Title
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Journal
Journal of Neuroimmune Pharmacology, 15(4)

ISSN
1557-1890

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Publication Date
2020-12-01

DOI
10.1007/s11481-020-09908-9

Peer reviewed

Journal of Neuroimmune Pharmacology
https://doi.org/10.1007/s11481-020-09908-9

ORIGINAL ARTICLE

Immune and Inflammatory Determinants Underlying Alzheimer's Disease Pathology

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Received: 13 November 2019 / Accepted: 13 February 2020

Abstract

This study examines the link between peripheral immune changes in perpetuation of the Alzheimer's disease (AD) neuropathology and cognitive deficits. Our research design using human AD patients and rodent model is supported by past evidence from genomic studies. We observed an active immune response against Aβ as indicated by the increased Aβ specific IgG antibody in the serum of AD and patients with mild cognitive impairments as compared to healthy controls. A similar increase in IgG and decrease in IgM antibody against Aβ was also confirmed in the 5xFAD mouse model of AD. More importantly, we observed a negative correlation between reduced IgM levels and cognitive dysfunction that manifested as impaired memory consolidation. Strong peripheral immune activation was supported by increased activation of microglia in the brain and macrophages in the spleen of AD mice compared to wild type control littermates. Furthermore, inflammatory cytokine IL-21 that is involved in antibody class switching was elevated in the plasma of AD patients and correlated positively with the IgG antibody levels. Concurrently, an increase in IL-21 and IL-17 was observed in spleen cells from AD mice. Further investigation revealed that proportions of T follicular helper (Tfh) cells that secrete IL-21 are increased in the spleen of AD mice. In contrast to Tfh, the frequency of B1 cells that produce IgM antibodies was reduced in AD mice. Altogether, these data indicate that in AD the immune tolerance to Aβ is compromised leading to chronic immune/inflammatory responses against Aβ that are detrimental and cause neuropathology.

Keywords Alzheimer's disease · Inflammation · Aβ · IL-21 · Tfh · Cognition

Introduction

Alzheimer's disease (AD) has become the most common form of dementia in the elderly, affecting over 5 million people in the United States alone. Unfortunately, current therapies are not very effective. Hence, there is an urgent need to improve our understanding of the mechanisms that drive the development and progression of AD. A growing body of work now indicates that age-related cognitive decline is caused by age-related inflammation (Heppner et al. 2015). In fact, it is likely that inflammation is a key trigger for Mild Cognitive Impairment (MCI) and its evolution to AD. Recent evidence from genomic studies has highlighted the role of inflammation and the immune system in the etiology of AD (Heneka et al. 2015a, b; Heppner et al. 2015).

AD is characterized by the deposition of amyloid beta (Aβ) protein and formation of neurofibrillary tau tangles. Aβ is a self-protein that is present in healthy individuals. However, in AD abnormalities including defective clearance lead to accumulation of Aβ. The excess Aβ aggregates and forms oligomers as well as fibrils that are deposited in the brain as plaques, leading to neurodegeneration and pathology associated with AD. The defective clearance of Aβ is believed to

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11481-020-09908-9) contains supplementary material, which is available to authorized users.

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Published online: 24 February 2020

enhance inflammation though the underlying mechanisms are not well understood. The immune system plays a major role in preventing inflammatory responses against self-antigens such as Aβ (Banchereau and Steinman 1998). Antigen presenting cells (APCs) such as dendritic cells and macrophages take up foreign antigens, but this uptake also induces the upregulation of activation markers and the secretion of pro-inflammatory cytokines. The APCs then present the antigen to T cells in the lymph node to initiate adaptive immune T and B lymphocyte responses. The CD4 T cells proliferate and aid B cells in removing the antigen while CD8 T cells kill infected cells. The initial encounter of B cells with antigens results in the production of IgM class antibodies. Once help arrives in the form of CD4 T cells, an isotype switch occurs where the IgM antibody class switches to IgG or another isotype. Further, the B cell receptor rearranges, enhancing the affinity of IgG for the antigen and facilitating its clearance. In contrast, the uptake of self-antigens by APCs does not result in their activation or inflammatory cytokine secretion. Therefore, there is no downstream T and B cell activation. The tolerance to Aβ appears to be compromised in AD, as studies indicate that AD patients display strong Aβ-specific T cell responses (Cao and Zheng 2018; Monsonego et al. 2003). An increase in Aβ antibody-secreting B cells has also been observed in AD (Gaskin et al. 1993; Sollvander et al. 2015). Our previous studies also indicate that the immune system loses its tolerance to Aβ in AD patients resulting in chronic ongoing immune/inflammatory responses against the antigen (Agrawal et al. 2018). In addition to increased immune activation, emerging evidence suggests that these immune cells can affect neuroinflammation. Recently, CD4 T cells were demonstrated to enhance the expression of MHC-II on microglia and affect neuroinflammation (Mittal et al. 2019).

The objective of the present study is to use the 5xFAD mouse model of AD to determine possible mechanisms responsible for the increased inflammatory/immune responses and to determine whether these changes correlate with cognitive impairments. A clearer understanding of the link between peripheral immune changes and cognitive dysfunction has the potential for development of novel circulatory biomarkers for AD.

Materials and Method

Human Serum Samples

De-identified serum samples from AD (Alzheimer's disease) and MCI (mild cognitive impaired) patients as well as age- and sex-matched healthy controls (HC) were obtained from the Alzheimer's disease research center (ADRC) core at UCI. Participants enrolled in the UCI ADRC cohorts undergo, at minimum, a comprehensive annual evaluation that includes a neurological and physical examination, neuropsychological assessment, brain imaging, lumbar puncture, blood and diagnostic tests, and an interview with a study partner. Table 1 provides the description of the samples.

Mice

All animal experimentation procedures were performed in accordance with the guidelines provided by NIH and approved by the University of California Irvine Institutional Animal Care and Use Committee. Animals were maintained in standard housing conditions (20 °C ± 1 °C; 70% ± 10% humidity; 12 h:12 h light and dark cycle) and provided ad libitum access to standard rodent chow (Envigo Teklad 2020x) and water. Early and late stage male and female AD mice and their wild type littermate controls were evaluated at approximately 3–4 and 8–9 months of age, respectively and described henceforth as 4 mo and 8 mo ages. In both cases the animals were stratified by age to maintain equivalent age distributions between experimental groups.

Behavioral Testing

For one week prior to behavior, testing the lead investigator, blinded to the animal groupings, handled all mice for habituation for 2–3 min per mouse each day. Independent investigators, blinded to the experimental groups, scored all behavior files. Male mice were tested separately from female mice.

For the FE test two contexts (A and B) were used to determine whether mice could learn and then extinguish conditioned fear responses over the course of 5 days (Acharya et al. 2019; Chang et al. 2009). The conditioning testing chamber (context A; 17.5 × 17.5 × 18 cm; Coulbourn Instruments) had a steel grid floor and the scent of 10% acetic acid in water, while the extinction chamber (context B) had a smooth Plexiglas floor, additional stimulus lighting and the scent of

Table 1 Description of AD, MCI cohort

	AD	MCI	HC
Number of subjects	26	26	26
Age Range (Years)	75–87	74–86	75–85
Age Mean (Years)	79.6	79.6	79.3
Gender			
Male	13	13	13
Female	13	13	13
MMSE scores	6 to 25	21–30	27–30
Mean+/-S.D. MMSE	17.93+/−5.5	27.5+/−2.4	29.6± 0.8
CDR (sum)	4.5 to 13	0.5–4.5	0–0.5
Mean+/-S.D. CDR	8± 3.1	1.97± 1.3	0.07+/−2.1

MMSE- The Mini–Mental State Examination

CDR- The Clinical Dementia Rating

10% almond extract in water. Each test chamber was disinfected in between testing trials. Digital cameras were mounted in the ceiling of each chamber and connected via a quad processor for automated scoring of freezing (FreezeFrame, Coulbourn Instruments). For each mouse, the fear conditioning protocol for day 1 used context A and started with a 120 s pre-fear conditioning habituation followed by 3 pairings of a 120 s, 80 dB, 16 kHz white noise conditioned stimulus (CS) co-terminating with a 1 s, 0.6 mA foot shock (US) presented at 2 min intervals (day 1, T_1-T_3). For extinction training, starting on day 2, each mouse was placed in context B and allowed to acclimate for 2 min followed by extinction training that was comprised of 15 non-reinforced 120 s CS presentations at 5 s intervals. Fear extinction data is presented as the average of 5 tones. Extinction training was repeated daily for 2 additional days. Subsequently, retention testing was performed on day 5 where each mouse was returned to context B and following a 2 min acclimation period freezing was assessed during three non-US reinforced CS tones (16 kHz, 80 dB, lasting 120 s) at 2 min intervals. Extinction memory was calculated as the percentage of time spent freezing during the test. After behavior studies were completed, immunohistochemical and immunological analyses were performed on subsets of the same mice that had been used in the behavior studies.

Aβ Antibody Assay

Previously collected plasma samples from aged and young human subjects and serum samples from AD, MCI and age-matched HC were assayed for the presence of Aβ42 specific antibodies using an in-house ELISA as described (Agrawal et al. 2018; Qu et al. 2014). Plasma from mice was assayed in a similar manner to human plasma except that the secondary IgG and IgM antibodies used for detection were anti-mouse.

Flow Cytometry and Cytokine in Mouse Spleen

Spleen was collected from 5xFAD mice and their littermate controls at 4 months and 8 months of age. Single cell spleen suspensions were stained with antibodies specific to T follicular helper cells (Tfh) ($CD4^+CXCR5^+PD\text{-}1^+$), B1 cells ($CD19^+CD5^+CD43^+$), or macrophages ($CD11c^-$, $CD11b^+$, $MHC\text{-}II^+$). The antibodies were obtained from Biolegend (San Diego, CA). Analysis was done using Flow Jo software (Treestar, Ashland, OR).

For cytokine analysis, spleen cells (1×10^6/ml) were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma) and ionomycin (1 μg/ml; Sigma) in RPMI medium containing 10% FBS**.** Supernatants collected after overnight stimulation were assayed for 15 analytes including, IL-6, TNF-α, IFN-γ, IL-17, IL-22, IL-10, IL-1β, IFN-α, CCL-2, CXCL-10, CCL-5, GMCSF, IL-1α, IL-33, IL-21 using magnetic bead based kit (Thermo Fisher Scientific).

Immunohistochemistry of Brain Samples

Mice were deeply anesthetized using isoflurane and euthanized via intracardiac perfusion using 4% paraformaldehyde (ACROS Organics; NJ) in 100 mM phosphate buffered saline (PBS; pH 7.4, Thermo Fisher Scientific)(Acharya et al. 2016). Brains were cryoprotected (10–30% sucrose gradient over 2–3 days) and sectioned coronally into 30 μm using a cryostat (Leica Microsystems, Germany). For each endpoint, 4 representative coronal brain sections of the amygdala and medial prefrontal cortex (mPFC) regions from each of the 4 animals per experimental group were selected at approximately 15 section intervals and stored in PBS. For the immunofluorescence labeling of microglial activation marker CD68, rat anti-mouse CD68 (1:500; AbD Serotec) primary antibody was used with Alexa Fluor 594 secondary antibody (1:500). Tissues were then DAPI nuclear counterstained and sealed in slow fade/gold antifade mounting medium (Life Technologies).

Thioflavin S Staining

Sections were rehydrated in an ethanol series (100%, 95%, 70%, 50%) and then incubated in a 0.5% thioflavin S solution in 50% ethanol for 10 min. Tissues were rinsed twice in 50% ethanol and then rinsed twice in PBS. Sections were mounted and sealed with slow fade/gold antifade mounting medium (Life Technologies).

Confocal Microscopy, Image Processing and 3D Quantification

The stained coronal brain sections were scanned using a confocal microscope (Nikon Eclipse Ti C2) equipped with a 40× PlanApo oil-immersion lens (1.3 NA, Nikon) and an NIS-Elements AR interface (v4.30, Nikon). 30 z stacks (1024 bit depth) at 0.5 μm from three different fields (318 × 318 × 24 μm) were imaged in each section in the areas of interest. The digitized z stacks were deconvoluted using the AutoQuant software (version X3.0.4, Media Cybernetics, Rockville, MD). An adaptive, 3D blinded method was used to create deconvoluted images for direct import into the Imaris module (version 8.1.2, Bitplane, Inc., Zurich, Switzerland). The 3D algorithm-based surface rendering and quantification of fluorescence intensity for each marker was carried out in Imaris at 100% rendering quality. In the case of CD68, each channel was analyzed separately where 3D surface rendering detects immunostained puncta or nuclear staining (DAPI) satisfying pre-defined criteria, for the puncta size (0.5 to 1 μm) verified visually for accuracy. Using deconvoluted confocal z

stack volume from the control group (WT) as a baseline for the minimum thresholding, a channel mean intensity filter was applied and used for all the experimental groups for each batch of molecular markers. The pre-set parameters were kept constant throughout the subsequent analysis of immunoreactivity for each antigen. To maintain uniformity among the varying number of puncta for each individual time point and/or antigen analyzed, the number of puncta per 318 × 318 × 24 μm was normalized to WT control and data was expressed as mean immunoreactivity (percentage) relative to WT controls.

Cytokine Assay

Plasma samples from AD, MCI and healthy controls were assayed for IL-6, TNF-α, IFN-γ, IL-17, IL-22, IL-10, IL-1β, IFN-α and Granzyme B using a Magpix multiplex kit (Thermo Fisher) following the manufacturer's instructions. IL-21 was assayed by specific ELISA (RnD Systems).

Statistical Analysis

Graph Pad Prism software was used for statistical analysis. Unpaired Student's t test was used for measuring significance within groups. For comparisons between three or more groups, one-way ANOVA followed by Tukey's test was used. Spearman's test was used for correlation. Values of $p < 0.05$ were considered significant. All tests were two tailed with 95% confidence interval.

Results

Aβ (1–42) Peptide Specific Antibodies of IgM Isotype Are Decreased in 5xFAD Mice Compared to Littermate Controls

We have previously reported (Agrawal et al. 2018) an increase in Aβ (1–42) peptide specific antibodies of IgG isotype in AD subjects as compared to HC displayed here as (Fig. 1A). The ratio of IgM to IgG is significantly decreased in AD patients compared to both MCI and healthy controls. Increased IgG against an antigen is an indication of an immune/inflammatory response against that antigen. We also analyzed the data to determine differences between males and females. However, there was no significant difference in the levels of IgM and IgG antibodies in AD, MCI and controls. To confirm that AD mice display similar changes in Aβ specific IgM and IgG antibodies as humans, serum from 5xFAD mice and littermate controls of 4 and 8 months of age was collected and ELISA was performed as for human samples. Aβ specific IgM antibodies were significantly decreased in the serum of early and late AD mice compared to controls (Fig. 1B; $p < 0.05$). Concomitantly an increase in IgG antibodies in early and late AD mice was also observed, but the difference was significant only at the late AD stage (Fig. 1B; $p < 0.05$). Here also, we did not observe a significant difference between males and females at both early and late time points. The 5xFAD model displays a fast AD pathology therefore changes including Aβ plaques are apparent as early as 4 months of age (Supplementary Fig. 1). These data also suggest that 5xFAD is serve as a good pre-clinical model, as they display similar immunological changes as those observed in humans. Elevated IgG antibodies also indicate an ongoing immune/inflammatory response against Aβ in the AD mice.

Correlation of IgM and IgG Responses with Cognitive Function

To link AD neuropathology and the peripheral immune response, mice were evaluated for cognitive function. Fear extinction (FE) memory refers to the active process of memory consolidation including dissociating a learned response to a prior adverse event. In this test, mice are first taught to expect a foot-shock after a tone is played, causing them to freeze in fear of the anticipated averse event. During the following three days, the mice undergo extinction training where they repeatedly hear the tone, but receive no foot-shock. Cognitively intact animals are able to abolish that fear memory and continue moving about instead of freezing, indicating minimal stress and anxiety. However, the AD mice showed severely impaired memory consolidation as indicated by an increased percent of test time spent freezing compared to the aged-match WT mice (Fig. 2A; $p = 0.01$). The WT mice exhibited a gradual decrease in freezing behavior (data not shown) over the extinction training whereas AD mice maintained higher freezing levels. These behavior data clearly link the neuropathological hallmarks of AD with cognitive dysfunction. Importantly, the cognitive function of AD mice (impaired fear memory consolidation) showed a strong negative correlation with reduced IgM levels (Fig. 2B; $r = -0.74014$, $p = 0.04$). On the contrary, the correlation between the IgG levels and freezing behavior was not significant ($r = -0.48$, $p = 0.23$). Together, these data demonstrate a correlation between immune/inflammatory responses in periphery and neuro-pathological changes in the AD brain.

Macrophages/Microglia Display Increased Activation in 5xFAD Mice Compared to Littermate Controls

To confirm that the immune system is activated in AD mice compared to WT controls, innate immune cell

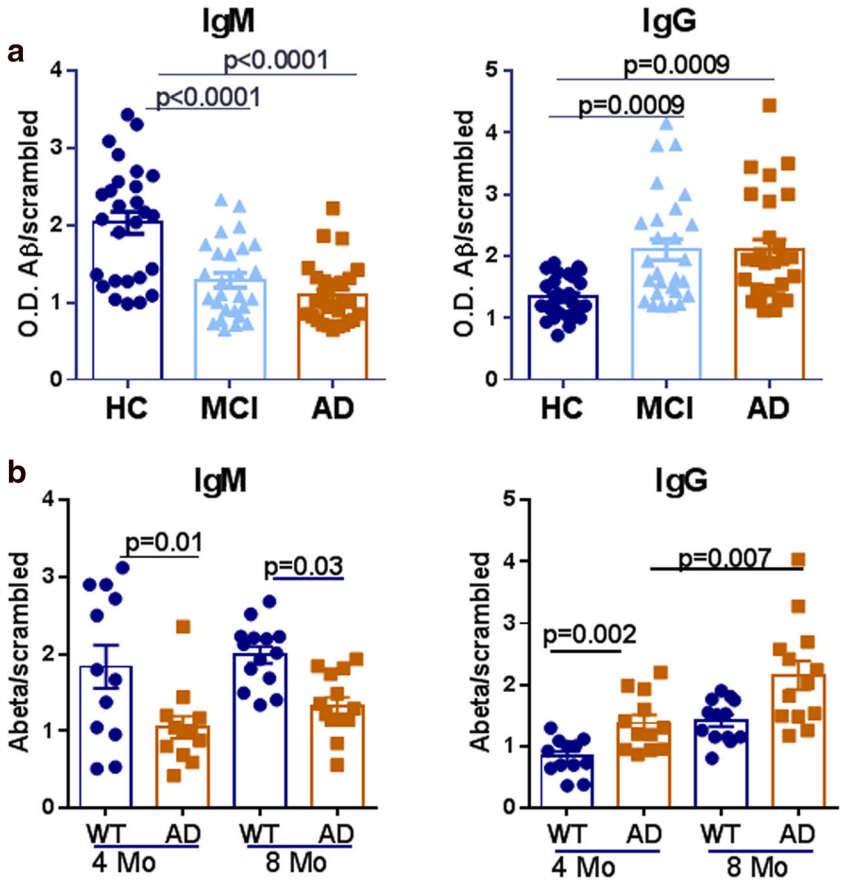

Fig. 1 Aβ (1–42) peptide specific antibodies of IgM isotype are decreased in AD patients and in 5xFAD mice compared to littermate controls. The levels of Aβ peptide (1–42) specific antibodies, as well as Aβ scrambled peptide, were measured in the plasma samples of AD, MCI and age matched healthy controls (HC). (**A**) Dot plots depict the Aβ specific IgM to IgG antibodies. Mean± S.D. of 26 AD patients, 26 MCI patients and 26 HC. Equal number of males and females in each group. (**B**) The levels of Aβ peptide (1–42) specific antibodies as well as Aβ scrambled peptide were also measured in the plasma samples of 5xFAD mice compared to WT littermate controls at 4 and 8 months of age. Dot plots depict the ratio of Aβ specific IgG and IgM antibodies to scrambled peptide antibodies. Mean ± S.D. For 4 mo: $N = 7M, 2F$ mice per group; 8 mo: $N = 7M, 7F$ mice per group. P values are derived from one way ANOVA in A and Student's t test in B

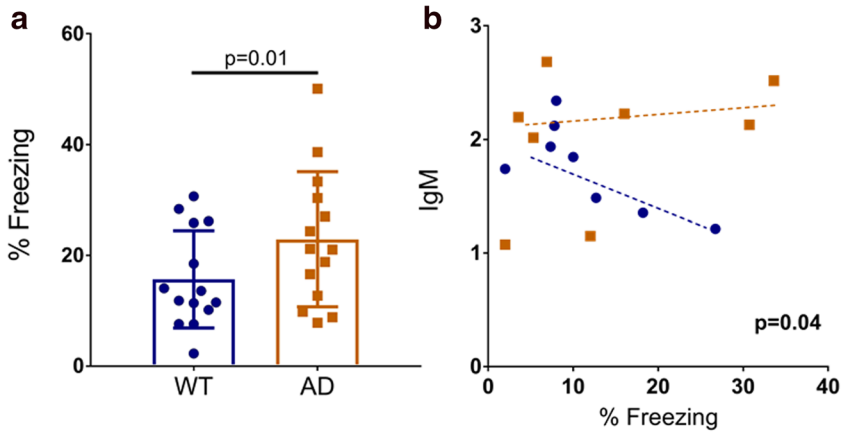

Fig. 2 Correlation of IgM and IgG response with cognitive function. (**A**) The cognitive testing of 6–8 month old WT and AD mice using the Fear Extinction (FE) memory test, showed significant cognitive impairments as indicated by elevated freezing during the extinction test. (**B**) The AD-related memory deficits correlated with the reduced IgM levels. AD mice showing higher freezing display higher IgM levels. Mean ± SE ($N = 14$ mice per group, A; $N = 8$, mice per group B). P values are derived from Student's t test (**A**) and Pearson's test (**B**)

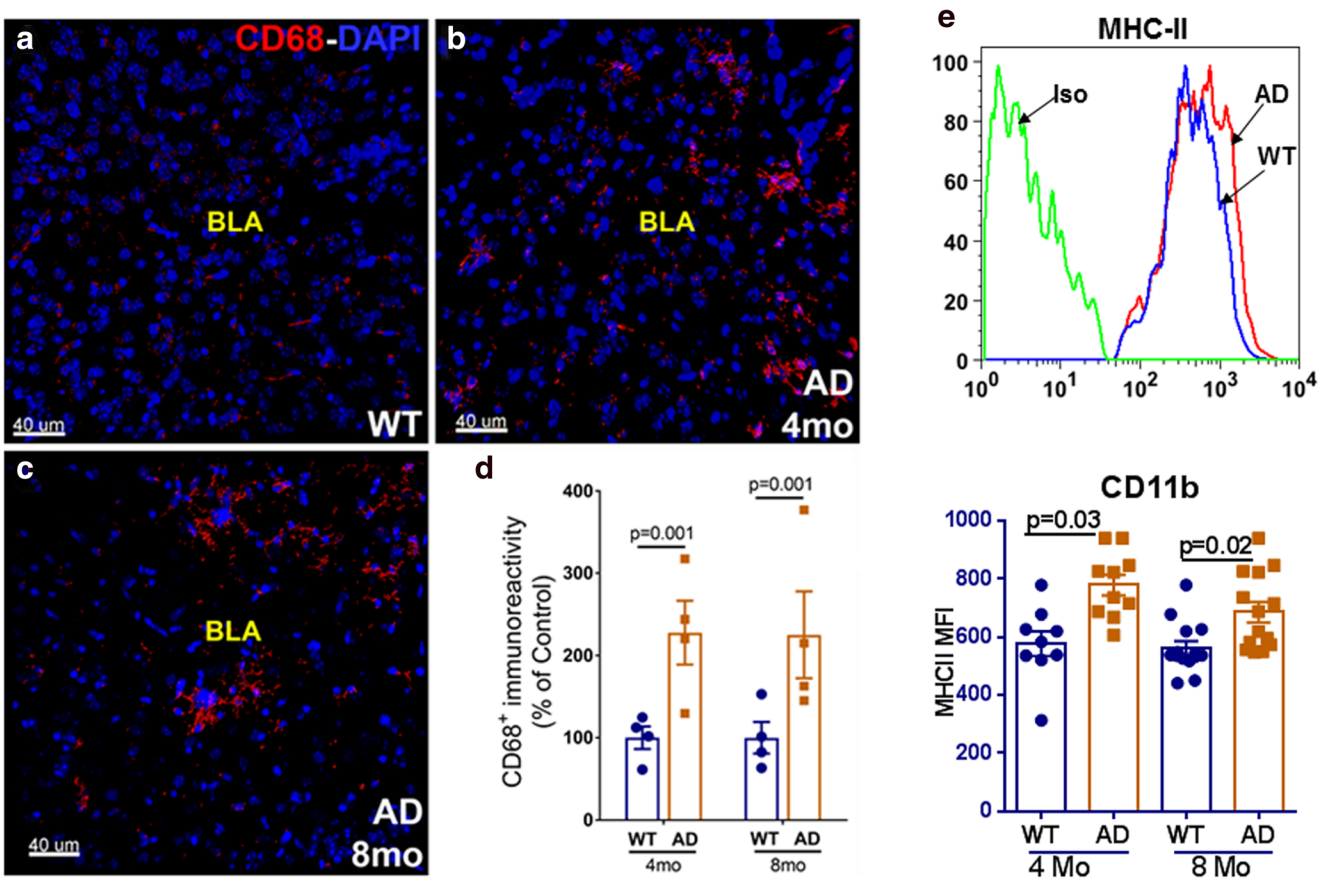

Fig. 3 Macrophages/microglia display increased activation in 5xFAD mice compared to littermate controls. (**A-C**) Representative high-resolution confocal micrographs from the basal lateral amygdala (BLA) of WT and AD mouse brains (red, CD68; blue, DAPI nuclear counter-stain; Scale 40 μm). (**D**) Quantification of CD68 immunohistochemical staining demonstrated that compared to controls, AD mice had increased microglial activation at both 4 and 8 months of age as compared to WT controls where data are Mean ± SE ($n = 4$ mice per group); *$p < 0.01$. (**E**) Dot plot depict the MFI (Mean fluorescence intensity) of MHC-II on macrophages in the spleen of AD and WT mice at 4 and 8 months of age. Histogram is representative of macrophage-MHC staining. For 4 mo: $n = 7$M, 2F mice per group; 8 mo: $n = 7$M, 7F mice per group. *P* values are derived from Student's t test

activation was also evaluated. It is well established that microglia in the brain are over activated in AD, and as expected the brains of AD mice showed a significant elevation in activated microglia (the resident immune-cell) at both the early and the late stages of disease progression compared to controls in the amygdala (Fig. 3A-D; $p = 0.01$) and mPFC (data not shown). To explore whether this activation of macrophages is also observed in the periphery, flow cytometry was used to determine the level of activation of macrophages in the spleen. The level of expression of MHC-class II on macrophages was found to be significantly higher on splenic macrophages from AD mice at both at 4 months and 8 months as compared to controls (Fig. 3E; $p = 0 < 0.05$). This increased level of MHC-II is indicative of activation, as the upregulation of MHC-II is associated with antigen presentation (Steinman 2012) and priming of T and B cell responses. These data indicate that periphery and brain reflect similar AD-related changes.

Inflammatory Cytokines Are Increased in AD Patients and 5xFAD Mice

Increased inflammation is usually associated with an ongoing immune response; therefore, we determined the level of inflammatory cytokines in the plasma from AD, MCI and HC. As is evident from Fig. 4A, we observed a significant increase in IL-21 levels in MCI and AD patients compared to controls. IL-21 is a highly inflammatory cytokine secreted primarily by T follicular helper cells (Tfh) cells and/ or Th17 cells (Tangye 2015). It is involved in class switching of antibody isotypes from IgM to IgG (Recher et al. 2011). For this reason, we performed correlation analysis between IL-21 and IgG in AD and MCI groups. There was significant correlation between IgG levels and IL-21 in both MCI ($r = 0.40$; $p = 0.03$) and AD groups ($r = 0.45$; $p = 0.01$). These data indicate that IL-21 may play a role in the increased IgG response in AD and MCI patients. None of the other cytokines tested demonstrated a significant difference between the three groups.

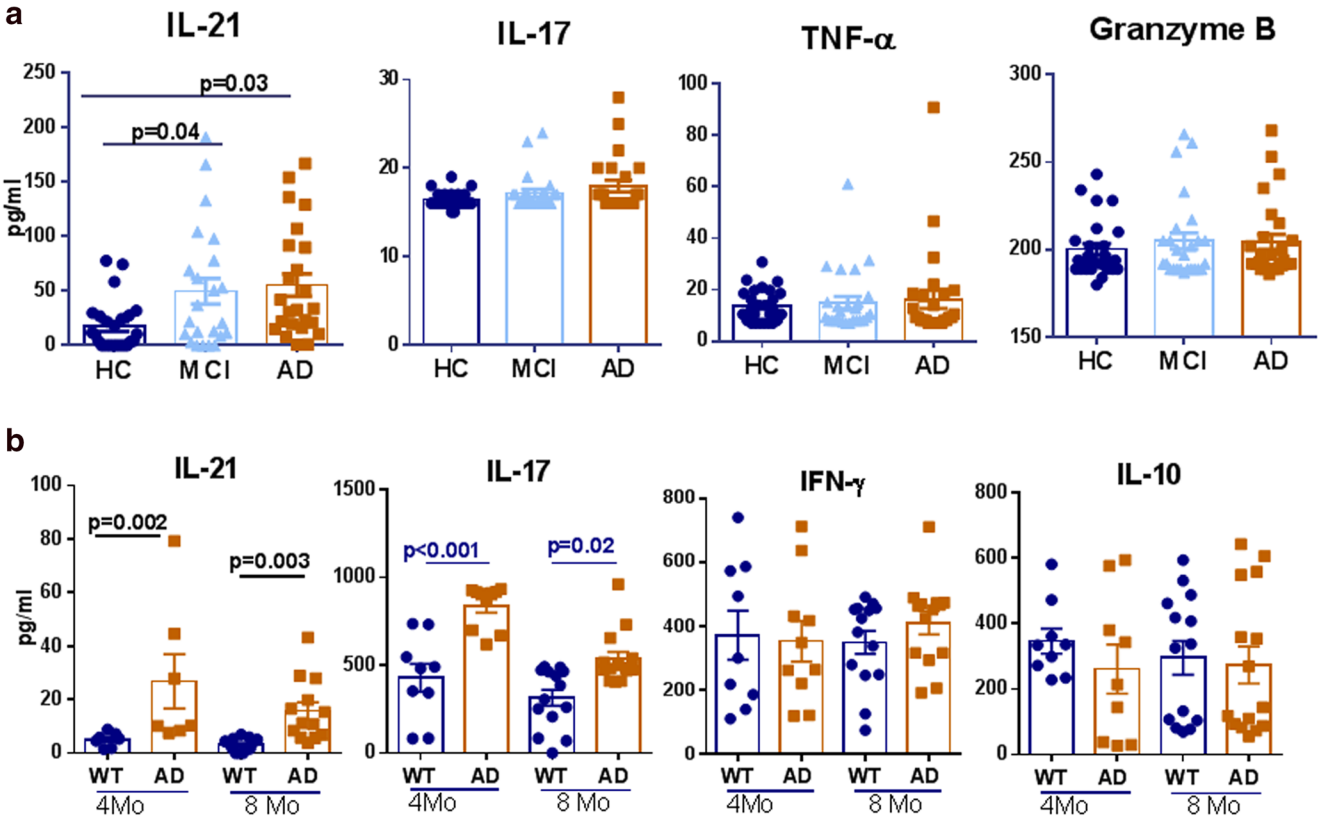

Fig. 4 Inflammatory cytokines are increased in AD patients and 5xFAD mice. **(A)** Dot plots depict the levels of cytokines in the plasma of human AD, MCI and HC samples. Mean ± S.D. of 26 AD patients, 26 MCI patients and 26 HC. (**B**) Dot plots display the levels of cytokines secreted by PMA, ionomycin stimulated spleen cells from 5xFAD mice and WT littermate controls at 4 and 8 months of age. For 4 mo: 7M, 2F mice per group; 8 mo: 7M, 7F mice per group. *P* values are derived from Student's t test

Next, T cell cytokine secretion was compared in spleen cells from AD and control mice to determine the nature of Th cell bias in AD. The spleen cells were stimulated overnight with PMA and ionomycin and the supernatant was assayed for 15 cytokines, chemokines, and growth factors via multiplex (Elahi et al. 2018). Of these IL-17 and IL-21 displayed significantly increased secretion in AD mice (Fig. 4B; $p < 0.05$). These changes are similar to what was observed in humans. These in vivo mouse data once again indicate ongoing immune/inflammatory responses in AD.

Tfh Cells Are Increased and B1 Cells Are Decreased in 5xFAD Mice Compared to Littermate Controls

Tfh cells play a crucial role in B cell proliferation and switching of IgM antibody isotype to IgG and other isotypes as well as antibody secretion (Crotty 2014). Furthermore, IL-21 is the signature cytokine of Tfh cells; therefore, we compared the percentages of Tfh cells in the spleens of AD and WT mice. The percentage of Tfh cells was significantly increased in the spleens of AD mice as compared to controls at both 4 and 8 months of age (Fig.5A; $p < 0.05$). The presence of Tfh cells indicates an active, ongoing immune response. The increase in Tfh cells in AD mice was much more significant at the early time point suggesting that the immune response starts at the early stage of AD. These data indicate potential involvement of Tfh cells in the increased IL-21 and IgG antibodies observed in AD patients and mice.

One of the mechanisms of clearance of self-antigens such as Aβ is via IgM antibody. A special subset of B cells called B1 cells specializes in producing IgM antibodies, which are often polyreactive. B1 cells are spontaneous IgM producers at homeostasis and are crucial for clearance of self-antigens without overt inflammation (Prieto and Felippe 2017). For that reason, the proportion of B1 cells in the spleen of AD and WT mice were compared by flow cytometry. A significant decrease in the number of B1 cells was observed in AD mice as compared to WT (Fig. 5B; $p < 0.05$). In addition, a significant correlation between B1 cells and IgM was observed between B1 cells and IgM levels in WT mice while no correlation was apparent in AD mice ($p = 0.001$, $r = 0.97$). These data support our previous observation of a decrease in IgM antibodies in AD patients and AD mice and suggest that it could be linked to a decrease in B1 cells.

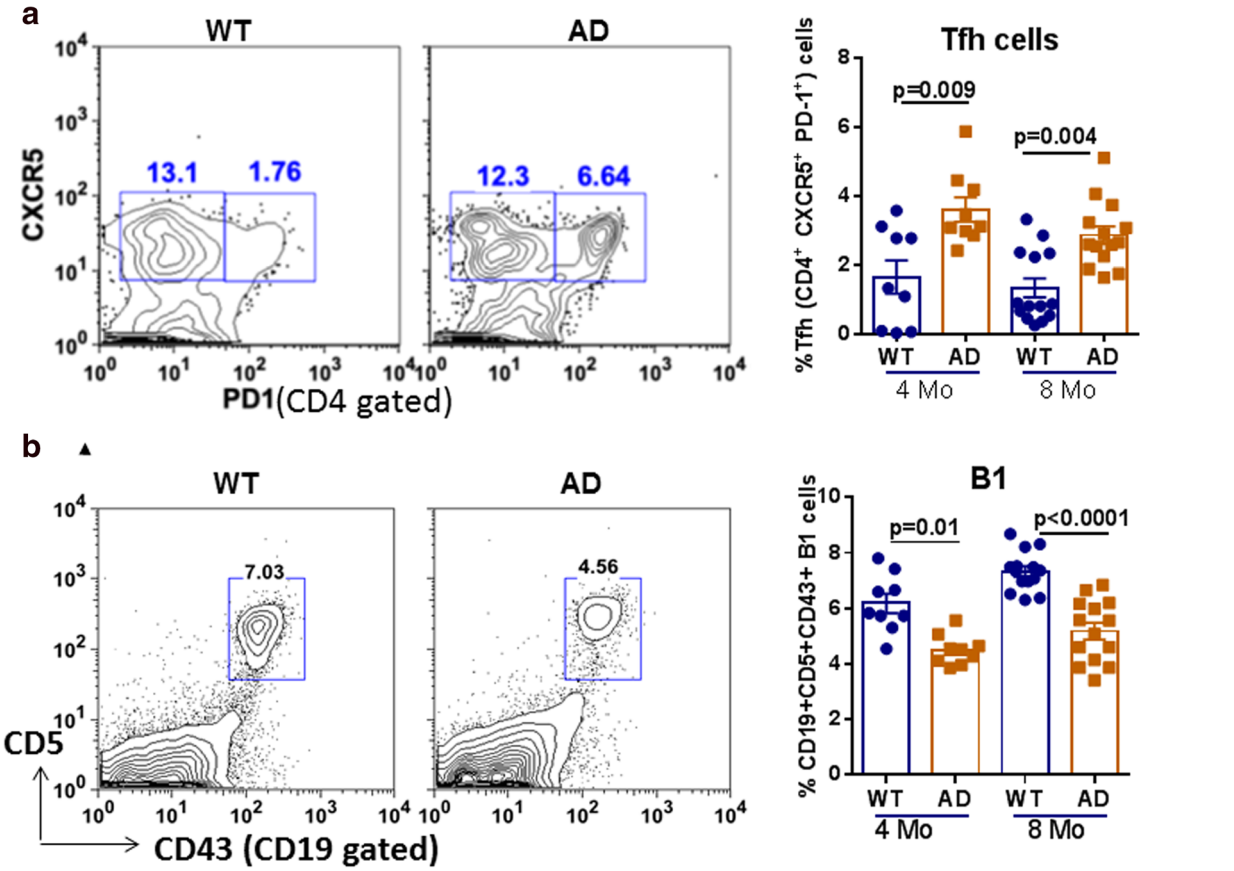

Fig. 5 Tfh cells are increased and B1 cells are decreased in 5xFAD mice compared to littermate controls. **(A)** Spleen cells were stained for Tfh cells ($CD4^+$, $CXCR5^+$, $PD\text{-}1^+$) and analyzed by flow cytometry. Contour plots depict the percentage of Tfh cells in AD and WT mice. Dot plots depict the % of $CD4^+$, $CXCR5^+$, $PD\text{-}1^+$ Tfh cells at 4 and 8 months of age. (**B**) Spleen cells were stained for B1 cells (CD19 + CD5 + CD43+) and analyzed by flow cytometry. Contour plot depicts the percentage of B1 cells in AD and WT mice. Dot plot depicts the percentage of B1 cells at 4 and 8 months of age. Mean +/-S.E. For 4 mo: 7M, 2F mice per group; 8 mo: 7M, 7F mice per group. *P* values are derived from Student's t test

Discussion

The present study demonstrates that the proportions and/or activation of peripheral immune system cells is significantly increased in AD patients and mice compared to controls. More importantly, this immune activation is linked to cognitive dysfunction.

We have previously demonstrated (Agrawal et al. 2018) and shown here (Fig. 1A) that AD patients display increased IgG response against Aβ compared to healthy controls indicative of an inflammatory immune response. We observe a similar increase in IgG in the mouse model (Fig. 1B) indicating that human and mice parameters are alike. A probable scenario for the increase in IgG is that the continuous exposure of the immune system to high levels of Aβ, possibly due to impaired clearance, leads to breaking of immune tolerance and immune reactivity to Aβ. The chronic activation due to Aβ enhances inflammation and increases neuropathology. This is supported by our data (Fig. 2A-C) where an inverse correlation between low IgM and cognitive dysfunction tests was observed. In addition, the activation of macrophages in the spleen and their counterpart microglia in the brain was also increased in AD mice compared to controls, indicative of an inflammatory response in the brain and in the periphery (Fig. 3A-E). The concomitant changes in the brain and in the periphery suggest the possibility of developing a blood-based biomarker for diagnosis of AD.

The level of inflammatory cytokine, IL-21 was increased in the circulation of AD and MCI patients compared to HC as demonstrated previously (Agrawal et al. 2018) and here in Fig. 4A. Furthermore, it correlated positively with IgG levels in both MCI and AD subjects. IL-21 belongs to γC family of cytokines along with IL-2, IL-15 etc. It is produced primarily by CD4 T cells particularly the Tfh subset in the germinal centers where it acts on B cells to mediate class switching and sustain production of antibodies via differentiation of B cells to plasma cells (Kishida et al. 2007; Recher et al. 2011; Tangye 2015). IL-21 also acts in an autocrine manner and promotes the development of Tfh cells that may be reason that increased proportions of Tfh cells are observed in the spleen of AD mice compared to controls (Fig. 5A). In addition, IL-21 is a highly inflammatory cytokine that can enhance the

differentiation of IL-17, producing Th17 cells (Spolski and Leonard 2014). We do find increased secretion of IL-17 and IL-21 in spleen cells from AD mice (Fig. 4B) indicating increased differentiation towards Th17 cells. Th17 cytokines including IL-17, IL-21 and IL22 are reported to be increased in AD patients (Zhang et al. 2013). Furthermore, both IL-21 and IL-17 have been implicated in numerous autoimmune inflammatory diseases (Sarra et al. 2010; Spolski and Leonard 2014; Ueno et al. 2015; Zhang et al. 2013) including multiple sclerosis (Okada and Khoury 2012). More recently, IL-21 was found to be highly elevated in the mouse brain after cerebral ischemia (Clarkson et al. 2014). Thus IL-21 may not only be promoting antibody class switching, but also enhancing inflammation in AD subjects.

Interesting changes in the proportions of both Tfh and B1 cells in AD mice were also observed in this study (Fig. 5A, B). Tfh cells are found with in the germinal centers and an increase in their percentages is indicative of an ongoing immune response (King et al. 2008). Tfh cells are essential for generation of optimal antibody responses; however, an excessive Tfh cell response can result in breakdown of tolerance. For example, increased numbers and activity of Tfh cells have been reported in lupus (Blanco et al. 2016; Yang et al. 2016). Similarly, increased frequencies of Tfh cells and B cell plasmablasts have been found in peripheral blood and cerebrospinal fluid (CSF) from multiple sclerosis patients with relapsing–remitting or secondary progressive forms of the disease (Fan et al. 2015; Puthenparampil et al. 2019). Furthermore, elevated levels of IL-21 in plasma and CSF were observed to correlate with disease severity. An increase in activated Tfh cell frequencies in circulation is also found in another neurological autoimmune disorder, Neuromyelitis Optica Spectrum Disorders (NMOSD) (Li et al. 2015). These patients also displayed increased IL-21 levels in both CSF and plasma. It is therefore plausible that the increased proportions of Tfh cells as observed in AD mice are not only a source of IL-21 but may also be playing a role in breakdown of tolerance against Aβ. Future studies with human subjects will explore whether the frequencies of Tfh cells and IL-21 levels in circulation can be used as potential biomarkers for AD.

Another immune cell that displayed significant changes in AD mice compared to littermate controls was the B1 subset. Antibodies are secreted by B lymphocytes that can be divided into two major subsets, B1 and B2 (Montecino-Rodriguez and Dorshkind 2012; Rothstein et al. 2013; Silverman et al. 2000). B1 and B2 subsets display different phenotypic markers, ontogeny and functions. B2 are the conventional B lymphocytes that are capable of producing various subtypes (IgG, IgE) of high affinity antibodies via class switching. In contrast, the principal function unique to B1 cells is spontaneous, constitutive secretion of antibody, termed *natural antibody* that appears in the absence of infection or immunization. B1 cells plays a critical role in housekeeping removal of cellular debris and self-proteins (Rothstein et al. 2013). Natural antibody is predominantly IgM, and it is estimated that 80–90% of resting serum IgM is derived from B1 cells (Rothstein et al. 2013). More importantly, serum IgM is preferentially produced by B1 cells in the spleen. Natural antibody tends to be polyreactive, autoreactive, and anti-microbial at relatively modest affinity (Montecino-Rodriguez and Dorshkind 2012; Rothstein et al. 2013; Silverman et al. 2000). The effectiveness of B1 cell natural antibody may depend, in part, on that polyreactivity that provides the means for a single antibody to heteroligate different and possibly widely spaced, surface antigens, thereby increasing effective avidity. Further, IgM effectively binds the complement component C1q that also helps in clearance (Chen et al. 2009). One of the major homeostatic functions of the natural antibodies is to help in the removal of autoantigens such as Aβ (Gronwall et al. 2012). In this regard, natural autoantibodies against Aβ have also been demonstrated to play a protective role against AD (Puli et al. 2014; Qu et al. 2014). Autoantibodies against varying Aβ epitopes have been detected at higher levels in the blood of control subjects as compared to AD patients (Qu et al. 2014). Thus, reduced proportions or function of B1 cells may contribute to the decreased IgM in antibodies observed in AD patients and mice.

Conclusions

In summary, our data indicate immune tolerance to Aβ is compromised and a strong immune /inflammatory response against Aβ is generated in AD. This may be a compensatory mechanism to clear Aβ but the increased inflammation leads to neuropathology in the brain. Furthermore, we also report that the peripheral immune changes are linked to cognitive dysfunction and some of those peripheral immune changes, such as IL-21 and Tfh, may be developed as biomarkers for AD.

Acknowledgements We are grateful to UCI-ADRC for providing the serum samples from AD, MCI patients and controls.

Author Contributions J.E.B. maintained the 5xFAD mouse colony and performed molecular analyses and analyzed data. M.A.A. performed mice behavior experiments, analyzed data and prepared Figs. L.A.A. performed mouse behavior experiments. C.M. helped with processing of mouse tissues for staining. S.A. performed the human serum experiments and flow cytometry of mice cells. A.A. designed the study, analyzed the data, prepared the figures and wrote the manuscript. All authors were involved in discussions and writing of manuscript.

Funding Information The project described was supported by AG045216, AG064709 to A.A. and the California Institute for Regenerative Medicine DISC1–10079 to J.E.B. The UCI-ADRC is funded by NIH/NIA Grant P50 AG16573. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Data Availability All relevant raw datasets analyzed during the current study are freely available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate All procedures performed in studies involving human participants were in accordance with the ethical standards of the University of California Irvine Institutional Research Board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Since deidentified human serum samples were obtained from ADRC, the research was considered non-human subjects by UCI-IRB. Informed consent from subjects was obtained by ADRC.

Mice All animal experimentation procedures were performed in accordance with the guidelines provided by NIH and approved by the University of California Irvine Institutional Animal Care and Use Committee.

Abbreviations AD, Alzheimer's disease; MCI, Mild Cognitive Impairment; HC, Healthy Controls; Aβ, Amyloid beta; APC, Antigen presenting cells; Tfh, T follicular helper cells; ADRC, Alzheimer's disease research center; FE, Fear Extinction; CS, conditioned stimulus; PMA, phorbol 12-myristate 13-acetate; WT, Wild type control mice; CSF, cerebrospinal fluid; NMSOD, Neuromyelitis Optica Spectrum Disorders

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