C16 peptide shown to prevent leukocyte infiltration and alleviate detrimental inflammation in acute allergic encephalomyelitis model

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Abstract

Integrins are important adhesion receptors for leukocytes binding to endothelial cellular adhesion molecules. Previous studies have suggested that blocking relevant integrins might prevent leukocyte infiltration and suppress clinical and pathological features of neuroinflammatory disease. Experimental autoimmune encephalomyelitis (EAE), a rodent model of Multiple sclerosis (MS), is characterized by chronic inflammatory disorder of the central nervous system in which circulating leukocytes enter the brain and spinal cord leading to inflammation, myelin damage and subsequent paralysis. To prove this hypothesis and explore a promising application for MS treatment, the effects of C16, an αβ3 integrin-binding peptide, were tested in vitro and in vivo by transendothelial assay, electron microscopy observation, multiple histological and immunohistochemical staining. The results showed C16 inhibited transendothelial migration of the C8166-CD4 lymphoblast cells, and alleviated extensive spinal cord and brain infiltration of leukocytes and macrophages in the EAE model. Furthermore, a significant amelioration of astrogliosis and a dramatic decrease in demyelination and axonal loss were observed in C16 treated animals. The attenuating inflammatory progression may improve the regional environment and trigger further neuroprotective effects on myelin and axons, all this suggests that C16 peptide may be a promising therapeutic agent for multiple sclerosis.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune inflammatory disorder that occurs in rodent central nervous systems (CNS). It is widely used as an animal model of multiple sclerosis (MS), a progressive inflammatory autoimmune disease of the CNS, in which an excess of circulating leukocytes entering the CNS results in pathogenesis (Stanislaus et al., 2004). Focal leukocyte infiltration produces inflammation followed by demyelination, axonal loss and defective remyelination, leading to hindlimb weakness and paralysis (Desai and Barton, 1989). The inflammatory reaction in the CNS is driven by activated auto-reactive immune cells penetrating the CNS and damaging myelin antigens (Kent et al., 1995). The vulnerability of the CNS and the progression of deficits from caudal to rostral have been attributed to the increased permeability of the blood brain barrier (BBB) and activated macrophage or microglia mediated demyelination (Claudio et al., 1989; Paul and Bolton, 1995; Yin et al., 2010).

In CNS inflammation, the up-regulation of surface cell adhesion molecules in peripheral blood cells is a prerequisite for their interaction with activated ependymal and endothelial cells, and their crossing of the blood–brain barrier (Schenkel et al., 2004). Integrins, a family of heterodimeric membrane proteins serving as adhesion receptors for various extracellular matrix components, are primarily expressed on leukocytes (Alon and Luscinskas, 2004). The β2 integrins are important in the process of rolling leukocytes binding to endothelial cellular adhesion molecules (Stanislaus et al., 2004), moreover, they are also critically involved...
in transporting recently arrested monocytes to sites of subsequent transendothelial migration (TEM). Blockade of either leukocyte integrins or their endothelial ICAM ligands results in disoriented leukocyte locomotion from its original attachment site, as well as prevention of the leukocyte TEM process (Alon and Luscinskas, 2004). Expression of α4-integrin was also observed on leukocytes in peripheral blood in the EAE model, and the antibody against α4 integrin was found to prevent leukocyte infiltration and suppress clinical and pathological features of EAE in guinea pigs (Kent et al., 1995).

Integrin αvβ3 is the most promiscuous member of the integrin family, allowing endothelial cells to interact with laminin, a large extracellular matrix protein (Brooks et al., 1994; Weerasinghe et al., 1998). The synthetic C16 peptide, representing a functional laminin domain, selectively binds to αvβ3 and αvβ1 integrin (Ponce et al., 1999). Our previous work has shown that C16 peptide attenuated monocyte transmigration across an endothelial cell layer to the same extent as blocking antibodies against αvβ3 integrins in vitro (Han et al., 2010). The immunoreactivity of αv and β3 on endothelial cells, but not on infiltration cells, was detected following spinal cord injury, suggesting that αvβ3 integrin on endothelial cells plays a role in the transmigration (Han et al., 2010). Furthermore, we confirmed in vivo that an intravenous injection of C16 alleviated the infiltration of extravasated leukocytes, activated microglia and extravasated macrophages (a major contributor to demyelination) in a spinal cord injury model. In general, reduction of infiltration of such cells can increase undamaged tissue and improve function in rodent injury models. The fact that αvβ3-blocking antibodies also reduce transmigration suggests that C16, by binding to αvβ3, interferes with the binding of a leukocyte ligand required for transmigration that plays a dynamic role in preventing leukocyte entrance into the CNS (Han et al., 2010).

Drugs known to relieve inflammation and affect the immune system have become the primary focus for treatment of multiple sclerosis. Initially, corticosteroids, interferons and mitoxantrone therapy were widely used (Nguyen et al., 1997; Van der Meide et al., 1998; Floris et al., 2002; Stanislaus et al., 2004; Garay et al., 2007, 2008). However, these treatments cause numerous side effects associated with immunosuppression. The characteristic of being able to prevent leukocyte infiltration without affecting the systemic leukocyte count implies that C16 peptide may be considered as a prime potential therapeutic agent for MS treatment (Han et al., 2010). To prove this hypothesis and explore a promising application for MS treatment, the present study was designed to determine the effects of C16 peptide in lymphocytes transmigration in vitro and in vivo, as well as evaluate its possible effects on the demyelination, axonal loss, neuronal death and astrogliosis in the acute rodent EAE model.

Fig. 1. α5β1 and αvβ3 integrins labeled on blood vessels in EAE rats. Traverse section through the lumbar spinal cord. In EAE rats at week 2 post-immunization, α5 (A; green), β1 (D; green) and αvβ3 integrin (G; green) immunoreactivity appeared in PECAM-labeled endothelial cells (B, E, H; red) which could be seen in the merged image (C, F, I; Yellow). Bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
2. Methods

2.1. Transendothelial migration assay

Briefly, human umbilical vein endothelial cells (HUVEC), were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and plated on permeable filters in 24-well transwell culture plates with 5 μm diameter pores (Costar, Cambridge, MA) at 4 × 10^5 cells per well. They were grown for 72–96 h to reach confluence at 37 °C in RPMI 1640 medium supplemented with 10% foetal calf serum (GIBCO, New York, NY). Afterward, the insert chamber of the Transwell system was loaded with 2 × 10^6 CB166-CD4 T lymphoblasts (a human T cell line immortalized by human T cell leukemia virus, ATCC, Manassas, VA) on endothelial cells. 300 μl of medium were placed into the lower chambers of the Transwells with or without 100 ng/ml of Pro-interleukin-16 (IL-16, NovoProtein Scientific, NJ), which was previously known as lymphocyte chemoattractant factor (LCF), for 18 h at 37 °C. C16 peptide (KAFDITYVRLKF; synthesized by Shanghai Science Peptide Biological Technology Co., Ltd, China) was added into the medium of the insert chamber at concentration of 100, 200 and 600 μM, and SP3 peptide (RFSVAVSSHYPFWSR, an inactive scrambled form of a αβTβ-peptide without effects on neural precursor migration in vitro; synthesized by Shanghai Science Peptide Biological Technology Co., Ltd, China) was added at 600 μM. Peptides were centrifuged to remove any precipitates before addition. The vehicle served as the control. CB166-CD4 cells were allowed to transmigrate for 18 h at 37 °C and the number of CB166-CD4 cells that crossed the endothelial cell layer was counted. The value was calculated from three wells among each concentration, and all assays were repeated three times.

2.2. Animals and EAE induction

A total of 30 adult male Lewis rats were obtained from the Laboratory Animal Services Centre of the Zhejiang University. Of these, 6 were taken as normal control and the remaining 24 were randomly assigned into a vehicle control group and a C16 treatment group (12/pair group). Experiments were carried out in accordance with NIH Guidelines for the Care and Use of Laboratory Animals, with approval from the animal subjects committee at the Zhejiang University. EAE was induced in the rats for C16 treatment and the vehicle control group with intradermal, hind footpad or nuchal injection of 0.2 ml: 1:1 mixture of guinea pig spinal cord homogenate (GPSCH) and complete Freund adjuvant (CFA), containing 0.5 mg of heat killed Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI). The rats in the normal control group were injected with CFA emulsified 1:1 with 0.9% saline. Beginning on day 7, the animals were weighed and assessed for clinical signs of disease on a daily basis. Disease severity was assessed using a scale ranging from 0 to 5: grade 0 – no signs, grade 1 – partial loss of tail tonicity, grade 2 – loss of tail tonicity, grade 3 – unsteady gait and mild paralysis, grade 4 – hindlimb paralysis and incontinence, and grade 5 – moribund or death (Yin et al., 2010). The EAE model was generally considered a success as its score exceeded 2, and scoring of the study was continued until the time of sacrifice.

2.3. Intravenous injection

C16 peptide was dissolved in distilled water with 0.3% acetic acid. The peptide solution was sterilized through a 0.22-μm disc filter and neutralized to pH 7.4 with sterilized NaOH. This solution was buffered by adding an equal volume of sterile phosphate-buffered saline and the final concentration was 2 mg/ml. The vehicle group was prepared in the same manner without adding the peptide. The first dose of 0.5 ml C16/vehicle treatments, via intravenous injection of the tail vein, was given immediately after receiving EAE induction to the C16 treatment and vehicle control groups. Then the solutions were injected intravenously each day for a period of two weeks.

2.4. Perfusion and tissue processing

Animals of vehicle control and C16 treatment groups were sacrificed at 2 weeks and 8 weeks (6 per time point) post immunization. Rats were anesthetized with sodium pentobarbital and perfused intracardially with cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The whole eye balls, spinal cord and brain tissues were carefully harvested and dissected. 1 cm of the lumbar spinal cord and a half of the brain of each animal were fixed in the same fixative for 4 h, and then transferred into 30% sucrose in PBS until the tissue sinks to the bottom of the container. Twenty μm-thick sections were cut on freezing microtome through the coronal plane of brain, transverse plane of spinal cord and eye balls using a Leica cryostat, then mounted onto 0.025% poly-lysine-coated slides. All sections were collected for histological assessment, and immunohistological and immunofluorescent staining. The remains of the central nervous tissue were fixed in 5% glutaraldehyde solution and examined by transmission electron microscope.

2.5. Histology assessment

Hematoxylin and eosin (H&E) staining and cresyl violet (nissl) staining were employed to assess inflammation and neuron survival respectively. Neuron counts from both spinal cord anterior horns were performed and restricted to the neurons with a well-defined nucleus as well as a cell body with enrichment of endoplasmic reticulum. Digital images were collected using a Nikon TE-300 microscope in 3 vision fields/per section with ×200 magnification under bright field. An assessment of the severity of inflammatory cell infiltration was done by a conventional H&E staining following scale as described (Ma et al., 2010): 0, no inflammation; 1, cellular infiltrates only around blood vessel and meninges; 2, mild cellular infiltrates in parenchyma (1–10/section); 3, moderate cellular infiltrates in parenchyma (11–100/section); and 4, serious cellular infiltrates in parenchyma (100/section).

Luxol fast blue (LFB) staining was used to evaluate the degree of demyelination. Sections were immersed in graded ethanol solutions and placed in a 0.1% LFB solution for 1.5 h at 65 °C, and then placed in ethanol and differentiated for 15–20 min in 0.05% lithium carbonate. Finally, they were dehydrated in graded ethanol, immersed in xylene, mounted, and coverslipped. Digital photomicrographs were obtained at ×40 magnification. Demyelination was scored as follows using the following scale (Yin et al., 2010): 0, normal white matter; 1, rare foci; 2, a few areas of demyelination; 3, confluent perivascular or subpial demyelination; 4, massive perivascular and subpial demyelination involving one half of the spinal cord with presence of cellular infiltrates in the CNS parenchyma; and 5, extensive perivascular and subpial demyelination involving the whole cord section with presence of cellular infiltrates in the CNS parenchyma.

Bielschowsky silver staining was performed to estimate the situation of axonal loss. The sections were placed in 20% silver nitrate at room temperature in the dark for 15 min. The solution was washed off with distilled water and replaced by ammoniacal silver solution for 10 min in the dark. Then ammonium hydroxide/formaldehyde was added to stain slides until the tissue turned brown with a gold background, and was followed by treatment with gold chloride and sodium thiosulphate. Axonal loss was assessed using the following scale (Yin et al., 2010): 0, no axonal loss; 1, a few foci of superficial axonal loss which involves less than 25%
of the tissues; 2, foci of deep axonal loss that encompassed over 25% of the tissue; and 3, diffuse and widespread axonal loss.

2.6. Immunohistochemical staining

Slides were warmed for 20 min on a slide warmer, a ring of wax was applied around the sections with a PAP pen (Invitrogen, Carlsbad, CA). After rinsing in 0.01 M Tris-buffered saline (TBS) for 10 min, the sections were permeabilized and blocked with 0.3% Triton X-100/10% normal goat serum in 0.01 M PBS for 30 min, then incubated with polyclonal rabbit antibodies anti-CD4 (1:500, AbCam, Cambridge, MA), anti-caspase 3 (1:500; Cayman Chemical, Ann Arbor, MI) and mouse anti-myelin basic protein (MBP, 1:500, AbCam, Cambridge, MA) overnight at 4°C. Sections were incubated with secondary biotinylated goat anti-rabbit/mouse IgG antibody (1:400; Vector Laboratories, CA) for 1 h at room temperature, followed by an avidin–biotin peroxidase complex (ABC kit, Thermo Fisher Scientific, CA). After incubation for 5 min with 0.02% DAB and 0.003% H2O2 in 0.005 Tris–HCl, the sections were counterstained with hematoxylin. Primary antibody omission controls were used in order to confirm further the specificity of the immunohistochemical labeling. Five sections from the cortex, hippocampi and anterior horns of spinal cord of each animal were randomly selected and images were photographed under ×200 magnification in 3 vision fields/section, and the caspase-3 immunoreactive cells counted.

2.7. Immunofluorescence and double immunofluorescent staining

The sections were pretreated with the same method described above, incubated with primary monoclonal mouse antibodies CD45, CD68/ED1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal rabbit anti-glia fibrillary acidic protein (GFAP, 1:200, Thermo Fisher Scientific, Waltham, MA) overnight at 4°C, then washed with PBS and incubated with 1:200 TRITC (Rhodamine)-conjugated goat anti-mouse or FITC-conjugated goat anti-rabbit IgG secondary antibodies for 1 h at 37°C (Invitrogen, CA). The sections were finally coverslipped with antifade Gel/Mount aqueous mounting media (SouthernBiotech, AL). All control sections were incubated in PBS without primary antibodies. Immunoreactive areas of GFAP, CD45 and CD68 were analyzed with NIH image software.

The sections from time points of week two post immunization were processed with double-immunofluorescent staining for platelet endothelial cell adhesion molecule (PECAM/CD31) and αvβ3 or α5β1 integrin. In brief, after blocking non-specific staining with 10% goat serum in TBS containing 0.3% Triton X-100 (TBST) for 1 h at room temperature, sections were incubated overnight at 4°C in TBST containing 5% goat serum and mouse anti-PECAM (1:100, Santa Cruz, CA). The sections were then incubated in TBST containing 5% goat serum and 1:200 TRITC-conjugated goat anti-mouse IgG secondary antibodies (Invitrogen, Carlsbad, CA). Afterward, for double staining, sections were incubated overnight at 4°C in TBST containing 5% goat serum and rabbit anti-αvβ3 integrin (1:500, ebioscience, San Diego, CA), anti-α5β1 integrin (1:500, ebioscience, San Diego, CA), and anti-β1 integrin (1:500, Epitomics, Inc. Burlingame, CA), followed by an incubation in 1:200 of FITC (Fluorescein)-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Finally, the sections were coverslipped with antifade Gel/Mount aqueous mounting media (SouthernBiotech, Birmingham, AL). In between steps, sections were washed 3 times for 10 min each in TBS.

Fig. 3. Diffuse infiltration of inflammatory cells was observed in the spinal cord of the vehicle treated EAE rats, which was attenuated in C16 treated EAE rats. H&E stained traverse section through the lumbar spinal cord, bar = 100 μm. Normal control group (A), vehicle treated EAE rats (B, F), and C16 treated EAE rats (C, G) at week 2 post-immunization; Vehicle treated EAE rats (D) and C16 treated EAE rats (E) at week 8 post-immunization; The arrow denoted “perivascular cuffing” of inflammatory cells in vehicle treated EAE rats (F), but surrounding blood vessels were less severely infiltrated by inflammatory cells in C16 treated EAE rats (G) at week 2 post-immunization. C16 treatment reduced inflammatory cells evaluated by four points inflammation score (H), a P < 0.05 versus normal control; b P < 0.05 versus vehicle treated EAE rats at week 2 post-immunization group; c P < 0.05 versus vehicle treated EAE rats at week 2 post-immunization group; d P < 0.05 versus vehicle treated EAE rats at week 8 post-immunization.
2.8. Processing for electron microscopy

10% glutaraldehyde fixed tissues were washed 3 times with 0.1 M PB. Post-fix the tissues placed in 1% osmium tetroxide at 4 °C overnight, then washed 3 times with 0.1 M PB. After post-fixation, the following steps were done in an EM processor with agitation at room temperature; dehydrating the tissues in graded ethanol (30%, 50%, 70%, 80%, 90%, 95% ethanol) for 5 min each followed by three changes of absolute ethanol for 10 min each; after two changes in 1, 2-propylene oxide (PO) for 15 min each, immersing the tissues in a 1:1 PO: Epon mixture for 1 h, then overnight in pure Epon and finally embedding the tissues in pure Epon and keeping at 60 °C for 3 days. The Epon embedded tissues were cut into 90 nm sections with a diamond knife on an ultracut microtome and collected on a 200 mesh copper grid. Lead citrate (approximately 3%) and 8% uranyl acetate were filtered before use. The grids were stained with lead citrate droplets for 20 min in a Petri dish, washed 3 times with distilled water. Then the sections were stained on droplets of 8% uranyl acetate inside a Petri dish at room temperature for 20 min, washed 3 times with distilled water, and then the grids were ready for electron microscopic analysis. Images were captured first at low resolution (×6200), and then imaged at higher magnification (×15,000—×30,000) in different regions of the white matter.

2.9. Statistical analysis

Data was presented as Mean ± Standard deviation (SD). Kruskal–Wallis nonparametric one-way analysis of Variance (ANOVA) was used for data presented as percentages. Differences between clinical scores and histological scores were analyzed with Mann–Whitney tests. Data was analyzed by SPSS 13.0 software and P values less than 0.05 were considered statistically significant. All statistical graphs were performed with GraphPad Prism Version 4.0 (GraphPad Prism Software, Inc. CA).

3. Results

3.1. αvβ3 integrin was expressed in blood vessels of EAE rats

The double-immunofluorescent staining for PECAM (endothelial cells specific marker, red channel) and αvβ3 or α5β1 integrin (green channel) showed an evident co-localization in the blood vessels of EAE rats (Fig. 1C, F, I).

3.2. C16 attenuated T lymphocytes transmigration in vitro

Lymphocyte transmigration across an endothelial cell layer in a two-compartment culture system was measured to determine the cellular and integrin targets of C16 that might play a role in leukocyte infiltration.

Without the attracting ability of IL-16, administration of the C16 peptide reduced the T lymphocytes transmigration to 54% of vehicle control at 200 μM and to 32% at 600 μM, which exhibited significant difference compared with vehicle control.

![Fig. 4. C16 treatment attenuated leukocyte infiltration in CNS both at week 2 and week 8 post-immunization. (A–I): TRITC conjugated CD45 immunofluorescent staining of the traverse section through the lumbar spinal cord. Normal control group (A), vehicle treated EAE rats (B, C) and C16 treated EAE rats (D) at week 2 post-immunization. Vehicle treated EAE rats (E), and C16 treated EAE rats (F) at week 8 post-immunization. (G–I): H&E staining of traverse section for eyeball showing C16 treatment inhibition of leukocyte infiltration and alleviation the uveitis in EAE rats. Normal control group (G), vehicle treated EAE rats (H, retinal folding and detachment, and serous subretinal bleeding) and C16 treated EAE rats (I: mild uveitis and retinitis) at week 2 post-immunization, bar — 100 μm.](image-url)
Fig. 5. C16 treatment attenuated macrophage and lymphocyte extravasation in CNS both at week 2 and week 8 post-immunization. Traverse section through the lumbar spinal cord. (A–G): TRITC conjugated CD68 immunofluorescent staining, normal control group (A), and vehicle treated EAE rats (B–C) at week 2 post-immunization. (White arrow in C denoted extensive perivascular cuffing of CD68+ inflammatory cells). (D–E): C16 treated EAE rats at week 2 post-immunization. The arrow denoted CD68 positive inflammatory cells were diffusely distributed (E). Vehicle treated EAE rats (F) and C16 treated EAE rats (G) at week 8 post-immunization. (H–L): CD4 immuno-staining, counterstained with hematoxylin. Normal control group (H), vehicle treated EAE rats (I), and C16 treated EAE rats (J) at week 2 post-immunization. Vehicle treated (K) and C16 treated (L) EAE rats at week 8 post-immunization, bar = 100 μm.
When the lymphocyte chemoattractant factor, an important inflammatory-promoting factor in EAE, was loaded in the chambers of the transwells, the C16 peptide treatment still inhibited the C8166-CD4+ lymphocytes transmigration significantly when compared with the vehicle control ($P < 0.05$). The higher concentration (600 μM) C16 treatment showed a higher capacity to reduce the attracting ability of IL-16 and superior blocking effect to 200 μM ($P < 0.05$). Similarly, no visible effect could be found with SP3 treatment ($P > 0.05$, Fig. 2A).

At a concentration of 100 μM, the C16 peptide did not show a visible effect on lymphocyte transmigration with or without the lymphocyte chemoattractant factor IL-16 ($P < 0.05$, Fig. 2A).

### 3.3. C16 postponed the onset of motor symptoms and alleviated the disease progression in EAE rat model

In the vehicle-treated rats ($n = 6$), disease symptoms appeared on day 9–10 post immunization. The acute phase of the disease began with a sharp increase in motor symptoms which peaked at week 3 post immunization with an average clinical score of 4.2 ± 0.4 (Fig. 2B). Thereafter, animals maintained a steady clinical score with only modest variability (clinical score 3–4) for 3 weeks, then displayed gradually recovery. At week 8 post immunization, the clinical score of survival vehicle-treated animals returned to a mean level of 2 (Fig. 2B). Animals treated with C16 showed a similar disease course to the vehicle group, but with an obvious delay in the onset of clinical signs, in which motor symptoms began on day 17–18 and peaked at week 4 post immunization with an average clinical score of 3.3 ± 0.5, then dropped to a mean level of 1.5 at week 8 post immunization (Fig. 2B). Animals treated with C16 ($n = 6$) exhibited significantly reduced disease severity, from first to third week post immunization ($P < 0.05$), especially at the second week post immunization ($P < 0.01$, Fig. 2B).

### 3.4. The C16 attenuated perivascular/parenchymal inflammation in vivo

On day 14 and day 21 post immunization, diffuse infiltration of inflammatory cells in the spinal cord of the vehicle treated EAE rats was confirmed by using H&E staining (Fig. 3B, F), whereas more diffuse but less severe perivascular and parenchymal infiltration of inflammatory cells (mixed with macrophages, microglia and lymphocytes) within the spinal cords and brains of the C16 treated EAE rats were observed (Fig. 3C, G). The inflammatory score of the vehicle-treated group was significant lower than that of the C16-treated EAE group both in week 2 and week 8 post immunization ($P < 0.05$, Fig. 3H).

For determination of the types of inflammatory cells, the immunostaining of CD45, a marker for extravasated leukocytes (pan-leukocyte marker), CD4 for T lymphocytes, and CD68, for activated microglia and extravasated macrophages was carried out. The results showed that cells positive for CD45 or CD68 all increased remarkably in both gray and white matter of the spine and brain of the vehicle treated EAE rats. An evident decline of leukocytes infiltration and macrophages extravasation was detected in C16 treated group (Figs. 4–6). Moreover, inflammatory infiltration was ascertained in the vitreous and retinal layers of eye balls in the vehicle treated group with significant clinical scores at both early and late time periods. In severe cases, retinal folding and detachment, and serous subretinal bleeding were revealed at week 2 post immunization (Fig. 4H). An inflammatory cell infiltration, mild uveitis and retinitis were also found in the C16 treated rats in the onset stage of EAE (Fig. 4I), but recovered spontaneously at week 8 post immunization. Extensive perivascular cuffing formed by CD4+ lymphocytes can be found in the vehicle treated EAE rats, but not in C16 treated group (Fig. 5H–I). In the normal control animals, there was no evident inflammatory cells infiltration (Figs. 4A, G and 5A, H), hence corroborating the absence of a clinical score.

### 3.5. C16 treatment inhibited apoptosis and reduced neuron loss in the CNS of EAE rat model

Accompanied with severe inflammation, there was remarkable neuron loss in the anterior horn of spinal cords and motor cortices of the vehicle treated EAE rats (Fig. 7), especially at the late stage of clinical course. A remarkable increase in the number of the caspase-3 immunostaining cells in the spinal cords, motor cortices and hippocampi (Fig. 8). C16 treatment significantly reduced neuron loss and suppressed expression of neuronal caspase-3 in the CNS when compared with vehicle control (Figs. 7 and 8). Significant differences were found between these two groups at both week 2 and 8 post immunization ($P < 0.05$, Fig. 9B).

### 3.6. C16 treatment inhibited demyelination, prevented axos loss and reactive gliosis in the EAE rat model

Demyelination appeared during the manifestation of clinical signs in the active model of EAE. By using luxol fast blue (LFB)
Fig. 7. C16 treatment reduced neuron loss in spinal cord anterior horn (A, C, E, G) and hindlimb area of motor cortex (Ctx-HL, B, D, F, H) both at week 2 and week 8 post-immunization, cresyl violet (nissl) staining. Vehicle treated EAE rats (A, B) and C16 treated EAE rats (C, D) at week 2 post-immunization. Vehicle treated EAE rats (E, F) and C16 treated EAE rats (G, H) at week 8 post-immunization, bar = 100 μm. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)
staining for total myelin, massive perivascular and confluent demyelinated area presented in the parenchyma of CNS in the vehicle treated EAE rats (Fig. 10B, C, E, I, K). However, in the C16 treatment group at the same time point, only rare foci or a few areas of demyelination were determined (Fig. 10D, F, J, L). In addition to LFB staining, immunohistochemistry for MBP, one of the major central myelin proteins, also exhibited extensive and severe demyelination in vehicle control rats (Fig. 11B–D, G–H). However, the group with C16 injection showed a distinct increase in myelin protein level at both week 2 and 8 post immunization (Fig. 11E–F, J–L).

Transmission electron microscopy (TEM) examination further revealed that a considerable amount of the myelin sheath displayed splitting, vacuolus, and loose and fused change in the vehicle control group (Fig. 12C, G–H). Meanwhile, the axons were covered by disrupted myelin sheaths and in some places they had even disappeared (arrow in Fig. 12C). The neurons showed apoptotic signs of a shrunken nucleus with condensed, fragmented and marginated nuclear chromatin (Fig. 12D, I). Some vacuolated myelin sheaths were also found in C16 treated EAE rats, and the corresponding axons also had also shrunk (Fig. 12E). However, the neurons had kept a relatively normal ultrastructure (Fig. 12F, L). At week 8 post immunization in the vehicle control group, some myelin lamellae were still undergoing vesicular disintegration, but others were remyelinated (Fig. 12H). In the meantime, more remyelinated fibers appeared in C16 treated EAE rats (Fig. 12J, K).

At the chronic stage, the Bielschowsky staining impregnation revealed the axons to have swelling, deformed and ovoid formation (Fig. 13E). Likewise, axon lesions in the C16 treated group were less frequent and had markedly reduced destruction (Fig. 13F).

To assess whether our treatments could inhibit EAE-induced reactive gliosis at chronic stage, we examined expression of GFAP, a marker for astrocytes, at 8 weeks post-immunization. The GFAP expression and glia scar formations were significantly decreased in the C16 treated group (Fig. 14E, F versus H, I, Fig. 15C, P < 0.05). Furthermore, the values of myelination and axonal loss scores also demonstrated prevention of demyelination and axons loss in C16 treated rats in comparison with vehicle treatment (P < 0.05, Fig. 15A, B).
4. Discussion

Previous studies have revealed that C16 appears to exert anti-inflammatory activity mediated by specific integrins. As we aimed to ascertain whether endothelial cells in the CNS of a rat EAE model would respond to C16 peptide, the expression of αvβ3 and α5β1 integrin was assessed in induced EAE in Lewis rats after the onset of motor symptoms (week 2 post immunization). Our data showed that αvβ3 and α5β1 integrins are expressed in the endothelial cells of blood vessels (labeled by its specific marker: PECAM). Therefore, we deduced that C16 peptide might target against αvβ3 and α5β1 integrins, which may be involved in the process of the leukocyte infiltration that occurs in EAE.

Our results of in vitro transendothelial migration assay further corroborated that, by binding to integrins in endothelial cells and subsequently interfering with the leukocyte ligand required for transmigration, C16 peptide can prevent the transmigration of T lymphocytes at certain concentrations, and even counteract the effect of chemoattractant IL-16 at higher concentrations. Since previous studies have showed the scrambled C16 might act as an antagonist of αvβ3 and α5β1, and reverse C16 undergoes some binding activity with endothelial cells in culture, therefore, we selected SP3 peptide, which has no effect on migration, as the control peptide instead of the C16-based one (Ponce et al., 1999, 2001). In the present study, SP3 peptide, as an inactive scrambled peptide, is not capable of binding to αvβ3 nor α5β1 integrins (Jacques et al., 1998; Han et al., 2010).

Among a variety of EAE models in rodents, the acute EAE model induced in Lewis rats is a well-established model of MS, characterized by a single peak of paralysis after which animals recover spontaneously (Almolda et al., 2011). The utilization of this model gives us an opportunity to elucidate the induction, peak and resolution of the inflammation-based immune response of MS.

In acute EAE models, the induced animals rapidly exhibited a very strong degree of leukocyte infiltration throughout their brains and spinal cords after EAE onset. Several studies have demonstrated that macrophage/microglia activation and infiltration are essential for the development of clinical EAE (Ravich and Banati, 2004; Bhasin et al., 2007; Ponomarev et al., 2007). In particular, the activated microglia may produce a proinflammatory milieu, strip off myelin from axons, phagocytize myelin via receptor mediated pathways, disrupt the blood–brain barrier (BBB) integrity and attract and activate T lymphatic cells and monocytes which could augment the destruction of myelin (Carson, 2002; Yin et al., 2010). Macrophage cells could also differentiate into microglia-like cells, which were antigen presentation cells to the encephalitogenic T cells, and induce exacerbation of micro-environment (Ponomarev et al., 2004, 2005). Therefore, targeting neuroinflammatory reaction has been an important remedial point to protect the motor neurons and alleviate the clinical motor symptoms. This has been verified in numerous studies which traced the therapeutic effects through disruption of the leukocyte infiltration and macrophage/microglia activation (Mangas et al., 2008; Fang et al., 2011). Our data has demonstrate that the widespread perivascular and parenchymal infiltration of leukocytes, lymphocytes (labeled by pan-leukocyte marker CD45 and lymphocytes marker CD4), activated microglia and extravasated macrophages (labeled by CD68) in the CNS of EAE rats were all significantly suppressed by consecutive intravenous injections of C16. The inflammatory scores were also clearly decreased compared with that of the vehicle control.

Following trauma and inflammation to the CNS, astrocytes undergo hypertrophy and/or proliferation to become reactive astrogliosis. In general, GFAP positive reactive astroglia often accumulate within and at the margins of demyelinating lesions areas (Balasingam et al., 1994), and contribute to the inflammatory response in MS and EAE by synthesizing proinflammatory cytokines and chemokines, or by presenting peptide antigens to T lymphocytes (Balasingam et al., 1994). A long term result of the astrocytic reaction could be the formation of a glial scar at the lesion site, which may inhibit axonal regeneration or remyelination (Banerjee et al., 2007). Our observation revealed that C16 treatment evidently reduced the GFAP positive reactive astroglia at the chronic or recovery stage (week 8 post immunization) of acute EAE model, which was in accordance with the more rapid recovery of locomotor function, and TEM revealed more remyelination of axons in this group.

In our vehicle control EAE rats, extensive demyelination was confirmed by reduced LFB staining and focalized loss of MBP immunoreactivity. With electron microscopy, abundant loose, fused and fragmented spires of myelin sheath were observed. Axonal damage and subsequent axonal loss were also prominent in the spinal cords and brain cortices of EAE rats, which indicates a destructive inflammatory process lies behind the clinical signs such as motor impairment and paralysis. However, in the C16 treated group, significant decrease of demyelination areas and relief of axonal damage were detected at both week 2 and week 8 post immunization. Meanwhile, a notable decline in the expression of active caspase-3, an enzyme critically involved in the execution of the mammalian apoptotic cell death program, was detected in C16 treated EAE rats, which correlated with the increase of neurons that survived in both
Fig. 10. C16 treatment prevented demyelination in spinal cord and brain cortex at both week 2 and week 8 post-immunization, Luxol fast blue staining, counterstained with cresyl violet (nissl) staining. (A–F): Traverse section through the lumbar spinal cord. Normal control group (A), vehicle treated EAE rats (B, C) and C16 treated EAE rats (D) at week 2 post-immunization. (C is magnified image from the framed region in B, depicting the inflammatory cells infiltrated demyelination area). Vehicle treated (E) and C16 treated (F) EAE rats at week 8 post-immunization. (G–L): Horizontal section of the hindlimb area of motor cortex of each group. Normal control group (G, H), vehicle treated EAE rats (I) and C16 treated EAE rats (J) at week 2 post-immunization. Vehicle treated (K) and C16 treated (L) EAE rats at week 8 post-immunization, bar = 100 μm. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)
Fig. 11. C16 treatment protected myelination, MBP immuno-staining, counterstained with hematoxylin. Coronal sections of motor cortex: Normal control group (A), vehicle treated EAE rats (B, C, D), and C16 treated EAE rats (E, F) at week 2 post-immunization; Vehicle treated (G, H, I) and C16 treated (J, K, L) EAE rats at week 8 post-immunization, bar = 100 μm.
Fig. 12. TEM observation demonstrated prevention of myelination or axon loss, and inhibition of neuronal apoptosis in C16 treated rats. (A, B): Normal myelinated axons (A) (dark rings shape myelin sheath surrounding axon), and normal neuron (B) in normal control group. In the vehicle treated EAE rats at week 2 post-immunization (C, D), a considerable amount of the myelin sheath displayed splitting, vacuoles, and loose and fused change (C), component of axon has disappeared (arrow in C). The neurons showed apoptotic signs of a shrunken nucleus with condensed, fragmented and marginated nuclear chromatin (D). In C16 treated EAE rats, week 2 post-immunization (E, F), some vacuolated myelin sheaths were observed, and shrunken axons could be found (E), but the ultrastructure of neuron was still kept relatively normal (F). In vehicle treated EAE rats at week 8 post-immunization (G–I). The most impressive myelin lamella abnormalities were myelin cleavage, splitting, and fragmentation. Some myelin lamellae undergoing vesicular disintegration, others undergoing remyelinating (arrow in H), many apoptotic neurons (I). C16 treated EAE rats at week 8 post-immunization (J–L). Neurons maintained relatively normal ultrastructure in comparison with vehicle treated EAE rats (L), and more fibers were being remyelinated (arrow in J,K), bar = 2 μm.
Fig. 13. C16 treatment alleviated axonal loss revealed by Bielschowsky staining at week 8 post-immunization. Traverse sections through the lumbar spinal cord in normal control (A), vehicle treated EAE (B), and C16 treated EAE (C) rats. Axons orderly arranged in normal control group (D), but showed swelling, deformed and ovoid formation in the vehicle treated EAE group (E). Axon lesions in C16 treated group were less frequent (F), bar = 100 μm.
Fig. 14. C16 treatment inhibited reactive gliosis revealed by FITC-conjugated GFAP immunofluorescent staining at week 8 post-immunization, traverse section through the lumbar spinal cord. (A–C): Normal control group. (D–F): vehicle treated EAE rats. (G–I): C16 treated EAE rats. Normal astrocytes shape (B). Reactive astrogliosis forms gila scar both in anterior (E) and posterior (F) column of spinal cord in EAE rats, but such gila scar formations were significantly decreased in C16 treated group (H, I). Bar = 100 μm.
the brain and spinal cord anterior horn (shown by Nissl staining and cell counting). All these phenomena may be ascribed to the amelioration of the inflammatory milieu and improvement of the CNS micro-environment. Accordingly, the application of C16 also postponed the time point of EAE motor symptoms onset and depressed the clinical scores of the peak stage of EAE, thus suggesting it as a potential therapeutic agent for MS treatment.

Although our drugs have only been administered for 14 days, evident sustained neuroprotective effects were still confirmed at week 8 post immunization, which suggests that C16 peptide has a long-lasting effect.

5. Conclusion

Our present study has demonstrated that C16 treatment obtained beneficial effects through relieving inflammation-based immune response in vitro and in vivo. Although we elucidated that such a protective effect may be a consequence of the improvement in micro-environment, the underlying molecular mechanisms still need further investigation. Experiments along these lines are currently in progress in our laboratory.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

SH and MF designed the experiments and drafted the manuscript. ZYH and SCL participated in its design and coordination, YYS, JY and BBPW performed the experiments, HD analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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