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Neurological Functional Recovery After Thymosin Beta4 Treatment in Mice with Experimental Auto Encephalomyelitis

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Abstract

In the present study, we hypothesized that thymosin beta 4 (Tbeta4) is a potential therapy of multiple sclerosis (MS). To test this hypothesis, SJL/J mice (n=21) were subjected to experimental autoimmune encephalomyelitis (EAE), an animal model of MS. EAE mice were treated with saline or Tbeta4 (6 mg/kg, n=10) every 3 days starting on the day of myelin proteolipid protein immunization for total 5 doses. Neurological function, inflammatory infiltration, oligodendrocyte progenitor cells (OPCs) and mature oligodendrocytes were measured in the brain of EAE mice. Double immunohistochemical staining was used to detect proliferation and differentiation of OPCs. Tbeta4 was used to treat N20.1 cells (premature oligodendrocyte cell line) in vitro, and proliferation of N20.1 cells was measured by bromodeoxyuridine (BrdU) immunostaining. Tbeta4 treatment improved functional recovery after EAE. Inflammatory infiltrates were significantly reduced in the Tbeta4 treatment group compared to the saline groups (3.6±0.3/slide vs 5±0.5/slide, $p<0.05$). $NG2^+$ OPCs (447.7±41.9/mm^2 vs 195.2±31/mm^2 in subventricular zone, 75.1±4.7/mm^2 vs 41.7±3.2/mm^2 in white matter), $CNPase^+$ mature oligodendrocytes (267.5±10.3/mm^2 vs 141.4±22.9/ mm^2), $BrdU^+$ with $NG2^+$ OPCs (32.9±3.7/mm^2 vs 17.9±3.6/mm^2), $BrdU^+$ with $CNPase^+$ mature oligodendrocytes (18.2±1.7/mm^2 vs 10.7±2.2/mm^2) were significantly increased in the Tbeta4 treated mice compared to those of saline controls ($p<0.05$). These data indicate that Tbeta4 treatment improved functional recovery after EAE, possibly, via reducing inflammatory infiltrates, and stimulating oligodendrogenesis.

Keywords

experimental autoimmune encephalomyelitis; thymosin beta 4; oligodendrocyte progenitor cells; oligodendrocytes; oligodendrogenesis

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Introduction

Thymosin beta 4 (Tbeta4) is a highly conserved, 43-amino acid acidic peptide that was first isolated from bovine thymus tissue. The most prominent physiological function of Tbeta4 is the regulation of actin polymerization which is necessary for cell motility and organogenesis (Crockford, 2007). Recently, studies found that the intracellular function of Tbeta4 is not limited to simple actin-sequestration (Yarmola et al., 2007). The healing properties of Tbeta4 have been found in the skin (Philp et al., 2004a, Philp et al., 2004b) and cornea (Fine, 2007, Guarnera et al., 2007), and more recently it has been shown that Tbeta4 facilitates cardiac repair after infarction by promoting cell migration and myocyte survival (Cavasin, 2006). Tbeta4 is an essential paracrine factor of endothelial progenitor cells (Bock-Marquette et al., 2004, Hinkel et al., 2008, Kupatt et al., 2008) which mediate cardioprotection (Srivastava et al., 2007), and Tbeta4 promotes vasculogenesis and angiogenesis (Smart et al., 2007a, Smart et al., 2007c) after ischemic injury. Tbeta4 is an anti-inflammatory agent (Sosne et al., 2007a, Sosne et al., 2007b), inhibits proliferation and induces differentiation and apoptosis of leukemic cells (Philp et al., 2004b, Huang et al., 2006). Safety, tolerability and efficiency of Tbeta4 are being evaluated in clinical patients with acute myocardial infarction (Crockford, 2007) and other diseases (Fine, 2007, Guarnera et al., 2007, Smart et al., 2007b).

Tbeta4 is widely distributed in mammalian tissues including the nervous system. The presence of this peptide in the nervous system suggests a role for Tbeta4 in neuroprotection, synaptogenesis, axon growth, cell migration and plastic changes (Yang et al., 2008, Popoli et al., 2007), However, the effects of Tbeta4 on the oligodendrogenesis and suppression of inflammatory infiltration in the CNS have not been studied.

Oligodendrocytes are myelin producing cells which wrap around the axon. Multiple sclerosis (MS) is an immune-mediated demyelinating disease that recognizes myelin peptide determinants and initiates attacks directly against myelin constituents and causes myelin destruction (Lucchinetti et al., 2000, Hemmer et al., 2002). Multiple lesions of inflammation and demyelination are major pathological changes in the MS/EAE (Lucchinetti et al., 2000, Hemmer et al., 2002, Kuhlmann et al., 2002). Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS (Youssef et al., 2002).

Treatment approaches to MS/EAE include preventing damage from occurring and repairing residual damage. Besides suppression of the immune onslaught, oligodendrogenesis and remyelination are prerequisites for sustained functional recovery (Keirstead and Blakemore, 1999), reducing axonal loss and diminishing disability (Waxman, 1998). Oligodendrogenesis/ remyelination occurs in MS/EAE lesions (Suzuki et al., 1969, Prineas et al., 1993, Raine and Wu, 1993) which leads to the characteristic profile of remitting-relapsing disease (Kerschensteiner et al., 2004). Mature oligodendrocytes in the adult mammalian CNS are post-mitotic and are unable to proliferate in response to injury (Keirstead and Blakemore, 1997, Minagar, 2007). Once oligodendrocytes are lost in the adult CNS, they are not replaced by surviving mature oligodendrocytes (Keirstead and Blakemore, 1997, Carroll et al., 1998, McTigue and Tripathi, 2008). The repair process is mediated by oligodendrocyte progenitor cells (OPCs) located in the adult CNS and lesions, which contribute to the remyelination (Raine et al., 1988, Carroll and Jennings, 1994, Gensert and Goldman, 1997, Scolding et al., 1998, Wolswijk, 1998,Wolswijk, 2002). OPC proliferation is evident during demyelination (Raine et al., 1988, Carroll and Jennings, 1994, Gensert and Goldman, 1997, Carroll et al., 1998, Scolding et al., 1998, Wolswijk, 1998, Reynolds et al., 2002, Wolswijk, 2002), and endogenous OPCs represent a viable target for therapies intended to enhance remyelination in MS patients. Unfortunately, oligodendrogenesis /remyelination are often incomplete and not sufficient to recover neurological function and stop or delay disease relapse. During progression of the disease, repeated demyelination exhausts OPCs, and this failure of remyelination is one of the

main reasons for clinical deficits in demyelinating disease (Carroll et al., 1998, Keirstead and Blakemore, 1999)

There is as yet no cure for MS. Many medications have serious side effects and some carry significant risks (Aktas et al., 2007). In the present study, we hypothesize that Tbeta4 is a potential treatment which reduces the inflammatory infiltration and promotes oligodendrogenesis and improves functional recovery in the EAE mice.

Experimental procedures

All experimental procedures have been approved by the Institutional Animal Care and Use Committee of Henry Ford Health System.

1. EAE model and groups

EAE was induced in female SJL/J mice (8–10 week old, Jackson Laboratory, Bar Harbor, ME) by subcutaneous injection with 100ug myelin proteolipid protein (PLP) (p139–151, SynPep Corporation, Dublin, CA) dissolved in 50ul complete Freund's adjuvant (Difco Laboratories, Livonia, MI). Pertussis toxin (List Biological laboratories, Inc. Campbell, CA) 200ng in 0.2ml phosphate buffered saline (PBS) was injected into the mouse tail vein on the day of immunization and 48 hours later. A 0~5 functional scoring scale is widely used to measure neurological function of the EAE animal model (Youssef et al., 2002, Zhang et al., 2005). Mice were scored daily for clinical symptoms of EAE, as follows: 0, healthy; 1, loss of tail tone; 2, ataxia and/or paresis of hindlimbs; 3, paralysis of hindlimbs and/or paresis of forelimbs; 4, tetraparalysis; 5, moribund or dead. The higher score the more severe the disease. Mice were randomly divided into: **Tbeta4 treatment group** (n=10): Tbeta4 (RegeneRx, Bethesda, MD) was dissolved in saline, and administered at a concentration of 6 mg/kg i.p. on the day of PLP immunization, and then every 3 days (6 mg/kg IP) for 4 additional doses. The employed dose was based on our study of Tbeta4 treatment in stroke animal (data not shown). **Saline treatment group** (n=11): Saline (0.1ml) was injected i.p. on the day of immunization, and every 3 days for 4 additional doses as EAE controls. We tested the neurological functions of EAE mice treated with Tebeta4 or saline daily until 30 days after clinical symptom onset. In addition, mice were administered bromodeoxyuridine (BrdU, Sigma) 100 mg/kg ip daily for 7 days initiated on the disease onset day.

Mice with the EAE symptoms were randomized into either the saline treated group or the Tbeta4 treated group with the neurological assessments performed daily up to 30 days using the 0~5 scale. To study the Tbeta4 effect on neurological recovery in EAE, we calculated the median and cumulative neurological scores up to day 30 and compared the group difference using the nonparametric Wilcoxon Rank sum test, given that neurological scores were not normal. We used the cumulative of neurological scores (the sum of all the daily neurological score to 30 days) to measure the total deficits/recovery over the 30 days, and the neurological score at 30 day to measure the recovery at day 30.

2. Tissue Preparation

EAE mice treated with Tbeta4 or saline were euthanized at 30d after disease onset. Anesthetized mice were intracardiac perfused with saline and followed by 4% paraformaldehyde. Brains (bregma +1.54mm---bregma-0.54mm) were fixed in 4% of paraformaldehyde and divided into 2 serial blocks which were then embedded in paraffin. Four coronal slides (6μm thick) at intervals of 100 μm were cut from each block.

3. Histopathology and Quantification

Slides were stained with hematoxylin and eosin (HE) to detect inflammatory infiltrates adjacent to vessels in the brain of EAE mice.

OPCs and oligodendrocytes were identified by antibodies NG2 (1:100, Chemicon, Temecula, CA) and 2′,3′ cyclic nucleotide 3′ phosphodiesterase (CNPase, 1:100, Chemicon, Temecula, CA), respectively. A mouse monoclonal antibody against BrdU (1:100, DAKO, Carpinteria, CA) was employed to identify cell proliferation. Doubleimmunostaining for BrdU and NG2 was used to demonstrate the proliferation of OPCs. Double immunostaining CNPase and BrdU was performed to identify the differentiation of OPCs.

Immunostaining was performed following standard protocols. Slides were treated first with the primary antibody, and then with the antibody conjugated to fluorescein isothiocyanate (FITC, Jackson ImmunoResearch, West Grove, Pennsylvania). These slides were then treated with a second primary antibody, and then incubated with antibody conjugated to Cy3 (Jackson ImmunoResearch). Negative control slides for each animal received identical preparations for immunostaining, except that primary antibodies were omitted.

For each animal, the numbers of vessels with inflammatory infiltrates were detected in the total brain slides, and the immunoreactive cells were measured in 10 fields of the white matter, or total length of subventricular zone (SVZ), in each 6-μm thick slide digitized under a 40x microscope (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970 MD) interfaced with Micro Computer Imaging Device (MCID) image analysis system (Imaging Research Inc.Cambridge, England). The numbers of vessels are presented as numbers per slide. The numbers of immunoreactive cells were calculated and divided by the measured areas, and presented as numbers per mm^2.

Data are presented as mean ± SE. Significance between the two groups was examined using ANOVA analysis. A value of $p < 0.05$ was considered significant.

4. In Vitro Oligodendrocyte Proliferation

To further examine whether Tbeta4 is involved in oligodendrogenesis, we employed a premature oligodendrocyte cell line (N20.1, generously provided by Dr. Anthony Campagnoni, University of California at Los Angeles) to measure cell proliferation. N20.1 cell line was obtained from mouse primary cultures of oligodendrocytes conditionally immortalized by transformation with a temperature-sensitive large Tantigen. N20.1 cells are widely used to investigate cell proliferation and differentiation, and they are useful models to study the cellular and molecular mechanisms involved in the development, maturation and possibly formation of myelin by oligodendrocytes in the mammalian brain (Paez et al., 2004.

N20.1 cells were employed to mimic in situ OPCs. N20.1 cells were incubated in the presence of Tbeta4 (0, 25, 50ng/ml). N20.1 cells were grown in Dulbecco's modified Eagle's medium/F12 with 1% fetal bovine serum and G418 (100 μg/ml) at 39°C, and then were divided into 3 groups: (a) regular cell culture medium for control; (b) 25ng/ml Tbeta4; (c) 50ng/ml Tbeta4. For the cell proliferation study, N20.1 cells were treated for 24h, and 20ug/ml BrdU was added to the cell cultures for 2h prior to termination of incubation.

Immunocytochemistry was performed with BrdU antibody to determine proliferating N20.1 cells. BrdU immunopositive cells were measured by counting 10 random fields in each well with 6 wells per group. The results are presented as a percentage (positive cells divided by total cells). One-way analysis of variance followed by Student-Newman-Keuls test was performed. The data are presented as means ± SE. A value of $p<0.05$ is considered significant.

Results

1. Tbeta4 Treatment Improves Neurological Functional Recovery in EAE Mice

21 Mice with the EAE symptoms were randomized into either the saline treated group (n=11) or the Tbeta4 treated group (n=10) with the neurological assessments performed daily up to 30 days, using the 0~5 functional scale. Significant therapeutic Tbeta4 effects were detected as early as day 11 after EAE onset. Nearly 50% relative functional recovery based on the cumulative cores up to 30 days was observed in the Tbeta4 treated group, compared to the saline controls ($p<0.01$). At day 30, the median neurological score was 1 in the Tbeta4 treated group and 2 in the control treated group, with a significant difference between the two groups ($p<0.01$) (Figure A). Tbeta4 treatment initiated on the PLP immunization day, also significantly delayed the EAE onset compared with that in the saline treatment (9±2.1 vs 6.8±0.8, $p<0.05$), implying that Tbeta4 is a potential treatment for EAE animal which can delay the EAE onset and decrease the severity of disease.

2. Tbeta4 Treatment Reduces Inflammatory Infiltrates in the Brain of EAE Mice

Using H&E staining, inflammatory infiltrates adjacent to vessels in the brain of EAE mice were evident. The numbers of vessels containing inflammatory cell infiltration were significantly reduced in Tbeta4 treatment group compared with the EAE control group (3.58 ±1.25 vs 4.97±1.52, $p<0.05$) (Figure B~D). Since EAE is an autoimmune disease, these data imply that Tbeta4 may prevent and reduce EAE damage by attenuating immune reaction.

3. Tbeta4 Treatment Increases Oligodendrocyte Progenitor Cells and Mature Oligodendrocytes in the Brain of EAE Mice

Given the significant and robust improvement in neurological outcome, we sought to address whether Tbeta4 treatment affects oligodendrogenesis and remyelination which play critical roles in the MS/EAE recovery process. As indicated by the OPC marker NG2 immunostaining, the $NG2^+$ cells were mainly located in the SVZ and white matter, and significantly increased in the Tbeta4 treatment group compared with the control group (447.6±125.8 vs 195±93.1/mm^2, 75.1±14 vs 41.7±9.6/mm^2, $p<0.05$, respectively) at the 30d after disease onset (Figure E~I).

The mature oligodendrocytes, the $CNPase^+$ cells, were mainly located in the white matter, and these cells were significantly increased in the Tbeta4 treatment group compared with the control group (267.5±30.9 vs 141.4±72.5/mm^2, $p<0.05$) at 30d after disease onset (Figure J~L).

4. Tbeta4 Treatment Increases Oligodendrogenesis in the Brain of EAE Mice

Using proliferating cell marker (BrdU) and OPC marker (NG2) double immunostaining, we found proliferating OPCs ($BrdU^+$-$NG2^+$ cells) present in the EAE CNS, and Tbeta4 enhanced this proliferation (Figure M~O, Q). Using the proliferating cell marker (BrdU) and mature oligodendrocyte marker (CNPase) double immunostaining, $BrdU^+$ and $CNPase^+$ cells were present in the EAE CNS. After Tbeta4 treatment, $BrdU^+$-$CNPase^+$ cells were significantly increased compared with saline treatment (Figure P~Q). Since the mature oligodendrocytes are post-mitotic and are unable to proliferate (Keirstead and Blakemore, 1997, Minagar, 2007), these data imply that increased $BrdU^+$-$CNPase^+$ cells after Tbeta4 treatment derive from proliferating OPC differentiation.

5. Tbeta4 Treatment Promotes Oligodendrocyte Cell Culture Proliferation

Given the significant oligodendrogenesis effect of Tbeta4 in vivo, we sought to address whether Tbeta4 treatment promotes oligodendrogenesis in N20.1 cells in vitro. N20.1 cell proliferation, measured by BrdU immunostaining, was significantly increased in the Tbeta4 treatment groups

(16.7±6.5% in 25ng/ml, 18.3±6.7% in 50ng/ml) compared with normal group (9.6±3.1%, $p<0.01$).

Discussion

We demonstrate for the first time that Tbeta4 treatment of EAE initiated before disease onset significantly improves neurological functional recovery. Concomitant with this neurological benefit, are the significant reduction of inflammatory infiltration and induction of oligodendrogenesis. These data suggest that Tbeta4 treatment is a potential pharmacological therapy for MS patients.

Since EAE is an immune-mediated response that recognizes and attacks myelin peptide determinants, that causes myelin destruction and axonal loss (Lucchinetti et al., 2000, Hemmer et al., 2002), a reasonable first step in combating EAE is to suppress the immune onslaught. Blood-brain barrier (BBB) breakdown and the subsequent inflammatory cell infiltration have been recognized as important events in the pathogenesis of EAE/MS (Li et al., 2004). Our data demonstrated that the infiltration of inflammatory cells was significantly reduced in the Tbeta4 treatment group compared with the EAE control group. This may due to the suppression of inflammation induced by PLP, reduction of BBB damage and decrease of inflammatory cell infiltration, all of which contribute to the alleviation of disease. Tbeta4 is an anti-inflammatory agent (Sosne et al., 2007a) with immunomodulatory properties (Badamchian et al., 2003) and down-regulates local inflammation (Girardi et al., 2003). Tbeta4 significantly suppresses the secretion of interleukin-8 (Reti et al., 2008), and the activation of nuclear factor-kappa b, which is the mediator of the inflammatory process initiated by pro-inflammatory cytokines (Sosne et al., 2007b) (Sosne et al., 2007a). These results have important clinical implications for the potential role of Tbeta4 as an anti-inflammatory agent. Further studies are required to measure other indices of immune reaction, such as Th1 and Th2 cytokines, and to evaluate the immunomodulatory role of Tbeta4 after EAE. In addition, further investigations on the inflammatory cell infiltration (e.g. using CD45 antibody) in the EAE CNS with and without Tbeta4 treatment is warranted.

Tbeta4 treatment of EAE mice by reducing inflammation in the CNS may reduce damage to oligodendrocytes and thereby promote neurological functional benefit. However, while considerable progress has been made in the recent years with the development of anti-inflammatory and immunomodulatory therapies, there are currently no effective repair therapies routinely used in MS patients (Rodriguez, 2003, Zhao et al., 2005, Aharoni et al., 2008). Clinical trials have clearly demonstrated that an anti-inflammation treatment approach alone is insufficient in preventing or ameliorating permanent and accumulating MS deficits (Confavreux et al., 2003). Oligodendrogenesis/remyelination is an important therapeutic goal (Keirstead and Blakemore, 1999, Chari and Blakemore, 2002, Stangel and Hartung, 2002, Bruck et al., 2003, Ohori et al., 2006, Tripathi and McTigue, 2007, McTigue and Tripathi, 2008). There is a highly plastic remyelination response of the CNS to the MS/EAE lesions (Suzuki et al., 1969, Prineas et al., 1993, Raine and Wu, 1993). Self repair capacity of CNS after MS/EAE leads to the remitting-relapsing characteristics of function. This repair process is mediated by a population of cells located throughout the gray and white matter in the adult CNS, that has often been referred to as adult OPCs (Raine et al., 1988, Althaus et al., 1992, Vick et al., 1992, Prayoonwiwat and Rodriguez, 1993, Carroll and Jennings, 1994, Gensert and Goldman, 1997, Keirstead et al., 1998, Scolding et al., 1998, Wolswijk, 1998, Wolswijk, 2002, Jiang et al., 2008).

We then investigated the effects of Tbeta4 on oligodendrogenesis/remyelination. Widespread death of oligodendrocytes occurs in MS/EAE leading to demyelination (Lucchinetti et al., 2000, Hemmer et al., 2002). How to induce and strengthen the endogenous response such as

oligodendrogenesis to MS/EAE of the CNS is the primary goal of the present study. Oligodendrogenesis consists of two sequential events; first, OPCs proliferate after damage, and second, proliferated OPCs differentiate to mature oligodendrocytes to remyelinate axons, leading to functional recovery. NG2 immunostaining demonstrated OPCs are present in the SVZ and white matter of the EAE CNS, and Tbeta4 treatment significantly increased $NG2^+$ cells, and proliferating OPCs ($BrdU^+$-$NG2^+$) in these regions, suggesting that Tbeta4 treatment promotes the first step of oligodendrogenesis. $CNPase^+$ cells and $BrdU^+$-$CNPase^+$ cells significantly increased after Tbeta4 treatment, implying that Tbeta4 promotes OPC differentiation into myelinated oligodendrocytes. This hypothesis is also supported by our in vitro data that Tbeta4 significantly increased N20.1 cell proliferation.

In summary, our data indicate that Tbeta4 treatment improves neurological functional recovery after EAE in mice, possibly via, reducing inflammatory infiltrates and by increasing oligodendrogenesis. However, we note, that in the present study, the pathways involved in the Tbeta4 treatment activatation of oligodendrogenesis were not investigated.

Comprehensive list of abbreviations

Tbeta4, thymosin beta 4
MS, multiple sclerosis
EAE, experimental autoimmune encephalomyelitis
OPC, oligodendrocyte progenitor cell
BrdU, bromodeoxyuridine
CNS, central nervous system
PLP, myelin proteolipid protein
PBS, phosphate buffered saline
HE, hematoxylin and eosin
CNPase, 2′,3′ cyclic nucleotide 3′ phosphodiesterase
FITC, fluorescein isothiocyanate
SVZ, subventricular zone
MCID, Micro Computer Imaging Device

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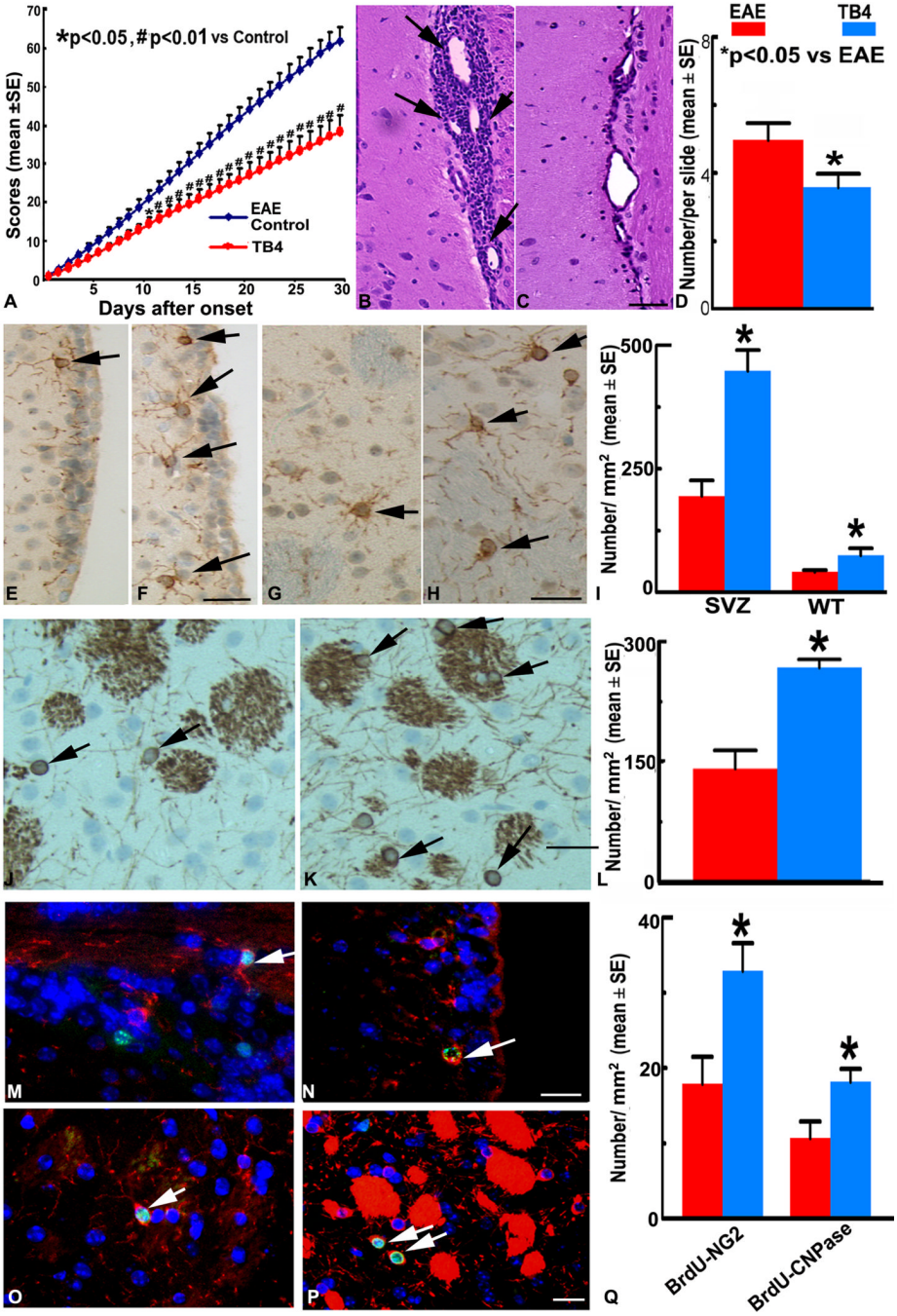

Figure 1.

The neurological response of EAE mice treated with or without Tbeta4. The significant therapeutic Tbeta4 effects were detected as early as day 11 after EAE onset. Nearly 50% relative functional recovery was observed in the Tbeta4 treated group, compared to the saline controls with $p<0.01$ for either the median score or the cumulative score up to 30 days. **B~D**. H&E staining show inflammatory infiltrates adjacent to vessels (black arrows) in the brain of EAE control mice (B) and Tbeta4 treated mice (C). Quantitative data (D) show the numbers of vessels containing inflammatory infiltrates were significantly reduced in the Tbeta4 treatment group compared with the saline group ($p<0.05$). **EμI**. The DAB staining by NG2 shows the OPCs in the SVZ (E~F) and the white matter (G~H) of EAE mice treated by saline

(E, G), and Tbeta4 (F, H). Quantitative data show that $NG2^+$ cells (I) were significantly increased at 30d after EAE onset in the Tbeta4 treatment group compared to that in the saline group. (**J~L**) The staining by CNPase shows the mature oligodendrocytes in the white matter of EAE mice treated by saline (J) and Tbeta4 (K). Quantitative data (L) show that $CNPase^+$ cells were significantly increased at 30d after EAE onset in the Tbeta4 treatment group compared to that in the saline group. (**M~Q**) Double immunofluorescence staining (nuclei, DAPI, blue) indicated that $BrdU^+$ cells (FITC, green) were colocalized with $NG2^+$ OPCs (CY3, red) in the corcpus collasum (M), SVZ (N) and striatum (O), $BrdU^+$ cells were also colocalized with $CNPase^+$ mature oligodendrocytes (CY3, red) in the white matter (P). Quantitative data (Q) show that $BrdU^+$-$NG2^+$ cells and $BrdU^+$-$CNPase^+$ were significantly increased at 30d after EAE onset in the Tbeta4 treatment group compared to that in the saline group. Scale bars in B~C=50 μm, E~H, J~K, M~P=25 μm.