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Exercise training alters autoimmune cell invasion into the brain in autoimmune encephalomyelitis

Liel Hamdi1,*, Hanan Nabat1,*, Yehuda Goldberg1, Nina Fainstein2, Shir Segal1, Efrat Mediouni1, Yarden Asis1, Olga Touloumi3, Nikolaos Grigoriadis3, Abram Katz4, Tamir Ben-Hur2 & Ofira Einstein1

1Department of Physical Therapy, Faculty of Health Sciences, Ariel University, Ariel, Israel
2Department of Neurology, The Agnes Ginges Center for Human Neurogenetics, Hadassah – Hebrew University Medical Center, Jerusalem, Israel
3B’ Department of Neurology, AHEPA University Hospital of Thessaloniki, Greece
4Astrand Laboratory, The Swedish School of Sport and Health Sciences, GIH, Stockholm, Sweden

Correspondence
Ofira Einstein, Department of Physical Therapy, Faculty of Health Sciences, Ariel University, Ariel 40700, Israel. Tel: 972-3-9066398; Fax: 972-3-9066268; E-mail: ofirae@ariel.ac.il

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*These authors contributed equally to this work.

Abstract

Background: The mechanisms by which exercise training (ET) elicits beneficial effects on the systemic immune system and the central nervous system (CNS) in autoimmune neuroinflammation are not fully understood. Objectives: To investigate (1) the systemic effects of high-intensity continuous training (HICT) on the migratory potential of autoimmune cells; (2) the direct effects of HICT on blood–brain-barrier (BBB) properties. Methods: Healthy mice were subjected to high-intensity continuous training (HICT) by treadmill running. The proteolipid protein (PLP) transfer EAE model was utilized to examine the immunomodulatory effects of training, where PLP-reactive lymph-node cells (LNCs) from HICT and sedentary donor mice were analyzed in vitro and transferred to naive recipients that developed EAE. To examine neuroprotection, encephalitogenic LNCs from donor mice were transferred into HICT or sedentary recipient mice and the BBB was analyzed. Results: Transfer of PLP-reactive LNCs obtained from HICT donor mice attenuated EAE severity and inflammation in recipient mice. HICT markedly inhibited very late antigen (VLA)-4 and lymphocyte function-associated antigen (LFA)-1 expression in LNCs. Transfer of encephalitogenic LNCs into HICT recipients resulted in milder EAE and attenuated CNS inflammation. HICT reduced BBB permeability and the expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in CNS blood vessels. Interpretation: HICT attenuates EAE development by both immunomodulatory and neuroprotective effects. The reduction in destructive CNS inflammation in EAE is attributed to systemic inhibition of autoreactive cell migratory potential, as well as reduction in BBB permeability, which are associated with reduced VLA-4/VCAM-1 and LFA-1/ICAM-1 interactions.

Introduction

A large body of evidence indicates beneficial outcomes of exercise training (ET) among multiple sclerosis (MS) patients and in experimental autoimmune encephalomyelitis (EAE), the animal model of MS.1 In a series of studies, we utilized the passive transfer EAE model that enabled differentiation between systemic immunomodulatory effects and direct neuroprotective effects of different training paradigms of ET. We first demonstrated peripheral-systemic immunomodulatory effects of moderate-intensity continuous training (MICT) in transfer EAE.2 Interestingly, MICT did not result in a direct protective effect on the central nervous system (CNS) from encephalitogenic T cells.2 Subsequently, we found that high-intensity continuous training (HICT) induced superior benefits than MICT in preventing systemic autoimmunity in EAE.3 Moreover, in addition to its superior systemic immunomodulatory effect, HICT also resulted in a direct protective effect on the CNS against autoimmune neuroinflammation.4 HICT protected the CNS against EAE development by reducing microglial-derived reactive oxygen species (ROS)
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Materials and Methods

Experimental animals

Female SJL/JCrHsd mice (6–7 weeks 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 PLP \textsuperscript{139–151} transfer EAE model in mice was utilized as previously described.\textsuperscript{2,4,17} To assess the modulatory effects of ET on systemic autoimmunity, we examined in vivo and in vitro the encephalitogenicity of lymph nodes cells (LNCs) from mice that underwent the HICT program prior to proteolipid (PLP) immunization, compared with LNCs from sedentary (SED) mice (Fig. 1A). To that end, healthy mice were subjected to a defined HICT treadmill-running program. This was followed by their immunization with a PLP peptide. Thereafter, their draining inguinal lymph nodes were removed. LNCs were stimulated in culture with PLP peptide and the encephalitogenic cells were injected to naïve recipient mice, which developed EAE. Another group of recipient mice were injected with PLP-reactive encephalitogenic cells from SED mice and served as controls. This approach enabled assessment of whether treadmill running of the donor mice modulated the systemic autoimmune process: (1) in vivo by examination of the clinical severity and CNS inflammation of EAE in recipient naive SJL mice, followed by injection of encephalitogenic cells from trained- vs. sedentary donor mice; and (2) in vitro, at the day of PLP-reactive LNCs removal or following secondary activation in vitro by the PLP auto-antigen, using chemokine receptor and integrin gene expression and surface integrin expression analyses.

To assess the direct neuroprotective effects of ET, healthy mice were subjected to a defined HICT treadmill-running program, followed by injection of PLP-reactive, encephalitogenic cells from donor mice (Fig. 1B). Sedentary mice were injected with the same PLP-reactive encephalitogenic cells and served as controls. Here we examined whether HICT program of the recipient mice prior to transfer of encephalitogenic cells attenuated the severity of EAE and CNS inflammation via direct neuroprotective effects. BBB analysis was performed 5 days after transfer of encephalitogenic PLP-reactive cells, prior to appearance of clinical symptoms (pre-EAE) (see below).

Treadmill exercise training

Healthy mice underwent 6-wks of HICT treadmill running, including pre- and post-training performance tests on a 5-lane treadmill designed for mice (Panlab Harvard Apparatus, USA) as previously described.\textsuperscript{3,4,17}

Transfer experimental autoimmune encephalomyelitis (EAE)

The PLP \textsuperscript{139–151} transfer EAE model was utilized as previously described.\textsuperscript{2,4,17} EAE was induced either in naïve recipient mice by transfer of encephalitogenic cells from PLP-immunized HICT (HICT-Tr-EAE, \(n = 7\)) and SED (SED-Tr-EAE, \(n = 10\)) mice (Fig. 1A), or in recipient formation, neurotoxicity, and pro-inflammatory responses. Thus, our and other studies\textsuperscript{2–9} highlight a favorable outcome for extensive ET. Nevertheless, the mechanism of ET on modulation of the systemic immune system and direct protection of the CNS are not fully elucidated.

It is widely accepted that in MS and EAE, the pathological process is triggered by infiltration of autoreactive cells, which are targeted against antigens of the myelin sheath, through the blood–brain barrier (BBB) into the CNS. This initiates a neuroinflammatory response, involving BBB disruption\textsuperscript{10} and recruitment of further autoimmune cells.\textsuperscript{11–15} The induction of cell surface molecules, such as integrins on the leucocyte and adhesion molecules on the endothelial cells of the BBB, play a major role in determining the pattern and route of leucocyte emigration. Additionally, studies suggest that ET reduces BBB permeability as it induces antioxidative mechanisms, reduces oxidative stress and has anti-inflammatory effects.\textsuperscript{16} Hence, decreased inflammatory cell infiltration into the CNS tissue in trained mice may be attributed to either an attenuated systemic immune response, including a limitation in the ability of inflammatory cells to migrate to the CNS, or sustained capacity of the BBB to restrict the trans-migration of autoimmune encephalitogenic cells. We therefore hypothesized that HICT attenuates disease progression in EAE by reducing autoimmune cell migratory potential and by attenuating BBB permeability.

Thus, the aim of the current study was to investigate the effects of HICT on migration and infiltration of autoreactive immune cells into the CNS in EAE. The chronic-relapsing transfer EAE model enabled us to examine the systemic effects of HICT on the migratory properties of encephalitogenic immune cells as well as the direct effects of HICT on BBB integrity in EAE. To assess the mechanisms for these changes we focused on very late antigen (VLA)-4 / vascular cell adhesion molecule (VCAM)-1 and lymphocyte function-associated antigen (LFA)-1 / intercellular adhesion molecule (ICAM)-1 interactions.
HICT (HICT-EAE, n = 8) and SED (SED-EAE, n = 12) mice by transfer of encephalitogenic cells obtained from PLP-immunized donor mice (Fig. 1B). Recipient mice developed EAE and were scored daily for neurological symptoms up to 30 days after EAE induction as follows: 0 - asymptomatic; 1 - partial loss of tail tonicity; 2 -
atactic tail; 3 - hind leg weakness, difficulty to roll over, or both; 4 - hind leg paralysis; 5 - four leg paralysis; 6 - death due to EAE.

**Histopathology analyses**

Twelve days after encephalitogenic cell transfer (at the acute phase of disease), a group of SED-EAE, HICT EAE, SED-Tr-EAE and HICT-Tr-EAE mice were sacrificed for histopathological analysis \( (n = 5/\text{group}) \) as previously described.\(^2\) Serial paraffin-embedded transverse sections were obtained from mid-cervical, mid-thoracic and mid-lumbar levels of the spinal cords. Immunohistochemistry was performed in adjacent serial sections for macrophages (rat anti-mouse Mac3, 553322, 1:800, BD Pharmingen) and T cells (monoclonal rabbit anti–CD3, RM-9107-SO; 1:800, Thermo-Scientific). For each staining, the white matter of three sections per mouse were quantified in a blind fashion, one section per spinal cord level. Mac3+ and CD3+ cells were counted in the perivascular infiltrates and parenchyma and reported as total average number of each cell type per square millimeter. All pathology measurements were performed by using the Image J software analysis (ver. 1.51H, NIH, USA).

**In vitro analyses of encephalitogenic LNCs**

Lymph nodes were excised from HICT or SED mice at 10 days after PLP immunization (Fig. 1A). LNCs were cultured for 72 h as single cell suspensions with 10 µg/ml PLP peptide as previously described.\(^2\) LNCs at day of excision and following 72 h in culture were analyzed for chemokine receptor gene determination [using real-time polymerase chain reaction (RT-PCR), \( n = 5/\text{group} \)] and integrin expression (using flow cytometry, \( n = 8/\text{group} \)).

**Integrin and chemokine receptor gene determination**

Total RNA was prepared using the RNeasy Plus Mini Kit (QIAGEN) from LNCs that were excised from mice 10 days after PLP immunization \( (n = 5/\text{group}) \). cDNA was prepared from 300 ng total RNA using qScript cDNA Synthesis Kit (Quanta Biosciences), according to manufacturer’s instructions. Semiquantitative real-time PCR was performed using the PerfeCTa SYBR Green FastMix, ROX (Quanta Biosciences).

**Flow cytometry for integrin expression**

LNCs from HICT and SED mice were analyzed for integrin expression following their activation in vitro for 72 h in culture with PLP peptide, using flow cytometry \( (n = 8/\text{group}) \). The following antibodies were used: FITC-labeled anti-CD4 (anti- mouse CD4, 100405, BioLegend) and PE-labeled anti-VLA-4 (anti- mouse CD49d, 103607, Biolegend), APC-labeled anti-LFA-1 (anti-human CD11a/CD18, 363409, BioLegend), FITC-labeled anti-F4/80 (anti- mouse F4/80, 123107, BioLegend), PE-labeled anti-B220 (anti-CD45R, 45–0452-82, eBioscience). The fraction of VLA-4+ or LFA-1+ cells out of total LNCs was calculated. Double staining with anti-VLA-4 and anti-CD4 or anti-F4/80 was used to identify the fraction of VLA-4+ T cells or macrophages, respectively. Double staining with anti-LFA-1 and anti-CD4 or anti-B220 was used to identify the fraction of LFA-1+ T cells or B cells, respectively. In all flow cytometry experiments, cells were pre-coated with anti-mouse CD16/CD32 (BD Pharmingen), as an Fc blocker, to block non-specific binding. In early experiments antibodies were tested for their specificity with an isotype control. All samples were analyzed in a Cytomics FC 500 apparatus (Beckman Coulter, Life Science) using the CXP analysis software (ver. 2.1; Informer Technologies, Inc).

**Blood-brain-barrier analysis**

The BBB permeability was evaluated in spinal cords, 5 days after transfer of encephalitogenic PLP-reactive cells, prior to appearance of clinical symptoms (pre-onset EAE mice, Fig. 1B). BBB permeability was assessed by 5-(and 6)-tetramethylrhodamine biocytin (Biocytin-TMR, Invitrogen, Thermo Fisher Scientific) extravasation (HICT Pre-EAE, \( n = 5 \); SED Pre-EAE, \( n = 5 \); naïve controls, \( n = 3 \)), and adhesion molecules and tight junction molecules expression (HICT Pre-EAE, \( n = 5 \); SED Pre-EAE, \( n = 5 \)). All immunofluorescence analyses were performed by using the Image-Pro Plus software (Media Cybernetics).

**Biocytin-TMR leakage assessment**

The integrity of the BBB was examined by the biocytin-TMR extravasation method. At 5 days following transfer of encephalitogenic cells (pre-onset EAE) a total of 1 mg of Biocytin-TMR diluted in 100 µL PBS per mouse was injected into the tail vein of HICT Pre-EAE \( (n = 5) \), SED Pre-EAE \( (n = 5) \), naïve controls \( (n = 3) \). Thirty minutes after injection, animals were sacrificed by intracardial perfusion with 4% paraformaldehyde. Spinal cords were extracted and serial 10 µm longitudinal frozen sections were prepared. Nuclear counterstain was performed using DAPI (Vector Laboratories). Biocytin-TMR extravasation was quantified by measuring the mean fluorescence intensity (MFI) in spinal cord sections. Images of three sections from each spinal cord, 60 µm apart, were captured uniformly using identical laser intensity, exposure times,
and magnification, and thresholded according to spinal cord from biocytin-TMR- injected (positive control) and non-injected naïve mice (negative control). Areas that exceeded the threshold levels were defined as leakage area. Group average MFI ± SEM was calculated and reported as ratios compared to MFI of naïve mice sections (blood–brain-barrier leakage index – BBLI).

Adhesion molecules and tight junction molecules expression assessments

Immunofluorescence was performed on spinal cords of HICT Pre-EAE and SED Pre- EAE mice (n = 5/group) for ICAM-1 (monoclonal anti-mouse ICAM-1, sc-107, 1:200, Santa cruz), VCAM-1 (monoclonal anti-mouse VCAM-1, sc-13160, 1:800, Santa cruz), occludin (monoclonal anti-mouse occludin, sc-133256, 1:600, Santa cruz) and claudin-4 (monoclonal anti-mouse claudin-4, sc-376643, 1:800, Santa cruz). To identify blood vessels, sections were double stained with CD31 (rat anti-mouse CD31, 550274, 1:800, BD-Pharmingen) and nuclear counterstain was performed using DAPI. Ten microscopic field images (×40 magnification) of three sections in each spinal cord at 60 μm apart were captured as described above. Stained areas around blood vessels in each image were marked. Group average MFI ± SEM was calculated and reported as ratio to stained area in spinal cords of naïve mice.

Statistical analyses

Normality of distribution of variables was tested by the Shapiro–Wilk test followed by the appropriate statistical test for comparison. Two experimental groups were compared using the unpaired Student’s t test or the two-tailed Mann–Whitney test, according to the normality test. When comparing ≥2 mean values, a one-way analysis of variance (ANOVA) was used. If a significant F ratio was found, then the Newman–Keuls multiple comparison test was used to identify the location of significance. Each experiment was repeated 2–3 times for consistency. 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 (SEM).

Results

HICT induces systemic immunomodulation in donor mice to attenuate the clinical severity and acute CNS inflammation in recipient EAE mice

Transfer of encephalitogenic cells derived from PLP-immunized HICT donor mice induced a markedly milder clinical course of EAE in recipient mice, compared to mice that received encephalitogenic cells from PLP-immunized SED donor mice (Fig. 2A,B). Average maximal clinical score of disease was decreased by >60% (Fig. 2C), the burden of disease was ~80% lower (Fig. 2D), and the number of relapses decreased by ~70% in HICT-transfer than in SED- transfer EAE mice (Fig. 2E). Although the average onset of disease in the HICT- transfer EAE group was delayed, this delay was not statistically significant (Fig. 2F).

Next, we examined whether the attenuated disease in recipient mice induced by encephalitogenic cells from HICT mice was associated with reduced neuroinflammation. Inflammatory infiltrations were assessed at the peak of the acute relapse (at day 12 post-transfer, Fig. 2G–L). CD3+ T cell (Fig. 2G,H) and Mac3+ macrophage (Fig. 2J, K) infiltrations in HICT-transfer EAE mice were markedly diminished (Fig. 2G,H,I,K). CD3+ T cell (Fig. 2H) and Mac3+ macrophages (Fig. 2K) infiltrations were decreased by 65% and 76%, respectively, in HICT-transfer EAE mice (Fig. 2L).}

HICT induces reductions in integrin and chemokine receptor expression in PLP-reactive encephalitogenic LNCs

Since EAE mice that were transferred with HICT- derived encephalitogenic cells exhibited reduced inflammatory infiltrations in vivo, we investigated the effects of training on the migratory potential of PLP-reactive LNCs by assessment of integrin and chemokine receptor expression in vitro (Fig. 3A). First, integrin and chemokine receptor gene expression of freshly isolated LNCs were examined (Fig. 3B–G). mRNA levels of VLA-4 and LFA-1 integrins were not affected in LNCs from PLP-immunized mice versus SED controls (Fig. 3B,C), nor were the mRNA levels of CX3C chemokine receptor (CXCXR)-3, C-C chemokine receptor (CCR)1 and CCR2 affected (Fig. 3D–F). However, HICT virtually abolished the increases in CCR5 mRNA levels (Fig. 3G).

Then, integrin expression in LNCs following re-stimulation with PLP was examined (Fig. 3H–M). The fractions of VLA-4+ LNCs (Fig. 3H) and VLA-4+, CD4+ T cells (Fig. 3I) were reduced by ~50% and 40%, respectively, in HICT PLP-immunized mice. The fraction of VLA-4+, F4/80+ macrophages was not affected (Fig. 3I). HICT also resulted in an ~30% reduction in the fraction of LFA-1- expressing CD4+ T cells (Fig. 3L) but did not affect LFA-1 expression in either total LNCs (Fig. 3K) or B220+ B cells (Fig. 3M).

In conclusion, HICT reduces the capacity of autoreactive cells to migrate into the CNS, in association with marked reduction in VLA-4, LFA-1 and CCR5 expression.
Figure 2. High-intensity continuous training (HICT) inhibits the encephalitogenicity of lymph node (LN)-derived cells to attenuate the clinical course and tissue inflammation upon their transfer to recipient mice in transfer model of experimental autoimmune encephalomyelitis (EAE). (A) Experimental design to investigate the systemic immunomodulatory effects of HICT on the clinical severity and inflammatory infiltration into the central nervous system (CNS) in transfer EAE model. Healthy mice were subjected to a HICT treadmill-running program, or sedentary (SED) period followed by proteolipid (PLP) immunization. Their LNs were removed, and encephalitogenic lymph node cells (LNCs) were generated and injected to naive recipient mice, which developed EAE. Clinical course (B) and clinical parameters (C–F) of transfer EAE in mice that received PLP-reactive encephalitogenic LNCs from trained mice (HICT-transfer EAE, n = 7) or from sedentary mice (SED-transfer EAE, n = 10). The severity of EAE was scored according to a 0–6 scale. Transfer of encephalitogenic LNCs derived from HICT, PLP-immunized mice to naive recipients induced a significantly milder EAE course (B–F). Mice were sacrificed for histopathology analysis for CNS inflammatory infiltrations at 12 days post EAE induction, at the acute phase of disease (n = 5/group, G–L). Evaluation of CD3+ T cells (G, H) and Mac3+ macrophages (J, K) on cross sections of the spinal cords of mice that were injected with encephalitogenic LNCs derived from trained mice (HICT-transfer EAE, n = 7) or from sedentary mice (SED-transfer EAE, n = 10). In HICT-transfer EAE there were less CD3+ T cells (H, I) and Mac3+ macrophages (K, L) infiltrations within the CNS than in SED-transfer EAE (G, J, I, L). Scale bars = 100 μm; Data are mean ± SE. **p < 0.01, ****p < 0.0001.
HICT induces direct CNS protection from autoreactive encephalitogenic cells to attenuate the clinical severity and CNS acute inflammation in recipient EAE mice

To study the direct effects of HICT on the CNS, encephalitogenic cells were transferred to trained and sedentary recipient mice (Fig. 4A). Encephalitogenic cells from donor mice induced a milder clinical course of EAE in HICT recipient mice (HICT EAE) compared with sedentary mice (SED EAE, Fig. 4B). The day of onset was significantly delayed by 3 days in the HICT group versus SED group (Fig. 4C), and the overall burden of disease was ~50% lower in HICT mice than in control SED mice (Fig. 4D). HICT induced a small, nonsignificant reduction in the average maximal clinical score (~30%, Fig. 4E) and in the number of relapses (30%, Fig. 4F) compared to SED mice. We also examined whether HICT protected the CNS from the destructive acute inflammatory process (Fig. 4G–L). SED EAE mice exhibited extensive acute inflammation (Fig. 4G, J), whereas HICT EAE mice exhibited substantially less inflammation (Fig. 4H, K). The CD3+ T- cells (Fig. 4G, H) and Mac3+ macrophages (Fig. 4J, K) infiltrations were decreased by ~30% (Fig. 3I) and ~35% (Fig. 3L), respectively.

HICT reduces BBB permeability to autoreactive encephalitogenic cells

As HICT had a direct protective effect on the CNS following transfer of encephalitogenic cells, resulting in reduced CNS inflammatory infiltrates, we assessed whether BBB integrity was associated with this observation. Since systemically administered PLP-reactive encephalitogenic cells encountered the CNS that had already been modulated by ET, we examined the BBB permeability and the expression of tight-junction and adhesion molecules expression at day 5 post-encephalitogenic cell transfer, a time point of initial invasion of autoimmune cells into the CNS, and prior to clinical onset (Fig. 5A). Biocytin-TMR extravasation in HICT healthy mice was similar to that of naïve healthy mice (data not shown). EAE induction markedly increased extravasation of biocytin-TMR in spinal cords of sedentary EAE mice (Fig. 5C, I, E) versus naïve controls (Fig. 5G, H, I). However, training reduced the increase by ~40% (Fig. 5E).

To examine the underlying mechanism by which HICT affected the profile of endothelial cells of blood vessels in spinal cords at 5 days after transfer of encephalitogenic T cells, CD31+ endothelial cells in spinal cords of naïve healthy mice and HICT and SED pre EAE mice were double stained with occludin (Fig. 6A–C), claudin-4 (Fig. 6E–G), VCAM-1 (Fig. 6I–K), and ICAM-1 (Fig. 6M–O). Immunofluorescent analysis indicated that occludin expression in CD31+ endothelial cells was reduced by ~40% following transfer of encephalitogenic T cells in SED pre-EAE mice (Fig. 6B, D) but only by ~20% in HICT pre-EAE (Figs. 6C, D) mice compared to naïve healthy mice (Fig. 6A, D). Thus, HICT diminished the reduction in occludin expression in HICT pre-EAE mice by ~50% compared to SED pre-EAE mice. Transfer of encephalitogenic cells had no effect on claudin-4 expression in SED pre-EAE mice (Fig. 6F, G, I) versus naïve controls (Fig. 6E, H). However, encephalitogenic T cells in HICT induced a ~15% reduction in claudin-4 expression (Fig. 6G, H, I).

Finally, the basal expression of both VCAM-1 and ICAM-1 in naïve mice (Fig. 6J, N respectively) was markedly increased following encephalitogenic T cell transfer in SED pre-EAE mice (~60%, Fig. 6K, O, M, Q). These increases were completely abolished in HICT pre-EAE mice.

In summary, HICT prevented the reduction in occludin expression, and prevented the induction of VCAM-1 and ICAM-1 expression in CD31+ endothelial cells, to reduce the breach in BBB integrity following transfer of encephalitogenic cells, resulting in reduced CNS infiltration with T cells and macrophages.

Discussion

In the current study we investigated the systemic versus direct effects of training on autoimmune neuroinflammation using the transfer EAE model. The key findings of the study are: (1) HICT attenuated autoimmune neuroinflammation and clinical severity of EAE; (2) the effects of HICT are mediated by systemic immunomodulation and direct neuroprotection of the CNS; and (3) the positive effects of HICT are associated with diminished autoimmune cell migration and invasion and maintained BBB integrity.

Previous studies have shown that ET modulates the immune system, reduces neurodegeneration and promotes neuroprotection in different models of EAE. However, these studies used active EAE models that could not distinguish between the effects of training on the systemic immune system versus direct protective effects on the CNS to reduce encephalitogenicity. The transfer EAE model enables this distinction. The current work corroborates the beneficial effects of HICT in attenuation of EAE and further demonstrates the superior effects of high-intensity training on both systemic immunomodulation and direct neuroprotection in autoimmune neuroinflammation. Moreover, the current study extends our previous findings by demonstrating additional mechanisms that
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A. Encephalitogenic LNC transfer

Trained / Sedentary donor mice

HICT / SED period

LNCs

Integrin & Chemokine receptor expression

Recipient EAE mice

B. VLA-4 Relative Expression

C. LFA-1 Relative Expression

D. CKR3 Relative Expression

E. CCR1 Relative Expression

F. CCR2 Relative Expression

G. CCR5 Relative Expression

H. VLA-4+ fraction of total LNCs (%)

I. VLA-4+ fraction of CD4+ T cells (%)

J. VLA-4+ fraction of F4/80+ macrophages (%)

K. LFA-1+ fraction of total LNCs (%)

L. LFA-1+ fraction of CD4+ T cells (%)

M. LFA-1+ fraction of B220+ B cells (%)
Exercise alters brain autoimmune cell invasion

Figure 3. Inhibitory effects of high-intensity continuous training (HICT) on integrin and chemokine receptor expression on lymph node cells (LNCs) derived from proteolipid (PLP) immunized mice. (A) Experimental design to investigate the immunomodulatory effects of HICT on the systemic autoimmune cells. Healthy mice were subjected to a HICT treadmill-running program, or sedentary (SED) period, followed by PLP immunization. Their LNCs were removed and analyzed in vitro for integrin and chemokine receptor expression. B-G: Real time-PCR analysis of freshly isolated LNCs from HICT and SED mice (n = 5/group) for mRNA levels of integrins very late antigen (VLA)-4 (B) and lymphocyte function associated antigen (LFA)-1 (C) and chemokine receptor mRNA levels of CXC chemokine receptor (CXCR)-3 (D), C–C chemokine receptor (CCR)-1 (E), CCR2 (F), CCR5 (G). Flow cytometry analysis of VLA-4 (H–J) and LFA-1 (K–M) expression on total LNCs (H, K), CD4+ T cells (I, L) F4/80+ macrophages (J) and B220+ B cells (M) after 72 h of in vitro stimulation with PLP peptide (n = 8/group). HICT in PLP- immunized mice induced a marked reduction in mRNA levels of CCR5 in LNCs (G), fraction of VLA-4+ LNCs (H) and CD4+ T cells (I) and in the fraction of LFA-1+ T cells (L). Data are mean ± SE. B–G: Relative expression to SED group = 1. *p < 0.05, **p < 0.01.

can account for the systemic immunomodulatory and direct neuroprotective effects of ET in EAE.

In the first part of the current study, we demonstrate that transfer of PLP-reactive encephalitogenic LNCs obtained from HICT mice results in reduction of immune cell infiltrations in the CNS of recipient mice. The induction of immune responses of T cells to brain-derived antigens occurs in peripheral lymphoid organs.54–56 We therefore hypothesized that ET may interfere with the migratory potential of LNCs in the peripheral lymphoid system upon immunization with the PLP peptide. Our findings demonstrate that HICT inhibits several key molecules involved in encephalitogenic LNC migration into the CNS. HICT in PLP-immunized mice decreases mRNA levels of CCR5 chemokine receptor, and the expression of VLA-4 in LNCs and both VLA-4 and LFA-1 in T cells after PLP re-stimulation in vitro.

The role of VLA-4 integrin in leukocyte recruitment to the inflamed CNS during EAE has been extensively investigated.57 Early studies showed that blockade of VLA-4 prevented disease development in rats and mice.58,59 Moreover, VLA-4 integrin was shown to mediate initial recruitment of encephalitogenic T cells, as well as the secondary infiltration of inflammatory cells, thereby being involved in initiating and sustaining CNS inflammation.60 The role of LFA-1 in the development of EAE was also widely studied. Targeting LFA-1 integrin could block or delay the onset of EAE.41,42 Transfer of LFA-1−/− T cells to wild type mice caused less severe disease than transfer of T cells to wild-type controls.61 Disruption of CCR may lead to impaired monocyte function, including chemokine-directed chemotaxis, and CCR manipulation may aid in inflammatory disease resistance.43,44 CCR5 is implicated in immune cell migration and cytokine release in the CNS and play a role in the pathogenesis of MS and EAE.45,46 It was shown that mice lacking CCR5 were resistant to EAE, failed to develop mononuclear cell infiltrates, and displayed decreased pro-inflammatory responses in the CNS and spinal cord.47 Taken together, our findings suggest that the inhibitory effect of HICT on the expression of VLA-4, LFA-1 in PLP-reactive encephalitogenic LNCs contribute to the decrease in inflammatory infiltration into the CNS and attenuation of acute CNS inflammation, tissue injury and clinical severity of EAE. Interestingly, HICT did not affect the mRNA levels of VLA-4 and LFA-1 in freshly isolated LNCs after PLP stimulation. This points to the essential role of re-stimulation of LNCs with PLP in vitro to generate potent encephalitogenic LNCs. Moreover, VLA-4 in F4/80+ macrophages and LFA-1 in B220+ B cells were not affected by HICT, suggesting that the inhibitory effect of HICT is targeted to the T cell populations.

With respect to neuroprotection, we demonstrate that training did not prevent the formation of encephalitogenic LNCs, which were obtained from donor mice, but rather protected the CNS in trained recipient mice and prevented neuroinflammation. We therefore hypothesized that the positive effect of HICT on the CNS could be attributed to modulation of the BBB. Brain endothelial cells are characterized by structured tight junctions that are formed primarily by endothelial-specific proteins.48,49 Occludin and claudin-4 play a crucial role for tight junction formation and BBB function. Additionally, inflamed CNS endothelial cells upregulate expression of both VCAM-1 and ICAM-1. VLA-4-VCAM-1 and LFA-1-ICAM-1 interactions are critically involved in the firm arrest and adherence of CD4+ T cells to the inflamed endothelium of the CNS vessels.50,51 In vitro and in vivo studies stress the importance of VLA-4 integrin binding to VCAM-1 in the adhesion of T cells to the vasculature of the brain white matter.52–55 Moreover, LFA-1-ICAM-1 interactions dictate the polarization and crawling of CD4+ T cells into the inflamed CNS vessels. The effect of inhibiting ICAM-1, the LFA-1 ligand, during EAE was also studied.56,57 EAE development was significantly attenuated in ICAM-1−/− mice, as well as by targeting ICAM-1 with a staphylococcal-derived anti-adhesive protein.59

First, our results indicate that training did not alter BBB permeability in naive healthy mice (data not shown). However, following exposure to encephalitogenic LNCs, HICT induced a substantial decrease in BBB permeability to biocytin-TMR tracer, indicating reduction in BBB disruption. Additionally, HICT partially reversed the
decrease of the tight junction molecule occludin. Moreover, HICT markedly inhibited the expression of the adhesion molecules VCAM-1 and ICAM-1 in the CNS of EAE mice. Thus, our findings indicate that HICT does not affect the integrity of the BBB independent of disease, but modulates the properties of the BBB and maintains the integrity of the BBB following EAE induction to attenuate neuroinflammation.

Interestingly, Souza et al. reported decreases in the expression of occludin and claudin-4 in control sedentary EAE mice, that were both upregulated following training.30 Our results are supportive of this effect on...
occludin, as transfer of encephalitogenic LNCs to SED mice caused a significant reduction in occludin expression, whereas HICT resulted in a partial reversal of the decrease. However, in our study claudin-4 was not affected by encephalitogenic LNC transfer in SED mice, and HICT even reduced the expression of claudin-4. This discrepancy could probably be contributed to the differences in strain of mice, EAE model and different ET programs that were utilized in both studies. These results underscore the importance of identifying the mechanisms whereby training impacts the neuroprotective effects in autoimmune neuroinflammation.

Previous studies have reported that oxidative stress might account for development of endothelial injury during MS development. It is now accepted that ROS are produced upon interaction of monocytes with brain endothelium, leading to tight-junction alterations, cytoskeleton rearrangements, loss of BBB integrity, and subsequent extravasation of leukocytes into the CNS. Additionally, it has been demonstrated that ET in MS preserved the levels of occludin in the spinal cord of mice, by inhibiting ROS and oxidative stress. Consistent with these findings was our recent observation of reduced ROS formation in HICT-derived microglia.

In summary, the data suggest that BBB preservation, and specifically the re-establishment of occludin by HICT, contribute to reducing resident microglia cytotoxicity.

Figure 5. High-intensity continuous training (HICT) directly reduces blood–brain-barrier (BBB) permeability in recipient mice in a transfer model of experimental autoimmune encephalomyelitis (EAE). (A) Experimental design to investigate the direct effects of HICT on the BBB in transfer EAE model. Healthy mice were subjected to HICT treadmill-running program or sedentary (SED) period and injected with proteolipid (PLP)-reactive encephalitogenic lymph node cells (LNCs) from donor mice. At 5 days post encephalitogenic LNC transfer (pre-EAE stage) spinal cords of HICT (HICT Pre-EAE, n = 5) and SED (SED Pre-EAE, n = 5) recipients were analyzed for 5-(and 6)-tetramethylrhodamine biocytin (biocytin-TMR) extravasation. Biocytin-TMR fluorescence in longitudinal spinal cord sections of naïve (B, B1), SED Pre-EAE (C, C1) and HICT Pre-EAE (D, D1) mice. Transfer of encephalitogenic LNCs markedly increased biocytin-TMR extravasation in spinal cords of SED Pre-EAE (C, C1) compared to naïve controls (n = 3; B, B1, E). Biocytin-TMR extravasation was significantly lower in HICT Pre-EAE mice (D, D1, E) compared to SED Pre-EAE mice. Biocytin-TMR – red; Dapi – blue. E: BBB leakage index (BBBLI) = relative to naïve controls (n = 3). Data are mean ± SE. **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 6. High-intensity continuous training (HICT) induces an increase in tight junction molecule expression and decrease in adhesion molecule expression in recipient mice in a transfer model of experimental autoimmune encephalomyelitis (EAE). (A) Experimental design to investigate the direct effects of HICT on tight junction and adhesion molecule expression in spinal cords blood vessels in transfer EAE model. Healthy mice were subjected to HICT treadmill-running program or sedentary (SED) period and injected with proteolipid (PLP)-reactive encephalitogenic lymph node cells (LNCs) from donor mice. At 5 days post encephalitogenic LNC transfer (pre-EAE stage) spinal cords of HICT (HICT Pre-EAE, n = 5) and SED (SED Pre-EAE, n = 5) recipients were analyzed for the expression of occludin (B–E) and claudin-4 (F–I) tight junction molecules and vascular cell adhesion molecule (VCAM)-1 (J–M) and intercellular adhesion molecule (ICAM)-1 (N–Q) expression. Immunofluorescence analysis of CD31+ endothelial cells indicated that transfer of encephalitogenic LNCs in SED Pre-EAE mice induced a reduction in occludin expression (C, E) and increase in VCAM-1 (K, M) and ICAM-1 (O, Q) expression, compared to naïve mice (n = 3; B, J, N, respectively). Occludin expression was significantly higher in HICT Pre-EAE mice (D, H, L, P) compared to SED Pre-EAE mice (D, E) and naïve mice (E). HICT restored VCAM-1 and ICAM-1 to control levels (L and M, P and Q, respectively). Claudin-4 expression was not affected by encephalitogenic LNCs in SED Pre-EAE mice (F, I) compared to naïve mice (F, I). Occludin, claudin-4, VCAM-1, ICAM-1 – red; CD31 – green; Dapi – blue; arrows – double stained area, arrowheads – only CD31 staining. Data are mean ± SE. ***p < 0.001, ****p < 0.0001.
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Although the identification that CNS endothelial cells are the site of the BBB proper, it has become apparent that endothelial cells require contacts with various CNS cell types to establish BBB characteristics. Live cell imaging studies of trans-endothelial migration across ICAM-1 and ICAM-2-deficient brain endothelial monolayers have shown that CD4+ T cell may undergo diapedesis in an ICAM-independent manner, suggesting that other adhesion molecules may function in a redundant manner. Thus, further studies are required to investigate the effects of ET on other factors that are involved in BBB function in autoimmune neuroinflammation.

Finally, in the present study, the burden of disease (area under curve) of HICT- transfer EAE (Fig. 2B) was lower than that of HICT EAE (Fig. 4B). These findings suggest that ~70% of the beneficial effects of training on disease progression derive from systemic immunomodulation and ~30% from direct protection of the CNS. If this estimate is correct, one could argue that modulating the encephalitogenicity of LNCs prior to their invasion into the CNS is a preferable approach than inducing CNS protective mechanisms. Accordingly, we suggest that future research and therapy approaches should focus primarily on the immunomodulatory effects of ET.

In conclusion, HICT induces both systemic immunomodulatory and direct neuroprotective effects in an experimental model of autoimmune neuroinflammation. Autoimmune cell migration and infiltration into the CNS serve as a key therapeutic target for neuroimmunomodulation and its modulation by HICT may reduce CNS inflammation and subsequently reduce tissue injury and clinical severity. Our results indicate that ET affects the migration and invasion of encephalitogenic LNCs by affecting both the systemic immune system and the CNS. HICT inhibits systemically the expression of VLA-4 and LFA-1 on PLP-reactive encephalitogenic cells, as well as directly reduces the expression of VCAM- and ICAM-1 in endothelial cells on CNS blood vessels. The combined inhibitory effect of HICT on VLA-4-VCAM-1 and LFA-1-ICAM-1 interactions contributes to the decrease in inflammatory infiltration into the CNS and attenuation of acute CNS inflammation, tissue injury and clinical severity of EAE. In-depth understanding of the cellular and molecular mechanisms underlying the beneficial effects of ET on EAE is essential for designing effective clinical treatments in MS patients and other patients with autoimmune diseases.

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Conflict of interest

The authors declare no competing financial interests.

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