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Bowman-Birk Inhibitor Suppresses Autoimmune Inflammation and Neuronal Loss in a Mouse Model of Multiple Sclerosis

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Abstract

The Bowman-Birk inhibitor (BBI) is a soybean-derived serine protease inhibitor. BBI concentrate (BBIC) is an extract enriched with BBI, but predominantly contains other ingredients including several protease inhibitors. We previously found that BBIC administration to Lewis rats with experimental autoimmune encephalomyelitis (EAE) significantly suppresses disease. In the present study we determined whether BBI mediates the suppressive effects of BBIC in EAE, evaluated its potential neuroprotective effects, and investigated mechanisms of BBI action. We tested effects of purified BBI on clinical and histopathological parameters of EAE in two models (relapsing/remitting EAE in SJL/J mice and chronic EAE in C57BL/6 mice). Effects of BBI were compared to BBIC in relapsing/remitting EAE, and effects of BBI on neuronal survival were examined during acute optic neuritis. Treatment with BBI in both EAE models significantly improved EAE disease parameters (onset, severity, weight loss, inflammation and demyelination). BBI significantly reduced the incidence of optic neuritis and prevented loss of retinal ganglion cells. In most experiments proliferation of immune cells derived from BBI-treated mice was significantly lower relative to control groups. Using Boyden's chamber assay we found that BBI inhibited invasiveness of activated splenocytes through the matrigel barrier. BBI also induced higher production of EAE-suppressive cytokine IL-10 by immune cells. These results demonstrate that BBI is the active component of BBIC that ameliorates clinical EAE. BBI reduces inflammation and attenuates neuronal loss, making it an excellent candidate for oral therapy in MS. BBI likely ameliorates EAE by inhibiting multiple pathways involved in disease pathogenesis.

Keywords

Multiple sclerosis; EAE; serine proteases; Bowman-Birk inhibitor; neuroprotection; neuroimmunology

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1. Introduction

Multiple Sclerosis (MS) is an incurable inflammatory demyelinating disease of the central nervous system (CNS) [1]. Therapies for MS rely on manipulation of the immune system, but with often modest effectiveness on reducing clinical episodes or permanent neurological disability, requiring frequent injections, and with sometimes significant side effects [2]. Introduction of a novel treatment that can be administered orally, and acts synergistically with existing therapies would be highly beneficial for the treatment of MS [3].

EAE is an animal model of MS commonly used for studying disease mechanisms and testing potential therapies [4]. EAE is induced by immunization with myelin proteins, leading to development of myelin-specific T cells. These autoreactive T cells traffic into CNS parenchyma where they initiate an inflammatory cascade through recruitment of other cells such as monocytes and neutrophils. Accumulation of immune cells within CNS leads to myelin damage, axonal loss and clinical deficit in affected animals [5].

The role of proteases in inflammatory responses, mainly, the plasminogen/plasmin system and the matrix metalloproteinases (MMPs) have been extensively studied [6]. Emerging data indicate that serine proteases play crucial regulatory roles in inflammatory processes through a number of different mechanisms, including direct modification of chemokine and cytokine activity, activation of receptors, cleavage of adhesion molecules, induction of CXCL2, CXCL8, CCL2, MCP-1 and IL-8 expression, activation of TLR4, and induction and activation of MMPs [7], [8] and [9]. Proteases can directly contribute to inflammatory damage in EAE through disruption of the blood brain barrier (BBB) [10] and parenchymal injury [11]. Mechanisms of protease-mediated damage in EAE include the generation of MMPs from inactive precursors [12] and intra- and extracellular cleavage of myelin proteins [13] and [14]. Patients with acute MS have elevated MMP levels in cerebrospinal fluid, blood, and brain tissue [15], [16], [17], [18], [19] and [20].

BBi is a small (8 kD) soybean-derived protein that belongs to a family of serine protease inhibitors [21]. BBi molecules have two different protease inhibitory sites; one having high binding affinity toward trypsin-, and another one toward chymotrypsin-like proteases [21]. Proteases inhibited by BBi include trypsin, chymotrypsin, elastase, cathepsin G, and mast cell chymase [21], [22] and [23]. BBi has both anti-carcinogenic [24] and anti-inflammatory effects [25]. BBi is resistant to degradation in the digestive system and a large proportion is adsorbed into the circulation [26], crucial features for an orally administered drug.

BBi concentrate (BBiC) is soybean extract enriched in BBi that has been investigated in preclinical studies and clinical trials in patients with benign prostatic hyperplasia, oral leukoplakia and ulcerative colitis [27]. Importantly, BBiC has shown a favorable safety profile, opening a possibility for its use as an oral therapy for MS [28], [29] and [30]. Fractionation of BBiC demonstrates proteins comprise only one third of its dry weight and BBi represents only a fraction of the proteins. BBiC, in addition to BBi, contains another four protease inhibitors with trypsin inhibitory activity, but unlike BBi they do not inhibit chymotrypsin [27]. Thus BBi, a potentially active component of BBiC, represents only a minor ingredient within this complex mixture, suggesting other components of BBiC might produce effects difficult to distinguish from those of BBi.

Previously we found that BBiC suppresses EAE in Lewis rats with no apparent adverse effects [31]. The goal of the present study was to determine whether BBi mediates the suppressive effects of BBiC in EAE, and to investigate potential mechanisms of its action. The use of pure BBi allows for dissection of its effects without interference by other components present in BBiC. We conducted these studies in mice because of advantageous smaller size of the animals, especially from the perspective of dosing with pure BBi. In addition, the mouse system offers

a number of genetically modified strains, different EAE models and reagents that are not available in rat.

BBI administration intraperitoneally or orally significantly reduced clinical and histopathological severity of EAE in two mouse models. Importantly for therapy of MS, BBI ameliorated disease even when treatment was initiated after disease onset. In addition to reducing CNS inflammation, BBI also prevented neuronal loss associated with optic nerve inflammation in EAE. These improvements were associated with reduced numbers of immune cells that infiltrated CNS during disease and with increased IL-5 and IL-10 secretion. Taken together, our data strongly support BBI as a candidate drug for oral therapy of MS.

2. Methods

2.1. Mice and EAE induction

Mice were purchased from Jackson Laboratories (Bar Harbor, ME), and housed at the Thomas Jefferson University animal care facility. For EAE induction, C57BL/6 female mice were injected subcutaneously with 100 µg of myelin oligodendroglial protein peptide (MOG_{35–55}) in CFA containing 1 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Pertussis toxin (200 ng) was given intraperitoneally (i.p.) on days 0 and 2 post-immunization (p.i.) [32]. EAE in SJL/J mice was induced using the same protocol, except proteolipid protein peptide (PLP_{139–151}) immunogen was used.

2.2. Cytokine measurements

Splenocytes and lymph node cells were cultured at a density of 2.5×10^6 cells/ml in RPMI 1640 medium containing 10% FCS in the presence of 30 µg/ml PLP_{139–151} (SJL/J mice) or MOG_{35–55} (C57BL/6 mice), 5 µg/ml Con A, or 1 µg/ml anti-CD3 and 1 µg/ml anti-CD28. Cytokine production was assayed from cell culture supernatants using Cytometric Bead Array (CBA, BD Biosciences). Concentrations of IL-4, IL-5 and IL-10 were re-assayed using ELISA kits (BD Bioscience).

2.3. T cell proliferation assays

Spleen or lymph node cells were cultured in 96-well plates at a density of 2.5×10^5 cells/ml for 60 h in the presence of 30 µg/ml PLP_{139–151} (SJL/J mice) or 30 µg/ml MOG_{35–55} (C57BL/6 mice), 5 µg/ml Con A or 1 µg/ml anti-mouse anti-CD3/anti-CD28. During the last 12 h of culture, cells were pulsed with 0.5 µCi of ³H-thymidine, then harvested on nitrocellulose filter paper and ³H-thymidine incorporation was measured using a beta-counter (Applied Biosystems, Foster City, CA).

2.4. Isolation of CNS cells and flow cytometry

Mononuclear cells (MNCs) from the CNS of immunized mice were isolated by Percoll gradient centrifugation based on modified published methods [33] and [34]. Briefly, mice were transcardially perfused with PBS. Spinal cords were mechanically dissociated through a 100 µm cell strainer, and the pellet was fractionated on a 30/60% Percoll gradient. Microglia and infiltrating MNCs were recovered from the 30/60 interface and used for flow cytometric analyses. Flow cytometric analysis of single cell suspensions of spleen cells for surface and intracytoplasmic staining was performed as described [32].

2.5. Quantification of neuronal survival in optic neuritis

Retinal ganglion cells (RGCs) were labelled and quantified as described previously [35]. 2.5 µl of 1.25% hydroxystilbamidine (Fluorogold, Molecular Probes, Eugene, OR) in PBS was injected stereotactically into each superior colliculus one week prior to immunization.

Following sacrifice, eyes and optic nerves were removed and fixed in 4% paraformaldehyde. Dissected retinas were flat mounted on glass slides, viewed by fluorescent microscopy (Nikon Eclipse E600), and photographed at 20X magnification in 12 standard fields: 1/6, 3/6, and 5/6 of the retinal radius from the center of the retina in each quadrant. RGCs were counted by a blinded investigator using Image-Pro Plus 5.0 software (Media Cybernetics, Silver Spring, MD, USA). Optic nerves were embedded in paraffin, and cut into 5 μ m longitudinal sections. Nerves were stained with hematoxylin and eosin, and the presence of inflammatory cell infiltration was assessed. Eyes with any level of optic nerve inflammation, scored on a 4-point scale, were considered to have optic neuritis.

2.6. Invasion assay

The Boyden's chamber invasion assay was performed according to the manufacturer's instructions (BD Biosciences) using 24 well plates. Briefly, the upper chamber was filled with 300 μ l of RPMI 1640 (Cellgro, Herndon, VA) and the lower chamber with 700 μ l of RMPI containing 5% FCS and 50 ng/ml of Stromal cell-derived factor 1 alpha (SDF-1 α). Splenocytes harvested from naive C57BL/6 mice were activated in vitro for 48 h with 5 μ g/ml of Con A, placed in the upper chamber at density of 1.5×10^6 cells/well and incubated for 24 h. Depending on the experiment, different concentrations of BBI or BBIC were added to each chamber. After incubation, cells in the lower chamber were counted.

2.7. Statistical analysis

Non-parametric Mann-Whitney U test was used for comparisons of average daily clinical scores, pathological lesion numbers and severity indices, proliferative responses, and cytokine levels between different groups.

3. Results

3.1. BBI suppresses EAE in SJL/J mice

The effectiveness of BBI versus BBIC on EAE in SJL mice was compared. Treatment with 1 mg/day of BBI or 3 mg/day of BBIC i.p. from day 5 to 20 p.i. had identical ameliorating effects on clinical course of EAE. Disease onset was delayed (Fig. 1A) and cumulative, maximal and average clinical scores were significantly lower compared to PBS-treated mice (Fig. 1B). Loss of body weight in BBI and BBIC treated groups was also delayed until disease onset (Fig. 1C). At the end of the experiment we determined by ELISA that both BBI and BBIC elicited strong humoral responses against BBI (data not shown). Nonetheless, this experiment demonstrated that highly purified BBI ameliorates EAE in the SJL/PLP₁₃₉₋₁₅₁ model similar to BBIC.

Proliferative responses of spleen cells from BBI and BBIC treated mice to PLP₁₃₉₋₁₅₁ were significantly lower than PBS treated mice. In response to Con A stimulation, lymphocyte proliferation was also lower in BBI and BBIC treated mice, however, these differences did not reach statistical significance (Fig. 1D).

3.2. BBI suppresses EAE in C57BL/6 mice

Intraperitoneal delivery of BBI induced high levels of anti-BBI antibodies, whereas Maki et al. showed that oral application of BBI does not elicit significant antibody responses [36]. We corroborated this observation in several experiments (data not shown), and in the following experiments administered BBI by oral gavage.

C57BL/6 mice immunized with MOG₃₅₋₅₅ were treated with two doses of BBI (0.1 or 1 mg) from day 6 to 20 p.i. Both doses significantly reduced clinical EAE, however, the higher dose was more effective (Fig. 2A). We observed delayed onset of EAE and significant reduction of clinical parameters (cumulative, maximal and average scores) in the 1 mg BBI treatment group

(Fig. 2B). These animals also lost significantly less body weight than animals treated with PBS (Fig. 2C).

The number of spinal cord infiltrating MNCs in both BBI treated groups was 60 % reduced in comparison with PBS treated mice (Fig. 2D). Flow cytometric analysis of these MNCs demonstrated that composition/ratio of different cell types ($CD4^+$, $CD8^+$ T cells, macrophages and B cells) was not significantly affected by BBI treatment compared to control (data not shown).

The size of demyelinated areas and the number of inflammatory foci was significantly reduced in spinal cords in the 1 mg/day BBI treatment group compared to PBS treated mice (Fig. 3A, 3B, 3C, 3D). We also observed a trend of reduced demyelination and inflammation in the 0.1 mg treatment group that did not reach statistical significance.

3.3. Early treatment with BBI reduces clinical EAE

In order to study at which phase of EAE induction BBI exerts effects, after immunization of C57BL/6 mice we administered BBI (1 mg/day) by oral gavage from day 1 to day 8 p.i. Early BBI treatment significantly reduced clinical EAE (Fig. 4A). Both inflammation and demyelination were significantly reduced in the BBI treated group (Fig. 4B, 4C) in comparison with PBS treated mice.

Separate groups of mice ($n=7$) were immunized with MOG₃₅₋₅₅ and treated with 1 mg/day of BBI from day 1 to day 8, and then sacrificed on day 9, 14 and 21 p.i. BBI treatment reduced the number of spinal cord infiltrating MNCs of these mice more than 60 % at all three time points in comparison to PBS treated mice (data not shown).

Both spleen and lymph node lymphocyte proliferation in response to MOG₃₅₋₅₅ or anti-CD3/CD28 antibodies was significantly lower in BBI-treated mice sacrificed on day 14 p.i. (Fig. 4D, 4E). In the group sacrificed at day 21, splenocyte proliferation was not reduced, while in lymph node cultures, proliferation after MOG₃₅₋₅₅ stimulation remained significantly reduced in BBI treated mice compared to the PBS treatment (Fig. 4F, 4G).

We also determined cytokine production in response to MOG₃₅₋₅₅ and the mitogen Con A after 72 h of culture. Both spleen and lymph node cells from BBI treated mice showed a significant increase in the production of IL-5 and IL-10 for groups sacrificed at day 9, 14 and 21 p.i. Data for mice sacrificed 14 days p.i. are shown (Fig. 4H, 4I). There was no significant difference in any of the other cytokines tested (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF- α , IFN- γ , MCP-1) at any time point.

To study the effect of BBI on EAE after disease onset, MOG₃₅₋₅₅ immunized mice were treated with 0.1 or 1 mg of BBI from day 13 p.i. At the time treatment was initiated mice already had EAE symptoms (average clinical score of 2). BBI treatment rapidly stopped further progression of disease at both dosages, while PBS treated group continued to accumulate clinical deficits (Fig. 5). However, this improvement did not reach statistical significance.

3.4. Incidence of optic neuritis and evaluation of RGC loss in R/R EAE mice treated with BBI

To determine whether BBI treatment attenuates neuronal loss in EAE, retinal ganglion cell (RGC) survival was assessed during acute optic neuritis. Mice were treated with PBS or BBI by oral gavage daily from day 6 until sacrifice at day 14 or day 18 p.i.

The incidence of optic neuritis (ON) was lower in BBI-treated EAE mice than placebo-treated EAE mice (Fig. 6A), as 75% (9/12) of optic nerves from placebo-treated EAE mice had histologic evidence of ON, whereas only 30% (3/10) eyes from BBI-treated EAE mice

developed ON at day 14; and 62.5% (5/8) of eyes from placebo-treated EAE mice had ON, whereas only 25% (2/8) eyes from BBI-treated EAE mice developed ON at day 18. In addition, all of the few eyes from BBI-treated mice that did develop ON at either time point had only mild inflammation (graded 1.0 ± 0.0 , $N = 5$), which was significantly less inflammation than in placebo treated eyes (1.43 ± 0.51 , $N = 14$; $p = 0.008$).

Significant loss of RGCs occurred in EAE ON eyes treated with placebo ($*p < 0.05$) as compared to control eyes at both day 14 and day 18 (Fig. 6B), similar to prior studies [35]. The few eyes that did develop ON with BBI treatment had no significant loss of RGCs. EAE eyes that did not develop ON (placebo- or BBI-treated) had no loss of RGCs compared with control eyes (data not shown), similar to prior studies.

3.5. BBI induces IL-5 and IL-10 production in naïve C57BL/6 mice

Splenic and lymph node cells derived from EAE mice treated with BBI showed increased production of IL-5 and IL-10 (Fig. 4H, 4I). To study the effect of BBI on proliferation and cytokine production in the absence of inflammation, we treated naïve C57BL/6 mice with 1 mg/day of BBI for 7 days and sacrificed them at day 8. Spleen cells from BBI and PBS treated mice showed identical proliferative responses irrespective of whether BBI was present or absent in the cell culture medium (data not shown). Consistent with our previous observation with immunized mice, activation of splenocytes from BBI treated mice lead to increased production of IL-5 and IL-10 in cell cultures (Fig. 7A), while concentrations of other cytokines remained the same between the two groups.

Splenocytes from previously untreated C57BL/6 mice upon *in vitro* activation in the presence of BBI also produced higher levels of IL-5 and IL-10 (Fig. 7B) while proliferative response remained the same irrespective of BBI presence (data not shown).

3.6. BBI inhibits invasiveness of splenocytes in the Boyden's chamber assay

To measure the effect of BBI on invasiveness of immune cells *in vitro*, we used the Boyden's chamber (BD BioCoat™ Angiogenesis Systems). In this model, upper and lower wells are separated by a layer of matrigel, a natural protein gel similar to a basement membrane. Naïve splenocytes were activated with Con A and then placed in the upper well containing media with BBI. Numbers of cells that migrated to the lower well, containing chemoattractant SDF1- α , was determined after 24 h. BBI dramatically reduced splenocyte invasion at concentrations of 0.1, 0.01 and 0.001 mg/ml ($p = 0.013$, $p = 0.006$ and $p = 0.013$ respectively) (Fig. 8). Addition of anti-BBI antisera in the upper well abrogated inhibitory effects of BBI on cell migration, while non-BBI-specific anti-sera had no effect (data not shown). Viability of activated splenocytes was not affected by the presence of BBI, as demonstrated by exclusion of trypan blue dye. Thus, differences in cell invasiveness can not be attributed to toxicity of BBI.

4. Discussion

Our previous study demonstrated efficacy of the soybean-derived extract, BBIC, in the treatment of a rat EAE model [31]. Here we demonstrate that pure BBI has a beneficial effect in two mouse EAE models. Direct comparison of BBI and BBIC showed identical effects on disease course, suggesting that BBI is an active component of BBIC.

Daily intraperitoneal administration of BBI to SJL mice immunized with PLP₁₃₉₋₁₅₁ lead to delayed disease onset and milder EAE symptoms. However, repeated injections of BBI induced strong anti-BBI antibody responses, confirming previous observations that this route of administration is immunogenic [36]. Considering that oral application is preferred for treatment of MS patients, and the low immunogenicity of BBI administered orally, we conducted

subsequent experiments using oral gavage. BBI in C57BL/6-MOG₃₅₋₅₅ EAE showed similar effects as in SJL/J-PLP₁₃₉₋₁₅₁ EAE. Taken together, beneficial effects of BBI and BBIC in rat monophasic, mouse relapsing, and chronic EAE models, as well as optic neuritis, indicate that BBI is a potential treatment for patients with different types of MS. It is also encouraging that BBI has beneficial effects in a variety of treatment regimens, at the time of disease induction, during disease onset, or during clinically established disease. Though optimal dosing and timing of BBI treatment remain to be evaluated, our data indicate that BBI can be used for both preventive and therapeutic types of treatment. The well established non-toxic nature of BBI would likely allow its long term application [28], [29] and [30].

While BBI reduces CNS inflammation and ameliorates EAE, its exact mechanism of action remains unclear. BBI might exert its effect in EAE by more than one mechanism by inhibiting different serine proteases. Theoretically, BBI can impact development of autoimmune response at the periphery after immunization, diminish invasiveness of immune cells through the BBB, dampen effector mechanisms of immune cells within the CNS or curtail recruitment of additional inflammatory cells into the CNS. Our data indicate that BBI may act on all the above mentioned aspects of disease development. Suggestive of the possibility that BBI affects more than one pathway involved in EAE pathogenesis is that both 0.1 and 1 mg doses lower the number of CNS infiltrating cells to the same extent, but the higher dose is more effective in suppressing clinical disease. Perhaps, both BBI concentrations efficiently inhibit CNS invasion by immune cells, but only the higher dose affects additional pathways required to more successfully ameliorate clinical disease. Consistent with this view are our in vitro data showing that even low concentrations of BBI inhibit invasiveness of immune cells in Boyden's chamber assay as effectively as much higher concentrations.

Our data support the view that BBI ameliorates EAE through its action on the immune system without acting as an overt immunosuppressant [25]. BBIC inhibits activation of T cells reactive to MBP in vitro and ex vivo from immunized rats [31] possibly due to inhibition of antigen processing because BBI potently inhibits proteosomal chymotrypsin-like activity [37]. However, we observed similar T cell inhibition in systems where PLP₁₃₉₋₁₅₁ and MOG₃₅₋₅₅ peptides were used. Contrary to MBP, a full length protein, these peptides do not require significant processing to be presented in the context of MHC class II. Serine protease inhibitors can block the generation of extracellular immunogenic peptides [38], but it is unlikely that this affects presentation of PLP₁₃₉₋₁₅₁ and MOG₃₅₋₅₅ peptides as mitogenic stimulation with Con A or anti-CD3/CD28 antibodies led to lower proliferation of T cells. BBI may inhibit processing and presentation of endogenous myelin antigens within CNS, leading to decreased CNS inflammation. However, this should not affect the magnitude of myelin specific responses within peripheral lymphoid organs, where activation of T cells relies on exogenous peptides used as immunogens. Thus, it seems unlikely that BBI ameliorates EAE by inhibiting processing of myelin antigens used for immunization.

One potential mechanism of BBI-mediated suppression of CNS inflammation might be inhibition of BBB breakdown by immune cells, resulting in reduced infiltration into CNS. MMPs are produced by inflammatory cells and directly act on basement membrane increasing permeability of BBB [10]. MMPs are typically produced as enzymatically inactive proenzymes and activated by proteolytic cleavage initiated by serine proteases [12], [39], [40], [41] and [42]. Thus, serine protease inhibitors can prevent conversion of pro-MMPs to enzymatically active forms. BBI inhibits generation of active MMP-1 and MMP-9 in vitro [43] and [44], and BBIC reduces MMP-2 and -9 activity in supernatants of spleen cells [31]. Consistent with the hypothesis that BBI inhibits invasiveness of immune cells through BBB, in Boyden's chamber assay BBI reduced migration of activated splenocytes. Our published data suggest the mechanism underlying this effect may rely on inhibition of conversion of pro-MMPs into enzymatically active forms [31], [43] and [44]. Extrapolated to in vivo, it is likely that BBI can

diminish immune cell trafficking across BBB, consistent with our observation that mice treated with BBI have dramatically lower numbers of CNS infiltrating MNCs than control animals.

A previous study demonstrated that BBI can enter inflamed CNS [31], likely due to breakdown of BBB by immune cells. Accessibility of inflamed CNS regions to BBI provides the basis for direct in situ effects of BBI on infiltrating cells and their effector mechanisms. Strikingly, even treatment initiated after mice already developed symptoms rapidly ameliorated disease. Rapid effects of BBI at this disease stage (day 14 p.i., average score 2) when spinal cord is already infiltrated with immune cells indicates that BBI directly affects effector mechanisms of these cells, possibly by inhibiting serine proteases that activate tissue damaging MMPs.

Our findings also suggest BBI may ameliorate EAE by altering immune cell cytokine production. Supernatants from in vitro activated splenocytes showed that BBI increases production of IL-5 and IL-10. IL-5 is a Th2 cytokine that does not have apparent suppressive effects on EAE, since mice deficient for this cytokine have susceptibility to EAE similar to wild type mice [45]. However, in our system increased production of IL-5 may potentially lead to immune deviation of anti-myelin responses toward Th2 type, which are known to positively correlate with suppression of EAE and recovery from the disease. Unlike for IL-5, strong suppressive effects of IL-10 on EAE are well documented [46], [47], [48] and [49]. Simultaneous upregulation of IL-5 and IL-10 has been associated with suppression of EAE [50], [51] and [52], but it remains unclear if IL-5 plays a role in the suppression, or observed effects can be fully attributed to IL-10. Increased IL-10 and possibly IL-5 production likely represent one mechanism underlying amelioration of EAE by BBI.

Thus, various mechanisms of BBI acting to reduce autoimmune responses at periphery, altering cytokine production, preserving BBB, and affecting effector mechanisms likely work in combination to mediate the potent suppression of EAE by BBI. In addition to ameliorating clinical severity of EAE and reducing histopathological inflammation and demyelination, BBI also attenuated neuronal loss during acute optic neuritis in EAE. Axonal damage and loss of neurons leads to permanent neurological dysfunction in MS and EAE [53], and current immunomodulatory therapies have limited efficacy in preventing neuronal damage [54]. The ability of BBI to prevent both inflammatory and neurodegenerative aspects of EAE suggest that it may be useful for treating both acute MS exacerbations as well as long-term neurological dysfunction. In all, past and current results suggest BBI is an excellent candidate for an oral therapy for MS.

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Fig. 1.

Effect of BBI and BBIC on EAE in SJL/J mice. Female 8 weeks old SJL/J mice were immunized with PLP₁₃₉₋₁₅₁ and i.p. injected with 0.2 ml of BBIC (3 mg/mouse/day), BBI (1 mg/mouse/day) or PBS (n = 7 per group), from day 5 to day 20 p.i. (A) Mice were scored daily for clinical disease. Data represent average daily scores $\pm$ SE. (B) Cumulative score, maximal score and average maximal score from the day of immunization until day of sacrifice (day 22 p.i.). Values represent average $\pm$ SE. (C) Mouse body weight was measured from day 5 p.i. until sacrifice, and represented as average per group $\pm$ SE. (D) Spleen cells were harvested on day 22 p.i. and activated with 30 μ g/ml of PLP₁₃₉₋₁₅₁ or 5 μ g/ml Con A. Proliferation is shown as CPM and the values represent average $\pm$ SE. In all the figures: *p < 0.05; **p < 0.01.

Fig. 2.

Effect of BBI on EAE in C57BL/6 mice. Female 8 weeks old C57BL/6 mice were immunized with MOG₃₅₋₅₅ and treated with BBI (0.1 or 1 mg/mouse/day) or PBS (n = 7 per group) by oral gavage from day 5 to day 20 p.i. Mice were scored daily for clinical disease and sacrificed on day 21 p.i. (A) Data represent average daily scores $\pm$ SE. (B) Cumulative score, maximal score and average maximal score from the day of immunization until day of sacrifice. Values represent average $\pm$ SE. (C) Average body weight. Values represent average $\pm$ SE. (D) Columns represent the total number of MNCs isolated from pooled spinal cords from each treatment group. (E) Spleen cells were harvested after sacrificing the mice, and analyzed by flow cytometry after surface staining.

Fig. 3.

Effect of BBI on histopathological parameters of EAE. C57BL/6 mice immunized with MOG₃₅₋₅₅ and treated with BBI (0.1 or 1 mg/day) or PBS from day 6 to day 20 p.i. (n=7), were sacrificed on day 21 p.i. and spinal cords were harvested after perfusion. 10 μ m sections were stained with H&E (inflammation) or LFB (myelin stain). Representative microphotographs for (A) inflammation and (B) demyelination are shown. (C) Quantitative pathological examination of the number of inflammatory foci, and (D) the percentage of demyelinated area in the spinal cord of C57BL/6 mice treated with BBI or PBS. Columns refer to mean values and bars to SE. * $P < 0.05$, ** $P < 0.01$.

Fig. 4.

Effect of early BBI administration on EAE. Female 8 weeks old C57BL/6 mice were immunized with MOG₃₅₋₅₅ (n = 7 per group) and treated by oral gavage with BBI (1 mg/day) or PBS from day 1 to day 8 p.i. and sacrificed at day 21 p.i. (A) Mice were scored daily for clinical disease. Data represent average daily scores $\pm$ SE. (B) Quantitative pathological examination of the number of inflammatory foci (H&E staining) and (C) the percentage of demyelinated area (LFB staining) in the spinal cords. Columns refer to mean values and bars to SE (n = 7 per group). * P < 0.05, ** P < 0.01. (D) Spleen and (E) lymph node cells proliferation from C57BL/6 mice immunized with MOG₃₅₋₅₅ and treated by oral gavage with BBI (1 mg/day) or PBS from day 1 to day 8 p.i. Mice were sacrificed on day 14 and their

immune cells cultured in the presence of 30 µg/ml MOG₃₅₋₅₅ or 1 µg/ml of anti mouse anti-CD3 and anti-CD28 antibodies. Columns refer to mean values of CPM and bars to SE (n = 5 per group). (F) and (G) are analogous to (D) and (E) except mice were sacrificed 21 days post immunization. (H) IL-5 and (I) IL-10 concentrations in the cell culture supernatants of splenocytes and lymph node cells from BBI and PBS-treated mice cultured in the presence of MOG₃₅₋₅₅ or 5 µg/ml of Con A for 72 h. IL-5 and IL-10 concentrations were determined by ELISA. Data represent average ± SE of triplicate values; * p < 0.05, ** p < 0.01, *** p < 0.001.

Fig. 5. BBI ameliorates ongoing EAE. Female 8 weeks old C57BL/6 mice were immunized with MOG₃₅₋₅₅ and treated by oral gavage with BBI (0.1 and 1 mg/day) or PBS (n = 6 per group) from day 13 p.i. (average clinical score 2) to day 24 p.i. Mice were scored daily for clinical disease. Data represent average daily scores $\pm$ SE.

Fig. 6. BBI reduces incidence of optic neuritis and attenuates loss of neurons. Female 6–7 weeks old SJL/J mice were injected with Fluorogold to label RGCs. Mice were immunized one week later (Day 0) with PLP_{139–151} and treated with oral BBI (1 mg/mouse/day) or placebo (PBS), from day 6 p.i. until sacrifice at day 14 or day 18. (A) BBI treatment reduced the incidence of optic neuritis. At day 14, 75% of optic nerves from placebo-treated EAE mice had optic neuritis, whereas only 30% of eyes from BBI-treated EAE mice developed optic neuritis. Similarly, at day 18, 62.5% of placebo-treated versus 25% of BBI-treated eyes developed optic neuritis. (B) Significant loss of RGCs occurred in optic neuritis eyes treated with placebo (* $p < 0.05$) as compared to control (non-EAE) eyes at both day 14 and day 18. Eyes that did develop optic neuritis with BBI treatment had no reduction in RGC numbers as compared to control eyes.

Fig. 7.

BBI induces production of IL-5 and IL-10 in spleen cells. (A) Naïve 8 weeks old female C57BL/6 mice were treated for 7 days with 1 mg/day of BBI or PBS by oral gavage and sacrificed on day 8 post initiation of the treatment. Spleen cells were harvested and activated with anti-CD3/anti-CD28 antibodies in the presence of BBI (0.1 mg/ml) ($n = 6$ per group). (B) Splenocytes from naive C57BL/6 mouse were activated with anti-CD3/anti-CD28 antibodies in the presence of BBI (0.1 mg/ml), or vehicle (PBS). IL-5 and IL-10 concentrations were determined by ELISA in culture supernatants collected 96 h post stimulation. Data represent average $\pm$ SE of triplicate values; * $p < 0.05$, ** $p < 0.01$.

Fig. 8.

BBI inhibits invasiveness of immune cells in Boyden's chamber assay. Splenocytes harvested from naive C57BL/6 mouse were activated in vitro with 5 $\mu\text{g/ml}$ of Con A for 48 h. Activated cells were placed ($1.5 \times 10^6/300 \mu\text{l}$ RPMI) in the upper well of Boyden's chamber in the presence of BBI (100, 10 and 1 $\mu\text{g/ml}$). Bottom wells contained RPMI with 5 % fetal calf serum and 50 ng/ml of SDF1- α . After 24 h cells from the lower well were collected and counted. Columns refer to the mean $\pm$ SE of the number of cells. * $p < 0.05$, ** $p < 0.01$.