High-Intensity Exercise Training Protects The Brain Against Autoimmune Neuroinflammation: Regulation of Microglial Redox and Pro-Inflammatory Functions

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Research

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Abstract

Background: Exercise training induces beneficial effects on neurodegenerative diseases, and specifically on multiple sclerosis (MS) and its model experimental autoimmune encephalomyelitis (EAE). However, it is unclear whether exercise training exerts direct protective effects on the central nervous system (CNS), nor are the mechanisms of neuroprotection fully understood. In this study, we investigated the direct neuroprotective effects of high-intensity continuous training (HICT) against the development of autoimmune neuroinflammation and the role of resident microglia.

Methods: We used the transfer EAE model to examine the direct effects of training on the CNS. Healthy mice performed HICT by treadmill running, followed by injection of encephalitogenic proteolipid (PLP)-reactive T-cells to induce EAE. EAE severity was assessed clinically and pathologically. Brain microglia from sedentary and HICT healthy mice, as well as 5-days post EAE induction (prior to onset of disease) were analyzed *ex vivo* for reactive oxygen species (ROS) and nitric oxide (NO) formation, mRNA expression of M1/M2 markers and neurotrophic factors, and secretion of cytokines and chemokines.

Results: Transfer of encephalitogenic T-cells into HICT mice resulted in milder EAE, compared to sedentary mice, as indicated by reduced clinical severity, attenuated T-cell and neurotoxic macrophage/microglial infiltration, and reduced loss of myelin and axons. In healthy mice, HICT reduced the number of resident microglia without affecting their profile. Isolated microglia from HICT mice after transfer of encephalitogenic T-cells exhibited reduced ROS formation and released less IL-6 and monocyte chemoattractant protein in response to PLP-stimulation.

Conclusions: HICT protects the CNS against autoimmune neuroinflammation by reducing microglial-derived ROS formation, neurotoxicity and pro-inflammatory responses involved in propagation of autoimmune neuroinflammation.

Background

Recent evidence highlights a favorable outcome for extensive exercise training in the treatment of Parkinson's Disease (PD) [1, 2], Alzheimer Disease (AD) [3, 4], stroke [5] and mood and anxiety disorders [6]. Even in the absence of pathological conditions, exercise training has been shown to improve cognitive function and spatial memory through increased regional neurogenesis and plasticity in mice [7]. Accordingly, there is a large body of evidence indicating beneficial outcomes of exercise among multiple sclerosis (MS) patients [8, 9]. Although the favorable effects of exercise on brain health are well accepted, a thorough understanding of the neuroprotective effects of exercise on autoimmune neuroinflammatory diseases is still lacking.

We recently demonstrated peripheral-systemic immunomodulatory effects of moderate-intensity continuous training (MICT) in transfer experimental autoimmune encephalomyelitis (EAE), an animal model used for the study of autoimmune-mediated disease of the central nervous system (CNS) [10]. MICT reduced the encephalitogenicity of autoreactive T cells and attenuated the clinical severity of

transfer EAE. We further demonstrated that high-intensity continuous training (HICT) induced superior benefits in preventing systemic autoimmunity in EAE as compared to MICT [11]. Interestingly, we showed that MICT did not result in a direct protective effect on the CNS from encephalitogenic T cells [10]. This prompted us to investigate whether HICT, in addition to its superior systemic immunomodulatory effect. will also induce a protective effect directly on the CNS against autoimmune neuroinflammation, as well as potential mechanisms whereby training may induce direct neuroprotection.

Accumulating data indicate that microglia are pivotal in mediating neuroinflammation, demyelination, and neurodegeneration in MS and EAE [12]. Toxic activation of microglia is apparent during early and late MS and EAE, and correlates with axon and oligodendrocyte pathology [13]. Microglial modulation is considered to promote CNS well-being by exercise training [14]. These findings prompted us to examine whether microglia play a role in training-mediated neuroprotection.

Here, we investigate the direct effects of HICT on neuroprotection and the development of autoimmune neuroinflammation in EAE, employing the transfer EAE model, whereby we administer encephalitogenic T cells into HICT and sedentary (SED) mice. We show that HICT protects the brain from encephalitogenic T cells, resulting in reduced neuroinflammation and tissue injury. HICT results in reduced toxic and pro-inflammatory activation of resident microglia.

Methods

Experimental animals

Female SJL/JCrHsd mice (6–7 wks of age) were purchased from Envigo Inc, Israel. Animal experimentation was approved by the Institutional Animal Care and Use Committee. The studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Experimental design

The proteolipid (PLP) $_{139-151}$ transfer EAE model in mice was utilized as previously described [10, 11, 15]. This model enables isolation of the direct effects of HICT on the CNS, as indicated by induction of EAE following transfer of encephalitogenic T cells (Fig. 1A). Healthy mice were subjected to a 6-week treadmill high-intensity continuous training (HICT) program. PLP-reactive, encephalitogenic lymph node-T cells from donor mice were injected into trained mice, 72 h prior to the last exercise bout. Sedentary (SED) mice were injected with the same PLP-reactive encephalitogenic T cells and served as controls. We examined whether the treadmill running program of the recipient mice prior to transfer of encephalitogenic T cells induced direct neuro-protective effects on the CNS as follows: (1) by *in vivo* clinical and pathological severity of EAE following transfer of encephalitogenic T cells (Fig. 1A); and (2) by *ex vivo* functional analysis of resident microglia isolated from HICT and SED mice at two time points: from healthy mice, prior to transfer of encephalitogenic T cells (Fig. 4A) and from EAE mice 5 days after transfer of PLP, prior to infiltration of systemic immune cells into the CNS and to clinical onset of EAE (pre-onset EAE, Fig. 5A).

Treadmill exercise training

Of the various exercise protocols that we have employed to date [MICT, HICT and high-intensity interval training (HIIT)], HICT was found to be most effective in attenuating disease progression via systemic immunomodulation [11] (and data not shown). Therefore, healthy mice underwent 6-wks of treadmill running and performance tests on a 5-lane treadmill designed for mice (Panlab Harvard Apparatus, USA), using the HICT protocol we established earlier [10, 11]. The running speed in the HICT protocol was based on exhaustion speed performance tests, as previously described [11].

Transfer experimental autoimmune encephalomyelitis (EAE)

The PLP $_{139–151}$ transfer EAE model in mice was utilized as previously described [15]. EAE was induced in recipient HICT (n = 10) and SED (n = 10) mice by a transfer of encephalitogenic lymph node (LN) - T cells obtained from PLP-immunized donor mice (Fig. 1A). Recipient EAE mice developed clinical signs of EAE 7–10 days post transfer of encephalitogenic T cells and were assessed daily for neurological symptoms for up to 50 days after EAE induction.

Histopathology analyses

At twelve days (at the acute phase of disease) and 50 days (at the chronic phase of disease) after encephalitogenic LN- T cell transfer, groups of SED (n = 6 for each time point) and HICT (n = 6 for each time point) EAE mice were sacrificed for histopathological analysis as previously described [10]. Serial paraffin-embedded transverse sections were obtained from mid-cervical, mid-thoracic and mid-lumbar levels of the spinal cords. Sections were stained with hematoxylin and eosin (H&E), Luxol fast blue (LFB)/nuclear fast red and Bielschowsky silver impregnation, to assess inflammation, demyelination and axonal damage, respectively. Immunohistochemistry was performed in adjacent serial sections for macrophages (rat anti-mouse Mac3, 553322, 1:800, BD Pharmingen), T cells (monoclonal rabbit anti–CD3, RM-9107-SO; 1:800, Thermo-Scientific) and amyloid precursor protein (APP; monoclonal mouse anti-APP, MAB 348; 1:2000; Millipore). Goat anti-rat (sc2041 Santa Cruz), goat anti-rabbit (BA 1000, Vector) and goat anti-mouse (BA9200, Vector) were used as secondary antibodies appropriately. The 3,3'-diaminobenzidinetetrahydrochloride was used as chromogen and sections were counterstained with hematoxylin. Immunofluorescence was performed for Iba-1 (polyclonal rabbit anti-IBA1, 019-19741,Wako), inducible nitric oxide synthase (iNOS; monoclonal mouse, sc-7271, Santa Cruz), arginase –1 (Arg-1 (polyclonal goat, sc-18354, Santa Cruz). Anti-rabbit CF488A (20012, Biotium), anti-goat CF555 (20039, Biotium) and anti-mouse CF555 (20030, Biotium) were used as secondary antibodies appropriately. Sections were mounted with Dapi (23004, Biotium). For each staining, the whole white matter of three sections per mouse was quantified, one section per spinal cord level. The number of immune cells in perivascular infiltrates were counted in H&E stained sections, and reported as the total average number per square millimeter. Mac3+, CD3 + cells, and double positive Iba-1+, iNOS + and Iba1+, Arg-1 + were counted in the perivascular infiltrates and parenchyma, and reported as total average number of each cell type per square millimeter. Demyelination was assessed by calculating the area of

LFB loss. For the quantification of acute axonal injury, APP + axonal swellings and spheroids were counted, and the average of APP + profiles per square millimeter was calculated. For chronic axonal damage, the area of reduced axonal density in Bielschowsky silver staining was assessed. All pathology measurements were performed by using the Image J software analysis (ver. 1.51H, NIH, USA).

Ex vivo analyses of CD11b+ microglia

Microglia were isolated (see below) from HICT and SED mice brains 48 h after the last exercise bout (n = 5–8/group). Microglia were isolated from healthy mice (Fig. 4A) or 5 days following transfer of encephalitogenic PLP-reactive T cells (transfer occurred 72 h before last bout of exercise), prior to appearance of clinical symptoms (pre-onset EAE mice; Fig. 5A). This time point was selected according to our preliminary results confirming that no inflammatory infiltrates were present in the brain tissue (data not shown). Freshly isolated CD11b + microglia were seeded on poly-L-lysine –covered 96 well plates and were activated over-night with either 200 ng/ml lipopolysaccharide (LPS, E. coli O111:B4, Sigma-Aldrich; for healthy- derived microglia) or 10 µg/ml PLP peptide (BioBasic; for pre-onset EAE- derived microglia). Activated microglia were analyzed for reactive oxygen species (ROS) and nitric oxide (NO) formation [(using enzyme-linked immunosorbent assay (ELISA)], cytokine gene determination [real time-PCR (RT-PCR)] and cytokine and chemokine secretion (MAGPIX System).

Microglia isolation

Brain tissue from HICT and SED healthy or pre-onset EAE mice were dissociated to single cell suspension, using the Neural Tissue Dissociation Kit (Miltenyi Biotec). Myelin was removed using Percoll (GE Healthcare) followed by microglia isolation using CD11b microbeads and MS columns (Miltenyi Biotec) according to manufacturer instructions. Degree of microglia enrichment was assessed by CD11b (BD Bioscience, M1/70) staining and flow cytometry analysis (Beckman Coulter). In all experiments at least 85% of isolated cells expressed CD11b.

Nitric oxide (NO) and reactive oxygen species (ROS) formation

NO formation was assessed (on 25×10^6? cells/well) using Greiss Reagent System according to manufacturer's protocol (Promega, G2930) and quantified using an ELISA plate reader (Tecan Spark 10M). ROS formation was measured (on 5×10^6 cells/ well) using DCFDA dye according to manufacturer's protocol (abcam, AB-ab113851) and quantified with an ELISA plate reader (Beckman Coulter DTX 880 multimode detector).

Cytokine gene determination

Total RNA was prepared using the RNeasy Plus Mini Kit (QIAGEN) from microglia. cDNA was prepared from 300 ng total RNA using qScript cDNA Synthesis Kit (Quanta Biosciences). Reaction mixture included 1 µl of cDNA, 300 nM of appropriate forward and reverse primers (Agentek) and 5 µL PerfeCTA SYBR

Green FastMix ROX (Quanta Biosciences) to a total volume of 10 µL. Gene amplification was carried out using the StepOnePlus real-time PCR system (Applied Biosystems).

Cytokine and chemokine secretion assessment

Cytokine and chemokine concentrations in LPS or PLP -stimulated microglia supernatants were measured with MILLIPLEX® MAP mouse high sensitivity magnetic bead panels, according to manufacturer instructions (EMD Millipore Corp., MO, United States). The Luminex xMAP® technology was utilized, based on immunoassay performed on the surface of fluorescent-coded magnetic beads MagPlex®-C microspheres. Acquisition and data analysis were performed using Luminex analyzer (MAGPIX®) software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analyses

For performance tests, the values before and after training for each experimental group were compared using Student's paired *t* test. For clinical parameters and microglia analyses, experimental groups were compared using unpaired Student's *t* tset. For pathology parameters, the experimental groups were compared using the two-tailed Mann-Whitney test. Data were analyzed in GraphPad Prism software v.5. Differences were considered statistically significant at $p < 0.05$. All data are presented as mean ± standard error of mean (SE).

Results

High-intensity continuous training (HICT) improves physical performance

Based on performance test conducted before and at the end of the training program, HICT significantly improved both maximal speed (~ 10%, 44.5 ± 0.7 vs. 47.0 ± 0.6 cm/s, $p < 0.05$) and exercise tolerance (~ 100%, 15:09 ± 0:20 vs. 29:39 ± 2:08 min:s, $p < 0.001$). No significant performance changes were noted in SED mice (exhaustion speed: 42.0 ± 1.2 vs. 42.0 ± 1.3 cm/s, $p > 0.05$; exercise tolerance: 14:25 ± 1:10 vs., 17:±1:32 min:s, $p > 0.05$).

Training induces direct CNS protection from autoreactive encephalitogenic T cells

The transfer of PLP-reactive encephalitogenic T cells from donor mice induced a milder clinical course of EAE in HICT recipient mice, as compared to SED mice (Figs. 1B and C). The day of onset was significantly delayed by ~ 3 days in the HICT group ($p < 0.01$) vs. SED group and the overall burden of disease was significantly lower (> 40%, $p < 0.05$) in HICT mice than in control SED mice (Fig. 1C). Although the average maximal clinical score of disease in the HICT group was decreased by almost 20%, the decrease was not statistically significant (Fig. 1C).

Demyelination and acute axonal injury were assessed at the peak of the acute relapse (day 12 post-transfer, Fig. 2A-F). LFB staining showed a significant > 20% reduction in the extent of demyelination in HICT EAE mice vs. control SED EAE mice (Figs. 2A-C). APP immunohistochemistry at the acute phase showed > 30% reduction in the number of injured axons (Fig. 2D-F). Demyelination and chronic axonal loss were evaluated also at the chronic phase of EAE (day 50 post-transfer, Fig. 2G-L). At this stage, the protective effect of HICT was even more pronounced. In HICT EAE mice the area of demyelination (Figs. 2G-I) and chronic axonal loss (Figs. 2J-L) were reduced by ~ 50%.

Since the severity of tissue damage in EAE is related to the autoimmune inflammatory process [16–18], we examined whether HICT protected the CNS from the destructive inflammatory process. SED EAE mice exhibited extensive acute inflammation (Figs. 3A, D, G), whereas HICT EAE mice exhibited substantially less inflammation (Figs. 3B, E, H). The overall immune cell, CD3 + T- cell and Mac3 + macrophages infiltrations were decreased by 20–50% (Figs. 3C, F, I). Evaluation of neuroinflammation at the chronic phase of disease (day 50 post-transfer, Figs. 3J-R) revealed no significant differences in inflammatory cell counts between the two experimental groups.

Training modulates microglial inflammatory and neurotoxic properties

The *in vivo* experiments showed that HICT induced CNS protection against the PLP-reactive encephalitogenic T cells and their destructive effects on the CNS. Since training protected the CNS directly, we examined the effect of HICT on the CNS innate immune system. Microglia mediate the recruitment and activation of systemic immune cells, and induce neurotoxicity in chronic neuroinflammatory and neurodegenerative disorders [19]. We therefore examined the microglial profile *in vivo* at the acute phase of EAE. HICT induced a marked ~ 60% decrease in the number of neurotoxic Iba1+, iNOS+ (M1 type) microglia compared to SED control (Figs. 4A-C). No differences were noted in the number of M2 type Iba-1+, Arg-1 + microglia between HICT and SED EAE mice (Figs. 4D-F).

Since systemically administered PLP-reactive encephalitogrenic T cells encountered the CNS milieu that had already been modulated by exercise training we next characterized microglia at the end of the training or sedentary period, before the transfer of encephalitogenic T cells. To that end, CD11b + microglia were isolated for *ex vivo* evaluation. (Fig. 5A). The total number of CD11b + microglia was significantly reduced by HICT vs. SED controls (> 30%, Fig. 5B). Moreover, HICT decreased the mRNA level of iNOS (~ 50%, Fig. 5C) in isolated CD11b + microglia, but did not affect mRNA levels of Arg1 and CD206 (Fig. 5C), nor brain derived neurotrophic factor (BDNF) or nerve growth factor (NGF; Fig. 5D). Also, the ability of CD11b + microglia from HICT mice to respond to LPS stimulation *ex-vivo* was not affected when compared to SED mice, in terms of reactive oxygen species (ROS; Fig. 5E) and nitric oxide (NO; Fig. 5F) formation, as well as release of cytokines (Figs. 5G-K) and chemokines (Figs. 5L-N). These findings indicate that HICT did not alter the ability of microglia to respond to an infectious challenge.

Then, the response of brain microglia to the transfer of encephalitogenic T cells was examined at days 5 post-transfer, a time point of initial invasion of autoimmune cells into the CNS, and prior to any clinical

manifestations (Fig. 6A). Noteworthy, induction of EAE by T-cell transfer in HICT mice restored brain content of microglia to control levels (Fig. 6B), indicating normal reactivity. However, when re-stimulated with PLP in culture, microglia derived from HICT mice exhibited marked reduction in ROS formation compared to SED mice (~ 40%, Fig. 6C). Reduction in ROS formation was also observed when HICT-derived microglia were stimulated with LPS in culture, compared to control (> 30%, Fig. 6E). HICT did not affect NO formation by microglia after PLP (Fig. 6D) or LPS (Fig. 6F) stimulation *in vitro*, compared to SED controls.

Finally, we examined the effect of training on the inflammatory profile of microglia isolated from the brain at 5 days after transfer of encephalitogenic T cells and following PLP re-stimulation *in vitro*, as reflected by measuring cytokine and chemokine secretion (Figs. 6G-N). HICT mice -derived microglia exhibited reduced interleukin (IL) − 6 (> 30%, Fig. 6G) and monocyte chemoattractant protein (MCP)- 1 (~ 25%, Fig. 6L) release. There was no difference in the release of other cytokines (Figs. 6H-K) / chemokines (Figs. 6M, N).

Discussion

We investigated the direct effects of training on autoimmune neuroinflammation using the transfer EAE model. High-intensity training provided direct protection to the CNS from autoimmune neuroinflammation, resulting in attenuated disease progression, and reduction in inflammation- driven demyelination and axonal loss. The neuroprotective effect appears to be mediated, at least partly, by modulating the CNS innate immune system and reducing the microglial neurotoxic and pro-inflammatory response to T-cell mediated autoimmune neuroinflammation. Our study provides the first demonstration of direct neuroprotective effect of training on autoimmune neuroinflammation and points to the critical role of training intensity in this process.

Earlier studies suggested various mechanisms to account for the beneficial effects of training on EAE [20–26]. However, these studies used active EAE models that could not distinguish between the effects of training on the systemic immune system vs. direct protective effects on the CNS to reduce encephalitogenicity. The transfer EAE model used in the present study enables this distinction.

In our previous work, we utilized the PLP-transfer EAE model to show that moderate-intensity training of recipient mice did not affect the clinical course nor the CNS pathology of EAE [10]. Here we show that when recipient mice undergo high-intensity training, their CNS is protected from the deleterious effects of encephalitogenic T cells, resulting in milder tissue pathology and clinical symptoms of EAE. Thus, training intensity is paramount for inducing direct neuroprotection against autoimmune neuroinflammation.

Interestingly, the inflammatory process was attenuated in HICT mice only during the acute phase. Since the training program ended soon after (72 h) injection of encephalitogenic T cells, it is possible that the protection of training on the CNS inflammatory process is a short-term effect. Alternatively, the lack of attenuation of neuroinflammation at the chronic phase reflects the observed partial spontaneous

resolution of neuroinflammation after the acute relapse. However, most tissue injury occurs in the early phase of EAE, and was therefore reduced in HICT mice in both the acute and chronic phases.

Microglia play a crucial role in the maintenance of CNS homeostasis in health and disease [27]. Activated microglia produce a wide range of inflammatory and toxic mediators, which contribute to the recruitment of immune cells and the spread of the inflammatory response in the CNS of MS and EAE [28–30]. The observation that a decrease in microglial reactivity has been associated with a beneficial effect in EAE [31–33] led to the perception of microglia as detrimental cells in EAE pathogenesis. Thus, we hypothesized that microglia are potential key components to mediate the training- induced neuroprotection in the CNS. Indeed, HICT induced a substantial decrease in the number of iNOS + microglia in EAE mice. Interestingly, HICT did not affect the number of Arg-1 + microglia. These findings suggest that training reduces the neurotoxic profile of microglia, rather than inducing their shift to M2 phenotype.

Earlier studies demonstrated that microglial reactivity precedes the onset of EAE, and that inhibition of their activation suppresses the development and maintenance of inflammatory lesions in the CNS [34]. We therefore further examined the profile and characteristics of microglia *ex vivo* at the end of the training program and following EAE induction, but prior to infiltration of encephalitogenic T cells into the CNS. The current study suggests that HICT modulates microglia phenotype following EAE induction to attenuate neuroinflammation. In our experimental paradigm, training did not prevent the formation of encephalitogenic T cells, which were obtained from donor mice, but rather affected brain innate immune cells in trained recipient mice, and prevented neuroinflammation and tissue destruction.

Importantly, both over-activation and suppression to a mal-functional state of microglia are deleterious to brain health [35, 36]. Restoration of microglial homeostasis, rather than their elimination or total inhibition, is the preferred therapeutic target. Accordingly, our findings in healthy mice demonstrate that training reduces the number of microglial cells, without affecting their ability to produce immune mediators, nor affecting their M1/M2 phenotype. Furthermore, training did not induce a general suppressive effect on microglia derived from mice prior to onset of EAE. Rather, training induced modulation of microglial neurotoxic phenotype, as indicated by reducing ROS, IL-6 cytokine and MCP-1 chemokine production in response to PLP and LPS stimulation. The lack of a general suppressive effect of training on microglial function is important in enabling the still activated, HICT-derived microglia to participate in their homeostatic and regenerative roles.

A key finding in the current study was the reduction in PLP-mediated secretion of the pro-inflammatory cytokine IL-6 and the MCP-1 chemokine and ROS formation by HICT pre-onset EAE microglia. Studies indicate that intrinsic production of IL-6 in the brain is necessary for the induction of EAE, and plays an important role in modulating the development and progression of the disease [37–39]. IL-6 is necessary for the induction of cerebrovascular adhesion molecules, which are crucial for leukocyte trafficking to the CNS in EAE [40]. MCP-1 is detected prior to the onset of inflammation in EAE and plays an important role for the influx of inflammatory cells into the CNS [41–43]. There is a positive correlation between the

expression of MCP-1 in the CNS and the degree of CNS inflammation and severity of EAE [44]. Taken together, our finding suggests that the inhibitory effect of HICT on IL-6 and MCP-1 secretion by microglia contribute to the decrease in inflammatory infiltration into the CNS and attenuation of acute CNS inflammation, tissue injury and clinical severity of EAE.

The positive effects of HICT on disease progression in the present study is not associated with a decrease in microglial NO formation in response to PLP stimulation. In contrast, the ROS response to PLP was markedly decreased by HICT. NO and ROS play a central role in microglial neurotoxicity [19]. Indeed, excessive formation of ROS contributes to the death of neurons of the mammalian CNS in various neurodegenerative diseases [45, 46]. Accordingly, ROS have been implicated as a mediator of demyelination and axonal injury in both EAE and MS [47, 48]. The formation of ROS is dependent on the simultaneous production (starting with generation of the superoxide anion (O_2^{*-}), which leads to the formation of other ROS species, such as H_2O_2 and hydroxyl radicals) and removal of ROS (generally achieved by antioxidant enzymes such as superoxide dismutase, glutathione peroxidase [using reduced glutathione, GSH] and catalase) [49]. Exercise results in increased production of ROS, especially at high intensities [49, 50], and it is therefore unlikely that the decreased formation of ROS following HICT is due to a decreased capacity to produce ROS. On the other hand, it is well documented that exercise training increases the activity of antioxidant enzymes and the levels of GSH in skeletal muscle [51]. Similarly, exercise training also increases antioxidant defense systems in some but not all rodent brain cell populations [52–54]. Therefore, we hypothesize that the decreased ROS response to PLP-stimulation in HICT microglia derives from increased removal of ROS species owing to elevated antioxidant defense systems [55]. If this hypothesis is correct, then one would expect that administration of exogenous antioxidants to EAE-SED mice would mimic the effects of HICT on disease progression. Indeed, a recent study using experimental conditions comparable to those of the current study demonstrated that administration of a mitochondria-targeted antioxidant (Mito-Q) to EAE-SED mice diminished clinical progression of the disease [56]. Moreover, the increased mRNA levels of CD11b and IL6 in spinal cords of EAE-SED mice were markedly decreased by administration of Mito-Q. Additionally, neurodegeneration and neuroinflammation in spinal cord sections of EAE-SED mice were also diminished by Mito-Q. Taken together, our results, together with those from the literature, suggest that HICT attenuates disease progression by inhibiting ROS formation in microglia. Although the emphasis of microglia in mediation of the beneficial effects of exercise on neuroprotection is supported by considerable experimental evidence [14], our findings do not preclude a positive effect of exercise training on other brain cell populations as well.

Conclusions

High-intensity training induces a direct neuroprotective effect in an experimental model of autoimmune neuroinflammation. CNS microglia serve as a key therapeutic target for neuroinflammation, and their modulation by HICT may reduce their neurotoxic and pro-inflammatory properties. These findings may

also be applicable to other neurodegenerative diseases driven by microglial neurotoxicity, such as AD and PD.

List Of Abbreviations

AD: Alzheimer Disease

APP: amyloid precursor protein

Arg-1: arginase -1

BDNF: brain derived neurotrophic factor

CNS: central nervous system

EAE: experimental autoimmune encephalomyelitis

H&E: hematoxylin and eosin

HICT: high-intensity continuous training

HIIT: high-intensity interval training

IL: interleukin

iNOS: inducible nitric oxide synthase

LFB: Luxol fast blue

LN: lymph node

LPS: lipopolysaccharide

MCP: monocyte chemoattractant protein

MICT: moderate-intensity continuous training

MS: multiple sclerosis

NGF: nerve growth factor

NO: nitric oxide

PD: Parkinson's Disease

PLP: proteolipid

ROS: reactive oxygen species

SED: sedentary

TNF: tumor necrosis factor

Declarations

Ethics approval: All experiments were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals and were approved by the Ariel University Institutional Animal Care and Use Committee.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and analyzed are available from the corresponding author on reasonable request.

Competing interests: The authors declare no competing financial interests.

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Authors' contributions: YZ designed research studies, conducted experiments, acquired data, analyzed data and wrote the first draft. NF designed research studies, conducted experiments, acquired and analyzed data. OT conducted experiments, acquired and analyzed data. YG, LH, SS, HN, SZ conducted experiments. NG and AK analyzed data and critically reviewed the manuscript. TBH designed research studies, analyzed data and critically reviewed the manuscript. OE designed research studies, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

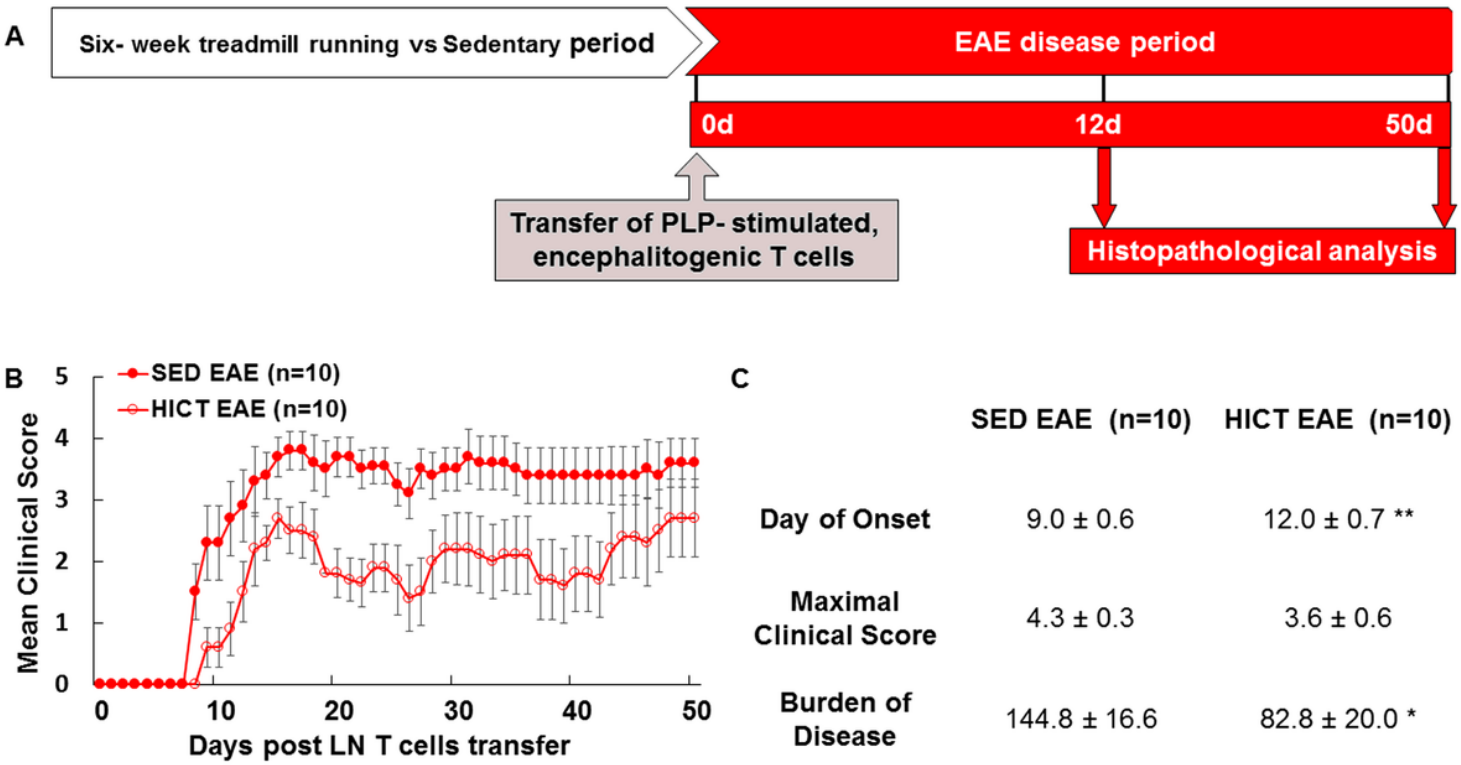
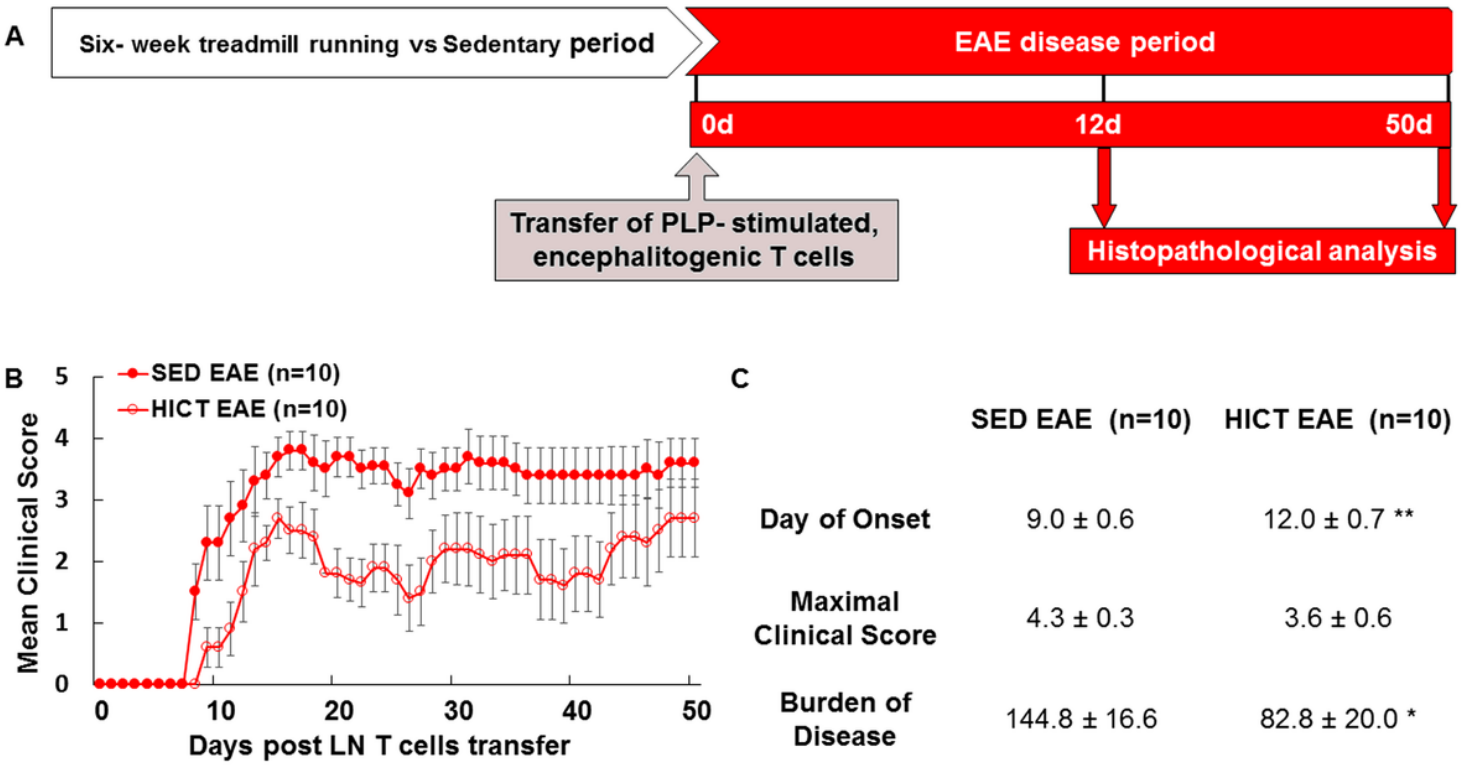


Figure 1

High-intensity training attenuates the clinical course of recipient mice in a transfer model of EAE. (A) Experimental time line to investigate the direct effects of exercise training on central nervous system in transfer EAE model. Healthy mice were subjected to a 6 week- high-intensity continuous training (HICT) treadmill-running program and served as recipient mice to further develop EAE. Another group of naïve donor mice were immunized with PLP peptide, and after 10 days their lymph node-T cells were removed and stimulated in culture with PLP peptide. The encephalitogenic T cells were injected into either HICT, 72 h prior to last exercise bout, or sedentary control recipient mice, which developed EAE and were scored daily for neurological symptoms up to 50 days post-transfer. Mice were sacrificed for central nervous system histopathology analyses at 12 days and 50 days post EAE induction, at the acute phase and chronic phase of disease, respectively. Clinical course (B) and clinical parameters (C) of EAE in recipient sedentary (SED EAE, n=10) and HICT (HICT EAE, n=10) mice following transfer of encephalitogenic T cells. The severity of EAE was scored on a 0–6 scale. Transfer of encephalitogenic T cells to HICT recipients induced a significantly milder EAE course. DOO – day of onset, MCS – maximal clinical score, BOD – burden of disease. Data are mean ± SE. *p<0.05, **p<0.01 vs. SED EAE.



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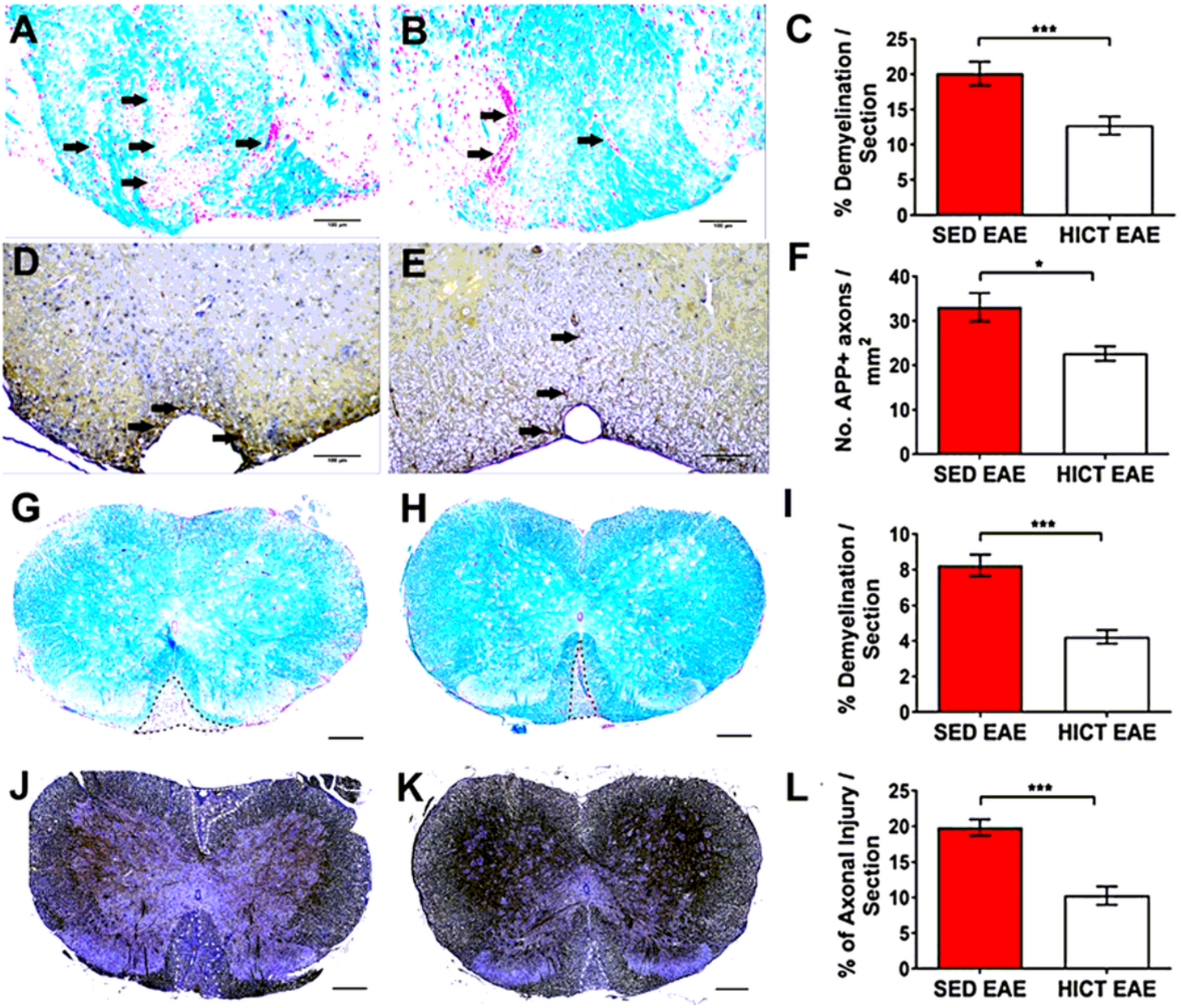

Figure 2

Reduction of tissue pathology in the spinal cords of high- intensity trained EAE mice. Evaluation of demyelination (A-C, G-I) and axonal damage (D-F, J-L) on cross sections of the spinal cords of sedentary (SED EAE: A, D, G, J, n=6) and high-intensity continuous trained (HICT EAE: B, E, H, K, n=6) mice that were injected with encephalitohenic T cells and developed EAE. A-F –12 days post EAE induction, acute phase of disease; G-L –50 days post EAE induction, chronic phase of disease. A, B: Arrows – indicate areas of demyelination; D, E: Arrows indicate amyloid precursor protein (APP) + injured axons; G, H: Dashed lines – indicate area of demyelination; J-K: Dashed lines indicate area of axonal loss. C, F, I, L – quantification of tissue pathology in spinal cord white matter. Luxol fast blue (LFB) histochemistry with periodic acid schiff (PAS) counterstaining showed reduction in the area of demyelination in HICT EAE (B, H) vs. SED EAE (A, G) at the acute (C) and chronic (I) phases of disease. In HICT EAE there were less APP + injured axons (E, arrows) than in SED EAE (D) at the acute phase (F). Bielschowsky staining at the chronic phase

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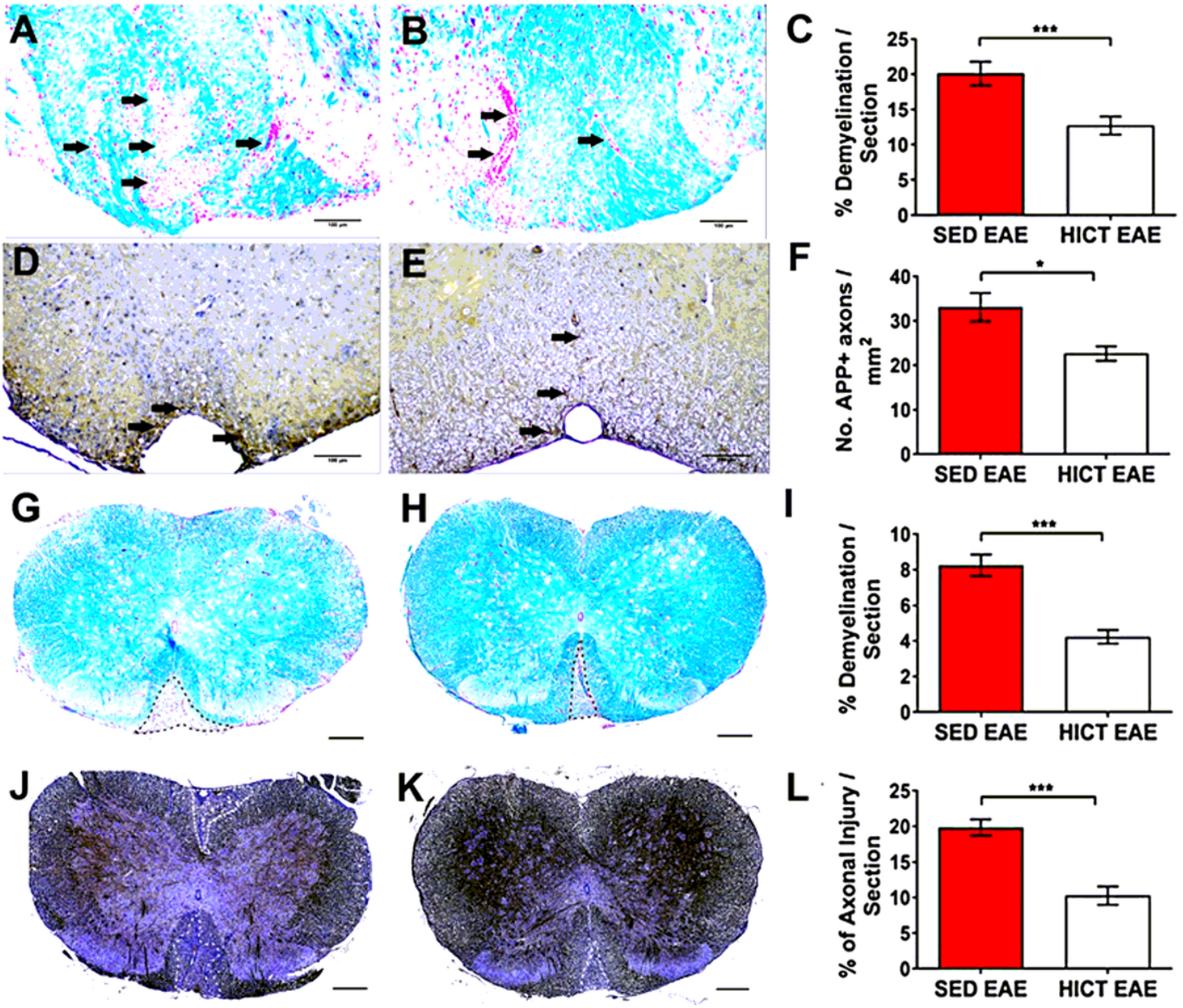

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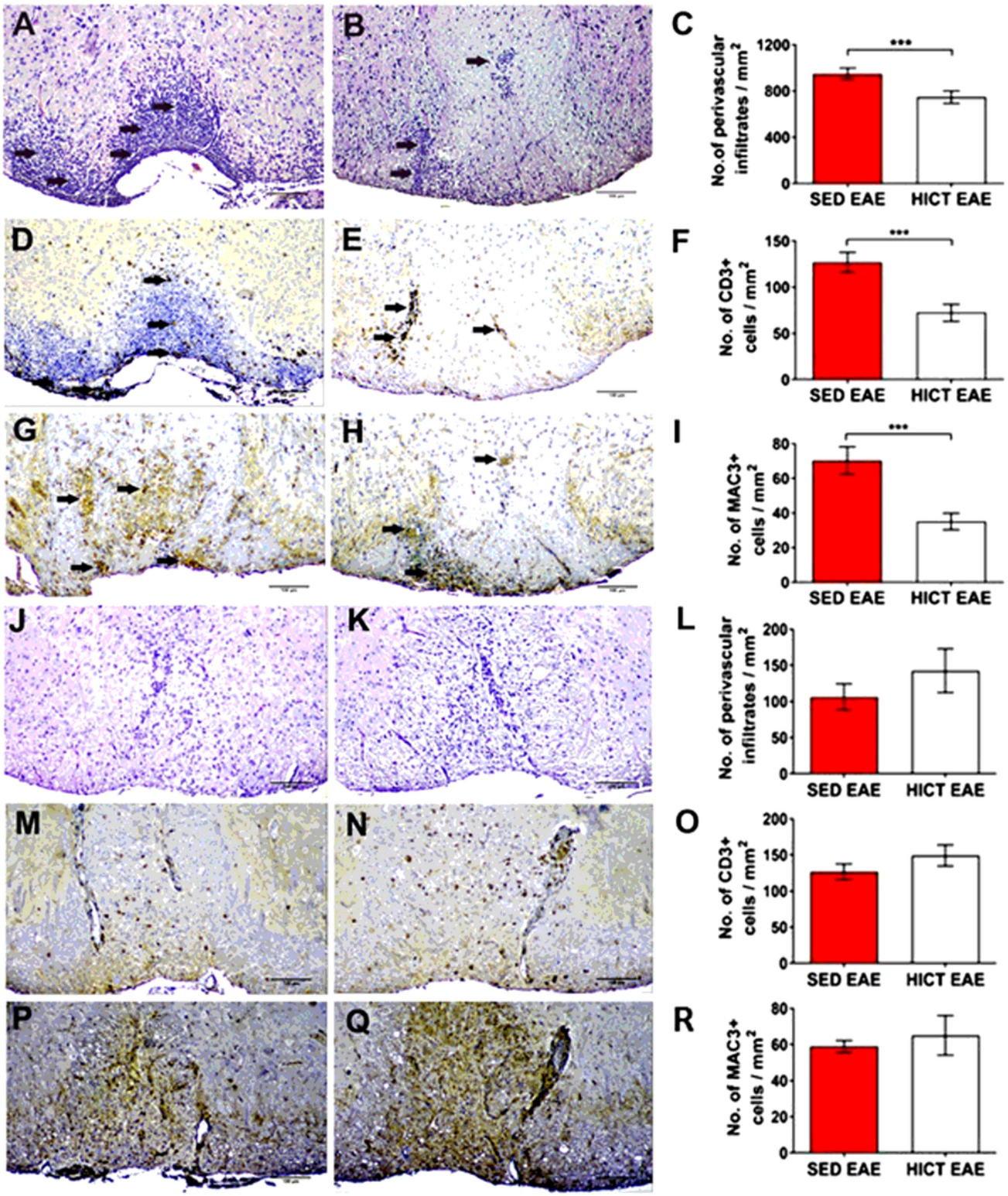
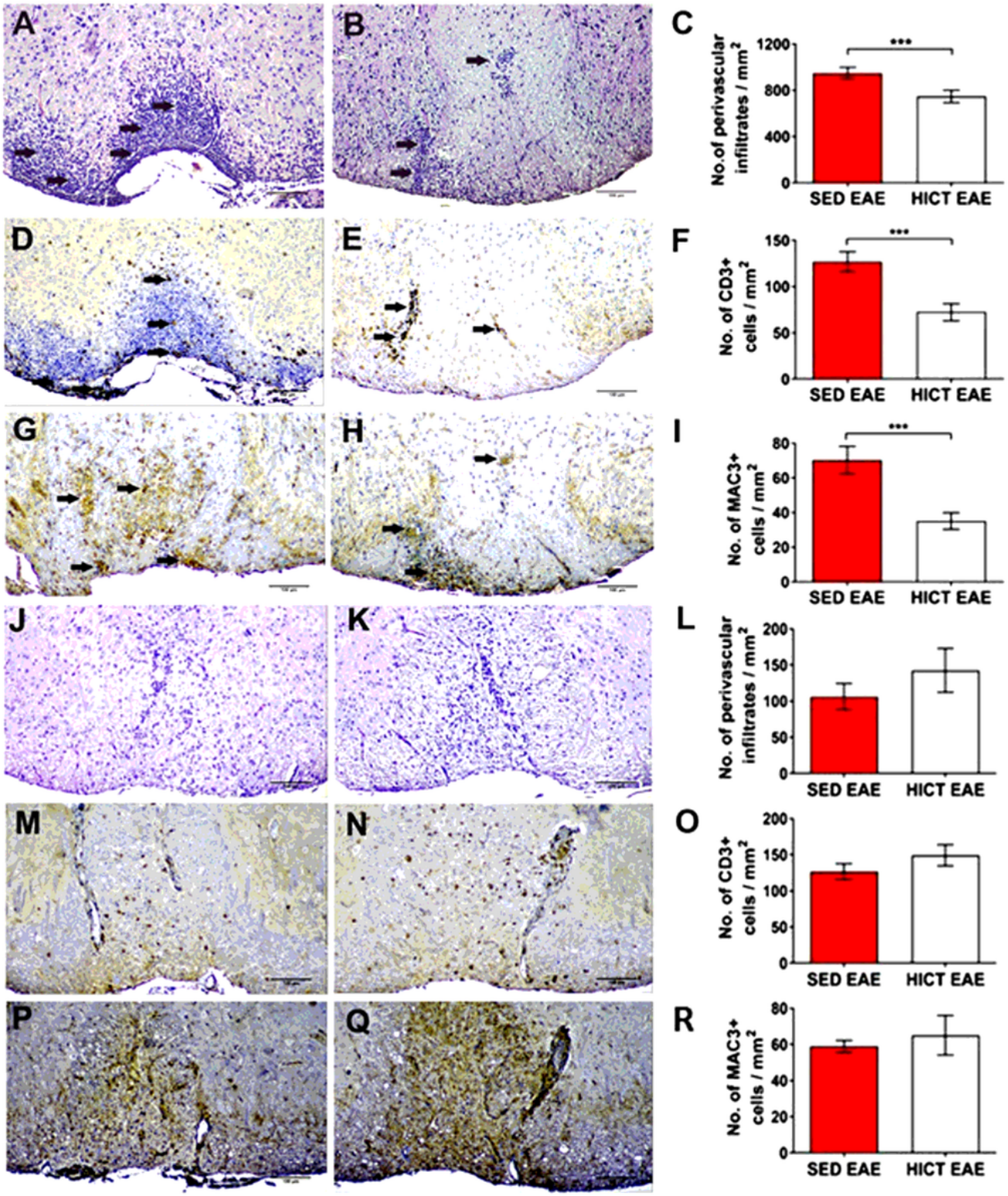


Figure 3

Reduction of acute autoimmune inflammation in the spinal cords of high-intensity trained EAE mice. Immunohistochemistry for inflammatory infiltrates (A-C, J-L), CD3 T cells (D-F, M-O) and Mac3 macrophages (G-I, P-R) on cross sections of the spinal cords of sedentary (SED EAE: A, D, G, J, M, P, n=6) and high-intensity continuous trained (HICT EAE: B, E, H, K, N, Q, n=6) mice that were injected with encephalitogenic T cells and developed EAE. A-I –12 days post EAE induction, acute phase of disease, J-R – 50 days post EAE induction, chronic phase of disease. A, B: arrows – indicate perivascular infiltrates; D, E: arrows – indicate perivascular CD3+ T cells; G, H: arrows – indicate Mac3+ macrophages. C, F, I, L, O, R – counts of inflammatory cell types in spinal cord white matter. At the acute phase of disease, in HICT EAE mice there was a significant reduction in total perivascular immune cell infiltrations (B, C), in CD3+ T cells (E, F) and Mac3+ macrophages (H, I) vs. SED EAE mice (A, D, G, respectively). Similar numbers of perivascular immune cell infiltrations (L), CD3+ T cells (O) and Mac3+ macrophages (R) were counted in SED EAE (J, M, P) and HICT EAE (K, N, Q, respectively) mice at the chronic phase of disease. Scale bars = 100 μm. Data are mean ± SE. $p < 0.05$, ***$p<0.001$.



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R – 50 days post EAE induction, chronic phase of disease. A, B: arrows – indicate perivascular infiltrates; D, E: arrows – indicate perivascular CD3+ T cells; G, H: arrows – indicate Mac3+ macrophages. C, F, I, L, O, R – counts of inflammatory cell types in spinal cord white matter. At the acute phase of disease, in HICT EAE mice there was a significant reduction in total perivascular immune cell infiltrations (B, C), in CD3+ T cells (E, F) and Mac3+ macrophages (H, I) vs. SED EAE mice (A, D, G, respectively). Similar numbers of perivascular immune cell infiltrations (L), CD3+ T cells (O) and Mac3+ macrophages (R) were counted in SED EAE (J, M, P) and HICT EAE (K, N, Q, respectively) mice at the chronic phase of disease. Scale bars = 100 μm. Data are mean ± SE. p < 0.05, ***p<0.001.

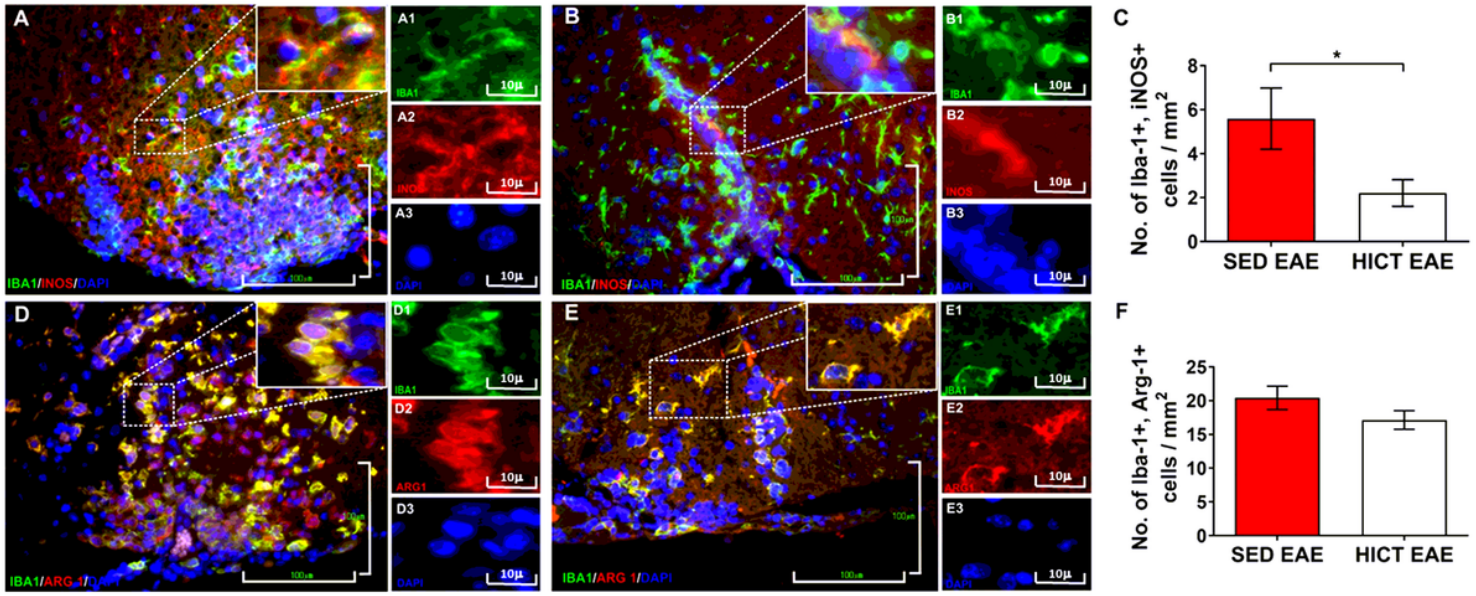
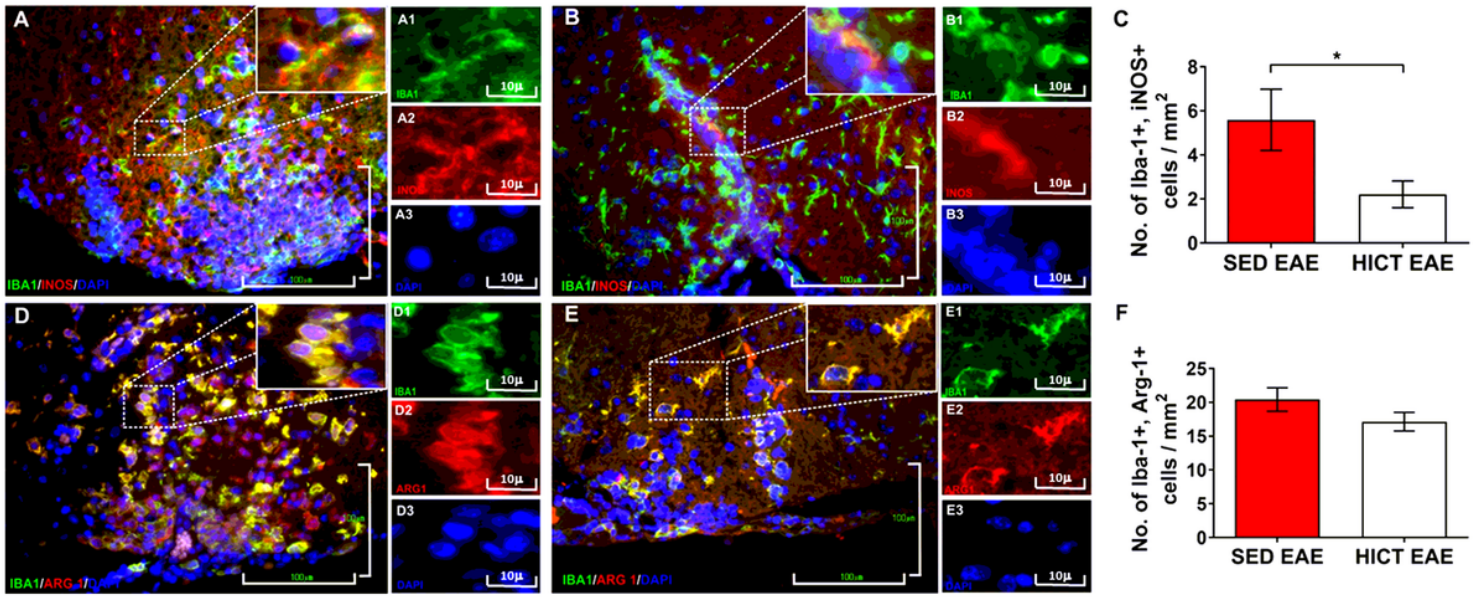


Figure 4

Reduction of neurotoxic microglia in the spinal cords of high-intensity trained EAE mice. Double immunofluorescent stainings for Iba-1 and inducible nitric oxide synthase (iNOS) M1 phenotype microglia (A,B) and Iba-1 and arginase-1 (Arg-1) M2 phenotype microglia (C, D) on cross sections of the spinal cords of sedentary (SED EAE: A, A1-3; D, D1-3; n=6) and high-intensity continuous trained (HICT EAE: B, B1-B3; E, E1-3; n=6) mice at 12 days post EAE induction by injection with encephalitogenic T cells (acute phase). C, F – counts of double positive cells in spinal cord white matter. In HICT EAE mice, there was a significant reduction in double positive Iba-1+, iNOS+ M1 type toxic microglia (B) vs. SED EAE mice (A, C). Similar numbers of double positive Iba-1+, Arg-1+ M2 type microglia were counted in SED EAE (D) and HICT EAE (E, F). Iba-1: A, A1, B, B1, D, D1, E, E1 – green; iNOS: A, A2, B, B2 – red; Arg-1: D, D2, E, E2 – red; nuclear staining with DAPI: A, A3, B, B3, D, D3, E, E3 - blue. Scale bars: A, B, D, E = 100 μm; A1-3, B1-3, D1-3, E1-3 = 10 μm. Data are mean ± SE. p < 0.05.



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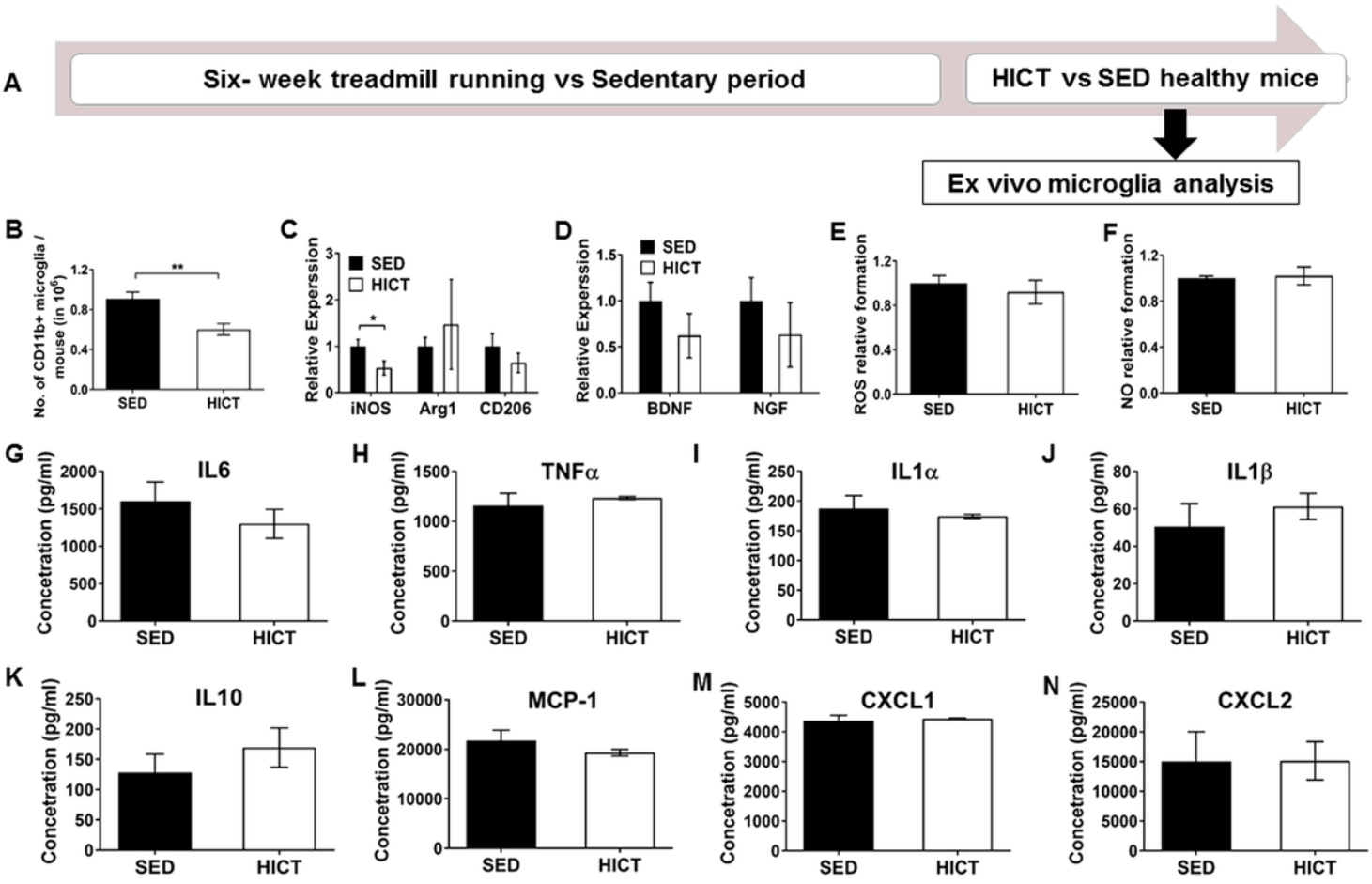
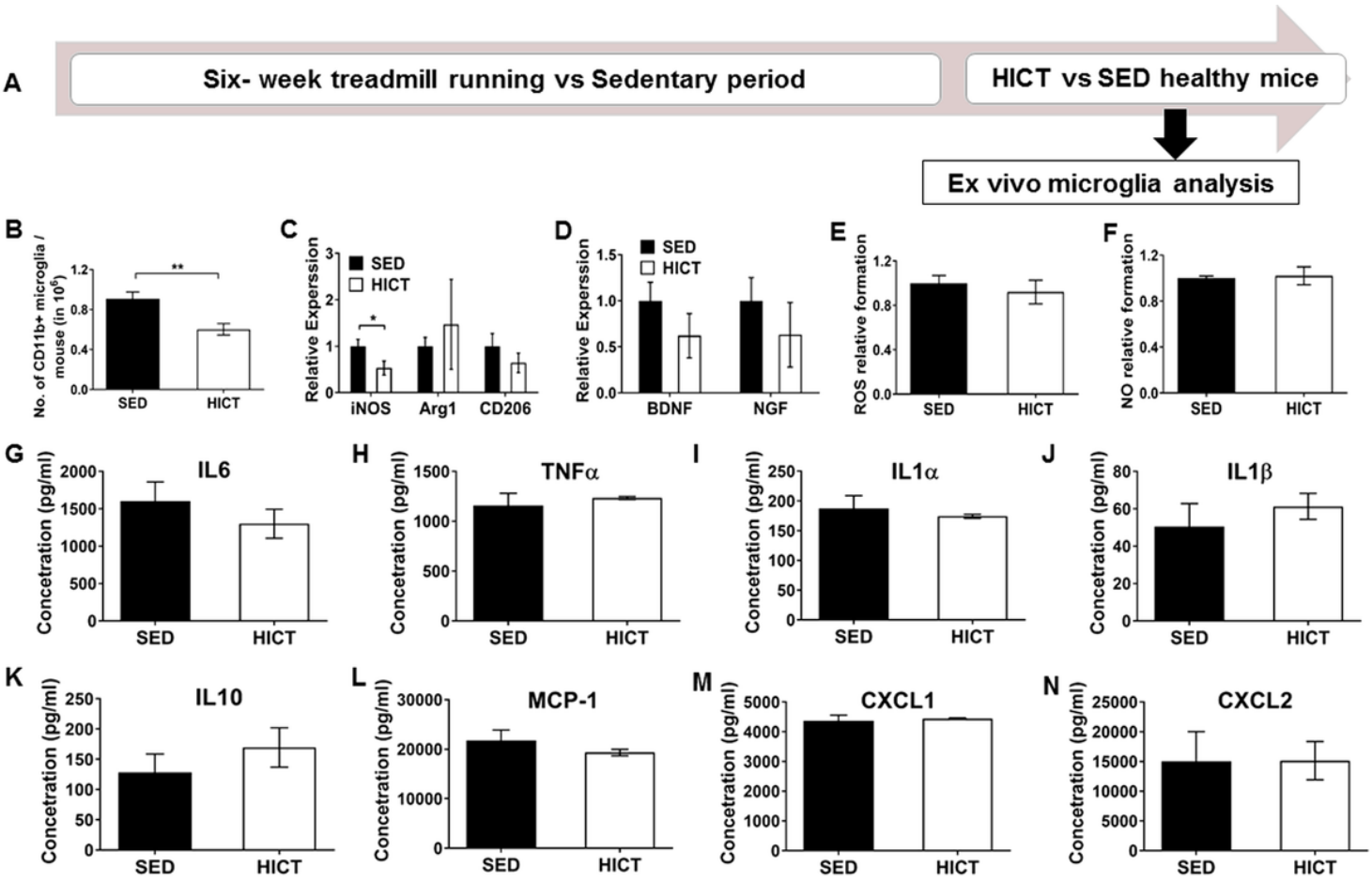


Figure 5

Decreased levels but preserved function of brain microglia in healthy high- intensity trained EAE mice. A: CD11b+ microglia were isolated from brain of mice following 6 weeks of high-intensity continuous training (HICT), 48 h after the last exercise bout, or sedentary period (SED) and analyzed ex vivo on day of isolation (B-D) or after additional 24 h in culture with lipopolysaccharide (LPS) stimulation (E-N, n=5-8/group). B: Number of CD11+ microglia per mouse at day of microglia isolation. mRNA levels of inducible nitric oxidase synthase (iNOS), arginase (Arg)-1 and CD206 M1/M2 phenotype markers (C) and brain- derived neurotrophic factor (BDNF) and nerve growth factor (NGF, D) in CD11b+ microglia at day of isolation. Levels of reactive oxygen species (ROS, E) and nitric oxide (NO, F) in supernatants of LPS-stimulated CD11b+ microglia cultures. G-N: protein concentrations of interleukin (IL)-6 (G), tumor necrosis factor (TNF)- α (H), IL-1α (I), IL-1β (J), IL-10 (K), monocyte chemoattractant protein (MCP-1, L), CXCL-1 (M), CXCL-2 (N) in supernatants of LPS- stimulated CD11b+ microglia cultures. HICT induced reduction in the total number of CD11b+ microglia (B) and in iNOS mRNA level in CD11b+ microglia (C) on day of isolation. Training did not affect Arg-1 and CD206 (C), BDNF and NGF mRNA levels, ROS (E) and NO (F) formation and cytokine and chemokine secretion (G-N) in CD11b+ microglia in response to LPS stimulation in vitro. Data are mean ± SE. C-F: Relative expression to SED group = 1. *p<0.05, ** p<0.01.



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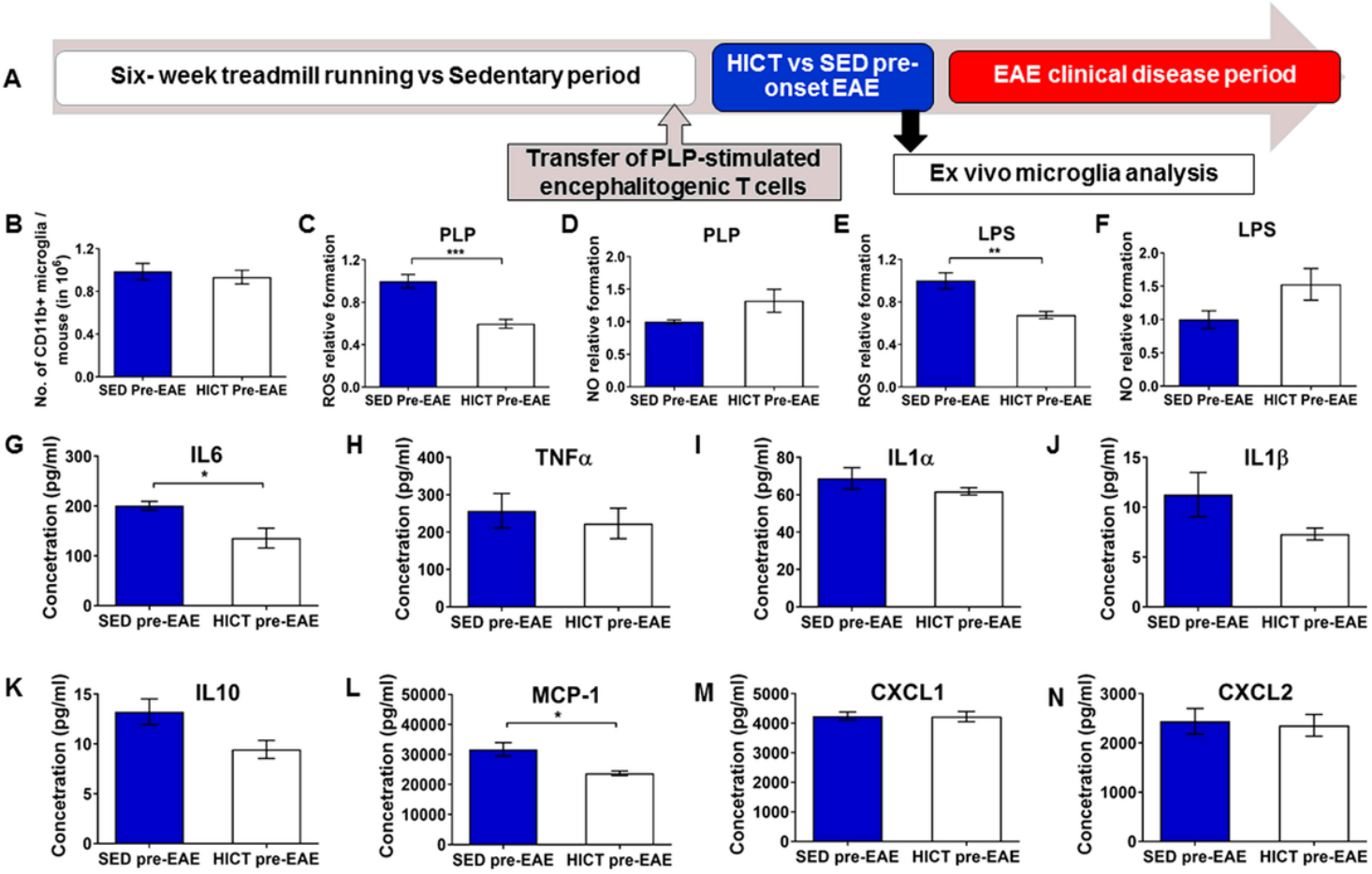
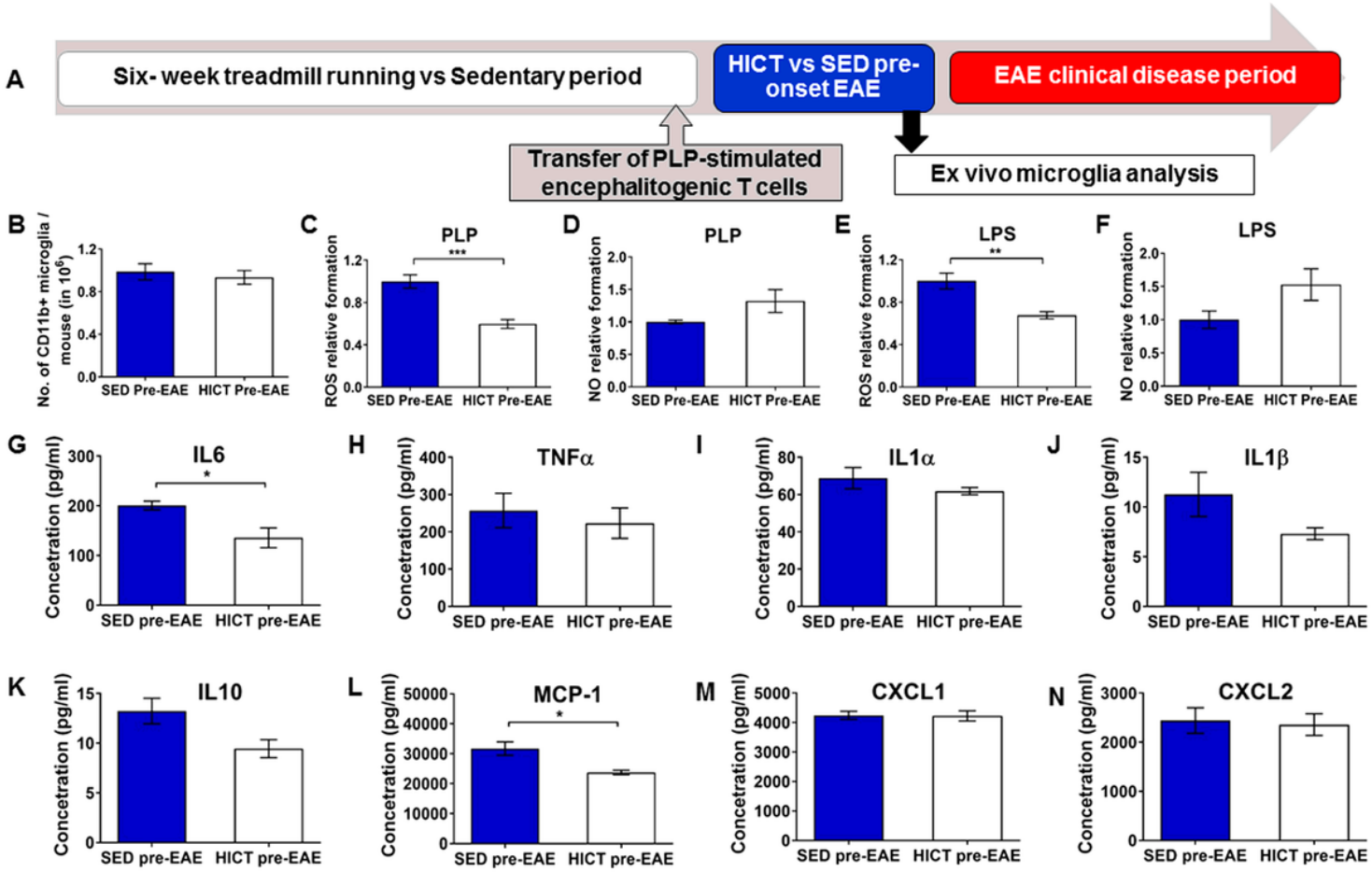


Figure 6

Reduced cytotoxic and proinflammatory properties in trained- derived microglia following transfer of encephalitogenic T cells. A: Mice were trained for 6 weeks. 72 h prior to last exercise bout, encephalitogenic T cells were injected into trained or sedentary control recipient mice. In general, mice develop clinical signs of experimental autoimmune encephalomyelitis (EAE) at 7–10 days post transfer of encephalitogenic T cells. Five days post-transfer (48 h after the last exercise bout), prior to clinical onset of EAE, PLP-reactive CD11b+ microglia were isolated from brains of high-intensity continuous trained (HICT pre-EAE) or sedentary control (SED pre-EAE) mice (n=5-8/group). Isolated CD11b+ microglia were counted at day of isolation (B) and analyzed ex vivo after 24 h in culture with PLP (C, D, G-N) or lipopolysaccharide (LPS, E-F) stimulation. Levels of reactive oxygen species (ROS; C, E) and nitric oxide (NO; D, F) in supernatants of PLP- (C, D, respectively) or LPS- (E, F, respectively) stimulated- CD11b+ microglia cultures. G-N: protein concentrations of interleukin (IL)-6 (G), tumor necrosis factor (TNF)- α (H), IL-1α (I), IL-1β (J), IL-10 (K), monocyte chemoattractant protein (MCP-1, L), CXCL-1 (M), CXCL-2 (N) in supernatants of CD11b+ microglia cultures stimulated with PLP. Training, followed by PLP- reactive encephalitogenic T cells transfer, induced reduction in ROS formation by CD11+ microglia in response to PLP- (C) and LPS- (E) stimulation in vitro; and in pro-inflammatory IL-6 cytokine (G) and MCP-1 chemokine (L) secretion after 24 h of PLP stimulation in culture. Training did not affect the total number of CD11b+ microglia on day of isolation (B), the formation of NO in response to PLP (D) or LPS (F) stimulation in vitro, nor the secretion of TNF-α (H), IL-1α (I), IL-1β (J), IL-10 (K), CXCL-1 (M) and CXCL-2

(N) after 24 h of PLP in culture. Data are mean ± SE. C-F: Relative expression to SED group = 1. *p<0.05, ***p<0.001.



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