ORIGINAL RESEARCH

Transfer of a healthy microbiota reduces amyloid and tau pathology in an Alzheimer's disease animal model

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ABSTRACT

Objective Cerebral amyloidosis and severe tauopathy in the brain are key pathological features of Alzheimer's disease (AD). Despite a strong influence of the intestinal microbiota on AD, the causal relationship between the gut microbiota and AD pathophysiology is still elusive.

Design Using a recently developed AD-like pathology with amyloid and neurofibrillary tangles (ADLP^{APT}) transgenic mouse model of AD, which shows amyloid plaques, neurofibrillary tangles and reactive gliosis in their brains along with memory deficits, we examined the impact of the gut microbiota on AD pathogenesis.

Results Composition of the gut microbiota in ADLP^{APT} mice differed from that of healthy wild-type (WT) mice. Besides, ADLP^{APT} mice showed a loss of epithelial barrier integrity and chronic intestinal and systemic inflammation. Both frequent transfer and transplantation of the faecal microbiota from WT mice into ADLP^{APT} mice ameliorated the formation of amyloid β plaques and neurofibrillary tangles, glial reactivity and cognitive impairment. Additionally, the faecal microbiota transfer reversed abnormalities in the colonic expression of genes related to intestinal macrophage activity and the circulating blood inflammatory monocytes in the ADLP^{APT} recipient mice.

Conclusion These results indicate that microbiota-mediated intestinal and systemic immune aberrations contribute to the pathogenesis of AD in ADLP^{APT} mice, providing new insights into the relationship between the gut (colonic gene expression, gut permeability), blood (blood immune cell population) and brain (pathology) axis and AD (memory deficits). Thus, restoring gut microbial homeostasis may have beneficial effects on AD treatment.

Significance of this study

What is already known on this subject?

- Alzheimer's disease (AD) is an age-related neurodegenerative disease characterised by accumulation of beta-amyloid plaque, neurofibrillary tau tangles and neuroinflammation throughout the brain; memory deficits are also a common feature of AD.
- Human studies using patients with AD have shown that gut microbial diversity is different between patients with AD and age-matched controls, and that probiotics improves cognitive functions in patients with AD.
- Studies using several animal models of AD including 5×FAD mouse have also found a correlation between gut microbiota and amyloid pathology; however, the causal association between gut microbiota and tau tangles is not studied.

What are the new findings?

- Altered intestinal microbiota, loss of epithelial barrier integrity, and intestinal and systemic inflammation were shown in AD-like pathology with amyloid and neurofibrillary tangles (ADLP^{APT})mice, a recently developed transgenic AD mouse model showing beta-amyloid (Aβ) and tau pathologies together in the brain.
- Long-term, frequent transfer and transplantation of faecal microbiota of healthy wild-type mice alleviated Aβ deposition, tau pathology, reactive gliosis and memory impairment in ADLP^{APT} mice.
- Specifically, long-term transfer of healthy faecal microbiota to ADLP^{APT} mice turned back intestinal macrophage activity and circulating blood Ly6C^{hi} monocyte population, as similar levels to those of wild-type mice.
- It provides new insights into the relationship between the gut (colonic gene expression, gut permeability), blood (blood immune cell population) and brain (pathology) axis and AD.

INTRODUCTION

Alzheimer's disease (AD) is characterised by accumulation of beta-amyloid (Aβ) and hyperphosphorylated tau protein throughout the brain, together with synaptic dysfunction.[1] In addition, changes in inflammatory signalling occur in the AD brain, as revealed by activated microglia and astrocytes.[2] These features lead to cognitive impairment and memory loss.[3] Although the neuropathological hallmarks of AD are well documented, there is no effective treatment capable of stopping or delaying AD progression. Therefore, we need to consider new approaches to AD treatment based on various pathological pathways.

The gut microbiota is recognised as a forgotten modulator of host physiology and metabolism,[4] and the influence of the microbiota extends to brain development and cognitive function.[5] A possible

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- The restoration of intestinal microbiota and the maintenance of healthy microbiota contribute to slowing down AD pathogenesis and cognitive impairment with age.
- Changing or maintaining the environment of intestinal microbiota is one of the factors to consider in the clinical treatment for patients with AD or cognitively impaired subjects and clinical prevention for ageing population.

link between the gut microbiota and neurodegenerative disease has been reported in Parkinson's disease and amyotrophic lateral sclerosis.[6 7] The microbiome studies on a transgenic AD model and patients have attempted to refine previous knowledge about AD pathogenesis and to characterise AD pathology differently. Alterations in the composition and biodiversity of the intestinal microbiota are observed in mouse AD models and human patients with AD.[8 9] APP/PS1 mice treated with antibiotics (ABX)[10] and 3×TgAD mice treated with probiotics[11] manifested reduced brain Aβ deposition with an altered pattern of peripherally circulating cytokines, chemokines and gut hormones. Furthermore, Aβ pathology is transmissible via a transplant of the altered microbiota to germ-free *APP*-transgenic mice,[8] suggesting that the gut microbiota is a hidden player in early AD pathogenesis. Nevertheless, specific signalling mediators or mechanisms behind even a remote contribution of the gut microbiota to AD pathogenesis including tau tangles are unclear at present.

In this study, we examined the effects of the gut microbiota on the pathogenesis of AD in a recently developed murine transgenic AD model, AD-like pathology with amyloid and neurofibrillary tangles (ADLP^{APT}), showing both Aβ and tau pathologies in the brain.[12] We found that alterations in gut microbial community composition and the loss of epithelial barrier integrity induce systemic chronic inflammation in the pathological condition manifested by ADLP^{APT} mice. Of note, frequent transfer and transplantation of faecal microbiota from healthy wild-type (WT) mice mitigated Aβ plaque and tau tangle formations, and memory deficit as well as reactive gliosis in ADLP^{APT} mice. In addition, the modulation of the gut microbiota changed the colonic gene expression and the peripheral immune cell population. Our results elucidate a critical role of the gut microbiota in AD pathogenesis and suggest that aberrant immune responses act as a potential regulator of remote communication between the host brain and the gut microbiota.

METHODS

Behavioural test

Spontaneous alternation in Y-maze is a behavioural test for short-term spatial learning and memory.[13] Contextual fear-conditioning is a test for long-term spatial learning and memory function.[14] Open-field test is commonly used to assess anxiety-like behaviours in rodents.[15]

16S rRNA gene-based community analysis

Faecal metagenomic DNA was extracted, and hypervariable regions of 16S ribosomal RNA (rRNA) gene were sequenced with a 454 pyrosequencing GS FLX Titanium (Roche) and Illumina MiSeq sequencing (Illumina).

Frequent transfer and transplantation of faecal microbiota

Fresh faecal matters of WT mice were orally provided to ADLP^{APT} mice for 16 weeks for frequent faecal microbiota transfer (FMT), and to ABX-pretreated ADLP^{APT} mice for 4 weeks for faecal microbiota transplantation.

Aβ ELISA analysis

ELISA was performed for measuring Aβ levels in the cerebral cortex of ADLP^{APT} mice.[16]

Immunohistochemistry

Free-floating tissue slices were stained with the appropriate primary antibodies and Alexa fluorophore-conjugated secondary antibodies for confocal imaging.

Database submission

The 16S rRNA amplicon and colonic RNA-Seq data sets were deposited to the European Nucleotide Archive under accession number PRJEB24580.

The detailed procedures and additional methods are described in the online supplementary appendix.

RESULTS

Community-level alterations in the intestinal microbiota of ADLP^{APT} mice

To test whether the neuropathological changes in the AD brain are associated with alterations in the gut microbiota, we studied the composition of the gut microbiota in ADLP^{APT} mice at the age of 8 months when severe Aβ and tau pathologies are present. Using 454 pyrosequencing of 16S rRNA gene amplicons, operational taxonomic units (OTUs)-based community comparisons of the gut microbiota between WT and ADLP^{APT} mice were conducted by principal coordinate analysis on the basis of Bray-Curtis (OTU abundance) and Jaccard (OTU occurrence) dissimilarities. The gut microbiota of ADLP^{APT} mice was separated from that of WT mice in terms of the occurrence ($p=0.03$) and abundance ($p=0.019$) of the OUT-level bacterial species (figure 1A, B), indicating that ADLP^{APT} mice have an altered gut microbiota.

To figure out whether these microbial community alterations are initiated before AD pathologies, we monitored the composition of the gut microbiota of ADLP^{APT} mice at the ages of 2, 4 and 6 months. By means of Illumina MiSeq sequencing of 16S rRNA gene amplicons, the gut microbiota of ADLP^{APT} mice was distinguished from that of WT mice significantly at the age of 2 months in terms of OTU occurrence ($p=0.017$) and abundance ($p=0.017$). Although this separation weakened at the age of 4 months, that is, at the onset of AD-related pathologies (OTU occurrence, $p=0.263$; OTU abundance, $p=0.207$), the separation became recurrent at the age of 6 months when AD pathologies were in progress (OTU occurrence, $p=0.042$; OTU abundance, $p=0.143$; online supplementary figure 1A, B). These observations suggested that ADLP^{APT} mice have an altered gut microbiota early in life, and the alterations in the gut microbiota may be attributed to the transgenic AD-related modifications.

The bacterial diversity of ADLP^{APT} mice was slightly higher than that of WT mice at all ages, although it did not reach statistical significance (online supplementary figure 1C). To determine specific bacterial groups associated with AD development, we identified bacterial taxa discriminating the gut microbiota of WT and ADLP^{APT} mice by linear discriminant analysis effect size (LEfSe) analysis.[17] A few bacterial genera specific to WT or ADLP^{APT} mice were identified, but they varied with age, and no bacterial groups commonly specific

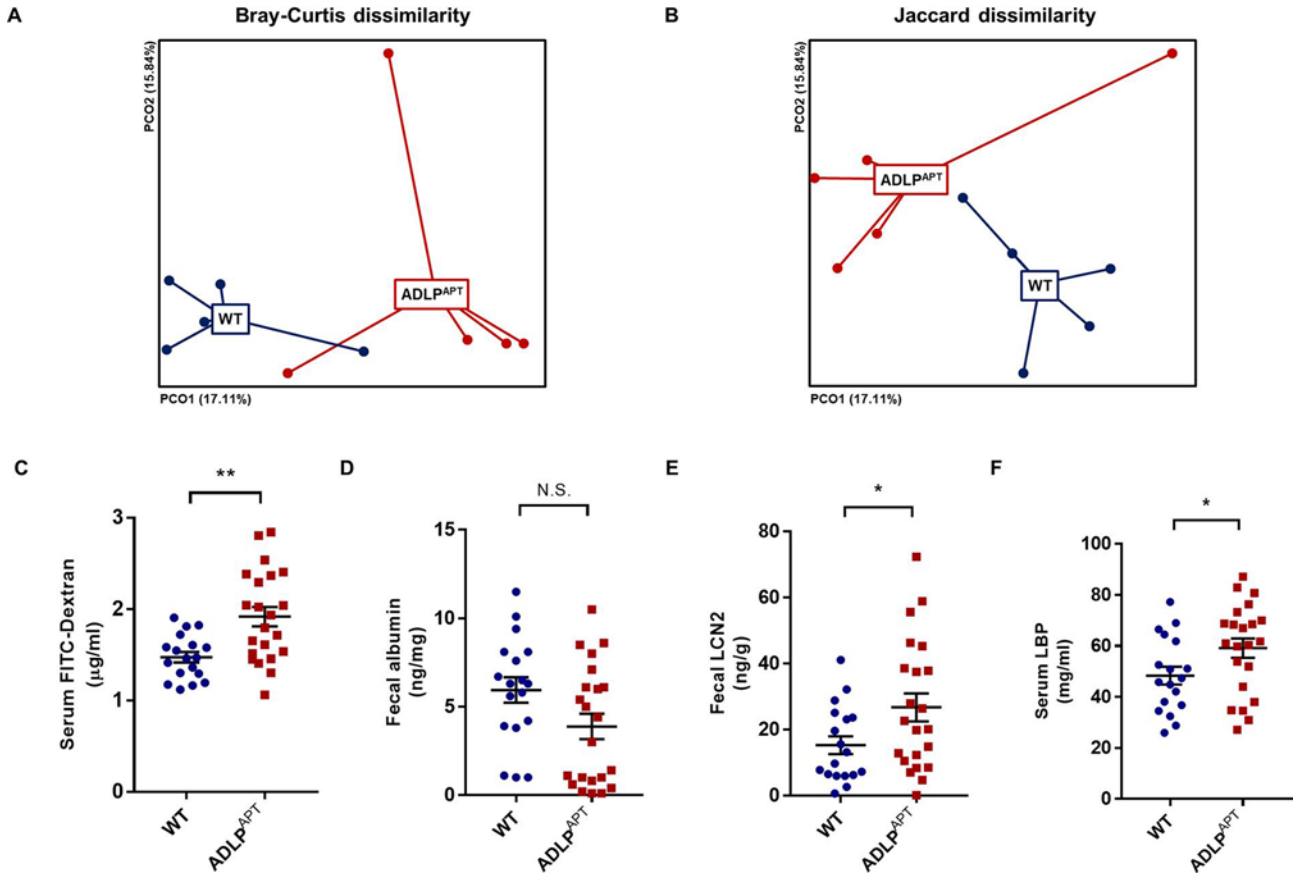

Figure 1 The alteration of gut microbiota composition and high intestinal permeability along with the intestinal and systemic inflammation in ADLP^{APT} mice. The bacterial community composition of 8-month-old WT (n=5) and ADLP^{APT} (n=5) mice was compared by PCoA on the basis of (A) Bray-Curtis dissimilarity (p=0.03) and (B) Jaccard dissimilarity (p=0.019). Statistical significance was evaluated by ANOSIM with 999 permutations. (C) High intestinal permeability of ADLP^{APT} mice was observed as compared with WT mice by the FITC-dextran method (p=0.0014). (D) Similar concentrations of faecal albumin were detected in WT and ADLP^{APT} mice (p=0.0516). (E) High concentrations of faecal lipocalin 2 (LCN2) conducive to intestinal inflammation were detected in ADLP^{APT} mice compared with WT mice (p=0.0360). (F) High concentrations of serum lipopolysaccharide-binding protein (LBP) conducive to systemic inflammation were found in ADLP^{APT} mice compared with WT mice (p=0.0454). The WT (n=18) and ADLP^{APT} (n=22) mice at the age of 6 months were used. All data are mean±SEM; *p<0.05, **p<0.01, according to two-tailed Student's t-test. ADLP^{APT}, AD-like pathology with amyloid and neurofibrillarytangles; ANOSIM, analysis of similarities; FITC, fluorescein isothiocyanate; PCO1 and PCO2, principal coordinates; PCoA, principal coordinate analysis; WT, wild-type.

to ADLP^{APT} mice were identified (online supplementary figure 2A–F). These data suggested that community-level alterations (rather than changes in a single bacterial taxon) in the intestinal microbiota may be associated with AD development in ADLP^{APT} mice.

Chronic low-grade intestinal and systemic inflammation in ADLP^{APT} mice and high gut permeability in ADLP^{APT} mice

To determine the effect of the altered gut microbiota in ADLP^{APT} mice on the gut barrier functions, we measured intestinal permeability of WT and ADLP^{APT} mice by a fluorescein isothiocyanate (FITC)-dextran method.[18] We found that serum concentration of FITC-dextran in ADLP^{APT} mice was significantly higher than that in WT mice, pointing to an increase in the intestinal permeability of ADLP^{APT} mice (figure 1C). We measured faecal albumin levels of WT and ADLP^{APT} mice, but no difference was observed (figure 1D), suggesting that high intestinal permeability of ADLP^{APT} mice may not be attributed to cellular injury. In addition, a high concentration of faecal lipocalin 2 representing intestinal inflammation[19] was demonstrated in ADLP^{APT} mice compared with WT mice (figure 1E), suggesting that chronic low-grade inflammation is present in the ADLP^{APT} intestine. A loss of intestinal barrier integrity in ADLP^{APT} mice enables a flow directed from the intestinal lumen to the peripheral organs through the portal vein. In this regard, we measured serum lipopolysaccharide-binding protein (LBP) level as a marker of systemic dissemination of microbiota-derived compounds causing systemic inflammation.[20] We found elevated levels of serum LBP in ADLP^{APT} mice compared with WT mice (figure 1F), pointing to possible systemic inflammation caused by an influx of gut microbiota-derived antigens including lipopolysaccharide (LPS) into the circulatory system of ADLP^{APT} mice. Altogether, these results provided evidence that microbial components such as LPS from the altered gut microbiota of ADLP^{APT} mice are likely to contribute to AD pathogenesis through a permeable gut barrier.

Frequent transfer of faecal microbiota attenuates the cognitive impairment, Aβ plaque burden, tau pathology and reactive glial activation in ADLP^{APT} mice

To clarify implications of the altered gut microbiota for AD pathogenesis, we transferred faecal materials of WT mice almost daily to ADLP^{APT} mice for 4 months (FMT) (figure 2A). The FMT largely affected the altered gut

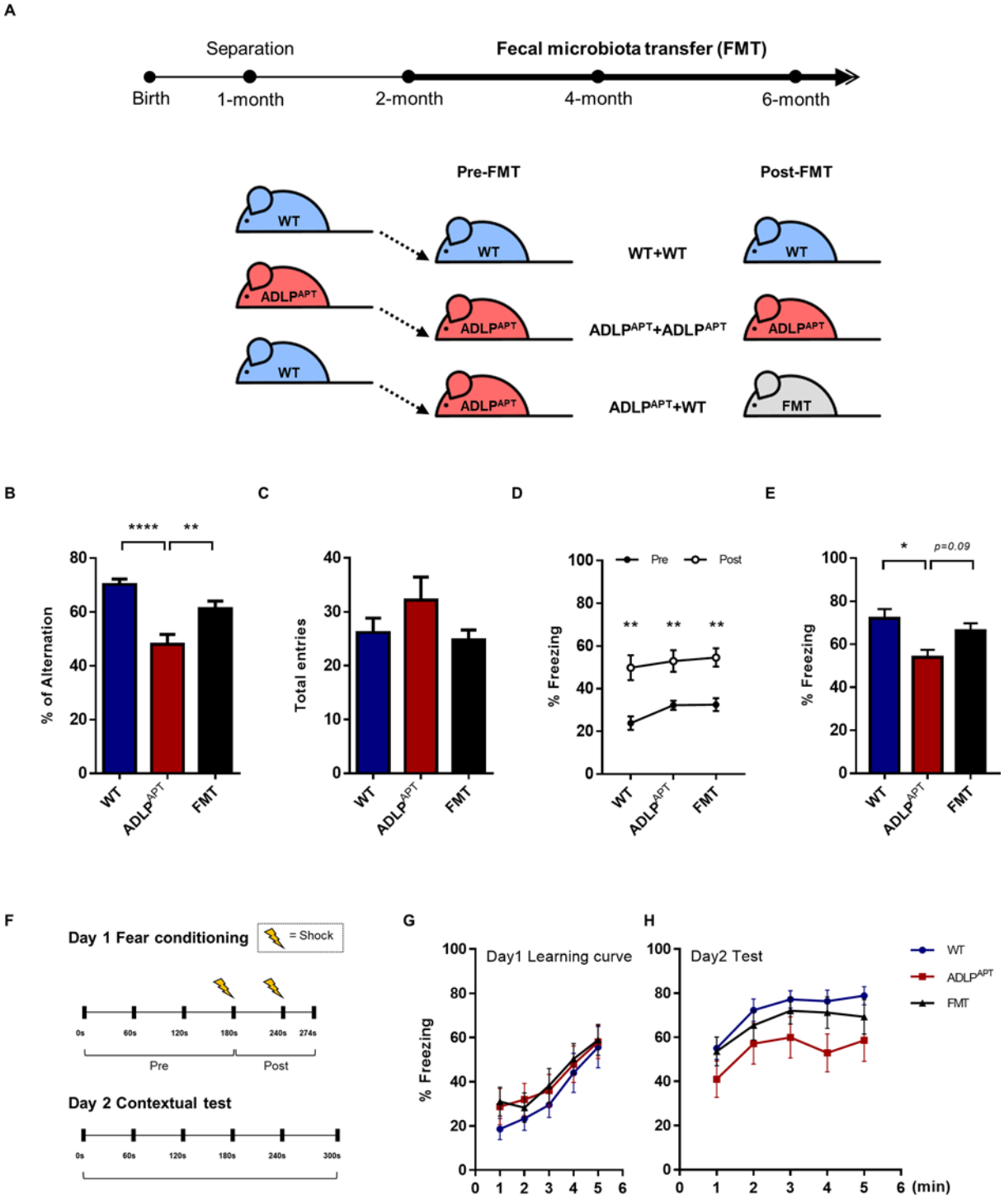

Figure 2 Improved memory deficits in ADLP^{APT} mice after FMT. (A) Study design. The animals were separated by genotype within 4 weeks after birth. After that, 4 or 5 mice of each group were raised in the same cage for 6 months under the identical specific pathogen-free conditions. The WT (n=16) and ADLP^{APT} (n=14) groups received the faecal extract from healthy WT mice and diseased mice (strain ADLP^{APT}), respectively. Another ADLP^{APT} group (FMT, n=16) received the faecal extract from WT donors to directly change gut microbial composition. All three groups of the 8-week-old mice underwent oral delivery of faecal microbiota 5 days a week for 4 months, and their faeces were collected as indicators of the gut microbiota every 2 months. The 6-month-old animals subjected to the experiment were euthanised, after which blood, colon and brain samples were collected. (B) Percentage of spontaneous alternations performance and (C) number of total entries of WT mice (n=16), ADLP^{APT} mice (n=13) and FMT mice (n=16) at the age of 6 months in the Y-maze task. (D) Percentage of time spent freezing during the 180 s before foot-shock exposure (pre) and during 90 s after foot-shock exposure (post) in the fear-conditioning, and (E) percentage of time spent freezing in the contextual test of WT mice (n=16), ADLP^{APT} mice (n=13) and FMT mice (n=16) at the age of 6 months. (F) Schematic diagram of the contextual fear-conditioning test. (G) The learning curves during the fear-conditioning (day 1) and (H) percentage of time spent freezing during the contextual test (day 2). Data represent mean±SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, according to one-way analysis of variance followed by Tukey's multiple comparison test. ADLP^{APT}, AD-like pathology with amyloid and neurofibrillarytangles; FMT, faecal microbiota transfer; WT, wild-type.

microbiota of ADLP^{APT} mice. The composition of gut microbiota of FMT mice was significantly changed after 2 months of FMT (4-month-old mice) ($p<0.05$); finally, it had similarities with both the microbiota of WT and ADLP^{APT} mice after 4 months of FMT (6-month-old mice) (WT, $p=0.186$; ADLP^{APT}, $p=0.201$) (online supplementary figure 3). We conducted two behavioural tests to assess spatial learning and memory function.[21] In the Y-maze, spatial short-term memory impairment was significantly rescued in FMT mice, compared with ADLP^{APT} mice, with no significant differences in the number of total entries (figure 2B, C). We next evaluated FMT mice in the contextual fear-conditioning assay. While all groups in the fear-conditioning test showed similar freezing behaviours after foot-shock exposure (figure 2D–G), FMT mice showed a tendency to improve long-term memory impairment in the contextual test (figure 2E, H). These data suggest that continuous exposure to the healthy gut microbiota may improve the impaired cognitive function of ADLP^{APT} mice.

To identify an influence of the healthy gut microbiota on Aβ plaque deposition, we detected the Aβ plaque burden in ADLP^{APT} mice. The total plaque area of the frontal cortex (figure 3A, B) and hippocampus (figure 3G, H) was significantly smaller in FMT mice than in ADLP^{APT} mice. Moreover, ELISA analysis showed that, compared with ADLP^{APT} mice,

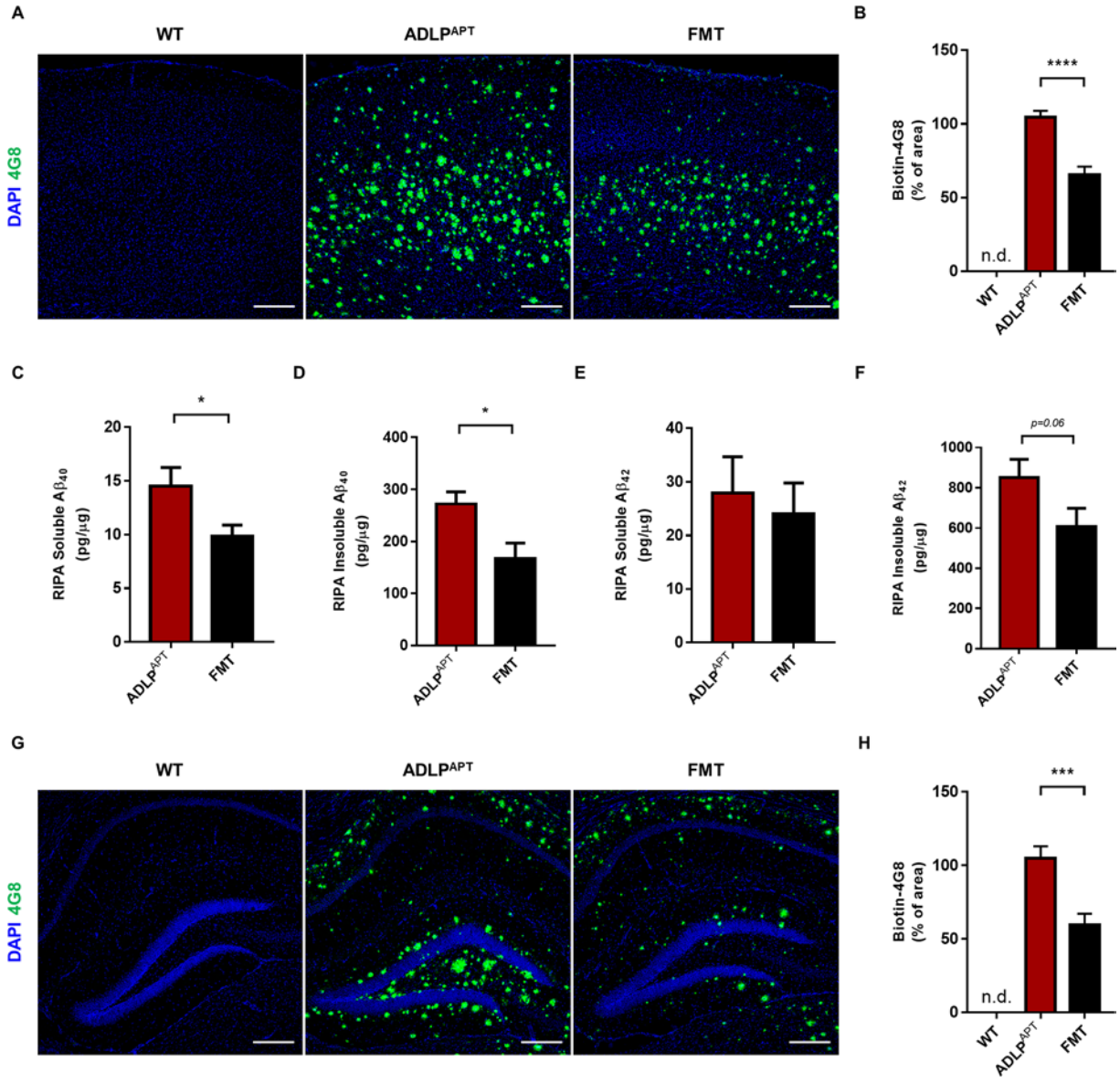

Figure 3 Reduced Aβ plaque burden in ADLP^{APT} mice after FMT. Immunostaining for Aβ plaque burden in the brain slices from 6-month-old ADLP^{APT} mice after FMT treatment. Representative confocal images of (A) Aβ plaque (Biotin-4G8) staining and (B) quantification of Aβ-positive area proportion in the frontal cortex of ADLP^{APT} mice (n=14) and FMT mice (n=16). ELISA analysis for measuring Aβ levels in the brain lysates from 6-month-old ADLP^{APT} mice after FMT treatment. (C–F) RIPA soluble and insoluble Aβ levels in the cerebral cortex of ADLP^{APT} mice (n=14) and FMT (n=16). Representative confocal images of (G) Aβ plaque (Biotin-4G8) staining and (H) quantification of the Aβ-positive area proportion in the hippocampus of ADLP^{APT} mice (n=14) and FMT mice (n=16). Data represent mean±SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, according to two-tailed unpaired t-test. Scale bars in A and G: 200 µm. Aβ, beta-amyloid; ADLP^{APT}, AD-like pathology with amyloid and neurofibrillarytangles; DAPI, 4',6-Diamidino-2-Phenylindole; FMT, faecal microbiota transfer; n.d., non-detection; RIPA, radioimmunoprecipitation assay; WT, wild-type.

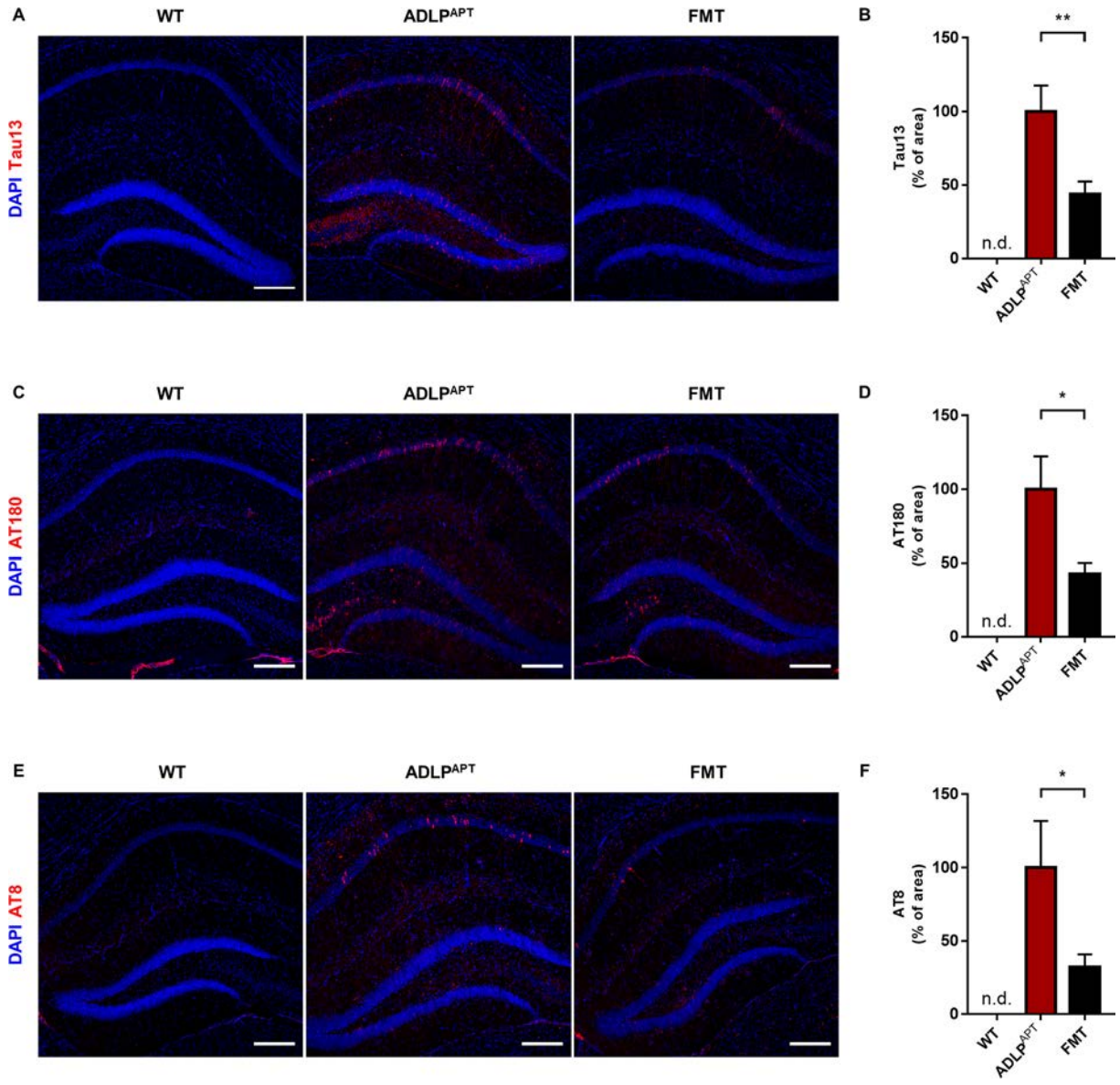

Figure 4 Reduced aggregation of tau in the hippocampus of ADLP^{APT} mice after FMT. Immunostaining for tau pathology in the brain slices from 6-month-old ADLP^{APT} mice after FMT treatment. Representative confocal images of (A) human tau (Tau13) staining and (B) quantification of the Tau13-positive area in the hippocampus of ADLP^{APT} mice (n=14) and FMT mice (n=16). Representative confocal images of (C) PHF-tau (AT180) staining and (D) quantification of the AT180-positive area in the hippocampus of ADLP^{APT} mice (n=14) and FMT mice (n=16). Representative confocal images of (E) PHF-tau (AT8) staining and (F) quantification of AT8-positive area in the hippocampus of ADLP^{APT} mice (n=14) and FMT mice (n=16). Data represent mean±SEM; *p<0.05, **p<0.01, according to two-tailed unpaired t-test. Scale bars in A, C and E: 200 µm. ADLP^{APT}, AD-like pathology with amyloid and neurofibrillarytangles; DAPI, 4',6-Diamidino-2-Phenylindole; FMT, faecal microbiota transfer; n.d., non-detection; PHF, paired helical filament; WT, wild-type.

both soluble and insoluble $A\beta_{40}$ levels of the cerebral cortex were significantly decreased in FMT mice (figure 3C, D). Besides, FMT mice showed tendency to amelioration of $A\beta_{42}$ aggregation (p=0.0683) (figure 3E, F).

Next, we found that the FMT-induced changes of the gut microbiota decreased pathological tau aggregates in the hippocampus of ADLP^{APT} mice (figure 4A, B). Using the AT180 (hyperphosphorylation at the Thr231 site representing an early event of tau pathology) and AT8 (hyperphosphorylation at the Ser202/Thr205 site representing a middle or late stage of tau pathology) antibodies, we found significant reduction of both AT180-detected and AT8-detected paired helical filament-like tau in ADLP^{APT} mice by FMT (figure 4C–F). These results suggested that exposure to the healthy gut microbiota could weaken both Aβ deposition and tau pathology in the brain.

As gliosis in the central nervous system (CNS) is a well-established pathological sign of AD,[22] we identified activated microglia stained with ionised calcium-binding adaptor molecule (Iba1),[23] and hypertrophic reactive astrocytes stained with glial fibrillar acidic protein (GFAP).[24] Compared with ADLP^{APT} mice, both Iba1-positive microglia and GFAP-positive astrocyte (figure 5A–C) dramatically decreased in the frontal cortex of FMT mice, indicating that gliosis was attenuated by the transfer of the healthy gut microbiota, which in turn may reduce the immunopathological responses in the brains of ADLP^{APT} mice.

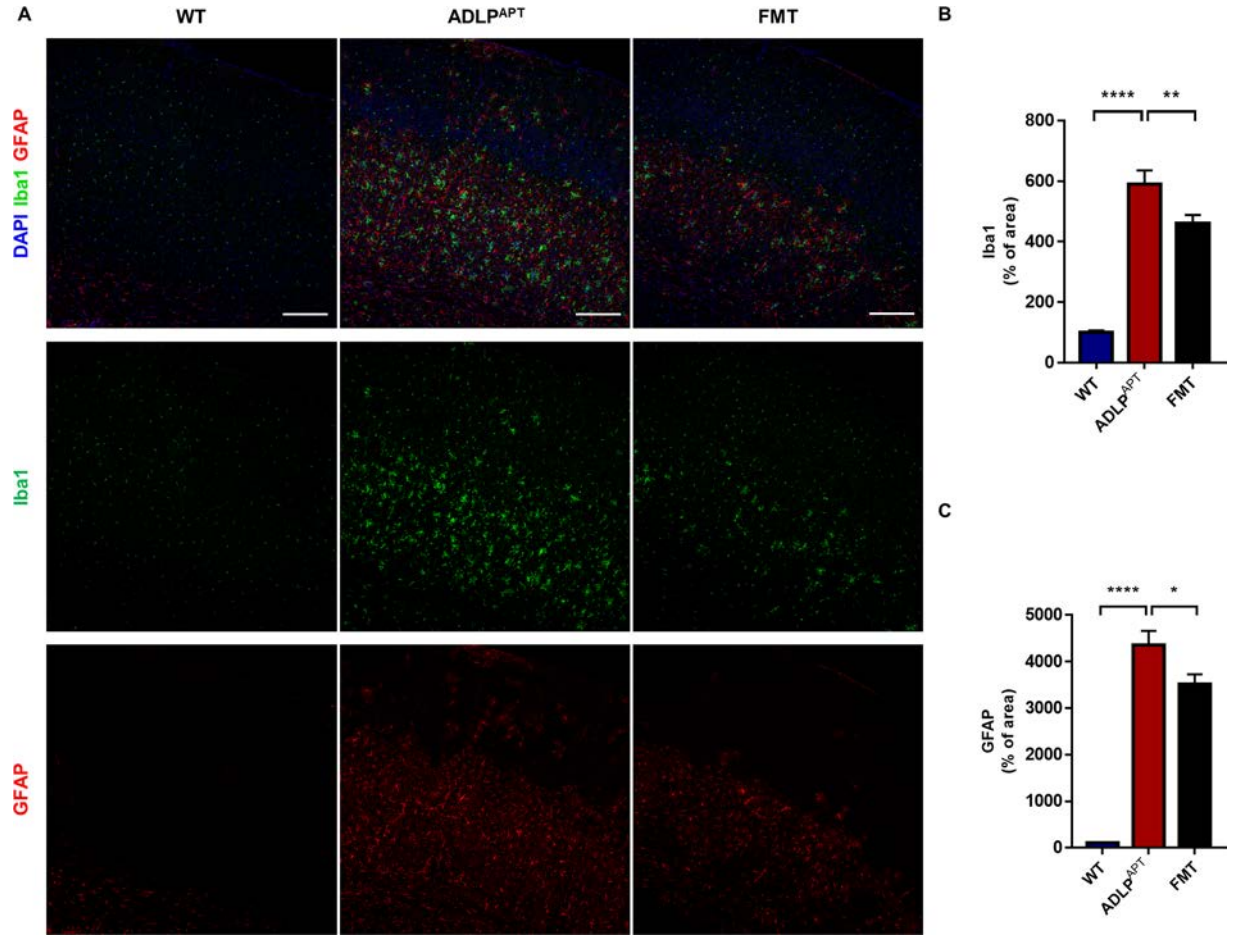

Figure 5 Suppression of microgliosis and astrogliosis in ADLP^{APT} mice after FMT. Immunostaining for gliosis in the brain slices from 6-month-old ADLP^{APT} mice after FMT treatment. Representative confocal images of (A) microgliosis (Iba1) and/or astrogliosis (GFAP) staining in the frontal cortex of WT mice (n=16), ADLP^{APT} mice (n=14) and FMT mice (n=16). Graphs represent the quantification of (B) Iba1-positive and (C) GFAP-positive area. Data represent mean±SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, according to one-way analysis of variance followed by Tukey's multiple comparison test. Scale bars in A: 200 µm. ADLP^{APT}, AD-like pathology with amyloid and neurofibrillarytangles; FMT, faecal microbiota transfer; GFAP, glial fibrillar acidic protein; Iba1, ionised calcium-binding adaptor molecule; WT, wild-type.

Microbiota transplantation improves behavioural impairment, Aβ plaque burden, tau pathology and reactive glial activation in ABX-treated ADLP^{APT} mice

To further clarify the microbiota-induced suppression of AD pathologies, we transplanted faecal microbiota of either WT or ADLP^{APT} mice into recipient ADLP^{APT} mice pretreated with five types of ABX (online supplementary figure 4A–C). First, we confirmed that ABX suppressed microbial activities effectively (online supplementary figure 4B), but no changes were observed in Aβ plaque deposition (online supplementary figure 5A–E, and H), tau tangle formation (online supplementary figure 5F, I and J) and gliosis (online supplementary figure 5G, K). The transplantation of WT faecal microbiota transformed the composition of gut microbiota of ABX-treated ADLP^{APT} mice; the gut microbiota of two groups of recipient ABX-treated ADLP^{APT} mice were separated from each other in accordance with those of the donor mice (p=0.003, OTU occurrence; OTU abundance, p=0.059) (online supplementary figure 6A–C, and E). Interestingly, the ADLP^{APT} microbiota-transplanted ABX-treated ADLP^{APT} mice showed higher gut permeability, compared with the WT microbiota-transplanted ABX-treated ADLP^{APT} mice (online supplementary figure 6D). The WT microbiota-transplantation also ameliorated hyperactivity in ABX-treated ADLP^{APT} mice (online supplementary figure 7).

We next tested whether the faecal microbiota transplantation affects AD pathogenesis in the brain. Donor ADLP^{APT} mice and ADLP^{APT} microbiota-transplanted ABX-treated ADLP^{APT} mice showed similar levels of Aβ plaque, whereas the WT microbiota transplantation delayed Aβ plaque deposition in the frontal cortex of ABX-treated ADLP^{APT} mice (online supplementary figure 8A, B). A decrease in the AT180-detected tau (online supplementary figure 8C, D) and a tendency to decrease in the AT8-detected tau (online supplementary figure 8E, F) were observed in ABX-treated ADLP^{APT} mice after WT microbiota transplantation, but not after ADLP^{APT} microbiota transplantation. In addition, the difference in the gliosis was observed between the ABX-treated ADLP^{APT} mice receiving WT or ADLP^{APT} microbiota (online supplementary figure 9A–C). Taken together the results with ABX-treated ADLP^{APT} mice indicated that re-establishing the healthy gut microbiota may alleviate the intestinal barrier integrity and AD pathologies in the brain of ADLP^{APT} mice.

FMT reverses colonic transcriptional changes associated with tissue degeneration and intestinal macrophage activity in ADLP^{APT} mice

To elucidate the gut-associated mechanisms underlying FMT-induced reversal of AD-related pathologies in ADLP^{APT} mice,

we performed RNA-Seq analysis on the colonic tissue of WT, ADLP^{APT} and FMT mice at the ages of 6 months. Thus, 3.1±0.3 Gb (mean±SD) of high-quality reads per mouse were obtained, which yielded 19 257±115 gene assignments. Using differential expression analysis of the colonic transcriptome data, we regarded genes as upregulated in ADLP^{APT} mice if their expression levels were significantly higher than those of WT mice; we regarded genes as downregulated in ADLP^{APT} mice if their expression levels were significantly lower than those of WT mice (online supplementary figure 10A, B). Consequently, we found 78 upregulated genes and 136 downregulated genes in ADLP^{APT} mice compared with WT mice ($q<0.05$; figure 6A and online supplementary table 1), indicating that the colonic transcriptome of ADLP^{APT} mice is altered. To figure out how FMT treatment affects colonic transcriptome of ADLP^{APT}, we tracked changes in the upregulated and downregulated genes by comparing gene expression levels of FMT mice with those of WT mice (online supplementary figure 10C, D). Among the 78 genes upregulated in ADLP^{APT} mice, we found no significant differences in the expression of 60 genes in FMT mice. In the case of the 136 downregulated genes, 83 genes showed no significant differences between ADLP^{APT} and FMT mice (figure 6B), indicating that the majority of the colonic genes with altered expression levels were normalised by FMT. On the basis of the expression levels of differentially expressed (DE) genes, FMT mice clustered closer to WT mice than to ADLP^{APT} mice (figure 6C).

By gene ontology analysis, we found that the altered DE genes of ADLP^{APT} mice are associated with ageing-related tissue degeneration and deterioration of mucosal immunity (figure 6D and online supplementary table 2). Specifically, the genes upregulated in ADLP^{APT} mice contribute to decreased cell proliferation, cellular senescence and cell growth arrest, which are well-characterised ageing-related phenotypes.[25] The downregulation of genes related to mitochondrial and ribosomal activities in ADLP^{APT} mice may lead to aberrations in ATP synthesis and protein synthesis, which are considered key features of ageing and neurodegenerative diseases.[26 27] Furthermore, ADLP^{APT} mice showed downregulation of genes involved in mucosal immune responses, many of which are related to intestinal macrophage activity and the monocyte–macrophage axis. In the intestine, intestinal macrophages that are continually replenished by circulating blood Ly6C^{hi} monocytes contribute to gut homeostasis including tissue homeostasis.[28] Thus, the comparative analysis of the colonic transcriptome revealed that FMT reversed gut-associated abnormalities such as ageing-induced tissue degeneration and macrophage-mediated immune dysfunction in ADLP^{APT} mice.

FMT returns the oversized population of Ly6G^{-}Ly6C^{+}CD115^{+} myeloid cells in ADLP^{APT} mice to the WT level

To identify blood immune cell populations that are related to the AD pathologies affected in ADLP^{APT} mice by FMT, we compared profiles of peripheral blood leucocytes (PBLs) in WT, ADLP^{APT} and FMT mice (online supplementary figure 11A). Because colonic transcriptome data showed the altered gene expression associated with intestinal macrophages, we focused on changes in CD11c^{-}CD11b^{+} myeloid cells. In flow cytometric analysis, fractional changes of CD11c^{-}CD11b^{+}Ly6G^{-}Ly6C^{+}CD115^{+} inflammatory monocytes were detected according to the changes in disease severity. Indeed, the proportion of inflammatory monocytes was significantly higher among the PBLs of ADLP^{APT} mice (63.87% on average) than among those of WT mice (42.28% on average) or FMT mice (39.94% on average) (figure 7A, B), indicating covariation of inflammatory monocytes with disease severity in ADLP^{APT} mice. However, FMT did not affect other myeloid cells (online supplementary figure 11B–D). These data provided evidence that the gut microbiota-associated signals could contribute to AD pathogenesis in the brain of ADLP^{APT} mice in which the circulating inflammatory monocytes may facilitate as a cellular mediator.

DISCUSSION

The objective of this study was to investigate the potential influence of the gut microbiota on AD pathogenesis. This study reveals that alterations of gut microbiota composition early in the life of ADLP^{APT} mice give rise to chronic intestinal inflammation and to the loss of epithelial integrity, subsequently leading to systemic inflammation. These gut-associated abnormalities coincided with Aβ deposition, tau pathology, reactive gliosis and cognitive impairment in ADLP^{APT} mice; monocyte recruitment and intestinal macrophage dysfunction appear to be strongly associated. Of note, the pathogenic features of ADLP^{APT} mice were significantly attenuated by the normal microbiota either frequently transferred or transplanted in ADLP^{APT}. Thus, our findings are noteworthy for supporting the notion that gut–brain bidirectional communication affects the development of AD neuropathology.

Differences of the gut microbiota composition in ADLP^{APT} mice from that of WT mice started to show early in the life (at 2 months of age) and were more pronounced in aged mice, suggesting that alterations in the gut microbiota can be attributed to the transgenic modification. The AD transgene-induced microbial alterations did not reach division-wide or phylum-level changes; few discriminant bacterial taxa for ADLP^{APT} mice were detected, but these bacterial taxa were not confined to a specific bacterial group across different ages. These observations imply that complicated signalling cascades via the gut–brain axis are influential, but are not a strict determinant of particular members of the gut microbiota. These also point to the importance of community ecology, microbe–microbe interactions or microbial functional capacity (rather than the importance of a single bacterial taxon) for AD pathogenesis. It is worth noting in this context that FMT-induced modifications towards the microbiota pattern of WT mice appeared to ameliorate amyloidosis, tau pathology, reactive gliosis and cognitive impairment in ADLP^{APT} mice. While Minter *et al*[10] employed ABX cocktails to suppress microbial activities in APP/PS1 mice, Bonfili *et al*[11] tested a probiotic mixture in 3×TgAD mice. Recently, Sun *et al*[29] treated faecal matter of WT mice to APP/PS1 mice. Nevertheless, different manipulations of the gut microbiota in several AD model mice have similar consequences on pathophysiological features, suggesting that gut microbial dysbiosis can be regarded at least as an accelerator of AD.

The colonic gene expression pattern in ADLP^{APT} mice was also different from that of WT mice, and many of these genes participate in inflammatory responses of monocyte chemotaxis and macrophage activation. The gut-resident macrophages have been found to be crucial for the initiation of immune responses for microbiological surveillance,[28] and to perform a principal function in the clearance of apoptotic or senescent cells and in epithelial integrity and remodelling.[30 31] As circulating Ly6C^{hi} monocytes locally mature into intestinal macrophages, monocyte replenishment (eg, the C-C motif chemokine ligand 2–C-C chemokine receptor 2 (CCL2–CCR2 axis)) is the essential process for the maintenance of intestinal macrophages.[32] Therefore,

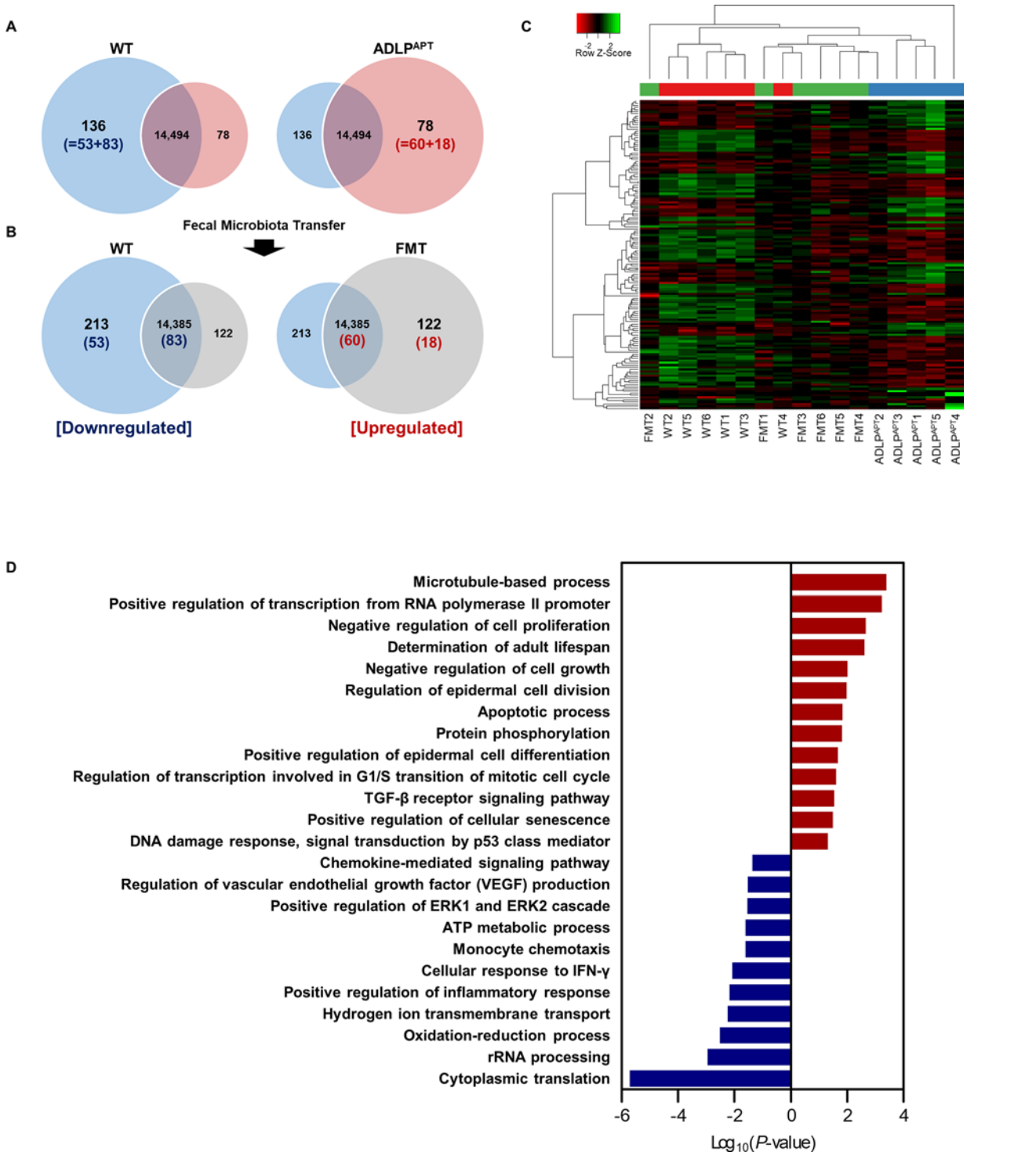

Figure 6 The reversal of the altered colonic gene expression in ADLP^{APT} mice after FMT. (A) RNA-Seq analysis revealed differentially expressed (DE) colonic genes upregulated or downregulated in ADLP^{APT} mice (n=5) as compared with WT mice (n=6) in a Venn diagram. (B) The majority of the DE genes were recovered in FMT mice (n=6). (C) The profile of the DE genes in WT, ADLP^{APT} and FMT mice was visualised on a heat map. The gene profiles of the three groups were clustered on the basis of Euclidean distance by the UPGMA method. (D) Bar graphs represent the gene ontology terms of the DE genes categorised by biological processes. ADLP^{APT}, AD-like pathology with amyloid and neurofibrillarytangles; ATP, adenosine triphosphate; DE, differentially expressed; ERK, extracellular signal-regulated kinase; FMT, faecal microbiota transfer; IFN-γ, interferon gamma; rRNA, ribosomal RNA; TGF-β, transforming growth factor beta; UPGMA, unweighted pair group method with arithmetic mean; WT, wild-type.

downregulation of genes related to chemokine-mediated monocyte recruitment may cause incomplete replenishment of monocytes, thereby causing a deficiency of intestinal macrophages in ADLP^{APT} mice. In this regard, downregulation of interferon-γ signalling[33] and extracellular signal-regulated kinase signalling[34] pathways of macrophage activation may be attributed to perturbations in immune and epithelial homeostasis in the intestine of ADLP^{APT} mice. Interestingly, the majority of ADLP^{APT} mouse-specific altered DE genes returned to almost normal expression by FMT; these macrophage-mediated changes may further

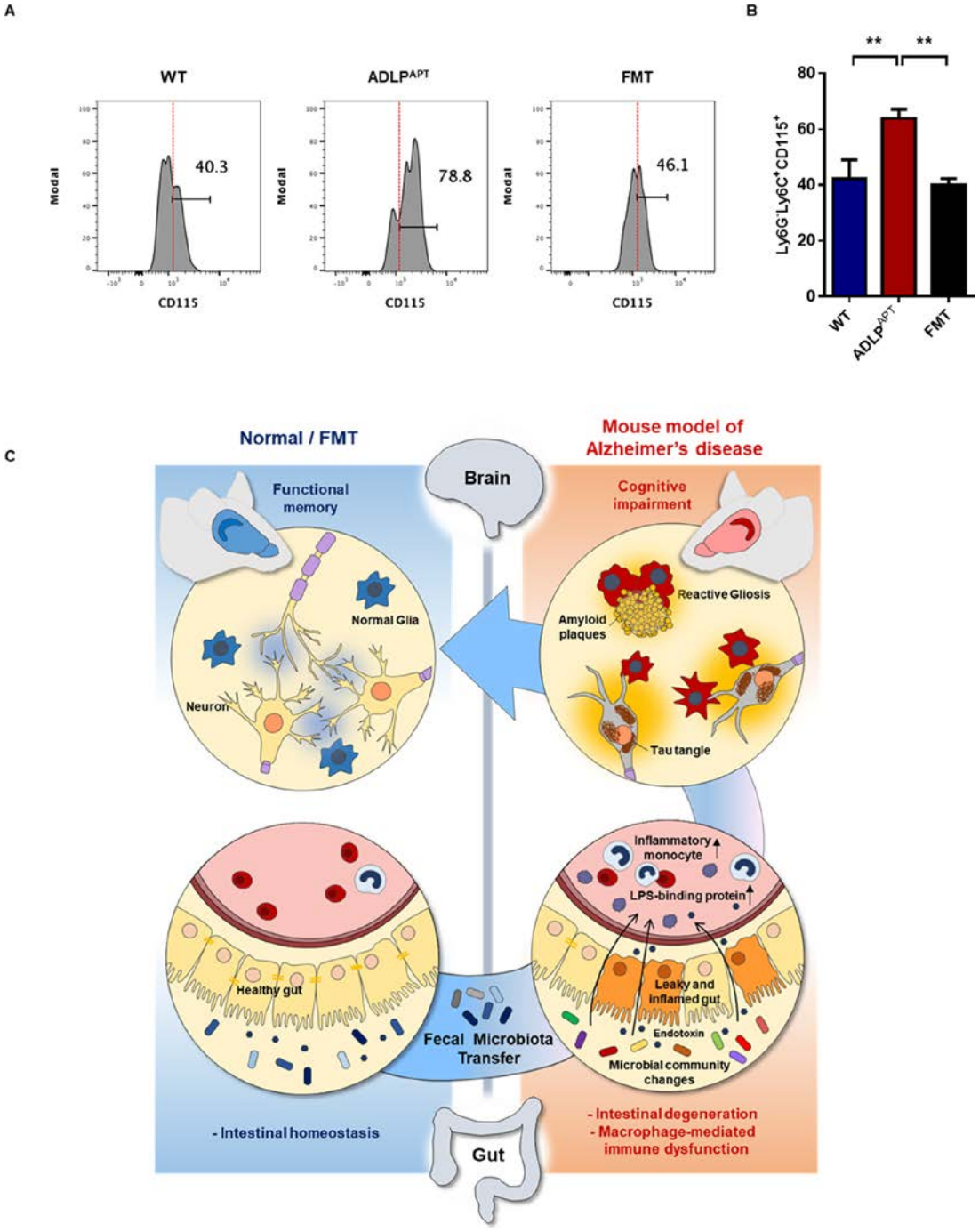

Figure 7 FMT induces alteration of a peripheral immune cell population. Flow cytometric analysis of PBLs of 6-month-old ADLP^{APT} mice after FMT treatment. Representative (A) flow cytometry histograms and (B) a graph of the proportion (%) of CD11c$^-$CD11b$^+$Ly6G$^-$Ly6C$^+$CD115$^+$ subsets in the WT mice (n=10), ADLP^{APT} mice (n=9) and FMT mice (n=10). Data represent mean±SEM; *p<0.05, **p<0.01, according to one-way analysis of variance followed by Tukey's multiple comparison test. (C) A schematic diagram for this study. The alterations of gut microbial community and leaky gut in ADLP^{APT} mice increased LPS-binding protein and inflammatory monocyte in the blood. Transfer of a healthy intestinal microbiota ameliorates AD pathologies including amyloid plaques, tau tangles and reactive gliosis in the brains of ADLP^{APT} mice. AD, Alzheimer's disease; ADLP^{APT}, AD-like pathology with amyloid and neurofibrillarytangles; FMT, faecal microbiota transfer; LPS, lipopolysaccharide; PBLs, peripheral blood leucocytes; WT, wild-type.

contribute to the suppression of cerebral amyloidosis, tauopathy, reactive gliosis and cognitive deficits in ADLP^{APT} mice by means of circulating Ly6C$^+$ monocytes.

These gut-associated changes may mutually contribute to the increased gut barrier permeability and chronic inflammation seen in the intestine of ADLP^{APT} mice; these problems are considered potential risk factors of AD.[35] In order to determine differential contribution of Aβ-related and tau-related neurodegeneration to gut barrier function, we also evaluated the intestinal permeability in ADLP mice expressing solely Aβ pathology (ADLP$^{APP/PS1}$ mice) or tau pathology (ADLP^{Tau} mice). The high gut permeability shown in ADLP^{APT} mice was more associated

with Aβ plaques than with tau-related pathology (online supplementary figure 12A). Furthermore, the high gut permeability is considered a prominent feature associated with Aβ deposition in the brain in that it was similarly observed in App knock-in mouse models of AD[36] (online supplementary figure 12B). This microbiota-associated intestinal barrier dysfunction has been observed in elderly people,[37] aged mice[38] and *Drosophila* flies[39] and are thought to be a trigger of ageing-associated chronic, low-grade, systemic inflammation. On the basis of the colonic transcriptome data and the faecal albumin level, the intestinal barrier dysfunction of ADLP^{APT} mice may be characterised by epithelial cell senescence and dysfunction of macrophage-mediated barrier integrity, rather than cellular damage to gut epithelial layers. These findings are similar to the results of Thevaranjan *et al*[40] suggesting that the gut leakiness in aged mice is due to paracellular permeability. Moreover, continuous anomalies in intestinal immune responses themselves can trigger a microbial imbalance,[41] thereby causing a vicious cycle of chronic inflammation. Given that intestinal permeability precedes systemic inflammation,[40] the transfer of microbiota-derived compounds from the intestine to the blood circulation is a strong driver of systemic inflammation, which subsequently mediates tissue damage with age.[42] Indeed, ADLP^{APT} mice showed high serum LBP concentration, an increase of which correlates with systemic inflammation in the elderly.[43] In particular, an alteration of the inflammatory monocytes was returned to the similar state of WT mice by FMT, suggesting that ADLP^{APT} mice are systemically exposed to gut-associated bacterial endotoxins, such as LPS. Considering that the circulating microbial endotoxins increase monocyte frequencies in the circulation by inducing monocyte emigration from the bone marrow,[44] metabolic endotoxaemia in ADLP^{APT} mice might provide an opportunity for the Ly6C^{+} inflammatory monocytes to circulate into the bloodstream. The increment of inflammatory monocytes in the periphery could affect AD pathogenesis by infiltrating the brain directly or producing signalling molecules to the brain.[45] Therefore, the role of circulating monocytes to the brain in AD remains for further investigation.

High gut permeability can deteriorate the blood–brain barrier, and CNS is easily exposed to the contents of the altered gut microbiota.[46] LPS has been found in the brain and participates in AD pathology. In vitro incubation of LPS and the Aβ peptide augments Aβ fibrillogenesis,[47] and in vivo injection of bacterial LPS causes cognitive deficits in WT mice and transgenic AD mouse models, with the formation of Aβ deposits and tau aggregation in the hippocampus.[48 49] In addition, LPS colocalised with Aβ plaques is observed in postmortem brain tissue from patients with AD.[50] Considering that Aβ expression has been described for antimicrobial activity against microbial infection in brain,[51] a permeable intestinal barrier and the following systemic inflammation may account for Aβ plaque burden in ADLP^{APT} mice. According to recent study revealing that 5×FAD mice lost their phagocytic functions during the tolerant phase of microglia,[52] it is expected that chronic translocation of microbiota-derived metabolites and/or bacteria into the circulation contributes to the metabolic defects of microglia in ADLP^{APT} mice.

Recently, FMT received a great deal of attention regarding treatment of diseases such as *Clostridium difficile* infection, IBD and autism.[53 54] In this study, we first examined the effects of FMT on both Aβ and tau pathologies in the mouse model of AD. Of note, microbiota replacement by healthy gut microbiota alleviated Aβ, tau and reactive glial pathology in the brain (figure 7C). On the basis of these findings, we suggest that abnormal systemic factors in AD are new therapeutic targets in this disease. Therefore, modulation of peripheral systems including the microbiota may provide a novel perspective on the AD pathogenesis.

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