SHORT COMMUNICATION

MyD88 Is Dispensable for Cerebral Amyloidosis and Neuroinflammation in APP/PS1 Transgenic Mice

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Activated microglia are associated with amyloid plaques in transgenic mouse models of cerebral amyloidosis and in human Alzheimer disease; yet, their implication in Alzheimer disease pathogenesis remains unclear. It has been suggested that microglia play dual roles depending on the context of activation, contributing negatively to disease pathogenesis by secreting proinflammatory innate cytokines or performing a beneficial role via phagocytosis of amyloid beta (Aβ) deposits. Toll-like receptors, most of which signal through the adaptor protein myeloid differentiation factor 88 (MyD88), have been suggested as candidate Aβ innate pattern recognition receptors. It was recently reported that MyD88 deficiency reduced brain amyloid pathology and microglial activation. To assess a putative role of MyD88 in cerebral amyloidosis and glial activation in APPswe/PS1 (APP/PS1) mice, we crossed MyD88-deficient (MyD88−/−) mice with APP/PS1 mice, interbred first filial offspring, and studied APP/PS1 MyD88+/−, APP/PS1 MyD88+/+, and APP/PS1 MyD88−/− cohorts. Biochemical analysis of detergent-soluble and detergent-insoluble Aβ1-40 or Aβ1-42 in brain homogenates did not reveal significant between-group differences. Furthermore, no significant differences were observed on amyloid plaque load or soluble fibrillar Aβ by quantitative immunohistochemical analysis. In addition, neither activated microglia nor astrocytes differed among the three groups. These data suggest that MyD88 signaling is dispensable for Aβ-induced glial activation and does not significantly affect the nature or extent of cerebral β-amyloidosis in APP/PS1 mice. (Am J Pathol 2014, 184: 2855–2861; http://dx.doi.org/10.1016/j.ajpath.2014.07.004)

Alzheimer disease (AD) is an insidious public health threat characterized by deposition of β-amyloid as senile plaques, formation of neurofibrillary tangles, and large-scale cortical neuronal loss leading to dementia. In addition to these pathognomonic features of the disease, AD patients exhibit low-level chronic neuroinflammation. This is hallmarked by the spatial and temporal occurrence of activated microglia with amyloid beta (Aβ) deposits. Yet, the mechanisms by which microglia recognize and respond to Aβ accumulation remain unclear. Current evidence suggests that there are varied forms of activated microglia in AD, some of which are detrimental and others beneficial.2 Because microglial activation is a complex continuum of varied responses,7 it stands to reason that a wide array of immune molecules may orchestrate microglial responses to Aβ. Ultimately, a clearer understanding of the pathways leading to beneficial microglial responses and clearance of misfolded proteins could open new avenues for AD treatment.

Numerous recent studies have proposed that Toll-like receptors (TLRs) play a role in the microglial response to Aβ and, more specifically, that aggregated Aβ can activate microglia via TLRs.3–4 Most TLRs (except TLR3) signal through the adaptor protein myeloid differentiation factor 88 (MyD88), suggesting that it may play an important role in microglial activation in response to cerebral amyloid accumulation. To test this possibility, two recent studies crossed MyD88 knockout mice with APP/PS1 mouse models of cerebral amyloid deposition and examined effects on cognitive deficits and AD-like pathology. In one study, it was reported

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that MyD88 deficiency of the doubly transgenic APPswe/PS1dE9 mouse reduced cerebral amyloid pathology and microglial activation and decreased expression of CX3CR1 in 10-month-old animals.12,13 Lim and coworkers13 suggested that inhibiting MyD88-associated TLR signaling would alter the microglial activation state, and they reported less cerebral amyloid deposition in this cross. However, their findings were perplexing given previous reports showing that activation of TLRs leads to decreased amyloid load and increased Aβ phagocytosis, leading to the hypothesis that MyD88 deficiency would either cause buildup of amyloid or have no effect on amyloid levels in APP/PS1 mice.6,11,13–15 Another recent study published findings more consistent with this hypothesis, demonstrating that APPswe/PS1A246E mice heterozygous for MyD88 had accelerated spatial learning and memory deficits and increased levels of soluble Aβ oligomers. These results led the authors to conclude that MyD88-mediated activation of microglia was protective in the context of cerebral amyloid deposition.16 In an attempt to clarify the uncertainty surrounding this critical question, we crossed APPswe/PS1dE9 (APP/PS1) mice with MyD88 knockout (MyD88−/−) mice (both on a C57BL/6 background) and analyzed APP/PS1 MyD88+/+, APP/PS1 MyD88+/−, and APP/PS1 MyD88−/− cohorts for Alzheimer-like pathology at 15 months of age.

Materials and Methods

Animals

MyD88 knockout mice (MyD88−/− mice) in a mixed C57BL/6 × 129 background were gifted by Shizuo Akira (Osaka University, Osaka, Japan) and were backcrossed to C57BL/6 mice for >10 generations. B6.Cg-Tg(APPswe,PSEN1dE9) 85Db/J mice (APP/PS1 mice) were purchased from The Jackson Laboratory (Bar Harbor, ME) and were crossed with MyD88−/− mice to generate APP/PS1 MyD88+/− offspring. First filial APP/PS1 MyD88+/− mice were then crossed with MyD88−/− mice. For this study, these second filial offspring (mouse groups: APP/PS1 MyD88+/+, n = 9; APP/PS1 MyD88+/−, n = 9; and APP/PS1 MyD88−/−, n = 6) were aged for 15 months, at which point they were euthanized for analysis of AD-like pathology. All the animal protocols used for this study were approved by the Yale University Institutional Animal Care and Use Committee (New Haven, CT).

Tissue Isolation and Preparation

Animals were deeply anesthetized with 2% to 4% isoflurane and were perfused intracardially with sterile ice-cold phosphate-buffered saline. Brains were extracted and quartered. The two front quarters were snap frozen for protein extraction and biochemical analysis, and the rear quarters were fixed in 4% paraformaldehyde overnight for subsequent OCT and paraffin embedding, according to previously published methods.17,18

Biochemical Analysis

We performed biochemical analysis of Aβ peptides according to a two-step extraction method.17,19 Briefly, one front brain quarter was randomly selected, weighed, and homogenized with 500 μL of ice-cold lysis buffer and centrifuged at 15,000 × g for 15 minutes. The supernatant (triton-soluble fraction) was removed and the pellet was treated with 10× volume of 5 mol/L guanidine HCl (pH 8.0) overnight to extract detergent-insoluble isoforms. Aβ species were quantified in detergent-soluble and detergent-insoluble fractions using Aβ1-40,42 enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Carlsbad, CA), with no dilution for triton-soluble fractions and a 1:500 dilution for guanidine HCl fractions. Total protein levels were determined by bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL) and were used to normalize Aβ ELISA results.

Immunohistochemical Analysis, Microscopy, and Quantitative Image Analysis

Coronal sections (10 μm) were sliced at 50-μm intervals using a Leica RM2125 microtome (Leica Microsystems, Buffalo Grove, IL) and were mounted on glass slides. Sections were then dewaxed in xylenes, hydrated in a graded series of ethanol, and boiled in antigen retrieval buffer (Dako, Glostrup, Denmark) for 30 minutes. Sections were then blocked with serum-free protein block (Dako) and hybridized overnight at 4°C with one or more of the following primary antibodies: Iba1 (dilution 1:250; Wako Chemicals USA Inc., Richmond, VA); 4G8 (dilution 1:200; Covance Inc., Princeton, NJ); OC-oligomer conformation antibody [dilution 1:1000; a gift from Dr. Charles G. Glabe (University of California, Irvine, CA)]; CD45 (Abcam Plc., Cambridge, UK), and glial fibrillary acidic protein (dilution 1:200; Dako). Sections were then washed and incubated with the appropriate fluorophore-conjugated secondary antibodies (dilution 1:200; Invitrogen). For amyloid burden, sections were directly stained with 1% Thioflavin S (ThioS; Sigma-Aldrich, St. Louis, MO) according to standard practice. All the sections were cooverslipped with ProLong Gold mounting medium (Dako) before imaging. Stained sections were viewed and imaged using a Zeiss Axio Image M2 with an attached ApoTome camera (Carl Zeiss Microscopy, Jena, Germany). Quantitative histologic analysis was performed using previously published methods.17,18 Images of four 10-μm sections (150 μm apart) through each anatomical region of interest (hippocampus or cortical areas) were captured and image analysis was performed using ImageJ version 1.45 (NIH, Bethesda, MD). For each image, a threshold optical density was obtained that discriminated staining from background, and each field was manually edited to eliminate artifacts. For β-amyloid and ThioS burden, along with astrocytosis and microgliosis analyses, data are reported as integrated densities. Similar analysis using the percentage of immunolabeled area captured (positive pixels) divided by
the full area captured (total pixels) gave comparable results. Each immunohistochemical analysis was performed by a single examiner (T.M.W., D.G., or K.R.-Z.) in a blinded manner.

Immunoblotting and Densitometric Analysis

One front brain quarter was randomly assigned to homogenization in ice-cold lysis buffer and was centrifuged at 15,000 \( \times \) g for 15 minutes. The supernatant was collected for Western blot analysis. Protein concentrations were determined by bicinchoninic acid protein assay, and 25 to 50 \( \mu \)g of protein was electrophoresed on Mini-PROTEAN TGX gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad Laboratories). Membranes were blocked with blocking buffer (Tris-buffered saline + 0.1% Tween-20 + 5% nonfat dried milk) for 30 minutes and were then incubated overnight at 4°C with one of the following primary antibodies: 22C11 (Millipore, Billerica, MA), 6E10 (Chemicon, Temecula, CA), or CX3CR1 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed and hybridized with the appropriate horseradish peroxidase conjugated secondary antibody (dilution 1:3000; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature in blocking buffer. Proteins were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific). The membranes were stripped and reprobed with antibody to actin (diluted 1:1000; Millipore). The resulting protein bands were scanned using an Epson Perfection V750 Pro scanner (Epson Electronics, Schaumburg, IL), and the integrated density of each protein band was determined using ImageJ version 1.45. To normalize for protein loading, the integrated density of each band was divided by the integrated density of the actin band in the same lane from the same membrane.

Statistical Analysis

All the experiments were performed by an examiner blinded to sample and subject identities (T.M.W., D.G., or K.R.-Z.), and code was not broken until the analyses were completed. Because significant differences were not detected between males and females, both sexes were combined within groups. Data are presented as means ± 1 SEM. For single mean comparisons, the Levene test for equality of the variance and then Student’s \( t \)-test for independent samples were performed. In instances of multiple mean comparisons, one-way analysis of variance was used, followed by post hoc comparison of the means using Bonferroni or Dunnett T3 methods (where appropriateness was determined by the Levene test for equality of the variance). \( P < 0.05 \) was considered significant. All the analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Power analyses were conducted using Power and Precision software version 2 (Biostat, Englewood, NJ).

Results

Cerebral β-Amyloidosis

If MyD88 played a significant role in the microglial response to \( \beta \)-amyloid, then MyD88 deficiency might significantly alter β-amyloidosis in brains of \( APP/PS1 \) mice. However, biochemical analysis of human \( A\beta_40 \) and \( A\beta_42 \) in triton-soluble and triton-insoluble brain extracts and 5 mol/L guanidine HCl–soluble extracts from aged \( APP/PS1 \ MyD88^{+/+} \), \( APP/PS1 \ MyD88^{+/-} \), and \( APP/PS1 \ MyD88^{-/-} \) mice revealed no significant between-group differences in either \( \beta \)-amyloid species (Figure 1). To assess the effect of knocking out \( MyD88 \) on amyloid plaque load in \( APP/PS1 \) mice, we performed a quantitative histologic analysis of diffuse amyloid plaques after staining with human \( \beta \)-amyloid antibody (4G8). The results did not reveal significant differences in amyloid burden among the three groups of mice in three brain regions classically associated with AD in humans: the cingulate cortex, hippocampus, and entorhinal cortex (Figure 2, A and B). In addition, we examined levels of soluble oligomeric fibrillar \( \beta \)-amyloid using a conformation-specific OC antibody\(^\text{20,21}\) and also analyzed fibrillar amyloid plaques by ThioS staining. Complementing the data presented previously herein, neither immunostaining with OC antibody nor staining with ThioS revealed significant between-genotype differences in any of the three brain regions examined (Figure 2, C–E).

Microgliosis and Astrogliosis

Progressive amyloid accumulation in \( APP/PS1 \) mice is associated with neuroinflammation, typically earmarked by

![Figure 1](https://example.com/figure1.png)

**Figure 1**  MyD88 deficiency does not alter amyloid beta (Aβ) oligomer levels in \( APP/PS1 \) mice. Two-step–extracted brain homogenates from \( APP/PS1 \ MyD88^{+/+} \) (white bars), \( APP/PS1 \ MyD88^{+/-} \) (gray bars), and \( APP/PS1 \ MyD88^{-/-} \) (black bars) mice were assayed for triton-soluble human \( A\beta_{40} \) (A) and \( A\beta_{42} \) (B) or for 5 mol/L guanidine HCl–soluble human \( A\beta_{40} \) (C) and \( A\beta_{42} \) (D). All the animals from each group were included in each analysis (\( APP/PS1 \ MyD88^{+/+}, n = 9 \); \( APP/PS1 \ MyD88^{+/-}, n = 9 \); and \( APP/PS1 \ MyD88^{-/-}, n = 6 \)). Bars represent means ± SEM.
plaque-associated reactive microglia and astroglia. Based on the present results indicating no differences in Aβ/β-amyloid abundance between MyD88-sufficient and MyD88-deficient animals, we did not expect differences in gliosis among the three genotypes. However, it could still be possible that microglia deficient in MyD88 were altered in number or activation status in response to, but not affecting, cerebral Aβ load. On quantitative analysis of Iba1 staining for activated microglia in cingulate cortex, hippocampus, and entorhinal cortex, the percentage of area covered by Iba1⁺ cells did not differ among the three genotypes, indicating that MyD88 deficiency did not affect microgliosis in APP/PS1 mice (Figure 3, A and B). Moreover, quantitative analysis for CD45 immunoreactivity, a common marker for activated microglia, and glial fibrillary acidic protein, a marker for reactive astrocytes, did not produce between-genotype differences in the three brain regions surveyed (Figure 3, C and D).

**APP Processing**

To control for the possibility that MyD88 deficiency altered amyloid precursor protein (APP) expression or APP metabolism, biochemical analyses were performed. As expected, the results indicated that APP processing was unaltered among the three genotypes of mice. Furthermore, the amyloidogenic APP β-C terminal fragment also remained unchanged (Figure 4, A–C), indicating that MyD88 deficiency was unable to alter amyloidogenic APP metabolism.

**Fractalkine Receptor Expression**

Several studies have shown a role for CX3CR1, the fractalkine receptor, in the context of microglial responses to cerebral amyloidosis.22,23 In addition, a recent report showed decreased CX3CR1 expression in MyD88-deficient mouse models of cerebral amyloid deposition.12 To eliminate the possibility that the microglia in the cohort of MyD88-deficient animals had altered CX3CR1 expression, we performed Western blot analysis on brain homogenates from all three genotypes using CX3CR1 antibody, followed by densitometric analysis. However, no significant between-group differences were detected (Figure 4, A and D), indicating that MyD88 deficiency had no obvious effect on microglial CX3CR1 expression in APP/PS1 mice.

**Discussion**

Although the innate immune response is thought to play a central role in AD progression, the specific role of microglia in this process is still uncertain. There are multiple studies *in vivo* and *in vitro* suggesting that TLRs play a role in cerebral Aβ clearance.4,6,7,24–26 For example, a recent study examining the effects of knocking out Tlr2 in...
**APP**swe/PS1(A246E) mice found decreased amyloid plaque burden and increased soluble Aβ.

In another study, APPswe/PS1dE9 mice homozygous for a destructive mutation in Tlr4 (Tlr4<sup>dE9/dE9</sup>) were found to have increased diffuse and fibrillar Aβ deposits as well as increased soluble and insoluble Aβ abundance.

Although mounting evidence points to TLRs impacting AD-like pathology in mouse models and Aβ-induced neurotoxicity in vitro, studies examining the role of MyD88 in microglial responses to Aβ have been contradictory. For example, MyD88-deficient APPswe/PS1dE9 and APPswe/PS1(A246E) mice with reduced MyD88 expression have previously been reported to have reduced amyloid plaque burden. Yet, APPswe/PS1(A246E) mice with attenuated MyD88 expression were also found to have increased levels of soluble Aβ and accelerated spatial memory deficit in the T-water maze, whereas MyD88-deficient APPswe/PS1dE9 mice had decreased soluble Aβ and improved spatial learning in the Morris water maze.

These conflicting results raise at least two different scenarios: signaling through TLRs via MyD88 in response to increasing Aβ burden results in detrimental inflammation that ultimately aggravates disease; and signaling through MyD88 is protective against AD-like disease. To clarify this, we crossed APPswe/PS1dE9 mice with MyD88 knockout mice and examined aged offspring. The results indicate that MyD88-deficient APPswe/PS1dE9 mice are not significantly different from MyD88-sufficient APPswe/PS1dE9 mice on measures of AD-like pathology. Thus, in contrast to the two other reports, we conclude that signaling through TLRs via MyD88 does not significantly affect the nature or extent of AD-like pathology in aged APP/PS1 mouse brains.

The differing results between the present study and previous reports could be accounted for by differences in the transgenic lines used and/or breeding anomalies. Whereas Lim et al. used the same APP/PS1 transgenic and MyD88-deficient mouse lines on a pure C57BL/6 background as reported herein, another previous study used a different line of transgenic mice [APPswe/PS1(A246E)], making it difficult to directly compare these results to theirs. In addition, both of the aforementioned studies had difficulty
producing APP/PS1 MyD88−/− animals from their crosses of MyD88−/− mice with APP/PS1 animals, a problem that we did not encounter in this report. Another difference between these studies and ours is the age of the mice. Previous reports showed that MyD88 deficiency impacted AD-like pathology in APP/PS1 transgenic mice at 9 months of age.12,16 According to the present results, if there is such an effect, it is quantitatively minor at 15 months of age. One explanation is that MyD88 deficiency slows amyloid buildup during the early stages of amyloidosis in APP/PS1 mice and that at some point the amyloid burden reaches a threshold that MyD88-deficient microglia are unable to mitigate. In addition, differences in tissue dissection, sampling, and extraction may account for some of the discrepancies between reports. It is also important to consider statistical power to detect a significant difference, if one were, in fact, present. In this regard, a formal statistical power analysis was conducted and revealed that this study has 80% power to detect a 40% difference in plaque load at a significance level of 0.05. Furthermore, note that Lim et al used fewer mice in their study (n = 6 per group), and they were able to detect significant differences (effect size, 30% to 40%; P < 0.05) between genotypes in amyloid load, indicating that sizable differences can be detected between groups with these numbers. Last, it deserves mentioning that the present methods have been validated17,18 and have produced significant differences in plaque load and gliosis in several other experimental studies on mouse models of cerebral amyloidosis. Whatever the reason for discrepancy, if MyD88 deficiency does not affect amyloidosis in aged APP/PS1 mice, it is not likely to be clinically relevant.

It is becoming clear that a wide array of activated microglial phenotypes exist, some of which are detrimental and others beneficial, in the context of AD. In addition, the nature of the stimulus can largely affect the type of microglial response that is mounted.1,2 Based on these results, we conclude that blocking proinflammatory innate immune responses by making APP/PS1 mice deficient for MyD88 does not significantly affect AD-like pathology. However, rebalancing inflammation by blocking key immunosuppressive factors may be more effective at remediating AD-like pathology. For example, the present group previously showed that genetic ablation of innate immune TGF-β–Smad 2/3 signaling leads to resolution of cerebral amyloidosis, which does not come at the cost of damaging neuroinflammation.18 Other studies currently ongoing in the Town laboratory also indicate that boosting proinflammatory responses can reduce the extent of β-amyloidosis in APP/PS1 mice.

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