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# Beneficial Effects of Blueberries in Experimental Autoimmune Encephalomyelitis

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**ABSTRACT:** Experimental autoimmune encephalomyelitis (EAE) is an animal model of autoimmune disease that presents with pathological and clinical features similar to those of multiple sclerosis (MS) including inflammation and neurodegeneration. This study investigated whether blueberries, which possess immunomodulatory, anti-inflammatory, and neuroprotective properties, could provide protection in EAE. Dietary supplementation with 1% whole, freeze-dried blueberries reduced disease incidence by >50% in a chronic EAE model ($p < 0.01$). When blueberry-fed mice with EAE were compared with control-fed mice with EAE, blueberry-fed mice had significantly lower motor disability scores ($p = 0.03$) as well as significantly greater myelin preservation in the lumbar spinal cord ($p = 0.04$). In a relapsing−remitting EAE model, blueberry-supplemented mice showed improved cumulative and final motor scores compared to control diet-fed mice ($p = 0.01$ and 0.03, respectively). These data demonstrate that blueberry supplementation is beneficial in multiple EAE models, suggesting that blueberries, which are easily administered orally and well-tolerated, may provide benefit to MS patients.

**KEYWORDS:** *experimental autoimmune encephalomyelitis, EAE, dietary supplementation, blueberry, Vaccinium ashei, mouse, myelin, MOG, PLP*

## INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a demyelinating autoimmune disease that can be induced in most mammalian species. EAE presents with pathological and clinical features very similar to those of multiple sclerosis (MS), making it amenable for mechanistic and intervention studies.[1−4] Each EAE model has several common pathologic and clinical features of MS[3,4] and leads to an ascending, flaccid paralysis beginning with tail tone and hind limb paralysis.[5] Active immunization with myelin oligodendrocyte glycoprotein (MOG) or MOG35−55 peptide in C57BL/6 mice yields a chronic monophasic disease, whereas immunization of SJL mice with proteolipid protein (PLP) peptide 139−151 produces a chronic relapsing−remitting disease.[5,6] Even though EAE models have proven useful in investigating various aspects of MS, MS is a heterogeneous disease and no single EAE paradigm is sufficient to predict whether efficacious animal therapies will be translatable to human treatments.[4,5] Therefore, utilization of different mouse models of EAE helps to reproduce the heterogeneity found in varied MS presentations.[4] In both MS and EAE, T cell activation and proliferation with subsequent migration through the blood−brain barrier lead to glial activation, production of pro-inflammatory cytokines and reactive oxygen and nitrogen species, followed by eventual demyelination and axonal damage.[3−5] Interventions that reduce or suppress one or more of these events should reduce both clinical and pathological symptoms and, thus, are candidates for clinical evaluation.[3,4]

Recent work in the area of polyphenolics as possible preventive agents in EAE, and therefore with potential in human MS, is very encouraging. Polyphenolics may reduce inflammation and neuronal damage by altering antigen presentation[7] or through suppression of myeloid cell infiltration into the central nervous system (CNS).[8] A dose-responsive, protective effect shown in both passively and actively induced relapsing and remitting EAE suggests that polyphenolics function through pathways other than by only immunosuppression.[9] Furthermore, different polyphenolics have been shown to ameliorate EAE symptoms by blocking interleukin (IL)-12 signaling in T lymphocytes, thereby reducing inflammation and neural damage.[10,11] A suggested common mechanism involves inhibition of NFκB signaling.[12] However, not all isolated polyphenolics show improvement in the EAE model and may even delay recovery.[13] Therefore, although botanical extracts that are rich in flavonoid compounds hold great potential in the prevention or treatment of EAE/MS, each botanical model needs to be carefully characterized.

Consumption of flavonoid-rich foods such as blueberries has been suggested to limit neurodegeneration associated with neurodegenerative disease and to prevent loss of age- and neural damage-related cognitive function.[14−20] Blueberries, specifically, are protective in models of Parkinson's disease (PD),[21] ischemic heart damage,[22] stroke,[23,24] and cognitive loss

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from normal aging as well as the oxidative insult of $\gamma$ rays simulating space travel.[19] Blueberries, through their antioxidant and anti-inflammatory properties, may block oxidative stress-induced cell death.[16,25−32] Another mechanism by which blueberries also may mediate beneficial effects is through suppression of pro-inflammatory cytokines IL1$\beta$ and TNF$\alpha$ and their primary mediator, NF$\kappa$B.[19,20,33,34] It has been well documented that blueberries can suppress injury-induced increases in inflammatory mediators.[19,20,27,35−37] However, blueberries have not yet been tested in EAE models. Therefore, in the current study, we evaluated the therapeutic potential of blueberries as a prospective supplement to support the health of animals subjected to two different models of EAE. Positive findings in both models are encouraging and are now being extended to the mechanistic studies.

## MATERIALS AND METHODS

**Diet Preparation.** A large quantity of freeze-dried Tifblue blueberries (*Vaccinium Aashei*) was obtained from Van Drunen Farms (Momence, IL), shipped to Harlan Teklad (Madison, WI), ground under low-heat conditions to a powder, and stored in vacuum-packed aliquots at −20 °C. Earlier studies, including our own, utilized water-soluble Tifblue extract included at 2% of the diet and observed beneficial effects.[16,21,27] However, a dose-response study in our PD model[21] revealed that a 1% inclusion of whole freeze-dried Tifblue blueberries gave a maximal suppression of injury-induced inflammatory mediators such as TNF$\alpha$ (McGuire and Hejna, unpublished data). Therefore, the 1% whole, freeze-dried berries were used in this experiment. These berries contained approximately 32.5 mg/g total phenolics and 14.8 mg/g anthocyanins on a dry matter basis. Diets were prepared by Harlan Teklad as previously described.[21] Briefly, NIH-31 (Harlan Teklad 7017) was used as a fixed-formula control diet containing 4.7% fat, 18% protein, and 46.5% available carbohydrate and supplemented with standard mineral and vitamin mixes. The blueberry-supplemented diet was made by replacing 1% of the ground corn (w/w) in the control diet with 1% freeze-dried blueberry powder. Both diets were calculated to be isocaloric and made from a common basal mix at the same time, thus differing only in blueberry supplementation.

**Animals and Induction of EAE.** Two EAE models were used in the current study: a chronic form of EAE in C57BL/6 mice and a relapsing−remitting form of EAE in SJL mice to more fully represent the heterogeneous nature of EAE.[5] Each model of EAE utilized leads to an ascending, flaccid paralysis.[5] All procedures were completed in accordance with National Institutes of Health guidelines on the care and use of laboratory animals for research purposes and were approved by the local IACUC committee.

*Chronic EAE in C57BL/6 Mice.* Eight-week-old female mice were obtained from Charles River Breeding (Cambridge, MA). Mice were housed under a 12 h light/dark cycle in microisolator cages contained within a laminar flow system to maintain a pathogen-free environment. Mice were placed on standard NIH-31 chow until 3 days before the beginning of the immunization protocol. At 3 days (−3d) before the immunization protocol, mice were randomly assigned to one of two dietary treatment groups, either (1) NIH-31 chow (nonsupplemented diet) or (2) NIH-31 chow with 1% corn replaced with 1% whole freeze-dried blueberries (blueberry-supplemented). These experimental diets were continued throughout the study. A chronic form of EAE was induced in C57BL/6 mice using synthetic myelin oligodendrocyte glycoprotein peptide 35−55 (MOG35−55), MEVGWYRSPFSRVVH-LYRNGK (purchased from Anaspec, San Jose, CA). Mice were injected subcutaneously with an emulsion containing 300 $\mu$g of MOG35−55 dissolved in 100 $\mu$L of phosphate-buffered saline (PBS), mixed with 100 $\mu$L of complete Freund's adjuvant (CFA) containing 500 $\mu$g of *Mycobacterium tuberculosis* (Difco, Detroit, MI). The animals then received an intraperitoneal injection of 200 ng of pertussis toxin (List Biochemicals, Campbell, CA) in 200 $\mu$L of PBS. Two days later, the mice received a second pertussis toxin injection and 1 week later, a booster MOG35−55 injection. Mice were monitored daily, and clinical signs of EAE were graded by an experienced investigator, blinded to treatments, on an every-other day basis beginning with the day of the booster injection. Because EAE is a progressive, ascending paralysis, mice were removed from the home cage and evaluated for tail tone, ambulation, limb weakness, and righting response. Clinical signs were scored on a 5 point scale: grade 0, no clinical signs; 1, limp tail and/or impaired righting; 2, paresis of one hind limb; 3, paresis of two hind limbs; 4, moribund; 5, death. The number of mice exhibiting motor scores of 1.0 or greater versus the total mice on the treatment was used to calculate disease incidence. Animals were sacrificed 60 days after initial immunization, as previously described.[38,39]

*Relapsing−Remitting EAE in SJL Mice.* Eight-week-old female mice were obtained from Charles River Breeding and housed as previously discussed. Relapsing−remitting EAE was induced by immunizing SJL female mice with PLP peptide 139−151 (Anaspec) emulsified in CFA (Difco) in combination with two injections of pertussis toxin, 200 ng per injection, in PBS (List Biochemicals). All mice were fed the standard NIH-31 chow diet. On day 39, after the first relapse−remission cycle, the mice were placed into one of two dietary treatment groups ($n$ = 9 per group) balanced for the mean daily EAE score. The first group remained on the nonsupplemented NIH-31 chow diet, and the second group was fed the 1% blueberry-supplemented diet. Each group of mice remained on these diets through the end of the experiment. After the animals were switched to experimental diets, feed was replaced and motor signs were evaluated on an every other day basis.

**Immunochemistry Staining.** Mouse brains and spinal columns were fixed in 4% paraformaldehyde in 0.1 M PBS at pH 7.6 overnight at 4 °C.[39] Brains were frozen and sectioned at 30 $\mu$m, and spinal cords were collected, sectioned at a thickness of 14 $\mu$m, and thaw-mounted (37 °C for 5−10 min) onto precleaned SuperFrost slides (Fisher Scientific, Pittsburgh, PA). Sections were subject to immunochemistry or immunofluorescence staining according to the following protocols. For light level immunohistochemistry, the sections were washed three times with PBS (1 min each time), and then endogenous peroxidase activity was quenched by incubation with incubation in 1% $H_2O_2$ solution in neutral PBS for 10 min. Blocking for nonspecific staining was accomplished by incubation for 30 min with 10% normal goat serum (Vector Laboratories, Burlingame, CA) with 0.25% Triton X in TBS (TBST; Sigma, St. Louis, MO) for 1 h. Sections were then incubated overnight with rat anti-mouse myelin basic protein (MBP, 1:300, Abcam, Cambridge, MA) in PBS at 4 °C. Sections were washed extensively in TBST and incubated with biotinylated goat anti-rat IgG, mouse adsorbed (1:2500, Vector Laboratories) at room temperature for 1 h, followed by an ABC perioxidase reaction (Vectastain Elite ABC Staining Kit, anti-goat IgG, Vector Laboratories). Color was developed by using a chromagenic reaction with 3,3′-diaminobenzidine (ImmPACT DAB Peroxidase Substrate, Vector Laboratories). Sections were coverslipped with Permount (Fisher Scientific) and examined using an Olympus BX50 microscope (Tokyo, Japan). MBP was quantified by image analysis for the light level immunoreactivity visualized with DAB. Images were captured of at least nine sections through the lumbar spinal cord. The ventral and lateral white matter areas were traced, and the average density of MBP in the white matter was quantified with Image J (NIH). In this manner, a mean average density of MBP white matter staining was determined for each mouse and was used for statistical analyses. For immunofluorescence staining, sections were blocked for nonspecific staining using 10% bovine serum albumin (BSA) in PBS for 1 h. Sections were incubated overnight with rat anti-mouse MBP (1:300, Abcam) in PBS at 4 °C. Sections were washed extensively in PBS and incubated with the Alexa Fluor 555 goat anti-rat IgG (1:500, Invitrogen, Carlsbad, CA). The resultant slides were viewed and photographed using an Olympus BX50 microscope (Olympus America Inc.) equipped with a digital camera system (Qimaging Retiga 2000R). These images were used to visualize the location of the EAE lesions in the lumbar cord.

*Statistical Analysis.* Data are expressed as the mean ± SEM. Independent sample $t$ tests and one-way and repeated measures ANOVAs were performed as appropriate to determine significant

differences among experimental groups (SPSS19, IBM Corp., Armonk, NY). Post hoc comparisons among individual groups were accomplished using Fisher's LSD method. $p < 0.05$ was considered to be statistically significant.

## RESULTS AND DISCUSSION

**Chronic EAE.** Dietary supplementation with 1% freeze-dried blueberries significantly reduced the incidence of disease (Figure 1A), from 11 of 12 animals exhibiting motor deficits

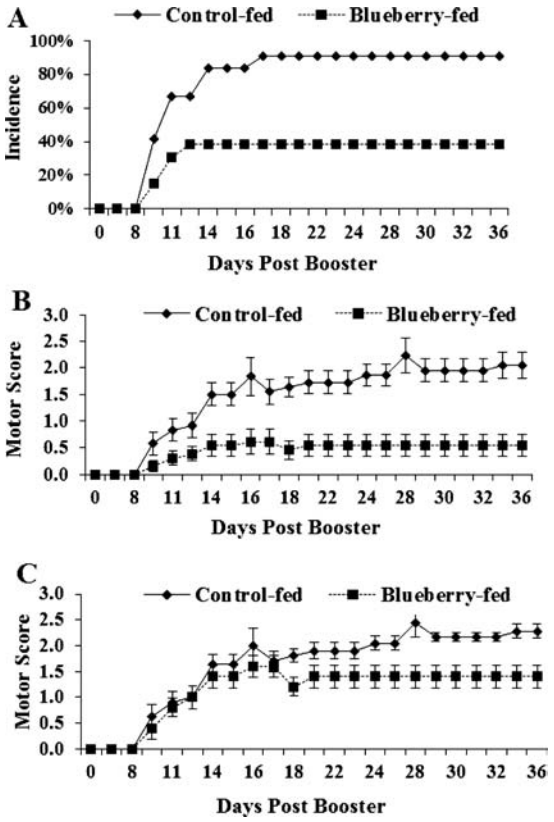


**Figure 1.** Incidence and clinical severity of MOG-induced EAE is decreased by blueberries: (A) effect of diet on incidence of EAE (% animals with EAE motor deficits; control diet ($n = 12$), blueberry diet ($n = 13$), $p < 0.01$); (B) effect of diet on clinical motor disability score for all mice (mean ± SEM; control diet ($n = 12$), blueberry diet ($n = 13$), $p = 0.02$); (C) clinical scores for motor disability for only those mice showing active signs of motor deficits (mean ± SEM; control diet ($n = 11$), blueberry diet ($n = 5$); average disease score at the end of the experiment ($p = 0.03$)).

(92%) in the nonsupplemented control group to 5 of 13 animals exhibiting EAE-associated motor deficits (39%) in the blueberry-supplemented group ($p < 0.01$). Mortality in the control group was 16%, 2 of 12 mice; however, no mice in the blueberry-supplemented group succumbed to EAE. There was no difference in the average day of disease onset between the two groups (day 11.7 ± 0.8 versus day 10.8 ± 0.4, control-fed versus blueberry-supplemented, respectively) (Figure 1B). However, there was a significant difference in the cumulative disease score, which represents overall motor disability across the course of the experiment, between the control group (30.21 ± 4.2) and the blueberry-supplemented group (10.08 ± 3.8; $p = 0.02$) as determined by two-way ANOVA (Figure 1B). When the subset of blueberry-fed mice that exhibited motor signs indicative of active EAE ($n = 5$) was compared with control-fed mice with active EAE ($n = 11$), the average disease score on the last day of the experiment, day 36 postbooster, was significantly lower in the blueberry-fed mice than in the nonsupplemented mice (2.77 ± 0.4 and 1.40 ± 0.2, control versus blueberry-supplemented, respectively, $p = 0.03$), indicating less severe physical disability in blueberry-supplemented mice at this time point (Figure 1C). Therefore, blueberry supplementation led to a decrease in both disease incidence and clinical severity in this chronic EAE model.

A characteristic hallmark of EAE, and of MS itself, is pathological demyelination, which results in motor deficits. To determine whether the protection conferred by blueberry feeding in the subset of blueberry-fed mice that exhibited motor deficits was the result of a reduction in demyelination, we performed an immunohistochemical analysis for MBP. The lumbar spinal cord from three mice from each of the following groups was immunostained for MBP: (1) control-fed with EAE motor deficit signs, (2) blueberry-fed with EAE motor deficit signs, and (3) blueberry-fed without EAE motor deficits ($n = 3$ for each group). Sections of lumbar spinal cord evidenced areas of hypodense MBP staining in both groups of mice with motor deficits, indicating demyelinating lesions within the spinal cord white matter (Figure 2a, arrowheads). A comparison of lumbar

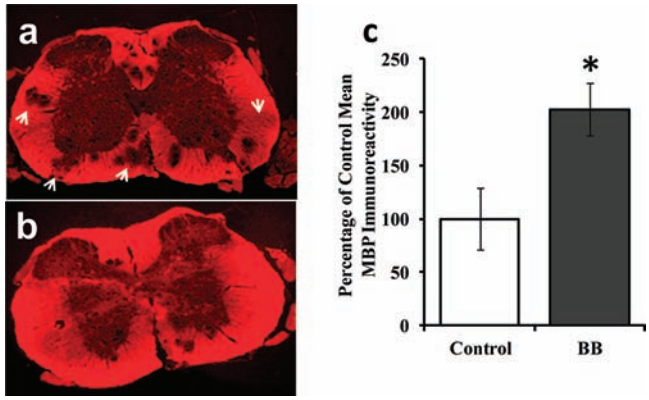


**Figure 2.** MOG-induced demyelination in the spinal cord is decreased by blueberries: MBP immunofluorescence in (a) control-fed and (b) blueberry-fed animals (lesions are indicated by arrow heads); (c) quantification of MBP (data represent the mean ± SEM for control-fed ($n = 3$) and blueberry-fed ($n = 6$) mice ($p = 0.04$)).

MBP immunoreactivity (MBPir) in control-fed ($n = 3$) versus blueberry-fed mice ($n = 6$) revealed a significant sparing of myelin in blueberry-fed mice, consistent with less overall motor dysfunction in blueberry-fed mice as seen in Figure 1B (Figure 2c; $p = 0.04$; 202.86 ± 24.3 versus 100.00 ± 29.2%, blueberry-fed versus control-fed). To determine whether a blueberry-associated preservation of white matter within the spinal cord could account for the blueberry-associated protection in motor symptoms evidenced in the subset of blueberry-fed mice as seen in Figure 1C, MBPir in the subsets of blueberry-fed mice, those with and without motor signs, and in control-fed mice was subjected to one-way ANOVA. Control-fed animals tended to have lower MBP immunoreactivity, suggesting greater lesion area, than did either of the two blueberry groups (100.0 ± 29.2, 198.12 ± 43.0, and 207.61 ± 32.7 for control-fed with motor signs, blueberry-fed with motor signs, blueberry-fed without motor signs, respectively). These data suggest that one of the beneficial effects of blueberry may be suppression of demyelination.


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**Relapsing–Remitting EAE.** To confirm these findings in an additional, independent model, a long-term treatment model was utilized. One of the gold standards for potential MS treatments is that they have efficacy during ongoing disease. In particular, the prevention of relapse is a desired outcome. The use of PLP as an immunogenic peptide in the SJL mouse produces a relapsing–remitting disease that is useful in this regard. Following the induction of EAE, mice were allowed to undergo a second relapse–remit cycle and were then divided into two groups ($n$ = 9) with one remaining on the NIH-31 chow diet and the other placed on NIH-31 diet supplemented with 1% freeze-dried blueberries. The cumulative disease scores from day 39, when experimental diets were introduced, to the end of the experiment at day 66 postbooster were 32.78 ± 5.7 in the control mice and 20.67 ± 5.3 in the blueberry-fed group (Figure 3). Although the blueberry-supplemented mice had a

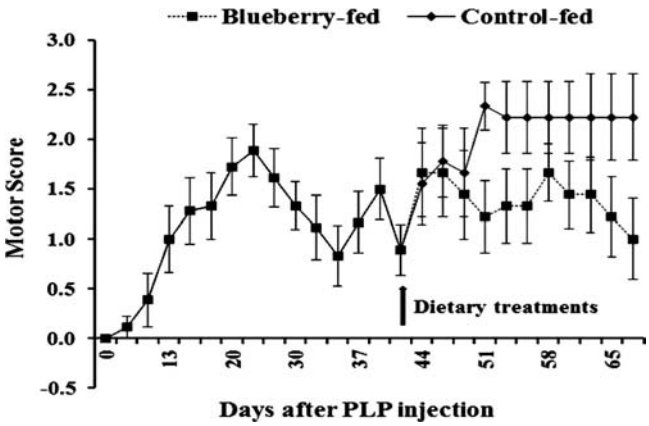


**Figure 3.** Motor function is improved in mice with relapsing–remitting EAE. Data represent the mean ± SEM of $n$ = 9 mice per treatment ($p$ = 0.06).

41% reduction in cumulative disease score, the difference did not reach significance. This is most likely due to the high variability in the severity of relapse in both groups. However, when the final disease score was compared, the control group score of 2.33 ± 0.4 tended to be more severe than that of the blueberry-supplemented group of 1.11 ± 0.5 ($p$ = 0.06). Two-way, repeated measures ANOVA of animals with a disease score of ≥3 at the point of intervention ($n$ = 5) revealed a decrease of 60% cumulative disease ($p$ = 0.01; 30.8 ± 4.5 and 12.6 ± 2.6) and a >80% reduction in final disease scores ($p$ = 0.03; 2.4 ± 0.4 versus 0.4 ± 0.4, control diet versus blueberry-supplemented diet, respectively). These data strongly suggest that dietary supplementation with blueberries not only reduces disease incidence but can also attenuate the clinical severity and reduce the severity of relapses.

Our results revealed that dietary supplementation with whole, freeze-dried blueberries not only decreased the overall incidence of motor deficits in a mouse model of MS (EAE) but also decreased motor deficit in blueberry-fed mice that did exhibit EAE motor signs when compared with control diet-fed mice. Furthermore, we confirmed that blueberry-fed mice evidenced significantly less demyelination in the ventral and lateral lumbar spinal cord. Although we examined demyelination in the spinal cord via immunohistochemistry, a quantification of myelin alteration needs to be determined utilizing other techniques.

The underlying mechanisms of beneficial blueberry effects may involve multiple cellular and molecular components. First, we previously showed that dietary supplementation with blueberries significantly improved both the survival and efficacy of transplanted embryonic dopamine neurons in a rat model of Parkinson's disease,[21] which may involve reduced inflammation and/or direct neuroprotective properties. We[40] and others[41] have shown that suppression of TNF$\alpha$ improves survival of embryonic dopamine neurons, and blueberries have been shown to reduce TNF$\alpha$ production.[34,37,42] Our data also suggest that a 1% dose of blueberries is sufficient to suppress injury-induced expression of TNF$\alpha$ (McGuire and Hejna, unpublished data). Other investigators have shown alterations of IL12 signaling in T cells, further implicating polyphenolic alterations in inflammation underlying reduced inflammation and damage in EAE.[10,11] Certainly, isolated anthocyanins have been shown to be protective in acute inflammatory responses in the mouse brain in other neurodegenerative models[43] where suppression of TNF$\alpha$, IL1$\beta$, and IL6 resulted from anthocyanin treatment. Therefore, alterations in pro- and anti-inflammatory cytokines may be involved in the protective effect of blueberries.

Recent studies have demonstrated that both polyphenolics and isolated flavonoids can be protective in EAE through mechanisms involving modulation of T cell responses, which can involve both antigen presentation or infiltration of immune cells into the CNS.[7,10,11] Several studies with both berries and isolated polyphenolics revealed a berry-mediated alteration in the activation and inflammation state of glial cells.[35−37,44] We postulate that bioactive compounds in blueberries may mediate an immunomodulatory effect on interactions between antigen-presenting cells and T cells, which may shift the T cell response from a pro-inflammatory toward an anti-inflammatory response including interactions within the brain on glial cell activation. From a mechanistic perspective, inflammatory response markers in the CNS, including T cell infiltration, MHC II expression, and cytokine and chemokine production, as well as activation of glial cells, all remain to be determined using qualitative and quantitative approaches. Because it is possible that different components of the immune system will not respond in the same dose-responsive fashion to individual nutrients within berries,[33] it will be particularly important to consider dose-responsive effects of dietary blueberry components on immune system function. We also hypothesize that individual components of the immune system will have differential responses to individual dietary supplements, especially in a disease model such as EAE, where there are multiple regulatory points that could be targeted such as antigen presentation, T cell priming, T cell reactivation, and cytokine production. Therefore, it is essential to test the efficacy of blueberry polyphenolics in several models of EAE such that regardless of where in the immune pathway they are suppressive, we will be able to determine whether blueberry supplementation is a viable treatment strategy in MS. One important and continuing direction of our berry work is to determine the dose responsiveness of blueberries on several different aspects of MS-related immune function. For example, we are investigating antigen presentation, T cell proliferation both in the peripheral circulation and in the CNS, glial cell activation in the brain and the effect of macrophage function in demyelination and remyelination.

Lastly, it has been shown that blueberry supplementation increases production of brain-derived neurotrophic factor

(BDNF),[45] a suggested mechanism of action for the MS treatment glatiramer acetate, which appears to protect neurons and areas of axonal injury.[46] As a continuation of the current studies, our laboratory is actively conducting in vivo and in vitro studies to investigate these possible immune, growth factor, and glial mechanisms.

It also is very encouraging that blueberry supplementation, at a level approximately equal to 1 cup of blueberries per day for a human,[42] showed beneficial effects in both our mouse models of MS/EAE, suggesting that berries may be beneficial for the treatment as well as prevention of MS. However, care must be taken to expand these studies across multiple doses and types of berries as well as several different models because no single EAE paradigm is sufficient to predict whether efficacious animal-tested therapies will translate to efficacy in human trials.[47] In summary, the present study suggests that dietary supplementation with blueberries may be a valuable approach to reduce the incidence and clinical severity of EAE and provide an insight for a clinical trial for its therapeutic potential for MS patients.

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## ABBREVIATIONS USED

EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; CNS, central nervous system; MBP, myelin basic protein; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; CFA, complete Freund's adjuvant.

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