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NAD+ attenuates central nervous system demyelination in experimental autoimmune encephalomyelitis mice

Bin Zheng[1], Jianhua Ma[2], Xiao Deng[3], Jiangang Pan[4], Feng Liao[4]

[1]Department of Neurosurgery, The Second Affiliated Hospital of Shandong First Medical University, Taian, Shandong Province, China, [2]Department of Vascular Surgery, People's Hospital of Feicheng, Feicheng City, Taian, Shandong Province, China, [3]Department of Rehabilitation Medicine, The Second Affiliated Hospital of Guangdong Medical University, Xiashan District, Zhanjiang, Guangdong Province, China, [4]Department of Neurology, Affiliated Hospital of Guangdong Medical University, Xiashan District, Zhanjiang, Guangdong Province, China

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Abstract

Nicotinamide adenine dinucleotide (NAD+) supplementation attenuates demyelination in the experimental autoimmune encephalomyelitis (EAE) model. The aim of the study was to confirm the therapeutic effect of NAD+ on the EAE model and investigate its protective mechanism. Mice were divided into 3 groups: EAE, EAE + NAD+, and Control (Ctrl). EAE and EAE + NAD+ groups were induced with myelin oligodendrocyte glycoprotein (MOG) to initiate the demyelination process. The EAE + NAD+ group received an NAD+ injection at a dosage of 250 mg/kg/day. Clinical, neuroinflammation, and neurodemyelination scores were monitored. At the peak of onset, animals were euthanized, and mRNA expression level in the spinal cord was tested. NAD+ supplementation promoted the conversion of regulatory T cells (Tregs) into T helper 17 (Th17) cells with increased concentrations of NAD+. NAD+ alleviated neuroinflammation, attenuated central nervous system (CNS) demyelination, and improved the disease score of EAE mice. NAD+ promoted expression of the cytokine interleukin 17A (IL-17A).

Key words: *experimental autoimmune encephalomyelitis, IL-17A, multiple sclerosis, NAD+, neuroinflammation, Th17 cell, Treg cell.*

Introduction

Multiple sclerosis (MS) and its experimental autoimmune encephalomyelitis (EAE) murine models are characterized by inflammatory processes that are associated with auto-reactive immune cell infiltration into the central nervous system (CNS) [26,46]. Nicotinamide adenine dinucleotide (NAD^+), a metabolic coenzyme pre-sent in various metabolic pathways, plays a key role in preserving mitochondrial function. NAD^+ governs the process of aging, regulates gene expression, maintains calcium homeostasis, and regulates cell death [45]. NAD^+ depletion is linked to various neurodegenerative diseases [5]. Notably, neurons display susceptibility to damage that is associated with NAD^+ deficiency in MS [35]. Conversely, NAD^+ inhibits the stress-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway and the activation of pro-inflammatory T helper (Th) cell subsets, such as Th17 and Th1 [41], which are implicated in MS pathology. The disease initiation and progression are driven by pro-inflammatory $CD4^+$ T cells, specifically Th1 and Th17 differentiated cells. These cells play a key role in the breakdown of the blood-brain barrier (BBB) through production of the signature cytokines interferon γ (IFN-γ) and interleukin (IL)-17, along with

other cytokines and chemokines. Through infiltration of the CNS, these cells facilitate monocyte recruitment, activate macrophages, and induce other cell-mediated effector responses, resulting in neuronal demyelination [24]. Regulatory T cells (Treg cells) maintain self-tolerance and prevent autoimmunity. They are characterized by the expression of $CD4^+$ $CD25^+$ $FoxP3^+$ and/or $CD4^+$ $FoxP3^+$ markers. Impairment of Treg cells, either through a decrease in their numbers, functional defects, or their conversion into inflammatory effector cells, contributes to the development of autoimmune diseases. Treg cells exert their regulatory function in neuroinflammation by controlling the number and activity of effector cells *via* various mechanisms. These include cell-to-cell contact-dependent suppression, the secretion of inhibitory cytokines, including IL-10 and transforming growth factor β (TGF-β), modulation of antigen-presenting cell function, cytolysis, metabolic disruption, and the induction of other suppressor cells. In the context of EAE, antigen-specific Treg cells generated in the peripheral immune system in tolerogenic responses ameliorate or prevent the development of the disease [14]. In this regard, there is a need for novel therapeutic agents that effectively reduce the pro-inflammatory Th1 and Th17 subsets and promote the anti-inflammatory Th2 and Treg subsets as a potential treatment approach for MS. The NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is such a complex comprising multiple proteins that facilitate the conversion of the precursor of inflammatory factors, such as pro-IL-1 and pro-IL-18, into their active forms, IL-1 and IL-18, respectively. The dysregulated NLRP3 inflammasome is involved in various neurological disorders, including MS [1].

In this study, we used EAE mice to investigate the potential mechanism of NAD^+ that attenuates the clinical symptoms and reduces the severity of EAE. Our findings indicate that NAD^+ mitigates the severity of EAE by conversion of Treg cells into Th17 cells.

Material and methods

Animals

Twenty-one female C57BL/6 mice, aged 8-10 weeks and weighing 16-22 g, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were housed in a temperature-controlled room maintained at 24 ±2°C with a 12-hour light and dark cycle. The mice had unrestricted access to food. All experiments, including animal experiments, were conducted at the Department of Neurosurgery. The experiments were performed in accordance with the guidelines for animal care and use, and the experiments were approved by the Experimental Ethics Committee of The Second Affiliated Hospital of Shandong First Medical University. The methods used in the study adhere to the ARRIVE guidelines (https://arriveguidelines.org) for reporting animal experiments.

Experimental autoimmune encephalomyelitis

The study included a total of 21 mice. The mice were then divided into three groups: the healthy control (Ctrl) group included seven mice (n = 7), the EAE induction group included seven mice (n = 7), and the EAE + NAD^+ group included seven mice (n = 7). The mice were injected with a mixture of 0.25 mg of myelin oligodendrocyte glycoprotein (MOG35-55) and complete Freund's adjuvant (CFA) for induction of the EAE model. Afterward, the mice were administered intraperitoneal injections of 500 ng of pertussis toxin twice.

Nicotinamide adenine dinucleotide

The NAD^+ treatment involved the administration of a diluted solution of 250 mg/kg/day of NAD^+ in phosphate-buffered saline (PBS) to the EAE mice in the NAD^+ group (n = 7). The NAD^+ treatment was initiated at the time of inoculation and was administered daily until the study was concluded. The mice received daily subcutaneous injections of PBS from the inoculation day in the control and EAE model groups.

Disease

The mice exhibited initial signs of disease onset at 14 days post-immunization (dpi), and the disease reached its peak at 20 dpi. A semi-quantitative assessment was conducted with the help of a 5-point scoring scale to evaluate paralysis [22]. The scoring scale included categories: 0 indicated no clinical signs, 1 indicated a paralyzed tail, 2 indicated paresis, 3 indicated paraplegia, 4 indicated paraplegia with weakness, and 5 indicated a moribund state. A score of ±0.5 was assigned to accommodate mice that showed characteristics that overlap between the adjacent score levels in instances where the assessment fell in between those grades.

Euthanasia

The animals were euthanized when they reached the peak of their clinical score. Next, the mice were anesthetized using a combination of MOG35-55, curdlan, CFA, and pertussis toxin to conduct RT-qPCR and transcriptome analyses.

Tissue

The spinal cords of EAE and healthy Ctrl mice were meticulously extracted during the disease's peak.

The extracted spinal cords were immediately frozen in liquid nitrogen and subsequently ground using a Trizol reagent. RNA extraction was conducted using the chloroform-isopropanol-ethanol method, and the resulting RNA was dissolved in RNase-free water.

RNA and cDNA

The extraction of RNA was carried out using the triazole method (G3013, Servicebio, Wuhan, China). The concentration of mRNA was performed by qPCR using the qPCR master mix (G3332, Servicebio, Wuhan, China) following the instructions provided by the manufacturer. The extraction of RNA was performed from isolated Treg cells after cultivation. Treg cells were homogenized in a lysis buffer (0.5 ml) and passed through a column. Next, after several washes, the RNA was converted into complementary DNA (cDNA) using First Strand cDNA Synthesis Kits (G3332, Servicebio, Wuhan, China) with an equal volume of RNA used for the synthesis of cDNA. The relative mRNA levels were performed using the $2^{-\Delta\Delta CT}$ method.

RT-qPCR

The concentration and quality of RNA were assessed using a NanoDrop spectrophotometer. Reverse transcription of RNA was performed using the SweScript RT II First Strand cDNA Synthesis Kit (Servicebio, Wuhan, China) following the instructions provided by the manufacturer. The resulting cDNA was then used as a template for gene expression analysis using RT-qPCR. SYBR Green served as a fluorescence indicator, and ROX served as a fluorescence reference. The concentration of primer for amplification was set at 0.2 µM, and Taq DNA Polymerase was used. The RT-qPCR procedure involved a 10-minute pre-degeneration step at 95°C, followed by 35 cycles of amplification. Next, the melting curves were analyzed. Further, the cycle threshold (CT) value was also compared to an internal reference to evaluate the expression level of the gene of each target RNA. The primers used are presented in Table I.

Statistical analysis

GraphPad Prism 9.5.1 software (GraphPad Software Inc., La Jolla, CA, USA) was used for data analysis. The results are presented as the mean ± standard deviation (SD). Statistical comparisons of clinical sign scores were performed using the Mann-Whitney *U* test. For comparisons of multiple groups, a one-way analysis of variance (ANOVA) followed by the Mann-Whitney *U* test was conducted. Statistical significance was defined as a significance level of $p < 0.05$.

Results

NAD+ improves neurological function in EAE

To assess the potential effect of NAD+ treatment on reducing EAE severity, we compared the scores of neurological symptoms in Ctrl mice and EAE mice that received a daily intraperitoneal injection of NAD+ at a dosage of 250 mg/kg (EAE + NAD+). The treatment was administered from the 2nd day to the 20th dpi. The score of neurological severity reached its peak at 2.5 on day 14, indicating occurrences of paraplegia, tail weakness (Fig. 1A), reduced spontaneous activity, and weight loss. The EAE + NAD+ group exhibited a sig-

Table I. Primers used in RT-qPCR

Gene	Primers	Sequence	Primer length
NLRP3	Forward (5' to 3')	5'-TAAGAACTGTCATAGGGTCAAAACG-3'	25
	Reverse (5' to 3')	5'-GTCTGGAAGAACAGGCAACATG-3'	22
TGF-β	Forward (5' to 3')	5'-GCCCTGGATACCAACTATTGCTTCA-3'	25
	Reverse (5' to 3')	5'-TAGGGGCAGGGTCCCAGACAGAAGT-3'	25
IFN-γ	Forward (5' to 3')	5'-CCATGTCAGAAGACTCTTGCGT-3'	22
	Reverse (5' to 3')	5'-TGTCAACGAAGAGAACTTGGTCA-3'	23
IL-4	Forward (5' to 3')	5'-CTGGTCCATGCTCCTGCTG-3'	19
	Reverse (5' to 3')	5'-GGACGGACGAAGATGCCTAG-3'	20
IL-6	Forward (5' to 3')	5'-AAGGAGTGGCTAAGGACCAAGAC-3'	23
	Reverse (5' to 3')	5'-AGTGAGGAATGTCCACAAACTGAT-3'	24
IL-10	Forward (5' to 3')	5'-CCTCAGACTACCTCAACCGTTCC-3'	23
	Reverse (5' to 3')	5'-AGGCTCCCTCTTCAGGACCAG-3'	21
IL-17	Forward (5' to 3')	5'-CCTCAGACTACCTCAACCGTTCC-3'	23
	Reverse (5' to 3')	5'-AGGCTCCCTCTTCAGGACCAG-3'	21
GAPDH	Forward (5' to 3')	5'-CCTCGTCCCGTAGACAAAATG-3'	21
	Reverse (5' to 3')	5'-TGAGGTCAATGAAGGGGTCGT-3'	21

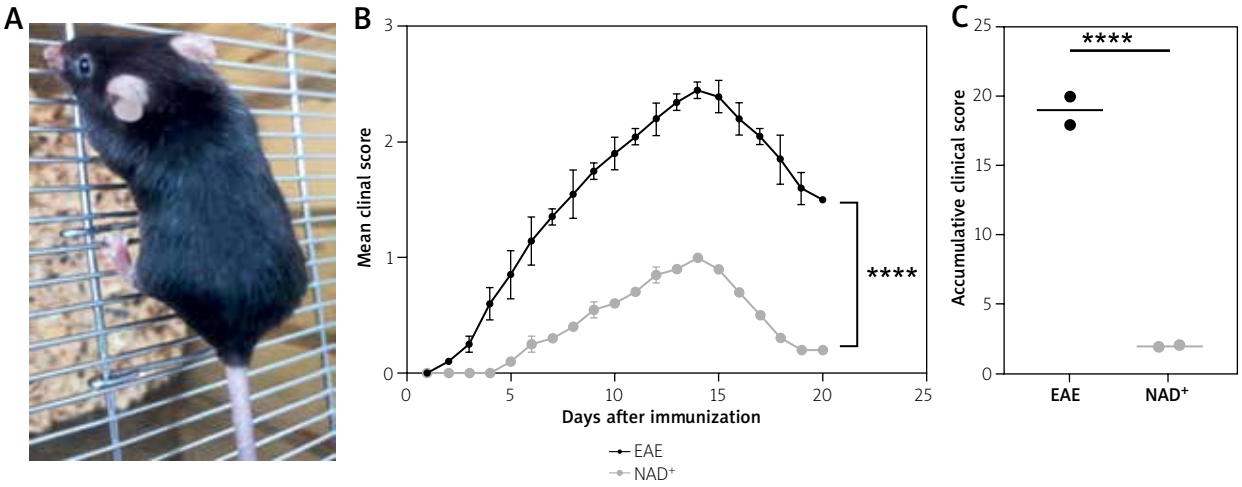

Fig. 1. NAD+ treatment showed a mitigating effect on the clinical severity of experimental autoimmune encephalomyelitis (EAE). **A**) Mice in the EAE model group that received post-immunization saline injections exhibited paralysis in the tail and bilateral limbs. The administration of NAD+ resulted in a significant delay in the increase of the clinical score (**B**) and a reduction in the cumulative score (**C**). The cumulative score represents the total neurological scores recorded from the 2nd day to the 20th dpi following EAE induction in mice, showing a notable preservation of neurological function (n = 7). The neurological function and cumulative scores were significantly lower compared to those in the EAE model group ($p < 0.0001$).

nificant delay in the onset of neurological symptoms compared to the EAE group (13.71 ±0.755 vs. 17.43 ±0.876, respectively) ($p < 0.0001$). The EAE + NAD+ group showed significantly lower neurological function scores and cumulative severity compared to the EAE group ($p < 0.0001$) (Fig. 1B, C).

NAD+ reduces inflammatory factors in EAE

The NLRP3 inflammasome plays a key role in promoting activation of inflammatory factors. Inhibition of NLRP3 expression may provide protection against demyelination and axonal damage induced by EAE. In order to investigate the effect of NAD+ on the NLRP3 inflammasome and its protective effects in the development of EAE, we compared the expression levels of NLRP3 in tissues from the spinal cord in Ctrl, EAE, and EAE + NAD+ groups at 20 dpi. The pro-inflammatory factor NLRP3 exhibited significant expression in tissue from the spinal cord of mice in the EAE group compared to the Ctrl group, indicating the presence of substantial neuroinflammation. However, in the EAE + NAD+ group, NLRP3 expression was lower than in the EAE model group (Fig. 2). The x-axis of the figure reflects the different conditions or treatments, while the y-axis reflects the relative mRNA expression level of the genes. The relative mRNA expression levels are plotted on the y-axis, ranging from 0 to 2.

NAD+ promotes Treg conversion into Th17 cells

To determine whether NAD+ provides the ability to induce the conversion of Tregs into Th17 cells, we analyzed the cytokine profiles of the $CD4^+$ $CD25^+$ $Foxp3^+$ cells that differentiated into $CD4^+$ $IL\text{-}17A^+$ cells. We analyzed mRNA and cytokine levels that are specific to Th1, Th2, Treg, and Th17 cells using RT-PCR in isolated Tregs treated with concentrations of NAD+ at different time points. IL-17A mRNA (Fig. 3F) levels increased in a dose-dependent manner with NAD+. Concurrently, the mRNA levels of TGF-β and the cytokine IL-10, which are associated with Tregs, dramatically decreased (Fig. 3A, E). Likewise, the mRNA levels of IL-4 and IL-6, which are associated with Th2 cells, decreased in a dose-dependent manner with NAD+ (Fig. 3C, D). Notably, we found a slight increase in IFN-γ mRNA levels (Fig. 3B), a representative Th1 cytokine, which is dose-independent of NAD+. The results suggest that NAD+ promotes expression of the IL-17A cytokine.

Discussion

Our findings indicate that NAD+ reduced the severity of symptoms and damage of white matter caused by EAE in mice. EAE is a used model for MS as it mimics various signs and symptoms associated with the disease. Our study showed that the benefits of NAD+ are dependent on the induction of the disease and involve

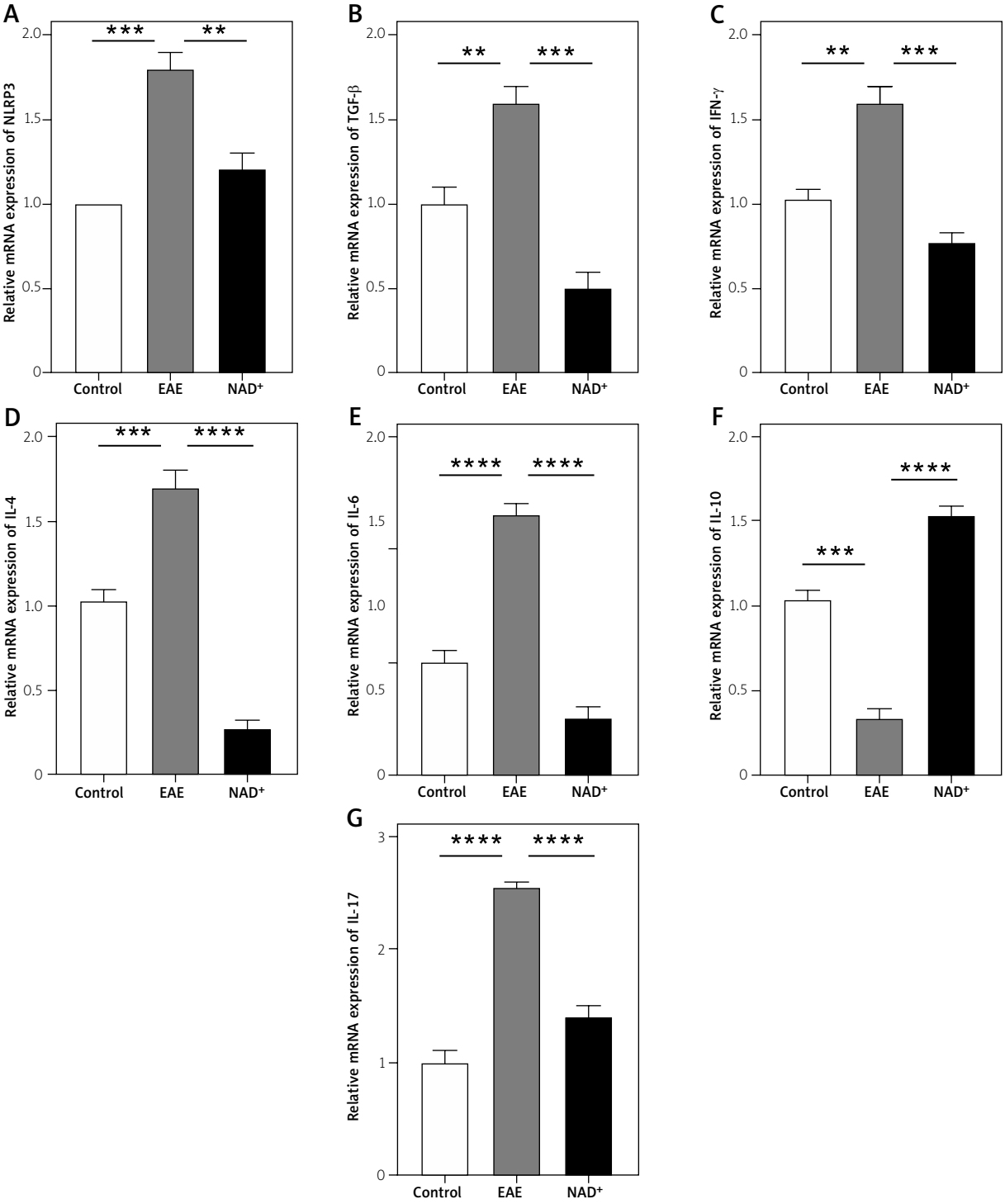

Fig. 2. NAD+ treatment exerted a suppressive effect on pro-inflammatory signaling in experimental autoimmune encephalomyelitis (EAE). The relative expression levels of NLRP3 (**A**), Treg (TGF-β) (**B**), Th1 (IFN-γ) (**C**), Th2 (IL-4, IL-6, and IL-10) (**D-F**), and Th17 (IL-17A) (**G**) in the spinal cord of EAE mice were compared in the groups at 20 dpi using RT-qPCR (n = 7). Treatment of NAD+ attenuated the increased levels of NLRP3 and the pro-inflammatory cytokines IL-17 and IFN-γ in the spinal cord of the EAE mice model. NAD+ treatment increased the expression level of the anti-inflammatory cytokine IL-10. Statistical significance is defined as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

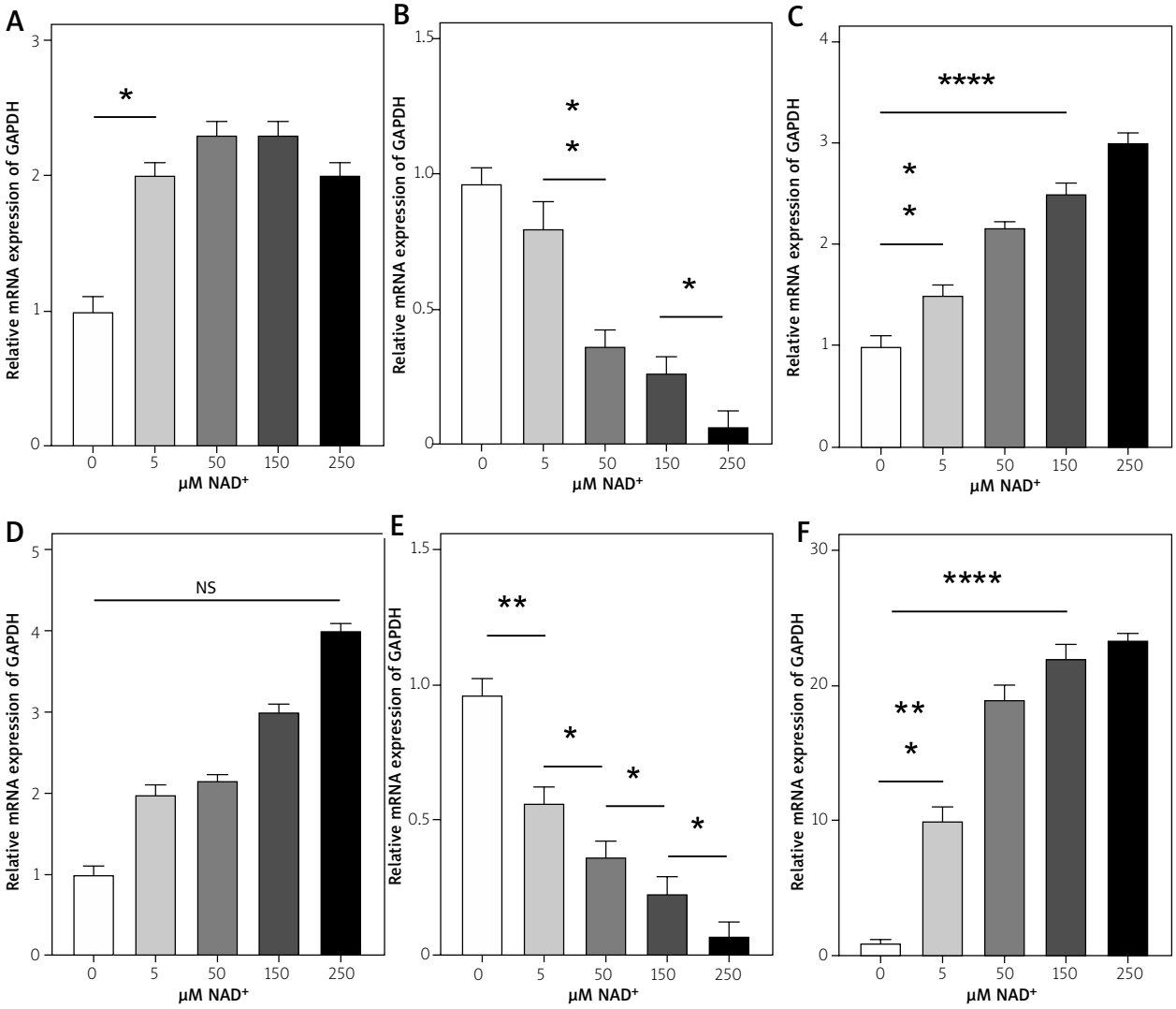

Fig. 3. NAD^+ exerts an effect on Treg conversion into Th17 cells. Tregs were treated with increased concentrations of NAD^+. The mRNA and cytokine levels of Tregs (TGF-β) (**A**), Th1 (IFN-γ) (**B**), Th2 (IL-4, IL-6, and IL-10) (**C-E**), and Th17 (IL-17A) (**F**) were analyzed using RT-PCR ($n = 7$). The data are expressed as mean ± SD. Statistical significance is defined as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and NS, not significant.

downstream signaling of pro-inflammatory cytokines. NAD^+ provides the potential to increase the production of IL-17, even without conditions that promote the differentiation of Th17 cells and with high concentrations of IL-2. Our study demonstrated that NAD^+ promotes Treg cell conversion into IL-17A-producing cells regardless of the cytokine environment.

The role of inflammation-driven synaptic abnormalities as a key pathological mechanism is increasingly acknowledged in MS [8]. Patients with MS manifest notable upregulation of activated Th1 and Th17 subsets with significant production of pro-inflammatory cytokines IL-1 and IL-17, which are increased in patients with MS [7,12,13,28]. Thus, IL-1β promotes the opening of the BBB and blood-spinal cord barrier (BSCB) [2]. IL-1β also promotes the proliferation of neuroinflammatory cells in the CNS, such as macrophages, microglia, and reactive astrocytes [25], and contributes to the differentiation of the native $CD4^+$ T cells into Th17 cells [30]. In contrast, IL-10 exerts immunosuppressive effects and offers protection in case of tissue damage [4]. Microglia, the primary immunocompetent phagocytic cells present in the CNS, migrate to the site of infection [9], neurodegenerative disease, or dysregulation and exhibit increased pro-inflammatory gene expression in lesions and white matter in patients with

MS [40]. Regarding EAE with demyelinated lesions, activated microglia lead to neuronal death by producing inflammatory mediators, including TNF-α, IL-1, and other cytokines that are implicated in MS-related damage [17]. Microglia gained attention in NLRP3 inflammasome research and provide potential for the expression of various inflammation-sensing proteins. NAD+ reduces microglial cell activation, neuroinflammation, and neurodemyelination in EAE mice. NAD+ modulates the differentiation of Th1 and Th17 cells, decreases NLRP3, TGF-β, IFN-γ, IL-4, IL-6, and IL-17 production, and enhances IL-10 production. NAD+ alleviates the pathology of EAE by inducing a shift in the cytokine profile towards an anti-inflammatory state.

NAD+ could contribute to a decrease in p62 expression and a reduction in disease severity. Autophagy plays a negative regulatory role in inflammation by preventing the excessive amplification of inflammatory factors [29], which contribute to demyelination [27]. Autophagy inhibitors, such as 3-methyladenine (3-MA), intensified neuroinflammation and neurodemyelination and also delayed EAE remission. In contrast, NAD+ decreased the expression of NLRP3 and the extent of demyelination injury and promoted the expression of autophagic proteins [42]. Thus, the suppression of autophagy reduced the benefit of NAD+ in both inflammation reduction and cellular protection in mice with EAE. NAD+ mitigates EAE severity through the promotion of autophagy, inhibition of the inflammatory response by degradation of the NLRP3 inflammasome, and the prevention of pro-inflammatory cytokine activation. Autophagy is a type of cellular degradation and recycling pathway that is highly conserved and serves to maintain cellular homeostasis in response to various stressors, including deficiency of nutrients [10]. It is a cellular process responsible for breaking down and recycling components and plays a key role in maintaining cellular balance during stressful conditions, such as nutrient deficiency [43].

The release of inflammatory cytokines initially occurs in inactive forms that require caspase-1-mediated cleavage in a cytosolic complex called the inflammasome to attain their full bioactivity [18]. Among different types of inflammasomes, the NLRP3 inflammasome is extensively studied in the development of various inflammatory conditions such as MS. The NLRP3 inflammasome with elevated activation is related to poor prognosis in MS. Notably, individuals with gain-of-function mutations in NLRP3 display MS high comorbidity [31], indicating that a constantly active NLRP3 inflammasome may serve as a risk factor in MS. The absence of components of the NLRP3 inflammasome protects against demyelination and functional impairments in the EAE mouse model of MS [11,15]. Intriguingly, NAD+ significantly decreased the expression of NLRP3 in the spinal cord of mice with EAE and prevented activation of pro-inflammatory factors, such as TGF-β, IFN-γ, IL-4, IL-6, IL-10, and IL-17A. This reduction in NLRP3 and suppression of pro-inflammatory factors contributed to the alleviation of EAE severity by maintaining the integrity of the BBB and BSCB and preventing excessive leukocyte infiltration into the white matter. Among different types of inflammasomes, the NLRP3 inflammasome is extensively studied in the development of various inflammatory conditions [23].

Our findings indicate that NAD+ provides the ability to promote conversion of Treg cells into IL-17A-producing T cells. The increase in the population of IL-17A+ T cells in mice treated with NAD+ may be attributed to Treg conversion into IL-17A+ cells. Notably, ATP and NAD+ activate the P2RX7 receptor at low levels [32,33]. However, there is a difference between ATP and NAD+ in terms of their requirements for inducing Treg conversion into IL-17A-producing cells. ATP requires IL-6 [6] to facilitate this conversion. NAD+ has a higher affinity for purinergic receptors, potentially contributing to a potent response and the activation of additional signaling pathways.

We found that an increase in the dosage of NAD+ enhances the neurological impairments in EAE mice. Due to the widespread presence of NAD+ as a coenzyme, its anti-inflammatory and therapeutic benefits involve various signaling pathways. Previous work supports the present study in favor of neurological conditions [3,19-22,34,38,39,44,47]. Further investigations are necessary to comprehensively understand these mechanisms and gather more evidence supporting the effectiveness of NAD+ as a prospective treatment for MS.

Limitations

The findings are based on the EAE mouse model, which mimics certain aspects of human MS [37] but does not fully replicate the complexity of the human disease. Therefore, caution should be exercised when extrapolating the results to human patients. The study focused on a specific dosage of NAD+ (250 mg/kg/day) and examined its effects in a controlled experimental setting. The applicability of these findings to different dosages or treatment regimens and diverse patient populations remains to be determined. The study suggests that NAD+ promotes Treg cell conversion into Th17 cells, but the exact molecular mechanisms in this process are not fully investigated. The study did not investigate the long-term effects of NAD+ supplementation or the stability of the improvements. Human studies are needed to validate the findings and determine the relevance of NAD+ supplementation in clinical settings.

Future perspectives

NAD^+ plays a vital role as a metabolite in various cellular processes, such as cellular bioenergetics, genomic stability, mitochondrial homeostasis, adaptive stress responses, and cell survival. Several enzymes that depend on NAD^+ play a key role in synaptic plasticity and neuronal stress resistance. By gaining a deeper understanding of the molecular and cellular mechanisms underlying NAD^+-mediated neuronal resilience, new strategies could be developed to promote healthy brain aging and potentially treat various neurological conditions [16]. Understanding the molecular pathways involved in this process provides valuable insights into the therapeutic potential of NAD^+ supplementation. Determining the optimal dosage and treatment duration of NAD^+ supplementation is important for maximizing its therapeutic effects. Examining the durability of the observed improvements in neuroinflammation, demyelination, and disease scores over an extended period will provide valuable information on the long-term benefits of NAD^+ treatment. Translating the findings from animal models to clinical settings is a key step. Exploring the synergistic effects of NAD^+ supplementation with other therapeutic approaches may enhance treatment outcomes. Investigating the combination of NAD^+ with existing immunomodulatory drugs or other neuroprotective agents may lead to improved therapeutic strategies for demyelinating disorders. Identifying reliable biomarkers and predictive factors associated with the response to NAD^+ supplementation may aid in patient selection and treatment monitoring. Machine learning methods could be implemented to predict diseases [36]. Future studies could focus on identifying biomarkers that could help predict treatment response and disease progression, enabling personalized therapeutic approaches.

Conclusions

NAD^+ treatment attenuates the severity of EAE by promoting Treg conversion into Th17 cells with increased concentrations of NAD^+. NAD^+ suppresses inflammatory responses and inflammatory cell infiltration that cause demyelination in the CNS.

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Disclosures

The experiments were performed at the Department of Neurosurgery and were approved by the Experimental Ethics Committee of The Second Affiliated Hospital of Shandong First Medical University, No. 706, Taishan Street, Taian 271000, Shandong Province, China. All methods were carried out in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

The authors report no conflict of interest.

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