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Microglia Lacking E Prostanoid Receptor Subtype 2 Have Enhanced Aβ Phagocytosis yet Lack Aβ-Activated Neurotoxicity

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Experimental therapies for Alzheimer's disease (AD) are focused on enhanced clearance of neurotoxic Aβ peptides from brain. Microglia can be neuroprotective by phagocytosing Aβ; however, this comes at the cost of activated innate immunity that causes paracrine damage to neurons. Here, we show that ablation of E prostanoid receptor subtype 2 (EP2) significantly increased microglial-mediated clearance of Aβ peptides from AD brain sections and enhanced microglial Aβ phagocytosis in cell culture. The enhanced phagocytosis was PKC-dependent and was associated with elevated microglial secretion of the chemoattractant chemokines, macrophage inflammatory protein-1α and macrophage chemoattractant protein-1. This suggested that microglial activation is negatively regulated by EP2 signaling through suppression of prophagocytic cytokine secretion. However, despite this enhancement of Aβ phagocytosis, lack of EP2 completely suppressed Aβ-activated microglia-mediated paracrine neurotoxicity. These data demonstrate that blockade of microglial EP2 is a highly desirable mechanism for AD therapy that can maximize neuroprotective actions while minimizing bystander damage to neurons. ***(Am J Pathol 2005, 166:1163–1172)***

Microglia are thought to play an important role in pathogenesis of several neurodegenerative diseases in which they become activated, displaying both innate immune response and increased phagocytic activity. A robust innate immune response occurs in association with Aβ-containing plaques in brain of patients with Alzheimer's disease (AD) that is characterized by activation of complement, increased secretion of several cytokines and chemokines, increased production of reactive oxygen and nitrogen species, and increased production of prostaglandin (PG) E2,[1–4] some combinations of which can be neurotoxic. Indeed, Aβ is one of handful of endogenous ligands known to activate innate immunity and thereby is proposed to contribute to AD initiation and early progression.[5,6] Consistent with this hypothesis, several large observational studies have repeatedly observed that long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a decreased incidence of AD,[7,8] although other mechanisms of action for some NSAIDs have been proposed.[9]

However, as demonstrated by numerous examples from other organs, activated tissue macrophages need not be exclusively deleterious. In fact, double-transgenic mice that overexpress an AD-causing mutant form of human APP as well as a natural inhibitor of complement C3 suggest that the innate immune response in AD may in part be beneficial by enhancing clearance of Aβ peptides from plaques through phagocytosis.[10] Thus, an emerging consensus hypothesizes that aggregated Aβ stimulates a presumably beneficial microglial phagocytic response while at the same time activating a neurotoxic glial innate immune response; if overwhelming, microglial phagocytosis fails to remove aggregated Aβ and the protracted innate immune response becomes neurotoxic.[11] This model of AD pathogenesis implies two apparently mutually exclusive therapeutic strategies: stimulate microglia to enhance Aβ clearance and suppress microglial activation to dampen bystander damage to neurons.

PGE_2, a product derived from arachidonic acid by cyclooxygenase (COX) and specific synthases, is a potent autocrine and paracrine factor that is distinct from other eicosanoid products of COX because of multiple G-protein coupled receptor subtypes, EP1, EP2, EP3, and EP4 that are linked to functionally antagonistic second messenger systems.[12] All EP receptor subtypes are expressed on varying cells in brain; microglia express

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EP1 and EP2.[13] Because of this complexity, PGE_2 has been conflictingly reported to mediate neurotoxicity and to be neuroprotective, as well as to enhance and suppress macrophage phagocytosis.[14–16] Thus, increased PGE_2 in the central nervous system, as occurs early in AD,[17] may have both pro- and anti-inflammatory actions and may significantly modulate microglial phagocytosis. Here, we tested the hypothesis that ablation of one microglial PGE_2 receptor, EP2, may enhance microglial Aβ phagocytosis while suppressing bystander damage to neurons from Aβ activation of microglia.

Materials and Methods

Animals and Materials

Mice homozygous for disruption of the gene that encodes the EP2(EP2−/−) were backcrossed >12 generations to the BALB/c genetic background. BALB/c wild-type (WT) control mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were maintained in a temperature-controlled specific pathogen-free facility with a strict 12-hour light/dark cycle and with free access to food and water. All experiments were performed exactly as approved by the University of Washington Institutional Animal Care and Use Committee. Antibodies 4G8 and 6E10 (specific for $A\beta_{1\text{-}17}$ and so does not recognize the p3 fragment of APP) were from Signet Laboratories (Dedham, MA). CD11b was from Serotec (Raleigh, NC). Antibodies against microtubule-associated protein 2 (MAP-2) and neuronal nuclei (NeuN) were from Chemicon (Temecula, CA). Alexa fluorescent-labeled secondary antibodies were from Molecular Probes (Eugene, OR). 4,6-Diamidino-2-phenylindole-containing mounting medium was from Vector Laboratories (Burlingame, CA). Poly-D-lysine was from BD Biosciences (Bedford, MA). Papain and DNase I were from Worthington Biochemical (Lakewood, NJ). Synthetic $A\beta_{1\text{-}42}$ was from Bachem (Torrance, CA) and fluorescein-labeled $A\beta_{1\text{-}42}$ was from rPeptide (Athens, GA). AH6809, butaprost, and 17-phenyl trinor prostaglandin E2 (PTPE2) were from Cayman (Ann Arbor, MI). SC51089 was from Biomol (Plymouth Meeting, PA). Forskolin and bisindolylmaleimide (BIM) were from Calbiochem (La Jolla, CA). Culture media, heat-inactivated fetal bovine serum, bovine calf serum, and penicillin/streptomycin were from Invitrogen (Carlsbad, CA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Ex Vivo *Studies*

Frozen AD hippocampus was sliced into 10-μm-thick sections using a cryostat and sections were mounted onto poly-D-lysine-coated coverslips. Sections were stored at −80°C and, to minimize the variation among sections used for WT and EP2−/− microglia, consecutive sections were selected. Primary microglia cultures were derived from cortices of P1 to P3 neonates. Cells were dissociated using enzyme solution containing Dulbecco's modified Eagle's medium (DMEM), ethylenediamine tetraacetic acid (0.5 mmol/L), L-cysteine (0.2 mg/ml), papain (15 U/ml), DNase I (200 μg/ml) followed by trituration. Culture medium (DMEM with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) was changed after 24 hours of initial seeding. Microglia at the 14th day *in vitro* (DIV) were separated from the underlying astrocytic monolayer by gentle agitation using their differential adhesive properties and were seeded on hippocampal sections at 1×10^5 cells per section in microglial culture medium for 2 hours followed by an additional 48-hour incubation in serum-free DMEM containing penicillin and streptomycin. To determine purity of microglia, 1×10^4 cells were cultured on chambered slides with treatment of 100 nmol/L of aggregated $A\beta_{1\text{-}42}$ for 24 hours followed by cytochemistry analysis using a microglial marker, CD11b. Percentage of positive microglia was normalized by 4,6-diamidino-2-phenylindole nuclei counterstaining. From six independent experiments, purity of microglia was 99.4 ± 0.2%. For immunohistochemical analysis, cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and subjected to formic acid (88%) treatment before application of antibodies against CD11b (1:50) and Aβ (4G8, 1:200). Mounting medium containing 4,6-diamidino-2-phenylindole was used to label nuclei. Images were randomly selected using Leica confocal/two-photon imaging system. A representative image with a comparable number of microglia between WT and EP2−/− was compared for reduction of Aβ plaques. All images were subjected to quantification of CD11b using MetaMorph computer-assisted software. Data were presented as average positive area in arbitrary units per microglia on top of human brain sections. For Western blot analysis, cultures were lysed with 8 mol/L urea containing proteinase inhibitor cocktail. Lysates with equal amount of protein were subjected to 16.5% Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot using 6E10 antibody. Enhanced chemiluminescence was used to reveal Aβ species and intensity of the corresponding bands were quantified using the GS-710 imaging densitometer (Bio-Rad, Hercules, CA). Six separate experiments were performed for each analysis.

Microglial Aβ Phagocytosis

At DIV 14, microglia were seeded in six-well plates at 1.5×10^5 cells per well in microglial culture medium. After 2 hours of seeding, medium was changed with 1.5 ml of serum-free DMEM containing penicillin and streptomycin. To make aggregated Aβ for phagocytosis, Aβ was dissolved in 2 mmol/L NaOH and was diluted into 10 μmol/L of stock solution (100×) in PBS followed by incubation at 37°C for 2 hours and monitored by turbidity. For studying signaling pathways involved in Aβ phagocytosis, some microglia were pretreated for 1 hour with AH6809 (20 μmol/L), butaprost (1 to 50 μmol/L), SC51089 (10 μmol/L), PTPE2 (20 μmol/L), forskolin (50 μmol/L), or BIM (10 μmol/L) before treatment with aggregated fluorescein-labeled $A\beta_{1\text{-}42}$ (100 nmol/L). After 24 hours of incubation of Aβ, cells were washed with PBS followed by trypsinization (0.05%) and centrifugation.

Cells were then resuspended in PBS and subjected to flow cytometry. Flow cytometer (FACScan; BD Biosciences, San Jose, CA) set at ungated 10,000 cells per measure was used to analyze Aβ uptake as an indication of Aβ phagocytosis. Data were analyzed using Summit Mo-Flo software (Cytomation, Fort Collins, CO). Phagocytosis was presented as the percentage of mean fluorescein isothiocyanate intensity in WT controls treated with Aβ. Some cells were plated onto chambered slides at 1×10^4 for assessing cellular uptake of Aβ using a fluorescent microscope (TE200; Nikon, Melville, NY).

Aβ-Activated Microglia-Mediated Neurotoxicity

For primary neuronal cultures, embryonic (E16 to E17) cortices from WT mice were extracted, partially digested with proteases, and gently disrupted before plating at 5×10^5 cells per well in poly-D-lysine/laminin (BD Biosciences) coated 24-well plates. One hour after seeding, medium was replaced with neurobasal medium with B-27 supplement, penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mmol/L). Cytosine arabinoside (2.5 μmol/L) was added on the second day. Purity for neuronal cultures was 98 ± 1% as measured by NeuN antibody. DIV 14 primary microglia derived from WT and EP2−/− mice were plated at 1×10^5 per well of WT neurons (DIV 6). To make aggregated Aβ for neurotoxicity assay, 1 mg of $Aβ_{1-42}$ was dissolved in 20 μl of dimethyl sulfoxide and was then diluted into PBS to make 500 μmol/L of stock solution followed by 37°C incubation for 24 hours. Freshly prepared, preaggregated Aβ at 12 μmol/L was added into co-culture medium containing DMEM, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin for 24 hours. Cultures were washed twice with PBS and subjected to fixation with 4% paraformaldehyde in PBS at 4°C for 1 hour. Terminal dUTP nick-end labeling (TUNEL) assay (Roche Applied Science, Indianapolis, IN) was performed per the manufacturer's instructions, followed by fluorescent immunocytochemistry using MAP-2 antibody to identify neurons. Neurotoxicity was presented as percentage of co-positivity for both TUNEL and MAP-2 in all MAP-2-positive neurons.

Cell Count and Levels of Secreted Cytokines

To measure cell proliferation, microglia from WT and EP2−/− were initially seeded at 1×10^5 cells per 24-well culture plate in serum-containing medium. After 2 hours of incubation, some cultures were changed to serum-free conditions. After an additional 24 hours of incubation, cells were washed with PBS and were trypsinized followed by cell count. Six separate experiments were performed to obtain cell count. For cytokine analysis, culture medium from serum-free condition was collected from WT and EP2−/− microglial cultures and were stored at −20°C until measurements were performed. Cytokine/chemokine membrane array (Ray Biotech, Norcross, GA) were used to screen for 32 molecules: interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL12, IL-12p70, IL-13, IL-17, interferon-γ, six conserved cytokines (6Ckine), cutaneous T cell-attracting chemokine, eotaxin, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, cytokine-induced neutrophil chemoattractant (KC), leptin, macrophage chemoattractant protein (MCP)-1, MCP-5, macrophage inflammatory protein (MIP)-1α, MIP-2, MIP-3β, RANTES (regulated on activation, normal T cell expressed and secreted), stem cell factor, thymus and activation-regulated chemokine, tissue inhibitors of matrix metalloproteinases-1, tumor necrosis factor-α, soluble tumor necrosis factor receptor type I, thrombopoietin, and vascular endothelial cell growth factor. Enzyme-linked immunosorbent assay kits for mouse MCP-1 (Pierce, Rockford, IL), MIP-1α (Biosource Int., Camarillo, CA), and macrophage colony-stimulating factor (Pierce) were used according to the manufacturers' instructions. Levels of cytokines were obtained at pg per ml of culture medium from 10^5 microglia cultivated at basal conditions.

Results

Enhanced Phagocytosis of Aβ from Senile Plaques in Human Hippocampus by EP2−/− Microglia ex Vivo

We first used a human *ex vivo* model by incubating primary mouse microglia on top of hippocampal sections from patients who died with AD and participated in the rapid autopsy program at the University of Washington. Confocal images showed abundant Aβ-immunoreactive (IR) material in the distribution of senile plaques in an AD tissue section that was incubated with WT microglia (Figure 1A) and, similar to the results of others, no substantial change from a consecutive tissue section that was incubated without WT microglia (not shown). In contrast, incubation of the next tissue section with EP2−/− microglia (Figure 1B) markedly reduced Aβ-IR material in tissue. Although several possible mechanisms exist to explain the reduction of Aβ-IR material in human hippocampus caused by EP2−/− microglia, EP2−/− microglia displayed morphological features of Aβ phagocytosis, including pseudopodia engulfing Aβ-IR material (arrow in inset of Figure 1B), that were not present in WT microglia incubated with AD tissue. Moreover, EP2−/− microglia incubated with AD tissue contained Aβ-IR material (Figure 1D) inside phagocytic vacuoles as indicated by intracellular compartments that were IR for CD11b, an indicator for microglial activation (Figure 1E).[18] In addition, DNA fragments, likely released from necrotic cells in the tissue section, were also seen within these vacuoles (Figure 1F). Co-localization among Aβ, CD11b, and DNA fragments is shown in Figure 1C.

Microglial Activation and Reduction of Ab_{1-40} and Ab_{1-42} in Human Hippocampus by EP2−/− Microglia ex Vivo

To quantify disappearance of Aβ peptides in this *ex vivo* model, samples were extracted and subjected to West-

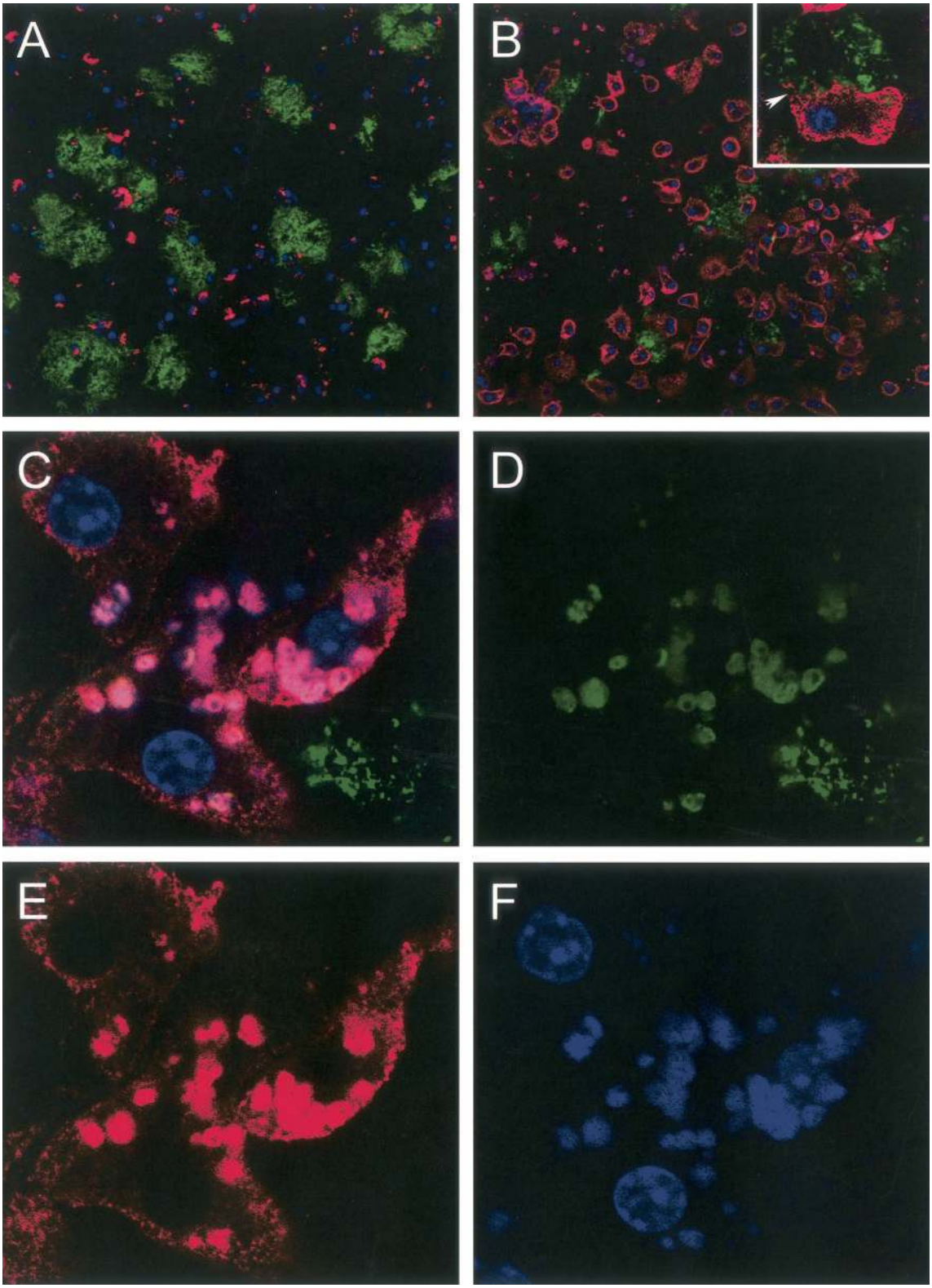

Figure 1. Enhanced phagocytosis of Aβ from senile plaques in human hippocampus by EP2−/− microglia *ex vivo*. AD brain sections with primary microglial cultures from WT or EP2−/− mouse cerebrum were fixed and subjected to immunofluorescent labeling. Representative confocal images containing similar number of microglia are presented. **A:** Tissue with Aβ-IR material (green) in the pattern of senile plaques, WT microglia (red), and nuclei (blue); the density and pattern of Aβ-IR plaque material in tissue incubated with WT microglia was no different from tissue sections incubated without microglia (not shown). **B:** Consecutive tissue section incubated with EP2−/− microglia showed marked disappearance of Aβ-IR material. Pseudopodia engulfing Aβ-IR plaque material were also seen in EP2−/− microglia (**inset**, **arrow**). At a higher magnification, EP2−/−-activated microglia contained Aβ-IR material (**D**) inside phagocytic vacuoles as indicated by intracellular compartments IR for the integrin marker CD11b (**E**). In addition, DNA fragments were also seen within these vacuoles (**F**). **C** is a merge of **D**, **E**, and **F**. Original magnifications: ×250 (**A**, **B**); ×1000 (**C–F**).

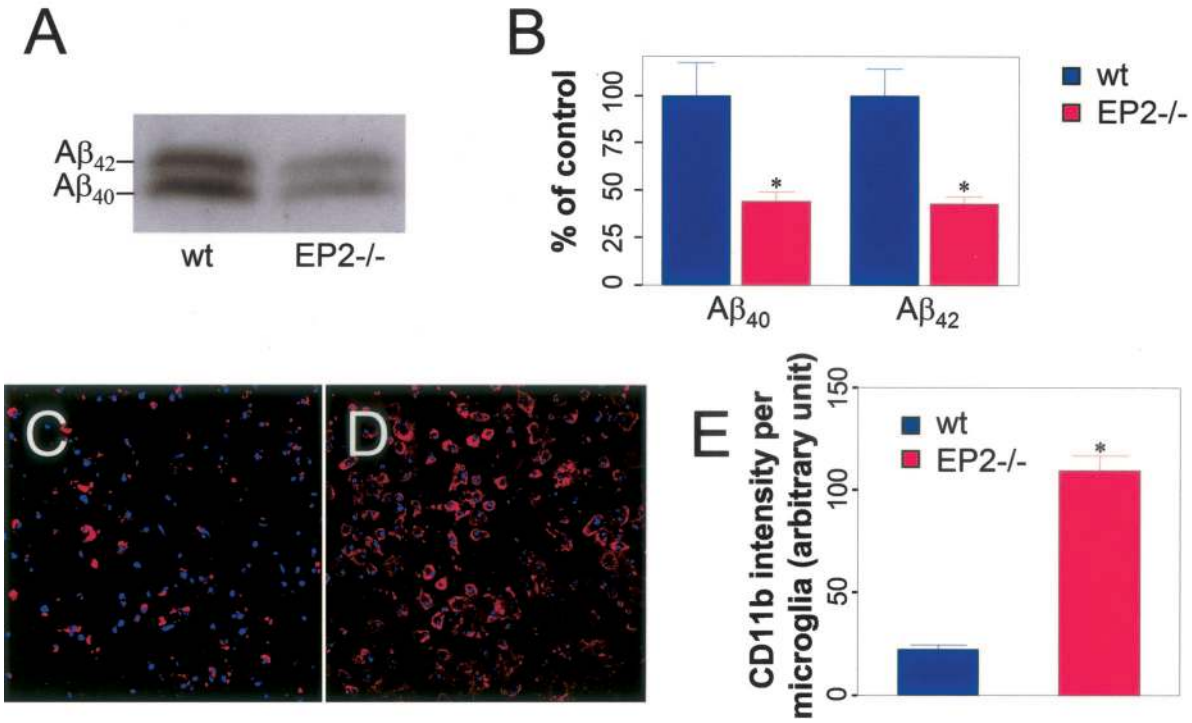

Figure 2. Microglial activation and reduction of Ab_{1-40} and Ab_{1-42} in human hippocampus by EP2−/− microglia *ex vivo*. AD brain sections with primary microglial cultures from WT or EP2−/− mouse cerebrum were subjected to urea extraction followed by Western blot analysis (representative blot in **A**). There was no difference in band density for $A\beta_{1-40}$ or $A\beta_{1-42}$ when tissue sections incubated without microglia were compared to sections incubated with WT microglia (not shown). **B:** $A\beta_{1-40}$ was reduced 44 ± 5% ($n = 6$, $P < 0.05$) and $A\beta_{1-42}$ was reduced 43 ± 4% ($n = 6$, $P < 0.01$) in human hippocampus incubated with EP2−/− microglia compared to tissue incubated with WT microglia. Intensity of CD11b immunoreactivity per microglial cell was evaluated by confocal images from WT (**C**) and EP2−/− (**D**) on human hippocampal slices and quantified (**E**); fluorescence intensities (in arbitrary units) in WT microglia and EP2−/− microglia were 23 ± 2 ($n = 5$) and 110 ± 7 ($n = 10$, $P < 0.01$), respectively. Original magnifications, ×250 (**C**, **D**).

ern blot analysis. $A\beta_{1-40}$ and $A\beta_{1-42}$ levels from AD tissue sections were determined from the corresponding band density using densitometry. We found that, similar to the results of others, tissue incubated with WT microglia had $A\beta_{1-40}$ and $A\beta_{1-42}$ levels that were no different from consecutive sections incubated without microglia (not shown). In contrast, tissue sections incubated with EP2−/− microglia had $A\beta_{1-40}$ reduced to 44 ± 5% ($P < 0.05$, $n = 6$) and $A\beta_{1-42}$ reduced to 43 ± 4% ($P < 0.01$, $n = 6$) after 48 hours compared to consecutive tissue sections incubated with WT microglia (Figure 2, A and B); by inspection, minimal clearance of Aβ immunoreactivity had occurred by 24 hours. Estimation of CD11b IR per microglial cell incubated with human brain tissue slices was performed using confocal images from WT (Figure 2C) and EP2−/− (Figure 2D). Data showed that CD11b IR in WT or EP2−/− microglia was 23 ± 2 arbitrary fluorescence units ($n = 5$) or 110 ± 7 arbitrary fluorescence units ($n = 10$, $P < 0.01$), respectively (Figure 2E); these data associate reduction of Aβ burden in AD brain section with a standard measure of microglial activation. Although the total number of microglia present on AD tissue were equivalent regardless of genotype at the end of incubation (159 ± 17, $n = 5$, WT microglia in ×250 fields; and 165 ± 22, $n = 10$, EP2−/− microglia in ×250 fields), they were not similarly distributed; EP2−/− microglia tended to aggregate around Aβ-IR plaques whereas WT microglia were more evenly dispersed across the tissue section, perhaps suggesting enhanced chemoattaction in EP2−/− microglia. In combination, these observations showed that EP2−/− microglia had enhanced phagocytic activity toward fibrillar Aβ peptides and reduced Aβ peptide tissue burden in human hippocampal slices much more effectively than WT microglia. Although the precise mechanisms of reduced Aβ tissue in these experiments have not been determined, we speculate, as have most others[5,19,20] that this occurs by degradation of phagocytosed protein.

Microglial Phagocytosis of Aggregated Fluorescein-Labeled $A\beta_{1-42}$

To characterize better the enhanced $A\beta_{1-42}$ phagocytosis by EP2−/− microglia, primary cultures of microglia from WT and EP2−/− mice were incubated with aggregated $A\beta_{1-42}$ labeled with fluorescein and analyzed by microscopy and flow cytometry. Consonant with our *ex vivo* studies described above, EP2−/− microglia (Figure 3B) had greater $A\beta_{1-42}$ uptake compared to WT (Figure 3A). Some EP2−/− microglia displayed phagocytosed Aβ that exceeded that observed in WT microglia (arrow in Figure 3B), whereas other EP2−/− microglia appeared similar to their WT counterparts, suggesting a heterogeneous population of EP2−/− microglia with a subpopulation of cells with enhanced phagocytic properties for $A\beta_{1-42}$. A representative scatter plot from flow cytometric analysis showed that granularity and size were not differ-

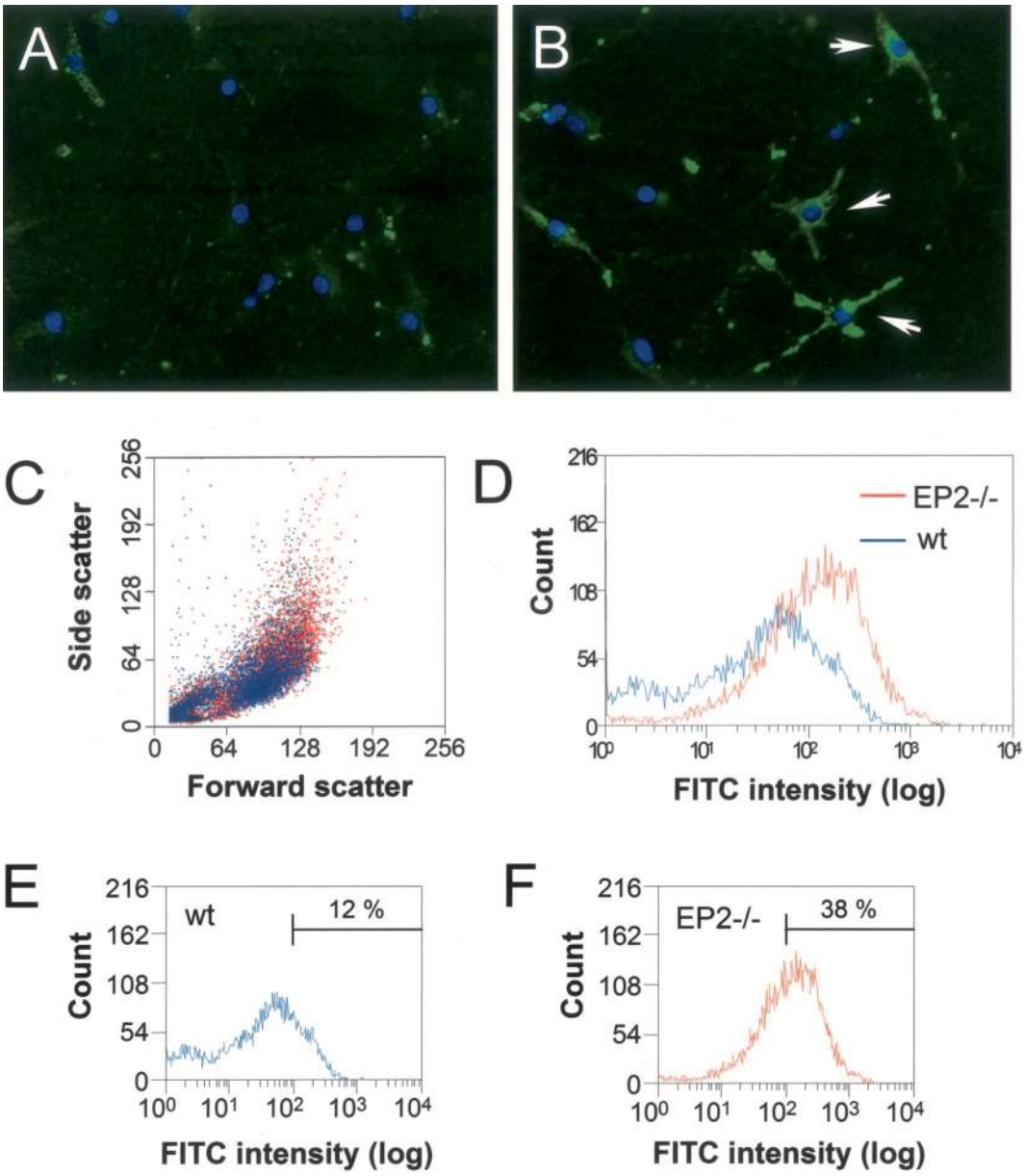

Figure 3. Microglial phagocytosis of aggregated fluorescein-labeled A$\beta_{1\text{-}42}$. Phagocytosis of A$\beta_{1\text{-}42}$ was quantified by flow cytometry; some microglia were cultivated in chamber slides for fluorescent analysis. Representative fluorescence images (green is fluorescein label, nuclei are blue) of primary microglia cultures from chamber slides with WT mice (**A**) or EP2−/− mice (**B**) showed an increased proportion of cells with enhanced fluorescence intensity in EP2−/− microglia compared to WT. Representative flow cytometric scatter plot (**C**) showed that granularity and size were not different between WT (blue) and EP2−/− (red) microglia. Flow cytometric histogram in log scale (**D**) showed a shift toward higher fluorescent intensity in EP2−/− microglia compared to WT. Defining high A$\beta_{1\text{-}42}$ fluorescent intensity as that greater than the peak value for EP2−/− microglia, we determined the percentage of the tested cells with high-fluorescent intensity between WT and EP2−/− microglia; representative histograms are shown in **E** and **F**. Original magnifications, ×200 (**A**, **B**).

ent between WT and EP2−/− microglia (Figure 3C). Flow cytometric data (Figure 3D) showed a shift toward higher fluorescent intensity in EP2−/− microglia compared to WT after incubation with fluorescein-labeled A$\beta_{1\text{-}42}$. Using a cutoff of the peak value for EP2−/− microglia, the percentage of tested cells with high-fluorescent intensity in WT (Figure 3E) or EP2−/− microglia (Figure 3F) was 12 ± 1% (n = 10) or 38 ± 1% (n = 10), respectively ($P <$ 0.001). However, there was overlap between microglia of the two genotypes at low A$\beta_{1\text{-}42}$ uptake (Figure 3D). These data support the hypothesis that a subpopulation of EP2−/− microglia has enhanced phagocytic properties for A$\beta_{1\text{-}42}$ and suggest that ablation of EP2−/− relieves an inhibitory mechanism thereby leading to a greater proportion of cells with heightened phagocytic activity for A$\beta_{1\text{-}42}$.

Signaling Pathways for Enhanced A$\beta_{1\text{-}42}$ Phagocytosis in EP2−/− Microglia

We next investigated the signaling events that underlie augmented A$\beta_{1\text{-}42}$ phagocytic activity in EP2−/− microglia using the same flow cytometric technique (Figure 4).

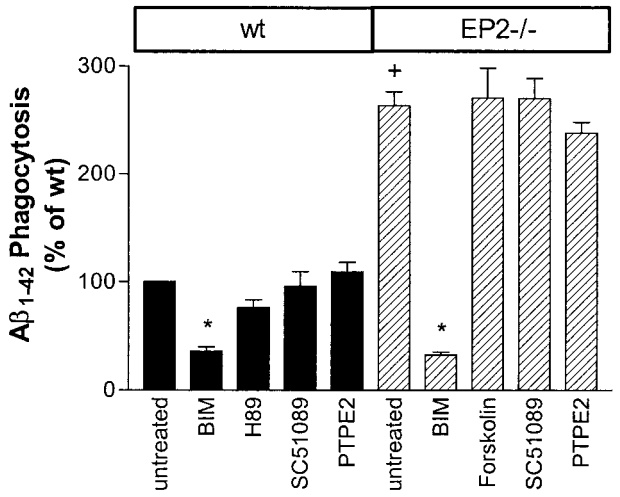

Figure 4. Signaling pathways for enhanced $A\beta_{1\text{-}42}$ phagocytosis in EP2−/− microglia. Fluorescein-labeled $A\beta_{1\text{-}42}$ phagocytosis was quantified by flow cytometry as above. Data from WT microglia are in **solid bars** and data from EP2−/− microglia are in **striped bars**. Basal $A\beta_{1\text{-}42}$ uptake in EP2−/− microglia ($n = 16$) was 263 ± 13% of WT microglia ($n = 7$; t-test, $^{+}P < 0.0001$). Drugs were used to test the involvement of specific signaling pathways. Analysis of variance for five treatment groups of WT microglia had $P < 0.0001$ with Bonferroni-corrected posttests having $P < 0.001$ for untreated versus BIM but $P > 0.05$ for all other comparisons to untreated WT microglia. Analysis of variance for five treatment groups of EP2−/− microglia had $P < 0.0001$ with Bonferroni-corrected posttests having $P < 0.001$ for untreated versus BIM but $P > 0.05$ for all other comparisons to untreated EP2−/− microglia.

Phagocytosis of $A\beta_{1\text{-}42}$ was 2.5- to 3-fold greater in EP2−/− microglia compared to WT ($P < 0.0001$). WT and EP2−/− microglia had 36 ± 4% ($P < 0.001$) and 35 ± 4% ($P < 0.001$) of WT basal $A\beta_{1\text{-}42}$ uptake when incubated with the PKC inhibitor BIM at 10 μmol/L, suggesting that all of the enhanced $A\beta_{1\text{-}42}$ phagocytosis observed in EP2−/− microglia and part of basal $A\beta_{1\text{-}42}$ phagocytosis in WT microglia are PKC-dependent. A PKA inhibitor (H89) was used in WT microglia and a PKA activator (forskolin) was used in EP2−/− microglia to determine whether this signaling pathway was contributing to the enhanced $A\beta_{1\text{-}42}$ uptake. H89 did not enhance $A\beta_{1\text{-}42}$ uptake in WT microglia and so did not replicate the EP2−/− phenotype, and forskolin did not reverse the enhanced $A\beta_{1\text{-}42}$ phagocytosis observed in EP2−/− microglia. We next investigated the effects of the relatively selective EP1 antagonist, SC51089 at 10 μmol/L, or EP1 agonist, PTPE2 at 20 μmol/L, on $A\beta_{1\text{-}42}$ phagocytosis by microglia. We found that both drugs were without effect in either WT or EP2−/− microglia, suggesting that EP1 activation or blockade does not significantly alter microglial $A\beta_{1\text{-}42}$ phagocytosis. Finally, butaprost, a relatively selective EP_2 agonist, did not alter $A\beta_{1\text{-}42}$ uptake in WT microglia (98 ± 3% at 1 μmol/L, 104 ± 2% at 10 μmol/L, and 92 ± 2% at 50 μmol/L; $n = 6$ for each condition), suggesting that stimulation of EP2 does not have the opposing effect of ablating EP2. These data indicated that basal Aβ uptake in WT microglia is EP2-independent and EP2-mediated suppression of microglial $A\beta_{1\text{-}42}$ phagocytosis is primarily, if not completely, PKC-dependent.

Basal Activation of EP2−/− Microglia

As seen in Figure 1, enhanced Aβ peptide phagocytosis by EP2−/− microglia in human tissue sections was associated with morphological features associated with activation. Although this may result from stimulation of microglia by tissue components, it also may result, at least in part, from constitutive activation of EP2−/− microglia; here we investigated the latter (Table 1). One feature of microglial activation is increased proliferation. We investigated this by counting primary microglia after 24 hours in culture after plating under identical conditions and observed no difference between WT and EP2−/− microglia. Another indicator of activation is cytokine and chemokine secretion. Conditioned culture medium was screened for secretion of 32 cytokines and chemokines using a membrane array that has the advantage of simultaneously measuring multiple molecules but the disadvantage of relative insensitivity. Only two chemokines, MCP-1 and MIP-1α, were sufficiently elevated in conditioned medium from EP2−/− microglial cultures to be detected by cytokine array, whereas WT microglia had no detectable cytokines or chemokines when simultaneously assayed (data not shown). Data from enzyme-linked immunosorbent assay confirmed that MCP-1 and MIP-1α were significantly greater in EP2−/− than WT-conditioned medium ($P < 0.001$) (Table 1). Macrophage colony-stimulating factor, reported to increase microglial phagocytosis of Aβ,[21] was not detected by our membrane array or by enzyme-linked immunosorbent assay (limit of detection of 5 pg/ml) of conditioned medium from WT or EP2−/− microglia. Thus, even without specific exogenous stimuli, EP2−/− microglia displayed some features of an activated phenotype with increased secretion of MCP-1 and MIP-1α compared to WT microglia, but not increased proliferation by cell count. Moreover, these

Table 1. Basal Activation of EP2−/− Microglia

	WT microglia ($n = 6$)	EP2−/− microglia ($n = 6$)
Cell number (serum)	$10 \pm 1 \times 10^4$	$10 \pm 1 \times 10^4$
Cell number (serum-free)	$8 \pm 1 \times 10^4$	$9 \pm 1 \times 10^4$
MIP-1α (pg/ml)	1089 ± 49	2227 ± 122*
MCP-1 (pg/ml)	633 ± 8	1112 ± 35*

Proliferation of primary cultures of mouse cerebral EP2−/− microglia were compared to WT microglia by counting microglia after 24 hours of incubation in medium either containing or free of serum supplement at initial cell number of 1×10^5; no significant difference between microglia from mice of different genotypes was observed ($P > 0.05$). Cytokine/chemokine array pointed to MIP-1α and MCP-1 as detectably increased in conditioned medium from primary EP2−/− microglia compared to WT; ELISA data for both chemokines are presented. Data are mean ± SEM for $n = 6$ in each condition (*, $P < 0.001$).

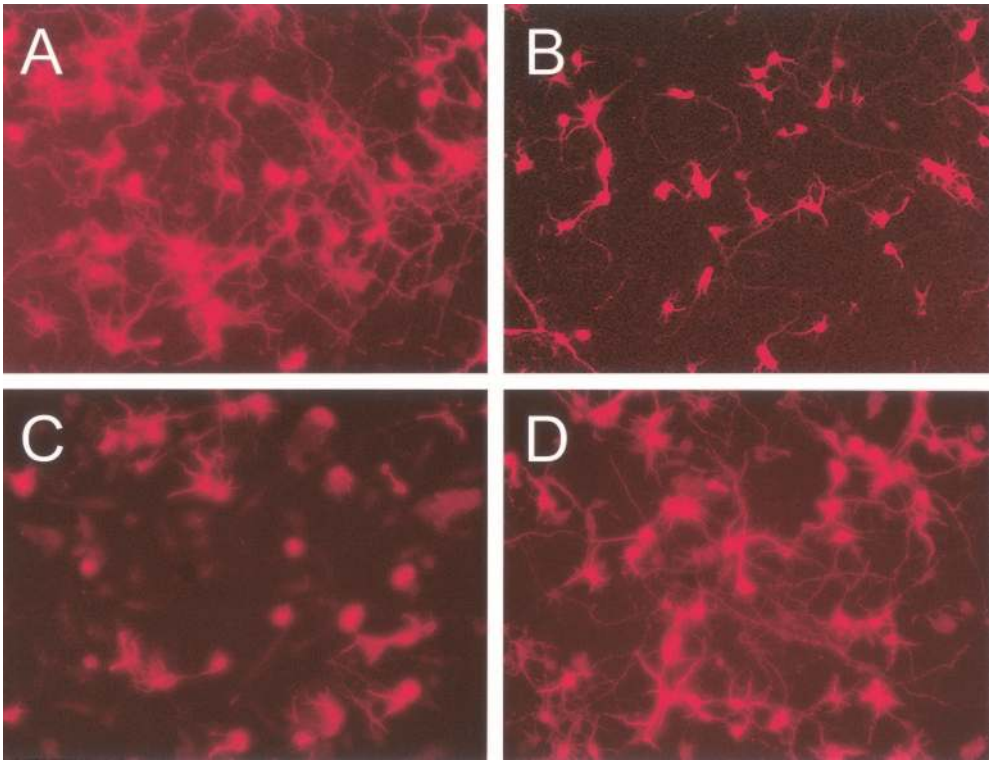

Figure 5. Morphological changes in primary neurons by Aβ toxicity. To test whether Aβ-activated EP2−/− microglia had enhanced paracrine neurotoxicity, co-cultures of primary WT neurons (DIV 6) and microglia (DIV 14) from either WT or EP2−/− mice were used for assessment of morphological changes in neurons using MAP-2 antibody. Results showed that neurons alone without treatment (**A**) displayed extensively branched dendrites, whereas exposure of neurons alone to 12 μmol/L of aggregated A$\beta_{1\text{-}42}$ (**B**) was associated with decreased dendrite length as assessed by MAP-2 IR; even greater A$\beta_{1\text{-}42}$-initiated dendritic atrophy was observed in cultures that combined neurons and WT microglia (**C**). Strikingly, cultures that contained neurons and EP2−/− microglia (**D**) showed no evidence of neurotoxicity by morphological assessment and were comparable to what was observed with neurons alone without A$\beta_{1\text{-}42}$ exposure. Original magnifications, ×200.

data indicate that at least some chemoattractant chemokines, namely MCP-1 and MIP-1α, are regulated by EP2 signaling.

Neuroprotection from Aβ Toxicity by EP2−/− Microglia

Although enhanced A$\beta_{1\text{-}42}$ phagocytosis and reduction of Aβ peptide burden in AD tissue seen with EP2−/− microglia is desirable, if it comes at the cost of increased activation with increased neurotoxicity, then it is not clear if this is a true benefit to patients with AD. To test whether Aβ-activated EP2−/− microglia had enhanced paracrine neurotoxicity, we used primary cultures of WT neurons and microglia from either WT or EP2−/− mice. The final dimethyl sulfoxide concentration in these experiments was 0.1% and was nontoxic to primary microglia or neurons. As expected from the results of others,[22–24] exposure of neurons alone to aggregated A$\beta_{1\text{-}42}$ was associated with dendrite atrophy (Figure 5B) and decreased neuron number as assessed by MAP-2 IR (Table 2). Even greater A$\beta_{1\text{-}42}$-initiated dendrite atrophy and neuron loss was observed in cultures that combined neurons and WT microglia (Figure 5C and Table 2). These results were confirmed in the remaining MAP-2 IR cells that showed increased TUNEL positivity after exposure to A$\beta_{1\text{-}42}$ and still further increased TUNEL positivity in MAP-2 IR cells in cultures containing neurons and WT microglia (Table 2). Strikingly, cultures that contained neurons and EP2−/− microglia showed no evidence of neurotoxicity

Table 2. Neuroprotection from Aggregated A$\beta_{1\text{-}42}$ by EP2−/− Microglia

Neurons	Microglia	MAP-2-IR cells (%, n = 15 each)	TUNEL and MAP-2-IR cells (%, n = 15 each)
WT	none	67 ± 5*	196 ± 11*
WT	WT	56 ± 4*†	259 ± 12*†
WT	EP2−/−	102 ± 4	59 ± 5‡

Primary neuronal cultures (DIV 6) with or without primary microglia (DIV 14) were exposed to 12 μmol/L of aggregated A$\beta_{1\text{-}42}$ for 24 hours. Cultures were then fixed and the total number of MAP-2-IR cells and the remaining number of MAP-2 IR cells that were TUNEL-positive were determined. There was no difference among the number of MAP-2 IR cells in neurons only, neuron plus WT microglia, and neuron plus EP2−/− microglia cultures when exposed to vehicle rather than A$\beta_{1\text{-}42}$ (not shown). Data were presented as percent of A$\beta_{1\text{-}42}$-nonexposed neuron-only cultures.

*$P < 0.001$ compared to corresponding culture not treated with A$\beta_{1\text{-}42}$.

†$P < 0.05$ compared to neuron-only cultures exposed to A$\beta_{1\text{-}42}$.

‡$P < 0.05$ compared to corresponding culture not treated with A$\beta_{1\text{-}42}$.

by either of these endpoints or morphological assessment (Table 2 and Figure 5D). Indeed, by one measure, TUNEL positivity, co-culture with EP2−/− microglia was associated with apparent neurotrophism. The lack of $A\beta_{1\text{-}42}$-mediated neurotoxicity in EP2−/− microglia/WT neuron co-cultures strongly suggests that, although $A\beta_{1\text{-}42}$ can be directly toxic to isolated neurons in culture, when combined with microglia the major neurotoxins are paracrine factors secreted by $A\beta_{1\text{-}42}$-activated microglia. WT microglial activation by Aβ yielded the expected large increase in COX-2 and iNOS as determined by Western blot; primary and secondary products of both enzymes can cause neurotoxicity.[25–27] Activation of EP2−/− microglia under identical conditions resulted in COX-2 and iNOS induction that were 16 ± 1% and 43 ± 4%, respectively, of WT microglia ($n = 3$ for each, $P < 0.01$ for both). Although the mechanisms by which microglia may damage neurons are more complex than simply COX-2 and iNOS products, these data indicate at least two mechanisms by which Aβ-activated EP2−/− microglia may be less neurotoxic.

Discussion

Microglia have been proposed to play both neuroprotective and neurotoxic roles in AD pathogenesis. On one hand, phagocytosis of Aβ peptides by activated microglia is thought to be neuroprotective by limiting exposure of neurons to toxic forms of these proteins. On the other hand, exposure of microglia to Aβ peptides initiates an innate immune response with secretion of several neurotoxic factors. Which of these processes is dominant at different points in AD pathogenesis remains obscure; however, it is clear that identifying therapeutic targets that enhance microglial Aβ phagocytosis while also suppressing microglia-mediated neurotoxicity would be a major advance. Our data show that microglial EP2 is such a therapeutic target and demonstrate that it is possible to separate microglial activation with its associated neurotoxicity from enhanced Aβ phagocytosis.

As a justification for Aβ vaccination trials, others have shown that culturing WT rodent or human microglia on human AD brain sections that had been incubated with anti-Aβ antibodies results in clearance of Aβ from tissue and appearance of Aβ-IR material in intracellular phagosomes. Presumably this anti-Aβ antibody-enhanced microglial phagocytosis of tissue Aβ is because of opsonization and Fc receptor-dependent phagocytosis in *ex vivo* systems,[28–30] although this has been challenged as the mechanism of action of Aβ vaccination *in vivo* in which non-Fc receptor-dependent phagocytosis is also involved.[20,31] It is important to note that without incubation of tissue with anti-Aβ antibodies and presumed opsonization of tissue Aβ, WT microglia do not phagocytose Aβ or clear Aβ from human tissue sections *ex vivo*.[29] To our knowledge, ours is the first demonstration of genetically altered microglia achieving similar Aβ clearance from human tissue sections without involvement of antibody binding as anti-Aβ opsonization followed by WT microglia reported by others.

Recently, others have shown that EP2 on rodent alveolar macrophages suppresses Fc receptor-mediated phagocytosis of bacterial pathogens.[14] Importantly, EP2-mediated suppression of Fc receptor-mediated phagocytosis by alveolar macrophages was cAMP-dependent and the suppressed phagocytic phenotype was restored in EP2−/− macrophages by treatment with forskolin; the participation of EP2 in Fc receptor-independent macrophage phagocytosis is not yet reported. Similar to these investigators, we also observed an EP2-dependent suppressive effect on microglial Aβ phagocytosis; however, this was dependent on PKC activation and was not modified by drugs that activate or inhibit adenylate cyclase. Importantly, we observed that inhibition of PKC in EP2−/− microglia restored the suppressed phagocytic phenotype in EP2−/− microglia to WT levels, strongly suggesting that postreceptor signaling cascades were intact in EP2−/− microglia. Because Aβ phagocytosis is thought to be, at least in part, opsonin-independent and mediated by a group of receptors including formyl peptide receptor, scavenger receptors, and receptor for advanced glycation end-products,[19] these data raise the possibility that, in contrast to Fc receptor-dependent phagocytosis, EP2-mediated suppression of Fc receptor-independent phagocytosis may be mediated by PKC-dependent mechanisms. The exact effectors responsible for this novel mechanism underlying negative regulation of Aβ phagocytosis through EP2 signaling are not entirely clear; however, the mechanism is likely related, at least in part, to increased secretion of prophagocytic β chemokines, such as MIP-1α and MCP-1. Because β chemokine signaling during inflammation requires PKC activity,[32,33] one possibility is that downstream effectors of EP2 activation suppress phagocytosis by inhibiting β chemokine signaling, activity of PKC isoforms, or both.

If enhanced Aβ phagocytosis resulted from generalized activation of microglia with correspondingly enhanced innate immune response and increased bystander damage to neurons, then the utility of microglial EP2 as a therapeutic target would be unclear. Previously, we have observed in a murine model of activated cerebral innate immune response from intracerebroventricular injection of lipopolysaccharide that neuronal oxidative damage is completely suppressed in EP2−/− mice;[34] however, interpretation of these data are confounded by microglial and neuronal expression of EP2. Others have shown that peroxisome proliferator-activated receptor γ (PPARγ) agonists can suppress EP2 expression in lung carcinoma cell lines[35] and that PPARγ agonists also suppress Aβ-stimulated microglial paracrine neurotoxicity.[36] Therefore, EP2 may be critical in microglia-mediated neurotoxicity. Here we specifically determined that ablation of microglial EP2 only, without any genetic alteration to neurons, completely suppressed $A\beta_{1\text{-}42}$-mediated neurotoxicity. Moreover, as suggested by results from the TUNEL assay, EP2−/− microglia may even be mildly neurotrophic. Thus, EP2−/− microglia possess the highly desirable complementary traits of enhanced Aβ phagocytosis without enhanced bystander damage to neurons. Given the complications of Aβ vaccination,[37] these results point to suppression of microglial EP2 as a perhaps safer but similarly efficacious means of reducing Aβ burden in human brain.

In conclusion, our study provides new insights into the central role of EP2 in regulation of microglial Aβ phagocytosis and Aβ-stimulated microglia-mediated paracrine neurotoxicity. Critically, we showed that ablation of microglial EP2 achieved the highly desirable combination of enhancing Aβ phagocytosis while at the same time completely suppressing bystander damage to neurons. If these results can be extended to human microglia, then these data indicate blockade of microglial EP2 signaling as a means of maximizing beneficial while minimizing deleterious effects of microglia in AD brain.

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