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Effects of moderate- versus high- intensity swimming training on inflammatory and CD4$^+$ T cell subset profiles in experimental autoimmune encephalomyelitis mice

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory neurodegenerative disease of the central nervous system (CNS). Evidence about experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, has been shown to modulate disease parameters within exercise intervention. However, these initial studies weren't carried out intensity of exercise in mice. This study explored the impacts of different-intensity swimming training on EAE mice. Female mice were given access to swimming with predetermined weight (moderated-intensity (ME) group is 0% body weight; high-intensity (HE) group is 4% body weight) for 6 weeks, were immunized to induce EAE and then continued swimming until sacrificed. Compared to non-exercise mice, ME training didn't affect EAE clinical symptoms and neuropathology. However, HE swimming attenuated EAE clinical scores, reduced infiltrating cells and demyelination of spinal cords. Analysis of CD4$^+$ T cell subsets from CNS of EAE showed the reduction of Th1 and Th17 populations and an increase of Treg in HE, not ME mice. Accordingly, HE training lead to a decrease of IFN-γ and IL-17 and an increase of IL-10 and TGF-β. Of note, HE, not ME, swimming induced an increase of brain derived neurotrophic factor in the CNS of EAE. Moreover, HE training upregulated Treg and downregualted antigen-specific T cell proliferation and Th1 and Th17 populations from draining lymph node cells. These results suggest that HE swimming training might have benefits on attenuating the progression and pathological hallmarks of EAE, thus representing an important non-pharmacological intervention for improvement of chronic inflammation or T-cell mediated autoimmunity.

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating, infiltrating inflammatory disease of the central nervous system (CNS) and affects approximately 2.3 million people worldwide and 400,000 cases in the United States (Markowitz, 2013). MS is a heterogeneous disease and the predominantly clinical symptoms are sensory changes including myasthenic, fatigue, spasticity and lose the part of CNS function such as coordination problems when relapsed (Motl, 2013). Although there is currently no cure for MS, there are treatments available that are aimed at modifying the disease course and managing related symptoms. The common therapies are aimed at modulating inflammation and immune response, yet are only moderately effective and have profound side-effects. Etiology of MS is still unclear. Genetic predisposition is a key risk factor and environmental factors are also involved. Physical activity represents a modifiable environmental factor that could potentially have a positive impact on autoimmune diseases.

MS and experimental autoimmune encephalomyelitis (EAE), an animal model of MS, are believed to be inflammation and T-cell mediated autoimmunity that primarily targets the myelin glycoproteins surrounding axons in the CNS (Filippi et al., 2018; Robinson et al., 2014). Results from human studies suggest that physical activity has the health benefits for persons with MS in improving some symptoms, preventing complications, and possibly being neuroprotective in MS (Cortese et al., 2018; Heine et al., 2015; Mokhtarzade et al., 2017; Motl and Sandroff, 2015; Sandroff et al., 2016). Recently, Deckx and

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coworkers have shown the immunomodulating effects of 12 weeks of combined endurance and resistance on the increase of regulatory plasmacytoid dendritic cells, which is positively correlated with type 1 regulatory T cells (Tr1) in patients with MS (Deckx et al., 2016; Mokhtarzade et al., 2017). In accordance with this study, 8-weeks of upper and lower limb aerobic interval training could not affect serum IL-10. However, aerobic interval training inhibits proinflmmatory mediators leptin and TNF-α while increases blood adiponectin levels in women with MS (Deckx et al., 2016; Mokhtarzade et al., 2017). In rat and mouse EAE models, exercise has also been reported to have no significant effect (Patel and White, 2013) or attenuate rat EAE clinical symptoms (Bernardes et al., 2013; Einstein et al., 2018; Souza et al., 2017). Exercise activates mechanisms that lead to inhibit auto-Ag-specific T cell proliferation and the production of inflammatory cytokines, increase or do not change brain derived neurotrophic factor (BDNF), and change $CD4^+$ T cell subsets in the CNS or peripheral lymphoid tissues. In addition, a disease-modifying neuroprotective potential has also been observed in EAE by delaying dendritic spine loss, increasing neural progenitor cell proliferation, and reducing overall symptoms of EAE (Bernardes et al., 2016; Magalon et al., 2007; Rossi et al., 2009). Although these reports are inconsistent, physical activity has been suggested to have the health benefits on improving MS or EAE symptoms. However, this discrepancy may be due, in part, to different kind of exercise, such as running or swimming, in addition to widely altering training conditions including intensity, duration, or exercise status.

Regular moderate-intensity exercise can enhance the antigen-specific immune response which might contribute to being against pathogen infection (Molanouri Shamsi et al., 2017; Simpson et al., 2012; Wang et al., 2012). While prolonged, exhaustive training and strenuous endurance exercise may impair immune function which is associated with an increased risk of infection and is mediated possibly by inhibiting NK cell activity, IFN-γ production, but increasing the number of regulatory T cells (Treg) and IL-10 production (Handzlik et al., 2013; Simpson et al., 2015). Although exercise such as prior regular swimming (Bernardes et al., 2016), strength and endurance exercise (Souza et al., 2017), motorized treadmill or voluntary wheel (Rossi et al., 2009) training could attenuate EAE clinical symptoms, it is still unclear if regular- or exhausted- intensity exercise has the similar or different protective effect on EAE and the underlying mechanisms.

Swimming exercise is a well-controlled physical training protocol. Swimming training has been demonstrated to be capacity of enhancing muscular endurance (Shei et al., 2016), retarding the tumor development (Almeida et al., 2009), promoting antiinflammation and neuroprotective effects in mice (Bernardes et al., 2013). In addition, swimming may induce a more neuroprotective effects via enhancing the molecular adaptation when compared with running (Deforges et al., 2009; Goes et al., 2014). Exercise effect on BDNF, an important molecular mediator of neuronal survival, tends to increase in patient serum with MS when immersed in water compared to training on land (Bansi et al., 2013). In addition, the thermal effect on immune function is significant (Butler et al., 2013) but few studies, especially different intensity exercise, have performed systematic investigations between regular and exhaustive swimming exercise and the inflammatory responses of EAE during the acute phase of the disease, which may contribute to providing important information regarding the biological basis of MS progression and management of the disease.

The present study therefore explored the relationship between exercise intensity and immune response associated with EAE. Given the reported motorial intensity dependent differences in cytokine profiles, $CD4^+$ T cell subsets, and neurotrophic factors in EAE, we also assessed inflammatory levels in mice with this disease.

2. Materials and methods

2.1. Animals

C57BL/6 female mice (6–8 weeks of age) were obtained from Nanjing Biomedical Research Institution of Nanjing University (Nanjing, China). Animals were maintained at a controlled environment with a 12:12 h light:dark cycle and were provided with water and food ad libitum. After the intervention of exercise and induction with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (more detail subsequently), mice were sacrificed by CO_2 asphyxiation followed by exsanguination and tissues were collected post-mortem. All experimental procedures were carried out in compliance with the National Institutes of Health Guidelines on Laboratory Research and Guild for the Care and Use of Laboratory Animals (Eighth Edition, 2011) and approved by the Institutional Animal Care and Use Committee of Huaihe Hospital at Henan University.

2.2. Physical exercise protocol

The exercise protocol was performed in a swimming pool following protocols as described previously (Almeida et al., 2009; Bernardes et al., 2013), which was maintained at a controlled temperature (31 ± 1 °C), for 50 min/day, 5 day/week, over 6 weeks. Briefly, the initial swimming for first week, mice were subjected to progressive adaptation for 4 days in the swimming pool and a progressive load test (day 5), which consisted of an increasing workload corresponding with 2% of the animal's body weight added every 3 min until exhaustive. The intensitiy of the the following endurance training was set at 80% of the maximal weight obtained in the progressive load test. The maximal weight carried by the animal in the progressive load test was converted to a percentage of the animal's body weight. The mice were weighed weekly, and a new workload was determined using the previously calculated value. The test and workload adjustments were described previously (Almeida et al., 2009). The average workload was set up an average of 4% of the animal's body weight (HE). The moderate-intenstity (ME) mice were trained without 0% workload of the animal's body weight. The non-exercise (control) mice were kept in their cages. Two different experiments were performed, shown schematically in Fig. 1. Experiment 1: Following progressive adaptation for the first week, mice were randomized to three groups (n = 12/group): non-exercise (Control), moderate-intensity swimming exercise (ME), and high-intensity swimming exercise (HE). After exercise for 6 weeks, mice were immunized to induce EAE described as in the section of "*Induction and clinical assessment of EAE*". Mice were then monitored for 30 days to record symptom scores. At the end of study on day 30, spinal cords were collected to evaluate inflammatory response and myelin destruction. Experiment 2: Mice (n = 24/group) were given the same swimming exercise as mentioned above and then were sacrificed on day 14 after induction of EAE to access the inflammatory cytokine profiles, BDNF, and $CD4^+$ T cell subsets. To control for the stress associated with the water environment, the non-exercise mice were put on a flat open surface inside the swimming pool for the same time as the exercise groups. To prevent the stress associated with the activation of the body temperature control mechanism after swimming, the mice were gently dried using a towel.

2.3. Induction and clinical assessment of EAE

After the completion of 6 weeks of exercise according to their respective program, the EAE model was induced as described previously (Niu et al., 2018) and the exercise program continued post-immunization until sacrificed. Briefly, mice were immunized by *s.c.* injection in the flanks with 200 μg of $MOG_{35–55}$ peptide (synthesized by Synpeptide Co., Ltd., Shanghai) in 200 μl Complete Freund's Adjuvant (CFA) containing 5 mg/ml heat-killed Mycobacterium tuberculosis (Sigma-

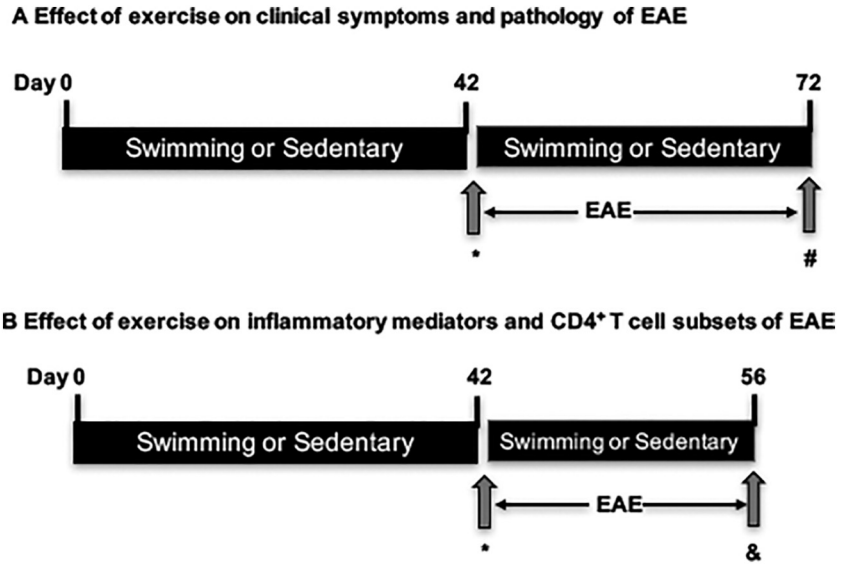

Fig. 1. Study design of the protocol. Following progressive adaptation for the first week, mice were randomized to three groups: non-exercise (Control), moderate-intensity swimming exercise (ME), and high-intensity swimming exercise (HE). After exercise for 6 weeks, mice (n = 12/group) were immunized to induce EAE (*). Mice were then monitored for 30 days to record clinical symptom. Twenty-four hours after the last exercise (#), spinal cord was collected to evaluate inflammation and myelin destruction (A). Seventy-two mice in three groups (n = 24/group) were given the same swimming exercise as mentioned above and then were sacrificed on day 14 (&) after induction of EAE to access the inflammatory cytokine profiles, BDNF, and $CD4^+$ T cell subsets (B).

Aldrich, St. Louis, MO) and *i.p.* injection with 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) in 0.2 ml PBS on day 0 and 2. Animal weight and clinical score were monitored daily after symptom appearance. The scores were defined as follows: 0 = no clinical signs, 1 = tail paralysis (or loss of tail tone), 2 = tail paralysis and hind-limb weakness, 3 = hind-limb paralysis and 4 = complete hind-limb paralysis and frontlimb weakness.

2.4. Histopathological analysis

Mice were euthanized 24 h after the last swimming training and perfused with 10% paraformaldehyde via intracardiac route. Spinal cord was collected, fixed with paraformaldehyde, and embedded in paraffin. Cross-sections were stained with hematoxylin and eosin (H&E) for inflammation and with Luxol Fast Blue (LFB) for demyelination assessment. The sections were observed under microscope, and the number of inflammatory cells and the percentage of demyelinated whilte matter was counted and calculated with the Image-Pro Plus software.

2.5. Isolation of infiltrating cells from the CNS

Mice were euthanized on day 14 post-immunization and perfused with cold PBS via

intracardiac route. Brain and spinal cord were removed and pooled for mononuclear cell isolation using Neural Tissue Dissociaton Kits (MACS Miltenyi Biotec, Auburn, CA). The isolated cells were counted for evaluation of total infiltrating cells, and then were directly analyzed for $CD4^+CD25^+Foxp3^+$Treg or re-stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (both from Sigma-Aldrich) in the presence of monensin (GolgiStop, BD Biosciences) for 4 h to determine the expression of Th1 ($CD4^+$IFN-γ^+) and Th17 ($CD4^+$IL-$17A^+$) by flow cytometry as described previously (Wang et al., 2013).

2.6. Lymph node cell preparation

After immunization for 14 days, mice were sacrificed and the draining lymph nodes (LN) were collected to prepare single cell suspension as described previously (Wang et al., 2013). One part of LN cells was directly stained with fluorescence-conjugated antibodies to determine the frequency of Treg cells and another LN cells were cultured with $MOG_{35–55}$ peptide and re-stimulated with PMA and ionomycin in the presence of monensin for the final 4 h, to determine the populations of Th1, Th2, and Th17 cells with the flow cytometry method as described below.

2.7. Immune parameter evaluation in CNS

On day 14 post-immunization, the mice were euthanized to collect brains and spinal cords and stored in liquid nitrogen. Next, the tissue homogenates were prepared as previously described (Bernardes et al., 2013) and the supernatants were stored at −80 °C. The level of BDNF, IL-1β, (both from R&D Systems), IFN-γ (BD Bioscience), TGF-β, IL-17A, IL-6, and IL-10 (all from eBioscience) in tissue homogenates was determined using ELISA kit in accordance with the manufacturer's instructions.

2.8. Flow cytometry

Different florescence-conjugated antibodies for flow cytometry were as follows: anti-CD3, anti-CD4, anti-CD25, anti-IL-4, anti-IL-17, anti-IFN-γ, anti-Foxp3, and their respective isotype antibodies (all from eBioscience). After cells were blocked with anti-CD16/CD32 (BD Bioscience) and then stained with florescence-conjugated antibodies, they were acquired using flow cytometry or fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) and stained with fluorochrome-labeled antibodies. Foxp3 staining was performed using the Mouse Foxp3 Buffer Set (eBiosciences). Flow cytometric measurements were conducted using a Mindray Bricyte E6 flow cytometer (Shenzhen, China), and data were analyzed with the use of FlowJo7.6 software (Treestar, Ashland, OR, USA).

2.9. Statistics analysis

All values in the figures and text are presented as means ± SEM. Statistical analysis was carried out by one-way or two-way ANOVA followed by Tukey's HSD *post-hoc* test using GraphPad Prism 6.0 software. Significance was set at $P < .05$.

3. Results

3.1. Effect of moderate- and high- intensity swimming training in the development of the clinical signs of EAE

To evaluate the effects of ME or HE swimming training on the disease development of clinical EAE, the exercise and non-exercise mice immunized with $MOG_{35–55}$ were monitored until 30 days post-EAE induction. Following the swimming training, we did not observe any

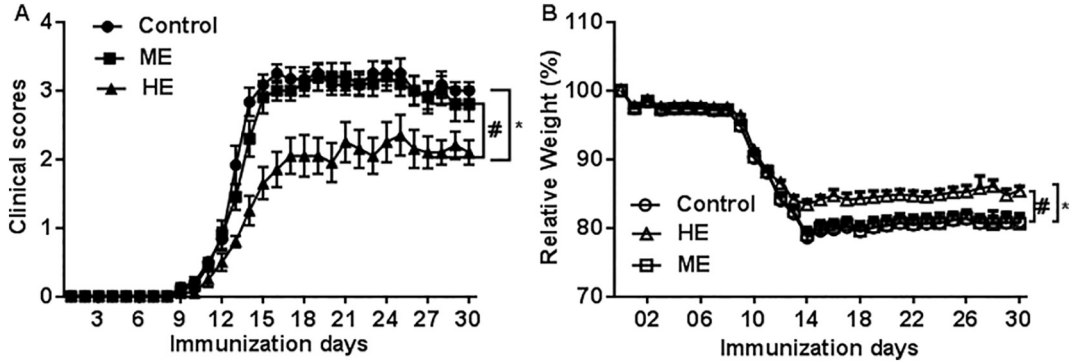

Fig. 2. Clinical assessment of different-intensity exercise EAE mice. After induction of EAE following exercise for 6 weeks, mice were monitored daily to record clinical symptom as described in the "*Materials and Methods*" section (A). The body weight was weighted daily to evaluate the effect of exercise on body weight changes (B). Data are expressed as mean ± SEM (n = 16/group). Significant difference was determined by two-way ANOVA followed by Tukey's test. $^{*}P < .01$ compared to the control. $^{\#}P < .01$ compared to the ME. Control, non-exercise; ME, moderate-intensity swimming exercise; HE, high-intensity swimming exercise.

difference of the incidence of EAE among exercise and non-exercise groups, but HE swimming exercise significantly attenuated disease severity of EAE mice (Fig. 2A). While such effect was not observed in ME swimming exercise mice. Of note, HE swimming training kept a higher body weight than that of ME or non-exercise mice (Fig. 2B) in the whole study. These results indicate that HE swimming training may have the ability to attenuate the clinical signs of EAE.

3.2. *Effect of moderate- and high- intensity swimming training in the inflammatory infiltration and CD4$^+$ T cell subpopulations in the CNS of EAE mice*

MS and its animal model EAE are mainly caused by inflammation in the CNS, and inflammation is identified as T-cell mediated autoimmunity (Karussis, 2014). To access the effect of ME and HE swimming training on the infiltrating inflammatory cells and demyelination in the CNS of EAE mice, spinal cord was sectioned and stained with H&E for evaluation of infiltrating cells and LFB for assessment of myelin destruction. In agreement with clinical signs, immune cell infiltration (Fig. 3A) and demyelination (Fig. 3B) of spinal cord were attenuated in HE swimming EAE mice; while ME swimming training did not change immune cell infiltration and demyelination.

To further evaluate total infiltrating cells and T cell subpopulations in the CNS, spinal cord and brain were collected on day 14 post-EAE induction (peak immune-pathological response), and total infiltrating cells and CD4$^+$ T cell subsets were determined using flow cytometry. In accordance with histopathological changes, following swimming exercise, total infiltrating cells in the CNS of EAE mice significantly decreased in HE training group and no change was observed in ME training group when compared with non-exercise group (Fig. 4A). Further analysis of CD4$^+$ T cell subsets showed that HE training resulted in a reduction of Th1 (Supplementary Fig. 1A and Fig. 4B) and Th17 (Supplementary Fig. 1A and Fig. 4C) populations, and an increase of Treg (Supplementary Fig. 1B and Fig. 4E) when compared with non-exercise or ME mice. While such effects were not observed in ME training mice compared to non-exercise mice.

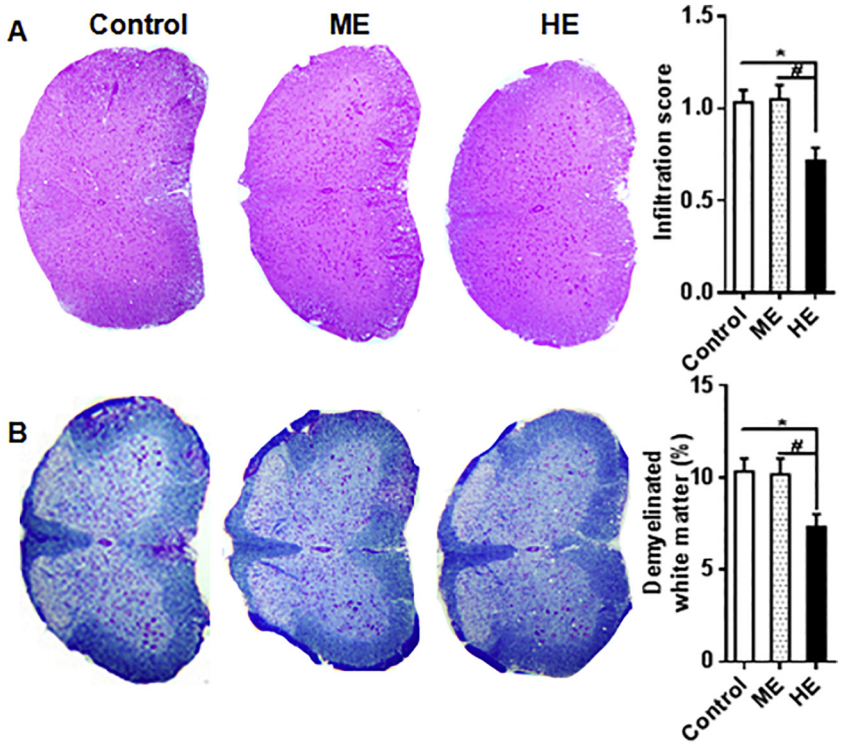

Fig. 3. Histopathological analysis of different-intensity exercise EAE mice. Twenty-four hours after the last exercise, exercise or non-exercise EAE mice were euthanized and spinal cord was collected. Sections of the fixed samples were obtained and stained with H&E (A, left panel) or Luxol fast blue (LFB) (B, left panle) for assessment of inflammation or demyelination, respectively. Each section is the representative of 12 mice in each group. The number of inflammatory cells (A, right panel) and the percentage of demylinated white mattere (B, right panle) were counted and calculated as described in the "*Materials and Methods*" section. Data are expressed as mean ± SEM (n = 12/group). Significant difference was determined by one-way ANOVA followed by Tukey's HSD *post-hoc* test. $^{*}P < .05$ compared to the control. $^{\#}P < .05$ compared to the ME. Control, non-exercise; ME, moderate-intensity swimming exercise; HE, high-intensity swimming exercise. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

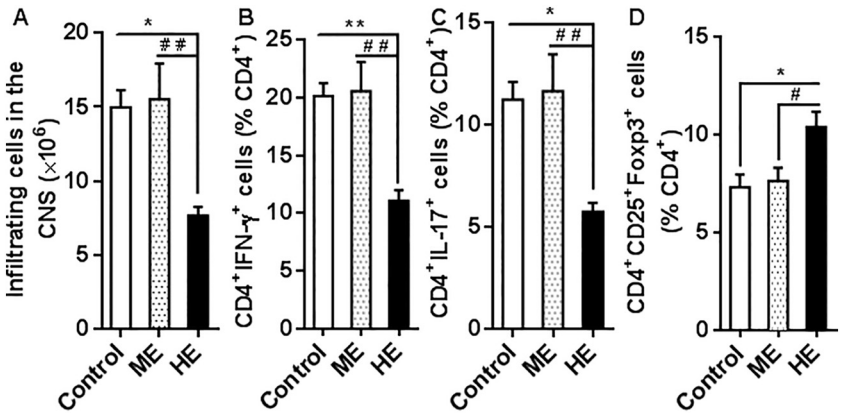

Fig. 4. Analysis of infiltrating inflammatory cells and $CD4^+$ T cell subsets of different-intensity exercise EAE mice. On day 14 post-immunization, mice were euthanized and brain and spinal cord were collected. The mononuclear cells from the brain and spinal cord were isolated, and total infiltrating cells (A), Th1 (B), Th17 (C), and Treg (D) populations were determined on gated $CD4^+$ T cells using flow cytometry. Data are expressed as mean ± SEM (n = 12/group). Significant difference was determined by one-way ANOVA followed by Tukey's HSD *post-hoc* test. * $P < .05$ compared to the control. $^{\#}P < .05$, $^{\#\#}P < .01$ compared to the ME. Control, non-exercise; ME, moderate-intensity swimming exercise; HE, high-intensity swimming exercise.

3.3. Effect of moderate- and high- intensity swimming training on cytokine profiles and BDNF level in the CNS of EAE mice

Cytokines play crucial roles in the T-cell mediated autoimmunity. Anti-inflammatory cytokines including IL-10 and TGF-β has the protective effects on MS and EAE; while proinflammatory cytokines including IL-1β, IFN-γ, IL-6, and IL-17A promote autoimmune development. To investigate the role of inflammatory cytokines in the exercise-mediated immune response of EAE, the cytokine profiles and BDNF levels were assessed in spinal cord and brain homogenates from non-exercise and exercise mice. HE swimming training significantly decreased the concentration of IFN-γ (Fig. 5A), IL-17A (Fig. 5B), and IL-1β (Fig. 5D) and increased the IL-10 (Fig. 5E), TGF-β (Fig. 5F) and BDNF (Fig. 5G) level in brain from EAE mice compared to non-exercise or ME EAE mice. Simlar to this effect observed in brains, HE swimming training decreased IFN-γ (Fig. 6A), IL-17A (Fig. 6B) concentration and increased TGF-β (Fig. 6C) and BDNF (Fig. 6D) level in spinal cord from EAE mice. However, ME swimming training did not affect these cytokine concentrations in addition to IL-1β (Fig. 5D) in brain when compared with non-exercise mice. In addition, we also determined cytokine IL-6 concentration in brain and no significance difference in the level of IL-6 (Fig. 5C) was observed among all groups.

3.4. Effect of moderate- and high- intensity swimming training on CD4+ T helper cell subsets in the lymph node of EAE mice

Next we wanted to determine whether different-intensity swimming training also impacted the priming and expansion of pathogenic T cells in peripheral tissues. Draining LN cells from EAE mice were collected on day 14 post-immunization to evaluate $MOG_{35–55}$-specific T cell proliferation and $CD4^+$ T cell subsets. We did not observe any difference for the percentage of $CD4^+$ and $CD8^+$ T cell population among all groups (data not shown). Interestingly, HE swimming mice had a lower T cell proliferation (Fig. 7A), Th1 (Supplementary Fig. 2A and Fig. 7B) and Th17 (Supplementary Fig. 2A and Fig. 7C) populations and a higher Treg (Supplementary Fig. 2C and Fig. 7E) when compared with non-

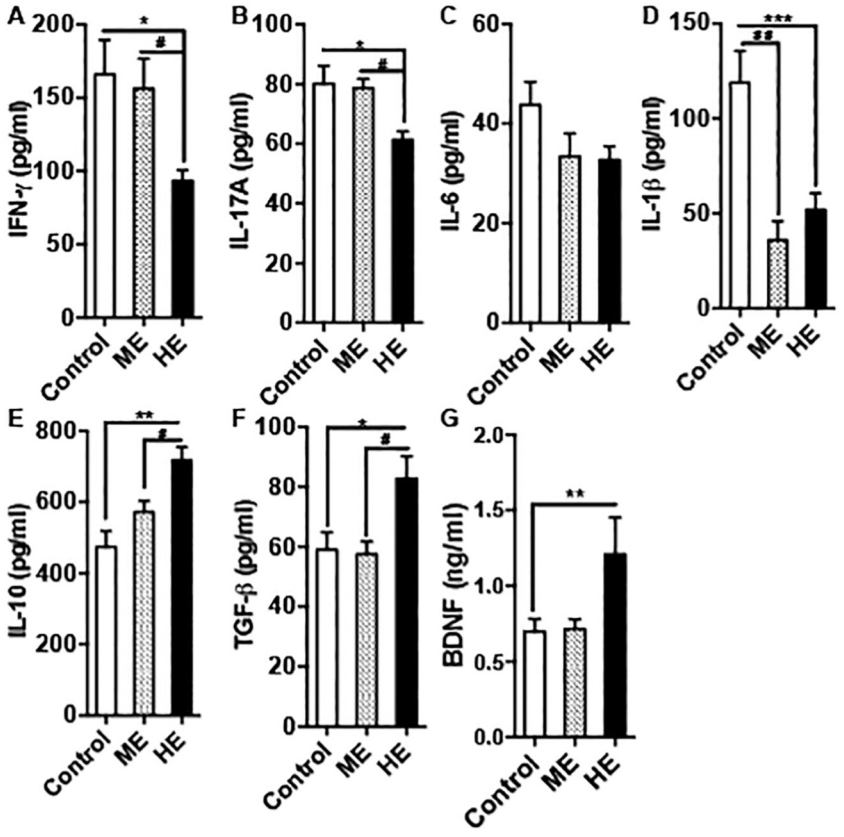

Fig. 5. Assessment of inflammatory cytokines and BDNF in brain of of different-intensity exercise EAE mice. On day 14 post-immunization, mice were euthanized and brain was collected and homogenized to measure cytokines IFN-γ (A), IL-17A (B), IL-6 (C), IL-1β (D), IL-10 (E), TGF-β (F), and BDNF (G). Data are expressed as mean ± S.E.M. (n = 12/group). Significant difference was determined by one-way ANOVA followed by Tukey's test. *$P < .05$, **$P < .01$ compared to the control. $^{\#}P < .05$, $^{\#\#}P < .01$ compared to the ME. Control, non-exercise; ME, moderate-intensity swimming exercise; HE, high-intensity swimming exercise.

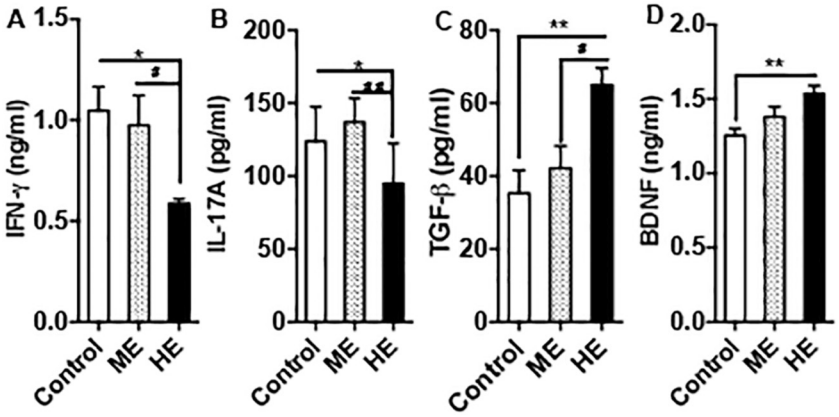

Fig. 6. Assessment of inflammatory cytokines and BDNF in spinal cord of exercise and non-exercise EAE mice. On day 14 post-immunization, mice were euthanized and spinal cord was collected and homogenized to measure cytokines IFN-γ (A), IL-17A (B), TGF-β (C), and BDNF (D). Data are expressed as mean ± S.E.M. (n = 12/group). Significant difference was determined by one-way ANOVA followed by Tukey's test. $^{*}P < .05$, $^{**}P < .01$ compared to the control. $^{\#}P < .05$, $^{\#\#}P < .01$ compared to the ME. Control, non-exercise; ME, moderate-intensity swimming exercise; HE, high-intensity swimming exercise.

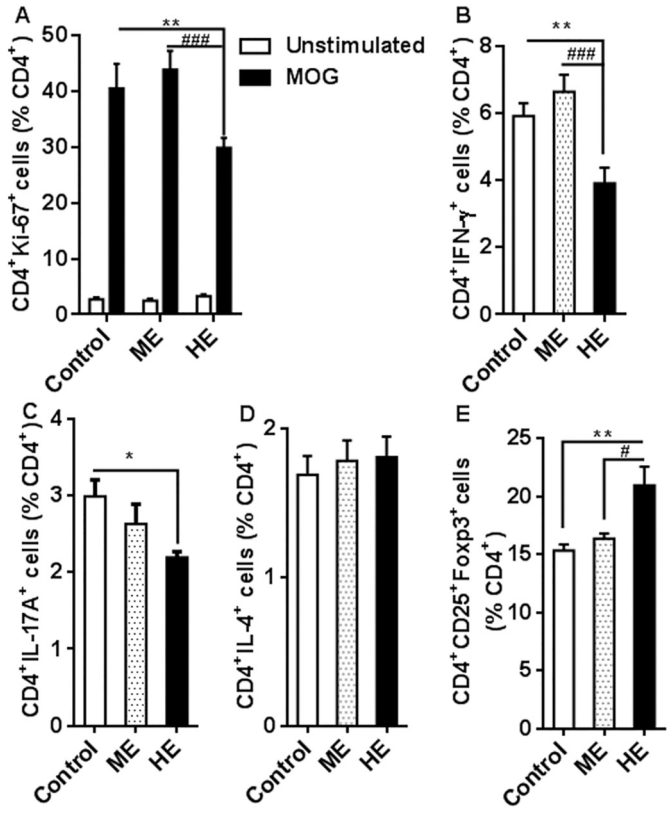

Fig. 7. Evaluation of T cell proliferation and $CD4^+$ T cell subsets in the lymph node of exercise and non-exercise EAE mice. Draining lymph node cells were isolated on day 14 post-immunization. Cells were re-stimulated with $MOG_{35–55}$ peptide and cell proliferation with the Ki-67 expression (A) and the populations of Th1 (B), Th17 (C), Th2 (D), and $CD4^+CD25^+Foxp3^+$ Treg (E) cells were determined on gated $CD4^+$ T cells by flow cytometry. Data are expressed as mean ± SEM (n = 12/group). Significant difference was determined by one-way ANOVA followed by Tukey's HSD *post-hoc* test. $^{*}P < .05$, $^{**}P < .01$ compared to the control. $^{\#}P < .05$, $^{\#\#\#}P < .001$ compared to the ME. Control, non-exercise; ME, moderate-intensity swimming exercise; HE, high-intensity swimming exercise.

exercise or ME mice, but they had the same Th2 population (Supplementary Fig. 2B and Fig. 7D) with non-exercise or ME mice. Furthermore, we did not find any difference for these immune parameters between ME and non-exercise mice. Finally, compared to ME training mice, HE swimming training inhibited T cell proliferation and Th1 cells while increased Treg.

4. Discussion

Based on our previously demonstrated enhancement of cell-mediated immunity (CMI) in a regular moderate intensity exercise program via an increase of proinflammatory responses while impairment of CMI via an increase of antiinflammatory responses in a prolonged, exhaustive high-intensity exercise program (Wang et al., 2012), in the present study, we evaluated the modulation of immune parameters that are known to influence disease activity in EAE mice, namely, cellular and immune-modulators of adaptive immunity, following different-intensity swimming programs. This hypothesis is supported by studies investigating the effect of physical activity in MS (Bansi et al., 2013; Deckx et al., 2016) and its EAE animal models (Bernardes et al., 2013; Einstein et al., 2018).

MS and EAE have been characterized by inflammatory cell infiltration and demyelination in CNS which is mediated via unbalancing proinflammatory Th1/Th17 cells and antiinflammatory Treg (Axtell et al., 2010; Stoycheva et al., 2015). It has been known to have less number of Treg and more number of Th1 and Th17 cells at the site of local inflammation in the CNS from MS patients (Axtell et al., 2010; Jafarzadeh and Nemati, 2018). Thus, modulation of specific autoimmune responses (Treg vs Th1/Th17), rather than inhibition of global immune system, is crucial for efficient control disease with minimizing the unnecessary suppression of systemic immune surveillance.

Evidence has demonstrated that physical activity has the ability to modulate Th17/Treg balance. Strenuous endurance exercise could promote alterations of Treg and Th17 population, e.g., increasing Th17 cells while decreasing Treg in the blood of marathon and half-ironman triathlon athletes (Perry et al., 2013). However, Souza et al. has demonstrated that strength exercise, not endurance exercise, could induce an increase of Treg in splenocytes during peak phase in trained EAE mice (Souza et al., 2017). Further, strength and endurance exercise decreased the concentration of IL-17 in spinal cord of EAE mice. In addition, trained non-EAE mice did not affect Treg. These results indicate that exercise internsity may have the ability to modulate the specific autoimmune responses.

In this study, we did not find any differences following 10 weeks of regular ME swimming training for improvement of EAE clinical symptoms and CNS histopathology compared to non-exercise group, which is inconsistent with findings by other that might be due to different-intensity swimming program (Bernardes et al., 2013). Furthermore, regular ME training also did not affect total infiltrating cells, Th17 and Treg populations in the CNS and lymph node of EAE mice. However, HE swimming training attenuated EAE clinical symptoms and histopathology, suppressed proinflammatory Th17 and Th1 cells while promoted antiinflammatory Treg cells in the CNS of EAE mice when compared with non-exercise groups. In addition, suppression of $MOG_{35–55}$-specific Th17 and Th1 cells secreting IL-17 and IFN-γ, repectively, and promotion of Treg were also observed in peripheral

lymphoid organs in HE training program. These data suggest that HE swimming training might have the ability to migrate autoimmune diseases. In addition, ME mice trained with 0% workload of the animal's body weight might be able to progressively adapt to this intensity during the late training periods, so we observed little effect of this intensity used in this study on the development of EAE. Current data indicate that sufficient motorial intensity could improve the EAE development in an swimming program. A progressive load test with lower workload of the animal's body weight may be considered in mice exercise protocol in future study.

Proinflammatory cytokines such as IL-1β and IL-6 could promote Th17 development (Bruggemann et al., 2015); while anti-inflammatory TGF-β could induce anti-inflammatory Treg development and Treg are the resources of TGF-β and IL-10 (Lee et al., 2017). Therefore, alteration of proinflammatory cytokines such as IL-1β, IL-6, IL-17, and IFN-γ, as well as antiinflammatory mediators such as IL-10 and TGF-β, may contribute to attenuating neurodegeneration and disability (Goverman, 2009). Although we did not find alteration of the levels of IL-17, IFN-γ, IL-6, IL-10, and TGF-β in the CNS following regular ME swimming program, a decrease of the IL-1β concentration in brain was observed compared to non-exercise EAE mice. Furthermore, we did not find any difference for IL-6 in brain among all groups, but HE swimming training resulted in a reduction of IL-1β, IL-17, and IFN-γ while an increase of IL-10 and TGF-β in the CNS of EAE mice. These data confirm and extend the study done by Souza et al. (Souza et al., 2017) and Alvarenga-Filho et al. (Alvarenga-Filho et al., 2016), showing that endurance or combination of physical activity decreases the production of IL-17 and IFN-γ in the CNS of EAE mice or in the plasma of MS patients, respectively. Furthermore, during the acute phase of EAE, HE swimming training increased Treg in the CNS, even as inhibited the proinflammatory cytokines and increased antiinflammatory mediators, such as IL-10 and TGF-β. In addition, the populations of Treg in HE swimming EAE mice were higher than those of non-exercise or ME mice, which is similar to Golzari et al.'s data in patients with MS (Golzari et al., 2010), demonstrating that strength training has the ability to increase the frequency of Treg and inhibit the proinflammatory cytokines during the acute, not chronic phase of EAE. Thus, we suggest that physical activity, to the intensity and to a less extent duration, appear to modulate the frequency of $CD4^+$ T cell subsets, especially in the lymphoid organs during the early, acute, and chronic phase. However, whether it is indeed needs to be further determined.

It is believed that stress hormones such as glucocorticoid, catecholamine, and prostaglandin E2 (PGE2) might be regarded as an immune-modulator for the response to exercise. The plasma concentration of glucocorticoid, catecholamine, and PGE2 increase proportionally during exercise. While they could directly or indirectly affect T cell functions via their respective receptors, i.e., dexamethasone and hydrocortisone have the antiinflammatory effects on the reduction of IL-12, IFN-γ, and IL-17 production (Alvarenga-Filho et al., 2017; Yang et al., 2009; Zhao and Ding, 2018) and the increase of IL-10 and Treg (Roquilly et al., 2014; Wang et al., 2015); PGE2 could inhibit T-cell secreting IFN-γ (Sreeramkumar et al., 2012) and IL-17 (Chen et al., 2009). In addition, the production of neurotransmitters such as dopamine and serotonin appears to be elevated following physical activity (Russell et al., 2014) while they can impact different functions of T cells (Alvarenga-Filho et al., 2016). Thus, these results suggest that hormones or other immune-modulators may be involved in altering the immune balance between proinflammatory/antiinflammatory cytokines and $CD4^+$ T cell subsets.

Exercise intensity has been shown to impact the elevation of BDNF levels in the CNS while BDNF has been indicated to be involved in the positive effect of physical activity (Bernardes et al., 2013; Monnier et al., 2017; Pedard et al., 2019). Bernardes et al.'s study has demonstrated the elevation of BDNF levels in brain and spinal cord on day 14 from exercise-trained EAE mice (7% workload of animal weight) (Bernardes et al., 2013). Furthermore, short-term exercise training (Monnier et al., 2017) or downhill and uphill Training (Pedard et al., 2019) could also increase the BDNF levels and induce BDNF signaling activation in rats. In accordance with these reports, our data demonstrated that high-, not moderate-, intensity could increase the levels of BDNF in brain and spinal cord of EAE mice. In the CNS, neurons are the major source of BDNF which could promote neuronal growth and survival via binding to its TrkB receptor. Furthermore, BDNF treatment has a beneficial effect on EAE (Makar et al., 2009). Most important, physical activity increases BDNF/TrkB signaling in brain (Baranowski and MacPherson, 2018; Kim et al., 2016). These results further suggest that exercise intensity may modulate the elevation of BDNF levels and BDNF-TrkB signaling in the CNS. Furthermore, activated immune cells are capacity of releasing BDNF in peripheral blood (Kerschensteiner et al., 1999). In the present study, we could not detect the BDNF levels from the blood among all groups. This indicates that the elevation of BDNF from exercise-mice may not be derived from immune cells. Thus, we will plan a specific in depth study in the future to determine the role of BDNF and BDNF signaling in HE swimming program's effect on $CD4^+$ T cell subsets involving altered pro−/anti- inflammatory $CD4^+$ T cell subset balance.

In summary, we showed that regular ME swimming training during the development of EAE, prior to disease onset, is not effective in attenuating the symptom and pathological change of EAE, as well as does not affect inflammatory cytokines and $CD4^+$ T cells subsets. However, prolonged, exhaustive HE swimming program attenuates the disease severity and pathology of EAE. This effect is associated with the reduction of inflammatory infiltration and the altering immune-balance of pro−/anti- inflammatory mediators and $CD4^+$ T cell subsets in the CNS. These results strongly suggest a promising potential for using HE swimming program as a preventive/therapeutic program in MS and possibly in other T-cell mediated autoimmune diseases as well. This study has also revealed a novel mechanism to help us understand the health beneficial effect of different-intensity swimming program. Future studies are needed to determine the efficacy by which exercise may be neuroprotective in MS and to demonstrate whether the mechanism for EAE in this study can also be applied to other autoimmune diseases.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2018.12.005.

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