The American Journal of Pathology, Vol. 177, No. 1, July 2010

DOI: 10.2353/ajpath.2010.090840

Neurobiology

Suppressed Accumulation of Cerebral Amyloid β Peptides in Aged Transgenic Alzheimer's Disease Mice by Transplantation with Wild-Type or Prostaglandin E_2 Receptor Subtype 2-Null Bone Marrow

C. Dirk Keene,* Rubens C. Chang,* Americo H. Lopez-Yglesias,* Bryan R. Shalloway,* Izabella Sokal,* Xianwu Li,* Patrick J. Reed,* Lisa M. Keene,* Kathleen S. Montine,* Richard M. Breyer,† Jason K. Rockhill,‡ and Thomas J. Montine*

From the Departments of Pathology, and Radiation Oncology,‡ University of Washington, Seattle, Washington; and the Vanderbilt University Medical Center,† Nashville, Tennessee*

A complex therapeutic challenge for Alzheimer's disease (AD) is minimizing deleterious aspects of microglial activation while maximizing beneficial actions, including phagocytosis/clearance of amyloid β (Aβ) peptides. One potential target is selective suppression of microglial prostaglandin E_2 receptor subtype 2 (EP2) function, which influences microglial phagocytosis and elaboration of neurotoxic cytokines. To test this hypothesis, we transplanted bone marrow cells derived from wild-type mice or mice homozygous deficient for EP2 ($EP2^{-/-}$) into lethally irradiated 5-month-old wild-type or APP*swe*-PS1ΔE9 double transgenic AD mouse model recipients. We found that cerebral engraftment by bone marrow transplant (BMT)-derived wild-type or $EP2^{-/-}$ microglia was more efficient in APP*swe*-PS1ΔE9 than in wild-type mice, and APP*swe*-PS1ΔE9 mice that received $EP2^{-/-}$ BMT had increased cortical microglia compared with APP*swe*-PS1ΔE9 mice that received wild-type BMT. We found that myeloablative irradiation followed by bone marrow transplant-derived microglia engraftment, rather than cranial irradiation or BMT alone, was responsible for the approximate one-third reduction in both Aβ plaques and potentially more neurotoxic soluble Aβ species. An additional 25% reduction in cerebral cortical Aβ burden was achieved in mice that received $EP2^{-/-}$ BMT compared with mice that received wild-type BMT. Our results provide a foundation for an adult stem cell-based therapy to suppress soluble Aβ peptide and plaque accumulation in the cerebrum of patients with AD. ***(Am J Pathol 2010, 177:346–354; DOI: 10.2353/ajpath.2010.090840)***

Alzheimer's disease (AD), the most common dementing neurodegenerative disease,[1] is a major public health burden for older Americans.[2] Amyloid β (Aβ) peptides are pleotropic molecules that are directly neurotoxic and stimulate liberation of cytotoxic cytokines through activation of microglia innate immune response.[3] However, activated microglia phagocytosis and degradation of Aβ species is key to cerebral Aβ homeostasis.[4] Thus, an important but complex therapeutic challenge is balancing deleterious and beneficial aspects of microglial activation in AD.[5] One proposed mechanism of microglial modulation is prostaglandin E_2 signaling, especially through activation of the E prostanoid receptor subtype 2 (EP2).[6] Cultured microglia lacking EP2 ($EP2^{-/-}$) show enhanced phagocytosis of Aβ from human brain explants and reduced paracrine neurotoxicity.[7] *In vivo* experiments with $EP2^{-/-}$ mice have shown reduced accumulation of cerebral Aβ in a transgenic mouse model of AD,[7–9] as well as suppressed oxidative damage to neurons following innate immune activation.[7,10–12] However,

Supported by NIH grants P50AG05136, T32AG000258, R01AG024011, and the Nancy and Buster Alvord Endowed Chair in Neuropathology. EP2 null mice were generated with support from GM015431 (R.M.B.).

Accepted for publication March 15, 2010.

Supplemental material for this article can be found on *http://ajp.amjpathol.org*.

Address reprint requests to C. Dirk Keene, M.D., Ph.D., Harborview Medical Center, Box 359645, 325 Ninth Ave, Seattle, WA 98104. E-mail: cdkeene@u.washington.edu.

because EP2 is expressed by several cell types in brain, including microglia and neurons, the importance of microglial-specific EP2 has not been established. To address this gap in our knowledge, bone marrow cells from EP2$^{-/-}$ mice were transplanted into APP*swe*-PS1ΔE9 mice.

Circulating bone marrow transplant (BMT)-derived cells can selectively replace resident microglia,[13] and up to 30% of microglia can be derived from donor marrow in wild-type mice recipients up to a year after transplantation.[14,15] Moreover, engraftment of brain appears qualitatively more efficient in recipient AD mice than in wild-type controls.[16,17] The reasons for the apparent higher engraftment are not clear, but may be in response to chronic low level immune activation in AD mouse brains.[16,17] Some investigators have shown BMT-derived microglia associated with Aβ deposits *in vivo*, and that transgenic AD mouse BMT recipients have reduced Aβ plaque burden.[17] Although previous data addressed potential mechanisms by which BMT-derived microglia might promote clearance of Aβ peptides,[18] the results of these studies were confounded by the effects of preconditioning brain irradiation; it is possible that the reduced Aβ plaque burden was caused by irradiation-induced alteration of Aβ production or clearance rather than BMT-derived microglia. In the current studies, we robustly quantify microglial engraftment in brains of APP*swe*-PS1ΔE9 mice. In addition, we control for the potential confounder of irradiation-mediated Aβ peptide suppression by evaluating Aβ in mice that received cranial-specific irradiation with or without BMT. Finally, we test the hypothesis that BMT with cells from EP2$^{-/-}$ mice would enhance cerebral bone marrow derived microglia engraftment and clearance of Aβ peptides from cerebrum of APP*swe*-PS1ΔE9 mice.

Materials and Methods

Animals

All protocols were approved by the University of Washington Institutional Animal Care and Use Committee. All mice were obtained from Jackson Laboratories (Bar Harbor, ME). Bone marrow transplant recipient mice were female B6C3F1/J and either hemizygous APP*swe*-PS1ΔE9 double transgenic mice or their nontransgenic littermates as wild-type controls. Bone marrow donor mice were male C57BL/6 mice hemizygous for green fluorescent protein (GFP) and either homozygous deficient in prostaglandin E_2 receptor EP2 (EP2 knockout)[19] or wild-type controls. Green fluorescent protein expression is under control of the β-actin promoter and cytomegalovirus enhancer. The APP*swe* transgene encodes a mouse-human hybrid transgene containing the mouse sequence in the extracellular and intracellular regions, and a human sequence within the Aβ domain with Swedish mutations K594N and M595L. The PS1ΔE9 transgene encodes exon-9-deleted human presenilin-1. Both transgenes are coexpressed under control of the mouse prion promoter with plaque deposition beginning at 5 to 6 months of age.[20,21]

Bone Marrow Harvest and Transplantation

BMT followed an established protocol.[22] Briefly, recipient female mice were either lethally irradiated (11 Gy single dose at 2 Gy per minute to whole body from Cesium-137 source [JL Shepherd, Model 81-14, San Fernando, CA]) or had the same radiation dose applied to the head only with 5 cm of lead shielding (equivalent to 7.7 half-value layers = less than 0.5% of the total dose) to the neck, body, and tail 24 hours before an IV (retroorbital sinus) infusion of unfractionated bone marrow cells (5×10^6/mouse) obtained aseptically from femurs of 10-week-old male GFP-expressing EP2$^{-/-}$ or wild-type mice. Bone marrow cells were flushed from femurs and tibias and pooled with R10 media (RPMI with 10% fetal bovine serum), passed through a 25-gauge needle, filtered through a 70-μm nylon mesh, centrifuged, and erythrocytes lysed with ammonium chloride potassium lysing buffer. Cells were washed and resuspended in sterile PBS to a concentration of 5×10^6 nucleated cells per 200 μl. All unshielded mice that received lethal levels of irradiation received transplants. Transplanted mice were housed in autoclaved cages in a specific pathogen free facility.

In the first study, recipient mice were anesthetized 6 months after transplantation, splenectomy was performed, and then mice were perfused transcardially with ice-cold PBS. After perfusion, the brain was removed rapidly, divided by mid-sagittal section, and the right cerebral hemisphere was immersed in 4% paraformaldehyde. The left half of the brain was dissected rapidly into cerebral cortex, hippocampus, and rhombencephalon, flash frozen in liquid nitrogen, and stored at −80°C. In the second study, recipient mice were anesthetized 8 months after transplantation, the spleens removed, and the mice were transcardially perfused with ice-cold 4% paraformaldehyde. The whole brain was harvested and postfixed in 4% paraformaldehyde at 4°C. Fixed brain tissue from both studies was frozen in OCT by immersion in isopentane cooled in liquid nitrogen, and then stored at −80°C. All assays were performed by operators blinded to experimental conditions.

Peripheral Engraftment

Fresh spleens were processed for flow cytometry to assess hematopoietic engraftment following established methods.[23] Cells were resuspended at 2 to 3×10^6/ml, and 200 μl of cell suspension was incubated in a 96-well plate with specific antibodies for 20 minutes at room temperature in the dark. Fluorochrome-conjugated monoclonal antibodies include markers for B cells (B220), T cells (CD3), and macrophages (F480). Fcγ antibody was added before staining with specific antibodies to block nonspecific binding. Stained cells were washed with Fluorescence Activated Cell Sorting (FACS) buffer (PBS with 0.2% bovine serum albumin and 0.1% NaN3) three times before analysis on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Table 1. Whole Body Irradiation Preconditioning Is Required for Peripheral Engraftment of Donor Bone Marrow

Recipient genotype	Treatment (Radiation/BMT)	% of total cells derived from spleen			
		GFP$^+$ cells	GFP$^+$ T cells	GFP$^+$ B cells	GFP$^+$ macrophages
GFP	No XRT/No BMT	97.5 ± 1.0	33.3 ± 1.0	53.6 ± 0.6	2.4 ± 0.2
APP*swe*-PS1ΔE9	No XRT/No BMT	0.6 ± 0.2	0.8 ± 0.4	0.9 ± 0.6	0.7 ± 0.7
	HO XRT/GFP BMT	0.7 ± 0.5	1.1 ± 0.7	0.7 ± 0.6	0.6 ± 0.3
	HO XRT/No BMT	0.7 ± 0.5	0.8 ± 0.5	0.7 ± 0.5	0.7 ± 0.4
	WB XRT/GFP BMT	85.7 ± 10.7	27.9 ± 11.2	43.9 ± 1.8	1.6 ± 0.2

Flow cytometric analysis of splenic engraftment in APP*swe*-PS1ΔE9 mice. Data are average ± SD of four to six mice in each group. All mice were harvested at 11 months of age. At 5 months of age, recipient mice were untreated (No XRT, No BMT), received head only (HO) irradiation (XRT) followed by wild-type/GFP BMT (HO XRT/GFP BMT), received head only XRT without BMT (HO XRT/No BMT), or received whole body (WB) XRT followed by wild-type/GFP BMT (WB XRT/GFP BMT). WB XRT without BMT is lethal and was not performed. Age-matched GFP-heterozygous mice without radiation or BMT were used as controls (No XRT/No BMT). Total GFP positive cells and GFP positive T lymphocytes (CD3), B lymphocytes (B220), and macrophages (F480) were quantified.

Immunofluorescence and Central Nervous System Engraftment

Coronal sections from paraformaldehyde-fixed brain tissue were cut at 40 μm on a cryostat. Every sixth section was used for each immunostaining paradigm. Immunofluorescence staining was performed according to previously published protocols.[24] Primary antibodies included anti-Iba-1 (Wako, Richmond, VA; 1:500), anti-Aβ peptides (10D5; 1-28, Athena Neurosciences, San Francisco, CA; 1:1000), and anti NeuN (Invitrogen, Carlsbad, CA; 1:200); species-appropriate secondary antibodies were conjugated to Cy3 (1:400) and Cy5 (1:200; Jackson Immunoresearch, West Grove, PA). Prolong-Gold Anti-Fade with 4′,6-diaminido-2-phenylindole (DAPI) (Invitrogen) was used for coverslipping and nuclear counterstain.

To quantify Iba-1-immunopositive microglia and bone marrow transplant-derived cells (GFP), sections were analyzed by using systematic random sampling.[25] Every sixth brain section (240 μm apart) was analyzed at 400× magnification by using a Nikon inverted fluorescence microscope (Mellville, NY) and StereoInvestigator software (MicroBrightField, Williston, VT). A fractionator was used with a counting frame measuring 150 μm × 150 μm applied every 500 μm in bilateral hippocampi or unilateral cortex. Cells were assessed as Iba-1-positive, GFP-positive, or double labeled. Nonirradiated, nontransplanted age matched wild-type, and APP*swe*-PS1ΔE9 mice were used as nontreated controls.

Volume estimates of the hippocampal pyramidal cell layer and dentate gyrus stained with NeuN were determined by using every sixth coronal section through the entire hippocampus by using the Cavalieri principle and StereoInvestigator software (MicroBrightField).[26,27]

Immunohistochemistry and Plaque Assessment

Sections (40 μm) through hippocampus were cut on a cryostat, and every sixth section (average 14 per mouse) was processed for immunohistochemistry with rabbit polyclonal anti-Aβ (Invitrogen, number 44136) exactly according to our previously published protocol.[28] Stained sections were scanned (Nikon Super Coolscan 4000 ED) and the images were analyzed separately for cerebral cortex and hippocampus by using Image J software (NIH, Bethesda, MD). The percent area occupied by Aβ-immunoreactive (IR) plaques was averaged over all sections for each mouse, and averaged values from each mouse were used in statistical analyses.

Soluble Aβ Quantitation

Quantification of detergent soluble (DS) $A\beta_{40}$ and $A\beta_{42}$ followed published procedures from our lab.[29,30] Luminex assays for soluble Aβ peptides were performed on material solubilized in 10 mmol/L Tris, 1 mmol/L ethylene glycol bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid, 1 mmol/L dithiothreitol, and 10% sucrose, pH 7.5, plus 1% Triton X-100 using reagents from Biosource (Camarillo, CA) according to the manufacturer's instructions. Standards were provided by the manufacturer, and standard curves were generated from 30 to 3000 pg/ml Aβ species with a detection limit of 30 pg/ml.

Results

Our first series of experiments was performed to confirm reduction of Aβ plaque burden in cerebrum from APP*swe*-PS1ΔE9 mice transplanted with wild-type bone marrow, as has been observed by others,[17] and to determine whether cranial irradiation (XRT) was sufficient for the observed effects. Table 1 presents results from spleen engraftment for four groups of APP*swe*-PS1ΔE9 mice transplanted at 5 months and sacrificed at 11 months of age. Mice received the following treatments: no irradiation, no BMT (No Tx); 11 Gy whole body (WB) irradiation followed by wild-type/GFP BMT (WB XRT/BMT); 11 Gy head only (HO) XRT followed by wild-type/GFP BMT (HO XRT/BMT); and 11 Gy HO XRT without BMT (HO XRT). WB XRT without BMT is lethal and was therefore not performed. There were expected levels of spleen engraftment in the WB XRT/BMT group, but, as expected, no significant engraftment of spleen in the HO XRT/BMT group, because only the head was irradiated. Six months after irradiation, fur in unshielded areas had turned gray irrespective of spleen engraftment, confirming extent of radiation in the respective groups (Figure 1A).

Figure 1B shows results for Aβ-immunoreactive plaque burden in cerebral cortex and hippocampus. Figure 1C

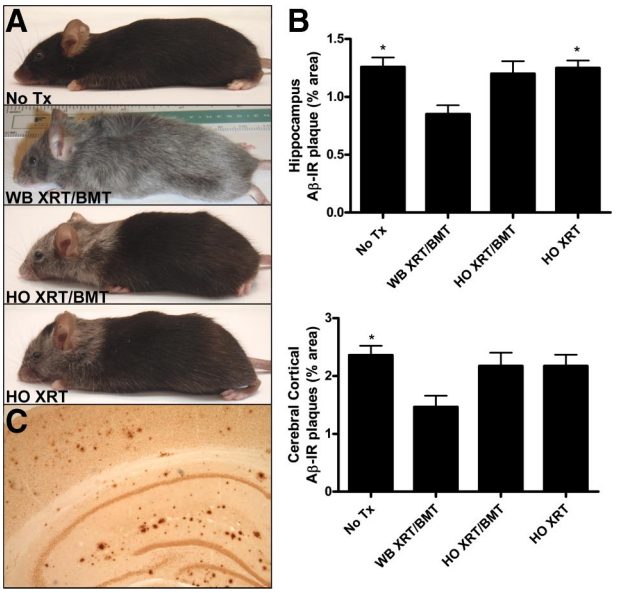

Figure 1. Cerebral irradiation is not sufficient for suppression of Aβ plaque formation. Data are from the same groups of mice. Eleven-month-old APP*swe*-PS1ΔE9 mice received whole body irradiation with bone marrow transplant (WB XRT/BMT), head only irradiation with bone marrow transplant (HO XRT/BMT), or head only irradiation without bone marrow transplant (HO XRT). Control APP*swe*-PS1ΔE9 mice received no irradiation and no BMT (No Tx). Bone marrow was isolated from wild-type/GFP donors at five months of age. **A:** Coat color demonstrates radiation exposure distribution. **B:** Average (±SEM) percent area of Aβ-IR plaques were determined for hippocampus (analysis of variance $P < 0.01$) and cerebral cortex (analysis of variance $P = 0.01$). Bonferroni-corrected post tests had $^{*}P < 0.05$ when compared with WB XRT/BMT. **C:** Photomicrograph shows a representative partial section of the scanned image from hippocampus and overlying cerebral cortex from a No Tx mouse after immunohistochemical staining for Aβ peptides (Supplemental Figure S1, see *http://ajp.amjpathol.org* for representative sections from the other three groups).

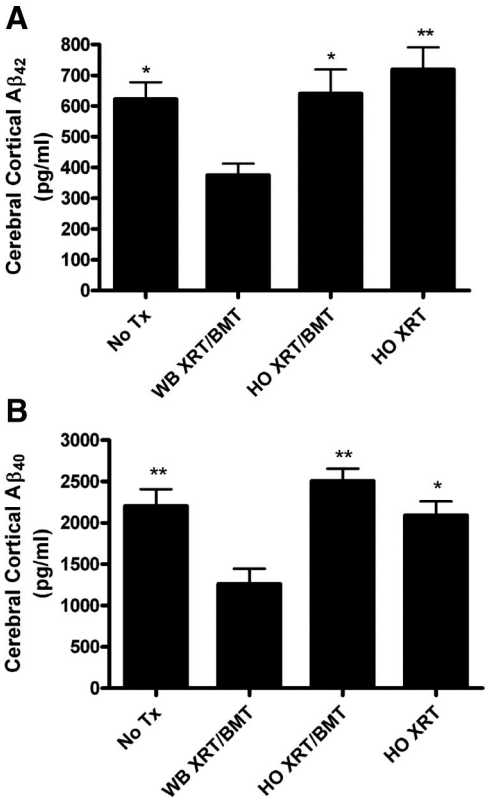

Figure 2. Soluble Aβ peptides in cerebral cortex are reduced after BMT. Data are concentrations of Triton X-100 extractable $A\beta_{42}$ (**A**) or $A\beta_{40}$ (**B**) peptides in cerebral cortex extracts of APP*swe*-PS1ΔE9 mice at 11 months of age. Mice were treated as described in Figure 1. Data are the average ± SEM pg/ml from four to six mice in each group: no irradiation or BMT (No Tx), whole body (WB) irradiation followed by bone marrow transplant from wild-type/GFP donor (WB XRT/BMT), head only (HO) XRT followed by BMT from wild-type/GFP donor (HO XRT/BMT), and head only XRT with no BMT (HO XRT). One-way analysis of variance for cerebral cortical $A\beta_{42}$ or $A\beta_{40}$ each had $P < 0.01$. Bonferroni-corrected post tests had $^{*}P < 0.05$ or $^{**}P < 0.01$ when compared with WB XRT/BMT.

shows a representative partial section of hippocampus and overlying cerebral cortex from a No Tx mouse after immunohistochemical staining for Aβ peptides (Supplemental Figure S1, see *http://ajp.amjpathol.org* for representative sections from the other three groups). A reduction of approximately one-third in the burden of Aβ-IR plaque was observed in both cerebral cortex ($P < 0.05$) and hippocampus ($P < 0.05$) of APP*swe*-PS1ΔE9 mice in the WB XRT/BMT as compared with nonirradiated, nontransplanted (No Tx) mice. Importantly, we also observed significantly lower Aβ-IR plaque burden in cerebral cortex and hippocampus when comparing WB XRT/BMT with either HO XRT/BMT ($P < 0.05$ for hippocampus and $P < 0.05$ for cerebral cortex) or HO XRT groups ($P < 0.05$ for hippocampus and $P < 0.05$ for cerebral cortex); however, neither of the HO XRT groups was significantly different from the No Tx group in either brain region. Aβ-IR plaques are mixtures of detergent-insoluble fibrillar Aβ species and DS Aβ species. Although previous work has concentrated on the former, more recent data has focused attention on DS Aβ species as containing more neurotoxic forms of these peptides. To determine the effect of BMT on DS Aβ species, we quantified the concentration of $A\beta_{40}$ and $A\beta_{42}$ in Triton X-100 extracts of hippocampus and cerebral cortex in the four groups shown in Figure 1. Although we did not observe significant differences in soluble $A\beta_{40}$ or $A\beta_{42}$ levels in hippocampus (one-way analysis of variance for $A\beta_{40}$ or $A\beta_{42}$ each had $P \geq 0.05$; $n \geq 4$ for each group), there were significant differences in cerebral cortex (Figure 2, A and B). Specifically, soluble $A\beta_{40}$ and $A\beta_{42}$ were significantly reduced by approximately one-third to one-half in WB XRT/BMT mice compared with No Tx mice ($P < 0.05$ for $A\beta_{42}$ and $P < 0.01$ for $A\beta_{40}$). Both HO XRT groups had significantly greater levels of soluble Aβ isoforms than WB XRT/BMT but did not differ from control APP*swe*-PS1ΔE9 (No Tx) mice. These morphometric and biochemical data show that Aβ peptide accumulation in aged APP*swe*-PS1ΔE9 mice can be suppressed by BMT at 5 months of age in mice with whole body irradiation. Although we cannot fully exclude an independent effect of WB XRT in the observed reductions in Aβ levels and plaques, our results demonstrate that irradiation confined to the brain is not sufficient to reduce the accumulation of Aβ peptides or plaques in aged APP*swe*-PS1ΔE9 mouse cerebrum.

We explored the possibility of radiation-induced parenchymal damage further by determining the volume of hippocampal pyramidal layer and dentate gyrus, reasoning that this would capture neuron loss as well as atrophy. Our volume (mm^3) estimates for the hippocampal pyramidal layer were 0.75 ± 0.05 for No Tx, 0.66 ± 0.03 for

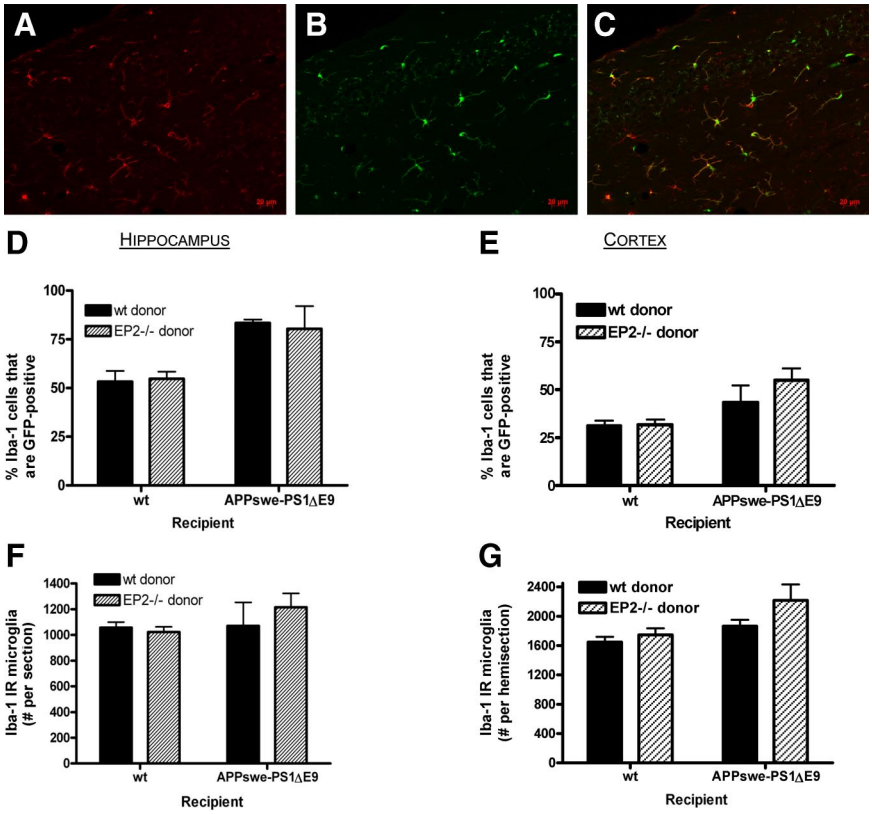

Figure 3. Mouse hippocampus and cortex engraftment by BMT-derived microglia. Wild-type ($n = 10$) or APP*swe*-PS1ΔE9 mice ($n \geq 4$) received whole body irradiation followed by BMT from wild-type/GFP or EP2$^{-/-}$/GFP mice at five months of age and then were sacrificed at 13 months of age. Mice were perfusion-fixed and then sections probed with anti-Iba-1 primary antibody and visualized with Cy3-conjugated secondary antibody. **A–C:** Photomicrographs (×200) of representative CA1 field of hippocampal pyramidal layer in an APP*swe*-PS1ΔE9 mouse that received wild-type/GFP BMT and stained for Iba-1 (red; **A**), GFP fluorescence (green, **B**), and co-localization of GFP and Iba-1 (merged, **C**). **D** and **E:** Data are the mean (+SEM) percent of those Iba-1 IR cells that were also GFP-positive. Two-way analysis of variance had $P < 0.01$ for genotype of the recipient mouse, $P > 0.05$ for genotype of the donor mouse, and no significant interaction for both hippocampus (**D**) and cortex (**E**). **F** and **G:** Data are total Iba-1 positive cells per section in wild-type and APP*swe*-PS1ΔE9 recipient mice after wild-type or EP2$^{-/-}$ BMT. There was no statistically significant difference in Iba-1 IR microglia for recipient or donor genotype comparisons for hippocampus (**F**), but two way analysis of variance had $P < 0.05$ for host genotype and $P = 0.05$ for donor genotype with no significant interaction comparisons of total cortical Iba-1 IR microglia (**G**).

WB XRT/BMT, 0.75 ± 0.04 for HO XRT/BMT, and 0.66 ± 0.03 for HO XRT ($P > 0.05$ by analysis of variance). Our volume (mm^3) estimates for hippocampal dentate gyrus were 0.41 ± 0.02 for No Tx, 0.35 ± 0.03 for WB XRT/BMT, 0.38 ± 0.05 for HO XRT/BMT, and 0.36 ± 0.02 for HO XRT ($P > 0.05$ by analysis of variance). Thus, we found no differences in the volume of hippocampal dentate gyrus and pyramidal cell layers between groups.

Given the above results, our second series of experiments focused on WB XRT/BMT. Our first goal was to determine the level of brain engraftment using wild-type donor marrow under the conditions that led to reduced cerebral burden of Aβ peptides and plaques. In this series, mice received a lethal dose of whole body irradiation followed by transplantation with wild-type bone marrow at 5 months of age, and were then sacrificed 8 months later (13 months of age). Engraftment of spleen in these mice was not significantly different from the data presented in Table 1 (not shown). Next we examined BMT-derived microglial engraftment in hippocampus and cortex by assessing the percentage of double labeled Iba-1/GFP positive cells compared with the total number of Iba-1 immunoreactive cells (Figure 3; A–C show representative photomicrographs). In wild-type recipients of wild-type BMT, ~50% of hippocampal and ~30% of cortical Iba-1 positive microglia were BMT derived (GFP positive), and this was significantly increased to ~80% and ~50% engraftment in APP*swe*-PS1ΔE9 host hippocampus (Figure 3D) and cortex (Figure 3E), respectively ($P < 0.05$). Although the total number of hippocampal Iba-1 IR microglia (Figure 3F) was not significantly increased in APP*swe*-PS1ΔE9 versus wild-type, significantly more cortical Iba-1 positive microglia (Figure 3G) were identified in APP*swe*-PS1ΔE9 host mice compared with wild-type ($P < 0.05$). There was no significant change in the total number of Iba-1 IR cells between APP*swe*-PS1ΔE9 mice that did or did not receive irradiation and BMT (not shown). Greater than 96% of all GFP-positive cells were Iba-1 IR. No GFP-positive cells co-localized with NeuN, a marker for mature neurons (not shown). As observed by many others, using filters for GFP, no fluorescent cells were identified in any brain sections from mice that did not receive BMT (not shown). Our results are broadly consistent with those of other investigators who have evaluated mouse brain after long-term (>6 months) peripheral engraftment from BMT after whole body irradiation.[15] Interestingly, APP*swe*-PS1ΔE9 mice showed more efficient cortical and hippocampal engraftment than wild-type recipients. Other authors have reported recently that engraftment of APP*swe*-PS1ΔE9 mice by wild-type/GFP BMT is about 1.4 times the engraftment in wild-type mice.[16,17] Our results confirmed this finding and showed that, under the conditions used in our experiment, ~80% of resident microglia in APP*swe*-PS1ΔE9 hippocampus and ~50% in APP*swe*-PS1ΔE9 cortex can be replaced with BMT-derived cells. The mechanism for increased engraftment of hippocampus and cortex in APP*swe*-PS1ΔE9 mice versus wild-type is unknown but thought to derive from inflammatory molecules secreted by brain in this chronic disease model.[16,17]

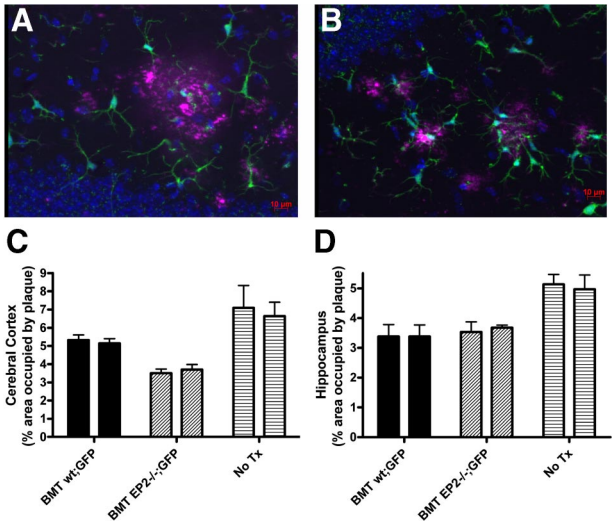

Figure 4. Suppression of Aβ plaque formation with wild-type/GFP bone marrow transplant or EP2$^{-/-}$/GFP BMT into irradiated APPswe-PS1ΔE9 mice. Fluorescence photomicrographs (×400) of hippocampus (including dentate gyrus) for anti-Aβ (purple), DAPI (blue), and GFP (green). **A** is BMT from wild-type/GFP donor into whole body irradiated APP*swe*-PS1ΔE9 recipient at five months of age with sacrifice at 13 months of age. **B** is BMT from EP2$^{-/-}$/GFP donor into whole body irradiated APPswe-PS1ΔE9 at same ages. **C** and **D** show mean (±SEM) cerebral cortical (**C**) or hippocampal (**D**) area occupied by Aβ-immunoreactive plaque in APPswe-PS1ΔE9 mice at 13 months of age; **right** and **left** columns represent data from **right** and **left** structures, respectively. One group was not irradiated and received no BMT (No Tx), whereas the others received whole body irradiation followed by BMT at five months of age from wild-type/GFP donor (BMT wild-type/GFP) or EP2$^{-/-}$/GFP donor (BMT EP2$^{-/-}$/GFP); each group had four or five mice. Two-way analysis of variance (group versus side) had $P < 0.001$ for group but $P > 0.05$ for side and interaction. Averaging **right** and **left** data for each animal and then performing one-way analysis of variance had $P < 0.001$ for cerebral cortex with Bonferroni-corrected post-tests having $P < 0.05$ for wild-type/GFP BMT versus EP2$^{-/-}$/GFP BMT, $P < 0.01$ for wild-type/GFP BMT versus No Tx, and $P < 0.001$ for EP2$^{-/-}$/GFP BMT versus No Tx. Similar one-way analysis of variance for hippocampal area occupied by Aβ-immunoreactive plaque had $P < 0.05$, and Bonferroni-corrected post-tests with $P < 0.05$ for each BMT group versus No Tx, but $P > 0.05$ for wild-type/GFP BMT versus EP2$^{-/-}$/GFP BMT.

The second series of studies also tested the hypothesis that whole body irradiation followed by BMT with cells derived from EP2$^{-/-}$/GFP mice would engraft brain and more efficiently clear Aβ peptides than wild-type donor cells. In wild-type recipients of EP2$^{-/-}$ BMT, ~50% of hippocampal and ~30% of cortical Iba-1 positive microglia were BMT derived (GFP positive), and this was significantly increased to ~80% and ~50% engraftment in APP*swe*-PS1ΔE9 host mice hippocampus (Figure 3D) and cortex (Figure 3E), respectively ($P < 0.05$). There was no effect of donor genotype (wild-type versus EP2$^{-/-}$) on hippocampal or cortical microglia engraftment or total hippocampal Iba-1 IR cells (Figure 3, D–F). However, significantly more cortical Iba-1 IR cells were identified in APP*swe*-PS1ΔE9 mice that received EP2$^{-/-}$ BMT compared with APP*swe*-PS1ΔE9 that received wild-type BMT ($P = 0.05$; Figure 3G). Moreover, BMT-derived microglia from wild-type donors appeared to be more commonly associated with Aβ deposits (Figure 4A) compared with nontransplanted control APP*swe*-PS1ΔE9 mice, and this effect appeared to be further bolstered in mice who received EP2$^{-/-}$ bone marrow (Figure 4B).

Next we compared right and left cerebrum directly by determining Aβ plaque area; we focused on determining plaque area from each side to permit direct comparison with previous studies.[16,17] There was no statistical difference between right and left sides, so we averaged these values for each mouse. APP*swe*-PS1ΔE9 mice that received neither irradiation nor BMT (No Tx) had 8.2 ± 0.6 percentage of cerebral cortical area occupied by Aβ-IR plaque. In APP*swe*-PS1ΔE9 mice transplanted with wild-type/GFP bone marrow, the area occupied by Aβ-IR plaque was 66% of the No Tx group ($P < 0.01$). By contrast, this area was 44% of the no BMT group in APP mice with EP2$^{-/-}$/GFP donor cells ($P < 0.001$ compared with No Tx and $P < 0.05$ compared with wild-type/GFP BMT). Similar results from BMT were obtained in the hippocampus, although in this location there was no significant difference between wild-type/GFP BMT and EP2$^{-/-}$/GFP BMT. These results showed that BMT with EP2$^{-/-}$ cells lead to comparable levels of brain engraftment as wild-type cells, but total cortical microglia were increased in association with a further reduction in the burden of cerebral cortical Aβ-IR plaques.

Discussion

Hematopoietic stem cells from a BMT repopulate marrow to generate a high percentage (> 85%) of donor circulating blood cells as well as cells that take up residence in virtually every organ, including brain. We confirmed the finding of several other laboratories that the vast majority of brain BMT-derived cells are phenotypically microglia.[14–17] Although some authors have reported that a very small number of neurons may also be BMT-derived,[13] we did not observe such an event. Whether these BMT-derived microglia arise from chronically circulating cells or cells acutely engrafted into brain that then expand over time is the focus of elegant experimentation.[31,32] The literature on murine BMT reports percentages on the replacement of resident microglia with BMT-derived microglia that appear to be increasing with increasing probe sensitivity (from *in situ* hybridization for Y chromosome markers to β-galactosidase to GFP-expressing cells) and with increasing time from BMT. Broadly, studies of microglia engraftment in mouse brain following BMT with less than 6 months between BMT and sacrifice reveal that 2% to 20% of microglia are BMT-derived.[14] We are aware of one study that extended the engraftment period to 1 year; they observed 30% engraftment of microglia in mouse brain.[15] Thus, between 1% and 5% of resident brain microglia per month apparently can be replaced by BMT-derived microglia in wild-type mice. Our results with wild-type BMT into wild-type mice were consistent with these estimates from the literature.

Two previous studies have investigated BMT in murine models of AD; both used wild-type donor cells.[16,17] We chose to perform BMT in mice older (5 months) than typical peripheral engraftment experiments (2 months) because we wanted the transplant to coincide with the age of onset for Aβ plaque formation in APP*swe*-PS1ΔE9 transgenic mice, a situation that perhaps more closely mimics any future clinical application. Our data robustly quantified the noted observation of others that replace-

ment of cerebral microglia after wild-type BMT is much more efficient in transgenic mouse models of AD than wild-type mice.[16,17] The reason for more efficient replacement of microglia in APP*swe*-PS1ΔE9 mice is not clear, but is proposed to be in response to chronic low-level immune activation in brains of diseased transgenic mice that could augment either engraftment or expansion. Our data also confirmed that WB XRT followed by BMT reduces Aβ plaque accumulation in these transgenic mice.[17] Although our data do not specifically address the contribution of increased Aβ clearance versus reduced Aβ deposition, we favor increased clearance based on prior studies from our lab,[7] as well as the observation that BMT results in increased microglia surrounding cerebral plaques that can be further increased by using donor EP2$^{-/-}$ bone marrow (Figure 4, A–D). Importantly, we expanded this finding to show suppression of cortical DS Aβ peptide accumulation, a mixture that contains the proposed neurotoxic forms of Aβ peptides. Indeed, other authors have recently shown that DS Aβ species and fibrillar (insoluble) Aβ species are phagocytized and degraded by microglia via different mechanisms.[4] Because the major neurotoxic species of Aβ peptides are thought to be DS forms, these data are a critical addition to our understanding of the therapeutic potential of BMT-derived microglia. It is important to note that we cannot fully exclude an independent effect of whole body irradiation on Aβ levels because myeloablative radiation is lethal without BMT. However, we were able to demonstrate that cranial irradiation was not sufficient to explain the reduced accumulation of Aβ peptides, a previously omitted control that is essential to interpretation of results. In aggregate, our results using wild-type BMT showed that cerebral microglial replacement was more efficient in APP*swe*-PS1ΔE9 mice, and that WB XRT plus BMT was necessary to reduce age-related accumulation of cerebral Aβ plaques and DS Aβ peptides.

To our knowledge, the studies described here are the first to use genetically modified BMT cells in an effort to augment clearance of cerebral Aβ peptides. We selected EP2$^{-/-}$ BMT because our group has previously demonstrated that primary murine cultures of EP2$^{-/-}$ microglia demonstrate enhanced phagocytosis of synthetic Aβ peptides and Aβ peptides in tissue slices from AD brain.[7] Moreover, our group has previously demonstrated that APP*swe*-PS1ΔE9 mice on an EP2 null background accumulate less cerebral Aβ plaques and peptides; however, interpretation of these experiments is confounded by EP2 expression by neurons as well as microglia and data suggesting that neuron biosynthesis of Aβ peptides may be reduced in APP*swe*-PS1ΔE9 on an EP2 null background.[9] Results from the studies reported here indicated that at least some of the reduction in age-related cerebral cortical Aβ plaque accumulation in APP*swe*-PS1ΔE9/EP2$^{-/-}$ mice was due to enhanced clearance from EP2$^{-/-}$ microglia, although a significant reduction in Aβ in this study is seen with wild-type donor BM, suggesting additive mechanisms of action by donor marrow and donor marrow that lacks EP2. Experiments to determine the role of age of BMT donor, as well as specific immune mechanisms underlying wild-type BMT-mediated Aβ reduction, are ongoing. Interestingly, we did not observe a significant effect of EP2$^{-/-}$ BMT on age-related hippocampal Aβ plaque accumulation, and no differences in hippocampal DS Aβ were identified. The significance of this anatomical difference is unclear, but we speculate that it may indicate variation in regional BMT-derived microglial activity or in the balance between production and clearance of Aβ peptides in these two regions. Alternatively, we found significantly increased cortical, but not hippocampal, Iba-1 IR cells in APP*swe*-PS1ΔE9 that received EP2$^{-/-}$ BMT compared with those receiving wild-type BMT, which may underlie differences in hippocampal plaque and DS Aβ peptides. Studies aimed at differentiating these processes are ongoing.

The goal of the current study was to further characterize bone marrow transplantation-mediated reduction in cerebral Aβ and to evaluate the role of innate immune activation in this process. Evaluation of functional outcomes, such as behavioral measures, and associated neuropathological correlates, such as neuron loss and toxicity, synaptic integrity, astrogliosis, and finer indices of neuroinflammation, are ongoing and will be critical to understanding the functional relevance of Aβ-based therapies. Although behavioral endpoints were not pursued in the current study, several other groups have associated reduced Aβ peptide burden in transgenic mice with improved behavior. For example, Aβ vaccine protocols also reduce mouse hippocampal and cerebral Aβ plaque burden by about 50%, and this is associated with significant improvement in Morris water maze performance.[33,34] Because we observed similar reductions in cerebral Aβ plaque burden in these studies, we anticipate improved performance on future behavioral tests and neuropathological correlates of behavior. However, interpretation of these results may be challenging. For instance, human Aβ vaccine trials, which evolved from the murine studies described above, were halted prematurely due to complications of meningoencephalitis[35,36] that were not apparent in experimental models. Moreover, postmortem examination of some of the vaccinated patients revealed decreased cerebral Aβ even though no significant differences in clinical performance were identified.[37] Thus, comparison of results from experimental (mouse) and observational (human) studies may shed new insight into the pathophysiology of these uniquely human diseases. Although no gross brain injury or reaction, such as hemorrhage or reduction in volume of hippocampal pyramidal layer or dentate gyrus, was observed, these are coarse measures and it remains to be determined whether subtle stress accompanies BMT or enhanced clearance of Aβ peptides. If we do observe subtle neuron damage accompanying wild-type BMT into APP*swe*-PS1ΔE9 mice, then we will be especially interested in the results from EP2$^{-/-}$ BMT into APP*swe*-PS1ΔE9 mice, because activated EP2$^{-/-}$ microglia have greatly reduced capacity to produce paracrine damage to neurons.[11]

In summary, we have shown that engraftment with donor cells was necessary for wild-type WB XRT and BMT-mediated reduction in age-related cerebral Aβ

plaque formation in APP*swe*-PS1ΔE9 mice, and that this was accompanied by reduced cerebral concentration of the proposed neurotoxic forms of Aβ peptides. Further reduction in cerebral cortical Aβ burden was achieved with $EP2^{-/-}$ BMT, suggesting that part of the cerebral Aβ lowering effect observed in APP*swe*-PS1ΔE9 mice that lacked $EP2^{-/-}$ receptors[9] derived from enhanced phagocytosis by $EP2^{-/-}$ microglia. Our results provide a well-controlled foundation for an adult stem cell-based therapy to reduce Aβ peptide accumulation in the brains of patients with AD.

Acknowledgments

We thank Elaine Raines, Carole Wilson, and Jingjing Tang (University of Washington) for reviewing the article and excellent transplantation and flow cytometry advice and assistance, Xiaoping Wu (Benaroya Research Institute, Seattle) for assistance with flow cytometry, and Aimee Schantz and Amy Look (University of Washington) for administrative support.

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