Myeloid differentiation factor 88-deficient bone marrow cells improve Alzheimer’s disease-related symptoms and pathology

Wenlin Hao,1,2,* Yang Liu,1,2,* Shirong Liu,1,2 Silke Walter,1,2 Marcus O. Grimm,1,2 Amanda J. Kiliaan,3 Botond Penke,4 Tobias Hartmann,1,2 Claudia E. Rübe,5 Michael D. Menger6 and Klaus Fassbender1,2

1 Department of Neurology, University of the Saarland, 66421 Homburg/Saar, Germany
2 German Institute for Dementia Prevention (DIDP), University of the Saarland, 66421 Homburg/Saar, Germany
3 Department of Anatomy and Department of Cognitive Neuroscience, Donders Institute for Brain, Cognition, and Behavior, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands
4 Department of Medical Chemistry, Albert Szent Gyorgyi Medical University, Szeged, Hungary
5 Department of Radiation Therapy and Radiation Oncology, University of the Saarland, 66421 Homburg/Saar, Germany
6 Institute for Clinical and Experimental Surgery, University of the Saarland, 66421 Homburg/Saar, Germany

*These authors contributed equally to this work.

Correspondence to: Dr Yang Liu,
Department of Neurology,
University of the Saarland,
Kirrberger Straße,
66421 Homburg/Saar, Germany
E-mail: a.liu@mx.uni-saarland.de

Alzheimer’s disease is characterized by extracellular deposits of amyloid β peptide in the brain. Increasing evidence suggests that amyloid β peptide injures neurons both directly and indirectly by triggering neurotoxic innate immune responses. Myeloid differentiation factor 88 is the key signalling molecule downstream to most innate immune receptors crucial in inflammatory activation. For this reason, we investigated the effects of myeloid differentiation factor 88-deficient bone marrow cells on Alzheimer’s disease-related symptoms and pathology by establishing bone marrow chimeric amyloid β peptide precursor transgenic mice, in which bone marrow cells differentiate into microglia and are recruited to amyloid β peptide deposits. We observed that myeloid differentiation factor 88-deficient bone marrow reconstruction reduced both inflammatory activation and amyloid β peptide burden in the brain. In addition, synaptophysin, a marker of neuronal integrity, was preserved and the expression of neuronal plasticity-related genes, ARC and NMDA-R1, was increased. Thus, myeloid differentiation factor 88-deficient microglia significantly improved the cognitive function of amyloid β peptide precursor protein transgenic mice.

Myeloid differentiation factor 88-deficiency enhanced amyloid β peptide phagocytosis by microglia/macrophages and blunted toxic inflammatory activation. Both the expression of amyloid β peptide precursor protein and amyloid β peptide degrading enzymes and also the efflux of amyloid β peptide from brain parenchyma were unaffected by myeloid differentiation factor 88-deficient microglia. By contrast, the activity of β-secretase was increased. β-Secretase is expressed primarily in neurons, with relatively little expression in astrocytes and microglia. Therefore, microglial replenishment with myeloid differentiation factor 88-deficient bone marrow cells might improve cognitive functions in Alzheimer’s disease mouse models by enhancing amyloid β peptide phagocytosis and reducing inflammatory activation. These results could offer a new therapeutic option that might delay the progression of Alzheimer’s disease.
**Introduction**

Growing evidence suggests that inflammatory processes driven by microglia/macrophages contribute to the pathogenesis of Alzheimer’s disease (Akiyama et al., 2000; Wyss-Coray, 2006). Increased expression of inflammatory mediators has been observed in post-mortem Alzheimer’s disease brains (Eikelenboom and Stam, 1982; McGeer et al., 1988) and positron emission tomography studies have shown an association between microglial activation and Alzheimer’s disease progression (Cagnin et al., 2001; Edison et al., 2008; Okello et al., 2009). Moreover, epidemiological studies consistently link the use of non-steroidal anti-inflammatory drugs with reduced risk for later Alzheimer’s disease. (McGeer et al., 1996; in t’ Veld et al., 2001; Szekely et al., 2004) and there is evidence that the protective benefit of non-steroidal anti-inflammatory drugs arises from their anti-inflammatory effects (Szekely et al., 2008), apart from their possible inhibitory effects on the amyloid β peptide (Aβ) processing (Weggen et al., 2001; Eriksen et al., 2003). In the animal model of Alzheimer’s disease that over-expresses Aβ precursor protein, activation and recruitment of microglia can be visualized around Aβ deposits even before neuronal damage occurs (Benzing et al., 1999; McGowan et al., 1999; Bolmont et al., 2008; Meyer-Luehmann et al., 2008), further arguing for a role of neuroinflammation in progressive neurodegeneration in Alzheimer’s disease.

Recent studies, however, challenge the view that microglia play a solely detrimental role in Alzheimer’s disease. Paradoxically, microglia also exert beneficial effects in Alzheimer’s disease pathophysiology. Microglia can clear Aβ plaques via phagocytosis (Liu et al., 2005; Simard et al., 2006; Town et al., 2008), especially when therapeutically enhanced by vaccination against Aβ (Bard et al., 2000, McLaurin et al., 2002; Koenigsknecht-Talboo et al., 2008). It has even been argued that defective Aβ plaque clearance by mononuclear phagocytes might be a mechanism for the most common, ‘sporadic’ form of Alzheimer’s disease (Fiala et al., 2007).

As key, innate immune-effector cells of the central nervous system, microglia are armed with an extensive repertoire of pattern recognition receptors, including CD14 and toll-like receptors that are essential for detection and rapid elimination of invading microorganisms (Akira et al., 2001; Bbsbi et al., 2002). Upon ligand binding, CD14/toll-like receptor signals are transduced via a common adaptor protein, myeloid differentiation factor 88 (MyD88), resulting in activation of AP-1 and NF-κB transcription factors that regulate the expression of numerous important immune response genes (Akira et al., 2001). We and other researchers, have shown that CD14, toll-like receptor 2 and toll-like receptor 4 on microglia interact with aggregated Aβ, thereby triggering microglial inflammatory activation and stimulating microglia-mediated clearance of Aβ (Fassbender et al., 2004; Liu et al., 2005; Tahara et al., 2006; Jana et al., 2008; Richard et al., 2008; Reed-Geaghan et al., 2009). Thus MyD88, the molecule that controls signalling for these important innate immune receptors, could be a key determinant for the detrimental and beneficial effects of microglia in Alzheimer’s disease pathophysiology.

In this study, we created MyD88-deficient bone marrow chimeric Aβ precursor protein transgenic mice to examine the role of the key, innate immune protein, MyD88, on symptoms and pathological features of Alzheimer’s disease as a potentially relevant therapeutic target in this disease.

**Materials and methods**

**Mice**

*APP Terry* Aβ precursor protein transgenic mice in a C57BL6/129 background (continuously interbred for over three generations) were provided by D. Westaway (University of Toronto; Chishti et al., 2001). *APP Terry/PSTAG* mice were obtained from D. Borchelt (University of Florida; Jankowsky et al., 2001) and kept in C57BL/6 background (Hooijmans et al., 2009). MyD88 knockout (MyD88−/−) mice in a C57BL/6 background were provided by S. Akira (Osaka University). Green fluorescent protein (GFP) transgenic (C57BL/6-Tg UBC-GFP 30Scha/J) and C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, USA). All animal experiments were approved by the regional ethical committee in Saarland, Germany.

**Cell isolation and culture**

Bone marrow cells were isolated from medullar cavities of the tibia and femur of 8-week-old female MyD88−/−, C57BL/6 or green fluorescent protein-transgenic mice. For bone marrow transplantation, cells were washed and suspended in ice-cold phosphate buffered saline. Bone marrow-derived macrophages were cultured in Dulbecco’s modified Eagle medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% foetal calf serum (PAN Biotech, Aidenbach, Germany) and 20% L929 cell-conditioned medium (DSMZ, Braunschweig, Germany) in 75 cm² flasks (BD, Heidelberg, Germany) according to a published protocol (Takahashi et al., 2007).

**Preparation of Aβ peptides**

The 42-amino acid form of human Aβ (Aβ42) was provided by B. Penke. Fluorescent Aβ was prepared by mixing fluorescein isothiocyanate-conjugated Aβ42 (Bachem, Heidelberg, Germany) and unlabelled Aβ42 at a ratio of 1:4. Aggregated Aβ was prepared using an established method (Dahlgren et al., 2002). Endotoxin concentrations of peptide samples were <0.01 EU/ml as determined by the Limulus amoebocyte lysate (LAL) assay (Lonza, Basel, Switzerland). The oligomeric conformations of fluorescein isothiocyanate-conjugated and non-conjugated forms of Aβ42 peptides were the same as confirmed by western blot and electron microscopy.
Flow cytometric analysis of fluorescein isothiocyanate-Aβ42 internalization in primary macrophages

Primary bone marrow-derived macrophages were cultured in a 24-well plate (BD) at a density of 2 x 10⁵ cells per well and treated with fluorescein isothiocyanate-conjugated Aβ42 (0.5, 5 or 10 μM) for 0, 1, 3, 6 and 24 h. Macrophages were washed and detached. The mean fluorescence intensity of fluorescein isothiocyanate-labelled Aβ42 was immediately determined by BD FACSCanto™ II flow cytometry (Franklin Lakes, USA). In order to monitor the inflammatory activation during Aβ internalization, supernatants were collected for ELISA detection of tumour necrosis factor α (TNF-α) using a kit from R&D Systems (Wiesbaden, Germany).

Bone marrow transplantation

On the day of transplantation, 3-month-old TgCRND8 mice or 6- or 9-month-old APPswPS1dE9 mice (recipients) were exposed to a 10 Gy total-body irradiation given as split doses of 2 x 5 Gy with a 4 h interval using a linear accelerator (r-source). Donor bone marrow cells (1 x 10⁷ per mouse) derived from MyD88−/−; C57BL6 or green fluorescent protein mice were then administered via the tail vein to each recipient. Transplanted mice were housed in autoclaved cages and treated with antibiotics in drinking water (0.2 mg/ml trimethoprim and 1 mg/ml sulphamethoxazole, both from Sigma, Schnelldorf, Germany) for three weeks.

Barnes maze test

The Barnes circular maze is a brightly lit open disk with 40 holes in the perimeter. Mice are motivated using distal cues to escape from the maze by finding the one hole in 40 that leads to a small dark recessed chamber (Barnes, 1979; Mayford et al., 1996). The test involved five days of acquisition training with two trials per day and was performed according to the published protocol (O’Leary and Brown, 2009). In order to reduce the stress for mice, no additional aversive stimuli were given. For each trial, latency to enter the escape hole and distance travelled were recorded by EthoVisionXT (V6.1) tracking software (Noldus Information Technology, Wageningen, The Netherlands). The experimenter, blind to the genotype of bone marrow cells, recorded errors, which were defined as searches of any hole that did not have the chamber beneath it. Searches included nose pokes and head deflections over the hole. Finally, the latency, total distance and number of errors were averaged from the two trials per day.

Tissue collection

Animals were sacrificed three months after bone marrow transplantation by inhalation of isofluorane (Abbott, Wiesbaden, Germany). Whole blood was collected via intracardiac puncture and kept in ethylenediaminetetraacetic acid-containing Eppendorf tubes. Mice were then rapidly perfused transcardially with ice-cold phosphate buffered saline and the brain removed and divided. The left hemi-brain was immediately fixed in 4% paraformaldehyde (Sigma) for immunohistochemistry. A 0.5 mm thick piece of tissue was sagittally cut from the right hemi-brain and homogenized in Trizol (Invitrogen) for RNA isolation. The remainder of the right hemi-brain was used to prepare a single cell suspension or snap frozen in liquid nitrogen for biochemical analysis.

Flow cytometric analysis of blood cell lineages after bone marrow reconstruction

To examine the degree of chimerism of bone marrow cells, green fluorescent protein-positive white blood cells were directly detected by flow cytometry after green fluorescent protein-transgenic bone marrow reconstruction. To identify cell types after MyD88-deficient and wild-type bone marrow reconstruction, the blood was co-stained with fluorescein isothiocyanate-conjugated rat anti-mouse CD45 (30-F11) and Alexa647-conjugated rat anti-mouse CD11b (M1/70), PE-conjugated rat anti-mouse Ly-6G (1A8) or rat anti-T cell antigen receptor β chain (H57-597) antibodies, all of which are from Pharmingen, Heidelberg, Germany. After 1 h incubation on ice, the blood cells were treated with BD FACS Lysing Solution (Pharmingen) and measured with flow cytometry.

Flow cytometric analysis of CD45-positive microglia in the brain

Hippocampal and cortical tissues including the grey and white matter were carefully dissected to prepare a single cell suspension using a 24 gauge needle, 2 ml syringe and 70 μm mesh (BD) according to the published protocol (Babcock et al., 2008; Hagihara et al., 2009). After pelleting cells by centrifugation, 200 μl of blocking buffer containing 25 μg/ml rat anti-mouse CD16/CD32 antibody (2.4G2, Pharmingen), 10% foetal calf serum and 0.1% sodium azide was added to prevent non-specific binding. Cells stained with Alexa647-conjugated CD11b antibody or isotype controls were used to identify the CD11b-positive cell population. After side scatter and CD11b gating, positive staining with fluorescein isothiocyanate-conjugated CD45 antibody was determined based on fluorescence levels compared with the relevant isotype control.

Brain homogenization and Aβ/AB precursor protein ELISA

The brain was homogenized as previously described (Maier et al., 2008). Briefly, frozen hemispheres were bounce-homogenized in a carbonate buffer (sodium carbonate buffer: 100 mM Na₂CO₃, 50 mM NaCl, pH 11.5) containing protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and centrifuged at 16 000 x g 80 min at 4 °C. The supernatant (sodium carbonate buffer-soluble fraction) was collected and stored at -80 °C. The pellets were resuspended in the sodium carbonate buffer plus 1% Triton-X (sodium carbonate buffer plus Triton), sonicated for 5 min in a 4 °C water bath and centrifuged at 16 000 g for another 30 min at 4 °C. The supernatant was collected and stored at -80 °C as the sodium carbonate buffer plus Triton-soluble fraction. The pellets were extracted for a third time using an ice-cold guanidine buffer (5 M guanidine-HCl/50 mM Tris, pH 8.0) to produce the guanidine-soluble fraction. Protein concentrations of all samples were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). Aβ42/40 and Aβ precursor protein ELISA kits (Invitrogen) were used to determine Aβ concentrations in three different fractions of brain homogenates and in plasma and Aβ precursor protein concentrations in the sodium carbonate buffer plus Triton-soluble fraction. In each
case except plasma, the result was normalized to the protein concentration of the sample.

**β-Secretase 1 activity assay**

The activity of β-secretase 1 was quantified in sodium carbonate buffer plus Triton soluble brain homogenate using SensiZyme β-secretase 1 Activity Assay Kit (Sigma). In this assay, β-secretase 1 in the tissue extract is caught by a β-secretase 1-specific antibody coated on a 96-well plate. The β-secretase 1 activity was expressed as nanogram per millilitre relative to the pure β-secretase 1 protein standard.

**Evaluation of synaptophysin protein levels**

Synaptophysin levels in sodium carbonate buffer plus triton-soluble brain homogenates were quantified with a capture ELISA as described previously (Oakley et al., 2006). A SY38 monoclonal synaptophysin antibody (1.75 μg/ml; Millipore, Schwalbach/Ts, Germany) was used to coat plates and a rabbit polyclonal synaptophysin antibody (0.125 μg/ml; Abcam, Cambridge, USA) was used to detect synaptophysin. Because purified synaptophysin was not available, the results are shown in optical density (OD) values adjusted by protein concentration in the same sample.

**Immunohistochemistry**

Paraformaldehyde-fixed brains were embedded in paraffin and serial 2 μm thick sagittal sections were mounted on glass slides. Immunohistochemical staining was performed on these sections with the VectaStain Elite ABC kit (Vector Laboratories, Burlingame, USA) to demonstrate the neuropathological changes. The following primary antibodies were used: rabbit anti-Iba-1 (1:500, Wako Chemicals, Neuss, Germany) and mouse anti-αβ antibody (1:50, clone 2F/3D, Dako, High Wycombe, UK). Diaminobenzadine (Sigma) was used as the chromogen. All images were acquired by Olympus AH-2 light microscopy.

To quantify αβ deposits by morphometry, we used computer-assisted image analysis capable of colour segmentation and automation via programmable macros (AnalySIS, Soft Imaging System, Puchheim, Germany) with ×20 objective. αβ deposits labelled with Cy5 were imaged after excitation with a 635 nm laser. Thereafter, Z-stack scanning from –5 to +5 μm was performed under the excitation of 488, 561 and 635 nm lasers. From each section, five randomly chosen areas were analysed. The images of Alexa488 (green fluorescent protein), Cy3 (Iba-1) and Cy5 (αβ) from the same layer were colour-coded and merged with ImageJ (http://rsb.info.nih.gov/ij/) and Adobe Photoshop (V9.0.2, Adobe Systems Incorporated, San Jose, USA). The total number of Iba-1-positive cells, the number of Iba-1-positive cells co-localizing with αβ deposits and the number of green fluorescent protein-positive cells were counted.

To identify the cellular origin of β-secretase 1 protein, the MyD88-deficient bone marrow chimeric αβ precursor protein transgenic mouse brain was co-stained with rabbit anti-β-secretase 1 antibody (anti-B690, Merck KGaA, Darmstadt, Germany; Yan et al., 2001), and with antibodies against different cellular markers: mouse anti-NeuN (for neurons, clone A60, Millipore), mouse anti-MHC II (for microglia, clone OX6, Abcam) and mouse anti-GFAP (for astrocyte, clone 2A5, Abcam). Relevant Alexa488 or Cy3-conjugated second antibodies were used. The β-secretase 1 expression was analysed under confocal microscopy.

**Quantitative reverse transcription-polymerase chain reaction for analysis of gene transcripts**

Total RNA was isolated from the brain homogenate in Trizol. Reverse transcription and quantitative polymerase chain reaction on the 7500 Fast real-time polymerase chain reaction system (Applied Biosystems, Foster City, USA) were performed using our established protocol (Liu et al., 2009). Taqman® gene expression assays obtained from Applied Biosystems were used to measure 18s RNA and transcripts of the following genes: TNF-α, interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), chemokine (C-C motif) ligand 2 (CCL2), interleukin-4 (IL-4), interleukin-10 (IL-10), neprilysin (NEP), insulin-degrading enzyme (IDE), scavenger receptor A (SR-A), CD36, receptor for advanced glycation end Products (RAGE), low-density lipoprotein receptor-related protein 1 (LRP1), activity-regulated cytoskeleton-associated protein (Arc), NMDA receptor subunits NR1 (NMDS-R1), and β-secretase 1.

**Statistics**

Data shown in the figures are presented as mean ± SEM. For multiple comparisons, we used one-way or two-way ANOVA followed by Bonferroni’s, Tukey’s Honestly Significant Difference or Tamhane’s T2 post hoc test (dependent on the result of Levene’s test to determine the equality of variances). Two-independent-samples t-test was used to compare means for two groups of cases. All statistical analysis was performed on Statistical Package for the Social Sciences 15.0 for Windows (SPSS, Chicago, USA). Statistical significance was set at P < 0.05.
Results

Bone marrow-derived cells are recruited to cerebral Aβ deposits in bone marrow chimeric Aβ precursor protein transgenic mice

Bone marrow-derived cells are able to migrate to the brain and specifically differentiate into microglia (Hickey and Kimura 1988; Priller et al., 2001; Simard et al., 2006), especially under the conditions of diseases or direct irradiation (Mildner et al., 2007). In order to investigate the pathogenic effect of MyD88 in microglia, we reconstructed MyD88-deficient bone marrow in Alzheimer’s mice. After bone marrow transplantation, we observed that both groups of TgCRND8 Aβ precursor protein transgenic mice receiving MyD88-deficient or wild-type cells showed the same tendency to increase in body weight (Fig. 1A). Similarly, the percentages of different cell lineages [CD11b+, T-cell antigen receptor (TCR)+] in the white blood cells were not altered by MyD88-deficient bone marrow reconstruction (Fig. 1B). To examine the degree of chimerism of bone marrow cells, we transplanted some of our TgCRND8 Aβ precursor protein transgenic mice with donor cells derived from green fluorescent protein transgenic mice. The body weight (BW) of mice receiving MyD88-deficient and wild-type bone marrow cells increased with the same tendency (n ≥ 12 per group). In order to track bone marrow-derived cells, some TgCRND8 Aβ precursor protein transgenic mice were transplanted with donor cells derived from green fluorescent protein transgenic mice. The location relevant to Aβ and differentiation of bone marrow-derived cells in the brain parenchyma was investigated by immunofluorescent staining with antibodies against green fluorescent protein, Iba-1 and Aβ. Brain sections were analysed by confocal microscopy. Co-localization of green fluorescent protein and Iba-1 was shown by yellow fluorescence (arrow) and co-localization of all three molecules by white fluorescence (arrow head), superimposing fluorescent images of green fluorescent protein (green), Iba-1 (red) and Aβ (blue). wt = wild-type; ko = MyD88-deficient.

Bone marrow-reconstructed Aβ precursor protein transgenic mice, we did not detect IgG leakage into the brain tissue, which indicated the intactness of blood–brain barrier (data not shown).
MyD88-deficient bone marrow reconstruction improves cognitive function of Aβ precursor protein transgenic mice

We examined pathogenic effects of MyD88-deficient bone marrow-derived microglia on neuronal function with the Barnes maze test. TgCRND8 Aβ precursor protein transgenic mice (three months post-transplantation with wild-type bone marrow) spent significantly more time (Fig. 2A, P < 0.001), travelled longer distances (Fig. 2A, P < 0.001) and made more errors (data not shown, P < 0.001) before reaching the escape hole than did their wild-type littermates after a similar bone marrow reconstruction. The efficacy of Barnes maze as a cognitive test for APP<sub>swe</sub>/PS1<sub>de9</sub> transgenic mice was validated by O’Leary and Brown (2009). After successful bone marrow reconstruction with MyD88-deficient bone marrow cells, both TgCRND8 mice at three months post-transplant and APP<sub>swe</sub>/PS1<sub>de9</sub> transgenic mice at 12 months post-transplant reached the escape hole in significantly faster times and shorter distances compared with the respective wild-type bone marrow-transplanted Aβ precursor protein transgenic mice (Fig. 2A and B, P < 0.05). Similarly, we observed that MyD88-deficiency in bone marrow cells markedly reduced errors for TgCRND8 mice engaged in searching for the escape hole (data not shown, P < 0.001).

We further measured synaptophysin in the brain homogenate by ELISA to evaluate the neuronal integrity. We observed 0.016 ± 0.001 (OD-value/mg of wet brain) in TgCRND8 Aβ precursor protein transgenic mice (n = 16), which was significantly less than 0.019 ± 0.001 in their wild-type littermates (n = 8, t-test versus Aβ precursor protein mice, P = 0.004). Interestingly, measurements of synaptophysin levels showed that the protein level in MyD88-deficient bone marrow chimera TgCRND8 Aβ precursor protein mice was 1.39 ± 0.12-fold higher than in the wild-type bone marrow Aβ precursor protein control mice (P = 0.003, Fig. 2C). Moreover, transcription of the Arc and NMDA-R1 genes, both of which are involved in neuronal plasticity (Dickey et al., 2003; Bramham et al., 2008; Richard et al., 2008), was significantly upregulated in TgCRND8 Aβ precursor protein transgenic mice with MyD88-deficient bone marrow reconstruction compared with Aβ precursor protein transgenic mice with wild-type bone marrow reconstruction (P < 0.05, Fig. 2D). In the APP<sub>swe</sub>/PS1<sub>de9</sub> mice, we did not observe any effects of MyD88-deficient bone marrow reconstruction on synaptophysin levels or transcription of Arc and NMDA-R1 genes (data not shown).

MyD88-deficient bone marrow reconstruction attenuates neuroinflammation in Aβ precursor protein transgenic mice

As inflammatory activation contributes to Alzheimer’s disease pathogenesis, we investigated whether MyD88-deficient bone marrow reconstruction reduces inflammatory activities in the brain. We measured recruitment of microglial cells in the hippocampus by counting Iba-1 positive cells in TgCRND8 mice. The two groups differed significantly in microglial recruitment, with 122.37 ± 4.01 cells/mm<sup>2</sup> in wild-type bone marrow-reconstructed Aβ precursor protein transgenic mice compared to 111.17 ± 3.38 cells/mm<sup>2</sup> in MyD88-deficient bone marrow-reconstructed Aβ precursor protein transgenic mice (P = 0.043, Fig. 3A). Microglial morphology was similar in the two groups (Fig. 3A insert). We used flow cytometry to count CD45<sup>+</sup>/CD11b<sup>+</sup> cells, which represent peripherally recruited and resident activated...
We observed that more CD45+ microglia were recruited in Aβ precursor protein transgenic mice than in their wild-type littermates after bone marrow reconstruction (Fig. 3B, P = 0.002). In bone marrow-reconstructed Aβ precursor protein mice, MyD88-deficiency in bone marrow cells significantly reduced CD45+/CD11b+ cells in the Alzheimer's disease mouse brain (one-way ANOVA followed by post hoc tests, P < 0.05, n = 4 per group). (C) Inflammatory gene transcripts were measured by quantitative reverse transcription polymerase chain reaction. Transcription of TNF-α and CCL-2 in TgCRND8 mice, and TNF-α and interleukin-1β in APPsw/PS1dE9 mice was significantly lower in MyD88-deficient-bone marrow-reconstructed mice than in the wild-type-bone marrow controls (t-test, P < 0.05, n = 13 per group). FITC = fluorescein isothiocyanate; IL = interleukin; iNOS = inducible nitric oxide synthase.

Figure 3 MyD88-deficient bone marrow-derived cells reduce inflammatory activities in the brain. Three months after bone marrow reconstruction with MyD88-deficient (ko) and wild-type (wt) cells, the brains of TgCRND8 Aβ precursor protein (APP) transgenic mice were analysed for inflammatory activation. (A) Microglial recruitment was investigated by performing immunohistochemistry with antibodies against Iba-1. Positive staining appears brown. The number of Iba-1 positive cells was significantly more in the hippocampus of Aβ precursor protein transgenic mice reconstructed with wild-type-bone marrow cells than with MyD88-deficient-bone marrow cells (t-test, P < 0.05, n ≥ 13 per group). Microglial morphology shown in the inserts did not differ between these two mouse groups. (B) CD45-positive microglia were calculated using flow cytometry. Following wild-type bone marrow reconstruction, the percentage of CD45+ cells among the CD11b+ cell population was higher in Aβ precursor protein transgenic mice than in their wild-type littermates. Compared with wild-type bone marrow reconstruction, MyD88-ko bone marrow reconstruction significantly reduced CD45+/CD11b+ cells in the Alzheimer's disease mouse brain (one-way ANOVA followed by post hoc tests, P < 0.05, n = 4 per group). (C) Inflammatory gene transcripts were measured by quantitative reverse transcription polymerase chain reaction. Transcription of TNF-α and CCL-2 in TgCRND8 mice, and TNF-α and interleukin-1β in APPsw/PS1dE9 mice was significantly lower in MyD88-deficient-bone marrow-reconstructed mice than in the wild-type-bone marrow controls (t-test, P < 0.05, n ≥ 13 per group). FITC = fluorescein isothiocyanate; IL = interleukin; iNOS = inducible nitric oxide synthase.
transplantation. As shown in Fig. 3C, levels of TNF-α and CCL-2 transcripts in TgCRND8 Aβ precursor protein mice and TNF-α and interleukin-1β in APPsw/PS1dE9 mice were significantly reduced by MyD88-deficient bone-marrow reconstruction as compared with wild-type bone marrow reconstruction (P < 0.05, Fig. 3C). Transcription of anti-inflammatory cytokine interleukin-10 did not differ between the two sets of mice, and interleukin-4 transcription was not detectable in any of the animals.

**MyD88-deficient bone marrow reconstruction reduces cerebral Aβ in Aβ precursor protein transgenic mice**

To analyse the effects of bone marrow reconstruction on Aβ load in the brain, we first determined the Aβ profile in MyD88-deficient bone marrow chimeric Aβ precursor protein transgenic animals by western blots (Supplementary Fig. 1). We separated brain homogenate from MyD88-deficient and control TgCRND8 mice into three fractions (sodium carbonate buffer, sodium carbonate buffer plus Triton and guanidine chloride-soluble). The sodium carbonate buffer fraction consisted primarily of monomers, the sodium carbonate buffer plus Triton fraction consisted of oligomers, and the guanidine fraction was enriched with high molecular-weight aggregates. We measured the amounts of cerebral Aβ in TgCRND8 Aβ precursor protein transgenic with and without wild-type bone marrow reconstruction. We found the concentration of Aβ42 in the guanidine fraction to be markedly reduced following bone marrow transplantation (P < 0.001, Fig. 4A), consistent with the results recently reported by Keene’s group (2010). In the comparison with wild-type bone marrow reconstruction, the concentration of Aβ42 in the guanidine fraction was markedly reduced following bone marrow transplantation (P < 0.001, Fig. 4A), consistent with the results recently reported by Keene’s group (2010). In the comparison with wild-type bone marrow reconstruction, the concentration of Aβ42 in the guanidine fraction was markedly reduced following bone marrow transplantation (P < 0.001, Fig. 4A), consistent with the results recently reported by Keene’s group (2010). In the comparison with wild-type bone marrow reconstruction, the concentration of Aβ42 in the guanidine fraction was markedly reduced following bone marrow transplantation (P < 0.001, Fig. 4A), consistent with the results recently reported by Keene’s group (2010). In the comparison with wild-type bone marrow reconstruction, the concentration of Aβ42 in the guanidine fraction was markedly reduced following bone marrow transplantation (P < 0.001, Fig. 4A), consistent with the results recently reported by Keene’s group (2010). In the comparison with wild-type bone marrow reconstruction, the concentration of Aβ42 in the guanidine fraction was markedly reduced following bone marrow transplantation (P < 0.001, Fig. 4A), consistent with the results recently reported by Keene’s group (2010). In the comparison with wild-type bone marrow reconstruction, the concentration of Aβ42 in the guanidine fraction was markedly reduced following bone marrow transplantation (P < 0.001, Fig. 4A), consistent with the results recently reported by Keene’s group (2010). In the comparison with wild-type bone marrow reconstruction, the concentration of Aβ42 in the guanidine fraction was markedly reduced following bone marrow transplantation (P < 0.001, Fig. 4A), consistent with the results recently reported by Keene’s group (2010). In the comparison with wild-type bone marrow reconstruction, the concentration of Aβ42 in the guanidine fraction was marked...
chimeric TgCRND8 mice, we observed that the concentrations of both Aβ40 and Aβ42 in the sodium carbonate buffer plus Triton fraction and Aβ42 in guanidine fraction were significantly lower following MyD88-deficient bone marrow reconstruction (P < 0.05, Fig. 4A). In the guanidine fraction, the concentration of Aβ40 was also reduced in MyD88-deficient bone marrow chimeric mice, compared with control animals, although the difference did not reach statistical significance (P > 0.05, Fig. 4A). In the sodium carbonate buffer fraction, the concentrations of Aβ40 and Aβ42 were nearly the same between the two bone marrow chimeric mouse groups (P > 0.05, Fig. 4A). Immunohistochemical staining showed that the levels of Aβ deposits in the TgCRND8 Aβ precursor protein transgenic mouse brain receiving MyD88-deficient bone marrow cells (0.35 ± 0.04% of region of interest) were significantly decreased as compared with levels in the control brain (0.68 ± 0.10% of region of interest; P = 0.047, Fig. 4B).

We further confirmed the Aβ-reducing effect of MyD88-deficient bone marrow cells by measuring Aβ concentrations in the brain of 13-month-old bone marrow chimeric APPsw/ePS1dE9 mice. The concentrations of Aβ42 in both sodium carbonate buffer plus Triton and guanidine fractions were significantly reduced by MyD88-deficient bone marrow mouse cells (sodium carbonate buffer plus Triton: 0.368 ± 0.016 → 0.243 ± 0.059 and guanidine: 84.911 ± 9.362 → 66.653 ± 8.396 ng/mg wet brain; t-test, P = 0.043 and 0.037, respectively; n = 7 per group).

**Modulation of Aβ metabolism is not the mechanism by which MyD88-deficient bone marrow cells reduce Aβ levels**

After observing that MyD88-deficient bone marrow reconstruction reduced both inflammatory activation and Aβ load, we investigated the mechanism responsible for Aβ reduction. To determine whether Aβ production was altered in our bone marrow chimeric Alzheimer’s disease model, we measured Aβ precursor protein level and β-secretase 1 activity. As shown in Fig. 5A, Aβ precursor protein amount in sodium carbonate buffer plus Triton-soluble brain homogenates were not significantly different between the two groups of mice reconstructed with MyD88-deficient and wild-type bone marrows (MyD88-deficient bone marrow: 0.31 ± 0.01 and wild-type bone marrow: 0.34 ± 0.03 μg/mg brain, P = 0.467). Interestingly, β-secretase 1 activity (cleaving Aβ precursor protein to produce Aβ) was significantly increased in MyD88-deficient bone marrow chimeric mice compared with wild-type control mice (P = 0.013, Fig. 5B). To confirm this result, we performed β-secretase 1 activity assays in brains from 6-month-old MyD88 or toll-like receptor 2-deficient and wild-type mice without Aβ precursor protein transgenic expression and observed that β-secretase 1 activity was significantly elevated in MyD88 or toll-like receptor 2-deficient mice compared with C57BL6 controls (Supplementary Fig. 2). In order to clarify the biological significance of increased β-secretase 1 activity, we investigated the cellular origin of β-secretase 1. As shown in Fig. 5C, we observed that β-secretase 1 was mainly expressed in neurons, but was also seen in astrocytes and microglia, especially around Aβ deposits. We detected β-secretase 1 transcription at low levels in cultured macrophages. Significantly higher levels of β-secretase 1 transcript were seen in MyD88-deficient macrophages compared with the wild-type control; however, this transcriptional difference disappeared after treatment with 10μM aggregated Aβ42 (data not shown). Levels of β-secretase 1 transcription were similar in...
microglia isolated from brains of 6-month-old MyD88-deficient and wild-type mice (isolation according to Cardona et al., 2006; data not shown). Thus, elevated β-secretase 1 activity could result from cross-talk between bone marrow-derived and resident microglia or from the talk between microglia and neurons. MyD88-deficient bone marrow reconstruction did not inhibit Aβ precursor protein processing.

We further used quantitative reverse transcription polymerase chain reaction to measure expression of Aβ-degrading enzymes, neprilysin and insulin-degrading enzyme (Leissring et al., 2003; Miners et al., 2008). There was no increase in transcription of NEP and IDE genes in Aβ precursor protein transgenic mice reconstructed with MyD88-deficient bone marrow (Fig. 5D), suggesting that MyD88 deficiency has no effect on Aβ catabolism.

MyD88-deficient bone marrow reconstruction does not affect Aβ transportation across the blood–brain barrier

Deane and colleagues (2004) reported that altered communication of Aβ between the brain interstitial fluid and peripheral blood stream affects cerebral Aβ load. Thus, we measured plasma levels of Aβ and expression of RAGE and LRPI in the brain, two receptors that act as shuttles to transport Aβ across blood–brain barrier (Shibata et al., 2000; Deane et al., 2003, 2004). As shown in Fig. 6, neither plasma Aβ40/42 concentrations (Fig. 6A), nor receptor expression in the brain (Fig. 6B) was affected by MyD88-deficient bone marrow reconstruction (P > 0.05).

MyD88-deficient bone marrow reconstruction enhances microglial recruitment towards Aβ deposits and cerebral expression of Aβ-interacting receptors

We hypothesized that microglial clearance of Aβ could be the mechanism to reduce cerebral Aβ. To test this hypothesis, we used confocal microscopy to investigate the relationship between Aβ deposits and microglia. We could only count microglial cells co-localizing with Aβ deposits, as technical difficulties prevented reliable identification of intracellular Aβ in the whole brain. However, we clearly observed the co-localization of Aβ and microglia (Fig. 7A). Recruitment of microglial cells to Aβ deposits appeared to be enhanced in MyD88-deficient bone marrow chimeric mice compared with wild-type control mice (P = 0.069, Fig. 7B). Furthermore, we observed that MyD88-deficient bone marrow reconstruction significantly increased the transcription in the brain of the receptors, scavenger receptor A and CD36 (P < 0.05, Fig. 7C), which are known to be involved in Aβ phagocytosis (El Khoury et al., 1996, 2003).

MyD88-deficiency significantly enhances phagocytosis of aggregated Aβ42 by macrophages after a long-term incubation

As it is difficult to show the effects of MyD88 on microglial uptake of Aβ in vivo, we performed Aβ internalization assays in cultured MyD88-deficient and wild-type bone marrow-derived macrophages. Macrophage internalization of Aβ was detectable within 1 h after administration of aggregated fluorescein isothiocyanate-labelled Aβ42, and levels of internalized Aβ increased as the incubation time increased (Fig. 8A and B). Treatment with cytochalasin D, a known inhibitor of phagocytosis (Liu et al., 2005), blocked the internalization of aggregated Aβ within 2 h, indicating the phagocytic pattern of Aβ42 internalization (data not shown).

In comparing the phagocytic capacities of MyD88-deficient and wild-type macrophages, we observed that MyD88-deficiency did not reduce Aβ phagocytosis following treatment with Aβ42 aggregates at concentrations of 0.5 and 5 μM (data not shown). Notably, following treatment with aggregated Aβ42 at 10 μM, we observed that Aβ42 uptake over a 6 h interval was significantly increased in MyD88-deficient macrophages compared with wild-type macrophages (P = 0.033, Fig. 8A and B). This difference became even greater when Aβ42 treatment was prolonged (24 h, P = 0.044, Fig. 8B). No differences in autofluorescence were observed between MyD88-deficient and wild-type macrophages.

To investigate the relationship between Aβ phagocytosis and inflammatory activation, we monitored TNF-α secretion from Aβ42-phagocytosing macrophages. TNF-α release from MyD88-wild-type macrophages treated with 10 μM Aβ42 started to...
increase significantly after 6 h, whereas no significantly elevated secretion of TNF-α was detected from MyD88-deficient macrophages (Fig. 8C). Aβ42 at 5 μM triggered TNF-α secretion at a lower level (data not shown). Internalization of Aβ42 aggregates at 0.5 μM did not cause TNF-α secretion within the time frame studied (1–24 h) in either cell type, in corroboration with earlier observations (Liu et al., 2005).

To analyse cell damage from high concentrations of Aβ, we used 10 μM aggregated Aβ42 for cell treatment in experiments to measure caspase-3/7 activity and leakage of lactate dehydrogenase into the extracellular medium as indicators of apoptosis and necrosis of macrophages. We did not observe significant cytotoxicity of Aβ42 on macrophages within 24 h (Supplementary Fig. 3).

To confirm the effects of MyD88-deficiency on macrophage phagocytosis of Aβ, we examined the effect of toll-like receptor 2 deficiency on Aβ phagocytosis by macrophages. Toll-like receptor 2 is a receptor upstream of MyD88 and we found that toll-like receptor 2-deficiency similarly increased Aβ internalization (Supplementary Fig. 4).

Discussion

In the pathophysiology of Alzheimer’s disease, microglia are key players, although they behave like a double-edged sword; on one hand, they promote neuronal degeneration by releasing neurotoxic inflammatory mediators; on the other hand, they can clear Aβ plaques by phagocytosis (Akiyama et al., 2000; Bard et al., 2000; Selkoe, 2002; Liu et al., 2005; Wyss-Coray, 2006). Here, we show that depleting MyD88 in bone marrow cells of Aβ precursor protein transgenic mice simultaneously shifts both phagocytic clearance of Aβ and neuroinflammation in the beneficial direction. Furthermore, MyD88 deficiency improves spatial learning and contextual memory deficits in Aβ precursor protein transgenic mice.
by manipulating bone marrow cells. Bone marrow-derived cells have been observed to migrate to the brain and differentiate into microglia (Priller et al., 2001; Simard et al., 2006). These peripherally recruited cells serve immune functions similar to those of their resident counterparts (Turrin et al., 2007). This concept potentially has considerable clinical relevance and has already been translated into clinical practice in patients with amyotrophic lateral sclerosis (Appel et al., 2008). As a therapeutic approach, a genetically engineered MyD88 gene inserted into bone marrow cells is preferable to a pharmacological inhibitor of MyD88, as the latter non-specifically targets MyD88-expressing cells and could have toxic effects on neuronal and endothelial cells. Here we used 3-month-old TgCRND8 and 6- to 9-month-old APP<sub>swr</sub>/PS1<sub>de3</sub> mice with already marked plaque pathology (Chishti et al., 2001; Kim et al., 2009) as animal models of symptomatic Alzheimer's disease, in order to better model the clinical intervention. The results of this study raise the possibility that depleting MyD88 in bone marrow cells could become a therapeutic strategy for patients with Alzheimer's disease.

The inhibition of neuroinflammation by recruited MyD88-deficient bone marrow cells corroborates findings from earlier studies on the role of innate immune receptors, e.g. CD14, toll-like receptor 2 and toll-like receptor 4, in Aβ-triggered microglial activation (Fassbender et al., 2004; Walter et al., 2007; Jana et al., 2008; Reed-Geaghan et al., 2009). Notably, these innate immune receptors all depend on MyD88 in transduction of inflammatory signals (Akira et al., 2001).

Our study showed that bone marrow reconstruction with wild-type cells following whole body irradiation reduces cerebral Aβ in Aβ precursor protein transgenic mice, which was also recently reported by Keene's group (2010). This Aβ-reducing effect is unlikely to be due to the irradiation, as head irradiation alone without bone marrow manipulation does not change Aβ amount in the brain (Keene et al., 2010). Interestingly, we demonstrated that MyD88-deficiency augments this bone marrow cells-mediated Aβ reduction and probably enhances the microglial phagocytosis of Aβ. In Aβ precursor protein transgenic mice reconstructed with MyD88-deficient bone marrow, the total number of microglia was decreased, but recruitment of microglial cells at sites of Aβ deposits was not impaired, and Aβ phagocytosis-related receptors, such as scavenger receptor A and CD36 (El Khoury et al., 1996, 2003) were even upregulated in the brain. In principle, this upregulated scavenger receptor A and CD36 could even facilitate microglia to adhere to Aβ, although this hypothesis needs to be tested further. Direct evidence of phagocytic clearance as the mechanism for reducing Aβ load comes from the observation that Aβ phagocytosis was markedly enhanced in cultured MyD88-deficient macrophages, while the inflammatory responsiveness to Aβ was nearly completely shut down.

Alternatively, inflammation could have modulated expression of Aβ precursor protein and β-secretase 1 as previously reported (Grilli et al., 1995; He et al., 2007; Buggia-Prevot et al., 2008). However, we observed that MyD88-deficient microglia did not decrease but instead increased β-secretase 1 activity, consistent with our observations that systemic knockout of MyD88 markedly increases both β-secretase 1 and γ-secretase activity in the brain. We observed that β-secretase 1 is mainly expressed in neurons, but also is seen in astrocytes and microglia. We further demonstrated that MyD88-deficiency does not significantly alter β-secretase 1 expression in adult brain-derived microglia or cultured Aβ-activated macrophages. Thus, bone marrow-derived microglia themselves are unlikely to contribute to the elevated activity of cerebral β-secretase 1; however, it remains a possibility that the interaction between bone marrow-derived and resident microglia and the cross-talk between microglia and neurons could modify Aβ precursor protein processing in the brain. Furthermore, neprilysin and insulin-degrading enzyme, considered as Aβ-degrading enzymes (Leissring et al., 2003; Miners et al., 2008), were not upregulated in the brain of the MyD88-deficient animal. Therefore, neither decreased Aβ production nor increased Aβ degradation can explain the reduced cerebral Aβ load in our experiments.

Another possibility is that altered transport of Aβ between peripheral circulation and brain parenchyma could affect cerebral Aβ load. However, compared with control animals, Aβ precursor protein transgenic mice with MyD88-deficient bone marrow reconstruction showed changes neither in plasma Aβ levels nor in cerebral expression of RAGE and LR1P, involved in transport of Aβ across the blood–brain barrier (Shibata et al., 2000; Deane et al., 2003, 2004).

Previous studies suggest that inflammatory activation inhibits phagocytosis in mononuclear phagocytes (Koenigsknecht-Talbo and Landreth, 2005; Townsend et al., 2005; Zelcer et al., 2007; Hickman et al., 2008). In both patients with Alzheimer’s disease and aged Aβ precursor protein transgenic mice, decreased Aβ clearance correlates with higher cytokine production (Fiala et al., 2005, 2007; Hickman et al., 2008). Therefore, the reduction of Aβ load in our Aβ precursor protein transgenic mice reconstructed with MyD88-deficient bone marrow could be caused by enhanced microglial phagocytosis of Aβ due to lack of suppression by inflammatory activation.

Activation of toll-like receptors 2, 4 and 9, the upstream receptors of MyD88, has been shown to increase Aβ phagocytosis (Irizarren et al., 2005; Chen et al., 2006; Tahara et al., 2006; Scholtzova et al., 2009). However, in those experiments, the increased Aβ phagocytosis occurred after prestimulation by toll-like receptor ligands. Upon Aβ challenge, phagocytosis, indeed, occurs earlier than inflammatory activation (Liu et al., 2005), with phagocytosis even serving to trigger inflammatory activation (Halle et al., 2008). Moreover, toll-like receptor-triggered phagocytosis was observed not to be strictly dependent on MyD88 (Kong and Ge, 2008).

It is still controversial which cells (resident microglia or bone marrow-derived microglia) are more important for Aβ removal. Resident microglia have limited effects on formation and maintenance of Aβ deposits (Grathwohl et al., 2009). Bone marrow-derived microglia have been reported to be more efficient than their resident counterparts in eliminating Aβ deposits (Simard et al., 2006). However, in our bone marrow chimeric Alzheimer’s disease model, we observed that the number of recruited bone marrow cells was much lower than that of their resident counterparts. It is even possible that recruited bone marrow cells cross-talk with resident microglia, especially around the Aβ deposits, thereby enhancing their phagocytotic capacity.
In conclusion, this study demonstrates that replenishment of microglia with MyD88-deficient bone marrow-derived precursor cells improves cognitive function of the animal model of Alzheimer’s disease, potentially by suppressing detrimental neuroinflammation while, in parallel, enhancing beneficial Aβ clearance. Engineering the MyD88 gene in host bone marrow cells could feasibly offer a therapeutic option to prevent progression of Alzheimer’s disease.

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