cells and specific to Th1 immunity, as revealed at both the protein and gene levels.

Dopaminergic agonists and antagonists have been used previously to study the role of dopamine on EAE progression. Although by distinct mechanisms, both dopamine agonists, such as pramipexole [22] and bromocriptine [23], and antagonists, such as SCH23390 [24], risperidone and clozapine [25,26], reduced the severity of symptoms and immune response in EAE mice. Although we cannot exclude the participation of other neurotransmitters that are also affected by modafinil, such as noradrenaline, the collective data suggest that the effects of modafinil on EAE may be related to dopamine [27]. In line with this hypothesis, previous studies have shown that depletion of central dopamine induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exacerbated the severity of EAE symptoms [28], whereas the monoamine oxidase (MAO) inhibitor phenelzine, which increases brain dopamine levels, decreased the severity of EAE [29].

Concomitantly, the analysis of immune cells in the spleen of EAE animals revealed that the percentage of T $CD4^+$ (T helper) cells was reduced in modafinil-treated animals, whereas the percentage of T $CD8^+$ (T cytotoxic) cells was unaffected. On the other hand, modafinil increased the percentage of $CD11b^+CD86^+$ and $CD11b^+$MHC II^+ cells, as well as the surface expression of CD86 and MHC class II, markers of activated $CD11b^+$ cells. However, we could not determine whether modafinil promoted changes in the M1/M2 polarization of activated macrophages in the current model, which would help to reveal whether the increased activation of $CD11b^+$ cells is related to classically

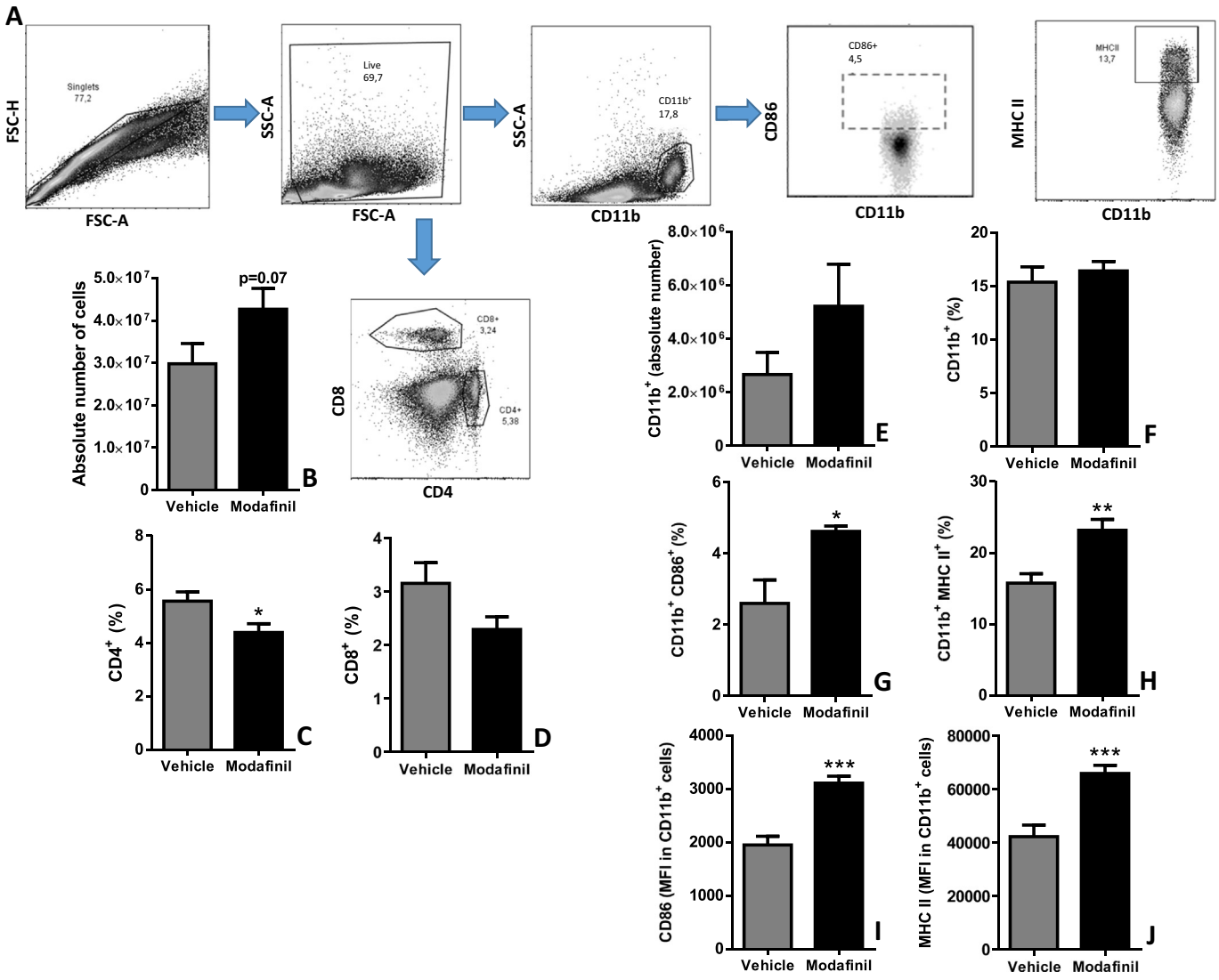

Fig. 5. Modafinil treatment increases activation of $CD11b^+$ cells in the spleen of EAE mice. Adult female mice (n = 10/group) were immunized with $MOG_{35–55}$ and, after the onset of EAE symptoms, treated with modafinil for 5 consecutive days. Following the last drug treatment, all mice were subjected to collection of spleen and isolation of cells, followed by flow cytometry. The figure presents (A) the gating strategy, and the mean values (± SEM) of (B) absolute number of immune cells, and percentages of (C) $CD4^+$ cells and (D) $CD8^+$ cells. The figure also illustrates the mean values of (E) absolute number of $CD11b^+$ cells, and the percentages of (F) $CD11b^+$ cells, (G) $CD11b^+CD86^+$ cells and (H) $CD11b^+MHC\ II^+$ cells, as well as the mean fluorescence intensity of (I) CD86 and (J) MHC II in $CD11b^+$ cells. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

activated (M1, pro-inflammatory) or alternatively activated (M2, anti-inflammatory) macrophages. These results therefore need to be interpreted with caution.

Although contradictory, the effects of modafinil on immune cells of the CNS *versus* spleen in EAE animals corroborate our previous findings indicating an anti-inflammatory effect in the CNS [11] and a pro-inflammatory effect in splenic immune cells [12]. It is possible to speculate that the effects of modafinil on neuroinflammatory models are mediated by the modafinil-induced increase in brain dopamine, which negatively affects the activation of immune cells, such as T cells and macrophages [30,31]. In this respect, a microdialysis study demonstrated that modafinil promotes a fast and significant increase in brain dopamine content in a dose dependent fashion [32]. This hypothesis is corroborated by a previous study that showed that the pharmacological blockage of dopaminergic D1 receptors prevents some of the modafinil-mediated effects in inflammation-induced sickness behaviors [11].

Taken together, the data presented here provides evidence that therapeutic modafinil treatment improves EAE symptoms, and this effect is correlated with the reduction in Th1 cells and Th1-related cytokines and genes in the CNS. To our knowledge, this is the first pre-clinical study that links the improvement in MS-related fatigue reported previously in modafinil-treated patients [13] to an anti-inflammatory effect of the drug. Our study, therefore, opens a new avenue of clinical research regarding the therapeutic effect of modafinil on MS and indicates that, in addition to improving fatigue, modafinil treatment has immunomodulatory properties and may be useful as adjuvant treatment for MS patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105809.

Funding

This work was supported by grants from the São Paulo State Foundation-FAPESP (Grants #2013/18921-5 to AZ and #2011/18703-2 and #2017/26170-0 to JPP).

Declaration of competing interest

None.

References

[1] H. Bastuji, M. Jouvet, Successful treatment of idiopathic hypersomnia and narcolepsy with modafinil, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 12 (5) (1988) 695–700.

[2] Y.C. Kuan, D. Wu, K.W. Huang, N.F. Chi, C.J. Hu, C.C. Chung, K.W. Tam, Y.H. Huang, Effects of modafinil and armodafinil in patients with obstructive sleep apnea: a meta-analysis of randomized controlled trials, Clin. Ther. 38 (4) (2016) 874–888.

[3] M.L. Andersen, E. Kessler, K.S. Murnane, J.C. McClung, S. Tufik, L.L. Howell, Dopamine transporter-related effects of modafinil in rhesus monkeys, Psychopharmacology 210 (3) (2010) 439–448.

[4] N.D. Volkow, J.S. Fowler, J. Logan, D. Alexoff, W. Zhu, F. Telang, G.J. Wang, M. Jayne, J.M. Hooker, C. Wong, B. Hubbard, P. Carter, D. Warner, P. King, C. Shea, Y. Xu, L. Muench, K. Apelskog-Torres, Effects of modafinil on dopamine and dopamine transporters in the male human brain: clinical implications, Jama 301 (11) (2009) 1148–1154.

[5] J.C. Jung, Y. Lee, J.Y. Son, E. Lim, M. Jung, S. Oh, Simple synthesis of modafinil derivatives and their anti-inflammatory activity, Molecules 17 (9) (2012) 10446–10458.

[6] M. Raineri, B. Gonzalez, B. Goitia, E. Garcia-Rill, I.N. Krasnova, J.L. Cadet, F.J. Urbano, V. Bisagno, Modafinil abrogates methamphetamine-induced neuroinflammation and apoptotic effects in the mouse striatum, PLoS One 7 (10) (2012) e46599.

[7] M. Raineri, V. Peskin, B. Goitia, I.R. Taravini, S. Giorgeri, F.J. Urbano, V. Bisagno, Attenuated methamphetamine induced neurotoxicity by modafinil administration in mice, Synapse 65 (10) (2011) 1087–1098.

[8] J.A. Aguirre, A. Cintra, J. Hillion, J.A. Narvaez, A. Jansson, T. Antonelli, L. Ferraro, F.A. Rambert, K. Fuxe, A stereological study on the neuroprotective actions of acute modafinil treatment on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced nigral lesions of the male black mouse, Neurosci. Lett. 275 (3) (1999) 215–218.

[9] K. Fuxe, A.M. Janson, L. Rosen, U.B. Finnman, S. Tanganelli, M. Morari, M. Goldstein, L.F. Agnati, Evidence for a protective action of the vigilance promoting drug modafinil on the MPTP-induced degeneration of the nigrostriatal dopamine neurons in the black mouse: an immunocytochemical and biochemical analysis, Exp. Brain Res. 88 (1) (1992) 117–130.

[10] Y.L. Xiao, J.M. Fu, Z. Dong, J.Q. Yang, F.X. Zeng, L.X. Zhu, B.C. He, Neuroprotective mechanism of modafinil on Parkinson disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Acta Pharmacol. Sin. 25 (3) (2004) 301–305.

[11] A. Zager, W.N. Brandao, R.O. Margatho, J.P. Peron, S. Tufik, M.L. Andersen, B.R. Kornum, J. Palermo-Neto, The wake-promoting drug Modafinil prevents motor impairment in sickness behavior induced by LPS in mice: role for dopaminergic D1 receptor, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 81 (2018) 468–476.

[12] A. Zager, W.N. Brandao, R.O. Margatho, D.S.G. Cruz, J.P. Peron, S. Tufik, M.L. Andersen, M. Moresco, F. Pizza, G. Plazzi, B.R. Kornum, J. Palermo-Neto, Increased interferon-mediated immunity following in vitro and in vivo Modafinil treatment on peripheral immune cells, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 81 (2018) 297–305.

[13] W.S. MacAllister, L.B. Krupp, Multiple sclerosis-related fatigue, Phys. Med. Rehabil. Clin. N. Am. 16 (2) (2005) 483–502.

[14] H. Shangyan, L. Kuiqing, X. Yumin, C. Jie, L. Weixiong, Meta-analysis of the efficacy of modafinil versus placebo in the treatment of multiple sclerosis fatigue, Mult Scler Relat Disord 19 (2018) 85–89.

[15] A. Zager, J.P. Peron, G. Mennecier, S.C. Rodrigues, T.P. Aloia, J. Palermo-Neto, Maternal immune activation in late gestation increases neuroinflammation and aggravates experimental autoimmune encephalomyelitis in the offspring, Brain Behav. Immun. 43 (2015) 159–171.

[16] D.B. McKim, M.D. Weber, A. Niraula, C.M. Sawicki, X. Liu, B.L. Jarrett, K. Ramirez-Chan, Y. Wang, R.M. Roeth, A.D. Sucaldito, C.G. Sobol, N. Quan, J.F. Sheridan, J.P. Godbout, Microglial recruitment of IL-1 beta-producing monocytes to brain endothelium causes stress-induced anxiety, Mol. Psychiatry 23 (6) (2018) 1421–1431.

[17] H. Neumann, Molecular mechanisms of axonal damage in inflammatory central nervous system diseases, Curr. Opin. Neurol. 16 (3) (2003) 267–273.

[18] M. Rodriguez, Effectors of demyelination and remyelination in the CNS: implications for multiple sclerosis, Brain Pathol. 17 (2) (2007) 219–229.

[19] D.A. Brown, P.E. Sawchenko, Time course and distribution of inflammatory and neurodegenerative events suggest structural bases for the pathogenesis of experimental autoimmune encephalomyelitis, J. Comp. Neurol. 502 (2) (2007) 236–260.

[20] A.C. Murphy, S.J. Lalor, M.A. Lynch, K.H. Mills, Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis, Brain Behav. Immun. 24 (4) (2010) 641–651.

[21] R. Vajdi-Hokmabad, M. Ziaee, S. Sadigh-Eteghad, S. Sandoghchian Shotorbani, J. Mahmoudi, Modafinil improves catalepsy in a rat 6-Hydroxydopamine model of Parkinson's disease; possible involvement of dopaminergic neurotransmission, Adv Pharm Bull 7 (3) (2017) 359–365.

[22] V. Lieberknecht, S.C. Junqueira, M.P. Cunha, T.A. Barbosa, L.F. de Souza, I.S. Coelho, A.R. Santos, A.L. Rodrigues, A.L. Dafre, R.C. Dutra, Pramipexole, a dopamine D2/D3 receptor-preferring agonist, prevents experimental autoimmune encephalomyelitis development in mice, Mol. Neurobiol. 54 (2) (2017) 1033–1045.

[23] P.N. Riskind, L. Massacesi, T.H. Doolittle, S.L. Hauser, The role of prolactin in autoimmune demyelination: suppression of experimental allergic encephalomyelitis by bromocriptine, Ann. Neurol. 29 (5) (1991) 542–547.

[24] K. Nakano, T. Higashi, K. Hashimoto, R. Takagi, Y. Tanaka, S. Matsushita, Antagonizing dopamine D1-like receptor inhibits Th17 cell differentiation: preventive and therapeutic effects on experimental autoimmune encephalomyelitis, Biochem. Biophys. Res. Commun. 373 (2) (2008) 286–291.

[25] D. O'Sullivan, L. Green, S. Stone, P. Zareie, M. Kharkrang, D. Fong, B. Connor, A.C. La Flamme, Treatment with the antipsychotic agent, risperidone, reduces disease severity in experimental autoimmune encephalomyelitis, PLoS One 9 (8) (2014) e104430.

[26] L.K. Green, P. Zareie, N. Templeton, R.A. Keyzers, B. Connor, A.C. La Flamme, Enhanced disease reduction using clozapine, an atypical antipsychotic agent, and glatiramer acetate combination therapy in experimental autoimmune encephalomyelitis, Mult Scler J Exp Transl Clin 3 (1) (2017) (p. 2055217317698724).

[27] M. Levite, F. Marino, M. Cosentino, Dopamine, T cells and multiple sclerosis (MS), J. Neural Transm. (Vienna) 124 (5) (2017) 525–542.

[28] E. Balkowiec-Iskra, I. Kurkowska-Jastrzebska, I. Joniec, A. Ciesielska, A. Muszynska, A. Przybylkowski, A. Czlonkowska, A. Czlonkowski, MPTP-induced central dopamine depletion exacerbates experimental autoimmune encephalomyelitis (EAE) in C57BL mice, Inflamm. Res. 56 (8) (2007) 311–317.

[29] C.A. Benson, G. Wong, G. Tenorio, G.B. Baker, B.J. Kerr, The MAO inhibitor phenelzine can improve functional outcomes in mice with established clinical signs in experimental autoimmune encephalomyelitis (EAE), Behav. Brain Res. 252 (2013) 302–311.

[30] W. Jiang, Y. Huang, F. He, J. Liu, M. Li, T. Sun, W. Ren, J. Hou, L. Zhu, Dopamine D1 receptor agonist A-68930 inhibits NLRP3 Inflammasome activation, controls inflammation, and alleviates histopathology in a rat model of spinal cord injury, Spine (Phila Pa 1976) 41 (6) (2016) E330–E334.

[31] Y. Yan, W. Jiang, L. Liu, X. Wang, C. Ding, Z. Tian, R. Zhou, Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome, Cell 160 (1–2) (2015) 62–73.

[32] L. Ferraro, S. Tanganelli, W.T. O'Connor, T. Antonelli, F. Rambert, K. Fuxe, The vigilance promoting drug modafinil increases dopamine release in the rat nucleus accumbens via the involvement of a local GABAergic mechanism, Eur. J. Pharmacol. 306 (1–3) (1996) 33–39.