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Blueberry Opposes β-Amyloid Peptide-Induced Microglial Activation Via Inhibition of p44/42 Mitogen-Activation Protein Kinase

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Abstract

Alzheimer's Disease (AD) is the most common age-related dementia, with a current prevalence in excess of five million individuals in the United States. The aggregation of amyloid-beta (Aβ) into fibrillar amyloid plaques is a key pathological event in the development of the disease. Microglial proinflammatory activation is widely known to cause neuronal and synaptic damage that correlates with cognitive impairment in AD. However, current pharmacological attempts at reducing neuroinflammation mediated via microglial activation have been largely negative in terms of slowing AD progression. Previously, we have shown that microglia express proinflammatory cytokines and a reduced capacity to phagocytose Aβ in the context of CD40, Aβ peptides and/or lipopolysaccharide (LPS) stimulation, a phenomenon that can be opposed by attenuation of p44/42 mitogen-activated protein kinase (MAPK) signaling. Other groups have found that blueberry (BB) extract both inhibits phosphorylation of this MAPK module and also improves cognitive deficits in AD model mice. Given these considerations and the lack of reduced Aβ quantities in behaviorally improved BB-fed mice, we wished to determine whether BB supplementation would alter the microglial proinflammatory activation state in response to Aβ. We found that BB significantly enhances microglial clearance of Aβ, inhibits aggregation of $A\beta_{1-42}$, and suppresses microglial activation, all via suppression of the p44/42 MAPK module. Thus, these data may explain the previously observed behavioral recovery in PSAPP mice and suggest a means by which dietary supplementation could mitigate an undesirable microglial response toward fibrillar Aβ.

Introduction

Approximately 5.1 million people have Alzheimer's disease (AD) in the United States.[1] It is the most common age-related progressive dementia, and widespread synaptic injury and loss has been consistently correlated with its characteristic neurocognitive deficits. Indeed as the disease progresses, so does neuron cell loss.[2] Amyloid-beta (Aβ) is a 37- to 43-amino-acid peptide generated by cleavage from a large transmembrane precursor, the amyloid precursor protein (APP). The aggregation of Aβ into fibrillar amyloid plaques is a key pathological event in the development of AD.[3] Importantly, these amyloid structures have been demonstrated both *in vitro* and *in vivo* to be neurotoxic and synaptotoxic.[4–6] Thus, neuroinflammation may be one of the most important mediators of subsequent histopathologic changes resulting in affective dysregulation and cognitive decline in AD. Although several mechanisms may be crucial in amyloid-induced neurtoxicity and neuroinflammation, *in vitro* studies indicate that a self-potentiating cycle of Aβ peptide-associated brain inflammation is critical.

Indeed aggregated forms of Aβ peptide activate microglial intracellular signaling cascades, leading to production of cytokines and chemokines or "pro-inflammatory microglial activation," a central phenomenon in AD neuroinflammation.[7] These activated cells have caused synaptic pruning and neuronal damage.[8,9] In addition many proinflammatory cytokines also confer increased amyloidogenic processing of APP by neurons,[10] further fueling the inflammatory cycle in the AD brain. Indeed, the major cellular mediators associated with inflammation in and around amyloid plaques seem largely to be activated microglia, and to a lesser extent reactive astrocytes.[11,12] Compared to the healthy average brain, abundant activated microglia are typically localized in or

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around amyloid plaques of the AD neocortex, and microglial processes both border and penetrate plaque cores.[13,14]

These activated microglia express proinflammatory cell-surface markers, including major histocompatibility complex II (MHC II).[15] MHC II is overexpressed in areas of microglial proliferation around plaques in AD brain, while downregulated in control brain.[16–18] The mechanism underlying this fibrillar, Aβ-induced activation into an MHC II-expressing, proinflammatory phenotype is not clear presently. However evidence exists that Aβ interacts with several receptors present in microglia plasma membranes, including integrins, receptor of advanced glycation end products (RAGE), the serpin enzyme complex (SEC) receptor,[19] and scavenger receptors (SR), which mediate activation of intracellular tyrosine kinase pathways.[20,21] This, in turn, results in mitogen-activated protein kinase (MAPK) activation, which causes phosphorylation of cAMP response element-binding protein (CREB). This terminal event then increases transcription of genes related to cytokine and complement components synthesis as well as release of intracellular Ca^{++} stores and reactive oxygen species (ROS).[22] Taken together, these data suggest Aβ-induced activation of microglia involves clustering of cell-surface receptors, which then cause MAPK activation, ultimately leading to synaptic and neuronal damage.

Previously, it was demonstrated that blueberry (BB) supplementation reversed the deleterious effects of aging on motor behavior and neuronal signaling in senescent rodents.[23] It has also been demonstrated that BB-fed (from 4 months of age) PSAPP mice (a transgenic line carrying mutant APP and presenilin 1 transgenes) showed no deficits in Y-maze performance (at 12 months of age) with no alterations in Aβ‴ burden.[24,25] Because the BB supplementation yielded these beneficial effects on several deficits in behavior as well as neuronal signaling in the senescent and AD mouse models, an addition of BB may be of benefit in reducing the proinflammatory Aβ-mediated microglial activation. Findings from our laboratory suggest that this activation occurs through a p44/42 MAPK-dependent pathway.[26,27] Given these considerations and the lack of reduced Aβ quantities in behaviorally improved BB-fed mice,[25] we wished to determine whether BB supplementation would alter the microglial proinflammatory activation state in response to Aβ. We found that BB enhances microglial clearance of Aβ, inhibits aggregation of A$\beta_{1–42}$, and suppresses microglial activation, all via suppression of the p44/42 MAPK module. Thus, these data may explain the previously observed behavioral recovery in PSAPP mice[25] and suggest a means by which dietary supplementation could mitigate an undesirable microglial response toward fibrillar Aβ.

Materials and Methods

Materials

Mouse anti-human Aβ monoclonal antibody (BAM-10) was purchased from Sigma (St. Louis, MO). A$\beta_{1–42}$ and fluorescein isothiocyanate (FITC)-conjugated A$\beta_{1–42}$ were obtained from Biosource International (Camarillo, CA). Orange fluorescing cyanine dye, Cy3, was purchased from Amersham (Piscataway, NJ) for conjugation with the A$\beta_{1–42}$ peptide. Blueberry (freeze-dried power) was obtained from Van Drunen Farm (Momence, IL). Purified FITC-anti-mouse MHC class II antibodies were obtained from PharMingen (San Diego, CA). DuoSet™ mouse tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D systems (Minneapolis, MN). Mouse interleukin-6 (IL-6) ELISA kit was obtained from eBioscience (San Diego, CA). Antibodies for phospho-p44/42 (pp44/42, Thr202/Tyr204) MAPK and total p44/42 MAPK were obtained from Cell Signaling Technology (Beverly, MA) as well as cell lysis buffer and sodium dodecyl sulfate (SDS) blue loading buffers. PD98059 (a specific MEK1/2 inhibitor) was obtained from Calbiochem (La Jolla, CA). PD98059 was dissolved in dimethylsulfoxide (DMSO) before adding to complete cell medium. DMSO alone was used as a solvent control, which did not differ from the untreated controls presented. Thioflavin T and bacterial lipopolysaccharide (LPS) were obtained from Sigma (St. Louis, MO), and LPS was dissolved in complete cell culture medium. Anti-rabbit horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) secondary antibodies and western blotting luminol reagent were obtained from Pierce (Rockford, IL). Immun-Blot polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad systems (Minneapolis, MN).

Murine primary cell culture

Breeding pairs of BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were housed in the animal facility at the University of South Florida Health Science Center. Murine primary culture microglia were isolated from mouse cerebral cortices and grown in complete RPMI 1640 medium according to previously described methods.[26] Briefly, cerebral cortices from newborn mice (1–2 days old) were isolated under sterile conditions and kept at 4°C prior to mechanical dissociation. Cells were grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-mercaptoethanol. Primary cultures were kept for 14 days so that only glial cells remained. Microglial cells were isolated by shaking flasks at 200 rpm in a LabLine™ Incubator-Shaker. More than 98% of these glial cells stained positive for Mac-1 (Boehringer Mannheim, Indianapolis, IN).

MTT assay

For cell proliferation analysis, primary mouse microglia were seeded at 2×10^4 cells/well ($n = 6$ for each condition) in 96-well tissue culture plates containing 100 μL of complete RPMI 1640 medium. These cells were treated for 24, 48, and 72 hr with BB extract at different concentrations (25 μg/mL, 50 μg/mL, and 100 μ/mL). Cell proliferation as determined by MTT assay (MTT kit, Sigma). BB extract did not show high activity on proliferation of primary mouse microglial cells in culture (data not shown).

Thioflavin T measurement

Thioflavin T measurement was performed by according to previously described methods[28] with slight modifications. For Aβ aggregate-formation assay, Aβ (20 μM) dissolved in 50 mM Tris-HCl buffer (pH 7.4) with a test BB extract (25 μg/mL, 50 μg/mL, and 100 μ/mL) was incubated at 37°C for 0, 12, 24, 48, and 72 hr. At the end of the incubation, 10

μM thioflavin T in 50 mM glycine/NaOH (pH 9.0) was added to the mixture. Fluorescence of thioflavin T bound to Aβ aggregates was measured with a microplate reader (SpectraMax®, Molecular Devices) with an excitation wavelength of 442 nm and emission wavelength of 485 nm after incubation for 30 min at room temperature. Percentage inhibition was calculated by comparing these fluorescence values with those found in control solutions with no BB.

Microglial phagocytosis assays

Primary mouse microglia were seeded at 1×10^5 cells/well ($n = 6$ for each condition) in 24-well tissue culture plates containing 0.5 mL of complete RPMI 1640 medium. "Aged" FITC-Aβ_{1-42} is created by preaggregating Aβ_{1-42} prior to adding to microglial cells. Specifically, FITC-Aβ_{1-42} was first diluted in complete medium to a final concentration of 20 μM and vortexed. The mixture was then pH-adjusted to 5.0 with 1 M HCl and incubated at 37°C for 24 hr, as previously described.[29] These cells were then treated for 2 hr with 500 nM "aged" FITC-Aβ_{1-42}. In the presence of FITC-Aβ_{1-42}, microglial cells were then co-treated with BB (50 μg/mL). Some of these cells were treated with PD98059 (5 μM), BB (50 μg/mL), or PD98059 and BB for 1 hr prior to LPS (50 ng/mL), or BAM-10 (2.5 μg/mL), in the presence of FITC-Aβ_{1-42} for 2 hr. Microglial cells were then rinsed three times in Aβ-free complete medium, and the media were exchanged with fresh Aβ-free complete medium for 10 min to allow for removal of nonincorporated Aβ and promote concentration of Aβ into phagosomes. Extracellular and cell-associated FITC-Aβ were quantified using an MSF (SpectraMax®, Molecular Devices) with an emission wavelength of 538 nm and an excitation wavelength of 485 nm. A standard curve from 0 to 600 nM of FITC-Aβ was run for each plate. Total cellular proteins were quantified using the Micro BCA Protein Assay (Pierce, Rockford, IL). The mean fluorescence values for each sample at 37°C and 4°C at the 2-hr time point were determined by fluorometric analysis. Relative fold change values were calculated as: mean fluorescence value for each sample at 37°C/mean fluorescence value for each sample at 4°C. In this manner, both extracellular and cell-associated FITC-Aβ were quantified. Considering nonspecific adherence of Aβ to the plastic surface of culture plates, an additional control without cells was carried out through all of experiments above, and the mean values have been normalized to these controls. An incubation time of less than 4 hr did not change the amount of Aβ peptide detected in the supernatant, which is consistent with a previous report.[30] To determine the extent to which cell death might have influenced the phagocytic activity in the various treatment groups, we performed the lactate dehydrogenase (LDH) assay on the relevant supernatant. Data showed that there was no significant cell death occurring over the 3-hr time frame in any of the treatment groups (data not shown, $p < 0.05$).

Fluorescence microscope examination

"Aged" FITC-A$\beta 1_{-42}$ was prepared according to methods described above. Microglial cells were cultured at 1×10^5 cells/well in 24-well tissue culture plates with glass inserts. In the presence of FITC-Aβ_{1-42} (1 μM), microglial cells were co-treated with BB (50 μg/mL) at 37°C for 2 hr. Additionally, in parallel 24-well tissue culture plates, microglial cells were incubated at 4°C with the same treatment as above. Following treatment, these cells were washed five times with ice-cold phosphate buffered saline (PBS) to remove extracellular Aβ and fixed for 10 min at 4°C in 4% (wt/vol) paraformaldehyde (PFA) diluted in PBS, followed by staining with 4′,6-diamidino-2-phenylindole (DAPI) at 4°C for 15 min. Finally, sections were mounted with fluorescence mounting medium containing Slow Fade antifading reagent (Molecular Probes, Eugene, OR), and then viewed under an Olympus IX71/IX51 microscope equipped with a digital camera system (40×).

Fluorescence confocal microscopy

In parallel with the fluorescence microscope examination, slides were viewed with a Leica DMI6000 inverted microscope, TCS SP5 confocal scanner, and 63X/1.4NA Plan Apochromat oil immersion objectives (Leica Microsystems, Germany). Excitation wavelengths of 488 nm (for FITC) and 405 nm (for DAPI) were used to generate fluorescence emission in green (for Aβ_{1-42}) and blue (for nuclei), respectively. Images were captured with photomultiplier detectors at 3× zoom and were prepared with the LAS AF software, version 1.6.0, build 1016 (Leica Microsystems, Germany). Differential interference contrast (DIC) images were also captured using the 488 laser line.

Primary microglial cells (seeded in 24-well tissue culture plates at 5×10^4 cells/well) were treated with Cy3-Aβ_{1-42} peptide (300 nM) in the presence or absence of BB (50 μg/mL) at 37°C for 48 hr, Cy3-Aβ_{1-42} was prepared according to previous methods.[29] Following treatment, these cells were washed, fixed, and permeabilized in 0.2% Triton X-100, 5% horse serum for 1 hr. This was followed by staining with FITC–anti-mouse MHC class II antibody (2 μg/mL) incubated overnight at 4°C. Slides were analyzed using the same fluorescence confocal microscope equipped as above.

TNF-α and IL-6 ELISA

Primary cultured microglial cells were plated in 24-well tissue culture plates (Costar, Cambridge, MA) at 5×10^4 cells/well for 24 hr. They were then stimulated for 16 hr with LPS (50 ng/mL), Aβ peptides (1 μM), or LPS + Aβ peptides in the presence or absence of pretreatment (1 hr) with BB (50 μg/mL), PD98059 (5 μM), or appropriate controls. Cell-free supernatants were collected and assayed by TNF-α (R&D Systems, Minneapolis, MN), and IL-6 (San Diego, CA) ELISA kits in strict accordance with the manufacturer's instruction. The BCA Protein Assay (Pierce, Rockford, IL) was performed to measure total cellular protein from each of the cell groups under consideration just before quantification of cytokine release.

Western immunoblotting

Murine primary culture microglia were plated in six-well tissue culture plates at a density of 8×10^5 cells/well. These cells were incubated for 30 min with or without LPS (50 ng/mL), Aβ_{1-42} (1 μM), in the presence or absence of pretreatment (1 hr) with BB (50 μg/mL), PD98059 (5 μM), or appropriate controls. After treatment, microglial cells were washed in ice-cold phosphate-buffered saline (PBS) three times. Next the cells were lysed with 1× SDS sample buffer

(62.5 mM Tris-HCI [pH 6.8], 2% SDS, 10% glycerol, 50 mM dithiothreitol), sonicated for 15 sec, and then heated at 100°C for 5 min. The cell lysates were centrifuged at 12,000 rpm (4°C) for 5 min, and the protein concentration of the supernatant was measured by BCA Protein Assay System (Pierce). Western blotting of phosphorylated p44/42 MAPK was performed according to the manufacturer's instructions using phospho-specific antibodies. Briefly, proteins were electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to immunoblotting PVDF membranes (Bio-Rad). The membranes were blocked for 1 hr at room temperature in Tris-buffered saline (TBS, Bio-Red) and 0.1% Tween-20 with 5% nonfat dry milk, and then were incubated with primary antibodies overnight at 4°C. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, the protein bands were detected with a Super Signal west Femto Maximum Sensitivity Substrate (Pierce) and BIOMAX-MR film (Eastman Kodak Co.). For detection of total p44/42, the membranes were stripped with Restore Western blot Stripping Buffer (Pierce) followed by incubation with specific antibodies.

Statistical Analysis

All experiments were performed at least three times, and the representative results were shown. Data were analyzed using analysis of variance (ANOVA) followed by *post hoc* comparisons of means by Bonferroni, or were analyzed using the Student *t*-test. A value of $p < 0.05$ was considered to be significant.

Results

BB enhances microglial phagocytosis of Aβ$_{1–42}$ peptide

Microglial phagocytosis of Aβis most likely a terminal event leading to removal of β-amyloid from the brain parenchyma.[31] We have found that microglia demonstrating this phagocytic phenotype are polarized toward an anti-inflammatory state characterized by attenuated p44/42 MAPK activity.[7,26,27] Because BB was previously shown to enhance cognitive performance in rodent models of AD and without coincident reduction of parenchymal amyloid, we sought to determine whether microglial phagocytosis was modulated by BB.[24] Thus "aged" FITC-tagged Aβ$_{1–42}$ (500 nM) was added to primary cultured microglial cells for 2 hr in the absence (control), presence of BB, positive control BAM-10 (2.5 μg/mL) or IgG isotype-control (2.5 μg/mL). As a control for nonphagocytic incorporation of Aβ by microglia, microglial cells were incubated at 4°C in parallel cell culture plates under the same treatment conditions described above. Cell supernatants and lysates were analyzed for extracellular and cell-associated FITC-Aβ using a fluorometer. As shown in Fig. 1A (top and bottom panel) , BB enhances microglia phagocytosis of Aβ$_{1–42}$ peptide, a result that was further verified by quantitative immunofluorescence assay (Fig. 1B). In a parallel experiment, results further showed microglial phagocytosis of Aβ$_{1–42}$ peptide was localized within the cytoplasm of microglial cells (Fig. 1C).

BB inhibits the formation of Aβ$_{1–42}$ aggregation

With regard to the inhibitory effect of BB on Aβ fibril formation, time dependencies and concentration dependencies were examined by using the thioflavin T method (Fig. 2A) , which detects mainly mature β-pleated sheet amyloid fibrils.[32] Figure 2 shows data for the thioflavin T assay, with incubation of 20 μM Aβ$_{1–42}$ peptides in 50 mM Tris-HCl buffer (pH 7.4) with or without BB extract (25 μg/mL, 50 μg/mL, and 100 μg/mL). After incubation at 37°C for different time points, BB (50 μg/mL and 100 μg/mL) significantly inhibited Aβ aggregation ($p < 0.05$, or $p < 0.001$, Student *t*-test) at the 48 hr point by approximately 75% and 60% respectively, whereas BB (25 μg/mL, 50 μg/mL, and 100 μg/mL) further inhibited Aβ aggregation ($p < 0.05$, or $p < 0.001$, Student *t*-test) at the 72-hr point by approximately 82% (25 μg/mL), 65% (50 μg/mL), and 57% (100 μg/mL), respectively. In this experiment, BB was shown to inhibit peptide aggregation in a clear, time- and concentration-dependent manner.

BB suppresses microglial activation and enhances microglial phagocytosis of Aβ$_{1–42}$ peptide through a p44/42 MAPK-dependent pathway

Previous studies have shown that activation of MEK1/2 and downstream p44/42 MAPK is involved in TNF-α and IL-6 production in macrophages, monocytes, and microglia after activation of these cells with a variety of stimuli, including LPS and CD40 ligand.[27,33–36] Moreover, BB extract inhibited the production of inflammatory mediators from LPS-activated BV2 microglia.[37] These data led us to ask whether the observed effect of BB on opposing microglial activation might be mediated via activation of the MAPK module. Thus, we analyzed p44/42 MAPK phosphorylation status in microglial cell lysates after treatment with LPS (50 ng/mL) or Aβ$_{1–42}$ (1 μM) or appropriate controls for 30 min. Results showed that phosphorylation of p44/42 was induced within 30 min after treatment with LPS or Aβ$_{1–42}$. Furthermore, we observed that treatment microglial cells with PD98059 (PD, 5 μM; a selective inhibitor of MEK1/2) or BB (50 μg/mL) for 1 hr prior to treatment with LPS (50 ng/mL) or Aβ$_{1–42}$ (1 μM) for 30 min, result in significant reduction of activation of LPS or Aβ-induced p44/42 MAPK (Fig. 3A,B). To determine whether BB and activation of p44/42 MAPK was responsible for TNF-α and IL-6 production after co-treatment of microglia with LPS and Aβ$_{1–42}$ peptide, we treated microglia with BB or PD98059 before stimulation with LPS and Aβ$_{1–42}$ peptide. Production of TNF-α and IL-6 were markedly decreased compared with appropriate controls within 16 hr after treatment with BB, PD98059, and LPS plus Aβ$_{1–42}$ peptides. Namely, either BB or PD abrogated LPS- or LPS/Aβ$_{1–42}$-induced production of TNF-α or IL-6 (Fig. 3C, top and bottom panels). These observations prompted us to investigate the role of p44/42 MAPK signaling in the BB- mediated microglial phagocytosis of Aβ$_{1–42}$ peptide. Microglial cells were pretreated with BB or PD98059 (5 μM) alone, or a combination of PD98059 and BB for 1 hr, then co-treated with "aged" FITC-tagged Aβ$_{1–42}$ (500 nM) for 2 hr in the absence (control) or presence of LPS. Cell culture supernatants were collected and cell lysates were prepared to measure Aβ by fluorometer (Fig. 3D, top and bottom panels). Results show that cross-linking BB boosts microglial phagocytosis of Aβ$_{1–42}$ peptide, which was enhanced by inhibition of p44/42 activation. Whereas, the combination of BB and LPS/PD did not confer additional effects

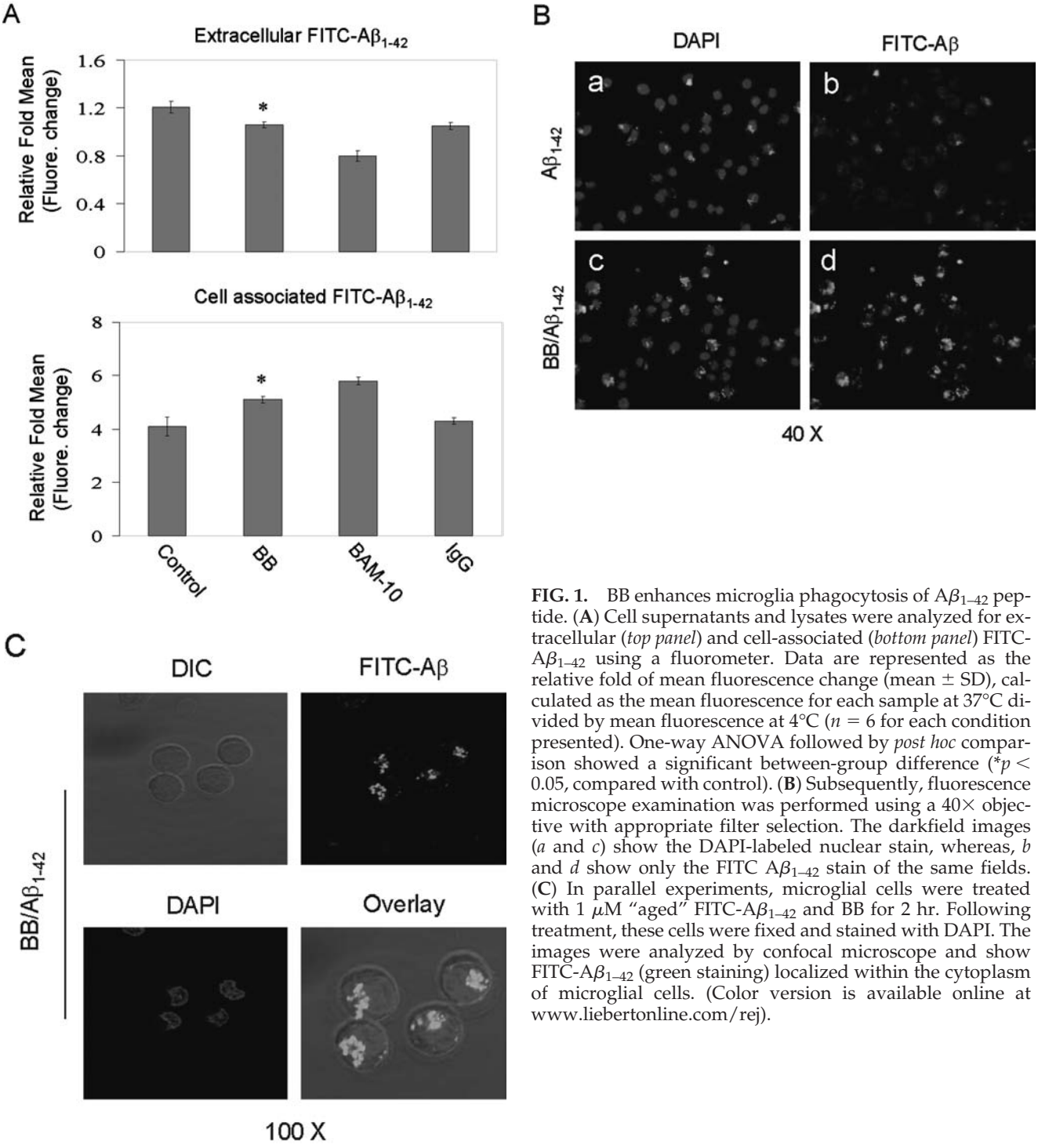

FIG. 1. BB enhances microglia phagocytosis of A$\beta_{1–42}$ peptide. (**A**) Cell supernatants and lysates were analyzed for extracellular (*top panel*) and cell-associated (*bottom panel*) FITC-A$\beta_{1–42}$ using a fluorometer. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as the mean fluorescence for each sample at 37°C divided by mean fluorescence at 4°C ($n = 6$ for each condition presented). One-way ANOVA followed by *post hoc* comparison showed a significant between-group difference ($^*p < 0.05$, compared with control). (**B**) Subsequently, fluorescence microscope examination was performed using a 40× objective with appropriate filter selection. The darkfield images (*a* and *c*) show the DAPI-labeled nuclear stain, whereas, *b* and *d* show only the FITC A$\beta_{1–42}$ stain of the same fields. (**C**) In parallel experiments, microglial cells were treated with 1 μM "aged" FITC-A$\beta_{1–42}$ and BB for 2 hr. Following treatment, these cells were fixed and stained with DAPI. The images were analyzed by confocal microscope and show FITC-A$\beta_{1–42}$ (green staining) localized within the cytoplasm of microglial cells. (Color version is available online at www.liebertonline.com/rej).

on microglial phagocytosis of A$\beta_{1–42}$ peptide compared with LPS/PD98059. These data collectively suggests that BB suppresses microglial activation and enhances microglial phagocytosis of A$\beta_{1–42}$ peptide through a p44/42 MAPK-dependent pathway.

BB inhibits co-localization of microglial MHC class II and A$\beta_{1–42}$ peptide

MHC class II is expressed by microglial cells in the frontal cortex and hippocampus of normally aging individuals, and levels of expression are markedly increased in these brain regions in AD cases. The impairment of MHC II function results in a significant reduction of microglia-associated central nervous system (CNS) inflammation,[38] suggesting the elevated level of MHC class II expression is strongly associated with a microglial proinflammatory response. We have shown that CD40 ligation increases MHC II- Aβ peptide complexes[35] or cross-linking of CD45RB inhibits MHC class II- Aβ co-localization, as detected by fluorescence microscopy.[27] To examine further whether BB could inhibit formation of immunogenic MHC II-Aβ peptide complexes on

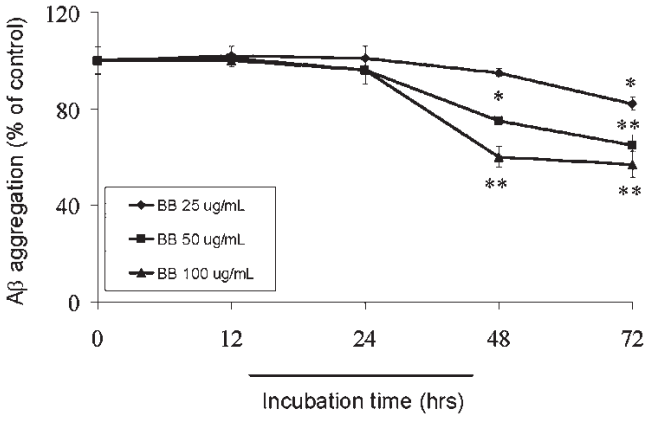

FIG. 2. BB inhibits formation of Aβ_{1-42} aggregation. Effect of different BB concentration on Aβ_{1-42} aggregation was assessed by the thioflavin T method. Reaction mixtures containing 20 μM of Aβ_{1-42}, 50 mM Tris-HCl buffer (pH 7.4), and different concentrations of BB extract were incubated at 37°C for indicated time points. Aβ aggregation was expressed as a percentage of control, which was observed in the absence of BB extract. Values represent the means ± SD from three independent experiments. (*) $p < 0.05$, (**) $p < 0.001$ compared with the BB-untreated control.

the cell surface, we treated microglia with BB (50 μg/mL) in the presence or absence of "aged" Cy3- Aβ_{1-42} peptide (300 nM) for 48 hr, followed by immunofluorescence staining with FITC-conjugated anti-mouse MHC class II antibody. Results show that BB inhibits MHC class II- Aβ co-localization as detected by fluorescence confocal microscope equipped (Fig. 4).

Discussion

The proinflammatory activation state of microglia seems to be a pervasive link between the majority of neurodegenerative diseases, including human immunodeficiency virus (HIV)-associated dementia (HAD),[39,40] multiple sclerosis (MS),[41,42] amytropic lateral sclerosis (ALS),[43,44] stroke,[45,46] and AD.[9,47] Because it is a common link, the study of compounds, especially those that can be easily taken as a part of diet, is attractive as most of these diseases come with advancing age, suggesting a significant epidemiologic problem.

However, current pharmacological attempts at reducing neuroinflammation mediated via microglial activation have been largely negative in terms of slowing AD progression.[48] These anti-inflammatory drugs have included nonsteroidal anti-inflammatory drugs (NSAIDS), prednisone, COX-2 inhibitors (celecoxib, rofecoxib, and nimesulide), and hydroxychloroquine.[49–52] These trials have failed for various reasons. The COX-2 inhibitors have not been in clinical use long enough for epidemiological data to accumulate, but their failure could be fairly well forecasted because COX-2 is highly expressed in normal pyramidal neurons, including those of AD cases.[53] Thus, these drugs will largely affect vulnerable pyramidal neurons and not microglia. Also animal neurotoxicity was observed in models following COX-2 inhibition.[54] Hydroxychloroquine confers side effects of toxicity, neuronal, and retinal damage.[55] Indeed no epidemiological or immunohistochemical evidence exists to support a role for this 4-aminoquinoline in neuroinflammation. NSAIDs have been shown *in vitro* to reduce the neurotoxicity of activated microglia toward neuronal cells,[56] and the original NSAID study by Rogers and colleagues seemed to reflect this notion. This trial demonstrated an arrest of mental deterioration over a 6-month period in AD patients compared to nontreated AD patients.[48] However, follow-up studies using NSAIDS have not been as promising. For example, a trial of the mixed COX inhibitor diclofenac combined with misoprostyl, demonstrated nonstatistically significant arrest of mental deterioration in drug compared with placebo patients.[57] However, a Japanese trial did show a significantly slower cognitive decline compared to placebo where cases were treated with a combination of estrogen and vitamin E, in addition to the NSAID.[58] It is also important to note that gastrointestinal and adverse side effects can be a barrier to patient compliance with such agents, particularly with chronic use in the elderly.[59]

Thus, natural compounds specifically aimed at blocking microglial activation may be more efficacious at ameliorating microglial-associated neuropathology in AD. In this study, we focused on identifying a specific intracellular signaling target, p44/42 MAPK, which when activated, could enhance microglial activation downstream of the extracellular pro-inflammatory mediators Aβ peptide and/or LPS. Our rationale for such investigation was three fold. First, it has been reported that in macrophages, LPS induces protein tyrosine phosphorylation of several proteins, including the p42 and p44 MAPKs in mice, similar to the manner in which fibrillar Aβ activates human moncytes.[22] As mice require a proinflammatory stimulus to mimic human microglia responses to Aβ, LPS was co-administered. Second, if we could inhibit microglial activation by Aβ peptide in the presence of LPS, the self-potentiating inflammatory cycle resulting from intracellular signal transduction cascades could be suppressed. Third, our previous experiments indicate that inhibition of p44/42 MAPK attenuated the microglial proinflammatory MHC II-expressing phenotype.[36]

Our data show that microglia can be activated after treatment with Aβ peptides and LPS into a proinflammatory state as evidenced by significantly increased secretion of TNF-α IL-6 (Fig 3C), decreased Aβ phagocytosis (Fig 3D), and increased phosphorylation of p44/42 MAPK (Fig 3A,B). Because BB conferred behavioral improvement in PSAPP without mitigation of amyloid plaques in previous studies, we next determined if BB was a negative regulator of the proinflammatory response of microglia to Aβ and LPS. Data showed that BB markedly reduced microglial of p44/42 MAPK activation resulting from Aβ and LPS co-treatment (Fig 3A,B).

These data are in line with previous experiments in our[7] and other[60] laboratories that strongly suggest an additional role in microglial Aβ responses, which may act to balance the proinflammatory microglial activation described above.[61] Consistent with this hypothesis, we did observe increased microglial Aβ cell association activity under these conditions, suggesting that BB stimulates non-MHC-II-expressing (Fig. 4), pro-phagocytic microglial phenotype (Figs. 1B,C and 3D).[7] In accordance with this, co-treatment of Aβ-

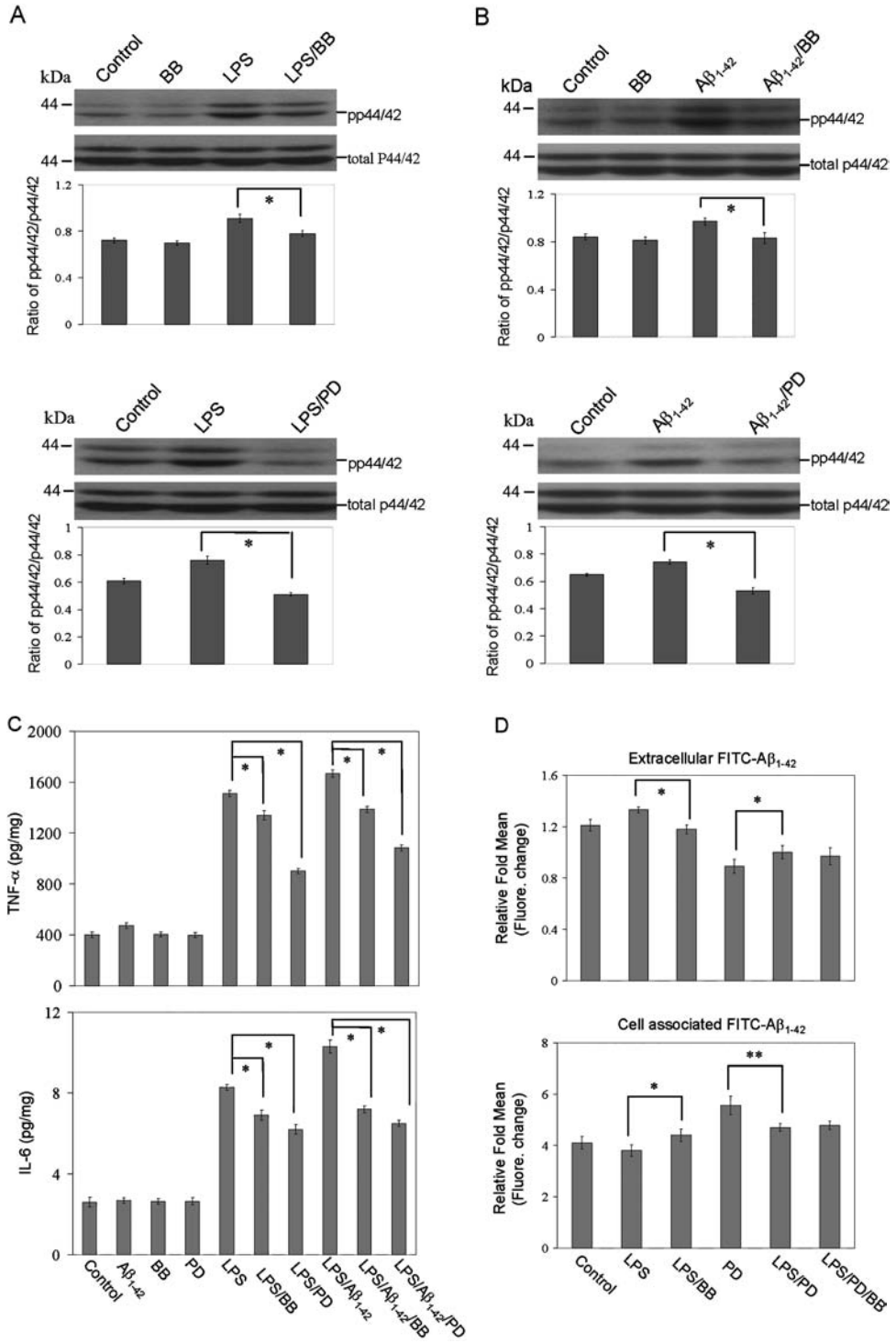

FIG. 3. BB suppresses microglial activation and enhances microglial phagocytosis of A$\beta_{1–42}$ peptide through a p44/42 MAPK-dependent pathway. Microglial treatment conditions are indicated and are further described in the Materials and Methods section. Cell lysates were analyzed by western immunoblotting using specific antibodies that recognize phosphorylated or total p44/42 MAPK at the indicated time point (**A** or **B**, *top panels*). Phosphorylation of p44/42 MAPK after treatment with LPS or A$\beta_{1–42}$ peptides was inhibited by PD98059 (**A** or **B**, *bottom panels*). Histograms below the immunoblots represent the mean band density ratio $\pm$ 1 SD (pp44/42/total p44/42 MAPK; $n = 3$ for each condition presented, $^*p < 0.05$). (**C**) Microglial activation is evidenced by mean TNF-α and IL-6 release $\pm$ 1 SD ($n = 3$ for each condition presented; $^*p < 0.001$). (**D**) Microglial phagocytosis of A$\beta_{1–42}$ peptide after pretreatment with PD98059, BB or PD98059, and BB for 1 hr then co-treated with "aged" FITC-tagged A$\beta_{1–42}$ or LPS. Supernatants and cell lysates were analyzed for extracellular (*top panel*) and cell-associated (*bottom panel*) FITC- A$\beta_{1–42}$ using a fluorometer ($^*p < 0.05$, $^{**}p < 0.001$). For **A– D**, one-way ANOVA followed by *post hoc* Bonferroni testing was used. *Note:* PD, PD98059; pp, phosphorylated.

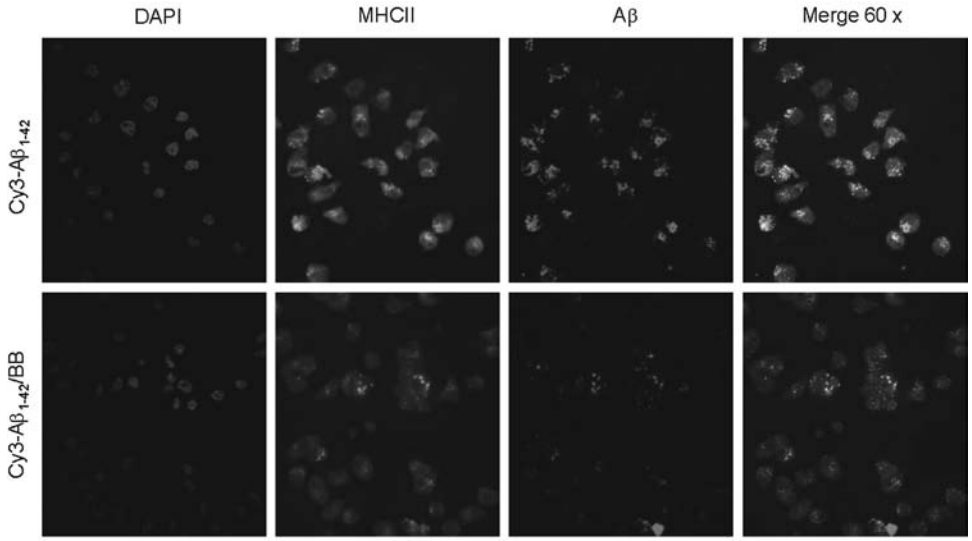

FIG. 4. BB inhibits microglia MHC class II-Aβco-localization. To examine microglia MHC II-Aβpeptide complex formation on the cell surface, microglia were treated with "aged" Cy3- Aβ_{1-42} peptide (300 nM) in the presence or absence of BB for 48 hr followed by staining with FITC-anti-mouse MHC class II antibody and confocal microscopy. *Note:* Red indicates Aβ-positive; green indicates MHC class II-positive; yellow indicates the co-localization of MHC class II and Aβ; blue indicates DAPI nuclear stain of the same fields. Original magnification, 60× for top and bottom panels. (Color version is available online at www.liebertonline.com/rej).

and LPS-activated microglia with BB resulted in statistically significant reduction of microglial IL-6 and TNF-α secretion (Fig. 3C), other important mediators of neuronal damage in AD.[62,63] As an additional possible anti-amyloid mechanism, we sought to establish whether BB could also inhibit Aβ aggregation *in vitro* and found that all doses tested (25, 50, and 100 μg/mL) aggregation was significantly inhibited after 48 and 72 hr (Fig. 2). Together, these results suggest that BB is a viable approach for both downregulating Aβ-induced microglial activation and possibly attenuating Aβ conglomeration into plaques.

It is important to note, however, that use of microglial expression of MCH II as a marker of activation is limited. Microglia usually remain in a ramified resting form, but age may alter this quiescent state, as is seen by upregulated MHC II expression.[64,65] It has already been suggested that MCH II expression is a marker of cell aging, in addition to, or even instead of, being an activation marker.[66,67] Future studies will be required to correlated CD45 (another marker for microglial activation)-immunostaining, in addition to the effect of aging which may be expressed by the MHC class II immunoreactivity.

Previously, it has been shown that dietary supplements with BB extracts reduce some neurological deficits in aged animal models.[68,69] Catechin is the major flavonoid found in blueberries (*Vaccinium ashei Reade*), with 387 mg/100 grams fresh weight. Blueberry extract also contains epicatechin, ranging from 34 to 129 mg/100 grams fresh weight, whereas total anthocyanins range from 84 to 113 mg/100 grams fresh weight.[70] Joseph and colleagues showed that dietary supplementation for 8 weeks with BB extracts reversed cognitive deficits in Morris water maze performance tests in 19-month-old rats.[23] This effect seemed to occur via downregulaton of the proinflammatory nuclear factor-κB (NF-κB) module, as the aged rats with BB extracts diet had significantly lower levels than aged control diet.[64] Additional evidence was seen in PSAPP mice, which develop age-dependent AD-like senile plaques.[24] These mice, when supplemented with BB extracts (2% of diet) from 4 to 12 months of age, displayed Y-maze performance similar to that of nontransgenic mice and significantly better than that of nonsupplemented transgenic mice.[25] However in contrast to our *in vitro* microglial phagocytosis experiments, the examination of the brain of these mice revealed blueberry extracts had no affect on Aβ peptide production, deposition, or quantity.[25] This would suggest that the blueberry induced prophagocytic property of microglia may not be enough to compensate for the strong amyloidogenic APP processing in the PSAPP mouse strain.

Thus, taking previous these previous studies and current findings into account, we suggest that BB extract supplementation might prevent cognitive deficits most likely through an antimicroglial activation mechanism, which results in the protection of neurons and synapses from toxic inflammatory mediators, without a necessary reduction in parenchymal Aβ.

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